Iron controls the adaptive immune response

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Abstract

Adaptive immune responses require dynamic cellular reconfigurations as antigen-specific lymphocytes proliferate, acquire effector functions and generate immunological memory. Several metabolic pathways are co-opted during this process, but specific metabolites identified as important substrates are rarely systemically limiting. Iron deficiency affects a billion people worldwide and a mutation in transferrin receptor that disrupts the ability of lymphocytes to acquire iron causes immunodeficiency. However, to date if and how serum iron deficiency disrupts adaptive immunity has not been investigated. Here we demonstrate that genetic defects in iron homeostasis, such as the TfrcY20H/Y20H mutation and IRP1/2 deficiency, cell-intrinsically disrupt adaptive immunity. We find that T-cell metabolic activity including mitochondrial ATP generation is impaired by limiting iron acquisition in vitro, concomitant with reduced proliferation, suggesting that iron is a key 'immunometabolite'. We then provide in vivo evidence that transient and physiological serum iron deficiency caused by increased activity of the iron regulatory hormone hepcidin profoundly reduces the magnitude of the primary antigen specific CD8, CD4 and B-cell responses to multiple immunisation platforms, and alters the differentiation trajectory of CD8 T-cells such that memory and secondary recall responses are impaired even after iron status has been normalised. Low serum iron levels also disrupt protective T- and B-cell responses to experimental influenza virus infection, increasing lung viral load, inflammation and neutrophil infiltration, and prolonging weight loss in infected animals. Overall, these findings reveal an

unexpected sensitivity of adaptive immunity to serum iron availability and may have implications for control of infections and vaccination programmes in developing-world populations where iron deficiency is highly prevalent and the infectious burden is considerable.

More generally we suggest that manipulating iron availability may provide a tool to control the amplitude and quality of adaptive immune responses.

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Declaration of authorship

The majority of the work presented in this thesis was developed and performed by myself under the supervision of Professor Hal Drakesmith at the Weatherall Institute of Molecular Medicine, University of Oxford. Experiments performed by our collaborators were conceptualised and analysed as a joint effort and are clearly indicated in the thesis. No part of this thesis has been submitted for examination for a degree at this or any other university.

Joe Frost

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Glossary

2-OG	2-oxogluterate
4-OHT	4-hydroxytamoxifen
DCYTB	duodenal cytochrome oxidase B
DMT1	Slc11a2, divalent metal transporter 1
EPO	erythropoietin
ERFE	erythroferrone
EtOH	ethanol
FPN	ferroportin
GC	Germinal Centre B cell
HFE	Human homeostatic iron regulator protein
HIF1a	Hypoxia inducible factor 1 alpha
HJV	hemojuvelin
IL	interleukin
IRE	iron response element
IRP1	Aco1, iron regulatory protein 1
IRP2	Ireb2, iron regulatory protein 2
LMIC	low- and middle-income country
LSEC	liver sinosoidal endothelial cell
mHep	mini-hepcidin
NOS	nitric oxide synthase
NRF2	nuclear factor erythroid 2- related factor 2
PCBP	poly(rC)-binding protein
PMN	polymorphonuclear leukocytes
STEAP3	Six-membrane epithelial antigen of the prostate three
TET	ten-eleven-translocation
TF	transferrin
Tfh	T follicular helper cell
TFR1	tfrc, CD71, transferrin receptor 1
TLR	toll like receptor

Introduction

1.1 Iron is essential for cellular function

Multiple families of proteins exploit the unique chemical properties of iron to facilitate key biological chemical reactions; a handful of key examples will be discussed below. The iron, oxygen and 2-oxogluterate (2OG) dependent dioxygenases are a family of enzymes which regulate diverse and important biological processes (Loenarz and Schofield, 2008). Family members include: collagen prolyl-hydroxylase, which is important for the biosynthesis of collagen (Hutton et al., 1967); the prolyl-4-hydroxylase domain proteins (PHD) which sense oxygen and regulate the cellular transcriptional response to hypoxia (Schofield and Ratcliffe, 2004); the JmjC family of histone demethylases and the ten-eleven-translocation (TET) family of DNA demethylases place the 2OG-dependent-dioxygenases and iron as key players in epigenetic regulation (Klose et al., 2006); and fatty acid metabolism involves two 2OG-dependent-dioxygenases in mammals, Trimethyllysine hydroxylase and γ-butyrobetaine hydroxylase, which play distinct and important roles in carnitine biosynthesis (Vaz and Wanders, 2002). Lipid metabolism is further linked to iron via a the iron-dependent desaturase enzymes (Shanklin et al., 2009).

Attempts to inorganically recapitulate the chemical reactions central to cellular metabolism highlight the importance of iron's catalytic properties for life (Muchowska et al., 2019). The centrality of iron to oxidative metabolism has been appreciated for nearly 100 years (Warburg, 1925). There are a total of 12 Iron-sulphur (Fe-S)

complexes in complex I-III of the mitochondrial electron transport chain, while the citric acid cycle enzymes succinate dehydrogenase (complex II) and aconitase also contain Fe-S clusters (Rouault, 2015). Fe-S clusters also indirectly support metabolism as lipoic acid synthetase is required for the biosynthesis of lipoic acid, an essential co-factor for the mitochondrial enzymes pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and branched chain keto acid dehydrogenase (Mayr et al., 2011). As such, inducible disruption of Fe-S cluster biogenesis unsurprisingly has a profound effect on cellular metabolism (Crooks et al., 2018). Many proteins involved in DNA synthesis and repair including primase (Klinge et al., 2007), eukaryotic DNA polymerase (Netz et al., 2011) and the repair helicases xeroderma pigmentosum protein D and Fanconi anaemia protein J (Rudolf et al., 2006) include Fe-S clusters (Gari et al., 2012). This observation, alongside the key importance of iron in the catalytic activity of ribonucleotide reductase (Thelander et al., 1983) demonstrates the importance of iron for the eukaryotic cell cycle and division. Fe-S clusters, and regulation of their biogenesis, also regulate cellular iron homeostasis by determining the activity of the cytoplasmic aconitase aka iron regulatory protein 1 (IRP1), the importance of which for cellular iron homeostasis will be discussed more extensively later in this thesis (Walden et al., 2006). Genetic diseases of defective Fe-S cluster biogenesis reinforce the importance of this prosthetic group for cell function (Rouault, 2015).

In addition to free iron and iron in Fe-S complexes, iron set within the protoporphyrin ring of haem plays an essential role in cellular biochemistry by facilitating biological

reactions with oxygen (Poulos, 2014). Haem plays an important role not only in systemic oxygen delivery; for example in biosynthesis of prostaglandins by prostaglandin endoperoxidase synthase; xenobiotic detoxification by cytochrome P450 enzymes; NO production by the nitric oxide synthase enzymes iNOS, eNOS and nNOS in immunity and signaling; indolamine and tryptophan metabolism by indolamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase; electron transfer in mitochondria depends on the haem groups of complex II, III and IV working alongside Fe-S clusters (Kim et al., 2012; Poulos, 2014).

1.2 Iron homeostasis

Iron is interesting among micronutrients in that total body levels are maintained appropriately not through the regulated excretion of iron, but by modulating uptake through the intestine, particularly the duodenum (Ganz, 2011). The landmark discovery in our understanding of iron homeostasis was the identification of a small peptide hormone synthesized by the liver, hepcidin. The production of hepcidin was induced by iron loading (Pigeon et al., 2001), hepcidin deficient mice and humans showed profound iron accumulation (Nicolas et al., 2001; Roetto et al., 2003) and hepcidin injection was sufficient to cause a decrease in serum iron (Rivera et al., 2005). So hepcidin is a hormone that responds to iron levels and is required for iron homeostasis, but how does it control iron levels?

The only mammalian cellular exporter is ferroportin (FPN), expressed particularly on the basolateral surface of duodenal enterocytes and red-pulp (and similar)

macrophages (Abboud et al., 2000; Donovan et al., 2000; McKie et al., 2000). Constitutive global and enterocyte specific KO of ferroportin in mice is embryonic lethal, with inducible KO in enterocytes leading to progressive anaemia and enterocyte iron loading, firmly establishing ferroportin as the protein which transports iron from the enterocyte into the circulation (Donovan et al., 2005). Constitutive macrophage specific KO of ferroportin resulted in disrupted iron recycling and anaemia due to failure to supply sufficient iron to the erythron (Zhang et al., 2011). The link between hepcidin and ferroportin was found in 2004 when it was discovered that hepcidin treatment caused internalization and degradation of ferroportin blocking iron export and facilitating intracellular iron accumulation (Nemeth et al., 2004b). Subsequently mutations in ferroportin preventing hepcidin binding and hepcidin induced degradation were identified and these recapitulate hepcidin deficiency causing severe iron loading in mice and man (Altamura et al., 2014; Drakesmith et al., 2005b; Schimanski et al., 2005). Combining these observations with data concerning the relative flux of iron in the body suggests a situation where the majority of the body's iron requirements (20-25mg/day) are met through the degradation of senescent erythrocytes by red-pulp macrophages and subsequent release of iron, with a small, but important (1mg/day) contribution of dietary uptake to balance out the unregulated loss of iron (Drakesmith et al., 2015).

A grasp of cellular iron homeostasis is important to complete the picture of systemic iron homeostasis. Ferroportin controls iron efflux from the cell, but uptake of iron by the intestine is also critically dependent on apical uptake via the divalent metal

transporter 1 (DMT1) (Fleming et al., 1997; Gunshin et al., 2005a, 1997), facilitated by the reductase duodenal cytochrome B (DCYTB) (Gunshin et al., 2005b; McKie et al., 2001). For many cells uptake of iron from circulating transferrin by the transferrin receptor is critical for iron acquisition (Levy et al., 1999); iron is delivered into the acidifying endosome where ferrireductases such a Six-transmembrane epithelial antigen of the prostate 3 (STEAP3) reduce it for uptake into the cytosol via DMT1 (Muckenthaler et al., 2017). In the cytosol there is a ill-defined labile iron pool, which by poorly understood mechanisms is delivered to the sites of utilization, such as the mitochondria or iron-containing proteins like proyl-hydroxylases, whilst minimizing the possibility that it might cause oxidative damage to the cell. The moonlighting RNA binding proteins poly(rC)-binding protein 1 and 2 (PCBP1 and PCBP2) have been proposed to chaperone the labile iron pool (Nandal et al., 2011; Shi et al., 2008). Because of its reactivity free iron in the cell is tightly regulated and excess iron is sequestered within ferritin. The regulation of cellular iron homeostasis will be discussed in more depth in a subsequent chapter when we consider it in the context of lymphocyte physiology. In brief, IRP1 and IRP2 are two RNA-binding proteins that regulate translation of various iron associated gene mRNAs, via RNA stem-loop structures, in order to balance uptake, storage and release of iron as per the iron requirements of the cell (Muckenthaler et al., 2017).

To maintain systemic iron homeostasis, excess uptake from the diet needs to be prevented when the body is iron sufficient and promoted when the body is iron deplete, whilst fine tuning iron release from and flux through the storage tissues of

the liver and red-pulp macrophages to keep serum iron constant in the face of enhanced epithelial sloughing and erythropoietic iron demands. This is achieved through regulation of hepcidin expression in the liver by whole body iron status. Liver-produced hepcidin then controls absorption of iron from the diet by inhibiting ferroportin expressed by enterocytes. The different forms of hereditary haemochromatosis (genetic iron loading), have helped identify key molecular regulators of hepcidin expression including HFE, hemojuvelin (HJV) and transferrin receptor 2 (TFR2) (Papanikolaou et al., 2004). Administration of bone morphogenetic proteins (BMPs) is sufficient to induce hepcidin expression *in vitro* and *in vivo* (Babitt et al., 2007). The physiological importance of BMP signaling for iron homeostasis is highlighted by the discovery that the hemochromatosis associated gene HJV was found to be a co-receptor for BMP signaling in hepatocytes (Babitt et al., 2006). Levels of type I BMP signaling in the liver reflects iron status, with iron loading inducing hepcidin expression, SMAD1/5/8 phosphorylation and a slew of BMP responsive genes including inhibitor of DNA binding 1 *(Id1*), Atonal BHLH Transcription Factor 8 (*Atoh8*), *Smad6* and *Smad7* (Kautz et al., 2008). Deficiency of the genes encoding BMP6 and SMAD4 was subsequently found to recapitulate the hepcidin suppression and unrestrained iron loading observed in *Hjv* KOs, suggesting that BMP6 is an endogenous regulator of hepcidin expression and iron homeostasis (Andriopoulos et al., 2009; Wang et al., 2005). Recently it was found that increased total body iron stores induced BMP6 via the production of iron induced reactive oxygen species (ROS) in BMP6 secreting liver sinusoidal endothelial cells (LSEC), in an Nuclear factor erythroid 2-related factor 2 (NRF2) dependent manner (Canali et al., 2017; Lim et al., 2019). Interestingly LSEC BMP2 production has also been suggested to be important

for systemic iron homeostasis, but unlike BMP6, production of BMP2 does not change dynamically to reflect iron status (Koch et al., 2017).

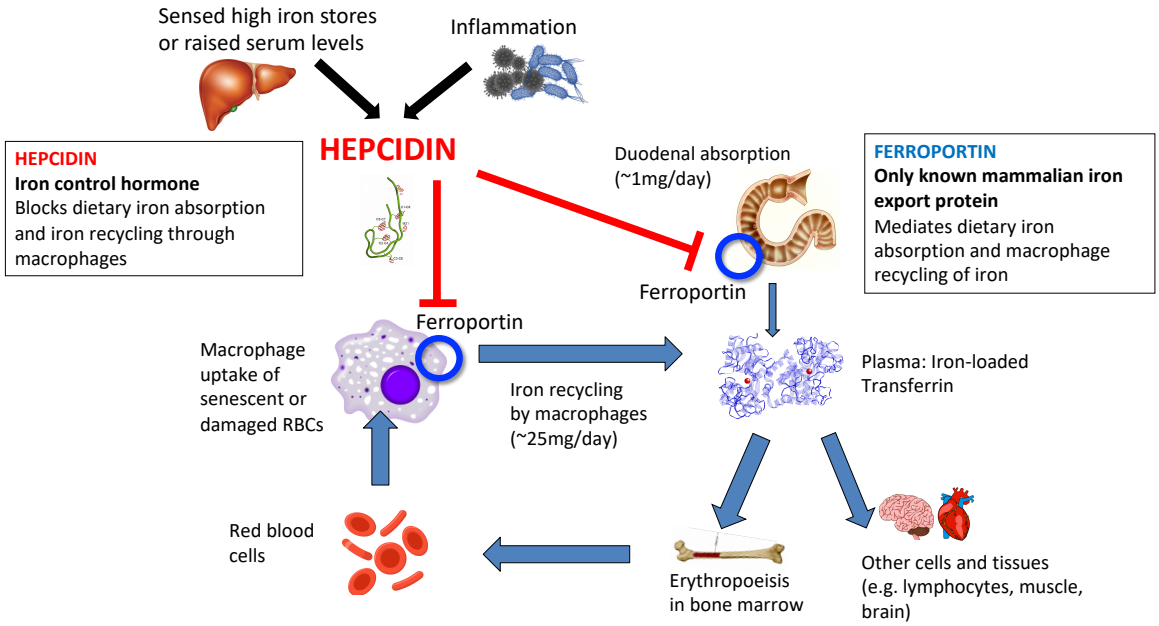
Whilst BMP6 production and signaling via HJV to control hepcidin transcription has been proposed to reflect longer term changes in total body iron content, responses to transient changes in transferrin saturation have been proposed to control hepcidin via a complex involving TFR1, TFR2 and HFE (Corradini et al., 2011; Schmidt et al., 2008a). TFR2 and HFE are thought to modulate sensitivity of hepatocytes to BMP signaling such that increased transferrin saturation increased SMAD1/5/8 phosphorylation and hepcidin expression (Ganz, 2011).

Cell type specific KOs suggest that hepatic hepcidin production is essential for maintaining systemic iron balance (Zumerle et al., 2014). However a role for non-hepatic hepcidin in local iron regulation, in tissues such as heart has been proposed (Lakhal-Littleton et al., 2016, 2015). Whether other tissues exploit hepcidin to locally regulate iron fluxes remains unclear.

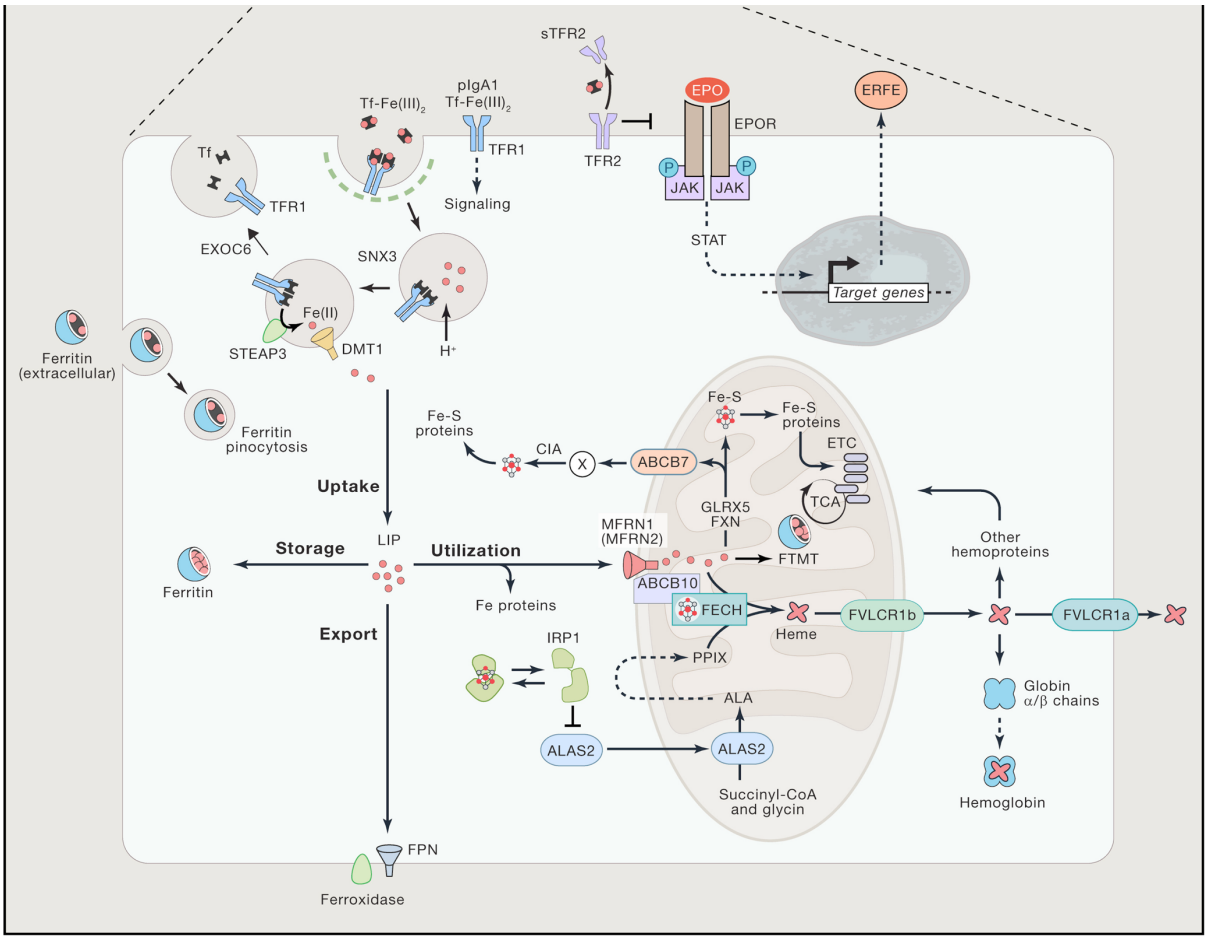
In addition to systemic regulation of iron flux out of the duodenal epithelia by hepcidin, the organismal response to iron deficiency is also critically dependent on local integration of signals. Iron deficiency transcriptionally upregulates expression of DMT1, DCYTB and Ferroportin in enterocytes, dependent on the local stabilization of HIF2α by intracellular iron deficiency. (Mastrogiannaki et al., 2009; Shah et al., 2009; Taylor et al., 2011). The IRPs also play an important role in modulating iron uptake at

the intestine through repressing ferritin accumulation which has the potential to sequester iron and limit flux (Galy et al., 2013). Thus, multiple regulatory mechanisms exist within and acting upon duodenal enterocytes to control iron flux from the diet into the circulation.

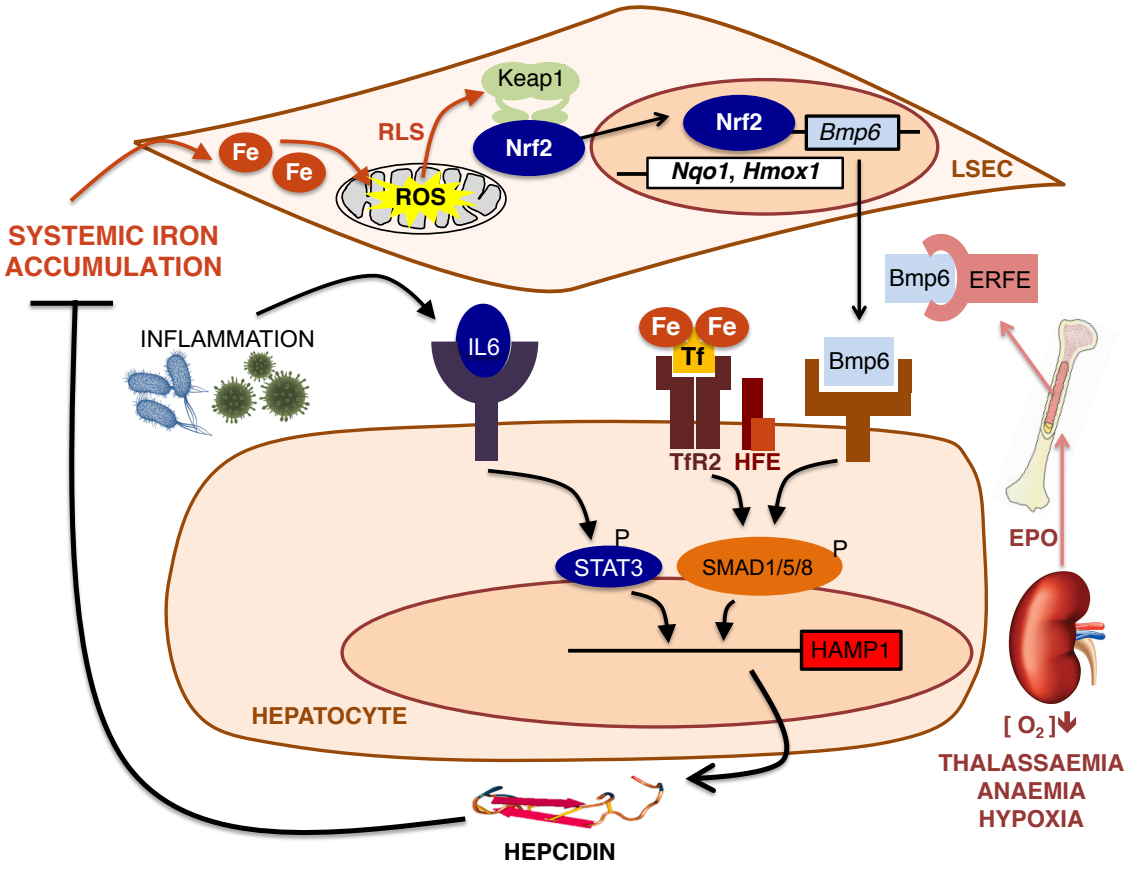
One of greatest acute challenges to iron homeostasis is the requirement to supply sufficient iron to rapidly make new red blood cells after phlebotomy, or to recover from hemolysis, adapt to high altitude or in response to erythropoietin (EPO) administration; unsurprisingly these situations are associated with suppressed hepcidin in order to increase iron release into plasma. Hepcidin suppression during recovery from hemolysis was inhibited by irradiation, therefore suggesting that a factor produced by hematopoietic cells is important (Vokurka et al., 2006). A number of putative erythroid regulators of hepcidin have been proposed, but the most definitive evidence supports a central role of erythroferrone in the suppression of hepcidin by enhanced erythropoiesis (Kautz et al., 2015, 2014). No direct receptor for erythroferrone is known, instead erythroferrone is proposed to act as a ligand trap for BMP6, binding it and preventing its interaction with its receptor on hepatocytes, so inhibiting a key signal that activates hepcidin transcription (Arezes et al., 2018).



Schematic summarising the systemic flux of iron through the body and the role of hepcidin and ferroportin in regulating it.



Schematic summarising the path and usage of iron in an erythroid cell. The iron physiology of other cells is thought to be analogous, aside from ALAS1 replacing ALAS2 and haemoglobin not being a major destination for iron. Fe, iron. Tf, Transferrin. TFR1, transferrin receptor 1. TFR2, transferrin receptor 2. FPN, ferroportin. LIP, labile iron pool. DMT1, divalent metal transporter 1. EXOC6, exocyst complex component 6. SNX3, sorting nexin 3. IRP1, iron regulatory protein 1. ALAS2, 5-aminolevulinate synthase 2. MFRN1, mitoferrin 1. FECH, ferrochelatase. PPIX, protoporphyrin IX. FXN, frataxin. GLRX5, glutaredoxin 5. FTMT, mitochondrial ferritin. CIA, cytosolic iron sulfur cluster assembly. ETC, electron transport chain. TCA, tricarboxylic acid cycle. FLVCR, feline leukaemia virus subgroup C cellular receptor family member. ERFE, erythroferrone. EPO, erythropoietin. TFR2, transferrin receptor 2. This image was reprinted with modifications from Cell, Vol 168, issue 3, Muckenthaler et al, Red Carpet for Iron metabolism, pages 344-361, Copyright 2017, with permission from Elsevier. (Muckenthaler et al, 2017).



Schematic summarising the control of hepcidin expression. Fe, iron. ROS, reactive oxygen species. KEAP1, kelch-like ECH associated protein 1. NRF2, nuclear factor erythroid 2-related factor 2. *Nqo1*, NAD(P)H Quinone Dehydrogenase 1. *Hmox1*, Haem oxygenase 1. Bmp6, Bone morphogenetic protein 6. ERFE, erythroferrone. EPO, erythropoietin. Tf, Transferrin. TfR2, transferrin receptor 2. HFE, Human homeostatic iron regulator protein. IL6, interleukin 6.

1.3 Hepcidin and hypoferremia of inflammation

Early in an inflammatory response a myriad of systemic physiological responses are induced, collectively called the acute phase response. Central to the acute phase response is the production of a battery of acute phase proteins by the liver in response to cytokines, such as interleukin (IL)-6, made by immune cells reacting to the infection. An interesting aspect of the acute phase response is a reduction in serum iron concentration and transferrin saturation and among these acute phase proteins is hepcidin (Armitage et al., 2011; Ganz and Nemeth, 2015; Nemeth et al., 2004a). Hepcidin, as discussed, targets ferroportin for degradation blocking the recycling of erythrocyte-derived iron out of hemophagocytic macrophages and favouring iron sequestration within the reticuloendothelial system. Chronically raised hepcidin results in a block in uptake of iron at the duodenum (Ganz, 2011). Hepcidin is required for inflammatory hypoferremia in mice (Armitage et al., 2016a; Deschemin and Vaulont, 2013; Gardenghi et al., 2014; A. Kim et al., 2014) and correlates with hypoferremia during human viral and bacterial infections (Armitage et al., 2014; Darton et al., 2015).

This inflammatory induction of hepcidin is thought to provide an innate defence mechanism against pathogens, starving them of iron (Arezes et al., 2015), but may also starve host cells in a similar manner to dietary iron deficiency (Scrimshaw and SanGiovanni, 1997). The evidence of inflammatory hypoferraemia and hepcidin production as being a protective response to infection primarily comes from

siderophilic extracellular bacteria such as *Vibrio vulnificus* and the 09 serotype of *Yersinia enterocolitica* (Arezes et al., 2015; Stefanova et al., 2018). However endogenous and therapeutic hepcidin had no effect on the growth of a *Y. enterocolitica* (O8), which encodes its own siderophore containing pathogenicity island, or *Staphylococcus aureus* (Stefanova et al., 2017). However what appears to be a simple story of hepcidin limiting the accessibility of non-transferrin bound iron to prevent opportunistic colonisation by pathogens which have made limited investment in iron acquisition, is made more complicated by research suggesting the hepcidin response can limit growth of pathogenic strains of *Escherichia coli* and *Klebsiella pneumoniae* both of which encode siderophores (Michels et al., 2017; Stefanova et al., 2018). Also, of note is that all of these models use constitutive hepcidin KO mice, making it hard to distinguish the effect of constitutive iron loading from the effect of hepcidin mediated hypoferraemia.

The importance of iron and hepcidin for intracellular bacterial infections *in vivo* is complicated. Two studies suggest that experimental iron loading, hepcidin deficiency and iron deficiency do not alter *Mycobacterium tuberculosis* growth (Harrington-Kandt et al., 2018; Stefanova et al., 2017). It has been proposed that raised hepcidin can lead to increased intra-macrophage iron levels, which in turn might facilitate growth of intracellular bacteria such as *Salmonella*. An 'unhelpful' hepcidin response has been demonstrated for *Salmonella typhimurium* infection (D.-K. Kim et al., 2014), but this is controversial and downregulation of ferroportin on *Salmonella* infected macrophages has been proposed to be hepcidin independent (Willemetz et al., 2017).

Furthermore, it has been suggested that hepcidin does not in fact increase intracellular iron availability for *Salmonella,* rather hepcidin prevents the ferroportin mediated export of iron into the *Salmonella* containing vacuole. This reduction in iron delivery to the *Salmonella* containing vacuole does not iron starve the pathogen and instead impairs ROS mediated host defence facilitating intracellular *Salmonella* growth (Lim et al., 2018).

Hepcidin is not the only acute phase protein related to iron homeostasis, hemopexin the plasma haem scavenger is also induced during infection. Depriving pathogens of haem through sequestration by hemopexin may also play a role in innate defence against some infections (Sakamoto et al., 2017).

The highly unfortunate demonstration that iron supplementation of iron deficient populations can increase the rate of malaria and diarrheal disease demonstrates how iron deficiency can be protective, but the role of hepcidin in this protection is unknown (Sazawal et al., 2006; Soofi et al., 2013). Beyond bacterial infection evidence for the protective role of hepcidin mediated hypoferremia is less clear. Broadly iron availability seems to be a positive modifier of fungal infections, but most fungal pathogens encode complex mechanisms for acquiring iron and hypoferraemia alone is unlikely to deprive them of iron (Matthaiou et al., 2018). In malaria infection iron restricted erythropoiesis suppresses blood stage *Plasmodium* growth (Clark et al., 2014) and raised hepcidin driven by ongoing blood stage infection has been proposed to cause hypoferraemia and liver iron deprivation sufficient to inhibit the liver stage

of a second superinfecting parasite (Portugal et al., 2011). The importance of iron in viral infection will be discussed more extensively in the later chapter on influenza infection, and has been reviewed previously (Drakesmith and Prentice, 2008).

1.4 Iron deficiency

Iron deficiency anaemia, the most documented clinical manifestation of iron deficiency, effects an estimated one sixth of the world's populations (Lopez et al., 2016a; Miller, 2013); iron deficiency without associated anaemia could be even more prevalent. Groups particularly at risk of iron deficiency are young children, non-pregnant and pregnant women, particularly in low and middle income countries (Kassebaum et al., 2014). Iron deficiency anaemia is thought to engender a mostly reversible negative impact on fitness and general wellbeing, however effects of iron deficiency on cognitive development can be irreversible and have long-term consequences (Armitage and Moretti, 2019; Georgieff, 2011). Iron availability may impact on growth rate, but conversely growth rate influences iron homeostasis (Armitage et al., 2019) and therefore further investigations are required to investigate how iron supplementation influences growth.

Iron deficiency can have a complex aetiology, most obviously it can result from insufficient dietary iron to balance physiological demands (including growth, menstrual loss and provisioning the placenta and foetus) (Armitage and Moretti, 2019; Lopez et al., 2016a), which physiologically results in low systemic iron in all compartments, and is termed absolute iron deficiency. It is also appreciated, as

discussed above, that the inflammatory response to infection, cancer and autoimmune disease can lead to raised hepcidin, which causes a 'functional' iron deficiency as dietary uptake is blocked and iron is sequestered in macrophages, contributing to development of anaemia (Weiss and Goodnough, 2005). A combination of dietary iron deficiency and raised hepcidin due to chronic inflammatory signals, intriguingly from respiratory infections, appears to drive iron deficiency anaemia in African children (Prentice et al., 2019) and likely a similar combined picture of diet and inflammation drive iron deficiency in the elderly (Busti et al., 2014).

Raised hepcidin and the dietary block of iron absorption complicates the use of iron supplementation as a solution for iron deficiency and anaemia. In the context of poor iron absorption, iron supplements increase gut luminal iron availability which can promote pathological changes in the gut microbiota (Paganini and Zimmermann, 2017). An association of iron supplementation with diarrheal disease has also been noted (Soofi et al., 2013). Furthermore iron deficiency anaemia seems to favour resistance to malarial infection, and attempts to resolve anaemia by iron supplementation in the absence of anti-malarial prophylaxis can increase the severity of malaria (Sazawal et al., 2006). The reciprocal interaction between inflammation, infection and iron homeostasis makes effective resolution of iron deficiency a complex problem, lacking a clear-cut answer and a clear understanding of the pathophysiological consequences of iron deficiency is required (Pasricha et al., 2018a).

1.5 Iron deficiency and the immune response

Understanding what regulates immunity in a global health context is important when infectious disease is the greatest contributor to under-5 mortality globally (H. Wang et al., 2016). Indirect evidence for an interaction between iron deficiency and immunity is provided by the increased prevalence of iron deficiency in low-and-middle income countries (LMICs) compared to high-income countries (Lopez et al., 2016a; Vos et al., 2017) and how this might relate to the observed phenomenon of reduced vaccine efficacy in LMIC settings. Reduced efficacy of a live attenuated influenza vaccine (Victor et al., 2016), relatively reduced T cell responses to an experimental malaria vaccine (Bliss et al., 2017) and impaired rotavirus vaccine efficacy (Madhi et al., 2010) in LMIC settings compared to high-income countries have been documented. Obviously iron deficiency is very unlikely to be the only or even the main contributor to iron impairment, but its prevalence makes its importance a determinant of immunity worth considering.

Historically there has been significant interest in the immunological phenotype associated with iron deficiency, almost wholly focusing on nutritional iron deficiency. A number of reviews have been written on the subject (Oppenheimer, 2001; Scrimshaw and SanGiovanni, 1997), here key historical findings will presented as well as more modern advances in the field. Comparison between iron deficient cases with iron replete controls is often troubled by the association of iron deficiency with other nutrient deficiencies, pre-existing inflammation and socio-economic status; one approach to reduce the number of confounders is the iron supplementation of one

arm of an iron deficient cohort. Furthermore, analysis is complicated by the lack of a standardised definition of iron deficiency with a combination of serum iron, soluble transferrin receptor, serum ferritin and potentially in the future serum hepcidin used to diagnose different categories of iron deficiency.

The ex vivo response of lymphocytes from iron deficient individuals to mitogen stimulus has been reported to be impaired (Chandra and Saraya, 1975). Experiments examining the capacity of splenocytes from animals subjected to dietary iron deficiency to proliferate *ex vivo* have yielded mixed results, dependent on the severity of the iron deficiency regime, with some work demonstrating that lymphocytes from iron deficient animals show reduced *in vitro* responses to mitogens (S. R. Kuvibidila et al., 2001; Kuvibidila and Porretta, 2003). Reduced *in vitro* splenocyte responses to T cell mitogens were accompanied with minor reductions in secretion of IL-10, interferon (IFN)-γ and IL-4 (Kuvibidila et al., 2003, 2012, 2010; Omara and Blakley, 1994). Other evidence suggests iron deficiency *in vivo* has little impact on subsequent activation of lymphocytes, whilst iron deficient culture conditions *in vitro* significantly reduce proliferation (Mainou-Fowler and Brock, 1985).

Some effects of prolonged iron deficiency on unstimulated lymphocytes have been noted, which might be responsible for impaired *ex vivo* responses. The role for iron in lymphocyte development, suggested by disrupted TFR1 activity blocking thymocyte development (Brekelmans et al., 1994; Macedo et al., 2004; Ned et al., 2003), is supported by observations in mice subjected to iron deficient diets presenting with

thymic atrophy associated with reduced thymocyte cell division (Kuvibidila et al., 2001). Thymic atrophy is also reported in rat models of iron deficiency (Kochanowski and Sherman, 1982).

Prolonged iron deficiency in animals has also been suggested to impair activation-associated increases in protein kinase C activity and hydrolysis of phosphatidyl inositol 4-5 bisphosphate, both important aspects of the signalling cascades downstream of activation (Klecha et al., 2005; Kuvibidila et al., 1999, 1998), and to decrease lymphocyte cluster of differentiation 28 expression (Kuvibidila and Porretta, 2003). Significantly reduced serum interferon-IFN-γ, IL-12 and IL-4 was detected in mice fed iron deficient diet for 7-8 weeks relative to control and pair fed animals (Kuvibidila et al., 2012; Kuvibidila and Warrier, 2004). A shorter three week course of low iron diet was not observed to perturb resting cytokine profiles (Bonaccorsi-Riani et al., 2015). Complementing this work is the observation of reduced serum IFN-γ and production of IFNγ by ex vivo stimulated leukocytes in children defined as having low serum iron concentration and low C reactive protein (CRP) (Contreras et al., 2016). In a separate cohort iron supplementation of patients with IDA was associated with higher serum IL-2 and IFNγ (Suega and Bakta, 2010).

More recently analysis of peripheral blood lymphocyte subsets in iron deficient individuals has given a range of outcomes. In children, iron deficiency associates with a decreased proportion of T cells expressing CD4 and CD8 relative to CD1a, taken to indicate fewer mature effector cells (Attia et al., 2009). Decreased absolute numbers

of CD4+ T cells and reduced CD4:CD8 ratio in iron deficiency anaemia groups have also been observed; iron supplementation resolved these abnormalities (Das et al., 2014). Reduced lymphocyte counts, in particular T cell counts, have also been noted in iron deficient adults (Keramati et al., 2011).

Historically, efforts have been made to distinguish how iron deficiency specifically affects both the humoral response to antigen—the reactive antibody titre— or how cellular immunity, in particular cytotoxic lymphocyte responses, are perturbed. This is an extremely important line of inquiry as it relates directly to how iron deficiency may impact on vaccination regimes.

A number of studies have found no association between iron deficiency and reduced general (Das et al., 2014; Sadeghian et al., 2010) and vaccine specific antibody titres (Chandra and Saraya, 1975), however the small sample size of these studies prevents a concrete statement that iron deficiency does not impact on the quality of the antibody response. Recently in a Chinese cohort the presence of iron deficiency at the time of sampling correlated with a reduced anti-measles antibody titre (Jiang et al., 2019). However, this correlation was only observed in samples taken from individuals over the age of 10, and it is not possible to link iron status at that age with the development of anti-measles immunity to a vaccine given many years earlier (at which point iron status was not determined). Accompanying animal data suggests that 5 weeks of low iron diet from 3-weeks of age results in a profound B cell lymphopenia

and a defect in humoral immune response, potentially via an effect on B cell cycle entry due to altered histone demethylase activity (Jiang et al., 2019).

Classically, one approach for assaying cellular immune responses *in vivo* was through examination of contact hypersensitivity responses or the local response to injected antigen. Immune responses to cutaneous injection of antigen were less frequent in an iron deficient cohort, suggesting that iron deficiency might suppress delayed hypersensitivity responses (Chandra and Saraya, 1975), an observation supported in animal models where severe dietary iron deficiency attenuated the response to the hapten dinitroflurobenzene and sheep red blood cells (Kuvibidila et al., 1981; Omara and Blakley, 1994).

In some studies, iron deficiency associates with a skew away from Th2 type cytokines and towards Th1 type cytokines upon *ex vivo* stimulation of lymphocytes from children (Jason et al., 2001). Mechanistically iron can suppress sensitivity to IFNγ through iron induced downregulation of interferon-γ receptor 2 in activated T lymphocytes and T cell leukaemia lines (Regis et al., 2005). Iron supplementation also antagonizes IFNγ induced gene expression in *ex vivo* human monocytes (Oexle et al., 2003). Mice deficient for the acute phase protein haemopexin, which is involved in the handling of free haem, show decreased mercury induced autoimmunity associating with reductions in T cell surface CD71 expression and sensitivity to IFN-γ; increased iron availability was suggested to be the source of the abnormality (Fagoonee et al., 2008). In addition hemopexin deficient mice were suggested to a generalised defect in

lymphocyte development and impaired ability to develop adaptive immune responses (Huntoon et al., 2008). Conversely more severe disease and enhanced Th17 responses was noted in haemopexin deficient mice in a model of experimental autoimmune encephalitis (Rolla et al., 2013).

One potential explanation for the observed effects of iron deficiency on T helper cell polarisation is the coupling between differentiation and cell proliferation; expression of stereotypical Th2 cytokine Il-4 required at least three cell divisions (Bird et al., 1998). Therefore, conditions, such as reduced iron availability, that restrict cell proliferation might especially suppress or delay Th2 type responses.

In more recent work, three weeks of low iron diet was found to attenuate the T cell dependent acute hepatitis and liver damage induced by injection of the plant lectin Concanavalin A (ConA) (Bonaccorsi-Riani et al., 2015; Tiegs et al., 1992). The phenotype correlated with a reduced proportion of T cells expressing IFNγ, and reduced hepatic and systemic inflammatory cytokines. The authors suggest that iron deficiency associated suppression of hepatic lymphocyte activation might contribute to the previously observed reduced frequency of spontaneous tolerance towards transplanted livers with low iron status, as lymphocyte activation facilitates clearance of reactive lymphocytes (Bohne et al., 2012). Iron status may therefore modulate auto-immune and anti-transplant immune responses (Schaefer et al., 2014).

Changes in iron intake are known to affect the gut microbiota of humans and laboratory animals (Guus Am Kortman, 2015; Paganini and Zimmermann, 2017; Zimmermann et al., 2010) and the extent to which the microbiota shapes the immune system, and immune responses, is being increasingly appreciated (Hagan et al., 2019; Hooper et al., 2012), therefore it is important to note that even in mice where the microbiota has been depleted, iron deficiency still attenuates Concanavalin A induced hepatitis (Bonaccorsi-Riani et al., 2015). How iron modulates immune response through altered interaction with the microbiota is yet to be fully explored (Guus Am Kortman, 2015).

Beyond numerous studies using animal models suggesting that for some pathogens, iron restriction provides an innate defence against infection (Drakesmith and Prentice, 2012), there are a few examples where iron deficiency was unhelpful. In a rat model of helminth infection, dietary iron deficiency resulted in delayed kinetics of worm expulsion from the small intestine and prolonged disruption of mucosal histopathology, which was partially ameliorated by injection of iron dextran (Bolin et al., 1977). Rats are normally resistant to secondary infection after clearance of primary infection naturally or with anthelminthic drugs. Iron deficiency impairs rapid clearance of secondary infection (Duncombe et al., 1979) and antagonized the ability of adoptively transferred lymph node cells from rats which have recovered from primary infection to protect naïve recipients from severe infection (Cummins et al., 1978). The influence of iron on immunity has not only focussed on infectious disease, iron has been implicated as promoting rheumatic disease, but detailed mechanistic studies and

clinical trials are lacking (Baker and Ghio, 2009). Iron loading has also been proposed to enhance experimental autoimmune encephalitis in mice by promoting pro-inflammatory cytokine production (Wang et al., 2018).

TFR1 expression is notably also a hallmark of activated lymphocytes, providing further evidence that iron is important for adaptive immune responses. Treatment with TFR1 blocking antibodies prevents mitogen induced DNA synthesis and proliferation of T and B cells (Neckers and Cossman, 1983), an observation mainly attributed to reduced activity of the iron dependent enzyme ribonucleotide reductase and a dearth of deoxyribonucleotides for DNA synthesis (Thelander et al., 1983; Yu et al., 2007). Iron deficiency may alternatively prevent entry into S phase by affecting cell cycle regulators; iron chelation reduces accumulation of cyclin dependent kinase 1 (CDK1) in activated lymphocytes (Terada et al., 1993). Links between iron and the cell cycle have been explored in the context of cancer and cell lines (Sanchez et al., 2006; Yu et al., 2007). Whilst B cell immunoglobulin secretion is not prevented by anti-TfR antibody treatment, net production is decreased as a consequence of reduced proliferation (Neckers et al., 1985, 1984). The importance of TfR during *in vitro* activation provides evidence that iron acquisition is a key cellular requirement for the mounting of an adaptive immune response.

The *in vivo* significance of iron uptake for adaptive immune function was recently demonstrated by Raif Geha and colleagues (Jabara et al., 2016) as the causal mutation in two families with unexplained combined immunodeficiency mapped to the

transferrin receptor. Affected individuals had normal circulating T, B and natural killer (NK) cells, but decreased memory B cells and low serum IgG. *Ex vivo* analysis of patient T and B cells indicated reduced capacity to proliferate in response to a range of stimuli and a block on B cell class switching. The mutation was found to reduce the efficiency of transferrin endocytosis; introduction of the same mutation into a mouse model recapitulated the deficiency in TFR1 internalization and *ex vivo* lymphocyte responses. Increasing the non-transferrin bound iron (NTBI) in the culture medium rescued the defect in *ex vivo* activation, demonstrating that a deficiency in iron acquisition was likely responsible for the severely impaired adaptive immune response of affected individuals.

1.6 Iron overload and adaptive immunity

Whilst iron deficiency is by far the most prevalent disrupted state of iron homeostasis worldwide, iron overload is clinically observed in hereditary haemochromatosis (HH), a family of genetic diseases where dysregulation of the hepcidin/ferroportin axis leads to unrestrained dietary iron uptake (Pietrangelo, 2004). Iron overload is observed in many patients with thalassemia. Iron overload, if severe, can lead to liver cirrhosis, arthropathy, cardiac complications and damage to the endocrine system (Ajioka and Kushner, 2003; Kremastinos Dimitrios T. et al., 2010). The immune phenotype of haemochromatosis and transfusion-dependent β-thalassemia has also received some attention; a subset of patients present with abnormally low circulating numbers of CD8+ T cells often correlating with more severe iron overload (Cunningham-Rundles et al., 2000; Porto and De Sousa, 2007). Iron homeostasis dependent and independent

effects of the *HFE* mutation on lymphocyte activation have been proposed to cause altered T cell homeostasis (Costa et al., 2015; Reuben et al., 2014), however analysis of the effect of *HFE* mutations on immunity is complicated by the position of *HFE* adjacent to the MHC. Ex vivo mitogenic responses of PBMCs were enhanced in thalassaemic patients after one or two cycles of iron chelation therapy (Cunningham-Rundles et al., 2000). Furthermore a reciprocal role for lymphocytes, in particular CD4 T cells, in modulating systemic iron homeostasis has also been suggested (Pinto et al., 2014; Porto and De Sousa, 2007).

Notably experimental iron overload has also been demonstrated to modulate the immune response, but the picture is confused; iron loading increased susceptibility in a murine model of *Candida* infection (Mencacci et al., 1997), but reduced immunopathology in a murine malaria model (Van Den Ham et al., 2015), and increased T cell activity and pathology in experimental autoimmune encephalitis (Wang et al., 2018). The effect of iron overload on immunity is a complicated one with multiple confounders such as linkage within the MHC for *HFE* and the immunomodulatory effects of multiple red blood cell transfusions. The literature suggests that too much iron can be as bad as too little iron.

1.7 Summary paragraph

Iron deficiency is the most prevalent micronutrient deficiency globally, affecting a billion people worldwide primarily in LMIC countries (Lopez et al., 2016). Iron deficiency has a complex aetiology, driven by both low dietary iron availability and

perturbation of systemic iron homeostasis due to unresolved inflammation (Armitage and Moretti, 2019; Pasricha et al., 2018a; Prentice et al., 2019). Despite iron being an essential part of cellular biochemistry, involved in processes as diverse as metabolism and DNA replication, the impact of iron deficiency on human health beyond anaemia is poorly understood (Armitage and Moretti, 2019). The global prevalence of iron deficiency co-incides with regions where infectious disease is a major driver of high mortality (Wang et al., 2016), but vaccine efficacy is lower compared to high-income countries. Uncovering and resolving the drivers of reduced vaccine efficacy could have important implications for global health; iron deficiency impairing adaptive immunity is one candidate. A number of studies in humans, animal models and *in vitro* have been conducted to investigate if iron deficiency can indeed impair adaptive immunity. However, it is hard to come to a firm decision as these studies have been under powered, suffered from poor experimental design and lacked detailed assessment of lymphocytes involved in the adaptive immune response. Recently a mutation in transferrin receptor that disrupts the ability of lymphocytes to acquire iron was found to cause immunodeficiency (Jabara et al., 2016) providing firm evidence that iron is essential for adaptive immune responses in human. However, to date if and how serum iron deficiency disrupts the magnitude and quality of antigen-specific adaptive immunity has not been investigated.

1.8 Unanswered questions

1. Can reduced serum iron concentrations limit the availability of iron to lymphocytes and therefore impair the development of an antigen specific adaptive immune responses to a vaccine *in vivo*?
2. If iron deficiency does impair adaptive immunity, what are consequences of this on vaccine efficacy in a global health context?
3. Can iron supplementation improve the quality of adaptive immune responses *in vivo*?
4. How do low serum iron levels and the adaptive immune responses interact in the context of infection with different pathogens, with different requirements for iron?
5. What are the specific molecular pathways within lymphocytes which are altered by iron restriction?
6. Beyond the transferrin receptor, what is the importance of the other mediators of cellular iron homeostasis in lymphocyte physiology?

1.9 Aims of thesis

In this thesis I aim to address:

1. If genetic disruption of iron uptake results in a cell intrinsic defect in adaptive immunity *in vivo*
2. The importance of the intracellular iron homeostatic system controlled by IRP1/2 for CD8 T cell function
3. The extent to which iron restriction *in vitro* alters CD8 T cell physiology, with a focus on metabolism

4. If iron deficiency *in vivo* alters the primary antigen specific CD8, CD4 and B cell response to immunisation
5. If transient iron restriction during the primary immune response alters the nature of CD8 T cell memory and secondary immune response
6. The impact of iron restriction on the outcome of influenza virus infection

Methods

2.1 Mice

Unless otherwise stated, animal procedures were performed under the authority of UK Home Office project and personal licenses in accordance with the Animals (Scientific Procedures) Act 1986, and were approved by the University of Oxford ethical review committee. Mice were housed in individually ventilated cages and fed ad-libitum with a standard diet containing 188ppm iron (SDS Dietex Services, diet 801161)

WT C57BL/6JOlaHsd mice were ordered from Envigo. C57BL/6 mice were used for immunisation experiments investigating the effect of serum iron deficiency on immunity. C57BL/6 mice were also as recipient mice for bone marrow chimera experiments and as a source of cells for *in vitro* experiments.

WT B6.SJL-Ptprca Pepcb/BoyJ (congenic C57BL/6 CD45.1 expressing mice) were bred in house at the University of Oxford.

Tfrc$^{Y20H/Y20H}$ mice were a kind gift from Raif Geha, Boston Children's Hospital, Harvard Medical School. *Tfrc*$^{Y20H/Y20H}$ were bred at the University of Oxford.

Tfrc$^{Y20H/Y20H}$ and B6.SJL-Ptprca Pepcb/BoyJ congenic WT controls were used as bone marrow donors for the chimerism experiments. *Tfrc*$^{Y20H/Y20H}$ and WT C57BL/6 mice as controls were used for *in vivo* immunisation experiments and *in vitro* T cell culture experiments.

OT-I CD45.1: Transgenic C57BL/6.Tg(TcraTcrb)1100Mjb (OT-I) mice crossed with BL6 congenic strain B6.SJL-Ptprca Pepcb/BoyJ in house were a kind donation from Vincenzo Cerundolo, University of Oxford. OT-I CD45.1 mice were used as a source of antigen specific donor T cells for *in vivo* experiments and to provide a source of murine CD8 T cells for *in vitro* experiments.

OT-II CD45.1/CD45.2 heterozygous mice. Transgenic B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II) mice were a kind donation from Oliver Bannard, University of Oxford, and crossed to congenic homozygous B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) to generate mice heterozygous for the Tg(TcrbaTcrb)425Cbn transgene, Pepc and Pepb alleles: OT-II CD45.1/CD45.2 heterozygous. OT-II CD45.1/CD45.2 heterozygous mice were used as a source of antigen specific donor T cells for *in vivo* experiments.

