Thesis

“Hematopoietic stem cells in experimental models of Systemic Lupus Erythematosus (SLE)”

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Medical School Graduate Program
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University of Crete
2016-2017

Διπλωματική Εργασία

“Αρχέγονα Αιμοποιητικά Κύτταρα σε Πειραματικά Μοντέλα Συστεμικού Ερυθηματώδη Λύκου (ΣΕΛ)”

Μαρίνα Αναστασίου

“Εργαστήριο Αυτοανοσίας και Φλεγμονής”
Υπεύθυνος: Δ. Μπούμπας

Μεταπτυχιακό πρόγραμμα Ιατρικής Σχολής
“Μοριακή Βάση Νοσημάτων του Ανθρώπου”
Πανεπιστήμιο Κρήτης
2016-2017

Acknowledgements

First and foremost I would like to thank my family for all their love and support throughout the years. Next, I feel obligated to thank Dr. Boumpas who graciously offered me the opportunity to join his lab for the past year and for helping me out with eveyrthing I needed. My deepest appreciation has to go to Maria Grigoriou, who patiently taught and adviced me. Next, I would like to thank Aggelos Banos for supervising the projects I was involved in and for encouraging me to think and act critically. Also, I am thankful to Panos Verginis, Katerina Hatziioannou and Themis Alissafi for all the amazing discussions and the depth of immunological knowledge they so eagerly share with all the students in the lab. Special thanks go to my fellow Master- and labmates Lydia Kalafati and Roza Barouni for assisting me in every way imaginable way and for contributing in making the last two years the most productive, intellectually stimulating and fun of my entire life. I would be remise had I not thanked George Bertsias for participating in the evaluation of this project.

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Abbreviations

BM	Bone marrow
CFU	Colony-forming unit
HSCs	Hematopoietic Stem Cells HSCs
HSPCs	Hematopoietic Stem/Progenitor Cells
LSK	Lin- Sca-1+ c-Kit+
SLE	Systemic Lupus Erythematosus

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Abstract

The bone marrow consists of numerous cell populations including the Hematopoietic Stem Cells (HSCs) which give rise to all blood cells, including granulocytes, lymphocytes, monocytes and endothelial cells, which, in turn, participate in the pathogenesis of Systemic Lupus Erythematosus (SLE). The working hypothesis of this project is that SLE pathogenesis can be traced all the way back to HSCs due to both intrinsic and extrinsic stress effects. This body of work consists of observational data regarding HSCs by focusing on key aspects of their nature: enumeration, proliferation, differentiation and function. Previous studies have already shown that in lupus mice HSCs overproliferate, which results in BM hypercellularity. We have found significantly increased frequencies as well as absolute numbers of HSCs in the BM of lupus (NZBxNZW)/F1 mice with established disease as compared to young (NZBxNZW)/F1 mice or to C57/BL6 mice. Phenotypic hematopoietic progenitor cell analysis points to a trend in lupus-prone mice; they appear to have disturbances in lineage determination even at that point in differentiation between disease stage and different strains. Targeted studies have been curiously contradictory regarding the effect of pro-inflammatory cytokines such as interferons in the expansion and function of HSCs and their progenitors while, at the same time, pointing to an inhibitory effect on hematopoiesis. Our project looks into the effects of type I interferons on murine HSCs - as defined by the widely used LSK phenotype-in the physiological setting of aging and disease progression and on HSC culture in vitro. This project's previous findings include global gene expression analysis of HSCs originating from lupus mice, which revealed various differentially expressed genes including transcription factors crucial for hematopoiesis like Cebpα. Building on that, our subsequent data provide observations that reinforce our original hypothesis with statistically significant evidentiary support. The ultimate goal is to better appreciate the immune response during inflammatory, autoimmune diseases and gain knowledge on how HSC change –both functionally and in pure numbers- through the course and the development of murine lupus using an interferon-dependent model that has been known to best resemble the actual autoinflammatory milleu characterising the full-blown active lupus observed in humans.

Περίληψη

Ο μυελός των οστών αποτελείται από πλήθος κυτταρικών πληθυσμών συμπεριλαμβανομένων και των αρχέγονων αιμοποιητικών βλαστοκυττάρων, τα οποία δίνουν γένεση σε όλα τα κύτταρα του αίματος συμπεριλαμβανομένων των κοκκιοκυττάρων, των λεμφοκυττάρων, των μονοκυττάρων και των ενδοθυλιακών κυττάρων, που με τη σειρά τους συμμετέχουν στην παθογένεση του Συστεμικού Ερυθηματώδους Λύκου (ΣΕΛ). Η υπόθεση αυτής της εργασίας είναι ότι η παθογένεση του ΣΕΛ μπορεί ήδη να εντοπιστεί από τα αρχέγονα αιμοποιητικά κύτταρα λόγω εσωτερικών και εξωτερικών παραγόντων. Αυτή η εργασία συμπεριλαμβάνει δεδομένα που προέρχονται από την παρατήρηση ειδικών εκφάνσεων της φύσης των βλαστοκυττάρων αυτών: πολλαπλασιασμός/συχνότητα, διαφοροποίηση και λειτουργία. Προηγούμενες μελέτες έχουν ήδη δείξει ότι τα ποντίκια με προδιάθεση για ΣΕΛ έχουν αρχέγονα αιμοποιητικά βλαστοκύτταρα με αυξημένη ικανότητα πολλαπλασιασμού, γεγονός που οδηγεί σε αυξημένη κυτταροβρίθεια του μυελού των οστών. Παρατηρήθηκαν αυξημένες συχνότητες και απόλυτες τιμές αρχέγονων αιμοποιητικών βλαστοκυττάρων στο μυελό των οστών σε πειραματικό μοντέλο ΣΕΛ (NZBxNZW)/F1 συγκριτικά με ποντίκια του ίδιου στελέχους που δεν εμφάνιζαν την ασθένεια αλλά και με ποντικια από άλλο στέλεχος (C57/BL6). Ανάλυση των άμεσα διαφοροποιημένων προγονικών κυττάρων αποκαλύπτει μια τάση στα ποντίκια του ΣΕΛ: φαίνεται να έχουν διαταραγμένη επιλογή πορείας διαφοροποίησης, φαινόμενο που σχετίζεται με την ηλικία, το στάδιο της ασθένειας και το στέλεχος των πειραματοζώων. Στοχευμένες μελέτες φαίνεται πως είναι διχασμένες ως προς το αποτέλεσμα προφλεγμονωδών κυτταροκινών όπως των ιντερφερονών στη διαίρεση και τη λειτουργικότητα των αρχέγονων αιμοποιητικών βλαστοκυττάρων και των άμεσων απογόνων τους, ενώ ταυτόχρονα δείχνουν πως η επίδραση τέτοιων ουσιών είναι ανασταλτική για την αιμοποίηση. Η παρούσα εργασία ελέγχει την επίδραση της ιντερφερόνης α στο φαινότυπο των αρχέγονων αιμοποιητικών βλαστοκυττάρων κατά το γήρας και το ΣΕΛ, χρησιμοποιώντας διαφορετικά στελέχη ποντικιών. Προηγούμενα αποτελέσματα του εργαστηρίου σχετίζονται με αλληλούχιση του μεταγραφώματος των αρχέγονων αιμοποιητικών βλαστοκυττάρων από ποντίκια με ΣΕΛ τα οποία δείχνουν διαφορική έκφραση ορισμένων γονιδιων –και μάλιστα μεταγραφικών παραγόντων- όπως το Cebpa, που είναι σημαντικά για την αιμοποίηση. Βασισμένοι σε αυτά, τα επακόλουθα δεδομένα ενισχύουν την αρχική μας υπόθεση. Ο τελικός σκοπός είναι να καταλάβουμε καλύτερα τις ανοσολογικές αποκρίσεις κατά τη φλεγμονώδη αντίδραση, στα αυτοάνοσα νοσήματα και να σχηματίσουμε μια σαφή εικόνα για το πως τα αρχέγονα αιμοποιητικά κύτταρα αλλάζουν ποιοτικά και ποσοτικά κατά την ανάπτυξη του ΣΕΛ σε γενετικά επιρρεπή ποντίκια-μοντέλα αυτοανοσίας που εξαρτώνται από ιντερφερόνη για να αναπαραστήσουν το χαρακτηριστικό φλεγμονώδες μικροπεριβάλλον που χαρακτηρίζει το ΣΕΛ στο μυελό των οστών και μοιάζει με τον ανθρώπινο.

1.**Introduction**

1.1 Systemic Lupus Erythematosus (SLE)

SLE is the result of recurrent activation of immune system components and is accompanied by production of autoantibodies and cytokines that promote systemic and local inflammation and tissue damage. SLE requires genetic susceptibility and siblings of patients are 30 times more likely to develop SLE. According to observational studies there is only 20% concordance for SLE in homozygotic twins, which is indicative of epigenetic (heritable, reversible, cell specific) and environmental effects –and the interplay between the two-determining at the very least the initiation of the disease. Genome-wide association studies have identified more than 40 lupus associated loci (Frangou, Bertsias and Boumpas*). LncRNAs are increasingly accused of modulating immune responses and, to an extent, contribute to onset and severity of autoimmune disorders in a cell-type specific manner. But the limited effect-size of the susceptibility loci that have been identified till now elevated the argument in favour of environmental over genetic factors that are coaxing towards SLE onset. (14). Voulgarelis and colleagues mentions that BM necrosis, stromal alterations, hypocellularity, dyspoiesis, and distortion of BM architecture contribute to bone marrow failure in SLE (32)

1.2 Murine Lupus strains

NZB are crossed with NZW mice to get the (NZBxNZW)/F1 mice. This is discussed in greater detail in the *Material and Methods* section.

New Zealand White (NZW) inbred mice were developed in 1952 (25). Following infection with LCM and polyoma virus NZW mice had lower viral titers and “lesser” infection compared to NZB and (NZBxNZW)/F1 mice, which tends to correlate with less severe "autoimmune" disease. The etiology behind the NZW genetic background that allows for this evolutionary advantage to surface remains unclear (30). Despite the autoimmune background that accompanies the NZW strain, autoantibodies (albeit at lower levels), nephritis and relatively shortened lifespan, these mice hardly ever die from end-stage renal disease but do develop mesangial glomerulonephritis. NZW mice

have an allele for the CD22 gene, which results in an abnormally spliced mRNA which gives out a protein that is both unusually upregulated upon LPS stimulation and associated with BCR engagement. Apart from CD22, disease in NZW mice is linked to the MHC locus. NZW mice have a more pronounced CD4 population with decreased Treg proportion in the periphery compared to B6 and BALB/c murine strains (25;30).

(NZBxNZW)/F1 mice most closely resemble human SLE. Female mice of this strain develop glomerulonephritis on average at about 8 months of age. An increasing body of evidence points to T regulatory cells (Tregs) as an interesting group that when is eliminated, glomerunonephritis is accelerated during the preactive phase in autoimmune-prone female (NZB×NZW)/F1 mice (10). Zhou et al summarizes that all lupus-prone mice have high IFN signature, whereas others claim that IFN signature in these mice is either weak or absent, with the exception of the pristane model, which exhibits strong IFN signature (36). Murine models of active SLE exhibit an expansion of their LSK (Lin^- $Sca1^+$ $c\text{-}Kit^+$) population which phenotypically marks the HSC fraction. (36)

1.3 Hematopoietic Stem Cells and SLE

The hematopoietic system relies on HSCs to replenish primarily hematopoietic progenitors and secondarily fully differentiated blood cells from embryonic development to adulthood through a process that is referred to as “hematopoiesis” or “hemopoiesis”. (23). Balancing the proverbial scale between self-renewal and multi-lineage differentiation requires a type of homeostatic co-ordination that is both tightly regulated and, at the same time, easily tipped towards stem cell exhaustion and uncontrolled production of either myeloid or lymphoid cells (29)**.** To get any terminally differentiated cells, HSCs first have to differentiate into Hematopoietic Progenitor cells (HPCs). These HPCs can be produce cells that follow either the lymphoid or the myeloid differentiation pathway. During pathological conditions there may be bias towards commitment to one pathway. As Goodell eloquently puts it: “Lineage biasing of HSCs may be mediated by differential responses of HSC subtypes”.

