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ΑΛΛΗΛΟΥΧΙΣΗ ΤΟΥ RNA ΚΑΙ ΑΝΑΛΥΣΗ ΤΗΣ ΓΟΝΙΔΙΑΚΗΣ ΕΚΦΡΑΣΗΣ ΤΩΝ ΑΙΜΟΠΟΙΗΤΙΚΩΝ ΣΤΕΛΕΧΙΑΙΩΝ ΚΥΤΤΑΡΩΝ ΣΤΟΝ ΣΥΣΤΗΜΑΤΙΚΟ ΕΡΥΘΗΜΑΤΩΔΗ ΛΥΚΟ

ΜΑΡΙΑ ΓΡΗΓΟΡΙΟΥ

ΒΙΟΛΟΓΟΣ, MSc

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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ΑΘΗΝΑ

NATIONAL AND KAPODISTRIAN UNIVERSITY OF ATHENS, ATTIKON UNIVERSITY HOSPITAL,
4TH DEPARTMENT OF INTERNAL MEDICINE
HEAD: DIMITRIOS T. BOUMPAS

RNA-SEQUENCING AND TRANSCRIPTOME ANALYSIS OF HEMATOPOIETIC STEM CELLS IN SYSTEMIC LUPUS ERYTHEMATOSUS

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DOCTORAL THESIS
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Ο Όρκος του Ιπποκράτη

Ὄμνυμι Ἀπόλλωνα ἰητρὸν, καὶ Ἀσκληπιὸν, καὶ Ὑγείαν, καὶ Πανάκειαν, καὶ θεοὺς πάντας τε καὶ πάσας, ἵστορας ποιεύμενος, ἐπιτελέα ποιήσειν κατὰ δύναμιν καὶ κρίσιν ἐμὴν ὅρκον τόνδε καὶ ξυγγραφὴν τήνδε.

Ἡγήσασθαι μὲν τὸν διδάξαντά με τὴν τέχνην ταύτην ἴσα γενέτῃσιν ἐμοῖσι, καὶ βίου κοινώσασθαι, καὶ χρεῶν χρηίζοντι μετάδοσιν ποιήσασθαι, καὶ γένος τὸ ἐξ ωὐτέου ἀδελφοῖς ἴσον ἐπικρινέειν ἄῤῥεσι, καὶ διδάξειν τὴν τέχνην ταύτην, ἢν χρηίζωσι μανθάνειν, ἄνευ μισθοῦ καὶ ξυγγραφῆς, παραγγελίης τε καὶ ἀκροήσιος καὶ τῆς λοιπῆς ἀπάσης μαθήσιος μετάδοσιν ποιήσασθαι υἱοῖσί τε ἐμοῖσι, καὶ τοῖσι τοῦ ἐμὲ διδάξαντος, καὶ μαθηταῖσι συγγεγραμμένοισί τε καὶ ὠρκισμένοις νόμῳ ἰητρικῷ, ἄλλῳ δὲ οὐδενί.

Διαιτήμασί τε χρήσομαι ἐπ' ὠφελείῃ καμνόντων κατὰ δύναμιν καὶ κρίσιν ἐμὴν, ἐπὶ δηλήσει δὲ καὶ ἀδικίῃ εἴρξειν.

Οὐ δώσω δὲ οὐδὲ φάρμακον οὐδενὶ αἰτηθεὶς θανάσιμον, οὐδὲ ὑφηγήσομαι ξυμβουλίην τοιήνδε. Ὁμοίως δὲ οὐδὲ γυναικὶ πεσσὸν φθόριον δώσω. Ἁγνῶς δὲ καὶ ὁσίως διατηρήσω βίον τὸν ἐμὸν καὶ τέχνην τὴν ἐμήν.

Οὐ τεμέω δὲ οὐδὲ μὴν λιθιῶντας, ἐκχωρήσω δὲ ἐργάτῃσιν ἀνδράσι πρήξιος τῆσδε.

Ἐς οἰκίας δὲ ὁκόσας ἂν ἐσίω, ἐσελεύσομαι ἐπ' ὠφελείῃ καμνόντων, ἐκτὸς ἐὼν πάσης ἀδικίης ἐκουσίης καὶ φθορίης, τῆς τε ἄλλης καὶ ἀφροδισίων ἔργων ἐπί τε γυναικείων σωμάτων καὶ ἀνδρῴων, ἐλευθέρων τε καὶ δούλων.

Ἃ δ' ἂν ἐν θεραπείῃ ἢ ἴδω, ἢ ἀκούσω, ἢ καὶ ἄνευ θεραπηίης κατὰ βίον ἀνθρώπων, ἃ μὴ χρή ποτε ἐκλαλέεσθαι ἔξω, σιγήσομαι, ἄῤῥητα ἡγεύμενος εἶναι τὰ τοιαῦτα.

Ὅρκον μὲν οὖν μοι τόνδε ἐπιτελέα ποιέοντι, καὶ μὴ ξυγχέοντι, εἴη ἐπαύρασθαι καὶ βίου καὶ τέχνης δοξαζομένῳ παρὰ πᾶσιν ἀνθρώποις ἐς τὸν αἰεὶ χρόνον. παραβαίνοντι δὲ καὶ ἐπιορκοῦντι, τάναντία τουτέων.

Curriculum Vitae

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EDUCATION

December 2015- Present	*PhD Candidate* 4th Department of Internal Medicine, National and Kapodistrian University of Athens, Athens, Greece Supervisor: Prof Dimitrios Boumpas (boumpasd@uoc.gr)
2013- 2015	*MSc in "Molecular Biology and Biomedicine* Joint Graduate Programme with Department of Biology/Medicine, University of Crete, Heraklion Crete, Greece Grade: 9,50/10
2008- 2013	*BSc in Biology, Biomolecular Sciences and Biotechnology Direction* Faculty of Biology, University of Crete, Heraklion Crete, Greece ▪ Grade: 7,02/10
2001- 2008	*High School of Archanes, Crete, Greece* ▪ Grade: 18,23/20

EXPERIENCE

December 2015- Present	*PhD Thesis* "RNA sequencing and Transcriptome analysis of Hematopoietic Stem Cells in Systemic Lupus Erythematosus" Laboratory of "Inflammation and Autoimmunity" D. Boumpas, BRFAA, Athens, Greece http://www.bioacademy.gr/lab/boumpas?lang=gr
2014- 2015	*MSc Thesis* "Gene expression study of hematopoietic stem cells in an experimental model of Systemic Lupus Erythematosus" Laboratory of "Inflammation and Autoimmunity" D. Boumpas, BRFAA, Athens, Greece http://www.bioacademy.gr/lab/boumpas?lang=gr Grade: 10/10
24/08/2014- 28/10/2014	*Two-month internship in Bioinformatics* Subject "Analysis of rheumatoid arthritis expression data" Laboratory of "Bioinformatics and Computational Biology"

	P. Pavlidis, J. Iliopoulos, Medical School, University of Crete, Heraklion Crete, Greece http://pop-gen.eu/wordpress/people/pavlos-pavlidis, http://cbg.med.uoc.gr Grade: 10/10
05/05/2014- *05/08/2014*	*Two-month internship in Immunobiology* Subject "Gene expression study of hematopoietic stem cells in an experimental model of Systemic Lupus Erythematosus (SLE)" Laboratory of "Inflammation and Autoimmunity" D. Boumpas, BRFAA, Athens, Greece http://www.bioacademy.gr/lab/boumpas?lang=gr Grade: 10/10
04/03/2014- *04/05/2014*	*Two-month internship in Neurobiology* Subject "Use of molecullar biology techniques to generate plasmid construct for: A. Suppression of gene expression through RNA interference (RNAi) in C. Elegans, B. Creation of a translational reporter for expression analysis in C. Elegans" Laboratory of "Neurogenetics and Aging" N. Tavernarakis, IMBB-ITE, Heraklion Crete, Greece http://www.tavernarakislab.gr/index-gr.html Grade: 9/10
20/01/2013- *20/04/2013*	*Erasmus Internship in Genetics* Subject "The genetics of stuttering" Medical Genetics Program, Tommerup Group N. Tommerup, Department of Cellular and Molecular Medicine (ICMM), University of Copenhagen, Copenhagen, Denmark http://icmm.ku.dk/english/research/research_program/genetics/ Grade: 9/10
2011-2012	*BSc Thesis* "Study of the interaction between the soluble MHC class II and T lymphocytes using acoustic biosensors" Biosensors Lab in collaboration with Immunobiology Lab (Athanassakis Irene) E. Gizeli, IMBB-FORTH and Department of Biology, University of Crete, Heraklion Crete, Greece http://biosensorslab-forth.gr/ Grade: 10/10

AWARDS/FELLOWSHIPS

2016-2019	IKY Fellowship (Operational Programme «Human Resources Development, Education and Lifelong Learning» in the context of the project "Strengthening Human Resources Research Potential via Doctorate Research" (MIS-5000432), implemented by the State Scholarships Foundation)
2013	Erasmus Placement Internship
2008	Excellence Awards "Ranked 8th out of 120 Department of

	Biology, University of Crete («Δες την ψηφιακά»)
2008	Excellence and Progress Awards, Ranked 2nd, High School

PEER-REVIEWED PUBLICATIONS

28/11/2019	Grigoriou M., Banos A, et al. Transcriptome Reprogramming and Myeloid Skewing in Hematopoietic Stem and Progenitor Cells in Systemic Lupus Erythematosus. *Annals of the Rheumatic Diseases* (doi: 10.1136/annrheumdis-2019-215782)
12/2019	Kefallinou D, Grigoriou M, Boumpas DT., Gogolides E., Tserepi A., Fabrication of a 3D microfluidic device towards bone marrow-on-a-chip, Micro and Nano Engineering (*in preparation*)
2019	Frangou E., Garantziotis P., Grigoriou M., Banos A., Bertsias G, Dermitzakis E., Filia A., Boumpas DT., Comparative transcriptome profiling of target-tissues in experimental lupus model reveals novel molecular pathways (*in preparation*)

ORAL PRESENTATIONS

2019	"Is SLE a Stem Cell Disease?", 45th Annual Panhellenic Medical Conference, Athens, Greece
2018	"Transcriptome Analysis of Hematopoietic Progenitor Cells in an Experimental Model of Systemic Lupus Erythematosus: Disorders of the Myeloid Lineage", 26th Panhellenic Congress of Rheumatology, Athens, Greece
2017	"Hematopoietic Stem Cells in Systemic Lupus Erythematosus", 2nd SLE School, National Institute of Research, Athens, Greece

POSTER PRESENTATIONS

2019	"Transcriptome reprogramming and myeloid skewing in hematopoietic stem and progenitor cells in systemic lupus erythematosus", 70th Hellenic Society of Biochemistry and Molecular Biology, Athens, Greece
2017	"RNA-seq profiling of Hematopoietic Stem Cells in Murine Systemic Lupus Erythematosus: Validation and Functional characterization", 37th European Workshop for Rheumatology Research, Athens, Greece
2015	"The role of Hematopoietic Stem Cells (HSCs) in Systemic Autoimmunity", 66th Hellenic Society of Biochemistry and Molecular Biology, Athens, Greece
2015	"The role of Hematopoietic Stem Cells (HSCs) in Systemic Autoimmunity", 3rd Hellenic New Scientists Conference, Athens
2015	"The role of Hematopoietic Stem Cells (HSCs) in Systemic Autoimmunity", 35th European Workshop of Rheumatology Research, Budapest, Hungary
2012	"Immunomodulation mediated by soluble MHC class II antigens", 63th Hellenic Society of Biochemistry and Molecular Biology, Heraklion Crete, Greece

WORKSHOPS

08-12/07/2019	The 2019 Lectures in Biology and Chemistry: Genome Editing, FORTH, Heraklion Crete, Greece
20-22/06/2019	1st Summer Bioethics Course, Stavros Niarchos Foundation Cultural Center, Athens, Greece
2-11/10/2017	3rd Laboratory Animal Science European Union Function Course, Athens, Greece
2014	Laboratory safety, FORTH, Heraklion Crete, Greece
02/2013-05/2013	Medicine through the Microscope, Lifelong Learning Program, Center of Continuing Education and Training of the National and Kapodistrian University of Athens

PARTICIPATION IN CONFERENCES

2019	45th Annual Panhellenic Medical Congress, Athens, Greece
2018	26th Panhellenic Congress of Rheumatology, Athens, Greece
2017	2nd SLE School, National Institute of Research, Athens, Greece
2017	37th European Workshop for Rheumatology Research, Athens, Greece
2015	66th Hellenic Society of Biochemistry and Molecular Biology, Athens, Greece
2015	3rd Hellenic New Scientists Conference, Athens, Greece
2015	1st Symposium in Clinical Genetics, Athens, Greece (12 CME)
2015	35th European Workshop of Rheumatology Research, Budapest
2014	9th European Lupus Meeting, Athens, Greece
2013	9th Hellenic Congress of Immunology, Athens, Greece (15 CME)
2012	63th Hellenic Society of Biochemistry and Molecular Biology, Heraklion Crete, Greece
2010	1st International Congress of Proteomics, Heraklion Crete, Greece

ADDITIONAL SKILLS

Organizational/Teaching skills	✓ Laboratory assistant within the framework of the MSc (2013-2014) ✓ Supervision of undergraduate (1 student for BSc thesis, 2 students for BSc internship) and graduate students (2 student for MSc thesis, 2 students for MSc internship, 1 PhD candidate) within the framework of the PhD
Technical skills	✓ Excellent handling of laboratory animals/FELASA holder (*M. musculus*, *R. Norvegicus*, *C. elegans*) ✓ Immunology techniques: tissue/cell isolation from human/animals, ELISA, cell culture, hematopoietic cell biology, immunofluorescence preparation/analysis, FACS/sorting preparation/analysis ✓ Molecular techniques: PCR, qPCR, isolation of DNA/RNA/protein, electrophoresis of DNA/RNA/protein, construct of plasmid vectors, plasmid cloning ✓ Handling and analysis of acoustic biosensors (SAW, QCM) ✓ Bioinformatics: analysis of DNA-seq, RNA-seq, Methyl-seq

data

Informatics skills	✓ European Computer Driving License (ECDL) ✓ Basic knowledge of R ✓ Software: Windows, Macintosh, Linux

PERSONAL SKILLS

Languages	Greek (native speaker) English (excellent command/ECPE) German (basic communication skills/Zertifikat Deutsch B1)
Interests	Volunteering: First Aid Assistant, Training Program «First aid for citizens», 02/2004

RESEARCH INTERESTS

Biology has always impressed me, especially through the tuned functions in a living organism. Immunobiology is a field that encompasses balanced recognition and response. I am particularly interested in how this balance is disturbed leading to autoimmunity and chronic inflammatory diseases, such as Systemic Lupus Erythematosus.

An intriguing sequence of events is the origin of a cellular disorder, the stage of differentiation at which the cells are being "activated" and how they finally behave in a target-tissue. In order to approach these questions, the contact with the great world of the molecular biology is inevitable. How and why the molecular landscape of the cells changes during autoimmunity, both at chromatin and gene expression level, are major niches of investigation throughout my research life.

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Table of Contents

Abbreviations

ACR	American College of Rheumatology
ANA	Anti-nuclear Antibodies
APCs	Antigen-presenting cells
α-MEM	Minimum Essential Medium Eagle-alpha modification with nucleosides
BM	Bone Marrow
BMMCs	Bone Marrow Mononuclear Cells
BMoC	Bone-marrow-on-a-chip
B6-Y	C57BL/6 Young mice (3 months old)
B6-O	C57BL/6 Old mice (6-9 months old)
CGI	CpG islands
CMPs	Common Myeloid Progenitors
CLPs	Common Lymphoid Progenitors
DAPI	4'6-diamidin-2-phenilindole
DEGs	Differentially Expressed Genes
DCs	Dendritic Cells
DMEM	Dulbecco's Modified Eagle Medium
DMRs	Differential Methylation Regions
eBM	engineered Bone Marrow
F1-P	NZB/W F1-Prediseased mice (3 months old)
F1-L	NZB/W F1-Lupus mice (6-9 months old)
FACS	Fluorescence-Activated Cell Sorting
FDR (q-value)	False Discovery Rate (adjusted p-value)
FBS	Fetal Bovine Serum
FC	Fold Change
γ-H2AX	phospho-H2A.X
GMPs	Granulocyte/macrophage Progenitors
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis

GWAS	Genome-Wide Association Studies
HC	Healthy Controls
HSCs	Hematopoietic Stem Cells
HSPCs	Hematopoietc Stem/Progenitor Cells
IMDM	Iscove's Modified Dulbecco's Medium
IL-3	Interleukin 3
IL-6	Interleukin 6
IFN-α	Interferon alpha
IRF	Interferon Regulatory Factor
IFNAR	Type I IFN Receptor
LoC	Lab-on-a-Chip
LN	Lupus Nephritis
LT	Long-Term
Methyl-seq	DNA Methylation sequencing
MSCs	Mesenchymal Stem Cells
NGS	Next-Generation Sequencing
NZB	New Zealand Black
NZW	New Zealand White
NZB/W F1	New Zealand Black and White hybrid 1 (F1 generation)
NoC	BM Niche-on-a-chip
OoC	Organ-oon-a-chip
PBS	Phosphate Buffer Saline
PB	Peripheral Blood
PBMCs	Peripheral Blood Mononuclear Cells
PDMS	Poly(dimethyl-siloxane)
Pen/Strep	Penicillin Streptomycin
PGA	Physician Global Assessment
RNA-seq	RNA sequencing
RNEA	Regulatory Network Enrichment Analysis
RRBS	Reduced Representation Bisulfite Sequencing
SCF	Stem Cell Factor
SEM	Scanning Electron Microscope
SLE	Systemic Lupus Erythematosus

SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SLICC	Systemic Lupus International Collaborating Clinics
ST	Short-Term
TPO	Thrombopoietin

Abstract

Systemic Lupus Erythematosus (SLE) is a prototypic autoimmune disease that affects mainly women and typically exhibits manifestations in multiple organs including skin, joints, kidneys and nervous system. It is a complex disease resulting from the interaction of heritable (genetic and epigenetic), hormonal and environmental factors. Immune complexes, auto-antibodies and all blood cells (auto-reactive lymphocytes, dendritic cells, etc) are involved in clinical manifestations. *Hematopoietic Stem and Progenitor Cells* (HSPCs) are multipotent cells giving rise to all blood cell lineages, both myeloid and lymphoid. We reasoned that the aberrancies of immune cells observed in SLE could be traced back to HSPCs. The aim of the study is to further investigate the role and function of bone marrow-derived HSPCs in SLE.

Bone marrow (BM) samples from female NBZW/F1 lupus-prone mice and their age-matched controls were used. HSPCs defined as *Lin$^-$Sca-1$^+$c-Kit$^+$ hematopoietic progenitors* (LSK). Transcriptomic analysis of LSK from diseased lupus mice demonstrated a strong myeloid signature accompanied with expanded frequencies of common myeloid progenitors (CMPs) -but not of common lymphoid progenitors (CLPs)- reminiscent of a "trained immunity" signature. CMP profiling revealed an intense transcriptome reprogramming with suppression of granulocytic regulators as major regulators such as *Cebpe*, *Cebpd* and *Csf3r* are downregulated in lupus mice. The data are indicative of a differentiation arrest and disturbed myelopoiesis. Despite the differentiation arrest, frequencies of BM neutrophils were markedly increased in diseased mice suggesting an alternative granulopoiesis pathway.

In humans, hematopoietic progenitors were defined and isolated as CD34$^+$ BM cells. In SLE patients with severe disease pattern, CD34$^+$ demonstrated enhanced proliferation, cell differentiation and transcriptional activation of cytokines and chemokines that drive differentiation towards myelopoiesis thus mirroring the murine data. Comparative analysis of human and murine transcriptional profiles revealed that murine CMP and human CD34$^+$ cells shared an attenuated myeloid signature consistent with a differentiation arrest.

Collectively, these data demonstrate priming of HSPCs and aberrant regulation of myelopoiesis in SLE, potentially contributing to persistent inflammation and risk for flare. The data point out the crucial role of BM homeostasis in SLE pathogenesis therefore the construction of *in vitro* BM simulating system is emerging. This system will provide easily further mechanistic and functional investigation with fewer samples from animal models and human samples.

Περίληψη

Ο *Συστηματικός Ερυθηματώδης Λύκος* (ΣΕΛ) είναι μία πρωτότυπη αυτοάνοση νόσος που επηρεάζει κυρίως τις γυναίκες και τυπικά έχει εκδηλώσεις σε πολλά όργανα, συμπεριλαμβανομένων του δέρματος, των αρθρώσεων, των νεφρών και του νευρικού συστήματος. Είναι μία πολύπλοκη ασθένεια που προκύπτει από την αλληλεπίδραση κληρονομικών (γενετικών και επιγενετικών), ορμονικών και περιβαλλοντικών παραγόντων. Τα ανοσοσυμπλέγματα, τα αυτοαντισώματα και όλα τα κύτταρα του αίματος (αυτο-δραστικά λεμφοκύτταρα, δενδριτικά κύτταρα) εμπλέκονται σε κλινικές εκδηλώσεις. Τα Α*ιμοποιητικά Στελεχιαία και Προγονικά Κύτταρα* (ΑΣΠΚ) είναι πολυδύναμα κύτταρα που παράγουν όλα τα κύτταρα του αίματος, τόσο μυελικά όσο και λεμφικά. Θεωρήσαμε, λοιπόν, ότι οι ανωμαλίες των κυττάρων του ανοσοποιητικού συστήματος που παρατηρούνται στον ΣΕΛ θα μπορούσαν να εντοπιστούν στα ΑΣΠΚ. Σκοπός της μελέτης ήταν να διερευνηθεί περαιτέρω ο ρόλος και η λειτουργία των ΑΣΠΚ που προέρχονται από των μυελό των οστών στον ΣΕΛ.

Σε αυτήν την εργασία, χρησιμοποιήθηκαν δείγματα μυελού των οστών (ΜΟ) από θηλυκά NBZW/F1 ζωικά πρότυπα λύκου και αντίστοιχα υγιή πειραματόζωα. Τα ΑΣΠΚ ορίστηκαν ως *Lin^{-}Sca-1^{+}c-Kit^{+}* (LSK). Η μεταγραφική ανάλυση των ΑΣΠΚ από πειραματόζωα με ΣΕΛ έδειξε ισχυρή μυελική υπογραφή συνοδευόμενη από αυξημένη συχνότητα μυελικών προγονικών κυττάρων (CMPs) –αλλά όχι λεμφικών προγονικών κυττάρων (CLPs)- γεγονός που θυμίζει την υπογραφή «ανοσολογική εκπαίδευση». Το μεταγραφικό προφίλ των μυελικών προγονικών κυττάρων αποκάλυψε έντονο επαναπρογραμματισμό με καταστολή των ρυθμιστών της δημιουργίας κοκκιοκυττάρων, καθώς μείζονες ρυθμιστές όπως οι *Cebpe*, *Cebpd* και *Csf3r* βρέθηκαν να υποεκφράζονται στο ΣΕΛ. Τα δεδομένα είναι ενδεικτικά αναστολής της διαφοροποίησης και διαταραχής της μυελοποίησης. Παρά ταύτα, τα ουδετερόφιλα στον ΜΟ βρέθηκαν σε αυξημένη συχνότητα στα πειραματόζωα με ΣΕΛ το οποίο υποδηλώνει ότι υπάρχει εναλλακτική οδός για τη δημιουργία κοκκιοκυττάρων.

Στους ανθρώπους, τα ΑΣΠΚ ορίστηκαν και απομονώθηκαν από δείγματα ΜΟ με βάση τον δείκτη CD34. ΑΣΠΚ από ασθενείς με σοβαρή μορφή ΣΕΛ εμφάνισαν αυξημένο πολλαπλασιασμό, διαφοροποίηση και μεταγραφική ενεργοποίηση των κυτοκινών και χημειοκινών που οδηγούν στη διαφοροποίηση προς μυελική σειρά, αντικατοπτρίζοντας έτσι τα δεδομένων των μυών. Συγκριτική ανάλυση του ανθρώπινου μεταγραφικού προφίλ των ΑΣΠΚ με των πειραματοζώων, έδειξε ότι τα μυελικά προγονικά κύτταρα ποντικού και τα ανθρώπινα CD34^{+} κύτταρα μοιράζονται την ίδια μυελική υπογραφή που υποδηλώνει αναστολή της διαφοροποίησης.

Συνολικά, τα δεδομένα αποδεικνύουν την ενεργοποίηση των ΑΣΠΚ και την παθολογική διαφοροποίηση προς την μυελική σειρά στο ΣΕΛ, συμβάλλοντας ενδεχομένως στην διαιώνιση της φλεγμονής και σε ενδεχόμενη έξαρση της νόσου. Τα δεδομένα επιβεβαιώνουν το κρίσιμο ρόλο της ομοιόστασης στο ΜΟ για την παθογένεια του ΣΕΛ και αναδεικνύεται η ανάγκη για κατασκευή ενός συστήματος προσομοίωσης του ΜΟ *in vitro*. Το σύστημα αυτό θα παρέχει τη δυνατότητα για περαιτέρω μηχανιστική και λειτουργική διερεύνηση με τη χρήση λιγότερων δειγμάτων από ζωικά πρότυπα και ανθρώπους.

1. Introduction

Systemic Lupus Erythematosus

Systemic lupus erythematosus is a complex chronic inflammatory autoimmune disease characterized by various manifestations like arthritis, skin rashes, oral ulcers, serositis, glomerulonephritis, nervous system involvement and the production of antinuclear antibodies (ANA) i.e. anti-Sm, anti-dsDNA, anti-Ro, anti-La (Bertsias et al., 2010; Tsokos, 2011). It is easily confused with other disorders due to its diverse clinical manifestations (Figure 1.1). The disease severity can vary from mild to moderate and severe, and potentially could be a fatal disease. In Europe, annual incidence ranges between 1 and 4.9 per 100,000 and prevalence ranging from 28 to 97 per 100,000 population (Laustrup et al., 2009; Rees et al., 2016). SLE mainly affects women between 10 and 50 years of age with female/male ratio about 9/1 (Christou et al., 2018; Ortona et al., 2016).

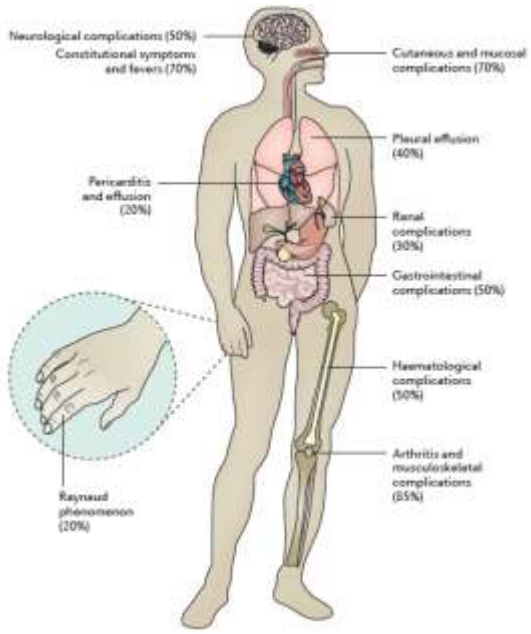

Figure 1.1. The multifaceted SLE. Clinical heterogeneity of SLE includes different organs that can be affected, and in parallel each organ can have different manifestations. Adopted by (Kaul et al., 2016).

Systemic autoimmune diseases result from interactions between genes and environmental triggers that build on overtime until clinical symptoms appear. A complex interplay between innate and adaptive immunity lies at the core of most of autoimmune diseases (Figure 1.2). This interplay is not static, since initial inflammatory cascades might change as organ damage accumulates. Furthermore, these diseases can be heterogeneous regarding the type of organs involved, clinical course and response to treatment (Banchereau et al., 2013).

The pathogenesis of SLE hinges on loss of tolerance and sustained autoantibody production (Tsokos et al., 2016). The two predominant cell types involved in the adaptive immune system -B and T cells- are both essential for the development of lupus. Many genetic loci have been implicated in the pathogenesis including genes encoding for complement, nuclear antigens, B and T cell dysregulation. B cells are pathogenic in SLE because of the

autoantibodies and cytokines that they produce. T cells drive the systemic and intra-renal activation of B cells (Mohan and Putterman, 2015).

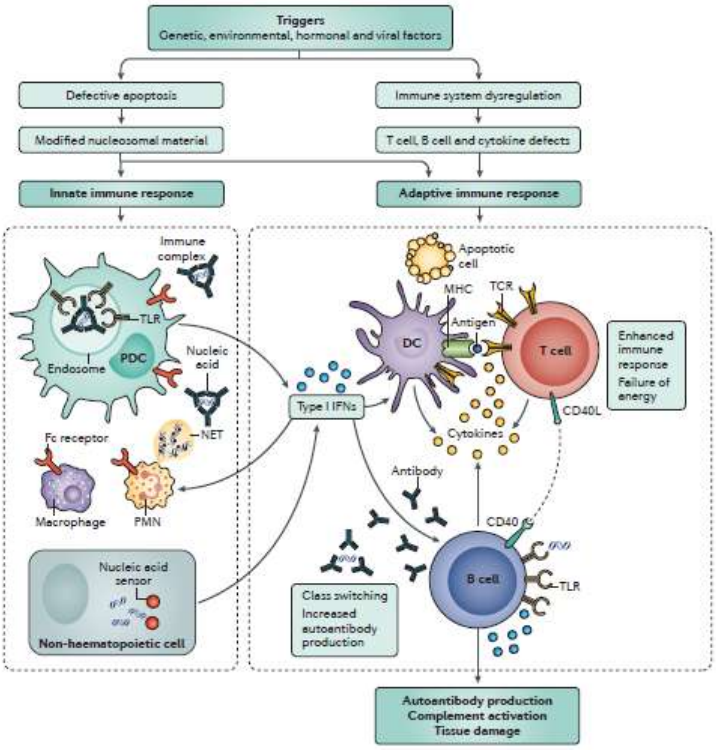

Figure 1.2. Overview of the pathogenesis of SLE. Immune system in SLE is affected by genetic, environmental, hormonal, epigenetic and immuneregulatory factors, sequentially or simultaneously. Their action results in the generation of autoantibodies, immune complexes, inflammatory cytokines, and defective apoptosis. All may initiate and amplify inflammation and damage to various target-organs which may be affected further by local factors. Adopted by (Kaul et al., 2016)

In the early stages of disease, dendritic and other myeloid cells, activate T cells, and produce key mediators such as B cell activating factor. This effect results in the activation of the adaptive immune system. Activation of the systemic immune system leads to the generation of effector T cells and autoantibodies that subsequently target organs such as kidneys resulting in lupus nephritis (LN). These processes together with numerous soluble mediators elicit chronic inflammation within glomerular and tubulointerstitial sites in the kidneys (Mohan and Putterman, 2015). A traditional view of LN pathogenetic mechanisms implicates activation of the complement system by deposition of immune complexes in the glomerulus, recruitment of myeloid cells (particularly neutrophils) and the release of enzymes from neutrophil granules and reactive oxygen intermediates from macrophages (Golbus and McCune, 1994).

Another key concept in SLE pathogenesis is the imbalance between apoptotic cell production and disposal of apoptotic material (Figure 1.2). Nuclear antigens are typically not accessible to the immune system, but during the course of apoptosis, cell membrane forms blebs that pinch off from the cell containing fragmented cellular material and nuclear antigens (Moser et al., 2009). Normally, the debris is cleared rapidly and would not be accessible to the immune system. However, impaired clearance of apoptotic cells and/or their products triggers and potentially activates the adaptive immune response (Kaul et al., 2016). Immune complexes are formed in large amounts as ANA formed and they are not cleared promptly. Levels of ANA in serum mostly tend to reflect disease activity (Rahman and Isenberg, 2008).

In SLE, hematological aberrations such as neutropenia, lymphopenia, thrombocytopenia, anemia and neutrophil dysfunction are common (Keeling and Isenberg, 1993). Leukopenia occurs in approximately 50% of SLE patients and may be attributed to neutropenia, lymphopenia or their combination. Cytopenias may be due to several etiologies; disease activity, bone marrow failure, drug toxicity, peripheral destruction, tumor infiltration and sepsis have all been implicated.

The mortality rate in SLE patients is about 2–3 times greater than that of the normal population(Fathollahi et al., 2018). The leading causes of death include cardiovascular disease, respiratory disease, malignancies, infection, active SLE disease, cerebrovascular disease and renal failure. (Mak et al., 2012; Thomas et al., 2014; Voss et al., 2013; Wang et al., 2015). The mortality rate has diminished dramatically because of the development of new therapeutics, however the continuously decreasing slope pattern has dramatically reduced indicating the lack of development of new therapeutics with higher efficiencies (Mak et al., 2012; Ruiz et al., 2014).

Current protocols for the treatment of SLE have been used for a long time and include non-steroid anti-inflammatory drugs, corticosteroids, mycophenolate mofetil, azathioprine, cyclophosphamide, cyclosporine. Recently, a class of biological drugs have been added, such as belimumab which affects B cell maturation by targeting B Lymphocyte Stimulator (BLyS) and prevents immunoglobulin production by these cells (Borba et al., 2014; Thanou and Merrill, 2014). There is a large need in both diagnosis and treatment of SLE. The heterogenous and flaring nature of the disease makes assessment of drug efficacy in clinical trials difficult.

