Master thesis

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Understanding transcriptional and epigenetic regulation of macrophage activation in neonatal mice

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MOLECULAR BIOLOGY AND BIOMEDICINE

Summary

Macrophages are key regulators of innate immunity through their different activation phenotypes, M1 and M2, that they acquire by responding to diverse signals. Differentiation and activation of macrophages require the timely regulation of gene expression including transcription factors and epigenetic modifications. The M1 macrophage phenotype, that is, the state of activated pro-inflammatory macrophages, is characterized by increased glycolysis, induction of the pentose phosphate pathway, conversion of arginine to nitric acid by the inducible Nitric Oxide Synthase (iNOS), fatty acid synthesis and oxidation. Anti-inflammatory M2 macrophages exhibit increased glutamine uptake and catabolism, Arginase-1 metabolism of arginine, oxidative phosphorylation, enhanced fatty acid oxidation and glycolysis. Metabolism also regulates macrophage cell activation, mainly through the PI3K / AKT / mTOR pathway, whereas in recent years, epigenetic regulators appear to play an important role in regulating metabolism and therefore in macrophage activation. Previously performed siRNA screen, identified histone modifying enzymes being important for M1/M2 polarization. Earlier experiments in the laboratory have shown the different metabolic profiles of AKT1 - / - and AKT2 - / - macrophages as well as the possible differential role of PHF2 and PHF8, two histone demethylases, in macrophage activation.

The purpose of this thesis was to study the role of PHF2, PHF8 and other epigenetic factors, in the regulation of macrophage metabolism in both adult and neonatal mice. The expression and role of histone modifying enzymes in the metabolic profile of macrophage-deficient mice for AKT1 or AKT2 kinase was also studied. In addition, other epigenetic factors were screened at the mRNA level to determine potential differential expression between adults and neonates. In the present work, primary macrophages from adult and neonatal mice were used, whose metabolic profile was examined using molecular techniques such as Elisa, real time PCR, Western Blot. We showed that neonatal macrophages inefficiently induced key components of proinflammatory reaction upon infection, such as Inducible Nitric Oxide Synthase, Tumor Necrosis Factor alpha and Interleukin-12 while they maintained higher basal levels for the first two. We demonstrated that neonatal macrophages induced much higher expression of Interleukin-10, an anti-inflammatory cytokine, upon TLR4 stimulation compared to adult macropahges. However, this difference was not evident when other anti-inflammatory regulators were examined, such IRAKm, Arginase 1, Fizz1, while CCAAT Enhancer Binding Protein beta, which appeared to be slightly higher in neonates upon LPS treatment. Transcription factors of the Interferon Regulatory Factors family such as IRF5 and IRF4 were identified to be higher in adults in unstimulated macrophages. IRF5, a regulator of M1 pro-inflammatory activation, was induced only in adults after TLR4 triggering. IRF4, a key regulator of M2 macrophages, was induced at later stages of activation and at comparable levels between adults and neonates. We also identified differences in the post-transcriptional regulators miR-155, which is higher in neonates both basally and 6 hours after LPS stimulation, and miRNA-147a, which was slightly elevated in neonatal macrophages compared to adult 6 hours following LPS stimulation. Furthermore, we showed that epigenetic regulation of innate immune response might vary due to the differential expression of the methyltransferases MLL1 and SETD7 as well as the histone demethylases JMJD3, PHF8 and PHF2. Finally, we demonstrated that the epigenetic factors SETD7, MLL1, MLL3, HDAC1, SIRT6 were regulated by the AKT pathway since they were differentially expressed in Akt1 or Akt2 deficient primary murine macrophages. In this master thesis, we propose some transcriptional, post-transcriptional and epigenetic regulators that might be responsible for the insufficient innate immune response of neonates.

Περίληψη

Τα μακροφάγα είναι οι βασικοί ρυθμιστές της έμφυτης ανοσίας μέσω των διαφορετικών φαινοτύπων ενεργοποίησής τους, M1 και M2, τους οποίους αποκτούν μετά την απόκρισή τους σε διάφορα σήματα. Η διαφοροποίηση και η ενεργοποίηση των μακροφάγων απαιτούν την έγκαιρη ρύθμιση της γονιδιακής έκφρασης, συμπεριλαμβανομένων μεταγραφικών παραγόντων και επιγενετικών τροποποιήσεων. Ο M1 φαινότυπος, δηλαδή η ενεργοποίηση των προ-φλεγμονωδών μακροφάγων, χαρακτηρίζεται από αυξημένη γλυκόλυση, διέγερση του μονοπατιού φωσφορικής πεντόζης, μετατροπή αργινίνης σε νιτρικό οξύ από την Επαγώγιμη Συνθάση νιτρικού οξέος (iNOS), σύνθεση λιπαρών οξέων και οξείδωση. Τα αντιφλεγμονώδη μακροφάγα M2 εμφανίζουν αυξημένη πρόσληψη και καταβολισμό γλουταμίνης, υψηλό μεταβολισμό αργινίνης από την Αργινάση-1, οξειδωτική φωσφορυλίωση, αυξημένη οξείδωση λιπαρών οξέων και γλυκόλυση. Ο μεταβολισμός ρυθμίζει επίσης την ενεργοποίηση των μακροφάγων, κυρίως μέσω την οδού PI3K / AKT / mTOR, ενώ τα τελευταία χρόνια, οι επιγενετικοί ρυθμιστές φαίνεται να παίζουν σημαντικό ρόλο στη ρύθμιση του μεταβολισμού και συνεπώς στην ενεργοποίηση των μακροφάγων. Προηγούμενο πείραμα siRNA screen, ταυτοποίησε ορισμένες πρωτεΐνες τροποποίησης ιστονών που φάνηκε να είναι σημαντικές για την πόλωση προς M1 ή M2. Παλαιότερα πειράματα στο εργαστήριο χαρακτήρισαν τα διαφορετικά μεταβολικά προφίλ των μακροφάγων σε AKT1 - / - και AKT2 - / - ποντίκια, καθώς και τον πιθανό διαφορετικό ρόλο των PHF2 και PHF8, δύο απομεθυλασών ιστονών, στην ενεργοποίηση των μακροφάγων.

Ο σκοπός αυτής της διπλωματικής εργασίας ήταν να μελετήσει τον ρόλο των PHF2, PHF8 και άλλων επιγενετικών παραγόντων, στη ρύθμιση του μεταβολισμού των μακροφάγων τόσο σε ενήλικα όσο και σε νεογνά ποντίκια. Μελετήθηκε επίσης η έκφραση και ο ρόλος των πρωτεϊνών τροποποίησης ιστόνης στο μεταβολικό προφίλ ποντικών με ανεπάρκεια μακροφάγων για AKT1 ή AKT2 κινάση. Επιπλέον, άλλοι επιγενετικοί παράγοντες εξετάστηκαν σε επίπεδο RNA για διαφορική έκφραση μεταξύ ενηλίκων και νεογνών. Στην παρούσα εργασία, χρησιμοποιήθηκαν πρωτογενή μακροφάγα ενήλικων και νεογνών ποντικών, των οποίων το μεταβολικό προφίλ εξετάστηκε χρησιμοποιώντας μοριακές τεχνικές όπως Elisa, PCR σε πραγματικό χρόνο, Western Blot. Δείχνουμε ότι τα νεογνικά μακροφάγα είναι ανεπαρκή όσον αφορά την επαγωγή βασικών συστατικών της προφλεγμονώδους αντίδρασης κατά τη μόλυνση, όπως η Επαγώγιμη Συνθάση νιτρικού οξέος (iNOS), ο παράγοντας νέκρωσης όγκου άλφα (TNFa) και η ιντερλευκίνη-12 (IL-12) ενώ διατηρούν υψηλότερα βασικά επίπεδα για τα πρώτα δύο. Δείχνουμε επίσης ότι τα νεογνά προκαλούν πολύ υψηλότερη έκφραση της ιντερλευκίνης-10 (IL-10), μια αντιφλεγμονώδη κυτοκίνη, μετά από διέγερση TLR4 και σε σύγκριση με τους ενήλικες. Ωστόσο, αυτή η διαφορά δεν είναι ορατή όταν εξετάζονται και άλλοι αντιφλεγμονώδεις ρυθμιστές, όπως IRAKm, Arginase 1, Fizz1, ενώ το CCAAT Enhancer Binding Protein beta (c/EBPb) φαίνεται να είναι ελαφρώς υψηλότερος στα νεογνά μετά τη διέγερση από λιποσακχαρίτη (LPS). Οι μεταγραφικοί παράγοντες της οικογένειας Interferon Regulatory Factors όπως οι IRF5 και IRF4 εντοπίστηκαν σε υψηλότερα επίπεδα στους ενήλικες σε μη διεγερμένη κατάσταση, με τον πρώτο, ο οποίος ρυθμίζει την προ-φλεγμονώδη ενεργοποίηση των M1, να αυξάνεται μόνο σε ενήλικες μετά τη σηματοδότηση TLR4 ενώ ο IRF4, ένας βασικός ρυθμιστής των μακροφάγων M2, αυξάνεται σε μεταγενέστερη χρονική περίοδο και σε ίσα επίπεδα μεταξύ ενηλίκων και νεογνών. Εντοπίσαμε επίσης διαφορές στους μετα-μεταγραφικούς ρυθμιστές miR-155 που είναι υψηλότερο στα νεογνά τόσο σε βασικά επίπεδα όσο και 6 ώρες μετά τη διέγερση με LPS΄ και miR-147a το οποίο είναι ελαφρώς αυξημένο στα νεογνά σε σύγκριση με τους ενήλικες 6 ώρες μετά από LPS. Επιπλέον, δείχνουμε ότι η επιγενετική ρύθμιση της έμφυτης ανοσοαπόκρισης μπορεί να ποικίλει λόγω της διαφορικής έκφρασης των μεθυλοτρανσφερασών MLL1 και SETD7 καθώς και των απομεθυλασών ιστονών JMJD3, PHF8 και PHF2. Τέλος, αποδείξαμε ότι οι επιγενετικοί παράγοντες SETD7, MLL1, MLL3, HDAC1, SIRT6 ρυθμίζονται από το μονοπάτι AKT, δεδομένου ότι εκφράζονται διαφορετικά σε πρωτογενή μακροφάγα ποντικού Akt1 ή Akt2 Knock-Out. Σε αυτήν την μεταπτυχιακή εργασία, προτείνουμε μερικούς μεταγραφικούς, μετα-μεταγραφικούς και επιγενετικούς ρυθμιστές που ενδέχεται να είναι υπεύθυνοι για την ανεπαρκή έμφυτη ανοσοαπόκριση των νεογνών.

Contents

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List of abbreviations

Akt	Protein kinase B
APS	Ammonium Persulphate Solution
Arg1	Arginase 1 protein
BCA	bicinchoninic acid assay
BGG	Bacillus Calmette–Guérin
BMDMs	Bone Marrow Derived Macrophages
bp	base pairs
BSA	Bovine Serum Albumin
c/EBPb	CCAAT Enhancer Binding Protein beta
CARM1/PRMT4	Coactivator Associated Arginine Methyltransferase 1
Chip	Chromatin immunopercipitation
CPB	CREB-binding protein
CREB	cAMP response element-binding protein
DMEM	Dulbecco's Modified Eagle Medium
ECL	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
Ezh	Enhancer of Zeste
FBS	Fetal Bovine Serum
Fizz1	Found in inflammatory zone 1 / Resistin Like molecule alpha1
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HRP	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
iNOS	cytokine-inducible Nitric Oxide Synthase
IRAKm	Interleukin 1 Receptor Associated Kinase M
IRF	Interferon Regulatory Factor
Jmjd3	Jumonji domain-containing protein D3
KDM2B	Lysine Demethylase 2B
KDM3B	Lysine-specific demethylase 3B
KO	Knock-out
L	Liter
LCCM	L Cell Conditioned Medium (L-929 cells)
LPS	Liposaccharide
M	Molar
mA	miliamper
MAPK	Mitogen-activated protein kinase
me	methylation
me1	monomethylation
me2	dimethylation
me3	trimethylation
mir / miRNA	microRNA
mL	mililiter
MLL	Mixed lineage leukemia protein
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
mTORC	mammalian target of rapamycin complex

NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanograms
nm	nanometer
NO	Nitric Oxide
ns	Not significant
ox- LDL	oxidazed low-density lipoprotein
PAMP	Pathogen Associated Molecular Patterns
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PHF2	PHD Finger Protein 2
PHF8	PHD Finger Protein 8
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PPAR	Peroxisome proliferator-activated receptor
PVDF	polyvinylidene difluoride
qPCR	quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay buffer
rpm	rounds per minute
RT	Room Temperature
SDS	Sodium dodecyl sulfate
seq	sequencing
SETD7	SET domain containing lysine methyltransferase 7
siRNA	small interfering RNA
SIRT6	Sirtuin 6
Temed	Tetramethylethylenediamine
TGF	Transforming Growth Factor
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor
ug / µg	microgram
uL / µL	microliters
Utx	Ubiquitously transcribed tetratricopeptide repeat, X chromosome
w/v	weight/volume
WFI	Water For Injections
β-glucan	beta glucan

Introduction

Innate immunity is the first, non-specific defense against pathogens intrusion. It takes place immediately after infection and although it has been long proposed that it possesses no immunological memory it has been recently shown that can be trained. Cell types involved in innate immunity and their main functions are summarized in Figure 1. Innate immune response is activated through Toll-like receptors (TLRs) that bind a variety of microbial antigens, such as liposaccharide LPS (Ulvitech & Tobias, 1995), called pathogen associated molecular patterns (PAMPs) (Medzhitov, 2001). The resulting inflammatory response is necessary for the elimination of the pathogens. However, excessive inflammation or sudden and large scale production of cytokines (cytokine storm) may cause severe tissue damage, sepsis and sometimes even mortality. To avoid such events and maintain balance between beneficial and harmful effects of inflammation, a negative feedback loop is required for the inhibition and resolution of inflammatory response. Innate immunity is also essential for the activation of acquired immunity which depends on antigen recognition and memory adaptation.

Cell	Image	% in adults	Nucleus	Functions	Lifetime	Main targets
Macrophage*		Varies	Varies	• Phagocytosis • Antigen presentation to T cells	Months – years	• Various
Neutrophil		40-75%	Multi-lobed	• Phagocytosis • Degranulation (discharge of contents of a cell)	6 hours – few days	• Bacteria • Fungi
Eosinophil		1-6%	Bi-lobed	• Degranulation • Release of enzymes, growth factors, cytokines	8-12 days (circulate for 4-5 hours)	• Parasites • Various allergic tissues
Basophil		< 1%	Bi- or tri-lobed	• Degranulation • Release of histamine, enzymes, cytokines	Lifetime uncertain; likely a few hours – few days	• Various allergic tissues
Lymphocytes (T cells)		20-40%	Deeply staining, eccentric	T helper (Th) cells (CD4+): immune response mediators Cytotoxic T cells (CD8+): cell destruction	Weeks to years	• Th cells: intracellular bacteria • Cytotoxic T cells: virus infected and tumour cells • Natural killer cells: virus-infected and tumour cells
Monocyte		2-6%	Kidney shaped	Differentiate into macrophages and dendritic cells to elicit an immune response	Hours – days	• Various

Figure 1: Characteristics and function of cells involved in innate immunity (Warrington, Watson, Kim, & Antonetti, 2011)

Inflammation involves a variety of immune cell populations and signaling molecules that initiate a cascade of actions that lead to the induction of proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukins, chemokines, reactive oxygen and nitrogen species, antimicrobial peptides production, along with phagocytosis events (Takeuchi & Akira, 2010). Cytokines are signaling proteins that are secreted by the donor cell and bind to the receptor of the recipient cell, initiating signaling. They can regulate multiple biological functions such as innate and acquired immunity and inflammation. They allow communication between the immune system and host tissue cells, regulating cell death of inflammatory tissues. IL-1α/β, TNFα/β, IL-6, IL-11, IL-18, IFN-γ are known proinflammatory cytokines while IL-10, TGFβ, IL-1ra are anti-inflammatory cytokines.

Endotoxin tolerance is the state in which macrophages exhibit reduced activation upon re-stimulation with bacterial endotoxin or lipopolysaccharide (LPS) even at mortal doses. TNFα and IL-6 mRNA levels are reduced in LPS-tolerant macrophages after LPS treatment (West & Heagy, 2002).