HSCs differentiation occurs either naturally or it can be induced due to stress. Apart from aging, radiation, and chemical treatments, exposure to chronic infections can turn otherwise quiescent HSCs to activate their cell cycle program and proliferate, thus induce pre-term aging, terminal differentiation or what is termed an early-on senescent phenotype. The explanation for this lies in the fact that all the above come with inflammatory signals (15).

SLE presents with inflammation and is oftentimes accompanied by hematologic abnormalities (Bashal, 2013). Hypocellularity alone crucially contributes to bone marrow dysfunction and it all can be explained by chronic immune activation in SLE (32). Furthermore, SLE patients have pronounced IFNα in multiple cell types and tissues and Holmes et al. found that a potential mechanism involves boosted α-mediated JAK-STAT signalling in HSCs which exhausts their precursor pool and results in hematologic abnormalities in SLE (12).

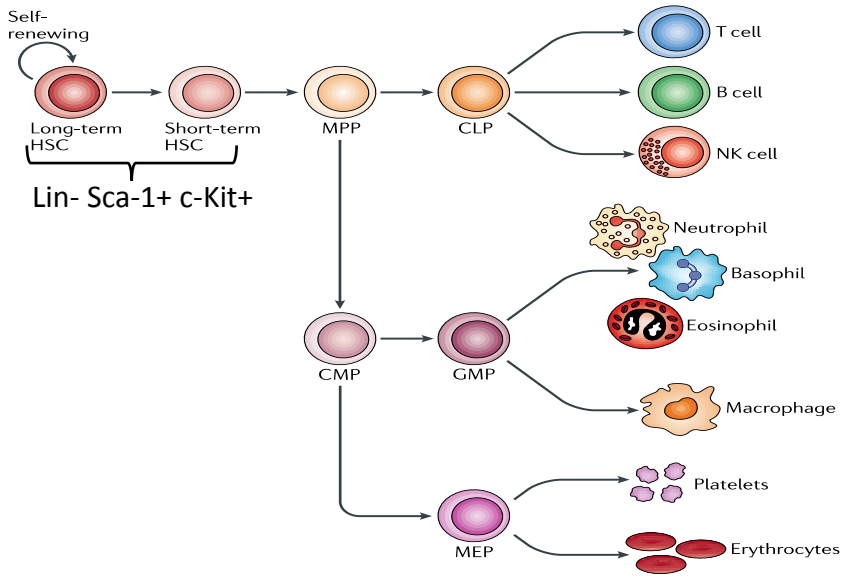

Figure 1 Differentiation tree adapted from Goodell et al., 2011, Nat Reviews Immunology" the hematopoietic tree is traditionally thought to divide along two main branches: myeloid and lymphoid"(15)

Hematopoietic abnormalities have been accused of a plethora of autoimmune diseases. It has been postulated that HSCs in SLE and other myeloproliferative and/or autoinflammatory conditions exhibit an aged phenotype. However how HSC aging affects SLE onset, flares, disease severity and response to therapy remains poorly explained. In any case, the ultimate goal is to shed light on mechanisms that either reprogram these cells to the point of rejuvenation or on treatments that selectively deplete the most exhausted HSCs in the bone marrow pool to attenuate the overall aging immune phenotype, thus preventing hematopoietic malignancies and, to an extent, autoimmune diseases such as SLE.

1.3.1 HSC regulation – myelopoiesis

Commitment towards the hematopoietic differentiation of HSCs and other less potent progenitor cells to myeloid cells such as granulocytes, monocytes, erythrocytes and platelets is termed myelopoiesis. Myelopoiesis starts in the bone marrow but terminal differentiation occurs in either the blood or the peripheral organs. In general, murine myeloid cells can be phenotyped and sorted through flow cytometry in the murine system because, on their surface, they have elevated levels of the GPI-anchored protein Gr1 as well as the integrin CD11b (Mac-1). (O'Connor, 2016**)

Bone marrow common myeloid progenitors (CMPs) can rapidly accelerate myelopoiesis and produce neutrophils in response to septic shock (31).

1.3.1.1 IFNα

Essers and colleagues demonstrated for the first time that acute exposure to type I interferons causes HSCs to proliferate rapidly *in vivo* (4). On the other hand, Pietras showed that addition of IFNα failed to enhance HSC proliferation *in vitro* at a concentration of 100 ng/ml, a result validated by the use of methylcellulose cultures of purified HSCs (22). The ladder group also noted that this antiproliferative effect of IFNα on HSCs does not extend to the equally stimulating IFNy but seems to be a restricted property of type I interferons. All these allow room for the hypothesis that interferons act

in distinct ways on HSCs. [HSC proliferation after 12 h of culture with 100 ng/ml IFN-γ was also inhibited (22, Pietras' unpublished data)].

Specifically, following prolonged "chronic" exposure to type I interferons *in vivo*, bone marrow HSCs tend to transiently proliferate, an observation most likely attributed to a breakdown in mechanisms safeguarding their quiescent state. This temporary proliferative capability of HSCs is followed by immediate return to quiescence and, therefore, a state in which HSCs run low danger of exhaustion and apoptosis, thus maintaining the HSC pool despite the intense stimulation. After all, under any setting, HSCs' hyperproliferation should always be seen as the prequel of their slow decline in numbers and functionality over time.

Elegant studies have concluded that chronic administration of type I interferons seems to render HSCs proapoptotic but not antiproliferative. Type I interferons in culture force HSCs to behave, arguably, in an opposite way. *In vitro* IFNα-treated HSCs do not proliferate. To add to that, they are not protected from apoptosis. The cell cycle of IFNα-treated HSCs doesn't seem to be affected in culture which, by itself, both paints culturing HSCs in an unfavorable light when it comes to studying cell cycle phases undergoing treatments and fails to mimic what is really occurring in the bone marrow under the very same stimulus. So, type I interferons block proliferation *in vitro* but not *in vivo*.

However, the most significant drawback of *in vitro* versus *in vivo* studies of HSCs in response to type I interferon stimulation is the fact that, in culture, one cannot monitor cellular physical relocation. In the bone marrow, Kunisaki and colleagues showed that type I IFN–exposed HSCs move away from quiescence-enforcing periarteriolar niche cells, which, by extension, results in HSCs acquiring a proliferative phenotype that cannot possibly extent *in vitro*, in which case bone marrow niches aren't represented (16).

Another explanation of why IFNα causes HSCs to proliferate *in vivo* but not *in vitro*, as hypothesized by Yamazaki, is that, in the ladder, quiescent mechanisms get inactivated when the cells are removed from the marrow and this is irrespective of the presence or absence of a proliferative stimulus in culture (33). Under normal conditions, HSCs are out of cell cycle but stress in their microenvironment can induce this “exhaustion inducing” process.

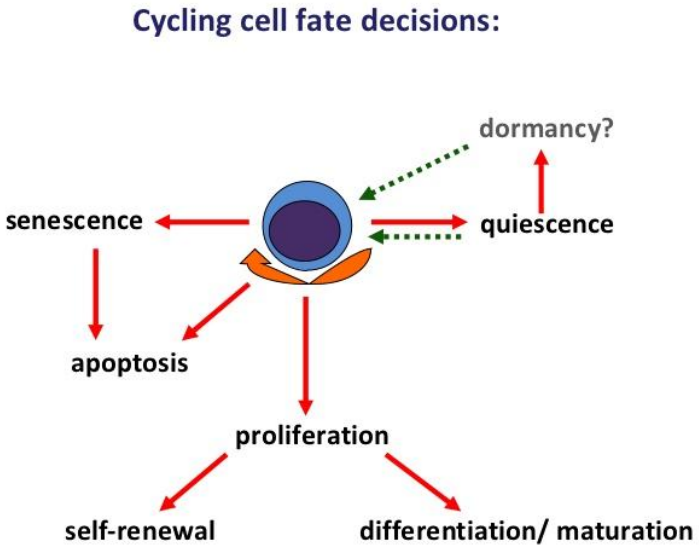

Figure 2 Fate decisions on HSC cell cycle

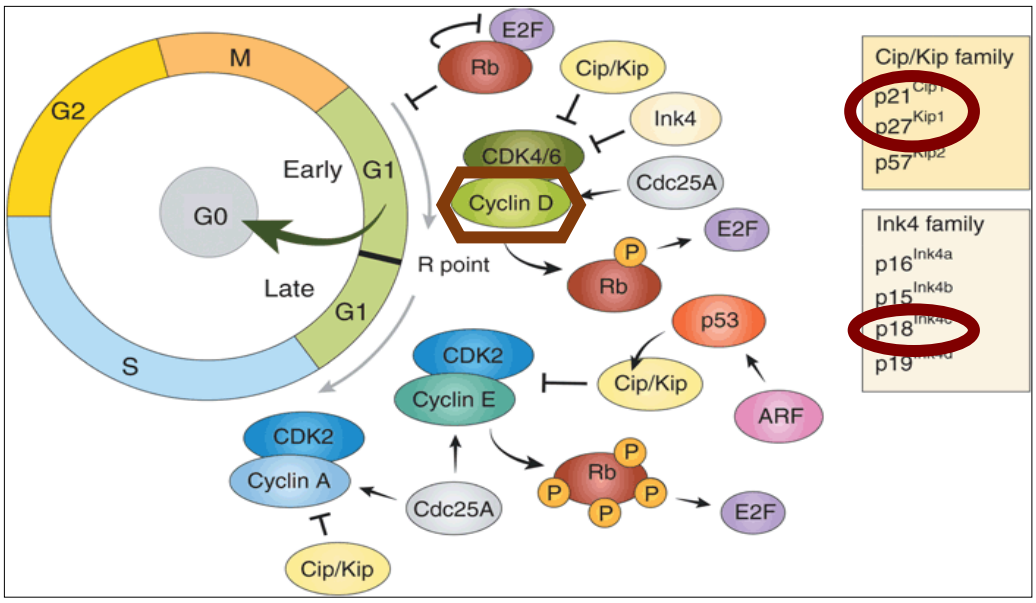

Figure 3 Cell cycle phases are under tight regulatory control

1.3.2 HSC aging and exhaustion

The physiological process of aging particularly effects HSCs' self-renewal, regeneration, differentiation and overall functionality. HSC aging, which has been attributed to both intrinsic and extrinsic stress factors, comes with immune-remodelling, reactive oxygen species accumulation, multiple disease manifestations and increasingly diminishing organ functions (28). The mechanisms of HSC aging are no longer as elusive as they used to be partly due to increased appreciation of this difficult-to-study, rare cell subset and partly because they are now being conceptualized and depicted through the looking glass of cell senescence, clonal selection and epigenetic shifting.

1.4 RNA-seq in SLE

The above mentioned complex mechanisms are now being studied using advanced bioinformatics analyses. Within the last few years microarrays have been replaced by the far more sophisticated RNA-sequencing.