Inducible systemic hepcidin-knockout (*iHamp1-KO* or *Hamp1*$^{flox/flox}$ *Rosa26-CreERT2*) mice were generated in our lab as described (Armitage et al. 2016). Mice with LoxP sites flanking exon 2 and 3 of *Hamp1* were crossed with *Rosa26-CreERT2* mice which globally express, under the Gt(ROSA)26ser promoter, a Cre recombinase/estrogen receptor fusion protein which on tamoxifen treatment translocates to the nucleus.

Crossing of *Hamp1*$^{flox/flox}$ *Rosa26-CreERT2*$^{+/-}$ with *Hamp1*$^{flox/flox}$ *Rosa26-CreERT2*$^{-/-}$ generated litters of Cre+ *Hamp1*$^{flox/flox}$ *Rosa26-CreERT2*$^{+/-}$ (*iHamp1-KO*) or Cre- *Hamp1*$^{flox/flox}$ *Rosa26-CreERT2*$^{-/-}$ (*iHamp1-Ctrl*) for use in immunisation experiments examining the effect of iron loading on immune responses.

Aco1$^{flox/flox}$, *Ireb2*$^{flox/flox}$, *Rosa26-CreERT2* negative, termed IRP1/2$^{flox/flox}$ Cre -ve and *Aco1*$^{flox/flox}$,*Ireb2*$^{flox/flox}$, *Rosa26-CreERT2* positive animals, termed IRP1/2$^{flox/flox}$ Cre +ve were generated by Bruno Galy, DKFZ, Heidelberg.

2.2 Mouse genotyping

2.2.1 *iHamp1-KO* mice genotyping

PCR on ear tissue was used to genotype mice. Ear tissue was lysed in 75µl of alkaline lysis buffer (25mM NaOH, 0.2mM EDTA, pH= 12) at 95˚C for 2 hours. Lysate was neutralised with 75µl of 40mM Tris HCL (pH= 5.5). 5µl of Lysate provided template for PCR genotyping with MyTaqRed mix (Bioline, BIO-25044) and 0.2µM primers in a final volume of 25µl.

Primers for *iHamp1-KO* mouse genotyping PCR:

i) Cre-Forward (5'-CGTTTTCTGAGCATACCTGGA-3')

ii) Cre-Reverse (5'-ATTCTCCCACCGTCAGTACG-3')

PCR cycling conditions:

1) Initial denaturation: 5 mins 95˚C

2) Denaturation: 30 seconds at 95˚C
3) Annealing: 30 seconds at 60˚C
4) Extension: 45 seconds at 72˚C
5) Repeat steps 2 to 4 for a total of 35 cycles
6) Final extension: 10 mins at 72˚C

Cre-positive *iHamp1-KO* amplicon is 472bp

2.2.2 IRP1 genotyping procedure and primers

PCR on frozen cell pellets using Extracta DNA Prep for PCR (Quanta Bio, Extra DNA prep for PCR – Tissue 95091-002). 1µl of Lysate provided template for PCR genotyping with PCR Master Mix FASTSTART (Roche, ref: 04 710 452 001) and 1µM primers in a final volume of 25µl.

Primers for *IRP1* allele genotyping PCR:

i) Primer Delta RecIRP1_C (5´-tactgtagcaaaaatgctttgtctctg-3´)
ii) primer B38 (5´-tctatccctgaggtcggtaggc-3´)
iii) primer P1flxCRErev (5´-GTCATTTTTCTCATTTCTTGAGCATTAG-3´)

PCR cycling conditions:

1) Initial denaturation: 1 mins 94˚C
2) Denaturation: 30 seconds at 94˚C
3) Annealing: 1 minute at 60˚C
4) Extension: 1 minute at 72˚C
5) Repeat steps 2 to 4 for a total of 35 cycles
6) Final extension: 5 mins at 72˚C

Unrecombined floxed allele fragment is 296bp, recombined floxed allele fragment is 214bp.

2.2.3 IRP2 genotyping procedure and primers

PCR on frozen cell pellets using Extracta DNA Prep for PCR (Quanta Bio, Extra DNA prep for PCR – Tissue 95091-002). 1µl of Lysate provided template for PCR genotyping with PCR Master Mix FASTSTART (Roche, ref: 04 710 452 001) and 1µM primers in a final volume of 25µl.

Primers for *IRP1* allele genotyping PCR:

i) Primer A52 (5´-ggcttcaatagtcttcataccacg-3´)

ii) primer P2floxfwd (5´-CTGAAAGACTGACCCTTCTTGTTC-3´)

iii) primer Delta-RecIRP2-B (5´-TGAGTGGTGCCTGCATTTTAAG-3´)

PCR cycling conditions:

1) Initial denaturation: 1 mins 94˚C
2) Denaturation: 30 seconds at 94˚C
3) Annealing: 1 minute at 60˚C
4) Extension: 1 minute at 72˚C
5) Repeat steps 2 to 4 for a total of 35 cycles
6) Final extension: 5 mins at 72˚C

Unrecombined floxed allele fragment is 288bp, recombined floxed allele fragment is 199bp.

2.4.1 Injected substances

Mini-hepcidin: An appropriate mass of mini-hepcidin PR73 (Arezes et al., 2015) (da-TH-Dpa-bhPro-RCR-bhPhe-Ahx-Ida(Hexadecylamine)-NH2) was dissolved in 80% ethanol and then mixed with 60mg of Purebright SL220/ Sunbright DSPE-020CN (NOF). The control solution was Purebright SL220 dissolved in ethanol. The ethanol was evaporated off using a vacuum chamber warmed to 50˚C. The resultant gel was stored up to 24 hours at 4˚C and re-dissolved in an appropriate volume of water to give a mHep concentration of 1mM: 100nMoles of mini-hepcidin in 100ul of water was injected per mouse per dose.

Ferric ammonium citrate (FAC) (F5879 Sigma Aldrich)**.** FAC was dissolved to 3mg/ml in PBS, 100ul was injected intraperitoneally into mice (300µg/mouse).

Tamoxifen. *iHamp1-KO or iHamp1-Ctrl* mice were injected i.p. with 1mg tamoxifen (Sigma, T5648) in 90% corn oil/ 10% ethanol on day 2 post immunisation.

2.5.1 Immunisations

Modified Vaccinia Virus Ankara expressing Ovalbumin: MVA-OVA was a kind gift from Simon Draper, University of Oxford. Virus was diluted to 10^7 PFU/ml in PBS, 100ul was injected sub-cutaneously into the flank of mice.

Adenovirus expressing Ovalbumin: AdOVA was a kind gift from Simon Draper, University of Oxford. Virus was diluted to 10^10 VLP/ml in PBS, 100ul was injected sub-cutaneously into the flank of mice.

BMDCs were derived as described in cell culture. On day 7 of culture BMDCs were activated with 1µg/ml LPS (ultrapure K12 LPS, cat. No. tlrl-peklps, Invivogen) overnight and the following morning pulsed with 0.5µM B8R peptide (AS-64688 Tebu-bio. Aa sequence TSYKFESV) for 2 hours before washing and injecting 2*10^6 BMDCs i.v. per mouse.

For OVA protein immunisations mice received 200µg (OT-I) or 800ug (endogenous responses) of OVA (endofit Ovalbumin vac-pova, Invivogen), 25µg of MPLA (vac-mpla, Invivogen) and 1µg of alpha-galactosylceramide (gift from Vincenzo Cerundolo or KRN7000 Enzo Biotechnology).

2.6.1 Adoptive transfers

For experiments involving response of OT-I CD8 T cells, red cell lysed suspensions from an OT-I CD45.1 spleen was obtained and the frequency of OT-I CD8 T cells evaluated by flow cytometry. The concentration of whole spleen suspension was adjusted to 50,000 CD8 T cells/ml and 100ul of cell suspension in PBS was injected i.v.

For experiments involving response of OT-II CD4 T cells, red cell lysed suspensions from an OT-I CD45.1 spleen was obtained and the frequency of OT-II CD4 T cells assayed by flow cytometry. The concentration of whole spleen suspension was adjusted to 500,000 CD4 T cells/ml and 100ul of cell suspension in PBS was injected i.v.

2.7.1 Mixed bone marrow chimeras

For mixed bone marrow chimerism experiments a mix of 60% Tfrc$^{Y20H/Y20H}$ CD45.2 (or WT CD45.2) bone marrow was combined with 40% CD45.1 bone marrow to test the capacity of Tfrc$^{Y20H/Y20H}$ cells to reconstitute and contribute to immune responses relative to wild type cells. Male CD45.2 recipients at 8-weeks of age received were lethally irradiated with 4.5 Gy for 300 seconds, followed by a 3-hour rest, and a subsequent 4.5 Gy dose for 300 second. Mice received 2 million total bone marrow cells from the specified mice, after 9-weeks of rest peripheral blood was taken to analyse reconstitution by flow cytometry. To prevent bacterial infection recipient mice received antibiotics in their drinking water (0.16mg/mL Enrofloxacin (Baytril), Bayer Coporation).

2.8.1 Cell culture

Complete R10. RPMI with 10% FCS (sigma), glutamine (2mM), penicillin (100U/ml), streptomycin (0.1mg/ml) and Beta-mercaptoethanol (55µM). All centrifugation steps are 400g at 4˚C for 5 mins.

BMDCs. Red cell lysed bone marrow homogenates were cultured for 7-8 days at 1.5*10^6 live cells/ml in complete R10 with 100ng/ml FLT3L (550702 Cat. No. Biolegend). Cells were washed and re-cultured with 50ng/ml FLT3L, 1µg/ml (lipopolysaccharide) LPS or 0.5mM CpG ODN-1585 (tlrl-1585 Invivogen) to activate.

Iron modified culture medium. Pannexin NTS serum substitute (P04-95080; Pan Biotech) lacking iron was ordered. Iron free Pannexin NTS serum substitute was added at 10% v/v to RPMI alongside glutamine (2mM), penicillin (100U/ml), streptomycin (0.1mg/ml) and Beta-mercaptoethanol (55µM) to make iron free complete medium. To modify the iron content of the iron free complete medium, holotransferrin (T0665 Sigma Aldrich) was added at the specified concentration. Apotransferrin (T1147 Sigma Aldrich) was also added to iron free complete medium to maintain the total transferrin concentration of the medium at 1.2mg/ml.

CD8 T cells. Single cell suspensions were made from cervical, inguinal, axillary and brachial lymph nodes by mechanical dissociation through a 40µm cell strainer. When proliferation was analysed lymph node suspensions were resuspended in 500ul of PBS with 5µM Invitrogen Cell Trace Violet (C34557) and stained at 37˚C for 8 minutes before quenching with 12ml of complete medium, washing and proceeding to T cell isolation. CD8 T cells were isolated from the lymph nodes of WT C57BL/6, *Tfrc*$^{Y20H/Y20H}$, OT-I CD45.1 using the Stem Cell Technologies CD8 T cell isolation kit (19853, Stem Cell Technologies) and Easy Plate Magnet, and from IRP1/2$^{flox/flox}$ Cre -/+ ve mice using the

Miltenyi Biotech negative CD8 T cell isolation kit (130-104-075, Miltenyi Biotech) as per manufactures instructions.

Flat bottom tissue culture treated plates were coated with 5μg/ml of anti-mouse CD3 (145-2C11) for 2 hours at 37˚C and then washed with PBS. CD8 T cells were plated in complete medium with a final concentration of 100U/ml mouse IL-2 and 1μg/ml anti-mouse CD28 (37.51).

Cultures involving *Tfrc*$^{Y20H/Y20H}$, WT C57BL/6 cells or IRP1/2$^{flox/flox}$ Cre -/+ ve were carried out in complete R10 medium. Cultures examining the effect of reduced iron concentration on WT T cells from C57BL6/ or OT-1 CD45.1 mice were performed in iron free complete medium.

Final concentrations of CD8 T cells were 500,000/ml for analysis at 72hrs post activation for proliferation analysis or 700,000/ml for analysis at 24hrs post activation for activation analysis.

For culture of IRP1/2$^{flox/flox}$ Cre -/+ ve cells were treated with either 4-hydroxytamoxifen (4-OHT) at 100nM or a comparable volume of pure ethanol concurrent with the activation mixture of soluble aCD28, IL-2 and plate-bound aCD3. When cell proliferation was to be analysed, after 24hrs of culture cells were harvested, spun down and resuspended in fresh medium with 100U/ml mouse IL-2 and 1μg/ml anti-mouse CD28 to remove 4-OHT or EtOH and re-plated in wells coated with anti-

mouse CD3. Cells were cultured for an additional 48hrs and proliferation was evaluated by flow cytometry.

For iron rescue experiments iron sulfate ($FeSO_4$) or ferric ammonium citrate (FAC) was added at the indicated concentration from the start of culture and replenished in the case of the IRP1/2$^{flox/flox}$ Cre -/+ ve experiments after 24hrs when 4-OHT/EtOH was removed.

2.9.1 Tissue processing

Mice were euthanised in increasing CO_2 concentrations. ~3-mm^2 Liver pieces were harvested in RNAlater (AM7020 Thermofisher Scientific) for RNA extraction and gene expression analysis. 10mm^2 Liver pieces were snap frozen for non-haem iron measurements. For histology Liver slices across the lobe (<5mm) were placed in 4% paraformaldehyde in phosphate buffer for 48hours.

Analysis of spleen and lymph node populations by flow cytometry. Single cell suspensions of lymph nodes or spleen were made by mechanical dissociation through a 40μm cell strainer. Red cells were lysed in spleen suspensions using Tris ammonium chloride (ACT) Red cell lysis buffer (2.06g/L Tris base and 7.47g/L NH_4Cl , 1L H_20, adjusted to pH 7.2).

Lung tissue processing from Flu mice. The large single left lung lobe was placed in 3ml of R10 on ice for flow cytometry (day 8 experiment) or placed in 4% paraformaldehyde in PBS for histological analysis (day 10 experiment). The right lung lobes were divided in two and snap frozen for RNA analysis etc.

Isolation of Lung lymphocytes (Flu experiments). Lungs were minced with scissors and digested for 30 minutes in 4ml of digestion medium: RPMI, 40U/ml DNAse I (Sigma-Aldrich, catalog number: D5025) and 0.4mg/ml of Collagenase IV (Sigma-Aldrich, catalog number: C5138). Single cell suspensions were made by crushing through a 70µM cell strainer. Cells were pelleted and red cell lysed with ACT Red cell lysis buffer. A 40%/80% Percoll (GE Healthcare 17-0891-02) layer isolation was then used to enrich for lymphocytes in the lung suspension. Enriched lymphocytes were then washed and counted before flow staining.

For analysis of peripheral blood cell populations up to 100µl of blood from a tail bleed (recovery) or cardiac puncture (immediately after euthanasia) placed in a BD microtainer EDTA tube (Beckton Dickinson). For red blood cell parameters, a 100µl of whole blood was ran through a haematology analyser. For analysis of leukocytes 100µl of whole blood was mixed with 1ml of ACT Red Cell lysis buffer and incubated at room temperature for 20 minutes. The blood solution was spun down at 400g for 5 minutes, supernatant removed, and the leukocyte pellet transferred to a round bottom 96 well plate for flow cytometric staining.

For serum analysis up to 400µl of blood obtained by cardiac puncture was placed in a BD microtainer SST tube (Beckton Dickinson). Serum was obtained by spinning the clotted blood sample was spun at 8,000g for 5 minutes and stored at -80˚C. Serum iron was quantified using the Abbott Architect c16000 automated analyzer (Abbott Laboratories) and the MULTIGENT Iron Kit at Oxford John Radcliffe Hospital, UK.

2.10. Influenza virus

2.10.1 Influenza virus propagation

Influenza virus production and quantification were carried out by our collaborator Tiong Tan. Influenza virus (A/X-31 (H3N2) (a reassorted virus containing the HA & NA from A/Aichi/2/1968 (H3N2) and internal genes of PR8 (Kilbourne, 1969)) originally produced in embryonated eggs, were propagated in MDCK-SIAT1 cells as previous described (Powell et al 2012). Haemagglutination assays and as $TCID_{50}$ were used to quantify virus. Haemagglutination Assays were performed as previously described (Powell et al 2012). In brief, HA titer was measured by adding 50µl of 1% (vol/vol) human red cells (adjusted so that a 1:2 dilution gave an optical density at 600 nm [OD600] of 1.8) to dilutions of virus in 50 ul PBS in V-bottomed 96-well plates. Hemagglutination was read at 1h by loss of teardrop formation after tilting the plate.

The $TCID_{50}$ was determined in MDCK-SIAT1 infected with a ½-log dilution series of viruses in VGM (Viral Growth Media; DMEM, 1% BSA, 10mm HEPES buffer and Penicillin/Streptomycin for 1 hour in 8-replicates on a 96-well flat-bottomed plate,

then 150 µl of VGM with TPCK-treated trypsin (1 µg/mL) were added and incubated for 48hours at 37C. TCID50 was calculated using the method of Reed and Muench (Reed and Muench, 1938)

2.10.2 Influenza Microneutralisation assay

Microneutralization assays MN assays were performed as described (Powell et al., 2012) by our collaborator Tiong Tan with minor modification. In brief, S-eGFP (X-31) (single cycle influenza virus expressing eGFP) were titrated to give saturating infection of $3X10^4$ MDCK-SIAT1 cells on a 96-well plate. Murine serum samples from each mouse collected on day of cull were heat inactivated for 30 min at 56˚C. Dilutions of sera were incubated with S-eGFP and incubated for 2 hours (5% CO_2, 37°C). Indicator MDCK-SIAT1 cells ($3X10^4$ per well) were then added and the mixtures were incubated overnight. A virus-only control and a cells-only control were included for each plate. Plates were incubated for 24 hours (5% CO_2, 37°C). Post-incubation, 10% formalin was added to each well and plates were incubated at 4°C for 30 min. For MN assays using S-eGFP, formalin was removed and plate was washed 3 times with 200 µL PBS. Wells were then filled with 100 µL and GFP intensity was quantified using a ClarioStar plate reader and titres reported as fold-dilution of samples that resulted in 50% reduction of the GFP signal.

2.10.3 Mouse challenge with influenza

Mice were anaesthetised with aerosolised isoflurane and infected with influenza virus X-31 at the specified dose intranasally. Virus suspension was pipetted drop wise onto the nares such that the virus is inhaled.

2.11 Vaccinia virus

1.11.1 Vaccinia virus challenge

Vaccinia virus experiments were carried out in collaboration with Lise Chauveu. The thymidine kinase negative vaccinia virus based off the western reserve background expressing HIV-1 HXB.2 Gag (vVK1) was from the NIH AIDS reagent program (Karacostas et al., 1989), kind gift from Professor Persephone Borrow. For vaccinia virus challenge, mice were injected intraperitoneally with $3x10^6$ PFU in 100µL PBS. Weight was monitored daily for 5 days then mice were humanely killed by inhalation of carbon dioxide and cervical dislocation and ovaries were collected for virus titration.

1.11.2 Vaccinia virus plaque assay

Ovaries collected in D0 (DMEM, 1% PenStrep) were homogenized using glass beads in screw cap tubes and a vibrating tissue homogeniser. Samples were then subjected to 3 freeze-thaw cycles between 37°C and dry ice and sonicated 3 times for 30s with 30s intervals on ice. Supernatants containing virus were collected in new tubes after centrifugation at 10,000 rpm for 3 min at 4°C.

143B cells were seeded in 12 well plates at a density of $0.25x10^6$ cells per well in 1mL D10 (DMEM, 10% FCS (sigma), glutamine (2mM), penicillin (100U/ml), streptomycin (0.1mg/ml)). The next day, log serial dilutions of samples were prepared in D0 (DMEM,

glutamine (2mM), penicillin (100U/ml), streptomycin (0.1mg/ml)). Cells supernatant was replaced with 550ul of diluted samples and incubated for 2h at 37°C, swirling plates every 30 min to avoid drying. Virus containing samples were then removed and cells were covered in 1.5mL of D10, 1% P/S, 0.5% carboxymethylcellulose (CMC). 48h after infection, cells were carefully washed with PBS and fixed in 4% formaldehyde for 20 min at RT before staining with 0.5% crystal violet.

2.12 Flow cytometry

2.12.1 surface staining

Tissue cell suspensions or *in vitro* activated cells at appropriate time points after activation cells were transferred to a 96 well round bottom plate, spun down and washed with 200µl of PBS. Cells were stained with appropriate concentrations of FC receptor block, fluorophore conjugated antibodies and a fixable live dead dye in 40µl of PBS for 20 minutes at 4˚C in the dark. Cells were washed and ran directly on an Invitrogen Attune or BD LSR Fortessa. For some experiments, cells were fixed for 10 minutes in 100ul of 4% paraformaldehyde at 4˚C in the dark before washing and resuspension for analysis or resuspended in saponin based perm buffer to stain intracellular antigens.

Tetramer staining using reagents from the NIH tetramer facility was carried out at room temperature for 30 mins in FACs buffer (PBS with 2% FCS and 0.04% Sodium Azide) prior to antibody staining

Cells were stained with Mitotracker Green (M7514, Thermofisher Scientific) and Mitotracker Deep Red (M22426, Thermofisher Scientific) at 100nM or 2NBDG (N13195 Thermofisher Scientific) at 100µM in complete media (either iron deficient medium for serum iron deficiency cultures or R10 for *Tfrc*$^{Y20H/Y20H}$ cultures) for 30mins at 37˚C.

2.12.2 Intracellular cytokine staining

For detection of intracellular cytokines splenocytes were plated at 2 million cells/200µl in complete media in a round bottom 96 well plate with 0.1µg/ml SIINFEKL peptide for OVA specific responses or 0.5µg/ml B8R peptide for Vaccinia specific responses. After 30mins 5µg/ml Brefeldin A was added and cells were cultured for a further 4 hours before surface/intracellular staining for flow cytometry.

For detection of MVA specific CD4 responses by ICS, day 7 BMDC were plated at 0.3*10^6 cells per well of a 96 well plate with 50ng/ml FLT3L. BMDCs were either left unstimulated, non-specifically stimulated with 0.5µM of CpG or infected with MOI of 1 of MVA-OVA overnight. The following day the cells were washed and 2 million splenocytes from unimmunised or MVA immunised mice were added. After 30mins 5µg/ml Brefeldin A was added and cells were cultured for a further 4 hours before surface/intracellular staining for flow cytometry. Cytokine secreting CD4 T cells were defined as CD154+ cytokine+ (compared to unstimulated CD4 on CpG pulsed DCs or CD4s from naïve mice).

2.13 RNA isolation, cDNA synthesis and qRT-PC

RNA was isolated by lysing a 3mm^2 liver chunk in 700µl of RLT+ buffer or a single whole lung lobe in 2ml of RLT+ buffer. 350µl of lysate was used for RNA extraction as per the manufactures instructions using Qiagens RNA-easy Plus kit (Qiagen, 74136). cDNA was synthesised using High capacity RNA-to-cDNA kit (Thermofisher Scientific, 4387406). qPCR was undertaken on 1-5ng/ml of cDNA using Taqman gene expression master mix (Thermofisher Scientific, 4369016) and expression assays as in (Armitage et al., 2016b).

2.14 Tissue iron measurements

Tissues were dried for 3 hours at 100˚C, and the dry mass of tissue piece was measured. Tissue pieces were digested in 10% trichloroacetic acid/ 30% hydrochloric acid for 20 hours at 65˚C. Non-haem iron content was determined by reaction of the acid digested tissue with chromogen reagent containing 0.1% (w/v) bathophenanthrolinedisulphonic acid (Sigma, 146617) and 0.8% thioglycolyic acid (Sigma, 88652) measuring absorption at 535nm and comparing to a standard curve of ferric ammonium citrate as in (Armitage et al., 2016b)

2.15 Histology

Spleen sections (0.5mm long) and a single lung lobe were fixed overnight in an excess volume of 4% paraformaldehyde in PBS, before transferring into PBS for storage at 4˚C ready for shipping to Dr. Tiago L. Duarte (Instituto de Investigação e Inovação em Saúde, Porto, Portugal) who carried out the rest of the histological analysis. Samples were embedded in paraffin and sectioned. Following deparaffinisation with xylene and hydration by passage through a grade of alcohols, 3µM sections were stained with haematoxylin-eosin or Perl's Prussian blue reaction for ferric iron using standard procedures.

2.16 Seahorse metabolic flux analysis

ATP production rate and the relative contribution of mitochondrial and glycolytic metabolism to this was calculated using the Agilent Seahorse XF real-time ATP rate assay kit (103592-100) and an Aligent XF96 instrument. Metabolic flux analysis of lymphocytes was carried out in collaboration with Barbara Kronsteiner-Dobramysl.

The methodological approach for seahorse metabolic flux analysis was performed as described with minor modifications (van der Windt et al., 2016). The sensor cartridge was hydrated with 200µl of dH2O per well overnight at 37˚C in a humidified incubator without CO2. On the day of the assay, the water was replaced by 200ul of XF Calibrant solution (Agilent) per well and incubated under the conditions above for at least one hour prior to running the assay. The culture wells of the 96 well plate were coated

with 30µl of poly-D-lysine (100µg/ml in H_2O, A-003-E Sigma) for one hour at 37C, washed with H_2O, allowed to dry completely and stored at 4-8˚C until use.

In vitro activated CD8 T cells were cultured in iron restricted conditions as described in cell culture. After 48 hours of culture the CD8 T cells were washed and resuspended in XF assay medium (XF RPMI pH 7.40 103576-100 supplemented with 2mM L-Glutamine 103579-100, 1mM Sodium pyruvate 103578-100 and 10mM Glucose 103577-100 all from Agilent) and counted. The concentration was adjusted to 3 million viable cells/ml and 50µl of the cell suspension, equivalent to 150,000 cells, was aliquoted per well. The plate was spun at 400g, 5 mins with a low deceleration setting and T cells were allowed to settle and adhere for 30min at 37˚C without CO2 in a humidified incubator. An additional 130µl of XF assay medium was carefully added to each well so as not to disturb the monolayer and the cells were incubated under the conditions above for another 30-40min. The formation of a monolayer of T cells was checked under a light microscope before starting the assay.

Metabolic inhibitors oligomycin, and rotenone and antimycin A (Rot/AA) were made up to 10X stock concentrations according to the manufacturer's instructions. 20µl of Oligomycin were added to each port A on the cartridge and 22µl of Rot/AA were added to each port B, which resulted in a final concentration of 1.5uM Oligomycin and 0.5uM Rot/AA per well. The run settings for the Agilent Seahorse XF real-time ATP rate assay kit was selected on the XF96 instrument as recommended by the manufacturer and the assay was run.

Glycolytic ATP production rate was calculated through the measurement of extracellular acidification (ECAR), aka the rate of proton production (pmol H^+ /min). The stoichiometry of glycolysis generates one molecule of ATP per proton/molecule of lactate produced. GlycoATP (pmol ATP/min) = glycolytic ECAR (H^+/min)

Mitochondrial ATP production rate was derived from the oxygen consumption rate (OCR). The mitochondrial OCR associated with ATP production (OCR_{ATP}) (pmol O_2 /min) rate was calculated as the total OCR - the OCR after addition of the ATP synthase inhibitor Oligomycin. The mitoATP production rate is then derived from an experimentally calculate coefficient that for a broad range of cell lines 2.75 molecules of ADP are phosphorylated for each atom of oxygen. So mitochondrial ATP (pmol ATP/min) = 2 (pmol O/pmol O_2) X 2.75 (pmol ATP/pmol O_2) X OCR_{ATP} (pmol O_2 /min).

Total ATP production rate is simply the sum of glycoATP + mitoATP production rates

2.17 RT-qPCR taqman assays

Hypoxanthine-guanine phosphoribosyltransferase	*Hprt*	Mm01545399_m1
Bone morphogenetic protein 6	*Bmp6*	Mm01332882_m1
Hepcidin	*Hamp1*	Mm04231240_s1
Inhibitor of DNA-binding protein 1	*Id1*	Mm00775963_g1
Sons of mothers against decapentaplegic 6	*Smad6*	Mm00484738_m1
Sons of mothers against decapentaplegic 7	*Smad7*	Mm00484742_m1
Interferon gamma	*Ifng*	Mm01168134_m1
Interleukin 2	*Il2*	Mm00434356_m1
Interleukin 6	*Il6*	Mm99999064_m1
Interleukin 10	*Il10*	Mm01288386_m1
Serum amyloid A1	*Saa1*	Mm00656927_g1
Granzyme B	*Gzmb*	Mm00442837_m1
Tumour necrosis factor alpha	*Tnf*	Mm00443258_m1

2.18 Antibodies used for flow cytometric analysis of lymphocyte populations

Biolegend	Fluorophore	Target	Clone	Catalogue code
	Purified	CD16/32	93	101302
	FITC	CD8	53-6.7	100705
	FITC	CD3	17A2	100203
	FITC	CD71	R17217	113805
	FITC	CD11b	M1/70	101205
	FITC	CD4	GK1.5	100405
	FITC	CD45.1	A20	110706
	FITC	CD62L	MEL-14	104405
	FITC	IgM	RMM-1	406505
	FITC	GL7	GL7	144603
	FITC	GZMB	GB11	515403
	FITC	CD44	IM7	103005
	FITC	CD25	PC61	102005
	FITC	TCRb	H57-597	109205
	FITC	TNFa	MP6-XT22	506303
	PerCP/Cy5.5	CD4	GK1.5	100433
	PerCP/Cy5.5	CD8	53-6.7	100733
	PerCP/Cy5.5	TCRb	H57-597	109227
	PerCP/Cy5.5	IgD	11-26c2a	405709
	PerCP/Cy5.5	B220	RA3-6B2	103235
	PerCP/Cy5.5	CD90.2	30-H12	105337
	PE	CD71	R17217	113807
	PE	CD43	1B11	121207
	PE	CD69	H1.2F3	104507
	PE	CD62L	MEL-14	104407
	PE	CD98 (4F2)	RL388	128207
	PE	CD127	SB/199	121111
	PE	CD138	281-2	142503
	PE	CD154	MR1	106505
	PE	CD185 (CXCR5)	L138D7	145503
	PE	CX3CR1	SA011F11	149005
	PE	IL-2	JES6-5H4	503807

Biolegend	Fluorophore	Target	Clone	Catalogue code
	PE/Cy7	CD71	R17217	113811
	PE/Cy7	CD8	53-6.7	100707
	PE/Cy7	CD279/PD-1	RMPI-30	109109
	PE/Cy7	IFNg	XMG1.2	505825
	PE/Cy7	KLRG1	2F1/KLRG1	138415
	PE/Cy7	CD98	RL388	128213
	PE/Cy7	Tcrb	H57-597	109221
	PE/Cy7	CD69	H1.2F3	104511
	PB	CD3	17A2	100213
	PB	CD8	53-6.7	100728
	PB	CD44	IM7	103019
	PB	Tcrb	H57-597	109225
	PB	GL7	GL7	144613
	BV421	CD27	LG.3A10	124233
	BV421	CD71	R17217	113813
	BV412	CD127	A7R34	135027
	BV421	CD138	281-2	142507
	BV421	TNFa	MP6-XT22	506328
	BV605	CD8	53-6.7	100744
	BV605	B220	RA3-6B2	103244
	BV605	CD45.1	A20	110737
	BV605	IL-2	JES6-5H4	503829
	BV785	CD44	IM7	103041
	BV785	CD8	53-6.7	100749
	BV785	CD3	17A2	100231
	BV785	CD45.1	A20	110743

Biolegend	Fluorophore	Target	Clone	Catalogue code
	AF648	GZMB	GB11	515046
	AF648	IgG1	RMG1-1	406617
	APC	CD4	GK1.5	100411
	APC	CD44	IM7	103011
	APC	CD25	PC61	102011
	APC	CD45.1	A20	110713
	APC	CD62L	MEL-14	104411
	APC	CX3CR1	SA011F11	149007
	APC	IFNg	XMG1.2	505809
	APC	IL-2	JES6-5H4	503809
	APC	Tcrb	H57-597	109211
	APC	CD45.1	A20	110713
	APC	B220	RA3-6B2	103211
	APC/Cy7	B220	RA3-6B2	103224
	APC/Cy7	CD11b	M1/70	101226
BD				
	PE/Cy7	CD95	Jo2	557653

Lymphocyte-intrinsic iron regulation is required for adaptive immunity

3.1 Introduction

The Iron regulatory proteins -1 and -2 (IRP1, IRP2) are ubiquitously expressed RNA-binding proteins that respond to fluctuations in cellular iron levels and, in turn, modulate the expression of specific iron metabolism genes (Muckenthaler et al., 2017; Rouault, 2006; Wilkinson and Pantopoulos, 2014). The IRPs bind stem loop structures, called iron regulatory elements (IRE), located at the 3' or 5' UTRs of target RNAs. *TFR1* mRNA contains multiple IREs in the 3' UTR, so that IRP binding in iron starved conditions prevents Regnase-1 endonuclease-mediated degradation of *TFR1* mRNA. This facilitates translation and enhances expression to facilitate iron uptake (Yoshinaga et al., 2017) . DMT1 mRNA also contains a 3' UTR IRE, but the physiology of the regulation of divalent metal transporter 1 (DMT1) by the IRE/IRP is poorly characterised (Galy et al., 2013). IRPs bind a single IRE at the 5' UTR of target mRNAs, including ferritin heavy and light chains (*FTH* and *FTL)*, 5-aminolevulinate synthase 2 *ALAS2*, *FPN,* aconitase 2 (*ACO2*) and *HIF2*α. Suppression of their translation in iron-starved cells enhances protein expression in contexts of iron sufficiency/overload. These handful of genes are the best defined targets of IRP1/2, however, 'omics' approaches have defined many more possible targets (Sanchez et al., 2011) and the full scope of the physiologically relevant IRP1/2 regulome remains to be defined.

Despite being homologues, IRP1 and IRP2 are regulated by distinct mechanisms. In iron replete conditions when biogenesis of iron-sulfur clusters (Fe-S) is unrestricted, IRP1 binds a Fe-S and is active as the cytoplasmic aconitase converting citrate to isocitrate. In iron deficient conditions IRP1 tends to lose its Fe-S cluster and undergoes a structural change which favours its activity as an RNA binding protein (Rouault, 2006). IRP2 does not have an Fe-S binding site and instead its activity is regulated by iron-dependent protein degradation. In iron replete cells IRP2 interacts with F-box/LRR-repeat protein 5 (FBXL5), which facilitates ubiquitination of IRP2 and proteasomal degradation. In iron deficient cells, FBXL5 loses its N-terminally bound iron and is degraded, resulting in IRP2 accumulation (Salahudeen et al., 2009; Vashisht et al., 2009). IRP2 accumulation is also favoured in hypoxic conditions as low oxygen tensions disfavour the association of FBXL5 with the cytosolic Fe-S cluster assembly complex, an interaction which was found to facilitate FBXL5-mediated degradation of IRP2 (Mayank et al., 2019).

Irp1/2 double KO is embryonic lethal (Smith et al., 2006), whereas single KOs of the individual IRPs have relatively mild phenotypes. This suggests that the IRPs are partially redundant in function, with the degree of redundancy depending on the tissue in question and the target mRNA. *Irp2*-/- mice have a relatively mild microcytosis phenotype with alterations in systemic iron distribution (Galy et al., 2005). *Irp2*-/- mice have also been reported to suffer from neural degradation, however, the severity of this phenotype differs between reports (Galy et al., 2006; Ghosh et al., 2006; LaVaute et al., 2001). *Irp1*-/- mice initially seemed to be grossly

phenotypically normal, with some evidence of higher ferritin expression in the kidney and brown fat (interestingly these are the tissues with the highest IRP1 expression) (Meyron-Holtz et al., 2004). However, upon closer analysis, *Irp1*-/- animals show transient polycythaemia at 6-weeks of age, likely to due to a lack of IRP1-mediated suppression of HIF2α, driving relatively elevated EPO expression (Anderson et al., 2013).

Cell type specific double KOs have elucidated the key role of the IRPs in iron homeostasis. Constitutive KO of *Irp1/2* in intestinal cells dramatically reduced viability and demonstrates an absolute requirement of the IRPs for intestinal function and development (Galy et al., 2008). Hepatocyte specific *Irp1/2* deletion resulted in liver failure and early death, likely driven in part by mitochondrial dysfunction. *Irp1/2* KO livers showed disrupted citric acid cycle and electron transport chain function, as well as reduced iron sulphur cluster (ISCU) and haem protein synthesis (Galy et al., 2010). *Irp1/2* KO enterocytes also showed evidence of mitochondriopathy by histology and cardiomyocyte specific *Irp1/2* KO resulted in an impaired capacity to respond to stress conditions associated with mitochondrial dysfunction, further supporting a link between appropriate cellular iron homeostasis and sustaining metabolic integrity (Galy et al., 2008; Haddad et al., 2016). Tamoxifen-induced KO of IRPs in intestinal cells in adult mice does not generate a lethal phenotype and instead revealed a role in locally co-ordinating iron absorption (Galy et al., 2013), suggesting that IRP1/2 may be particularly important during growth and organ development.

The importance of IRP1 and 2 for immunity has not been widely investigated. Selective disruption of the IRPs in myeloid cells was shown to impair innate defence mechanisms against *Salmonella* infection, in part through reduced LCN2 production and increased ferritin expression (Nairz et al., 2015). Whilst FBXL5 global knock out is embryonic lethal (Moroishi et al., 2011), *Fbxl5* KO and IRP2 stabilisation in haematopoietic stem cells have been shown to disrupt self-renewal and repopulation capacity *in vitro* and *in vivo* (Muto et al., 2017) indicating that appropriate IRP activity is essential for stem cell function.

IRP1/2 deficiency disrupts physiology through a myriad of direct and indirect pathways. The most obvious phenotype is cellular iron deficiency due to an inability to maintain maximal expression of transferrin receptor and perhaps DMT1 (Galy et al., 2010). The transferrin receptor has also been proposed to have signalling activity and a role in cellular physiology independent of iron uptake (Chen et al., 2015; Coulon et al., 2011; Senyilmaz et al., 2015). In the absence of IRP1/2 activity ferritin H and L translation are uninhibited. The effect of ferritin overexpression on cell physiology is poorly understood, but mutations in the Ferritin L IRE in humans lead to hyperferritinemia and cataract syndrome, suggesting that accumulation of ferritin L can disrupt cell function, in this case in the retina (Beaumont et al., 1995). Through its capacity to sequester iron, ferritin overexpression may exacerbate the cellular iron deficiency associated with *Irp1/2* KO (Wu et al., 1999). LPS-induced Ferritin H expression in dendritic cells (DCs) has been proposed to deplete the labile iron pool and facilitate activation-associated HIF1α stabilisation (Siegert et al., 2015). Ferritins

also plays key cytoprotective, anti-oxidant and metabolic regulatory roles (Weis et al., 2017). Constitutive ferritin H KO is embryonic lethal (Ferreira et al., 2000) and inducible systemic ferritin H KO in adult mice rapidly leads to death (Blankenhaus et al., 2019).

As mentioned, one key role of IRP1/2 in maintaining cellular iron levels is enhancement of transferrin receptor expression in the context of iron deficiency. One way to address the importance of iron in lymphocytes for cellular function is by disrupting transferrin receptor activity. However, *Tfrc*-/- cells fail to progress beyond the DN stage of thymocyte development and have an ill-defined effect on B cell development, but may be able to differentiate to the IgM+ stage (Ned et al., 2003). Differences in the severity of the T vs B cell phenotype may relate to a role of TFR1 in T cells unrelated to uptake, or the capacity of B cells to access an alternative iron source. Complementary approaches to disrupting transferrin receptor function such as atransferrinaemic mice also show reduced thymic cellularity and a developmental block in thymocyte differentiation around the DN3/4 stage (Macedo et al., 2004). A similar block around DN3 is observed when thymus cultures are treated with transferrin receptor-blocking antibodies (Brekelmans et al., 1994). The block in DN differentiation is consistent with the DN3/4 stage being metabolically active and proliferative as cells undergo β-selection (Boudil et al., 2015; Douglas et al., 2001). The effect of atransferraemia on B cell development and non-erythroid haematopoiesis is unknown, as is the effect on immunity. The developmental defect of *Tfrc*-/- mice is in contrast to the apparently normal peripheral lymphocyte profile of patients and mice

with the *Tfrc*$^{Y20H/Y20H}$ mutation (Jabara et al., 2016). In contrast to gene deletion, the substitution of the tyrosine in the YXXΦ endocytosis domain impairs, but does not abolish, TFR1 endocytosis and is therefore proposed to decrease the efficiency of iron acquisition. Patients with the *Tfrc*$^{Y20H/Y20H}$ mutation exhibited immunodeficiency with increased infection risk and failure to develop antibody responses to routine vaccines. T and B cells from patients and mice with the *Tfrc*$^{Y20H/Y20H}$ mutation fail to proliferate *in vitro,* but this defect can be rescued by supplying non-transferrin bound iron, arguing that the effect of *Tfrc*$^{Y20H/Y20H}$ is due to impaired iron uptake and not a signalling defect.

In the context of defective transferrin-bound iron uptake due to reduced surface expression of TFR1 (due to IRP1/2 KO) or impaired endocytosis (in *Tfrc*$^{Y20H/Y20H}$ (Jabara et al., 2016)) the capacity of cells to take up iron independently of the transferrin receptor must be considered. The capacity of some tissues to develop in the absence of the transferrin receptor (see below) indicates the existence of alternative iron uptake pathways.

LCN2 has the capacity to bind exogeneous and endogenous iron-carrying siderophores (Bao et al., 2010). LCN2 can be endocytosed by receptors Megalin and 24p3, presumably liberating its iron in the endosome (Devireddy et al., 2005; Hvidberg et al., 2005). Examining kidneys from chimeric embryos of *Tfrc* WT and KO cells indicated that the capsule and stroma could develop independently of TFR1, these regions also expressed low levels of TFR1 in wildtypes and instead efficiently

endocytosed ferritin L chains. Iron acquisition from ferritin was proposed to be mediated by scavenger receptor class A member 5 (SCARA5) (Li et al., 2009). T-cell immunoglobulin mucin family member 2 (TIM-2) has also been suggested to be capable of ferritin H uptake in mice, and is highly expressed by B cells among other cell types (Chen et al., 2005), however TIM-2 lacks an ortholog in humans. Human TFRC has also been proposed to act as a Ferritin-H uptake receptor (Li et al., 2010) and may facilitate ferritin H uptake by activated human lymphocytes. Ferritin is then thought to be degraded in the endosome to release iron for uptake via DMT1. The physiological importance of iron acquisition from ferritin or LCN2/siderophore complexes by these pathways and the significance of the apparent lack of conservation in non-transferrin-bound iron uptake between species remains unclear.

Uptake of iron from the endosome into the cytoplasm is thought to occur via DMT1 (Slc11a2) (Muckenthaler et al., 2017)**.** *Dmt1* KO pups are born, but show growth retardation and progressive severe anaemia proposed to be due to a combination of defective erythroid and intestinal iron uptake (Gunshin et al., 2005a). Despite there being no known redundant parallel pathways for uptake of transferrin-bound iron into the cytoplasm, the phenotype of *Dmt1* KO mice is less severe than embryonic lethality of *Tfrc* KO mice. The importance of DMT1 for iron uptake by haematopoietic cells other than erythrocytes is unknown and warrants further investigation. A key aspect of DMT1 transport of iron is that it takes place optimally at the low pH of the endosome. The search for plasma membrane-localised iron transporters, which could direct import of non-transferrin bound free iron into the cell, has focussed on divalent

cation transporters, Zrt and Irt like protein (ZIP) 14 or 8 (Liuzzi et al., 2006; Wang et al., 2012). The importance of ZIP14 *in vivo* has been explored in the context of tissue iron loading in hemochromatosis models (Jenkitkasemwong et al., 2015). However, neither ZIP14 nor DMT1 were found to be required for uptake of non-transferrin bound iron (specific $Fe_3Citrate_3$) by human CD4 or CD8 T cells (Arezes et al., 2013). Endolysosomal Haem response gene 1 (HRG1) mediates cellular haem uptake in *Caenorhabditis elegans,* is important for erythropoiesis in *Danio rerio* and recycling of haem iron by murine macrophages (Rajagopal et al., 2008; White et al., 2013). The importance of HRG1 mediated haem uptake or ZIP14/ZIP8 mediated NTBI uptake for iron acquisition in cells other than macrophages and liver/pancreas respectively remains to be defined.

The seemingly selective sensitivity of lymphocytes to the $Tfrc^{Y20H/Y20H}$ mutation and unexpected resistance of erythroid cells (affected humans and mice were only mildly anaemic) was proposed to be driven by the much higher expression of STEAP family ferrireductases (in particular STEAP3) in erythroid cells compared to resting lymphocytes (Jabara et al., 2016). The STEAP family of metalloreductases facilitate reduction of transferrin bound Fe^{3+} to Fe^{2+} to facilitate uptake into the cell via DMT1 (Ohgami et al., 2006, 2005). *Steap3* KO indicated the primary sensitive tissue was the erythron (Ohgami et al., 2005). However, bone marrow chimeras of *Steap3* WT and KO indicate an iron uptake-dependent cell intrinsic defect in B cell proliferation *in vitro* (Jiang et al., 2019). In the future it will be interesting to explore how expression of the STEAP family changes during lymphocyte activation and development, and whether

this contributes to the relative specificity of the *TfrcY20H/Y20H* mutation to mature lymphocyte activation.

3.1.2 Chapter Aims

Interestingly, IRE binding activity increases during *in vitro* lymphocyte activation and is suppressed by iron loading, suggesting a regulatory activity of IRPs in lymphocytes (Testa et al., 1991; Wu et al., 1999). Among the many genes that IRP1 and IRP2 regulate, the expression of transferrin receptor (of TFR1 or CD71) has been shown to be essential for lymphocyte function (Jabara et al., 2016). Whilst ferritin H KO disrupts lymphocyte homeostasis likely due to the toxic effects of an increased unbuffered NTBI pool (Vanoaica et al., 2014), the effect of ferritin overexpression on lymphocyte function remains unknown. The IRPs are thus strong candidate regulators of lymphocyte activation and adaptation to iron deficiency, but their precise role in lymphocytes is unknown. Therefore, we proposed to investigate the importance of IRP1 and IRP2 for CD8 T cell function.

Furthermore, we wanted to address the importance of iron uptake via TFR1 (proposed to be essential for lymphocyte iron uptake and a key target of IRP1/2) for adaptive immune responses *in vivo* by exploiting the $Tfrc^{Y20H/Y20H}$ hypomorphic knock in mouse (Jabara et al., 2016).

The aims of this chapter are as follows:

1. To inducibly KO T cell *Irp1/2* during *in vitro* activation, while minimising direct Cre effects
2. To investigate the effect of *Irp1/2* deletion on T cell activation prior to the first division
3. To investigate the effect of *Irp1/2* deletion on T cell proliferation
4. To investigate the role of defective TFR1 mediated iron uptake in lymphopoiesis and adaptive immune responses by generating mixed bone marrow chimeras of $Tfrc^{Y20H/Y20H}$ and congenic WT bone marrow

3.1.3 Experimental Approach

All IRP1/2 work was carried out at DKFZ Heidelberg, in collaboration with Dr. Bruno Galy and Michael Bonnadona (DKFZ, University of Heidelberg).

To permit inducible KO of IRP1 and IRP2, mice homozygous for floxed *Aco1* (IRP1) and *Ireb2* (IRP2) alleles ($Aco1^{flox/flox}$, $Ireb2^{flox/flox}$) were crossed with a transgenic line globally expressing a tamoxifen inducible CRE (*Rosa26-CreERT2*). $Aco1^{flox/flox}$, $Ireb2^{flox/flox}$, *Rosa26-CreERT2* positive animals were termed $IRP1/2^{flox/flox}$ Cre +ve , whilst $Aco1^{flox/flox}$, $Ireb2^{flox/flox}$, *Rosa26-CreERT2* negative litter mate controls were termed $IRP1/2^{flox/flox}$ Cre -ve.

Lymphocytes were isolated from lymph nodes, labelled with Cell Trace violet proliferation dye and CD8 T cells were enriched by Miltenyi MACs mediated negative isolation. T cells were activated with aCD3/aCD28 in the presence of 4-

hydroxytamoxifen(4-OHT), to induce CRE activity, or ethanol control conditions for 24hrs after which the 4-OHT or EtOH was washed off the cells. In pilot experiments (data not shown) we attempted to maintain naive cells in IL-7 concurrent with 4-OHT or EtOH and then stimulate with aCD3/CD28 to KO *Irp1/2* prior to activation, but found that, in line with published literature (Rathmell et al., 2001), IL-7 maintained cells had good viability but poor mitogenic responses to stimulation. Therefore, in our experimental scheme, deletion took place concurrent with the first 24hrs of activation.

Cells were activated for a further 48hrs, to resolve proliferation through the dilution of cell trace violet (Quah et al., 2007), depending on the experiment. When iron was used to rescue proliferation $FeSO_4$ was added at the indicated concentration from the start of culture and replenished after 24hrs when 4-OHT/EtOH was removed and media was replaced. Flow cytometry was used to evaluate T cell proliferation.

For mixed bone marrow chimerism experiments a mix of 60% Tfrc$^{Y20H/Y20H}$ CD45.2 (or WT CD45.2) bone marrow was combined with 40% CD45.1 bone marrow to test the capacity of Tfrc$^{Y20H/Y20H}$ cells to reconstitute and contribute to immune responses relative to wild type cells. 2 million bone marrow cells in total were i.v. injected into a lethally irradiated CD45.2 recipient. After 9-weeks peripheral blood was taken to analyse reconstitution by flow cytometry. Mice were then immunised with either 800µg of ovalbumin (OVA) protein, 25µg of MPLA and 1µg of alpha-galactosyl ceramide intravenously (OVA and adjuvant immunisation) or 1X10^7 PFU of MVA-OVA

subcutaneously. Mice were culled one week after immunisation and the immunisation induced immune response was evaluated by flow cytometry.

3.2 Results

3.2.1 *In vitro* model of deletion of IRP1/2 alleles in CD8 T cells

1A

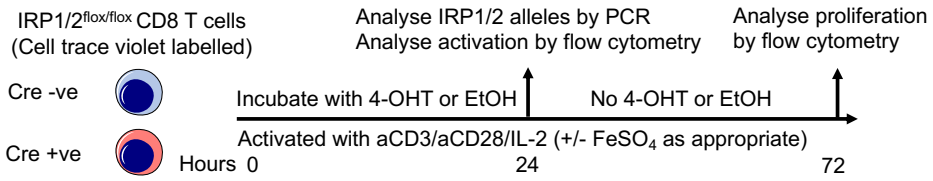


Figure 3.1. Experimental scheme for *in vitro* IRP1/2 KO experiments (1A)

CD8 T cells from IRP1/2$^{flox/flox}$ Cre +ve and IRP1/2$^{flox/flox}$ Cre -ve mice were activated as described in Fig. 3.1A for 24 hours in the presence of 4-OHT or EtOH. To confirm that 24hrs of 4-OHT was sufficient to KO IRP1/2 in the majority of CD8 T cells, genomic PCR and gel electrophoresis was used to evaluate the size of the IRP1/2 alleles in CD8 T cells from different treatments. Genomic PCR for the smaller, faster migrating, recombined alleles of *Aco1* and *Ireb2* in activated CD8 T cells indicate variable background recombination in Ethanol treated IRP1/2$^{flox/flox}$ Cre +ve CD8 T cells; 24hrs treatment with 100nM 4-OHT results in the recombined alleles dominating indicating

that our treatment regime is sufficient to KO IRP1 and IRP2 in the majority of cells (2A and 2B).

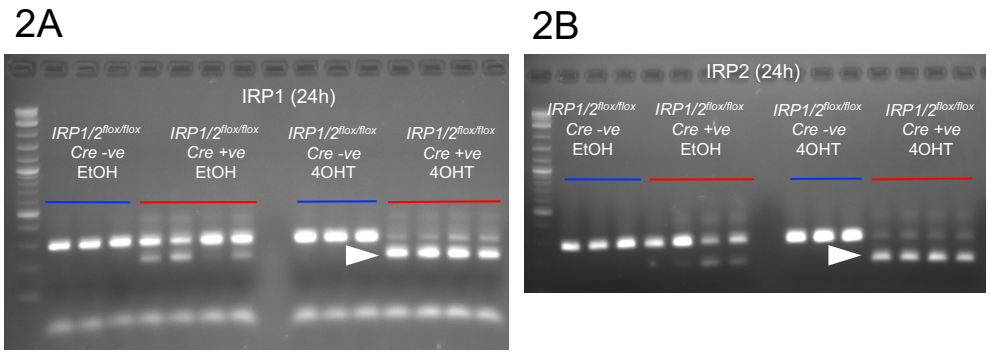


Figure 3.2. Genomic PCR and gel electrophoresis results showing IRP1 alleles (2A) and IRP2 alleles (2B) after 24 hours of activation and treatment with EtOH or 4-OHT. The smaller migrating bands are the recombined 'KO' alleles.

