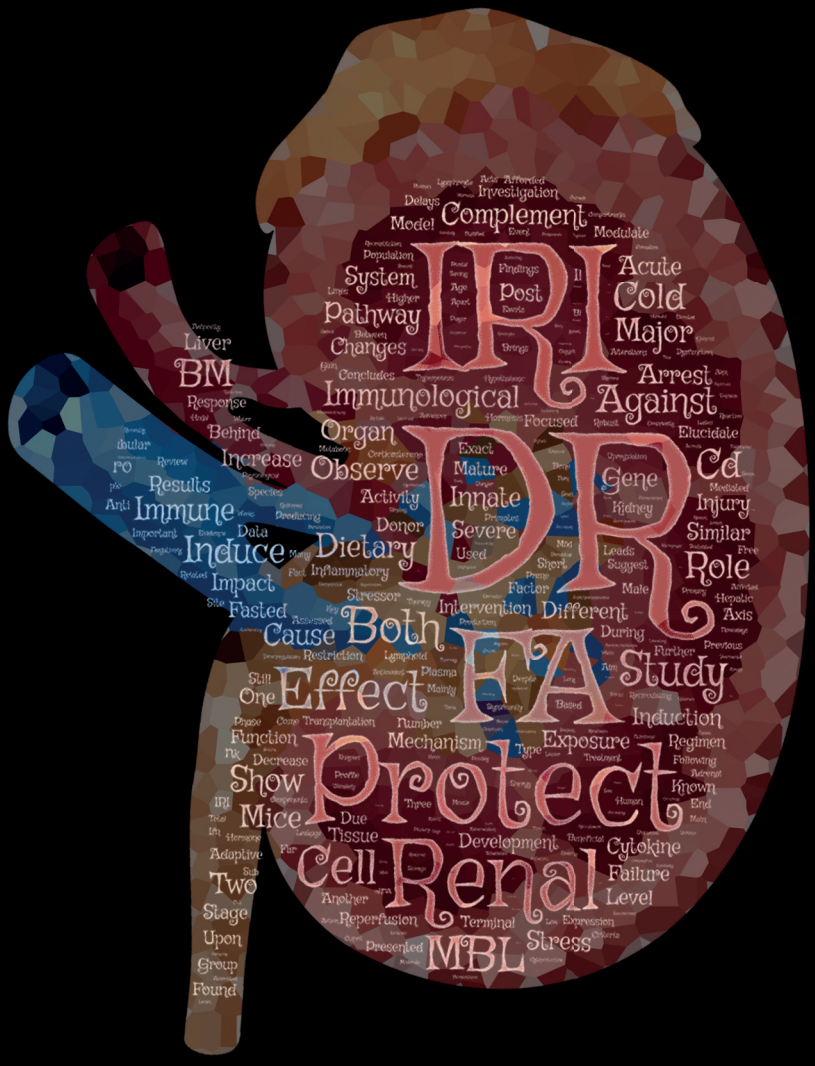
Immunomodulation by Dietary Restriction in Renal Ischemia/Reperfusion Injury



Shushimita

IMMUNOMODULATION BY DIETARY RESTRICTION IN RENAL ISCHEMIA/ REPERFUSION INJURY

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Dedicated to my love Geo and my family

CHAPTER 1

General introduction

GENERAL INTRODUCTION

Dietary restriction is a moderate reduction in daily calorie intake (20-40% reduction) without causing malnutrition. It has been shown convincingly by many studies that dietary restriction plays a crucial role in not only extending lifespan and improving health span but also in deceleration of several age-associated diseases including cancer, diabetes, hypertension and chronic inflammation. In addition, dietary restriction has proved beneficial in solid organ transplantation by decreasing ischemia reperfusion injury. Several mechanisms have been proposed explaining the beneficial effects of dietary restriction including hormones, nutrient sensing signaling pathways, mTOR pathway and the immune system. Both ischemia reperfusion injury and immuno-modulation by dietary restriction involve adaptive and innate immune system.

In this chapter, ischemia reperfusion injury, dietary restriction/intervention and the immune system are briefly introduced. This is followed by research aim and thesis outline.

ISCHEMIA REPERFUSION INJURY

Ischemia reperfusion injury (I/RI) is initiated by deprivation of tissue oxygen (ischemia) leading to accumulation of cellular waste, nutrient deprivation, and an excess of anaerobic metabolites. Subsequent restoration of blood flow (reperfusion) to the ischemic tissue leads to oxygen free radical generation and various other adverse

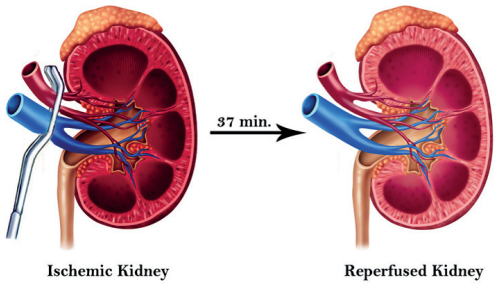

Fig.1: Induction of renal ischemia reperfusion injury. *Ischemia induced by clamping both the renal pedicles refers to the deprivation of blood flow and oxygen to the kidney. This deprivation is induced for 37min in male C57bl/6 mouse model while reperfusion is the process when the blood flow and oxygen is restored back into the kidney. Ischemia is characterized by ROS formation while reperfusion is characterized by infiltration of immune cell and acute tubular necrosis.*

reactions that damage the tissues [1] (acute oxidative stress) followed by inflammation and dysfunction of the transplanted organ [2–4] (Fig.1). I/RI is a key detrimental event in clinical conditions such as sepsis, cardiovascular surgery, trauma, various forms of infarction, and organ transplantation. It is a multifactorial antigen-independent inflammatory condition which has both immediate and long-term effects on the allograft [5]. Successful renal transplantation, considered to be the only real treatment for the people with end stage renal disease, is negatively affected by I/RI. I/RI is the major cause of ischemic acute renal failure, and is responsible for causing renal cell death, renal failure, which also result in delayed graft function and renal graft rejection [3]. Acute kidney injury (AKI), which is the functional consequence of I/RI, is associated with substantial morbidity and health care expenditures [6,7]. Despite advances in renal replacement therapy, the morbidity induced by I/RI remains high without specific therapy.

Preventive and therapeutic measures in I/R injury are needed to reduce the severity of graft dysfunction and failure thus allowing safe expansion of the donor pool with marginal donor kidneys that have suffered more initial injury before organ retrieval. Unfortunately, current treatment for renal I/RI is still primarily supportive, and experimental therapies aimed at minimizing I/RI have been applied in animal models generally. In order to better design the therapeutics for clinical renal I/RI, detailed knowledge on the pathophysiological mechanisms leading to ischemic acute graft injury after transplantation is required.

The pathophysiology of I/RI is multifactorial and only partially understood. Although inflammation [8] is regarded as a crucial event in the development of tissue injury and graft dysfunction in renal I/RI, highly complex cascade of events, including free reactive oxygen species (ROS) formation, endothelial dysfunction, and immune activation also play a major role in the pathophysiology [3,5,9–11]. Parenchymal damage occurs from both direct microvascular dysfunction from hypoxia and the subsequent inflammatory response. Acute ischemia leads to oxygen deprivation and adenosine triphosphate depletion resulting in direct parenchymal damage through tissue necrosis. Upon restoration of blood flow to the ischemic tissue, a “no-reflow” phenomenon occurs [12]. Capillaries and microcapillaries are not perfused, potentiating further tissue damage. In the light of donor organ shortage, extended criteria donors are increasingly being accepted for transplantation, with an increased risk for I/RI.

Based on animal experiments, many individual factors, such as cytokines, the innate and adaptive immune system and the complement have been suggested to be involved in the inflammatory response. The ensuing inflammatory response following I/RI is considered to increase the damage causing activation of both the innate and adaptive immune system. Different immunological players involved in I/RI, such as leukocyte adhesion

molecules, lymphocytes, regulatory T lymphocytes, and most importantly the complement system [9,10,13,14] have been studied and documented as one of the hallmarks of renal I/RI [13,15,16]. Acute ischemia causes activation of endothelium resulting in increased permeability and increased expression of adhesion molecules. These primed endothelial cells are more adhesive, and upon reperfusion, inflammatory cells attach to the endothelium [17]. Reactive oxygen species, cytokine, chemokines and adhesion molecules are generated, secreted, and released, augmenting the inflammatory response [5,18–22]. The combination of vascular permeability, and increased cellular signaling augment the recruitment and infiltration of circulating leukocytes into the post-ischemic tissue. This inflammatory response has been shown to result in tissue destruction and organ dysfunction in both experimental models and human data [23].

DIETARY RESTRICTION/INTERVENTION

Dietary restriction (DR), a moderate reduction in daily calorie intake (20–40% reduction) without causing malnutrition, has been known as an intervention that plays a key role in extending life-span [24], delaying ageing [25] was first reported by C.M. McCay in 1935 [24]. DR can be achieved either by restricting the food intake or by employing fasting regimens, with ad libitum access to water. Life-long DR associated with extended longevity have been reported in a variety of species like mice, rats, flies, fish, worms, and yeast [25–32]. Importantly, long-term DR also extends life span in non-human primates [33] and improves health in humans [34,35]. Apart from having an effect on longevity, there is accumulating evidence that DR delays the onset of various aging-related diseases, including cancer, diabetes, atherosclerosis, cardiovascular disorders, kidney disease, autoimmune disease and neuronal loss associated with Parkinson's and Alzheimer's disease [36–39]. Besides the beneficial long-term effect of DR on life span, short term DR (fasting) has also proved beneficial in improving stress resistance. Preoperative short-term fasting (FA) (1-4 days) has improved protection against organ injury after solid organ transplantation such as ischemia-reperfusion injury (I/RI) of both kidney and liver [40,41]. FA has also shown to delay the growth of cancer cells [39]. Prevention of many ageing-related diseases by DR and FA has been linked to immunology. Many of the beneficial effects of both DR and FA (commonly known as dietary restriction) on ageing-related diseases have been attributed to its anti-inflammatory qualities [42]. Dietary interventions extend life span not only by reducing reactive oxygen species but also by delaying age-related immune deficiencies, such as slowing down thymic involution and declining the production of lymphocytes [43]. Reduced serum cytokine levels (IL-6, IL-1β, and TNF-α) have been reported in case of DR mice prior to induction of myocardial I/RI [44]. In addition, the production of TNF-α upon stimulation with LPS was found to be decreased by DR [45] suggesting that DR dampen the inflammatory response.

Overall, short-term DR and FA increase stress resistance in animals and possibly in humans, and the protective effect involves upregulation of cytoprotective molecules such as hemoxygenase-1 (HO-1) [46] and decrease in the production of pro-inflammatory cytokines. The immunomodulatory effects of the two dietary interventions remain elusive, and warrants further investigations to find out the mechanism of protection by DR against detrimental effects of I/RI.

HORMESIS/COLD EXPOSURE

Dietary restriction, as such, is considered a mild stressor commonly referred to as hormesis. Hormesis can be defined as a life-supporting beneficial effect resulting from the cellular responses to mild, repeated stress [47]. Hormesis enhances processes that protect against the action of damaging agents and also promotes processes that repair the damage once it occurs. There is evidence that an increased ability to cope with damage enhances longevity, probably by retarding the damage from ageing processes [48,49]. The pathway by with DR enhances protective and repair processes has not yet been delineated. However, some hormesis pathways have been linked to the DR induced protection and repair processes such as increase in sir2 deacetylase activity, enhanced transcription of the stress-response genes for eg. HSP70 and elevated levels of plasma corticosterone and glucocorticoids [50]. Thus, hormetic effect induced by DR and FA could be hypothesized to be one of the underlying mechanisms by which these dietary interventions ameliorate I/RI. Hormesis has been known to be promoted by different types of stress that induce different mechanisms. Another stress model that has shown to have hormetic effects, and has proved to increase longevity is cold exposure [51]. Cold exposure is a commonly used method to increase metabolism, since it results in activation of brown adipose tissue and gives the white adipose tissue more brown adipose tissue-like characteristics. In mice, cold exposure induces hypothalamic-pituitary-adrenal axis activity, since it results in elevated plasma corticosterone concentrations [52]. DR and FA are also postulated to induce the hypothalamic-pituitary-adrenal axis activity, since plasma corticosterone concentrations are increased [53]. Since, both the dietary interventions and cold exposure induce hypothalamic-pituitary-adrenal axis activity studies have been performed in this thesis to correlate with I/RI survival and to investigate the changes in immunological profile of these three stress models.

IMMUNE SYSTEM

Classically the immune system is divided into adaptive and innate components with distinct roles and functions. The innate immune system is characterized as the non-specific interaction with the antigen, whereas adaptive immunity is characterized by

antigen specificity and immunological memory [54]. During the process of I/RI the immune system is activated as a result of infiltration of the immune cells to the organs leading to inflammation and are highly involved in causing AKI and allograft dysfunction. The key components of the immune system are discussed further in the below paragraphs.

Adaptive Immunity

Approximately 450 million years ago the adaptive immune response is assumed to have developed. This assumption is based on the fact of insertion of a transposon that carried the primitive variants of the recombinase activating genes, RAG-1 and RAG-2, into the germ line of jawed vertebrates [55]. The adaptive immune response is considered to be the second line of defense where it responds to the antigenic challenge with specificity that requires antigen receptors, which can recognize millions of molecular structures. At rest, the population of lymphocytes expressing antigen receptors is relatively low. Upon re-exposure to a particular antigen there is massive expansion of the specific lymphocytes. This leads to generation of memory cells that store information and upon antigenic challenge the rapidity and vigor with which the organism responds is augmented. The adaptive response is localized with precise homing mechanisms of lymphocytes [19,56].

Lymphocyte development

All blood cells, including lymphocytes, originate from a small number of self-renewing and pluripotent stem cells (HSCs) in the mouse bone marrow (BM) by successive expansions in cell number [57]. The HSCs later become committed to common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). During the time of development the CLP progenitors become segregated into B-lymphocyte, T-lymphocyte, and NK cells or dendritic cells while CMPs give rise to granulocyte-macrophage progenitors [58–60]. A schematic presentation of the stages of development has been represented in Fig.2.

B cell development in bone marrow

The humoral immune response is an important aspect of immunity and is mediated by secreted antibodies produced by the B cells (B lymphocyte lineage). It is well known that B cell development is a highly regulated process, which takes place in bone marrow (BM) [61,62]. B cells develop from multipotent hematopoietic stem cells which arise from common lymphoid progenitors (CLPs) and which in turn generate B cell precursors. These B cell precursors lack cell surface expression of a set of lineage molecules (including CD45R/B220 B lineage marker, CD11b/Mac-1 and GR1 myeloid/granulocyte/macrophage lineage markers, and the Ter119 erythroid lineage marker) [61,63]. Immediately after the CLP stage, B lineage restrictions are broken, and are recognized by the expression of B220 (an isoform of CD45), known as pro-pre B cell lineage. In BM it develops from CLP and

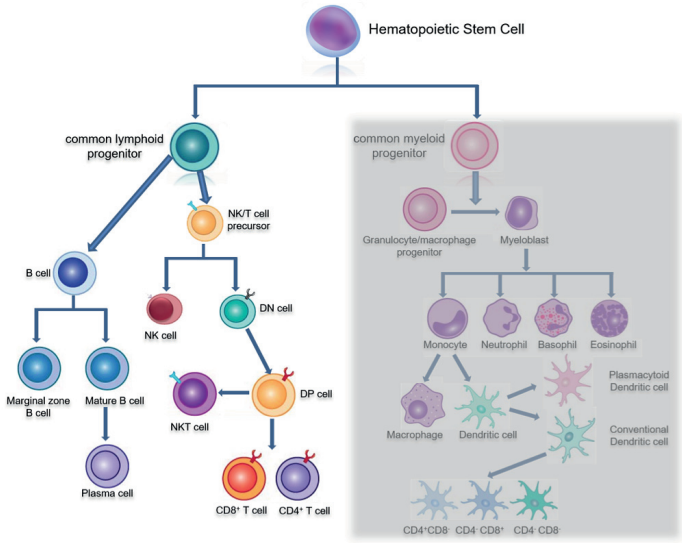

Fig.2: Schematic overview of hematopoiesis. *Hematopoietic stem cells (HSCs) give rise to all blood cells while retaining the capacity of self-renewal. HSCs become developmentally restricted in a stepwise fashion, ultimately leading to asymmetric division into lymphoid and myeloid progenitor cells. These lymphoid and myeloid cells then proliferate and differentiate into T or B cells, or myeloid cells such as monocytes, macrophages or dendritic cells, respectively. In this thesis the lymphoid progenitor cells have been studied.*

starts differentiating from pro-B cells, pre-B cells, and immature B cells to recirculating mature B cells. In the BM the differentiation starts with pro-pre B cells which express B220 without any expression of immunoglobulin (Ig). The pro-pre B cells further express B220, CD19 and CD43 again without any Ig expression and are named as pro B cells [61,64]. The pro B cells undergo heavy chain gene rearrangement, which expresses an additional surface marker CD2, from then on known as pre B cells. The pre B cells undergo light chain gene rearrangement and also express IgM (the first cell surface marker to appear on the B cells) to form immature B cells. The immature B cells referred to as by the cell surface expression of B220, CD19, CD2, and IgM undergo changes in RNA processing, along with expression of the second surface marker (IgD) and are referred to as recirculating mature B cells. These cells are ready to migrate to other peripheral lymphoid organs. The recirculating mature B cells after stimulation with an antigen becomes activated B cells and express most of the cell surface markers of various isotypes. The activated B cells undergo further class switching and differentiation in the secondary lymphoid organs like spleen and lymph nodes, and form plasma and memory B cells which are the mostly expressed Ig of the various isotypes, and which have the capacity to fight with invading micro-organisms time and again by secreting antibodies [61,65–67] (Fig.3).

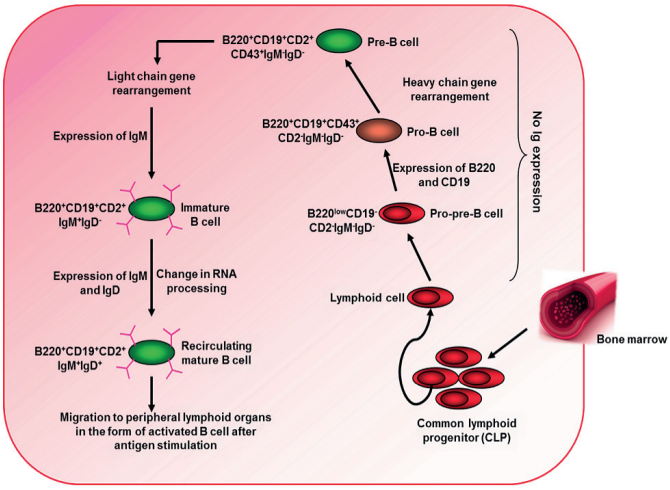

Fig. 3: B cell development in the bone marrow. *B cell development in bone marrow takes place in discrete stages starting from pro-pre B cells to recirculating mature B cells based on the surface markers expression. The pre-B cells are distinguished from pro-B cells on the basis of heavy chain rearrangement while the recirculating mature B cells are distinguished from immature B cells based on the expression of both IgM and IgD surface markers. The new recirculating mature B cells migrate to the periphery in the form of naïve mature B cells that recirculate throughout the body, including the bone marrow.*

T cell development in Thymus

T cells form a unique cell type of all the lymphocyte cell population because of requirement of a different dedicated organ, the thymus. The development of T lymphocytes known as thymopoiesis takes place in the thymus in a tightly regulated movement of hematopoietic cells from cortex, cortico-medullary junction, and finally to medulla [68,69].

As soon as the hematopoietic precursors leave bone marrow they enter the inner cortex region of thymus as lymphoid progenitors and start the multiple stages of T cell differentiation, which results in generation of immature thymocytes and mature T cells. The lymphoid progenitor cells, lacking the proper expression of the T-cell receptor (TCR), migrate from inner cortex to outer cortex by differentiating into double negative stages (DN) [70]. In murine models, it has been reported that there are four DN stages, unlike humans who have only three. These four DN stages are distinguished by expression of different surface markers such as $CD44^{+}CD25^{-}$ for DN1, $CD44^{+}CD25^{+}$ for DN2, $CD44^{-}CD25^{+}$ for DN3 and $CD44^{-}CD25^{-}$ for DN4 phenotypic stages [71,72]. After the thymocytes have passed through from DN2 to DN4 there is expression of pre-TCR due to rearrangement of the TCR- chain. Depending upon expression of pre-TCR, cells proliferate from DN4 to double positive (DP) stage while the thymocytes, which could not properly

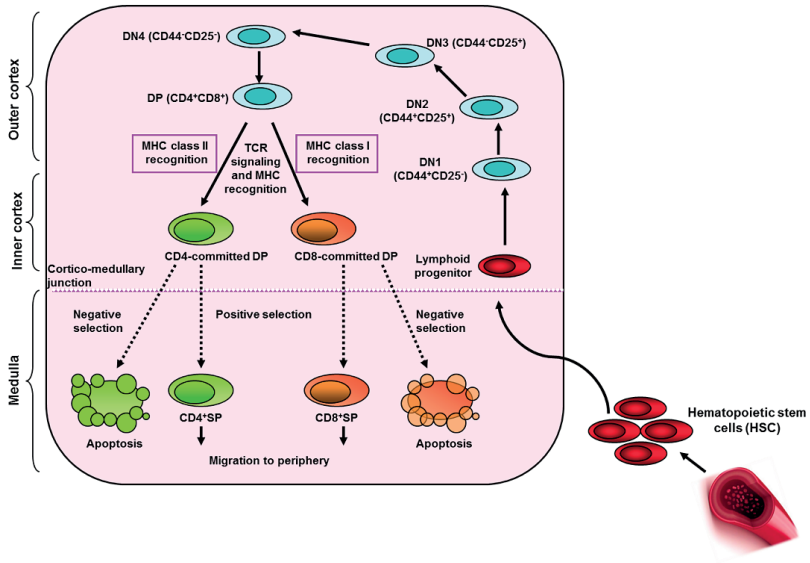

Fig. 4: T cell development stages in thymus. *T cell development takes place in thymus into αβ and γδ T cell lineages. Based on the cell surface marker expression and rearrangement of TCRα and TCRβ loci the T cell development stages undergo four distinct stages of development. The stages are DN (double negative), ISP (immature single positive), DP (double positive), and SP (single positive).*

express pre-TCR, undergo apoptosis and are negatively selected [70,73]. The DP stage thymocytes then undergo TCR- rearrangement and hence form the $TCR^{+}CD4^{+}CD8^{+}$ (DP) thymocytes. These cells interact with the epithelial cells of the cortical region where there is high expression of both MHC class I and II. The TCR of DP cells interact with both MHC I and II and if there is no proper signaling or too much signaling, the cells are negatively selected and undergo apoptosis [74]. When there is interaction of DP thymocytes with MHC I then they differentiate into $CD8^{+}$ single positive (SP) T cells while interaction of DP with MHC II leads to differentiation into $CD4^{+}$ SP T cells [70,74,75]. Once these cells have been committed to specific cell types, they are ready to migrate from medulla to the peripheral region as mature T cells [70].

Lymphocyte function

Naïve B lymphocytes after undergoing their development in the BM are generally divided into three functionally distinct mature B cell subsets categorized as marginal zone (MZ) B cells, follicular (FO) B cells, and B1-B cells [76,77]. These three B cell subsets differ in their origin and development, location, migration ability, and the likelihood of being activated in a T cell dependent or independent manner. Apart from mediating

humoral immune responses, B cells also secrete cytokines and have the potential to present antigen to naïve T cells.

Marginal zone and follicular B cells

Immature B cells migrating from BM to the spleen undergo further maturation into mature B cells in the spleen. In this maturation stage the immature B cells undergo transient transitional T1 and T2 B cell stages to either follicular (FO) or marginal zone (MZ) B cells [78,79].

MZ B cells are considered to be the major population of innate-like lymphocytes of the spleen, a unique lymphoid area located at the interface between the circulation and the immune system [80]. These MZ B cells are strategically located at the interface between the circulation and the white pulp of the spleen, where they provide a first line of defense by rapidly producing IgM and class-switched IgG antibodies in response to infections by blood-borne viruses and encapsulated bacteria. In rodents, MZ B cells are sessile and express high levels of IgM, CD21 and CD1d (to facilitate the presentation of lipid antigens to invariant natural killer T cells) [80,81]. These cells also express lower levels of IgD, CD23 and B220. Once the immune complexes are injected they bind rapidly to B cells in the MZ, and the high levels of CD21 on MZ B cells facilitate the transport of immune complexes from the circulation to the splenic follicles. In mouse the MZ B cells primarily express a non-mutated B cell receptor (BCR) and enters the circulation only to shuttle antigens to the follicle. These cells also have the ability to self-renew and to survive for as long as the life span of the host [82].

FO B cells are the most mature recirculating cells that home only to B cell follicles in the secondary lymphoid organs – spleen, lymph nodes and Peyer's Patches [78]. The B cell follicles are always adjacent to T cell zone and therefore follicular B cells are particularly well suited to participate in T cell-dependent immune responses to protein pathogens [82]. These FO B cells have the ability to differentiate into long-lived plasma cells upon antigen exposure and help $CD4^+$ T cells that secrete high-affinity and class-switched antibodies (IgG, IgE or IgA) [61,78,79]. The class-switch of the antibodies takes place in discrete structures within the B cell follicles known as germinal centers, where B cells undergo massive antigen dependent proliferation, class switch recombination, and somatic hypermutation. The FO B cells express high levels of IgD, CD23 and lower levels of IgM, and CD21 than MZ B cell and no detectable CD1d [62] (Fig.5).

Innate Immunity

The innate immune system, also known as the first line of defense, posts an immediate immune response and lacks immunologic memory. The mammalian innate immune system comprises of plasma proteins (such as complement), cells (neutrophils, macrophages and natural killer cells) and physical barriers (such as skin). The complement

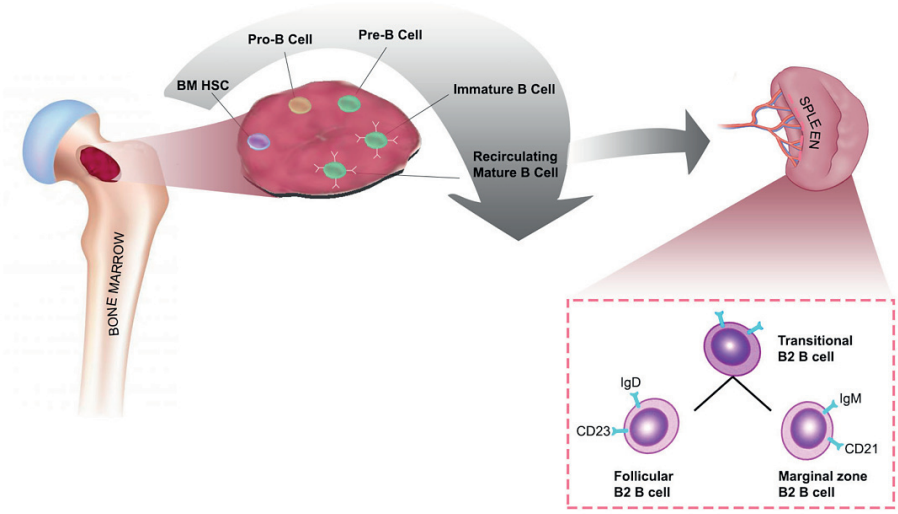

Fig. 5: Schematic overview of B cell fate in bone marrow and spleen. *After undergoing B cell development in bone marrow, the recirculating mature B cells circulate to spleen. In the spleen these cells undergo maturation from transitional B cells to either marginal zone or follicular B cells based on surface marker expression.*

system is one of the key players combating against pathogens and during the course of sepsis, cardiovascular diseases and other conditions such as I/RI in transplantation.

The Complement system

The complement system, first described in the late 1800s,[83] serves as an essential component of the innate immune system that serves a major role in host defense against invading pathogens, and at the same time is involved in effective clearance of apoptotic and necrotic cells. It is a complex cascade, consisting of approximately thirty plasma and membrane bound proteins. They are graded into different pathways depending on the type of activation mechanism; the classical pathway (CP), activated through C1-complex (Igs, phospholipids, pentraxins, etc.), the lectin pathway (LP), activated via MBL and ficolins (modified terminal sugars, etc.), and the alternative pathway activated on unprotected surfaces, amplifies CP/LP, and can be directed specifically via properdin. Each pathway recognizes certain molecular patterns, and has different activation and regulation pathways that converge at the level of C3, the central component of the complement system. The activation and deposition of C3 generates and initiates membrane attack complex (MAC) that leads to cytolysis of the target cell [84,85].

Classical pathway

The classical pathway (CP) is phylogenetically the most recent component of the complement system, arising in tandem with antibodies [86]. The CP augments antibody mediated immunity through binding of the C1 complex to the Fc-tails of the immunoglobulins (i.e. IgG or IgM bound to their antigen) [87,88]. The functional C1 complex comprises of one C1q complex with two pairs of C1r and C1s serine proteases ($C1qr^2s^2$) [89]. Upon binding of C1 complex to naturally pentameric IgM or membrane bound hexamerised IgGs the complement system is activated. The activated C1 complex cleaves C4 into C4b, and C2 into C2a, which initiates the CP complement cascade by forming the C4b2a complex (C3 convertase) [90]. The C3 convertase binds and cleaves C3 to C3b [91] (the initiation factor for terminal pathway) and C3a (a weak anaphylatoxin). Cleavage of C3 by CP results in formation of C5 convertase (C4bC2aC3b), and activation of terminal pathway by cleavage of C5 into C5a and C5b. C5a is a potent anaphylatoxin, which induces local inflammation, recruits inflammatory cells and modulates inflammatory cell responses. Terminal pathway results in formation of C5b-9 (MAC) [84]. The MAC complex forms a transmembrane pore on cell surfaces, lysing pathogens and promoting apoptosis on damaged cells.

Lectin pathway

The lectin pathway (LP) has four known LP specific initiation complexes: mannose binding lectin (MBL), ficolin-M (FCN1), ficolin-L (FCN2) and ficolin-H (FCN3). The LP is initiated through binding of oligomeric pattern recognition protein complex and cleavage of C4 by complex associated serine proteases. The LP dependent complement activation is mediated by MBL associated serine proteases (MASP1-3) (having the similar role as the C1 associated proteases C1r and C1s) which facilitate the cleavage of C4 and C2 into C2a and C4b that form the Mg2+ dependent CP/LP C3-convertase [91]. MBL is formed from trimers of identical peptides the main targets of which are terminal sugar residues including mannose, glucose, N-acetylglucosamine and N-acetylmanosamine. With a very low affinity for monosaccharides, MBL has high avidity for pathogen-associated molecular patterns due to multiple binding stalks in one functional molecule [92]. A number of MBL mutations affecting both promoter and encoding gene are known which have been found to result in highly variable serum concentrations from low nanogram range to mid-microgram range, and hence accounts for the variations in measurement of serum MBL concentrations [93].

Alternative pathway

The alternative pathway (AP) is phylogenetically the most primitive component of the complement system, and is central in the overall function of the complement system [86,94]. Unlike classical and lectin pathway, the alternative pathway is continuously activated, and does not require C4 for its activation. The alternative pathway can alternatively be activated by continuous hydrolysis of C3 to $C3(H_2O)$, properdin, and by

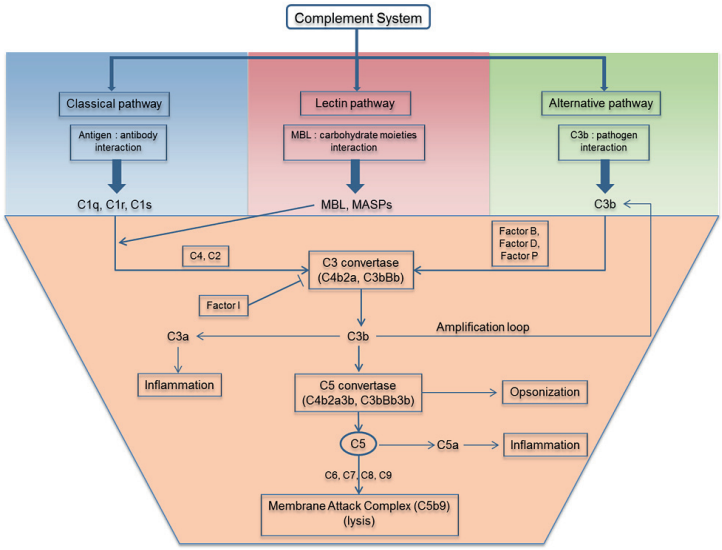

Fig. 6: Schematic representation of the complement cascade. *The central component of the complement cascade, C3 is activated by three pathways namely classical, lectin and alternative pathway. The three pathways get activated in distinct ways, such as antigen-antibody interaction for classical pathway, carbohydrate moieties-MBL for lectin pathway and direct binding of C3b to the activating surface for alternative pathway. All three pathways converge at the level of C3 and C5 convertase to form anaphylatoxin C5a, which modulates local inflammation, and recruits inflammatory cells, and the membrane attack complex (C5b-9), which creates a transmembrane pore on the activation site, effectively lysing pathogens and promoting apoptosis of nucleated cells.*

amplification of CP and LP [95]. The C3(H_2O) and factor B (fB) bind together with Mg2+, and fB is cleaved and activated by fD (fD) to form short-lived but highly active, minor AP C3 convertase C3(H_2O)Bb [96]. This C3 convertase cleaves C3 to C3a and C3b, which forms the major C3bBb complex and is stabilized by properdin causing increase in the

lifetime of this complex. Properdin is a unique regulator of complement activation through its ability to stabilise the AP C3 convertase up to 5-10 fold by forming C3bBbP complex [97,98]. AP is central in amplifying CP and LP mediated complement activation through recruitment of fB to the C3b fragments generated by CP/LP C3 convertase. It is suggested that up to 80% of overall activation of CP may originate from AP mediated amplification loop [99]. After formation of C3 convertase the downstream activation pathway resembles that of the classical and lectin pathway leading to formation of MAC, and finally phagocytosis of the apoptotic or necrotic cells.

Terminal Pathway

A central event in the complement activation is the cleavage of C5 by C5 convertases to form C5a and C5b. The terminal complement pathway is activated by formation of C5a and C5b-9. C5a is a potent anaphylatoxin that recruits inflammatory cells to the site of activation and modulates the local inflammation. The terminal complement complex, also known as membrane attack complex (MAC) or C5b-9, is a pore-forming complex that directly lyses the cells. The C5 convertase initiates activation of terminal pathway by forming C4bC2aC3b and C3bBbC3b. the cleavage of C5 to C5a and C5b allows binding of C6 [100], which then allows sequential binding of C7, C8 and C9. C8 forms the first MAC component to breach the bilayer membrane, resulting in weak lytic complex [101,102]. The binding of C8 to C5b-7 provides an oligomerisation site for 10-18 C9 proteins which form a membrane spanning pore capable of cell lysis [103–105].

Effectors and regulators of complement activation

The three main effectors of complement activation are opsonins, anaphylotoxins and the C5b-9 complex. While opsonins mediate endocytosis via complement receptors Cr1 and Cr2 [103,104], anaphylotoxins promote inflammation and chemotaxis through receptors such as C3aR and C5aR [108,109]. The C5b-9 complex on the other hand lyse unprotected cells and promote apoptosis of injured cells [110,111]. The regulators of complement activation (RCAs) or complement control proteins (CCPs) are soluble, membrane bound or transmembrane protein modulators of complement activation. They form an essential part in limiting the complement activation on healthy host cells and in directing the activation on damaged host cells or unprotected pathogen surfaces. Regulation of CP and LP is mediated by C1-inhibitor (C1INH), C4 binding protein (C4BP), and in part through the alternative pathway regulators decay accelerating factor (DAF, CD55), and factor I which have a C4b specificity. The C4-binding protein forms the second major regulator of CP and LP. C4BP has natural binding capacity for apoptotic cells together with protein S, CRP and DNA, and a central role in facilitating clearance of damaged tissues. C4BP has decay acceleration activity towards the C4bC2a C3-convertase, thus limiting the activation of both classical and lectin pathways [112].

AP which can get activated independently is essential for amplification of CP and LP. Therefore, AP RCAs are essential in modulating the complement system activation *in vivo*. Factor H is a multifunctional soluble serum AP RCA which binds to host cell surfaces and inhibits AP activation through cofactor activity with factor I (fI), and has a decay accelerating activity specific for AP C3-convertase C3bBb [113]. FI further facilitates the cleavage and deactivation of cell surface C3b into C3c, C3d, C3dg and iC3b, with similar activity towards C4b [114]. Cleavage of C3b to iC3b inhibits binding of fB, and therefore formation of AP C3-convertase and AP-amplification. Factor I has cofactor activity with other proteins such as DAF and fH and it processes iC3b further to soluble C3c and membrane bound C3dg and C3d [115].

The complement activation is also modulated on cell surfaces by membrane proteins such complement receptor 1 (Cr1, CD35), decay accelerating factor (DAF, CD55), and membrane cofactor protein (MCP, CD46). Both Cr1 and MCP have cofactor activity with fI which accelerates the decay of C3- and C5-convertases, whereas DAF has only C3-convertase specificity [116]. The terminal pathway activation results in formation of C5b-9 lytic complex [103]. The membrane protein MAC-IP (CD59) inhibits the membrane attack complex pore formation, and hence regulates the inadvertent lysis by C5b-9 complexes [75–77].

RESEARCH AIM AND THESIS OUTLINE

The aim of this thesis is to explore the immune factors and processes involved in protection against renal I/RI by two dietary interventions, namely 2 weeks of 30% dietary restriction (DR), and 3 days of water-only fasting (FA). In previous studies we have shown that both these dietary interventions induce robust protection against the detrimental effects of I/RI. However, the exact mechanism of action of these dietary interventions is unknown and needs to be explored. **Chapter 2** of this thesis provides an overview of the effects of the dietary restriction on the immune system. While **chapter 3** of this thesis explores the immunological changes taking place immediately after both dietary interventions without any ischemic insult to the kidney. This chapter illustrates that major changes are taking place in the adaptive immune system especially with respect to the B and T cell development after both DR and FA. In **chapter 4**, an important factor of the innate immune system, the complement system (Mannan-Binding Lectin), is assessed following the dietary interventions and renal I/RI. In this chapter, we investigated whether mannan-binding lectin mechanistically plays an important role in DR induced protection against renal I/RI. The overall impact of both DR and FA has been studied on the complement system in **chapter 5**. In this chapter the systemic, renal and hepatic changes taking place after DR and FA have been presented. While in chapter 3 immunological changes immediately after DR and FA have been explored, in **chapter 6** immunological changes post renal I/RI have been investigated. This chapter demonstrates the major adaptive immunity changes taking place post DR, FA and renal I/RI. Since it is known that DR and FA cause major metabolic changes, in **chapter 7** the major metabolic changes after DR and FA have been studied. Another hormetic mouse model, similar to DR, i.e. cold exposure has been studied to explore whether it would induce similar protection against renal I/RI as DR/FA do, and whether this is associated with similar metabolic, immunologic, and molecular phenomena. In **chapter 8** the results of the studies performed in this thesis are summarized and discussed along with an insight on future perspectives. **Chapter 9** summarizes the findings of the studies reported in this thesis.

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CHAPTER 2

Dietary restriction and its impact on immune system: relevant modulation in the protection against ischemia/ reperfusion injury

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ABSTRACT

Optimum but balanced food intake maintains healthy growth and disease-free life span. However, imbalanced food intake and over-nutrition promotes obesity, diabetes, malignancy, osteoporosis, infectious diseases, etcetera. In 1936, McCay reported that dietary restriction prevents weight gain and extends lifespan in rodents. Dietary restriction (DR), referred to as reduction in daily calorie intake without causing malnutrition, has shown to have impact on immune function by studies performed in mice. Several mechanisms have been proposed behind the advantageous effects of DR such as 1) attenuation of oxidative damage, 2) alteration of the growth hormone IGF-1 axis, 3) alteration of the glucose-insulin system, 4) hormesis and 5) delay in senescence. Reported changes in innate and adaptive immunity due to DR have also been noted recently and been investigated. To address the question of role of immunity in the advantageous effects of DR, several groups initiated long-term studies in rodents and non-human primates in the 1980s. Here, we review published data describing the impact of DR on the aging immune system of rodents and non-human primates, and discuss the data that delineate similarities and differences in the effects of DR upon adaptive and innate immunity.

INTRODUCTION

Dietary restriction (DR), defined to as the reduction in daily calorie intake without causing malnutrition, has been shown to retard aging and extending life span in early 1930s by McCay et al., [1]. Since then, similar observations have been reported in a variety of species like mice, rats, flies, fish, worms, and yeast [2–9]. DR has been defined in two different ways; long-term DR (for a period of more than 6 months) and short-term DR (for less than a month usually 2-4 weeks or 72hrs of water-only fasting) [10]. Apart from having an effect on longevity, there is accumulating evidence that DR delays the onset of various aging-related diseases, including cancer, diabetes, atherosclerosis, cardiovascular disorders, kidney disease, autoimmune disease and neuronal loss associated with Parkinson's and Alzheimer's disease [11–14]. It has been demonstrated that short-term DR not only proves beneficial for life-span extension in several species but also proves beneficial in protection against organ injury, such as ischemia-reperfusion injury (I/RI) of both kidney and liver [15,16]. Both the short (2-4weeks)- and long-term DR (6 months) have proved to be beneficial in not only rodents but also non-human primates [17] such as rhesus monkey (*Macaca mulatta*) [18] and has shown to improve health in humans [19,20].