The NZB/W F1 Experimental Model of SLE

The oldest classical model of SLE is the NZB/W F1 which is generated by crossing a female NZB mouse with a male NZW mouse (Dubois et al., 1966; Perry et al., 2011). The parental strains, NZB and NZW, develop mild autoimmunity such as hemolytic anemia in NZB (Crampton et al., 2014). This hybrid F1 model produces autoantibodies (ANA, anti-dsDNA), develops immune complex glomerulonephritis and mild vasculitis. Also, they develop splenomegaly, lymphadenopathy and hypergammaglobulinemia (Crampton et al., 2014). Female F1 mice do develop primarily the disease compared to male, similar to humans (Richard and Gilkeson, 2018), being a very useful model for studying SLE. Its main drawback is the long disease progression as it develops after 6 months of age in female mice with 50% mortality at 9 months. F1 model exhibits a weak interferon signature after 6 months of age. Disease could be exacerbated by IFN-I over-expression with accelerated (15 to 20 weeks earlier) anti-dsDNA autoantibody production, increased proteinuria, and worse glomerulonephritis (Mathian et al., 2005). Although this model develops partially the disease, it could provide insights of the pathogenesis while mice have the advantage of the importance of genetic susceptibility factors, as in human SLE.

The first experiments which suggest the role of hematopoietic progenitor cells in the etiology of autoimmune disease were conducted by Morton and Siegel (Morton and Siegel, 1974a, b). In lethally irradiated NZB and H-2 compatible wild-type mice, they reciprocally transferred fetal liver, spleen, or bone marrow cells. They observed that transfer of NZB derived cells only into normal recipients induced autoantibody production and glomerulonephritis (Theofilopoulos and Dixon, 1985a). Lupus HSCs or lymphoid precursors are inherently abnormal and express all that is necessary for the emergence of autoimmunity. In conclusion, hematopoietic stem cells (female/male) are capable of driving the disease to irradiated recipient mice. However, female hematopoietic stem cells led to earlier disease onset compared to male HSCs, after being transferred (David et al., 2014).

Hematopoiesis

Hematopoiesis is the lifelong process by which all the cells of the blood system are produced in a hierarchical manner from a restricted rare population of hematopoietic stem cells (HSCs) (Bhatia et al., 1998). Ernst Haeckel first used the word stem cell ('Stammzelle') in 1868. As a Darwinist, he used it to refer to the primordial unicellular organism from which all multicellular life descended. He put forward the concept of a common progenitor of red and white blood cells as well as a common precursor of myeloid and lymphoid leukaemic cells. The stem-cell concept has thus been framed into a tree-like model, in which multipotent stem cells give rise to their progeny through an ordered series of branching steps.

During development, the first definitive HSCs appear in the aorta–gonad–mesonephros (AGM) region of the embryonic mesoderm followed by the yolk sac, fetal liver and thymus (Tavian et al., 2010). Later in gestation, HSCs migrate to the developing bone marrow (BM) within the trabecular region of long bones, where they reside in the adult organism (Lim et al., 2013). Within the BM, HSCs represent a very small fraction (~0.1%) of the overall cell content (Figure 1.3).

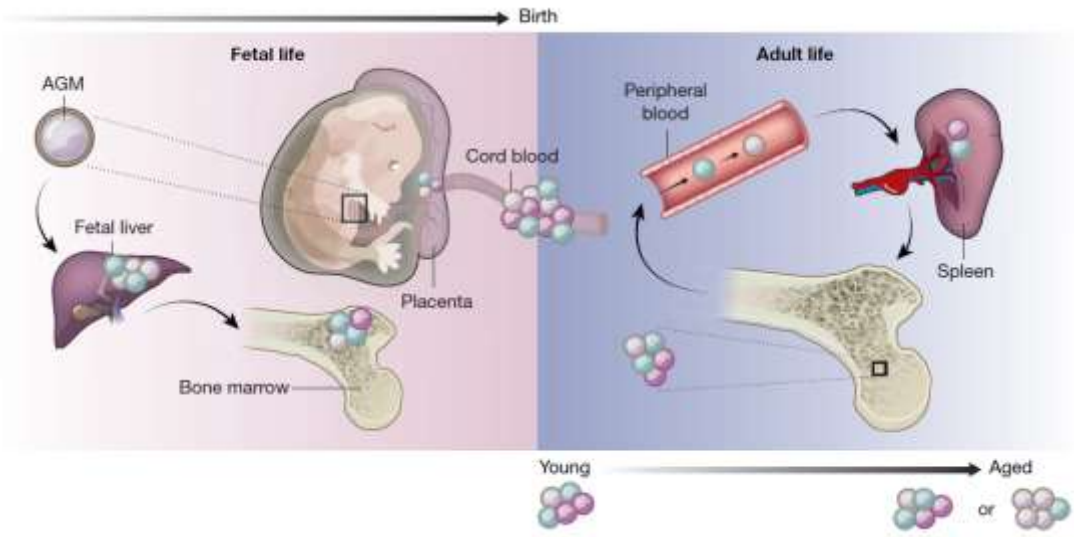

Figure 1.3. Hematopoiesis. HSPC compartment from fetal to adult life. HSPCs are found in various sites in the body across a lifetime. Adopted by (Laurenti and Gottgens, 2018).

Historically, HSPCs have been defined on the basis of two essential properties: self-renewal and multipotency. They give rise to progenitor cells that become gradually lineage restricted cells and ultimately differentiate into all mature lineages of blood (Figure 1.4). As HSPCs continuously replenish cells that are lost, they must self-renew to maintain their number over the lifetime of an organism (Pietras et al., 2011b). Homeostasis within the hematopoietic system depends on the replacement of the immune effector cells by hematopoietic precursors (King and Goodell, 2011b). By contrast, progenitors are defined by the absence of extended self-renewal and a restricted lineage differentiation capacity (most often bi- or unilineage). Followed by several further branching steps on either side of the tree progressing from multi- to bi- and finally to unipotent progenitor cells (Figure 1.4).

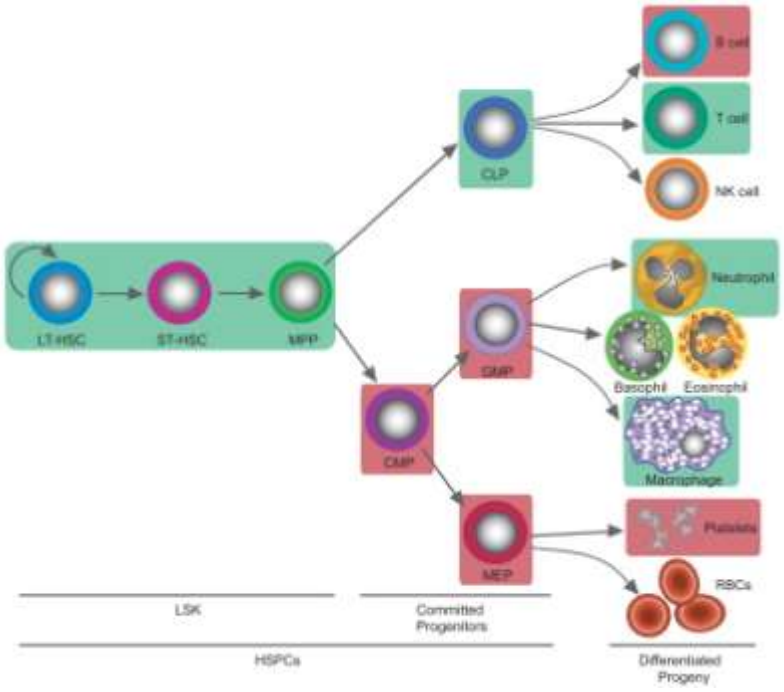

Figure 1.4. The Hematopoietic Tree. Hematopoiesis branching from HSPCs gives rise to all blood cell lineages. Adopted by (Baldridge et al., 2011).

Studies of the HSPC compartment in adult mice indicate that it is composed of two kinetically distinct subpopulations, with turnover rates of 5 weeks and 21 weeks (termed activated and dormant HSCs), respectively (Wilson et al., 2008)(Figure 1.3). Physiologically, under steady state, HSPCs proliferate at a very low rate and most HSPCs are kept in G_0 phase of the cell cycle, in quiescent state. Disruption of HSPC quiescence leads to premature exhaustion of the stem cell pool and causes hematological failure under stress conditions. Thus, HSPC self-renewal and quiescence have to be constantly balanced to maintain a stable HSPC pool that is capable of producing blood cells for the lifetime of the organism (Pietras et al., 2011a). The properties of HSPCs that are known to be targeted by this developmental ''switch'' include their cycling status, their speed of expansion after transplantation, and their granulopoietic activity (Bowie et al., 2006, 2007b).

The field widely accepts that, both in mouse and human, HSPCs that repopulate in transplantation assays for more than 16 weeks after a primary transplantation and at least in a second round of transplantation are considered to be long-term HSCs (LT-HSCs) (Kiel et al., 2005; Oguro et al., 2013). If these cells can produce all differentiated cell types and engraft transiently in primary (and in some cases secondary) transplants, they are referred as intermediate HSCs, short-term HSCs (ST-HSCs) or multipotent progenitors (MPPs) (Benveniste et al., 2010), depending on the length and robustness of the graft produced (Geiger et al., 2013). The evidence so far suggests that lineage choice occurs earlier than previously thought. Probably as early as within the phenotypic HSC populations, as shown for dendritic cells by Lee et al. (Lee et al., 2017a, b). Different HSC subtypes have been defined using a variety of criteria to distinguish their mature blood cell outputs. The Muller-Sieburg group has identified different HSPC -subsets as ''myeloid-biased'' (My-bi), ''lymphoid-biased'' (Ly-bi), or ''balanced'' (Bala), based on a measurement of the predominant lineages within the total output of donor-derived blood cells (Muller-Sieburg et al., 2012). Moreover, Haas et al. showed that under inflammatory conditions, a special stem cell-like megakaryocyte-committed population is expanded, while sharing many

features with HSPCs (Haas et al., 2015). Additionally, microfluidics-based analyses of the transcriptomes of individual cells in a highly purified HSC population have suggested that further subsets may exist based on their nonrandom pattern of expression of certain genes (Glotzbach et al., 2011)

LSK compartment

Fluorescence-activated cell sorting subsequently facilitated the purification of HSCs, with a landmark study from Spangrude et al. that demonstrated the use of positive and negative selection (Spangrude et al., 1988). In the adult BM there is low frequency of HSCs, with two to five HSCs per 10^5 total BM cells (Geiger et al., 2013). In mice, the population of enriched HSCs exhibits a phenotype of $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ markers and represent approximately 0.05% of the adult BM cells (Seita and Weissman, 2010). Within the LSK compartment, they represent a very rare subset (~0.005%) of BM nucleated cells, the $CD150^{+}CD48^{-}$LSK (Kiel et al., 2005).

Moreover, there are three subpopulations within the LSK compartment that demonstrate differential expression of CD34 antigen. CD34 is a glycoprotein on the surface of hematopoietic stem/progenitor cells (HSPCs). $CD34^{+}$ cells have the capability to produce all types of blood cell types, derived either from BM or from peripheral blood (Civin et al., 1996; Krause et al., 1996). $CD34^{low/-}$ population support LT lymphomyeloid reconstitution and $CD34^{+}$ cells support only ST reconstitution (Osawa et al., 1996). c-Kit (tyrosine kinase receptor) and Flt3 are expressed and function in early hematopoiesis as well as their respective ligands SCF and FL, both in humans and mice (Adolfsson et al., 2001).

In an adult human, there are about 3,000–10,000 HSPCs, which probably divide only once every 3 months to once every 3 years (Catlin et al., 2011). Humans produce an estimated 1.4×10^{14} mature blood cells per year (Dancey et al., 1976). The BM cellularity decreases with age, although the number of immunophenotypic human HSPCs has been shown to actually increase with age (Pang et al., 2011). The HSPC function clearly declines with age; therefore larger population of HSPCs may be required for maintaining normal hematopoiesis in healthy elderly individuals. On the cellular level, old human HSPCs compared to young human HSPCs are less quiescent, with a larger fraction undergoing cell division, while they have accumulated more oxidative DNA damage. Both of these factors can limit the ability of old human HSPCs individually to effectively self-renew and reconstitute the hematopoietic system. Gene expression profiling of human HSPCs has shown that myeloid genes are mostly up-regulated and lymphoid genes are mostly down-regulated with age (Bhatia et al., 1997).

In some strains of mice, the HSPCs actually expand with advancing age and this age-dependent expansion of HSPCs is a transplantable, cell-autonomous property of HSPCs (Morrison et al., 1996; Sudo et al., 2000). Accumulation of DNA damage within HSPCs has been proposed as a mechanism that contributes to age-associated decrease of their functionality. A study found that mice defective in mediators of DNA-damage regulators do not manifest changes in the size or composition of their HSPC pool under homeostatic conditions. However, when transplanted, their HSPCs did show increased apoptosis, reduced reconstituting activity, and decreased self-renewal (Rossi et al., 2007). These findings evidence that normal HSPCs do accumulate DNA damage with age. Accumulation of

oxidative DNA damage appears to have a similar influence on human HSPCs (Yahata et al., 2011).

Recent published reports described a novel property of HSCs to control their own fate, by converting danger signals into versatile cytokine signals for regulation of stress hematopoiesis. In this regard, many studies have provided direct and indirect evidence of cytokine receptor expression in murine LSK cells, including IL-6Rα, IFN-γR, TNFR1 and TNFR2 (Figure 1.5)(Baldridge et al., 2010; Baldridge et al., 2011)

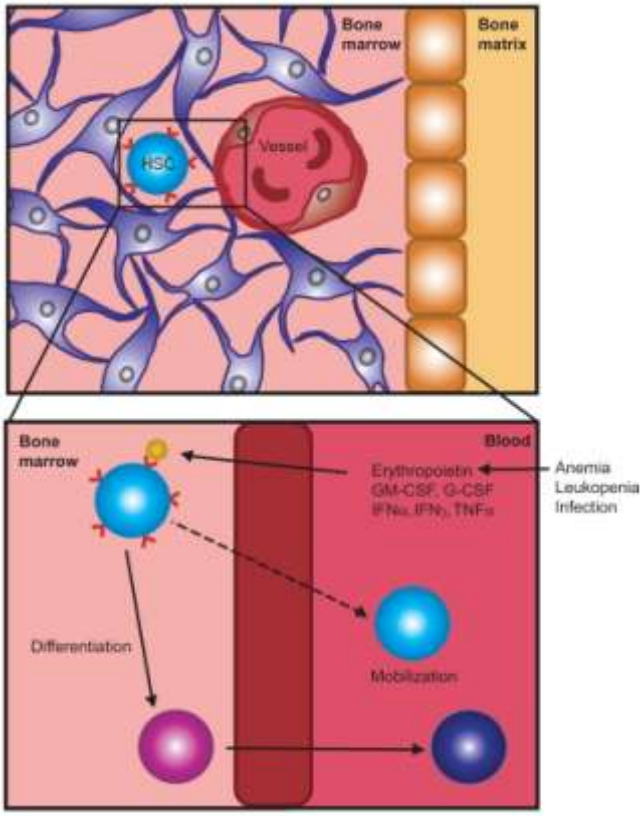

Figure 1.5. HSCs life within the BM niche. HSCs reside in quiescence in the BM niche amidst stromal cells (purple). Circulating factors i.e. cytokines reach the BM via small sinusoidal vessels. (upper panel). When systemic stress, stimulate production of cytokines, these factors reach and stimulate HSCs to proliferate, mobilize into circulation and/or differentiate into committed progenitors and immune effector cells (lower panel). Adopted by (Baldridge et al., 2011).

Hematopoiesis and Inflammation

HSPCs are first responders to both acute and chronic infection. Infection leads to changes in the HSC niche that contribute to alterations in HSPC biology. Pro-inflammatory cytokines released during infection are crucially important to HSPC regulation. These cytokines appear to be required for the maintenance of the appropriate number of HSPCs and for the proliferation and differentiation of HSPCs, both under homeostatic conditions and in response to stress (King and Goodell, 2011b). Most of the cells in the BM of adult mice are quiescent (i.e. in a dormant or G_0 state). Thus, in the absence of any unusual perturbation, they are resistant to drugs or other treatments that specifically target dividing cells.

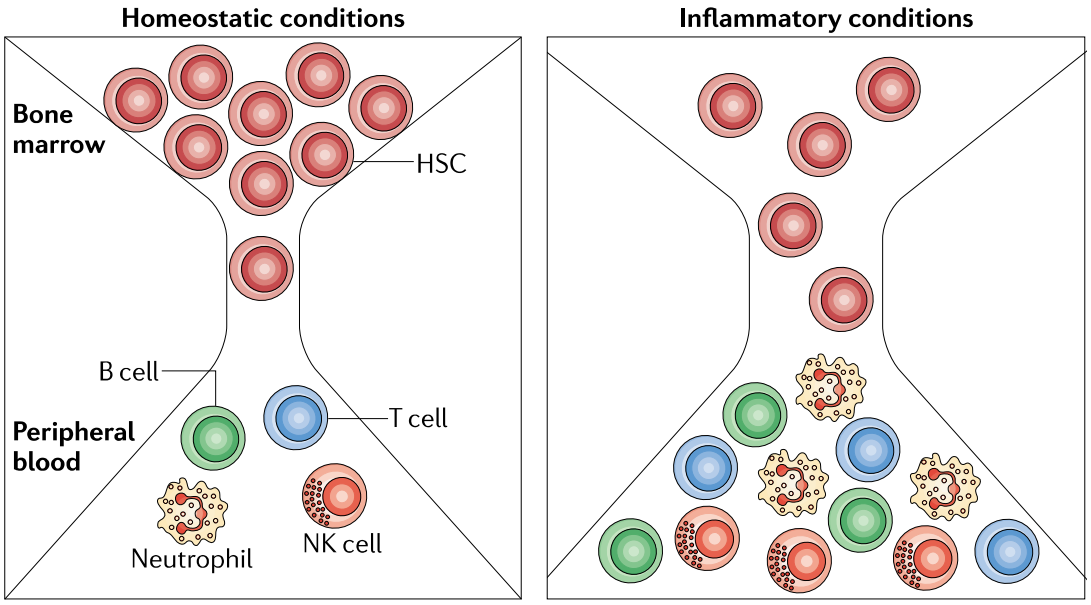

Figure 1.6. HSPC response under steady state and inflammatory conditions. HSCs are constantly called upon to manufacture different types of blood cells in order to maintain the status quo for normal homeostasis or under conditions of stress such as infection or injury when specific subsets of leukocytes must be rapidly expanded. Adopted by (King and Goodell, 2011b).

Inflammatory conditions increase proliferation and self-renewal among LSK compartment. HSPCs divide as part of the primary immune response rather than simply to replace depleted progeny pool (Figure 1.6). They can also be "pulled" towards cell division following the depletion of committed progenitor populations from the BM. HSPCs adapt to inflammation by a combination of cell-intrinsic mechanisms (transcriptional, epigenetic and metabolic) and cell-extrinsic mechanisms (soluble growth factors, cytokines, microbial ligands and adhesive interactions) (Mitroulis et al., 2018a). HSPC responses can be beneficial in promoting the elimination of an infection, however if HSPC activation is chronically sustained, this could cause impairment of their function and exhaustion, or lead to their contribution to the chronicity of inflammatory pathologies (Nahrendorf, 2018).

HSPCs can bypass several steps of the traditional tree-like hematopoietic hierarchy via direct differentiation into myeloid progenitor cells through asymmetric division giving rise to progeny cells with distinct fates; such as a stem cell and a lineage-committed cell. Upon stress (like myeloablation or inflammation), HSPCs rapidly respond to replenish myeloid

cells through a process called 'emergency myelopoiesis' at the expense of lymphopoiesis (Schultze et al., 2019). Especially, the rapid adaptation of HSPCs that leads to peripheral blood neutrophilia is called 'emergency granulopoiesis' (Manz and Boettcher, 2014). During emergency granulopoiesis, G-CSF induces proliferative and lineage-specification signals by acting mainly on CMPs and GMPs (Figure 1.7), which expand and form well-defined clusters that differentiate into granulocytes (Herault et al., 2017; Hirai et al., 2006).

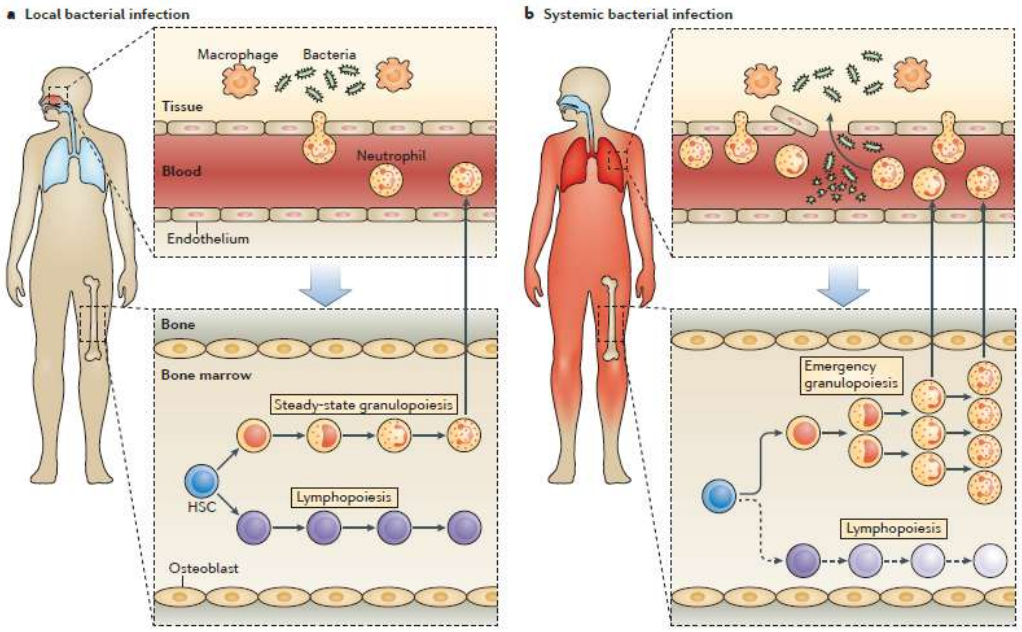

Figure 1.7. Steady-state and emergency granulopoiesis under bacterial infection. (a)During a localized bacterial infection, tissue-resident immune cells such as macrophages and recruitment of neutrophils fight infection which leads to its rapid resolution, thus preventing bacterial dissemination. (b) Under systemic bacterial infection, bacterial dissemination occurs and neutrophils are consumed in large quantities, with pre-existing neutrophil pools being used. To counterbalance neutrophil depletion and provide a supply of urgently needed neutrophils to combat systemic bacterial spread, 'emergency granulopoiesis' is initiated, which is characterized by the large-scale de novo generation of neutrophils from myeloid progenitors in the bone marrow. Adopted by(Manz and Boettcher, 2014).

According to Manz et al. 'emergency granulopoiesis' is used to describe a microbial infection-driven process and the term 'reactive granulopoiesis' is used to describe enhanced granulopoiesis in the absence of microbial infection. The last can be initiated by non-infectious stimuli such as chemical agents (for example, 5-FU), physical insults (for example, trauma or ionizing radiation) or autoimmune disorders.

Another emerging term in the context of inflammation is 'trained immunity' which is a form of adaptation that enhances the response of innate immune cells to secondary challenges independently of adaptive immunity (Netea et al., 2016). Trained innate immunity operates in HSPCs as well. Trained immunity -induced by the injection of β-glucan into mice- leads to the expansion of myeloid-biased HSPCs conferring a favorable outcome onto secondary systemic challenge with LPS and protection against chemotherapy-induced myelosuppression. Mitroulis et al. resolved a historic paradox about the long-term effect of

trained immunity on myeloid cells with a relatively short lifespan (Mitroulis et al., 2018b). Thus, infectious or inflammatory challenges can epigenetically imprint an 'inflammatory memory' onto HSPCs which drives myelopoiesis. Subsequently, increased numbers of inflammatory myeloid cells enhance inflammation, which in turn perpetuates HSPC-mediated myelopoiesis (Figure 1.8). HSPCs play central role in the host response in chronic diseases and the adaptation to inflammation generates a destructive feed-forward loop as Chavakis et al. proposed (Chavakis et al., 2019).

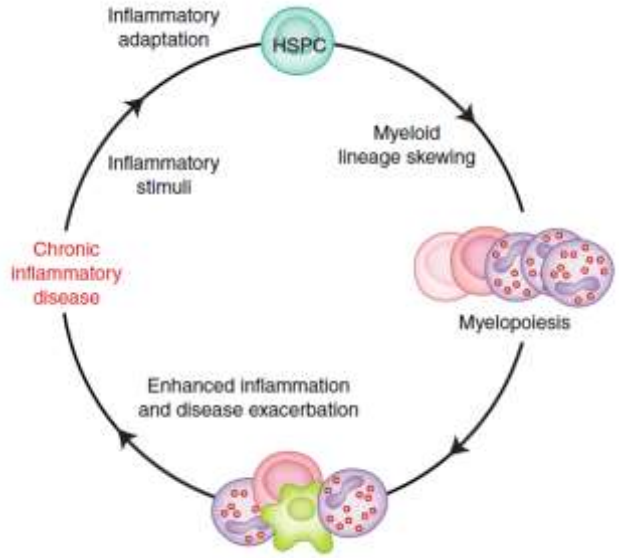

Figure 1.8. From adaptation to inflammation and to chronic inflammatory disease. HSPC response under inflammatory stimuli, promotes myelopoiesis which in turn leads to enhanced inflammation. Adopted by (Chavakis et al., 2019).

Bone Marrow Niche

The term 'niche' for the specific HSC bone-marrow microenvironment was first coined by Schofield, who proposed that HSCs are in intimate contact with bone, and that cell–cell contact was responsible for the apparently unlimited proliferative capacity and inhibition of maturation of HSCs (Schofield, 1978). Maintenance of HSCs and regulation of their self-renewal and differentiation *in vivo* is thought to depend on their specific microenvironment, which has been historically called the hematopoietic inductive microenvironment or 'stem-cell niche' (Wilson and Trumpp, 2006). Stroma cells –mesenchymal stem cells, vascular endothelial cells and osteoblasts– play crucial role in organizing the BM niche (Smith and Calvi, 2013).

The physical location of HSCs close to the bone surface was first shown in 1975 (Lord et al., 1975). Morphological evidence for the presence of HSC niches in close association with the endosteum was provided more recently when HSC or hematopoietic progenitor activity and/or phenotype were shown to localize close to the endosteal lining of bone-marrow cavities in trabecular regions of long bones, whereas more differentiated hematopoietic progenitors were found mainly in the central bone-marrow region (Kiel et al., 2005; Lord et al., 1975; Nilsson et al., 2001)

A stem-cell niche can be defined as a spatial structure in which stem cells are housed and maintained by allowing self-renewal in the absence of differentiation. An individual stem cell can give rise to two non-identical daughter cells, one maintaining stem-cell identity and the other becoming a differentiated cell. There are two mechanisms by which this asymmetry can be achieved, depending on whether it occurs pre- (divisional asymmetry), or post- (environmental asymmetry) cell division (Figure 1.9). It is possible that HSCs could undergo both divisional and environmental asymmetric divisions; therefore both mechanisms could be used in parallel by independent HSCs to direct non-stem-cell daughters to distinct cell fates.

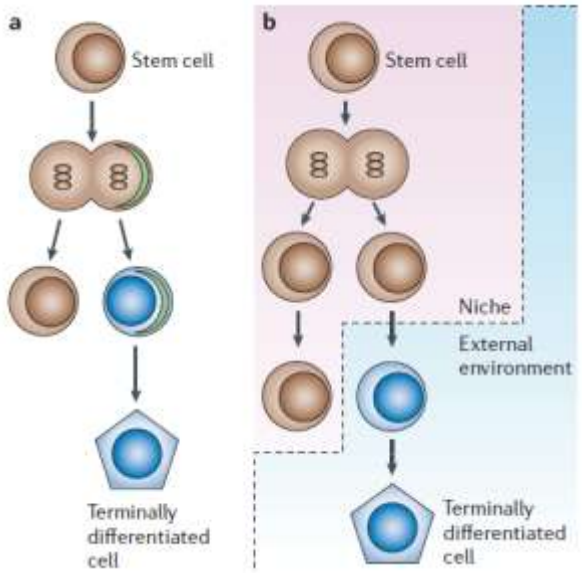

Figure 1.9. Asymmetric cell division model. (a) During divisional asymmetry, cell-fate determinants are asymmetrically localized to only one of the two daughter cells (one stem-cell, one differentiated cell). (b) During environmental asymmetry, after division, one of the two identical daughter cells remains in the self-renewing niche while the other relocates to the differentiation - promoting niche. Adopted by (Wilson and Trumpp, 2006).

BMECs are known to support the survival, proliferation and differentiation of myeloid and megakaryocyte progenitors (Avecilla et al., 2004; Kopp et al., 2005; Rafii et al., 1997) whereas primary bone-marrow stromal cells release factors that inhibit megakaryocyte maturation (Delehanty et al., 2003). It is probable that the pool of HSCs located in the vascular and self-renewing endosteal niches are freely exchanged to maintain homeostasis in a constantly changing hematopoietic environment. In addition, HSCs that are located in the self-renewing endosteal niche produce multipotential progenitors (MPPs) by divisional and/or environmental asymmetry. These cells give rise to myeloid-cell lineages as well as lymphocyte precursors. B-cell progenitors are uniformly distributed throughout the bone marrow attached to CXCL12-expressing fibroblasts (in the B-cell niche) (Hirose et al., 2002; Tokoyoda et al., 2004).

The HSC niche is a highly complex ecosystem that sustains HSC function, promoting survival and long-term maintenance of the HSC pool (Baryawno et al., 2017). The vast majority of HSC are located in the BM, only a small percentage of HSCs, approximately 1%, are released in the periphery (blood and spleen) with circadian-clock controlled patterns (Inra et al., 2015; Mendez-Ferrer et al., 2008). The first step in the trafficking of HSCs from BM is the release of these cells from their anchoring within the stem cell niche. This anchoring is mediated predominantly by two pathways: VCAM1 and CXCR4/CXCL12 (Calvi and Link, 2015). CXCL12/CXCR4 signaling plays a critical role in maintaining a pool of HSCs in a cell-autonomous manner, supporting the survival and/or self-renewing divisions of HSCs (Nie et al., 2008; Sugiyama et al., 2006). The translocation of HSCs is accompanied with an increase in their proliferation, suggesting that signals emanating from the BM niche where HSCs reside determine the balance between quiescence and self-renewal.

LT-HSCs mainly serve as a reserve pool that can be reactivated in response to stress or injury and might even be stored in a separate 'quiescent-storage' niche (Ohlstein et al., 2004) (Figure 1.10). In cases of mobilization such as myeloablative agents, HSCs are released into the circulation, enter the cell cycle to re-establish hematopoiesis and migrate to putative HSC niches in the spleen and liver. After repair, they return to their BM niches and become quiescent again (Randall and Weissman, 1997; Venezia et al., 2004). The release of HSCs not only occurs during mobilization but is also observed during homeostasis, when a small number of HSCs are constantly released into the circulation (Wright et al., 2001). Homing can be defined as recruitment of circulating HSCs to the BM microvasculature and subsequent transendothelial migration into the extravascular hematopoietic cords of the BM.

In summary, a complex combination of migration, adhesion, proteolysis and signaling occurs at the interface between HSCs and the endosteal BM niche (Figure 1.10). In addition, signals originating from the periphery can influence HSC homing, retention and mobilization, therefore determining whether a niche is silent or whether HSCs exit the niche in response to stress.

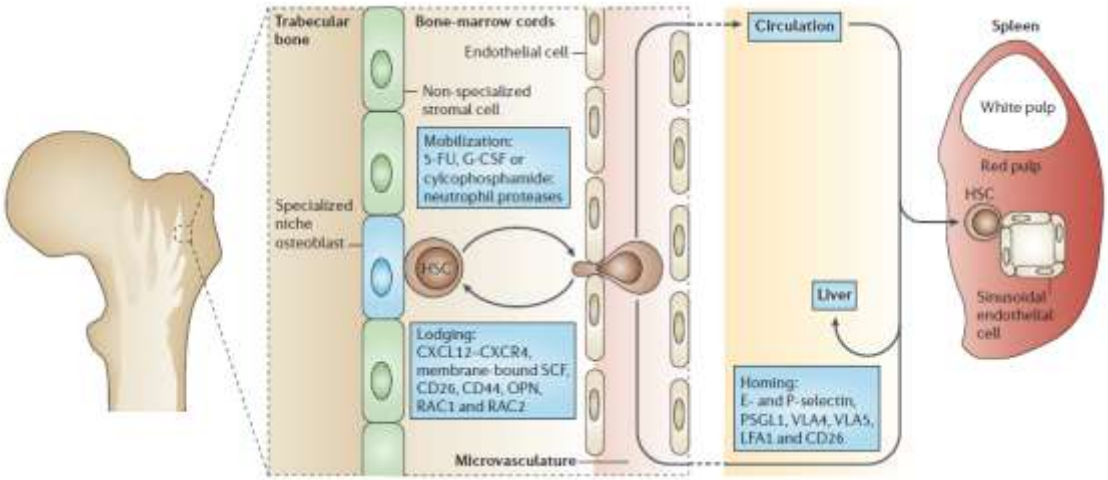

Figure 1.10. Schematic diagram showing mobilization, homing and lodging in BM. Adopted by (Wilson and Trumpp, 2006).

Mesenchymal Stem Cells

One major component of BM niche is comprised of the mesenchymal stem cells (MSCs). They are non-hematopoietic cells derived from the mesoderm with the potential to differentiate into bone, fat, and cartilage *in vitro* (Kfoury and Scadden, 2015). MSCs have been shown to differentiate into pericytes, myofibroblasts, BM stromal cells, osteocytes, osteoblasts and endothelial cells (Figure 1.11), all of which constitute the functional components of the HSC niche that support hematopoiesis (Muguruma et al., 2006).

