



**A Conserved Glucocorticoid-Linked Transcriptional Program Underlies
Obesity, Weight Loss, and Monogenic Immune Dysregulation**

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Declaration

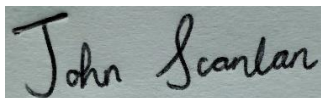
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John Scanlan BSc, MSc

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To my Mam and Dad, I would like to dedicate this thesis to you both. When one is young, one doesn't recognise the simple impressions made by your parents that are compounded and grow into something much greater as you get older. As I get older, I recognise the gifts you have given not just me but all your children. Your love of learning, books and curiosity about the world has left an indelible mark on me as a person. For your continued support of me I am eternally grateful. Thanks.

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Abstract

This thesis set out to address fundamental questions in obesity immunology: why immune cells adopt a persistently activated inflammatory state in obesity, whether that state is reversible, and what molecular circuitry governs the transition between disease and health. A consistent finding was that the gene *TNFAIP3*, that encodes the protein product (A20), a critical negative regulator of NF- κ B, is significantly downregulated in lymphocytes across tissues and in humans and mice in obesity, with expression restored following weight loss. This pattern links obesity to chronic NF- κ B activation and aligns with phenotypes observed in *TNFAIP3* knockout systems and in A20 haploinsufficiency (HA20), a genetic disorder where uncontrolled inflammation is a hallmark. Multi-omic analyses, genetic associations, and cross-species comparisons converge on a model in which reduced A20 lowers the threshold for lymphocyte activation and promotes persistent inflammatory tone, while restoration with weight loss resets this immune setpoint toward health.

A second central theme is the role of glucocorticoids and the glucocorticoid receptor. Weight loss and fasting induce transient rises in cortisol, which activate a glucocorticoid driven anti-inflammatory program including *TNFAIP3*, *NFKBIA*, *DUSP1*, *ZFP36*, *TSC22D3*, and *DDIT4*. This network of genes includes NF- κ B and AP-1 members suggesting co-operation at shared enhancers to terminate inflammation, reprogram metabolism, and direct lymphocyte trafficking via *CXCR4*. In obesity, reduced enhancer accessibility, impaired cortisol regeneration through *HSD11B1*, and altered AP-1 composition degrade this network, likely explaining the failure of immune resolution.

Finally, by comparing obesity to the genetic disorder A20 haploinsufficiency, I show that both states converge on a glucocorticoid receptor driven regulatory network, despite obesity being environmentally acquired and A20 haploinsufficiency being inherited. That convergence implies that obesity may phenocopy aspects of A20 haploinsufficiency at the gene-regulatory level.

Overall, this thesis provides multiple insights into transcriptional signatures associated with obesity, weight loss, fasting and A20 haploinsufficiency and their effects on lymphocyte regulation and has implications for how we think about immunity, metabolic disease and weight loss interventions.

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Abbreviations

11 β -HSD	— 11 β -hydroxysteroid dehydrogenase
ACTH	— Adrenocorticotrophic Hormone
ADC	— Antibody–Drug Conjugate
AMP	— Adenosine monophosphate
AMPK	— AMP-activated protein kinase
AP-1	— Activator Protein-1 (FOS/JUN transcription factor complex)
BC	— Before Christ
BMI	— Body Mass Index
C/EBP β	— CCAAT/Enhancer-Binding Protein beta
CCA	— Canonical Correlation Analysis
ChIP-Seq	— Chromatin Immunoprecipitation followed by sequencing
CPM	— Counts Per Million
CRD	— Calorie-Restricted Diet
CRP	— C-reactive protein
CX3CR1	— Chemokine (C-X3-C motif) receptor 1
CXCR4	— Chemokine (C-X-C motif) receptor 4
CXCL12	— C-X-C motif chemokine ligand 12 (SDF-1)
DC	— Dendritic Cell
DDIT4	— DNA Damage-Inducible Transcript 4 (REDD1)
DIO	— Diet-Induced Obesity
DREAM	— Downstream Regulatory Element Antagonist Modulator
DUSP1/2	— Dual-Specificity Phosphatase 1/2
eQTL	— Expression Quantitative Trait Locus
FFA	— Free Fatty Acid
FKBP4 / FKBP5	— FK506-binding proteins (GR chaperone complex)
GLMPCA	— Generalised Linear Model PCA
GLP-1	— Glucagon-Like Peptide-1
GLUT4	— Glucose Transporter 4 (SLC2A4)
GC	— Glucocorticoid
GCR	— Glucocorticoid resistance
GR	— Glucocorticoid Receptor
GRE	— Glucocorticoid Response Element
GRE	— Glucocorticoid Response Element

GTEx — Genotype-Tissue Expression (project)
 HA20 — A20 haploinsufficiency
 hdWGCNA — High-dimensional Weighted Gene Co-expression Network Analysis
 HFD — High-Fat Diet
 HPA (axis) — Hypothalamic–Pituitary–Adrenal axis
 HPA (atlas) — Human Protein Atlas
 HSP — Heat-Shock Protein
 HVG — Highly Variable Gene
 IFN γ — Interferon-gamma
 IL-6 / IL-10 / IL-17 — Interleukin-6 / -10 / -17
 ILC — Innate Lymphoid Cell
 IR — Insulin Resistance
 IRS — Insulin Receptor Substrate
 ITAM — Immunoreceptor Tyrosine-based Activation Motif
 JIA — Juvenile Idiopathic Arthritis
 kME — Module eigengene-based connectivity
 kNN — k-Nearest Neighbour
 KO — Knockout
 LFD — Low-Fat Diet
 LINCS — Library of Integrated Network-based Cellular Signatures (L1000)
 LPS — Lipopolysaccharide
 MAIT — Mucosal-Associated Invariant T cell
 MCP-1 — Monocyte Chemoattractant Protein-1 (CCL2)
 MHO — Metabolically Healthy Obese
 MNN — Mutual Nearest Neighbours
 MR — Mineralocorticoid Receptor
 MUO — Metabolically Unhealthy Obese
 NF- κ B — Nuclear Factor kappa-B
 NFKBIA — NF- κ B Inhibitor Alpha (I κ B α)
 NIK — NF- κ B-inducing kinase
 NK cell — Natural Killer cell
 nGRE — Negative Glucocorticoid Response Element
 NPX — Normalised Protein eXpression (Olink unit; log₂-scaled)
 NR3C1 — Gene encoding the Glucocorticoid Receptor
 nTPM — Normalised Transcripts Per Million

OTU — Ovarian Tumour (deubiquitinase) domain
 OXPHOS — Oxidative Phosphorylation
 PCA — Principal Component Analysis
 PCD — Programmed Cell Death
 PBMC — Peripheral Blood Mononuclear Cell
 pDC — Plasmacytoid Dendritic Cell
 PCK1 — Phosphoenolpyruvate Carboxykinase 1
 PEA — Proximity Extension Assay (Olink)
 PPI — Protein–Protein Interaction
 PTM(s) — Post-translational Modification(s)
 PTSD — Post-Traumatic Stress Disorder
 RA — Rheumatoid Arthritis
 ROS — Reactive Oxygen Species
 sQTL — Splicing Quantitative Trait Locus
 S1PR5 — Sphingosine-1-phosphate Receptor 5
 scRNA-seq — Single-cell RNA sequencing
 SLE — Systemic Lupus Erythematosus
 SNP — Single Nucleotide Polymorphism
 T2D — Type 2 Diabetes
 TF — Transcription Factor
 TGFβ — Transforming Growth Factor-beta
 TOM — Topological Overlap Matrix
 TNF / TNFα — Tumour Necrosis Factor / TNF-alpha
TNFAIP3 — Tumour Necrosis Factor Alpha-Induced Protein 3 (A20)
 TNFR — Tumour Necrosis Factor Receptor
 Treg — Regulatory T cell (you use “T-Reg” in places; pick one and standardise)
 UMAP — Uniform Manifold Approximation and Projection
 USF1 — Upstream Stimulatory Factor-1
 ZFP36 — Zinc Finger Protein 36 (TTP)

1.0 - Chapter 1 - Literature Review

This literature review covers key areas relevant to this thesis: the historical framing of obesity as a disease, the immunometabolic mechanisms that sustain chronic low-grade inflammation, and the interplay between natural killer (NK) cell dysfunction, NF- κ B signalling, and glucocorticoid pathways. Together, these sections provide the context for understanding how obesity perturbs immune balance and how interventions may restore it.

1.1 - First Discussion on Obesity

‘Corpulence is not only a disease itself, but the harbinger of others’¹, this was noted by the father of medicine, Hippocrates around 400 BC. Thus, obesity or corpulency as it was often known as has long been referenced in literature and associated with ill-health. Several hundred years after Hippocrates observations, the Greek physician Galen is thought to have adapted many of Hippocrates ideas and developed them further. Galen also made associations between obesity and ill-health, and he described obesity as a pathological condition resulting from gluttony and lack of exercise. Galen recognised morbid obesity and named it as ‘Polisarkia’ and believed that obesity results from the surplus of ‘bad humours’². Whilst several of his ideas on physiology and pathology have been proven incorrect, many of his treatments are still recommended today including weight loss interventions through diet. Galen dedicated a whole body of work to diet, ‘*De Victu Attenuante*’, translating literally as ‘On the Slimming Diet’ where he advocates for exclusion of foods such as milk, cheese, wine, and honey as they were deemed ‘extremely fattening’². He also advocated for non-strenuous exercise in the form of walking, swimming and a ball game, as methods to lose weight².

Galenic ideas on obesity were used throughout the Middle Ages and Renaissance where obesity was stigmatised and even assigned to be one of the seven deadly sins by the Catholic church. In medieval theology, gluttony was seen as both morally dangerous and physiologically disruptive, posing a unique challenge because the desire for food is natural and inescapable. As John Cassian observed, “we can never rid ourselves of the proximity and service of gluttony,” and later writers like Peraldus viewed it as the gateway to both bodily sickness and spiritual decay, necessitating moderation rather than mere abstinence³. Additionally, obesity or being overweight was referred to in Shakespeare’s work, for example in Hamlet, Queen Gertrude describes her son, Hamlet, as ‘fat and scant of breath’, linking excess weight to health issues. Thus, at this stage obesity had been associated with ill-health and been stigmatised both through religious teachings and in popular writings at the time.

With the scientific revolution came further advancements in our understanding of obesity, in 1727, Thomas Short, released one of the first scientific books to study obesity as a health problem, ‘A Discourse Concerning the Causes and Effects of Corpulency’. Obesity was then recognised as a risk for gout, sleep problems and sudden death ⁴. Then, 136 years later, William Banting, releases the first popularised weight loss diet in ‘Letter of Corpulence’. Despite an active lifestyle and numerous medical treatments, including Turkish baths, rowing, and starvation diets, he found no lasting relief. Eventually, by eliminating bread, sugar, milk, beer, and potatoes, he lost over 30 pounds in a few months. Banting framed his experience not as a matter of vanity, but as a public health concern, arguing that corpulence was both misunderstood by the medical profession and treatable through common-sense dietary changes.

It could be argued that modern immunology was born just nineteen years later after Banting’s ‘*Letter of Corpulence*’, when Elie Metchnikoff discovered phagocytes and proposed the idea of innate immunity. The field of immunometabolism, integrating immune function and metabolic regulation, emerged from converging observations made over more than a century. As early as 1884, physicians observed that infections like meningitis could induce hyperglycaemia, revealing a metabolic response to immune challenge ⁵. As immunology advanced throughout the 20th century, so did an understanding of obesity. By the 1940s, obesity had shifted from being mainly a social or aesthetic concern to being classified as a metabolic disorder linked to dietary changes, hormonal changes and diabetes. By the 1960s, obesity was associated with insulin resistance and chronic metabolic dysfunction ⁶ while immune cell infiltration into adipose tissue was observed in animal models ⁷. However, the molecular links were not understood until the 1980s, when conditioned media from lipopolysaccharide (LPS) -stimulated macrophages was shown to impair insulin signalling in adipocytes ⁷. A landmark point came in 1993, when Gökhan Hotamisligil and colleagues demonstrated that tumour necrosis factor (TNF), an inflammatory cytokine, was overexpressed in the adipose tissue of obese rodents and contributed to insulin resistance ⁸. In 1994, leptin was discovered as a major metabolic regulator responsible for controlling satiety to food consumption ⁹. Thus, by the mid-1990s, recognition of the links between obesity and immunity had emerged. Obesity was recognised as an established disease, this will be discussed in detail later in the introduction..

1.2 - Natural Killer (NK) Cells and their Dysregulation in Obesity

NK cells are the central immune population examined in this thesis, chosen both for their important roles in cytotoxicity and immune regulation, and because a rich literature shows that obesity produces consistent dysfunction in NK cell numbers, phenotype, and effector function.^{10,11} NK cells are effector type 1 innate lymphoid cells, known for their ability to kill virally infected and cancer cells via cytotoxic mechanisms¹². NK cells typically kill their targets through the targeted secretion of lytic granules, which contain pore forming perforin and granzyme proteins which induce apoptosis in the infected or cancerous cell¹³. NK cells rely upon the central metabolic pathways of oxidative phosphorylation (OXPHOS) during homeostasis but switch to glycolysis to meet the energy demands to kill tumour cells and carry out their effector function¹⁴. NK cell activation is tightly managed by balancing activating and inhibitory signals provided by the expression of germline coded receptors¹⁵. The balance of these signals can change in response to changes in environmental cues, promoting either inhibition or activation of the cell. Loss of self-identifying molecules such as HLA, results in a loss of an inhibitory signal from killer-cell immunoglobulin like receptors expressed on the surface of NK cells, leading to the activation of the NK cell and the lysis of the target cell¹⁶.

In obesity, humans and mice display functional deficits in NK cells and have an increased risk of cancer and infections¹⁰. It is no surprise that since obesity is a metabolically altered state, that it would induce dysfunction in NK cells. It was shown that obesity induces lipid metabolism in NK cells, after just one week of high fat diet (HFD), lipid handling and metabolism genes are significantly upregulated when compared to standard fat diet (SFD) fed mice¹⁰. In humans, NK cells are also highly dysfunctional as shown by both quantitative and functional impairment: individuals with obesity have fewer circulating NK cells compared to lean controls, and those NK cells display reduced cytotoxicity and cytokine production in of altered metabolism, including elevated expression of lipid-uptake markers such as CD36, intracellular lipid accumulation, and dysregulated metabolic pathways, coupled with decreased expression of effector molecules like granzymes and perforin¹⁰. NK cell dysfunction contributes to obesity-related comorbidities and occurs even in the absence of T2D, i.e., in obese individuals with or without diabetes.¹¹ Natural killer (NK) cells within adipose tissue can promote metabolic disease through multiple routes. For example, in mice fed a high-fat diet, NK cells adopt an activated phenotype and produce interferon- γ (IFN- γ), genetic ablation of IFN- γ attenuates the polarisation of adipose macrophages toward a pro-inflammatory (M1) state, thereby reducing inflammation and insulin resistance.¹⁷

Although NF- κ B is a well-established regulator of lymphocyte biology, its role in natural killer cells has been far less studied than in T or B cells. Using patients with monoallelic NFKB1 mutations, Lougaris *et. al.* show that the canonical NF- κ B pathway is in fact critical for NK cell biology¹⁸. Their data demonstrate that NFKB1 haploinsufficiency leads to impaired NK cell maturation, evidenced by reduced expression of NKp46, KIRs, CD57, CD62L, and CXCR1¹⁸. Functionally, NFKB1-mutated NK cells display defective degranulation against target cells, reduced interferon- γ production after IL-12/IL-18 stimulation, failure to up-regulate the activation marker NKp44 under IL-2 stimulation, and diminished proliferative responses¹⁸. Together, these findings indicate that, although under-characterised, the NF- κ B pathway, particularly NFKB1, plays an essential role in human NK cell maturation, cytotoxicity, cytokine production, and IL-2-driven activation¹⁸. NF- κ B signalling in NK cells will be explored in greater detail later in this chapter, but the key take-home message from current evidence is that the canonical pathway is indispensable for NK cell development and effector functions in humans.

1.3 - NF- κ B Signalling in Obesity: Activation and Inflammatory Pathways

NF- κ B is an ancient, evolutionary conserved pathway fundamental to immunity, inflammation, apoptosis and differentiation¹⁹. NF- κ B and I κ B homologues have been observed in *Drosophila melanogaster* and *Carcinoscorpius rotundicauda* (horseshoe crab), the most ancient arthropod which has survived relatively unchanged for ~550 million years¹⁹. As more complex biological organisms evolved over time NF- κ B became intrinsically linked to many processes beyond regulation of inflammation, one of which is metabolism whose link will be described later in this section^{20 21}.

NF- κ B proteins of which there are 5, exist as transcription factors, vital for the biological functions stated previously²². NF- κ B transcription factors can homodimerise or heterodimerise to carry out their function, resulting in 15 possible dimer variations, in resting cells these dimers are sequestered in the cytoplasm by inhibitor I κ B proteins which bind to the dimer, preventing translocation to the nucleus²². The canonical NF- κ B pathway is shown in figure 1.1

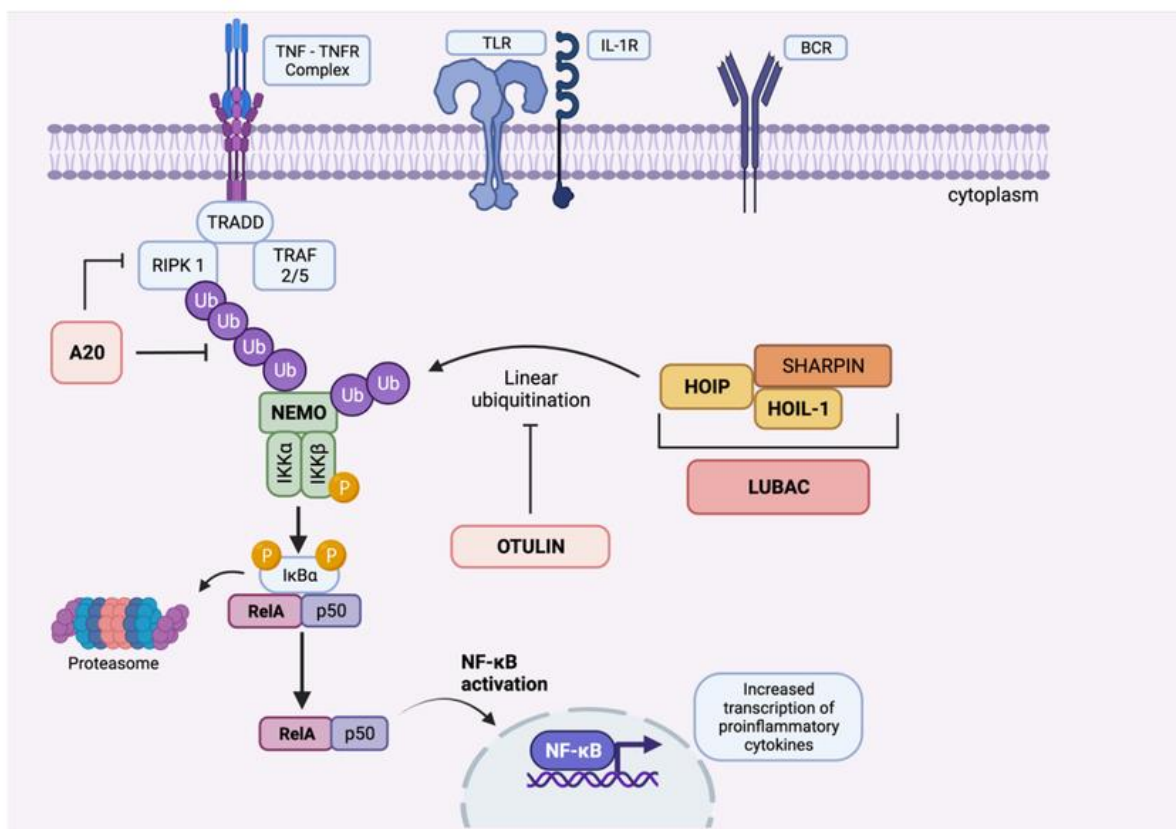


Figure 1.1 - Canonical NF- κ B signalling and regulation by ubiquitination - Schematic representation of the canonical NF- κ B signalling pathway downstream of inflammatory receptors including the TNF–TNFR complex, Toll-like receptors (TLRs), IL-1 receptor (IL-1R), and the B-cell receptor (BCR). Receptor engagement leads to recruitment of adaptor proteins such as TRADD and TRAF2/5 and activation of RIPK1, which becomes modified by K63-linked and linear (M1-linked) ubiquitin chains. These ubiquitin scaffolds promote recruitment of the IKK complex (IKK α , IKK β , and NEMO), resulting in phosphorylation of I κ B α . Phosphorylated I κ B α is subsequently ubiquitinated and degraded by the proteasome, allowing NF- κ B heterodimers (RelA/p50) to translocate to the nucleus and induce transcription of pro-inflammatory genes. Linear ubiquitination is mediated by the linear ubiquitin chain assembly complex (LUBAC), composed of HOIP, HOIL-1, and SHARPIN, and enhances signal propagation through NEMO. This process is counterbalanced by deubiquitinases including OTULIN, which removes linear ubiquitin chains, and TNFAIP3 (A20), which terminates signalling by editing ubiquitin chains on RIPK1 and TRAF proteins. Together, these opposing ubiquitination and deubiquitination events tightly regulate the magnitude and duration of canonical NF- κ B activation. Figure taken from ²³

However, overnutrition and metabolic stress provide continuous inflammatory stimuli which lead to the activation of the NF- κ B pathway which occurs *via* phosphorylation of inhibitor I κ B proteins by I κ B Kinase (IKK) and the subsequent translocation of the dimer to the nucleus where it initiates transcription, typically of pro-inflammatory cytokines ²¹. Metabolic stress typically activates NF- κ B *via* plasma fatty acids, hyperglycaemia or endotoxins that engage pattern recognition receptors like Toll Like Receptor 4 (TLR4) ²⁰.

Thus, obesity can create a consistent feedforward loop of NF- κ B activation. Free fatty acids (FFAs) released by expanded (hypertrophic) adipocytes can directly activate NF- κ B via TLRs²⁴. NF- κ B drives expression of target genes that mediate cell proliferation and release of antimicrobial molecules and cytokines which lead to further activation of the immune response²⁵. For example, in the liver, FFAs activate TLR4 in Kupffer cells resulting in reduced β -oxidation and more lipid droplet formation. In addition to NF- κ B activation via TLRs, the adipose tissue due to its hypertrophic state experiences significant internal stress and stress induced by infiltrating immune cells²⁶. This is a complex interplay, and it ultimately creates a self-sustaining chronic inflammatory environment that drives metabolic dysfunction. As the adipocytes undergo hypertrophy to expand to meet the supplies of energy it's obtaining, so does the dysfunctional state, characterised by hypoxia and necrosis²⁷. The immune landscape undergoes a dramatic shift in obesity where macrophages account for up to 40-60% of immune cells, a drastic change to the 10% observed in lean adipose tissue²⁷. Additionally, a type of structure known as crown-like structure is observed in obesity, these crown-like structures form when macrophages attach to necrotic adipocytes²⁸. These macrophages are highly pro-inflammatory, sometimes described as M1 macrophages, with expression of the pro-inflammatory and NF- κ B activating cytokines, Tumour Necrosis Factor (TNF) and Interleukin-6 (IL6). T cells are also affected both functionally and by numbers of cells present in the adipose tissue. More pro-inflammatory T cells are present in obese adipose tissue and regulatory T (T-reg) cells decline which are critical for homeostasis via expression of anti-inflammatory interleukin-10 and transforming growth factor beta (TGF β)²⁹. Th1 cells, characterised by production of interferon- γ are increased in adipose tissue and their subsequent interferon- γ production further activates macrophages. Similarly, Th17 cells are increased leading to more interleukin-17 (IL-17) production²⁹. Dendritic cells further activate T cells via interleukin-12 (IL-12) and interleukin-18 (IL-18) expression which primes T cells whilst B cells produce autoantibodies and IL6²⁹. These changes create a pro-inflammatory environment and are recognised as the hallmark biomarkers of obesity-associated inflammation, commonly referred to as chronic low-grade inflammation or 'metainflammation'. NF- κ B is a central transcriptional driver of the M1 macrophage program, and indeed many M1 markers such as TNF, IL-1 β and IL-6 are NF- κ B targets.³⁰ The recruitment of monocytes and their polarisation into M1 macrophages works in a reinforcement loop: NF- κ B-dependent cytokines from M1 macrophages further amplify local NF- κ B activation and can recruit even more immune cells including neutrophils and T lymphocytes³¹. Upon arrival these T cells in obese adipose skew toward pro-inflammatory

subsets previously mentioned such as Th1 and Th17 which in turn produce cytokines that activate NF- κ B in macrophages and adipocytes, sustaining a chronic inflammatory loop ³².

NF- κ B activity in metabolic tissues like adipose and liver doesn't just have a local effect, these changes affect the body systemically and exacerbate metabolic problems such as type 2 diabetes (T2D) and insulin resistance (IR).²¹ By 2050, T2D and IR are expected to be the leading cause of death worldwide,²¹ highlighting the need to have a better understanding of the implications of NF- κ B activation. T2D begins with IR and eventually reduced insulin production from the pancreatic β -cells responsible for insulin production. Interestingly, the involvement of NF- κ B in T2D was first implicated in 1901 when salicylates such as aspirin, widely used for rheumatic conditions like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), were found to improve glucose tolerance in patients ³³. Mechanistically, it was shown that salicylates inhibit NF- κ B activity, likely through direct inhibition of IKK β , meaning I κ B is not phosphorylated and NF- κ B cannot translocate to the nucleus and carry out its transcriptional activities.^{34 35} The effects of salicylates were so profound that IR could be reversed in murine models and that mice heterozygous (+/-) for IKK β were protected from diet-induced obesity (DIO).³⁴ The mechanism by which products of NF- κ B lead to IR and T2D are well characterised. TNF impairs insulin signalling by promoting the serine phosphorylation of insulin receptor substrate (IRS) proteins which reduces downstream activity of PI3K-AKT, which ultimately leads to translocation of GLUT4 (glucose transporter) to the plasma membrane, allowing glucose to enter a cell.³⁶

1.4 - A20 (*TNFAIP3*): A multi-functional protein

A central player in mediating NF- κ B activity and central to this thesis is the gene tumour necrosis factor alpha-induced protein 3 (*TNFAIP3*), which encodes the protein, A20. *TNFAIP3* was discovered in 1990 by Opinari *et.al* and was described as 'rapidly' induced (15 minutes) and transiently induced following TNF treatment ³⁷. It was found to be a 790 amino acid protein ³⁷. Their conclusion in this paper remarked that A20 may be a TNF-inducible transcription factor ³⁷. However, thirty-five years later the understanding of A20 is much clearer and it was described as a 'jack of all trades' ³⁸. A20 has been studied primarily for its anti-inflammatory role it plays through negative regulation of NF- κ B and inflammation. As described previously NF- κ B is known for its pro-inflammatory functions which can be initiated through a vast array of stimuli, when these stimuli activate NF- κ B, A20 is rapidly induced and acts as a negative regulator of NF- κ B signalling ³⁹. The significance of A20 is highlighted through a multitude of single nucleotide polymorphisms

(SNPs) identified in severe inflammatory diseases such as rheumatoid arthritis (RA), SLE, psoriasis ⁴⁰. In 2016, heterozygous germline loss-of-function mutations in *TNFAIP3* were first identified as the cause of a novel autosomal-dominant autoinflammatory disorder, now termed A20 haploinsufficiency (HA20). These mutations result in a single functional copy of *TNFAIP3*, leading to diminished A20 expression and impaired negative regulation of the NF- κ B pathway ⁴⁰. Further evidence through animal models shows that whole body knockout (KO) of *TNFAIP3* results in severe inflammation, cachexia and ultimately premature death ⁴¹. Unsurprisingly, *TNFAIP3* KO mice were hypersensitive to NF- κ B inducing stimuli such as LPS and TNF and were more susceptible to TNF mediated programmed cell death (PCD) highlighting another role for A20 in protection against apoptosis ⁴¹.

The genetic structure of human *TNFAIP3* and its surrounding region as mentioned has several disease associations ⁴². *TNFAIP3* locus is at 6q23 and is composed of nine exons. The 790 amino acids include an N-terminal cysteine protease ovarian tumour protease (OTU) domain and seven C-terminal zinc finger motifs. Together these domains perform a 2-step process to dampen NF- κ B signalling, these two steps revolve around deubiquitination and ubiquitination. Ubiquitination is a post translational modification which is central to the precise regulation of NF- κ B signalling ⁴³, operating alongside phosphorylation to control signal amplitude and duration, the 76-aa ubiquitin is covalently attached to the lysine residues of target proteins. This post-translational modification begins with the stepwise activity of E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases, which serve to attach ubiquitin. Ubiquitin itself can form chains *via* its seven internal lysines (K6, K11, K27, K29, K33, K48, K63) or its N-terminal methionine (M1), creating polyubiquitin structures that encode functional signals beyond just proteasomal targeting ³⁹. Signalling pathways involving A20 and the mechanisms by which it controls these pathways are shown in Figure 1.2

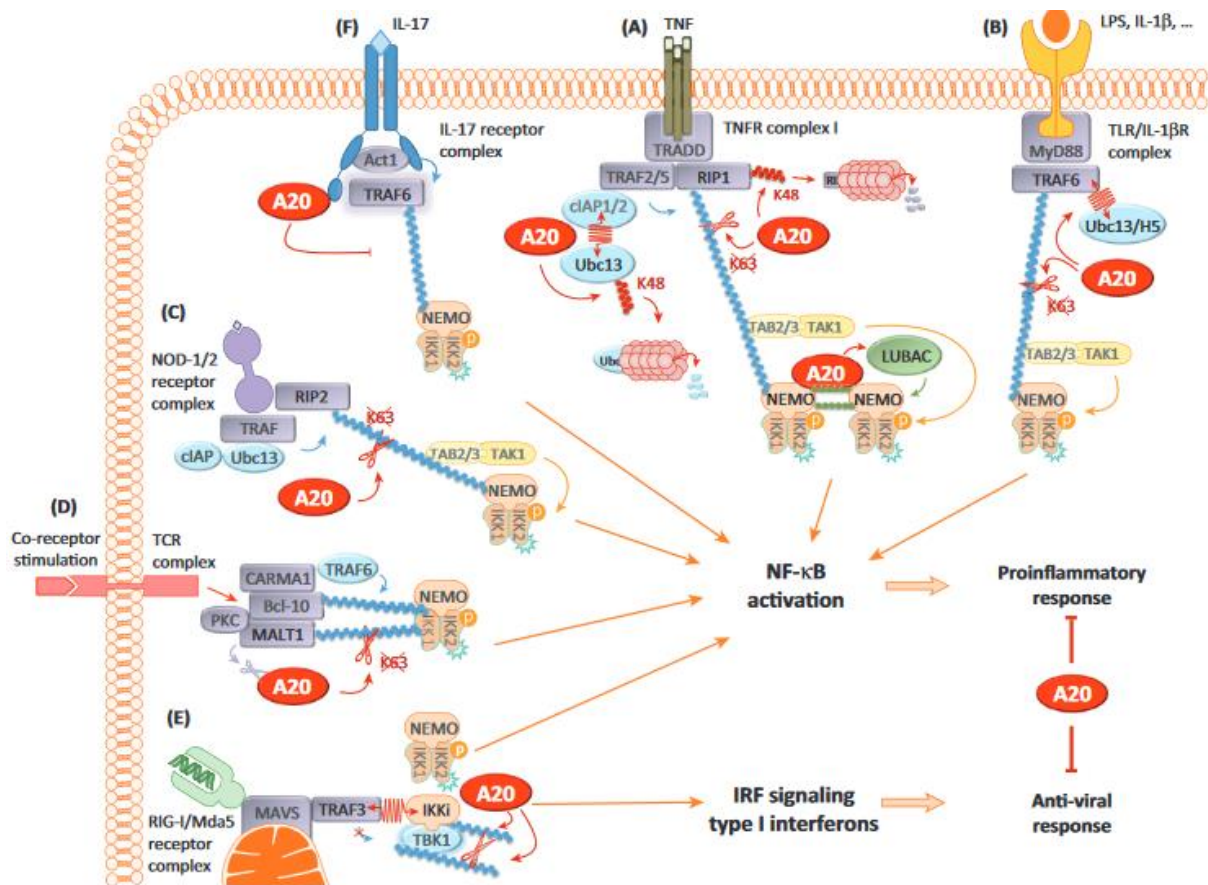


Figure 1.2 - Mechanisms by which A20 (*TNFAIP3*) restrains inflammatory signalling. This schematic illustrates major signalling pathways regulated by A20 across several receptor contexts: (A) TNF receptor – A20 removes K63-linked ubiquitin chains and adds K48-linked ubiquitin to RIP1/NEMO, destabilizing signalling complexes; (B) TLR/IL-1 receptor – A20 opposes TRAF6-mediated ubiquitination to attenuate downstream NF-κB activation; (C) NOD1/2 receptor – A20 disrupts RIP2 ubiquitination within the NOD receptor complex; (D) TCR/BCR (co-receptor) signalling – A20 inhibits MALT1/TRAF6 ubiquitination within the CBM complex; (E) RIG-I/MAVS antiviral pathway – A20 regulates TBK1/IKKε activity to limit type I interferon induction; (F) IL-17 receptor – A20 binds IL-17R complex components and limits TRAF6 signalling. Downstream of all these pathways, A20 acts as a brake on NF-κB activation and helps restore homeostasis. Figure taken from ⁴⁴

In phase 1 of shutting off NF-κB, the N-terminal of A20 acts as the deubiquitinase ⁴⁵, this involves the removal of K63-linked ubiquitin chains from key signalling proteins like receptor interacting protein kinase 1 (RIPK1) and TRAF6, this disrupts NF-κB signalling downstream of stimuli like TNF ^{42 45}.

In phase 2, the ZnF4 domain of A20 acts as an E3 ubiquitin ligase, attaching K48-linked chains to proteins like RIPK1 which labels them for degradation by the proteasome ⁴⁵. The ZnF7 domain binds linear M1-linked ubiquitin chains which helps stabilise inhibitory complexes around key signalling proteins like NF-κB essential modulator (NEMO) ⁴⁶. Upon stimulation with TNF, A20 is rapidly recruited to NEMO and linear ubiquitin chain assembly

complex (LUBAC), A20 inhibits LUBAC-induced NF- κ B *via* ZnF7⁴⁶. It was shown that ZnF7 polypeptide on its own could inhibit NF- κ B activation⁴⁶. This is how A20 has been conventionally recognised, however, some recent evidence suggests that the deubiquitinase activity is limited and that A20's anti-inflammatory properties primarily arise from its interaction with ubiquitin chains on receptor complex on TNF receptor 1 (TNFR1) and TLRs *via* ZnF4 and ZnF7⁴⁷.

Since NF- κ B activation occurs across a multitude of cell types and *via* various mechanisms, it is necessary to discuss how A20 plays a role across these various signalling mechanisms. MALT1 is required for T cell antigen receptor-induced signalling to NF- κ B and is essential for T cell activation and proliferation⁴⁸. T cell antigen receptor stimulation induced the recruitment of A20 into a complex with MALT1 and the subsequent cleavage of A20 impairing its NF- κ B inhibitory function⁴⁸. Interestingly, MALT1 is also expressed in natural killer (NK) cells, myeloid cells and mast cells and some non-immune cells⁴⁹, meaning a similar process may occur in a multitude of cells. In NK cells, key innate immune cells that fight viruses and tumours⁵⁰, MALT1 is required for NF- κ B activation *via* immunoreceptor tyrosine-based activation motif (ITAM) – containing signalling chains. Many of the proinflammatory cytokine genes that are induced by NK-cell activation, including TNF and interferon- γ are regulated by NF- κ B and thus cleavage of A20 for activation of NF- κ B is likely important.⁵⁰

Another mechanism by which A20 operates is downstream of IL17 signalling, A20 is induced by IL-17 in an NF- κ B dependent manner and acts as a feedback inhibitor to suppress IL-17-mediated inflammatory gene expression. It does so by binding and deubiquitinating TRAF6, thereby inhibiting downstream NF- κ B and MAPK (particularly JNK) signalling.⁵¹

TNF activation of NF- κ B is the most studied mechanism for NF- κ B activation. The shutting down of TNFR-1 mediated activation of NF- κ B involves several steps to dampen signalling. During signalling, RIPK1 is modified with lysine-63-linked ubiquitin chains, which are removed by A20. A20 then promotes the addition of lysine-48-linked ubiquitin chains to RIPK1, targeting it for proteasomal degradation⁵². However, A20 doesn't act alone on its substrate, RIPK1 (or TRAF6 in LPS signalling), there is evidence that A20 forms a complex 15-30 minutes post TNFR1 stimulation with Tax1-binding protein (TAX1BP1), RING finger protein 11 (RNF11) and Itch^{53 54 55}. In murine embryonal fibroblasts (MEFs) deficient for TAX1BP1 or ITCH and in RNF11, A20 cannot interact with RIPK1 when TNFR1 is stimulated with TNF^{53 54 55}. A20 can also interact with ABIN proteins, who are also involved

in the inhibition of NF- κ B⁵⁶. Three distinct ABIN proteins exist (ABIN1, ABIN2 and ABIN3) all bind to A20⁵⁶. Overexpression of ABINs inhibit NF- κ B activation via TNF and other stimuli⁵⁶.

1.5 - Regulation of *TNFAIP3* Expression

Interestingly, the expression of A20 is itself controlled by NF- κ B where NF- κ B dampens its own activity via upregulation of anti-inflammatory mediators like A20 and I κ B α ⁵⁷. The initial characterisation of A20's transcriptional programme came in 1992, when it was shown that TNF triggered the binding of NF- κ B in two distinct sites within *TNFAIP3*'s promoter region⁵⁸. Several more binding sites have been characterised since then within the promoter region. In macrophages it was demonstrated that the transcription factor CCAAT/enhancer-binding protein beta (C/EBP β) participated in A20 transcriptional regulation in response to LPS stimulation, C/EBP β itself was upregulated by MAPK p38⁵⁹. Orphan nuclear receptor estrogen-related receptor- α (ERR α) was shown to upregulate *TNFAIP3* expression and thus negatively regulate TLR signalling⁶⁰. Downstream regulatory elements antagonist modulator (DREAM) has been shown to play a key role as a transcriptional repressor of *TNFAIP3*⁶¹. DREAM deficient mice showed persistent *TNFAIP3* expression and unchanged A20 response in response to endotoxin⁶¹. Conversely, when the transcription factor upstream stimulatory factor-1 (USF1) binds to DRE-associated E-box in the *TNFAIP3* gene, *TNFAIP3* becomes activated in response to inflammation⁶¹. This reveals opposing pro-inflammatory and anti-inflammatory roles for DREAM and USF1 respectively. Importantly, in this work Dream^{-/-} mice produced much less of NF- κ B transcribed IL6, MCP-1 and TNF α ⁶¹, hallmark signatures of obesity-induced inflammation and perhaps linking DREAMs ability to repress A20 with the chronic low-grade inflammation observed in obesity.

Interestingly, one of the downstream targets of NF- κ B is a micro RNA (miR) miR-125b, which binds to *TNFAIP3* mRNA transcripts preventing it from being transcribed, thereby prolonging NF- κ B activity⁶². ROQUIN, encoded by the gene RC3H1, is part of the DNA damage response and binds preferentially short-lived and DNA damaged-induced mRNAs highlighting its role in the DNA damage response⁶³. Interestingly, it was found that ROQUIN binds *TNFAIP3* mRNA, reducing the stability and half-life of transcripts⁶³. This is seen to have a fine-tuning effect rather than full activation of NF- κ B⁶³.

At the protein level, cell-extrinsic factors control A20 protein stability, for example, high glucose levels can target A20 for proteasomal degradation⁶⁴. Shrikande *et. al* showed that

high glucose levels did not impact *TNFAIP3* transcription or mRNA stability in human coronary endothelial and smooth muscle cell cultures ⁶⁴. However, they did show that diabetic and non-diabetic mice challenged with LPS showed significantly different levels of A20 protein. Non-diabetic mice had 2 to 3 fold higher A20 protein levels compared to the their diabetic counterparts, despite the diabetic mice having higher mRNA levels of *TNFAIP3* ⁶⁴. It is thought that reactive oxygen species may degrade A20 as is shown in the case of multiple ubiquitinating enzymes ⁶⁵.

1.6 - Glucocorticoids

Glucocorticoids (GCs) are potent anti-inflammatory steroid hormones produced by the adrenal cortex that are essential for the homeostatic control of various biological functions ⁶⁶. Though glucocorticoids are produced by the adrenal cortex, they are primarily under the control of the hypothalamic-pituitary adrenal (HPA) axis ⁶⁶. Their function is regulated at a tissue-level by 11 β -hydroxysteroid dehydrogenases (11 β -HSDs) and they signal through the glucocorticoid receptor (GR) encoded by NR3C1 ⁶⁶. 11 β -HSD1 is present in most cells and is responsible for the conversion of cortisone into its active form, cortisol, which can bind the GR. 11 β -HSD2 carries out the opposing function, converting active cortisol to inactive cortisone, thus preventing GR activity ⁶⁶. GCs have been widely studied across a variety of diseases and cell types, and synthetic versions of GCs, such as dexamethasone have been used as treatments for rheumatoid arthritis, inflammatory bowel disease and asthma.⁶⁷ So profound was the introduction of glucocorticoids for treatment of patients with rheumatoid arthritis that it led to the award of the Nobel Prize for Physiology or Medicine in 1950 to Edward Kendall, Philip Hench, and Tadeus Reichstein.⁶⁸ Thus, GCs have been recognised for a long time as potent inhibitors of inflammatory disease. At a pathway level GCs can inhibit signalling in key inflammatory pathways like NF- κ B and AP-1.⁶⁷ The GC receptor complex operates via two main mechanisms, transactivation and transrepression.⁶⁷ In transactivation, the GC receptor complex acts through binding of the GR to glucocorticoid response elements (GREs), leading to increases in gene transcription, this typically occurs with GR dimers. In transrepression, monomeric GRs don't directly bind to DNA, but 'tether' to specific factors, interfering with downstream pro-inflammatory signalling pathways.⁶⁷ Transrepression is believed to be the mechanism by which the anti-inflammatory mechanism of GR works, blocking transcription at pro-inflammatory sites.⁶⁹ Glucocorticoids can affect the expression levels of 20% of all genes in immune cells.⁷⁰ Though, this expression varies significantly between stage of differentiation and cell type.^{70,71} NF- κ B can cooperate with the GR to carry out transcription of anti-inflammatory genes such as *TNFAIP3*. NF- κ B and

GR were found co-occupying an intronic *TNFAIP3* locus, the same study showed they also upregulated the A20 binding partner *TNIP1* and the potent sequestering protein NFKBIA, this was shown in airway epithelial cells.⁷² In another study carried out on human bronchial epithelial cells, the synthetic GC, Budesonide, was shown to cause a sharp increase in *TNFAIP3* mRNA within 1-2 hours, classifying it as an early GR target gene in these cells.⁷³ Thus, across a number of non-immune cell types it has been shown that GCs can upregulate *TNFAIP3* mRNA levels.

Given the context of this thesis and the setting of obesity and weight loss, it is important to consider their effect on glucocorticoid signalling. Importantly, a variety of growth factors, neuropeptides, cytokines and adipokines can affect adrenal secretions. Adrenocortical cells express receptors for each of these factors.⁷⁴ Insulin has been shown to inhibit cortisol secretion from bovine adrenocortical cells⁷⁵ and glucagon inhibits cortisol from human adrenocortical cells.⁷⁶ Glucagon is the peptide hormone produced in the pancreas as a key regulator of glucose homeostasis.⁷⁶ The gut derived hormone glucagon-like peptide 1 (GLP-1) was also shown to inhibit glucocorticoid release from rat adrenal cortex in response to adrenocorticotrophic hormone (ACTH) by impairing the later stages of GC synthesis.⁷⁶ Interestingly, mice overexpressing 11 β -HSD1 in adipose tissue develop visceral obesity, insulin resistance, dyslipidaemia, and hypertension.⁷⁷ 11 β -HSD1 inhibition has been to increase glucose tolerance and reduce food intake and weight gain hyperglycaemic mouse models.^{78,79} 11 β -HSD1 knockout mice are resistant to hyperglycaemia when on an obesogenic diet and crucially show significantly reduced expression of the hepatic enzyme phosphoenolpyruvate carboxykinase (PCK1). PCK1 is a rate-limiting enzyme in gluconeogenesis, catalysing one of the irreversible steps required to generate glucose. Its downregulation in 11 β -HSD1-deficient mice highlights the role of glucocorticoids in sustaining hepatic glucose output despite hyperglycaemia, thereby linking GC signalling directly to gluconeogenic flux.⁸⁰ Glucocorticoids are essential regulators of lipid homeostasis, but chronic excess drives ectopic lipid deposition in tissues like skeletal muscle and liver, which exacerbates insulin resistance and contributes to the metabolic syndrome.⁸¹ Ultimately, the effects of GCs are highly cell and context dependent and no one conclusion can be extrapolated from one setting to another.

1.7 - GR Mechanism of action

Glucocorticoids are synthesised in the zona fasciculata of the adrenal cortex and are lipophilic, meaning they can freely diffuse into cells across the cell membrane.⁸² In the cytoplasm, glucocorticoids bind the GR, a transcription factor encoded by NR3C1. The GR

is bound by a complex in the cytoplasm consisting of mainly heat shock proteins (HSPs), immunophilins (FKBP5 and FKBP4) that prevent degradation of the GR and can enhance ligand binding capacity.⁸³ When GCs bind to the GR, the complex structure changes allowed for the GR to break free of the complex and translocate to the nucleus where it will carry out its mechanisms via three potential mechanisms.

The primary mechanism of GR and best described is transactivation, which occurs when a genes transcription increases in response to GR homodimers binding to GC response elements (GREs). GR dimer affinity for GREs is modulated by the exact GRE DNA sequence itself, which can influence recruitment of coactivators and the outcome of chromatin remodelling. In practice, many genomic GR binding sites deviate from the canonical GRE motif, so their variable sequence context likely underlies differential GR binding strength and consequent regulation of target genes.⁸⁴ ChIP-exo (a high-resolution chromatin immunoprecipitation method using exonuclease trimming to pinpoint binding sites at near single-nucleotide precision) confirms that GR dimers can engage highly degenerate GRE sequences beyond the canonical motif.⁸⁵

A second and alternative mechanism of action occurs through the binding of monomeric GR to a sequence which contains half a GRE. This occurs in conjunction with another transcription factor with which binds the other half of the DNA regulatory element, this can be thought of as a hybrid transcriptional mechanism where GR and another transcription factor, such as NF- κ B both bind simultaneously in close proximity to a DNA regulatory element⁸². This can result in gene activation or repression.⁸⁵

The previous two mechanisms involved the action of GR *via* dimers and sometimes in conjunction with another transcription factor without direct interactions. The final mechanism occurs *via* physical interactions between the GR and another transcription factor, e.g. NF- κ B. This is believed to be the primary mechanism of transrepression, although this is debated.⁸⁴

An important concept to understand how the GR works is whether it operates via its monomeric or dimeric form.⁸⁶ The classical Monomer–Dimer Concept proposed that glucocorticoid (GC) effects arise from dimer-mediated transactivation (TA) of metabolic genes such as G6P, PEPCK, and SCD1–3, whereas the anti-inflammatory effects are mediated by monomer-dependent transrepression of AP-1 and NF- κ B.⁸⁷ Evidence supporting this model came from mice carrying a point mutation (A458T) that disrupts dimerisation, these mice showed impaired GRE-driven transcription but preserved

repression of AP-1 and NF- κ B activity. However, subsequent work revealed that this is overly simplistic, some side effects like hyperglycaemia and visceral obesity, are indeed TA-dependent and rely on dimerisation, but others, such as osteoporosis, persist even in mice incapable of forming GR homodimers.⁸⁸ Additionally, GR dimerisation has proven indispensable for protection in acute inflammatory settings, with mice incapable of GR dimerisation being highly susceptible to septic shock and TNF-induced lethality,⁸⁴ whereas wild-type mice are protected by exogenous GCs. In contrast, chronic inflammation models show less dependence on dimerisation. Thus, GR functions cannot be cleanly split into monomer = therapeutic and dimer = side effects, instead, the balance between transactivation and transrepression, DNA-binding site architecture, and disease context together determine GC transcriptional outcomes.⁸⁴ Mechanism of GR action and co-operation are shown in Figure 1.3.

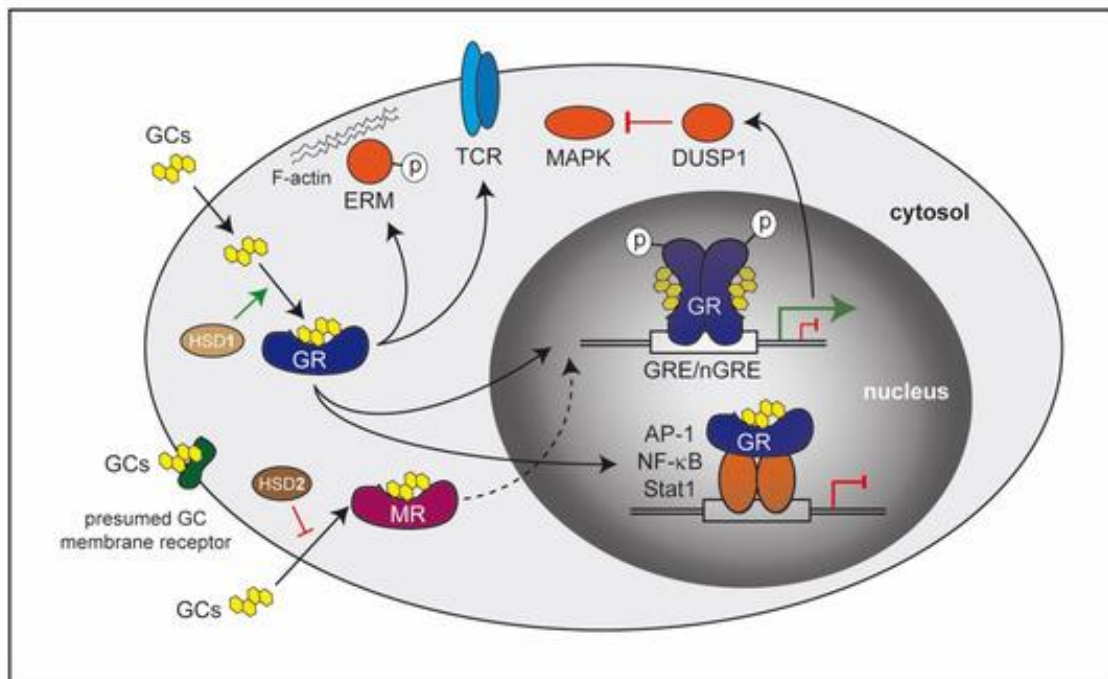


Figure 1.3 - Schematic overview of glucocorticoid (GC) / glucocorticoid receptor (GR) / mineralocorticoid receptor (MR) actions in controlling inflammation - Glucocorticoids (natural or synthetic) cross the cell membrane and bind cytosolic GR, which can be locally activated by 11 β -HSD1 (HSD1) or inactivated by 11 β -HSD2 (HSD2) in some cell types. Upon ligand binding, GR undergoes conformational changes, dissociates from chaperones, and translocates to the nucleus, where it binds GREs or negative GREs to regulate transcription. GR also modulates inflammation by tethering to transcription factors such as AP-1, NF- κ B, or STAT1 and can repress or activate target genes. Figure taken from ⁸⁹

1.8 - Cooperation of NF- κ B and GR

Glucocorticoid resistance (GCR) is defined as an unresponsiveness to the therapeutic effects, including the anti-inflammatory effects of glucocorticoids and GR.⁸⁶ This can be inherited or can arise often through the management of inflammatory diseases *via* the use of glucocorticoids where chronic usage eventually leads to resistance. The glucocorticoid receptor (GR) and NF- κ B represent two of the most influential transcriptional regulators at the interface of endocrine and immune control. Their crosstalk has been studied for decades because of the apparent paradox between their canonical roles: GR, a steroid hormone receptor, exerts anti-inflammatory and immunosuppressive effects, whereas NF- κ B functions as a master activator of pro-inflammatory and stress-responsive genes.⁹⁰ Far from being limited to mutual antagonism, evidence now points to a nuanced interplay where these two transcription factors can antagonise, cooperate, or even synergistically regulate overlapping sets of genes depending on context, stimulus, and chromatin architecture.^{91 92} Understanding the molecular mechanisms of this crosstalk is central to deciphering how endocrine-immune balance is maintained in health and disrupted in disease. Initial models emphasised the antagonistic relationship between GR and NF- κ B. Glucocorticoids, through GR, suppress transcription of NF- κ B-dependent inflammatory genes such as IL6 and IL8, while NF- κ B activation can in turn reduce GR-mediated transcription at glucocorticoid response elements (GREs).⁹³ Mechanistically, this antagonism was attributed to “tethering” interactions: GR physically associates with DNA-bound p65 or p50 and inhibits NF- κ B transactivation without directly binding κ B DNA motifs.⁹⁴ Conversely, p65 can inhibit GR activity at GREs, suggesting a reciprocal inhibitory dynamic.⁹² Additional layers of inhibition include competition for limiting cofactors such as CBP/p300 and modulation of chromatin accessibility at inflammatory promoters. Collectively, these mechanisms provided a molecular rationale for the potent anti-inflammatory efficacy of synthetic glucocorticoids.

Although antagonism dominates much of the literature, genome-wide studies have revealed that GR and NF- κ B can also cooperate. Using ChIP-Seq and Pol II occupancy mapping, Rao *et al.* (2011) demonstrated that coactivation of GR and NF- κ B by glucocorticoid and TNF treatment does not simply sum their independent transcriptional programs.⁹¹ Instead, coactivation generates a distinct transcriptional repertoire in which ~43% of regulated genes are unique to the dual-stimulus condition.⁹¹ At the chromatin level, this reflects the emergence of novel co-occupied sites enriched for both GRE and κ B motifs, suggesting that cooperative binding reconfigures local regulatory landscapes. Importantly, coactivation not only repressed subsets of NF- κ B-driven pro-inflammatory genes but also synergistically

induced other genes, including *TNFAIP3*.⁹¹ These data underscore that GR–NF-κB crosstalk is not exclusively inhibitory but can promote unique gene expression patterns essential for fine-tuned stress and immune adaptation. At the molecular level, GR–NF-κB interactions occur both prior to and after nuclear translocation. In the cytoplasm, GR influences the binding of IκB to NF-κB complexes, stabilising NF-κB in its inactive state and reducing nuclear translocation.⁹²

Chaperones such as Hsp90 and immunophilins (FKBP5 and FKBP4), which regulate GR transport into the nucleus, also interact with NF-κB pathway components, providing additional nodes for cross-regulation. Inside the nucleus, GR exerts both transrepression, and transactivation (inducing NF-κB inhibitors such as IκBα), creating a dual regulatory system

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The integrity of GR–NF-κB cooperation is vital for maintaining homeostasis. When this balance is disrupted, the consequences manifest across multiple disease domains. In chronic stress, reduced GR sensitivity or altered receptor isoform expression diminishes glucocorticoid action, leading to unchecked NF-κB-driven inflammation. Bekhbat et al. (2017) highlight how this endocrine-immune imbalance contributes to neuropsychiatric disorders such as depression and post-traumatic stress disorder (PTSD), where GR resistance permits sustained NF-κB activation in brain and immune circuits⁹⁵. Similarly, in inflammatory and autoimmune diseases, impaired GR signalling fails to resolve NF-κB-mediated cytokine production, prolonging inflammatory cascades⁹⁶. Conversely, excessive glucocorticoid signalling may over-suppress NF-κB activity, impairing host defences and tissue repair. These findings emphasise that the pathology does not arise simply from NF-κB “overactivity” or GR “underactivity,” but from the dysregulation of their cooperative balance.

Glucocorticoid-mediated resolution of inflammation typically entails suppression of NF-κB activity, yet the mechanisms preserving anti-inflammatory homeostasis under such suppression have been less well understood. In their landmark study, Altonsy et al. (2014) unveil a sophisticated mechanism whereby glucocorticoid receptor (GR) and NF-κB cooperate, rather than antagonise, to sustain expression of *TNFAIP3*. Using airway epithelial cells treated with dexamethasone and TNF, the authors found that although GR robustly represses classic pro-inflammatory targets (e.g., IL1β, IL8), expression of anti-inflammatory genes such as *TNFAIP3* and *NFKB1A* is selectively spared or even augmented under co-

stimulation, despite similar occupancy of GR and NF- κ B across both repressed and induced loci.⁷²

Of crucial importance to this thesis, Altonsy *et al.* identified an intronic enhancer within the *TNFAIP3* gene that is co-occupied by GR and NF- κ B and mediates synergistic transcriptional activation, as evidenced by reporter assays showing that dexamethasone with TNF together drive expression beyond the sum of their individual effects. The functional cooperation depends on the presence of a working GR binding site (GBS) in that enhancer.⁷² These findings support a model whereby glucocorticoids preserve essential anti-inflammatory feedback circuits, such as *TNFAIP3* expression, through context-dependent GR–NF- κ B cooperation, thereby enabling both effective inflammation suppression and controlled resolution.

1.9 - Cortisol levels in obesity and weight loss

In obesity, circulating cortisol concentrations often remain within the normal range, potentially due to higher metabolic clearance⁶⁶, yet local tissue-specific alterations in glucocorticoid action emerge as critical determinants of pathology. Increased 11 β -HSD1 activity in liver and adipose tissue enhances local cortisol levels, promoting gluconeogenesis, adipogenesis, and insulin resistance but not obesity.⁹⁷ Genetic studies underscore this link, as 11 β -HSD1 knockout mice are resistant to diet-induced hyperglycaemia and display reduced expression of gluconeogenic enzymes.⁸⁰ Furthermore, glucocorticoid receptor (GR) expression itself is dysregulated in obesity, with increased GR in liver and skeletal muscle contributing to insulin resistance and features of the metabolic syndrome.⁹⁸ Antagonism or selective inactivation of hepatic GR improves glucose tolerance and reduces hyperglycaemia in diabetic rodent models, highlighting GR as a potential therapeutic target.⁹⁹ Weight loss, whether induced by caloric restriction, exercise, or bariatric surgery, exerts complex effects on glucocorticoid metabolism. Some studies report reduced adipose tissue 11 β -HSD1 expression following dietary weight loss, correlating with reductions in BMI, while others find no change.⁶⁶ Similarly, bariatric surgery consistently reduces glucocorticoid exposure, evidenced by decreased 11 β -HSD1 activity and shifts toward cortisone accumulation in adipose depots, though whether these effects reflect weight loss *per se* or gastrointestinal manipulation remains unresolved. Exercise introduces additional complexity, while acute training transiently activates the hypothalamic-pituitary-adrenal axis and raises circulating glucocorticoids acting as a stressor, consistent chronic voluntary exercise reduces tissue-specific GR and 11 β -HSD1 expression in muscle and liver, improving insulin sensitivity and glucose tolerance.¹⁰⁰

1.10 - Obesity, Inflammation, and the Therapeutic Effects of Weight Loss

Obesity is recognised not only as a state of energy imbalance but as a condition characterised by chronic, low-grade systemic inflammation¹⁰¹. Adipose tissue in obesity undergoes profound remodelling: hypertrophic adipocytes release free fatty acids (FFAs) and stress signals, while increased immune cell infiltration transforms adipose tissue into a metabolically active and pro-inflammatory organ²⁹. This chronic activation of innate immune pathways drives the secretion of cytokines including TNF- α , IL-6, and MCP-1, alongside acute-phase reactants such as C-reactive protein (CRP)²⁸. These inflammatory mediators contribute to systemic insulin resistance, endothelial dysfunction, and heightened cardiovascular risk, thereby linking excess adiposity to metabolic syndrome, type 2 diabetes, and atherosclerosis²⁶. At the molecular level, adipose expansion in obesity activates pattern recognition receptors such as Toll-like receptors (TLRs) on adipocytes and macrophages, with saturated FFAs serving as key ligands. TLR activation triggers NF- κ B and JNK signalling, leading to transcription of pro-inflammatory cytokines. In parallel, adipocyte stress induces hypoxia and cell death, further amplifying immune infiltration and cytokine release. Importantly, systemic biomarkers of inflammation mirror these tissue-level events: plasma CRP, IL-6, and TNF- α are consistently elevated in obese individuals compared with lean controls¹⁰². This low-grade but chronic inflammatory burden plays a causal role in metabolic complications, making inflammation both a hallmark and a mechanistic driver of obesity-associated disease¹⁰¹.

1.11 - Weight Loss as an Anti-Inflammatory Strategy

Given the role of inflammation to obesity-related pathology, weight loss represents a primary therapeutic avenue to dampen inflammatory signalling and restore immune homeostasis in obesity. Evidence consistently shows that reductions in adiposity, whether achieved through hypocaloric diets, exercise, or bariatric surgery, lower systemic markers of inflammation in proportion to the amount of weight lost¹⁰³. These anti-inflammatory benefits are now considered one of the major mechanisms by which weight loss reduces cardiovascular risk and improves insulin sensitivity¹⁰⁴. Diet-induced weight loss provides the clearest evidence for this relationship. Tchernof *et al.* (2002) demonstrated that caloric restriction leading to fat loss produced marked reductions in circulating CRP in obese women, directly linking weight loss to improvements in systemic inflammation¹⁰⁵. This finding was supported by Forsythe *et al.* who reviewed numerous intervention studies and concluded that dietary weight loss consistently reduces inflammatory cytokines and improves insulin sensitivity, independent of macronutrient composition¹⁰³. These results highlight that the reduction in

fat mass itself, rather than the specific diet employed, is the critical determinant of inflammatory improvements. These observations were reinforced in a large systematic review of 76 clinical studies comprising 6,742 patients, concluding that weight loss reliably reduces circulating CRP, IL-6, and TNF- α across diverse populations ¹⁰⁶. Importantly, even modest weight loss of 5–10% of body weight was sufficient to elicit significant improvements in inflammatory markers ¹⁰⁶. This degree of weight loss is achievable and sustainable in many clinical and lifestyle settings, underscoring the translational relevance of these findings. Together, these studies demonstrate that dietary weight loss not only improves metabolic endpoints such as glucose tolerance and lipid profiles but also exerts systemic anti-inflammatory effects that likely contribute to reduced long-term morbidity and mortality.

Beyond dietary interventions, bariatric surgery provides an alternative pathway to sustained weight loss and inflammatory resolution, particularly in severe obesity where diet and lifestyle modification may not suffice. Surgical interventions such as Roux-en-Y gastric bypass and sleeve gastrectomy result in profound and durable reductions in systemic inflammation ¹⁰⁷. These reductions extend beyond those observed with diet alone, with consistent decreases in CRP, IL-6, and TNF- α , and correlate with improvements in cardiovascular risk factors and type 2 diabetes remission ¹⁰⁷. Mechanistically, bariatric surgery exerts unique anti-inflammatory effects through modulation of gut-derived signals, including changes in bile acid metabolism, GLP-1 secretion, and the gut microbiota, which together reshape systemic immune responses ¹⁰⁷. Thus, while weight loss *per se* is central, surgical interventions may amplify the anti-inflammatory response through endocrine and metabolic reprogramming that is not fully replicated by diet alone.

Exercise-based interventions also contribute to reductions in systemic inflammation, though the mechanisms differ from those of weight loss. Acute exercise transiently increases pro-inflammatory cytokines, but prolonged periods of exercise training reduces basal inflammatory tone by lowering visceral adiposity, improving insulin sensitivity, and modulating myokine secretion ¹⁰⁸. When combined with dietary restriction, exercise produces additive effects, further reducing CRP and cytokine levels beyond those achieved by weight loss alone ¹⁰⁶.

The systemic effects of weight loss on inflammation are clinically meaningful. CRP is a well-validated biomarker of cardiovascular risk, and reductions in CRP following weight loss have been consistently documented across interventions. Tchernof *et al.* observed

reductions in CRP that paralleled decreases in adiposity, emphasising the causal link between fat mass and systemic inflammation.¹⁰⁵ These reductions occur regardless of dietary composition, underscoring the central role of energy balance and adiposity rather than macronutrient distribution ¹⁰³. In parallel, reductions in IL-6 and TNF- α lower the inflammatory burden on endothelium and liver, improving vascular function and reducing hepatic insulin resistance. These systemic anti-inflammatory effects are now recognised as key mediators of the broad health benefits of weight loss. The therapeutic implications extend to chronic disease prevention and management. Obesity-related inflammation is a major contributor to type 2 diabetes, cardiovascular disease, and certain cancers.^{102,109} By attenuating systemic inflammation, weight loss interventions reduce the incidence and progression of these conditions. Bariatric surgery reduces long-term cardiovascular events and mortality, effects that are at least partly attributable to reductions in inflammatory mediators.¹⁰⁷ Dietary weight loss, though potentially less dramatic, provides a feasible first-line strategy to shift patients away from inflammatory and metabolic risk trajectories. Importantly, the degree of inflammatory resolution achieved through weight loss may serve as a biomarker for therapeutic response, guiding personalised intervention strategies.

1.12 – A20 Haploinsufficiency

A20 haploinsufficiency (HA20) is a monogenic autoinflammatory disorder caused by heterozygous loss-of-function mutations in *TNFAIP3*, leading to insufficient A20 activity and failure to appropriately terminate inflammatory signalling. The disease was first described in 2016 and was based on the genotyping of six unrelated families with early-onset systemic inflammation and Behçet-like features, with patient cells showing enhanced I κ B α degradation, p65 (RelA) nuclear translocation, and heightened NF- κ B dependent cytokines.⁴⁰ Beyond NF- κ B dysregulation, patient PBMCs also demonstrated constitutive NLRP3 inflammasome activation with caspase-1 cleavage and increased secretion of mature IL-1 β and IL-18, reinforcing the concept that HA20 sits at the intersection of hyper-responsive TNF/NF- κ B signalling and inflammasome priming.¹¹⁰ Since 2016, subsequent systematic reviews have expanded the case numbers and clinical scope of HA20, but the core pathobiology of A20 loss is the unleashing of innate inflammatory programs.¹¹¹ Many patients first accrue polygenic labels, Behçet's disease, Juvenile Idiopathic Arthritis (JIA)/Rheumatoid Arthritis (RA), Periodic Fever, Aphthous stomatitis, Pharyngitis, and Adenitis (PFAPA), autoimmune thyroiditis, Systemic Lupus Erythematosus (SLE), Crohn's Disease, before a genetic test can establish the disease is HA20.¹¹¹ Disease expression is heterogeneous, even within families carrying the same variant, implying modifying

influences from other genes and environment.¹¹¹ The typical day-to-day manifestation of HA20 is mucosal pain, fevers, rashes, and arthritis. Beyond obvious phenotypic manifestations, profiling in a 34-patient cohort revealed restricted BCR and TCR diversity with enrichment for V-genes classically seen in lymphomas (e.g., IGHV4-34, TRBV20-1), driven by a feed-forward TNF/A20/NF- κ B loop, notably, anti-TNF therapy reversed these repertoire distortions in longitudinal sampling, although some patients still progressed to overt lymphoma.¹¹²

At the molecular level, A20 normally edits ubiquitin signals to brake pro-inflammatory cascades. In HA20, defective deubiquitination of K63-linked chains on key adaptors (TRAF6, NEMO, RIP1) following TNF stimulation magnifies canonical NF- κ B signalling, producing exaggerated transcription of cytokines and chemokines. The same loss of A20 also licenses inflammasome activity, patient cells display spontaneous and stimulus-sensitive NLRP3 activation with caspase-1 processing and secretion of IL-1 β /IL-18; pharmacologic NLRP3 blockade with MCC950 attenuates these signals *ex vivo* ⁴⁰. Reviews synthesising human and murine work converge on a two-pronged inflammatory mechanism in HA20, hyper-responsive TNF/NF- κ B and NLRP3 priming, culminating in excess IL-1 β , IL-6, IL-18 and TNF- α ¹¹².

These pathway shifts leave fingerprints in circulating immune cells. In the original cohort, patients had increased CD14+ and CD16+ inflammatory monocytes, and skewed T-helper cells leading to increased IL17A production consistent with innate–adaptive crosstalk downstream of A20 loss. More recently, human repertoire analyses indicated that permissive antigen receptors preferentially couple to NF- κ B signalling when A20 is scarce, helping explain why TNF blockade can normalise clonal metrics in HA20 ¹¹². In short, HA20 activates an inflammatory architecture in which TNF/NF- κ B and NLRP3 amplify one another, with cellular consequences that extend from myeloid activation to lymphocyte selection.

1.13 – Overall Aims of the Thesis

The overarching aim of this thesis is to define the molecular and transcriptional mechanisms that link obesity to chronic immune activation, and to determine how these processes are reversed by weight loss. In particular, this work focuses on the regulation of the NF- κ B inhibitory protein A20 (encoded by TNFAIP3) and its role in shaping immune cell function across metabolic states.

Building on evidence that obesity induces persistent low-grade inflammation and immune dysfunction, this thesis aims to investigate whether reduced expression of TNFAIP3 represents a conserved immunometabolic feature of obesity, whether this alteration is reversible with weight loss, and how it is embedded within broader transcriptional and endocrine regulatory networks.

Specifically, the aims of this thesis are to:

1. Characterise the expression of TNFAIP3 across obesity, weight loss, and metabolic disease, using integrated human and murine transcriptomic and proteomic datasets, with a focus on immune cell populations, particularly natural killer (NK) cells.
2. Define the transcriptional and epigenetic networks associated with TNFAIP3 regulation, identifying co-expressed gene programs, upstream transcription factors, and chromatin features that distinguish obese, lean, fasted, and weight-loss states.
3. Investigate the role of glucocorticoid signalling in regulating anti-inflammatory gene programs, including TNFAIP3, and assess how alterations in glucocorticoid–NF- κ B cooperation may contribute to immune dysregulation in obesity.
4. Compare obesity-associated immune transcriptional states with A20 haploinsufficiency (HA20), a monogenic inflammatory disorder, to determine whether obesity phenocopies aspects of genetically impaired A20 function at the gene-regulatory level.

Together, these aims seek to establish TNFAIP3 as a central immunoregulatory node linking metabolic state, endocrine signalling, and immune homeostasis, and to provide a unified framework for understanding how obesity and weight loss remodel immune function through conserved transcriptional mechanisms.

2.0 – Chapter 2 - Methods

This chapter outlines the key experimental and computational approaches employed throughout the thesis. To maintain consistency with the structure of the thesis, the methods are described sequentially, following the order of the main Results chapters. Chapter 3 methods are presented first, followed by those for Chapter 4 and Chapter 5. Within each section, experimental procedures, data acquisition, preprocessing steps, and analytical frameworks are detailed. Where appropriate, common pipelines and statistical approaches used across multiple datasets are described once to avoid redundancy, with dataset-specific deviations highlighted in the relevant subsections.

2.1.0 – Methods used in Chapter 3

This work exploited several mouse and human datasets to elucidate how obesity, weight loss and metabolic disease affects the expression of the key anti-inflammatory gene *TNFAIP3* relative to lean expression levels. This allows hypothesis exploration and generation across a variety of conditions and cell types to gain a better understanding of what metabolic changes can affect *TNFAIP3* expression levels in a variety of settings. In this chapter multiple human and mice datasets are used of various modalities including bulk RNA-seq, single cell RNA-seq and proteomics. These datasets, along with the computational methods used throughout this chapter are described in detail in this section.

In human studies, body mass index (BMI) is commonly used to categorise adiposity. Individuals with a BMI of 20–24.9 kg/m² are typically considered lean or within a healthy weight range, those with a BMI of 25–29.9 kg/m² are classified as overweight, and a BMI of 30 kg/m² or greater is used to define obesity.

In mice, obesity in mice is typically defined relative to control animals using experimentally induced measures of body weight and adiposity. One of the most common approaches is the diet-induced obesity (DIO) model, in which rodents (often C57BL/6J mice) are fed a high-fat or energy-dense diet, typically containing ~45–60 % of calories from fat, for a prolonged period.

Below is a table describing the datasets, two of these datasets are publicly available and are accessible through the links shown in table 1.

Table 2.1 – Publicly available datasets used in the thesis – both datasets are available on the GeoDatasets website under the accession numbers shown below.

Dataset Name	Accession Number	Link
MD2 scRNA-seq	GSE182233 (GeoDatasets)	<u>Link to GeoDatasets</u>
Weinstock scRNA-seq	GSE141036 (GeoDatasets)	<u>Link to GeoDatasets</u>

Table 2.2 – Datasets of Chapter 3 with Key

Dataset	Organism	Cell Types	Cohort	Intervention & Sampling	Tissue	Modality		Mouse
HD1		NK & gamma deltas	Lean vs Obese (n = 2)	1 Timepoint				Human
HD2		Immune Cells	Obese Females (n=4)	Sampled before caloric restriction and after 6 weeks of 1100 calories per day				Blood
HD3		Non-Immune and Immune	T2D Obese vs Metabolically Healthy Obese (n=5)	1 Timepoint	 Subcutaneous and Omentum Adipose			Liver
HD4		Immune Cells	T2D lean and obese vs metabolically healthy lean and obese (27 total)	1 Timepoint				Muscle
MD1		Immune Cells	T2D Obese vs Metabolically Healthy Obese (n=5)	Obese=8 weeks HFD Lean=8 weeks SFD WL=6weeks HFD then 2 weeks SFD				Spleen
MD2 (Hasty)		Immune Cells	Lean, Obese and WL Mice (n = 5)	Obese=9 weeks SFD then 18 weeks HFD / Lean=27 weeks SFD / WL=18 weeks HFD then 9 weeks SFD				Adipose
MD3 (Weinstock)		Immune Cells	Obese and WL Mice (n = 5)	Obese=24 weeks HFD WL=24 weeks HFD then 70% of ad-libitum calories				scRNA-Seq
MD4		NK & CD8 T Cells	Obese and Lean (n = 4 & 3 respectively)	Obese=8 weeks HFD Lean=8 weeks SFD				RNA-Seq
MD5		NK	Obese and Lean (n = 8 & 4 respectively)	Obese=10 weeks HFD Lean=10 weeks SFD				Proteomics

Table 2.2 Acronyms - NK – Natural Killer (cells) - T2D – Type 2 Diabetes - PBMC – Peripheral Blood Mononuclear Cells - WL – Weight Loss - HFD – High-Fat Diet - SFD – Standard-Fat Diet - RNA-seq – RNA sequencing

2.1.1 - Datasets

Description of mouse and human datasets utilised in Chapter 3 are depicted in an overview in Table 2.1 and Table 2.2. Several of these datasets are used throughout the thesis including in the next chapters also.

2.1.2 – HD1 - Human Dataset 1: Lean & Obese – Bulk RNA-seq dataset

Peripheral blood was collected from four human donors (two obese and two lean) by members of the Kellis lab in MIT and shared with the Lynch lab. Three immune cell subsets were isolated by flow cytometric sorting: CD56 bright NK cells, CD56 dim NK cells, and $\gamma\delta$ T cells. Following sorting, bulk RNA sequencing was performed on each population, this approach captures total RNA from pooled cells, generates cDNA libraries, and sequences them to measure gene expression at the population level rather than at single cell resolution. RNA-seq data were normalised (normalisation method not specified) prior to downstream analysis and these normalised data were saved in an excel file. It is not known if these obese patients were metabolically healthy or metabolically unhealthy. Therefore, given that there is only two obese and two lean and that the metabolic health of these individuals are not known, no major conclusions should be made from this dataset and should be treated as an initial highly preliminary piece of evidence as to what *TNFAIP3* levels are in human NKs and $\gamma\delta$ s.

Information on this dataset is difficult to get as the person who worked in collaboration with the Kellis lab has left the Lynch lab and no metadata was shared with the excel sheet of normalised RNA-seq data.

2.1.3 – HD2 - Human Dataset 2: Weight loss PBMCs – scRNA-seq

All data collection and processing was performed by a former member of the Lynch lab, Dr. Hannah Prendeville. Study participants were recruited from St Columchille’s Hospital, Loughlinstown in collaboration with Dr. Johan Meurling and Prof. Donal O’Shea. All patients were obese and self-admitted to the hospital to undergo a 6-week-long dietary restriction program, which consisted of a daily caloric intake of 1100 calories. During the program, patients were not allowed to leave the hospital grounds. Patients were provided with six helpings of full-fat milk daily in addition to Multibionta (multi-vitamins, minerals, and biotic culture) and were encouraged to drink at least 2 L of water each day. Patients were monitored by a physiotherapist once a week and were encouraged to attend an exercise class on Fridays. Patients aimed to reach 8000 steps a day, but this varied depending on the patient's own ability. Patient blood samples were collected upon admission (week 0) and after 6 weeks. Peripheral blood mononuclear cells (PBMCs) were isolated for functional analysis of cytotoxic and innate lymphocytes and single-cell RNA sequencing.

Single-cell RNA sequencing was performed using the Nextera XT assay on the NovaSeq instrument. The raw sequencing data was pre-processed to remove adapter contamination and low-quality reads using standard procedures. After preprocessing, the data was aligned to the reference human genome and processed using the Cell Ranger pipeline. The resulting gene-barcode matrices were used for downstream analysis.

Patient demographic and clinical characteristics, including patient number, age, initial weight, final weight, initial BMI, final BMI, type 2 diabetes mellitus (T2DM), smoking status, and depression status, were recorded for each patient.



Figure 2.1 – Diagram of 6-week caloric restriction program undertaken: 4 obese female patients underwent 6 weeks of 1100 calories per day. scRNA-seq samples were taken prior to weight loss and after 6 weeks of weight loss

Metadata for the patients involved in this study are shown below in Table 2.3.

