Interactions between B cells and $CD8^+$ T cells in multiple sclerosis

Ayman Rezk

Microbiology and Immunology
McGill University, Canada
Submitted in August 2019

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctorate of Philosophy

Table of Contents

Abstract 5

Resumé 7

Acknowledgements 9

Contribution to original knowledge 10

Contributions of authors 11

List of abbreviations: 12

Chapter 1: Introduction and literature review 15

1.1 Introduction to Multiple Sclerosis 16

1.1.1 Clinical presentation of multiple sclerosis 16

1.1.2 Genetic factors in multiple sclerosis 17

1.1.3 Environmental factors and multiple sclerosis 18

1.2 From $CD4^+$ T cell-mediated disease to disease of B cell:$CD4^+$ T cell interactions 19

1.2.1 Imbalance between $CD4^+$ effector and regulatory T cells in MS 19

1.2.2 Targeting B cells in multiple sclerosis 27

1.2.3 B cell antibody-dependent functions in multiple sclerosis 29

1.2.4 B cell antibody-independent functions in multiple sclerosis 32

1.3 Implication of $CD8^+$T cells in the MS central nervous system 39

1.3.1 Opposing roles for CD8+ T cells in neuroinflammation 41

1.4 Mucosal associated invariant T (MAIT) cells 43

1.4.2 MAIT cell development 44

1.4.3 MAIT cell anti-microbial and anti-viral functions 46

1.4.4 MAIT cells in autoimmune diseases 47

1.5 Summary 49

Preface: Chapter 2 50

Chapter 2: Simultaneous detection and isolation of multiple, viable, distinct cytokine-secreting B cells 51

Abstract 52

Introduction 53

Materials and Methods 54

Cell Isolation and culture 54

Flow cytometry 55

Enzyme-linked immunosorbent assay (ELISA) 56

Quantification of mRNA expression by quantitative, real-time polymerase chain reaction 56

Statistics 56

Results 56

Detection of single low frequency cytokine-secreting human B cells *in vitro* 56

Isolation of low frequency cytokine-secreting human B cells using CSA 56

Simultaneous isolation of two low-frequency cytokine-secreting human B cells 57

Concurrent detection of three cytokine-secreting B cell subpopulations 58

Discussion **58**

Disclosures **60**

Acknowledgments **60**

Figure 1. Cytokine expressing human B cells detected using intracellular cytokine staining versus a cytokine-secretion assay optimized for detection of low-frequency cytokine-secreting B cells. **61**

Figure 2. Validation of the cytokine secretion assay as an approach for isolating viable low-frequency cytokine-defined human B cells. **62**

Figure 3. Concurrent isolation of two low-frequency cytokine-secreting B-cell subpopulations using the cytokine secretion assay. **64**

Figure 4. Simultaneous detection and isolation of three cytokine-secreting B cell subpopulations using the cytokine secretion assay. **66**

Supplementary figure 1. Detection and isolation of viable TNF-secreting B cells using the cytokine secretion assay **67**

Preface: Chapter 3 ..... ***68***

Chapter 3: B cells regulate CD8$^+$ MAIT cell effector functions: implications for multiple sclerosis ***69***

Abstract **70**

Introduction **71**

Materials and methods **72**

Human subjects and ethics approval 72

Cell isolation 72

B cell and T cell activation 73

Flow cytometry 73

Enzyme-linked immunosorbent assay (ELISA) 74

PBMC immunophenotyping prior to and following B cell depletion 74

Statistical analysis 74

Results **74**

Human B cells differently impact CD8$^+$ T cell subset proliferation 74

Human B cells enhance CD8$^+$ T cell cytotoxic potential 76

Human B cells modulate CD8$^+$ T cell cytokine production 76

B cell depletion therapy shifts the distribution of blood CD8$^+$ CD161$^-$ and MAIT cells 77

CD8$^+$ T cell pro-inflammatory cytokine production is diminished after B cell depletion therapy 78

Discussion **78**

Disclosures **81**

Acknowledgments **81**

Figure 1. B cells reciprocally regulate non-MAIT and MAIT CD8$^+$ T cell proliferation. **83**

Figure 2. B cells reciprocally modulate the proliferation of FACS-sorted non-MAIT and MAIT cells **85**

Figure 3. B cells enhance CD8$^+$ T cell subset cytotoxic potential. **86**

Figure 5. Circulating MAIT cells are reduced post-B cell depletion in MS patients. **89**

Figure 6. CD8$^+$ T cell pro-inflammatory cytokine production is diminished following B cell depletion therapy 90

Figure S1. Purity confirmation of MACS-sorted CD8+ T cells and B cells. 91

Figure S2. Human mucosal associated invariant T (MAIT) cells recognize vitamin B metabolites presented on MR1 and can be distinguished by the expression of CD161 and TCR-Vα7.2. 92

Figure S3. Purity confirmation of FACS-isolated CD8+ T cell subsets. 93

Figure S4. Distinct capacities for B cell subsets to regulate CD8+ T cell proliferation. 95

Figure S5. Flow cytometry gating strategy used to distinguish various CD8+ T cell subsets in PBMC. 97

Figure S6. CD8+ T cell cytotoxic profile is not impacted after B cell depletion. 98

Supplemental Table 1. Patient characteristics 99

Supplemental Table 2. Immune phenotyping panels 100

Supplemental Table 3. Staining reagents used in immunophenotyping 101

Supplementary Table 4. Cell subset counts pre- and on-treatment with anti-CD20 103

Supplementary Table 5. Cell subset frequencies pre- and on-treatment with anti-CD20 104

Chapter 4: Discussion, conclusion and future directions 105

4.1 General discussion 106

4.2 Human cytokine-defined B cells – novel therapeutic targets in MS? 106

4.3 Dual B cell functions: regulatory and helper cells 107

4.4 B cell: CD8+ T cell interactions beyond MS 110

4.5 Conclusion and future directions 111

References 112

Appendix figure 1 150

Appendix figure 2 152

Abstract

Previous studies have identified functionally distinct human B cell sub-populations based on distinct cytokine expression profiles and characterized the functional capacities of such B cell subsets to modulate T cell and myeloid cell effector functions. Such antibody-independent B cell functions have emerged as highly relevant to disease activity in multiple sclerosis (MS), as evidenced by the ability of B-cell depleting therapy (anti-CD20 monoclonal antibody) to robustly reduce new relapsing disease activity while having minimal to no impact on the abnormal antibody profile within the central nervous system (CNS) of patients. While these results have been interpreted to reflect B cell:$CD4^+$ T cell interactions, we noted that B-cell depleting therapy results in reduced proliferation and pro-inflammatory cytokine responses of both $CD4^+$ and $CD8^+$ T cells, suggesting that B cells and $CD8^+$ T cells may also interact in disease-relevant ways. It is noteworthy that in MS, $CD8^+$ T cells are actually more abundant in the inflamed MS CNS than are $CD4^+$ T cells. Furthermore, among the CNS-infiltrating T cells in MS, pathologists have identified presence of clonally expanded mucosal-associated invariant T cell receptor (MAIT) cells expressing pro-inflammatory effector molecules. The overall aim of my thesis is to characterize antibody-independent interactions between human B cells and CD8+ T (both MAIT and non-MAIT) cells, and their possible implication in MS pathophysiology.

Chapter 1 introduces multiple sclerosis by summarizing the genetic and environmental factors associated with disease development, highlighting the roles of $CD4^+$ T cells and B cells (both antibody dependent and independent functions) in disease pathogenesis and the implication of $CD8^+$ T cells, including MAIT cells, in MS neuroinflammation.

Advancing our understanding of the functional response profiles of distinct cytokine-defined B cell subsets requires isolating them in a viable state. However, a major impediment to date is that the permeabilization and fixation process required for intracellular cytokine staining, and thus identification of cytokine-producing B cells, results in loss of cell viability. Other challenges include the low frequency of cytokine-expressing B cells in the circulation and the often-limiting volumes of samples available for study. In Chapter 2, I set out to optimize an approach for the simultaneous isolation of multiple rare cytokine-secreting viable human B cells. I demonstrate that

the cytokine-secretion assay can be developed and applied for the detection and isolation of multiple highly-purified, viable, low-frequency cytokine-defined B cells (i.e. cells secreting GM-CSF or IL-10), and that isolated cell fractions are amenable for downstream applications including gene expression profiling and *in vitro* culture. The assay can be multiplexed, wherein up to three cytokines can be simultaneously detected on B cells, without any impact on cell purity, though a variable loss in yields requires assay optimization. Overall, this approach is a stepping-stone in enabling future investigations into the biology of these cytokine-defined B cell subsets including their impact on responses of other cells.

In chapter 3, I examine the interactions between B cells and both $CD8^+$ MAIT and non-MAIT cells using a series of *in vitro* experiments complemented by deep immunophenotyping of $CD8^+$ T cells within peripheral blood mononuclear cells obtained prior to and after B cell depletion therapy in patients with MS. I found that B cells have opposing effects on different $CD8^+$T cell subsets, namely induction of proliferation, pro-inflammatory cytokine responses, and cytotoxicity of MAIT cells, yet suppression of non-MAIT cell proliferation and pro-inflammatory cytokine production. *In vivo*, B cell depletion in MS patients results in reduced counts and pro-inflammatory responses of MAIT cells.

Overall, these observations provide novel insights into fundamental functions of human B cells in the modulation of $CD8^+$ T cell effector responses, in both health and disease. They also add to our understanding of the complex cellular networks involving the balance between pro- and anti-inflammatory functions of different cell types and their interactions in the pathogenesis of multiple sclerosis by invoking a B cell:$CD8^+$ T cell axis (and particularly the role of B cell: MAIT cell interactions), which may be therapeutically targeted by B cell depletion. Further characterization of cytokine-defined B cell responses and their interactions with $CD8^+$ MAIT and non-MAIT cells could enable the development of more focused therapies that selectively impact these interactions for the treatment of autoimmune diseases.

Resumé

Les lymphocytes B acquièrent la capacité à produire des cytokines, et celle-ci est nécessaire pour contrôler les fonctions des lymphocytes T and cellules myéloïdes. Les fonctions des lymphocytes B indépendante de leur capacité à produire des anticorps s'est révélées importante dans le contexte de la sclérose en plaque, compte tenu des bienfaits cliniques des thérapies visant les lymphocytes B à base d'anticorps monoclonal dirigé contre CD20. En fait, ces thérapies ont eu un effet minimal sur les niveaux des anticorps présent au sein du système nerveux central. Nos études ont démontré que l'appauvrissement du nombre total des cellules B démunirait les fonctions effectrices des lymphocytes T CD4 et CD8, ce qui indique qu'au-delà des interactions bien décrites des lymphocytes B and T CD4, il 'y a peut-être lieu pour une interaction entre les lymphocytes B and T CD8. Il s'avère que les lymphocytes T CD8 sont plus nombreux que les lymphocytes T CD4 dans le cerveau des patients atteints de la sclérose en plaque, et que parmi les lymphocytes T CD8, des cellules dénotées MAIT produisant des cytokines pro-inflammatoires subsistent par une expansion clonale. Le but de mon projet de thèse est de décrire l'interaction entres les lymphocytes B et T CD8, et son implication dans la pathologie de la sclérose en plaque.

La production des cytokines par les cellules B est utile pour démarquer certains sous-types dotés de fonctions uniques. Malgré ceci, les caractéristiques de ces sous-types de cellules B restent inconnues due à l'inconvénient majeure de l'approche couramment utilisée pour mesurer l'expression des cytokines par la cytometrie en flux qui nécessite la fixation and perméabilisation des cellules. Par conséquent, cette approche s'est soldée par la perte de la viabilité des cellules. Autres défis consistent en un taux bas des sous-types des cellules B définies par la production des cytokines dans le sang et un volume du sang restreint et disponible pour la recherche. Dans le chapitre 2, j'ai développé une approche alternative pour l'isolation des cellules B produisant des cytokines. L'approche, connue comme « cytokine secretion assay », est utilisée dans le but d'isoler des cellules B capable de sécréter plusieurs cytokines rares telles qu'IL-10 et GM-CSF. Cette méthode a l'avantage de permettre l'isolation des cellules vivantes, ce qui est important pour le séquençage de prochaine génération et la culture des cellules *in vitro*. La détection de trois cytokines en parallèle est faisable, mais l'optimisation de cette manipulation est nécessaire afin de

réduire la perte de signale. En tout, cette méthode permettra des avances considérables dans notre compréhension de la biologie des cellules B produisant des cytokines.

Dans le chapitre 3, j'examine les interactions parmi les cellules B et les sous types de lymphocytes T CD8, notamment cellules non-MAIT et MAIT. A cette fin, j'ai établis un système de co-culture de cellules B et T, et examiné le phénotype et la fonction des lymphocytes T CD8 dans le sang chez les patients traités avec l'anticorps monoclonal dirigé contre CD20. Mes observations démontrent que les cellules B interagissent différemment avec les sous types de lymphocytes T CD8. En fait, elles induisent la prolifération, la production des cytokines pro-inflammatoires et potentiel cytotoxique de cellules MAIT, mais elles suppriment la prolifération et la production des cytokines pro-inflammatoire de cellules non-MAIT. D'autant plus, l'appauvrissement des cellules B chez les patients traités réduit le nombre de cellules MAIT dans la circulation and leur production de cytokines pro-inflammatoires.

L'ensemble des mes travaux présentés dans cette thèse a approfondi notre compréhension du rôle de lymphocytes B, notamment leur capacité de contrôler les fonctions effectrices de lymphocytes T CD8 dans le contexte pathologique et physiologique sain. Cela sert a démontré que l'axe cellulaire lymphocytes B : T CD8 est pertinent dans la pathologie de la sclérose en plaque, et peut être visé par les thérapies ciblant les cellules B. Les futures recherches sur cette interaction cellulaire peuvent s'achever au développent des nouvelles thérapies dans le contexte des maladies auto-immunitaire y compris la sclérose en plaque.

Acknowledgements

The thesis presented here reflects what for me has been an amazing scientific journey that started several years ago and still continues to this day. It is humbling to look back, and realize how far I have come. I hope it is the launchpad for a fulfilling and rewarding scientific career. Many wonderful individuals have made this journey fun and exciting to whom I wish to say – THANK YOU!

Dr. Amit Bar-Or – I am forever grateful that you took a punt on me and provided me with the opportunity to work on cutting-edge and exciting projects. You have continuously challenged my thinking and provided great mentorship, which made me a better scientist. I have learnt a great deal from you, and I continue to do so. Words cannot express my gratitude towards you!

Dr. Jack Antel – thank you for your guidance, mentorship and support!

Drs. Irah King and Connie Krawczyk – I deeply appreciate your guidance and critical minds which have been important on this journey.

Dr. Rui Li – my brother in arms! We have collaborated on numerous fun and challenging projects, and during this time, I have learnt so much from you! Thank you for everything. I am certain that there is much more to come.

Dr. Giulia Fadda – you have been an inspiration for me! I have had a chance to see you grow as a scientist, and your dedication and enthusiasm to science are simply amazing. Our friendship has provided me with so much more – thank you, my dear!

I am grateful for past and present lab members - your support and critical minds have been instrumental in this journey!

This journey would not have been possible without the support of my dearest family and friends!

Contribution to original knowledge

The key findings of this thesis are:

1. Newly developed cytokine secretion assay can overcome the limitation of the intracellular cytokine staining and be used for the detection and isolation of viable low as well as high frequency cytokine-secreting human B cells. Isolated fractions are highly purified and enriched for the corresponding cytokines, and can be integrated in downstream assays including next generation sequencing or cell culture.
2. The cytokine secretion assay can be multiplexed, allowing for the simultaneous detection and isolation of up to three cytokines, a particular advantage when dealing with limiting sample volume.
3. Human B cells can function as helper cells to activate MAIT cells. B cells enhance MAIT cell proliferation, cytotoxic potential, and cytokine production *in vitro*.
4. B cells can function as suppressors of $CD8^+$ non-MAIT cells that down-regulate their proliferation and cytokine production.
5. B cell depletion therapy results in a shift in the distribution of circulating $CD8^+$ T cell subsets, with a particular reduction in MAIT cells.
6. $CD8^+$ non-MAIT and MAIT cell pro-inflammatory cytokine production is diminished following B cell depletion in multiple sclerosis patients.

Contributions of authors

Chapter 1: Introduction and literature review
A.R wrote the literature review

Chapter 2: Simultaneous detection and isolation of multiple, viable, distinct cytokine-secreting B cells

Authors: Ayman Rezk, Rui Li, Alexandre Prat, Jennifer Gommerman and Amit Bar-Or

AR generated all hypotheses, designed and performed all the experiments, analyzed the complete data sets and wrote the manuscript

RL assisted with human cell isolation and helpful research discussions

J.G and A.P were involved in research plan and discussion and helped with manuscript revision

A.B.O. provided the overall supervision, generated hypotheses, guided the experimental plan, analyzed the data and wrote/revised the manuscript.

Chapter 3: B cells regulate $CD8^+$ MAIT cell effector functions: implications for multiple sclerosis

Authors: Ayman Rezk, Koji Shinoda, Rui Li, and Amit Bar-Or

AR generated all hypotheses, designed and performed all the experiments, analyzed the complete data sets and wrote the manuscript

KS assisted with data generation

RL assisted with data generation and helpful research discussions

A.B.O. provided the overall supervision, generated hypotheses, guided the experimental plan, analyzed the data and wrote/revised the manuscript.

Chapter 4
A.R wrote the discussion

List of abbreviations:

5-A-RU: 5-amino-6-D-ribitylaminouracil

5-OE-RU: 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil

5-OP-RU: 5-(2-oxypropylideneamino)-6-D-ribitylaminouracil

ADP: Adenosine diphosphate

APC: Antigen presenting cells

APRIL: A proliferation inducing ligand

ATP: Adenosine triphosphate

BAFF: B cell activation factor

BALT: Bronchus-associated lymphoid tissues

BBB: Blood brain barrier

BCR: B cell receptor

CCL: CC chemokine ligand

CCR: CC chemokine receptor

CD: Cluster of differentiation

CIS: Clinically isolated syndrome

CNS: Central nervous system

CSF: Cerebrospinal fluid

CTLA-4: Cytotoxic T-lymphocyte-associated protein 4

EAE: Experimental autoimmune encephalomyelitis

EBNA-1: Epstein-Barr nuclear antigen 1

EBV: Epstein-Barr virus

ELISA: Enzyme linked immunosorbent assay

FOXP3: Forkhead box 3

GATA3: GATA binding protein 3

GF: Germ-free

GM-CSF: Granulocyte macrophage colony stimulating factor

GWAS: Genome-wide association studies

HLA: Human leukocyte antigen

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

IRA: Innate response activator

iTreg: Inducible regulatory T cells

MAIT: Mucosal-associated invariant T cells

MBP: Myelin basic protein

MCAM: Melanoma cell adhesion molecule

MHC: Major histocompatibility complex

MOG: Myelin oligodendrocyte glycoprotein

MR1: Major histocompatibility complex, class I related

MRI: Magnetic resonance imaging

MS: Multiple sclerosis

NaCl: Sodium chloride

OCB: Oligoclonal bands

OR: Odd risk

PLP: Myelin proteolipid protein

PMA: Phorbol 12-myristate 13-acetate

PPMS: Primary progressive multiple sclerosis

RAR-related orphan receptor gamma

RIS: Radiologically isolated syndrome

RRMS: Relapse-remitting multiple sclerosis

RTE: Recent thymic emigrant

SNP: Single nucleotide polymorphism

SPF: Specific-pathogen free

SPMS: Secondary progressive multiple sclerosis

STAT: Signal transducer and activator of transcription

STIM: Stromal interaction molecule

T-bet: T-box transcriptional factor

Tconv: Conventional T cells

TCR: T cell receptor

Tfh: Follicular helper T cells

TGF: Transforming growth factor

Th: Helper T cells

TLR: Toll-like receptor

TNF: Tumor necrosis factor

Tr1: Type 1 regulatory T cells

TRAJ: T cell receptor alpha joining

Treg: Regulatory T cells

Chapter 1: Introduction and literature review

1.1 Introduction to Multiple Sclerosis

Multiple sclerosis is an immune-mediated and neurodegenerative disease of the central nervous system[1-4]. Over two million individuals worldwide are diagnosed with the disease, which is associated with a major social and economic burden[5,6]. The disease is most often diagnosed in individuals aged 20 to 40 years old, though pediatric-onset disease has been observed[6,7]. Disease prevalence and incidence has steadily increased in the last five decades. In particular, female incidence has more than doubled in the last 50 years, which can explain the increasing female to male ratio estimated at 2:1 to 3:1[8-11]. Latitudinal gradient of prevalence is still present in Europe and North America; however, recent epidemiological studies denote a disappearing gradient across the United States and elsewhere[10]. These relatively rapid changes in geographical and gender prevalence are likely driven by environmental rather than by genetic factors[12].

1.1.1 Clinical presentation of multiple sclerosis

Several clinical phenotypes have been described, including radiologically isolated syndrome (RIS), clinically isolated syndrome (CIS), relapsing-remitting multiple sclerosis (RRMS), secondary progressive multiple sclerosis (SPMS), and primary progressive multiple sclerosis (PPMS)[3,13,14]. RIS patients are asymptomatic yet have magnetic resonance imaging (MRI) lesions suggestive of MS[3,13,14]. CIS is diagnosed in patients who experience a single clinical event suggesting CNS demyelinating disease, but do not yet meet the full criterion for dissemination in time for formal MS diagnosis[3,13,14]. Both RIS and CIS are now essentially thought of as 'early MS' when patients present with typical MRI appearance and spinal fluid features (such as the presence of 'clonally-restricted' immunoglobulin in the cerebrospinal fluid known as oligoclonal bands) seen in MS. Overall, RRMS is the most common clinical presentation found in 85% of patients, and is typically characterized by periods of clinical exacerbation (relapses) with complete or partial recovery and interim stability (remission) [3,13,14]. The majority of RRMS patients then develop progressive clinical worsening, a disease course defined as SPMS, with a median time to progression estimated at 19 years[3,13,14]. Finally, approximately 15% of patients experience gradual disease worsening from clinical onset with no frank relapses and remissions, which is referred to as PPMS[3,13,14].

1.1.2 Genetic factors in multiple sclerosis

Multiple sclerosis is a complex disease, in which an interplay between genetic and environmental factors underlies disease risk[15-17]. Indeed, monozygotic twins have a higher rate of concordance (20-30%) than dizygotic twins (2-5%) or second- and third-degree relatives[18-21]. Sibling recurrence risk was estimated at 7-fold in the population of Sweden[22-24]. Human leukocyte antigen (HLA) variants within the major histocompatibility complex (MHC) gene were first described in MS in the 1970's[15]. In fact, HLA haplotypes are a major determinant of disease risk, as individuals carrying the MHC class II variant HLA-DRB1*15.01 have three-fold increased risk, whereas individuals carrying the MHC class I variant HLA-A*02 are relatively protected (odd risk=0.6)[25-28]. Further, the DRB1*15 allele is more prevalent in children of European ancestry diagnosed with MS compared to normal controls or children presenting with acquired demyelinating syndromes that are not MS[29,30].

The genetics of MS have been extensively examined in genome wide association studies (GWAS), which have identified over 200 single nucleotide polymorphisms (SNPs) conferring increased risk of MS. These SNPs generally map to noncoding regions of the genome and are notably shared with other autoimmune conditions[25,26,31-33]. The majority of candidate genes linked to these SNPs encode proteins that function in the immune system, such as *IL2R*, *IL7R*, *CD86,* etc., and have an influence on T cells, B cells and monocytes[15,25,26,31,34,35]. Individual variants have rather small effects on disease risk, and such variants are present in non-diseased individuals where they have probably evolved to provide the host with a fitter immune system to fight pathogens[36]. With few exceptions, the effects of these SNPs on gene function are not well understood[37-43]. One prominent example is rs1800693, which influenced influenced the splicing *TNFRSF1A*, resulting in the production of a novel soluble form of the TNF receptor[39]. This receptor inhibited TNF signaling in a similar fashion to TNF blocking drugs, which has been shown to dramatically increase disease activity in MS patients[39,44-46]. Another example is an insertion-deletion variant in *TNFSF13B* encoding for BAFF, termed BAFF-var, that was associated with increased MS risk in the Sardinian population but no other European populations[47]. BAFF-var was associated with increased circulating B cell numbers, decreased circulating monocyte numbers, and increased levels of soluble BAFF, IgG, IgA and IgM in the Sardinian population[47]. These studies showcase the consequence of individual SNP variant on the immune system, however, their collective impact

on the immune dysregulation observed in MS is unknown as well as the putative interactions between genetic and environmental factors in dictating disease risk.

1.1.3 Environmental factors and multiple sclerosis

Classical epidemiological studies have revealed key roles for environmental factors in disease risk. These factors include low levels of vitamin D, lack of sun exposure, obesity in adolescence, tobacco smoking or second-hand tobacco exposure, and early Epstein-Barr virus (EBV) infection or development of clinical infectious mononucleosis[12,16,48]. The environmental contributions to disease risk have also been ascertained in migrant studies. In fact, the overall disease risk in migrants is intermediate between the risk in countries of origin and residence and depends on age at migration[49-54]. Many of the implicated environmental factors can regulate immune responses and can interact with the HLA alleles to modulate disease risk[12].

1.1.3.1 Vitamin D and sun exposure

Vitamin D conversion into active metabolites depends on ultraviolet B radiation from sun exposure, and in countries at high latitude, sun exposure is rather limited[12,17]. In fact, individuals exposed to higher sunlight have a reduced MS risk[55-57] and disease risk declines with increasing levels of 25(OH)D[30,58-60]. In addition, polymorphisms in genes involved in vitamin D metabolism are associated with disease risk[61]. Vitamin D metabolites may act as immunomodulatory agents to regulate pro-inflammatory functions of both T cells and B cells[62,63].

1.1.3.2 Obesity

Obesity in early life is associated with greater than two-fold increase in risk of pediatric and adult-onset multiple sclerosis, and there is a notable interaction between obesity with HLA alleles resulting in further increased disease risk (OR=14)[12,64-68]. Obesity is associated with low grade inflammation, increased levels of leptin, and reduced levels of vitamin D, all of which can instigate detrimental pro-inflammatory immune responses[12,16].

1.1.3.3 Tobacco smoking

Active or passive tobacco smoking is linked to increased disease risk, with heavy smokers at higher risk (OR=1.3 to 1.6)[69-73]. Like obesity, smoking interacts with HLA alleles, resulting in increased disease risk (OR=14)[12]. In one proposed model, smoking causes lung irritation and inflammation

and induces the formation of bronchus-associated lymphoid tissue, where self-reactive immune cells are activated and traffic to the CNS[12,16]. This is based on the observation that encephalitogenic T cells were licensed in the lungs before migrating to the CNS in experimental autoimmune encephalomyelitis (EAE), the commonly used animal model of MS neuroinflammation[74].

1.1.3.4 EBV infection

EBV, a double stranded DNA γ-herpesvirus, eventually infects more than 90% of the general population, and more than 99% of the MS population[12,16,75]. EBV seropositivity was also more prevalent in children with MS than in age- and sex-matched controls, and viral reactivation was more often observed in pediatric MS patients[30,76-79]. Individuals who experience clinical infectious mononucleosis due to EBV have greater than two-fold increased disease risk, as demonstrated in a meta-analysis of case-control studies and in the Danish population[75,80,81]. In a nested case control study, EBV seronegative individuals seroconverted before MS disease onset[82]. Another body of work showed that MS patients had higher levels of antibodies against EBV proteins (such as EBNA-1), as well as a higher frequency of T cells specific for EBV antigens in the blood and CSF[83-87]. In that regard, it has been hypothesized that molecular mimicry between EBV and CNS antigens can drive pathogenic T cell and B cell responses in the CNS[75]. Others observed that the frequency of EBV-specific $CD8^+$ T cells was reduced in MS patients, resulting in impaired control of EBV infection[88-92]. Other hypotheses regarding a link between EBV infection and immune pathogenesis in MS include bystander damage in the CNS, EBV-induced aberrant B cell cytokine response and $\alpha\beta$ Crystallin 'mistaken self'[93]. In the mouse model experimental autoimmune encephalomyelitis (EAE), latent γ-herpesvirus 68 infection resulted in a more severe disease course that was associated with increased $CD4^+$ as well as $CD8^+$ T cell infiltration into the CNS and a skewed Th1 response, though the implication for MS remains uncertain[94]

1.2 From $CD4^+$ T cell-mediated disease to disease of B cell:$CD4^+$ T cell interactions

1.2.1 Imbalance between $CD4^+$ effector and regulatory T cells in MS

The prevailing concept of MS pathophysiology has highlighted a central role for T cells, and in particular $CD4^+$ T cells, in the cellular immune cascades leading to tissue injury in the MS CNS[4]. Much of this thinking has been shaped over the years by findings in the commonly used mouse model of MS, EAE[95]. Described over 80 years ago, in this model, animals can be actively immunized with myelin proteins or peptides along with complete Freund's adjuvant,

Mycobacterium tuberculosis and pertussis toxin[96,97]. The animals develop a disease characterized by demyelination in the spinal cord and brain, with appearance of perivascular lesions and inflammatory infiltrates, gliosis, partial remyelination, and in some instances, presence of immunoglobulin in the lesions and CSF[95]. Further emphasizing the role of T cells in the animal model were observations that thymectomized rats failed to develop the disease, as well as the ability to passively transfer disease into naïve animals by injecting activated lymph node cells (or activated CNS-reactive T cells) from immunized (diseased) animals[98-103]. Seminal observations made in EAE have been extended with the notion that $CD4^+$ helper cells are instrumental in MS pathophysiology. While EAE is not actually MS in animals, there is ample evidence for over-activated $CD4^+$ T cells in peripheral blood as well as the brain in MS patients[4].

It is now well appreciated that, in general, $CD4^+$ T cells can acquire distinct functional phenotypes upon encountering antigen-presenting cells (APC) in lymphoid tissues, which can be relevant in both health and disease states [104,105]. Indeed, three signals are necessary for optimal naïve T cell differentiation upon APC encounter, including TCR engagement with peptide loaded MHC molecules (signal 1), co-stimulatory signals such as CD80 and CD86 (signal 2), and polarizing cytokines (signal 3)[104,105]. These polarizing cytokines serve to reflect the nature of the perceived insult, thus providing a mean to mount the appropriate immune response to differing host infections. IL-12, a heterodimer of IL-12p40 and IL-12p35, binds its receptor on T cells and induces their differentiation into type 1 T helper (Th1) cells that express the master transcriptional factor T-bet and produce IFN-γ, TNF, and GM-CSF[104,106]. IL-4 induces T cell differentiation into Th2 cells that express the transcriptional factor GATA3 and produce IL-4, IL-5, and IL-13[104,106]. Th17 cells have emerged as distinct from the original dichotomy (Th1 versus Th2), and can be induced by a combination of IL-1β, IL-6, IL-23 and TGF-β and maintained by autocrine IL-21 production[107-116]. Th17 cells express the transcriptional factor RORγt, and produce IL-17, IL-21, and GM-CSF. These are not hard-wired phenotypes, as a degree of plasticity has been characterized reflecting the impact of the inflammatory microenvironment in which T cells operate[104,117].

Th17 cells are characterized by higher expression of brain-homing receptors (CCR6, CCR2 and CCR5) and adhesion molecules (CD49d, CD146 (MCAM), CD6)[118-126]. IL-17 expression was

elevated in lesions obtained from post-mortem MS cases compared to controls, and higher numbers of IL-17$^+$ T cells were found in active compared to inactive areas of MS lesions[127-129]. Th17 cells can engage in cross-talk with brain resident cells including astrocytes and blood brain barrier (BBB) endothelial cells. Human blood brain barrier endothelial cells express the cell surface receptors for IL-17 and IL-22, and exposure to IL-17 and IL-22 leads to disruption of the tight junctions and secretion of CCL2[130,131]. This allows Th17 cells and other CNS-recruited cells to migrate more efficiently across the barrier[121,130]. This interaction with the BBB also enhances IL-17 and IL-22 expression in T cells[121,130]. Fetal human astrocytes stimulated *in vitro* with IL-17 and TNF express increased levels of inflammatory molecules such as IL-6 and CCL20, which can sustain local inflammation by recruiting CCR6-expressing cells (such as Th17 cells among others)[132]. CCR6 deficiency abrogated EAE disease induction, and this was associated with reduced T cell infiltration into the CNS through the choroid plexus[121]. It follows that the frequency of Th17 cells was increased in the cerebrospinal fluid (CSF) compared to peripheral blood, and that it was higher in MS patients compared to controls[119,131,133-136]. IFN-γ producing Th17 cells, known as Th17.1 cells, were found in MS brain, showed preferential accumulation in the CSF, and possessed enhanced migratory capacities across endothelial cells[129,137]. Not surprisingly, MS-derived Th17 and Th17.1 cells showed a distinct functional phenotype from normal controls cells, including lower expression of granzyme B and IL-10[136].

A pathogenic role for GM-CSF in neuroinflammatory disease was suggested by *McQualter et al.* wherein GM-CSF deficient mice were resistant to EAE induction[138]. Immune cell infiltration into the brain was reduced, as was the proliferation and effector functions of splenic immune cells. Similarly, adoptive transfer of CSF2$^{-/-}$ Th1 or Th17 cells into naïve mice failed to induce EAE[139,140]. Therapeutic intervention with anti-GM-CSF antibody at disease initiation or at disease peak resulted in clinical benefits[138,140]. In this model, GM-CSF induced the expression of a pro-inflammatory gene signature in monocyte-derived dendritic cells that perpetuated the inflammatory response in CNS[141-143]. Indeed, GM-CSF-stimulated monocyte-derived dendritic cells up-regulated the expression of CCL6, CCL24, and CCL17 to enhance further myeloid cell recruitment into CNS and expressed IL-1β and IL-23, which supported pathogenic T cell generation in peripheral lymph nodes and compromised the blood brain barrier integrity[141-143]. In humans, the frequency of GM-CSF-producing CD4$^+$ T cells was elevated in both MS peripheral

blood and CSF, with a preferential accumulation of these cells in the CSF and lesions[144-149]. In contrast to murine T cells, human naïve T cells express GM-CSF under Th1 and not Th17 polarizing conditions, and are dependent on IL-2-STAT5 axis and IL-12 for optimal differentiation[144,147]. Further, a polymorphism in *IL2RA* (rs2104286) was associated with an increased propensity for $CD4^+$ T cells to produce GM-CSF *ex vivo* and *in vitro* cultures[147].