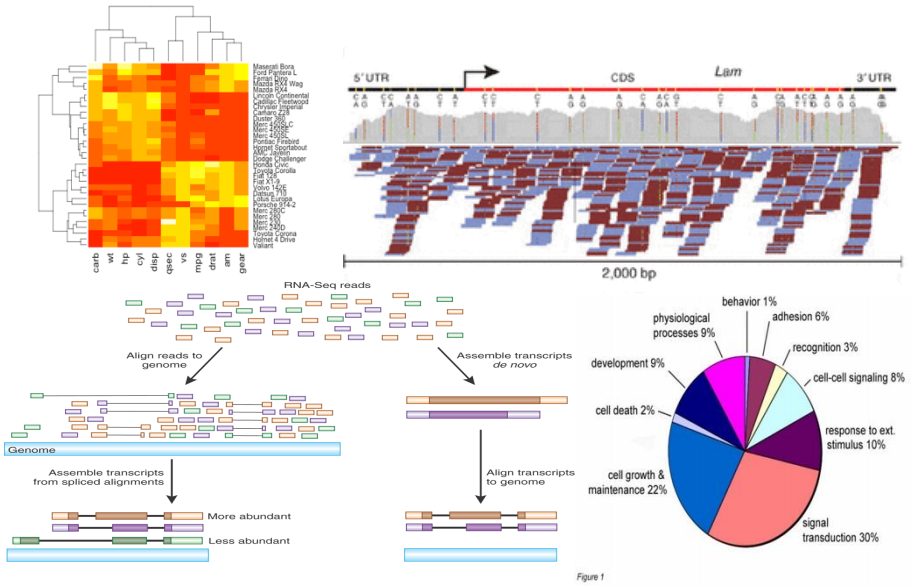

Figure 4 RNA sequencing: transcripts are aligned with reference transcriptome, gene expression levels are estimated and differentially expressed genes (DEGs) are identified. Conclusions can be visualized with heatmaps and preliminary results can be subjected to GO analysis to identify specific biological pathways that are particularly affected by the combinational effect of DEGs. (20)

We took advantage of this technique to study the transcriptome of the previously mentioned phenotypes of HSCs of SLE patients and lupus mice and their respective controls. Based on our RNA-seq analyses we chose all our initial studying targets.

1.5 CAAT-binding protein alpha (Cebpα)

Cebpα impacts the process of myeloid differentiation via distinct patterns of layered regulation. More specifically, it has been proposed that the "PU.1 to Cebpα" ratio is necessary and enough to instruct terminal differentiation of myeloid progenitor cells. In PU.1 deficient mice, inducible expression of these two master regulators concluded that the lower the above mentioned ratio, the more pronounced the incline of the myeloid progenitors to differentiate towards granulocytes (19).

The mechanism behind this intertwined relationship between these master (co)-regulators lies, in part, in their close proximity. Co-localization of PU.1 with Cebpα results in the reciprocal regulation of both their expression, with Cebpα binding to the SIP1 promoter and the -14kb enhancer to enhance PU.1 expression or Cebpα

displacing cofactor c-Jun to repress PU.1 expression. Additionally, it has been shown that certain interactions like dimerization of Cebpα with c-Jun and c-Fos weaken the affinity for Cebpα target genes without affecting PU.1 specificity, thus tipping the scale towards terminal monocytic differentiation. But yet, one begs the question: What dictates the enhancement or repression activity on PU1 and Cebpα promoters? (19;34)

A notable reduction in bone marrow cellularity toppled by a nearly 70-fold in LSK frequency was observed by Ye et al., 2013 when they conditionally ablated Cebpα (19). On another note, Cebpα deficiency, malfunction or attenuation inhibits the transition from CMP to the granulocyte/monocyte progenitor, thus contributing to the loss of granulocytes in the bone marrow (34).

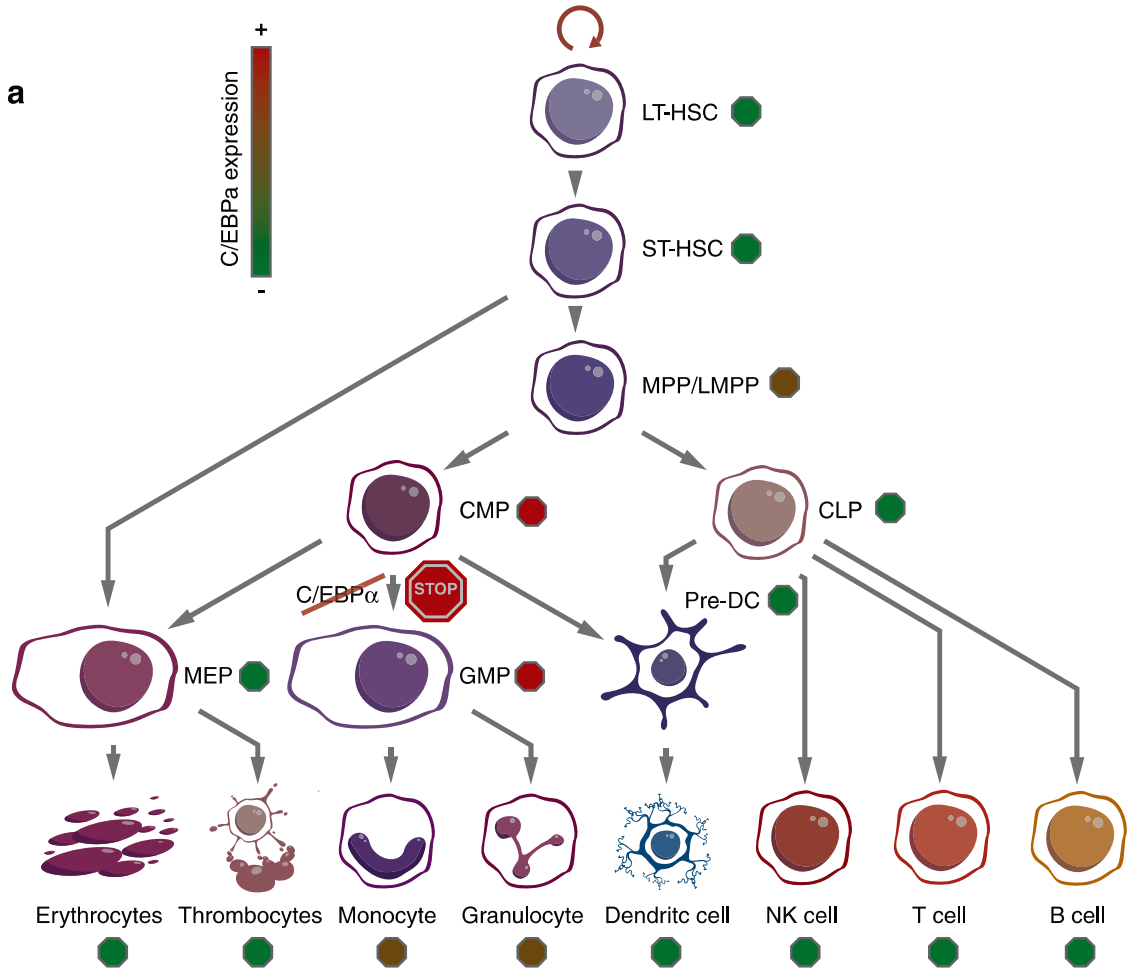

Figure 5 Cebpα expression-Granulocytic signature

2. Materials and Methods

2.1 Mice

All mice were bred, kept and cared for in the BRFAA Animal Facility. We used C57/BL6 (young mice ≈ 2-3 months and old mice ≈ 7-9 months old) as our control group. These mice are resistant to inflammation and are widely used in immunological experiments. We also used parental strains NZB and NZW (age-matched mice) for mating purposes to create a sustainable (NZBxNZW)/F1 colony. Additionally, for the purposes of this project, NZW mice served as a secondary control group with similar genetic background to our lupus-prone (NZBxNZW)/F1 mice. The (NZB/NZW)/F1 mice are generated by crossing NZB female mice with NZW male mice. This is the only heterozygous strain that inherits disease-related gene loci from both NZW and NZB mice and develops severe lupus symptoms. This is evidenced and monitored by periodic proteinuria measurements using metabolic cages and sticks that detect different protein levels in the urine. Protein levels higher than 100mg/dl indicate a nephritic (NZB/NZW)/F1.

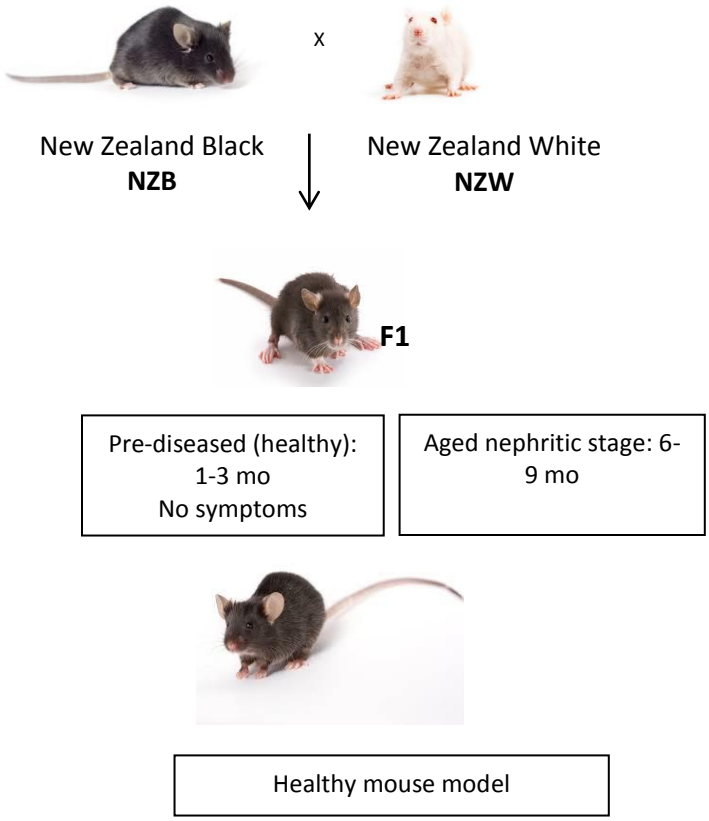

Figure 7 Colony Maintenance of F1 lupus prone mice

2.2 Bone Marrow Isolation

BM cells were flushed from femurs, tibias and humorae with 5% FBS/PBS using an insulin syringe (27G), followed by use of a 23G syringe to ensure single cell suspension. Ammonium chloride [(NH_4Cl), pH-7.4] was used as hemolysis buffer to lyse erythrocytes ((n+1)ml, n=animals, 2min, 25^{o}C). Cell suspensions were centrifuged and resuspended in 5% FBS/PBS so as to use appropriate staining to isolate stem cell populations. To distinguish between cellular populations, extracellular staining of the cells in 500ul 5%FBS/PBS for 20min at 4^{o}C was done. The stained cells were washed and collected in 3ml of 5% FBS/PBS buffer using a 70um mesh.

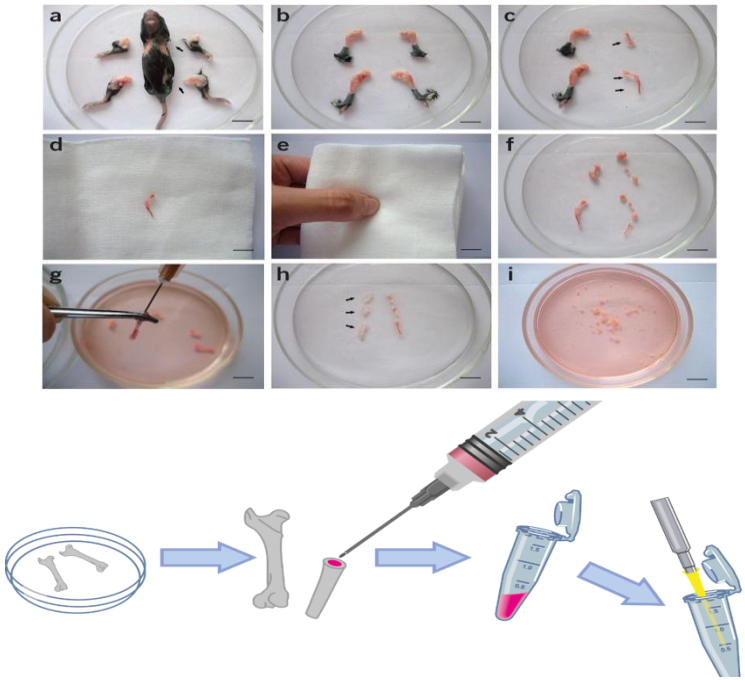

Figure 8 Bone marrow isolation following murine sacrifice

2.3 MACS lineage cell enrichment kit (Miltenyi Biotec, 130090858)

In order to maximize the number of cells that pass through the FACS sorter each time, we used this kit to enrich the lineage negative population and, by extension, our sample's LSK population. MACS column was used in order to decrease the number of cells and the time for the FACS sort. This MACS column is used to deplete the Lin^+ cells, therefore decreasing the total number of cells and enriching the Lin^- cells. Bone marrow cells were counted and appropriate columns were picked. Kit buffer (0.5%BSA-2mMEDTA-PBS, pH=7.2) and biotin antibody cocktail were added. The mix was incubated for 10min at 4-8^{o}C. Extra buffer was added to accommodate the subsequent addition of anti-biotin microbeads. The mix was incubated for 15min at 4-8^{o}C. Cells were washed with buffer and resuspended in 1ml of buffer so as to be sorted through magnetic separation using the LS column. The column was washed with buffer thrice with 3ml to reach equilibrium (equilibration). Cell suspension was made to pass through the column filter. The column was washed thrice with 3ml buffer. The flow-through

(enriched with Lin- cells) was collected and subsequently stained for Sca-1^+ and c-Kit^+. To validate kit performance, flow-through was stained for Lin^- markers as well.