Table 2.3 - Metadata for human participants in 6-week caloric restriction diet

Patient	Age	Initial weight (kg)	Final Weight (kg)	Initial BMI	Final BMI	T2Dm	HTN	HDL
1	33	177	166	65	61	No	No	No
2	53	159	149	62	59	Yes	Yes	No
3	51	202	192	68	65	No	No	No
4	41	186	174	80	74	Yes	Yes	No

Footnote: BMI – Body Mass Index; T2DM – Type 2 Diabetes Mellitus; HTN – Hypertension; HDL – High-Density Lipoprotein

2.1.4 – HD3 - Human Dataset 3: MHO vs MUO - Bulk RNA-seq

All data collection and processing was performed by a former member of the Lynch lab, Dr. William Trim. Fifty-eight individuals undergoing voluntary bariatric surgery gave written informed consent to donate abdominal subcutaneous and omental adipose tissue and liver for the present study. Ethical approval was granted for the collection of human specimens for the purposes of this research project (PHS IRB: 2019P001128) and was conducted in accordance with the Declaration of Helsinki. Specimen collection was carried out at St. Vincent's University Hospital and St. Michael's University Hospital, Dublin, Ireland in a consecutive fashion between July 2020 and June 2021. Patient specimens and clinical data were disconnected from any identifiable information and all data were assigned an anonymised code. Samples were collected at once following the end of the required bariatric surgery and tissue specimens were immediately transferred into pre-warmed (37°C) unsupplemented Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich; Darmstadt, DE) for transport back to the laboratory for processing.

Patient stratification: Patients were grouped into either obese without metabolic syndrome (Ob) or obese with type-II diabetes mellitus (Ob+T2D), with the latter designated by a clinical diagnosis of T2D along with glycated haemoglobin (HbA1c) ≥ 39 mmol/mol (or $\geq 5.7\%$) measured as part of routine pre-surgical blood assessments. The use of an HbA1c cut-off of $\geq 5.7\%$ (pre-diabetes threshold) versus 6.5% (diabetes threshold) in combination with a clinical diagnosis of T2D is due to the limitations of the 6.5% threshold for diagnostic purposes in individuals who have only recently presented with symptoms of T2D. Therefore, the more relaxed threshold in combination with a clinical diagnosis of T2D was used here.

Adipose tissue and liver processing: Whole adipose tissue (1–12g) was extracted from HBSS transport solution, where the tissues were washed over 100 μ m gauze with pre-warmed (37°C) phosphate buffered saline (PBS; Thermo-Fisher Scientific Inc.; Leicestershire, UK) to wash out remaining blood. Samples were then weighed and partitioned as follows: One adipose explant (0.5–5g) was immediately partitioned for enzymatic digestion (described below). Remaining tissue was either snap-frozen in liquid nitrogen and transferred to -80°C until analysis or placed into CellSTOR® CS10 cryopreservation media (STEMCELL Technologies™; Cambridge, UK) and frozen at a set-rate to -80°C in isopropyl alcohol-containing receptacles (Mr.Frosty™) until analysis. A small section of tissue was also placed into 10% neutral buffered formalin at room temperature (RT) for imaging analysis (described below). Liver tissue was immediately placed into CellSTOR® CS10 cryopreservation media and frozen at a set-rate to -80°C in isopropyl alcohol-containing receptacles (Mr.Frosty™)

until analysis. Adipose tissue digestion: Adipose explants were extensively mechanically minced and placed into 6mL pre-warmed (37°C) adipose tissue media (referred to as adipose buffer hereon in) at pH 7.4 containing 5.5mM D-glucose, 140mM sodium chloride, 4.7mM potassium chloride, 1.25mM magnesium sulfoxide heptahydrate, 2.5mM calcium chloride dihydrate, 10mM N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), 2.5mM sodium phosphate monobasic dihydrate (all Sigma-Aldrich; Darmstadt, DE), and 2% bovine serum albumin (BSA) fraction V (Sigma-Aldrich; Darmstadt, DE), spiked with type-1 collagenase (250U/mL) (Worthington Biochemical; NJ, US). Samples were agitated at 180–200rpm on an orbital shaker at 37°C for 45–60 minutes. Digestions were terminated with the addition of 2mM EDTA (final concentration) (Sigma-Aldrich; Darmstadt, DE). Following digestion, samples were passed through a 400µm gauze and centrifuged at 100 x g for 1 minute at RT. The remaining post-digestion solution was centrifuged at 300 x g for 7 minutes at RT, and was thereafter incubated with 9 volumes erythrocyte lysis buffer (155.1mM ammonium chloride, 9.9mM potassium bicarbonate, 0.1mM EDTA; all Sigma-Aldrich; Darmstadt, DE) for 10 minutes at RT. Post-lysis, samples were topped up with adipose buffer, centrifuged once more at 300 x g for 7 minutes at RT and the supernatant discarded before topping up with adipose buffer for filtering through 100µm gauze, and centrifuged once more at 300 x g for 7 minutes at RT before being resuspended in PBS containing 2mM EDTA and 2% FBS (referred to hereon in as FACS buffer) for flow cytometry analysis.

For Bulk-RNA-sequencing: A total of 10 patients were selected, split evenly into five per cohort (metabolically healthy obese and metabolically unhealthy obese), and paired omental, subcutaneous adipose tissue and liver tissue samples were used for bulk-RNA-seq.

2.1.5 – HD4 - Human Dataset 4: PBMCs – MHO vs MUO - scRNA-seq

All data collection and processing were carried out by members of the Lynch Laboratory in Boston in collaboration with hospital staff. This study was conducted at Brigham and Women's Hospital, Boston, from August 2019 to December 2019. The participants were categorised into five groups: obese with T2D, obese pre-diabetic, obese non-T2D, lean pre-diabetic and lean non-T2D.

All patient procedures took place at Brigham and Women's Hospital. In total, 33 volunteers were recruited for the study. The breakdown of the groups is as follows:

Obese T2D: eight participants (three women, five men) with a BMI range of 30–45 kg m⁻² and haemoglobin A1c (HbA1c) levels between 5.7% and 9%. It is important to note that one participant was not adhering to their prescribed metformin regimen. Another individual had a BMI marginally below the initial target (29.8 kg m⁻²). Obese pre-diabetic: three participants (two men, one woman), including one individual with a BMI slightly below the initial target (28.3 kg m⁻²). Obese non-T2D: 11 participants (three men, eight women), with BMI ranges primarily within 30–45 kg m⁻², including one individual with a BMI slightly below the initial target (29 kg m⁻²). Lean pre-diabetic: one participant (one man) with a BMI of 19.6 kg m⁻². Lean non-T2D: ten participants (two men, eight women), all within the targeted BMI range for lean controls.

Patients attending the diabetes clinic at Brigham and Women's Hospital were recruited prospectively. All patients provided written informed consent. This study was reviewed and approved by the Brigham and Women's Hospital institutional review board (IRB 2020P002554 and 2019P001128).

Recruitment

Volunteers for this study were recruited from the diabetes clinic and/or the weight management clinic at Brigham and Women's Hospital for the obese groups with or without T2D. In addition, flyers placed in clinical and non-clinical areas within the hospital were used to recruit lean control subjects.

The study included men and women aged 18–75 years. For lean controls, Caucasian and African American individuals were required to have a BMI of 18.5–24.9 kg m⁻², whereas Asian adults needed a BMI of 18.5–23.0 kg m⁻². The obese cohorts, whether with or without pre-diabetes or diabetes, had inclusion criteria specifying a BMI of 30–45 kg m⁻² and HbA1c levels between 5.7% and 9.5%. Individuals diagnosed with diabetes were primarily

managed with metformin, with some also receiving insulin. The study excluded current smokers, individuals who were pregnant or planning pregnancy during the study, individuals with serious uncontrolled cardiovascular, nervous system, pulmonary, renal or gastrointestinal diseases; individuals with serologic evidence of HIV, hepatitis B or hepatitis C; individuals with a history of tuberculosis; individuals with liver disease characterised by elevated hepatic enzymes; or individuals with any hematologic abnormalities within the last 12 months, including a white blood count outside the range of 3,000–14,000 cells per microlitre, lymphocyte count below 500 cells per microlitre, platelet count below 150,000 cells per microlitre, haemoglobin levels less than 10 g dl⁻¹ or neutrophil count below 2,000 cells per microlitre. Additional exclusion criteria were poor glycaemic control (HbA1c over 9.5% for individuals in the diabetes group), major psychiatric illness or ongoing alcohol or drug abuse.

Table 2.4 - Metadata for metabolically healthy vs metabolically unhealthy scRNA-seq dataset

Subject ID	BMI	Age	Sex	Medications (Y/N)	Diabetic Status	Weight Status
2019FG27	18.77	67	Female	Yes	-	lean
2019BT34	20.80	21	Female	No	pre-diabetic	lean
2019EM25	21.27	21	Female	No	-	lean
2019FS22	22.37	45	Female	Yes	-	lean
2019SS18	22.47	28	Female	Yes	-	lean
2019SL23	23.12	52	Female	No	pre-diabetic	lean
2019EM12	24.04	34	Male	No	-	lean
2019LF24	24.40	38	Male	Yes	-	lean
2019NN31	28.31	34	Male	No	pre-diabetic	overweight
2019SE28	29.80	48	Male	No	+	overweight
2019MC13	31.30	66	Female	Yes	pre-diabetic	obese
2019NS11	32.00	46	Female	Yes	-	obese
2019CE02	32.60	59	Male	Yes	+	obese
2019QN07	33.40	38	Female	Yes	-	obese
2019KN19	34.82	45	Male	No	pre-diabetic	obese
2019QC10	34.90	45	Female	Yes	-	obese
2019JL33	34.91	67	Female	Yes	-	obese
2019TL26	34.92	72	Male	Yes	+	obese
2019BN05	35.50	34	Male	Yes	-	obese
2019BC29	35.65	66	Male	No	pre-diabetic	obese
2019UT15	35.90	62	Male	Yes	+	obese
2019QN06	42.30	53	Female	Yes	+	obese
2019TT03	42.90	56	Male	Yes	+	obese

2.1.6 – MD1 - Mouse Dataset 1: Mouse Model of Obesity and Weight Loss – scRNA-seq

All data collection and processing were carried out by a previous member of the Lynch lab, Dr. Hannah Prendeville. A mouse model of diet-induced obesity (DIO) was used to investigate how obesity and weight loss affect immune cell populations across multiple tissues. Mice were fed either a high-fat diet (HFD, 60% kcal from fat) to induce obesity, or a standard-fat diet (SFD, 12% kcal from fat) as a control. For weight loss modelling, mice were initially fed HFD and then switched to SFD for two weeks. Mice were weighed weekly.

Single-cell RNA-sequencing (scRNA-seq) was performed on live CD45⁺ immune cells isolated from blood, gonadal (epididymal) adipose tissue, liver, and muscle. CD45⁺ is a sorting marker that is positively expressed on all immune cells except erythrocytes and platelets.

All mice were male, with 5 mice per group unless otherwise noted. Mice were euthanised by cervical dislocation.

Blood was collected into EDTA tubes via heart puncture, and live CD45⁺ cells were sorted using flow cytometry prior to sequencing. Gonadal adipose tissue and liver were freshly dissociated, with samples processed for scRNA-seq; only gonadal adipose and liver samples maintaining sufficient viability after cryopreservation and shipping were sequenced. Calf muscle tissue was freshly dissociated using collagenase digestion followed by density gradient enrichment (Lymphoprep) to isolate immune cells, and live CD45⁺ cells were subsequently sorted for scRNA-seq. For blood and muscle collected in later experiments, only live CD45⁺ cells were selected for sequencing. The experimental design is depicted in Figure 2.3.

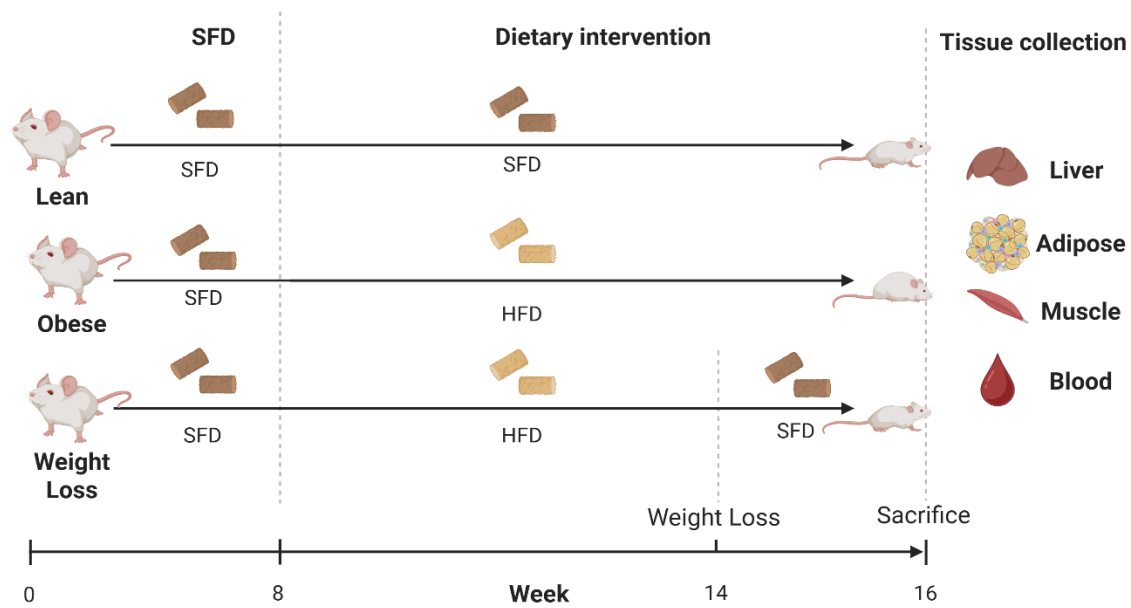


Figure 2.2 – MD1- In house scRNA-seq model of lean, obese and weight loss model across tissues: liver, adipose, muscle and blood

Footnote: SFD – Standard Fat Diet; HFD – High Fat Diet

2.1.7 – MD2 - Mouse Dataset 2: Hasty Lab Mouse Model of Obesity and Weight Loss

Male C57BL/6J mice were obtained from Jackson Laboratories at seven weeks of age and housed under standard conditions (23 °C, ~30% humidity, 12-hour light/dark cycles), the number of mice used was 4 per group (n = 4). After a one-week acclimation period, mice were randomly assigned to one of the four pre-defined 27-week protocols to receive either a high-fat diet (HFD, 60% kcal from fat; Research Diets #D12492) or a low-fat diet (LFD, 10% kcal from fat; Research Diets #D12450B) for 9-week intervals, repeated for three cycles totalling 27 weeks. This protocol generated metabolically distinct groups: chronically lean (LFD), chronically obese (HFD), and weight-cycled (alternating LFD/HFD), as previously described. A cohort of female mice underwent the same dietary cycling. Body weight and food intake were recorded weekly. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Vanderbilt University.

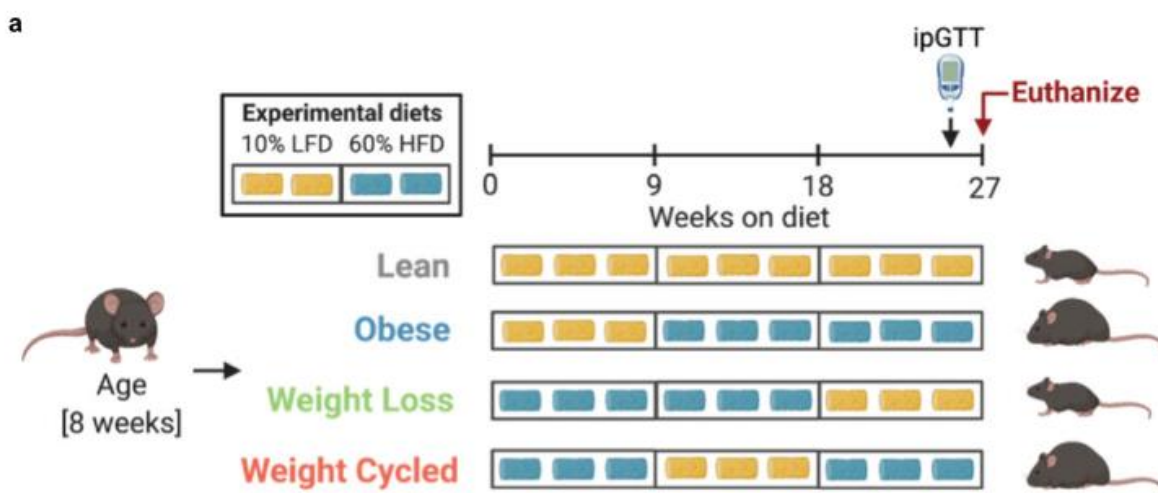


Figure 2.3 – Study design utilised showing the different 27 week feeding regimes, lean, obese, weight loss and weight cycling mice make up this cohort, scRNA-seq was performed on the adipose tissue after this study ¹¹³

Footnote: LFD - Low Fat Diet; HFD – High Fat Diet ; ipGTT – Intraperitoneal Glucose Tolerance Test; Each Box in the figure represents a 3 week period

Isolation of Stromal Vascular Fraction and Immune Cells

At the study endpoint, mice were euthanised by isoflurane overdose followed by cervical dislocation and perfused with 20 mL PBS. Epididymal adipose tissue (eAT) was excised, minced, and enzymatically digested in 6 mL of 2 mg/mL collagenase type II (Worthington LS004177) at 37 °C for 30 minutes. Following digestion, suspensions were filtered (100 µm, then 35 µm), red blood cells lysed with ACK buffer, and washed for downstream labelling.

The stromal vascular fraction (SVF) was blocked with anti-mouse Fc Block and stained with TotalSeq-C hashtag antibodies (BioLegend) for sample demultiplexing and with anti-CD45 microbeads (Miltenyi Biotec) for magnetic enrichment. Biological replicates were pooled and CD45⁺ immune cells isolated using an AutoMACS Pro Separator with the “possel_s” program. CITE-seq antibodies targeting cell surface markers were used for simultaneous transcriptomic and protein profiling. DAPI staining enabled exclusion of dead cells, and DAPI⁻ singlets were selected for single-cell sequencing.

Single-Cell RNA Sequencing and Library Preparation

Sorted CD45⁺ viable immune cells were processed using the 10x Genomics Chromium Controller (5' Single Cell Gene Expression + Feature Barcoding assay) targeting 20,000 cells per dietary group (~5,000 per biological replicate). Libraries were sequenced on an Illumina NovaSeq 6000 (PE-150) to a depth of approximately 50,000 reads per cell. All samples were processed and sequenced on the same day to minimise batch effects. Library preparation and sequencing were performed by the Vanderbilt Technologies for Advanced Genomics (VANTAGE) Core Facility. All this work was carried out by Cottam *et. al.*¹¹³

2.1.8 – MD3 - Mouse Dataset 3: Caloric Restriction scRNA-seq Dataset - Weinstock

Adipose scRNA-seq dataset obtained from the GeoDatasets – [GSE141036](#), this scRNA-seq data was generated and made publicly available by the Fisher lab in New York University Langone Health.¹¹⁴ This data was first accessed and analysed in the summer of 2023.

Animal Models and Diets

Male C57BL/6J mice, aged six weeks, were purchased from Jackson Laboratories and acclimated for two weeks in a specific pathogen-free (SPF) facility under standard conditions (25 °C, 12-h light/dark cycle). Mice were assigned to one of two dietary regimens to induce metabolic perturbation. The obese group was fed a high-fat, high-cholesterol diet (60% kcal from fat, 0.3% cholesterol; Research Diets) ad libitum for 24 weeks. To further enhance hyperlipidemia, mice received weekly intraperitoneal injections of 5 mg/kg low-density lipoprotein receptor antisense oligonucleotide (Ionis Pharmaceuticals). A caloric restriction (CR) group was established by administering 70% of the ad libitum HFD consumption starting in week 24 for two additional weeks until week 26. The number of mice used in this experiment was not specified.

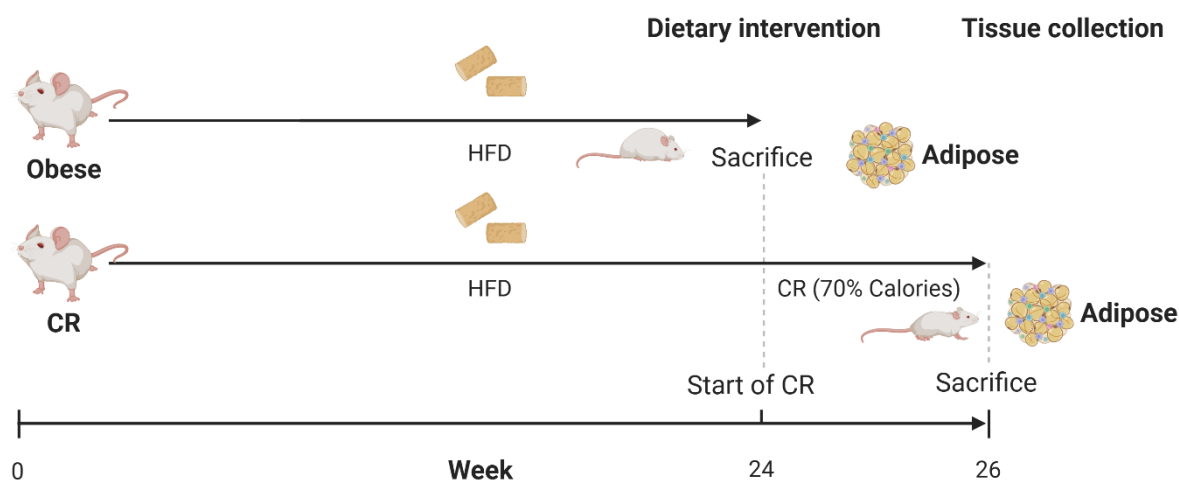


Figure 2.4 – Study design utilised showing the different obese and caloric restriction groups in this cohort, scRNA-seq was performed on the adipose tissue after this study

Tissue Collection and Stromal Vascular Fraction Isolation

At the experimental endpoint, mice were euthanised and perfused with 10 mL of sterile saline to eliminate peripheral blood contamination. Perigonadal visceral adipose tissue (VAT) was dissected, rinsed in ice-cold PBS, and mechanically minced into ~2–3 mm fragments. Tissues were enzymatically digested in a cocktail containing 1 mg/mL bovine

serum albumin, 0.77 mg/mL Liberase, 15.8 mU hyaluronidase, 25 mU DNase I, and 1.5 μ M Ca^{2+} in Hanks' Balanced Salt Solution. Digestion was performed using the gentleMACS Octo Dissociator (Miltenyi Biotec) with the "mr_adipose_01" program, run three times. The resulting single-cell suspensions were filtered through a 100 μ m strainer and washed with PBS.

Cell Sorting and Single-Cell RNA Sequencing

Live CD45⁺ immune cells were isolated from the stromal vascular fraction via fluorescence-activated cell sorting (FACS). Cells were stained with a viability dye and antibodies targeting CD45 (BV510), Fcgr4 (BV785), and CD31 (PE/Dazzle 594). Debris and dead cells were excluded through forward/side scatter and UVB gating. Approximately 200,000 viable CD45⁺ cells per sample were loaded onto a 10x Genomics Chromium Controller for single-cell 3' gene expression profiling. Libraries were prepared using standard Chromium Single Cell 3' v2 chemistry and sequenced according to manufacturer protocols.

Read Processing and Quality Control

Sequencing reads were processed using the Cell Ranger software suite (v2.2, 10x Genomics). FASTQ files were generated using cellranger mkfastq and aligned to the mm10 mouse genome using cellranger count. UMI counts per gene were calculated, and low-quality cells were filtered based on thresholds for high mitochondrial gene content, low total UMI counts, and abnormal gene detection levels indicative of doublets or apoptotic cells. The average reads per cell were 79,505 in obese mice and 106,584 in CR mice. All this work was carried out by Weinstock et. al ¹¹⁴.

2.1.9 – MD4 - Mouse Dataset 4: Natural Killer Cell Proteomics: Tumours & Stimulation with IL2 & IL12

To investigate the impact of obesity on natural killer (NK) cell function, a diet-induced obesity (DIO) model was employed. Mice were fed a high-fat diet (HFD) for a duration of 10 weeks, a time frame consistent with prior studies of metabolic dysregulation in immune cells. At 10 weeks old mice were subcutaneously injected with B16-F10 cells. In this experiment, two different obesogenic diets were used: four mice were rendered obese through a palm oil-based HFD and four mice through a butter fat-based HFD. These mice were pooled together as a single “obese” group for comparative analysis against lean controls, as both diets produced a similar obese phenotype in terms of weight gain and metabolic outcomes.

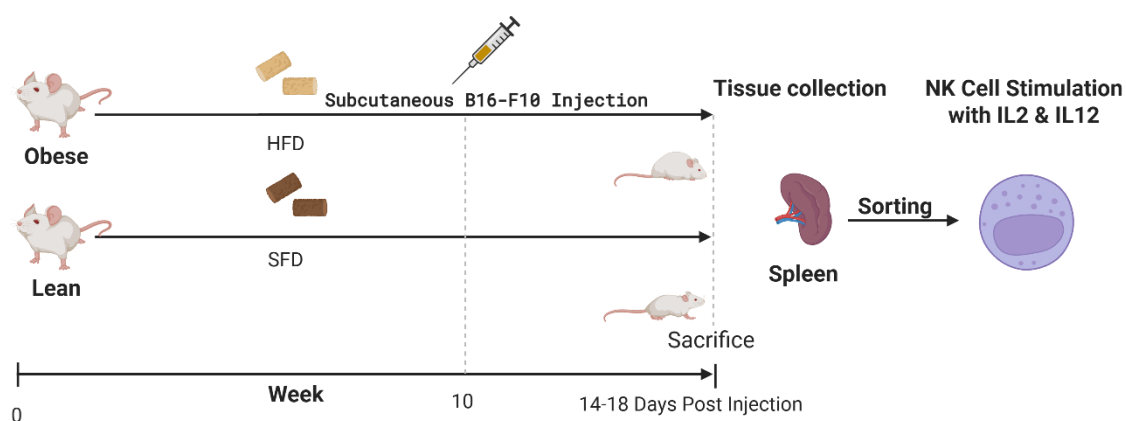


Figure 2.5 – Study design utilised showing the obese and lean B16 cancer model used, proteomics was performed on the sorted splenic NK cells after this study

Footnote: SFD – Standard Fat Diet; HFD – High Fat Diet; B16-F10; A C57BL/6 mouse-derived melanoma cell line commonly used as a syngeneic tumour model in immuno-oncology research; IL2 & IL12 – Interleukin-2 and Interleukin-12

Following the dietary intervention, mice were sacrificed, and splenic NK cells were isolated ex vivo using magnetic bead-based cell separation. Purified NK cells were then stimulated overnight with recombinant mouse IL-2 (10 ng/mL) and IL-12 (20 ng/mL) to mimic inflammatory activation conditions. After stimulation, cells were processed for downstream proteomic profiling.

This approach allowed for the assessment of obesity-induced alterations in NK cell protein expression in a physiologically relevant, activated state. Notably, these mice bore subcutaneous tumours as stated, providing a context in which both metabolic and tumour-

associated immune dysregulation could be assessed. Weight gain and tumour volume were measured prior to euthanasia and confirmed the establishment of the obese, tumour-bearing phenotype. Proteomic results were later compared with transcriptomic data (RNA-seq) to validate consistent molecular signatures across platforms.

2.1.10 - MD5: Natural Killer Cell and CD8+ T Cell Proteomics: HFD and SFD

Murine Cell Isolation and Sorting
Spleens were harvested from C57BL/6 mice maintained on either a standard fat diet (SFD) or a high fat diet (HFD), length of each diet was 8 weeks long and was 45% lard for HFD. Mice were sacrificed after diet intervention, and spleens were mechanically dissociated by mashing through a mesh in RPMI media. Cell suspensions were topped up to 10 mL with RPMI and centrifuged at 1400 RPM for 4 minutes. Red blood cells were lysed by adding 1 mL of distilled water and swirling for ~3 seconds, followed by immediate quenching with 10 mL RPMI. After a second centrifugation (4 minutes, 1400 RPM), supernatant was removed and cells were resuspended in RPMI. Fc receptors were blocked using 25 µL Fc Block (1:100 dilution) for 5 minutes in the dark. Cells were stained with an antibody master mix consisting of CD3, CD4, CD8, NK1.1, and a viability dye (each at 1:200 dilution, except viability at 1:500), and incubated for 20 minutes in the dark.

CD8⁺ T cells (CD3⁺, TCRβ⁺, CD8α⁺) and NK cells (CD3⁻, NK1.1⁺) were sorted using a BD FACS Aria cell sorter. Cells were isolated from a total of 14 samples: 3–4 mice per group for each diet and cell type, comprising CD8⁺ T cells and NK cells from both SFD (n=3) and HFD (n=4) groups. Sorted cells were washed in HBSS, snap-frozen as dry pellets, and stored at -80 °C prior to proteomic analysis.

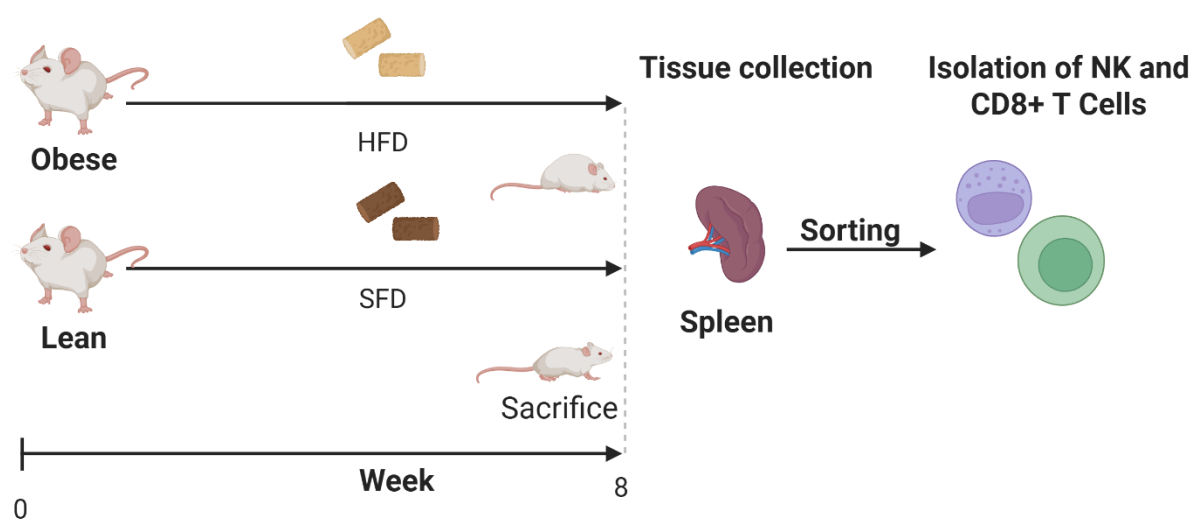


Figure 2.6 – Study design utilised showing the obese and lean model used, proteomics was performed on the sorted splenic NK cells after this study

Footnote: SFD – Standard Fat Diet; HFD – High Fat Diet

Proteomic Sample Preparation and Mass Spectrometry
Dry cell pellets were submitted to the FingerPrints Proteomics Facility (University of

Dundee). Protein samples were processed using S-Trap columns and quantified via MicroBCA assay. Proteins were digested with trypsin and subjected to peptide quantification prior to analysis.

Mass spectrometry was performed using Data Independent Acquisition (DIA). Peptide identification and quantification were conducted using Spectronaut software. Protein alkylation with iodoacetamide was used during sample processing to ensure complete reduction and alkylation of cysteines. This wet lab work was carried out within the Finlay lab by Carrie Corkish, Cathal Keane and Cristhiane Favero de Aguiar.

2.1.11 - Computational Methods in Chapter 3

The computational methods used in this chapter are typically basic Seurat and Scanpy pipelines that have been established for the processing of scRNA-seq data. All methods used are discussed in detail below.

Note: Where ‘()’ is observed throughout the text, this refers to a specific function used in R.

2.1.12 - Preprocessing Methods

scRNA-seq datasets were processed on the Illumina platform and subsequently processed with Cell Ranger which created the 10x file format with three separate files, barcodes.tsv (unique cell identifiers), features.tsv and matrix.mtx. Using the R programming language these can be loaded into the scRNA-seq processing package called Seurat. ‘Mouse Dataset 2’ was already processed and available to download in an R object format (.rds) and no processing was required as it had already been processed. ‘Mouse Dataset 3’ was available in (.csv) format and could be loaded into R as a raw counts matrix which could then undergo the same subsequent processing as 10x files once loaded in.

Loading Data into Seurat and Creation of Seurat Object

Data was loaded into Seurat using the Read10X function. Where data was in a different format (MD3 – Weinstock (csv)), alternative methods were used such as read_csv function. CreateSeuratObject was then used to turn the matrices into a Seurat object with associated metadata.

scRNA-seq Quality Control

For each dataset, we evaluated key quality control (QC) metrics to identify and exclude low-quality cells, multiplets, and other technical artifacts. I calculated QC metrics per cell: number of detected genes (nFeature_RNA) as low values may indicate empty droplets or dead cells, unusually high values may reflect doublets. Total number of transcripts (nCount_RNA) was used alongside gene counts to identify outliers. Percentage of mitochondrial transcripts (percent.mt) was used as elevated values may reflect stressed or dying cells. The mitochondrial percentage was computed using Seurat's PercentageFeatureSet() function, targeting genes beginning with "MT-". Visual inspection of QC metrics was carried out using VlnPlot() and FeatureScatter() to assess relationships between nCount_RNA, nFeature_RNA, and percent.mt, helping to guide filtering thresholds. Mitochondrial cutoffs were set following recommendations in the paper “Systematic determination of the mitochondrial proportion in human and mice tissues for

single-cell RNA-sequencing data quality control”.¹¹⁵ This paper provided a framework for mitochondrial DNA QC and how to set thresholds. Cells were also retained based on thresholds for gene count, these varied by dataset based on sequencing depth etc. These thresholds were adjusted as needed depending on the dataset to account for biological differences, platform-specific variation, and experimental design. Typically cutoffs for mitochondrial percentage were chosen using the paper on mitochondrial percentage cut-offs for scRNA-seq data.¹¹⁵

Log-Normalisation of Gene Expression

Normalisation of gene expression was performed using Seurat’s `NormalizeData()` function, which applies a global-scaling method known as `LogNormalize`. In this procedure, the raw UMI count for each gene in each cell is divided by the total number of transcripts detected in that cell (i.e., total UMI counts per cell), multiplied by a scaling factor (default = 10,000), and log-transformed using natural logarithms. This step mitigates variability due to differences in sequencing depth and allows for meaningful comparisons of gene expression levels across cells.

Identification of Highly Variable Features (Genes)

To focus downstream analysis on the most informative genes, highly variable genes (HVGs) were selected using Seurat’s `FindVariableFeatures()` function with the (variance-stabilising transformation) method. This algorithm models the mean–variance relationship of gene expression and identifies genes that exhibit the greatest biological variability across the dataset, independent of sequencing depth. The top 2,000 variable genes were retained and used in subsequent steps including dimensionality reduction and clustering.

Data Scaling and Standardisation

The `ScaleData()` function was used to scale and centre the data prior to principal component analysis (PCA). Each gene’s expression was centred to have a mean of zero and scaled to unit variance (standard deviation = 1) across all cells. This Z-score transformation removes the influence of genes that differ in absolute expression range and ensures that highly expressed genes do not dominate the PCA. Optionally, known sources of unwanted variation (e.g., mitochondrial percentage, cell cycle scores) can be regressed out during this step, although no such regression was applied unless otherwise stated.

Principal Component Analysis (PCA): Principal Component Analysis (PCA) is a statistical method used to reduce the dimensionality of datasets with many intercorrelated

variables while retaining the most important information. It transforms the original data into a new set of variables called principal components, which are uncorrelated and ordered by the amount of variance they explain in the data.

PCA begins with a matrix in which the rows are observations (cells) and the columns are variables (gene expression levels). The first step is usually to centre each variable by subtracting its mean, often one also standardises to unit variance (especially when variables are on different scales). The PCA is then performed on this transformed matrix X . The result is a new set of orthogonal axes (principal components) that capture descending proportions of the total variance.

The mathematical foundation of PCA lies in Singular Value Decomposition (SVD) or the eigen decomposition of the covariance or correlation matrix of X .

The first principal component captures the direction of maximum variance in the data. Each subsequent component captures the highest remaining variance while being orthogonal to the previous components. The eigenvalues (squared singular values) quantify how much variance is explained by each component.

The results of PCA include:

- Factor scores: coordinates of observations in the new space.
- Loadings: correlations between original variables and components.

In essence, PCA reveals hidden structures in the data, simplifies interpretation, and is widely used in exploratory data analysis, especially when visualising high-dimensional data in 2D or 3D.

The breakdown and inner workings of the PCA algorithm was examined using the paper ‘Principal Component Analysis’ from Abdi *et. al.*¹¹⁶ Interestingly, the original ideas underpinning PCA came from a 1901 paper by Pearson, ‘On lines and planes of closest fit to systems of points in space’. Pearson also developed correlation coefficient now known as ‘Pearsons r ’.¹¹⁷ All information described here pertains to that PCA was used for both the metabolomics and in the preprocessing of scRNA-seq data in every scRNA-seq dataset. PCA was implemented via two separate mechanisms, in python via the scikit-learn library. In R, PCA was implemented using the in-built Seurat RunPCA function.

Construction of the Shared Nearest Neighbour Graph

Using the selected principal components (typically PCs 1–30), a k-nearest neighbor (kNN) graph was constructed via the `FindNeighbors()` function. Each cell is connected to its k most similar cells (in PCA space), and edges are weighted by Jaccard similarity, forming the basis for graph-based clustering. This step transforms the reduced dimensional space into a cell–cell similarity graph used to define local cell communities.

Graph-Based Clustering

Cell clustering was performed using Seurat’s `FindClusters()` function, which implements the Louvain modularity optimisation algorithm (or Leiden if specified). This community detection algorithm partitions the kNN graph into discrete clusters of transcriptionally similar cells by maximising modularity, a measure of edge density within versus between clusters. A resolution parameter (e.g., 0.5) controls the granularity of clustering, with higher values producing more clusters. These clusters were interpreted as distinct immune cell subtypes based on known marker gene expression profiles.

Nonlinear Dimensionality Reduction for Visualisation: UMAP

For visualisation, Uniform Manifold Approximation and Projection (UMAP) was applied using the `RunUMAP()` function. UMAP is a nonlinear manifold learning algorithm that embeds high-dimensional data into two dimensions while preserving both local and global data structure. UMAP was run using the same PCA dimensions (e.g., PCs 1–30) as input. The resulting two-dimensional embeddings were used to visualise clusters and annotate cell types based on marker expression.

Wilcoxon Rank-Sum Test for Differential Expression:

To identify genes differentially expressed between conditions or cell types, the Wilcoxon Rank Sum Test (also known as the Mann-Whitney U test) was employed, as implemented in the Seurat R package. This non-parametric test evaluates whether the distributions of gene expression differ significantly between two independent groups of cells. Rather than comparing means, the test ranks all expression values and assesses whether one group tends to have higher or lower ranks than the other. This approach is well-suited to single-cell RNA-seq data, which often exhibit non-normal distributions and a high frequency of zero values. Genes were considered differentially expressed based on a $\log_2$ fold change threshold and false discovery rate (FDR)-adjusted p-value. The Bonferroni FDR adjusted p-value works like this: For each gene, Wilcoxon rank sum tests gives a raw p-value telling us whether its expression differs between the two groups. Because many genes are tested at once, Seurat

also reports an adjusted p-value (called `p_val_adj`) to correct for multiple comparisons. Seurat uses a Bonferroni correction, adjusting each raw p-value for the total number of genes tested. This method is more conservative (stringent) than other corrections like FDR (false discovery rate), so it reduces the chance of false positives. All statistical testing where appropriate uses adjusted p-value.

2.1.13 - Integration Methods

To harmonise data generated from distinct single-cell RNA sequencing (scRNA-seq) experiments and minimise batch effects, Canonical Correlation Analysis (CCA) was employed as a key dimensionality reduction and integration strategy. CCA is a multivariate statistical technique designed to identify and align correlated gene expression patterns across datasets by maximising the correlations between linear combinations of variables (in this case, genes) from different conditions or batches. This approach is particularly well-suited for scRNA-seq data integration because it focuses on shared sources of biological variation while minimising dataset-specific technical noise.¹¹⁸

In this study, CCA was applied using the implementation available in the Seurat R package (version 4.1.3), which identifies canonical components based on variable features that are consistently expressed across datasets. Specifically, we selected genes that were highly variable in each dataset, this method to find HVGs is described above in the section ‘Identification of highly variable genes’, these HVGs are then used as input features for CCA. The analysis was conducted across datasets representing different experimental conditions (e.g., lean, obese, and weight loss), as well as across human and murine samples, where applicable.

Following identification of canonical correlation vectors, cells were projected into a shared low-dimensional space defined by the top canonical components. These components represent the dimensions where datasets exhibit the most correlated expression patterns. Subsequent alignment and correction of batch effects were achieved through mutual nearest neighbour (MNN) anchoring, enabling more accurate clustering and comparative analysis across conditions.

CCA was chosen over other integration methods due to its ability to preserve biological heterogeneity while correcting for technical variability, making it particularly useful for datasets with substantial overlap in cell populations but differing sequencing depth, donor variability, or library preparation protocols.

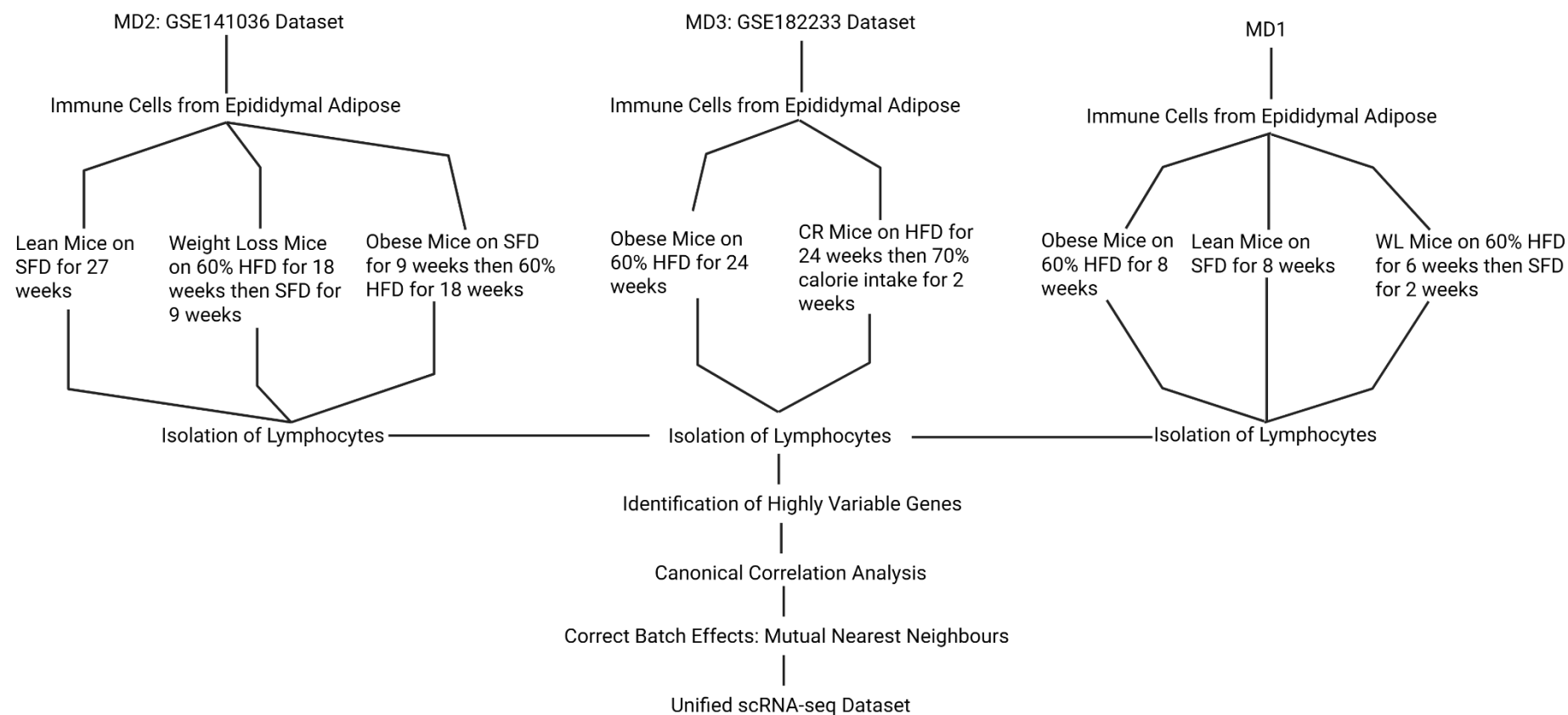


Figure 2.7 - Workflow for integrating multiple scRNA-seq datasets. Highly variable genes (HVGs) are selected from each dataset, canonical correlation analysis (CCA) identifies shared expression patterns, and mutual nearest neighbour (MNN) anchoring aligns the datasets to correct for batch effects across conditions such as lean, obese and weight loss groups

Footnote: CCA – Canonical Correlation Analysis; HVGs – Highly Variable Genes; MNN – Mutual Nearest Neighbour; SFD – Standard Fat Diet; HFD – High Fat Diet; scRNA-seq – Single-cell RNA sequencing.

2.1.14 Downstream scRNA-seq Analysis

A variety of visualisation methods were utilised throughout this chapter to show data in a meaningful clear way. PCA plots as described above were utilised to show variations between treatment groups along principal components, this was typically PC1 and PC2 as the two principal components that would capture the most variation within the data.

Volcano plots were utilised using the `ggvolc` R package. To make these plots, differential expression using Wilcoxon rank sum test was performed between the two conditions of interest. The resulting dataframe contains a list of genes that are differentially expressed between the conditions ranked by adjusted p-value and then by $\log_2$ fold change. This dataframe is given to the `ggvolc` package which plots the data as a volcano plot.

Violin plots are used throughout the thesis and were created using the `VlnPlot` function in Seurat, this function allows for plotting of multiple genes or single genes and can be split by treatment.

Custom bar plots are sometimes utilised, e.g. to show cell proportions differences or $\log_2$ fold changes across multiple cell types. These plots were created using `ggplot2`, `dplyr` and `datatable`.

Pseudobulking of scRNA-seq data

Pseudobulking is a computational approach used in single-cell RNA sequencing (scRNA-seq) analysis to aggregate gene expression data across cells within defined groups, such as individuals, conditions, or cell types. Instead of treating each cell as an independent observation, pseudobulking sums or averages expression counts for each gene across cells from the same group, effectively creating a “bulk-like” expression profile per sample. This approach helps reduce the noise and variability inherent in single-cell data and enables the use of well-established bulk RNA-seq statistical frameworks, such as `DESeq2` or `edgeR`, for differential expression analysis. Pseudobulking is particularly useful when comparing gene expression across biological replicates or conditions while preserving the resolution of specific cell populations.

2.1.15 - Proteomic Dataset Analysis

All analyses were performed in R (4.1.3) using the packages `data.table`, `ggplot2`, `reshape2`, `dplyr`, `plotly`, and `limma` for data handling, visualisation, and statistical analysis. Raw protein copy-number data from the file `126_2025_TCD_CC-copy-numbers.txt` were imported with `fread()`. Only gene identifiers and copy-number columns were retained, and gene names were cleaned by removing missing values, trimming whitespace, and converting to upper case. Duplicate gene entries were removed to create a non-redundant matrix with genes as rows and samples as columns.

An outlier sample (NK-HFD-4) was excluded prior to normalisation. Copy numbers were $\log_2$ -transformed with a pseudocount of 1 to stabilise variance, and genes with excessive zeros or zero variance across samples were filtered out. Sample names were simplified by removing prefixes and suffixes and standardising cell-type labels.

The processed matrix was reshaped into long format using `melt()` to allow generation of boxplots and exploratory plots with `ggplot2`. A custom function extracted expression values for selected genes, added group annotations (obese versus lean), and produced Prism-ready wide-format tables. This cleaned dataset was written to CSV for downstream plotting in GraphPad Prism or within R/`ggplot2`. Principal component analysis (PCA) was then performed on the transposed, filtered $\log_2$ matrix using `prcomp()` with centring and scaling. Metadata for each sample (cell type and diet) were combined with PCA scores to visualise clustering by experimental condition.

2.1.16 - Bulk RNA-seq analysis / GraphPad Prism

Bulk RNA-seq was received already in a normalised state and ready for analysis (HD1 and HD3). Analysis was done in prism or R using ggplot2 and dplyr. Visualisation of bulk-seq data usually involved stratifying patients depending on treatment groups and then performing significance testing in R or prism depending on the package being used.

GraphPad Prism

GraphPad Prism v.10 was utilised to make barplots and perform statistical testing in some instances. Any data utilised in prism graphs had been previously processed in R or Python.

R Packages used:

All analyses were conducted in R version 4.1.3 (2022-03-10) on a Windows 10 x64 platform. The Seurat pipeline and associated single-cell analyses were performed using the Seurat R package (version 4.3.0) and SeuratObject (version 4.1.3). Additional packages used included ggplot2 (3.5.1) for plotting, data.table (1.14.8) for data handling, and SeuratDisk (0.0.0.9020) for cross-format single-cell data conversion, primarily used for conversion of .h5ad files used in python to .h5 files used in R (Seurat).

2.1.17 – Conventions

Gene names are written in italicised format and will either be fully in uppercase for humans e.g. *TNFAIP3* or lowercase for mice *Tnfaip3*. When speaking generally about the gene, for example when referring to what has been observed collectively in humans and mice it will be referred to as *TNFAIP3*.

Protein names are not italicised.

Log₂ fold change: Log₂ fold change (log₂FC) is a standardised way of representing changes in gene expression in single-cell RNA-seq (scRNA-seq) data and in other omics data. It expresses the ratio of expression between two conditions (e.g. obese vs lean) on a logarithmic scale base 2. A log₂FC of 1 indicates a doubling in expression, while a log₂FC of -1 indicates a halving. This convention allows both upregulation and downregulation to be represented symmetrically and makes large changes more interpretable. Throughout this work, log₂FC values are used to describe differential gene expression, enabling consistent comparison across genes and datasets.

2.2.0 – Methods used in Chapter 4

New methods, previously not seen in Chapter 3 were utilised in this to uncover mechanisms regulating *TNFAIP3*, the primary new method in this chapter focused on high dimensional weighted gene co-expression network analysis (hdWGCNA), which is required for finding modules or groups of genes co-regulated together. All methods are explained in detail below.

2.2.1 Datasets and Databases

Several additional datasets and databases were utilised to uncover the mechanisms controlling *TNFAIP3*'s expression. New datasets will be assigned codes using the same convention as in the previous results chapter, e.g. MD6, MD7, HD6. Details of the datasets are detailed below in Table 2.5.

Table 2.5 - Datasets used in Chapter 4

Dataset	Organism	Cell Types	Cohort	Intervention & Sampling	Tissue	Modality		
								Mouse
								Human
HD7		Immune Cells	Healthy (n = 4)	Methylprednisolone (22.7 µM) vs vehicle; Blood Taken - 2 h & 6 h post-treatment				Blood
HD2		Immune Cells	Obese Females (n=4)	Sampled before caloric restriction and after 6 weeks of 1100 calories per day				Plasma
MD2 (Hasty)		Immune Cells	Lean, Obese and WL Mice (n = 5)	Obese=9 weeks SFD then 18 weeks HFD / Lean=27 weeks SFD / WL=18 weeks HFD then 9 weeks SFD				Tumour
MD6		Adipocytes	Lean, Obese (12 or 25 wk), Weight-loss (Obese→chow for varying periods)	Diet manipulations; ATAC-seq libraries from epi-adipose				Spleen
MD7		NK Cells	<i>Ad-Libitum</i> Fed vs Fasting Mice	<i>Ad libitum</i> vs calorie-restricted + 24–48 h fasting				Adipose
HD6		Plasma Proteins	12 Healthy Adults	Water Only 7-day Fast				scRNA-Seq
								RNA-Seq
								Proteomics
								ATAC-Seq

Table 2.5 Acronyms - NK – Natural Killer (cells) - WL – Weight Loss - HFD – High-Fat Diet - SFD – Standard-Fat Diet - RNA-seq – RNA sequencing

2.2.2 - MD6 - GSE236575 - Adipose tissue ATAC-seq, lean, obese and weight loss

ATAC-seq was performed by Hinte *et. al*¹¹⁹ on epididymal adipose tissue (epiAT) from C57BL/6N mice. Obesity was induced by high-fat diet feeding (60% kcal from fat) for 12 or 25 weeks, while control mice were maintained on chow (10% kcal from fat). For the weight loss group, mice were switched from high-fat diet to chow for varying periods, with short term weight loss and long-term weight loss regimes (. ATAC-seq libraries were prepared from nuclei isolated from snap-frozen epiAT and sequenced on an Illumina NovaSeq 6000 (paired-end, 150 bp), this work was carried out by Hinte *et. al* and made publicly available on GeoDatasets under accession number GSE236575. I obtained the raw ATAC-seq peak files (BED format) and associated metadata were obtained from the aforementioned GEO accession number GSE236575. Metadata included diet group assignment (SFD, HFD, WL), sample identifiers, and file paths. Each BED file was loaded in R and converted to GRanges objects using a custom function to standardise strand information and include peak scores. To enable cross-sample comparison, peak scores were normalised to counts per million (CPM) per sample by dividing each peak's score by the total score of all peaks in that sample, then scaling by 10^6 . Genomic coordinates of nine exons from the *Tnfrsf25* gene (based on the mm10 reference genome) were defined manually and converted into GRangesList objects. For each exon, overlaps with normalised ATAC-seq peaks were computed using `subsetByOverlaps()` from the GenomicRanges package. The total normalised score of peaks overlapping each exon was calculated per sample. To map functional domains of the A20 protein onto genomic exons, domain start and end positions in amino acids were translated to nucleotide coordinates within the coding sequence. These were mapped to genomic coordinates using Ensembl exon annotation (via biomaRt) for transcript ENSMUST00000019997. The final data frame included sample ID, diet group, exon identity, normalised accessibility score, and Pfam domain annotation. Boxplots of exon-level chromatin accessibility were generated using ggplot2, with jittered data points overlaid and faceting by exon. Colours were assigned per diet group using a custom palette. Figures were saved as high-resolution PNG files (600 dpi) for inclusion in the thesis.

All analysis was performed in R (version 4.3.0), and the following packages were used: GenomicRanges, ggplot2, ggpubr, dplyr, tidyr, readr, and biomaRt.

2.2.3 - MD7 - Mouse Dataset 7: Fasting NK Cells Mice - GSE267467

C57BL/6 mice were maintained under specific pathogen-free conditions and placed on either a standard chow diet (ad libitum), a high-fat diet (HFD), or a calorie-restricted chow

diet (CRD). For fasting experiments, mice on CRD were additionally subjected to a 24–48 h fasting period with access to water only. Age- and sex-matched mice were used across experimental groups. Body weight and food intake were monitored throughout. To assess circulating glucocorticoid levels, serum corticosterone was measured using a commercially available ELISA kit (Thermo Fisher). Blood was collected from mice in 1.5 ml Eppendorf tubes without heparin and allowed to clot at room temperature for 30 minutes. Samples were centrifuged at $13,000 \times g$ for 10 minutes, and serum was collected for analysis. In selected experiments, spleen and bone marrow tissue homogenates were also processed to assess local corticosterone content. Single-cell RNA sequencing was performed on sorted NK cells using the 10x Genomics Chromium 3' v3 platform. CD49b⁺NK1.1⁺ NK cells were isolated by flow cytometry from pooled spleen, bone marrow, and tumour samples of either ad libitum-fed, HFD-fed, or CRD + fasting mice. Prior to encapsulation, cells were stained with barcoded Total-Seq B antibodies (BioLegend) for sample multiplexing. Equal numbers of cells from each condition were mixed and resuspended at 700–1,300 cells/ μ l in PBS + 1% BSA, with viability >80% confirmed by trypan blue exclusion. Approximately 15,000 cells were loaded per lane. Libraries were prepared according to the 10x manufacturer's protocol and sequenced on an Illumina NovaSeq S4 (R1: 28 cycles; i7: 8 cycles; R2: 90 cycles). Raw reads were processed using Cell Ranger (10x Genomics). Low-quality cells with >20% mitochondrial transcripts and potential doublets were excluded. Normalization was performed using scran, clustering applied with the Louvain algorithm ($k = 30$), and imputation with MAGIC. All the previous work was carried out by Delconte *et. al* and made publicly available on GeoDatasets at GSE267467.

Single-cell RNA sequencing data were obtained from the GeoDatasets under accession number GSE267467. Raw count matrices and metadata were downloaded and imported into R (version 4.3) for analysis. Data were processed using Seurat v5.0. Cells were pooled into two groups according to their reported feeding status: ad libitum-fed versus fasted. Differential expression between ad libitum and fasted NK cells was performed in Seurat using the Wilcoxon rank-sum test, with default parameters unless otherwise specified. Genes were considered significantly differentially expressed at $p < 0.05$ after multiple testing correction (Benjamini–Hochberg). Differential expression results were visualised using ggvolc, an R package for creating enhanced volcano plots. Volcano plots displayed log₂ fold change against $-\log_{10}$ adjusted p -value, highlighting significantly up- or downregulated genes between feeding regimes.

2.2.4 - HD5 - Human Dataset 5: Treatment of immune and non-immune cells with methylprednisolone - GSE112101

RNA-seq data from nine highly pure primary human cell populations exposed in vitro to the glucocorticoid methylprednisolone or vehicle was obtained from GeoDatasets (GSE112101). The dataset comprised four hematopoietic cell types (circulating B cells, CD4⁺ T cells, monocytes, and neutrophils) and five non-hematopoietic cell types (endothelial cells, fibroblasts, myoblasts, osteoblasts, and preadipocytes), each isolated from a separate cohort of four unrelated healthy donors (biological replicates BR1–BR4). Hematopoietic cells were purified from peripheral blood using magnetic selection (negative selection for B cells, CD4⁺ T cells, and neutrophils; positive selection for monocytes), while non-hematopoietic cells were cultured until the early plateau growth phase before treatment. For hematopoietic cells, B cells, CD4⁺ T cells, and monocytes were incubated overnight before exposure, and neutrophils were cultured for four hours prior to treatment. Methylprednisolone was dissolved in ethanol (vehicle) and applied at a final concentration of 8,500 µg/L (22.7 µM) for methylprednisolone or 0.07% (15.57 mM) for ethanol. For each cell type, samples were collected at two time points after treatment (2 h and 6 h) into TRIzol reagent and frozen at –80 °C before RNA extraction and sequencing. This data was made publicly available by Franco *et. al.* at accession number GSE112101 on the GeoDatasets website ⁷¹.

Raw read count data were downloaded from GeoDatasets and restricted to CD4⁺ T cells and monocytes for analysis. Columns corresponding to these cell types were identified by name pattern matching and subset from the full dataset. Gene identifiers were assigned as row names, and raw counts were transformed to $\log_2(\text{count} + 1)$ values to stabilise variance and improve comparability between samples. Sample metadata were parsed from the column names, which followed the format *CellType_Time_Treatment_Donor*, to extract cell type, time point, treatment condition (methylprednisolone, MP; vehicle, VH), and donor ID.

Differential expression analysis was performed for CD4⁺ T cells using the limma package with a paired design, treating donor as a blocking factor to account for within-donor correlations. Treatment was coded as a factor with VH as the reference level. A design matrix was constructed, and the ‘duplicateCorrelation’ function was applied to estimate the intra-donor correlation. The model was fitted using lmFit with donor blocking and the estimated correlation structure, followed by empirical Bayes moderation (eBayes). All genes were ranked by moderated t-statistics for the methylprednisolone versus vehicle comparison, and the complete results table was extracted with the topTable function.

For genes of interest, including *TNFAIP3*, expression was visualised separately in each cell type by pooling the two time points, generating individual and combined plots, and saving them at publication resolution (600 dpi) for inclusion in figures.

2.2.5 - Public Database 1: ChIP-Seq Atlas

Transcription factor–target relationships were obtained using ChIP-Atlas (<https://chip-atlas.org>), a curated database of ChIP-Seq experiments from public repositories. The Target Genes website function was used to retrieve experimentally supported binding targets for transcription factors of interest. Briefly, the transcription factor of interest (e.g., *NR3C1* (Glucocorticoid Receptor)) was entered into the query interface, the “Target Genes” option was selected, and all available binding targets were downloaded. The resulting *NR3C1* target gene list was cross-referenced with genes identified in the hdWGCNA human network, created from NK Cell data, this was done to see what genes corresponded present in the network corresponded with the glucocorticoid receptor, *NR3C1*. For each overlapping gene, binding scores provided by ChIP-Atlas (reflecting aggregated binding signal strength) were extracted, ranked from highest to lowest, and visualized as a heatmap to assess relative transcription factor binding across target genes.

2.2.6 - Public Database 2: Gene Perturbation Reference via LINCS L1000

The Library of Integrated Network-based Cellular Signatures (LINCS) L1000 database was used to obtain reference gene expression signatures following genetic perturbation. For a given gene of interest (e.g., *CXCR4*), the search interface was used to retrieve lists of genes significantly upregulated or downregulated upon knockdown or knockout of that gene in relevant cell lines. These perturbation signatures were used as a reference to compare against genes of interest identified in the present study, aiding in the interpretation of potential regulatory and functional relationships.

2.2.7 - Public Database 3: eQTL and sQTL Analysis - Genotype-Tissue Expression (GTEx)

The Genotype-Tissue Expression (GTEx) project is a public human tissue resource linking genetic variation to gene expression and splicing across many tissues. In GTEx, RNA-seq data are normalised and residualised to remove technical and biological covariates (e.g. PEER factors, genotype PCs), and then cis-eQTLs are mapped by regressing gene expression residuals on nearby variant dosages (0/1/2) within ± 1 Mb using linear models (FastQTL) and adjusted for multiple comparisons. gtexportal.org Similarly, GTEx identifies sQTLs by modelling allele-dependent splicing variation against genotypes. In this thesis, I retrieved *TNFAIP3* eQTLs and sQTLs from the GTEx portal, and then overlaid the

variant coordinates onto my chromatin/accessibility datasets to hypothesise cis-regulatory impacts in immune cell and obesity contexts.¹²⁰

2.2.8 - HD6 - Human Dataset 6: 7-Day Fasting Proteomics Dataset

12 apparently healthy, non-smoking adults of mainly white-European ancestry (all but one participant). Exclusion criteria included any known disease and a percent body fat below 15% for females and 12% for males. No statistical methods were used to pre-determine sample size; however, the sample size is comparable to that used in our previous work. All participants provided written informed consent, and the study protocol was approved by the Norwegian School of Sport Sciences ethics committee (#15-220817).

Participants underwent a stringent 7-day fasting regimen in which only water intake was allowed. Fasting blood samples were collected in the morning 2 days prior to study onset, daily during the fasting period, and after 3 days of ad libitum food intake. Venous blood was drawn into EDTA tubes, kept on ice, centrifuged at 3,500 g for 10 min at 4 °C, transferred to Protein LoBind tubes, and stored at –80 °C until analysis.

Plasma protein profiling was performed using the Olink Explore 3072 platform, covering 2,923 unique protein targets across eight panels. This platform uses proximity extension assays (PEA), in which matched antibody pairs are labelled with complementary oligonucleotides. Upon binding to their target protein, the oligonucleotides hybridise and are amplified, with quantification by next-generation sequencing. Results were expressed as Olink's normalised protein expression (NPX) values, which are log₂-scaled.

Olink's standard quality control and normalization procedures were applied, including the use of incubation, extension, amplification, and plate controls. All NPX values, including those below the assay limit of detection, were retained to maintain a complete data matrix. This data was made available by Pietzner *et al* on the Zenodo repository¹²¹.

Processed values were downloaded, exported and analysed in Python v3.10.2. Subsequent filtering, statistical comparisons, and data wrangling were implemented in Python. Final processed datasets were exported to GraphPad Prism for graphing and figure preparation.

2.2.9 - Computational Methods used in Chapter 4

Additional computational methods are deployed in this chapter that were not used in Chapter 3. Any new methods utilised are detailed below.

2.2.10 – High Dimensional Weighted Gene Co-expression Network Analysis (hdWGCNA)

To uncover coordinated gene programmes in human natural killer (NK) cells during a weight loss intervention, I implemented hdWGCNA,¹²² a framework for co-expression network inference from high-dimensional single-cell data. hdWGCNA was applied to a Seurat object previously generated from PBMCs sampled before and after weight loss, and adipose tissue immune cells from lean, obese and weight loss mice as described in Chapter 3, Section 3.2.1 Datasets.

A pre-integrated .rds object was loaded into R and updated for compatibility with Seurat v5 using `UpdateSeuratObject()`. The scaled RNA assay expression matrix was explicitly converted into a standard matrix (`as.matrix`) to ensure consistency with downstream computations. Data were normalised using `NormalizeData()` and scaled using `ScaleData()`.

To enable cell-type-specific network construction, high-resolution identities were collapsed to broader categories by combining $CD56^{\text{bright}}$ and $CD56^{\text{dim}}$ NK cells under a unified “NK Cells” label. Each cell was annotated by timepoint (“Week 1 – Pre-WL” or “Week 6 – WL”) using string matching within the `orig.ident` metadata field. Although $CD56^{\text{bright}}$ and $CD56^{\text{dim}}$ subsets differ functionally, they were combined here to increase cell numbers and to allow identification of broader, conserved co-expression networks. It is important to note that $CD56^{\text{dim}}$ NK cells are also composed of several subsets of NK cells.

Gene selection was performed using `SetupForWGCNA()`, retaining genes expressed in at least 5% of NK cells. To reduce sparsity and stochastic dropout effects, transcriptionally similar cells were aggregated into metacells using `MetacellsByGroups()`, which applies a k-nearest neighbours algorithm to a GLMPCA-reduced space. A gene expression matrix for NK cells was then constructed with `SetDatExpr()`.

Pairwise Pearson correlations were computed and transformed into an adjacency matrix via soft thresholding, where the power (β) was selected in a data-driven manner using `TestSoftPowers()` to approximate a scale-free topology. Adjacency matrices represent finite graphs in which nodes are genes. To increase biological relevance and reduce spurious associations, a Topological Overlap Matrix (TOM) was computed with `ConstructNetwork()`. Unlike raw correlations, TOM incorporates both direct and shared neighbours, producing

more robust and modular structures. Hierarchical clustering of the TOM-derived dissimilarity matrix defined gene modules using the Dynamic Tree Cut algorithm.

Each module was summarised by its eigengene, defined as the first principal component of the module's expression matrix, using `ModuleEigengenes()`. Module eigengenes (MEs) were retrieved in both raw and harmonised forms using `GetMEs()`. Intramodular connectivity was assessed with `ModuleConnectivity()`, computing kME (eigengene-based connectivity) values for each gene. Hub genes were identified as those with the highest kME values using `GetHubGenes()`. Modules were visualised with `PlotKMEs()`.

Modules were scored per cell using UCell (`ModuleExprScore()`), a rank-based method robust to dropout, and visualised with `ModuleFeaturePlot()` and `ModuleRadarPlot()`. This systems-level approach enabled identification of co-regulated gene programmes underlying NK cell transitions during weight loss.

To integrate transcriptomic co-expression with protein-level interactions, module properties were combined with curated protein–protein interaction (PPI) data from the STRING database (version 11.5), restricted to *Homo sapiens* (taxon ID 9606). Interactions were retrieved using the STRINGdb R package and filtered to a confidence threshold of 400 or above. Protein identifiers were mapped to gene symbols, and unmatched entries were excluded. The PPI network was further restricted to genes expressed in our dataset.

The co-expression network, represented by the TOM, was aligned with the STRING-derived adjacency matrix. The element-wise product of these matrices produced an integrated TOM–PPI matrix, preserving only co-expression links also supported at the protein level. This yielded a biologically enriched representation of gene connectivity.

Comparative heatmaps were generated for each module using both the TOM and TOM–PPI matrices. For each gene, both kME and PPI degree (number of STRING-supported neighbours) were computed. Genes with high values for both were designated as robust hub genes. Scatter plots of kME versus PPI degree were used to highlight these dual hubs.

Ego-centric subgraphs were constructed for specific genes of interest such as *TNFAIP3* and *CX3CRI*, encompassing all genes within one or two edges. Subgraphs were visualised with force-directed layouts, with module membership indicated by colour and hub status by node size.

Module eigengenes were compared across groups such as pre- versus post-weight loss using Wilcoxon rank-sum tests. Differential module expression results were displayed in volcano plots, with $\log_2$ fold change against $-\log_{10}$ adjusted p-value.

To interpret module function, gene set enrichment was performed using Enrichr with the GO Biological Process 2023 database, accessed via the hdWGCNA package. The top enriched terms for each module were visualised in dot plots, where dot size reflected gene set size and colour indicated significance.

All plots were generated at publication-quality resolution in PNG format. Layouts used consistent node sizing, colour coding and annotation. A fixed random seed ensured reproducibility. Filtering thresholds and parameters were documented in full, and both standalone and comparative visualisations were assembled into multi-panel layouts to enable cross-module comparisons.

2.3.0 – Methods used in Chapter 5

This chapter uses computational techniques that were previously described in earlier chapters and are appropriate to the analysis required. The new techniques used are visualisation techniques and these are explained in figure legends.

2.3.1 - Datasets

Two new scRNA-seq PBMC datasets are utilised in this chapter (HD7 and HD8) with the primary aim of comparing HA20 to obesity. The third dataset used is the primary human obesity / weight loss dataset seen in previous chapters (HD2). Additionally, the proteomics dataset on NK cells and CD8 T cells are utilised for cross-referencing transcripts in this chapter with protein measurements from obese and lean mice (MD4). An overview of the datasets used are captured in Table 2.6.

Table 2.6 – Datasets used in Chapter 5

Dataset	Organism	Cell Types	Cohort	Intervention & Sampling	Tissue	Modality		Mouse
HD7		Immune Cells	HA20 patients with germline TNFAIP3 mutations (n=2 and healthy controls (n = 1)	1 Timepoint				Human
HD2		Immune Cells	Obese Females (n = 4)	Sampled before caloric restriction and after 6 weeks of 1100 calories per day				Blood
HD8		Immune Cells	1 Healthy Adult	1 Timepoint				Spleen
MD4		NK & CD8 T Cells	Obese and Lean (n = 4 & 3 respectively)	Obese=8 weeks HFD Lean=8 weeks SFD				scRNA-Seq
								Proteomics

Table 2.6 - Acronyms - NK – Natural Killer (cells) - T2D – Type 2 Diabetes - PBMC – Peripheral Blood Mononuclear Cells - WL – Weight Loss - HFD – High-Fat Diet - SFD – Standard-Fat Diet - RNA-seq – RNA sequencing

2.3.2 – HD7 - Human Dataset 7: A20 Haploinsufficiency Dataset

Peripheral blood was obtained from individuals with germline *TNFAIP3* (HA20) mutations and healthy controls after written informed consent. Collection and use of samples from HA20 patients and 36 healthy controls were approved by the Ethics Commission Halle (Saale) (project no. 2014-75) and by the National Human Genome Research Institute (NHGRI) Institutional Review Board. All procedures adhered to the Declaration of Helsinki. PBMCs were isolated by density-gradient centrifugation. Freshly isolated or cryopreserved PBMCs were used for single-cell encapsulation. Single-cell RNA-seq (and, where available, paired V(D)J) libraries were prepared on the 10x Genomics Chromium platform using the manufacturer's chemistry for PBMCs (5' kit v2 with human BCR/TCR amplification where applicable). Libraries were sequenced on an Illumina platform to recommended depth. Raw BCL files were demultiplexed and converted to FASTQ, reads were aligned to the GRCh38 reference and UMIs/cell barcodes were quantified with the Cell Ranger pipeline. Resulting gene-by-cell count matrices were imported into R as Seurat objects. Upstream QC (empty-drop removal, ambient RNA correction, low-quality cell filtering) followed the originating dataset's published criteria, the analysis in this dataset began from pre-processed Seurat objects. All this work was carried out by Schultheiß *et. al* and the data was made available at the EMBL-EBI ArrayExpress website under accession numbers E-MTAB-12485 and E-MTAB-12491¹²³.

The scRNA-seq data was downloaded from the website above using the accession numbers provided and the data was loaded into R version 4.3.0. Three Seurat objects were integrated: a HA20 PBMC dataset (HA20), the human weight loss scRNA-seq data described in previous chapters, and in addition to this a small publicly available PBMC dataset was integrated as an additional healthy control dataset, this is due to the A20 haploinsufficiency dataset only containing one healthy control individual. A standard pre-processing and integration methods were used as described in chapter 3. All downstream processing and analysis methods have been previously described in previous chapter methods.

2.3.3 – HD8 - Human Dataset 8: 3K PBMC Healthy Dataset

The scRNA-seq data were obtained from the 10x Genomics “3 k PBMCs from a Healthy Donor” dataset (commonly referred to as “pbmc3k”), freely available via the 10x Genomics support portal. These peripheral blood mononuclear cells were derived from a single healthy donor and captured using the Chromium Single Cell 3' Gene Expression platform (GEM-based microfluidics). Approximately 2,700 cells were detected after sequencing; the libraries

were sequenced on an Illumina NextSeq 500. The sequencing configuration included a 98 bp read for transcript capture, alongside indexing reads encompassing the sample barcode (8 bp), the GEM (cell) barcode (14 bp), and a 10 bp UMI. Raw sequencing data were processed using the Cell Ranger pipeline, generating feature-barcode (UMI) count matrices.

The resulting filtered count matrix was imported into R (version 4.3.0) and loaded using the `Read10X()` function, followed by construction of a Seurat object, as documented in the Seurat guided clustering tutorial. No new preprocessing methods were used beyond the standard steps already applied in previous chapters. All downstream analyses, including QC filtering, normalisation, dimensionality reduction, clustering, and cell-type annotation, follow the same pipelines and methods established in earlier chapters.

2.3.4 – Integration Method

To investigate shared transcriptional programs across genetic and environmentally driven immune dysregulation, three single-cell RNA-sequencing (scRNA-seq) datasets were integrated, peripheral blood mononuclear cells (PBMCs) from individuals with germline *TNFAIP3* haploinsufficiency (HA20) and healthy controls (E-MTAB-12485, E-MTAB-12491), PBMCs from the previously seen human weight loss cohort (described in Chapter 3), and the 10x Genomics “3k PBMC” dataset from a healthy donor, included to provide an additional reference control due to the limited number of healthy individuals in the HA20 dataset.

PBMCs from HA20 patients were collected under informed consent and ethical approval from the Ethics Commission Halle (Saale) (project no. 2014-75) and the NHGRI Institutional Review Board. Sample collection, processing, and scRNA-seq library preparation were performed as described by Schultheiß et al. Briefly, PBMCs were isolated by density-gradient centrifugation, and 5' scRNA-seq (and, where available, paired V(D)J) libraries were prepared on the 10x Genomics Chromium platform and sequenced to recommended depth on an Illumina platform. Count matrices generated using Cell Ranger were obtained from ArrayExpress and imported into R (version 4.3.0) as Seurat objects.

The 3k PBMC healthy dataset was downloaded from the 10x Genomics support portal. Approximately 2,700 PBMCs were sequenced on the Chromium Single Cell 3' Gene Expression platform and processed using Cell Ranger with the `--cells=3000` parameter. Filtered count matrices were imported into R and converted into Seurat objects following the Seurat guided clustering workflow.

The human weight loss dataset was incorporated as described in Chapter 3. In brief, PBMCs from obese individuals before and after 6 weeks of caloric restriction (1100 calories per day) were profiled using the 10x Genomics Chromium platform and analysed with the same preprocessing pipeline (quality control filtering, normalisation, clustering, and annotation).

To integrate data generated from distinct experiments and minimise batch effects, Canonical Correlation Analysis (CCA) was used as implemented in Seurat (version 4.3.0). Highly variable genes were selected independently from each dataset and used as input features to identify canonical components capturing shared sources of variation. Cells were projected into a shared low-dimensional space defined by the top canonical components, followed by mutual nearest neighbour (MNN) anchoring to align datasets and correct for technical variation. The resulting integrated object was used for downstream analyses, including differential gene expression, module scoring, and network mapping, as described in earlier chapters.

3.0 - Chapter 3 – Characterisation of *TNFAIP3* levels in obesity, weight loss and T2D

Aims: This chapter aims to comprehensively characterise *TNFAIP3* expression across single-cell and bulk RNA-sequencing datasets, as well as at the protein level using proteomics, in both human and murine models. Expression patterns were examined across lean, obese, and weight loss conditions, and in the context of T2D, to better understand how *TNFAIP3* is altered in obesity, weight loss and metabolic disease. Understanding how *TNFAIP3* behaves across immune cell subsets and tissues in metabolic disease can reveal whether chronic inflammation in obesity reflects a failure of this regulatory axis and whether weight loss can restore it. Such knowledge would identify a mechanistic link between metabolic stress and persistent immune activation, highlight potential biomarkers of immune resetting during weight loss.