Macrophages

Macrophages are a type of white blood cells, members of the innate immune system and are responsible for the detection and destruction of pathogens through phagocytosis or recruitment of other cells. They are the main effectors of innate immune system, but also important for clearance from cellular debris, development, tissue repair and homeostasis. They are responsible for initiation of inflammation by releasing signaling molecules called cytokines, but also its resolution. Tissue-resident macrophages differentiate during development from the yolk sac, fetal lever and bone marrow (X. Zhang, Zhivaki, & Lo-Man, 2017). Precursors of macrophages that reside in bone marrow are hematopoietic stem cells (Figure 2).

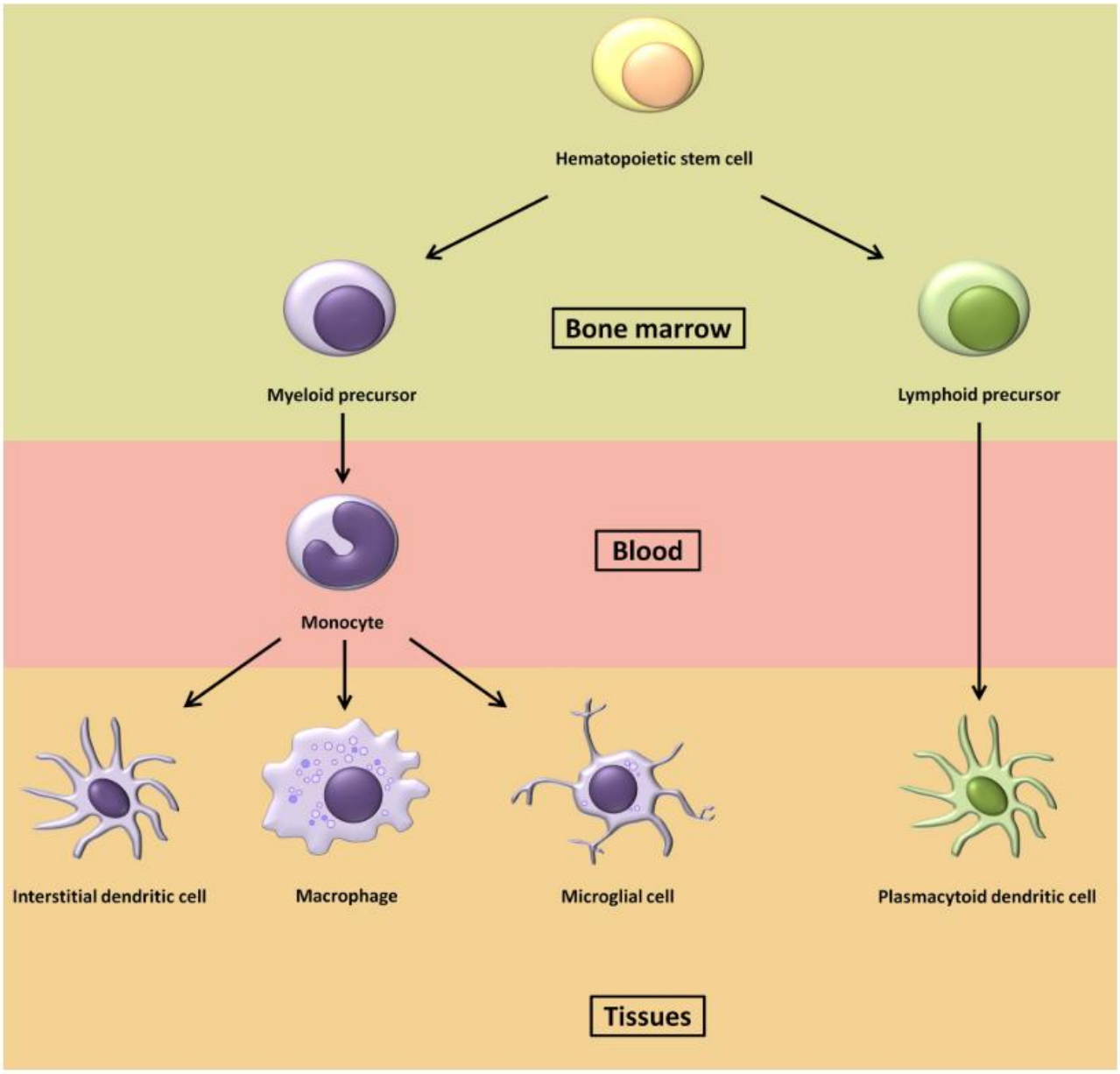

Figure 2: All cells from the monocyte-macrophage lineage derive from a same progenitor multipotent cell, the hematopoietic stem cell (HSC). The HSC, located in the bone marrow, may differentiate either into a myeloid or a lymphoid precursor, setting up the divergence between the myeloid (blue) and plasmacytoid (green) lineage. The myeloid precursor is then able to migrate into the blood stream and to differentiate into a monocyte. Monocytes migration to specific tissues and their differentiation occur upon a stimulation of a different cytokines, interleukins and/or other factors cocktail. Depending to the location, the monocytes become either interstitial dendritic cells, macrophages or micro- glial cells. Lymphoid precursor runs parallel with the myeloid one, but can directly differentiate into another type of dendritic cell, the plasmacytoid dendritic cell (Le Douce, Herbein, Rohr, & Schwartz, 2010).

Macrophage polarization

Activated macrophages can acquire different phenotypes broadly characterized as, M1-like and M2-like, also known as alternative activated macrophages. While M1 macrophages inhibit cell proliferation and cause tissue damage, M2 promote cell proliferation and wound healing. M1 activation usually occurs after LPS or IFNγ stimulation, leading to the expression and secretion of pro-inflammatory cytokines, such as IL-1α/β, TNFα, IL-6, IL-12, IL-11, IL-18, IFN-γ and nitric oxide (NO) which follows the expression of the cytokine-inducible Nitric Oxide Synthase iNOS. TNF-α (Tumor Necrosis Factor-alpha) is a potent multifunctional cytokine which can exert regulatory, inflammatory and cytotoxic effects on a wide range of normal lymphoid and non-lymphoid cells and tumor cells. TNF-α is secreted by macrophages, monocytes, neutrophils, T-cells (primarily CD4+ T cells), NK-cells, and many transformed cell lines. Lipopolysaccharides (LPS) are components of the outer membrane of Gram negative bacteria. LPS create a strong immune response after binding Toll-like-receptor 4 (TLR4), promoting secretion of pro-inflammatory cytokines and nitric oxide. LPS can also activate a non-canonical pathway leading to the creation of inflammasome in an TLR4-independent caspase 11-dependent manner (Molloy, 2013). The main role of M1 macrophages is to eliminate pathogens. M2 activation is usually triggered by IL-4, IL-12, IL-13, immunocomplexes, and glycocorticoids, initiating the production of many M2 markers responsible for the resolution of inflammation, such as IL-10 which is an inhibitory cytokine, Arginase1, Ym1, Mrc1, found in inflammatory zone 1 Fizz1 (Beschin, Brys, Baetselier, Raes, & Noe, 2002) and other transcriptional regulators such IRF4, IRAKm, cEBP/β. M2s are responsible for building extracellular matrix and prevent tissue damage (Gratchev et al., 2001). The metabolic profile of M2 macrophages includes suppression of the inducible nitric oxide synthase (iNOS) that catalyzes the production of NO and L-citrulline from L-arginine; instead, an alternative reaction involves arginase, an enzyme that catalyzes the conversion of L-arginine to ornithine and urea (Munder, Eichmann, & Modolell, 1998)

Efficient immune response and metabolism are two of the most important selection determinants that

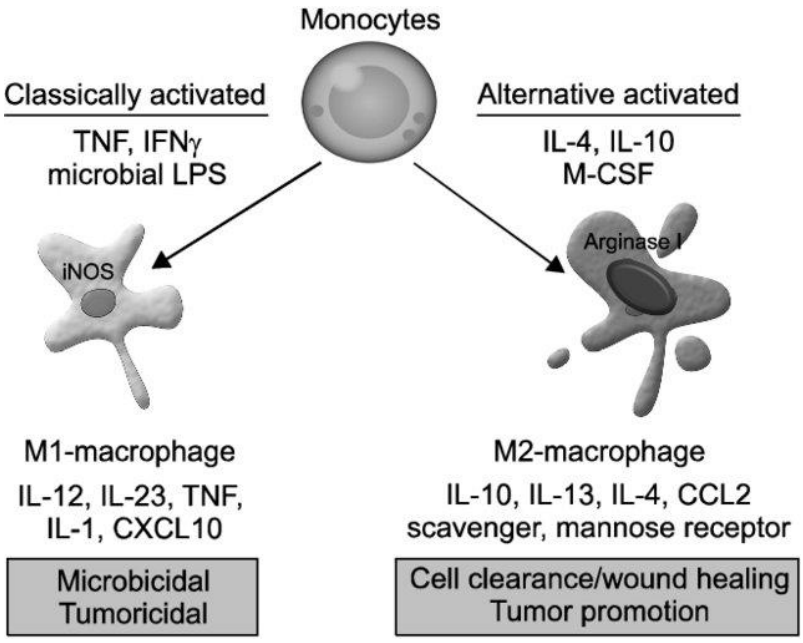

Figure 3: Macrophage activation and polarization. "Classically activated" M1 macrophages are activated by TNF, IFNγ or bacterial products such as microbial lipopolysaccharide (LPS), express high levels of IL12, IL-23, TNF, IL-1 or CXCL10 and low levels of IL10. By contrast, "alternatively activated" M2 macrophages are activated by IL4, IL10, IL13, M-CSF or glucocorticoid hormones, express high levels of IL10, IL-13, IL-4, CCL2, scavenger receptor-A or mannose receptor and low levels of IL12. Functionally, M1 macrophages are microbicidal or tumoricidal and M2 macrophages play a role in cell clearance/wound healing or tumor promotions (Cho, 2013).

contributed to the successful human evolution. A variety of molecules play a role in both nutrient metabolism and inflammatory activation. For instance, tumor necrosis factor-α (TNFa) is a pro-inflammatory cytokine that can also disrupt the insulin signaling cascade (Hotamisligil, Shargill, & Spiegelman, 1993). Obesity is a well characterized metabolic state of energy imbalance that may result in mild chronic inflammation promoting insulin resistance and diabetes. Insulin resistance is also promoted locally upon tissue inflammation. Metabolic plasticity of macrophages is crucial for their activation phenotype, leading to

differential innate immune response. For instance, M2 polarized macrophages highly express Arginase 1 which metabolizes L-arginine to L-ornithine and urea. Ornithine is an intermediate to the amino acid proline which is essential for collagen protein synthesis. The aforementioned example supports the tissue remodeling and extracellular matrix maintenance features of M2 macrophages. On the other hand, M1 macrophages use arginine for the production nitric oxide synthase which catalyzes nitric oxide generation, a hallmark of proinflammatory phenotype (Lumeng, DeYoung, Bodzin, & Saltiel, 2007). mTOR is a major pathway in macrophage metabolism. mTORC1 stimulates the expression of the transcription factor c/EBPb. Akts (Akt1, Akt2, Akt3) serine-threonine kinases regulate a variety of cellular processes such as survival, proliferation differentiation, metabolism. Each Akt has unique functions. Akt1 promotes M2 polarization while deletion of Akt1 promotes M1 polarization. Akt2 lacking macrophages possess a M2-like phenotype with reduced mir-155 expression and LPS response (Arranz et al., 2012; Ieronymaki et al., 2019). The reduced mir-155 levels lead to increased levels of the transcription factor c/EBPb, one of the main regulators of M2 polarization.

Metabolism regulates macrophage cell activation, mainly through the PI3K / AKT / mTOR pathway. PI3K/Akt pathway is also pivotal for inhibition of LPS signals such as TNFα expression and secretion (Guha & Mackman, 2002). Inhibition of PI3-kinase has been proved to be essential for iNOS and IL-12 expression after LPS stimulation (Fukao & Koyasu, 2003; Panan, Raymond, & Singh, 1999). PI3K regulates the extend of primary activation, early after TLR signaling, since it's activated from LPS and downstream activates Akt kinases.

Transcriptional and post-transcriptional regulation of macrophage polarization

Successful macrophage activation is tightly linked with coordination of macrophage transcriptional regulation, involving a variety of transcription factors. IRAKm is a transcription factor activated upon Toll-like receptor (TLR) signaling and regulates the inhibition of TLR (Kobayashi et al., 2002), dampening the inflammatory response. CCAAT/enhancer binding protein c/EBPb is a transcription factor involved in immune and inflammatory responses by activating anti-inflammatory genes, such as IRAK-m, thus characterized as an M2 marker. IRAKm promoter can be bound by c/EBPb after removal of a silencing H3K27 trimethylation catalyzed by enhancer of Zeste 2 (Ezh2), part of the polycomb recessive complex 2. Upon LPS stimulation, this H3K27me3 is removed, allowing IRAKm expression (Lyroni et al., 2017). c/EBPb is also known to induce M2 markers such as Arginase 1, IL-10 and mir-146a, while it's deletion inhibits M2 polarization (Ruffell et al., 2009). Irf4 is a master regulator of M2 polarization since it also inhibits TLR signaling (Satoh et al., 2010). Fizz1 is another secreted protein which serves as a good marker for alternative activated macrophages (Beschin et al., 2002).

Micro RNAs are short non-coding molecules that regulate gene expression through binding to complementary mRNAs, thus regulating mRNAs translation and turnover. miRNA expression is activated in response to specific extracellular signals. A number of miRNAs have been identified to act upon LPS stimulation and play a role to the inflammatory phenotype. Some microRNAs, such as mir-155 and mir-146a, regulate the inflammatory response and endotoxin tolerance (Doxaki, Kampranis, Eliopoulos, Spilianakis, & Tsatsanis, 2015). Mir-155 targets c/EBPb which regulates Arg1 expression and M2 polarization. Mir-155, initially identified as an oncogene (Eis et al., 2005), is strongly induced downstream of TLR signaling in response to LPS, promoting TNFα expression (O'Connell, Taganov, Boldin, Cheng, & Baltimore, 2007; Tili et al., 2007). Mir-155 is regulated by Akt1 after LPS stimulation in macrophages (Androulidaki et al., 2010). miRNA-146a is a negative regulator of the innate immune response. It is expressed in response to LPS, TNF, IL-1β or other TLR ligands and suppresses both NF-κB and IRF3 that are responsible for M1 polarization. Mir-155 targets c/EBPb which regulates Arg1 expression and M2 polarization. (Niu & Schulert, 2019). Mir-146a has been reported to promote M2 polarization by inducing nuclear receptor protein PPAR-γ (Huang et al., 2016). Molecular roles of mir-155 and mir-146a are summarized in Figure 4 (Niu & Schulert, 2019).

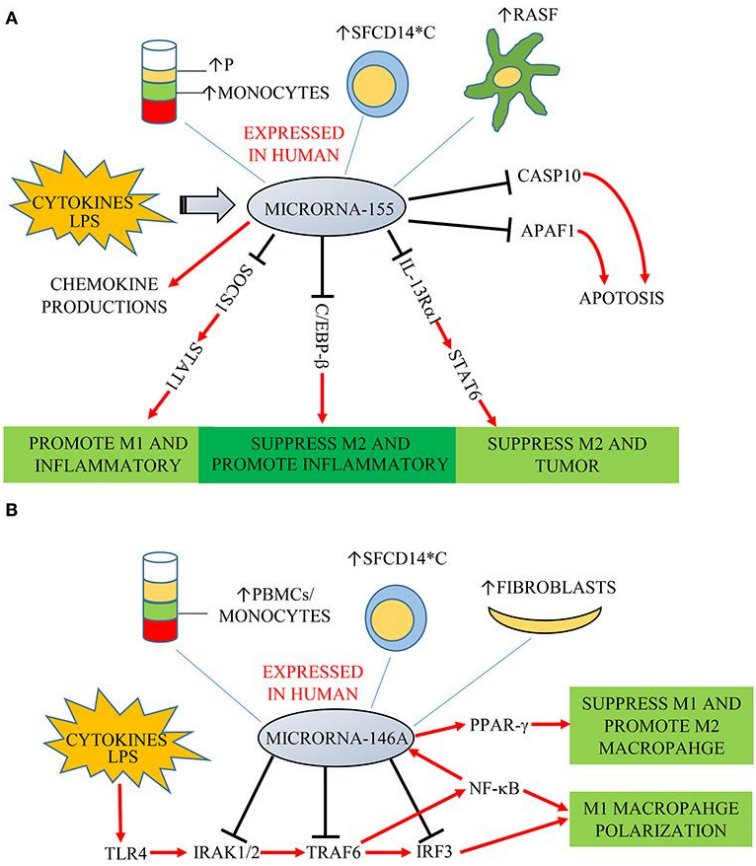

Figure 4: (A) MiR-155 is induced by cytokines and LPS, and overexpression increases chemokine production. SOCS1, IL-13Rα1, and C/EBP-β are key target genes of miR-155. SOCS1 is a negative regulator of STAT1. MiR-155 decreased SOCS1 expression, increasing signaling through STAT1 to promote M1 macrophages and suppress M2 macrophages to promote inflammatory responses. MiR-155 could also directly target C/EBP-β to suppress M2 macrophages. MiR-155 directly targets IL-13Rα1 and decreases the levels of IL-13Rα protein, resulting in decreased activation of the M2-promiting STAT6. MiR-155 is also associated with decreased expression of two predicated miR targets that mediate apoptosis: CASP10 and APAF1. (B) MiR-146a is induced by cytokines and LPS through the NF-κB pathway. It controls TLR4 signaling through a regulatory loop: the upregulation of miR-146a by caused by activated NF-κB; miR-146a reduces the expression of its targets including TRAF6, IRAK1, IRAK2, and IRF3; which limits activity of both NF-κB and IRF3 pathways.