The mechanism behind the beneficial effects exerted by DR to prolong life span or protection against aging related diseases and I/RI have not been illustrated so far. Several theories of mechanism of action have been put forward, however; the limited data available so far do not point to a single gene, pathway, or molecular mechanism underlying the protective effects of short-term DR. Instead, these studies highlight a range of potential mechanisms. Instead, these studies highlight a range of potential mechanisms.

The effects of DR on growth hormones, glucocorticoid and hormone mediated anti-aging actions of DR have been studied thus far [21–24]. In a study from 2003, Everitt has shown that pituitary growth hormones (GH) play an important role in aging [25] and that DR causes a reduction in the growth hormone secretion which lowers the oxidative damage of tissues, attenuates inflammatory responses [25,26] and which thereby, reduces the age-related pathologies and extends lifespan [27]. Apart from the decreased GH secretion, several other modulations have been observed at the molecular level due to DR. These modulations include: increased hepatic glucocorticoid receptor expression, which helps in controlling the glucocorticoid-mediated metabolic responses leading to resistance to stress, such as trauma, heat shock and drug toxicity [28], decreased plasma glucose, insulin, immunosenescence, and inflammation without any irreversible side effects [29].

Systemic changes involving the innate immune system and endocrine hormones are also observed. These include immunomodulation via increased anti-inflammatory

cytokine production and/or decreased expression of pro-inflammatory cytokines, chemokines and cell-surface adhesion molecules [30,31]; and reduced serum IGF-1 levels [32,33].

The nutrient sensing signaling pathways have been found to play an essential role in exerting its beneficial effects including mTOR [34,35]. In the context of nutrients, the essential amino acid deprivation, reduced serum IGF-1 levels require the amino acid deprivation sensor GCN2 [33].

In this review we summarize our understanding on how different dietary interventions (DR and Fasting) have an effect on modulating the immune system. We have illustrated the important role played by immune system in delineating the beneficial effects of DR.

EFFECT OF DR ON IMMUNITY

One of the major hallmarks of short-term DR is reduction in total body fat. This is speculated to increase the life span and also maintain immune function by significantly increasing lymphocyte function such as mitogen-activated lymphocyte proliferation in mice which was not only demonstrated by McCay et al., [1] but also by Walford et al., [36]. Along with attenuating age-related decline in mitogen-induced lymphocyte proliferation, DR attenuates cytokine production, antibody response to sheep red blood cells and also NK cell activity. The immune modulating effect of DR is not only limited to proliferation and cytokine production but also to the changes in percentage and phenotypic expression of lymphocytes. The percentage of lymphocyte populations such as $CD3^+$, $CD8^+$ T cells and NK cells ($OX8^+OX19^-$) were found to be increased in 8 months old DR Lobund Wistar rats compared to the ad libitum fed rats. DR also prevents a rise in memory T cells and thus maintains a higher number of naive T cells in aged mice and in this way prevents a decline in aging immune system [37]. Shushimita et al have however observed a decline and arrest of the T cell development stages [38].

A decrease in immunologic functions due to aging, known as immunosenescence, has been proposed as the major predisposing factor for increase in morbidity and mortality of animal species. During DR, the expression of IL-2, IL-2R a T cell receptor and NFAT (nuclear factor of activated T cell) transcription factor is increased in rodents, which proves to be beneficial in aging by preserving better immune function through the influence on age-sensitive immune parameters (such as lymphocyte proliferation, cytokine production and antigen presentation). Restoration of the immunologic functions thus contributes to the reduction in pathology and maintenance of healthy conditions during aging [37]. Therefore, the immunological status of animals fed a dietary restricted diet is assumed to be superior to the immunological status of the non-restricted animals.

Studies performed by the author Jolly [39] in 2004 proposed a few mechanisms for the protective effect of DR on immune function in aging and disease models. One of the mechanisms proposed was via protection of the immune cells from oxidative damage. He showed that when C57Bl/6 mice were dietary restricted by 40% it prevented age-associated increases in the cellular peroxidises in splenic lymphocytes and also blunted age-associated susceptibility of lymphocytes to hydrogen peroxide-mediated apoptosis. DR also improves lymphocyte function by maintaining the activity of the signal transduction pathways, which are important for proliferation, and/or by reducing the increased susceptibility to age-dependent increases in apoptosis. The second mechanism proposed was the alteration of specific lymphocyte populations. One of the important actions of long term DR is the delay in thymic involution but other thymic hormones also assist in regulating immune function. DR brings about its effects by bringing changes in the development of specific T-lymphocyte subsets rather than on bulk T-lymphocyte maturation. Forty-percentage DR in young mice for 14days increased the CD45RA receptor-positive CD4 T-lymphocyte population in mouse blood, LN and Spleen. Shushimita et al. have however observed that CD4 T cell population was decreased significantly due to 14days of DR [38].

The third mechanism examining the action of DR on immune function was based on the genetic level [40] where the effect of DR was examined on hypoxanthine guanine phosphoribosyl transferase on splenic lymphocytes of rats to test for gene mutation rates. It was found that 40% DR reduced the frequency of mutations in splenic T lymphocytes and the type of mutations that were affected were primarily small sequence mutations, same as those seen after free radical damage. This genetic level mechanism could therefore be linked to the ability of DR to increase antioxidant defence mechanisms protecting immune cells from free radical attack [41].

In the following paragraphs, a detailed overview of the effects of DR on the components of immune system is highlighted.

Effect of DR on adaptive immunity

Advanced age is known to be associated with decline in most physiological functions, including those of the innate and adaptive immune systems. The major hallmark of immunological changes occurring due to aging has been the loss of naïve lymphocytes and accumulation of memory cells [41]. The major changes taking place in the adaptive immunity due to DR have been reported with the roles played by B and T cells.

T-lymphocytes

The vast majority of experiments examining the role of DR on immune function have been performed with a focus on T lymphocytes. Various investigations have illustrated that DR delays age-related impairment of the immune system and especially T cell function [43].

Poetschke et al. also observed enormous alterations in the thymic and splenic architecture and showed a severe reduction in thymic and splenic size and cellularity in the DR mice [44], confirmed by Shushimita et al [38]. In addition to the decreased thymic size and cellularity, a strong depletion in the thymocyte subsets, particularly the $CD4^+CD8^+$ double positive T cells attributed to DR were observed. There was also evidence of decreased thymocyte development and proliferative capacity based on the percentages of $CD44^+$ and $CD25^+$ thymocytes showing alterations in the thymopoiesis. Thymopoiesis occurs in discrete stages starting with double negative stages (DN). In mice, it has been reported that there are four DN stages distinguished by the expression of CD25 and CD44 cell surface markers [45,46]. The double negative (DN) stages starting with DN1 ($CD44^+CD25^-$), was increased in the DR-induced mice indicating a delay in T cell commitment. The percentages of thymocytes within the $CD44^-25^{-\,(DN4)}$ subset were also significantly decreased under DR treatment. Thus, chronic DR increases the relative percentage of the pro-T cell $CD44^+25^-$ thymocyte subset and decreases the percentage of the $CD44^-25^-$ proliferative thymocyte population, indicative of a delay in thymocyte maturation. The authors show that DR exerts its effects through three interrelated mechanisms: (i) increased cell death within the immature thymocyte compartment; (ii) decreased thymocyte proliferative capacity; (iii) delayed thymocyte maturation [44]. Similar results have also been shown by Shushimita [38] and Fernandes G et al., [43] where the authors demonstrated that food restriction helps in preventing or delaying the age-associated decline in the T-cell mediated immune responses. The reason behind this was reported to be slow production of pro-inflammatory cytokines, which prevents a rise in the memory T cells populations. Maintenance of naïve T cell populations, proliferative capacity of the T cells, and modulation of immunity to infectious agents by DR have also been reported by Messaoudi et al. in rodents [47], and the reason behind this maintenance has been validated to be an increase in thymic involution.

In 1998, Harrison and colleagues had shown that acute DR helps in retaining circulating naïve CD4 and CD8 T cell populations in peripheral blood and spleen [48]. They also presented that DR is responsible in delaying the rate of thymic aging. They observed a significant increase in the percentage of the $CD8^+CD4^+$ double positive (DP) thymocytes upon DR. No change was observed in the $CD8^-CD4^-$ double negative (DN) precursors of DP cells, nor in the $CD8^+CD4^-$ and $CD8^-CD4^+$ single positive (SP) thymocytes. Interestingly, recent studies performed in our own laboratory demonstrated a significant decline in the $CD4^+CD8^+$ DP thymocytes as well as a significant decrease in the DN thymocytes, indicative of arrested thymopoiesis [38].

Studies performed in non-human primates (Rhesus macaque) have also reported similar changes as in rodents. Reduction in splenic lymphocyte numbers, loss of naïve CD4 and CD8 T cells from circulation and to a slower extent from lymph nodes, and replacement

of memory phenotype cells in secondary lymphoid organs which are incapable of immediate cytokine production have been described [17].

Apart from maintaining the naïve T cell population, DR also maintains the proliferative capacity of the T cells [49]. Zugich and Messaoudi have postulated a thorough explanation on how DR maintains the proliferative capacity of T cells and hence has not been covered fully in this review [47].

The immunological changes observed in the rodent studies is not just limited to the particular species but is also extended to higher animal species such as non-human primates. The progressive loss of naïve CD4 and CD8 T cells from the circulation and lymph nodes due to aging seem to be replaced by the memory phenotypes as reported by Pitcher et al. [50]. However, due to the effect of DR, naïve T cells as well as their proliferative capacity are maintained [47].

All these immunological changes occurring in the different restricted groups indicate that dietary interventions indeed modulate the immune system. A more in-depth investigation is required to increase our understanding of immunomodulation by DR in several disease conditions such as in an I/RI model where DR has proved to be highly protective [15].

B-lymphocytes

Although not many studies have been performed to study the effect of DR on B cell, a few studies show similar changes as that observed in the T cell populations. From these studies it has been illustrated that DR reduces the number of circulating B cells. Study by Tanaka et al., showed that starvation (a form of short-term DR, otherwise known as fasting) induces alterations in B cell development in the bone marrow of mice. Experimentally it was revealed that starvation (for up to 48hrs) led to a significant decrease in the proportion of $B220^{+}$ B cells including the B cell development stages such as pro-B, Pre-B, and immature B cells. However, the mature B cells were increased significantly due to this short-term fasting [51]. Studies from our own research reveal a remarkable decrease in the B cell numbers of the bone marrow due to both DR and fasting of up to 72hrs [38]. We have also shown that both DR and starvation decrease the early B cell development stages while increasing the recirculating mature B cells in bone marrow. Upon further investigation of the spleen, less B cell population and mature cells including marginal zone and follicular B cells were observed due to DR and fasting [38]. It has been proposed that corticosterone plays a role in the starvation-induced alteration of B cell development because starvation increases corticosterone production as a physiologic adaptation to energy deprivation through the activation of the hypothalamus–pituitary adrenal axis [52,53]. Additional studies are required to elucidate the involvement of humoral mechanisms in the alteration of B-cell development in the bone marrow under starvation conditions.

Effects of DR on innate immunity

NK cells

Apart from extending life span and decreasing the incidence of cancer, and severity of autoimmune diseases in laboratory animals, DR can also increase their susceptibility to infection. Several studies have suggested that these outcomes are linked to the effects of DR on the immune system and on the innate immune compartments. These studies describe how DR affects natural killer (NK) cell responses. DR in mice has been shown to alter the distribution of NK cell subsets to the peripheral tissues, spleen and lymph nodes; mice subjected to DR had fewer mature NK cells and increased frequencies of $CD127^+$ NK cells. This resulted in the mice on the restricted diet having NK cells that were less responsive to cytokine-mediated activation, although their NK cells could still mediate cytotoxic activity against target cells. Such altered NK cell function following DR in mice may contribute to some of the immunological phenomena observed in these animals [54].

Yamazaki et al., compared the proportion and cytolytic activity of NK cells in mice that were fed ad libitum versus mice on 40% DR for 4 weeks. The authors found that the relative proportion of NK cells ($DX5^+CD3^-$) in the spleen was lower in the 40% DR mice than in AL mice. Also, they demonstrated that the younger mice (11 weeks old), when subjected to 40% DR, were deprived of NK cell cytolytic activity, whereas this effect was not observed in 22 weeks old mice under the same circumstances [55]. Other research groups have investigated the important role of DR in infectious state. They performed experiments on influenza virus infection in C57Bl/6 mice and found that during influenza infection, NK cell cytotoxicity was lowered in 3 weeks 40% DR mice at day 1 in lung and at day 2 in spleen when compared to that in AL mice [55]. This research group has also found out that short term DR alters the NK cell maturation and function in C57Bl/6 mice [54] while the mice are fine. They have shown that there is a reduced frequency of NK cells in peripheral tissues and they exhibit an altered phenotype in the spleen of DR mice. Furthermore, it has also been shown that DR mostly influences the homeostasis of mature NK cells such as significant reduction in the $CD11b^+$ mature NK cell population in spleen. It had been demonstrated long back in the 1980's that DR reduces the NK activity without harm to the mice and without infectious problems [56], which could be an important contributing factor in protection against IRI as NK cell infiltration is an important feature of I/RI.

Neutrophils and Macrophages

The effects of polymorphonuclear neutrophils (PMNs) that represent the first line of defence, acting to eliminate invading bacteria at the site of infection have not been studied in detail in relation to DR. A few studies have shown that in response to an inflammatory stimulus, PMNs marginate, adhere to endothelium and migrate to local inflammatory sites early in the inflammatory process. It is also known that exudative

rather than circulating PMNs play the most important role in host defence at local sites. There have been no studies stating the effect of DR on the expression of adhesion molecules on the circulating PMNs or on chemokine production at local inflammatory sites.

Knowledge regarding the effects of DR on macrophage (MΦ) function is sparse and inconclusive. In 1983, Wing and Barczynski demonstrated that starvation of over 48 or 72hrs enhances immune function in mice by up-regulation of peritoneal macrophage (PMΦ) bactericidal activity. It was demonstrated that mice fasted for 72hrs followed by infection with *Listeria monocytogenes* led to survival of 95% as compared to that of 5% in the AL fed mice. It was also demonstrated that alveolar macrophage (AMΦ) phagocytosis and bacterial challenge was increased in the rats fed DR [57]. This study hence demonstrated that DR leads to increased phagocytosis and hence had a higher capacity to fight against the invading pathogens as compared to that of the normal fed animals. It was also observed by Jolly et al. that DR affects multiple signal transduction pathways in macrophages [39] without any conclusive effects in I/RI condition.

Complement system

DR has also been described to impact on the complement system. The complement system is crucial in host immune defense, and previous studies have suggested that adipose tissue serves as an important site of production for some complement proteins. Although studies are sparse that have been studied the effect of DR on complement system, Pomeroy et al., studied the effect of body weight and DR on serum complement proteins in starvation induced anorexia patients. Anorexia has been defined as a serious eating disorder in which the patients severely restrict calorie intake leading to an immense weight loss, far below ideal body weight. Lower serum complement proteins have been reported in the anorectic patients in this study; serum complement levels of proteins such as C3, Factor D, C1q, C2, C4, Factor H and Factor I were found to be significantly lower than in the control patients. The reason behind lower levels of Factor D has been attributed to the loss of adipose tissue. The lower levels of the other serum complement proteins have been attributed to the less production of these complement factors as stated by Pomeroy et al [58]. Hence DR causes a decrease in the complement levels due to the decreased amount of adipose tissue considered being the site of production of some of the complement factors. DR studies performed in our own laboratories have highlighted the crucial role played by mannan-binding lectin (MBL) in protection against renal I/RI. We found that both short term DR and fasting significantly reduced the circulating levels of MBL as well as its mRNA expression in liver; the sole production site of MBL. Upon reconstitution of MBL, the protection afforded by DR was abolished, whereas in the FA group the protection persisted. Vascular leakage and extravasation of MBL following reperfusion is known to play a pivotal role in induction of renal I/RI and exposure of epithelial cells to MBL immediately following reperfusion is the primary culprit of tubular injury. Another important aspect observed was that the protection by FA was not

mediated by prevention of vascular leakage suggested that FA had an effect on tubular cell homeostasis and resistance to tubular injury, independent of MBL [59]. In another study we assessed the functionality of serum complement with pathway specific functional complement assays together with focused analysis of hepatic and renal expression of key complement proteins and regulators. The results showed that FA, and to lesser extent DR, reduced the systemic complement activities of all the three pathways, with most radical impact on the terminal pathway, and only moderate impact on the initiation pathways. Upon further investigation into hepatic expression, major reduction of gene expression of terminal pathway components was observed. However, only FA resulted in systemic downregulation of terminal pathway components while no effect of DR was observed. A significant upregulation of complement factor C3 was observed in both liver and kidney tissues after both DR and FA suggesting a compensatory mechanism akin to acute phase response (shushimita et al, submitted).

Effect of DR on cytokines and chemokines

Apart from the changes described above, DR also brings about changes in the various cytokines and chemokines. In (NZB x NZW) F1 mouse strain, Jolly experimentally showed that DR reduces the plasma levels of pro-inflammatory cytokines like IFN-γ, IL-12, IL-6 and TNF-α and also increases the levels of immunosuppressive anti-inflammatory cytokines such as TGF-β and IL-10 [39]. Similarly, Pahlavani showed that DR helps in maintenance of IL-2 production by old T cells by reversion of age related decline in MAPK and calcineurin activation [60]. Yamazaki et al. investigated the production of the cytokines like IFN-γ and IL-4 and found that compared with the T cells of the 11 weeks old AL mice, the T cells from the 11 weeks old 40% DR mice produced less IFN-γ and higher levels of IL-4 [56]. Similarly Chandrasekar et al., reported that restricting dietary calories prevents salivary-gland abnormalities and modulates expression of transforming growth factor-β1, IL-6, and TNF-α in major salivary glands of autoimmune, lupus-prone female mice [61]. Spaulding et al., demonstrated that calorie restriction significantly attenuates age-related dysregulation of TNF-α and IL-6 generation in mice [62]. Hence, DR reduces the pro-inflammatory cytokines while increasing the levels of anti-inflammatory cytokines, as demonstrated in various studies. Since, pro-inflammatory cytokines contribute to acute and chronic inflammation post reperfusion, decrease by DR serves as a major contributor of protection against I/RI [63,64].

CONCLUSION

Caloric restriction is the most efficacious intervention method known thus far that increases median and maximum lifespan in laboratory animals. The increase in longevity with DR is directly correlated with the decrease in the age-associated diseases such as infections, autoimmunity, and cancer. Thus, the observation that reduced caloric intake is associated with increased longevity and reduced pathology in experimental animals has provided a rationale for the immune-enhancing effect of DR. As indicated by the various publications summarized above, the overwhelming majority of the reported studies indicate that DR modulates the immune function and restores or delays the immunosenescence in not only laboratory animals, but also in higher species such as non-human primates. We have shown how the adaptive and innate immunity are modulated by DR and conclude that DR preserves the naïve T cell population as well as maintains the proliferative capacity of the T cells which deteriorates with aging. With respect to B cell population it has been hypothesized that DR causes a decline in the early B cell developmental cells while increasing the population of mature B cells in bone marrow. How this is advantageous in exerting the beneficial effects is something that warrants further investigation.

Much work remains to elucidate the real mechanisms underlying these effects in experimental models. Moreover, there are questions and concerns which remains to be answered such as if there are immediate translational potential, and whether clinical applications are foreseeable [65]. Currently, clinical trials are ongoing (unpublished) to study the mechanisms of DR, and to see whether special diet mimicking DR can protect against the side-effects of chemotherapy, while maintaining its anti-cancer effect. What are the functional biomarkers of DR that will help us to cross the species boundaries (from rodents to humans)? How long DR should be given in humans compared to mice? Will humans remain immunocompetent? How much body weight loss is anticipated in humans when DR is administered? If we could answer some of these concerns then it would be more convenient for clinical trials to be performed (which are going on, but only with modified diets) and we would be closer than ever to translate short-term DR from the laboratory to the clinic.

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CHAPTER 3

Dietary restriction and fasting arrest B and T cell development and increase mature B and T cell numbers in bone marrow

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ABSTRACT

Dietary restriction (DR) delays ageing and extends life span. Both long- and short-term DR, as well as short-term fasting provide robust protection against many "neuronal and surgery related damaging phenomena" such as Parkinson's disease and ischemia-reperfusion injury. The exact mechanism behind this phenomenon has not yet been elucidated. Its anti-inflammatory actions prompted us to thoroughly investigate the consequences of DR and fasting on B and T cell compartments in primary and secondary lymphoid organs of male C57Bl/6 mice. In BM we found that DR and fasting cause a decrease in the total B cell population and arrest early B cell development, while increasing the number of recirculating mature B cells. In the fasting group, a significant reduction in peripheral B cell counts was observed in both spleen and mesenteric lymph nodes (mLN). Thymopoiesis was arrested significantly at double negative DN2 stage due to fasting, whereas DR resulted in a partial arrest of thymocyte development at the DN4 stage. Mature $CD3^+$ T cell populations were increased in BM and decreased in both spleen and mLN. Thus, DR arrests B cell development in the BM but increases the number of recirculating mature B cells. DR also arrests maturation of T cells in thymus, resulting in depletion of mature T cells from spleen and mLN while recruiting them to the BM. The functional relevance in relation to protection against organ damage needs to be determined.

INTRODUCTION

Dietary restriction (DR), a moderate reduction in daily calorie intake (20-40% reduction) without causing malnutrition, has been known as an intervention that plays a key role in extending life-span [1], delaying ageing [2] and also in many ageing-related diseases such as diabetes, atherosclerosis, cardiovascular disorders, kidney disease, autoimmune disease and neuronal loss associated with Parkinson's and Alzheimer's disease [3]. Both long-term (dietary intervention for more than six months) and short-term (maximum of four weeks) DR, have shown to be beneficial in predicting long-term health and in reducing the rate of cardiovascular disease and insulin sensitivity [4]. Long-term DR has not only proven to be effective in mice [5] but also in various other species like rats [6], flies [7], worms [8], yeast [9,10], fish [11], non-human primates [12,13], and in humans [14,15]. Short-term fasting, another type of DR, has also proven to be beneficial in promoting stress resistance as well as longevity in model organisms and in delaying the growth of cancer cells [16]. Prevention of many ageing-related diseases by DR and fasting has been linked to immunology. Many of the beneficial effects of DR on ageing-related diseases have been attributed to its anti-inflammatory qualities [17]. DR extends life span not only by reducing reactive oxygen species but also by delaying age-related immune deficiencies, such as slowing down thymic involution and declining the production of lymphocytes [18].

No recent data have explicitly shown the effect of DR on the immune system in a broad perspective, but we have demonstrated that short-term DR and fasting have a robust protective effect on ischemia-reperfusion injury (IRI) of both kidney and liver in mice. IRI has been known to be one of the most important inevitable consequences of solid organ transplantation and has a negative impact on both short- and long-term graft survival leading to acute organ failure. Following renal and hepatic IRI, the production of pro-inflammatory cytokines and the subsequent infiltration of the organs by lymphocytes that follows IRI was significantly blunted [19]. Collectively these data strongly imply that the immune system is an important factor in the protective features of DR and fasting. Therefore, we set out to investigate the impact of dietary interventions on the immune system in the same mouse model (10-12 weeks old), but in the absence of IRI. We thoroughly investigated the consequences of DR and fasting on B and T cell development in bone marrow (BM) and thymus respectively. We also studied B cell differentiation in the spleen and mesenteric lymph nodes (mLN) and T cell subtypes in BM and secondary lymphoid organs after DR and fasting.

MATERIALS AND METHODS

Animals

Ten to 12 weeks old C57Bl/6 male mice, weighing 20-25g, were purchased from Harlan, Horst, the Netherlands. The mice were kept under normal laboratory physiological conditions (temperature 20–24oC, relative humidity 50–60%, 12hr light/12hr dark) with 3-4 animals per cage having free access to food (Hope Farms, Woerden, the Netherlands) and water until the start of experimental procedures. All the experimental procedures were performed after approval by the university animal experiments committee (Dutch Ethical Committee, Protocol no. 105-12-12) under the Dutch National Experiments on Animals Act, compiled with Directive 86/609/EC (1986) of the Council of Europe.

Dietary regimen

Mice were randomly divided into three groups (n=8/group): ad libitum (AL), two weeks 30% DR (DR) and three days water-only fasting (FA). The first group of AL mice was allowed free access to food and water. In the second group with 30% dietary restriction (4 animals/cage) food intake was weighed daily during the first week (normal consumption is approximately 9.48 g per day) and 30% DR was performed by providing 70% of the food determined by the intake of the previous week. The food was provided at the same time at the end of the day to avoid any discrepancies. The mice in the three-day fasting group were transferred into a new clean cage (to restrain them from eating their own faeces) with free access to water but no food. The fasting regimen also started at the end of the day just before the beginning of the active period of the animals.

Cell isolation and staining

After dietary interventions, mice were exsanguinated by cardiac puncture and thymus, spleen, mLN and bones (bone marrow) were harvested and processed to make cell suspensions. Thymus, spleen and mLN were mashed and passed through 100µm Nylon cell strainers (BD FalconTM, BD Biosciences Europe, Erembodegem, Belgium). The cell suspensions were made in RPMI-1640 medium (Lonza Europe B.V., Verviers, Belgium) supplemented with 10% FCS (Lonza Europe B.V., Verviers, Belgium) and 1% penicillin/streptomycin (InvitrogenTM/Gibco®, Bleiswijk, the Netherlands). To make BM cell suspensions, femur and tibia were crushed using a mortar and pestle followed by re-suspending in RPMI-1640 medium. The total number of live cells were then counted using a Casy TT counter and analyzer (Innovatis, Roche Diagnostics Nederland B.V., Almere, Netherlands) and prepared for flow cytometric analyses.

Flow cytometric analyses

For all lymphoid organs, 2x106 cells were plated in a 96-well plate for each staining. The plate was then centrifuged at 1360 rpm for 3 min. After discarding the supernatant, cells

were stained with appropriate mixture of antibodies for staining of B and T cell populations and incubated for 20 min at 4oC. The cells were then washed with 200µl of FACS buffer and treated with a secondary staining wherever required. After staining the cells with antibodies as mentioned in Table 1, data were acquired on a FACS LSRIITM flow cytometer (BD biosciences,) and analysed using FlowJoTM (Tree Star, Ashland, OR, USA) research software. Dapi and aqua live-dead staining were used to gate on the live cells. These live events were then recorded for the lymphocytes based on forward and side scatter.

Chemicals and reagents used

Monoclonal antibodies directed against mouse leukocyte populations were obtained from BD Biosciences (BD Biosciences) and eBioscience (Campus Vienna Biocenter 2, Vienna, Austria) unless mentioned otherwise. These monoclonal antibodies labeled with different antibody fluorochrome combinations according to the cell development stages are presented in Table 1.

Statistical analysis

Non-parametric paired sample T-tests were performed on the three experimental groups using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.), while the graphs were plotted using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA). P-values ≤0.05 were considered to be statistically significant.

Table 1 shows the list of different antibody combinations according to the cell development stages in the various lymphoid organs.

Cell Development	Developmental stage	Antibody combination
B cell development (BM)	Total B cells	$CD19^{+}B220^{+}$
	Pro-B cells	$CD43^{+}CD2^{-}IgM^{-}IgD^{-}$
	Pre-B cells	$CD43^{-}CD2^{+}IgM^{-}IgD^{-}$
	Immature B cells	$IgM^{+}IgD^{low}$
	Recirculating mature B cells	$IgM^{+/low}IgD^{high}$
B cell lymphoid population (Spleen)	Total B cells	$CD19^{+}B220^{+}$
	Pro-pre B cells	$IgM^{low}IgD^{low}$
	Immature B cells	$IgM^{+}IgD^{-}$
	Mature B cells	$IgM^{+}IgD^{-}$
	Marginal zone B cells	$CD21^{+}CD23^{-}$
	Follicular zone B cells	$CD21^{-}CD23^{+}$
T cell development (Thymus)	Double Negative stages (DN)	$CD3^{-}CD4^{-}CD8^{-}$
	DN1	$CD44^{+}CD25^{-}$
	DN2	$CD44^{+}CD25^{+}$
	DN3	$CD44^{-}CD25^{+}$
	DN4	$CD44^{-}CD25^{-}$
	Immature Single Positive (ISP)	$CD8^{+}CD3^{-}CD69^{-}$
	Double Positive (DP)	$CD3^{-/low}CD4^{+}CD8^{+}$
	$CD8^{+}$ Single Positive (SP)	$CD3^{+}CD8^{+}CD4^{-}$
	$CD4^{+}$ Single Positive (SP)	$CD3^{+}CD4^{+}CD8^{-}$
T cell lymphoid population (Spleen)	Total T cell population	$CD3^{+}NK1.1^{-}$
	$CD3^{+}CD4^{+}$ SP	$CD3^{+}CD4^{+}CD8^{-}$
	Memory T cells	$CD4^{+}CD8^{-}CD62L^{low}$
	Naïve T cells	$CD4^{+}CD8^{-}CD62L^{+}CD25^{-}$
	Naïve Tregs	$CD4^{+}CD8^{-}CD62L^{+}CD25^{+}$

RESULTS

Effect of dietary restriction and fasting on body weight and cellularity of lymphoid organs

In the AL group the body weight increased by an average of 9% (from 24.5 +/- 1.2 to 26.7 +/- 0.9 g) (Fig.1A), while the mean body weight of mice on DR decreased by an average of 6% (from 25.5 +/- 0.9 to 24.2 +/- 0.8 g) during the two-week observation period. Three days of fasting reduced weight by approximately 20% (from 26.1 +/- 2.1 to 20.8 +/- 0.8 g). Despite the significant weight loss, no mortality or morbidity was observed solely due to the dietary regimens.

The total number of cells in the femur and tibia of the AL mice was 60x106, of the DR group $63x10^6$, and the FA group $60x10^6$ (Fig.1B). While in the thymus the total number of cells was significantly reduced in the DR group ($43x10^6$ cells) and in the FA group ($19x10^6$ cells) compared to the AL group ($83x10^6$ cells) (Fig.1C). The total number of cells in the spleen was significantly increased in the DR group ($69x10^6$ cells), compared to the AL group ($61x10^6$ cells) while it was significantly reduced in FA group ($31x10^6$ cells) (Fig.1D). In mesenteric lymph nodes (mLN), the total cell count in the DR group was not different from the AL group ($6.8x10^6$ cells vs. $7.1x10^6$, respectively) while in the FA group, it was found to be significantly decreased ($2.9x10^6$) compared to both AL and DR groups (Fig.1E).

The ratio of organ cellularity to the weight of the organ was calculated and was found to be significantly decreased in both the DR and FA group for the spleen as well as the thymus. In the spleen, the ratio in the FA group is also significantly lower than in the DR group. For the thymus, DR and FA ratios are not significantly different. The results of B and T cell numbers and specific subtypes do not always reflect the patterns of the ratios, and thus the proportional reduction of organ weight does not explain all immunological results. An interesting finding is that the organ weight/body weight ratio actually shows an increase for DR and FA, with a significant difference between DR and FA (the relative organ weight in the FA group being higher) (Fig 1F and G). No reliable weights could be obtained for the mLN, as the number of available mLNs (after dissection from the mesenteric fat) varied per animal.

Fig 1:

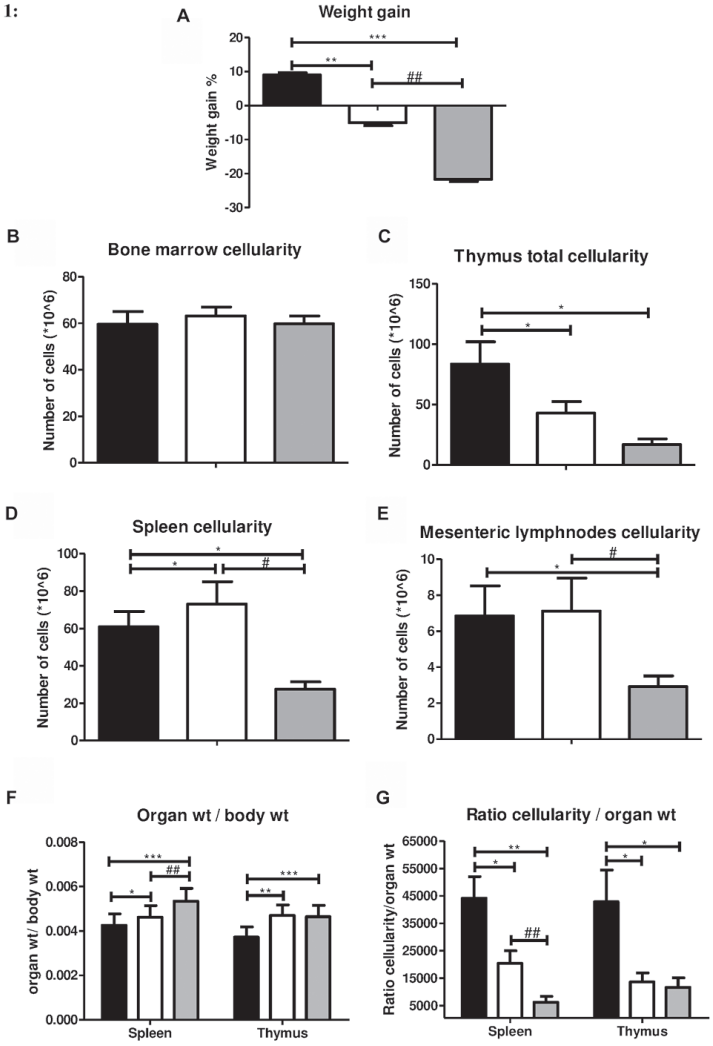

Figure 1: The effect of DR and FA on body weight and cellularity of lymphoid organs. *A) Both DR and FA cause a significant reduction body weight while B, C, D, and E show that total cellularity changes take place due to the two dietary interventions. F) shows the ratio of spleen and thymus organ weight to the body weight of the mice. G) shows the ratio of spleen and thymus cellularity to their respective organ weights. Fig 1A ** = p<0.001, *** = p<0.0005, ## = p<0.001. Fig 1C, D, and E *, # = p<0.05. Fig 1F * = p<0.05, **,## = p<0.005, *** = p<0.0001. Fig 1G * = p<0.05, **,## = p<0.005. n=8/group Ad libitum, 2 weeks 30% DR and 3 days fasting groups are represented by black, white and grey box, respectively. BM – 1 hind leg*

Dietary restriction and fasting modulate B cell development

B-lymphocytes develop in the BM from a common lymphoid progenitor cell, they start differentiating from pro-B cells ($CD43^+CD2^-IgM^-IgD^-$) through pre-B cells ($CD43^-CD2^+IgM^-IgD^-$), and immature B cells (IgM^+IgD^{low}) to recirculating mature B cells ($IgM^{+/low}IgD^{high}$) (Table 1). The total number of $CD19^+B220^+$ B cells found in the AL and DR group was $8x10^6$ while in FA group it was significantly reduced to $3.4x10^6$ (Fig.2A). The population of surface IgM^- B cell precursors in AL mice was found to be $5x10^6$ cells while in DR and FA mice this population was reduced significantly to $3.5x10^6$ and $0.5x10^6$, respectively. Out of the total number of surface IgM^- B cell precursors, the number of $CD43^+CD2^-IgM^-IgD^-$ pro-B cells was calculated to be $0.5x10^6$ in the AL group and 0.4x106 in the DR group. After FA pro-B cells were significantly reduced to $0.04x10^6$ cells (Fig.2B). The $CD43^+CD2^+IgM^-IgD^-$ pre- B cell population was 4x106 cells in the AL group and was significantly reduced to $2.5x10^6$ cells in the DR group. After FA these numbers were reduced by ten-fold to $0.4x10^6$ cells (Fig. 2C).

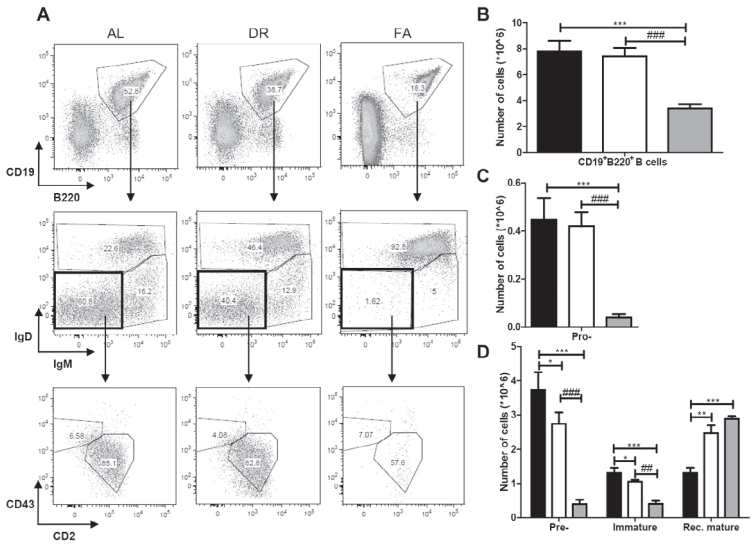

Figure 2: Effects of dietary restriction on B cell development phenotype in the BM. *A) The first panel of FACS plots represents the plots of CD19 and B220 from the three dietary regimen groups. The second panel shows IgD and IgM populations from $CD19^+B220^+$ B cell fraction with IgM^-IgD^- populations (pro-B + pre-B cells), IgM^+IgD^{low} (immature B cells) and $IgM^{+/low}IgD^{high}$ (recirculating mature) B cells. The third panel represents the $CD43^-CD2$ profiles of IgD^-IgM^- fractions and distinguishes between pro-B ($CD43^+CD2^-IgM^-IgD^-$) and pre-B ($CD43^-CD2^+IgM^-IgD^-$). B) Represents the total B-lineage population ($CD19^+B220^+$) in BM. C, D) shows the changes in the B cell development phenotype where both DR and FA cause significant phenotypic alternations in the development stages. Fig 2B and C *** = p<0.0002, ### = p<0.0004. Fig 2D *, # = p<0.05, **, ## = p<0.004, *** = p<0.0003. n=8/group. Ad libitum, 2 weeks 30% DR and 3 days fasting groups are represented by black, white and grey box, respectively.*

We then investigated the phenotypic changes in the immature-, and recirculating mature B cell populations. The total number of immature B cells ($IgM^{+}IgD^{low}$) was $1.4x10^6$ in AL mice, $1.1x10^6$ cells in DR, and $0.41x10^6$ cells in FA mice. The recirculating mature B cell ($IgM^{+/low}IgD^{high}$) population was 1.5x106 in the AL group while it was significantly increased to $2.7x10^6$ and $3x10^6$ cells in the DR and FA groups respectively (Fig.2D).

Thus, DR and FA both cause a significant reduction in the pro-B, pre-B and immature-B cell population and a significant increase in recirculating mature B cells in the BM. Compared to DR, FA has a more pronounced effect.

Dietary restriction and fasting reduce specialized B cell subsets in secondary lymphoid organs

In the spleen we investigated B cell subtypes such as marginal zone (MZ) and follicular (FO) B cells as well as IgM and IgD low as well as high populations (Table 1). The total $CD19^{+}B220^{+}$ B cell population was $32x10^6$ cells in the AL group and $29x10^6$ in the DR group. However, in the FA group the number of B cells was significantly reduced to $11x10^6$ (Fig.3B). The population of $IgM^{low}IgD^{low}$ cells out of the total fraction of $CD19^{+}B220^{+}$ B cells in the AL group was $1.8x10^6$ and $1.2x10^6$ in the DR group. In the FA group it was significantly reduced to $0.4x10^6$ cells. The number of $CD19^{+}B220^{+}IgM^{+}IgD^{-}$ immature B cells in the AL group was $5.8x10^6$, whereas the count in the DR group was $4x10^6$. In the FA group the population was significantly reduced to $1.2x10^6$ cells. The population of $CD19^{+}B220^{+}IgM^{+}IgD^{+}$ mature B cells in the AL and DR groups was similar in size (~$24x10^6$ cells and $21x10^6$ cells, respectively), whereas this population was reduced significantly to $10x10^6$ cells in the FA group (Fig.3C). We also investigated the $CD19^{+}B220^{+}CD5^{+}$ B1 B cell population, which was found to be $1.4x10^6$, $1.3x10^6$ and $1.1x10^6$ cells in AL, DR and FA groups, respectively (data not shown). B cell subtypes such as $CD21^{+}CD23^{-}$ MZ and $CD21^{-}CD23^{+}$ FO B cells were also measured in the dietary restricted groups and were all found to be decreased after fasting, while no significant changes were observed after DR (Fig.3D).

Thus, fasting causes a significant reduction in the total $CD19^{+}B220^{+}$ B cell population as well as in IgM, IgD, marginal zone (MZ) and follicular B cell sub-populations in the spleen, while DR has no significant effect. However, distribution over mature/immature on MZ/FO is not affected.