3.2.2 Effect of IRP1/2 deletion at 24 hours

Flow cytometric analysis of CD8 T cells after 24hrs of activation indicates that the percentage of cells expressing early activation marker CD69 in 4-OHT compared to EtOH treatment condition was not significantly different between IRP1/2$^{flox/flox}$ Cre +ve and IRP1/2$^{flox/flox}$ Cre -ve (3A), indicating that inducible KO of *Irp1* and *Irp2* does not alter the early signalling events downstream of TCR ligation that mediate T cell activation. However, the ratio of CD71 expression for 4-OHT treated cells compared to the EtOH treatment condition was significantly reduced in IRP1/2$^{flox/flox}$ Cre +ve relative to IRP1/2$^{flox/flox}$ Cre -ve indicating that even at this early time point (after activation but before cells have divided) *Irp1/2* KO interferes with appropriate upregulation of CD71 expression (3B). Normalised absolute CD71 expression was also significantly lower in IRP1/2$^{flox/flox}$ Cre +ve cells treated with 4-OHT compared to

IRP1/2$^{flox/flox}$ Cre -ve cells (3C), as demonstrated by representative histograms of CD71 expression (3D).

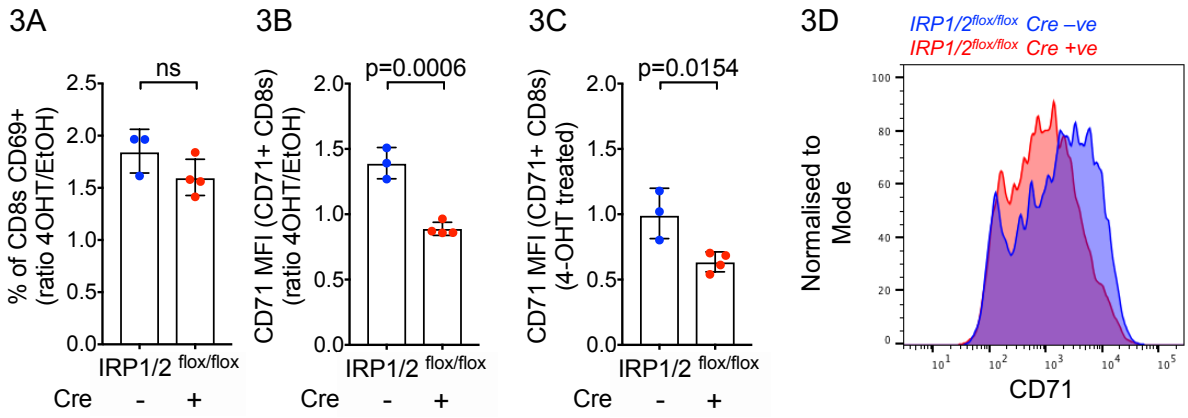


Figure 3.3. Flow cytometric analysis of the ratio of CD69+ cells (3A) and CD71 MFI (3B) between the 4-OHT and EtOH treated conditions for each genotype. Relative absolute CD71 MFI for CD8 T cells treated with 4-OHT (3C), representative histograms of absolute CD71 expression (3D). All at 24 hours post activation. T test.

3.2.3 Effect of IRP1/2 deletion at 72 hours and rescue with non-transferrin bound iron

To examine the effect of IRP1 and IRP2 deficiency on T cell proliferation *in vitro* we activated CD8 T cells of both genotypes for 24hrs in the presence of 4-OHT or EtOH, washed the cells and re-cultured them without 4-OHT or EtOH for an additional 48hrs. Overlays of the CTV histograms from the 4-OHT and EtOH conditions indicate that 4-OHT treatment of IRP1/2$^{flox/flox}$ Cre -ve cells did not significantly alter proliferation. In contrast, 4-OHT treatment of IRP1/2$^{flox/flox}$ Cre +ve cells (deletion of IRP1 and IRP2) strongly suppressed *in vitro* proliferation (4A). Furthermore, 4-OHT treatment of IRP1/2^{WT} Cre+ve cells had a minimal effect on proliferation, suggesting that the 4-OHT

phenotype is related to recombination of *Irp1/2* loci and not a direct Cre effect (data not shown). Finally, 4-OHT treatment of IRP1/2$^{flox/flox}$ Cre +ve significantly reduced CD71 expression at this timepoint relative to IRP1/2$^{flox/flox}$ Cre -ve (5A and 5B).

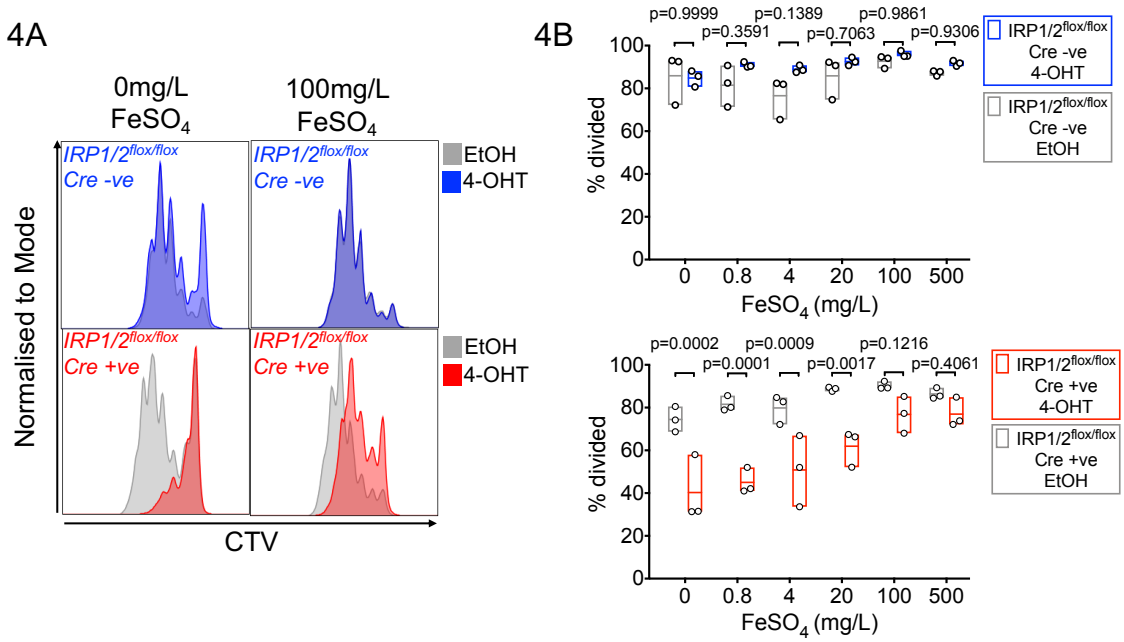


Figure 3.4. Representative histograms of CD8 T cell CTV profiles (4A) at 72 hours post activation. Effect of genotype, treatment and iron on % of CD8 T cells divided (4B). Anova, multiple comparisons for treatment at each iron level.

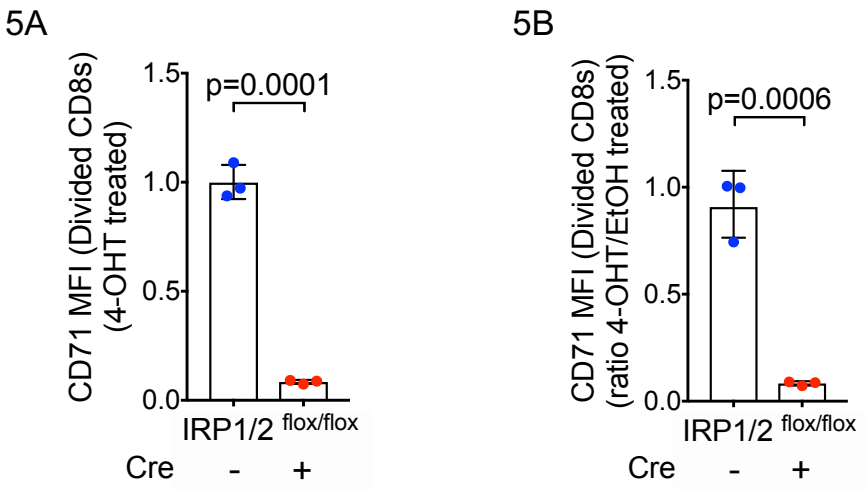


Figure 3.5. Normalised absolute CD71 expression of 4-OHT treated CD8 T cells (5A) and relative CD71 MFI of 4-OHT treated compared to EtOH treated CD8s (5B) at 72 hours post activation.

Our data indicate that IRP1 and IRP2 are required for appropriate expression of CD71, and thus support CD8 T cell proliferation *in vitro*. The obvious hypothesis is that deficiency of IRP1 and IRP2 results in inappropriately low expression of CD71 and failure to acquire sufficient iron. However, IRP1/2 deficiency can have other direct effects, such as overexpression of ferritin, and CD71 has been proposed to be important for processes other than iron acquisition. To investigate if the IRP1/2 deficiency phenotype was due to disrupted iron acquisition cells treated with EtOH or 4OHT were also supplemented with iron sulphate. Iron sulphate will provide high levels of non-transferrin bound iron to the cells which are able to be taken up independently of CD71. Whilst iron supplementation tended to improve proliferation of cells regardless of genotype and treatment condition, the extent to which iron supplementation improved proliferation was the greatest for 4-OHT treated IRP1/2$^{flox/flox}$ Cre +ve. 4-OHT treated IRP1/2$^{flox/flox}$ Cre +ve cells showed profoundly defective proliferation at 0mg/L of Iron sulfate, whereas at 100mg/L iron sulfate proliferation was nearly normalised (4A and 4B). This result further supports our hypothesis that 4-OHT treatment of IRP1/2$^{flox/flox}$ Cre +ve disrupts proliferation through recombination of the IRP1/2 alleles leading to insufficient CD71-mediated iron acquisition, and not due to a 'Cre effect'.

3.2.4 The *Tfrc*$^{Y20H/Y20H}$ mutation results in an iron-dependent inhibition of CD8 T cell proliferation *in vitro*

The mutation potential provides a complementary genetic model to investigate how disrupted iron acquisition alters adaptive immunity. We isolated CD8 T cells from *Tfrc*$^{Y20H/Y20H}$ and WT mice, and then activated with aCD3, aCD28 and IL-2 in standard complete RPMI culture medium. Wells also received a titration of $FeSO_4$ comparable to that used on the IRP1/2 KO cells. We confirmed published results (Jabara et al., 2016) and found that the *Tfrc*$^{Y20H/Y20H}$ substitution inhibited CD8 T cell proliferation (resolved as dilution of cell trace violet labelling), but that this could be rescued with a similar concentration of NTBI as rescued the proliferation of IRP1/2 KO (6A and 6B).

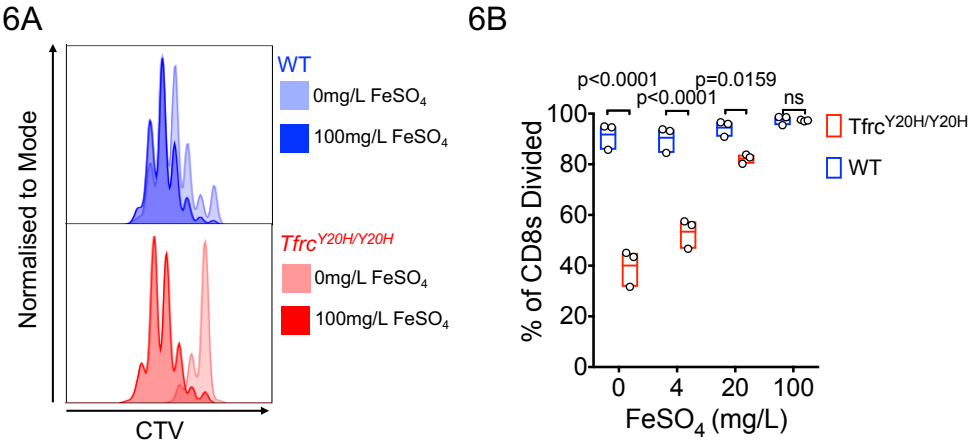


Figure 3.6. WT and *Tfrc*$^{Y20H/Y20H}$ activated for 72hours in the presence or absence of $FeSO_4$. Representative CTV profiles (6A). Effect of genotype and iron on % of CD8 T cells divided (6B). Anova, multiple comparisons for treatment at each iron level.

3.2.4 The *Tfrc*$^{Y20H/Y20H}$ mutation does not seem to disrupt lymphopoiesis *in vivo*

Results from studies of the *Tfrc*$^{Y20H/Y20H}$ mutation suggest that uptake of iron from serum transferrin is essential for adaptive immune function. However, this

interpretation is based on results obtained *in vitro* or *in vivo* involving a globally expressed hypomorphic *Tfrc* allele (Jabara et al., 2016). The patient phenotype could be driven by expression of the hypomorphic *Tfrc* allele on non-lymphocytic cells essential for the adaptive immune response such as dendritic cells, macrophages or stromal cells. To test the cell intrinsic importance of iron uptake for adaptive immunity, mixed bone marrow chimeras of WT bone marrow and bone marrow from mice homozygous for the hypomorphic $Tfrc^{Y20H/Y20H}$ allele were made (7A). The ratio of $Tfrc^{Y20H/Y20H}$: WT and WT: WT lymphocytes in peripheral blood on day 63 post immunisation were non-significantly different suggesting that the *Tfrc* Y20H mutation did not result in a competitive defect during lymphopoiesis consistent with observations of no obvious disruption in peripheral lymphocyte profiles in affected patients and $Tfrc^{Y20H/Y20H}$ mice (Jabara et al., 2016) (8A, 8B and 8C).

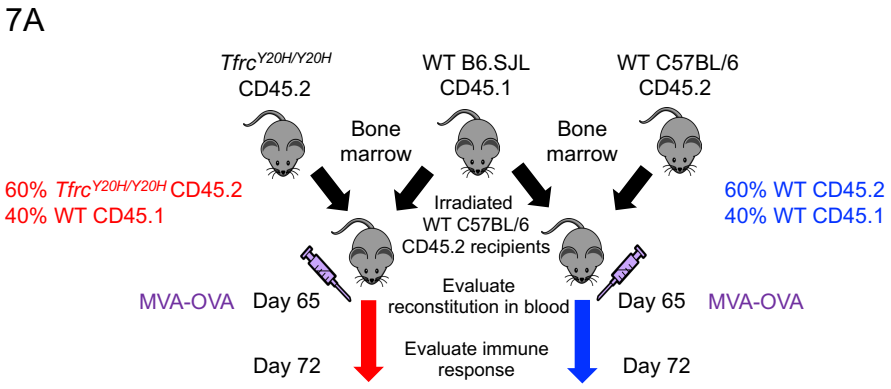


Figure 2.7. Experimental scheme for WT and *Tfrc*$^{Y20H/Y20H}$ mutant bone marrow chimera experiments (7A)

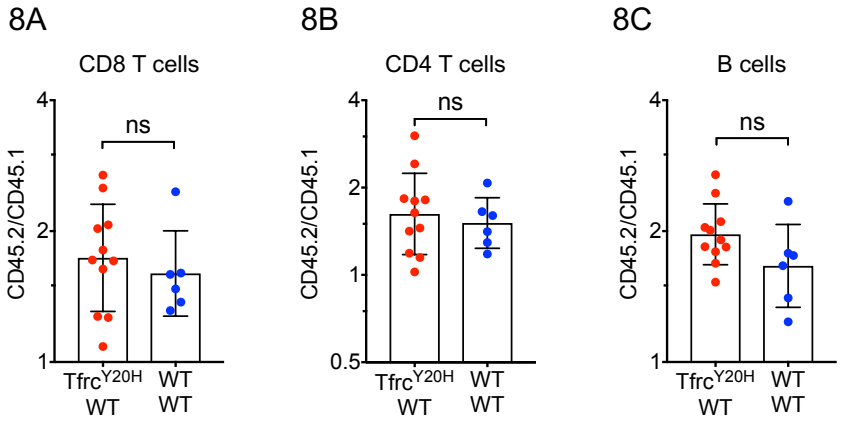


Figure 2.8. Ratio of CD45.2/CD45.1 cells within peripheral blood CD8 T cells (8A), CD4 T cells (8B) and B cells (8C) at 9 weeks after chimerism. T test.

3.2.5 The Tfrc$^{Y20H/Y20H}$ mutation results in a cell intrinsic disruption of adaptive immunity *in vivo*

We then immunised mice subcutaneously with MVA-OVA and examined adaptive immune response 7 days post immunisation. The ratio of *Tfrc*$^{Y20H/Y20H}$: WT cells in bystander naïve CD44- CD8 T cells was then compared to the ratio in immunisation induced H-2Kb B8R tetramer positive, vaccinia specific, CD44+ CD8 effector cells. The ratio of *Tfrc*$^{Y20H/Y20H}$: WT cells was significantly lower in effector cells compared to

naïve cells suggesting that the *Tfrc*$^{Y20H/Y20H}$ mutation bestows a cell intrinsic competitive defect in the ability of CD8 T cells to contribute to the adaptive immune response (9A). Similar results were found when the ratio of *Tfrc*$^{Y20H/Y20H}$: WT cells was compared between naïve CD44- CD4 T cells and T follicular helper cells (defined as CXCR5+ PD-1+ CD44+ CD4 T cells) (9B), and follicular B cells (IgD+ B220+ B cells) and germinal centre B cells (GL7+ CD95+ IgD- B220+ B cells) (9C). Representative gating schemes for each antigen specific population can be found in supplementary figures S2, S3 and S4. These results were recapitulated for protein in adjuvant immunisation (9D, 9E and 9F).

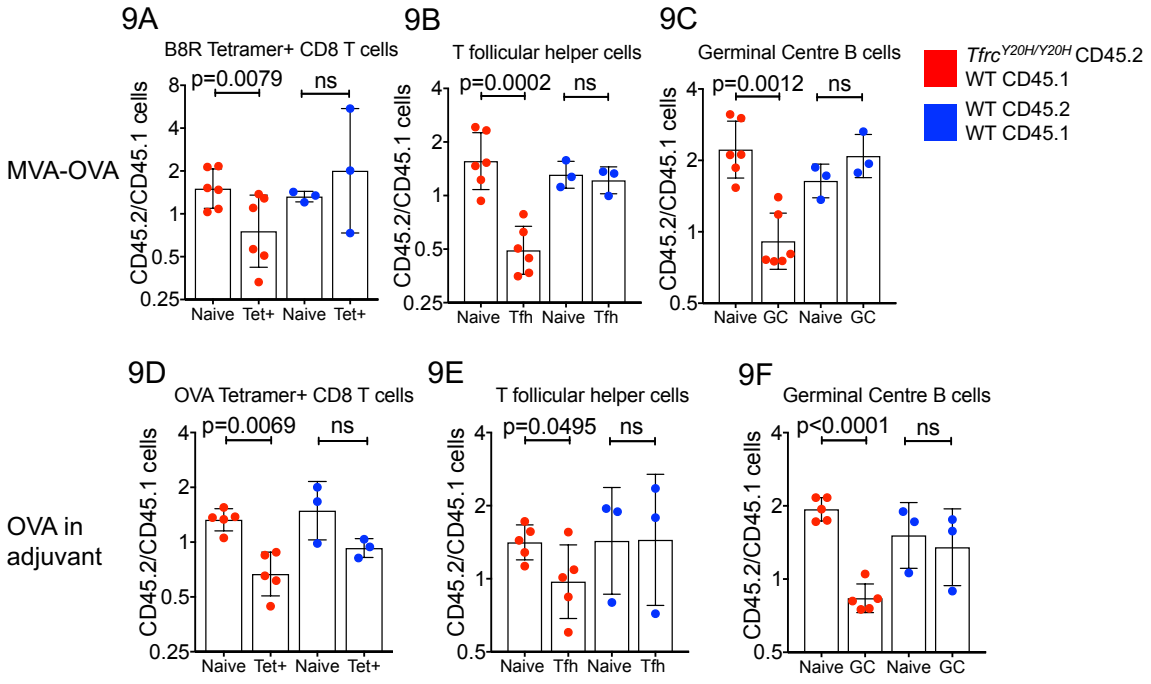


Figure 2.9. Comparison of the ratio of CD45.2/CD45.1 cells within naïve and effector splenic CD8 T cells (9A), dLN naïve and Tfh CD4 T cells (9B) and dLN naïve follicular and GC B cells (9C) at 10 weeks after chimerism and 1 week after MVA-OVA immunisation. Comparison of the ratio of CD45.2/CD45.1 cells within naïve and effector splenic CD8 T cells (9D), splenic naïve and Tfh CD4 T cells (9E) and splenic naïve follicular and GC B cells (9F) at 11 weeks after chimerism and 1 week after OVA in adjuvant immunisation. Paired T test.

3.3. Discussion

We have demonstrated that KO of IRP1/2 concurrent with the first 24hrs of T cell activation is sufficient to severely disrupt CD71 expression and proliferation of CD8 T cells. The proliferative defect of IRP1/2 KO cells could be rescued with 100mg/L of $FeSO_4$ suggesting an inability to maintain high levels of CD71 expression might be limiting iron uptake. The same concentration of $FeSO_4$ was also sufficient to rescue proliferation of *Tfrc*$^{Y20H/Y20H}$ CD8 T cells where iron uptake is disrupted through reduced efficiency of TFR1 endocytosis (here CD71 expression is much higher than WT controls – consistent with cellular iron deficiency). It is worth noting that it is unclear if the rescue is due to direct uptake of NTBI or that addition of NTBI super-saturates available (bovine) transferrin such that even with reduced transferrin receptor activity sufficient iron can be acquired. These results highlight a key role of IRP1/2 in synergising with transcriptional mechanisms of metabolic remodelling (such as MYC driven increases in CD71 expression (Preston et al., 2015)) to maintain iron acquisition even under control conditions *in vitro*. It will be interesting to further characterise the dynamic regulation of the activity of the IRP1/2 system during T and B cell activation to facilitate rapid proliferation and cellular remodelling (Wu et al., 1999).

One drawback of our approach was the need for 4-OHT treatment concurrent with activation, due to poor activation profiles after 24hrs culture with IL-7. 4-OHT treatment was limited to the first 24hrs as it is increasingly being reported that hyper-activity of the Cre recombinase can induce a DNA damage response, cGAS/STING-mediated IFN response and growth arrest (Loonstra et al., 2001; Pépin et al., 2016).

Finding the optimal balance of dose and duration of 4-OHT treatment is key to maximise recombination efficiency but minimise direct Cre effects. We also found a low level of recombination in vehicle treated CreERT2+, possibly suggesting heterogeneity within our populations. However, vehicle treated CreERT2+ cells are indistinguishable from WT cells in terms of CD71 expression and proliferation, therefore, this low level of recombination does not materially affect our conclusions. Furthermore, incomplete KO even after 24hrs highlights that any phenotype observed in the 4-OHT treated IRP1/2 KO population may be an underestimation of the KO phenotype due to the presence of CD8 T cells with unrecombined IRP1/2 alleles which could proliferate at the expense of KO cells.

The next step would be to generate transgenic mice where IRP1/2 can be disrupted inducibly in the peripheral T cell compartment *in vivo*. The capacity of IRP1/2 KO cells to respond to immunisation or infection would be measured. The need to take results *in vivo* is highlighted by the more severe defect of *Tfrc*$^{Y20H/Y20H}$ *in vitro* than *in vivo* (*in vitro Tfrc*$^{Y20H/Y20H}$ fail to proliferate more than a couple of times, whereas *in vivo* we see a perhaps 3-fold competitive defect). These differences likely reflect how the availability of different iron sources differs between niches *in vivo* and *in vitro* culture medium. Two standard approaches to achieve this would be to generate mixed bone marrow chimeras of congenic IRP1/2$^{flox/flox}$ Cre +ve and IRP1/2$^{flox/flox}$ Cre -ve cells or crossing the IRP1/2$^{flox/flox}$ allele onto a CD4-Cre, *Lck*-Cre or *Gzmb*-Cre background to analyse their importance at different points in T cell development. An alternative approach to determine the role of various proteins essential for iron homeostasis in T

cells would be to exploit newly developed protocols for efficient CRISPR/Cas9 mediated gene KO in T cells (Seki and Rutz, 2018). By targeting genes encoding proteins including the IRPs, FBXL5, DMT1, HRG1, Zip14 and Zip8 in transgenic OT-I T cells, WT and KO T cells could be adoptively transferred into a recipient mouse and the relative importance of a target gene for CD8 T cell responses could be followed *in vivo* following immunisation.

It would also be desirable to uncover which aspects of T cell physiology are impaired by IRP1/2 deficiency. T cell function is intrinsically linked to dynamic changes in metabolism and high metabolic rates are required to support rapid proliferation (Phan et al., 2017). *Irp1/2* deficiency is known to lead to metabolic defects in other tissues and many metabolic enzymes utilise iron as a cofactor (Galy et al., 2010). A number of complementary approaches could be exploited to investigate if *Irp1/2* deficiency impairs T cell metabolism. Extracellular flux analysis using Seahorse technology could be exploited to analyse whether altered glycolytic or mitochondrial respiratory activity prior to the first cell division could underlie the proliferative defect of IRP1/2 deficiency. Furthermore, the concentration of ATP and other metabolites could be measured in activated WT and KO T cells.

On immunisation of $Tfrc^{Y20H/Y20H}$ (CD45.2) and WT CD45.1 bone marrow chimeras we found a de-enrichment of $Tfrc^{Y20H/Y20H}$ cells in the pool of responding T-cells (antigen specific CD8s, Tfh) or B cells (GC B cells) relative to frequency of $Tfrc^{Y20H/Y20H}$ cells in the bystander naïve pool. As both wild type and $Tfrc^{Y20H/Y20H}$ cells were responding in the same environment having equal exposure to WT stroma (from the recipient) and

WT donor haematopoietic cells including dendritic cells and macrophages we interpret this data as showing that the *Tfrc*$^{Y20H/Y20H}$ mutation has a cell-intrinsic effect on adaptive immune cell function *in vivo.* The *in vivo* consequences of the *Tfrc*$^{Y20H/Y20H}$ were not addressed in the publication which first proposed an impact on adaptive immunity (Jabara et al., 2016). Of note is that our approach of transferring mutant or WT CD45.2 cells into CD45.2 donors may in fact mean we are under-estimating the impact of the *Tfrc*$^{Y20H/Y20H}$ mutation on the T cell compartment. A subset of peripheral T cells are relatively radio-resistant and can remain after irradiation. Therefore, some of the CD45.2 cells responding to immunisation in *Tfrc*$^{Y20H/Y20H}$ may, in fact, be recipient-derived and WT. We would like to extend our analysis of *Tfrc*$^{Y20H/Y20H}$ /WT mixed bone marrow chimeras to examine how perturbed transferrin-bound iron uptake alters the function of other immune cell subsets (such as dendritic cells, NK cells and ILCs).

The creation of mixed bone marrow chimeras using *Tfrc*$^{Y20H/Y20H}$ (CD45.2) and WT CD45.1 bone marrow also demonstrated that, despite lymphopoiesis being perturbed by complete *Tfrc* KO (Ned et al., 2003) and involving highly proliferative stages [eg the DN3 stage] with high transferrin receptor expression, even in a competitive situation the *Tfrc*$^{Y20H/Y20H}$ did not perturb T or B lymphopoiesis. A lack of a lymphoid development phenotype had been suggested from analysis of human patients (Jabara et al., 2016), but it is plausible that in this situation some relaxation of selection or homeostatic feedback, involving proliferation in the periphery, compensates for impaired proliferation/iron acquisition by *Tfrc*$^{Y20H/Y20H}$ progenitors during

development, to ensure the formation of a functional peripheral T and B cell compartment. Therefore, it would be interesting to explore the TCR and BCR repertoire of naïve cells in the context of iron deficiency or the *Tfrc*$^{Y20H/Y20H}$ mutation. The lack of competitive defect of *Tfrc*$^{Y20H/Y20H}$ cells in lymphopoiesis might also relate to B and T cell progenitors having, unlike mature cells, partially redundant iron uptake mechanisms to compensate for reduced TFR1 activity. However, the phenotype of the complete *Tfrc* KO argues against these iron uptake mechanisms being completely redundant (Ned et al., 2003). Furthermore, in the bone marrow or thymic microenvironment the form and level of available extracellular iron might reduce the impact of the *Tfrc*$^{Y20H/Y20H}$ allele. Or, as with respect to erythroid cells and STEAP3, it might be that mature lymphocytes lack high levels of some protein(s) expressed by progenitors, which mitigate the impact of the *Tfrc*$^{Y20H/Y20H}$ mutation.

Alternatively, it may simply be that the iron requirements of activation-induced proliferation and metabolic remodelling to support effector functions are greater, and more perturbed by the *Tfrc*$^{Y20H/Y20H}$ mutation than lymphocyte development. Finally, we must consider that an aspect of the specificity of the *Tfrc*$^{Y20H/Y20H}$ mutation to mature lymphocytes might be some lymphocyte specific role of TFRC signalling. The mutated YXXΦ endocytosis domain has also been proposed to be involved in signalling downstream of TFRC (Coulon et al., 2011). But rescue of the proliferative defect of cells with *Tfrc*$^{Y20H/Y20H}$ by iron supplementation argue against this. To test these hypotheses an extensive phenotyping of lymphopoiesis in mixed and standard bone marrow chimeras will be required, alongside a characterisation of the expression of

alternative iron import proteins and compensating factors in different subsets. Functionally, *in vitro* models of thymocyte and B cell development (such as culture of bone marrow progenitors with OP9-DL1 feeder cells (Schmitt and Zúñiga-Pflücker, 2002) could demonstrate the resistance of lymphocyte progenitor cells to the *Tfrc*$^{Y20H/Y20H}$ mutation in the absence of the *in vivo* microenvironment.

In the next chapter, comparison of the early activation events of CD8 T cells, with a focus on metabolic remodelling, from WT and *Tfrc*$^{Y20H/Y20H}$ mice will be used as a model of iron-limited T cell activation to complement an *in vitro* model of iron deficiency.

In vitro investigations of the effect of iron restriction on T cell physiology

4.1 Introduction

4.1.1 Immunometabolism

Quiescent naïve CD8 and CD4 T cells are proposed to fuel themselves primarily through oxidative phosphorylation. Adaptive immunity necessitates rapid cell proliferation and effector functions which must be fuelled by greatly enhanced metabolic activity. TCR activation and IL-2 signalling induces a number of signalling events, including an initial wave of amino acid uptake, which facilitates activation of MYC and the mTORC1-dependent uptake of further nutrients and metabolic reprogramming to facilitate blastogenesis and proliferation driving the T cell out of quiescence (Chapman et al., 2019; Preston et al., 2015; Sinclair et al., 2013a; Wang et al., 2011). The strength of TCR signalling has been proposed to control IRF4 activity, which then modulates metabolism, coupling the quality of initial activation signal the T cell receives to survival and proliferation (Man et al., 2013; Nayar et al., 2014). T cell metabolism controls the size of the T cell response *in vivo* (Man et al., 2013).

A marked hallmark of activated T cell metabolism is the engagement of aerobic glycolysis where despite sufficient oxygen glucose is metabolised to lactate (Buck et al., 2015). Aerobic glycolysis or 'Warburg metabolism' is also a hallmark of cancer cells and is proposed to fuel rapid biosynthesis and proliferation. Uptake of glucose has

been proposed to be limiting for T cell activation (Jacobs et al., 2008). Whilst mitochondrial metabolism increases with a smaller fold increase than glycolysis during T cell activation, oxidative metabolism is also essential for T cell proliferation and activation, providing ATP and synthesising multiple key metabolites (Bailis et al., 2019; Ron-Harel et al., 2016; van der Windt et al., 2012a). Mitochondrial activity has also been proposed to be a key regulator of CD8 T cell longevity and differentiation towards memory cells, this will be discussed more in the chapter concerning iron and T cell memory.

The nutrient sensing kinase mTORC1 has emerged as a central regulator of metabolic reprogramming; it not only facilitates the activation of T cells, but drives ongoing effector differentiation of CD8 and CD4 T cells (Chapman et al., 2019; Delgoffe et al., 2011). mTORC1 has been suggested to control 10% of the cytotoxic CD8 T cell proteome, in particular regulating expression of cytotoxic and metabolic machinery (Hukelmann et al., 2016). Primarily mTORC1 is thought to respond to amino acid availability and growth factor signals (Kim and Guan, 2019a). T cell function and metabolism can also be modulated by oxygen sensing through the activity of the HIF pathway (Doedens et al., 2013; Finlay et al., 2012). Detection of energy stress, in particular increased ratios of AMP:ATP, activating the kinase AMPK can also modulate CD8 T cell differentiation (Rolf et al., 2013). Needless to say, other pathways also facilitate the response of CD8 T cells to nutrients, and mTORC1, HIF1α and AMPK extensively cross-regulate each other's activity. As such, CD8 T cells are highly

metabolically active cells which have the capacity to dynamically respond to their external environment (Ramsay and Cantrell, 2015).

Appropriate and sufficient metabolic activity is not only required for proliferation, but also regulates and supports differentiation to fully competent effector cells and cytokine production. Failure to support a sufficient level of metabolic activity, either due to an integration of signals that instruct the cell to suppress metabolism, or because of environmental nutrient restriction, can drive T cell dysfunction in the context of chronic infection and cancer (Bengsch et al., 2016; Scharping et al., 2016).

4.1.2 Iron and metabolism

The central importance of iron for metabolism is not a new concept. The observation that the addition of iron accelerates oxidative metabolism was proposed by Otto Warburg in 1925 (Warburg, 1925) and recent attempts to define the inorganic (prebiotic) origin of the citric acid cycle reactions rely on iron as the key agent of catalysis (Muchowska et al., 2019). These ideas are supported when the presence of iron in the enzymes of metabolism are examined, because whilst in mammals none of the 10 enzymes of glycolysis require iron to function, iron is prevalent in the electron transport chain and is found in the active sites of two citric acid cycle enzymes: aconitase and succinate dehydrogenase.

Experimental perturbation of iron homeostasis has been shown to disrupt metabolism *in vitro* and *in vivo,* and supports a particular sensitivity of oxidative metabolism and mitochondrial function to iron deprivation. Treatment of erythroleukemia cells with iron chelators suppressed oxidative metabolism and induced a compensatory increase in glycolysis (Oexle et al., 1999) and a similar shift was observed upon IRP2 KO in MEFs *in vitro* (Li et al., 2019). KO of IRP1 and IRP2 results in hepatic failure, proposed in part to be due to disrupted mitochondrial metabolism (Galy et al., 2010). Inducible inhibition of iron-sulfur cluster formation disrupted metabolism in HEK293 cells directly by impairing key iron-requiring enzymes such as cytosolic and mitochondrial aconitase and mitochondrial complex II (succinate dehydrogenase). This break in oxidative metabolism led to an accumulation of citrate which was proposed to firstly cause increased lipid biosynthesis and lipid droplet formation, but also reduced glycolytic flux through feedback inhibition of rate limiting enzyme phosphofructokinase 1 (Crooks et al., 2018). Disrupted Fe-S cluster biogenesis also dysregulates metabolism indirectly: lipoic acid synthase (LIAS) requires a Fe-S cluster to function. Reduced LIAS activity impairs lipoylation of a number of key metabolic enzymes including pyruvate dehydrogenase which require this modification to function (Crooks et al., 2018). Treatment of human monocyte derived macrophages with the cell permeable iron chelator deferiprone led to a similar relative enhancement of glycolysis and suppression of the citric acid cycle, particularly due to reduced mitochondrial aconitase activity. Iron chelator treatment resulted in citrate accumulation, increased synthesis of fatty acids and a more anti-inflammatory macrophage phenotype (Pereira et al., 2019). The comprehensive analysis of cellular metabolism in Crooks et al and Pereira et al demonstrates how the multiple links

between metabolic pathways results in unpredictable and complex effects of iron restriction on metabolism.

In terms of lymphocyte biology, disruption of BH4 synthesis and redox homeostasis in T cells disrupted activation and immune responses in *in vivo*; part of this phenotype was related to BH4 deficiency influencing mitochondrial iron homeostasis and metabolic activity (Cronin et al., 2018). These results support the hypothesis that iron restriction (genetically or environmentally) could disrupt lymphocyte metabolism and function.

4.1.3 Chapter Aims

The effect of genetically disrupting iron homeostasis on lymphocyte proliferation through the TfrcY20H/Y20H mutation or inducible IRP1/2 KO made us wonder if wild-type lymphocyte function was sensitive to iron availability. To investigate this, we developed an *in vitro* model of T cell activation in the context of serum iron deficiency, where we replaced the ill-defined iron content of fetal calf serum with a defined serum substitute that was complete aside from lacking iron and transferrin. Into this 'iron deficient medium' we titrated in holo-transferrin, whilst maintaining apo-transferrin at a constant level. The *Tfrc*$^{Y20H/Y20H}$ model provides an orthogonal model of genetic iron restriction, instead of environmental iron restriction, to confirm that iron acquisition was important for CD8 T cell function.

The aims of this chapter are to investigate to what extent iron restriction *in vitro* affects:

1. The early activation and proliferation of CD8 T cells
2. Differentiation and effector function of CD8 T cells
3. ATP synthesis, mitochondrial health and metabolic function
4. Activity of the mTORC1 pathway

4.2 Results

4.2.1 Iron restriction impaired T cell proliferation and acquisition of effector function *in vitro*

CD8 T cells were cultured as depicted in figure 1A. Iron was provided through a titration of holo-transferrin (transferrin carrying iron) against apo-transferrin (iron free transferrin). Total transferrin was maintained at a concentration of 1.2mg/ml, close to the concentration in peripheral blood (2-4mg/ml (Bhagavan, 2002)) and in line with published models of *in vitro* iron deficiency (Byrnes et al., 2016). The holo-transferrin concentrations utilised (0.0002mg/ml to 0.625mg/ml) cover a spectrum of conditions where viability was not impaired, but at the lowest concentrations proliferation was (1E). Whilst the lowest of these holo-transferrin concentrations are proportional to extremely low transferrin saturations, we do not know how low transferrin saturation can go in circumstances of iron deficiency in the tissue fluid of

metabolically active tissues such as lymph nodes, which is the physiologically relevant source of iron for immune cells.

Low iron availability appeared not to interfere with early CD8 T cell activation, with a similar % of CD8s upregulating the early activation marker CD69 within 24 hours (1B) (a process which is predominantly transcriptional regulated by NF-κB, AP-1 and ERG1 (Cibrián and Sánchez-Madrid, 2017)). In contrast the expression level of CD71 on CD69+ activated T cells is significantly increased by culture in low iron levels (1C), indicative that cellular iron deficiency was likely beginning to manifest itself even within this short period of culture.

In vitro iron deficiency seemingly also prevented entry into the cell cycle and DNA synthesis. Cells synthesising DNA/progressing through the cell cycle were resolved by using DAPI to stain intracellular nucleic acids; the % of CD8s which had higher than 2N level of DNA content at 24hours, indicative of cells which were in the S/G2/M phases of the cell cycle, was reduced at low iron levels (1D).

Over 2-3 days in the culture CD8 T cells divide multiple times, this can be resolved through the dilution of cell trace violet signal. Reduced serum iron concentrations *in vitro* reduced the number of divisions completed in 72 hours (1E and 1F). Concomitantly increased CD71 expression was detected in divided CD8 T cells cultured in low iron conditions, up to a point, before CD71 expression dropped off at the lowest iron conditions (1G).

Iron deficiency also impaired acquisition of effector functions by CD8 T cells *in vitro*, with CD8 T cells in iron deficient cultures expressing lower levels of Granzyme B (Gzmb) (1H) and the high affinity IL-2 receptor subunit, CD25 (1I). Expression of CD25 has been correlated with effector properties (Pipkin et al., 2010; Widjaja et al., 2017).

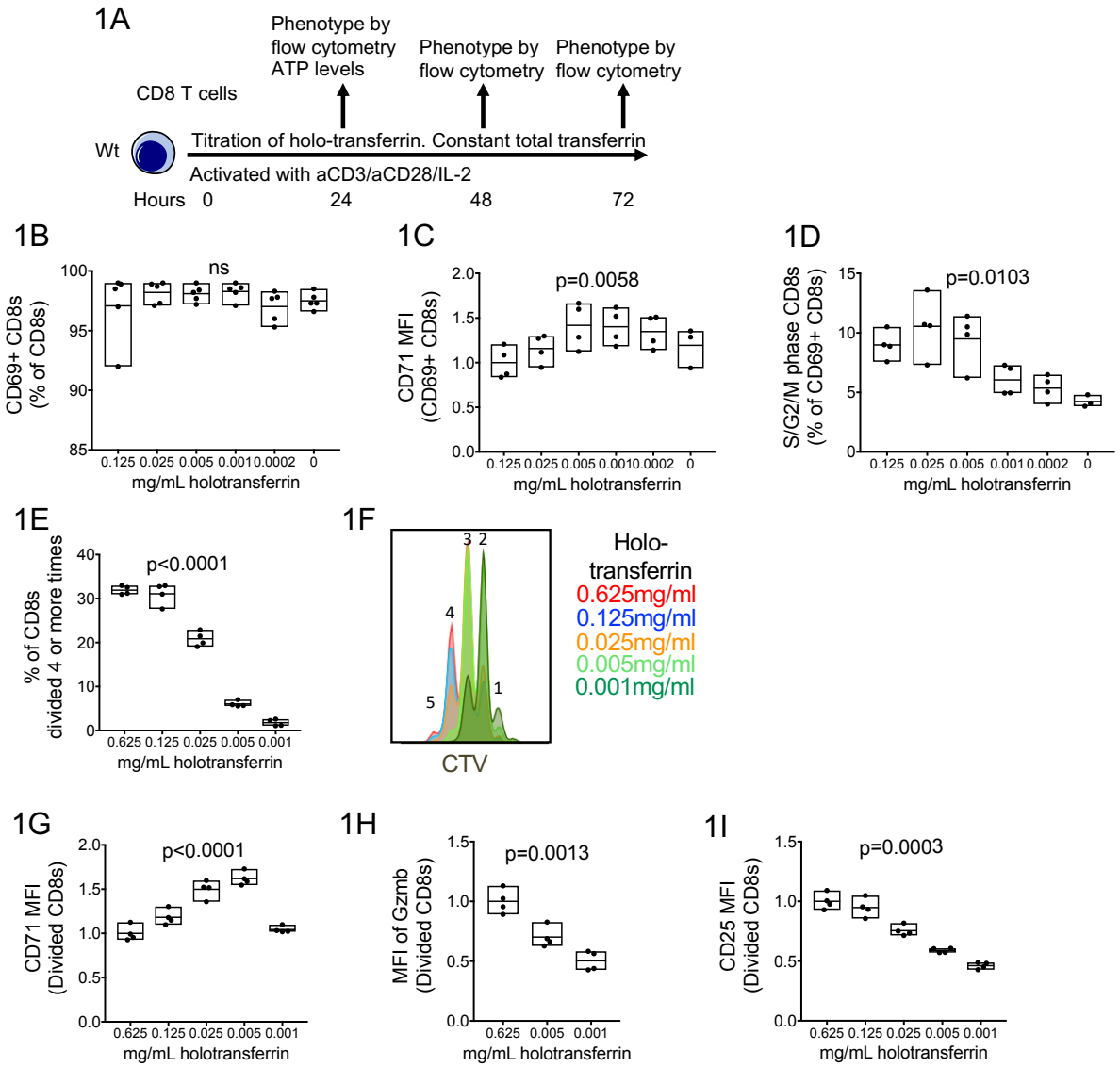


Figure 4.1. Experimental scheme for *in vitro* iron deficiency experiments (1A). CD69 (1B), CD71 (1C) and higher than G1 DNA content (1D) (resolved by DAPI staining of fixed cells) was measured by flow cytometry in cells activated for 24 hours. Flow cytometry was used to detect dilution of cell trace violet staining, indicative of division in cells activated for 72 hours(1E). Example Cell trace violet histograms showing dilution of cell trace violet signal with each successive round of division, numbered to indicate the number of divisions each peak represents (1F). CD71, Granzyme B and CD25 expression were measured by flow cytometry on cells activated for 48 hours.

4.2.2 Iron restriction disrupted T cell metabolism

As discussed, iron plays a role in multiple enzymes, in particular those of the mitochondrial electron transport chain. The hypothesis that iron deficiency might impair CD8 T cell metabolism was supported by reduced total ATP content of equal number of T cells activated in iron deficient conditions compared to iron sufficient conditions for 24hours as measured by an enzymatic luminescent assay (ATP content normalised to value at 0.625mg/ml 2A and absolute luminescence 2B)). We took 48hour activated CD8 T cells which had be grown under different iron condition, aliquoted multiple technical replicates of equal cell numbers and in collaboration with Barbara Kronsteiner-Dobramysl, we used Seahorse metabolic flux based analysis to resolve the contribution of mitochondrial and glycolytic metabolism to ATP production. By measuring OCR (oxygen consumption rate) and ECAR (extracellular acidification rate) and using the known molar ratio between O_2 consumed or protons produced with ATP production, ATP production by oxidative phosphorylation and glycolysis respectively can be calculated. Iron restriction suppressed the rate of mitochondrial ATP production (2C and 2F) and there was a trend towards reduced rate of total ATP production which was significant when each biological replicate was normalised to itself (2E and 2H). There was no significant difference in the rate of glycolytic ATP production (2D and G).

Finally, consistent with iron deficiency disrupting mitochondrial function we found that the ratio of signal from mitochondrial voltage dependent dye (Xiao, 2016) (mitotracker deep red) to mitochondrial content dependent dye (mitotracker green)

was reduced by iron deficiency (2I), indicating that at this timepoint iron deficiency inhibits mitochondrial activity, but not amount of mitochondria. The mitochondrial dysfunction of CD8 T cells expanded in iron deficient conditions could also be expressed as the % of cells with depolarised mitochondria (2J).

BH4 was recently proposed to play a role in securing iron uptake into the mitochondria and facilitating T cell activation. However, no effect of iron deficiency on BH4 levels were found at 24 hours post activation suggesting that reduced iron uptake does not reciprocally interact with the BH4 synthesis pathway (2K).

Defective energy production correlated with reduced levels of mTORC1, assayed by levels of pS6 (which is phosphorylated by the direct mTORC1 target S6K (Kim and Guan, 2019b)), at 24hrs and 48hrs after activation (3A, 3B and example histograms 3C). Iron deficiency also resulted in decreased expression of amino-acid transporter CD98 (3D and 3E) and reduced forward scatter (3F and 3G, indicating reduced blasting) and consistent with disrupted mTORC1 signalling (Sinclair et al., 2013a) (3D).

In parallel to mTORC1, Hif1α has been proposed to be a modulator of CD8 T cell function interfacing with the metabolism and the cellular environment (Doedens et al., 2013). HIF1α can be stabilised in normoxic conditions by iron deficiency as the function of the prolyl-hydroxylases which negatively regulate HIF1α levels is not only oxygen dependent, but iron dependent (Siegert et al., 2015). We measured HIF1a levels by flow cytometry. Addition of Dimethyloxalylglycine (DMOG) (a PHD inhibitor)

should stabilise HI1α and in line with this we observed increased signal in DMOG wells, confirming our staining was detecting HIF1α. Iron deficiency resulted in a small increase in HIF1α signal, but this difference was minor compared to the positive control DMOG treated wells (3E and 3F). Further experiments are required to investigate the role of HIF1α in the lymphocyte response to iron deficiency.

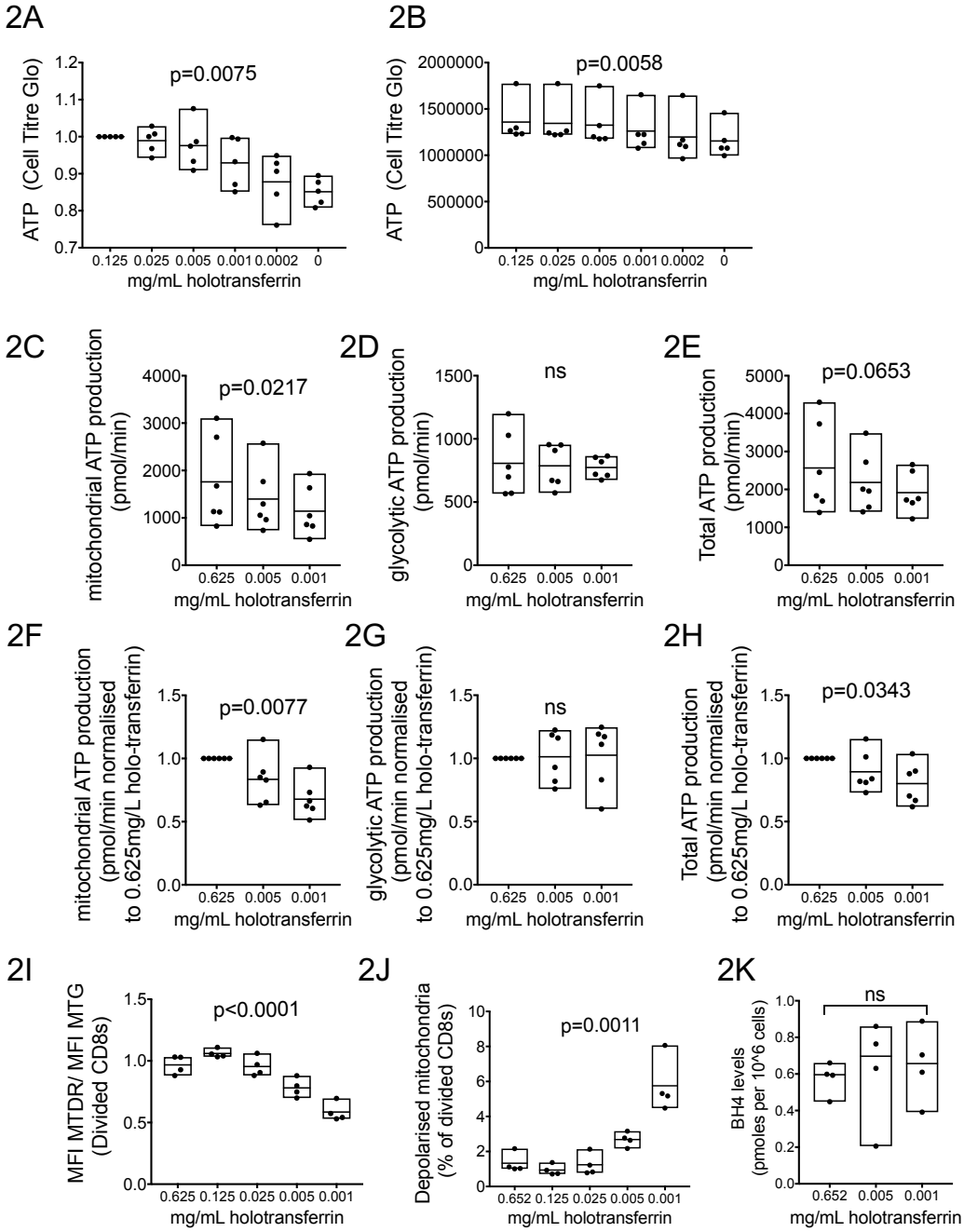


Figure 4.2. ATP content was measured using Cell Titre Glo luminescent assay for equal numbers of CD8 T cells per condition after 24 hours of activation (2A and 2B). The OCR and ECAR of 48 hour activated CD8 T cells was measured using a seahorse metabolic flux analyser, these data were used to generate mitochondrial, glycolytic and total ATP production rates (2C, 2D, 2E, 2F, 2G and 2H). Each data point is a biological replicate from a single mouse, representing the average of 2-5 technical replicate wells, after excluding any wells showing a poor monolayer. The MFIs of mitotracker deep red and mitotracker green were measured by flow cytometric analysis of 72 hour activated cells and ratio the of MTDR to MTG was calculated (2I). The % of CD8 T cells with depolarised mitochondria resolved by MTDR and MTG staining of 72 hour activated cells was also measured by flow cytometry (2J). BH4 levels were detected by HPLC in 24 hour activated cells (2K). One way ANOVA, effect of iron.