3.1 – Chapter 3 Introduction

Obesity is an established risk factor for a wide spectrum of metabolic disorders, cancers, neurodegenerative diseases, and a diminished quality of life.¹⁰⁴ At its core, the pathophysiology of obesity is driven by chronic, low-grade inflammation, a state perpetuated by cytokine imbalances and persistent activation of the NF- κ B signalling pathway.²¹ This occurs *via* a number of pro-inflammatory mechanisms, for example, NF- κ B activated due to excess free fatty acids (FFAs) drive production of cytokines like TNF, IL-1 β and IL-6 which interfere with insulin receptor substrates impairing insulin signalling.³⁶ To understand the importance of NF- κ B in metabolic disease development, one can look at how inhibition of NF- κ B via therapeutic or genetic interventions leads to improvements in systemic inflammation and metabolic readouts, e.g. salicylates inhibit NF- κ B and reverse many of the metabolic issues caused by NF- κ B.³⁵ These inflammatory processes disrupt cellular homeostasis, leading to widespread metabolic dysfunction. From an evolutionary perspective, natural selection favoured traits that enhanced survival during periods of nutrient and energy scarcity, as prolonged caloric excess was historically common.¹²⁴ Mechanisms to store energy efficiently and prioritise survival under conditions of hypoglycaemia were advantageous. For example, while short-term hyperglycaemia is tolerable, hypoglycaemia poses an immediate threat to survival, leading to the evolution of robust systems for energy storage and utilisation.¹²⁵ However, in modern societies where caloric surplus is widespread, these adaptive mechanisms have become counter-productive, contributing to the global epidemic of metabolic disorders associated with obesity.¹²⁵ In the context of metabolism and immunity, these two systems are deeply intertwined.¹²⁴ A well-

functioning immune system relies on adequate energy resources, while immune signalling pathways regulate key metabolic processes.

In obesity, the dysregulation of metabolism profoundly impacts the immune system, impairing its ability to combat infections and cancer effectively while simultaneously driving chronic inflammatory states that exacerbate comorbidities such as type 2 diabetes and cardiovascular disease.¹²⁶ Central to this inflammatory cascade is the NF- κ B pathway, a master regulator of pro-inflammatory gene expression.²¹ The ubiquitin-editing enzyme A20, encoded by the *TNFAIP3* gene, serves as a critical negative regulator of NF- κ B signalling, ensuring the timely resolution of inflammation and maintaining immune homeostasis.¹²⁷

TNFAIP3 and its encoded protein, A20, are expressed in all immune cells, as well as in most CD45-negative cells (non-immune cells), underscoring its essential role across various cells and tissues. However, its expression in immune cells is noticeably higher especially in lymphocytes populations as depicted in Figure 3.1.

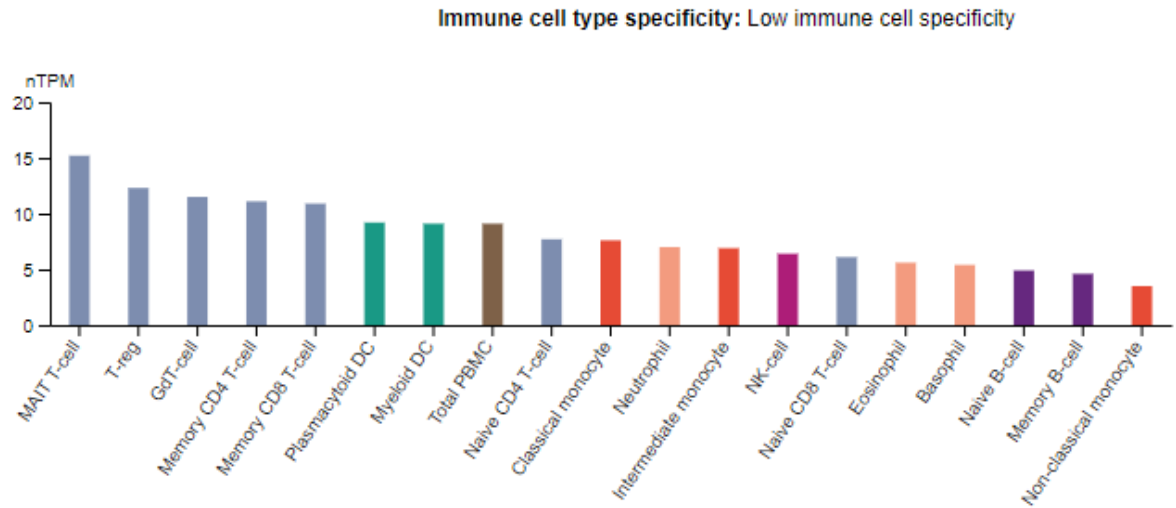


Figure 3.1 – Expression of *TNFAIP3* (A20) mRNA (nTPM) across sorted human immune cell populations, retrieved from the Human Protein Atlas (HPA) immune cell RNA dataset. *TNFAIP3* shows relatively low immune cell specificity, with highest expression observed in MAIT cells, Tregs, and $\gamma\delta$ T cells, and moderate expression across most other lymphocyte and myeloid subsets. Data reflect normalised transcripts per million (nTPM) as reported by the HPA.¹²⁸

Dysregulation of A20 has been implicated in a host of inflammatory and metabolic diseases, making it a pivotal molecule in understanding the immune-metabolic interface in obesity. Moreover, A20’s dynamic regulation in response to metabolic changes, such as those occurring in obesity and weight loss, highlights its importance as both a mediator and potential biomarker of metabolic status. GWAS studies have highlighted A20 as being

associated with numerous conditions such as rheumatoid arthritis (RA) and juvenile idiopathic arthritis, systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), coeliac disease, psoriasis, type I diabetes, Sjogren's syndrome, coronary artery disease, rheumatic heart disease, and systemic sclerosis.^{129,42} As well as SNPs being linked to multiple inflammatory and autoimmune conditions, loss of function mutations in *TNFAIP3* led to a newly described autoinflammatory disease known as A20 haploinsufficiency in 2016.⁴⁰ A20 haploinsufficiency is a severe childhood-onset inflammatory disease characterised by heterozygous germline loss of function mutations in *TNFAIP3*, this results in a variety of clinical manifestations with early-onset systemic inflammation showing as fever, ulcers, skin lesions and increased inflammatory markers (IL17, TNF, IL-6) in the blood.^{123, 40}

The aim of this chapter was to investigate *TNFAIP3* in obesity by characterising the expression patterns of *TNFAIP3* and its encoded protein A20 in immune cells across various tissues under distinct metabolic conditions: lean, obese, and during weight loss states driven by caloric restriction or fasting. This analysis was done by exploiting several different publicly available datasets of humans and mice, as well as data generated from human volunteers participating in weight loss trials and in house *in vivo* experiments. In doing so this work highlights the changes in expression levels of this gene and lays the groundwork for further exploration of its mechanisms, particularly within T and NK cells. Establishing how *TNFAIP3* expression levels change in response to obesity and weight loss, provides key insights into cell dysfunction in obesity and how it can be restored in weight loss.

3.2.0 – Chapter 3 Results

To determine whether *TNFAIP3* expression is altered in obesity, we first examined its levels in sorted lymphocyte populations from lean and obese humans. NK cells and $\gamma\delta$ T cells were selected as representative innate-like lymphocytes because of their established sensitivity to metabolic cues and their prominent roles in tissue immunoregulation. This exploratory analysis provided an initial indication that *TNFAIP3* is downregulated in obesity, motivating a broader and more detailed investigation across additional cell types, tissues, and species.

3.2.1 - *TNFAIP3* is downregulated in obesity across species and tissues

To investigate the impact of obesity on *TNFAIP3* in blood lymphocytes, I analysed a cell-sorted bulk RNA-sequencing dataset comprising of NK cells and $\gamma\delta$ T cells. Lean and obese human donors were sampled to identify differences in gene expression. *TNFAIP3* expression was downregulated in obese donors across both NK and $\gamma\delta$ T cells. Due to the small sample size (n=2 per group), statistical testing was not performed. However, visualisation tentatively suggested a trend, warranting the analysis of additional datasets to confirm these observations. As mentioned in the methods section, it is not known whether these individuals were metabolically healthy or unhealthy.

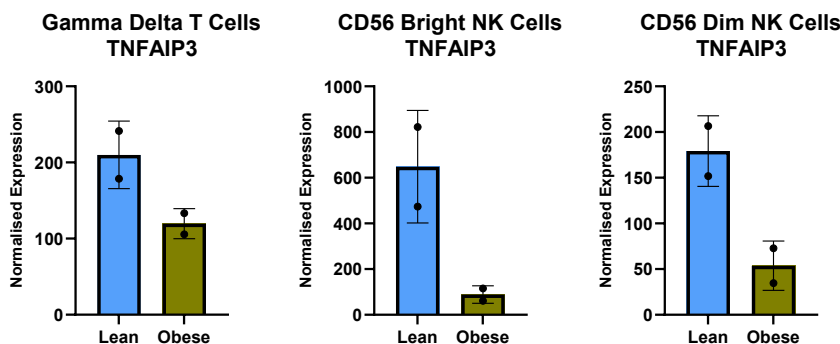


Figure 3.2 – Bulk RNA-seq dataset of human cell subsets from blood, CD56 Dim NK Cells, CD56 Bright NK Cells and Gamma Delta T Cells showing *TNFAIP3* expression in lean and obese groups (n = 2)

3.2.2 - *TNFAIP3* is downregulated in immune cells in obesity in mice in adipose, liver and blood

A scRNA-seq experiment was designed to explore the effects of obesity on *Tnfaip3* expression in immune cells in mice. A weight loss group was also included as well as the lean control group, the groups consisted of 8-week-old mice on standard-fat diet (SFD), high-fat diet (HFD) and the weight loss group consumed HFD for 6 weeks before returning to SFD for 2 weeks to lose weight, as shown in methods section 2.1.6. Immune cells (CD45+

cells) from blood, adipose, liver and muscle were sorted and scRNA-seq was performed on these cells.

Metabolomics was also performed but for the purpose of this study it is not necessary to discuss this other than showing that adipose tissue exhibited the highest degree of differences between groups at a metabolomic level and thus could also be a centre of metabolically driven inflammatory changes. The PCA plot in Figure 3.3, highlights the grouping of the mice by group indicating clear metabolic signatures for each condition in the adipose. Whilst patterns existed in the liver and blood tissues at a metabolomic level, the differences were not as pronounced as adipose tissue shown in Figure 3.3. Liver and plasma metabolomics are shown in Supplemental Figure 3.1 at the end of the document. The observed variation was primarily driven by changes in fatty acids such as oleic acid which was significantly upregulated in weight loss versus lean and obese mice. The observed variation was primarily driven by changes in fatty acids: in particular, oleic acid was markedly higher in weight loss mice than in lean or obese mice ($p < 0.0001$, one-way ANOVA with post-hoc comparisons) shown in Supplemental Figure 10.3. Whilst metabolomic datasets are not analysed again within the dataset, I think it is important to note that distinct metabolic profiles can occur within short time frames but most importantly, two weeks of weight loss can alter the metabolome in the adipose tissue. Given that the metabolome and the immune system are intrinsically linked, this is important observation.

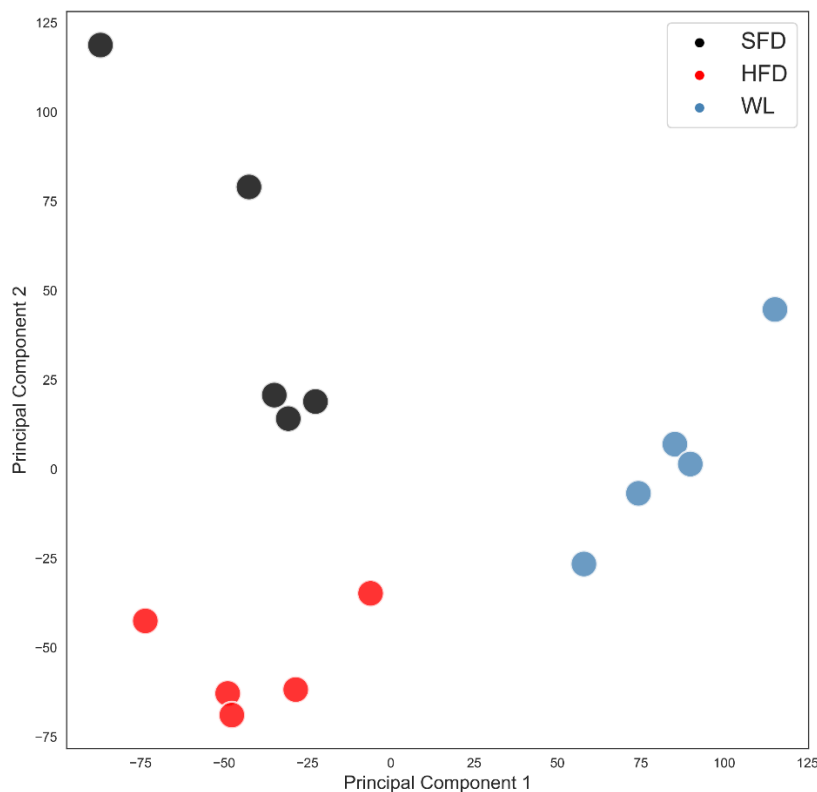


Figure 3.3 – Principal component analysis (PCA) of adipose tissue metabolite profiles revealing separation by diet group. Each point represents one mouse, coloured by group: black = lean (SFD), red = obese (HFD), blue = weight loss (WL). Axes show the first two principal components. Clustering indicates that weight loss mice form a distinct metabolic state compared to lean and obese mice.

Given the altered metabolome differences between groups as depicted in Figure 3.3, changes in inflammatory profiles were likely given known immune responses to changes in metabolism.¹²⁶ To elucidate the key changes at across all immune cells at the transcriptional level, differential gene expression was performed on all cells from lean mice vs all cells from obese mice to look for changes in key changes in the NF- κ B pathway. NF- κ B related genes were taken from an established database (KEGG) and overlaid onto the volcano plot in Figure 3.4. *Tnfaip3* was significantly downregulated in obese mice. Several other key NF- κ B genes were also trending down in obesity, suggesting a downregulated NF- κ B response including key transcription factors such as *Nfkb1* (Figure 3.4).

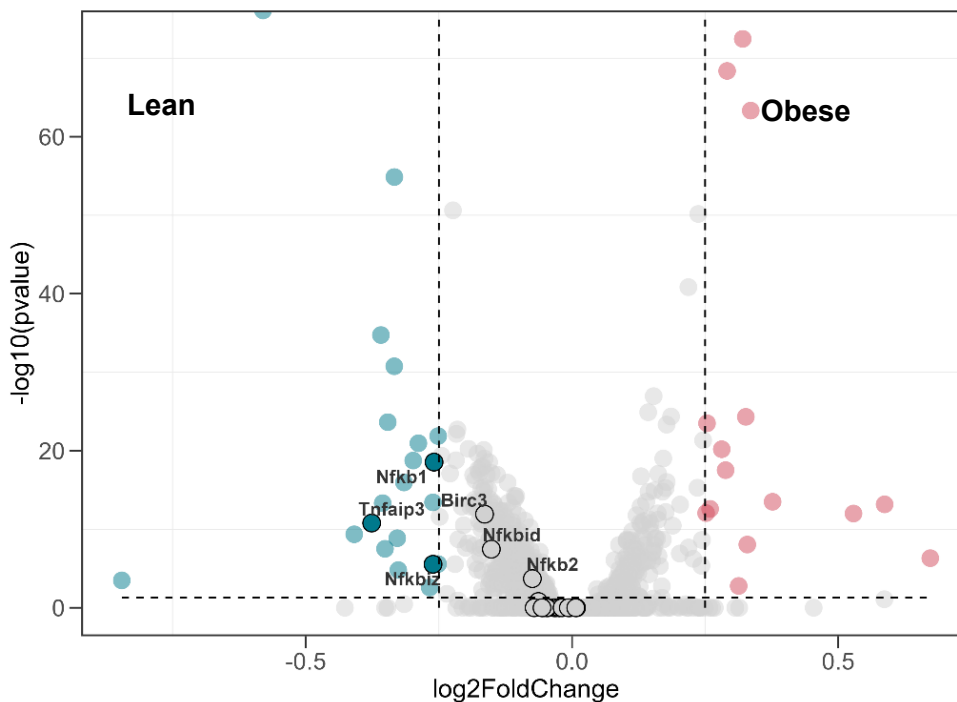


Figure 3.4 – Volcano plot showing differentially upregulated and downregulated NF- κ B genes at global transcriptome level in adipose tissue CD45⁺ cells, genes with a negative log₂ fold change are downregulated in obesity versus lean immune adipose immune cells. All NF- κ B genes are shown with a black ring around it and if significantly altered also include a label with the gene name – MD1 Dataset

Significantly downregulated genes in obesity included *Tnfaip3*, a negative regulator of NF- κ B, along with *Nfkb1* and *Nfkbiz*, both key transcriptional regulators within the NF- κ B pathway. Additionally, genes such as *Birc3*, *Nfkbid*, and *Nfkb2* trended toward downregulation, though these did not reach statistical significance. These genes are also involved in inflammatory signalling and NF- κ B modulation, suggesting a broader suppression of this regulatory axis in obesity. *Tnfaip3* being a central anti-inflammatory modulator of NF- κ B signalling was chosen to investigate in further detail given its significant roles across many diseases, apoptosis and inflammatory signalling. *Tnfaip3* expression was ubiquitous across the immune cell population in adipose tissue, except for in neutrophils which seemed to produce very little *Tnfaip3* despite been shown to express it in the Human Protein Atlas in Figure 3.1. Innate lymphoid cells (ILCs) along with Cd4⁺ and Cd8⁺ T cells produced large amounts of *Tnfaip3* whilst B cells and myeloid cells typically produced much less. The results require careful consideration, on one hand *Tnfaip3* is downregulated and therefore, one may expect an upregulation of several key pro-inflammatory NF- κ B genes. However, there seems to be a dysregulation at all levels of signalling, both pro and anti-inflammatory genes suggesting a general disruption caused by obesity. Additionally, NF- κ B is regulated post translationally via PTMs such as phosphorylation and thus it is no surprise not to see significant changes in NF- κ B transcripts.

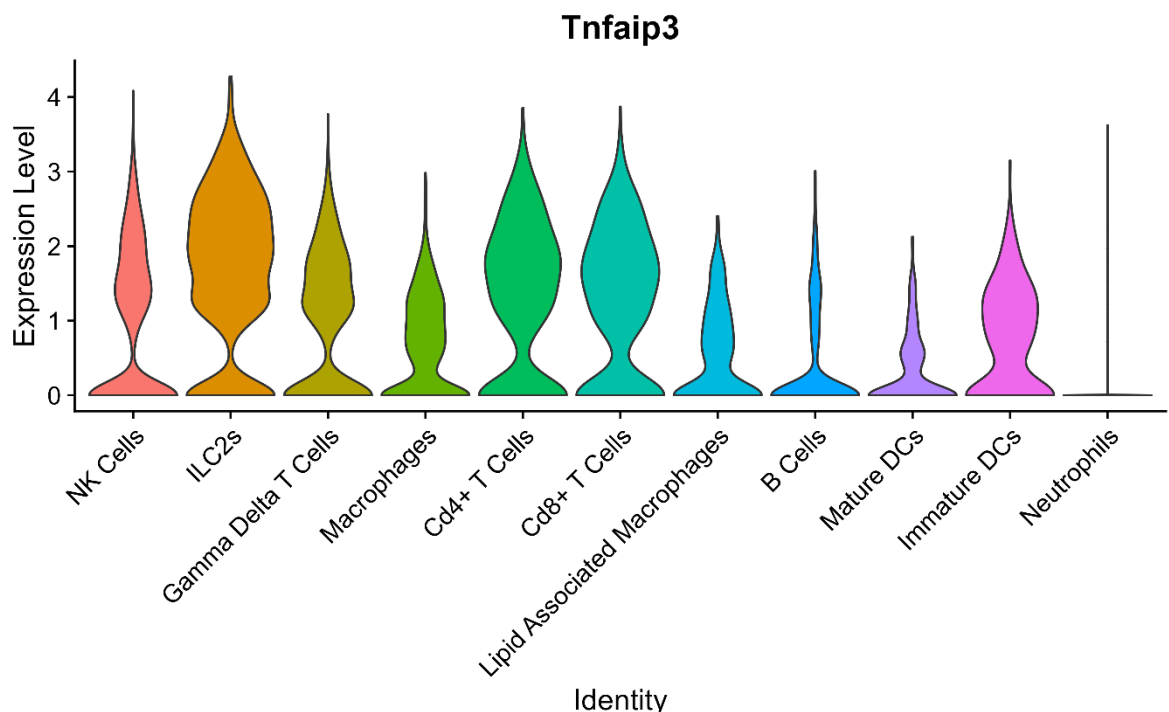


Figure 3.5 – Violin plot showing *Tnfaip3* expression across CD45⁺ cells in adipose tissue of MD1 dataset, each cell type is shown in a different colour simply to highlight differences, colour does not signify anything other than showing different cell types

Liver and blood expression also exhibited a downregulated expression of *Tnfaip3* in obesity as depicted in Figure 3.6 and Figure 3.7. The differences here are subtle but still clear. Liver immune cells produced significantly less *Tnfaip3* in general compared to immune cells in adipose and blood, and this is irrespective of what treatment group or cell type the cells were examined suggesting all immune cells in the liver produce less. Despite low expression of *Tnfaip3* transcripts, obesity drives an even lower expression when compared to lean cells.

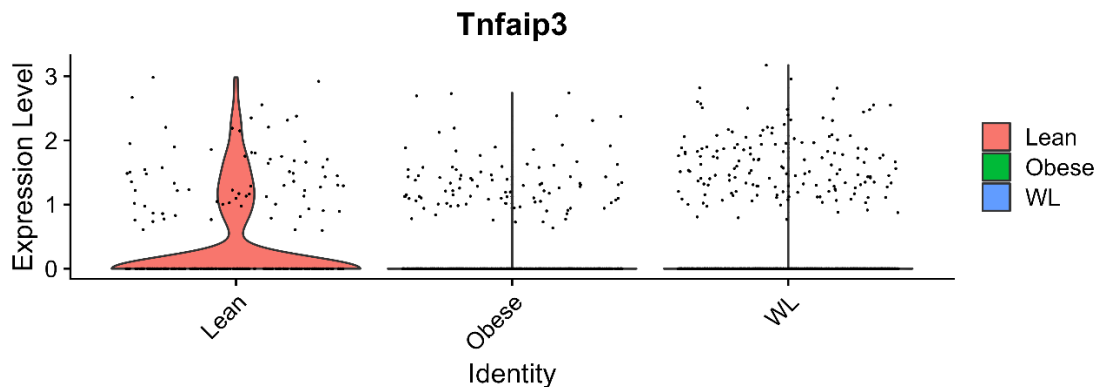


Figure 3.6 – *Tnfaip3* expression in liver CD45+ cells grouped by treatment groups, lean, obese and weight loss – MD1 Dataset, the dots represent cells and their expression levels. No violin is produced in Obese or weight loss mice showing a downregulation versus lean mice

Blood immune cells produced large amounts of *Tnfaip3* and demonstrated a downregulated expression pattern in keeping with adipose tissue and liver, plot shown in Figure 3.7. Analysis of the immune cells from muscle showed there was no significant changes across any cell type in muscle. Positive and negative $\log_2$ fold-change values were symmetrically distributed around zero, indicating minimal overall biological shift in *Tnfaip3* expression, indeed, the fold-changes observed were extremely small in magnitude. These data, therefore, indicate that *Tnfaip3* is not altered in resident immune cells in muscle tissue.

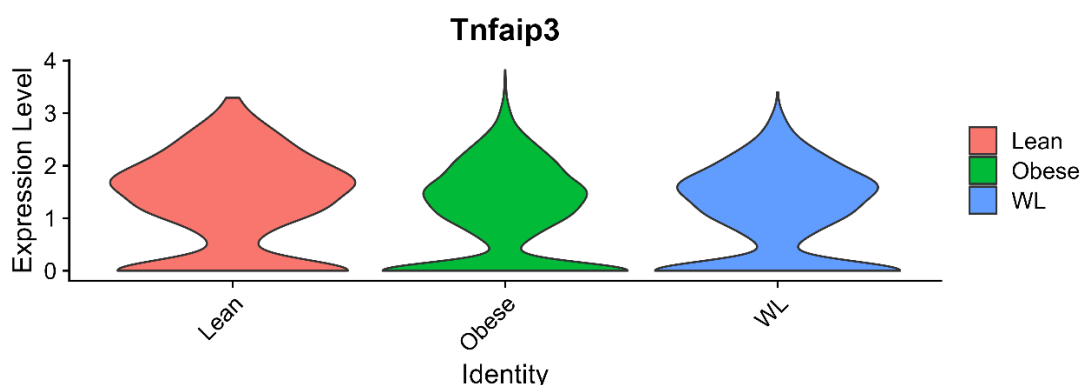


Figure 3.7 – *Tnfaip3* expression levels in CD45+ immune cells in the blood grouped by treatment group – MD1

The results from this experiment show *Tnfaip3* is downregulated in obesity as shown in human NK cells and $\gamma\delta$ T cells from blood but also in three tissues in mice with the biggest changes in expression being observed between lean and obese CD45+ immune cells in the adipose tissue. Significant metabolome differences were also observed in the adipose tissue. Therefore, the changes could be considered systemic with varying degrees of expression changes across groups.

3.2.3 - Integration of murine adipose scRNA-seq datasets

Whilst the results seemed to have a strong indication that *Tnfaip3* is indeed downregulated across multiple tissues in mice and in blood in humans, I sought to explore *Tnfaip3* expression in publicly available scRNA-seq datasets containing immune cells from adipose tissue in lean, obese and weight loss immune cells. This would both confirm levels of *Tnfaip3* are downregulated via independently generated datasets but also to explore the effects of weight loss on *Tnfaip3* expression. To do this the in-house adipose scRNA-seq dataset (MD1) introduced in the previous section was integrated with the adipose datasets GSE141036 (MD3) and GSE182233 (MD2) in Seurat to create a unified adipose cell atlas of lean, obese and weight loss lymphocyte cells (excluding B cells). Integration allowed for multiple datasets to be brought together and allows one to look at shared cell states across different types of diet and length of time on diet. This added robustness to the findings. The weight loss method in each dataset is varied, with one dataset having a 70% caloric restriction HFD diet of what the mice were eating during their HFD *ad libitum* phase meaning the same diet (HFD) was used but just calorie restricted. The in-house dataset involved a switch from HFD to SFD which induces weight loss over a two-week period. The third dataset utilised a switch from HFD to LFD which also induced weight loss over a 9-week period.

Seurat in R was utilised to create a unified scRNA-seq dataset, with only lymphocyte populations kept (excluding B cells). The integration method is shown in Figure 2.7 in the methods section. 8 cell clusters were identified using known gene markers as shown in Figure 3.8. All cell clusters were verified using a variety of additional markers (not shown), cross-referenced against literature.

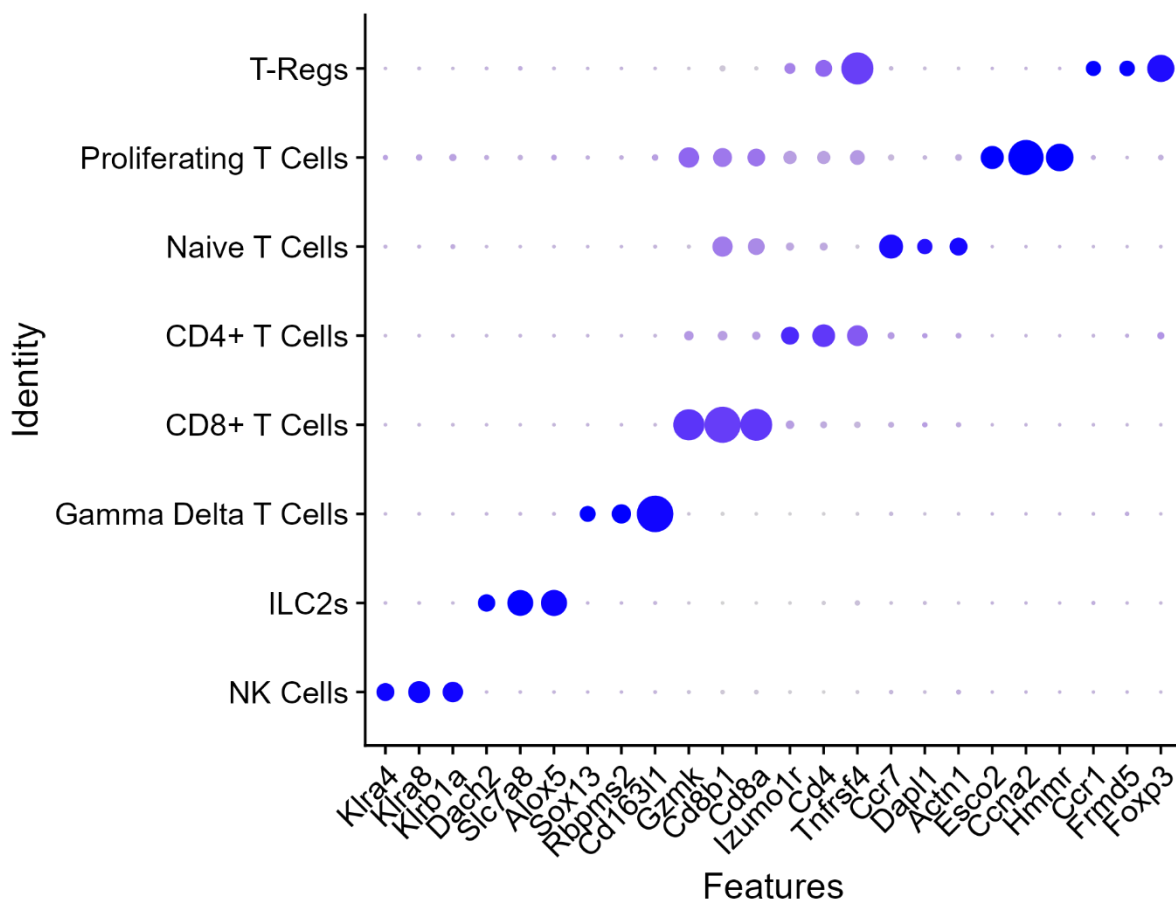


Figure 3.8 – Dot plot showing the top three marker genes per cell cluster (determined via cluster-wise differential expression) in the integrated scRNA-seq dataset. Each row is a cluster, each column a marker gene; dot colour represents the average expression level within that cluster, and dot size reflects the percentage of cells in that cluster expressing that gene. Clustering is independent of condition (Lean, Obese, Weight Loss).

Across groups (lean, obese and weight loss), the proportions of cell types change significantly, many of these changes have been established and captured in the literature previously such as loss of T-Regs in obesity and expansion of Cd8+ T cells in obesity in adipose. Along with these losses or gains of cell populations, there were also significant transcriptional shifts in the populations, ILCs in lean adipose tissue undergo a transcriptional shift with progression of obesity characterised by high expression of the gene *Txnip* which has been associated with redox reactions and glucose metabolism. Changes in cell proportions are highlighted in Figure 3.9.

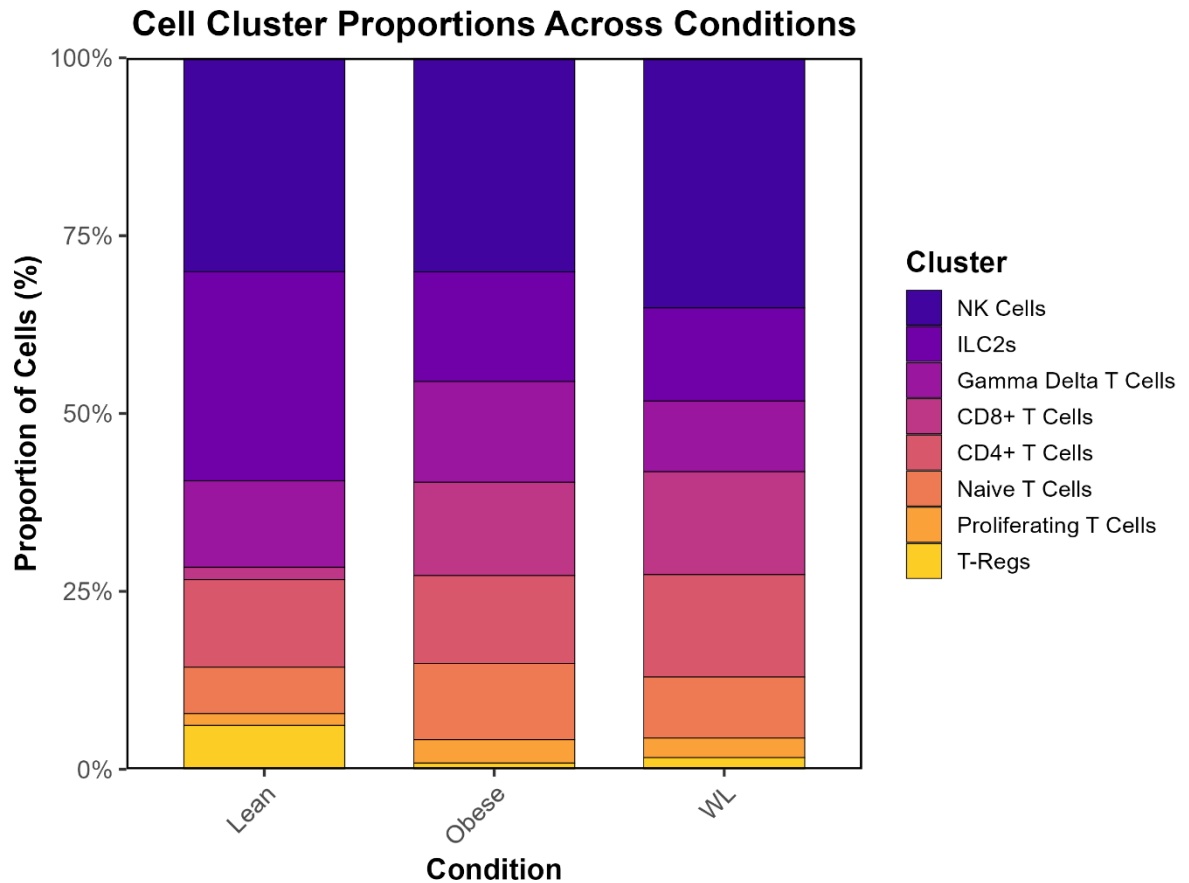


Figure 3.9 – Cell cluster proportions across metabolic conditions in adipose tissue - Stacked bar plot showing the relative proportions of immune cell clusters identified from integrated single-cell RNA-seq data of human adipose tissue. Data were combined from three independent datasets (MD1, MD2, and MD3) and grouped by condition: Lean, Obese, and Weight Loss (WL). Major immune populations are displayed as a proportion of total cells within each condition, including NK cells, ILC2s, $\gamma\delta$ T cells, CD8⁺ T cells, CD4⁺ T cells, naïve T cells, proliferating T cells, and T regulatory cells (T-regs).

In the integrated dataset, the clusters with the highest expression of *Tnfaip3* were T-Regs and ILC2s (Figure 3.10) which also happen to be the clusters with the biggest reduction in cell proportions in obesity in the adipose tissue as shown in Figure 3.9.

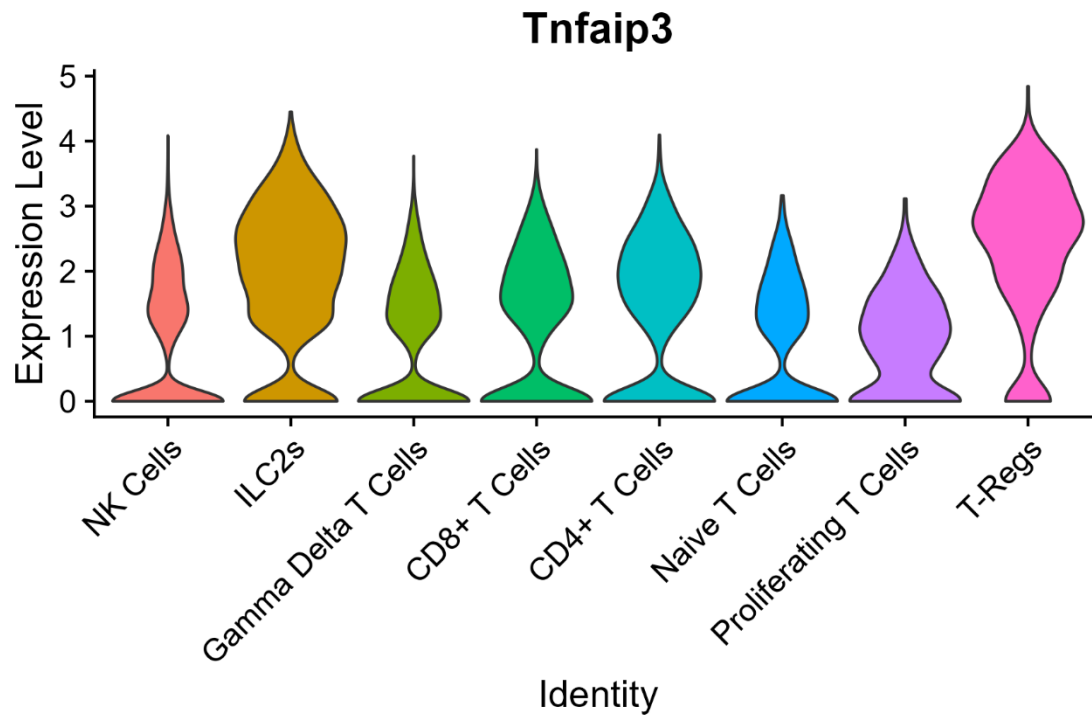


Figure 3.10 – *Tnfaip3* expression across cell clusters in integrated dataset, this includes all conditions, colour is used to depict different cell types

3.2.4 - Validation of *Tnfaip3* expression in lean and obese immune cells

The integrated dataset as stated contains cells from lean, obese and weight loss mice from three datasets, the lab generated scRNA-seq dataset (MD1) and publicly available datasets GSE141036 (MD3) and GSE182233 (MD2). At a cell cluster level, differential expression was performed across all cell types and $\log_2$ fold changes calculated to understand at a cellular level what is occurring, plot shown in Figure 3.11. The biggest downregulation of *Tnfaip3* across these cell types (adjusted p -value = 0.014, Wilcoxon Rank Sum Test) was seen in T-regs which are known to die prematurely in obese adipose. ILC2s also saw a large downregulation of the gene (adjusted p -value = 0.02, Wilcoxon Rank Sum Test), these were the only clusters with significantly downregulated *Tnfaip3*. However, across the whole integrated dataset the trend is that of a downregulated one. These results show that across multiple datasets the phenotype is consistent and *Tnfaip3* is downregulated across multiple cell types in murine adipose tissue.

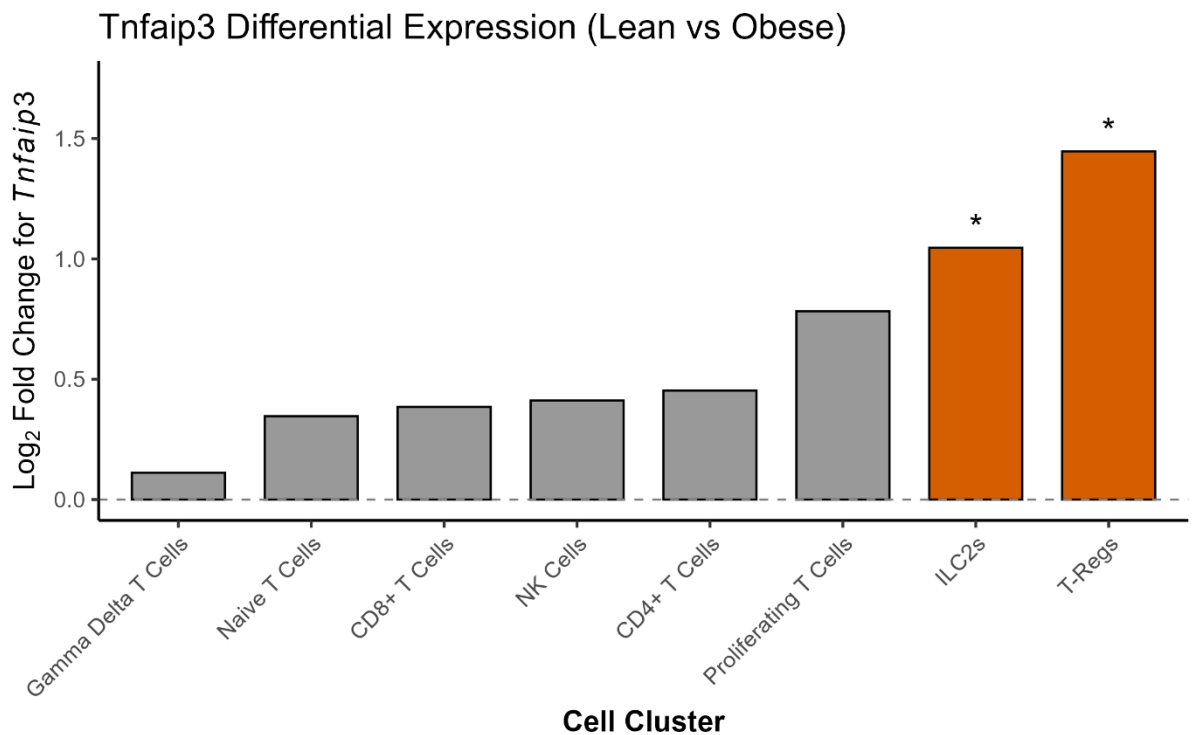


Figure 3.11 – *Tnfaip3* expression in cell types in integrated scRNA-seq dataset in lean versus obese cells calculated using Wilcoxon rank sum test – positive log₂ fold changes represent that *Tnfaip3* is upregulated in this cell type in lean cells versus obese, statistically significant changes in *Tnfaip3* expression are depicted with coloured bars with a ‘*’ to highlight significance (adjusted-p value < 0.05)

3.2.5 – Weight Loss Restores *TNFAIP3* in murine adipose CD45+ Immune Cells

Weight loss is a proven intervention strategy for improving circulating inflammatory markers associated with obesity ¹⁰⁶. Utilising the integrated dataset differential gene expression was performed to check if weight loss recovered *Tnfaip3* expression across the cell types. Significant improvements were observed in Cd4+ T cells, Cd8+ T cells and in ILC2s whilst T-Regs showed a large but non-significant upregulation, likely due to the low number of T-Regs present for the statistical analysis in obesity and weight loss (Figure 3.12).

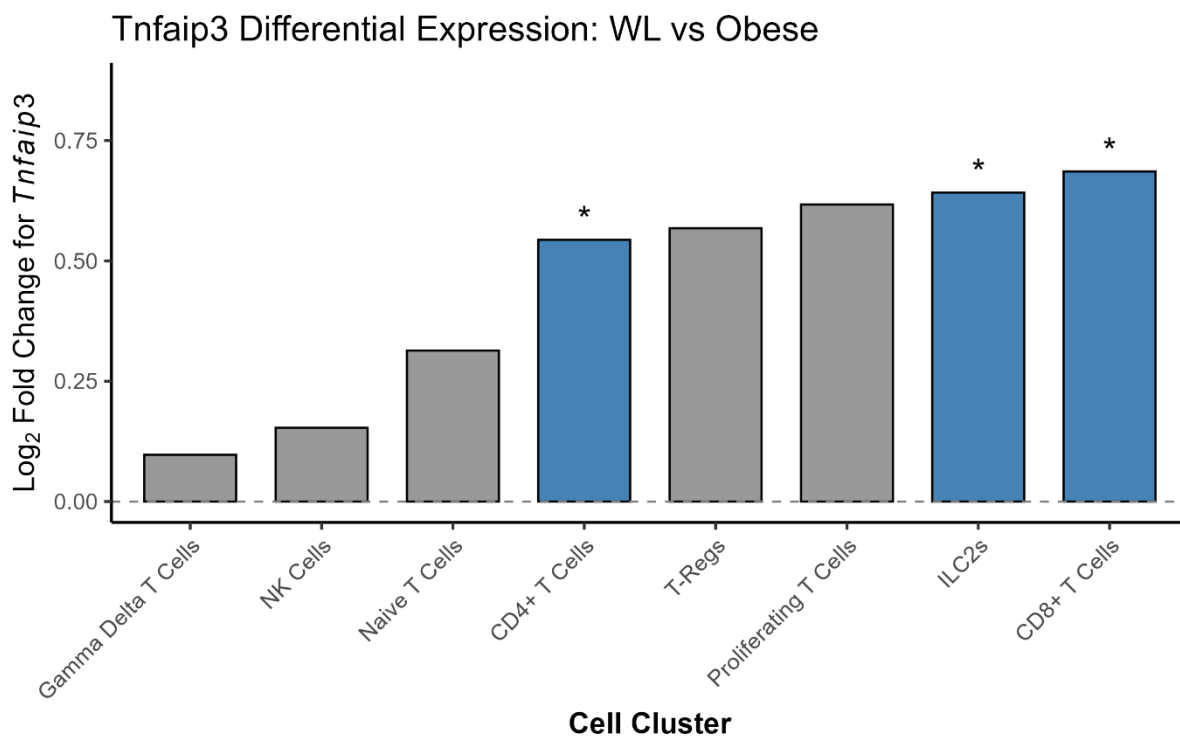


Figure 3.12 – *Tnfaip3* expression in cell types in integrated scRNA-seq dataset in weight loss versus obese cells calculated using Wilcoxon rank sum test – positive log₂ fold changes represent that *Tnfaip3* is upregulated in this cell type in weight loss cells versus obese, statistically significant changes in *Tnfaip3* expression are depicted with coloured bars with a ‘*’ to highlight significance (adjusted-p value < 0.05)

Given that the MD2 dataset was the longest duration of weight loss out of the three datasets, it was analysed separately to check the impact of longer-term weight loss (9 weeks) versus shorter periods, for example, two weeks for the dataset generated in-house (MD1). Viewing changes in this individual dataset paints a very different picture to the integrated dataset. In the integrated dataset, Gamma Delta T cells had the smallest upregulation of *Tnfaip3* in response to weight loss, however, in the MD2 dataset they had the largest change (Figure 3.13). Similarly, NK cells were also significantly altered with respect to *Tnfaip3* in the MD2 dataset but not in the integrated dataset. This highlights that dataset specific changes are present, and that integration can dilute some of the findings, this is a limitation of integration, however, a strength of integration is its increased cell numbers which is important where cell counts are very low as is the case with T-Regs. Integration of heterogeneous datasets can introduce a Simpson’s-paradox effect ¹³⁰, whereby subgroup-specific expression patterns (e.g. gene upregulation in one dataset) are diluted or even reversed in the pooled analysis. Despite this, the upregulation is present in every cell type.



Figure 3.13 - *Tnfaip3* expression in cell types in MD2 (Hasty Dataset) only - scRNA-seq dataset in weight loss versus obese cells calculated using Wilcoxon rank sum test – positive log₂ fold changes represent that *Tnfaip3* is upregulated in this cell type in weight loss cells versus obese, statistically significant changes in *Tnfaip3* expression are depicted with coloured bars with a ‘*’ to highlight significance

3.2.6 - Weight Loss in human PBMCs upregulates *TNFAIP3* (HD2)

As demonstrated already in this chapter, *TNFAIP3* is downregulated in immune cells in obesity in human PBMCs (HD1: n=2) and recovered at least in murine adipose tissue with weight loss (MD1, MD2, MD3). To validate that human weight loss also recovered *TNFAIP3* expression an experiment was designed (HD2) with 4 obese female patients undergoing 6 weeks of severe caloric restriction in a controlled hospital setting (1100 calories per day). These individuals all had a BMI > 40. The patients underwent significant weight loss, improvements in fasting blood glucose and HbA1c, highlighting the significant metabolic benefits of the caloric restriction (Figure 3.14).

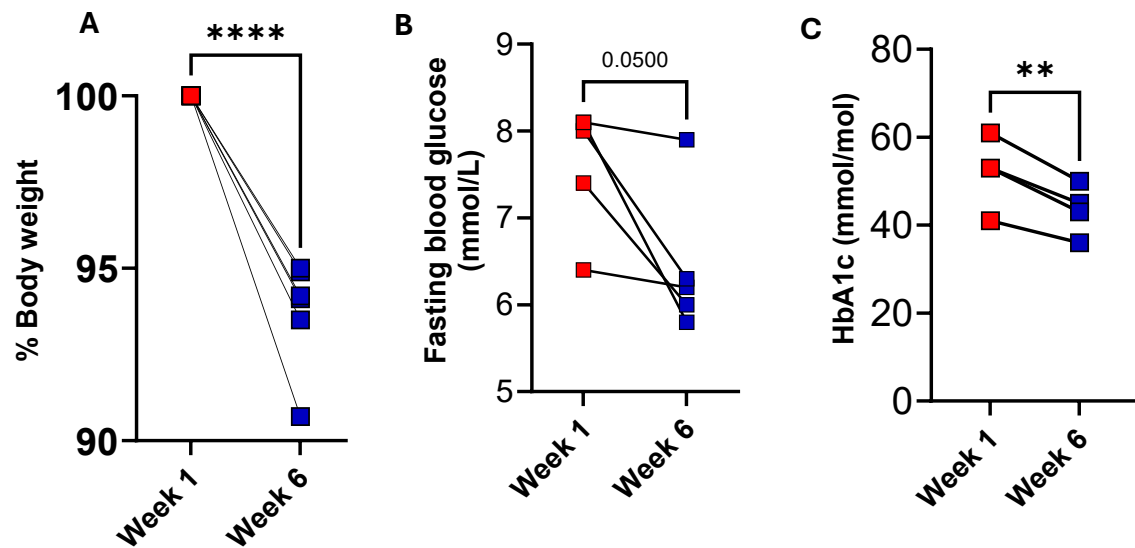


Figure 3.14 – Human caloric restriction experiment, changes in body weight, fasting blood glucose and HbA1c – paired t-tests carried out ($p < 0.05 = *$, $p < 0.01 = **$, $p < 0.0001 = ****$) comparing pre-weight loss with post-weight loss conditions

PBMC samples were taken before and after weight loss and scRNA-seq data was generated for each timepoint. The processing steps are detailed in the methods section describing HD2. Figure 3.15 shows a UMAP of the cell types sequenced within the dataset. Cell types were annotated using extensive matching of cell cluster markers with known gene markers in literature.

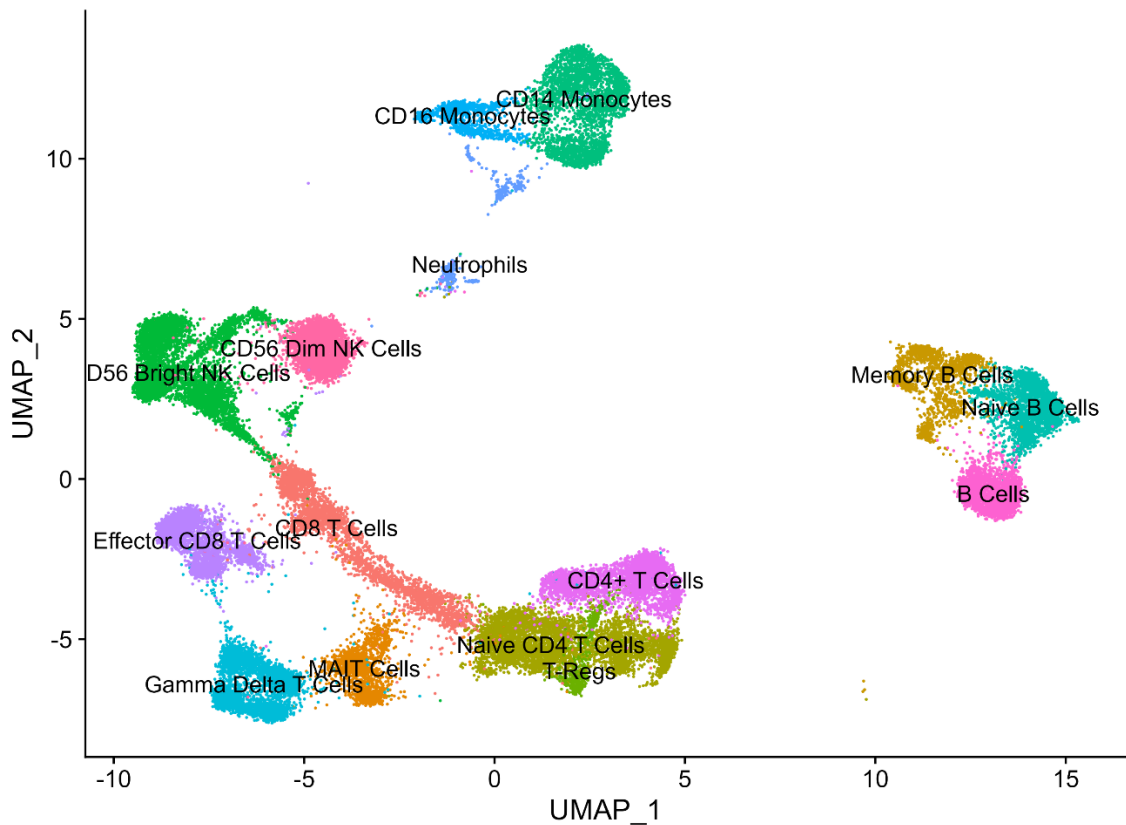


Figure 3.15 – UMAP projection of single-cell RNA-sequencing (scRNA-seq) data from the human caloric restriction study (HD2). Each point represents a single cell, coloured by its assigned cell-type cluster, positioned according to its transcriptional similarity in UMAP space

As well as this caloric restriction intervention resulting in significant weight loss a robust upregulation of *TNFAIP3* in PBMCs was also observed. Notably, *TNFAIP3* expression increased across every immune cell subset in this dataset, with Naïve B Cells showing a contrasting pattern of upregulation in obesity that was not reversed by weight loss. The scale of the $\log_2$ fold changes is significantly higher here compared to the mice integrated dataset. This is likely due to how severe caloric restriction was and the length of time of it, restoring the *TNFAIP3* that was likely lost in obesity based on previous observations. It is important to note these patients had class 3 obesity where the BMI is greater than 40. Figure 3.16 shows a volcano plot representing the results of differential expression comparing all cells at week 1 (before weight loss) and at week 6 (after weight loss) with only *TNFAIP3* highlighted. *TNFAIP3* was the most significantly restored gene with weight loss along with the chemokine receptor *CXCR4* (*CXCR4* will be discussed in detail in later chapters).

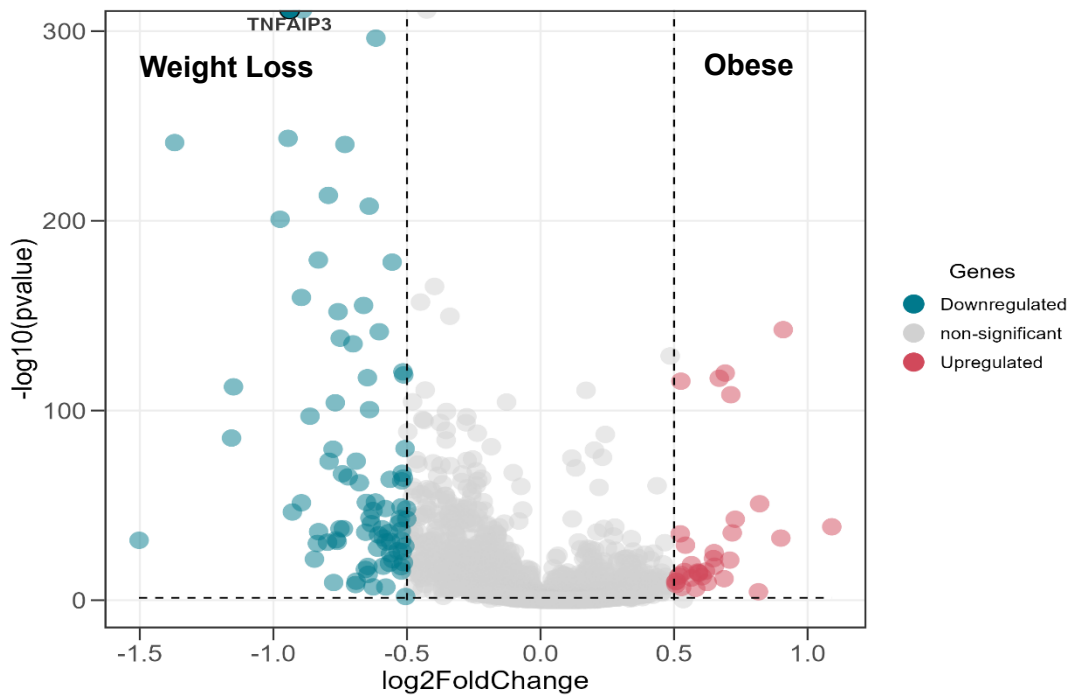


Figure 3.16 – Volcano plot depicting differential expression pre-weight loss and post-weight loss with *TNFAIP3* expression highlighted – negative $\log_2$ fold change values represent genes upregulated in weight loss versus obesity

Significant changes in *TNFAIP3* in response to fasting are only associated with lymphocyte cell clusters. $\gamma\delta$ T Cells experienced the highest $\log_2$ fold change followed by CD56 Dim NK cells, CD4+ T cells and two CD8+ T cell clusters. T-regs whilst exhibiting an increase in *TNFAIP3*, did not achieve significance, this is likely due to the low cell numbers sequenced in this dataset, potentially since T-Regs are known to be reduced in obesity. The myeloid cells experienced upregulation of *TNFAIP3*, but this was not significant, interestingly, one group of B cells experienced downregulation of *TNFAIP3* in response to caloric restriction.

These results are shown in figure 3.17.

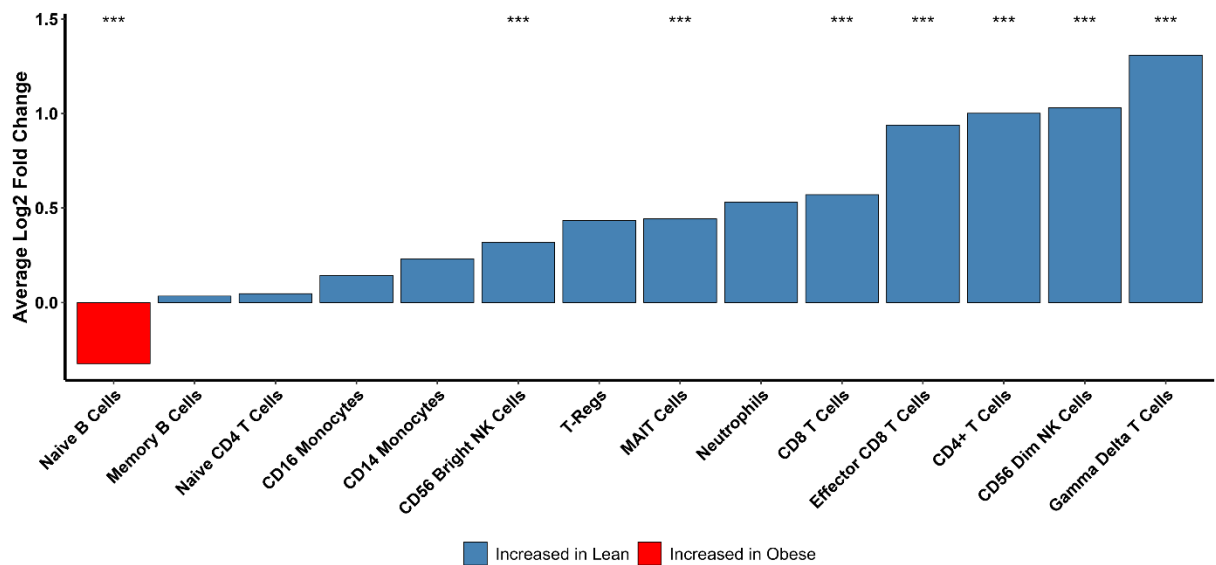


Figure 3.17 – *Tnfaip3* expression in cell types in HD2 - scRNA-seq dataset in weight loss versus obese cells calculated using Wilcoxon rank sum test – negative log₂ fold changes represent that *Tnfaip3* is upregulated in this cell type in weight loss cells versus obese, statistically significant changes in *Tnfaip3* expression are depicted with coloured bars with a ‘*’ to highlight significance, significance was less than 0.0001 across every cell type that happened to be significant and thus all happen to have ‘***’

3.2.7 – MD4 - A20 is downregulated at the protein level in obesity in splenic NK cells

Previous observations of *TNFAIP3* had been made at the RNA level, and therefore differences at the protein level might not mirror those seen across lean, obese, and weight loss states. Because transcript and protein levels do not always correlate, proteomic analysis was performed to confirm whether the transcript-level findings were also reflected at the protein level. Initially, a proteomics dataset consisting of sorted splenic NK Cells from obese and lean mice was analysed for expression levels of A20 protein. Results highlight a significant downregulation of A20 protein in obesity (shown in Figure 3.18 however, there are numerous caveats to this experiment as these NK cells were stimulated with IL2 and IL12 and the mice had B16-F10 tumours. Given this model contrasts with previous models utilised for transcriptomic analysis where no stimulations of cells or cancer were present, this model is not a fair protein measurement to validate previous observations. However, it is still interesting to note that in this alternative model of obesity with cancer and NK cell stimulation, A20 downregulation matches what had been observed at the transcriptomic level.

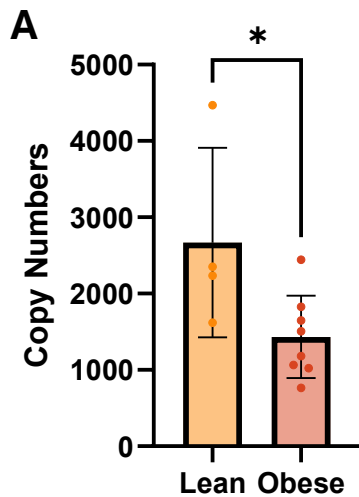


Figure 3.18 – A20 protein expression in splenic NK cells from lean and obese mice. Splenic NK cells from lean (standard fat diet, SFD) and obese (high fat diet, HFD) mice were stimulated with IL-2 and IL-12 for 20 hours prior to proteomic analysis. Mice were injected with B16-F10 cells at week 10 of the diet regimen. A20 protein copy numbers were significantly reduced in NK cells from obese compared to lean mice ($p = 0.035$, Student's t -test). Bars represent mean $\pm$ SD with individual data points overlaid.

3.2.8 - MD5 - Proteomics – A20 NKs Spleen – No Stimulation

In addition to the proteomics experiment shown in Figure 3.18, another proteomics experiment was carried out in NK cells and CD8⁺ T cells isolated from the spleen of mice on HFD for 8 weeks or on SFD for 8 weeks. These cells were not stimulated nor was there any cancer present and thus, provide a different perspective to the other proteomics dataset. As shown in the PCA plot in Figure 3.19, separation by diet and cell type occurred with PC1 capturing the greatest variance (65.9%) which is expected with two cell types present. PC2 captures 8.8% variance mainly separates NK cells by diet with no major separation of CD8⁺ T cells along PC2.

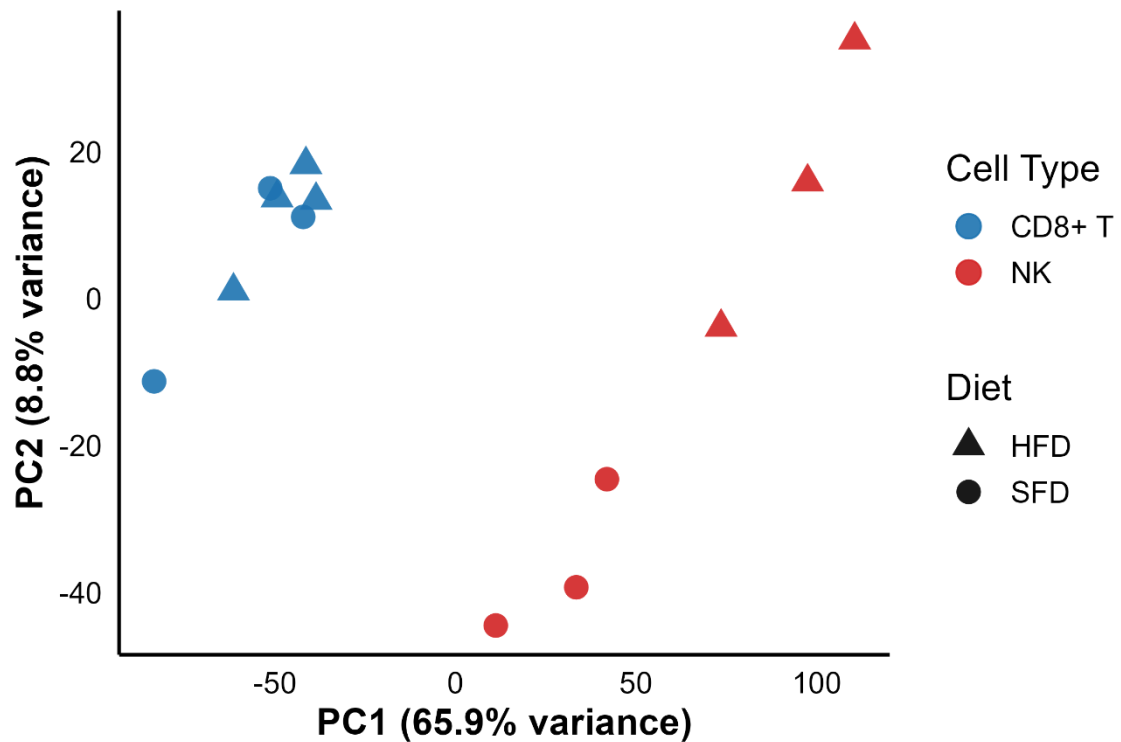


Figure 3.19 – PCA of proteomics, cell types and diets depicted using red for NK cells, SFD mice are represented using circles and HFD mice are represented using triangles – MD5

Analysis of A20 expression revealed a downregulation of A20 in obese CD8⁺ T Cells and NK Cells (Figure 3.20). In CD8⁺ T cells isolated from spleens of SFD and HFD mice, A20 expression was slightly lower in HFD mice compared with SFD controls, but this difference did not reach statistical significance ($p = 0.2103$). In contrast, NK cells displayed a marked reduction in A20 expression in HFD mice relative to SFD mice, with a strong trend toward significance ($p = 0.0645$). These findings indicate that high-fat diet is associated with a pronounced downregulation of A20 in splenic NK cells but only a modest, non-significant reduction in CD8⁺ T cells.

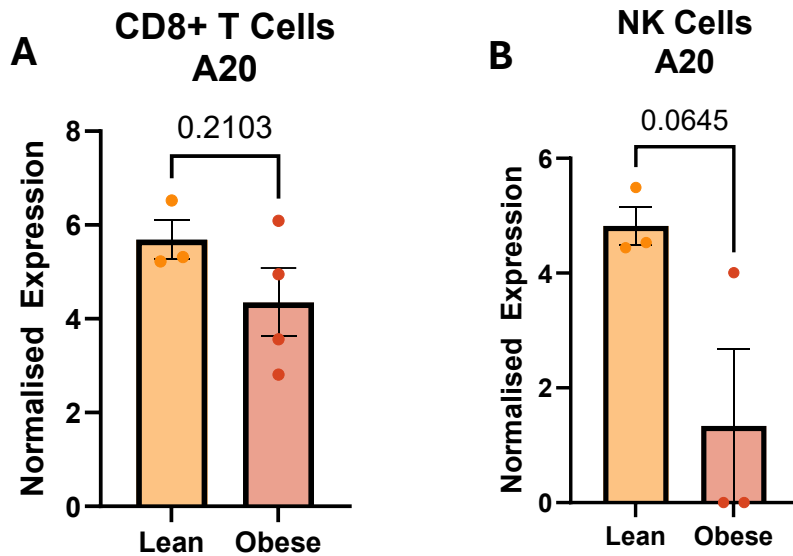


Figure 3.20 – MD5 - A20 expression in CD8⁺ T cells and NK cells from lean and obese mice. A) A20 expression in CD8⁺ T cells was compared between lean and obese mice ($p = 0.2103$, Student's t -test). B) A20 expression in NK cells was compared between lean and obese mice ($p = 0.0645$, Student's t -test). Data are presented as mean $\pm$ SEM with individual data points overlaid.

3.2.9 - HD3 - T2D & *TNFAIP3* Expression – Contrasting Results

Metabolic diseases such as type 2 diabetes (T2D) frequently co-occur with obesity due to an interplay of factors including insulin resistance, chronic low-grade inflammation, adipose tissue dysfunction, and altered immune-metabolic signalling. To elucidate what were the driving factors behind *TNFAIP3* expression in obesity, a bulk-RNA sequencing dataset consisting of 5 metabolically healthy obese (MHO) and 5 metabolically unhealthy obese (MUO) people was analysed with fat tissue sampled from omentum and subcutaneous tissue along with liver tissue shown in Figure 3.21. These data include non-immune and immune cells and thus cell-specific responses could not be analysed and this is the first dataset that includes non-immune cells. However, at a global level no significance and no trend can be observed across any tissue. In keeping with Figure 3.21 (Liver), *TNFAIP3* expression also appears to be low in the liver in humans. In liver and omentum there was a non-significant downregulation of *TNFAIP3* in MUO, the opposite was observed in subcutaneous adipose. Thus, no major conclusions can be drawn from this other than that there is variation across tissues and T2D does not appear to have a significant effect on *TNFAIP3* levels at a tissue level.

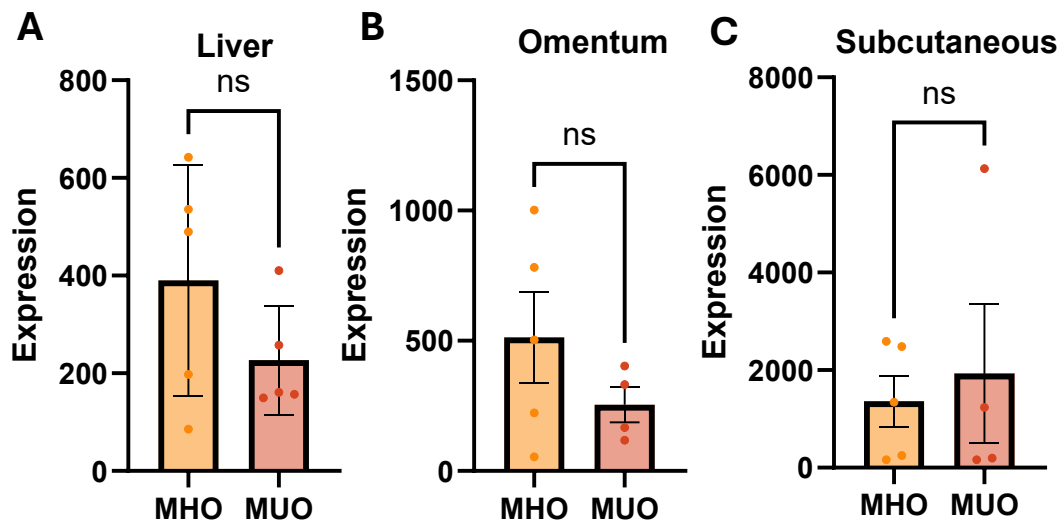


Figure 3.21 – A) *TNFAIP3* expression in liver, groups split by metabolically healthy obese vs metabolically unhealthy obese B) *TNFAIP3* expression in subcutaneous adipose, groups split by metabolically healthy obese vs metabolically unhealthy obese C) *TNFAIP3* expression in omentum, groups split by metabolically healthy obese vs metabolically unhealthy obese - (Student's *t*-test). Data are presented as mean $\pm$ SEM with individual data points overlaid

In subcutaneous tissue shown in Figure 3.21, panel C, the plots shows an outlier point with higher *TNFAIP3* than all metabolically healthy and metabolically unhealthy participants. With removal of outliers, it is hard to make any solid conclusions from these data.

3.2.10 - HD4 - Human scRNA-seq PBMC Dataset: Lean and Obese with and without T2D

A human PBMC dataset was then analysed, comprising patients classified into one of the following groups: lean non-diabetic, lean pre-diabetic, obese diabetic, or obese non-diabetic. The data was already processed and provided with cluster labels. Participant numbers varied considerably between groups, for instance, there were 6 lean non-diabetics but only 2 lean pre-diabetics. Another important consideration is that many patients, particularly those with diabetes, were on medications such as metformin and glucocorticoids which can have significant effects on both inflammatory and metabolic readouts. Particularly relevant to the this chapter is the use of synthetic glucocorticoids as medication, two individuals were on glucocorticoid medication out of the 23 in the dataset, one with BMI in a healthy range one in the obese range, however, these individuals also took substantial amounts of other medications, and it is difficult to uncouple the effects of glucocorticoids on *TNFAIP3* expression. Cell types shown in Figure 3.22.

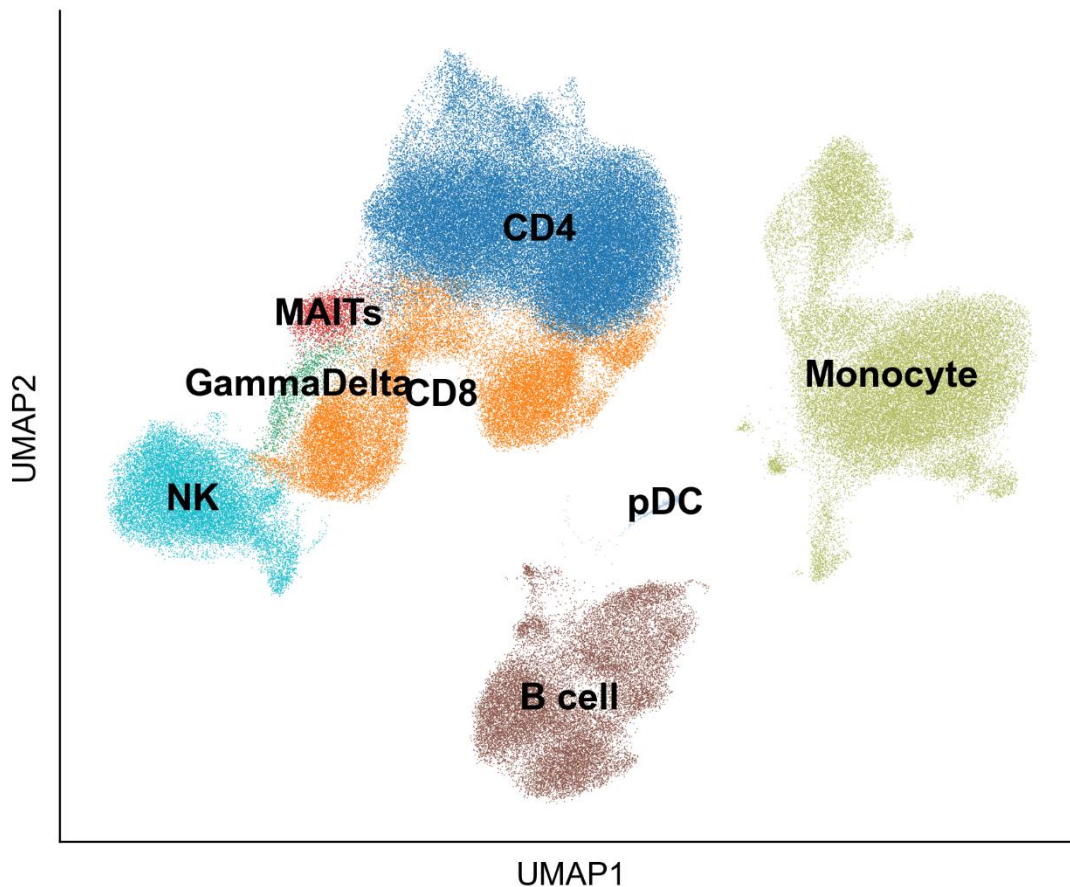


Figure 3.22 - UMAP projection of single-cell RNA-sequencing (scRNA-seq) data from the HD4 cohort. Each point represents an individual PBMC, coloured by its assigned broad cell-type cluster. The dataset includes cells from lean and obese individuals spanning diabetic, pre-diabetic, and non-diabetic states, positioned according to transcriptional similarity in UMAP space.

More precise cell labels were also present in the dataset's metadata in the Seurat Object (not shown) providing more granular cell types. These cell types were utilised to get lineage specific comparisons for *TNFAIP3* across cell types. For a fair comparison and to glean insight into obese and lean diabetics, cells from obese and lean participants were split and then further subdivided into T2D+ or T2D- or in the case of lean donors, T2D- or pre-diabetic. For every cell cluster in the dataset the differential expression was calculated between the diabetic and non-diabetic group. Cell clusters without enough cells (less than 200) to perform differential expression were excluded, as a result only 6 cell types remained after applying this threshold. The results of lean non-diabetic versus lean pre-diabetic are shown in Table 3.4. All cell types tested exhibited positive and significant upregulation of *TNFAIP3* in non-diabetic participants versus pre-diabetic as shown in Table 3.1.

Table 3.1 – Adjusted p-values and log fold changes for *TNFAIP3* expression in immune cell subsets (lean non-diabetics vs lean pre-diabetics) - cell clusters with over 200 cells per condition (Lean T2D- and Lean Pre-Diabetic) were required to undergo differential expression

Cell Type	Gene	Adjusted p-value	Log FC
CD4 TCM	<i>TNFAIP3</i>	2.64e-07	0.656711
CD4 Naive	<i>TNFAIP3</i>	7.41e-06	0.854405
NK	<i>TNFAIP3</i>	2.13e-06	1.084322
CD8 TEM	<i>TNFAIP3</i>	3.9e-07	1.089498
CD8 Naive	<i>TNFAIP3</i>	1.62e-05	0.9753
B Naive	<i>TNFAIP3</i>	1	0.285435

All T and NK cells were pooled to create a visualisation of expression, Figure 3.23, which shows higher *TNFAIP3* expression in non-diabetic lean participants versus pre-diabetic lean participants as would be expected based on the differential expression results shown in Table 3.1.