Analysis of B cell sub populations in the mLN revealed significant changes in the populations of $CD19^{+}B220^{+}$ B cells, which were observed to be $2.5x10^6$, $2x10^6$ and $0.6x10^6$ in AL, DR and FA, respectively (Fig.S1).

Thymic T cell development is arrested by dietary restriction and fasting

T cell development (thymopoiesis) and differentiation occur in discrete stages starting with double negative stages (DN) in the thymus. In mice, it has been reported that there

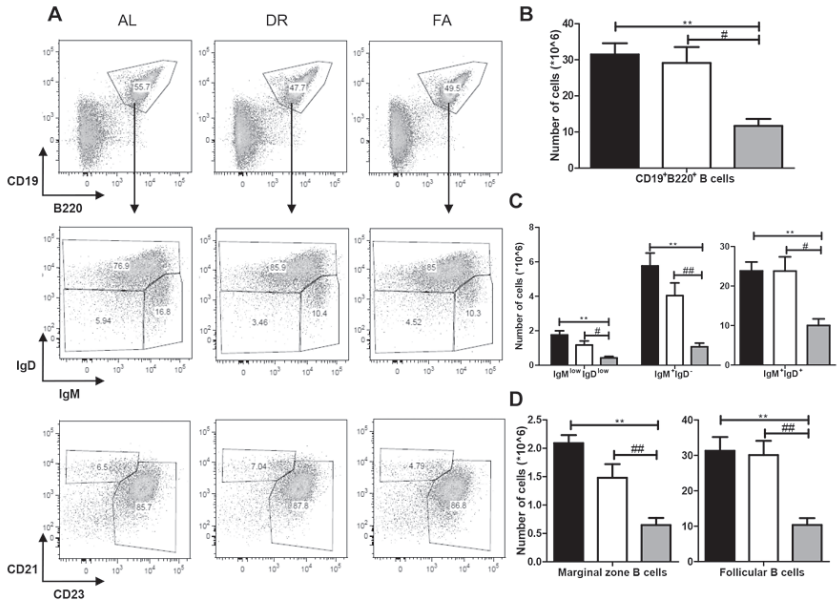

Figure 3: Effects of dietary restriction on the splenic B cell subtypes. *A) In the first panel, FACS plots of splenic cells plotted against CD19 and B220 in the dietary interventions groups, while the second panel shows the IgM/IgD profiles of gated $CD19^+B220^+$ fractions. The third panel represents the CD21/CD23 profiles of the $CD19^+B220^+$ fractions. B) Shows the total $CD19^+B220^+$ B cell cellularity in spleen. C) Shows that $IgM^{low}IgD^{low}$ was significantly affected by FA but not by DR. A similar trend was also observed with respect to cellularity of immature IgM^+IgD^- and mature IgM^+IgD^+ populations. D) Represents the cellularity changes in $CD21^+CD23^-$ marginal zone and $CD21^-CD23^+$ follicular B cells. Fig 3B ** = p<0.001, # = p<0.05. Fig 3C # = p<0.05 **, ## = p<0.001. Fig 3D ** = p<0.002, ## = p<0.005. n=8/group. Ad libitum, 2 weeks 30% DR and 3 days fasting groups are represented by black, white and grey box, respectively.*

are four DN stages distinguished by the expression of CD25 and CD44 surface markers (Table 1): $CD44^+CD25^-$ for DN1, $CD44^+CD25^+$ for DN2, $CD44^-CD25^+$ for DN3 and $CD44^-CD25^-$ for DN4 phenotypic stages. When we gated the $CD4^-CD8^-$ total DN population, we found a decreased number in both DR and FA groups ($1.5x10^6$, $1x10^6$ respectively) compared with $2.6x10^6$ in the AL group. We observed the same trend when we gated the total DN $CD3^-$ T cell population, which was found to be decreased in DR and FA groups from $1.8x10^6$ in AL to $1x10^6$ and $0.7x10^6$, respectively. We studied the DN stages from this DN $CD3^-$ T cell population. No significant changes were observed in the population of DN1 stage while the other DN stages were found to be decreased due to both DR and FA (Figure 4A, 4C). The size of the DN2 population in the AL group was $0.2x10^6$ while this was $0.14x10^6$ in the DR group and $0.04x10^6$ in FA group. Similarly, the DN3 population in DR and FA was reduced from $0.44x10^6$ cells in the AL group to $0.35x10^6$ cells in the DR group to $0.14x10^6$ cells in the FA group. The DN4 populations

also showed a similar decrease with 1x10^6 cells, 0.41x10^6 cells and 0.34x10^6 cells in AL, DR and FA group respectively (Fig.4C).

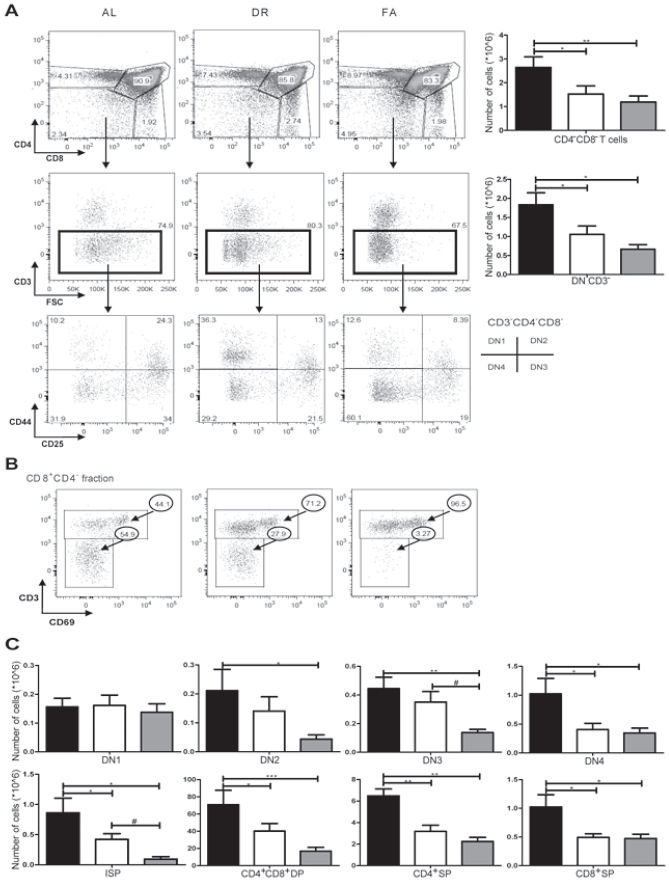

Figure 4: Effects of dietary restriction on T cell development phenotype in thymus *A) FACS plot of CD4 against CD8 in the first panel and the corresponding CD4$^-$CD8$^-$ cellularity graph. The second panel represents the CD3/FSC FACS profiles of CD4$^-$CD8$^-$ DN fractions along with the corresponding cellularity graph of DN CD3$^-$. The third panel of plots shows CD44/CD25 profiles of DN CD3$^-$ fraction. This panel shows the DN1-DN4 stages in the dietary intervention groups. B) FACS plots show the expression of CD3/CD69 profiles of CD8$^+$CD4$^-$ fractions representing the CD3$^-$CD69$^-$ISP and CD3$^+$CD8$^+$ SP cells C) The upper and lower panel of graphs show the phenotypic changes taking place during the development stages starting from DN stages to CD3$^-$CD69$^-$ ISP, CD4$^+$CD8$^+$ DP and CD4$^+$ SP and CD8$^+$ SP. Fig 4A and C *, # = p<0.05, ** = p<0.001, *** = p<0.007. n=8/group. Ad libitum, 2 weeks 30% DR and 3 days fasting groups are represented by black, white and grey box, respectively.*

After the DN stages, thymocytes which start CD8 upregulation progress to immature single positive (ISP) $CD8^+CD3^-CD69^-$ thymocytes. The number of $CD8^+CD3^-CD69^-$ ISP cells in the thymus was $0.86x10^6$, $0.42x10^6$, $0.09x10^6$ in AL, DR and FA groups, respectively, while that of mature CD8 single positive (SP) was $1x10^6$ in the AL group, $0.5x10^6$ in both DR and FA groups (Fig.4B, 4C).

Once the cells have passed through the ISP stage they progress from double positive (DP, $CD4^+CD8^+$) to single positive (SP) stage ($CD4^+CD8^-$ or $CD4^-CD8^+$). The number of DP cells was $71x10^6$ in the AL group and was significantly decreased in the DR ($40x10^6$) and FA groups ($17x10^6$). We also found a significant decrease in SP $CD4^+$ T cells from $7x10^6$ in the AL group to $3.2x10^6$ in the DR group and to $2.2x10^6$ in the FA group. Similarly, the SP $CD8^+$ T cell population was found to be $2x10^6$, $1x10^6$ and $0.7x10^6$ in AL, DR and FA groups, respectively (Fig.4C). Thus, DR and FA both cause a significant inhibition of thymocyte development, with severely reduced numbers of thymocyte subpopulations from the DN2 and DN4 stage onwards.

Fasting reduces the number of $CD3^+NK1.1^-$ T cells and Tregs in the spleen and mesenteric lymph nodes

We investigated naïve, memory and naïve regulatory T cells (Tregs) which are known to be distinct cell populations residing in the spleen. We first gated on the true $CD3^+NK1.1^-$ T cell (to distinguish $CD3^+$ T cells from NKT^+ cell population, Fig.5A). The total $CD3^+NK1.1^-$ T cell population in AL group was $16x10^6$ cells, and $27x10^6$ cells in the DR group. In the FA group there was a significant reduction to $10x10^6$ cells (Fig.5B). Out of the total $CD3^+NK1.1^-$ T cell population we plotted for $CD4^+CD8^-$ T cells (Fig.5C) and found that there was a significant decrease in the FA group ($5x10^6$, respectively) as compared to $9x10^6$ in the AL group, while counts in the DR group remained unchanged. The same could be demonstrated for $CD4^-CD8^+$ T cell population, which was found to be increased in the DR group and decreased in the FA group in comparison to the AL group ($6x10^6$, $10x10^6$ and $5x10^6$, respectively). When we plotted CD62L/CD25 profiles from CD4+CD8- fractions, we found that the percentages of CD62Llow memory T cells were decreased in the DR group, while no significant difference was observed in the FA group. However, with respect to $CD62L^+CD25^-$ naïve T cells, we found an increased percentage in the DR group and a decreased percentage in the FA group, although differences were small. For $CD62L^+CD25^+$ naïve Treg, we found a significantly increased percentage in the FA group (Fig.5D). The T- to B cell ratio in the spleen was significantly increased in the FA group while no changes were observed in the DR group (Fig.S2). Thus, FA causes a significant reduction in the $CD3^+NK1.1^-$ T cell population together with an increase in the number / percentage of Tregs. T cells in the mLN showed a similar trend as observed in the spleen (Fig.S3).

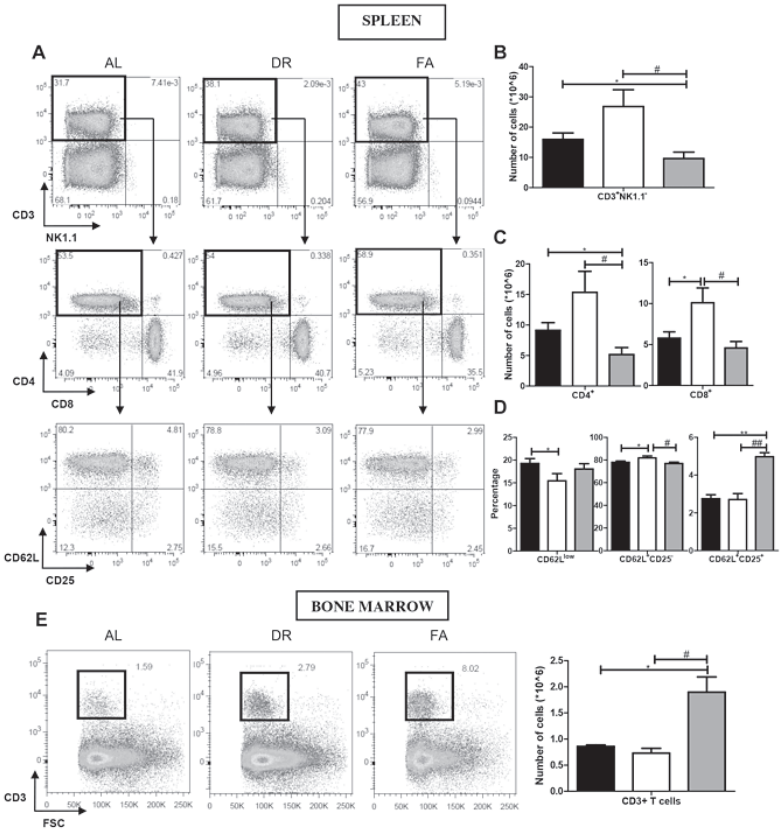

Figure 5: Effects of dietary restriction on spleen and BM T cell lymphoid populations. *A) The first panel represents the FACS plot of the total CD3⁺NK1.1⁻ T cell population in spleen. The second panel of FACS plots show the CD4/CD8 profiles of the gated total CD3⁺NK1.1⁻ fractions while the third panel of FACS plots represents the CD62L/CD25 profiles of the total CD4⁺CD8⁻ fractions. B) The graph represents the total CD3⁺NK1.1⁻ T cell cellularity. C) Shows the total number of CD4⁺ and CD8⁺ T cells. D) Shows the percentages of CD62L^{low} memory T cells, CD62L⁺CD25⁻ naïve T cells and CD62L⁺CD25⁺ Tregs populations. E) Shows the CD3⁺ T cell changes taking place in BM. Fig 5B * = p<0.01, # = p<0.05. Fig 5C *** = p<0.0005. Fig 5D *,# = p<0.05, **,## = p<0.001. Fig 5E *,# = p<0.02. n=8/group. Ad libitum, 2 weeks 30% DR and 3 days fasting groups are represented by black, white and grey box, respectively.*

Dynamics of T cells in bone marrow

Although the total number of CD3⁺ T cells in BM is normally quite low, we found significant changes in both of the dietary intervention groups. The total number of CD3⁺ T cells in BM in the AL group was 0.86x10^6, which was decreased to 0.73x10^6 cells in the DR group and significantly increased to 1.9x10^6 cells in the FA group (Fig.5E)

DISCUSSION

Dietary restriction has not only shown to be effective in reducing the major inflammatory disturbances in ageing-related diseases but has also shown to increase stress resistance and suppress organ damage and inflammation following toxic or ischemic insults. We previously investigated the effect of two weeks of 30% DR and three days of fasting on IRI in the kidney and liver and found that the inflammatory reaction was strongly suppressed [19]. To assess the involvement of the immune system in the beneficial effects of DR, we investigated the effect of these dietary restriction regimes on lymphoid and myeloid cell populations in all the major primary and secondary lymphoid organs, in the present study. The regimen of two weeks of 30% DR and three days of fasting causes a decrease in the total $CD19^+B220^+$ B cell population in BM. These dietary interventions also cause depletion of all B cell development stages, and induce an increase in IgM^+IgD^+ B cells in the BM, which are B cells that return to the BM after they have completed their maturation process in the periphery [20]. Since we see an increased phenotypic population of B cells in the BM due to both dietary interventions, we hypothesize that this is related to the depletion of the same set of B cells from the spleen and mLN and suggests either recruitment of peripheral B cells to the BM or that the increase may be due to changes in cellular migration or homing. Apparently, dietary interventions are causing BM to save more energy by halting the production of more B cells and concomitantly directing the BM to spare its energy to maintain its metabolic imbalances.

DR and FA both induced a depletion of immature, transitional B cells ($CD19^+B220^+IgM^+IgD^-$) as well as mature B cell populations ($CD19^+B220^+IgM^+IgD^+$) in the spleen. We have also observed that FA causes significant depletion of marginal zone and follicular B cells. The same holds true for B cell changes taking place in mLN. Hence, all the splenic B cell sub-populations were observed to be significantly reduced in the FA group compared to both AL and DR, demonstrating that FA has a more pronounced effect on the B cell population in the spleen as compared to that of DR. The reason behind FA having a more pronounced effect than DR is not yet clear. We hypothesize that these are two different types of dietary interventions in which the mice are subjected to two weeks of 30 % DR and three days of water-only fasting. One explanation may be the difference and duration of the two interventions. The weight loss observed in the DR group is greater during the first few days after which the weight is returned to normal suggesting that they acclimatize and adjust to the amount of food they receive. However, the mice in the FA group constantly lose weight for three days and we have noticed the fact that they also dehydrate themselves by not drinking water. We have learned that both these dietary interventions are two different types of stressors leading to pronounced immunological changes due to FA and in a lesser extent due to DR. The exact mechanism behind this phenomenon still merits further investigation, as both interventions lead to robust protection of renal IRI [19]. It is not yet clear where the depleting B cells from spleen and

mLN migrate to during the event of dietary interventions but one of the hypotheses is that these cells are (partially) recruited to the BM, as mentioned before. Alternatively these cells may move to the Peyer's patches, which are a component of the gut-associated lymphoreticular tissues (GALT), and are the major site of antigen uptake to induce mucosal secretory-IgA antibody responses [21].

In the thymus both DR and FA cause major changes in T cell development. Both these dietary interventions cause depletion of the T cells starting at the DN stage and progressing through the ISP and DP stages. Thus, dietary interventions arrest the development and maturation of T cells and hence arrest thymopoiesis. FA also causes an increase in mature T cells in the BM which is an unusual phenomenon, and we are currently investigating the explanation thereof.

We have already mentioned the beneficial effects of dietary interventions not only in ageing-related diseases but also in organ transplantation. Information currently recovered from studies, especially in the field of organ transplantation (IRI), demonstrates that there is an enormous amount of infiltration and activation of the lymphoid cells causing damage to the organ. We find that dietary interventions cause a depletion in pro-, pre-, and immature B cell populations, while they increase the population of recirculating mature B cells in BM which may be a mechanism by which DR and FA cause robust protection. From the various IRI models using mice with defects in T/B cell maturation, we have also learned that these mice are protected against severe effects of IRI. Rabb et al. showed that athymic nu/nu mice were protected from acute kidney injury and upon adoptive transfer of T cells into these mice renal injury was reconstituted following IRI. This demonstrated that T cell deficiency conferred protection from acute kidney injury in this strain [22]. Several other groups have also illustrated that specific T cell subtypes for examples, RORγt+ T cells [23], CD4/CD8$^{-/-}$ [24] and γδ T [25] cells play an important role in the pathophysiology of IRI. We are currently investigating the effects of DR and FA on different unconventional T cell subsets and whether these effects may be related to the protection by dietary interventions.

Besides the importance of T cells in IRI, Rabb's group has shown that B cell deficient mice (μMT (Igh-6tm1Cgn) mice) are protected against IRI as well, thereby confirming the role of B cells in IRI [26]. We are not aware of published studies that address different B cell subtypes and their role in IRI. From our studies we conclude that there is a reduction in both B (all subtypes) and T cells in the spleen induced by DR and FA (which may be protective against renal IRI), in accordance with Rabb's findings.

Tanaka et al. [27] stated that deprivation of nutrition induces atrophy of the thymus and spleen and also reduces the number of circulating B and T cells which is what we observed in our mouse model of DR and FA. The major factors responsible for these changes in B and T lymphocyte population are likely hormones such as corticosterone, for which

serum levels are high in DR and significantly higher after FA [28]. Leptin, which has all the characteristics of a pro-inflammatory cytokine, has been shown to link nutritional status with immune responses and has been demonstrated to play a major role in altering the B cell development during FA [27,29]. A recent study by Fujita et al., also report the role played by leptin in B cell homeostasis by inhibiting apoptosis. They showed that after FA for 60 hours there is an increased rate of B cell apoptosis to ~14%. However, after leptin administration during the FA period, a proportional reduction in the apoptotic cells was observed. This can most likely be attributed to the stabilization of metabolic and endocrine disturbances induced by FA regimen [30]. Together these data suggest that apoptosis may be a mechanism by which the loss of lymphocytes during DR can be explained.

The mechanism behind life-span extension through DR has been primarily reported to be due to the nutrient-sensing pathways [31]. As reported by these authors, many of the mutations that extend life span decrease activity of nutrient-signaling pathways, including the mammalian target of rapamycin (mTOR) pathways suggesting induction of a physiological state similar to that resulting from periods of food shortage. Other research groups have also highlighted the importance of the mTOR pathway in increasing the life span through DR via treatment with rapamycin which forms an inhibitory complex with TOR kinase and prevents proteins synthesis and cell growth [32,33]. A recent publication by Yilmaz OH. et al. has demonstrated that DR/CR acts by inhibiting activity of mTOR complex 1, of the intestinal stem cells thereby resulting in promoting a more favourable stem-cell microenvironment [34]. However, no mechanism is known with respect to the effect of DR on B and T cell development. A study by Zhang et al. has shown that mTOR inhibition affects T cell differentiation and function by a decrease in the number of thymocytes and CD4+, CD8+ T cell populations in spleen. It is also shown that inhibition of mTOR causes alterations in T cell activation, trafficking, or homeostasis and differentiation. mTOR inhibition does not only affect T cells; also B cells are affected by mTOR inhibition. Experiments performed in mTOR inhibition model showed that both the numbers and percentages of B220+ B cells have been reduced in the spleen and BM [35], one of the phenomena that we observed in our DR and FA model. This suggests that alterations in B and T cell populations observed due to DR and FA may act through the inhibition of mTOR although a thorough mechanistic study needs to be performed.

Engagement of both the innate and adaptive immunity in IRI has been comprehensively reviewed in the article by Rabb et al. Based on numerous studies highlighted in the article, a robust inflammatory process engages cells and elements of both the innate and adaptive immune responses in causing initial organ injury and mediating long-term structural changes, suggesting a complex role for the immune system [36]. We hypothesize that DR and FA act as a low-level stressor inducing basic protective mechanisms that modulate inflammatory responses to harmful danger signals as well as bringing about alterations in B and T cell development. These dietary interventions also cause recruitment

of recirculating mature B cells and $CD3^+$ T cells to the BM, possibly contributing to the organ saving more of its energy by not producing more of the B cells and thereby directing the BM to more energy consumption/storage. Hence, both DR and FA cause major alterations in B and T cell development and differentiation in primary and secondary lymphoid organs. The immunological effects observed due to DR are not the same as those observed due to FA, although both DR and FA are protective against the adverse effects of renal IRI. These different immunological outcomes suggest that different mechanisms of action may play a role in the protection against IRI, and that the immunological effects of the dietary interventions may not be crucial in the protective effect nor that these are bystander effects. This clearly merits further investigation.

It was shown by Fernandes et al. in a murine model that DR caused an increase in lifespan, even after infection with murine AIDS [37]. Influenza infection has been known as one of the diseases that has a major impact on elderly human health. Effros et al. demonstrated that DR had a protective effect against influenza infection in aged mice [38]. This implies that the function of the immune system in diet restricted mice is not compromised, and that the changes observed due to dietary interventions in our model with respect to B and T cell development may be associated with improved immune function. There have been several more recent studies highlighting the effect of influenza infection in DR treated mice in which decreased survival in DR mice has been observed [39,40]. This raises questions about the relevance of the major phenotypic immunological changes taking place in different immune compartments. The functional consequences of these phenotypic changes are currently unknown. In this context, it has been shown by Pahlavani et al. that DR significantly enhances lymphocyte function as assessed by mitogen-induced lymphocyte proliferation, and attenuates age-related decline in immunologic responses such as cytokine production, antibody response to sheep red blood cells and NK cell activity. Consistent with our findings, it has been reported that DR delays a rise in memory T cells during ageing and thus retains a higher number of naive T cells in aged mice and in this way delays immunosenescence [41].

Dietary restriction is the most efficacious and non-invasive method of causing an increase in lifespan of laboratory animals. The decrease in age-associated diseases is directly correlated to the increased longevity due to DR. Only a few studies have shown that DR brings about these protective effects by modulating changes in the immune system by means of delaying immunosenescence and also by stress resistance. However, the mechanism by which DR exerts its protective effect remains unclear. We have shown that two different short-term dietary interventions cause alterations in all lymphoid compartments. When compared to two weeks of 30% DR, three days of short-term fasting has a more pronounced effect. Both DR and FA halt B and T cell development and also cause recruitment of recirculating mature B and T cells to the BM, which has not been observed previously. Whether these major immunological changes observed after dietary interventions are a bystander effect or directly mechanistically related remains to

be elucidated. Our laboratory is currently conducting research projects determining the functional consequences of these immunological changes and how these alterations may be involved in the beneficial effects of DR on life-span and acute stress resistance.

ACKNOWLEDGEMENT

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SUPPLEMENTARY MATERIALS

Supplemental fig.1

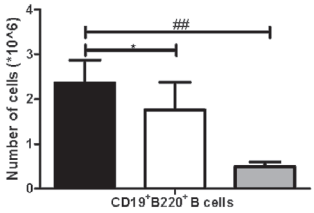

Figure S1: The effect of DR and FA on CD19+B220+ B cells in mesenteric lymph nodes. *Both FA and DR cause a significant decrease in the total B cell as compared to the AL group. * = p<0.05, ## = p< 0.005. n=6/group. Ad libitum, 2 weeks 30% DR and 3 days fasting groups are represented by black, white and grey box, respectively.*

Supplemental fig.2

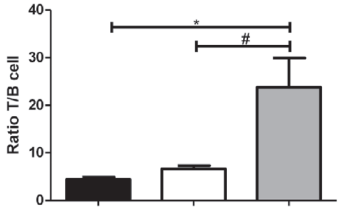

Figure S2: The effect of DR and FA on T/B cell ratio in spleen. *FA causes a significant increase in the T/B cell ratio while DR has no effect as compared to the AL group. *, # = p<0.05. n=8/group. Ad libitum, 2 weeks 30% DR and 3 days fasting groups are represented by black, white and grey box, respectively.*

Supplemental fig.3

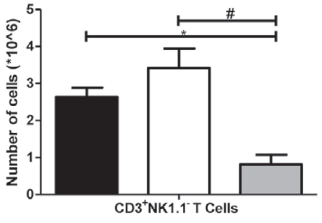

Figure S3: The effect of DR and FA on mesenteric lymph node T cell lymphoid population. *DR does not cause any significant change while FA causes a significant reduction in the total $CD3^{+}NK1.1^{-}$ T cell population as compared to the AL group. *,# = p<0.05. n=6/group Ad libitum, 2 weeks 30% DR and 3 days fasting groups are represented by black, white and grey box, respectively.*

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CHAPTER 4

Mannan-binding lectin is involved in the protection against renal ischemia/ reperfusion injury by dietary restriction

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ABSTRACT

Preoperative fasting and dietary restriction offer robust protection against renal ischemia/reperfusion injury (I/RI) in mice. We recently showed that Mannan-binding lectin (MBL), the initiator of the lectin pathway of complement activation, plays a pivotal role in renal I/RI. Based on these findings, we investigated the effect of short-term DR (30% reduction of total food intake) or three days of water only fasting on MBL in 10-12 weeks old male C57/Bl6 mice. Both dietary regimens significantly reduce the circulating levels of MBL as well as its mRNA expression in liver, the sole production site of MBL. Reconstitution of MBL abolished the protection afforded by dietary restriction, whereas in the fasting group the protection persisted. These data show that modulation of MBL is involved in the protection against renal I/RI induced by dietary restriction, and suggest that the mechanisms of protection induced by dietary restriction and fasting may be different.

INTRODUCTION

Evidence that long-term dietary restriction (DR; a moderate reduction in calorie intake (20-40%) without causing malnutrition) exerts several beneficial effects in improving health and life-span exists since 1935 [1]. DR has proved beneficial in lowering the incidence of many age-related diseases such as cancer [2,3], cardiovascular diseases, diabetes [4], and abdominal obesity [5]. However, the mechanisms by which DR induces the protective effects have not been elucidated so far. Several mechanisms have been proposed until now, highlighting pathways such as the insulin/insulin-like growth factor signaling pathway, sirtuins [6,7], mTOR pathway [8,9], and nutrient sensing signaling pathways [10–12].

In addition, short-term DR regimens offer robust protection against a wide variety of acute stressors, such as acetaminophen induced liver toxicity [13]. We have shown that both 14 days of 30% DR as well as 3 days of preoperative fasting protect against renal and hepatic ischemia/reperfusion injury [14]. Ischemia/reperfusion injury (I/RI) is a key detrimental event in clinical conditions such as sepsis, cardiovascular surgery, trauma, various forms of infarction, and organ transplantation. It is a multifactorial antigen-independent inflammatory condition which has both immediate and long-term effects on the allograft [15]. I/RI exerts its deleterious effects by inducing renal cell death, renal failure, and may result in delayed graft function and renal graft rejection [16]. Acute kidney injury, which is the functional consequence of I/RI, is associated with substantial morbidity and health care expenditures [17,18]. Despite advances in renal replacement therapy, the mortality of patients with renal I/RI and morbidity of transplantation related renal I/RI remain high without specific therapy.

Different immunological players (both of the innate and adaptive immune system) involved in I/RI have been studied, such as leukocyte adhesion molecules, lymphocytes, regulatory T lymphocytes, and the complement system [19–22]. Several studies have documented the activation of complement system as one of the hallmarks of renal I/RI [21,23,24].

The complement system is one of the central components of innate immunity, consisting of three activation pathways: the classical, alternative and lectin pathway (MBL pathway). Involvement of the MBL (Mannose Binding Lectin) pathway in the pathogenesis of renal I/RI has been demonstrated by several studies in rats, and deposition of MBL in the kidney has been observed after I/RI [25–27]. In MBL-deficient mice, the lack of MBL has been shown to be important in protecting against the adverse effects of renal I/RI with significantly less renal damage [26]. Recently, we demonstrated a pivotal role for MBL in the pathogenesis of renal I/RI; MBL was shown to be directly cytotoxic to tubular epithelial cells independent of complement activation. Upon reperfusion of the ischemic kidney, vascular leakage exposed tubular epithelial cells to circulation-derived MBL,

which contributed to tubular injury [28]. Together these data prompted us to investigate the role of MBL in the protection afforded by dietary restriction. We here show that modulation of MBL levels is involved in the protection induced by DR, but not by fasting.

MATERIALS AND METHODS

Animals

Male C57/Bl6 mice (10-11 weeks old), purchased from Charles River Laboratories (Maastricht, the Netherlands), were kept at specific-pathogen free and normal physiological conditions (temperature 20-24°C, relative humidity 50-60%, 12hr light/dark period) to acclimatize for one week. Free access to food and water was allowed to these mice until the start of the experimental procedures. All the experimental procedures were performed after the approval of the university animal experiments committee (Dutch Animal Ethical Committee, Protocol no. 105-12-12) in accordance with the Dutch National Experiments on Animals Act, complied with Directive 2010/63/EU of the Council of Europe.

Dietary regimen

Mice were divided in three groups; ad libitum (AL), 2 weeks 30% dietary restriction (DR) and 3 days water-only fasting (FA) with n=6 animals/group. AL mice were allowed free access to food and water while the DR (n=3 per cage) group's food intake was weighed daily for the first week and 30% DR was carried on by providing 70% of the food that the mice consumed in the previous week (approximately 7.7 g per day). At the start of the experiment with the FA group, mice were transferred into a new clean cage at the end of the day with free access to water but no food.

Experimental set up

After the mice had finished the three dietary regimens, they were divided in two different experimental groups to answer the following questions of our study.

Question I: The main aim of this phase of the experiment was to elucidate the effect of DR and FA on the MBL pathway activity and on the MBL complement factor production in kidney and liver. In this phase, mice were divided into the three dietary intervention groups (n=6/group) and were fed AL, DR and FA. Afterwards, the dietary intervened mice were sacrificed through exsanguination by cardiac puncture. The blood was stored in a serum separator tube, containing a gel separator and clot activator, and the tube was put on ice for serum isolation. This freshly drawn blood was centrifuged at 3000g for 10 min at 4oC, after which the serum was aliquoted, stored at -80oC and used further for the MBL ELISA assay. Furthermore, kidney and liver tissues were harvested for histology and quantitative PCR (qRT-PCR).

Question II: The second aim combined the dietary interventions (AL, DR, and FA) along with induction of renal I/RI. In this particular phase of the study, MBL was administered using human MBL (hMBL) in the dietary intervened renal I/RI induced mice and in AL fed sham-operated mice to study the effect of intraperitoneally-administered MBL in the dietary intervention mediated protection against renal I/RI.

Quantification of MBL by ELISA

For the measurement of MBL-A and -C concentrations, ELISA kit from Hycult biotech (Uden,The Netherlands) (MBL-A catalog no. HK208-02 and MBL-C catalog no. HK209-01) were used in which the microtiter wells were pre-coated with the respective antibody. Samples were diluted (1:500 for MBL-A and 1:1000 for MBL-C) followed by addition of the antibodies and reagents according to the manufacturer's instructions.

Induction of renal I/RI

Induction of renal I/RI was performed bilaterally. Bilateral occlusion was performed after the dietary interventions. Mice were anaesthetized by isoflurane inhalation (5% isoflurane initially followed by maintenance on 2.5% with oxygen). Body temperature of the mice was maintained by placing them on heating pads until recovery from anesthesia. A midline abdominal incision was followed by localization of the left renal pedicle and dissection of the renal artery and vein. The left kidney was occluded using an atraumatic microvascular clamp for 37 min. For bilateral occlusion, the procedure was repeated immediately on the right kidney. After the sign of ischemia (purple color) was observed, the wound was covered with phosphate-buffered saline (PBS)-soaked cotton and the animals were placed under an aluminum foil blanket for the maintenance of body temperature. The clamps were released after 37 min of ischemia, and restoration of blood-flow was confirmed when the kidney regained its normal color. The abdominal wound was closed in two layers using 5/0 sutures followed by subcutaneous injection of 0.5 mL PBS for maintenance of fluid balance and were kept warm under a heat lamp.

Animal care and humane endpoints

Following DR, FA and surgery, animals were weighed and monitored daily for signs of distress. Animals with decreased body weight of 15% in 2 days along with disturbed behavior and/or locomotion, excessive bleeding while performing surgery and eye abnormality, lethargy, rufled fur and tremors, were euthanized. The animals were euthanized through exsanguination by heart puncture under isoflurane anesthesia. Before surgery all the animals received 0.05 mg/kg of Buprenorphine every 12 hours for 2 days as pain-relieving treatment.

Isolation of RNA and cDNA synthesis

Snap frozen liver tissues (n=6/group of AL, DR and FA mice) were used for RNA extraction using Trizol reagent (Invitrogen) (a monophasic solution of phenol and guanidine isothiocyanate). The tissues were homogenized using ultra thurrax followed by addition

of chloroform, which upon centrifugation for 15min at 12000g and 4oC, separated the solution into an aqueous phase containing the RNA and an organic phase. The top RNA layer was further precipitated using isopropyl alcohol (2-(iso) proponal). 75% ethanol was used to recover DNA and other proteins and to allow RNA to be free from DNA and proteins. After RNA extraction, the quality of RNA (260nm/A280nm) was assessed by measuring the RNA concentration on nanodrop machine (Thermo Scientific, Netherlands). Values obtained between 1.9 and 2.1 were considered to be ideal for the extracted RNA. Long-term storage of the RNA samples was done at -80oC. DNAse treatment of the RNA samples was also performed on 2.2µg of RNA, using DNAse and DNAse buffer, and run on PCR machine (30min at 37oC) followed by stopping the reaction by addition of DNAse stopmix and running the program DNAse off (10min at 65oC). First-strand cDNA synthesis was performed on 2µl of the already prepared cDNA with random primer, using the SuperScript™ II Reverse Transcriptase kit (Invitrogen, Paisley, UK). A total volume of 25µl was used for various real time RT-PCR reactions.

Quantitative (RT)-PCR

Specific primer sequences for mouse Mannan Binding Lectin-A (MBL-A) and Mannan Binding Lectin-C (MBL-C) were as follows: MBL-A (forward, 5'-CAG GGT CAC AAA CCT GTG AG 3'- ; reverse, 5'-TGC AAC TTG TTG GTT AGC TG 3'-), MBL-C (forward, 5'-GAC CTT AAC GAA GGT GTT CA 3'- ; reverse, 5'-CAG TTT CTC AGG GCT CTC AG 3'-). Before the real-time PCR could be performed, each primer sets were first tested for the appropriate annealing temperature by running the temperature gradient on Bio-Rad iQ cycler ranging from 50°C to 64°C. Each of the PCR products was then separated on 3% agarose (for smaller fragments) and on 2% agarose (for larger fragments) TBE gel containing ethidium bromide. PCR was performed using Bio-Rad iQ SYBR Green Supermix (Biorad) containing 0.2 µM of each primer. For each RT-PCR, reactions were prepared for both the complement genes as well as the negative control, which were processed the same way as cDNA but without addition of SuperScript™ II Reverse Transcriptase as a control for genomic contamination. Housekeeping gene for liver (β2-microglobulin) was used to normalize the data with the experimental samples. Real-time Relative quantitative PCR (RT-PCR) was carried out using the Bio-Rad MyiQ Single Color Real-Time PCR Detection System to detect amplification of the PCR products, and the accompanying Optical System Software v1.0 was used to analyse data. Expression of each gene was normalized against mRNA expression of the housekeeping gene β2-microglobulin (liver). Data was analysed using Gene Expression Analysis for iCycler Real-Time PCR Detection System (Biorad) and calculated using ΔΔCt formula ($1.8^{-(\Delta Ctsample-\Delta Ctcontrol)}$). All the samples were tested in duplicate at least two times to confirm the data.

Preparation and reconstitution of human MBL (hMBL)

MBL was purified from human serum as previously described [29]. Before reconstitution of MBL, approximately 200µl of blood was drawn from the tail of all the experimental mice as a control of the effect of hMBL reconstitution. Reconstitution with 20µg hMBL was performed in DR and FA mice (n=6) immediately after induction of renal I/RI. To assess the effect of hMBL on normal kidney function, also AL-fed sham-operated animals received hMBL. The purified hMBL was dissolved in 0.2ml PBS and administered intra-peritoneally (i.p.). DR and FA control group was placebo treated and administered with PBS (i.p.) after induction of I/RI. Subsequently, all mice were monitored for 7 days post-operation (Fig.1). In another experiment, AL, DR and FA mice were reconstituted with hMBL (i.p.) and sacrificed 6 hours post reperfusion. Kidney tissues were harvested from these mice and snap-frozen for hMBL staining.

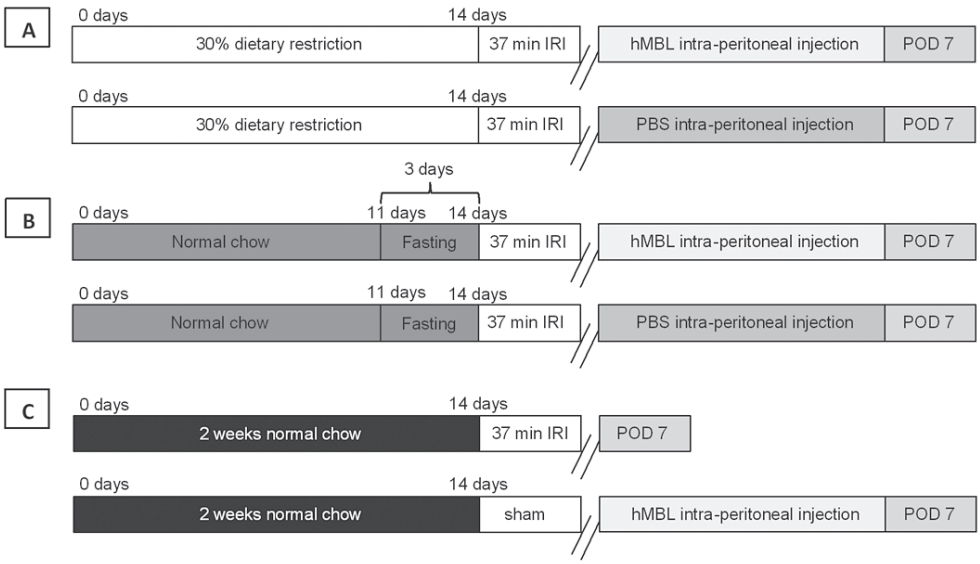

Figure 1: hMBL reconstitution experimental setup. *Three groups of experimental animals divided further into subgroups were used for the reconstitution experiment. A) Shows the first group of DR animals where after 2 weeks period of DR, I/RI was induced and was either infused with hMBL or PBS (control counterpart). B) Shows the second group of FA where after the fasting period of 3 days renal I/RI was induced followed by infusion of either hMBL or PBS. C) Shows the third group of ad libitum controls, which followed the normal chow for a period of 2 weeks. After 2 weeks one of the subgroups underwent renal I/RI while the other subgroup was sham-operated followed by infusion of hMBL. All the three groups were monitored for 7 days post-operation and sacrificed at the end of seventh day.*

hMBL measurement in serum

Circulating levels of hMBL after reconstitution (t=6hrs) were assessed by sandwich ELISA. In brief, 96-wells ELISA plates (Nunc Bioscience, Belgium) were coated with a monoclonal antibody to MBL (mAb 3E7, Hycult Biotech, Uden, The Netherlands). Mouse serum samples were incubated in the coated wells and bound hMBL was detected with a digitonin (DIG)-conjugated monoclonal anti-MBL antibody (mAb 3E7; Hycult Biotech) followed by detection with horseradish peroxidase (HRP)-conjugated sheep anti-DIG (Roche Diagnostics, Mannheim, Germany). Enzyme activity was detected using 2,2-azino-bis3-ethylbenzthiazoline-6-sulphonic acid (Sigma Chemical Co., St Louis, MO, USA). The optical density was measured at 415 nm using a microplate reader (Model 680; Biorad, Philadelphia, PA, USA).

hMBL staining of kidney tissue

Mouse kidney sections (5 µm) of snap-frozen kidneys were air dried and acetone-fixed. Presence of hMBL was assessed using anti-human MBL (mAb 3E7; Hycult Biotech) followed by HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.). The staining was visualized using Nova RED (Vector Labs, Peterborough, United Kingdom). Micrographs were made using a microscope (Leica, DMI6000, Rijswijk, The Netherlands).