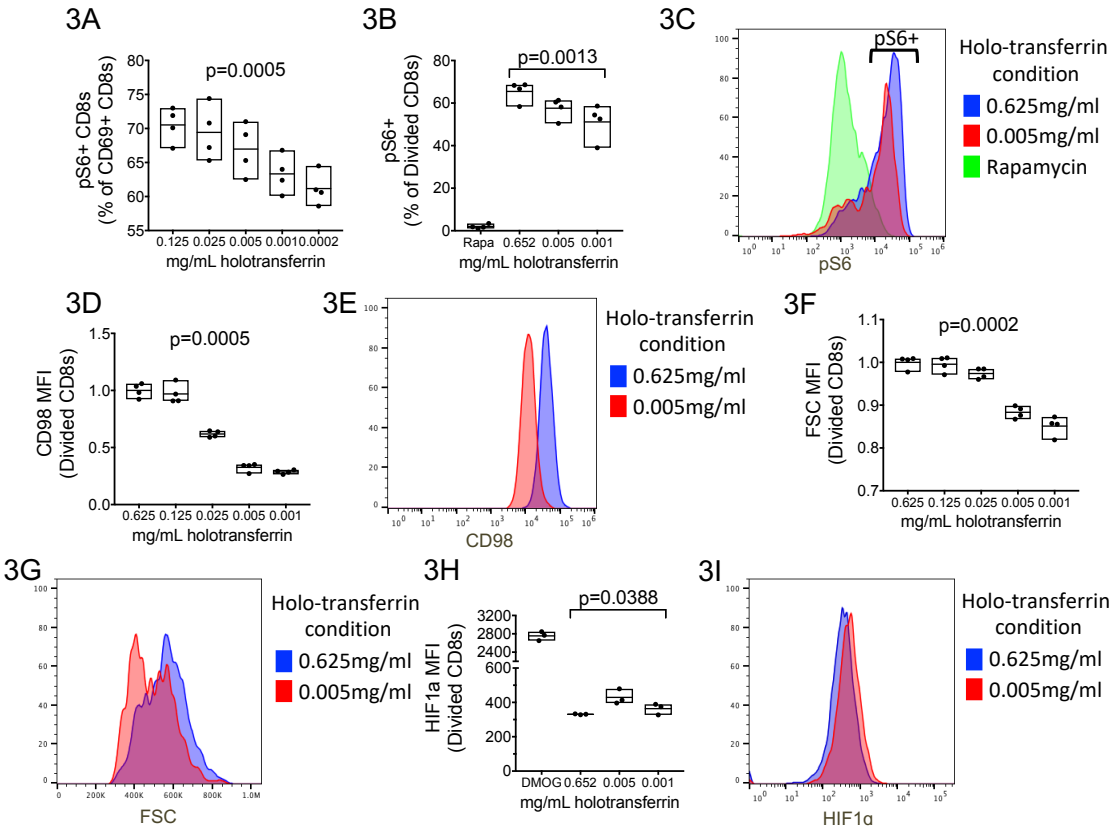


Figure 4.3. % of CD8 T cells positive for pS6 was measured by flow cytometry in cells activated for 24 (3A) and 48 (3B) hours, example pS6 histograms for divided CD8 T cells after 48hours of iron deficiency (3C). Relative CD98 expression(3D) and example CD98 expression histograms of divded cells (3E); relative FSC (3F) and FSC histograms of divided cells (3G); absolute HIF1α expression (3H) and HIF1α expression histograms (3I) D and E) was measured by flow cytometry on 48 hour activated cells. One way Anova, effect of iron.

4.2.3 Genetically impaired iron uptake recapitulates the defects of reduced iron availability

As an alternative approach to examine how impaired iron acquisition disrupts the physiology of CD8 T cells, we exploited mice with the *Tfrc*$^{Y20H/Y20H}$ mutation, which as discussed in chapter 3 show reduced efficiency of transferrin receptor mediated iron acquisition. The *Tfrc*$^{Y20H/Y20H}$ mutation impairs lymphocyte division, however we showed that supplementation with 100mg/L $FeSO_4$ could rescue proliferation *in vitro,* presumably by providing iron in an accessible form and rescuing the otherwise crippling cellular iron deficiency. As the profound inhibition of proliferation over 48-72 hours by the *Tfrc*$^{Y20H/Y20H}$ mutation made detailed phenotyping inappropriate, we focussed on 24 hours after activation, prior to the onset of cell division, where cell numbers and viability between genotypes would be more comparable (4A). To examine how defects in iron acquisition disrupted CD8 T cell physiology we compared the extent to which 100mg/L $FeSO_4$, which we had previously shown rescued *Tfrc*$^{Y20H/Y20H}$ cell proliferation to WT levels, improved various parameters in *Tfrc*$^{Y20H/Y20H}$ compared to WT cells. We expected that $FeSO_4$ would have a minimal effect in WT cells which were competent for iron uptake compared to *Tfrc*$^{Y20H/Y20H}$ cells where it should ameliorate any cellular iron deficiency.

There was no difference in the effect of $FeSO_4$ on CD69 expression between genotypes suggesting that impaired iron uptake had little effect on early activation events (4B). However, when total ATP content was measured by chemiluminescence assay (CellTitre Glo) for equal numbers of plated cells, both $FeSO_4$ and Ferric Ammonium

Citrate improved ATP levels to a greater extent in *Tfrc*$^{Y20H/Y20H}$ cells compared to WT cells (4C and 4D).

Similarly, when mitochondrial health was examined by flow cytometry both the ratio of voltage dependent MTDR: voltage insensitive MTG improved by $FeSO_4$ to a greater

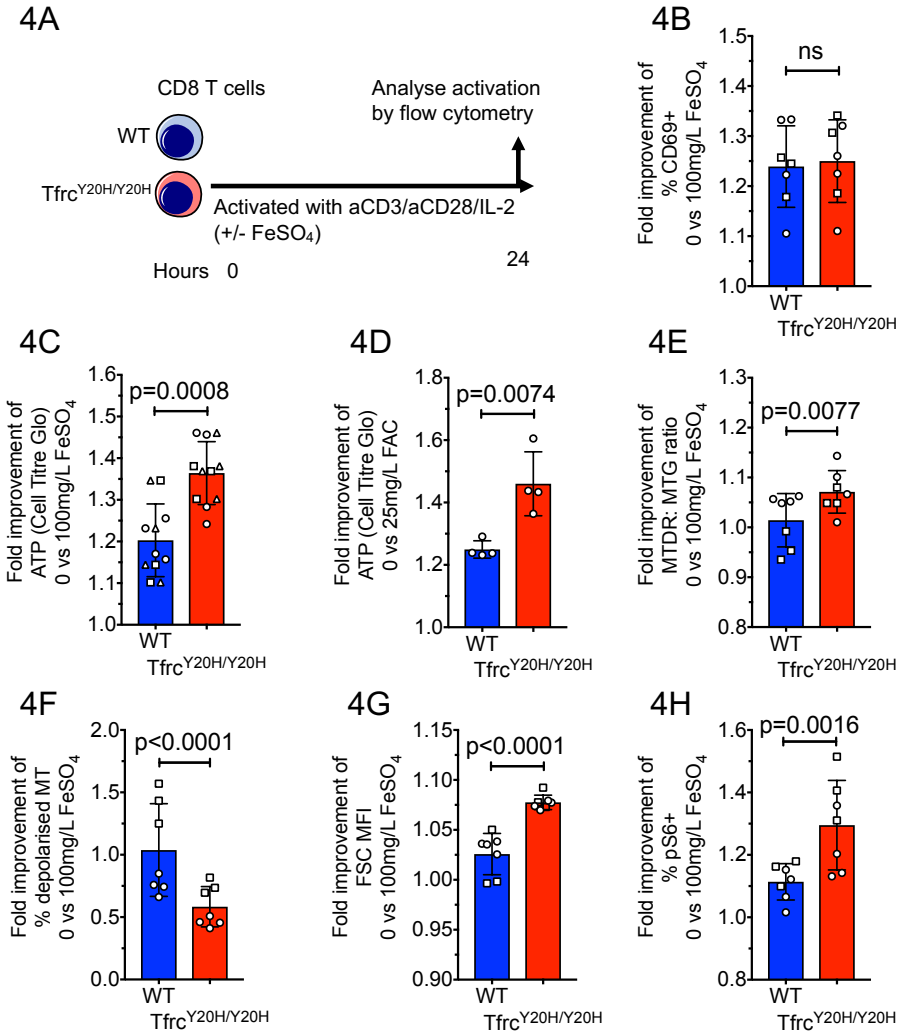


Figure 4.4. Experimental scheme for 24 hour *Tfrc*$^{Y20H/Y20H}$ experiments (4A). % of CD69+ cells was measured by flow cytometry (4B). ATP production was measured using Cell Titre Glo kit for cells cultured +/- $FeSO_4$ (4C) or Ferric ammonium citrate (4D). The MFIs of mitotracker deep red and mitotracker green were measured by flow cytometric analysis and ratio the of MTDR to MTG was calculated (4E). The % of CD8 T cells with depolarised mitochondria resolved by MTDR and MTG was also measured by flow cytometry (4F). FSC MFI and the % of pS6 expressing cells was measured by flow cytometry. (4B, E, F, G and H Combination of two independent experiments). (4C combination of three independent experiments). Two-way ANOVA of experiment and genotype, reported p value is genotype effect.

extent in *Tfrc*$^{Y20H/Y20H}$ than in WT cells (4E). Similarly, $FeSO_4$ reduced the frequency of viable CD8 T cells with low MTDR signal (that indicates depolarised mitochondria) (4F). These results indicate that disrupted transferrin bound iron uptake might be limiting mitochondrial biosynthesis, energy production and mitochondrial metabolism in these cells.

Iron supplementation of *Tfrc*$^{Y20H/Y20H}$ *in vitro* similarly resulted in greater improvements in FSC (4G) and the % of cells which were pS6+ (4H) than in WT cells indicating that mTORC1 activity may be limited by disrupted iron uptake by the *Tfrc*$^{Y20H/Y20H}$ mutation.

4.2.4 Disruption of Nrf2-/- does not alter sensitivity of CD8 T cells to iron availability *in vitro*

We show above that iron restriction disrupts CD8 T cell mitochondrial health and ATP production. However, ATP production is not the only function of the mitochondria and some publications suggest that generation of ROS by mitochondria is essential for activation/proliferation of CD4 T cells (Sena et al., 2013). Therefore, to address if altered ROS production contributed to the phenotype of iron deficient CD8 T cells we isolated CD8 T cells from Nrf2-/- mice and WT controls. Nrf2 is a ubiquitously expressed transcription factor with a central role in regulating the tolerance of cells to oxidative and xenobiotic stress, furthermore it is also emerging as a negative regulator of immune responses (Suzuki and Yamamoto, 2017). Nrf2 deficiency would be expected to result in lower expression of ROS detoxifying enzymes and elevated ROS

levels. We hypothesised that if altered ROS played a part in the T cell response to iron deficiency then Nrf2-/- cells would be less sensitive to the effects of iron deficiency.

We isolated CD8 T cells from WT and Nrf2-/- mice and activated in our standard activating conditions. We first examined the effect of Nrf2 genotype on activation in our iron deficient culture model. There was no significant difference between Nrf2-/- and WT mice in the extent to which decreasing extra cellular iron concentrations reduced proliferation (expressed as the % of cells which had divided) in 72hours (5A).

In addition, we found that Nrf2-/- mice showed the same changes in CD25 MFI (5B) (decreasing as serum iron levels drop) and CD71 MFI (5C) (increasing and then decreasing as serum iron levels decrease) as WT mice. Finally given our hypothesis that ROS might be important for the response to iron deficiency we examined how cellular staining with H2DCFDA, a fluorescent probe for total cellular ROS (Kalyanaraman et al., 2012), changed with extracellular iron concentration in the two groups. Cellular H2DCFDA fluorescence intensity increases on treatment with the pro-oxidant Tert-Butylperoxide (5D), which acted as a positive control. We found that H2DCFDA decreased as iron availability decreased in both genotypes (5E), however H2DCFDA is an imperfect sensor of cellular ROS and has been noted to respond directly to the levels of redox active metals, including iron, in a cell (Kalyanaraman et al., 2012). Therefore, our results suggesting H2DCFDA signal decreases significantly with iron deficiency and in fact increases on culturing of cells with addition $FeSO_4$ (5F and 5G), may in fact not indicate altered ROS levels, but in fact be a read out of altered

intracellular iron levels. Interestingly we also stained 24hour activated *Tfrc*$^{Y20H/Y20H}$ and WT cells with H2DCFDA in a separate experiment and found that $FeSO_4$ supplementation increased H2DCFDA signal more in *Tfrc*$^{Y20H/Y20H}$ than WT mice, consistent with defective iron uptake either impairing ROS production or simply resulting in less redox active Fe in the cell (5H).

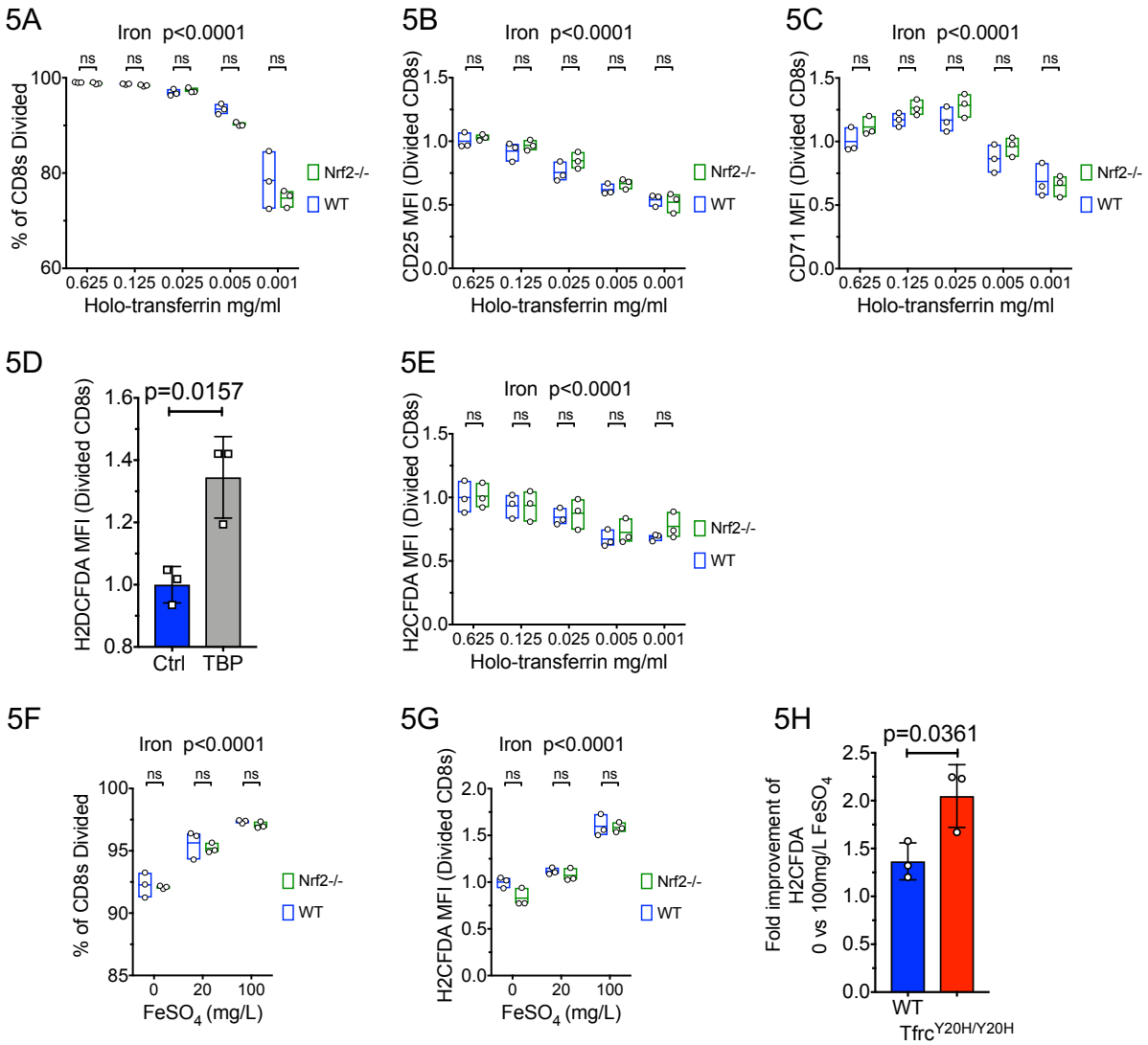


Figure 4.5. % of CD8 T cells which had divided after 72hours of culture in iron free medium with titration of holo-transferrin resolved by analysis of cell trace violet dilution by flow cytometry (5A). MFI of CD25 (5B), CD71 (5C), H2DCFDA (5D and 5E) measured by flow cytometry. % of CD8 T cells which had divided after 72hours after culture in R10 +/- $FeSO_4$ resolved by analysis of cell trace violet dilution by flow cytometry (5F). MFI of H2DCFDA (5G) in cells culture +/- $FeSO_4$ measured by flow cytometry. MFI of H2DCFDA measured for WT and *Tfrc*$^{Y20H/Y20H}$ cells (5H).

4.3 Discussion

Here we present a novel *in vitro* model of physiological serum iron deficiency; to bypass the unavoidable (variable) iron content of fetal calf serum, we instead utilise a

modification of a serum free media supplement which was custom produced to lack iron and transferrin. By titrating in different amounts of holo-transferrin, whilst maintaining total transferrin concentrations constant we can mimic the effects of hypoferremia on immunity *in vitro*. We also used CD8 T cells isolated from mice with the *Tfrc*$^{Y20H/Y20H}$ mutation where the efficiency of iron uptake is genetically inhibited. Both these models are advantageous compared to previous work using small molecule chelators, as chelators can non-physiologically remove iron directly from the intracellular iron pool, disrupt redox homeostasis and interact with other metal ions (Richardson and Ponka, 1998).

We found that restricting iron uptake inhibited proliferation of CD8 T cells *in vitro,* disrupted acquisition of effector functions and perturbed cellular metabolism, but did not disrupt the early transcriptional activation event of increasing CD69 expression. Iron restriction likely interacts with the cell cycle at multiple points, hitting mitogenic signalling, disrupting expression of cyclins and cyclin dependent kinase inhibitors, reducing deoxynucleotide synthesis and altering the catalytic activity of multiple enzymes involved in DNA replication, including the DNA polymerases (Netz et al., 2011; Yu et al., 2007). Proteomic and whole transcriptome analysis will likely be required to fully define the disruption of the cell cycle in iron deficient immune cells.

The reduced expression of CD25 we observe on iron deficient cells could reflect disrupted differentiation, with its expression positively correlating with effector potential and negatively correlating with expression of memory markers such as

CD127/IL7R *in vitro* (Pipkin et al., 2010). CD25 not only mediates IL-2 signalling, the expression of CD25 is enhanced by IL-2, generating a positive feedback loop where IL-2 promotes more IL-2 signalling to drive proliferation and differentiation. Reduced CD25 in our *in vitro* cultures could be argued to reflect less net IL-2 production and paracrine signalling because of fewer CD8 T cells. However, for this reason, all cultures were supplemented with a high dose of 100U murine recombinant IL-2/ml. It is possible therefore that iron deficiency produces a cell intrinsic defect in T cell IL-2 signalling which erodes effector differentiation, potentially through failure to increase Blimp1 expression (Pipkin et al., 2010). It would be interesting to examine if iron restriction had a more severe effect on T cell function in the absence of additional IL-2 supplementation and how iron restriction impairs the early wave of IL-2 secretion prior to division.

Our data suggests that in conditions of both genetic and environmental iron deficiency T-cell ATP production is restricted and that this results specifically from a defect in mitochondrial ATP production. We detect reduced mitochondrial membrane potential relative to mitochondrial mass and more depolarised mitochondria. This is of note, as whilst glycolysis is not directly iron dependent, iron is prevalent in the electron transport chain and citric acid cycle enzymes. Our finding that mitochondrial metabolism is particularly sensitive to iron deprivation is in line with published research in cell lines using chelators and where iron homeostasis has been disrupted genetically (Cronin et al., 2018; Galy et al., 2010; Li et al., 2019; Oexle et al., 1999). However, in our hands CD8 T cells do not undergo a compensatory increase in

glycolytic metabolism, as has been suggested for other cell types. Iron can clearly interface with metabolism in a range of ways directly and indirectly and the outcome of iron deficiency on metabolism likely depends on cell type and circumstance.

Whether the reduction in ATP production we observe in iron deficient and *Tfrc*$^{Y20H/Y20H}$ T cells is sufficient to disrupt cell function remains to be seen. The established model is the majority of activated T cell energy production is supplied by a shift to oxidative glycolysis (Buck et al., 2015). However mitochondrial derived ATP maybe important in some circumstances such as at the immunological synapse (Baixauli et al., 2011) and biogenesis of mitochondria is an important hall mark of T cell activation (Fischer et al., 2018). Metabolic insufficiency has been suggested to drive dysfunction of T cells in other physiological situations such as in the context of chronic viral or anti-tumour immune responses and in such cases even slightly sub-optimal metabolism could have severe consequences on adaptive immunity (Bengsch et al., 2016; Scharping et al., 2016). Furthermore enhancing mitochondrial electron transport chain activity and metabolic fitness *in vivo* improves differentiation to memory and cytokine secretion (Champagne et al., 2016).

Interestingly, for Th1 CD4 cells generalised inhibition of oxidative metabolism through inhibition of the TCA cycle impairs cytokine production and proliferation. However specific inhibition of complex I disrupted proliferation, whilst complex II inhibition prevented inflammatory cytokine production (Bailis et al., 2019). Whether these observations hold true for CD8 T cells is yet to be shown, and it will be interesting to

explore the differential contribution of ETC complexes to T cell function in the context of the perturbation of the ETC by iron deficiency.

In addition their capacity to make ATP, mitochondria are increasingly being thought of as essential for the function of T cells and other proliferating cells independent of their role in ATP production (Desdín-Micó et al., 2018). Production of mitochondrial ROS has been demonstrated to be key for T cell function (Kamiński et al., 2012), in particular it has been suggested that mitochondrial ROS facilitates IL-2 production, linking back to the observed changes in effector differentiation and CD25 expression (Fischer et al., 2018; Sena et al., 2013). Mitochondrial remodelling during CD4 T cell activation has been suggested to be important for enhanced one carbon metabolism to fuel purine and thymidine synthesis (Ron-Harel et al., 2016). Glutamine uptake is essential and highly upregulated on T cell activation, in part this will provide fuel for the citric acid cycle (Carrio et al., 2004). Mitochondria play essential roles in the biosynthesis of metabolites such as aspartate, which is essential for nucleotide biosynthesis, by providing electron acceptors (Birsoy et al., 2015; Sullivan et al., 2015). The upshot of this is that our observation of reduced ATP production and mitochondrial health in iron restricted CD8 T cells might be indicative of mitochondrial dysfunction, but may not be responsible *per se* for our defective T cell phenotype. Measurement of mitochondrial ROS and metabolite profiling of iron deficient cells, directly examining the activity and expression of the electron transport chain, as well as examining the ability of metabolite supplementation (Sullivan et al., 2015) to bypass the iron deficient phenotype may address if and how mitochondrial dysfunction is

contributing to T cell dysfunction in iron deficiency. However for CD4 T cells it has been noted that genetic disruption of mitochondrial function through deletion of key mitochondrial transcription factor Tfam reduces proliferation, but aberrantly enhances the Th1 pro-inflammatory phenotype, in part through disrupted lysosomal function in these cells (Baixauli et al., 2015)

Negative regulation of the mTORC1 pathway by iron restriction has been noted in developing red blood cells, the brain and cell lines (Knight et al., 2014; Ndong et al., 2009; Ohyashiki et al., 2009; Watson et al., 2016); we demonstrate reduced CD8 T cell mTORC1 in both cases of environmental iron restriction and cells expressing the hypomorphic $Tfrc^{Y20H/Y20H}$. In cell lines the suppression of mTORC1 by iron deficiency was proposed to be via the protein DNA damage-inducible transcript 4 (DDIT4, also known as REDD1) which was transcriptionally induced by iron deficiency (Ohyashiki et al., 2009; Watson et al., 2016). Despite DDIT4 being a HIF1a target gene, the induction of DDIT4 by iron deficiency was reported to be independent of increased HIF1a activity (Watson et al., 2016). HIF1α has however been reported to contribute to the suppression of mTORC1 activity in hypoxic germinal centre B cells (Cho et al., 2016). Furthermore reduced ATP production due to mitochondrial dysfunction in the context of iron deficiency could hypothetically activate AMPK which in turn can suppress mTORC1 (Gwinn et al., 2008; Inoki et al., 2003). Finally reduced mTORC1 in the context of reduced IL-2 is interesting as IL-2 is a major regulator of mTORC1 activity in CD8 T cells (Rollings et al., 2018). Therefore altered DDIT4 production, AMPK activity, IL-2 signalling or amino acid uptake could all contribute to mTORC1 suppression in iron

deficient cells (Gwinn et al., 2008; Ohyashiki et al., 2009; Rollings et al., 2018; Shapiro Jason S et al., 2017; Watson et al., 2016). It remains to seen whether mTORC2 signalling is modulated by iron deficiency.

Altered mTORC1 activity underlie some of the defects in effector differentiation, such as reduced Gzmb expression, that we observe in iron deficiency (Pollizzi et al., 2015). In cancer cells, nutrient restriction induces mTORC1 inhibition and growth arrest, and in the absence of mTORC1 inhibition cells die because their activity does not match nutrient availability (Inoki et al., 2003). mTORC1 suppression therefore might play an important part in the survival of iron deficiency through modulation of cellular anabolism and direct regulation of cellular iron homeostasis (Bayeva et al., 2012). The levelling off of CD71 expression at very low iron levels could possibly reflect a reduction in mTORC1 activity reducing CD71 transcription or translation, but also reduced mTORC1 activity has been proposed to post transcriptionally inhibit CD71 through tristetraprolin (TTP or ZFP36) (Bayeva et al., 2012). Therefore, it will be important to carry out experiments testing if lymphocytes that have hyperactive mTORC1 signalling will be more likely to die during iron deficiency.

Suppression of CD98 expression by iron deficiency may contribute to impaired T cell function as CD98 KO T cells show impaired clonal expansion *in vitro* and *in vivo* (Cantor et al., 2011). Canonically CD98 or SLC3a2 forms complexes with Slc7a5,6,7 or 8 to form the System L family of large neutral amino acid (LNAA) transporters and one would expect reduced CD98 expression to disrupt amino acid uptake (Ren et al., 2017), but

this could be formally proven by examining kynurenine uptake as a proxy for LNAA uptake (Sinclair et al., 2018). It remains unclear if altered CD98 expression is upstream or downstream (or both) of suppressed mTORC1 activity in iron deficiency. If CD98 expression reflects System L mediated amino acid uptake more generally, which could be resolved for example with the recently published kynurenine uptake assay (Sinclair et al., 2018), it might be that reduced amino acid transporter expression and amino acid uptake is driving reduced mTORC1 activity in CD8 T cells (Kim and Guan, 2019a; Sinclair et al., 2013). Unpublished work supports the concept that iron influencing amino acid uptake may in turn regulate mTORC1 activity (Shapiro Jason S et al., 2017). However, of note is that CD98 also shows integrin binding activity; the disrupted proliferation of CD98 KO cells can be almost rescued with a CD98 construct lacking amino acid transport, but not integrin binding activity suggesting CD98's essential role in T cells might be related to cell-cell interactions (Cantor et al., 2011); in which case one would assume another amino acid transporter can compensate for CD98 deficiency.

Another aspect of metabolic adaptation to nutritional stress we have yet to explore in this work is autophagy, a process where cytoplasmic contents (organelles and proteins) are delivered to the lysosome for degradation (Das et al., 2012). Studying autophagy in activated lymphocytes is complicated because of its role in T cell development and naïve T cell survival (Pua et al., 2007). There is also some asymmetry in the sensitivity of CD8 vs CD4 subsets (Murera et al., 2018; Puleston et al., 2014). Autophagy is generally thought to be less active in anabolic effector cells (Adams et

al., 2016), with the particularly catabolic memory and naïve cells being more dependent. mTORC1 negatively regulates autophagy through inhibition of the autophagy promoting kinase ULK1, coupling nutrient sensing with autophagy. It is plausible that reduced mTORC1 activity in iron deficient T cells results in enhanced autophagy which might play a role in metabolic adaptation to iron restriction. Autophagy also plays a direct role in iron homeostasis as degradation of ferritin and liberation of stored iron is dependent on autophagy ('ferritinophagy') and the specialised cargo adaptor NCOA4 (Mancias et al., 2015, 2014; Quiles del Rey and Mancias, 2019). It will be of interest to explore whether ferritinophagy and release of iron stores plays a role in fuelling initial lymphocyte activation and adaptation to iron deficiency, especially on entering iron deprived microenvironments (eg a tumour, or foci of infection) after acquiring a store of iron in a more favourable niche (lymph node or spleen). One point of note is that the mechanism for Ferritin/NCAO4 targeting to the lysosome in ferritinophagy is unclear (Goodwin et al., 2017) and therefore the phenotype of ATG5, 3 or 7 deficiencies used to examine autophagy in many studies may not reflect a deficiency in ferritinophagy.

We also present preliminary evidence for a small increase in HIF1α levels in iron deficient CD8 T cells. HIF1α is a major regulator of cellular metabolism (Gordan et al., 2007), but it does not seem to play a role in the early metabolic remodelling observed during activation of CD8 T cells (Wang et al., 2011). However genetic stabilisation of HIF1α activity does modify CD8 T cell differentiation and metabolism (Doedens et al., 2013) and HIF1α KO disrupts acquisition of effector functions and anti-tumour

immunity (Palazon et al., 2017). It is unclear what role HIF1α activation plays in the adaptation of cells to iron deficiency and more specifically if HIF1α stabilisation is driving the phenotype of iron deficient immune cells.

We also considered how altered ROS production might contribute to the phenotype of iron deficient CD8 T cells; the limitation of the H2DCFDA probe for reactive oxygen species (Kalyanaraman et al., 2012) does not exclude the possibility that Nrf2-/- CD8 T cells experience increased ROS compared to WT cells, however our results do not suggest that Nrf2-/- CD8 T cells have altered sensitivity to iron deficiency. However, our results do support the idea that any change in intracellular iron levels (reduced transferrin bound iron, NTBI supplementation and the *Tfrc*$^{Y20H/Y20H}$ mutation) does reduce the signal from H2DCFDA. Whether the H2DCFDA signal reflects iron directly detected or is due to the role of iron in the cellular physiology of ROS production remains to be seen and could be addressed with alternative ROS reporters. The activation defect of mitochondrial Complex III deficient (Uqcrfs1−/−) T cells could be partially rescued by exposure to increased ROS through the addition of galactose oxidase and galactose, so it may be that exposure to increased ROS can ameliorate some of the phenotype of T cells stimulated under iron deficient conditions (Sena et al., 2013). A more extensive characterisation of ROS levels in iron deficient T cells using multiple approaches and how modulating ROS affects the phenotype of iron deficient T cells is therefore required.

Our results also more generally suggest that Nrf2 is not essential for CD8 T cell proliferation *in vitro* and furthermore does not modulate expression of CD25 or CD71. However Nrf2 has been proposed to modulate T cell activity in graft vs host disease (Tsai et al., 2018), the proinflammatory phenotype of macrophages (Mills et al., 2018) and anabolism of cancer cells (Mitsuishi et al., 2012). Our *in vitro* phenotyping of CD8 T cells was very superficial and *in vitro* T cell culture fails to recapitulate many aspects of *in vivo* T cell differentiation, therefore it would be of interest to investigate further the role of Nrf2 in T cell immunity *in vivo* with a particular focus on immunometabolism, anti-cancer and infection immunity.

One final note is whilst the perturbation of CD8 T cell physiology by iron deficiency has been mostly discussed here in the context of driving dysfunction, it is important to recognise that these changes may in fact represent a physiological adaptive stress response to iron deficiency to minimise the long-term consequences of this extremely common physiological state on adaptive immunity. For example, inhibition of mTORC1 during glucose deprivation is essential for avoiding cell death (Inoki et al., 2003). This concept will be explored further in the chapter relating to 'iron and immune memory'. It would be interesting to explore in the future the pathways which mediate tolerance and adaptation of the immune response to iron deficiency.

Serum iron deficiency inhibits the primary immune response

5.1 Introduction

5.1.1 Serum iron deficiency and anaemia

In the previous chapter we demonstrated that reduced transferrin saturation *in vitro*, mimicking serum iron deficiency, resulted in impaired CD8 T cell proliferation, metabolic activity and effector function. In Gambian infants serum iron levels decline rapidly from birth such that by 2-months of age they are <5µmol/L (Armitage et al., 2019). In similarly aged children from high income countries however serum iron levels remain stable at levels similar to that observed in adults (Ritchie et al., 2002). Iron deficiency anaemia is estimated to effect one sixth of the world's population and is particularly prevalent in children and pre-menopausal women (Kassebaum et al., 2014; Lopez et al., 2016a; Miller, 2013), with a distribution coinciding with regions demonstrating poor vaccine efficacy (Kollmann, 2013). The longitudinal data of Gambian infant serum iron and prevalence of iron deficiency anaemia suggests that near undetectable serum iron levels might be a widespread pathophysiological phenomenon in LMIC infants. As serum transferrin-bound iron, continuous with iron in the tissue fluid, is the physiological supply of iron for the body's cells, a question is whether low serum iron affects the adaptive immune response and efficacy of vaccination?

Serum iron deficiency and anaemia in Gambian infants has been shown to be driven by a combination of persistently raised hepcidin redistributing iron and blocking dietary iron uptake in the context of already low dietary iron availability (Pasricha et al., 2014; Prentice et al., 2019). The high level of iron in standard mouse chow and the predisposition of mice to iron load upon weaning onto solid food, makes mouse models of iron deficiency using iron deficient diet impractical. Chronic dietary iron deficiency also results in anaemia, raised erythropoietin and splenic stress erythropoiesis in mouse models which could conceivably modulate immunity (Cho et al., 2016; Purroy et al., 2017). Finally chronic iron deficiency could perturb adaptive immunity through effects on lymphopoiesis (Jiang et al., 2019). For these reasons and the physiological relevance of hepcidin as a driver of serum iron deficiency in humans, we decided to utilise injection of hepcidin as a way to acutely induce serum iron deficiency *in vivo* to investigate how low levels of serum iron, due to raised hepcidin, alter the adaptive immune response to immunisation. Hepcidin injection reduces serum iron by inducing degradation of ferroportin on reticuloendothelial macrophages, preventing the recycling iron derived from the degradation of senescent red blood cells (Rivera et al., 2005). Ongoing iron consumption by cells, significantly the erythron, depletes the serum of iron. Because of the short half-life of hepcidin *in vivo* we exploited a mimetic of hepcidin, termed minihepcidin which is analogous to the N-terminal 9 amino acids of hepcidin, but includes modified amino acids to improve its pharmacokinetic properties. Minihepcidin shows increased potency and the capacity to durably reduce serum iron for at least 24 hours even in mice on standard diets (Arezes et al., 2015; Ramos et al., 2012); and then investigated

the effect of the induced low serum iron state on T-cell and B-cell responses, both in terms of magnitude, effector functions and differentiation.

5.1.2 Control of CD8 T cell differentiation

As discussed in previous chapters, dynamic changes in T cell metabolism are essential for supporting activation, proliferation and differentiation (Chapman et al., 2019). Key questions in T cell biology are what controls the maturation of a naïve cell into a fully competent effector cell and then what determines if a responding T cell dies during contraction or eventually contributes to immune memory, and what type of memory cell will it make. How iron deficiency affects memory responses will be discussed in more detail in the next chapter, but it is important to consider here the various factors which regulate the CD8 T cell response *in vivo* and how they might be modulated by reduced iron availability. Various models for generating diversity in the effector and memory pools of T cells have been proposed (Kaech and Cui, 2012).

Effector and multiple subsets of memory cells can be derived from a single naïve donor cell (Stemberger et al., 2007), arguing against diversity of the T cell response originating from diversity in the pool of naïve cells. After TCR stimulation the naïve T cell has been proposed to undergo asymmetric division resulting in one daughter cell with an effector bias and one with a less differentiated memory bias (Chang et al., 2007), in part underlined by asymmetric amino acid uptake and mTOR activity (Pollizzi et al., 2016).

Other models of CD8 T cell differentiation suggest a situation of decreasing potential where naïve cells loose functions like 'stemness', longevity and proliferative function, but gain effector functions as they terminally differentiate driven by ongoing stimuli. Evidence for this model includes the decreased likelihood that cells receiving TCR stimulation late in the response will become memory (Sarkar et al., 2008), whilst naïve cells which become involved in the immune response closer to its end have an altered differentiation profile (D'Souza and Hedrick, 2006). Furthermore memory CD8 T cells which arise after secondary challenge (secondary memory CD8s) are suggested to favour a more differentiated effector memory state than primary CD8 memory T cells (Nolz and Harty, 2011).

A separate, but not necessarily mutually exclusive concept is that the strength and integration of different signals either drives the differentiation of CD8 T cells down one of a number of different parallel differentiation paths to become either an effector or surviving memory cell (Kaech and Cui, 2012). Biologically this has generated a great deal of interest in defining the CD8 effector cells which are the precursors of memory versus those effector cells which will die after the primary response; it seems that expression of the interleukin 7 receptor (IL7R or CD127) and lack of killer cell lectin receptor G1 (KLRG1) defines cells with a 10 fold greater chance of becoming memory (Joshi et al., 2007). This signal integration model is supported by observations that different ratios of IL7R+ KLRG1- (MPECs) and IL7R- KLRG1+ (SLECs) are observed in different tissues, for different infections (Plumlee et al., 2013). Furthermore, after

single cell transfer of antigen specific cells, it has been suggested that clones with a larger burst size might favour a KLRG1+ phenotype (Plumlee et al., 2013). Also cells expressing a memory precursor-like phenotype are less proliferatively active (Kinjyo et al., 2015), posing a question regarding how nutrient availability and proliferation might regulate differentiation.

Lineage tracing of effector CD8 T cells suggest that acquisition of effector functions such as Gzmb and IFNg expression does not prevent, and may be a prerequisite step along the path to, memory formation (Bannard et al., 2009; Harrington et al., 2008). Furthermore, analysis of the DNA methylation profile of naïve, memory and effector CD8 T cells (both those biased and not biased to become memory), suggests that T cell differentiation involves the dynamic methylation/suppression of naïve associated genes during the effector phase and then re-expression of these naïve associated genes in memory cells (Youngblood et al., 2017).

Mechanistically the lineage choices of CD8 T cells seems to be driven by the activity of a number of transcription factors which sometimes co-operate and sometimes mutually antagonise each other to favour effector or memory cells (Kaech and Cui, 2012). Both T-bet and eomesodermin (EOMES) are induced to some degree in effector cells, with a level of reciprocal expression; T-bet is key for effector differentiation, whilst EOMES drives the differentiation of memory cells (Joshi et al., 2007; Takemoto et al., 2006). BLIMP1 also favours effector differentiation during primary and secondary infection, but is additionally important for secondary expansion of memory

cells (Kallies et al., 2009) and seems to be locked in another regulatory network with the memory favouring transcription factor BCL6 (Ichii et al., 2004). BACH2 seems to restrain terminal effector differentiation with KO cells having reduced capacity for primary clonal expansion and secondary response (Roychoudhuri et al., 2016).

IRF4 is required for expansion and differentiation of effector cells (Yao et al., 2013), in part driving expression of lineage defining transcription factors T-bet and Blimp1. IRF4 co-operates with BATF in this process (Kurachi et al., 2014). The strength of initial TCR stimulus may control the level of IRF4 expression and in turn regulate the metabolism, clonal burst size and capacity of cells to secrete cytokines on restimulation (Man et al., 2013; Nayar et al., 2014).

This differentiation programme does not take place in isolation, with external signals from cytokines and direct cell-cell interactions determining the outcome of the CD8 T cell response. IL-12 has been shown to positively regulate CD8 effector differentiation and SLEC differentiation in response to viral challenge (Joshi et al., 2007). The cytokines essential for supporting CD8 T cell responses may also differ between immune stimuli, with IL-27 being essential for adjuvanted protein responses, but dispensable for CD8 T cell responses to viral infection (Klarquist et al., 2018).

5.1.3 Humoral immunity

The humoral immune response begins with the activation of naïve B cells in the B cell follicle of a secondary lymphoid organ. Unprocessed soluble antigen can diffuse into the follicle. In contrast particulate antigens, such as immune complexes, bacteria or viruses must be transported in, presented by and retained on the surface of macrophages, dendritic cells or resident follicular dendritic cells (Batista and Harwood, 2009). The activated B cell will then endocytose antigen, present it on MHC-II and form long term stable interactions with activated antigen-specific CD4 T cells at the interfollicular region (Qi et al., 2008). Reciprocal co-stimulation between antigen specific B and T cells drives clonal expansion of both populations in the secondary lymphoid organ.

Early in the humoral immune response B cells choose one of two paths (Paus et al., 2006): to either migrate out of the follicle and contribute to the first wave of antibody production through the early extra follicular plasmablast response (Nutt et al., 2015), or to remain in the follicle and form a germinal centre (GC) (De Silva and Klein, 2015). The specialised substructures which constitute germinal centres form from four days after immunisation with model antigens in mice (Kerfoot et al., 2011). In germinal centres antigen specific B cells proliferate rapidly, undergoing iterative rounds of somatic hypermutation of the B cell receptor (BCR) and clonal selection for cells expressing a BCR with the highest affinity for the antigen of interest (De Silva and Klein, 2015). Over a number of days this process of affinity maturation produces GC B cells, expressing a BCR with a higher affinity for the antigen of interest, which can then go on to differentiate into high quality antibody secreting plasma cells or memory B cells

(Nutt et al., 2015). This fate choice is under the control of key lineage transcription factors; expression of BLIMP1 and IRF4 controls plasma cell differentiation (Nutt et al., 2015), whilst BACH2 favours the memory B cell fate (Shinnakasu et al., 2016). It is interesting that similar networks of transcription factors regulate the differentiation fate of B cells and T cells.

The CD4 T cell subsets that interact with B cells during the humoral response and support formation of the germinal centre are termed T follicular helper cells (Tfh). Tfh are defined by expression of the transcription factor BCL6 and surface expression of PD-1 and CXCR5 (Johnston et al., 2009). Interestingly BCL6 is also a key lineage transcription factor of germinal centre cells (Dent et al., 1997). T cells seem to acquire a Tfh-like phenotype early in the response as they help B cells, with the subset of cells which migrate into the follicle along with the GC B cell precursors retaining this phenotype (Kerfoot et al., 2011). GC B cells and Tfh are reciprocally dependent on each other for maintenance: any cell intrinsic defect in GC B cell differentiation limits the size of the Tfh population and vice versa (King, 2011).

Similar to T-cells, the rapid proliferation, differentiation and effector functions (production of antibody) by B-cells requires increases in glycolytic and oxidative metabolism (Boothby and Rickert, 2017). Increased glucose uptake and glycolysis fuel the biosynthesis of metabolic intermediates including ribonucleotides via the pentose phosphate pathway. Mitochondrial remodelling also takes place facilitating increased oxidative energy production and biosynthesis in the mitochondria (Waters et al.,

2018). Of relevance to our exploration of iron in the adaptive immune responses is that haem biogenesis in the mitochondria has been proposed to be a major regulator of B cell differentiation, through haem mediated suppression of BACH2 favouring plasma cell differentiation (Jang et al., 2015; Tsui et al., 2018). The importance of haem as a regulator of T cell differentiation still requires further exploration.

Similar to T cells, mTOR has emerged as a central regulator of B cell responses. mTOR inhibition disrupted the GC B cell response, reducing the magnitude and quality of the antibody response (Ye et al., 2017). In particular within the GC, mTOR is induced in B cells by T cell help, facilitating nutrient acquisition and biomass accumulation to fuel the B cell for subsequent rounds of proliferation expanding the selected clone. mTOR therefore plays a central role in clonal selection and affinity maturation in the GC (Ersching et al., 2017). Signalling via PKCβ has been proposed to regulate plasma cell and GC B cell differentiation through the induction of mTOR and mitochondrial metabolism (Tsui et al., 2018). Co-stimulatory signals from GC B cells received via ICOS induce mTOR activity in Tfh. This Tfh intrinsic mTOR activity and induction of anabolism is essential for their differentiation and maintenance (Zeng et al., 2016). Hypoxia in the GC microenvironment has also been proposed to regulate GC B cell function through HIF1α and mTOR (Cho et al., 2016).

5.1.4 Chapter Aims

Previous chapters showed that iron restriction impairs CD8 T cell expansion, metabolism and acquisition of effector functions *in vitro*, *in vivo* development of

quality adaptive immune responses depends on the complex interactions between metabolism, lineage defining transcription factors and epigenetic regulators. We found that genetic disruption of iron uptake also severely disrupted adaptive immune function. A major aim of this body of work is to investigate to what extent the severe serum iron deficiency observed in LMIC infants (Armitage et al., 2019; Lopez et al., 2016a), impairs adaptive immune responses *in vivo*.

We utilise injection of mini-hepcidin PR73 to prevent export of iron out of recycling reticuloendothelial macrophages, and induce functional serum iron deficiency (Arezes et al., 2015; Ramos et al., 2012). Methodologically we mainly focus on three vaccination platforms. Two transgenic replication deficient viruses: an adenovirus based vector and Modified vaccinia virus Ankara, both expressing OVA (AdHu5-OVA and MVA-OVA); these vaccine platforms induce strong cellular and humoral immunity and are being explored as possible candidate vectors for malaria vaccines (Draper et al., 2011; C. Wang et al., 2016). Given the possibility that iron deficiency might alter viral replication directly (Drakesmith and Prentice, 2008) (discussed in a future chapter) we felt it was important to use replication deficient viruses for these experiments. We also selected an OVA and adjuvant immunisation involving whole ovalbumin (OVA) protein mixed with Monophosphoryl Lipid A (MPLA), a TLR4 ligand, and α-galactosylceramide (αGC). αGC is a synthetic lipid antigen which activates iNKT cells which in turn activate DCs via CD40/CD40L interactions (Fujii et al., 2004; Hermans et al., 2007). This immunisation is therefore broadly analogous to protein immunisation involving protein, a TLR ligand and CD40 stimulation (Klarquist et al.,

2018). To resolve the cellular immune response to immunisation we focus on: the endogenous antigen specific CD8 T cell response, resolved using MHC-I tetramers and identified by flow cytometry using the gating describe in supplementary figure S2; the response generated from congenic adoptively transferred OVA-specific OT-I CD8 T cells, gated as in supplementary figure S1; the endogenous GC B cell response, gated as in supplementary figure S3; the endogenous T follicular helper cell response gated as in supplementary figure S4.

Specifically, we will investigate:

1. Mini-hepcidin injection as a model of transient serum iron deficiency
2. If mini-hepcidin injection and/or low iron diet perturb adaptive immunity and the dependence of this on serum iron deficiency
3. The extent to which serum iron deficiency disrupts primary CD8, CD4 and B cell responses to immunisation

5.2 Results

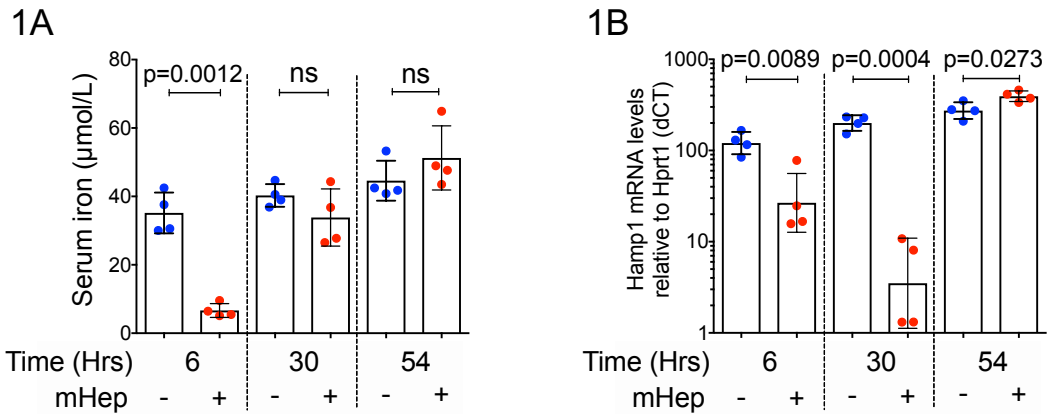


Figure 5.1. Mice were injected with mini-hepcidin or vehicle control and culled 6, 30 or 54 hours after injection. Serum iron concentration (1A) and hepatic hepcidin mRNA (1B) were measured. ANOVA, reported multiple comparisons vehicle vs mHep for each time point.

5.2.1 Mini-hepcidin injection causes transient serum iron deficiency

To develop a model of acute and transient serum iron deficiency we decided to utilise the mini-hepcidin PR73. Mini-hepcidins have been previously shown to cause reduced serum iron concentration *in vivo*, but we wished to confirm this for our supplier and in our animal house. Mice were injected with 100nmol of mini-hepcidin PR73 or an appropriate amount of vehicle control and culled at 6, 30 and 54 hours. Mini-hepcidin treatment significantly reduced serum iron concentration at 6 hours post injection and there was a trend towards lower serum iron at 30 hours (1A); liver hepcidin mRNA expression was significantly reduced at 6 hours and even lower by 30 hours in the mini-hepcidin treated group (1B). By 54 hours after injection hepcidin mRNA expression was slightly raised in in the mini-hepcidin treated group, relative to the control group. Hepatic hepcidin mRNA expression indirectly reflects serum iron deficiency, as reduced serum iron levels will be sensed through HFE, TFR2 and TFR1

reducing SMAD1/5/8 phosphorylation and hepcidin transcription (Corradini et al., 2011; Ganz, 2011).

5.2.2 A combination of mini-hepcidin injection and dietary iron deficiency inhibit adaptive immunity

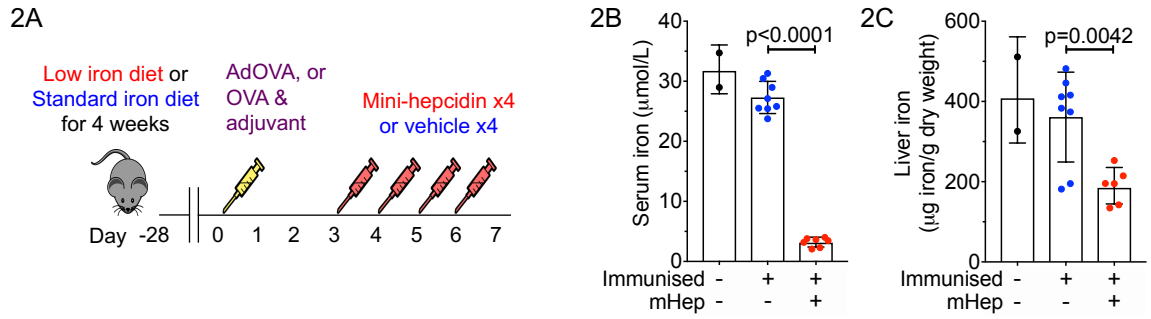


Figure 5.2. Experimental scheme for mouse treatment (2A). Serum iron concentration (2B) and liver iron content (2C) were measured from samples collected on day of cull, 7 days after immunisation. T test.

To first address how limited extracellular iron availability might alter adaptive immune responses *in vivo* we exploited a mouse model of severe serum iron deficiency. 3-week old C57BL/6 male mice were purchased, rested for 1-week and then placed on a low iron (2ppm) diet or a nutritionally matched normal (200ppm) iron control diet for 4-weeks. At 8-weeks of age mice were immunised with an AdHu5-OVA vector in a first experiment or OVA protein in adjuvant in a second experiment. From day 3 post immunisation mice in the low iron diet group were injected with mini-hepcidin (mHep) to induce serum iron deficiency, whilst mice in the control diet group received vehicle control (2A).

The mini-Hepcidin/low iron diet group had profoundly reduced serum iron levels (2B) and liver iron stores at 9 weeks of age (2C). Alteration of iron status did not change total splenic cellularity (data not shown). Endogenous OVA specific CD8 effector T cells responses to AdHu5-OVA were detected with a H-2K^{b} tetramers loaded with the immunodominant OVA epitope SIINFEKL. mini-Hepcidin/low iron diet significantly reduced the magnitude of the antigen specific CD8 T cell response to both AdHu5-OVA (3A and B) and OVA protein immunisation (3C). Antigen specific CD8 T cells can also be defined as CD8 T cells which synthesise IFNγ or TNFα after *ex vivo* restimulation with SIINFEKL peptide, the frequency of SIINFEKL specific cytokine producing cells was also reduced by low iron diet/mini-hepcidin (data not shown). Significantly increased CD71 expression on antigen-specific CD8 T cells likely reflects cellular iron deficiency (3D).

We also investigated the magnitude of the humoral response for the OVA protein immunisation. The mHep/low iron diet group had significantly reduced immunisation induced OVA specific IgG titres, germinal centre B cell, T follicular helper cell, plasma cell responses (4A, B, C and D). Significantly raised CD71 expression was detected in each subset of responding lymphocytes (4E, F and G).