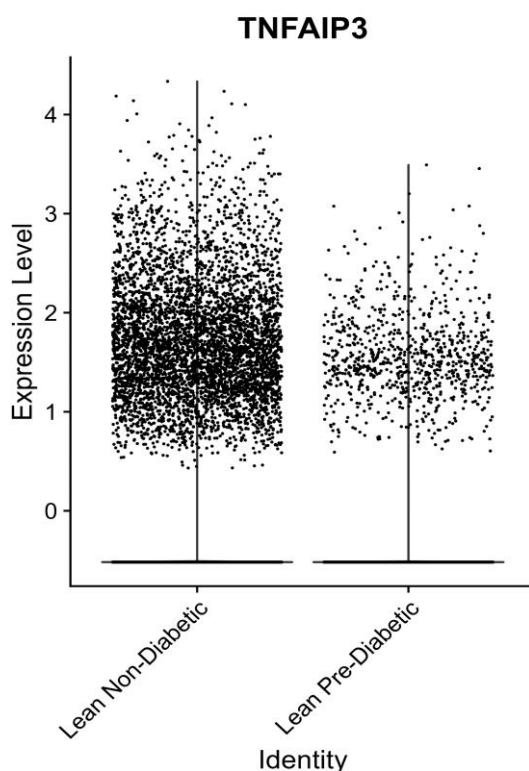


Figure 3.23 – *TNFAIP3* expression - Lean Non-Diabetics versus Lean Pre-Diabetics, all T and NK cell populations pooled together, each dot represents a cells expression of *TNFAIP3*

This same differential expression analysis was repeated on obese diabetics versus obese non-diabetics. Again, there were very significant differences in CD4+ and CD8+ T cell populations which followed the same trend as in lean participants, that is, that *TNFAIP3* is significantly downregulated in diabetic patients compared to non-diabetic participants. Unlike the lean participants, there was no significant changes in NK Cells. The effects of diabetes on *TNFAIP3* expression seem to affect T cell populations the most based on adjusted p-values as shown in these two Tables with CD4+ and CD8+ T cell populations being most affected but also NK cells in lean participants. Several additional cell clusters passed the threshold of cells (200 cells) required to perform differential expression and thus Table 3.2 contains additional cell clusters compared to Table 3.2.

Table 3.2 – Adjusted p-values and log fold changes for *TNFAIP3* expression in immune cell subsets (obese non-diabetics vs obese diabetics) – cell clusters with over 200 cells per condition (Obese T2D+ and Obese T2D-) were required to undergo differential expression

Cell Type	Gene	Adjusted P-Value	Log FC
CD4+ TCM	<i>TNFAIP3</i>	8.14 e-50	0.78
CD14+ Mono	<i>TNFAIP3</i>	0.59	-0.11
CD4+ Naive	<i>TNFAIP3</i>	1.14e-11	0.75
NK Cell	<i>TNFAIP3</i>	0.85	0.41
B Naive	<i>TNFAIP3</i>	1	-0.06
CD8+ Naive	<i>TNFAIP3</i>	0.695	0.46
CD8+ TEM	<i>TNFAIP3</i>	1.84e-18	1.38
CD16+ Monocyte	<i>TNFAIP3</i>	1	0.27
B Intermediate	<i>TNFAIP3</i>	1	0.56
T-Reg	<i>TNFAIP3</i>	0.0592	0.81
CD4+ TEM	<i>TNFAIP3</i>	2.45e-07	0.72
B Memory	<i>TNFAIP3</i>	1	0.41

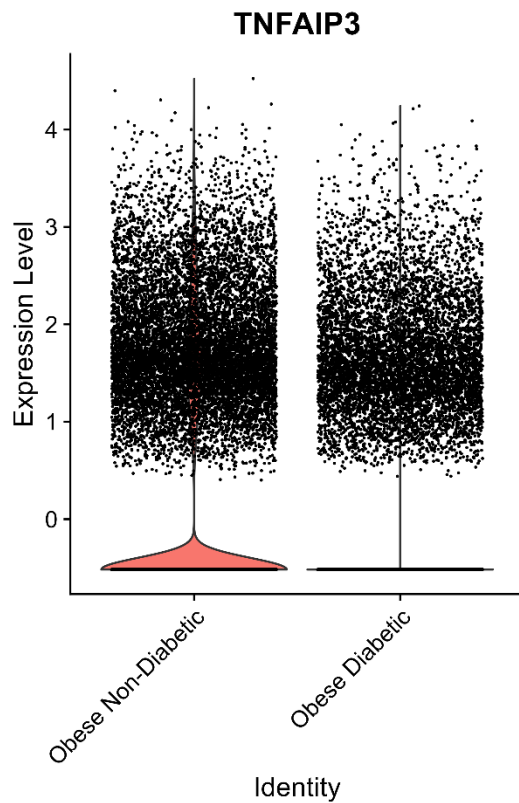


Figure 3.24 – *TNFAIP3* expression – Obese Non-Diabetics versus Obese Diabetics - all T and NK cell populations pooled together; each dot represents a cells normalised expression of *TNFAIP3*

As an additional test, pseudobulking was performed (described in methods), where total transcripts for each gene are aggregated for each individual in the dataset. Counts for *TNFAIP3* were summed for every patient and compared versus that person's blood glucose readout as was available in the metadata. Non-significant negative correlations were observed between total *TNFAIP3* counts per patient versus blood glucose (Figure 3.25).

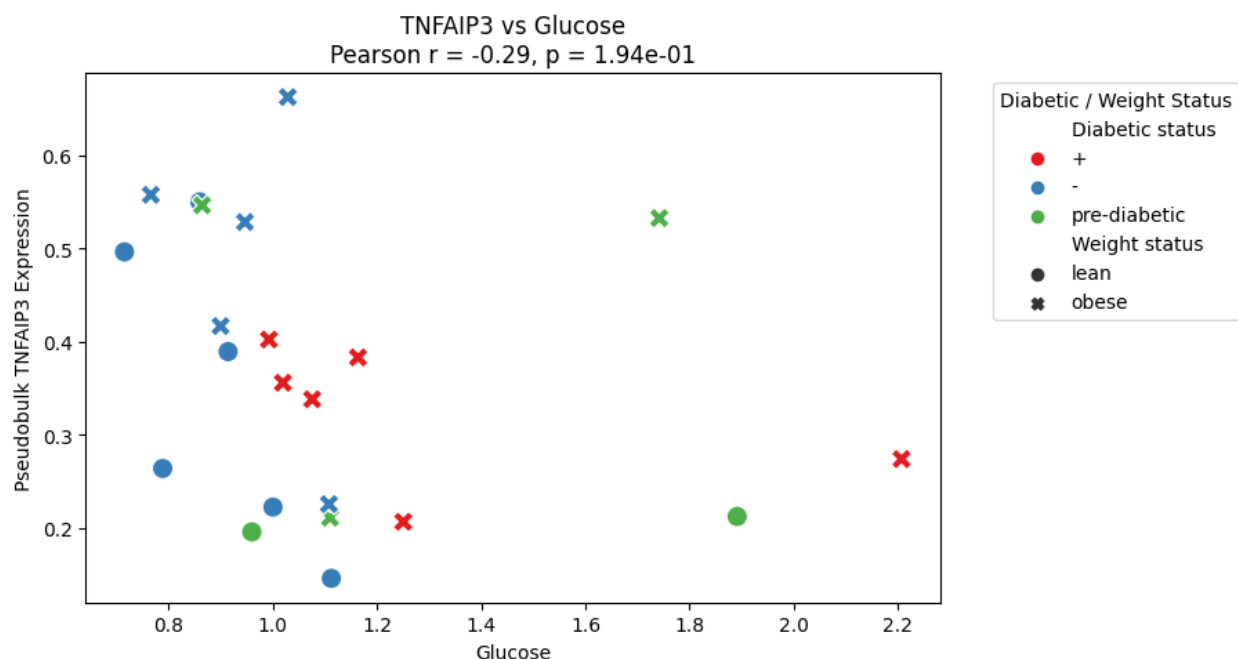


Figure 3.25 – Pseudobulk *TNFAIP3* expression versus blood glucose levels in individual patients. Each point represents one patient’s pseudobulk *TNFAIP3* expression plotted against their blood glucose level. Colours indicate diabetic status (red = diabetic, blue = non-diabetic, green = pre-diabetic), while shapes distinguish weight status (circles = lean, crosses = obese). The Pearson correlation coefficient (r) and p -value are shown above the plot.

As expected, pseudobulking *TNFAIP3* levels in T and NK cells for each patient led to a lower p -value but still non-significant result with a higher negative correlation in Figure 3.26, this was expected as the significant changes observed previously primarily occurred in T and NK cells. This was done to exclude non-T and NK Cells and see if there was a significant correlation. This is merely adding weight to the idea that diabetes seems to have a greater effect on T and NK cells expression of *TNFAIP3*. Myeloid cells for example, seem to be less responsive to diabetes with respect to *TNFAIP3* expression.

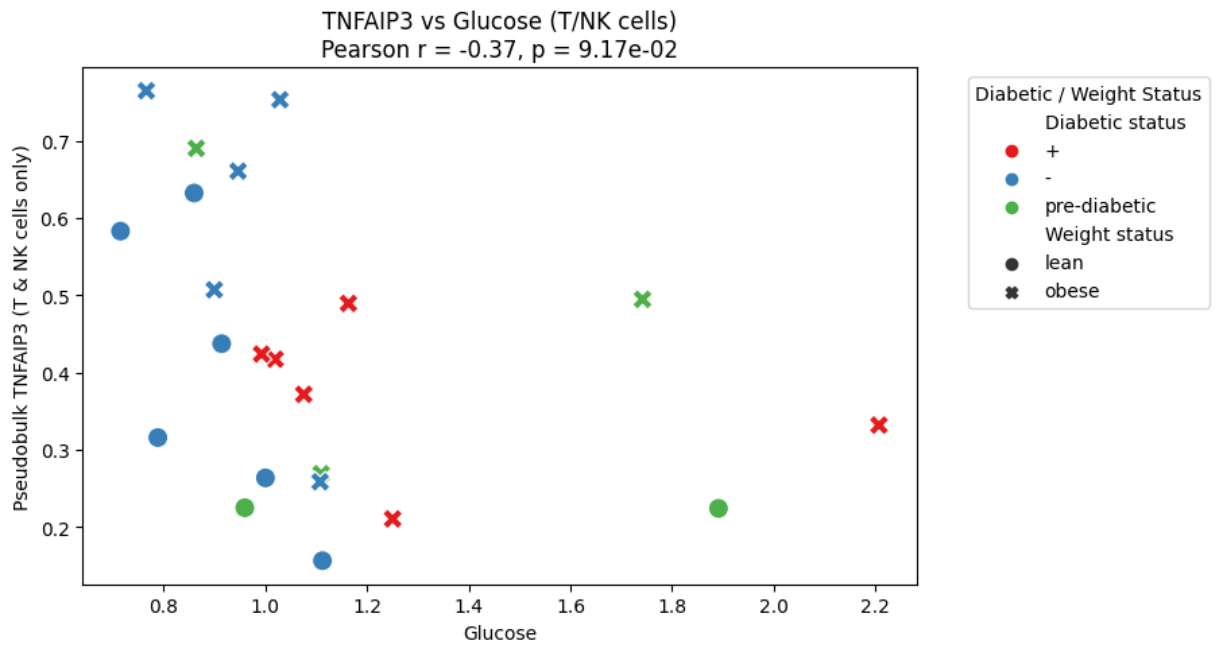


Figure 3.26 – Pseudobulk *TNFAIP3* expression in T and NK cells versus blood glucose levels in individual patients. Each point represents one patient’s combined pseudobulk *TNFAIP3* expression from T and NK cells plotted against their blood glucose level. Colours indicate diabetic status (red = diabetic, blue = non-diabetic, green = pre-diabetic), while shapes distinguish weight status (circles = lean, crosses = obese). The Pearson correlation coefficient (r) and p -value are shown above the plot.

Overall, these analyses suggest that the presence of diabetes may exert a modest influence on *TNFAIP3* expression, but this effect is cell-type specific. The most consistent changes were observed in CD4⁺ and CD8⁺ T cells, with weaker or absent effects in NK and myeloid populations. Bulk tissue data did not reveal these differences, highlighting the need for cell-resolved analyses. It is also important to note that many participants were taking multiple medications, including agents known to affect inflammatory and metabolic pathways, which may confound these observations.

3.3.0 – Chapter 3 Discussion

The results presented in this chapter establish a consistent and robust pattern of *TNFAIP3* downregulation in immune cells during obesity across both human and murine datasets. As a key negative regulator of NF- κ B signalling and inflammatory homeostasis,¹³¹ *TNFAIP3* was found to be consistently downregulated in immune cell populations in humans and mice in obesity, particularly within lymphocyte populations such as NK cells, T cells, and innate lymphoid cells. This downregulation was evident in both blood and tissue-resident immune compartments and most obvious in adipose tissue of mice, this coincided with observed metabolomic data shown distinct metabolomes driven by obesity and subsequent weight loss. Integration of multiple murine adipose scRNA-seq datasets further validated this phenotype and revealed that weight loss can partially or fully restore *TNFAIP3* expression across multiple cell types. Importantly, proteomic analysis supported the transcriptomic findings, suggesting that *TNFAIP3* dysregulation extends to the protein level. Moreover, stratification of human samples by diabetic status highlighted that *TNFAIP3* suppression is most strongly associated with T2D within T and NK cell populations. Together, these findings support a model in which obesity and metabolic dysfunction lead to a systemic, cell-type-specific suppression of *TNFAIP3*, potentially undermining immune regulation and contributing to chronic inflammation.

3.3.1 – *TNFAIP3* as a Core Immunometabolic Regulator

A20, encoded by *TNFAIP3*, acts as a molecular brake on pro-inflammatory cascades by terminating NF- κ B activation in response to a wide range of immune stimuli¹³². This function is critical in preventing prolonged or inappropriate inflammation. While the canonical anti-inflammatory role of A20 is well established, the findings in this chapter highlight that *TNFAIP3* expression is not static, it is dynamically regulated in response to metabolic states, such as obesity and weight loss. This suggests that *TNFAIP3* is sensitive to shifts in the metabolic environment. Although further mechanistic work is required to establish causality and a mechanism, the consistent pattern of *TNFAIP3* downregulation in obesity and its restoration following weight loss points to a potential regulatory role in the immunometabolic adaptations that accompany these conditions. The only previous research carried out on *Tnfaip3* in a metabolic setting was by Catrysse *et. al.* who showed that deletion of A20 specifically in myeloid cells paradoxically protected mice from diet-induced obesity and insulin resistance, despite creating a highly inflammatory milieu within metabolic tissues. In their model, A20-deficient macrophages displayed a distinct immunometabolic program characterised by impaired mitochondrial respiration, increased palmitate utilisation

and upregulation of Irg1/itaconate production. This metabolic rewiring of macrophages was proposed to drive fatty acid clearance from the circulation and limit adipose tissue expansion during high-fat feeding. These findings highlight that A20 can exert cell-type specific effects in metabolic disease: while myeloid loss of A20 promotes a lean phenotype through altered macrophage metabolism, the downregulation of *TNFAIP3* observed here in lymphocytes during obesity may instead contribute to immune dysregulation rather than protection. Together, this underscores the emerging idea/hypothesis that changes in *TNFAIP3* expression levels, and ultimately the production of A20, functions as a dynamic node at the interface of immunity and metabolism, but its consequences depend critically on cellular context.³⁰

My analyses demonstrate that *TNFAIP3* is ubiquitously expressed across immune cell populations, including T cells, NK cells, and ILC2s, in both humans and mice. This widespread expression underscores its fundamental role in maintaining immune cell equilibrium. Crucially, *TNFAIP3* expression is not fixed, it is dynamically modulated in response to metabolic state. In obese states, *TNFAIP3* expression is consistently downregulated, particularly in adipose resident immune cells such as T-Regs and ILC2s which have been shown to be cell types essential for limiting inflammation and preserving tissue function. This loss of *TNFAIP3* likely contributes to the chronic, low-grade inflammatory state characteristic of obesity, where immune cells become dysregulated and shift toward pro-inflammatory phenotypes.

Conversely, weight loss restores *TNFAIP3* expression, reinforcing the idea that its regulation is tightly linked to nutrient status and metabolic inflammation. These findings highlight *TNFAIP3* as a sensor of metabolic stress within the immune system and as a link between classical inflammatory signalling and immunometabolic reprogramming. Therefore, A20 may serve not only to restrain inflammation but also to reflect broader changes in immune function that occur in response to metabolic shifts, particularly those seen in obesity and weight loss.

3.3.2 - Obesity-Associated Downregulation of *TNFAIP3* in Immune Cells

A consistent finding across analysed datasets is the downregulation of *TNFAIP3* in obesity, observed in both human and murine immune cells across multiple tissues. This suppression was most pronounced in adipose tissue, a key site of metabolic and immunological crosstalk. Through scRNA-sequencing of murine CD45⁺ immune cells from adipose tissue, liver, blood, and muscle, I identified a marked reduction in *Tnfaip3* expression in obese mice,

particularly in T regulatory cells and ILC2s, immune populations known for their roles in tissue repair, inflammation resolution, and metabolic homeostasis.

The vulnerability of these regulatory populations to *Tnfaip3* downregulation suggests a mechanism by which obesity drives a pro-inflammatory shift in the immune landscape. T-Regs and ILC2s are central to maintaining an anti-inflammatory environment in adipose tissue¹³³; their dysfunction or loss, as evidenced here by both transcriptional downregulation of *TNFAIP3* and decreased abundance, could act as a tipping point, permitting unchecked activation of effector T cells and macrophages but also leading to apoptotic loss of these cells as *TNFAIP3* protects cells from apoptosis.³⁸ Based on two *in-vivo* *Tnfaip3* knockout models in mice where CD4⁺ T cells and NK cells both had *Tnfaip3* knocked out in independent studies we can infer as to what is likely happening in obesity when *Tnfaip3* is chronically downregulated in these cells. These papers showed that A20 restrains lymphocyte activation–metabolism axes. In CD4⁺ T cells, *TNFAIP3* limits mTOR signalling and is required for TCR-induced autophagy; its loss elevates mTOR activity, impairs mitophagy, increases ROS, and reduces cell survival defects that are partially rescued by mTOR inhibition.¹³⁴ In NK cells, A20 similarly restrains mTOR and TNF pathways; genetic deletion causes hyperactivation with heightened protein synthesis and glucose uptake but results in spontaneous cell death and severe NK lymphopenia, which can be mitigated by rapamycin and by TNF blockade.¹³⁵

Taken together, if *TNFAIP3* is downregulated in lymphocytes during obesity (as observed here), a reasonable inference is that mTOR tone rises while autophagy capacity falls, predisposing T cells to mitochondrial stress/ROS and reduced viability, and pushing NK and CD4⁺ T cells towards a hyperactivated state. This could help explain a state of dysfunctional immunity, excessive signalling and poor cellular fitness during obesity. Weight loss likely drives a restoration of *TNFAIP3* potentially normalising mTOR–autophagy balance and rescuing lymphocyte survival and homeostasis.

Interestingly, this pattern is not universal across all tissues. While liver and blood showed modest reductions in *Tnfaip3* expression, muscle-resident immune cells displayed no meaningful differences between lean and obese states. This tissue specificity of *TNFAIP3* expression likely reflects the metabolic changes in that tissue in response to obesity or weight loss, for example, adipose tissue had the most altered metabolome across groups and saw the biggest shift in *TNFAIP3* expression.

3.3.3 – *TNFAIP3* Upregulated with Diet-Induced Weight Loss

Following the consistent downregulation of *TNFAIP3* in obesity, one significant finding from this chapter is its robust re-upregulation in response to weight loss, both in murine models and human patients. In mice, diet-induced weight loss led to significant upregulation of *Tnfaip3* in key immune cell populations, particularly in adipose-resident T cells and ILC2s. Notably, T-Regs, despite their reduced abundance in obesity, exhibited a large, though statistically underpowered, increase in *Tnfaip3* expression in response to weight loss. This suggests that restoration of *TNFAIP3* may be one of the key transcriptional changes associated with immune rebalancing following metabolic improvements driven by weight loss / caloric restriction.

This reversal of obesity-induced suppression was not restricted to mice. In a controlled clinical setting, obese human subjects undergoing 6 weeks of caloric restriction also demonstrated a broad upregulation of *TNFAIP3* across PBMC subsets, further validating the gene's sensitivity to metabolic context. While most cell types showed this recovery, plasmacytoid dendritic cells (Naïve B Cells) emerged as an exception, maintaining *TNFAIP3* expression in obesity that was not reversed with weight loss, pointing to potential cell-type-specific regulation mechanism in Naïve B Cells.

The ability of immune cells to re-express *TNFAIP3* with weight loss underscores its role as a metabolic response gene that may facilitate the return to homeostasis. This suggests potential therapeutic avenues aimed at restoring *TNFAIP3* function to recalibrate immune responses in obesity and its comorbidities. The mechanisms around loss and restoration of *TNFAIP3* are explored in detail in later chapters.

3.3.4 – Is T2D Affecting Expression of *TNFAIP3*?

The analysis of *TNFAIP3* expression in individuals with varying metabolic health states reveals a complex and somewhat inconclusive picture due to a variety of factors. Two datasets analysed, HD3 and HD4, both had individuals that were T2D+ and T2D-, however, it is difficult to uncouple the effects of medication in these individuals where in many participants there was more than one type of medication been taken. The influence of medications is particularly important given what is learned in later chapters about the influence of glucocorticoids on *TNFAIP3* expression. While no significant differences were detected in bulk adipose or liver tissue between metabolically healthy and unhealthy obese individuals, these data may obscure important immune cell-specific dynamics as were observed in the scRNA-seq analysed later in the results section. The inclusion of both

immune and non-immune cells in the bulk tissue datasets likely reduces any signal from responsive cell types such as T cells or NK cells, which have been shown elsewhere in this chapter to be key responders to metabolic shifts.

In contrast, the human PBMC dataset, which allowed for cell-type-specific analysis, suggests that *TNFAIP3* expression is lower in diabetic individuals compared to non-diabetics, in both lean and obese groups. This aligns with previous findings from murine and human weight loss datasets showing that *TNFAIP3* is responsive to metabolic perturbation and may reflect underlying immune activation states. However, the interpretation of these data is complicated by substantial variation in group sizes and the use of medications such as metformin and glucocorticoids in participants. Metformin and other antidiabetic therapies are known to influence inflammatory and metabolic pathways, including NF- κ B and AMPK signalling, which could in turn impact *TNFAIP3* expression.¹³⁶

These findings underscore the critical need to control for pharmacological interventions when studying immunometabolic markers like *TNFAIP3*. Without stratifying or adjusting for medication effects, it becomes difficult to discern whether observed differences are disease-driven, treatment-driven, or a combination of both.

3.3.5 – The Implication: Why *TNFAIP3* levels matter

While the established literature has already characterised *TNFAIP3* as a key negative regulator of NF- κ B signalling¹²⁹, the findings presented in this chapter add novel insights into how its expression is dynamically regulated by metabolic state, with important implications for immune cell function in obesity and weight loss. Across multiple independent datasets in both humans and mice, *TNFAIP3* was found to be consistently downregulated in obesity in immune cell populations in humans and mice, particularly in lymphocyte subsets. In humans all cell types except Naïve B Cells upregulated *TNFAIP3* in response to caloric restriction. Similarly, the MD2 dataset had the longest period of weight loss relative to the other mice datasets and revealed the greatest upregulation of *Tnfaip3* across lymphocytes. I hypothesize that the change in expression is relative to the severity of the caloric restriction / weight loss, in other words, the more severe the caloric restriction, the greater the effect on *TNFAIP3* expression. This hypothesis is based off MD2 and HD2 datasets where caloric restriction / weight loss was most severe. These changes were apparent in adipose tissue across the three mice datasets MD1, MD2 and MD3 which is a key site of immunometabolic cross-talk in obesity,¹⁰² but extended to other compartments

such as liver and blood. Importantly, the downregulation of *TNFAIP3* was not just a marker of altered inflammation but is likely contributing to the immune dysfunction seen in obesity.

Regulatory lymphocyte populations such as Tregs and ILC2s, both of which are known to limit adipose inflammation and promote tissue repair ¹³³, exhibited large reductions in *TNFAIP3* expression. Given that A20 controls both NF- κ B activity and cell death thresholds ^{137,134,135}, the loss of *TNFAIP3* in these populations may have profound consequences. A20-deficient CD4 T cells and NK cells have been shown to be more prone to apoptosis and hyperactivation in response to antigen or cytokine stimulation ^{134 135}, suggesting that the observed loss of expression in T-Regs may compromise their survival or suppressive capacity. In obesity, where these cells are already reduced in number, downregulation of *TNFAIP3* may tip the balance towards chronic inflammation or its loss may be the driver of cell-death in T-Regs.

Moreover, *TNFAIP3* loss may affect not only immune regulation, but also the integrity of antigen-driven responses. In CD8⁺ T cells and NK cells, reduced *TNFAIP3* could contribute to exaggerated or prolonged effector responses, cytotoxic dysfunction, or even premature exhaustion. A20 signalling normally acts to terminate T cell receptor (TCR)-induced inflammatory responses ⁴⁸. Recent findings have shown that the paracaspase MALT1, a critical mediator of TCR signalling, directly cleaves A20 at arginine 439, thereby diminishing its NF- κ B inhibitory activity and promoting T cell activation. ⁴⁸ Given that NK cells also express MALT1, this mechanism could be occurring in NK cells as well. In obesity, the reduction in A20 expression may amplify this effect, effectively removing a crucial checkpoint on TCR-driven NF- κ B activation. This could lead to heightened T cell activation, proliferation, and survival of pro-inflammatory T cell subsets, further exacerbating inflammation. These findings raise the possibility that restored *TNFAIP3* expression in weight loss may help preserve specific pro-inflammatory lymphocyte populations through its anti-apoptotic function. While typically considered anti-inflammatory, A20 may in this context contribute to the survival of previously activated or pathogenic immune cells, potentially influencing immune memory or relapse dynamics post-obesity as memory of obesity has been shown in other cell types.¹¹⁹

An additional layer of relevance is the known role of *TNFAIP3* in regulating multiple cell death pathways beyond apoptosis, including necroptosis and pyroptosis ¹²⁷. These forms of lytic cell death are increasingly recognised as contributors to adipose tissue inflammation and immune activation ¹³⁸. A20 controls these pathways by modulating the ubiquitination of

RIPK1 and other adaptor proteins, suggesting that its downregulation could not only permit NF- κ B activation but also sensitise cells to inflammatory cell death ¹³⁹. This could amplify local cytokine release and immune infiltration in metabolic tissues, compounding the immune dysfunction of obesity.

The observation that *TNFAIP3* expression is restored in response to weight loss is particularly important in this context. It reinforces the idea that *TNFAIP3* is metabolically regulated, potentially through nutrient-sensing pathways such as AMPK, which is known to be upregulated during energy deficit and to suppress NF- κ B signalling.¹³⁶ Given that AMPK activation is associated with anti-inflammatory effects and is induced by adiponectin, ghrelin, metformin, and caloric restriction, all of which are relevant in the context of weight loss.¹³⁶ It is plausible that re-expression of *TNFAIP3* reflects a broader restoration of immune-metabolic homeostasis.

Finally, genetic evidence supports the concept that even partial reductions in *TNFAIP3* function can have pathological consequences. As described in the literature, haploinsufficiency of *TNFAIP3* leads to early onset autoinflammatory disease, highlighting the importance of maintaining adequate expression of A20 to prevent immune dysregulation ¹²³. Although the downregulation observed in obesity is not due to germline mutations, the convergence of transcriptomic suppression and chronic inflammation observed here suggests that this may represent a form of phenocopying, driven by obesity.

In summary, this chapter demonstrates that *TNFAIP3* is not only dynamically regulated by metabolic state but may also serve as a molecular link between energy sensing and immune homeostasis. Its suppression in obesity may impair the regulatory and survival capacities of key immune cells, exacerbating chronic inflammation. Conversely, its re-expression following weight loss suggests a potential mechanism by which immune equilibrium is restored. These findings support the idea that *TNFAIP3* is not just a downstream responder to inflammation but may actively shape the immune landscape in response to metabolic signals.

3.4.0 – Chapter 3 Conclusion

This chapter establishes *TNFAIP3* as a gene whose expression is tightly coupled to the host's metabolic state. Across multiple datasets in both humans and mice, and in diverse immune cell subsets, *TNFAIP3* is consistently downregulated in obesity, a state characterised by chronic low-grade inflammation and disrupted immune regulation. Importantly, this suppression is reversible, as demonstrated by the robust re-expression of *TNFAIP3* in response to weight loss achieved through either dietary restriction or caloric intervention.

The widespread expression of *TNFAIP3* across immune populations, including T-regs, CD8+ T cells, CD4+ T cells, NK cells, ILC2s, and $\gamma\delta$ T cells, and its strong transcriptional regulation in metabolically active tissues such as adipose, underscores its pivotal role in maintaining immune cell equilibrium. Its downregulation in obesity likely contributes to the pro-inflammatory phenotype of these cells and exacerbates systemic metabolic dysfunction. The observed rebound in *TNFAIP3* expression in these same populations during weight loss highlights its responsiveness to metabolic cues and its possible role in reinstating immune homeostasis. Collectively, these data position *TNFAIP3* not only as a molecular biomarker of metabolic inflammation, but also as a functional mediator of the immunological restoration that accompanies weight loss due to its ability to inhibit NF- κ B, apoptosis and other cell death mechanisms. *TNFAIP3* encodes for a central node at the intersection of immunity and metabolism, acting as sensor and mediator, and offering a potential therapeutic target for reversing obesity-associated immune dysfunction. These findings lay a solid foundation for mechanistic exploration of *TNFAIP3* loss and recovery in immune cells in future chapters. However, establishing a causal role for *TNFAIP3* in lymphocytes in diverse tissues and in conditions such as obesity and weight loss is not trivial as dysregulation in obesity doesn't just occur at a metabolic level, it also occurs at an immunological level. Therefore, to decouple what is driven by metabolic cues or driven by inflammation is difficult given that both co-occur in obesity. To begin to separate these factors and probe causal relationships, I examined A20 haploinsufficiency, integrated transcriptional and epigenetic datasets, and applied advanced computational approaches to elucidate the drivers of *TNFAIP3* expression.

3.5.0 - Chapter 3 Limitations

The HD1 dataset, the data obtained was normalised and processed, how this was carried out is unclear and provides a major limitation for that dataset. However, the data was mainly used observationally, and no central ideas or hypotheses were generated from this dataset that could not be sufficiently tested by other means.

In the T2D diabetes datasets HD3 and HD4 there is either no account of the medications (HD3) or there is an account of the medications in (HD4). Whilst there are significant differences in *TNFAIP3* expression in several cell types between T2D+ and T2D- cell comparisons, it is difficult to account for the effects of medications as in many instances, participants are taking a wide variety of medication and thus it is not possible to account for these potential effects.

4.0 - Chapter 4 – Transcriptional and Epigenetic Regulation of *TNFAIP3* in response to Obesity and Weight Loss

Aim: This chapter aims to define the regulatory landscape of *TNFAIP3* in the context of obesity and weight loss. Specifically, I examine which factors alter *TNFAIP3* expression and identify genes consistently co-expressed with it across metabolic states. To do this, I integrate established knowledge of *TNFAIP3* with analyses of chromatin accessibility, genetic regulation (eQTLs), transcriptomic and proteomic datasets. Finally, I explore how weight loss, fasting, and glucocorticoid signalling converge on *TNFAIP3* regulation, with the goal of disentangling canonical NF- κ B feedback from higher-order regulatory mechanisms that may explain its suppression in obesity. This research provides mechanistic insights into the role obesity, weight loss, acute fasting and glucocorticoid treatment play in altering transcriptional programs in immune cells.

4.1 – Chapter 4 Introduction

TNFAIP3 (A20) is a central negative regulator of inflammation, normally induced by NF- κ B to terminate immune activation¹³². Chapter 3 demonstrated that in obesity, despite persistent NF- κ B signalling, the expression of the regulator of NF- κ B signalling, *TNFAIP3*, remains low. This suggests that a breakdown of the canonical feedback loop contributes to the inflammation associated with obesity. The mechanisms underlying this paradox remain unresolved and likely involve additional layers of regulation beyond NF- κ B alone.

TNFAIP3, also known as A20, is a ubiquitin-editing enzyme (see structure Figure 4.1) that plays a central role in limiting inflammation and maintaining immune homeostasis. It functions primarily as a negative regulator of NF- κ B and other pro-inflammatory signalling pathways by removing K63-linked ubiquitin chains and promoting K48-linked ubiquitination of key signalling intermediates.¹³⁷ Multiple separate domains have been identified responsible for deubiquitination, interacting with other components of the ubiquitination machinery and for lysosomal targeting (Figure 4.2). Evidence points to a complex network of transcriptional and epigenetic control of *TNFAIP3* expression. AP-1 factors (*FOS*, *JUN*), *USF1*, *KCNIP3*, and glucocorticoid receptor signalling have all been implicated in *TNFAIP3* regulation,^{132,72,57} while chromatin remodelling in adipose tissue during obesity and weight loss suggests that accessibility at the locus itself may be altered. In parallel, *TNFAIP3* lies within one of the most eQTL-dense regions of the genome, where context-dependent variants can modulate expression under inflammatory or metabolic stress. Such interactions highlight how obesity may unmask genetic predispositions to *TNFAIP3* dysregulation.

These converging possibilities, transcription factor occupancy, chromatin state, and genetic variation, highlight why *TNFAIP3* cannot be understood through transcriptomics alone. In this chapter, I integrate evidence across single-cell and bulk RNA-seq, proteomics, ATAC-seq, and eQTL analyses, alongside co-expression network modelling, to map the regulatory landscape of *TNFAIP3*. The goal is to determine whether its suppression in obesity reflects a simple failure of NF- κ B induction or arises from higher-order regulatory blocks that can be reversed by weight loss.

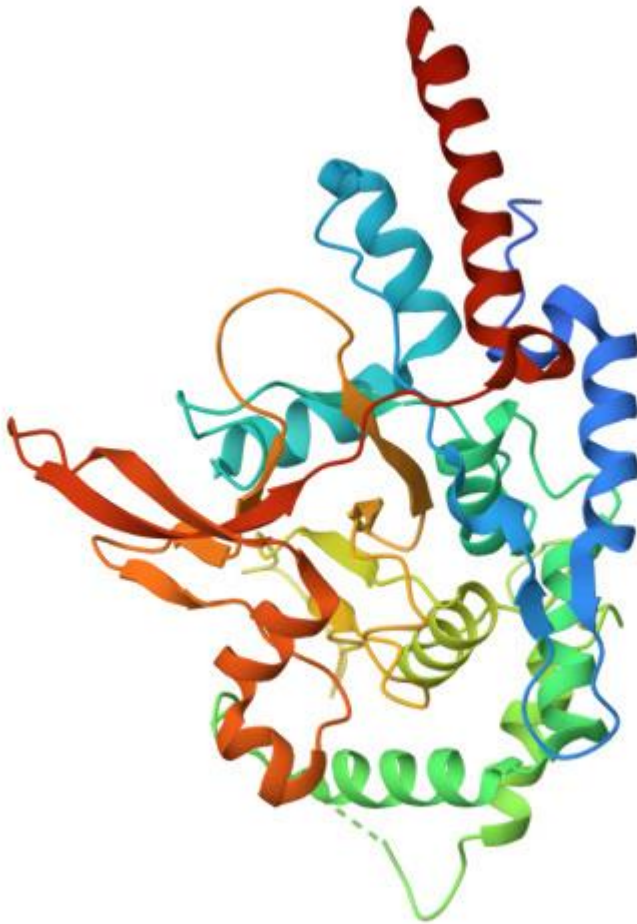


Figure 4.1 – Three-dimensional structure of A20 (*TNFAIP3*) from the Protein Data Bank. Ribbon representation of the full-length A20 protein showing the N-terminal OTU deubiquitinase domain and C-terminal zinc finger regions.

Domains of the A20 protein are shown below in Figure 4.2 highlighting the multi-functional capacity of the A20 protein.

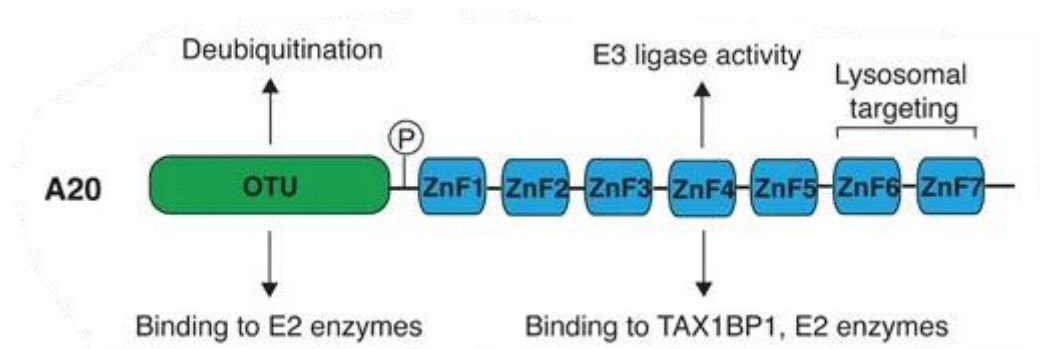


Figure 4.2 – Domain organisation and functional regions of A20 (*TNFAIP3*). The N-terminal ovarian tumour (OTU) domain mediates deubiquitination and binding to E2 ubiquitin-conjugating enzymes. The C-terminal region contains seven zinc finger (ZnF) motifs with distinct functions: ZnF1–2 contribute to E2 enzyme and TAX1BP1 binding; ZnF4 confers E3 ubiquitin ligase activity; and ZnF6–7 is implicated in lysosomal targeting. Phosphorylation sites (P) within the linker region modulate A20 activity.

4.2.0 – Chapter 4 - Results

Chapter 3 established that *TNFAIP3* levels and A20 are responsive to changes in the metabolic environment, with obesity leading to reduced expression across most immune cell types in humans and mice in multiple tissues. Building on these observations, Chapter 4 investigates the regulatory landscape underlying this suppression, integrating transcriptomic, epigenomic, genetic, and proteomic evidence. The aim here is not only to catalogue expression changes but to identify the factors and pathways that shape *TNFAIP3* regulation, and to determine whether its downregulation in obesity reflects a failure of canonical NF- κ B feedback or additional, higher-order regulatory constraints. By combining single-cell and bulk RNA sequencing, proteomics, ATAC-seq, and eQTL analyses, alongside co-expression network modelling, this chapter provides a systems-level perspective on how *TNFAIP3* is controlled in lean, obese, and weight loss states.

4.2.1 Expression Quantitative Trait Locus Analysis (eQTL)

eQTLs are regions of genomes associated with variations in the expression levels of specific genes. Here, I sought to find regions within the genome that affect expression levels to identify causal regulatory mechanisms. If a known *TNFAIP3* eQTL overlaps an open chromatin region in your immune cells and/or obesity datasets, it points to a likely cis-regulatory element. If that same enhancer becomes closed or repressed in obesity, it suggests that *TNFAIP3* expression will likely be reduced at an mRNA level.

Obesity → chromatin changes at enhancer → reduced transcription factor binding at eQTL locus → suppression.

Interestingly, of the 63 eQTLs associated with *TNFAIP3* on the GTEX database, 55 of the eQTLs affected expression within the brain, the rest were related to testis, skin and one related to blood.

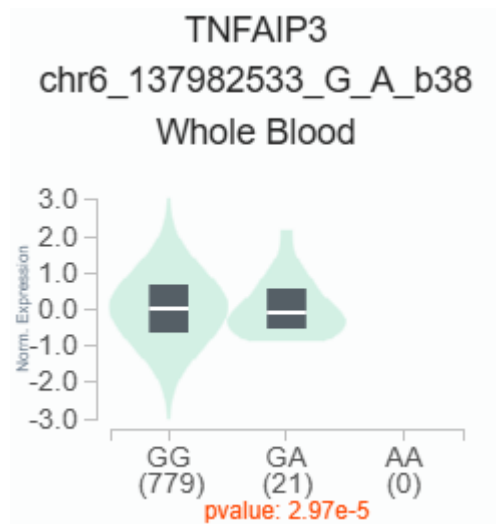


Figure 4.3 – The SNP rs12205708 (G → A) in the *TNFAIP3* locus is associated with reduced expression of *TNFAIP3* in blood. Genotype groups (G/G, G/A, A/A) are plotted on the x-axis, with normalised expression on the y-axis. Statistical significance was assessed by linear regression under an additive genetic model and *t*-test of the regression coefficient (data mined from the GTEx portal). Negative effect of the “A” allele is indicated by lower median expression in A carriers.

The SNP in Figure 4.3, rs12205708, is about 120 kilobases downstream of the *TNFAIP3* transcription start site and the only blood related eQTL identified for *TNFAIP3*. As blood is the primary tissue studied in humans in this thesis, note was taken of this SNPs position for future work in ATAC-seq analyses carried out on MD6 dataset later on in this chapter. As human genetic data was not analysed in this thesis, beyond recognising that SNPs exist that can modulate expression of *TNFAIP3*, no links can be made between eQTLs and expression of the gene in obesity or weight loss.

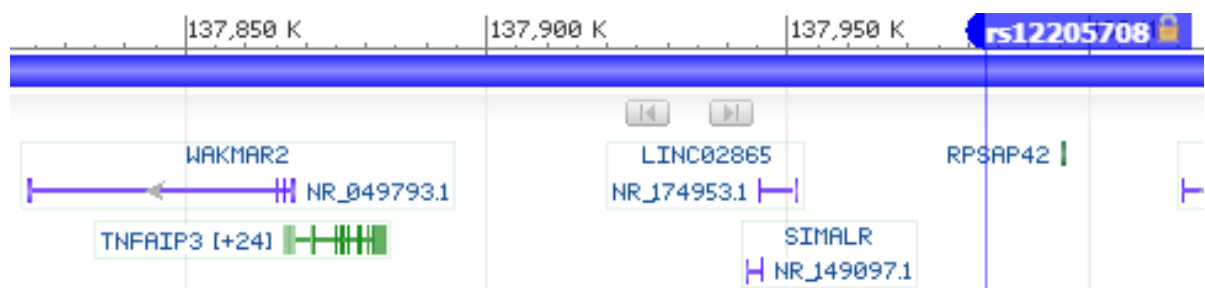


Figure 4.4 – *TNFAIP3* distance from eQTL (rs12205708), plot taken from GTEx portal showing *TNFAIP3* position in its local genetic environment, visualised using the GTEx portal

4.2.2 Splicing Quantitative Trait Loci Analysis (sQTLs) – *TNFAIP3* sQTLs

In addition to eQTLs, another genetic trait that can be analysed are splicing quantitative trait loci (sQTLs) which are explored for potential variants that can alter functionality of the

TNFAIP3 region. 717 sQTLs have been identified for *TNFAIP3* across the human body as discovered on the Ensembl website. Given that A20 has multiple roles within the body as shown in Figure 4.2 (A20 functions by domain), functions could vary hugely across individuals depending on whether there are alternative splicing mechanisms in different tissues or diseases. Common splice variants are shown in Table 4.1.

Table 4.1 – *TNFAIP3* splice variants transcript table

Transcript ID	Name	bp	Protein	Biotype
ENST00000612899.5	<i>TNFAIP3</i> -206	4665	790aa	Protein coding
ENST00000421450.2	<i>TNFAIP3</i> -203	4689	790aa	Protein coding
ENST00000433680.2	<i>TNFAIP3</i> -204	4533	790aa	Protein coding
ENST00000237289.8	<i>TNFAIP3</i> -201	4432	790aa	Protein coding
ENST00000420009.6	<i>TNFAIP3</i> -202	3861	790aa	Protein coding
ENST00000711061.1	<i>TNFAIP3</i> -209	4683	108aa	Nonsense mediated decay
ENST00000698330.1	<i>TNFAIP3</i> -208	336	No protein	Protein coding CDS not defined
ENST00000698329.1	<i>TNFAIP3</i> -207	5432	No protein	Retained intron
ENST00000485192.2	<i>TNFAIP3</i> -205	4966	No protein	Retained intron

Table 4.1 Legend – *TNFAIP3* splice variants identified using the Ensembl portal

Unless Smart-seq2 is used for scRNA-seq, it is very difficult to tell which splice variants are present in the data. Thus, no further analysis can be done around splice variants except hypothesise which variants of the gene could be transcribed given chromatin accessibility which will be covered in the ATAC-seq analysis.

4.2.3 - *TNFAIP3* is co-expressed within a highly anti-inflammatory network of genes

TNFAIP3's restoration with weight loss is a good indicator of cell health in immune cells, however, the context within which it is being expressed is important, for example, what other genes are being coexpressed with *TNFAIP3* matter as it may uncover the mechanisms and changes occurring within the cell. To uncover this, hdWGCNA was utilised which is an R package allowing for identification of groups of genes and modules of gene that are co-expressed together in scRNA-seq data. Downstream of finding groups of genes that are co-expressed, string analysis can be applied to integrate co-expressed genes with known protein-protein interactions and build networks to visualise how these genes would interact at a protein level. This establishes biologically grounded groups of genes that have been established as interactors at a protein level. Thus, *TNFAIP3*s coexpressed targets can be established and this establishes a biological context within which the gene is changing.

For this analysis the human weight loss dataset (HD2) detailed in section 2.1.7 in Chapter 2 was utilised along with the MD2 public dataset.

Initial steps for both datasets involved taking the processed scRNA-seq data and isolating the cells of interest, in my case NK cells were chosen. In NK cells, *TNFAIP3* levels change in response to obesity or weight loss and as NK cells are established in literature to be dysregulated metabolically with several cytotoxic defects in obesity in both humans and mice and thus were chosen. Figure 4.5 was shown previously in Figure 3.15 and is placed into this chapter again as a reminder and to highlight the cells isolated for hdWGCNA analysis (CD56^{bright} and CD56^{dim} NK cells).

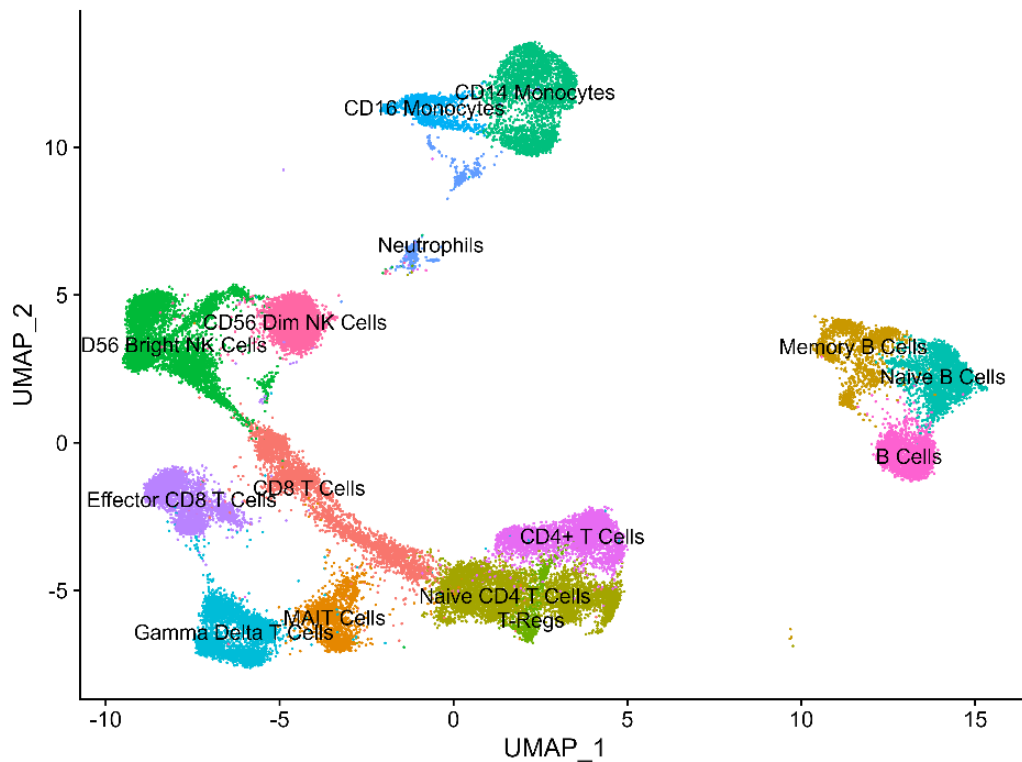


Figure 4.5 – UMAP projection of single-cell RNA-sequencing (scRNA-seq) data from the human caloric restriction study (HD2). Each point represents a single cell, coloured by its assigned cell-type cluster, positioned according to its transcriptional similarity in UMAP space.

NK cells were isolated from both datasets and aggregated into metacells as described in the methods, these were then used as the input expression matrix to create the network. The network was then created for both the human and mice NK cells. Modules of genes are identified after construction of the network. These modules of genes are groups of genes that are highly coexpressed together within the cell type used to create the network. For this analysis both pre-weight loss and after weight loss NK Cells were used and CD56^{Bright} NK Cells along with CD56^{Dim} NK Cells were used together. This was to get adequate numbers of cells to form the network despite their being subtleties in their expression.

In humans 5 gene modules were identified as shown in Figure 4.6.

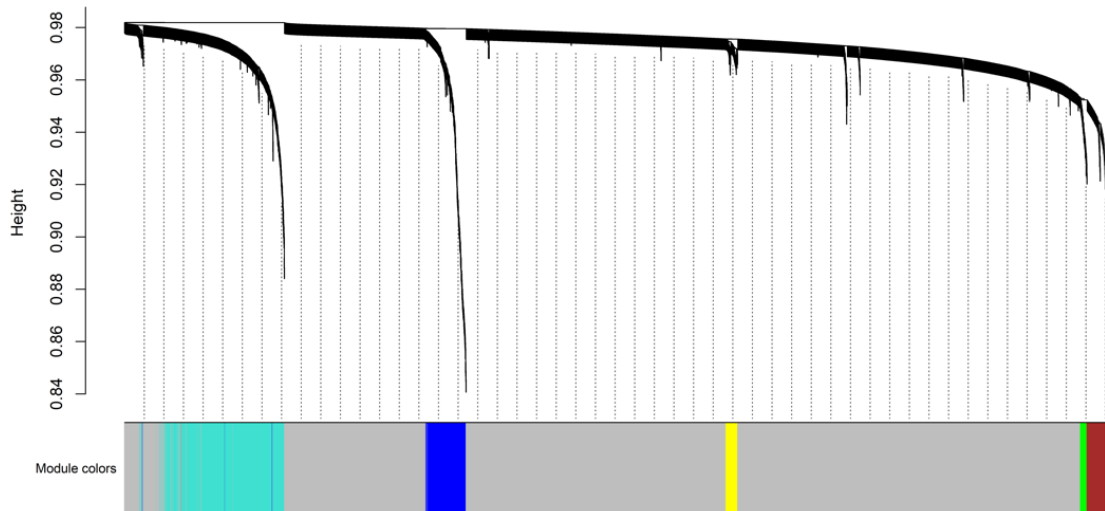


Figure 4.6 – Hierarchical clustering dendrogram of genes from NK cells generated using high-dimensional WGCNA (hdWGCNA). Each branch represents a gene, and the clustering tree groups genes according to topological overlap. The coloured bar beneath the dendrogram indicates module assignment, where each colour corresponds to one of the five identified gene co-expression modules (turquoise, blue, yellow, green, and brown). Genes not assigned to a module are shown in grey

To get an understanding of the underlying biology of each module, pathway analysis was run on the genes classified into each module shown in Figure 4.7. The hdWGCNA analysis resolved five distinct co-expression modules, each reflecting a different layer of cellular function (Figure 4.6). The pathway analysis (Figure 4.7) revealed that the turquoise module was enriched for antigen processing and NK cell degranulation, pointing to a core cytotoxic program likely driving immune effector activity. The blue module mapped strongly to translation, cytoplasmic and peptide biosynthesis pathways, representing a transcriptionally active ribosomal and protein-production hub. In contrast, the brown module carried signatures of mRNA export and post-transcriptional gene regulation. The yellow module was enriched for cell–cell junction assembly and microtubule localisation, reflecting cytoskeletal reorganisation and intercellular contact processes (Figure 4.7). Finally, the green module displayed enrichment for apoptotic regulation and mRNA tail shortening according to the pathway analysis, suggesting a stress-responsive transcriptional program linked to RNA metabolism and cell fate control. Together, these modules outline a structured set of different immune and metabolic processes, with clear implications for how cellular states adapt under obesity and weight loss conditions.



Figure 4.7 – Gene Ontology (GO) Biological Process enrichment analysis of NK cell hdWGCNA modules. Genes from each of the five hdWGCNA modules (turquoise, blue, brown, yellow, green) were submitted to the GO Biological Process 2023 database using the Enrichr package. The y-axis lists the top enriched biological processes for each module, while the x-axis indicates module identity. Point colour represents the statistical significance of enrichment ($-\log_{10} p$ -value), with warmer colours indicating stronger enrichment. Point size reflects the degree of enrichment ($\log_{10}$ enrichment score).

Central hub genes for each module were identified; hub genes are genes that are generally considered to have higher connectivity and are more central to that module's functionality. The kME is the metric used, which represents a genes connectivity within the module (Figure

4.7). The only hub genes that will be important for this chapter are green hub genes which will be explored in detail throughout this chapter. Genes identified in the green module such as *DUSP2*, *ZFP36* and *CXCR4* are discussed in detail in later sections of this chapter and showcased in Figure 4.8 as central to the green modules connectivity.

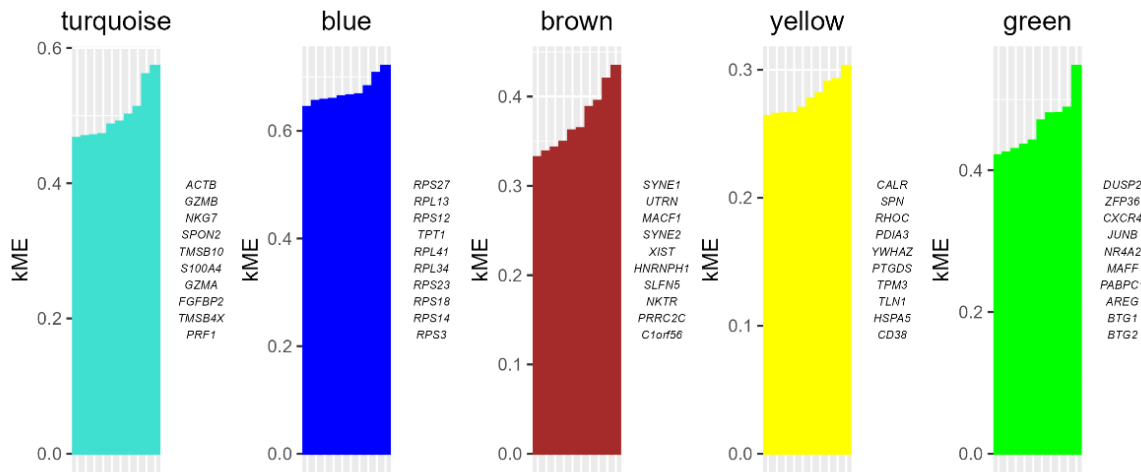


Figure 4.8 – Module membership (kME) of top hub genes in each hdWGCNA module identified from NK cells. Bars represent the eigengene connectivity (kME) values of genes within each module (turquoise, blue, brown, yellow, green), which quantify how strongly each gene correlates with the corresponding module eigengene. The highest-ranking hub genes for each module (listed alongside the bars) show the strongest module membership and are candidate drivers of each module’s co-expression pattern – HD2

Modules (groups) of genes, like individual genes, can also be found to be differentially expressed between two conditions (pre weight loss and post weight loss). In humans, obese vs weight loss differential module expression revealed that the green module, blue module, yellow and brown module were associated (upregulated) with weight loss in NK cells, the turquoise cytotoxic module was more associated with obesity in NK cells (Figure 4.9).

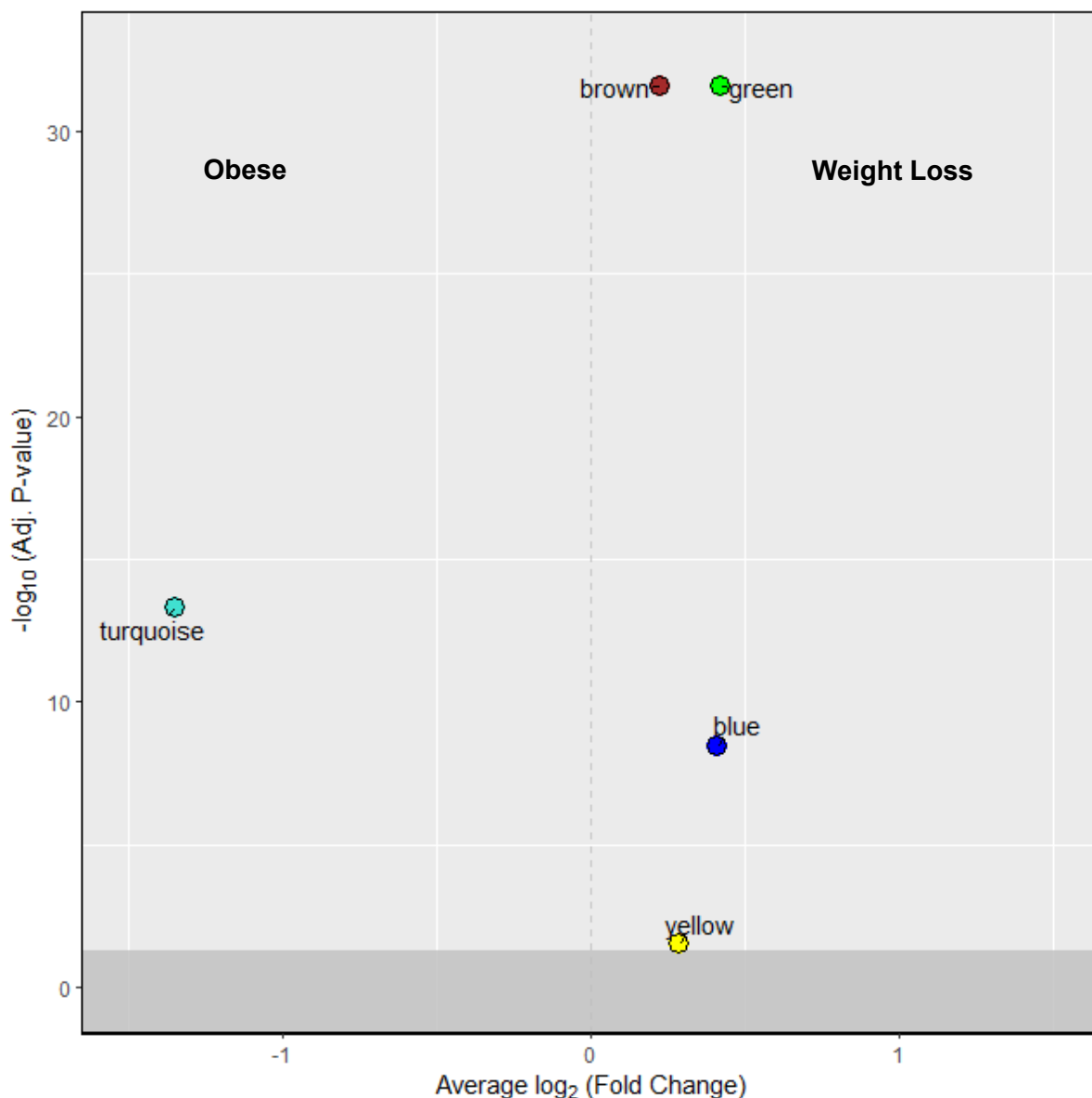


Figure 4.9 – Volcano Plot with Log₂ fold change of hdWGCNA module eigengene expression between obese and weight-loss NK cells. Each coloured point corresponds to one hdWGCNA module (yellow, blue, green, brown, turquoise). The x axis represent the log₂ fold change (weight loss vs. obesity) for each module eigengene. Positive values indicate modules upregulated in NK cells after weight loss, whereas negative values indicate modules upregulated in NK cells during obesity. Statistical significance of differential module expression was assessed using the Wilcoxon rank-sum test.

TNFAIP3 was identified to belong to the green module. The green module consisted of 50 genes. To gain a better understanding of the overall functions of the green module, the co-expression network was overlaid with protein-protein interaction (PPI) interaction networks from stringDB. By adding PPI to the co-expression matrix generated from the original scRNA-seq matrix, one gains a biologically grounded network of interacting proteins as

shown in Figure 4.10. The addition of established PPI interactions helps remove noise which is naturally present in the co-expression network by integrating with PPI from stringDB.

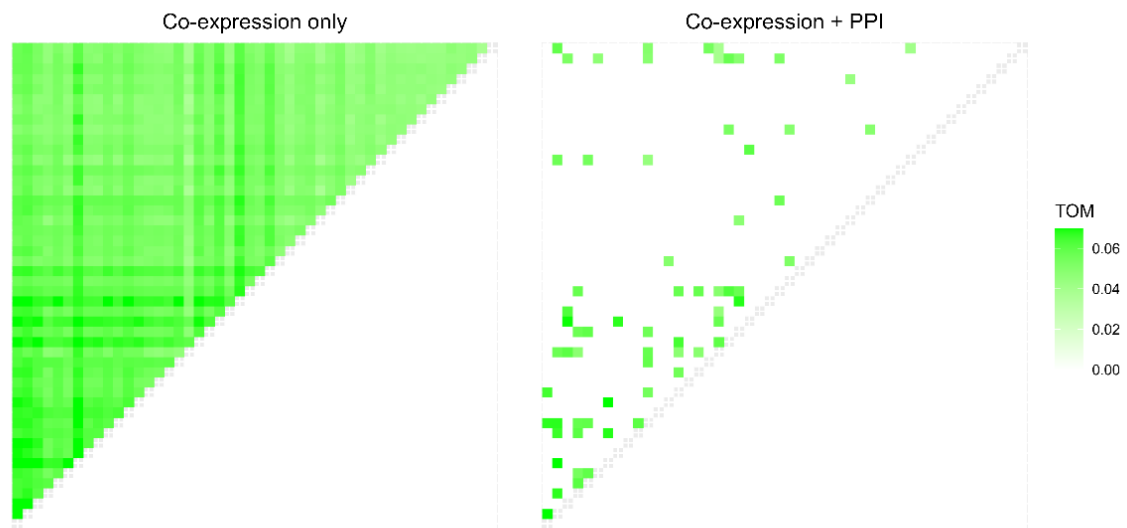


Figure 4.10 - Topological overlap heatmaps of the green hdWGCNA module derived from NK cells. The left panel shows the topological overlap matrix (TOM) calculated using gene co-expression only. The right panel shows the TOM recalculated after integrating both gene co-expression and protein–protein interaction (PPI) data. Each square represents the strength of connectivity between a pair of genes (TOM value; color scale at right), with darker green indicating higher topological overlap. Integration of PPI data (right) highlights a more selective, higher-confidence set of gene–gene relationships compared with co-expression alone (left).

Plotting of the network reveals an interesting set of interactions. When applying PPI to the co-expression network, naturally a lot of genes co-expressed in the scRNA-seq expression matrix will not have been validated to interact at the protein level and thus they do not get included in the final biological network created from the integrated string PPI and co-expression data (Figure 4.10). One established interaction is enough for to be included in the final co-expression and PPI network. One is at risk of losing novel biological interactions here, similarly whilst they may not interact at a protein level, the interaction could occur at another regulatory level. This is especially possible given the number of transcription factors present in the green module. String analysis does not include transcription of a gene as an interaction, the interaction must occur at the protein level.

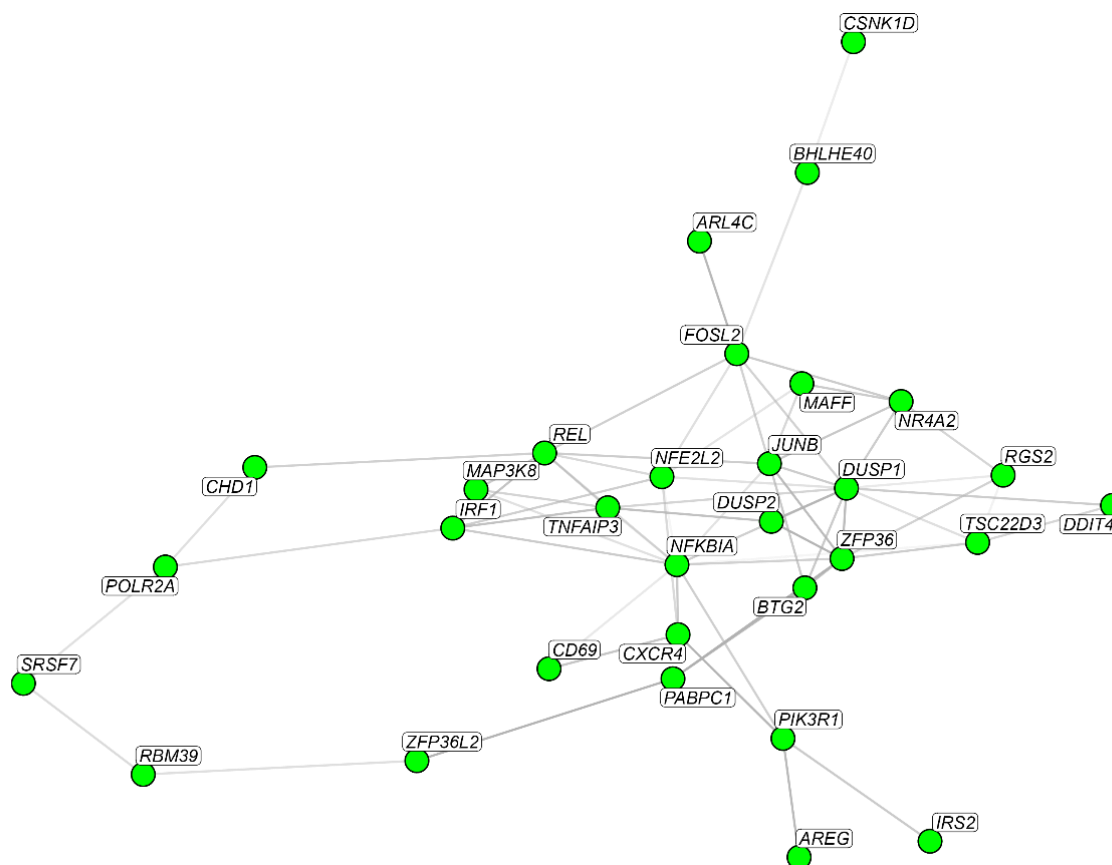


Figure 4.11 – Global structure of co-expression & PPI integrated network in green module built using human CD56^{dim} and CD56^{bright} NK cells as input to hdWGCNA – the 50 genes within the green network are uploaded to StringDB and any interactions at a protein level are retained, a network of established interactions can then be built

In the protein–protein interaction (PPI) network focused on *TNFAIP3* (A20), several well-characterised interactors emerge, providing mechanistic insight into its regulatory context. *NFKBIA* and *REL* are tightly associated with *TNFAIP3*, consistent with A20's established role in attenuating NF- κ B signalling through feedback inhibition pathways. A20 typically exerts this effect by removing K63-linked ubiquitin chains and promoting K48-linked ubiquitination of key signalling intermediates such as RIP1 and TRAF6, thereby terminating upstream receptor signalling and limiting sustained NF- κ B activation ¹³¹. Upstream signalling kinases such as MAP3K8 are also connected to *REL* and *NFKBIA*, placing *TNFAIP3* at the nexus of the MAPK–NF- κ B axis and showing the level of crosstalk that occurs between inflammatory pathways. Other phosphatases, including *DUSP1* and *DUSP2*, are also detected in the network, these phosphatases modulate MAPK activity and are known to be involved in resolving inflammatory signalling, suggesting synergistic regulation with A20. Collectively, this network highlights a tightly integrated signalling module in which

TNFAIP3 interacts with both canonical NF- κ B components and MAPK regulators, reflecting its dual role in shutting down inflammation at multiple molecular nodes.

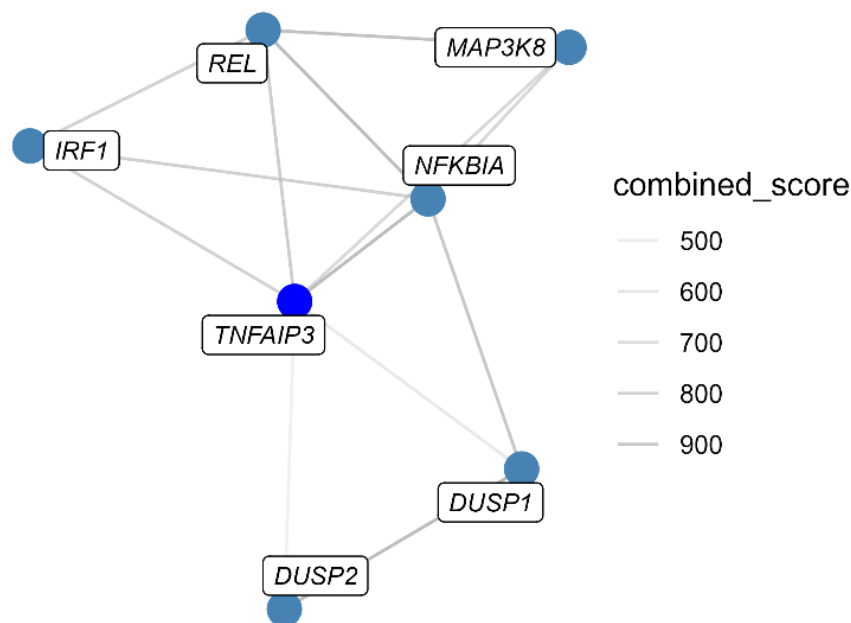


Figure 4.12 – Local structure of PPI network in green module around *TNFAIP3* – score represents a confidence score, higher scores represent higher confidence in the interaction with 400 used as the lowest possible retention interaction score – all genes are established genes with interaction score above 400 with *Tnfaip3* in the green network

Of course, changes in PPI will only capture proteins with established interactions, however, beyond PPI, many important biological interactions are happening at the transcription factor and chromatin accessibility level which is highlighted here as there are several genes that work via chromatin modifications. By plotting the PPI network with the additional genes missing from the PPI network using their co-expression metrics and their top 2 correlated genes we can build a full network of all the genes in the green module and get a sense of what is interacting at protein level and what may be occurring at a more genetic level. The genes are shown as having blue edges which means this interaction exists at a co-expression level derived from the scRNA-seq data, the black edges are established to interact at a protein level on the String database. *ARL4C* and *PABPC1* emerge as hub genes in the co-expression section as several genes are linked to these two genes (Figure 4.11). *ARL4C* is known to regulate cytoskeletal remodelling and membrane dynamics through actin reorganisation. Its involvement in cell migration and Wnt/TGF- β -driven pathways suggests that transcriptional upregulation of *ARL4C* in this context may reflect shifts in cellular motility or activation states.

PABPC1, a central regulator of mRNA stability and translation, also stands out as a co-expression hub. Given its role in global protein synthesis and mRNA decay, high co-expression connectivity with *PABPC1* likely indicates coordination of protein expression programs across the module, potentially in response to metabolic or inflammatory cues.

In the expanded co-expression network identified via hdWGCNA, one metabolically relevant node stands out prominently: *IRS2*, the insulin receptor substrate-2 (Figure 4.11), which is part of the green module. *IRS2* is a critical mediator of insulin and IGF-1 signalling that propagates downstream activation of PI3K-AKT and MAPK pathways, essential for glucose uptake, lipid metabolism, and cell survival. Obesity is known to suppress *IRS2* expression, especially in myeloid and adipose tissue macrophages, leading to impaired IL-4 driven M2 polarization and systemic insulin resistance. In contrast, the elevated *IRS2* observed with weight loss suggests restoration of insulin sensitivity and re-establishment of anti-inflammatory programs. Additional interactors around *TNFAIP3* in the same network, particularly *PIK3R1* (the regulatory subunit of PI3K), further link *TNFAIP3* related inflammation control to metabolic insulin signalling pathways. Collectively, the presence and upregulation of *IRS2* and *PIK3R1* in this network highlight a convergence of inflammatory and metabolic regulatory circuits that are reversed following weight loss.

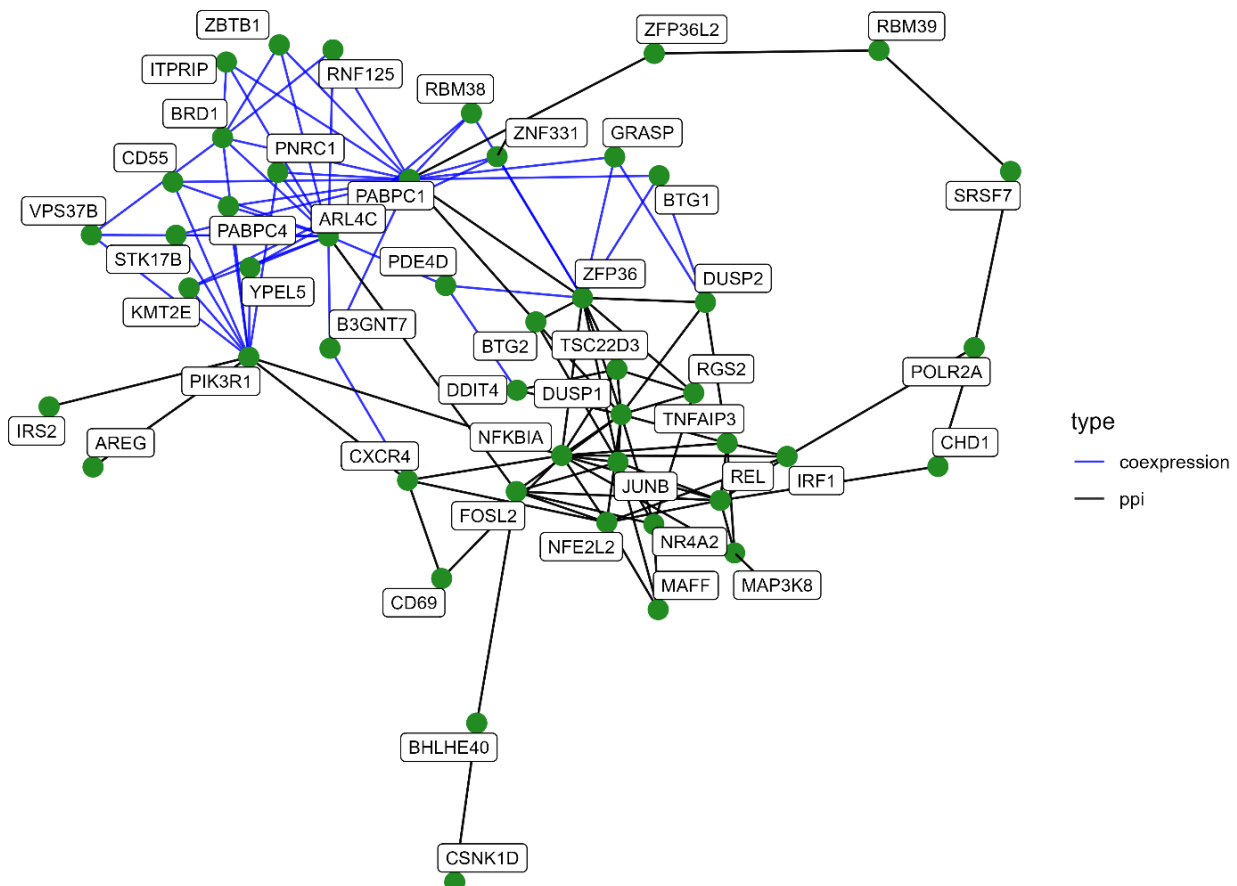


Figure 4.13 – Combined protein–protein interaction (PPI) and co-expression network centred on *TNFAIP3* (A20) in NK cells. Nodes represent genes (green circles), with black edges indicating curated PPI connections and blue edges indicating co-expression links. Genes present in the PPI database are connected by their known physical or functional interactions. Genes absent from the PPI database were added back into the network by linking each to its top two co-expressed partners to preserve module structure. This hybrid approach highlights both established A20 interactors (e.g., *NFKBIA*, *REL*, *JUNB*) and additional co-expression partners, providing a broader view of the regulatory landscape surrounding *TNFAIP3*.

4.2.4 - The Cytotoxic Program of Gene Regulation in Obesity

The cytotoxic turquoise module was upregulated in NK cells in obesity as shown in Figure 4.9. This module is significantly larger than the green module with a total of 821 genes, compared to 50 genes for the green module (data not shown). This module was characterised by high levels of cytotoxic genes such as *PRF1*, *GZMA* and *GZMB*. The chemokine receptor *CX3CR1* is of interest as a trafficking gene for NK cells and was upregulated in obesity in NK cells in contrast to *CXCR4* which is upregulated after weight loss from obesity as shown by their expression in a volcano plot of CD56^{dim} NK cells shown later in Figure 4.23 (Figure is more relevant to later sections). Whilst the green module was small, with only 50 genes, the turquoise module captures a broader and generally cytotoxic response. Given *CX3CR1* is the most significantly upregulated gene in obesity prior to weight loss (Figure 4.23), a local network was plotted around this gene network revealing *CX3CR1* as a major hub, connecting to numerous immune effector and signalling molecules (Figure 4.14). Prominent interacting partners included cytotoxic mediators (*PRF1*, *GZMA*, *GZMB*) and adhesion/integrin molecules (*ITGB2*, *ITGAE*), suggesting a potential role in coordinating NK cell trafficking and cytotoxic function in NK cells. Chemokines and chemokine receptors (*CCL3*, *CCL4*) were strongly represented, indicating a chemotactic signalling axis anchored by *CX3CR1*. Several key regulatory proteins, such as *CASP1* (inflammatory caspase), *TYROBP* (DAP12 adaptor protein), and *FCGR3A* (CD16), further link *CX3CR1* to innate immune activation and antibody-dependent cytotoxicity.