The aforementioned changes in T cell responses were observed in adult-onset MS patients, wherein the biological onset likely preceded the clinical onset by many years and aberrant immune functions can be secondary to ongoing injury or chronic inflammation[3]. In contrast, pediatric-onset multiple sclerosis offers a unique window into disease relevant immune functions given the likely closer time proximity between biological and clinical onset[7]. In this setting, the frequency of blood $CD4^+$ T cell population co-expressing the brain homing receptors CCR2 and CCR5 was found to be increased and produced elevated levels of IFN-γ and IL-17 in children with MS at the first clinical presentation but not in normal controls and in children with monophasic acquired demyelinating syndrome (serving as 'other CNS inflammatory disease' controls)[150].

The antigen(s) recognized by T cells in the context of multiple sclerosis remain elusive. Myelin proteins are attractive candidates, and indeed, earlier studies have found that T cell clones derived from MS patients were reactive to myelin basic protein (MBP) and myelin oligodendrocytes glycoprotein (MOG)[151-160]. However, similar $CD4^+$ T cell reactivity was also observed in normal controls[151-160]. Furthermore, *Banwell et al*. demonstrated that T cells of children with multiple sclerosis and remote CNS injury exhibited similarly increased reactivities to CNS-derived antigens including MBP and MOG, making the point that abnormally increased T cell proliferative responses to CNS antigens may be seen as a consequence of and not necessarily the cause of CNS injury[161]. It remains possible that myelin-reactive T cells in MS are functionally distinct from the control cohort counterparts in that these T cells did not require co-stimulation for *in vitro* expansion and produced elevated levels of pro-inflammatory cytokines, including IFN-γ, IL-17, and GM-CSF, along with decreased IL-10 levels[162-165]. Other candidate antigens may be relevant, including axoglial apparatus molecules, as such proteins were enriched in the CSF of children with MS compared to controls at the first clinical presentation[166].

Planas et al. recently identified IFNγ producing $CD4^+$ T cell clones present in the CSF of multiple sclerosis patients that recognized a ubiquitous self-antigen known as guanosine diphosphate (GDP)-L-fucose synthase[167]. This antigen was expressed in white and gray matter of post-mortem brain tissue samples[167]. The human protein had 40% sequence similarity with the bacterial homologue, and T cell clones were reactive to bacterial peptides, though T cell cross-reactivity was not examined in this study[167]. Similarly, $CD8^+$ T cell clones isolated from a MS patient recognizing $PLP_{45\text{-}53}$ in the context of HLA-A3 were (cross) reactive to another ubiquitous antigen known as glycerolphosphatidylcholine phosphodiesterase 1[168]. This theme extends to rheumatoid arthritis where T cells and antibodies isolated from patients recognized 60S ribosomal protein L23a (RPL23A)[169]. Altogether, this data supports a model wherein a secondary immune response is directed to ubiquitous antigens released from tissues following primary injury instigated in part by a defective immune cell repertoire.

In the normal state, effector T cells do not function in isolation but rather are controlled by cellular regulatory mechanisms to prevent immune pathologies. In this regard, regulatory T cells provide a key cellular mechanism aimed at preventing excessive effector T cell functions[170-172]. This is exemplified by the systemic autoimmunity observed in humans and mice carrying mutations in the gene encoding the transcriptional factor forkhead box P3 (FOXP3)[173-175]. FOXP3 is highly expressed in $CD4^+CD25^+$ regulatory T cells and is required for their suppressive functions[176-178]. Scurfy mice carrying a Foxp3 mutation have a deficiency in $CD4^+CD25^+$ regulatory T cells, accompanied by excessive T cell proliferation, multi-organ immune cell infiltration, and elevated serum cytokine levels that result in neonatal and adult death[176-179]. Similar outcomes were observed in athymic nude mice receiving CD25-depleted T cells or in mice with acute depletion of regulatory T cells[180-182]. In the absence of regulatory T cells, T cell reactivity was directed toward self-antigens and not microbial or food antigens[183-185].

Regulatory T (Treg) cells develop from T cells recognizing self-antigens but are not eliminated by negative selection in the thymus, and as such, their TCR repertoire is biased to self-antigens[170,172,186,187]. Inducible Treg (iTreg) cells can be induced *in vitro* or can develop from naïve T cells in peripheral tissues such as the gut lamina propria[184,188,189]. Regulatory T cells display an anergic phenotype to T-cell receptor stimulation, which can be overcome by co-stimulatory signals

such as CD28 and high IL-2 dose[190-194]. Regulatory T cells maintain peripheral tolerance through multiple suppressive mechanisms that act on antigen-presenting and/or effector T cells. These mechanisms include competition for co-stimulatory signals on antigen presenting cells via CTLA-4, consumption of locally-produced IL-2, degradation of ATP and generation of adenosine, production of immunosuppressive molecules such as IL-10 or IL-35, and cytotoxicity[170,171]. Further, certain mechanisms are more relevant for the maintenance of tolerance in tissues, such as IL-10 production. Indeed, IL-10-deficient mice exhibited inflammation in the colon and the central nervous system, and specific IL-10 depletion in regulatory T cells resulted in spontaneous colitis and exacerbated EAE [195-202]. In this context, IL-10 signaling in T cells was required for the suppression of pathogenic Th17 cell responses as well as the generation of type 1 regulatory T cells (Tr1 cells)[198,199,203-205].

Human regulatory T cells include $FOXP3^{+}CD25^{hi}CD127^{lo}CD4^{+}$ T cells, and more recent mass cytometry and single-cell RNA-seq analysis have emphasized the presence of discrete regulatory T cell populations in human blood[190-193,206-210]. Regulatory T cells are functionally heterogenous, with certain subsets exerting greater suppressive effects on effector T cells, such as $CD45RA^{+}CD25^{hi}FOXP3^{lo}$ cells and $CD45RA^{-}CD25^{hi}FOXP3^{hi}$ cells, whilst others acquire a capacity to produce cytokines, such as $CD45RA^{-}CD25^{hi}FOXP3^{lo}$ cells[211-214]. Indeed, cytokine-producing regulatory T cells have been characterized in the circulation of normal individuals and patients and are enriched within the memory T cell pool[215-218]. Th1-like, Th2-like, and Th17-like regulatory T cells co-express FOXP3 and T-bet, GATA-3, and RORγT, respectively; produce prototypical cytokines such as IFN-γ, IL-4, and IL-17; and express characteristic Th cell chemokine receptors[215-218]. Th-like regulatory T cells retain suppressive functions; however, they cannot be suppressive and produce cytokines simultaneously[216,217]. Regulatory T cell sensitivity to CD3, CD28, and cytokine receptor signaling is comparatively low, which may repress cytokine expression in these cells[219]. Also, Th-like regulatory T cells in humans differ from $T\text{-}bet^{+}$, $GATA3^{+}$, and RORγT^{+} regulatory T cells in mice, which fail to produce the corresponding cytokines IFN-γ, IL-4, and IL-17 under acute inflammation[220-224]. The acquisition of T-bet, GATA3, and RORγT appears necessary for optimal maintenance and recruitment of murine regulatory T cells to the site of inflammation, where these cells serve to regulate Th1 and Th17

cells[220-224]. Nonetheless, regulatory T cells can produce inflammatory cytokines in the context of chronic inflammation, such as cancer or CNS neuroinflammation[225,226].

In autoimmune diseases, defects in regulatory T cells have been observed, including a reduction in the frequency of circulating cells, aberrant and/or loss suppressive mechanisms, and resistance of effector T cells to suppression[227]. In multiple sclerosis, thymic output is reportedly diminished such that the number of recent thymic emigrant (RTE) T cells is reduced[228-234]. This includes reduced frequency of circulating RTE regulatory T cells and a relative increase in the frequency of memory regulatory T cells[228,231] while the frequency of total circulating regulatory T cells is unchanged[234-238]. Further, it is now well documented that regulatory T cells from MS patients fail to suppress the expansion of autologous or allogenic effector T cells in antigen-specific or polyclonal culture systems[234,235,237,239]. This deficient suppressive function has been linked to reduced FOXP3 expression[240]. FOXP3 serves to maintain regulatory T cell hypo-responsiveness, to amplify and stabilize the expression of suppressive soluble mediators (such as *IL10*, *Ebi3, GzmB),* as well as the ectonucleotidases (*Entpd1, Nt5e*), and to repress the expression of pro-inflammatory cytokines (such as *Il2*, *Il4*, *Il17a,* and *Ifng)*[241,242]. In line with a reduction in FOXP3 expression, CD39 expression on regulatory T cells isolated from MS patients was reduced, and the function of CD39-expressing regulatory T cells was altered[243]. CD39 is an ectonucleoside triphosphate diphosphohydrolase required for the conversion of extracellular ATP into adenosine[244-246]. Paracrine or autocrine adenosine signals through A2AR and has multiple effects on T cells that include negative regulation of TCR signaling in effector T cells as well as amplification of CD39, CD73, and FOXP3 expression in regulatory T cells[246]. *In vitro*, CD39-expressing regulatory T cells can inhibit the secretion of IL-17 and IFN-γ in effector T cells, a regulatory mechanism defective in MS patients[243,247]. The differentiation of T cells into type 1 regulatory T cells, characterized by IL-10 production upon CD2 or CD46 engagement, was less efficient in MS patients compared to normal controls[37,248,249].

The frequency of Th1-like, but not Th17-like, regulatory T cells was increased in the blood of MS patients compared to normal controls, whilst the frequency of IL-10$^+$ regulatory T cells was reduced[250,251]. This resulted in an increased ratio of IFN-γ^+/IL-10$^+$ regulatory T cells in patients compared to controls. Th1-like Treg cells displayed a similar methylation pattern in Treg-cell

specific demethylated region (TSDR) than non-IFN-γ producers, demonstrating that these cells represent bone-fide Treg cells[250]. Their differentiation was dependent on IL-12 and on PI3K-AKT-FOXO1-FOXO3 pathways and could be antagonized by TIGIT signaling[250-253]. In MS, the suppressive capacity of Th1-like Treg cells was deficient, and this could be normalized by utilizing an IFNγ neutralizing antibody or by a TIGIT agonistic antibody[250,253].

Recent observations in pediatric-onset MS mirrored the abnormalities in regulatory T cells seen in adult-onset MS. Regulatory T cells from children with MS expressed lower FOXP3 levels than controls (including children with acquired-demyelinating syndrome) and possessed an impaired suppressive capacity[150]. In addition, effector T cells from children with MS were resistant to suppression by control regulatory T cells, suggesting that aberrant effector $CD4^+$ T cell functional responses can contribute to the cascade of events leading to tissue injury early in the disease[150].

Environmental factors can modulate the balance between effector and regulatory T cells. Salt, melatonin, and gut microbiota are emerging as putative regulators. Sodium chloride enhanced IL-17 production in effector $CD4^+$ T cells *in vitro* and *in vivo* and, in parallel, impaired Treg cell suppressive capacity by promoting Th1-like Treg phenotype and reducing IL-10 production[251,254-256]. However, the pathophysiologic relevance remains uncertain, as there was no association between the average 24-hour urine sodium levels with clinical or MRI outcomes over follow-up, nor with conversion to clinically definite MS[257]. In addition, the total dietary sodium intake at baseline was not associated with the risk of developing MS in the Nurses' Health Study I and II[258]. *Farez et al.* found that melatonin suppressed human Th1 and Th17 cells and enhanced Tr1 cells *in vitro* and in EAE mouse model[259]. In MS patients, urine melatonin levels were highest in autumn and winter, during which time patients typically see reduced rates of relapse[259]. In regards to the gut microbiota, several studies have observed that the diversity in fecal microbiota was similar between MS patients and controls[260-263]. However, there were subtle changes in the relative abundance of certain phyla and gena, such as an increased abundance of *Euryarcheaota*, *Verrucomicrobia*, *Methanobrevibacter*, *Akkermansia, and Acinetobacter* with a reduced abundance of *Parabacteroides*, *Butyricinomas*, *Prevotella,* and *Sutterella*[260-263]. In mouse models, the EAE disease course was attenuated in germ-free mice compared to conventionally colonized mice or specific pathogen free mice[264,265]. In germ-free mice, pro-inflammatory Th1 and Th17 cell

responses were reduced in the gut and spinal cord concomitant with increased T cell regulatory functions and reduced dendritic cell effector functions in secondary lymphoid tissues[264,265]. Disease susceptibility was restored in germ-free mice colonized with segmented filamentous bacteria[265]. Similarly, transfer of MS patients' microbiota into germ-free mice exacerbated EAE disease course compared to normal controls' microbiota, and this was associated with reduced FOXP3 and IL-10 expression in T cells in mesenteric lymph nodes[263]. Transfer of MS-affected twins' microbiota into EAE spontaneous mouse model resulted in a more severe disease course compared to the transfer of microbiota from healthy twins[261]. In parallel *in vitro* studies, *Acinetobacter calcoaceticus* and *Akkermansia muciniphila* enhanced IFN-γ production in human T cells, whilst *Parabacteroides distasonis* induced IL-10 production in T cells[263]. Further, there was an inverse correlation between the frequency of intestinal IL-17 producing T cells and the relative abundance of *Prevotella* strains in MS patients and controls[266]. Disease modifying therapies may in part act by modifying the gut microbiota, as patients receiving interferon-beta-1a or glatiramer acetate had an increased relative abundance of *Prevotella* and *Sutteralla* compared to untreated patients[260]. *In vivo*, therapeutic intervention with *Prevotella histicola* in an EAE mouse model resulted in decreased CNS infiltration by Th1 and Th17 cells and increased frequencies of tolerogenic dendritic cells and regulatory T cells in peripheral lymphoid tissues[267]. Finally, in a case-control study, *Tremlett et al.* found that the overall diversity of the gut microbiota was similar between pediatric-onset MS patients and controls[268-270]. However, the relative abundance of *Actinobacteria*, *Desulfovibrio*, *Bifidobacterium,* and *Christensenellaceae* was increased in patients' fecal microbiota, with a decreased abundance of *Lachnospiraceae* and *Ruminococcaceae*[269,270]. In pediatric MS patients, the frequency of blood Th17 cells was negatively correlated with the relative abundance of Bacteroides but was positively correlated with Firmicutes' abundance[268]. Altogether, the dysregulated T cell functions observed in patients with multiple sclerosis may in part be controlled by a distinct gut microbial community.

1.2.2 Targeting B cells in multiple sclerosis

Initial observations on the deposition of immunoglobulin (Ig) in brain lesions and the abnormal presence of CSF oligoclonal Ig bands in MS patients were suggestive of a pathological antibody-dependent role for B cells in the disease course and provided the initial rational for targeting B cells in patients[271]. Two of the main strategies used to target B cells in clinical trials of MS, anti-

CD20 antibody (rituximab, ocrelizumab, ofatumumab) and transmembrane activator and CAML interactor (TACI)-Ig fusion protein (atacicept), had contrasting outcomes[272-277]. A growing understanding over the recent years of the relevant modes-of-action of these two therapies has contributed important novel insights into antibody-independent B cell functions that are now thought to play a role in MS pathophysiology, including their ability to present antigens to T cells, secrete pro- and anti-inflammatory cytokines that modulate both T cell and myeloid cell responses, and contribute to the formation of ectopic lymphoid structures[271].

CD20 is a cell surface receptor expressed at high levels on pre-B cells, transitional, naïve, and memory B cells but absent on progenitor B cells and antibody secreting/plasma cells[271]. Anti-CD20 antibody binds to CD20, and can deplete B cells via complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity, antibody-dependent cell-mediated phagocytosis, and induction of apoptosis[271] (with the latter likely the least relevant *in vivo*). In two phase 3 clinical trials enrolling relapsing-remitting MS patients, intravenous anti-CD20 antibody (ocrelizumab) showed substantial (over 95%) decrease in new MRI disease activity compared to patients receiving β-interferon[274]. Clinical benefits observed in MS patients treated with B cell depletion therapy (BCDT) occurred whilst CSF IgG index, IgG concentration, and oligoclonal band numbers were unaffected[272,278-280]. This was highly suggestive that some pro-inflammatory antibody-independent function/s of B cells is/are involved in the pathological cellular cascades leading to new waves of focal inflammation and disease relapses in MS. Indeed, mechanistic studies have since demonstrated exaggerated pro-inflammatory B cell responses in untreated MS patients with the capacity to induce pro-inflammatory T cell and myeloid cell functions[271]. In keeping with this, depletion of B cells using anti-CD20 resulted in diminished T cell and myeloid cell effector responses [271,281,282]. Further, the functional profile of reconstituting B cells in patients was distinct from pre-treatment in that pro-inflammatory cytokine production by the reconstituting B cells was reduced concomitant with increased anti-inflammatory cytokine production [271,281,283].

In contrast to the benefit of anti-CD20, atacicept, a fusion protein of TACI and Ig, increased annualized relapse rates compared to placebo in a phase II safety and efficacy trial in MS patients[277]. TACI is a cell surface receptor that binds B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), two B cell pro-survival factors[284,285]. BAFF also binds to

BAFF-R, whilst APRIL binds to BCMA[284,285]. Subsequent analysis showed that whilst transitional and naïve B cells as well as plasmablasts/plasma cells were strongly affected by this treatment, memory B cells were spared[271,286,287]. The striking contrast in clinical outcomes of atacicept and ocrelizumab serves to highlight the diverse and functionally distinct features of B cell subsets in either activating or regulating immune responses. The subsequent sections provide further detail of both antibody-dependent as well as antibody-independent functions of B cells that are thought to be relevant in MS pathophysiology. In the context of antibody-independent B cell functions, both pro-inflammatory (and pathogenic) functions of memory B cells and potential regulatory (and beneficial) functions of naïve B cells and plasma cells will be reviewed.

1.2.3 B cell antibody-dependent functions in multiple sclerosis

A question common to MS and other autoimmune diseases in which autoantibodies are considered as potential contributors to pathophysiology is whether such abnormal antibodies are generated in a very focused way to particular target-organ antigens (e.g. CNS in MS) or whether a broader abnormality of B cell tolerance exists. In the normal state, two mechanisms of B cell tolerance (central and peripheral) exist to limit the development of potentially harmful autoreactive antibodies[288]. Central tolerance occurs during early B cell development and results in purging of up to 75% of potentially auto-reactive B cells in the bone marrow[288]. Intact B-cell receptor and toll-like receptor mediated signaling are required for this process, as individuals with mutations in BTK, PTPN22, IRAK-4, MYD88, and UNC-93B show defects in central as well as peripheral tolerance[288]. Peripheral tolerance serves as a second checkpoint to control auto-reactive clones that have escaped selection in the bone marrow[288]. This process appears to depend on regulatory T cells and BAFF concentrations. IPEX patients, patients with mutation in CD40L or MHC-II, and CD3- or AIRE-deficient patients have defects in peripheral B cell tolerance reflected in polyreactive mature naïve B cells[289-291]. *Kinnumen et al.* reported a loss of peripheral (but not central) tolerance, associated with defective regulatory T cell function in MS patients[292,293]. Mature naïve B cells of patients were polyreactive and tended to recognize antigens present in white matter extracts[292].

In MS, pathology studies have identified abnormal antibody deposition suggestive of binding to particular CNS target-antigens[271,294]. Surface and cytoplasmic immunoglobulins have been reported in myeloid cells close to myelin sheaths at demyelinating plaque margins in MS, hinting

to a pathogenic role for antibodies[295,296]. In a classical study by *Lucchinetti et al.,* four demyelinating lesion patterns emerged in post-mortem brain tissues from MS patients, with pattern II most commonly found among patients examined[297,298]. Pattern I and II associated with inflammatory infiltrates, whereas loss of oligodendrocytes was prominent in pattern III and IV[297,298]. Immune infiltrates in pattern II lesions were composed of T cells and macrophages around blood vessels as well as the deposition of IgG and complement C9neo antigen at sites of active myelin destruction[297,298]. Ig bound to myelin fragments could be observed within macrophages and at the active plaque edge[297,298]. C9neo antigen was associated with degenerating myelin and myelin degradation products[297,298]. Ig-containing plasma cells could also be found in active MS lesions[297,298].

In addition, >95% of MS patients have oligoclonal immunoglobulin bands restricted to the CSF (i.e. not overlapping with serum) with a predominance of IgG bands, though IgM bands are also observed in a subset of patients[3,299-305]. Increased intrathecal IgG synthesis rates are found in 96.2% of MS patients, with lower rates in controls, and there is overlap between CSF immunoglobulin proteomes and immunoglobulin transcriptomes of CSF B cells, which is further indicative of local immunoglobulin synthesis[300,306]. This is also supported by the capacity for CSF clonally-expanded plasma cells to produce IgG that comprise the oligoclonal bands in the CSF[307,308].

Recombinant antibodies produced from plasma cells isolated from CSF of MS patients were found to cause myelin loss and astrocyte activation in spinal cord explant cultures when added in the presence of complement [309]. Continued efforts have been made to determine the target antigen(s) recognized by CSF Igs, though it has been difficult to demonstrate antibodies recognizing myelin antigens. Recent work suggested that that CSF Igs of MS patients recognized ubiquitous intracellular proteins (e.g. MKNK1/2, FAM84A, and AKP17A) that were not brain specific, but were more likely generated secondary to tissue injury[310]. Indeed, serum antibodies against FAM84A antigens were more prevalent in inflammatory bowel disease patients than in controls[311]. *Von Budingen et al.* demonstrated that monoclonal antibodies derived from clonally expanded CSF plasma cells showed reactivity to myelin and intracellular filaments but failed to react to classical myelin antigens including MOG, MBP, PLP, or common viral antigens[307]. *Ho et al.* identified CSF antibodies with higher reactivity to 17 lipids in MS patients, targeting a phosphate group in

phosphatidylserine and oxidized phosphatidylcholine derivatives[312]. Myelin phospholipids were reduced in MS lesions compared to non-MS lesions[312]. *In vivo* experiments revealed that phospholipid injection into mice immunized with $PLP_{139-151}$ attenuated disease course[312]. None of the observations above have been firmly replicated and, overall, the exact antigen(s) recognized by auto-antibodies present in the CNS of MS patients remain rather elusive.

There has also been interest in measuring anti-CNS (including anti-myelin) antibodies in the periphery of MS patients for potential use as biomarkers of diagnosis and/or prognosis. Numerous studies however have failed to consistently find abnormal prevalence or titers of anti-myelin (e.g. anti-MBP and anti-MOG) antibodies in MS patients[313-315]. In other cases, initial and promising findings could not be replicated by independent groups in subsequent studies, such as the case for anti-KIR4.1 antibody. Work by Hemmer's group showed that serum levels of antibodies to KIR4.1 were elevated in MS patients compared with controls and were more prevalent in MS patients compared with those with other neurological diseases (46.9% versus 0.9% of patients, respectively)[316]. KIR4.1 is an ATP-sensitive inward rectifier potassium channel expressed on oligodendrocyte cell bodies and astrocyte processes[316]. Injection of KIR4.1 into mice led to rapid loss of KIR4.1 expression on glial cells, altered GFAP expression on astrocytes, and activation of the complement cascade, which can lead to demyelination and/or lack of remyelination[316]. However, two multi-center studies subsequently failed to detect anti-KIR4.1 antibody in the serum (or CSF) of over 500 MS patients in ELISA or cell-based assays, calling into question the initial observations [317,318].

Another important question in MS pertains to the relationship between B cells within the CNS and circulating B cells in the periphery. It has long-since been recognized that intrathecal B cells in MS patients are clonally expanded, show signs of somatic hypermutation indicative of antigen-driven B cell maturation in the CNS, and can persist over many years in the same patient[319-326]. A bi-directional flow of B cells between the periphery and the brain is now recognized in MS patients[327,328]. Initial findings demonstrated that there was a degree of sharing in VDJ sequence between CSF and peripheral blood B cell clones[324,325,329,330]. In addition, specific peripheral blood immunoglobulin-class switched memory B cells were clonally-related to CSF B cells, and the progression of somatic mutations suggested the initial antigen encounter occurred in the periphery, with further maturation taking place within the CNS[327,328]. This was supported by paired brain and

cervical draining lymph nodes tissue analysis, which identified that identical B cell clones were present in both tissues, with founding members of clones more frequently present in the draining lymph nodes[327,328]. Finally, identical B cell clones populating the CNS could be found in distinct regions within the CNS, including the meninges, inflammatory plaques, normal appearing white matter, and CSF[323].

1.2.4 B cell antibody-independent functions in multiple sclerosis

1.2.4.1 Antigen presentation to T cells

B cells recognize conformational epitopes via the B cell receptor, which is subsequently internalized[331,332]. Antigenic epitopes are then processed and presented on MHC class I or class II molecules to T cells along with co-stimulatory signals[331,332]. Cognate interactions occur between B cells and T cells when the interacting cells recognize the same antigen[331,332]. These cognate interactions are thought to be critical in EAE pathogenesis. Mice with TCR and BCR restricted to MOG spontaneously developed EAE-like symptoms, with demyelination and inflammatory infiltrates observed in the spinal cord and optic nerve[333,334]. Meanwhile, selective MHC-II ablation in B cells rendered mice immunized with MOG protein resistant to EAE, as Th1 and Th17 cell functions were diminished. Partial susceptibility was then restored upon injection of anti-MOG antibody[335].

In MS patients, expression of CD40, CD80, and HLA-DR was increased on B cells[336,337]. In both controls and MS patients, polymorphism in CD40 (rs4810845*T) resulted in reduced CD40 and IL-10 expression in B cells, whilst polymorphism in CD86 (r9282641) enhanced its expression on B cells[43]. Recent work has highlighted the potential role of B cell:T cell interactions in MS including the observation that patients with MS harbored both B cells and T cells that were more prone to auto-proliferation, akin to the autologous-mixed lymphocyte reaction (MLR). Auto-MLR was first described over 40 years ago in a study by *Opelz et al.*, wherein human T cell-enriched fractions proliferated in co-culture with B-cell enriched fractions from the same persons in the absence of exogenous antigens[338]. Similar outcomes can be observed in co-culture of T cells and dendritic cells[185,339-341].

With this background, *Jelcic et al*. re-demonstrated that co-cultured autologous B cells and T cells undergo proliferation in the absence of exogenous antigen or stimulus, but noted higher responses in MS patients carrying HLA-DR15[342]. This process has been termed autologous proliferation or

auto-proliferation. The proliferating MS T cells had a classical and non-classical Th1 phenotype, whereas proliferating B cells from the same patients were class-switched memory B cells[342]. In contrast to naïve B cells, memory B cells were more potent inducers of T cell auto-proliferation[342]. Pharmacological inhibition of TCR and BCR as well as HLA-DR neutralization abrogated cell proliferation, indicating that this response was antigen-specific[342]. Auto-proliferating T cell clones were found to be clonally expanded in the blood and brain of MS patients[342]. These clones recognized an antigen derived from RASGRP2, a protein expressed in B cells and in cortical grey matter[342]. Therapeutic intervention with rituximab resulted in reduced T cell auto-proliferation, whilst natalizumab (anti-VLA-4 antibody) increased the response[342]. This study highlights a pathogenic role for memory B cells in multiple sclerosis by provision and presentation of self-antigens to self-reactive $CD4^+$ T cells in the periphery and subsequent T cell trafficking to the CNS where they can mediate local inflammation.

1.2.4.2 B cell pro-inflammatory cytokine responses

B cells can further regulate pro-inflammatory T cell functions by producing pro-inflammatory (e.g. GM-CSF, IL-6, TNF, LT, IL-15) or anti-inflammatory (e.g. IL-10, IL-35) cytokines that serve to directly modulate T cells and/or indirectly affect T cells through modulation of myeloid cell responses[271]. While both murine and human B cells can produce T cell prototypical cytokines *in vitro* and *in vivo*[271,343], results have not always been consistent across species. In mice, B cells can produce IFN-γ, IL-4 and IL-17, which regulates T cell and myeloid cell functions[343]. B cells engaged in a cognate interaction with polarized Th1 cells produced IFNγ *in vitro*[344,345]. Additional cytokines such as IL-12, IL-18, and IFN-γ were required for optimal generation and maintenance of these B cells[344,345]. *In vivo*, IFN-γ producing B cells promoted pro-inflammatory T cell and myeloid cell functions. For instance, IFN-γ producing B cells induced TNF and iNOS activity in myeloid cells in mice infected *Listeria monocytogenes*[346]. In addition, selective IFN-γ ablation in B cells conferred resistance to arthritis in an experimental model[347]. This protective effect was associated with increased regulatory T cell frequency and decreased frequencies of Th1 and Th17 cells in the spleen[347]. IL-4 producing B cell generation was dependent on IL-4 derived from Th2 cells and IL-4R signaling, and in turn, Be-2 cells enhanced Th2 cell functions in mice infected with *Heligmosomoides polygyrus*[345,348,349].

Splenic B cells isolated from mice infected with *Trypanosoma cruzi* produced IL-17 at early time points post-infection, thus providing a rapid and supplemental source of this cytokine[350]. IL-17-producing B cells were positioned outside the splenic B cell follicles in proximity to the T cell zone and had a plasmablast/plasma cell phenotype ($CD138^{+}GL7^{-}B220^{int}$)[350]. IL-17 production in B cells was dependent on CD45 and BTK signaling but was independent from AHR and RORγT[350]. Human tonsillar B cells secreted IL-17 in culture with *Trypanosoma cruzi* supplemented with IL-10 and BAFF[350]. IL-17 expression could also be induced in human tonsillar B cells by stimulation with IL-6, IL-23, and TGFβ[351]. Potential roles for those cytokine-producing B cells in CNS neuroinflammation remain unknown.

Regarding human B cell cytokine responses, *Duddy et al*. provided early evidence that human B cells stimulated with dual signals (BCR cross-linking antibody together with CD40 engagement) produced more LTα, TNF and less IL-10[283,352]. This double signal is meant to model B cell stimulation with its antigen and T-cell co-stimulation, respectively[331,332]. Meanwhile, stimulation through CD40 alone (as a proxy to bystander activation via co-stimulation in the absence of B cell antigen) enhanced B cell expression of IL-10 with little or no expression of LTα and TNF[283,352]. This paradigm subsequently served to reveal that compared to normal control B cells, untreated MS B cells secreted increased LTα, TNF, and IL-6 levels and reduced IL-10 levels, resulting in higher LT/IL-10, TNF/IL-10, and IL-6/IL-10 ratios[282,283,353]. Studies into the mechanism underlying B-cell cytokine regulation revealed that CD40L and BCR cross-linking antibody induced expression of miR-132, a negative regulator of the NAD-dependent deactylase sirtuin 1 (SIRT1)[354]. This miR-132-SIRT1 axis was found to be dysregulated in MS B cells, such that miR-132 knock-down or pharmacological activation of SIRT1 normalized the abnormal levels of secreted TNF and LTα in MS B cells[354]. B cell-derived TNF, LTα, and IL-6 enhanced T cell proliferation *in vitro* and *in vivo*[282,355] As an *in vivo* proof-of-principle that B-cell derived cytokine can influence T cell responses and CNS inflammation, in mice immunized with MOG_{35-55} or protein, selective ablation of IL-6 in B cells resulted in reduced EAE disease severity, which was associated with diminished Th17 but not Th1 CNS-reactive T cell responses[355]. Thus, the therapeutic benefit of B cell depletion therapy can in part be due to normalizing the levels of secreted pro-inflammatory cytokines such as TNF, IL-6, and LT by MS B cells[355,283].

B cells can also modulate myeloid cell functions by producing granulocyte-macrophage colony-stimulating factor (GM-CSF), with prominent roles identified in mice in contexts of sepsis and neuroinflammation[281,356-359]. Splenic GM-CSF producing B cells, termed innate response activator (IRA), were located in the red pulp of mice challenged with LPS[356]. Gene expression analysis positioned this population as distinct from other B cell subsets, including plasma cells and marginal zone or follicular B cells[356]. IRA cells differentiated from a B1a B cell precursor and required TLR-4, MyD88, and autocrine GM-CSF for full maturation[356]. Mature IRA B cells expressed IL-3 but lacked the expression of IL-1β, IL-6, TNF, and IL-10[356]. Mouse IRA B cells were phenotyped as IgM^{hi} $CD23^{lo}$ $CD43^{+}$ $CD93^{+}$ IgD^{lo} $CD21^{lo}$ $CD138^{+}$[356]. Selective ablation of GM-CSF from B cells rendered mice more susceptible to cecal ligation and puncture (CLP) sepsis model, with high levels of pro-inflammatory cytokine levels in the serum and peritoneum associated with inefficient bacterial clearing by neutrophils[356]. In contrast, IL-3 producing IRA cells instigated a cytokine storm by inducing myelopoiesis of Ly-6^{hi} monocytes and neutrophils, enhancing host susceptibility to sepsis[359]. Finally, IRA B cells migrated from the pleural space to the lung parenchyma and provided the host with protection against pneumonia by producing IgM in a GM-CSF dependent mechanism[357]. In humans, splenic GM-CSF producing B cells had a memory phenotype ($CD27^{+}CD43^{-}$)[356].

In humans, *Li et al*. described a circulating GM-CSF producing B cell population that differs from murine IRA cells and was characterized by high production of pro-inflammatory cytokines TNF and IL-6 and lacked IL-10 production[281]. *Ex vivo* $GM\text{-}CSF^{+}$ human B cells were phenotyped as memory rather than naïve B cells ($CD24^{+}$ $CD25^{hi}$ $CD27^{+}$ $CD38^{lo}$ $CD43^{-}$ $CD49^{hi}$ $CD86^{hi}$[281]). Their optimal differentiation from naïve B cells was dependent on three signals, namely BCR, CD40L, and IL-4 or TGF-β[281]. Surprisingly, STAT5 and STAT6 signaling cooperated to induce GM-CSF expression in B cells whilst antagonizing IL-10 expression[281]. Secreted products from GM-CSF-producing human B cell culture enhanced IL-12 and IL-6 secretion from monocyte-derived macrophages and reduced secreted IL-10 levels[281]. IL-12 and IL-6 are important modulators for Th1 and Th17 cell differentiation, respectively, suggesting that B cells can indirectly modulate T cell effector functions via their impact on myeloid cells. Also, naïve human B cells primed with CD40L, BCR cross-linking antibody, and IL-4 upregulated co-stimulatory molecules such as CD80 and CD86 and were more efficient at presenting antigens to T cells *in vitro* and driving their

polarization to Th1 and Th17 phenotypes in an IL-6-dependent manner[360]. Importantly, in untreated MS patients, the frequency of blood GM-CSF$^+$ B cells was increased compared to controls concurrent with reduced IL-10 frequency, resulting in an increased GM-CSF/IL-10 ratio[281]. In addition, GM-CSF-producing B cell culture supernatants from MS patients induced higher secretion of IL-12 and IL-6 from monocyte-derived macrophages compared to normal controls, and this was correlated with higher B cell GM-CSF levels[281]. Thus, another mechanism by which BCDT may so effectively limit new MS relapses is by diminishing the ability of B cells to stimulate myeloid cells via GM-CSF secretion and in turn further contribute to diminished responses of pro-inflammatory T cells. *Knier et al.* recently showed that CNS-infiltrating, GM-CSF-producing B cells enhanced microglia activation and contributed to EAE disease severity within the CNS[358]. In a regulatory feedback loop, GM-CSF$^+$ B cells induced Ly6G$^+$ cell differentiation into PMN-MDSCs in a STAT3-dependent mechanism, which served to prevent B cell accumulation in the inflamed CNS[358].