2.4 Flow Cytometry

The markers mentioned below were used for LSK/HSC compartments by fluoresence-activated cell sorting analysis using the BD FACSAria II cell sorter:

- PE/Cy7 anti-mouse CD150 (SLAM) [BioLegend] (1:100)
- PE/Cy7 anti-mouse/human CD11b [BioLegend] (1:200)
- PE/Cy7 anti-mouse Ly-6G/Ly-6C (Gr-1) [BioLegend] (1:200)
- PE/Cy7 anti-mouse CD3e [BioLegend] (1:200)
- FITC anti-mouse/human CD45R/B220 [BioLegend] (1:200)
- FITC anti-mouse TER-119 [BioLegend] (1:200)
- FITC anti-mouse CD16/CD32 (FcγII/IIIR) [BioLegend] (1:200)
- FITC anti-mouse CD19 [BioLegend] (1:200)
- APC anti-mouse Ly-6A/E (Sca-1) [BioLegend] (1:200)
- APC/Cy7 anti-mouse CD48 (Alexa Fluor 700) (SLAM) [BioLegend] (1:100)
- PE anti-mouse CD117 (c-Kit) [BioLegend] (1:200)
- PerCP anti-mouse IL-7Ra [BioLegend] (1:200)
- PercP 7-AAD Viability Staining Solution [BioLegend] (fixed amount per sample)
- Pacific Blue anti-mouse Ki-67 [BioLegend] (1:50)
- DAPI [BioLegend] (1:200)

2.5 Cell Cultures

Sorted LSK cells were cultured in IMDM [IMDM lipid-free medium supplemented with 2% FBS, 10.000U/ml penicillin (Gibco) 10.000ug/ml streptomycin (Gibco), 50ng/ml rm-IL6 (Immunotools), 20ng/ml rm-IL3 (Immunotools), 20ng/ml rm-SCF (Immunotools), 20ng/ml rm-TPO (Immunotools), 50mM β-mercaptoethanol (Gibco), 2mM L-glutamine (Gibco). Cells were cultured into 96-well round-bottom plates at 37^oC, 5% CO_2.

- Stimulation with freshly isolated murine peripheral blood serum: After 24hrs each well was stimulated with 2,5% serum from one of the following mice: C57/BL6,

F1-H, F1-L mice. Phenotypic profiling and gene expression analysis was followed

- Stimulation with rmIFNα(carrier-free) [BioLegend]: after 6hrs and 12hrs cells were stimulated with 100ng/ul of rmIFNα.

2.6 Colony-Forming Cell Assay (MethoCult^{TM})

1-2x10^3 sorted LSK cells were resuspended in IMDM medium and injected in MethylCellulose media and plated in duplicate in 35-mm tissue culture dishes. Plates were incubated at 37^{o}C, 5% CO_2 and were periodically observed under the microscope. After 9-10 days colonies were scored for characterization as CFU-G, CFU-M, CFU-GM and CFU-GEMM. Semi-solid methylcellulose cultures permit the clonal expansion of each single progenitor cell to remain spatially isolated from other colonies within the culture dish, so they may be well-separated, easily identified and ultimately counted and calculated as a percentage of the total number of grown colonies (GMP=CFU-GM, granulocytes-CFU-G, monocytes/macrophages=CFU-M, CMP=CFU-GEMM), that exist simultaneously in each culture dish.

2.7 RNA extraction

Samples were mixed with 100ul lysis buffer (RA1) and 2ul TCEP. Samples were stored at -80^{o}C. 100ul of 70% ethanol was mixed with each sample prior to each being transferred to a blue-ring column. Room temperature centrifugations at the indicated speed were followed by the addition of the indicated volume of MDB buffer. An additional centrifugation superseded the addition of the indicated amount of freshly prepared rDNAse/RDD buffer into the column. Samples were incubated at room temperature for 15 min. RPE was added twice with centrifugation in between. RNAse-free H_2O was added into each column as each was transferred to a 2ml RNAse-free sterile collection tube and centrifuged at full speed to elute the RNA. RNA was quantitatively and qualitatively measured.

2.8 Reverse Transcription

Total RNA extracted from each sample was used for cDNA synthesis using SuperScript® II Reverse Transcriptase Kit (Invitrogen).

- Sample RNA
- oligoDT (500ng/ul)
- dNTPs (10mM)
- sterile RNAse-free H_2O
- 5x First-Strand Buffer
- 0.1M DTT
- RT enzyme

cDNA program:

- Heating at 62^{o}C (5min)
- Incubation at 42^{o}C (2min)
- Incubation at 4^{o}C (5min)
- Incubation at 42^{o}C (50min)
- Heating at70^{o}C (15min)

2.9 Real-Time PCR

RT- PCR was performed using the iTaq^{TM} Universal SYBR® Green Supermix (Biorad). Sample mRNAs Cts were normalized to the corresponding HPRT ones to determine expression. For each primer set gradient PCR was performed to determine optimal conditions. Experiments were performed at T_m 61^{o}C with 40 cycles

Table 1RT-PCR primer list

Gene	Forward primer	Reverse primer
Ccnd1 (Cyclin D)	GCG TAC CCT GAC ACC AAT CTC	GAG CAG AAG TGC GAA GAG GAG
HPRT	GTG AAA CTG GAA AAG CCA AA	GGA CGC AGC AAC TGA CAT
Cdkn2c (p18)	CCT TGG GGG AAC GAG TTG G	AAA TTG GGA TTA GCA CCT CTG AG
Cdkn1a (p21)	CCT GGT GAT GTC CGA CCT G	CCA TGA GCG CAT CGC AAT C
Cebpα	CAA GAA CAG CAA CGA GTA CCG	GTC ACT GGT CAA CTC CAG CAC
IFNα	TCT GAT GCA GCA GGT GGG	AGG GCT CTC CAG ACT TCT GCT CTG
IFNαR	AGC CAC GGA GAG TCA ATG G	GCT CTG ACA CGA AAC TGT GTT TT

ISG15	AGC AAT GGC CTG GGA CCT AAA	AGC CGG CAC ACC AAT CTT

2.10 Statistical Analysis

Data are processed and plotted using the GraphPad Prism 5.0 software. Statistical analysis for comparisons between two groups was performed using Student's t-test.

3. Results

In an attempt to delineate what is different within the LSK compartment of the bone marrow of the murine model of SLE (NZWxNZB)/F1, this project continued building upon previous results. The aim was to pinpoint any defect or gain-of-function in the LSK compartment in SLE mice and determine if it is the lupus microenvironment or cell-intrinsic properties that make the difference, without excluding the possibility that it could be a little of both. It is also important to interpret all these results under the prism of what would be of benefit to lupus patients.

3.1 LSK Isolation

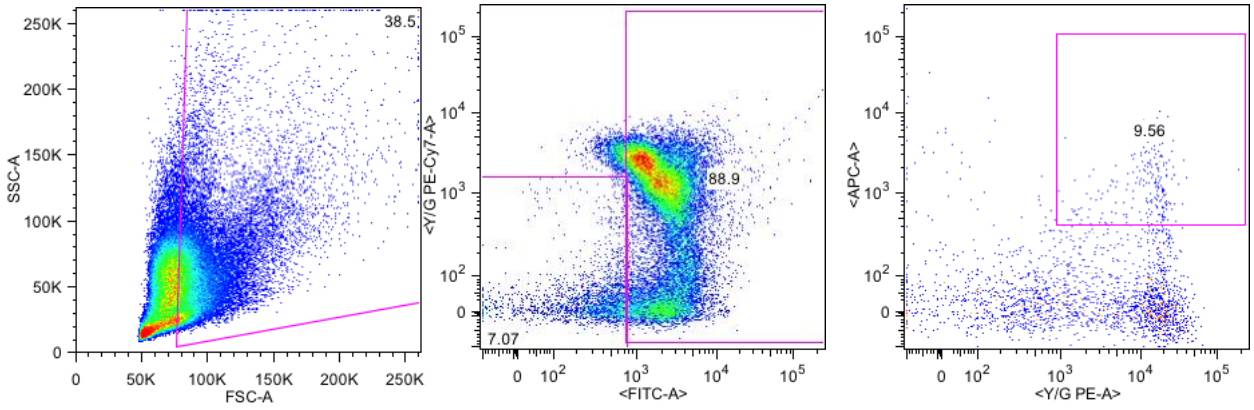

Figure 9 MACS representative flow cytometry cell sorting plot area

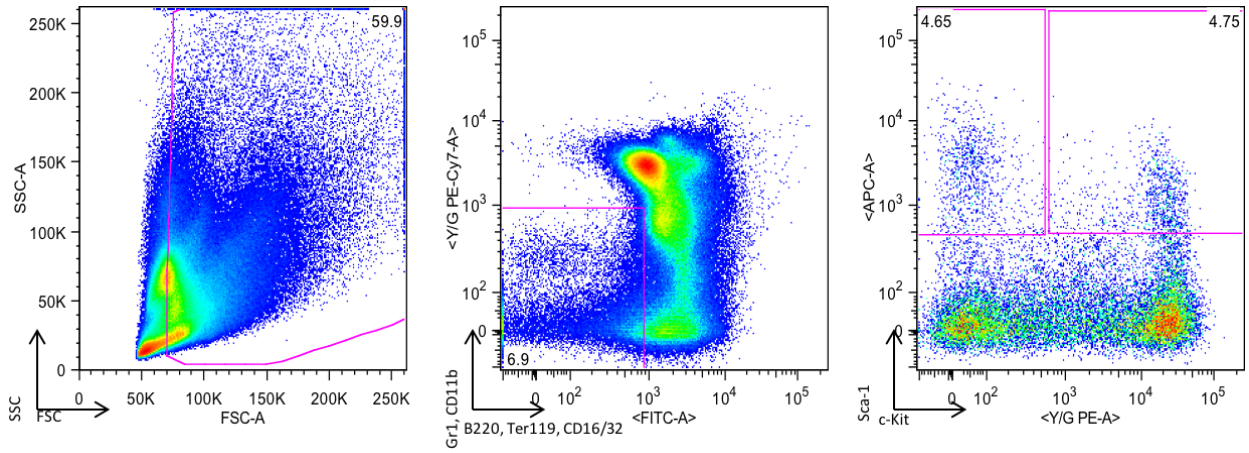

Figure 10 LSK gating strategy

The analysis was done as depicted in Figures 9 and 10. Bone marrow cells (in Figure 9 we have an enrichment for the Lineage ˉ population) were phenotyped based on their size and granularity. Using the appropriate Lin- markers (B220, Ter119, CD16/32, Gr1, CD11b), the negative population was selected. Gating on that population, the double positive for Sca-1, c-Kit was selected, profiled and used for further experiments and analysis. This population represents the LSK compartment.