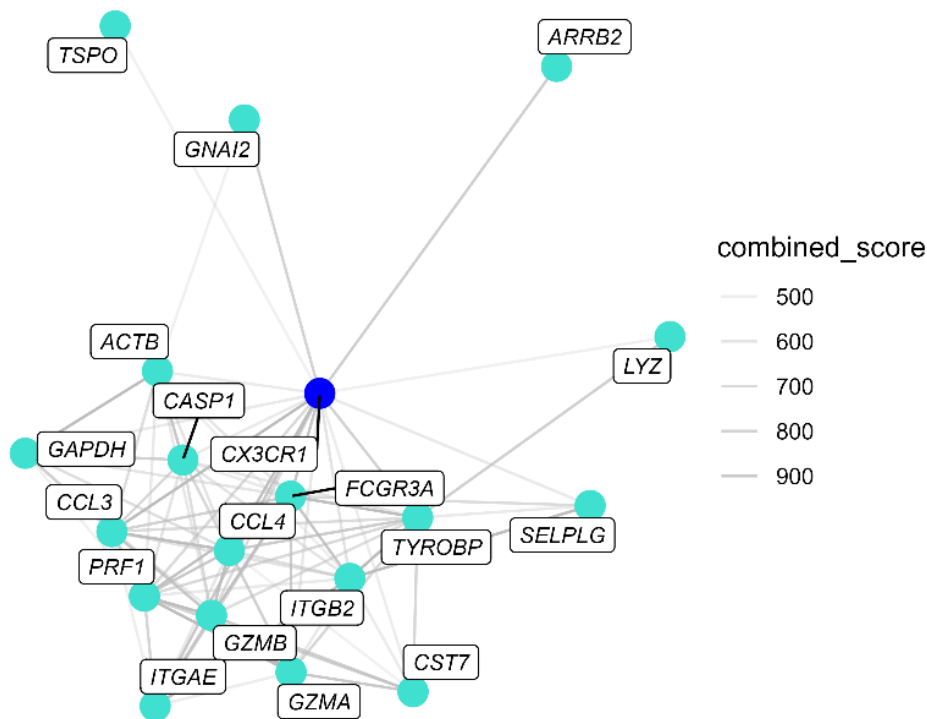


Figure 4.14 – Local network centred around the *CX3CR1* trafficking gene revealing a highly cytotoxic network of genes: created from PPI and co-expression of the turquoise module - network created from the integration of PPI and co-expression data from the original scRNA-seq

In the broader context of the cytotoxic turquoise module associated with obesity, metabolic genes were altered significantly; particularly there was an upregulation of genes of the OXPHOS pathway and for several glycolytic genes (Figure 4.15). This included elevated expression of core mitochondrial respiratory chain components such as *NDUF6B*, *NDUFA4*, *COX6A1*, *COX17*, *ATP5MC*, and *UQCRI0*, together with mitochondrial transporters like *VDAC1* and *VDAC2*. At the same time, glycolytic enzymes including *GAPDH*, *LDHA*, and

TP11 were also enriched in this module, pointing to coordinated shifts in both oxidative phosphorylation and glycolysis (Figure 4.15).

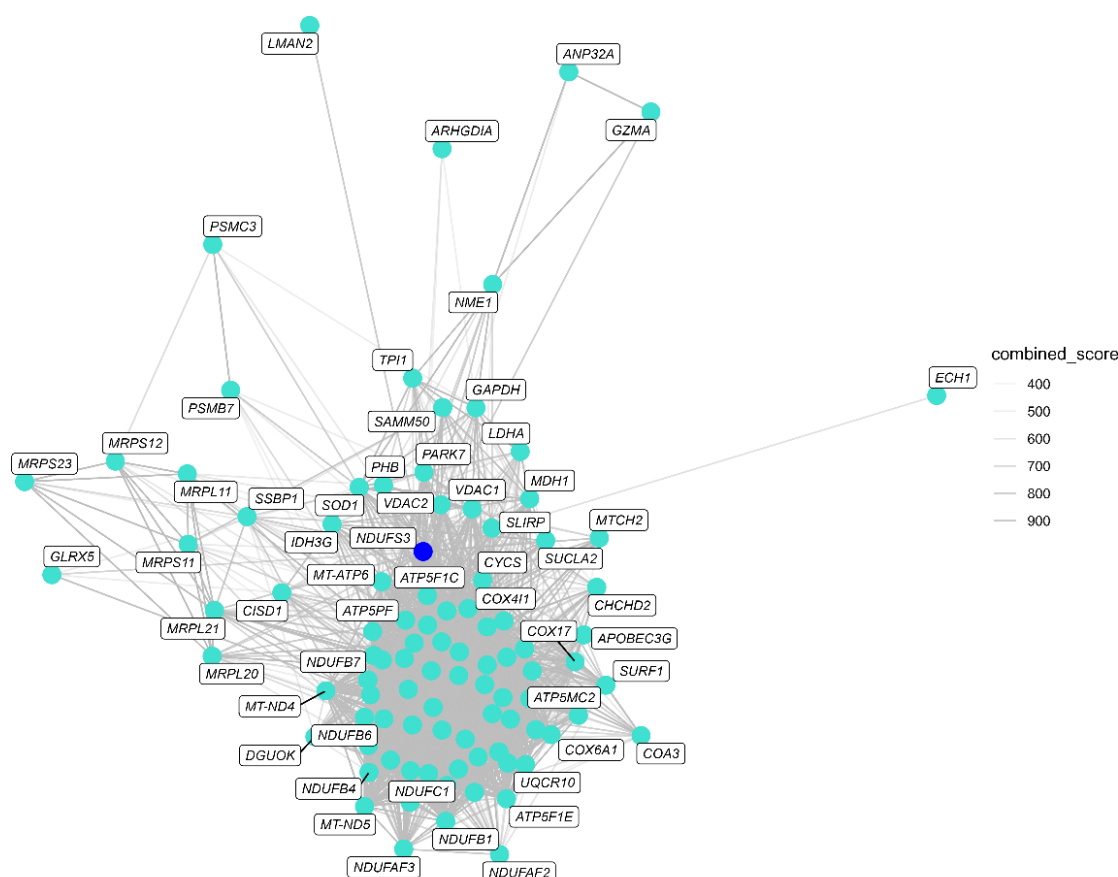


Figure 4.15 – Hub of metabolic genes identified in the turquoise module highlighting coupling of metabolic genes to cytotoxic output – network created from the integration of PPI and co-expression data from the original scRNA-seq

4.2.5 - Transcription Factor Analysis via MotifScan and xgboost

To investigate upstream regulatory drivers of *TNFAIP3* expression, I used MotifScan, which applies the motifmatchR algorithm to scan promoter regions of all expressed genes within the input scRNA-seq matrix for occurrences of transcription factor motifs. This function annotates each gene's promoter within the matrix, enabling integration of motif presence with gene expression data. Using this pipeline, I performed an unbiased motifmatchR analysis on all genes in the dataset, screening for transcription factor motifs using the JASPAR database.

Motif analysis combined with correlations present in scRNA-seq matrix using the hdWGCNA package revealed the top 10 transcription factors most strongly associated with *TNFAIP3* expression: *IRF1*, *FOSL2*, *REL*, *JUNB*, *BHLHE40*, *CREM*, *KLF3*, *FOXX1*, *SP3*,

and *FOS*. Among these, *IRF1* showed the strongest correlation (Cor = 0.648) and highest motif gain score (0.377), suggestive of a prominent role in *TNFAIP3* regulation. Members of the AP-1 family (*FOSL2*, *JUNB*, *FOS*) and NF-κB family (*REL*) also featured prominently, consistent with *TNFAIP3*'s known role in inflammatory and stress-response signalling. To appear as a transcription factor on this list, a few conditions must be met. e.g. a promoter region must be available for the transcription factor to bind to, and the gene must be expressed in the original scRNA-seq matrix. Thus, genes may have a motif detected using hdWGCNAs function 'MotifScan', a transcription factor may be highly correlated in the expression matrix, it does not mean that the top hit, *IRF1*, is necessarily driving *TNFAIP3* expression.

Table 4.2 – Top 10 predicted transcription factors involved in *TNFAIP3* regulation in human NK cells

<i>TF</i>	Gene	Gain	Cover	Frequency	Cor
<i>IRF1</i>	<i>TNFAIP3</i>	0.37684	0.094	0.094	0.647618
<i>FOSL2</i>	<i>TNFAIP3</i>	0.144329	0.078	0.078	0.610364
<i>REL</i>	<i>TNFAIP3</i>	0.177472	0.084	0.084	0.602113
<i>JUNB</i>	<i>TNFAIP3</i>	0.092134	0.042	0.042	0.564988
<i>BHLHE40</i>	<i>TNFAIP3</i>	0.088757	0.138	0.138	0.556711
<i>CREM</i>	<i>TNFAIP3</i>	0.004251	0.024	0.024	0.35298
<i>KLF3</i>	<i>TNFAIP3</i>	0.017973	0.072	0.072	0.34194
<i>FOXX1</i>	<i>TNFAIP3</i>	0	0	0	0.293609
<i>SP3</i>	<i>TNFAIP3</i>	0	0	0	0.2851
<i>FOS</i>	<i>TNFAIP3</i>	0	0	0	0.283903

In addition to looking at the top transcription factors outputted from the model, some additional transcription factors known to be regulators of *TNFAIP3* were examined to explore what genes they were correlated to (*KCNIP3*, *USF1* and all many of these genes are understandably the genes found in Figure 4.11, the green module (network), as these were partially built on co-expression. However, interesting NK cell receptors genes show up here that have not shown up in previous analyses. *IL18R1* was the top target of *NFKB1* and 10th

for *NFKB2*, *IL18R1* is associated with increased expression of interferon- γ , a key cytokine associated with NK cells. The crosstalk between transcription factors is significant as several of them can bind to the promoter of many shared genes.

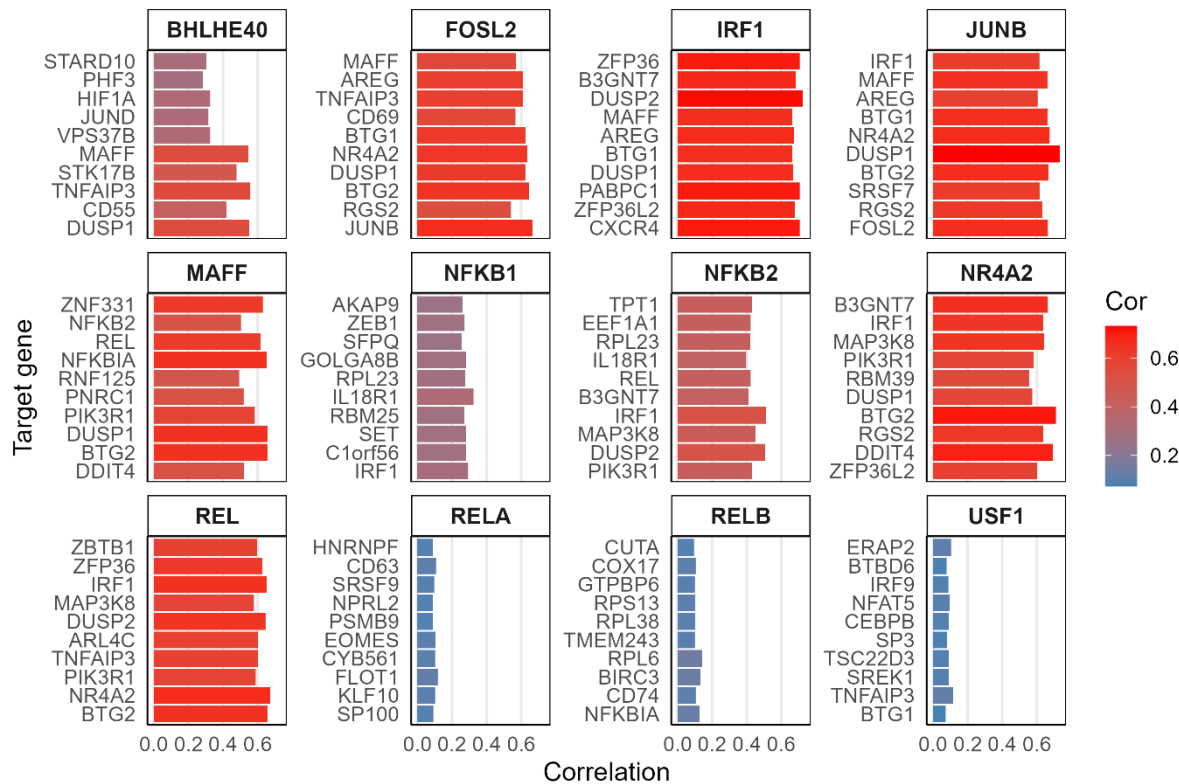


Figure 4.15 – Transcription factors from green module & five NF- κ B transcription factors - Top 10 Correlated Targets with established motifs, genes ordered from top to bottom for each transcription factor based on feature importance from an xgboost model, correlation is simply showing how correlated expression is between the transcription factor and the target gene in the co-expression matrix. Calculated using original scRNA-seq input matrix and motif scan, data fed into xgboost model to calculate top transcription factor targets in data

The bar plots in Figure 4.15 show the top target genes for a transcription factor based on the transcription factor regulatory network analysis. Target genes are ranked by the predicted interaction strength from XGBoost (Gain) multiplied by the sign of the correlation between the transcription factor and the target gene from the co-expression matrix. To clarify that expression was increased in all the transcription factors in the green module, a dotplot was used to show increases in NK cell expression across these transcription factors (Figure 4.16).

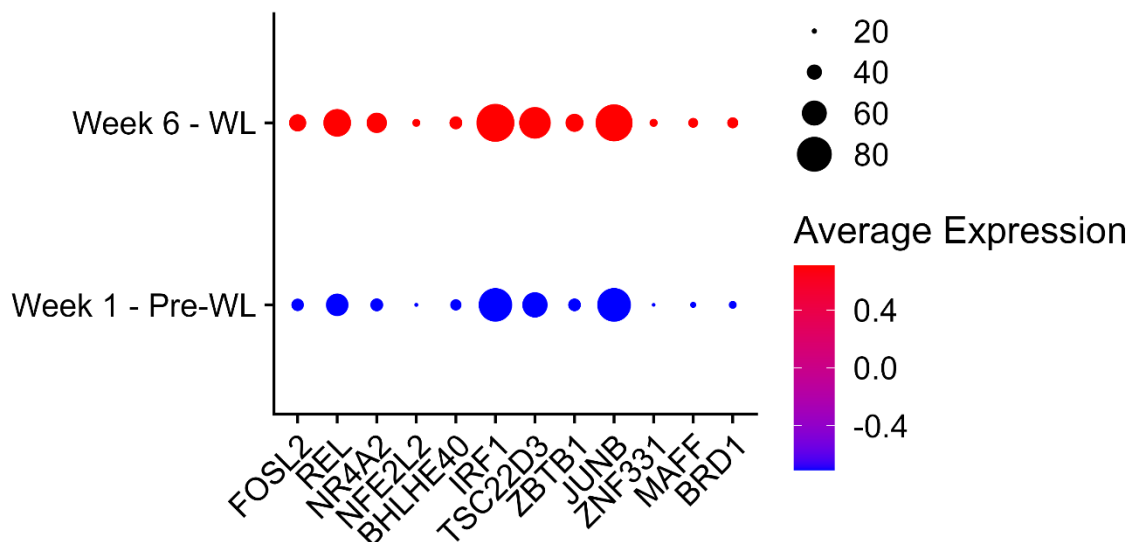


Figure 4.16 – Expression of transcription factors from the green hdWGCNA module in NK cells before and after weight loss. Each point represents a transcription factor, with point size proportional to the percentage of NK cells expressing the gene and point colour indicating the average expression level (red = higher expression, blue = lower expression). Data are shown for NK cells at Week 1 (pre-weight loss; blue) and Week 6 (post-weight loss; red), illustrating transcriptional changes in module-associated transcription factors over the weight loss intervention.

4.2.6 - MD2 - Mouse hdWGCNA results – Adipose Tissue NK Cells

hdWGCNA analysis can be attempted on integrated mouse and human data together, where gene names are standardised, and consensus variation is looked for across datasets. This was attempted and the results were spurious, therefore, analysis on the MD2 dataset was completed in isolation allowing for an analysis untainted by the technical variation that could be introduced whilst doing a consensus analysis. The MD2 dataset was chosen for several reasons, the most important being it was the longest period of obesity and weight loss, more representative of the human dataset. Secondly, there are more NK cells (1325) in this dataset than the other mouse datasets, which improves downstream co-expression own.

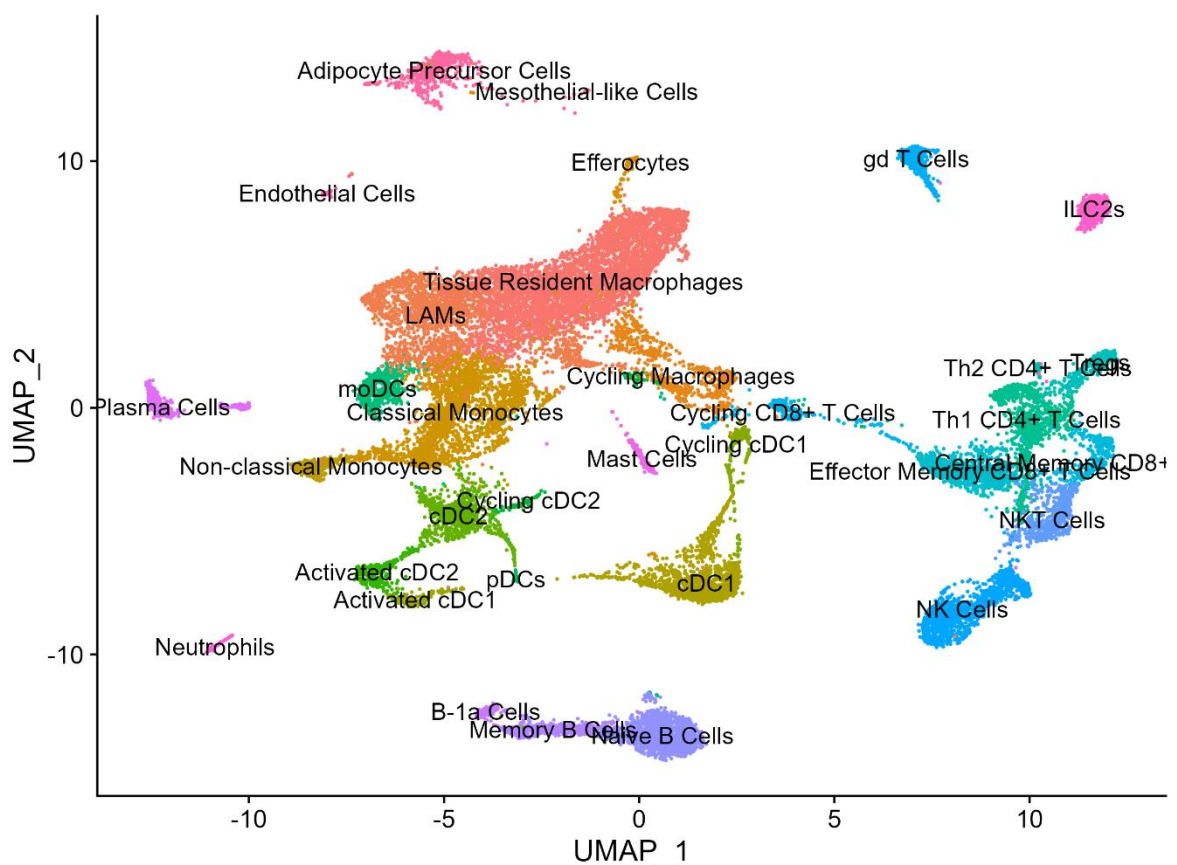


Figure 4.17 – UMAP projection of single-cell RNA-sequencing (scRNA-seq) data from the mouse weight loss study (MD2). Each point represents a single cell, coloured by its assigned cell-type cluster, positioned according to its transcriptional similarity in UMAP space.

The hdWGCNA package was used using the same procedures as used for human dataset above. NK Cells were isolated in the MD2 dataset and fed into the hdWGCNA pipeline. This analysis of murine NK Cells revealed 7 modules of coexpressed genes. Module sizes varied significantly in the number of genes present in the dataset (Figure 4.18).

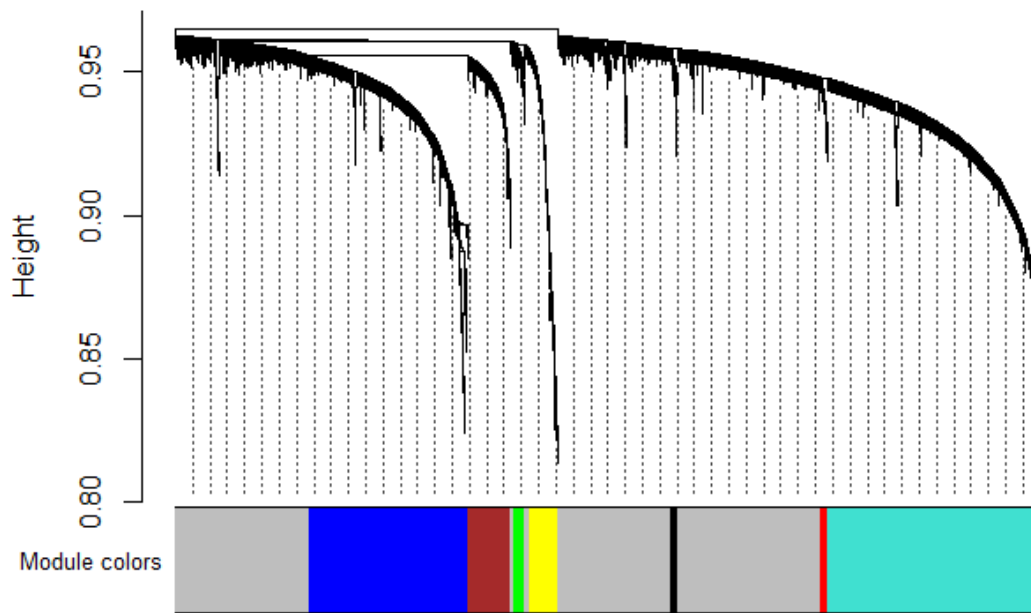


Figure 4.18 – Hierarchical clustering dendrogram of genes from NK cells generated using high-dimensional WGCNA (hdWGCNA) from the MD2 dataset. Each branch represents a gene, and the clustering tree groups genes according to topological overlap. The coloured bar beneath the dendrogram indicates module assignment, where each colour corresponds to one of the seven identified gene co-expression modules (turquoise, blue, yellow, green, black, red, brown). Genes not assigned to a module are shown in grey.

It is important to note that colours are assigned randomly and that green module in human doesn't have any link to the green module in mice, these are arbitrarily assigned colours for each hdWGCNA analysis. In mice, green module genes are strongly enriched for DNA replication and repair processes, including mitotic DNA replication, DNA-templated DNA replication initiation, and double-strand break repair via break-induced replication. These pathways consistently appear as the top enriched terms; thus, the green module represents a highly proliferative or active cell cycle NK cell state which is upregulated in weight loss.

Table 4.3 - Average $\log_2$ fold change, expression percentages, and adjusted p -values for hdWGCNA modules in NK cells comparing weight loss versus obese individuals.

Module	Average Log ₂ Fold Change	pct.1	pct.2	Adjusted p-value
yellow	-0.28	0.37	0.55	2.85e-121
brown	-0.63	0.48	0.64	2.92e-120
turquoise	-1.89	0.4	0.56	1.11e-110
blue	-1.16	0.47	0.6	1.76e-80
black	0.73	0.49	0.38	2.06e-59
red	0.84	0.45	0.37	3.05e-33
green	0.23	0.28	0.33	2.30e-13

Table 4.3 Legend - Negative $\log_2$ fold change values indicate lower module eigengene expression in weight-loss NK cells relative to obese NK cells; positive values indicate higher expression in weight-loss NK cells. pct.1 and pct.2 refer to the proportion of cells in the weight loss and obese groups, respectively, expressing module genes. Adjusted p-values are from Wilcoxon rank-sum tests corrected for multiple testing

Pathway analysis revealed that the turquoise module, which is downregulated in weight loss compared to obesity ($\log_2FC = -1.89$), shows a robust signal for biosynthetic and translational activity (Figure 4.19). Enriched processes include broad translation, cytoplasmic translation, peptide biosynthesis, and macromolecule biosynthesis, pointing to modules engaged in protein production and high metabolic turnover. As above, the hub genes for each module were also determined through calculating kME values (Figure 4.20).

The blue module is also downregulated with weight loss versus obesity ($\log_2FC = -1.16$) and is associated with protein kinase regulation and stress response pathways, such as positive regulation of Tau protein kinase activity, stress granule assembly, and regulation of fibroblast apoptotic process, along with mitochondrial electron transport. These suggest involvement in stress adaptation, signal transduction, and mitochondrial function.

Genes in the brown module, likewise, downregulated in weight loss ($\log_2FC = -0.63$), are enriched for immune regulatory functions, including positive regulation of leukocyte apoptotic process, negative regulation of sprouting angiogenesis, cellular response to oxygen levels, and positive regulation of myeloid cell apoptotic process. This module may capture NK cell responses to microenvironmental cues or programmed cell death regulation.

By contrast, the red module is upregulated with weight loss ($\log_2FC = +0.84$) and stands out for pathways tied to NK-specific immunity and lymphocyte proliferation: notably positive regulation of NK T cell proliferation, regulation of NK T cell proliferation, positive regulation of receptor binding, B cell mediated immunity, and cell junction disassembly. These terms highlight roles in receptor signaling, intercellular interactions, and immune proliferation.

The yellow module, downregulated with weight loss ($\log_2FC = -0.28$), is enriched for innate and humoral immune responses, including myeloid leukocyte mediated immunity, humoral immune response mediated by circulating immunoglobulin, negative regulation of myeloid leukocyte mediated immunity, regulation of prostaglandin biosynthetic process, and dendritic cell chemotaxis. This suggests that yellow module genes are involved in both

promoting and restraining inflammation, with roles in antibody-associated pathways and dendritic cell recruitment.

Finally, the black module is upregulated in weight loss ($\log_2FC = +0.73$) and displays enrichment for centriole biogenesis and cell division processes: positive regulation of centriole replication, regulation of centriole elongation, and dendritic cell chemotaxis. This overlap of cell cycle-related centriole replication and immune cell chemotaxis points to modules regulating both cell proliferation machinery and migratory behaviour.

Taken together, green, turquoise, and black modules emphasize cell cycle, translation, biosynthesis, and centriole replication pathways, suggesting states of proliferation and metabolic activation in NK cell subsets (Figure 4.19, 4.20). Blue and brown modules capture stress signalling, apoptotic regulation, and mitochondrial and kinase-mediated responses, indicative of activation or inflammatory stress states. Red and yellow modules are enriched for immune effector functions, including NK and B cell proliferation, receptor-mediated activation, humoral signalling, and inflammation regulation (Figure 4.19, 4.20).

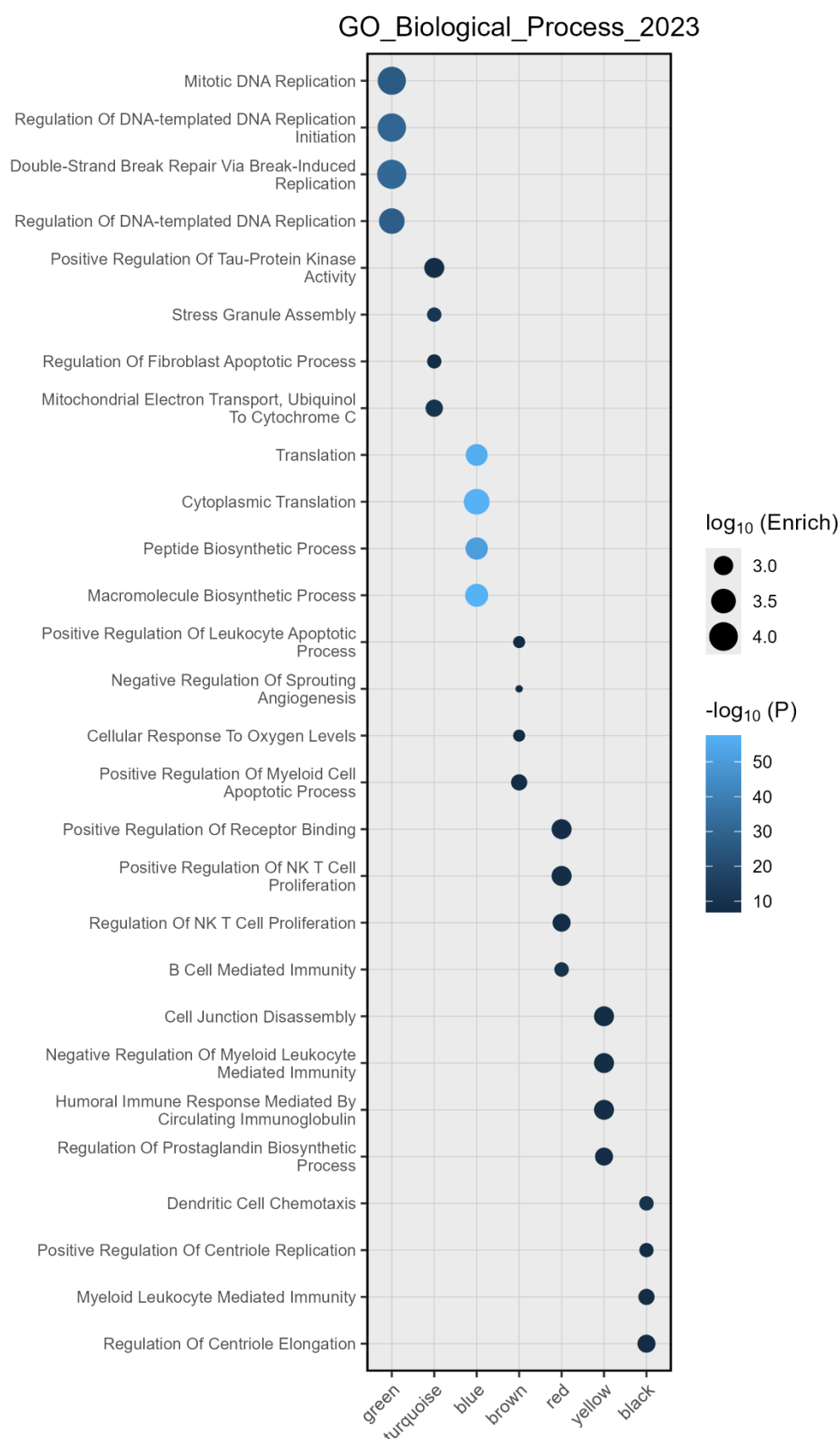


Figure 4.19 – Mouse hdWGCNA results - Gene Ontology (GO) Biological Process enrichment analysis of NK cell hdWGCNA modules. Genes from each of the seven hdWGCNA modules (turquoise, blue, brown, yellow, green, red and black) were submitted to the GO Biological Process 2023 database using the Enrichr package.

The y-axis lists the top enriched biological processes for each module, while the x-axis indicates module identity. Point colour represents the statistical significance of enrichment ($-\log_{10} p\text{-value}$), with warmer colours indicating stronger enrichment. Point size reflects the degree of enrichment ($\log_{10}$ enrichment score).

Consistent with the human NK Cells is a substantial change in metabolic and ribosomal genes being significantly altered in obesity. By chance the blue module in both humans and mice represents mainly ribosomal genes and interestingly these modules are significantly downregulated in obesity in humans and mice NK cells as shown in Table 4.3 for MD2 and in Figure 4.9 for HD2.

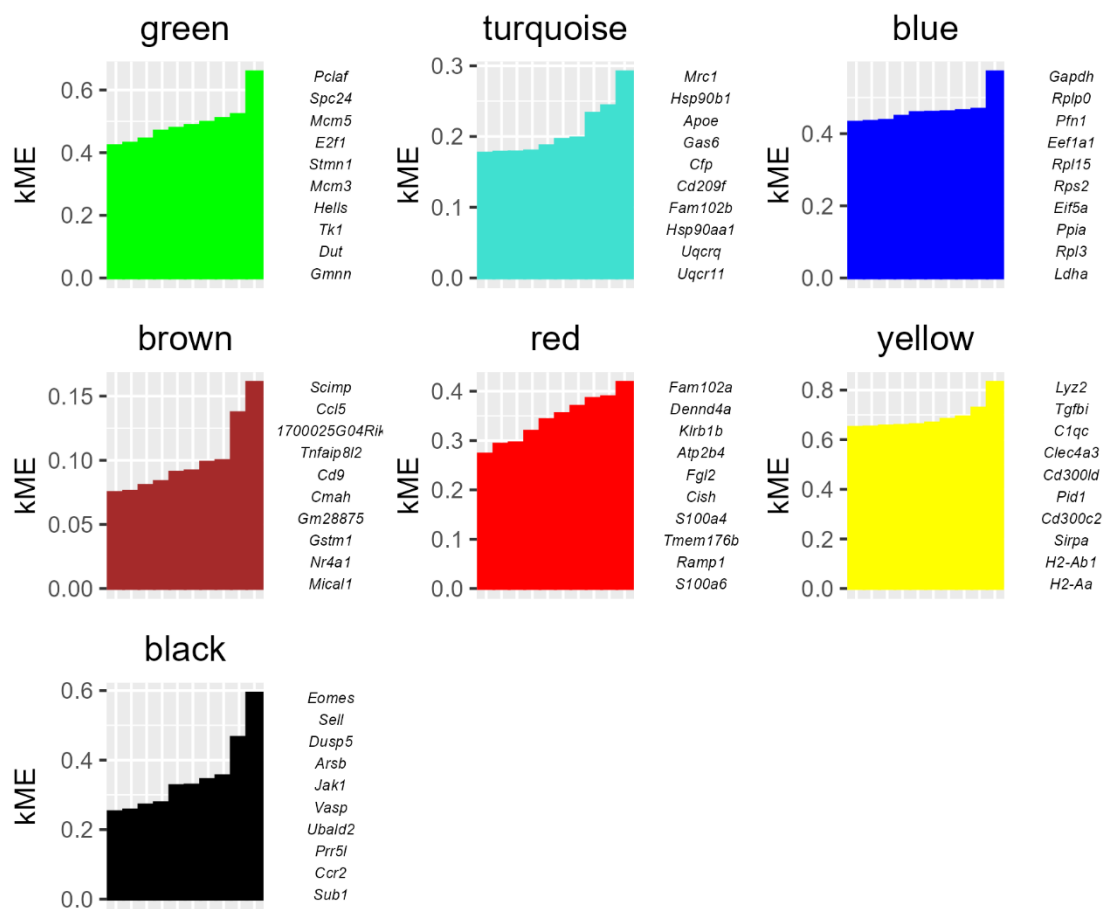


Figure 4.20 – Module membership (kME) of top hub genes in each hdWGCNA module identified from NK cells. Bars represent the eigengene connectivity (kME) values of genes within each module (turquoise, blue, brown, yellow, green, black, red), which quantify how strongly each gene correlates with the corresponding module eigengene. The highest-ranking hub genes for each module (listed alongside the bars) show the strongest module membership and are candidate drivers of each module's co-expression pattern

Tnfrsf3 was present in the red module, and after combining the red module with known PPI the local network structure around *Tnfrsf3* was determined. The local PPI subnetwork

around *Tnfaip3* includes *Tab2*, *Traf3ip2*, *Birc2*, *Ptk2b*, and *Tnfaip8* (Figure 4.21, Figure 4.22). *Tab2* serves as an adaptor that couples ubiquitinated signalling intermediates to TAK1/IKK complexes, driving NF- κ B activation downstream of cytokine receptors. *Traf3ip2* (Act1) similarly links cytokine receptors to both NF- κ B and JNK pathways via TRAF proteins, suggesting coordinated regulation of inflammatory signalling in NK cells. *Birc2* (cIAP1/2), as an inhibitor-of-apoptosis protein with E3 ubiquitin ligase activity, interfaces with TRAF proteins to scaffold TNF receptor signalling complexes, modulating survival and NF- κ B outputs. The presence of *Ptk2b*, a calcium-responsive tyrosine kinase involved in MAPK and cytoskeletal signalling, indicates potential cross talk between adhesion pathways and inflammatory regulation in NK cells. Finally, *Tnfaip8*, a lipid-binding anti-apoptotic protein, is positioned to coordinate survival signalling and maintain NK cell viability under inflammatory stress.

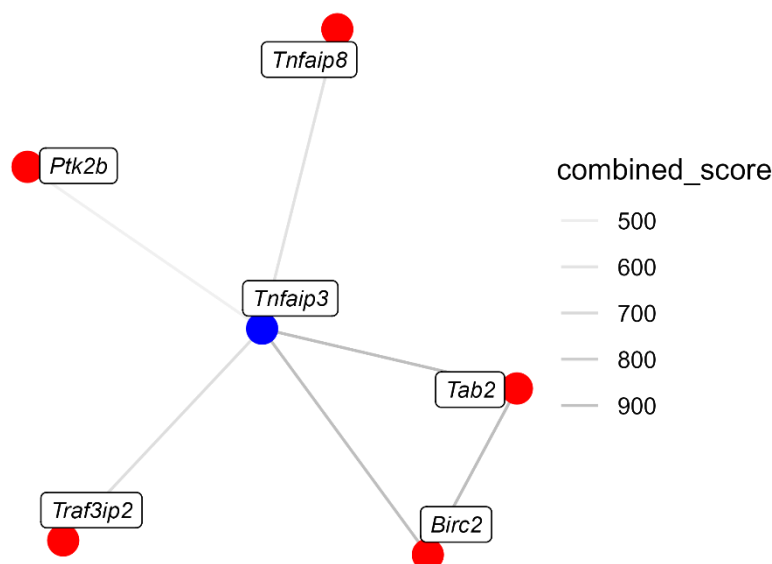


Figure 4.21 – MD2- Local structure of PPI network in red module around *Tnfaip3* – score represents a confidence score, higher scores represent higher confidence in the interaction with 400 used as the lowest possible retention interaction score – all genes are established genes with interaction score above 400 with *Tnfaip3* in the green network

In murine NK cells, the red module reveals a densely interconnected hub of chemokine signalling, cytokine receptors, integrin-mediated adhesion, PI3K pathway components, and cytoskeletal regulators. Prominently featured in this module is *Cxcl12*, a key homeostatic chemokine likely influencing NK cell positioning or retention in tissues. This chemokine is surrounded by multiple integrin subunits (e.g., *Itga4*, *Itgb1*, *Itgb7*) and adhesion-linked molecules such as *Lcp2*, *Zyx*, *Lpxn*, and *Ptk2b*.

Within the same cluster, *Il7r* and *Il2rb* co-occur, reflecting coordinated expression of γ -chain cytokine receptors. Their proximity to *Pik3cd*, the leukocyte-enriched catalytic subunit of PI3K, suggests a direct signalling axis driving survival, proliferation, or activation. This indicates that PI3K–Akt signalling may be a central node activated downstream of cytokine and chemokine engagement in NK cells, PI3K genes were also present in the human *TNFAIP3* (green) network.

Also visible are signalling adaptors and kinases (e.g. *Jak2*, *Mmp9*, *Smad2/Smad3*) co-expressed within the module, hinting at intersecting pathways including cytokine-mediated JAK–STAT, TGF- β modulation, and extracellular matrix remodelling. The presence of TNFRSF-related molecules and matrix metalloproteinase *MMP9* might reflect dynamic regulation of NK cell migration or tissue remodelling. This integrated module suggests that in murine NK cells, chemotactic signals, adhesion receptors, cytokine responsiveness, and PI3K-mediated transduction are tightly co-expressed, potentially enabling coordinated regulation of NK cell trafficking, positioning, and responsiveness to environmental cytokine and chemokine inputs. An expanded network around *Tnfaip3* is shown in Figure 4.22.

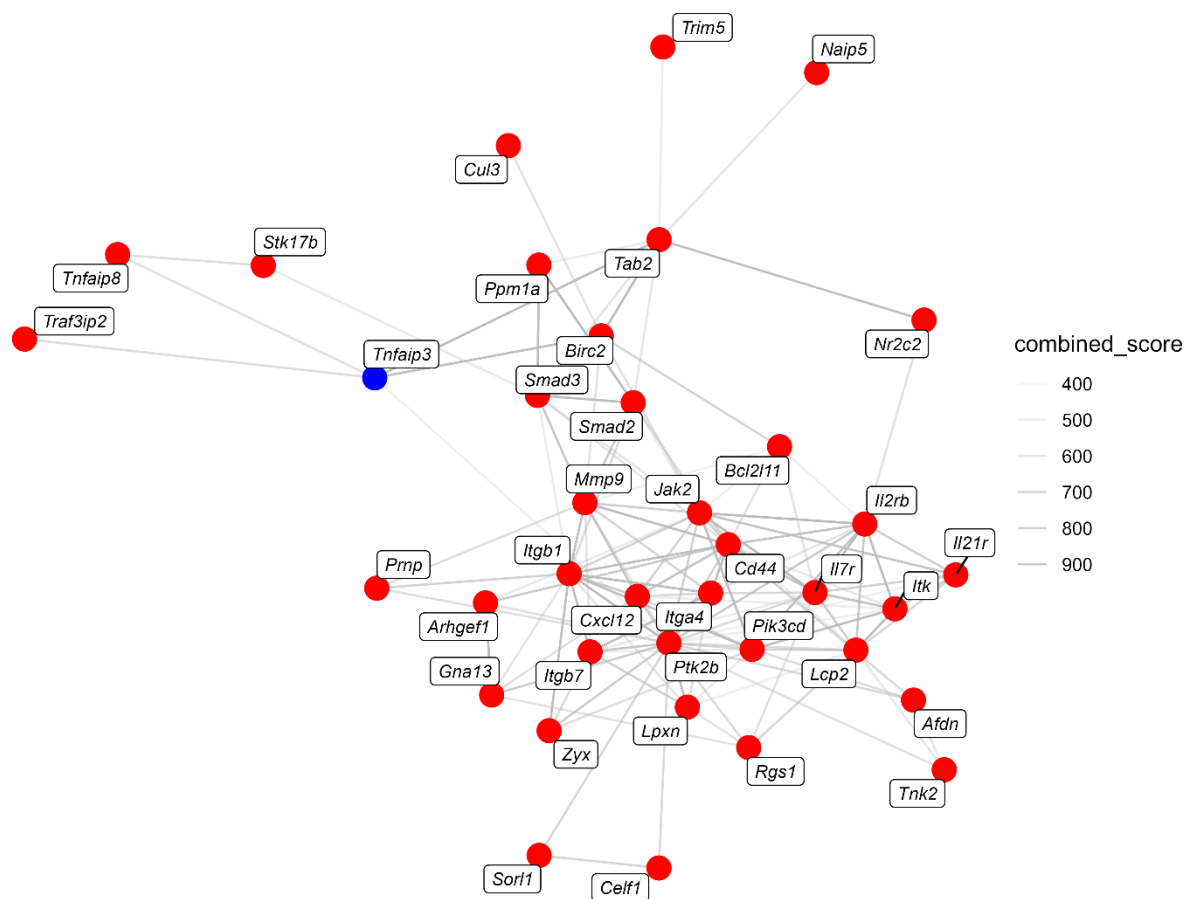


Figure 4.22 – Expanded *Tnfaip3* co-expression and PPI network in mouse NK cells from adipose tissue - MD2

With respect to the transcription factor analysis, the results seem more random compared to humans. A number of transcription factors were identified to contain motifs within the *Tnfaip3* promoter region in mice. However, none of these have been described in literature to play a role in the regulation of *Tnfaip3*. Thus, these findings are spurious and given that there were less cells used to construct this network compared to the human network, it raises the possibility of inaccurate results.

Table 4.4 – Transcription factors associated with *TNFAIP3* expression in murine NK cells from adipose

Gene	Gain	Cover	Frequency	Correlation
<i>Arid3b</i>	0.13	0.12	0.12	0.23
<i>Arntl</i>	0.07	0.1	0.1	0.01
<i>Rarg</i>	0.03	0.04	0.04	0.15
<i>Rxra</i>	0.16	0.11	0.11	0.3
<i>Smad3</i>	0.12	0.1	0.1	0.3
<i>Stat2</i>	0.03	0.05	0.05	0.04
<i>Stat4</i>	0.05	0.07	0.07	-0.05
<i>Stat5a</i>	0.06	0.06	0.06	-0.08
<i>Stat5b</i>	0.1	0.1	0.1	0.1
<i>Stat6</i>	0.15	0.13	0.13	0.1

4.2.7 - Trafficking Receptors in NK Cells – Divergent States in Obese and Fasted States

Trafficking receptors are vital controllers of NK cell location and fate. Differential expression analysis revealed opposing chemokine receptor signatures in NK cells across metabolic states. In obesity, *CX3CR1* emerged as the most strongly upregulated gene, suggesting enhanced recruitment towards *CX3CL1* (fractalkine). Conversely, in weight loss, *CXCR4* was the most upregulated gene, indicating a potential shift toward NK cells with a *CXCR4*-driven trafficking or homing phenotype and towards *CXCL12*. This reciprocal regulation highlights a chemokine receptor switch between obesity and weight loss, suggesting distinct migratory and functional programs in NK cells across these conditions. Genes up and downregulated in obesity and weight loss in CD56^{Dim} NK Cells are shown in Figure 4.23.

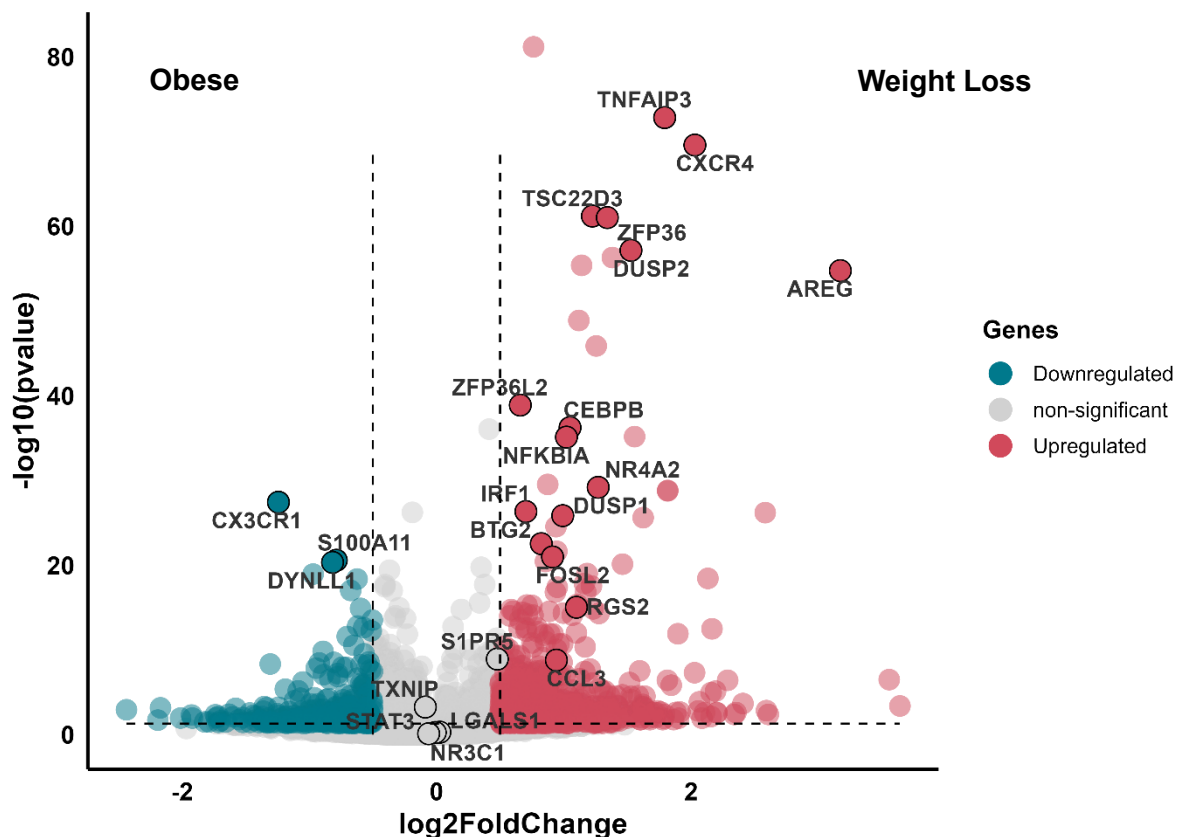


Figure 4.23 – HD2- CD56^{Dim} NK Cells - Differential gene expression in CD56^{Dim} NK cells comparing weight loss versus obesity. Volcano plot showing results of Wilcoxon rank-sum testing. Each point represents a gene; red points indicate genes significantly upregulated after weight loss ($\log_2$ fold change $\geq +0.25$, adjusted $p < 0.05$), blue points indicate genes significantly upregulated in obesity ($\log_2$ fold change ≤ -0.25 , adjusted $p < 0.05$), and grey points indicate genes not meeting these thresholds. Selected key genes are labelled.

The Library of Integrated Network-Based Cellular Signatures (LINCS) L1000 dataset is a large-scale resource of gene-expression signatures generated from thousands of perturbations (genetic knockouts, knockdowns, and small molecules) across diverse human cell lines. It enables rapid inference of how genes respond to specific molecular perturbations. The LINC L1000 was checked for *TNFAIP3* as a response gene. When *CXCR4* is knocked out, *TNFAIP3* expression is downregulated, suggesting *CXCR4* is one mediator of *TNFAIP3* expression.

Table 4.5 – LINC L1000 - Knockout of *CXCR4* across an array of cells leads to downregulation of *TNFAIP3*

Symbol	Name	Standardized Value
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	-0.0064309

NK cells split into two clusters during clustering and processing steps of the HD2 scRNA-seq dataset. The gene for CD56 (*NCAM1*) was utilised to differentiate which cluster was bright and dim for CD56, this is shown in Figure 4.24.

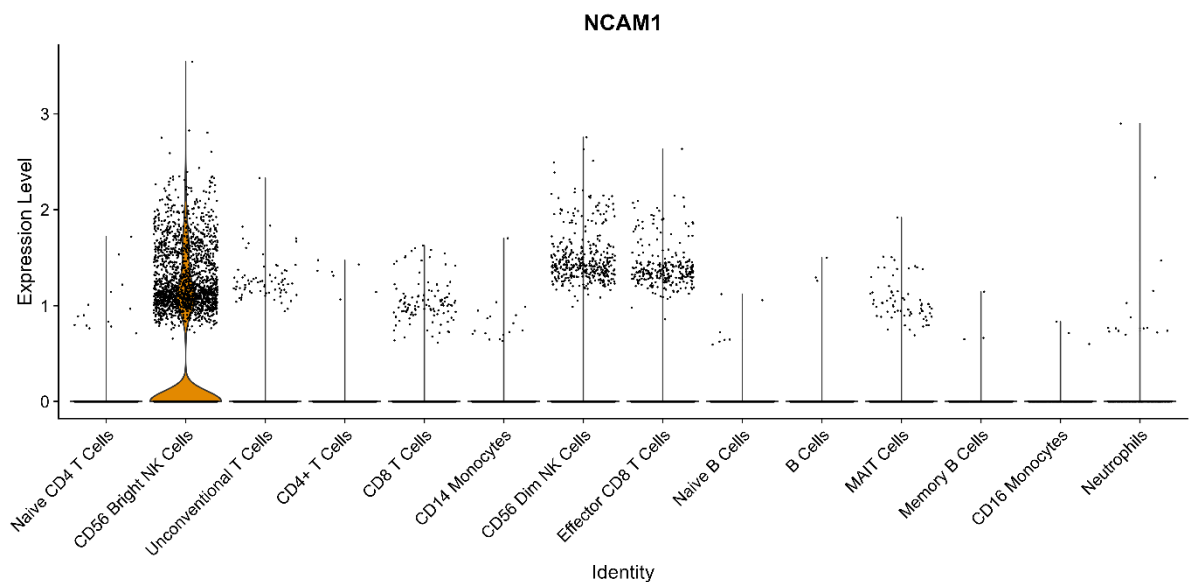


Figure 4.24 – HD2 - Human weight loss Dataset – Violin plot used for the identification of CD56 (*NCAM1*) bright NK cells – each dot represents a cell, whilst the y-axis represents the normalised expression level of that gene in each cluster

Visualisation of expression in CD56^{dim} NK Cells shows a clear shift in the cluster of *CX3CR1* to *CXCR4* (Figure 4.25). This separation within the cluster highlights how NK cells can

undergo a phenotypic shift in weight loss from obesity. Panel A represents more of an obese phenotype whilst Panel B represents a transcriptional shift in CD56^{dim} NK Cells after weight loss.

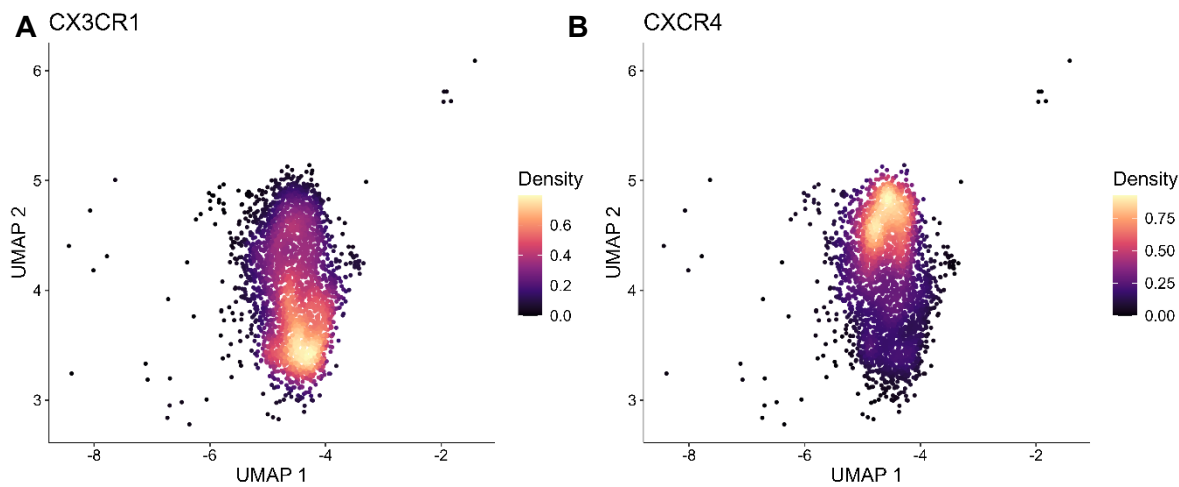


Figure 4.25 – Density UMAP plots of *CX3CR1* (Panel A) and *CXCR4* (Panel B) expression within CD56^{dim} NK cells. Each panel shows the same UMAP of CD56^{dim} NK cells coloured by expression density of the indicated gene. Warmer colours indicate higher local cell density of expressing cells. *CX3CR1* and *CXCR4* are enriched in distinct regions of the same NK cell cluster, highlighting subpopulation heterogeneity within CD56^{dim} NK cells.

The CD56^{bright} NK cells had generally had the exact same transcriptional profile as CD56^{dim} NK cells but what did differ greatly was the expression of AP-1 transcription factors (*JUN*, *FOSB*, *FOS*) which were very much upregulated in obesity in CD56^{bright} NK cells. Metabolically, CD56^{bright} NK cells had an abundance of metabolic genes in the top differentially expressed genes. *MT-AP6*, *MT-CO1* were both upregulated with weight loss whilst the amino acid transporter *SLC38A2* was significantly up in obesity versus weight loss (Figure 4.26). Conserved here in CD56^{bright} NK cells, like in CD56^{dim} NK cells is the upregulation of *CXCR4*, *TNFAIP3*, *NR4A2* and *AREG*. Unique to the bright population was the expression of *MYOM2* which seems to have an uncharacterised function in NK cells and was observed to be the most significantly upregulated gene in weight loss versus obesity. Unlike CD56^{dim} NK cells, *CX3CR1* was not significantly changed in CD56^{bright} NK cells in obesity.

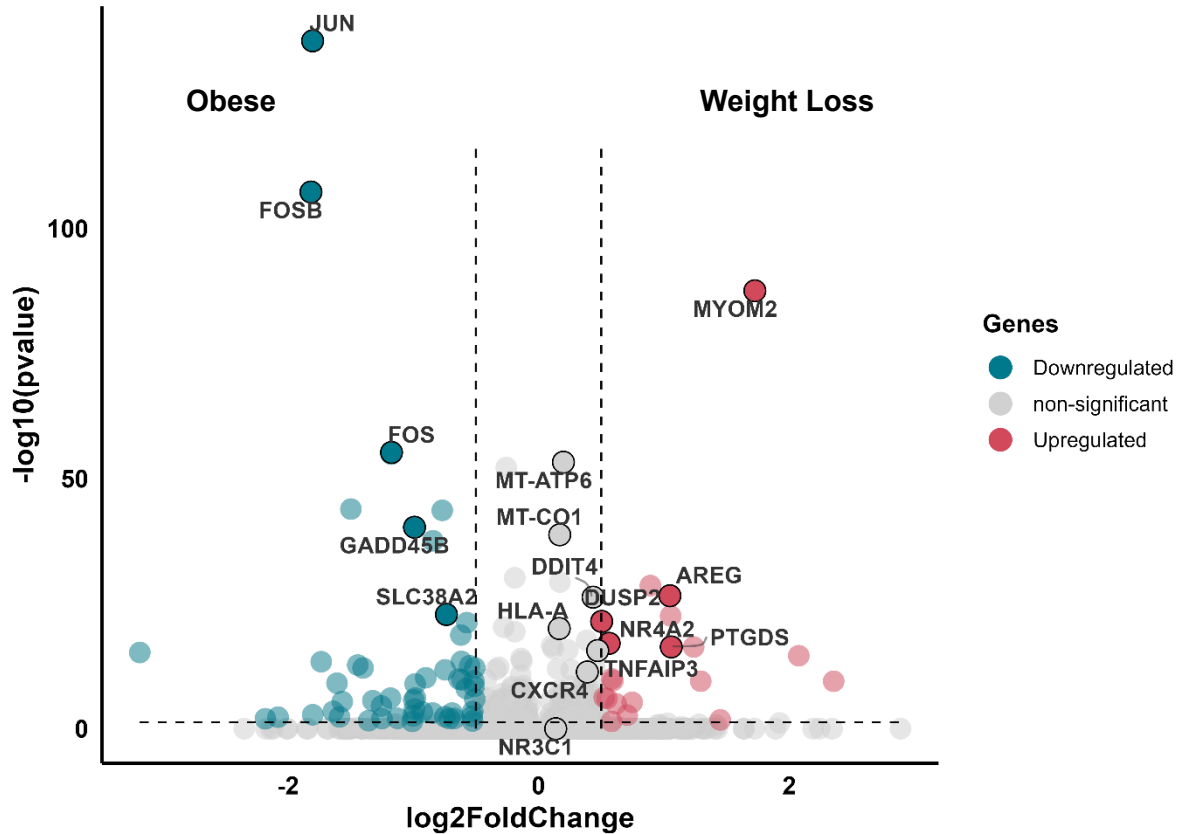


Figure 4.26 – HD2 - CD56^{Bright} NK Cells - Differential gene expression in CD56^{Bright} NK cells comparing weight loss versus obesity. Volcano plot showing results of Wilcoxon rank-sum testing. Each point represents a gene; red points indicate genes significantly upregulated after weight loss ($\log_2$ fold change $\geq +0.25$, adjusted $p < 0.05$), blue points indicate genes significantly upregulated in obesity ($\log_2$ fold change ≤ -0.25 , adjusted $p < 0.05$), and grey points indicate genes not meeting these thresholds. Selected key genes are labelled.

When examining the phenotype of murine adipose NK cells, the differential expression analysis between obese and weight loss NK cells had a very similar transcriptional phenotype to human NK cells. Despite the different species and tissue, the response is conserved across human and mice NK cells suggesting hallmark signatures of obesity and weight loss (Figure 4.27). *Cxcr4* was substantially upregulated in weight loss and there was a non-significant trend towards *Cx3cr1* downregulation in weight loss. Meanwhile, the expression of *Slpr5* was significantly downregulated in weight loss versus obesity.

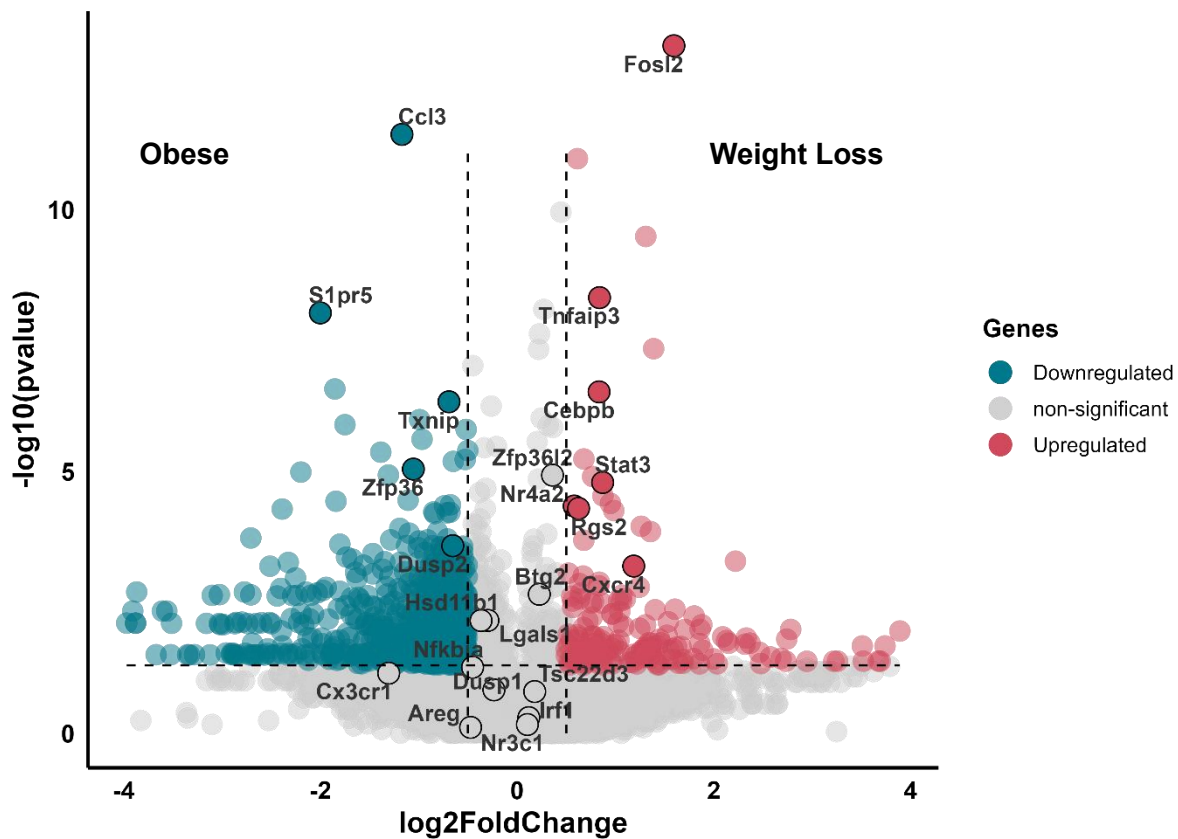


Figure 4.27 – MD2 – Differential Expression on NK cells from adipose tissue - Volcano plot showing results of Wilcoxon rank-sum testing. Each point represents a gene; red points indicate genes significantly upregulated after weight loss ($\log_2$ fold change $\geq +0.25$, adjusted $p < 0.05$), blue points indicate genes significantly upregulated in obesity ($\log_2$ fold change ≤ -0.25 , adjusted $p < 0.05$), and grey points indicate genes not meeting these thresholds. Selected key genes are labelled.

For all the comparisons performed above gene expression data was compared for NK cells in obese mice/human vs NK cells after weight loss. However, a vital question to consider is whether trafficking receptors expression are indicators of obesity and recovery or whether this pattern of expression signature reflects a response to fasting. To investigate the impact of fasting on the expression of the identified trafficking receptors a separate dataset was utilised with sequencing data for NK cells with and without 48 hour fasting from spleen, tumour and bone marrow. The NK cells from these tissues different locations, were combined and examined together to find a general signature of fasting in NK cells. Interestingly, despite different tissues of origin, the transcriptional signature of fasting was extremely similar to that observed in the weight loss models, both human and murine; for example, the top differentially expressed genes in fasting were *TNFAIP3*, *CXCR4* amongst many other upregulated genes. In contrast, *CX3CR1* and *SIPR5* were significantly increased

in expression in the fed state, i.e in *ad libitum*. In other words *CX3CR1* and *S1PR5* are downregulated in NK cells upon 48 hours fasting. These data suggest that these changed in trafficking receptors are linked to the feeding state and not necessarily related to inflammatory conditions associated with obesity.

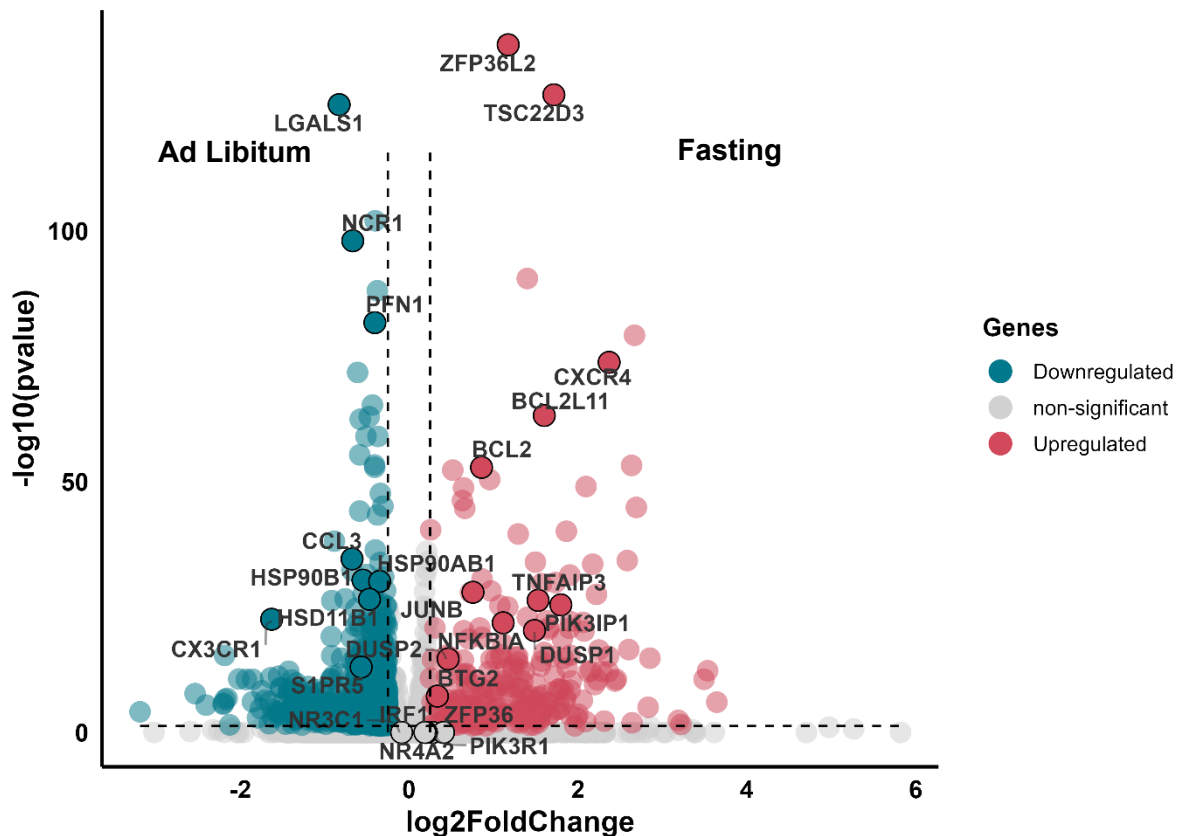


Figure 4.28 – MD7 - Fasting vs Ad libitum Fed Mice - NK Cells – Data shows differential gene expression in NK cells comparing fasting versus *ad libitum* fed mice. NK cells were isolated from spleen, tumour and bone marrow. Volcano plot showing results of Wilcoxon rank-sum testing. Each point represents a gene; red points indicate genes significantly upregulated after fasting ($\log_2$ fold change $\geq +0.25$, adjusted $p < 0.05$), blue points indicate genes significantly upregulated in *ad libitum* ($\log_2$ fold change ≤ -0.25 , adjusted $p < 0.05$), and grey points indicate genes not meeting these thresholds. Selected key genes are labelled.

Next, to further investigate the expression of *CXCR4* during fasting, another human dataset was analysed, HD6, wherein 12 individuals underwent a water only fast for 7 days. This study provided serum proteomic data for circulating factors in the serum but did not analyse NK cells directly. Therefore, these data were used to assess the expression levels of *CXCL12* (Stromal Derived Factor-1), the ligand for *CXCR4*. Indeed, during fasting there was a significant increase in the circulating levels of *CXCL12* (Figure 4.29). Together, these data

show that both the circulating ligand (*CXCL12*) and the NK cell expressed chemokine receptor (*CXCR4*) are upregulated in response to fasting.

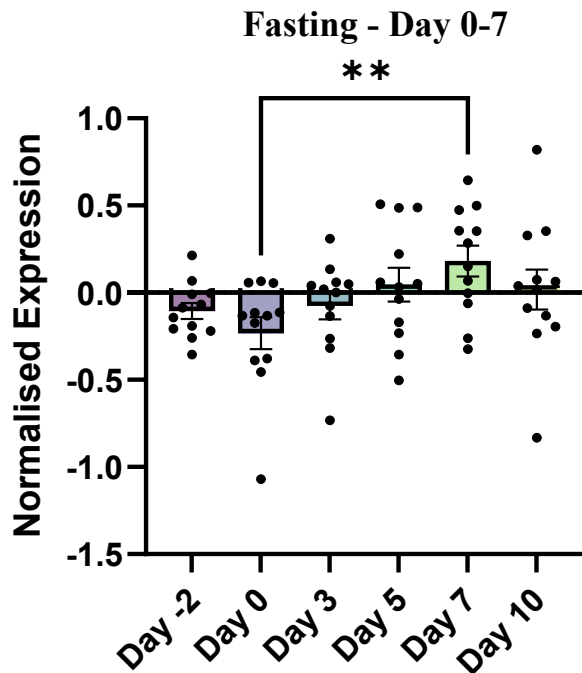


Figure 4.29 – *CXCL12* levels across a 12-day human fasting–refeeding protocol. Measurements were taken at pre-fasting baseline timepoints (Days –2), at the fasting initiation (Day 0), during fasting at day 3 and day 5, at fasting completion (Day 7), and following refeeding (Day 10). Data are presented as mean $\pm$ SEM (n = 12 participants). Paired t-tests comparing Day 0 and Day 7 values were performed; asterisks denote significant differences ($p < 0.05$, $p < 0.01$ vs. Day 0).

4.2.8 - ATAC-Seq – Adipocyte Analysis

ATAC-seq provides insight into how accessible chromatin is, thus providing insight into the regulatory landscape of the genome, including potential transcription factor binding, enhancer and promoter activity, and the dynamic regulation of gene expression across conditions or cell types. It is important to note that ATAC-seq signal over exonic regions does not directly reflect classical regulatory element activity, as promoter and enhancer accessibility are the primary determinants of transcriptional control. Consequently, aggregating peak counts over exons may be influenced by transcriptional elongation or gene body chromatin dynamics rather than changes at bona fide regulatory sites. Such data could indicate if the differences observed in the expression of *TNFAIP3* are likely driven by chromatin alterations. Utilising the publicly available dataset, MD6, GSE236575, the accessibility of the *TNFAIP3* locus was assessed in adipocytes. While this dataset was for

adipocytes and not immune cells, the data was of interest because it contained data for lean, obese and weight loss samples and so might be informative to how these metabolic states impact upon the chromatin at the *TNFAIP3* region. An additional reason to use this dataset is the current understanding that *TNFAIP3* also has important functions in adipocytes including regulating metabolic responses and adipogenesis ¹⁴⁰.

This ATAC-seq dataset is described in the datasets section in methods, section 2.2.2. For this analysis, peak numbers were aggregated per exon region (Table 4.6) for each diet, irrespective of the time spent on each diet, to get an understanding of the general response per diet. Exon locations were obtained from UCSC genome browser website using the reference genome: GRCm38/mm10 (Table 4.6). The data showed that there was little variability in the accessibility of exons 1 to 9 of *Tnfaip3* in the SFD condition, thus no major changes were observed across SFD mice adipocytes (Figure 4.30).

Table 4.6 – Exon locations in the mm10 genome alignment obtained from UCSC genome browser.

Exon	Start	End
Ex1	19011481	19011963
Ex2	19008145	19008335
Ex3	19007182	19007329
Ex4	19006863	19007033
Ex5	19005551	19005731
Ex6	19004457	19005331
Ex7	19003576	19003757
Ex8	19000910	19003015
Ex9	19015153	19015410

Genome locations were validated using the visualisation software tool: IGV browser, which allows for ATAC-seq bed files to be loaded directly into the browser and receive visualisations instantly. The regions in Table 4.6 mapped onto the *Tnfaip3* region, confirming these genome locations obtained from UCSC align correctly with the bed files alignment and genome build mm10. Exon level analysis revealed that exons 6 and 7 of *Tnfaip3* exhibit near complete loss of chromatin accessibility in HFD mice, but locus accessibility is restored after weight loss. This suggests dynamic epigenomic regulation within this gene region that is responsive to overall metabolic state of the mice. Although exons themselves are not inherently functional units, these specific exons fall within the zinc-finger (ZnF) protein–protein interaction region of the A20 protein. Therefore, altered chromatin accessibility here could feasibly impact protein functionality via altered transcription or splicing. While mouse-specific SNPs in these exons are not well documented, leveraging variation datasets

(e.g., Sanger Mouse Genomes) and *in silico* annotation tools could help assess the potential impact of variations overlapping exons 6–7. Thus, although downstream effects, such as alternative splicing or altered transcript levels are highly speculative, it is plausible that these observed changes in Figure 4.30 could affect expression. Specifically, that in the absence of access to the locus at exon 6 and 7, it is likely more difficult for the cell to generate any *Tnfaip3* mRNA due to less chromatin accessibility. In sum, these data indicate that obesity induces context-specific chromatin remodelling within the *Tnfaip3* gene, specifically at functionally relevant positions in the *Tnfaip3* locus, and this may modulate A20 expression or isoform composition in metabolic states.

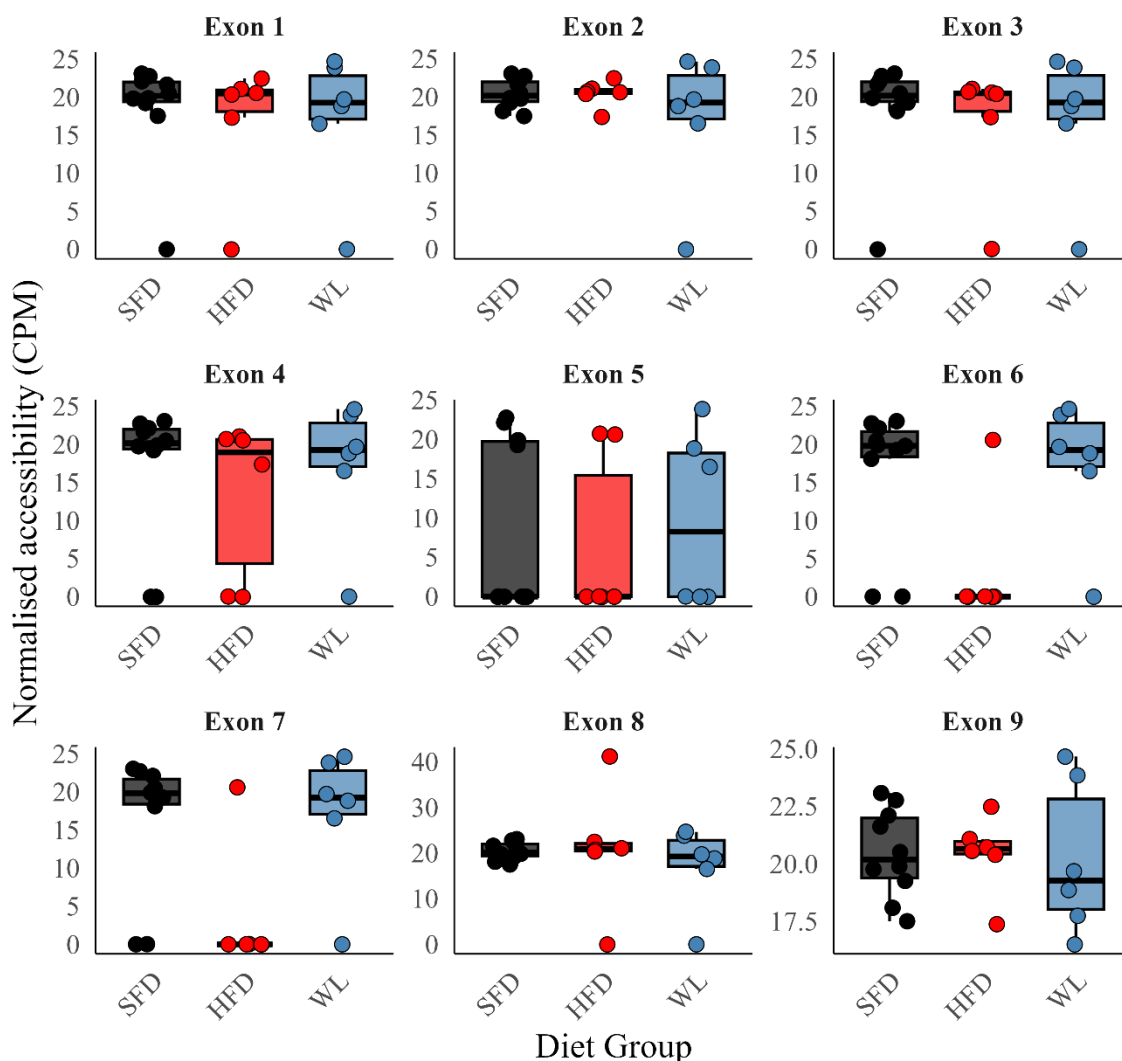


Figure 4.30 - Normalised read counts across *Tnfaip3* exons in adipocytes under different metabolic conditions. Box-and-jitter plots show normalised counts per million (CPM) mapped to each exon of *Tnfaip3* (Exons 1–9) in adipocytes from mice fed a standard-fat diet (SFD; lean), a high-fat diet (HFD; obese), or subjected to weight

loss (WL). Each point represents an individual mouse; boxes indicate median and interquartile range. These data illustrate exon-level expression differences in *Tnfaip3* across metabolic states in adipocytes.