Epigenetic regulation of macrophage polarization

Epigenetic modifications, including histone methylation and acetylation, are important for the regulation of gene expression, regulating gene promoters' accessibility. During recent years, epigenetics has quickly emerged as critical aspect of immunological pathways. Known epigenetic factors have been identified to be involved in macrophage polarization. Epigenetic changes allow macrophages to switch from one fate to the other, giving them the appropriate plasticity to function according to external stimulations. Although little is still known, macrophage activation is proposed to be epigenetically regulated through histone modifications, making gene promoters accessible or not to transcription machinery.

Histone marks have a major role in cell differentiation and identity, possibly contributing in macrophage plasticity. Histone modifications have been shown to control expression of genes responsible for M1-M2 polarization and innate immunity. H3K4 trimethylation is known for active transcription while H3K9, H3K27, H4K20 trimethylations are characterized as silencing (Barski et al., 2007). Enhancer of zeste 2 (Ezh2) is one of the main players of the Polycomb repressive complex-2, involved in H3K27me3 (Schuettengruber, Chourrout, Vervoort, Leblanc, & Cavalli, 2007); while Jmjd3 and Utx act as H3K27 demethlases. Jmjd3 expression is induced in macrophages upon bacterial products, such as LPS, or inflammatory cytokine stimulation (Santa, Totaro, Prosperini, Notarbartolo, & Testa, 2007). In particular, Jmjd3 is recruited to transcription start sites of highly transcribed genes, such as Irf4, that carry H3K4me3 (Satoh et al., 2010), after TLR stimuli. Irf4 is crucial for M2 phenotype downstream of Jmjd3.

Neonatal innate immunity

Early stages of life, usually defined as the period from the fetus until the first few years of life in humans, and are associated with increased susceptibility to infection, inefficient protection and insufficient or excessive activation of the immune system. Human neonates are considered up to the age of four weeks after birth. During this period, immune regulation ensures vital functions, organ development, microbial colonization tolerance and avoidance of tissue damage. Neonates depend a lot in their innate immune system since they have not yet developed adaptive immunity and lack immunological memory. Excessive inflammation and sepsis are often observed in neonates, making them less competent to recover from microbial infections. Infection is a leading cause of mortality, especially in preterm infants. However, the cause of their susceptibility remains unclear. Studies have shown that newborns are able to appropriately upregulate TLR expression, excluding this to be a main cause of susceptibility (J. Zhang, Yang, Levy, & Chen, 2010). Yet, downstream effects of TLR signaling, such as oxygen radicals, are strikingly altered. Newborns appear to have much lower Toll-like receptor (TLR) induced response and reduced pro-inflammatory cytokine production (Kollmann, Levy, Montgomery, & Goriely, 2012). Neonatal macrophages also respond differently when it comes to cytokine production upon LPS (TLR4) stimulation. It's been shown that neonatal peritoneal macrophages produce higher IL-6 and IL-10 (Kollmann et al., 2009) while less IL-1a compared to adults (Winterberg et al., 2015). Other studies in innate immune cells identified IL-23 and IL-1β to be higher. IL-27, a heterodimeric cytokine of the IL-12 family, is also elevated in neonatal macrophages (Kraft, Horzem-, Davis, & Robinson, 2013), while IFN-α, IL-12, IFN-γ and TNFα are less produced in response to toll-like receptor ligands (Kollmann et al., 2009).

Transcriptional regulation upon TLR signaling is also different in neonates. A well-studied family of such transcription factors is the interferon response factor (IRF) including IRF3, IRF4, IRF5, IRF7. Nuclear translocation of IRF7 is impaired in neonates. IRF5 nuclear translocation along with the activation of major transcription factors such as NF-κB, is essential for expression of IL-12. IRF3 requires the coactivator CREB-binding protein (CBP) in order to associate with DNA. IRF3-CBP-DNA complex formation appears to be decreased in neonates (Aksoy et al., 2007). This example reveals that chromatin remodeling might be responsible for differential regulation of molecular components of innate immunity. Emerging evidence suggest a potential role of epigenetic mechanisms in disease and immune system maturation during early

life. Understanding the remodeling of immune networks from neonates to adulthood might allow us to develop better immune intervention and vaccine strategies.

Neonatal mice

Trained immunity

Immunological memory was assumed for a long time to be a unique feature of adaptive immunity. However, literature of the last decade suggests that cells of the innate immunity show characteristics of memory such as better protection against reinfection. Trained immunity is the biological process in which the innate immune system exhibits adaptive characteristics (Netea et al., 2020). Endogenous or exogenous stimuli continuously challenge and reprogram innate immune cells causing an altered response when they undergo a second stimulation after returning to a non-activated state. The secondary response might be stronger or weaker than the primary response. For instance, β-glucan, LPS or BCG (Bacillus *Calmette–Guérin*) vaccine can induce trained immunity programs. Trained immunity in innate immune cells is mainly mediated by epigenetic alterations of transcription pathways, allowing them to react with stronger, faster and even qualitatively different expression patterns when challenged with a secondary stimulus non-specific to the original one. Evidence suggest that chromatin organization alterations could regulate this altered responsiveness. Development of trained immunity could be particular beneficial for host survival in early life and might affect the risks of infection.

Aim of the thesis

Infant mortality due to impaired immune defense in still common globally. The nature of this work is to elucidate transcriptional or epigenetic mechanisms that regulate weaker immune response in neonates. In particular, we tried to identify the differences in the expression levels of major innate immunity transcription or epigenetic regulators, between adult and neonates. It has been proposed that distinct epigenetic landscapes might underlie the unique responses of neonatal immune system. Previous work of the lab through siRNA screen, identified some transcription factors and histone modification proteins that are significant for macrophage polarization (Figures 5, 6). Thus, we tried to elucidate if they play a role in this differential immune response in neonates or are involved in the metabolic pathway of Akt kinase. We also asked whether neonatal incompetence against infections is due to lack of training. Hence, we wanted to investigate whether training of neonatal macrophages could lead to a better response when exposed to a secondary infection.

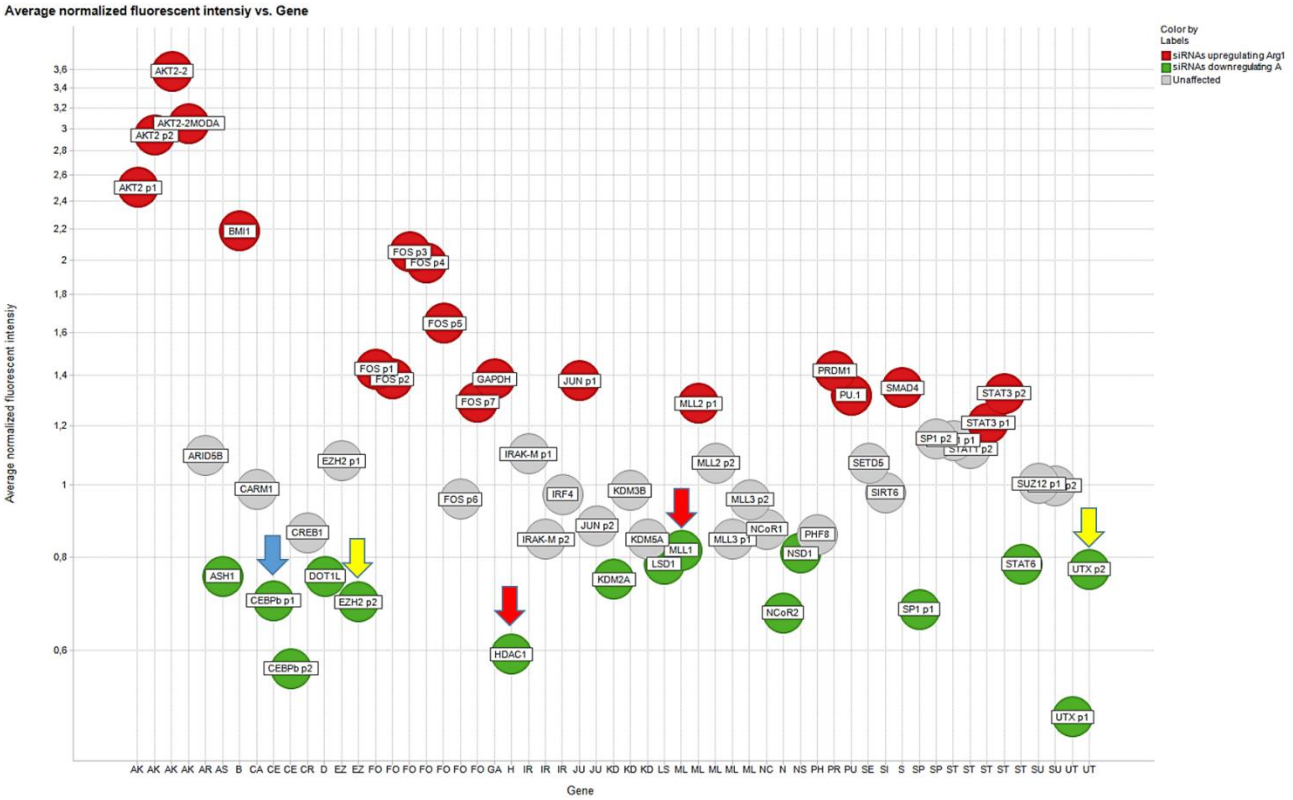

Figure 5: Scatter plot showing Arginase 1 immunoflurescent signal in macrophages treated with 100ng/ml LPS for 48 hours following transfection with indicated siRNAs. Red siRNAs upregulating Arg1, green downregulating Arg1 while gray siRNAs do not affect them.

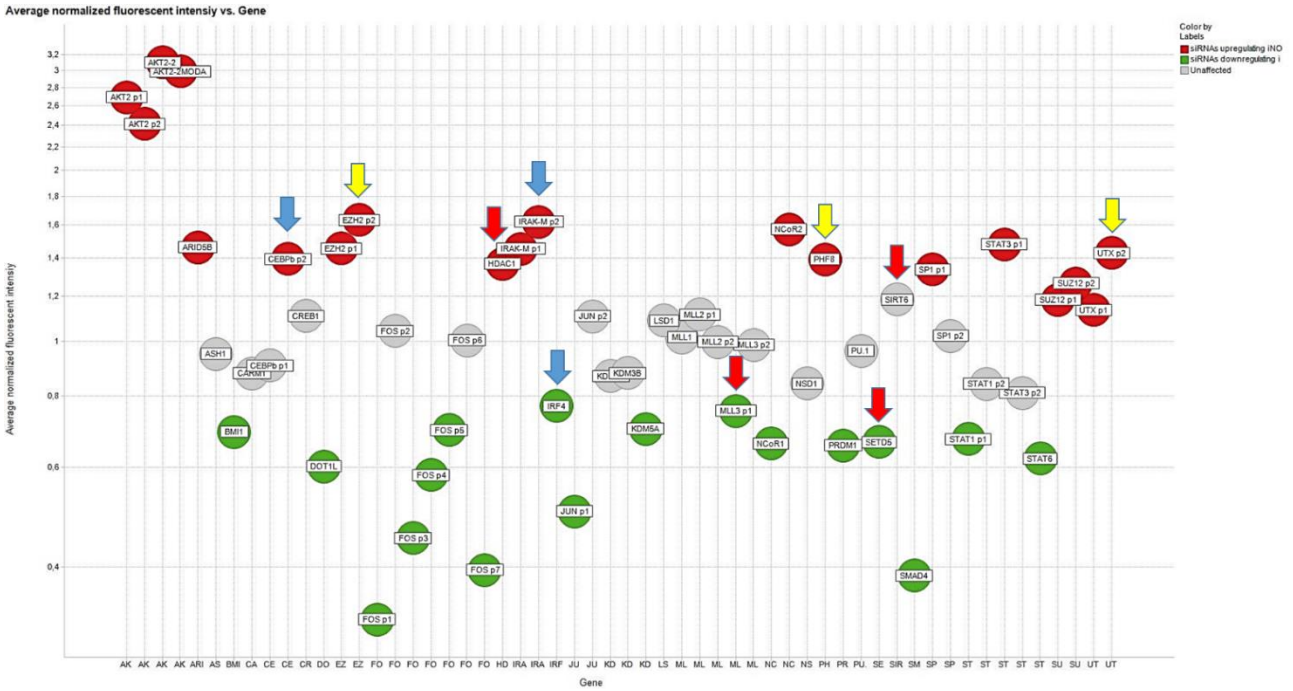

Figure 6: Scatter plot showing iNOS immunoflurescent signal in macrophages treated with 100ng/ml LPS for 48 hours following transfection with indicated siRNAs. Red siRNAs are upregulating iNOS, green downregulating iNOS while grey siRNAs do not affect them.

Materials and methods

Buffers

- Cell culture medium: DMEM Low Glucose with 1% antibiotic penicillin-streptomycin and 10% FBS final (filtered and heat inactivated)
- RIPA solution (50ml): 500ul Tris-HCl pH 8, 1ml 0.5M EDTA, 1.4ml 5M NaCl, 2.5ml 20% TitonX, 5ml 10% Na-deoxycolate (wear a mask), 0.5ml 10% SDS
- 10X Tris-Glycine 1L: 30.275g Tris base, 144g Glycine
- Running Buffer 1L: 100ml 10X Tris-Glycine, 10ml 10% SDS
- Transfer buffer 1L: 100ml 10X Tris-Glycine, 10ml 10% SDS, 200ml Methanol
- PBS 1X
- PBS-T: Add 500ul Tween20 in 500ml 1X PBS
- Mild Stripping (0.5L) pH 2.2: 7.5g Glycine, 0.5g (or 10ml of 10% solution) SDS
- Harsh Stripping (0.5L): 10ml 10% SDS, 6.25ml Tris-HCl pH 6.8 0.5, 33.75ml dH_2O, 0.4ml β-mercaptoethanol
- PonceauS Staining Solution: 0.1%(w/v) Ponceau S in 5%(v/v) acetic acid

Primary macrophages isolation

Thioglycolate broth is an enriched growth medium for bacteria, which is used to recruit macrophages to the peritoneal cavity of mice, after injected intraperitoneally, without activating them (Leijh, Zwet, Kuile, & Furth, 1984).

We injected 4 days old neonates and adults with thioglycolate intraperitoneally. Four days later, we sacrificed the mice. To begin with, we aliquoted low glycose medium with 1% ampicillin-streptomycin in 50mil falcons (in cell culture laminar hood, under sterile conditions). Then we sedated animals with sevofluraneand made sure they had no reflexes. We sprayed the abdomen with ethanol and wiped well. For neonates, we made two holes opposite from where we will inject DMEM intraperitoneally, diving the needle in and up, using a 21g needle. We placed a petri dish below so that we would collect macrophages there. We injected 8-9ml medium using a 27g 5ml needle holding neonate above petri dish. We then performed peritoneal lavage so that the cells will come out onto the petri dish and transferred in a 50ml falcon using a pipette. Cells were kept in ice until we collected all neonatal macrophages. We collected cells by centrifugation at 1000rpm for 5 minutes. It is important to clean the working area with ethanol often.

Since we wanted to isolate bone marrow cells, we proceed by cutting neonates dorsally epidemically and pulled softly in order not to break any bones. We separated the leg from the skin and cut with scissors by the spinal cord vertically to the femur. Muscle and fat were discarded from the bone which was placed in PBS.

Concerning adults, we used 16ml medium per mouse (2x8ml) using a 10ml syringe. Mice were sacrificed by cervical dislocation. Equipment was cleaned with ethanol and again we cut abdomen epidemically, tearing epidermis apart. We then injected 8ml medium into peritoneal area. Syringe was then taken out, but not the needle, while a 50ml falcon was placed beneath the needle. We palpated abdomen in order for the medium with peritoneal macrophages to come out, and repeated once more with 8ml fresh medium. Again, cells were centrifuged 1000rpm for 5 minutes.

