

*Neuro-Immune Elements of
Inflammatory Disease*

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Interactions between the immune system and the nervous system are currently underappreciated, assumed to play minor mechanistic roles in disease pathogenesis. In contrast, our laboratory has demonstrated the importance of this relationship with significant impact, initially in Type 1 Diabetes (T1D). The experiments presented here build on our previous work to provide insights into the etiology of Multiple Sclerosis (MS) and Type 2 Diabetes (T2D).

Transient Receptor Potential Vanilloid-1 (TRPV1) is an ion channel expressed on peripheral sensory afferent neurons that fundamentally control T1D pathogenesis. Here we show that mice with genetic ablation of TRPV1 are protected from EAE progression, attributable to reduced central nervous system (CNS) leukocyte passage. The pathogenic role of TRPV1 in permeabilizing the blood-CNS barriers may also translate to MS, as patients with progressive disease show a significant mutation bias within the TRPV1 gene.

We were simultaneously intrigued by the growing worldwide obesity epidemic, and we observed that obese mice develop more severe EAE compared to lean mice. This was mechanistically linked to an expansion of T_H17 cells, driven by sustained rises of IL-6 in obese mice. This research implies new therapeutic opportunities for the many obese patients with diverse autoimmune diseases.

Finally, the immune system, obesity, and T2D are functionally linked, and we contributed to research that uncovered a large presence of immune cells in adipose tissue that drive insulin resistance. Manipulation of T cells and B cells affects local inflammation as well as whole-body insulin resistance and glucose homeostasis. Intriguingly, auto-antibodies in insulin resistant individuals are specific for a number of unique proteins, including glial fibrillary acidic protein

(GFAP), initially shown by our laboratory to play a key role in T1D progression. We further characterized the autoimmune and neuronal progression elements that steer disease pathogenesis, and observed that administration of a vaccine containing GFAP is able to dramatically reduce weight gain and insulin resistance in mice.

The data presented in this thesis provide a number of novel, mechanistic observations linking the immune and nervous systems in disease, and implies several potential avenues for treatment.

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Publications

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Abbreviations

ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
APC	Antigen Presenting Cell
APOE	Apolipoprotein E
APRIL	A Proliferation-Inducing Ligand
AS160	AKT Substrate of 160 kDa
ASIC	Acid-Sensing Ion Channel
ATP	Adenosine Triphosphate
b	Base
B6	C57BL/6
BBB	Blood-Brain Barrier
BCR	B Cell Receptor
BDNF	Brain-Derived Neurotrophic Factor
BLyS	B Lymphocyte Stimulator
BMI	Body Mass Index
BP	Blood Pressure
BSA	Bovine Serum Albumin
BSCB	Blood Spinal Cord Barrier
C	Celsius
cal	Calorie
cAMP	Cyclic Adenosine Monophosphate
CCL	C-C Motif Chemokine Ligand
CCPGSMS	Canadian Collaborative Project on the Genetic Susceptibility to Multiple Sclerosis
CCR	C-C Motif Chemokine Receptor
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFA	Complete Freund's Adjuvant
CGRP	Calcitonin Gene-Related Peptide
Ci	Curie

CLS	Crown-Like Structure
CNS	Central Nervous System
cpm	Counts per Minute
CREB	cAMP Response Element-Binding Protein
CRP	C-Reactive Protein
CSF	Cerebrospinal Fluid
CTLA	Cytotoxic T-Lymphocyte Antigen
CXCL	C-X-C Motif Chemokine Ligand
CXCR	C-X-C Motif Chemokine Receptor
d	Day
Da	Dalton
DIO	Diet-Induced Obesity
DRG	Dorsal Root Ganglion
EAE	Experimental Autoimmune Encephalomyelitis
EBV	Epstein Barr Virus
ECM	Extracellular Matrix
EDSS	Expanded Disability Status Scale
ELISA	Enzyme-Linked Immunosorbent Assay
F(ab')₂	Fragment Antigen-Binding
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
Fc	Fragment Crystallizable
FOXO1	Forkhead Box Protein O1
FoxP3	Forkhead Box P3
g	Gram
GA	Glatiramer Acetate
GAP	GTPase-Activating Protein
Gd-DTPA	Gadolinium Diethylenetriaminepentacetate
GFAP	Glial Fibrillary Acidic Protein
GLP-1	Glucagon-Like Peptide-1
GLUT	Glucose Transporter
GOSR1	Golgi SNAP Receptor Complex Member 1

GSK-3	Glycogen Synthase Kinase-3
GTT	Glucose Tolerance Test
GWAS	Genome-Wide Association Study
H&E	Hematoxylin and Eosin
HbA1c	Haemoglobin A1c
HDL	High-Density Lipoprotein
HFD	High-Fat Diet
HLA	Human Leukocyte Antigen
hr	Hour
HRP	Horseradish Peroxidase
IAPP	Islet Amyloid Polypeptide
IBD	Inflammatory Bowel Disease
ICAM	Intercellular Adhesion Molecule
IFA	Incomplete Freund's Adjuvant
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin-Like Growth Factor-1
IKK-β	Inhibitor κ B Kinase β
IL	Interleukin
IR	Insulin Resistant
IRS	Insulin Receptor Substrate
IS	Insulin Sensitive
ITT	Insulin Tolerance Test
IVIG	Intravenous Immunoglobulin
JNK	Jun N-Terminal Kinase
KCNJ11	Potassium Inwardly-Rectifying Channel Subfamily J Member 11
L	Litre
LADA	Latent Autoimmune Diabetes of the Adult
LDL	Low-Density Lipoprotein
LFB	Luxol Fast Blue
LN	Lymph Node
M	Molar

mAb	Monoclonal Antibody
MadCAM	Mucosal Addressin Cell Adhesion Molecule
MAG	Myelin-Associated Glycoprotein
MBP	Myelin Basic Protein
MCP-1	Monocyte Chemotactic Protein-1
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
MODY	Maturity-Onset Diabetes of the Young
MOG	Myelin Oligodendrocyte Glycoprotein
mol	Mole
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
MSSS	Multiple Sclerosis Severity Score
mTOR	Mammalian Target of Rapamycin
NCD	Normal Chow Diet
NF-κB	Nuclear Factor κ B
NGF	Nerve Growth Factor
NK	Natural Killer
NO	Nitric Oxide
NOD	Non-Obese Diabetic
OVA	Ovalbumin
PAI-1	Plasminogen Activator Inhibitor-1
PANEM	Pericytes, Astrocytes, Neurons, Endothelial Cells, Microglia
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PD-L1	Programmed Cell Death 1 Ligand 1
PDK1	Phosphoinositide-Dependent Protein Kinase 1
PDX-1	Pancreatic and Duodenal Homeobox-1
PECAM	Platelet Endothelial Cell Adhesion Molecule
PI3K	Phosphatidylinositol 3-Kinase
PIP3	PtdIns-3,4,5-Trisphosphate
PKB	Protein Kinase B

PKC	Protein Kinase C
PLP	Proteolipid Protein
PMA	Phorbol 12-Myristate 13-Acetate
PML	Progressive Multifocal Leukoencephalopathy
PNS	Peripheral Nervous System
PP	Primary Progressive
PPAR-γ	Peroxisome Proliferator-Activated Receptor- γ
PtdIns	Phosphatidylinositol
PTP	Protein Tyrosine Phosphatases
PTX	Pertussis Toxin
RAG1	Recombination Activating Gene 1
RANTES	Regulated and Normal T Cell Expressed and Secreted
RD	Regular Diet
RNA	Ribonucleic Acid
ROR	Retinoic Acid Receptor-Related Orphan Receptor
RP	Relapsing Progressive
rpm	Rotations Per Minute
RPMI	Roswell Park Memorial Institute medium
RR	Relapsing Remitting
RTX	Resiniferatoxin
S6K1	S6 Kinase 1
S100β	S100 Calcium Binding Protein β
SAT	Subcutaneous Adipose Tissue
SH2	Src Homology 2
SI	Stimulation Index
SNAP	Soluble NSF Attachment Protein
SNP	Single Nucleotide Polymorphism
SOCS	Suppressor of Cytokine Signalling
SP	Secondary Progressive
sP	Substance P
STAT	Signal Transducer and Activator of Transcription
SVC	Stromal Vascular Cell

SYK	Spleen Tyrosine Kinase
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
Tac1	Tachykinin, Precursor 1
TCR	T Cell Receptor
TGF	Transforming Growth Factor
T_H	T Helper
TIMP	Tissue Inhibitor of Metalloproteinase
TLR	Toll-Like Receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour Necrosis Factor
TRPV1	Transient Receptor Potential Vanilloid 1
TZD	Thiazolidinedione
U	Unit
UCP2	Uncoupling Protein 2
VAT	Visceral Adipose Tissue
VCAM	Vascular Cell Adhesion Molecule
VLA-4	Very Late Antigen-4
wk	Week

CHAPTER 1

Introduction

1. Multiple Sclerosis

Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) characterized by demyelination and axonal damage. It was first reported by renowned French neurologist Jean-Marie Charcot (1) and significant discoveries into its aetiology and pathogenesis have subsequently been made. Both genetic and environmental factors contribute to the development of disease. Neuronal injury leads to a wide range of symptoms that significantly impact quality of life, and exact a large physical, social, and economic burden on those affected and their families.

1.1. Diagnosis and clinical features

Diagnosis of MS can be difficult, particularly in its earlier stages, and a definitive diagnostic test does not exist. Instead, patient history, clinical examination, radiographic and laboratory findings, and the distribution and progression of neuronal lesions are all interpreted. The need for comprehensive and standardized diagnostic criteria lead to the establishment of the Poser system (2). Using this system, a patient is described as “definite” MS, “probable” MS, or not MS, based on the history of attacks and clinical evidence of lesions. Although these designations are often still used, the Poser system has been superseded by the McDonald system, due to advances in gadolinium enhanced magnetic resonance imaging (MRI) technology. The dissemination of lesions in both time and space are also taken into consideration (3). MS is considered likely if the patient has suffered distinct bouts of neurological dysfunction two or more times accompanied by lesions in the myelinated regions of the CNS, or one attack supported by radiological and laboratory evidence such as conduction delays in an evoked potential test (4).

Disease manifests in a wide range of deficits that depend on the location of demyelination within the CNS. For example, lesions in the spinal cord are associated with paresis, whereas those in

the optic nerve are associated with impaired vision. A significant proportion of lesions, possibly up to 80-90%, do not manifest as clinical symptoms (5). The number of lesions is therefore a poor correlate of disability, and *vice versa*. Additional symptoms include fatigue, diplopia, loss of coordination and balance, impaired speech, bowel and bladder dysfunction, and pain (6).

Attempts have been made to quantify the disability in MS. The Expanded Disability Status Scale (EDSS) assigns patients a value between zero and ten based on their symptoms in eight functional systems (7). EDSS scores increase with disability, and those with an EDSS score of greater than or equal to five show impaired ambulatory ability. More recently, the Multiple Sclerosis Severity Score (MSSS) was shown to quite accurately predict disease progression, following a single assessment of disability (8). The MSSS value represents the percentage of MS patients from a collection of almost ten thousand individuals with similar disease duration but a lower EDSS score.

1.2. Prognosis

The clinical course of MS is highly variable and it remains difficult to establish an accurate prognosis. Approximately 15% of patients develop primary progressive (PP) disease, characterized by steady deterioration with only occasional plateaus or temporary minor improvements (9). However, the majority of MS patients have a milder, relapsing remitting (RR) disease, with acute attacks followed by periods of remission. Approximately 50% of RR MS patients will enter a stage of steady deterioration known as secondary progressive (SP) MS within ten years of diagnosis (10), and this number increases to more than 80% after twenty five years (11). An additional group of patients, roughly 5%, fall into a hybrid classification known as relapsing progressive (RP) MS. These individuals show more rapid worsening of disease, similar to PP MS, but with atypical attacks, and without the characteristic remissions of RR MS patients (4, 9).

The course of MS typically evolves over several decades, but is widely variable. Factors associated with a good prognosis include female sex, early age of onset, optic neuritis or sensory

attacks, complete recovery from attacks, and long periods between attacks (12). Factors associated with poor prognosis include male sex, cerebellar or motor attacks, incomplete recovery from attacks, and frequent attacks (12). On average, patients present with EDSS scores of six at fifteen years post-diagnosis. At this point in time, 15% of patients require the use of a wheelchair, while 25% will have only minor disability (13). Based on progression, MS is often distinguished as either a mild form of disease referred to as “benign”, or a more progressive form termed “malignant”, including the often fatal Marburg variant. Taken together, the life expectancy of patients with MS is estimated to be 5 to 10 years less than the unaffected population (14). Approximately two-thirds of patients with MS will die as a result of the disease and its related complications (15).

1.3. Epidemiology

The global estimated median incidence of MS is 2.5 per 100 000 people per year, and the global estimated median prevalence is 30 per 100 000 people (16). However, disease is not equally distributed throughout the world, with an impressive latitude bias reminiscent of other autoimmune diseases and rather closely overlapping with Type 1 Diabetes (T1D) (17). Areas with higher disease burden include Northern Europe, Canada, and Southeastern Australasia (15), although it has been suggested that this regional bias is shrinking. Prevalence is likely underestimated because of the considerable number of people with MS who are currently undiagnosed or misdiagnosed.

MS is typically considered a disease of younger adults, diagnosed most often in the third and fourth decade of life. However, 2% of patients present before the age of ten, and 5% before the age of sixteen (18). Many children may go undiagnosed following an attack early in life that they retrospectively recall at a later time in life, and therefore pediatric MS may be more common than stated. In children, distinguishing MS from acute disseminated encephalomyelitis or other disorders with CNS involvement often requires time to observe the subsequent natural history (19).

Like other autoimmune conditions, a gender bias exists in MS, though it is not as dramatic as in diseases such as systemic lupus erythematosus. Females show a two times greater susceptibility to RR MS, but this bias is not present in cases of PP MS (18, 20). This sex bias seems to exert effects at multiple levels, as the offspring of mothers with MS have a greater risk of developing disease compared to the offspring of fathers with MS (21), which is curiously the exact opposite of the equally enigmatic situation in T1D. It is noteworthy that while females are affected by MS more often than males, females routinely have a more fortunate prognosis.

Both the prevalence and incidence of MS are believed to be increasing in a number of regions in the world, including Europe and Canada (22, 23). This increase seems to be overrepresented in women (24, 25). Although a mechanistic explanation is lacking, it is likely environmental in nature, considering the short time frame in which the change has developed.

In Canada, the incidence and prevalence of MS is also heterogeneous and highly dependent on location within the country, with an almost two-fold difference between the province of Quebec and Atlantic Canada (26). Overall, the prevalence in Canada ranks among the highest in the world, with roughly 200-250 individuals affected per 100,000 (27, 28); it is estimated that about 70,000-85,000 Canadians are afflicted by this debilitating disease.

1.4. Genetics

MS incidence cannot be explained by Mendelian inheritance, as the risk in siblings does not match that of classic dominant or recessive diseases. Instead, similar to other autoimmune diseases, MS is a polygenic disease in which multiple genes make small but important contributions.

Although numbers differ between studies, the concordance rate for monozygotic twins is estimated to be between 20% and 40%, compared to a rate of approximately 3.5% in dizygotic twins (29-31). Measuring concordance for MS prospectively is difficult, given the significant risk to develop disease at an older age. Nevertheless, studies following cohorts for longer periods of time

have reached similar estimates (32). Disease risk of dizygotic twins does not differ significantly from non-twin siblings, while both groups are at higher risk than more distant relatives and the general population (33).

Genome-wide association studies (GWAS), comparing the frequency of alleles between unrelated cases and controls, have consistently shown linkage to only one gene, the Major Histocompatibility Complex (MHC) on chromosome 6 (34). More recent studies have revealed associations of disease with regions on chromosome 5 and chromosome 10, later shown to be linked to the Interleukin (IL)-7 receptor- α chain (35) and the IL-2 receptor- α chain (36), respectively. Larger GWAS have increased the number of candidates to almost one hundred, the majority of which are intimately connected to immune and T helper cell function (37). These include adhesion molecules, cytokines and their receptors, chemokines, and immune receptors or accessory molecules.

Additionally, analyses have been made stratifying patients depending on the severity of their disease. Such comparisons could provide valuable insights into the highly variable course and severity of MS. Although associations are more modest and do not reach typical GWAS significance, a number of novel genes that participate in neuronal processes, calcium ion transport, and interferon signalling were implicated (38).

Unlike the very diverse human population, the most commonly used mouse model for MS, experimental autoimmune encephalomyelitis (EAE), allows for analysis of inbred animals that have an extremely homogeneous genetic framework. Deletion or over-expression of a pathologically relevant gene can demonstrate its involvement in disease susceptibility or disease course. Alternatively, the genome of susceptible and resistant strains can be compared by fine mapping and further breeding for gene candidates which can then be validated. More than thirty five EAE risk loci have been identified in this manner, linked to different characteristics of disease. These risk loci

were identified by crossing susceptible SJL mice to resistant B10.S mice, or susceptible B10.RIII mice to resistant RIIS mice (39), in which parental strains express the same MHC haplotype but the remainder of their genetic background is diverse. A number of genes in EAE risk loci are located on chromosome 11 and are directly immune-related, including chemokines and cytokines such as IL-3, IL-4, IL-5, and IL-13 (40, 41), although a number of these loci are gender-selective, with disease effects in either male or female mice (42).

1.4.1. Human leukocyte antigen

The MHC, or Human Leukocyte Antigen (HLA), is central to adaptive immune system activation. It was implicated in MS pathogenesis shortly after the realization that it played a key role in transplant rejection (43, 44). Interestingly, these early studies linked MS to the MHC Class I proteins, and recent studies continue to demonstrate their importance in altering MS risk (45). Yet subsequent publications showed stronger associations to MHC Class II proteins (46, 47). Extended haplotypes that contain HLA-DRB1*1501 show the most powerful association to disease, increasing the relative risk of an individual to develop MS by approximately three-fold. This association has been mapped further to the Class II extended haplotype DRB1*1501-DQA1*0102-DQB1*0602, increasing susceptibility to MS when all three alleles are present together (48). Additionally, the combined effect of haplotypes should be considered, as the risk of carrying a copy of DRB1*15 is more than doubled when present as a DRB1*15/DRB1*08 genotype (17). Other associations within the DR region have been proposed, but these effects appear weaker than those of DRB1*15.

The HLA class II region can also be associated with a reduction, as opposed to an increase, in disease risk (49). These alleles include DRB1*14 and DRB1*11, and exhibit dominant protection over DRB1*15 when inherited together. DRB1*01 and DRB1*10 are also thought to be protective

but only in the presence of DRB1*15. Protective HLA alleles are more frequently observed in people of Asian descent, where MS is relatively uncommon.

The cause of association between HLA-DRB1 and MS is not fully understood, though it is likely explained by its ability, or lack thereof, to strongly bind disease-associated peptide chains of proteins found in myelin such as myelin basic protein (MBP) (50-52). Interestingly, the overall contribution of MHC to MS risk is weaker than that in other autoimmune diseases such as T1D. Nevertheless, it remains the most prominent gene set to influence susceptibility. This is also the case in EAE, where MHC class II molecules are the strongest predictors of disease risk. In mice, it has been hypothesized that MHC haplotypes are not strictly permissive or resistant, but rather work on a gradient of disease regulation including high, intermediate, and low responders based on studies in MHC congenic Lewis rats (53).

1.5. Environment

The large discordance of MS in monozygotic twins is strong evidence that the environment plays a large role in disease incidence. These environmental triggers have been extensively studied, and possibly set the threshold for genetic penetrance (54). The age at which genetic and environmental factors interact appears to be very important (15). While these factors seem to influence disease incidence on their own, there is little evidence for additive or multiplicative effects (55).

The effects of the environment are difficult to study using mouse models, as external inputs are kept to a minimum due to stringent conditions within animal facilities. Nevertheless, studies examining Lewis rats purchased from different animal facilities revealed variation in their incidence and severity of disease, suggestive of an environmental effect (56). Additionally, the month of EAE induction seems to interact with both sex and age in a complex manner to contribute to the appearance of lesions and clinical disease (57). Studies have also examined the influence of

microorganisms on disease penetrance and severity. Mice maintained under non-physiological germ-free conditions display dramatically attenuated EAE compared to conventionally colonized mice. This is linked to decreased interferon (IFN)- γ and IL-17 expression in the intestine and the spinal cord, which can be reversed following colonization with segmented filamentous bacteria (58). These results were confirmed in a spontaneous model of EAE, where animals are also protected from disease in germ-free conditions, show decreased IL-17 production, and effects are negated following re-colonization with conventional flora from specific pathogen-free mice (59). Therefore, it appears that the progression and severity of EAE is modified by input from the environment in addition to the genome.

1.5.1. Infectious agents

For decades it has been hypothesized that an infectious agent influenced the incidence of MS. Indeed, viral infections may be associated with relapses in patients (60). Direct infection of the nervous system gained interest due to the antigen spreading hypothesis, in which an infection within the CNS by a neurotropic virus could also initiate an immune response against myelin components. However, there remains no conclusive evidence that a virus or bacterium causes disease, or that there is a chronic infection of the nervous system during MS.

Studies looking at viruses are largely correlative rather than causative. One candidate is Epstein Barr virus (EBV). As measured by immunoglobulin (Ig) G antibody to EBV, prior infection can be detected in approximately 90% of the general population, but close to 100% of MS patients (61). Given the already high frequency of EBV infection in adults, the pediatric MS population represents a valuable research opportunity in this context. In children, it is more likely that MS patients have been exposed to EBV than healthy controls (62, 63). EBV has been suggested to increase MS incidence through molecular mimicry, as peptides from EBV have been shown to elicit responses from MBP₈₅₋₉₉-specific T cell clones (64), and crystal structure analysis revealed a

similarity between EBV₆₂₇₋₆₄₁ complexed with DRB5*0101 and MBP₈₅₋₉₉ complexed with DRB1*1501 (65). However, no direct evidence supporting the EBV hypothesis has emerged to date.

Other pathogens of interest have included Chlamydia pneumonia, human herpes virus 6, cytomegalovirus, and herpes simplex virus 1 (66), but reports are inconsistent and conflicting.

1.5.2. Nutrition and diet

The striking geographical distribution of MS prevalence has been proposed to be a result of vitamin D production following exposure to solar ultraviolet B radiation, which is more intense at the equator. High circulating levels of vitamin D levels are associated with a lower risk of multiple sclerosis, particularly before the age of twenty (67). There is also an inverse relationship between 25-hydroxyvitamin D levels and the hazard of relapse (68). Several studies, including those from our lab, have suggested that high doses of vitamin D are well tolerated, and may reduce the number of relapses (69). Administration of vitamin D in people with a family history of MS might serve as an effective, non-toxic prophylactic treatment.

A correlation between cow milk consumption and the development of MS was reported long ago also in an attempt to explain the geographic distribution of disease (70). Indeed, a significant correlation between liquid cow milk and MS prevalence exists (71). The mechanism behind this association is thought to relate to molecular mimicry between cow milk antigens and CNS antigens. The cow milk hypothesis derives from other autoimmune diseases such as T1D, where our lab co-founded the near global 'TRIGR' T1D primary prevention study now running for more than ten years (72). In MS, it has been demonstrated that antibody specific for the extracellular Ig-like domain of myelin oligodendrocyte glycoprotein (MOG) cross-reacts with a homologous N-terminal domain of the bovine milk protein butyrophilin, and butyrophilin-specific antibody can be sequestered in the cerebrospinal fluid (CSF) of patients (73). Furthermore, MS patients display

abnormal T cell reactivity to several other cow milk proteins, including a specific epitope of bovine serum albumin different from the major T1D epitope 'ABBOS' (74, 75).

1.6. Pathogenesis

The pathogenesis of MS has been studied in detail and progress has been made in our understanding of the disease process, although this is difficult given that MS pathology is extremely variable and unpredictable. MS heterogeneity has been classified into four defined patterns following examination of biopsy and autopsy specimens depending on the contribution from different immune cells, antibody and complement deposition, myelin loss, and oligodendrocyte death (76). Intriguingly, patterns differ when comparing patients, though individuals often appear to be homogeneous for one pattern throughout.

In general, pathogenesis implicates myelin-specific T cells, which, following activation in the periphery, migrate to the CNS to initiate damage. An increase in proinflammatory cytokines and chemokines results, including IFN- γ , IL-23, tumour necrosis factor (TNF)- α , lymphotoxin, regulated and normal T cell expressed and secreted (RANTES/CCL5), C-X-C motif chemokine ligand (CXCL)-10, and IL-8. There is subsequent activation of resident CNS cells such as microglia and astrocytes, as well as recruitment of other leukocytes including monocytes, CD8⁺ T cells, B cells, and mast cells. These cell types orchestrate the formation of the inflammatory lesion. Conversely, lesion resolution includes a switch towards T_H2 cytokines such as IL-10 and transforming growth factor (TGF)- β and the secretion of growth factors by both resident cells and T cells. While this sequence of events is almost certainly true, the exact molecular and biological processes that shape this process must be complex and remain enigmatic, as they are in most chronic-progressive autoimmune disorders.

Hallmarks of MS consequently include demyelination and inflammatory lesions that lead to plaque formation. This process is traditionally thought to occur in the white matter of the CNS, as it

is rich in myelin and myelin-producing oligodendrocytes. But additional evidence suggests that CNS damage is more complex and widespread, and also involves grey matter pathology even in early stages of disease that, despite being variable between patients, can often be found within the cortex. These cortical lesions have fewer infiltrating leukocytes, suggesting that neuronal degeneration may play a large role (77). Grey matter demyelination can also be seen in the deep grey matter nuclei, cingulate gyri, cerebellum, thalamus, and spinal cord (78). Post-mortem analysis suggests that grey matter lesions can even exceed white matter lesions in more progressive forms of disease (78, 79).

Concepts of MS pathogenesis, while valuing the importance of T cell-mediated demyelination, must therefore consider a broad array of immune mechanisms, axonal damage and widespread grey matter pathology (80). A schematic of MS pathogenesis is shown in **Figure 1.1**.

1.6.1. Autoantigens

In contrast to other neurological diseases such as myasthenia gravis, but reminiscent of T1D, the MS field has been plagued by the lack of a single autoantigen sufficient to cause disease. Focus has centred on reactivity to myelin antigens including MBP, proteolipid protein (PLP), MOG, and some non-myelin-specific proteins. While reactivity to several antigens exists, epitope spreading may expand the number of targets, to include additional epitopes or additional antigens.

Activation of myelin-specific autoreactive T cells in the periphery occurs for reasons and in locales unknown. Disease-associated HLA molecules may preferentially bind myelin self peptides leading to T cell presentation and expansion after the initial myelin damage – ‘the original sin’. Alternatively, these HLA molecules may bind to a limited set of peptides producing less complete thymic negative selection. The processing and loading of peptides may also be hypo- or hyper-functional in MS patients, or a defect could lie within the T cell, for example in its ability to induce anergy following successful MHC-T cell receptor (TCR) engagement.

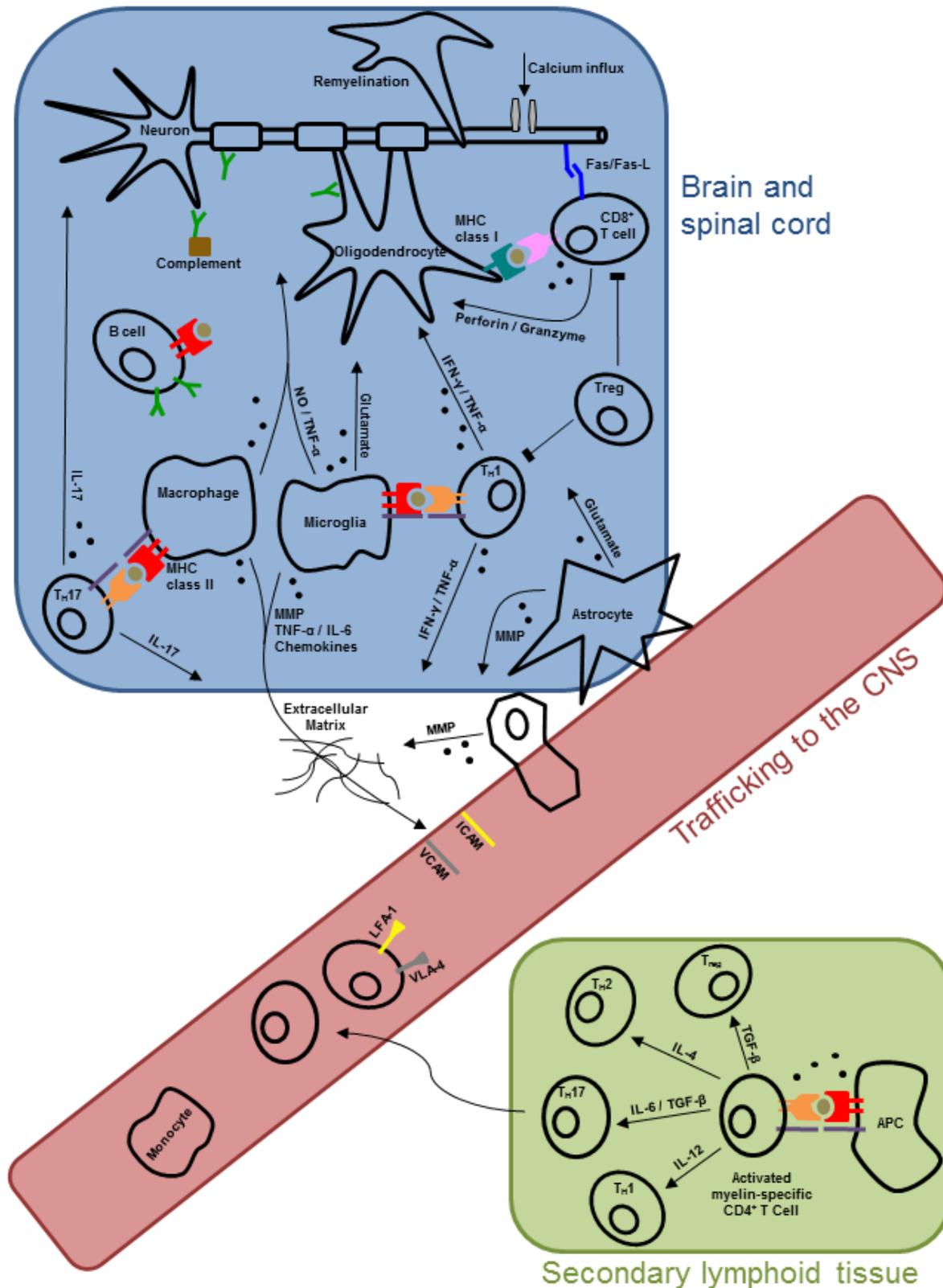


Figure 1.1 – Visual representation of MS pathology. Activation in the periphery is followed by homing to the CNS, reactivation, and formation of inflammatory lesions that damage brain and spinal cord tissue and result in clinical symptoms.

1.6.2. Impairment of the blood-brain barrier

Cells activated in the periphery must then overcome the blood-spinal cord barrier (BSCB) and blood-brain barrier (BBB) to extravasate into the CNS *via* the classic steps of tethering, arrest, and transmigration. The BSCB and BBB serve to maintain nutrient homeostasis for the CNS while guarding against toxic substances. Under normal conditions, tight junctions and low adhesion molecule expression inhibit cellular extravasation into CNS tissue, while extrusion pumps and selective transporters control the permeation of small molecules. The CNS has traditionally been considered an immune privileged site, because inflammation within the brain and spinal cord was assumed to be deleterious. In fact, T cells, macrophages and dendritic cells all actively patrol the healthy CNS for harmful agents, though they are present in small proportions relative to the periphery. Approximately 80% of immune cells found in the CSF of healthy individuals are T cells, indicative of their propensity to access the CNS (81). Conversely, B cells do not cross an intact barrier and are only thought to appear after inflammation has started, along with antibodies and complement. In MS, peripherally activated autoreactive T cells gain the essential surface molecules necessary to home and adhere to the surface of the BSCB and BBB, and those isolated from the CSF express higher levels of C-X-C motif chemokine receptor (CXCR) 3, but similar levels of C-C motif chemokine receptor (CCR) 1, CCR2, CCR3, CCR5, and CCR6 (82).

A small number of myelin-specific T cells access the perivascular space of the CNS, unlike non-myelin-specific T cells which re-enter circulation after failing to be activated in the CNS (83). Cells that have recognized antigen again are further activated and begin formation of a lesion. It has been shown that dendritic cells are central to this process in mice, as expression of MHC class II exclusively on dendritic cells is sufficient to develop EAE (84). The highest numbers of immune cells within the CNS are located in areas where the integrity of the tight junction barrier is reduced. These include the choroid plexus at the blood-CSF barrier at which CSF is produced following the

filtration of serum and subsequent secretion of solutes on the ventricle-facing membrane, the post-capillary venules at the pial surface of the brain, and post-capillary venules that enter the parenchyma directly (85). Vessels in the choroid plexus and subarachnoid space display increased P-selectin, E-selectin, and intercellular adhesion molecule (ICAM)-1 immunoreactivity (86), while the role of additional molecules such as vascular cell adhesion molecule (VCAM)-1, mucosal addressin cell adhesion molecule-1 (MadCAM-1), and platelet endothelial cell adhesion molecule (PECAM)-1 should also be considered. Additionally, the expression of CCR6 on T cells facilitates their entry into the choroid plexus, as its ligand C-C motif chemokine ligand (CCL) 20 is highly expressed by epithelial cells at this location (87). Much less is known about extravasation into the spinal cord (85), as infiltration is sparse in MS. However, BSCB integrity is critically compromised during EAE, where the most impressive perivascular inflammatory cell infiltration can be seen in the white matter of the spinal cord, to a greater extent than the white matter of the brain. Studies in mice have confirmed the importance of integrin expression on the surface of both endothelial and T cells, as mice deficient in ICAM-1 are dramatically protected from disease (88).

The BSCB and BBB lose their integrity during or after lesion formation, and this process is augmented by matrix metalloproteinase (MMP) release from a number of cell types including monocytes, T cells, microglia, astrocytes, and oligodendrocytes. MMPs are a family of endopeptidases which work to breakdown and reshape the extracellular matrix (ECM) of the basement membrane below the layer of endothelial cells. It has been demonstrated that serum MMP-9, but not tissue inhibitor of metalloproteinase (TIMP)-1, levels are elevated in RR MS patients compared to healthy controls, and high MMP-9 levels precede the appearance of new gadolinium-enhanced lesions (89). Others have shown that MMP-9 is increased in the CSF of both RR MS and, to a lesser extent, PP MS patients, while no differences are observed in the levels of MMP-2, MMP-3, TIMP-1, or TIMP-2 (90). Further elucidation of the role of each MMP is required,

as it has been hypothesized that some MMPs may have beneficial roles. The ECM components also dictate T cell extravasation, as infiltration positively correlates with sites in the CNS expressing laminin alpha 4 and negatively correlates with laminin alpha 5 expression (91).

It should be noted that in addition to MMP activity and extracellular matrix proteins, BSCB and BBB breakdown can also be exacerbated by a host of proinflammatory molecules, oxygen and nitrogen radicals, and neuropeptides. Both TNF- α and IFN- γ can upregulate adhesion molecules on endothelial cells, thereby increasing the efficiency of extravasation (92). Astrocytes maintain BSCB and BBB integrity under healthy conditions, but can also become polarized to release inflammatory cytokines such as IL-6, leading to endothelial cell activation (93).

1.6.3. CD4⁺ T cells

A role for CD4⁺ T cells in MS is supported by the association of certain HLA class II molecules with disease risk, and by their ability to influence processes such as antibody production and CD8⁺ T cell activation. It was recognized early on that MBP-specific CD4⁺ T cells can be identified in both MS patients and healthy controls (94). However, the reported frequency of autoreactive T cells varies greatly. Tissue culture techniques have shown MBP-specific frequencies of approximately 1 per 10⁶-10⁷ peripheral blood mononuclear cell (PBMC) (95), while enzyme-linked immunospot assays estimate the number to be one to two orders of magnitude higher (96). Unfortunately, tetramer-based assays do not work well for autoreactive HLA class II-restricted T cells, likely due to low affinity TCR interactions. More recent methods have estimated numbers to be 1 per 10⁴ PBMC or higher (97). High avidity T cells that respond to low antigen concentrations are also increased in MS patients (97). Additionally, it appears that the activation of CD4⁺ T cells in MS patients occurs at lower thresholds. For example, myelin-specific CD4⁺ T cells are less dependent, or entirely independent, of the interaction between CD28 and CD80/CD86 (98, 99).

CD4⁺ T cells of MS patients also show inefficient inhibition following cytotoxic T-lymphocyte antigen (CTLA)-4 stimulation (100).

The cytotoxic ability of CD4⁺ T cells, and subsequent neuronal destruction, is not as evident as that of CD8⁺ T cells. While MBP-specific CD4⁺ T cells can initiate both perforin and Fas/Fas-ligand-mediated cytotoxicity of MBP expressing targets (101, 102), it is unlikely that direct lysis of oligodendrocytes or neurons involves CD4⁺ T cells because these cells do not express HLA class II. The phenotype of CD4⁺ autoreactive T cells is largely skewed toward a T_H1-type proinflammatory response and during acute attacks the predominant pattern of cytokine secretion includes higher levels of IFN- γ and TNF- α and reduced levels of IL-10 (96, 97, 103). In mice, IFN- γ ⁺ T cells can be found in the CNS of symptomatic animals and can induce MHC class II expression within the CNS, upregulate chemokine production to subsequently recruit innate immune cells, activate macrophages, or mediate cell death directly (104). Yet, IFN- γ deficient mice still develop EAE and display massive infiltration complete with lymphocytes, macrophages, and granulocytes (105). On the other hand, TNF knock-out mice exhibit delayed disease onset and severe EAE resolved faster than in wild type mice, while leukocytes were unable to form perivascular cuffs in the CNS (106).

More recently the role of CD4⁺ T_H17 cells in pathogenesis has been recognized. There are increased numbers of circulating T_H17 cells in MS patients experiencing an acute relapse (107). Furthermore, in acute lesions, genomic profiling of infiltrating T cells revealed the upregulation of TCR α and β chains, retinoic acid receptor-related orphan receptor γ (ROR γ) transcription factor, and cytokines that mediate T_H17 cell expansion (108). Elevated levels of IL-17 and numbers of IL-17⁺ T cells can be found in the blood, CSF, and active lesions (109, 110). T_H17 cells have the ability to open the blood-brain barrier, cause axonal damage and neuronal death, and recruit other lymphocytes to the CNS (111), providing further evidence that this subset plays a role in disease progression. The role of CD4⁺ T_H17 cells in EAE has also been thoroughly investigated. T_H17 cells

were shown to be expanded by IL-23, and are pathogenic in passive transfer of EAE distinct from T_H1 cells (112). IL-17 regulates chemokine expression in the CNS during EAE, and anti-IL-17 antibodies reduce disease severity (113). Unfortunately, targeting this cell subset in MS patients using an IL-12/IL-23 p40 neutralizing antibody was unable to reduce the cumulative number of lesions in patients (114).

It has been suggested that T_H1 and T_H17 cells elicit different forms of encephalitis, further defining the role of CD4⁺ T_H cell subsets. For example, while the avidity of TCR and MHC interaction may determine the ratio of T_H1 to T_H17 cells produced systemically, only T_H17 cells mediate parenchymal inflammation while both T helper subsets infiltrate the meninges (115). In support of this idea, passive transfer of EAE following *in vitro* polarization of cells with either IL-12 or IL-23 yields clinically indistinguishable disease, but histology and immune profiling suggest two very different pathologies (116). Others have proposed a more cooperative role in which T_H1 cells have an increased capacity to enter the non-inflamed CNS, and in turn recruit T_H17 cells (117).

A role for T_H2 cells has also been established in EAE, where they are thought to be protective, as their induction using IL-4 results in improved clinical disease, reduced demyelination, and inhibited proinflammatory cytokine expression in the CNS (118). While signal transducer and activator of transcription (STAT) 6 deficient mice, which display a T_H1 bias, develop severe EAE, STAT4 deficient mice, which display a T_H2 bias, are protected (119). However, disruption of the IL-4 gene does not affect the course of EAE, suggestive of redundant roles for this cytokine (120).

Alternatively, immunoregulatory CD4⁺ T (Treg) cells, which can suppress T cell proliferation and function via cell to cell contact and cytokine-mediated mechanisms (121), show a significant dampening of their effector function when comparing cells from the peripheral blood of MS patients to healthy controls (122). Treg-1 cells, which are defined by high levels of IL-10 production, produce less IL-10 when isolated from MS patients and cannot suppress proliferation

following stimulation of CD4⁺ T cells (123). Additionally, high avidity myelin-specific T cells that encounter their antigen in the periphery are induced by Treg cells to adopt an anti-inflammatory phenotype characterized by high levels of IL-10 and TGF- β production (124). The importance of IL-10 is demonstrated in mice in which IL-10 deficiency exacerbates EAE due to increased IFN- γ and TNF- α production, while increased IL-10 expression prevents disease (120). It remains to be determined if Treg cells exert their effects primarily in the periphery or within the CNS. A high proportion of CD4⁺ T cells within the CNS express IL-10 during remission of disease and these cells can inhibit proliferation *in vitro* (125). It is possible that they mediate T cell apoptosis as a mechanism to eliminate them from the CNS compartment in the white and grey matter, as demonstrated by several forms of EAE (126). The role of Tregs is supported by GWAS studies that implicated CD25 in MS risk (36).

1.6.4. CD8⁺ T cells

Although our current understanding of MS continues to position CD4⁺ T cells as the central mediators of disease, the role of cytotoxic CD8⁺ cells cannot be neglected. In addition to CD4⁺ T cells, CD8⁺ T cells from patients with MS target several CNS autoantigens, to a greater extent than healthy individuals, and CD8⁺ T cell responses from MS patients are functionally distinct (127). Post-mortem examination of active demyelinating lesions in the brain, as well as the CSF and blood, reveal significant infiltration and clonal expansion of CD8⁺ T cells (128, 129). Indeed, CD8⁺ T cells are up to ten times more prevalent in MS brain tissue than CD4⁺ T cells (128, 130). Memory CD8⁺ T cells show an increased response to MBP peptides presented by HLA-A2 *in vitro*, and produce large amounts of IFN- γ and TNF- α (131).

It is important to recognize that, except for microglia, resident CNS cells do not express MHC class II. However, higher levels of MHC class I can be induced on neurons and oligodendrocytes, particularly following cytokine stimulation or during viral infections (132, 133),

providing the basis for interaction with CD8⁺ T cells. Oligodendrocyte cell death is believed to occur by apoptosis triggered by either Fas/Fas-ligand interactions or perforin and granzyme secretion (134, 135). Conversely, apoptosis induced in neurons may be predominantly mediated by Fas/Fas-ligand binding (136).

The encephalitogenic potential of myelin-specific CD8⁺ T cells has been verified in EAE. MOG-specific CD8⁺ T cells induce massive demyelination and autoreactive cells can still be detected many months after transfer (137). MBP-specific CD8⁺ T cells can also transfer disease in recipient mice and infiltration and demyelination are located primarily in the brain rather than the spinal cord, a similarity to MS not observed in most EAE models (138).

1.6.5. B cells

The function of B cells in encephalitis has been difficult to determine due to the fact that many EAE models lack a prominent B cell component. In MS, B cells and mature plasma cells can be detected in plaques and in areas of active demyelination (139). B cells have been shown to establish follicles within the meninges during SP MS and likely drive intrathecal antibody production. These follicles are associated with younger age at disease onset, irreversible disability, more pronounced demyelination, and microglia activation (140). A continuous humoral response within the CNS may explain elevated IgG levels and the presence of oligoclonal bands following electrophoresis of MS patient CSF, both of which are often used in the diagnosis of disease.

Oligoclonal bands in the CSF are suggestive of a scenario in which a limited number of B cell clones contribute to disease. Indeed, autoantibodies to myelin proteins such as MBP are detected in plaques and areas of active demyelination (141). In addition to those targeting MBP, anti-MOG antibodies are elevated in the serum of MS patients and have been shown to induce death of MOG-expressing cells *in vitro* (142, 143). Anti-MOG antibodies appear to be more prevalent among patients with very early onset MS, especially patients less than ten years of age, and these

purified antibodies bind myelin and glial cells in the human CNS (144). Antibodies bound to myelin could facilitate demyelination by macrophage-mediated phagocytosis or through the binding and activation of complement. The relevance of other non-myelin antigens remains an outstanding question. A number of potential targets have been described, such as $\alpha\beta$ -crystallin, an anti-inflammatory heat shock protein abundant in active lesions that is targeted by antibodies in both the CSF and serum of MS patients (145).

However, the importance of these antibodies in disease progression remains in question, as only a small proportion of them have demonstrated pathogenicity (146). Antibodies targeting MBP and MOG were thought to correlate with MS progression after an initial demyelinating event (147) but this finding was not reproduced and their role as a disease predictor remains uncertain (148). Studies in animals have also produced conflicting results. Intravenous administration of MOG-specific autoantibodies at disease onset may increase passive EAE severity (149), yet B cell deficient mice show more rapid disease onset and increased severity compared to controls, suggestive of an immunomodulatory role (150). Furthermore, while immunization in mice with myelin antigens can result in the induction of an antibody response, EAE cannot be generated by antibodies alone and antibody is not required for the transfer model (151).

The importance of infiltrating B cells could also lie in their ability to serve as antigen presenting cells (APC) for autoreactive T cells and provide re-stimulation. This role in pathogenesis is supported by the anti-CD20 B cell depleting monoclonal antibody Rituximab. Encouragingly, a clinical trial using Rituximab dramatically reduced both lesions and relapses in RR MS patients (152). The known lack of effect of Rituximab on plasma cells, as well as the speed of results, suggested that the effect was due to altered antigen presentation to T cells or cytokine production, rather than decreased autoantibody production.

1.6.6. Innate immune system and microglia

The innate immune system is thought to play a fundamental role in the initiation of disease in the periphery, as well as in the progression of disease alongside its CNS counterparts at sites of lesions. Dendritic cells are not always included in the scope of studies examining MS pathology. However, as the most effective APC within the periphery, dendritic cells play a role in the activation of pathogenic lymphocytes. SP MS patients have an increased percentage of CD80-expressing IL-12- and TNF- α -producing dendritic cells but a decreased percentage expressing programmed cell death 1 ligand 1 (PD-L1) compared to RR MS patients or controls. A higher percentage of dendritic cells from SP MS and RR MS patients also express CD40 compared to controls (153). Dendritic cells from MS patients have been shown to secrete elevated levels of IL-23, and *in vitro* inhibition of IL-23 and IL-12 gene expression was associated with increased IL-10 and decreased TNF- α production (154).

CNS-infiltrating macrophages, differentiated from precursor monocytes, as well as resident microglia, are present in large quantities in demyelinating lesions. Indeed, microglia activation may be a key event in MS. It is known to occur very early in lesion development through examination of the brain tissue of MS patients who died during the onset of a relapse or shortly thereafter (155). Macrophages, in addition to microglia, express an activated phenotype, and are capable of producing a broad spectrum of cytokines such as TNF- α that can inhibit the growth and branching of neurites *in vitro* (156). Macrophages and microglia also contribute to T cell reactivation within the CNS through MHC class II and TCR interactions. For example, mice with CD40 deficient microglia show reduced EAE severity and an inability to maintain a robust anti-myelin response within the CNS (157). Phagocytes are also equipped with the enzymatic machinery to generate damaging reactive species such as nitric oxide (NO). NO is found in high concentrations within inflammatory lesions and is highly expressed by macrophages and microglia (158, 159). It is

possible that NO affects MS progression through a number of mechanisms including disruption of the BBB, direct injury to oligodendrocytes, and axonal degeneration or impaired conduction (160).