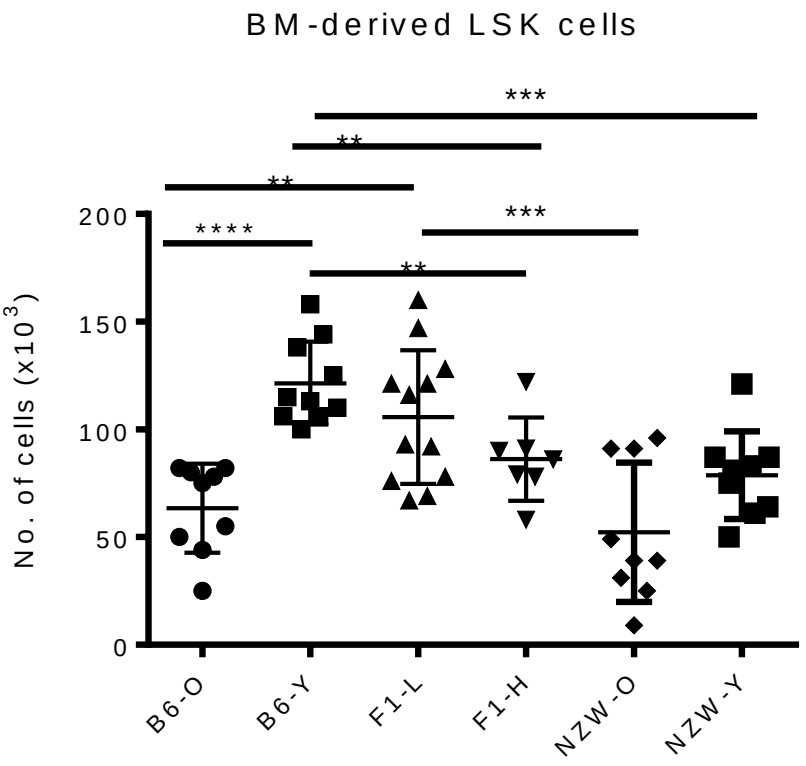

Figure 11 Bone marrow cellularity, LSK cellularity

LSK cells at higher number in B6 and NZW age-matched “young” mice and they decrease over age (Figure 11). In respect to B6 mice, Porto and colleagues report the polar opposite finding with LSK cellularity increasing with age in their B6 colony (24). Regarding (NZWxNZB)/F1 mice, we see an increase in LSK in the nephritic lupus mice, which reinforces our original hypothesis and is in agreement with the literature.

appear to be significantly enhanced in F1 old mice compared to F1 young (Figure 9). Also, we observed similar phenomenon in the Lin-Sca-1+c-Kit- compartment, where cells contain early myeloid-committed precursors. They were significantly increased compared to F1 young and their counterpart C57/BL6 old (Figure 10). (24)

3.2 Analysis of intermediate –expressing c-Kit population

It has been substantiated that reduced densities of Kit expression correlate with loss of “stemness.”. Our gating strategy resulted in a consistently identifiable ckit intermediate

population of cells that were gated on Lin$^-$ (data not shown).

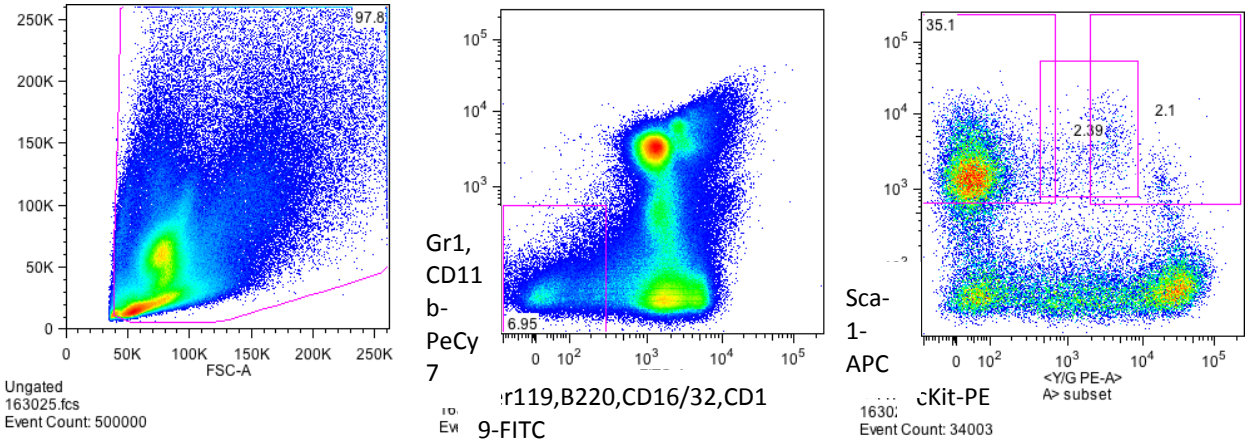

Figure 12 Gating strategy of c-Kit intermediate population of LSK cells

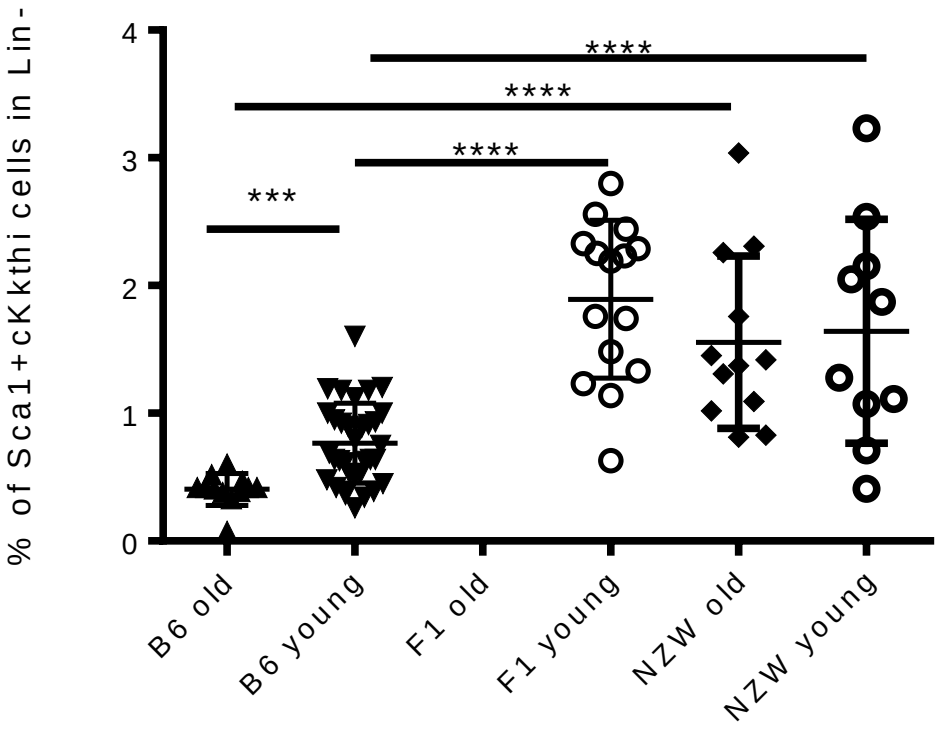

Figure 13 Percentages of c-Kit hi population of LSK cells

Based on the gating strategy depicted on Figure 12 (representative flow cytometry analysis from LSK from a NZW young mouse), we exerted the analysis on Figure 13, which combines results across all strains. It is evident that the c-Kit intermediate

population experiences statistically significant changes between strain and age/disease groups.

To our knowledge there is limited information in the literature regarding the change in the total cell numbers in the bone marrow of lupus-prone mice as disease progresses so we hypothesized that the observed changes may reflect or be associated with differences in the bone marrow cellularity.

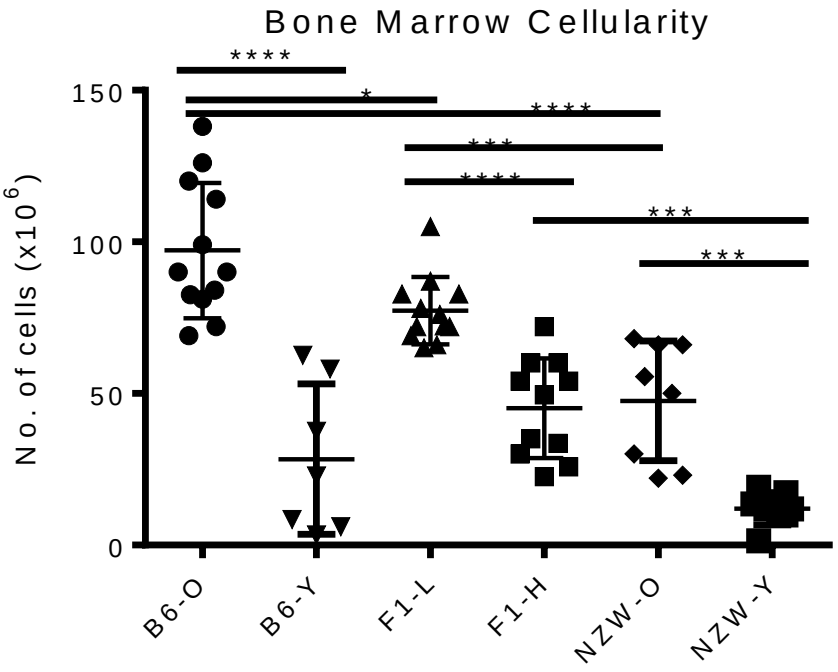

Figure 14 bone marrow cellularity across all used strains

Our results show that old female mice in all strains examined (B6, F1, NZW) appear to have statistically significant enhancement in their bone marrow cellularity (Figure 14). This can be explained physiologically because aging is associated with overall low grade inflammation which promotes cell proliferation. It is also worth noting that, when we compare total numbers of bone marrow cells among young female mice across all strains it is evident that F1 healthy mice have higher cellularity within their bone marrow when compared to young mice of the NZW white parental strain, perhaps indicating that F1 healthy bone marrow is under some inflammation-induced

stress long before lupus onset. Furthermore, B6 old mice appear to have the most drastic increase in their bone marrow cellularity across all strains.

3.3 Gating strategy for Hematopoietic Progenitors

We next asked whether changes in the LSK compartment could be reflected in the early steps of the differentiation process by looking into the directly generated committed progenitor. When looking into the percentages of the common myeloid and common lymphoid progenitors (CMPs and CLPs respectively) within the lineage negative bone marrow cells across all three strains at two developmentally distinct stages (puberty and adulthood), one can draw several key conclusions.

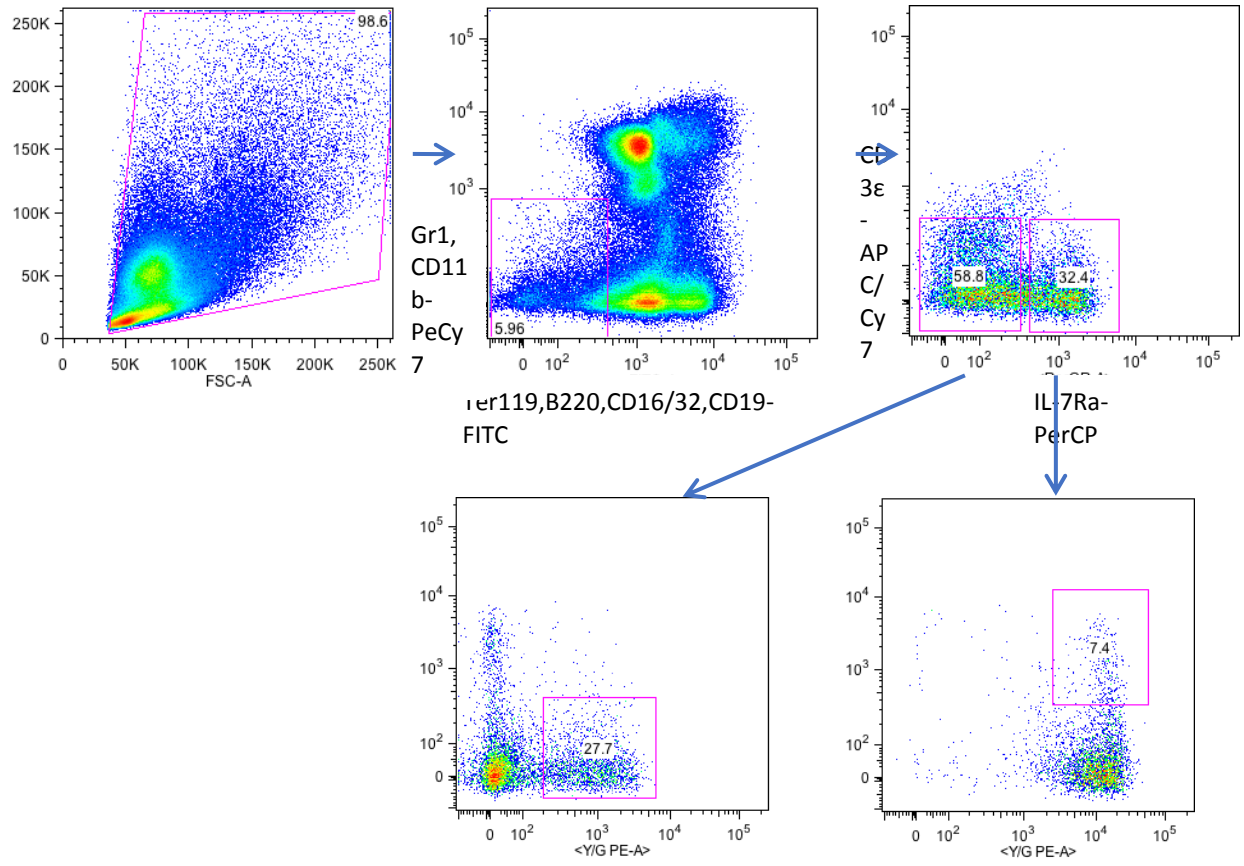

Figure 15 Gating strategy for Hematopoietic Progenitor Isolation

As depicted in Figure 15, stained bone marrow cell suspension was phenotyped based on the cells' size and granularity. Using the appropriate Lin- markers (B220, Ter119,