For the isolation of bone marrow cells, epidermis was torn dorsally, so that bones were revealed. We cut in parallel with the spinal cord and discarded fat and muscles in order to isolate the femur bone which was placed in PBS.

Primary macrophages cell culture

Peritoneal cells counting and calculation

We worked in cell culture room under sterile conditions. After centrifugation the 50ml falcons containing primary macrophages at 1000rpm for 5 min, supernatant was discarded and 10ml of fresh medium was added. Cells were resuspended and 10ul were counted supplemented with 5ul Trypan blue (stains dead cells) and 5ul 8% acetic acid (kills erythrocytes), in a Hemocytometer. For RNA-collection experiments we used 500.000 cells per well in a 24-well-plate. For protein collection experiments, we plate either 1 million cells per well in a 12-well-plate or 3 million cells per well in a 6-well-plate depending on the number of cells we had, and how many conditions we wanted to examine. The second option is preferred.

Bone marrow derived macrophages

We isolated femur bone from neonates and adults C57BL/6 mice and placed them in 1X PBS. After this step we worked in cell culture room under sterile conditions. We prepare a 12-well-plate with wells with EtOH and wells with PBS and sterilized the femur by diving it in EtOH for 1 minute and then wash in PBS for another minute. Then we cut top and bottom epiphysis and injected the medullary cavity with medium using an insulin syringe; and repeated until bone became whitened. Next we centrifuge 1000rpm for 5min and prepared 100ml of medium with 30% LCCM differentiation medium (supernatant of L929 cell culture). After centrifugation, supernatant was discarded and 15ml of the aforementioned medium was added. Cells were resuspended and cultured in a 15ml flask. Five days later we added another 15ml medium with 30% LCCM and left it for 10 days, when we collected cells in a falcon by scratching the bottom of the flask.

Stimulate peritoneal macrophages

Macrophages can be activated toward different phenotypes depending on the stimulus. IL-4 and IL-10 induce M2 phenotype, while LPS induces M1. We used the following stimuli in specific final concentrations: IL-4: 10ng/ml, IL-10: 100ng/ml, LPS: 100ng/ml, IFNγ: 50ng/ml

Diluted aliquots were kept in the fridge for use in the following one or two days. We tested the conditions: IL-4 for 48 hours, IL-10 for 48 hours, IL-4 for 24 hours, IL-10 for 24 hours, LPS for 24 hours, LPS+IFNγ for 24 hours, LPS for 6 hours, LPS+IFNγ for 6 hours, Basal (no stimulus).

Bone Marrow Derived Macrophages (BMDMs) were only stimulated with 100ng/ml LPS final concentration for 0, 6 or 24 hours.

Collect cells for RNA extraction

Supernatants were collected and stored in -80°C for testing for macrophage metabolites. Well-plate was washed once or twice with 1X PBS and 200ul Trizol (TRI Reagent) were added in each well of the 24-well-plate, which was then placed on a shaker for 5 minutes. Then, we scratched the bottom of the wells, collected lysate in a tube and stored at -80°C. We had also plated one extra well for BCA quantification of supernatants. This well was collected with RIPA buffer.

Collect cells for protein extraction

Initially, 800µl RIPA buffer was prepared (800µl RIPA, 32µl Protein Inhibitor 25X, 0.8µl phosphatase inhibitor 1000X). Supernatant was discarded and plate was washed twice with 1X PBS. 100µl RIPA buffer was added per well for 12-well-plates or 150ul for 6-well-plates that were then left on the shaker for 5 minutes. Again we scratch the bottom of the wells, collected lysate in a tube and stored at -80°C.

RNA extraction

Samples were defrosted on ice and vortexed. 200µl Chloroform /ml Trizol (e.g. 40ul for 200ul Trizol) were added per sample which was then vortexed or mixed by flickering and incubated at room temperature for 5 minutes. We centrifuged the samples at 11000rpm for 15 minutes at 4°C, transfer the upper phase into fresh tubes and added 500ul Isopropanol per ml Trizol (e.g. 100ul). Samples were incubated at room temperature

for 10 minutes or overnight at -20°C. Subsequently, we centrifuged 14000rpm for 10 minutes at 4°C and kept the pellet. 400ul of EtOH were added and samples were mixed my flickering. We centrifuged at 7000 rpm at 4°C and washed once more with 75% EtOH, which was then discarded and pellet was left to dry for approximately 20 minutes. Samples were resuspended in 15-20ul RNase free water and incubated for 5 minutes at 56°C before concentration measurement in nanodrop.

cDNA synthesis

For the cDNA synthesis we used PrimeScript™ RT reagent Kit (Perfect Real Time). Concentrations per reactions are shown in Table. Master mix was made prior to use.

Reagent Amount	Final conc.
5X PrimeScript Buffer (for Real Time)	2 µl
Oligo dT Primer (50 µM)	0.5 µl
Random 6 mers (100 µM)	0.5 µl
PrimeScript RT Enzyme Mix I	0.5 µl
total RNA	
RNase Free dH2O	
Total	**10 µl**

In order to calculate how much RNA and H_2O is needed per reaction, we estimate the number of ul required for 500ng or 800ng RNA per sample, depending on the concentrations. Then we calculate how much water is needed to make the reaction up to 10ul. First, we add the appropriate RNA and water in PCR tubes for each sample, and then 3.5ul from the master mix was added. Spin down shortly and put the tubes in a PCR machine with the following program:

- 37°C for 15 min (Reverse transcription)
- 85°C for 5 sec (Inactivation of reverse transcriptase with heat treatment)
- Hold at 4°C

Real Time PCR (qPCR)

Real Time PCR was performed using StepOnePlus™ Instrument (96 Wells). We used Quantitation – Comparative Cτ (ΔΔCτ) experimental properties with a SYBR Green Reagents detector, including a melting curve. Real Time PCR program included initialization of holding stage at 95°C for 3 minutes. During cycling stage, step 1 was at 95°C for 10 seconds while step 2 at 60°C for 30 seconds. After the latter step, there was data collection from the detector. Cycling stage was repeated for 40 cycles. Melt curve stage included 15 seconds at 95°C, 1 minute at 60°C and a slow increase at 95°C for 15 seconds with a 1% rate. Melt curve stage was continuous and contained data collection. Reaction volume was set to 10µL.

Real Time PCR Reaction (10µl)	
Kapa mix (KAPA SYBR® FAST qPCR Master Mix 2X)	5 µL
Primer Forward (10mM)	0.2 µL
Primer Reverse (10mM)	0.2 µL
RNase Free Water	2.6 µL
cDNA	2 µL

Protein sample preparation

Since we also tested proteins that bind chromatin, we sonicated samples for 20sec at 50% once. Then we performed a BCA assay to estimate protein concentrations in our samples. To begin with, we took a 96-well flat bottom plate and added 10ul of every BSA concentration (1000ug/ml, 500uh/ml, 250ug/ml, 125ug/ml, 62.5ug/ml) and 15ul WFI per well in duplicates; and 5ul of every sample and 20ul WFI per so that all wells

were up to 25ul. We also added 25ul WFI for blank. We also prepared BCA working reagent 50:1 and added 175µl per well. Plate was incubated for 30 minutes at 37°C and absorbance was measured at 562nm. Analysis was performed in Microsoft Excel by deducting average blank measurement from all samples. Standard curve was determined in GraphPad Prism 7 using XY analysis and concentrations were calculated taking into consideration that we added 10ul of BSA while only 5ul of sample in the BCA assay. Thus, measurements were doubled to calculate ug/ml. ug/ml was converted to ug/ul by dividing with 1000.

Western Blot

Minimum of 20ug protein was filled up to 25ul for small wells or 50ul for 10-wells gel with Water for Injections. Loading dye 6X was added to each sample. Samples were then, boiled in the heatblock at 100°C for 5 minutes and after spinned down stored at -20°C or loaded onto the gel. The day of the run, gel cassette was prepared and checked for any leakage. The molecular weights of the proteins of interest led us to pick 8% as an optimal concentration for the running gel.

8% polyacrylamide running gel (10ml)	5% stacking gel (5ml)
2.6ml 30% polyacrylamide	850ul 30% polyacrylamide
2.5ml 1.5M Tris pH 8.8	625ul 1.5M Tris pH 6.8
4.7ml WFI	3.4ml WFI
100ul 10% SDS	50ul 10%SDS
100ul 10% APS	50ul 10% APS
10ul Temed (at the end)	5ul Temed

Gel solution of running gel was mixed by inverting the falcon and poured. 1ml Isopropanol was added to straighten the top of the gel and was let to dry. Gel solution of stacking gel was prepared after running gel was dry and isopropanol was discarded. Combs were placed and gel was either stored in a wet tissue paper in an aluminum foil at 4°C or used immediately.

Run western blot gel

Gel Cassette Sandwich was placed into the slots at the bottom of each side of the Electrode Assembly. Inner Chamber Assembly was lowered into the Mini Tank and inner chamber was filled with running buffer all the way to the top. Outer chamber was filled with enough buffer to submerge the bottom of the gel and comb was carefully removed. We loaded 5ul of pre-stained protein marker and samples onto the gel slowly to allow them to settle evenly on the bottom of the well. Electrical leads were inserted into a suitable power supply with the proper polarity. We ran the gel at 60 Volt until the samples have crossed the stacking gel and then turned to 100 Volt. After electrophoresis was completed, we turned off the power supply and carefully removed the tank lid; lifted out the Inner Chamber Assembly and discarded or stored the running buffer for more uses (max 3). We used the green BioRad Gel Releaser spatula to separate the two glass plates of the Gel Cassette Sandwich and discarded the stacking gel and cut a small piece from the top right corner of the separating gel to mark the orientation.

Transfer

Using the foam fiber pad as guide, we cut the PVDF membrane and six whatman filter papers to approximately match the dimensions of the gel. We soaked the membrane in methanol for 2 minutes and then incubated membrane, filter papers and fiber pads in cold transfer buffer for 5 min. Cassette was placed inside a bowl containing cold transfer buffer with the black side down and fiber pad - filter papers - gel sandwich was placed on the black side with the gel facing up. We soaked membrane in methanol for a few minutes to activate it and briefly in transfer buffer and placed it on top of the gel. A tube or a roller was used to gently roll out air bubbles. We then placed three filter papers on top of the membrane and removed air bubbles. After making sure that gel was towards the black side, we closed and locked the cassette with the white latch and placed it in module taking care to match the colors: the black side of the cassette must face the black side of the module. An ice pack was placed inside the tank which was filled with transfer buffer,

along with a small stir bar to help maintain even buffer temperature and ion distribution in the tank. We put the lid on being careful to match the colors of the electrodes (black should be negative) and ran the blot at 300-400mA for 1 hour or at 40mA overnight in cold room. Upon completion of the run we turned off the power source, disassembled the sandwich and removed the membrane. The presence of the protein marker on the blot is the first sign of a successful transfer.

Ponceau Staining

To address the effectiveness and the quality of the transfer, we stained the membrane with Ponceau S before proceeding to antibody staining. We placed the membrane into a small plastic container, covered it with Ponceau S staining solution and incubated for 5 min (or up to 1 hour) at room temperature with gentle agitation. Staining solution was recollected for future use (it can be reused up to 10 times) and membrane was rinsed with dH_2O until the background is clear. Protein bands should appear as red lines. To remove the staining, we either rinsed with dH_2O. When everything was satisfactory we proceeded with blocking.

Blocking

Blocking buffer with 5% BSA (w/v) in PBS-T or 5% milk powder (w/v) in PBS-T was prepared. When we wanted to detect very low concentrations of protein, given that the primary antibody has not background, we used 1-3% blocking. Blocking was performed by incubating the membrane at room temperature for 1 hour or overnight at 4^{o}C with gentle agitation, and then washed with PBS-T once for 15 min and twice for 5 min at room temperature with gentle agitation. Membrane was cut if necessary to use multiple antibodies simultaneously.

Antibody probing

PBS-T from washes was discarded and membrane was covered with the desired primary antibody solution properly diluted in milk or BSA (according to antibody data sheet instructions). This step was performed with the membrane in a small plastic container or in a small plastic bag (in order to use less volume of antibody). Membrane was incubated overnight at 4°C (cold room) with gentle agitation or for 40min to 1 hour if it is a highly expressed protein (e.g. actin). The following day, we recollected the primary antibody solution in order to be reused and transferred the membrane in a small plastic container, which was washed with PBS-T once for 30 min, once for 10 min and twice for 5 min at room temperature with gentle agitation. PBS-T from washes was discarded and membrane was covered with secondary antibody solution properly diluted (according to antibody data sheet instructions) and incubated for 1 hour at room temperature with gentle agitation. Secondary antibody solution was discarded and membrane was washed with PBST-T once for 30 min, once for 10 min and once for 5 min at room temperature with gentle agitation. We also perform one last wash with PBS for 5 min at room temperature with gentle agitation. When mouse anti-actin antibody had a lot of background, we tried dilution 1:4000 in 3% milk in PBS-T and probed for 1 hour of less. When an antibody was very weak but gave no background, we tried dilutions of 1:500 or 1:250 in 0.5% or 1% BSA in PBS-T.

Visualization (Biorad)

For membrane visualization we used BioRad Gel Doc XR+ System. We placed the membrane in the machine and poured 1ml ECL (photosensitive) per membrane after making sure that camera is on for at least 20 minutes. We opened ImageLab program and selected gel Chemi-Hi resolution and XL; set 1 to 300sec and to take 100 images and ran protocol. When saturation started to appear, we saved images and stopped the run. We then set to colorimetric and took a picture in which the pre-stained marker is visible.

Membrane Stripping

Membrane stripping can be performed many times in order to probe with many antibodies, but the potential signal may be weaker and the background higher after each round of stripping. Depending on whether we wanted a mild or a harsh stripping we used the following protocols with the corresponding buffers. It is

proposed to first try mild stripping and if there is still a signal from the antibody, proceed to harsh. After stripping, membrane is ready for blocking or storage.

Homemade Mild Stripping

We placed the membrane in a small plastic container, added enough stripping buffer to cover it completely and incubated at room temperature for 5-10 min with gentle agitation. Then, buffer was discarded and incubation was repeated for 5-10 min with fresh stripping buffer. Buffer was discarded and membrane was washed with PBS twice for 10 min and twice for 5 minutes with PBS-T at room temperature with gentle agitation.

Homemade Harsh Stripping

Buffer was warmed to 50°C prior to use. Membrane was covered with buffer in a small contained which has a lid and incubated 50°C for up to 45 min with mild agitation. Then, buffer was discarded and membrane was rinsed under running water for 1 to 2 minutes and washed PBS-T for 5 min at room temperature with mild agitation. Traces of β-mercaptoethanol can damage the antibodies, thus proper washed are required.

Thermofisher stripping

According to the manufacturer's instructions we incubated membrane for 5min at 37°C with mild agitation, washed with PBS and PBS-T.

Eliza mouse TNF-α (BioLegent)

Since we tested cell culture supernatants, we had to dilute some of the samples in order to be within the standard curve. We diluted 1:25 sample:culture medium all samples that were treated with LPS.