An interesting further consideration is whether the therapeutic benefit of BCDT in MS patients may extend into the B cell reconstitution phase after discontinuation of BCDT. In fact, reconstituted B cells following BCDT in patients with MS produced less GM-CSF and concomitantly higher IL-10 levels compared to B cells prior to depletion, and this resulted in persistence of the diminished pro-inflammatory myeloid cell responses (i.e. the myeloid cell production of IL-6 and IL-12 remained reduced even as B cells reconstituted)[281]. These observations may reflect a restored balance between pro- and anti-inflammatory cytokine production by the reconstituted MS B cells, raising the specter of immune-regulation by B cells.

1.2.4.3 B cell anti-inflammatory cytokine production

Seminal work by *Katz et al.* and *Neta et al.* demonstrated that B cells could exert a suppressive effect on T cells in a delayed hypersensitivity model through an antibody-independent mechanism[361,362]. *Wolf et al.* showed that mice that were deficient in B cells suffered from an exacerbated EAE disease course that was associated with enhanced Th1 cell responses[363], suggesting an anti-inflammatory role for B cells in CNS inflammation. In subsequent work, selective deletion of IL-10 from B cells worsened EAE disease course, which was improved by transfer of wild-type B cells[364-372]. The absence of IL-10 expression by B cells resulted in increased disease severity in experimental models of arthritis, lupus, colitis, and neuroinflammation, but not

in systemic autoimmunity in a similar fashion to IL-10 deficiency in regulatory T cells[373-378]. Thus, IL-10 is necessary in inflammatory contexts as an important mechanism for maintaining tissue homeostasis and preventing immune pathologies. As a corollary, regulatory B cells or plasma cells could hamper the host immune response to infections and tumors[372,379-384]. Regulatory B cells suppressed the effector functions of $CD4^+$ and $CD8^+$ T cells as well as enhanced the generation of regulatory T cells[271,343,385]. These effects can be mediated by direct interactions between B cells and T cells as well as B cells and myeloid cells[271,343,384,385]. The suppressive mechanisms include secretion of anti-inflammatory cytokines, such as IL-10, IL-35, and TGF-β, as well as expression of cell surface negative-regulators, such as PD-L1, GITR-L, CD39, and CD73[271,343,384,385].

Most of the work to date considering regulatory B cells has focused on IL-10-producing B cells. IL-10 is a heterodimer cytokine produced by T cells, myeloid cells, NK cells, and B cells[386]. It signals through IL-10RA and IL-10RB receptors and induces STAT3 and to a lesser extent STAT1 and STAT5 phosphorylation[386,387]. IL-10 is generally viewed as an immunosuppressive cytokine given its inhibitory effect on T cells and myeloid cells[386]. However, it should be remembered that IL-10 can also stimulate other immune cells, such as B cells and mast cells[386]. IL-10 expression is inducible in several B cell populations during inflammation. In mice, these include gut IgM^{int} $CD1d^{hi}$ $CD21^{int}$ $CD23^{hi}$ $CD62^{lo}$ B cells, spleen and peritoneal B1a cells characterized as $IgM^{hi}CD23^{lo}CD43^+CD93^-$, transitional 2 marginal zone precursor cells characterized as IgM^{hi} $CD21^+$ $CD23^{hi}$, splenic B10 cells characterized as IgM^{hi} IgD^{lo} $CD1d^{hi}$ $CD5^+$ $CD21^{hi}$ $CD23^{lo}$ $CD24^{hi}$ $CD43^+$ $CD11b^{hi}$, plasma cells characterized as $LAG3^+$ $CD138^{hi}$ $CD1d^{hi}$ $CD22^-$ $CD43^{lo}$ $CD71^{lo}$ $CD200^{hi}$ Fas^{lo} MHC-II^{lo} B220lo, IgA+ plasma cells/plasmablasts, as well as plasmablasts characterized $CD138^+$ $CD44^{hi}$ $CD38^{lo}$ $CD43^+$ $CXCR4^{hi}$ $CXCR5^{lo}$ $B220^{lo}$ [372,374,383,388-392]. In humans, *ex vivo* IL-10-producing B cells are enriched in transitional (phenotyped as IgM^{hi} IgD^{hi} $CD19^+$ $CD5^{hi}$ $CD1d^{hi}$ $CD10^+$ $CD24^{hi}$ $CD27^-$ $CD38^{hi}$) and in memory (phenotyped as $CD19^{hi}CD24^{hi}CD25^{hi}CD27^{hi}$) B cells[377,393-395]. Furthermore, *in vitro* CD40L-stimulated naïve as well as transitional B cells produced higher levels of IL-10 than memory B cells[283,377]. In the context of human autoimmunity, regulatory B cell functions are reportedly impaired in MS, rheumatoid arthritis, diabetes, and SLE patients[271,282,283,377,385,394,396,397]. The lack of lineage-specific transcriptional factor(s) and cell surface markers may underscore the plastic and transient nature of IL-10 expression in B cells.

Given the therapeutic implications, how IL-10 expression is regulated by B cells has been a topic of growing interest. IL-10 production by B cells is positively regulated by signaling through BCR, CD40, CD1d, TIM-1, TLRs (MyD88, TLR2, TLR4 and TLR9), and cytokine receptors such as IL-21R[282,283,364,366,371-374,382,393,398,399]. To date, only one negative regulator of IL-10 has been described, known as Toso[400]. Toso, an Fc receptor for IgM, was shown to regulate the BCR activation threshold, and its selective ablation in B cells resulted in enhanced IL-10 production upon exposure to BAFF, LPS, anti-CD40, or anti-IgM[400]. Perturbation of the gut microbiota using antibiotics also resulted in a reduction in the numbers of IL-10 producing B cells in the spleen; however, SPF and gnotobiotic mice showed no difference in splenic IL-10-producing B cells[390,401], suggesting that the impact of the gut microbiota on regulatory B cell functions remains to be fully elucidated. Unlike T cells and myeloid cells, the transcriptional regulation of IL-10 expression by B cells remains elusive. Sequential TLR and BCR ligation induced NFAT activation in a $Ca2^{+}$ dependent fashion[369]. STIM-1 and STIM-2 are calcium sensors located to the endoplasmic reticulum and are necessary for productive BCR signaling[369]. Selective STIM-1 and STIM-2 deletion in B cells impaired IL-10 production[369]. BCR and TLR signaling also induced ERK and STAT3 phosphorylation, and pharmacological inhibition of these pathways reduced IL-10 expression by B cells[402,403]. Whilst master transcriptional factor(s) for regulatory B cells are lacking, two transcriptional factors have emerged as positive regulators of IL-10. Interferon regulatory factor 4 (IRF4) was induced by sequential ligation of TLR and BCR in plasmablasts and bound an upstream region from the IL10 transcriptional start site[392]. Selective deletion of IRF4 in B cells resulted in diminished IL-10 secretion[392]. Hypoxia-inducible factor 1-alpha (HIF1a) was induced by BCR or TLR signaling and cooperated with phosphorylated STAT3 to induce IL-10 expression in $CD1d^{hi}CD5^{+}$ B cells[404]. HIF1a also regulated the expansion of $CD1d^{hi}CD5^{+}$ B cells in a glycolysis-dependent mechanism[404].

More recent investigation identified a capacity for B cells to produce the immune-regulatory cytokine IL-35. Murine $CD138^{+}CD22^{-}$ plasma cells and $CD1d^{hi}CD5^{+}CD19^{+}B220^{lo}$ cells produced IL-35 upon stimulation[372,405,406]. *Wang et al.* showed that treatment of naïve B cells with IL-35 induced IL-35 and IL-10 expression, and acquisition of this regulatory phenotype was necessary to suppress effector Th1 and Th17 responses and induce regulatory T cells, which culminated in

reduced disease activity in experimental autoimmune uveitis[405]. IL-35 signaling in B cells is dependent on IL-12Rβ2 and IL-27Ra as well as STAT1 and STAT3 phosphorylation[405,406]. *Shen et al.* found constitutive IL-12p35 expression in naive B cells, whilst Ebi3 induction was dependent on BCR, CD40, and TLR4 signaling[372]. Selective IL-12p35 or Ebi3 deletion in B cells resulted in failure to recover from EAE and a reduced susceptibility to Salmonella infection[372]. In both instances, effector Th1 and Th17 as well as myeloid cell functions were enhanced, without any functional impact on regulatory T cells[372,405]. Human IL-35-producing B cells remain to be identified.

Together, studies in humans and animals have established important antibody-independent functions of B cells that are highly relevant to MS pathophysiology, including B cell functions as APCs to T cells and as producers of cytokines that can either activate or regulate T cells and myeloid cells. The abnormal pro-inflammatory responses of B cells in untreated MS patients likely explains the observation that BCDT is highly effective at limiting new relapses, as B cell depletion results in diminished pro-inflammatory responses of both T cells and myeloid cells[271,282,355,360]. While this body of work has strongly implicated interactions between B cells and $CD4^+$ T cells, our own lab reported that $CD8^+$ T cell responses are also diminished post-B cell depletion in MS patients, raising the possibility that B cell-$CD8^+$ T cell interactions are also relevant in MS[282]. It is noteworthy that unlike EAE, which is largely a $CD4^+$ T-cell mediated disease, the pathology of MS lesions demonstrates a predominance of $CD8^+$ T cells over $CD4^+$ T cells, with the $CD8^+$ T cells in the MS lesions also exhibiting clonal expansion[95,294]. The following sections will expand on the roles of $CD8^+$ T cells, including a subset known as mucosal-associated invariant T (MAIT) cells, in MS and EAE.

1.3 Implication of $CD8^+$T cells in the MS central nervous system

Classical histopathological observations of post-mortem tissues from MS patients demonstrate that $CD8^+$ T cells, and not $CD4^+$ T cells, predominate among T cells in the perivascular space of veins, tissue parenchyma, and normal appearing white matter in both acute and progressive disease[86,297,298,407-413]. The predominance of $CD8^+$ T cells was not unique to the MS CNS, as they could also be observed in brain tissues from inflammatory controls[413,414]. Nonetheless, these findings challenge the view that MS is solely a $CD4^+$ T cell-mediated disease. CNS-infiltrating

$CD8^+$ T cells have an effector memory phenotype, characterized by the expression of CD45RO (and lack of CCR7), PD-1, CTLA-4, and TIM-3[86,413-416]. In addition, a subset expressed CD69 and/or CD103 and lack S1P1 expression, consistent with tissue resident memory cell phenotype[86,413-416]. Infiltrating $CD8^+$T cells appeared activated and expressed pro-inflammatory molecules such as IFN-γ, TNF, IL-17, granzyme B, and perforin[86,413-416]. The secretion of cytokines by $CD8^+$ T cells may modulate brain resident cells, including glial cells and myeloid cells, and could promote local inflammation and tissue injury. This is supported by findings indicating a positive correlation between the expression of APP on axons, a marker for axon transection and damage, with the numbers of $CD8^+$ T cells and myeloid cells in lesions[417,418]. $CD8^+$ T cells could also be found in close proximity to glial cells in white matter lesions[86,411]. In addition, MHC-I was constitutively expressed on microglia, macrophages, and endothelial cells and to a lesser degree on astrocytes, oligodendrocytes, neurons and axons. Among the CNS-infiltrating $CD8^+$ T cells in the MS CNS are MAIT cells. MAIT cells can be found in multiple locations in the MS CNS, including perivascular and parenchymal lesions as well as in meningeal infiltrates[419-424]. Additionally, MAIT-cell activating cytokines (IL-18 and IL-23) were expressed in MS lesions[422]. A major focus of this thesis relates to the effects of B cells on MAIT cells, which are described in greater detail in subsequent sections.

Studies employing CDR3 spectratyping on single cells demonstrated that MS brain infiltrating $CD8^+$ T cells were clonally expanded, with a high degree of sharing among cells present in demyelinating lesions, NAWM, and CSF, and less so with blood T cells[410,425-434]. This suggested that $CD8^+$T cells locally responded to antigenic stimulation and/or to bystander inflammation. The former is supported by the expression of TCR downstream signaling molecule NFAT in $CD8^+$ T cells present in active lesions[413]. Clonally expanded $CD8^+$ T cells expressing the MAIT cell invariant TCR (Vβ1-Jα2.3-Vα7.2) were also found in MS brain lesions and in blood samples, and the canonical Vα7.2 MAIT cell clones identified in brain lesions were found among circulating $CD8^+$ T cells even 18 years later, showing signs of clonal expansion[423]. However, the antigenic specificity of $CD8^+$ T cells (both MAIT and non-MAIT) in MS remains elusive. Some studies found that $CD8^+$ T cell clones isolated from MS CSF and lesions were not reactive to CNS antigens such as MBP, MOG, and MAG[86,435,436], while others found that $CD8^+$ T cells could lyse myelin-pulsed target cells, including oligodendrocyte cell lines, and could secrete pro-inflammatory

cytokines[437-444]. It is unclear if these functional responses are different in magnitude in MS patients.

A potentially MS-relevant interaction between $CD8^+$ T cells and B cells is supported by observations that human B cells stimulated with CD40L expressed IL-15 *in vitro* and enhanced $CD8^+$ T cell cytotoxic functions as well as cell migration across the inflamed blood brain barrier[445]. In MS lesions, brain-infiltrating $CD8^+$ T cells were located in proximity to IL-15 expressing cells, which could enhance granzyme B expression[446]. Potentially relevant to the observation that TACI-Ig (atacicept) treatment worsened MS disease activity is the observation that murine splenic B cells isolated from TACI-Ig treated mice upregulate IL-15 expression and, conversely, that BAFF suppresses IL-15 expression by B cells[447]. Taken together, these findings support a potentially MS-relevant role for B cell:$CD8^+$ T cell interactions. The actual roles, however, of $CD8^+$ T cells and their subsets (e.g. both MAIT and non-MAIT) in MS remains unclear, with some studies attributing pro-inflammatory and pathogenic properties to $CD8^+$ T cells, while other studies suggest they may have regulatory roles in the context of CNS inflammation. The following sections will explore these different roles of $CD8^+$ T cells and their potential implication in MS.

1.3.1 Opposing roles for CD8+ T cells in neuroinflammation

The functions of $CD8^+$ T cells in the context of neuroinflammation have predominantly been examined in the EAE mouse model, with contrasting observations indicating both regulatory and pathogenic roles for $CD8^+$ T cells in the course of the disease.

1.3.1.1 CD8+ T cell pathogenic functions in neuroinflammation

Huseby et al. demonstrated that adoptive transfer of MBP-specific $CD8^+$ T cells into C3H mice (or C3H SCID mice) resulted in CNS inflammatory injury[448]. In line with MS pathology, these mice displayed perivascular cuffs around capillaries and venules, inflammatory brain lesions in the white matter of the cerebellum, and meningeal inflammation specifically in severe cases[448]. This disease was attenuated in mice treated with anti-IFN-γ antibody, but not with anti-TNF antibody, findings that are also consistent with results from MS clinical trials[448]. Notably, the same cannot be said about CD4-induced EAE, as results in murine experiments failed to predict outcomes in clinical trials of MS patients [448]. Similar pathogenic $CD8^+$ T cell effector functions were observed in other EAE mouse models[449-454]. Class I MHC (HLA-A2 and HLA-A3) influence

$CD8^+$ T cell functions in the course of $CD8^+$ T-cell mediated disease. HLA-A3 / 2D2 TCR double transgenic humanized mice immunized with PLP45-53 exhibited mild disease with a fraction developing a more severe late disease course[455]. Demyelination, axonal transection, and degeneration were observed[455]. Immune cells including T cells and myeloid cells infiltrated the spinal cord, the cerebellum, and the optic nerve, with an enrichment for $CD8^+$ T cells in early disease and $CD4^+$ T cells in late disease[455]. $CD4^+$ T cells and/or B cells drove the late disease, as MHC-$II^{-/-}$ and $Rag2^{-/-}$ mice failed to develop this phase[455]. In contrast, HLA-A3/HLA-A2/2D2 TCR triple transgenic mice failed to develop early and late disease, without evidence of demyelination or axonal and neuronal degeneration[455]. This may relate to HLA-A2 mediating negative selection of 2D2-TCR CD8+ T cells in these mice, resulting in reduced peripheral accumulation of these cells and protection from disease[455]. More recently, $CD8^+$ T cells were shown to drive relapsing remitting-like disease in MOG_{35-55} immunized mice injected with plasma extracellular vesicles isolated from donor EAE mice [456]. Disease was dependent on $CD8^+$ T cells and on the enrichment of fibrinogen on extracellular vesicles[456,457]. Fibrinogen injection into MOG_{35-55} immunized mice enhanced T cell and myeloid cell recruitment into the CNS[456-458]. In addition, fibrinogen activated macrophages and microglia via CD11b/CD18 and enhanced their antigen presentation capacities to facilitate local T cell IFNγ production[456-458]. Even in $CD4^+$ T-cell induced EAE, MBP-specific $CD8^+$ T cells were primed by CNS infiltrating Tip^+ dendritic cells and oligodendrocytes, upon which these cells potentiated tissue damage[459]. $CD8^+$ T cells, and in particular Tc17, could provide help to $CD4^+$ T cells and were required for initiation of typical MOG_{37-50} or MOG_{35-55} CD4-induced EAE[416,460]. Together, the animal model-based studies suggest that $CD8^+$ T cells can both drive various aspects of CNS inflammation (including playing a role in either early or later phases of $CD8^+$ T-cell mediated EAE) as well as cooperate with $CD4^+$ T cells in $CD4^+$ T cell-mediated EAE. Supporting a pro-inflammatory role of $CD8^+$ T cells in the human disease are reports of increased frequencies of GM-CSF^+ and IL-17^+ $CD8^+$ T cells in blood and/or CSF of MS patients[146,148,460,461].

1.3.1.2 $CD8^+$ T cell regulatory functions in neuroinflammation

Suppressor $CD8^+$ T cells have been described in mice that have recovered from EAE or have received oral MBP tolerization. Splenic $CD8^+$ T cells isolated from Lewis rats recovering from EAE induced by adoptive transfer of $CD4^+$ MBP-specific T cells lysed the CNS-reactive T cells

in vitro and suppressed their encephalitogenic property *in vivo*[462,463]. Further, the $CD4^+$ T cell TCR repertoire was modulated by $CD8^+$ T cells in recovering mice, such that it was more diverse in these animals compared with $CD8^+$ T-cell deficient mice[463]. Similarly, MBP-immunized mice were resistant to second disease induction unless $CD8^+$ T cells were removed[464,465]. In addition, optimal *in vivo* tolerization with MBP was dependent on $CD8^+$ T cells and their production of TGF-β[466-468]. Suppressor CD8+ T cells downregulated the expression of co-stimulatory molecules on antigen-presenting cells to indirectly inhibit $CD4^+$ T cell effector functions, while also directly targeting $CD4^+$ T cells in certain contexts[468-474]. More recent studies identified a murine $CD8^+$ regulatory T cell population that suppressed follicular helper T cells in a Qa-1-dependent mechanism[475-477]. Analogous human $CD8^+$ T cells have been characterized that co-express KIR2DL2/3 or KIRDL1, CD44, CD122, and Helios. Their implication in neuroinflammatory disorders remains uncertain[475,478,479].

1.4 Mucosal associated invariant T (MAIT) cells

Mucosal associated invariant T (MAIT) cells are innate-like T cells that, as noted previously, express the semi-invariant TRAV1.2-Jα12/20/33 and a restricted TCR-β repertoire[480-482]. *Porcelli et al.* described a $CD4^-CD8^-$ (double-negative; DN) T cell population that expressed Va7.2-Jα (IGRJa14) paired with Vβ2, 8, 11, or 13 in human blood[483]. Lantz's group found that this population was conserved in three species, including human, mice, and cattle[484]. These cells had an oligoclonal TCR-Vβ repertoire and were present in MHC-II- and TAP-deficient patients as well as in MHC-I- or CD1d-deficient mice, indicating that these cells are neither selected by nor dependent on classical MHC molecules[484]. Indeed, further investigation revealed that MAIT cells were absent in β2-microglobulin and MHC related protein 1 (MR1)-deficient mice[484,485]. An evolutionary biology analysis found that MR1 appeared 170 million years ago and was a highly conserved molecule across mammalian species but was lost in three instances (carnivores, armadillos, and lagomorphs)[486-488]. Interestingly, genes encoding TRAV1-2 and MR1 were interconnected, such that MR1 was absent in species lacking TRAV1-2[486]. Further, MR1 molecules isolated from different species were cross-reactive, in that human MAIT cells can be activated by antigen-loaded rat, mouse, or bovine MR1 molecules[487].

Whilst MR1 ligands remain incompletely elucidated, cells infected with bacteria or fungi were shown to induce MAIT cell activation *in vitro* and *in vivo* in an MR1-dependent mechanism[489-491]. *Kjer-Nielsen et al.* elegantly demonstrated that riboflavin (vitamin B2) metabolites such RL-6,7-diMe, RL-6-ME-7-OH, and rRL-6-CH_2OH could bind to the MR1 antigen-binding pocket and activate MAIT cells, whilst 6-formyl-pterin (6-FP), a folic acid metabolite, could bind to MR1 but fails to induce MAIT cell activation[492]. Further, 5-amino-6-D-ribitylaminouracil (5-A-RU) reacted with glyoxal and methylglyoxal to form the pyrimidine adducts 5-(2-oxypropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU), two activating MAIT cell ligands [493,494].

The TCR-β repertoire on MAIT cells is restricted to certain chains including TRBV6 and TRBV20[495-497]. Nonetheless, recent evidence points to a role for TCR-β chains in regulating the magnitude of MAIT cell activation in response to different antigens. For instance, diclofenac metabolites could activate TRBV6-1 but not TRBV6-4 or TRBV20 expressing cells[498]. Likewise, TCR-Vβ8, 13.1 and 13.6 expressing MAIT cells produced lower amounts of TNF and IFNβ in response to stimulation with *Escherichia coli*-pulsed monocytes *in vitro*[497]. This denotes a clonal heterogeneity in MAIT cell population in regard to their capacity to mount a response against pathogens and to respond to therapeutics.

1.4.2 MAIT cell development

MAIT cells develop in the thymus like conventional T cells and are selected by MR1-expressing double positive cortical lymphocytes[499-503]. The antigen(s) required for successful selection have not been defined but appear not to be derived from gut microbiota[501]. Analysis of murine and human thymus using MR1 tetramer loaded with 5-OP-RU revealed three developmental stages that give rise to effector, terminally differentiated MAIT cells[501,504]. In mice, $CD24^+CD44^-$ T cells represented stage 1 and had the potential to differentiate into stage 2 ($CD24^-CD44^-$) and stage 3 ($CD24^-CD44^+$)[501,504]. Stage 1 and stage 2 cells lacked expression of the transcriptional factors PLFZ, T-bet, and RORγT and could not produce effector cytokines (IFN-γ and IL-17) upon *in vitro* stimulation[501,504]. The transition from stage 1 to stage 2 was dependent on MR1[501,504]. Gut microbiota, PLFZ, and miRNAs were indispensable for the differentiation from stage 2 to stage 3, at which point MAIT cells acquired the expression of PLFZ, T-bet, or RORγT and produced

effector cytokines such IFN-γ or IL-17[501,504]. A recent analysis found that PLFZ was highly expressed in innate T cells and was associated with the expression of all cytokine and chemokine receptor genes[505]. The frequencies of NKT and MAIT cells were correlated in blood; however, these two innate-like T cell subsets competed for resources, as CD1d deficient mice had increased MAIT cell numbers in the periphery[501,505-507].

In adult humans, $CD27^{-}CD161^{-}$, $CD27^{+}CD161^{-}$, and $CD27^{+/-}$ $CD161^{+}$ T cells represented stage 1 to 3 MAIT cells, respectively[501,508]. Stage 3 was represented by effector MAIT cells that express high levels of PLZF, T-bet, and RORγT and produce effector cytokines (IFN-γ and TNF)[501,508]. Circulating stage 3 MAIT cells in adult blood are found within $CD4^{-}CD8^{+}$ and DN T cell fractions, and they are phenotypically characterized as effector memory T cells with diverse chemokine receptor expression, including CCR2, CCR6, CCR5, CXCR4, CXCR6, and CCR9 [497,508-512]. Their expression of diverse chemokine receptors is reflective of an enhanced migratory capacity to diverse tissues in inflamed conditions[480,513]. Functional heterogeneity among MAIT cell subpopulations was associated with TCR-β usage and could be denoted by cell surface markers, such as CD56 and CD94[497,508,514].

There is also phenotypic and functional diversity among MAIT cells found in fetal tissues as well as cord and adult blood[502,503,506,509]. In fetal thymus, spleen, and mesenteric lymph nodes, MAIT cells have a naïve phenotype, express CD62L and CCR7, express low levels of IL-18R and PLZF, and do not respond to *in vitro* stimulation[502,503,506,509]. In contrast, MAIT cells found in fetal small intestine, liver, and lung are mature, lack expression of lymphoid tissue homing receptors, express high levels of IL-18R and PLZF, and respond to *in vitro* stimulation by expanding and producing cytokines[502,503,506,509]. MAIT cells present in cord blood have a naïve phenotype, whilst in adult blood, they display a memory phenotype[502,503,506,509,515]. MAIT cell frequency is higher in adults than neonates, reaching a peak in the third decade of life and thereafter decreasing[506,516-518]. In human adults, MAIT cell frequency varies between 0.1 to 15% in blood, and up to 45% in liver[495,502,503,505,506,509,519,520].

The signals necessary for MAIT cell peripheral expansion remain largely unknown, but recent studies in individuals with monogenic primary immunodeficiencies provided some clues. In fact,

MAIT cell numbers were diminished in individuals with heterozygous loss of function mutation in STAT3 that resulted in autosomal-dominant hyper IgE syndrome[521-523]. Residual circulating MAIT cells secreted diminished levels of IL-17, but not IFN-γ, TNF, and granzyme B, in spite of normal RORγT and PLZF expression[521]. Peripheral MAIT cells were absent in patients with bi-allelic RORC mutation, which contributed to increased susceptibility to *Candida* and Mycobacterium infections[524]. MAIT cell deficiency was also observed in patients with loss of function mutations in IL-12RB1, IL-21R, IL-12RB2, and IL-23R that resulted in Mendelian susceptibility to mycobacterial disease[523,525]. X-linked inhibitor of apoptosis (XIAP) deficiency resulted in reduced MAIT and iNKT cell numbers in patients with X-linked lymphoproliferative syndrome[526]. Normal MAIT and iNKT cells have a pro-apoptotic functional phenotype characterized by high expression of active caspse-3 and caspase-7[526]. The XIAP protein negatively regulates caspase-3 and caspase-7 activity, thereby promoting iNKT and MAIT cell survival[526].

Environmental factors influence the frequency of circulating MAIT cells. Indeed, MAIT cell number was correlated between monozygotic and dizygotic twins at birth, was higher in females than males across the age span, and was lower in CMV seropositive individuals and active smokers[505,506,516-518,527,528]. Additionally, MAIT cell frequency in neonates was not influenced by month of birth or birth delivery method and was not correlated with mother's MAIT cell frequency[506]. Given that the gut microbiota is indispensable for MAIT cell development, it is plausible that its composition regulates the magnitude of MAIT cell peripheral expansion as well as their effector functions[485,501,502].

1.4.3 MAIT cell anti-microbial and anti-viral functions

Similar to conventional $CD8^+$ T cells, MAIT cells require three signals to acquire full effector functions necessary for eliminating pathogen-infected cells, namely TCR signaling, co-stimulatory signals and innate cytokines[480,529-531]. Innate cytokines produced upon pathogen receptor stimulation by invading pathogens serve as checkpoint to regulate MAIT cell responses against commensal bacteria[532]. MAIT cells can produce IFNγ and granzyme B in response to innate cytokines and thus contributing to anti-viral immune responses in a bystander fashion [491,533-536].

MAIT cells contribute to tissue immunity by ongoing migration from the periphery and/or acquisition of tissue-residence signature. In fact, MAIT cells are present and can rapidly expand in the liver, gut, lung, and genital mucosa upon pathogen exposure[485,490,495,519,534,536-544]. MAIT cells can contribute to mucosal immunity, as they can produce elevated levels of IL-17 and IL-22, two cytokines critical for the maintenance of barrier integrity, in the colon and female genital mucosa[540,541,545]. Further, recent evidence indicated that MAIT cells can acquire tissue-residency program in the liver and the skin, and thus they are strategically positioned in tissues and are poised to produce effector molecules critical for host defense against invading pathogens, similar to tissue-resident conventional T cells[480,546-548].

The rapid-fire nature of MAIT cell responses is integral to an effective anti-pathogen immune response. Recently, *Gutierrez-Arcelus et al*. showed that MAIT cell innate characteristics were ingrained in their gene expression[505]. The authors sequenced conventional $CD4^+$ and $CD8^+$ T cells, MAIT cells, iNKT, V-δ1, V-δ2, and NK cells and found a continuous 'innateness' gradient that separated the different immune cell subsets, with conventional T cells on one end and innate T cells on the other[505]. Over 1500 genes were associated with this innateness gradient, such as perforin, granzyme B, granulysis, CCL3, CCL4, CCL5, XCL1, XCL2, and IFNγ[505]. In fact, the sum expression of 37 cytokines and chemokines followed the innateness gradient, with higher expression in MAIT cells compared with conventional T cells[505]. In contrast, 'adaptiveness' was associated with proliferation and ribosome biogenesis[505]. POLR1D, an RNA I polymerase component involved in ribosomal RNA transcription, was associated with adaptiveness, whilst POLR2G and POLR2K, RNA polymerase II components involved in transcribing mRNA, were associated with innateness[505]. Altogether, this data suggests that T cell adaptiveness is associated with cell growth, while their innateness is associated with rapid effector functions.

1.4.4 MAIT cells in autoimmune diseases

The observation that MAIT cells express diverse chemokine receptors that effectively license them to migrate into multiple different tissues in inflammatory conditions suggests that they may respond in both the normal context and in an autoimmune disease context in a relatively antigen-agnostic fashion (as far as the organ is concerned) and mediate their effects mainly through bystander mechanisms[480,513]. However, it cannot be ruled out that certain TCRs on MAIT cells

recognize endogenous (self) antigens that can trigger a response to self. This is supported by recent evidence for a broader MR1 ligand library that extended beyond riboflavin and folate metabolites to include organic compounds as well as the finding that MR1-restricted T cells were reactive towards hydrophilic molecules in THP-1 lysate and the MR1 molecule itself[498,549,550].

The roles of MAIT cells in autoimmune disease may include both pro-inflammatory and anti-inflammatory/regulatory effects[513,551]. MAIT cells exhibit fast effector functions initiated by TCR-dependent and/or independent signaling that include release of pro-inflammatory cytokines and cytotoxic granules. While these effector functions may be protective as part of normal anti-pathogen immunity, these effects could be detrimental in the autoimmune disease context. It is conceivable that the dysregulated immune cellular networks described in MS can set in motion aberrant MAIT cell functions that contribute to local CNS inflammation and tissue injury. However, as detailed below, MAIT cells may also play acquiescing roles in the autoimmune disease context.

In type 1 diabetes, the frequency of blood MAIT cells were lower in children with recent onset disease compared to controls[552]. MAIT cells produced elevated levels of TNF, IL-4, and granzyme B, and the frequency of granzyme B-producing MAIT cells correlated with age at diagnosis and HbA1c%[552]. In NOD mice, MAIT cells seemed to have two opposing roles—maintaining gut barrier permeability by producing IL-17 and IL-22 and promoting inflammatory infiltrates into pancreatic islets. $MR1^{-/-}$ mice had a more severe diabetic disease course, suggesting that the protective role prevailed in this model[552]. This was further supported by a histopathology study, which found no evidence for MAIT cell infiltration into pancreatic islets in biopsy tissues from recent onset T1D subjects (3-9 weeks from diagnosis)[553]. In contrast, reduced peripheral blood frequencies of MAIT cells were associated with accumulation of these cells in target organs of other immune-mediated conditions, including celiac disease, inflammatory bowel disease, systemic lupus erythematosus, rheumatoid arthritis, type 2 diabetes, and obesity[554-560]. In inflammatory bowel disease, SLE, and obesity, blood MAIT cells produced elevated levels of IL-17 compared to controls[554-560].

In multiple sclerosis, MAIT cells can be found, as noted above, among immune-cell infiltrates in multiple sub-compartments within the MS CNS[419-424]. There are inconsistent reports of MAIT cell frequencies in peripheral blood of MS patients, with some studies identifying increased frequencies, while others reported no differences or decreased frequencies in the circulation of patients compared to controls[420-422,561]. The factors underlying this discrepancy in different studies are unknown.

The increased propensity of MAIT cells to produce pro-inflammatory mediators, their presence in perivascular lesions, and their sustained depletion in patients benefiting from durable disease quiescence after aggressive bone marrow transplantation in MS all suggest that $CD8^+$ MAIT T cells have a disease promoting function in MS[150,419,420]. More recently, *Mexhitaj et al.* demonstrated increased frequency and pro-inflammatory cytokine (e.g. IFNγ and IL-17 secretion) responses of blood MAIT cells in children with MS soon after their initial presentation compared to children with monophasic inflammatory demyelinating disease and normal controls[150]. The strength of studies in children compared to adults is the time proximity between biological and clinical disease onset, which reduces the influence of chronic inflammation on circulating immune cell functional phenotype[7]. Of note, it is entirely plausible that MAIT cells play different roles (i.e. both pro- and anti-inflammatory) at different stages of the disease course.

1.5 Summary

The clinical success of B cell depletion therapies targeting CD20, in spite of no changes in CSF immunoglobulin synthesis and levels, emphasizes the contribution of B cell antibody-independent functions to the pathological cascade leading to new inflammatory disease activity in MS. While B cell engagement in bi-directional interactions with $CD4^+$ T cells has been the focus of many recent studies, histopathological studies demonstrate a predominant $CD8^+$ T cell infiltrate, including a population of $CD8^+$ T cells termed MAIT cells, that may interact with B cells in the CNS of MS patients. Our lab's observation that BCDT results in reduced $CD8^+$ T cell proliferation and pro-inflammatory cytokine production further hints at *in vivo* interactions between B cells and $CD8^+$ T cells, which may be disease-relevant. However, little is known about the nature of such putative interactions which are the focus of this thesis.