To investigate regulatory changes at the *Tnfaip3* locus, I mined the differentially enriched enhancer regions provided by Hinte *et al.* for histone post-translational modifications (hPTMs).¹¹⁹ These hPTMs serve as epigenetic indicators of chromatin activity: H3K27ac (histone H3 lysine 27 acetylation) marks active enhancers, while H3K4me1 (histone H3 lysine 4 monomethylation) marks poised or active enhancer regions, depending on context and co-occurrence with other marks. Enhancer regions associated with *Tnfaip3* displayed dynamic regulation in response to both obesity and weight loss (Table 4.7). The comparisons span multiple time points (short-, medium-, and long-term) and conditions (obese vs. control; weight loss vs. control), allowing inference of temporal changes in enhancer activation. Firstly, obesity (ob) showed time dependent changes in the activity of *Tnfaip3* enhancer regions. In the short-term, *Tnfaip3* enhancer regions showed a modest increase in H3K4me1 signal (~17 kb upstream of the TSS). With prolonged obesity (medium- and long-term), enhancer regions downstream of the gene (+13 kb and +27 kb) exhibited significant reductions in H3K4me1 signal, indicating reduced accessibility and repressive chromatin remodelling.

Across all weight loss conditions when compared to lean controls, there was a trend toward increased activation at with positive log fold changes in H3K27ac or H3K4me1 signal (Table 4.7). Notably, a consistent increase in H3K4me1 was observed at a site ~45 kb upstream of the *Tnfaip3* transcription start site (TSS) in both medium- and long-term weight loss comparisons to lean mice that reached statistical significance (FDR < 0.05) in the long-term condition. These data suggest that in weight loss there may be a rebound effect where there is a increased *Tnfaip3* compared to mice that never experience obesity, i.e. lean controls.

Taken together, these findings suggest that while early obesity may initially trigger an adaptive response at *Tnfaip3* enhancers, sustained metabolic stress leads to epigenetic silencing, potentially contributing to its transcriptional suppression. Then following weight loss the *Tnfaip3* enhancers become reactivated, maybe even to a greater extent than lean controls. The data support a model where weight loss reactivates enhancers near *Tnfaip3*, while chronic obesity leads to their repression. This is consistent with the idea that *Tnfaip3*, a key negative regulator of NF- κ B signalling, becomes progressively dysregulated in obesity due to changes in enhancer accessibility. Although some enhancer changes fall just outside

traditional thresholds of significance (e.g., FDR ~0.06–0.07), they provide biological directionality and align with transcriptional data.

Table 4.7 – *Tnfaip3* enhancer regions changes mined from MD7 dataset

Gene	logFC	FDR	Dir	hPTM	Feature	DToTSS	Comparison	Time
<i>Tnfaip3</i>	1.986	0.061	up	H3K27ac	Enhancer	-50887	WL vs Lean	Long Term
<i>Tnfaip3</i>	1.542	0.071	up	H3K27ac	Enhancer	-45038	WL vs Lean	Long Term
<i>Tnfaip3</i>	1.374	0.051	up	H3K4me1	Enhancer	-44826	WL vs Lean	Medium Term
<i>Tnfaip3</i>	1.407	0.044	up	H3K4me1	Enhancer	-44826	WL vs Lean	Long Term
<i>Tnfaip3</i>	-1.392	0.001	down	H3K4me1	Enhancer	27082	Obese vs Lean	Medium Term
<i>Tnfaip3</i>	-1.757	0.018	down	H3K4me1	Enhancer	13829	Obese vs Lean	Long Term
<i>Tnfaip3</i>	1.487	0.019	up	H3K4me1	Enhancer	-16938	Obese vs Lean	Short Term

4.2.9 - NF- κ B and AP-1 signalling in obesity

NFKB is the first described family of transcription factors to regulate *TNFAIP3* expression. Thus, a deep exploration of levels of NF- κ B transcripts across lean, obesity and weight loss conditions would provide an understanding of whether transcriptional changes to NF- κ B genes occur in obesity and weight loss, which might contribute to changes in *TNFAIP3*. Several of the datasets analysed previously were reinvestigated with the intention of examining the expression of NF- κ B and AP-1 family transcription factor expression, change during obesity and weight loss. Interestingly, the expression of NF- κ B transcription factor genes did not change during obesity. Rather, significant changes in the expression of the AP-1 family (*FOS*, *FOSB*, *FOSL1*, *FOSL2*, *JUN*, *JUNB*, *JUND*) during obesity and weight loss.

Firstly, the MD2 mouse adipose dataset was used with all cells combined to get a global insight into transcriptome changes (Figure 4.31) and showed that several AP-1 transcription factors are more highly expressed in immune cells in adipose tissue of lean mice compared to obese mice. These included *Jund*, *Fosb*, *Fosl2* and *Fos* upregulated along with *Nfkb1*. In

contrast, the expression of non-canonical NF- κ B transcription factor *Relb* increased in obesity. In NK cells specifically, the findings are very similar to those for all adipose immune cells with increased *Jund* and *Fosl2* in lean mice, whereas *Relb*, *Nfkb2* and *Fos* were increased in expression in NK cells in obese adipose tissue (Figure 4.31)

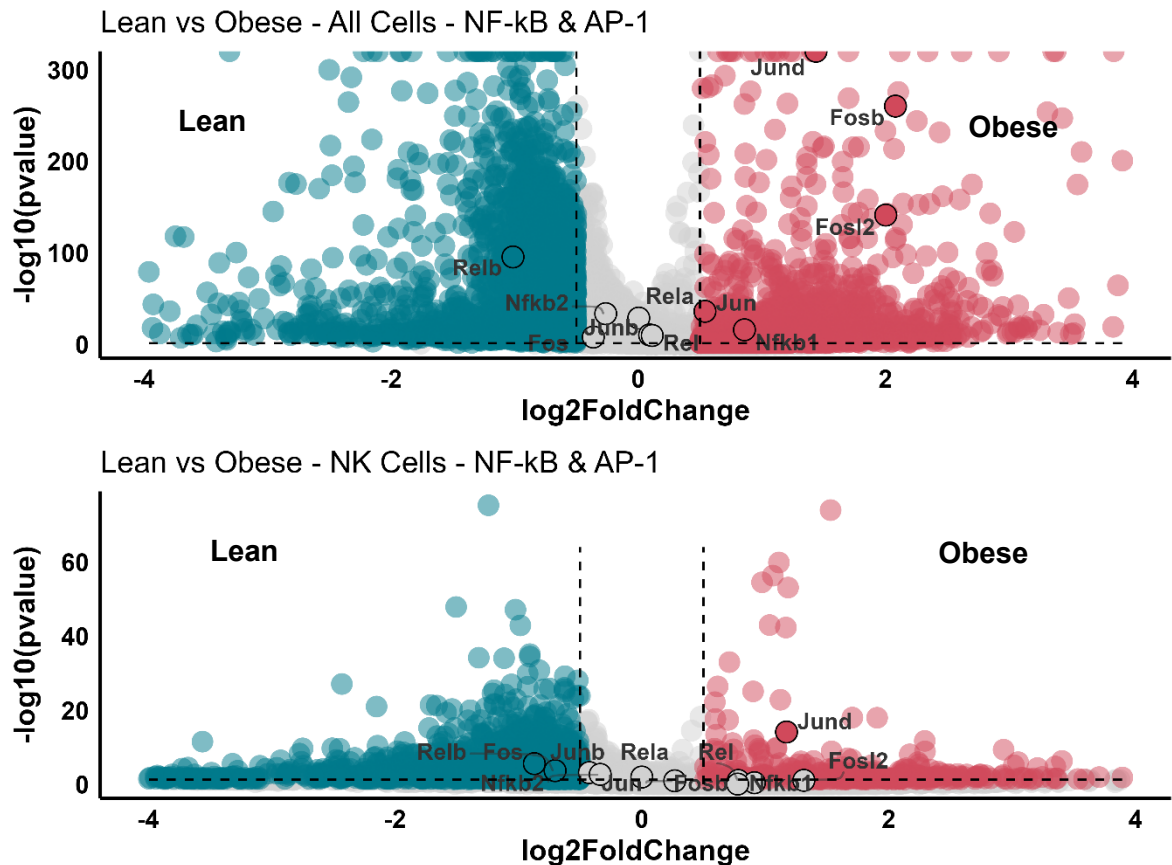


Figure 4.31 – MD2 - Lean vs Obese – Panel A – NK Cells, NF- κ B and AP-1 genes in obesity versus lean, Panel B – All Cells, NF- κ B and AP-1 genes in obesity versus lean

Next the expression of NF- κ B and AP-1 genes was compared for obese mice versus mice after weight loss (Figure 4.32). The data show that *Fosl2* expression is significantly higher in global adipose immune cells, and in isolated NK cells, in mice after weight loss. In contrast, *Fos* was significantly higher in expression in adipose NK cells in obese mice compared to mice after weight loss. The key takeaway seems to be *Fos* is associated with obesity and *Fosl2* is associated with lean and weight loss conditions.

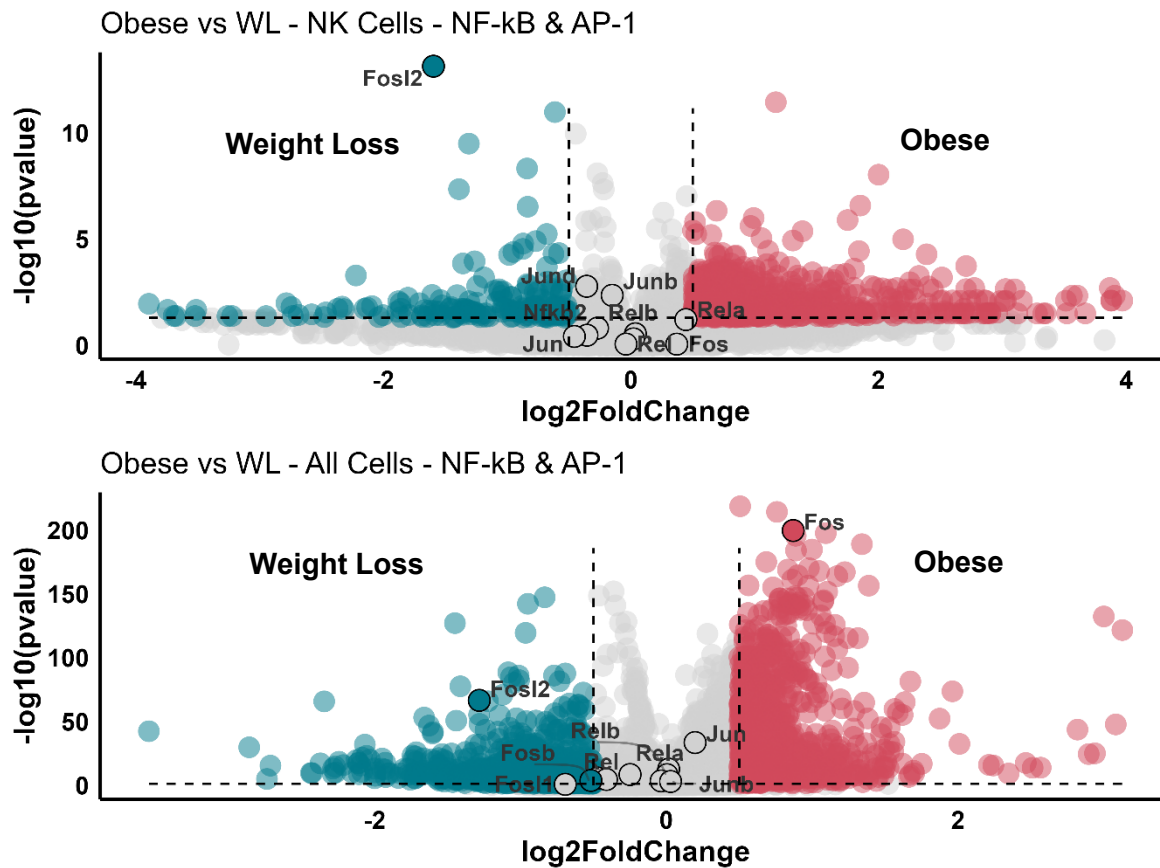


Figure 4.32 – MD2 – NF-κB and AP-1 transcription factor responses in MD2 – Volcano plots show differential gene expression for the contrast obese vs weight loss (WL) using the two-sided Wilcoxon rank-sum test. (A) NK cells only from adipose (B) All immune cells from the same dataset. The x-axis is $\log_2$ fold-change and the y-axis is $-\log_{10}(p\text{-value})$. Interpretation: negative $\log_2\text{FC}$ indicates higher expression in WL than Obese; positive $\log_2\text{FC}$ indicates higher expression in Obese than weight loss.

In human cells comparisons of obesity and weight loss showed similar changes in transcription factor expression. Increased *FOSL2* expression was associated with weight loss in all cells and CD56^{dim} NK cells, but not in $\text{CD56}^{\text{bright}}$ NK cells. In $\text{CD56}^{\text{bright}}$ NK cells the expression of *FOSB*, *FOS* and *JUN* were significantly associated with obesity, a phenotype that was also captured in the overall transcriptome for PBMC. Interestingly, in CD56^{dim} NK cells none of the AP-1 family transcription factors were expressed at higher levels with obesity but rather had several AP-1 and NF-κB family transcription factors increased in expression in the weight loss group. These observations are captured in Figure 4.33.

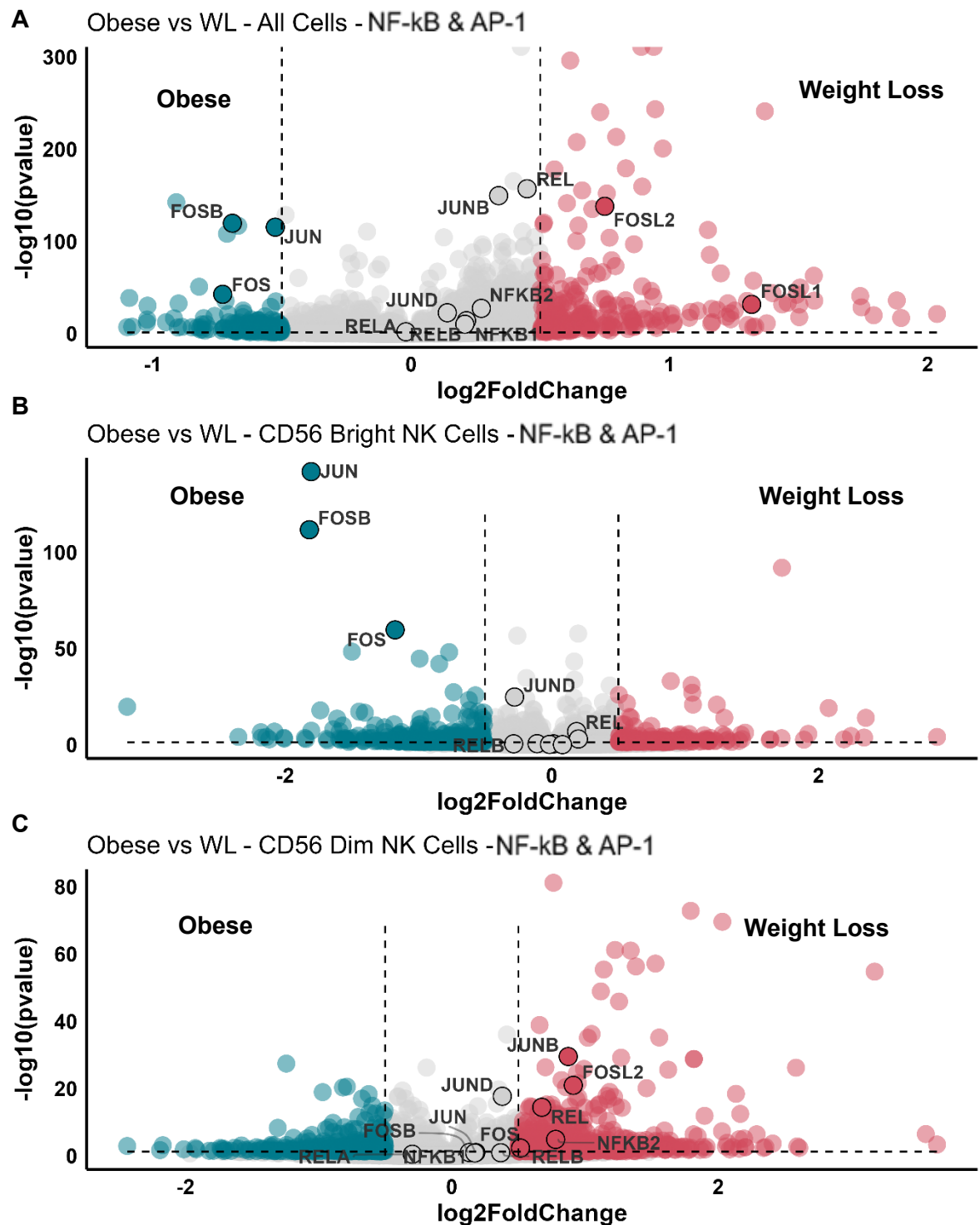


Figure 4.33 – Differential expression of NF-κB-related genes in NK cells and all PBMCs following weight loss. Volcano plots showing differential gene expression between obese (blue) and weight loss (red) conditions in the HD2 dataset, calculated using the Wilcoxon rank sum test. (A) All immune cells, (B) CD56^{Bright} NK cells, and (C) CD56^{Dim} NK cells. Genes belonging to the NF-κB pathway are highlighted.

Next, to understand what was occurring at a protein level and check if the transcriptional observations were the same at protein level, the proteomic data from obese and lean splenic

NK cells in mice were examined for NF- κ B and AP-1 activity changes (Figure 4.34). In CD8⁺ T cells there is a general trend towards the downregulation of all NF- κ B genes, but none of these changes were significant. The only AP-1 family transcription factor detected in the proteomics was *Jund* and there were no differences in *Jund* copy number in any condition. NK cells showed a distinct downregulation of NF- κ B transcription factors in NK cells with ($p < 0.05$) for *Rel*, *Rela* and *Nfkb2*.

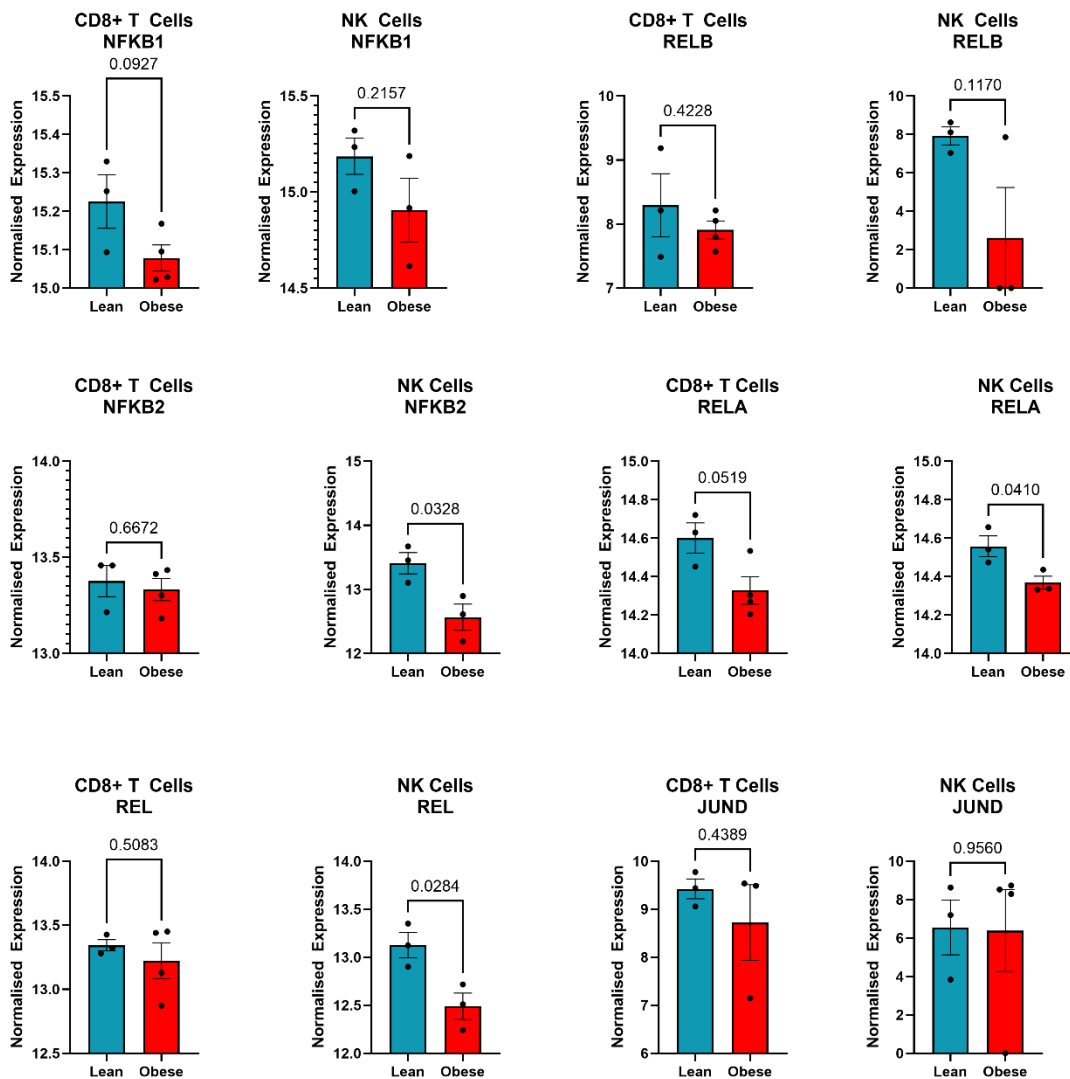


Figure 4.34 – Bar graphs show Normalised proteomics expression (mean ± SEM) of *Nfkb1*, *Relb*, *Nfkb2*, *Rela*, *Rel*, and *Jund* in splenic CD8⁺ T cells and splenic NK cells isolated from lean (blue) and obese (red) mice. Each dot represents an individual biological replicate. P values were calculated using Student's unpaired two-tailed *t*-test between lean and obese groups for each gene and cell type, with the exact *p* values shown above the bars.

4.2.10 – Glucocorticoid signalling may contribute to the induction of *TNFAIP3* expression

From the hdWGCNA analysis on human NK cells, it became apparent that *TNFAIP3* was highly co-expressed with several glucocorticoid receptor (*NR3C1*) target genes. Thus, I sought to investigate whether glucocorticoids contribute to increased *TNFAIP3* expression in fasting and weight loss. In this section the data shows in immune cells that *TNFAIP3* is regulated by the glucocorticoid receptor (GR). I also show (Figure 4.35), that established canonical gene targets of GR are upregulated after weight loss (adjusted p-value < 0.05) for all genes in Figure 4.35.

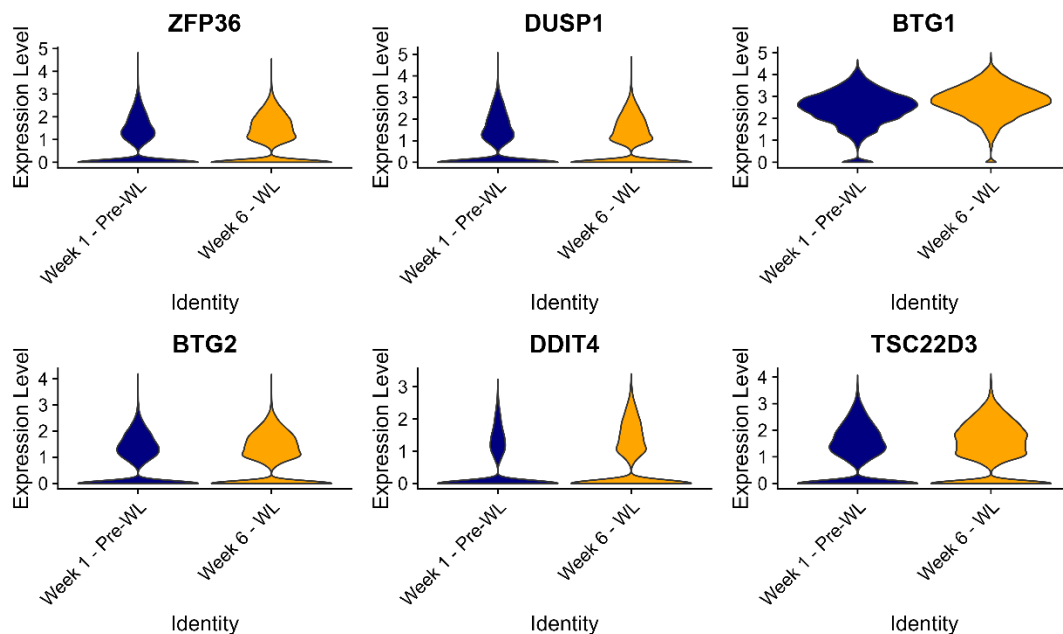


Figure 4.35 – HD2 - Expression of GR-associated anti-inflammatory genes in NK cells before and after weight loss. Violin plots showing expression levels of *ZFP36*, *DUSP1*, *BTG1*, *BTG2*, *DDIT4*, and *TSC22D3* in NK cells from obese individuals at baseline (Week 1, Pre-WL) and after 6 weeks of weight loss (Week 6, WL). These genes belong to the “green module” identified in co-expression analysis, which also contains *TNFAIP3*. Across individuals, weight loss is associated with induction of this glucocorticoid-responsive, anti-inflammatory network, consistent with restoration of immune resolution capacity.

Data from the HD2 dataset revealed that among the genes upregulated in weight loss were a number of glucocorticoid receptor regulated genes and these were established as canonical GR targets using literature, the genes include *TSC22D3*, *ZFP36*, *DUSP1*, *BTG1*, *BTG2* and *DDIT4* (Figure 4.36)^{141 142 143 144 145}. This information was used to inform further analysis and hypothesise about the potential impact of GR on expression of key genes identified to be co-expressed with *TNFAIP3*. Analysis of the MD5 proteomics experiment, revealed the

protein copies for the glucocorticoid receptor, NR3C1, were not significantly changed in CD8 T cells or NK cells in obesity, but the protein responsible for the conversion of inactive cortisone to cortisol, HSD11B1, was significantly downregulated in CD8⁺ T Cells (Figure 4.36). NR3C1 whilst not significantly down in obesity in NK Cells still displayed a significance value worth considering and is suggestive of differences (p-value = 0.1043). These decreases likely result in reduced levels of glucocorticoid receptor activity in obesity in both cell types. Were cortisol and glucocorticoid receptor activity regulators of *Tnfaip3* transcription, this reduction in HSD11B1 would be a candidate mechanism to explain reduced *Tnfaip3* in obesity.

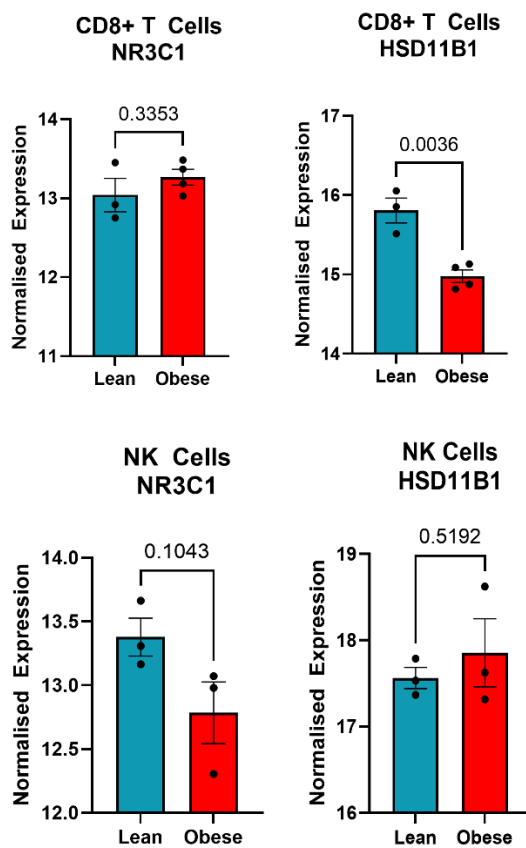


Figure 4.36 – Expression of glucocorticoid-related genes in splenic NK cells and CD8⁺ T cells from lean and obese mice. Normalised expression levels of NR3C1 and HSD11B1 were measured in sorted splenic NK cells and CD8⁺ T cells from lean (blue) and obese (red) mice. Comparisons between groups were performed using Student's t-test.

Next, a chip atlas website (<https://chip-atlas.org/>) was mined to leverage information from their database of ChIP-Seq experiments. The data for several of the central hub genes in the green human module of genes identified in hdWGCNA was collated into a list and cross-

references against Chip-Atlas binding data for *NR3C1* (Figure 4.11). The genes on the y axis were ranked by summed *NCR3I* binding activity across every experiment (x-axis) with the highest ranked targets across the top (Figure 4.38). *TNFAIP3* was ranked 4th from the top of this list and in all experiments *TNFAIP3* had a higher affinity rate for *NR3C1* than *TSC22D3*, an established *NR3C1* target also called GILZ (Glucocorticoid Induced Leucine Zipper). Thus, these data strongly support that *TNFAIP3* is a target of the glucocorticoid receptor, *NR3C1*.

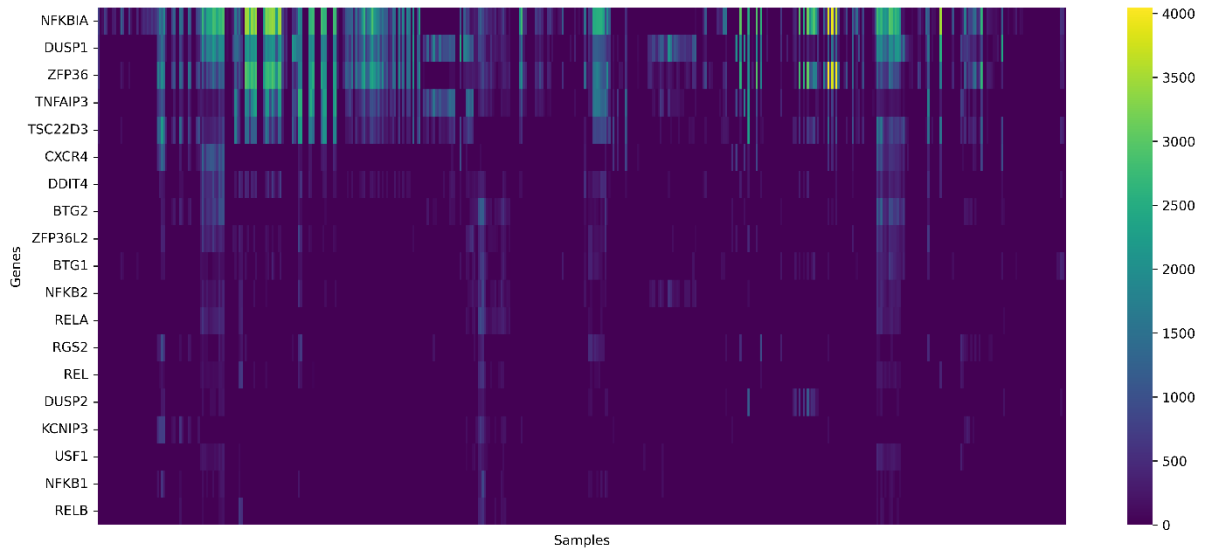


Figure 4.37 – *NR3C1* gene targets obtained from chip-atlas and cross-referenced versus the gene network in Figure 4.11. Genes are ranked by total activity across all ChIP-Seq datasets. Genes ranked by total activity from top to bottom.

To further validate *TNFAIP3* as a target of glucocorticoids, a public dataset was utilised, GSE112101, labelled as HD5 in the methods section. In this study, primary human cell types were treated with methylprednisolone, and this resulted in a consistent upregulation of *TNFAIP3* expression in several immune cell subsets (Figure 4.38). Across the nine primary cell types examined, *TNFAIP3* was significantly induced in B cells ($p = 0.0177$), CD4⁺ T cells ($p = 1.08 \times 10^{-8}$), fibroblasts ($p = 0.0472$), monocytes ($p = 0.00319$), and neutrophils ($p = 4.27 \times 10^{-7}$) when compared with vehicle-treated controls (Figure 4.38). The most pronounced induction was observed in CD4⁺ T cells and neutrophils, where methylprednisolone treatment led to a clear upward shift in expression levels across all donors. In contrast, endothelial cells ($p = 0.306$), myoblasts ($p = 0.452$), osteoblasts ($p = 0.353$), and preadipocytes ($p = 0.608$) did not display statistically significant changes in *TNFAIP3* expression following treatment, suggesting that these effects are immune cell

specific. These results demonstrate that methylprednisolone induced the transcription of *TNFAIP3* mRNA in multiple immune cell types, suggesting a cell type-specific glucocorticoid responsiveness that is most marked in lymphoid and myeloid populations.

Expression of *TNFAIP3* across all cell types

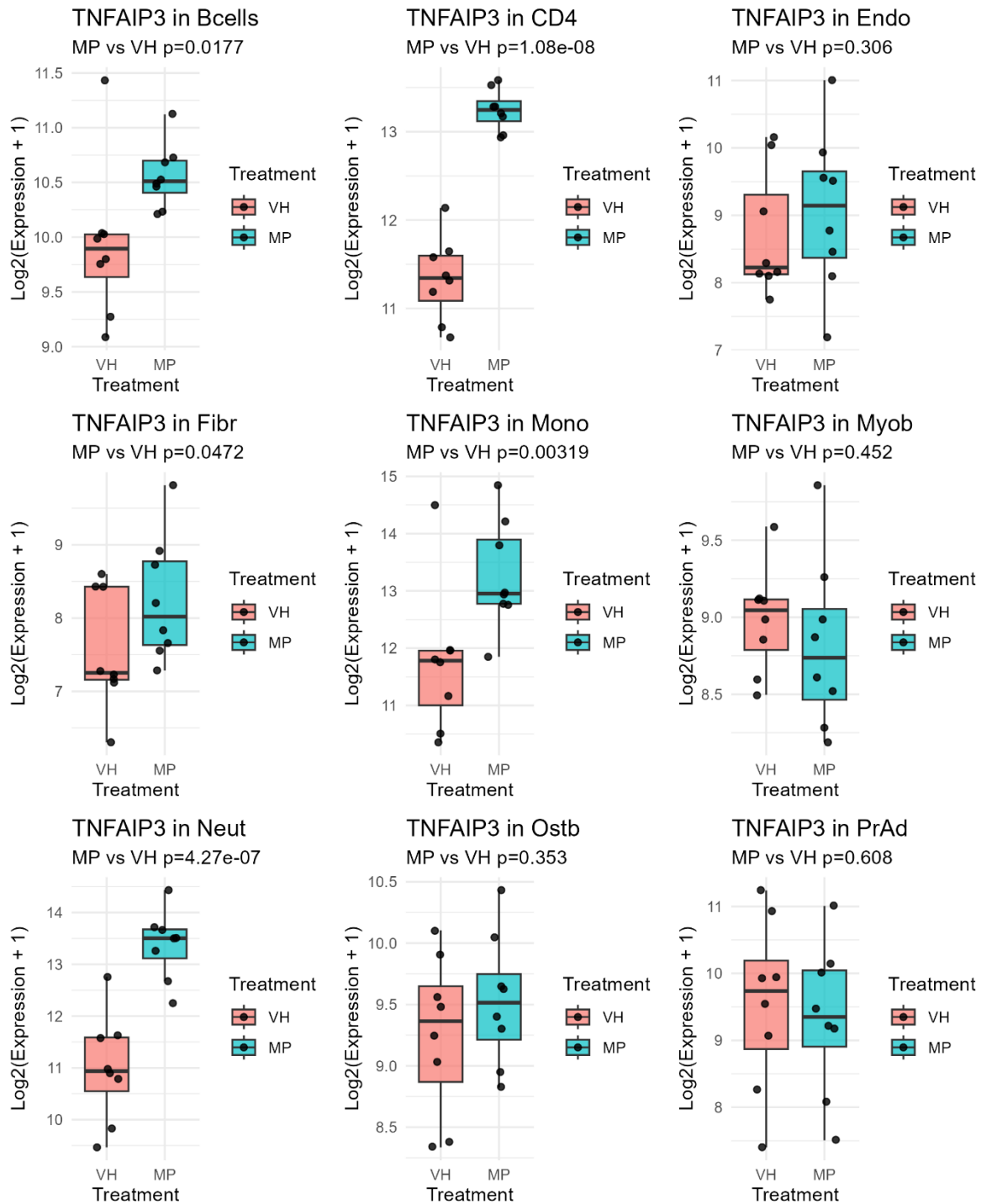


Figure 4.38 – HD5 - *TNFAIP3* expression in immune and non-immune cell populations following treatment with vehicle (VH) or methylprednisolone (MP). Expression values are shown for paired samples, with statistical comparisons performed using paired t-tests between VH and MP conditions for each cell type. Data

are presented as mean $\pm$ SEM, with individual sample points displayed. $p < 0.05$ was considered statistically significant.

To create a gene set of glucocorticoid receptor (GR; *NR3C1*) response genes, GR target genes were identified using the two aforementioned sources, ChIP-Atlas binding data for *NR3C1* and the publicly available dataset GSE112101 (HD5), which profiled transcriptional responses to glucocorticoid treatment. Genes overlapping between these datasets were considered high-confidence GR targets. We next projected these GR target genes onto the human NK cell scRNA-seq network created with hdWGCNA (Figure 4.11), and further integrated the outputs with PPI data. This overlay revealed a network enriched for anti-inflammatory regulators known to be GR targets (Figure 4.39). Notably, several canonical glucocorticoid-induced genes, highlighted in blue nodes, were central to this network. These included *TNFAIP3*, *NFKBIA*, *DUSP1*, *DUSP2*, and *ZFP36*, all of which function as negative feedback regulators of inflammatory signalling pathways.

The integration of these GR targets emphasised a coordinated transcriptional module that dampens pro-inflammatory responses. Including *TNFAIP3*, *NFKBIA* acts as a key inhibitor of NF- κ B signalling, while *DUSP1* and *DUSP2* deactivate MAP kinase pathways, and *ZFP36* promotes the degradation of inflammatory transcripts. The presence of these genes within highly connected regions of the NK cell network suggests that glucocorticoids exert their immunomodulatory effects by reinforcing an anti-inflammatory circuit that is intrinsic to NK cell regulatory modules.

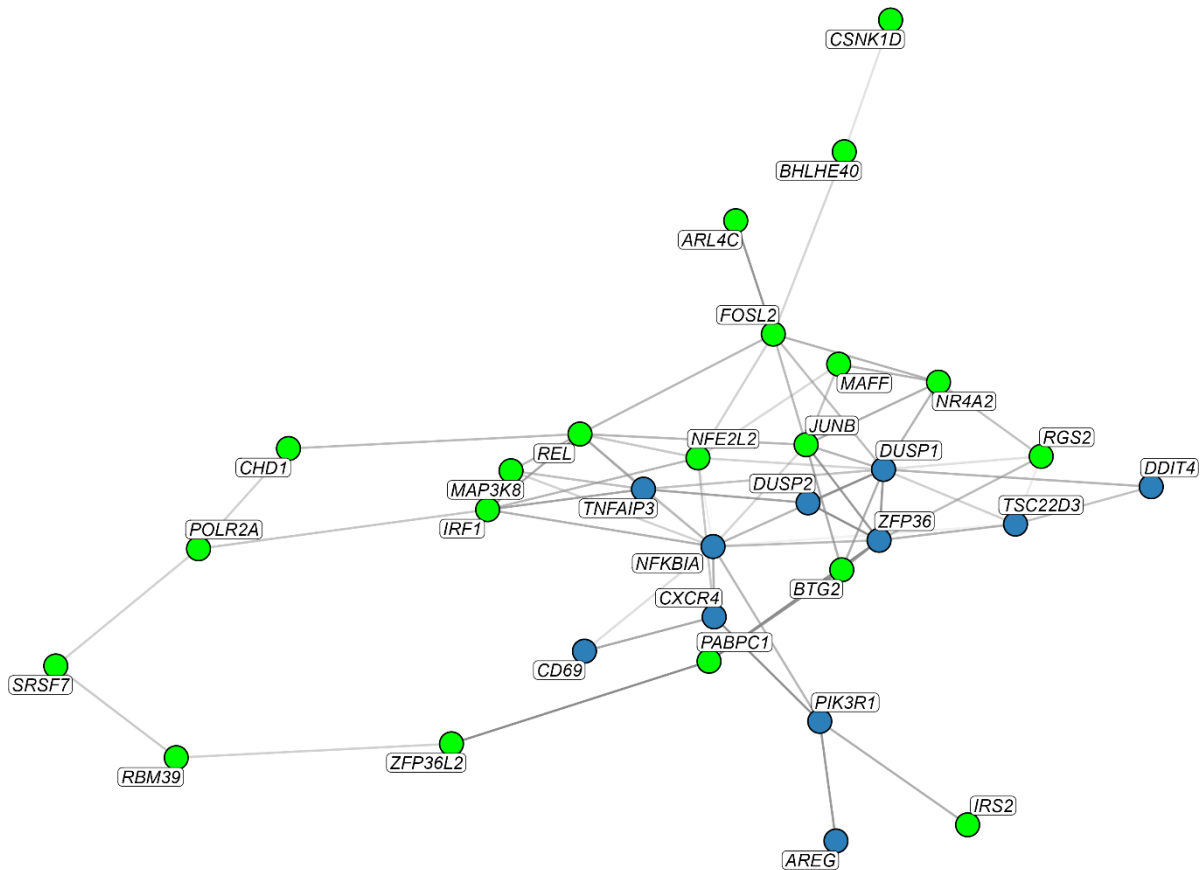


Figure 4.39 - Integration of glucocorticoid receptor (GR/NR3C1) target genes into the NK cell gene coexpression–protein interaction network. Genes identified as glucocorticoid receptor targets from ChIP-Atlas (NR3C1 binding) and from the differential expression dataset used in Figure 4.38 (vehicle vs methylprednisolone treatment) were overlaid onto the weighted gene co-expression network constructed from the human NK cell scRNA-seq matrix using hdWGCNA, combined with protein–protein interaction (PPI) data. Highlighted nodes indicate putative GR target genes within the NK cell network, providing insight into how glucocorticoid signalling intersects with NK cell–specific regulatory modules.

As discussed above, it is important to consider what component of the transcriptional changes are in response to fasting, and in fact independent of obesity and associated inflammation. Thus, to establish if the response we have observed is linked to weight loss / caloric restriction from obesity or if this response can occur independently of obesity a publicly available dataset was utilised Delconte *et. al.*¹⁴⁶ All NK cells present in the dataset were pooled by condition, fasting vs fed (*ad. Libitum*) and differential gene expression analysis was performed. Genes identified as GR responsive genes (Figure 4.39) were cross referenced against differentially expressed genes from the fasting dataset. This approach revealed that fasted mice that were never obese (lean prior to intervention) showed the same

transcriptional response, involving GR regulated genes, that was observed in the human NK cells from subject that underwent weight loss.

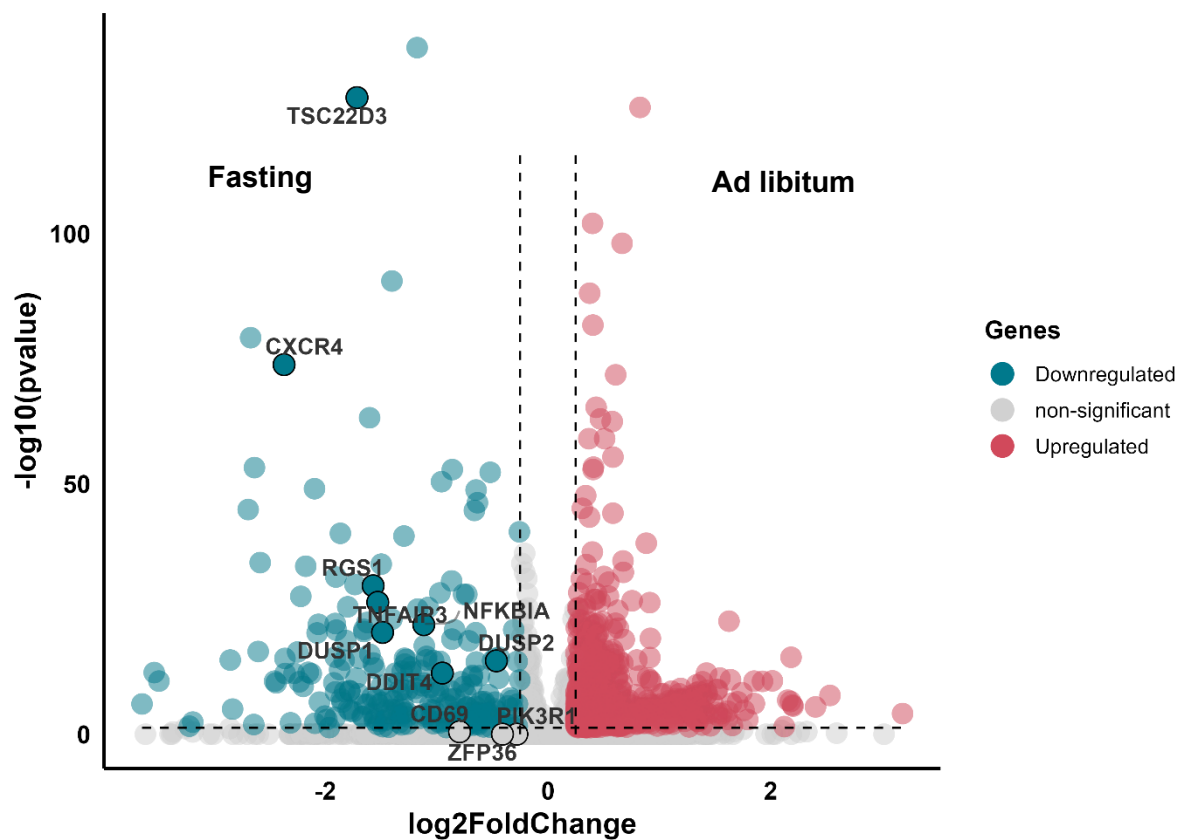


Figure 4.40 – MD7 - Differential expression of glucocorticoid response genes in NK cells from spleen, tumour, and bone marrow. Single-cell RNA-seq data from murine NK cells isolated from spleen, tumour, and bone marrow were analysed to compare ad libitum-fed versus fasted conditions. Differential expression was assessed using the Wilcoxon rank sum test. Genes previously identified as glucocorticoid receptor (GR) response targets are highlighted.

This finding is important, it shows that in NK cells in mice that were never obese or on a HFD, that the same transcriptional signature is observed. That is that GR target genes identified from HD2 and cross referenced with literature and the publicly available dataset (HD5) are upregulated in NK cells in fasted mice showing the response independently from obesity for the first time.

A future interesting avenue to explore is the expression of heat shock proteins, including *HSPA5*, *HSPA8*, and *HSP90AA1*, known negative regulators of GR transcriptional activity. These heat shock proteins bind the GR in the cytoplasm preventing translocation to the nucleus. Interestingly on the LINCS 1000 website, following deletion of *TNFAIP3* there

were increased expression in a number of heat shock proteins (Table 4.8). This might suggest that one role of *TNFAIP3* may be the downregulation heat shock protein expression, which might in turn enhance the cells ensitivity to GR activity.

Symbol	Name	Standardised Value
<i>HSPA5</i>	Heat shock 70kDa protein 5	0.00876296
<i>HSP90AA1</i>	Heat shock protein 90kDa alpha	0.00761798
<i>HSP90AB1</i>	Heat shock protein 90kDa alpha	0.00643867
<i>HSPA8</i>	Heat shock 70kDa protein 8	0.00599014

Table f - Heat Shock Protein Genes Are Upregulated Following *TNFAIP3* Knockout in the LINCS L1000 Dataset

4.3.0 – Chapter 4 Discussion

This discussion focuses on the variety of mechanisms by which *TNFAIP3* can be regulated with a focus on glucocorticoid regulation as the key driver of transcriptional changes. Glucocorticoid signalling will be the main discussion point as it is the most impactful and novel insight into its mechanism.

4.3.1 – Transcriptional and Post-Transcriptional Complexity in *TNFAIP3* Control

The regulation of *TNFAIP3* is complex, involving transcriptional, post-transcriptional, and post-translational layers that interact with both genetic variation and environmental context. eQTL analysis, even without direct genotype-phenotype mapping in this thesis, provides an important conceptual idea. Germline variation at distal enhancers can significantly modulate *TNFAIP3* transcription, sometimes over 100kb from the gene body. If such an enhancer lies within an obesity-sensitive chromatin region, closing under chronic inflammation or metabolic stress, it could represent a mechanism that can alter *TNFAIP3* expression in obesity → altered enhancer accessibility → reduced transcription factor occupancy (e.g., NF-κB, AP-1, GR) → *TNFAIP3* suppression.

Most GTEx identified *TNFAIP3* eQTLs appear in brain tissue, potentially reflecting sample size and tissue representation biases rather than exclusive neuronal regulation. However, the single blood-associated eQTL highlights that cell-type-specific regulatory mechanisms exists and may be revealed more clearly in purified immune populations under stimulatory conditions. This aligns with evidence that *TNFAIP3* is rapidly inducible by NF-κB following inflammatory signalling. In obesity, where chronic low-grade inflammation sustains NF-κB activation, paradoxical downregulation of *TNFAIP3* in T and NK cells suggests that inflammation alone is insufficient for induction, perhaps because accessibility at key enhancers is diminished or because repressive transcription factors are favoured in the metabolic dysregulation occurs.

sQTL analysis is highly speculative like eQTL analysis and no conclusions can be drawn from it other than recognition the high *TNFAIP3* potential for isoform diversity to influence *TNFAIP3* function. Given that A20's domains mediate distinct biochemical activities, deubiquitinase function in the OTU domain, E3 ligase activity in zinc fingers, alternative splicing could re-balance these activities in a cell- and context-specific manner. While technical limitations (3' scRNA-seq chemistry) prevent isoform quantification here, it is plausible that metabolic state could shift splice isoform usage via chromatin, and RNA-binding-protein-mediated changes in co-transcriptional splicing. From a grand-scheme

perspective, the interplay between genetic architecture (eQTLs, sQTLs) and environmental modulation (obesity, weight loss) positions *TNFAIP3* as a candidate for gene, environment interaction in immune regulation. Germline variation may set a baseline capacity for *TNFAIP3* induction or isoform balance, but chromatin state, shaped by metabolic health, may determine whether that capacity is realised. Glucocorticoids, for instance, are potent inducers of *TNFAIP3* as shown in this chapter, but this induction is likely blunted if chromatin is inaccessible. Similarly, obesity-associated hyperglycaemia and dyslipidaemia can alter histone acetylation/methylation patterns, potentially influencing both NF- κ B-driven transcription and GR recruitment.

Together, these layers suggest that restoration of *TNFAIP3* expression with weight loss could reflect not only the removal of inflammatory stressors but also the reopening of key enhancer elements, normalisation of transcription factor networks, and potential rebalancing of splice isoform production. Future studies integrating immune-cell-specific QTL mapping, obesity-stratified chromatin accessibility data, and transcription factor binding assays will be critical to disentangle how inherited variants interact with metabolic state to fine-tune *TNFAIP3*'s immunoregulatory role.

4.3.2 - Co-expression and Network Context of *TNFAIP3* in NK Cells

The co-expression analysis provides an insight into the regulatory neighbourhood in which *TNFAIP3* operates, moving beyond single-gene analysis to examine the broader transcriptional and protein–protein interaction (PPI) environment. *TNFAIP3*'s placement within the green module, a module upregulated with weight loss suggests that its restoration is part of a coordinated shift towards immune homeostasis and metabolic recalibration in NK cells.

The PPI overlay reveals a mechanistically coherent hub in which *TNFAIP3* is flanked by *NFKBIA* and *REL*, positioning it at the heart of the NF- κ B negative feedback loop. This is consistent with *TNFAIP3*'s canonical role in terminating inflammatory signalling by deubiquitinating RIPK1 and TRAF6, thereby dampening NF- κ B-driven transcription. Importantly, the network also connects *TNFAIP3* to MAP3K8 and dual-specificity phosphatases *DUSP1* and *DUSP2*, linking it to the MAPK stress-response axis. This dual positioning, spanning both NF- κ B and MAPK nodes, reinforces the idea that *TNFAIP3* acts as an inflammation “off switch”, with the ability to terminate responses across different signalling cascades. In the context of obesity, where persistent low-grade activation of NF- κ B and MAPK pathways is a defining feature, suppression of *TNFAIP3* likely extends the

half-life of pro-inflammatory signalling, sustaining NF- κ B signalling. While the PPI network captures validated protein-level relationships, co-expression analysis highlights genes linked to chromatin remodelling and transcriptional regulation that would be invisible to PPI-centric approaches. This is significant, as obesity has been repeatedly shown to remodel immune cell chromatin landscapes, particularly at enhancer elements controlling anti-inflammatory genes. In this green module, several transcription factors and chromatin regulators show high co-expression connectivity, suggesting that *TNFAIP3* induction after weight loss may require both transcription factor activation (e.g., NF- κ B, GR, AP-1) and permissive chromatin states at key regulatory elements.

4.3.3 - Metabolic–inflammatory crosstalk: IRS2 and PI3K signalling

An interesting finding within the green network is the presence of *IRS2* and *PIK3R1*. *IRS2* propagates downstream activation of PI3K–AKT and MAPK pathways, both essential for glucose uptake, lipid metabolism, and cell survival ¹⁴⁷. Obesity has been shown to suppress *IRS2*, impairing M2 macrophage polarisation and systemic insulin sensitivity ¹⁴⁸, while weight loss restores its expression. The co-occurrence of *IRS2* and *TNFAIP3* within the same module suggests a tight functional coupling between anti-inflammatory signalling and metabolic insulin sensitivity in NK cells. Given that chronic inflammation drives insulin resistance and vice versa, the parallel upregulation of these genes with weight loss may represent a repair mechanism, in which restored metabolic signalling accelerates inflammatory resolution. In obesity, the combination of *TNFAIP3* suppression and *IRS2* loss likely shifts NK cells into a dysfunctional, pro-inflammatory state. Weight loss appears to reverse this trajectory by reactivating an anti-inflammatory, metabolically competent gene network in which *TNFAIP3* is a central player. In sum, *TNFAIP3*'s restoration with weight loss does not occur in isolation, it is embedded in a highly anti-inflammatory, metabolically integrated gene network that bridges NF- κ B/MAPK resolution, RNA stability control, cytoskeletal reorganisation, and insulin signalling. This places *TNFAIP3* not merely as a brake on inflammation, but as a node in a broader gene–environment–metabolism regulatory circuit that can be tipped towards immune health or dysfunction by changes in metabolic state.

4.3.4 - Glucocorticoid signalling as a likely driver of TNFAIP3

A20/TNFAIP3 is classically framed as an NF- κ B–induced negative regulator, but the co-expression wiring in our hdWGCNA points to an additional axis: glucocorticoid receptor (GR/*NR3C1*) signalling. Links between *TNFAIP3* and GR signalling have been well described particularly in airway epithelial cells ¹⁴⁹. *TNFAIP3* upregulation in immune cells

has never been described in literature based on my searches. In the network of genes, *TNFAIP3* clustered with a group of canonical GR targets, *TSC22D3*, *ZFP36*, *DUSP1*, *BTG1/2*, *DDIT4*, whose expression rose with weight loss. That pattern is difficult to dismiss as coincidence, it suggests that physiological glucocorticoids (GCs) contribute to *TNFAIP3* re-induction in the “resolution” state. Further analysis of the dataset revealed several more GR target genes in the GSE112101 (HD5) dataset that coincide with the human network of genes established in Figure 4.11, where 9 human cell types were treated with synthetic glucocorticoid (methylprednisolone).

Before diving into the network and the implications it is important to consider the general and wide-ranging effects of glucocorticoids especially within clinical contexts. The most important clinical observation is that chronic glucocorticoids can actually induce diabetes mellitus or ‘steroid diabetes’.⁸⁷ Within 7 days of healthy individuals taking synthetic glucocorticoids, insulin resistance and impaired glucose homeostasis were observed¹⁵⁰. This raises many important questions for obesity, fasting and metabolic disease. Whilst glucocorticoids are excellent treatments for inflammatory disorders, the metabolic ill effects are important considerations and thus research is being done to find GCs capable of the anti-inflammatory effects without induction of ‘steroid diabetes’. Steroid diabetes occurs due to GCs increasing transcription of gluconeogenic enzymes in the liver resulting in increased glucose output, even in fed state which can overwhelm the normal insulin-mediated suppression⁸⁷. Clinically, GCs vary significantly in half-life and potency: Relative to short-acting hydrocortisone (half-life of 8 h), the intermediate-acting prednisolone (half-life of 16–36 h) and methylprednisolone (half-life of 18–40 h), which are the most commonly used glucocorticoids, are 4–5-fold more potent in their anti-inflammatory effect than hydrocortisone. The long-acting dexamethasone and betamethasone (both with a half-life of 36–54 h) are 25-fold more potent than hydrocortisone, which is the synthetic equivalent of cortisol (natural GC)¹⁵¹. It is important to note the differences between potency and half-life of synthetic GCs and natural cortisol levels going forward. Observed in this thesis is a natural diet-induced response to weight loss / fasting. Based on the wealth of literature, it is safe to say that fasting elicits this stress response that drives cortisol expression, ultimately leading to increases in glucose production and this is a natural and important physiological response to ensure glucose levels are adequate to fuel the body in fasting or calorie restricted conditions. Where glucocorticoid treatment has negative effects for the individual is likely due to scenarios where usage of synthetic glucocorticoids is in people that have not fasted and do not need additional glucose required for homeostasis.

Taken together, the data support a coherent model in which physiological glucocorticoids act as a proximal driver of *TNFAIP3* (A20) induction during fasting and weight loss, and in which the capacity for that induction is gated by local cortisol metabolism dictated by *HSD11B1* activity amongst other probable mechanisms which will be discussed throughout the rest of this section. The fasting analysis of the Delconte *et. al* dataset is highly informative. Pooling NK cells by condition (fasted versus ad libitum) and testing differential expression shows that the same set of GR-responsive genes highlighted by our hdWGCNA/PPI overlay (Fig. 40) is mobilised in fasted, non-obese mice (Fig. 41). This indicates that the signature we observe after human weight loss is not merely a by-product of reversing obesity but can be elicited by the glucocorticoid response driven by caloric restriction. Consistent with this, the transcriptomic analysis of demonstrates that a short pharmacologic pulse of methylprednisolone, induces *TNFAIP3* across multiple primary human immune subsets (B cells, CD4⁺ T cells, monocytes, neutrophils), with minimal effects in several stromal lineages, underscoring that *TNFAIP3* behaves as a cell-type-restricted, GR target. Mining ChIP-Atlas across hundreds of experiments further reinforces *TNFAIP3* as a GR target by showing recurrent *NR3C1* occupancy at the *TNFAIP3* locus. GR occupancy across all Chip-Atlas datasets had a great summed occupancy at the *TNFAIP3* locus than canonical GR target such as *TSC22D3* (GILZ).

A key aspect of this model is that GR abundance itself is not the limiting factor. While we do not observe state-dependent changes in *NR3C1* protein levels in CD8⁺ T cells, we do see a reduction in NK cells. However, I interpret this reduction because of the broader defect in protein synthesis seen in obese NK cells, where most proteins are downregulated rather than as a targeted, context-specific downregulation of *NR3C1*. That apparent paradox resolves when accounting for two upstream layers of control: ligand availability/bioactivation and enhancer accessibility. First, *HSD11B1*, regenerating cortisol from cortisone, is reduced in obese NK and CD8⁺ T cells, plausibly lowering intracellular GC tone even when circulating steroids are adequate. Second, our adipocyte ATAC-seq identifies weight-loss-associated reopening and activation of enhancer-like elements near *TNFAIP3*, whereas chronic obesity is associated with loss of H3K4me1 and reduced accessibility at related regions. Because GR preferentially binds accessible half-site GREs and often relies on assisted loading at pre-opened enhancers, the chromatin changes seen with weight loss create a permissive landscape in which brief physiological GC pulses can efficiently transactivate *TNFAIP3*; in

obesity, the same pulses are expected to be less effective because the relevant enhancers are harder to access.

These glucocorticoid effects also affect the trafficking and metabolic reprogramming we observe in NK cells. Weight loss and fasting are accompanied by a shift toward a *CXCR4* phenotype and increased circulating *CXCL12*, while the obesity state favours *CX3CR1*. In that context, GR activation appears to reinforce a “resolution” program: it co-induces *TNFAIP3*, *NFKB1A* and *DUSP1/2*, curtails MAPK/NF- κ B signalling bursts, and aligns with recovery of ribosomal/translation modules and the *FOSL2*-dominant AP-1 composition we associate with weight loss. The functional logic is straightforward: when the organism is fasting or losing weight, NK cells need to relocate to supportive niches, conserve fuel and avoid unnecessary inflammatory expenditure.

An important nuance is that GR and NF- κ B can cooperate and oppose each other⁷², and that cooperation is likely context-dependent and vulnerable to breakdown in obesity. At nearby regulatory elements, GR and NF- κ B have been shown to co-occupy enhancers and co-activate genes that dampen inflammation, *TNFAIP3* is a prime candidate, given its placement at the interface of these pathways and the cooperative GR/NF- κ B regulation reported in other cell types. In obesity, certain changes likely erode this productive partnership: enhancer elements near *TNFAIP3* may be less accessible as shown in adipocytes, limiting both NF- κ B and GR loading. The net effect is that even when NF- κ B activity is present, and even when systemic GCs are not frankly low, cooperative GR–NF- κ B induction of A20 is blunted, contributing to the paradox of persistent inflammatory tone with inadequate negative feedback. Weight loss reverses both impediments: enhancers reopen, and intracellular GC tone rises (via fasting physiology and restored *HSD11B1*), allowing GR and NF- κ B to re-engage cooperatively at *TNFAIP3* and re-establish tight control of signal termination, this is evident in adipocytes and could potentially be conserved in immune cells.

The heat-shock observations provide an additional regulatory layer worth consideration. In the LINCS L1000 resource, *TNFAIP3* knockout is associated with upregulation of multiple HSPs (e.g., *HSPA5*, *HSPA8*, *HSP90AA1*), these are the genes that bind cytosolic GR and regulate ligand-dependent nuclear translocation of GR. This could simply reflect compensatory stress after loss of A20’s anti-inflammatory function, but it also raises the possibility of a feedback loop.

There are caveats. GC biology is circadian¹⁵²; sampling time will influence apparent associations. Pharmacologic ligands used in reference datasets (methylprednisolone,

dexamethasone) are more potent and longer acting than endogenous cortisol. The methylprednisolone effects on *TNFAIP3* are robust in multiple immune subsets but not universal across stromal lineages, so generalisation beyond immune cells needs direct validation.

Despite these limits, the simplest working model consistent with our data is that glucocorticoids are a principal upstream driver of *TNFAIP3* induction in fasting and weight loss, acting through *NR3C1* and likely with NF- κ B to reinstate rapid negative feedback on inflammatory signalling. This allows one to make clear, testable predictions: GR occupancy and probable cooperation with NF- κ B at *TNFAIP3* enhancers should increase in NK populations during weight loss; acute GR antagonism during fasting should attenuate A20 induction and prolong NF- κ B activity without changing *NR3C1* abundance; manipulating *HSD11B1* should tune *TNFAIP3* inducibility at fixed systemic GC levels. Conceptually, this places *TNFAIP3* at the centre of a gene–environment interaction in which metabolic state (fasting/weight loss vs obesity) governs enhancer accessibility and steroid bioactivation, thereby deciding whether the GR–NF- κ B partnership functions as an efficient anti-inflammatory switch.

4.3.5 - The Cytotoxic Program of Gene Regulation in Obesity

The turquoise module represents a broad cytotoxic transcriptional programme preferentially expressed in NK cells in the obese state as shown in Figure 4.14 with *CX3CR1* as the hub gene, in contrast to the smaller, anti-inflammatory green module associated with weight loss. With 821 genes, the turquoise module is substantially larger than the green module and is characterised by the upregulation of key cytotoxic effectors, including *PRF1*, *GZMA*, and *GZMB*, which are central to NK cell–mediated target killing. While these molecules are essential for immune surveillance, their sustained elevation in obesity is consistent with a chronically activated NK cell phenotype, which in adipose tissue can contribute to persistent inflammation, recruitment of pro-inflammatory myeloid cells, and local insulin resistance. However, many of the changes around *GZMB* and other cytotoxic markers in obese and weight loss states are subtle. The changes seen in the green *TNFAIP3* module are much greater in response to weight loss (measured by log₂ fold change), therefore, the same value should not be given to observations in the turquoise cytotoxic module due to the subtlety of changes.

As mentioned, one of the most notable genes within this module is *CX3CR1*. *CX3CR1*'s prominence within the network suggests it may serve as a central organiser of the cytotoxic

programme, linking effector function with tissue localisation. Given its potential role in shaping NK cell behaviour in metabolically inflamed adipose tissue, *CX3CR1* will be discussed in greater detail later in this chapter.

Metabolically, the turquoise module is also distinguished by the upregulation of oxidative phosphorylation (OXPHOS) genes alongside several glycolytic enzymes, reflecting a dual-fuel metabolic profile that can sustain prolonged NK cell activation and cytotoxicity. While such metabolic flexibility supports rapid immune responses, chronic engagement in obesity may predispose NK cells to bioenergetic strain, altered effector capacity, and persistent inflammatory output.

In sum, the turquoise module reflects a highly cytotoxic, metabolically active NK cell state that dominates in obesity and likely contributes to the maintenance of adipose tissue inflammation. The transition away from this programme with weight loss, towards the anti-inflammatory and metabolically integrated profile of the green module, suggests that these transcriptional states are dynamic and reversible, with implications for targeting NK cell function in metabolic disease.

4.3.6 - Mouse hdWGCNA Results

An attempt was made to directly integrate the human and mouse scRNA-seq datasets into a consensus hdWGCNA network by harmonising orthologues. However, this produced unstable results, reflecting both technical differences in the datasets and distinct tissue contexts. The human analysis was derived from circulating NK cells, whereas the murine dataset originated from adipose tissue NK cells using obesity and weight loss conditions (MD2). Because adipose tissue is a lipid-rich, cytokine- and hypoxia-exposed microenvironment, it imposes unique pressures on gene expression programmes. To avoid technical artifacts from consensus analysis, the mouse dataset ($n \approx 1,325$ NK cells) was analysed independently with the same hdWGCNA workflow as used for the human data, allowing methodological comparability without obscuring biology. Module colour labels are specific to each run and not shared across species.

The murine adipose NK-cell network resolved into seven robust modules. Several were dominated by proliferative and biosynthetic states, the green module was enriched for DNA replication and repair, while the turquoise module contained signatures of translational activity and peptide synthesis. A black module reflected centriole and cell cycle regulation. Other modules highlighted stress adaptation: the blue module contained stress granule, ribosomal and mitochondrial electron transport genes, while the brown module was enriched

for leukocyte apoptosis regulation and responses to oxygen levels. A red module captured receptor-mediated signalling, trafficking, adhesion and cytokine pathways.

As in the human dataset, translational and ribosomal programmes were suppressed in obesity and re-activated with weight loss, indicating that restoration of protein synthesis is a conserved feature across species in obesity and weight loss. However, the position of *Tnfaip3* within the network differed markedly between species. In humans, *TNFAIP3* clustered within a small, anti-inflammatory module enriched with GR target genes, strongly upregulated in weight loss. In contrast, in mouse adipose NK cells, *Tnfaip3* was embedded in the red module alongside chemokine receptors, adhesion molecules and PI3K-coupled cytokine pathways.

Integration of STRING PPI data into the mouse red module revealed mechanistic partners that situate *Tnfaip3* at the interface of inflammatory regulation and apoptosis. These included TRAF adaptors (*Tab2*, *Traf3ip2*) and *Birc2*, all within A20's canonical NF- κ B regulatory space, but also adhesion- and cytoskeleton-associated proteins such as *Ptk2b* (PYK2), *Zyx*, and integrin subunits (*Itga4*, *Itgb1*, *Itgb7*). Cytokine receptors (*Il7r*, *Il2rb*) and PI3K catalytic subunit *Pik3cd* formed a survival and proliferation axis, while *Jak2* and SMAD2/3 linked to TGF- β and γ -chain signalling. *Cxcl12* emerged as a chemokine hub, placing *Tnfaip3* within a circuit coupling chemokine retention, adhesion, cytokine responsiveness and inflammatory restraint.

The contrast with the human network is clear. In humans, *TNFAIP3* was part of a focused anti-inflammatory module closely tied to weight loss induction. In mice, *Tnfaip3* was integrated into a broader tissue-contextual programme, in which inflammatory shutdown is linked to cell adhesion, positioning, and cytokine responsiveness within adipose. This likely reflects both tissue and species context: adipose-resident NK cells require coordination between positional control and signal restraint, whereas circulating human NK cells reflect systemic anti-inflammatory programming. However, some of these changes could reflect technical aspects, the human dataset used significantly more NK cells for hdWGCNA ($n = 6000$), thus co-expression in humans is likely more robust. The most likely explanation is glucocorticoid levels may not reach the same levels in weight loss in adipose as in other tissues like blood.

Despite these differences, both species show that obesity is associated with suppression of ribosomal and translational capacity, with restoration following weight loss. Thus, while the wiring of *TNFAIP3* differs, the shared theme is that resolution of metabolic stress restores

both translational activity and regulatory modules that prevent excessive inflammatory activation.

4.3.7 - Trafficking Receptors in NK Cells – Divergent States in Obese and Fasted States

A consistent theme across our human and murine analyses is that NK cells remodel their trafficking “postcode” as metabolism changes. In humans, obesity is dominated by *CX3CR1*, the most strongly upregulated gene in our differential analyses, whereas following weight loss the axis flips toward *CXCR4* as the most upregulated receptor. This signals a re-routing of NK-cell trafficking. *CX3CR1*, via its ligand fractalkine (*CX3CL1*), promotes vascular patrolling and adhesion to inflamed endothelium, a behaviour strongly linked to adipose tissue inflammation and monocyte/macrophage recruitment in obesity ¹⁵³. By contrast, *CXCR4*, via *CXCL12*, is the canonical retention cue for marrow and stromal niches, pointing toward redistribution into supportive microenvironments as organisms exit an obesogenic state ¹⁴⁶.

Two independent lines of evidence support the mechanistic significance of this inversion. First, LINCS L1000 perturbation data suggest that *CXCR4* knockout reduces *TNFAIP3* (A20) expression, raising the possibility that *CXCR4* engagement feeds into A20-mediated NF-κB restraint. This is associative and context-dependent, but it offers a plausible link between a homing receptor and an anti-inflammatory rheostat. Second, our human proteomics time course shows *CXCL12* rises during fasting and falls on refeeding, temporally mirroring the transcriptional rise of *CXCR4*, creating the ligand–receptor pairing expected to promote marrow retention.

The murine data argue that these are not human blood–specific findings. In adipose NK cells from the MD2 cohort, *Cxcr4* increases with weight loss, *Cx3cr1* trends down, and *Slpr5* an egress receptor along sphingosine-1-phosphate gradients also decreases. This pattern closely matches an independent dataset spanning spleen, tumour, and marrow under 48-hour fasting, where *Cxcr4* and *Tnfaip3* were upregulated while *Cx3cr1* and *Slpr5* fell. The convergence across tissues and species points to a conserved response induced by weight loss/fasting or obese states.

This interpretation is strongly consistent with the recent work of Delconte *et al.* who demonstrated that fasting redistributes NK cells from the periphery into the bone marrow, enhances anti-tumour capacity, and requires *CXCR4* and *SIPR5* signalling for this redistribution. Antagonising *CXCR4* (AMD3100) or deleting *Slpr5* impaired marrow entry during fasting and inhibited fasting-induced tumour control, despite similar tumour NK-cell

numbers. Mechanistically, fasting increased glucocorticoids and fatty acids, driving *CPT1A*-dependent fatty-acid oxidation in splenic NK cells, while bone marrow residency exposed NK cells to IL-12-producing myeloid cells that primed *STAT4* signalling and IFN- γ competence. Notably, marrow entry required both *S1PR5* (for egress into circulation) and *CXCR4* (for local retention), coupling trafficking with metabolic and cytokine reprogramming.

Thus, the obese *CX3CR1* NK cell phenotype and the weight-loss/fasted *CXCR4* NK cell phenotype are not simply expression markers but represent alternate routing mechanisms. In obesity, NK cells transcriptionally adopt a cytotoxic output, whereas in fasting/weight loss, NK cells are tethered to *CXCL12*-rich niches that enable metabolic refuelling, transcriptional resetting, and *TNFAIP3* re-expression.

4.3.8 - ATAC-seq – Adipocyte analysis

ATAC-seq provides a direct readout of chromatin accessibility and thus may provide insight into regulations across lean, obese and weight loss conditions. Although adipocytes are mechanistically distinct from immune cells, *TNFAIP3* (A20) is functionally relevant in adipose biology, implicated in adipogenesis and metabolic signalling¹⁴⁰, and the public dataset we analysed contained matched lean, obese (high-fat diet, HFD) and weight loss (WL) conditions. That combination makes adipocytes a useful parallel system to ask a simple but important question: does metabolic state reconfigure the regulatory landscape at the *Tnfaip3* locus in a way that could plausibly generalise to immune cells?

Exon coordinates for *Tnfaip3* (mm10) retrieved from UCSC and verified in IGV against the ATAC-seq BED files to ensure the correct genome was being used. Aggregating accessibility over exons (to average over local peak heterogeneity and reduce sample-to-sample noise), a striking pattern was observed: exons 6 and 7 show near-complete loss of accessibility in HFD adipocytes, with accessibility restored in weight loss. These exons map to the zinc-finger region of A20 that mediates protein–protein interactions and E3 ligase functions. By themselves, exons are not classical regulatory elements, and one needs to be careful around inferring functionality from exons. Nevertheless, closure within the gene region is not the typical signature of a uniformly down-regulated gene; it could hint at localised chromatin remodelling that could intersect with co-transcriptional splicing or RNA processing. Any alternative splicing possibilities remain a hypothesis.

Beyond accessibility changes in *Tnfaip3*, enriched histone post-translational modifications (hPTMs) curated by Hinte et al were analysed. Focus was placed on H3K27ac as a mark of

active enhancers and H3K4me1 as a mark of poised/active enhancer chromatin (context-dependent). Several features were consistent and biologically cohesive within this experiment and with observations made from scRNA-seq data. Across weight loss contrasts, increased enhancer activity was observed upstream of *Tnfaip3*, including a site ~45 kb 5' of the TSS that gained H3K4me1 in both medium- and long-term weight loss, and additional H3K27ac gains at ~45–51 kb 5' in long-term WEIGHT LOSS. In the obesity contrasts, we saw a biphasic pattern: a short-term increase in H3K4me1 ~17 kb 5', followed by significant losses of H3K4me1 at +13 kb and +27 kb downstream with prolonged HFD. Several weight loss effects sat just above conventional FDR cut-offs (0.06–0.07), but the directionality was uniformly positive and matched the exon-level reopening and the independent scRNA-seq observation that *Tnfaip3* expression rebounds with weight loss. Taken together, the enhancer data support a model in which chronic obesity progressively represses elements around *Tnfaip3*, whereas weight loss reactivates a subset of upstream enhancers that likely contribute to its transcriptional restoration.

Mechanistically, the chromatin changes in the enhancer neighbourhood around *Tnfaip3* in adipocytes is a plausible contributor for mRNA transcript differences.

How far can this be generalised to immune cells? Two lines of reasoning argue the adipocyte data are informative rather than incidental. First, the temporal logic, early accommodation, late repression with HFD, reopening with weight loss, mirrors what has been observed in immune compartments at the transcript and protein level. The safest interpretation is therefore mechanistic rather than literal element reuse: obesity compresses regulatory capacity at *Tnfaip3*, weight loss re-expands it.