Friedenstein and colleagues were the first who identified multipotential stromal precursor cells. They demonstrated that the rodent BM contains cells that have the ability to form fibroblastoid colonies (CFU-F) when cultured on plastic, make bone, and reconstitute the hematopoietic microenvironment when transplanted subcutaneously (Friedenstein et al., 1974; Friedenstein et al., 1982; Friedenstein et al., 1968). Cells having CFU-F and multi-lineage *in vitro* differentiation capacity were demonstrated present in many tissues other than the BM such as adipose tissue, umbilical cord, placenta, amniotic fluid, dental pulp, skeletal muscle, tendons, and synovial membrane. Later, due to their multipotentiality, Caplan introduced the 'mesenchymal stem cell' term (Caplan, 1991), and because they were first found in the BM, they were termed "marrow stromal cells".

After several studies, the International Society for Cellular Therapy (ISCT) had to issue a nomenclature clarification restricting the use of the term Mesenchymal Stem Cells for cells that meet the stem cell criteria and recommending the term Multipotent Mesenchymal Stromal Cells for the fibroblast-like plastic adherent cells regardless of the tissue of origin. This clarification kept the widely recognized acronym (MSC) in use (Horwitz et al., 2005). A year later, ISCT issued the minimal criteria for defining MSCs, requiring adherence to plastic under standard culture conditions; the expression of CD105, CD73, and CD90; the lack of expression of CD45, CD34, CD14 or CD11b, and CD79a or CD19; and HLA-DR surface molecules in addition to the ability to differentiate into the osteogenic, chondrogenic, and adipogenic lineages *in vitro* (Dominici et al., 2006).

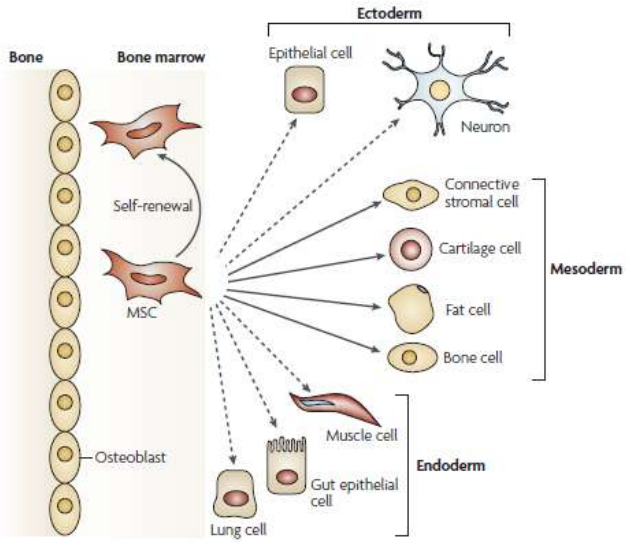

Figure 1.11. **Properties of MSCs.** MSCs can self-renew (curved arrow) and differentiate (straight, solid arrows) towards the mesodermal lineage. It is reported their ability to transdifferentiate into cells of other lineages (ectoderm, endoderm) which is controversial *in vivo* (dashed arrows). Adopted by (Uccelli et al., 2008).

MSCs have the ability to interact with immune cells both in innate and adaptive immune systems (Figure 1.12). In addition, MSC-mediated immunosuppression is dependent on the combined reaction of chemokines, inflammatory cytokines, and effector factors secreted by MSCs, as well as the microenvironment and strength of the inflammatory stimulus. Studies have shown that stromal cells, at all stages of maturation, have anti-proliferative features that are in common with physiological stromal-cell niches, including the HSC niche. A number of groups in the last few years have extensively reported that human MSCs inhibit the effector functions of both T and B cells, the generation of dendritic cells, and the proliferation of natural killer (NK) cells in response to interleukin (IL)-2 (Bartholomew et al., 2002; Di Nicola et al., 2002; Jiang et al., 2005; Li et al., 2008). Collectively, these data characterize MSCs as active suppressors of inflammation that effectively modulate the immune response at many levels.

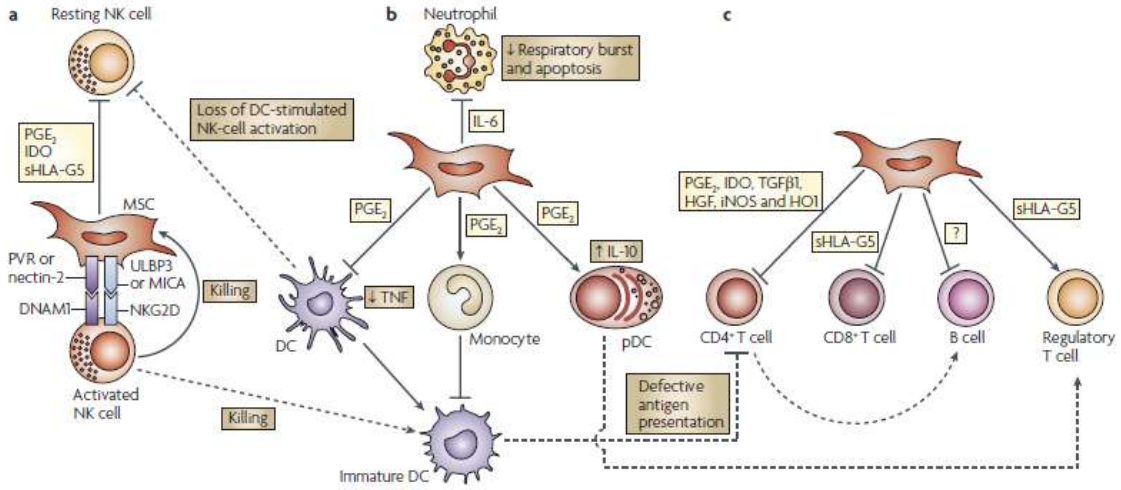

Figure 1.12. Possible interactions between MSCs and cells of the innate and adaptive immune system. Adopted by (Uccelli et al., 2008)

BM-derived MSCs serve as an important component of the niche supporting HSCs, while among them there are distinct subgroups which have different functions in regulating HSCs (Kunisaki et al., 2013a). Most of them are located in the perivascular space and are associated with arteriole or sinusoidal blood vessels, producing key HSC niche factors such as Cxcl12 and SCF (Kitl) (Morrison and Scadden, 2014). They are identified by expression of leptin receptor (Lepr) (Ding and Morrison, 2013; Ding et al., 2012), Nestin (Nes)(Mendez-Ferrer et al., 2010), or NG2 (Cspg4)(Kunisaki et al., 2013b). $NG2^+$ and $Nestin^+$ perivascular MSCs have been proposed as critical regulators of HSC function through production of Cxcl12 and Kitl. Under homeostasis, HSCs reside in perisinusoidal or periarteriolar locations predominantly found in close proximity to $Lepr^-$ cells, also shown to produce Cxcl12 and Kitl. A small subgroup of MSCs ($NG2^+$ $LepR^-$ cells), which belong to small arterioles close to the endosteal region, was shown to contribute to arteriolar niches maintaining HSC quiescence. The other part of MSCs ($NG2^-$ $LepR^+$ cells), which are adjacent to sinusoids, is thought to form perisinusoidal niches proliferating HSCs in the BM. The distribution of HSCs between these two kinds of niches changes depending on the activation of their cell cycle. The prevailing model indicates a structurally unique niche in the BM made of MSC-HSC pairings, tightly regulated by local input from the surrounding microenvironment and by long-distance cues from hormones and cytokines.

Organ-on-a-Chip

Microfluidics have emerged in the early 1979 as the science and technology of manipulating minute amounts of fluids constrained to structures with characteristic dimensions at the sub-millimeter scale. It entails the physics, handling and precise control of fluids ranging from 10^{-6} to 10^{-15} liters volume that are geometrically constrained to channels of dimensions at the order of mere to hundreds of micrometers (Whitesides, 2006). Having adopted the already established expertise from the field of microelectronics, it was not long until the microfluidic technology was implemented in biochemical analysis, with the advent of Lab-on-a-chip (LoC) systems, the devices that functionally integrate in an automated way one or more laboratory processes on a single chip format (Geschke et al., 2008).

During the last decades, the LoC devices have become renowned in life sciences, leveraging the miniaturization-driven advantages. In addition to their evident assets of low consumption of biological samples, cost-effectiveness and versatility (Geschke et al., 2008), they manage to offer better mimicry of the cellular microenvironment (Sackmann et al., 2014). In particular, the high surface area-to-volume ratio (S/V) facilitates the dominance of capillary forces and surface tension over gravity, giving rise to scaling effects for a more realistic mirroring of the extracellular matrix. Additionally, laminar flow regime is established, allowing for better control of the spatiotemporal variations of the cellular microenvironment (Sackmann et al., 2014). Added to these, their ergonomy, diagnostic speed and portability have all in all made them indispensable tools in both biological and medical research. .

Within the last decade, microfluidics technology paved the way to a novel, promising era with the upraise of organ-on-a-chip devices (OoC), the epitome of biomimetic systems at the interface of micro-engineering and cellular biology (Huh et al., 2011). OoC devices aim to reproduce the function of biological organs or tissues as realistic models using microscale technologies. Cells are grown inside the chambers and channels to generate tissues or complete organs to emulate their biology, and integrative physiology (Wikswo, 2014). In general, OoC allows the performance of *in vitro* experiments with better controlled parameters -such as pressure, flow rate, pH, osmotic pressure, nutrient content and toxins presence- replicating the physiological environment at the cellular level (Yum et al., 2014).

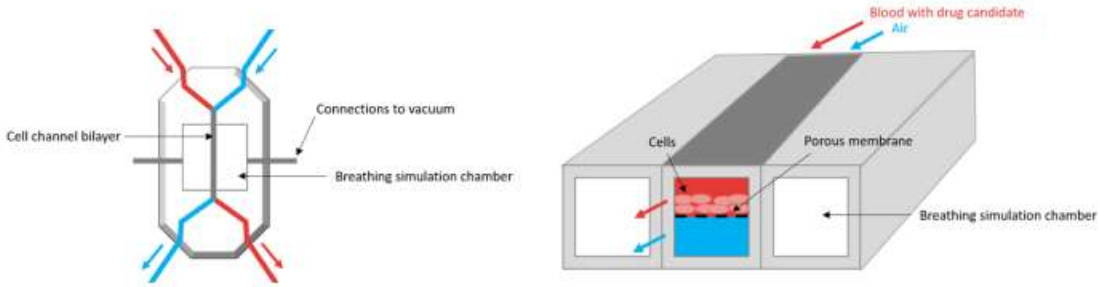

Figure 1.13. Example of OoC. Adopted by http://sitn.hms.harvard.edu/flash/2018/organs-chips-promising-future-therapeutic-drugs/

OoC devices satisfy the emerging need for new approaches to model complex human-relevant conditions (Henderson et al., 2013; Seok et al., 2013), given the limitations of the currently used methods in biomedical research, the static *in vitro* cell cultures and animal testing. In specific, conventional static cell cultures fail to reconstitute the dynamics of the cellular microenvironment (Tan et al., 2003). On the other hand, these microengineered co-culture models offer *in vivo* recapitulation of the complex interactions between different cell types that are mediated by the provision of various soluble and insoluble factors of the extracellular matrix, such as cytokines, nutrients, growth factors and hormones, as well as the formation of intercellular junctions, all of them dexterously controlled within the OoC (El-Ali et al., 2006; van der Meer and van den Berg, 2012). As for laboratory animals, although they are capable of simulating the physiological complexity at a whole-organism level, they are seen through increased scrutiny and skepticism regarding their scientific validity and translatability to humans (Mak et al., 2014). Systematic studies on the predictive value of animal models have demonstrated a poor correlation between animal data and human outcomes owing to substantial interspecies differences in key disease pathways and disease-induced changes in gene expression profiles.

OoCs are highly beneficial in terms of biomedical detection, study and characterization, since they offer direct real-time visualization and quantitative high-resolution analysis of diverse biological processes by employing their microfluidic properties and optical transparency. Owing to their small size, the absence of turbulence and mixing between neighboring streams, generates abrupt step gradients on the micrometer scale that delivers gradients of chemicals across the diameter of a single cell (Takayama et al., 2001). In this way, chemical gradients are sustained with complex structures over many hours to days to study cell motility in response to chemotactic stimuli (Selimovic et al., 2011). In addition, the compartmentalized channel design allows independent fluidic access to different tissue types within a single device as well as parametric control of environmental factors. Conditioned media from different chamber can be sampled and analyzed for their metabolites and other secretory products, which could aid in the identification and development of novel biomarkers for efficacy, toxicity or disease processes (Bhatia and Ingber, 2014).

The next generation of OoC is the 'human-on-a-chip' system (Figure 1.14), the development of a complex organ model with multiple cell types to mimic the *in vivo* organ system which will predict body-level drug responses that reflect both complex organ-level effects and organ–organ interactions (Sung et al., 2013). The most common approach to create this type of system is to link multiple microengineered organ models via a fluidic network that allows for their functional integration and interaction in a physiologically relevant manner. In these systems, the incorporation of BM model provides an immune-response element to the integrated human-on-a-chip microsystem.

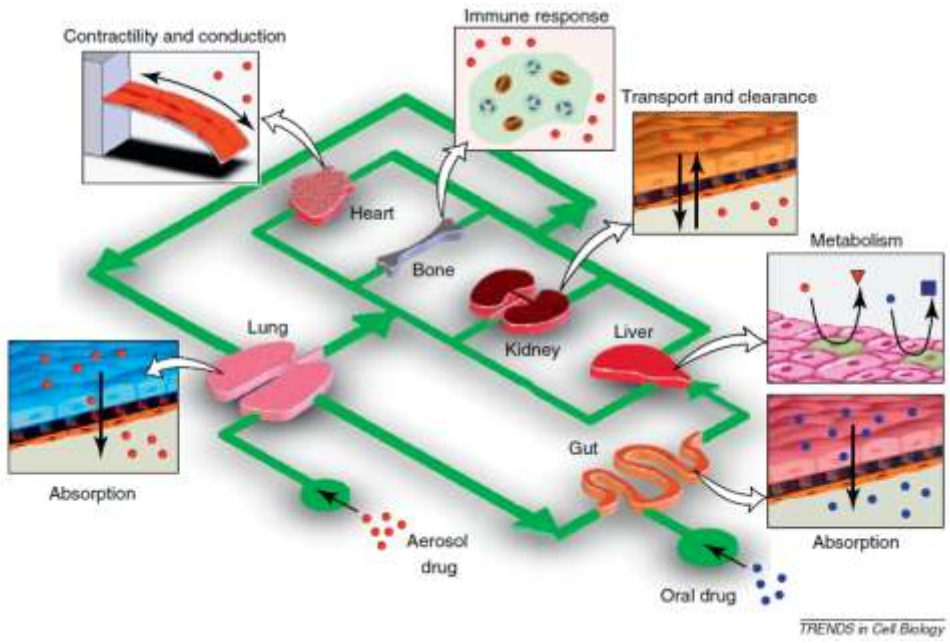

Figure 1.14. Human-on-a-chip model. Different organs are integrated into a single microdevice and internally linked via a microfluidic system mimicking dynamic process of drug absorption, distribution, metabolism and excretion. It can be used to study the absorption of inhaled aerosol drugs (red) from the lung to microcirculation, to measure cardiotoxicity, transport and clearance in the kidney, metabolism in the liver, and immune-cell contribution to these responses (BM). Drug substances (blue) can also be introduced into the gut compartment to investigate the interplay between orally administered drugs, molecular transporters and metabolizing enzymes expressed in the integrated organs. Adopted by (Huh et al., 2011).

Bone marrow-on-a-chip

One system of major biomedical interest for realistic simulation is BM, with the bone marrow-on-a-chip (BMoC) serving as a platform to study the tissue's physiological response as the hub of the mammalian immune system in an appropriately controlled environment. One of its primary applications is to record the effect of drugs on organ function or the toxicity of various chemicals on tissues. In recent years, several attempts have been made to engineer *in vitro* BM, inspired by the natural structure, though many issues need to be addressed in order to realistically recapitulate the tissue physiology and reproduce the viable mechanism of hematopoiesis.

The onset for the construction of a hematopoietic BM system was given by Torisawa et al., who first engineered a hybrid *in vivo/in vitro* BMoC system. More specifically, they initially implanted a scaffold-supported microfluidic cavity into a mouse and allowed it to be populated by murine cells. The formatted engineered BM (eBM) was afterwards removed and placed in a similarly shaped microfluidic chamber that was proved to support the maintenance of primitive hematopoietic cells (Torisawa et al., 2014). More recently, Siebert et al. described an *in vitro* 3D microfluidic system that modeled the BM, but the biology of the system was significantly simplified by the inclusion of a single niche cell type (MSCs) to test the ability of the device to support primitive HSPCs (Sieber et al., 2018). Later, another study was published by Aleman et al., who developed a human BM niche-on-a-chip (NoC)

platform with 4 major distinct bioengineered niche constructs connected within a single, closed, recirculating microfluidic device system. This platform allows the study of the impact of each individual niche on the homing and lodging/retention of circulating HSPCs, as well as the effects the distinct niches exert upon each another (Aleman et al., 2019). These studies highlight the great number of factors that influence the development of artificial BM.

In this context, our goal is to construct BMoC that pioneers the purely *in vitro* and scaffold-free development of hematopoietic BM. This system aims to study SLE given its hematologic manifestations, recapitulating the structural and physiological properties of the hematopoietic niche. Finally, as a clinical application, the SLE-BMoC can be used as a platform to prove the contribution of HSCs to SLE and them as a therapeutic target.

Multi-omics

Transcriptomics

RNA sequencing (RNA-seq) refers to techniques used to determine the sequence of RNA molecules. It includes high-throughput sequencing of cDNA molecules obtained by reverse transcription from RNA within a biological sample in an effort to determine the primary sequence and relative abundance of each RNA molecule (Figure 1.15). RNA-seq measures global transcriptional profiles, quantitative measurement of mRNA and non-coding RNAs and can also detect splicing variants (Banchereau et al., 2013). In addition, paired-end RNA-seq can be particularly advantageous for fusion identification because of the increased physical coverage it offers (Ozsolak and Milos, 2011).

The transcriptome is the complete set of transcripts in a cell and their quantity is specific to developmental or other condition. Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues as well as for understanding the development and disease. In addition, other issues that RNA-seq is addressing include the determination of the transcriptional structure of genes in terms of their start sites, 5'-3' ends, splicing patterns and other post-transcriptional modifications and to quantify the changing expression levels of each transcript during development and under different conditions (Wang et al., 2009).

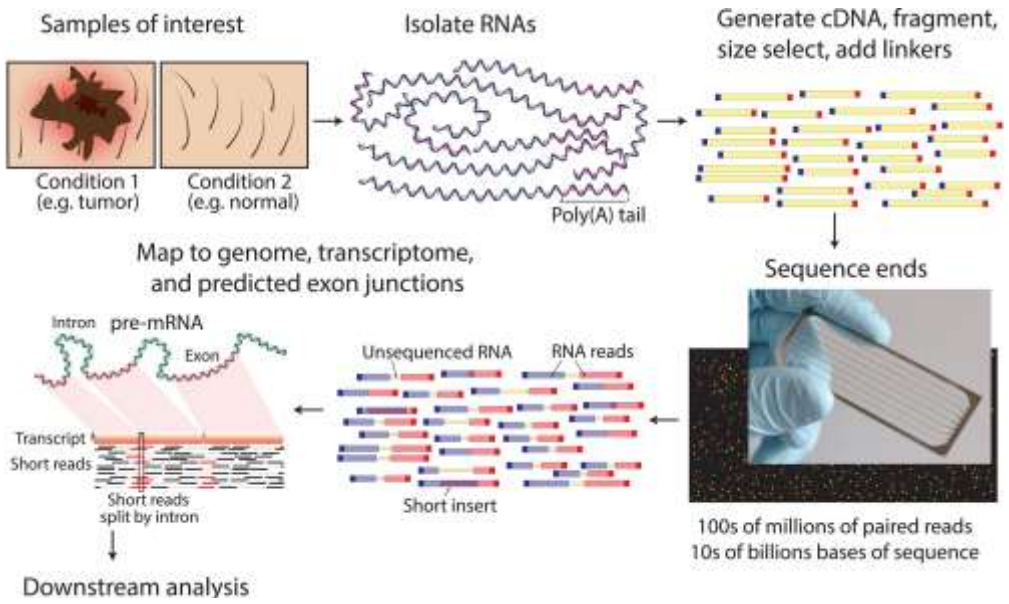

Figure 1.15. Example of sample preparation and RNA-seq workflow.

RNA-seq is the first sequencing based method that allows the entire transcriptome to be surveyed in a very high-throughput and quantitative manner. This method offers both single-base resolution for annotation and digital gene expression levels at the genome scale. High-throughput sequencing of RNA allows counting and exquisitely resolving tens of thousands of RNA transcripts and isoforms within a single assay (high quality reads), with potentially far greater impact on clinical diagnostic tests. Moreover, it enables direct measurement of a single transcript altered in one of a multitude ways through alternative

promoter usage, rare splicing events and gene fusion, epitranscriptome modifications, allele-specific expression or nonsense-mediated decay. This multivariate information opens up clinical applications that are beyond the scope and capability of microarrays (Van Keuren-Jensen et al., 2014). Genome-wide transcriptome analysis in cancer and other multifactorial diseases provides a global view of the expressed elements and networks that reshapes the biology of normal cells in their transformation and progression to aggressive diseased cells. Through RNA-seq, it is possible to define global transcriptome signatures that are not restricted to gene expression patterns, and lead therefore to a molecular characterization of the disease at higher resolution.

Epigenomics

Gene expression is regulated by DNA accessibility by transcription factors which is regulated by epigenetic changes (DNA methylation, histone modifications, chromatin remodeling, non-coding RNAs). Epigenetics refer to the study of durable changes in gene expression that are not accompanied by alterations to the nucleotide sequence. Epigenetic processes include DNA methylation, post-translational histone modifications, histone variants and noncoding RNAs that regulate gene expression. DNA methylation regulates gene expression by serving as a platform for repressive protein binding. DNA methylation was the first epigenetic change identified in patients with SLE. Procainamide and other drugs known to induce lupus-like features are inhibitors of DNA methylation(Chen et al., 2017). It is important to understand how epigenetic changes can contribute to rheumatic diseases because epigenetic patterns are plastic during inflammatory processes.

DNA methylation is a key epigenetic modification that provides heritable information not encoded in the nucleotide sequence. 5-Methylcytosine is the only known covalent modification of DNA in vertebrates (Jeltsch, 2002). Mammalian DNA methylation serves a wide range of functions including regulation of gene expression, genomic imprinting and X-chromosome inactivation. It contributes to genomic stability and acts as a defense mechanism against transposable elements (Jaenisch and Bird, 2003; Robertson and Wolffe, 2000). DNA methylation in CpG islands (CGIs), their borders and other genomic *cis*-regulatory elements (CREs) mediates chromatin inaccessibility, regulates transcription, maintains genomic stability and plays a vital role in cell lineage commitment (Zhang and Cao, 2019).

High resolution DNA methylation map is based on various method, one of them called Reduced Representation Bisulfite Sequencing (RRBS), which selects specific size of restriction fragments to generate a 'reduced representation' of the genome of a strain, tissue or cell type (figure 1.16). The sequence-specific detection of 5mC is the chemical derivative of DNA using sodium bisulfite, a method termed bisulfite sequencing , which was first reported in a seminal study by Frommer et al. in 1992 (Frommer et al., 1992). Genomic DNA is digested to completion using a restriction enzyme. After size-selection, an adapter is added. The DNA is denatured and unmethylated cytosines are bisulfite-converted to uracil. Sodium bisulfite efficiently deaminates unmethylated cytosine to uracil without affecting 5-methyl cytosine. The two resulting C-poor strands are no longer complementary to each other. Primers specific for the converted adapter sequence are used to fill-in the second (G-poor) strand and for PCR amplification. PCR products are cloned and sequenced. Sequences generated from RRBS libraries are projected onto the genome and analyzed in silico (Meissner et al., 2005).

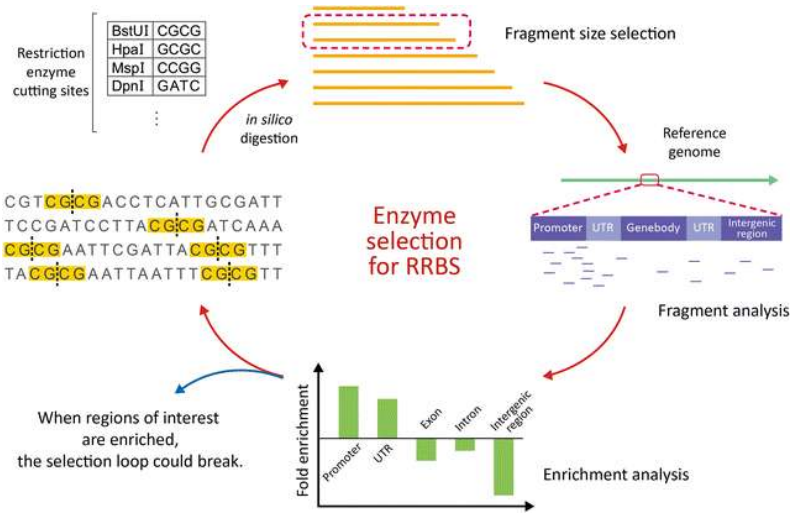

Figure 1.16. Schematic flow-chart of RRBS procedure. Adopted by (Hsu et al., 2017)

Transcriptomic studies have revealed disease-related pathways and novel pathogenic cell types in rheumatic diseases. On the other hand, epigenomic studies have revealed memory-related phenomena that might help to explain the chronicity of disease. These approaches have been extensively applied to the study of fundamental biological processes, including immune and autoimmune responses (Banchereau et al., 2017; Ermann et al., 2015). These technologies have led to the development and rapid application of methods to assess gene expression, chromatin accessibility and structure and DNA methylation at the genome-wide level (Figure 1.17) (Donlin et al., 2019). NGS techniques have the potential to provide insight into the interaction of environmental and genetic factors in the pathogenesis of rheumatic diseases. Effective coupling of NGS information with clinical data could elucidate the fundamentals of rheumatic disease pathology and substantially contribute to the application of precision medicine in rheumatology.

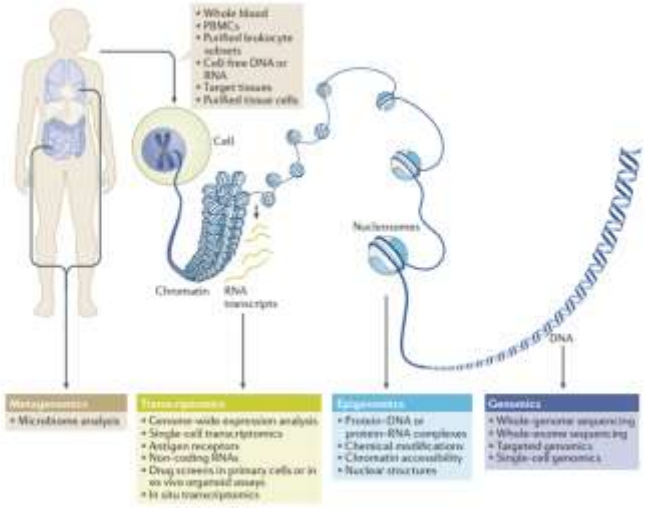

Figure 1.17. Overview of NGS applications. Adopted by (Donlin et al., 2019)

2. Purpose of the Study

Autoimmune diseases come from interactions between environmental, epigenetic and genetic factors that result in downstream perturbations of complex and interactive biological networks. It is important to note that existing murine lupus models have not yet led to the development of specific treatments for human lupus while many drugs used for treatment can cause serious adverse effects (Pascual et al., 2010). New therapies should be tailored to individuals in patient subgroups with distinct clinical and serological features. HSPCs are used for treatment, either as autologous or allogeneic transplantation. The results are not yet encouraging; however autologous HSPC transplantation is a major chance for refractory SLE patients (Marmont du Haut Champ, 2012)

The implication of BM cells in the pathogenesis of SLE has already been highlighted. Nakou et al. showed the role of apoptosis and granulopoiesis between active and inactive SLE patients using microarrays (Nakou et al., 2008). A major question is whether BM cells adapt to systemic inflammation and specifically how HSCPs change their output due to chronic inflammation.

The *main goal of this study* is to focus on the most primitive population of the immune system, the *HSPCs*, and to elucidate their *role in the pathogenesis of the disease*. Using a murine lupus model, the molecular profile and landscape of HSPCs was characterized during the progression of the disease, while the transcriptional stamp of HSPCs between SLE patients and healthy controls was identified. In parallel, noting how important the BM function is, we are trying to develop an *in vitro* BM simulating system in order to fully understand the interactions in the lupus niche which will be helpful for testing potential drug targets in the future.

3. Materials and Methods

Mice

C57BL/6 mice were purchased from the Jackson Laboratory. NZB/OlaHsd and NZW/OlaHsd mice were purchased from Envigo. C57BL/6 and NZB/W F1 female mice were used (12 and 28–36 weeks old). NZB/W F1 mice were considered as diseased (Lupus), when exhibiting ≥100ng/dl of urine protein level (Vlachou et al., 2016). All animals were maintained in the animal facility of the Biomedical Research Foundation of the Academy of Athens (4948/19-07-2016).

Flow cytometry and cell sorting

Single-cell suspensions were prepared from murine bone marrow, peripheral blood mononuclear cells (PBMC), or spleen and were stained with conjugated antibodies against mouse (catalog/clone): Ter119 (116206/TER-119), CD16/32 (101306/93, 101317/93), Gr1 (108406/RB6-8C5), B220 (103206/RA3-6B2), CD3e (100330,145-2C11), CD34 (119321/MEC14.7, 128608/HM34), Il7Rα (121114/SB/199), CD135 (135313/A2F10), CD150 (115909/TC15-12F12.2), CD48 (103426/HM48-1), Sca-1 (122512/E13-161.7, 108127/D7), c-Kit (105808/2B8), Ly6G (127608/1A8), Ly6C (128032/HK1.4), CD11c (117318/N418), CD11b (101212/M1/70), Ki-67 (652422/16A8, 652425/16A8), Annexin V/Annexin V Binding Buffer (640917/422201), 7-AAD Viability Staining Solution (420404) (Biolegend). For cell cycle intracellular staining, cells were fixed and stained using the Foxp3 Fixation & Permeabilization Kit (Molecular Probes) according to the manufacturer's instructions. $CD11b^-$ $Gr\text{-}1^-$ $Ter119^-$ $B220^-$ $CD16/32^-$ $Sca\text{-}1^+$ $c\text{-}Kit^+$ (LSK) and $CD11b^-$ $Gr\text{-}1^-$ $Ter119^-$ $B220^-$ $CD16/32^-$ $CD34^+$ $Sca\text{-}1^+$ $c\text{-}Kit^+$ (CMP) cells were isolated from bone marrow suspension (tibia, femur and brachial were used) and sorted on a FACS ARIA III (Becton Dickinson Biosciences). Cell purity was above 95%. Data were analyzed with FlowJo software.

Human subjects

Bone marrow aspirates and peripheral blood samples, collected in EDTA-coated tubes, were obtained from patients with SLE and healthy individuals after informed consent. All patients met the 1999 American College of Rheumatology revised criteria for the classification of SLE(Hochberg, 1997). Exclusion criteria included: a) intake of morning glucocorticoid and/or immunosuppressive treatment; b) recent (within the last month) treatment with pulse intravenous methyl-prednisolone or cyclophosphamide; c) pregnancy; d) active infection or malignancy; e) concomitant auto-inflammatory or rheumatic disease. Active SLE was defined based on physician's assessment and/or SLE Disease Activity Index, whereas severity of SLE was based on the presence of British Isles Lupus Assessment Group (BILAG) combined with physician's assessment at any time during the course of the disease (group A manifestations defined as severe disease, group B as moderate disease and group C-E as mild disease)(Gladman et al., 2002; Isenberg et al., 2005; Petri et al., 2012) . Clinical and laboratory characteristics of the patients included in the study are summarized in Table 1 (see Appendix-Table 1).

Table 1. Clinical and demographic characteristics of SLE patients	
Sex, female/male	8/2
Age, mean ± SD	47.3 ± 16.02
SLEDAI (mean ± SD)	
High SLEDAI≥12	14 ± 1.41
Low SLEDAI<12	4.5 ± 2.34
Severity pattern	
Moderate SLE	4/10
Severe SLE	6/10
Activity pattern	
Active SLE	8/10
Inactive SLE	2/10
History of Immunosuppressive Therapy	6/10
Nephritis	4 /10
NPSLE	1/10
Serositis	3/10
Arthritis	8/10
** Active SLE was defined based on physician's assessment and/or SLE Disease Activity Index. Controls included 3 healthy bone marrow donors (1 man and 2 women), 24–50 years of age.* SLEDAI, SLE disease activity index; NPSLE, neuropsychiatric SLE	

Mononuclear cell isolation and processing

Human bone marrow mononuclear cells (BMMCs) and PBMCs were isolated using Histopaque 1077 (Sigma-Aldrich) (400 g, 30 minutes, room temperature). Mononuclear cells were washed, erythrocytes were lysed with RBC buffer (420301, Biolegend), and stained with conjugated antibodies against human: CD34 (343606/561), CD38 (356605/HB-7), CD45RA (HI100/304106), CD90 (328123/5E10), CD49f (313624/GoH3), CD10 (312217/HI10a), CD123 (306017/6H6), CD127 (351316/A019D5), CD4 (317428/OKT4), CD8 (344714/SK1), CD66b (305118/G10F5), CD14 (HCD14/325604), CD16 (3G8/302056), CD19 (HIB19/302241), CD25 (BC96/302604), HLA-DR (L243/307618). For neutrophils characterization, peripheral blood after erythrolysis was used. $CD34^+$ cells were isolated from BMMCs using magnetic beads (StemCell Technologies, 18056).