Preface: Chapter 2

As part of my overarching interest in studying B cell:CD8 T cell interactions, I wished to understand whether functionally distinct B cells have differing effects on (or respond differently to) $CD8^+$ T cells. To do so, I needed to develop a technique that would enable isolation and better characterization of viable, functionally distinct B cell subpopulations. One of the important functional responses of B cells relates to their capacity to produce different cytokine profiles, which has been found to be an important mechanism by which B cells regulate $CD4^+$ T cell and myeloid cell effector functions in autoimmunity and infectious diseases. Human B cells can produce pro-inflammatory cytokines such as GM-CSF, TNF, and IL-6 as well as anti-inflammatory cytokines such as IL-10. Abnormalities in both the circulating numbers and cytokine responses of B cells have been demonstrated in several disease contexts; however, our understanding of the development and differentiation of such cytokine-defined B cells and how they may differentially impact other cells remains limited. This is due in part to a lack of validated and specific cell surface markers that allow their isolation in a pure and viable state. In this chapter, I show that the cytokine secretion assay represents an attractive approach for the detection and isolation of viable cytokine-secreting B cells from humans *ex-vivo*. I demonstrate the assay can be multiplexed, allowing for the isolation of B cells simultaneously expressing up to three distinct cytokines, including both low frequency and high frequency cytokine-secreting B cells. I further show that the cytokine-secreting B cells isolated using this approach are amenable to gene expression profiling and *in vitro* culture. Altogether, this work highlights the utility of a multiplexed cytokine secretion assay in simultaneously isolating B cell sub-populations defined by multiple different cytokines and, ultimately, providing an alternative avenue for characterizing these populations in depth using next-generation sequencing and other platforms.

Chapter 2: Simultaneous detection and isolation of multiple, viable, distinct cytokine-secreting B cells

Ayman Rezk[1,2], Rui Li[1,2], Alexandre Prat[3], Jennifer Gommerman[4] and Amit Bar-Or[1,2,5, §]

[1]Neuroimmunology Unit, Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada
[2]Center for Neuroinflammation and Experimental Therapeutics (CNET) and Department of Neurology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
[3]Neuroimmunology Research Lab, CRCHUM, Faculty of Medicine, University de Montreal, Montreal, Quebec, Canada
[4]Department of Immunology, University of Toronto, Toronto, Ontario, Canada
[5]Division of Neurology, Children's Hospital of Philadelphia, Perelman School of Medicine, University of Pennsylvania, PA, USA

[§] Corresponding Author:
Prof. Amit Bar-Or MD, FRCPC
Melissa and Paul Anderson President's Distinguished Professor
Director, Center for Neuroinflammation and Neurotherapeutics
Chief, Multiple Sclerosis Division, Department of Neurology
Perelman School of Medicine, University of Pennsylvania
Adjunct Professor of Neurology, Montreal Neurological Institute, McGill University
Perelman Center for Advanced Medicine (PCAM)
South Pavilion, 7th Floor, Rm 763
3400 Civic Center Blvd, Philadelphia, PA 19104, USA
amitbar@upenn.edu

Author contributions

AR generated all hypotheses, designed and performed all the experiments, analyzed the complete data sets and wrote the manuscript

RL assisted with human cell isolation and helpful research discussions

J.G and A.P were involved in research plan and discussion and helped with manuscript revision

A.B.O. provided the overall supervision, generated hypotheses, guided the experimental plan, analyzed the data and wrote/revised the manuscript.

Abstract

Secreted cytokines by B cells have now been shown to modulate both $CD4^+$ T cell and myeloid cell functions, with relevance in host immunity and disease pathogenesis. However, a current limitation to further functional characterization of these cytokine-secreting B cells (in the absence of specific subset-defining cell-surface markers that would enable sorting viable cells) is that that their detection using intracellular cytokine measurements results in non-viable cells. An alternative approach to isolating viable cytokine-defined B cells could be the use of a cytokine secretion assay, based on capturing cytokine secreting cells using a bi-specific cell surface antibody for CD45 and a specific cytokine, thereby persevering cell viability and integrity. However, cytokine capture assays have not been developed for simultaneous sorting of multiple viable human cytokine-secreting immune cell subpopulations, which is especially challenging for low-frequency cell subsets. We therefore set out to optimize a multi-parametric cytokine secretion assay that would address this gap. We first show that low-frequency GM-CSF^+, IL-10^+ and higher frequency TNF^+ B cells can be individually isolated using cytokine-capture assays, yielding highly purified B cell populations (confirmed by unique expression of *CSF2*, *IL-10,* and *TNF RNA,* respectively) that are viable and able to secrete high levels of their corresponding cytokines upon further activation. We demonstrate successful co-staining of a high (TNF) and low (GM-CSF or IL-10) frequency B cell subpopulations, with minimal loss of signal compared with single staining. In contrast, co-staining with two low-frequency (GM-CSF and IL-10) can result in significant competition for CD45 and loss of signal that requires careful assay optimization. Finally, we show for the first time a triple detection and isolation of TNF^+, GM-CSF^+, and IL-10^+ B cells. We conclude that the cytokine secretion assay is suitable for multi-parametric fluorescent activated cell sorting of distinct, viable, cytokine-defined B cell sub-populations for further investigations including RNA-profiling and *in vitro* functional assays.

Introduction

Cytokines have an essential role in immune cell development and function, shaping the nature and the magnitude of immune responses in health and disease[562-564]. Deleterious consequences are observed in individuals with monogenic mutations in genes encoding for molecules involved in cytokine signaling[523]. Cytokine secretion is a common feature of immune cells, such as B cells. Indeed, B cells are multifunctional and capable not only of differentiation into immunoglobulin secreting cells, but they also function as antigen presenting cells and modulators of inflammatory responses through cytokine secretion[271,332,343]. The latter function has emerged as essential for bi-directional interactions with T cells and myeloid cells and has been implicated in infectious and autoimmune diseases and their therapies[271,343].

Human B cells can secrete multiple cytokines in a context-dependent manner, including pro-inflammatory cytokines such as GM-CSF, TNF, and IL-6 as well as anti-inflammatory cytokines such as IL-10[271,343]. It is notable that certain cytokines are commonly expressed in *ex vivo* human blood B cells including IL-6 and TNF, representing high frequency cells, while others such as GM-CSF and IL-10, have a limited expression and represent low frequency circulating cells[281]. Expression of particular cytokines can be enriched in certain B cell subpopulations; however, unique cell surface markers are still lacking to distinguish them[281,377,389,392,393]. The absence of unique cell surface markers for cytokine-producing B cells has hampered further investigations into their biology including gene expression profiles, regulatory networks controlling their development and maintenance, contributions to disease states, and therapeutic manipulation. While detecting the actual cytokine/s expressed by the B cells has remained the only approach for studying these cytokine-defined B cell subpopulations[281,372,377,383,389,392,393], use of intracellular cytokine staining involves cell fixation and permeabilization which results in loss of cell viability and integrity, seriously limiting cell usage in downstream applications[565].

The cytokine-secretion assay (herein referred to as CSA) was designed to overcome this limitation and to allow for the isolation of viable cytokine-defined immune cells[566,567]. CSA relies on bispecific antibodies (bsAbs) bound to the cell surface that capture secreted cytokines on viable cells, thereby preserving cell viability and integrity, in contrast to intracellular cytokine staining[565-567]. In essence, CSA can be divided into four major steps: (1) polyclonal or antigen-specific

activation of cells; (2) labelling of cells with bsAbs that binds to cell surface antigen (CD45) and a particular cytokine; (3) secretion and capture of cytokines; and (4) detection of cytokine-secreting cells with fluorochrome-conjugated anti-cytokine antibodies for flow cytometry analysis[566,567]. However, cytokine capture assays have more typically applied to high frequency cells and, in particular, have not been used for simultaneous isolation of multiple low frequency cytokine secreting cells. The latter would be particularly beneficial when sample volumes and cell numbers are limiting, such as in many human cellular immunology contexts. In this study, we adapted CSA for the simultaneous detection and isolation of viable human cytokine-secreting immune cells, and particularly low frequency B cell subpopulations. We found that this approach allows for the simultaneous isolation of both low and high frequency cytokine secreting B cells, which have high purity and viability and are amenable to subsequent *in vitro* culture or to gene expression profiling. We also show that while simultaneous detection and isolation of up to three viable cytokine-secreting B cell subpopulations is feasible using CSA, assay optimization is required to minimize cell losses and optimize the ability to detect each subset when assessed in combination. Overall, our study provides a newly adapted multiplexing CSA approach for isolating multiple viable human cytokine-secreting immune cells that can be used for multiple applications.

Materials and Methods

Cell Isolation and culture

Fresh blood was obtained from healthy individuals recruited from the Montreal Neurological Institute and Hospital (MNI/H), McGill University, and the University of Pennsylvania. All subjects provided informed consent as approved by the corresponding institutional ethics review boards. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Ficoll-Paque density gradient centrifugation (GE Healthcare) and a strict standardized protocol[150]. B cells were selected from PBMC using CD19 microbeads (Miltenyi Biotec) according to manufacturer's recommendations. Typical purities routinely assessed by flow cytometry were >98%. B cells were cultured in serum-free X-Vivo medium (Lonza) and plated in flat-bottom 24-well plates at a concentration of $2x10^6$ cells/well in a total volume of 1000µl of medium. Cells were activated with phorbol 12-myristate 13-acetate (PMA; 20ng/ml, Sigma-Aldrich) and Ionomycin (500ng/ml, Sigma-Aldrich) for 4 hours. Golgi stop (Monensin, BD Biosciences) was added at the start of stimulation for intracellular cytokine staining (ICS).

Flow cytometry

Intracellular cytokine staining of human B cells was performed as previously described[281]. Briefly, cells were stained with LIVE/DEAD fixable Aqua dead cell stain (Thermo Fisher Scientific) for 20 minutes on ice, following which cell-surface marker staining was performed using mouse anti-human CD20 (BD Biosciences; clone: 2H7) and mouse anti-human CD3 (BD Biosciences; clone: UCHT1). Cells were then fixed and permeabilized using fixation/permeabilization buffer (BD Biosciences). Rat anti-human GM-CSF (clone: BVD2-21C11), rat anti-human IL-10 (clone: JES3-19F1), and mouse anti-human TNF (clone: MAb11) antibodies (BD Biosciences) or matching isotype controls were added, and cells were incubated for 30 minutes on ice. Cells were washed and resuspended in FACS buffer (PBS/1%FCS) until analysis on a FACS LSR Fortessa (BD Biosciences).

For the cytokine-secretion assay, cells were harvested and washed once in ice-cold MACS buffer (PBS/2mM EDTA/5%BSA). B cells were then resuspended in serum-free X-VIVO medium and labelled with capture antibodies for GM-CSF (final dilution 1:20), IL-10 (final dilution 1:10), or TNF-alpha (final dilution 1:10) (Miltenyi Biotec) for 10 minutes on ice. Cells were further diluted in pre-warmed medium at $1x10^5$ cells/ml and incubated while rotating (using a MACS rotor) at 37C°/5% CO_2 for 45 minutes. B cells were then placed on ice for 10 minutes to terminate the secretion phase. Cells were subsequently washed twice in ice-cold MACS buffer before labelling with detection antibodies for GM-CSF (final dilution 1:20), IL-10 (final dilution 1:10), or TNF (final dilution 1:10) along with mouse anti-human CD20 (clone: 2H7) and mouse anti-human CD3 (clone: UCHT1) antibodies. We employed the anti-human TNF antibody (clone: MAb11) conjugated to BUV395™ (BD Biosciences) as a detection antibody. Of note, we also validated an alternative detection antibody for GM-CSF (clone: BVD2-21C11) that performs equally well as the reagent provided in the manufacturer's kit (data not shown). Where indicated, mouse anti-human CD45 antibody (ThermoFisher Scientific; clone: HI30) was added to measure the expression of unbound CD45 following capture phase. In other experiments we used anti-human TNF antibody (MAb11; 1:50) and anti-human GM-CSF antibody (BVD2-21C11; 1:100) as alternative detective reagents. B cells were washed once in ice-cold MACS buffer and analyzed on BD LSRFortessa™ (BD Biosciences). Data analysis was performed with FlowJo Software (TreeStar).

Enzyme-linked immunosorbent assay (ELISA)
Cytokine levels in cultured supernatants were measured by OptEIA ELISA kit (GM-CSF, IL-10, and TNF; BD Biosciences) following the manufacturer's protocols.

Quantification of mRNA expression by quantitative, real-time polymerase chain reaction
Total RNA extraction was performed using RNeasy Plus Micro kit (Qiagen, Valencia, CA), following manufacturer's protocols. The RNA was stored -80C and used to generate single-stranded cDNA in a standard reverse transcription (RT) reaction using high-capacity cDNA reverse transcription kit with RNase inhibitor (Thermo Fisher Scientific). Analysis of gene expression was performed using the following TaqMan® probes: *CSF2 (Hs00929873_m1), IL10 (Hs00961622_m1), and TNF (Hs01113624_g1). 18s (Hs03003631_g1)* was used as a housekeeping gene. Fold-change calculations were performed using the –ΔΔCT method.

Statistics
All values are expressed as mean ± SEM for individual measurements made across multiple experiments. Student's paired t-test or one-way ANOVA with a Tukey post-hoc test was used for statistical comparisons between two groups. GraphPad Prism 6 was used to perform all the statistical analyses. P values of 0.05 or less were considered statistically significant.

Results

Detection of single low frequency cytokine-secreting human B cells *in vitro*
We first employed the cytokine secretion assay (CSA) in parallel with the intracellular cytokine staining (ICS) to determine its suitability for the detection of low-frequency cytokine-producing B cells. B cells isolated from PBMC were stimulated with PMA and ionomycin for 4 hours with or without monensin A for ICS and CSA, respectively. Upon stimulation, B cells produce IL-10 and GM-CSF, which can be detected by either approach (Fig.1A and B). Additionally, we found that CSA performed equally as well as ICS for the detection of IL-10$^+$ (CSA: 2.22 ± 0.59 % *vs.* ICS: 0.81 ± 0.28%, p=0.1620 Fig.1C) and GM-CSF$^+$ (CSA: 5.49± 1.18 % *vs.* ICS: 2.65 ± 0.60 %, p=0.2514 Fig.1C) B cells.

Isolation of low frequency cytokine-secreting human B cells using CSA
We next assessed the ability to sort the low-frequency cytokine-defined B cell subpopulations and their degree of enrichment following isolation. In line with published work, we found that co-

staining of IL-10 and GM-CSF in B cells by ICS revealed that IL-10$^+$ and GM-CSF$^+$ B cells were two mutually exclusive subpopulations (Fig.2a)[281]. B cells stimulated with PMA and ionomycin for 4 hours were used to sort IL-10$^+$ or GM-CSF$^+$ cells using fluorescence-activated cell sorting (FACS). We determined the purity of sorted fractions by flow cytometry, with typical enrichment confirmed as >95% (Fig.2b). As part of the purity confirmation, we found that *Il10* and *CSF2* expression were strikingly enriched in the sorted IL-10$^+$ and GM-CSF$^+$ B cells, respectively (Fig.2c). We next demonstrated that the sorted cells were able to respond to subsequent restimulation, with induction of secreted IL-10 or GM-CSF (measured by ELISA) confirmed in culture supernatants of the respectively sorted sub-populations (Fig.2d). The concordance between the *ex vivo* detection and ICS demonstration of mutually exclusive expression of IL-10 and GM-CSF, coupled with the qPCR and ELISA readouts obtained post-CSA isolation and subsequent activation of the sorted sub-populations, is reassuring and indicates that the activation used for the CSA did not modify the cytokine profile of the cells to now express cytokines that were not expressed by these cells when assessed immediately *ex vivo*. These initial experiments demonstrate that sorted low-frequency cytokine-producing B cells are of high purity and quality and can be integrated into downstream applications such as gene expression arrays, proteomic arrays, or *in vitro* cell cultures.

Simultaneous isolation of two low-frequency cytokine-secreting human B cells

We next sought to adapt the CSA for the concurrent detection and isolation of two low-frequency cytokine-secreting B cell sub-populations. Multiplexed CSA can in principle allow for efficient use of limited cell numbers obtained from rare and/or limited clinical samples. Therefore, we tested the impact of combining two capture reagents on the detection and purity of IL-10$^+$ and GM-CSF$^+$ B cells. Compared with single captured B cells, the presence of a second capture reagent resulted in slight reductions in the frequencies of IL-10 (single: 3.83 $\pm$ 0.84 % *vs.* double: 2.88 $\pm$ 0.65 %, $p<0.0001$) and GM-CSF$^+$ (single: 5.25 $\pm$ 1.26 % *vs.* double 4.28 $\pm$ 1.08%, $p=0.0139$) B cells (Fig.3a, b). In addition, the mean fluorescence intensity of either population was consistently reduced in the presence of a second capture reagent, suggesting that dual staining resulted in a reduction of the binding of cytokine-specific capture antibody on a cell basis. Multiplexing did not appear to adversely impact cell purities of the cytokine-captured sub-populations obtained post-FACS isolation (Fig.3c).

Concurrent detection of three cytokine-secreting B cell subpopulations

We next wished to assess the feasibility of simultaneous sorting of both low-frequency as well as higher frequency cytokine-defined B cell subpopulations. IL-6 and TNF are both expressed by relatively higher frequencies of circulating human B cells compared to IL-10 and GM-CSF expressing populations[281]. After first validating the detection and isolation of TNF^+ B cells using CSA (Fig.S1), we then integrated TNF capture into our multiparametric CSA to test the feasibility of simultaneous detection of three cytokine-secreting B cell subsets of differing frequencies. $IL\text{-}10^+$, $GM\text{-}CSF^+$, and TNF^+ B cells could all be detected simultaneously, as shown on t-SNE plot (Fig.4a). Next, we evaluated the impact of co-staining two or three cytokines on the detection of cytokine-positive B cells. In these experiments, we diluted cells in a final volume suitable for single TNF detection to minimize detection of non-specific cytokine-secreting cells. For IL-10, co-staining with GM-CSF (-24.02 $\pm$ 2.34 %, $p<0.0001$) or with GM-CSF and TNF (-27.89 $\pm$ 2.42 %, $p<0.0001$) resulted in greater loss of signal than with TNF (-11.29 $\pm$ 2.56%, $p=0.0046$; Fig.4b). There was a variable degree of reduction in the frequency of $GM\text{-}CSF^+$ B cells when combined with IL-10 (-22.72 $\pm$ 7.18%, $p=0.0528$), TNF (-14.31 $\pm$ 6.88%, $p=0.2381$) or IL-10 and TNF (-34.00 $\pm$ 6.31%, $p=0.0029$; Fig.4b). For TNF, co-staining with IL-10 (-14.27 $\pm$ 2.08%, $p=0.0006$) and/or GM-CSF (-20.54 $\pm$ 2.46%, $p=0.0001$) caused a modest decrease in yield, with greater loss with IL-10 and GM-CSF (-26.15 $\pm$ 3.24%, $p=0.0002$; Fig.4c). Nonetheless, $IL\text{-}10^+$, $GM\text{-}CSF^+$ and TNF^+ cell fractions isolated in a four-way FACS isolation along with triple negative cells showed excellent purity (Fig.4c) and viability (data not shown). Also, sorted and re-stimulated $IL\text{-}10^+$ and $GM\text{-}CSF^+$ cells secreted IL-10 and GM-CSF respectively, while all sorted subsets secreted TNF (Fig.4d). Thus, simultaneous detection and isolation of low and high frequency B cell subsets is feasible using CSA, with relatively greater impact on the yield of the low-frequency vs. the high-frequency subpopulations.

Discussion

Cytokine-secreting B cells, including low frequency subsets, orchestrate immune responses in infectious and inflammatory diseases and, consequently, there is a growing interest in their detection and isolation in order to better define the molecular mechanisms driving their development and functions[271,343]. Such efforts have been constrained by the lack of validated and unique cell surface markers to detect cytokine-secreting subpopulations, which has limited their isolation in a viable state suitable for downstream applications[281,377,389,392,393].

In this study, we have adapted and validated the use of cytokine-secretion assay (CSA) for the detection and isolation of viable low-and high-frequency cytokine-secreting human B cells. We have shown that CSA performed equally as ICS in the detection of cytokine-producing B cells *ex-vivo*, and could be used for the isolation of single low- and high-frequency cytokine-secreting cells. We further extended the utility of this approach by demonstrating that dual and triple simultaneous detection and isolation of distinct cytokine secreting cells could be achieved without loss of purity, though with variable reductions in cytokine detection. Sorted cytokine-secreting cells were viable, and can be integrated in other assays including gene expression analysis and *in vitro* cell culture.

We have insofar demonstrated the utility of the cytokine secretion assay by utilizing PMA and ionomycin for the induction of cytokine secretion by B cells *ex-vivo*. These compounds activate protein kinase C (PKC) and $Ca2^{+}$/calmodulin dependent signaling pathways which mobilize NFAT and its partners AP1/Fos/Jun[568]. Treatment with PMA and ionomycin can imprint a gene signature on immune cells, characterized by up-regulation of genes encoding for cytokines and chemokines as well as it can induce chromatin changes[569-571], and as such we recognize that this mode of activation may not always be optimal for certain downstream applications. Alternatively, the cytokine-secretion assay could be validated for the detection of antigen-specific B cells *ex-vivo* or upon *in vitro* expansion. In fact, *Pinder et al*. adapted the principle of this assay and developed a flow cytometry-based Ig capture assay for the detection of live antibody-secreting cells *ex-vivo* in recently immunized individuals[572]. Whether antigen-specific human B cells also produce cytokines *ex-vivo* requires further investigation.

It is arguable that the diminished signals observed upon CSA multiplexing was caused by the saturation of surface CD45. The capture reagents are bi-specific antibodies that bind to CD45 for cell anchoring, and upon saturation of CD45 by a mixture of multiple distinct (two or more) bispecific antibodies, the level of individual cytokine-capture reagent can be diminished. This can result in diminished yields but preserved purities. Titration of the capture reagents is warranted and could help optimize CSA assays developed for different combinations of cytokines, though may not entirely resolve this issue.

Proper dilution of cells in the appropriate medium during the secretion phase is important to reduce false positive and background signals. The appropriate cell concentration in this assay is dependent on the expected frequency of cytokine-secreting cells, such that higher frequencies of positive cells require greater cell dilution[566,567]. This should be kept in mind when performing dual and triple staining, as it follows that cells should be diluted according to the highest expected frequency of cytokine-secreting cells in the mixture to reduce non-specific binding during the capture phase.

In summary, we present a method for the simultaneous isolation of viable low- and high-frequency cytokine secreting human B cells and we highlight its strengths and limitations. This newly adapted and validated CSA provides an opportunity to carry out in-depth analyses of low-frequency functionally-distinct cytokine-defined immune cell subpopulations, with multiple applications including reliable RNA expression-profiling and use in a range of functional assays.

Disclosures

The authors have no financial conflicts of interest

Acknowledgments

We would like to thank all members of the Canadian B cells in MS team. We also acknowledge Camille Stegen for help with cell sorting. This study was supported by a Collaborative Team grant from the Research Foundation of the MS Society of Canada (RF-MSSC) to the Canadian B cells in MS Team (A.B.-O., A.P., and J.L.G.)

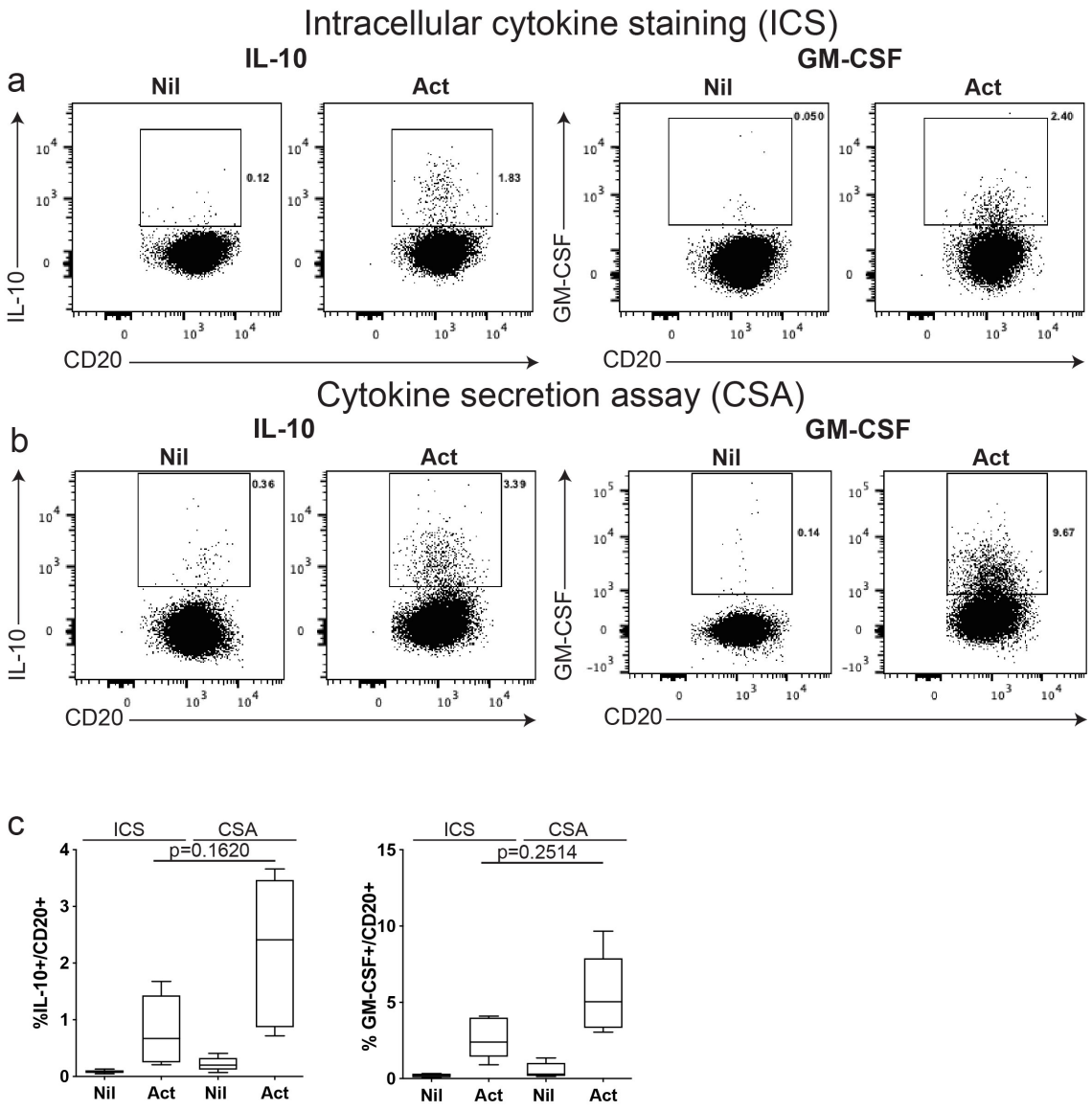


Figure 1. Cytokine expressing human B cells detected using intracellular cytokine staining versus a cytokine-secretion assay optimized for detection of low-frequency cytokine-secreting B cells. Human B cells were isolated from peripheral blood mononuclear cells (PBMC) using CD19-microbeads (purity routinely confirmed by flow cytometry as >97%). Cells were left unstimulated (Nil) or briefly activated (Act) with PMA (20ng/ml) and ionomycin (500ng/ml) for 4 hours before assessing IL-10 and GM-CSF expression by either intracellular cytokine staining (ICS) or cytokine-secretion assay (CSA). Representative FACS profiles are shown for ICS **(a)** and CSA **(b)**, with results of n=5 independent experiments summarized in **(c)**.

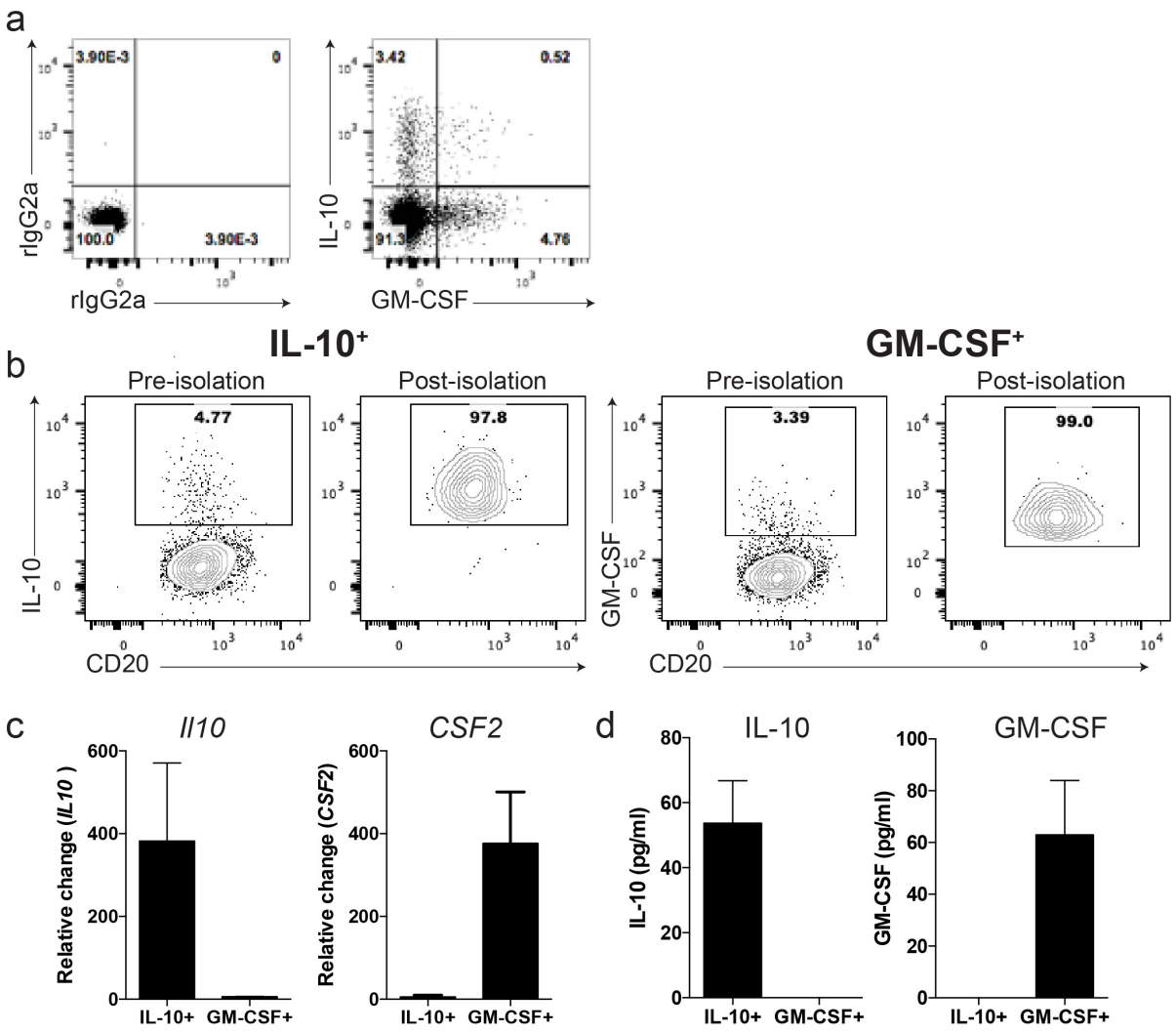


Figure 2. Validation of the cytokine secretion assay as an approach for isolating viable low-frequency cytokine-defined human B cells. Purified human B cells were activated with PMA and ionomycin for 4 hours prior to detecting the expression of either IL-10 or GM-CSF using the cytokine secretion assay. **(a)** *Ex vivo* co-concurrent intracellular cytokine staining (ICS) confirms essentially mutually exclusive expression of IL-10 and GM-CSF by distinct B cells subpopulations. **(b)** Representative cytokine-secretion assay (CSA) FACS profiles prior to and following sorting of either IL-10+ or GM-CSF+ human B cells (BD FACSAria). **(c)** RNA expression levels for IL10 (left) and CSF2 (right) measured by qPCR in freshly sorted CSA-captured and sorted IL-10+ and GM-CSF+ B cells (n=3). **(d)** Levels of IL-10 and GM-CSF secreted by viable CSA-captured and sorted IL-10+ and GM-CSF+ B cells; 5000 cells/well were

rested overnight and re-stimulated with PMA and ionomycin for 12 hours (n=3). Data shown are the mean +/- SEM (error bars). Statistical analysis carried out with paired Student's t-test. ND, non-detectable.

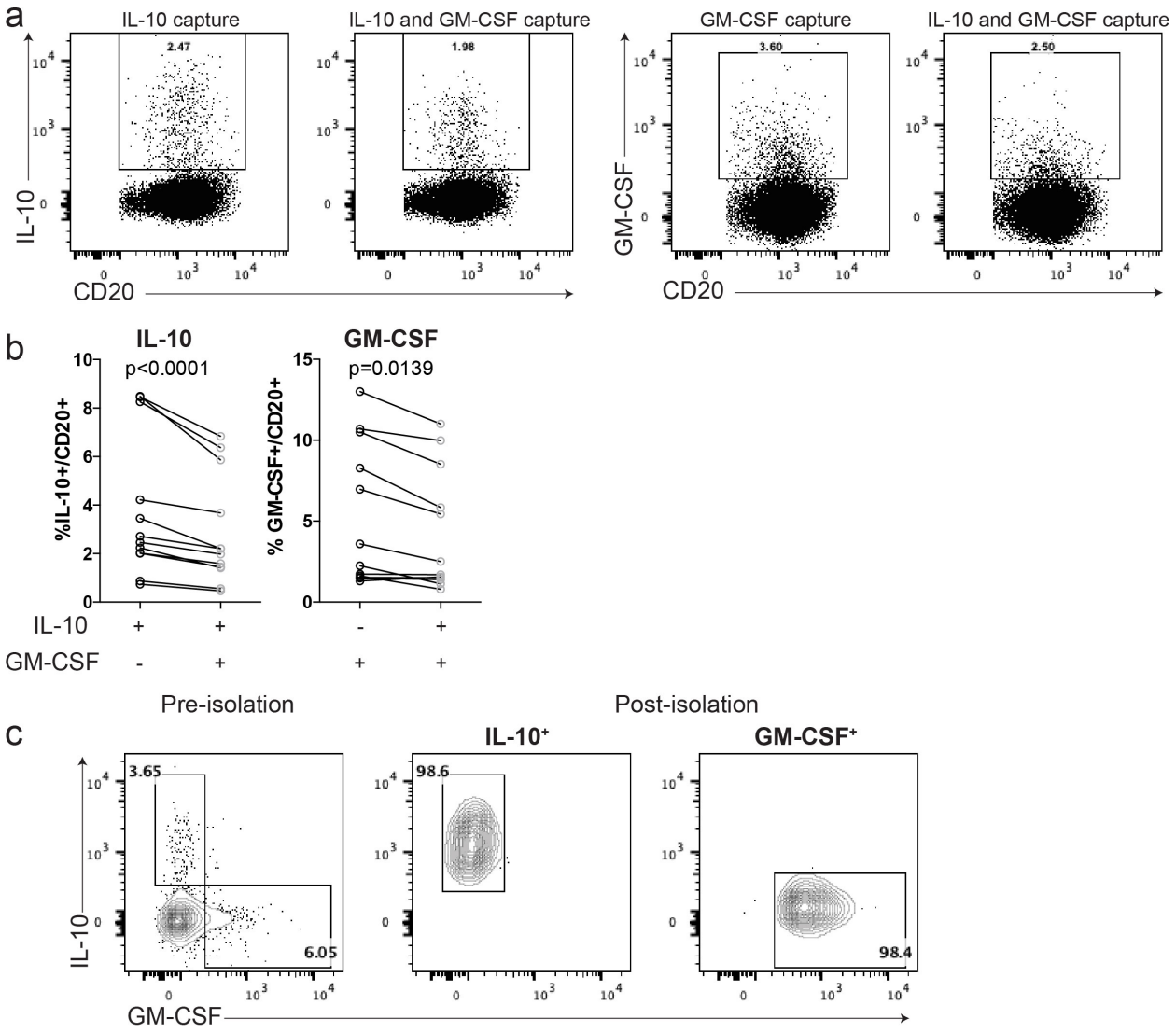


Figure 3. Concurrent isolation of two low-frequency cytokine-secreting B-cell subpopulations using the cytokine secretion assay. Human B cells were activated with PMA (20ng/ml) and Ionomycin (500ng/ml) for 4 hours before carrying out the cytokine secretion assay. The frequencies of live IL-10$^+$ and GM-CSF$^+$ cells were examined following ex-vivo B cell stimulation and either single cytokine capture (IL-10 or GM-CSF alone), or dual cytokine capture (both IL-10 and GM-CSF simultaneously); **(a)** staining from representative experiment; **(b)** summary of n=12 independent experiments. **(c)** A representative staining of IL-10$^+$ and GM-CSF$^+$ B cells, prior to and following isolation using simultaneous dual cytokine-capture. Statistical analysis carried out with paired Student's t-test.