The role of natural killer (NK) cells in MS remains controversial. An inverse correlation between NK cell cytotoxic activity and disease has been reported (161). Interestingly, NK cell-mediated lysis appears to fluctuate depending on the phases of disease, becoming reduced prior to and during acute attacks, but remaining normal during stable remissions (162).

1.6.7. Axonal loss and neurodegeneration

In addition to demyelination, axonal loss is an important feature of a typical lesion (163, 164). Axonal loss can occur through a number of mechanisms, including direct transection by CD8⁺ T cells (165) followed by neurodegeneration that results in the further loss of axons (166). CD4⁺ T_H17 cells have also been demonstrated to play an integral role in this process (167). As neurons compensate for altered conduction velocities, there is an increased entry of sodium into cells leading to eventual slowing of nerve conduction. This is followed by a reversal in sodium-calcium exchange, expelling sodium and bringing calcium into the cell in large quantities, leading to calcium-mediated injury. Increased glutamate production by cells in proximity to dystrophic axons also promotes entry of calcium into oligodendrocytes and neurons in a process termed glutamate excitotoxicity (168). Finally, macrophage-derived NO can also induce focal axonal degeneration via energy failure believed to be a result of mitochondrial dysfunction (169).

The relationship between neurodegeneration and demyelination remains uncertain, due to the lack of a strong correlation between inflammation and neurodegeneration. It is possible that axonal injury may occur independently.

1.6.8. Regeneration

The CNS, like many other tissues, has the capacity for limited regeneration. This process parallels inflammation and phagocytosis, but also occurs in later progressive phases when

inflammation is less prominent. Following CNS damage, oligodendrocyte progenitor cells migrate to the sites of lesions and extend processes towards demyelinated axons in an attempt to remyelinate the neurons (170). This process can be very effective, particularly in early disease stages, with an estimated 20% of MS patients displaying plaques that show evidence of remyelination (171). Nevertheless, the original thickness of compact myelin is not restored and conduction velocities remain subnormal. Repaired myelin also differs from mature myelin in its composition, containing a different abundance of myelin protein isoforms or post-translational modifications (172). This process of remyelination accounts for the appearance of shadow plaques in MS patients. Neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and insulin-like growth factor-1 (IGF-1), are produced by cells such as oligodendrocytes and may help to prevent axon loss and preserve myelination (173). While recruitment of precursor cells to lesions is not affected in MS patients, the ability of these cells to differentiate into myelin-producing oligodendrocytes remains problematic. Ultimately, remyelination is unable to fully compensate for extensive demyelination and disease course progresses (174).

1.7. Therapies

MS has proven to be a very challenging disease to treat, despite decades of intensive research, with very few drugs proven to effectively decrease relapses and slow overall progression. Given the role of the immune system in MS pathogenesis, it is not surprising that most therapies target immune function, in various phases ranging from activation, to trafficking, to effector function. Early drugs such as cyclophosphamide and prednisone served as proof of principle of immune involvement and had some efficacy in limiting disease progression (175, 176). These drugs may still be used to treat active relapses, but due to their side effects, more specific drugs are required. Most investigational therapies continue to focus on the T cell, but as an appreciation for processes such as neurodegeneration has grown, additional avenues have become appealing (177).

Unfortunately, a lack of significant progress in the management of MS, coupled with the debilitating nature of disease, has led to desperate, often unsupported, attempts to treat disease (178). Currently, the role of cerebrospinal venous insufficiency is being evaluated. However, results show very low specificity of obstructed veins in MS patients, and early clinical trials suggest little effect following surgery (179-182).

This section will focus on only some of the major approved therapies for MS. It should be noted that treatments often define success by the reduction of relapses in patients. However, whether relapses are related to, or are independent of, overall disease progression remains an ongoing debate (11, 183), and this should be considered when examining the achievements of current therapies. The ideal therapy for MS should halt progression, reverse neurological deficits, and prevent disease.

1.7.1. Glatiramer acetate

Glatiramer acetate (GA), also known as Copaxone, consists of a random mixture of L-amino acids tyrosine, lysine, alanine, and glutamic acid. These amino acids combine to form peptide chains that average in length from 40 to 100 residues. GA was initially designed to resemble MBP, a suspected autoantigen at the time. Surprisingly, GA exhibited protective properties rather than initiating encephalitis following immunization (184). The rather non-toxic GA subsequently underwent clinical testing in the following decades (185, 186), showing success in decreasing relapses (187), and was eventually approved in 1995 for the treatment of MS.

The mechanism of action of GA remains a mystery, though most theories focus on alterations in autoreactive T cell populations. GA binds MHC class II and subsequently may affect presentation to T cells (188, 189). Modification of the affinity of interaction between the APC and T cell can lead to a T_H2 skewed response in which anti-inflammatory cytokines and neurotrophic factors are released (190-192). GA is not believed to accumulate in the CNS at high concentrations, and therefore this process may be working in the periphery. In this scenario, T_H2 cells would then

migrate to the CNS alongside pathogenic cells and dampen inflammation through mechanisms of bystander suppression such as cytokine secretion. Additionally, GA can generate an increased number of FoxP3⁺ Treg cells (193) and exposure to GA causes Treg cells to secrete higher levels of IL-10 (194). These observations serve as potential explanations behind the therapeutic effects of GA. Unfortunately, even in those patients with a definitive response to therapy, drug efficacy eventually wanes.

1.7.2. Interferon- β

Interferon- β (IFN- β) is a pleiotropic cytokine with antimicrobial and anticancer properties. IFN- β was first shown to have efficacy in the treatment of MS with the publication of two landmark reports (195, 196). These studies showed that interferon- β -1b (IFN- β -1b) was effective in reducing the rate of exacerbations in RR MS. This was accompanied by a decrease in the number and frequency of lesions as assessed by MRI and follow-up showed a one-third reduction in exacerbation rate with no significant increase in lesion burden up to five years following initiation of treatment (197). An additional clinical trial, which used another form of recombinant IFN- β , interferon- β -1a (IFN- β -1a), also showed efficacy (198) and further substantiated the ability of IFN- β to limit MS pathology. Following these results, IFN- β was approved for the treatment of RR MS.

The mechanism of action of IFN- β remains unknown. It was initially chosen as a therapeutic because it possesses anti-viral activity that was predicted to reduce the frequency of suspected virus-precipitated relapses. However, additional data demonstrated that IFN- β can skew immune responses towards an anti-inflammatory phenotype. IFN- β inhibits the proliferation of T cells and reduces the production of IFN- γ and its consequent upregulation of MHC class II (199). PBMC from patients with MS stimulated *in vitro* with MBP secrete greater quantities of IL-4 and IL-10 in the presence of IFN- β (200). IFN- β can also downregulate the production of IL-17 (201), and reduces IL-23 mRNA expression in PBMC (202), suggesting that the action of IFN- β may also

involve T_H17 cells. Additionally, IFN- β downregulates the expression of adhesion molecules on T cells, impairing their ability to bind to the endothelium and extravasate into the CNS to cause damage (203, 204). MS patients receiving IFN- β treatment have decreased serum levels of MMP-9, which correlate closely with improvements in relapse frequency, disease score, and lesions detected by MRI (205). IFN- β may therefore act at several levels of immune activation, including antigen presentation, systemic cytokine profiles, and trafficking into the CNS.

1.7.3. Anti-VLA-4

The integrin $\alpha_4\beta_1$, also known as very late antigen-4 (VLA-4), is an integrin dimer expressed on the surface of all leukocytes except neutrophils. VLA-4 mediates adhesion to endothelial surfaces by binding to its receptor VCAM-1 (206). In MS, this process is crucial in allowing cells to cross the BBB. Activated autoreactive leukocytes expressing high levels of VLA-4 interact with activated endothelium expressing VCAM-1, resulting in diapedesis.

The efficacy of a monoclonal antibody against VLA-4, Natalizumab, was assessed in two early independent clinical trials, one of which involved co-administration with IFN- β -1a (207-209). These studies showed that Natalizumab reduced the progression of disability, decreased the rate of relapse, and reduced the number of lesions in MS patients. Unfortunately, dosing of Natalizumab was suspended by the manufacturers when three cases of progressive multifocal leukoencephalopathy (PML) were identified (210) and later determined to have been caused by reactivation of latent John Cunningham virus. As of recently, a total of thirty five cases of PML had been recorded and the risk to those being treated was estimated to be 1 in 1000 (211). These adverse effects continue to limit the use of Natalizumab as a therapeutic for MS. Natalizumab is available to treat highly active RR MS with a high incidence of recent relapses, and often only in patients who do not respond well to IFN- β .

2. Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is the primary animal model used to study MS. It was first described almost a century ago following injections of human spinal cord extract into rabbits (212), and a short time later in monkeys (213). The impetus for the creation of EAE was the encephalitis and polyneuritis produced in people immunized with a rabies vaccine that contained spinal cord material. The robustness of EAE has been improved upon many times, including the introduction of an adjuvant in addition to the spinal cord homogenate (214). Since these early experiments, EAE has been adapted to mimic almost all clinical features of MS, including progressive disease, relapsing disease, and disease with opticospinal involvement (146). A number of variables, including the high reproducibility of disease, short time course, and suitability of multiple animals and strains, have allowed a myriad of studies on EAE to be published in a relatively short period of time. EAE has consistently accounted for approximately 20-30% of all MS-related publications over several decades (215).

2.1. Induction and disease course

In general, EAE is induced following subcutaneous injection of a relevant CNS autoantigen emulsified in Complete Freund's Adjuvant (CFA). CFA contains a combination of mineral oil and *Mycobacterium tuberculosis* strain H37Ra that is highly immunogenic when emulsified with antigen. It triggers a T_H1 and T_H17 response depending on cytokine microenvironments within the draining lymph nodes, but not a T_H2 response. Immunization is often accompanied by intraperitoneal injections of pertussis toxin (PTX). EAE can also be passively transferred following injection of peripheral autoreactive lymphocytes into recipient mice, indicative of their pathogenicity.

The EAE model has allowed for the rapid identification of encephalitogenic proteins in myelin, as well as their immunodominant peptides, rather than earlier whole spinal cord homogenate preparations. These proteins are both major and minor components of myelin, and include MBP, PLP, MOG, myelin-associated glycoprotein (MAG), and S100 calcium binding protein β (S100 β).

Due to the artificial induction of EAE, efforts have been made to establish a spontaneous model of disease. These attempts often focus on alterations to the MHC-TCR interaction. For example, transgenic mice expressing a humanized HLA-DRB1*15 and anti-MBP₈₅₋₉₉ TCR develop spontaneous T cell infiltration in the CNS and resulting paralysis (216). Alternatively, approximately 35% of MOG-specific TCR transgenic mice develop spontaneous optic neuritis (217). If both TCR and B cell receptor (BCR) are considered together, for example in mice engineered to express a TCR recognizing MOG₃₅₋₅₅, and the heavy chain of a BCR recognizing a conformational epitope on MOG, 50% of mice develop chronic disease, characterized by infiltrates and lesions in both the spinal cord and optic nerve (218). Spontaneous T cell priming in all of these transgenic mice likely results from a unique scenario of uncontrolled activation, made possible through increased co-stimulatory signals or a paucity of Treg cells.

The course of EAE depends on the animal species, strain, and protein or peptide used to induce disease. The encephalitogenic PLP₁₃₉₋₁₅₁ peptide induces a relapsing remitting EAE in SJL mice, whereas MOG₃₅₋₅₅ triggers a chronic progressive EAE in C57BL/6 mice. These two models are most often used for transgenic studies due to the widespread use of these strains of mice, though EAE can also be induced in other strains or species such as the non-obese diabetic (NOD) mouse and the Lewis rat. Although one model of EAE does not encompass the entire pathology of MS, each aspect of MS can be found in one of the many EAE models.

EAE is acute and symptoms appear within days of immunization. This is contrasted by MS, in which the appearance of symptoms occurs subtly after a much greater period of time and after significant alterations in the CNS have occurred. Though disease course can vary significantly based on the immunization protocol, symptoms in EAE are quite similar. Mild symptoms include weight loss, fatigue, limp tail, and an abnormal righting reflex, while more severe symptoms include unilateral or bilateral paralysis in the hind limbs, ascending paralysis to the forelimbs, and death. Some EAE models report additional symptoms such as tremor, ataxia, and spasticity.

Based on these indications, scoring systems for disease severity were created and continue to evolve. Unfortunately, no universal measure is used in the EAE field, and existing scales vary from between five and ten points. More complex scoring systems with smaller intervals between points have more power to detect mild disease or discrete changes in symptoms.

2.1.1. Myelin oligodendrocyte glycoprotein

MOG is a 218 amino acid member of the immunoglobulin superfamily (219). The sequence of MOG is highly conserved among species, with the greatest area of diversity occurring in the transmembrane domain (220). It is encoded for on chromosome 6p22.1 in close proximity to the distal end of MHC class I (221). MOG contains two hydrophobic domains, a possible intracytoplasmic phosphorylation site, and undergoes glycosylation *in vivo*.

The function of MOG remains unknown, although it has been hypothesized that MOG serves as an adhesion molecule that acts during later stages of myelination to form contacts between fibres. Its expression on the surface of myelin and membership in the Ig superfamily support this view (222). Myelin sheaths in the periphery, which lack expression of MOG, do not exhibit similar close contact compared to those in the CNS. Alternatively, it has been proposed that MOG is directly involved in the immune response. MOG from the CNS, but not the peripheral nervous system (PNS), effectively binds to C1q in a region close to the Ig binding site (223, 224).

MOG composes a relatively minor component of myelin in comparison to MBP or PLP. Nevertheless, it is able to induce one of the most robust and reproducible forms of EAE. The most encephalitogenic portion of MOG is contained in the extracellular, Ig-like domain ranging from amino acids 24 to 98. More specifically, the N-terminus domain of MOG is effective in inducing EAE in SJL mice and the Lewis rat, while the C-terminus domain induces EAE in SJL, C57BL/6, and NOD/Lt mice (225).

2.1.2. *Pertussis toxin*

Pertussis toxin is one of several toxins produced by the bacterium *Bordetella pertussis*, the pathogen that causes whooping cough. It consists of A and B subunits, of which A is enzymatically active while B dictates receptor binding (226). The physiological receptor for PTX remains unknown, however several candidates have been proposed. Upon binding, PTX is thought to enter the cell directly through interactions between its hydrophobic N-terminus and phospholipids of the cell membrane. Following its cleavage, intracellular PTX can then irreversibly ADP-ribosylate the α subunit of G proteins (227). G proteins are central to the conversion of extracellular signals to intracellular responses. To mediate these signals, G proteins reside on the intracellular face of the cell membrane in heterotrimeric complexes, consisting of an α , β , and γ subunit. Activation leads to the dissociation of the α subunit, which then alters several effector enzymes or ion channels and their downstream secondary messengers (228). PTX has been shown to inhibit Gi or Go proteins specifically, due to their accessible ribosylation sites (229).

PTX was first employed in the murine EAE model, where it increased the incidence and severity of EAE (230), followed by Lewis rats, where it was shown to produce an acute form of disease (231). Several years later it was shown that PTX was associated with increased CNS vascular permeability during EAE, possibly through sensitization to the effects of histamine (232, 233). Alternatively, there is a large body of evidence that PTX is more likely involved in amplifying

the acute immune response with little direct effect on BBB and BSCB integrity. PTX has mitogenic effects on T cells similar to other molecules such as concanavalin A (234). It is also able to stimulate the production of IFN- γ and IL-2 by T cells, and increases the expression of co-stimulatory molecules such as CD28 on T cells and B7-1 and B7-2 on antigen presenting cells (235). PTX effects appear to be dependent on toll-like receptor (TLR) 4, since lymphocyte rolling and adhesion does not occur following PTX injection in TLR4 deficient mice, and since PTX upregulates signalling pathways common to the well-known TLR4 agonist, lipopolysaccharide (236). In fact, PTX is superior to several TLR ligands in its ability to induce T cell proliferation and T_H1 polarized responses (237).

2.2. *Is EAE a good model for MS?*

EAE is the leading animal model for MS and has allowed for many insights into the pathogenesis of organ-specific autoimmune disease. EAE is mediated by a pathogenic autoimmune response, which is not surprising given that it is induced using an inflammatory immunization. Important early experiments showed that thymectomy of rats at birth rendered them resistant to developing EAE later in life (238), while adoptive transfer of lymph node cells from immunized mice into naïve recipient mice resulted in encephalitis (239). Cells in the periphery have similar cytokine signatures and proliferative capacities as those that infiltrate the CNS, and their avidity to antigen is not enriched once in the CNS (240), demonstrating the importance of the BSCB and BBB. Importantly, pathogenesis shows many similarities to MS, as activated myelin-specific T cells must transit from the periphery into the CNS, undergo reactivation, and trigger further innate immune cell recruitment and demyelination.

A major criticism of EAE is that it relies too heavily on inflammatory processes, and does not address the pathophysiological mechanisms that underlie neurodegeneration. However, other models of disease which are induced by toxic agents or induced by viruses also have many

shortcomings. For example, the toxic cuprizone model causes demyelination but does not involve inflammation or axonal damage, while Theiler's murine encephalomyelitis virus provides insights into how viral infections induce CNS damage but does not produce a robust and reproducible disease.

The strongest support in favour of the relevance of EAE is the fact that the majority of treatments for MS originate from EAE studies, and all currently approved drugs for MS also show efficacy in EAE. EAE allows for further research on established MS treatments to gain insight into the mechanisms of therapeutic action and to improve dosing regimens to decrease adverse events. However, this is not a trivial process. For example, given our understanding about the properties of IFN- γ and MS aetiology at the time, clinical trials employing IFN- γ administration began optimistically. But these trials proved unsuccessful when many patients experienced exacerbations rather than protection (241). TNF- α blockade also showed promise when transfer of EAE could be dramatically dampened using an anti-TNF- α /lymphotoxin antibody (242), and subcutaneous injection of a soluble TNF receptor prevented chronic relapsing EAE in SJL mice (243). Yet, results from EAE studies again did not translate in humans, and TNF neutralization increased the frequency and severity of exacerbations (244). EAE is also unable to predict all toxicities and side effects of new therapies. This was illustrated by the therapeutic anti-VLA-4 monoclonal antibody Natalizumab, which showed efficacy in EAE (245) but had unpredictable adverse effects in some MS patients.

Therefore, though EAE has proven to be a very useful model for MS, it is important to understand its limitations when translating EAE research to MS treatments. Similarities and differences between MS and EAE are highlighted in **Table 1.1**. Despite some differences, it cannot be refuted that EAE has been invaluable to our understanding of MS pathogenesis and for the treatment of patients with MS (246).

	<i>EAE</i>	<i>MS</i>
Disease Course	<ul style="list-style-type: none"> • Relapsing-remitting and progressive forms of disease • Acute, develops over days • Induced 	<ul style="list-style-type: none"> • Relapsing-remitting and progressive forms of disease • Long-term, develops over years • Spontaneous
Pathology	<ul style="list-style-type: none"> • Lesions predominantly in the spinal cord; symptoms largely related to motor function • Inflammation and demyelination are most prominent 	<ul style="list-style-type: none"> • Lesions predominantly in the brain; symptoms vary greatly based on lesion location • Inflammation, demyelination, axonal loss, and neurodegeneration
Genetics	<ul style="list-style-type: none"> • Inbred, genetically similar animals • MHC class II linked to risk of disease • Several risk loci determined through breeding and fine mapping • Increased female susceptibility is dependent on strain 	<ul style="list-style-type: none"> • Outbred, genetically diverse people • MHC class II linked to risk of disease • Several gene candidates detected by GWAS • Increased female susceptibility in RR MS
Environment	<ul style="list-style-type: none"> • Environmental inputs important, such as colonization with bacteria 	<ul style="list-style-type: none"> • Possible association with viruses and nutrients such as EBV and vitamin D
Immune Response	<ul style="list-style-type: none"> • Clonal myelin-specific lymphocytes • CD4⁺ and CD8⁺ T cells found in lesions • Role of B cells unknown but likely contribute to disease severity • Complement deposition 	<ul style="list-style-type: none"> • Clonal myelin-specific lymphocytes • CD8⁺ T cells dominate in active lesions • Importance of B cells attributed to APC function or cytokine secretion • Complement deposition
Treatment	<ul style="list-style-type: none"> • Many approaches are effective, most of which are immunosuppressive or immunomodulatory 	<ul style="list-style-type: none"> • Few drugs show efficacy, most of which are immunosuppressive or immunomodulatory

Table 1.1 – Similarities and differences between EAE and MS.

3. Transient Receptor Potential Vanilloid-1

The sensory nervous system is responsible for detecting and communicating environmental stimuli. Sensory neurons are present peripherally in almost all organs and the proteins they express equip them with specialized receptive properties. A major subtype of sensory neurons, nociceptors, have the unique ability to respond to a broad range of dangerous physical and chemical stimuli, though notably only when the stimulus is capable of causing tissue damage (247). A protein central in this detection is transient receptor potential vanilloid-1 (TRPV1), and its activation has been linked to numerous pathologic conditions.

3.1. Structure

TRPV1 is a member of the TRP ion channel family first identified in *Drosophila melanogaster*, which leads to blindness in flies when mutated (248). Following cloning of the TRP locus, it was determined that this family consists of intracellular N- and C-terminus, and a pore structure within the cell membrane. The pore structure is formed by six transmembrane domains and one intramembrane domain (249). TRPV1 contains a lengthy N-terminus containing three ankyrin-repeat domains, and a C-terminus containing a TRP domain (250), while the entire protein is 838 amino acids in length. TRPV1 is believed to form functional homotetramers, although more recent evidence also supports heteromer formation involving TRPV2 and TRPV3 (251, 252).

3.2. Expression

Nociceptor cell bodies are found in the trigeminal, nodose, and dorsal root ganglia, from which they project axons centrally to the dorsal horn of the spinal cord. TRPV1 expression can be robustly detected in these locations, particularly the dorsal root ganglia (DRG) (253, 254). These nociceptive neurons can be both C fibers or A δ fibers, which differ in their diameter, myelination, and consequently their conduction velocities. TRPV1 is predominantly expressed on a large

population of C fibers which also contain the immune-modulating peptides substance P (sP) and calcitonin gene-related peptide (CGRP) (255). Expression is not located within a particular region of the neuron, but rather it is expressed ubiquitously throughout.

Peripheral expression of TRPV1 has been the subject of intensive research, whereas TRPV1 expression in the CNS has only been confirmed within the last decade through the advent of improved technologies and reagents. Within the CNS, TRPV1 is expressed in the spinal cord dorsal horn and within areas of the brain. Other groups have suggested TRPV1 expression in cell types other than neurons, such as microglia and astrocytes, and that activation of TRPV1 in these cells can mediate cell toxicity or production of reactive species (256-258). TRPV1 expression has also been demonstrated within hematopoietic cells (259, 260), though this is not conclusive (261). The function of central TRPV1 remains poorly understood. Expression patterns suggest that it might play a role in pain processing and/or thermoregulation.

TRPV1 expression is altered in several diseases such as irritable bowel syndrome and diabetic retinopathy (262, 263). Inflammation or tissue injury has been shown to increase the number of non-myelinated C fibers that express TRPV1 (264). These conditions are associated with pain likely in part due to increased TRPV1 firing.

3.3. Activation and function

For many years, the vanilloid compound capsaicin was known to activate sensory neurons (255, 265). Physiological recordings showed that capsaicin could depolarize neurons by promoting the influx of cations (266, 267). However, it remained uncertain if the capsaicin receptor had intrinsic ion channel activity, or if it promoted ion influx following downstream signalling. It was later determined that capsaicin binds selectively to TRPV1, following the transfection of cells with complementary deoxyribonucleic acid (cDNA) pools from a DRG library and subsequent monitoring for intracellular calcium influx (253). A structurally-related vanilloid, resiniferatoxin

(RTX), also binds TRPV1 and exhibits an even greater potency for activation (268). Both capsaicin and RTX have been instrumental in our understanding of TRPV1 function, yet TRPV1 is a unique ion channel given its ability to also integrate a number of chemical and physical stimuli apart from binding vanilloid compounds. TRPV1 is activated by temperatures greater than 43°C, a threshold that closely correlates to heat-evoked pain sensation in mammals (269). Additionally, protons that reduce extracellular pH to below physiological levels of 7.6 can open the TRPV1 channel (270). This decrease in pH can occur following tissue damage associated with inflammation or ischemia. Temperature and pH can cooperate to activate TRPV1 in conditions where the channel is normally closed (269). Finally, endogenous lipids with a similar structure to vanilloids may activate TRPV1 (271, 272), in addition to animal venom (273).

Upon activation, TRPV1 transports divalent cations such as Ca^{2+} into the intracellular space, while transport of monovalent cations such as Na^+ , K^+ , Li^+ , Cs^+ , and Rb^+ , protons, and large polyvalent cations occurs in a more selective manner (267). Depolarization of TRPV1⁺ neurons leads to afferent signal transduction, relayed through sensory ganglia including the DRG, from peripheral tissues to the CNS via eventual vesicular glutamate release. Additionally, peptidergic TRPV1⁺ neurons that contain the neuropeptides sP and CGRP are uniquely able to efferently secrete these proteins at synapses, a process that increases plasma extravasation and vasodilation, and promotes inflammation.

The effects of capsaicin are notable when administered at high systemic doses shortly after birth, resulting in selective and irreversible C fiber loss that renders these animals unresponsive to TRPV1 stimulation as adults (255). This persistent desensitization may result from cellular toxicity due to prolonged calcium influx or a lack of sufficient survival signals from factors such as NGF. However, these mice may retain some remnant TRPV1 expression in the periphery and in particular, the CNS. Two independent groups simultaneously created knock-out mice by genetically ablating

TRPV1 (274, 275). These mice are viable, fertile, and have unaltered sensory ganglion development, though altered inflammatory and thermogenic responses are observed in TRPV1^{-/-} mice following injection of capsaicin. These mice conclusively demonstrate that TRPV1 signalling has important effects on nociception, inflammation, and temperature regulation.

Due to their central role in pain transduction, intensive efforts have been devoted to the creation of TRP family agonists and antagonists that could potentially treat hyperalgesia and other disorders such as urinary incontinence. Given that TRPV1 modulators influence the pain response and the regulation of body temperature, it is a challenge to provide desensitization and reduce acute pain while limiting hyperthermia (276). Further complicating this challenge, TRPV1 agonists and antagonists such as capsaizepine are less selective at high concentrations, also activating or inhibiting other members of the TRP family (277, 278).

Under pathological conditions such as asthma, T1D, inflammatory bowel disease (IBD), and arthritis (279-281), dysregulated neuropeptide release in TRPV1⁺ neurons is believed to contribute to pathogenesis, representing a connection between the immune system and the nervous system that could potentially be exploited for the development of new therapeutics. The role of TRPV1 in other diseases such as MS also warrants further attention.

4. Type 2 Diabetes

Diabetes Mellitus encompasses a group of disorders characterized by the inability to control blood glucose levels. It can be further classified into subtypes, the most common being T1D and Type 2 Diabetes (T2D). More recently, CNS insulin resistance associated with, or a hallmark of, Alzheimer's Disease has been termed Type 3 Diabetes. Maintaining proper glucose homeostasis within tight dose and time limits requires precise coordination between numerous organs, including

the pancreas, brain, liver, muscle, and adipose tissue. Though each subtype of diabetes possesses a unique etiology, they ultimately converge on defects in insulin production and function, and glucose utilization. Complications from T2D are debilitating and affect the quality and length of life of patients. The rising incidence of obesity in the world makes T2D a huge financial threat to already struggling health care systems.

4.1. Diagnosis and clinical features

A number of symptoms are considered in addition to laboratory analyses in order to reach a conclusive diagnosis of T2D. Individuals with disease present with hyperglycemia, a result of poor use of available insulin or the eventual decrease in insulin secretion in later stages of disease. An individual is considered a T2D candidate when fasting plasma glucose values exceed 7.0 mmol/L or if non-fasting plasma glucose values exceed 11.1 mmol/L (282). A glycated haemoglobin A1c (HbA1c) concentration of 6.5% or higher is also useful in diagnosis (283). Additionally, plasma glucose spikes beyond 11.1 mmol/L following oral glucose challenge is a strong indicator of diabetic glucose homeostasis. Evaluation often also includes family history of T2D, past history of cardiovascular disease, and parameters such as blood pressure, serum cholesterol, and renal function.

Although patients become highly insulin resistant during T2D, insulin levels are not very useful in diagnosis, because they can range from decreased to normal or increased, depending on the stage of disease and the degree of adiposity. Unlike T1D, which is relatively acute and life threatening if left untreated, T2D develops over a longer period of time and can be asymptomatic for many years before diagnosis. In fact, in younger individuals T2D remains largely undiagnosed (284). T2D is usually preceded by a metabolic syndrome of variable length that mimics some or several T2D symptoms, but is definitely reversible, while T1D is not.

4.2. Prognosis

Following a diagnosis of T2D, a number of factors must be optimally controlled in order to manage disease and reduce long-term complications. This includes monitoring blood glucose and lipid concentrations, blood pressure, body weight, and screening for other health problems. Unfortunately, glycemic control becomes increasingly difficult as time progresses due to expansion of β -cell pools and then progressive β -cell failure. Diabetes is not often listed as the primary cause of death, but is a major contributor to mortality. Of the total excess global mortality, an estimated 5.2% is directly attributable to T2D, and this number is greater in developed nations (285).

Complications from T2D result from the toxicity associated with uncontrolled glucose levels, which affects both the micro and macro vascular systems. This results in substantial morbidity and mortality due to cardiovascular, renal, and visual system complications (286). Patients who are diagnosed in the prediabetes/metabolic syndrome stage, are at increased risk not only of diabetes but also have an 11% chance of developing serious cardiovascular disease within ten years (287).

In many developed nations such as Canada and the USA, diabetes is the leading cause of blindness among adults. It leads to approximately 40% of all end-stage renal diseases which exacerbate demand for kidney dialysis or transplant. T2D is also responsible for the majority of non-traumatic lower limb amputations and is associated with non-alcoholic fatty liver disease (288, 289). It has been suggested that some types of cancer may be more prevalent in T2D patients (290), though further research is required. Complications appear to vary based on race, with Asian populations suffering from a greater number of strokes and renal failure.

4.3. Epidemiology

The estimated worldwide prevalence of diabetes among adults was estimated to be 285 million people in 2010, representing a staggering 6.4% of the world adult population (291). T2D

accounts for the majority of these diabetes cases in North America and around the world: almost 90% compared to approximately 10% for T1D. Diabetes and stages of prediabetes can be regularly observed in almost every ethnic population in the world. In Canada, the incidence of T2D is estimated at approximately 600 per 100 000 people per year. In 2008, approximately 2.4 million Canadians had been diagnosed with a form of diabetes (289).

T2D onset occurs most often in adulthood. Approximately half of all individuals affected with T2D fall within the working ages of twenty five to sixty five, although T2D is typically diagnosed after the age of forty. Until recently, T2D was rarely diagnosed in children, though this is no longer true (284). Unfortunately, due to higher rates of obesity and decreased physical activity in youth, diagnosis in children and adolescents is rapidly increasing. Indeed, the incidence of T2D in young people now exceeds that of T1D in some ethnic groups (292), representing up to 80% of all pediatric diabetes cases (293).

A noticeable gender bias is not present in T2D, though there are striking differences between male and female patients when analyzing energy expenditure and storage. Males typically store fat in the upper body including the abdomen, while females store fat in the thighs due to lower basal fat oxidation (294). This correlates to larger visceral adipose tissue (VAT) depots in males and larger subcutaneous adipose tissue (SAT) depots in females.

The prevalence and incidence of T2D are rapidly increasing, almost certainly due to lifestyle changes given the short time frame of the T2D explosion. Without significant prevention and control programmes, this will continue to occur (295). By 2030, the prevalence of diabetes is expected to reach a staggering 439 million people or 7.7% of the world population, an increase largely contributed by developing countries (291). However, these numbers are believed to omit a large number of people who are diabetic but undiagnosed, possibly as many as one third of all diabetic individuals.

4.4. Genetics

T2D cases are complex and vary in their underlying molecular pathogenesis. Hence, identifying genetic variants that contribute to disease poses a significant challenge. The heritability of T2D is demonstrated by twin studies, in which monozygotic twins show concordance rates of up to 90% (296-298). Measuring concordance for T2D is a difficult undertaking, as many develop the disease at an older age (299). The strong genetic component of T2D can also be demonstrated by the offspring of a diabetic parent, who have close to a 40% chance of developing disease, compared to a risk in the general population of approximately 7%. If both parents have T2D this risk increases further (300).

Linkage analyses and focused candidate gene studies have been effective in identifying genes that are responsible for monogenic forms of T2D, and evidence is compelling. An estimated 1-2% of cases are inherited in Mendelian fashion, including maturity-onset diabetes of the young (MODY), insulin resistance syndromes, mitochondrial diabetes, and neonatal diabetes (301). For example, almost all cases of MODY can be explained by one of several mutations in genes encoding hepatocyte nuclear factors, insulin transcriptional regulators, or glucokinases (302, 303). Patients with monogenic disorders appear clinically similar to patients with common T2D, making them difficult to distinguish.

In the majority of T2D cases, a number of genes are thought to make contributions to increasing disease risk. Early studies examined those genes already implicated in monogenic diabetes, with some commonalities shown (304). However, the first definitive association with common T2D involved a variant of peroxisome proliferator-activated receptor- γ (PPAR- γ) (305). This mutation was shown to have modest effects, but occur at a high frequency and therefore substantially influence T2D risk. Variants of potassium inwardly-rectifying channel subfamily J member 11 (KCNJ11), an adenosine triphosphate (ATP)-sensitive potassium channel expressed by

β -cells that controls insulin secretion following potassium influx, also increases T2D risk to a similar extent (306). The application of GWAS rapidly expanded the number of gene candidates that influence T2D incidence. These studies confirmed prior links to the transcription factor TCF7L2, and implicated a number of other genes including cyclin-dependent kinases, additional hepatocyte nuclear factors, and the insulin receptor substrate-1 (IRS-1) (307-309). A large number of these genes relate to Wnt signalling, zinc transport, and cell cycle regulation, and their function within the β -cell emphasize the core role of β -cell physiology in disease. Consistently, genetic variants that predispose to T2D largely act through insulin secretion, rather than insulin action. It is also interesting that in multifactorial T2D, a large proportion of polymorphisms lie outside of coding regions, and are therefore believed to influence transcription rather than protein function (310). It should be noted that genetic variants differ between obese and lean individuals with T2D, but this is difficult to accurately detect and subclassify (311). In fact, these lean T2D patients may possess elements of latent autoimmune diabetes of the adult (LADA), a form of late onset T1D.

The majority of T2D susceptibility alleles are believed to have a low penetrance, increasing overall risk of disease by a small amount. The list of gene candidates continues to expand, as only 10% of the heritability of T2D can be explained by the susceptibility loci identified to date. The remaining heritability may be contributed by a large number of less common variants with a low allele frequency difficult to detect with current approaches. In addition, the discordance among monozygotic twins differs considerably in different environments, making it important to also consider how the surroundings of an individual affect disease incidence.

4.5. Environment

The environment plays a fundamental role in the incidence and progression of T2D, with dramatic effects. While intra-familial risk of T2D is increased, families and ethnic groups often share common behaviours. Indeed, risk is highest in families with at least two affected siblings,

regardless of parental disease status, suggestive of environmental influences (300). In mice, it has been suggested that one environmental factor could be the microbiota of the gut and their effect on innate immunity, as mice genetically deficient in TLR5 have an altered commensal flora and exhibit hyperlipidemia, hypertension, insulin resistance, and increased adiposity (312).

The rapid increase in T2D prevalence is a result of relatively recent changes in our diet and behaviour. Dietary shifts have occurred from foods that are typically unprocessed, low energy, and high in fibre, to diets that are more readily available, highly processed, high energy, and high in sugar and fat (313, 314). Concurrently, increased use of technology reduces energy expenditure and contributes to a sedentary lifestyle (315). The effect of these environmental factors on disease incidence is illustrated by the rise in diabetes following migration of isolated populations to urban centers (316).

More recently, it has been realized that fetal and neonatal programming may play significant roles in contributing to susceptibility to T2D. Children with low birth weight or intrauterine growth retardation are at greater risk of developing T2D later in life (317). In discordant pairs of monozygotic twins, it is often the twin with lower birth weight that develops disease (318). There is also evidence that gestational diabetes predisposes to diabetes and hyperglycemia in the offspring (319). Fetal malnutrition may alter metabolic pathways in favour of increased nutrient absorption and storage. Mechanisms are not well understood, though intrauterine programming likely involves epigenetic modifications.

4.5.1. Nutrient excess and obesity

Obesity is undoubtedly the greatest environmental risk factor for T2D. The growing diabetes epidemic is paralleled by an equally substantial obesity epidemic, and the two are often difficult to differentiate (320). More than 60% of T2D cases can be linked to obesity or periods of excessive weight gain (321, 322), and this number increases disproportionately in the severely obese (323).

Globally, about 1 billion people are now overweight or obese, outnumbering, for the first time in history, those who are underweight. In the USA, one of the most obese countries in the world, approximately two thirds of adults are overweight and one third are obese (324). This increase may finally be plateauing in developed countries, but continues in less developed countries (325). Of great concern is the number of obese children, estimated at approximately 10% worldwide and growing rapidly (326).

It has been shown that not all adipose tissue is equally pathogenic. Increased visceral deposits are associated with an increased risk of T2D (327), in addition to predisposing to cardiovascular disease (328). Alternatively, the distinct effects of subcutaneous fat are demonstrated following transplantation into the visceral cavity of mice, which results in a decrease in body weight, total fat mass, glucose levels, and insulin levels (329). These differences in the distribution and characteristics of fat depots may help to explain why many chronically overnourished and overweight individuals do not develop diabetes at all or develop it very late in life.

4.6. Pathogenesis

The regulation of whole-body glucose homeostasis is intricately controlled by insulin secretion and responsiveness by tissues such as the liver, muscle, adipose tissue, and β -cells. Under healthy conditions, intrinsic energy sensors within cells determine energy balance and regulate cellular functions through proteins such as phosphatidylinositol (PtdIns) 3-kinase (PI3K) and mammalian target of rapamycin (mTOR). Additionally, pancreatic β -cells secrete sufficient insulin in non-pathological conditions to meet constantly changing variations in demand.

T2D ultimately involves progressive β -cell failure following an increase in insulin demand. The need for high levels of insulin secretion results from insulin resistance in these key metabolic tissues. However, this greatly oversimplifies the pathogenesis of disease. T2D is heterogeneous and

results from the dysfunction of diverse molecular pathways. A diagram of the development of T2D is provided in **Figure 1.2**.

4.6.1. Insulin secretion and signalling

Insulin secretion in β -cells occurs downstream of glucose binding to the glucose transporter (GLUT) 2. The subsequent metabolism of glucose leads to the generation of ATP through mitochondrial oxidation, and results in the closure of ATP-sensitive K^+ channels. This leads to depolarization of the plasma membrane, the opening of Ca^{2+} channels, and release of insulin-containing granules (330).

Insulin signalling is then initiated following ligation of insulin with the insulin receptor on the surface of various cell types. Binding activates the endogenous tyrosine kinase activity associated with the β -subunit of the insulin receptor. The insulin receptor substrates (IRS) 1 and 2 are subsequently phosphorylated (331). In both muscle and adipose tissue, the phosphorylated IRS adaptor proteins bind to other proteins containing src homology 2 (SH2) domains, such as PI3K (332). PI3K is recruited to the plasma membrane and converts inositol phospholipid PtdIns-4,5-bisphosphate (PtdIns (4,5) P2) to PtdIns-3,4,5-trisphosphate (PIP3). Increased amounts of PIP3 then interact with protein kinase B (PKB), recruiting PKB to the plasma membrane where it is phosphorylated in two locations by phosphoinositide-dependent protein kinase 1 (PDK1) and mTOR. Phosphorylated PKB in turn phosphorylates the AKT substrate of 160 kDa (AS160) protein and glycogen synthase kinase-3 (GSK-3) (333), the latter resulting in a reduction in its activity.

In the end, glycogen synthase is activated due to the dephosphorylation of its serine residues normally targeted by active GSK-3, and conversion to glycogen ensues. Similarly, phosphorylation of AS160 inhibits its associated Rab GTPase-activating protein (GAP) domain, allowing for Rab protein-dependent translocation of GLUT-containing vesicles to migrate to the cell surface and

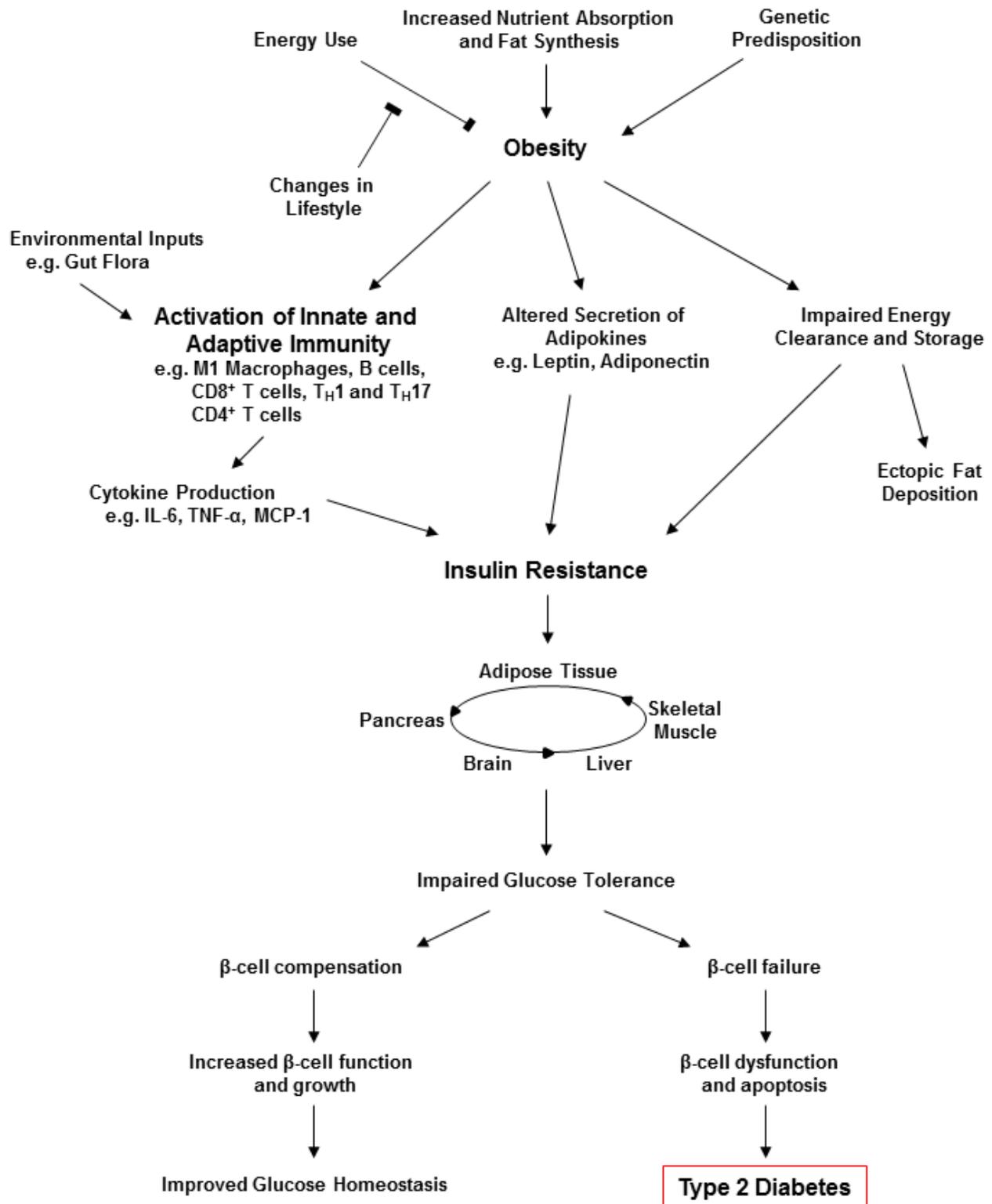


Figure 1.2 – Emerging relationship between inflammation, obesity, and T2D.

promote glucose uptake. Though expression of each GLUT isoform is tissue-restricted, GLUT4 predominates in both muscle and adipose tissue (334).

Following diffusion of circulating glucose through GLUT proteins, glucose is rapidly phosphorylated by glucokinase in the liver and hexokinase in other tissues to form glucose-6-phosphate, which can either enter the glycolysis or glycogen pathways. Insulin signalling is terminated via dephosphorylation of the tyrosine residues on the insulin receptor and IRS proteins by protein tyrosine phosphatases (PTP) (335).

4.6.2. Insulin resistance

Insulin resistance involves defects in insulin signalling, leading to the inability of insulin to suppress hepatic glucose production and impaired glucose uptake into peripheral tissues (336). From rodent models, it is known that alterations in key metabolic tissues occurs rapidly, as soon as 3 days after initiating over-feeding (337). Alternatively, obese T2D patients exhibit rapid improvements in insulin sensitivity and glucose tolerance before considerable weight loss has occurred (338). The importance of insulin signalling in the liver has been demonstrated by liver-specific insulin receptor deficient mice, which show a dysregulated metabolic phenotype (339). Similar results are observed when parallel studies are conducted in other key metabolic locales such as adipose tissue (340). Surprisingly, a lack of insulin receptor signalling in muscle does not result in similar alterations to whole-body glucose homeostasis (341), though impaired responsiveness of skeletal muscle is a precondition for T2D. A lack of insulin signalling in β -cells, via selective deletion of the insulin receptor in mice, or of IRS-1 in β -cells *in vitro*, leads to progressive glucose intolerance and insulin secretory defects (342, 343).

Mechanistically, impaired insulin signalling can occur at a number of steps in the insulin pathway. Serine phosphorylation, rather than tyrosine phosphorylation, of the IRS proteins occurs at numerous locations and negatively affects downstream signalling in the majority of these cases

(335). These serine phosphorylation sites may represent a necessary negative feedback loop in healthy individuals that is overridden in T2D patients due to uninhibited serine kinase activation. These protein kinases include Jun N-terminal kinase (JNK), inhibitor κ B kinase β (IKK β), mTOR complex 1, and protein kinase C (PKC) family members such as PKC- θ (344-347). Intermediates of triglyceride synthesis have been shown to lead to kinase activation (348). The IRS proteins may also be regulated at the protein expression level, as reduced levels of IRS1 have been reported in adipocytes of T2D patients (349).

Further downstream of insulin ligation, a number of regulatory subunits of PI3K, which include p85, p55, and p50, can impair insulin signalling and are found in increased levels in insulin resistant and overfed individuals (350, 351). Additionally, downstream targets of mTOR such as S6 kinase 1 (S6K1) negatively regulate insulin signalling through phosphorylation of IRS-1. This is demonstrated by mice with genetic deletion of S6K1, which are protected from obesity and have enhanced insulin sensitivity (352).

Mitochondria mediate β -oxidation of free fatty acids and it has been hypothesized that their function may be perturbed in insulin resistance, particularly in skeletal muscle. While evidence suggests that mitochondrial dysfunction correlates closely to insulin resistance in T2D patients, opposing reports suggest that this may be a secondary consequence of obesity and insulin resistance (353, 354). It is possible that increased lipid concentrations overload β -oxidation pathways following the accumulation of lipid intermediates.