CD16/32, CD19, Gr1, CD11b), the negative population was selected and, gating on that population cells were separated based on their expression of CD3e and IL-7Ra on their surface. Based on that plot the CD3e -, IL-7Ra + cells were selected and, gating on those, we can positively select CLPs based on their expression of Sca-1 and c-Kit. CLPs were profiled and used for further analysis. Gating on the double negative cells on the CD3e/IL-7Ra plot and analyzing for Sca-1 and c-Kit, we can isolate CMPs as Sca-1 -, c-Kit + cells, profile them and use them for further experiments and analysis.

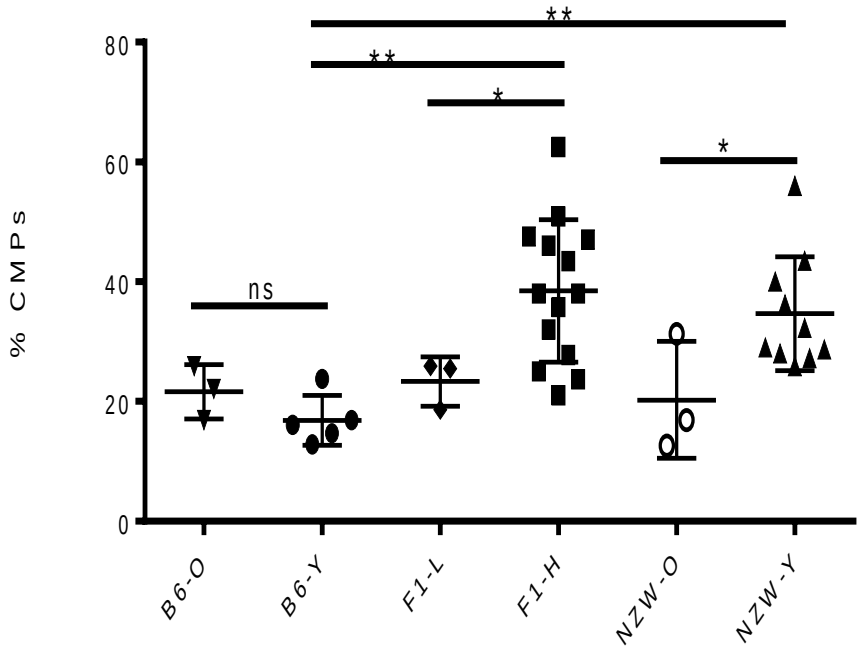

Figure 16 Myeloid Hematopoietic Progenitors' percentages in the lin- population of the bone marrow

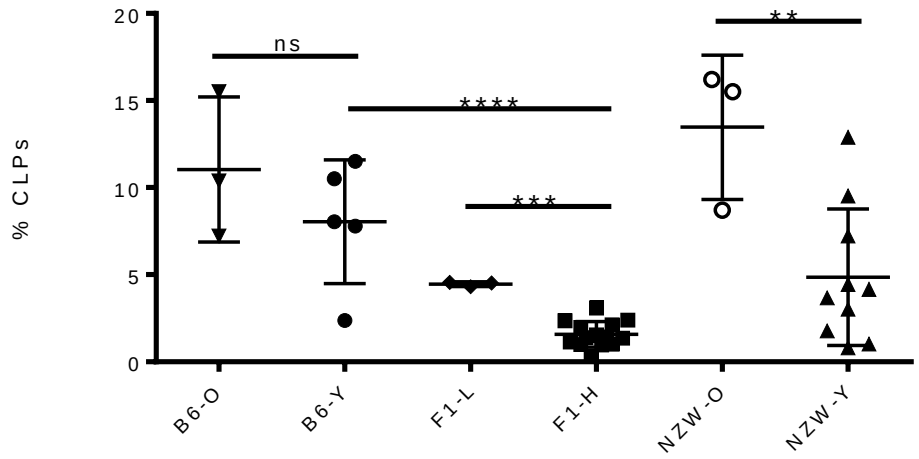

Figure 17 Lymphoid Hematopoietic Progenitors' percentages in the lin- population of the bone marrow

Different regulatory events involved in early stages of HSC commitment are key to homeostasis because there is not a consistent pattern characterizing CMP and CLP commitment changes as depicted in Figures 16 and 17. In the B6 strain there does not appear to be a statistically significant shift in the percentages of either CMPs or CLPs as ageing progresses. On the other hand, ageing and disease progression come hand-in-hand with both statistically significant decreases in the percentage of CMP cells and statistically significant increases in the percentage of CLP cells in the bone marrow of lupus-prone mice (F1 and NZW). This additional finding highlights the genetic contribution of parental strain NZW in respect to bone marrow composition changes under physiological conditions and underlines the importance of this parental strain to always be used as a control group.

3.4 Gene expression analysis

3.4.1 Cell cycle

We chose to study the cell cycle of the LSK population because it is an important aspect of HSC biology. Our hypothesis here is that the cell cycle of HSCs in lupus mice is deregulated, leading to exit from quiescent state and enhanced proliferation. That would most certainly explain the changes observed in the percentages of LSK cellularity.

For the study of cell cycle within the LSK compartment, the following cell cycle genes were chosen: Cell cycle enhancer Cyclin D: Cyclin D-Cdk4/6 (kinase 4/6) complex activity controls the transition to G1 cell cycle phase and is thought to be a prominent regulator of the LSK cell cycle under physiological conditions. Even though the Cyclin D family has multiple members (Ccnd1, Ccnd2, Ccnd3), all differentially expressed in HSCs at one point or another, cell cycle analysis of the LSK compartment in this project, focuses on relative expression changes of Cyclin D1 (Ccnd1). Cell cycle inhibitor p21: from the members of the CIP/KIP family of cell cycle genes this project examines mainly p21 which primarily prevents transition into the S phase by acting as a Cyclin E-Cdk2 (kinase 2) complex inhibitor (23). Cell cycle inhibitor p18: p18-INK4C prevents the activation of the CDK kinases and therefore functions by negatively regulating the transition to G1.

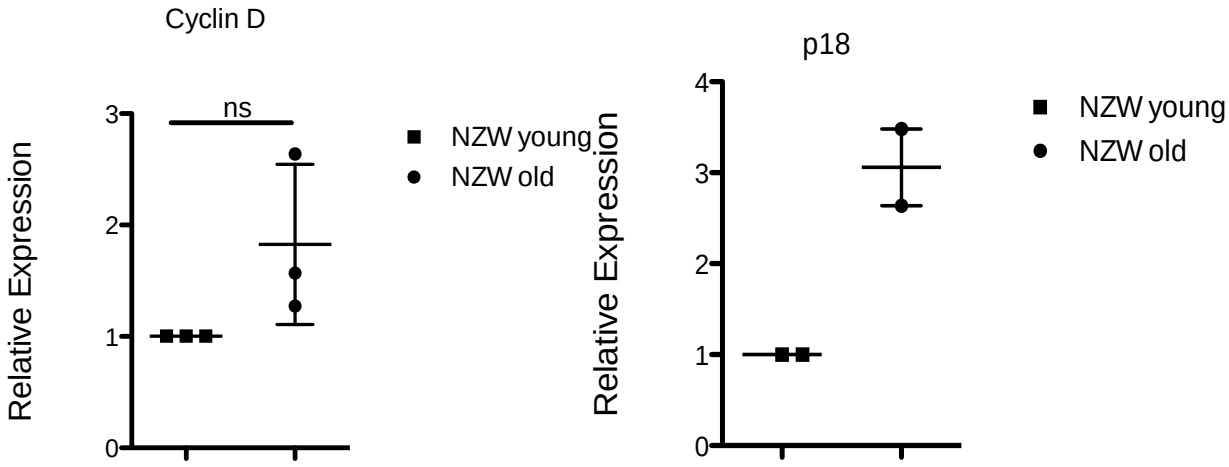

Figure 18 Cell cycle analysis in LSK cells of NZW young and old mice

Cell cycle analysis by Real Time PCR of sorted LSK cells from female mice of parental strain NZW at two developmentally distinct stages under no stimuli and/or treatments produced inconclusive results (Figure 18). It appears that there is no cell cycle enhancement from young to old mice of the NZW parental strain. Preliminary results from our datasets seem to show that Cyclin D is upregulated at the LSK cells of F1 mice with active lupus compared to both age-matched B6 mice and LSK from young F1 healthy mice (data not shown). The above graph shows a tendency of Cyclin D to be

upregulated in LSK from NZW old mice but, most likely the small sample group prevents us from drawing definitive conclusions.

To make sure that we are accurately appreciating the LSK cell cycle accurately, we would periodically use the Ki-67 assay (Figure 19) to calculate the percentage of cells being in each cell cycle phase.

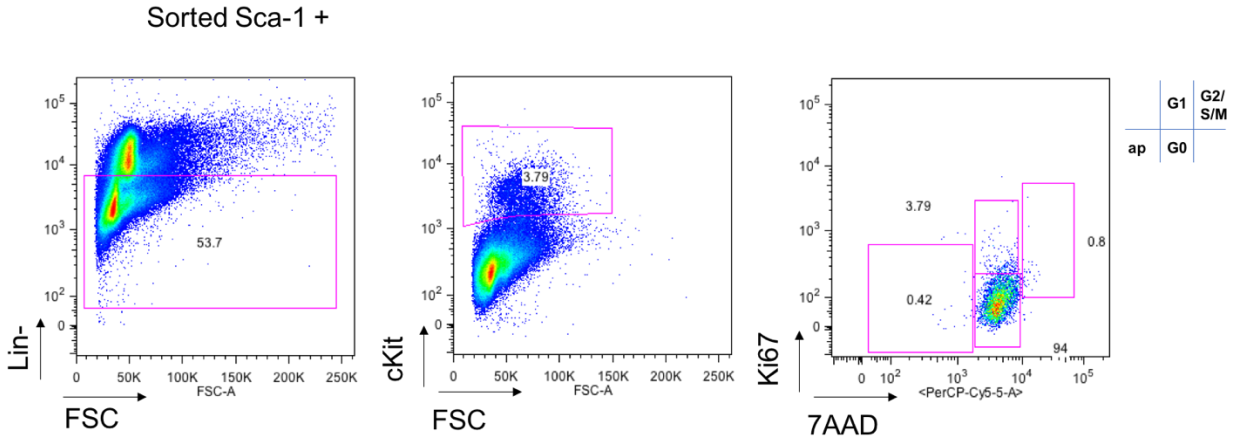

Figure 19 Ki-67 analysis of LSK cells

3.4.2 Cebpα-IFNα signalling

Cebpα is one of the genes that is differentially expressed in the lab's next generation sequencing study between F1 pre-diseased and lupus mice. It is also one of the most prominent master regulators of the myeloid differentiation processes. Since, lupus is associated with enhanced myelopoiesis, it elevates Cebpα to a gene worth looking into in lupus-prone mice.