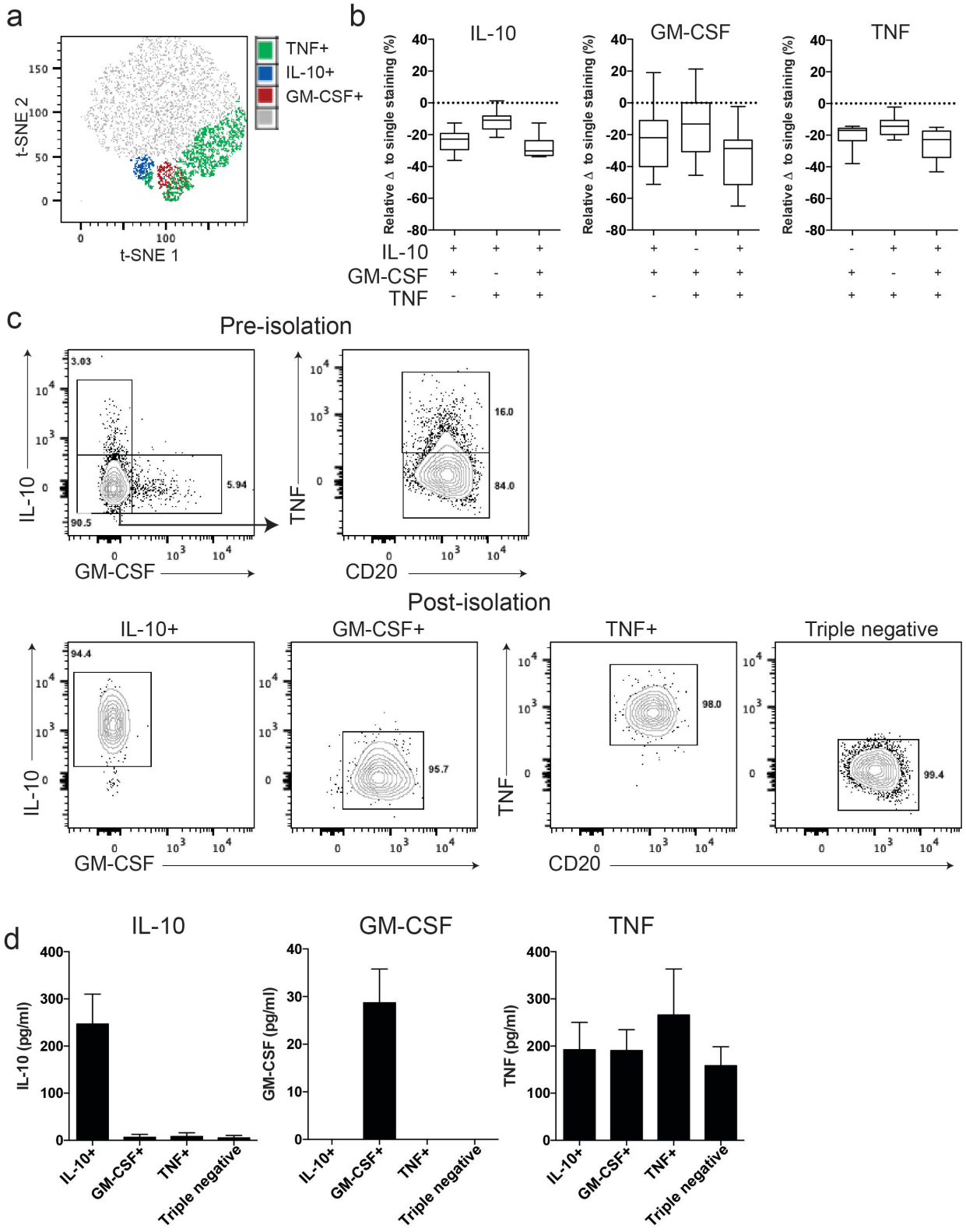
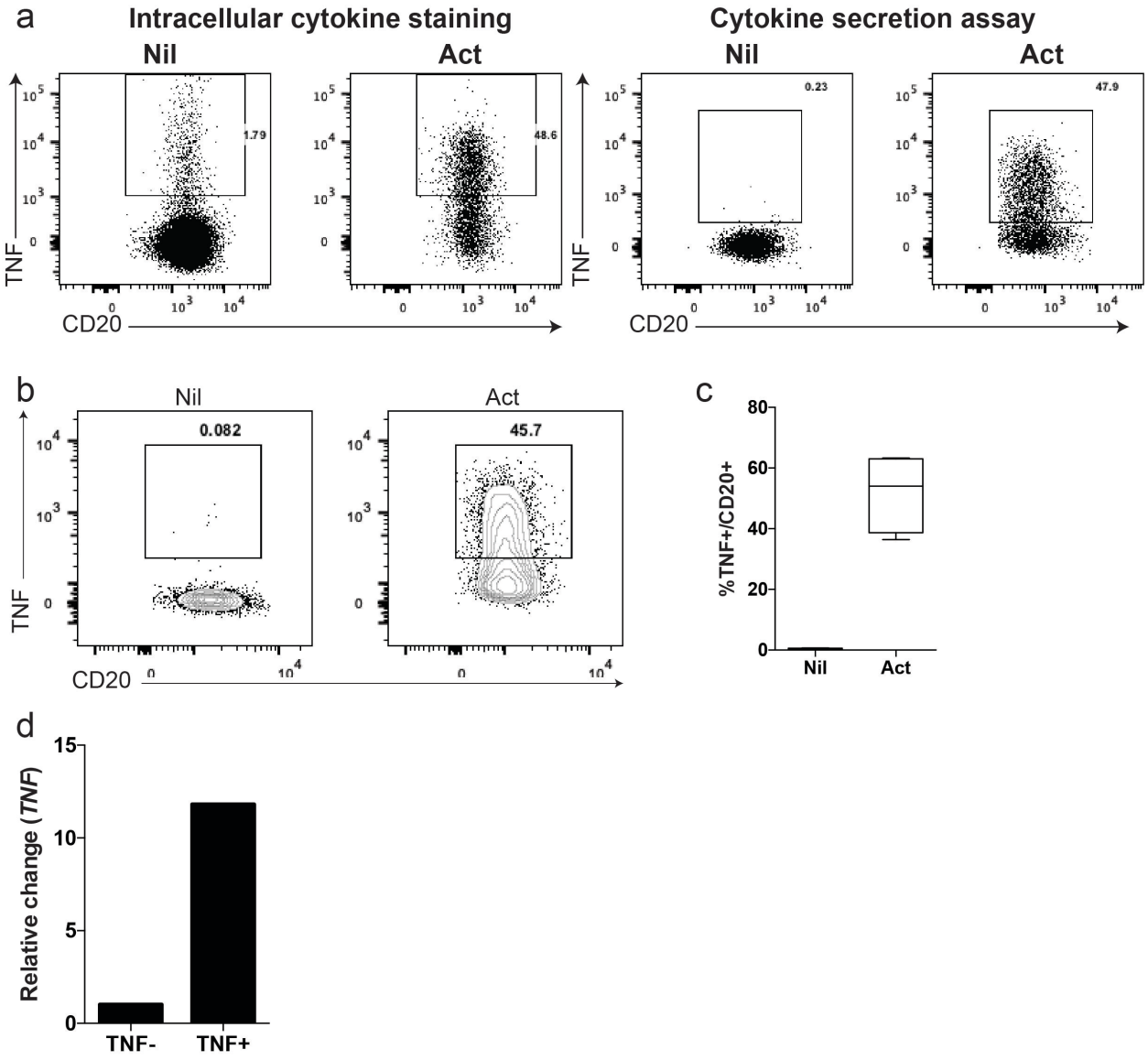

a
TNF+
IL-10+
GM-CSF+
t-SNE 2
t-SNE 1
b
IL-10
GM-CSF
TNF
Relative Δ to single staining (%)
IL-10
GM-CSF
TNF
c
Pre-isolation
IL-10
GM-CSF
TNF
CD20
3.03
5.94
90.5
16.0
84.0
Post-isolation
IL-10+
GM-CSF+
TNF+
Triple negative
94.4
95.7
98.0
99.4
d
IL-10
GM-CSF
TNF
IL-10 (pg/ml)
GM-CSF (pg/ml)
TNF (pg/ml)
IL-10+
GM-CSF+
TNF+
Triple negative

Figure 4. Simultaneous detection and isolation of three cytokine-secreting B cell subpopulations using the cytokine secretion assay. (a) Concurrent detection of three cytokine-secreting B cell populations in one sample visualized on t-SNE plot. **(b)** Relative change in the frequency of IL-10, GM-CSF and TNF in samples stained with different combinations of capture and detection reagents, as compared to individual staining (n=9). Data are shown as box with 5-95%whiskers. (c) A representative staining of IL-10^{+}, GM-CSF^{+} and TNF^{+} B cells, prior to and following isolation using simultaneous triple cytokine-capture. (d) Sorted B cell sub-populations were rested overnight and re-stimulated with PMA and ionomycin for 12 hours. Levels of secreted cytokines were assessed by ELISA. Data shown are the mean +/- SEM (error bars). Statistical analysis carried out with paired Student's t-test.



Supplementary figure 1. Detection and isolation of viable TNF-secreting B cells using the cytokine secretion assay. (a) Representative FACS profile of TNF production by B cells upon short-stimulation detected by ICS (left) or CSA (right). **(b)** B cells were left unstimulated or stimulated with PMA and ionomycin for 4 hours prior to detection of TNF-secreting B cells using CSA as shown in a representative experiment and summarized in (c, n=4 independent experiments). **(d)** RNA expression for *TNF* measured by qPCR in freshly sorted TNF^+ or TNF^- CSA-captured B cells, and represented as relative change to TNF^- (n=1).

Preface: Chapter 3

In the previous chapter, I described a cytokine-secretion assay developed and optimized for the purpose of detecting and isolating viable, functionally-distinct, low- and high-frequency cytokine-secreting B cells, which will enable in-depth characterization of these B cell subpopulations as well as assessment of their functional properties in culture. We have previously defined several abnormalities of B cells in untreated MS patients that we have implicated in the abnormal induction of pro-inflammatory responses of $CD4^+$ T cells and myeloid cells. In keeping with these *ex-vivo* and *in vitro* observations, we have also shown that B-cell depletion with anti-CD20 monoclonal antibody, which is very effective at limiting new MS activity, diminishes $CD4^+$ T cell and myeloid cell pro-inflammatory responses in patients. An intriguing observation is the parallel reduction in $CD8^+$ T cell pro-inflammatory cytokine responses in the same treated patients. This suggests that B cells and $CD8^+$ T cells can interact *in vivo* and raises the possibility that such interactions are relevant to MS pathophysiology and possibly also to the therapeutic effect of ani-CD20 therapy in MS. Both conventional $CD8^+$ T cells as well as mucosal-associated invariant T (MAIT) cells have been identified within the MS brain where they are clonally-expanded and express pro-inflammatory/effector molecules. My overarching hypothesis is that human B cells can interact with and modulate $CD8^+$T cells in ways that will explain the diminished $CD8^+$T cell responses seen in patients undergoing B cell depletion. Surprisingly little is known about the potential for human B cell: $CD8^+$T cell interactions, and it is essentially unknown whether and how functionally distinct B cells will have a different impact on $CD8^+$T cells or on their subsets. My work on this part of the project integrates a series of observations from *in vitro* experiments demonstrating that differentially activated human B cells can have a distinct impact on human conventional versus innate-like $CD8^+$ T cells. I further demonstrate the *in vivo* consequences that B cell depletion has on $CD8^+$T cell subsets in patients with MS. Together, my results provide novel fundamental insights into the complexity of potential interactions between functionally distinct human B cell subsets and distinct $CD8^+$T cell subsets and point to *in vivo* interactions between B cells and $CD8^+$T cells that appear to be relevant to disease pathophysiology and potentially to therapeutic targeting of such interactions in patients with MS.

Chapter 3: B cells regulate CD8$^+$ MAIT cell effector functions: implications for multiple sclerosis

Ayman Rezk[1,2,4], Koji Shinoda[2], Rui Li[1,2], and Amit Bar-Or[1,2,3,4 §]

[1] Department of Neurology, Perelman School of Medicine, University of Pennsylvania, PA, USA
[2]Center for Neuroinflammation and Experimental Therapeutics (CNET) and Department of Neurology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
[3]Division of Neurology, Children's Hospital of Philadelphia, Perelman School of Medicine, University of Pennsylvania, PA, USA
[4] Neuroimmunology Unit, Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada

[§] Corresponding Author:
Prof. Amit Bar-Or MD, FRCPC
Melissa and Paul Anderson President's Distinguished Professor
Director, Center for Neuroinflammation and Neurotherapeutics
Chief, Multiple Sclerosis Division, Department of Neurology
Perelman School of Medicine, University of Pennsylvania
Adjunct Professor of Neurology, Montreal Neurological Institute, McGill University
Perelman Center for Advanced Medicine (PCAM)
South Pavilion, 7th Floor, Rm 763
3400 Civic Center Blvd, Philadelphia, PA 19104, USA
amitbar@upenn.edu

Author contributions

AR generated all hypotheses, designed and performed all the experiments, analyzed the complete data sets and wrote the manuscript

KS assisted with data generation

RL assisted with data generation and helpful research discussions

A.B.O. provided the overall supervision, generated hypotheses, guided the experimental plan, analyzed the data and wrote/revised the manuscript.

Abstract

B cells carry out antibody-independent functions that serve to orchestrate other cellular immune responses, important for both host protective immunity and in the context of inflammatory diseases. Among these, bi-directional interactions between B cells and $CD4^+$ T cells have been well-described. Indeed, pro-inflammatory $CD4^+$ T cell responses are diminished by depleting B cells with anti-CD20 antibody, which is highly effective at limiting new disease activity in patients with multiple sclerosis (MS). However, initial studies in patients undergoing the same *in vivo* B cell depletion indicated that $CD8^+$ T cell function may also be affected, suggesting *in vivo* interactions take place between these two immune cell subsets and that such interactions may be relevant to the pathophysiology and potential therapeutic targeting in the context of autoimmune disease. Here we set out to study potential interactions between B cells and both conventional $CD8^+$ T cells (non-MAIT) and innate-like $CD8^+$ T cell cells known as mucosal-associated invariant (MAIT) cells. We find that, *in vitro*, human B cells can suppress the proliferation of conventional $CD8^+$ T cells yet significantly promote MAIT cell expansion both polyclonally and at the antigen-specific level. This differential B cell effect on $CD8^+$ T cell subsets is rather dependent on the differentiation stage and activation of the B cells. Activated B cells also differentially enhanced $CD8^+$ T-cell subset cytotoxicity, an effect that did not require cell:cell contact, as it could be mediated through B-cell secreted products. B cells further modulated cytokine production in $CD8^+$ T cells by selectively enhancing IFNγ production by MAIT cells, while suppressing GM-CSF and TNF production by non-MAIT cells. Finally, B cell depletion using anti-CD20 monoclonal antibody in patients with MS resulted in an *in vivo* shift in the profile of circulating $CD8^+$ T cells, including a reduction in MAIT cells and diminished $CD8^+$ T cell pro-inflammatory functions including cytokine production, that mirrored aspects of our *in vitro* findings. Together, these findings reveal the capacity of activated human B cells to reciprocally impact distinct $CD8^+$ T cell subsets and indicate that *in vivo* cross-talk between B cells and $CD8^+$ T cells may be relevant to inflammatory disease activity. The particular capacity of B cells to induce activation and effector responses of MAIT cells may be especially relevant to MS pathophysiology and possibly to the therapeutic mode of action of anti-CD20 therapy in patients.

Introduction

B lymphocytes are multifunctional cells with a capacity to secrete immunoglobulins, present antigens, and produce cytokines[271,332,343]. The latter two functions are essential for bi-directional interactions between B cells and $CD4^+$ T cells as well as with invariant natural killer T (iNKT) cells[271,343,573,574]. B cells can produce pro- as well as anti-inflammatory cytokines depending on integration of signals downstream of B-cell receptor (BCR), co-stimulatory CD40L, toll-like receptors, and cytokine receptors [281,372,383,391,393]. This integration allows B cells to acquire inflammatory or suppressive (regulatory) functions in a context-dependent manner[271,343]. Accordingly, B cell depletion or the manipulation of cytokine production by B cells can modulate functions of different cells such as $CD4^+$ T cell subsets, inducing them to exhibit enhanced or diminished inflammatory responses[271]. B cell:CD4 T cell interactions appear to have notable *in vivo* relevance for infectious diseases, autoimmune diseases, and other inflammatory conditions[281,282,342,360,372,382]. How responses of $CD8^+$ T cells and their subsets may be modulated by B cells, and what the relevance of such modulation may be in the context of human disease and therapeutics, remain poorly-defined[382,575-578].

$CD8^+$ T cells are phenotypically and functionally heterogeneous. Among $CD8^+$ T cells, a subset known as mucosal-associated invariant T (MAIT) cells is emerging as relevant for host defense against pathogens and inflammatory diseases[480,513,551]. MAIT cells are an innate-like T cell subset expressing TRAV1 (TCR-Vα7.2) and a restricted TCR-β repertoire that recognize conserved microbial antigens derived from vitamin B metabolites and presented on MHC-related protein 1 (MR1)[485,492,493,497,508]. In this regard, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) is a MAIT cell activating antigen that is commonly used as part of the MR1-tetramer to identify MAIT cells[493,542]. Upon antigen recognition, MAIT cells release pro-inflammatory cytokines and cytotoxic granules necessary for the elimination of pathogen-infected target cells[480,529]. Their transcriptome is distinct from conventional $CD8^+$ T cells, yet similar to iNKT cells, with enhanced expression of genes encoding for cytokines, cytotoxic molecules, and chemokine receptors[505,511]. In humans, MAIT cells circulate in the blood, representing 0.1 to 15% of $CD8^+$ T cells, and traffic to the liver, lung, and gut, where they can acquire tissue-residence signature[502,505,507,510]. MAIT cells have also been implicated in autoimmune disease, including multiple sclerosis, where they have been found to be clonally expanded and expressing pro-

inflammatory effector molecules within the inflamed central nervous system of patients[150,419,420,422,423,561].

Since B cells have been known to engage in productive bi-directional interactions with iNKT cells, serving to modulate iNKT effector functions[573,579-583], and given the similarities between iNKT and MAIT cell transcriptional profiles[505,507], it is tempting to speculate that B cells and MAIT cells may also be 'interacting partners' *in vivo*. In this study, we sought to investigate the interactions between human B cells and both conventional and innate-like $CD8^+$ T cell subsets using two complementary approaches: a series of *in vitro* experiments assessing interactions between functionally distinct human B cells and $CD8^+$ T cell subsets and *ex vivo* functional immunophenotyping of $CD8^+$ T cells within peripheral blood mononuclear cells (PBMC) obtained from MS patients prior to and after *in vivo* B cell depletion therapy (BCDT) with anti-CD20 antibody. We hypothesized that B cells can stimulate MAIT cell pro-inflammatory functions, which would then be attenuated following B cell depletion. Our study highlights the capacity of B cells to differentially regulate MAIT and conventional non-MAIT cell pro-inflammatory responses *in vitro* and *in vivo*.

Materials and methods

Human subjects and ethics approval

All subjects provided written informed consent as approved by the Montreal Neurological institute and Hospital Ethics Review Board and the University of Pennsylvania Institutional Review Board. Normal controls were recruited at the Montreal Neurological institute and University of Pennsylvania. Ocrelizumab-treated patients were recruited at the Hospital of the University of Pennsylvania. Patient demographics are described in supplemental table 1.

Cell isolation

Peripheral blood mononuclear cells were isolated from whole blood by Ficoll-Paque density gradient centrifugation (GE Healthcare), as described previously[150]. B cells were positively selected using CD19 microbeads (Miltenyi Biotec), and $CD8^+$ T cells were negatively selected using CD8+ T cell isolation kit (Miltenyi Biotec) according to manufacturer's instructions. Typical post-isolation purities assessed by flow cytometry were >95% (Figure S1).

B cell and T cell activation

Isolated CD8+ T cells were labelled with CellTrace Violet (ThermoFisher Scientific) to monitor cell proliferation, resuspended in X-VIVO medium (Lonza) and seeded at 1 x 10^5 cells per well in U-bottom 96-well plates. In parallel, isolated B cells were either left unstimulated or activated overnight (16-18 hours) with either of three activation conditions: CpG DNA (1uM, ODN2006; InvivoGen) alone, CD40L (1ug/ml; Enzo Life Sciences) alone, or a combination of CD40L with goat anti-human BCR F(ab)'2 fragment antibody (10ug/ml; Jackson ImmunoResearch Laboratories) and recombinant IL-4 (20ng/ml, R&D systems). B cells were then harvested and washed in X-VIVO medium prior to co-culture with autologous T cells, where they were added into co-culture at 2x10^5 cells per well in a total culture volume of 200ul. Isolated T cells were polyclonally activated with beads coated with anti-CD2, anti-CD3, and anti-CD28 antibodies (Miltenyi Biotec). Where indicated, neutralizing antibodies targeting IL-10 (JES3-19F1, 2.5ug/ml), TGF-R (1D11, 2.5ug/ml), IL-12p35 (27537, 2.5ug/ml), (all from R&D systems), PD-L1 (29E.2A3, 2.5ug/ml) (Biolegend), and matching isotype controls were added to cell culture. Recombinant IL-2 (1-100U/ml; Sigma Aldrich) was used where indicated.

Flow cytometry

Expression levels of T cell and B cell surface and intracellular proteins were quantified by flow cytometry using fluorochrome-conjugated antibodies: CD3 (SK7, 1:50), CD8 (RPA-T8, 1:50), CD20 (2H7, 1:20), CD25 (M-A251, 1:100), CD27 (M-T271, 1:50), CD38 (HIT2, 1:20), CD69 (FN50, 1:20), CD71 (M-A712, 1:20), CD107a (H4A3, 1:40), CD161 (REA631, 1:200), CD138 (clone, 1:50), IL-2 (MQ1-17H12, 1:50), IL-6 (MQ2-6A3, 1:400), IL-10 (JES3-19F1, 1:50), IFNγ (B27, 1:50), TNF (MAb11, 1:400), GM-CSF (BVD2-21C11, 1:400), granzyme B (GB11, 1:400), perforin ($\delta G9$, 1:50), and TCR-Va7.2 (3C10, 1:50). Phorbol 12-myristate 13-acetate (PMA; 10ng/ml; Sigma Aldrich), ionomycin (1ug/ml; Sigma Aldrich) and Golgi Stop (monensin; BD Biosciences) were added on day 3 for the last 5 hours of culture in order to detect intracellular cytokine production. CD107a and Golgi Stop were added on day 3 for the last 5 hours of culture to quantify cell degranulation. Cells were harvested and stained with a viability dye (Live/Dead fixable aqua dead cell stain, ThermoFisher Scientific) as per manufacturer's instructions. Where indicated, MR1 tetramers loaded with 5-OP-RU or 6-Ac-FP were used to confirm the identity of MAIT cells. Cell surface staining using pre-mixed antibody cocktails was performed at room temperature for 30 minutes, with the use of Brilliant buffer (BD Biosciences) to mitigate non-

specific interactions among brilliant violet conjugated antibodies. Cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences). Intracellular molecules were stained using pre-mixed antibody cocktails for 30 minutes on ice. Cells were resuspended in PBS prior to flow cytometry data acquisition on LSRFortessa. Data analysis was carried out using FlowJo (Tree Star).

Enzyme-linked immunosorbent assay (ELISA)

Levels of secreted cytokines in culture supernatants were measured by OptiEIA ELISA kits for IFNG, GM-CSF, TNF, IL-10, and IL-6 (all from BD Biosciences), according to the manufacturer's instructions.

PBMC immunophenotyping prior to and following B cell depletion

PBMC were isolated from whole blood obtained from MS patients prior to and on-treatment (3 months post-treatment) with the anti-CD20 monoclonal antibody ocrelizumab. PBMCs were then cryopreserved using standardized operating procedures developed and validated for all steps of sample procurement, processing, cryopreservation, storage, and thawing[150]. Thawed PBMC samples with cell viability of less than 70 % were excluded from analysis. Functional immunophenotyping was performed in batch by a blinded operator. Each batch included pre- and on-treatment PBMC samples and an internal control sample used consistently across batches. Multiparameteric flow cytometry panels were developed and validated on fresh and cryopreserved PBMC before their application to the patient cohort (Supplemental Tables 2 and 3).

Statistical analysis

All values were expressed as the mean measurements across experiments $\pm$ standard errors of the means. Statistical testing across groups employed as appropriate either one-way ANOVA with a Dunn's post-hoc test, two-way ANOVA with Tukey's post-hoc test or two-tailed ratio paired t test, using GraphPad Prism version 6. P values less than 0.05 were considered statistically significant.

Results

Human B cells differently impact $CD8^+$ T cell subset proliferation

To examine potential interactions between human B cells and $CD8^+$ T cells, we set up an *in vitro* co-culture system, wherein autologous polyclonally activated $CD8^+$ T cells were co-cultured with unstimulated B cells or with B cells pre-activated with either CpG (toll-like receptor 9 agonist),

CD40L, or a combination of CD40L, BCR-cross-linking antibody, and IL-4 (herein referred to as 40X4). These stimuli have been previously shown to induce distinct human B cell cytokine profiles and cell surface phenotypes[281-283]. We defined conventional MAIT cells as $CD3^+CD8^+$ T cells that were $CD161^{hi}$ TCR-Vα7.2$^+$ and defined non-MAIT cells as $CD3^+CD8^+$ T cells that were $CD161^{-/int}$ TCRVα7.2$^{+/-}$. As expected, *ex vivo* $CD161^{hi}$ TCRVα7.2$^+$ $CD8^+$ T cells recognized 5-OP-RU presented on MR1 tetramers, whilst $CD161^{-/int}$ TCRVα7.2$^{+/-}$ cells did not (Fig.S2). Upon activation, MAIT cells proliferated less vigorously than conventional non-MAIT cells (Fig.1a), which may reflect a higher TCR threshold and/or a requirement for additional co-stimulatory signals to enter the cell cycle[505,584-586]. We found that pre-activated B cells efficiently suppressed conventional non-MAIT cell proliferation, while they significantly enhanced MAIT cell proliferation (Fig.1a-b). The B cells exerted suppression of non-MAIT cell proliferation across a range of strengths of T cell stimulation and across a range of B cell to T cell ratios, with stronger suppression observed at early time points in the co-culture (Fig.1c-e). Unstimulated B cells also suppressed non-MAIT cells, albeit they were less potent than pre-activated B cells. In sharp contrast, unstimulated and pre-activated B cells stimulated MAIT cell proliferation with greater strength of T cell stimulation and with higher B cell: T cell ratios, effects that were observed at later time points in the co-culture (Fig.1c-e). In contrast to earlier studies, we found that MAIT cells were not more prone to cell apoptosis than non-MAIT cells upon activation (data not shown)[526,587] and that B cell effects on $CD8^+$ T cell subset proliferation were independent of any changes in $CD8^+$ T cell survival (data not shown).

While these initial experiments suggested that B cells have contrasting effects on non-MAIT versus MAIT cell proliferation, MAIT cells are known to be effector memory cells, while the non-MAIT cells included both naïve and memory $CD8^+$ T cells. For a better comparison, we therefore examined the impact of B cells on sorted MAIT cells and sorted naïve ($CCR7^+CD45RO^-$), effector memory ($CCR7^-CD45RO^+$), and terminal effector ($CCR7^-CD45RO^-$) $CD8^+$ T cell subsets (Fig.S3). We found that pre-activated B cells suppressed naïve T cell proliferation and enhanced MAIT cell proliferation (Fig.2a-c), while they did not consistently modulate terminal effector and effector memory proliferation (Fig.2a-c).

Human B cells are functionally heterogeneous, with naïve and transitional B cells in particular possessing regulatory functions[271,283,377]. To determine whether the capacity of B cells to differentially modulate $CD8^+$ T cell subset proliferation was similar for all B cell subsets, we isolated transitional ($CD24^{hi}CD38^{hi}$), naïve ($CD27^-IgD^+$), class-switched memory ($CD27^+IgD^-$), and non-class switched memory ($CD27^+IgD^+$) B cells by FACS and co-cultured them with autologous $CD8^+$ T cells. We observed that while all B cell subsets enhanced MAIT cell proliferation, the naïve and transitional B cells were more effective than the memory B cell subsets at suppressing non-MAIT T cell proliferation (Fig.S4). Our data highlights functional heterogeneity among conventional and innate-like T cells that can be differentially regulated by B cells.

Human B cells enhance $CD8^+$ T cell cytotoxic potential

MAIT cells, similar to conventional $CD8^+$ T cells, are poised to release cytotoxic granules in a TCR dependent and/or independent manner[491,529,533]. We sought to extend our initial findings on $CD8^+$ T cell proliferation and examined the capacity of B cells to modulate non-MAIT and MAIT $CD8^+$ T cell cytotoxic phenotypes *in vitro*. Activated non-MAIT and MAIT cells markedly up-regulated the expression of granzyme B, perforin, and CD107a (Fig.3a-c). B cells pre-activated with CpG induced non-MAIT cell degranulation and granzyme B expression but not perforin expression (Fig.2a-c). In contrast, all pre-activated B cells amplified MAIT cell cytotoxic potential (Fig.2a-c). Further, soluble factors present in supernatants of B cells could recapitulate the changes in non-MAIT and MAIT cell cytotoxicity observed in co-culture (data not shown). Altogether, our data reveal that B cells can regulate $CD8^+$ T cell subset cytotoxicity in a context-dependent manner.

Human B cells modulate $CD8^+$ T cell cytokine production

We next assessed the levels of secreted cytokines in culture supernatants and found substantially increased IFNγ and decreased GM-CSF levels in co-culture supernatants of $CD8^+$ T cells and B cells pre-activated with CpG (Fig.4a-b), with minor reduction in TNF levels in co-culture supernatants of CD8+ T cells and B cells pre-activated with CD40L or 40X4. We next employed intracellular cytokine staining to examine the production of these cytokines in $CD8^+$ T cell subsets. Whilst the frequency of IFN-γ^+ CD8+ T cell subsets did not significantly differ upon co-culture with B cells (data not shown), the mean fluorescence intensity of IFN-γ^+ non-MAIT and MAIT cells was significantly increased in co-culture with CpG-primed B cells (Fig.4d-e). This data

combined with the proliferative response of non-MAIT cells demonstrate a dissociation between these two effector functions, suggesting that non-overlapping mechanisms of regulation are exerted by B cells on non-MAIT cells. It is notable that MAIT cells express higher levels of IFN-γ than non-MAIT, suggesting a greater contribution to overall secreted levels. Further, the frequency of GM-CSF-producing non-MAIT cells, and to a lesser extent MAIT cells, was decreased in co-culture with pre-activated B cells (Fig.4f-g). A similar trend of change was observed for TNF (Fig.4h-i). Altogether, our data suggests that B cells can differentially regulate cytokine production by non-MAIT and MAIT cells.

B cell depletion therapy shifts the distribution of blood $CD8^+$ $CD161^-$ and MAIT cells

To validate our *in vitro* observations on the effects of B cells on $CD8^+$ T cell functional responses, we turned to peripheral blood mononuclear cells (PBMC) obtained from patients with multiple sclerosis treated with B cell depletion therapy (BCDT, ocrelizumab). BCDT has demonstrated significant clinical efficacy in reducing new disease activity in patients with multiple sclerosis[273,274]. Samples collected prior to and after treatment initiation were analyzed using validated multi-parametric flow cytometry panels that included relevant cell surface markers, cytokines, and cytotoxic molecules for use on cryopreserved PBMC (Table 2 and 3). The gating strategy used in this analysis is presented in Fig.S5.

Analysis of major T cell subsets revealed that B cell depletion did not impact the absolute cell counts of circulating $CD8^+$ T cell subsets, including naïve, central memory, effector memory and terminal effector cells (Fig.5a-d and summarized in table 4), though we observed a minor shift in $CD8^+$ T cell subset distribution with an increased frequency of naïve T cells (41.52 ± 7.51 pre- to 48.33 ± 7.80 on-treatment, p=0.0076, table 5) and a decreased frequency of effector memory T cells (25.68 ± 4.40 pre- to 21.65 ± 4.06 on-treatment, p=0.0105, table 5). Further, and in line with our *in vitro* $CD8^+$T cell proliferation data, we found that B cell depletion resulted in a decreased absolute cell counts (Fig.5f) and frequency of MAIT cells (4.78 ± 0.84 pre- to 3.85 ± 0.71 on-treatment, p=0.0472, table 5).