4.6.3. β -cell compensation and failure

Increased insulin demands are initially countered by increased secretion. This process is poorly understood, but it is believed that β -cells gain increased secretory function and increase in total mass to maintain normal glucose levels (355, 356). The factors that stimulate β -cells to respond with greater effectiveness are likely a consequence of increased nutrient supply. For example,

glucagon-like peptide-1 (GLP-1) and its receptor on β -cells are increased following high-fat feeding and may enhance β -cell function (357). Both glucose and GLP-1 are stimuli for insulin gene transcription and may therefore be involved in the aspect of β -cell compensation that involves an increase in insulin biosynthesis and subsequent secretion (358).

There remains considerable debate as to the ability of β -cells to effectively self-renew or expand through differentiation of other cell types. Though animal models have provided evidence in favour of this theory, their propensity in humans has been questioned. Again, increased nutrient and growth factor supply, such as glucose and free fatty acids, is believed to promote β -cell survival (359). Insulin, IGF-1, and IGF-2 increase the expression of the pancreatic and duodenal homeobox-1 (PDX-1) protein, which increases β -cell proliferation and survival (360). This occurs following signalling through PKB phosphorylation and inactivation of forkhead box protein O1 (FOXO1) (361). GLP-1 can also activate IRS-2 and PKB via the cAMP response element-binding (CREB) protein (362).

However, β -cell compensation cannot continue indefinitely. This period of increased insulin demand is associated with cumulative β -cell damage and apoptosis leading to the eventual loss of β -cell mass and a diminished secretory capacity of those cells that remain (363, 364). For example, β -cells isolated from T2D patients post-mortem are unable to reverse hyperglycemia when transplanted into immunodeficient diabetic mice (365). At the time of T2D diagnosis, it is estimated that there is at least a 50% loss of β -cell function (355). It remains uncertain as to when β -cell failure occurs, though evidence suggests that it may be long before the onset of prediabetes when glycemia remains normal (366). The propensity for failure could be one factor that determines why only a subset of insulin resistant individuals progress to T2D.

β -cell failure is believed to be a result of acquired defects following exposure to hyperglycemic conditions in genetically susceptible individuals. While elevated levels of glucose

and free fatty acids initially evoke protective pathways, they can also overwhelm and induce cell death (367). Analysis of isolated islets from T2D patients and normal controls revealed striking differences in gene expression of a number of proteins related to insulin signalling and glucose metabolism that may correlate to failure (368). The accumulation of islet amyloid polypeptide (IAPP) has been proposed to cause β -cell cytotoxicity. IAPP deposits can be observed in the islets of many T2D patients, although the presence of IAPP does not correlate well with disease severity (369). Additionally, elevated metabolism through oxidation results in increased mitochondrial membrane potential, superoxide production, and activation of uncoupling protein 2 (UCP2) (370). While UCP2 helps to dampen this increased membrane potential through the generation of heat, it does so at the expense of ATP production and hence insulin secretion. Finally, high demands for insulin synthesis remain chronically elevated, and this may eventually overwhelm the protein folding capacity of the endoplasmic reticulum leading to stress and unfolded protein responses (371).

4.6.4 Obesity and obesity-induced inflammation

Obesity is the largest risk factor for T2D, and therefore much of the related T2D research focuses on the biology of adipose tissue and its impact on whole-body insulin action and glucose metabolism. Obesity results from an imbalance in energy uptake relative to energy expenditure. The result is increased storage of energy in the form of fat, particularly in adipose tissue itself. While the size of adipocytes has been noted to dramatically increase in size, adipocyte numbers are thought to be determined early in life (372, 373).

Obesity also leads to elevated levels of lipid intermediates and triglycerides within additional insulin-sensitive tissues, the extent of which negatively correlates to insulin sensitivity (374). It is these lipid metabolites that are believed to mediate the detrimental effects of obesity. Conversely,

weight loss leads to a reduction in hepatic lipid content and improvement in hepatic insulin resistance (375).

During obesity, and corresponding periods of energy excess, hormones and cytokines bridge immune, neuronal, and metabolic tissues to generate systemic alterations in insulin signalling. Enlarged adipocytes are central to this process and are more prone to secrete a number of factors, including leptin, adiponectin, resistin, visfatin, and retinol-binding protein-4, more familiarly known as adipokines. Adiponectin, for example, increases fatty acid oxidation in skeletal muscle and is decreased in obese T2D patients (376, 377). The importance of adipocytes is linked to these adipokines in addition to the sequestration of lipids as stored triglycerides. In the absence of adipose tissue, fasting hyperinsulinaemia and impaired glucose disposal is observed in humans and mice (378, 379). Conversely, transplantation of fat tissue into lipodystrophic mice restores insulin sensitivity (380), indicative of its potentially beneficial effects.

Obesity has been linked to inflammation and the immune system for many years. Salicylates, whose anti-inflammatory properties are derived from inhibition of prostaglandin and thromboxane synthesis, were shown in 1876 to dramatically reduce glucosuria in T2D patients (381). The relationship between obesity and inflammation is also demonstrated by the prominent insulin resistance that contributes to sepsis mortality, a result of massive innate immune system stimulation (382).

More recently it was determined that C-reactive protein (CRP), a non-specific marker of acute inflammation, is significantly upregulated in obesity and T2D (383). Additionally, both obesity and insulin resistance are associated with elevated levels of TNF- α , IL-6, and IL-8 (384-386). The pathogenicity of IL-6 is demonstrated by improvements to insulin resistance of obese mice following its neutralization (387). Similarly, TNF- α administration worsens disease in lean rats (388), and TNF- α deficient mice show improved systemic insulin resistance despite similar

adiposity (389). It is believed that the effects of TNF- α are mediated by suppressor of cytokine signalling (SOCS)-3 activation (390). TNF- α leads to the eventual serine phosphorylation of IRS proteins, known to impair insulin signalling. Immune-mediated insulin resistance is also attributed to the actions of other kinases such as JNK and IKK β , which are activated following inflammation-induced stimulation of the activator protein-1 (AP-1) and NF- κ B pathways. JNK activity correlates with obesity and insulin resistance in T2D patients and in animal models, and mice are protected from disease in its absence (344). IKK β activity is also increased in obese and insulin resistant mice (391), and overexpression in lean mice with a constitutively active hepatocyte-specific transgene causes systemic insulin resistance (387).

In the obese state, a large proportion of inflammatory cytokines are derived from adipose tissue, demonstrated by increased levels of TNF- α mRNA and protein detection in supernatants *in vitro* following explanted adipose culture (392). Early research in mice demonstrated that adipose tissue infiltrating macrophages are the main source of these cytokines (393, 394). In lean adipose tissue, macrophages are present in low numbers, isolated and widely dispersed among adipocytes, likely contributing to the removal of cell debris and tissue remodelling during natural turnover (395). In non-obese humans and mice these macrophages are believed to be predominantly of an anti-inflammatory phenotype (395, 396). Following high-fat diet consumption, macrophages rapidly infiltrate into adipose tissue prior to systemic signs of insulin resistance (393). Macrophage presence positively correlates with adipocyte size and body mass in obesity, organizing in crown-like structures around large, dying adipocytes in multiple mouse models as well as in human subjects (397). Close to 50% of the total cell content in adipose tissue of obese mice is estimated to be contributed by macrophages, compared to 10% in lean mice (394). Macrophage recruitment to adipose tissue is mediated in part by monocyte chemoattractant protein-1 (MCP-1) (398), though additional chemokines and their receptors are likely involved. Similarities between macrophages

and adipocytes extend beyond cytokine production. Both cell types express PPAR- γ , which plays a role in lipid accumulation and is a target of insulin sensitizing therapies.

In addition to adipose tissue-resident macrophages described here, other immune cell types, in addition to other tissues such as the liver and muscle, are also involved. For example, neutrophils are also rapidly, but temporarily, recruited to adipose tissue following initiation of high-fat diet (399). While early evidence demonstrated that both B cells and T cells are present in adipose tissue in lean fat (400), subsequent publications showed a notable presence of NK cells, NKT cells, and T cells within different fat depots (401). The presence of lymphoid cells is impressive, as they constitute roughly 10-15% of the stromal vascular cell fraction of VAT of mice. Interestingly, lymphocyte accumulation in VAT is more prevalent and inflammatory than in SAT, further illustrating the difference between adipose depots. NKT cells are present in adipose tissue, and to a greater extent in the liver, but their ability to significantly modulate glucose homeostasis appears limited (402).

Weight gain is accompanied by the development of a proinflammatory T_H1 and T_H17 phenotype, which overwhelms Treg cells and exacerbates inflammation (397, 403). Following IFN- γ stimulation, there is a sustained loss of insulin-stimulated glucose uptake in adipocytes and downregulation of the insulin receptor, IRS-1, and GLUT4 (404). Additionally, IFN- γ deficiency protects mice from insulin resistance despite no differences in weight gain (405). IL-17 production from T_H17 cells positively correlates to HbA1c levels in T2D patients, and T cells from obese T2D patients produce greater amounts of IL-17 than obese non-diabetic controls (406). IL-17 deficient mice are more glucose tolerant and insulin sensitive following exposure to high-fat diet, consistent with a pathogenic role for T_H17 cells (407). Treg cells are also present in high proportions in adipose tissue and display a significantly different transcription profile compared to conventional Tregs, including a massive upregulation in IL-10 expression (408).

It is possible that inflammatory changes following high-fat diet result from hypoxia due to adipocyte enlargement (409, 410). Hypercaloric diets may therefore represent the initial pathogenic event that establishes a low-level proinflammatory milieu and insulin resistance within adipocytes, hepatocytes and skeletal muscle, and this may help to explain the transition from symptoms associated with the metabolic syndrome to hyperglycemia (411).

4.6.5. Central nervous system-mediated metabolic control

As mentioned, altered glucose homeostasis and insulin resistance arises as a consequence of the dysregulation of normal complex inter-organ communication. A body of evidence exists demonstrating the importance of the CNS in the regulation of energy and glucose homeostasis. The brain receives afferent neuronal signals from organs to obtain information about the periphery. For example, following lipid detection in the intestine, neuronal signals are relayed to the brain and then efferently to the liver, leading to altered hepatic glucose production and establishing an inter-organ axis of communication (412). Alternatively, the brain can directly sense hormones and nutrients such as glucose, leptin, and insulin. Mice lacking the insulin receptor in the brain develop obesity and mild insulin resistance, suggestive of a role for the brain in whole-body metabolic homeostasis (413). This effect relies primarily on the arcuate nucleus and its coordination of hepatic glucose output, and the ventromedial nucleus and its role in glucose uptake (414). High levels of both insulin and leptin receptors are expressed in the arcuate nucleus (415, 416). Leptin, which signals predominantly through STAT3, is known to decrease triglyceride synthesis and lipogenesis, while enhancing β -oxidation (417). Its importance is exemplified by the *ob/ob* C57BL/6 mouse, which develops severe adiposity due to genetic inactivation of the leptin gene (418). These roles position the hypothalamus as a key modulator of insulin resistance and glucose control (419).

4.7. Therapies

Our lack of understanding of the pathophysiology of T2D continues to hamper efforts to develop effective therapeutics. Massive public health approaches are now required due to the high prevalence and incidence rates and subsequent health care costs. The timing of diagnosis and initiation of treatment is crucial, as effective management of prediabetes can reduce or delay T2D incidence by up to 60% (420, 421). Prediabetes treatment often involves lifestyle interventions, but adherence is a challenge.

Interestingly, the effectiveness of many treatments is measured by the ability to tightly control plasma glucose concentrations, yet evidence is conflicting if this correlates with improved outcomes. Results of three large trials involving patients with established T2D showed no effective reduction in overall mortality or cardiovascular disease-related mortality when randomized to intensive glycemic management compared to conventional glycemic control (422-424). Nevertheless, intensive glycemic control may delay or slow the onset and progression of microvascular complications including nephropathy, retinopathy, and neuropathy (423, 425).

A number of treatment options are available for T2D patients, including modifications to lifestyle, drugs that modify insulin action and secretion, and surgery. Not surprisingly, some of these rely on lowering body weight in order to treat T2D. The following section will focus on different approaches to treating T2D and highlight a number of medications in the process. The ideal treatment for T2D would consider the age of the patient, stage of their disease, degree of β -cell dysfunction, extent of vascular damage, and aim to reverse β -cell pathology and insulin resistance.

4.7.1. Changes in lifestyle

A number of changes can be made to an individual's lifestyle that have important therapeutic effects, specifically in terms of obesity. A balanced diet rich in fibre, grains, and legumes, in addition to aerobic exercise, can facilitate weight loss. These interventions also lead to reductions in

T2D, hypertension, glycated HbA1c, and triglycerides (426). Weight loss is associated with a disproportional loss of VAT, which may explain the improvement in insulin sensitivity (427). Conversely, the removal of subcutaneous adipose tissue through liposuction does not result in improvements in aspects of the metabolic syndrome (428).

The major challenge with these voluntary options is patient adherence, and therefore more drastic measures may sometimes be taken. Bariatric surgery has increasingly become a choice among obese individuals with T2D and has become minimally invasive. Though there are four major bariatric surgery procedures, the Roux-en-Y gastric bypass and the laparoscopic adjustable gastric band are performed most often (429). Improvement or remission of T2D after partial gastrectomy has been demonstrated (430). As the rates of obesity continue to rise, the benefits of surgery, including sustained improvements in glycemic control in morbidly obese T2D patients, become more appealing (431). More recent studies have shown that obese T2D patients who undergo bariatric surgery also have reduced hypertension, cholesterol levels, cardiovascular disease, and overall mortality compared to those who do not have surgery (432-434). These dramatic reductions in morbidity and mortality have led to the inclusion of bariatric surgery as a viable and cost-effective treatment option (435).

Bariatric surgery should ideally take place early in T2D progression, before irreversible loss of β -cell mass has occurred. However, mechanisms explaining how bariatric surgery rapidly alters homeostatic mechanisms are poorly understood. Research suggests that traditional theories involving decreased food passage and absorption are oversimplified. The effects of bariatric surgery likely result from reduced inflammatory and nutrient toxicity following sustained weight loss, in addition to unique factors that override normal biological mechanisms to regain the lost weight (436).

4.7.2. Increased insulin levels

Given a lack of insulin secretion or insulin action, it would seem straightforward to administer exogenous insulin as a treatment for T2D, as in T1D. In some cases insulin is used for T2D patients who become insulin deficient or have poorly controlled disease, and it may also be an option for those in very early stages of disease when used acutely (437). Both long-acting and short-acting formulations are available for tighter glucose control, increased ease of use, and better patient compliance (438). However, insulin does not reverse any pathology associated with T2D and dosing remains a problem; insulin can cause weight gain in some patients or hypoglycaemia when used inappropriately (439). Therefore, though this is an increasingly popular treatment option, it may not represent a long-lasting solution.

Sulfonylureas are a popular oral medication used to treat T2D. They bind to and close ATP-sensitive K^+ channels expressed on the surface of β -cells. This disrupts the hyperpolarized membrane potential and stimulates increased insulin release through the opening of voltage-sensitive Ca^{2+} channels, independent of glucose concentration (440). Sulfonylureas rapidly reduce blood glucose levels compared to other agents. The use of sulfonylureas is associated with modest weight gain and may result in hypoglycaemia requiring medical attention (441, 442). Sulfonylureas have no effect on disease progression and have been suggested to accelerate β -cell decline through increased cellular exhaustion, calling their usefulness into question (441). Their efficacy also becomes limited as T2D progresses and the β -cell mass is reduced.

Finally, the observation that ingestion of nutrients provided a greater stimulus for insulin secretion than intravenous challenge led to the incretin concept (443), in which eating induces the release of multiple gastrointestinal hormones that subsequently increase insulin release. One such hormone, GLP-1, is significantly increased within minutes of eating and prior to the passage of food through the gut, likely the result of endocrine and neural signals. The binding of GLP-1 to its

receptor on the surface of β -cells leads to rapid increases in cyclic adenosine monophosphate (cAMP) and intracellular calcium, followed by insulin exocytosis (444). GLP-1 receptor ligation also renders β -cells resistant to apoptosis and increases their survival (445). Notably, this incretin effect is significantly reduced or absent in patients with T2D likely due to reduced effectiveness of GLP-1 signalling (446). Therefore, interest focused on the generation of GLP-1 receptor agonists to treat T2D patients and these agents have shown efficacy in lowering blood glucose and treating disease when administered intravenously (447, 448). Longer periods of infusion effectively improve insulin secretion, insulin sensitivity, and glycated haemoglobin (449). It is recommended that GLP-1 receptor agonists be used as early in the disease process as possible, as patients taking them typically show modest weight loss, delayed gastric emptying, and decreased appetite. Efforts continue to reduce peak levels of the drug, reduce nausea, and create longer-acting formulations to provide uniform and sustained GLP-1 receptor activation with fewer injections (450). Further elucidation of the effects of GLP-1 related drugs on cardiovascular morbidity and mortality (451), as well as their long-term safety, is required.

4.7.3. Altered metabolism and increased insulin sensitivity

Metformin is the most established first-line treatment for T2D, and is recommended upon diagnosis alongside modifications to lifestyle. Metformin works through stimulation of AMP-activated protein kinase, an enzyme central to cellular energy metabolism (452), and decreases hepatic glucose production (453). While metformin was previously approved in select countries, its use has expanded exponentially following evidence of its effectiveness in decreasing glycated HbA1c, diabetes-related mortality, myocardial infarction, and overall mortality (454). Notably, metformin administration does not result in weight gain and rarely causes hypoglycaemia, unlike many other T2D medications. Adverse events are usually related to its gastrointestinal effects, particularly at high doses and at initiation of treatment.

Thiazolidinediones (TZD) act through enhancing insulin sensitivity in peripheral tissues and reducing hepatic glucose production. The use of TZDs remains infrequent despite their proven efficacy compared to other T2D drugs (441). This is due to concerns of increased cardiovascular morbidity and mortality (455), though results remain conflicting and further research is required (456). The mechanism of action of TZDs involves the activation of PPAR- γ , also a known genetic risk locus for T2D. TZDs are the only drugs that act on adipose tissue, but their exact mode of action is complex. TZD treatment results in a redistribution of adipose tissue, from visceral to subcutaneous depots, and decreases in lipolysis, TNF- α secretion, and free fatty acids (457, 458).

5. Diet-Induced Obesity

Diabetes is a complex, multiorgan disease involving the pancreas, liver, muscle, adipose tissue, gut, and brain, indicative of the requirement for an *in vivo* approach in animal models to observe the complex interaction between the tissues involved. Nevertheless, lower organisms can prove valuable for some aspects of T2D biology, since many pathways are highly conserved (459). Animals such as cats, pigs, and primates have been used to study T2D, but their size, availability, and cost limit their use. Rodents are therefore most often used to mimic human T2D, and a number of models have been created. Their phenotypes arise in some cases following genetic manipulation, and either spontaneously or following environmental inducers such as chemicals. Given that obesity is the major environmental risk factor for T2D, it is important that the animal model used to study disease develop obesity as a precursor to eventual diabetes.

5.1. Induction and disease course

Diet-induced obesity (DIO) is currently the most widely used animal model for T2D. Diets rich in fat induce obesity, and subsequently diabetes, in various animal species, including rats and

mice (460, 461). Indeed, there is a positive correlation between the levels of fat in the diet and weight gain (462). Generally, diets composed of 30-80% fat as a proportion of total energy intake are used.

The C57BL/6 mouse remains lean and euglycemic when raised on a low-fat diet, maintaining glucose homeostasis with normal levels of insulin. However, once placed on a high-fat diet, animals develop increased adiposity, characterized by selective deposition of fat in the mesentery, an observation that is consistent with increased risk of T2D in humans with greater abdominal obesity (463). Lipid-derived metabolites also accumulate outside of adipose depots in mice, including skeletal muscle, heart, and liver. While C57BL/6 mice do eat slightly more food than other strains on high-fat diet such as the A/J mouse, this does not account for the additional diet-induced weight gain (464). Additionally, obese C57BL/6 mice are as active as their lean counterparts and almost three times as active as other strains (465). It is therefore likely that obesity in C57BL/6 mice results from increased feed efficiency, a greater degree of weight gain relative to calories consumed.

T2D in C57BL/6 mice is characterized by hyperinsulinemia, insulin resistance and glucose intolerance (466). Hyperglycemia can be observed after approximately one month of high-fat diet consumption. This is in stark contrast to leptin deficient ob/ob mice, which do not exhibit fasting or basal hyperglycaemia. Disease in C57BL/6 mice worsens with time, likely due to the effects of increasing obesity. After sixteen weeks of high-fat diet, mice exhibit adipocyte hyperplasia and hypertrophy that corresponds to a two to three fold increase in absolute fat mass (467). Interestingly, the metabolic syndrome at this stage remains largely reversible. Despite a low islet mass relative to total body mass, C57BL/6 mice have a large number of small sized islets that can effectively expand when stressed (468).

In the obese C57BL/6 mouse, a range of phenotypic and diagnostic tests are employed, and often a comparative approach is taken to analyze age- and sex-matched individuals to look for statistically significant differences. These tests are further outlined in **Table 1.2**. They include the intraperitoneal and oral glucose tolerance tests (GTT), in which a glucose load is administered to fasted mice relative to body weight, and subsequent changes in blood glucose levels are measured over a designated period of time. Similarly, the insulin tolerance test (ITT) involves administration of a known dose of insulin relative to body weight, followed by blood glucose monitoring. The GTT and ITT are robust and reproducible tests that indicate the efficiency of glucose disposal as well as insulin secretion and action. Standardized protocols are also used for measuring metabolic rate by indirect calorimetry, food intake, and physical activity. Urine and plasma, often from fasted animals, can be biochemically analysed for factors relevant to glucose homeostasis such as insulin, adrenaline, leptin, and glucagon. Additionally, fat and lean mass composition, as well as bone mineral density, can be examined by imaging technologies such as X-ray absorptiometry and MRI.

5.2. Relevance to human T2D

There are clear differences when studying human and mouse metabolism that should be considered when translating results, some of which have been demonstrated by analogous genetic abnormalities that elicit unexpectedly different phenotypes. For example, mice lacking the insulin receptor are born with normal body weight but die in infancy due to ketoacidosis, while humans with similar null mutations are born underweight and do not develop ketoacidosis (469). Additionally, it is believed that diabetes in C57BL/6 mice appears as a consequence of the inability to significantly increase β -cell mass in response to obesity-induced insulin resistance, a process that is possible in human patients. Furthermore, except for monkeys and cats, animals do not display islet pathology such as amyloidosis (470).

<i>Test</i>	<i>Output</i>
Measurement	<ul style="list-style-type: none"> • Body weight • Body length
Blood <ul style="list-style-type: none"> • (Non-fasting) 	<ul style="list-style-type: none"> • Non-fasting glucose • Non-fasting insulin • Red and white blood cell count • Hormones (e.g. leptin, glucagon, glucagon-like peptide-1, ghrelin) • Cytokines (e.g. TNF-α, IL-6)
Blood <ul style="list-style-type: none"> • (Fasting) 	<ul style="list-style-type: none"> • Fasting glucose • Fasting insulin
Glucose Tolerance Test <ul style="list-style-type: none"> • (Oral or Intraperitoneal) 	<ul style="list-style-type: none"> • Glucose • Insulin • Glucose normalization
Insulin Tolerance Test	<ul style="list-style-type: none"> • Glucose • Insulin • Insulin sensitivity
Metabolic Cage	<ul style="list-style-type: none"> • Food intake • Water intake • Fecal production • Urine production • Activity
Indirect Calorimetry	<ul style="list-style-type: none"> • Volume O₂ • Volume CO₂ • Respiratory exchange ratio • Heat
Quantitative Magnetic Resonance Imaging	<ul style="list-style-type: none"> • Fat mass • Lean mass
Blood Pressure	<ul style="list-style-type: none"> • Blood pressure

Table 1.2 – Common tests to assess T2D and obesity in mice.

Nevertheless, the C57BL/6 mouse is a particularly good model for human metabolic syndrome because it features many similarities to human disease following exposure to nutrient excess (466), including hyperglycemia, hyperinsulinemia, and hypertension. This also occurs gradually, similar to T2D in humans. Conversely, other strains of mice such as the A/J mouse or the C57BL/KsJ are relatively resistant to the effects of high-fat diet (463, 471). Due to the range of metabolic parameters altered by high-fat diet, the pathogenesis of T2D complications such as atherosclerosis and nephropathy can also be studied in obese C57BL/6 mice (472). The countless number of transgenic and knock-out strains on the C57BL/6 background have also provided valuable insights into the role of specific proteins on glucose metabolism and β -cell health during T2D progression.

Overall, the C57BL/6 mouse has proven to be a valuable tool for T2D research due to the ease of genetic manipulation, phenotypic testing of endocrine and metabolic changes, and elucidation of pathological mechanisms. It is sensitive to both genetic and environmental risk factors, in contrast to other strains such as the ob/ob mouse which is largely genetically determined. Its many advantages have made it possible to screen anti-diabetic therapeutics prior to translation in humans (473).

6. Glial Fibrillary Acidic Protein

Glial fibrillary acidic protein (GFAP) was discovered following its purification from the brain tissue of MS patients, which involved fibrous reactive astrocytes and demyelinated axons (474). It is now known to be one of the key intermediate filaments within astrocytes, and its expression can also be detected in a number of peripheral tissues. The function of GFAP continues

to be elucidated and its importance is exemplified by the fatal and progressive Alexander disease that results from mutations within the GFAP gene (475).

6.1. Sequence and structure

The cytoskeleton is composed of, in ascending thickness, microfilaments, intermediate filaments, and microtubules. The intermediate filaments are approximately 8-12 nm in diameter and can be further divided into six classes based on sequence homology (476). GFAP belongs to the type III class and is the major intermediate filament protein in astrocytes. Intermediate filaments are arranged into an amino-terminal “head” domain, a central and highly conserved “rod” domain, and a carboxy-terminal “tail” domain (477). During assembly, two GFAP molecules form a parallel dimer structure, which then joins with another dimer in an anti-parallel fashion to form a tetramer. These protofilaments further wind around each other in a rope-like fashion in groups of approximately eight to create the filament structure (478). GFAP can also naturally associate with vimentin, another intermediate filament expressed in astrocytes (479). However, mice with genetically inactivated vimentin demonstrate that it is possible for GFAP to form filaments on its own, though these filaments show abnormally compact organization in these mice (480). Phosphorylation of GFAP can occur at six different sites within the head and tail domain and this impacts its filament assembly and disassembly (481). These phosphorylation sites are conserved evolutionarily between species, suggestive of their importance.

The GFAP gene was first cloned in mice using a cDNA library that was screened with GFAP-specific polyclonal antiserum (482). It was later found to map to chromosome 17q21 in humans and is composed of nine exons and eight introns. Transcription produces mature mRNA approximately 3kb in length. Interestingly, a number of isoforms of GFAP have been identified. They vary quite significantly in length due to alternative splicing and different start sites (477). These isoforms are thought to be expressed in an astrocyte subset-specific manner but the factors

that control their individual expression are unknown. Some isoforms lack considerable portions of the head or tail domains, and they are therefore assumed to have altered polymerization and assembly kinetics, or may be unable to form proper filaments altogether (477). The discovery of these isoforms may lead to the retrospective analysis of prior literature to determine which GFAP isoforms are most important for each function.

The GFAP gene contains close to one hundred reported DNA base changes. These polymorphisms are all associated with Alexander disease, a neurological condition in which patients develop megalencephaly accompanied by spasticity and mental retardation (475, 483). It is believed that mutations in GFAP increase its function or expression, causing cytoplasmic protein aggregates known as Rosenthal fibres.

6.2. Expression

GFAP was initially believed to be an astrocyte-specific intermediate filament. More recently it has been shown to be expressed in a number of non-glial and non-CNS cells, including enteric glia (484), Schwann cells (485, 486), and possibly lymphocytes (487). Its role in the gut is particularly intriguing, as transgenic mice expressing herpes simplex virus thymidine kinase under control of the mouse GFAP promoter develop fulminant and fatal jejuno-ileitis following administration of ganciclovir (488).

Transcription of GFAP is highly regulated by its promoter and other binding elements, as well as epigenetic mechanisms such as phosphorylation and DNA methylation. While vimentin is the major intermediate filament protein in the neonatal brain, GFAP dominates in the adult brain. However, the time at which GFAP expression begins is debated, with estimates ranging from between nine to twenty five gestational weeks (489, 490). Increased GFAP expression later in life may result from progressive damage to cells by reactive species (491).

Astrocytes are involved in a number of CNS pathologies including brain damage and during CNS degenerative conditions such as Alzheimer's disease (T3D) and MS (492). During stress or damage, astrocytes undergo hypertrophy of their processes known as reactive gliosis and increase their production of intermediate filaments including GFAP.

6.3. Function

The highly conserved sequence of GFAP across vertebrate species suggests that it plays a critical role in the physiology of the CNS. Despite its usefulness as a marker for astrocytes, relatively little is understood about its function. In order to gain further insight into its role, a number of transgenic mice have been created with varying levels of GFAP expression. Mice deficient in GFAP were created simultaneously by several laboratories. It was hypothesized that such a deletion would be embryonically lethal, given that fundamental cellular processes of astrocytes rely heavily on the cytoskeleton, including their positioning around blood vessel walls, at neuronal synapses, and in proximity to nodes of Ranvier. Yet, GFAP deficiency does not affect viability, life span, sexual reproduction, or motor capabilities (493, 494).

GFAP deficient mice do not lack intermediate filaments, likely due to other integral proteins such as vimentin, and these other intermediate filament proteins are able to initiate reactive gliosis. However, data suggest that GFAP is important for astrocyte-neuron interactions and synaptic efficiency. There is a deficiency in cerebellar long-term depression in GFAP knock-out mice (495) while the induction of hippocampal long-term potentiation may be enhanced (493).

GFAP deficiency does not affect responses to certain types of injury, including scrapie infection (494). However, in EAE, GFAP knock-out mice develop more severe disease, possibly due to a lack of reactive gliosis-mediated protective mechanisms (496). In addition, astrocytes in mice that carry extra copies of the GFAP gene are hypertrophic and have intermediate filament aggregates within their cytoplasm that closely resemble the Rosenthal fibres seen in Alexander

disease (497). Evidence therefore suggests that GFAP plays an important role in diseases involving cellular damage and stress.

7. Thesis Objectives

Cross-talk between the immune system and the nervous system is common, and helps to maintain normal physiology. Conversely, aberrations in this interaction are observed in the context of a variety of diseases, indicative of its importance. Thus, investigation into this relationship often yields unexpected results with clinical implications.

Immune modulation by the nervous system is largely mediated by cytokines and neuropeptides. For example, both sP and CGRP can dramatically alter lymphocyte subsets and their activation status (498). In T1D, a critical element of pathogenesis relates to pancreatic sP deficiency, reflective of mutations in the NOD mouse TRPV1 gene, which is highly polymorphic in humans as well (279). It is plausible that similar processes, involving TRPV1⁺ neurons or other cell types, exist in diseases such as MS, where leukocyte infiltration and subsequent injury within the CNS occurs in close proximity to TRPV1 expression (Chapter 2).

Neuronal destruction in both MS and EAE is primarily immune-mediated, dependent on a number of cell types. Both the nature of T cell priming in the periphery and capacity of T cell subsets within the CNS are a strong determinant of pathology. T_H17 cells have been shown to mediate key aspects of disease progression (110, 111). Their differentiation and expansion is linked to cytokines such as IL-6, which is, intriguingly, elevated in obesity (385). Changes associated with the obese state, such as nutrient excess and chronic inflammation, have been linked to several disorders though the link with MS requires further understanding (Chapter 3).

Obesity is the strongest environmental factor for T2D. Rapid expansion of adipose tissue is associated with the infiltration of a variety of leukocytes, and subsequent insulin resistance and β -cell failure is mechanistically linked to immune activation. Lymphocytes in obese mice display limited receptor diversity (397), suggestive of a cognate autoimmune response. In T1D, autoantigens include those primarily expressed in neuronal tissue such as GFAP (485), an additional example of neuro-immune interaction. In T2D, analyzing the role of lymphocytes and their receptor specificities may enable immunomodulation (Chapter 4).

Collaborative studies examining the intersection of the immune system and nervous system, such as those presented in this thesis, will continue to advance our understanding of inflammatory diseases.

CHAPTER 2

TRPV1 Gates Tissue Access and Sustains Pathogenicity in Autoimmune Encephalitis

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Contributions: The experimental mouse studies were done by myself (GP) with technical assistance by PW, and advice, training, and supervision by several senior members in the Dosch lab (HMD, JY, SW, HT, YM, IA) and the Salter lab (MWS, XJL). MRI experiments were performed in collaboration with the Henkelman lab (RMH, LSC, CLL). MS genetic studies were done by the Ebers lab in Oxford, UK (GCE, SVR, GCD, ADS).

Abstract

Multiple Sclerosis (MS) is a chronic-progressive, demyelinating condition whose therapeutic needs are unmet, its pathoetiology elusive. We report that Transient Receptor Potential Vanilloid-1 (TRPV1), expressed in a major sensory neuron subset, controls severity and progression of experimental autoimmune encephalomyelitis (EAE) in mice and likely in primary progressive MS. TRPV1^{-/-} B6 congenics are protected from EAE. Increased survival reflects reduced central nervous system (CNS) infiltration, despite indistinguishable T cell autoreactivity and pathogenicity in the periphery of TRPV1-sufficient and -deficient mice. The TRPV1⁺ neurovascular complex defining the blood-CNS barriers promoted invasion of pathogenic lymphocytes without the contribution of TRPV1-dependent neuropeptides such as substance P. In MS patients, we found a selective risk-association of the missense rs877610 TRPV1 single nucleotide polymorphism (SNP) in primary progressive disease. Our findings indicate that TRPV1 is a critical disease modifier in EAE and we identify a predictor of severe disease course and a novel target for MS therapy.

Introduction

Multiple sclerosis (MS) is a debilitating inflammatory disease of the central nervous system (CNS), characterized by progressive demyelination and axonal damage (10). Its etiology remains unclear, though tissue damage is autoimmune in nature, with several genetic disease risk loci mapped to T cell function (36, 499, 500). A key step in disease is thought to be impairment of the blood-brain barrier (BBB), a logical, but elusive target for therapeutic intervention (501). This physical barrier is established early during embryogenesis and involves a neurovascular unit of pericytes, astrocytes, neurons, endothelial cells, and microglia (PANEM) (502). Normally, tight junctions and low vascular adhesiveness greatly inhibit extravasation into CNS tissue (501). Disruption of the BBB triggers a complex cascade of events in which the interactive PANEM tissue elements respond to secreted mediators of immune or neuronal derivation (111, 503, 504).

A blood-tissue barrier exists in peripheral tissues, although it is not as restrictive as the BBB. Extravasation into peripheral tissues can be induced by activating Transient Receptor Potential Vanilloid-1 (TRPV1), a prominent member of the TRP ion channel superfamily (505). TRPV1 is expressed on the major subset of sensory afferent neurons that integrate noxious stimuli including heat and acidity (253, 272, 273). The outcome of peripheral TRPV1 activation and consequent cation influx in TRPV1⁺ sensory neuron is two-fold; (i) electric afferent signal transduction to the CNS generating pain perception, and (ii) local efferent release of neuropeptides such as substance P (sP) and calcitonin-gene related peptide (CGRP). Long perceived as largely a heat sensor, TRPV1 is emerging as a major controller of pleiotropic functions in different tissue milieus. Abnormal TRPV1 activity can dramatically alter the severity and progression of peripheral inflammatory conditions including Type 1 Diabetes (T1D), colitis, and arthritis, through mechanisms largely mediated by its local efferent secretory function: too little in T1D, too much in colitis and arthritis (279-281).

TRPV1 is also expressed within CNS regions, including the spinal cord dorsal horn and areas of the brain (506-510). TRPV1 function in these locations remains a topic of debate. Broad expression patterns have fostered the view that central TRPV1 plays a role in pain processing and thermoregulation, and independent studies have suggested that PANEM cells may all express TRPV1, characterizing the BBB as a TRPV1-rich microenvironment (258, 507, 509). Direct activation of TRPV1 by application of the agonist capsaicin directly to the brain disrupts BBB integrity (511), and peripheral TRPV1 stimulation also opens the BBB and blood spinal cord barriers (BSCB) (512), supporting recent evidence that TRPV1 may modify cytokine signalling during CNS neuro-inflammation and implying a disease-modifying role (513).

We investigated this possibility with a focus on EAE-autoimmunity. TRPV1^{-/-} mice are dramatically protected from disease developing minimal and delayed disease onset, reduced clinical scores and reduced demyelination. TRPV1 signalling effectively increases BSCB and BBB permeability, actively promoting inflammatory cell extravasation which is minimal in the absence of TRPV1. A model is provided to explain how TRPV1 signalling in the PANEM complex may be sustained at body temperature. We conducted parallel studies of patients from the Canadian Collaborative Project on Genetic Susceptibility to Multiple Sclerosis (CCPGSMS), a large and lengthy population-based study (514-516), focusing on the extremely polymorphic TRPV1 locus (517). We found a significant missense single nucleotide polymorphism (SNP) selection bias in patients with a very progressive disease course, indicating that the unexpected core role of TRPV1 may extend to patients with MS.

Methods

Mice

All mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in our vivarium in a pathogen-free, temperature-controlled environment on a 12hr light/dark cycle. Experiments were performed using age- and gender-matched mice under approved protocols and in agreement with animal ethics guidelines.

Induction and transfer of EAE

EAE was induced in mice 6-8wk old. For active immunization, mice were immunized in each flank with 50 µg of MOG₃₅₋₅₅ peptide (Alpha Diagnostic) emulsified in CFA (1:1) (Sigma-Aldrich). Where indicated, 200 ng pertussis toxin (Sigma-Aldrich) was given intraperitoneally on the day of EAE induction and 48hr later. Passive immunization was performed as described (518). Briefly, donor mice were immunized as described above. Draining lymph node cells were removed 8d post-immunization and cultured in the presence of 5 µg/mL MOG₃₅₋₅₅ peptide for 72 hr. 1×10^7 cells were then injected intravenously into recipient mice along with 200 ng of pertussis toxin (Sigma-Aldrich) intraperitoneally on the day of injection and 48hr later. Animals were followed for a minimum of 24d and disease was scored using the following scale: 0 - asymptomatic; 1 - limp tail; 2 - abnormal righting reflex and/or hind limb weakness; 3 - unilateral hind limb paralysis; 4 - bilateral hind limb paralysis; 5 - moribund or death.

Histology and immunofluorescence

Spinal cord and brain tissue was dissected at peak disease, 16-18d post-active immunization and 24d post-passive immunization. Tissue was fixed for up to 72hr in 10% buffered formalin before staining with hematoxylin and eosin (H&E) or luxol fast blue (LFB, myelin stain). Infiltration was scored by a blinded certified pathologist using the following scoring system: 1 -

normal, with intravascular non adherent leukocytes; 2 - intravascular adherent leukocytes; 3 - perivascular infiltration. Demyelination was quantified using ImageJ software analysis.

Immunoblotting

Mouse spinal cord and brain tissue was dissected and snap-frozen. Extracted total protein was electro-fractionated on 10% acrylamide gels (Invitrogen), blotted, and probed overnight at 4°C with rabbit anti-TRPV1 antibody (1:200, Calbiochem) and rabbit anti- β -actin antibody (1:1000, Cell Signaling). The following day, bands were visualized using the Western Lightning-ECL kit (Perkin Elmer).

Proliferation and cytokine secretion

4×10^5 splenocytes or inguinal lymph node cells were incubated in 96-well plates with HL-1 media (Lonza) and stimulated with MOG₃₅₋₅₅ for 72hr. For proliferation experiments, 1 mCi of [³H] thymidine was added for 18hr, prior to harvest and liquid scintillation counting. T cell and B cell specific proliferation was measured as described above following stimulation with 1 μ g/mL α -CD3 plus 0.25 μ g/mL α -CD28, or 1 μ g/mL α -CD40 (BD Pharmingen). For cytokine analysis, supernatants were collected after 72hr of culture. IFN- γ , IL-10, and IL-4 (BD Biosciences), IL-12 and TNF- α (eBioscience), and IL-17 (R&D Systems) were measured by ELISA according to the manufacturer's protocols.

Flow cytometry

CNS cells were isolated as previously described (403). 1.5×10^6 splenocytes or CNS cells were incubated at 4°C for 15min in 100 μ L 2% FBS/PBS with 10 μ g/mL Fc-blocker (eBioscience). Cells were incubated for 30min with the following fluorescent antibodies: CD11b (1:100), CD3 (1:100), CD4 (1:75), CD8 (1:100), B220 (1:100), IgM (1:100) (eBioscience), CD11c (1:100), CD62L (1:150), CD44 (1:100) (BD Biosciences), or CD45.2 (1:75) (Invitrogen). Samples were run

on an LSR-II flow cytometer (BD Biosciences) and FACS plots were analyzed using FlowJo software (Tree Star Inc.).

Gene expression microarray analysis

For cDNA gene arrays, RNA from brain and spinal cord was isolated from mice 8d post-immunization using TRIzol Reagent (Invitrogen), followed by PureLink RNA mini kit (Invitrogen). Gene arrays used the Mouse Gene 1.0 ST array (Affymetrix) and analysis by Expression Console (Affymetrix). Predicted and unknown genes were excluded from analysis.

Blood spinal cord/brain barrier analysis

Evans Blue dye flux into brain and spinal cord tissue was determined as described (512). Briefly, 4 mL/kg of 2% Evans Blue dye (Sigma-Aldrich) was injected intravenously and after 2hr animals were perfused with PBS. The spinal cord and supraspinal tissues were immediately dissected, removing the dura mater. The lumbar spinal cord, thoracic spinal cord, brain stem, cerebellum, and frontal cortex were further dissected and weighed. Tissue was incubated in formamide (Sigma-Aldrich) at 60°C for 72hr, and dye concentration in extracts was determined by spectrophotometry (620nm) in a 96-well plate reader.

To measure permeability of large molecule (infrared-labelled IgG), 5 µg/g of donkey anti-goat IgG, IRDye 800 CW labelled (Li-COR Biosciences) was injected intravenously and after 30min animals were perfused with PBS. Spinal cord and supraspinal tissues were immediately dissected and scanned with an Odyssey Infrared Imager (Li-COR Biosciences).

For magnetic resonance imaging, a multi-channel 7.0 T magnet was used to acquire in vivo 2D T1-weighted MR images (100 µm in-plane resolution) of the thoracic and lumbar spinal cord. Mice were injected with a bolus of Gadolinium Diethylenetriaminepentacetate (Gd-DTPA) (1.5 mmol/kg) intraperitoneally and imaged at 1% isoflurane according to established protocols. Quantitative measurements of signal intensity were determined by normalizing the spinal cord

intensity to the average intensity of two phantoms included in each coil (microcapillary tubes containing 1% Gd-DTPA in agar).

Human subjects

All subjects used in the study were ascertained through the ongoing CCPGSMS, as described (514-516). DNA and anonymized clinical charts from a total of one hundred and sixty-three sporadic MS patients were identified for analysis. The disability of the sporadic MS patients was carefully assessed and recorded at study entry with the Expanded Disability Status Scale (EDSS) determined by neurologists involved in the CCPGSMS. Here, patients were classified as having either “benign” or “malignant” MS based on EDSS scores sustained or achieved over designed time intervals. The benign MS cases (n=112) fell into the relapsing-remitting clinical subtype where minimal disability (i.e. $EDSS \leq 3$) was sustained over a period >20yr from disease onset. In contrast, the malignant MS cases (n=51), a subgroup of MS patients acquiring significant disability (i.e. $EDSS > 6$; the need for a walking aid or worse) within 5yr of disease onset, had the primary progressive form of the disease.

Genotyping

Total genomic DNA, extracted from whole blood as part of the CCPGSMS (519), was used to type SNPs. Genotyping of SNPs was performed using the Sequenom MassEXTEND protocol (www.sequenom.com). Only conservative and moderate genotyping calls were accepted in this study. Samples having aggressive or low probability quality genotypes were reanalysed.

Statistical analysis

Statistical significance between two means was assessed by Mann-Whitney and unpaired t-tests, and Welch correction was employed where appropriate. Analysis of curves was performed using two-way ANOVA. Human data was analyzed using the PLINK analysis package (520).

Statistical significance was two tailed and set at 5%, with error bars showing a single standard deviation.

Results

TRPV1 is pathogenic in EAE

To determine the role of TRPV1 in EAE progression, we induced disease in B6 mice, and TRPV1^{+/-} and TRPV1^{-/-} congenics. Spinal cord TRPV1 expression in heterozygous mice was 35% of wild type B6 levels, providing a valuable intermediate (**Figure 2.1.A**). In the classical MOG-EAE model, both B6 and TRPV1^{+/-} congenics progressed to severe disease rapidly, while TRPV1^{-/-} mice exhibited strong protection (**Figure 2.1.B**). High proportions (~60%) of wild type and TRPV1^{+/-} mice were moribund and euthanized before the end of the 24d observation period, whereas only few TRPV1^{-/-} mice reached that stage, later in the disease course (**Figure 2.1.C-D**). TRPV1 expression levels thus correlated with disease penetrance, day of disease onset, maximum clinical disease scores, and mortality (**Table 2.1 and Figure 2.1.B-D**).

TRPV1^{-/-} mice generate systemic pathogenic autoreactivity but fail to infiltrate the CNS

B6, TRPV1^{+/-} and TRPV1^{-/-} mice are immunocompetent and show no obvious differences in the composition of secondary lymphoid tissues (**Figure 2.2.A-D**). Nevertheless, we investigated if TRPV1 expression influenced the efficacy of MOG₃₅₋₅₅ immunization, conceivably explaining different disease susceptibilities. Draining lymph node (**Figure 2.3.A**) and spleen cells (**Figure 2.3.B**) of immunized B6, TRPV1^{+/-} and TRPV1^{-/-} mice proliferated to a similar extent when stimulated with a 5-log range of MOG₃₅₋₅₅ peptide doses, suggesting similar effector cell pool sizes and affinity spectra in the three mouse lines. No differences were observed following polyclonal T or B cell activation by α -CD3 plus α -CD28 or α -CD40 antibodies (**Figure 2.3.C**). MOG₃₅₋₅₅-induced cytokine secretory profiles were also comparable in the three strains, with no differences found in those relevant to T_H1, T_H17, or T_H2 responses (**Figure 2.3.D-I**).

	<i>Incidence</i>	<i>Mean Day of Onset</i>	<i>Mean Maximum Score</i>
C57BL/6	20/20 (100%)	9.9 ± 0.390	4.425 ± 0.171
TRPV1 ^{+/-}	20/20 (100%)	10.8 ± 0.427	4.325 ± 0.196
TRPV1 ^{-/-}	19/20 (95%)	14.7 ± 0.831	2.675 ± 0.352

Table 2.1 – Summary of EAE severity in B6, TRPV1^{+/-}, and TRPV1^{-/-} mice.