Kidney function assessment

Renal function was determined by measuring urea level in serum samples using QuantiChrom assay kits based on the improved Jung and Jaffe methods (DIUR-500; Gentaur, Brussels, Belgium). The assay was measured in a 96-well format at 520 nm on a Varioskan multimode microplate reader (Thermo Scientific B.V., Breda, The Netherlands).

Stastistical analysis

All the data are represented as means with standard error of mean. Non-parametric paired sample T-tests were performed on the three experimental groups using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.), while graph plotting was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA). Significance was defined as a p-value ≤ 0.05.

RESULTS

DR and FA decrease serum MBL-A and -C concentrations

To assess the effect of DR and FA on the MBL pathway, we first measured the serum concentrations of both MBL-A and MBL-C through ELISA. After DR or FA, the serum concentrations of MBL-A were 15.4±0.95 µg/ml and 12.4±0.96 µg/ml respectively, which was significantly lower compared to AL fed mice (19.9±1.25 µg/ml) (Fig.2A). Similar to MBL-A concentrations, MBL-C concentrations were also significantly decreased in both DR (89.4±4.24 µg/ml) and FA (49.5±3.33 µg/ml) groups as compared to AL fed mice (109.6±4.34 µg/ml) (Fig.2B).

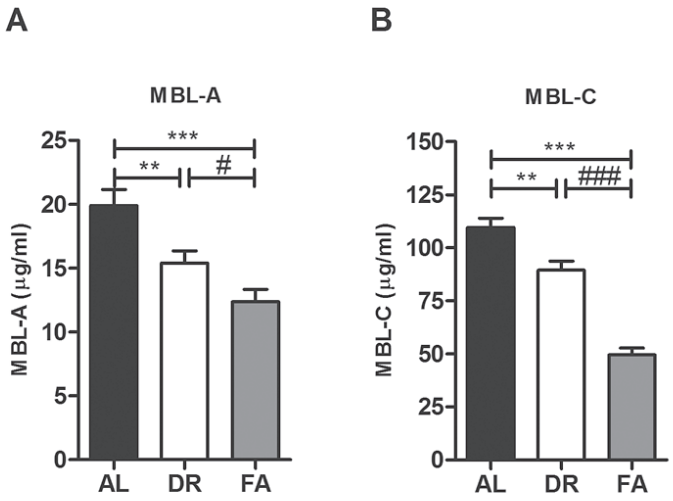

Figure 2: Serum MBL-A and -C concentrations after DR and FA. *A) MBL-A concentrations were reduced after DR (15.4 µg/ml) and FA (12.4 µg/ml) as compared to AL fed mice (19.9 µg/ml). B) Also the MBL-C concentration after DR (89.4 µg/ml) and FA (49.5 µg/ml) was reduced as compared to AL fed mice (109.6 µg/ml). Fig 2A ** = p≤0.009, *** = p≤0.0001, # = p<0.03. Fig 2B ** = p≤0.002, ***, ### = p≤0.0001. n=8/group*

DR and FA cause reduction in mRNA expression of MBL-A and -C in liver

Since the liver is the major source of MBL production, to further elucidate the effect of DR and FA, we investigated the mRNA expression levels of both MBL-A and -C in liver tissue through qRT-PCR. The mRNA expression of MBL-A was significantly reduced in the FA group, and not in the DR group (Fig.3A). However, mRNA expression of MBL-C was significantly reduced in the DR group and not in the FA group (Fig.3B).

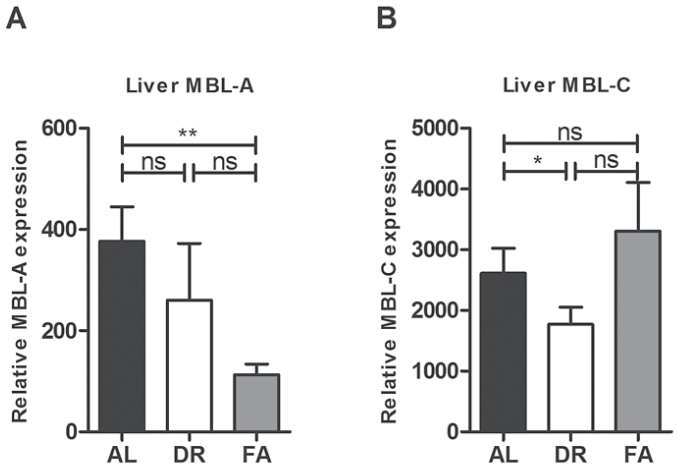

Figure 3: Liver mRNA expression of MBL-A and -C after DR and FA. *A) Shows that after FA the MBL-A mRNA expression is reduced as compared to AL while B) Shows that after DR MBL-C mRNA expression is reduced as compared to AL fed mice. Fig 3A ** = p≤0.005. Fig 3B * = p≤0.01. n=6/group.*

Intraperitoneal administration of MBL breaks the protection against I/RI by DR, but not FA and reinstates renal dysfunction after I/RI

To study the functional effect of the reduced MBL-A and -C mRNA expression and protein concentration in circulation, we performed renal I/RI in mice that underwent FA and DR and subsequently reconstituted with purified human MBL (hMBL), which is able to activate mouse complement as well [30]. Administration of MBL after reperfusion was confirmed by assessing hMBL in circulation 24hrs after intraperitoneal infusion and induction of renal I/RI (Fig.4). Normal levels of hMBL were measured in both the DR (5.29±0.77µg/ml) and FA group (5.4±1.07µg/ml) that received hMBL, whereas the levels of hMBL in PBS-injected animals in these groups were below the detection limit of 0.05 ng/ml.

Induction of renal I/RI resulted in marked renal dysfunction in the ad libitum (AL) group as shown by a significant increase in serum urea levels 24 hours after reperfusion (Fig.5). In addition, 85% of the animals had to be sacrificed because of morbidity indicative of irreversible kidney failure demonstrated by buildup of toxic waste products (urea and creatinine), including weight loss, loss in body temperature, ruffled fur, decreased activity and a hunched body posture. We previously showed that serum urea levels and morbidity correlate with histological tubular damage and inflammation [14].

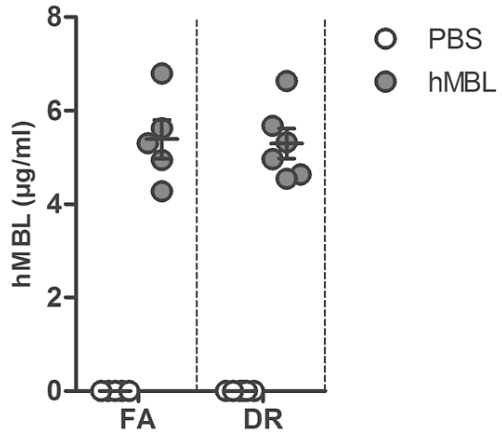

Figure 4. Serum hMBL levels after infusion. *Twenty-four hours post infusion of hMBL, hMBL levels were measured in the serum of the experimental mice. After 24 hours the serum levels of the hMBL were reinstated in the mice confirming presence of injected hMBL in circulation. In the control animals (where only PBS was injected) hMBL levels were measured to be below the detection limit of 0.05ng/ml (n=6/group).*

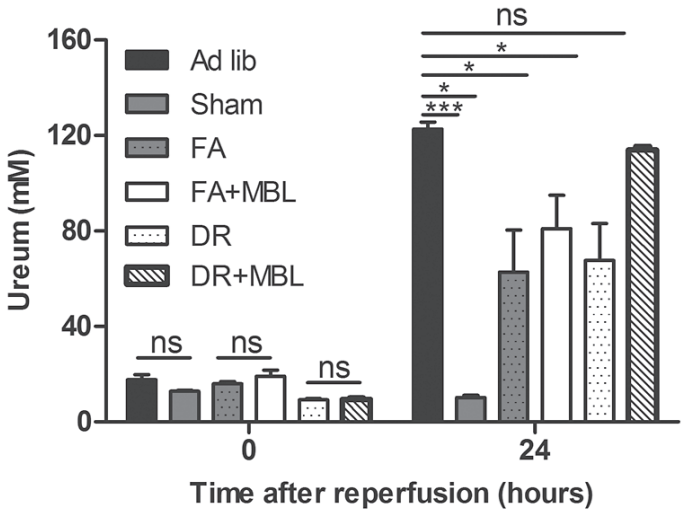

Figure 5. Renal function by serum urea concentration. *Renal function as measured by serum urea concentration of the experimental animals before (t=0) and after (t=24) induction of I/RI and infusion of hMBL. The urea concentration of the DR animals infused with hMBL were found to be highly elevated as compared to its control counterpart as well as compared to FA and AL groups. Fig 5 * = p≤0.05, *** = p≤0.0001. n=6/group*

Both DR and FA preserved renal function and prevented morbidity associated with acute kidney injury following reperfusion. Importantly, administration of purified hMBL broke this protection in the DR group, but not in the FA group (Fig.6), strongly suggesting that the protection by DR is dependent on downregulation of MBL. In the DR group, serum urea levels and. mortality were not different from those in AL fed mice, whereas in FA mice administration of hMBL did not affect urea levels or survival. Sham-operated mice showed no significant difference in renal function after administration of hMBL (Fig.5).

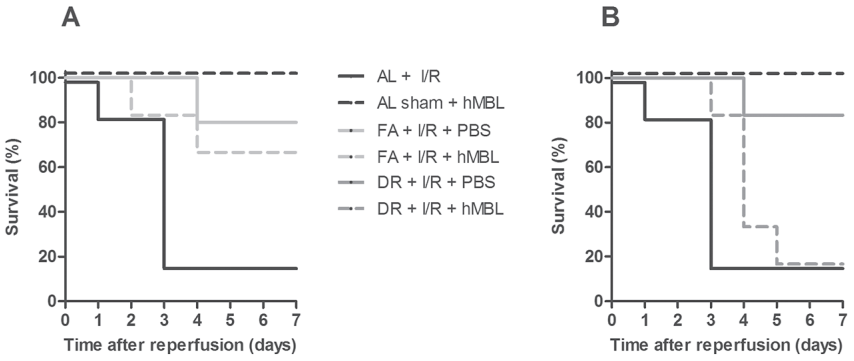

Figure 6. Survival curves of animals after infusion of hMBL and induction of renal I/RI*. A) Represents the survival curves of AL and FA mice after induction of renal I/RI and infusion of hMBL. The bold line in black shows the survival curve of ad libitum control (AL control). The mortality observed in the AL mice was higher than that of the hMBL infused I/RI induced FA mice. Mortality was observed until day 7 post-surgery. B) Infusion with hMBL followed by renal I/RI led to a significant death in DR animals as compared to their control counterparts as analyzed by Kaplan-Meier analysis (log rank test, P < 0.02) (n=6/group).*

DR and FA do not prevent vascular leakage and extravasation of MBL

We previously demonstrated that vascular leakage and extravasation of MBL following reperfusion plays a pivotal role in the induction renal I/RI and that exposure of epithelial cells to MBL immediately following reperfusion is the primary culprit of tubular injury [31]. To assess whether DR or FA, as a protective mechanism, prevent vascular leakage, we here also studied the localization of hMBL after reconstitution and induction of I/RI. Staining of hMBL in the kidney 6hrs after infusion of hMBL and induction of I/RI revealed clear extravasation and interstitial presence of hMBL (Fig.7) in both the DR and FA group. In contrast, kidneys from sham-operated animals only showed staining of hMBL in glomeruli and peritubular capillaries, reflecting circulating hMBL (Fig.7).

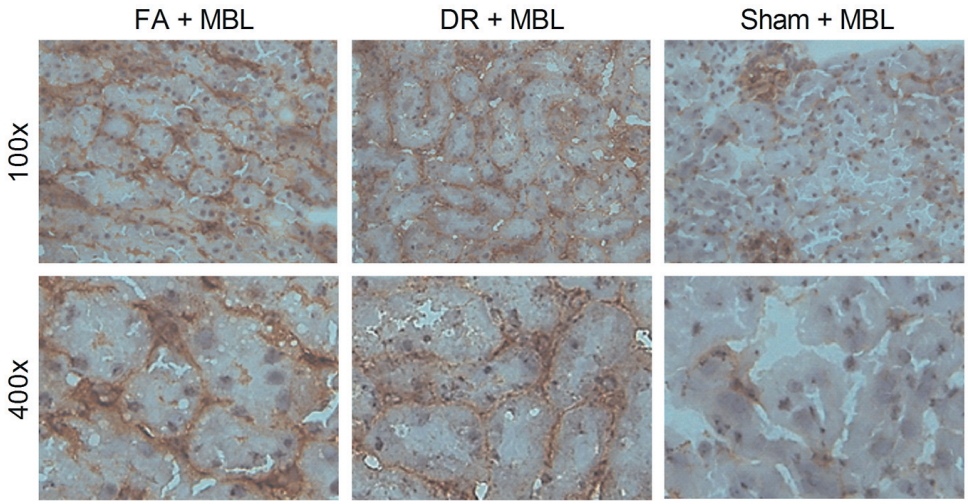

Figure 7. Localization of MBL after infusion of MBL and induction of renal I/RI. *The tissue staining of FA and DR groups 6hrs after infusion of hMBL and renal I/RI shows vascular leakage and extravasation of MBL while in the sham-operated group this is not observed.*

DISCUSSION

The present study demonstrates the involvement of MBL in the protection against renal I/RI by DR. In a mouse model of renal I/RI subjected to DR and FA, we showed that both MBL-A and -C serum concentrations were significantly decreased when compared to those in control AL fed mice. We observed that the protection against renal I/RI by DR was broken by reconstitution of hMBL, while the protection by FA was maintained, indicating that MBL may be involved in the protection against renal I/RI, at least in the DR group.

Several studies have shown that complement plays an important role in the pathophysiology of renal I/RI. In conditions such as myocardial [32], skeletal muscle [31], gastrointestinal [33], cerebral I/RI [34] and renal I/RI [28], accumulating evidence suggests complement activation as an important contributor to the tissue necrosis following ischemia. In case of myocardial infarction and stroke, activation of complement in the area of tissue infarction has been found and inhibition of complement reduces the extent of tissue destruction [35]. Several molecular and cellular pathways of injury caused by complement have been studied which highlight the role played by the terminal pathway products C5a and C5b-C9 [36] as well as the classical and the alternative pathway [21]. More importantly, the role played by the MBL pathway has been studied in

detail [21,26,28,37]. Findings from these studies indicate the detrimental role played by MBL in recognizing structures on the reperfused kidneys after ischemia, which is implicated in tissue injury after I/RI. Also, the study by Orsini et al.[37] establishes that MBL plays a pivotal role in the pathogenesis of not only renal I/RI, but also brain I/RI. Inhibition of MBL using structurally different inhibitors led to protection against I/RI.

MBL recognizes endogenous ligands presented in post-ischemic kidneys, resulting in complement activation during acute kidney injury. In MBL-deficient mice, decreased ischemic damage and better protection against I/RI have been observed. In case of myocardial I/RI, studies have shown that MBL absence imparts significant protection against infarction, as well as that adding MBL restored injury following ischemia [38]. Also, studies by Kristensen et al. show that MBL deposition contributes to the induction of functional damage in renal I/RI; when renal I/RI was induced in MBL double knock-out mice, the mice were found to be protected against I/RI [26]. Recently, we have shown that MBL mediates renal I/RI independent of complement activation. We demonstrated that depletion of MBL in rats preserves renal function following I/RI. When we investigated whether the complement system is also activated during this phase we found that this protection is independent of complement activation. This observation could be because of the kinetics of tubular injury, which is observed at 2-5hrs while complement deposition becomes apparent only after 24hrs. Despite complete inhibition of terminal pathway, no protection by anti-C5 treatment was observed. Taken together, these studies clearly demonstrate the crucial role of MBL in the pathophysiology of I/RI [28].

Vascular leakage and extravasation of MBL following reperfusion play a pivotal role in the induction of renal I/RI and exposure of epithelial cells to MBL immediately following reperfusion is the primary culprit of tubular injury [31]. In the present study, we investigated the localization of hMBL after reconstitution and induction of I/RI and found that there was clear extravasation and interstitial presence of hMBL in both the DR and FA group. These findings indicate that DR does not protect against renal I/RI by preventing vascular leakage and subsequent exposure of tubular cells to MBL, but by decreased circulating levels of MBL. The observation that protection by FA is not mediated by preventing vascular leakage suggests that FA has an effect on tubular cell homeostasis and resistance to tubular injury [14], independent of MBL.

I/RI causes inflammation during the course of reperfusion [39]. Both DR and FA reduce inflammation [40] and cytokine production during I/RI and hence prevent further damage to the organ. We have shown that FA causes reduction in the markers of inflammation such as IL-6 and P-selectin to a significantly lower degree after I/RI as compared to AL [14]. Hence, we hypothesize that the effect of FA on reducing inflammation is greater than that of DR which could lead to DR breaking the protection upon hMBL

infusion while FA not. This observation indicates that there may be different mechanisms of action of DR and FA and as observed by us previously [41]. This could be discussed based on the fact that the method of dietary intervention is different in both "sub-acute" DR (2 weeks of 30% dietary restriction) and more severe and acute FA (3 days of water-only fasting) groups. Also, the decrease in MBL concentrations as observed in FA group is approximately 50% while that observed in DR group is approximately 25% when compared to that of AL group. The liver MBL-A mRNA levels are significantly downregulated due to FA while this is not the case with MBL-C expression. This implies that in the FA model the protection is specific for MBL-A (which mimics hMBL), and there might be differential roles for MBL-A and -C. In the rat I/RI studies what we have shown is that specific depletion of MBL-A was sufficient for protection (28). This could also mean that the protection observed by the DR group mostly involves MBL-A and not MBL-C. This however needs further investigation. The other hypothesis is that the fasting regimen is more robust and may not be nullified by the amount of hMBL used for reconstitution, whereas the DR induced protection is. To add to this, we may hypothesize that the different timelines for the dietary regimens (3 days fasting vs. 14 days DR) may explain the differences in MBL-A and –C mRNA expression in the liver.

In summary, DR and FA are non-invasive methods to induce robust protection against renal I/RI [14]. Here we have specifically highlighted the role played by MBL in protection against renal I/RI and have shown that dietary interventions attenuate the circulating levels of MBL. Restoration of MBL levels breaks the protection against I/RI induced by DR, which underscores the role of MBL in the pathophysiology of I/RI, and may suggest a pivotal role for MBL in its mechanism of action. Furthermore, translation of our novel findings should be carried out in humans, in whom we previously demonstrated the clinical feasibility of DR [42]. Ultimately, we aim to develop DR mimicking agents to ensure more straightforward clinical applicability.

DISCLOSURES

The authors of this manuscript have no conflicts of interest to disclosure

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CHAPTER 5

Dietary restriction modulates hepatic and renal expression of complement system genes in mice

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ABSTRACT

Dietary interventions such as, dietary restriction (DR) and fasting (FA), are non-invasive and robust methods of protection against ischemia reperfusion injury, through upregulation of cytoprotective genes and amelioration of oxidative stress. We have recently shown that dietary restriction reduce serum MBL levels conveying partial protection against renal ischemia reperfusion injury. However, the overall impact of DR and FA on liver derived serum complement or to intrarenal expression of complement has not been studied so far. Here, we assessed the functionality of serum complement with pathway specific functional complement assays and analysed hepatic and renal expression of key complement proteins and regulators. Our results show that especially FA reduces hepatic expression of terminal pathway complement genes, but not the expression of C3 as central component of the complement cascades. This results in reduced C6 and C9 concentrations in serum, and impaired functional activity of the terminal pathway of complement. Furthermore, intrarenal expression of C3, and deposition of C3 activation fragments within kidneys was markedly increased upon FA, raising questions about the role of intrarenal C3 in the absence of inflammation and injury. Together our results show that dietary restriction results in modulation of the complement system which may be associated with the increased stress resistance observed after DR and FA.

INTRODUCTION

Experimental models of controlled dietary intervention such as dietary restriction (DR) and fasting (FA), have been shown to improve stress resistance against organ injury. For example, preoperative DR with 20-40% reduction in calorie intake or 1-3 days water-only FA reduces renal and hepatic ischemia/reperfusion injury (I/RI) through upregulation of key genes responsible for cytoprotection and amelioration of oxidative stress [1-3]. Furthermore, long term DR and short term FA has been shown to result in major physiological changes in rodents, including changes in hormonal levels and modulation of hepatic serum protein production [4-6].

The complement system consists of more than 30 serum and membrane bound proteins, and it is one of the major contributors to the pathogenesis of renal and hepatic I/RI [7]. The complement system can be initiated through three different pathways; the classical (CP), lectin (LP) and alternative pathway (AP), which all converge at the level of C3 activation. This initiates the terminal pathway (TP) and results in formation of proinflammatory anaphylatoxins C3a and C5a, and the terminal membrane attack complex C5b-9 [8,9]. The main source of complement, like for most serum constituents, is the liver, whereas the functional impact of systemic complement occurs locally or after extravasation of complement [8,10,11]. However, local secretion of soluble complement and expression of membrane regulators of complement activation (RCA) have been shown to be important contributors in complement-mediated injuries [11-14].

Partial amelioration of hepatic I/RI has been demonstrated with therapeutic targeting of CP and LP activation [15], specific targeting of C5a and C5b-9 formation [16,17], or total inhibition of systemic complement [18]. However, in contrast to this, recent studies have also established an essential role for complement in liver repair and regeneration, which accounts for the negative impact of total inhibition of complement [19,20].

Both systemic and local complement components have been demonstrated to aggravate renal I/RI. Genetic and therapeutic interventions have established that extravasation and activation of AP components and subsequent C5a and C5b-9 generation coupled with intrarenal expression of C3 and C5a receptors are all contributing factors to the pathogenesis of renal I/RI [21-25]. Additionally, a novel MBL-dependent, but complement activation independent mechanism of renal I/RI has been described, with evidence that MBL or MBL associated protein serine 2 (MASP-2) promotes injury [26-28].

We have recently demonstrated that both FA and DR lower the MBL concentration in mouse serum, resulting in partial amelioration of renal I/RI, and that reconstitution of MBL results in loss of protection [29]. The aim of this study was to further characterise the impact of FA and DR on the systemic and local complement production and activity.

Following experimental dietary interventions in 10-12 weeks old male C57bl/6 mice, we determined pathway specific functionality of serum complement at the level of C3 and C9 activation, followed by gene expression analysis of hepatic and renal complement genes. Our results show that especially FA reduces hepatic expression of terminal pathway complement genes, which is reflected as impaired functional activity of terminal pathway C5 – C9 and reduced C6 and C9 serum concentrations. Furthermore, intrarenal expression of C3, and activation of C3 within kidneys was markedly increased upon FA, raising questions about the role of C3 in repair and regeneration of kidneys in the absence of inflammation and injury.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (10-11 weeks old), purchased from Harlan (Horst, the Netherlands), were used for all the experimental settings. The animals were kept under standard laboratory conditions (12hr light/park period, 20-24^{o}C temperature, and relative humidity of 50-60%) under specific-pathogen free conditions. Free access to food and water were provided until the start of the experiment. Approval of the experimental procedure was in accordance with the Dutch National Experiments on Animals act complied with Directive 2010/63/EU of the Council of Europe.

Dietary interventions

Mice divided in three groups were fed *ad libitum* (AL) (normal chow, allowed free access to food and water), 30% dietary restricted (DR) for 2 weeks (mice were fed with only 70% of their normal chow) and water-only fasted for 3 days (FA) with n=6 animals/group. The mice in DR and FA groups were divided into n=3 animals/cage. At the start of the dietary interventions, animals were transferred into a clean cage in order to avoid eating their own faeces with free access to water.

Serum and Tissue collection

After the dietary interventions, blood was collected through exsanguination by cardiac puncture. The collected blood was stored in serum separator tube (tube containing a gel separator and clot activator) and kept immediately on ice to avoid inactivation of the complement proteins. These freshly drawn blood samples were then centrifuged at 3000g for 10 min at 4^{o}C, after which the serum samples were aliquoted, stored (-80^{o}C) until used further for complement activation ELISA assays. Furthermore, liver and kidney tissue was harvested from these mice and stored at -80^{o}C for further tissue analysis.

Functional complement pathway activities at the level of C3 and C9 activation

Measurement of functional mouse pathway activities was performed as described earlier [30]. In short, purified human IgM [31] (in-house, LUMC, Leiden, the Netherlands) was used to activate CP, mannan for LP (M7504, Sigma-Aldrich, St. Louis, United States) and LPS from strain Salmonella enteritidis for AP (HK4059, Hycult Biotech). Serum samples were diluted into BVB++ buffer for CP and LP (Veronal buffered Saline / 0.5 mM $MgCl_2$ / 2 mM $CaCl_2$ / 0.05% Tween 20 / 1% BSA, pH 7.5) and in BVB++/MgEGTA buffer for AP (BVB++ / 10mM EGTA / 5 mM $MgCl_2$). For C3 functional ELISAs deposition of mouse C3b/C3c/iC3b was detected with biotinylated rat anti-mouse C3b/C3c/iC3b mAb clone 2/11 (HM1065, Hycult Biotechnology) [30] and Streptavidin-HRP conjugate (Hycult Biotechnology). Deposition of mouse C9 for these functional pathway ELISAs was quantified with Digoxigenin conjugated rabbit anti-mouse C9 (in-house, LUMC) and anti-DIG-POD, Fab fragments (Prod.no. 11207733910, Roche Diagnostics GmbH, Mannheim, Germany) diluted in PBT (PBS / 1% BSA / 0.05% Tween20). TMB Plus2 was used as substrate for C3-functional ELISAs (Cat.no. 4395 Kem-En-Tek), TMB XTRA was used for C9 functional ELISAs (Cat.no. 4800, Kem-En-Tek). The Colorimetric substrate incubation was 15-30 min at room temperature and stopped with 50 µl 1M H_2SO_4 and read at 450 nm with a BioRad 550 instrument (Tokyo, Japan).

Measurement of Serum complement components

Mouse C6 and C9 were measured as described elsewhere (Kotimaa et al. in preparation). In brief C6 was captured with Rabbit pAb anti-mouse recombinant C6 and detected with Rabbit anti-mouse rC6-DIG. Mouse C9 was captured with Rabbit pAb anti-mouse recombinant C9 and detected with Rabbit anti-mouse rC9-DIG. Rabbit anti DIG-POD (Roche Diagnostics) and TMB Plus2 (Kem-En-Tek) or ABTS (Sigma-Aldrich) was used to quantify each ELISA.

Immunostaining for activated C3 and C9

5µm sections of mouse kidney tissues (n=6/group) were acetone fixed and blocked for endogenous peroxidase activity using PBS, azide and H_2O_2 for a period of 20 min. Following blocking of endogenous peroxidase activity, the tissues were stained with 1/50 diluted rat anti-mouse C3b/C3c/iC3b (HM1065, Hycult Biotech) in PBS/1% BSA overnight. This was followed by incubation with 1/300 diluted mouse anti-rat kappa-DIG (kind gift from Prof. N.A.Bos, University Medical Centre Groningen.) secondary antibody for 1hr followed by 1/500 diluted sheep anti-DIG-POD (11207733910, Roche Diagnostics GmbH) for 1hr. The staining was visualized by embedding the tissues in Nova RED (protocol from Vector lab cat. SK-4800) followed by counter-staining with hematoxylin for a few seconds. Immunohistochemistry quantification was performed by assessing 10 consecutive high power fields (HPFs; magnification ×200) on each section in a blinded fashion. The positive regions in each image were quantified using image J software.

For immunostaining of C9 a similar protocol as for C3 was followed, but using the rat anti-mouse C9-DIG in 1/100 dilutions in PBS/1% BSA overnight as the primary antibody and 1/500 diluted anti-dig-POD as secondary antibody (Roche). Staining was visualized by embedding the tissues in Nova RED (protocol from Vector lab cat. SK-4800) followed by counter-staining with hematoxylin for a few seconds. Immunohistochemistry quantification was performed in a similar fashion as for C3.

Microarray analysis

To determine the effect of DR and FA on transcriptional levels of complement factors, kidney and liver tissue (4 mice per group) were collected immediately after AL, DR or FA and snap frozen in liquid nitrogen until further analysis. RNA isolation was done via QIAzol lysis Reagent and miRNAeasy Mini Kits (QIAGEN, Hilden, Germany), according to manufacturer's instructions protocol. The addition of wash buffers RPE and RWT (QIAGEN) was done mechanically by using the QIAcube (QIAGEN, Hilden, Germany) via the miRNeasy program and subsequently stored at -80°C. Measurement of RNA concentration was done using Nanodrop (Thermo Scientific) and quality assessment of the RNA with the 2100 Bio-Analyzer (Agilent Technologies, Amstelveen, the Netherlands) according to the manufacturer's instructions. The quality of the RNA was expressed as the RNA integrity number (RIN, range 0-10) and samples below RIN 8 were excluded from analysis. Hybridization to Affymetrix HT MG-430 PM Array Plates was done by the Microarray Department of the University of Amsterdam, the Netherlands according to their protocols. For each group, four to six biological replicates were used. Next, quality control was assessed and normalization done using the pipeline at the www.arrayanalysis.org website (Maastricht University, the Netherlands). Normalization was done using the Robust Multichip Average (RMA) algorithm and the MBNI custom CDF (http://brainarray.mbni.med.umich.edu/brainarray/default.asp) version #14 for this chip. The output after normalization consisted of data for 45141 probes, with several probes corresponding to the same Gene ID. The complete raw and normalized data and their MIAME compliant metadata have been deposited at GEO (www.ncbi.nlm.nih.gov/geo) (GSE65656).

Quantitative Polymerase Chain Reaction

qPCR was performed as described earlier [1]. In short, total RNA was extracted from frozen kidney and liver tissue using Ambion mirVana miRNA Isolation Kit and oligodT or hexamer primed cDNA synthesized using Superscript II (Invitrogen, California, United States) according to the manufacturer´s instructions. Quantitative real-time PCR was performed using a MyIQ (Biorad) with SYBR green incorporation. Relative expression was calculated using the equation: $1.8^{(\text{Delta CT sample - Delta CT Control})}$ [32]. Each sample was tested in duplo at least two times. The specific primer sequences used for mouse C3, C5 and C9 were as follows: C3 (forward, 5'-GGG GAC AAC CTC AAT GTC AA3'-; reverse, 5'-CCA GGC GAA ATG AAG GAA TA 3'-), C5 (forward, 5'AGG GTA CTT TGC CTG CTG AA 3'; reverse, 5'-TGT GAA GGT GCT CTT GGA TG 3'-), C9 (forward, 5'- CCA CCG AAG TAC CTG AAA AG 3'-; reverse, 5'-AGG AAA GTT GAC CTC AGC AC 3'-).

Statistical analysis

All the data are represented as means with standard error of mean. Non-parametric paired sample T-tests were performed using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.). The complement activity and individual factors were analysed using 1-way ANOVA, and the graphs were plotted using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA). P-value ≤0.05 were considered to be significant for quantitative PCR, serum and histological determinations, whereas ≤0.01 was used for microarray determination significance.

RESULTS

Effect of DR and FA on systemic complement activity

To investigate the impact of DR and FA on the systemic complement system, we measured functional activities of the classical, alternative and lectin pathways both at the level of C3 and C9 activation. DR did not impact any of the three pathways at the level of C3 activation (Fig. 1A-C). Also FA did not significantly affect C3 functional activity, although a trend of reduction was seen for CP (Fig. 1A) and AP (Fig. 1C). However, after FA all three pathways showed a significantly reduced activity at the level of C9 (Fig. 1D-F), suggesting a specific effect on the terminal pathway. In contrast, DR only showed a modest reduction, which was only significant for LP (Fig. 1E).

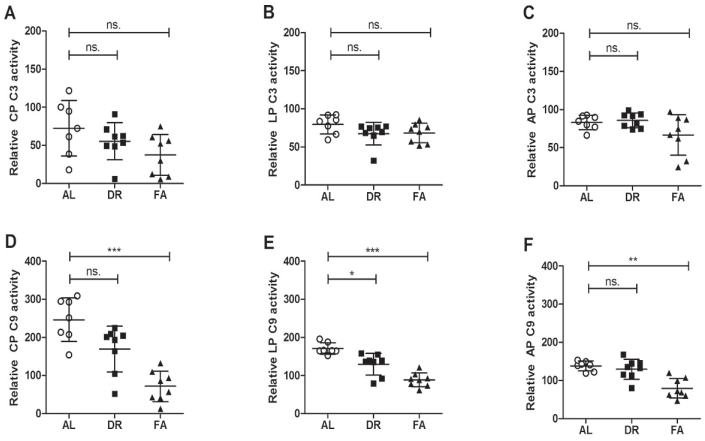

Figure 1: Determination of serum functional complement activities. *Pathway specific activity at the level of* ***A-C)*** *C3 and* ***D-F)*** *C9 activation was determined with functional complement ELISAs: classical (CP) was activated on IgM, lectin (LP) on mannan and alternative pathway (AP) on LPS coated ELISA plates. Deposition of mouse C3b/C3cb/iC3b and C9 were calculated as relative (AU/ml) to CD1 NMS standard serum activity. Ad Libitum (AL) group was used as a control group for dietary restriction (DR) and fasting (FA). Significance was determined with two-way ANOVA (ns.= not significant,*=p≤0.05, **=p≤0.01,***=p≤0.001)*

Effect of DR and FA on hepatic expression of complement genes

Since most complement components are produced by the liver, we analysed hepatic gene expression using microarray and compared mRNA expression levels following DR or FA with AL conditions. The hepatic expression of many complement factors showed a marked reduction following either DR or FA (Fig 2A, Supplementary table 1 and 2). FA induced significant (p≤0.01) downregulation of CP initiator C1q, LP initiator MBL-2, AP initiator properdin and complement regulators fH and fI, whereas DR impacted only C1q (Supplementary table 1). Moreover, FA downregulated all terminal pathway components with a 4.5 and 5 fold reduction in C6 and C9 (Fig 2B), whereas also DR impacted both C8 and C9 (Supplementary table 1). Interestingly, the expression of C3 was not inhibited by either FA or DR based on array analysis (Fig 2A).

A

Gene	Fold Change vs AL DR	FA
C1qa	-2.26	**-1.90**
C1qb	-2.11	-2.01
C1qc	-2.10	**-1.81**
C1ra / C1rb	-1.18	-1.29
C1s	-1.11	-1.29
C2	-1.08	-1.06
C4b	-1.17	-1.33
Mbl1	-1.28	-1.53
Mbl2	-1.07	**-1.36**
Masp1	1.15	-1.31
Masp2	-1.37	-1.04
Cfd	-1.09	-1.21
Cfh	-1.34	**-1.55**
Cfhr1	-1.29	-1.19
Cfhr2	-1.01	-1.55
Cfhr3	-1.30	-1.38
Cfi	-1.18	**-1.44**
Cfp	-1.80	**-1.97**
Cfb	-1.25	-1.30
C3	1.12	1.13
Hc (C5)	-1.14	**-1.52**
C6	-2.26	**-4.98**
C8a	-1.19	**-2.59**
C8b	**-1.81**	**-2.91**
C8g	-1.05	**-1.44**
C9	**-1.61**	**-4.34**

B

	Fasting			
			Mean absorbances	
Gene ID	p-value	Fold Change	AL	FA
C1qa	**0.001**	-1.9	173	92
C1qc	**0.010**	-1.8	92	51
Mbl2	**0.002**	-1.4	2515	1856
Cfh	**0.002**	-1.5	1069	691
Cfi	**0.0004**	-1.4	4761	3303
Cfp	**0.006**	-2.0	47	24
Hc (C5)	**0.002**	-1.5	3301	2176
C6	**0.00002**	-5.0	420	84
C8a	**0.00001**	-2.6	2991	1157
C8b	**0.00001**	-2.9	1002	345
C8g	**0.002**	-1.4	2624	1825
C9	**0.00001**	-4.3	4704	1084

Figure 2: Microarray analysis of hepatic complement gene expression. *Selection of complement system genes responsible for serum complement production were chosen for focused analysis of complement* ***A)*** *heatmap and fold change of gene expression against AL* ***B)*** *Expression of genes that were significantly (p≤0.01) modulated in FA vs AL. No major changes except for C1q was observed for DR. Table shows fold change vs AL, and the mean absorbance value of the representative microarray probeset.*

To further validate these findings, the C3 expression was analysed by qPCR showing that FA resulted in a significant 4-fold upregulation, whereas after DR C3 remained unchanged (Fig 3A). The qPCR determination of C5 (Hc) was in line with the array data, showing a modest but non-significant decrease compared to AL, but not DR (Fig 3B). Furthermore, a significant downregulation of C9 confirmed the array results from both DR and FA (Fig

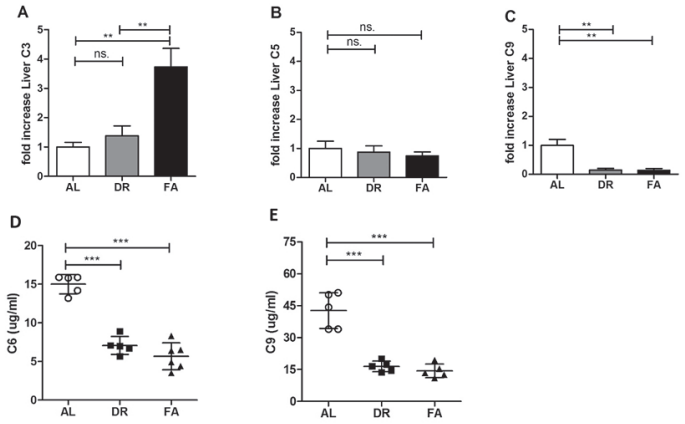

Figure 3: Quantitative PCR and serum protein analysis of hepatic expressed complement. *Verification of hepatic microarray expression was performed for* ***A)*** *C3,* ***B)*** *C5 and* ***C)*** *C9 with quantitative PCR. Serum levels of Terminal pathway specific serum proteins,* ***D)*** *C6 and* ***E)*** *C9, were determined from the sera of AL, DR and FA treated mice with sandwich ELISA. Significance was determined with two-way ANOVA. (ns. = not significant,*=p≤0.05, **=p≤0.01,***=p≤0.001).*

3C). The downregulation of terminal pathway specific components was further verified at the protein level for C6 and C9, which showed that serum levels of both were decreased 2 – 3 fold by both DR and FA (Fig 3D-E).

Effect of DR and FA on intra-renal complement factors expression

In view of the local complement activation, and its possible contribution to the protection against I/RI, we also performed microarray analysis of complement gene expression in the kidney. Intrarenal expression after DR did not reveal major modulation of expression of complement factors or regulators of complement activation (RCA) (Supplementary table 3). However, it should be noted that total renal expression levels (absorbance of specific probesets in microarray) is low for many of the complement related genes especially for the regulators. However, C3 was clearly expressed and showed a significant 4.5 fold upregulation (Fig 4A, Supplementary table 4).

To verify this finding, qPCR was performed, which showed that FA but not DR, resulted in a threefold increase in C3 mRNA expression levels (Fig 4B).

Renal histology following FA

To determine whether the intrarenal upregulation of C3 would result in intrarenal complement activation, immunohistological staining of C3b/C3c/iC3b were performed

and quantified. Interestingly, after FA treatment the kidneys had up to 50% higher intensity of C3 activation fragments (Fig 4C) without a similar increase in C9 deposition (Fig 4D).