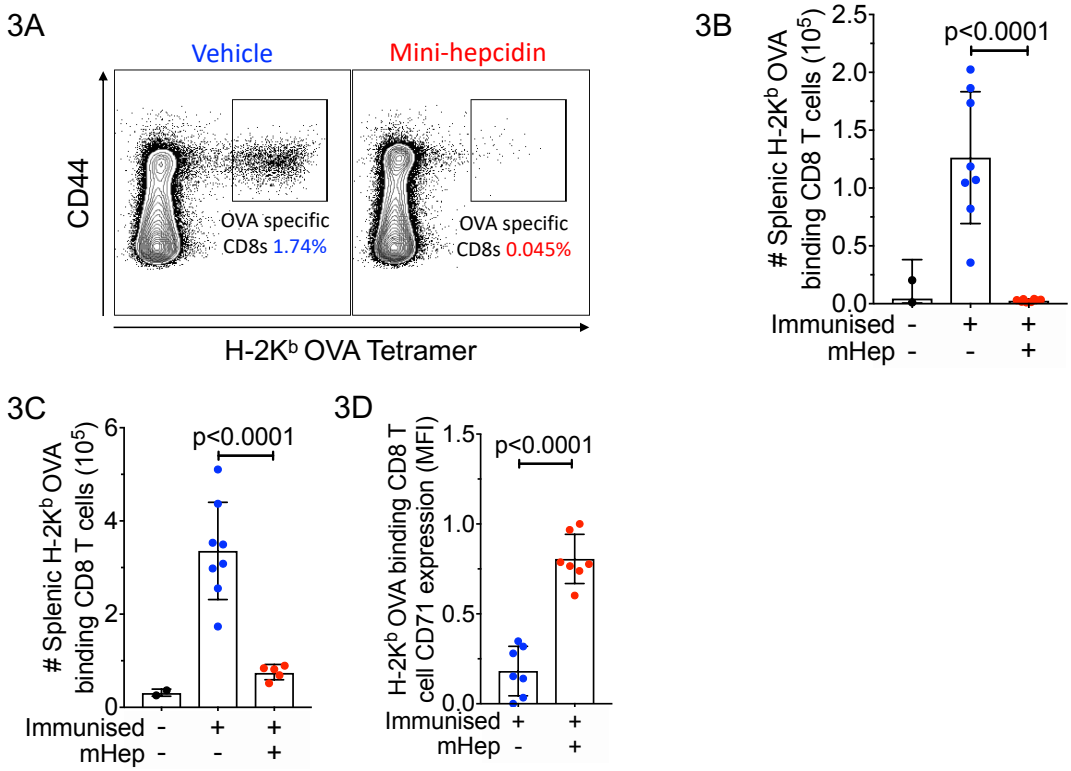


Figure 5.3. Endogenous OVA specific CD8 effector cells after AdHu5-OVA immunisation were detected with flow cytometry and enumerated as a % of total CD8s (3A) and total number per spleen (3B). Total number of endogenous OVA specific CD8 effector cells after OVA in adjuvant immunisation (3C) and CD71 expression of OVA specific CD8 effector cells (3D).

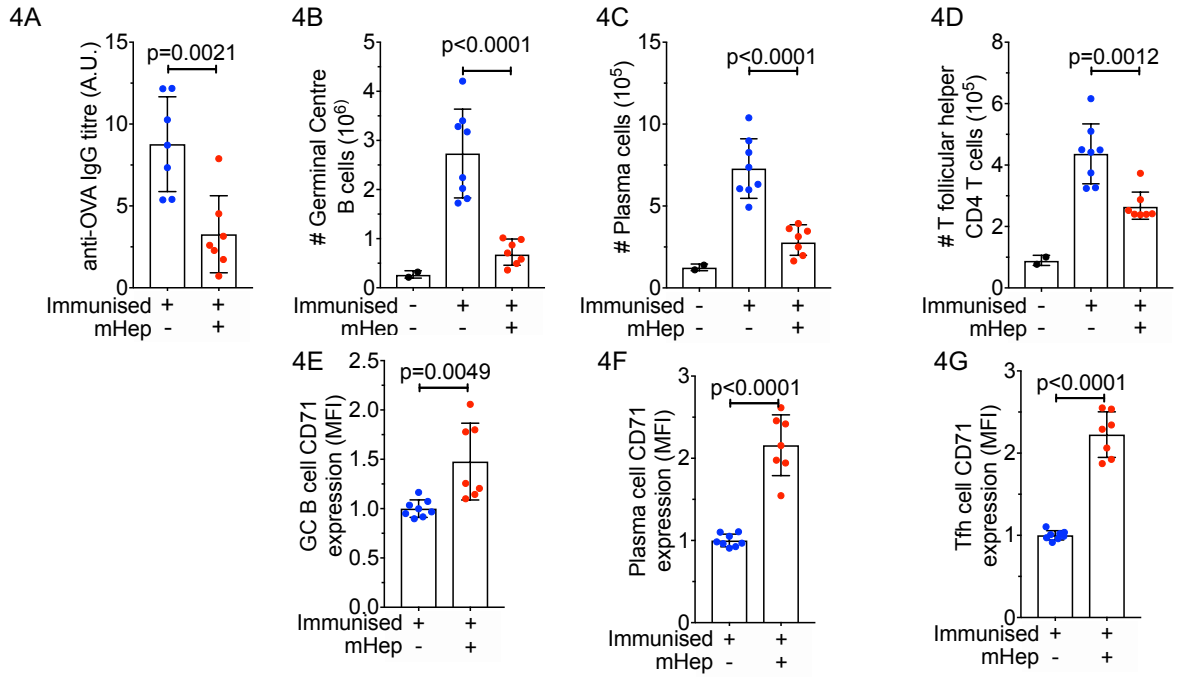


Figure 5.4. OVA specific antibody titres were measured by ELISA after OVA and adjuvant immunisation (4A). Splenic germinal centre (4B), plasma cell (4C) and T follicular helper cell (4D) number, and CD71 expression (4E, F, G) was detected by flow cytometry on day 7 after OVA and adjuvant immunisation. T test.

5.2.3 The effect of dietary iron deficiency alone on adaptive immune responses

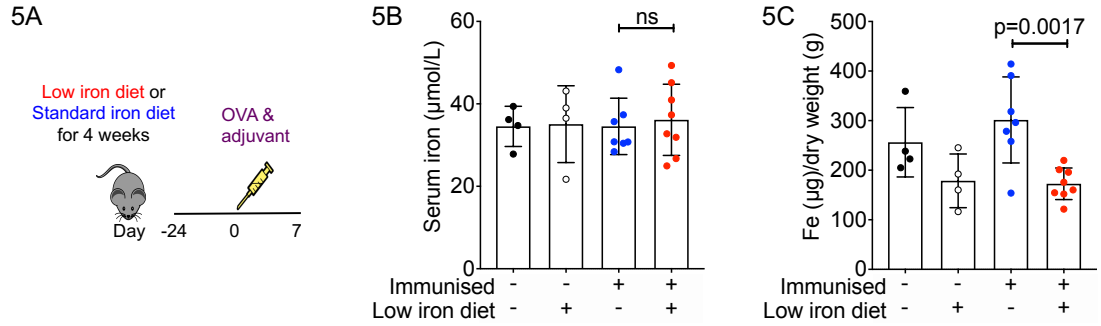


Figure 5.5. Experimental scheme for mouse treatment (5A). Serum iron (5B) and liver iron content (5C) was measured 7 days after immunisation. T test.

To determine if dietary iron deficiency alone was sufficient to perturb adaptive immune responses 3-week old male mice were purchased from Envigo and after resting for 1-week were placed on a low iron (2ppm) diet or a nutritionally matched normal (200ppm) iron control diet at 4-weeks of age. Mice were maintained on diet for almost 4 weeks, immunised with OVA protein and adjuvant, and maintained on diet for a further week before the primary immune response and iron indices were analysed (5A). A total of 5-weeks of low iron diet was sufficient to reduce liver non-haem iron stores (5C), but did not significantly reduce serum iron concentrations (5B). Consistent with unperturbed serum iron, the magnitude of the OVA-specific CD8 T cell and germinal centre B cell response was unchanged (6A and B). Furthermore OVA-specific effector CD8 T cells showed no difference in CD71 expression (6C), whilst germinal centre B cells exhibited a minor significant increase in CD71 compared to germinal centre B cells in mini-hepcidin treated animals (6D).

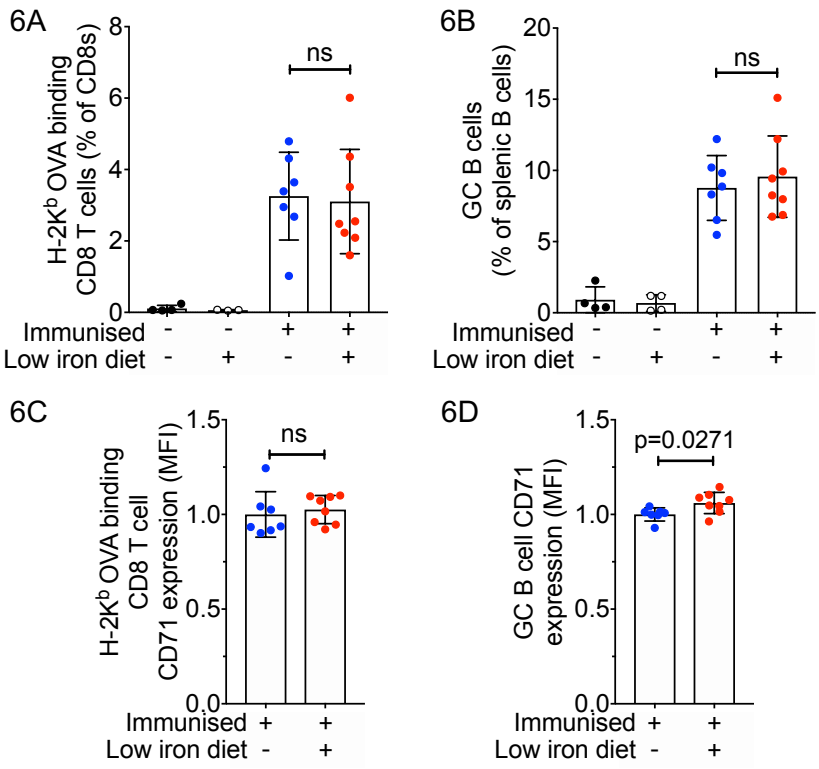


Figure 5.6. The frequency of splenic endogenous OVA specific CD8 effector cells (6A), germinal centre B cells (6B) and CD71 expression by these populations (6C and D) after OVA and adjuvant immunisation were detected with flow cytometry. T test.

5.2.4 Mini-hepcidin injection alone inhibits the T-cell response to immunisation, but this effect is reversed by iron supplementation

To test if mini-hepcidin injection alone could suppress adaptive immunity and to demonstrate that this was due to it reducing serum iron levels, mice which had received OT-I CD8 T cells on day -1 were immunised with OVA protein in adjuvant on

day 0 and injected with vehicle or mini-hepcidin and/or ferric ammonium citrate on day 3 and 4 post immunisation and culled on day 5 (7A).

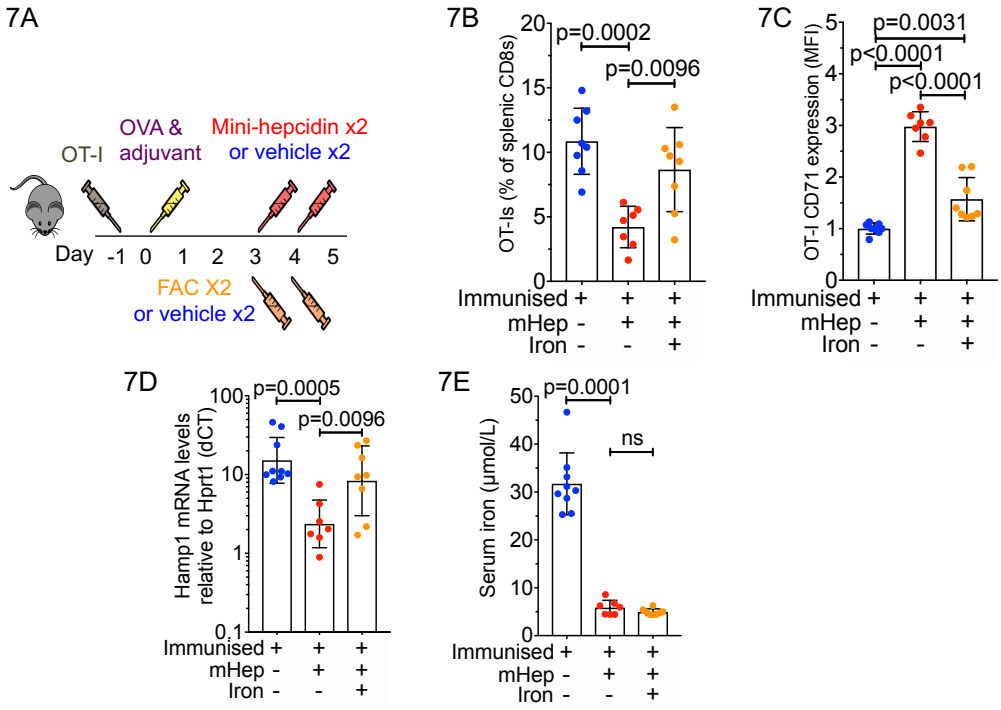


Figure 5.7. Experimental scheme of mouse treatment (7A). The frequency of splenic OT-I (7B) and CD71 expression (7C) after OVA and adjuvant immunisation were detected with flow cytometry. Liver hepcidin expression was measured by qRT-PCR (7D). Serum iron concentration was also detected (7E). One way ANOVA.

For this experiment, and where noted in the following experiments, the equivalent of 5000 congenic CD45.1 OT-I, OVA specific, TCR transgenic CD8 T cells were transferred into CD45.2 C57BL/6 recipients. Upon immunisation with OVA or the SIINFEKL peptide these transgenic T cells will expand and generate an effector CD8 population, which is easily and unambiguously detectable with using antibodies against the CD45.1 congenic surface marker. A low number of OT-Is was transferred as it has been published that this resulted in transgenic CD8 T cell differentiation which most resembled the endogenous response (Badovinac et al., 2007).

Two doses of mini-Hepcidin alone were sufficient to impair the immunisation-induced expansion of OT-I CD8 T cells (7B) and lead to an increase in OT-I CD71 expression (indicating cellular iron deficiency) (7C). Injection of ferric ammonium citrate alongside mini-Hepcidin, which would be expected to transiently increase iron availability, was sufficient to rescue OT-I frequency and cellular iron deficiency resolved by CD71 expression (7B and C). Supporting the hypothesis that FAC improves iron status, the FAC + mini-hepcidin group had significantly raised endogenous liver hepcidin mRNA expression compared to mini-hepcidin alone suggesting that the liver was sensing a less iron deficient environment (7D). Interestingly, mice receiving mini-hepcidin and mini-hepcidin + FAC had significantly decreased serum iron levels compared to control mice when culled suggesting that the added iron from FAC is rapidly taken up by cells (7E).

5.2.5 Effect of macrophage-targeted iron loading on T cell immunity

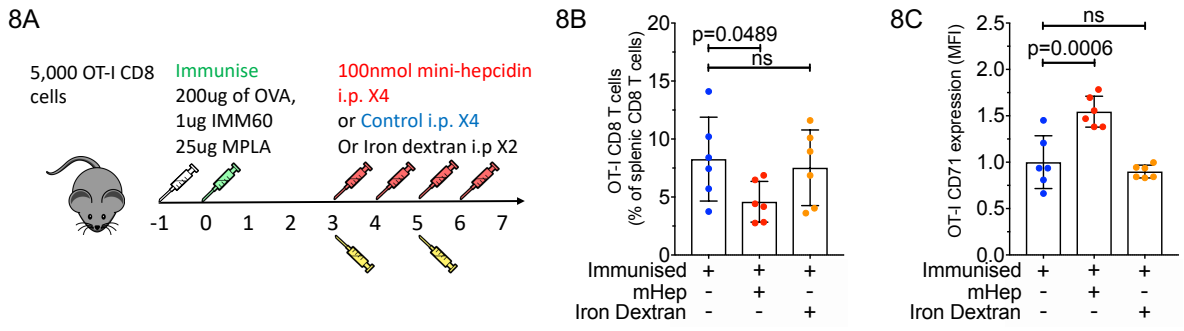


Figure 5.8. Experimental scheme of mouse treatment (8A). The frequency of splenic OT-I (8B) and CD71 expression (8C) after OVA and adjuvant immunisation were detected with flow cytometry. One way ANOVA.

Hepcidin and mini-hepcidin injection causes acute serum iron deficiency through degradation of ferroportin and blocking iron recycling out of macrophages of the reticuloendothelial system (Kupffer cells and red-pulp macrophages). Therefore mini-hepcidin injection would be expected to cause red pulp macrophage iron loading (Ramos et al., 2012). The role of red-pulp macrophages in the immune response to immunisation is unclear. However, to control for possible effects of differential red-pulp macrophage iron loading contributing to the phenotype of mini-hepcidin model (through altered cytokine production etc) (Corna et al., 2010; Costa da Silva et al., 2017; Pereira et al., 2019; Wang et al., 2009) we compared vehicle treated immunised mice, with mini-hepcidin treated immunised mice (red-pulp macrophage iron loading in the context of reduced serum iron), with iron dextran treated immunised mice (red-pulp macrophage iron loading in the context of unchanged/increased serum iron). We found that whilst the size of the antigen specific OT-I effector CD8 T cell response to immunisation was suppressed by mini-hepcidin injection, iron dextran had no effect on the magnitude of the CD8 T cell response (8B). Furthermore mini-hepcidin injection significantly increased OT-I CD71 expression, whilst iron dextran had no effect on CD71 expression (8C). Perl's staining on the spleen confirmed that mini-hepcidin resulted in minor accumulation of iron (purple staining specks) in the splenic red pulp compared to vehicle treated mice which had no detectable splenic iron deposits; iron dextran treated mice had even more extensive splenic iron loading (9A-D).

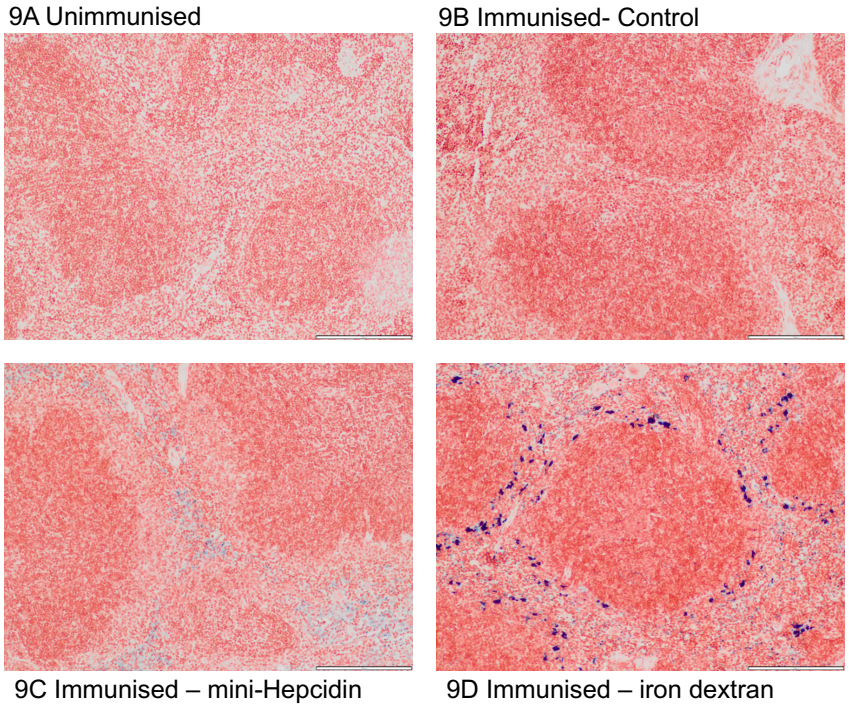


Figure 5.9. Perl's staining of spleen from mice treated as outlined in 8A. 9A-D groups as described. Magnification 100X, scale bar 200μm.

5.2.6 Effect of mini-hepcidin injection on priming of adaptive immunity *in vivo*

To address if immune suppression by mini-Hepcidin involved early events in the immune response, for example disrupted priming of adaptive immunity we injected mini-Hepcidin 3-hours prior to immunisation (10A). In this model despite initiation of

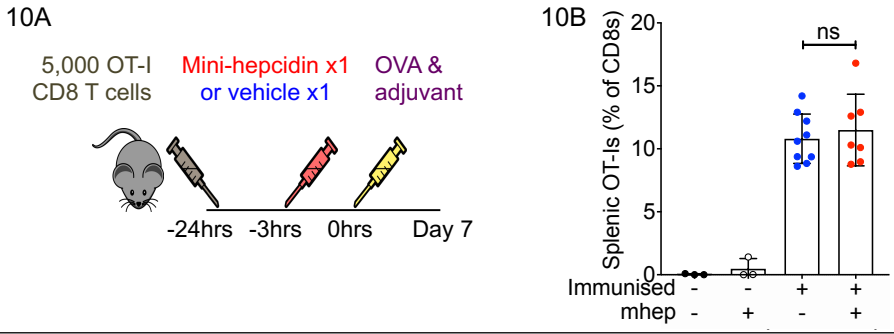


Figure 5.10. Experimental scheme of mouse treatment (10A). The frequency of splenic OT-I (10B) was measured after OVA and adjuvant immunisation were detected with flow cytometry. T test.

the adaptive immune response taking place 3 hours after mini-Hepcidin injection, and so assumed to be in the context of profound serum iron deficiency, there was no effect on the magnitude of the antigen specific CD8 T cell response (10B).

5.2.7 Direct effects of mini-hepcidin and hepcidin in CD8 T cell proliferation *in vitro*

To ask if hepcidin could directly perturb CD8 T cell proliferation we activated CD8 T cells *in vitro* and treated with either a titration of hepcidin, mini-hepcidin or the corresponding concentration of 'mini-hepcidin vehicle' (11A). Hepcidin or mini-hepcidin treatment did not significantly affect the percentage of CD8 T cells that had divided over 72hours (11B and 11D) or their CD71 expression (11C and 11E), arguing that hepcidin or mini-hepcidin has no effect on the proliferation or iron status of CD8 T cells; one may have expected hepcidin to downregulate T cell ferroportin (if they expressed a significant amount) resulting in iron sequestration, improved proliferation and reduced CD71 (Pinto et al., 2010).

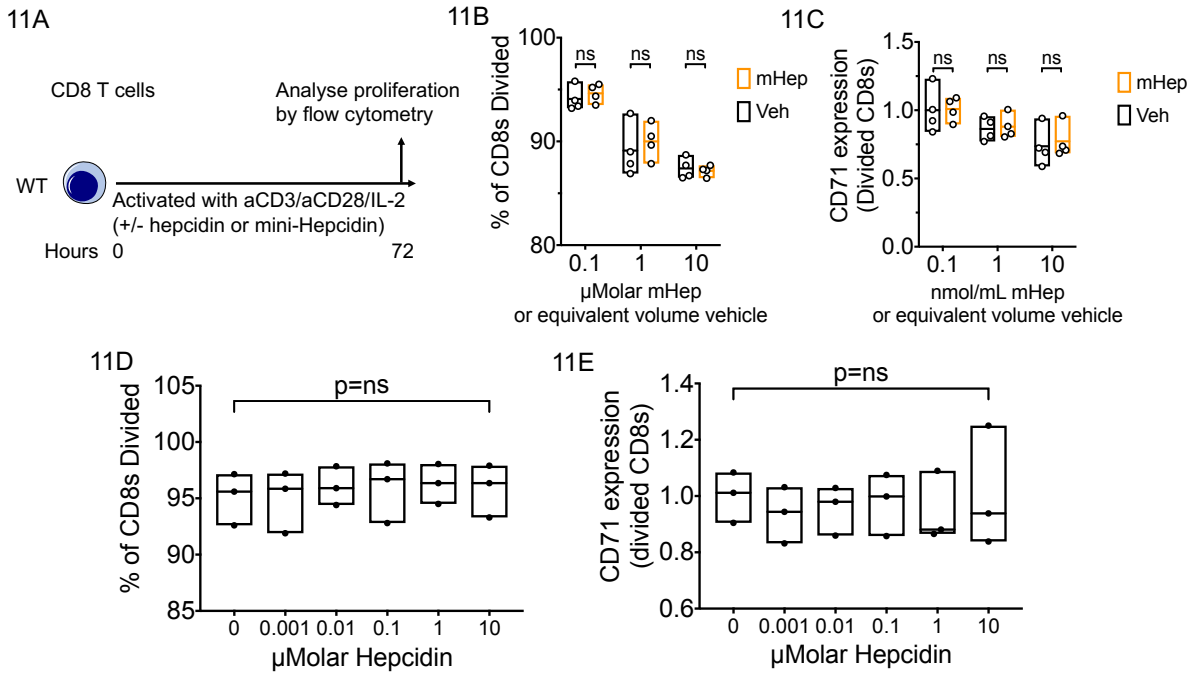


Figure 5.11. Scheme for *in vitro* CD8 T cell treatment (11A). % of CD8 T cells divided (according to dilution of cell trace violet) (11B) and CD71 expression measured (11C) measured by flow cytometry after treatment with mini-hepcidin or equivalent amount of vehicle control. % of CD8 T cells divided (according to dilution of cell trace violet) (11D) and CD71 expression measured (11E) measured by flow cytometry after treatment with hepcidin. Two way ANOVA, reporting multiple comparisons p value of minihepcidin vs vehicle 11B and 11C. One way ANOVA effect of hepcidin concentration 11D and 11E.

5.2.8 Mini-hepcidin injection alone perturbs primary CD8 T cell responses to a number of immunisations

Based on these model characterisation experiments we then went on to examine how mini-hepcidin injection and serum iron deficiency altered the phenotype of the primary immune response to a number of types of immunisations (scheme 12A). The magnitude of the endogenous OVA specific response to AdHu5-OVA in the spleen (12B) and peripheral blood (12C), the splenic OT-I response to MVA-OVA (12D), the splenic OT-I response to protein in adjuvant (12E) and the endogenous splenic vaccinia

specific (B8R peptide) response to MVA-OVA (12F) were all reduced by mini-hepcidin induced serum iron deficiency. Reduced serum

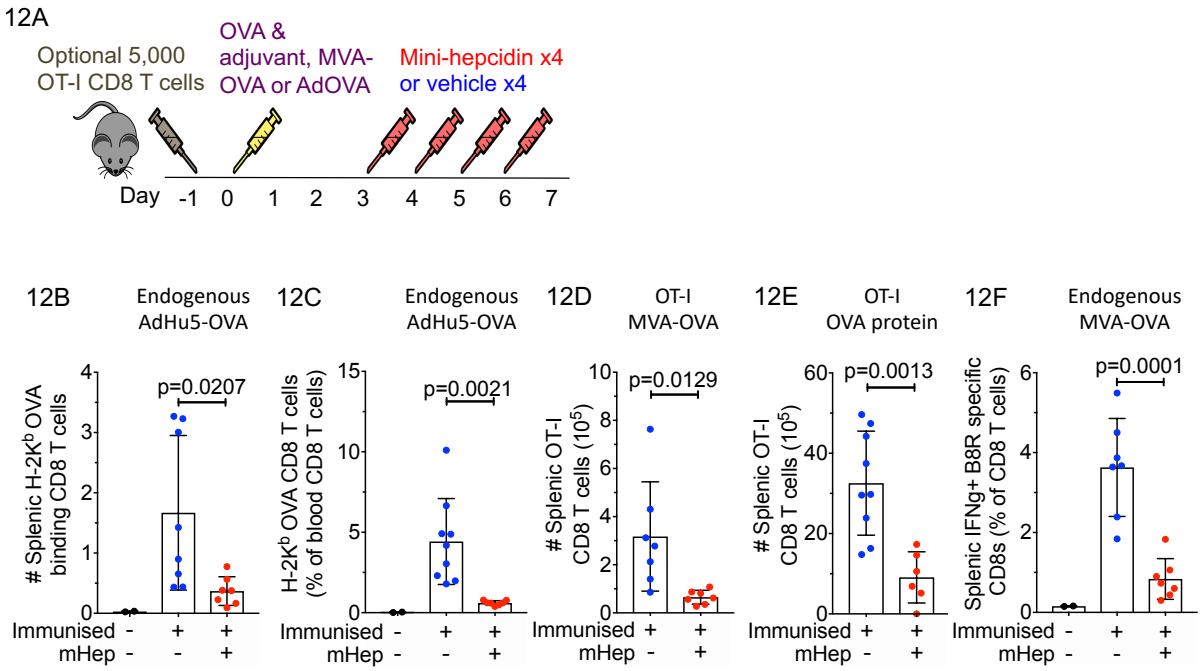


Figure 5.12. Experimental scheme for mouse treatment (12A). Number of splenic endogenous OVA specific CD8 T cells after AdHu5-OVA immunisation (12B). Frequency of endogenous OVA specific CD8 T cells as a % of total CD8s in peripheral blood after AdHu5-OVA immunisation (12C). Number of splenic OT-I OVA specific CD8 T cells after MVA-OVA immunisation (12D). Number of splenic OT-I OVA specific CD8 T cells after OVA and adjuvant immunisation (12E). Frequency of endogenous vaccinia (B8R epitope) specific CD8 T cells as a % of total CD8s, resolved by IFNγ production after *ex vivo* peptide restimulation, induced by MVA-OVA immunisation (12F). T test.

iron concentration (13A) and raised effector CD8 T cell CD71 (13B) were detected on day 7 post immunisation. In addition, we observed a defect in production of cytokines by OT-Is after *ex vivo* restimulation with their cognate peptide, SIINFEKL. Whilst there was no change in the percentage of OT-Is which produced IFNγ or TNFα, mini-hepcidin injection reduced the amount of IFNγ (13C) or TNFα (13D) produced by IFNγ or TNFα positive OT-I effector cells (respectively). Furthermore, significantly fewer OT-I

effector cells from mini-hepcidin treated animals produced IL-2 on *ex vivo* peptide stimulation.

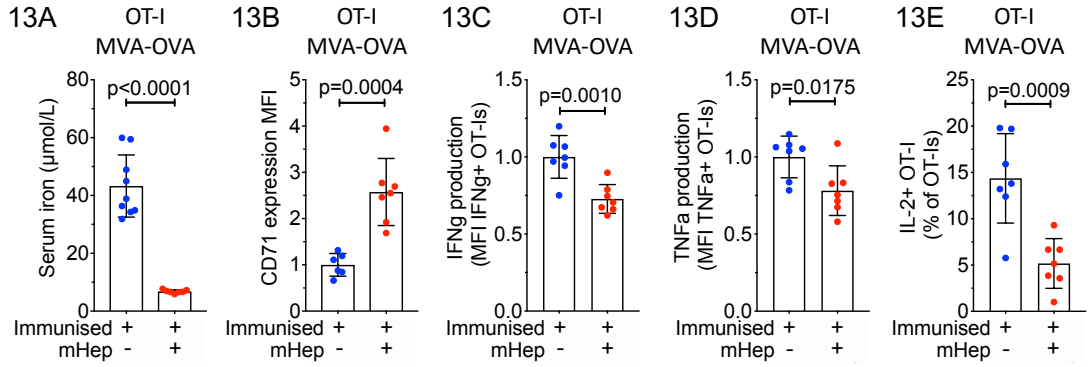


Figure 5.13. Serum iron (13A) measured 7 days after MVA-OVA immunisation, in line with experiment scheme 12A. Splenic OT-I antigen specific effector CD8 T cell CD71 expression measured by flow cytometry (13B) after MVA-OVA immunisation. MFI of IFNγ on IFNγ producing OT-Is (13C), MFI of TNFα on TNFα producing OT-Is (13D) and percentage of IL-2+ OT-Is (13E) after *ex vivo* peptide restimulation of splenocytes. T test.

5.2.9 Mini-hepcidin injection alone perturbs primary CD8 T cell differentiation

To resolve how serum iron deficiency alters the differentiation of responding CD8 T cells we subsetted effector cells using KLRG1 and CD127 to resolve KLRG1- CD127- early effector cells (EECs), KLRG1+ CD127- short lived effector cells (SLECs), KLRG1- CD127+ memory precursor cells (MPEC) and KLRG1+ CD127+ double positive effectors (Joshi et al., 2007; Obar et al., 2011). Mini-hepcidin treatment resulted in a reproducible skewing of CD8 T cell surface phenotype away from SLECs and towards the less differentiated MPEC and EEC subsets (14A-F). Whilst the majority of cells are CD127+ and very few are KLRG1+ after OVA in adjuvant immunisation (14F, and consistent with published literature (Klarquist et al., 2018) we found that iron

deficiency increased the frequency of effector cells which expressed CD62L, a marker downregulated during CD8 differentiation (14G).

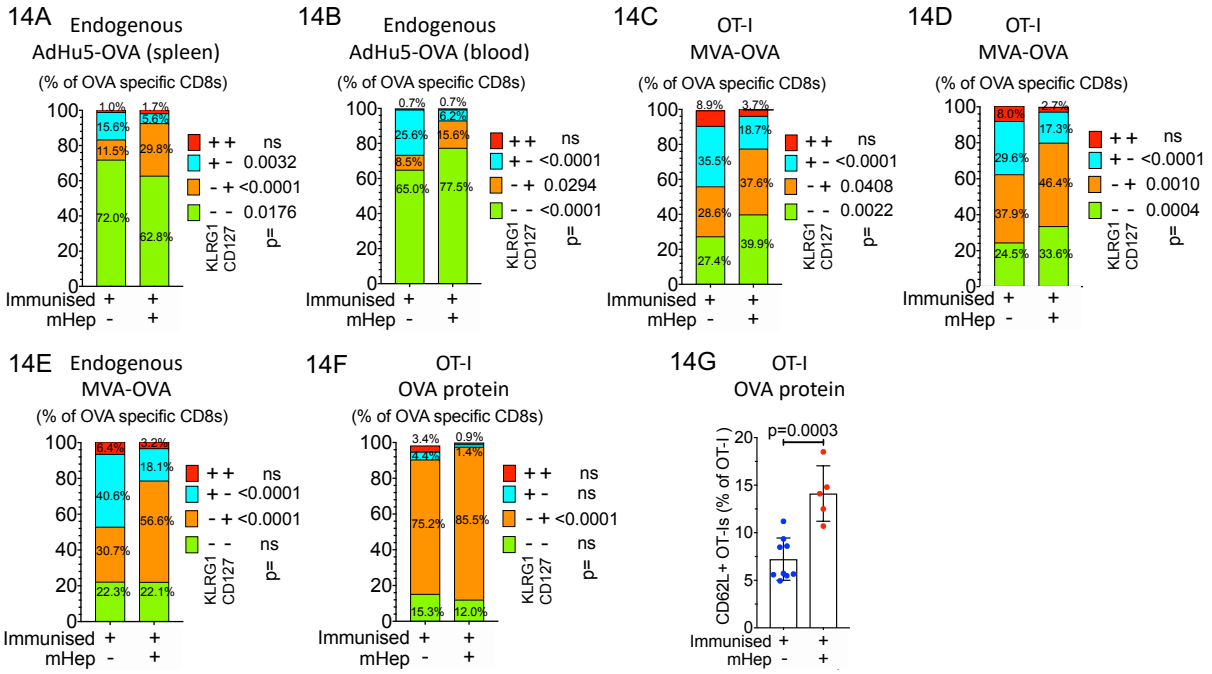


Figure 5.14. The relative frequencies of CD127- KLRG1-, CD127+ KLRG1-, CD127- KRLG1+, CD127+ KLRG1+ cells within the OVA specific CD8 T cell population for each immunisation. Profile of splenic endogenous OVA specific CD8 T cells after AdHu5-OVA immunisation (14A), endogenous OVA specific CD8 T cells in peripheral blood after AdHu5-OVA immunisation (14B), splenic OT-I OVA specific CD8 T cells after MVA-OVA immunisation (14C and 14D, independent experiments), splenic endogenous OVA specific CD8 T cells after MVA-OVA immunisation (14E), splenic OT-I OVA specific CD8 T cells after OVA and adjuvant immunisation (14F). The frequency of splenic CD62L+ OT-Is as a % of total OT-Is after OVA and adjuvant immunisation (14G).
14A-F frequencies on graph are mean of each group. Two way ANOVA, reporting multiple comparisons of effect of vehicle or mini-hepcidin on each subpopulation.
14G. T test.

5.2.10 Mini-hepcidin injection alone perturbs primary CD4 T cell and B cell responses to a number of immunisations

Mini-hepcidin injection also suppressed the magnitude of T follicular helper cell (15A) and germinal centre B cell response (15B) in the draining inguinal lymph node after

subcutaneous MVA-OVA immunisation. Furthermore, when MVA pulsed BMDCs were used as antigen presenting cells for bulk splenocytes (cytokine producing CD4 T cells resolved by flow cytometry using the gating scheme described in supplementary figure S5), from day 7 of MVA-OVA immunisation, the frequency of CD40L+ IFNg+, CD40L+ IL-2+ and CD40L+ TNFα+ Th1 CD4 effector cells (15C, 15D and 15E) was also significantly reduced by mini-hepcidin injection.

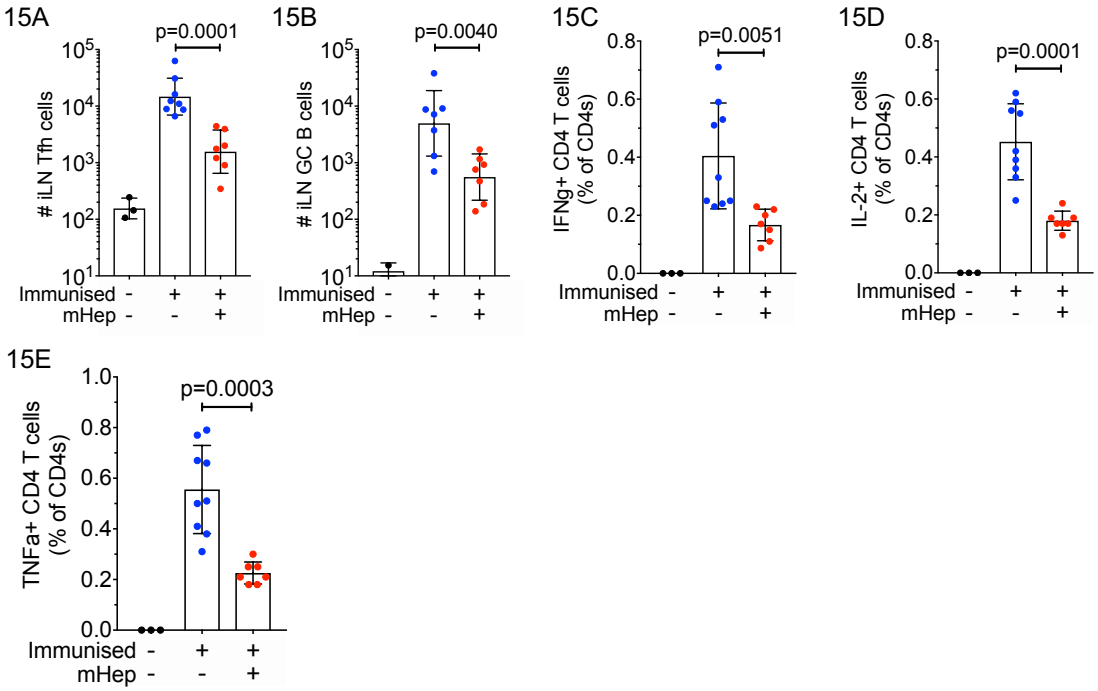


Figure 5.15. Number of T follicular helper cells (15A) and germinal centre B cells (15B) in the inguinal lymph node 7 days after MVA-OVA immunisation. Frequency of splenic CD40L+ IFNγ+ (15C), CD40L+ IL-2+ (15D) and CD40L+ TNFα+ (15E) CD4 effector cells as a percentage of CD4 T cells after restimulation with MVA-OVA pulsed bone marrow derived dendritic cells after MVA-OVA immunisation. T test.

To corroborate the suppression of CD4 T cell responses by mini-hepcidin injection the response of adoptively transferred OT-II cells after OVA protein in adjuvant immunisation was analysed. There were significantly fewer OT-II T follicular helper cells in the spleens of mini-hepcidin treated animals (16A). Furthermore, fewer of the

splenic OT-II CD4 effector cells had the capacity to secrete IL-2 (16B) or TNFα (16C) on restimulation with peptide *ex vivo.* Splenic germinal centre B cell responses induced by OVA protein in adjuvant were also suppressed by mini-hepcidin (16D).

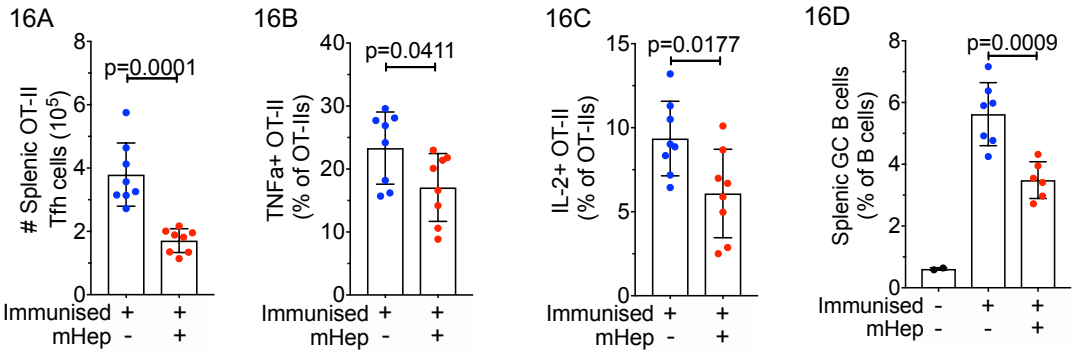


Figure 5.16. Number of splenic OT-II T follicular helper cells (16A) induced by OVA and adjuvant immunisation. Frequency of splenic TNFα+ (16B) and IL-2+ (16C) OT-II effector cells induced by OVA and adjuvant immunisation as a percentage of total OT-II CD4 T cells after *ex vivo* restimulation with peptide. Frequency of splenic GC B cells as a % of B cells after OVA and adjuvant immunisation (16D). T test.

5.2.11 Effect of Hepcidin inhibition on T cell immunity

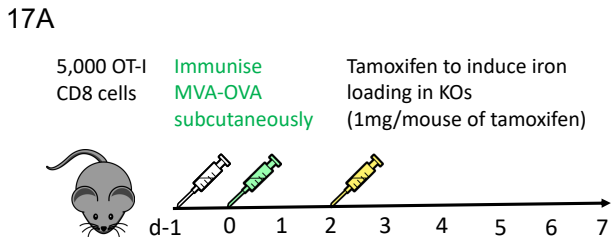


Figure 5.17. Experimental scheme for mouse treatment (17A)

Our results above indicate that injection of hepcidin and resulting hypoferremia suppresses primary CD8 T cells immune responses *in vivo*. To build upon these results we wanted to ask how inappropriately low hepcidin and iron loading altered the outcome of adaptive immunity. We exploited a mouse model established in the lab

carrying a ubiquitously expressed tamoxifen inducible Cre and a floxed Hepcidin allele, permitting global inducible hepcidin KO and iron loading (Armitage et al., 2016a). *Rosa26-CreERT2 +ve Hamp*$^{flox/flox}$ mice are termed iHampKO, whilst litter mate *Rosa26-CreERT2 -ve Hamp*$^{flox/flox}$, where tamoxifen injection does not disrupt the hepcidin loci, are termed iHampCtrl. iHampCtrl or iHampKO received 5000 naïve OT-I CD8s on day -1 and were immunised on day 0 with MVA-OVA, 2 days after immunisation all mice received an injection of tamoxifen to KO hepcidin in the iHampKO mice. Mice were culled on day 7 (17A).

iHampKO mice exhibited significantly reduced hepcidin mRNA consistent with disruption of the hepcidin loci by cre mediated recombination (18A). Reduced hepcidin production will result in elevated iron absorption and export of iron from reticuloendothelial macrophages; consistently we detected significantly raised serum iron (60μmol/L) concentrations in iHampKO mice (18B). In line with serum iron loading, serum transferrin levels were decreased in iHampKO (18C). Elevated Smad7

and Id1 mRNA indicated increased hepatic BMP signalling downstream of iron loading, consistent with previous findings (Kautz et al., 2008) (18D and 18E).

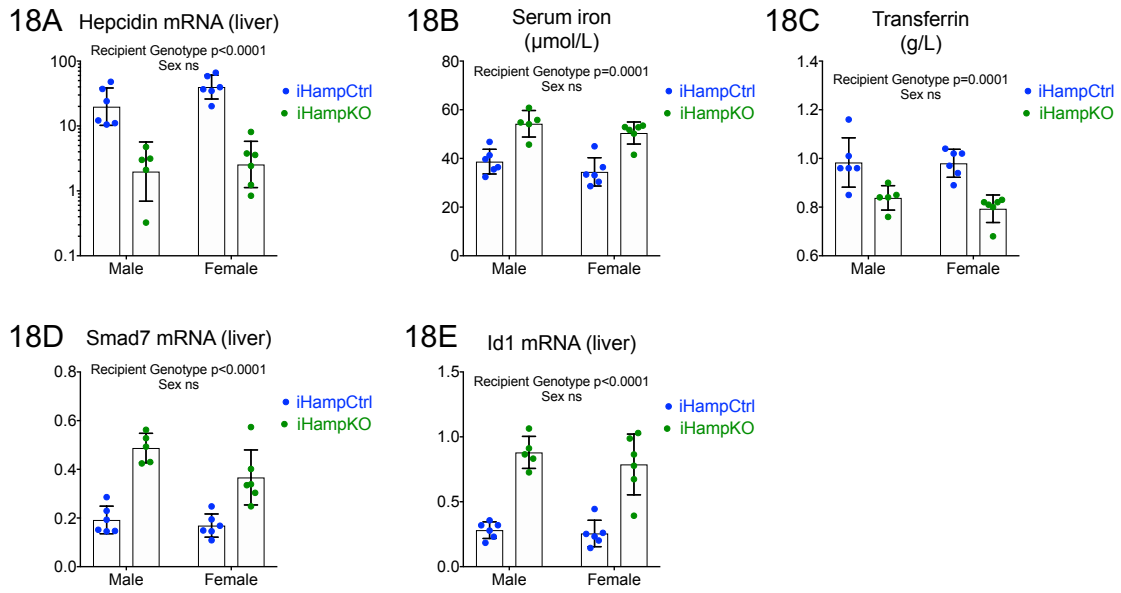


Figure 5.18. Liver Hepcidin mRNA (expressed a 2^{dCT} compared to Hprt) measured by qRT-PCR (18A). Serum iron concentration (18B) and serum transferrin concentration (18C) was also measured. Liver *Smad7* (18D) and *Id1* (18E) mRNA (expressed a 2^{dCT} compared to *Hprt*) measured by qRT-PCR. Two way ANOVA, p value for effect of genotype and sex reported.

MVA-OVA immunisation led to the expansion of an OT-I effector CD8 T cell population, this OVA specific CD8 T cell response was no different in magnitude between iHampKO or iHampCtrl mice in either the spleen or draining lymph node (19A and D). Increased serum iron due to hepcidin inhibition in iHampKO mice did however lead to significantly reduced CD71 expression in antigen specific CD8 T cells, even on day 7 post immunisation when most effector cells have finished dividing (19B and E). Most interesting was the finding that the percentage of OT-Is that expressed GZMB was significantly raised in the lymph nodes of iHampKO mice compared to iHampCtrls (19F). No effect of hepcidin deletion of Gzmb expression in splenic OT-I effector cells was found (19C). These results potentially suggest that increased iron availability

favours the acquisition of effector characteristics by CD8 T cells in draining lymph nodes.

The differentiation state of OT-I effector cells was also evaluated with CD127 and KLRG1 staining, and reduced frequencies of CD127- KRLG1- early effector cells were detected in iHampKO mice (20D and 20H). No alteration in the frequencies in other subsets of OT-I effector cells evaluated with these surface markers were found (20A, 20B, 20C, 20E, 20F and 20G). In line with the increased % of GZMB+ cells, fewer CD127- KLRG1- cells suggests that increased iron availability is favouring the differentiation of CD8 T cells.

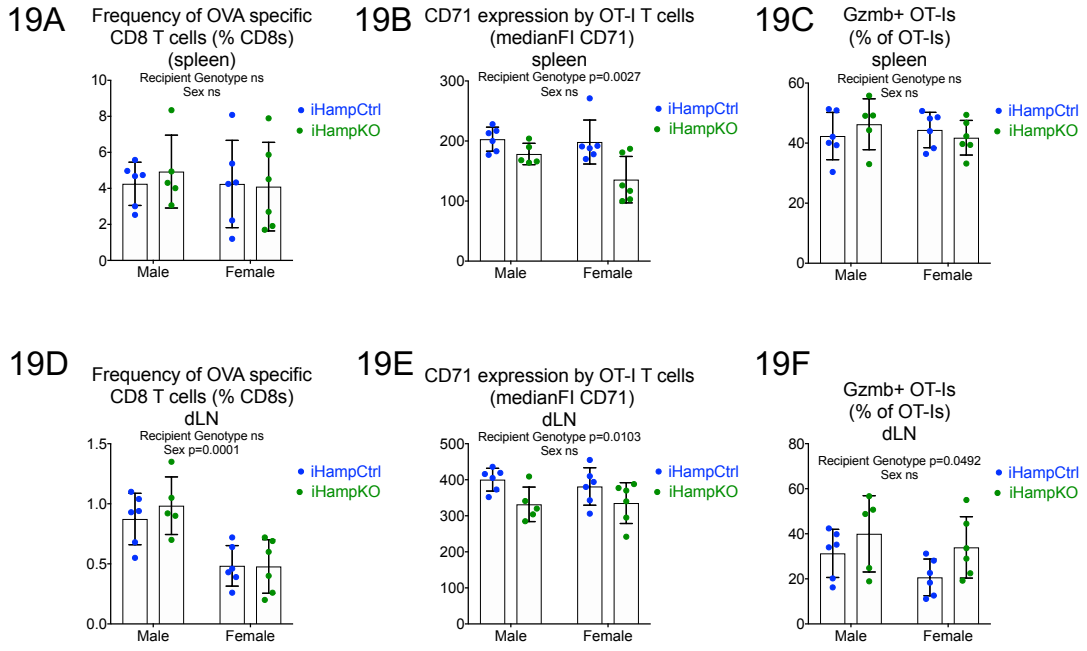


Figure 5.19. Frequency of splenic OT-I OVA specific effector cells as a % of CD8s (19A), OT-I CD71 expression (19B) and frequency of OT-Is expressing GZMB (as a % of OT-I) (19C) was measured by flow cytometry. The same parameters were measured for OT-Is in the draining inguinal lymph node (19D, 19E and 19F). Two way ANOVA, p value for effect of genotype and sex reported.

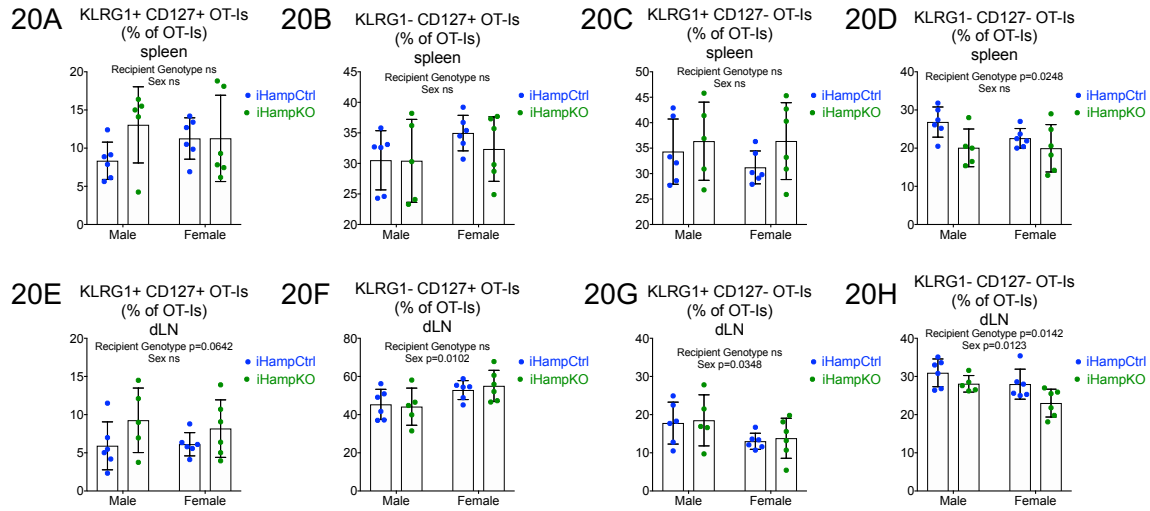


Figure 5.20. Frequency of KRLG1+ CD127+ (20A), KRLG1- CD127+ (20B), KLRG1+ CD127- (20C) and KLRG1- CD127- (20D) cells as a % of OT-Is in the spleen was measured by flow cytometry. The same parameters were measured for OT-Is in the draining inguinal lymph node (20E, 20F, 20G and 20H). Two way ANOVA, p value for effect of genotype and sex reported.