Before start following buffers were prepared:

- Wash buffer 500ml (250ul Tween-20, 500ml 1X PBS)
- Assay diluent 5X 20ml (4ml Assay Diluent 5X, 16ml PBS)
- Stop solution H_2SO_4 2N 1L (945.65ml dH_2O, 54.35ml from 18M stock solution)

We calculated how many wells we would need. We had all samples in duplicates, two wells for cell culture medium blank, two wells for Assay Diluent blank and 8 wells for the standard curve or 16 if we want the standard curve in duplicate. On the first day, we diluted Capture Antibody 200X in Coating Buffer (make 1X dilution from 5X, in Water For Injections) and added 100µL of this Capture Antibody solution to all wells that will be used of a 96-well flat bottom ELISA plate. The plate was sealed and incubated overnight in the fridge. On the second day, the plate was washed 4 times with 300 µL Wash Buffer per well and blot residual buffer by firmly tapping plate upside down on absorbent paper. All subsequent washes were performed similarly using a 200ul multi-pipet and pipet twice with 150ul each time. To block non-specific binding and reduce background, we added 200 µL Assay Diluent per well, sealed the plate and incubated at RT for 1 hour with shaking on a plate shaker. In the meantime, we prepared standard dilutions and appropriate sample dilutions. Plate was washed 4 times and 100 µL/well of standard dilutions and samples were added to the appropriate wells. To create the standard curve, we added 100ul Assay Diluent in each well, apart from the first one. We diluted TNFα in Assay Diluent so that the more concentrated well will have 2ng/ml TNFα. We made 300ul of 2ng/mL and added 200µL in the first well. Mixed by pipetting up and down and took 100ul and put them in the second well. We did the same procedure for the rest and discarded the final 100ul of the last well after mixing. Plate was sealed and incubated at RT for 2 hours or overnight in cold room with shaking. On the third day (or later), plate was washed 4 times with Wash Buffer and Detection Antibody was prepared by diluting Detection Antibody 200X in Assay Diluent. 100 µL of diluted Detection Antibody solution were added to each well, plate was sealed and incubated at RT for 1 hour with shaking. Plate was washed 4 times with Wash Buffer and Avidin-HRP was prepared by diluting Acidin-HRP 1000X in Assay Diluent. 100 µL of diluted Avidin-HRP solution were added to each well, plate was sealed and incubated at RT for 30 minutes

with shaking. Finally, plate was washed 5 times with Wash Buffer for 30 seconds to 1 minute for each wash and TMB Substrate Solution was prepared by mixing Substrate A and Substrate B 1:1. 100 µL of TMB Substrate Solution were added and plate was incubated in the dark for 15 minutes. Positive wells turned blue in color. Reaction was stopped by adding 100 µL of Stop Solution to each well. Positive wells turned from blue to yellow. We red absorbance at 450 nm within 15 minutes. For the analysis, we used Excel and GraphPad Prism; considering measurements as Dose-response-stimulation, log(agonist) vs response-variable slope (four parameters) and interpolated unknowns from standard curve.

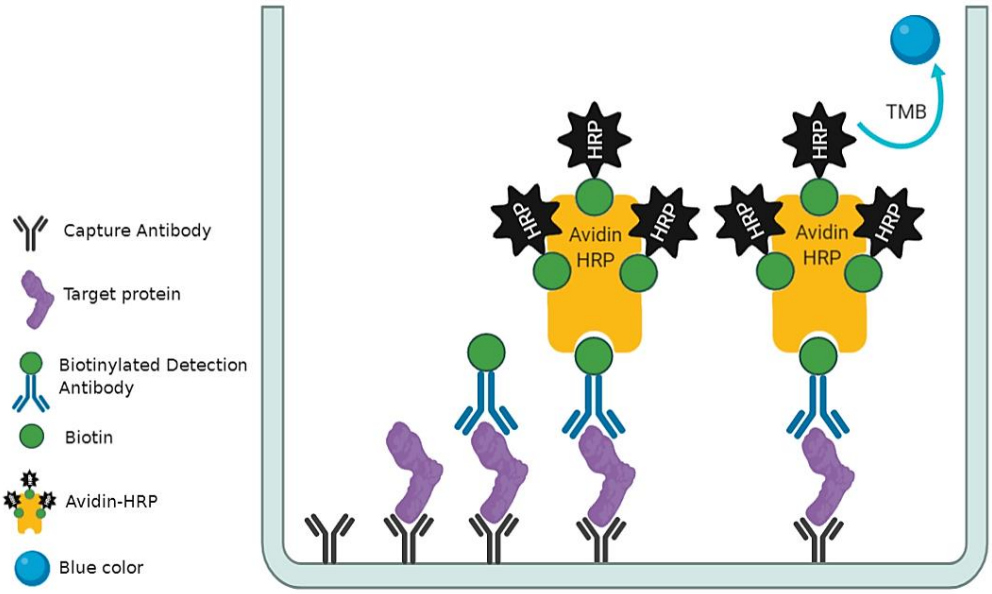

Figure 7: Capture antibody adheres at the bottom of the plate, then TNFa is bound; biotinylated detection antibody binds the target protein; Avidin HRP binds the biotin of the biotinylated detection antibody. TMB causes a reaction which leads to the production of blue color, which is then detected by the plate reader.

Nitride Measurement (Griess Reaction)

This assay was performed to measure Nitrite Oxide in the supernatant of primary macrophages cell culture. Cells were stimulated or not with LPS for different amount of time, while they originated either from adult or neonatal mice.

- NED Solution (0.1% N-1-napthylethylenediamine dihydrochloride in water)
- Nitride Standard (0.1M sodium nitride in water)
- Sulfanilamide Solution (1% sulfanilamide in 5% phosphoric acid)

We used a flat bottom 96-well plate and add 50ul of each experimental sample in duplicates, and dilution series for the Nitride Standard reference curve. Using a multichannel pipettor, we dispensed 50ul of the Sulfanilamide Solution to all wells. Incubated for 5 to 10 minutes at room temperature, protected from light. Then, we used again a multichannel pipettor to dispense 50ul of the NED Solution to all wells. Incubated at room temperature for 5 to 10 minute, protected from light. A purple color begun to form immediately. Absorbance was measured within 30 minutes in a plate reader at 540nm.

Animals

C57BL/6 mice where used as wild type controls. Macrophage-specific Akt1$^{-/-}$ mice and global Akt2$^{-/-}$ knockout mice were used and are referred here as Akt1 and Akt2 mice respectively. In particular, Akt1 conditional knock-out genotype is Lysozyme-CRE LoxP-Akt1-LoxP. All animals where housed at the University of Crete, School of Medicine.

Primers

Primers were designed using NCBI Primer-BLAST: https://www.ncbi.nlm.nih.gov/tools/primer-blast/

MLL1		Sequence (5'->3')	Length	Tm	GC%
	Forward primer	GTCCACCTAAGGAAGTCAGCAT	22	59.76	50.00
	Reverse primer	GACCTGTAGACACCAACCGC	20	60.67	60.00

MLL3		Sequence (5'->3')	Length	Tm	GC%
	Forward primer	CAGGAGGGCCTGCAAGATAC	20	60.18	60.00
	Reverse primer	TATCCTCCGGTTGGAGCTGA	20	60.03	55.00

SIRT6		Sequence (5'->3')	Length	Tm	GC%
	Forward primer	TGCAACCCACAAAACATGACC	21	59.86	47.62
	Reverse primer	GGAATCTCCAGCCCCAGATG	20	59.89	60.00

HDAC1		Sequence (5'->3')	Length	Tm	GC%
	Forward primer	GACAAACGCATCTCCATCTGC	21	59.94	52.38
	Reverse primer	AGTTCTTGCGACCACCTTCT	20	59.24	50.00

KDM3B		Sequence (5'->3')	Length	Tm	GC%
	Forward primer	TCATTCACCCTTTGGGCTGG	20	60.25	55.00
	Reverse primer	TTTGCTCTTCGCTACTGCTGA	21	60.00	47.62

CARM1/PRMT4		Sequence (5'->3')	Length	Tm	GC%
	Forward primer	CTGTGGCTGGAATGCCTACTG	21	61.02	57.14
	Reverse primer	AATGCCCGTGCTCATTATGGA	21	60.13	47.62
	Product length		149		

SETD7		Sequence (5'->3')	Length	Tm	GC%
	Forward primer	ACACCAAGAGGTTGACAGCAG	21	60.48	52.38
	Reverse primer	GCAGTTCGGAGTGAAGGAGT	20	59.68	55.00
	Product length		157		

We checked whether the primers would give non-specific products or primer dimers with PCR and visualized on an agarose gel.

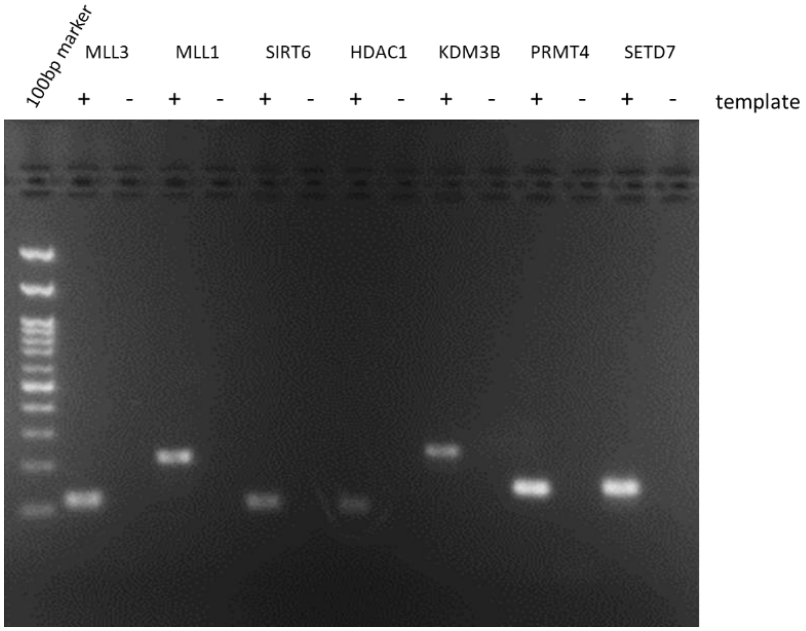

Figure 8: Agarose gel indicating that the newly ordered primers where specific for one product and do not make primer dimers since we got only one band per primer pair only when template was added.

B-glucan training

We cultured primary peritoneal macrophages as described above, in two 24-well plates. In each plate we tried different β-glucan buffers. We prepared 5mg/ml β-glucan in 10% NaOH and filtered it. This buffer was used in a final concentration of 5ug/ml per well in plate 1. We also prepared 5mg/ml in sterilized 1X PBS for cell culture and used in a final concentration of 5ug/ml per well in plate 2. Both plates were incubated for 24 hours at 37°C. The next day, we washed once wit 1X PBS and added fresh medium. Three days after initial beta glucan treatment, cells were incubated with LPS for 6 and 24 hours, and collected as described for primary macrophages. Cell culture supernatant was also collected and stored.

Statistical analysis

All statistical analysis between samples was performed assuming a Gaussian distribution. We used parametric unpaired t-test assuming that both populations examined each time have the same standard deviation. Graphs and statistical analysis were performed using GraphPad Prism 7.

Results

Transcriptional and epigenetic regulation of macrophage polarization in neonates

Macrophage isolation from neonates has many technical limitations since the number of cells is usually insufficient for in vitro experiments. Initially we tested which macrophage model would serve us better in order to study transcriptional and epigenetic regulation of macrophage activation; thus we isolated both thioglycolate peritoneal and bone marrow cells. Previous work of the lab showed that macrophages in neonates produce higher levels of IL-10 compared to adult mice. So, we checked mRNA levels of IL-10 in peritoneal and bone marrow derived macrophages and decided that the first ones would be a better model as both express higher levels of IL-10 than adult cells but bone marrow cells isolation was harder, more time consuming and gave lower number of cells.

After isolation of peritoneal, primary, murine macrophages we cultured and induced them with different stimuli. Then, cells were collected after specific hours of stimulation, and the transcriptional expression of M1 or M2 markers was studied using qPCR.

Transcriptional regulation

We stimulated thioglycolate derived peritoneal and bone marrow derived (BMDM) macrophages with LPS for 6 and 24 hours and IL-10 mRNA levels were validated using Real Time PCR.

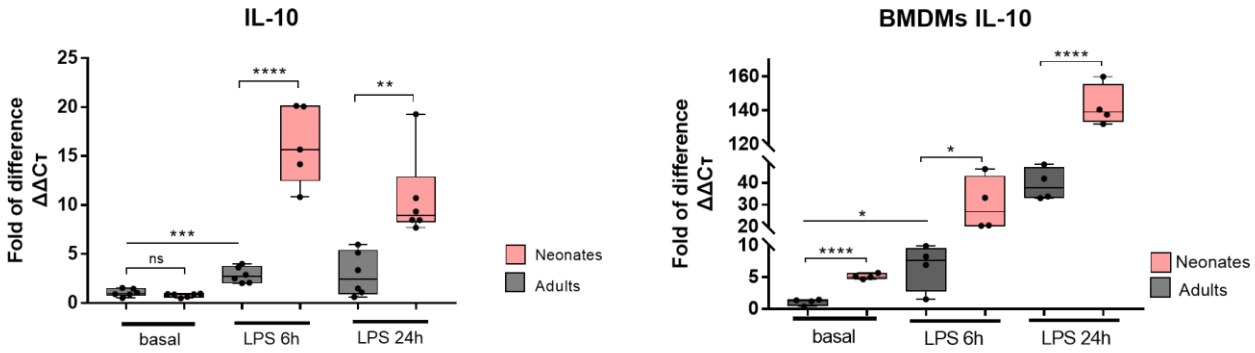

*Figure 9: mRNA levels of IL-10 in peritoneal (left) and bone marrow derived (right) macrophages basally and after 6 or 24 hours of 100ng/ml LPS induction, detected with Real Time PCR. IL-10 is significantly increased in neonates upon stimulation in both peritoneal and BMDMs. Peritoneal derived macrophages IL-10 mRNA detection experiment was performed twice with similar results, so this is an indicative graph. Dots represent the ΔΔCτ values. P-values were calculated with t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant.*

We observed that even though IL-10 levels in peritoneal macrophages were similar between adults and neonates basally, they were increased 15 times in neonates while only 3 times in adults 6 hours after stimulation. A decreased difference was also detected at 24 hours after LPS. Bone marrow derived macrophages showed higher IL-10 in neonates both basally and after stimulation, while levels seem to be more increased at 24 than 6 hours.

Then, we tested the expression pattern of pro-inflammatory markers upon LPS stimulation in adult and neonatal peritoneal macrophages. TNFa seem to be expressed and secreted in higher levels in neonatal macrophages compared to adults, in basal levels. However, adults express more TNFa 6 hours after LPS stimulation. Since IL-10 inhibits the expression of TNFa, it was anticipated that neonates with higher IL-10 after LPS would have less TNFa. Similar pattern was observed with iNOS detection after 6 hours of LPS (Figure 10C). iNOS mRNA was also examined after combinational stimulation with LPS and IFNγ (Figure 10D). The latter did not show any difference in neonates at 6 hours. IL-12 is a proinflammatory cytokine which was detected higher in adults both basally and 6 hours after stimulation.

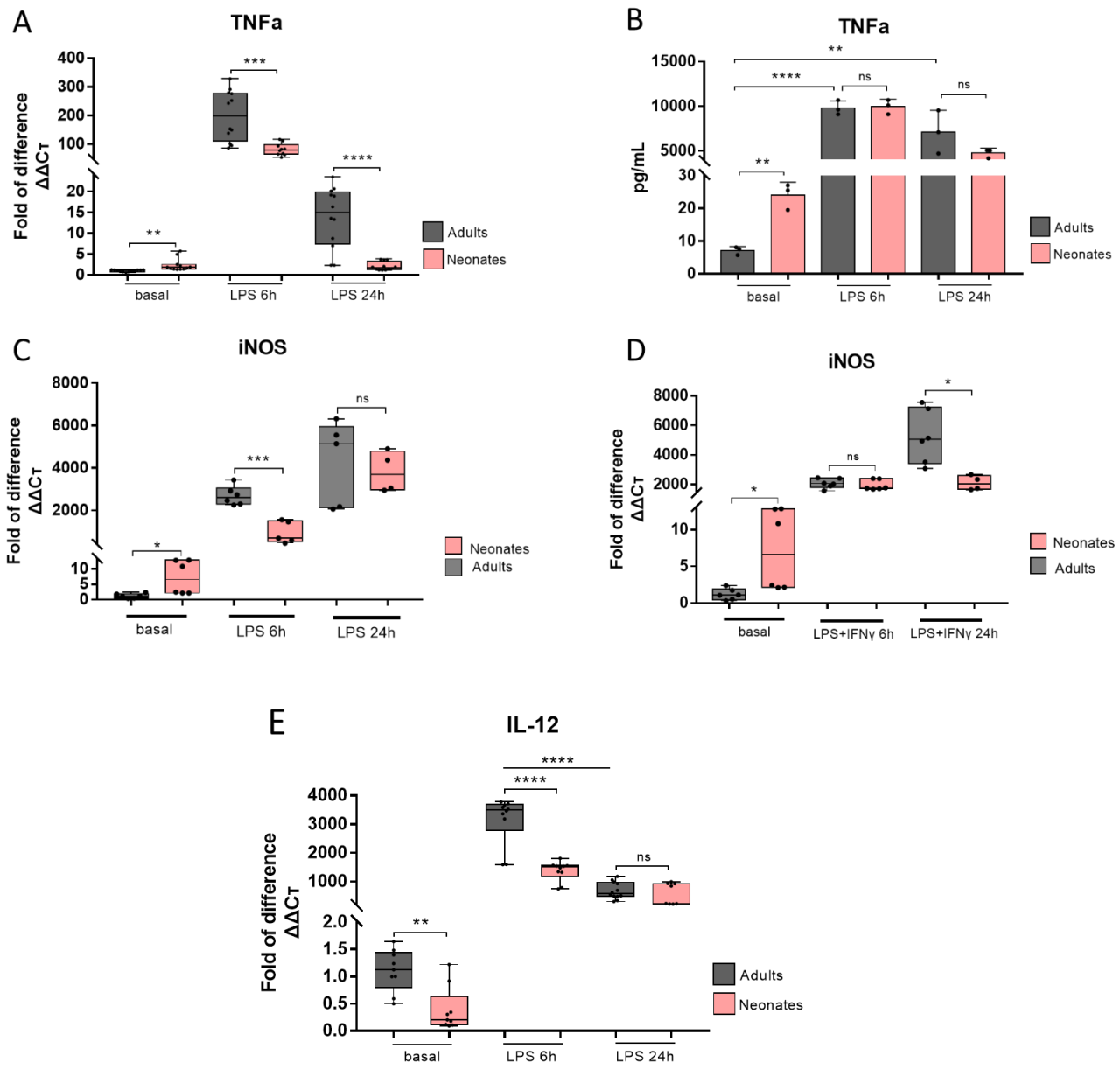

*Figure 10: Expression and secretion pattern of pro-inflammatory factors in peritoneal macrophages. A, mRNA levels of TNFa basally and after LPS detected with Real Time PCR. This experiment was performed twice and data were merged after normalized for adult basal. B, TNFa before and after LPS detected in cell culture supernatant with ELISA assay, normalized with BSA levels. C, mRNA levels of iNOS basally and after LPS induction. This is an indicative graph of the two replications of this experiment. D, mRNA levels of iNOS basally and afte LPS+IFNγ induction. E, mRNA levels of IL-12 before and after LPS induction for 6 and 24 hours. Experiment was performed twice and data were merged after normalized with the average of basal adults of each experiment. Dots represent ΔΔCτ values. P-values were calculated with t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant.*

Next, we examined transcription factors and one their cytoplasmic regulators known to regulate M2 polarization of macrophages such as cEBPb and IRAKm. We also checked the two most established markers for M2-like macrophages, Arginase 1 (Arg1) and Fizz1.