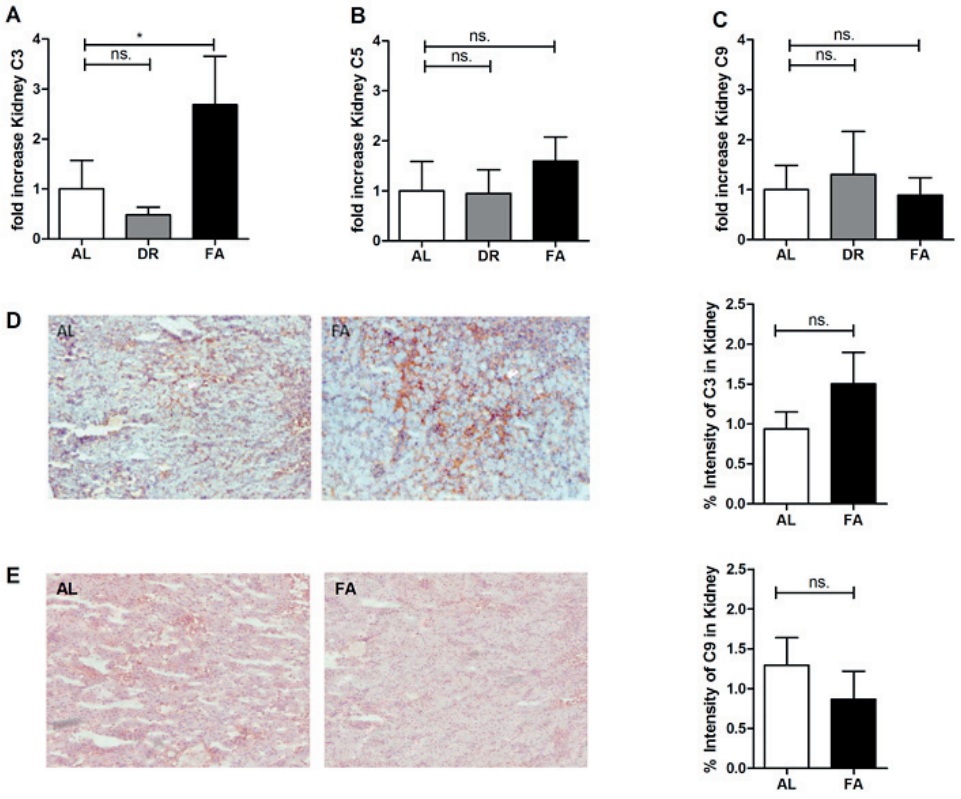

Figure 4: Intrarenal complement gene expression and focused analysis of intrarenal expression of complement. *Selection of complement system genes central to complement activation and regulation were chosen for focused analysis of complement* ***A)*** *Expression of genes that were significantly ($p \leq 0.01$) modulated in FA vs AL. No major changes for DR were observed. Table shows fold change vs AL, and the mean absorbance value of the representative microarray probeset. Verification of renal microarray expression was performed for* ***B)*** *C3 with quantitative PCR. Intrarenal expression and activation of C3 was evaluated with histological stains and quantification of* ***C)*** *C3b/C3c/iC3b and* ***D)*** *C9. Significance of change was determined with two-way ANOVA. Deposition of complement on renal tissue was performed by assessing 10 consecutive high power fields (HPFs; magnification ×200) on each section in a blinded fashion. The positive regions in each image were quantified using image J software. (ns. = not significant, *=$p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$)*

DISCUSSION

The purpose of this study was to further characterise the intriguing impact of dietary restriction on the complement system in mice. Our results clearly show that especially FA resulted in major modulation of serum complement that is due to specific modulation of terminal pathway specific complement factors. Interestingly, renal expression of complement factors was also regulated by FA. Short term DR and FA robustly protect against solid organ transplantation-associated I/RI, and be beneficial in protecting against acute trauma and sepsis [1,3].

Experimental models of I/RI have shown that the complement system has a major contribution in mediating or aggravating the injury [10,33,34]. Avtivation of the lectin and alternative pathway activation contribute to the pathogenesis of the injury [23,25,35], with consistent evidence that terminal pathway effectors C5a and C5b-9 are central in aggravating the ischemia injury [17,23,25].

Experimental dietary restriction in rats and clinical evidence on malnutrition have been shown to reduce liver synthesis of serum proteins and impairing bactericidal opsonic activity of serum, suggesting that the complement system is affected, but not directly determining levels of complement components [6,36,37]. In line with these results, we have recently shown that DR lowers serum MBL levels, and conveys partial protection in experimental renal I/RI in mice [29].

To better understand the impact of experimental dietary restriction on the hepatic, renal and systemic complement system in mice, we performed functional analysis of serum complement and analysed changes in local gene expression following dietary restriction. Our results on functional measurement of the three main pathways of complement showed that neither of the dietary interventions impacted systemic complement functionality at the level of C3 activation. However, determination at the level of C9 activation, which includes the whole terminal pathway C5 – C9 activation, showed that DR resulted in significant reduction of only LP, whereas FA reduced the terminal pathway activation by all three initiating pathways.

The liver is the main source of systemic complement, and is therefore the likely source of the observed modulation of serum complement activity following dietary restriction [11]. Analysis of the impact of FA and DR on the hepatic expression of key complement factors revealed that initiation factor MBL was downregulated, and although not predominantly produced by the liver, also properdin and C1q showed significantly lower expression. The transcriptome analysis suggested that the central complement components C3, C2 and C4 are not downregulated. Subsequent qPCR analysis showed that C3 expression was significantly upregulated after FA, but not after DR. However, the

most striking change in hepatic complement expression was the 1.5 – 4.9 fold downregulation of terminal pathway specific genes following FA, whereas also DR had significant impact on C6, C8 and C9 with 1.6 – 2.3 fold downregulation.

The hepatic expression of C5 and C9, determined with qPCR, reflected the findings from microarray data with a significant downregulation of C9 after DR and less prominent C5 downregulation in FA animals. Most importantly, these results were further verified with protein measurements of C6 and C9 in serum, which showed significant downregulation in both FA and DR.

Together these results suggest that the observed loss of terminal pathway activity after FA is a cumulative result of major downregulation of most terminal pathway complement factors, whereas after DR there is less uniform downregulation based on microarray data allowing partial function of the terminal pathway. However, our results cannot fully explain why DR shows higher functional activities than FA, as C6 and C9 were downregulated to the same degree, without further analysis of serum proteins especially regulators of complement activation such as fH and fI. Furthermore, the increase in C3 expression could also explain why downregulation of MBL [29], and possibly that of C1q and properdin were not readily detectable with the functional complement ELISAs at the level of C3 activation.

The underlying mechanisms to the hepatic expression changes are probably complex, however upregulation of C3 suggests a compensatory mechanism akin to acute phase responses [38]. Although C9 is also known as part of the acute phase response, it is also positively regulated by androgen hormones, such as testosterone, that have been demonstrated to decline during FA [4,39]. Further serum analysis of soluble regulators and other serum complement components and factors after dietary interventions would be required, especially for extrahepatically produced C1q and properdin, to fully understand which factors are affected [11,40,41]. Additionally, other functional assays such as opsonisation and haemolytic assays could be used to further investigate the functional differences observed here [42].

Microarray analysis of intrarenal complement expression revealed interesting modulation especially following FA, whereas DR did not show significant regulation of any complement genes. The global analysis was not sensitive enough to detect the expression of complement regulatory proteins or receptors, except for CD59 the regulator of terminal pathway, which showed no change. The expression of complement cell surface regulators and receptors is mostly cell type specific and can be masked in whole organ determinations [14,24,43].

The most remarkable finding was the marked upregulation of renal C3 following FA, which was analogous to the hepatic upregulation. The upregulation impacted the basal level of C3 activation within the kidney, as shown by increased C3b/C3c/iC3b staining, but we observed no evidence of C9 activation in otherwise healthy mouse kidneys. Therefore, the activation is limited to the level of C3, and the proinflammatory, and potentially injurious activation of terminal pathway was not observed probably due to intact terminal pathway inhibitor CD59 (MAC-IP) expression [22,44].

The intrarenal expression of complement, complement regulators and complement receptors has been identified as an important factor in the pathogenesis of renal injury [14,24,45]. Therefore, further research should also include detailed histological analysis of the key complement regulators such as CD55, CD59, CD46 and Crry to better understand whether dietary restriction induces renal protection through modulation of these key regulators of mouse complement.

Our finding that FA increased intrarenal C3 production and activation is counter-intuitive, as intrarenal C3 is sufficient in maintaining complement activation following renal injury [46]. However, the role of complement is not always injurious. In liver I/RI, complement exhibits intriguing an duality between injury and regeneration [20,47], with evidence that anaphylatoxins C3a and C5a are essential for regeneration [48]. Although the C3a-C3aR-axis has been shown to promote renal injury [49], it has not been evaluated whether C3a-C3aR axis also has a role in homeostatic repair of kidneys. Although highly speculative at this stage, C3a-C3aR has been shown to activate mesenchymal stromal cells (MSCs), which are responsible for homeostatic repair and regeneration of injured kidneys [50,51]. The C3a-C3aR axis is also an important factor for recruitment of MSCs [52]. Therefore, FA in absence of inflammatory stimuli such as ischemia and terminal pathway activation, could hypothetically result in increased repair of the normal kidney and preconditioning of the kidneys to better withstand I/RI.

In the context of renal I/RI, the FA-specific downregulation of terminal pathway components C5–C9 can in part explain the observed protection against hepatic and renal I/RI, especially as the central regulator of terminal pathway CD59 was intact. However, similar protective impact with DR could not be found, except for the low serum C6 and C9, which affect only C5b-9 formation in response to injury. The role of the compensatory mechanisms of hepatic and renal C3 upregulation in response to FA are intriguing and could hypothetically be linked to the emerging role of C3b and C3a in tissue regeneration.

Our results demonstrate a clear parallel to experimental models where systemic inhibition of serum complement results in amelioration of renal and hepatic I/RI [15,22,23,53,54].

Further research is necessary to characterise the impact of dietary restriction on complement in humans. Translation of the results of dietary interventions from animal studies to the clinical setting poses a challenge. However, the clinical feasibility of dietary restrictions have been reported [55] and future research is warranted not only for studies in healthy animals but also in humans. In addition, extension of these studies is needed, to encompass tissue specific expression of complement factors, regulators and receptors to better understand how dietary restrictions modulate the complement system in mice and men.

ACKNOWLEDGEMENT

We would like to thank Ngaisah Klar-Mohammad for her technical expertise on measuring complement factors from mouse sera.

SUPPLEMENTARY MATERIALS

Supplementary Table 1. Focused microarray analysis of dietary restricted mouse liver. Selection of complement system genes were chosen for analysis and the results described either as heatmap, relative fold change or with absolute probe specific intensity values. Dietary restricted (DR, n=4) mice were compared with ad libitum (AL, n=4) control group. Significance of change was determined with One-way ANOVA. Expression level above 50 absorbance units and significance below cut-off p≤0.01 was considered to be suitable for determination of change on the results.

				DR vs AL		Mean absorbances	
Heatmap	Gene ID	Gene	Probeset	Fold Change	p-value	AL	FA
	C1qa	**C1q**	**1417381_at**	**-1.90**	**1.27E-03**	**173**	**92**
	C1qb	C1q	1437726_x_at	-2.01	2.13E-02	55	27
	C1qc	**C1q**	**1449401_at**	**-1.81**	**9.92E-03**	**92**	**51**
	C1ra /// C1rb	C1r	1417009_at	-1.29	5.73E-02	360	280
	C1s	C1s	1424041_s_at	-1.29	1.89E-02	1137	883
	C2	C2	1441912_x_at	-1.06	7.12E-01	437	414
	C4b /// LOC675521	C4	1418021_at	-1.33	9.19E-02	1927	1448
	Mbl1	MBL1	1419578_at	-1.53	4.82E-02	1313	859
	Mbl2	**MBL2**	**1418787_at**	**-1.36**	**2.18E-03**	**2515**	**1856**
	Masp1	MASP1	1438602_s_at	-1.31	1.18E-01	140	107
	Masp2	MASP2	1420524_a_at	-1.04	9.15E-01	211	204
	Cfd	CFD	1417867_at	-1.21	1.45E-01	5	4
	Cfh	**CFH**	**1423153_x_at**	**-1.55**	**2.21E-03**	**1069**	**691**
	Cfhr1	CFHR1	1419436_at	-1.19	1.98E-01	1154	971
	Cfhr2	CFHR2	1425823_at	-1.55	2.64E-02	1344	867
	Cfhr3	CFHR3	1432330_at	-1.38	8.41E-02	18	13
	Cfi	**CFI**	**1418724_at**	**-1.44**	**3.62E-04**	**4761**	**3303**
	Cfp	**Properdin**	**1452279_at**	**-1.97**	**6.33E-03**	**47**	**24**
	C2 /// Cfb	CFB	1417314_at	-1.30	2.50E-01	872	668
	C3 /// LOC100048759	C3	1423954_at	1.13	2.58E-01	5474	6205
	Hc	**C5**	**1419407_at**	**-1.52**	**1.72E-03**	**3301**	**2176**
	C6	**C6**	**1449308_at**	**-4.98**	**2.20E-05**	**420**	**84**
	C8a	**C8**	**1428012_at**	**-2.59**	**1.23E-05**	**2991**	**1157**
	C8b	**C8**	**1427472_a_at**	**-2.91**	**1.23E-05**	**1002**	**345**
	C8g	**C8**	**1451625_a_at**	**-1.44**	**2.17E-03**	**2624**	**1825**
	C9	**C9**	**1422815_at**	**-4.34**	**7.95E-06**	**4704**	**1084**

Supplementary Table 2. Focused microarray analysis of fasted mouse liver. Selection of complement system genes were chosen for analysis and the results described either as heatmap, relative fold change or with absolute probe specific intensity values. Dietary restricted (DR, n=4) mice were compared with ad libitum (AL, n=4) control group. Significance of change was determined with One-way ANOVA. Expression level above 50 absorbance units and significance below cut-off p≤0.01 was considered to be suitable for determination of change on the results.

Heatmap	Gene ID	Gene	Probeset	DR vs AL Fold Change	DR vs AL p-value	Mean absorbances AL	Mean absorbances FA
	C1qa	**C1q**	**1417381_at**	**-1.90**	**1.27E-03**	**173**	**92**
	C1qb	C1q	1437726_x_at	-2.01	2.13E-02	55	27
	C1qc	**C1q**	**1449401_at**	**-1.81**	**9.92E-03**	**92**	**51**
	C1ra /// C1rb	C1r	1417009_at	-1.29	5.73E-02	360	280
	C1s	C1s	1424041_s_at	-1.29	1.89E-02	1137	883
	C2	C2	1441912_x_at	-1.06	7.12E-01	437	414
	C4b /// LOC675521	C4	1418021_at	-1.33	9.19E-02	1927	1448
	Mbl1	MBL1	1419578_at	-1.53	4.82E-02	1313	859
	Mbl2	**MBL2**	**1418787_at**	**-1.36**	**2.18E-03**	**2515**	**1856**
	Masp1	MASP1	1438602_s_at	-1.31	1.18E-01	140	107
	Masp2	MASP2	1420524_a_at	-1.04	9.15E-01	211	204
	Cfd	CFD	1417867_at	-1.21	1.45E-01	5	4
	Cfh	**CFH**	**1423153_x_at**	**-1.55**	**2.21E-03**	**1069**	**691**
	Cfhr1	CFHR1	1419436_at	-1.19	1.98E-01	1154	971
	Cfhr2	CFHR2	1425823_at	-1.55	2.64E-02	1344	867
	Cfhr3	CFHR3	1432330_at	-1.38	8.41E-02	18	13
	Cfi	**CFI**	**1418724_at**	**-1.44**	**3.62E-04**	**4761**	**3303**
	Cfp	**Properdin**	**1452279_at**	**-1.97**	**6.33E-03**	**47**	**24**
	C2 /// Cfb	CFB	1417314_at	-1.30	2.50E-01	872	668
	C3 /// LOC100048759	C3	1423954_at	1.13	2.58E-01	5474	6205
	Hc	**C5**	**1419407_at**	**-1.52**	**1.72E-03**	**3301**	**2176**
	C6	**C6**	**1449308_at**	**-4.98**	**2.20E-05**	**420**	**84**
	C8a	**C8**	**1428012_at**	**-2.59**	**1.23E-05**	**2991**	**1157**
	C8b	**C8**	**1427472_a_at**	**-2.91**	**1.23E-05**	**1002**	**345**
	C8g	**C8**	**1451625_a_at**	**-1.44**	**2.17E-03**	**2624**	**1825**
	C9	**C9**	**1422815_at**	**-4.34**	**7.95E-06**	**4704**	**1084**

Supplementary Table 3. Focused microarray analysis of dietary restricted mouse kidney. Selection of complement system genes were chosen for analysis and the results described either as heatmap, relative fold change or with absolute probe specific intensity values. Dietary restricted (DR, n=4) mice were compared with ad libitum (AL, n=4) control group. Significance of change was determined with One-way ANOVA. Expression level above 50 absorbance units and significance below cut-off p≤0.01 was considered to be suitable for determination of change on the results.

Heatmap	Gene ID	Gene	Probeset	DR vs AL Fold Change	DR vs AL p-value	Mean absorbances AL	Mean absorbances DR
	C1qa	C1q	1417381_at	-1.08	8.88E-01	81	77
	C1qb	C1q	1437726_x_at	-1.25	3.57E-01	40	32
	C1qc	C1q	1449401_at	-1.22	3.01E-01	80	64
	C1ra /// C1rb	C1r	1417009_at	-1.26	4.24E-01	31	28
	C1s	C1s	1424041_s_at	-1.20	5.60E-01	105	90
	C2	C2	1441912_x_at	1.36	1.47E-01	180	236
	C4b /// LOC675521	C4	1418021_at	-1.32	3.60E-01	46	33
	Mbl1	MBL1	1419578_at	-1.15	6.63E-01	40	35
	Mbl2	MBL2	1418787_at	1.02	9.61E-01	6	6
	Masp1	MASP1	1438602_s_at	-1.07	8.75E-01	14	13
	Masp2	MASP2	1420524_a_at	-1.05	8.71E-01	9	9
	Cfd	CFD	1417867_at	-3.46	4.40E-01	454	127
	Cfh	CFH	1423153_x_at	1.22	6.21E-01	522	627
	Cfhr1	CFHR1	1419436_at	1.03	8.96E-01	6	6
	Cfhr2	CFHR2	1425823_at	1.01	9.85E-01	9	9
	Cfhr3	CFHR3	1432330_at	1.09	7.34E-01	6	6
	Cfi	CFI	1418724_at	-1.09	8.29E-01	91	83
	Cfp	Properdin	1452279_at	-1.06	8.55E-01	12	11
	C2 /// Cfb	CFB	1417314_at	-1.03	9.49E-01	634	602
	C3 /// LOC100048759	C3	1423954_at	1.03	9.76E-01	137	133
	Hc	C5	1419407_at	1.03	9.36E-01	8	8
	C6	C6	1449308_at	-1.19	3.52E-01	10	8
	C8a	C8	1428012_at	-1.16	5.80E-01	204	173
	C8b	C8	1427472_a_at	-1.03	9.42E-01	7	7
	C8g	C8	1451625_a_at	1.18	2.68E-01	218	259
	C9	C9	1422815_at	1.15	6.24E-01	8	9
	C3ar1	C3aR	1419482_at	-1.11	7.88E-01	12	10
	C5ar1	C5aR	1439902_at	1.00	9.93E-01	7	7
	Cd46	MCP	1421586_a_at	1.05	9.09E-01	10	10
	Cd55	DAF	1443906_at	-1.42	5.31E-02	13	9
	Cd59a	MAC-IP	1429830_a_at	-1.12	8.93E-01	222	197
	Cr1l	Crry	1430131_at	1.25	3.88E-01	29	37
	Cr2	CR2	1425289_a_at	1.13	5.58E-01	7	8

Supplementary Table 4. Focused microarray analysis of fasted mouse kidney. Selection of complement system genes were chosen for analysis and the results described either as heatmap, relative fold change or with absolute probe specific intensity values. Dietary restricted (DR, n=4) mice were compared with ad libitum (AL, n=4) control group. Significance of change was determined with One-way ANOVA. Expression level above 50 absorbance units and significance below cut-off p≤0.01 was considered to be suitable for determination of change on the results.

				FA vs AL		Mean absorbances	
Heatmap	Gene ID	Gene	Probeset	Fold Change	p-value	AL	FA
	C1qa	C1q	1417381_at	-1.59	4.20E-03	125	79
	C1qb	C1q	1437726_x_at	-1.63	5.21E-04	48	29
	C1qc	C1q	1449401_at	-1.60	4.13E-04	110	69
	C1ra /// C1rb	C1r	1417009_at	-1.28	5.29E-03	53	41
	C1s	C1s	1424041_s_at	-1.42	6.52E-04	206	145
	C2	C2	1441912_x_at	1.01	9.54E-01	215	217
	C4b /// LOC675521	C4	1418021_at	-1.60	1.03E-02	46	29
	Mbl1	MBL1	1419578_at	1.15	1.09E-01	77	89
	Mbl2	MBL2	1418787_at	-1.04	8.16E-01	10	9
	Masp1	MASP1	1438602_s_at	-1.20	1.17E-01	23	20
	Masp2	MASP2	1420524_a_at	-1.14	2.83E-01	12	11
	Cfd	CFD	1417867_at	3.13	2.85E-01	82	257
	Cfh	CFH	1423153_x_at	1.08	3.34E-01	1079	1161
	Cfhr1	CFHR1	1419436_at	1.02	9.17E-01	7	7
	Cfhr2	CFHR2	1425823_at	1.04	7.74E-01	12	13
	Cfhr3	CFHR3	1432330_at	1.05	6.58E-01	7	7
	Cfi	CFI	1418724_at	-2.07	4.18E-03	132	64
	Cfp	Properdin	1452279_at	-1.11	2.47E-01	12	11
	C2 /// Cfb	CFB	1417314_at	-1.20	1.72E-01	729	608
	C3 /// LOC100048759	C3	1423954_at	4.52	9.85E-07	79	357
	Hc	C5	1419407_at	-1.05	7.67E-01	10	9
	C6	C6	1449308_at	-1.05	5.67E-01	9	9
	C8a	C8	1428012_at	-2.37	7.41E-05	328	139
	C8b	C8	1427472_a_at	-1.07	4.22E-01	10	10
	C8g	C8	1451625_a_at	1.51	1.29E-03	231	349
	C9	C9	1422815_at	-1.33	1.34E-01	12	9
	C3ar1	C3aR	1419482_at	-1.36	2.75E-02	18	13
	C5ar1	C5aR	1439902_at	-1.03	6.70E-01	7	7
	Cd46	MCP	1421586_a_at	1.47	8.82E-03	19	28
	Cd55	DAF	1443906_at	-1.41	7.88E-03	19	14
	Cd59a	MAC-IP	1429830_a_at	1.01	9.52E-01	333	336
	Cr1l	Crry	1430131_at	1.29	1.92E-02	50	65
	Cr2	CR2	1425289_a_at	-1.17	1.05E-01	19	16

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CHAPTER 6

Protection against renal ischemia/reperfusion injury through modulation of adaptive immunity induced by fasting

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ABSTRACT

Short-term fasting (FA) is one of the dietary interventions associated with protection against oxidative stress. It promotes potent changes in metabolic pathways and cellular processes, such as stress resistance, lipolysis and autophagy. Preoperative FA also has beneficial effects on ischemia/reperfusion injury (I/RI), but the exact mechanism behind this phenomenon has not yet been elucidated. In this study, we investigated the effect of FA on cytokine production and on the B and T cell development in mice, 6 and 24hrs after the induction of renal I/RI. In bone marrow (BM), we found that FA arrests B cell development after induction of renal I/RI, while increasing the number of recirculating mature B cells. Thymopoiesis was observed to be arrested significantly at the DN1 to DN2 transition. No major changes in $CD3^+$ T cell populations were observed in the spleen, while in BM the number of $CD3^+$ T cells was increased. Since cytokines play an important role in mediating I/RI after transplantation, pro- and anti-inflammatory cytokines were investigated. The production of pro-inflammatory cytokines by splenic cells, such as IFN-γ and IL-2, was decreased 6hrs post reperfusion in the FA mice but not anymore at 24hrs post reperfusion. No effect on anti-inflammatory cytokines was observed. Thus, FA arrests B and T cell development in BM and thymus, respectively, increases the number of recirculating mature B cells, and reduces the early inflammatory response after I/RI. Based on these findings, we hypothesize that the protective effect mediated by FA on I/RI is associated with an arrest in lymphocyte development and a decrease in pro-inflammatory cytokine production.

INTRODUCTION

Dietary restriction (DR), a reduction in nutrient and energy intake without malnutrition, encompasses a variety of interventions. DR is maintained by chronically reducing the food intake by 20-40%, while maintaining meal frequency. One form of short-term DR is fasting. Short-term fasting (FA) is defined as total absence of food intake during a number of days with free access to water. It promotes potent changes in metabolic pathways and cellular processes such as stress resistance, lipolysis and autophagy [1–4]. Findings from experimental animal studies, as well as emerging human studies indicate that DR provides an effective strategy to reduce weight, delay aging, and optimize health. In addition, it has been demonstrated that FA plays a role in cancer prevention and leads to a favorable outcome of treatment [5]. Positive effects have been shown on neurodegeneration, metabolic syndrome, inflammation and hypertension [6,7]. Importantly, FA also has beneficial effects on ischemia reperfusion injury (I/RI), which is one of the major hurdles to overcome in organ transplantation [8].

I/RI is initiated by deprivation of tissue oxygen and leads to accumulation of cellular waste, an excess of anaerobic metabolites, and the generation of oxygen free radicals. Together with these various adverse reactions that damage tissues during reperfusion, I/RI causes immense inflammation and dysfunction of transplanted organs [9–11]. I/RI, which has both immediate and long-term effects [12,13] is the major cause of ischemic acute renal failure and is responsible for causing acute kidney injury, associated with substantial morbidity and health care expenditures [3,6,7]. Preventive and therapeutic measures in I/RI are needed to reduce the severity of graft dysfunction and failure, allowing the safe usage of so-called extended criteria donor organs (organs of lower quality that have suffered more initial injury before organ retrieval) for transplantation. Unfortunately, there currently is no treatment for I/RI, although experimental therapies aimed at minimizing I/RI, such as machine perfusion, are entering the clinical arena [14].

The ensuing inflammatory response following I/RI involving both the innate and adaptive immune system is considered to increase the initial oxidative damage [13]. Different immunological players in I/RI-induced inflammation such as leukocyte adhesion molecules, lymphocytes, regulatory T lymphocytes, and most importantly the adaptive immune system [15,16] have been studied and documented as one of the hallmarks of renal I/RI.

Mitchell et al. [8] have reported the beneficial effects of FA on I/RI in kidney and liver in mice. They showed that FA induced an increase in stress resistance, involving upregulation of cytoprotective molecules such as hemoxygenase-1 (HO-1) and decrease in pro-inflammatory cytokine production following I/RI [17].

Recently, we have shown that FA alone without induction of renal I/RI increases recirculating mature B cells, and $CD3^+$ T cells in bone marrow (BM), as well as arrests thymopoiesis. In the spleen, we observed a decrease in both marginal zone and follicular B cells [18]. How these immunomodulatory effects of FA may contribute to the protection against I/RI remains elusive, and warrants further study. Therefore, in the current study, we have focused on the immunological changes taking place in BM, thymus and spleen of fasted mice after induction of renal I/RI.

MATERIALS AND METHODS

Animals

All experiments were performed in male C57Bl/6 mice purchased from Harlan, Horst, the Netherlands. The mice, weighing 20-25 g, were 10-12 weeks old and were kept at normal physiological conditions (temperature 20–24°C, relative humidity 50–60%, 12hr light/12hr dark) with three animals per cage, having free access to water and food (Hope Farms, Woerden, the Netherlands). Experiments were approved by the Erasmus MC Animal Medical Committee (Protocol no.105-12-12) under the under the Dutch National Experiments on Animals Act, compiled with Directive 86/609/EC (1986) of the Council of Europe.

Dietary regimen

Before induction of renal I/RI the animals were randomly divided into five groups of six mice each; ad libitum fed (AL), ad libitum fed sham operated (AL sham, undergoing only midline incision without induction of I/RI), ad libitum fed+I/RI (AL, undergoing renal I/RI), FA sham operated (three days of water-only FA) and FA+I/RI (three days of water-only FA, undergoing renal I/RI). For the FA groups, three animals per cage were kept (to avoid the animals from fighting and eating their own feces) and were transferred into a new clean cage (at the end of the day without access to any food while having free access to water). The mice were monitored daily for signs of distress and weight loss.

Induction of renal I/RI

After 72hrs of FA, renal I/RI was induced in I/RI groups. The mice were first anesthetized with 5% isoflurane, and were then maintained at 2.5% isoflurane. To maintain a normal body temperature, mice were put on a heating pad on the operation table. An abdominal midline incision was made and the kidneys were exposed. Both the renal pedicles were clamped using atraumatic microvascular clamps. Signs of ischemia by changing of the kidney color to purple was observed, after which the wound was covered with phosphate-buffered saline (PBS)-soaked cotton and the animals were placed under an aluminum foil blanket to maintain body temperature. After an ischemia time of 37 minutes, the clamps were removed and restoration of blood-flow was confirmed by the return of normal

kidney color (pink). The abdominal wound was closed in two layers using 5/0 sutures followed by subcutaneous injection of 0.5 mL PBS for maintenance of fluid balance and buprenorphine as analgesic. Mice were kept warm under a heat lamp.

Cell isolation and staining

After After a period of 6hrs and 24hrs, the mice were exsanguinated by cardiac puncture under anesthesia to draw blood, and primary and secondary lymphoid organs were harvested. Lymphoid cells were then processed as previously described [18]. For staining of blood, the erythrocytes were lysed using Osmotic lysis buffer (8.3g NH_4Cl end concentration 0.15M, 1.0g $KHCO_3$ end concentration 10mM, 200 ml 0.5M EDTA end concentration 0.1mM in 900 ml milliQ).

Flow cytometry

For all lymphoid organs, $2x10^6$ cells were plated in a 96-well plate for a particular stain. After the cells were plated, the next steps were followed as previously described with the same antibody staining scheme [18].

Chemicals and reagents

All the antibodies were of monoclonal origin directed against mouse leukocyte populations and obtained from BD Biosciences (BD Biosciences, city, country) or eBioscience (Campus Vienna Biocenter 2, Vienna, Austria), unless mentioned otherwise as depicted in Table 1.

Table 1 The different B and T cell phenotypes in lymphoid organs and their markers.

Organs & Stages in cell development	***Markers***
Bone Marrow	
B cells	$CD19^{+}B220^{+}$
Pre-B cells	$CD43^{+}CD2^{-}IgM^{-}IgD^{-}$
Pro- B cells	$CD43^{-}CD2^{+}IgM^{-}IgD^{-}$
Immature B cell	$IgM^{+}IgD^{low}$
Recirculating mature B cells	$IgM^{+/low}IgD^{high}$
T cells	$CD3^{+}$
Spleen	
B cells	$CD19^{+}B220^{+}$
early immature B cells	$IgM^{low}IgD^{low}$
Immature B cells	$IgM^{+}IgD^{-}$
Mature B cells	$IgM^{+}IgD^{+}$
Marginal zone B cells	$CD21^{+}CD23^{-}$
Follicular B cells	$CD21^{-}CD23^{+}$
Thymus	
Double Negative stages (DN)	$CD3^{-}CD4^{-}CD8^{-}$
DN1	$CD44^{+}CD25^{-}$
DN2	$CD44^{+}CD25^{+}$
DN3	$CD44^{-}CD25^{+}$
DN4	$CD44^{-}CD25^{-}$
Immature Single Positive (ISP)	$CD8^{+}CD3^{-}CD69^{-}$
Double Positive (DP)	$CD3^{-/low}CD4^{+}CD8^{+}$
CD8+ Single Positive (SP)	$CD3^{+}CD8^{+}CD4^{-}$

Elispot

The Elispot assays were performed using splenocytes. For this, spleen was mashed and passed through 100 µm Nylon cell strainers (BD Falcon™, BD Biosciences Europe, Erembodegem, Belgium). The cell suspension was made in RPMI-1640 medium (Lonza Europe B.V., Verviers, Belgium) supplemented with 10% FCS (Lonza Europe B.V.,

Verviers, Belgium) and 1% penicillin/streptomycin (Invitrogen™/Gibco®, Bleiswijk, the Netherlands). The total number of live cells were then counted using a Casy TT counter and analyzer (Innovatis, Roche Diagnostics Nederland B.V., Almere, Netherlands) and were further used for Elispot. The IFN-γ, IL-2, IL-4, and IL-10 Elispot assays (U-CyTech biosciences, Utrecht, the Netherlands) were used to determine the number of cytokine producing cells to phorbol 12-myrsitate 13-acetate (PMA) and ionomycin. After 40 hours of incubation with or without stimulus, the non-adherent cells were harvested and transferred in triplicate (IFN-γ: 1x10E4; IL-2: 1x10E5; IL-4 and IL-10: 2x10E5 cells/well) to the anti-mouse cytokine coated plate. The cells were incubated overnight in the absence or presence of PMA/ionomycin. Detection of spots was performed according to the protocol provided by the manufacturer (U-Cytech biosciences). The spots were counted automatically by using a Bioreader 3000 Elispot reader (BioSys, GmbH, Karben, Germany).

Statistics

Data are expressed as the mean ± SEM. Statistical analyses of the data on flow cytometry, immunohistochemistry, and Elispot was performed using Non-parametric Mann-Whitney U test using GraphPad Prism version 5.01 for windows (GraphPad Software, San Diego California USA). P-values ≤0.05 were considered to be statistically significant.

RESULTS

Fasting decreases pro-inflammatory cytokine production post renal I/RI

Since cytokines play an important role in mediating I/RI, we were interested to see whether FA influenced T cell function. To elucidate this we investigated the frequency by which splenocytes produce both pro- and anti-inflammatory cytokines in FA and I/RI conditions. The number of IFN-γ producing cells remained the same at 6hrs and 24hrs in the AL+sham group, while in the AL+I/RI group, an increase was seen at 6hrs compared to AL+sham ($p \leq 0.05$), which returned to comparable levels between all groups at 24hrs. Frequencies of IL-2 producing cells significantly ($p \leq 0.05$) increased in the AL+I/RI group compared to AL+sham, but the effect was waned off at 24hrs (Figure 1A, 1B). FA did not seem to have an effect on the anti-inflammatory cytokines IL-4 and IL-10 at both 6 and 24hrs post reperfusion (Figure 1C, 1D). There were no significant differences between groups.

Hence, these data show that FA results in a temporary reduction in Th1 cytokine producing cell frequencies, while having no impact on anti-inflammatory Th2 cytokines.

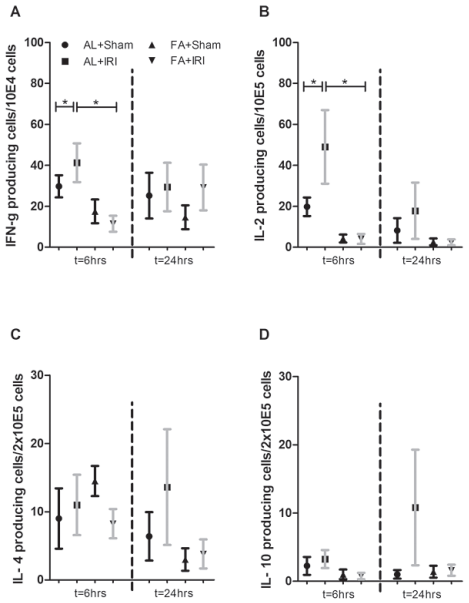

Figure 1. Effect of FA on cytokine profile post renal I/RI.

*In **Fig.1A** and **1B**, the number of IFN-γ and IL-2 producing cells is shown at 6 and 24hrs post reperfusion in both FA and AL fed mice. The numbers of anti-inflammatory cytokine producing cells are also depicted in **Fig.1C** and **1D** for IL-4 and IL-10.*

Fig.1A, 1B *≤ 0.05

For all the figures in this chapter

AL sham = ad libitum mice undergoing midline incision without induction of I/RI,

AL I/RI = ad libitum fed mice undergoing renal I/RI,

FA sham = three days of water-only FA mice, undergoing midline incision without induction of I/RI

FA I/RI = three days of water-only FA mice, undergoing renal I/RI

Fasting arrests B cell development in bone marrow up to 24hrs post renal I/RI

At first we investigated the B cell development stages in the BM after I/RI in fasted animals. FA had no effect on total $CD19^+B220^+$ B-lineage cell populations 6hrs post-reperfusion, but this population was significantly decreased 24hrs post-reperfusion, compared to the AL fed I/RI animals (Figure 2A). However, at 6hrs the FA+sham group had a significant decrease in the $CD19^+B220^+$ population as compared to that at 24hrs, whereas in FA+IRI at both 6 and 24hrs no differences were observed. We then investigated pro-, pre-, immature and recirculating mature B cells. Pro-B cells overall increase between 6 and 24hrs after I/RI in all groups. At 6hrs, pro-B cells ($CD43^+CD2^-IgM^-IgD^-$) in the FA+I/RI group were significantly lower than in the FA+sham group, but no difference was seen after 24hrs. The pro-B cell population was significantly increased 24hrs post reperfusion in the FA+I/RI group (Figure 2B), compared to the AL I/RI group. This accumulation of pro-B cells was accompanied by arrest of the pre-B cell population ($CD43^-CD2^+IgM^-IgD^-$) (Figure 2C). Pre-B cells showed an overall decrease between 6hrs and 24hrs in all groups. Both FA groups demonstrated lower percentages compared to AL mice. In the I/RI animals, a significant difference was seen in pre-B cell percentages between AL and FA at

both time points. Immature B cells ($IgM^{+}IgD^{low}$) (Figure 2D) of the FA+I/RI mice were significantly lower compared to the other 3 groups at 6hrs, and at 24hrs only compared to AL+I/RI mice. In the AL+I/RI group, immature B cells increased between 6 and 24 hrs. The most interesting finding was that in both the FA groups the recirculating mature B cells ($IgM^{+/low}IgD^{high}$) (Figure 2E) were found to be significantly increased at both 6hrs and 24hrs post reperfusion. No significant differences were found between the sham and I/RI induced animals in these B cell development stages.

Hence, FA arrests B cell development starting at pro-B cell stage while causing a significant increase in recirculating mature B cells, persisting at least 24hrs, and irrespective of I/RI.

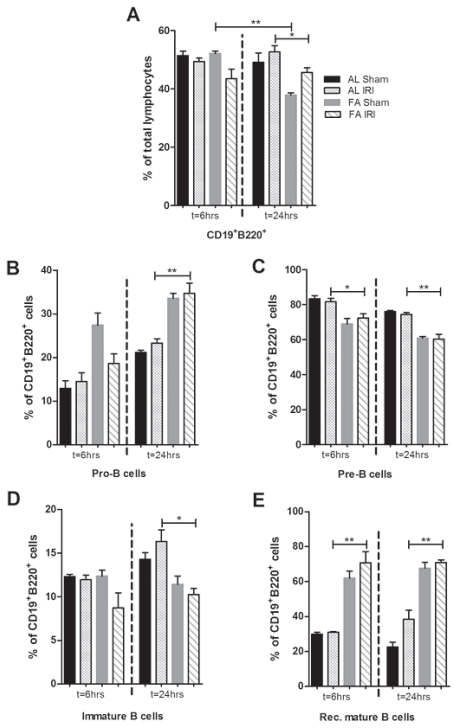

Figure 2. Effect of FA on B cell development phenotype in the BM post renal I/RI.

Fig.2A represents the total B cell lineage population ($CD19^{+}B220^{+}$) in BM in AL and FA fed mice 6 and 24hrs post induction of renal I/RI. **Fig.2B** and **2C** shows IgD and IgM populations from $CD19^{+}B220^{+}$ B cell fraction with $IgM^{-}IgD^{-}$ populations showing distinct pro-B and pre-B cells based on $CD43^{-}CD2$ profiles of $IgD^{-}IgM^{-}$ fractions and distinguishes between pro-B ($CD43^{+}CD2^{-}IgM^{-}IgD^{-}$) and pre-B ($CD43^{-}CD2^{+}IgM^{-}IgD^{-}$) cell populations. **Fig.2D** and **2E** represents the $IgM^{+}IgD^{low}$ (immature B cells) and $IgM^{+/low}IgD^{high}$ (recirculating mature) B cells at both 6 and 24hrs post induction of renal I/RI.

Fig. 2A *=p<0.05, **=p<0.005, 2B **=p<0.005, 2C *=p<0.05, **=p<0.005, 2D *=p<0.05, 2E **=p<0.005.

Fasting reduces B cell subsets in the spleen post renal I/RI

In the spleen, the total $CD19^+B220^+$ B cell population along with B cell subtypes, such as IgM and IgD low and high populations, as well as marginal zone (MZ) and follicular (FO) B cells were investigated. At 6hrs post reperfusion, a significant decrease between AL+sham and AL+I/RI groups as well as between AL+sham and FA+sham groups was observed. However, at 24hrs post reperfusion, no significant changes were observed in the total $CD19^+B220^+$ B cell population in the FA or AL animals (Figure 3A). At 6hrs post reperfusion the population of $IgM^{low}IgD^{low}$ (activated or class switched population generally low in frequency) cells out of the total fraction of $CD19^+B220^+$ B cells in the AL+sham group was 9.5%, while in the FA sham-operated mice it was found to be 6.2%; also in both the FA groups, a decrease at 24hrs post reperfusion is observed compared to 6hrs. Overall, a decrease in $IgM^{low}IgD^{low}$ population between 6 and 24hrs is observed, except for AL+I/RI where it remains the same (Figure 3B).

The percentage of $CD19^+B220^+IgM^+IgD^-$ immature B cells in the AL+sham group 6hrs post reperfusion was approximately 20% while, in the FA+sham group, this was significantly lower (13.8%), but was comparable to the FA+IRI group. No significant differences were observed 24hrs post reperfusion except for a decrease in the FA+I/RI group as compared to the AL+I/RI group (Figure 3C). No significant differences were observed in the population of $CD19^+B220^+$ IgM^+ IgD^+ mature B cells 6hrs post sham or post reperfusion. However, 24hrs post reperfusion, a significant increase in this mature B cell population was observed in the FA group (Figure 3D). B cell subtypes such as $CD21^+CD23^-$ marginal zone (MZ) and $CD21^-CD23^+$ follicular (FO) B cells were also measured in the dietary restricted groups and were found to be unaffected 6hrs post reperfusion while the MZ B cell population 24hrs post reperfusion was significantly decreased in the FA mice (Figure 3E). Similarly, no significant changes were observed in the FO B cells 6hr post reperfusion, while in the FA+I/RI group the FO B cells were higher compared to AL+I/RI at 24hrs post reperfusion due to FA (Figure 3F).