CD8$^+$ T cell pro-inflammatory cytokine production is diminished after B cell depletion therapy

We next examined the expression of pro-inflammatory cytokines and cytotoxic molecules expressed by non-MAIT (CD161$^{-/int}$ TCR-Vα7.2$^{+/-}$) and MAIT (CD161^{hi} TCR-Vα7.2$^+$) CD8$^+$ T cells. *Ex vivo*, MAIT cells produced higher levels of cytokines (TNF, IFNγ, and GM-CSF) compared to non-MAIT cells, but were mostly devoid of granzyme B and perforin (Fig.S5) as expected. The absolute cell counts and frequencies of granzyme- and perforin-expressing non-MAIT and MAIT cells were unchanged after B cell depletion (Fig.S6, table 4-5). Further, B cell depletion was associated with a reduction in the absolute cell counts of IFN-γ^+ (Fig.6a), GM-CSF$^+$ (Fig.6b) and TNF$^+$ (Fig.6c) MAIT cells, whilst only the numbers of GM-CSF$^+$ (Fig.6b) and TNF+ (Fig.6c) non-MAIT were reduced. Overall, our data indicate that B cell depletion results in reduced CD8$^+$ T cell subset pro-inflammatory cytokine production.

Discussion

In this study, we examined the capacity for human B cells to regulate CD8$^+$ T cells functions both *in vitro* and *in vivo*. Results of prior studies addressing interactions between B cells and CD8$^+$ T cells, mostly carried in animal systems, have yielded mixed reports. In fact, some studies highlighted B cell regulatory capacity that could suppress CD8$^+$ T cell effector functions, and others found an activating role for B cells, such as through MHC-I-dependent antigen presentation that enhanced CD8 T-cell functions[382,575-578,588]. Our *in vitro* studies using an autologous human system demonstrated the capacity for B cells to differentially impact distinct CD8$^+$ T cell subsets. B cells could induce substantial activation of MAIT cells, resulting in their enhanced proliferation, cytotoxicity, and IFNγ expression. In contrast, B cells substantially and consistently suppressed proliferation of conventional CD8$^+$ T cells, while enhancing their cytotoxicity under a limited set of conditions. By examining the effects of removing B cells *in vivo* in MS patients undergoing anti-CD20 therapy, we identified changes in circulating CD8$^+$ T cell subsets that corresponded with our *in vitro* findings. In particular, the demonstration that MAIT cell numbers and frequencies decreased in patients following the removal of B cells was consistent with the capacity we discovered for B cells to selectively activate and induce MAIT cell expansion. Given the highly pro-inflammatory nature of MAIT cells, we speculate that decreased peripheral activation of MAIT cells may represent a relevant aspect of the therapeutic mode-of-action of anti-CD20 therapy which has proven highly effective at limiting new MS relapses[348].

MAIT cells express diverse chemokine receptors that effectively license them to migrate into multiple different tissues under inflammatory conditions[480,513]. This enhanced migratory capacity suggested that they may become activated and relatively agnostic to the target organ or its unique antigens, and possibly mediate their effects principally through bystander mechanisms[480,513]. However, it may well be that certain TCRs on MAIT cells recognize endogenous (self) antigens that can trigger a response to self, given recent findings for a broader MR1 ligand library that extended beyond the initially identified riboflavin and folate metabolites and included other organic molecules [498,549,550]. Of interest in this regard are our observations that B cells could enhance MR1-restricted MAIT cell expansion, while suppressing the expansion of MR1-restricted non-MAIT cells. These findings extend a recent report that B cells can present antigen to MAIT cells in an MR1-restricted fashion[538] with the demonstration of contrasting effects of the B cells on different MR1-restricted $CD8^+$T cells. These observations support a model wherein B cells have the capacity to regulate $CD8^+$T cell proliferation while discerning among $CD8^+$ T cells even with similar antigenic specificity, presumably based on one or more other yet to be determined functional and/or phenotypic characteristic/s of the $CD8^+$ T cells.

Consistent with prior reports, we also noted a reduced capacity of MAIT cell to undergo TCR-driven proliferation compared to non-MAIT cells[505,584]. This may be explained by results of gene expression analysis of MAIT cells that found they expressed reduced levels of signaling molecules downstream of the TCR and were transcriptionally biased towards higher expression of effector molecules and lower expression of genes encoding for ribosomal proteins necessary for cell growth[505,584]. We also found that B cells could provide soluble growth factors to MAIT cells that acted in conjunction with high TCR strength of stimulation to further promote cell expansion. The co-stimulatory signals that promote MAIT cell expansion remain poorly understood. Innate cytokines can drive $CD8^+$ T cell proliferation in the absence of TCR signaling and can also serve to reduce the TCR threshold required for MAIT cell cycle entry[491,584-586,589,590]. Studies in patients with primary immunodeficiencies highlighted the importance of the IL-23-STAT3 axis in maintaining peripheral MAIT cell number and IFNγ production[521-523,525,591]. In a preliminary analysis, we found that B cells, particularly those pre-activated with CpG, selectively enhanced STAT3 phosphorylation in MAIT cells (data not shown). IL-23 expression in B cells has not been

reported, but like other cytokines expressed by these cells, the context of their interaction with other immune cells may be critical for its induction.

There have been inconsistent reports of MAIT cell frequencies in peripheral blood of adult MS patients (some identifying increased frequencies, with others reporting no differences or decreased frequencies compared to controls)[420-422,561]. While the factors underlying these discrepancies are not clear, they have raised questions about whether MAIT cells play pro-inflammatory or anti-inflammatory roles in MS. Our results, based on assessment of B cell: MAIT cell interactions using a combination of *in vitro* co-culture experiments and examination of the effects of *in vivo* B cell depletion on MAIT cell biology, support a pro-inflammatory role for MAIT cells in the pathophysiology of MS disease activity. These findings are consistent with the known propensity of MAIT cells to produce pro-inflammatory and cytotoxic mediators[480,529], the documented presence of clonally expanded MAIT cells within the perivascular inflammatory lesions of MS brains[419-423] (where MR1 and innate cytokines are expressed)[422], and their sustained depletion in patients benefiting from durable disease quiescence after aggressive bone marrow transplantation in MS[419]. It is noteworthy that we have recently observed increased frequency and pro-inflammatory cytokine (e.g. IFNγ and IL-17) responses of circulating MAIT cells in children developing MS soon after their initial presentation compared to children with monophasic inflammatory demyelinating disease and normal controls[150], which, taken together, further supports a pro-inflammatory role for MAIT cells in the disease.

MAIT cells are known to accumulate in mucosal tissues (such as liver, lung, and gut, where they strategically positioned to mediate early and efficient immune response against invading pathogens)[480,513]. Why MAIT cells would then migrate to the CNS, is not clear. It is noteworthy that MAIT cells may interact with IgA^+ plasma cells that express MR1[592]. The significance of such interaction is unknown, though in the context of neuroinflammation, *Rojas et al.* recently demonstrated that gut lamina propria IgA^+ plasma cells/plasamblasts could migrate to the central nervous system where they exerted IL-10-dependent suppressive functions that reduced local inflammation[391]. Whether MAIT cells follow similar trafficking patterns as IgA+ plasma cells, including a gut-CNS axis would be of interest for future study.

Overall, our study points to a capacity for human B cells to differentially regulate effector functions of distinct $CD8^+$ T cell subsets *in vitro* and *in vivo*. Particular interactions between B cells and MAIT $CD8^+$ T cells may contribute to the development of new MS disease activity in untreated patients. In turn, these interactions may represent a relevant therapeutic target for the highly effective B cell depleting therapies currently in use, and potentially for new therapies selectively targeting MAIT cell responses.

Disclosures

The authors have no financial conflicts of interest

Acknowledgments

We would like to thank all members of the Canadian B cells in MS team. We also acknowledge Camille Stegen for help with cell sorting. This study was supported by a Collaborative Team grant from the Research Foundation of the MS Society of Canada (RF-MSSC) to the Canadian B cells in MS Team (A.B.-O., A.P., and J.L.G.)

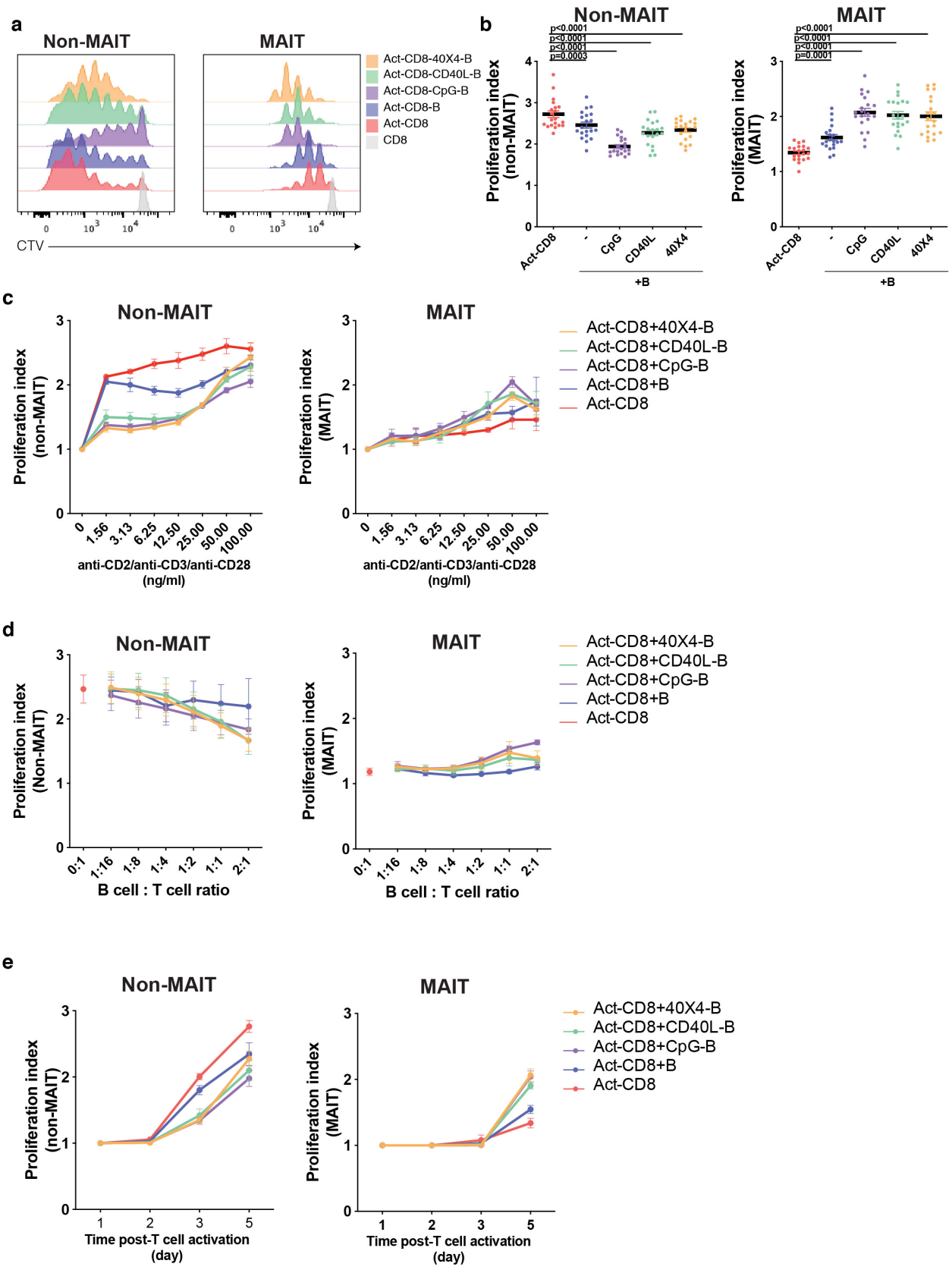

a
Non-MAIT
MAIT
Act-CD8-40X4-B
Act-CD8-CD40L-B
Act-CD8-CpG-B
Act-CD8-B
Act-CD8
CD8
CTV
b
Non-MAIT
MAIT
Proliferation index (non-MAIT)
Proliferation index (MAIT)
p<0.0001
p<0.0001
p<0.0001
p=0.0003
p<0.0001
p<0.0001
p<0.0001
p=0.0001
Act-CD8
-
CpG
CD40L
40X4
+B
c
Non-MAIT
MAIT
Proliferation index (non-MAIT)
Proliferation index (MAIT)
anti-CD2/anti-CD3/anti-CD28 (ng/ml)
Act-CD8+40X4-B
Act-CD8+CD40L-B
Act-CD8+CpG-B
Act-CD8+B
Act-CD8
d
Non-MAIT
MAIT
Proliferation index (Non-MAIT)
Proliferation index (MAIT)
B cell : T cell ratio
e
Non-MAIT
MAIT
Proliferation index (non-MAIT)
Proliferation index (MAIT)
Time post-T cell activation (day)

Figure 1. B cells reciprocally regulate non-MAIT and MAIT CD8$^+$ T cell proliferation. MACS-sorted CD8$^+$ T cells were activated with beads coated with anti-CD2, anti-CD3 and anti-CD28, and co-cultured with MACS-sorted autologous B cells (either unstimulated, or pre-activated with CpG, CD40L or CD40L + anti-IgM + IL-4) for up to 5 days before assessing T cell proliferation by flow cytometry. Non-MAIT were identified as CD161$^-$ TCR-Va7.2$^{+/-}$ and MAIT cells were identified as CD161^{hi} TCR-Va7.2$^+$. (a) Representative experiment demonstrating that non-MAIT cell proliferation is diminished in the presence of B cells, while MAIT cell proliferation is enhanced. (b) Summary of n=19 independent experiments. B cell regulation of CD8$^+$ T cell proliferation is dependent on (c) the strength of T cell stimulation (n=3); (d) the B cell to T cell ratio (n=4); and (e) the duration of T cell activation (n=5). All values are represented as mean $\pm$ SEM, and p-values were assessed by two-way ANOVA followed by Tukey's test.

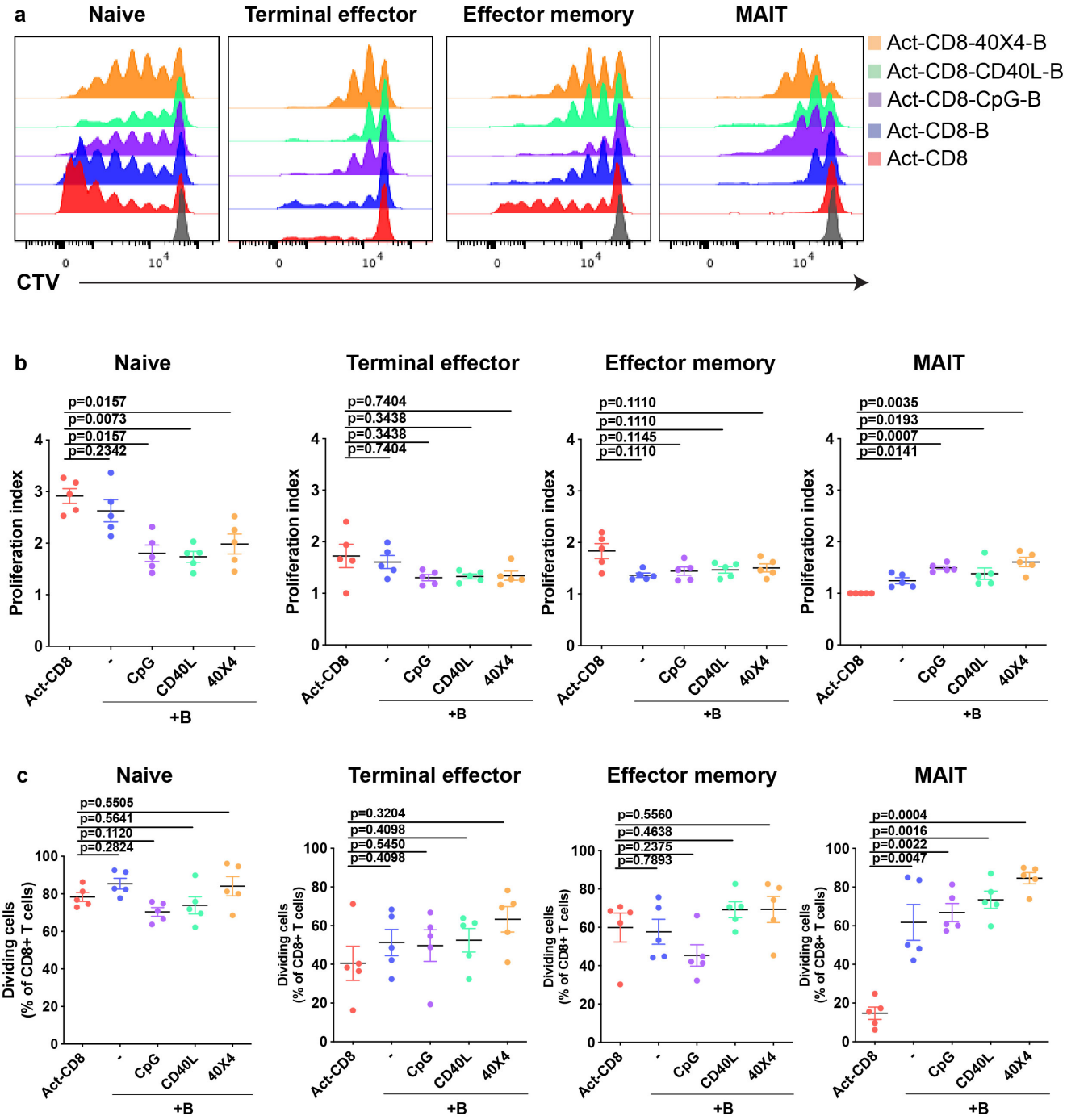

a
Naive
Terminal effector
Effector memory
MAIT
Act-CD8-40X4-B
Act-CD8-CD40L-B
Act-CD8-CpG-B
Act-CD8-B
Act-CD8
CTV
b
Naive
Terminal effector
Effector memory
MAIT
Proliferation index
p=0.0157
p=0.0073
p=0.0157
p=0.2342
p=0.7404
p=0.3438
p=0.3438
p=0.7404
p=0.1110
p=0.1110
p=0.1145
p=0.1110
p=0.0035
p=0.0193
p=0.0007
p=0.0141
Act-CD8
-
CpG
CD40L
40X4
+B
c
Naive
Terminal effector
Effector memory
MAIT
Dividing cells (% of CD8+ T cells)
p=0.5505
p=0.5641
p=0.1120
p=0.2824
p=0.3204
p=0.4098
p=0.5450
p=0.4098
p=0.5560
p=0.4638
p=0.2375
p=0.7893
p=0.0004
p=0.0016
p=0.0022
p=0.0047
Act-CD8
-
CpG
CD40L
40X4
+B

Figure 2. B cells reciprocally modulate the proliferation of FACS-sorted non-MAIT and MAIT cells. FACS-sorted $CD8^+$ T cell subsets were activated with beads coated with anti-CD2, anti-CD3 and anti-CD28, and co-cultured with MACS-sorted autologous B cells (either unstimulated, or pre-activated with CpG, CD40L or CD40L + anti-IgM + IL-4) for up to 5 days before assessing T cell proliferation by flow cytometry. (a) Representative experiment demonstrating that naive cell proliferation is diminished in the presence of B cells, while MAIT cell proliferation is enhanced. (b) Summary of n=5 independent experiments. All values are represented as mean $\pm$ SEM, and p-values were assessed by one-way ANOVA followed by Dunn's test.

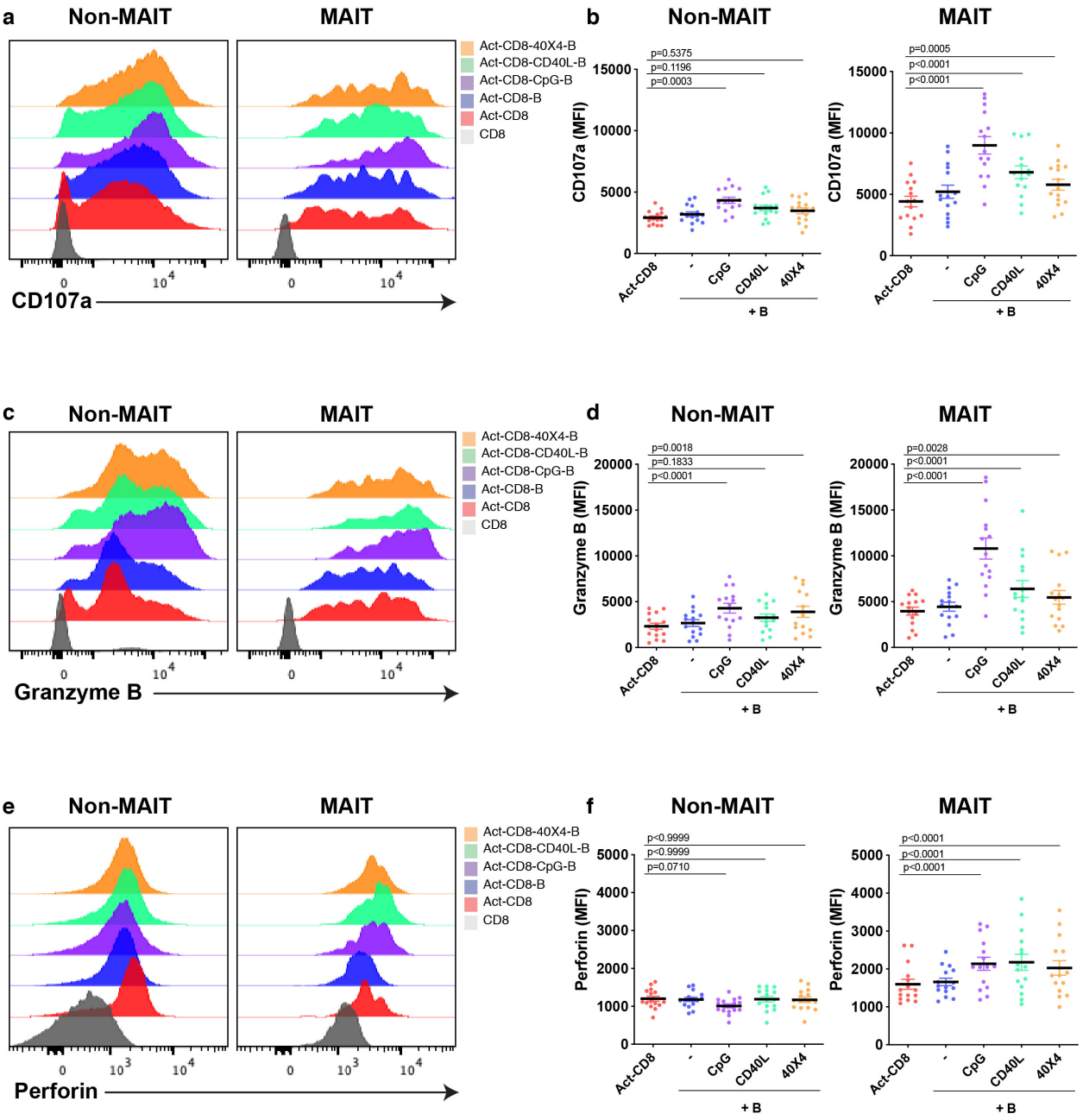


Figure 3. B cells enhance CD8$^+$ T cell subset cytotoxic potential. MACS-sorted CD8$^+$ T cells were activated with beads coated with anti-CD2, anti-CD3 and anti-CD28 and co-cultured with MACS-sorted autologous B cells (unstimulated or pre-activated with CpG, CD40L or CD40L + anti-IgM + IL-4) for 3 days before assessing the expression of CD107a **(a)**, granzyme B **(b)** and perforin **(c)** in both non-MAIT and MAIT cell. All values are represented as mean ± SEM, and p-values were assessed by two-way ANOVA with Tukey's test.

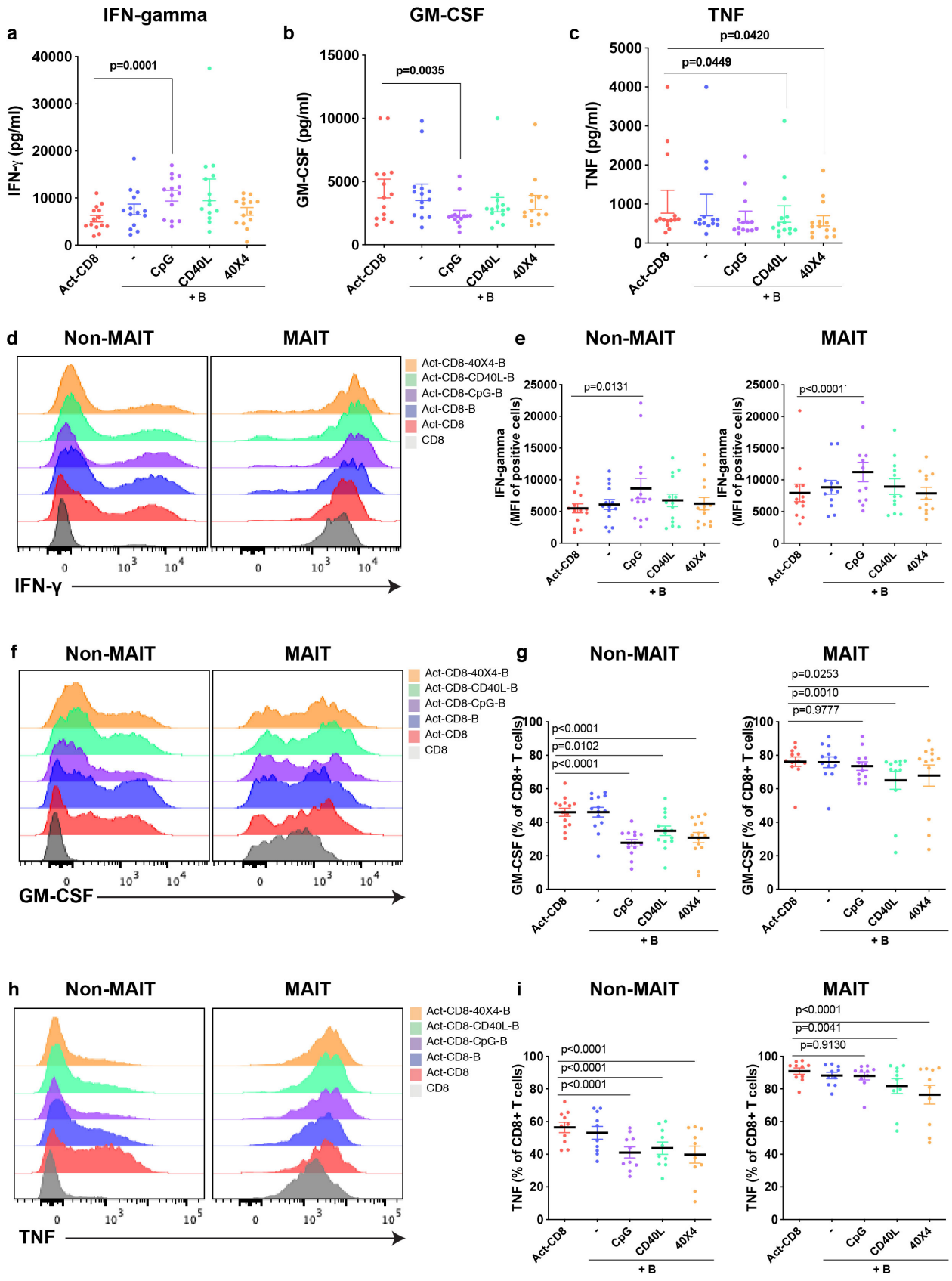

a
IFN-gamma
IFN-γ (pg/ml)
p=0.0001
b
GM-CSF
GM-CSF (pg/ml)
p=0.0035
c
TNF
TNF (pg/ml)
p=0.0420
p=0.0449
Act-CD8
-
CpG
CD40L
40X4
+ B
d
Non-MAIT
MAIT
Act-CD8-40X4-B
Act-CD8-CD40L-B
Act-CD8-CpG-B
Act-CD8-B
Act-CD8
CD8
IFN-γ
e
Non-MAIT
MAIT
IFN-gamma (MFI of positive cells)
p=0.0131
p<0.0001`
f
Non-MAIT
MAIT
GM-CSF
g
Non-MAIT
MAIT
GM-CSF (% of CD8+ T cells)
p<0.0001
p=0.0102
p<0.0001
p=0.0253
p=0.0010
p=0.9777
h
Non-MAIT
MAIT
TNF
i
Non-MAIT
MAIT
TNF (% of CD8+ T cells)
p<0.0001
p<0.0001
p<0.0001
p<0.0001
p=0.0041
p=0.9130

Figure 4. Pre-activated B cells shape $CD8^+$ T-cell subset cytokine production.

MACS-sorted $CD8^+$ T cells activated with beads coated with anti-CD2, anti-CD3 and anti-CD28 were co-cultured with MACS-sorted autologous B cells (unsimulated or pre-activated with CpG, CD40L or CD40L + anti-IgM + IL-4) for up to 5 days before assessing levels of secreted cytokines by ELISA and intracellular T-cell cytokine expression by flow cytometry. **(a-c)** Levels of secreted IFNγ were increased while levels of GM-CSF and TNF were decreased in co-culture supernatants of activated CD8+ T cells with pre-activated B cells (n=14). IFNγ expression was increased in both non-MAIT and MAIT cells co-cultured with CpG-B cells as shown in **(d)** and summarized in **(e, n=12).** The frequencies of $GM\text{-}CSF^+$ non-MAIT and MAIT cells were reduced in co-culture with pre-activated B cells as shown in **(f)** and summarized in **(g, n=12).** The frequencies of TNF^+ non-MAIT and MAIT cells were reduced in co-culture with pre-activated B cells as shown in a representative experiment **(h)** and summarized in **(i, n=11).** All values are represented as mean $\pm$ SEM, and p-values were assessed two-way ANOVA with Tukey's test.

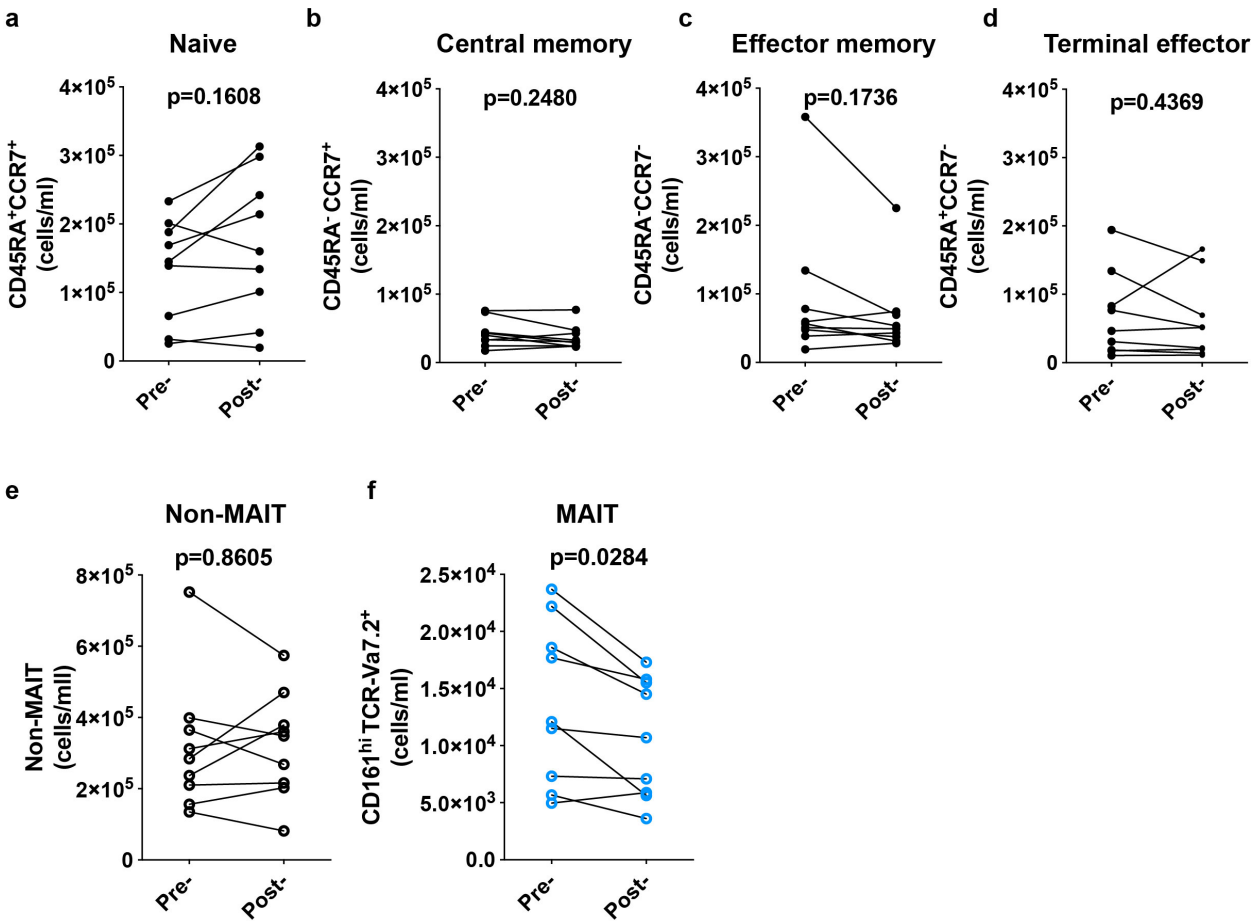


Figure 5. Circulating MAIT cells are reduced post-B cell depletion in MS patients. $CD8^+$ T cell subsets were quantified based on surface phenotype by flow cytometry within PBMC obtained prior to (Pre) and following (Post) B cell depletion. The gating strategy is shown in Figure S5. Absolute counts per volume of (a) $CCR7^+CD45RA^+$ (naïve), (b) $CCR7^+CD45RA^-$ (central memory), (c) $CCR7^-CD45RA^-$ (effector memory) and (d) $CCR7^-CD45RA^+$ (effector) CD8+ T cell subsets were quantified (n=9 donors). $CD8^+$ Non-MAIT and MAIT cells were identified based on the expression of CD161 and TCR-Vα7.2, such that $CD161^{-/int}$ TCR-V$\alpha7.2^{+/-}$ were considered non-MAIT cell and $CD161^{hi}$ TCR-V$\alpha7.2^+$ were considered MAIT cells. Cell counts of non-MAIT (e) and MAIT (f) were quantified (n=9 donors). P-values were assessed by two-tailed ratio paired t-test.