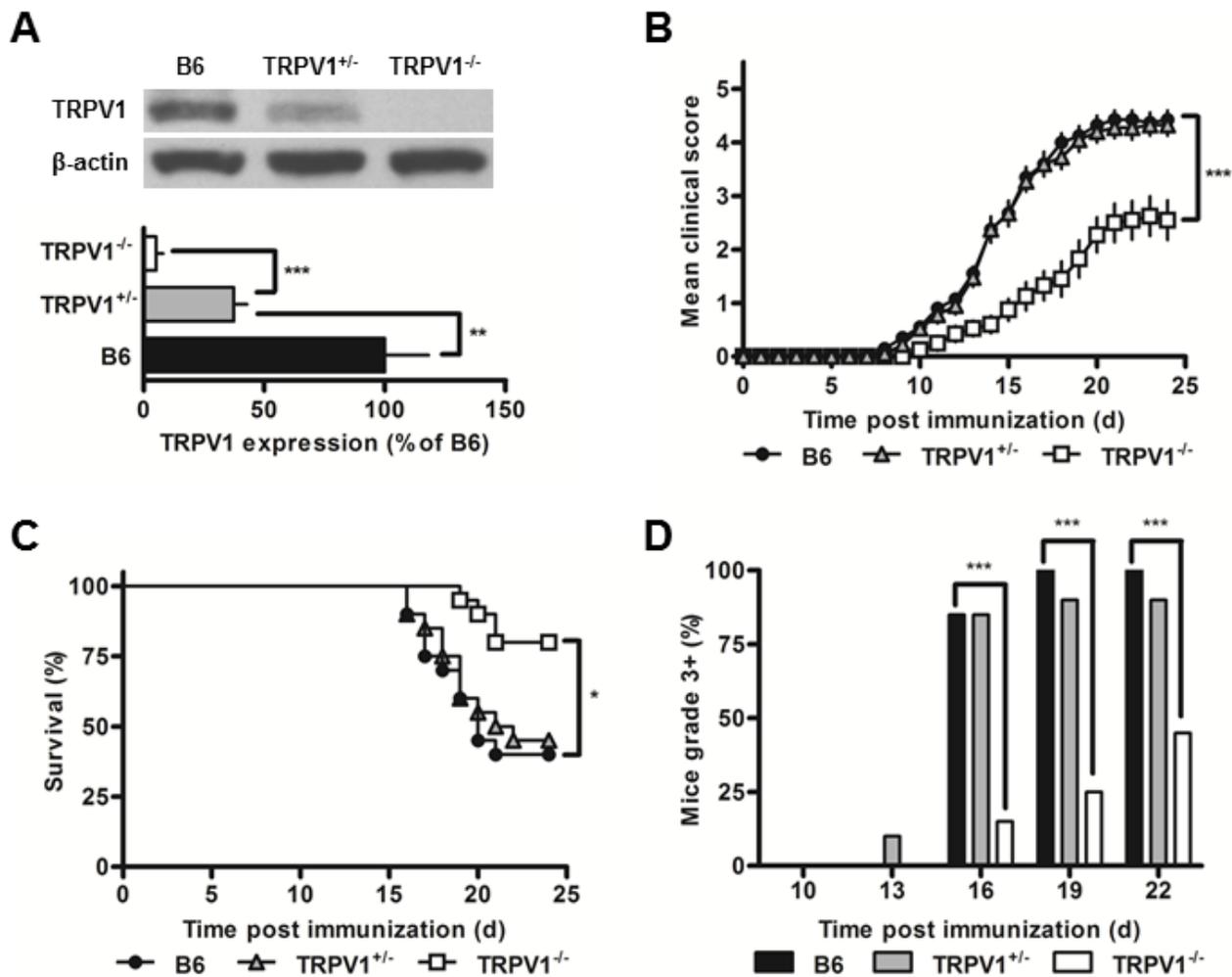


Figure 2.1 – TRPV1^{-/-} mice are protected from severe EAE. (A) Representative Western blot of TRPV1 protein in the spinal cord of B6, TRPV1^{+/-}, and TRPV1^{-/-} mice 6-8wk of age, with β-actin as a loading control. Quantification was performed by analysis of pixel density (n≥5 per group, **p<0.01, ***p<0.001). (B) Daily clinical scores of B6, TRPV1^{+/-}, and TRPV1^{-/-} mice following immunization with MOG₃₅₋₅₅ peptide and pertussis toxin (n=20 per group, ***p<0.0001). (C) Survival curve following induction of EAE (n=20 per group, *p<0.05). (D) Proportion of mice with severe EAE (n=20 per group, ***p<0.0001).

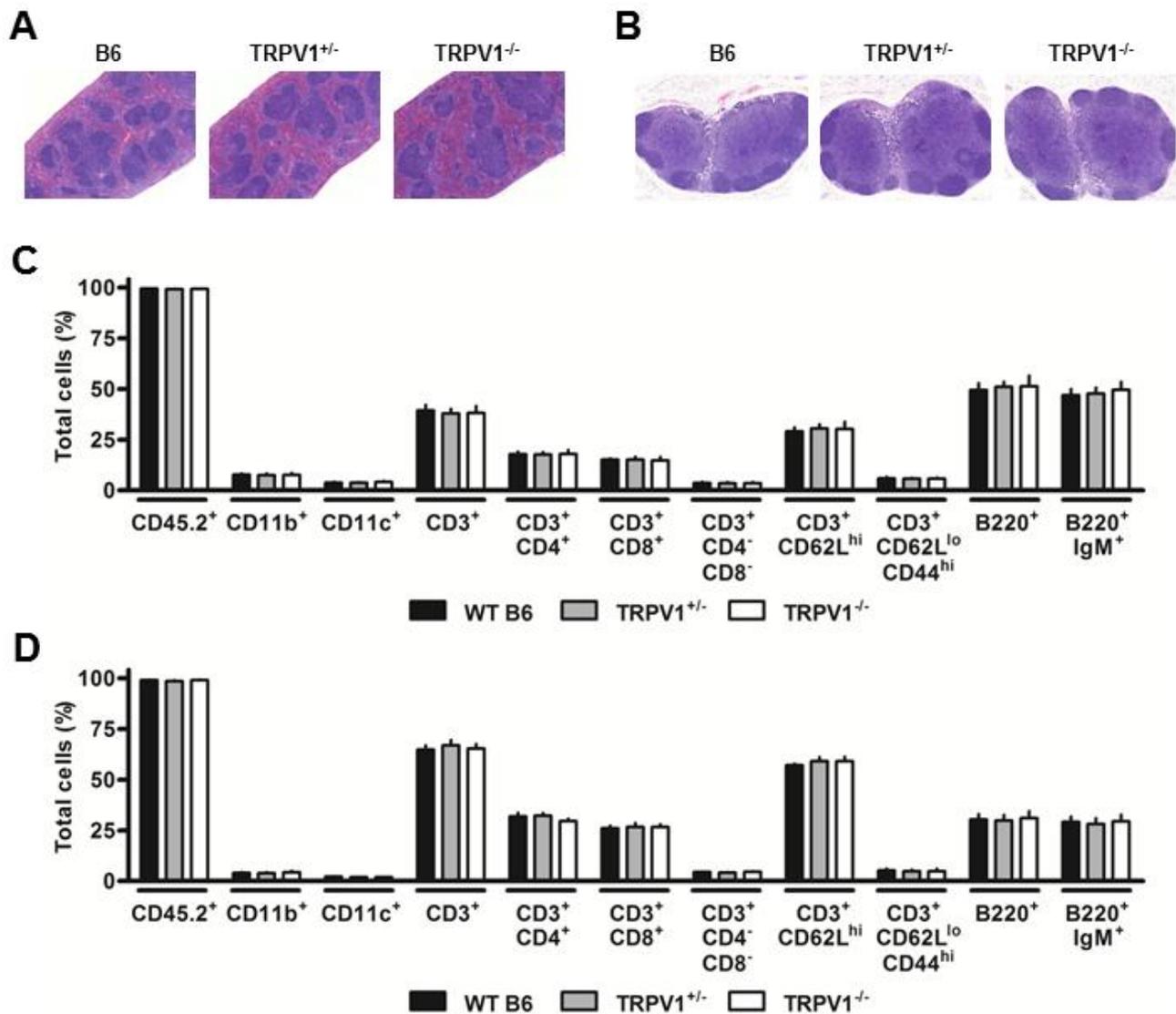


Figure 2.2 – TRPV1 congenic mice are immunocompetent. (A-B) Representative H&E stained spleen (A) and inguinal lymph node (B) sections from B6, TRPV1^{+/-}, and TRPV1^{-/-} mice. (C-D) Haematopoietic composition of B6, TRPV1^{+/-}, and TRPV1^{-/-} mice spleen (C, n=4 per group) and lymph node (D, n=4 per group).

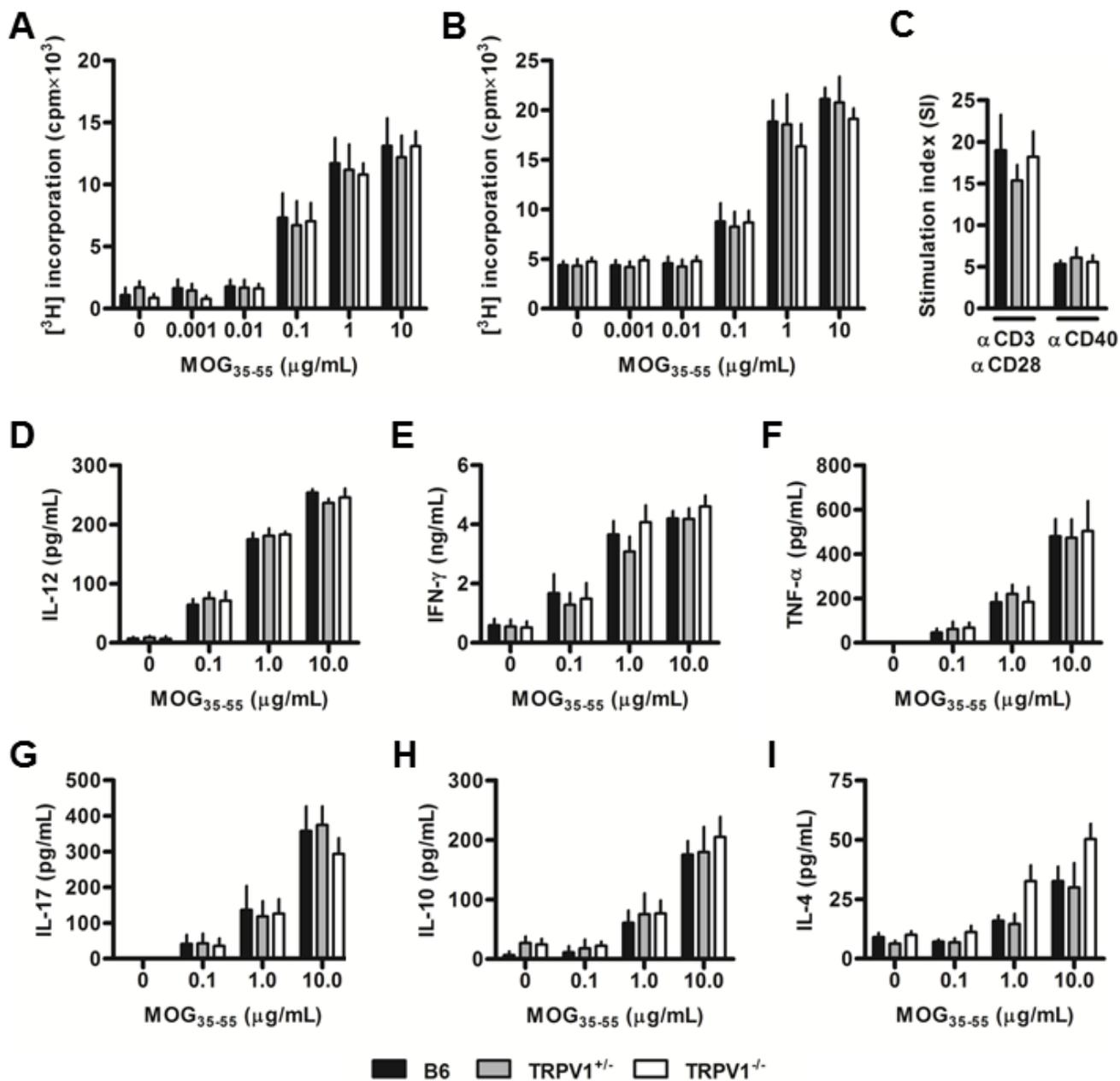


Figure 2.3 – TRPV1 expression does not affect the effectiveness of MOG₃₅₋₅₅ immunization. (A) Proliferation following MOG₃₅₋₅₅ peptide restimulation of inguinal lymph node cells (n=4 per group) from B6, TRPV1^{+/-}, and TRPV1^{-/-} mice sacrificed 8-10d post-immunization. (B) Proliferation following restimulation of splenocytes 16-18d post-immunization (n≥9 per group). (C) Mean stimulation index of splenocytes from naïve mice stimulated with α-CD3 and α-CD28 or α-CD40 antibodies (n=5 per group). (D-I) Production of IL-12 (D), IFN-γ (E), TNF-α (F), IL-17 (G), IL-10 (H), and IL-4 (I) by splenocytes following MOG₃₅₋₅₅ peptide restimulation (n≥5 per group).

In addition, cellularities were similar in spleen and draining inguinal lymph nodes before and after immunization (**Figure 2.4.A**), collectively confirming that the induction of anti-MOG₃₅₋₅₅ immune responses were indistinguishable. However, the ability of pathogenic MOG₃₅₋₅₅-activated cells to infiltrate the CNS was dramatically reduced in TRPV1^{-/-} mice (**Figure 2.4.B**). Differences were observed in absolute numbers of tissue-infiltrating cells including total lymphocytes, CD4⁺ and CD8⁺ T cells, and B cells (**Figure 2.4.B**). Interestingly, the relative proportions of lymphocyte subsets invading the CNS did not differ between B6 and TRPV1^{-/-} mice, including regulatory CD4⁺ Foxp3⁺ cells (**Figure 2.4.C**), demonstrating a role for TRPV1 in general tissue access rather than involvement in sublineage-selective interactions.

B6 mice sacrificed at peak disease displayed marked infiltration in the perivascular regions of both the brain and spinal cord that extended deeply into surrounding tissue, but much reduced infiltration was noted in TRPV1^{-/-} mice (**Figure 2.5.A-B**), corresponding well to the much reduced numbers extracted (**Figure 2.4.B**). Luxol fast blue staining of areas rich in compact myelin, revealed large lacunar areas of myelin loss in diseased wild type mice (**Figure 2.5.A**). In TRPV1^{-/-} mice, such lesions were small and sparse, equivalent to a ~3.5-fold reduction in demyelinated areas (**Figure 2.5.C**). As suggested by similar clinical disease scores, B6 and TRPV1^{+/-} mice were indistinguishable in their infiltration density, distribution, and demyelination. Protection in TRPV1^{-/-} mice correlated with the inability of infiltrating cells in these mice to penetrate deep into surrounding tissue. TRPV1^{-/-} mice showed a higher proportion of vessels in which leukocytes were non-adherent or adherent but intravascular and unable to leave the blood compartment (**Figure 2.5.D**), explaining disease protection and reduced demyelination.

Adoptive transfer of EAE fails in TRPV1^{-/-} mice

10⁷ *in vitro* MOG₃₅₋₅₅-restimulated cells from immunized B6 or TRPV1^{-/-} mice were intravenously injected into naive B6 mice to produce, within 3wk, a robust and severe form of EAE,

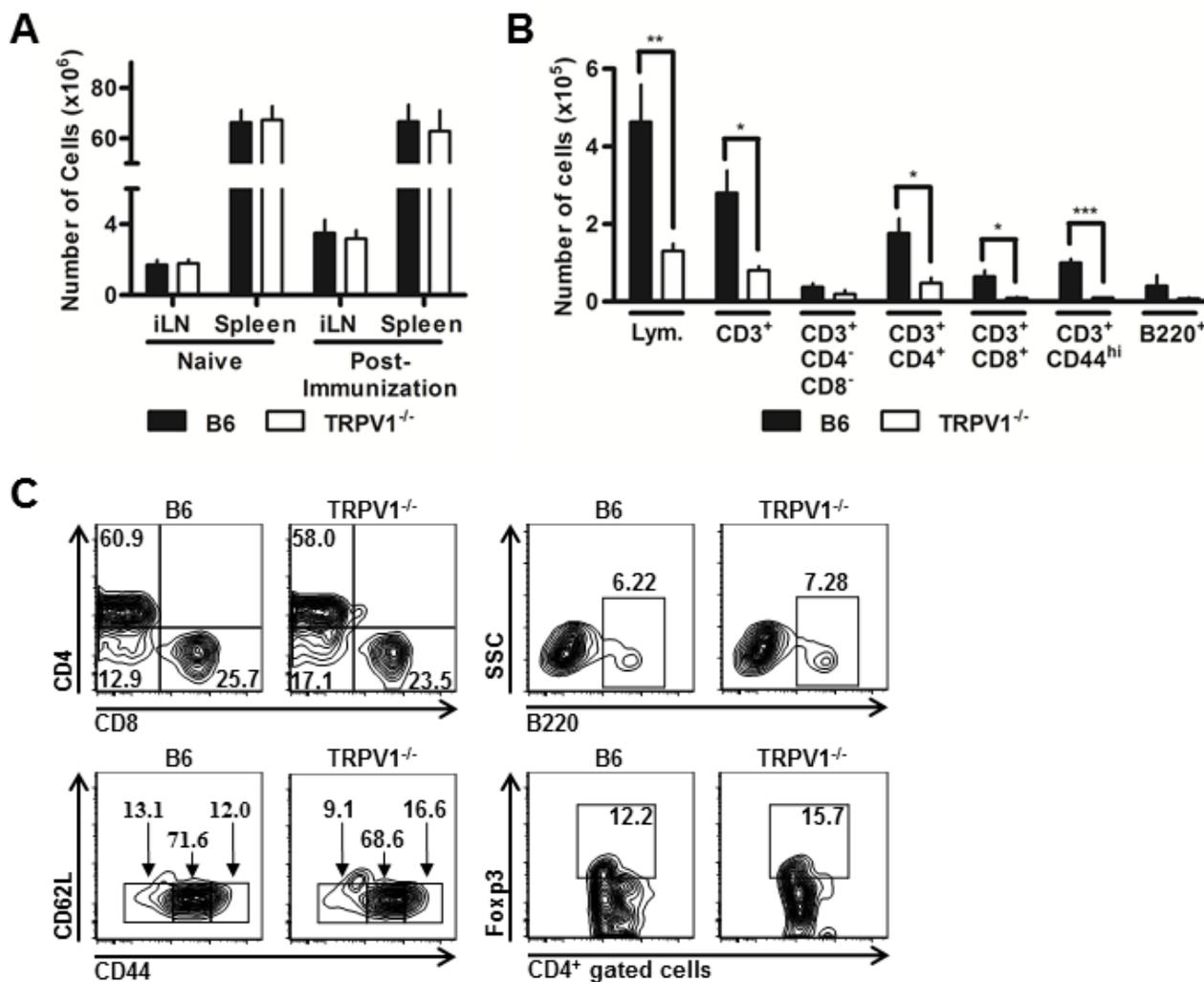


Figure 2.4 – TRPV1^{-/-} mice show reduced CNS infiltration. (A) Cell counts of inguinal lymph node and spleen prior to and following MOG₃₅₋₅₅ peptide immunization of B6 and TRPV1^{-/-} mice (n=9 per group). (B) Quantification of lymphocytes in the CNS by flow cytometry (n≥3 per group, *p<0.05, **p<0.01, ***p<0.001). (C) Representative FACS plot showing distribution of cells within the CNS; CD4⁺ and CD8⁺ cells previously gated on CD3⁺ cells (upper left), CD62L⁺ and CD44⁺ cells previously gated on CD3⁺ cells (lower left), B220⁺ cells (upper right), FoxP3⁺ cells previously gated on CD4⁺ cells (lower right).

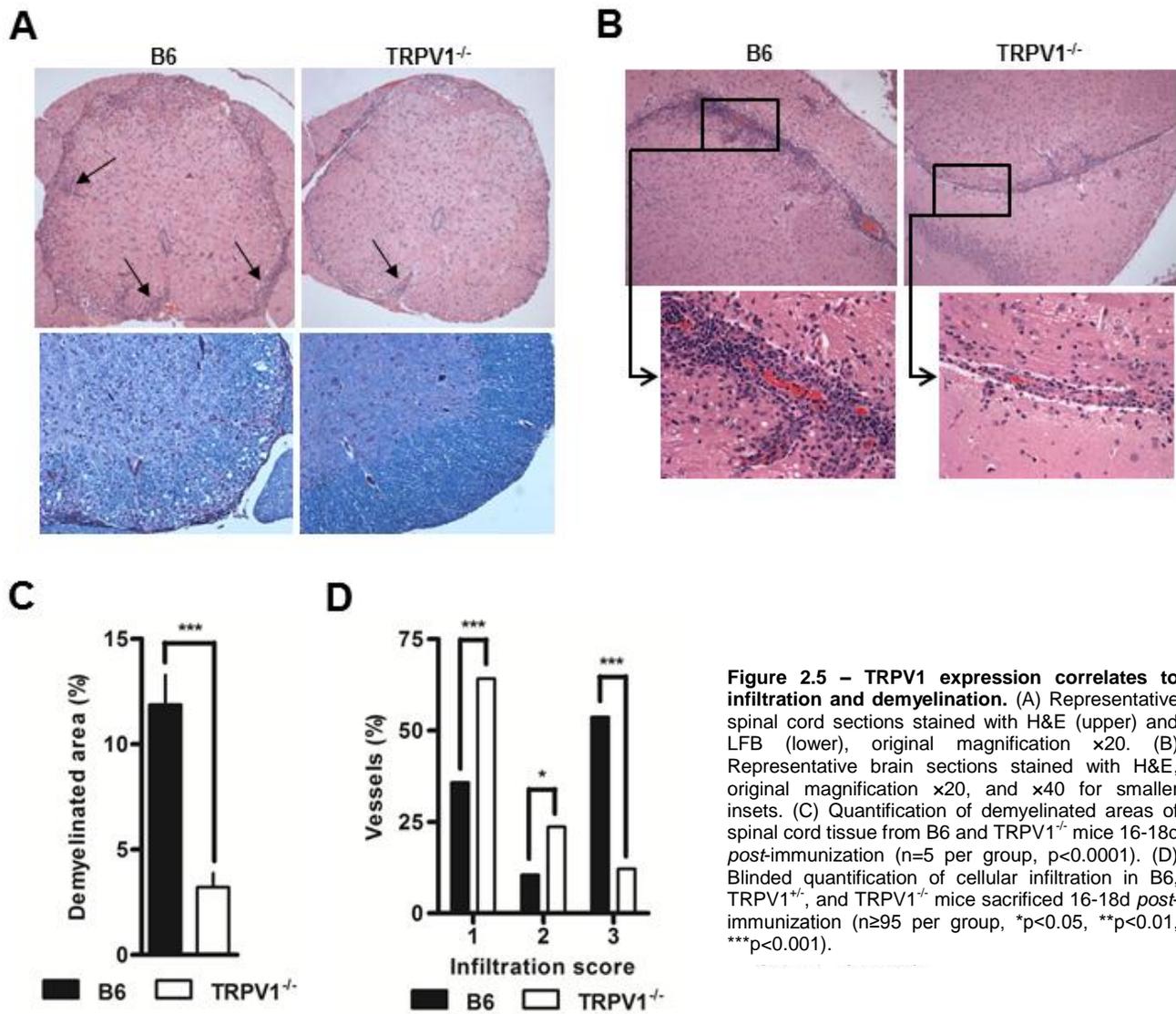


Figure 2.5 – TRPV1 expression correlates to infiltration and demyelination. (A) Representative spinal cord sections stained with H&E (upper) and LFB (lower), original magnification $\times 20$. (B) Representative brain sections stained with H&E, original magnification $\times 20$, and $\times 40$ for smaller insets. (C) Quantification of demyelinated areas of spinal cord tissue from B6 and TRPV1^{-/-} mice 16-18d post-immunization (n=5 per group, p<0.0001). (D) Blinded quantification of cellular infiltration in B6, TRPV1^{+/-}, and TRPV1^{-/-} mice sacrificed 16-18d post-immunization (n \geq 95 per group, *p<0.05, **p<0.01, ***p<0.001).

sustained over a 40d observation period (**Figure 2.6.A**). In contrast, injection of the same preparation of cells into TRPV1^{-/-} recipients, regardless of the donor, failed to generate similar disease penetrance (**Figure 2.6.A**). A majority of TRPV1^{-/-} recipients showed no clinical symptoms, and disease in mice with minor symptoms resolved quickly without progression. Intravenous adoptive transfer into wild type B6 mice generated lymphocyte infiltration in the brain and spinal cord (**Figure 2.6.B-C**), as early as day 4 *post*-injection, when greater total numbers of B220⁺ B cells and CD3⁺ T cells were already tissue-invasive, regardless of whether or not recipients were injected with pertussis toxin (**Figure 2.6.D-E**). Quantification of lymphocytes that were able to access the CNS revealed a >100% difference between B6 and TRPV1^{-/-} recipients (**Figure 2.6.F**). Given that TRPV1^{-/-} recipients are essentially unable to permit progressive pathogenicity, TRPV1 becomes the fundamental gate-keeper for *in vivo* disease development.

BSCB and BBB collapse in EAE is TRPV1-dependent and requires lymphocytes

Given the intimate proximity of TRPV1⁺ cellular PANEM elements at the BBB and BSCB, we monitored barrier integrity during EAE progression. We detected no increase in the permeability of Evans Blue prior to day 3 *post*-immunization (**Figure 2.7.A-B**). Evans Blue extravasation following injection of MOG₃₅₋₅₅ or of pertussis toxin alone was similar in brain and spinal cord regions of B6 and TRPV1^{-/-} mice, rarely, if at all, exceeding baseline day 0 levels (**Figure 2.7.A-B**). However, after immunization with MOG *plus* pertussis toxin, permeability was massive by day 11, particularly in the spine of B6 mice (**Figure 2.7.A-C**) and perhaps reminiscent of the recently discovered lumbar BSCB tissue entry pathway (521). Significantly lower leakage levels were seen in TRPV1^{-/-} mice, closely resembling those observed in lymphocyte-free recombination activating gene 1 (RAG1)^{-/-} B6 congenics (**Figure 2.8**). These observations extended to large molecule leakage, demonstrated by infrared-labelled IgG, with results comparable to Evans Blue extravasation (**Figure 2.7.D-E**).

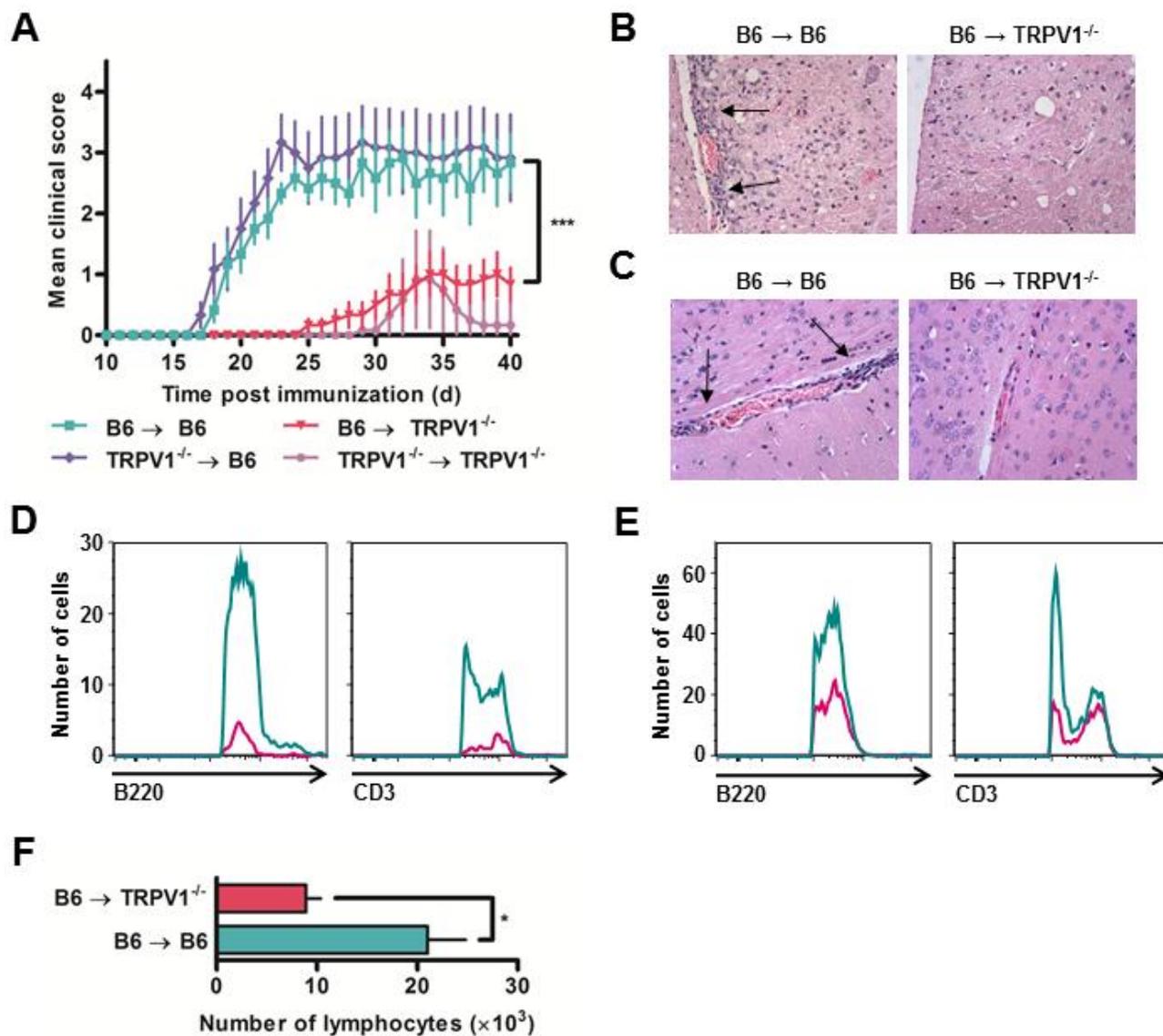


Figure 2.6 – TRPV1^{-/-} mice are protected from adoptive transfer of EAE. (A) Daily clinical scores of B6 and TRPV1^{-/-} recipient mice following the adoptive transfer of 1×10^7 donor cells ($n=6$ per group, $***p<0.0001$). (B-C) Representative H&E stained spinal cord (B) and brain (C) sections from B6 and TRPV1^{-/-} recipient mice 24d *post-transfer*, original magnification $\times 40$. (D-E) Number of B220⁺ and CD3⁺ cells within the CNS 4d *post-transfer* of B6 cells without (D) and with (E) injections of pertussis toxin. (F) Quantification of lymphocytes recovered from the CNS following adoptive transfer of B6 cells ($n=3$ per group, $*p<0.05$).

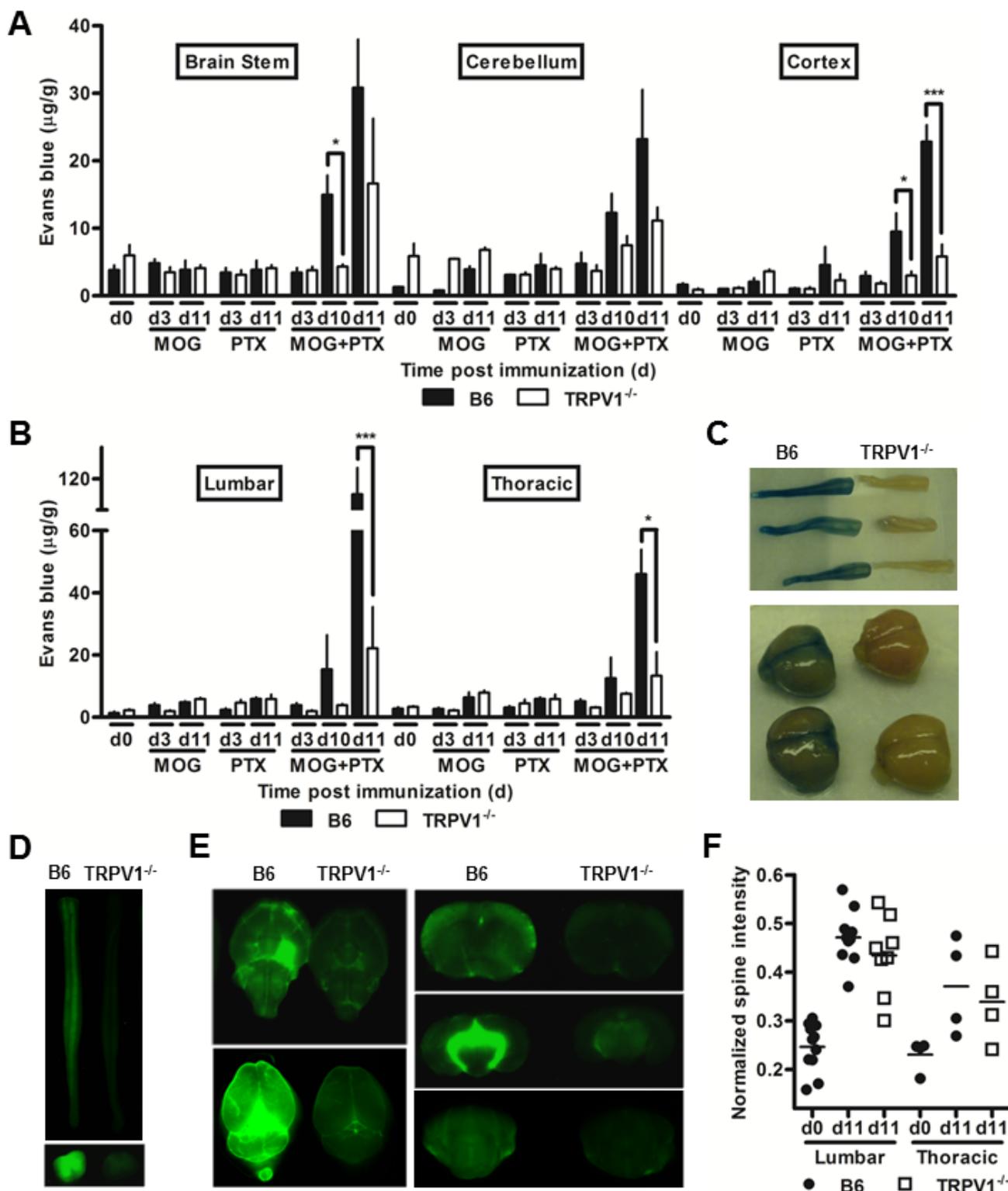


Figure 2.7 – TRPV1 increases the permeability of the BBB and BSCB. (A-B) Quantification of Evans Blue in regions of the brain (A, n=5 per group, *p<0.05, ***p<0.001) and the spinal cord (B, n=5 per group, *p<0.05, ***p<0.001) of B6 and TRPV1^{-/-} mice at indicated time points post induction of EAE, or post-injection with MOG₃₅₋₅₅ or pertussis toxin alone. (C) Representative images of brain and spinal cord tissue dissected from B6 and TRPV1^{-/-} mice 11d *post*-immunization following Evans Blue injection. (D) Representative images of B6 and TRPV1^{-/-} whole spinal cord (upper) and sectioned spinal cord (lower) following injection of infrared-labelled IgG 11d *post*-immunization. (E) Representative images of B6 and TRPV1^{-/-} whole brain (upper left: coronal and lower left: transverse) and sectioned brain (upper, middle, and lower right) following injection of infrared-labelled IgG 11d *post*-immunization. (F) Average intensity readings in the lumbar and thoracic regions of the spine following MRI of B6 and TRPV1^{-/-} mice 11d *post*-immunization.

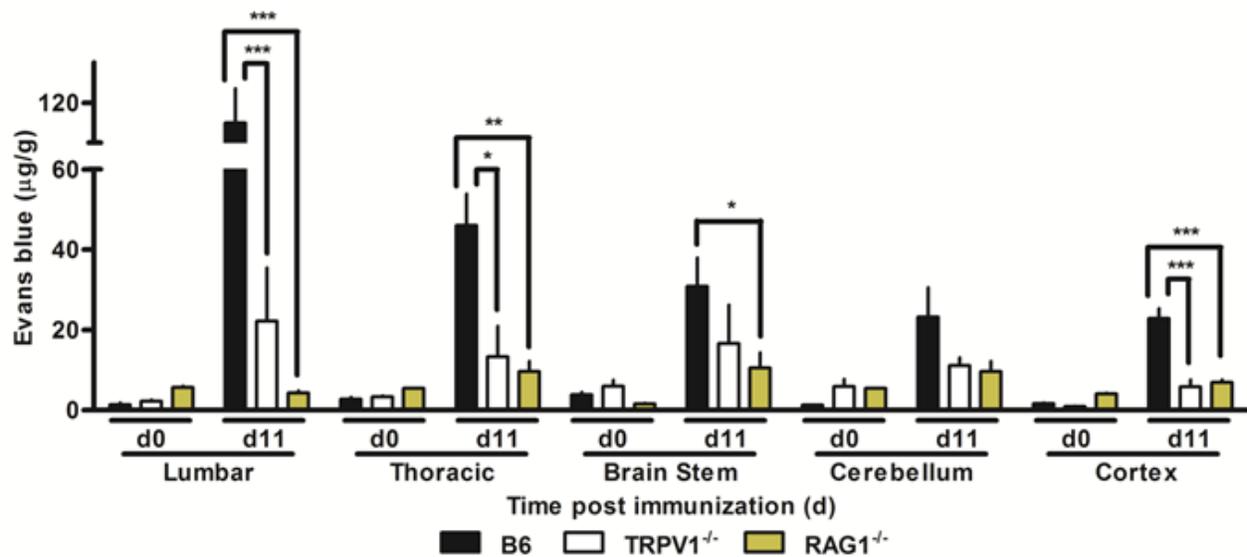


Figure 2.8 – Protection from BBB and BSCB breakdown in TRPV1^{-/-} mice is comparable to RAG1^{-/-} mice. Quantification of Evans Blue in regions of the spinal cord and the brain (n=5 per group, *p<0.05, **p<0.01, ***p<0.001) of B6, TRPV1^{-/-}, and RAG1^{-/-} mice at indicated time points *post*-immunization.

While large molecule permeability is dependent on transcytosis, we questioned the permeability of small molecules that normally depend on processes such as diffusion. The blood-spinal cord barrier is normally impermeable to magnetic resonance (MR) contrast agents such as the small (260Da) Gadolinium-DTPA reagent, but in cases of barrier disruption will appear hyperintense in T1-weighted MR images (522). MRI detected CNS edema sensitively but almost equally during EAE in B6 and in protected TRPV1^{-/-} mice, identifying a shared inflammatory element that is, however, self-limiting and without progressive tissue damage in the absence of TRPV1 (**Figure 2.7.F**).

We noted that the expression of numerous haematopoietic cell lineage genes is significantly upregulated compared to TRPV1^{-/-} mice, serving as an independent corollary of our data where B6 mice developed severe disease (**Figure 2.9.A-C**). B6 mice also showed increased transcription of numerous genes typically restricted to neuronal or non-hematopoietic tissue (**Figure 2.9.D**), providing a number of opportunities for future research investigating BSCB and BBB integrity. Thus, TRPV1-dependent BSCB and BBB compromise *per se* requires antigen, adjuvant, and lymphocytes, hallmarks of a cognate immune response.

A role for TRPV1, but not Tac1, in EAE and MS

In order to further elucidate the mechanism explaining protection seen in TRPV1^{-/-} mice, we induced active EAE in Tac1^{-/-} mice, which are deficient in sP, one of the main effector neuropeptides secreted following TRPV1 activation. Tac1^{-/-} mice did not have a comparable impact on disease, with clinical scores aligning very closely with B6 mice (**Figure 2.10**).

As we strive to identify links between mouse models and human disease, it was important that the studies described above be probed for possible parallels in MS patients. We chose a genomic approach, based on the extreme allelic polymorphism of the human TRPV1 locus (517), and analyzed a cohort of MS patients from the CCPGSMS trial (516). Measuring allelic variation in

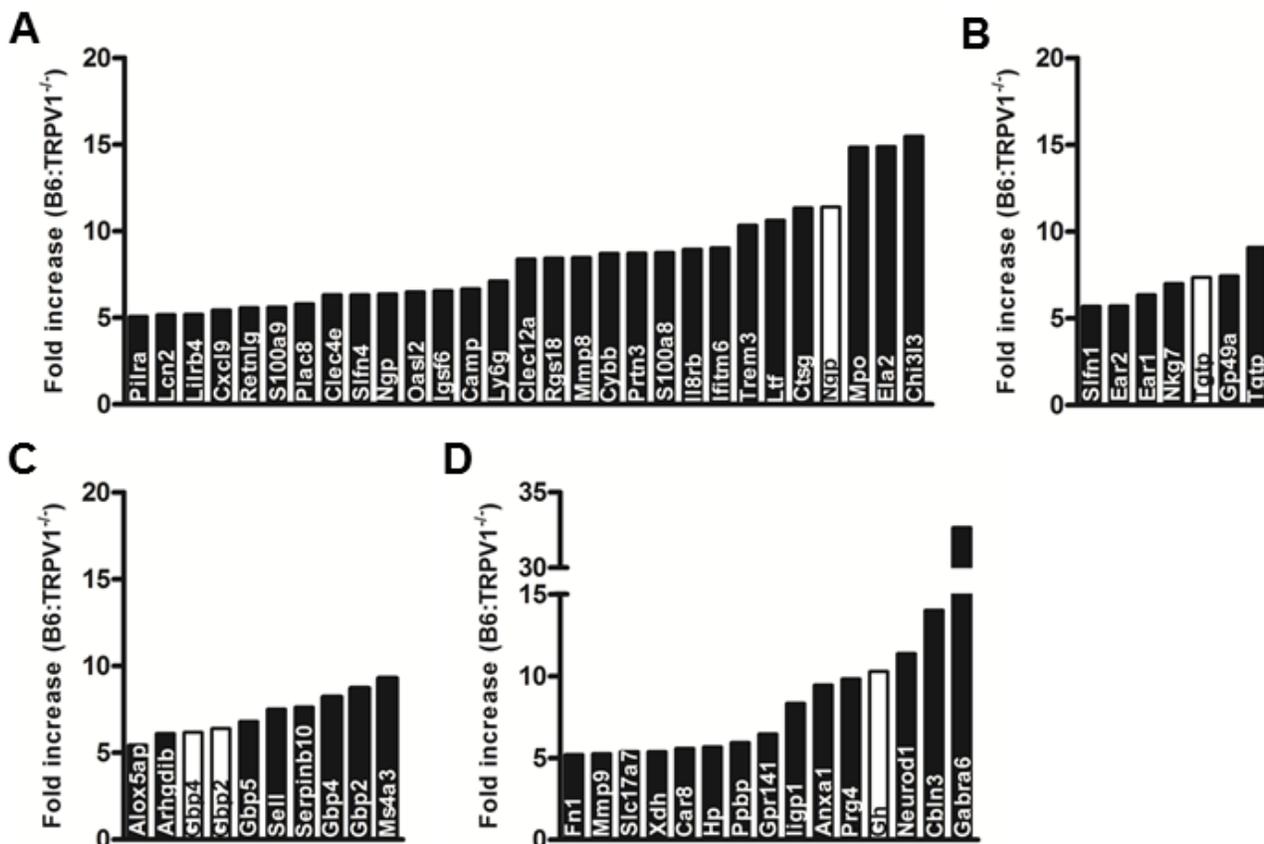


Figure 2.9 – Gene expression differences between B6 and TRPV1^{-/-} mice during early EAE. (A-D) Microarray results from the brain (white) and spinal cord (black) of B6 and TRPV1^{-/-} mice with a cut-off set at 5-fold or greater. Genes are segregated into those largely expressed by macrophages, dendritic cells, and neutrophils (A), T cells, B cells, NK cells, mast cells, and eosinophils (B), pan-immune (C), or predominantly non-immune (D). Pilra: paired immunoglobulin-like type 2 receptor alpha, Lcn2: lipocalin-2, Lilrb4: leukocyte immunoglobulin-like receptor subfamily B (with TM and ITIM domains) member 4, Cxcl9: chemokine (C-X-C motif) ligand 9, Retnlg: resistin like gamma, S100a9: S100 calcium binding protein A9, Plac8: placenta-specific 8, Clec4e: C-type lectin domain family 4 member E, Sifn4: schlafen 4, Ngp: neutrophilic granule protein, Oasl2: 2'-5' oligoadenylate synthetase-like 2, Igsf6: immunoglobulin superfamily member 6, Camp: cathelicidin antimicrobial peptide, Ly6g: lymphocyte antigen 6 complex locus G, Clec12a: c-type lectin domain family 12 member A, Rgs18: regulator of G protein signalling 18, Mmp8: matrix metalloproteinase 8, Cybb: cytochrome b-245 beta polypeptide, Prtn3: proteinase 3, S100a8: S100 calcium binding protein A8, Il8rb: interleukin 8 receptor beta, Ifitm6: interferon induced transmembrane protein 6, Trem3: triggering receptor expressed on myeloid cells 3, Ltf: lactotransferrin, CtsG: cathepsin G, Mpo: myeloperoxidase, Ela2: elastase 2, Chi3l3: chitinase 3-like-3, Sifn1: schlafen 1, Ear2: eosinophil-associated ribonuclease A family member 2, Ear1: eosinophil-associated ribonuclease A family member 1, Nkg7: natural killer cell group 7 sequence, Tgtp: T cell specific GTPase, Gp49a: glycoprotein 49 A, Alox5ap: arachidonate 5-lipoxygenase-activating protein, Arhgdib: rho GDP dissociation inhibitor beta, Gbp4: guanylate binding protein 4, Gbp2: guanylate binding protein 2, Gbp5: guanylate binding protein 5, Sell: L-selectin, Serpinb10: serpin peptidase inhibitor clade B (ovalbumin) member 10, Ms4a3: membrane-spanning 4-domains subfamily A member 3, Fn1: fibronectin 1, Mmp9: matrix metalloproteinase 9, Slc17a7: solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter) member 7, Xdh: xanthine dehydrogenase, Car8: carbonic anhydrase 8, Hp: haptoglobin, Pbbp: proplatelet basic protein, Gpr141: G protein-coupled receptor 141, ilgp1: interferon inducible GTPase1, Anxa1: annexin A1, Prg4: proteoglycan 4, Gh: growth hormone, Neurod1: neurogenic differentiation 1, Cbln3: cerebellin 3 precursor, Gabra6: gamma-aminobutyric acid receptor subunit alpha-6.

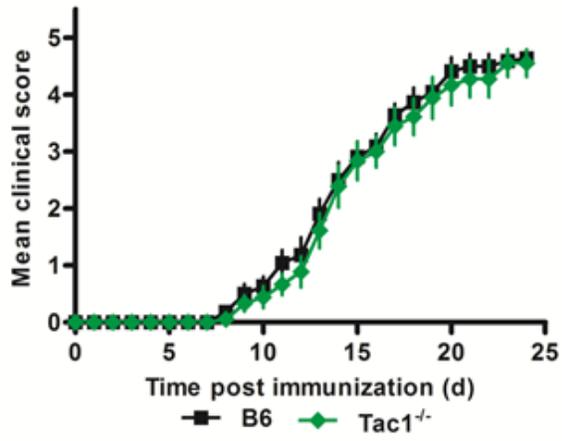


Figure 2.10 – Tac1^{-/-} mice are not protected from EAE. Daily clinical scores of B6 and Tac1^{-/-} mice following immunization with MOG₃₅₋₅₅ peptide (n≥9 per group).

TRPV1 and Tac1 genes, single nucleotide polymorphisms (SNPs) were compared in genomic DNA from MS cases at opposite extremes of long-term clinical outcomes, as assessed by the Expanded Disability Status Scale (EDSS). The MS cases selected represent the prognostic best 5%, classified as 'benign' MS, and the worst 5%, classified as 'malignant' MS. The difference between groups is dramatically reflected by mean age of onset and mean duration of disease (**Table 2.2**). Specific SNPs in the TRPV1 locus were either significantly over-represented in DNA of patients with malignant MS, or under-represented in the genomes of patients with benign MS (**Table 2.3**). Each possible scenario is being tested in larger DNA collections, but the data described here provide strong first evidence for a disease course-associated role of TRPV1 in MS. No significant disease association was found following the analysis of two SNPs present in the Tac1 gene (**Table 2.3**), in agreement with mouse studies.