In conclusion, FA+I/RI causes a decrease in the immature (IgM^+IgD^-) B-cells while an increase in the more mature (IgM^+IgD^+) B-cells in the spleen is seen. A relative reduction in MZ B cells was observed and a relative increase in FO B cells was detected upon FA+I/RI compared to AL+I/RI 24hrs post reperfusion. At 6hrs only minor differences were observed.

Thymopoiesis is arrested by Fasting

As we previously showed at t=0, FA significantly reduces the total number thymocytes persisting at least 24hrs, irrespective of I/RI. The number of thymocytes 6hrs post reperfusion in the AL+sham mice was found to be approximately $71.3x10^6$, which was significantly lowered to $19.1x10^6$ in FA+sham mice, lasting at least 24hrs post reperfusion (Figure 4A). Upon further investigation into the T cell phenotype development, which starts from $CD4^-CD8^-$ double negative (DN) T cells, a significantly higher proportion of

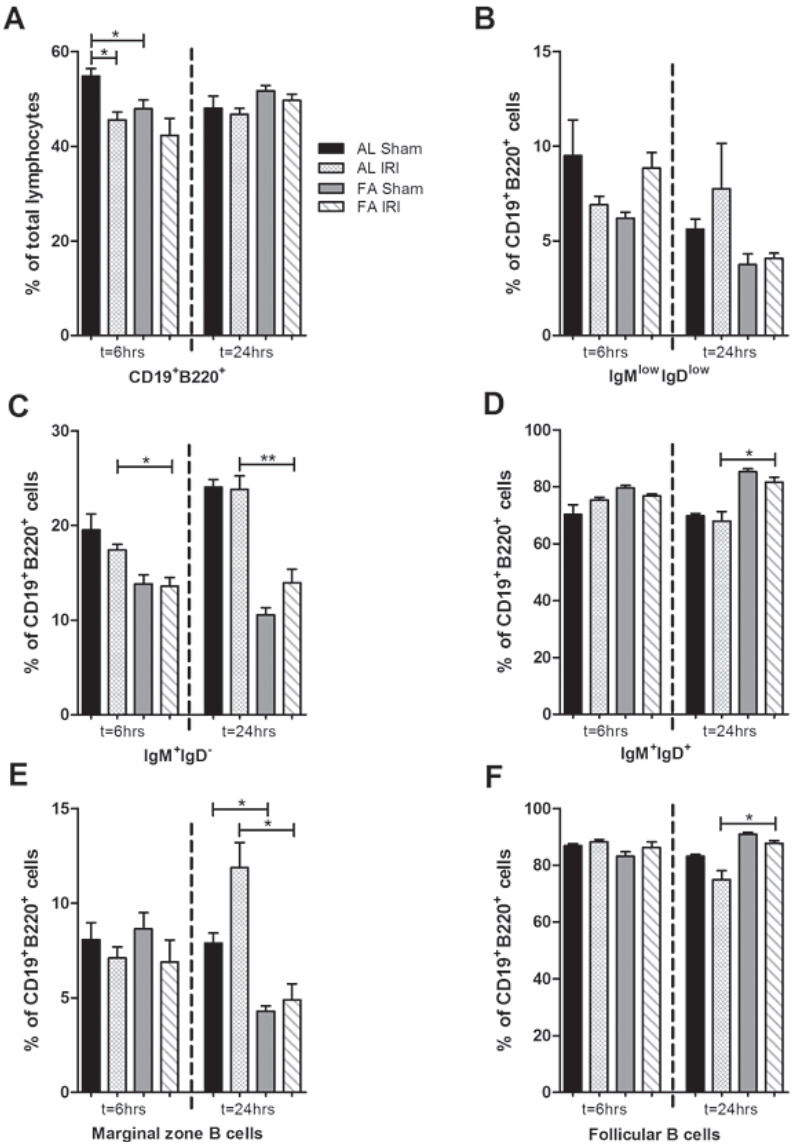

Figure 3. Effect of FA on splenic B cell phenotypes post induction of renal I/RI.

Fig.3A shows the total splenic $CD19^+B220^+$ B cell profile while **Fig. 3B**) Shows that $IgM^{low}IgD^{low\ population}$ of gated $CD19^+B220^+$ fractions was not affected due to both FA or AL diet while the cellularity of immature $IgM^+IgD^{-\ in\ Fig.3C}$ and mature IgM^+IgD^+ populations in **Fig.3D** was significantly affected by FA post induction of renal I/RI. **Fig.3E** and **3F**) represent the cellularity changes in $CD21^+CD23^-$ marginal zone and $CD21^-CD23^+$ follicular B cells.

Fig. 3A *=p<0.05, 3C *=p<0.05, **=p<0.005, 3D, 3E and 3F *=p<0.05.

this DN population was observed at both 6 and 24hrs post reperfusion, compared to the AL+sham mice (Figure 4B). Based on the expression of CD44 and CD25 this DN fraction was further categorized into four subsets. We found that the proportions of DN1 cells were higher 6hrs post reperfusion in the FA mice while no significant differences were observed 24hrs post reperfusion (Figure 4C). However, the DN2 population was lowered in the FA group, compared to the AL+I/RI mice (Figure 4D). Further down the developmental stages, the DN3 and DN4 populations were significantly lowered in the FA group 6hrs post reperfusion while no differences were observed 24hrs post reperfusion (Figure 4E, 4F).

After 6hrs, there was a relative accumulation of DN1 in the FA+I/RI group and all other populations decreased. This was similar at 24hrs, but differences were particularly seen in DN2 cells. No differences for the DN4 stage were observed. After the DN stages, the thymocytes start to upregulate CD8 and progress to immature single positive (ISP) $CD8^-CD3^-CD69^-$ thymocytes. The percentage of $CD8^-CD3^-CD69^-$ ISP cells in the thymus was significantly reduced in the FA groups both at 6 and 24hrs post reperfusion (Figure 4G). The double positive (DP) $CD4^+CD8^+$ cells, which comprise the major proportion of thymocytes were reduced significantly at both 6 and 24hrs post reperfusion (Figure 4H). However, both the CD4 and CD8 single positive stages (SP) were increased 6 and 24hrs post reperfusion in FA mice compared to the AL fed mice (Figure 4I, 4J).

Hence, in the current study, we observed that, in the FA groups, there is a significant arrest of T cell development already at the DN stage, leading to a substantial reduction of thymic size (due to a decrease in the largest population of DP cells), irrespective of I/RI. Only CD4 and CD8 SP cells in FA groups appear to be unaffected, since their proportions as well as absolute numbers remain stable or even rise after I/RI.

Fasting causes an influx of CD3+ T cells in spleen and bone marrow

Next, the proportions of $CD3^+$, $CD4^+$ and $CD8^+$ T cells were investigated in splenic tissue. Within the total lymphocyte population the proportion of $CD3^+NK1.1^-$ T cells (the true CD3 T cell population and not the NKT cell population) was significantly increased in FA+I/RI 24hrs post-reperfusion (Figure 5A) compared to 6hrs, in all groups, whereas no differences existed within the groups at 6hrs. The proportions of $CD4^+$ and $CD8^+$ in the $CD3^+$ T cell population were found to be unaffected by FA (Figure 5B, 5C). The percentage of $CD3^+$ T cells in BM is usually low. We found a significant increase in the percentage of these cells in FA animals both at 6 and 24hrs post reperfusion compared to that of the AL+I/RI mice (Figure 5D). This persisted in the context of renal I/RI, even at 24hrs post reperfusion.

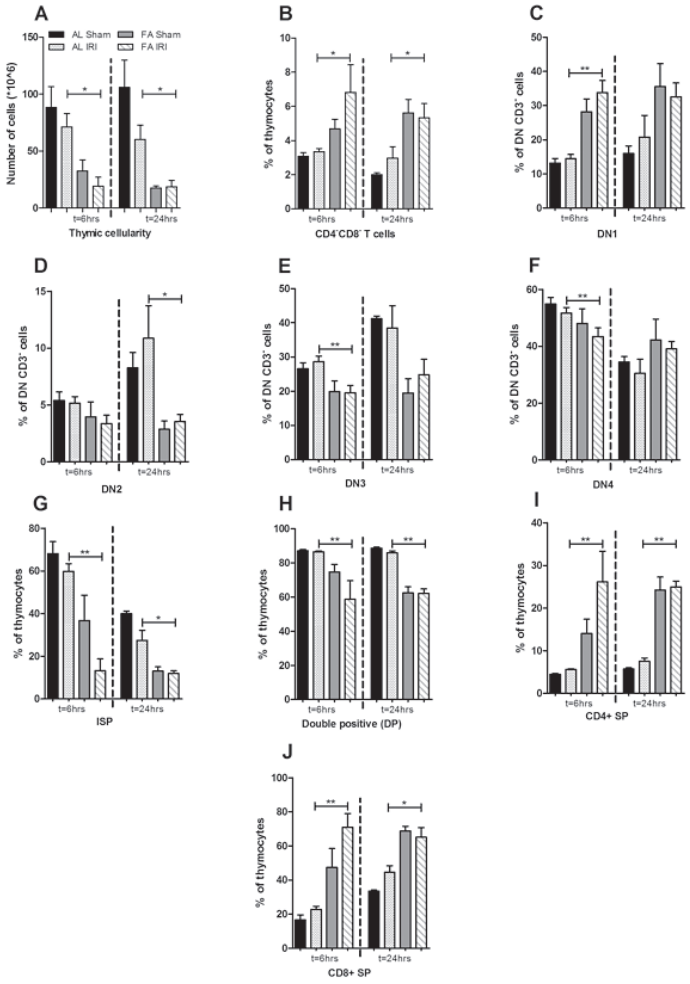

Figure 4. Effect of FA on T cell phenotype in Thymus due to FA post induction of renal I/RI.

Fig.4A shows the total thymic cellularity in the sham and ischemic thymus of both AL fed and FA mice. **Fig. 4B)** shows the $CD4^-CD8^-$ DN fractions of the total thymocytes. **Fig.4C, 4D, 4E** and **4F** shows the CD44/CD25 profiles $CD4^-CD8^-$ fraction represented by DN1, DN2, DN3 and DN4 stages. **Fig.4G, 4H, 4I** and **4J** shows the development stages based on the expression of CD3/CD69 representing $CD3^-CD69^-$ ISP, $CD4^+CD8^+$ DP and $CD4^+$ SP and $CD8^+$ SP populations.

Fig. 4A, B *=p<0.05, Fig. 4C **=p<0.005, Fig. 4D *=p<0.05, Fig. 4E, F, G, H, and I **=p<0.005, Fig. 4J *=p<0.05, **=p<0.005.

Hence, a significant increase in the total CD3+ T cell population in FA+I/RI mice was observed while the proportions of CD4+ and CD8+ T cells within the total CD3+ T cells remained stable in spleen. Similar to splenic CD3+ T cells, a significant increase in BM CD3+ T cells was seen in FA+I/RI mice.

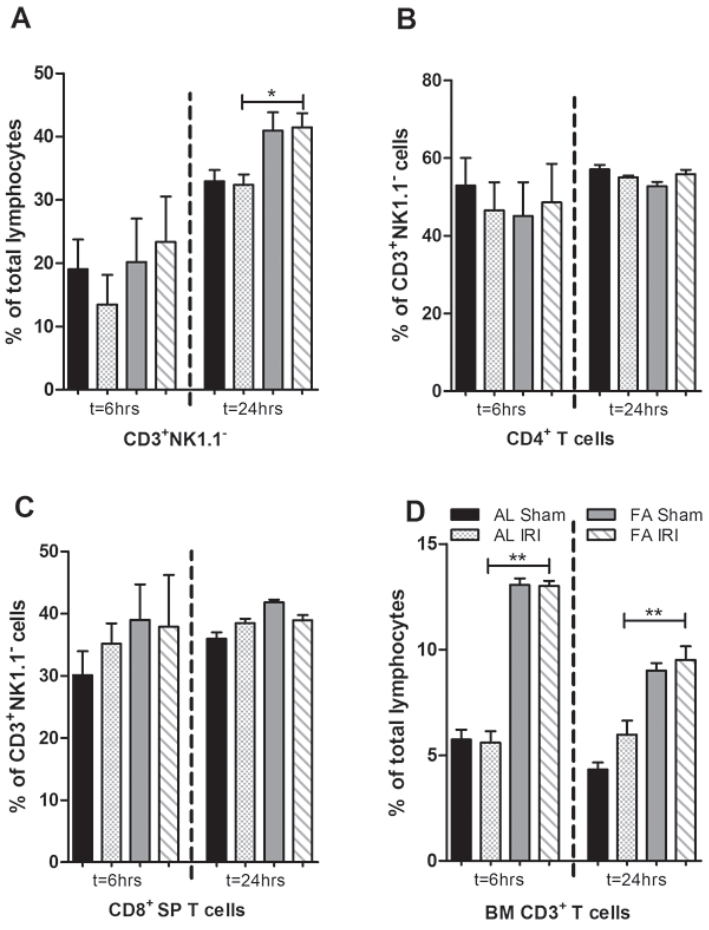

Figure 5. Effect of FA on T cell phenotype in Spleen and BM post induction renal I/RI.

Fig.5A shows the total CD3+NK1.1− T cell population in spleen. **Fig.5B** and **5C)** shows the total population of CD4+ and CD8+T cells out of the total CD3+NK1.1- T cell fraction. **Fig.5D)** shows the CD3+ T cell changes taking place in BM.

Fig. 5A *=p<0.05, Fig. 5D **=p<0.005.

DISCUSSION

Short-term DR is effective in reducing the major inflammatory changes in ageing-related diseases, but also induces resistance against inflammation induced by an ischemic insult. We have shown that three days of FA strongly reduces inflammation after both renal and hepatic I/RI [8]. In a previous study, we have highlighted the modulating effect of FA on the adaptive immune system without the induction of renal I/RI [18]. In the current study, we delineated the effects exerted by FA on the B and T cell phenotypes and cytokine production after the induction of renal I/RI.

We showed the cytokine production capabilities of the splenocytes of FA and AL fed mice post induction of renal I/RI. Several studies have highlighted the crucial role played by cytokines in the early induction of phase of I/RI during transplantation [19,20]. Out of the several cytokines, IFN-γ is a potent cellular immunomodulating cytokine that contributes to acute and chronic inflammation post I/RI [20,21]. I/RI experiments performed in IFN-γ KO animals have presented the highest impairment in renal function compared to their controls [22,23]. This shows that the Th-1 related cytokines such as IFN-γ and IL-2 are critically involved in renal I/RI. Upon investigation of these cytokines in the current settings we found a decrease in the number of IFN-γ and IL-2 producing cells in FA+I/RI groups 6hrs post reperfusion. No significant differences between the other experimental groups were observed 24hrs post-reperfusion. These results suggest that the effect exerted by FA occurs within the first 6hrs after induction of renal I/RI. The reason for this could be because of the fact that during the 6 and 24hrs post reperfusion, the mice were fed AL diet and hence the cytokine production capacity of the splenocytes was restored 24hrs post reperfusion. The decrease in the number of IFN-γ and IL-2 producing splenocytes could be an important contributor in the FA mediated protection against renal I/RI as these cytokines contribute to acute and chronic inflammation post reperfusion.

Next, we showed that at 24hrs post reperfusion, B cell development from the pro-pre B cell stage to the immature B cell stage is arrested by FA. Upon further investigation of the secondary lymphoid organs, we found that FA causes a decrease in the early mature B cell populations at both 6 and 24hrs post-reperfusion compared to the AL fed animals. FA also caused an increase in the mature and follicular B cells while causing a decrease in the marginal zone B cells 24hrs post-reperfusion. This arrest in B cell development stage in FA mice could partially explain the protection against renal I/RI. Based on previously published studies we know that the total B cell population is found to be increased three days post induction of I/RI [24]. It is also known that in B cell knock out (KO) mice, kidneys are functionally and structurally protected against I/RI compared to those of wild-type mice [24,25]. Studies performed by Renner et al. have demonstrated that mice completely deficient in mature B cells develop more severe I/RI than wild-type control mice [24]. Interestingly, in our study, the recirculating mature B cell populations were

increased in the FA groups 6 and 24hrs post reperfusion, to a similar extent as in our previous findings without induction of renal I/RI [18]. Whether the increased number of recirculating mature B cells are implicated in the immune regulation induced by FA in the form of B regulatory cells is something that needs further investigation. Splenic MZ B cells, which are known to be sufficient for I/RI induced tissue damage [26], were observed to be decreased after I/RI in FA mice.

T cell development is also arrested upon FA followed by induction of renal I/RI. We have reported earlier that FA causes a significant reduction in thymic cellularity and also arrested T cell development in the thymus [18]. Similar to our previous findings, we observed a significant reduction in the thymic cellularity. However, frequencies of $CD4^-CD8^-$ T cells and DN1 T cells were found to be increased post induction of I/RI at both 6 and 24hrs post reperfusion. The decreased frequencies of DN2 to DP T cell were due to the reduction in total thymic cellularity of the FA groups post induction of renal I/RI. Earlier studies have shown that CD4/CD8-deficient mice [27,28] have a marked functional protection from renal I/RI compared to wild-type, strain-matched controls [29,30]. This suggests that the protection induced by FA is associated with an arrest in the T cell development stages. The increase in $CD3^+$ T cells in BM upon induction, just as the influx of B cells in the BM and the arrest in B cell production, may contribute to the energy saving capacity of the organ induced by FA directing the BM to more energy maintenance instead of consumption.

Together, these observations direct towards a modulatory effect exerted by FA on the adaptive immune system that contributes to protection against renal I/RI.

FA is a robust and very efficacious method of not only preventing cancer but also in preventing neurodegeneration, inflammation and hypertension. FA causes major changes in the immune system and arrests both the B and T cell development in BM and thymus. FA also restores the animals' energy by maintaining an increased percentage of $CD3^+$ T cells in BM. Functionally, the frequencies of cytokine producing splenocytes (esp. those producing Th1 cytokines) are strongly diminished after FA and subsequent induction of I/RI, resulting in decreased inflammation. All these immunological changes may contribute to the protective mechanism of FA against renal I/RI, which does not seem to be exclusively regulated within the innate immune system [31], and is not just an hormetic phenomenon [32]. Further elucidation of this mechanism may result in the development of clinically relevant FA mimetics.

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CHAPTER 7

Protection against renal ischemia/reperfusion injury through hormesis? Dietary intervention versus cold exposure

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ABSTRACT

Dietary restriction (DR) and fasting (FA) induce robust protection against the detrimental effects of renal ischemia reperfusion injury (I/RI). Several mechanisms of protection have been proposed, such as hormesis. Hormesis is defined as a life-supporting beneficial effect resulting from the cellular responses to single or multiple rounds of (mild) stress. The cold exposure (CE) model is a stress model similar to DR, and has been shown to have hormetic effects and has proved to increase longevity. CE is considered to be the most robust method to increase metabolism through activation of brown adipocytes. BAT have been considered important in etiology of obesity and its metabolic consequences. Since DR, FA, and CE models are proposed to work through hormesis, we investigated physiology of adipose tissue and effect on BAT in these models and compared them to ad libitum (AL) fed mice. We also studied the differential effect of these stress models on immunological changes, and effect of CE on renal I/RI. We show similar physiological changes in adiposity in male C57Bl/6 mice due to DR, FA and CE, but the CE mice were not protected against renal I/RI. The immunophenotypic changes observed in the CE mice were similar to the AL animals, in contrast to FA mice, that showed major immunophenotypic changes in the B and T cell development stages in primary and secondary lymphoid organs. Our findings thus demonstrate that DR, FA and CE are hormetic stress models. DR and FA protect against renal I/IR, whereas CE could not.

INTRODUCTION

Ischemia-reperfusion injury (I/RI) is inevitable during organ transplantation, and is negatively correlated with clinical outcome and survival. In the light of donor organ shortage, extended criteria donors are increasingly being accepted for transplantation, with an increased risk for I/RI. Therefore, novel methods to prevent or reduce I/RI are of paramount importance. We have previously shown that both 14 days of 30% dietary restriction (DR) as well as 3 days of preoperative fasting (FA) protect against renal and hepatic I/RI in mice [1].

DR is considered a reduction in energy intake by 20-40% without causing malnutrition [2], and is one of the most robust, reproducible, non-invasive, clinically applicable and simple experimental manipulations that is known to extend both median and maximum lifespan in laboratory animals [3] and non-human primates [4]. The effect of DR has also proved to be highly beneficial in reducing many ageing related diseases, such as diabetes, autoimmune and respiratory diseases [5] along with improvement in kidney disease.

Over the last several decades, research has been focused intensively on trying to elucidate the mechanism behind the beneficial effects of DR. Several studies have demonstrated that DR helps in improving glucoregulatory function and insulin sensitivity along with decreased fasting glucose and insulin levels [6,7]. Other studies have highlighted the role of reduced T-lymphocyte production and proliferation, and slow thymic involution in the beneficial effects of DR [8,9]. We have recently shown that DR causes major immunophenotypic changes in the bone marrow (BM) and thymus, and arrests their B and T cell development [10].

From another point of view, DR and FA have been postulated to reprogram the immunological profile, and induce hypothalamic-pituitary-adrenal (HPA) axis activity, since plasma corticosterone concentrations are increased [11]. As such, DR can be considered a mild stressor commonly referred to as hormesis, defined as a life-supporting beneficial effect resulting from the cellular responses to single or multiple rounds of (mild) stress [12]. There is evidence that an increased ability to cope with damage enhances longevity, probably by retarding the damage from ageing processes [13,14]. Thus, induction of hormesis by DR and FA might be one of the underlying mechanisms by which these dietary interventions improve I/RI outcome.

Hormesis is promoted by different types of stress that induce different mechanisms. Another model that has shown to have hormetic effects, and has proved to increase longevity is cold exposure [15]. Cold exposure (CE) is a commonly used method to increase metabolism, since it results in activation of brown adipose tissue (BAT) and gives the white adipose tissue (WAT) more BAT-like characteristics. In mammals, BAT and WAT have opposite effects, but both are considered important in etiology of obesity and its

metabolic consequences [16]. While white adipocytes in WAT are mainly involved in energy storage in the form of triglycerides (TGs) that can be utilized during the periods of enhanced energy demand, brown adipocytes in BAT can oxidize TG-derived fatty acids to generate heat. BAT is capable to perform the latter since it contains uncoupling protein 1 (UCP1) that, when active, will uncouple mitochondrial oxidative phosphorylation from ATP synthesis. As a consequence, energy is released as heat [17].

In mice, CE also induces HPA axis activity, since it results in elevated plasma ACTH and corticosterone concentrations [18]. Thus, three different models, namely FA, DR and CE, induce HPA axis activity. However, only two of these preconditioning strategies have thus far been shown to ameliorate I/RI: DR and FA. On the other hand, only CE has been shown to result in activation of BAT and appearance of WAT with BAT-like characteristics. With respect to the immunological profiles, changes herein that might be of relevance for I/RI survival have only been studied in the FA and DR animal model [10].

We hypothesize that the temporary induction of stress in either one of the three models is crucial in the protection against I/RI. To study this, we compared the effects of FA, DR and CE on the HPA axis, and whether this correlates with I/RI survival. In addition, to compare the models in more detail, we investigated whether FA and DR affect BAT and whether CE affects the immunological profile and cytoprotective genes.

MATERIALS AND METHODS

Animal models and experimental setup

Male C57Bl/6 mice of 8 weeks, weighing 20–25g, were purchased from Harlan, Horst, the Netherlands. Mice were kept at normal laboratory physiological conditions with 3 animals per cage having free access to food (Hope Farms, Woerden, the Netherlands) and water until the start of experimental procedures.

For CE, the mice were acclimatized for 1 week under standard housing conditions before being housed individually, and put in a temperature-controlled climate chamber (Bronson, Nieuwkuijk, The Netherlands) with normal light/dark cycle. Body weights and food intake were measured before and after 24h-exposure to temperatures of either 23 or 4°C (n=8/group).

In the dietary intervention group, the animals were divided into 3 groups, namely, ad libitum (AL), 2 weeks 30% DR and 3 days water-only fasting (FA) (n=8/group). Dietary interventions were performed by Shushimita et al. as previously described [10]. However, for the AL group, mice were divided into two separate groups. One group had 4 animals/cage (Normal) while the other group had 1 mouse/cage (to mimic the cold- and warm-exposure experimental setup) referred to as normal separated. The experiments were

performed upon approval by the Dutch National Experiments on Animals Act, complied with Directive 2010/63/EU of the Council of Europe.

Tissue sample collection

Mice were terminated through exsanguination by cardiac puncture under isoflurane anesthesia. Plasma and tissues such as brown adipose tissue (BAT) and liver were collected and either immediately frozen and stored at -80°C or fixed in 4% paraformaldehyde, and after 24hrs stored in 70% ethanol, and later embedded in paraffin.

Serum corticosterone measurement

The serum corticosterone concentration of the experimental animals was measured by ELISA (Enzo Life Sciences, Antwerp, Belgium) according to manufacturer's instructions.

Histology of BAT

To perform the histological analysis of the BAT, hematoxylin and eosin staining was performed on the paraffinized tissues. The paraffin embedded tissues were cut into 8µm thick slices that were mounted on glass slides. The paraffinized tissue slides were deparaffinized by putting in xylene solution (2x, 5min each). The slides were then allowed to rehydrate using 100% alcohol (2x, 5min each), and were put in 96% alcohol for 5 min, followed by 70% alcohol for 5min. The slides were then washed in aquadest for 5min and stained with Mayer's hematoxylin solution for 2min. After hematoxylin staining, slides were washed in running tap water for 10min followed by counterstain with eosin solution for 4 min. The slides were then dehydrated in 96% alcohol (2x, 2min each) followed by 100% alcohol (2x, 2min each) and then cleared in xylene solution (3x, 2min each). The slides were then covered with PERTEX ® (Histolab, Gothenburg, Sweden), and a second glass.

Immunohistochemistry (IHC) for Ucp1

For Ucp1 IHC, the 8µm thick paraffin slices were mounted on 3-aminopropyltriethoxysilene coated slides. These slides were put at 60°C for 60min, after which the tissues were deparaffinized in xylene for 6min, and rinsed 2x with 100% ethanol. The endogenous peroxidase activity of the adipose tissues was blocked using 3% H2O2 in methanol for a period of 20min. After endogenous peroxidase blocking antigen retrieval was performed using fresh NaOH buffered citric acid, and heating it 3x for 5min followed by cooling down the slides to room temperature (RT). After antigen retrieval the slides were incubated with goat serum (5%) for 5min at RT, and were then incubated overnight with primary antibody rabbit polycolonal anti-Ucp1 (Sigma-aldrich) in 1:500 dilution, at 4oC. The following day slides were incubated with poly-HRP-GAM/R/RIgG (Immunologic, Duiven, the Netherlands) for 30min and Ucp1 was detected by 3,3'-diaminobenzidine (DAB) staining by incubating in a PBS solution with 0.075% w/v DAB solution and 0.25% v/v for 3min. The nuclear staining of the slides was performed using hematoxyline

followed by dehydration in 70%, 95%, 100% ethanol, and xylene. The slides were then covered with PERTEX ® (Histolab) and a second glass.

RNA isolation, cDNA synthesis and real-time PCR

Total RNA from mouse tissues was isolated using Tripure Isolation Reagent (Roche) according to the manufacturer's instructions. Genomic DNA was removed by DNAse treatment (Promega Benelux BV, Leiden, The Netherlands) for 30 minutes at 37 °C. Reverse transcription was performed using a cDNA synthesis kit (Roche) according to the manufacturer's instructions. Quantitative RT-PCR was performed using SYBRgreen mastermix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) with an ABI Prism 7900 Sequence Detection System. Sequences of the primers used are listed in Table 1. The expression of each gene was expressed in arbitrary units after normalization to the average expression level of the housekeeping genes Rn18s and beta2-microglobuline (B2m) using the $2^{-\Delta\Delta Ct}$ method [19].

Table 1 Primer sequences of the genes.

Gene	*Sequence, 5′ to 3′*	*Gene Bank acc. No.*	*Reference*
Atgl	F:GAGCCCCGGGGTGGAACAAGAT R: AAAAGGTGGTGGGCAGGAGTAAGG		
B2m	F:ATCCAAATGCTGAAGAACGG R:CAGTCTCAGTGGGGGTGAAT	NM_009735	
Cd36	F: CCTCCAGAATCCAGACAACC R: CACAGGCTTTCCTTCTTTGC		[30]
Fkbp5	F: ATTTGATTGCCGAGATGTG R: TCTTCACCAGGGCTTTGTC		[31]
Gr	F: CCGGGTCCCCAGGTAAAGA R: TGTCCGGTAAAATAAGAGGCTTG		
Leptin	F: CAGGATCAATGACATTCACACA R: GCTGGTGAGGACCTGTTGAT		
Lpl	F: GCTGGTGGGAAATGATGTG R: TGGACGTTGTCTAGGGGGTA		
Mcad	F: GATCGCAATGGGTGCTTTTGATAGAA R: AGCTGATTGGCAATGTCTCCAGCAAA		[32]
Pgc1a	F: CCCTGCCATTGTTAAGACC R: TGCTGCTGTTCCTGTTTTC	NM_008904	[33]
Prdm16	F: GACATTCCAATCCCACCAGA R: CACCTCTGTATCCGTCAGCA		
Rn18s	F: GTAACCCGTTGAACCCCATT R: CCATCCAATCGGTAGTAGCG	NR_003278	
Ucp1	F: GGCCTCTACGACTCAGTCCA R: TAAGCCGGCTGAGATCTTGT	NM_009463	[34]

Ucp1 Western blot

Protein was extracted from BAT as previously described [18]. Fifteen µg of protein was electrophoresed on a 10 % gel, and blotted on a nitrocellulose membrane. Membranes were blocked in PBS containing 3% nonfat powdered skim milk before an overnight incubation at 4°C with a rabbit polyclonal anti-UCP1 antibody (1:1000; Sigma) in PBS containing 0.1% Tween-20 and 3% nonfat powdered skim milk or a rabbit polyclonal anti- α-tubulin antibody (1:200; Santacruz, Heidelberg, Germany) in PBS containing 0.1% Tween-20 and 5% bovine serum albumin (BSA). Next, membranes were washed and incubated for 1 hour at room temperature with a goat-anti-rabbit IRDye 800 secondary antibody (1:10,000; Li-cor, Leusden, The Netherlands) in PBS containing

0.1% Tween-20 and 3% non-fat powdered skim milk. Ucp1 immunoreactivity was measured with an Odyssey fluorescence scanner (Li-cor, Leusden, The Netherlands) and was normalized for α-tubulin immunoreactivity in the same samples using Odyssey software.

Cell isolation for immunophenotyping

After the dietary interventions and CE, the mice were sacrificed by cardiac puncture under anesthesia, after which thymus, spleen and bones were harvested for further immunophenotyping. Both thymus and spleen were first processed to make single cell suspensions by mashing and passing them through 100µm Nylon cell strainers (BD Falcon™, BD Biosciences Europe, Erembodegem, Belgium). For bones, both femur and tibia were crushed using mortar and pestle, followed by suspension in RPMI-1640 medium (Lonza Europe B.V., Verviers, Belgium). The total number of cells was then counted on Casy TT counter and analyzer (Innovatis, Roche Diagnostics Nederland B.V., Almere, the Netherlands), and were prepared for flow cytometry.

Flow Cytometric analyses

For flow cytometric analyses, 2x106 cells were plated in a 96-well plate for each staining. Cells were further processed according to [10]. Also, stainings similar to our previous work were performed on these cells [10], making use of the same chemicals and reagents.

Renal Ischemia-reperfusion injury (I/RI)

After the dietary interventions and warm, cold exposure stress induction, the mice underwent bilateral induction of renal I/RI. For this, mice were first anesthetized by isoflurane inhalation (5% isoflurane followed by maintenance on 2.5% with oxygen). A midline abdominal incision was followed by localization of the left renal pedicle and dissection of the renal artery and vein. Both renal arteries and veins were occluded, using an atraumatic microvascular clamp for 37 min. Signs of ischemia (a.o. purple color) were observed, the wound was covered with phosphate-buffered saline (PBS)-soaked cotton and the animals were placed under an aluminum foil blanket for the maintenance of body temperature. After ischemia time of 37 min, the clamps were removed and restoration of blood-flow was confirmed when the kidney regained its normal color. The abdominal wound was closed in two layers using 5/0 sutures followed by subcutaneous injection of 0.5 mL PBS for maintenance of fluid balance and were kept warm under a heat lamp.

Statistical analysis

Paired sample T-tests were performed on all the experimental groups using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.), while the graphs were plotted using GraphPad Prism version 5.01 (GraphPad Software, San Diego California USA). Significance was based on p-values <0.05.

RESULTS

Dietary interventions and cold-exposure augment stress response

During the course of 2 weeks when the mice were undergoing 30% dietary restriction (DR), they lost approximately 5% of their body weight while the FA mice lost approximately 22% of their body weight during 72hrs of fasting. Since AL mice were having free access to food, they gained approximately 10% of their body weight. In the second group where mice were either kept at normal room temperature or at 4^{O}C for 24hrs, the AL mice did not gain or lose any weight, while the cold exposed mice lost approximately 1% of their body weight (data not shown).We measured the levels of stress in both the dietary intervention and CE stress models. In both DR and FA mice, serum corticosterone levels were found to be significantly elevated (Fig.1A). However, the same effect was not observed in CE mice compared to its control counterpart (Fig.1B). In line with this is the expression of the glucocorticoid receptor (GR) target gene *Fkbp5* in BAT under both manipulations. Fkbp5 expression was significantly 120-fold upregulated in FA (Fig.1C) while only a non-significant 2.5-fold upregulation was detected upon CE (Fig.1D).

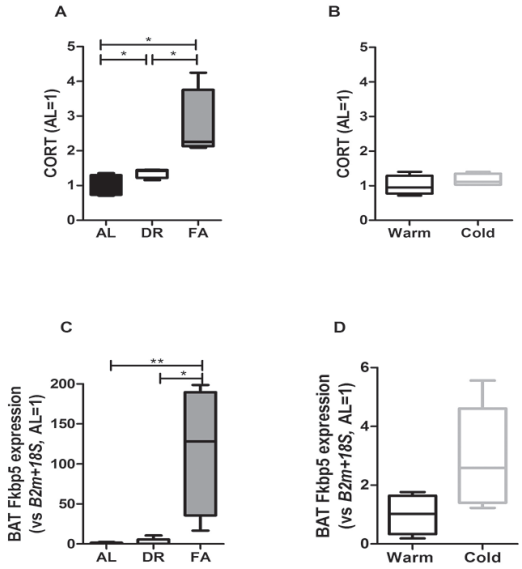

Figure 1. Effect of DR, FA and CE on stress hormone and stress related gene.

*In **Fig.1A** and **1B**, the level of stress hormone, corticosterone, has been depicted. It has been shown that FA causes a severe stress response as compared to that caused by cold exposure. **Fig.1C** and **1D** shows the stress related gene Fkbp5 and the effect of dietary interventions and cold exposure on this gene.*

Fig.1A *≤ 0.05, **≤ 0.001, Fig.1C *≤ 0.05, **≤ 0.001

*Fig.1A *≤ 0.05, **≤ 0.001, Fig.1C *≤ 0.05, **≤ 0.001*

Dietary interventions and cold-exposure differentially affect BAT activity
BAT weight, histology and Ucp-1 immunohistochemistry

The ratio of BAT:BW was not affected by DR however it was significantly upregulated by FA (Fig.2A). Compared to the AL group, BAT of the DR mice had larger lipid droplets (Fig.2B), suggesting reduced BAT activity. FA, however, resulted in denser BAT than the BAT of AL mice, suggesting more active BAT which was confirmed by the increased number of nuclei per area upon FA but not DR (Fig.2C). However, both immunohistochemistry and western blotting clearly revealed that FA resulted in a significant decline in BAT Ucp1 protein content (Fig.2D), indicating reduced BAT activity.

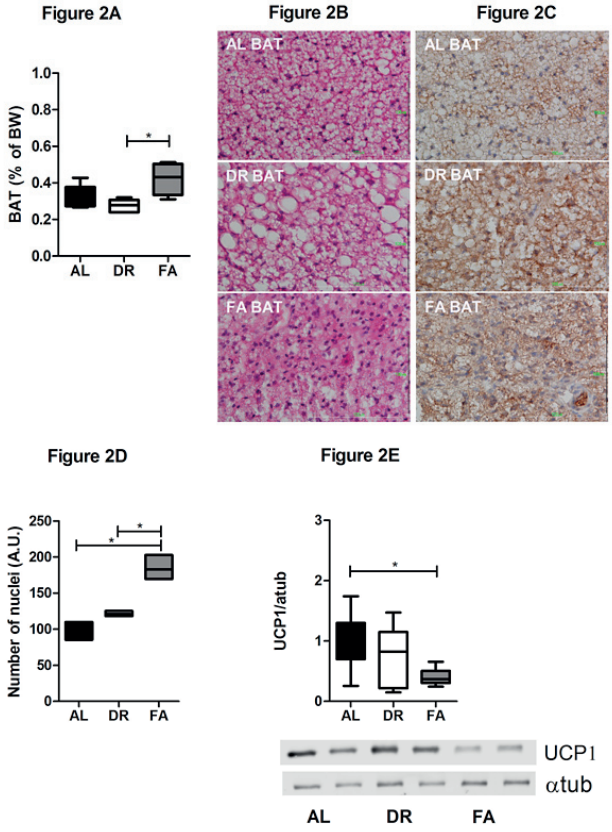

Figure 2. Effect of dietary interventions on histology and UCP-1 protein expression.

Fig.2A *represents the brown adipose tissue weight as compared to the percentage of body weight of the animal while* ***Fig.2B*** *shows the immunohistochemistry and Ucp-1 staining of the (BAT)*

In ***Fig.2C*** *number of nuclei per area of BAT has been presented, which is increased due to FA while the western blot data in* ***Fig.2D*** *shows significantly reduced expression due to FA.*

Fig.2A *≤ 0.05, Fig.2C **≤ 0.001, ***≤ 0.0001, Fig.2E *≤ 0.05.

*Fig.2A *≤ 0.05, Fig.2C **≤ 0.001, ***≤ 0.0001, Fig.2E *≤ 0.05.*

In the CE mice, the ratio of BAT to BW was not affected (Fig.3A), but BAT histology, BAT density and BAT Ucp1 protein content all showed induced BAT activity upon exposure to 4°C (Fig. 3B-E).

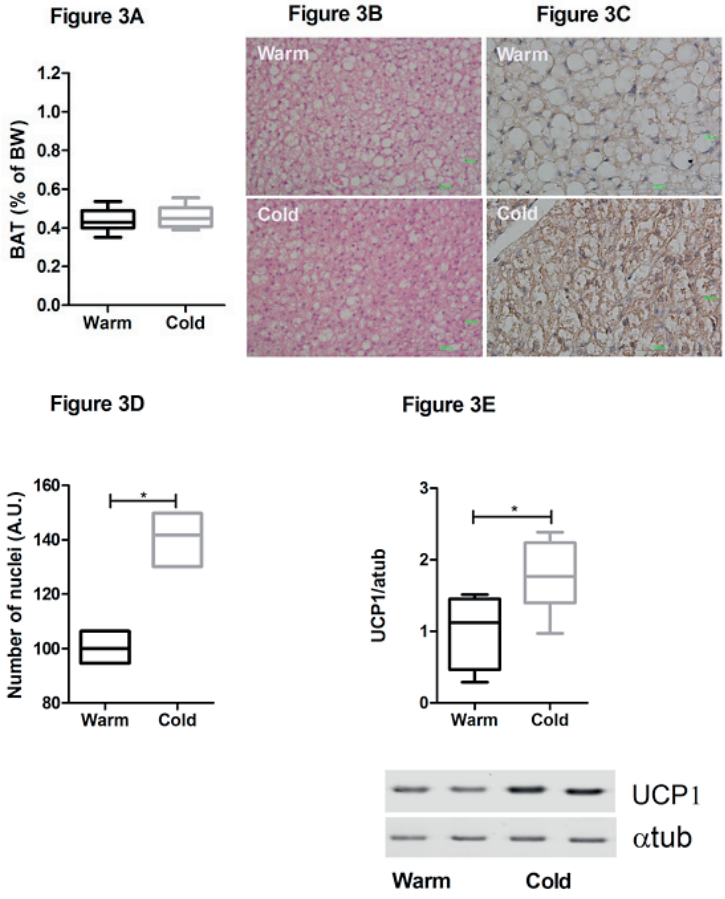

Figure 3. Effect of CE on histology and UCP-1 protein expression.

Fig.3A *represents the change in BAT in comparison to the body weight of the animal which is unaffected due to CE however, the histology and Ucp-1 expression shows more brown-like features, as represented in* ***Fig.3B, 3C****. This was further shown by the amount of nuclei measured in BAT, which was significantly increased in* ***Fig.3D*** *as well as by western blot,* ***Fig.3E,*** *which showed the similar increased expression.*

Fig.3D **≤ 0.005, Fig.3E *≤ 0.05.