4.3.9 - NF-κB and AP-1 signalling in obesity

Because *TNFAIP3* (A20) is a canonical NF-κB response gene, the obvious first hypothesis was that obesity should leave a strong transcriptomic footprint on NF-κB subunits themselves. Re-examining multiple datasets across species, tissues, and cell types complicates that intuition: at the mRNA level, NF-κB family members are not profoundly dysregulated in obesity or weight loss. Instead, the most reproducible signal is a reconfiguration of AP-1 composition, with a consistent enrichment of *FOS* (and, in some contexts, *FOSB/JUN*) in obesity and *FOSL2* (with *JUND* in mice) in lean/weight loss states.

This pattern is not paradoxical upon investigation as NF-κB activity is largely regulated post-translationally, controlled by IKK-dependent phosphorylation, IκB degradation, nuclear translocation, p100→p52 processing, and cofactor availability. Minute-scale pulses of

nuclear p65/RelA leave little necessity for changes in NF- κ B gene transcription. By contrast, AP-1 is an immediate-early transcriptional program whose transcripts (*FOS/JUN* family) are both sensitive and short-lived; their mRNA levels are therefore better reporters of upstream signalling flux (MAPK/JNK/p38, calcium) than NF- κ B subunit mRNAs are of IKK flux. In other words, the absence of large NF- κ B mRNA shifts does not argue against pathway activity; it argues that the readout observed via scRNA-seq, is insensitive to NF- κ B's activity changes, while AP-1 transcripts remain responsive to the same upstream milieu.

Across the observed datasets in this chapter, the mouse adipose (MD2) dataset and the human NK datasets show a coherent AP-1 response. In mouse adipose NK cells and in the global adipose transcriptome, Fos tracks with obesity, while *Fosl2* (*Fra-2*) and *Jund* rise in lean/weight loss. In human data, CD56^{bright} NK cells show an obesity-associated *FOS/FOSB/JUN* signature that mirrors the whole-tissue pattern, whereas CD56^{dim} NK cells do not enrich AP-1 factors in obesity but gain several AP-1 components in weight loss together with *NFKB2/REL*.

Functionally, what do changes in AP-1 mean? The *FOS*-high obesity state aligns with the cytotoxic/chemotactic, turquoise module and the *CX3CR1*-centric trafficking program we observed earlier: sustained JNK/p38 signalling from lipotoxic stress, ROS, cytokines (*TNF/IL-1*), and mechanical/adipose cues is exactly the upstream environment that drives *FOS/JUN*. FOS-rich AP-1 dimers favour rapid effector gene expression, matrix remodelling, and inflammatory chemokines, features we see in the obese NK program. Conversely, the *FOSL2/JUND*-biased weight loss state coincides with the anti-inflammatory green module, *CXCR4*-driven niche residency, restoration of ribosomal/translational capacity, and *TNFAIP3* re-expression.

The NF- κ B signal is better understood as one of gating rather than transcriptional abundance. Proteomic data provide an additional perspective. In obese splenic NK cells, global protein synthesis is markedly reduced, consistent with the generalised stagnation described earlier. Under these conditions, the absence of widespread NF- κ B or AP-1 protein differences likely reflects a limitation in translational capacity rather than a lack of upstream signalling. The detection of *JUND* in NK cells, despite this bottleneck, highlights that changes in AP-1 composition remain visible at the protein level. In CD8⁺ T cells, NF- κ B proteins trend downward without reaching statistical significance, consistent with a broad but modest suppression of biosynthetic output under obesogenic stress.

4.3.10 - MicroRNA regulation of *TNFAIP3* (A20): miR-125a and miR-221 as candidate modulators in metabolic disease

Another potential regulator of *TNFAIP3* is the evolutionary conserved microRNA known as hsa-miR-125a-5p. hsa-miR-125a-5p is conserved between human and mouse and has been validated to bind to the *TNFAIP3* 3'UTR,⁶² makes miR-125a an attractive candidate regulator at the interface of metabolism and inflammation. Prior work (observed in a Glasgow University PhD thesis by Ashleigh-Ann Rainey) reports that miR-125 overexpression reduces *Tnfaip3* in macrophages, consistent with direct post-transcriptional repression. Although this has not yet been demonstrated in obesity or T2D, the pattern is biologically plausible: *TNFAIP3* tends to be reduced in obesity, and miR-125a is up in some metabolic/inflammatory contexts, providing a mechanistic route by which miR-125a could lower *TNFAIP3* and extend NF-κB signalling.⁶²

Additionally, miRNAs converge on the same node. miR-22 and miR-221 have both been implicated in *TNFAIP3* downregulation; notably miR-221 is induced by hyperglycaemia in endothelial cells, where it impairs migration via c-Kit suppression, and antisense inhibition rescues function.¹⁵⁴ Given A20's central role in damping inflammatory signalling and protecting immune homeostasis, hyperglycaemia-driven miR-221 provides a credible path to *TNFAIP3* loss in obesity/metabolic disease, potentially compounding dysfunction observed in immune cells and altered cell numbers.

In sum, miR-125a-5p and miR-221 are credible post-transcriptional brakes on *TNFAIP3* that could amplify metabolic inflammation. The current evidence base is suggestive but not definitive in obesity/T2D; targeted, isoform-aware and RISC-anchored experiments should now test whether miRNA–A20 coupling is a driver (not just a passenger) of *TNFAIP3* suppression in metabolic disease.

4.4 – Chapter 4 Conclusion

This chapter asked why *TNFAIP3* (A20), a central brake on inflammatory signalling, falls in obesity yet rebounds with weight loss, and what that reveals about immune regulation across metabolic states. The most coherent answer from our multi-omic analyses is that physiological glucocorticoid (GC) signalling, acting through the glucocorticoid receptor (GR/*NR3C1*), is a principal upstream driver of *TNFAIP3* re-induction in immune cells during fasting and post-obesity weight loss. Mechanistically, GR can directly transactivate anti-inflammatory genes (e.g., *TSC22D3/GILZ*, *DUSP1/MKP-1*, *ZFP36*, *NFKBIA*) and modulate NF- κ B/AP-1 activity via tethering and composite enhancer interactions; the balance of these outputs is strongly cell-type and context dependent.

Two features help reconcile why this programme is muted in obesity but restored with fasting/weight loss. First, GC tone at the cell level is shaped by glucocorticoid metabolism with *HSD11B1*, which regenerates active cortisol from cortisone within tissues, obesity remodels *HSD11B1* in CD8 T cells and NK cells and this probably occurs in many immune cell subsets. This alters local GR ligand availability even when circulating glucocorticoid levels appears “normal.” Another plausible region is that A20 is actually required for management and regulation of heat shock proteins which bind the GR in the cytoplasm preventing it from translocation to the nucleus. With A20 downregulated in obesity heat shock proteins are likely more abundant and inhibit GR signalling.

Physiologically, fasting provides a glucocorticoid shift that re-engages this axis. Human studies show modest rises in cortisol with short-term fasting, and in vivo work demonstrates that fasting reprograms NK-cell trafficking and function through marrow retention cues and metabolic rewiring, conditions under which GR-responsive transcripts, including *TNFAIP3*, are favoured. These external data align with our finding that GR-target signatures are mobilised in fasted non-obese mice and during post-obesity weight loss in humans.

Clinically, the overlap between fasting/weight loss and glucocorticoid transcriptional signatures must be interpreted with care. Therapeutic GCs reproduce many of the same GR-dependent anti-inflammatory programmes but, when given chronically and given their extended half-life, this predictably drives insulin resistance and hyperglycaemia via increased hepatic gluconeogenesis, altered adipose and muscle substrate flux, and β -cell stress. This glucocorticoid-induced hyperglycaemia is common in practice and mechanistically well described. In contrast, fasting couples transient GC elevations with

concurrent energy deficit and counter-regulatory signals, a context that appears to favour immune resolution modules without sustained glucose increases.

As mentioned, synthetic GCs differ in potency and duration, so ligand choice and dosing schedule will shape GR's genomic occupancy and metabolic side-effects. If the therapeutic goal is to restore A20-centred feedback in immune cells without provoking steroid diabetes, strategies that mimic physiological glucocorticoid dynamics (short pulses; circadian-aligned dosing), leverage tissue-specific GC regeneration (targeted modulation of 11 β -HSD1), or exploit selective GR modulators could, in principle, favour anti-inflammatory transactivation at *TNFAIP3* while minimising systemic metabolic cost.

5.0 - Chapter 5 – Obesity Phenocopies A20 Haploinsufficiency

This chapter builds upon the observations from Chapter 3 and Chapter 4 to investigate A20 haploinsufficiency (HA20) and to explore whether a shared immune phenotype exists between HA20 and obesity. The work replicates the approach taken with obesity and weight loss, with a particular focus on two key questions:

1. Does obesity induce an immune state that resembles HA20 at the transcriptional level?
2. Is the expression of glucocorticoid signalling genes affected in A20 haploinsufficiency?

These questions are linked but not identical, the transcriptional profiles may converge in some respects and diverge in others. Capturing and defining the similarities is central to this chapter and hypothesising as to why.

5.1 - Chapter 5 Introduction

A20 haploinsufficiency (HA20) is a severe acquired inflammatory disease that arises as a result of heterozygous germline loss of function mutations in *TNFAIP3*.⁴⁰ HA20 phenotypes vary significantly but consistent across HA20 patients is systemic inflammation and inflammatory episodes that typically involve the skin, joints and gastrointestinal tract.¹²³ HA20 has also been likened to Behcet's disease which is defined by ulcers of the mucosa. HA20 is primarily driven by innate immune cells but evidence is growing showing its effect on B and T cells and how *TNFAIP3* loss-of functions variants plays a role in the disease's pathology.

Given the previous chapters work on the characterisation of *TNFAIP3* across settings and GR driven transcriptional programs, I wondered could the response observed in obesity or conversely restored with weight loss and fasting be observed in individuals with HA20? This research also allows for a decoupling of what transcriptional signatures are driven by loss of *TNFAIP3* versus what is driven by obesity and metabolic disease. However, confounding factors are present, for example, whilst HA20 disease arises due to LOF *TNFAIP3* variants, secondary inflammation drives further dysregulation further driving changes.

5.2.0 – Chapter 5 Results

Three datasets were integrated in this analysis, human weight loss dataset from previous chapters, a healthy control dataset and the publicly available scRNA-seq HA20 dataset. After integration of the datasets, comparisons of UMAPs were performed to assess if integration was successful, this is measured by overlap across datasets. As Figure 5.1 illustrates there is strong overlap across each dataset and cell cluster. This is to be expected given that all the data comes from human PBMCs and thus there should be high transcriptional similarity irrespective of disease present (HA20, Obesity). Figure 5.1 illustrates successful integration of the datasets and lays the foundation for downstream analysis and comparisons across datasets. Some of the datasets have higher sparsity and coverage due to lower cell numbers as shown in the pbmc3k UMAP.

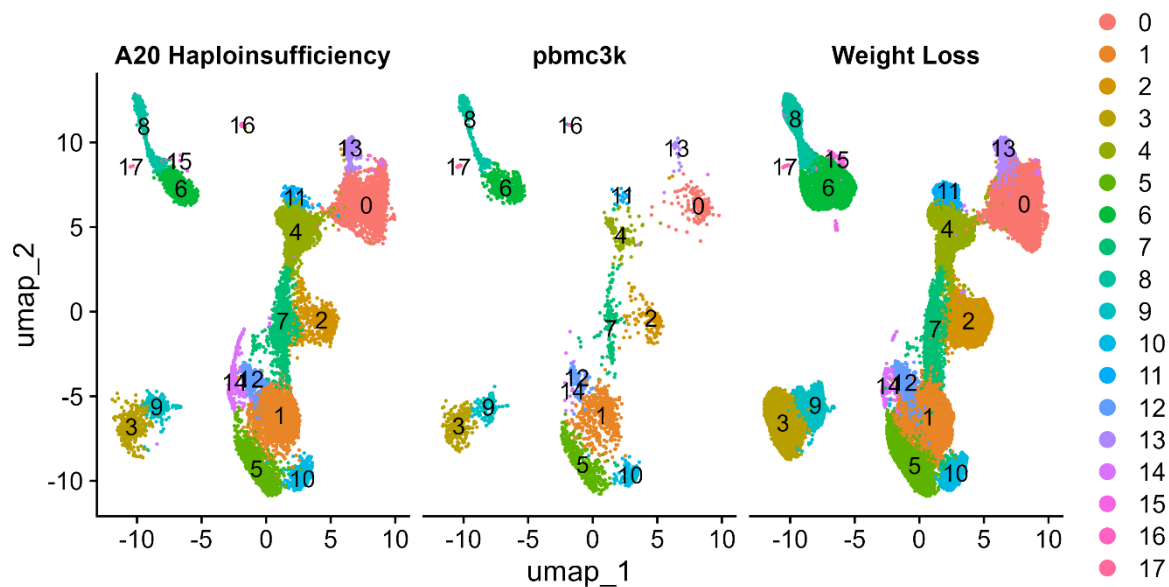


Figure 5.1 – UMAP projection of integrated scRNA-seq datasets, separated by dataset of origin: A20 haploinsufficiency (left), pbmc3k healthy donor reference (middle), and human weight loss PBMCs (right). Each point represents a single cell, coloured by cluster identity (0–17). The overall distribution of clusters is preserved across datasets, demonstrating successful integration despite biological and technical differences.

Cell counts varied across conditions and datasets (Table 5.1), however, there is no foreseen negative effects of this downstream as comparisons performed always had similar cell counts, for example, HA20 was always compared against healthy cell counts which are approximately equal, similarly with obese and weight loss conditions cell counts were consistent. Keeping comparisons consistent and not comparing across datasets ensures less technical effects affect analysis.

Table 5.1 – Cell Counts by Dataset and Condition:

Dataset	HA20	Healthy	Obese	WL
A20 Haploinsufficiency	7691	4741	0	0
pbmc3k	0	2700	0	0
Weight Loss	0	0	18269	19012

Within these three datasets are four separate conditions to be considered, healthy individuals, individuals with HA20 and individuals pre (obesity) and post weight loss. As a further control to check if there were any specific biological or technical effects as a result of integration, UMAPs were visualised based on condition (healthy, HA20, obese, weight loss). Across conditions, integration was successful as shown in Figure 5.2.

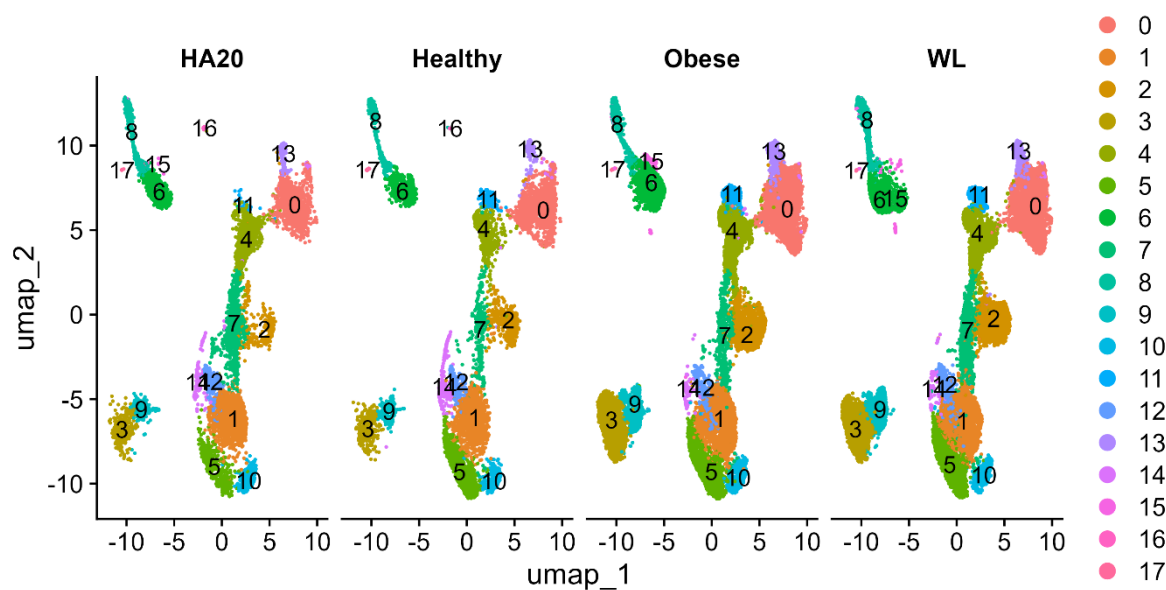


Figure 5.2 – Two-dimensional UMAP projections of integrated PBMC scRNA-seq data are shown, separated by condition: A20 haploinsufficiency (HA20, left), healthy control (second), obesity (third), and weight loss (WL, right). Each point represents a single cell, coloured according to cluster identity (0–17). The preservation of cluster structure and overlap across all four conditions indicates successful integration of cell conditions and provides a robust framework for downstream comparisons of HA20, obesity, and weight loss relative to healthy controls.

In addition to this, it was important to verify that *TNFAIP3* was downregulated in HA20. As expected *TNFAIP3* levels are significantly reduced in HA20 compared to Healthy, Obese and weight loss conditions. Whilst *TNFAIP3* is downregulated in obesity, there is still significantly more *TNFAIP3* produced in obesity when looking at the global PBMC

transcriptome. It is also important to note that HA20 expression, whilst very low, is not zero, there was simply not enough cells expressing *TNFAIP3* to produce a violin plot.

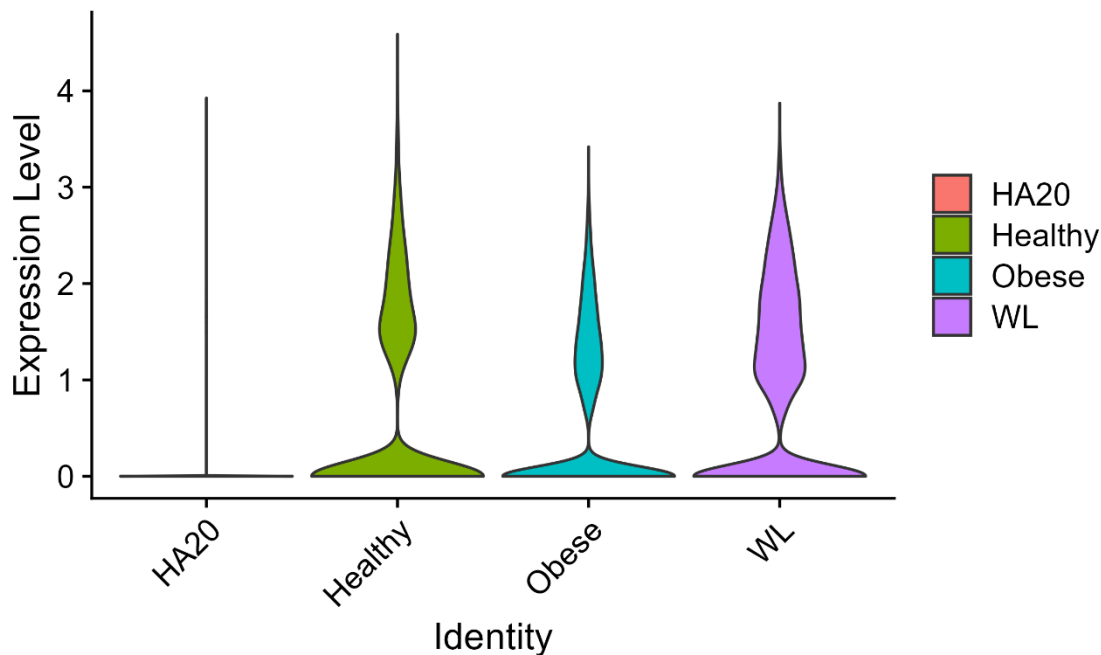


Figure 5.3 – Violin plots showing *TNFAIP3* expression levels in peripheral blood mononuclear cells (PBMCs) from individuals with A20 haploinsufficiency (HA20), healthy controls, obesity, and weight loss (WL). Expression is markedly reduced in HA20 compared with all other conditions, consistent with germline *TNFAIP3* loss-of-function mutations. Although *TNFAIP3* is also downregulated in obesity relative to healthy controls, its expression remains higher than in HA20. Weight loss restores *TNFAIP3* expression toward healthy levels.

After ensuring the data was correctly integrated, differential expression was performed across every cell cluster identified in the pre-processing steps. Differential expression revealed expression markers associated with each cluster and these were subsequently used to label each cell type in conjunction with old cell labels from previous analysis prior to integration. Some cell clusters shown in previous figures were removed due to resembling dying cells (high mitochondrial DNA transcripts) or the cell clusters had extremely low cell counts (cells < 250), these were removed. Depicted in Figure 5.4 is the final UMAP showing the integrated dataset with cell clusters labelled.

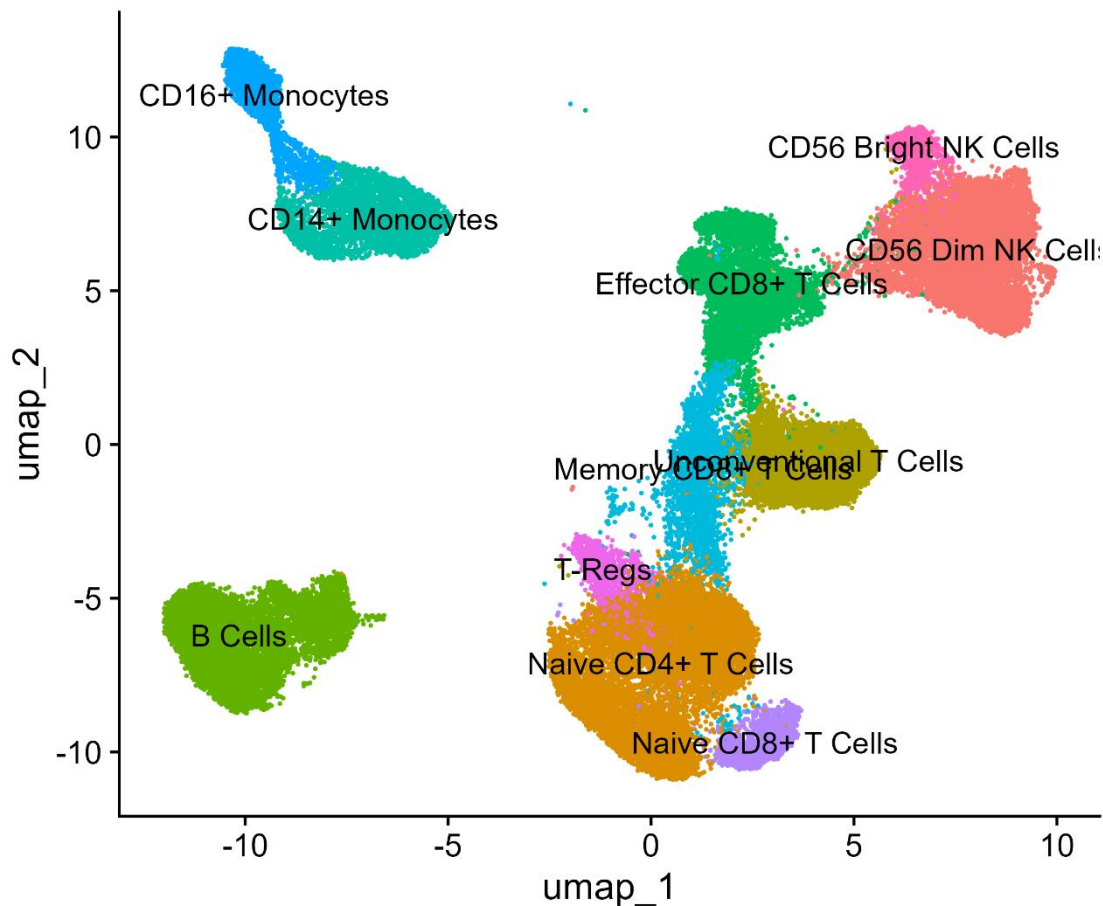


Figure 5.4 – Two-dimensional UMAP projection of the integrated single-cell RNA-seq datasets comprising A20 haploinsufficiency, healthy control (pbmc3k), and human weight loss PBMCs. Cells are coloured and annotated by major immune cell type, including CD14⁺ and CD16⁺ monocytes, B cells, naïve and memory T cells, regulatory T cells (Tregs), unconventional T cells, effector CD8⁺ T cells, and CD56^{bright} and CD56^{dim} NK cell subsets. The integration shows well-defined clustering of canonical immune populations across datasets, providing the foundation for subsequent comparative analyses of transcriptional states in HA20, obesity, and weight loss.

5.2.1 - A Global View of the A20 Haploinsufficiency Transcriptome

Initially, to get a general understanding of what changes occur in HA20 versus healthy controls, differential expression was performed between both conditions as shown in Figure 5.5. As expected *TNFAIP3* is highly significantly downregulated in HA20 versus healthy individuals. Interestingly, several genes downregulated in HA20 compared with healthy controls (*CXCR4*, *DUSP1/2*, *CD69*, *ZFP36*, *ARL4C*) overlapped with those also suppressed in obesity relative to weight loss, suggesting convergence of transcriptional programs between HA20 and obesity, this will be explored in detail later in the chapter. In addition to this several key metabolic genes were upregulated in healthy individuals versus HA20 (*MT-*

CO1, *MT-ND6*, *MT-CO1*) along with glycolytic genes *GAPDH* and *ALDOA*, suggestive of altered glycolysis and oxphos metabolism in HA20. Cytotoxic marker *GZMK*, this has been implicated as a marker of exhausted cell phenotype but seems to be very much associated with healthy phenotype in this scenario.

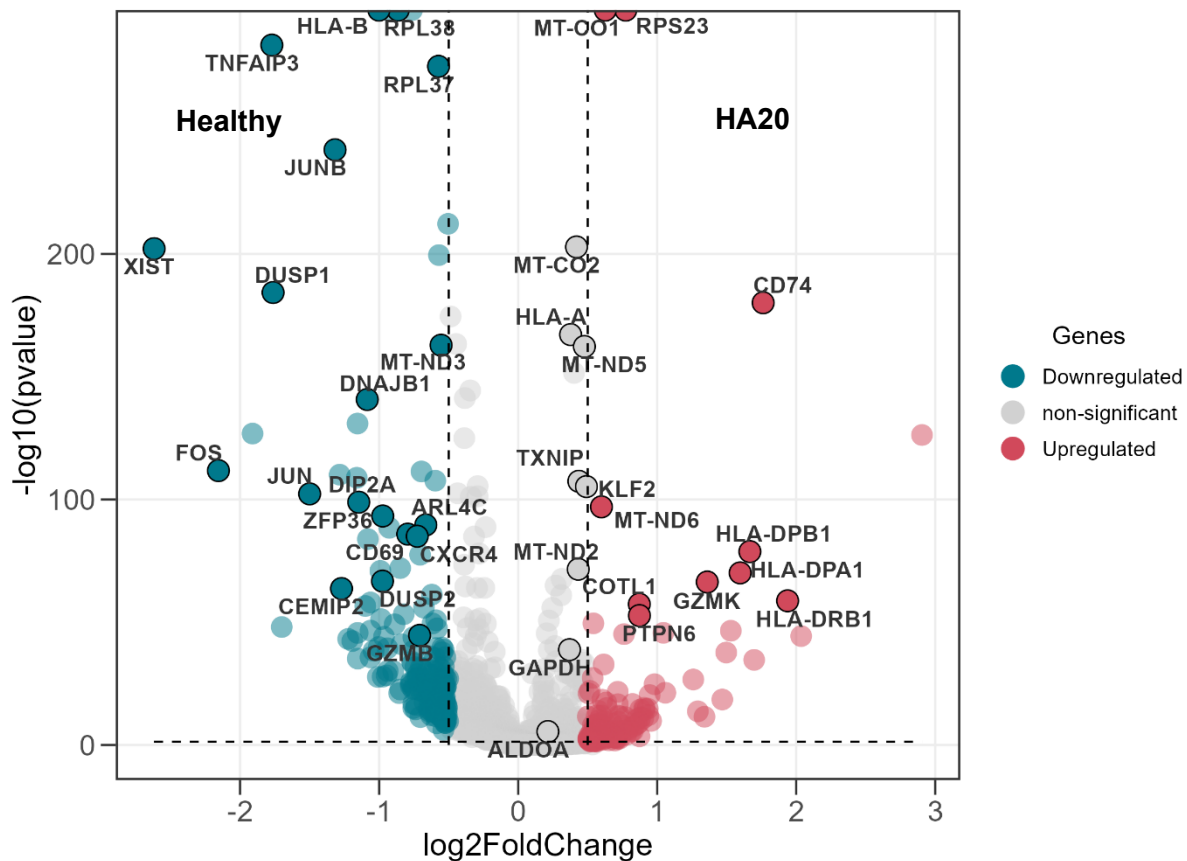


Figure 5.5 – Volcano plot of PBMCs from individuals with HA20 versus healthy controls. Each point represents a gene, with $\log_2$ fold change on the x-axis and $-\log_{10}$ adjusted p-value on the y-axis. Significantly upregulated genes are shown in red, downregulated genes in blue, and non-significant genes in grey. Cutoff threshold at 0.5 to capture moderately but significantly changed genes which still carry biological meaning

As described previously and established in literature, NF- κ B signalling and activity is also regulated at a post transcriptional level.¹⁵⁵ To confirm this in a different disease setting, HA20, NF- κ B genes were isolated from a Gene Ontology database and mapped onto the differential expression volcano plot, as shown in Figure 5.6. The only significantly altered NF- κ B gene that is altered in HA20 is *TNFAIP3* whilst *NFKBIZ* is also trending down. *BIRC3* was up in HA20 versus healthy. *NFKBIZ* operates in the nucleus as a co-regulator of NF- κ B signalling whilst *BIRC3*, like A20 has E3 ubiquitin ligase that prevents apoptosis, this could be a compensatory mechanism for the loss of *TNFAIP3*.

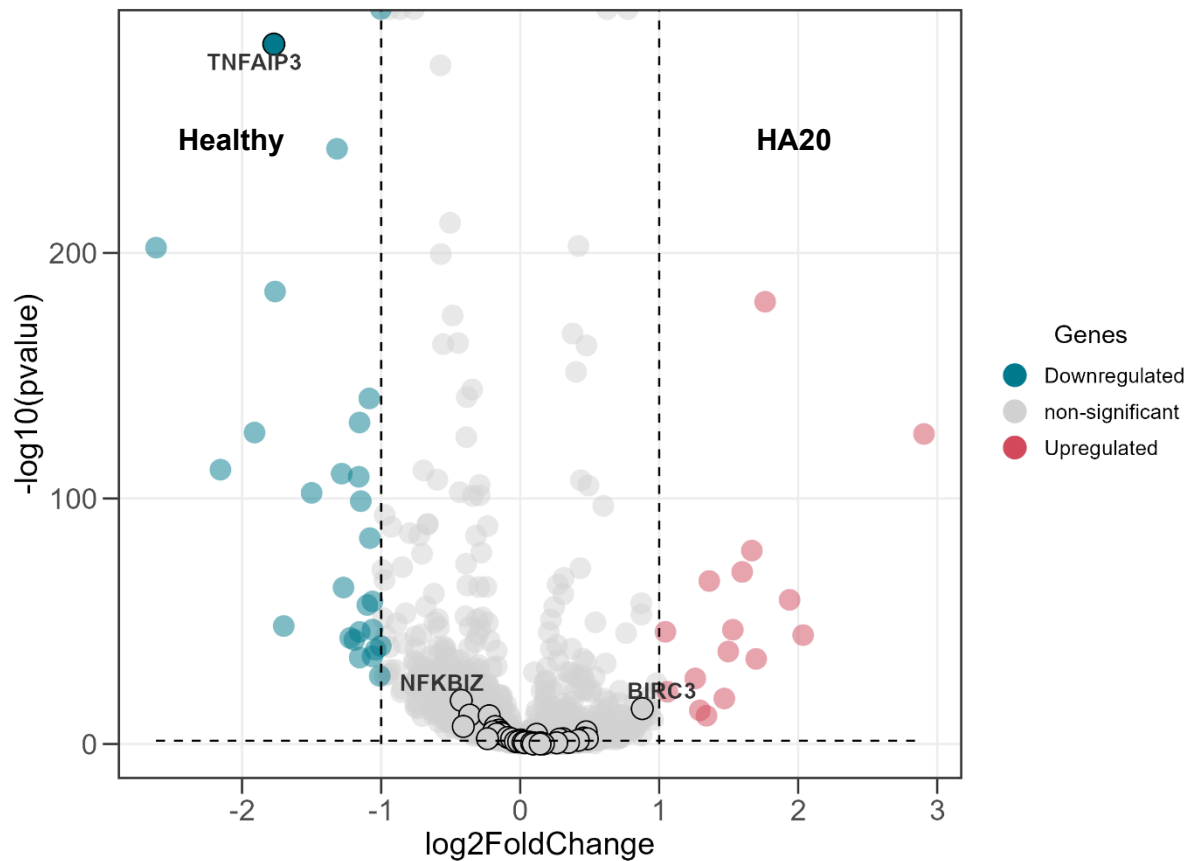


Figure 5.6 – Volcano plot showing differential expression of NF- κ B pathway-associated genes in PBMCs from individuals with A20 haploinsufficiency (HA20) compared with healthy controls. Each point represents a gene, with $\log_2$ fold change on the x-axis and $-\log_{10}$ adjusted p-value on the y-axis. Downregulated genes are shown in blue, upregulated in red, and non-significant genes in grey. *TNFAIP3* is significantly downregulated in HA20, consistent with haploinsufficiency, while other NF- κ B-related genes show more subtle alterations – cutoff threshold at 1 to highlight the extent of *TNFAIP3*’s $\log_2$ fold change

Pathway enrichment analysis of genes upregulated in HA20 compared with healthy controls identified several immune-related pathways (Figure 5.7). The most significantly enriched term was antigen processing and presentation, indicating heightened activity of major histocompatibility complex (MHC)-mediated antigen display. Other enriched pathways included Leishmaniasis, Epstein–Barr virus infection, and phagosome, all of which are related to host–pathogen interactions and innate immune activation. In addition, multiple adaptive immune pathways were upregulated, including hematopoietic cell lineage, allograft rejection, graft-versus-host disease, type I diabetes mellitus, rheumatoid arthritis, and autoimmune thyroid disease. Collectively, these findings demonstrate that HA20 is associated with transcriptional upregulation of pathways linked to antigen presentation, infection response, and autoimmune processes. This is in keeping with what would be

expected and with GWAS studies where SNPs in *TNFAIP3* have been associated with autoimmune diseases.

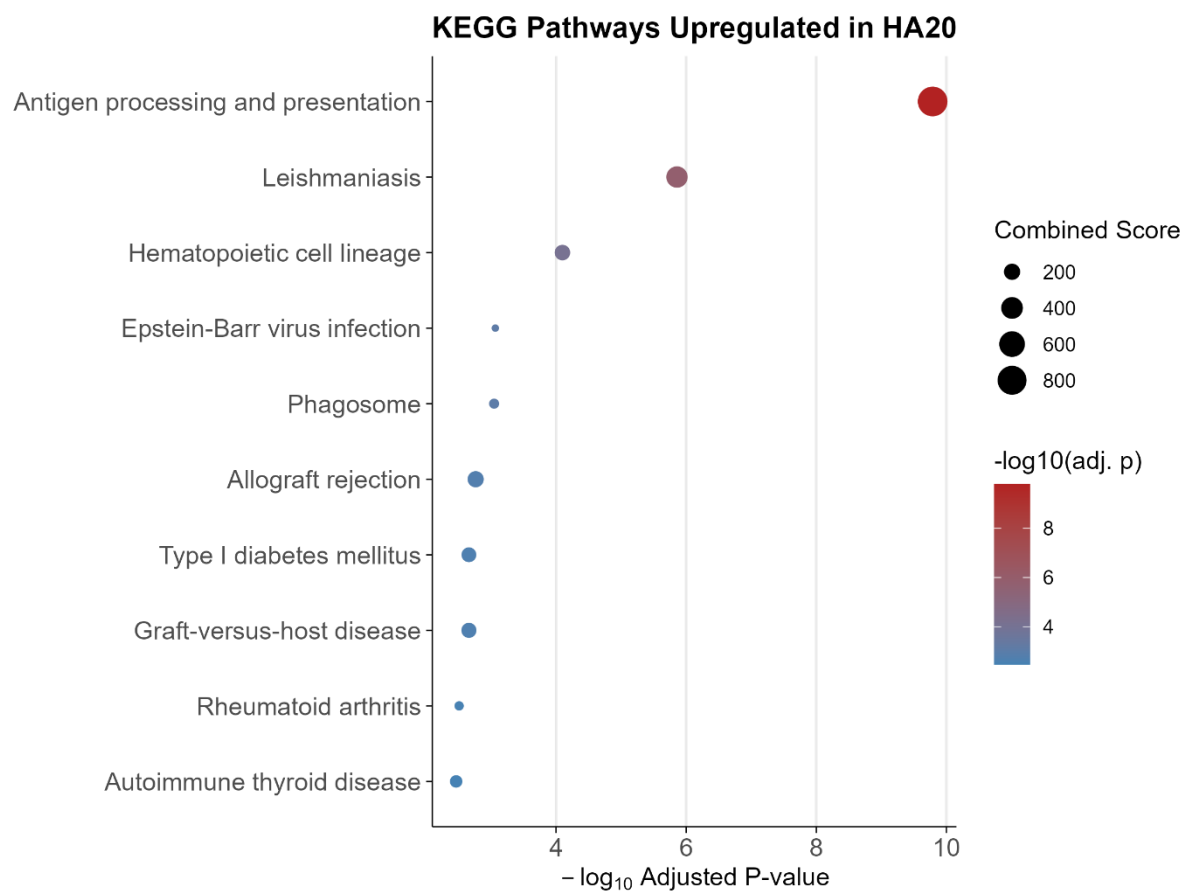


Figure 5.7 – KEGG pathway enrichment analysis of genes upregulated in PBMCs from individuals with A20 haploinsufficiency (HA20) compared with healthy controls. Dot size represents the number of genes contributing to each pathway, and colour indicates adjusted p-value significance.

Pathway enrichment analysis of genes downregulated in HA20 compared with healthy controls revealed suppression of several fundamental cellular and immune pathways as shown in Figure 5.8. The most significantly enriched downregulated pathway was the ribosome, suggesting reduced expression of genes involved in protein synthesis. Additional downregulated pathways included oxidative phosphorylation and non-alcoholic fatty liver disease, both linked to mitochondrial metabolism and energy production. Immune-related pathways were also diminished, including natural killer cell mediated cytotoxicity, the T cell receptor signalling pathway, and the TNF signalling pathway, pointing to impaired cytotoxic and pro-inflammatory signalling responses. Finally, pathways related to apoptosis and diabetic cardiomyopathy were also reduced. Collectively, these findings indicate that HA20

is associated with transcriptional downregulation of protein synthesis, mitochondrial metabolism, and key cytotoxic and inflammatory immune pathways.

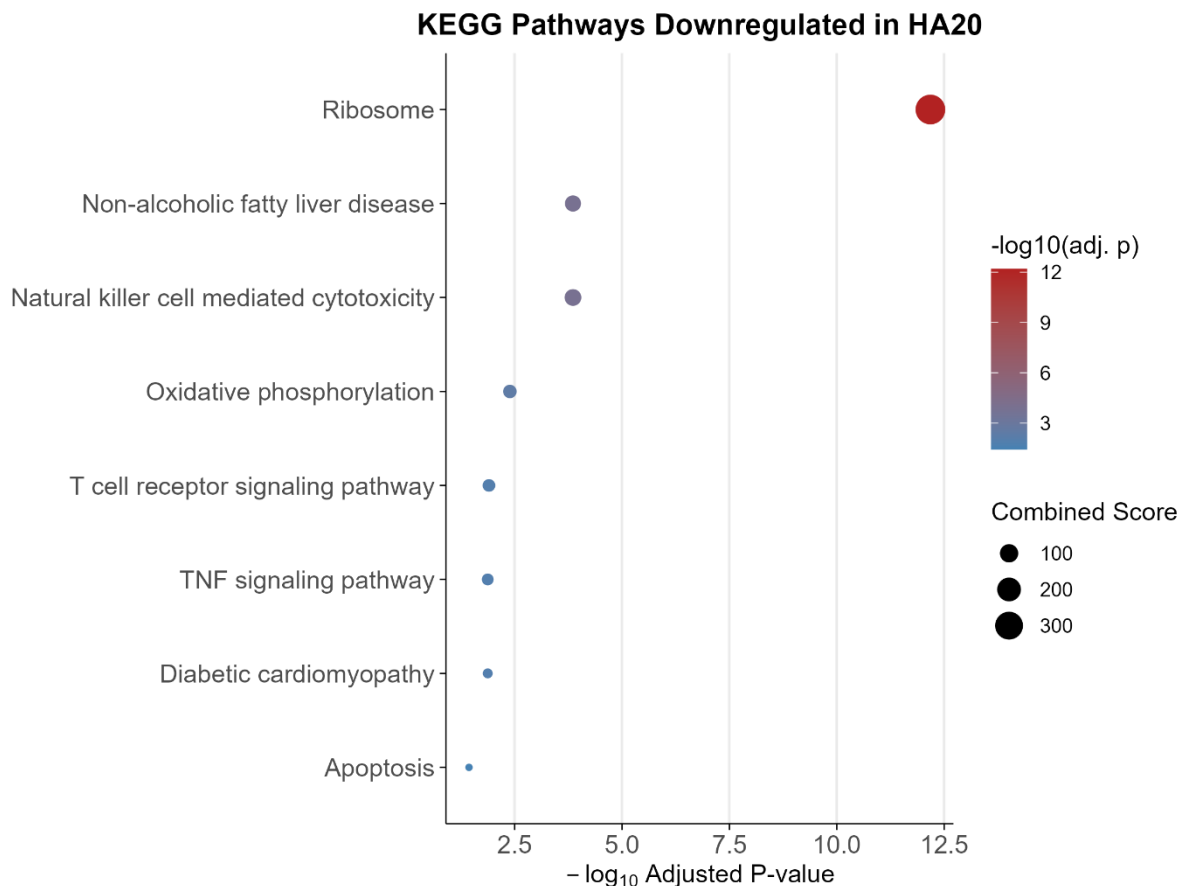


Figure 5.8 – KEGG pathway enrichment analysis of genes downregulated in PBMCs from individuals with A20 haploinsufficiency (HA20) compared with healthy controls. Dot size represents the number of genes contributing to each pathway, and colour indicates adjusted p-value significance.

As pathway analysis is biased towards pre-defined pathways, I also used STRING analysis to get further insight into genes upregulated and downregulated genes in HA20 and potential new regulatory mechanisms underpinning HA20.

To create gene signatures for genes either upregulated or downregulated and up in HA20, differential expression between HA20 and Healthy individuals was performed in every cell type. If a gene is upregulated in two or more cell types it was included in the String analysis to build a network of genes upregulated in HA20. Conversely to this, genes that were downregulated in at least two cell types were utilised to build a downregulated gene network in HA20.

A protein–protein interaction network was generated from genes upregulated in at least two immune cell types in HA20 using STRING (Figure 5.9). The network revealed several highly connected hubs. A cluster of mitochondrial-encoded genes (*MT-CO1*, *MT-CO2*, *MT-CO3*, *MT-ND2*, *MT-ND4*, *MT-ND5*, *MT-ND6*, *MT-ATP6*) formed a dense module, consistent with coordinated upregulation of mitochondrial respiratory chain components. This is interesting as 4 of these are Complex I genes, 3 are complex IV genes whilst *MT-ATP6* is a complex V gene. This is at odds with another mitochondrially encoded gene, *MT-ND3*, which is downregulated in HA20, suggestive of opposing mitochondrial alterations in HA20. A second major cluster was composed of ribosomal proteins (*RPS18*, *RPS19*, *RPS23*, *RPS5*, *RPLP0*, *RPLP1*), suggesting enhanced translational activity. Other well-connected nodes included antigen presentation genes (*HLA-A*, *HLA-E*, *HLA-DRA*, *B2M*, *CD74*), immune regulators (*STAT1*, *SOCS3*, *CISH*), and cytotoxic effector molecules (*GZMK*, *CTSW*, *GNLY*). Central bridging proteins such as *GAPDH* and *SELL* connected multiple modules. Together, these findings indicate that HA20 is characterised by coordinated upregulation of mitochondrial and ribosomal genes alongside immune activation modules, reflecting both metabolic and inflammatory reprogramming.

STRING PPI Network Around GAPDH

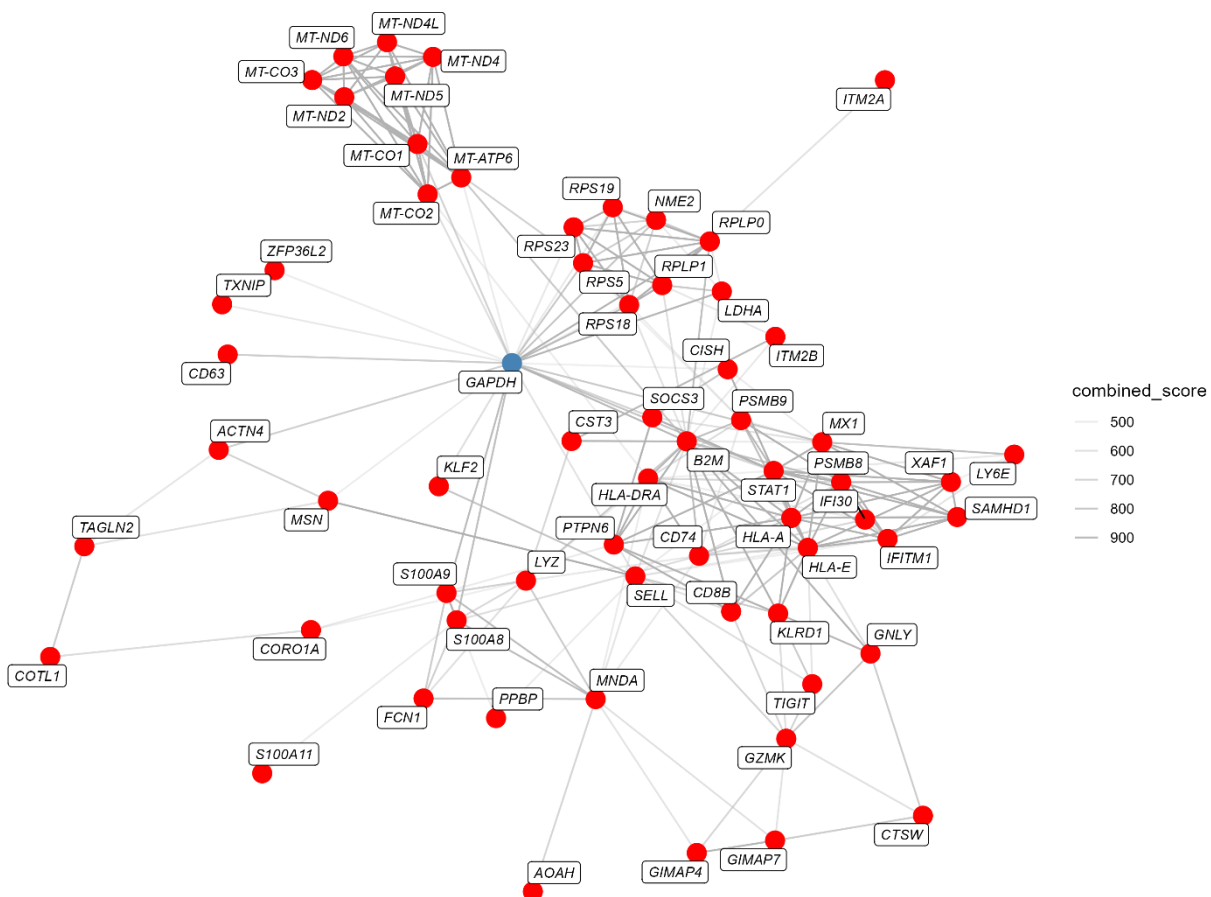


Figure 5.9 – Protein–protein interaction network generated using STRING from genes upregulated in at least two immune cell types in A20 haploinsufficiency (HA20) compared with healthy controls. Nodes represent individual genes, and edges represent known or predicted protein–protein interactions. The network reveals coordinated upregulation of mitochondrial, ribosomal, and immune-related modules, reflecting transcriptional reprogramming in HA20

The string network in Figure 5.9 was calculated off the basis of whether these genes were at least upregulated in two cell types in HA20, this could lead to limitations where certain cell types drive the whole signature. To account for this, the genes in Figure 5.9 were used to create a module score, an accumulated gene expression score by cell type, using this the gene signature could be visualised for every single cell type and split by disease type. This was carried out to ensure that the signature was consistent across cell types and not driven by a few similar clusters. As shown in panel A of Figure 5.10, there is a consistent upregulation of this gene set in HA20, from left to right the gene expression was ordered from highest to lowest.

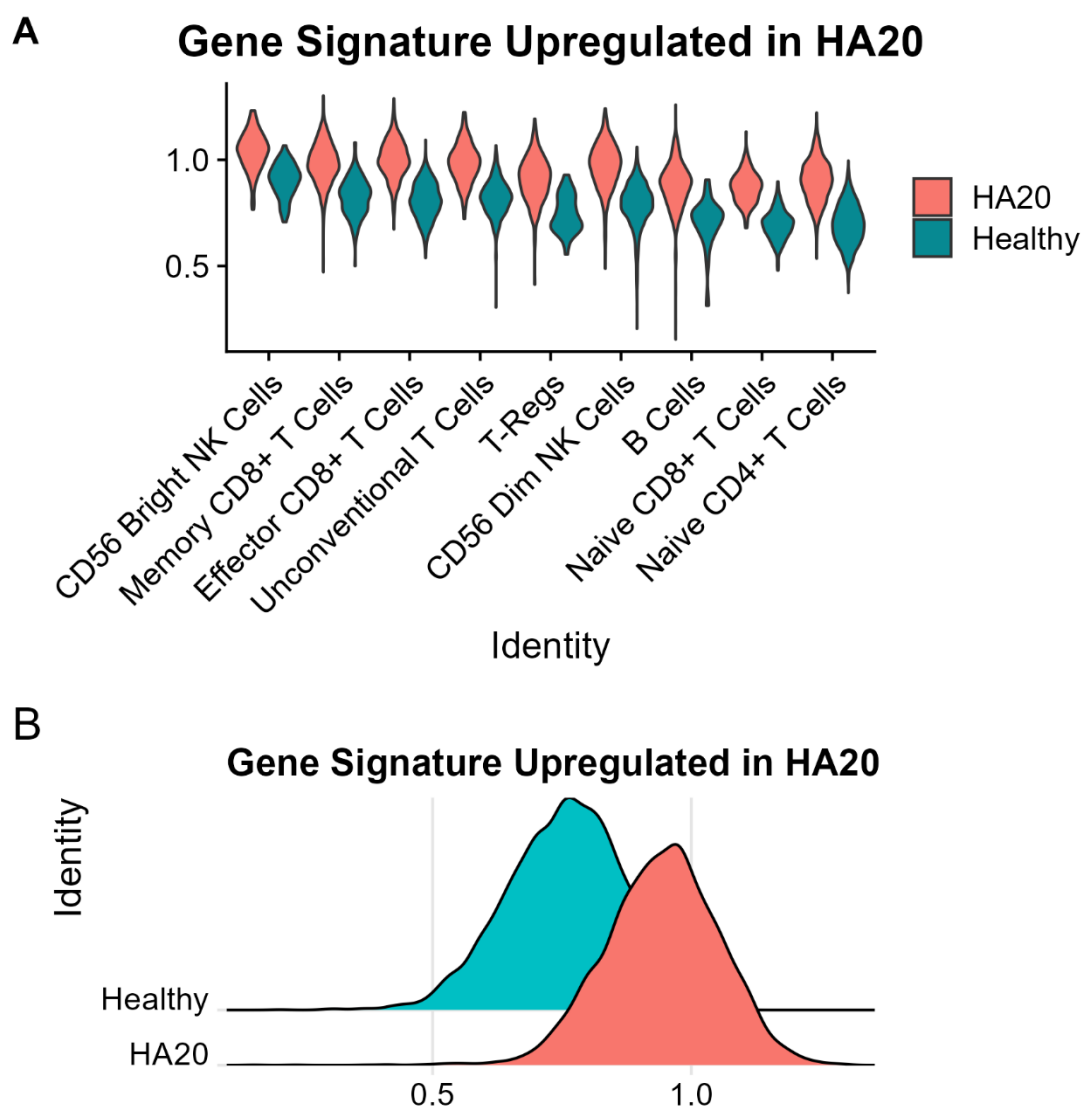


Figure 5.10 – Genes upregulated in HA20 – A) The gene signature previously calculated and shown in Figure 5.9 was used to create a module score and was then plotted by cell type and split to show healthy vs HA20 by disease status – B) All the cells in the dataset split by disease status showing a significant shift in this gene set in HA20

To further examine coordinated transcriptional changes in HA20, we constructed a STRING network of genes downregulated in at least two immune cell types in HA20, Figure 5.11. The network was highly interconnected, with central hubs formed by AP-1 transcription factors (*JUN*, *FOS*, *JUNB*, *JUND*) and immediate early response regulators (*DUSP1*, *DUSP2*, *ZFP36*, *BTG2*, *IER2*), reflecting broad suppression of stimulus-responsive signalling modules in HA20. Surrounding this hub, clusters of immune receptors and signalling genes were observed, including *CD247*, *CD69*, *CXCR4*, *ITGAM*, *ITGB1*, *CD5*, and KIR family genes, indicating reduced expression of pathways associated with T cell and

Genes downregulated in HA20 in the string network in Figure 5.11 was calculated off the basis of whether these genes were at least downregulated in two cell types in HA20, this could lead to limitations where certain cell types drive the whole signature. To account for this, the genes in the network in Figure 5.11 were used to create a module score, an accumulated gene expression score by cell type, using this the gene signature could be visualised for every single cell type and split by disease status (Figure 5.12). This signature was consistent across all cell types, highlighting that this gene set is reflective of the overall transcriptome in each cell type and thus, it can be concluded that the string networks in Figure 5.9 and Figure 5.11 are representative signatures of genes downregulated and upregulated in HA20 across PBMCs.

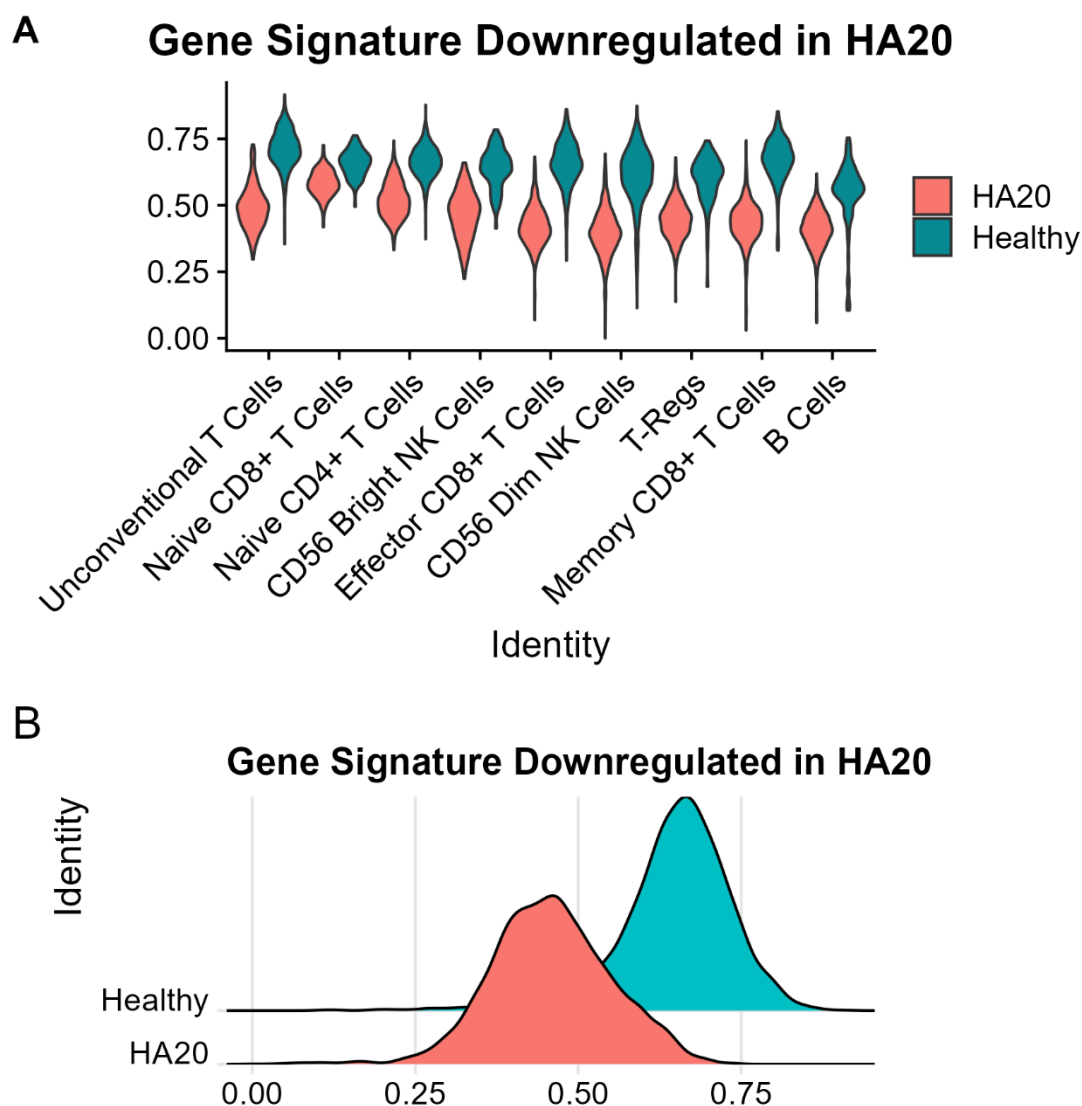


Figure 5.12 – Genes downregulated in HA20 – A) The gene signature previously calculated and shown in Figure 3.10 was used to create a module score and was then plotted by cell type and split to show healthy vs

HA20 by disease status – B) All the cells in the dataset split by disease status showing a significant downregulation of this gene set in HA20

5.2.2 - Glucocorticoid Signalling Genes

The inclusion of the glucocorticoid receptor (*NR3C1*) gene was observed in the previous network (Figure 5.11) and this gene has so far been lowly expressed across all transcriptomic dataset. Transcripts for *NR3C1* are low in this dataset again but there is a significant downregulation of *NR3C1* across the global transcriptome of the dataset, adjusted p value = 1.45×10^{-10} and $\log_2$ fold change of -0.32 highlighting a downregulation of *NR3C1* (GR) in HA20 (Figure 5.13).

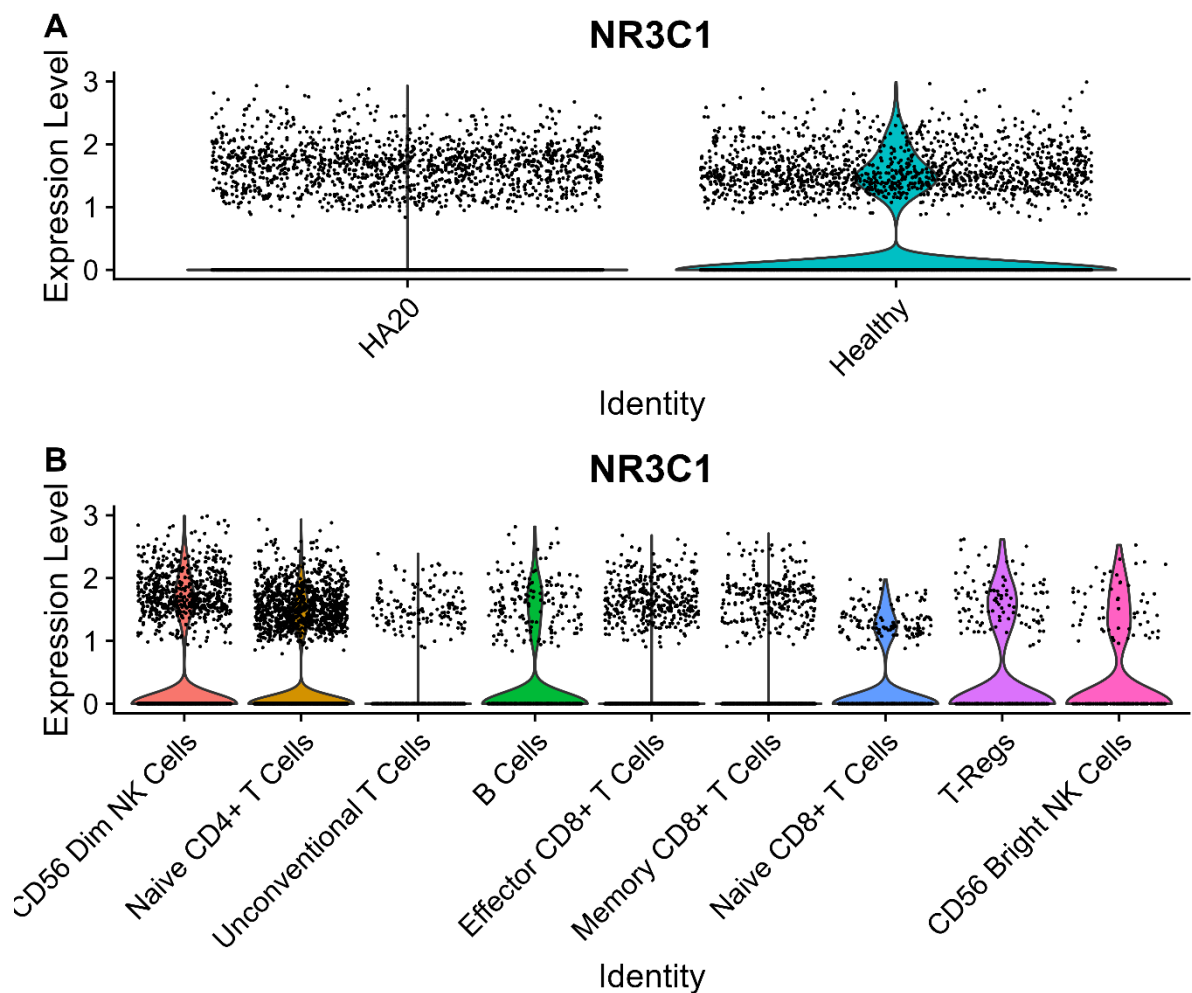


Figure 5.13 – (A) Violin plot showing *NR3C1* expression in PBMCs from individuals with A20 haploinsufficiency (HA20) compared with healthy controls. Expression of *NR3C1* is significantly reduced in HA20.

(B) Violin plots of *NR3C1* expression across annotated immune cell populations, including NK cell subsets, T

cell subsets, B cells, and regulatory T cells. Expression is consistently low across cell types in HA20 relative to healthy controls

I next aimed to elucidate if the transcriptional network of genes that was co-expressed after weight loss in humans, as shown in Figure 4.11 of Chapter 4 followed a similar transcriptional pattern to HA20. To do so, genes present in the Figure 4.11 network of genes from HD2, were labelled on the differential expression volcano plot of HA20 *versus* healthy differential expression and this analysis shows a clear loss of these genes in A20 haploinsufficiency as shown in Figure 5.14. Observing this same phenotype previously observed in obesity and weight loss, in HA20 raised some interesting questions as this transcriptional signature was noted for the first time outside of a weight loss / fasting / obese setting, this time in a disease setting created by germline LOF variants of *TNFAIP3*.

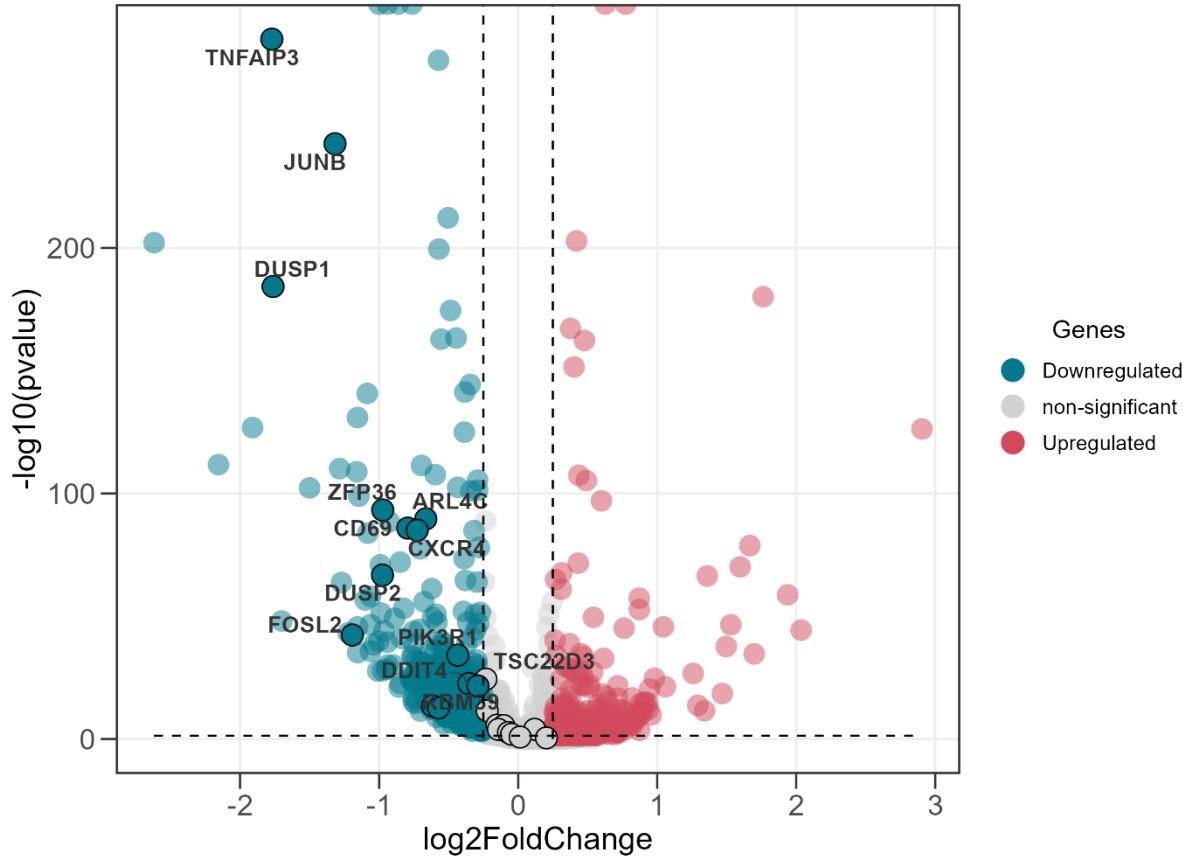


Figure 5.14 – Mapping of the hdWGCNA green network genes onto HA20 versus healthy differential expression - Volcano plot showing differential expression of PBMCs from HA20 compared with healthy controls, with genes from the green network defined in Chapter 4 (hdWGCNA) highlighted. Most of these genes, including *TNFAIP3*, are downregulated in HA20, indicating loss of this transcriptional program outside of the weight loss/fasting context and into a disease setting

Evident from the previous section was the high abundance of differentially expressed genes from the green network in Chapter 4 (Figure 4.11), and the directionality of these genes. Not

a single gene identified in the network from Figure 4.11 was upregulated in HA20, only lost in HA20 which raises questions of whether A20 plays a major role in maintaining this expression program and potentially plays a role in glucocorticoid signalling. To determine similarities between A20 haploinsufficiency and obesity differential expression was calculated for both datasets: Weight loss vs obesity differential expression was carried out and in the HA20 dataset differential expression was performed between healthy and HA20 condition using all the cells in each condition. Genes were then split into four quadrants based on differential expression across both datasets, for example, *TNFAIP3* is downregulated in HA20 and Obesity and thus is in the bottom left corner of Figure 5.15, this quadrant represents genes that are downregulated in obesity and HA20 and has a much greater number of genes than any other quadrants. Differentially expressed genes across both datasets were picked based on adjusted p value under 0.05.

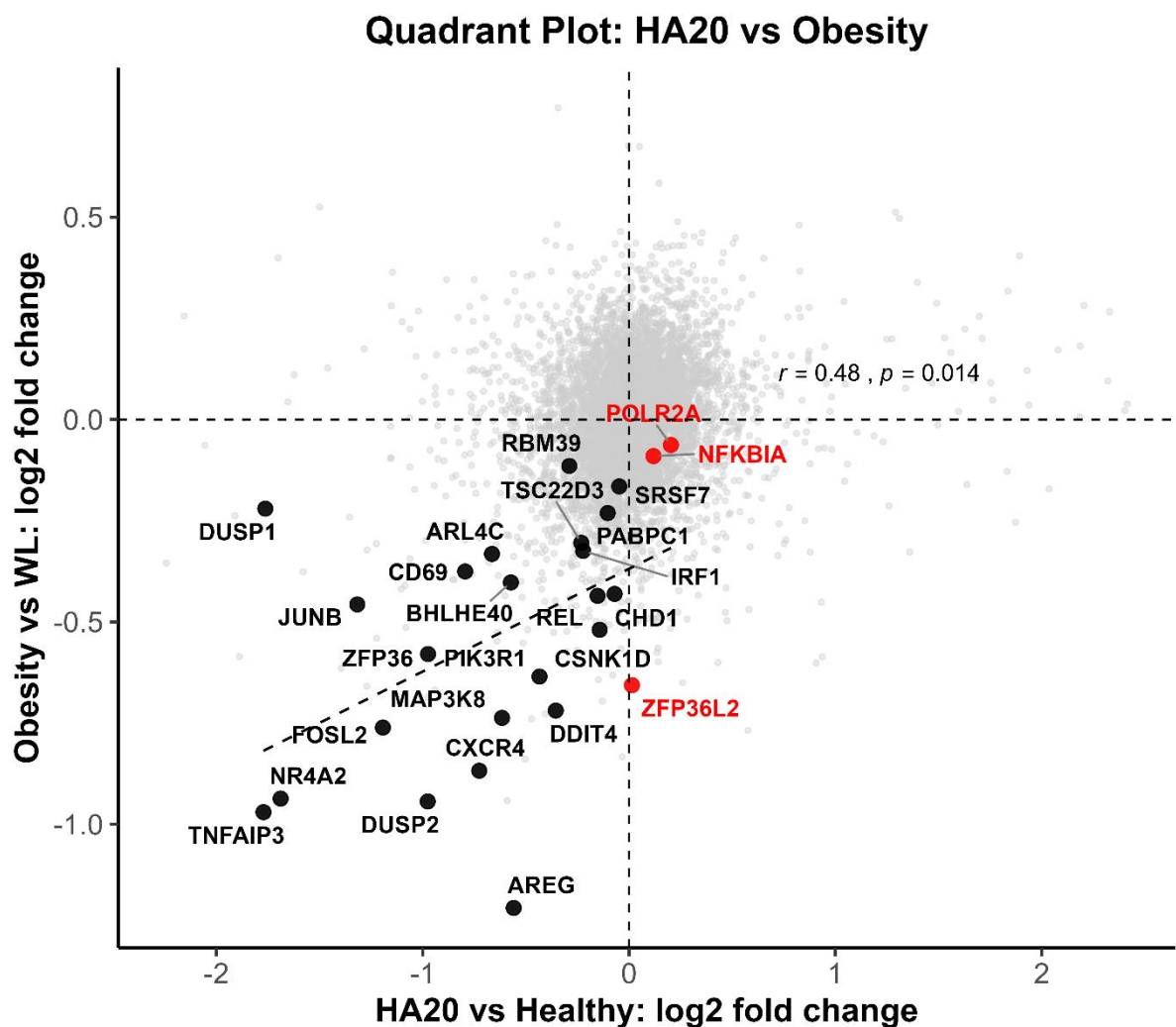


Figure 5.15 – Differential expression of green network genes in HA20 and obesity/weight loss datasets - Quadrant plot comparing differential expression of PBMCs from HA20 versus healthy controls (x-axis) with obesity versus weight loss (WL) (y-axis). Each point represents a gene, with genes from the green network defined in Chapter 4 highlighted (Figure 4.11). Genes in the lower-left quadrant, including *TNFAIP3*, are downregulated in both HA20 and obesity, indicating a shared loss of this transcriptional program. The correlation coefficient (r) and p-value indicate significant overlap between transcriptional responses in the two conditions

This network was built on NK cell co-expression and PPI in Chapter 4 from the HD2 dataset, however, conservation of gene responses had never been properly explored across other cell types in that chapter. Apparent from Figure 5.16 is that this response is observed across the global transcriptome in both datasets. To further examine this response at cell level, density plots were created to see which cell types had the greatest GR module scores (Figure 5.16).

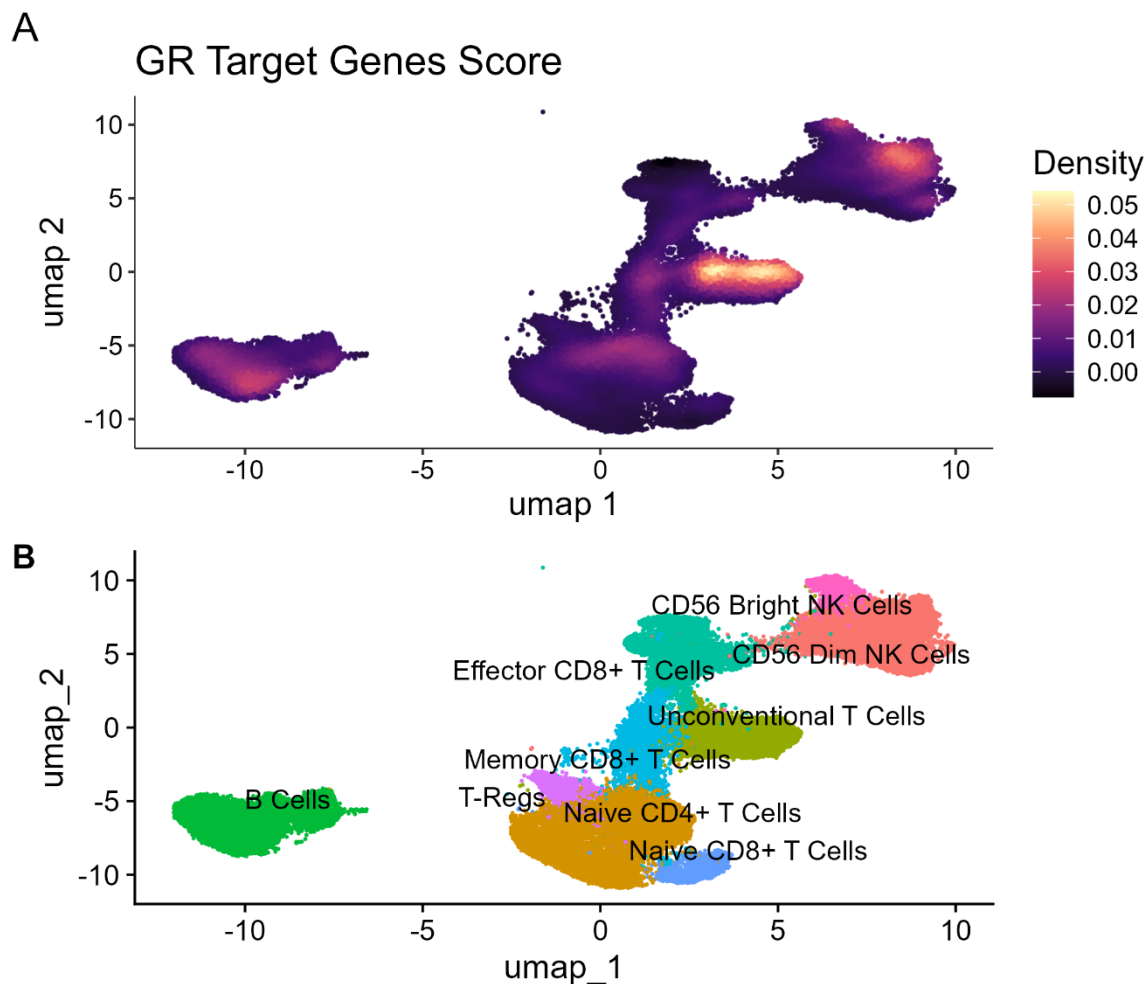


Figure 5.16 – GR module scores mapped onto the integrated UMAP. (A) UMAP visualisation of PBMCs showing the distribution of GR module scores, calculated from the glucocorticoid receptor (GR) target gene network defined in Chapter 4. Colour scale represents density of GR

module expression across the transcriptome.
(B) UMAP of the same dataset annotated by immune cell type, including NK cell subsets, T cell subsets, B cells, and regulatory T cells, providing context for cell type–specific variation in GR module scores.

Modules scores were plotted by condition per cell type for each dataset and a clear phenotype was observed across both datasets. In both the weight loss / obesity dataset and the HA20 dataset, there is a notably higher expression in weight loss and healthy conditions as shown in Figure 5.17. This reflects a conserved module of transcriptional regulation.