Hematopoietic stem cell culture

Isolated LSK cells were cultured into 96-well bottom-flat dishes IMDM supplemented with 2% FBS, 10.000U/ml penicillin (Gibco, 15140), 10.000ug/ml streptomycin (Gibco, 15140), 50ng/ml rm-IL6 (Immunotools, 12340063), 20ng/ml rm-IL3 (Immunotools, 12340033), 20ng/ml rm-SCF (Immunotools, 12343323), 20ng/ml rm-TPO (Immunotools, 12343613), 50mM β-mercaptoethanol (Gibco, 1480119), 2mM L-glutamine (Gibco, 25030-081). After 24hrs, cells are collected, splited and stimulated by serum from F1-P, F1-L or B6 mice. After 24hrs, BrdU assay, Cell Trace staining or Colony-forming cell assay is followed.

Isolated $CD34^+$ cells were cultured into 96-well bottom-flat dishes with StemSpan H3000 (Stem Cell Technologies, 09850), 10.000U/ml penicillin (Gibco, 15140), 10.000ug/ml streptomycin (Gibco, 15140), supplemented with cytokines (1/400) SCF (Immunotools, 11343323). TPO (Immunotools, 11344863), Flt3 (Immunotools, 11343303)
Cells are maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Colony Forming Assay

Cells were seeded in complete methylcellulose-based medium for the growth of hematopoietic progenitor cells in colony-forming unit assays. MethoCult™ (StemCell Technologies, M3434) was used for murine HSCs (LSK) and MethoCult™ (StemCell Technologies, H4434) for human HSCs. The seeding concentration was 1,500 cells/plate and 500 cells/plate in duplicates in 35-mm tissue culture dishes (Sigma, CLS430165) respectively. The enumeration and identification of colonies (CFU-M, CFU-G, CFU-GM, CFU-GEMM, BFU-E) was scored after 10-12 days in culture (at 37°C in 5% CO_2) under an inverted light microscope according to the manufacturer's instructions.

Mesenchymal stem cell isolation, characterization and culture

Human Bone Marrow-derived MSCs. Day 0: Seed the flow-through from the positive selection $CD34^+$ isolation column using α-MEM (containing Pen/Strep) with 20% FBS (heat inactivated) and 1% L-glutamine. *Day 1- Passage 0*: After 24hrs, discard all the non-adherent cells, wash 1-2 times with PBS and add fresh medium. Repeat, if needed, during the culture. When the cells reach confluency 80-90% (4-6 days), harvest the cells using 1x Trypsin/EDTA (5-7min, at 37°C). Centrifuge at 1200rpm, 7min, 25°C. Re-plating the cells, usually 1/100 dilution. [*Passage 1*] or freeze the cells (usually a T75 flask in one vial) in Freezing Medium (90% FBS-10%DMSO) and keep it at -80°C.
Mouse Bone Marrow-derived MSCs. Passage 0: Collect BM and resuspend cell pellet with CEM (IMDM with pen/strep, 1% L-glutamine and20% non-inactivated FBS). Plate 3-3.5 mice per T175 flask. Culture at 37°C, 5% CO_2. After 24 hours, remove media from the tissue culture and wash with sterile PBS without Mg and Ca ions. Replace with fresh MSC media. Replace media every 3-4 days. Cells should grow in colonies and the colonies should become confluent after 2-4 weeks of culture. [*Passage 1*] When cells become 70-80% confluent, harvest the cells by adding 1x Trypsin/EDTA (2 min, at 37°C). Centrifuge cells at 300 rcf for 5 min at room temperature. Replate 7.500, 10.000 or 15.000 cells/cm^2 in T175 flask with fresh medium(Soleimani and Nadri, 2009).
MSCs were stained with conjugated antibodies for human: CD105 (323205/43A3), CD73 (344005/AD2), CD90 (328123/5E10), CD45 (368508/2D1), CD34 (343606/561), CD11b (301330/ICRF44), CD19 (21270193S/ LT19), HLA-DR (21388993/ LT-DR)(Dominici et al., 2006) and mouse: CD105 (120407/ MJ7/18), CD73 (127224/ TY/11.8), CD0140a (135908/ APA5), Sca-1 (108127/D7), CD45 (103132/ 30-F11)(Houlihan et al., 2012).

Immunofluorescence

Cells were seeded in coverslips pretreated with poly-L-lysine (Sigma-Aldrich) for 15 min at 37°C and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature. Cells were permeabilized by using 0.5% Triton-X 100 (Sigma-Aldrich), 2% BSA, stained with mouse anti-phospho-H2A.X (γ-H2AX) antibody (1:200; 05-636; Millipore), and incubated with Alexa Fluor 555 conjugated anti-mouse IgG (1:500; A28180; Invitrogen).

DAPI staining (Sigma-Aldrich) was used for visualization for the nuclei. Samples were coverslipped with mowiol and visualized using a ×63 oil lens in a Leica SP5 inverted confocal live cell imaging system. The numbers of γ-H2AX puncta/cell were calculated using a macro developed in Fiji software as previously described (Alissafi et al., 2017).

RNA extraction, cDNA Synthesis & quantitative PCR analysis

RNA was extracted using the NucleoSpin RNA XS kit according to the manufacturer's instructions (Macherey-Nagel). First-strand cDNA synthesis was performed using PrimeScript Reverse Transcriptase (TaKaRa). qPCR was carried out using the KAPA SYBR FAST (Sigma-Aldrich). Relative expression of murine and human target genes was calculated by comparing them to the expression of the housekeeping gene *Hprt* and *GAPDH* respectively. The primers are listed below in Table 2:

Table 2. List of primers.

Organism	Gene	Forward Primer	Reverse Primer
Mouse	IFNα	TCTGATGCAGCAGGTGGG	AGGGCTCTCCAGACTTCTGCTCTG
Mouse	Hprt	GTGAAACTGGAAAAGCCAAA	GGACGCAGCAACTGACAT
Mouse	Isg15	AGCAATGGCCTGGGACCTAAA	AGCCGGCACACCAATCTT
Mouse	Cebpα	CAAGAACAGCAACGAGTACCG	GTCACTGGTCAACTCCAGCAC
Human	MX1	GTTTCCGAAGTGGACATCGCA	CTGCACAGGTTGTTCTCAGC
Human	IFNA	GGTGACAGAGACTCCCCTGA	CAGGCACAAGGGCTGTATTTCTT
Human	GAPDH	GCACCACCAACTGCTTAG	GCCATCCACAGTCTTCTG

RNA sequencing

LSK and CMPs were isolated from the bone marrow of NZB/W F1 and C57BL/6 mice using FACS ARIA III. $CD34^+$ human cells were isolated from BMMCs. RNA was extracted as mentioned above and libraries were generated using the Illumina TruSeq Sample Preparation kit v2. Single-end 75-bp mRNA sequencing was performed on Illumina NextSeq 500. Quality of sequencing was assessed using FastQC software (Andrews). Raw reads in fastq format were collected and aligned to the mouse genome (mm10 version) and human genome (hg38 version) using STAR 2.6 algorithm (Dobin et al., 2013). Gene quantification was performed using HTSeq(Anders et al., 2015) and differential expression analysis was performed using edgeR package (glmFit model) (Robinson et al., 2010) in R (Team, 2018). Genes with a False Discovery Rate (FDR) ≤0.05 and fold change (FC) ≥1.5 or ≤-1.5 were considered statistically significantly up- and downregulated, respectively. Heatmaps with hierarchical tree clustering and boxplots were created in R with an in-house developed script which is based on the ggplot package. Row tree cutting at height of 1.8 was used to obtain discrete clusters of genes with similar pattern of expression across samples. A set of specific gene signatures, which were manually curated from the literature, were retrieved from the RNA sequencing dataset. A signature was considered significant if >5% of genes had $p<0.05$. Venn diagrams were created using the Venny 2.1.0 online tool (Oliveros, 2015). Human-mouse overlap was tested using an online tool based on a normal approximation to the exact hypergeometric probability (Lund). For the human-mouse transcriptomic comparison, human genes were converted to mouse orthologous and mouse genes were converted to human orthologous. Only common genes and pathways were compared based

on their ID. Biological Processes, Molecular Functions and Cellular Components Gene Ontology terms and KEGG pathways were used.

Enrichment analysis

The significantly differentially expressed genes (DEGs) were used for *pathway and gene ontology (GO) analysis* using g:Profiler web-server (Reimand et al., 2007) and ClueGO plug-in in Cytoscape 3.7.0 (Bindea et al., 2009; Shannon et al., 2003). Immunological gene signatures were retrieved from GO-ImmuneSystemProcess-EBI-UniProt-GOA (ClueGO, updated on November 14, 2018). Statistically significant enriched pathways were considered those with Benjamini-Hochberg corrected p-value≤0.05 (two-sided hypergeometric test). *Regulator and transcription factor enrichment* was performed using Regulatory Network Enrichment Analysis (Chouvardas et al., 2016). Statistically significant factors were considered those with FC≥1 and p≤ 0.05. *Gene Set Enrichment Analysis (GSEA)* (Subramanian et al., 2005) was also performed in order to reveal enriched signatures in our gene sets based on the Molecular Signatures Database (MSigDB) v6.1, and in specific analyses based on publicly available data (see Results section). Gene sets were ranked by taking the –log10 transform of the p-value multiplied by the FC. Significantly upregulated genes were at the top of the ranked list and significantly downregulated genes were at the bottom of the ranked list. GSEA pre-ranked analysis was then performed using the default settings. Enrichment was considered significant by the GSEA software for FDR (q-value) <25%.

Genomic DNA extraction

Tissue/Cell digestion: To the frozen cell pellet add 200µl of each of solution A (25mM EDTA, 75mM NaCl) and B (10mM EDTA, 10mM Tris-HCl pH8.0, 1% SDS). Optional add 1µl of RNase (20mg/ml), vortex, briefly spin down and incubate at room temperature for 5-10min. Add 10µl of Proteinase K (20mg/ml), vortex and spin down briefly. Incubate in a shaking thermomixer @55°C (65°C for fixed material) overnight. Spin down briefly.
Phenol/Chloroform #1: Add 400µl Phenol/Choloroforn/Isoamylalcohol (24:1) mix (Invitrogen). Rotate overhead for at least 15min, then centrifuge 5min at room temperature, full speed. Transfer the upper, clear phase into a new tube.
Phenol/Chloroform #2: Add 400µl Phenol/Choloroforn/Isoamylalcohol (24:1) mix (Invitrogen). Rotate overhead for at least 15min, and then centrifuge 5min at room temperature, full speed. Transfer the upper, clear phase into a new tube.
Chloroform: Add 800µl Choloroforn/Isoamylalcohol (24:1). Rotate overhead for at least 15min, and then centrifuge 5min at room temperature, full speed. Transfer the upper, clear phase into a new tube.
Ethanol Precipitation: Add the followings: 1.5µl of Glycogen (20mg/ml), 0.1x Volume 3M NaAcetate (20µl), 2.5x Volume of ice-cold 100% ethanol (500µl). Invert the tube and incubate overnight at -20°C.
If fishing of DNA is possible: Use a 10µl pipette tip to fish the DNA. Quickly transfer it into a tube with ice-cold 70% ethanol. Pool all DNA into one tube and let it air dry until ethanol is gone. Add 50-200µl to the DNA and let it solve overnight at 4°C.
If fishing of DNA is not possible precipitate DNA by centrifugation: Centrifuge DNA at 13,000 rpm, at 4°C for 45min and remove supernatant. Wash the pellet with 1ml of ice-cold 70% ethanol. Centrifuge at 13,000 rpm, at 4°C for 40min and remove supernatant. Wash the pellet with 1ml of ice-cold 70% ethanol. Centrifuge at 13,000 rpm, at 4°C for 40min, remove

supernatant. Let the pellet air dry until ethanol is gone. Add 30-50µl to the DNA and let it solve overnight at 4°C or 1h at 50°C.

Reduced Representation Bisulfite Sequencing (RRBS)

Libraries for RRBS were prepared according to a protocol as previously described (Luetke-Eversloh et al., 2014) with minor modifications. Briefly, fresh frozen cell pellets (30,000-130, 000 cells) were lysed by overnight incubation at 55°C with 40 µl lysis buffer (10mM TrisHCl, 5mM EDTA) and 5 µl proteinase K (1 mg/ml; Merck Millipore, Burlington, USA). Proteinase K was then inactivated by incubation with 2 µl 4mM Pefabloc SC (Merck Millipore) for 1 h at RT. From each sample 26 µl were cut with 1µl HaeIII (10 U/µl; NEB, Ipswich, USA) overnight at 37°C, followed by A-tailing with Klenow fragment exo- (NEB) and ligation of sample-specific sequencing adaptors using T4-ligase (NEB). After bisulfite conversion with the EZ-DNA methylation Gold kit (Zymo research, Irvine, USA) samples were PCR amplified for 15-17 cycles and then purified with AMPure XP beads (Beckham Coulter, Brea, USA). NGS libraries were then sequenced for ca. 30 - 70 mio 100 bp single reads on an Illumina HiSeq 2500 machine.
Raw reads were trimmed with Trim Galore! (v0.4.2) (Bioinformatics, 2013), to remove adapter contaminations and 3' ends with base quality below 20 (Martin, 2011). RRBS reads were trimmed in RRBS mode and aligned to mm10 using the BWA (v0.6.2) (Li and Durbin, 2009) wrapper methylCtools (v0.9.2) (Hovestadt et al., 2014). Samtools (v1.3)(Li and Durbin, 2009) and Picard tools (v1.115)(Tools, 2015) was utilized for converting, merging and indexing of alignment files. Bis-SNP was used for SNP (dbSNP, v138) (Sherry et al., 2001) aware realignment, quality recalibration and methylation calls. Differential analysis was performed with RnBeads (Assenov et al., 2014). The resulting differentially methylated regions (DMRs) were annotated in R using the software packages ‚GenomicRanges' (Lawrence et al., 2013) and ‚TxDb.Mmusculus.UCSC.mm10.knownGene'(Team BC, 2018).

Bone-marrow-on-a-chip

The chip is made of (poly(dimethylsiloxane)) PDMS, an elastomeric material, well-suited for biological applications, due to its gas permeability contradicted to its impermeability to liquids, all along with its optical transparency down to 230 nm (Huh et al., 2013). The primary fabrication technique for the construction of the chip is *soft lithography* (Duffy et al., 1998), a process of replicating structures of the micrometer to nanometer scale from hard (silicon, metal, etc.) masters to elastomeric materials, such as PDMS.

The layout involves three PDMS layers of 76 x 26 mm^2, one serving as the upper cell culture chamber, separated from the lower chamber for medium perfusion with an intermediate PDMS porous membrane (Figure 3.1). The whole structure is mounted on a glass slide bonded with the upper chamber, to allow better cell observation by means of a microscope for real-time monitoring. As seen in Figure 2.1 , both the chambers are of cylindrical shape to approach the flow lines, the upper enclosing a 12-15uL volume, while the interfering porous membrane is chosen to have 10um thickness 6-8um pore size diameter to allow diffusion of nutrient material to the chamber without compromising cell stagnation in the culture chamber.

The first fabrication step includes the computer-aided design (CAD) of both the chambers and the photolithographic mask for the porous membrane with the aforementioned

architecture and dimensions. In specific, for the construction of the chambers' masters, these CAD files were used to transfer the designed microchambers to the aluminium substrate with 200um and 300um heights for the cell culture and the medium layer, respectively, using Computerized Numerical Control (CNC) Micromachining. For the construction of the membrane master, negative tone photolithography with SU-8 photosensitive resist on silicon wafer with the production of 6-8um diameter and approximately 30 um height SU-8 pillars on the silicon master (SU-8/silicon master).

The PDMS microchambers were then produced with soft lithography, by casting the PDMS prepolymer on the aluminum master and allowing it to cure at 70°C for more than an hour, bearing the complementary ("negative") to the master pattern. The PDMS porous membrane was produced by compressing a 10-um thickness PDMS layer against the micropatterned SU-8/silicon master.
Low pressure plasma of air composition was used for the successive sealing of PDMS layers and their stack with the glass (Si-O-Si covalent bonding). Atmospheric plasma employed for in-chamber hydrophilization (Liao et al., 2003). The process was completed by integrating interconnections into the input/output channels of each chamber ending in the glass roofing.

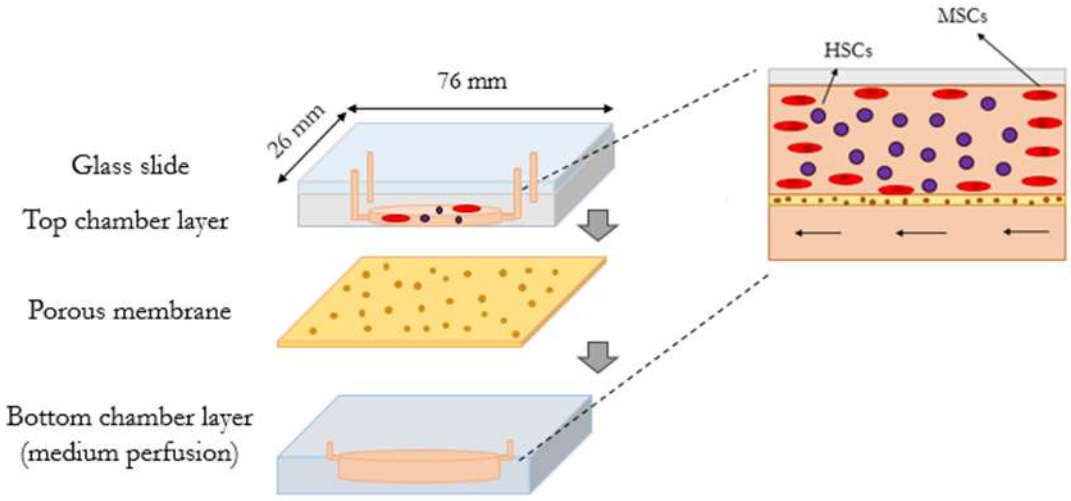

Figure 3.1. 3D schematic of the BMoC. Cross section in magnification indicates the cell culture and medium perfusion chambers.

Statistics

Statistical analyses were performed using an unpaired 2-tailed Student's t test while Mann-Whitney U test was used for the comparison of two groups. Data are presented as mean +/- SD. Differences were considered statistically significant at $P < 0.05$. All data were analyzed using GraphPad Prism v5 software.

Study approval

Informed consent was obtained from all patients and healthy individuals prior to sample collection (Athens, Greece, protocol 10/22-6-2017). All procedures in mice were in accordance with institutional guidelines and were reviewed and approved by the Greek Federal Veterinary Office (Athens, Greece, protocol 4948/19-07-2016).

Data Sharing Statement

Murine RNA-seq data have been deposited to GEO under accession number GSE128692. Human RNA-seq data have been deposited to EGA database under Study EGAS00001003679/Dataset EGAD00001005052.

4. Results

Profiling of hematopoiesis in murine model of SLE

The transcriptional profile of murine lupus LSK demonstrates myeloid skewing

To study whether HSPCs exhibit transcriptional alterations, the NZB/W F1 spontaneous model of SLE was used. It develops severe lupus-like phenotypes resembling the human disease with lymphadenopathy, splenomegaly, elevated serum antinuclear autoantibodies, and immune complex-mediated glomerulonephritis at 6-9 months of age (Dixon et al., 1978; Perry et al., 2011; Theofilopoulos and Dixon, 1985b). We used female NZB/W F1 mice at two time points: a pre-clinical stage (3-month-old, F1-Prediseased/F1-P) and at disease onset defined as >100ng/dl proteinuria (9-month-old, F1-Lupus/F1-L). Age-matched female C57BL/6 mice served as controls (B6-Young/B6-Y and B6-Old/B6-O respectively). Hematopoietic stem and progenitors cells were enriched in the c-Kit^{+} Sca-1^{+} compartment (LSK cells) within the lineage negative population (Lin^{-}) (King and Goodell, 2011a) (Figure 4.1).

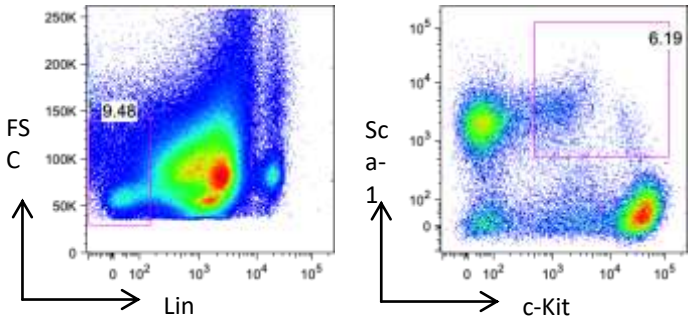

Figure 4.1. Gating strategy for sorting LSK cells. Representative fluorescence-activated cell sorting (FACS) plots for the identification of hematopoietic stem and progenitor cells (HSPCs). After gating for Lin^{-} cells, LSK cells were characterized as c-Kit^{+}Sca-1^{+}.

Next, we performed gene expression analysis of sorted LSK cells from NZB/W F1 lupus mice and C57BL/6 controls. 758 DEGs (|FC|> 1.5, FDR<0.05) between F1-P and F1-L mice were identified including enriched GO terms per cluster: (red) hematopoietic cell lineage, neutrophil degranulation and cell adhesion; (green/blue) lymphocyte activation, extracellular region, IGVH repertoire (Figure 4.2A). *Gene Set Enrichment Analysis (GSEA)* showed significant positive correlation with gene-sets related to immune activated state such as inflammatory response, activation of innate immune response and platelet degranulation in F1-L mice (Figure 4.2B). Importantly, these categories are crucial for the adaptation of stem cell phenotype to inflammation. Studies showed that stem cell-like megakaryocyte committed cells expanded within HSPCs under inflammatory conditions (Haas et al., 2015). *Gene ontology and pathway analysis* revealed that the DEGs were implicated in myeloid leukocyte-mediated immunity, cytokine secretion, granulocyte and neutrophil activation and migration (Figure 4.2C). Importantly, gene expression of key regulators showed that F1-L LSK had increased proliferation activity and a profound

myeloid signature (Figure 4.2D). IFN-associated genes were upregulated in F1-L LSK underscoring a strong type I IFN signature, a hallmark of active SLE (Crow, 2014) (Figure 4.2D).

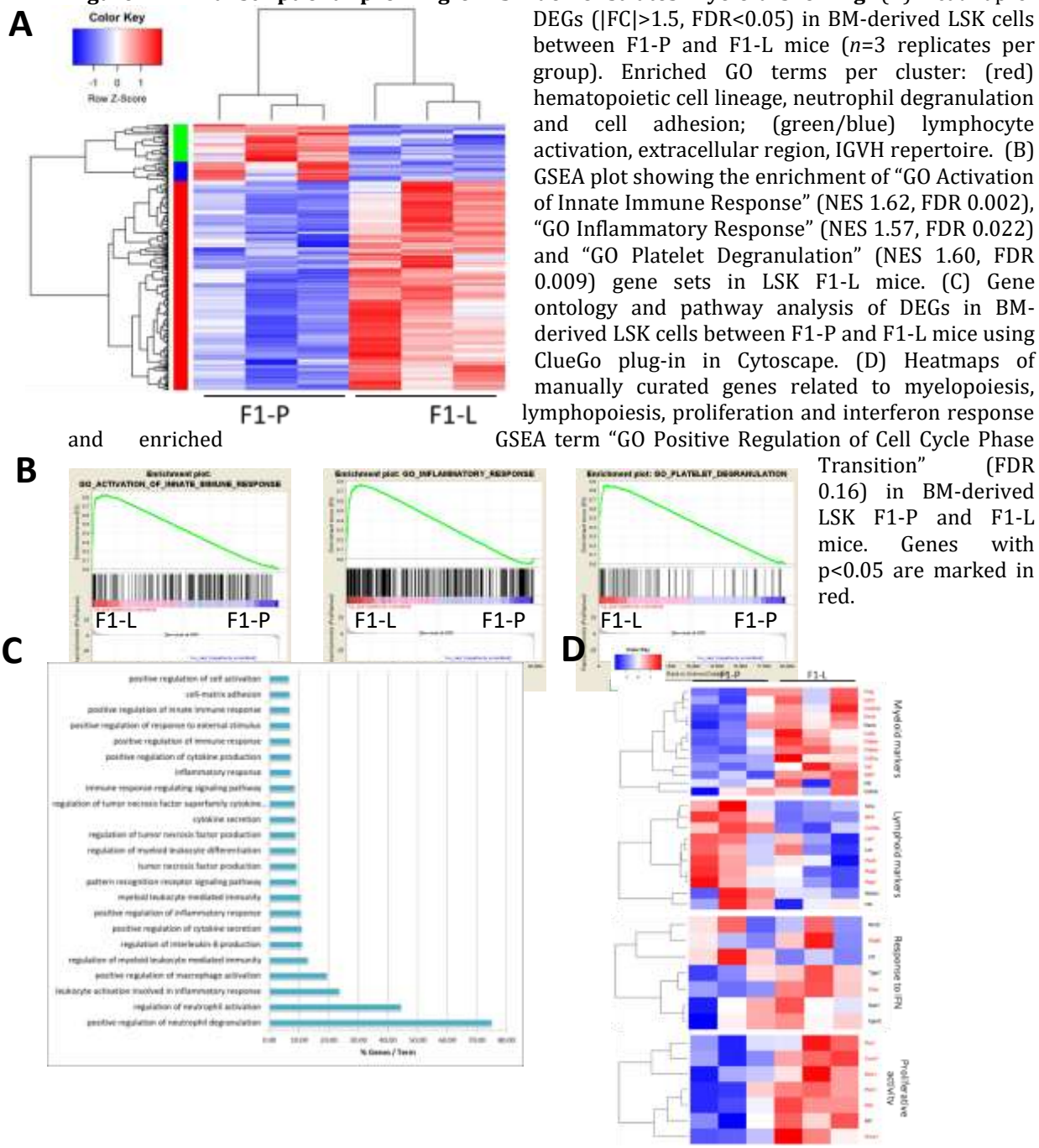

Figure 4.2. Transcriptional profiling of LSK demonstrates myeloid skewing. (A) Heatmap of DEGs (|FC|>1.5, FDR<0.05) in BM-derived LSK cells between F1-P and F1-L mice (*n*=3 replicates per group). Enriched GO terms per cluster: (red) hematopoietic cell lineage, neutrophil degranulation and cell adhesion; (green/blue) lymphocyte activation, extracellular region, IGVH repertoire. (B) GSEA plot showing the enrichment of "GO Activation of Innate Immune Response" (NES 1.62, FDR 0.002), "GO Inflammatory Response" (NES 1.57, FDR 0.022) and "GO Platelet Degranulation" (NES 1.60, FDR 0.009) gene sets in LSK F1-L mice. (C) Gene ontology and pathway analysis of DEGs in BM-derived LSK cells between F1-P and F1-L mice using ClueGo plug-in in Cytoscape. (D) Heatmaps of manually curated genes related to myelopoiesis, lymphopoiesis, proliferation and interferon response and enriched GSEA term "GO Positive Regulation of Cell Cycle Phase Transition" (FDR 0.16) in BM-derived LSK F1-P and F1-L mice. Genes with p<0.05 are marked in red.

Next, we asked whether this lineage bias of LSK represents a lupus-specific molecular signature. Publicly available datasets -encompassing CMP (Klimmeck et al., 2014) and granulocyte-macrophage progenitor (GMP) (Nilsson et al., 2016) signatures during hematopoiesis in wild-type mice- were used as reference transcriptomes. GSEA revealed a significant positive correlation of CMP and GMP signature gene sets within F1-L LSK cells (Figure 4.3). F1-L LSK were biased to differentiate towards the CMP-GMP axis.

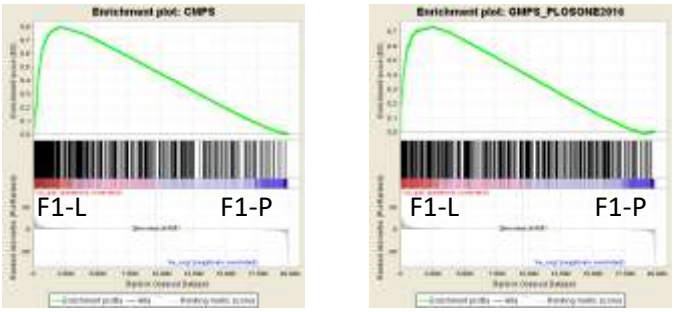

Figure 4.3. LSK are highly enriched towards myeloid lineage. GSEA plot showing the enrichment of CMP signature (NES 1.66, FDR<0.001) and GMP signature (NES 1.52, FDR <0.001) genes in LSK F1-L transcriptome.

To account for the possible effect of aging, we compared F1-L to the B6-O LSK expression profile. Indeed, F1-L LSK were more activated and tended to differentiate towards myeloid lineage (Figure 4.4A-B). Together, these transcriptomic data indicate activation of F1-L LSK -likely responding to type I IFN- increased proliferation and strong differentiation towards myeloid/granulocytic lineage (Forsberg et al., 2005).

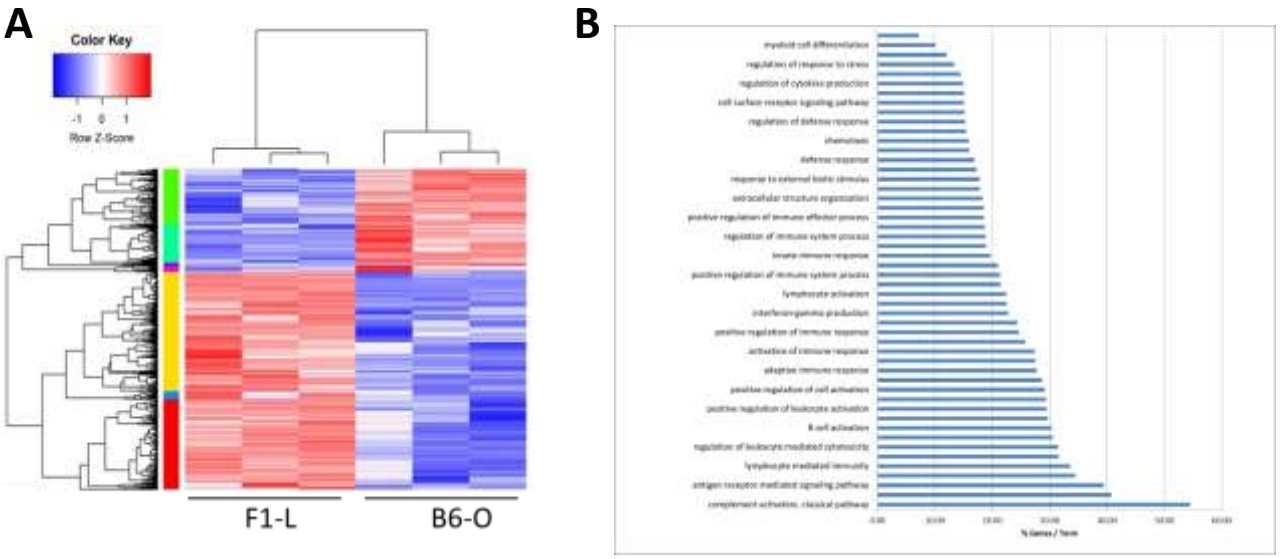

Figure 4.4. Lupus LSK profile is unique independently of their age. (A) Heatmap of DEGs in LSK cells from lupus NZB/W F1 and their age-matched C57BL/6 control mice. (B) Gene ontology and pathway analysis of DEGs in BM-derived LSK cells from lupus NBZ/W F1 and their age-matched C57BL/6 control using ClueGo plug-in in Cytoscape.

Gene expression changes are accompanied by phenotypic shift towards proliferative stress

To validate the transcriptomic data, we analyzed the LSK compartment and its subpopulations by flow cytometry. The frequency of LSK cells was increased by almost 2-fold in the BM of F1-L mice compared to F1-P (Figure 4.5A, B). We also searched within the LSK compartment (Kent et al., 2009) to identify the subpopulation(s) accounting for this increase. Short-term HSCs (CD48$^-$ CD150$^-$ LSK) and multi-potent progenitor (MPP) cells (CD150$^-$ CD48$^+$ LSK) had higher frequency in F1-L mice within the LSK compartment (Figure 4.5A, C).