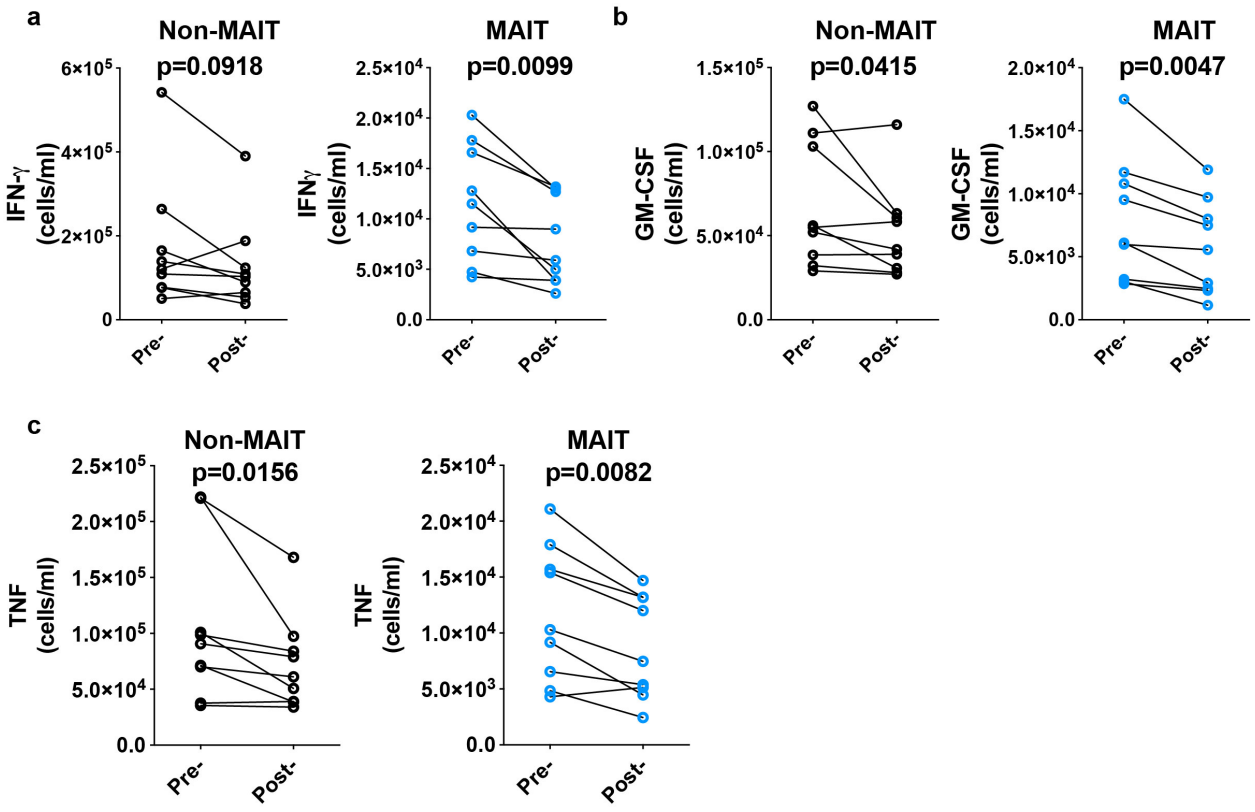


Figure 6. CD8$^+$ T cell pro-inflammatory cytokine production is diminished following B cell depletion therapy. Cryopreserved PBMC obtained from MS patients prior to (pre) and following B cell depletion (post) were short-term stimulated with PMA and ionomycin (cytokine production) for 5 hours to quantify non-MAIT (CD161$^-$ TCR-Va7.2$^{+/-}$) and MAIT (CD161^{hi} TCR-Va7.2$^+$) effector functions (a). P-values were assessed two-tailed ratio paired t-test.

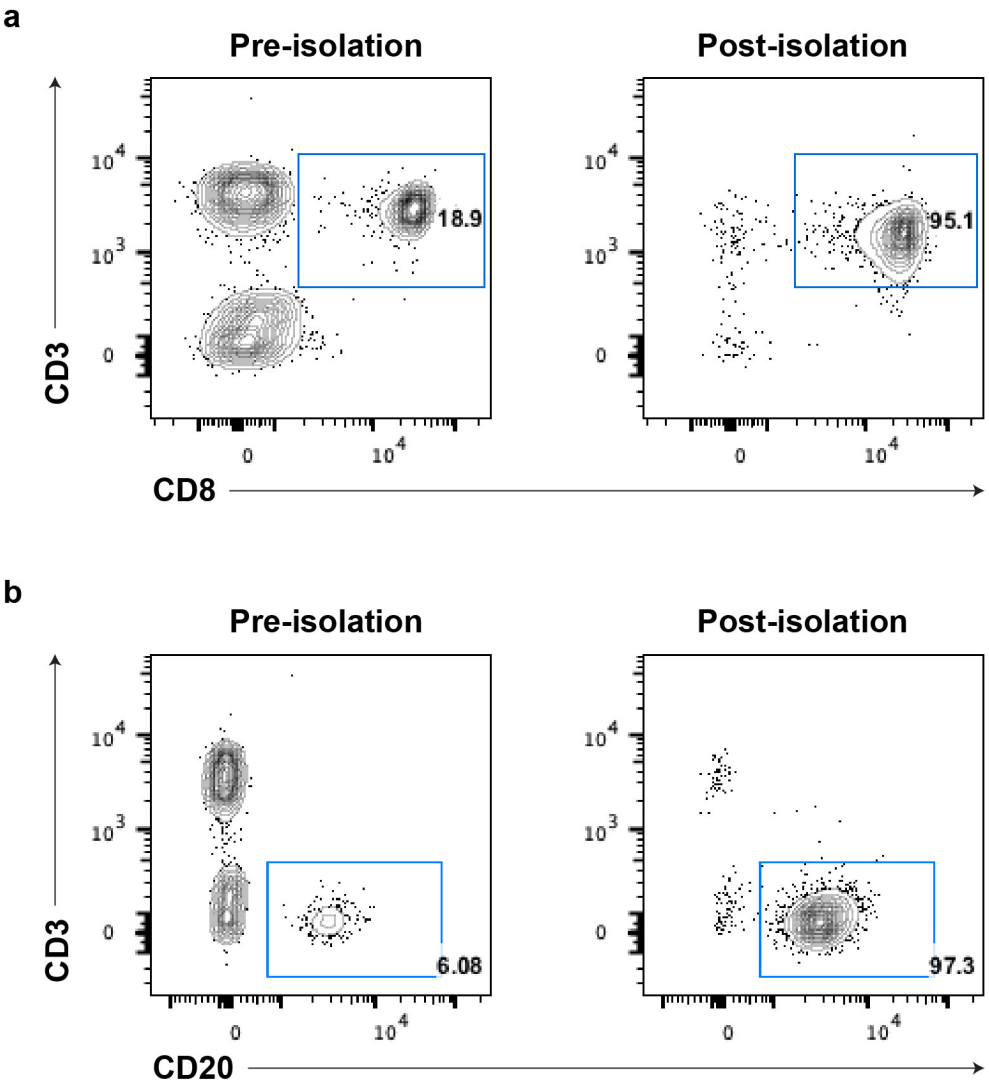


Figure S1. Purity confirmation of MACS-sorted CD8+ T cells and B cells.

$CD8^+$ T cells and B cells were routinely isolated from PBMC using magnetic microbeads (Miltenyi Biotec), and purity assessment was carried out by flow cytometry. Representative FACS staining of **(a)** $CD8^+$ T cells and **(b)** B cells pre- and post-isolation. Typical purities were >95%.

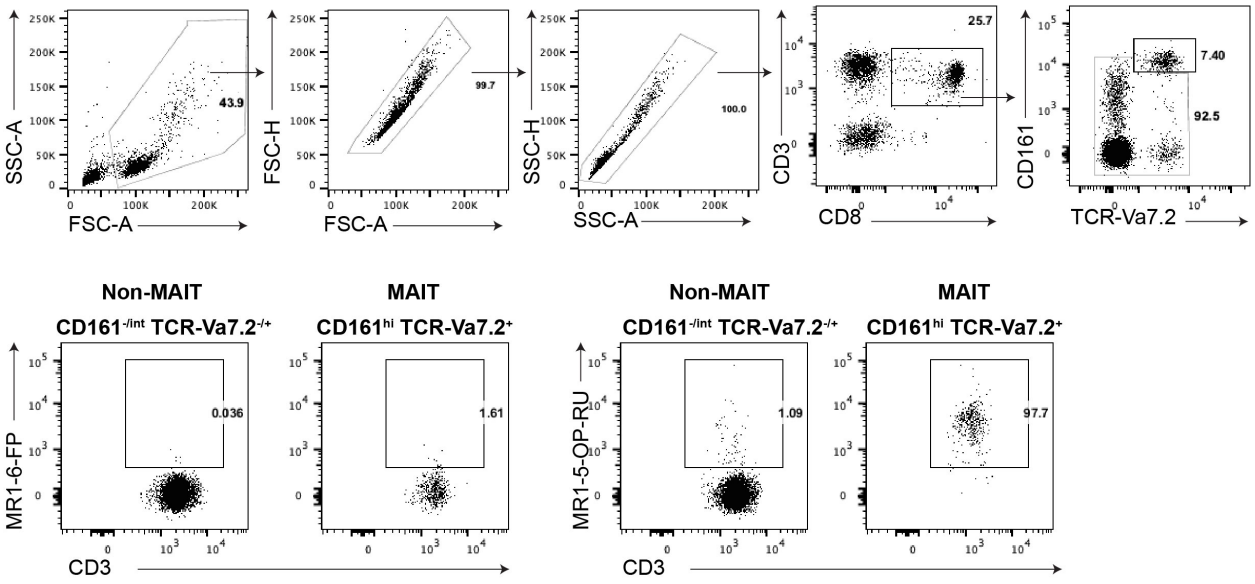


Figure S2. Human mucosal associated invariant T (MAIT) cells recognize vitamin B metabolites presented on MR1 and can be distinguished by the expression of CD161 and TCR-Vα7.2. Freshly isolated PBMC were labelled with MR1-5-OP-RU or MR1-6-Ac-FP followed by cell surface staining for CD3, CD8, CD161 and TCR-Vα7.2. Within the $CD8^+$ T cell population, two major subsets were identified based on CD161 and TCR-Vα7.2 expression namely $CD161^{-/int}$ TCR-V$\alpha7.2^{-+}$ and $CD161^{hi}$TCR-V$\alpha7.2^+$. While very few $CD8^+$ T cells bound to MR1-6-Ac-FP, $CD161^{hi}$ TCR-Va7.2$^+$ bound MR1-5-OP-RU with high affinity, thus distinguishing MAIT cells from other $CD8^+$ T cell subsets. Shown is a representative staining from n=3 independent experiments.

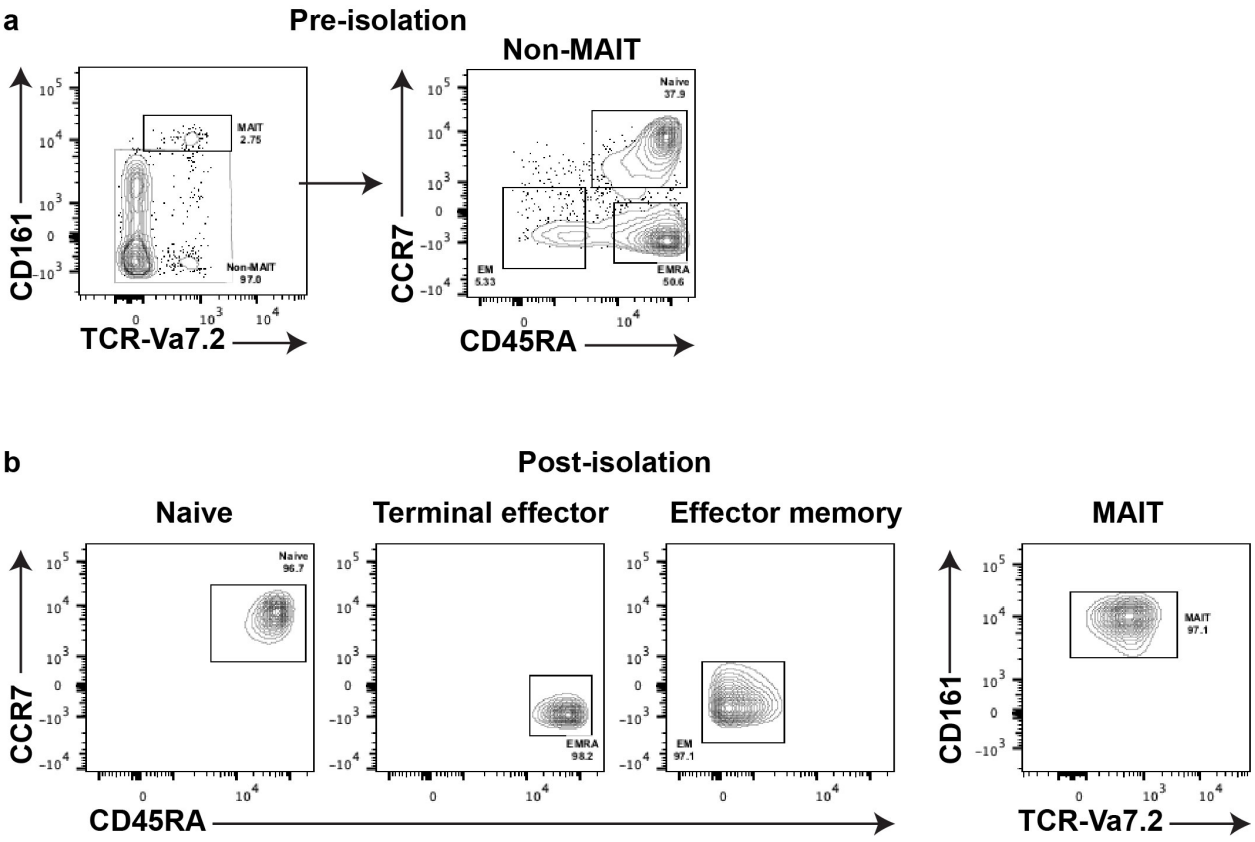


Figure S3. Purity confirmation of FACS-isolated CD8+ T cell subsets. MACS-sorted $CD8^+$ T cells were labeled with a cocktail of antibodies that included anti-CD3, anti-CD8, anti-CCR7, anti-CD45RA, anti-CD161 and anti-TCR-Vα7.2 prior to FACS isolation. **(a)** Representative gating strategy is shown, in which $CD161^{hi}$ TCR-$Va7.2^+$ (MAIT) and $CD161^{-/int}$ TCR-$Va7.2^{-/+}$ (non-MAIT) were first distinguished, and then non-MAIT cells were separated into additional three populations, namely $CCR7^+CD45RA^+$ (naïve), $CCR7^-CD45RA^+$ (terminal effector) and $CCR7^-CD45RA^-$ (effector memory). **(b)** Representative post-isolation confirmation purity is shown for all four sorted $CD8^+$ T cell populations, with typical purities >95%.

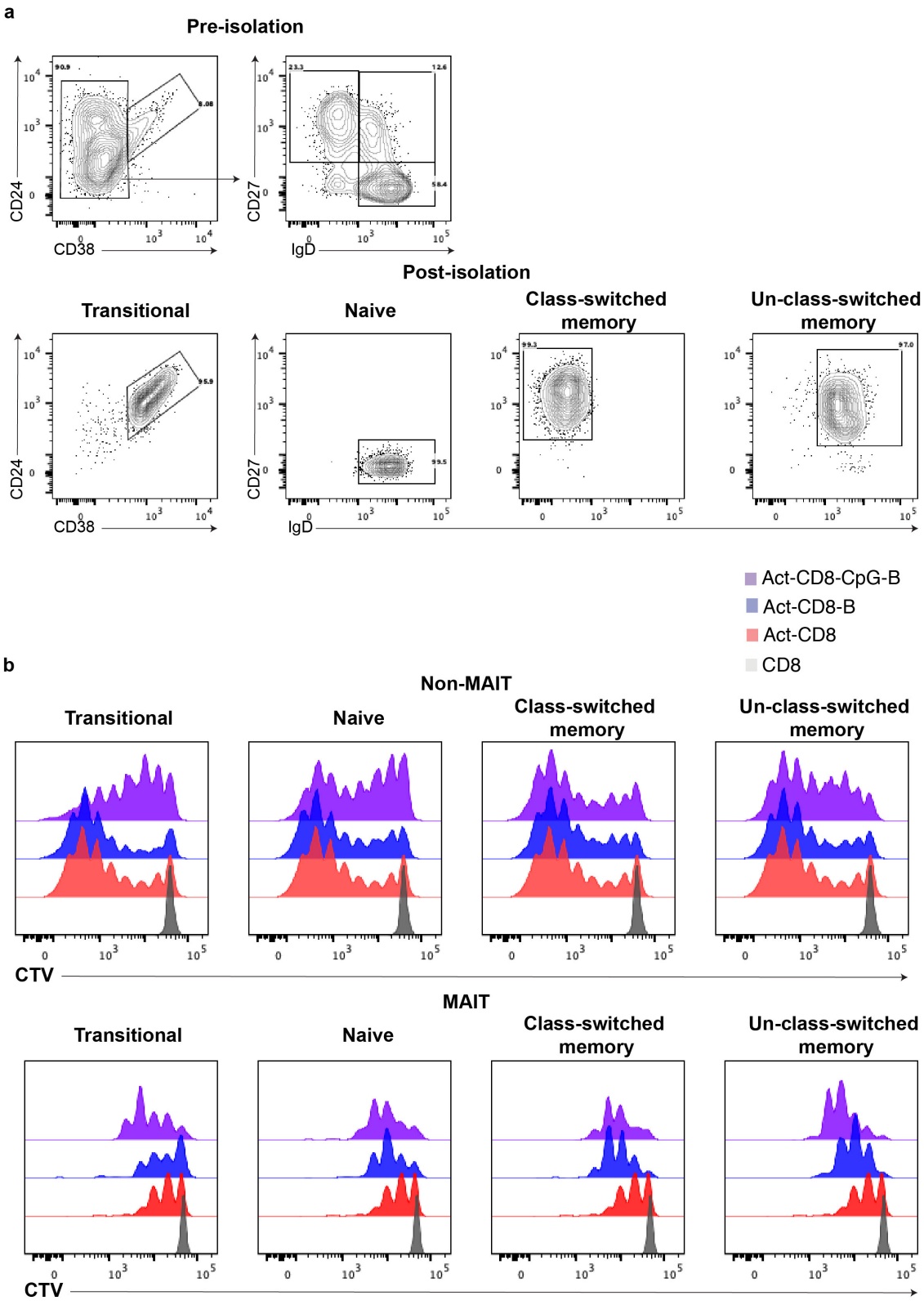

a
Pre-isolation
CD24
CD38
CD27
IgD
Post-isolation
Transitional
Naive
Class-switched memory
Un-class-switched memory
Act-CD8-CpG-B
Act-CD8-B
Act-CD8
CD8
b
Non-MAIT
Transitional
Naive
Class-switched memory
Un-class-switched memory
CTV
MAIT
Transitional
Naive
Class-switched memory
Un-class-switched memory
CTV

Figure S4. Distinct capacities for B cell subsets to regulate CD8+ T cell proliferation. MACS-sorted B cells were labeled with a cocktail of antibodies that included anti-CD20, anti-CD24, anti-CD38, anti-CD27 and anti-IgD prior to FACS isolation. **(a)** Top: Representative gating strategy is shown, in which $CD24^{+}CD38^{hi}$ (transitional) and $CD24^{-/+}CD38^{-}$ (mature) were first distinguished, and then mature B cells were sorted into three populations that included $CD27^{-}IgD^{+}$ (naïve), $CD27^{+}IgD^{+}$ (un-class switched memory) and $CD27^{+}IgD^{-}$ (class-switched memory). Bottom: Post-isolation purity confirmation is shown, with typical purities >95%. **(b)** MACS-sorted CD8+ T cells activated with anti-CD2, CD3 and CD28 were co-cultured with autologous FACS-sorted B cell subsets (naïve, transitional, class-switched and un-class switched memory B cells) that were either unstimulated or pre-activated with CpG, for 5 days before measuring T cell proliferation by flow cytometry. Representative experiment demonstrates that naïve and transitional B cells acquire suppressive capacities towards non-MAIT cells, and all B cells provide helper functions to MAIT cells (representative of four independent experiments).

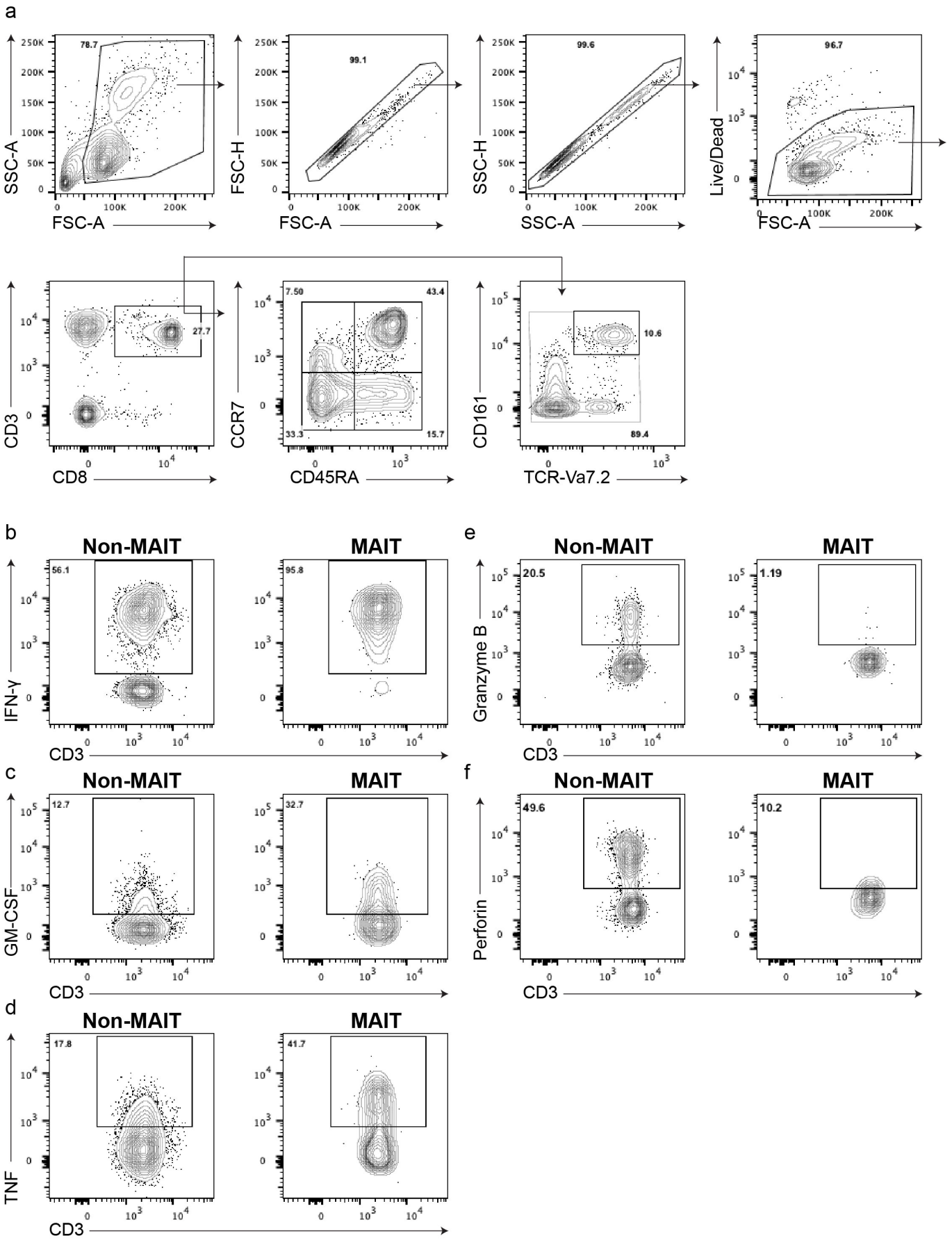

a
SSC-A
FSC-A
78.7
FSC-H
99.1
SSC-H
99.6
Live/Dead
96.7
CD3
CD8
27.7
CCR7
CD45RA
7.50
43.4
33.3
15.7
CD161
TCR-Vα7.2
10.6
89.4
b
Non-MAIT
MAIT
IFN-γ
CD3
56.1
95.8
e
Granzyme B
20.5
1.19
c
GM-CSF
12.7
32.7
f
Perforin
49.6
10.2
d
TNF
17.8
41.7

Figure S5. Flow cytometry gating strategy used to distinguish various CD8+ T cell subsets in PBMC. (a) PBMC were first gated based on FSC and SSC followed by exclusion of doublets and dead cells. $CD8^+$ T cells were gated as $CD3^+CD8^+$. Within CD8+ T cells, $CCR7^+CD45RA^+$ (naïve), $CCR7^+CD45RA^-$ (central memory), $CCR7^-CD45RA^-$ (effector memory) and $CCR7^-CD45RA^+$ (terminal effector) were identified. Alternatively, $CD161^{-/int}$ TCR-$Va7.2^{-/+}$ (non-MAIT) and $CD161^{hi}$ TCR-$Va7.2^+$ (MAIT) were identified. PBMC were stimulated for five hours with PMA, ionomycin in the presence of monensin A to examine intracellular expression of IFN-γ **(b)**, GM-CSF **(c)** and TNF **(d)** in non-MAIT and MAIT cells gated within PBMC as shown above. Expression of granzyme B **(e)** and perforin **(f)** were assessed in resting non-MAIT and MAIT cells gated within PBMC as shown above.

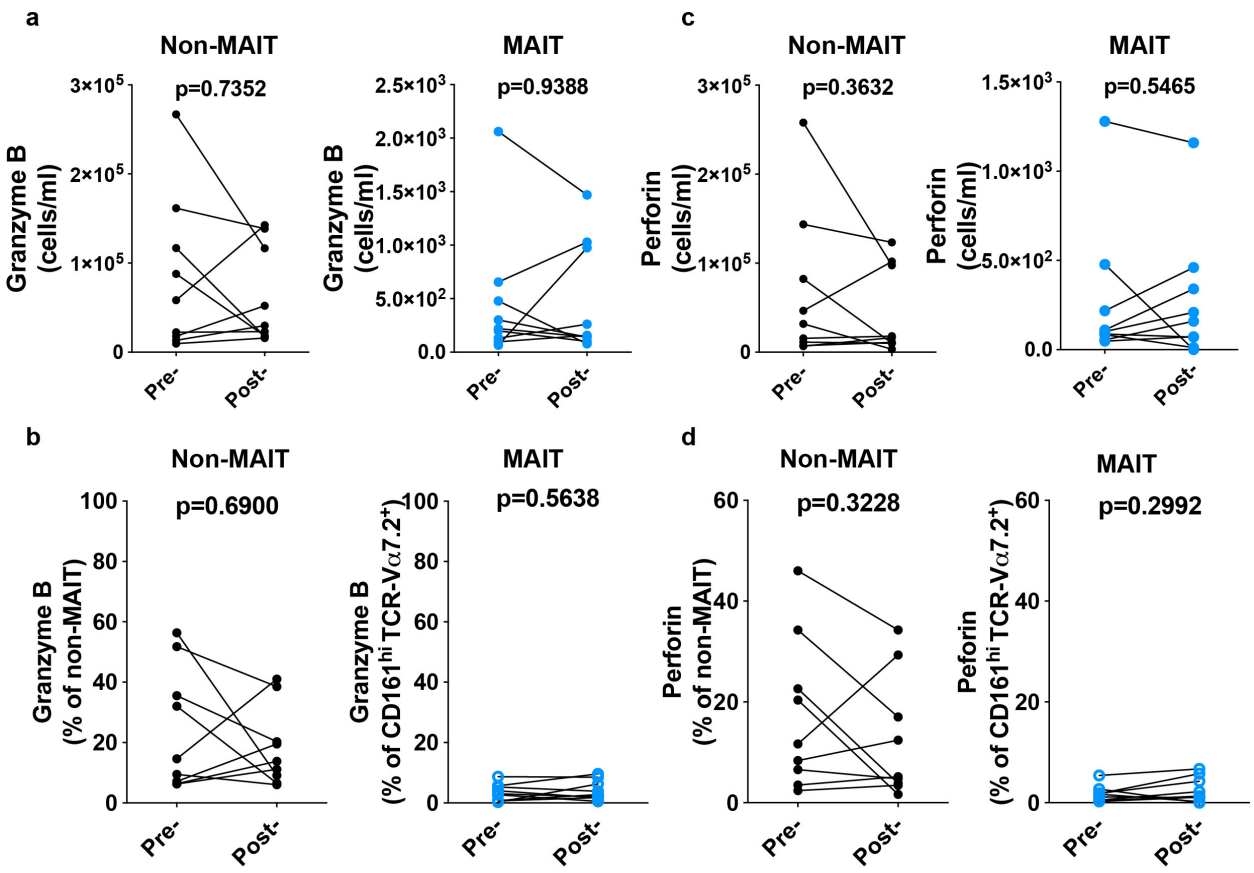


Figure S6. CD8+ T cell cytotoxic profile is not impacted after B cell depletion. $CD8^+$ T cell populations were quantified within PBMC obtained prior to (Pre) and after (Post) B cell depletion in MS patients. Absolute cell counts per volume of (a) granzyme B as well as (b) perforin expressing non-MAIT ($CD161^{-/int}$ TCR-Va7.$2^{-/+}$) and MAIT ($CD161^{hi}$ TCR-Va7.2^+) cells were quantified in n=9 donors. P-values were assessed two-tailed ratio paired t-test.

Supplemental Table 1. Patient characteristics

Characteristics	N= 9
Age (median, range)	42 (22-66)
Female (%, number)	45 (4/9)
Duration of MS (mean, range)	2.0 (0-9)
Previously treated (%, number)	0 (0/9)
EDSS score at baseline (median, range)	2.5 (1-4.5)

Supplemental Table 2. Immune phenotyping panels.

Laser	Blue (488nm)		Red (460nm)			Violet (405nm)					Yellow-Green (561nm)			UV (355nm)	
Filter	530/30	695/40	660/20	730/45	780/60	450/40	525/50	660/20	710/50	780/60	575/26	610/20	780/60	379/28	820/860
FC	FITC	PerCP-Cy5.5	APC	APC-R700	APC-H7	BV421	Aqua	BV650	BV711	BV786	PE	PE-CF594	PE-Cy7 (PE-Vio770)	BUV395	BUV805
Panel 1	CD45RA	CD57	CCR7	CD8	CD20	CD28	L/D	CD27	CD3	HLA-DR	Ki-67	KLRG1	CD95	CD38	CD4
Panel 2	GzK	TCR-Va7.2	CCR7	CD8	CD20	Perforin	L/D	CD56	CD3	CD45RA	GzA	GzB	CD161	NKG2C	CD4
Panel 3	TCR-Va7.2		CCR6	CD8	TNF	CCR2	L/D	IL-17	CD3	CD45RA	GM-CSF	CCR5	CD161	IFN-g	CD4

Supplemental Table 3. Staining reagents used in immunophenotyping

Antibody	Fluorochrome conjugation	Clone	Source
CD3	BV711	UCHT1	BD Biosciences
CD4	BUV805	SK3	BD Biosciences
CD8	APC-R700	RPA-T8	BD Biosciences
CD20	APC-H7	2H7	BD Biosciences
CD27	BV650	M-T271	BD Biosciences
CD28	BV421	CD28.2	BD Biosciences
CD38	BUV395	HB7	BD Biosciences
CD45RA	FITC	HI100	BD Biosciences
CD45RA	BV786	HI100	BD Biosciences
CD56	BV650	NCAM16.2	BD Biosciences
CD57	PerCP-Cy5.5	HNK-1	Biolegend
CD95	PE-Cy7	DX2	BD Biosciences
CD161	PE-Vio700	REA631	Miltenyi Biotec
CD197	Alexa-Fluor 647	3D12	BD Biosciences
GM-CSF	PE	BVD2-21C11	BD Biosciences
Granzyme A	PE	CB9	Biolegend
Granzyme B	PE-CF594	GB11	BD Biosciences
Granzyme K	FITC	GM26E7	Biolegend
HL-A-DR	BV786	G46-6	BD Biosciences
IFN-gamma	BUV395	B27	BD Biosciences
IL-17	BV650	N49-653	BD Biosciences
Ki-67	PE	Ki-67	Biolegend
KLRG1	PE-Vio615	REA261	Miltenyi Biotec
NKG2C	BUV395	134591	BD Biosciences
Perforin	BV421	$\delta G9$	BD Biosciences
TCR-Va7.2	PerCP-Cy5.5	3C10	Biolegend
TCR-Va7.2	FITC	3C10	Biolegend

TNF	APC/Cy7	MAb11	Biolegend
LIVE/DEAD Fixable Aqua Dead Cell Stain	N/A	N/A	Invitrogen

Supplementary Table 4. Cell subset counts pre- and on-treatment with anti-CD20

Cell subset	Pre (SEM)	On-treatment (SEM)	P-value
Total CD3$^+$ T cells	1.252e+006 (170109)	1.423e+006 (202085)	0.3297
CD8$^+$ T cells	331222 (62566)	333278 (49585)	0.9307
Naïve CD8$^+$ T cells	133144 (25112)	169244 (35200)	0.1608
Central memory CD8$^+$ T cells	42856 (6707)	36833 (5763)	0.2480
Effector memory CD8$^+$ T cells	93611 (34722)	67911 (20333)	0.1736
Terminal effector CD8$^+$ T cells	67944 (20644)	61600 (19358)	0.4369
Non-MAIT cells	316667 (61943)	322211 (49545)	0.8605
MAIT cells	13751 (2360)	10670 (1744)	0.0284
IFN-γ^+ non-MAIT cells	171711 (50784)	128956 (35796)	0.0918
IFN-γ^+ MAIT cells	11552 (1939)	7689 (1445)	0.0099
GM-CSF$^+$ non-MAIT cells	67089 (12237)	51644 (9329)	0.0415
GM-CSF$^+$ MAIT cells	7857 (1649)	5730 (1255)	0.0047
TNF$^+$ non-MAIT cells	105378 (23300)	72456 (14074)	0.0472
TNF$^+$ MAIT cells	11696 (2018)	8666 (1535)	0.0472
Granzyme B$^+$ non-MAIT cells	83892 (28849)	61646 (18253)	0.7352
Granzyme B$^+$ MAIT cells	468 (209)	484 (175)	0.9388
Perforin$^+$ non-MAIT cells	67080 (28162)	43638 (16233)	0.3632
Perforin$^+$ MAIT cells	274 (133)	276 (121)	0.5465

Mean and standard error of the mean for cell counts (cell/ml) of T cell subsets pre- and on-treatment are shown for n=9 donors. Ratio-paired t-test was used for statistical analysis.