Gene expression analysis

So far, the data suggest that BAT activity is not affected by DR but FA results in a declined BAT activity. As expected, CE resulted in a clear induced BAT activity. Next, we studied BAT activity in more detail by determining BAT expression of multiple genes. Indeed, all genes associated with BAT activity were upregulated upon CE (Fig.4A-F). With respect to the dietary interventions, expression of the gene encoding PR domain containing 16 (PRDM16) was not significantly increased (Fig.4A), while expression of the gene encoding peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1α) was increased upon FA (Fig.4B). Lipoprotein lipase (LPL) is involved in lipolysis of triglycerides into fatty acids that can be taken up by BAT. Interestingly, *Lpl* gene expression was significantly upregulated in both DR and FA groups (Fig.4C), while the gene encoding the fatty acid transporter CD36 was not affected in either DR or FA group (Fig.4D). Expression of the genes encoding the lipolytic enzymes adipose triglyceride lipase (ATGL) and medium-chain acyl coenzyme A dehydrogenase (MCAD) was not affected in the two dietary intervention groups (Fig.4E and 4F).

Leptin is a hormone made by fat cells that regulates the amount of fat stored in the body; its mRNA expression was significantly downregulated in both the DR and FA groups, but not changed upon CE (Fig.4G).

Effect of dietary interventions and cold-exposure on the immunophenotype in lymphoid organs

Effect of dietary intervention and CE on the primary lymphoid organs

The B cell development stages such as pro-B cells ($CD43^{+}CD2^{-}IgM^{-}IgD^{-}$), pre-B cells ($CD43^{-}CD2^{+}IgM^{-}IgD^{-}$), immature B cells ($IgM^{+}IgD^{low}$) and recirculating mature B cells ($IgM^{+/low}IgD^{high}$) were further investigated in the CE settings. Since no significant differences were observed due to DR, this group has been excluded in the immunophenotypic analysis. When we investigated the B cell development stage as compared to FA, the $CD19^{+}B220^{+}$ B cell population, pro-B cell population in the CE group was significantly increased and was similar as that of the AL fed mice (Fig.5A, 5B). A similar trend was observed with respect to the pre- and immature B cell populations (Fig.5C, 5D). We have seen in our previous findings that both the recirculating mature B cells and $CD3^{+}$ T cells were highly elevated due to FA as compared to the AL fed mice [10] however, the opposite observation was noted upon CE which was found to be the same as the AL fed mice (Fig.5E, 5F).

T cell development occurring in discrete stages in thymus starting from double negative (DN), double positive (DP) and single positive stage (SP) was also investigated. The four DN stages were first investigated and no significant changes were observed in the population of DN1 ($CD44^{+}CD25^{-}$) stage (Fig.6A) while DN2 ($CD44^{+}CD25^{+}$) stage was significantly reduced (Fig.6B). CE had no effect on the DN3 ($CD44^{-}CD25^{+}$) (Fig.6C) and

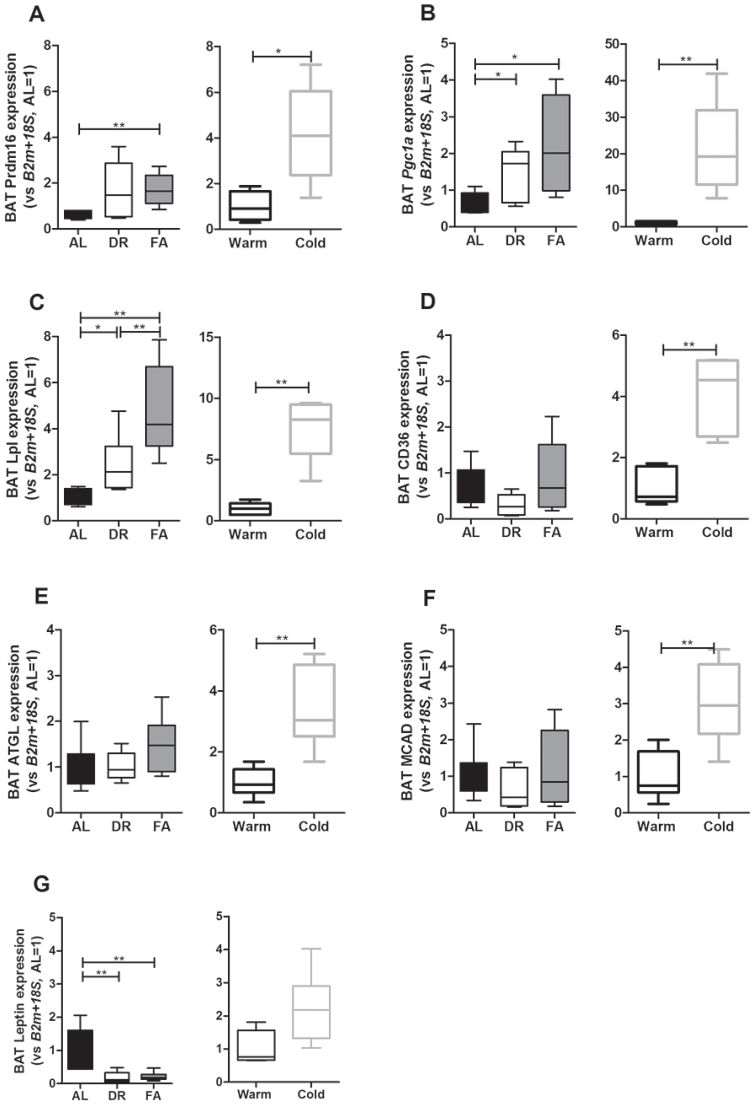

Figure 4. Effect of DR, FA and CE on the expression of metabolism related genes.

The mRNA expression analysis of genes involved in lipolysis has been represented in Fig4. The major lipolysis genes involved, like Lpl, CD36, ATGL and MCAD were upregulated due to CE while the differentiation and activation genes of BAT such as Prdm16 and Pgc-1α were increased, both in FA and CE. Leptin, a hormone made by fat cells that regulates the amount of fat stored in the body was significantly downregulated due to both the dietary interventions while no effect due to CE was observed.

Fig.4 *≤ 0.05, **≤ 0.005, ***≤ 0.0001

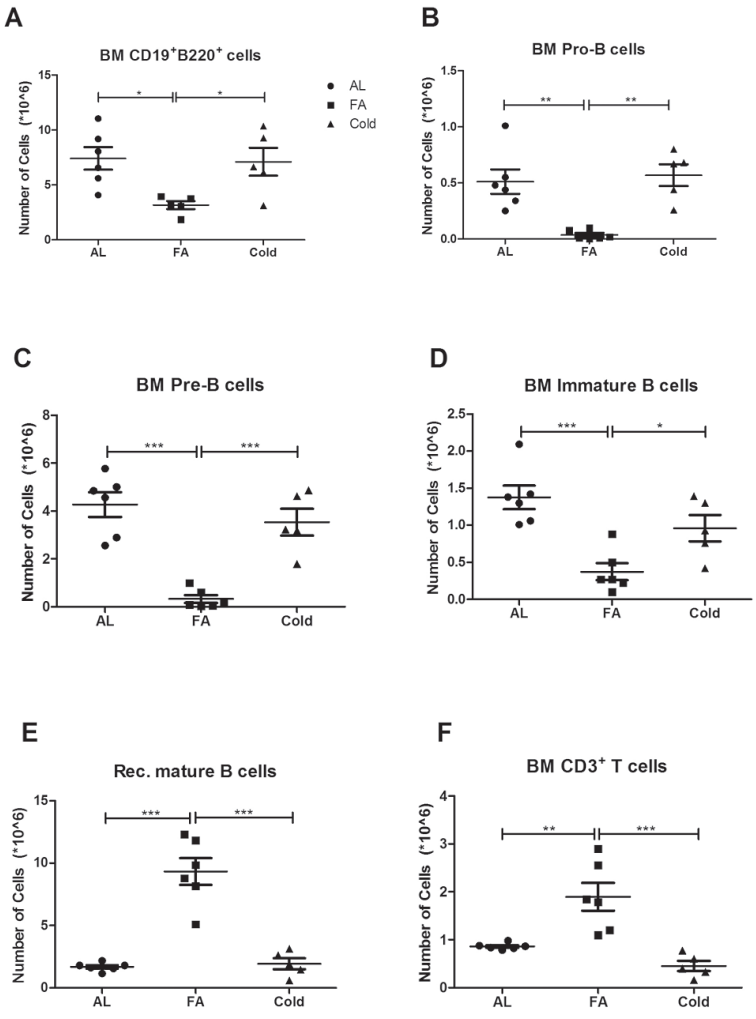

Figure 5. Effect of FA and CE on BM B cell development phenotype

*In **Fig.5** the B cell development phenotype in BM have been represented. At first the total B-lineage population ($CD19^+B220^+$) in BM has been shown followed by changes in the B cell development phenotype starting from Pro-B cells to recirculating mature B cell populations. FA causes a significant phenotypic alternation in the development stages while the B cell development populations remain largely unaffected by CE. The mature $CD3^+$ T cells also gets affected by FA but not by CE.*

Fig.5 *=p≤ 0.05, **≤ 0.001, ***=p<0.0005.

DN4 ($CD44^{-}CD25^{-}$) stages (Fig.6D) as compared to AL but these stages were significantly reduced by FA.

After the DN stages, the thymocytes progress to immature single positive (ISP) $CD8^{+}CD3^{-}CD69^{-}$ thymocytes. This population was significantly reduced in both the FA and CE animals (Fig.6E). The same was true for DP ($CD4^{+}CD8^{+}$) stage (Fig.6F). A significant decrease in the $CD4^{+}$ SP was observed due to FA however, a significant increase was observed in the CE animals (Fig.6G). The $CD8^{+}$ SP stage was significantly reduced due to both FA and CE (Fig.6H).

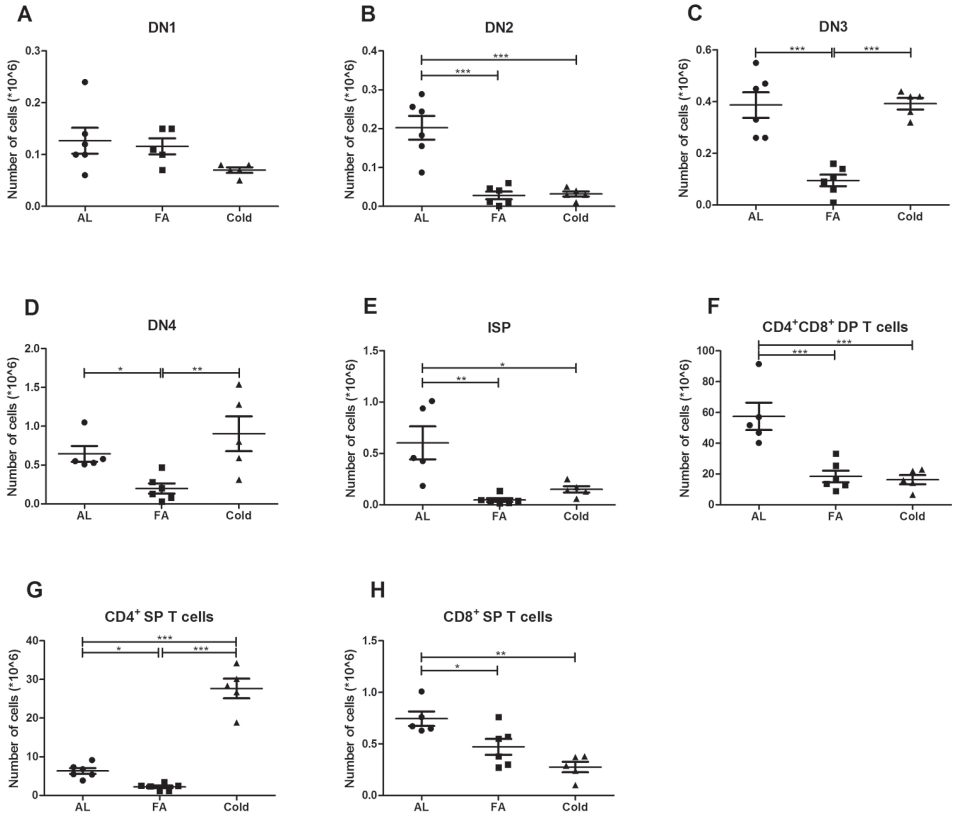

Figure 6. Effect of FA and CE on T cell development stages in Thymus

The T cell development phenotype stages in Thymus starting from DN1-DN4 stages has first been represented in the figures. This is followed by changes in the $CD3^{-}CD69^{-}$ISP and $CD4^{+}CD8^{+}$ DP population. Later changes in the CD4+ and CD8+ SP T cells have been represented.

*Fig.6 *=p≤ 0.05, **=p≤ 0.001, ***≤ 0.0001.*

Effect of dietary intervention and CE on secondary lymphoid organ

CE brought no change in the CD19+B220+ B cell population as compared to AL but the population was found to be increased significantly as compared to FA (Fig.7A).

The effect of FA and CE was also investigated on the splenic CD3+ T cell and was found to be not affected by CE but was significantly reduced by FA as compared to the AL fed mice (Fig.7B).

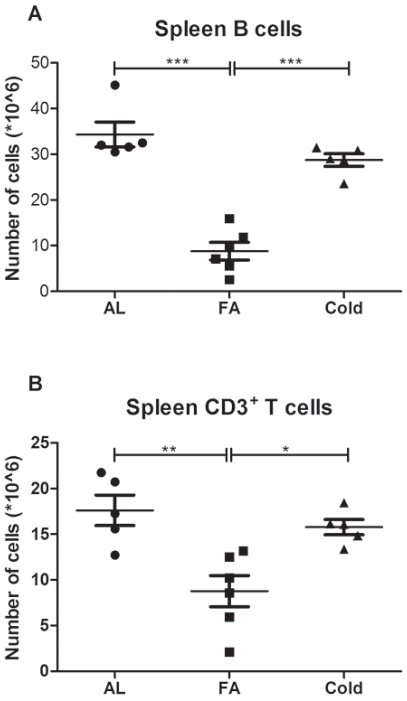

Figure 7. Effect of FA and CE on B and T cell phenotype in Spleen

The figure represents the Splenic B and T cells which are decreased due to the effect of FA, while CE has no effect on these populations.

Fig.7 *=p ≤ 0.05, **≤ 0.005, ***=p≤ 0.0001.

Induction of I/RI in dietary intervened and cold-exposed mice

So far, the data show that dietary interventions such as FA and DR have profound different effects on both BAT and the immunological phenotype than CE. However, all three models show phenotypic changes that might be beneficial for survival. Therefore, we decided to expose all three models to I/RI and determine whether they improve the outcome. Under the conditions of 37 min of bilateral induction of renal I/RI, 80% of the AL fed animals were killed or had to be sacrificed due to signs of morbidity (including excessive weight loss, ruffled fur and hunched back posture) by the fourth day after the surgery. In contrast, the pre-conditioned dietary intervened (DR and FA) groups were protected from mortality, weight loss and kidney dysfunction (Fig.8A). Bilateral renal I/RI induced in CE mice did not protect the mice from morbidity due to I/RI. In both warm and CE mice, approximately 50% of the operated animals had to be sacrificed due to signs of mortality and morbidity (Fig.8B) by day 3.

Figure 8A

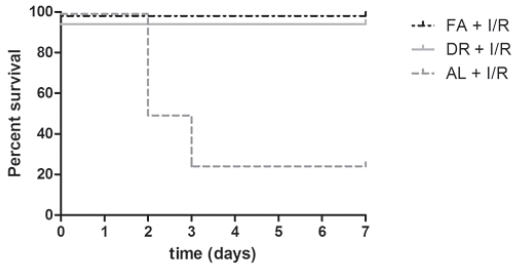

Figure 8B

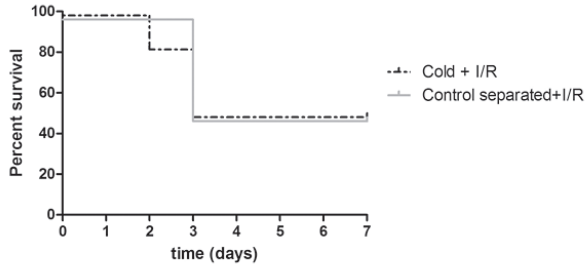

Figure 8. Surgical outcome of animals after dietary interventions and CE followed by renal I/RI.
Fig.8A *represents the survival curves of AL, DR and FA mice after induction of renal I/RI. The dotted line in grey shows the survival curve of AL control while the bold grey line represents the DR animals and the dotted black lines represent the survival curve of the FA animals. Mortality was observed until day 7 post-surgery.* ***Fig.8B*** *represents the survival curve of cold exposed mice followed by induction of renal I/RI. The bold grey line represents the survival of AL animals (n=1/cage, total n=8 animals), while the dotted black lines represent the CE animals (n=1/cage, total n=8 animals) induced with renal I/RI.*

DR and FA cause cytoprotection against oxidative stress, while CE does not

Finally, to understand the reason why CE mice did not survive renal I/RI while the DR and FA mice did, we checked the expression of genes that are important in cytoprotection. We investigated the stress proteins that confer cytoprotection against oxidative stress, like heme-oxygenase-1 (HO-1), and the antioxidant enzymes, such as superoxide dismutase (SOD-1 and SOD-2), glutathione reductase (Gsr) that underlies the protection from FA. The cytoprotective gene HO-1 was upregulated in FA, although this could not reach significant levels while in the CE, the expression was found to be low (Fig.9A). The antioxidant enzymes Gsr, SOD1 and SOD 2 were significantly upregulated in FA, while the expression in CE was significantly downregulated as compared to FA for all the three genes (Fig.9B, 9C & 9D).

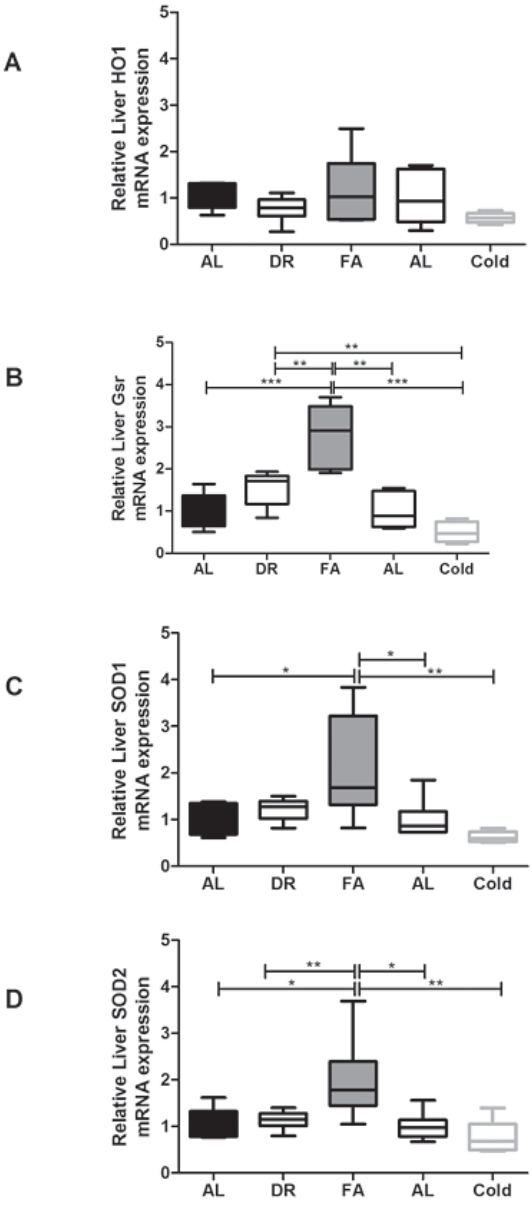

Figure 9. Effect of AL, DR, FA and CE on cytoprotective and antioxidant genes

Fig.9A *represents the effect of dietary interventions and CE on the cytoprotective gene HO-1, while* ***Fig.9B, 9C and 9D*** *represents the effect of experimental animals on the antioxidant enzymes SOD-1, SOD-2, and Gsr which underlies the protection from FA.*

Fig. 9 *=p ≤ 0.05, **=p≤ 0.001, ***≤ 0.0001

DISCUSSION

DR and FA protect against detrimental effects of I/RI [1], while CE has proven beneficial in treatment of obesity through activation of BAT [20]. Since dietary intervention and CE both exert hormetic effects, we investigated if CE could also have a beneficial effect against the damaging phenomena of renal I/RI in mice. Unfortunately, we did not observe full protection by CE as that observed by the dietary restriction regimens. To further answer the reason behind this failure in protection we investigated the changes in adiposity, stress hormonal levels, immunological changes and changes in the cytoprotective genes and antioxidant enzymes induced by both these mild stressors.

CE is a generally accepted method to activate BAT in animal models [21]. It has been shown that 24hrs exposure to 4°C resulted not only in increased BAT activity, but also induced HPA activity [18], making this a suitable model to study the hormetic effects of CE on I/RI survival. In the current study, we have demonstrated for the first time that not only CE, but also dietary interventions affect BAT. We observed that in the FA group, all the lipids have disappeared, as could be expected, since the animal very likely used all the fat stores to maintain itself. Of interest, the BAT Ucp1 protein content was reduced in the FA group compared to AL mice. A similar reduction of BAT Ucp1 protein content has been found by Muralidhara and Desautels [22] when they fasted mice up to 2 days and is in line with use of lipids for maintenance instead of thermogenesis. Although we did not find an induction of plasma corticosterone in the CE mice this time, previously, however, an induction in fecal corticosterone excretion was found in mice kept at 4°C for 24 hours [18]. Very likely, plasma corticosterone concentrations have peaked in the first hours after CE, but the differences between the 23°C and 4°C mice was not detectable anymore after 24 hours. The induction of BAT Fkbp5 mRNA expression upon CE, however, does show induced HPA activity upon CE.

Prdm16 and Pgc1α are important factors involved in differentiation and activity of brown adipocytes [23]. While CE clearly induced *Prdm16* and *Pgc1a* expression in BAT (as expected), both DR and FA also induced *Prdm16* and *Pgc1a* expression. This suggests that DR and FA promote brown adipocyte differentiation and activity in the DR group. We are unaware of studies investing the effects of (long term) FA on BAT *Pgc1a* expression, but our results are in line with previous findings that FA induces *Pgc1a* expression in murine liver [24,25] and heart [26]. BAT *Pgc1a* expression has been shown before to be enhanced by DR in mice [27].

BAT activity not only depends on Ucp1, but also upon the processes preceding heat production, e.g., extracellular triglyceride (TG) lipolysis, fatty acid uptake into the brown adipocyte, intracellular TG lipolysis and fatty acid beta-oxidation. Key enzymes in these processes are LPL, CD36, ATGL and MCAD, respectively. Analysis of the expression of these genes in our two experimental models showed that all four processes were

upregulated by CE, but that only LPL gene expression was regulated by DR and/or FA. Altogether, all data on our BAT analysis, it appears that while CE clearly induces BAT activity, DR seems to not affect BAT activity, while FA reduces BAT activity. Upon further analysis of BAT leptin, a significant reduction in the leptin levels is observed in DR and FA mice while no significant changes were observed in the CE mice. In light with this observation Robertson et al., [28] have recently shown that calorie restriction leads to reduction in serum leptin levels, which, upon supplementation during the pre-conditioning period, significantly reduced the beneficial effects of DR against renal I/RI. Hence, the failure to protect against renal I/RI due to CE could likely be explained by this observation.

Our findings also clearly illustrate different mechanisms of action induced by dietary restriction regimens and CE. And these differences in mechanisms could account for failure of CE to protect against renal I/RI, considering that dietary interventions could work via hormesis. However, upon further investigation of the immunomodulatory effects in both the dietary interventions and CE, we found that both the stress models exert different immunological effects. In correspondence with our previous findings that DR and FA both cause significant upregulation of recirculating mature B cells and $CD3^+$ T cells in the BM, no significant changes were observed in the CE animals. The other immunological effects of CE in the different immunological compartments resemble those seen in the AL group, indicating different mechanisms of actions. The differences in immunological effects, and differences in survival after I/RI could be related to the "severity" of the preconditioning regimen (FA seems to be more severe compared to 24hrs of CE). Further investigation into the antioxidant and cytoprotective genes indicate the protection against renal I/RI by FA and not by CE. HO-1 is an inducible stress protein which confers cytoprotection against oxidative stress in vitro and in vivo. Upregulation of HO-1 has been shown to confer protection against hepatic cold preservation injury (HO-1 overexpression protects the rat liver from I/R/I with extended cold preservation), and is likely to be also involved in the protective effect of FA [1] and not in the CE. Moreover, we show that the beneficial effects of FA are likely achieved by the overexpression of genes encoding for the antioxidant defense enzymes SOD1, SOD2 and Gsr at the baseline which could not be observed for the CE mice and which might be one of the reasons behind failure to protect against renal I/RI. However, one must keep in mind that shorter and/or milder forms of CE might have had beneficial effects on renal I/RI survival. For instance, a 3-hour acute cold exposure has been reported to induce presence of suppressor macrophages in the peritoneal fluid, a process that depended on cold-induced HPA activity [29].

In conclusion, our findings clearly demonstrate the metabolic changes induced by both the dietary interventions as well as CE. We show that there are similarities in the metabolic profiling of the stress related genes, while browning of the adipose tissue due

to the DR regimens was not observed upon quantification of Ucp-1. However, we did not observe a protective effect of CE against the detrimental effects of renal I/RI. Immunologically, there were differences between the two groups that could account for this failure. Also, downregulation of factors such as cytoprotective genes and genes encoding for the antioxidant defense enzymes could be the leading factors behind failure to protect against renal I/RI by CE mice. One puzzling fact which remains, is that both DR and FA induce robust protection against I/RI, whereas they both differ substantially in the different effects studied in this paper. This may indicate that the predominant mechanism behind the protection against I/RI yet remains to be elucidated.

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CHAPTER 8

Discussion and future perspectives

The studies in this thesis describe two novel treatments in amelioration of renal I/RI and its implications in modulating the immune system: dietary restriction and cold exposure.

IMMUNOMODULATION BY DIETARY RESTRICTION REGIMENS

In **Chapter 2** we reviewed published data describing the impact of DR on the aging immune system of the rodents including non-human primates. We also discussed the data that delineate similarities and differences on the adaptive and innate immunity due to the effects of DR. The various studies performed so far have not provided the exact mechanism behind the beneficial effects afforded by DR to prolong life span or to protect against aging related diseases. The limited data available we have found so far in this review do not point to a single gene, pathway, or molecular mechanism underlying the benefits of short-term DR. Instead, these studies have highlighted a range of potential mechanisms. In this chapter we have highlighted some of the proposed mechanisms of action of DR such as growth hormones, glucocorticoid [1–4], and reduction in oxidative damage which attenuates inflammatory responses [5,6]. Apart from these, several other modulations have been observed at the molecular level which include: increased hepatic glucocorticoid receptor expression, which helps in controlling the glucocorticoid-mediated metabolic responses leading to resistance to stress, such as trauma, heat shock and drug toxicity [7], decreased plasma glucose, insulin, immunosenescence, and inflammation without any irreversible side effects [8]. The overwhelming majority of the reported studies has indicated that DR modulates the immune function and restore or delay the immunosenescence not only in laboratory animals but also in higher species such as non-human primates. The mechanism by which DR alters immunosenescence remains unclear. The major focus of the chapter has been on the players of the immune system such as B, T cells, complement system, macrophages and neutrophils. How DR modulates these players of the innate and adaptive immunity have been extensively summarized.

Through the literature studies we conclude that DR preserves the naïve T cell population as well as maintains the proliferative capacity of the T cells, which deteriorates with aging. With respect to B cell population it has been hypothesized that DR causes a decline in the early B cell developmental cells while increasing the population of mature B cells in bone marrow. How this is advantageous in exerting the beneficial effects is something that warrants further investigation.

We have shown in **Chapter 3** how the adaptive immunity is modulated by DR and FA, and have concluded that these dietary interventions bring major immunological changes in the primary and secondary lymphoid organs. For this, we investigated the effect of 14days of 30% DR and 3 days of FA on the B cell development stages in bone

marrow, thymopoiesis in thymus and B and T cell subtypes in secondary lymphoid organ spleen. We found that both DR and FA caused a significant reduction in the early B cell development stages while the recirculating mature B cell (IgM^+IgD^+) population was significantly increased in the BM, which are B cells that return to the BM after they have completed their maturation process in the periphery. From this observation we hypothesized that this is related to the depletion of the same set of B cells from the spleen and mLN and suggests either recruitment of peripheral B cells to the BM or the changes taking place in cellular migration or homing. Apparently, dietary interventions are causing BM to save more energy by halting the production of more B cells and concomitantly directing the BM to spare its energy to maintain its metabolic imbalances. Lessons learned from various mice I/RI models with defects in T/B cell maturation have shown protection against severe effects of I/RI. It has been shown that B cell deficient mice (μMT (Igh-6^{tm1Cgn}) mice) are protected against I/RI, thereby confirming the role of B cells in I/RI [9]. We are not aware of published studies that address different B cell subtypes and their role in I/RI. Upon further analysis of the B cells in spleen a depletion of immature, transitional B cells ($CD19^+B220^+IgM^+IgD^-$), mature B cell populations ($CD19^+B220^+IgM^+IgD^+$) as well as depletion of marginal zone and follicular B cells was observed in spleen. It is not yet clear where the depleting B cells from spleen and mLN migrate to during the event of dietary interventions but one of the hypotheses is that these cells are (partially) recruited to the BM, as mentioned before. Alternatively these cells may move to the Peyer's patches, which are a component of the gut-associated lymphoreticular tissues (GALT), and are the major site of antigen uptake to induce mucosal secretory-IgA antibody responses [10]. In the thymus when the effect of DR and FA was investigated, interestingly we found that both the DR regimens arrested the development and maturation of T cells. An unusual phenomenon of increase in the mature T cell population was observed in the BM due to the DR regimens. Only a few studies have shown that DR brings about the protective effects by modulating changes in the immune system by means of delaying immunosenescence and also by stress resistance.

From our studies we conclude that there is a reduction in both B (all subtypes) and T cells in the spleen induced by DR and FA (which may be protective against renal I/RI), in accordance with Rabb's findings [9]. However, the mechanism by which DR exerts its protective effect remains unclear and we could only show the major phenotypic changes occurring due to DR regimens. In conclusion, it is unclear whether these major immunological changes observed after dietary interventions are a bystander effect or directly mechanistically related.

In **Chapter 4** we studied the effect of DR and FA on one of the most important components of innate immunity, i.e. the complement system. Of the three known pathways, the lectin pathway, which gets activated by Mannan Binding Lectin (MBL), has been shown to play a pivotal role in renal I/RI. In this chapter we showed that following dietary

interventions, vascular leakage and extravasation of MBL is the primary culprit of tubular injury post induction of renal I/RI. Investigation of the localization of hMBL after reconstitution and induction of renal I/RI showed a clear extravasation and interstitial presence of hMBL in both the DR and FA group. Findings from these indicate that DR does not protect against renal I/RI by preventing vascular leakage and subsequent exposure of tubular cells to MBL, but by downregulation of circulating levels of MBL. However, the observation that protection by FA is not mediated by preventing vascular leakage suggested that FA had an effect on tubular cell homeostasis and resistance to tubular injury [11], independent of MBL. We conclude that dietary interventions attenuate the circulating levels of MBL and upon restoration of MBL levels, the protection against I/RI induced by DR is rested, suggesting a pivotal role of MBL in its mechanism of action. It is known that I/RI causes inflammation during the course of reperfusion [12]. Both DR and FA reduce inflammation [13] and cytokine production during I/RI and hence prevent further damage to the organ. We have shown that FA causes reduction in the markers of inflammation such as IL-6 and P-selectin to a significantly lower degree after I/RI as compared to AL [11]. Hence, we hypothesize that the effect of FA on reducing inflammation is greater than that of DR which could lead to DR breaking the protection upon hMBL infusion while FA not. This observation indicates that there may be different mechanisms of action of DR and FA. This could be discussed based on the fact that the method of dietary intervention is different in both "sub-acute" DR (2 weeks of 30% dietary restriction) and more severe and acute FA (3 days of water-only fasting) groups. Also, the decrease in MBL concentrations as observed in FA group is approximately 50% while that observed in DR group is approximately 25% when compared to that of AL group. The other hypothesis is that the FA regimen is more robust and may not be nullified by the amount of hMBL used for reconstitution, whereas the DR induced protection is.

In conclusion, we have specifically highlighted the role played by MBL in protection against renal I/RI and have shown that dietary interventions attenuate the circulating levels of MBL. Restoration of MBL levels breaks the protection against I/RI induced by DR, which underscores the role of MBL in the pathophysiology of I/RI, and may suggest a pivotal role for MBL in its mechanism of action. Furthermore, translation of our novel findings should be carried out in humans, in whom we have previously demonstrated the clinical feasibility of DR [14].

In **Chapter 5**, to better understand the impact of dietary interventions on the hepatic, renal and systemic complement system in mice, we performed functional analysis of serum complement and analyzed changes in local gene expression following dietary interventions to uncover whether also other pathways or factors apart from MBL are affected by either DR or FA. The functional measurement of the three main pathways of complement showed that neither of the dietary interventions impacted systemic complement functionality at the level of C3 activation. In contrast, we observed that the

functional activities at the level of C9 were severely downregulated in all three pathways, suggesting a terminal pathway specific impact. Investigation of the hepatic expression of key complement factors revealed interesting modulation of the complement factor specific expression. In line with previous results shown in chapter 4, initiation factor MBL was downregulated, and although not predominantly produced by the liver, also properdin and C1q showed significantly lower expression. The array analysis revealed no major downregulation of complement components C3, C2 and C4. However, qPCR determination showed marked upregulation of C3 by FA but not by DR. The most striking change observed in hepatic complement expression was up to 5 fold downregulation of terminal pathway specific genes following FA and up to 2.3 fold downregulation of C6, C8 and C9 after DR. Together these results suggested a potential protection mechanism through terminal pathway specific downregulation of serum complement, which has been in part shown to mediate renal and hepatic I/RI [15]. In conclusion, our analysis of systemic complement and liver expression following dietary interventions clearly showed that initiation factors and terminal pathway components are downregulated, whereas C3 remains at normal levels, suggesting a specific modulation of complement. A marked upregulation of renal C3 following FA was observed and is counterintuitive as intrarenal C3 is sufficient in maintaining complement activation following renal injury [16]. However, the role of complement is not always injurious. In liver I/RI, complement exhibits intriguing duality between injury and regeneration [17,18], with evidence that anaphylatoxins C3a and C5a are essential for regeneration [19]. Although the C3a-C3aR-axis has been shown to promote renal injury [20], it has not been evaluated whether C3a-C3aR axis has a role in homeostatic repair of kidneys. What is known is that C3a-C3aR activates mesenchymal stromal cells, which are responsible for homeostatic repair and regeneration of injured kidneys [21,22]. Therefore, FA in absence of inflammatory stimulus such as ischemia and terminal pathway activation, could result in increased repair of the normal kidney and preconditioning of the kidneys to better withstand I/RI. In the context of renal and hepatic I/RI, the specific downregulation of terminal pathway components C5–C9 could explain the observed protection against hepatic and renal I/RIs, as lowered potential for C5a and C5b-9 formation as key regulators within kidney and liver were not affected. Our results demonstrated a clear parallel to experimental models where systemic inhibition of serum complement results in amelioration of renal and hepatic I/RIs [23–27].

Further research is however necessary to characterise dietary intervention impact on complement in human. Translation of the results of dietary interventions from animal studies to the clinic setting poses a challenge, however, the feasibility of dietary interventions have been reported and hence future research is warranted not only for studies in healthy animals but also in humans. Also expansion of the studies to encompass tissue specific expression of complement factors, regulators and receptors to better understand how dietary interventions modulate complement system in mice is needed.

In **Chapter 6** of this thesis, the effect of FA alone on the adaptive immune system have been studied in context with induction of renal I/RI. This chapter is a continuation of the studies reported in chapter 3 including induction of renal I/RI and assessing the immune compartment after 6 and 24hrs of reperfusion. Similar to the results shown in chapter 3 in this study also we showed that FA arrested B cell development 24hrs post reperfusion. Upon further investigation of the secondary lymphoid organ we found that FA caused a decrease in the early mature B cell populations at both 6 and 24hrs post-reperfusion as compared to that of the ad libitum fed animals. FA also caused an increase in the mature and follicular B cells while causing a decrease in the MZ B cells 24hrs post-reperfusion. What is known from the previously published studies [28] is that the total B cell population is found to be increased 3 days after induction of I/RI as compared to the control. It is also known that in the absence of B cells, kidney is functionally and structurally protected postischemic compared to those of wild-type mice [9,28]. Interestingly, the recirculating mature B cell populations were found to be increased in the FA groups 6 and 24hrs post-reperfusion which was observed to be the same in our previous findings without induction of renal I/RI [29]. Whether the increased number of recirculating mature B cells are implicated in the immune regulation induce by FA in the form of B regulatory cells is something that needs further investigation. Splenic MZ B cells which are known to be sufficient for I/RI induced tissue damage were observed to be less in FA induced I/RI mice as compared to those of ad libitum fed mice. Studies performed in CD4/CD8-deficient mice [30,31] have shown a marked functional protection from renal I/RI compared with wild-type, strain-matched controls [32,33] which is in line with our findings of arrest of T cell development by FA. The increase in $CD3^+$ T cells in BM upon induction of renal I/RI as also observed in chapter 3, possibly contributes to the energy saving capacity of the organ by not producing more of the B cells and thereby directing the BM to more energy consumption/storage. Upon investigation of the cytokine production capability of the splenocytes in the current settings of FA and AL, a decrease in the number of IFN-γ and IL-2 producing cells upon FA only at 6hrs post reperfusion was observed. No significant differences were observed 24hrs post-reperfusion.

In conclusion, FA causes major changes in the immune system and arrests both the B and T cell development in BM and thymus. FA also restores the animals' energy by maintaining an increased percentage of $CD3^+$ T cells in BM. Functionally, the frequencies of cytokine producing splenocytes are strongly diminished after FA and subsequent induction of I/RI. Based on the several observations at both 6 and 24hrs time points it could be concluded that the effect exerted by FA in causing protection against renal I/RI is very early, within 6hrs of induction. This is a novel finding and has not been reported thus far and could be a contributing factor in the protection against renal I/RI due to FA.

HORMETIC EFFECT OF DIETARY RESTRICTION REGIMENS AND COLD EXPOSURE

In **Chapter 7**, we have demonstrated the hormetic effect of dietary intervention and another stress model cold exposure (CE) that has shown to have hormetic effect, and has proved to increase longevity [34]. CE is a commonly used method to increase metabolism, since it results in activation of brown adipose tissue (BAT) and gives the white adipose tissue (WAT) more BAT-like characteristics. In mammals, BAT and WAT have opposite effects, but both are considered important in etiology of obesity and its metabolic consequences [35]. In mice both the dietary interventions and CE induce hypothalamic-pituitary-adrenal (HPA) axis activity and elevate plasma corticosterone concentrations. Since DR, FA and CE all induce HPA axis activity but only two of these preconditioning strategies have been shown to confer protection again renal I/RI we investigated if CE could also have a beneficial effect against the damaging phenomena of renal I/RI in mice. Unfortunately, we did not observe full protection by CE such as observed in dietary restriction groups. To further elucidate the reason behind this failure in protection, we investigated the changes in adiposity, stress hormonal levels, immunological changes and changes in the cytoprotective genes and antioxidant enzymes induced by both these mild stressors. We observed major changes in the adiposity of all the three groups. Our data on BAT analysis revealed that while CE clearly induced BAT activity, DR did not seem to affect BAT activity, while FA reduced BAT activity. Upon analysis of genes such as Fkbp5, Prdm16 and Pgc1a we observed a clear induction due to both dietary intervention and CE. This suggested that DR and FA promote brown adipocyte differentiation and activity in these groups. Our results hence, are in line with previous findings that FA induces Pgc1a expression in murine liver [36,37] and heart [38]. Upon further analysis of BAT leptin, a significant reduction in the leptin levels was observed in DR and FA mice while no significant changes were observed in the CE mice. In light with this observation Robertson et al., [39] have recently shown that calorie restriction leads to reduction in serum leptin levels, which, upon supplementation during the preconditioning period, significantly reduced the beneficial effects of DR against renal I/RI. Hence, the failure to protect against renal I/RI due to CE could likely be explained by this observation. Our findings clearly demonstrate the metabolic changes induced by both the dietary interventions as well as CE. We show that there are similarities in the metabolic profiling of the stress related genes. However, we did not observe a protective effect of CE against the detrimental effects of renal I/RI likely due to the immunological differences between these two groups. Upon further investigation of the immunomodulatory effects in both the dietary interventions and CE, we found that both the stress models exert different immunological effects. In correspondence with our previous findings that DR and FA both cause significant upregulation of recirculating mature B cells and $CD3^+$ T cells in the BM [29], no significant changes were observed in the CE animals. The other immunological effects of CE in the different immunological compartments resemble those seen in the AL group, indicating different mechanisms of actions. The differences in immunological

effects, and differences in survival after I/RI could be related to the "severity" of the preconditioning regimen (FA seems to be more severe compared to 24hrs of CE). Also, downregulation of factors such as cytoprotective genes and genes encoding for the antioxidant defense enzymes could be the leading factors behind failure to protect against renal I/RI by CE mice. One fact that remains unclear from this study is that both DR and FA induce robust protection against renal I/RI whereas they both substantially differ in the different effects studied in this chapter. This may indicate that the predominant mechanism behind the protection against I/RI yet remains to be elucidated.