<i>Clinical/Demographic Details</i>	<i>Benign MS</i>	<i>Malignant MS</i>
Sample Size (n)	112	51
Sex Ratio (F:M)	87:25 (3.48:1)	30:21 (1.43:1)
Mean Age of Onset (Years)	25.1	37.3
Mean Duration of Disease (Years)	26	3.6

Table 2.2 – Clinical and demographic data on benign and malignant MS patients.

<i>SNP</i>	<i>Allele Tested</i>	<i>Malignant Frequency</i>	<i>Benign Frequency</i>	<i>Chi</i>	<i>P (nominal)</i>	<i>Odds Ratio (95% CI)</i>
rs6465606	A	0.29	0.25	0.81	0.37	1.21 (0.8-1.84)
rs4526299	T	0.15	0.16	0.15	0.70	0.90 (0.54-1.52)
rs877610	T	0.09	0.04	5.43	0.02	2.43 (1.13-5.21)
rs8065080	C	0.30	0.34	0.53	0.47	0.86 (0.58-1.29)
rs224534	A	0.44	0.35	3.18	0.07	1.42 (0.97-2.07)

Table 2.3 – Tac1 and TRPV1 SNP frequencies in benign and malignant MS patients.

Discussion

Our inability to accurately predict the clinical course of MS, and subsequently tailor patient therapy, reflects our failure to fully understand disease etiology and pathogenesis. Here we identify TRPV1 as a major progression element in murine EAE and as a likely participant in MS.

We went to considerable efforts to determine if, as in T1D, the pathogenic role of TRPV1 had no detectable impact on disease-promoting autoimmune profiles, including numeric, functional and subset distributions of autoreactive lymphocyte lineages. TRPV1 may be expressed on antigen presenting cells (259, 261), but we conclude that it has negligible effects during the initiation and progression phases of EAE-autoimmunity. Conversely, the numeric size of T cell and B cell infiltrates in the CNS remains minuscule in the absence of TRPV1, despite equal pool sizes of disease-associated lymphocytes systemically. Similar results were observed following adoptive transfer of highly activated autoreactive cells, where B6 recipients developed acute disease and TRPV1^{-/-} recipients were almost entirely protected with sparse cellular infiltration while their lymphocytes were highly pathogenic in B6 hosts.

Our attention therefore shifted to the barriers that control lymphocyte egress into the CNS. Dramatic breakdown of BSCB and BBB integrity preceding peak disease was absent in TRPV1^{-/-} mice, suggesting that TRPV1 itself is a participant in the PANEM cluster that grants passage to effector cells. This effect on permeability was not all-encompassing, as small molecule permeability was still intact irrespective of TRPV1 expression. TRPV1 thus acts as an open-switch for tissue permeability gates in the nervous system, a view consistent with our previous observation of an extremely tight BBB in NOD mice (523), a mouse strain found years later to carry a hypofunctional TRPV1 mutant gene (279).

Genetic deletion of TRPV1 produced significant disease protection in B6 MOG₃₅₋₅₅ EAE, including day of onset, maximum clinical scores, and overall survival. Fittingly, one major genomic interval (*eae7*) that modulates the severity and duration of clinical signs during EAE (40, 42) includes TRPV1. It is likely that the contribution of TRPV1 in this locus is considerable, as precedence has been shown in another T cell-mediated autoimmune disease, T1D (*idd4.1*) (279). Intermediate disease scores in TRPV1^{+/-} mice support the idea that therapeutic targeting of TRPV1 by antagonists would, on face value, have a rationale, but might have to suppress significantly more than 65% of TRPV1 activity, i.e. the levels observed here in TRPV1^{+/-} heterozygotes.

Based on observations in the EAE model, we analyzed a total of 163 individuals from the CCPGSMS program who were stratified into two groups on opposite ends of the clinical spectrum, thus examining TRPV1 and Tac1 (sP-coding) genes as disease modifiers rather than determinants of overall disease risk, a comparison that generally yields more modest associations (38). Patients with the most severe progression showed an over-representation of certain TRPV1 SNPs. The nonsynonymous mutation rs877610 is located within the intracellular C-terminal domain of TRPV1 in proximity to known binding sites, while rs224534, which also results in an amino acid change, lies within an extracellular region between the first two transmembrane domains. It will take considerable sequencing efforts to more fully characterize the allelic heterogeneity of TRPV1 in general and in patient populations such as MS. Even though sP is functionally linked to TRPV1, Tac1 SNPs showed no bias in our patient population. In retrospect, our failure to find relevant Tac1 SNPs should not surprise, given our comparison within a disease population rather than comparing patients with normal controls as before (524, 525), and it coincides with the lack of EAE protection observed here in Tac1^{-/-} mice.

We have made several comparisons between encephalitis and T1D, given the relatively high prevalence, in some populations, of MS and T1D co-morbidity (526). The brain has the highest

energy budget among organs: 20% of the total energy for 2% of body mass (527). To use abundant glucose, the PANEM complex must sustain high volume one-way traffic of insulin that makes the PANEM complex an insulin-rich milieu comparable to some extent to pancreatic islets. Insulin receptor ligation by TRPV1⁺ terminals raises TRPV1 currents, resetting activation thresholds to room temperature (528, 529). Thus, there is potential for TRPV1 activation outside of its normal temperature constraints where specific TRPV1 alleles may confer a risk scenario. Alternatively, lipid TRPV1 agonists may also contribute to this process, as they have been reported to be upregulated in the brain and spinal cord during neuroinflammatory conditions such as EAE (530, 531).

Collectively, our data make TRPV1 a prominent factor controlling disease progression/severity in EAE. Although numerically limited, our human observations employed a stringently selected subset of a large and well-characterized, population-based patient cohort, linking differences in disease course to polymorphisms in the human TRPV1 locus. While considerable work will be required to characterize the TRPV1 locus in MS patients and their relatives, allele sequences are likely to be identified that can predict severe course and thus rationalize aggressive therapies. TRPV1 continues to be a challenging drug target in humans (532, 533), yet TRPV1-targeted treatments might have the potential to replace drug targeting of the immune system with its associated toxicities.

CHAPTER 3

Obesity Predisposes to IL-6 Dependent T_H17 Bias

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*These authors contributed equally to this work.

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Contributions: SW and GP designed and conducted the experimental mouse work, with constant help, advice, and supervision from senior members in the Dosch lab (HMD, YC, HT).

Abstract

Obesity is associated with numerous inflammatory conditions including atherosclerosis, autoimmune disease, and cancer. Although the precise mechanisms are unknown, obesity-associated rises in TNF- α , IL-6, and TGF- β are believed to contribute. Here we demonstrate that obesity selectively promotes the expansion of the T_H17 T cell sublineage, a population with prominent pro-inflammatory roles. Diet-induced obese (DIO) mice have larger CD4⁺ T_H17 cell pools and produce progressively more IL-17 than lean littermates in an IL-6-dependent manner. The T_H17 bias was associated with more severe experimental autoimmune encephalomyelitis (EAE), where DIO mice developed earlier and more progressive disease and histopathology, with increased IL-17 expression in target tissues. The well-described association of obesity with inflammatory and autoimmune disease is mechanistically linked to a T_H17 bias.

Introduction

The T_H17 cell lineage enhances protection against pathogens, including extracellular bacteria and fungi, which are not well protected against by the classic T_H1 or T_H2 effector pathways. T_H17 cells are characterized by expression of the retinoic acid receptor-related orphan receptor γ t (ROR γ t) transcription factor and the production of IL-17 family members, IL-21, and IL-22 (534). Both IL-17A and IL-17F, which exhibit a high degree of sequence homology, can induce the expression of proinflammatory cytokines such as IL-1, IL-6, and TNF- α , and C-X-C motif chemokine ligands (CXCL) such as CXCL8 and CXCL1 (535). IL-17 exerts its effects in a number of cell types due to wide receptor expression.

T_H17 cell differentiation was revealed, in part, by early studies examining mice deficient in selective IL-12 family member subunits, which allowed for the distinction of IL-12 versus IL-23 effects, and the role of the latter in T_H17 commitment (536). IL-23 was subsequently determined to be dispensable for T_H17 cell development, but effective in amplifying the T_H17 cell phenotype (537). Instead, TGF- β was shown to be critical for the development of T_H17 cells, and IL-6 decisively intervenes to override TGF- β signals from T regulatory (Treg) cell development to T_H17 cell development (538, 539). TGF- β and IL-6 therefore act cooperatively and independently to induce T_H17 lineage commitment.

In addition to their role in host defense, CD4⁺ T_H17 cells and their effector cytokines are key mediators of inflammatory and autoimmune diseases such as inflammatory bowel disease (IBD) and psoriasis (540, 541). Evidence also demonstrates a crucial, pathogenic role for IL-23 in solid tumour animal models (542). Yet the most conclusive evidence for disease modulation by T_H17 cells may come from studies in Multiple Sclerosis (MS) and its primary animal model, experimental

autoimmune encephalomyelitis (EAE), where T_H17 populations are critical to disease severity, progressive demyelination, and neuronal damage (110-112).

Epidemiological evidence has demonstrated the association between numerous proinflammatory conditions and obesity (543). The link with cancer is particularly strong, with obesity emerging as a premier risk factor (544). Men and women with a body mass index (BMI) greater than 40 are shown to have an increased risk of death from esophagus, colon and rectum, liver, gallbladder, pancreas, and kidney cancer (545). Obesity also predisposes to several autoimmune disorders, including IBD and psoriasis (546, 547), while the link to other diseases has not yet been investigated or properly established. Intriguingly, obesity in both humans and diet-induced obese (DIO) mice leads to an elevation in serum and tissue levels of IL-6 (385, 548). Adipocytes and adipose tissue-derived macrophages both contribute significantly to this increased IL-6 expression, estimated at approximately 33% and 20%, respectively (394).

We therefore explored the potential connection between obesity and T_H17 cell expansion and its functional sequelae. We show that T_H17 cells are expanded in obese mice. This process is IL-6 dependent, demonstrated by IL-6 deficient ($IL-6^{-/-}$) mice which do not exhibit a similar increase in T_H17 populations. Enhanced IL-17 production is relevant to autoimmune disease, as DIO mice progress to more severe EAE and increased central nervous system (CNS) infiltration. These novel results provide insights into the relationship between obesity, T_H17 cells, and autoimmune disease. Similar relationships should be examined in MS patients and considered for disease prevention or future treatments.

Methods

Mice

All experiments were performed in male C57BL/6J (B6) mice. WT and IL-6^{-/-} animals were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in our vivarium in a pathogen-free, temperature controlled, 12hr light and dark cycle environment. Animals were fed either a regular diet or a high fat diet composed of 60 kcal% fat (Research Diets). DIO mice received regular diet for the first 6wk of life and then high fat diet for the following 8-10wk. In EAE experiments, 16wk old DIO mice were maintained on high fat diet throughout the experiments. All studies reported here stringently followed approved protocols and were in agreement with animal ethics guidelines.

Diet and metabolic studies

Mice on regular diet and high fat diet were weighed regularly. After 8wk, we measured fasting blood glucose by glucometer readings (LifeScan Canada Ltd.) and insulin concentrations in serum (Crystal Chem ELISA). For glucose tolerance tests (GTT), mice were fasted for 16hr overnight and then injected intraperitoneally with 0.75 g of glucose (Sigma-Aldrich) per kilogram of body weight. For insulin tolerance tests (ITT), mice were given 0.75 U of human regular insulin (Eli Lilly) per kilogram of body weight. Glucose readings for both GTT and ITT were taken at times indicated using a glucometer. To measure the mass of VAT and SAT, fat pads were carefully dissected from mice and immediately weighed.

Induction of EAE

EAE was induced in 16wk old male B6 mice. Mice were immunized in each flank with 100 µg of MOG₃₅₋₅₅ peptide (Alpha Diagnostic) emulsified 1:1 in Complete Freund's Adjuvant (Sigma-Aldrich). In total, 200 ng of pertussis toxin (Sigma-Aldrich, Oakville, ON, Canada) was given

intraperitoneally on the day of EAE induction and 48hr later. Animals were followed for a minimum of 25d and disease was scored using the following scale: 0 - asymptomatic; 1 - limp tail; 2 - abnormal righting reflex and/or hind limb weakness; 3 - unilateral hind limb paralysis; 4 - bilateral hind limb paralysis; and 5 - moribund or death.

Histology

Fat pads used for adipocyte size quantification were removed, fixed for up to 72hr in 10% buffered formalin, and then stained with hematoxylin and eosin. Adipocyte diameter was measured with the straight-line tool in Image SXM software. At least 200 adipocytes were quantified from two different tissue sections per mouse.

Spinal cords and brains were removed 25d after EAE induction and were fixed for 24hr in 10% buffered formalin. Tissues were then stained with hematoxylin and eosin (H&E). Histology was scored by two blinded observers and the average score was used. The following scoring system for spinal cord histology was employed: 0 - unremarkable; 1 - focal mononuclear infiltration; 2 - mononuclear infiltration in <10% of white matter; 3 - mononuclear infiltration in 10–20% of white matter; and 4 - infiltration in >20% white matter.

Isolation of splenocytes and CNS mononuclear cells

Spleens were dissected from mice 25d after EAE induction and minced in a Stomacher blender. Red blood cells were lysed using pure water, followed by restoration of osmolarity using 10× PBS. Cells were then filtered and counted using a haemocytometer. Brains and spinal cords were removed 25d after EAE induction and minced in a Stomacher blender. The suspension was then incubated with 1 mg/ml collagenase (Sigma-Aldrich) for 1hr at 37°C with gentle resuspension every 5min. The suspensions were then pelleted and suspended in 4 mL of 30% Percoll (Sigma-Aldrich) and centrifuged at 1500 rpm for 15min over 4 mL of 70% Percoll. The mononuclear cells

at the 30%/70% Percoll interface were collected and washed two times in serum-free HL-1 medium, supplemented with 2 mM L-glutamine (Sigma-Aldrich) and antibiotics (Sigma-Aldrich).

Proliferation and cytokine secretion

4×10^5 inguinal lymph node cells or splenocytes were incubated for 72hr at 37°C in 96-well plates, pre-coated with 1 $\mu\text{g}/\text{mL}$ $\alpha\text{-CD3}$ plus 0.25 $\mu\text{g}/\text{mL}$ $\alpha\text{-CD28}$ (BD Pharmingen) or varying concentrations of MOG₃₅₋₅₅ peptide. In proliferation experiments, 1 μCi of [³H] thymidine was added for the last 18hr prior to harvesting and liquid scintillation counting. Alternatively, supernatants were collected after 72hr of culture and IFN- γ (BD Biosciences) and IL-17 and IL-13 (R&D Systems) were measured by ELISA according to the manufacturer's protocols.

Flow cytometry

1.5×10^6 splenocytes or lymph node cells were incubated at 4°C for 15min in 100 μL 2% FBS/PBS with 10 $\mu\text{g}/\text{mL}$ Fc-blocker (eBioscience). Cells were then incubated for 30min with the following fluorescent antibodies: CD4 (1:200), IL-17 (1:150), IFN- γ (1:100), and FoxP3 (1:100) (eBioscience). For intracellular IL-17 or IFN- γ staining, all cells were incubated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and 750 ng/mL ionomycin (Calbiochem) for 6hr in HL-1 media at 37°C and golgi blocked for the last 3hr (BD Bioscience). Samples were run on an LSR-II flow cytometer (BD Biosciences) and FACS plots were analyzed using FlowJo software (Tree Star Inc.).

Statistical analysis

Statistical significance between two means was assessed by Mann-Whitney and unpaired t-tests. Welch correction on t-tests was employed for sample sizes <6. Comparisons of curves were performed using two-way ANOVA or life tables. 2x2 tables were analyzed with Fisher's Exact test. Statistical significance was two tailed and set at 5%, all error bars show a single standard deviation.

Results

Obesity promotes T_H17 sublineage bias

Obesity results in abnormalities of numerous metabolic parameters including increased adiposity and defective insulin secretion and action. DIO mice showed significantly greater weight gain and visceral and subcutaneous adipose tissue (VAT and SAT, respectively) hypertrophy after 8wk on high-fat diet (HFD) (**Figure 3.1.A-C**). DIO mice also exhibited higher fasting glucose and insulin levels, and responded poorly following intraperitoneal challenge with glucose and insulin (**Figure 3.1.D-G**), typical of widespread glucose intolerance and insulin resistance.

To explore the relationship between obesity and T_H17 cells, we compared the proportions of CD4⁺ T_H17 cells in naïve spleen cells from DIO and regular diet (RD) mice. There were approximately 3× more IL-17 secreting CD4⁺ T cells in DIO mice compared to age-matched, non-immunized mice on regular diet (**Figure 3.2.A-B**). This expansion was T_H17 sublineage-selective, as DIO did not affect systemic pool sizes of CD4⁺, IFN-γ⁺ T_H1 cells, GATA-3⁺ T_H2 cells, or Foxp3⁺ T regulatory cells (**Figure 3.2.C-E**). The enlarged T_H17 cell pools in DIO mice were functional, with enhanced IL-17 production after *in vitro* splenocyte stimulation with α-CD3 plus α-CD28 despite comparable proliferation, while IFN-γ and IL-13 secretion was similar between RD and DIO cultures (**Figure 3.2.F-I**).

Obesity-driven T_H17 expansion is IL-6 dependent

Thus, DIO generates an immunological phenotype with selective expansion of the T_H17 sublineage. However, absolute frequencies of T_H17 cells were expectedly small (534, 549) and we therefore questioned whether this T_H17 sublineage bias is maintained and relevant during active T cell priming and expansion. We measured T_H17 and T_H1 cell expansion in draining lymph nodes

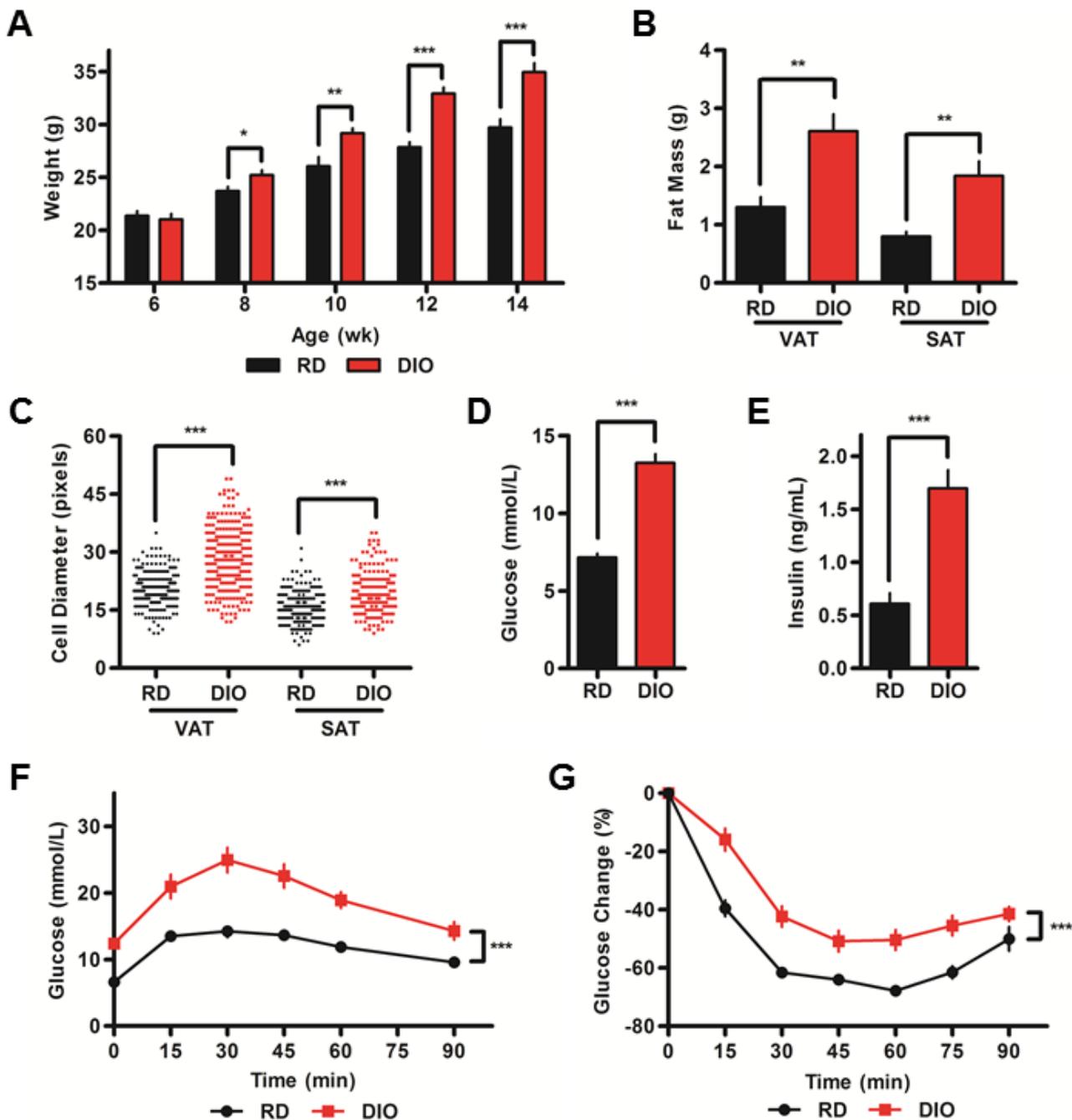


Figure 3.1 – DIO significantly increases weight gain and impairs glucose homeostasis. (A) Body weights of RD or DIO B6 mice 14wk old (n=10 per group, *p<0.05, **p<0.01, ***p<0.001). (B) Weight of epididymal VAT and inguinal SAT fat pads from 14wk old RD or DIO B6 mice (n=5 per group, **p<0.01). (C) Quantification of VAT and SAT fat cell diameter of 14wk old RD or DIO B6 mice (n≥200 per group, ***p<0.001). (D-E) Fasting glucose (D, n=10 per group, ***p<0.001) and fasting insulin (E, n=10 per group, ***p<0.001) of 14wk old RD or DIO B6 mice. (F-G) Glucose tolerance test (F, n=10 per group, ***p<0.001) and insulin tolerance test (G, n=10 per group, ***p<0.001) of 14wk old RD or DIO B6 mice.

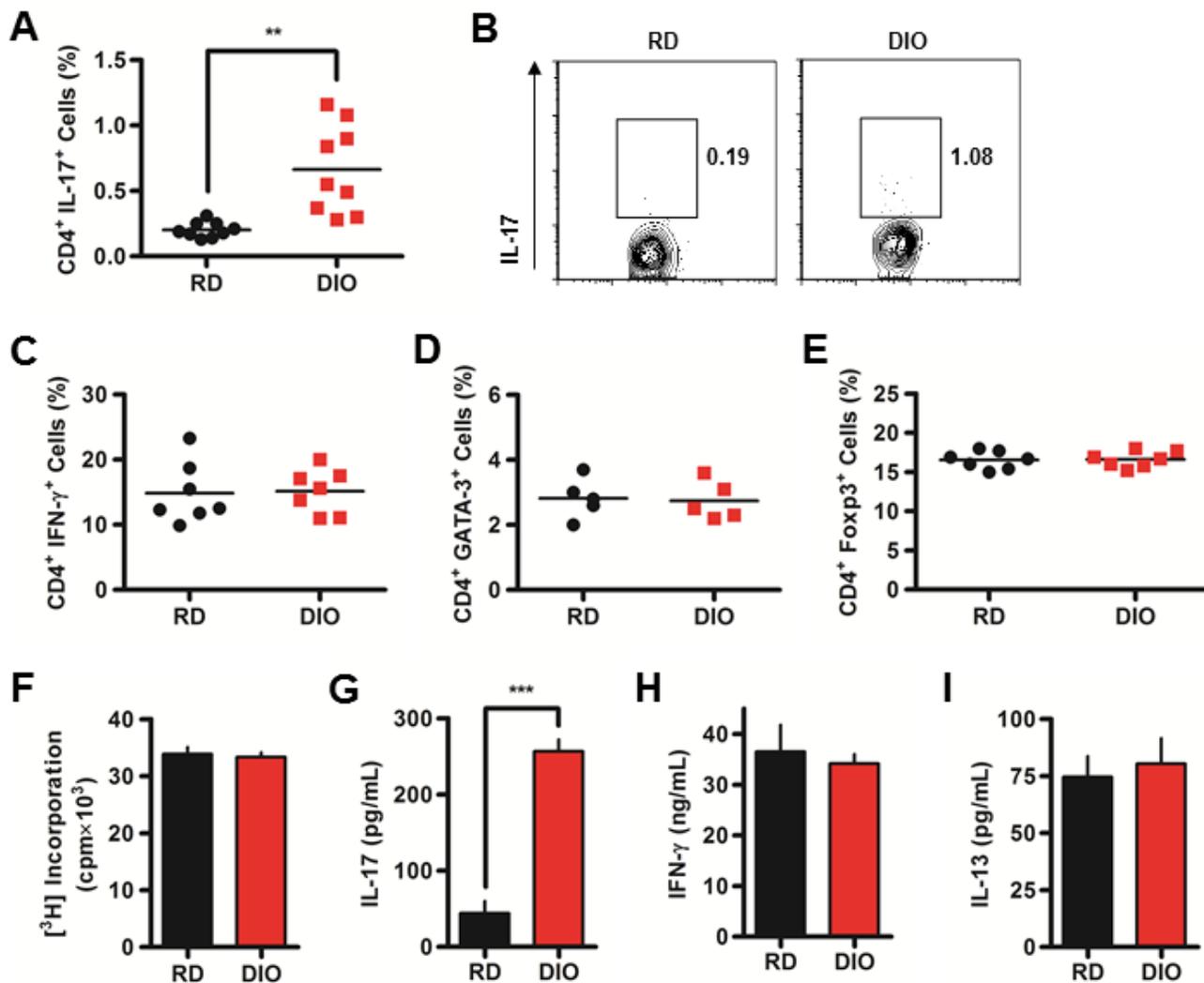


Figure 3.2 – Obesity is associated with a bias towards IL-17 production in naïve mice. (A-B) The percentage of CD4⁺ IL-17⁺ splenocytes from 16wk old RD and DIO mice (A, n=9 per group, **p<0.01), with representative FACS plots shown on the right (B). (C-E) The percentage of IFN-γ⁺ (C), GATA-3⁺ (D), and FoxP3⁺ (E) cells of 16wk old RD and DIO B6 mice, previously gated on CD4⁺ cells (n≥5 per group). (F) [³H]-thymidine incorporation following activation of purified splenic CD4⁺ T cells from RD and DIO mice stimulated with α-CD3 and α-CD28 or α-CD40 antibodies (n=5 per group). (G-I) Secretion of IL-17 (G), IFN-γ (H), IL-13 (I) by splenic CD4⁺ T cells from RD and DIO mice following α-CD3 and α-CD28 stimulation (n≥5 per group, ***p<0.0001).

following immunization of DIO and RD mice with a well characterized H-2^b binding peptide, myelin oligodendrocyte glycoprotein 35-55 (MOG₃₅₋₅₅) (550). Six days after immunization, lymphocytes from draining lymph nodes of DIO mice generated a dramatically increased pool size of CD4⁺ IL-17⁺ cells compared to lymph node cells from RD mice (**Figure 3.3.B and D**). In contrast, the CD4⁺ IFN- γ ⁺ T cell compartment was unaffected by DIO (**Figure 3.3.A and C**).

T_H17 development can proceed through both IL-6-dependent and IL-6-independent pathways, the latter driven by IL-21 and TGF- β (551, 552). We therefore placed IL-6^{-/-} mice on high fat diet and immunized with MOG₃₅₋₅₅ as above. Although DIO IL-6^{-/-} mice gained weight at similar rates as DIO WT mice (**Figure 3.3.E**), DIO IL-6^{-/-} mice did not develop the T_H17 bias exhibited by their DIO WT counterparts (**Figure 3.3.B and D**). These results indicate that obesity-induced T_H17 bias utilizes the IL-6-dependent pathway for T_H17 development.

DIO exacerbates EAE

We analyzed the clinical impact of DIO on EAE, given the prominent role of T_H17 cells in autoimmune encephalitis (111, 116). Both RD and DIO WT mice immunized with MOG₃₅₋₅₅ peptide showed initial clinical symptoms approximately 10d later. However, disease was progressively worse in DIO mice (**Table 3.1 and Figure 3.4.A**) and a greater proportion of mice became moribund and were euthanized (**Figure 3.4.B**). Approximately half of DIO mice reached disease scores greater than three by day 13 compared to minimal numbers of RD mice (**Figure 3.4.C**). DIO also worsened spinal cord inflammation with more pronounced inflammatory cell penetration in the white matter of mice with severe disease (**Figure 3.4.D-E**).

T cell recall responses to MOG₃₅₋₅₅ in EAE mice did not differ between DIO and RD mice (**Figure 3.5.A-B**), suggesting that the immunization was equally effective. However, T cells from DIO mice had significantly higher IL-17, but not IFN- γ , production than RD mice in the spleen,

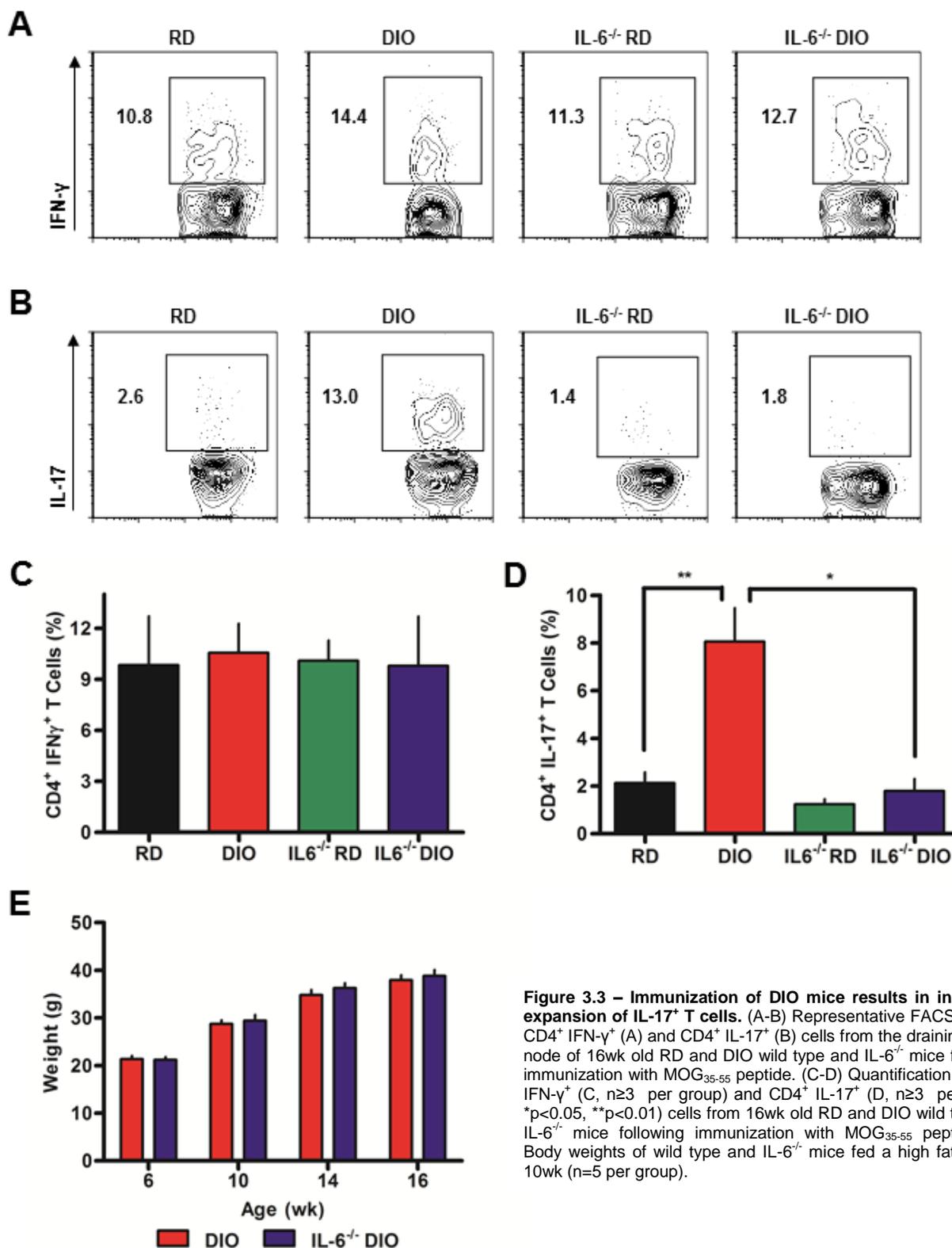


Figure 3.3 – Immunization of DIO mice results in increased expansion of IL-17⁺ T cells. (A-B) Representative FACS plots of CD4⁺ IFN-γ⁺ (A) and CD4⁺ IL-17⁺ (B) cells from the draining lymph node of 16wk old RD and DIO wild type and IL-6^{-/-} mice following immunization with MOG₃₅₋₅₅ peptide. (C-D) Quantification of CD4⁺ IFN-γ⁺ (C, n≥3 per group) and CD4⁺ IL-17⁺ (D, n≥3 per group, *p<0.05, **p<0.01) cells from 16wk old RD and DIO wild type and IL-6^{-/-} mice following immunization with MOG₃₅₋₅₅ peptide. (E) Body weights of wild type and IL-6^{-/-} mice fed a high fat diet for 10wk (n=5 per group).

	<i>Incidence</i>	<i>Mean Day of Onset</i>	<i>Mean Maximum Score</i>
RD	13/13 (100%)	10.462 ± 0.447	3.269 ± 0.466
DIO	14/14 (100%)	9.786 ± 0.239	4.286 ± 0.233

Table 3.1 – Summary of EAE severity in RD and DIO mice.

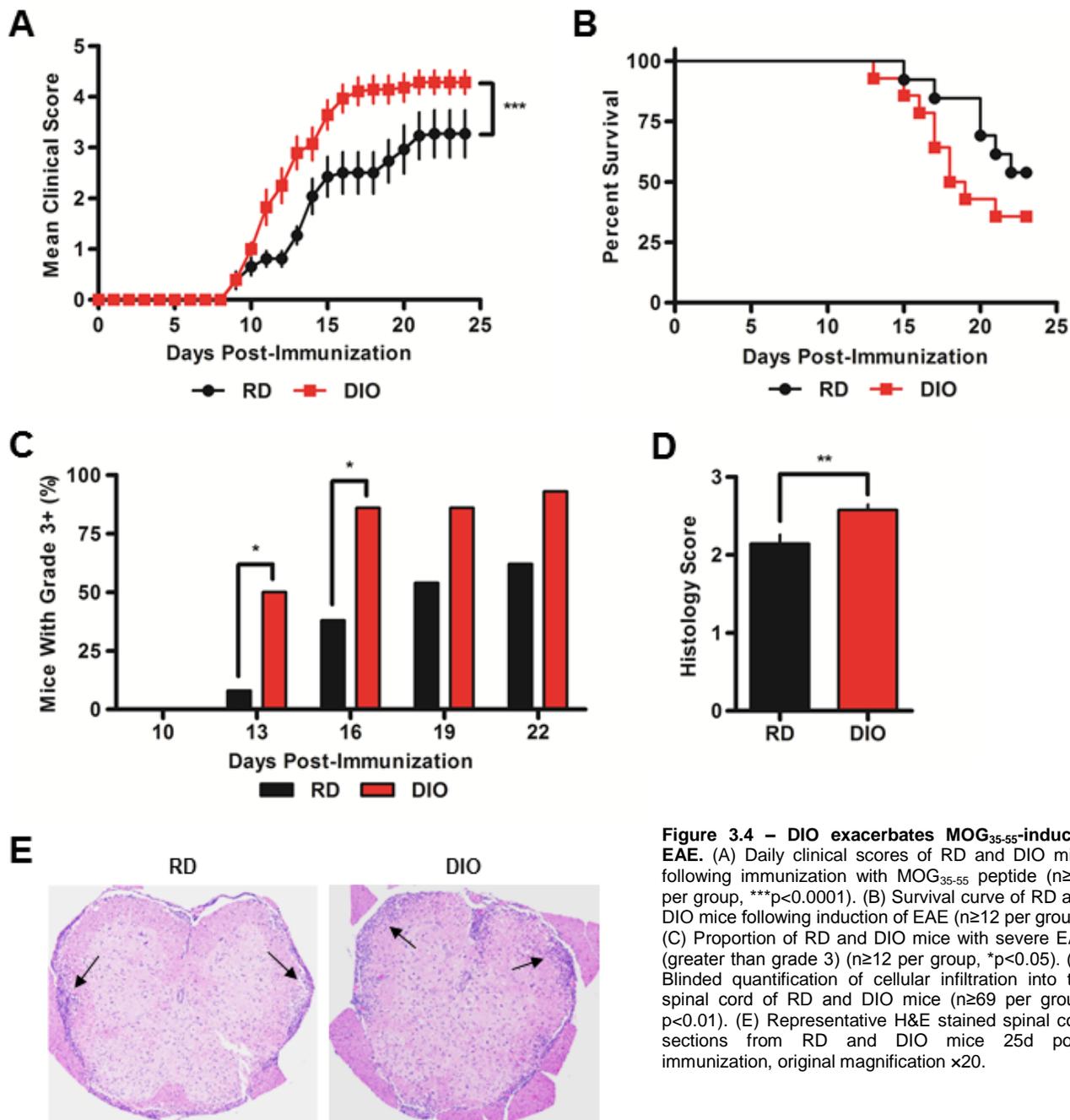


Figure 3.4 – DIO exacerbates MOG₃₅₋₅₅-induced EAE. (A) Daily clinical scores of RD and DIO mice following immunization with MOG₃₅₋₅₅ peptide (n≥12 per group, ***p<0.0001). (B) Survival curve of RD and DIO mice following induction of EAE (n≥12 per group). (C) Proportion of RD and DIO mice with severe EAE (greater than grade 3) (n≥12 per group, *p<0.05). (D) Blinded quantification of cellular infiltration into the spinal cord of RD and DIO mice (n≥69 per group, p<0.01). (E) Representative H&E stained spinal cord sections from RD and DIO mice 25d post-immunization, original magnification ×20.

draining inguinal lymph nodes (LN), and the CNS target tissue (**Figure 3.5.C-D**). Obesity-dependent T_H17 expansion was thus associated with progressively enhanced autoimmune disease and CNS lesions.

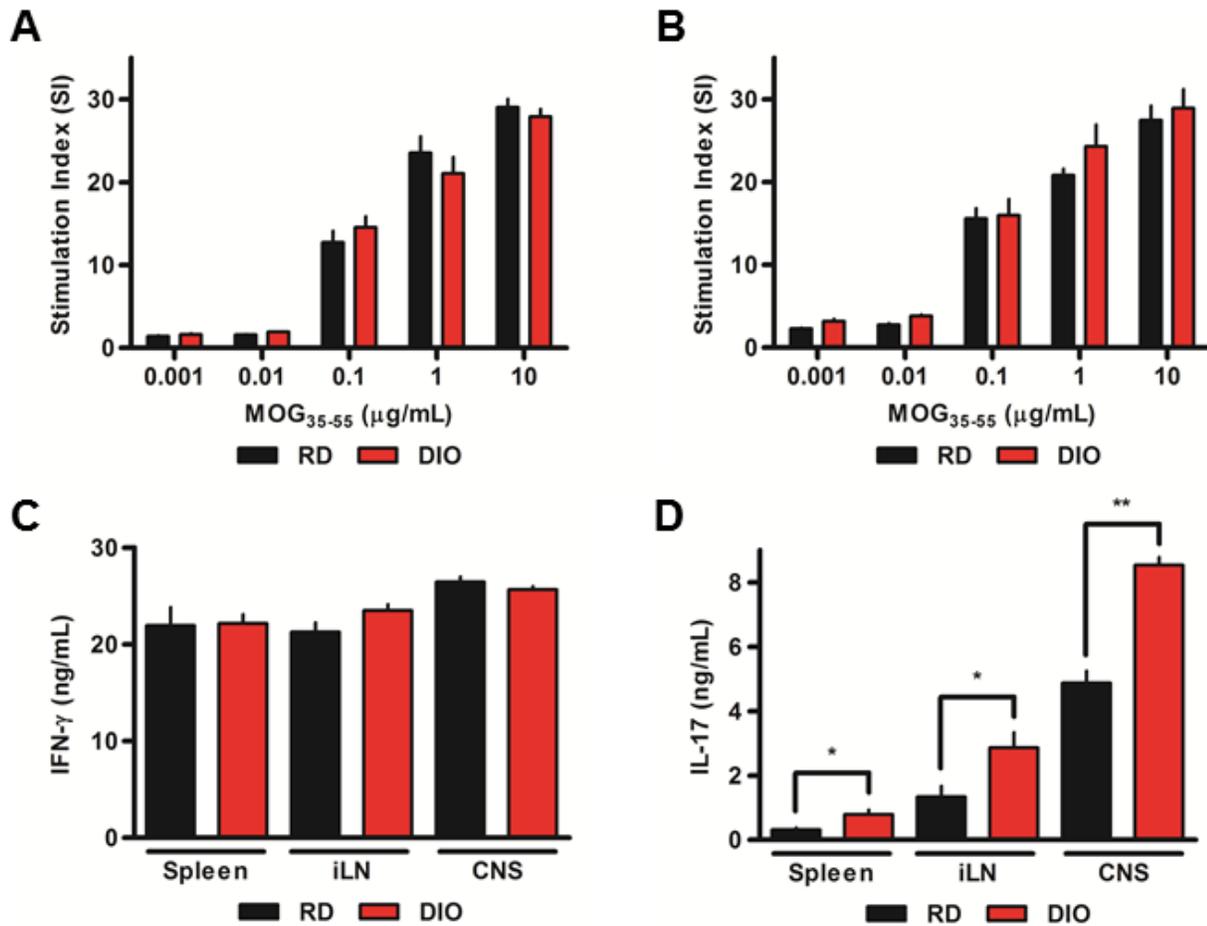


Figure 3.5 – Systemic and CNS-infiltrating lymphocytes produce increased levels of IL-17 during severe EAE. (A) Proliferation following MOG₃₅₋₅₅ peptide restimulation of inguinal lymph node cells (n=3 per group) from RD and DIO mice sacrificed 25d post-immunization. (B) Proliferation following restimulation of splenocytes 25d post-immunization (n=3 per group). (C-D) IFN-γ (C, n=4 per group) and IL-17 (D, n=4 per group, *p<0.05, **p<0.01) secretion by purified lymphocytes from the spleen, inguinal lymph nodes, and CNS following MOG₃₅₋₅₅ peptide restimulation.

Discussion

As the incidence of obesity increases worldwide, so will its complications and associated disorders. There is an established link between obesity and proinflammatory diseases including psoriasis, IBD, and asthma, all containing a significant T_H17 component (546, 547, 553, 554). In addition to autoimmune disease, obesity has been associated with risk to develop neoplastic disorders (544). IL-23 and its downstream T_H17 effector cells may promote cancer through the inhibition of anti-tumour CD8⁺ T cells, and through enhanced angiogenesis and production of tumour-promoting matrix metalloproteinase (MMP)-9 (542, 555).

Conversely, there is scant information on a conceivable linkage between obesity and MS, though a reduction in neurological disability was shown in patients on a low-fat diet compared to patients on a high fat diet (556). Data presented here suggest that this association is worth investigating, given the role for T_H17 effectors in the penetration of the blood-brain barrier and neuronal pathology (111).

T_H17 development requires signals that lead to increased intracellular levels of signal transducer and activator of transcription (STAT) 3. While several cytokines can promote T_H17 expansion (549), we demonstrate here that obesity-associated T_H17 expansion is fundamentally IL-6 dependent. IL-6 is produced in large amounts following innate immune activation and is critical in diverting uncommitted, naïve T cells to the T_H17 phenotype. In the absence of IL-6, uncommitted progenitor cells will default to the Treg pathway in the presence of TGF- β . IL-6 and TGF- β effects are therefore contextual, dependent on the surrounding cytokine milieu. Other cytokines may further amplify this role of IL-6 in obesity. Indeed, serum amyloid A, an acute phase protein highly elevated in overweight individuals, has been shown to increase dendritic cell IL-23 production *in vitro* (557).

We applied our observations and demonstrated that DIO and subsequent rises in IL-6 and T_H17 cells result in exacerbated inflammatory disease. DIO did not accelerate disease initiation, but rather worsened early disease progression. The high prevalence of obese patients with spontaneous proinflammatory disorders may indicate an additional role of the T_H17 axis in disease initiation that was not apparent in our disease model.

Additional observations from a small clinical study demonstrate that obese patients exhibit elevated IL-17 levels in serum (558), suggesting that our results in rodents are likely relevant to human disease. Future research may also determine whether the increased frequency of T_H17 effector cells in DIO mice causes autoimmune exacerbation directly or through involvement of other effector cells/mechanisms that could identify new therapeutic targets. DIO is reversible and it should be interesting to assess, to what extent and at which disease stage, weight loss can reduce T_H17 expansion and disease progression.

We have established an inter-organ system of communication with pathological implications.

CHAPTER 4 (A)

B Lymphocytes Promote Insulin Resistance through Modulation of T Lymphocytes and Production of Pathogenic IgG Antibody

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Contributions: This project was an intense collaboration between the Engleman lab and the Dosch lab. *In vivo* mouse studies were done by myself and members of the Dosch lab (HMD, SW, JY, GP, HT, PW) where the principal ideas originated, while antibody studies and flow cytometry were done at Stanford, USA (EGE, DAW, LS, PPW, MGD, MNA, HL, AG, MC, JK, TM, DBM). Anti-CD20 antibody was provided by TFT. I worked closely with senior members of the Dosch lab for this study, a follow-up to our initial work in *Nature Medicine* to which I also contributed significantly.

Abstract

Chronic inflammation characterized by T cell and macrophage infiltration of visceral adipose tissue (VAT) is a hallmark of obesity-associated insulin resistance and glucose intolerance. Here we show a fundamental pathogenic role for B cells in the development of these metabolic abnormalities. B cells accumulate in VAT in diet induced obese (DIO) mice, and DIO mice lacking B cells are protected from disease despite weight gain. B cell effects on glucose metabolism are mechanistically linked to the activation of proinflammatory macrophages and T cells and to the production of pathogenic IgG antibodies. Treatment with a B cell-depleting CD20 antibody attenuates disease, whereas transfer of IgG from DIO mice rapidly induces insulin resistance and glucose intolerance. Moreover, insulin resistance in obese humans is associated with a unique profile of IgG autoantibodies. These results establish the importance of B cells and adaptive immunity in insulin resistance and suggest new diagnostic and therapeutic modalities to manage the disease.

Introduction

Obesity and its associated metabolic abnormalities, including insulin resistance and type 2 diabetes (T2D), have reached epidemic proportions, adversely impacting health and global mortality rates (559). Multiple factors contribute to reduced insulin sensitivity, but chronic inflammation in visceral adipose tissue (VAT), which results in local and systemic increases in proinflammatory cytokines and adipokines is a major driver (391, 560). One of these drivers, macrophage infiltration of VAT, is a key event in the establishment of adipose inflammation and insulin resistance (393, 394). Classically activated, or CD11c⁺ CD206⁻ M1 macrophages, are elevated in VAT of DIO mice and produce pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α), interleukin (IL)-1 β , and IL-6 (561-563). T cells are also major participants in VAT inflammation, with pro-inflammatory CD8⁺ T cells and interferon- γ (IFN- γ)-producing CD4⁺ T cells contributing to inflammation, glucose intolerance, and insulin resistance in DIO mice (397, 405, 564). On the contrary, VAT-resident FoxP3⁺ regulatory T cells, which produce IL-10 and TGF- β , as well as IL-4- and IL-13-secreting T_H2 cells, can have protective roles (397, 408, 565). Notably, the clonal diversity of VAT T cells is highly restricted, which suggests that an active adaptive immune response expanding potentially autoimmune T cells occurs in obese VAT (397, 408, 565, 566).