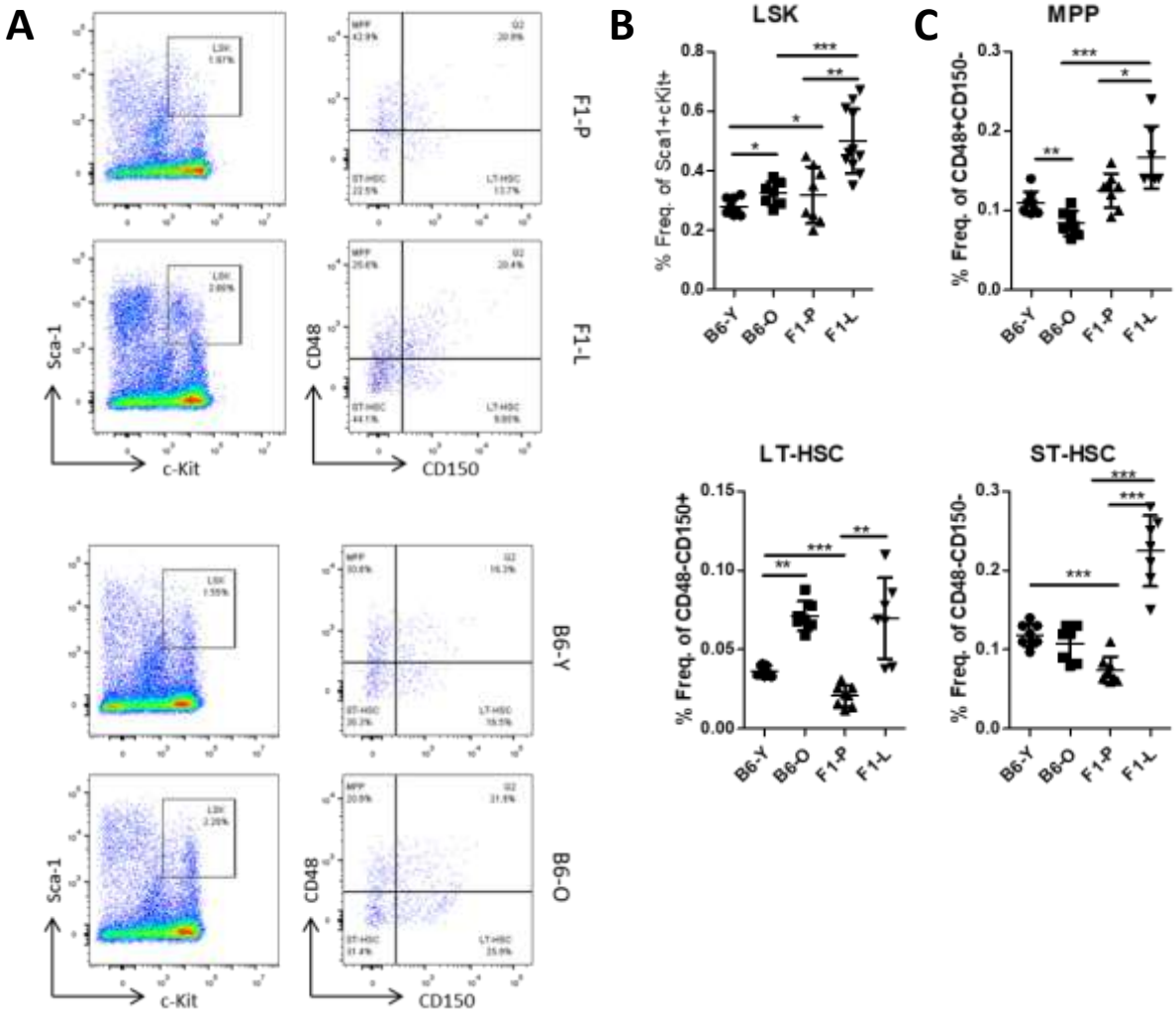

Figure 4.5. Phenotypic analysis of LSK and their subpopulations. (A) Representative flow cytometry analysis within LSK compartment. (B) Frequencies of LSK in BM of F1-P, F1-L and their age-matched C57BL/6 control mice (*n*=7-10). (C) LT-HSCs, ST-HSCs and MPPs in BM of F1-P, F1-L and their age-matched C57BL/6 control mice (*n*=7-10).

Next, we further investigated the cell-cycle of LSK cells. The HSPC population, during steady-state, is relatively quiescent, maintaining a low number of cycling cells that will differentiate and produce the various mature blood cells (Pietras et al., 2011a). Cell cycle

analysis revealed that lupus LSK cells display increased proliferation; F1-P LSK in the G1 phase were increased compared to B6-Y, while less F1-L LSK were in G0 phase compared to B6-O control (Figure 4.6A). The frequency of apoptotic LSK cells from NZB/W F1 mice was increased compared to B6 but there was no significant difference in F1-P versus F1-L stage (Figure 4.6B).

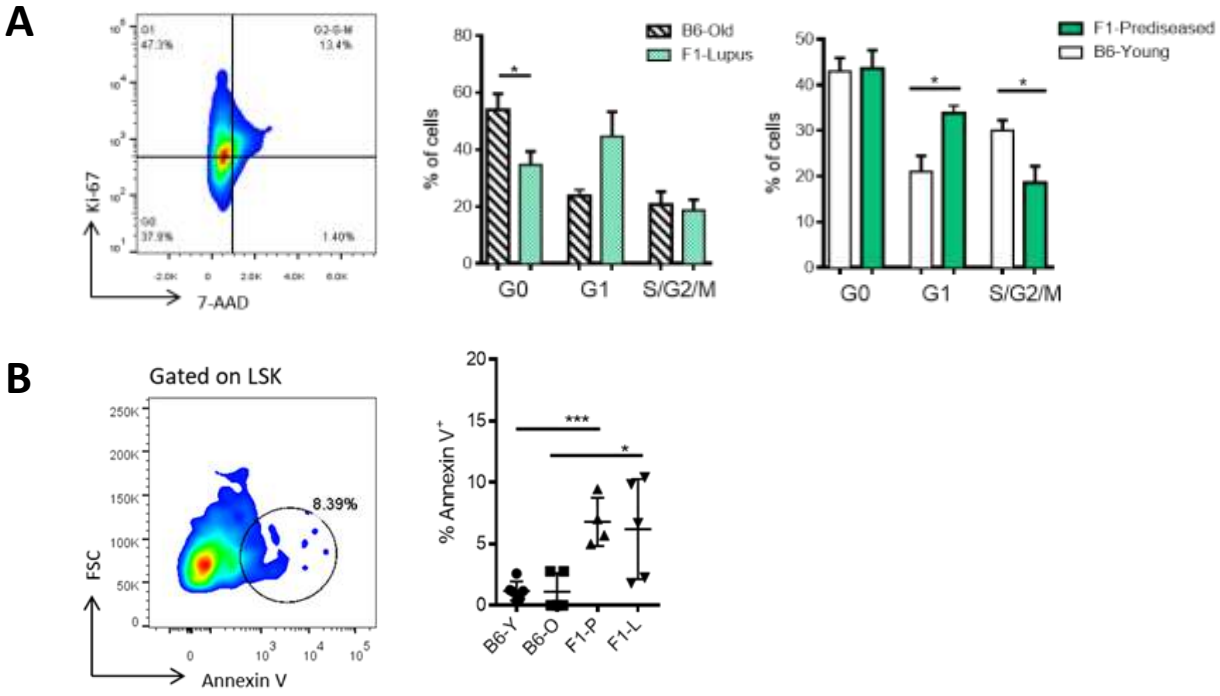

Figure 4.6. LSK demonstrate enhanced proliferation and apoptosis. (A) Representative flow cytometry plot of BM-derived LSK cell cycle analysis using Ki-67/7-AAD marker, and frequencies of cells in each different phase of cell cycle (G0, G1, S/G2/M) (*n*=4-6) . (B) Representative flow cytometry apoptosis analysis of BM-derived LSK cells and frequencies of Annexin V$^+$ cells (*n*=4-6, *P≤0.05, **P≤0.01, ***P≤0.001).

In view of the enhanced proliferation, γ-H2AX foci were measured as a marker of proliferative stress. Indeed, lupus LSK cells exhibited increased double-strand DNA breaks compared to B6 (Figure 4.7). Together these results suggest that LSK in F1-L mice are under replicative stress.

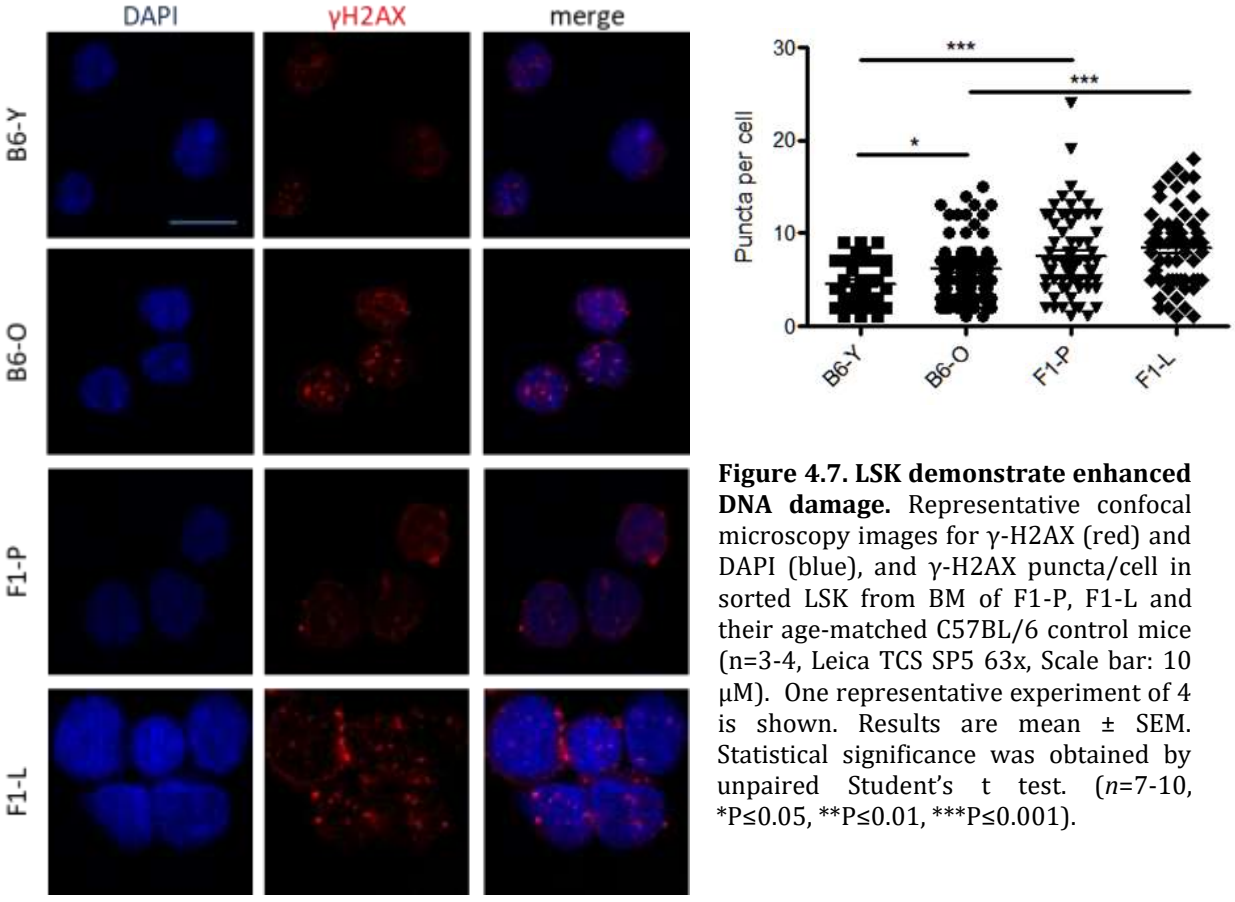

Figure 4.7. LSK demonstrate enhanced DNA damage. Representative confocal microscopy images for γ-H2AX (red) and DAPI (blue), and γ-H2AX puncta/cell in sorted LSK from BM of F1-P, F1-L and their age-matched C57BL/6 control mice (n=3-4, Leica TCS SP5 63x, Scale bar: 10 μM). One representative experiment of 4 is shown. Results are mean ± SEM. Statistical significance was obtained by unpaired Student's t test. (*n*=7-10, *P≤0.05, **P≤0.01, ***P≤0.001).

Given these data, we asked whether the CXCR4/CXCL12 axis is deregulated on LSK cells. CXCR4/CXCL12 signaling influences many aspects of HSPC biology including migration, retention within stem cell niches, proliferation, quiescence, apoptosis and DNA damage in HSCs (Nagasawa, 2014; Peled et al., 1999; Sugiyama et al., 2006; Zhang et al., 2016). In order to investigate this axis, the expression of CXCR4 was measured on LSK cells and we found increased CXCR4 expression at F1-L stage compared to F1-P (Figure 4.8). The data indicates that LSK upregulate this receptor in order to counterbalance the alterations in their quiescence and maintain their functions at F1-L stage.

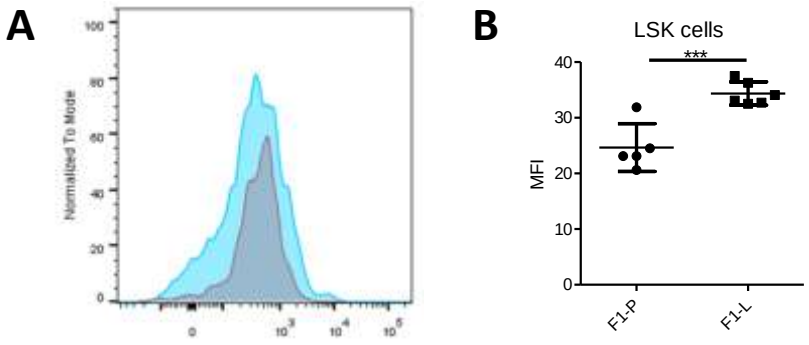

Figure 4.8. Phenotypic analysis of CXCR4 expression on the BM LSK. (A) Representative histogram plots of the CXCR4 expression on the LSK of F1-P (pink) and F1-L (blue) in the BM. (B) MFI of the CXCR4 expression on the LSK of F1-P and F1-L in the BM (*n*=4, *P≤0.05, **P≤0.01, ***P≤0.001).

Differentiation arrest of CMPs with lupus disease progression

To delineate the hematopoietic differentiation after LSK stage, we characterized the committed progenitors of each lineage. CMPs were increased by approximately 2.5-fold in contrast to GMPs which showed 2-fold decrease in F1-L versus F1-P mice (Figure 4.9A). There was no significant difference in common lymphoid progenitors (CLPs) in the lupus mice (Figure 4.9B).

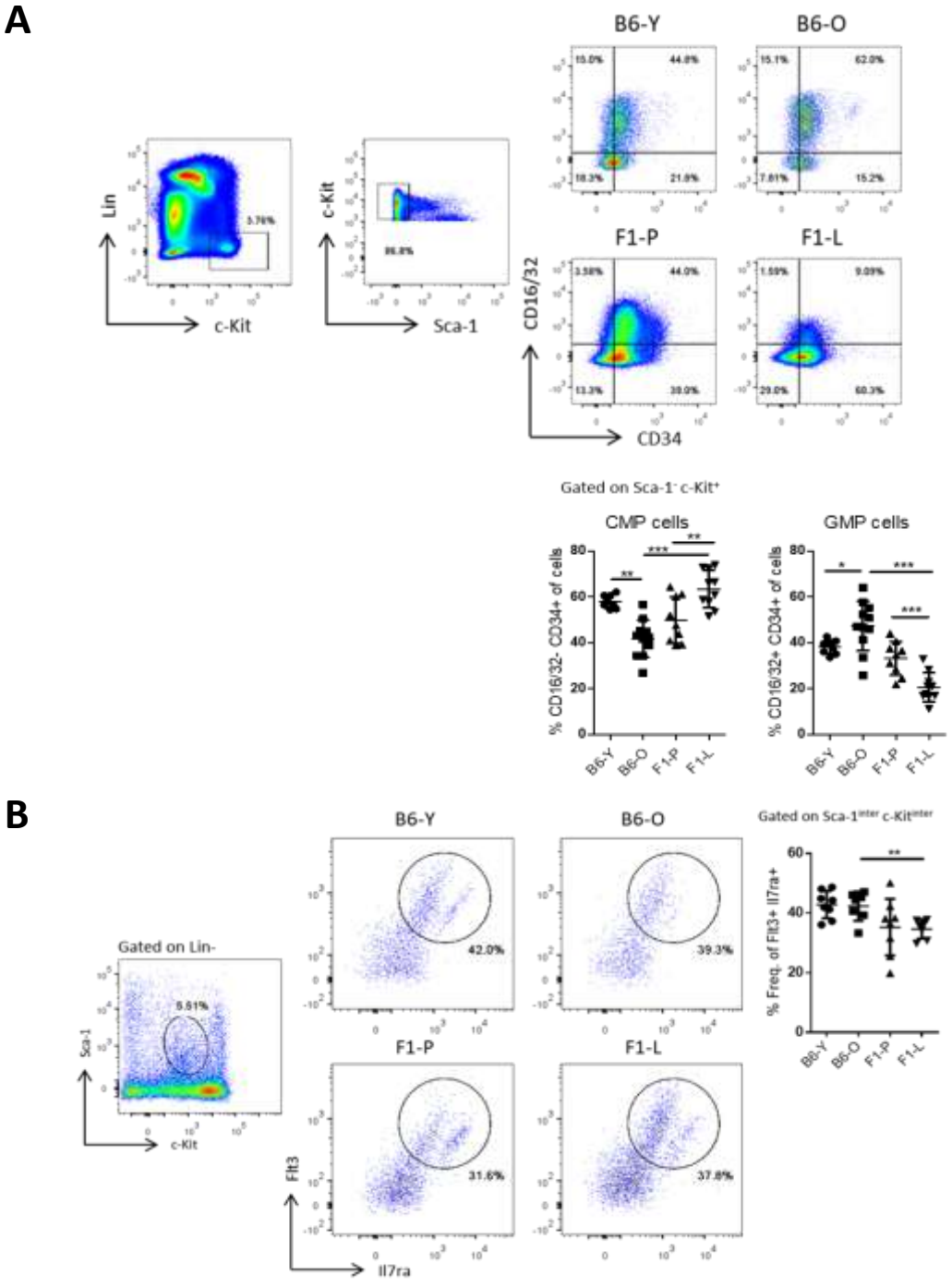

Figure 4.9. Phenotypic analysis of progenitors demonstrates increased CMP cells. (A) Representative flow cytometry analysis and frequencies of BM-derived committed myeloid progenitors (CMP) ($CD34^{+}CD16/32^{-}$) and granulocyte-macrophage progenitors (GMP) ($CD34^{+}CD16/32^{+}$) of F1-P, F1-L and their age-matched C57BL/6 control mice. (B) Representative flow cytometry analysis and frequencies of BM-derived committed lymphoid progenitors (CLP) (Lin^{-} $Sca\text{-}1^{inter}c\text{-}Kit^{inter}Flt3^{+}Il7r\alpha^{+}$) of F1-P, F1-L and their age-matched C57BL/6 control mice. (*n*=8-10, *P≤0.05, **P≤0.01, ***P≤0.001).

Both LSK and CMP frequencies were increased in F1-L mice, a profile reminiscent of emergency granulopoiesis (Manz and Boettcher, 2014). However, expression of *Cebpα* and *Cebpb*, the main regulators of emergency granulopoiesis, showed that there is not such regulation is lupus BM niche (Figure 4.10).

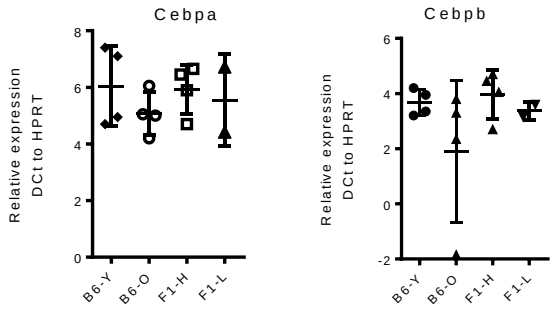

Figure 4.10. *Cebpα/b* expression at CMP stage (*n*=8-10, *P≤0.05, **P≤0.01, ***P≤0.001).

To further investigate the regulation of differentiation, we performed RNA-seq in CMPs from F1-P and F1-L mice. We found 721 DEGs with the majority of them (637 genes) downregulated at F1-L CMPs including enriched GO terms per cluster: (green) response to interferon-beta, nucleotide signaling; (blue) immune response/immunoglobulins; (red) cytokine signaling, neutrophil degranulation, hematopoietic cell lineage (Figure 4.11A). Myeloid markers were downregulated while proliferation markers were not differentially expressed between F1-P and F1-L CMPs (Figure 4.11B). DEGs were involved in pathways related to myeloid-mediated immunity, granulocyte activation, neutrophil migration and complement activation (Figure 4.11C). Thus, we searched in RNA-seq dataset for expression of specific granulocytic markers (Figure 4.11D). Chemokines and regulators of the IL-1 family -which is associated with differentiation and specifically with granulopoiesis (Herault et al., 2017; Mitroulis et al., 2018b; Pietras et al., 2016)- were downregulated in F1-L CMPs. Indicatively, the expression of main regulators such as Cebpe, Cebpd, Csf3r and Csf2rα was dampened in CMPs of F1-L stage (Figure 4.11D). Collectively, these results suggest an arrest of differentiation at the level of myeloid progenitors.

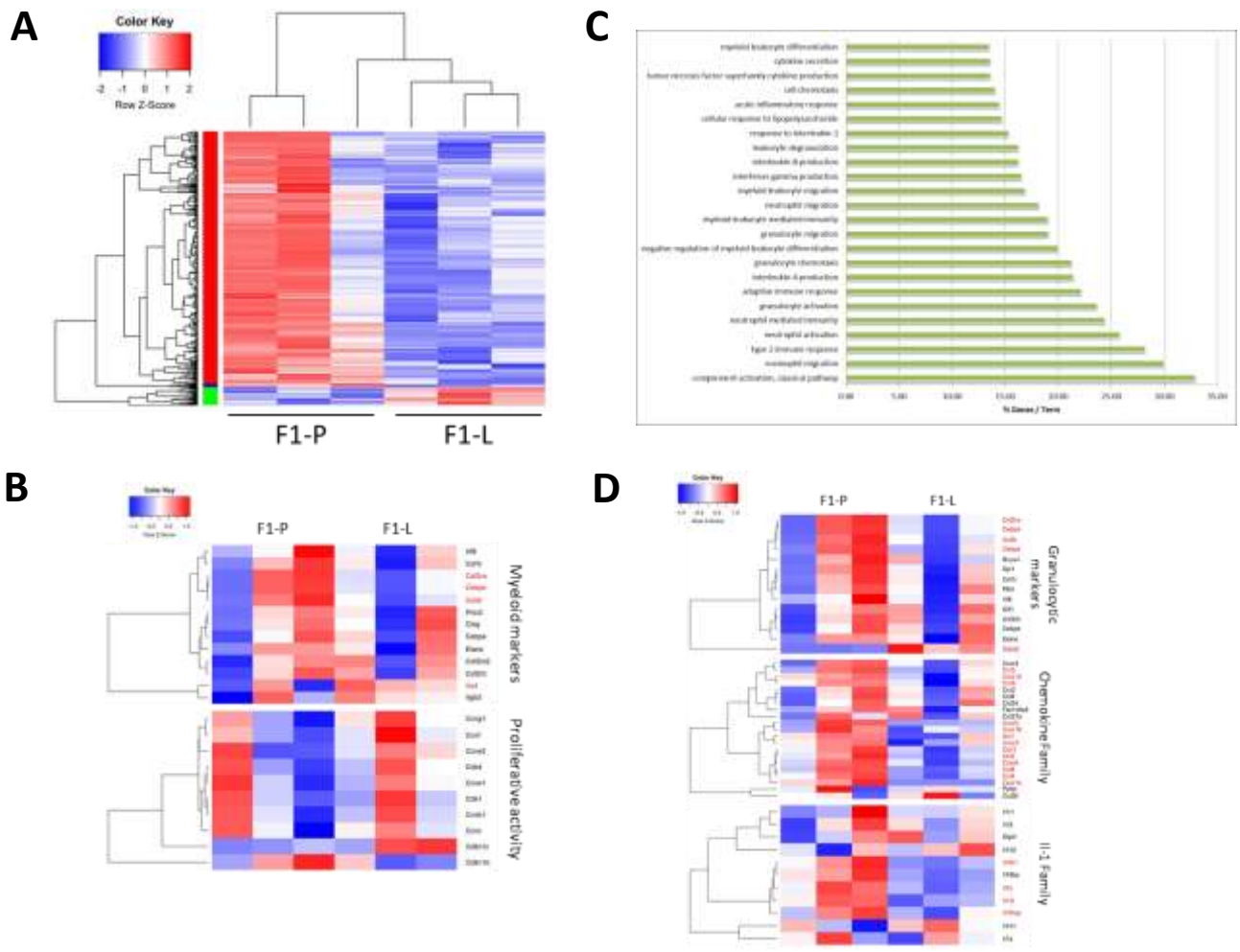

Figure 4.11. Attenuation of lupus CMP differentiation with the progression of the disease. (A) Heatmap of DEGs (|FC|>1.5, FDR<0.05) in BM-derived CMP cells between F1-P and F1-L mice (n=4 per replicate). Enriched GO terms per cluster: (green) response to interferon-beta, nucleotide signaling; (blue) immune response/immunoglobulins; (red) cytokine signaling, neutrophil degranulation, hematopoietic cell lineage. (B) Heatmaps of manually curated genes related to myelopoiesis and proliferation in BM-derived CMP F1-P and F1-L mice. Genes with $p<0.05$ are marked in red. (C) Gene ontology and pathway analysis of DEGs between BM-derived CMP cells F1-P and F1-L mice using ClueGo plug-in in Cytoscape. (D) Heatmap of manually curated genes related to granulopoiesis, chemokine-related and IL-1-related factors in BM-derived CMP of F1-P and F1-L mice. Genes with $p<0.05$ are marked in red.

Neutrophils are increased in the BM - but not in the periphery- of lupus mice: evidence of "granulocytic priming"

We then asked whether the terminally differentiated cells of myeloid lineage (monocytes/granulocytes) are decreased due to differentiation arrest at CMP level. T and B cells had similar frequencies during disease progression (Figure 4.12A, B). Neutrophils exhibited 1.6-fold increase in the F1-L mice compared to F1-P while there were comparable levels of monocytes in the BM (Figure 4.12A, B). Aging accounted for only an increase of 1.16-fold in neutrophils of control mice. This could be due to either extensive destruction of neutrophils in the periphery or migration to target tissues. Together these data suggest priming in the BM towards neutrophils.

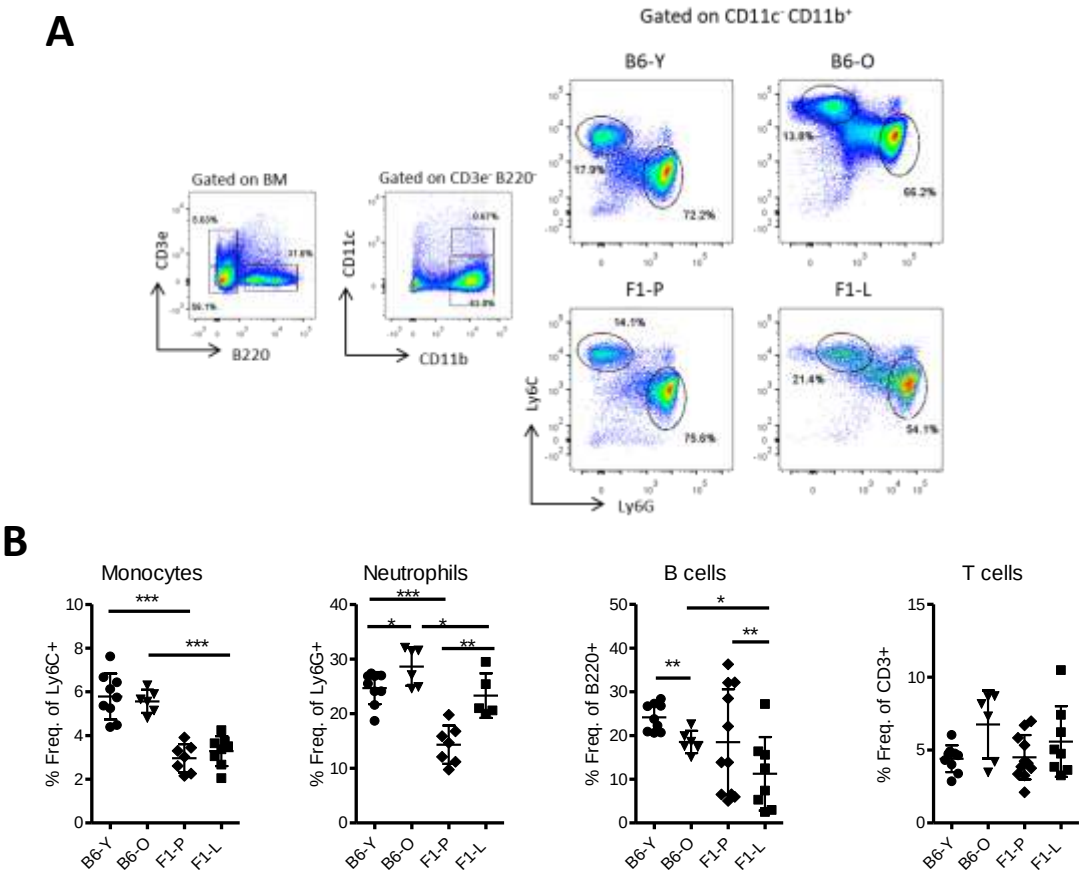

Figure 4.12. Neutrophils increase in the bone marrow of lupus mice. (A) Representative flow cytometry analysis of monocytes ($CD3e^-$ $B220^-$ $CD11b^+$ $Ly6C^+$) and neutrophils ($CD3e^-$ $B220^-$ $CD11b^+$ $Ly6G^+$) in BM of F1-P, F1-L and their age-matched C57BL/6 control mice. (B) Frequencies of monocytes, neutrophils, B and T cells in BM of F1-P, F1-L mice and their age-matched C57BL/6 control mice (*n*=6-10, *P≤0.05, **P≤0.01, ***P≤0.001).

Neutrophil homeostasis is maintained through a balance of neutrophil production, release from the bone marrow and clearance from the circulation (Christopher and Link, 2007). CXCR4 is a major receptor which along with CXCL12 plays a key role in maintaining

neutrophil homeostasis. It negatively regulates neutrophil release from the BM and it is dispensable for neutrophil clearance from the circulation (Eash et al., 2009). Phenotypic analysis of CXCR4 expression on the BM-derived neutrophils indicated that F1-L mice express less receptor than the F1-P (Figure 4.13). Our results suggest that neutrophils rapidly exit BM and enter periphery consistent with previous studies showing that neutrophils lacking CXCR4 have accelerated release from the bone marrow but normal clearance from the blood (Christopher and Link, 2007).

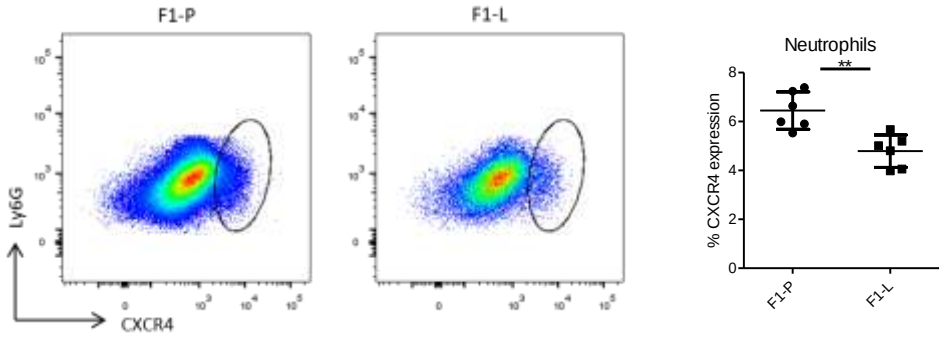

Figure 4.13. Phenotypic analysis of CXCR4 expression on the BM neutrophils. (A) Representative histogram plots of the CXCR4 expression on the neutrophils of F1-P and F1-L in the BM. (B) Frequencies of the CXCR4 expression on the neutrophils of F1-P and F1-L in the BM (*n*=4, *P≤0.05, **P≤0.01, ***P≤0.001).

Granulocytic differentiation is programmed to commence from LSK stage in lupus, adapting to an alternative granulopoiesis pathway

To investigate how "granulocytic priming" evolves during differentiation of hematopoiesis in lupus, we performed GSEA analysis using DEGs of lupus CMPs as a reference list. There was highly positive enrichment of the *"lupus CMP"* signature in the lupus LSK cells (Figure 4.14).

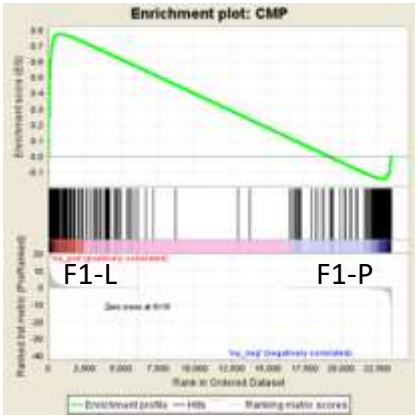

Figure 4.14. GSEA plot showing the enrichment of "lupus CMP signature" (NES 1.66, FDR<0.001) in LSK F1-L transcriptome.

Next, we sought how many common genes exist between DEGs at these two levels and how they are expressed. Interestingly, we found 327 common DEGs, which are mostly up-regulated in F1-L LSK, but downregulated in the F1-L CMPs (Figure 4.15A-B). Pathway analysis revealed that these common genes (cluster red on the heatmap, Figure 4.14B) were implicated in granulocyte migration, neutrophil activation and neutrophil apoptotic process (Figure 4.15C).