We conclude that preoperative dietary restriction and as well as CE induces metabolic changes and exhibit similar metabolic profile of the stress related genes. However, no protective effect of CE was seen against the detrimental effect of renal I/RI due to immunological differences between the dietary intervention and CE groups. Also, downregulation of factors such as cytoprotective genes and genes encoding for the antioxidant defense enzymes could be the leading factors behind failure to protect against renal I/RI by CE mice.

Hence, DR and FA are promising strategy to protect against renal I/RI in which immunology holds an important role. However, the exact mechanism of action of immune system in this protection against renal I/RI is not yet clear and warrants further exploration as innate immunity shows adequate role.

FUTURE PERSPECTIVES

DR and FA form the most robust, non-invasive and highly feasible method of protection against a range of diseases including detrimental effects against solid organ transplantation such as I/RI. We have shown several immunological players of both innate and adaptive immunity playing a key role in this protection mediated by DR. Studies performed in this thesis show that the important components of innate and adaptive immunity such as B cells, T cells and complement system play an important role in dietary preconditioning and also in DR mediated protection against renal I/RI. Although the data from the animal experiments have contributed much to our understanding of the benefits of DR and the immunological players in mediating these beneficial effects, translation of these findings to the clinic may be difficult due to the disparities between mice and humans.

The studies performed in this thesis are limited to experiments performed in healthy young mice. Humans waiting to undergo transplantation are mostly older and suffer from end-stage organ failure and co-morbidities. It is known that older rats and mice are more vulnerable to an ischemic insult of the liver [40,41], heart [42] and kidney [43]. DR has been shown to reduce ischemic stress in kidneys [43] and hearts of aged rats ex vivo. However, its effect on aged mice have not been elucidated so far. Further research is required to characterize the effects of DR on I/RI in these aged mice.

Another limitation to our study is the use of only male C57Bl/6 mice. Female mice are known to be more resistant to renal I/RI than males [44]. In addition, other male mouse strains such as Balb/C, and NIH Swiss are known to respond differently to the same ischemic insult caused to the kidney. Recently, experiments performed in aged-overweight male and female mice have shown protection against renal I/RI [45]. How DR affects the immunology in these mice would be valuable to know. Also, the effect of renal I/RI needs to be established in larger animals such as pigs, rats or monkeys which are more suitable models for translation to humans.

We have shown that reduced food intake by 30% for 2 weeks and 3 days of water-only FA has resulted in increased resistance against renal I/RI. Mice, however, have a metabolic rate approximately 7 times higher than humans [46]. This means that the dietary interventions in humans will have to be increased by 7 times to obtain similar results as those found in mice, which practically does not seem feasible. Nevertheless, the understanding of the underlying molecular mechanisms will open up future studies to explore more different stress models that could trigger the same protective effect. Hence, more focused stress model studies are required to better understand the beneficial effects during organ transplantation. Also more focused studies are needed to understand the similar mechanisms between human and mice so that the immunological changes observed in mice could be linked to human.

The other limitation of the studies performed on these mice is that the experiments have been performed in an I/RI model involving warm ischemic kidneys. In the clinic, usually the transplants undergo brief periods of warm ischemia and cold ischemia lasting up to 24hrs. This brief warm and cold ischemic condition should be tested in auto-transplant animal models involving both cold and warm ischemia times, more realistically reflecting the clinical situation and studies on how immune system plays an important role should be thoroughly investigated. Dietary intervention studies should also be performed on models relevant for vascular surgery, stroke, myocardial infarction, etc.

Dietary restriction leads to reduction in both calorie and nutrient intake, however, the contribution of calories or nutrients to the protective effect on renal I/RI is unknown. Verweij et al. have shown that the benefits of preoperative FA are not affected by the intake of calories via glucose water supplementation during FA [47]. Subsequently, diets lacking protein or even the essential amino acid tryptophan for 6–14 days resulted in similar protection against renal I/RI in mice [48–50]. Also amino-acid imbalance have resulted in extension of lifespan by DR in Drosophila [51]. Hence, specific nutrient restriction other than 30% DR or water-only FA is important and should be studied in detail to elucidate the mechanisms behind protection against renal I/RI.

DR and FA both shut down the B and T cell developmental stages in bone marrow and thymus respectively, which are hypothesized to be protective and presented in this thesis. Hence, it becomes interesting to understand how the immune system behaves after DR/FA in Rag-/- and athymic null mice in which the B and T cell compartment is not developed right from their birth.

In our studies we have shown that complement system plays an important role in DR mediated protection against renal I/RI. We have for the first time highlighted that DR downregulates the important complement proteins such as MBL, which might explain the reason behind protection. We however, have not shown the effect on the complement system in MBL deficient mice. It would be highly important to highlight the major immunological changes in these knockout mice and see if the mice are still protected against renal I/RI. We, in this thesis, have also presented the effects of dietary interventions on the other proteins of the complement system but have not been able to present the effects on other major immunological proteins in I/RI conditions and hence a complete overview of the effect of DR in I/RI condition with regards to innate immunity is lacking at the moment. It is therefore, required to acquire this knowledge to be able to fully understand the mechanism of protection.

Chemokine signaling [52–54] and adhesion molecules [55–57] play an important role in ischemia reperfusion injury. We in our studies have not investigated the effect of dietary regimens on these immunologically important molecules that help in recruitment of the immune cells to the site of injury. Hence, mechanistic studies are needed to elucidate the

molecular mechanisms by which these molecules modulate kidney injury and if they play an important role in protection.

Although we and others have shown that DR holds a key role in mediating protection against several diseases, this has not been fully confirmed in humans. Translation of the results to the clinical setting poses a challenge. Several randomized clinical trials have been performed indicating that humans on DR for 6 months to 2 years respond in similar ways to experimental mammalian models on long-term DR with respect to a variety of endpoints such as blood pressure, body weight, glucose metabolism, etc [58]. Rigorous studies to probe the effects of short-term DR in humans have not been completed to date. Nonetheless, there are several reports suggesting feasibility and efficacy of short-term DR in the clinical setting [14]. However, because ischemia times are short and complications are rare in living organ donor procedures, large numbers are required to observe any functional differences in a prospective clinical trial. Since, vascular surgeries have higher rates of complications, including heart attack and stroke, it may present a better opportunity in which the efficacy of short-term DR could be tested than living organ donor procedures [58]. Although 30% DR has been performed in humans for a period longer than 2 weeks but no study has been performed prior to surgery. If preoperative DR is performed in transplant patients the patients might become weak and hence may not be able to tolerate and/or respond to DR and the after-effects of surgery. Therefore future research should be performed in finding a mechanism, to develop a DR mimetic that could be easily translated to the clinic.

We agree, much has been and can be learned from mouse models, in particular as to how dietary interventions may alter pathways and phenotype. However, the data we generated would suggest that the extent that such changes in specific genes or pathways reflect importance in human transplant condition should be verified extensively. Since mice are not men a thorough investigation of the pathways and mechanisms taking place in human should also be interrogated. Clinically, we have shown the feasibility of DR but much focus and research is required to better understand how DR works in humans. Research should also be focused as to how long DR or FA should be translated from mouse to man and whether the immunological findings presented in this thesis are comparable to those in human studies.

One of the important questions that arose during the course of study is who should be dietary preconditioned whether it should be donor or the recipient or both (in case of transplantation). Scientifically, both the donor and the recipient should be preconditioned to be able to have maximum beneficial effect. However, in practicality only the live donors are motivated while the deceased donors are not be highly motivated to undergo dietary regimen. Hence, more dedicated study highlighting pros and cons of DR should be carried out so that the donor/recipient would not be worried to follow any dietary regimen before going for organ donation.

In conclusion, DR appears to be a robust and non-invasive way of protecting against renal I/RI. Several immunological players hold keys in this DR-mediated protection. Although we have tried to unravel some of the mechanisms behind this protection, further studies are warranted to fully understand the phenomenon. Also disparities between the action of DR and FA have been observed, indicating two potentially different mechanisms of action. This understanding could lead to development of a nutritional therapeutics that could benefit patients awaiting transplantations and also other forms of surgery. Although we are looking for DR mimicking agents still we need to join hands and come together to promote organ donation for betterment of human kind. Clinically, in human living kidney donors, DR before surgery has shown to be feasible, well tolerated and safe [14], however the robust effects on reducing I/RI as observed in mice is lacking and have not been elucidated yet. Hence, we are still far from DR mimetics and more clinical research is needed to translate the beneficial effect of preoperative diets from animals to humans. The scientific studies which we are conducting should be continued in a similar fashion which will help to increase the success rate and which will also help to further motivate people to donate their organs. Furthermore, scientific studies to increase the longevity of organ storage could play a crucial role and increase the success rate of organ transplantation. Currently, strategies to decrease I/RI are being tested experimentally and in clinical settings such as machine perfusion for better preservation of organs during prolonged cold ischemia time, ischemic pre- and post-conditioning where post-conditioning has been shown to be feasible and safe in patients undergoing kidney transplantation. Apart from these approaches administration of cells such as mesenchymal stem cells and regulatory T cells are being tested as a cellular therapy approach to have beneficial effects against I/RI.

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CHAPTER 9

Summary

SUMMARY

Kidney transplantation is considered to be the only curative treatment for people with end stage renal disease. Its results however, are negatively affected by the development of ischemia-reperfusion injury (I/RI) during transplantation. Nowadays, many deceased donor organs come from donors with higher age or comorbidity (extended criteria donor organs) that are more prone for I/RI. I/RI is one of the major causes of ischemic acute renal failure, and is the major cause of delayed graft function. Despite advances in the renal replacement therapy treatment of I/RI is still unsatisfactory. Several lines of evidence support free reactive oxygen species formation, endothelial dysfunction, and immune activation as the crucial event in the development of tissue injury and graft dysfunction during renal I/RI. Recently, mouse studies have shown that dietary restriction (DR) exerts beneficial effects against the detrimental effects of I/RI. Preoperative short term DR and fasting (FA) induce protection against I/RI in both kidney and liver. The exact mechanism behind protection afforded by DR has not been elucidated so far. However, DR regimens have been postulated to reprogram the immunological profile, and increase plasma corticosterone concentrations due to induction of hypothalamic-pituitary-adrenal axis activity. Apart from that, DR has also been considered a mild stressor that ultimately leads to protection, commonly referred to as hormesis and has been hypothesized to be responsible in ameliorating I/RI. Another type of hormesis cold exposure, has been known to induce hypothalamic-pituitary-adrenal axis activity since it results in elevated plasma adrenocorticotropic hormone and corticosterone concentrations.

This thesis describes the immunological changes following DR and FA, and after renal I/RI, with emphasis on both adaptive and innate immunity. We also studied cold exposure as stress model that could potentially induce protection against IRI.

Chapter 1 describes the general elements of the studies presented in this thesis. These elements include the main rationale of the study, i.e. renal I/RI, an inevitable phenomenon during organ transplantation, and the nutritional therapeutic DR and FA, which could be used to gain protection against renal I/RI. Since the aim of the thesis is immunolomodulation by DR, several immunological parameters have been described; mainly adaptive and innate immunity, and the major development stages occurring within these immunological systems.

Chapter 2 describes the immunological effects of DR. In this chapter, published data have been reviewed that describe the impact of DR on the aging immune system of rodents and non-human primates, and discusses the data that delineate similarities and differences of the effects of DR upon adaptive and innate immunity. In this chapter impact of DR on several immunological players such as T and B lymphocytes, NK cells, macrophages, neutrophils and most importantly complement system which is known as the major player of the innate immunity have been summarized. This review concludes

that DR modulates the immune function, and restores or delays the immunosenescence not only in laboratory animals, but also in higher species such as non-human primates.

In **Chapter 3,** we assessed the involvement of immune system in the beneficial effects of DR by investigating the effect of DR and FA on major lymphoid cell populations in all the major primary and secondary lymphoid organs of male C57Bl/6 mice. We found that both DR and FA caused a decrease in the total B cell population and arrested early B cell development in bone marrow (BM), while causing an increase in the number of recirculating mature B cells. Both DR and FA arrested thymopoiesis and caused an increase in the mature $CD3^+$ T cells in BM. We observed differences between the effects of DR and FA indicating discrepancies of the two types of dietary regimens. This chapter concluded that DR and FA acts as a low-level stressor inducing protective mechanisms that modulate inflammatory responses to harmful danger signals as well as bringing about alterations in B and T cell development. These dietary interventions also caused recruitment of recirculating mature B cells and $CD3^+$ T cells to the BM, possibly contributing to the organ saving more of its energy by not producing more of the B cells and thereby directing the BM to more energy consumption/storage.

In **Chapter 4**, we investigated one of the most important compartments within innate immunity, i.e. the complement system. Out of the three complement pathways, mannan-binding lectin (MBL) pathway was studied because this pathway plays a pivotal role in induction of renal I/RI. We found that both dietary regimens (DR and FA) induced a robust protection against renal I/RI, and significantly reduced the circulating levels of MBL as well as its mRNA expression in liver, the sole production site of MBL. We also established that upon reconstitution of MBL, the protection afforded by DR was abolished, whereas in the FA group the protection persisted. Vascular leakage and extravasation of MBL following reperfusion is known to play a pivotal role in induction of renal I/RI and exposure of epithelial cells to MBL immediately following reperfusion is the primary culprit of tubular injury. The observation that protection by FA was not mediated by prevention of vascular leakage suggested that FA had an effect on tubular cell homeostasis and resistance to tubular injury, independent of MBL. In this study also we observed that DR and FA have different mechanisms of action. This could be based on the fact that the method of dietary intervention is different in both "sub-acute" DR (2 weeks of 30% dietary restriction) and more severe and acute FA (3 days of water-only fasting) groups. Also FA regimen is more robust and may not be nullified by the amount of hMBL used for reconstitution, whereas the DR induced protection is. This chapter hence concluded that modulation of MBL is involved in the protection against renal I/RI induced by DR, and suggested that the mechanisms of protection induced by DR and FA may be different.

In **Chapter 5**, as a continuation of the previous study we focused our study on the overall complement cascade. In this study we assessed the functionality of serum complement with pathway specific functional complement assays together with focused

analysis of hepatic and renal expression of key complement proteins and regulators. Our results showed that FA, and to lesser extent DR, reduced the systemic complement activities of all the three pathways, with most radical impact on the terminal pathway, and only moderate impact on the initiation pathways. Upon further investigation into hepatic expression, major reduction of gene expression of terminal pathway components was observed. However, only FA resulted in systemic downregulation of terminal pathway components while no effect of DR was observed. A significant upregulation of complement factor C3 was observed in both liver and kidney tissues after both DR and FA. This upregulation of C3 could suggest a compensatory mechanism akin to acute phase response. The role of the compensatory mechanisms of hepatic and renal C3 upregulation in response to FA are intriguing and could hypothetically be linked to the emerging role of C3b and C3a in tissue regeneration.

In **Chapter 6**, we focused our study on illustrating impact of FA on adaptive immunity at two different time points after induction of renal I/RI. The studies presented in this chapter were a continuation of chapter 3 where we studied the immunomodulatory effect of DR and FA without induction of renal I/RI. For this study only FA was chosen since it showed the most pronounced effects in previous studies. This study was focused on the post-I/RI phase and mainly performed to observe how long the effects of FA lasted after induction of renal I/RI. Similar to our previous findings, we found that FA arrested B cell development after induction of renal I/RI, while increasing the number of recirculating mature B cells. Thymopoiesis was also observed to be arrested significantly and no major changes in $CD3^+$ T cell populations were observed in the spleen, while in BM the number of $CD3^+$ T cells was increased. All the immunological changes seen in this study were however, observed to return to their basal level 24hrs post induction of renal I/RI. Since cytokines play an important role in mediating I/RI after transplantation, in this study both pro- and anti-inflammatory cytokine production was investigated. The frequencies of cytokine-producing cells such as that of pro-inflammatory cytokines by splenocytes of fasted mice, such as IFN-γ and IL-2, was decreased 6hrs post reperfusion but not anymore at 24hrs post reperfusion. No effect of FA on anti-inflammatory cytokines was observed. Based on these findings, we hypothesized that the protective effect mediated by fasting on renal I/RI is associated with an arrest in lymphocyte development and a decrease in frequencies of pro-inflammatory cytokine-producing cells.

In **Chapter 7**, we investigated whether another stress model, i.e. cold exposure could induce protection against renal I/RI similar to DR. We showed similar physiological changes in adiposity in male C57Bl/6 mice due to DR, FA and cold exposure, but the cold-exposed mice were not protected against renal I/RI. To further elucidate the reason behind this failure in protection, we investigated the changes in adiposity, stress hormone levels, immunological changes and changes in the cytoprotective genes and antioxidant enzymes induced by both these mild stressors. We showed similarities in the

metabolic profiling of the stress related genes. However, immunologically, there were differences between the two groups (dietary intervention and cold-exposure) that could account for this failure. Also, downregulation of factors such as cytoprotective genes and genes encoding for the antioxidant defense enzymes could be the leading factors behind failure to protect against renal I/RI by cold-exposed mice. In conclusion, our findings show that, even though they may both be hermetic stimuli, the physiological effects of DR and cold exposure are different, and that cold exposure does not protect against I/RI.

Studies presented in this thesis have successfully elucidated the immunological effects of both DR and FA. We have shown that two different short-term dietary interventions cause alterations in all the lymphoid compartments. We showed arrest of the major development stages in both B and T cells. We also highlighted the role played by FA on MBL in the protection against renal I/RI. Furthermore, we showed that not only MBL but also other complement pathways such as terminal pathway play an important role in protection by DR and FA. Our studies show that another stress model, cold exposure, brings about major immunological and metabolic changes but does not induce protection against I/RI. In our studies we could show immunomodulation by dietary restriction but we have not been able to elucidate the exact mechanism behind protection by these dietary interventions. Therefore the role of these immunological effects of dietary interventions in relation to protection against renal I/RI warrants further investigation.

CHAPTER 10

Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

Niertransplantatie wordt beschouwd als de enige curatieve behandeling voor mensen met eindstadium nierfalen. De resultaten worden echter negatief beïnvloed door de ontwikkeling van ischemie-reperfusie schade (IRS) tijdens de transplantatie. Door een tekort aan orgaandonoren worden er tegenwoordig veel donororganen van oudere overleden donoren of donoren met co-morbiditeit (verruimde criteria voor donororganen) getransplanteerd, waarbij er een hoger risico is voor het ontwikkelen van IRS. IRS is een van de voornaamste oorzaken van acuut ischemisch nierfalen en is de belangrijkste oorzaak van vertraagde transplantaatfunctie. Er is nog geen goede behandeling van IRS. Meerdere onderzoeken tonen aan dat vrije zuurstofradicalen, endotheeldysfunctie en immuunactivatie als de cruciale gebeurtenissen in de ontwikkeling van weefsel schade en transplantaatdysfunctie tijdens renale IRS optreden.

Recente experimenten in muizen hebben aangetoond dat dieetrestrictie (DR) leidt tot vermindering van de nadelige gevolgen van IRS. Kortdurende DR en vasten (VA) voorafgaand aan inductie van IRS bieden bescherming tegen IRS in zowel nieren als de lever. Het exacte mechanisme achter deze bescherming door DR is tot nu toe nog niet ontrafeld. Er zijn aanwijzingen dat DR het immuunsysteem moduleert, en dat DR door activatie van de hypothalamus-hypofyse-bijnier-as de plasmaconcentratie van corticosteroïden verhoogt. Los daarvan wordt DR ook beschouwd als een milde stressopwekker die uiteindelijk leidt tot bescherming, een fenomeen dat meestal wordt beschreven als hormese, en één van de verantwoordelijke factoren voor de vermindering van IRS is. Een ander type van hormese is blootstelling aan koude. Hiervan is bekend dat het de activiteit van de hypothalamus-hypofyse-bijnier-as verhoogt, aangezien blootstelling aan koude resulteert in verhoogde adrenocorticotroop hormoon spiegels en corticosteron-concentraties in het plasma.

Dit proefschrift beschrijft de immunologische veranderingen na DR en VA al dan niet gepaard met IRS, met de nadruk op het verkregen-, en aangeboren immuunsysteem. Daarnaast hebben we de blootstelling aan koude bestudeerd als een model voor stress (en vergeleken met DR en VA), dat potentieel bescherming tegen IRS zou kunnen induceren.

Hoofdstuk 1 geeft een algemeen overzicht van de belangrijkste componenten van de studies beschreven in dit proefschrift. Het centrale onderwerp is het IRS in de nier, een onvermijdelijk fenomeen tijdens transplantatie. Ook beschrijven we hier DR en VA als mogelijke therapie voor IRS in de nier. Aangezien het doel van dit proefschrift is om te bestuderen hoe DR het immuunsysteem beinvloedt, wordt het immuunsysteem uitgebreid behandeld. We focussen hier op de ontwikkeling en het functioneren van het verkregen en aangeboren immuunsysteem.

Hoofdstuk 2 is een literatuuroverzicht dat de immunologische effecten van DR beschrijft. In dit hoofdstuk wordt beschreven hoe DR het ouder wordende immuunsysteem van knaagdieren en niet-humane primaten (apen) beïnvloedt, en wat de verschillen en overeenkomsten zijn in modulering van het adaptieve en aangeboren immuunsysteem door DR.

De invloeden van DR op de verschillende cellen van het immuunsysteem, zoals T en B lymfocyten, NK cellen, macrofagen, en neutrofielen worden uiteengezet. Daarnaast worden de effecten van DR op het complementsysteem, een zeer belangrijke speler in het aangeboren immuunsysteem, besproken.
Dit review concludeert dat DR de immuunfunctie kan moduleren, en daarbij de veroudering van het immuunsysteem kan vertragen, in zowel knaagdieren als in non-humane primaten.

In **hoofdstuk 3** beschrijven we de betrokkenheid van het immuun systeem bij de beschermende effecten die DR bewerkstelligt. We hebben hiertoe onderzocht hoe DR en VA de belangrijkste celpopulaties in primaire en secundaire lymfoïde organen van mannelijke C57Bl/6 muizen beïnvloeden. We hebben gevonden dat DR en VA beide leiden tot een afname in totale aantallen B cellen in secundaire lymfoïde organen door een blokkade in de vroege ontwikkeling in het beenmerg (BM), terwijl het aan de andere kant ook zorgt voor een verhoging van het aantal recirculerende mature B-cellen in het BM. Zowel DR als VA zorgen ervoor dat de T-cel ontwikkeling in de thymus wordt stopgezet, en voor een instroom van mature $CD3^+$ T-cellen in het BM. We hebben hierin duidelijke verschillen gevonden tussen de effecten van DR en VA; VA heeft een sterker effect dan DR. Dit hoofdstuk concludeert dat DR en VA acteren als milde stressoren die zorgen voor activatie van beschermende mechanismen en modulatie van de inflammatoire respons tegen schadelijke gevaarlijke signalen, wat lijdt tot veranderingen in de B- en T-cel ontwikkeling. Deze dieetinterventies zorgen er ook voor dat recirculerende mature B-cellen en $CD3^+$ T-cellen zich nestelen in het BM, zodat mogelijk energie kan worden bespaard in het BM, omdat er geen aanmaak van nieuwe cellen nodig is, en zo de beperkte energie in een situatie van DR of VA gebruikt kan worden voor andere processen.

In **hoofdstuk 4** onderzoeken we een van de belangrijkste compartimenten van het aangeboren immuunsysteem, het complement systeem. Van de 3 verschillende complement routes is hier de mannan-bindende-lectine (MBL) route bestudeerd, aangezien deze route een cruciale rol speelt bij het ontstaan van renale IRS. Beide dieetinterventies (DR en VA) bieden een robuuste bescherming bieden tegen IRS. Tijdens beide dieetinterventies is er sprake van een significante afname van de hoeveelheid circulerend MBL en mRNA expressie van het gen in de lever (waar MBL wordt geproduceerd). We hebben ook ontdekt dat de bescherming door DR verdwijnt wanneer MBL wordt teruggegeven, terwijl dit in de VA groep niet gebeurt. Extravasatie van MBL na

reperfusie speelt een cruciale rol in de inductie van IRS. Daarnaast leidt directe blootstelling van tubulusepitheel aan MBL bij reperfusie tot tubulaire schade. De observatie dat de bescherming door VA niet gemedieerd is door de preventie van vasculaire lekkage, suggereert dat VA een effect heeft op tubulaire cel homeostase en niet vatbaar is voor tubulaire schade, onafhankelijk van MBL. Dit is wederom een indicatie dat DR en VA wellicht een ander werkingsmechanisme hebben. Dit kan liggen aan het feit dat de methode van dieet interventie verschillend is; bij het "sub-acute" DR wordt gedurende 2 weken 30% minder voedsel gegeven, en bij het absolute VA wordt gedurende 3 dagen alleen water gegeven. Ook is de bescherming geinduceerd door VA robuuster en lijkt minder makkelijk omkeerbaar door de hoeveelheid MBL die wordt gegeven, terwijl dat wel lukt in de DR groep. Dit hoofdstuk concludeert dat modulatie van MBL van belang is in de protectie tegen renale IRS geïnduceerd door DR en suggereert dat het mechanisme van bescherming geïnduceerd door DR enerzijds en VA anderzijds kan verschillen.

In **hoofdstuk 5** als verlengstuk van hoofdstuk 4, zijn we meer gaan focussen op de gehele complementcascade. In deze studie hebben we de functionaliteit van het complementsysteem in het serum bepaald met route-specifieke functionele assays. Verder hebben we een gefocuste analyse van de expressie van belangrijke complement-eiwitten en regulatoren in de lever en de nieren uitgevoerd. Onze resultaten laten zien dat VA, en in mindere mate DR, de systemische complement activiteit reduceert van alle 3 de activatie initiatie-routes, met de meeste impact op de terminale complement factoren, maar een minder grote impact op de initiatie routes. Na verder onderzoek naar genexpressie in de lever werd een significante reductie van terminale route genen gevonden. Ondanks dat resulteerde alleen VA in systemische vermindering van de terminale route componenten, terwijl er geen effect van DR werd gevonden. Een significante opregulatie van complement factor C3 werd gezien in de lever en de nieren na DR en VA. Deze opregulering van C3 suggereert een compensatiemechanisme tegen een acute fase reactie. De rol van het compensatiemechanisme van opgereguleerd C3 inlever en nier in reactie op VA zijn erg interessant en kunnen hypothetisch gelinkt worden aan de rol van C3b en C3a in weefselregeneratie.

Hoofdstuk 6 legt nadruk op de impact van VA op het adaptieve afweersysteem Dit hoofdstuk bouwt verder op hoofdstuk 3 waar het immunmodulatoire effect van DR en VA zonder inductie van nier IRS werd bestudeerd. Voor deze studie is gekozen om alleen het VA protocol te gebruiken, aangezien het de meest uitgesproken effecten heeft gegeven in de vorige studies. Deze studie focust op de fase na IRS, met name om te bekijken hoe lang de effecten van VA aanhouden na de inductie van renale IRS. Net als in onze vorige experimenten hebben we gevonden dat VA de B-cel ontwikkeling blokkeert, ook na inductie van renale IRS, terwijl het een verhoging geeft van de aantallen recirculerende mature B-cellen. We hebben ook gevonden dat de thymopoeiese geblokkeerd is en er geen grote veranderingen zijn in de hoeveelheid $CD3^+$ T cel populaties in de milt, terwijl er meer van deze $CD3^+$ T cellen in het BM zijn. Al de immunologische

veranderingen zijn weer terug op hun basale levels 24 uur na inductie van de renale IRS. Omdat cytokines een belangrijke rol spelen in het ontwikkelen van IRS na transplantatie, hebben we in deze studie de pro- en anti-inflammatoire cytokine productie bestudeerd. De frequenties van cytokine producerende cellen zoals pro-inflammatoire cytokines zoals IFN-γen IL-2, door splenocyten van muizen in de VA groep, zijn 6 uur na re-perfusie verlaagd, maar 24 uur na reperfusie zien we dit niet meer. Er zijn geen effecten gevonden van VA op anti-inflammatoire cytokines. Gebaseerd op deze bevindingen beredeneren we dat het beschermende effect gemedieerd door VA op renale IRS geassocieerd is met een blokkade in de ontwikkeling van lymfocyten en een afname in de frequenties van pro-inflammatoire cytokine-producerende cellen.

In **hoofdstuk 7** bestuderen we of een ander stress model, namelijk blootstelling aan koude, bescherming tegen renale IRS kan induceren zoals DR dit doet. Na behandeling met DR, VA of koude hebben we dezelfde fysiologische veranderingen in hoeveelheid vetweefsel in mannelijke C57Bl/6 muizen aangetoond, maar de koude behandeling was niet beschermend tegen renale IRS. Om verder uit te zoeken waarom in dit model geen bescherming tegen renale IRS optrad, hebben we gekeken naar de veranderingen in hoeveelheid lichaamsvet, stress hormoon levels, immunologische veranderingen en veranderingen in cytoprotectieve genen en anti-oxidant enzymen geïnduceerd door DR, VA en koude. We hebben gelijkenissen aangetoond in de metabole profielen van deze stress gerelateerde genen. Desondanks waren er immunologische verschillen tussen de 2 groepen (dieet interventie versus koude blootstelling) die zouden kunnen leiden tot het falen van beschermen door koude blootstelling. Ook kan de lagere expressie van factoren als cytoprotectieve genen en genen die coderen voor anti-oxidant verdedigingsenzymen de leidende factor zijn van de afwezigheid van bescherming tegen renale IRS bij de aan koude blootgestelde muizen. Concluderend laten onze resultaten zien dat, ondanks dat beide methoden hormetische stimuli zijn, de fysiologische effecten van DR en koude behandeling verschillend zijn en dat blootstelling aan koude niet beschermt tegen IRS.

Studies gepresenteerd in dit proefschrift hebben in detail onderzocht wat de immunologische effecten van DR en VA zijn. We hebben laten zien dat 2 verschillende soorten kortdurende dieet veranderingen kunnen leiden tot indrukwekkende veranderingen in het lymfoïde compartiment. Zo hebben we de blokkade van belangrijke ontwikkelingsmomenten in de B- en T-cellen aangetoond na DR en VA. We hebben het effect van VA op MBL, dat mogelijk een rol speelt in de protectie tegen renale IRS aangetoond. Verder laten we zien dat niet alleen MBL, maar ook de andere complementroutes, zoals als de terminale complement route, belangrijk zijn in de bescherming gegeven door DR en VA. Onze studies laten zien dat een ander stress model, namelijk blootstelling aan koude, hoewel eveneens grote immunologische en metabole veranderingen teweeg werden gebracht, geen bescherming biedt tegen renale IRS.

In onze studies hebben we aangetoond dat dieetrestrictie immunomodulatie induceert, maar helaas hebben we niet het exacte mechanisme van bescherming tegen IRS door dieetrestrictie kunnen ontrafelen. Daarom is verder onderzoek nodig om de rol van de immunologische effecten van dieetinterventies in relatie tot bescherming tegen renale IRS te kunnen bepalen.

CHAPTER 11

Acknowledgement
Curriculum vitae
PhD Portfolio

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CURRICULUM VITAE

ABOUT THE AUTHOR

Shushimita was born on 2nd November, 1986 in Bihar, India. She obtained her high school diploma from D.A.V. Public School Patna, Bihar and then started her Bachelor studies in Biotechnology, Biochemistry and Genetics at T. John College, Bangalore University in 2005. After defending her bachelor studies from Bangalore, in August 2008 she began her master's studies at Wageningen University and Research Centre in Cellular and Molecular Biotechnology. In 2009 during her master's studies she was awarded the ABF (Anne van den Ban Fund) scholarship by the University. During summer holidays of 2009 she went to MIT, USA as a research scholar in the lab of Prof. Hidde Ploegh to carry out work on protozoan parasite Toxoplasma gondii. She studied molecular mechanism of antigen processing in *Toxoplasma gondii* and focused on understanding the relationship between the protozoan parasite *T.gondii* and hGBPs. She was so much inclined to the work that she continued with her research there and extended it for 6 months as a part of her master internship. In January 2010 she started her MSc. thesis at Cell Biology and Immunology group with Prof. Huub Savelkoul where she studied the switching of B cells from IgM to other immunoglobulin such as IgA and IgE. She graduated her MSc. studies in September, 2010 and immediately before graduating in August 2010 she started her doctoral research at Erasmus University Medical Center under supervision of Dr. Frank JMF Dor. Together with Dr. Frank Dor she initiated several collaborations within Erasmus MC and with LUMC, Leiden and Resenburg, Germany. This thesis documents her PhD research and published research work on "Immunomodulation by Dietary Restriction in Renal Ischemia-Reperfusion Injury".

PHD PORTFOLIO

ERASMUS UNIVERSITEIT ROTTERDAM

Name PhD Student: **Shushimita** Erasmus MC Department: **Surgery** Research School: **Erasmus Molecular Medicine Postgraduate School**	PhD Period: **2010-2014** Promotor: **Prof.dr. J.N.M .IJzermans** Supervisors: **Dr. F.J.M.F. Dor and Dr. R.W.F.de Bruin**

PHD TRAINING	YEAR	WORKLOAD/ ECTS
GENERAL COURSES AND ACADEMIC SKILLS		
WORKSHOP BASIC DATA ANALYSIS ON GENE EXPRESSION ARRAYS	2010	1.1
FLOW CYTOMETRY WORKSHOP	2010	0.4
BASIC INTRODUCTION COURSE ON SPSS	2011	1.0
ADVANCED MOLECULAR IMMUNOLOGY COURSE	2011	3.0
WORKING WITH RADIONUCLEOTIDES (5B)	2011	1.1
BIO MEDICAL SCIENTIFIC ENGLISH WRITING	2011	2.0
WORKSHOP ON PHOTOSHOP AND ILLUSTRATOR CS5	2013	0.3
WORKSHOP INDESIGN CS5	2013	0.15
SYMPOSIUM MUCOSAL IMMUNOLOGY	2013	0.5
NATIONAL CONFERENCES/PRESENTATIONS		
SIGNAL TRANSDUCTION, LEIDEN	2010	0.3
STAFDAG HEELKUNDE, ERASMUS MC, ROTTERDAM	2010	1.0
2ND IMMUNE TOLERANCE CONGRESS, AMSTERDAM (POSTER)	2011	1.0
STAFDAG HEELKUNDE, ERASMUS MC, ROTTERDAM (ORAL)	2011	1.0
MOLECULAR MEDICINE DAY, ROTTERDAM (ORAL)	2012	0.3
BOOTCONGRES, MAASTRICHT (ORAL)	2012	1.0
STAFDAG HEELKUNDE, ERASMUS MC, ROTTERDAM	2013	1.0
MOLECULAR MEDICINE DAY, ROTTERDAM (POSTER)	2013	0.3
BOOTCONGRES, ARNHEM (ORAL)	2013	1.0
MOLECULAR MEDICINE DAY, ROTTERDAM (POSTER)	2014	0.3
BOOTCONGRES, LEIDEN (POSTER)	2014	1.0
DUTCH COMPLEMENT SYMPOSIUM, NUNSPEET	2014	1.0

PHD TRAINING	YEAR	WORKLOAD/ ECTS
INTERNATIONAL CONFERENCES/PRESENTATIONS		
YIN YANG SYMPOSIUM ON IMMUNOLOGY, FRANCE	2010	1.0
EUROPEAN SOCIETY FOR SURGICAL RESEARCH, GERMANY (ORAL)	2011	1.0
BASIC SCIENCE SYMPOSIUM, USA (POSTER)	2011	1.0
EUROPEAN SOCIETY FOR ORGAN TRANSPLANTATION, UK (RAPID ORAL)	2011	1.0
EUROPEAN SOCIETY FOR SURGICAL RESEARCH, FRANCE (ORAL)	2012	1.0
METABLOLISM, DIET AND DISEASE CONGRESS, USA (POSTER)	2012	1.0
24TH INTERNATIONAL CONGRESS OF THE TRANSPLANTATION SOCIETY, GERMANY (POSTER)	2012	1.0
3RD EUROPEAN CONGRESS OF IMMUNOLOGY, UK (ORAL)	2012	1.0
BASIC SCIENCE MEETING, FRANCE (POSTER)	2013	1.0
AMERICAN TRANSPLANT CONGRESS, USA (POSTER)	2013	1.0
2ND INTERNATIONAL MEETING ON ISCHEMIA REPERFUSION INJURY IN TRANSPLANTATION, FRANCE (ORAL)	2014	1.0
TEACHING/OTHER ACTIVITIES		
SUPERVISING HLO STUDENT	2012-13	6
NEL KREEFT PRIZE, STAFDAG HEELKUNDE	2011	1
BEST ORAL PRESENTATION, BOOT CONGRESS	2012	1
FUNDING ERASMUS TRUSTFONDS, EUROPEAN SOCIETY FOR ORGAN TRANSPLANTATION	2011	1
FUNDING ERASMUS TRUSTFONDS, AMERICAN TRANSPLANT CONGRESS	2013	1

Kidney transplantation is considered to be the only curative treatment for people with end stage renal disease. Its results however, are negatively affected by the development of ischemia-reperfusion injury (I/RI) during transplantation. Nowadays, many deceased donor organs come from donors with higher age or comorbidity (extended criteria donor organs) that are more prone for I/RI. I/RI is one of the major causes of ischemic acute renal failure, and is the major cause of delayed graft function. Despite advances in the renal replacement therapy treatment of I/RI is still unsatisfactory. Several lines of evidence support free reactive oxygen species formation, endothelial dysfunction, and immune activation as the crucial event in the development of tissue injury and graft dysfunction during renal I/RI. Recently, mouse studies have shown that dietary restriction (DR) exerts beneficial effects against the detrimental effects of I/RI. Preoperative short term DR and fasting (FA) induce protection against I/RI in both kidney and liver. The exact mechanism behind protection afforded by DR has not been elucidated so far. However, DR regimens have been postulated to reprogram the immunological profile, and increase plasma corticosterone concentrations due to induction of hypothalamic-pituitary-adrenal axis activity. Apart from that, DR has also been considered a mild stressor that ultimately leads to protection, commonly referred to as hormesis and has been hypothesized to be responsible in ameliorating I/RI. Another type of hormesis cold exposure, has been known to induce hypothalamic-pituitary-adrenal axis activity since it results in elevated plasma adrenocorticotropic hormone and corticosterone concentrations. This thesis describes the immunological changes following DR and FA, and after renal I/RI, with emphasis on both adaptive and innate immunity. We also studied cold exposure as stress model that could potentially induce protection against IRI.

Niertransplantatie wordt beschouwd als de enige curatieve behandeling voor mensen met eindstadium nierfalen. De resultaten worden echter negatief beïnvloed door de ontwikkeling van ischemie-reperfusie schade (IRS) tijdens de transplantatie. Door een tekort aan orgaandonoren worden er tegenwoordig veel donororganen van oudere overleden donoren of donoren met co-morbiditeit (verruimde criteria voor donororganen) getransplanteerd, waarbij er een hoger risico is voor het ontwikkelen van IRS. IRS is een van de voornaamste oorzaken van acuut ischemisch nierfalen en is de belangrijkste oorzaak van vertraagde transplantaatfunctie. Er is nog geen goede behandeling van IRS. Meerdere onderzoeken tonen aan dat vrije zuurstofradicalen, endotheeldysfunctie en immuunactivatie als de cruciale gebeurtenissen in de ontwikkeling van weefsel schade en transplantaatdysfunctie tijdens renale IRS optreden. Recente experimenten in muizen hebben aangetoond dat dieetrestrictie (DR) leidt tot vermindering van de nadelige gevolgen van IRS. Kortdurende DR en vasten (VA) voorafgaand aan inductie van IRS bieden bescherming tegen IRS in zowel nieren als de lever. Het exacte mechanisme achter deze bescherming door DR is tot nu toe nog niet ontrafeld. Er zijn aanwijzingen dat DR het immuunsysteem moduleert, en dat DR door activatie van de hypothalamus-hypofyse-bijnier-as de plasmaconcentratie van corticosteroïden verhoogt. Los daarvan wordt DR ook beschouwd als een milde stressopwekker die uiteindelijk leidt tot bescherming, een fenomeen dat meestal wordt beschreven als hormese, en één van de verantwoordelijke factoren voor de vermindering van IRS is. Een ander type van hormese is blootstelling aan koude. Hiervan is bekend dat het de activiteit van de hypothalamus-hypofyse-bijnier-as verhoogt, aangezien blootstelling aan koude resulteert in verhoogde adrenocorticotroop hormoon spiegels en corticosteron-concentraties in het plasma. Dit proefschrift beschrijft de immunologische veranderingen na DR en VA al dan niet gepaard met IRS, met de nadruk op het verkregen-, en aangeboren immuunsysteem. Daarnaast hebben we de blootstelling aan koude bestudeerd als een model voor stress (en vergeleken met DR en VA), dat potentieel bescherming tegen IRS zou kunnen induceren

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