In contrast to macrophages and T cells, little is known about the role of B cells in the development of insulin resistance, despite evidence that such cells are recruited to adipose tissue shortly after the initiation of a high fat diet (HFD) (567) and that their activation is increased in patients with T2D (568). Here we show that B cells and IgG are key pathogenic effectors in the development of obesity-associated insulin resistance and glucose intolerance, but not of excess weight gain, in DIO mice. Manipulation of B cells, antibodies or their Fc receptors may yield promising new therapies for the management of insulin resistance and its associated co-morbidities.

Methods

Mice

All mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in a pathogen-free, temperature controlled environment on a 12hr light and dark cycle. All experiments were performed using male age-matched mice under approved protocols in agreement with animal ethics guidelines.

Metabolic studies

Animals were kept on a regular diet for the first 6wk of life, at which time they were either maintained on this diet, or switched to a high-fat (60 kcal% fat) diet (Research Diets). After 8wk, fasting blood glucose and insulin concentrations (Crystal Chem ELISA) were measured. For glucose tolerance tests, mice were fasted for 16hr and then injected with 0.75 g of glucose (Sigma-Aldrich) per kg of body weight. For insulin tolerance tests, mice were fasted for 4hr and then given 0.75 U of human regular insulin (Eli Lilly) per kg of body weight. VAT and SAT fat pads were carefully dissected and weights were recorded. Cell size was quantified by a blinded observer using ImageJ software analysis.

Isolation of VAT-associated immune cells and VAT lysates

We isolated VAT associated immune cells as described (397). We cultured 2.5×10^5 to 3.5×10^5 VAT derived stromal vascular cells for 12-16hr in a 96-well round bottom plate in RPMI supplemented with 10% fetal calf serum for cytokine measurements. We prepared VAT lysates by homogenizing VAT tissue in RIPA lysis buffer (Santa Cruz Technologies) followed by incubation on ice for 20min. We centrifuged the lysates at 15000 g for 10min at 4°C before protein quantification using a BSA protein quantification kit (Thermo-Scientific).

Antibody ELISA and ELISpot

We measured IgA, IgE, IgM and IgG subclasses IgG1, IgG2b, IgG2c and IgG3 in serum or VAT lysate by ELISA, using kits from Bethyl Laboratories. The frequency of spontaneous IgM- and IgG-producing B cells in spleen was determined using mouse IgG or IgM ELISpot^{PLUS} Kits (Mabtech).

Cytokine measurement

We measured serum cytokines by Luminex bead assay and cytokines in the supernatants of stromal vascular cell VAT cultures by ELISA according to the manufacturer's instructions (eBioscience). We acquired data on an LSR-II flow cytometer (BD Biosciences) and analyzed it using FlowJo software (Tree Star Inc.).

B cell and antibody purification

We mechanically dissociated spleens using 70 μ m cell strainers and selected B cells using a mouse B cell negative selection enrichment kit (StemCell Technologies). B cell purity was greater than 95%, as assessed by flow cytometry.

IgG was purified from mouse serum using a Melon Gel IgG Spin Purification Kit (Pierce Biotechnology). The flow-through from the column containing IgG was dialyzed against endotoxin-free PBS and further filtered to obtain sterile antibody solution. 150 μ g IgG in 200 μ L endotoxin-free PBS was passively transferred into B^{-/-} mice via intraperitoneal injections on days 1 and 3.

IgM was purified from mouse serum using an IgM Purification Kit (Pierce Biotechnology). IgM containing eluate was concentrated using Amicon Ultra-15 Centrifugal Filter device (Millipore). The concentrate was dialyzed in endotoxin-free PBS and filtered to obtain sterile antibody solution which was assessed for purity by ELISA (Bethyl Laboratories). 120 μ g IgM in 200 μ L endotoxin-free PBS was passively transferred into B^{-/-} mice via intraperitoneal injections on days 1 and 3.

F(ab')₂ fragments were made using a Pierce F(ab')₂ Preparation Kit (Thermo Scientific) according to the manufacturer's instructions.

Histology and immunofluorescence

We fixed and stained VAT as previously described (397). IgG (Vector) and IgM (Sigma) stains were performed according to the manufacturer's protocol.

Flow cytometry

1.5-2.0×10⁶ splenocytes or fat-associated cells were incubated at 4°C for 15min in 100 μL 2% FBS/PBS with 10 μg/mL Fc-blocker (eBioscience). Cells were incubated for 30min with fluorescent antibodies. We gated B cell subsets as B1a: CD19⁺IgM⁺IgD⁻B220^{-/lo} CD5⁺; B1b: CD19⁺IgM⁺IgD⁻B220^{-/lo} CD5⁻; B2/non-B1: all other CD19⁺ cells. Splenic MZ B cells were gated as: CD19⁺IgM⁺IgD⁻CD21⁺CD23⁻ while IgM⁺IgD⁻FC were defined as CD19⁺IgM⁺IgD⁻CD23⁺CD21⁻. Samples were run on an LSR-II flow cytometer (BD Biosciences) and FACS plots were analyzed using FlowJo software (Tree Star Inc.).

In vitro effects of HFD IgG

We positively selected monocytes/macrophages by CD11b microbeads (Miltenyi) from total VAT cells and then plated them at 1 x 10⁵ cells per well in 96-well ELISA plates coated with HFD IgG (50 ug/mL). We collected the supernatant after 24 hours.

B cell depletion with CD20 mAb

Mouse CD20 mAb (MB20-11, IgG2c isotype) was provided by Dr. Thomas Tedder. We suspended sterile CD20 mAb and isotype control (Southern Biotech) at 100 μg in 100 μL PBS and administered to HFD mice intravenously via retro-orbital injection. No significant differences were identified between isotype treated and PBS treated mice, and these data were pooled in the control population.

Human antibody array

We obtained sera from 32 age- and BMI-matched overweight to obese male subjects (mean age insulin resistant (IR): 55±10, insulin sensitive (IS): 54±7; mean BMI IR: 31.1 ± 2.4 kg/m²; IS: 31.1 ± 2.3 kg/m²). Insulin sensitivity was determined by a modified insulin suppression test and defined as either IR or IS based on steady-state plasma glucose levels falling in the top (IR) or bottom (IS) 40th percentile. Subjects were excluded based on the presence of major organ disease or change in physiological functioning, including heart failure, coronary artery disease, hepatic or renal disease, pregnancy, cancer, infection, recent weight gain or loss of more than 3 kg over 4wk, prior liposuction/bariatric surgery, and use of medications intended for weight loss or known to influence insulin sensitivity. Serum samples were obtained with the approval of the Stanford Internal Review Board for Human Subjects and with informed consent. We used ProtoArrays Version 5.0 (Invitrogen) with 1:500 diluted human sera run according to the manufacturer's instructions.

Immunoblotting

Recombinant purified human GOSR1, BTK (Novus) and human purified GFAP (US Biological) was electro-fractionated on SDS-PAGE and transferred onto a PVDF membrane. We probed the blots with patient serum samples (1:10 for GOSR1 and BTK, 1:100 for GFAP) or commercial anti-GOSR1 (1:160), anti-BTK (1:500) (Abcam), and anti-GFAP (1:500) (Biolegend) as positive controls. Bands were visualized using chemiluminescence (Invitrogen).

Statistical analysis

Statistical significance was assessed by Mann-Whitney and unpaired t-tests, and Welch correction was employed where necessary. Analysis of curves was performed using two-way ANOVA. Statistical significance was two tailed and set at 5%, with error bars showing a single standard deviation.

Results

B cells and antibodies in diet induced obesity

We analyzed early immune cell infiltration into epididymal VAT of 6wk old C57BL/6 mice fed a high fat diet (HFD, 60% kcal) for several weeks and compared the immune cell composition to age-matched C57BL/6 mice that were fed a normal chow diet (NCD) (**Figure 4.1.A**). HFD induced a significant accumulation of B cells in VAT by 4wk that was maintained after 6-12wk on HFD (**Figure 4.1.A**). This increase in B cell numbers included total B cells, B-1a cells, and B2 cells. Total T cell numbers were also increased by 4wk, and absolute numbers continued to rise while the mice were on a HFD, consistent with previous reports (397, 401, 567). Despite the increase in absolute B cell numbers in DIO VAT, the relative proportions of B1 and non-B1 subsets were unchanged (**Figure 4.1.A**). However, DIO VAT had increased numbers and proportions of class-switched mature B cells, such as IgG⁺ cells, a pattern suggesting an actively progressing immune process in this fat depot of obese mice (**Figure 4.1.B**).

To investigate the effects of HFD on systemic B cells, we analyzed spleens from age-matched 12-18wk old HFD and NCD fed mice. No significant differences were seen in total spleen cell counts or the percentages of naïve IgD⁺ B cells, marginal zone B cells, or IgM⁺IgD⁻ follicular B cells (**Figure 4.1.C**). However, in contrast to DIO VAT, DIO spleens contained reduced percentages of IgM⁺IgD⁻ cells (**Figure 4.1.C**). Consistent with these results, total spleen B cells from DIO mice showed reduced spontaneous production of IgM antibody, but elevated IgG secretion (**Figure 4.1.D**), suggesting that HFD induces a systemic humoral immune response.

We confirmed this systemic response by comparing concentrations of immunoglobulin isotypes in serum and VAT of NCD and HFD mice. DIO mice had reduced concentrations of serum IgA, and an increase in IgG2c (**Figure 4.1.E**), a pro-inflammatory isotype present in C57BL/6,

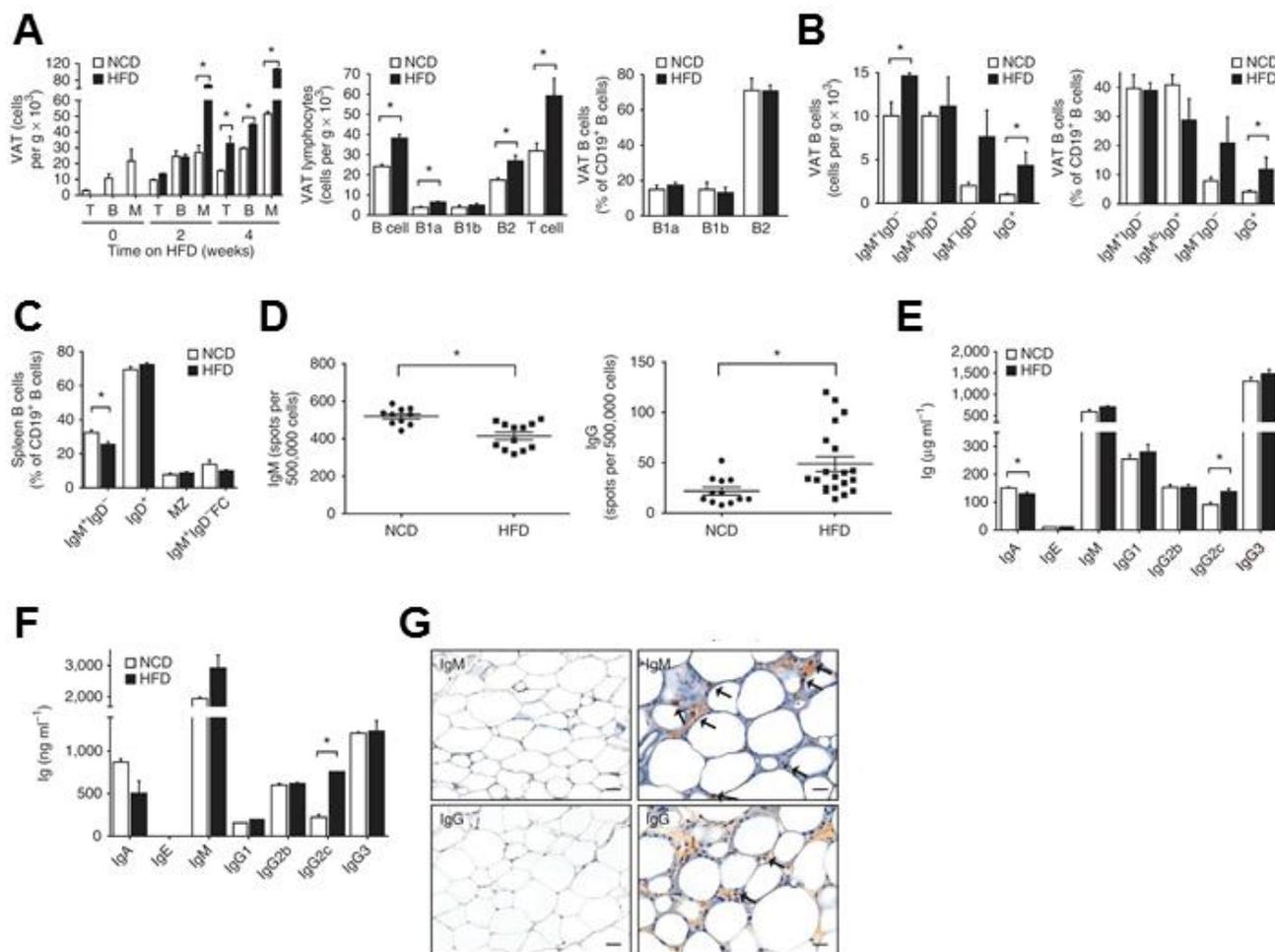


Figure 4.1 – B cell and antibody profile in DIO mice. (A) Time course of T cell (T), B cell (B) and macrophage (M) infiltration of VAT after initiation of HFD (left, 2 experiments, n=5 per group, *p<0.05). B cell subsets in VAT in response to 6-12wk of HFD in absolute numbers of B cells, B1a cells, B1b cells, non-B1 cells/ B2, and T cells (middle, 3 experiments, n=9 per group, *p<0.05), and in percentages of CD19⁺ cells (right, 3 experiments, n=9 per group). (B) VAT B cells in absolute numbers (left, 3 experiments, n=9 per group, *p<0.05) and proportion of CD19⁺ cells (right, 3 experiments, n=9 per group, *p<0.05). (C) Spleen B cell subsets in response to HFD (MZ, marginal zone; FC, follicular cells, n=5 per group, *p=0.01). (D) Spontaneous production of IgM (left, *p=0.0006) and IgG (right, *p=0.01) from mouse splenocytes. (E) Serum antibody concentrations in mice (n=10 per group, *p<0.05). (F) Antibody subtypes in VAT lysates from mice (2 experiments, n=5 per group, *p=0.0001). (G) IgM (top left) and IgG (bottom left) staining in VAT of DIO mice in regions of few and multiple CLSs (IgM top right; IgG bottom right). Arrows indicate antibody-stained cells. Scale bars 50 µm (left images) and 25 µm (right images).

C57BL/10, and NOD mice (569). VAT lysates from HFD-fed mice had higher concentrations of IgM compared to IgG and a marked (>3 fold) enrichment in pro-inflammatory IgG2c (**Figure 4.1.F**). Notably, antibody staining in VAT showed a preferred localization of IgG and IgM to regions of crown-like structures (CLS) (**Figure 4.1.G**) (570, 571). Many of these stained cells were at the interface of large mononucleate and multinucleate giant macrophages and dying adipocytes inside the CLSs. CLSs appeared bathed in tissue fluid enriched for IgM and IgG, whereas the remaining fat tissue showed either very weak or no Ig staining. Enrichment of IgG and IgM in CLSs implicates the involvement of antibodies in the clearance of dying adipocytes (570, 571).

B cells are pathogenic in glucose metabolism in DIO mice

To assess the effect of B cells on the regulation of obesity and insulin resistance, we investigated C57BL/6 immunoglobulin μ heavy-chain knockout mice (B^{null}), which fail to produce mature B cells, for their response to HFD started at 6wk of age (572). After 8wk of HFD, there was no difference in body weight or VAT adipocyte size (**Figure 4.2.A-B**), but DIO B^{null} mice had a lower ratio of VAT to subcutaneous adipose tissue (SAT) fat pad weight than DIO WT mice (**Figure 4.2.C**).

Compared to HFD-fed WT mice, 16wk old HFD-fed B^{null} mice had lower fasting glucose (**Figure 4.2.D**) and improved glucose tolerance (**Figure 4.2.E**). Similarly, HFD-fed B^{null} mice had reduced fasting insulin (**Figure 4.2.F**) and improved insulin sensitivity upon insulin challenge (**Figure 4.2.G**). B cell deficiency did not affect weight, fasting glucose, fasting insulin, or glucose and insulin tolerance in NCD mice (**Figure 4.2.D-G**), suggesting that metabolic influences of B cells require a HFD. To confirm that abnormal glucose metabolism in HFD-fed mice is directly attributable to B cells, age-matched 16-18wk old DIO B^{null} mice were reconstituted intraperitoneally with 1×10^7 total B cells from spleens of DIO WT mice. After 2-3wk, B cells reconstituted primarily in VAT over spleen (Appendix) and produced low concentrations of serum antibody (Appendix). B

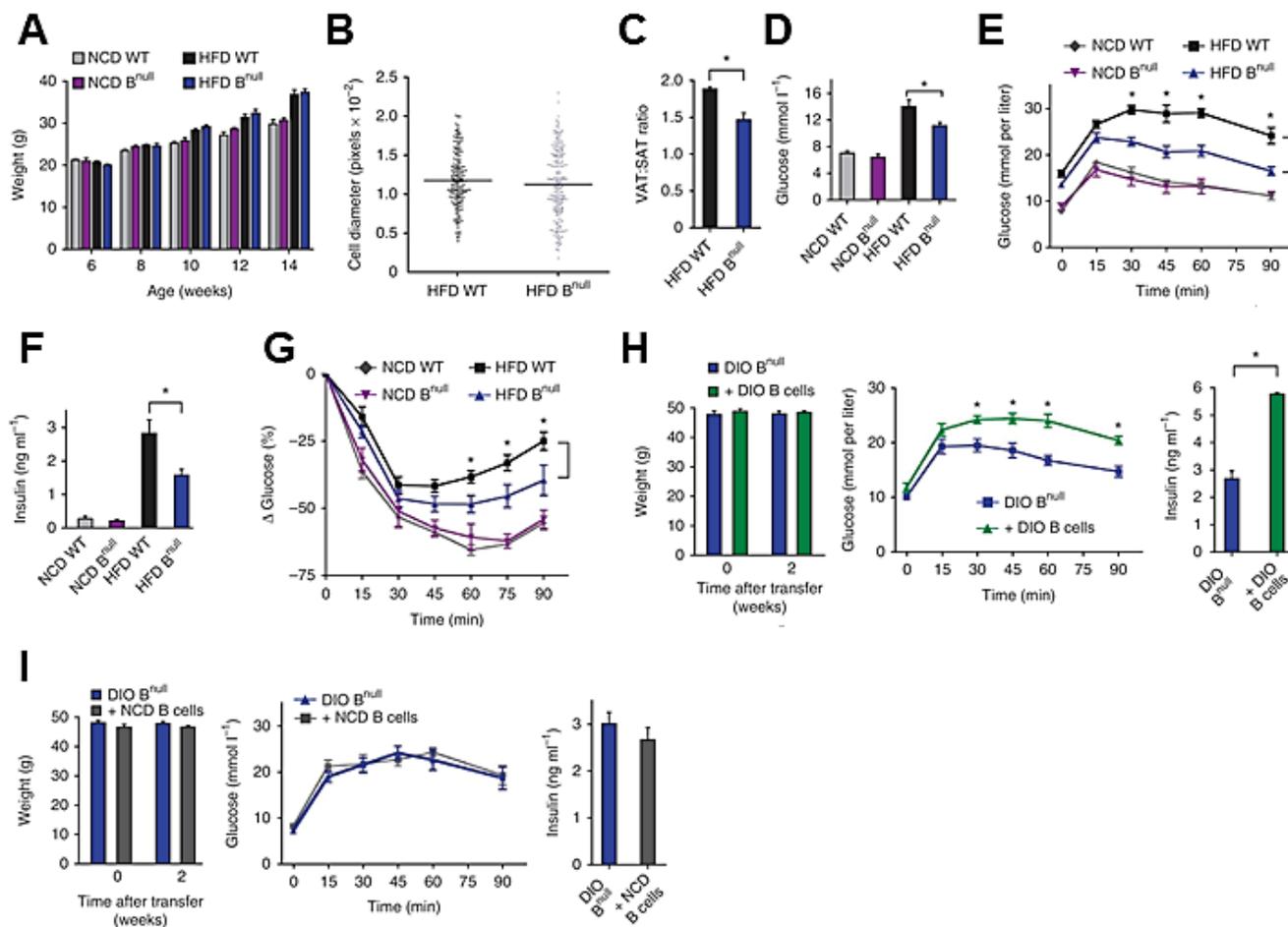


Figure 4.2 – B cell deficiency modulates glucose metabolism in DIO mice. (A) Body weights of WT and B^{null} mice over time (n=10 per group). (B) Relative fat cell diameter of 14-18wk old HFD mice (n=3 per group). (C) Ratio of epididymal VAT and SAT pad weights of mice on HFD (n=10 per group, *p=0.004) (D-E) Fasting glucose (D, n=10 per group, *p=0.04) and glucose tolerance test (E, n≥5 per group, *p<0.05) of WT or B^{null} mice on NCD or HFD. (F) Fasting serum insulin concentrations of 16wk old WT or B^{null} mice on NCD or HFD (n=10 per group, *p=0.04). (G) Insulin tolerance test (ITT) in WT or B^{null} mice on NCD or HFD (n=5 per group, *p<0.05). (H) Body weight (left, n=6 per group), GTT (middle, n=6 per group, *p<0.05), and fasting insulin (right, n=6 per group, *p=0.02) of DIO B^{null} mice 2wk after reconstitution with DIO WT B cells (representative of 3 experiments). (I) Body weight (left, n=5 per group), GTT (middle, n=5 per group), and fasting insulin (right, n=5 per group) of DIO B^{null} mice 2wk following reconstitution with NCD WT B cells (representative of 2 experiments).

cell transfer did not impact total weight (**Figure 4.2.H**). However, B^{null} mice reconstituted with B cells from DIO mice had worsened glucose tolerance (**Figure 4.2.H**) and higher fasting insulin levels (**Figure 4.2.H**) compared to control B^{null} recipients. B cells from NCD mice failed to promote impairment in glucose homeostasis (**Figure 4.2.I**) despite similar reconstitution profiles as B cells from DIO mice (Appendix), thus indicating that development of pathogenic B cells requires exposure to a HFD. Collectively, these results suggest a pathogenic role for HFD-derived B cells in the promotion of obesity-associated insulin resistance and glucose intolerance.

DIO B^{null} mice show reduced immune cell activation in VAT

Inflamed adipose tissue is a key feature of insulin resistance. As B cells reside in VAT and worsen metabolic parameters upon adoptive transfer, we examined the possibility that these cells promote inflammation in VAT. VAT total T cell and macrophage counts were similar in DIO WT and DIO B^{null} mice (**Figure 4.3.A**), but DIO B^{null} mice had fewer proinflammatory M1 macrophages (**Figure 4.3.B**). To examine the functional profiles of these immune cells, we measured cytokines known to affect insulin resistance (TNF- α and IFN- γ) (392, 405, 573). The supernatants of total VAT stromal vascular cell (SVC) cultures from DIO B^{null} mice had lower levels of IFN- γ compared to DIO WT mice (**Figure 4.3.C**). The number of VAT associated CD8⁺ T cells producing IFN- γ from DIO B^{null} mice was approximately 30% less compared to DIO WT CD8⁺ T cells (**Figure 4.3.D**). DIO B^{null} CD8⁺ T cells also expressed less of the cytotoxic activation marker CD107a (**Figure 4.3.D**). IFN- γ expression was variable in CD4⁺ T cells of DIO WT and B^{null} mice (Appendix).

Supernatants of VAT SVC cultures from DIO B^{null} mice also contained less TNF- α compared to DIO WT mice (**Figure 4.3.E**). Macrophages, especially M1 macrophages, are a major source of TNF- α , and its decrease in DIO B^{null} VAT is partly attributable to reduced numbers of macrophages producing this cytokine (**Figure 4.3.E**). In addition, fewer DIO B^{null} VAT

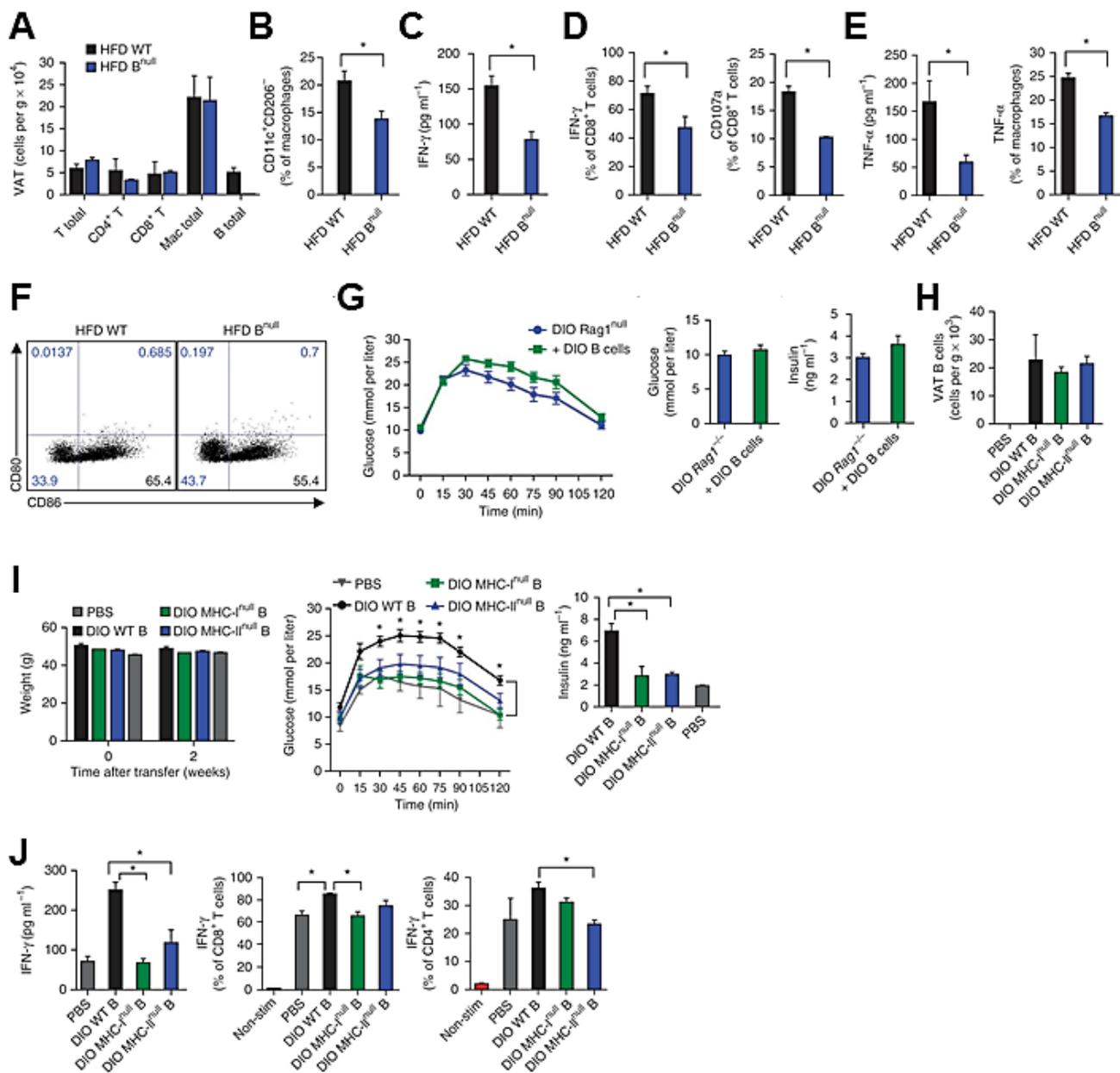


Figure 4.3 – B cells influence VAT T cell and macrophage function. (A) Numbers of cell subsets in VAT of 14-18wk old mice (4 experiments, n=10 per group). (B) Percentage of VAT macrophages (CD11b⁺F4/80⁺Gr-1) with M1 phenotype (3 experiments, n=8 per group, *p=0.049). (C) IFN- γ production from SVC cultures of VAT (3 experiments, n=9 per group, *p=0.02). (D) Intracellular IFN- γ staining of CD8⁺ T cells isolated from VAT (left, 4 experiments, n=10 per group, *p=0.04) and percentage of total VAT CD8⁺ T cells expressing CD107a (right, 2 experiments, n=6 per group, *p=0.02). (E) TNF- α production from VAT SVC cultures (left, 2 experiments, n=6 per group, *p=0.04) and intracellular staining of TNF- α in VAT macrophages (right, 2 experiments, n=6 per group, *p=0.02). (F) CD80 and CD86 expression on VAT macrophages (3 experiments, n=9 per group). (G) GTT (left, n=10 per group), fasting glucose (middle, n=10 per group) and fasting insulin (right, n=10 per group) of recipient DIO RAG^{null} mice 2wk after transfer of DIO B cells. (H) CD19⁺ B cells in VAT of B^{null} mice 2wk after reconstitution with DIO WT, DIO MHC-I^{null}, or DIO MHC-II^{null} B cells (3 experiments, n=9 per group). (I) Weights (left, 3 experiments, n=3 per group), GTT (middle, 3 experiments, n=3 per group, *p<0.05) and fasting insulin (right, 3 experiments, n=3 per group, *p<0.05) of recipient mice 2wk after transfer of DIO WT, DIO MHC-I^{null}, or DIO MHC-II^{null} B cells. (J) IFN- γ production from VAT SVC cultures (left, 2 experiments, n=6 per group, *p<0.05), and intracellular IFN- γ in VAT CD8⁺ T cells (middle, 2 experiments, n=6 per group, *p<0.05) and VAT CD4⁺ T cells (right, 2 experiments, n=6 per group, *p<0.05) isolated from recipient B^{null} mice receiving either PBS or DIO WT, DIO MHC-I^{null}, or DIO MHC-II^{null} B cells.

macrophages expressed co-stimulatory CD86, consistent with an overall decrease in macrophage activation in these mice (**Figure 4.3.F**).

Analysis of serum revealed that concentrations of resistin and plasminogen activator inhibitor-1 (PAI-1), both previously associated with insulin resistance (574, 575), were markedly lower in DIO B^{null} compared to DIO WT mice (Appendix). This result suggests that in DIO mice, B cells induce systemic as well as local (VAT) inflammation.

B cells modulate VAT associated T cells in vivo

B cell functions are influenced by other lymphocyte populations and vice versa (576). To begin to discern a role for T cells in facilitating B cell-mediated glucose intolerance, we reconstituted 16wk old HFD-fed recombination activating gene 1 (RAG1)^{null} mice, which lack lymphocytes, with 1×10^7 total DIO splenic B cells. After 2wk, despite reconstitution (Appendix), the B cells failed to worsen fasting glucose, insulin, and glucose tolerance, contrasting with transfers into HFD B^{null} mice, thereby suggesting that B cells require other lymphocytes to fully promote impairment of metabolic parameters (**Figure 4.3.G**).

B cells can activate CD8⁺ and CD4⁺ T cells by presenting antigen via major histocompatibility complex (MHC) class I and MHC class II, respectively. To determine if B cell-dependent T cell activation is involved in the promotion of glucose intolerance, we reconstituted age-matched 16wk old HFD B^{null} mice with 1×10^7 purified (>95%) splenic B cells from either DIO WT mice, or DIO mice lacking MHC class I (C57BL/6 MHC-I^{null}) or MHC class II (C57BL/6 MHC-II^{null}). Two weeks after intraperitoneal transfer, CD19⁺ B cells had successfully reconstituted VAT but not spleen in the DIO B^{null} mice (**Figure 4.3.H** and Appendix). There were no significant differences in weight after adoptive transfer (**Figure 4.3.I**). However, in contrast to recipients of B cell grafts from WT mice, recipients of B cells from either MHC-I^{null} or MHC-II^{null} mice did not develop glucose intolerance or hyperinsulinemia (**Figure 4.3.I**). Improved glucose homeostasis in

mice receiving MHC^{null} B cells compared to mice receiving WT B cells was associated with reduced total VAT SVC production of IFN- γ (**Figure 4.3.J**), attributable to either reduced number of VAT CD8⁺ T cells producing IFN- γ (MHC-I^{null} recipients, **Figure 4.3.J**) or reduced numbers of VAT CD4⁺ T cells producing IFN- γ (MHC-II^{null} recipients, **Figure 4.3.J**). The data show that B cell modulation of VAT T cells occurs in an MHC-dependent manner, likely through B cell antigen presentation to T cells, and that both MHC class I (CD8⁺ T cells) and class II (CD4⁺ T cells) are needed for the B cells to maximally affect glucose tolerance in the adoptive transfer model.

IgG antibodies are mediators of glucose intolerance

In addition to their functions in the modulation of T cell activation, B cells produce antibodies which are known regulators of immune function (577). Because obesity is associated with increased IgG production and IgG⁺ B cells in VAT, as well as with increased concentrations of systemic and local IgG2c (**Figure 4.1.B-F**), we next investigated a possible role of IgG in glucose intolerance. We purified IgG (>98% pure) from pooled sera of 16-24wk old HFD-fed (HFD IgG) or NCD-fed (NCD IgG) WT mice and injected it intraperitoneally into age-matched DIO B^{null} mice. One week after transfer, IgG was present in serum of all recipient mice (**Figure 4.4.A**). Within this time frame, antibody transfer had no effect on body weight (**Figure 4.4.B**). However, HFD IgG induced a dramatic worsening of glucose tolerance, which was absent in NCD IgG recipients (**Figure 4.4.C**), suggesting that pathogenic IgG specificities are induced during the course of a HFD but not during NCD. This antibody-mediated effect was associated with worsened fasting insulin, a hallmark of insulin resistance (**Figure 4.4.C**). Transferred antibodies also localized to VAT, where they were in close association with CLSs (Appendix). The effects of antibody transfer on glucose metabolism were transient and, as expected on the basis of normal IgG half life, by four weeks after transfer there was no difference in glucose tolerance or fasting insulin between IgG and control recipients (**Figure 4.4.D**).

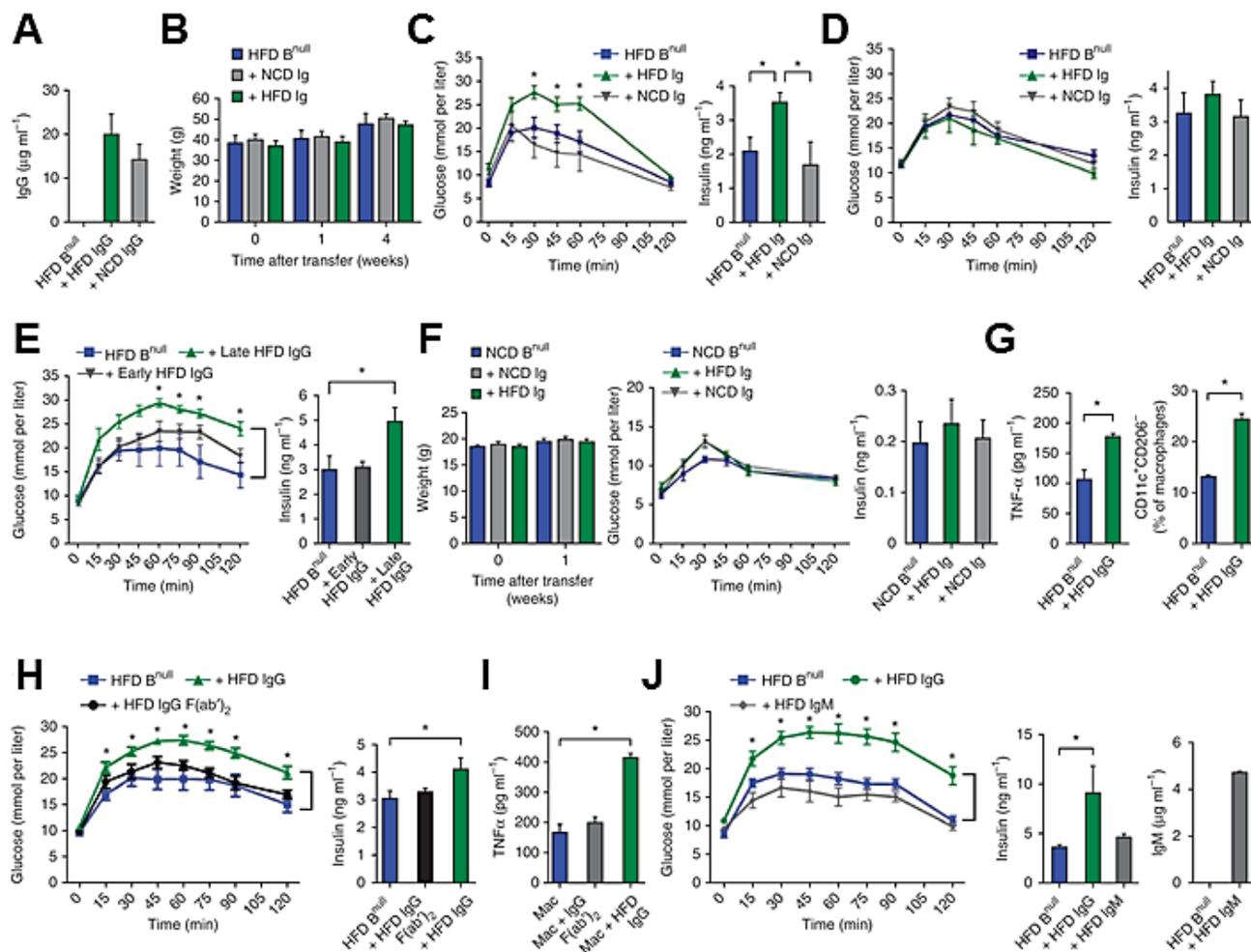


Figure 4.4 – HFD IgG induces abnormal glucose metabolism in recipient B^{null} mice. (A) Serum concentration of IgG in B^{null} mice 1wk after IgG injection (n=3 per group). (B) Body weights of HFD B^{null} recipient mice after IgG transfer (representative of 3 experiments, n=4 per group). (C) GTT (left, representative of 3 experiments, n=4 per group, *p<0.05) and fasting insulin (right, representative of 3 experiments, n=4 per group, *p<0.05) 1wk after the transfer of IgG into 16wk old HFD B^{null} mice. (D) GTT (left) and fasting insulin (right) 4wk after the transfer of IgG (representative of 2 experiments, n=4 per group). (E) GTT (left, n=5 per group, *p<0.05) and fasting insulin (right, n=5 per group, *p=0.048) 1wk after the transfer of late or early IgG. (F) Weights (left), GTT (middle), and fasting insulin (right) of 6wk old NCD B^{null} mice 1wk after IgG transfer (representative of 2 experiments, n=4 per group). (G) TNF-α from VAT SVC cultures (left, 2 experiments, n=6 per group, *p=0.04) and M1 macrophages in HFD B^{null} VAT 1wk after HFD IgG transfer (right, 2 experiments, n=6 per group, *p=0.007). (H) GTT (left, n=5 per group, *p<0.05) and fasting insulin (right, n=5 per group, *p=0.04) 1wk after transfer of HFD Ig or HFD F(ab)₂. (I) TNF-α from HFD B^{null} VAT macrophages stimulated *in vitro* with HFD IgG or HFD F(ab)₂ (n=3 per group, *p=0.007). (J) GTT (left) and fasting insulin (middle) of HFD B^{null} mice 1wk after receiving HFD Ig (n=5 per group, *P<0.05). Serum concentration of IgM in HFD B^{null} mice 1wk following IgM injection (right, n=3 per group).

To determine whether pathogenic antibodies arise early after the initiation of HFD, we purified IgG from 9-12wk old HFD-fed mice (early HFD IgG), as well as from 20-24wk old HFD-fed mice (late HFD IgG), and injected each type separately intraperitoneally into 20wk old HFD-fed B^{null} mice. We observed a much stronger effect on glucose tolerance and fasting insulin concentrations with late HFD IgG compared to early HFD IgG; this suggests a possible role for affinity maturation of antibody or late unmasking of HFD antigen in the process (**Figure 4.4.E**). To investigate whether HFD IgG-induced disease depends on HFD exposure in recipient mice, we transferred IgG purified from HFD mice into 6wk old lean mice. One week after transfer, there was little change in weight, glucose tolerance or fasting insulin levels (**Figure 4.4.F**), thus indicating that the effects of HFD IgG on glucose metabolism are dependent on the recipient's exposure to HFD.

To investigate the mechanism by which HFD IgG induces glucose intolerance, we first examined its effects on VAT and systemic inflammation. HFD IgG recipient mice had higher concentrations of TNF- α in VAT SVC cultures and more pronounced M1 macrophage polarization in VAT when compared to controls (**Figure 4.4.G**). In addition, these mice had elevated serum concentrations of proinflammatory mediators including monocyte chemoattractant protein-3, IL-6, and granulocyte macrophage colony-stimulating factor (Appendix). IgG antibodies, through their Fc portions, can bind Fc γ receptors (Fc γ Rs) on macrophages and directly induce macrophage oxidative burst, cytotoxicity and proinflammatory cytokine production (577). To determine whether the observed effect of HFD IgG antibodies on glucose intolerance is mediated through their Fc components, we generated F(ab')₂ fragments from HFD IgG and compared their effect on glucose metabolism to that of intact HFD IgG. One week after intraperitoneal transfer into age-matched DIO B^{null} mice, HFD IgG F(ab')₂ did not worsen glucose tolerance and fasting insulin compared to intact HFD IgG, thereby indicating that the pathogenic properties of HFD IgG are mediated through the Fc region (**Figure 4.4.H**). Consistent with these findings, macrophages isolated from VAT of DIO B^{null}

mice stimulated with HFD IgG show an Fc-dependent increase in TNF- α production *in vitro* (**Figure 4.4.I**). In contrast to HFD IgG, HFD IgM had no effect on metabolic parameters (**Figure 4.4.J**). These data point to an unexpected, pathogenic role for HFD-induced IgG antibody in promoting glucose intolerance and insulin resistance.

Insulin resistance is linked to distinct profiles of IgG

As HFD IgG can exert pathologic effects on insulin resistance in obese mice, we next examined whether IgG autoantibodies are present in insulin-resistant humans and, if so, whether they recognize a distinct cluster of antigenic targets. We probed Invitrogen ProtoArray V5.0 chips containing more than 9000 spotted antigens with serum from 32 age- and weight-matched, overweight to obese, otherwise healthy, male human subjects (**Table 4.1**). The two groups of subjects differed only in their insulin sensitivities, as determined by a modified insulin-suppression test, and individuals were defined as either insulin resistant (IR) or insulin sensitive (IS) on the basis of their steady-state plasma glucose (SSPG) concentration. We identified 122 IgG targets that differentially segregated with IR, whereas 114 targets segregated with IS. The ten antigens that were most highly associated with either IR or IS in our obese male subjects are shown in **Table 4.2**. Antibodies to the top three targets segregating with IR were validated in these subjects by Western blot (Appendix). Notably, in both groups the antigens are mostly intracellular proteins, many of which are expressed ubiquitously in tissues including immune cells, pancreas, nervous tissues, muscle, or fat.

B cell depletion ameliorates metabolic disease

To determine if manipulation of B cells can be exploited therapeutically in obesity-related insulin resistance, we treated HFD-fed WT mice with a depleting antibody specific to mouse CD20 (MB20-11) (578). We injected antibody 6-7wk after initiation of HFD and maintained the mice on a HFD. B cells, which were depleted >95% locally in VAT and systemically in spleen 8d after

	<i>IR</i>	<i>IS</i>	<i>P Value</i>
Age (Years)	55 ± 10	54 ± 7	0.83
Race (c/a/h/b)	11/5/0/0	15/1/0/0	0.17
BMI (kg/m ²)	31.1 ± 2.4	31.1 ± 2.3	0.99
Waist (cm)	109 ± 8	107 ± 9	0.70
Steady-State Plasma Glucose (mg/dL)	238 ± 32	87 ± 17	<0.001
Fasting glucose (mg/dL)	105 ± 9	98 ± 7	0.07
Systolic BP (mmHg)	131 ± 14	131 ± 22	0.99
Diastolic BP (mmHg)	79 ± 10	81 ± 10	0.61
Total Cholesterol (mg/dL)	194 ± 35	179 ± 40	0.34
Triglycerides (mg/dL)	165 ± 106	108 ± 62	0.12
LDL Cholesterol (mg/dL)	125 ± 24	110 ± 37	0.28
HDL Cholesterol (mg/dL)	40 ± 7	48 ± 11	0.05

Table 4.1 – Demographic and clinical characteristics of 32 BMI-matched males classified as insulin resistant or insulin sensitive used for protoarray analysis.

<i>Antigen</i>	<i>p Value</i>	<i>IR Prevalence</i>	<i>IS Prevalence</i>
GOSR	0.0000854	61.11%	11.11%
BTK	0.001224	50.00%	5.56%
GFAP	0.001224	50.00%	5.56%
ASPA	0.003399	44.44%	5.56%
NIF3L1	0.003399	44.44%	5.56%
PGD	0.003399	44.44%	5.56%
ALDH16A1	0.003399	44.44%	5.56%
KCNAB1	0.003399	44.44%	5.56%
RNA Polymerase	0.004574	61.11%	16.67%
GSTA3	0.004574	61.11%	16.67%
CTNNA1	0.001027	11.11%	61.11%
CDC37	0.001224	5.56%	50.00%
LGALS14	0.001224	5.56%	50.00%
BM88	0.002961	11.11%	55.56%
NCBP2	0.002961	11.11%	55.56%
PDDC1	0.003399	5.56%	44.44%
ALS2CR8	0.003399	5.56%	44.44%
PAFAH G Subunit	0.004574	16.67%	61.11%
XRCC4	0.006057	27.78%	72.22%
Influenza A Antigen (H3N2)	0.007749	44.44%	88.89%

Table 4.2 – Top ten antibody targets most strongly associated with insulin resistance (IR, upper rows) and with insulin sensitivity (IS, lower rows) in human male subjects.

injection (**Figure 4.5.A**), began to repopulate tissues by day 28 after injection; nonetheless, there was still >70% depletion. CD20-specific monoclonal antibody (mAb) treatment had no effect on weight (**Figure 4.5.B**) or total serum concentrations of IgG or IgM (**Figure 4.5.C**) by 28d after injection. However, treated mice showed improvements compared to control-treated mice in fasting glucose (**Figure 4.5.D**), glucose tolerance (**Figure 4.5.E**), and fasting insulin (**Figure 4.5.F**). Improved glucose tolerance persisted for more than 40d and diminished with the return of B cells (Appendix). Consistent with a role for B cells in altering the local VAT cytokine milieu, VAT SVCs of treated mice showed reduced concentrations of the key inflammatory mediators IFN- γ (**Figure 4.5.G**) and TNF- α (**Figure 4.5.H**); the latter change was attributable, at least partly, to reduced production by macrophages (**Figure 4.5.I**). Notably, IgG from CD20 mAb treated mice was unable to transfer metabolic disease (Appendix), suggesting that CD20-specific mAb treatment may lead to alterations in IgG function in addition to its other effects on B cells (579).