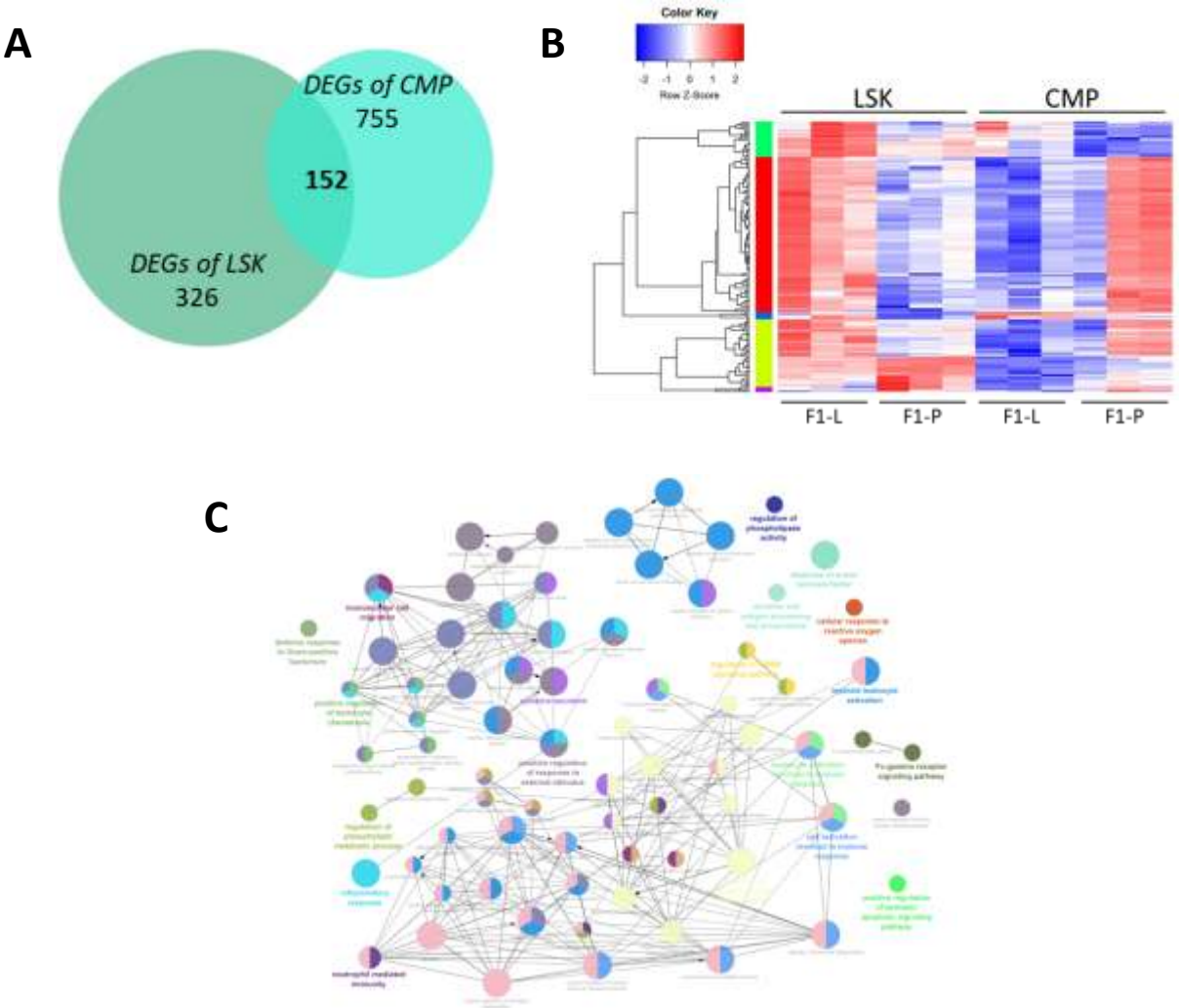

Figure 4.15. Comparison at the transcriptome level of lupus LSK and CMPs. (A) Venn diagram of the DEGs of LSK and CMP in BM of F1-P and F1-L mice. (B) Heatmap of the common DEGs. (C) Gene ontology and pathway analysis of the common DEGs using ClueGo plug-in in Cytoscape.

To this end, we performed a comparative analysis between the transcriptomic profile of murine lupus LSK and CMP. We used the RNEA algorithm to report enrichment of transcription factors and regulators by combining previous studies with our own data. This algorithm correlates the expression of DEGs with regulators and transcription factors found to be associated with them based on publicly available data. We identified 13 common differentially expressed transcription factors and regulators (Figure 4.16A), predominantly downregulated in the F1-L CMP stage (Figure 4.16B). These represent mainly transcription regulators of myeloid and granulocytic differentiation. Therefore, we looked into expression of major regulators of granulocytic and neutrophilic differentiation -such as *Cebpα*, *Cebpe*, *Irf8*, *Mpo* and *Elane*- which were found upregulated in F1-L LSK cells and downregulated in F1-L CMPs (Figure 4.16C). Collectively, these data indicate a deregulation of differentiation at the level of CMPs and "priming" of LSK cells towards granulocytes, indicative of an alternative granulopoiesis pathway.

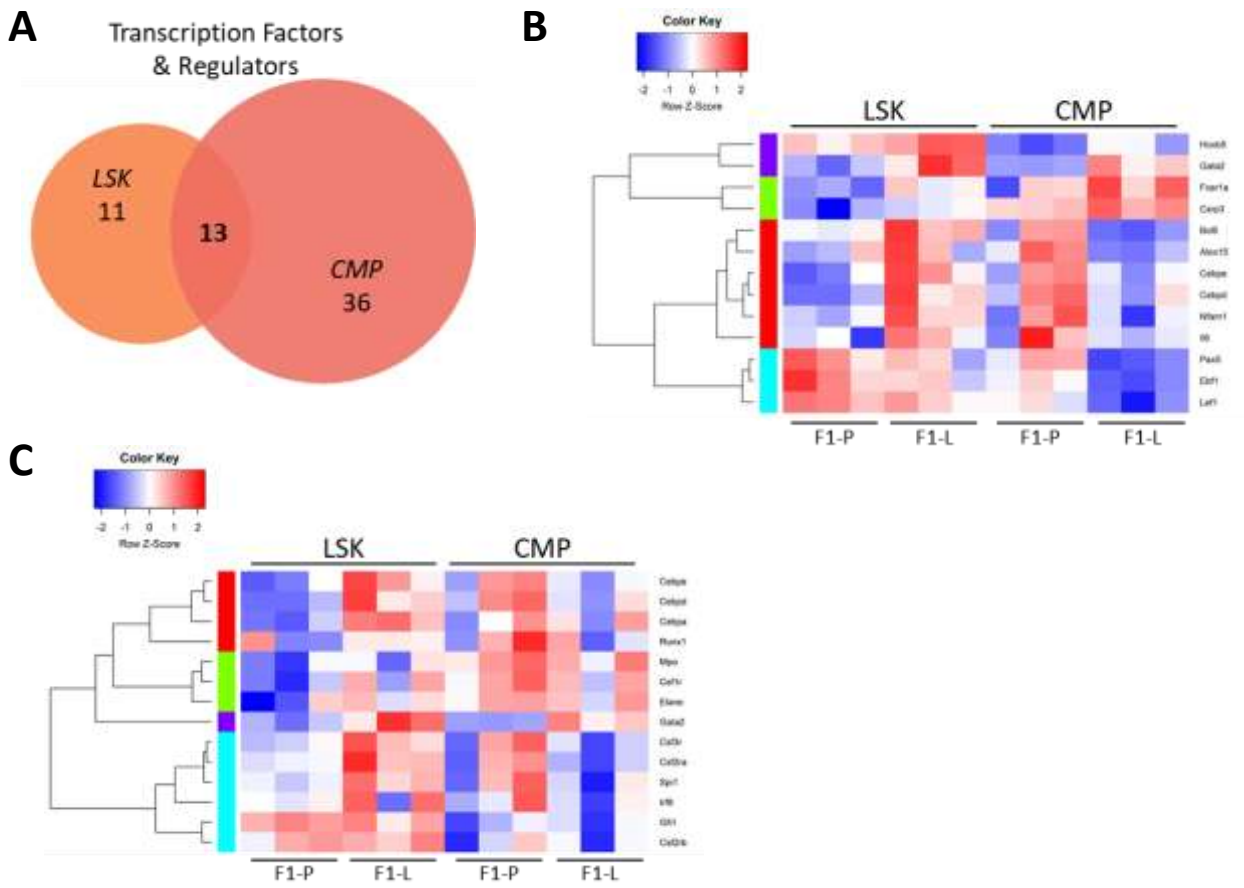

Figure 4.16. Comparison of regulators at the transcriptome level of lupus LSK and CMP. (A) Venn diagram of the deregulated transcription factors and regulators of LSK and CMP in BM of F1-P and F1-L mice based on RNEA algorithm. (B) Heatmap of the common deregulated factors and regulators. (C) Heatmap of differentially expressed granulocytic markers in BM-derived LSK and CMP cells of F1-P and F1-L mice.

Methylation

Approximately 70% of all expression changes between HSCs and early progenitors occur independently of lineage choice (Laurenti et al., 2013), following the fashion of methylation and chromatin accessibility (Corces et al., 2016; Ji et al., 2010). Methylation landscape of BM-derived HSPCs from F1-P (*n*=4) and F-L (*n*=6) mice was characterized by RRBS technology, where samples are generated by digesting genomic DNA with the restriction endonuclease MspI. This is followed by end-repair, A-tailing, adapter ligation and finally bisulfite conversion. Level of differential methylation on the site was computed. The CpG sites were grouped in categories: (a) regions containing the MspI restriction site, (b) promoters, (c) genes and (d) CpG islands (CGIs) and differential methylation was computed. Statistical significance was considered when FDR<0.05 except for CGIs where p-value<0.05 threshold was used. The number of differentially methylated regions, promoters, genes and CGIs are listed in Table 3.

Table 3. Differentially methylated sites across the genome in F1-L vs F1-P.

	Regions	Promoters	Genes	CGIs
Total	324	47	28	104
Hypermethylated	160	22	17	57
Hypomethylated	164	25	11	47

Typically, DNA methylation in promoter CGIs blocks transcription and promotes gene silencing (Doody et al., 2017; Hammaker and Firestein, 2018), whereas variable effects occur when CGIs in gene bodies, introns or enhancers are methylated (Jin et al., 2011). DNA methylation has been extensively analyzed in an attempt to uncover the underlying epigenetic mechanisms that control rheumatic diseases.

Statistically significantly differentially methylated regions (DMRs) and their closest associated genes in HSCPs between F1-P and F1-L are shown in Figure 4.17. Statistically significant differentially methylated CGIs are depicted on Figure 4.18.

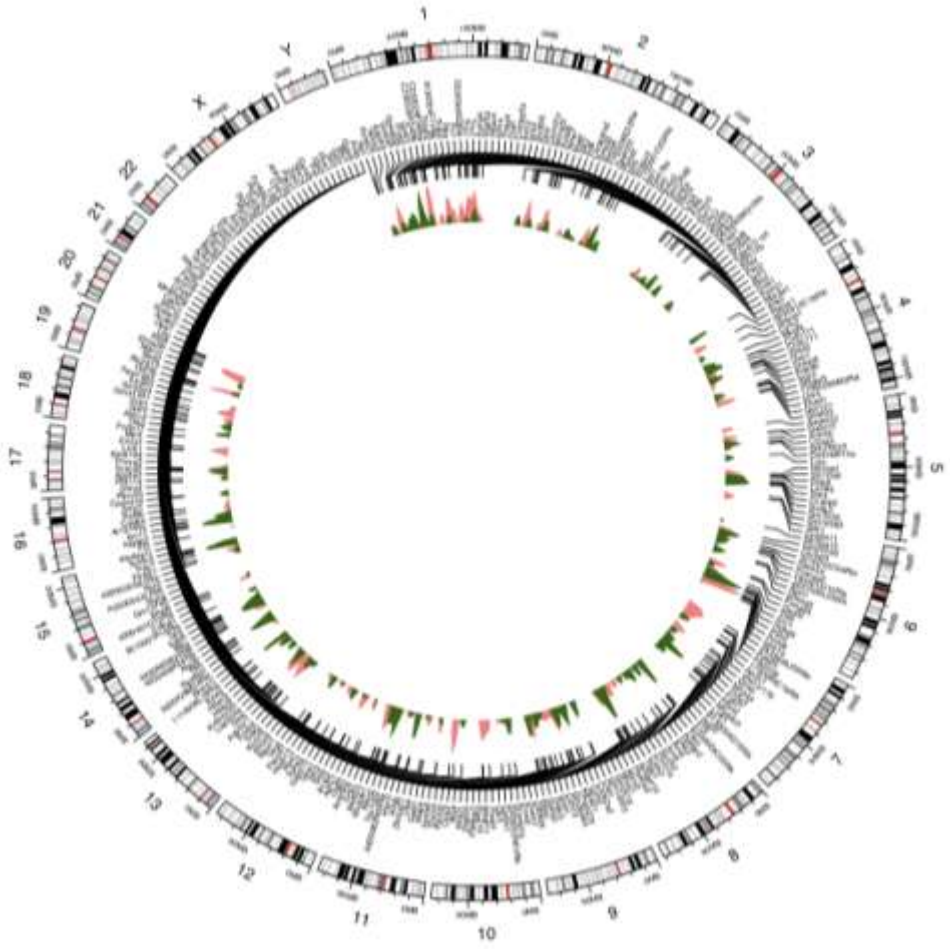

Figure 4.17. Circos plot showing the statistically significant DMRs (FDR<0.05) in HSPCs between F1-P and F1-L (green hypomethylation; pink hypermethylation in F1-L).

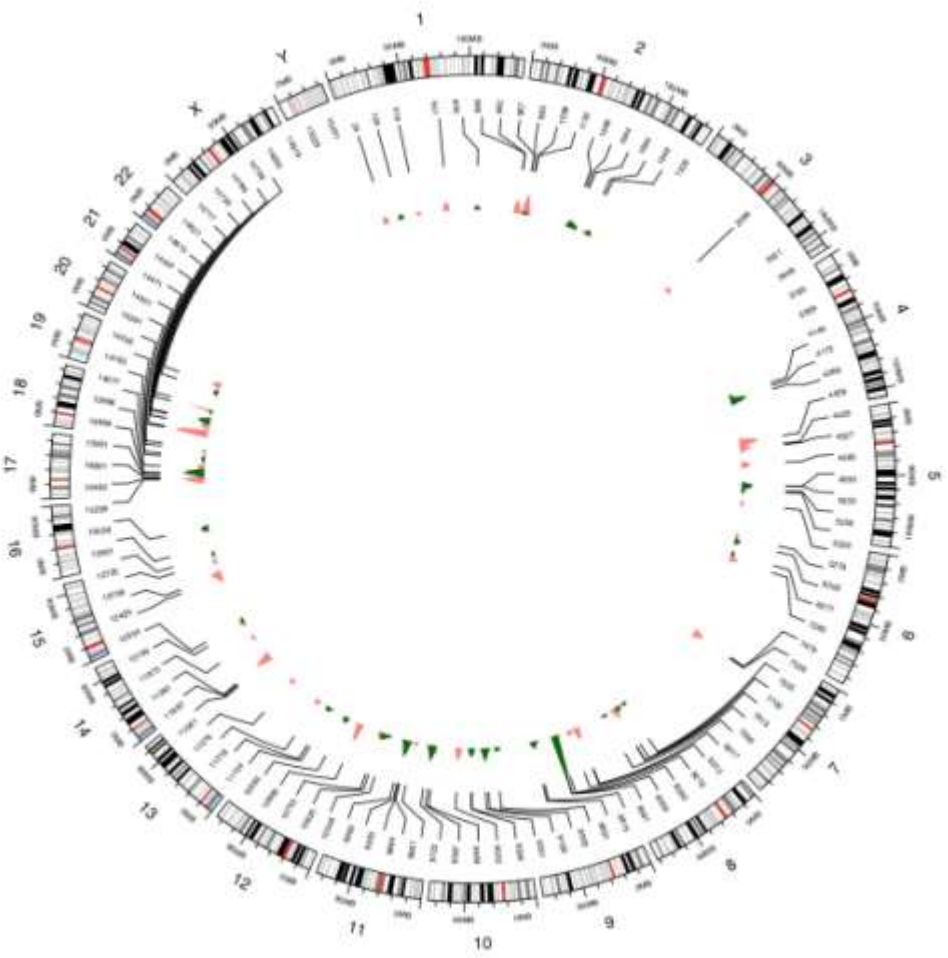

Figure 4.18. Circos plot showing the differentially methylated CGIs (p-value<0.05) in HSPCs between F1-P and F1-L (green hypomethylation; pink hypermethylation in F1-L).

Similarly, statistically significant differential methylated promoters and genes in HSPCs between F1-L and F1-P are shown in Figure 4.19 and 4.20 respectively.

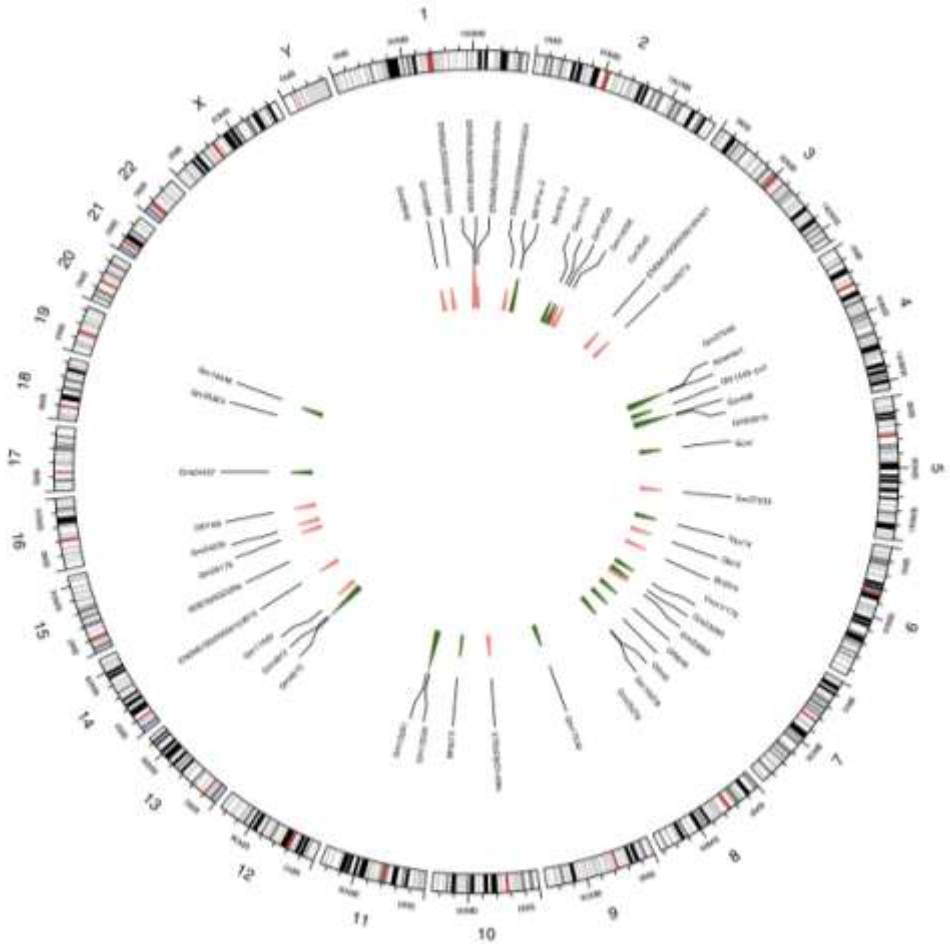

Figure 4.19. Circos plot showing the statistically significant differentially methylated promoters in HSPCs between F1-P and F1-L (green hypomethylation; pink hypermethylation in F1-L).

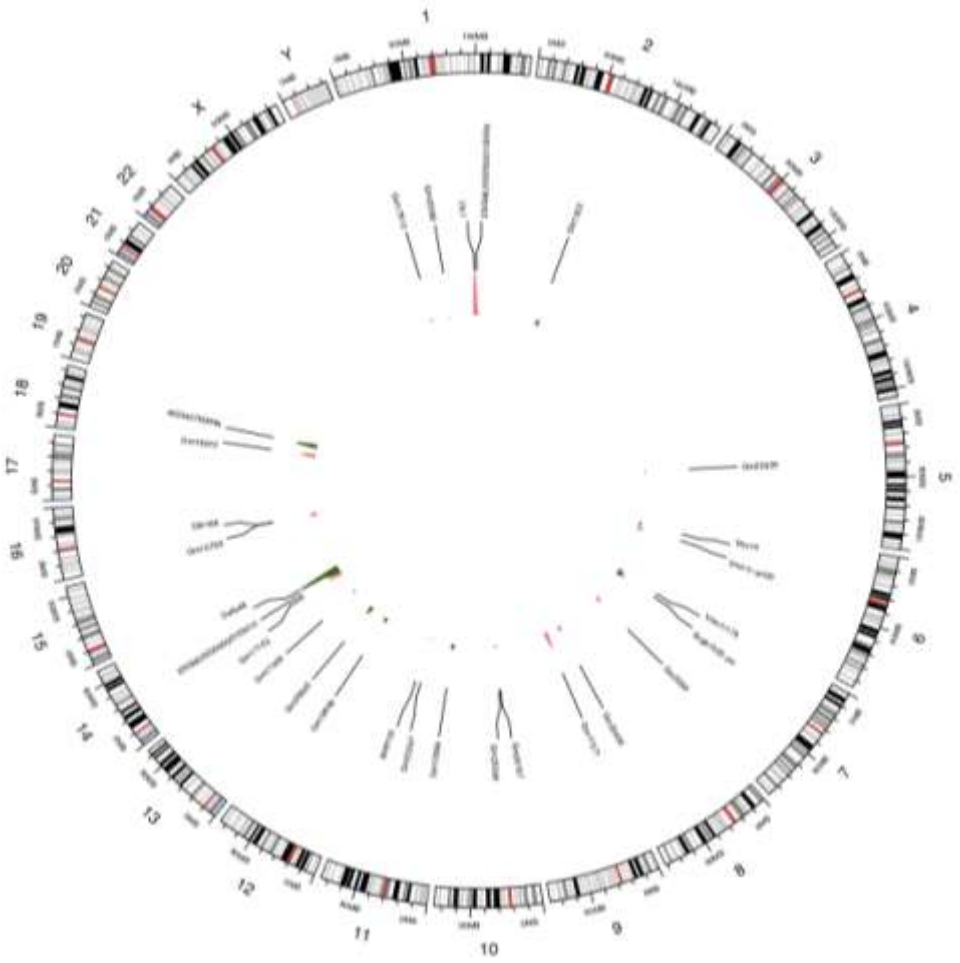

Figure 4.20. Circos plot showing the statistically significant differentially methylated genes in HSPCs between F1-P and F1-L (green hypomethylation; pink hypermethylation in F1-L).

Although the number of hypomethylated and hypermethylated regions and CGIs was limited (Figure 4.17 & 4.18), the genes which were associated with the differentially methylated promoters were mainly hypomethylated in the F1-L HSPCs which could be correlated with increased gene expression in lupus stage.

The genes which were associated with DMRs were used as input in order to identify enriched pathways and biological processes. LOLA software was utilized to perform enrichment analysis (Figure 4.21), thus facilitating interpretation of functional genomics and epigenomics data. This software provides functions for testing the overlap of sets of genomic regions with public and custom region (genomic ranges) databasets (Sheffield and Bock, 2016).

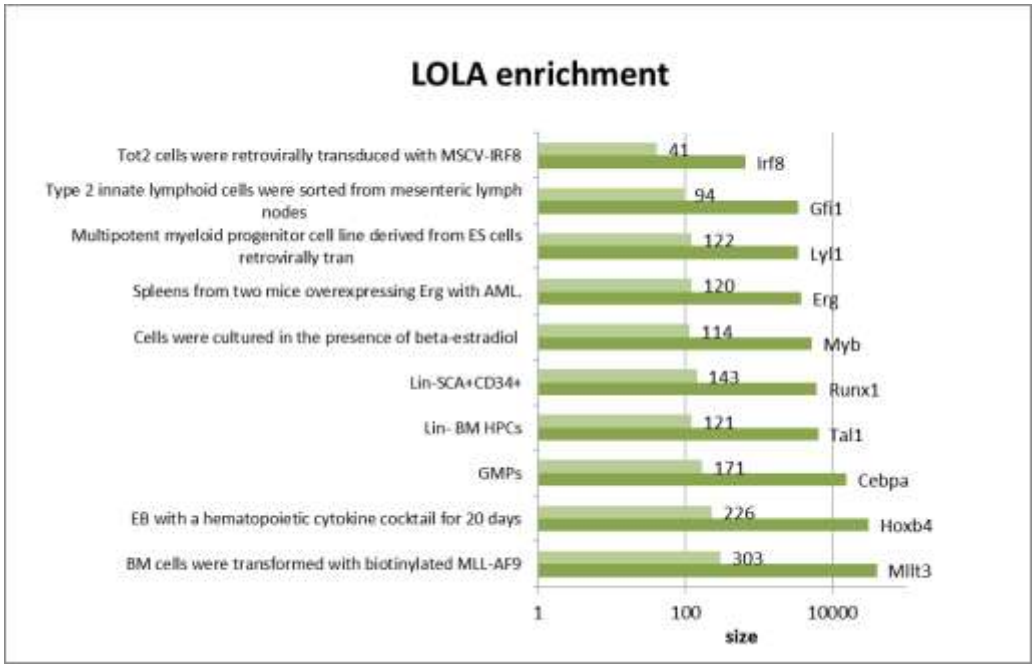

Figure 4.21. LOLA enrichment analysis of DMRs with publicly available datasets.

The enrichment analysis revealed various categories related to hematological and immunological functions. Specifically, DMRs showed positive enrichment within regions annotated to regions occupied by *Cebpα*, *Runx1*, *Gfi1* and *Irf8.* These results indicate differential methylation in SLE and specifically at genes characterized as major regulators for myeloid differentiation of HSPCs.

Next, integrative analysis between RRBS and RNA-seq data was performed. Additional analysis for the differential methylation was performed based on methylKit package (Akalin et al., 2012) as an alternative algorithm. Cross-section with Venn diagram between RNA-seq and RRBS data revealed 21 common genes as depicted on Figure 4.22. Those 21 genes are associated with differentiation of HSPC (i.e. *Tcf15*) and their interaction with the BM stroma (i.e. *Col18a1*, *Tgfbi*). It is important to highlight that these genes are simultaneously upregulated and hypo-methylated in F1-L. Although these are preliminary results, they are supportive to the biased differentiation of HSPCs towards myeloipoiesis in SLE, probably caused by the aberrant gene expression through epigenetic regulation of major players during hematopoiesis.

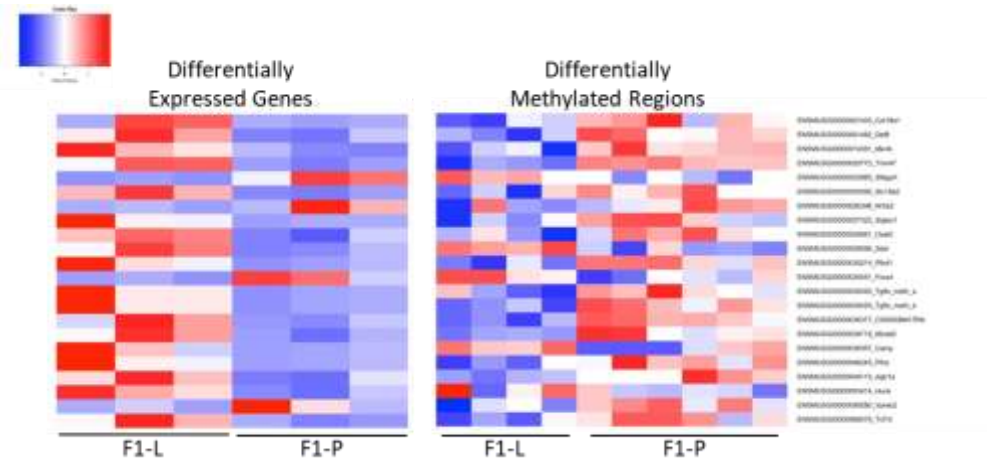

Figure 4.22. Heatmaps of common DEGs and DMRs in HSPCs from F1-L and F1-P mice.

Periphery

Based on previous results concerning the BM niche, we asked whether there are any HSPCs circulating in the periphery. Firstly, we searched for the progenitor cells in the peripheral blood (PB) and in the spleen. We found enhanced frequency of circulating LSK cells in the PB of mice with established disease compared to their control counterparts (Figure 4.22A). In contrast, there were comparable levels of LSK, CMP and GMP cells in the spleen (Figure 4.22B). Concluding, the data suggest that BM-derived LSK are activated, exit the niche and migrate to the periphery.

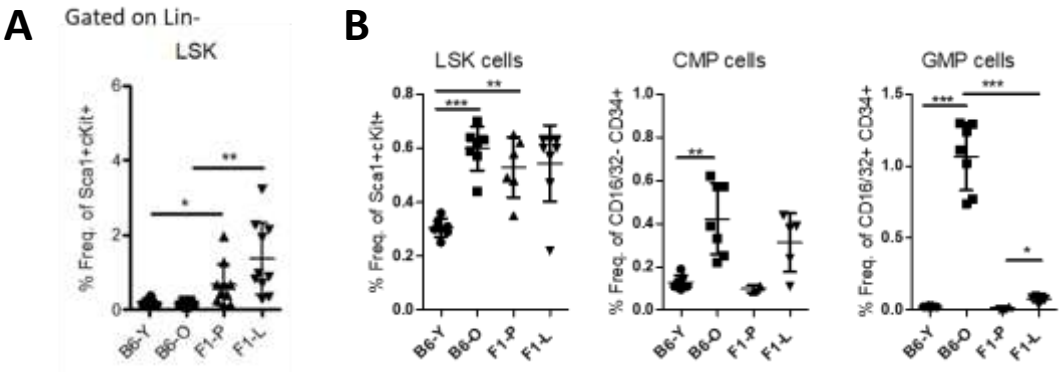

Figure 4.22. Phenotypic analysis of HSPCs and progenitor cells in the periphery. (A) Frequencies of LSK cells in PB of F1-P, F1-L mice and their age-matched C57BL/6 control mice. (B) Frequencies of LSK, CMP and GMP cells in spleen of F1-P, F1-L mice and their age-matched C57BL/6 control mice. (*n*=5-8, *P≤0.05, **P≤0.01, ***P≤0.001).

Next, we searched for the terminally differentiated cells in the periphery. As anticipated, there was marked decrease of neutrophils in the blood and spleen of F1-L mice. Monocytes were decreased only in the spleen whereas T cells were decreased only in the PB. B cells did not differ significantly in the periphery between lupus mice and their control counterparts (Figure 4.23A-B).

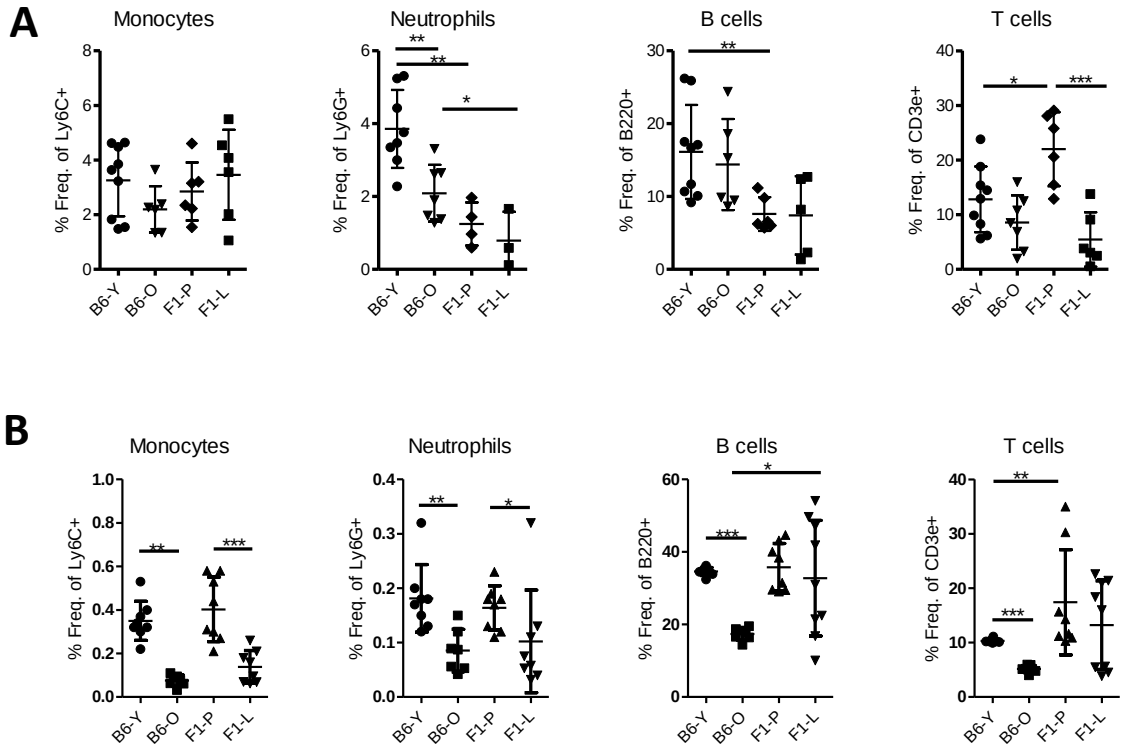

Figure 4.23. Phenotypic analysis of terminally differentiated cells in the periphery. (A) Frequencies of monocytes, neutrophils, B and T cells in PB and (B) spleen of F1-P, F1-L mice and their age-matched C57BL/6 control mice. (*n*=4-8, *P≤0.05, **P≤0.01, ***P≤0).

Profiling of hematopoiesis in humans

Transcriptional profiling of human CD34+ cells in SLE patients suggests an active proliferative state with myeloid skewing

Next, we asked whether we could trace the "*lupus LSK signature*" from F1 mouse model in human disease. To this end, we purified CD34+ cells from the BM of SLE patients and healthy controls (HC).

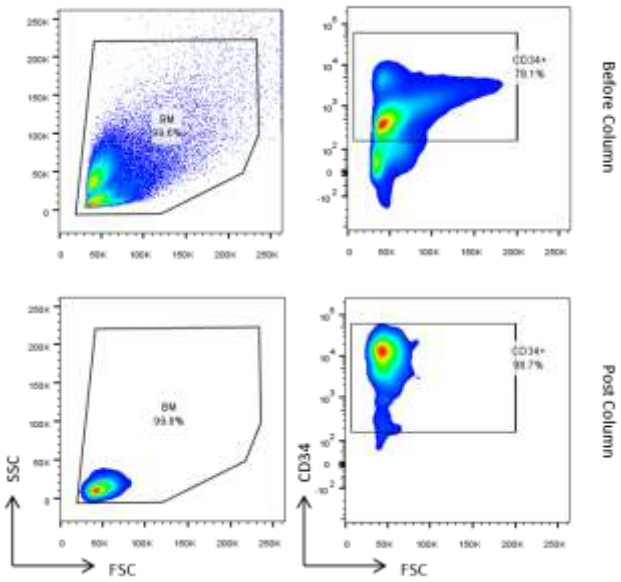

Figure 4.24. Representative flow cytometry analysis before and after CD34+ selection column.