5.3 Discussion

In this chapter we present results describing how serum iron deficiency inhibits diverse aspects of the adaptive immune response to immunisation *in vivo*. We first verified that repeated daily injections at 24-hour intervals with a standard dose of 100nmol of mini-hepcidin PR73 would be sufficient to maintain reduced serum iron (Ramos et al., 2012) during a primary immune response by monitoring iron status at multiple time points after a single injection of mini-hepcidin. The trend towards lower serum iron at 30 hours and significantly reduced liver hepcidin mRNA (reflecting the suppression of endogenous hepcidin expression by low serum iron) suggested to us that at 30 hours mice had only just started to recover serum iron, by which point the next mini-hepcidin injection (at 24 hours) would have taken effect to suppress serum iron. In addition, in subsequent experiments where mice were culled 24 hours after the last of four or two doses of mini-hepcidin we saw reduced serum iron concentrations.

We then utilised a severe model of serum iron deficiency combining both 4-weeks of low iron diet, to deplete iron stores, and mini-hepcidin injection, to acutely redistribute body iron and reduce its availability in the serum (Preza et al., 2011). We found that low iron diet and mini-hepcidin together profoundly inhibited the primary CD8 T cell, B cell and CD4 T cell response to immunisation; the CD8 T cell response in AdHu5-OVA immunised mice on low iron diet treated with mini-hepcidin was almost undetectable.

Given the strong phenotype of the diet/mini-hepcidin treatment, we wished to take apart our model and better understand what aspects of perturbed iron homeostasis influenced adaptive immunity. In our hands 5-weeks of 2ppm diet in male C57BL/6 mice starting at 4-weeks of age was insufficient to alter the magnitude of the primary adaptive immune response when no hepcidin was given. This is consistent with our hypothesis that it is serum iron concentration which determines iron availability in secondary lymphoid organs; 5-weeks of low iron diet did not reduce serum iron concentrations. This may be because a week or so eating standard mouse chow after weaning was sufficient to iron load our mice and during the 5-weeks on 2ppm diet the depletion of existing iron stores was sufficient to buffer serum iron and maintain an iron supply for adaptive immunity. Low iron diet, and depletion of liver iron stores, but minimal change in serum iron, induced a smaller increase in the CD71 expression than mini-hepcidin induced serum iron deficiency. Furthermore it is worth noting that functional iron restriction due to raised hepcidin, iron redistribution and a block on dietary uptake plays a major part in the iron deficiency anaemia observed in low- and middle-income countries (Prentice et al., 2019) and therefore an iron restriction model based around raised hepcidin maybe be more appropriate than simply reduced dietary iron.

The ability of parenteral iron supplementation to rescue the suppression of antigen specific CD8 T cell responses and the increase in CD71 expression induced by mini-hepcidin argues strongly that mini-hepcidin suppresses adaptive immunity through the induction of serum iron deficiency. Furthermore, we found that iron dextran

injection, which causes red-pulp macrophage iron loading in the context of unchanged/increased serum iron, had no effect on the magnitude of primary CD8 T cell response to immunisation. This result argues against mini-hepcidin perturbing adaptive immunity through sequestration of iron in red-pulp macrophages and alteration of macrophage function, and supports our hypothesis that it is mini-hepcidin induced serum iron deficiency during the expansion phase of the immune response that suppresses adaptive immunity. In addition, mini-hepcidin injection and serum iron deficiency concurrent with immunisation (i.e. during the priming phase, but before proliferation is induced) does not reduce the magnitude of the resultant CD8 T cell response. However, it would be important to carry out both deeper phenotyping of the quality of the CD8 T cell response on day 7 in this model, and focus on the effect of serum iron deficiency has on the innate immune response and DC function *in vivo*, to definitively demonstrate that innate immune priming of adaptive immunity is not perturbed by serum iron deficiency.

The finding that direct treatment of CD8 T cells with hepcidin or mini-hepcidin did not alter proliferation or CD71 expression also supports mini-hepcidin suppressing adaptive immunity via serum iron deficiency. One may have expected, in line with published literature concerning lymphocyte intrinsic production of hepcidin (Pinto et al., 2010), that hepcidin treatment would have improved lymphocyte iron retention, decreasing CD71 and enhancing proliferation. However, the published literature on hepcidin/ferroportin interactions modulating T cell proliferation concerns human T

cells, not mouse T cells and it is conceivable that T cell iron homeostasis may be subtly different between species.

Given the apparent robustness of our mini-hepcidin/serum iron deficiency model as a way of modulating adaptive immunity we then demonstrated that serum iron deficiency suppressed the magnitude of CD8 T cell responses induced by both viral vectors and protein-in-adjuvant. This is notable, because glycolytic metabolism of glucose has been proposed to be essential for expansion of effector cells in response to viral vectors, whilst aerobic mitochondrial metabolism of diverse substrates may be more important for protein-in-adjuvant vaccination (Klarquist et al., 2018). Therefore, serum iron deficiency disrupts CD8 T cell expansion regardless of the metabolic programme engaged, suggesting some converse process is disrupted.

Across a range of immunisation platforms we reproducibly observe an increased frequency of canonical CD127+ KRLG1- memory precursor cells (Joshi et al., 2007) during the primary response in serum iron deficient animals. This may be a physiological response to enrich for CD8 T cell subsets with better survival and maintain memory T cell frequency despite suppressed clonal expansion. Our investigations into the influence of serum iron deficiency during the primary response to immunisation on CD8 T cell memory will be presented in the next chapter. The observation that mTOR inhibition and AMPK activation favours memory differentiation (Araki et al., 2009; Pearce et al., 2009a), although perhaps only in secondary lymphoid organs (Sowell et al., 2014), and that deletion of FoxO1, a

transcription factor upregulated in the response to nutritional stress, disrupts differentiation of memory cells resulting in reduced expression of memory associated genes CD62L and CD127 and enhanced expression of effector genes Gzmb and IFNγ (Delpoux et al., 2017; Hess Michelini et al., 2013; Kim et al., 2013; Tejera et al., 2013) supports the idea that differentiation of CD8 T cells might adaptively respond to external nutrient availability to maintain the pool of memory cells.

However, it is important to consider that CD127 and CD62L, whilst both markers of memory precursor cells, are also highly expressed by naïve CD8 T cells, therefore the apparently skewing of CD8 T cells towards memory may also simply represent a general inhibition of differentiation by serum iron deficiency. In support of this is the observation that whilst CD127 and CD62L are correlates with memory potential (reflecting cytokine dependency and trafficking preferences), a functional property of memory and memory precursor cells is the capacity to secrete IL-2 (Sarkar et al., 2008), which we find to be strongly suppressed by iron deficiency. Our observations also likely link to findings in other studies that the increased burst size of a clone is linked to reduced memory precursor differentiation and enhanced effector differentiation, suggesting a reciprocal link between proliferation and differentiation of CD8 T cells (Jenkins et al., 2008; Plumlee et al., 2013). Division coupled differentiation of CD4 cells towards cytokine-production-competent effector cells (Gett and Hodgkin, 1998), and of B cells into plasma cells (Barwick et al., 2016) has also been proposed.

Many of the transcription factors which regulate differentiation also modulate effector capacity, for example IRF4 heterozygous T cells exhibit reduced capacity to make effector cytokines (Nayar et al., 2014). It will be important to investigate how the reduced effector cytokine production of CD8 T cells which develop during iron deficiency may reflect dysregulation of the differentiation trajectory. Further investigation of the expression of key transcription factors regulating differentiation and effector function, such as IRF4 and BLIMP1, in iron deficient cells will be important. How iron restriction might perturb effector functions through disrupted T cell metabolism also requires consideration; the enhanced metabolism of CD4 effector T cells supports maximal cytokine production at both the transcription and post-transcriptional level (Chang et al., 2013; Peng et al., 2016), and similar phenomena likely act in CD8 T cells (Gubser et al., 2013). Finally iron availability has been proposed to directly control the stability of the mRNA encoding GM-CSF and IL-2 in CD4 T cells in the context of EAE through the activity of PCBP1 (Wang et al., 2018). However, whilst we observe defective IL-2 production by CD8 and CD4 T cells in our *in vivo* models of serum iron deficiency, if this is mediated by PCBP1 remains unclear. It would be interesting to explore how PCBP1 KO CD8 or CD4 T cells expand and show modulated effector function in the context of serum iron deficiency as Wang et al only examines iron restriction *in vitro.*

Whilst the majority of our focus was on the CD8 T cell response to immunisation in the context of iron deficiency, we also conducted some analysis of the CD4 and B cell response. Serum iron deficiency reduced the magnitude of the germinal centre B cell

and T follicular helper cell response in the spleen after protein immunisation and in the draining lymph node after MVA-OVA immunisation. We found that the splenic plasmablast and early antigen-specific IgG response was impaired by serum iron deficiency.

Furthermore, the total frequency of MVA-OVA specific IFNg, TNFa and IL-2 producing Th1 CD4 effector cells and the % of OT-IIs which successfully differentiated into cytokine producing effector cells was significantly reduced by iron restriction. Whilst we have primarily focussed on the interactions between iron, metabolism, differentiation and function of CD8 T cells, and not conducted detailed examination of the influence of iron on CD4 T cells, the finding that CD4 effector function is impaired by iron restriction suggests an ubiquitous perturbation of effector differentiation in adaptive immune cells by iron deficiency.

Whilst our experiments using the *TfrcY20H/Y20H* model demonstrate that iron restriction can cell intrinsically impair adaptive immunity, in our mini-hepcidin serum iron deficiency model all immune cells are experiencing iron restriction, as is physiologically relevant. Therefore it is also worth considering the potential that serum iron deficiency impairing CD4 responses has the capacity to in turn influence CD8 T cell effector differentiation and memory formation through disrupted CD4 help (Bennett et al., 1998; Novy et al., 2011; Obar et al., 2010; Wilson and Livingstone, 2008). As noted, external signals from the myriad of other immune cells, including cytokines, also modulate CD8 T cell differentiation during infection. For example, both

CD8 and CD4 effector T cells show reduced capacity to secrete IL-2, and IL-2 signalling plays an important role in driving T cell proliferation, effector capacity and acquisition of memory potential (Obar et al., 2010; Pipkin et al., 2010).

Finally, we investigated the effect of increased serum iron concentrations on adaptive immunity through the inducible disruption of the hepcidin-ferroportin axis by tamoxifen treatment of iHampKO mice. Increased serum iron likely increased the cellular iron content of OT-I effector cells, as evidenced by reduced CD71 expression. One might expect that in more highly proliferating adaptive immune cells, such as CD8 T cells earlier in the response or GC B cells, hepcidin KO and iron loading might lead to a greater reduction in CD71. Whilst iron restriction was found to antagonise effector differentiation, we found that iron overload potentially promotes it: hepcidin inhibition reduces the frequency of OT-I effector cells with an immature CD127-ve KLRG1-ve phenotype and increases the frequency of OT-I effector cells in the lymph node which are GZMB positive. Why the frequency of GZMB+ OT-Is is only increased in the lymph node may reflect something particular about the interaction between iron availability and the lymphoid organ microenvironment.

Serum iron deficiency inhibits the formation of CD8 T cell memory

6.1 Introduction

6.1.1 Immunological memory

A fundamental part of the adaptive immune response is the formation of immunological memory to a particular pathogen after challenge (Ahmed and Gray, 1996; Kaech et al., 2002). On rechallenge with the pathogen the adaptive immune response will mount an anamnestic response, potentially clearing the pathogen rapidly and without overt symptoms of infection. This chapter primarily concerns the impact of acute serum iron deficiency on CD8 T cell memory responses. We will not consider the concept of innate memory or trained immunity where classically innate cells (such as monocytes, macrophages, NK cells or dendritic cells) can show enhanced responses on rechallenge (Netea et al., 2016). After the primary adaptive immune response to immunisation some pathogen specific T cells will survive and become long-lived memory cells. These are not only present at a greater frequency than the original antigen-specific naïve cells, but also exhibit enhanced functionality and can localise in barrier organs, meaning that on re-infection a response of greater magnitude can be mounted more quickly (Veiga-Fernandes et al., 2000).

Our understanding of what regulates CD8 T cell memory is more advanced than for CD4 T cell memory, in part because the experimental models for examining CD8 T cell responses are more amenable. In this chapter I will mostly consider CD8 T cell memory

unless otherwise noted. Whilst unclear for CD4 T cell memory, it seems that CD8 T memory cells can persist in the absence of continued antigen exposure (Lau et al., 1994), with memory cell numbers being maintained by IL-15 and perhaps IL-7 driven homeostatic proliferation (Tan et al., 2002). We have already introduced the control of CD8 T cell differentiation in the previous chapter concerning the effect of serum iron deficiency on the primary CD8 T cell response to immunisation.

6.1.2 Subsets in CD8 T cell memory

The definition of multiple subsets of memory cells with different functions and distribution has been a key part of assessing the quality of T cell memory. However these subsets are essentially a human classification of rationalising a continuum of heterogeneous T cells across a range of physiological situations (Jameson and Masopust, 2018). Subsetting of CD8 memory cells was first proposed for human peripheral blood CD8 T cells, splitting them into T central memory (CCR7+ CD62L+, Tcm) vs effector memory (CCR7- CD62L-, Tem) cells (Sallusto et al., 1999). CCR7 and CD62L are key molecules for entry of lymphocytes into lymph nodes and the different trafficking behaviour of central memory cells (which tend to localise in secondary lymphoid organs (SLO)) versus effector memory cells (enriched in the blood) became a central tenet of memory T cell subsetting (Weninger et al., 2001). Different cytokine production of profiles of human Tcm and Tem were proposed, with Tem showing enhanced immediate production of effector cytokines such as IFNγ (Sallusto et al., 1999). Mouse models were exploited to suggest that Tcm cells had an increased

capacity to maintain numbers long term, expand on secondary stimulation and protect against challenge with systemic pathogens (Wherry et al., 2003).

However this increased functionality of Tcm is slightly at odds with the observation that there are more memory cells in peripheral non-lymphoid tissues than in secondary lymphoid organs (Masopust et al., 2001). The more 'effector-like' memory cells of non-lymphoid tissues were subsequently found to include a subset of T resident memory cells (Trm) distinct from Tem and Tcm, which on entry into peripheral tissue during the primary immune response did not recirculate (Mueller and Mackay, 2016). T resident memory cells are diverse with different surface profiles and signalling requirements for their establishment depending on the tissue niche and infection (Hu et al., 2015; Zhang and Bevan, 2013) and it has even been shown that different routes of immunisation establish different Trm populations in the same tissues (Sheridan et al., 2014). Furthermore the picture of resident memory is complicated by the existence of non-circulating Trm within secondary lymphoid organs, with these further contributing to compartmentalised immunity, being enriched in specific draining lymph nodes after infection (Beura et al., 2018). These SLO Trm also demonstrate how arbitrary our memory subset definitions are given the phenotypic similarities with both peripheral Trm and some to Tcm.

Since the majority of human infections infect via barrier surfaces there has been significant interest in harnessing Trm for vaccination. Parabiosis experiments of challenged and un-challenged mice indicate that skin Trm provide much more

effective protection than circulating Tcm cells (Jiang et al., 2012). A number of preclinical papers have established that whilst systemic vaccination was ineffective, targeting antigen specific CD8 T cells to barrier surfaces and establishing Trm provided enhanced protection against HSV1 (Mackay et al., 2012), *Helicobacter pylori* (Liu et al., 2019), liver stage malaria infection (Fernandez-Ruiz et al., 2016) and *Chlamydia trachomatis* (Stary et al., 2015).

The definition of Trm as non-circulating memory cells in the periphery and Tcm as memory cells which circulate between the SLO and blood has to some extent confused the concept of Tem. However, a number of papers define T cells with a long-lived effector type profile and these have been proposed to mediate superior control against some infections (Bottcher et al., 2015; Gerlach et al., 2016; Olson et al., 2013), but even within these populations of effector like memory cells there is heterogeneity in function and trafficking behaviour (Gerlach et al., 2016). Furthermore despite the suggestion that Tcm exhibit enhanced function compared with Tem (Wherry et al., 2003), comprehensive analysis of T cells throughout multiple body compartments in humans suggest that CD8 Tem dominate (Sathaliyawala et al., 2013). The increasing acquisition of a more Tem like state by memory cells after repetitive immunisation has also been observed in mice and these secondary memory cells have been proposed to have particular functional properties that protect better against some pathogens, but not others (Nolz and Harty, 2011). The presence of 'innate-like-memory' and 'virtual-memory' T cells at high frequency is also noteworthy, these cells have a 'memory-like'

surface phenotype, but have never responded to cognate antigen stimulation (White et al., 2016).

Memory CD8 T cells have also been proposed not just to be less metabolically active than effector cells, but also to have a unique metabolic profile. Results from the laboratory of Erika Pearce have painted a picture whereby the enhanced mitochondrial capacity of memory T cells and a metabolic profile reliant on fatty acid oxidation is essential for their survival and ability to mount recall responses (Pearce et al., 2009a; van der Windt et al., 2012a). Mitochondrial fission and fusion have been shown to facilitate the metabolic remodelling which takes place during T cell differentiation, with fission favouring the aerobic glycolytic programme of effector cells and fusion being essential for efficient mitochondrial metabolism and formation of memory T cells (Buck et al., 2016). Autophagy has been observed to be particularly important in memory cells (compared to effector CD8s) to maintain healthy mitochondria and appropriate metabolic phenotypes (Puleston et al., 2014). Furthermore the capacity of memory CD8 T cells to mount immediate effector responses and rapidly secrete large quantities of cytokines is dependent on both enhanced glycolytic and mitochondrial metabolism, demonstrating the importance of metabolic health of immune cells for immunity (Bantug et al., 2018; Gubser et al., 2013). Other investigators have corroborated the idea that skewing CD8 T cell metabolism away from glycolysis with, the non-competitive hexokinase and competitive phosphoglucose isomerase inhibitor, 2-deoxyglycose (2DG) can favour T cell longevity and enhance a ‘memory-like’ phenotype (Sukumar et al., 2013). In

contrast enforced glycolytic activity, in this paper through over expression of phosphoglycerate mutase, limited T cell longevity. However, KO of VHL in CD8 T cells, which results in hyperactivation of HIF and enhanced glycolytic metabolism with concomitant suppression of mitochondrial respiration, did not prevent formation of long lived memory cells, secondary expansion and CD8 T cell memory protection against rechallenge with virus (Phan et al., 2016). One explanation put forward by Phan et al for these contrasting results is that enhancing energy production, either through improving 'energetically efficient' mitochondrial metabolism or by enhancing glycolytic activity favours memory formation.

Most existing work on memory T cell metabolism does not consider the relationship between metabolism and the functional heterogeneity of T memory cells. However it was recently found that Trm in the skin were shown to be uniquely reliant on extracellular fatty acid uptake and mitochondrial β-oxidation for their maintenance (Pan et al., 2017). For Tcm cells at least, enhanced fatty acid oxidation has been proposed to be fuelled, despite low levels of fatty acid uptake, by *de novo* synthesis from glycerol (Cui et al., 2015) and glucose (O'Sullivan et al., 2014). Furthermore, the memory cells formed in conditions of enforced glycolysis are proposed to metabolically resemble Tem more than Tcm (Phan et al., 2016), with the FAO dependent phenotype of memory cells proposed in other studies (van der Windt et al., 2012a) being suggested to mainly be a hallmark of Tcm or *in vitro* IL-15 expanded cells.

However, how acute changes in systemic nutrient availability influences different T cell memory subsets through their different metabolic requirements remains to be established. It is in this context that we sought to explore how altering plasma iron would influence T-cell memory.

6.1.3 Chapter aims

Data in the previous chapters support the idea that restricting serum iron availability profoundly inhibits the primary CD8 T-cell response, disrupting effector function and differentiation. One key translational focus of this work is the impact of pervasive iron deficiency, particularly during childhood, on vaccination programmes in low-and-middle-income countries. Effective vaccination requires persistent and high-quality immune memory. We hypothesised that serum iron-deficiency during primary immunisation, mimicking infant serum iron deficiency which resolves later in life (Armitage et al., 2019), would impact upon the quality and magnitude of the CD8 T-cell memory response even once iron status had normalised. OT-I and endogenous antigen specific CD8 T cell responses were resolved by flow cytometry using the gating schemes S1 and S2 respectively.

Specifically, we have examined:

1. If our regime of mini-hepcidin during primary immunisation affected serum iron at memory time points
2. How the magnitude and quality of the memory CD8 T-cell response is altered by serum iron deficiency during primary immunisation with protein-in-adjuvant vaccination

3. How the recall response to secondary immunisation is altered by serum iron deficiency during primary immunisation with protein-in-adjuvant vaccination
4. How the magnitude of CD8 T cell memory to vaccinia virus derived peptide loaded BMDC vaccine and its capacity to protect against viral challenge is altered by primary immunisation serum iron deficiency

6.2 Results

6.2.1 Serum iron deficiency during primary immunisation with protein-in-adjuvant immunisation is transient

To examine how serum iron deficiency affect the CD8 T cell immune response to the protein-in-adjuvant immunisation we adoptively transferred 5000 OT-I CD8 T cells into recipient C57BL/6 mice. Mice were then immunised with OVA protein in adjuvant and injected with mini-hepcidin or vehicle on days 3-6 post immunisation as in the previous chapter (1A). The frequency of antigen specific effector CD8 T cells in peripheral blood was significantly lower in mini-hepcidin treated animals on day 7 post immunisation (1B). Cytokine production by peripheral blood effector cells was assayed by stimulation with SIINFEKL ex vivo, and consistent with previous results fewer effector CD8 T cells in mini-hepcidin treated animals had the capacity to make IFNγ and IL-2. Cytokine positive cells also made significantly less TNFα and IL-2 on a per cell basis (1C-1G).

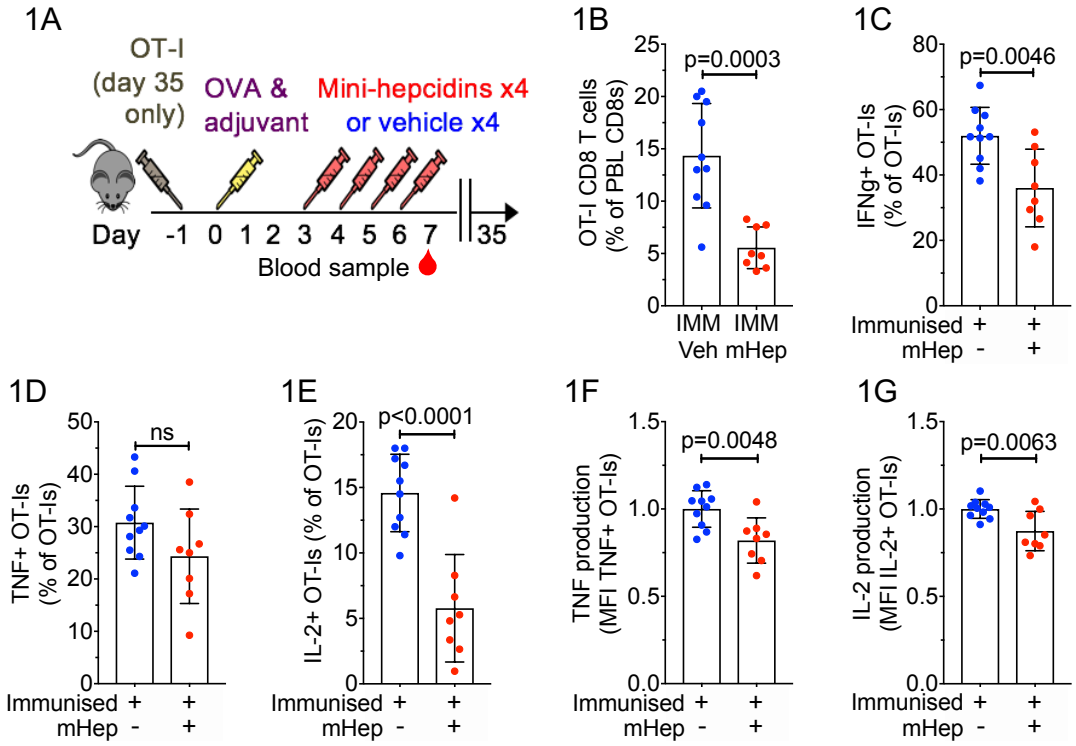


Figure 6.1. Experimental scheme for mouse treatment (1A). Frequency of OT-I CD8 effector cells as a % of total CD8 cells in peripheral blood (1B) and frequency of OT-Is which can secrete IFNγ (1C), TNFα (1D) or IL-2 (1E), and the median intensity of TNFα or IL-2 staining for TNFα+ or IL-2+ cells respectively (1F and 1G) on *ex vivo* peptide restimulation. All on 7 days post primary immunisation. T test.

Mice were then left for a further 4 weeks and culled on day 35 post immunisation. Serum iron levels were completely normalised on day 35, supporting the notion that the mini-hepcidin model only transiently perturbs systemic iron homeostasis (2A).

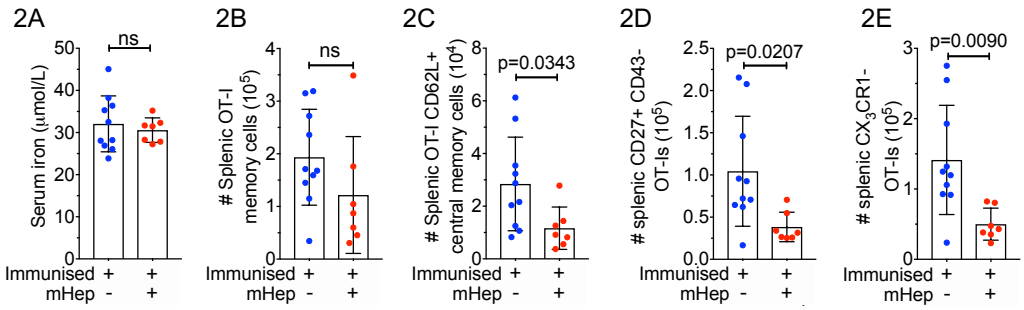


Figure 6.2. Serum iron concentration was measured (2A). Total number of OT-I memory cells (2B), and number of OT-I memory cells with a CD62L+ (2C), CD27+ CD43- (2D) or CX_3CR1- (2E) surface phenotype was measured by flow cytometry in the spleen. All on 35 days post primary immunisation as in 1A. T test.

6.2.2 Serum iron deficiency during primary immunisation with protein-in-adjuvant alters the profile of memory CD8 T-cells.

Mini-hepcidin treatment during primary immunisation resulted in a trend towards fewer OT-I memory cells in the spleen (p=0.1644) (2B). To characterise the profile of CD8 memory cells induced by immunisation in the context of serum iron deficiency, expression of CX3CR1, CD62L, CD27 and CD43 (1B11 isoform) was measured (Bottcher et al., 2015; Gerlach et al., 2016; Olson et al., 2013). We found a statistically significant decrease in the number of splenic 'central memory like' cells defined by three different gating schemes (CD62L+, CX_3CR1- and CD27+ CD43-) (2C-2E). Consistent results were found for central memory cells in peripheral blood as a % of total CD8s (data not shown)

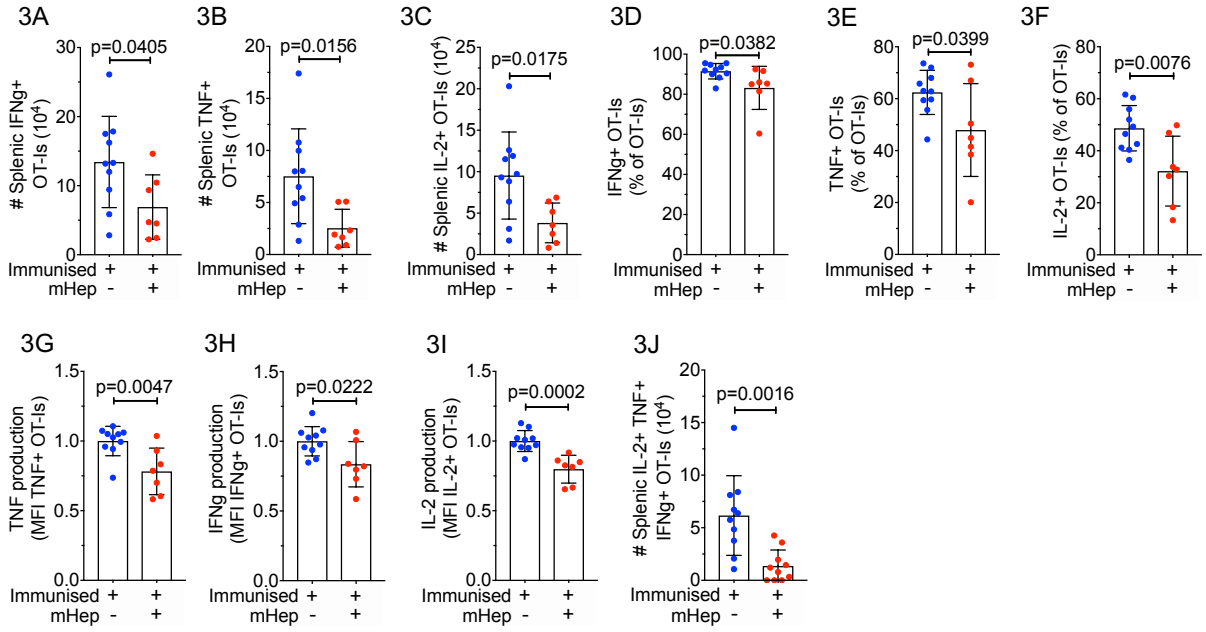


Figure 6.3. Cytokine production by splenic OT-I memory cells on day 35 post immunisation measured by intracellular cytokine staining and flow cytometry after restimulation *ex vivo* with SIINFEKL as in 1A. Total number of IFNγ+ (3A), TNFα (3B), IL-2+ (3C) OT-I memory cells and % of OT-I memory cells which can produce IFNγ (3D), TNFα (3E) and IL-2 (3F). MFI of TNFα (3G), IFNγ (3H) and IL-2 (3I) for TNFα+, IFNγ+ and IL-2+ cells respectively. Number of triple positive IL-2+, TNF+, IFNg+ memory cells per spleen (3J). T test.

Cytokine production by memory OT-I T cells was also assessed. The number and % of OT-Is that could synthesise TNFα, IFNγ or IL-2 was significantly reduced in mini-hepcidin treated mice (3A-F). The reduction in the frequency of cells which could secrete IL-2 was particularly strong, consistent with IL-2 production being a hallmark of central memory cells (Wherry et al., 2003) and the reduced frequency of central memory cells in mice treated with mini-hepcidin. Furthermore, median fluorescence intensity of anti-cytokine staining for cytokine positive cells was reduced for TNFα, IFNγ and IL-2 indicating that per cell production of cytokines by memory cells was also reduced by serum iron deficiency during primary immunisation (3G-I). These defects combine to result in a reduced frequency and number of polyfunctional OVA specific memory cells producing all three cytokines (3J). For a number of infections the polyfunctionality of T cells has been linked to quality of the T cell response (Betts et al., 2006; Boyd et al., 2015). These results suggest that serum iron deficiency during primary immunisation disrupts the differentiation and function of memory T cells.

6.2.3 Serum iron deficiency during primary immunisation with protein-in-adjuvant immunisation results in impaired secondary immunity.

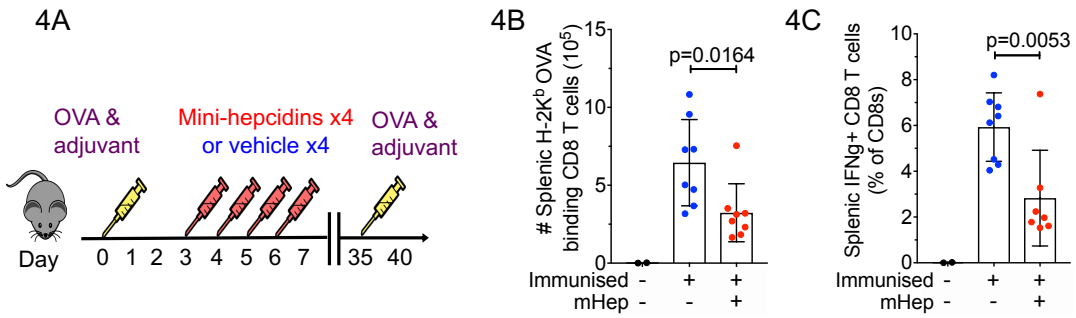


Figure 6.4. Experimental scheme for mouse treatment (4A). Number of endogenous OVA specific secondary CD8 effector cells in the spleen detected by OVA tetramer binding (4B) or frequency of splenic OVA specific CD8 effector cells detected by IFNγ+ production after *ex vivo* stimulation with SIINFEKL (4C). T test

Based on our findings that the quality of CD8 memory was reduced by mini-hepcidin treatment during primary immunisation, we decided to investigate how this scenario influenced the magnitude of the secondary immune response. For these experiments we chose to examine the endogenous SIINFEKL specific CD8 T cell response (rather than OT-I) because higher frequencies after boosting would render the enhanced detectability of OT-Is unnecessary. Mice were immunised with protein-in-adjuvant and treated with mini-hepcidin or vehicle on days 3-6. Mice were then boosted subcutaneously with protein-in-adjuvant on day 35 and the OVA specific CD8 recall response in the spleen was assessed on day 40 (4A). Mini-hepcidin treatment during primary immunisation significantly reduced the number and frequency of H-2K^b SIINFEKL tetramer binding CD8 T cells (4B). Similar results were observed when antigen specific CD8 T cells were detected via their capacity to synthesise IFNγ after SIINFEKL stimulation *ex vivo* (4C). Recall capacity and secondary expansion are

characteristics of good quality central memory-like CD8 memory responses (Wherry et al., 2003) and therefore these results looking at endogenous secondary responses are consistent with the phenotyping of OT-I memory responses in figure 2 and 3.

6.2.4 Serum iron deficiency during primary immunisation with BMDCs reduces frequency of memory CD8 T cells, but has no significant effect on control of virus.

Whilst frequency, surface phenotype and cytokine production are important phenotypes of memory T cells, the true test of quality of memory is ability to protect against infection. We immunised C57BL/6 mice against vaccinia virus using bone marrow derived dendritic cells, derived from FLT3L culture, pulsed with the immunodominant H-2K^{b} B8R$_{(20\text{-}27)}$ epitope to induce vaccinia specific CD8 T cells in the absence of vaccinia specific B-cells and CD4 T-cells so that we could focus on the contribution of impaired CD8 T cell immunity on protection (5A). It is worth noting that BMDCs will present non-self-peptides from FCS in the culture medium, which is likely to promote some CD4 help for the vaccinia specific CD8 response. Immunisation with the B8R epitope has previously been shown to be sufficient to protect mice from lethal challenge with vaccinia or ectromelia virus (mousepox) (Moutaftsi et al., 2009; Tscharke et al., 2005). As dendritic cell immunisation provides antigen ready-processed on antigen presenting cells, we hypothesised that the kinetics of the adaptive immune response would be slightly accelerated and for this experiment started mini-hepcidin or vehicle treatment on day 2 and ended it on day 6.

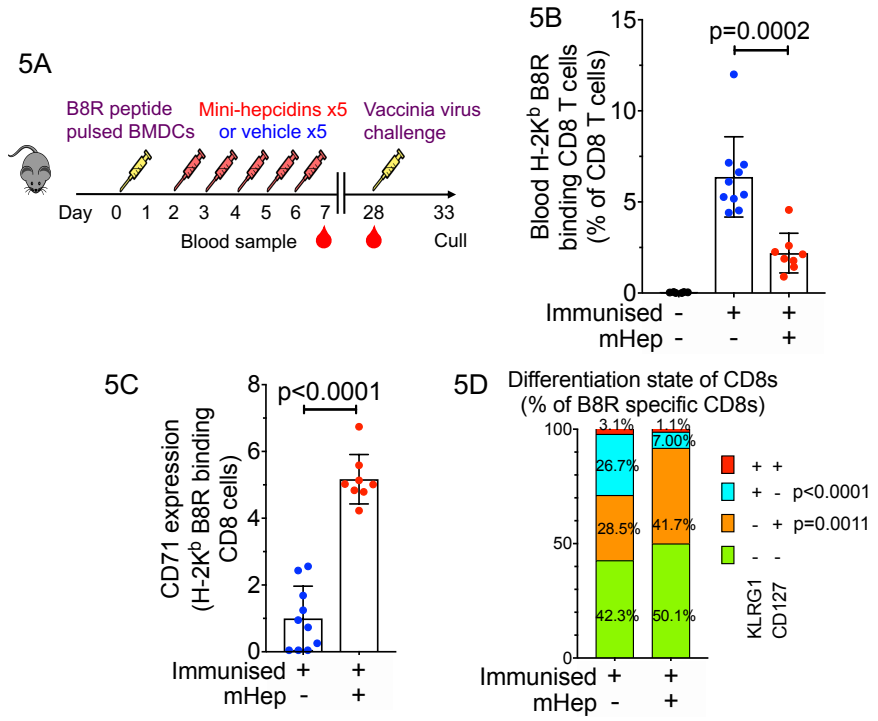


Figure 6.5. Experimental scheme for mouse treatment (5A). Frequency of endogenous vaccinia B8R specific CD8 effector cells in in peripheral blood as a % of total CD8s (5B). CD71 expression (5C) and differentiation state (frequency of KLRG1- CD127-, KLRG1+ CD127-, KLRG1- CD127+ and KLRG1+ CD127+) (5D) of B8R specific CD8 effector cells in blood. All on day 7 post immunisation. 5B and 5C T tests. 5D, Two way ANOVA, reporting p value for multiple comparisons between treatments for each T cell subset.

Detection of antigen specific CD8 T cells in peripheral blood using fluorescently labelled B8R loaded H-2K^b tetramers indicated that as expected mini-hepcidin treatment significantly inhibited the magnitude of the CD8 T cell response to immunisation on day 7 (5B). Furthermore, antigen specific effector CD8s showed the expected mini-hepcidin induced increase in CD71 expression (5C) and skewing of their surface phenotype from KRLG1+ CD127- to KLRG1- CD127+ (5D).

By day 28 post immunisation the frequency of antigen specific CD8s in peripheral blood had reduced, however there were still significantly fewer B8R specific CD8 T cells in the mini-hepcidin treated group compared to the control (6A). Therefore,

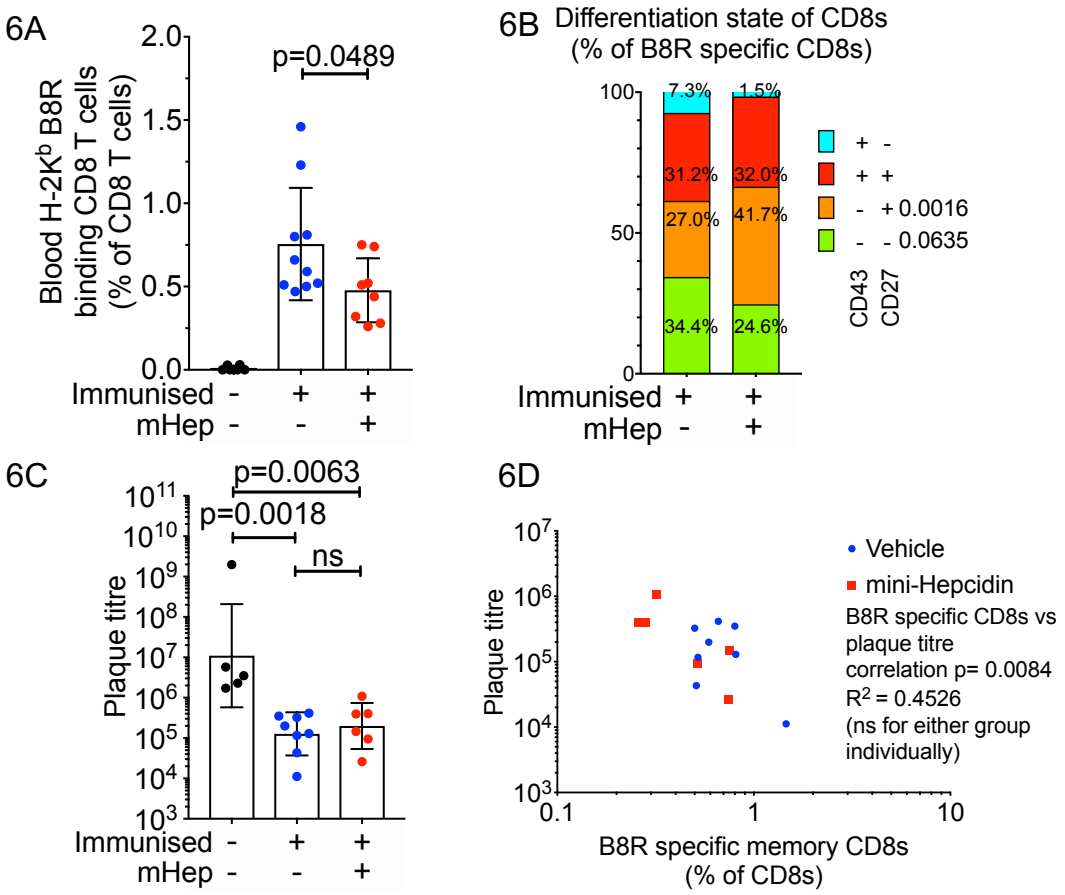


Figure 6.6. Frequency of endogenous vaccinia B8R specific CD8 memory cells in peripheral blood as a % of total CD8s (6A) and differentiation state (frequency of CD27- CD43-, CD27+ CD43-, CD27- CD43+ and CD27+ CD43+) of B8R specific CD8 memory cells in blood (6B) on day 28 post immunisation. Vaccinia virus plaque titre after titration of ovary homogenate on TK143 cells. Ovary homogenate from day 33 post immunisation (day 5 post virus challenge) (6C). Correlation of ovary viral load (plaque titre) on day 33 against frequency of vaccinia specific (B8R) specific CD8 memory cells on day 28. 6A T tests. 6B, Two way ANOVA, reporting p value for multiple comparisons between treatments for each T cell subset. 6C One way ANOVA. In two mice we failed to detect any vaccinia virus on day 5 post challenge, these mice are excluded from analysis on day 5 post challenge.

serum iron deficiency during primary immunisation with a dendritic cell-based immunisation is sufficient to reduce the magnitude of the memory response a month after immunisation. It has been suggested that expression of CD27 and CD43 on

memory CD8 T cells delineate a CD27 low subset with enhanced capacity to control vaccinia virus and listeria infection (Olson et al., 2013), we found that mice which experienced serum iron deficiency during the primary immune response to immunisation had a skewing of memory cell surface phenotype with more CD27+ CD43- cells and fewer CD27- CD43- , i.e. fewer of the presumed particularly protective subset (6B).

To investigate if the reduced frequency of antigen specific memory CD8 T cells in peripheral blood impacted upon immunity we challenged mice with $3X10^6$ PFU of TK-ve Vaccinia virus intraperitoneally and assayed virus load in the ovaries (a preferred niche of vaccinia virus replication) on day 5 post challenge (Olson et al., 2013; Xu et al., 2004). Whilst dendritic cell vaccination significantly reduced ovary viral titre relative to unimmunised mice, no difference in virus control was observed between mice treated with vehicle or mini-hepcidin during primary immunisation (6C). Interestingly however, given the reduced frequency of antigen specific memory cells in mini-hepcidin treated mice, there is a statistically significant inverse correlation between memory T cell frequency and ovary titre (6D).

6.3 Discussion

Whilst it is clear that iron restriction impairs the magnitude of primary CD8 T cell response the effect on the memory stage is more complex. Whilst the frequency of memory CD8 T cells clearly depends on the frequency of effector cells, it is also

determined by the frequency of effector cells which survive (Ahmed and Gray, 1996; Kaech et al., 2002). Interestingly, as presented in the previous chapter, we reproducibly observe that serum iron deficiency enriches for less mature effector cells with a more 'memory precursor like' surface phenotype' which may be a physiological response to buffer the size of the memory T cell response in the face of reduced clonal expansion.

Our results, however, do support the hypothesis that transient perturbation of nutrient availability during the primary response can have lasting consequences on the quality of immune memory, and the consequences of this on vaccination programmes in LMIC countries needs to be considered. We observed that serum iron deficiency during primary protein-in-adjuvant immunisation resulted in a reduced number of splenic 'central-memory-like' memory cells with an IL-2 producing, CD62L+ or CD27+ or CX_3CR1- phenotype (Jameson and Masopust, 2018). Whilst surface phenotype is only a proxy for function, the reduced number of central memory cells fits with reduced magnitude of recall response on secondary immunisation. Furthermore, a general defect in cytokine production by memory CD8 T cells originating from effector cells in mini-hepcidin treated animals suggests a more generalised disruption of memory cell physiology and differentiation. We have not explored what the lasting consequences on memory cell metabolism priming in low iron conditions might have, but it is clear that dysfunctional mitochondrial health could underlie a failure of memory cells to secrete cytokines or proliferate on secondary stimulation (Bantug et al., 2018; van der Windt et al., 2012a). This is further

supported by findings suggesting that enhancing mitochondrial electron transport chain activity improves the quality of memory T cell responses (Champagne et al., 2016).

In contrast to protein-in-adjuvant immunisation, for immunisation with peptide-pulsed bone marrow derived dendritic cells we observed a skewing of the surface phenotype of memory CD8 T cells away from a CD27- 'effector-like' subset with enhanced cytolytic capacity (Olson et al., 2013). It is possible that the skewing of effector CD8 T cells away from KLRG1+ subset during primary immunisation contributes to fewer of these long lived 'effector-like' cells (Herndler-Brandstetter et al., 2018).

In light of this it is worth considering the growing heterogeneity of CD8 T cell memory and that different subsets of CD8 memory cells appear to be particularly efficacious at protecting against different pathogens (Bachmann et al., 2005; Nolz and Harty, 2011; Olson et al., 2013; Pizzolla et al., 2017). Therefore, if serum iron deficiency modulates CD8 T cell differentiation, the impact may vary depending on the requirements for pathogen control. Therefore, whilst we did see suppressed memory CD8 T cell frequencies for our DC vaccination after iron deficiency during primary immunisation and an inverse correlation between CD8 T cell frequency and vaccinia titres on day 5 post infection, we did not detect an effect of iron restriction on protection against vaccinia infection. It is possible that the effect size of the impairment of T cell memory by low serum iron in this experiment was not enough to confer detectably altered

protection against infection given the large variability in ovary titres between mice in the same group. The ability of iron restriction to impair effector differentiation may mean that iron deficiency may more severely impair development of more effector like resident memory CD8 populations at mucosal surfaces. For a different immunisation/infection pair where defence at mucosal sites is important (Mackay et al., 2012; Stary et al., 2015), such as flu infection (Pizzolla et al., 2017), iron deficiency could plausibly cripple T cell mediated immunity and this warrants further investigation.

Serum iron deficiency suppressed the development of central memory like (CD62L+ or CD27+) CD8 T cells after protein-in-adjuvant immunisation but favoured the development of CD27- central memory like cells after dendritic cell vaccination. The differences in the effect of primary immunisation serum iron deficiency on memory cell surface phenotype in protein-in-adjuvant vs dendritic cell immunisation could relate to the differentiation pathways co-opted to support these CD8 T cell responses; protein-in-adjuvant vaccinations seem to induce a more rapid differentiation to a memory-like phenotype with increased early reliance on T-bet and EOMES (Klarquist et al., 2018). The observation that transient nutritional restriction in the form of low serum iron during the primary response impairs the functionality of CD8 memory cells makes the mechanism of this long term reprogramming interesting and suggests that the influence of altered iron availability of the epigenetics of T cells may be an interesting future line of inquiry (Etchegaray and Mostoslavsky, 2016; Phan et al., 2017). It has been suggested that memory cells have an epigenetic memory of their

life as an effector cell (Akondy et al., 2017). However failure to effectively suppress genes associated with effector function through histone methylation results in defective memory differentiation and preservation of 'stemness' (Pace et al., 2018).

A range of metabolic pathways intersect with epigenetic regulation of gene expression. Synthesis of Acetyl-CoA from citrate can modulate cellular epigenetic state, and T cell function, through modulation of histone acetylation (Peng et al., 2016). Defective synthesis of Iron-Sulphur cluster proteins has been noted to increase cellular citrate levels in cancer cells (Crooks et al., 2018), therefore perhaps acetyl-CoA accumulates in iron deficient T cells altering the profile of histone acetylation and perturbing differentiation. Sirtuin deacetylases, which can target histones, are NAD dependent, demonstrating how cellular energy balance can alter the epigenetic profile of cells (Houtkooper et al., 2012). S-adenosylmethionine, generated from one-carbon metabolism, is the methyl donor for histone and DNA methyltransferases (Phan et al., 2017). Finally both the DNA demethylases (Ten-eleven translocation enzymes) and JmjC domain containing histone demethylases are iron and α-ketogluterate dependent (Klose et al., 2006) and modulation of JmjC domain containing demethylase activity by iron chelation has been proposed to modify cell cycle behaviour in B cells (Jiang et al., 2019). The accumulation of the 2-dioxygenase enzyme inhibiting metabolite S-2-hydroxygluterate (S-2HG) during hypoxia modulates T cell function and epigenetic state through TET/JmjC enzyme inhibition (Tyrakis et al., 2016). S-2HG was induced by HIF1α activation, which could also take place during iron restriction; iron deficiency could therefore disrupt TET/JmjC enzymes both directly (as

they require iron to function) and indirectly (through mimicking hypoxia and causing S-2HG accumulation). It will be of interest to directly measure the epigenetic profiles of T cells which had developed in the presence of low iron and also utilise metabolomics to investigate how iron availability alters the production of epigenetic metabolites such as acetyl-CoA and S-adenosylmethionine.

The above work focusses on how low serum iron during the 'effector burst' affects the generation of CD8 T cell memory; however, the effect of low serum iron on other 'stages' of T cell memory could be interesting lines of inquiry to pursue. The phenotype of memory cells is determined in part during the contraction phase of the response, and alteration of mTOR signalling during this phase for example, can alter the quality of T cell memory (Araki et al., 2009). Furthermore, the number of circulating memory CD8 T cells is maintained through homeostatic proliferation (Tan et al., 2002) and resting memory T cells have unique metabolic profiles relying mainly on oxidative metabolism (Pan et al., 2017; Pearce et al., 2009b; van der Windt et al., 2012b).

Furthermore, as discussed in the previous chapter CD8 T cell responses do not develop in isolation. Whilst the literature is complex and the exact phenotype depends on the immunisation, it is clear that CD4 help is required for a fully functional CD8 T cell memory response. CD8 T cells with defective IL-2 signalling or primed in the absence of CD4 help generate memory cells with similar efficiency to WT cells, but show defects in secondary expansion (Sun and Bevan, 2003; Williams et al., 2006). The data we present indicates that the phenotypes of immune responses primed in low serum

iron conditions are complex, potentially due to the interactions of multiple cell types and the exact immunisation regime utilised.

Finally it would be interesting to extend our analysis to include CD4 memory cells and B cell memory, particularly in light of recent findings suggesting that severe dietary iron deficiency in mice reduces primary and secondary antibody responses to a model immunogen (Jiang et al., 2019). The exact pathways of CD4 differentiation from naïve to memory precursor to diverse memory cell subsets are more poorly defined than for CD8 T cells, in part because of the heterogeneity of CD4 helper subsets add an extra layer of complexity (Hale et al., 2013; Marshall et al., 2011). However given the importance of metabolic functionality for CD4 effector differentiation and memory cell function (Peng et al., 2016) it seems at least possible that low serum iron will influence the biology of CD4 T cell memory.

Serum iron deficiency inhibits the immune response to influenza virus

7.1 Introduction

7.1.1 Iron homeostasis and viral infection

Unlike for bacterial and fungal infections (Cassat and Skaar, 2013; Drakesmith and Prentice, 2012) the interactions between systemic iron homeostasis and the outcome of viral infection have been less extensively explored, with most focus to date on how iron directly influences viral replication and viral infection associated pathology with a particular focus on HCV and HIV infection (Drakesmith and Prentice, 2008). A number of viruses utilise the Transferrin receptor as an entry receptor (Drakesmith and Prentice, 2008), likely reflecting that iron acquiring, cycling, metabolically active cells with a high rate of biosynthesis are suitable targets for infection.