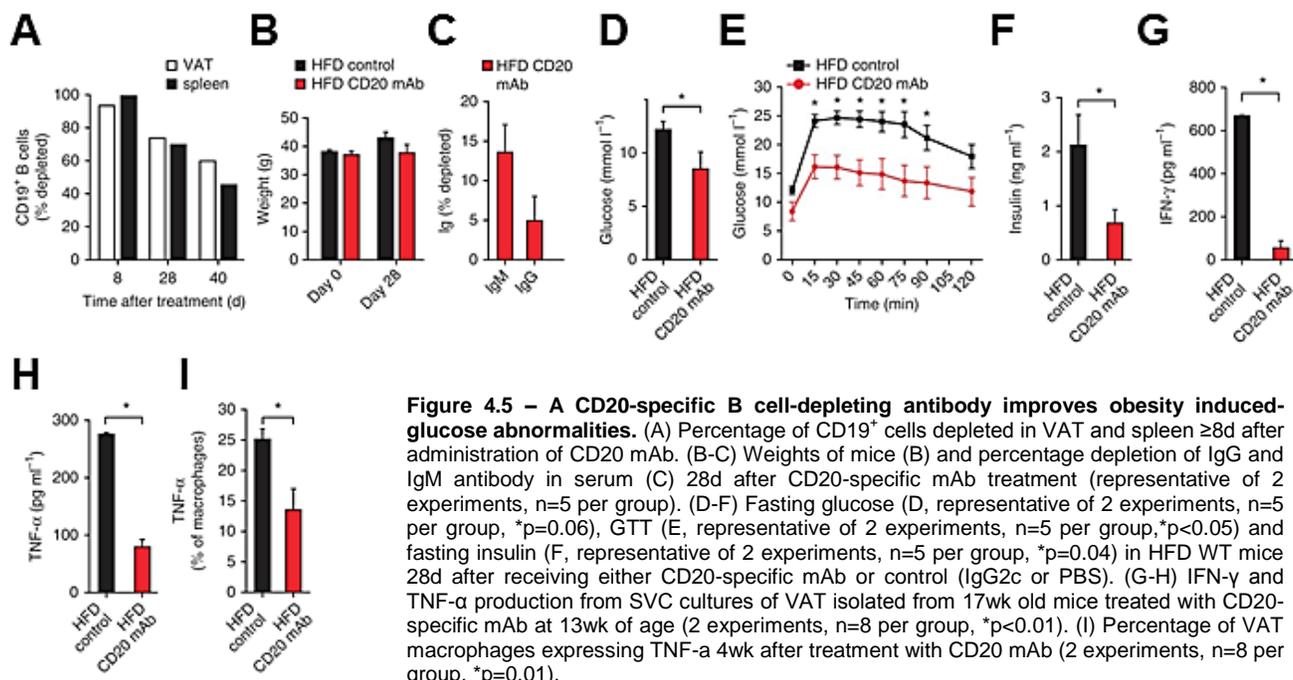


Figure 4.5 – A CD20-specific B cell-depleting antibody improves obesity induced-glucose abnormalities. (A) Percentage of CD19⁺ cells depleted in VAT and spleen ≥8d after administration of CD20 mAb. (B-C) Weights of mice (B) and percentage depletion of IgG and IgM antibody in serum (C) 28d after CD20-specific mAb treatment (representative of 2 experiments, n=5 per group). (D-F) Fasting glucose (D, representative of 2 experiments, n=5 per group, *p=0.06), GTT (E, representative of 2 experiments, n=5 per group, *p<0.05) and fasting insulin (F, representative of 2 experiments, n=5 per group, *p=0.04) in HFD WT mice 28d after receiving either CD20-specific mAb or control (IgG2c or PBS). (G-H) IFN-γ and TNF-α production from SVC cultures of VAT isolated from 17wk old mice treated with CD20-specific mAb at 13wk of age (2 experiments, n=8 per group, *p<0.01). (I) Percentage of VAT macrophages expressing TNF-α 4wk after treatment with CD20 mAb (2 experiments, n=8 per group, *p=0.01).

Discussion

We discovered a fundamental role for B cells in the pathogenesis of obesity-associated insulin resistance. Previous studies have identified other immune cells as metabolic controllers with pathogenic potential in obesity. In healthy non-obese individuals, VAT-resident regulatory T cells and T_H2 cells have a beneficial effect by reducing VAT inflammation. During DIO, these cells are overwhelmed by proinflammatory CD8⁺ and T_H1 cells, which promote insulin resistance and glucose intolerance (397). B lymphocytes can now be added to the list of immune cells participating in this process, in which they activate CD8⁺ and T_H1 cells and release pathogenic antibodies.

Consistent with a previous report (567), we show that B cell accumulation in VAT occurs early (by 4 weeks) after initiation of HFD. B cells worsen glucose tolerance, in part, by inducing MHC-dependent proinflammatory cytokine production by both CD4⁺ and CD8⁺ T cells. Similar mechanisms occur in models of cancer, infection, and autoimmunity (578, 580). As B and T cells are recruited early to VAT in response to HFD (564, 567), the data support a role for B cells in modulating T cell function in DIO VAT. Alternatively, T cells may function by inducing IgG class switching in B cells. Indeed, we observed elevated concentrations of proinflammatory IgG2c in serum and VAT of DIO mice. Class switching could also be influenced by lipids in the HFD VAT environment acting directly through TLRs on B cells. It is also possible that cytokines or antibodies produced by B cells can directly interact with and affect insulin sensitivity in adipocytes.

B cells also exacerbate metabolic disease through production of IgG. Although autoantibodies have not been previously recognized as playing a critical role in T2D, an estimated 10% of T2D patients have antibodies to islet cell antigens, and these antibodies are correlated with the need for insulin therapy (581). Almost one third of people with advanced T2D have autoantibodies that inhibit endothelial cell function (582). The presence of antibodies to glial

fibrillary acidic protein (GFAP), which predicts insulin resistance as shown by our array, is also reported at higher rates in T2D (583).

We show that transfer of IgG from DIO mice to DIO B^{null} mice induces rapid local and systemic changes in inflammatory cytokine production, and skews VAT macrophages to a proinflammatory M1 phenotype. These effects required exposure to a HFD, suggesting that factors related to diet, possibly including diet-induced conditioning or induction of target autoantigens, are required for antibodies to exert their effects on glucose metabolism. Furthermore, we show that HFD IgG antibodies induce insulin resistance through an Fc-mediated process. As DIO VAT is a site of increased apoptotic and necrotic load, and because antibodies concentrate in regions of VAT CLSs, it is conceivable that interactions between antibodies and FcRs on macrophages occur in VAT and promote clearance of apoptotic and necrotic debris and inflammation (584, 585). Identification of the precise FcR responsible for IgG effects on glucose metabolism warrants further investigation. Antibodies also fix complement and recently, the complement protein C3a and its receptor C3aR on macrophages, were identified as important mediators of insulin resistance (586).

We further show that insulin resistance in obese humans is linked to autoantibodies directed against a specific profile of self-antigens. Antibodies to one of the top three antigens linked to insulin resistance, GFAP, occur in approximately 30% of T2D patients (583). Interestingly, we detected antibodies to Golgi SNAP receptor complex member 1 (GOSR1) in more than 70% of obese insulin resistant males. GOSR1 is an essential component of the Golgi SNAP receptor complex, where it functions in trafficking proteins between the endoplasmic reticulum and the Golgi (587). It is unknown whether expression of this protein changes in response to endoplasmic reticulum stress, which is thought to be a prominent initiator of insulin resistance (588). Our array data also show distinct antigenic targets associated with insulin sensitivity, thereby raising the

possibility that some IgG antibodies may be protective. It will be important to validate antigenic targets in larger cohorts.

Finally, we show that depletion of B cells with a CD20-targeting mAb early in disease has therapeutic benefit in abnormal glucose metabolism. These results are consistent with a role for B cells early in disease pathogenesis, similar to observations in several autoimmune diseases (589). Recently, CD20-specific mAb was used in the treatment of atherosclerotic lesions in apolipoprotein E (ApoE)^{null} and low-density lipoprotein receptor (Ldlr)^{null} mice (590). In CD20-specific mAb experiments, beneficial anti-inflammatory effects are linked to reduced T cell activation. Consistently, we observed reduced concentrations of proinflammatory IFN- γ and TNF- α in VAT after CD20-specific mAb treatment.

Consistent with other reports, total serum levels of IgM and IgG following CD20-specific mAb treatment were not drastically changed despite a prominent therapeutic benefit (591). One possible explanation for this finding is that in our studies, CD20-specific mAb was administered by 6-7wk after HFD, just a few weeks after B cells substantially infiltrate VAT. Because HFD IgG became more pathogenic with longer exposure to HFD, we hypothesized that the antibodies present after early treatment were not fully pathogenic. This was verified by the inability of antibodies from animals treated with CD20-specific mAb to transfer metabolic disease. The lack of pathogenicity of HFD IgG from CD20-specific mAb treated mice at this time point could be a result of reduced affinity maturation, reduced class switching, or both (591).

Rituximab, a mAb specific for human CD20 and used in the treatment of rheumatoid arthritis as well as B cell malignancies, can cause both hyperglycemia and severe hypoglycemia (592). Other B cell- and antibody-modulating agents are either approved for human use or in clinical trials, including intravenous immunoglobulin (IvIg), transmembrane activator and calcium modulator and cyclophilin ligand (TACI) fusion proteins, and antibodies or small molecule

inhibitors to CD19, CD22, CD79a and b, B lymphocyte stimulator (BLyS), spleen tyrosine kinase (Syk), and a proliferation-inducing ligand (APRIL). Our findings suggest new possible uses for such agents, and agents that modulate FcR function and signaling, in the management of obesity-related abnormalities in glucose metabolism.

Collectively, our data support a model wherein early recruitment of B cells promotes VAT T cell activation and proinflammatory cytokine production, which potentiate M1 macrophage polarization and insulin resistance. B cells can also exert their detrimental effects systemically through the production of pathogenic IgG antibodies, which target distinct clusters of self proteins. Comparative mass sequencing of T and B cell antigen receptors in obesity-related insulin resistance is underway and promises to yield additional insights into the fundamental cause of this pervasive disease.

CHAPTER 4 (B)

Immunomodulation using GFAP Improves Obesity and Insulin Resistance

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Contributions: Mouse studies and histopathology were designed and done by myself (GP) with technical assistance by PW and RB. As is customary in our lab, I had advice and many helpful discussions by several senior members in the Dosch lab (HMD, HT, SW, YM, JY). Human proliferation assay experiments were performed by RC. Collaborative metabolic studies were done by the Drucker lab (DJD, LB).

Abstract

Inflammation plays a key role in the pathogenesis of obesity-related metabolic disease and Type 2 Diabetes (T2D). This response is ultimately driven by cognate autoimmunity *via* severely TCR-selected, fat-resident lymphocytes whose antigenic targets are unresolved. Here we show that insulin resistant T2D patients and obese C57BL/6 mice generate GFAP-specific autoimmunity. Systemic GFAP immunotherapy normalizes obesity-induced abnormalities in glucose homeostasis, insulin resistance, metabolic profile, and their progression. GFAP immunized mice exhibit decreased adipose tissue inflammation through lymphocytes, whose adoptive transfer into obese RAG1^{-/-} recipients results in improved glucose tolerance and insulin resistance. GFAP is one of likely few auto-antigens targeted by, and driving progression of, the immune response in obesity. Non-toxic systemic vaccination with GFAP deviates pathogenic lymphocyte pools and may be a lasting, affordable treatment option for early and later states of metabolic syndrome and T2D.

Introduction

The incidence of obesity is dramatically increasing worldwide, representing an epidemic of morbidity with few effective therapeutic options. Diet-induced obesity (DIO) is associated with chronic low-grade inflammation, including the activation of the NF- κ B pathway and an elevation in proinflammatory cytokines (384, 385, 391). It has been suggested that essentially systemic inflammation in liver, muscle, and adipose tissue may drive progression from obesity to insulin resistance and eventual overt Type 2 Diabetes (T2D) in those genetically permissive (393). Recently, several groups have independently demonstrated that leukocytes such as macrophages, neutrophils, NKT cells, CD4⁺ and CD8⁺ T cells, and B cells are active participants in this progressive process with potentially beneficial or detrimental effects on whole-body energy homeostasis (394, 397, 399, 401, 408, 564, 593, 594). Each of these cell types accumulate in insulin-sensitive tissues such as fat and contribute to local tissue inflammation.

We and others have provided strong evidence that these processes involve cognate recognition of a likely small number of target epitopes which drives severe selection of a narrow TCR- α and - β chain repertoires (397, 408, 566). Additionally, protein arrays have revealed several antigens differentially targeted by antibodies in insulin resistant and insulin sensitive patients (594). One protein, glial fibrillary acidic protein (GFAP), was originally linked to Type 1 Diabetes (T1D) and insulin resistance by our group as a key autoantigen targeted early in insulinitis (485, 595).

Autoimmune targeting of GFAP in diabetes is not obvious, given its role as an intermediate filament crucial for normal cell structure and signal transduction in mature astrocytes within the central nervous system (CNS) (474, 596), and in peripheral tissue such as enteric glia, Schwann cells, and possibly lymphocyte subsets (484, 486, 487). GFAP expression is upregulated following CNS injury and has been implicated in neurodegenerative conditions such as Parkinson's and

Alzheimer's disease (492, 597), while genetic mutations lead to the progressive and fatal Alexander disease (475). The GFAP gene is highly polymorphic, and though several isoforms are known to exist, their individual function is not well understood (477). Given its widespread expression and function, it is surprising that mice with a targeted deletion of the GFAP gene (GFAP^{-/-}) do not exhibit gross physical or neurological impairment, though changes are observed in synaptic signalling within the CNS (493).

Similarities between T1D and T2D are being increasingly recognized, including critical, pathogenic immune contributions in both diseases. We searched for possible GFAP autoreactivity in T2D and obesity models, asking if it was present and whether it could be linked to weight gain and insulin resistance. Anti-GFAP responses were detected in most T2D patients and they arise in B6 mice on hypercaloric diets. Immunization with GFAP in Incomplete Freund's Adjuvant (IFA) dramatically reduced weight gain despite continuous high fat diet. Treated mice showed improved glucose tolerance, increased insulin sensitivity, and their adipose tissue displayed decreased pro-inflammatory macrophage infiltration. Immunization with other self or non-self proteins was unable to elicit similar results. The effects of GFAP immunization are immune-mediated, as demonstrated by splenocyte transfer from immunized mice to recipient DIO RAG1^{-/-} mice. We successfully demonstrate that immunization can effectively treat obesity and insulin resistance.

Methods

Mice

All mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in our *vivarium* in a pathogen-free, temperature controlled environment on a 12hr light and dark cycle. All experiments were performed using male age-matched mice under approved protocols in agreement with animal ethics guidelines.

Immunizations and metabolic studies

Animals were kept on a regular diet for the first 6wk of life, at which time they were either maintained on this diet, or switched to a high-fat (60 kcal% fat) diet (Research Diets). At 6wk of age, mice were immunized intraperitoneally with 5 μ g of OVA (Sigma-Aldrich), actin (Cytoskeleton), or GFAP (Calbiochem) emulsified 1:1 in IFA (Sigma-Aldrich). After 8wk, fasting blood glucose and insulin concentrations (Crystal Chem ELISA) were measured. For glucose tolerance tests, mice were fasted for 16hr and then injected with 0.75 g of glucose (Sigma-Aldrich) per kg of body weight. For insulin tolerance tests, mice were fasted for 4hr and then given 0.75 U of human regular insulin (Eli Lilly) per kg of body weight. VAT and SAT fat pads were carefully dissected and weights were recorded. For transfer into RAG1^{-/-} mice, 1×10^7 splenocytes were injected intraperitoneally and glucose tolerance tests were performed on recipient mice 4wk after transfer. For immunizations with CFA, mice were injected with 5 μ g of MOG₃₅₋₅₅ peptide (Alpha Diagnostic) or GFAP (Calbiochem) emulsified in CFA (1:1) (Sigma-Aldrich), and 200 ng pertussis toxin (Sigma-Aldrich) was given i.p. on the day of immunization and 48hr later.

Oxymax and MRI studies

We placed mice in individual metabolic chambers with free access to food and water, measuring O₂ consumption, CO₂ output, heat, ambulatory and total activity over 17hr by indirect

calorimetry (Oxymax System, Columbus Instruments). We normalized all measurements to body weight. Total body fat and lean mass were measured using a whole-body magnetic resonance analyzer (Echo Medical Systems).

Histology and immunofluorescence

Fat, liver, brain, and spinal cord tissue was dissected and fixed for up to 72hr in 10% buffered formalin before staining with hematoxylin and eosin (H&E). Cell size and crown-like structures were quantified by a blinded observer using ImageJ software analysis. At least 180 total fat cells were counted from three different tissue sections of three different mice per group. For immunohistochemistry, we used paraffin-embedded sections stained with a CD3 (Sigma-Aldrich) or Mac-2 antibody (Cedarlane). Frozen pancreas sections were fixed in 4% paraformaldehyde, blocked with normal donkey serum, and probed with the following antibodies: rabbit anti-GFAP antibody (Covance), FitC-conjugated donkey anti-rabbit antibody (1:25, Jackson ImmunoResearch), guinea pig anti-insulin antibody (Dako), and Cy5-conjugated donkey anti-guinea pig antibody (1:300, Jackson ImmunoResearch).

Proliferation and cytokine secretion

4×10^5 human peripheral blood mononuclear cells (PBMC) or mouse splenocytes were incubated in 96-well plates in HL-1 media (Lonza) and stimulated with protein, 1 $\mu\text{g}/\text{mL}$ α -CD3 and 0.25 $\mu\text{g}/\text{mL}$ α -CD28, or 1 $\mu\text{g}/\text{mL}$ α -CD40 (BD Pharmingen) for 72hr. For proliferation experiments, 1 mCi of [^3H] thymidine was added for 18hr, prior to harvest and liquid scintillation counting. SI corresponds to the fold change above background proliferation. For cytokine analysis, supernatants were collected after 72hr of culture. IFN- γ , IL-6, and IL-10 (BD Biosciences), IL-17 and IL-13 (R&D Systems), and TNF- α (eBioscience) were measured by ELISA according to the manufacturer's protocols.

Anti-GFAP antibody assay

For detection of anti-GFAP antibodies, plates were coated overnight at 4°C with 2.5 µg/mL GFAP (Calbiochem) in PBS. The following day, plates were washed and blocked with 2% BSA (BioShop) for 1.5hr at room temperature. Samples were serially diluted in the plates in 3-fold dilutions and incubated for 2hr at room temperature. Plates were then probed with HRP-conjugated goat anti-mouse IgG (1:1000, Invitrogen) and colour was developed using a TMB substrate kit (Fisher Scientific).

Flow cytometry

$1.5-2.0 \times 10^6$ splenocytes were incubated at 4°C for 15min in 100 µL 2% FBS/PBS with 10 µg/mL Fc-blocker (eBioscience). Cells were incubated for 30min with the following fluorescent antibodies: F4/80 (1:100), CD3 (1:100), CD4 (1:75), CD8 (1:100), B220 (1:100), IgM (1:100), and FoxP3 (1:100) (eBioscience). Samples were run on an LSR-II flow cytometer (BD Biosciences) and FACS plots were analyzed using FlowJo software (Tree Star Inc.).

Immunoblotting

Mouse VAT, SAT, and brain tissue was dissected and snap-frozen. Extracted total protein or purified GFAP (Calbiochem) was electro-fractionated on 4-12% acrylamide gels (Invitrogen), blotted, and probed overnight at 4°C with serum or rabbit anti-GFAP antibody (1:1000, Abcam) and rabbit anti-β-actin antibody (1:1000, Cell Signaling). The following day, bands were visualized using Western Lightning-ECL kit (Perkin Elmer).

Mass spectrometry

GFAP (Calbiochem) was electro-fractionated on 4-12% acrylamide gels (Invitrogen) and stained with Coomassie Blue. Bands were manually excised and LCMS/MS results were analyzed using the Scaffold 3 Viewer (Proteome Software).

Statistical analysis

Statistical significance was assessed by Mann-Whitney and unpaired t-tests, and Welch correction was employed where necessary. Analysis of curves was performed using two-way ANOVA. Statistical significance was two tailed and set at 5%, with error bars showing a single standard deviation.

Results

Measurable anti-GFAP immune responses are present in humans and mice

We first measured GFAP autoreactivity in patients with T2D and in obese mice (583, 594). Peripheral blood mononuclear cells from 19 T2D patients were stimulated *in vitro* with the non-self protein ovalbumin (OVA), the self protein actin, or the self protein GFAP. Patients (~60%) exclusively recognised GFAP, but not OVA or actin (**Figure 4.6.A**). B6 mice with diet-induced obesity (DIO), insulin-resistance and near-diabetic hyperglycemia were compared to mice on regular diet (RD). Both RD and DIO mice had proliferative GFAP T cell responses (**Figure 4.6.B**), but DIO splenocytes produced greater amounts of the proinflammatory cytokines TNF- α , IL-6, and IFN- γ (**Figure 4.6.C**). These factors drive IgG production, pathogenic in obese mice (**Figure 4.4**). DIO mice showed robust anti-GFAP antibody responses, well above RD mice, that increased with time on high fat diet (**Figure 4.6.D**). The specificity of our antibody data were confirmed using B^{-/-} mice and mice deficient in GFAP (GFAP^{-/-}). In NOD mice, anti-GFAP autoimmunity promotes T1D progression. However, RD and DIO B6 mice did not display any signs of insulinitis, and the peri-islet Schwann cell mantle remains intact, unlike in NOD T1D (**Figure 4.6.E-G**) (485).

Mouse anti-GFAP IgG targeted a unique array of proteins of variable molecular weight, that differed based on the age and diet of mice (**Figure 4.7.A**). Purified GFAP protein comprises a number of bands that likely correspond to its numerous known isoforms (**Figure 4.7.B**). Analysis of these proteins revealed variable detection of regions of GFAP using mass spectrometry (**Figure 4.7.C**). Intriguingly, GFAP was also found to be expressed in adipose tissue, a primary site of obesity-induced inflammation that drives insulin resistance, in a dramatically isoform-selective manner that differs from the brain (**Figure 4.7.D**).

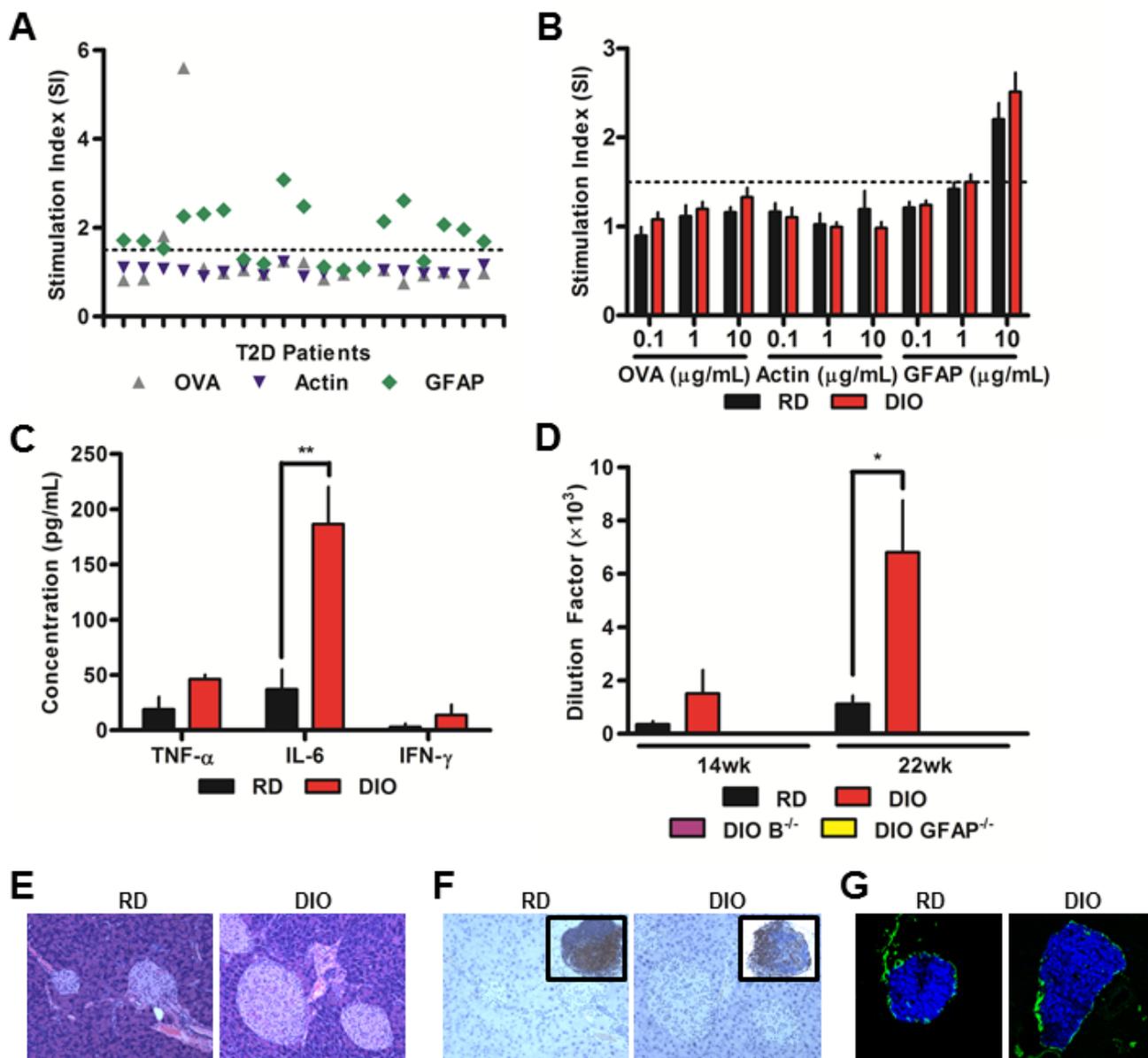


Figure 4.6 – GFAP autoreactivity is detectable in T2D patients and obese mice. (A) Stimulation index of peripheral blood from T2D patients following culture with OVA, actin, and GFAP (n=19). (B) Stimulation index of peripheral blood from naïve RD and DIO mice 14-22wk of age following culture with OVA, actin, and GFAP (n≥5 per group). (C) Splenocyte cytokine secretion following culture *in vitro* (n≥3 per group, **p<0.01). (D) ELISA for anti-GFAP IgG in RD and DIO wild type and B^{-/-} mice 14wk and 22wk of age (n=5 per group, *p<0.05). (E-F) Representative H&E (E) and CD3 (F) stained pancreas from RD and DIO mice 22wk of age. (G) Representative immunohistochemistry of pancreas sections of RD and DIO mice 22wk of age, showing GFAP (green) and insulin (blue).

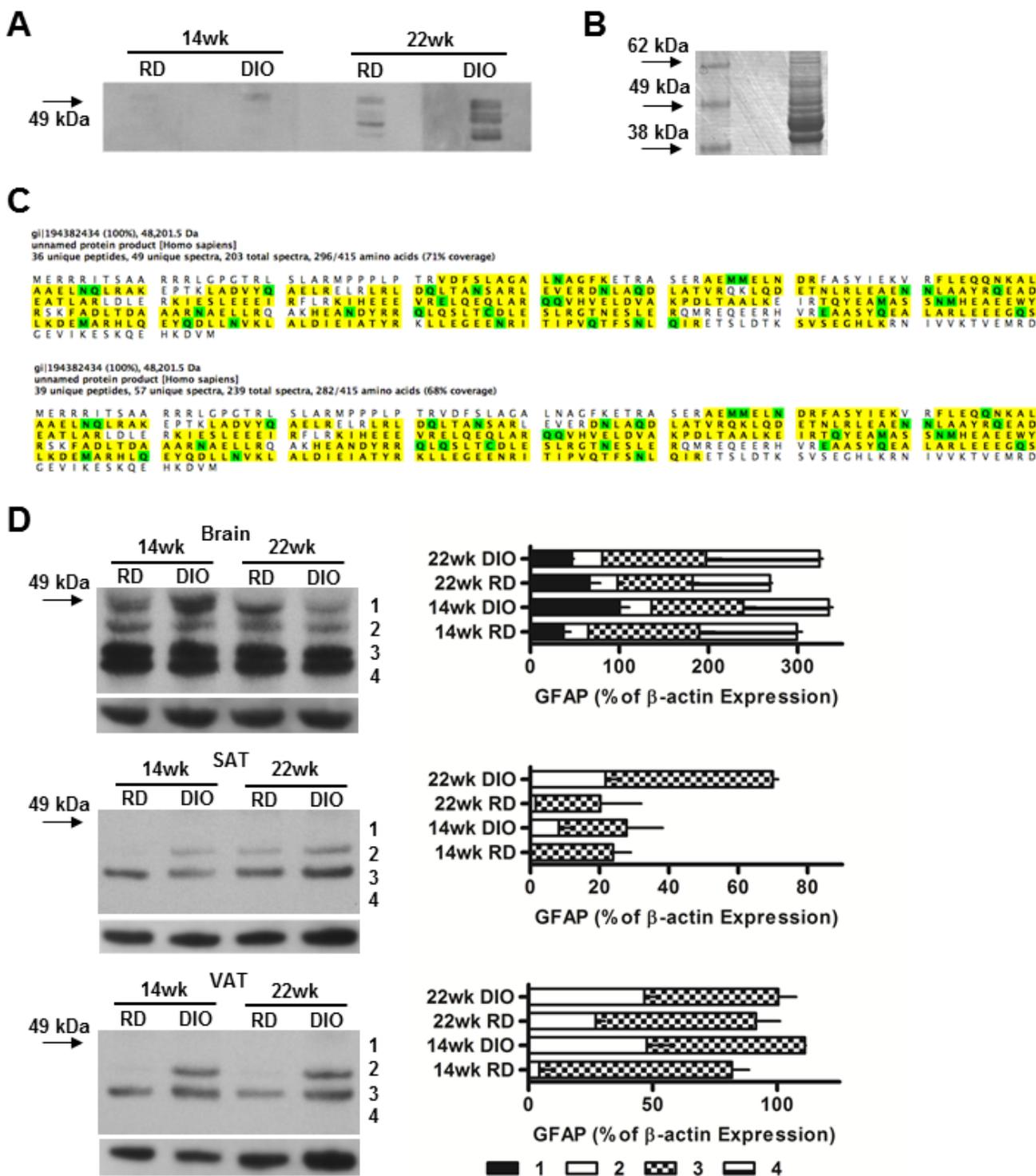


Figure 4.7 – DIO mice do not show restricted GFAP isoform reactivity and expression in adipose tissue. (A) Representative Western blot of GFAP protein probed with sera from naïve RD and DIO mice 14wk and 22wk of age. (B) Representative coomassie-stained gel showing band distribution from purified GFAP. (C) Mass spectrometry of GFAP purified protein showing the degree of amino acid overlap (highlighted) of two different bands with the known full-length sequence. (D) Representative Western blot (left) and quantification of bands by analysis of pixel density (right, n=3 per group) of GFAP protein in the brain (upper), SAT (middle), and VAT (lower) of naïve RD and DIO mice 14wk and 22wk of age, with β -actin serving as a loading control.

GFAP immunization limits adiposity and insulin resistance in DIO mice

Given the robust GFAP autoimmunity in B6 mice, we asked if immunotherapy could decrease weight gain and improve glucose homeostasis, as it is beneficial in NOD mouse T1D (485). We initially immunized mice on a regular diet at 6wk of age with a combination of IFA and OVA (RD^{OVA}), actin (RD^{Actin}), or GFAP (RD^{GFAP}). These immunizations were unable to significantly affect weight gain over the subsequent 8wks (**Figure 4.8.A-B**). There was no change in fat mass, fasting glucose, or fasting insulin in these mice (**Figure 4.8.C-E**), and immunized and non-immunized RD mice showed a similar capacity to maintain glucose homeostasis following challenge with glucose (**Figure 4.8.F**).

However, the obese state leads to changes in protein expression and modification, as well as the environment in which proteins are exposed to the immune system, thus potentially altering the response to proteins such as GFAP (**Figure 4.6.D-E** and **Figure 4.7.D**). Therefore, we immunized mice at 6wk of age, as they were switched to a high-fat diet. Immunization with IFA *plus* GFAP (DIO^{GFAP}) significantly reduced the weight gain associated with high-fat diet (**Figure 4.9.A**), while immunization with OVA (DIO^{OVA}) or actin (DIO^{Actin}) was ineffective. DIO^{GFAP} mice displayed weight gain with a trajectory dramatically different from DIO, DIO^{OVA}, and DIO^{Actin} mice (**Figure 4.9.B**). The accumulating fat mass was significantly reduced in SAT and in more pathogenic VAT depots of DIO^{GFAP} mice (**Figure 4.9.C**). 14wk old DIO^{GFAP} mice also showed controlled near normal fasting glucose and insulin levels, comparable to naïve RD mice, while DIO, DIO^{OVA}, and DIO^{Actin} mice bordered on diabetic readings (**Figure 4.9.D-E**). DIO^{GFAP} mice also showed a greater ability to normalize blood glucose levels following oral glucose challenge and an essentially normal insulin sensitivity (**Figure 4.9.F-G**).

Given the reduction in weight gain, we analyzed the adiposity of mice following immunization with GFAP in IFA. DIO^{GFAP} mice exhibited a reduction in SAT and VAT adipocyte

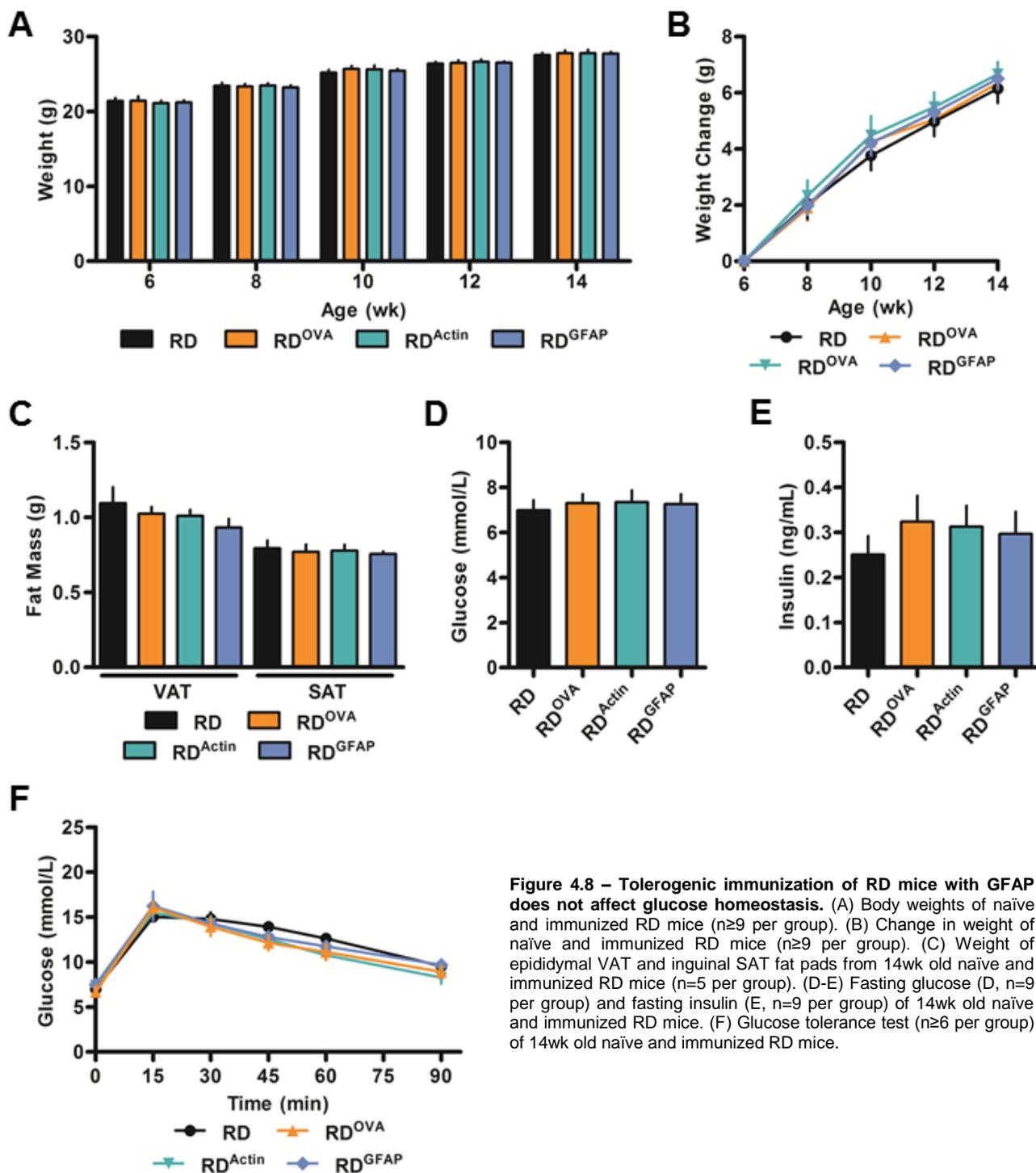


Figure 4.8 – Tolerogenic immunization of RD mice with GFAP does not affect glucose homeostasis. (A) Body weights of naïve and immunized RD mice (n≥9 per group). (B) Change in weight of naïve and immunized RD mice (n≥9 per group). (C) Weight of epididymal VAT and inguinal SAT fat pads from 14wk old naïve and immunized RD mice (n=5 per group). (D-E) Fasting glucose (D, n=9 per group) and fasting insulin (E, n=9 per group) of 14wk old naïve and immunized RD mice. (F) Glucose tolerance test (n≥6 per group) of 14wk old naïve and immunized RD mice.

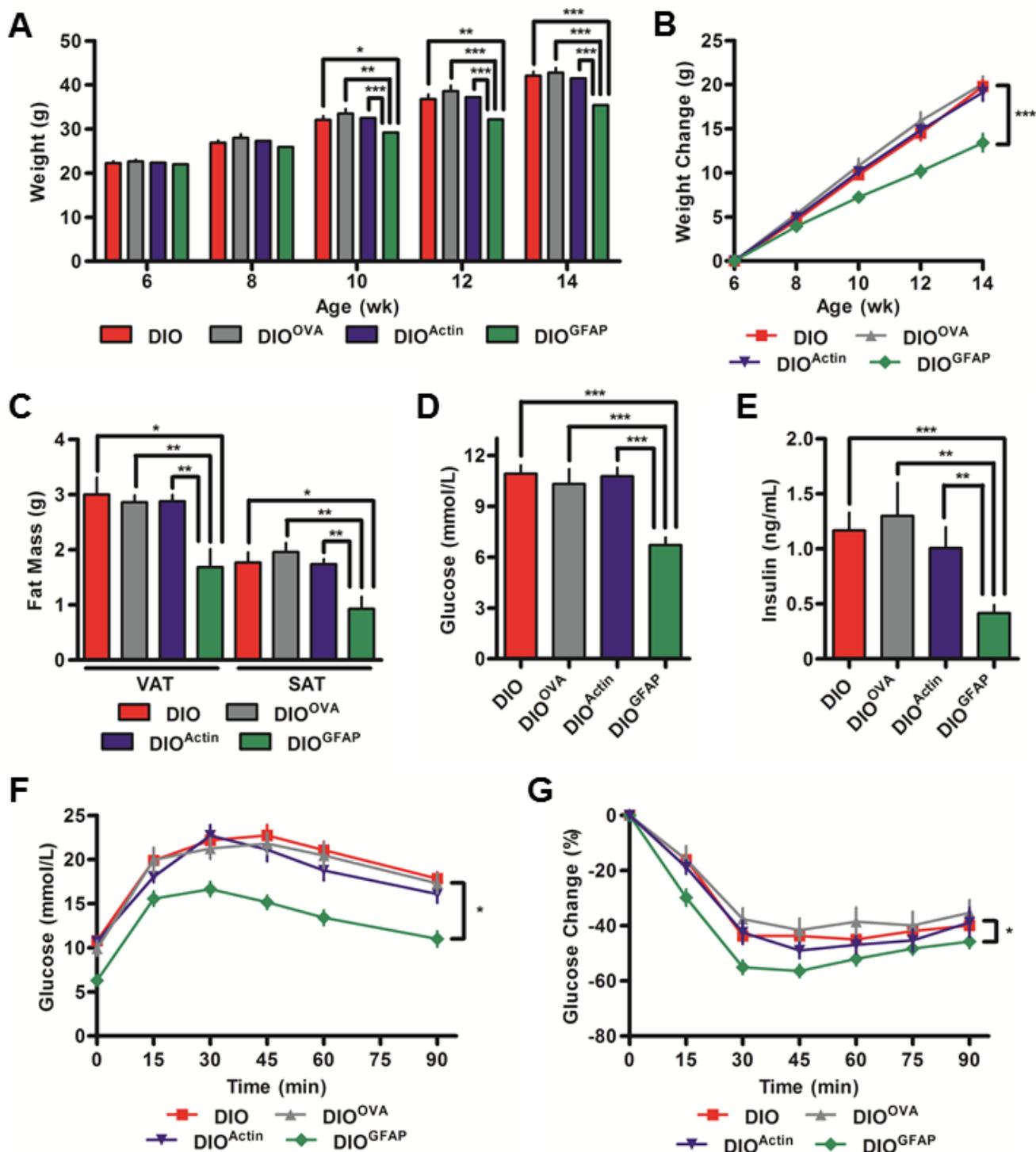


Figure 4.9 – Immunization of DIO mice with GFAP prevents weight gain and insulin resistance. (A) Body weights of naïve and immunized mice on high fat diet for 8wk (n≥12 per group, *p<0.05, **p<0.01, ***p<0.001). (B) Change in weight following placement on high fat diet (n≥12 per group, ***p<0.001). (C) Weight of epididymal VAT and inguinal SAT fat pads from 14wk old naïve and immunized DIO mice (n=6 per group, *p<0.05, **p<0.01). (D-E) Fasting glucose (D, n≥12 per group, ***p<0.001) and fasting insulin (E, n≥12 per group, **p<0.01, ***p<0.001) of 14wk old naïve and immunized DIO mice. (F-G) Glucose tolerance test (F, n≥10 per group, *p<0.05) and insulin tolerance test (G, n≥10 per group, *p<0.05) of 14wk old naïve and immunized DIO mice.

cell diameter that was not observed in DIO^{OVA} or DIO^{Actin} mice (**Figure 4.10.A and C**). DIO^{GFAP} mice also exhibited less macrophage infiltration in fat and fewer crown-like structures, indicative of reduced local inflammation known to contribute to obesity-associated insulin resistance (**Figure 4.10.B-C**). The liver of DIO^{GFAP} mice displayed less hepatosteatosis, with far less liposome inclusions (**Figure 4.10.C**), completing the wide range of physiological parameters related to the metabolic syndrome and T2D that were impacted positively by tolerogenic GFAP immunization.

GFAP is abundant in both peripheral and central neuronal tissue and we questioned if there was any leukocytic infiltration at these sites. Brain and peripheral pancreas tissue sections in immunized mice appeared normal and showed no invasive T cells (**Figure 4.11.A-B**). We were unable to induce CNS demyelination using GFAP in highly immunogenic Complete Freund's Adjuvant (CFA) (**Figure 4.11.C-E**), a powerful stimulant employed for immunization with myelin components in experimental models of encephalitis (403).

Consistent with the effects following immunization with GFAP, we observed dramatic changes in the metabolic profile of DIO^{GFAP} mice. Compared to DIO^{OVA} mice, DIO^{GFAP} mice consume more O₂, produce greater quantities of CO₂, and are significantly more active (**Figure 4.12.A-E**). These effects are thus strictly cognate and exclusive to the obese state as they are not observed in RD mice.

Leukocytes from GFAP immunized mice have reduced pathogenicity

We examined the immune profile of the RD and DIO immunized mice. Total cellularity in the spleen did not differ significantly between groups (**Figure 4.13.A**), and these cells showed an equal capacity to proliferate following T or B cell-specific stimulation (**Figure 4.13.B**). However, DIO^{GFAP} splenocytes are skewed away from producing inflammatory cytokines such as IFN- γ and IL-17 (**Figure 4.13.C**). As evidence of their lack of pathogenicity, transfer into obese RAG1^{-/-} mice (DIO^{RAG}) led to a measurable improvement from pre-transfer levels, while DIO^{OVA} cells led to a

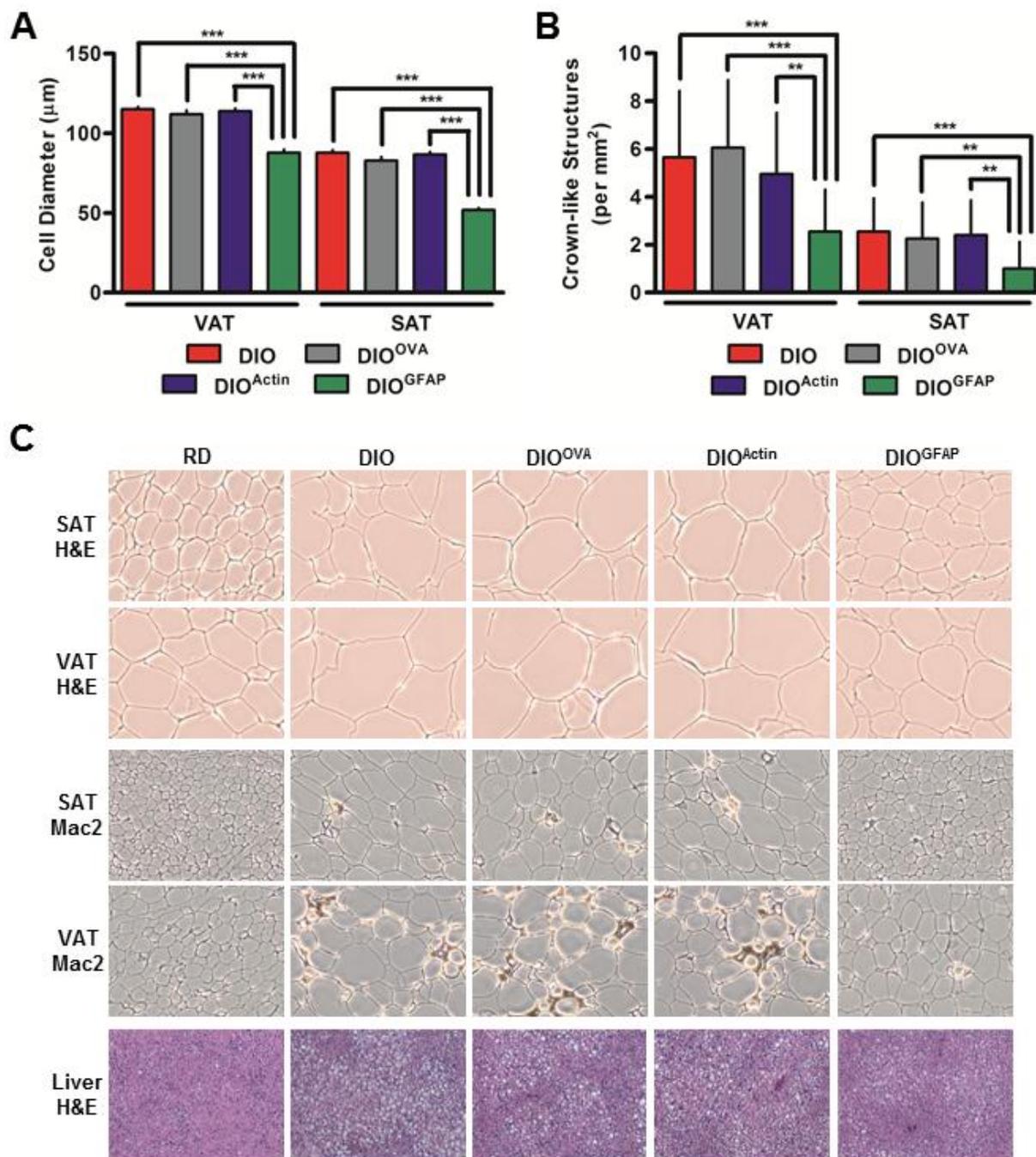


Figure 4.10 – GFAP immunization limits adiposity and local adipose inflammation. (A) Quantification of epididymal VAT and inguinal SAT fat cell diameter of 14wk old naïve and immunized mice on high fat diet (n=180 per group, ***p<0.001). (B) Quantification of crown-like structures in adipose tissue of 14wk old naïve and immunized mice on high fat diet (n=20 per group, **p<0.01, ***p<0.001). (C) Representative H&E and Mac-2 stained SAT, VAT, and liver sections from 14wk old mice.