In humans, CD34+ compartment comprises a cluster of 0.5-2% of BM cells including both stem and progenitor cells of different lineages (Strobl et al., 1993; Velten et al., 2017). BM-derived CD34+ cells were isolated with magnetic beads (Figure 4.24) and their expression profile was analyzed using RNA-sequencing sampling from female SLE patients and compared this to female HC (Christou et al., 2018; Ortona et al., 2016). We identified 2,364 DEGs between SLE patients and HC, which contained 832 upregulated and 1,532 downregulated genes (Figure 4.25A). Enriched GO terms per cluster include: (blue) Extracellular vesicle-mediated signaling in recipient; (turquoise) myeloid leukocyte migration, chemokine signaling pathway; (red) integrin-mediated signaling, regulation of cell-cell adhesion; (green) antigen processing and presentation, autoimmunity, abnormal inflammatory response; (yellow) cell surface receptor signaling pathway (Figure 4.25A). Lymphoid markers were downregulated in SLE, while expression of myeloid markers exhibited great variation within the patients. Combining these two panels, early hematopoiesis of human SLE is characterized by skewing towards myeloid lineage (Figure 4.25B). SLE CD34+ cells exhibited enhanced proliferation (Figure 4.25B) while GSEA exhibits significant positive correlation with gene sets related to 'Activation of ATR in response to replication stress', 'cell cycle' and 'DNA-dependent DNA replication' in SLE CD34+ cells (Figure 4.25C).

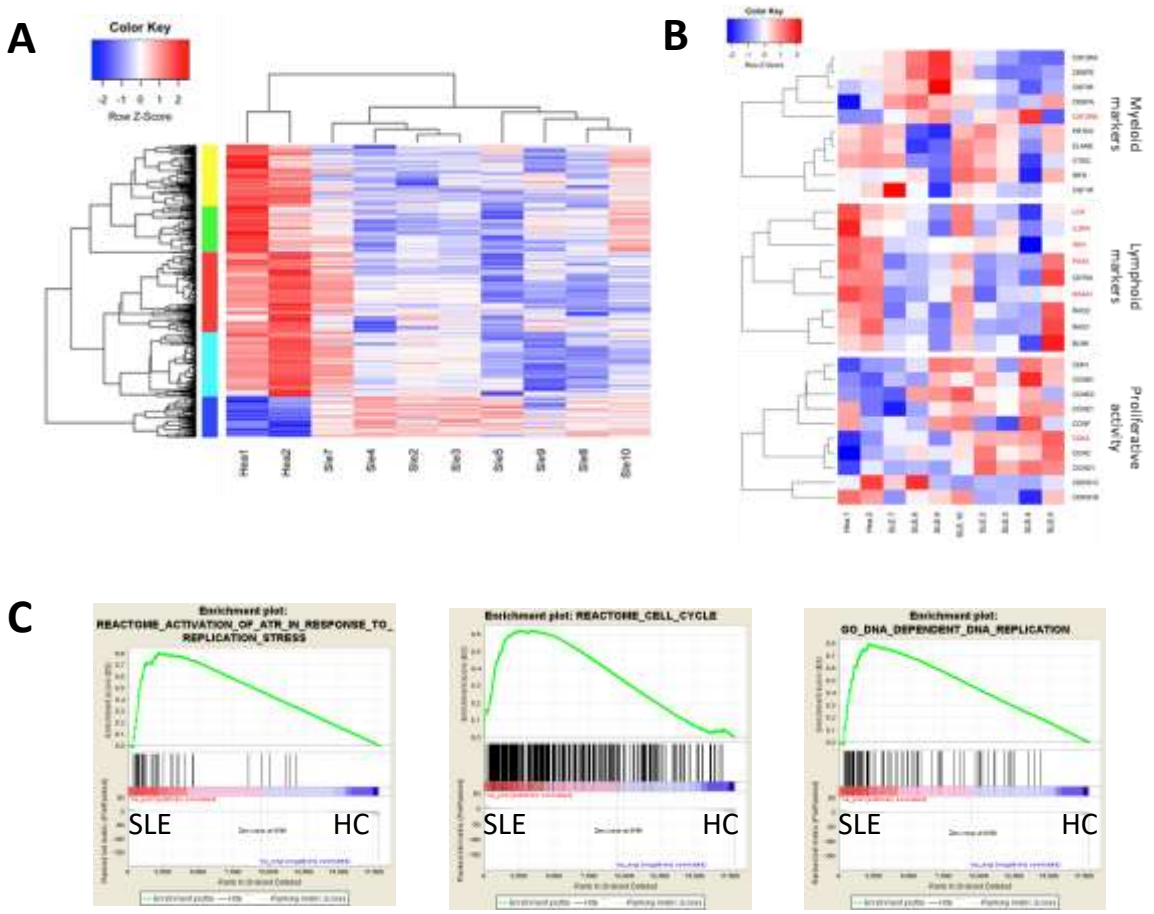

Figure 4.25. RNA-seq of human CD34⁺ cells in SLE patients suggests active proliferation. (A) Heatmap of DEGs in CD34⁺ cells isolated from BM of SLE patients (*n*=8) and HC (*n*=2). (B) Heatmaps of genes related to myelopoiesis, lymphopoiesis and proliferation in SLE patients and HC. Genes with p<0.05 are marked in red. (C) GSEA plot showing the enrichment of "Reactome: Activation of ATR in response to Replication Stress" (NES 1.73, FDR 0.056), "Reactome: Cell Cycle" (NES 1.81, FDR 0.002), "GO DNA dependent DNA Replication" (NES 2.03, FDR <0.001) gene sets in CD34⁺ SLE patient samples.

In view of enhanced proliferation (Walter et al., 2015), γ-H2AX was monitored to check if CD34⁺ cells are under proliferative stress; indeed lupus HSPCs exhibited increased double-strand DNA breaks (Figure 4.26). Collectively, CD34⁺ cells exhibited enhanced proliferation with increased DNA damage and a tendency to unbalanced differentiation towards the myeloid axis.

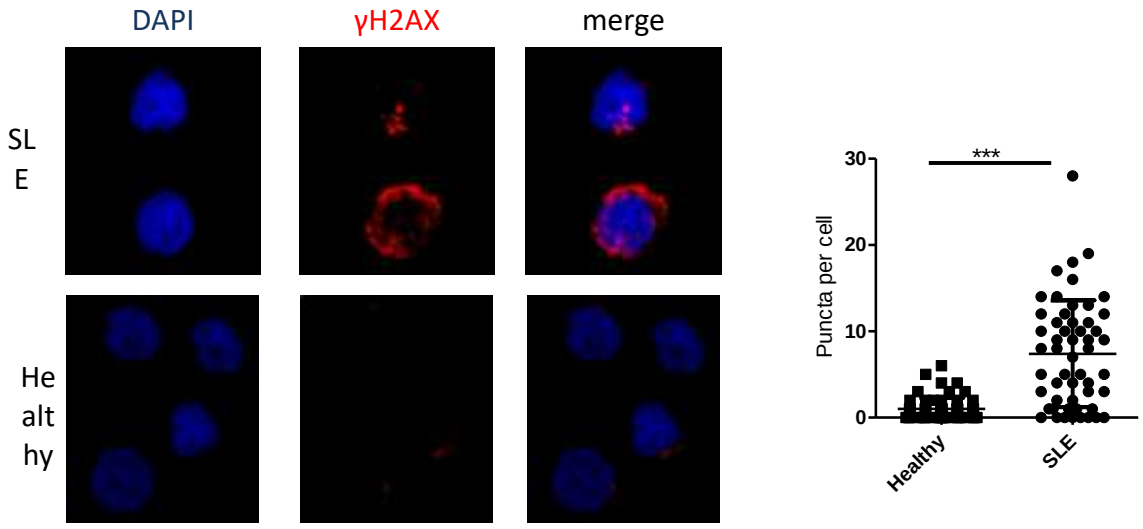

Figure 4.26. Representative confocal microscopy images for γ-H2AX (red), and DAPI (blue), and γ-H2AX puncta/cell in sorted CD34⁺ cells from BM of SLE patients (*n*=3) and HC (*n*=2) (Leica TCS SP5 63x, Scale bar: 10 μM). One representative experiment is shown. Results are mean ± SEM. Statistical significance was obtained by unpaired Student's t test (*P≤0.05, **P≤0.01, ***P≤0.001).

To further elucidate the differences of gene expression within SLE patients, we grouped them according to various parameters such as SLEDAI, nephritis, treatment, PGA score, activity and disease severity. Only the gene expression comparison based on disease severity (mild/moderate vs severe) revealed distinct profiles and had meaningful results about disease progression. We found 1,176 DEGs between severe and moderately severe patients with 867 upregulated and 309 downregulated genes (Figure 4.27A). Increased expression of myeloid markers as well as apparent downregulation of lymphoid markers indicate that patients with both severe and moderate disease are characterized by a bias towards myelopoiesis (Figure 4.27B). Notably, expression of specific markers for granulopoiesis –such as *CEBPZ, CEBPD, GATA2*- was increased in a subgroup of severe SLE patients compared to those with moderate disease (Figure 4.27B), a result consistent with our findings for the lupus LSK transcriptomic profile. GSEA revealed positive correlation with 'Cytokine-cytokine receptor interaction' and 'Positive Regulation of Locomotion' gene sets in patients with severe SLE as there was notable enrichment of secreted factors which interact with stem cells urging them to migrate (Figure 4.27C). Enrichment analysis using upregulated genes in severe SLE revealed an overrepresentation of chemotaxis and migration of granulocytes and neutrophilic GO terms (Figure 4.27D). Thus, HSPCs are activated and more proliferative in SLE patients compared to HC demonstrating a distinct transcriptional differentiation profile in patients with severe disease.

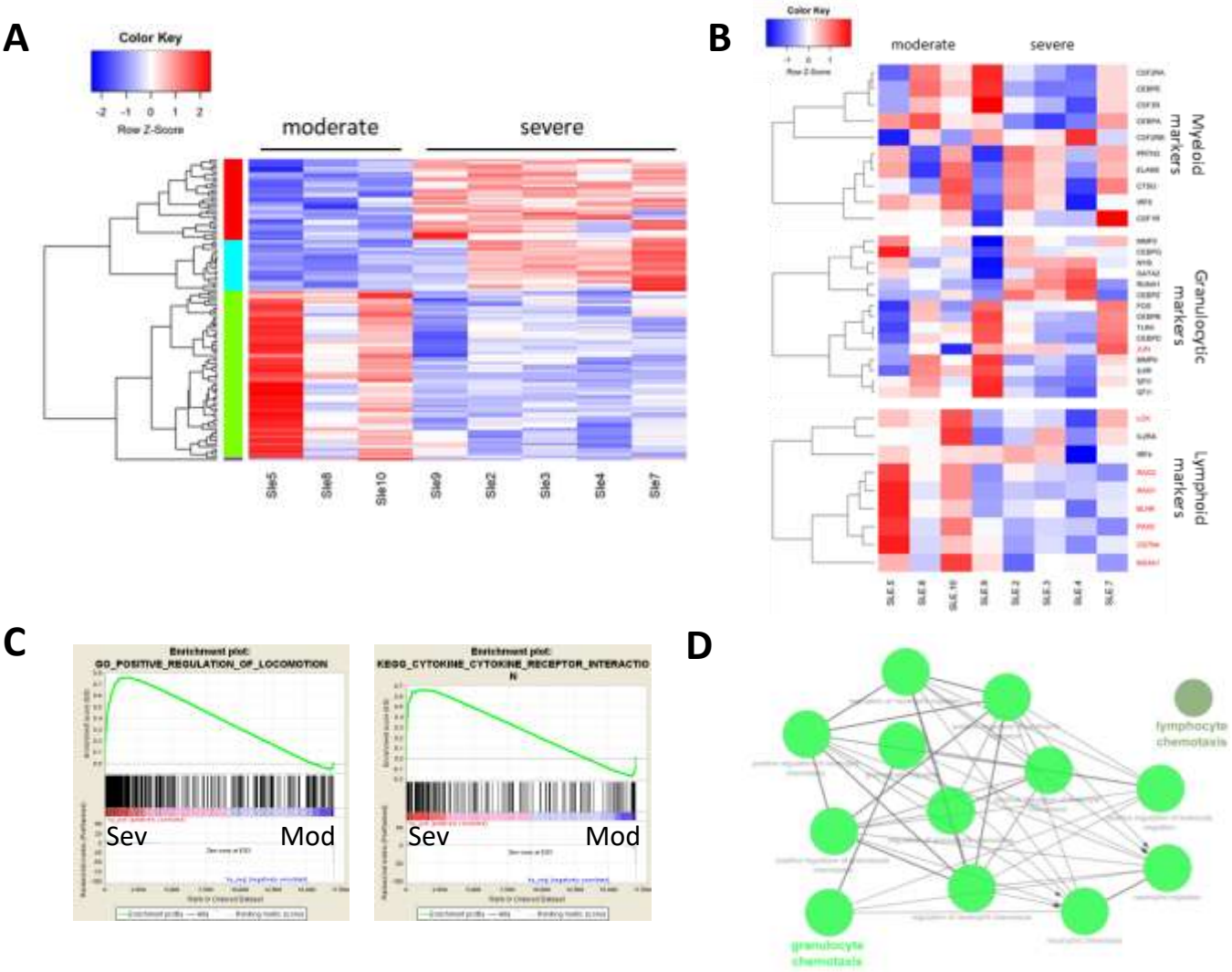

Figure 4.27. (A) Heatmap of DEGs in CD34⁺ cells isolated from BM of SLE with moderate (*n*=3) and severe (*n*=5) disease. (B) Heatmaps of genes associated with myeloid, lymphoid and granulocytic markers in CD34⁺ cells isolated from BM of SLE with moderate (*n*=3) and severe (*n*=5) disease. Genes with p<0.05 are marked in red. (C) GSEA plot showing the enrichment of "GO Positive Regulation of Locomotion" (NES 1.58, FDR 0.0053) and "KEGG Cytokine-Cytokine Receptor Interaction (NES 1.30, FDR 0.042) gene sets in CD34⁺ SLE severe patients. (D) Network of the upregulated genes associated with migration and chemotaxis of granulocytes and neutrophils using ClueGo plug-in in Cytoscape.

Periphery

Based on previous results concerning the SLE mouse model, we asked similarly whether there are any HSPCs circulating in the periphery. Using flow cytometry, we determined the frequencies of HSPCs and their subsets in the PB of healthy subjects and SLE patients. $CD34^+$ cells showed increased frequency in the PB of SLE patients (Figure 4.28A, B). Thus, we proceed to its subsets, the $CD34^+CD38^-$ population which represents more primitive cells, and the $CD34^+CD38^+$ which represents more differentiated cells. As it shown in Figure 4.28C, the enrichment comes from the $CD34^+CD38^-$ cells which were found to be increased specifically in the patients with more severe disease. These facts are consistent with our murine data and together propose that HSPCs in SLE are activated and highly immobilized cells. Similar profiling for the myeloid progenitor subsets showed no significant difference between healthy subjects and SLE patients (Figure 4.28D).

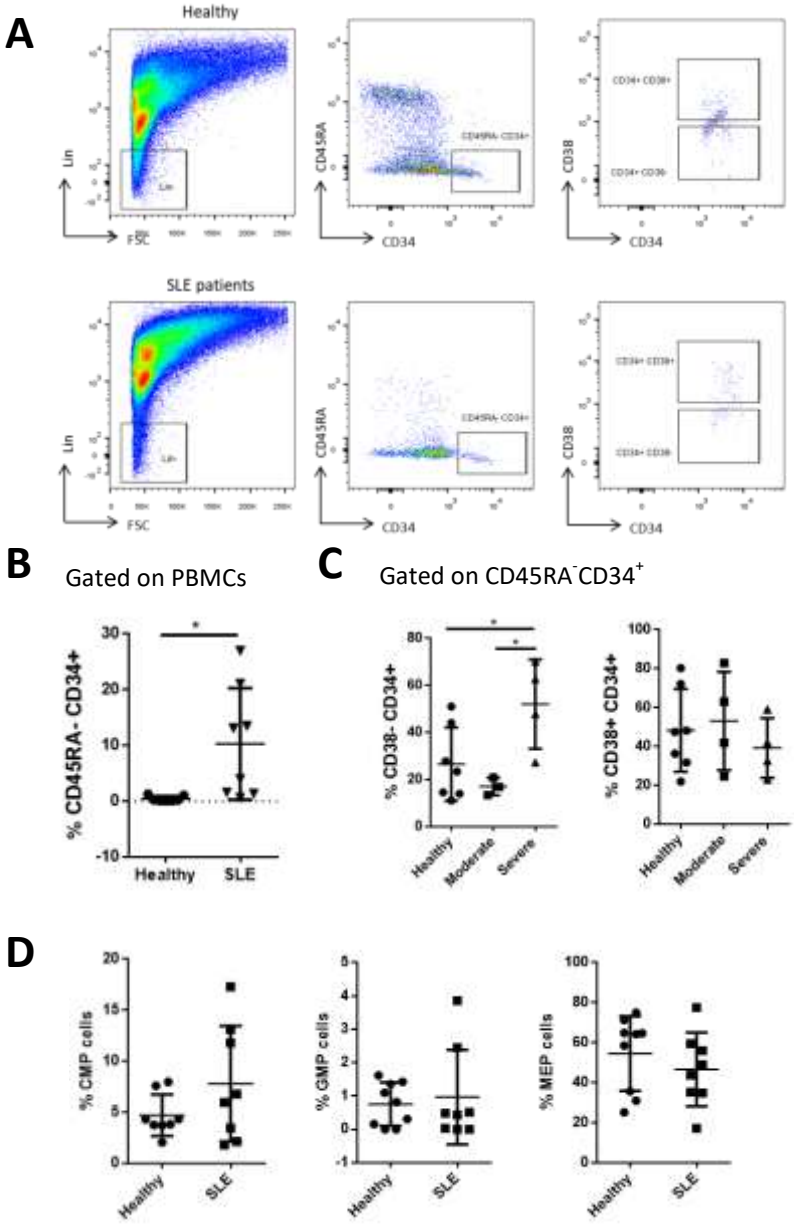

Figure 4.28. Phenotypic analysis of hematopoietic and progenitor cells in the peripheral blood.
(A) Representative flow cytometry analysis of HSPCs of HC and SLE patients.
(B) Frequencies of $CD34^+$ cells ($CD34^+CD38^-$) of HC and SLE patients.
(C) Frequencies of progenitor cells ($CD34^+CD38^+$) of HC and SLE patients with severe/moderate disease.
(D) Frequencies of CMP ($CD34^+CD38^+CD123^+CD45RA^-$), GMP ($CD34^+CD38^+CD123^+CD45RA^+$) and MEP ($CD34^+CD38^+CD123^-CD45RA^-$) cells of HC and SLE patients (n=4-9, *P≤0.05, **P≤0.01, ***P≤0.001).

Next, we further determined the frequency of each blood cell type in the PB from SLE patients and healthy subjects. Concerning the myeloid lineage cells, monocytes ($CD14^{+}$) and their subsets ($CD4^{+/-}CD16^{+/-}$) did not show any significant difference between SLE and healthy individuals (Figure 4.29 A&C). Neutrophils showed a slight not significant increase in SLE patients, but when the patients were grouped based on the severity of the disease, we noted that neutrophils were decreased at the moderate cases, and increased back to healthy levels when the disease was severe (Figure 4.28F). Between the lymphoid lineage subsets, pDCs ($CD14^{-}HLA\text{-}DR^{+}CD123^{+}$), Th ($CD4^{+}$), Treg ($CD4^{+}CD25^{+}CD127^{-}$) and Tc ($CD8^{+}$) had no significant differences between healthy subjects and SLE patients (Figure 4.29 B,D&E). However, Teff ($CD4^{+}CD25^{-}CD127^{+}$) cells were increased in patients (Figure 4.29 B&D) and B ($CD19^{+}$) cells showed decrease in SLE patients (Figure 4.29E) which is expected as previously described (Tsokos, 2001)

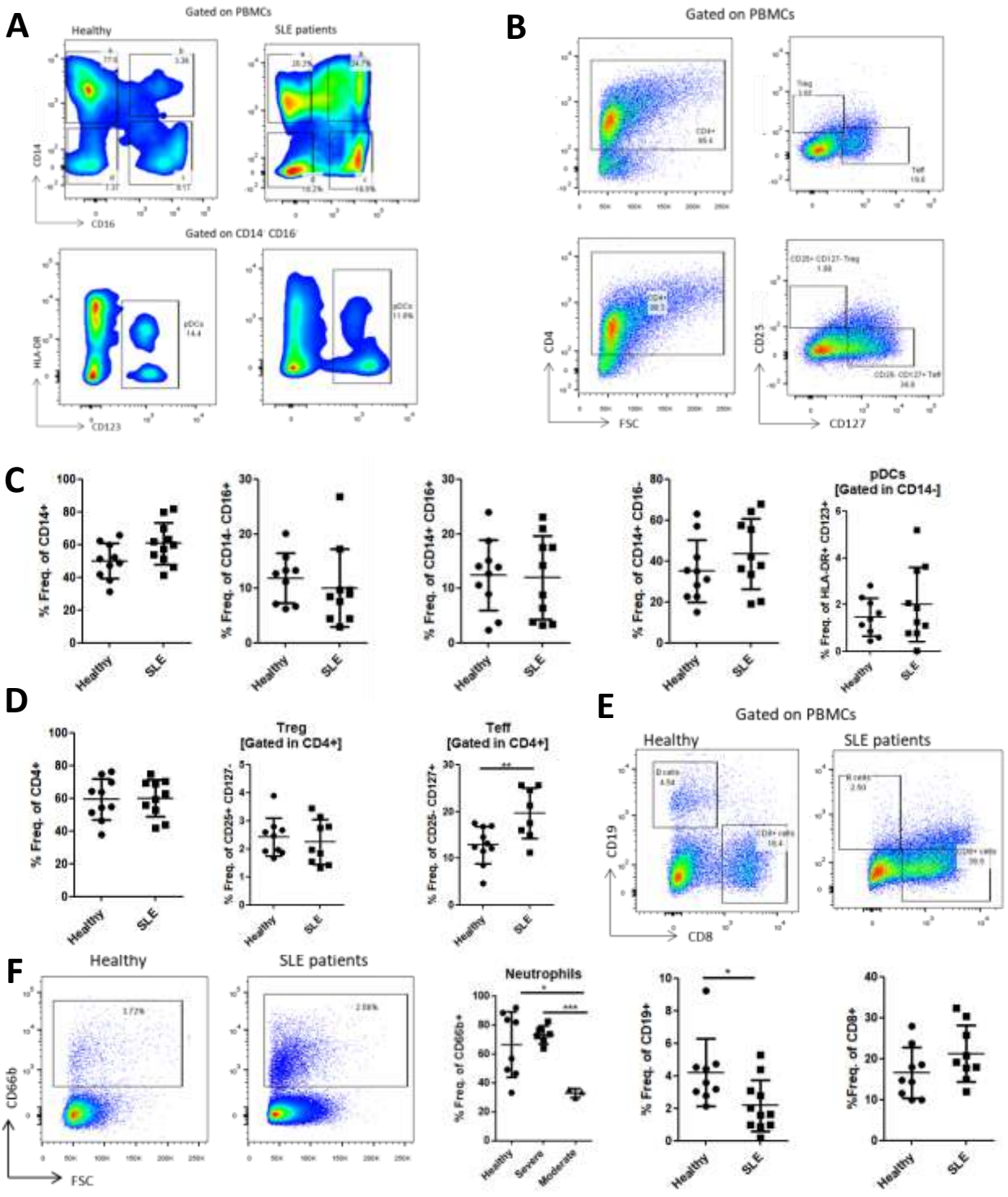

Figure 4.29. Phenotypic analysis of terminally differentiated cells in the peripheral blood. Representative flow cytometry analysis and frequencies of (A,C) pDCs, monocytes and their subsets, (B, D) Th, Treg, Teff, (E) Tc and B cells, and (F) neutrophils. (*n*=6-9, *P≤0.05, **P≤0.01, ***P≤0.001).

Comparison of human and murine hematopoietic stem and progenitor cells in SLE

Human lupus CD34+ with murine lupus CMP cell transcriptome reveal common attributes with evidence of differentiation arrest at the progenitor stage

To assess whether the results from mouse model can be corroborated in the context of human disease, we compared the human and mouse RNA-seq data based on previously described method (Ntougkos et al., 2017). Human BM-derived CD34+ cells include both the most primitive cells (HSPCs) as well as the progenitor cells (Strobl et al., 1993) which correspond to both LSK and CMP in the mouse model. Therefore human CD34+ data were compared to mouse LSK and mouse CMP data in parallel. There was a significant overlap of mouse LSK DEGs (6.7% with representation factor/RF=4.2, $p<2.7x10^{-29}$) and mouse CMPs DEGs (8.5% with RF=4, p-value$<4.7x10^{-18}$) with the humans CD34+ DEGs (Figure 4.30A & 4.31A, upper panel respectively) despite the interspecies variability and the high interpatient variability in humans. The expression of the common DEGs (26) between mouse LSK and human CD34+ cells revealed that the majority is downregulated in human SLE patients in contrast to the mouse model (Figure 4.30B). However, the expression of the common DEGs (54) between mouse CMPs and human CD34+ cells showed commonalities (Figure 4.30B). It was therefore shown that the SLE CD34+ transcriptomic profile is more similar to murine lupus CMPs than to LSK. This is likely due to the fact that the 'stem cell' LSK signature might be diluted in the heterogeneous CD34+ population in humans.

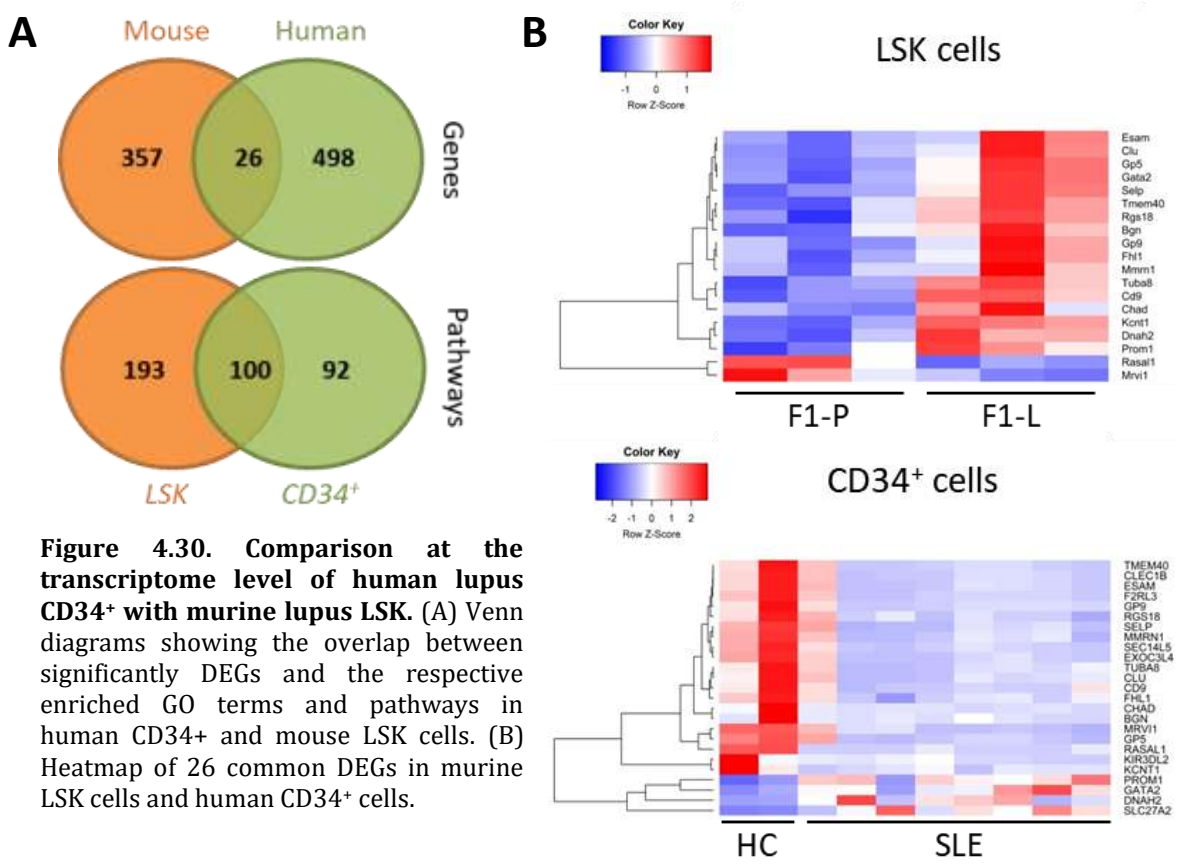

Figure 4.30. Comparison at the transcriptome level of human lupus CD34+ with murine lupus LSK. (A) Venn diagrams showing the overlap between significantly DEGs and the respective enriched GO terms and pathways in human CD34+ and mouse LSK cells. (B) Heatmap of 26 common DEGs in murine LSK cells and human CD34+ cells.

At pathway level, the overlap between mouse and human data was more prominent. Compared to gene level results, there was a 5-fold higher representation of the enriched pathways in LSK cells (RF=81, p<6.8x10-172) and a 2-fold higher representation of the enriched pathways in CMPs (RF=43.3, p<4.8x10-178) which were also found enriched in human HSPCs (Figure 4.30A & 4.31A, lower panel respectively). Key pathways such as cell activation, regulation of cell differentiation, immune system development and leukocyte migration were shared. Using RNEA algorithm to define associated regulators within CMP and $CD34^+$, we found 15 suppressed regulators in both lupus CMP and $CD34^+$ SLE cells. Among them, there were significant disease specific effectors such as IFNγ and IL-6 as well as regulators for the homeostasis-differentiation balance of stem cells like Lif, Ets1 and Pax5 (Figure 4.31C).

Next, we asked whether lupus LSK have more similarities with human $CD34^+$ when the patients' heterogeneity is diminished. To this end, correlation analysis performed between the murine lupus LSK and the human dataset based on disease severity. There was significant overlap both at gene level (RF=7.4, $p<8x10^{-8}$) and at pathway level (RF=86.2, $p<1.6x10^{-79}$) (Figure 4.32A). It is remarkable that the expression of the common DEGs (12) between mouse LSK and human $CD34^+$ cells have similar expression pattern. The majority of them are downregulated when the disease is at clinical stage, either in F1-L mice or in patients with severe disease (Figure 4.32B-C). The DEGs are associated with lymphoid differentiation at the BM niche, which means that the myeloid lineage is preferred.

Collectively, human $CD34^+$ cells from SLE patients display more commonalities in their transcriptomic profile with murine lupus CMPs rather than LSK. This comparison indicates an arrest at the progenitor level both in murine and human lupus hematopoiesis as important regulators for terminal differentiation are downregulated. However, human $CD34^+$ cells from severe SLE patients revealed similar expression pattern with murine lupus LSK cells, proposing that the myeloid lineage is favored when the disease is full-blown.

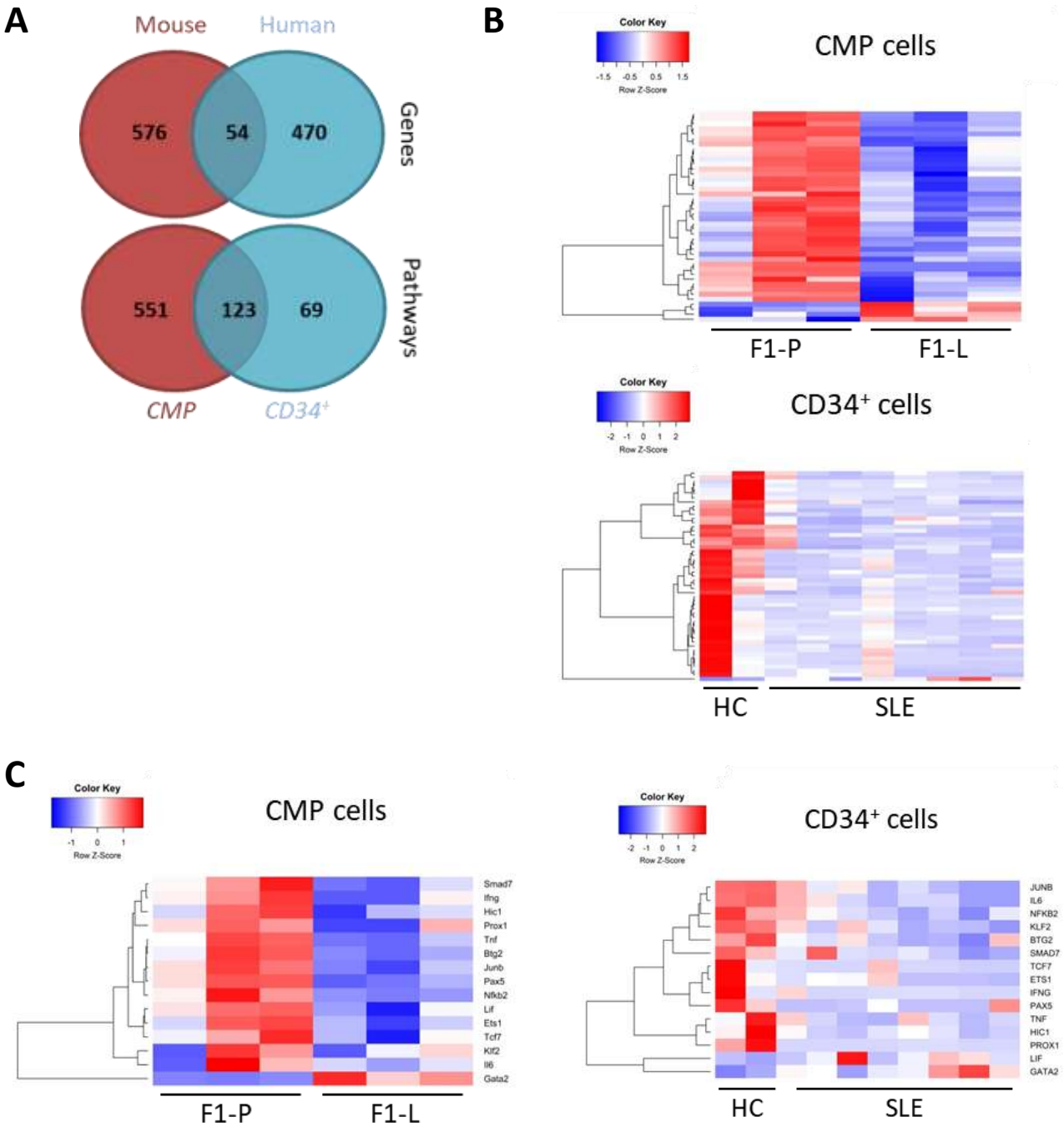

Figure 4.31. Comparison at the transcriptome level of human lupus CD34⁺ with murine lupus CMP. (A) Venn diagrams showing the overlap between significantly DEGs and the respective enriched GO terms and pathways in human CD34⁺ and mouse CMP cells. (B) Heatmap of 54 common DEGs in murine CMP cells and human CD34⁺ cells. (C) Heatmap of the enriched transcription factors and regulators in murine CMP cells and CD34⁺ cells based on RNEA algorithm.