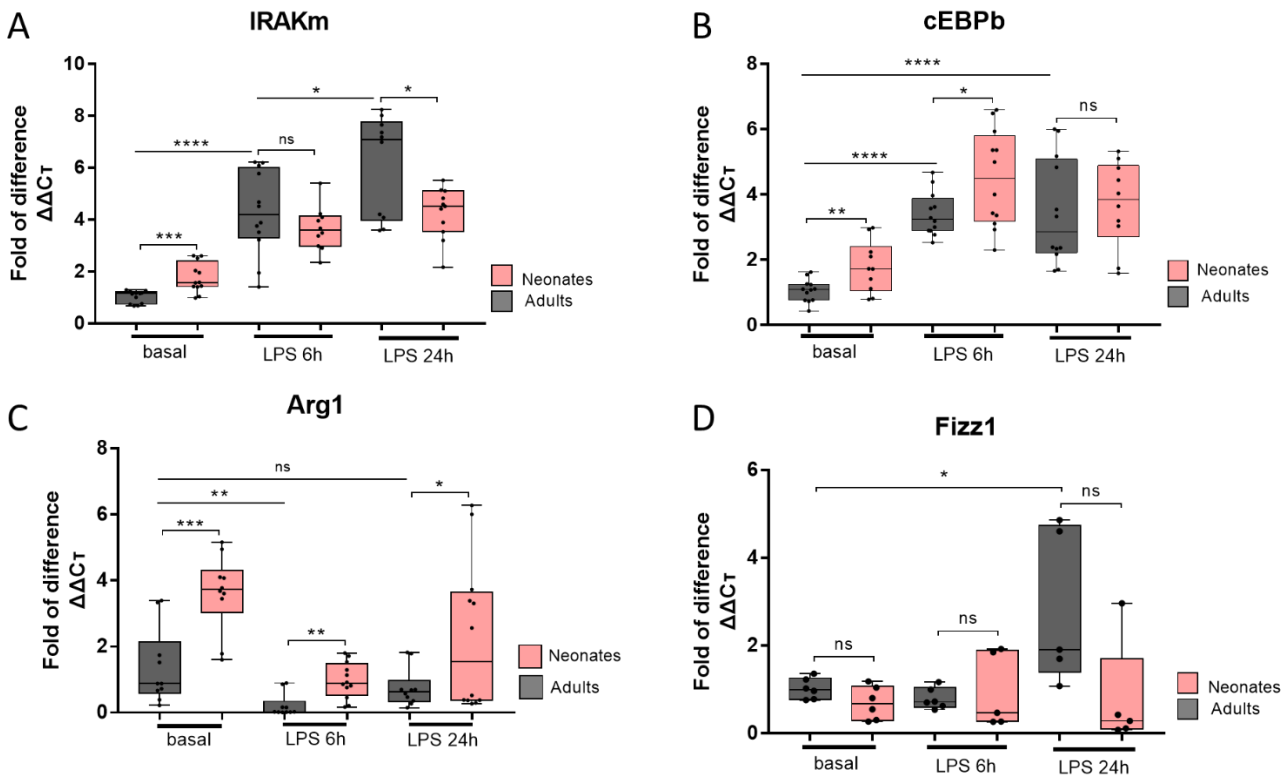

*Figure 11: mRNA levels of M2 markers. A, IRAKm mRNA is higher in neonates basally while upon LPS they reach similar levels. B, cEBPb is also higher in neonates basally while they increase in similar levels upon LPS. C, Arg1 is higher in neonates basally and is reduced at both adults and neonates 6 hours after LPS. For A, B, C experiment was performed twice and data were merged in the graph after normalized with adult basal. The two clusters depicted in A 24 hour adults in A or neonates in C are due to the differences between the two experiments. D, Fizz1 does not show differences between adults and neonates with the exception of a small increase in adults 24 hours after LPS. This is an indicative graph of the two experiments. Dots represent ΔΔCτ values. P-values were calculated with t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant.*

Both IRAKm and cEBPb were higher in neonates basally while they were increased after LPS stimulation approximately to the same level (Figure 11A, B). Arginase is the enzyme that catalases the hydrolysis of arginine into ornithine and urea. M2 macrophages are characterized by increased arginine metabolism by Arginase-1 (Arg1). Interestingly, Arg1 is higher in neonates at basal levels, while it is not increased in adults, as it would be expected. We suppose that Arg1 would be elevated later than 24 hours of LPS treatment (Figure 11C). Thus, we checked another M2 marker, Fizz1, which was increased 24 hours of stimulation only in adult murine macrophages (Figure 11D). It is possible that neonates delay upregulation of Fizz1 or use another mechanism.

Subsequently, we wanted to study M2 polarization after anti-inflammatory stimuli such as IL-4 and IL-10. Given that this kind of stimulation takes more time to be established, we tested for Arg1 expression at 24 and 48-hour time points. Here, we observed M2 polarization only in adults and only after IL-10 stimulation. Basally, we observed no significant differences between adults and neonates in Arginase-1 mRNA levels (Figure 12). The difference that is depicted in Figure 11C is lost here due to the fact that the experiment was not performed a second time to increase statistical significance.

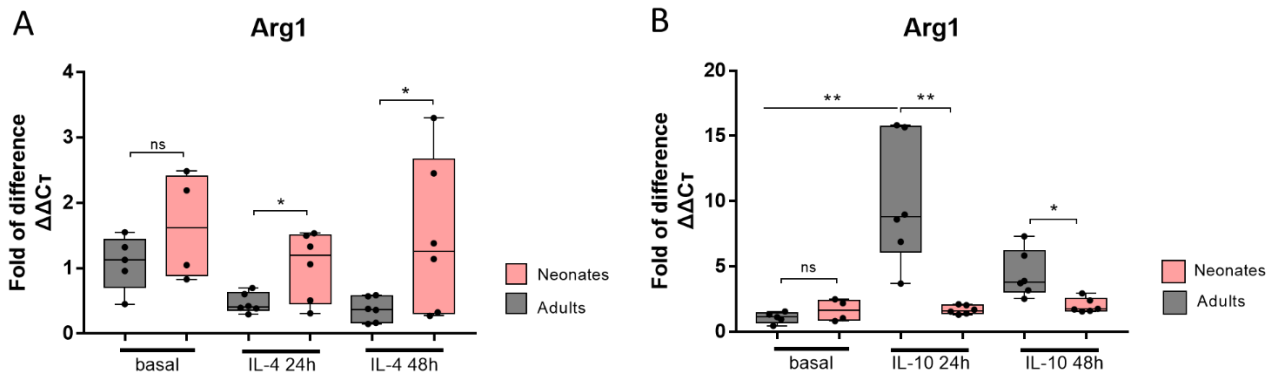

*Figure 12: mRNA levels of Arginase 1 after IL-4 (A) or IL-10 (B) stimulation for 24 and 48 hours. A, Similar levels of Arg1 mRNA between adults and neonates basally. 24 and 48 hours after IL-4 stimulation neonatal levels were a bit higher than adults but there is no statistical difference comparing with basal neonates. B, 24 hours after IL-10 stimulation, adult macrophages have an increased expression of Arg1 which remains higher than neonates at 48-hour time point. Dots represent ΔΔCτ values. P-values were calculated with t-test talking also in account an F-test. *p<0.05, **p<0.01, ns: not significant.*

Transcriptional and post-transcriptional regulation of macrophage polarization was also tested by measuring the levels of transcription factors such as IRF4 and IRF5 along with micro-RNAs such as mir-155 and mir-146a that are acknowledged for their role as master regulators in immune response.

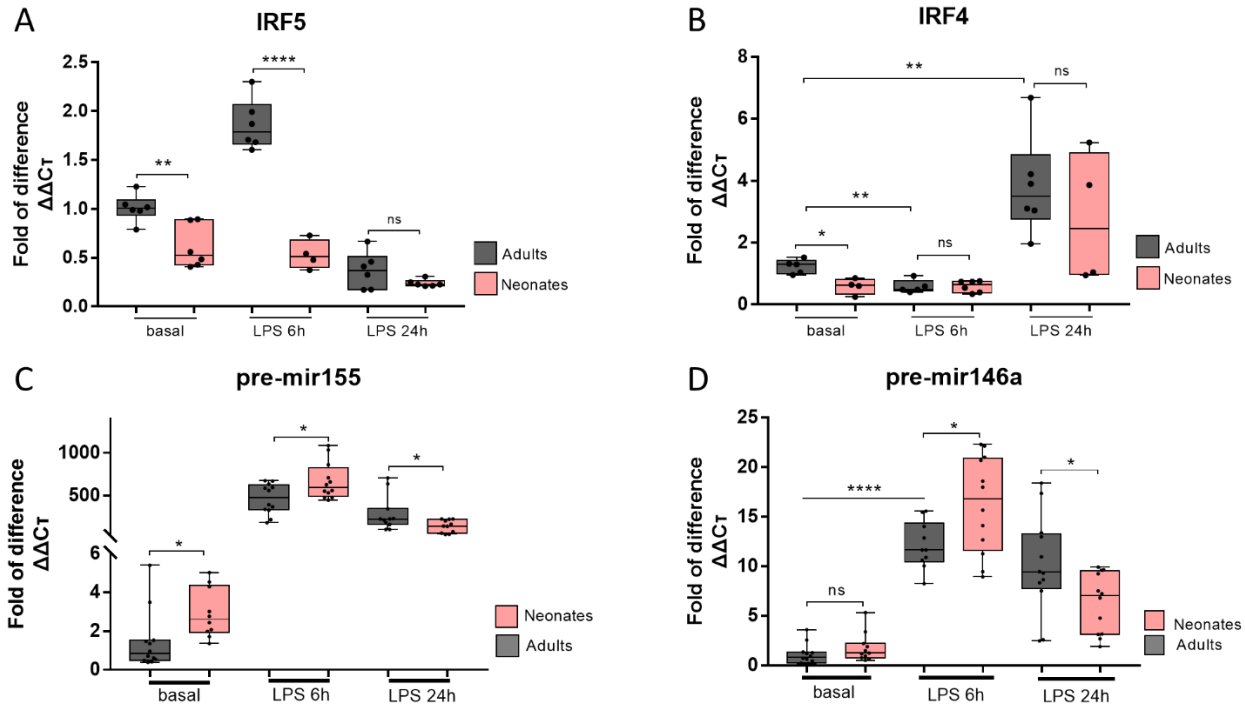

*Figure 13: mRNA levels of transcription regulators of M1 and M2 phenotypes. A, IRF5 mRNA is higher in adults both basally and 6 hours after LPS where there is also an increase. B, IRF4 is higher in adults basally and is increased in both adult and neonates in similar levels. C, pre-mir155 is higher in neonates basally and upon LPS for 6 hours while there is a small shift at 24 hours. pre-mir155 is highly increased after LPS. D, pre-mir146a is in equal levels in adult and neonates basally while it increases slightly more in neonates 6 hours after LPS while it decreases faster in neonates at 24 hours. Dots represent ΔΔCτ values. P-values were calculated with t-test talking. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant.*

Interferon regulatory factor 5 (IRF5), a transcription factor known to promote inflammation, is basally higher in adults and is increased 6 hours after LPS signal in adults. On the contrary, it's not increased in neonates at all. This is in agreement with our previous data since IRF5 is a master regulator of M1 polarization. IRF4 is less transcribed in neonates basally, which is sufficient with the fact that they express more pro-inflammatory markers. We also observed more pre mir-155 in neonates basally strengthening the profile of a more intense

pro-inflammatory phenotype initially but not that much induction. Basal neonatal levels are two times higher than adults. However, adult pre-mir-155 is increased around 320 times while neonatal around 230 times 6 hours after LPS stimulation. Pre-mir-146a did not appear to differ basally while it is increased at 6 hours and reduced faster at neonates.

Epigenetic regulation

Apart from the transcriptional regulation, we were also interested whether the epigenetic landscape affects macrophage activation. To this end, we studied epigenetic regulators that are acknowledged to be involved in innate immunity or inflammatory responses. Previous work of the lab identified Ezh2, Jmjd3, KDM2B, Phf2, Phf8 and Utx as putative epigenetic regulators of macrophage polarization. Some of them were also characterized by the literature to play a role in innate immune response. Initially we screened these genes in neonates and adults as for their relative mRNA levels prior to any stimulation. We noticed that they follow the same pattern in both neonates and adults.

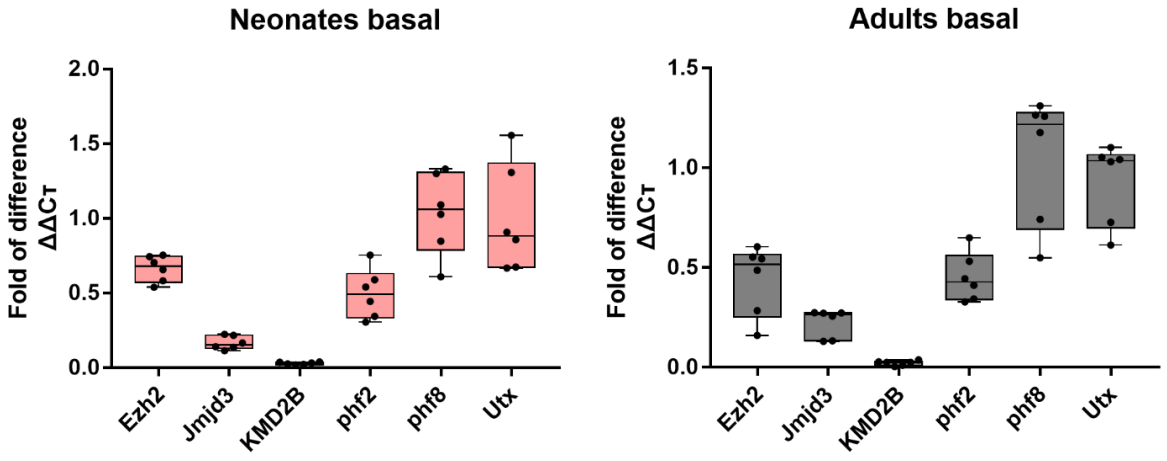

Figure 14: Comparative with each other mRNA levels of characterized epigenetic regulators: Ezh2, Jmjd3, Kdm2B, Phf2, Phf8, Utx, under not stimulated conditions. Values were normalized considering Phf8 average levels as 1. Dots represent ΔΔCτ values.