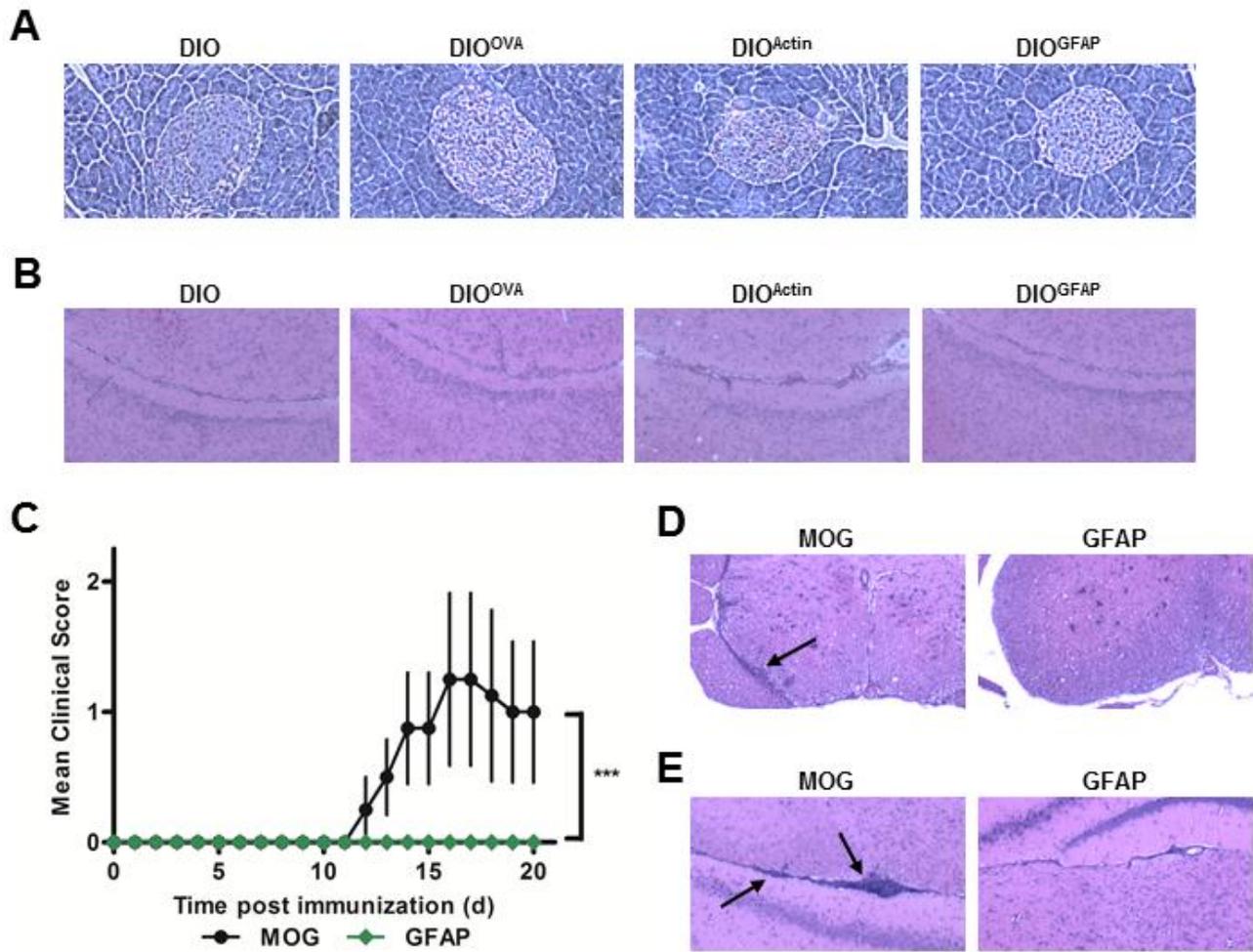


Figure 4.11 – Immunization with GFAP does not result in pancreas or CNS infiltration. (A-B) Representative CD3 stained pancreas (A) and representative H&E stained brain (B) sections from 14wk old naïve and immunized DIO mice. (C) Daily clinical scores of mice following immunization with MOG₃₅₋₅₅ peptide or GFAP and pertussis toxin (n=4 per group, ***p<0.001). (D-E) Representative H&E stained spinal cord (D) and brain (E) sections of MOG₃₅₋₅₅ peptide or GFAP immunized mice 20d post immunization.

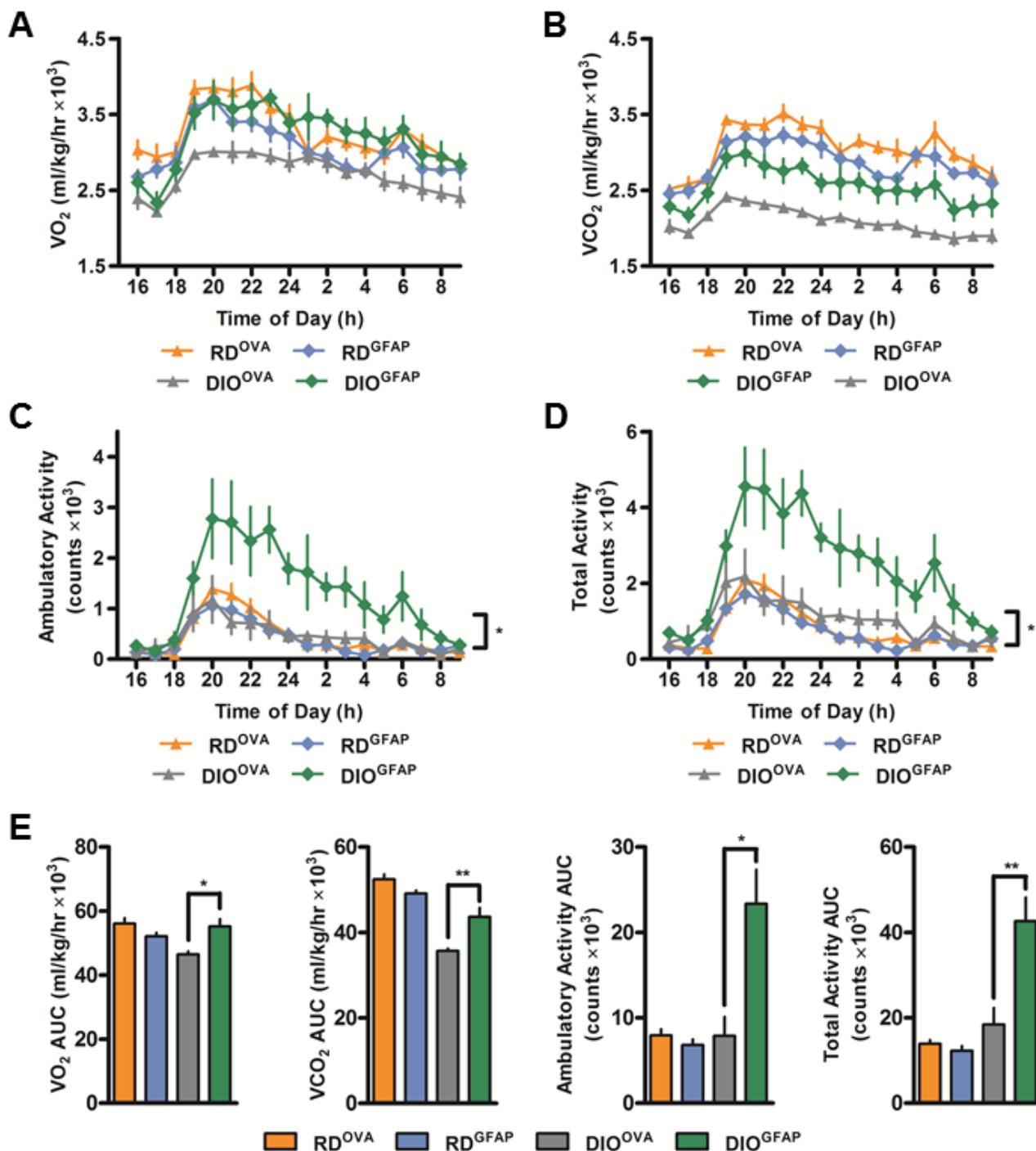


Figure 4.12 – GFAP immunization alters the metabolic profile of DIO mice. (A-D) VO_2 (A), VCO_2 (B), ambulatory activity (C), and total activity (D) of 14wk old immunized RD and DIO mice ($n \geq 5$ per group, * $p < 0.05$). (E) Quantification of the area under the curve of VO_2 , VCO_2 , ambulatory activity, and total activity readings ($n \geq 5$ per group, * $p < 0.05$, ** $p < 0.01$).

worsening of the ability to normalize glucose (**Figure 4.13.D-E**). Tolerogenic immunization with GFAP therefore likely functions through modulation of the proinflammatory and pathogenic anti-GFAP response.

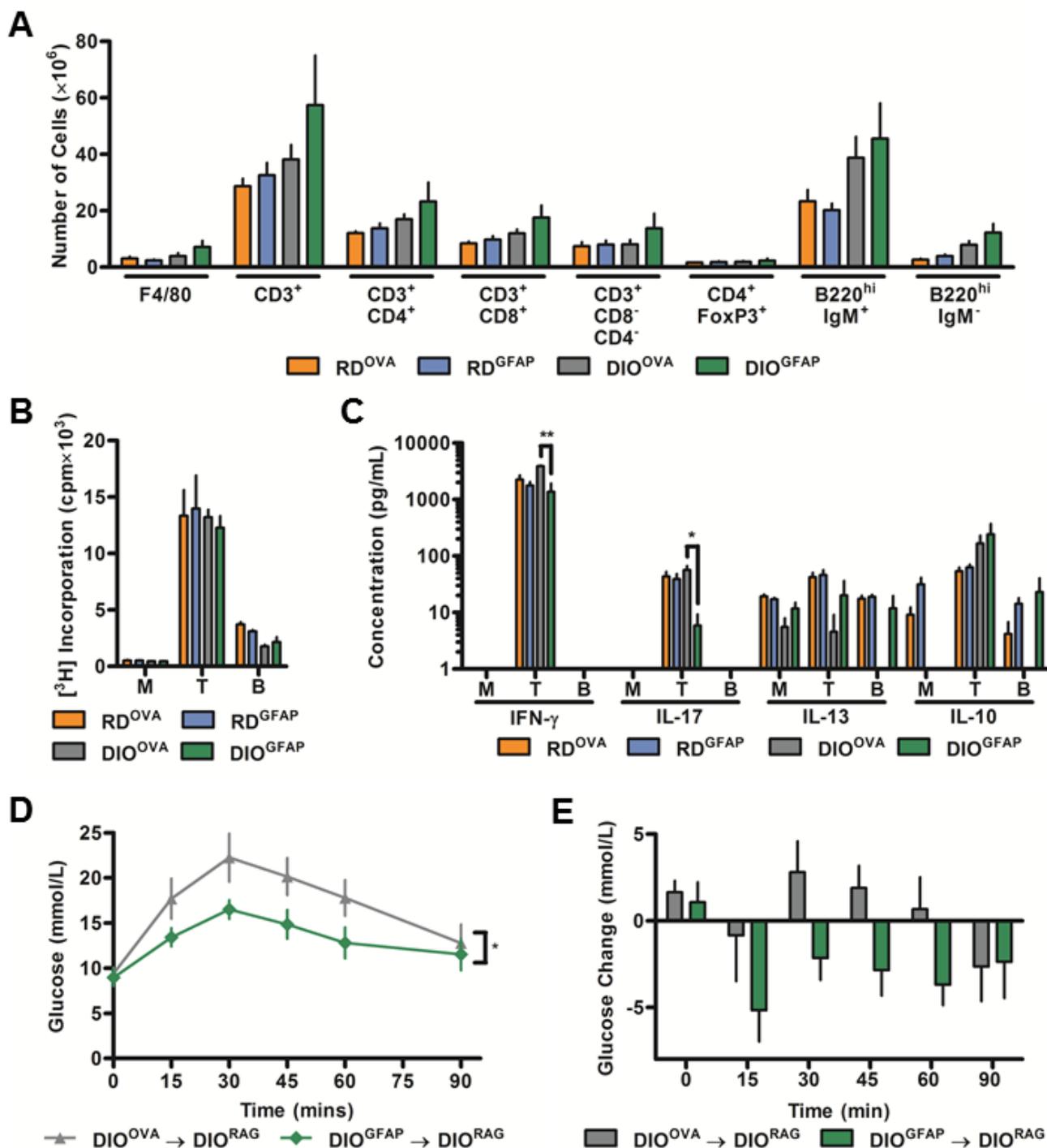


Figure 4.13 – Immunization with GFAP decreases splenocyte-derived proinflammatory cytokine production and prevents transfer of insulin resistance. (A) Cellular composition of the spleen of 14wk old immunized mice ($n \geq 3$ per group). (B) Mean stimulation index of splenocytes from immunized mice in media (M) or stimulated with α -CD3 and α -CD28 (T) or α -CD40 (B) antibodies ($n = 4$ per group). (C) Production of IFN- γ , IL-17, IL-13, and IL-10 by splenocytes following α -CD3 and α -CD28 or α -CD40 stimulation ($n \geq 4$ per group, * $p < 0.05$, ** $p < 0.01$). (D) Glucose tolerance test of RAG^{-/-} mice 4wk post-transfer of splenocytes from DIO^{OVA} or DIO^{GFAP} mice ($n = 4$ per group, * $p < 0.05$). (E) Change in glucose tolerance test readings comparing pre- and post-transfer readings ($n = 4$ per group).

Discussion

The ability of the adaptive immune system to regulate parameters of T2D is emerging as a major element of progressive metabolic pathogenesis, with a number of cell types actively recruited to adipose tissue during progressive obesity (397, 564, 594). Additionally, publications by us and others have documented an extreme T or B cell receptor bias in obesity and/or T2D (397, 408, 566, 594). Ultimately, it stands to reason that if the immunome plays a central, controlling role in metabolic diseases such as diabetes, it may be possible to harness the autologous immune system for therapy.

We were intrigued by the appearance of GFAP as an autoimmune target in obesity/T2D, given our previous publications linking it to diabetes pathogenesis. Analysis of the blood of T2D patients for peripheral anti-GFAP responses revealed a selective response to GFAP but not OVA or actin. We were also able to consistently detect anti-GFAP IgG levels in mice that increased with both age and adiposity. Given the 2-3wk half life of IgG in serum (598), it is likely that the GFAP response is both continuous and expanding progressively.

Adipose tissue rapidly becomes a site of chronic inflammation in the obese state and it is conceivable that innocuous self proteins, such as GFAP, may undergo alternative presentation to the immune system and relate to extreme T and B cell antigen receptor biases. We show for the first time that GFAP is expressed in subcutaneous and visceral fat depots in an isoform-selective manner, with the lipid-rich microenvironment driving selective isoform expression. Indeed, DIO GFAP isoforms targeted by anti-GFAP autoantibodies have a strikingly similar distribution as those selectively expressed in fat.

Our data provide strong evidence that GFAP autoreactivity is sufficiently major to elicit therapeutic effectiveness when altered. We discovered that immunization using IFA is effective at

preventing obesity and insulin resistance. DIO^{GFAP} mice showed fasting insulin and glucose levels that were comparable to control mice on regular diet, a dramatic and long-lasting outcome that is surprising given our immunization regimen of a single injection at 6wk of age. This effect is specific for GFAP, as other proteins were unable to produce analogous improvements in glucose homeostasis or behavioural modifications. Since RD^{GFAP} immunizations ultimately had no measurable ultimately metabolic effects, we conclude that obesity itself generates GFAP autoimmunity that is subsequently attenuated by GFAP-IFA immunization, sufficient to affect all measures of obesity progression measurable in our hands.

Immunization modulated the immunome, as demonstrated by protection following adoptive immune cell transfer in RAG-deficient mice. It is unlikely that any single immune or endocrine function, cell type, or repertoire can have the multiple target effects here ascribed to GFAP immunization. Considerably more work will be required to characterize the anti-GFAP response prior to vaccination, and how this is altered subsequent to our intervention. Vaccination also significantly altered the metabolic and behavioural profile of treated mice, impacting oxygen usage and carbon dioxide production, and dramatically increasing cage activity. In contrast, no significant changes were observed in the respiratory exchange ratio, indicative of similar carbohydrate and lipid metabolism.

Collectively, our studies identify multiple players and targets of the process driven by hypercaloric diet. We also identify a practical therapeutic escape strategy, mediated by mechanistic cytokine elements but we may remain ignorant if there is a singular, core pathogenic switch that sustains disease progression and is deviated or blocked by GFAP immunization.

CHAPTER 5

General Discussion

The data presented in this thesis bridges several fields, including immunology, neuroscience, and metabolism. In doing so, we have made several important novel discoveries that advance our understanding of inflammatory disease pathogenesis. Collectively, we hope that our work will impact future treatment avenues and improve the lives of Multiple Sclerosis (MS) and Type 2 Diabetes (T2D) patients. Current financial pressures on health care systems around the world, including Canada, continue to mount exponentially. The rise of major non-communicable diseases will soon overwhelm economies relative to gross domestic product (599), and therefore more effective alternatives to current therapies are required to maintain high standards of care.

The field of MS research is often looked at critically, due to a lack of tangible progress in effectively treating and preventing disease. Following diagnosis, MS patients face a lifelong decline in their quality of life due to decreased cognitive functions. While current therapeutics have shown efficacy in reducing relapses in patients and improving functional assessment scores, ultimately disease will progress and cannot be reversed.

MS is an inherently difficult disease to study. Symptoms arise after an unpredictable pre-disease period of time, subsequent to the initial insult, making it difficult to trace environmental factors critical to disease susceptibility. Additionally, at the current time, few experimental techniques exist to study the pathogenesis of disease *in vivo*. The vast majority of human MS research results from analysis of peripheral tissues and blood, non-invasive imaging, and *post-mortem* biopsies of central nervous system (CNS) tissue. Invasive techniques in living patients involving the brain and spinal cord are high-risk and therefore discouraged.

As an alternative, the MS field has come to rely heavily on the animal model experimental autoimmune encephalomyelitis (EAE). Given the intensive research surrounding EAE for many decades, much has been learned about the pathogenesis of encephalitis in rodents, from initiation to fulminant disease. These conclusions are often applicable to human disease due to many similarities

between EAE and MS. However, as in other diseases such as T2D, caution must always be taken when translating results from animal models (600).

Here, we show that transient potential receptor vanilloid-1 (TRPV1) plays a significant role in the promotion and progression of encephalitis. The extent to which TRPV1 gates CNS access was surprising to us, but parallels the impressive protection demonstrated in TRPV1^{-/-} mice. Although the link between TRPV1 and the blood-brain barrier (BBB) permeability has been hypothesized sporadically in the past, its role in encephalitis has not been previously examined. We were somewhat surprised to note that substance P (sP) was not mediating the TRPV1 effect, as neuropeptides are capable of skewing inflammatory responses towards different T helper profiles (601). While neurogenic inflammation may be protective in some settings of acute tissue damage, neuro-immune interactions can also promote pathological outcomes.

We also identify genetic variation in the TRPV1 gene that affects the severity of MS. This knowledge provides a number of potential opportunities including the identification of individuals at risk for progressive disease and the establishment of more accurate prognoses. Future research should determine if TRPV1 mutations render the protein hypofunctional or hyperfunctional, the former expected based on our mouse data. We have conducted collaborative *in vitro* transfection/activation experiments with the relevant human genomic variations, with a focus on both Multiple Sclerosis and Type 1 Diabetes (T1D), where TRPV1 also plays a fundamental role. Although conclusive data were generated in T1D-associated TRPV1 alleles, MS constructs did not yield consistent expression.

Given our experimental results, blockade of TRPV1 function represents an appealing therapeutic option for MS. Our early attempts to do so using the antagonist capsazepine were unsuccessful, although we believe that adjustments to the dose, timing, and possibly the antagonist itself may be prudent future approaches. Modulation of TRPV1 function has been considered for a

number of conditions related to pain, and TRPV1 antagonism in MS may consequently also address the MS-related pain that up to half of patients experience (602). However, targeting TRPV1 has been notoriously problematic due to the hyperthermia associated with antagonism, while long-term use may carry further unknown risks (603).

Future work examining TRPV1 and the BBB should examine the cell types mediating this phenotype. We have preliminary data confirming TRPV1 expression within astrocytes, as previously reported. This cell type plays a fundamental role in barrier integrity, and further investigation into astrocyte functions mediated by TRPV1 is warranted. Alternatively, neurons within the CNS that express TRPV1 may be activated by high insulin levels, local decreases in pH which also results in acid-sensing ion channel (ASIC) activation (177), or lipid metabolites upregulated in the brain and spinal cord during neuroinflammatory conditions such as EAE (530). Persistently active sodium channels and the subsequent shift in sodium and calcium balance within neurons can lead to axonal calcium overload and calcium-mediated toxicity (80).

Our work has also addressed the current obesity epidemic, a major worldwide health concern. Increased prevalence of obesity has resulted from dietary shifts and increasingly sedentary lives that reduce energy expenditure. These factors have important consequences on T2D, where obesity is clearly the greatest environmental risk factor. Indeed, the most commonly used mouse model of T2D, the diet-induced obese (DIO) mouse, is centered on weight gain. But the obese state can also promote numerous neuronal and hormonal changes that may or may not affect immune system activation. Additionally, the ability of adipocytes, once thought to be simple inert lipid storage depots, to produce and secrete immune modulators, is now realized.

We demonstrate that obesity has impressive effects on the immune system via IL-6, likely of both adipocyte and immune origin. IL-6 selectively expands systemic T_H17 cell populations in DIO mice in the naïve state compared to RD mice, and this increases significantly following active

priming of an immune response. As the proinflammatory properties of adipose tissue vary based on location, it would be interesting to determine if this T_H17 expansion is dependent on visceral adipose tissue to a greater extent than subcutaneous adipose tissue.

We questioned the clinical relevance of this discovery, and therefore examined autoimmune disease such as EAE, which relies heavily on a T_H17 -dependent component. Obese mice exhibit increased disease severity, increased histological scoring, and greater IL-17 production in the spleen, draining lymph nodes, and diseased CNS compared to mice on a regular diet.

While we were unable to investigate similar effects in MS due to a lack of patient and tissue access, other groups have independently reached similar conclusions subsequent to the publication of our study (604, 605). The growth of the obesity epidemic may therefore impact both incidence rates and the prognosis of MS, while further mechanistic understanding may lead to therapeutic possibilities. For example, IL-6 blockade in overweight and obese MS patients may reduce the severity of disease, or, alternatively, weight loss could help to slow progression.

Given the enormous social and financial burden of both obesity and T2D, there has been a dramatic increase in related research. This shift is necessary, as therapeutics for T2D are not able to prevent adverse outcomes, or reverse disease. Efficacy is often measured as a decrease in cardiovascular-related events, though reductions are generally, at best, modest. Similar to MS, T2D symptoms only arise after a lengthy asymptomatic period of disease progression, making it difficult to study environmental factors that increase disease incidence. Examination of T2D pathogenesis can be done through metabolic testing, though relevant tissue-specific assays may require more invasive techniques.

Although genetic risk loci for T2D do not directly implicate the immune system, the importance of inflammation in promoting insulin resistance has been consistently demonstrated, making it important to consider in disease pathogenesis.

We were the first group to demonstrate a definitive role for B cells and antibodies in obesity-related insulin resistance. B cell deficiency resulted in an impressive improvement in glucose tolerance and insulin resistance. This effect is, at least in part, mediated by antibody production, as we were able to transfer insulin resistance with IgG. This represents a novel and unexpected observation that changes our understanding of disease pathogenesis and it should add caution to the use of obese individuals as blood donors.

Transfer required donor IgG to come from DIO mice, once again demonstrating the importance of obesity-mediated conditioning, a requirement that we have observed in numerous contexts. We show that IgG antibodies induce insulin resistance through an Fc mediated process. Identification of the precise FcR responsible for IgG effect on glucose metabolism warrants further investigation. FcR γ chains in Fc γ RI, III, and IV can directly signal in macrophages to promote oxidative burst, cytotoxicity and pro-inflammatory cytokine production, fundamental processes that are thought to fuel insulin resistance in adipose tissue. In addition to our previous work on T cells in T2D and obesity, we have contributed fundamentally to the growing field that examines how the adaptive immune system and insulin resistance interact.

This work is also the first to describe specific clusters of autoantibodies linked to insulin resistance or insulin sensitivity in humans, which could form the basis for novel diagnostic and monitoring tests used in the clinic. Surprisingly, antigens targeted in insulin resistant individuals are not visceral adipose tissue (VAT) specific but show widespread expression in other tissues such as the pancreas, liver, nervous tissue, muscle, and hematopoietic cells. Isoform diversity and post-translational modifications of target autoantigens may also play an important role. These findings may potentially explain the longstanding mystery of why some people can become obese and remain insulin sensitive, and provide future avenues of research.

Antibodies and B cells play critical roles in autoimmune and inflammatory disease, including lupus, rheumatoid arthritis, and atherosclerosis. Traditionally, autoimmunity has been considered to be independent of T2D pathogenesis. While a proportion of diabetic patients exhibit features of both T1D and T2D, including insulin resistance, autoimmune destruction, and a loss of beta cell mass, these individuals are subclassified as latent autoimmune diabetes of the adult (LADA). Individuals with LADA also have detectable levels of serum anti-islet autoantibodies, and it would be valuable to determine the extent of GFAP targeting. Prior to our studies, the role of the immune system in T2D seemed largely innate, typical of autoinflammation (606), rather than true autoimmunity. Conversely, our data suggest that a high degree of adaptive immune involvement in T2D warrants further investigation. Insulin resistance is associated with changes in the pancreas, CNS, and adipose tissue, all of which could be the primary source of GFAP antigen exposure.

We show here that GFAP immunization effectively reduces adiposity and the development of insulin resistance following exposure to high fat diet. This effect was specific to GFAP and was not observed in mice consuming a regular diet. Immunization was also associated with dramatic changes to the metabolic profile of mice that requires further investigation to fully understand mechanistically. We will also strive to determine the cell type primarily responsible for the beneficial effects of immunization, information that could improve the injection regimen to be more powerful and long-lasting.

Vaccination and the subsequent activation of adaptive immunity has proven to be an incredibly effective means of preventing disease in general. Historically, there has been increased interest in determining if classical vaccination can also be applied to treat autoimmune disease. Instead of eliciting a classic T_H1 -skewed response dominated by $IFN-\gamma$ and cytotoxic T cell killing, these vaccines would aim to induce tolerance through regulatory T cells that secrete IL-10 and TGF- β (607). This approach dates back many decades (608) and more recently has shown efficacy in

treating allergy through the generation of Tregs and IL-10 production (609). We believe our approach using GFAP in DIO mice to be working through similar mechanisms of immune tolerance, as we observed greatly decreased T_H1- and T_H17-related cytokine production.

It is our hope that one day this approach may effectively treat patients with insulin resistance and T2D. The future of T2D therapy will likely need to contain a prominent immune-centric focus, with conceivably impressive results.

CHAPTER 6

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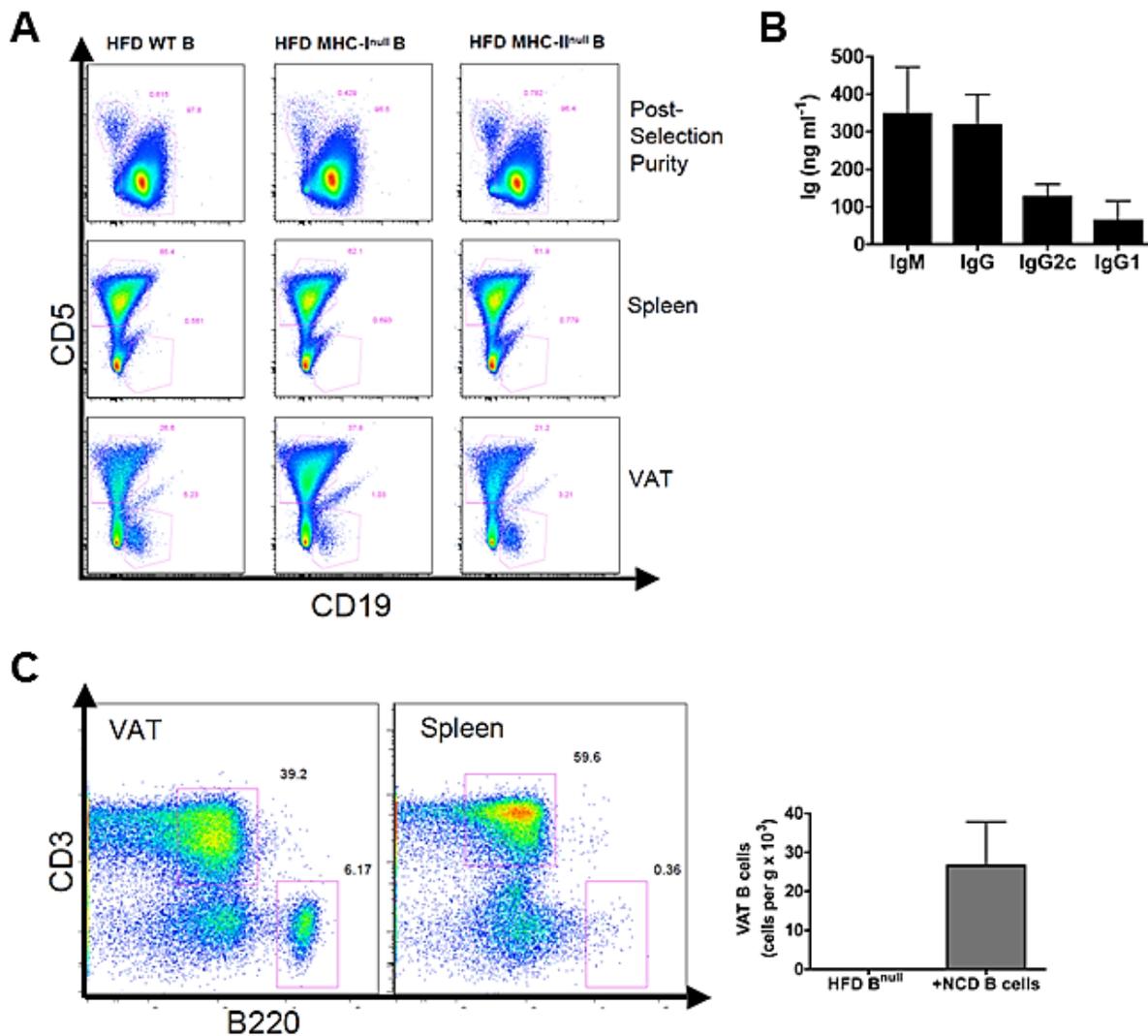
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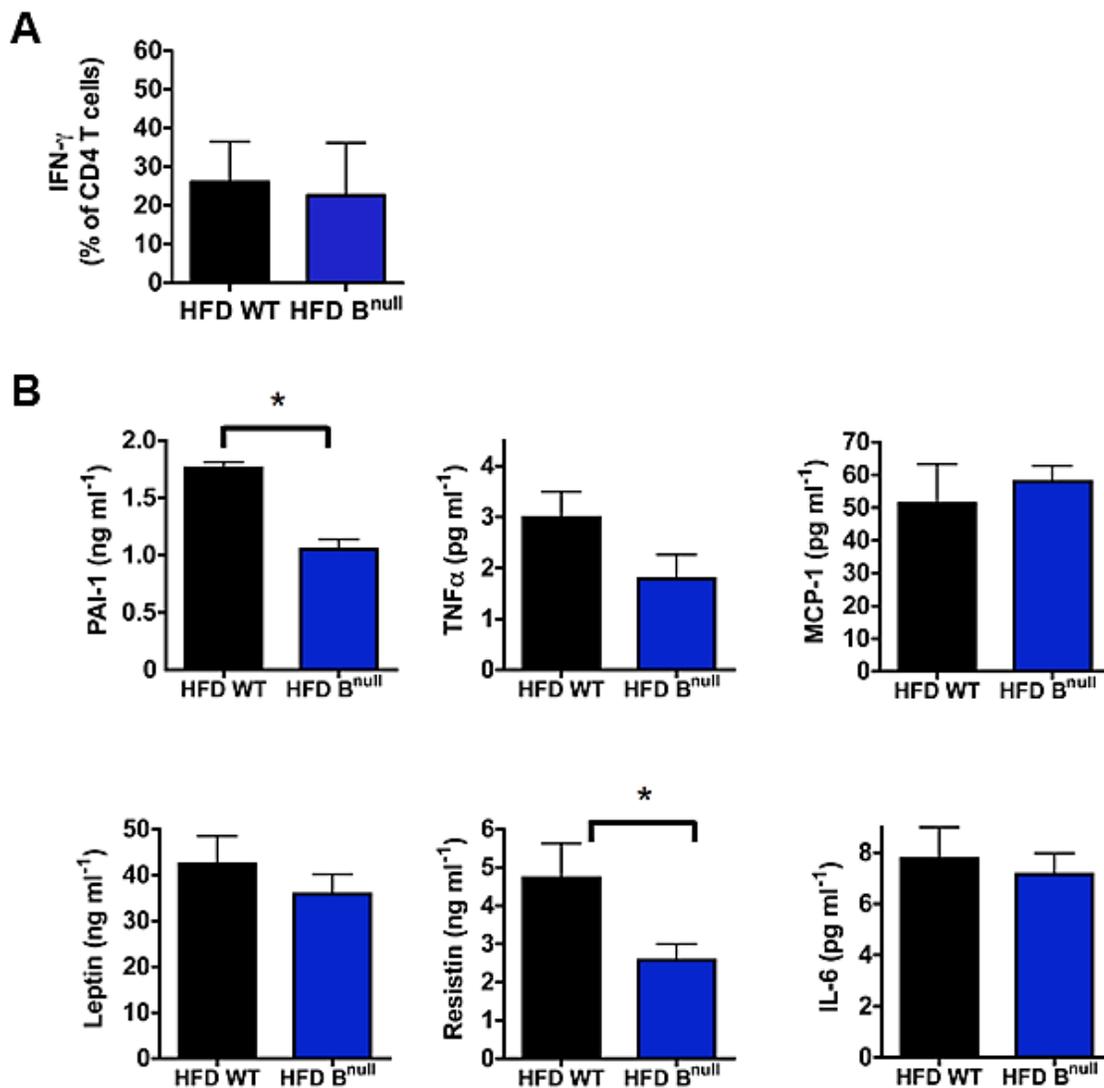
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CHAPTER 7

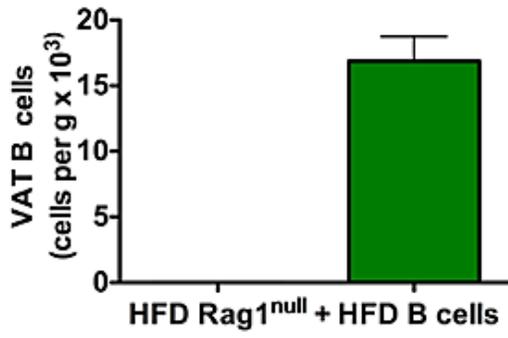
Appendix



Appendix Figure 1 – B cells traffic to VAT after intraperitoneal transfer. (A) FACS plots of purified B cells isolated by negative selection from DIO WT, MHC-I^{null}, or MHC-II^{null} mice (top row). 2wk after intraperitoneal injection into DIO B^{null} mice, CD19⁺ cells are not found in the spleen (middle row), but are found in VAT (bottom row) (3 experiments). (B) Antibody concentrations 3wk after HFD B cell transfer into DIO B^{null} mice (n=5). (C) B cell trafficking to VAT and spleen 3wk after intraperitoneal transfer of NCD B cells (2 experiments).

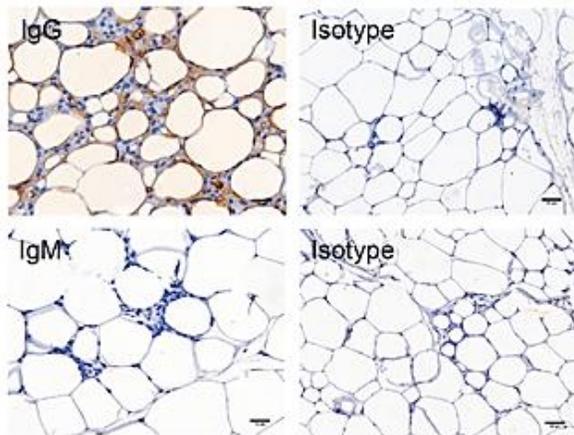


Appendix Figure 2 – Cytokine profiles in DIO B^{null} mice. (A) Intracellular IFN- γ staining of CD4⁺ T cells isolated from VAT of 16wk old HFD WT and HFD B^{null} mice (3 experiments, n=9 per group). (B) Serum concentrations of obesity and insulin resistance-associated adipokines and cytokines in 16wk old HFD WT and HFD B^{null} mice (n=5 per group, *p<0.05).

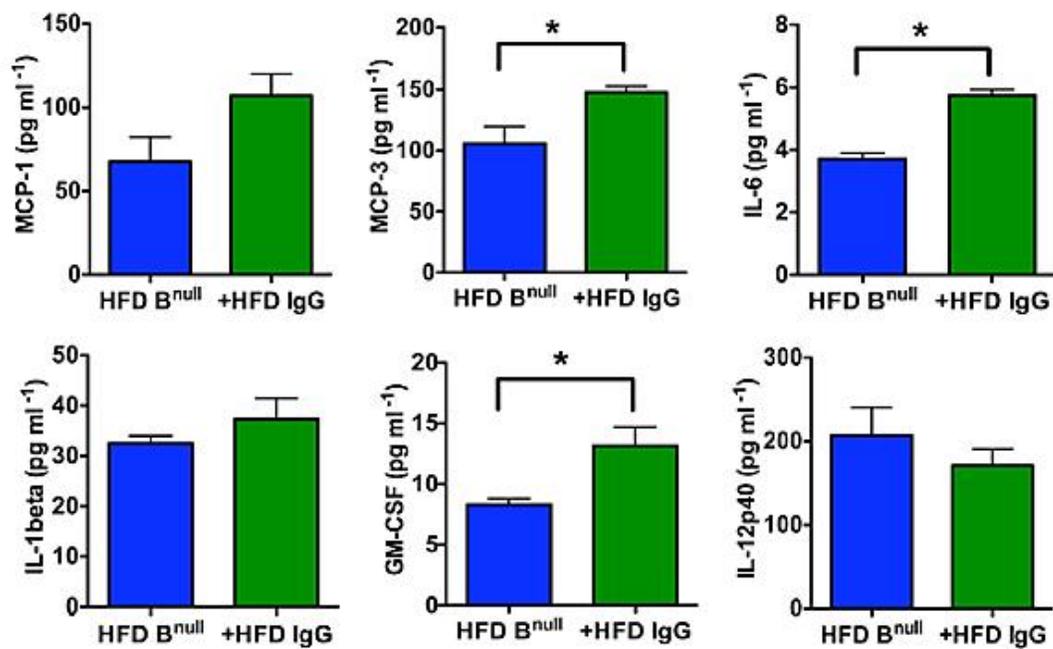


Appendix Figure 3 – B cells traffic to VAT in RAG1^{null} mice. B cell reconstitution of VAT in HFD RAG1^{null} mice 2wk after intraperitoneal transfer of 1×10^7 DIO B cells (n=3 per group).

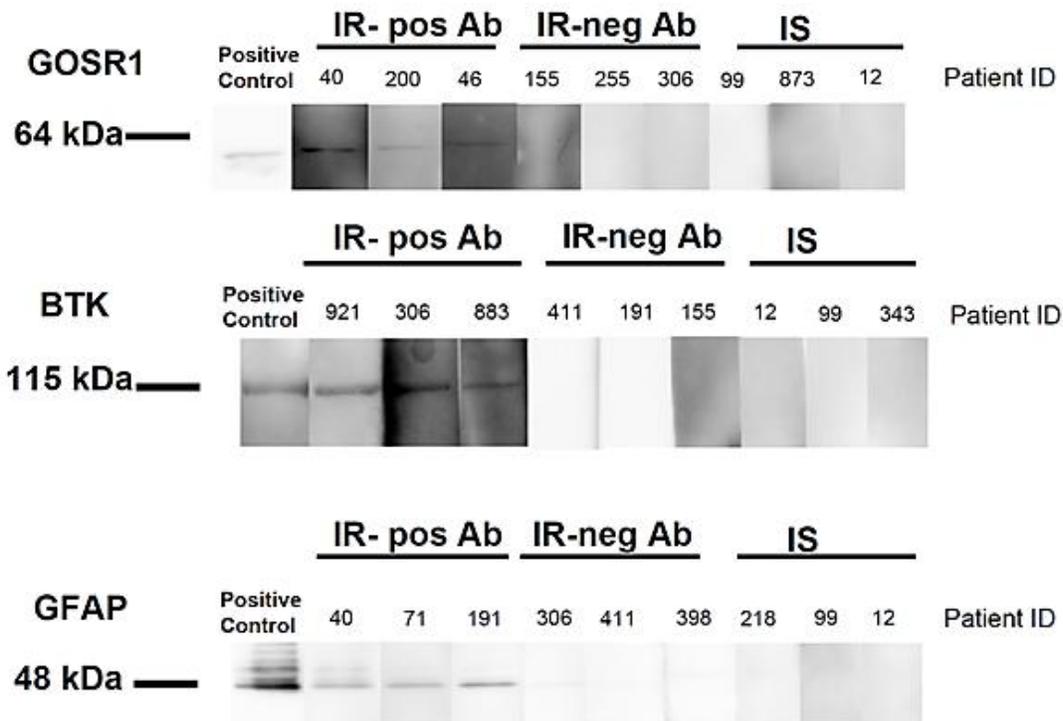
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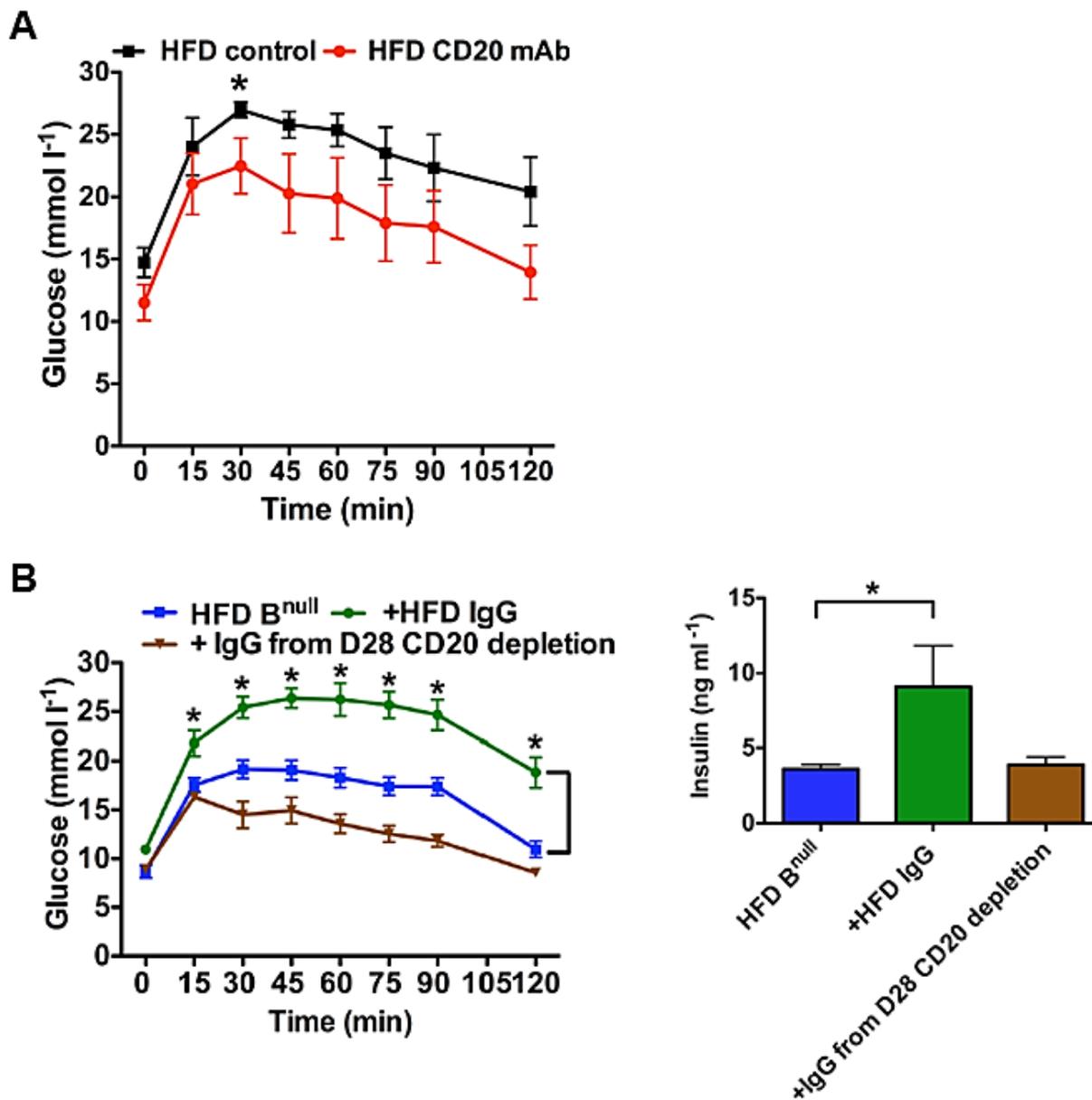
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Appendix Figure 4 – HFD IgG localization and effects on serum cytokines following intraperitoneal transfer. (A) IgG (top) and IgM (bottom) in VAT of DIO B^{null} mice 1wk after HFD IgG antibody transfer. (B) Serum concentrations of obesity and insulin resistance-associated cytokines 1wk after transfer of HFD IgG relative to PBS control (n=3 per group, *p<0.05).



Appendix Figure 5 – Validation of insulin resistant (IR) autoantibody targets in obese human subject serum. Shown are Western blots representing IR patients with higher levels of autoantibody (IR-pos Ab), no level of autoantibody (IR-neg Ab), or insulin sensitive (IS) patients which lack autoantibody against the chosen targets, which represent the top 3 antigens based on human array data.



Appendix Figure 6 – Additional treatment effects of anti-CD20 mAb in HFD mice. (A) GTT in HFD WT mice 40d after anti-CD20 mAb, or control IgG2c or PBS IgG (n=6 per group, *p<0.05). (B) GTT (left) and fasting insulin (right) of HFD B^{null} mice 1wk after transfer of serum antibodies from anti-CD20 mAb treated mice or HFD IgG (n=5 per group, *p<0.05).