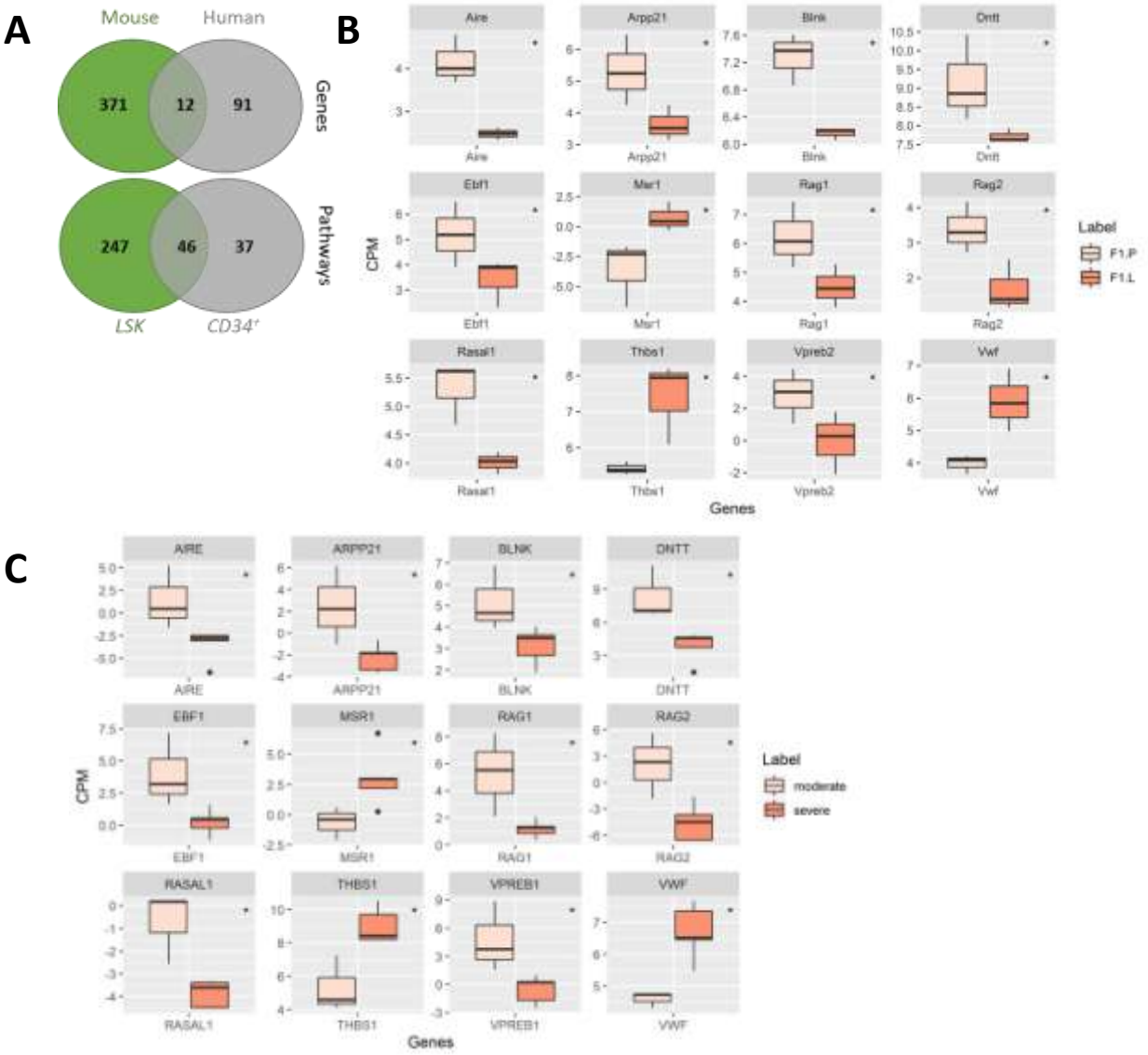

Figure 4.32. Comparison at the transcriptome level of severe human SLE CD34⁺ with murine lupus LSK. (A) Venn diagrams showing the overlap between significantly DEGs and the respective enriched GO terms and pathways in human CD34⁺ from severe and moderate patients and mouse LSK cells. (B) Boxplot of 12 common DEGs in murine LSK cells. (C) Boxplot of 12 common DEGs in severe and moderate SLE patients.

Bone marrow niche and microenvironment

Bone marrow niche is a complex system where several cell types exist with HSPCs. The interactions between the cells and the secreted factors make the BM milieu which is critical for the development and the differentiation of the hematopoietic cells, from the most primitive to the terminally differentiated cell.
We asked how the lupus microenvironment could influence the HSPCs. For this reason, wild-type LSK (B6-Y) were sorted and cultured *in vitro*. After 24h, different stimuli were added for 24h and then some basic properties of the cells were checked (Figure 4.33.).

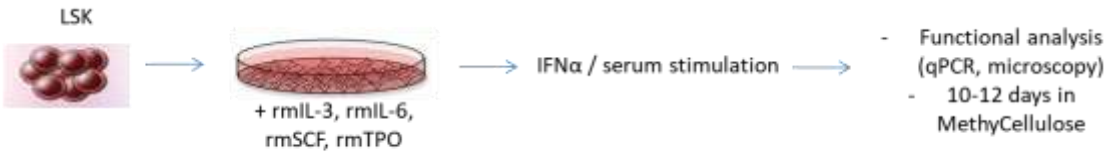

Figure 4.33. Schematic representation of the *in vitro* system. Sorted BM-derived LSK cells cultured *in vitro* with the appropriate cocktail of cytokines and medium to maintain their stemness. After 24hrs, the cells were stimulated *either* with serum (24 hrs) and transferred to the appropriate culture for CFU assay *or* with IFNα (12hrs) and proceed with functional analysis.

To recapitulate the BM milieu *in vitro*, we added serum isolated from peripheral blood of F1-P and F1-L mice -as it is the most representative and easily isolated material. The differentiation potential of wild-type HSPCs was measured after serum stimulation with CFU assay. F1-L serum drive differentiation towards myeloid lineage and more specifically towards granulocytes, as CFU-GM and CFU-G were increased whereas CFU-M were decreased (Figure 4.34). The results do not reach statistically significance, however the trend driven by F1-L serum is clear.

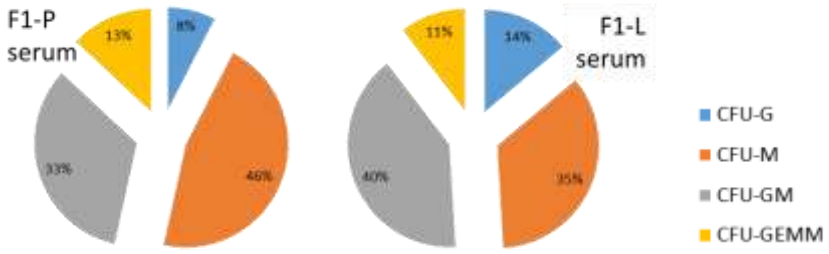

Figure 4.34. CFU assay after serum stimulation on wild-type LSK. Sorted B6-Y LSK stimulated with F1-P or F1-L serum, transferred to MethylCellulose for 10-12 days to form colonies.

Serum is mixed with many different cytokines and chemokines, therefore we used only IFNα stimulation as it is the most abundant and crucial cytokine in SLE (Panousis et al., 2019). Firstly, we measured the expression of *Isg15* gene, typical responsive gene in the IFN type I pathway, in order to ensure that LSK cells are responsive to IFNα stimulation in

accordance with Essers et al (Essers et al., 2009). LSK are responsive to IFNα stimulation even in the first six hours (Figure 4.35). Based on the results, we chose to use the concentration of 100ng/ml IFNα for 12 hours in culture which is considered as chronic phase (Mann et al., 2018) and is closer to the disease.

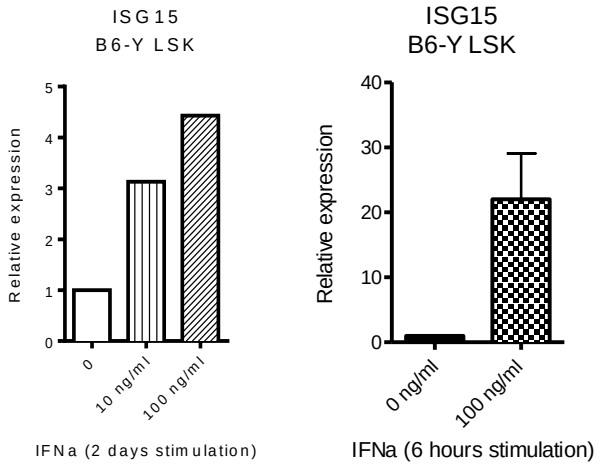

Figure 4.35. Gene expression of *Isg15* in B6-Y LSK after IFNα stimulation. Two different concentration were used (10ng/ml and 100ng/ml) at two different time points (6hrs and 2 days).

Based on transcriptomic results, lupus LSK have differentiation preference towards myeloid/granulocytic lineage. Among the up-regulated genes that are crucial regulators for this process is *Cebpα*. We asked whether IFNα could influence its expression. For this reason, F1-P, F1-L and B6-Y LSK cells were isolated and cultured *in vitro*. After 24 hrs in culture, IFNα was added as stimuli for 12 hours and *Cebpα* expression was measured. There were comparable levels among the three conditions (Figure 4.36) which could indicate that either IFNα alone cannot make the difference on *Cebpα* expression or a single dose is not enough.

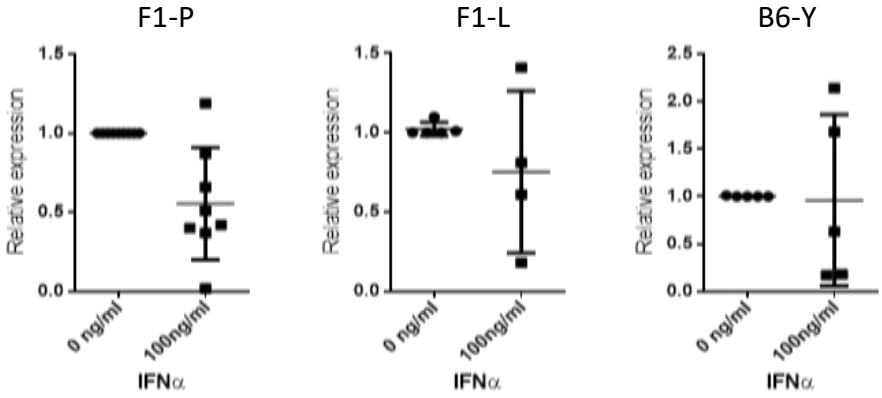

Figure 4.36. Gene expression of *Cebpα* in LSK cells after chronic IFNα stimulation (12hrs). F1-P (left), F1-L (middle) and B6-Y (right) LSK cells were cultured *in vitro*. Expression is normalized to HPRT.

However, there was great variation of the expression levels of *Cebpa* after IFNα stimulation. Therefore, we hypothesized that *Cebpa* may be regulated by IFNα pathway. *In silico* analysis of *Cebpα* promoter was performed using the program Genomatix in order to find possible

binding sites of transcription factors associated with IFNα stimulation (Figure 4.37). The analysis revealed that there are several binding sites of IFNα-associated factors. IRF4 belongs to those factors whose expression follows *Cebpα* expression during the progression of the disease. Based on the RNA-seq data, GO and pathway analysis revealed that there is enrichment of the myeloid factors at F1-L LSK cells compared to the F1-P where Irf4 and Cebpa are implicated as well. Taking into account all the results, it seems that there is a combination and an interplay between the stem cells and their niche that drive the disease.

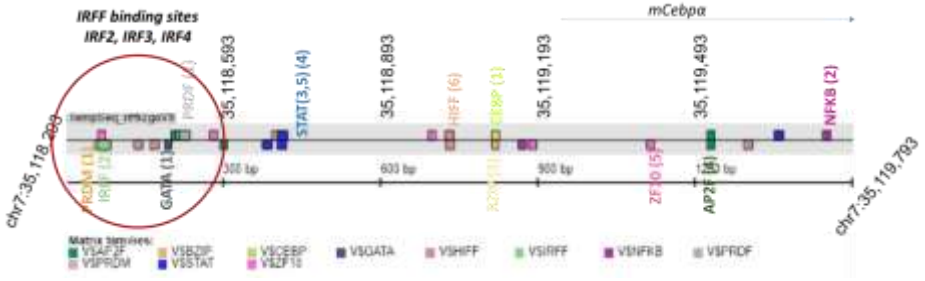

Figure 4.37. *In silico* promoter analysis of *Cebpα* gene using Genomatix tool.

Bone marrow-on-a-chip

The 3D microfluidic cell culture devices, lined by living cells, manage to recapitulate the heterogeneity of the cellular microenvironment by providing the essential mechanical cues and physicochemical gradients to promote cell organization into tissue-like structures and enable tissue-tissue interaction towards a faithful *in vitro* emulation of an organ's vital functional unit (Huh et al., 2011; Huh et al., 2013).

Our goal is to construct bone marrow-on-a-chip (BMoC), a novel 3D microfluidic device that pioneers purely *in vitro* and scaffold-free development of BM niche recapitulating hematopoiesis. Until now, BM models are either hybrid *in vivo*/*in vitro* models (Torisawa et al., 2014) or incorporate bone microstructure-mimicking matrices(Sieber et al., 2018). This system aims to study the hematopoiesis in the context of SLE milieu, recapitulating the structural and physiological properties of the hematopoietic niche. To this end, we collaborated with Dr. Gogolides team who were responsible for the technical part of the BMoC. In order to construct the final version of the chip, we first tested simple devices with microchambers. We asked whether the cells can grow inside them and how easily we can manipulate them.

Figure 4.38. Sealed microchambers (cell culture layer bonded with flat PDMS layer and glass slide) filled with human Umbilical Cord-derived MSCs.

The BM microenvironment is built up from the stroma, mainly derived from MSCs, and the marrow part consisting of the HSCs and their progeny (Uccelli et al., 2008). The first step was to culture efficiently MSCs in the microchamber in order to grow properly and create the stroma of the BM. In order to standardize the conditions, Umbilical Cord (UC)-derived MSCs (donored from Hellenic Cord Blood Bank-HCBB, BRFAA) were used, as MSCs from adult human donors are not easily available.

UC-derived MSCs have been injected inside the sealed microchambers at density of ~1.400 cells/μL and incubated in sterilized conditions (37^{o}C with 5% CO_2 supply) for a time period of 8 days (Figure 4.38). Medium renewal was performed every 24h. Optical observation has proved the successful viability and formation of BM tissue throughout the experiment, gradually developing and building up a 3D matrix (Figure 4.39). As depicted, MSCs are capable to attach and growth properly in the microchamber. Another important finding is that day 5 will be the timepoint when HSCs will be added on the chip, as the evolution of the 3D structure is stable after that day. Concluding, the data are very promising for on-chip growth and preservation of the BM stroma to further support HSCs seeding, and finally, study their contribution to SLE pathogenesis.

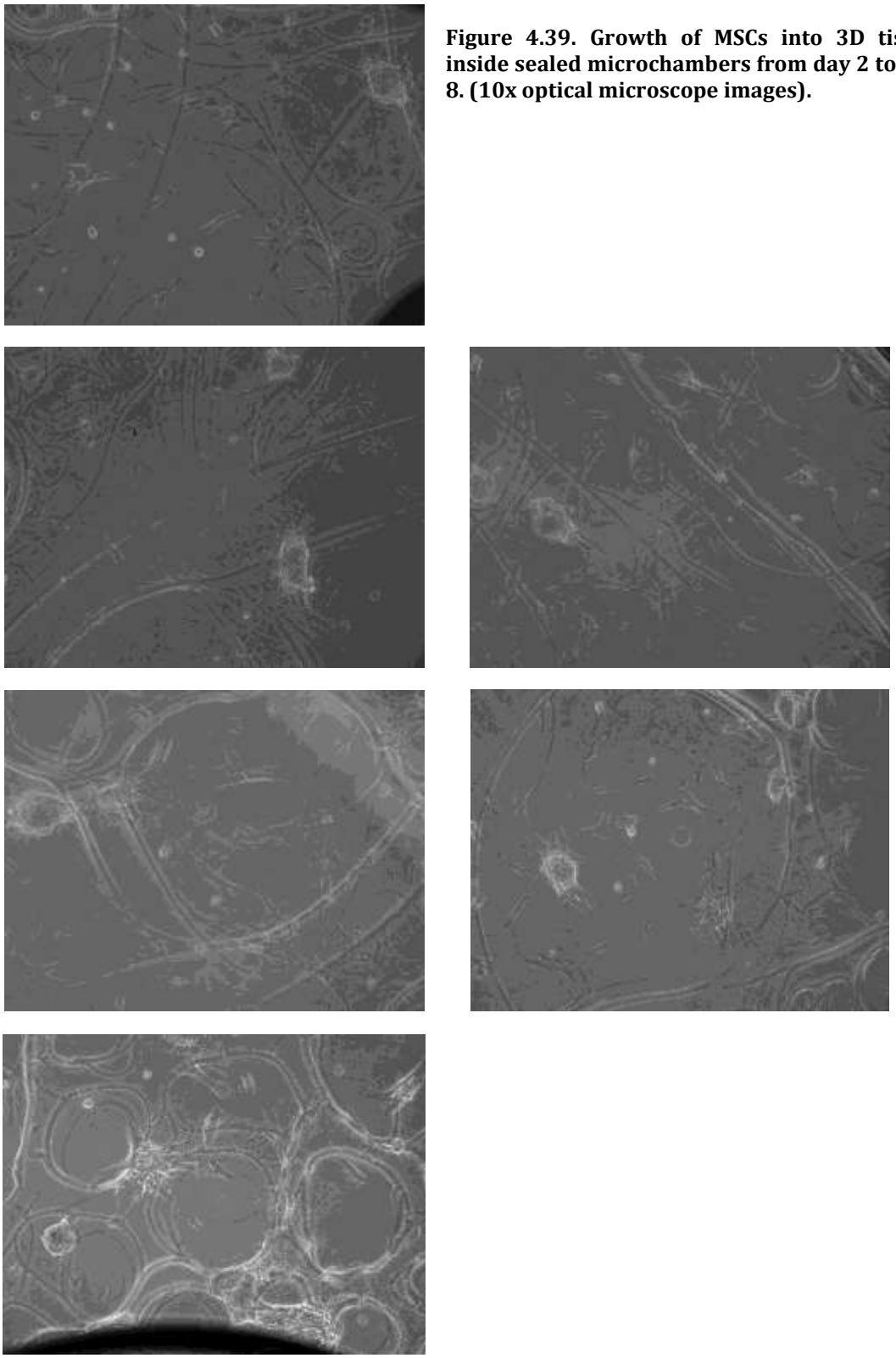

Figure 4.39. Growth of MSCs into 3D tissue inside sealed microchambers from day 2 to day 8. (10x optical microscope images).

5. Discussion

Blood and immune cells derive from HSPCs, which reside in the BM in quiescent state, being ready to respond to stress, such as severe infection, systemic inflammation, or iatrogenic myeloablation (Mendelson and Frenette, 2014). Recent data suggest significant heterogeneity within the HSPCs with evidence of early lineage segregation, lineage-biased existence and containment of lineage-restricted progenitors (Carrelha et al., 2018). These lineage-biased and lineage-restricted cells within the phenotypic HSPC compartment might serve as an emergency backup for stress conditions, capable of efficiently and specifically counter-balance the sudden loss of a particular lineage. Herein, we provide evidence of dysregulated differentiation during hematopoiesis in SLE. Transcriptomic data demonstrate enhanced activation and differentiation preference towards myeloid/granulocytic lineage after disease onset. NZB/W F1 exhibit detectable levels of type-I interferon compared to other SLE mouse models (Zhuang et al., 2015). We show that –indeed- IFN signature is present in the NZB/W F1 model and that lupus HSPCs can sense and respond to IFN. Chronic activation of the IFN-α pathway in HSPCs impairs their function whereas acute IFN-α treatment promotes the proliferation of dormant HSPCs (Essers et al., 2009).

Our flow cytometric analysis revealed enhanced proliferation of LSK in lupus mice and increase of their subpopulations. This finding is consistent with findings by Niu et al in a congenic lupus model where they found a genetic polymorphism on the *Cdkn2c* gene related to cell cycle (Niu et al., 2013). In the context of stem cell proliferation and activation, Walter et al showed direct response of HSPCs by exiting quiescence with concomitant DNA damage (Walter et al., 2015). In agreement to this, lupus LSK had more DNA damage compared to their controls which could be detrimental for their maintenance and self-renewal. Pronounced cell cycle entry and consequent proliferative stress may result in impaired HSC self-renewal potential (Behrens et al., 2014; Moehrle and Geiger, 2016). In addition to replicative stress, apoptosis may also take place in HSPCs within the highly inflammatory environment of lupus. Increased apoptosis of BM-derived $CD34^+$ cells in SLE patients have reported by Pyrovolaki et al. where they characterized a novel role for the CD40/CD40L in SLE mediating the Fas-apoptosis of progenitor cells (Pyrovolaki et al., 2009).

CXCR4/CXCL12 signaling is essential to confine HSPCs in the proper niche and controls their proliferation as showed by Nie et al (Nie et al., 2008). Another crucial role for CXCR4/CXCL12 axis is the prevention of ROS elevation, apoptosis and DNA damage in HSPCs (Zhang et al., 2016). In SLE, we found increased replication stress which results in increased DNA damage and apoptosis. In parallel, increased CXCR4 expression could indicate a compensatory mechanism for maintaining HSPC integrity due to systemic inflammation.

To confirm the transcriptomic results on LSK differentiation, we profiled CMPs. Physiologic myelopoiesis evolves through MPPs to lineage-restricted CMPs and then converges to GMPs (Akashi et al., 2000). Phenotypic analysis of progenitors showed increased frequency of CMPs, but decreased frequency of GMPs, as evidenced in RNA-seq by "dampening" of differentiation after the CMP stage. Myeloid skewing is –in part- expected due to inflammation and aging (Kovtonyuk et al., 2016) –both operant in our model. Our results suggest "priming" of LSK with a pronounced 'myeloid/granulocytic signature' but its downregulation as the differentiation evolves towards canonical myelopoiesis (Figure 5.1).

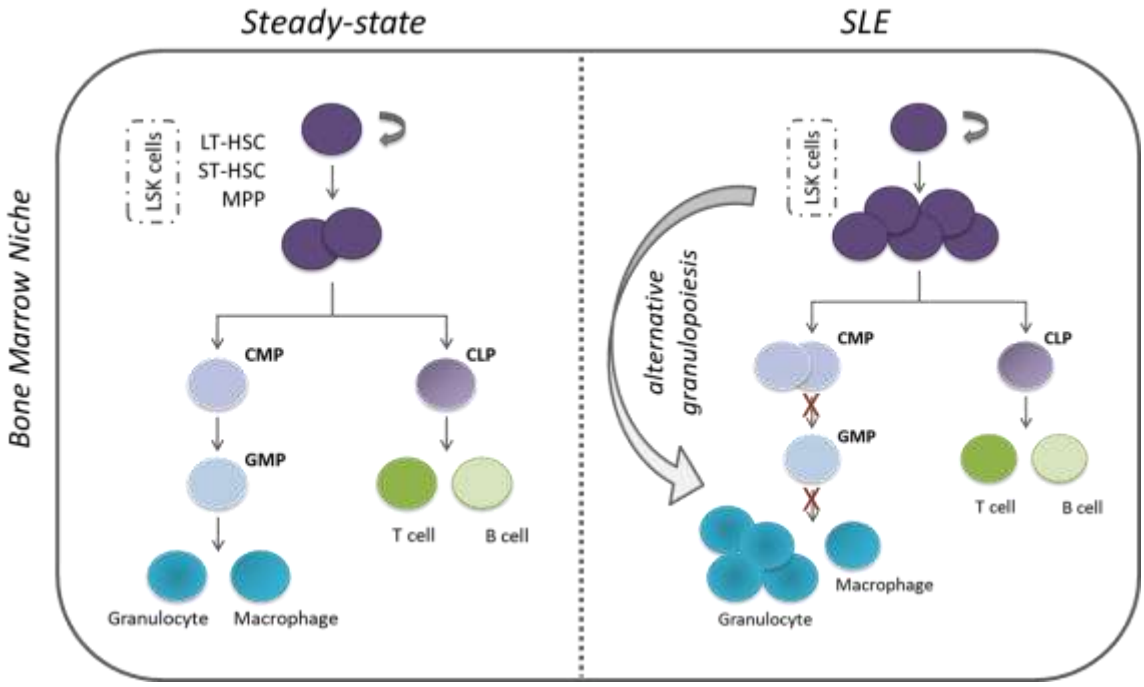

Figure 5.1. Proposed model of alternative granulopoiesis in SLE. LSK in SLE are pre-defined to differentiate towards the granulocytic lineage by skipping the CMP and GMP stages of the hematopoietic lineage.

Increased neutrophils in lupus BM suggest deregulation of homeostatic mechanisms in the level of CMPs with "priming" of LSK towards the granulocytic differentiation at the expense of lymphopoiesis. These results are consistent with our earlier findings of strong granulopoiesis signature in the BM by using DNA arrays (Nakou et al., 2008). "Priming" of LSK highly correlates with the signature of HSPCs after 'training' with β-glucan (Mitroulis et al., 2018b) strongly indicating that "SLE inflammatory milieu" promotes the immune training memory of BM progenitor cells (Figure 5.2). The dysregulated inflammatory responses in rheumatic diseases is the establishment of memory-like cellular phenotypes that can either promote or inhibit the responses of innate immune cells to environmental stimuli (Donlin et al., 2019). Accordingly, we found DMRs from lupus HSPCs overlapping with transcription factor binding sites relevant to hematopoietic development, including Cebpα (Figure 4.21). Innate immune memory, while beneficial to host defense against pathogens, could also lead to maladaptation of the immune system in chronic inflammation, leading to perpetuation of chronic inflammatory disorders and predisposing to flares in response to environmental stimuli such as infections or stress (Penkov et al., 2019).

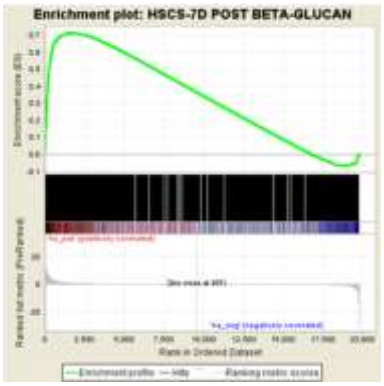

Figure 5.2. Trained immunity-like phenotype of F1-L LSK in the bone marrow. GSEA plot showing the enrichment of "HSCs 7 days post β-glucan' (NES 1.50, FDR<0.001) gene set in LSK F1-L mice. Trained immunity signature of HSCs was adopted by Mitroulis et al.

Myeloid cells are crucial for disease progression. In the periphery of lupus mice, we found increased circulating LSK, but decreased neutrophils. This could be due to either extensive destruction of neutrophils in the periphery or migration to target tissues. This might act as a positive feedback loop where inflammatory environment triggers priming and exit of HSPCs to periphery, driving them to increased myeloid output which in turn circulate and perpetuate the inflammation as proposed by Oduro et al. in an arthritis mouse model (Oduro et al., 2012). It is conceivable that neutrophils may migrate to the inflamed tissues hence their relative paucity in the periphery.

Neutrophils represent the most abundant leukocyte population and traditionally recognized as one of essential effector cells of the innate immune system in humans. Under basal conditions, the great majority of neutrophils reside in the BM. In response to infection or other stress, this pool of neutrophils can be mobilized into the blood, providing the host with a mechanism to rapidly increase neutrophil delivery to sites of infection. After maturation, neutrophils are retained in the BM through CXCR4 signaling, and CXCR2 signaling drives their release into the circulation (Devi et al., 2013). We found decreased CXCR4 expression in F1-L neutrophils which indicate their trend to migrate, but whether this expression is concomitant with CXCR2 expression as Evrard et al. showed (Evrard et al., 2018) needs more investigation. It is essential that neutrophil numbers in the blood are tightly regulated. Patients with SLE are characterized by a strong neutrophil and deregulated autophagy gene signature. Persistent neutropenia is associated with profound immunodeficiency, whereas excessive neutrophil infiltration and activation contributes to tissue damage in certain inflammatory disorders, such as rheumatoid arthritis. The release of neutrophil extracellular traps (NETs) represents a novel neutrophil effector function contributing to thromboinflammation and fibrosis in SLE (Frangou et al., 2019).

It has been assumed that various blood cell lineages arise via a hierarchical scheme -starting with HSPCs- and that their differentiation potential becomes increasingly restricted through oligopotent and then unipotent progenitors. However, recent work suggests a developmental shift to an adult "two-tier" hierarchy whereby HSPCs can generate restricted

subsets of terminally differentiated progeny, bypassing the stepwise progression through common progenitor stage (Notta et al., 2016). Yammamoto et al. proposed a revised model of hematopoietic differentiation with the existence of progenitors within the HSPC compartment, mostly myeloid-committed ones (Yamamoto et al., 2013). Our results are in agreement with this model. It is reasonable to assume that SLE LSK are already pre-defined to differentiate towards granulocytic lineage creating an alternative granulopoiesis pathway in the hematopoietic tree (Figure 5.1). In parallel, differentiation arrest at the intermediate stage of CMPs blocks flow of hematopoiesis towards GMPs.

Peripheral blood $CD34^+$ fraction contains cells with the same cell surface markers found in human BM samples (Cimato et al., 2016) which is in consistent with our results finding circulating HSPCs in murine and human periphery. Circulating HSPCs could perpetuate the systemic inflammation by producing primed myeloid cells. Previous studies from our group (Panousis et al., 2019) have shown that blood transciptome from SLE patients are characterized by a specific signature based on the course of the disease (susceptibility, activity, severity) which could facilitate to personalized care. However, focused analysis in children with SLE display unique signature related to granulopoiesis and induced by interferon in their periphery (Bennett et al., 2003). Conclusively, these suggest that gene expression pattern in SLE might not be that variable and could stem from BM niche.

In summary, we have presented evidence for deregulation of granulopoiesis and priming of HSPCs which may contribute to persistent inflammation in SLE and risk for flare once the disease is in remission. Myeloid skewing of HSPCs, associated with epigenetic tinkering, is also typical of HSPCs during aging (Dykstra et al., 2011; Yamamoto et al., 2013), contributing to decreased adaptive immunity and enhanced cardiovascular mortality of elderly population (Jaiswal et al., 2014; Jaiswal et al., 2017; Leins et al., 2018). Re-establishment of the appropriate lymphoid versus myeloid balance in systemic autoimmune diseases may improve immune function decreasing risk of infection or atherosclerosis and resolution of inflammation.

Appendix

Table 1. Detailed clinical and serological items for each SLE patient.

Sample ID	Age	Proteinuria (mg)	Serum albumin levels (g/dl)	ANA titer	Anti-dsDNA & titer	C3/C4 levels	Nephritis	NPSLE	Serositis	Arthritis	SLEDAI	Severity pattern	Medication at Bone Marrow Aspiration	Cumulative dose of Glucocorticoids over the last month	Past Immunosuppressive medication
SLE.2	54	0	3	1:640	positive moderate	low	-	-	-	Yes	15	Severe	PRE (15mg), HCQ (400mg), CYC (6g)	315 mg	AZA, MMF, RTX
SLE.3	62	0	4.5	1:640	positive low	low	-	-	-	Yes	5	severe	PRE (20mg)	3,935 mg	RTX
SLE.4	82	2300	3.5	1:160	negative	low	+	-	+	Yes	14	Severe	PRE (15mg)	3,230 mg	None
SLE.5	35	0	3.9	1:1280	negative	low	-	-	-	Yes	1	Moderate	HCQ (200mg)	0 mg	None
SLE.7	50	0	3.9	1:1280	positive low	low	History of LN	History of NPSLE	History of serositis	Yes	4	Severe	PRE (10mg), MMF (1g)	300 mg	CYC, AZA
SLE.8	28	0	4	1:640	negative	low	-	-	-	Yes	4	Moderate	HCQ (400mg)	0 mg	CYC, AZA
SLE.9	42	8800	3.5	1:640	positive high	low	+		+	Yes	15	Severe	HCQ (400mg), Rituximab (1g), CYC (500mg),	5,100 mg	None
SLE.10	46	0	4.2	1:80	negative	low	-	-	-	Yes	8	Moderate	HCQ (400mg)	112 mg	None

*Footnote: AZA, Azathioprine; MMF, Mycophenolate Mofetil; RTX, Rituximab; CYC, Cyclophosphamide; PRE, Prednisone; HCQ, Hydroxychloroquine

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