Then we moved to examine their mRNA levels after LPS stimulation. We also included more histone modification proteins that we considered important for innate immunity regulation or were known to be from the literature. Ezh2, MLL1, MLL3 and SETD7 are histone methyltransferases. SETD7 is an H3K4 methyltransferase that colocalizes with NFkB-p65 in human monocytes (Albuquerque, Batista, & Helguero, 2018). MLL1 is responsible for H3K4 trimethylation (Schuettengruber et al., 2007) and activates IL-6 expression in macrophages upon LPS stimulation (Minotti, Andersson, & Hottiger, 2015). MLL3 is another H3K4 metyltransferase leading to transcriptional activation.

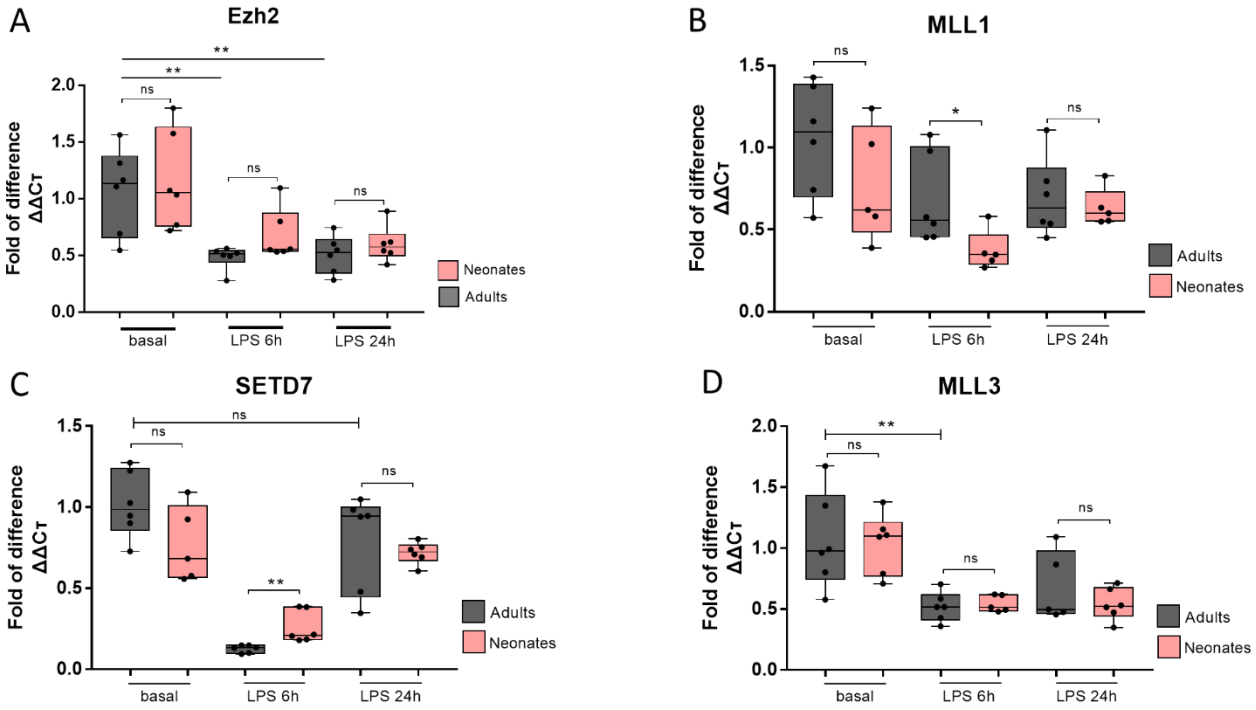

*Figure 15: mRNA levels of histone methyltransferases basally and upon LPS induction. Ezh2 mRNA is decreased upon LPS while there is no significant difference between adult and neonates. B, MLL1 decreases in neonates 6 hours after LPS stimulation. C, STED7 decreases 6 hours after LPS while at 24 hours return to basal levels. At 6 hours, adult SETD7 in significantly lower than neonates. D, MLL3 decreases upon LPS and there is no difference between adults and neonates. Dots represent ΔΔCτ values. P-values were calculated with t-test. *p<0.05, **p<0.01, ns: not significant.*

Ezh2 was reduced upon LPS while MLL1 results were inconclusive even though there is a small decrease at neonates in 6 hours. SETD7 appeared to be reduced in both adult and neonatal macrophages 6 hours after LPS, while it recovered at 24 hours. MLL3 also showed an interesting pattern with a decrease in 6 and 24 hours, non-age related.

Jmj3, Utx, KDM2B, Phf8 and Phf2 are histone demethylases. Jmjd3 is a histone H3K27me3 demethylase (Xiang et al., 2007), expressed in macrophages upon bacterial products and inflammatory cytokine stimulation (Santa et al., 2007). KDM2B is a H3K36 demethylase while Utx is another H3K27 demethylase. Utx is one of the genes that reside on X chromosomes but escape X-inactivation (Xu, Deng, Watkins, & Disteche, 2008). Plant homeodomain finger proteins Phf8 and Phf2 contain lysine demethylase (KDM). Phf8 demethylates H3K9me1/2, H3K27me2, H4K20me1 and acts as a transcription activator since H3K9Me1, H3K9Me2, H3K27Me2 and H4K20Me1 are epigenetic repressive marks. Phf2 demethylates H4K20me3, while phosphorylated Phf2 by protein kinase A (PKA) demethylates H3K9me2 (Daskalaki & Tsatsanis, 2018). Phf2 is known to initiate pro-inflammatory genes production (Stender et al., 2012) while Phf8 plays a role in M1 related genes inactivation.

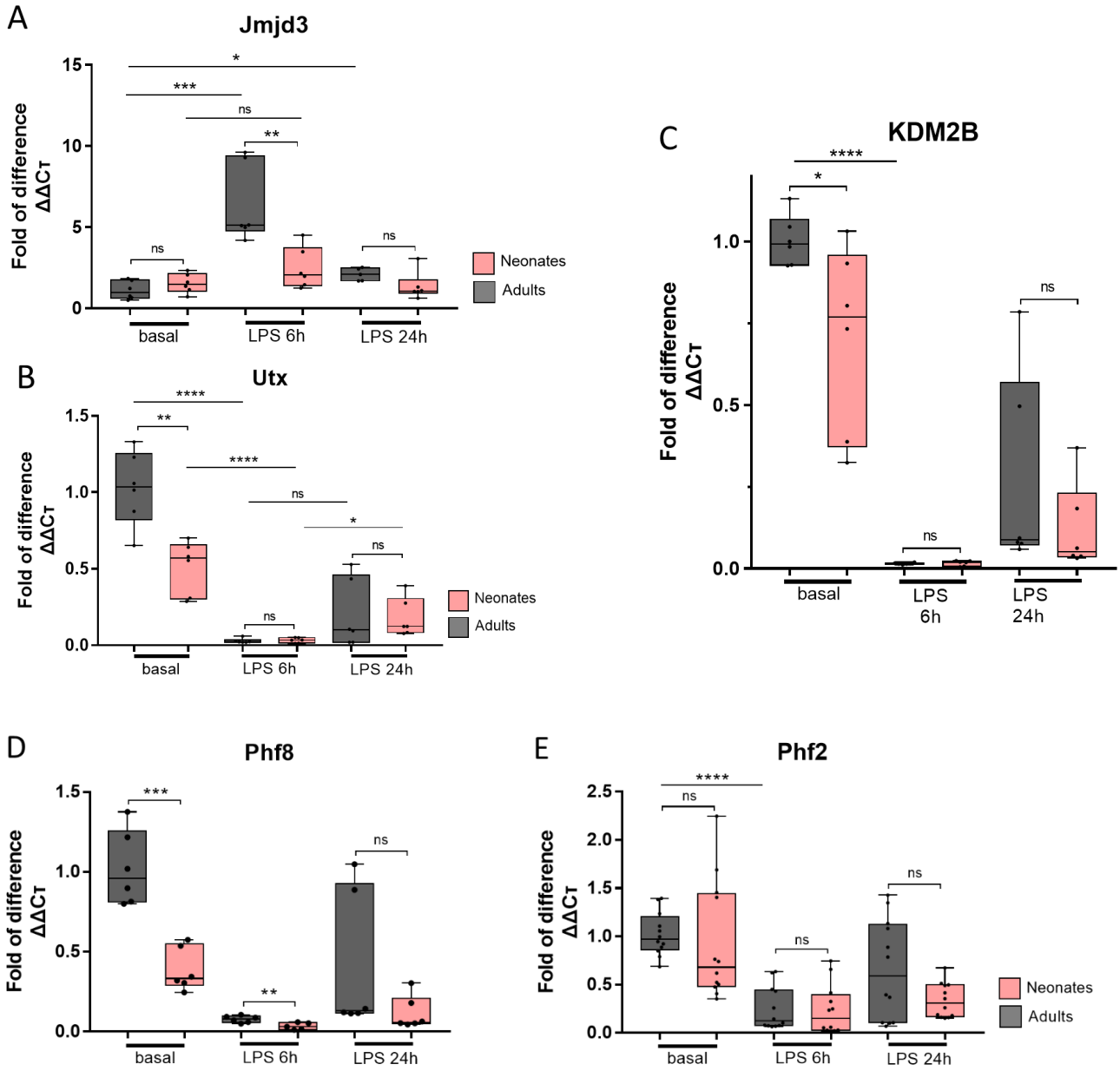

*Figure 16: mRNA levels of histone demethylases basally and upon LPS induction. A, Jmjd3 basal levels are equal between adults and neonates while 6 hours after LPS stimulation only adult Jmjd3 mRNA seems to increase while at 24 hours levels are returning back to basal. B, Utx is higher in adults basally and decreases significantly in both adult and neonates 6 hours after LPS while it begins to go back to basal levels at 24 hours. C, Kdm3B was higher in adults basally and decreases almost to non-detectable levels upon LPS stimulation for 6 hours while starts to return at basal levels in some of the biological replicates at 24 hours. D, Phf8 is higher in adults basally and decreases significantly in both adult and neonates at 6 hours LPS, maintaining the difference. E, Phf2 appears in equal levels between adult and neonates basally and decrease upon LPS stimulation for 6 hours. Experiment was performed twice and values were normalized for basal adults. Dots represent ΔΔCτ values. P-values were calculated with t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant.*

Jmjd3 seems to be upregulated only in adults after 6 hours of stimulation. Since Jmjd3 is a known regulator of macrophage polarization, we also tried to see what happens in the protein level. However, this was not achieved due to technical limitations. Utx seemed to be lower in neonates and generally reduced after LPS. Nevertheless, we could not appreciate this result since Utx is one of the genes that escape X-inactivation, thus exists in different doses between males and females. For adults we always used female mice in our experiments, but neonatal gender was hard to be determined at this developmental stage. Thus, we cannot be sure whether this difference is due to the fact that there are some male neonates. KDM2B was reduced 6 hours after LPS in almost undetectable levels and showed no significant difference in neonates. Phf8 was

reduced in neonates basally while both Phf8 and Phf2 appeared to decrease their mRNA levels after LPS stimulation. This result came in contrast with the literature. Thus, we considered appropriate to investigate these two epigenetic modifiers in protein level using western blot. We did not manage to visualize Phf8 due to technical reasons. Phf2 protein levels of adults and neonates basally and 24 hours after LPS stimulation are shown in Figure 17.

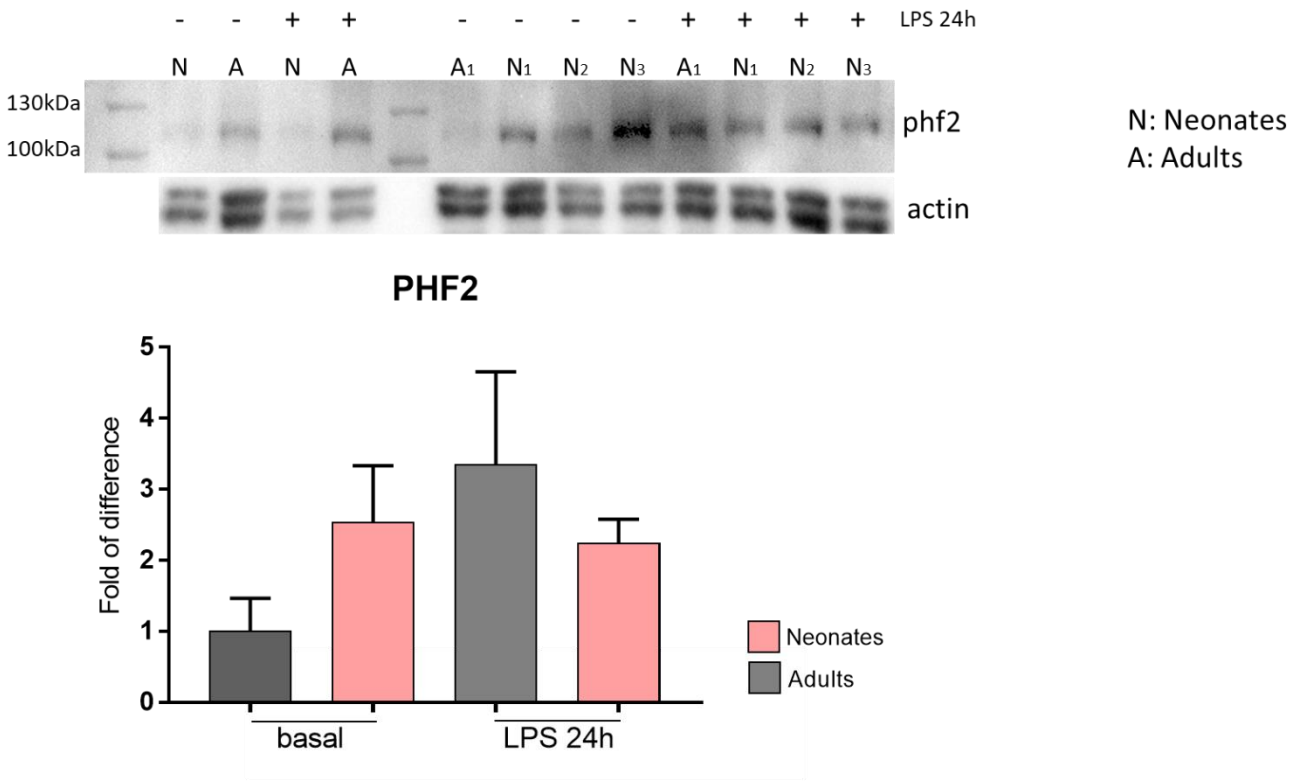

Figure 17: Western blot analysis using antibodies against mouse PHF2 and actin proteins in adults and neonates basally and 24 hours after LPS stimulus. PHF2 intensity was normalized with actin and analyzed with ImageJ. Protein extracts from two distinct experiments of primary macrophages cell culture were used. Experiment 1 includes one neonatal sample (N), and one from adults (A). Experiment 2 includes one adult sample (A1) and three neonatal (N1, N2, N3). Bottom figure includes values from both experiments and the standard deviation of the median in graphed.

We observed a clear increase in PHF2 protein levels in adults 24 hours after LPS stimulation. However, such increase was not detected in neonatal macrophages.

Epigenetic regulation of macrophages in Akt1 and Akt2 KO background

Previous work of the lab has explained the differential contribution of Akt1 and Akt2 kinases to macrophage polarization (Arranz et al., 2012). Thus, we considered that it would be useful to examine the expression levels some histone modification proteins that are known to play a role in innate immune response or macrophage polarization, in an Akt1 and Akt2 knockout background which exhibit a deficient metabolic profile of macrophages.

The histone methyltranferases studied were SETD7, MLL1 and MLL3. MLL1 siRNA showed a downregulation of Arginase 1 (Figure 5), while MLL3 or SETD7 siRNAs caused iNOS downregulation.