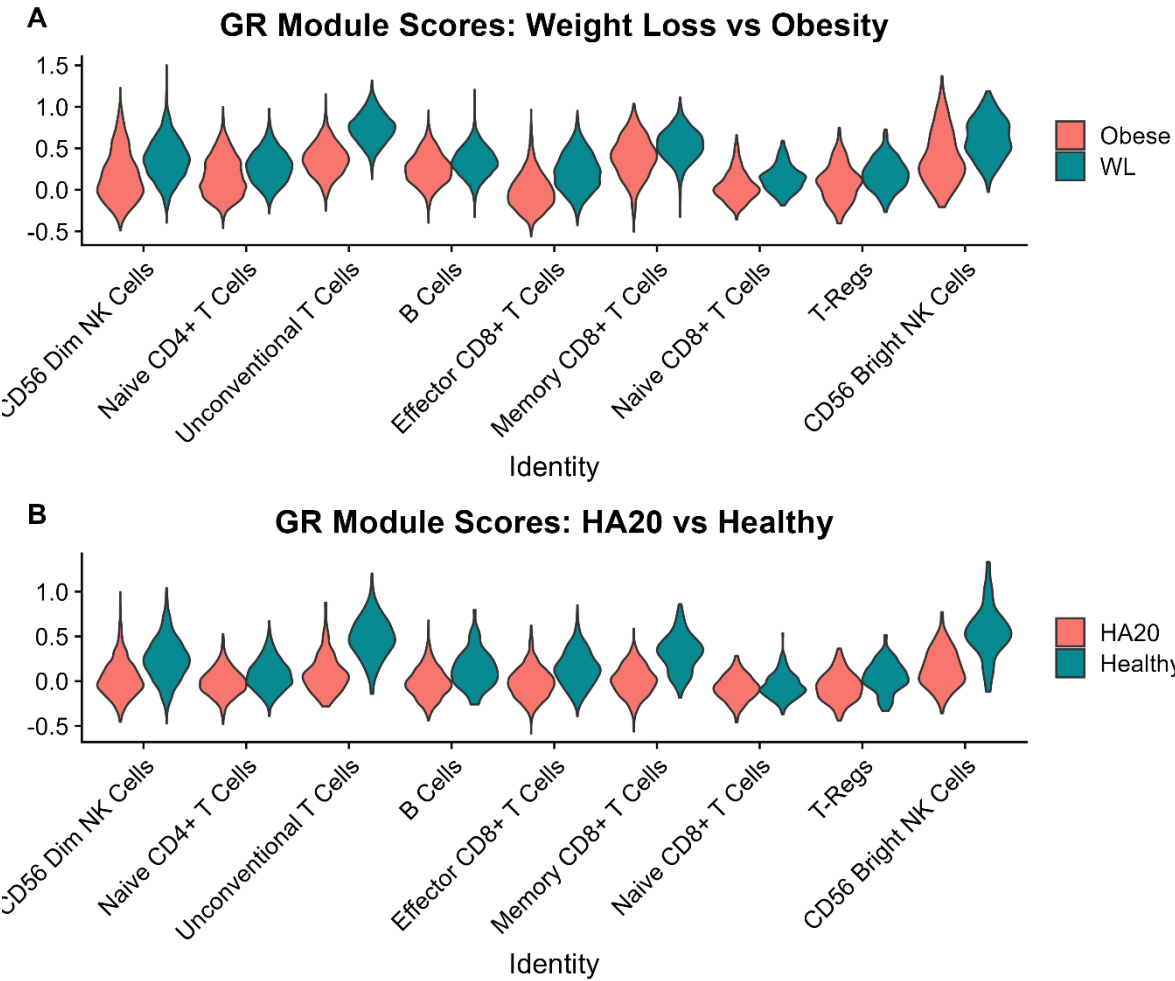


Figure 5.17 – GR Module scores computed from the gene network imputed in chapter 4 (Figure 4.11) in dataset HD2 A) Weight Loss Dataset, GR Module Scores Split Across Cell Types Comparing Pre and Post-Weight Loss B) HA20 Dataset, GR Module Scores Split Across Cell Types Comparing HA20 vs Healthy

5.3.3 Metabolic gene alterations in HA20 and Obesity

Alterations in metabolic genes is expected in conditions like obesity and weight loss / fasting where significant alterations to nutrient profiles occur, driving adaption of cells to match the environment. However, in the HA20 dataset there are genes downregulated in HA20 matching that of the obesity dataset suggesting changes driven by inflammation and not the nutrient / metabolite profiles. For example, *SLC2A3* (GLUT3) is significantly downregulated in both HA20 and the weight loss dataset. Another consistently altered metabolic gene is *PIK3R1*, *PIK3R1* is downregulated in obesity and HA20. In the proteomics data generated from CD8⁺ T cells and NK cells from lean and obese mice, *PIK3R1* and GLUT3 were both trending down in both cell subsets, *PIK3R1* is significantly down in NK cells in obesity whilst GLUT3 is almost significantly down in CD8⁺ T Cells. These genes are crucial for the immunometabolism of immune cells and the nutrients that can be utilised by the cell required for their effector functions (Figure 5.18).

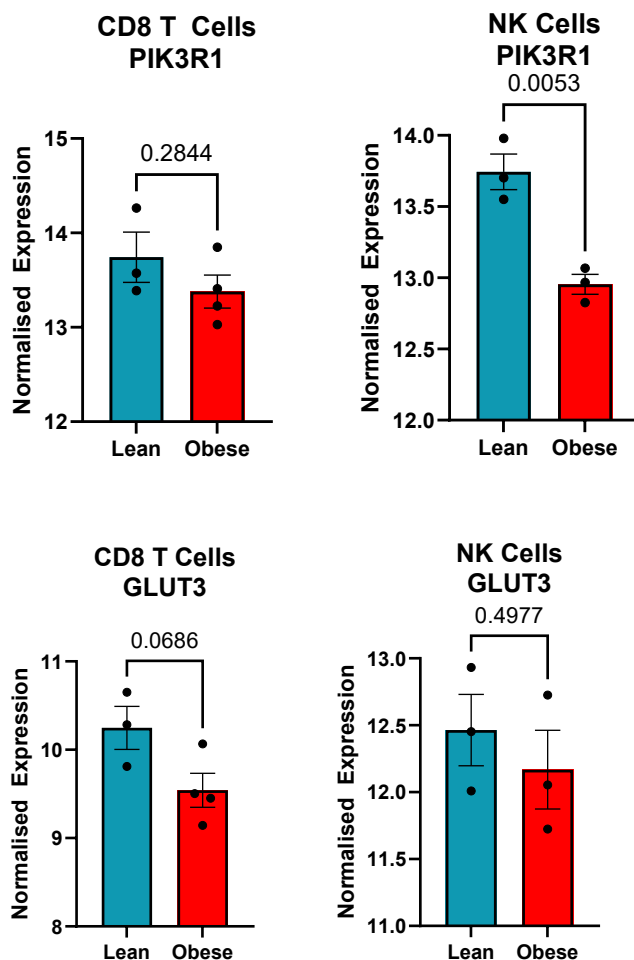


Figure 5.18 – Bar plots showing normalised protein expression of PIK3R1 (top) and SLC2A3/GLUT3 (bottom) in CD8⁺ T cells (left) and NK cells (right) from lean and obese mice. Each point represents an individual sample, with error bars indicating mean $\pm$ SEM.

To directly compare transcriptional changes in HA20 and obesity, a quadrant analysis was performed, here the $\log_2$ fold change in HA20 versus healthy controls (x-axis) is plotted against the $\log_2$ fold change in obesity versus weight loss (y-axis), shown in Figure 5.19 and only significant genes were plotted (adjusted p-value < 0.05).

This revealed consistent downregulation of several metabolic regulators in both conditions. *HIF1A*, a master transcription factor driving glycolysis and cellular adaptation to hypoxia, was suppressed, indicating impaired metabolic flexibility under both *TNFAIP3* haploinsufficiency and obesity. Similarly, *SNRK*, an AMPK-related kinase involved in energy sensing and stress adaptation, was reduced, pointing to a broader dysregulation of nutrient-sensing pathways. Downregulation was also observed for *NFE2L2* (NRF2), a central regulator of antioxidant defence and redox metabolism, suggesting a shared weakening of oxidative stress responses. Together, these alterations highlight convergence between HA20 and obesity on key regulators of glucose metabolism, mitochondrial adaptation, and redox balance. These data suggest that both HA20 and obesity drive a metabolic transcriptional state characterised by reduced hypoxia signalling, energy sensing, and antioxidant capacity, potentially limiting immune cell adaptability under stress.

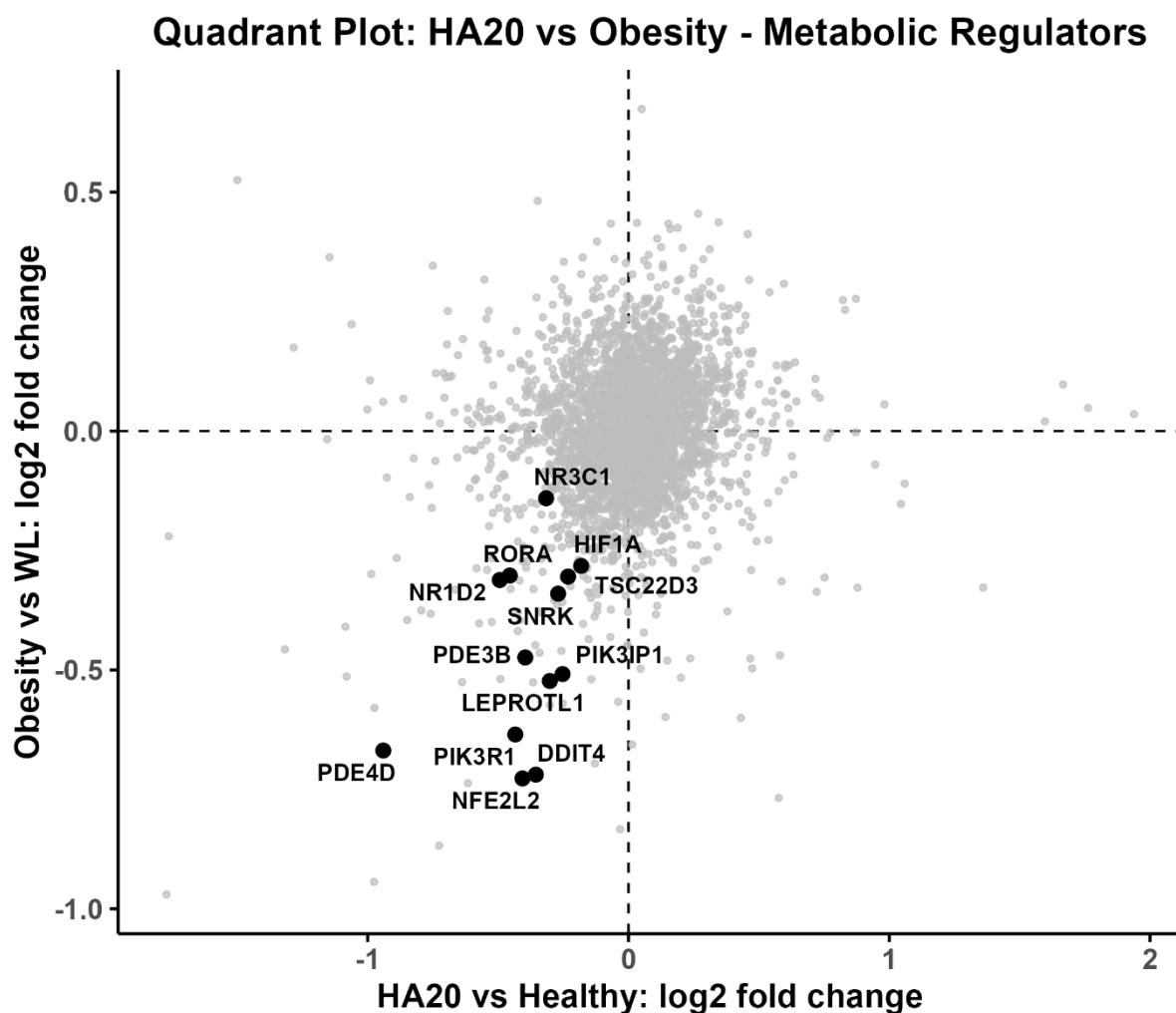


Figure 5.19 – Quadrant plot comparing differential expression in HA20 and obesity. Quadrant plot showing $\log_2$ fold change in HA20 versus healthy controls (x-axis) plotted against $\log_2$ fold change in obesity versus weight loss (y-axis). Each point represents a gene, with labelled examples indicating genes of interest.

Quadrant analysis revealed a clear overlap between HA20 and obesity, with consistent downregulation of multiple genes central to cellular metabolism, Figure 5.19. Several nutrient transporters were suppressed across both conditions, as stated previously, *SLC2A3* (GLUT3), but also *SLC38A1*, which mediates glutamine import and coordinates amino acid–glucose crosstalk through mTOR signalling. Their reduction suggests impaired substrate availability for both glycolysis and biosynthetic pathways. Key mitochondrial regulators were also consistently decreased. *CYCS* (cytochrome c) and *ISCA1*, which are essential for oxidative phosphorylation and electron transport chain integrity, were down, alongside *COQ10B*, required for coenzyme Q10 biosynthesis. *TOMM20*, a mitochondrial import receptor and marker of mitochondrial mass, was also diminished, together indicating a

collapse in mitochondrial functional capacity. Similarly, *MTERF4*, a regulator of mitochondrial transcription, was suppressed, reinforcing the impression of impaired OXPHOS and mitochondrial adaptation in HA20 and obesity.

Genes associated with lipid and cholesterol metabolism were also consistently downregulated. *IDII* (Figure 5.20), a key enzyme in cholesterol biosynthesis via the mevalonate pathway, and *ATP8A1*, a phospholipid flippase, point toward defective lipid homeostasis. *RAB18*, a lipid droplet-associated GTPase implicated in obesity and lipodystrophy, was also reduced, suggesting impaired lipid storage and trafficking.

Finally, regulators of cellular stress and redox balance were affected. *SORL1*, involved in endosomal trafficking and linked to insulin resistance, was suppressed, while *SELENOM*, an antioxidant selenoprotein, and *OXNADI*, a poorly characterised redox enzyme, were both downregulated, indicating a compromised oxidative stress response.

Altogether, whilst these are just a few genes, their shared downregulation across inflammatory conditions demonstrates that both HA20 and obesity converge on a transcriptional program marked by diminished glucose uptake, mitochondrial function, lipid handling, and antioxidant defence. Whilst HA20 is generally considered an inflammatory disorder from loss of A20, the shared metabolic repression suggests that immune dysfunction in HA20 and obesity is not only a biomarker of metabolic disease but can be driven by inflammatory disease also in the absence of any metabolic disease.

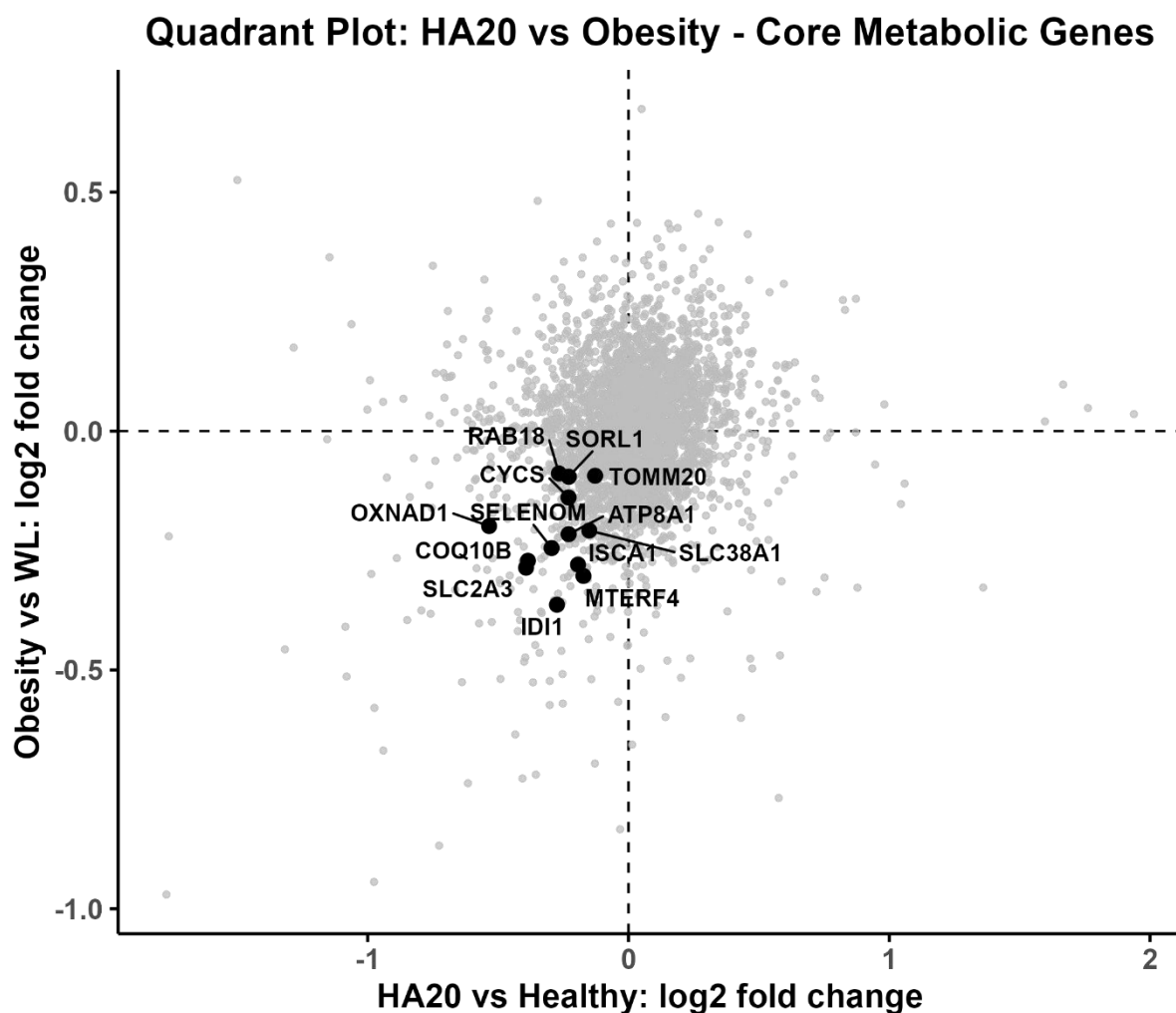


Figure 5.20 - Quadrant plot comparing HA20 and obesity transcriptional changes. Quadrant plot displaying log₂ fold change in HA20 versus healthy controls (x-axis) against log₂ fold change in obesity versus weight loss (y-axis). Each point represents a gene, with selected metabolic regulators labelled for reference.

The discordant quadrants in Figure 5.21 reveal that obesity and A20 haploinsufficiency, while converging on reduced *TNFAIP3* and impaired inflammatory braking, diverge with respect to some genes. Obesity preferentially engages stress-responsive cytotoxic effector programmes, whereas HA20 retains or induces pathways involved in immune trafficking and NF- κ B feedback control. These differences likely reflect distinct chromatin and cofactor environments shaped by metabolic stress versus genetic perturbation, reinforcing the concept that obesity represents a context-dependent failure of inflammatory resolution rather than a simple phenocopy of monogenic immune dysregulation.

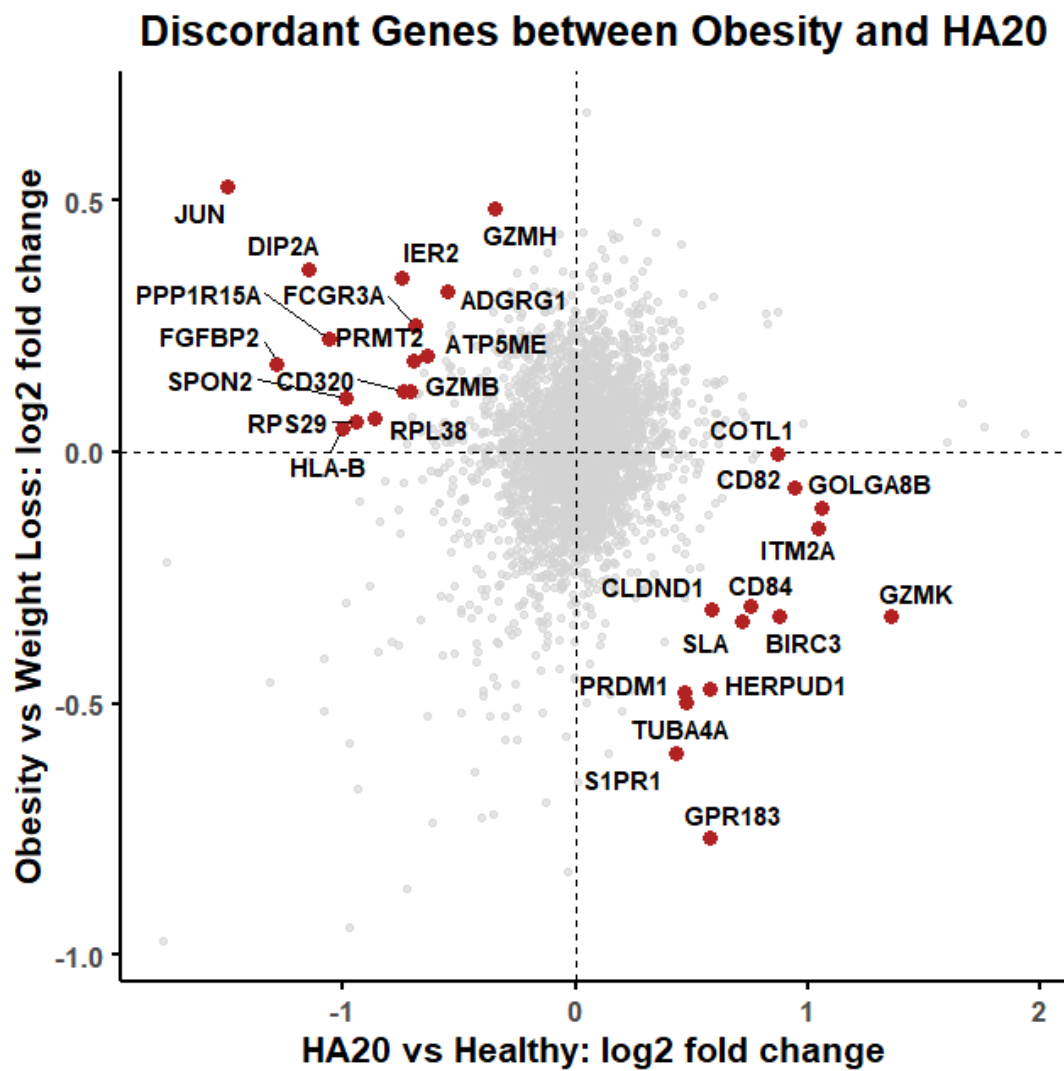


Figure 5.21 - Quadrant plot comparing HA20 and obesity transcriptional changes. Quadrant plot displaying log₂ fold change in HA20 versus healthy controls (x-axis) against log₂ fold change in obesity versus weight loss (y-axis). Each point represents a gene, discordant genes labelled for reference.

5.3.0 - Chapter 5 Discussion

Multiple chapters have culminated on the identification of a transcriptional signature driven by very different disease mechanisms namely obesity and HA20. This transcriptional signature is characterised by expression of key anti-inflammatory genes necessary for immune homeostasis with *TNFAIP3* being a central gene throughout this work. This signature is observed throughout this thesis is downregulated in obesity, and can be induced by fasting and weight loss independently of obesity. In this chapter, I show that individuals with A20 haploinsufficiency also have the same transcriptional signature. This signature occurs in A20 haploinsufficiency despite no changes to metabolic state suggestive that A20 itself or severe inflammation driven by cytokines like TNF, could play a crucial role in the manifestation of the phenotype observed in obesity and in A20 haploinsufficiency. A20 haploinsufficiency is driven by monogenic A20 failure whilst obesity is an environmentally driven phenotype culminating in loss of key genes for immune homeostasis. This observed phenotype is characterised by high expression of anti-inflammatory GR target genes such as *TNFAIP3*, *NFKBIA*, *TSC22D3* and *ZFP36* which were previously described in chapter 2. Based on the evidence presented in this chapter, one could argue that through the integration of scRNA-seq from HA20 PBMCs, a healthy control cohort, and the human obesity/weight loss dataset, and then applying matched differential analyses, that obesity at least partially phenocopies HA20 transcriptionally. The overlap is specific, across the whole transcriptome in each dataset, and concentrates on two mechanistically interpretable axes. Whilst the integration showed overlaps across shared cell types, the differential expression was performed within datasets meaning no technical effects were introduced into these analyses which form the basis of these findings. Crucially, this means that the findings observed are robust and independent from any potential technical effects.

To elaborate on these findings in more detail, firstly, glucocorticoid signalling is uniformly blunted in HA20, the same as in obesity. Across the global PBMC transcriptome, HA20 exhibits reduced *NR3C1* (GR) and diminished expression of canonical GR targets such as *TSC22D3*, *CXCR4*, *PIK3R1*, and *CD69*. The obesity dataset recapitulates the same direction of effect, whereas weight loss moves these signatures back toward the healthy state, which can also be induced with fasting independent of obesity (at least in NK cells) as shown in chapter 2. This is consistent with the idea that persistent inflammation constrains GR occupancy and inducibility through GR–NF- κ B/AP-1 crosstalk.⁸⁶ In HA20, loss of A20 prevents proper termination of NF- κ B activity and chronic inflammation characterised by systemic increases in cytokines such as TNF, IL17A and IL-6.⁴⁰ In obesity, chronic

metainflammation arises from a variety of factors culminating in the increase of cytokines like TNF. These mechanisms converge on the same transcriptional profile despite very different root causes.

Another shared transcriptional signature across A20 haploinsufficiency and obesity are changes in metabolic changes. Whilst this signature is less pronounced and likely secondary in overall importance to the altered GR transcriptional profile, immunometabolism is fundamental for immune cell responses.¹⁰⁹ The quadrant plots comparing HA20 vs. healthy with obesity vs. weight loss show a shared downregulation of metabolic regulators that includes nutrient transporters (*SLC2A3*/*GLUT3*, *SLC38A1*) which are required for glucose and glutamine transport into the cell, mitochondrial assembly and electron transport chain components (*CYCS*, *COQ10B*, *TOMM20*, *ISCA1*, *MTERF4*), genes involved in lipid and cholesterol handling (*RAB18*, *ATP8A1*, *ID1*), and redox/trafficking regulators (*SELENOM*, *OXNAD1*, *SORL1*). These genes are interesting due to their potential effect on the effector functions of immune cells. I also considered about whether these changes could affect how glucocorticoids are synthesised. As glucocorticoids are synthesised in the mitochondria, mitochondrial health may impact the glucocorticoid response. However, the synthesis of glucocorticoids only occurs in the zona fasciculata in the adrenal cortex, so unless the observed response in immune cells is highly conserved even in somatic cells in the adrenal cortex then no further thought should be invested in the synthesis of glucocorticoids in mitochondria in this thesis. Despite, this glucocorticoids in immune cells are heavily linked to mitochondria which shouldn't be a surprise as several stress responses controlled by GR actively need increased energy expenditure and 90% of cellular ATP is generated in the mitochondria.¹⁵⁶ Glucocorticoid response elements (GREs) have been identified in mitochondrial DNA sequences and the GR itself has been identified inside the mitochondria suggesting GR action on the mitochondrial genome.¹⁵⁷ Interestingly, acute versus chronic exposure to glucocorticoids has profoundly different effects, acute exposure is associated with induction of mitochondrial biogenesis and increase of enzymatic activity whilst chronic exposure leads to several mitochondrial defects, inhibition of cytochrome c oxidase (*COXI*, gene = *MT-COI*), increased ROS generation, mitochondrial structural problems, abnormal mitochondria biogenesis and increased cell death sensitivity.^{156,158} In keeping with this, *MT-COI* is the most significantly upregulated gene in healthy cells versus HA20 cells as shown in Figure 5.5. Additionally, *PIK3R1* has been consistently downregulated in HA20 and obesity transcriptionally. Proteomics in splenic murine CD8⁺ T and NK cells confirms similar protein-level decreases most notably *PIK3R1* in NK cells in obesity, aligning with

the transcriptomics whilst GLUT3 is trending downward in CD8⁺ T cells. The actual implications of these changes in metabolic genes at a transcriptional and proteomic level is hard to correlate with changes in effector functions directly, however, both STRING analyses (Chapter 2 and Chapter 3) have included *PIK3R1* in the networks suggesting its implicated as a GR target gene or is upregulated downstream of GR signalling or conversely suppressed by inflammatory signalling by cytokines like TNF.

The co-occurrence of altered or reduced GR target gene expression and altered metabolism is biologically coherent. GR is a master integrator of stress, circadian inputs, and metabolic reprogramming, when GR inducibility is impaired, cells fail to execute the resolution choreography that normally suppresses AP-1/NF- κ B bursts, restores mitochondrial function, rebuilds antioxidant tone, and re-establishes trafficking and adhesion programs. STRING networks and pathway analyses in this chapter reinforce that view, immediate-early brakes and canonical GR-NF- κ B targets (*DUSP1/2*, *ZFP36*, *BTG2*) are all reduced in HA20 and similarly suppressed in obesity versus weight loss. In chapter 2, the network of GR genes created was built using a co-expression matrix from NK cells. In this chapter, the whole lymphocyte transcriptome was examined together, a fascinating insight from this is even though the whole transcriptome is used for A20 haploinsufficiency analysis and comparison to obesity, the phenotype remains the same. This is suggestive of a conserved response at least across the lymphocyte population. In addition to this, as shown in Figure 5.16 and 5.17 the cell type with the largest module score was the cluster labelled ‘Unconventional T Cells’, this cluster consisted of two cell types with a very similar expression pattern, Mucosal associated invariant T (MAIT) cells and Gamma Delta T ($\gamma\delta$ T) Cells. This is suggestive of potentially higher responsive to glucocorticoids. CD56^{bright} NK cells also exhibited a higher GR module score when compared to CD56^{dim} NK Cells but this difference is subtle. A memory CD8⁺ T cell population also exhibited high GR module expression. However, the key takeaway from this analysis and comparison of GR module scores across cell types, obesity and HA20 is that in obesity and HA20, GR module scores are downregulated in every cell type in obesity vs weight loss and in HA20 vs healthy. Thus, this response whilst initially observed in NK cells in Chapter 3 is conserved across all lymphocyte populations and in different disease conditions.

Glucocorticoids provide systemic impacts and are vital for stress response, interestingly but not surprisingly another vital stimulus appears to be acting on immune cells, leptin. Leptin is a hormone, that is vital for regulating appetite and energy balance by signalling satiety to the hypothalamus, when fat stores increase (as in obesity), leptin levels rise suppressing

appetite and promoting energy expenditure. In obesity, leptin resistance often occurs where appetite suppression is not efficiently induced by leptin ¹⁵⁹. In Figure 5.19, *LEPROTL1* is shown to be downregulated in HA20 vs healthy individuals and obesity vs weight loss. *LEPROTL1* is a transmembrane protein that modulates cytokine receptor trafficking, including the leptin receptor. *LEPROTL1* restricts cell surface expression of leptin receptor (LEPR), thereby tuning leptin sensitivity. De Andrade et al. (2025) highlighted that the balance of leptin signalling is critical for NK cell function: physiological leptin promotes survival and cytotoxicity, but chronic hyperleptinemia in obesity drives NK cell dysfunction through leptin resistance ¹⁶⁰. Thus, a reduction in *LEPROTL1* expression during obesity and weight loss could reflect an adaptive mechanism to enhance leptin receptor availability at the cell surface in settings of chronically high leptin. However, this downregulation may paradoxically exacerbate signalling desensitisation by contributing to receptor overstimulation. In immune cells such as NK cells, this could further blunt cytotoxicity and IFN- γ responses, reinforcing impaired tumour and viral surveillance in obesity.

Other key metabolic genes are downregulated in obesity and HA20 such as *DDIT4*, *PDE4D* and *PDE4B*. Using Pan *et. al.* (2022) as a reference point to understand *DDIT4*, it has been observed that circulating *DDIT4* is significantly reduced in adults with hyperlipidaemia, and lower *DDIT4* tracks with higher BMI, insulin resistance, mTOR activation, and inflammatory markers (CRP, IL-6, TNF- α , MCP-1).¹⁶¹ Given that *DDIT4* is a GR target gene, this provides another mechanistic link between altered GR signalling and obesity. Similarly, the link to mTOR has been established with *TNFAIP3* where CD4+ T Cell and NK cell *TNFAIP3* KO in mice leads to mTOR activation, very much linking GR target genes to activated mTOR.^{134,135} With respect to the cAMP-specific phosphodiesterases (*PDE4D* and *PDE4B*), both of these are involved in the degradation of cAMP, regulation of cAMP is vital in NK cells, too much or too little impairs NK cell cytotoxicity providing another mechanism by which NK cells need cAMP levels tightly regulated for proper function, 80% cAMP decreases in NK cells caused a 90% decrease in cytotoxic capacity in NK cells.¹⁶²

Vitally, *NFE2L2* (NRF-2) is downregulated across both HA20 and obesity. This is important as NRF-2 is the cells master transcriptional regulator of antioxidant responses and is implicated in lipid metabolism.¹⁶³ In obesity, excessive fat accumulation and chronic inflammation elevate reactive oxygen species (ROS), which can in principle be regulated by NRF-2, literature shows that NRF-2 signalling often becomes blunted over time under chronic stress, contributing to worsening inflammation, dyslipidaemia, and cardiovascular vulnerability.¹⁶³ Within the context of HA20, systemic inflammation driven by unrestrained

NF- κ B activity likely suppresses NRF2-mediated resilience, thus, removing immune cells of antioxidant capacity and metabolic flexibility and mirroring known effects in obesity.

Based on the evidence presented, obesity is at least a partial phenocopy of HA20: HA20 represents a failure of an anti-inflammatory brake (*A20/TNFAIP3*), whereas obesity represents a failure driven by chronic inflammatory and endocrine load. Across datasets, the dominant shared signature is coordinated suppression, particularly of GR-responses and metabolic modules, while weight loss (and, in Chapter 3, fasting-like states) reverses these signatures, moving cells along the same latent axis in the opposite direction. The reversibility argues for biological plasticity and makes a strong case that the shared state can be therapeutically reprogrammed at least acutely in obesity via interventions like weight loss / fasting. In HA20, this is an acquired genetic life-long phenotype that can be managed only.

These findings have direct functional and translational implications. If the problem is a complete breakdown of GR signalling due to loss of A20, simply blocking cytokines may not restore health. Therapy should be reframed around restoring resolution capacity to produce *TNFAIP3*. This means enhancing GR activity rather than escalating steroid dose, reducing tonic NF- κ B inputs (dietary TLR ligands, adipose cytokines) and improving GRE access (e.g., chromatin/HDAC2 support, AP-1 alterations). In parallel, immune-metabolic re-priming interventions that increase glucose transport (GLUT3) and mitochondrial function (exercise mimetics, fasting-mimicking regimens, targeted NRF-2 activation), should shift cells toward the healthy state, consistent with the weight loss reversals observed here.

The data also suggest compact, interpretable biomarkers. A GR module score (NR3C1 targets such as *TSC22D3*, *CXCR4*, *PIK3R1*, *CD69*, *TNFAIP3*) and a transport/mitochondrial score (*SLC2A3*, *SLC38A1*, *TOMM20*, *COQ10B*, *ISCA1*, *CYCS*, *MT-COI*) together define a state-space in which healthy and weight-loss samples separate from HA20 and Obesity. Such scores could quantify distance to the healthy state during lifestyle or pharmacologic interventions and enable patient stratification.

Limitations remain in this chapter the HA20 cohort is very small (n=2), despite this, the shared transcriptional signature maps so tightly to what is observed in obesity, it is unlikely to be a coincidence. Similarly, this is mitigated by focusing on shared directions reproduced in an independent obesity/weight loss cohort. PBMCs may not fully mirror adipose or hepatic immune niches, nonetheless, the reproducible weight loss reversibility argues that the signals are biologically meaningful and would span tissues based on Chapter 3

observations in adipose tissue, validation in human adipose would strengthen generalisability. Finally, the mixed direction of individual ETC subunits in HA20 likely reflects compensatory or cell-type-specific rewiring.

5.4.0 – Chapter 5 - Conclusion

In summary, this chapter demonstrates that A20 haploinsufficiency and obesity converge on a shared immunometabolic state characterised by diminished glucocorticoid receptor (GR) signalling, loss of canonical GR targets, and suppression of metabolic regulators. What emerges is a reproducible signature that was first defined in NK cells (Chapter 4) but is now shown to extend across lymphocyte populations. Despite their different origins, monogenic loss of *TNFAIP3* in HA20 versus environmentally driven meta-inflammation in obesity, highlighting that obesity phenocopies HA20.

This overlap underscores a common breakdown in GR–NF- κ B cooperation, leaving immune cells unable to resolve inflammation and adapt metabolically. Crucially, weight loss reverses this state, highlighting its plasticity and therapeutic potential. Thus, the findings here position HA20 not just as a rare immunodeficiency but as a lens to understand the broader failure of immunometabolic resolution in obesity and point to strategies aimed at restoring GR competence and metabolic flexibility as a unifying route to immune homeostasis.

6.0 - Overall Discussion

This thesis set out to establish a few key underlying questions related to obesity and its pathology: why do immune cells in obesity adopt a persistently activated inflammatory state? Is that state reversible? What circuitry governs the transition between disease and health?

The first indicator as to what may be contributing to this inflammatory phenotype in obesity is the identification of a key negative regulator of inflammation, *TNFAIP3* (A20), which in Chapter 2 I identified as being consistently downregulated in obesity across tissues and immune cell types in humans and mice. This raised the question as to whether this gene, *TNFAIP3*, could be one of the mechanisms by which immune cells are chronically dysregulated in obesity? This finding, that *TNFAIP3* is downregulated in obesity, is significant to the study of obesity and its related co-morbidities as it highlights a mechanism by which NF- κ B could be chronically activated in obesity as NF- κ B's chronic activation in obesity has been well established.¹⁶⁴ However, *TNFAIP3* regulation in lymphocytes has not been well established in obesity, the expression and importance of *TNFAIP3* has only been established in the myeloid compartments in mice, where loss of *Tnfaip3* leads to chronic inflammation but protection against HFD-induced obesity and insulin resistance in mice.³⁰ Nothing has been established about lymphocytes when *TNFAIP3* is downregulated or knocked out in obesity. Using computational techniques *TNFAIP3* downregulation was explored using omics datasets generated within the lab or publicly available. Since NF- κ B is very much regulated post translationally via phosphorylation and other PTMs, no major conclusions could be made in lymphocytes via scRNA-seq data used in this dataset other than making predictions based off non-obese disease settings where *TNFAIP3* has been knocked out or is genetically downregulated as is the case in HA20.¹⁵⁵ KO models in murine CD4+ T Cells and NK cells independently revealed that *Tnfaip3* knockout leads to mTOR activation, increased apoptosis and severe chronic inflammation.^{135,134} Whole body *Tnfaip3* KO mice died prematurely due to chronic inflammation and cachexia.¹⁶⁵ Human GWAS studies are also very clear in establishing the effects of SNPs in the locus of *TNFAIP3*. SNPs in the *TNFAIP3* locus are associated with autoimmune diseases such as rheumatoid arthritis, psoriasis, SLE amongst others.³⁹ In 2016, HA20 was first described as a monogenic, inherited disease where an individual has a LOF variant of *TNFAIP3*, phenotypically this manifests as a disease resembling Behcet's disease.¹¹¹ Individuals with HA20 have increased circulating pro-inflammatory cytokines such as TNF, IL-6 and IL-17.¹¹¹ Based on all this

evidence, there is highly impactful implications for downregulation or loss of *TNFAIP3* in obesity based on these phenotypes established in literature.

Weight loss in obesity was associated with a significant restoration of *TNFAIP3*. I analysed multiple weight loss models varying both the duration and method of weight loss, for example, dietary switch (HFD to SFD), caloric restriction (e.g. mice receiving 70% of *ad libitum* calories), and fasting regimes. A 48-hour fast which was enough to significantly induce *Tnfaip3* expression levels in NK cells from multiple tissues in mice. This showed that even short-term fasting can induce *Tnfaip3* acutely even in the absence of obesity in mice. In humans, 6 weeks of severe caloric restriction highlighted a very significant increase in *TNFAIP3* as well across multiple cell types. The effects of the presence of T2D were also analysed, and it appears that there is at least a slight effect of T2D on *TNFAIP3* levels. However, it is difficult to uncouple the effects of medication on *TNFAIP3* levels. At a proteomic level in mice, it was confirmed that A20 is downregulated in murine CD8+ T cells and NK cells. In sum, Chapter 2 establishes that *TNFAIP3* is significantly downregulated in lymphocytes in obesity across tissues and species, and that weight loss restores its expression across diverse interventions. This pattern provides a mechanistic bridge between obesity and persistent NF- κ B dysregulation, with plausible crosstalk to mTOR signalling, apoptosis and other cell-death programs observed in *Tnfaip3* knockout systems. While our data are necessarily correlative at the lymphocyte level, the use of many multi-omic datasets, genetic evidence, and orthogonal disease models strongly supports a model in which reduced *TNFAIP3* lowers the threshold for inflammatory activation in obesity, and restoration with weight loss contributes to resetting this immune setpoint toward health.

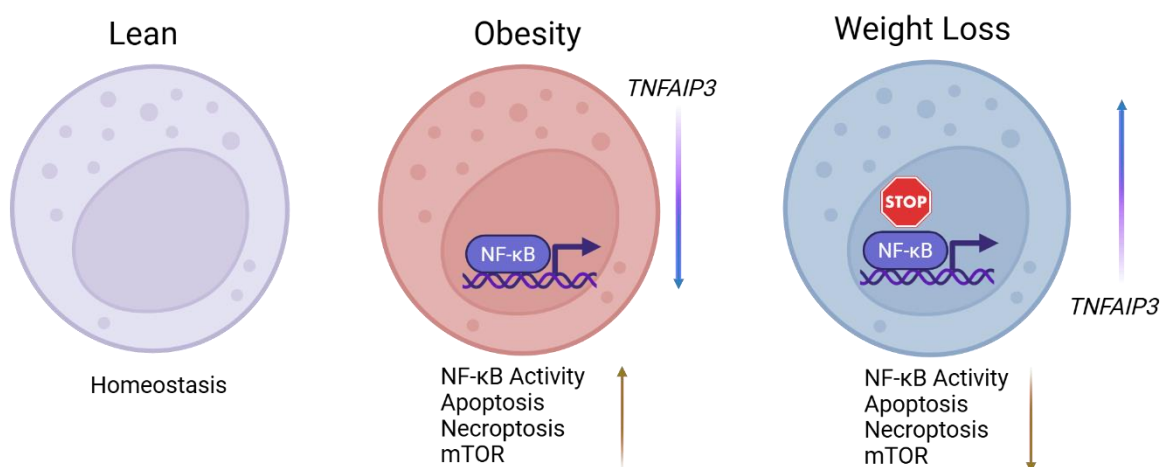


Figure 6.1 – Schematic for Chapter 2 - Proposed model of *TNFAIP3* (A20) regulation of NF- κ B activity and cell fate in lymphocytes across lean, obese, and weight loss states. Schematic representation of the working model developed in Chapter 2. In the lean state (left),

TNFAIP3 expression maintains immune homeostasis by restraining NF- κ B activation and limiting apoptosis and necroptosis. In obesity (middle), TNFAIP3 expression is downregulated, resulting in increased NF- κ B activity together with elevated apoptosis, necroptosis, and mTOR signalling. Following weight loss (right), *TNFAIP3* expression is restored, leading to suppression of NF- κ B signalling and partial reversal of apoptosis, necroptosis, and mTOR activation. This model highlights *TNFAIP3* as a potential mechanistic link between obesity and chronic immune activation.

Chapter 3 is primarily about glucocorticoids, their receptor (GR), and an inducible network that likely restores control over inflammatory signalling in response to GR signalling as occurs in weight loss and fasting. Chapter 2 introduced recurrent observations of reduced *TNFAIP3* in obesity and its recovery with weight loss, in chapter 2 this becomes part of a broader and more coherent program that includes *TNFAIP3*, *NFKBIA*, *DUSP1*, *ZFP36*, *TSC22D3*, *DDIT4* and other GR gene targets. The evidence points to glucocorticoid signalling through GR as the likely driver of this recovery program during fasting and weight loss, with important cooperation from NF- κ B, GR and AP-1 and likely involving the influence of chromatin accessibility as suggested by changes in adipocyte chromatin accessibility and the established importance of AP-1 and NF- κ B in the regulation of GR signalling.^{166,96} Whether the changes in chromatin accessibility observed in adipocytes are conserved in immune cells is a major leap to make but one worth considering. The network of anti-inflammatory genes identified in Chapter 3 carries a set of functional consequences that include faster termination of inflammation, relocation of lymphocytes, and restoration of translational capacity.

The central mechanism appears simple, cortisol rises during fasting and during times of energy deficit. GR binds accessible enhancers and promoters, and transactivates a set of genes that collectively promote an anti-inflammatory response. *TNFAIP3* removes ubiquitin chains from *RIPK1* and *TRAF6* and thereby dampens NF- κ B signalling. I κ B α (encoded by *NFKBIA*), sequesters RelA and other NF- κ B transcription factors in the cytosol.³⁸ *DUSP1* and *DUSP2* reduce MAPK activity and reduce the feed into AP-1.¹⁴² *ZFP36* destabilises inflammatory transcripts like *TNF* and *IL6*.¹⁶⁷ Two canonical GR targets, *TSC22D3* and *DDIT4* are important anti-inflammatory members of this network. *TSC22D3* directly suppresses NF- κ B and AP-1 activity, interferes with PI3K/AKT/mTOR and Ras signalling, and promotes lymphocyte quiescence and survival under stress.¹⁶⁸ *DDIT4* inhibits mTORC1 through the TSC1/2 complex, reduces protein synthesis, and shifts cells toward energy conservation, autophagy and oxidative-stress resistance during nutrient or oxygen limitation.¹⁶¹ The result is a network that likely does not completely silence immunity but

restores a higher threshold for activation and a tighter brake once activation has occurred. In addition, and perhaps paradoxically, cytotoxic capacity could be potentially enhanced after fasting despite the upregulation of multiple anti-inflammatory genes as is shown in *Delconte et. al.* work where NK cells were more functional after a 48 fast.¹⁴⁶ However, that work looked at fasting in lean mice and not in obesity.

Why this gene network fails in obesity is likely due to a reversal of what occurs during fasting. First, enhancer accessibility matters and is likely reduced as shown in adipocytes in obesity.¹¹⁹ In adipocytes obesity compresses enhancer landscapes at many loci¹¹⁹, including the region around *TNFAIP3* in adipose and potentially in immune cells. Second, ligand bioactivation matters where *HSD11B1* is responsible for the regeneration of cortisol from cortisone within cells.¹⁶⁹ In obese NK cells and CD8+ T cells, *HSD11B1* is reduced at a protein level as established in Chapter 3, which likely lowers intracellular cortisol levels even when circulating levels are adequate. A reduced ligand pool and a less accessible enhancer landscape make the same systemic glucocorticoid pulse less effective. The probable consequence is slower induction of A20 and its partners, and a longer tail of NF- κ B activity after each inflammatory insult. The longer consequence is a shift of the immune setpoint toward chronic activation. Additionally, other factors are unaccounted for like heat shock proteins which bind the GR in the cytoplasm preventing translocation.¹⁷⁰ These could potentially be elevated in the cytoplasm in obesity but were not analysed in this thesis.

The cooperation between GR and NF- κ B deserves significant consideration, GR is often described as antagonistic to NF- κ B due to its anti-inflammatory signalling. That is true for some promoters where tethering by GR suppresses RelA driven transcription.¹⁷¹ At a subset of enhancers GR and NF- κ B co-occupy the same regulatory elements and co-activate genes that limit inflammation. *TNFAIP3* and *NFKBIA* are established examples in non-immune cells and the same pattern fits the data in lymphocytes here.¹⁴⁹ In fasting and weight loss, when chromatin near these elements is open and intracellular cortisol is sufficient, along with adequate activity of *HSD11B1*, GR and NF- κ B likely work together to raise the expression of the genes that will then shut the pathway down. In obesity, those enhancers are likely closed as shown in adipocytes in Chapter 3 and when intracellular cortisol and *HSD11B1* activity are dysregulated, the partnership is affected and cannot carry out its anti-inflammatory action.

The network also includes a recurring trafficking signature. In humans with obesity, NK cells are enriched for *CX3CR1*. After fasting or weight loss, *CXCR4* rises and NK cells adopt a

phenotype that favours retention in marrow and other *CXCL12* rich niches.¹⁴⁶ Glucocorticoid signalling sits upstream of this inversion where *CXCR4* is a GR target gene, directly linking the GR to immune cell homing.¹⁷² Fasting increases cortisol and raises circulating and local *CXCL12*, the ligand for *CXCR4*. GR target genes that reduce inflammatory signalling are induced while the chemokine landscape tilts toward *CXCR4*. Bone marrow niches supply cytokines and metabolites that support translational recovery and the re-expression of negative regulators such as *TNFAIP3*. In energy deficit, the immune system conserves fuel, relocates immune cells to supportive niches, and prefers restraint over expenditure unless threat demands otherwise.¹⁷³

This spatial program generalises beyond NK cells and monocytes based on the conserved expression of *CXCR4* which is uniformly upregulated in the human dataset in response to caloric restriction.^{173,146} Monocytes use *CX3CR1* to adhere and patrol in response to vascular inflammation, linking *CX3CR1* to an inflammatory phenotype.¹⁷³ Endogenous glucocorticoid expression will likely increase the fraction of cells in niches with higher *CXCL12* expression gradients and this will favour conservation, repair, and controlled readiness.

These findings have important clinical considerations as synthetic glucocorticoids such as dexamethasone reduce inflammation but at the cost of insulin resistance and higher glucose output in individuals. The datasets in this thesis primarily explore diet-induced weight loss, a natural and evolutionary conserved occurrence that apply small and transient cortisol pulses that are primarily needed in the context of energy deficit or ‘fight or flight’ response to supply adequate glucose to fuel the organism.¹⁷⁴ That is a very different state to taking synthetic glucocorticoids in a fed state. This is one of the reasons why fasting or well-designed caloric restriction can calm immune tone without the metabolic side effects of long-acting steroids like dexamethasone. Targeted local enhancement of *HSD11B1* in immune cells, or the timing of brief GR agonism to the fasting window, may increase *TNFAIP3* induction without driving ‘steroid diabetes’.¹⁵¹ Conversely, blocking GR during fasting may blunt A20 induction and prolong inflammatory signalling even if GR protein levels do not change. Each of these hypotheses are testable. Therapeutically, a synthetic glucocorticoid specific to immune cells may hold huge benefits instead of systemically acting glucocorticoids which cause the metabolic effects in non-immune cells causing ‘steroid diabetes’.¹⁵¹ Based on the literature I could see one paper where this was attempted, the authors developed antibody-drug conjugates (ADCs) designed to deliver glucocorticoid agonists to immune cells. They engineered a version with a payload that allowed genuine,

cell-intrinsic activation of GR target genes in human B cells.¹⁷⁵ Approaches like this may result in more targeted and improved therapeutic outcomes from glucocorticoids without the metabolic side effects.

Circadian rhythm adds another layer where cortisol levels naturally vary over the course of a day.¹⁷⁶ Sampling time could change apparent associations in data, and interventions that align GR agonism with the peak of endogenous cortisol during an energy deficit may have disproportionate effects on *TNFAIP3* and its partners. Obesity is associated with disrupted sleep and altered circadian timing, which may further degrade the quality of the GR signal.^{176,177} However, due to the strength of the signal across multiple datasets, this is not believed to be having an effect here.

Ultimately in Chapter 3 I identify a network of genes co-expressed together that link immunity and metabolism via glucocorticoids and GR signalling. In obesity, reduced intracellular cortisol regeneration via *HSD11B1*, closed enhancers and potentially changes to co-factors needed for transrepression ultimately lead to a breakdown of GR signalling. In fasting and in sustained weight loss, this is reversed. GR and NF-κB meet at shared enhancers, this could in part be due to the breakdown of AP-1 homeostasis, this will be discussed in more detail in the next section, *TNFAIP3* and its partners are induced, *CXCR4* and *CXCL12* draw lymphocytes into niches where they can refuel and reset, and the immune setpoint moves back toward health.

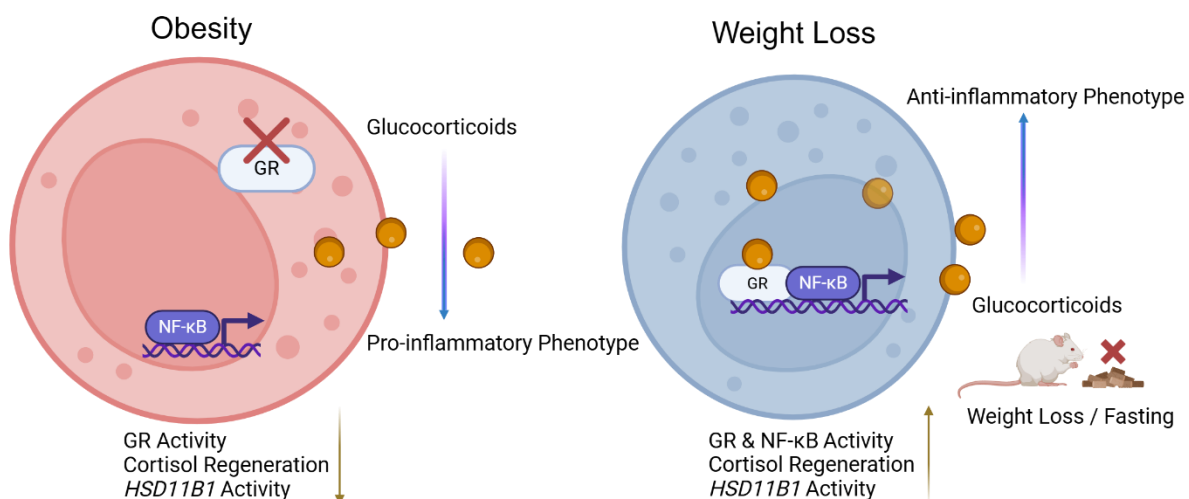


Figure 6.2 – Schematic for Chapter 3 - Proposed model of glucocorticoid receptor (GR) activity and NF-κB signalling in obesity versus weight loss. In obesity (left), reduced intracellular cortisol regeneration (↓HSD11B1 activity) and impaired GR activation lead to sustained NF-κB signalling and a blunted induction of anti-inflammatory genes. In weight loss or fasting (right), increased systemic glucocorticoids together with restored HSD11B1 activity allow GR to bind accessible chromatin and cooperate with NF-κB, inducing a

network of anti-inflammatory genes such as *TNFAIP3*, *NFKBIA*, *DUSP1* and *TSC22D3*. This model illustrates how the glucocorticoid–GR axis may reset immune tone and shorten NF- κ B activity after inflammatory stimuli during energy deficit.

Chapter 4 brings the glucocorticoid story full circle by placing HA20 beside obesity and asking whether a single genetic perturbation that disables A20 can recreate the same transcriptional state that emerges from chronic metabolic load as seen in obesity. Transcriptionally there is a clear and considerable overlap in the anti-inflammatory signature that is lost in HA20 and obesity as observed in Chapter 4. Across independent datasets and across lymphocyte lineages there is a shared signature that is downregulated in obesity, rises with fasting and weight loss, and is also diminished in A20 haploinsufficiency in the absence of metabolic change. This signature contains *TNFAIP3* itself along with a compact set of glucocorticoid receptor targets that act as immediate brakes on inflammatory signalling. It includes *NFKBIA*, *TSC22D3*, *ZFP36*, *DUSP1*, *DUSP2*, *DDIT4*, *CXCR4*, *PIK3R1* amongst others that together shorten the lifetime of inflammatory responses, stabilise stress responses, and reset trafficking.

Two main questions emerge from Chapter 4, either A20 is required to maintain a homeostatic glucocorticoid response, or the loss of A20 prolongs NF- κ B activity and associated cytokine release to such a degree that glucocorticoid inducibility is eroded through crosstalk and chromatin effects. The data in Chapter 4 favour the second explanation while not excluding the first but perhaps both mechanisms are potentially at play. In HA20 there is broad evidence of persistent inflammatory tone with elevations in *TNF* and other pro-inflammatory cytokines.¹¹¹ In obesity there is also persistent inflammatory tone arising from a multitude of sources.¹⁷⁸ In both settings the modules that contain classic glucocorticoid targets are reduced, and the same targets rise again with caloric restriction or fasting. This symmetry suggests that continued activation of NF- κ B and associated stress pathways constrains the capacity of GR to occupy enhancers and to induce anti-inflammatory genes. Where Chapter 3 established that fasting physiology and weight loss restore GR target genes, Chapter 4 shows that when A20 is downregulated that GR dysregulation is present independent of body weight.

The cooperation and tension between GR and NF- κ B helps explain our observations.¹⁶⁶ GR can repress some NF- κ B outputs by tethering, and at a subset of enhancers GR and NF- κ B co-occupy elements to induce anti-inflammatory genes such as *TNFAIP3* and *NFKBIA*. When A20 is lowered by genotype or chronic metabolic stress, NF- κ B activity persists and

cytokines remain elevated. This creates two problems for GR signalling: chronic inflammatory load reduces accessibility of relevant enhancers, and the cellular milieu shifts toward stress pathways and altered cofactor availability. The GR is not a pioneer factor and requires accessible chromatin, so its ability to induce anti-inflammatory brakes is degraded. Potentially, the dysregulation of the AP-1 transcription factor complex helps explain why HA20 and obesity converge on the same glucocorticoid module and why weight loss can restore it without altering genotype. Unlike NF- κ B, AP-1 family members (including FOS, FOSB, FOSL1/2, JUN, JUNB and JUND) show marked and state-specific changes in obesity and weight loss in our transcriptomic modules, suggesting shifts in AP-1 composition. This pattern aligns with findings from Biddie et al. (2011), who showed that AP-1 binds chromatin constitutively, maintains local accessibility, and is required for efficient GR recruitment to roughly half of its genomic targets; blocking AP-1 DNA binding collapses accessibility and reduces GR binding. Consistent with this insight, our data suggest that in obesity altered AP-1 dynamics could erode enhancer priming, thereby weakening cooperative induction of TNFAIP3 and its network partners even when NF- κ B levels are unchanged. Conversely, weight loss is associated with restoration of FOSL2 and other AP-1 factors, consistent with renewed chromatin priming and improved GR responsiveness. In the context of chronic metabolic stress, these shifts in AP-1 activity could degrade the chromatin environment required for efficient GR-driven anti-inflammatory responses. Future work profiling AP-1 occupancy and histone accessibility in immune cells across lean, obese, and weight-loss states will be critical to test this enhancer-priming hypothesis and further refine our understanding of the regulatory logic governing inflammatory resolution in metabolic disease.

CXCR4 appears again as a central gene determining where lymphocytes will reside when the glucocorticoid network is engaged. In obesity and in HA20 the *CXCR4* axis is subdued and *CX3CR1* is elevated in the disease states (HA20 and obesity). When fasting or undergoing weight loss increases endogenous cortisol, *CXCR4* rises along with *TNFAIP3* and other GR targets. Cells are then drawn back to supportive niches where they can conserve fuel, rebuild translational capacity, and reset their activation threshold. Chapter 4 shows that the same direction of change in the GR module is seen across lymphocyte populations in HA20 versus healthy and in obesity versus weight loss, and that the highest GR module scores belong to subsets such as unconventional T cells and CD56^{bright} NK cells.

The metabolic changes observed in Chapter 4 fit this interpretation without evoking alternative mechanisms as mitochondrial and transport genes trend down in HA20 and in

obesity and reverse with weight loss. *PIK3R1* and related elements in the insulin signalling axis are reduced in both states and appear within the same co-expression neighbourhoods as *TNFAIP3*.

The practical consequences of the HA20 comparison are obvious. If obesity partially phenocopies HA20 at the level of this network, then the same logic for intervention applies, adjusted for the fact that one condition is acquired and potentially reversible and the other is genetic and can only be managed. For obesity the lever is to restore the capacity for GR to occupy its sites and to cooperate with NF- κ B at shared enhancers. That means reopening enhancers through weight loss or exercise linked chromatin effects, restoring intracellular cortisol regeneration through *HSD11B1* in immune cells. For HA20, the mechanism is different. Blocking cytokines and lowering tonic NF- κ B inputs may be necessary but may not suffice if the cooperative GR-NF- κ B-AP-1 induction of brake genes is broken at the chromatin level. Strategies that improve GRE access and fine tune GR toward induction of brake genes rather than strong metabolic activation deserve testing. The observation that *CXCR4* and related niche signals move with the module suggests a second lever.

These findings also suggest compact biomarkers that are easy to compute and easy to interpret. A GR module score that includes *TNFAIP3*, *NFKB1A*, *TSC22D3*, *ZFP36*, *DUSP1*, *DDIT4*, *CXCR4*, *PIK3R1*, and *CD69* can be paired with a basic transport and mitochondrial score that includes *SLC2A3*, *SLC38A1*, *TOMM20*, *COQ10B*, *ISCA1*, *CYCS*, and *MT-CO1*. Together these scores place samples on a plane where obesity and HA20 occupy one quadrant and healthy and weight loss occupy the opposite quadrant. Movement on that plane during an intervention can be read as gain or loss of resolution capacity. Because the shared signature is conserved across lymphocyte types, the same plane can be applied at the level of bulk PBMCs or within sorted subsets when higher precision is needed.

There are limits to these analyses. The HA20 cohort is small and PBMCs may not reflect all tissue resident states. However, these constraints do not negate the central finding that the same GR centred module is reduced in HA20 and in obesity and rises with weight loss and fasting.

Taken together, this thesis supports a model in which obesity drives chronic immune activation not through irreversible immune reprogramming, but through erosion of a glucocorticoid-centred regulatory network that normally constrains inflammatory signalling. Central to this network is *TNFAIP3*, whose reduction lowers the threshold for NF- κ B activation and prolongs inflammatory responses across lymphocyte populations. Weight loss

and fasting restore this circuitry through endogenous glucocorticoid signalling, enhancer accessibility, and coordinated induction of anti-inflammatory brake genes. The convergence of obesity, weight loss, and A20 haploinsufficiency on the same transcriptional module suggests that immune dysregulation in obesity is mechanistically coherent, reversible, and governed by definable regulatory logic rather than stochastic dysfunction.

7.0 - Overall Conclusion

In summary, I identify *TNFAIP3* (A20) as a gene that is consistently downregulated in obesity and that plausibly underlies persistent NF- κ B activity in lymphocytes. I also show that *TNFAIP3* expression rebounds with weight loss, is inducible by fasting independent of obesity (at least in NK cells), and is concurrently reduced in HA20, linking genotype and metabolic stress through a shared failure of the same brake circuit. A coherent picture emerges from these observations, GR activity is renewed and induces a compact anti-inflammatory program (*TNFAIP3*, *NFKBIA*, *DUSP1*, *ZFP36*, *TSC22D3*, *DDIT4*, *CXCR4*). In obesity and HA20 this program is blunted, likely via reduced enhancer accessibility/cofactor context and potentially via dysregulated AP-1 signalling, so inflammatory bursts run longer and cells traffic to pro-inflammatory niches. The translational implications are important, leveraging endogenous, time-limited glucocorticoid signalling (fasting/energy deficit or weight loss physiology) as a means to restore this module without the fear of inducing ‘steroid diabetes’ that comes with taking synthetic glucocorticoids.

Following on from these insights the next questions are practical and testable. Recently, new weight loss interventions therapies have come to market and include the much-lauded GLP-1 agonists. The widespread use of these medications offers the potential to expand on the findings presented here. For example, do GLP-1-based weight loss regimens recapitulate the same GR-centred signature, or does fasting / weight loss have a unique signature? Can one target GR in immune cells (or boost immune-cell *HSD11B1*, or time brief GR doses to fasting windows to limit metabolic effects) without the negative metabolic side effects? Key to the successful exploration of these questions will be to apply state-of-the-art bioinformatic and statistical tools to human and animal cohorts, as exploited in this thesis.

Taken together, this thesis reframes obesity-associated immune dysfunction as a failure of inflammatory resolution governed by a defined, glucocorticoid-responsive regulatory circuit rather than as an irreversible state of immune activation. By identifying *TNFAIP3* and its associated network as a reversible, mechanistically coherent node linking metabolism, inflammation, and immune cell state, this work provides both a unifying framework for understanding immune dysregulation in obesity and a rational foundation for future therapeutic strategies aimed at restoring immune homeostasis rather than suppressing immunity outright.

8.0 – Future Work

The findings presented in this thesis identify TNFAIP3 and a glucocorticoid-centred regulatory network as a mechanistic link between metabolic state and immune tone. While the analyses presented are comprehensive and integrate multiple orthogonal datasets, they also motivate several clear and testable avenues for future work, spanning both experimental and computational approaches.

8.1 Future work arising from Chapter 3: TNFAIP3 regulation in obesity and weight loss

Chapter 3 establishes that TNFAIP3 is consistently downregulated in lymphocytes in obesity across species and tissues and is restored following weight loss and fasting. A key next step is to determine whether reduced TNFAIP3 expression is causally sufficient to drive lymphocyte dysfunction in metabolic disease. This could be addressed experimentally using conditional TNFAIP3 haploinsufficiency or inducible knockdown models in NK cells or CD8⁺ T cells, combined with high-fat diet feeding. Such models would allow direct testing of whether partial A20 loss lowers the activation threshold, prolongs NF- κ B signalling, or alters susceptibility to apoptosis and necroptosis in obesity. Complementary in vitro approaches using CRISPR interference or siRNA in primary human lymphocytes exposed to obesogenic stimuli (e.g. fatty acids or hyperglycaemia) could further dissect cell-intrinsic effects.

At a computational level, future analyses could integrate longitudinal human datasets to model within-individual changes in TNFAIP3 during weight loss, reducing inter-individual confounding and strengthening causal inference.

8.2 Future work arising from Chapter 4: Transcriptional and epigenetic control of TNFAIP3

Chapter 4 implicates chromatin accessibility, transcription factor context, and post-transcriptional regulation in shaping TNFAIP3 expression. A critical next step is to directly test whether the enhancer landscapes identified in adipocytes are conserved in immune cells. Single-cell ATAC-seq or multiome (scRNA-seq + scATAC-seq) profiling of immune cells from lean, obese, and weight-loss conditions would allow direct assessment of GR, NF- κ B, and AP-1 motif accessibility at TNFAIP3 and related loci.

In addition, the putative role of AP-1 composition in modulating GR responsiveness warrants mechanistic validation. Perturbation of specific AP-1 components (e.g. FOSL2 or

JUNB) followed by glucocorticoid stimulation could test whether AP-1 priming is required for induction of the anti-inflammatory GR module. Post-transcriptional regulation identified in this chapter, including candidate microRNAs and RNA-binding proteins such as RC3H1, also provides a tractable avenue for follow-up using reporter assays and RNA stability measurements.

8.3 Future work arising from Chapter 5: Obesity and A20 haploinsufficiency

Chapter 5 shows that obesity transcriptionally phenocopies A20 haploinsufficiency at the level of a compact GR-responsive module. Future work should explore whether therapeutic suppression of inflammatory tone in HA20 patients (e.g. cytokine blockade) restores GR responsiveness and chromatin accessibility at shared enhancers. Conversely, it will be important to determine whether chronic metabolic inflammation alone is sufficient to erode GR signalling in otherwise healthy immune cells, independent of TNFAIP3 genotype.

From a systems perspective, causal network modelling approaches could be applied to distinguish whether A20 loss primarily drives GR dysfunction or whether prolonged NF- κ B activity secondarily constrains GR action through chromatin remodelling and cofactor redistribution.

8.4 Translational and therapeutic directions

The convergence of obesity, fasting, weight loss, and HA20 on a shared GR-centred regulatory circuit suggests multiple translational opportunities. Future studies should assess whether emerging weight-loss therapies, particularly GLP-1 receptor agonists, recapitulate the same immune transcriptional signatures observed with caloric restriction and fasting. Comparative profiling of immune cells before and after GLP-1-mediated weight loss would help distinguish weight-loss-dependent effects from fasting-specific endocrine signals.

More broadly, strategies that selectively enhance glucocorticoid signalling in immune cells such as boosting immune-cell HSD11B1 activity, timing brief GR agonism to fasting windows, or using immune-targeted glucocorticoid delivery represent promising avenues to restore inflammatory resolution without inducing systemic metabolic side effects. These hypotheses are directly motivated by the data presented here and are amenable to experimental and computational testing.

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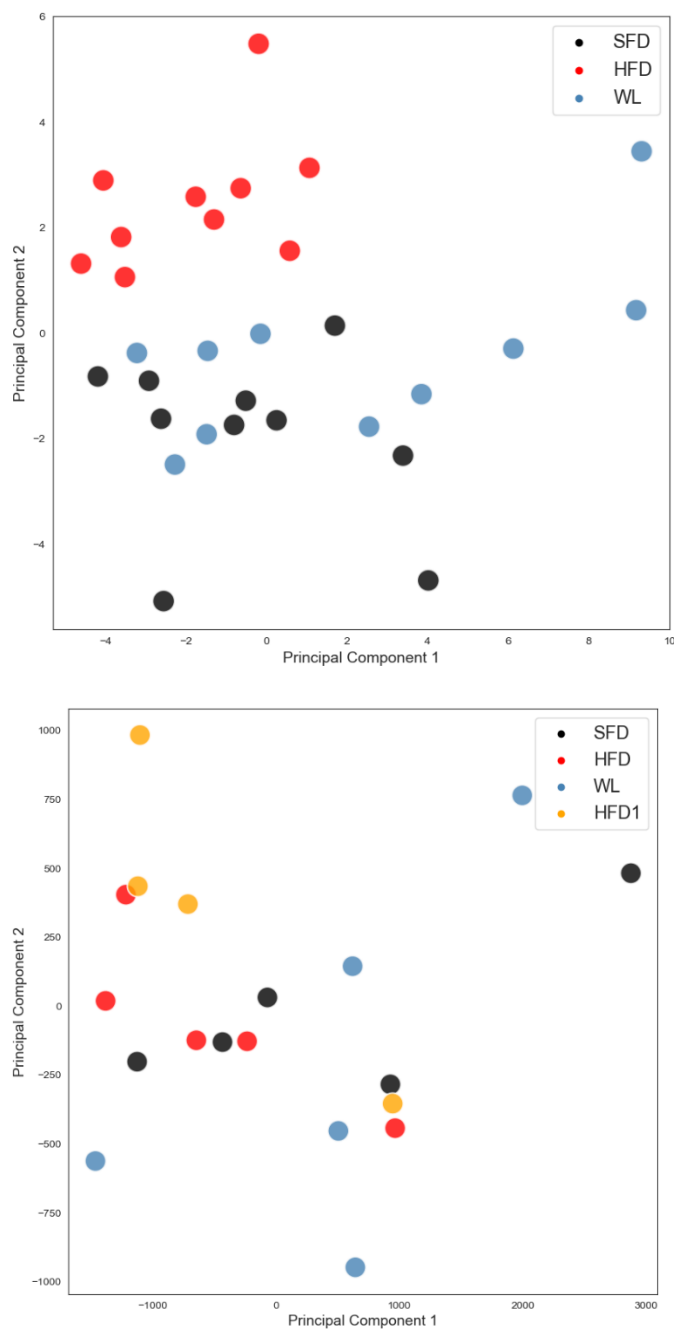
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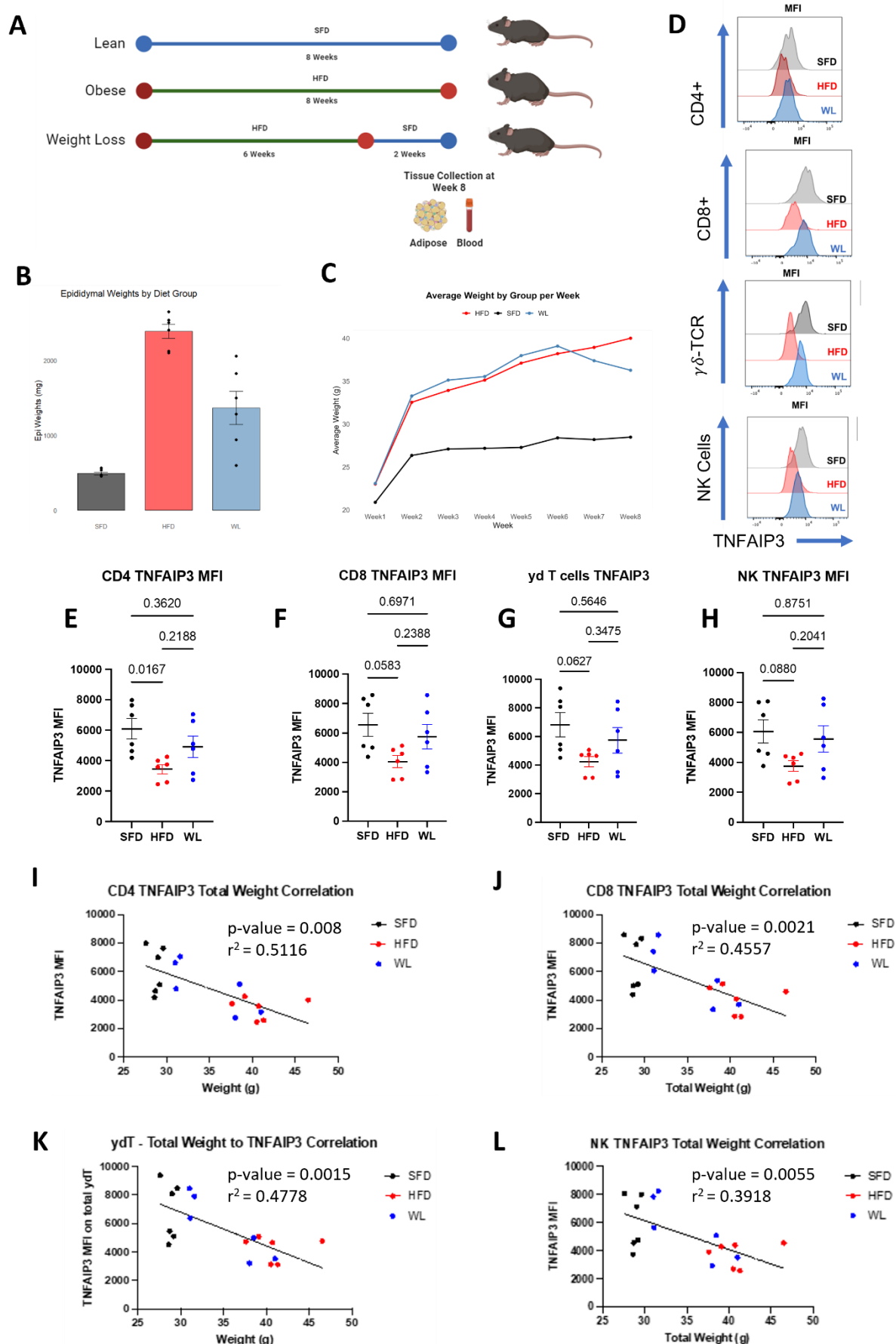
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10.0 - Supplemental Figures



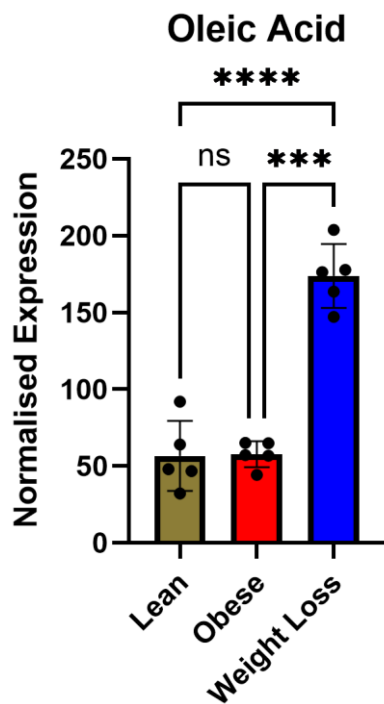
Supplemental Figure 10.1 – A) PCA of metabolite profiles in plasma, coloured by diet group. Points are individual mice: SFD (black), HFD (red), WL (blue). Axes denote PC1 and PC2. Group structure is weaker than in adipose, consistent with smaller global shifts in this compartment. B) Principal component analysis (PCA) of metabolite profiles coloured by diet group in Liver. Each point represents one mouse: SFD (black), HFD (red), and WL (blue), One Week HFD (orange). Axes show PC1 and PC2. Compared with adipose, separation among groups is weak, indicating subtler metabolic differences in this tissue.



Supplemental Figure 10.2 - (A) Study design. Mice were assigned to SFD (lean), HFD (obese), or weight loss (WL; 6 weeks HFD then 2 weeks SFD). Tissues were collected at week 8 (adipose and blood). - (B) Epididymal

adipose tissue (eWAT) weights by diet group. Each dot is one mouse; bars show mean $\pm$ SEM. - (C) Body-weight trajectories over the 8-week protocol for the three groups. - (D) Representative flow-cytometry histograms of intracellular *TNFAIP3* mean fluorescence intensity (MFI) showing relative shifts across SFD (grey), HFD (red), and WL (blue). - (E–H) Summary *TNFAIP3* MFI in blood lymphocyte subsets by diet group: (E) CD4 T cells, (F) CD8 T cells, (G) $\gamma\delta$ T cells, (H) NK cells. Points are individual mice; symbols denote mean $\pm$ SEM. - (I–L) *TNFAIP3* MFI versus total body weight for the same subsets: (I) CD4, (J) CD8, (K) $\gamma\delta$ T, (L) NK. Lines show least-squares linear fits; *p* values and r^2 from linear regression are displayed on each panel.

Supplemental Figure 10.3



Supplemental Figure 10.3 – Comparison of adipose oleic acid levels across metabolic groups by one-way ANOVA. Bar graph shows normalised expression of oleic acid in adipose tissue samples from the three diet groups (Lean, Obese, Weight Loss). Each dot is an individual sample; bars indicate mean $\pm$ SEM. Statistical differences among groups were assessed by one-way ANOVA, and pairwise comparisons are annotated (***, ****) with “ns” indicating non-significant.