HCV disrupts BMP signalling and suppress hepcidin expression (Eddowes et al., 2019), associating with increased liver iron content and liver disease progression (Fujita et al., 2007; Furutani et al., 2006). However, the evidence for iron directly influencing Hepatitis C virus replication is unclear, with conflicting literature suggesting that iron both enhances (Theurl et al., 2004) and suppresses HCV replication (Fillebeen and Pantopoulos, 2010).

The acute phase of HIV and norovirus infection is characterised by a transient acute phase response, with raised hepcidin and decreased serum iron (Armitage et al., 2014; Williams et al., 2019), although this viral associated hypoferremia appears to be less extreme than that observed in acute human typhoid infection (Darton et al., 2015). It has been suggested that the acute phase response to viral infections is different to bacterial infection (Slaats et al., 2016), but whether infection associated changes in iron homeostasis are generalisably different between bacterial vs viral infections is unclear, and seems unlikely. Patients chronically infected with HIV also display raised serum hepcidin relative to uninfected controls (Armitage et al., 2014). Macrophage iron loading in HIV has been associated with shorter survival times and increased incidence of AIDS-associated macrophage trophic secondary infections (Monyé et al., 1999)(McDermid et al., 2013). Furthermore host cell iron content has been proposed to have a direct positive effect on multiple aspects of HIV viral replication (Drakesmith and Prentice, 2008) and through the suppression of HFE expression by Nef, HIV is proposed to actively modulate host cell iron homeostasis to favour its replication (Drakesmith et al., 2005a).

Whilst the dependence of cellular biochemistry on iron is clear, how iron deficiency alters viral replication, tolerance of infection and most importantly to us the immune response to viruses as a general principle remains relatively unexplored.

7.1.2 Influenza virus

Influenza virus is a negative sense single stranded RNA virus spread by respiratory infections with the major burden of disease being seasonal epidemics resulting in 3-5 million cases of severe illness and 650,000 deaths per year ("CDC Press Releases," 2016; Krammer et al., 2018). Severe infection, particularly from pandemic or zoonotic viruses is characterised by extensive viral proliferation in the lower airways and a pathologically high production of cytokines. The virus is thought to primarily infect and replicate within respiratory epithelial cells, but can infect immune cells, predominantly macrophages, which can also generate infectious virus (DiPiazza et al., 2017; Manicassamy et al., 2010). Therefore, as a viral infection of huge global importance with well-established animal models and a large body of work on the immunological processes which contribute to control of viral infection, we chose influenza virus to investigate how serum iron deficiency altered the outcome of viral infection with a focus on immunity and immunopathology.

Little work has been conducted on the interaction between iron and viral replication, pathology or the immune response to influenza infection. A retrospective trial identified an association between iron deficiency and more severe disease in a cohort of children hospitalised with influenza (Lakhan et al., 2016). In animal models severe influenza infection results in a comparatively weak hepcidin and hypoferremia response when compared to canonical bacterial or TLR ligand models (Armitage et al., 2011; Rodriguez et al., 2014). In a rat model of vaccination with inactivated influenza virus dietary iron deficiency reduced virus specific antibody titres compared to control

animals. However, when iron deficiency animals were compared to pair-fed animals, fed the same mass of food each day as the iron deficient animals, iron deficient diet did not significantly alter antibody titres (Dhur et al., 1990) highlighting the advantages of a transient iron deficiency model.

7.1.3 immunity to influenza virus

Immune responses to influenza virus begin with innate recognition of viral infection through pattern recognition receptors detecting pathogen associated molecular patterns: RIG-I (5'-triphosphate RNA) and NLRP3 (infection associated cellular damage) detect replicating virus within infected cells, whilst TLR3 (dsRNA) and TLR7/8 (ssRNA) detect virally infected cells/viral particles in endosomes (Iwasaki and Pillai, 2014).

Activation of pattern recognition receptors induces production of a range of cytokines including type I interferons, which activate interferon stimulated genes including MX1, IFITM and PKR (Iwasaki and Pillai, 2014), which act in the cell intrinsic restriction of viral replication and make cells less hospitable for influenza virus replication. This induction of interferons is one of the first waves of transcriptional changes which takes place in the lung during influenza infection (Pommerenke et al., 2012).

Innate immune cell responses to influenza virus are dominated by both tissue resident immune cells, such as alveolar macrophages (Cardani et al., 2017), and cells recruited

from the circulation such as NK cells (Gazit et al., 2006), monocytes – which may differentiate into macrophages on reaching the lung (Hashimoto et al., 2007) - and neutrophils (Crowe et al., 2009; Tate et al., 2009; Zhu et al., 2019). This constellation of innate cells all contribute to survival from influenza infection, but when the innate response is too strong, often in cases of excessive viral replication, it can contribute to immunopathology (de Jong et al., 2006; Kobasa et al., 2007; Perrone et al., 2008; Short et al., 2018). Furthermore it is important to remember that the innate immune response is essential for induction of adaptive immunity and there is constant cross-talk between innate and adaptive arms of immunity through out infection (Iwasaki and Medzhitov, 2015). One example of this is the dynamic trafficking and activation of T cell responses by dendritic cells during flu infection (Ballesteros-Tato et al., 2010; Cabeza-Cabrerizo et al., 2019).

Data from mouse models demonstrate the importance of adaptive immunity for defence against influenza virus, but also the redundancy between these arms of immunity. CD8 T cells traditionally thought to play a major role in the defence against viral infection, have been shown to be important for optimal resolution of primary infection and immune memory to influenza virus. Antigen specific CD8 T cells accumulate in the lung after infection (Flynn et al., 1999) and CD8 mediated defence seems to primarily depend on targeted cytolytic processes: In context of CD4 depletion, the perforin and FAS mediated killing of target cells by CD8 T cells are essential for viral control (Topham et al., 1997). Even more profoundly mice deficient in TNF-related apoptosis inducing ligand (*Trail)*, a ligand expressed by CD8 T cells

which faciliates target cell killing, are profoundly sensitive to influenza infection, but adoptive transfer of TRAIL sufficient CD8 T cells can rescue this phenotype (Brincks et al., 2008). The importance of cytokine production by CD8 T cells, including IFNγ, of which CD8 T cells are a major producer in the infected lung, for viral clearance is unclear (Graham et al., 1993). However, and unexpectedly, production of anti-inflammatory IL-10 by lung effector T cells, predominantly CD8 T cells, was shown to play a role in resolution of lung inflammation and tolerance of infection (Sun et al., 2009).

There is some evidence from human infection models that influenza specfic CD8 T cells drive viral control on re-challenge (McMichael et al., 1983). Data from animal models has subsequently supported a model where lung resident CD8 T cell responses to conserved core intracellular antigens are central to immunity to heterosubtypic challenge with viruses of a different strain (where the different haemagglutinin (HA) and neuraminidase (NA) genes mean that pre-exisiting humoral immunity is ineffective (Slütter et al., 2017)). Whilst depletion of CD4 T cells seems to have little effect on viral clearance during primary infection, CD4 T cells appear to be required for optimal induction of CD8 T cell memory responses (Belz et al., 2002). CD4 memory T cells also might be important for optimal immunity to reinfection in humans (Wilkinson et al., 2012) and mice (Brown et al., 2006).

Studies using B cell deficient μMT mice demonstrate the importance of humoral immunity for clearance of primary infection (Graham and Braciale, 1997), with the

extreme susceptibility of CD8 depleted μMT mice demonstrating the partially redundant nature of adaptive immunity to influenza (Topham and Doherty, 1998). Whilst humoral immunity is thought to primarily bestow resistance to homotypic challenge with viruses expressing similar HA and NA proteins, a role for B cells in heterosubtypic memory has been proposed (Rangel-Moreno et al., 2008).

7.1.4 Chapter Aims

In previous chapters we have observed that injection of mini-hepcidin suppressed multiple arms of the adaptive immune responses to vaccination with protein and non-replicative viral vectors. Given the importance of adaptive immunity for clearance of influenza infection, we hypothesised that serum iron deficiency would suppresses influenza induced T and B responses (identified using the flow cytometry gating schemes in the supplementary information), and delay clearance of influenza virus from the lung, potentially resulting in more severe pathology of infection.

Specifically, we will address if mini-hepcidin mediated iron deficiency alters:

1. Influenza infection associated weight loss and recovery
2. Influenza induced adaptive immune responses in the lung, draining lymph node and spleen
3. Influenza virus RNA levels and inflammation associated gene expression in lung and liver
4. The clinical score of lung pathology

7.2 Results

7.2.1 Establishing an appropriate challenge dose of influenza virus

Different influenza virus strains result in different infection behaviours in different mouse strains, there is also batch to batch variability with viral preps and therefore it was important to titrate our virus *in vivo* to establish an infection model with a moderate amount of weight loss as a proxy for severity of infection. However, we also required a non-lethal model where weight loss did not exceed 20% so we could reliably follow infection to day 8 or 10, when adaptive immunity kicks in to clear infection (Pommerenke et al., 2012).

We chose the mouse adapted virus X31 strain (reassortment of PR8, with surface glycoproteins from the human of A/Aichi/2/68 (H3N2) strain (Kilbourne, 1969)) because it induces less severe disease than the parent strain PR8 and has been regularly used in mouse studies concerning the adaptive immune response to influenza. We challenged our mice with a titration of different hemagglutination units (HAU).

We chose a dose of 0.08 HAU for subsequent experiments as on average the mice reached their weight nadir on day 6 or 7, losing 10% of their starting weight. The recovery of weight on day 8 matches with when the literature indicates T cell immunity should peak in the lung (Pommerenke et al., 2012), we then subsequently

confirmed that a substantial number of flu NP-specific CD8 T cells were present on day 8 in the lungs of infected mice (data not shown).

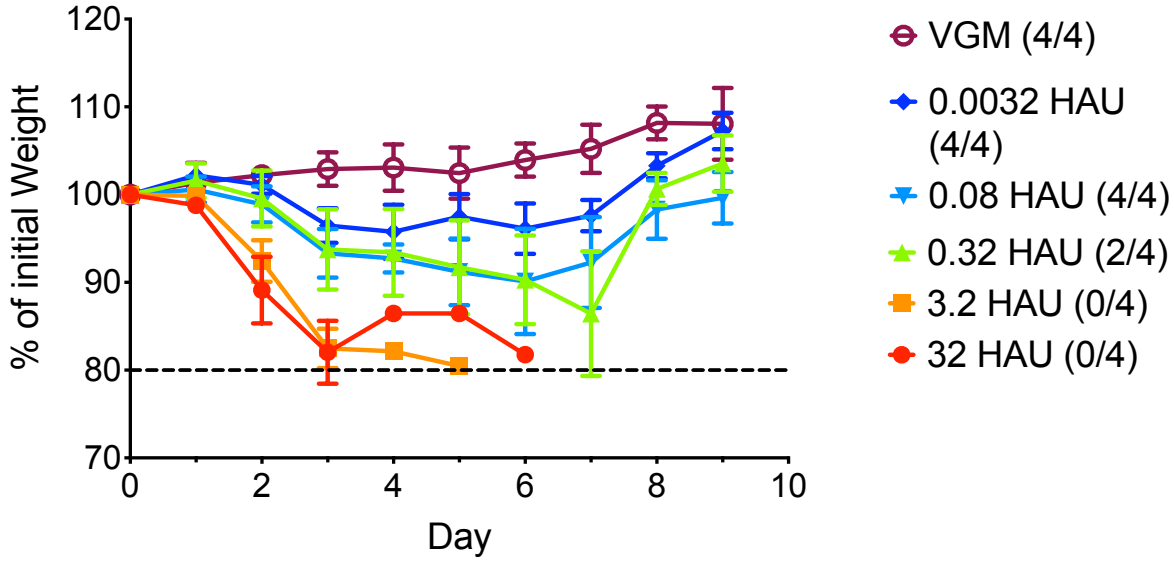


Figure 7.1. Mice were challenged with a titrated dose (HAU) of influenza virus X31 or viral growth medium (VGM) as an uninfected control and weight change was tracked for 9 days until recovery. Numbers in brackets indicates number of mice in each group which had to be killed before the end of the experiment.

7.2.2 Serum iron deficiency impairs adaptive immunity to influenza

In light of our previous work that serum-iron deficiency results in impaired adaptive immune responses to vaccines we investigated how mini-hepcidin injection altered the magnitude of influenza induced adaptive immune responses in the lung, spleen and mediastinal lymph node (mLN). In both experiments investigating the effect of mini-hepcidin mediated serum iron deficiency on influenza infection, mice were intranasally challenged with 0.08 HAU of X31 on day 0 and injected intraperitoneally daily with 100nmol of mini-Hepcidin or equivalent volume of vehicle from day 3 until the day prior to cull. In experiment 1 mice were culled on day 8 post infection and in experiment 2 mice were culled on day 10 post infection (experimental scheme 2A).

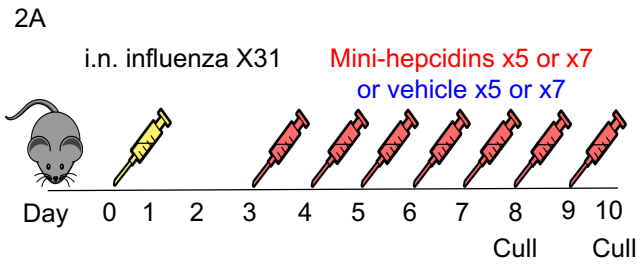


Figure 7.2. Experimental scheme for *in vivo* mouse experiments (2A)

To examine influenza specific CD8 T cells we utilised a fluorescently labelled MHC-I H-2D^{b} tetramer loaded with the immunodominant $NP_{366-374}$ epitope (NP specific CD8s) (Flynn et al., 1998). On Day 8 post infection we found a significantly reduced number and frequency of NP specific CD8s in the spleen (3A) and lungs (3D and 3E) of mini-hepcidin treated animals. CD71 expression on NP specific CD8s was also increased, consistent with mini-hepcidin treatment resulting in cellular iron deficiency within responding cells (3B and 3F). Similar results were found in the spleen on day 10 post infection (3G, H and I). The number of Granzyme B expressing CD44+ CD8 T-cells in the spleen on day 8 post infection was also significantly reduced by mini-hepcidin treatment; Granzyme B expressing CD8 T cells likely include virus specific effector CD8s and indicate that the effect of serum iron deficiency is not restricted to NP specific CD8s (3C).

We next focussed on the B cell response and found that whilst the total numbers of cells in mLN was not altered by mini-hepcidin treatment (4A), the number (4B) and frequency (4C) of germinal centre B cells (assessed as CD95+, GL7+, IgD- B cells) was

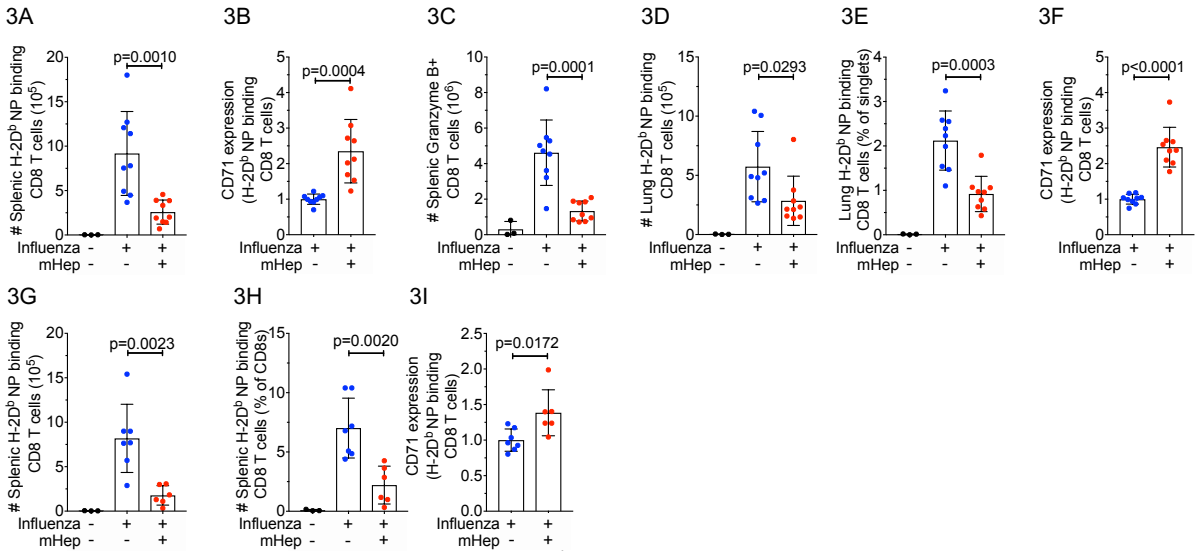


Figure 7.3. Number of influenza NP specific CD8 effector cells in the spleen (3A), CD71 expression by NP specific splenic CD8 effectors (3B) and the number of Granzyme B+ CD8 effector cells was measured by flow cytometry (3C) at day 8 after influenza infection. NP specific CD8 T-cell number (3D), frequency (3E) and CD71 expression was also measured in the lung on day 8 post infection. Splenic NP specific CD8 T cell number (3G), frequency (3H) and CD71 (3I) was also measured on day 10 after infection. T-test.

significantly reduced by mini-hepcidin treatment on day 8 after infection. Similarly, reduced numbers and frequency of GC B cells were observed on day 10 in the spleen (we could identify mLNs in all mice at day 10 post infection and therefore analysed the spleen at this time point). On both day 8 and day 10, mini-hepcidin treatment resulted in a significantly higher relative CD71 expression on GC B cells (4D and 4G).

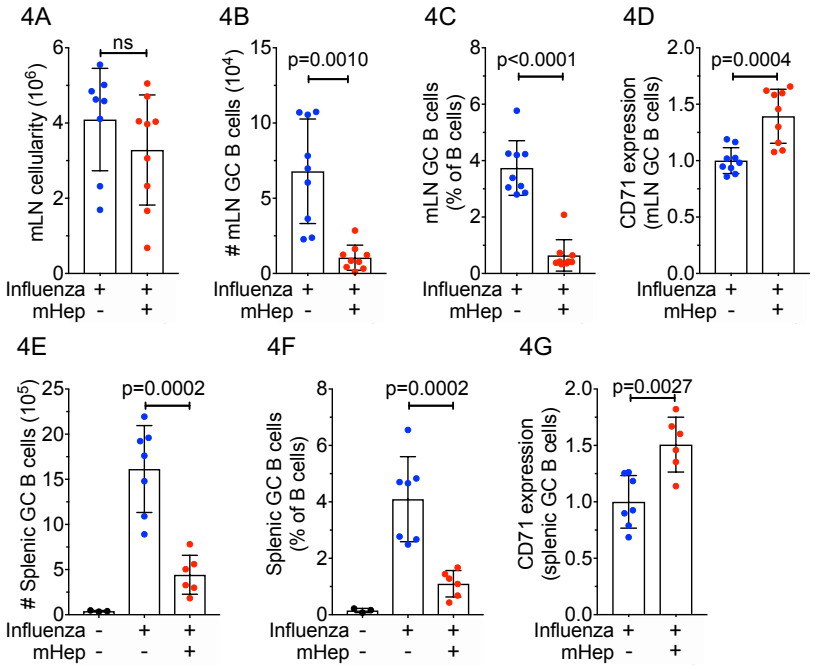


Figure 7.4. Mediastinal lymph node cellularity was measured (4A), as well as lymph node germinal centre B cell number (4B), frequency as a % of B cells (4C) and CD71 expression (4D) on day 8 after influenza virus infection. Splenic germinal centre B cell number (4E), frequency as a % of B cells (4F) and CD71 expression (4G) was measured on day 10 post influenza virus infection. T-test

Finally, consistent with an impairing of B cell immunity, we found that number and frequency of influenza induced T follicular helper cells (assessed as CD44+ CXCR5+ PD-1+ CD4 T cells) was significantly reduced by mini-hepcidin treatment on day 8 in mLN and day 10 in the spleen (5A, 5B, 5E and 5F). Tfh also increase CD71 expression in mini-hepcidin treated mice (5C and 5G). Furthermore, the infection induced increase in antigen-experienced CD44+ CD4 T-cells was also opposed by serum iron deficiency (5D and 5H).

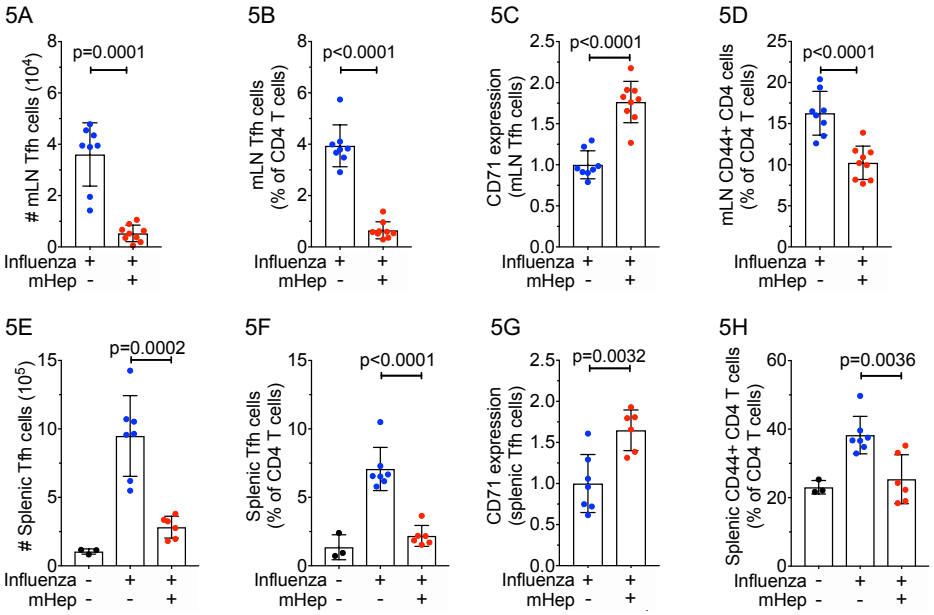


Figure 7.5. Lymph node T follicular helper cell number (5A), T follicular helper cell frequency as a % of CD4 T cells (5B), T follicular helper CD71 expression (5C) and frequency of CD44+ effector CD4s as a % CD4 T cells on day 8 post influenza virus infection (5D). Splenic T follicular helper cell number (5E), T follicular helper cell frequency as a % of CD4 T cells (5F), T follicular helper CD71 expression (5G) and frequency of CD44+ effector CD4s as a % CD4 T cells (5H) on day 10 post influenza virus infection. T-test.

7.2.3 Serum iron deficiency reduces the quality of the anti-influenza antibody response

In collaboration with Tiong Kit Tan (Townsend group, Weatherall Institute of Molecular Medicine, University of Oxford) we performed microneutralization (MN) assays to measure the X31 influenza neutralising antibody titres. The dilution of serum which resulted in a 50% reduction of infection of *in vitro* cells was calculated and plotted as the EC_{50}. Whilst there was no difference in the MN titre between mini-hepcidin treated and vehicle treated groups on day 8 (6A), this is quite an early time point to resolve the effect of humoral immunity on virus replication with µMT only

showing clearance defects from day 10 in published literature (Topham and Doherty, 1998). In the experiment which was continued until day 10 post infection there was a significantly lower MN titre in the mini-hepcidin treated mice, in fact only 1 of 6 mini-hepcidin treated mice had a titre above the limit of detection (6B). Therefore, serum iron deficiency resulted in severely impaired humoral immunity to influenza virus in line with our results looking at cellular B cell response by flow. It may be worth repeating the experiment once more to determine if the suppression of humoral immunity by mini-hepcidin is robust.

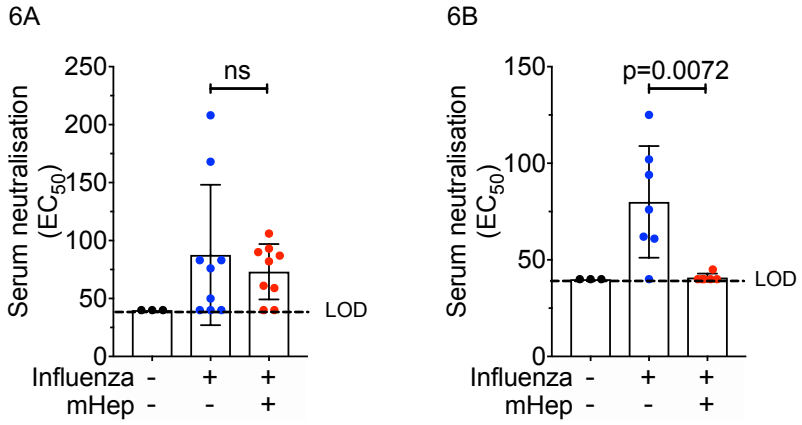


Figure 7.6. The dilution of heat-inactivated serum that completely neutralised viral replication (24hrs after plating) in MDCK cells in 50% of replicate wells was calculated and plotted as the EC_{50} (EC_{50} defined above). Neutralising antibody titres were measured for serum from day 8 post infection (6A) and day 10 post infection (6B). T-test.

7.2.4 Serum iron deficiency inhibits weight recovery after influenza virus infection

In both experiments control mice lost weight up until day 7 and then began to recover again, whereas mini-hepcidin treated mice had significantly lower weights from day 8 onwards suggesting that they were failing to recover from influenza infection. Mortality was non-significantly different between the two groups with two vehicle-treated and one mini-hepcidin treated mice having to be culled on day 7 and two mini-hepcidin treated mice having to be culled on day 8 of the 10 day experiment (7A and 7B).

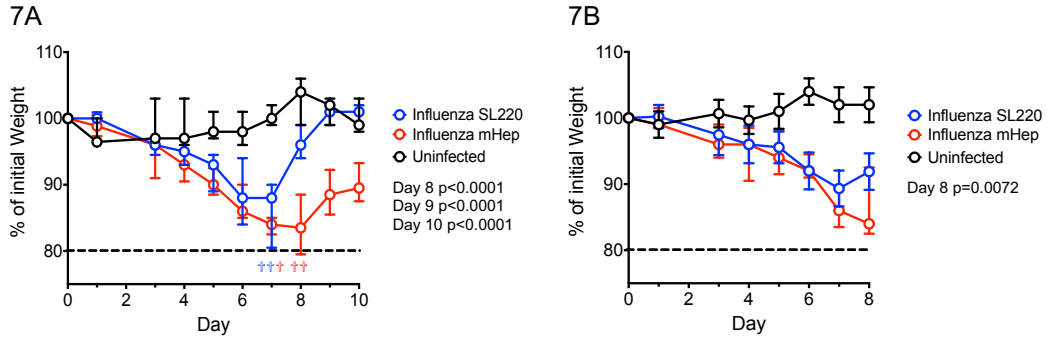


Figure 7.7. % of initial starting weight was measured throughout the 10 day (7A) and 8 day (7B) experiment, taking care to record weight at the same time each day to minimise variability associated with diurnal changes in weight. Crosses mark mice culled when the humane limit of 20% weight loss was reached. Anova, with multiple comparisons for each time point.

7.2.3 Serum iron deficiency increases levels of viral RNA in lung

On either day 8 or day 10 post infection RNA was isolated from whole lung lobes (to minimise expression artefacts due to patchy inflammation) and expression of

influenza NP RNA was used as a proxy for viral load. *Hprt* was chosen as an endogenous control gene as its expression was stable between control and infected lungs (not shown). As expected, no NP RNA transcripts were detected in uninfected mice. NP RNA expression was higher in mini-hepcidin treated animals on day 8 and day 10, consistent with the hypothesis that the inhibition of the adaptive immune response to influenza virus by serum iron deficiency resulted in impaired viral clearance (8A and 8B). Furthermore, on day 8 after infection there was a significant (negative) correlation between lung viral load and % of starting weight in mini-hepcidin treated mice and a nearly significant correlation in vehicle treated mice (8C). Relative expression of NP to the endogenous control gene *Hprt* was decreased on day 10, compared to day 8 suggesting that both groups of mice were clearing virus, but clearance was delayed in mini-hepcidin treated mice. No viral NP RNA was detected in the liver (data not shown).

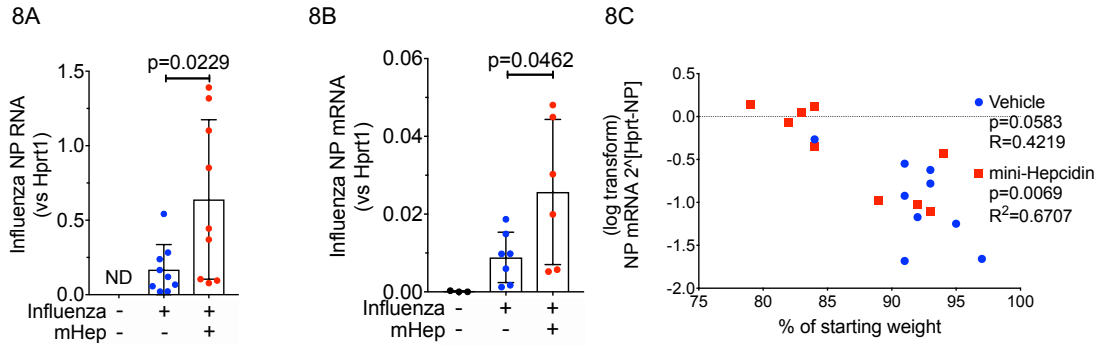


Figure 7.8. Influenza NP RNA in homogenates of a whole lung lobe was measured by qRT-PCR using TaqMan probes against NP on day 8 (8A) and day 10 (8B) post infection. Data presented as [2^(CT of *Hprt* - CT of NP)]. The correlation between viral load (Log-transformed of NP mRNA) and % of starting weight was also plotted for day 8 post infection (8C). 8A and 8B T-test. 8C Pearson correlation coefficient.

7.2.4 Histological analysis suggests serum-iron deficiency enhanced inflammation and lung damage

To examine the severity of influenza infection representative lung sections from day 10 after infection were analysed by H&E staining in collaboration with Tiago Duarte at the University of Porto. The uninfected group shows well-defined alveolar spaces clear of infiltrate, whereas in contrast both influenza infected groups shows focal airway inflammation that was absent in the uninfected group as expected. In the vehicle treated infected group, airway inflammation was scored to be mild to moderate, with inflammatory cells surrounded the perivascular/peribronchiolar compartment (arrows). In the mini-hepcidin treated infected group, perivascular/peribronchiolar inflammation was more severe. The mini-hepcidin infected group showed marked inflammatory infiltrate within alveolar parenchyma across large areas of the lung,

which was absent in the vehicle treated group (delineated by the dashed line at 40X) (9A).

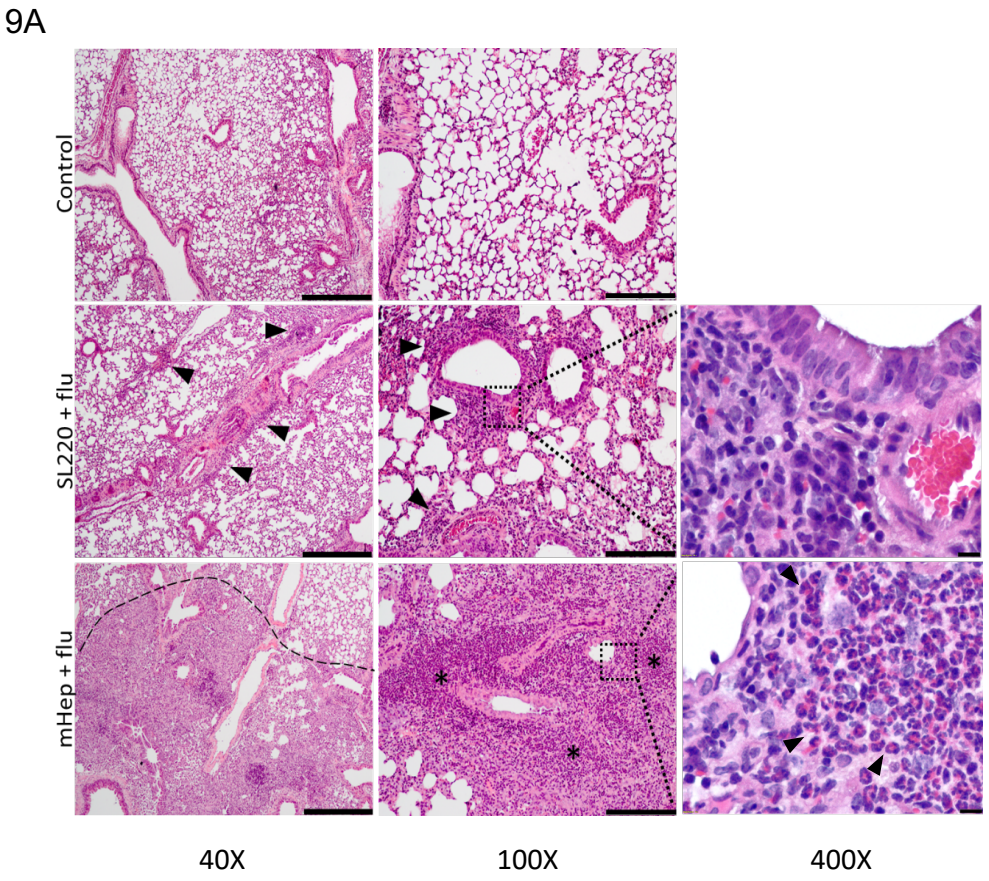


Figure 7.9. (9A) H&E staining of representative lung sections from mice on day 10 post influenza infection from each group. 40X, scale bar 500µm, arrows/area delineated by dotted line indicate inflammatory infiltrate. 100X, scale bar 200µm, inflammatory infiltrate indicated by arrows or stars. 400X, scale bar 10µM, arrows indicate frequent PMN with intense eosin staining cytoplasm .

In both infected groups, the inflammatory infiltrate was polymorphic, comprising lymphocytes, histiocytes and polymorphonuclear neutrophils (PMN) in variable amounts from area to area. Given the importance of inappropriate innate inflammation in the lung pathology of influenza we specifically focussed on the degree of neutrophilic infiltration. Cells with a PMN appearance (small intense purple and

pink staining cells, arrows at 400X in mini-hepcidin treated animals) were found to be dispersed with no aggregation in the vehicle treated infected group, whereas PMN were more frequent in the lungs of serum iron deficient infected mice, where they displayed multiple foci of aggregation in the perivascular/peribronchiolar areas, throughout the alveolar parenchyma and occasionally within the bronchiolar lumen (asterisks on 100X and numerous pink cells 400X mHep images) (9A). The increased frequencies of neutrophils in mini-hepcidin treated mice was confirmed by Leder staining that specifically stains the chloroacetate esterase producing neutrophils pink (arrows), but does not stain monocytes or lymphocytes. These signs of increased neutrophilic infiltration may indicate active inflammation (10A). Blinded scoring by a clinical pathologist supports increased inflammation (11A) and neutrophil infiltration (11B) in the lungs of mini-hepcidin treated influenza infected mice.

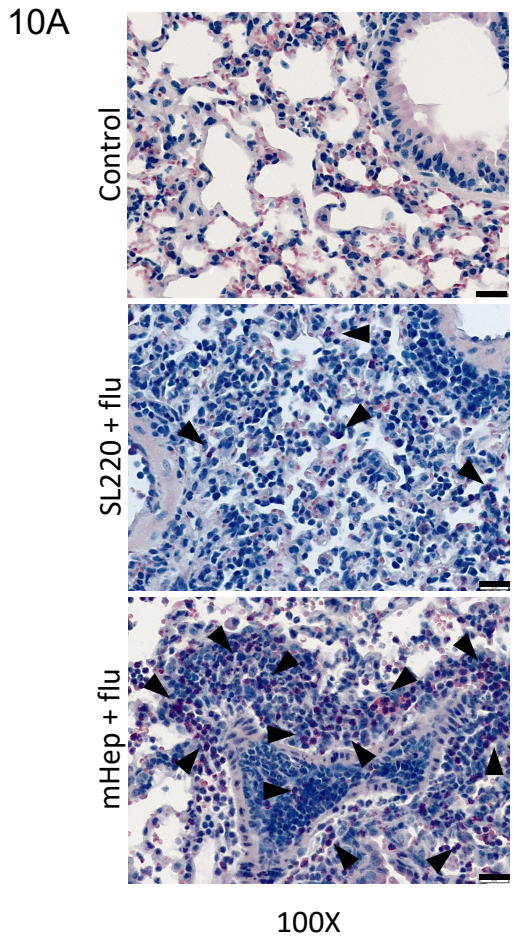


Figure 7.10. (10A) Leder staining of representative lung sections from mice on day 10 post influenza infection from each group. 100X, scale bar 20μm. PMN stain with pink cytoplasm, indicated by arrows.

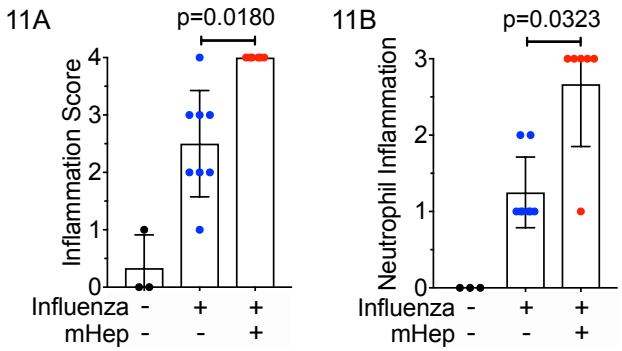


Figure 7.11. Summary inflammation score (11A) and neutrophil inflammation (11B) of H&E lung sections from each mouse scored by a blinded clinical pathologist. Kruskal-Wallis test

Orcein staining stains elastic fibres and indicates the increased size of the areas of lung where alveolar structure is disrupted in mini-hepcidin treated influenza infected mice are compared to SL220 vehicle treated infected mice (12A).

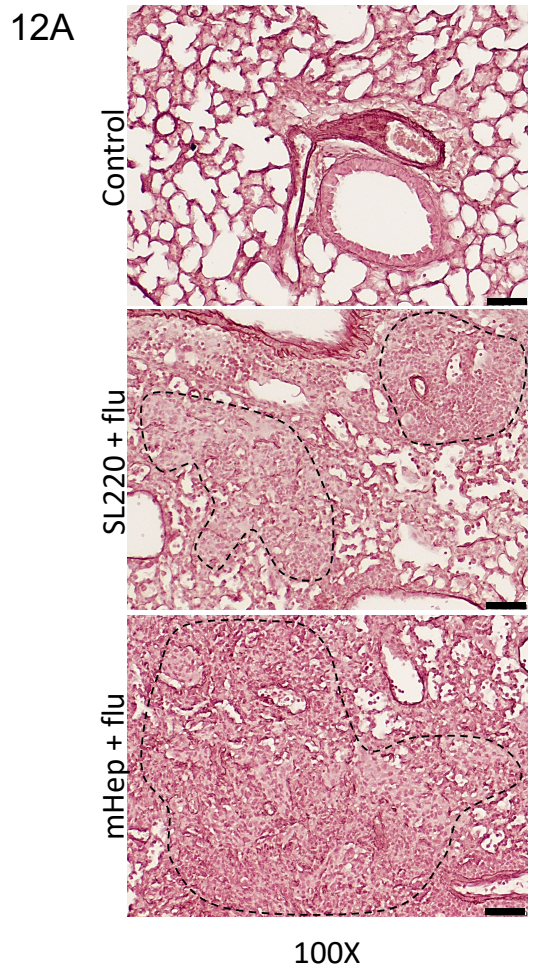


Figure 7.12. Representative lung sections stained with Orcein stain from day 10 post infection. 100X, scale bar 50µm.

7.2.5 Increased pro-inflammatory cytokine production in infected lungs of serum-iron deficient animals

We also measured the mRNA expression of a panel of inflammatory genes was measured in the lungs of infected mice. Expression of IL-6 and TNF-α mRNAs was increased in the lungs of mini-hepcidin treated animals on day 8 post infection (13A

and 13B), but not day 10 post infection (13E and 13F). IL-6 and TNF-α are pro-inflammatory cytokines which can be highly produced by cells of the innate immune system and over production of IL-6 and TNF-α has been linked to the pathologies of severe pandemic influenza infection (de Jong et al., 2006; Kobasa et al., 2007; Perrone et al., 2008). The differences in pro-inflammatory cytokine expression may have disappeared by day 10 if the wave of inflammation where IL-6 was being produced had resolved. Furthermore, the increased production of pro-inflammatory innate immune cytokines in whole lung lobe RNA is consistent with the enhanced innate immune inflammatory infiltrate observed by histological analysis.

In contrast no difference in whole lung mRNA expression of Granzyme B or IFN-γ was seen between vehicle and mini-hepcidin treated groups. This is not unsurprising as one may expect that at these late points the level of Granzyme B and IFN-γ mRNA in the lung might in part reflect the frequency of antigen specific CD8 T cells which by flow cytometry was reduced in the lung, as well as early infiltrating innate cells (such as NK cells) which may not have been reduced in frequency by mini-hepcidin treatment (13C, 13D, 13G and 13H).

On day 10, *Il10* mRNA production in the lung was also increased (13I). Whilst IL-10 is canonically anti-inflammatory, increased production may reflect feedback induction by increased lung inflammation. Enhanced IL-10 levels have been noted in the serum of individuals with more severe influenza infection (Yu et al., 2011).

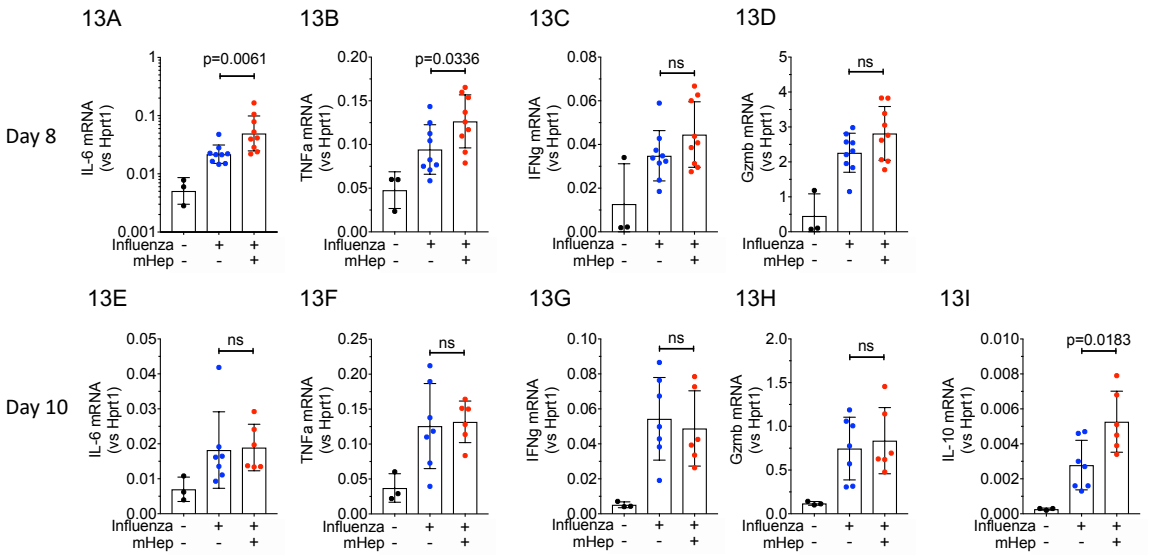


Figure 7.13. Inflammatory gene expression in whole lung lobe homogenate was measured by qRT-PCR using taqman probes against IL-6 (13A), TNFα (13B), IFNγ (13C) and Granzyme B (13D) on day 8 post infection. Similar analysis for IL-6 (13E), TNFα (13F), IFNγ (13G), Granzyme B (13H) and IL-10 (13I) was carried out on day 10 post infection. Data presented as [2^(CT of Hprt- CT of gene of interest)]. T-test.

7.2.6 Altered iron homeostasis and evidence of increased inflammation in the livers of serum-iron deficient animals infected with influenza virus

mRNA expression of key inflammatory and iron homeostasis genes was also analysed in small liver explants. Whilst IL-6 expression was not altered by mini-hepcidin treatment in the liver on day 8 (14A), there was statistically significantly higher *Il6* expression in the liver of mini-hepcidin treated infected animals on day 10 after infection (14F), consistent with persistent inflammation. The mRNA expression of acute phase protein SAA1 was however increased in serum-iron deficient animals on day 8 after infection (14B), with a possible trend towards increased expression on day

10 (14G). IL-6 is a major driver of acute phase gene expression and it may be that increased lung inflammation and IL6 production (6.2.5) is driving enhanced systemic inflammation reflected by increased liver *Saa1*.

We (and others) have previously shown that mini-hepcidin injection and serum-iron deficiency suppresses hepatic hepcidin and BMP target gene expression (such as ID1 (Kautz et al., 2008)), likely through reduced SMAD signalling induced by the serum iron sensing HFE/TFR2 pathway (Core et al., 2014; Gao et al., 2009; Schmidt et al., 2008b). Consistent with this influenza infected mini-hepcidin treated animals have significantly reduced *Hamp1* (14C and 14H) and *Id1* mRNA expression (14D and 14I). *Bmp6* mRNA expression is not altered by mini-hepcidin treatment (14F and 14J), likely

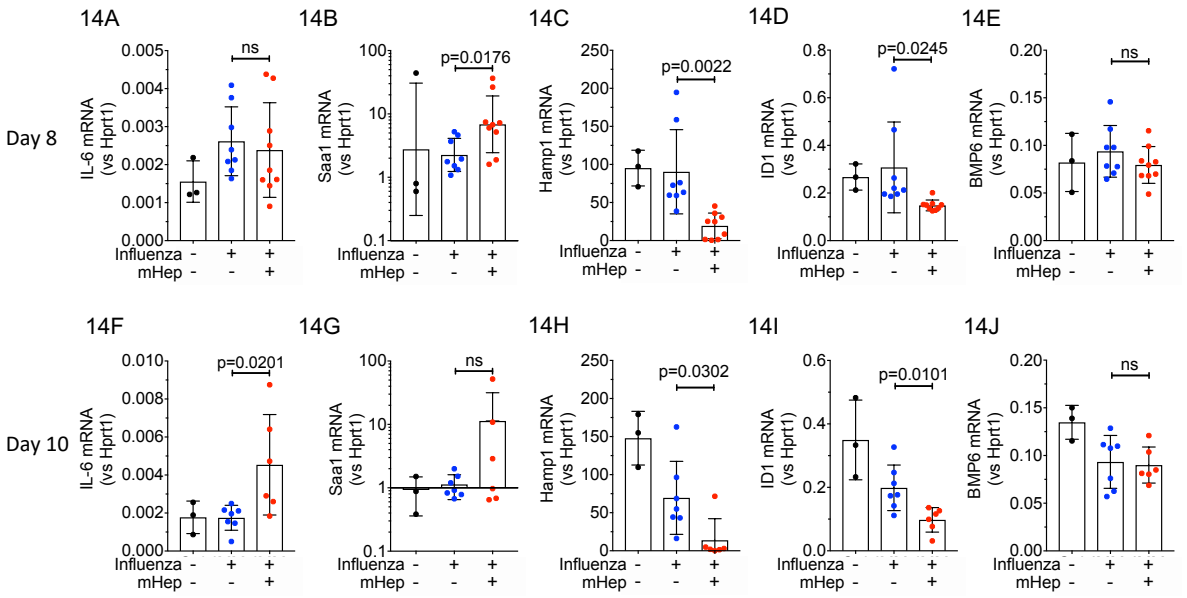


Figure 7.14. Gene expression in liver explants was measured by qRT-PCR using taqman probes against IL-6 (14A), Saa1 (14B), Hamp1 (14C), ID1 (14D) and BMP6 (14E) on day 8 post infection. Similar analysis for IL-6 (14F), Saa1 (14G), Hamp1 (14H), ID1 (14I) and BMP6 was carried out on day 10 post infection. Data presented as [2^(CT of Hprt- CT of gene of interest)]. T-test.

as iron stores are thought not to be altered by short-term treatment with mini-hepcidin.

7.3 Discussion

In previous chapters we have demonstrated that serum-iron deficiency mediated by mini-hepcidin injection strongly inhibits adaptive immune responses to protein-in-adjuvant immunisations and attenuated, replication deficient, viral vectors. Given on one hand the dogma that inflammatory hypoferremia and iron deficiency are protective against infection with some pathogens (Arezes et al., 2015; Drakesmith and Prentice, 2012; Gwamaka et al., 2012), but also the paucity of information regarding how systemic iron homeostasis influences the outcome of viral infections (Drakesmith and Prentice, 2008) and the possibility that iron deficiency might impair adaptive immune function, we utilised a non-lethal mouse model of influenza infection where adaptive immunity was important for viral control, to investigate how serum-iron deficiency influenced the outcome of a concurrent viral infection. Animals which lack B cells or CD4 and CD8 T cells fail to clear influenza infection and exhibit increased weight loss/impaired recovery of weight (Rangel-Moreno et al., 2008; Topham et al., 1997).

We imposed serum iron deficiency from day 3 of infection by injecting mini-hepcidins. Consistent with serum-iron deficiency inhibiting B and T-cell responses to immunisations, we found that the magnitude of CD8 T-cell, CD4 T-cell (Tfh) and

germinal centre B cell responses in the lung, spleen and mLN were significantly inhibited by mini-hepcidin injection. Raised CD71 expression by effector lymphocytes in mini-hepcidin treated mice is consistent with cellular iron deficiency. Not only did we find that cellular B-cell immunity was impaired, but on day 10 (but not day 8) the influenza neutralising antibody titre was reduced by mini-hepcidin treatment.

Consistent with observations in mice which lack B cells or CD4 and CD8 T cells (Rangel-Moreno et al., 2008; Topham et al., 1997), in mini-hepcidin treated mice, which exhibited a broad impairment of adaptive immunity to influenza virus, we observed a defect in weight recovery after the nadir on day 7/8 and increased detectable viral RNA in lung. One criticism of our experimental model is that measurement of viral RNA does not differentiate between infectious and non-infectious virus. The gold standard approach to demonstrate impaired viral control would be a plaque assay on lung homogenate or a 'tissue culture infectious dose 50' ($TCID_{50}$) assay (dilution of virus which results in 50% of wells being infected). In contrast WT mice are back to their starting weight by day 9 post infection, weight recovery on day 7 is concurrent with induction of adaptive immunity (Pommerenke et al., 2012). Defective immune control of viral replication and increased severity of infection is also a hallmark of severe influenza infection with pandemic virus strains (de Jong et al., 2006; Kobasa et al., 2007; Perrone et al., 2008). Furthermore, the increased viral RNA loads of mini-hepcidin treated animals argues against the suppressed adaptive immune response being driven by reduced antigen availability (Kaech and Ahmed, 2001).

Infection with high pathogenicity viruses results in high viral replication in lung and an exacerbated innate immune infiltrate which fails to clear the virus and ultimately results in immunopathology in the lungs (de Jong et al., 2006; Meunier et al., 2015). We decided to investigate the lung pathology of mini-hepcidin treated animals and found significantly more extensive inflammatory infiltrate in the lungs of mini-hepcidin treated animals on day 10 of infection, in particular infiltration into the alveolar space and presence of polymorphonuclear cells which was absent in vehicle treated animals. Neutrophils have a complex role in the immunopathology of influenza infection; their depletion from the beginning of infection can enhance the severity of infection (Tate et al., 2009). However, disruption of the IL-17 signalling axis which impairs neutrophil recruitment during infection improves the outcome of infection (Crowe et al., 2009). In addition, reduced lung pathology and improved weight loss are observed after infection of mice with BCL6 deficient neutrophils, which show reduced survival/recruitment to the lung, and also when neutrophils are depleted with antibodies later in the course of infection (Zhu et al., 2019).

Increased innate inflammation in mice with serum-iron deficiency was supported by analysis of lung inflammatory gene expression where on day 8, increased expression of innate immune cytokines IL-6 and TNF-α was noted. This likely has a systemic effect as mRNA expression of acute phase protein SAA1 was also increased in the livers of mini-hepcidin treated animals on day 8. Whilst increased viral load might contribute to enhanced innate inflammation in the lung, it has been shown that the adaptive immune response to influenza has a key role in control and resolution of infection

through production of IL-10 (Sun et al., 2009), however on day 10 of infection increased expression of *Il10* mRNA was detected in the lungs of mini-hepcidin treated animals. The contribution of impaired adaptive immunity in serum iron deficient animals to defective viral control vs a failure to directly control the innate immune response remains to be addressed.