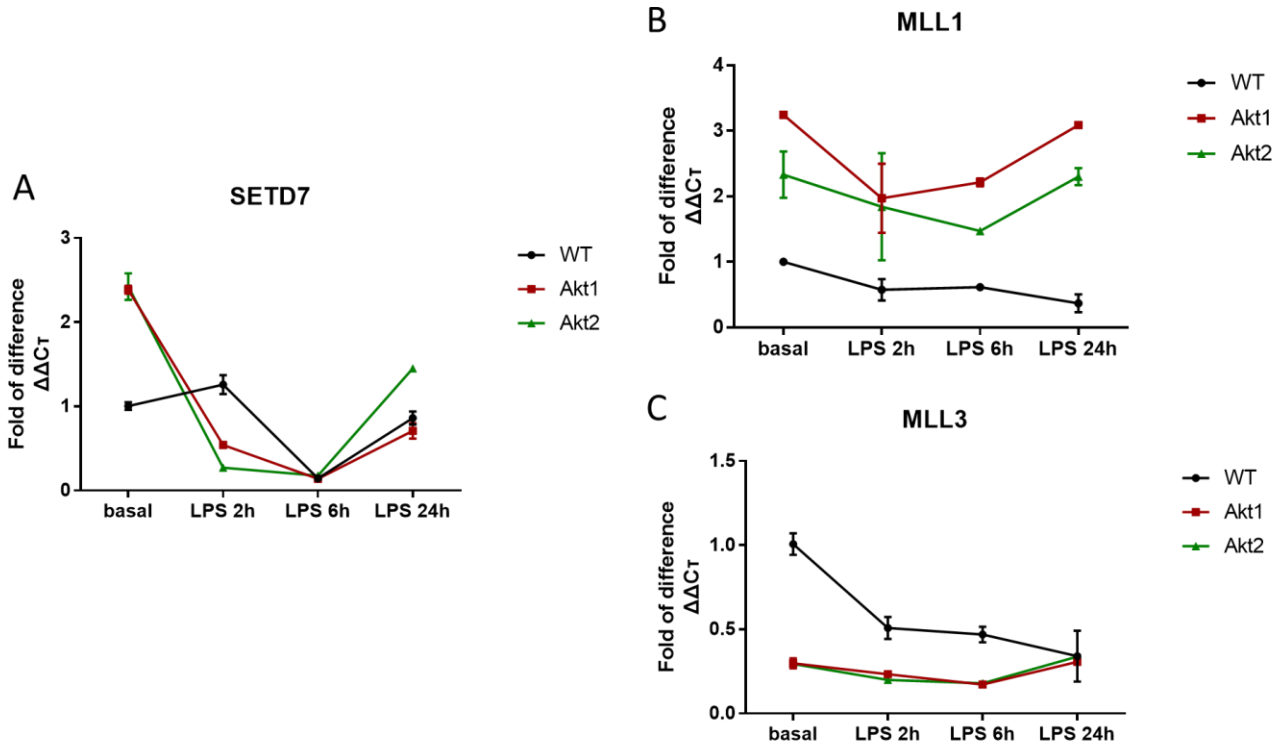

Figure 18: Comparative mRNA levels of histone methyltransferases basally and upon LPS stimulation for 2, 6 and 24 hours in wild type (WT) and Akt1 or Akt2 knockout mice. A, Setd7 levels are almost 2.5 times higher in Akt knockouts than WT, at unstimulated conditions. 2 hours after LPS stimulation Setd7 drops significantly in Akt knockouts, below the WT levels which remain unchanged. At 6 hours, all three genetic background mice have even more reduced Setd7 mRNA in equal levels with each other. At 24 hours, levels start to increase with Ak2 KO to be higher. B, MLL1 is lower in WT mice at all time-points. Setd7 levels in Akt1 mice are higher than Akt2 mice basally and after 6 hours of LPS stimulation. At 2 hours, there is a variation between the biological replicates of Akt mice, however there is a significant decrease compared with basal condition. This decrease at 2 hours is also significant in WT. At 6 hours, WT Sted7 levels remain unchained while there is not a statistically significant difference of Akt1 and Akt2 as well. 24 hours upon LPS, WT animals appear to decrease their Setd7 while Akts increase back to their basal levels. C, MLL3 basal levels are significantly higher in WT animals while decrease to half 2 hours after LPS and remains up to 6 hours for all three genotypes. At 24 hours, WT animals decrease Setd7 even more, while Akt KOs return to their basal levels. A,B,C, 2 animals were used and considered as biological replicates. Error bard depict the standard error of the mean. When variation between samples is very small, error bars are not visible.

Our results suggest that SETD7, MLL1 and MLL3 are regulated through the Akt pathway.

Histone deacetylases (HDACs) remove acetyl groups from histones, allowing negatively charged DNA to bind more strongly with histones. HDACs are known to regulate both innate and adaptive immunity. More specifically HDAC1 deacetylates c/EBPb while it downregulates promoter activities of other TLR responsive genes. HDAC1 mRNA is induced upon TLR4 stimulation as shown in Figure 19. Even though we observed the same rate of increase in all three genotypes, wild type mice had higher levels of HDAC1 at all time-points. SIRT6 is a class III deacetylase which promotes tissue inflammation. Its' mRNA levels are slightly higher in Akt knockouts basally. However, in wild type animals we observed a steadily increase after LPS stimulation while Akt knock outs appeared to decrease at 2 hours LPS before start recovering and increasing.

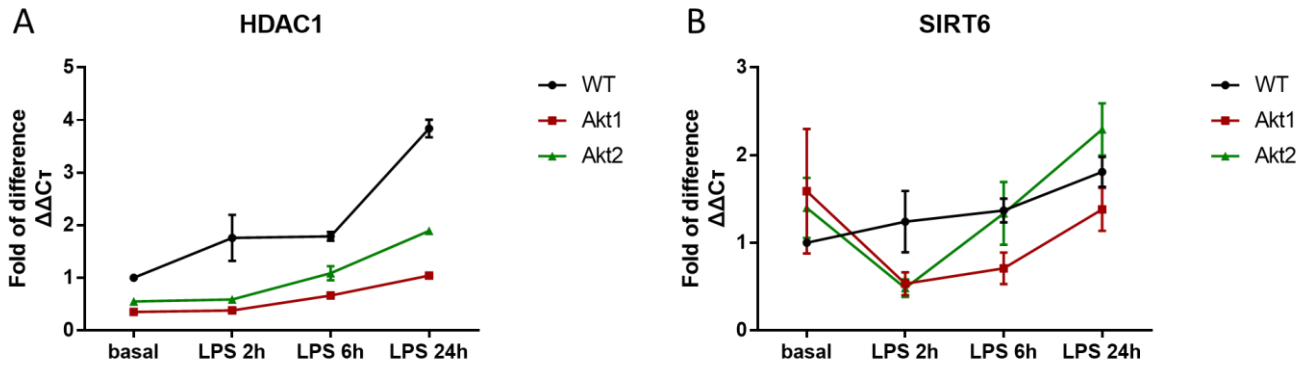

Figure 19: Comparative mRNA levels of histone deacetylases basally and upon LPS stimulation for 2, 6 and 24 hours in wild type (WT) and Akt1 or Akt2 knockout mice. A, Hdac1 mRNA increases upon LPS and keeps getting higher up to 24 hours in both WT and KO mice. WT levels are higher in all time-points, however increase with the same rate are the KO. Setd7 levels in Akt2 KO mice are higher than Akt2 KO at all time-points. A,B, 2 animals were used and considered as biological replicates. Error bard depict the standard error of the mean. When variation between samples is very small, error bars are not visible.

HDAC1 transcription seems to be enhanced through the Akt pathway.

Training

Based on two 2016 publications by Bekkering, Netea and co-workers (Bekkering et al., 2016; Netea et al., 2016), we tried to investigate if neonatal macrophages could be trained in order to acquire a phenotype more similar to adults. We choose beta glucan as the mean to training and 3-days rest as it seemed to have the most major difference. The experiment included 24 hours of beta glucan stimulation, 3 days rest and 6 hours stimulation with 100ng/mL LPS. According to the aforementioned work, we would expect higher production of TNFa when β-glucan (B-glu) training was mediated before LPS stimulation. However, we did not observe such event neither in adults nor in neonates (Figure 20). We noticed though again higher TNFa secretion in neonates basally.

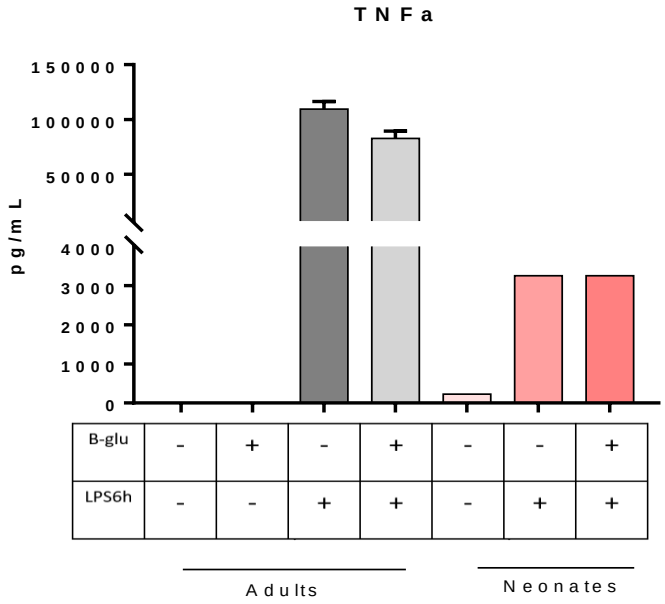

Figure 20: ELISA for TNFa with or without 5μg/mL β-glucan (B-glu) treatment, 3 days rest and additional treatment or not with 100ng/ml LPS for 6 hours in both adults and neonates. TNFa basal levels are undetectable in adult in contrast with neonates that were notably higher. B-glucan alone stimulation was only tested in adults and did not give any TNFa detection. When cells were trained with β-glucan and then treated with LPS we did not observe any significant difference comparing with LPS treatment alone neither in adults nor in neonates. Adult samples included 2 biological replicates while neonatal only one. Concentrations were calculated by normalizing with total BSA protein per sample.

Discussion

Neonatal stage of development represents a period of a relatively naïve immune system. Innate immune system includes the first responders to a massive exposure of new pathogens that will stimulate the adaptive immune system and the development of classic immune memory. Thus, the response of the innate immune system is critical for the initiation and maintenance of a coordinated defense mechanism. Evidence support that newborn immune system is immature and distinct from that of adults. TLRs are key elements of the innate immune response since they recognize pathogens and initiate the response. Our data suggest that neonatal innate immune response is quantitatively deficient compared to the adult, having a differential response as for the expression levels of cytokines, transcription factors and epigenetic regulators.

Comparing peritoneal thioglycolate and bone marrow derived macrophages, we observed that neonatal BMDMs had 5 times more IL-10 than adults basally. Since IL-10 is higher increased in neonates after stimulation, this could mean that bone marrow cells have technical limitations when differentiate to macrophages. This means that apart from differentiation, a small stimulation also takes place. Thus, basal levels on bone marrow macrophages are not completely unstimulated condition.

The observation of higher levels of TNFa and iNOS in neonates basally can be explained by the fact that during development, regeneration of tissues causes a kind of mild inflammation. However, upregulation of these factors after LPS is higher in adults. One hypothesis is that basal levels of TNFa in neonates cause a tolerance-like state which leads to less induction after LPS. Another hypothesis is that since IL-10 is expressed early and in high concentrations in neonates, it suppresses TNFa prematurely. Levy et. al. have claimed that an inhibitory factor, identified as adenosine, is present in neonatal blood plasma and possesses immunomodulatory properties. They showed that this neonatal adenosine system inhibits TNF-α production upon TLR stimulation, but preserves IL-6 production (Levy et al., 2006). Nevertheless, our primary macrophages cell culture system did not include the presence of blood plasma so there must be additional reasons to this lower TNFa expression in neonates.

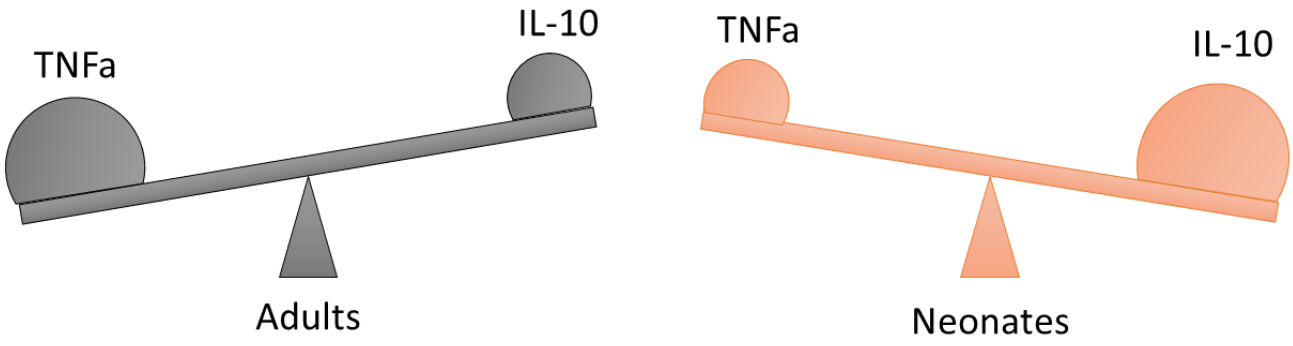

Figure 21: One hypothesis is that IL-10/TNFa ratio regulates the extend of the innate immune response. In adults, low IL-10 allows high levels of TNFa. However, in neonates, high IL-10 does not allow TNFa to reach adult levels.

High levels IL-10 in neonatal macrophages is usually correlated in the literature with an anti-inflammatory phenotype. However, our results point out that such statement is not accurate since only few of the genes involved in the anti-inflammatory response are differentially expressed in neonates compared to adults.

In Figures 16 and 17 phf2 mRNA and protein levels are presented upon LPS stimulation. However, when comparing 24 hours after LPS, mRNA seems to be reduced while protein is increased in adults only. These opposing results alert us that we should not always depend only on transcriptional regulation since usually proteins have a predominant role.

Bekkering *et. al* investigated and tried to optimize a protocol of monocyte trained immunity triggered by β-glucan, the bacillus Calmettee-Guérin (BCG) vaccine, and oxidized low-density lipoprotein (ox- LDL) as

primary stimulus. The effectiveness of their treatments were assessed in terms of the secondary cytokine response, the production of reactive oxygen species, cell morphology and induction of glycolysis. They claimed that monocytes primed with the aforementioned stimuli showed increased pro- and anti-inflammatory cytokine response upon restimulation with a different stimulus (Bekkering et al., 2016). According to their results, training with β-glucan for 24 hours and resting time 3 days appeared to have the most striking effect. Based on this study, we tried to recapitulate their experiment in the context of training neonatal primary peritoneal macrophages. We hypothesized that lack of training might be one the causes of neonatal incompetence to resolve microbial infection. However, we did not manage to replicate their results since pre-treatment with β-glucan did not alter the TNFa levels when stimulated with LPS (Figure 20). This could be due to technical limitation that were not resolved. It is possible that the thioglycolate derived macrophages cannot undergo training. A recent publication though, depicted the cytokine profiles to LPS following β-glucan priming, in human cord-blood monocytes. They showed that β-glucan priming led to a significant increase in the cytokine levels of IL-6, TNF and IL-10 in both adults and neonates that were re-stimulated with LPS (Namakula et al., 2020). However, cord blood monocytes are not equivalent to neonatal peripheral blood monocytes.

Future perspectives

Based on the results of this thesis, an interesting aspect would be to train neonatal macrophages and investigate whether their epigenetic background is altered. In particular, if epigenetic landscape simulates that adult one after training and triggered with a secondary stimulus. Longer rest periods after initial trigger could be tested. Another result that should be furtherly elucidated is why there is an opposite regulation of PHF2 protein and mRNA levels upon LPS stimulation. In addition, more epigenetic factors and time points should be investigated in the protein level, since their regulation regarding innate immunity might be post-transcriptionally or might change differently in mRNA and protein during time.

Another interesting idea might be an siRNA treatment of adult and neonatal macrophages with Jmjd3, Phf2 or other factors that appeared to be differentially expressed in neonates. Hypothesized that they affect M1-M2 polarization, we would expect to upregulate or downregulate iNOS or Arg1. It would be interesting to find some of those factors to have an influence only in adults or neonates or even an opposite effect.

A comprehensive analysis of gene expression could be also performed through an RNA-seq experiment for the following conditions of thioglycolate derived peritoneal macrophages: adult non-stimulated, neonate non-stimulated, adult stimulated with LPS for 6 hours, neonate stimulated with LPS for 6 hours. More time-point of LPS stimulation such as 2 hours or 24 hours could be added if there are resources available. The differentially expression patterns could be well-monitored with this approach and novel regulators of the neonatal innate immune system is possible to be identified. An ATAC-seq analysis of the same conditions might also support or reject the importance of histone modifications for the promoters' accessibility of immune system related genes. Differences between adults and neonates could reveal the epigenetic network that might be responsible for neonatal immune defects.

A Chip-seq experiment, in which chromatin immunoprecipitation will take place using antibodies against the histone modification proteins that appeared to play a role in macrophage metabolism or have a differential regulation in neonates, might reveal genes and regulatory elements with a non-described role in innate immunity. Gene promoters affected by the induction of histone modifications upon TLR4 signaling can be identified though Chip-seq.

To sum up with, the transcription and epigenetic factors that were identified to play a role in this differential innate immune response in neonates should be furtherly studied and validated. In addition to in vitro work, a bioinformatics approach could assist in both confirmation of the observed expression patterns and identification of new proteins that are important for macrophage metabolism.

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