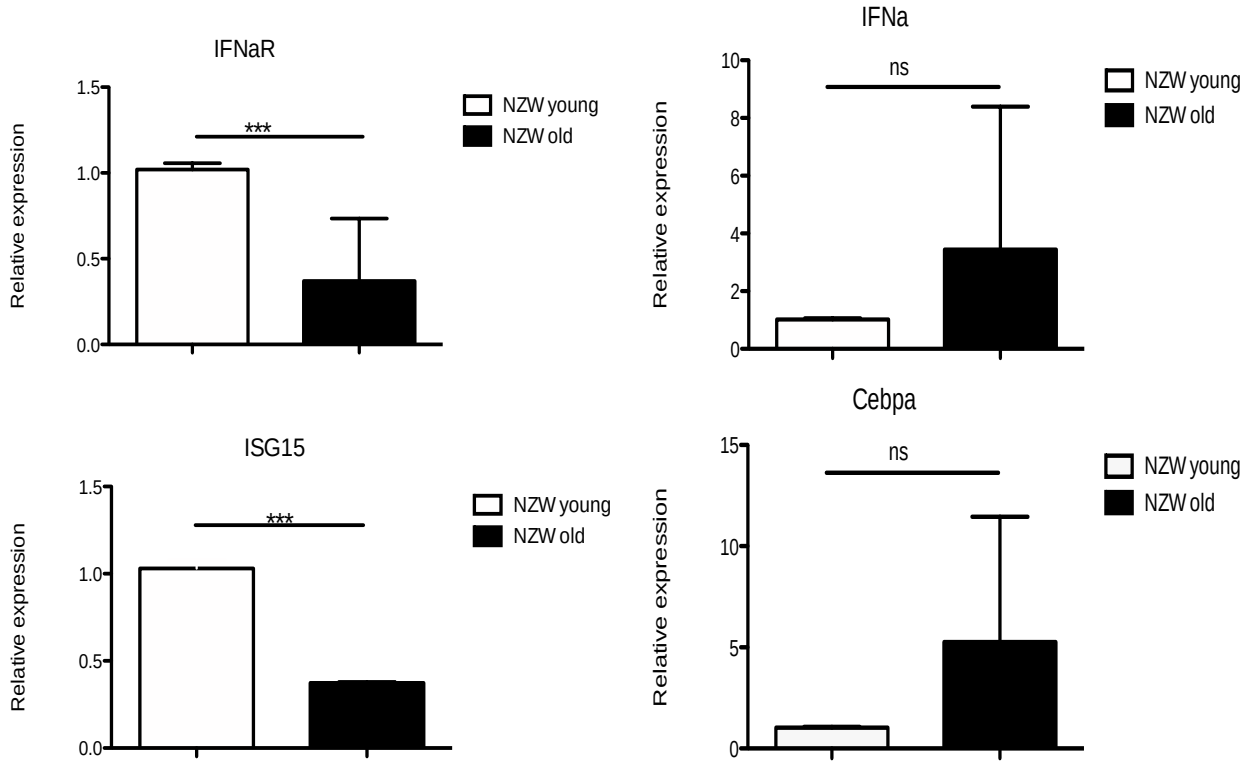

Figure 10 Cebpα and IFN-signaling analysis of the LSK compartment of NZW young and old mice

Our results show that there does not seem to be a statistically significant change in the relative expression of Cebpα in LSK cells of young and old NZW mice according to our results (Figure 20, panel down right). Goodel writes that Cebpα expression increases in B6 mice during development-age but our preliminary findings show an upregulation of Cebpα expression in old B6 mice compared to young (data not shown; 15). Additionally, our incomplete data in F1 mice demonstrate that Cebpα is downregulated in F1 lupus mice (data not shown). Further analysis is required in order to draw trustworthy conclusions.

Our RNA-seq analysis pointed to genes associated with inflammatory responses. Type I interferons are important for inflammatory responses and granulocytic differentiation. Interferon signature has been linked to active lupus and lupus flares in human SLE patients. As such, we sought to examine IFNα, IFNαR and ISG15 expression changes in young an old NZW mice. Even though we saw no changes in IFNα expression

between LSK NZW young and old mice (Figure 20, panel up right), we observed statistically significant changes in the expression of IFNαR and interferon response gene ISG15 (Figure 20, left panel). Both IFNαR and ISG15 expression seems to be downregulated in NZW old mice, with Perry et al., 2017 noting that overexpression of ISGs in humans is linked to ANA production and SLE severity (21).

3.5 Cell Cultures

LSK cells were cultured under such conditions that allowed them to maintain their phenotype intact (See Materials and Methods). In each experiment the setup was adjusted to the desired readout following IFNα stimulation. IFN-γ but not IFNαβ seems to mediate disease in the MRL*lpr* mice. (2). Also, in multiple tested lupus strains, both the spleen and BM IFNα expression levels were elevated.

3.5.1 Cell cycle changes in response to IFNα stimulation

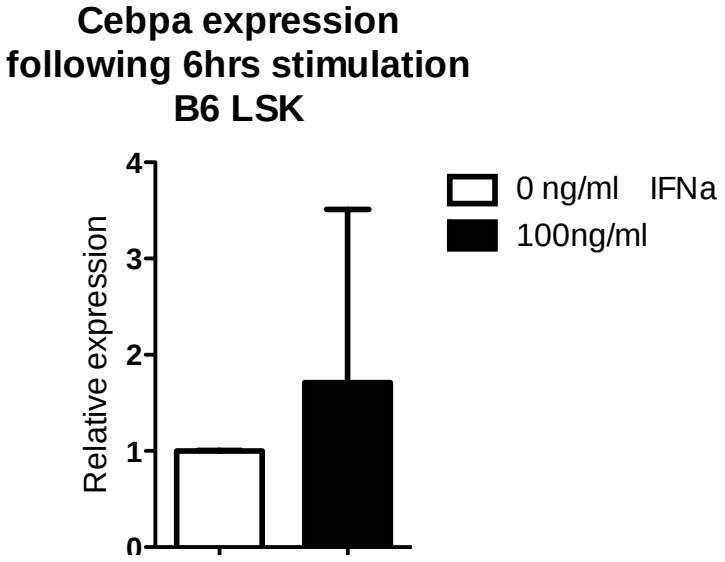

Figure 11 Cebpα expression following 6h IFNα stimulation in LSK cells from B6 young

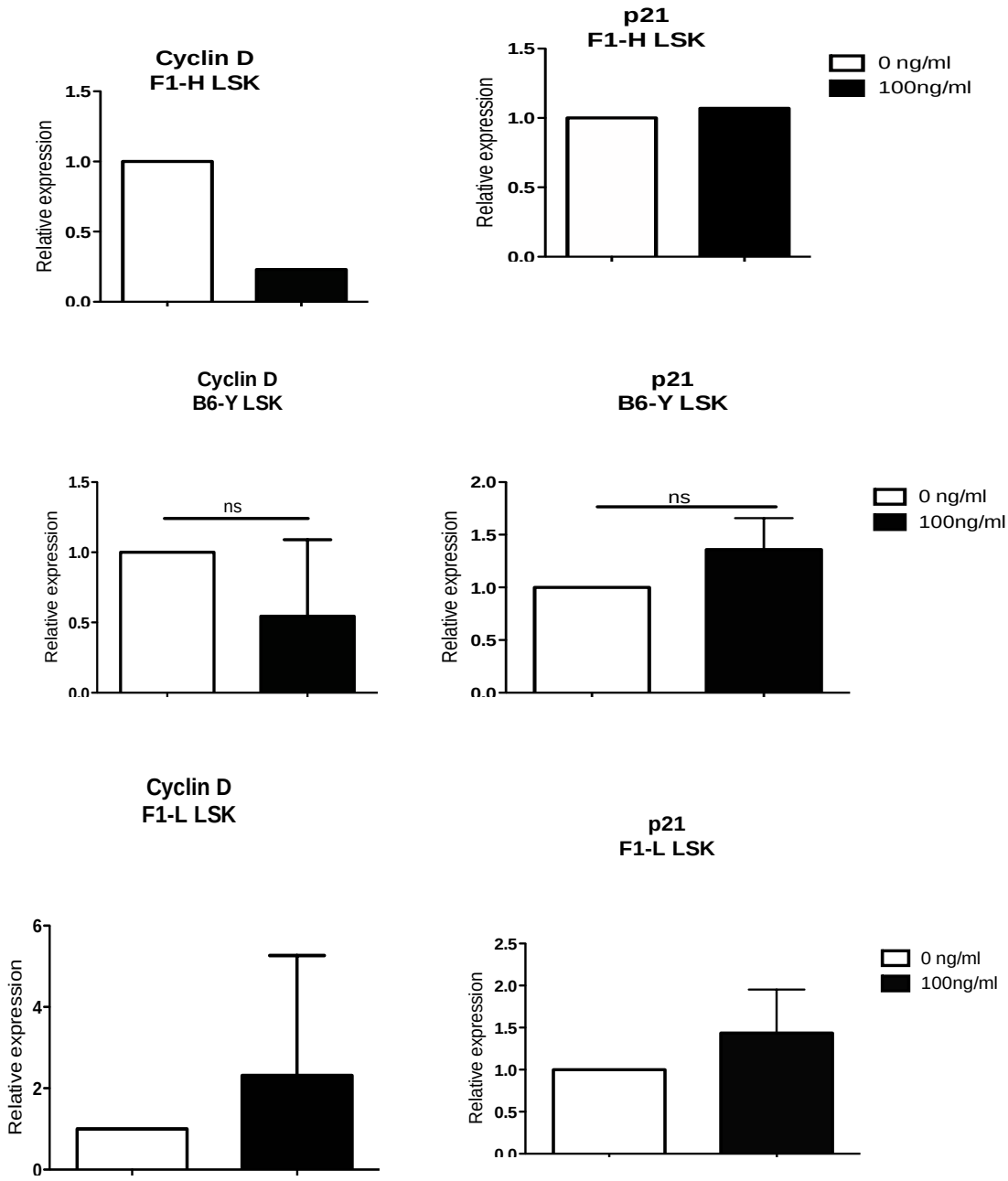

Figure 12 Cell cycle analysis of IFNα-treated LSK cells from B6 young, F1 pre-diseased and F1 lupus mice in vitro

LSK cells from B6 young, F1 pre-diseased and lupus mice were treated with 100ng/ml IFNα in culture for 6hours (Figure 21) and for 12 hours (Figure 22) and samples were analysed next to untreated controls for differential expression of cell cycle genes. We dismissed the 6 hour stimulation setup based on the combinational influence of our

inconclusive preliminary observations and the literature. The preliminary results depicted above show no tangible difference with inconsistences in regards to Cyclin D expression and p21 expression changes that point to cell cycle inhibition following treatment with IFNα. The p21 trend, however, is statistically insignificant, although more experiments are required to be able to conclusively state that our results are in agreement with the literature, which state that IFNα in vitro negatively impacts the LSK cell cycle. IFNα induces the expression of the cyclin-dependent kinase inhibitor p21 (11).