Our study does not determine whether it is the reduced B cell, CD4 T-cell or CD8 T-cell response that results in the observed phenotype. It is obvious how reduced titres of neutralising antibodies on day 10 may impair clearance, however in our day 8 experiment we still observed differences in viral load and weight loss between control and serum-iron-deficient mice despite no difference in neutralising antibody titres. We observe a quantitative defect in CD8 T cells and further investigations should be undertaken to examine if effector differentiation (cytokine production and transcription factor expression) during influenza infection is perturbed by iron restriction similarly to what we observe in the context of immunisation. Appropriate BLIMP1 expression by CD8 T cells has been noted to be required for immunity to influenza infection (Kallies et al., 2009). In addition, we did not measure the anti-viral Th1 biased CD4 response generated by influenza infection, this could be easily done by staining intracellular cytokines (IFNγ, TNFα and IL-2) in CD4 T cells after restimulating bulk spleen or lung lymphocytes with influenza pulsed dendritic cells, similar to the approach we took to look at the MVA-OVA induced CD4 response in the previous chapter.

Furthermore, it would be of interest to explore how the metabolic dysfunction we observe in effector CD8 T cells subjected to iron deficiency *in vitro* contributes to impaired immunity *in vivo*. Enhancing the metabolic fitness of CD8 T cells through deficiency in MCJ, an endogenous negative regulator of mitochondrial metabolism, improves cytokine secretion and the quality of CD8 T cell specific memory to influenza virus (Champagne et al., 2016). It will be interesting to explore how subtly inhibiting mitochondrial function of CD8 T cells through iron restriction alters immunity.

The data presented in this chapter therefore presents a model where serum-deficiency during an otherwise non-lethal influenza virus infection impairs the development of virus-specific cellular and humoral adaptive immunity. Impaired adaptive immunity likely contributes to impaired clearance of virus, which in turn drives persistent and more severe innate inflammation in the lung and systemically. A more severe inflammatory response to viral infection results in lung pathology and a failure to resolve and recover weight after infection. However, a direct effect of hepcidin and serum iron deficiency on the innate immune response is also possible. A detailed understanding of the interaction between iron availability, iron handling and immunophenotype of macrophages and other innate immune cells *in vivo* is lacking (Pereira et al., 2019). At steady state alveolar macrophages seem to heterogeneously express ferroportin and respond to changes in hepcidin (Neves et al., 2017) and no change in lung Perls' staining was observed in mini-hepcidin treated infected mice relative to infected controls (data not shown). However, alveolar macrophages have been observed to highly express CD71 and therefore may be sensitive to iron

availability (Allden et al., 2019). Recent results suggest that specific TLR stimulation of macrophages or monocytes can drive the development of hemophagocytic macrophages, which have similarities to red pulp macrophages, in inflammatory situations (Akilesh et al., 2019; Wang et al., 2019); the importance of iron homeostasis for the phenotype of these inflammatory hemophagocytic macrophages and what role they might play in a wider range of infections remains unclear however.

The complex micro-environment and numerous inflammatory myeloid cells in the influenza infected lung also more generally poses a point about iron availability in different tissue niches. During influenza infection how perturbed iron homeostasis relatively effects iron availability in the lung, lymph nodes and spleen, and how local cell populations contribute to available iron levels is unclear. Lymphocytes from all three tissues present with increased CD71 expression in mini-hepcidin treated animals compared to vehicle treated controls and CD8 T cell proliferation takes place not only in the spleen and MLN, but also in the lung (Lawrence et al., 2005).

If future experiments using the flu model were conducted it would be desirable to investigate if iron supplementation rescued the immunological, weight loss and lung pathology phenotype of mini-hepcidin treated animals. It would also be of interest to examine the influence iron supplementation alone or blockade of hepcidin on the course of influenza infection, perhaps the adaptive immune response could be enhanced by increased iron availability. Finally, it will be interesting to examine how

serum iron deficiency alters the trajectory of other viral infections which replicate in other tissue niches.

Discussion

8.1 Summary

The observation that hypomorphic mutations in the transferrin receptor lead to a profound immunodeficiency phenotype in affected patients (Jabara et al., 2016), combined with the global prevalence of iron deficiency (Lopez et al., 2016b) makes an understanding of how and to what extent iron restriction impairs the function of lymphocytes, and potentially disrupt vaccine efficacy, critically important. Historically studies in both humans and animal models have focussed on baseline immunological parameters and failed to examine antigen specific immune responses in any depth (Oppenheimer, 2001; Scrimshaw and SanGiovanni, 1997). The handful of studies examining humoral responses to vaccines in iron deficient individuals have been underpowered and confounded by the association of socio-economic status, inflammation and other nutritional deficiencies with iron deficiency.

We began in chapter three by confirming that genetic disruption of iron uptake impaired adaptive immune function. By exploiting the knock-in mouse model with the same *Tfrc*$^{Y20H/Y20H}$ substitution as the immuno-compromised patients we confirmed that impairing iron uptake resulted in a cell intrinsic defect in T and B-cell responses to immunization *in vivo*, without any obvious defect in haematopoiesis. We then demonstrated that in standard (iron sufficient) culture conditions the IRP1/2 system, the core regulator of cellular iron homeostasis, is essential for CD8 T cell proliferation and that this is likely due to a defect in iron uptake as proliferation can be rescued by supplying extra iron.

We next moved from disrupting iron acquisition by genetic means, to impairing acquisition of iron by a reduction in the availability of extracellular iron. In chapter four we used an *in vitro* model of decreased transferrin saturation to demonstrate that physiological iron restriction impairs proliferation, effector differentiation, mitochondrial metabolism and mTOR signalling in CD8 T cells. Furthermore, we recapitulated similar phenotypes in CD8 T cells from *Tfrc*$^{Y20H/Y20H}$ mice *in vitro*. These results provide the basis for thinking that iron restriction negatively impacts CD8 T cell metabolism and that we should consider iron as an additional 'immuno-metabolite'.

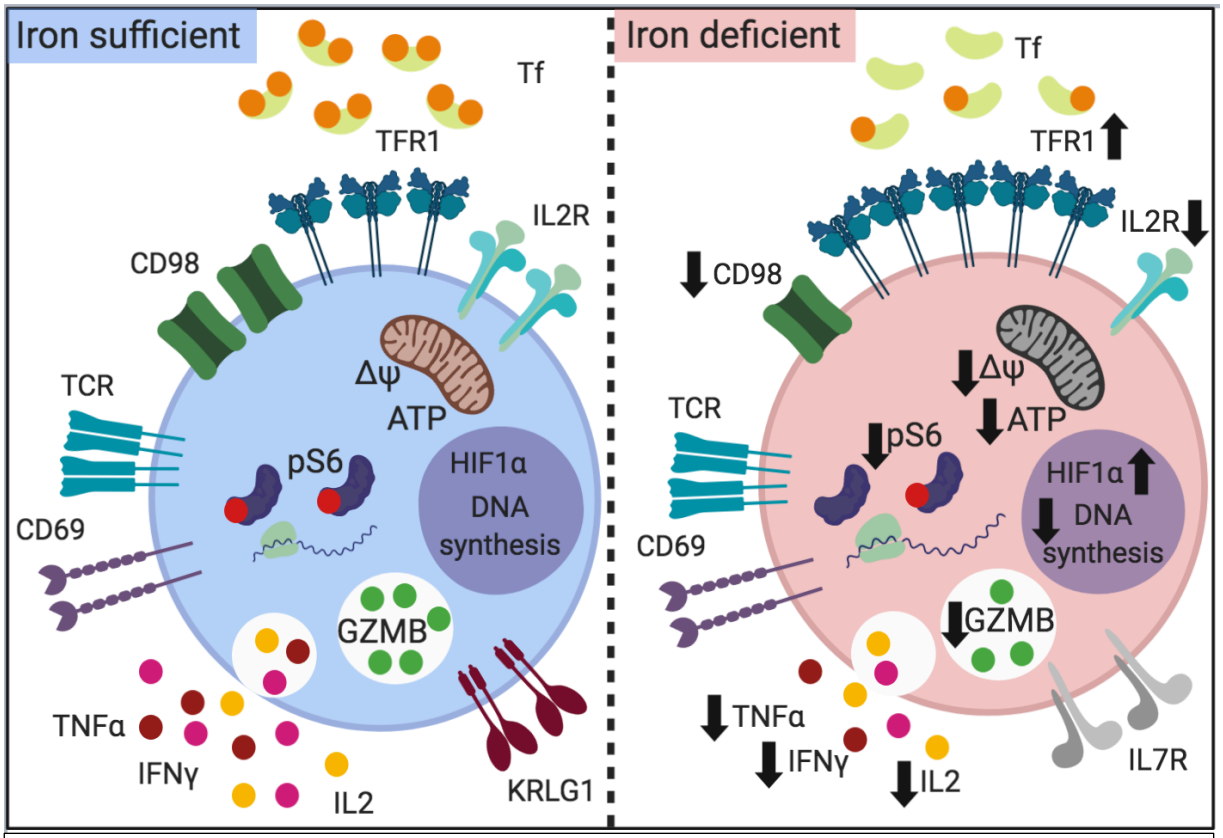
The key question however is whether serum iron deficiency can perturb the antigen specific lymphocyte response to immunisation *in vivo* and we attempted to answer this in chapter five. We found that low iron diet alone was suboptimal for inducing serum iron deficiency, so instead we opted to use mini-hepcidin injection to generate an acute transient hypoferremia during the primary immune response. Across a number of immunisation formats we observed that serum iron deficiency inhibited the magnitude of CD8, CD4 and B cell responses, accompanied by raised CD71 levels on responding lymphocytes indicative of cellular iron deficiency. We also confirmed that hepcidin injection inhibited CD8 T cell responses through reduced serum iron availability. More detailed phenotyping resolved a defect in the differentiation of CD8 T cells towards fully functional effector cells and reduction in *ex vivo* secretion of effector cytokines on restimulation. Finally, in an initial exploration of how increasing iron availability - via Cre-mediated excision of a floxed hepcidin gene - alters CD8 T cell

responses to immunisation, we found that CD8 T cell effector potential may be raised slightly by increasing iron availability. Repeating this experiment and inducibly disrupting hepcidin activity in the context of background iron deficiency might accentuate this phenotype, providing a useful model for exploring how iron supplementation might improve immunity.

In chapter six we extended our analysis to examine how serum iron deficiency during the primary adaptive immune responses influenced the quality of CD8 T cell memory and secondary immunity. For protein-in-adjuvant immunisation we observed that serum iron deficiency during the primary immune response significantly reduced the number of central memory CD8 T cells and the number of memory CD8 T cells competent for cytokine production on restimulation *ex vivo*. We also found that iron restriction during primary immunisation impaired the magnitude of the CD8 recall response upon secondary immunisation. Whilst we did not find evidence that iron restriction during primary immune responses was sufficient to significantly impair dendritic cell-based vaccination against vaccinia virus, we did find significantly fewer circulating memory cells prior to challenge. Further experiments are required to explore how iron restriction impacts on CD4 and B cell memory, and how iron restriction might disrupt vaccination induced protection against infectious challenge.

Finally, in chapter 7 we explored how serum iron deficiency influences the outcome of primary influenza virus infection. We observed that iron restriction reduced the magnitude of the influenza induced adaptive immune response, including the number

of NP-specific CD8 T-cells, which may have contributed to the detected increased levels of viral NP RNA in the lung. Increased persistence of virus may be behind the relative enhanced or unresolved innate inflammation in the lung of serum iron deficient animals, with increased cytokine production, neutrophil infiltration and larger areas of inflammation and tissue damage. Infected animals with serum iron deficiency, unlike control infected animals, also failed to recover body weight from day 8 of infection, correlating with the more severe lung pathology of mini-hepcidin treated animals. In chapter 7 we therefore provided evidence that serum iron deficiency can impair adaptive immunity and increase the severity of a viral infection where adaptive immunity is essential for its resolution.



Schematic summarising the phenotype of iron deficient T cells *in vitro* and *in vivo*. Tf, transferrin. TFR1, transferrin receptor 1. IL2R, IL-2 receptor. CD98, Slc3a2 (forms LAT1 in complex with SLC7a5). TCR, T cell receptor. GZMB, granzyme B. TNFα, tumour necrosis factor α. IFNγ, Interferon gamma. IL-2, interleukin 2. pS6, phosphorylated ribosomal protein S6 (mTORC1 target). HIF1α, hypoxia inducible factor α. IL7R, IL-7 receptor. KRLG1, Killer cell lectin-like receptor subfamily G member 1. ATP, adenosine triphosphate. Δψ , mitochondrial membrane potential. Figure generated on Biorender.

8.2 Future perspectives

8.2.1 Exploring the phenotype of iron deficient T cells

Our work hints at how a myriad of regulators of cell physiology could perhaps interact both transcriptionally and post-transcriptionally to generate the phenotype of iron restricted lymphocytes.

Increased binding of IREs by IRP1 and IRP2 post-transcriptionally facilitates cellular adaptation to iron deficiency, but our appreciation of cell type specific targets of IRP1/2 regulation beyond the core regulators of iron homeostasis is in its infancy (Sanchez et al., 2011) and it would be interesting to extend work defining IRP1/2 targets to lymphocytes. Further phenotyping of the metabolism and effector differentiation of IRP1/2 single and double KO cells *in vitro* and *in vivo* will be important. In addition to investigating the role of the IRPs in the adaptation to cellular iron deficiency, it will interesting to explore the extent to which the phenotype of iron deficient T cells is recapitulated by lymphocytes where IRP2 is hyperactive due to FBXL5 deficiency (Muto et al., 2017) or where a mutation in IRP1 increases IRE binding activity (Casarrubea et al., 2013),

PCBP1 and PCBP2 are RNA binding proteins that have also been proposed to regulate the intracellular trafficking of iron (Muckenthaler et al., 2017). PCBP2 has been proposed to generally enhance miRNA processing and activity during iron deficiency, modulating gene expression (Li et al., 2012). Whilst PCBP1 has been proposed to regulate inflammatory cytokine mRNA stability in CD4 T cells (Wang et al., 2018). We

have not considered the roles of PCBP1/2 in driving the phenotype of lymphocytes responding to serum iron deficiency in this thesis, it will be an important future line of inquiry. We could investigate how T cells deficient in PCBP1 or 2 respond to iron deficiency *in vitro* and *in vivo* and how this alters their proliferation, effector differentiation and cytokine production.

Iron deficiency would be expected to deactivate PHD proteins and lead to HIF stabalisation (Schofield and Ratcliffe, 2004). Further work is needed to explore the role of HIFs in the response of lymphocytes to iron deficiency vs hypoxia as we see evidence for an increase in HIF1α within iron restricted lymphocytes *in vitro*. The phenotyping of PHD deficient cells is informative (Doedens et al., 2013), but this reflects HIF1α over activation in the context of sufficient nutrients which is not the circumstance *in vivo*. It would be desirable to compare how WT and HIF1α, HIF2α or dKO T cells respectively respond to iron deficiency to probe the importance of this regulatory network to iron restricted immunity.

One of the most intriguing *in vitro* results was the inhibition of mTOR signalling in CD8 T cells activated *in vitro* in low iron conditions. Many of the phenotypes we see *in vivo*, including impaired effector differentiation, but the enhancement of some aspects of memory differentiation, are reminiscent of impaired mTOR signalling (Araki et al., 2009; Pollizzi et al., 2016, 2015) . As noted in chapter four it will be interesting to explore how cells with hyperactive mTOR signalling cope with serum iron deficiency *in vivo*. One could adoptively transfer a mix of WT and TSC complex subunit 1 or 2 KO

OT-Is, immunise and see how the phenotype and recovery of the two genotypes is different in the presence and absence of iron deficiency. One might hypothesise that cells with overactive mTOR signalling would fail to adapt their metabolism and proliferative activity to iron deficiency and show a greater survival defect during iron deficiency than wild type cells.

Furthermore, it is clear we need to explore in more depth the extent of the metabolic defects of iron-restricted lymphocytes and the functional consequences of this for adaptive immunity. For example, *in vitro* in chapter four we presented data supporting iron restriction disrupting mitochondrial health and oxidative phosphorylation. One of the most profound *in vivo* phenotypes we observe is a reproducible defect in IL-2 production on restimulation *ex vivo* in iron replete conditions. This could reflect a generalised differentiation defect, perhaps specific regulation of IL-2 production by iron (Wang et al., 2018) or the impaired mitochondrial health of CD8 T cells developing in the context of serum iron deficiency (Sena et al., 2013). It will be important in the future to explore the metabolic health of T cells derived from immunised animals directly *ex vivo*. Further links between iron and IL-2 signalling are suggested from our *in vitro* work: we observed reduced CD25 and Gzmb expression *in vitro* consistent with reduced IL-2 signalling (Pipkin et al., 2010), which is mimicked by the disrupted differentiation towards cells with an effector phenotype competent for the secretion of cytokines *in vivo*. It will also be interesting to further characterise the interaction between iron, metabolism and IL-2 production in CD4 T cells *in vitro* and *in vivo*, in

particularly because IL-2 production by CD4 T cells facilitates CD8 T cell responses and memory formation (Obar et al., 2010).

We have only scratched the surface of delineating how iron restriction, a common physiological phenomenon, alters the outcome of adaptive immune responses. Our superficial phenotyping hints at a number of paths to pursue, some of which are described above. Specifically following these leads in combination with hypothesis-free transcriptomic, metabolomic and proteomic approaches in CD8 T cells, but also CD4 T cells and B cells, will be important to fully characterise the role iron plays in lymphocyte function.

8.2.2 How does serum iron deficiency perturb immunity beyond T cells?

Many of the future directions for investigating CD8 T cell responses could be taken with the humoral immune response. It would be desirable to integrate *in vitro* and *in vivo* models to explore how iron deficiency alters humoral immunity through changes in B cell and CD4 T cell mTOR, HIF1α and mitochondrial metabolism (Cho et al., 2016; Ersching et al., 2017; Zeng et al., 2016). It would also be interesting to explore the haem/BACH2/B cell differentiation axis further in the context of restricted iron uptake (Jang et al., 2015; Tsui et al., 2018).

Whilst our experiments using the *TfrcY20H/Y20H* model demonstrate that iron restriction can cell intrinsically impair adaptive immunity, in our mini-hepcidin serum

iron deficiency model all immune cells are experiencing iron restriction, as is physiologically relevant. Therefore, in the future we would like to move away from a targeted approach where we just consider a handful of cell types, to a more unbiased approach, utilising techniques such as CyTOF and multiplex methods for measuring cytokines, where we can consider how iron deficiency alters multiple cell types and signals to influence the total profile of developing adaptive immunity throughout the response.

8.2.3 Innate hypoferremia vs adaptive immunity: translational perspective

A conceptual point to emerge from this thesis is the seeming conflict between the idea that iron deficiency can be both protective and unhelpful in the context of immunity. The hepcidin mediated hypoferremia of inflammation is a key evolutionarily conserved hallmark of the innate immune response and provides protection against some infections (Arezes et al., 2015). Furthermore iron supplementation, i.e. amelioration of iron deficiency, has been demonstrated to increase the risk of severe malaria, respiratory and diarrhoeal disease (Gera and Sachdev, 2002; Sazawal et al., 2006; Soofi et al., 2013). Oral iron supplementation also subsequently enhances the capacity of human serum to support bacterial growth *ex vivo* (Cross et al., 2015). However, our data supports the idea that lymphocyte responses are sensitive to serum iron availability and that hypoferremia can impair immunity to influenza virus. The idea that an immunological pathway can both help and hinder immunity, depending on the circumstance and pathogen is not new, Type I interferons for example are integral to resistance of viral infections, but can antagonise immunity to

bacteria (Auerbuch et al., 2004; Iwasaki and Pillai, 2014). However, the idea that a single nutrient can positively and negatively regulate immunity is an exciting concept to pursue in the future. One future avenue to explore might be that hypoferremia is a physiologically helpful response to bacterial, but not viral infections and how innate signals from different pathogens are integrated to generate an appropriate hypoferraemic response.

The first point for consideration is the temporal separation of hypoferremia and the adaptive immune response. Controlled challenge with typhoid in healthy western adults results in hepcidin induction and a drop in serum iron, with a nadir on day 2 post diagnosis and serum iron remaining low on day 4 post diagnosis (Darton et al., 2015). The average time to diagnosis in this study was approximately 7 days. Hypoferremia took place approximately day 9-11 days post challenge. Parallel studies by the same group suggest however that the typhi specific CD8 T cell response does not peak until 28 days after challenge, with no significant increase on day 14 post challenge (Gibani et al., 2019). Whilst the resolution of the expanding CD8 T cell response is limited, and the CD8 T cell response may initiate during the hypoferraemic phase, this at least hints towards a temporal separation between innate hypoferremia and induction of adaptive immunity in a controlled infection in man. One caveat of these data however is that patients are treated with antibiotics to resolve infection on diagnosis and therefore perhaps in the context of untreated infection and persistent bacteraemia hypoferremia is longer lasting and may overlap with adaptive immunity. In experimental human norovirus challenge models serum iron levels fall below

10µmol/L between day 2 and 4; serum iron levels were normalised on day 7, but no data in this study exists for days 5-6 (Williams et al., 2019). A bolus of LPS in humans generates a hypoferremia response which is still declining at 22 hours (Nemeth et al., 2004a).

During relatively mild experimental infection of mice with influenza virus hepcidin induction and mild hypoferremia has been observed (Armitage et al., 2011). It has yet to published however if the severe, dysregulated innate inflammatory response observed during infection with high pathogenicity viruses in man (de Jong et al., 2006) results in severe and persistent hypoferremia. One interesting angle to pursue in the future is the extent to which the inappropriate innate inflammation and hypoferremia during severe influenza (and other viral infections such as Ebola (Baize et al., 1999)) contributes to the disrupted development of adaptive immunity, failure to clear the virus and pathology.

Whilst it is possible for any given infectious challenge innate hypoferremia will be temporally distinct from the primary adaptive immune response in many circumstances, it is important to consider that on rechallenge the anamnestic adaptive immune response will be faster, increasing the likelihood that innate hypoferremia and adaptive immunity may overlap. Hypoferremia is not the only change in systemic metabolism that is driven by innate inflammatory responses. Infection induced release of acetate into the blood stream has been proposed to facilitate the immediate response of memory CD8 T cells to infection (Balmer et al., 2016) providing

a precedent for innate inflammatory changes in physiology interfacing with secondary adaptive immune responses. It would be interesting to explore to what extent the capacity of memory CD8 T cells to produce cytokines, remodel metabolism and rapidly divide is modulated by the innate hepcidin response; secondary challenge in the context of inducible hepcidin KO using the iHampKO mouse (Armitage et al., 2016b) could provide one way to model this.

Furthermore, it is unclear to what extent lymphocytes themselves and adaptive immunity in general regulate hepcidin and serum iron concentration during infection. Whilst inflammatory hypoferremia is thought of as an innate immune process, memory T cells (in particular) can modulate and amplify innate immune responses (Strutt et al., 2011, 2010) and it is unknown whether on secondary challenge with an infectious agent the hypoferraemic response is altered in magnitude. It is well established that after blood loss or in conditions of erythropoietic stress, systemic iron metabolism is remodelled with suppression of hepcidin facilitating iron availability to fuel increased erythropoiesis (Kautz et al., 2014). Whether adaptive immune cells actively modulate hepcidin expression to turn off the inflammatory hypoferraemic response and facilitate iron availability remains unknown. T cells might directly produce or indirectly favour the production of erythroferrone. Alternatively other cytokines produced during the adaptive immune response, such a TNFα could suppress hepcidin; there is some evidence for TNFα mediated hepcidin suppression in mouse models of colitis (Nemeth et al., 2004a; Shanmugam et al., 2012).

It has been noted that in conditions of massive T cell activation, such as during checkpoint blockade therapy, a depletion of aromatic amino acids from the serum is observed (Miyajima et al., 2017). Whilst induction of stress erythropoiesis transiently depletes serum iron and contributes to hepcidin suppression in this context (Artuso et al., 2019; Mirciov et al., 2018), whether lymphocyte activation can directly deplete serum iron is also unknown. Furthermore naïve T cells, particularly CD4 T cells, have been proposed to take up NTBI in iron loading situations and help to buffer the parenchyma from iron overloading (Pinto et al., 2014).

However as discussed, prevalent iron deficiency in children reflects in part chronic inflammation (Prentice et al., 2019), and therefore in this circumstance, which is likely both common now and has been over much of human history, there is potentially a much smaller kinetic separation between adaptive immunity and inflammatory hypoferremia. Furthermore, prevalent iron-deficiency is observed in the elderly (Busti et al., 2014) and cancer patients (Ludwig et al., 2013) and in both cases inflammatory increases in hepcidin expression likely play an important contributing role. Whether iron restriction impairs adaptive immunity, resulting in unresolved infection and continued defects in iron absorption in these models is unclear, but it clearly indicates how a twin approach targeting both the immune response and iron may be required in these pathologies (Pasricha et al., 2018a).

Hepcidin is not the only acute phase protein which can modulate systemic iron homeostasis, hemopexin and haptoglobin respectively bind free haem and

haemoglobin respectively. This sequestration of serum haem iron can mediate nutritional immunity against *Citrobacter rodentium* and *Escherichia coli* infection (Sakamoto et al., 2017) and protect against tissue damage during haemolysis (Smith and McCulloh, 2015). Haptoglobin and hemopexin deficient mice have been proposed to show both reduced and enhanced adaptive immune responses *in vivo* (Fagoonee et al., 2008; Huntoon et al., 2008; Rolla et al., 2013). The extent to which haem can provide an iron source for lymphocyte responses and whether sequestration of haem iron by haptoglobin or hemopexin is immuno-modulatory requires further investigation.

The trade-off between iron restriction as an innate protection against a subset of infections, but also as a limiting factor for the development of good quality adaptive immunity to vaccination poses important questions for the therapeutic implementation of iron supplementation (Armitage and Moretti, 2019; Pasricha et al., 2018b). Good sanitation, infection control and perhaps even limiting iron supplementation to the time of vaccination to minimise infection-enhancing 'off-target effects' could all play a part in improving iron limited vaccine responses in low- and middle-income countries.

It would be desirable to extend investigations of immunity in the context of hepcidin inhibition *in vivo* to address if: T cell memory or cytokine production is altered, the effect of iron overload in humoral immunity and whether pharmacological blockade of hepcidin, anti-BMP antibodies for example, can be exploited to experimentally

enhance adaptive immunity to vaccinations. Whilst the effect of *HFE* mutations on baseline CD8:CD4 ratios and lymphocyte activation phenotypes has been investigated extensively (Costa et al., 2015; Porto and De Sousa, 2007; Reuben et al., 2014), it is not well understood how *HFE* mutations alter the quality of adaptive immunity *in vivo*. It has been proposed that individuals heterozygous for the hypomorphic *HFE* allele C282Y show higher transferrin saturations, serum iron concentrations and haemoglobins (Beutler et al., 2003). What has not been explored however is if the C282Y *HFE* allele alters systemic iron homeostasis such to improve iron availability for adaptive immune responses, particularly in the context of prevalent iron deficiency which was likely the norm for most of human history. If funding was available it would be interesting to measure antibody titres to routine vaccinations in a large cohort of WT, C282Y heterozygous and C282Y homozygotes (yet to present with hemochromatosis, to avoid the confounding effects of severe iron overload and phlebotomy).

In context of improving vaccine efficacy and immunity to some infectious diseases it would be desirable to iron supplement or modulate hepcidin to improve iron supply to lymphocytes. However, the dependence of lymphocyte effector responses on iron supply also poses the question of could iron restriction, potentially through hepcidin injection, be a novel therapeutic approach for suppressing aberrant adaptive immune responses in the context of auto-immunity. It has been shown that iron restriction (genetic or dietary) can suppress and iron overload can enhance the development of EAE in mice (Mitchell et al., 2007; Wang et al., 2018). Furthermore, disruption of BH_4

synthesis, which has been proposed to interfere with lymphocyte iron homeostasis, reduced the severity of both experimental asthma and colitis models (Cronin et al., 2018).

8.2.4 Local regulation of iron availability

Another angle to the regulation of iron homeostasis by lymphocytes and how this links to the dependency of adaptive immunity on iron is the local regulation of iron availability. Lymphocyte responses in lymph nodes involve significant apoptosis and phagocytic clearance of dying cells by resident macrophages (Baratin et al., 2017; Rahman et al., 2010), these cells may have an unappreciated role in local nutrient, specifically iron, recycling. The role of stromal populations, such as the fibroblast reticular network in lymph nodes, regulating nutrient and iron availability is unknown. The concept that local heterogeneity in nutrient availability within lymph nodes can drive the outcome of immune responses is supported by observations showing that gradients of oxygen availability across the germinal centre exist and that HIF signalling in germinal centre B cells has important functional consequences on immunity (Cho et al., 2016). Similarly, as noted in the chapter on flu infection, we have a limited understanding of iron availability and trafficking in non-lymphoid tissues particularly during inflammatory situations. Macrophages have been proposed to be iron donors in the context of cancer in part through the production of LCN2 (a protein which binds endogenous and exogenous siderophores complexed to iron) (Mertens et al., 2018), although whether lymphocytes can exploit LCN2/siderophore iron as a source of iron remains to be shown. Intriguingly *Lcn2*-/- mice have poorer adaptive immune

responses to malaria suggesting LCN2 may play a role in supplying iron during inflammatory situations (Zhao et al., 2012). In inflammatory microenvironments local depletion of transition metals such as iron, zinc and manganese is an important innate immune defence (Cassat and Skaar, 2013; Ciraci et al., 2016); to what extent iron binding proteins regulated by innate immunity, such as lactoferrin, LCN2, haptoglobin, hemopexin and calprotectin, limit lymphocyte access to iron is unclear. Ultimately the lack of effective means of measuring the local availability of most nutrients *in vivo* is a significant limitation for the investigation of these questions (Kedia-Mehta and Finlay, 2019).

A particularly important circumstance to consider local iron availability is cancer; T cells battle for iron in a complex microenvironment of innate immune cells and proliferating cancer cells (Pfeifhofer-Obermair et al., 2018). Experiments have not been carried out to investigate how iron restriction affects the outcome of tumour immunotherapy. It is plausible that tumour cells have a relative advantage in iron acquisition and whilst growth rates are slowed slightly by iron restriction, T cell immunity is massively impaired, and the immunotherapy fails to clear the tumour. This concept of nutritional competition in the tumour microenvironment has been demonstrated for glucose (Chang et al., 2015). Dietary iron restriction seems to limit tumour growth *in vivo* (Hann et al., 1988). In contrast hepcidin seems to promote tumour growth in part through down regulation of cancer ferroportin and iron sequestration (Zhang et al., 2014). How this hepcidin mediated iron sequestration affects other cell types in the tumour microenvironment is unclear, but it hints to a

relative difference in the sensitivity of tumour cells vs immune cells to hepcidin mediated serum iron deficiency. Also of note is prevalent iron deficiency in cancer patients (Ludwig et al., 2013), in part likely driven by raised hepcidin, and it will be interesting to explore how hypoferremia correlates with the response to tumour immunotherapy using available cohorts of patients undergoing immunotherapy for melanoma. Furthermore, one could examine how mini-hepcidin injection or iron supplementation alters the outcome of experimental models of anti-PD1/anti-CTLA4 therapy or therapeutic cancer vaccination. Or alternatively one could examine tumour growth and quality of the anti-tumour T cell response after genetic modification of T cells or tumour cells to relatively enhance their capacity to compete for iron. The end goal of this work would to be understand if therapeutic modulation of iron homeostasis, such as via iron supplementation or by use of inhibitors of BMP6 or hepcidin, might enhance the efficacy of tumour immunotherapy.

Another angle which has not been explored in this thesis due to the predominant focus on a transient serum iron deficiency mode is the most commonly appreciated manifestation of dietary iron-deficiency: anaemia, and what this might mean for adaptive immunity. Whilst hypoxia clearly modulates immunity (Cho et al., 2016), it is unclear if iron deficiency commonly leads to sufficient anaemia to cause disrupted oxygenation of lymphoid organs and inflammatory sites, and to what extent this might alter immune responses. Furthermore at least in the context of chronic kidney disease erythropoietin is emerging as an anti-inflammatory modulator of T cell activity

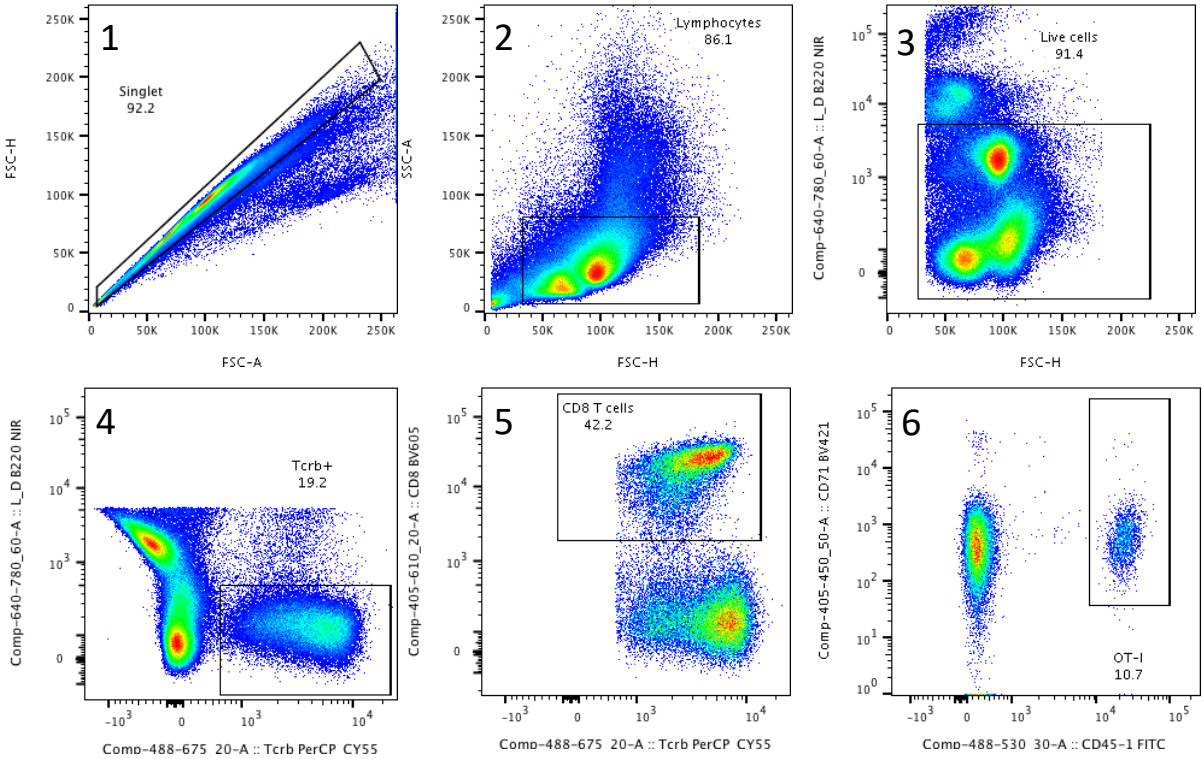
(Cravedi et al., 2014; Purroy et al., 2017), how this relates to adaptive immunity in more generalisable situations remains to be seen.

8.3 Final statement

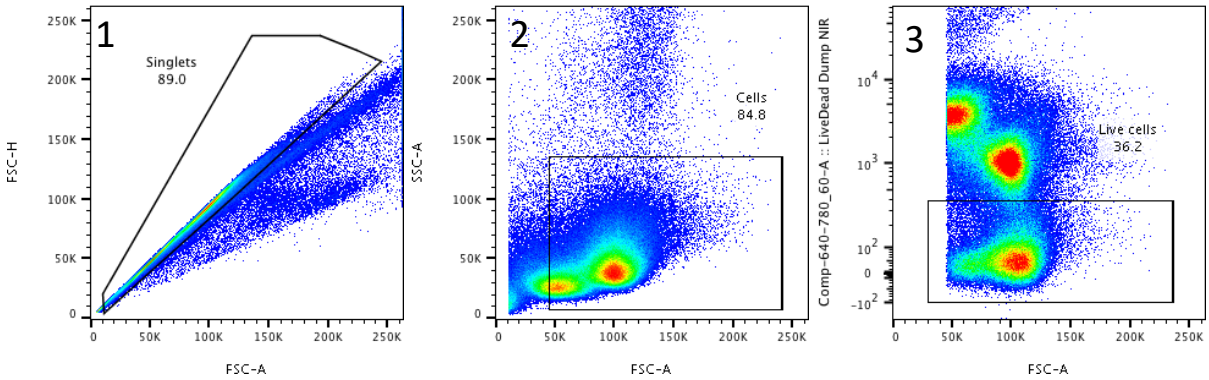
In this thesis we have demonstrated that iron is a critical nutrient for the development of effective adaptive immunity in the context of immunisation and infectious disease. We suggest that part of the requirement of lymphocytes for iron is to support high levels of metabolic activity including production of ATP by mitochondria. Future work will increase the depth of our understanding of how iron deficiency impairs lymphocyte function, more extensive phenotyping of the memory response and include more circumstances of primary and secondary infectious challenges. We are keen to investigate whether serum iron deficiency similarly impairs adaptive immune responses in humans and how targeted iron supplementation might be leveraged to improve vaccine efficacy.

Supplementary Figures: Flow Gating schemes

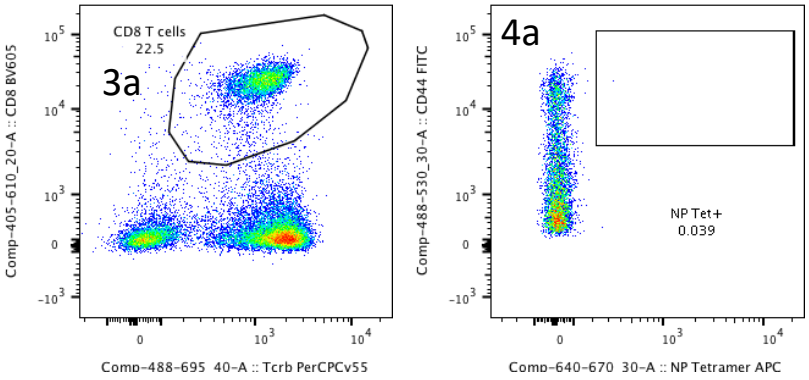
S1. OT-I gating scheme



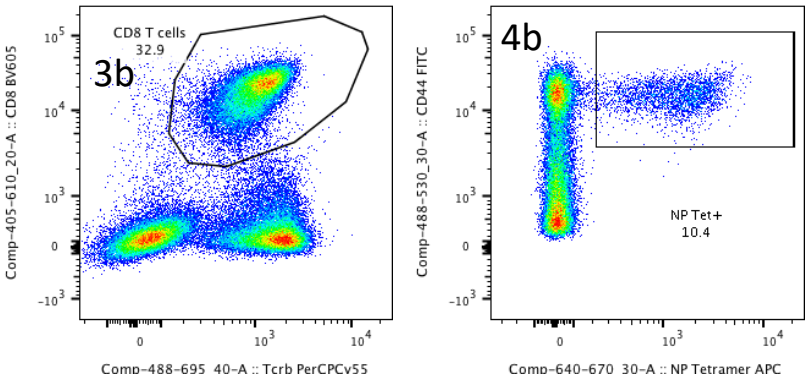
S2. Antigen specific CD8 gating scheme



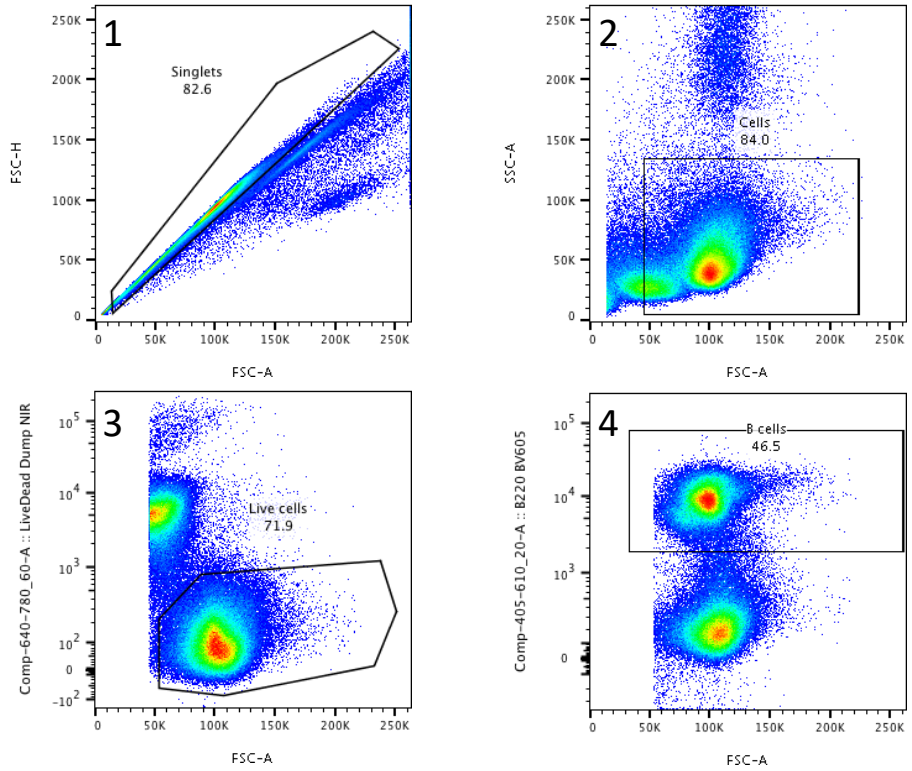
Naïve



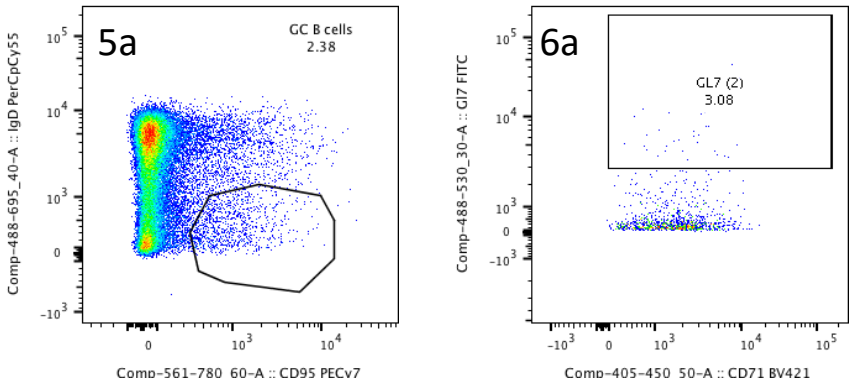
Influenza infected



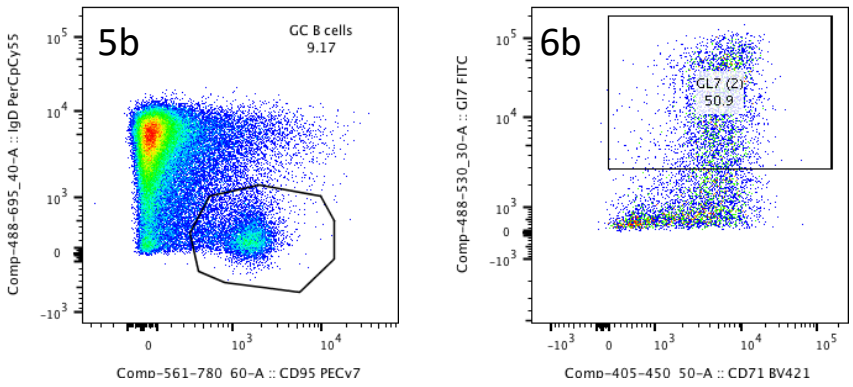
S3. Germinal centre B cell gating scheme



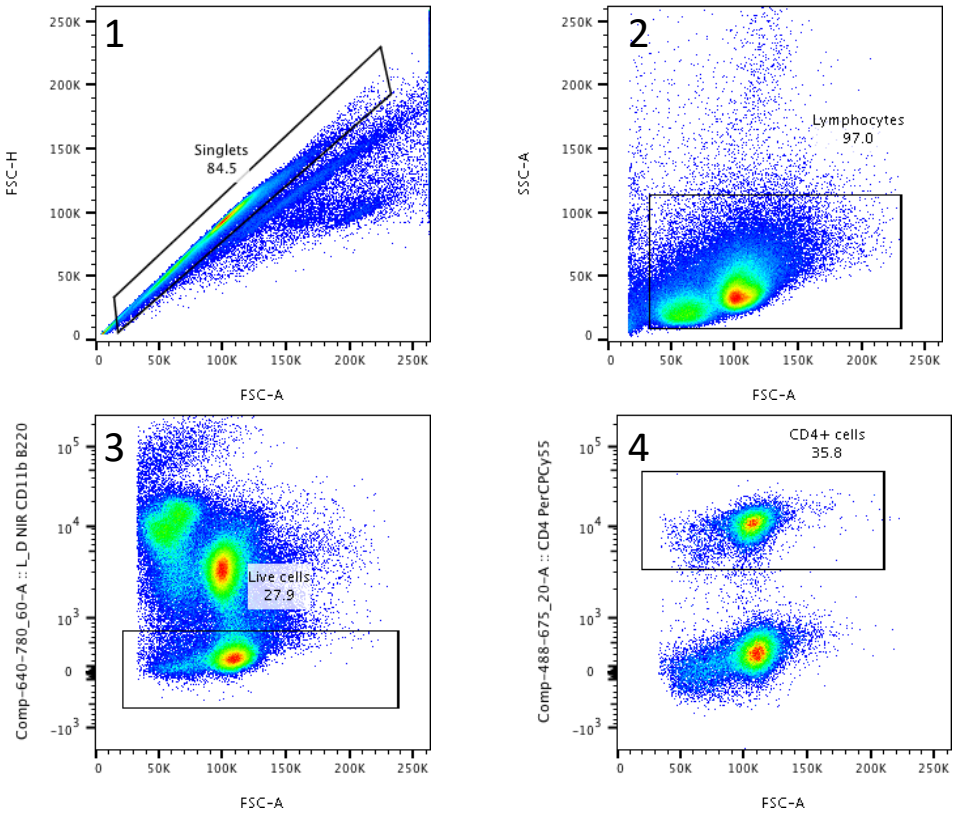
Naïve



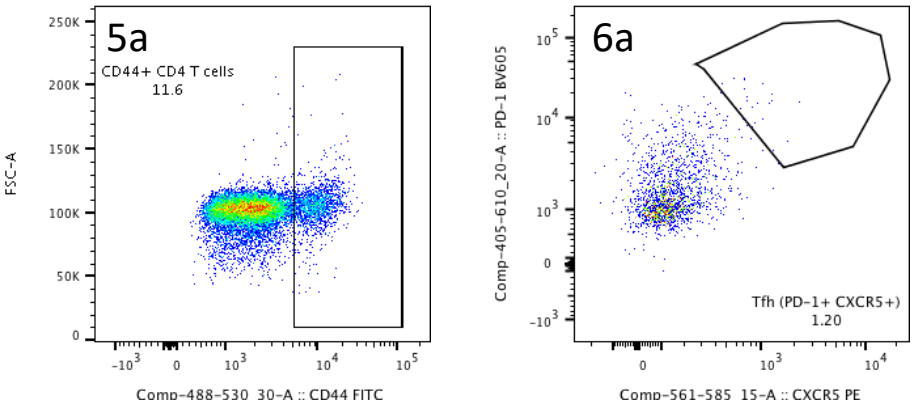
Immunised



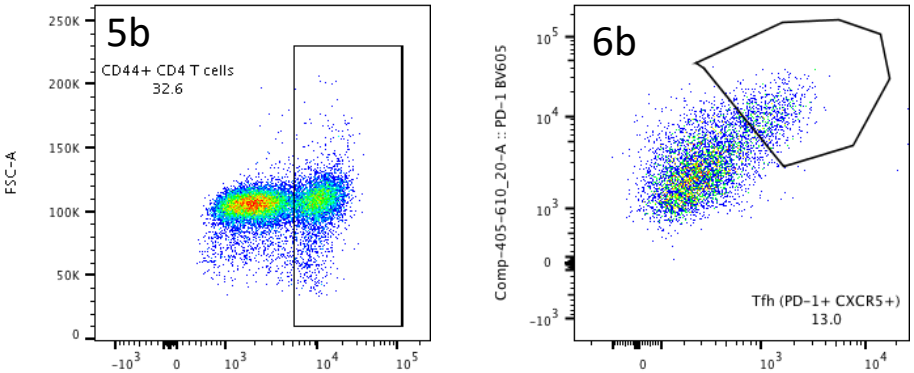
S4. T follicular helper cell gating scheme



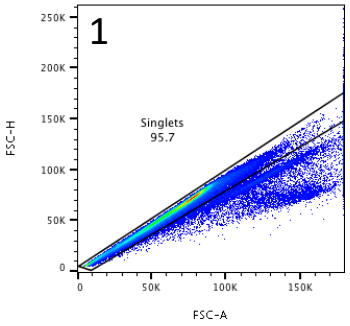
Naïve

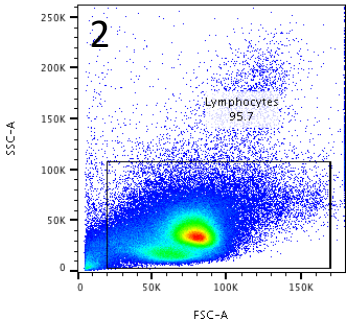


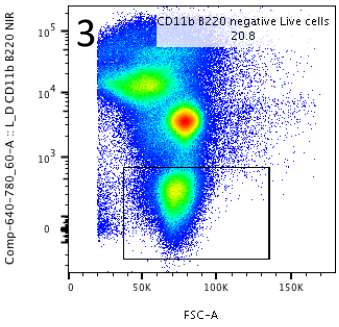
Immunised

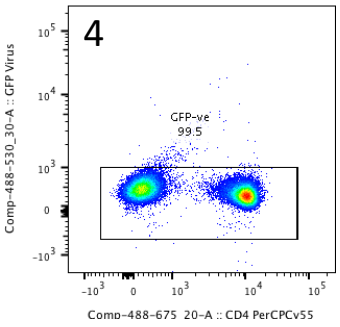


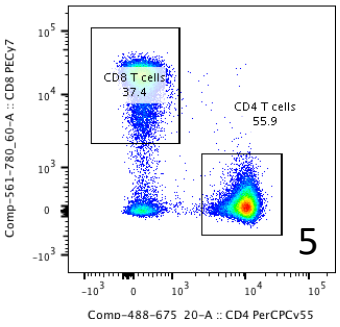
S5. Cytokine producing CD4 T cell gating scheme



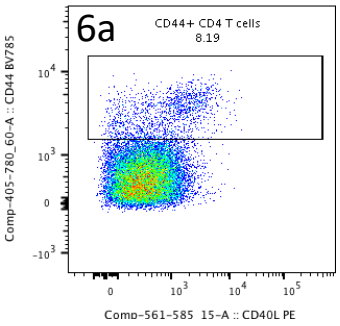


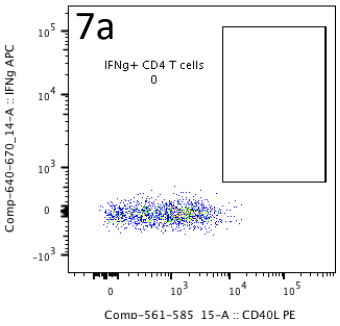




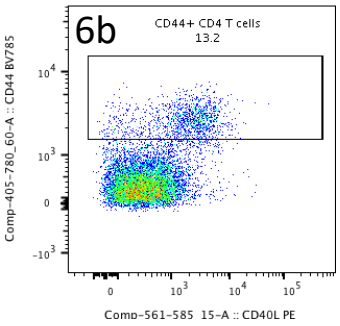


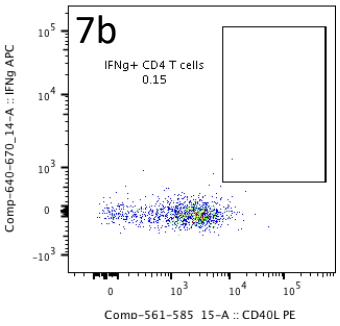
Un-immunised splenocytes and MVA-OVA pulsed DCs



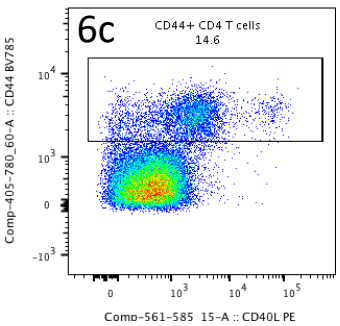


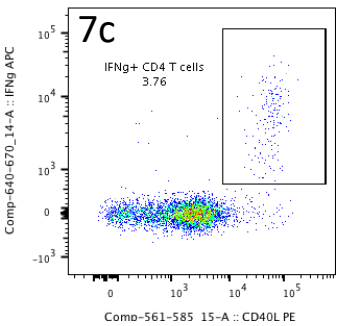
MVA-OVA immunised splenocytes and CpG pulsed DCs





MVA-OVA immunised splenocytes and MVA-OVA pulsed DCs





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