3.5.2 The impact of IFNα stimulation on Cebpα expression of cultured LSK cells

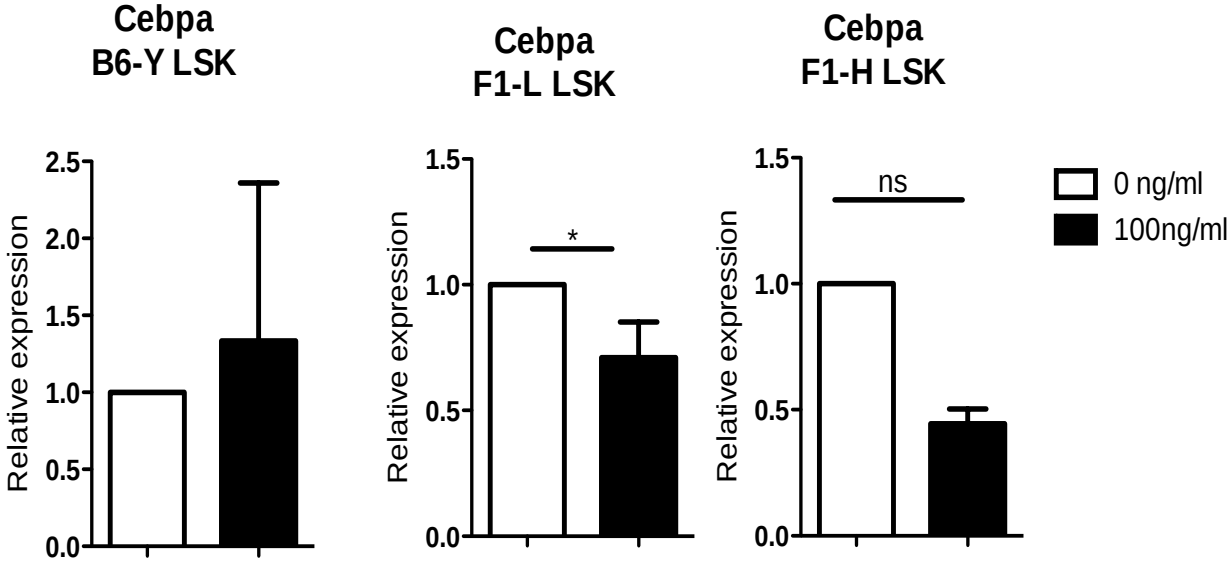

Figure 13 Cebpα expression analysis of IFNα-treated LSK cells from B6 young, F1 pre-diseased and F1 lupus mice in vitro

One of this project's working hypotheses is that IFNα may exert some of its observed effects by directly impacting Cebpα expression. To that end, we investigated whether in vitro IFNα-treated LSK cells from B6 young and F1 pre-diseased and lupus mice have a

differentially expressed Cebpα expression. Our results conclusively show that after 12h post-treatment with IFNα, lupus LSK downregulated their Cebpα expression, demonstrating that perhaps in these cells at this disease stage IFNα is a negative regulator of Cebpα expression.

It is worth noting that, following 12h IFNα treatments we noted decreased cell numbers. In agreement with the above observation Pietras and colleagues highlighted that IFNα treatments in vitro significantly decreased cloning efficiency and colony size. (22). These results will be discussed in the Conclusions section.

3.6 Assessment of LSK differentiation of NZW mice

Figure 14 Methylcellulose culture setup for LSK cells of NZW young and old mice

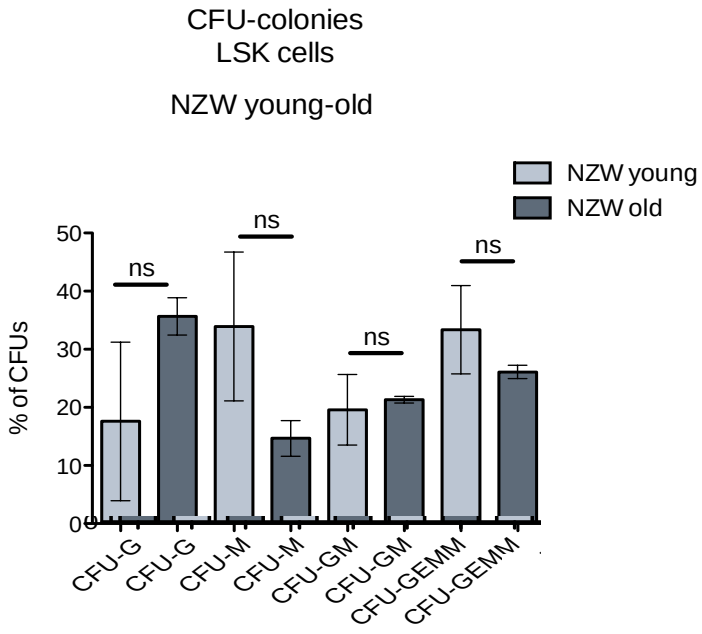

Figure 15 Colony forming unit scoring results from cultures of LSK cells from NZW young and old mice

Semisolid cultures did not show any statistically significant differences in terms of percentages of colony forming units between cultured LSK cells isolated from NZW young and old mice. The setup depicted on Figure 24 depicts how this assay works: sorted LSK (1000-2000 cells) are being mixed into the culture medium and this medium allows for clonal expansion of each cell which leads to the creation of colonies within the culture dish approximately 10 days later. Figure 25 is the analysis that followed of the ratios of each colony type as a percentage of the total number of counted colonies [***(GMP=CFU-GM, granulocytes-CFU-G, monocytes/macrophages=CFU-M, CMP=CFU-GEMM)***].

4. Discussion

HSC are important because they can regenerate the whole bone marrow if need be and because intrinsic hematologic abnormalities can be traced all the way back to these cells.

Our preliminary findings regarding the statistically significant changes in murine LT-HSCs in respect to age, health status and strain could have significant implications associated with other cell types. Effective neutralization of infection is directly linked to augmented production of neutrophils, which, is an immediate result of an LSK expansion in the bone marrow that forced these stem cells (particularly long-term repopulating HSCs and myeloid-restricted progenitor cells with long-term repopulating activity) to differentiate, mobilize and reach the spleen. Published research such as (7) paves the way for directly acting on HSCs to promote –or block when need be- differentiation into myeloid progenitors during emergency myelopoiesis. Additionally,emergency myelopoiesis may be the result of chronic IFNα signalling.

Expansion of LSK cells along with their differentiation and subsequent mobilization into the spleen, results in elevated production of neutrophils to control the infection. The percentages of LSK cells with high expression of c-Kit, as a portion of total Lin- cells, had statistically significant differences amongst different strains and diseased states. Grinenko and colleagues were the first to report differences in the clonal expansion capacities of HSCs expressing distinct levels of the c-Kit receptor using serial transplantation experiments (9). C-Kit ^{int} expressing-HSCs exhibited greater expansion capacities when compared to kit-^{hi}-HSCs, a finding pointing to a cell-intrinsic HSC clonal capabilities. Because the consecutive developmental transition from Kit^{int} to Kit^{hi} HSCs can be seen as a step towards differentiation commencement, it is also, by definition, significant for loss of expansion potential. Real time analysis following SCF stimulation by Grinenko points to inherent differences that can be found in processes associated

with cell cycling and cellular adhesion (9). Ye and colleagues showed that loss of Cebpα led to an expansion LT- HSCs, within the LSK compartment (34).

Furthermore, in respect to the overall bone marrow cellularity, B6 old mice appear to have the most drastic increase across all strains when compared with strain-matched developmentally teenage mice. That is indicative of two things. Firstly, the influence of aging alone in the bone marrow cellularity should not be underestimated and secondly, perhaps NZW parental strain is a more appropriate control to assess the age-associated changes in cellular compartments. The strong statistical differences between B6 and NZW young female mice should be considered best evidence when arguing the second point. It is noteworthy that, although our results are in agreement with the limited literature pertinent to (NZWxNZB)/F1 mice, they do not agree with Zhou and colleagues regarding B6. This groups mentions slightly decreased bone marrow cellularity in the B6 in ageing without any stimuli or treatment. Zhou and colleagues found a slight increase in the total bone marrow cell numbers in (NZWxNZB)/F1 mice (from 42 to 46 million cells), but this increase was observed neither in the MRL-lpr and B6.NZMSle1/2/3 lupus-prone mice nor the B6 mice, which served as the control non-lupus-prone strain (36).

Generally, attention should be paid to the fact that expansion of LSK cells can include myeloid progenitors retaining the LSK phenotype (7). Only 1 every 30 LSK cells is actually an HSC so, for any experiment focusing on this population, the use of the more specific SLAM (CD48−/CD150+) should be implemented to reaffirm the more used LSK phenotypic definition and validate experimental findings. This is particularly important for experiments that include analyses of cell cycling and proliferation as contamination by actively cycling myeloid progenitors with reacquired Sca-1 expression on their cell surface could very easily contaminate the results and affect a potential statistical significance.

LSK cells' cycle does not seem to change between NZW young and old female mice, as determined by the relative expression of Cyclin D and p18. Even though in the latter a trend is evidenced but a definitive conclusion cannot be drawn due to the paired

deficiency of a larger test sample and elevation to statistical importance that corroborates preliminary observations. More experiments should be added for statistical reinforcement and more cell cycle regulators should be looked into. IFNα induces the expression of the cyclin-dependent kinase inhibitor p21 (11).

Perry observed that overexpression of ISGs in humans is linked to ANA production and SLE severity (21). Preliminary analysis of LSK from the F1 and B6 strains has yet to offer conclusive results (data not shown). We observed a statistically significant upregulation of ISG15 in NZW old mice.

In the literature, (37) that lupus prone mice –with the exception of the pristane model- have weak or absent interferon signature. Our analyses point to NZW, the parental strain of the (NZWxNZB)/F1 mouse, being an IFN-dependent model. Zhou also observed that in the bone marrow (NZWxNZB)/F1 mice had increased IFNα as diseased progressed without being subjected to any treatment or exogenous stress(36).

To add to that, Zhou demonstrated that when IFN-inducible genes were tested in pDCs (which quickly respond to type I interferon stimuli) all MX1, IFIT2 and CXCL10 IFN response genes were upregulated in spleen and bone marrow pDCs in all tested lupus-prone strains. This was not the case for B6 mice. (36). Mathian and colleagues were the first to show that (NZB × NZW)/F1 mice infused with IFNα experienced accelerated lupus, thus proving that prolonged exposure of this cytokine in vivo causes premature deadly SLE in lupus prone mice. (17;13**).**

Also, SLE patients exhibit high levels of soluble TNF receptors (8) which could block endogenous TNFa. A genetic deficiency in TNF could lead to TNFa-mediated downregulation of the IFNαβ pathway. (NZWxNZB)/F1 mice have such a deficiency and maybe this is why the IFNα signature is present but not that easily detectable (2).

It is well documented that SLE patients have increased IFNα levels and strong IFNα signature, particularly during flares, in most cell types and tissues. Under physiological conditions and within the bone marrow, IFNα induces dormant HSCs to proliferate, and chronic IFNα stimulation leads to HSC dysfunction and finally exhaustion. Another

accepted generalization is that lupus patients exhibit granulopoietic/granulocytic signature. A highlight of this project's results can be summarized in the statement that downregulation of Cebpα in murine lupus HSCs, results in a pronounced expansion and enhanced proliferation, characteristics that result from IFNα-mediated (transcriptional) regulation on the Cebpα promoter.

5. Future plans

In addition to pursuing more complex bioinformatics analyses, it would be useful to mechanistically explore this project's findings regarding Cebpα, using lentiviral vectors that overexpress Cebpα. Once vectors are made (constructs that allow for Cebpα overexpression and others necessary for viral replication) and viruses are created, primary LSK cells will be transduced with the lentiviruses *in vitro* (efficiency will be monitored by Real Time PCR) to monitor if restoration of Cebpα levels will result in rescue of the lupus phenotype. The readout, in the LSK cells' case, will be change in the expression of cell cycle genes and colony distributions that most likely resemble the LSK cells of healthy controls (F1-prediseased) (LSK potential to proliferate and differentiate). Definitive experiment will always come down to bone marrow transplantation of lethally irradiated mice. A single bone marrow HSC can reproduce/recreate the entire immune system, if it is transplanted into a lethally irradiated animal (15). However, the particular nature of LSKs –and other rare stem cell populations for that matter- is that their behaviour -in terms of proliferation, differentiation and overall function- changes with their proximity to special niches within the bone marrow. As such, the most important results with the most applicable physiological meaning can only derive from well thought out in vivo studies. This would include LSK cells transduced with highly concentrated viruses with constructs for overexpressing Cebpα and transplanted to lethally irradiated SLE mice.

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