Supplementary Table 5. Cell subset frequencies pre- and on-treatment with anti-CD20

Cell subset	Pre (SEM)	On-treatment (SEM)	P-value
Total $CD3^+$ T cells	63.07 (0.9922)	72.54 (2.982)	0.0083
$CD8^+$ T cells	16.89 (1.863)	17.88 (2.476)	0.7890
Naïve $CD8^+$ T cells	41.52 (7.509)	48.33 (7.803)	0.0076
Central memory $CD8^+$ T cells	14.47 (2.203)	13.08 (2.335)	0.1183
Effector memory $CD8^+$ T cells	25.68 (4.398)	21.65 (4.055)	0.0105
Terminal effector $CD8^+$ T cells	18.33 (3.697)	16.88 (3.578)	0.2266
Non-MAIT cells	95.04 (0.8097)	95.98 (0.7238)	0.0189
MAIT cells	4.78 (0.8433)	3.86 (0.7173)	0.0472
IFN-γ^+ non-MAIT cells	51.76 (6.40)	39.64 (5.60)	0.0918
IFN-γ^+ MAIT cells	85.00 (2.80)	79.26 (2.30)	0.1104
GM-CSF^+ non-MAIT cells	23.10 (3.36)	18.29 (3.04)	0.0235
GM-CSF^+ MAIT cells	55.16 (2.93)	49.52 (4.29)	0.0954
TNF^+ non-MAIT cells	35.87 (5.92)	26.02 (4.62)	0.0472
TNF^+ MAIT cells	85.38 (1.57)	79.59 (2.36)	0.0984
Granzyme B^+ non-MAIT cells	24.40 (6.69)	18.44 (4.37)	0.6900
Granzyme B^+ MAIT cells	3.47 (0.93)	4.12 (1.08)	0.5638
$Perforin^+$ non-MAIT cells	17.33 (4.98)	12.45 (4.02)	0.3228
$Perforin^+$ MAIT cells	1.77 (0.53)	2.51 (0.83)	0.2992

Mean and standard error of the mean for cell counts (cell/ml) of T cell subsets pre- and on-treatment are shown for n=9 donors. Ratio-paired t-test was used for statistical analysis

Chapter 4: Discussion, conclusion and future directions

4.1 General discussion

In this thesis, I set out to study human B-cell functions of potential relevance to the common CNS inflammatory disease multiple sclerosis (MS). I focused on methods that would facilitate the study of functionally distinct (and particularly cytokine-defined) B cell subpopulations and on studies of antibody-independent functions of B cells (particularly B cell interactions with $CD8^+$ T cells). Following Chapter 1 (Introduction into MS pathophysiology), in Chapter 2, I optimized an approach for the isolation of viable human cytokine-secreting B cells that avoids the need for intracellular cytokine staining (which requires killing the cells), making it an exciting tool for investigating viable cytokine-secreting B cells in a range of applications. In Chapter 3, I showed that B cells can exert two opposing functions in their interactions with distinct $CD8^+$ T cell subsets, including interactions with MAIT cells, which may relevant to MS pathophysiology and to the therapeutic mode of action of B cell depleting therapy in MS.

4.2 Human cytokine-defined B cells – novel therapeutic targets in MS?

B cell cytokine production is undoubtedly involved in regulating adaptive and innate immune responses in host response to infection and inflammatory/chronic diseases[271,343]. B cells can secrete pro- and anti-inflammatory cytokines, and certain B cell populations possess a higher propensity to produce particular cytokines, such that human naïve and immature B cells can secrete higher levels of IL-10 than memory B cells[271,281,283,377]. Moreover, we and others have documented an imbalance between the production of pro- and anti-inflammatory cytokines by human B cells in MS patients, typified by an increased frequency of $GM\text{-}CSF^+$ B cells concomitant with a decreased frequency of $IL\text{-}10^+$ B cells [271,281,282]. Despite the relevance of cytokine-producing B cells to human diseases, there remain gaps in our understanding of their development and functions, in part due to a lack of unique cell surface markers, alone or in combination, which selectively encompass these cells. Our work presented in chapter 2 establishes a methodology that will serve as a foundation for further interrogating cytokine-secreting B cell subpopulations. This optimized approach for the isolation of viable and highly purified cytokine-defined human B cells will enable subsequent analyses such as next-generation sequencing and functional assays, which rely on intact, viable cells. We expect these studies will lead to the identification of transcriptional networks (including master transcriptional factors) and metabolic pathways that contribute to the regulation of GM-CSF or IL-10 expression in B cells. These future studies focusing on

interrogating cytokine-defined B cells should provide a platform for the development of more selective, and even personalized, therapeutics for patients with MS and beyond.

Even though anti-CD20 antibody therapy has proven highly efficacious in reducing new disease activity in MS, the long-term safety of such broad B cell depletion is not known[273,274]. Anti-CD20 rather non-selectively depletes both pathogenic and regulatory B cell sub-populations as well as other subsets required for host immunity against pathogens[271,278,279,281,283]. It is therefore conceptually more appealing to generate therapies that more selectively target pathogenic B cell sub-populations, such as those producing GM-CSF, while sparing other B cell subsets. One hopes that our new approach for isolating cytokine-secreting human B cells will facilitate discovery and development of such selective therapies.

For example, future work could examine whether distinct cytokine-producing B cell subsets utilize distinct metabolic pathways. Recent work has highlighted the differing metabolic requirements for effector and regulatory T cells[593] which has offered a therapeutic opportunity to selectively manipulate the balance between these subsets in anti-tumor immunity. The same could potentially be done with B cells in the context of MS and autoimmune diseases. In fact, dimethyl fumarate and dihydroorotate dehydrogenase inhibitor (teriflunomide), both approved therapies for MS, have recently been found to modulate T cell metabolic pathways in a way that reduces pro-inflammatory responses and prunes high-affinity T cells in MS patients[594,595]. It remains unknown if the metabolic demands of distinct B cell cytokine-producing subsets are similar to T cell subsets. If so, it might provide greater insights into the biology of cytokine-defined B cells and guide novel therapeutic targeting of key metabolic pathways that could restore the imbalance between pro- and anti-inflammatory T and B cell responses in multiple sclerosis.

4.3 Dual B cell functions: regulatory and helper cells

As part of our ongoing investigation of B cell antibody-independent functions, my PhD work has identified two novel roles for B cells in modulating conventional and innate-like $CD8^+$ T cell effector functions. In chapter 3, we found that, *in vitro*, B cells provided helper factors to MAIT cells, thereby enhancing their expansion, pro-inflammatory cytokine production, and cytotoxic potential. Therefore, we coin the term “MAIT-cell helper B cells” to describe the role of B cells in

this interaction. Our attempts to uncover these so-called helper factors have so far been unsuccessful, though my experiments indicate that they involve soluble factors that are generated as a result of cross-talk between the B cells and the MAIT cells.

My work assessing the effects of *in vivo* anti-CD20 treatment of MS patients revealed that B cell depletion therapy resulted in reduced circulating MAIT cell numbers and frequencies as well as IFNγ production, mirroring my *in vitro* observations. This favors a model wherein B cells engage in an *in vivo* productive interaction with MAIT cells to stimulate their expansion and pro-inflammatory effector functions. We recently reported that the MAIT cell frequency as well as their propensity to produce the proinflammatory cytokines IFNγ and IL-17 are abnormally elevated in the circulation of children with multiple sclerosis compared to children with monophasic CNS inflammatory disorders or normal controls[150]. Taken together, we speculate that B cells from multiple sclerosis patients instigate aberrant MAIT cell expansion and pro-inflammatory effector functions. The consequences of such interaction between B cells and MAIT cells may be relevant to propagating MS disease activity both in the periphery and within the CNS (where B cells and MAIT cells appear to occupy similar niches and are therefore likely to interact[413]) and may represent part of therapeutic mode of action of the highly effective B cell depleting approach in patients.

A reduction in MAIT cell numbers after anti-CD20 therapy in MS patients could alternatively be due to the expression of CD20 on T cells. In our preliminary experiments, we found that <5% of MAIT cells expressed CD20 *ex-vivo* in a cohort of normal controls, and that those cells produced higher levels of pro-inflammatory cytokines (data not shown). Future experiments could aim at investigating the expression of CD20 on MAIT cells in MS, the functional profile of $CD20^+$ MAIT cells including TCR-repertoire analysis, cytokine and cytotoxic molecule expression and its relevance to the depletion profile found in ocrelizumab-treated patients. These efforts would complement published work that documented CD20 expression on T cells in normal controls and MS patients. In fact, CD20 is found on a minor $CD4^+$ and $CD8^+$ T cell populations enriched for memory cells[596-598]. In MS, the frequency of circulating $CD20^+$ $CD8^+$ T cells is elevated compared to normal controls, and those cells can also be found within the CSF[596-598]. Further, two recent reports suggested that $CD20^+$ T cells may recognize myelin antigens, and display a Th1/Tc1

phenotype[598,599]. Though this data represents an intriguing phenomenon, it remains that the relevance of $CD20^+$ T cells to MS pathogenesis is uncertain.

In chapter 3, we also uncovered a regulatory role for B cells in modulating $CD8^+$ non-MAIT cell proliferation and cytokine production (GM-CSF and TNF). Our data indicates that B cell-derived soluble factors generated in cross-talk with $CD8^+$ T cells can increase the TCR activation threshold of non-MAIT cells. B cells exerted their suppressive effects on non-MAIT cells independently from IL-10, PD-1, adenosine, and IL-2 signaling (Appendix Figure 1) and without impacting on the survival of the non-MAIT cells (Appendix Figure 2). Further, a greater degree of suppression was exerted by transitional and naïve B cells than memory cells in keeping with published reports[271]. The molecular wiring that underlie the acquisition of suppressive functions by transitional and naïve B cells has not been investigated, as is the state of B cell regulation in MS.

One challenge in further extending our current observations into an *in vivo* model of CNS neuroinflammation such as classical MOG-induced EAE is the dependence of disease initiation on $CD4^+$ T cells with a less prominent role for CD8+ T cells[95]. Nonetheless, work by Goverman's group have shown that TCR-transgenic $CD8^+$ T cells, in conjunction with other immune cells such as $CD4^+$ T cells, can mediate inflammation within the CNS, which is aided by concurrent viral infections[448,452,459]. Whether B cells can impact disease course in those models through antibody-independent functions has not been explored. Similarly, infection with Theiler's encephalomyelitis virus can instigate CNS-localized inflammation in which $CD8^+$ T cells play dual roles – viral clearance and subsequent tissue injury[95]. B cell depletion results in a worsened disease course, associated with viral persistence within the CNS[600]. Thus, despite its relevance in dissecting aspects of CNS inflammation, the experimental model EAE does not mirror all aspects of the human disease, including relevant interactions among $CD8^+$ T cells and B cells.

In a similar vein, the relative contribution of MAIT cells to CNS inflammation remains uncertain. *Croxford et al*. hinted to a regulatory function for MR1-restricted T cells in EAE, however, these observations require further validation[601]. In addition, the advent of MR1-tetramer has greatly helped charting MAIT cell development and functions *in vivo*, bypassing the use of Vα19 transgenic mice[513,542]. Thus, it would now be ideal to characterize MAIT cell functional phenotype

during EAE clinical course in B cell replete and deficient mice, and conversely utilize MAIT cell-depleted mice (in MR1$^{-/-}$ or Traj33$^{-/-}$ mice) to infer their role(s) in this disease. An additional layer of complexity is the diversity of the gut microbiota and its influence on MAIT cells and EAE course. MAIT cell abundance is tightly linked to the presence of a diverse microbial community, such that germ-free mice are devoid of MAIT cells, and specific-pathogen free mice housed in different cages have varying numbers of MAIT cells[501,547,548]. It is interesting to note that It is also the case that germ-free mice exhibit a reduced EAE clinical score[261,263]. The intersection between those observations is unknown, as would be the effect of the microbiota on the functional phenotype of MAIT cells in MS patients.

4.4 B cell: CD8+ T cell interactions beyond MS

An emerging body of work suggests that MAIT cells can be found within the cellular infiltrate in human colon adenocarcinomas and esophageal adenocarcinomas, likely in response to local tissue inflammation[602,603]. MAIT cells may promote tumor immunity via release of pro-inflammatory and cytotoxic molecules in a bystander fashion[505]. Could B cells help promote MAIT cell effector functions within the tumor microenvironment? In fact, there is an increased recognition of an important role for B cells in tumor surveillance that can extend from their inherent capacity to secrete immunoglobulin – these functions can promote or inhibit productive tumor immunity[384]. Indeed, *Shalapour et al.* found that IgA^{+} plasma cells induced upon chemotherapy intervention can impede CD8^{+} T cell activation and effector functions in a prostate cancer mouse model[576]. Further, an increased B cell:CD8^{+} T cell ratio was associated with tumor metastasis in prostate cancer patients. Similarly, IgA^{+} plasma cells can promote CD8+ T cell exhaustion in hepatocellular carcinoma mouse model, in an IL-10 and PD-L1 dependent mechanism[577]. While these studies demonstrate the acquisition of regulatory roles by B cells within the tumor microenvironment, more recent observations suggest that the relationship between B cells and CD8^{+} T cells is more nuanced. In fact, the co-presence of B cells and CD8^{+} T cells, as well as the formation of tertiary lymphoid structures, within the tumors of metastatic melanoma patients treated with checkpoint blockade was associated with improved prognosis at baseline[604,605]. Parallel observations were made in soft-tissue sarcoma and lung cancer, in that the formation of tertiary lymphoid structures can predict improved patient survival and/or immunotherapy response[606-608]. The phenotype of tumor-infiltrating B cells is heterogeneous across different tumors, with B cells in tertiary lymphoid structures up-regulating known molecules such as Ki-67 and CXCR5[608]. These studies

hint to a positive role for B cells in tumor immunity, that may include antibody-dependent and independent functions. The extent and nature of any cellular interactions between B cells and other immune cells, including $CD8^+$ non-MAIT and MAIT cells, require further investigation. In sum, those observations serve to highlight the influence of the tissue microenvironment, disease context and treatment modality on B cell: T cell interaction(s), and the importance of studying biospecimen from patients to inform on novel insights that can be further dissected *in vitro* or otherwise.

4.5 Conclusion and future directions

B cells have emerged as an important cellular contributor to the pathogenesis of multiple sclerosis, in part by orchestrating other cellular responses via the production of cytokines. As mentioned above, the identity of cytokine-producing B cells remains enigmatic, and future studies should focus on deciphering their transcriptional programs using next-generation sequencing and interrogating their functions *in vitro* and *in vivo*. Our studies have added a potential new dimension to the cellular network involved in MS. We have shown that human B cells can modulate both innate (MAIT) and conventional $CD8^+$ T cell effector functions *in vitro* and *in vivo.* It will be important to define the signals involved in this cross-talk and to further characterize such interactions in healthy individuals and in untreated MS patients. It would be equally important to investigate the regulation of B cell functions by $CD8^+$ T cell subsets, as history has taught us that cellular immune interactions are rarely uni-directional.

References

1 Bar-Or, A. The immunology of multiple sclerosis. *Semin Neurol* **28**, 29-45, doi:10.1055/s-2007-1019124 (2008).

2 Steinman, L. Immunology of relapse and remission in multiple sclerosis. *Annu Rev Immunol* **32**, 257-281, doi:10.1146/annurev-immunol-032713-120227 (2014).

3 Filippi, M. *et al.* Multiple sclerosis. *Nat Rev Dis Primers* **4**, 43, doi:10.1038/s41572-018-0041-4 (2018).

4 Dendrou, C. A., Fugger, L. & Friese, M. A. Immunopathology of multiple sclerosis. *Nat Rev Immunol* **15**, 545-558, doi:10.1038/nri3871 (2015).

5 Group, G. B. D. N. D. C. Global, regional, and national burden of neurological disorders during 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Neurol* **16**, 877-897, doi:10.1016/S1474-4422(17)30299-5 (2017).

6 Owens, G. M. Economic burden of multiple sclerosis and the role of managed sare organizations in multiple sclerosis management. *Am J Manag Care* **22**, s151-158 (2016).

7 Waldman, A. *et al.* Multiple sclerosis in children: an update on clinical diagnosis, therapeutic strategies, and research. *Lancet Neurol* **13**, 936-948, doi:10.1016/S1474-4422(14)70093-6 (2014).

8 Orton, S. M. *et al.* Sex ratio of multiple sclerosis in Canada: a longitudinal study. *Lancet Neurol* **5**, 932-936, doi:10.1016/S1474-4422(06)70581-6 (2006).

9 Koch-Henriksen, N. & Sorensen, P. S. The changing demographic pattern of multiple sclerosis epidemiology. *Lancet Neurol* **9**, 520-532, doi:10.1016/S1474-4422(10)70064-8 (2010).

10 Koch-Henriksen, N. & Sorensen, P. S. Why does the north-south gradient of incidence of multiple sclerosis seem to have disappeared on the northern hemisphere? *J Neurol Sci* **311**, 58-63, doi:10.1016/j.jns.2011.09.003 (2011).

11 Koch-Henriksen, N., Thygesen, L. C., Stenager, E., Laursen, B. & Magyari, M. Incidence of MS has increased markedly over six decades in Denmark particularly with late onset and in women. *Neurology* **90**, e1954-e1963, doi:10.1212/WNL.0000000000005612 (2018).

12 Olsson, T., Barcellos, L. F. & Alfredsson, L. Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. *Nat Rev Neurol* **13**, 25-36, doi:10.1038/nrneurol.2016.187 (2017).

13 Klineova, S. & Lublin, F. D. Clinical Course of Multiple Sclerosis. *Cold Spring Harb Perspect Med* **8**, doi:10.1101/cshperspect.a028928 (2018).

14 Lublin, F. D. *et al.* Defining the clinical course of multiple sclerosis: the 2013 revisions. *Neurology* **83**, 278-286, doi:10.1212/WNL.0000000000000560 (2014).

15 Baranzini, S. E. & Oksenberg, J. R. The Genetics of Multiple Sclerosis: From 0 to 200 in 50 Years. *Trends Genet* **33**, 960-970, doi:10.1016/j.tig.2017.09.004 (2017).

16 Ascherio, A. & Munger, K. L. Epidemiology of Multiple Sclerosis: From Risk Factors to Prevention-An Update. *Semin Neurol* **36**, 103-114, doi:10.1055/s-0036-1579693 (2016).

17 Waubant, E. *et al.* Environmental and genetic risk factors for MS: an integrated review. *Ann Clin Transl Neurol*, doi:10.1002/acn3.50862 (2019).

18 Willer, C. J. *et al.* Twin concordance and sibling recurrence rates in multiple sclerosis. *Proc Natl Acad Sci U S A* **100**, 12877-12882, doi:10.1073/pnas.1932604100 (2003).
19 Hansen, T. *et al.* Concordance for multiple sclerosis in Danish twins: an update of a nationwide study. *Mult Scler* **11**, 504-510, doi:10.1191/1352458505ms1220oa (2005).
20 Hansen, T. *et al.* Risk for multiple sclerosis in dizygotic and monozygotic twins. *Mult Scler* **11**, 500-503, doi:10.1191/1352458505ms1202oa (2005).
21 Kuusisto, H. *et al.* Concordance and heritability of multiple sclerosis in Finland: study on a nationwide series of twins. *Eur J Neurol* **15**, 1106-1110, doi:10.1111/j.1468-1331.2008.02262.x (2008).
22 Westerlind, H. *et al.* Modest familial risks for multiple sclerosis: a registry-based study of the population of Sweden. *Brain* **137**, 770-778, doi:10.1093/brain/awt356 (2014).
23 Robertson, N. P. *et al.* Age-adjusted recurrence risks for relatives of patients with multiple sclerosis. *Brain* **119 (Pt 2)**, 449-455, doi:10.1093/brain/119.2.449 (1996).
24 Ebers, G. C., Sadovnick, A. D. & Risch, N. J. A genetic basis for familial aggregation in multiple sclerosis. Canadian Collaborative Study Group. *Nature* **377**, 150-151, doi:10.1038/377150a0 (1995).
25 International Multiple Sclerosis Genetics, C. *et al.* Risk alleles for multiple sclerosis identified by a genomewide study. *N Engl J Med* **357**, 851-862, doi:10.1056/NEJMoa073493 (2007).
26 International Multiple Sclerosis Genetics, C. *et al.* Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* **476**, 214-219, doi:10.1038/nature10251 (2011).
27 Barcellos, L. F. *et al.* HLA-DR2 dose effect on susceptibility to multiple sclerosis and influence on disease course. *Am J Hum Genet* **72**, 710-716, doi:10.1086/367781 (2003).
28 Barcellos, L. F. *et al.* Heterogeneity at the HLA-DRB1 locus and risk for multiple sclerosis. *Hum Mol Genet* **15**, 2813-2824, doi:10.1093/hmg/ddl223 (2006).
29 Disanto, G. *et al.* HLA-DRB1 confers increased risk of pediatric-onset MS in children with acquired demyelination. *Neurology* **76**, 781-786, doi:10.1212/WNL.0b013e31820ee1cd (2011).
30 Banwell, B. *et al.* Clinical, environmental, and genetic determinants of multiple sclerosis in children with acute demyelination: a prospective national cohort study. *Lancet Neurol* **10**, 436-445, doi:10.1016/S1474-4422(11)70045-X (2011).
31 International Multiple Sclerosis Genetics, C. *et al.* Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis. *Nat Genet* **45**, 1353-1360, doi:10.1038/ng.2770 (2013).
32 Andlauer, T. F. *et al.* Novel multiple sclerosis susceptibility loci implicated in epigenetic regulation. *Sci Adv* **2**, e1501678, doi:10.1126/sciadv.1501678 (2016).
33 International Multiple Sclerosis Genetics Consortium. Electronic address, c. c. y. e. & International Multiple Sclerosis Genetics, C. Low-Frequency and Rare-Coding Variation Contributes to Multiple Sclerosis Risk. *Cell* **175**, 1679-1687 e1677, doi:10.1016/j.cell.2018.09.049 (2018).
34 International Multiple Sclerosis Genetics, C. A systems biology approach uncovers cell-specific gene regulatory effects of genetic associations in multiple sclerosis. *Nat Commun* **10**, 2236, doi:10.1038/s41467-019-09773-y (2019).

35 International Multiple Sclerosis Genetics, C. Multiple sclerosis genomic map implicates peripheral immune cells and microglia in susceptibility. *Science* **365**, doi:10.1126/science.aav7188 (2019).
36 Chapman, S. J. & Hill, A. V. Human genetic susceptibility to infectious disease. *Nat Rev Genet* **13**, 175-188, doi:10.1038/nrg3114 (2012).
37 De Jager, P. L. *et al.* The role of the CD58 locus in multiple sclerosis. *Proc Natl Acad Sci U S A* **106**, 5264-5269, doi:10.1073/pnas.0813310106 (2009).
38 Gregory, S. G. *et al.* Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. *Nat Genet* **39**, 1083-1091, doi:10.1038/ng2103 (2007).
39 Gregory, A. P. *et al.* TNF receptor 1 genetic risk mirrors outcome of anti-TNF therapy in multiple sclerosis. *Nature* **488**, 508-511, doi:10.1038/nature11307 (2012).
40 Housley, W. J. *et al.* Genetic variants associated with autoimmunity drive NFkappaB signaling and responses to inflammatory stimuli. *Sci Transl Med* **7**, 291ra293, doi:10.1126/scitranslmed.aaa9223 (2015).
41 Ponath, G. *et al.* Enhanced astrocyte responses are driven by a genetic risk allele associated with multiple sclerosis. *Nat Commun* **9**, 5337, doi:10.1038/s41467-018-07785-8 (2018).
42 Kofler, D. M., Severson, C. A., Mousissian, N., De Jager, P. L. & Hafler, D. A. The CD6 multiple sclerosis susceptibility allele is associated with alterations in CD4+ T cell proliferation. *J Immunol* **187**, 3286-3291, doi:10.4049/jimmunol.1100626 (2011).
43 Smets, I. *et al.* Multiple sclerosis risk variants alter expression of co-stimulatory genes in B cells. *Brain* **141**, 786-796, doi:10.1093/brain/awx372 (2018).
44 TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. *Neurology* **53**, 457-465 (1999).
45 van Oosten, B. W. *et al.* Increased MRI activity and immune activation in two multiple sclerosis patients treated with the monoclonal anti-tumor necrosis factor antibody cA2. *Neurology* **47**, 1531-1534, doi:10.1212/wnl.47.6.1531 (1996).
46 Probert, L. TNF and its receptors in the CNS: The essential, the desirable and the deleterious effects. *Neuroscience* **302**, 2-22, doi:10.1016/j.neuroscience.2015.06.038 (2015).
47 Steri, M. *et al.* Overexpression of the Cytokine BAFF and Autoimmunity Risk. *N Engl J Med* **376**, 1615-1626, doi:10.1056/NEJMoa1610528 (2017).
48 Alfredsson, L. & Olsson, T. Lifestyle and Environmental Factors in Multiple Sclerosis. *Cold Spring Harb Perspect Med* **9**, doi:10.1101/cshperspect.a028944 (2019).
49 Alter, M., Kahana, E. & Loewenson, R. Migration and risk of multiple sclerosis. *Neurology* **28**, 1089-1093, doi:10.1212/wnl.28.11.1089 (1978).
50 Alter, M., Leibowitz, U. & Speer, J. Risk of multiple sclerosis related to age at immigration to Israel. *Arch Neurol* **15**, 234-237 (1966).
51 Dean, G. Multiple sclerosis in migrants to South Africa. *Isr J Med Sci* **7**, 1568 (1971).
52 Hammond, S. R., English, D. R. & McLeod, J. G. The age-range of risk of developing multiple sclerosis: evidence from a migrant population in Australia. *Brain* **123 (Pt 5)**, 968-974, doi:10.1093/brain/123.5.968 (2000).

53 Gale, C. R. & Martyn, C. N. Migrant studies in multiple sclerosis. *Prog Neurobiol* **47**, 425-448 (1995).
54 Kurtzke, J. F., Beebe, G. W. & Norman, J. E., Jr. Epidemiology of multiple sclerosis in US veterans: III. Migration and the risk of MS. *Neurology* **35**, 672-678, doi:10.1212/wnl.35.5.672 (1985).
55 Baarnhielm, M. *et al.* Sunlight is associated with decreased multiple sclerosis risk: no interaction with human leukocyte antigen-DRB1*15. *Eur J Neurol* **19**, 955-962, doi:10.1111/j.1468-1331.2011.03650.x (2012).
56 Bjornevik, K. *et al.* Sun exposure and multiple sclerosis risk in Norway and Italy: The EnvIMS study. *Mult Scler* **20**, 1042-1049, doi:10.1177/1352458513513968 (2014).
57 Tremlett, H., Zhu, F., Ascherio, A. & Munger, K. L. Sun exposure over the life course and associations with multiple sclerosis. *Neurology* **90**, e1191-e1199, doi:10.1212/WNL.0000000000005257 (2018).
58 Munger, K. L., Levin, L. I., Hollis, B. W., Howard, N. S. & Ascherio, A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA* **296**, 2832-2838, doi:10.1001/jama.296.23.2832 (2006).
59 Munger, K. L. *et al.* Preclinical serum 25-hydroxyvitamin D levels and risk of type 1 diabetes in a cohort of US military personnel. *Am J Epidemiol* **177**, 411-419, doi:10.1093/aje/kws243 (2013).
60 Salzer, J. *et al.* Vitamin D as a protective factor in multiple sclerosis. *Neurology* **79**, 2140-2145, doi:10.1212/WNL.0b013e3182752ea8 (2012).
61 Mokry, L. E. *et al.* Vitamin D and Risk of Multiple Sclerosis: A Mendelian Randomization Study. *PLoS Med* **12**, e1001866, doi:10.1371/journal.pmed.1001866 (2015).
62 Zeitelhofer, M. *et al.* Functional genomics analysis of vitamin D effects on CD4+ T cells in vivo in experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* **114**, E1678-E1687, doi:10.1073/pnas.1615783114 (2017).
63 Prietl, B., Treiber, G., Pieber, T. R. & Amrein, K. Vitamin D and immune function. *Nutrients* **5**, 2502-2521, doi:10.3390/nu5072502 (2013).
64 Munger, K. L., Chitnis, T. & Ascherio, A. Body size and risk of MS in two cohorts of US women. *Neurology* **73**, 1543-1550, doi:10.1212/WNL.0b013e3181c0d6e0 (2009).
65 Munger, K. L. *et al.* Childhood body mass index and multiple sclerosis risk: a long-term cohort study. *Mult Scler* **19**, 1323-1329, doi:10.1177/1352458513483889 (2013).
66 Hedstrom, A. K., Olsson, T. & Alfredsson, L. High body mass index before age 20 is associated with increased risk for multiple sclerosis in both men and women. *Mult Scler* **18**, 1334-1336, doi:10.1177/1352458512436596 (2012).
67 Wesnes, K. *et al.* Body size and the risk of multiple sclerosis in Norway and Italy: the EnvIMS study. *Mult Scler* **21**, 388-395, doi:10.1177/1352458514546785 (2015).
68 Gianfrancesco, M. A. *et al.* Obesity during childhood and adolescence increases susceptibility to multiple sclerosis after accounting for established genetic and environmental risk factors. *Obes Res Clin Pract* **8**, e435-447, doi:10.1016/j.orcp.2014.01.002 (2014).
69 Ascherio, A. & Munger, K. L. Environmental risk factors for multiple sclerosis. Part II: Noninfectious factors. *Ann Neurol* **61**, 504-513, doi:10.1002/ana.21141 (2007).

70 Sundstrom, P. & Nystrom, L. Smoking worsens the prognosis in multiple sclerosis. *Mult Scler* **14**, 1031-1035, doi:10.1177/1352458508093615 (2008).
71 Hedstrom, A. K., Baarnhielm, M., Olsson, T. & Alfredsson, L. Tobacco smoking, but not Swedish snuff use, increases the risk of multiple sclerosis. *Neurology* **73**, 696-701, doi:10.1212/WNL.0b013e3181b59c40 (2009).
72 Ramagopalan, S. V. *et al.* Association of smoking with risk of multiple sclerosis: a population-based study. *J Neurol* **260**, 1778-1781, doi:10.1007/s00415-013-6873-7 (2013).
73 Riise, T., Nortvedt, M. W. & Ascherio, A. Smoking is a risk factor for multiple sclerosis. *Neurology* **61**, 1122-1124, doi:10.1212/01.wnl.0000081305.66687.d2 (2003).
74 Odoardi, F. *et al.* T cells become licensed in the lung to enter the central nervous system. *Nature* **488**, 675-679, doi:10.1038/nature11337 (2012).
75 Ascherio, A. & Munger, K. L. EBV and Autoimmunity. *Curr Top Microbiol Immunol* **390**, 365-385, doi:10.1007/978-3-319-22822-8_15 (2015).
76 Alotaibi, S., Kennedy, J., Tellier, R., Stephens, D. & Banwell, B. Epstein-Barr virus in pediatric multiple sclerosis. *JAMA* **291**, 1875-1879, doi:10.1001/jama.291.15.1875 (2004).
77 Banwell, B. *et al.* Clinical features and viral serologies in children with multiple sclerosis: a multinational observational study. *Lancet Neurol* **6**, 773-781, doi:10.1016/S1474-4422(07)70196-5 (2007).
78 Pohl, D. *et al.* High seroprevalence of Epstein-Barr virus in children with multiple sclerosis. *Neurology* **67**, 2063-2065, doi:10.1212/01.wnl.0000247665.94088.8d (2006).
79 Yea, C. *et al.* Epstein-Barr virus in oral shedding of children with multiple sclerosis. *Neurology* **81**, 1392-1399, doi:10.1212/WNL.0b013e3182a841e4 (2013).
80 Nielsen, T. R. *et al.* Multiple sclerosis after infectious mononucleosis. *Arch Neurol* **64**, 72-75, doi:10.1001/archneur.64.1.72 (2007).
81 Thacker, E. L., Mirzaei, F. & Ascherio, A. Infectious mononucleosis and risk for multiple sclerosis: a meta-analysis. *Ann Neurol* **59**, 499-503, doi:10.1002/ana.20820 (2006).
82 Levin, L. I., Munger, K. L., O'Reilly, E. J., Falk, K. I. & Ascherio, A. Primary infection with the Epstein-Barr virus and risk of multiple sclerosis. *Ann Neurol* **67**, 824-830, doi:10.1002/ana.21978 (2010).
83 Lunemann, J. D. *et al.* Increased frequency and broadened specificity of latent EBV nuclear antigen-1-specific T cells in multiple sclerosis. *Brain* **129**, 1493-1506, doi:10.1093/brain/awl067 (2006).
84 Cepok, S. *et al.* Identification of Epstein-Barr virus proteins as putative targets of the immune response in multiple sclerosis. *J Clin Invest* **115**, 1352-1360, doi:10.1172/JCI23661 (2005).
85 Lossius, A. *et al.* High-throughput sequencing of TCR repertoires in multiple sclerosis reveals intrathecal enrichment of EBV-reactive CD8+ T cells. *Eur J Immunol* **44**, 3439-3452, doi:10.1002/eji.201444662 (2014).
86 van Nierop, G. P. *et al.* Phenotypic and functional characterization of T cells in white matter lesions of multiple sclerosis patients. *Acta Neuropathol* **134**, 383-401, doi:10.1007/s00401-017-1744-4 (2017).
87 Munger, K. L., Levin, L. I., O'Reilly, E. J., Falk, K. I. & Ascherio, A. Anti-Epstein-Barr virus antibodies as serological markers of multiple sclerosis: a prospective study among United

States military personnel. *Mult Scler* **17**, 1185-1193, doi:10.1177/1352458511408991 (2011).

88 Pender, M. P., Csurhes, P. A., Burrows, J. M. & Burrows, S. R. Defective T-cell control of Epstein-Barr virus infection in multiple sclerosis. *Clin Transl Immunology* **6**, e126, doi:10.1038/cti.2016.87 (2017).

89 Pender, M. P., Csurhes, P. A., Lenarczyk, A., Pfluger, C. M. & Burrows, S. R. Decreased T cell reactivity to Epstein-Barr virus infected lymphoblastoid cell lines in multiple sclerosis. *J Neurol Neurosurg Psychiatry* **80**, 498-505, doi:10.1136/jnnp.2008.161018 (2009).

90 Pender, M. P., Csurhes, P. A., Pfluger, C. M. & Burrows, S. R. Decreased CD8+ T cell response to Epstein-Barr virus infected B cells in multiple sclerosis is not due to decreased HLA class I expression on B cells or monocytes. *BMC Neurol* **11**, 95, doi:10.1186/1471-2377-11-95 (2011).

91 Pender, M. P., Csurhes, P. A., Pfluger, C. M. & Burrows, S. R. CD8+ T cells far predominate over CD4+ T cells in healthy immune response to Epstein-Barr virus infected lymphoblastoid cell lines. *Blood* **120**, 5085-5087, doi:10.1182/blood-2012-06-437285 (2012).

92 Pender, M. P., Csurhes, P. A., Pfluger, C. M. & Burrows, S. R. CD8 T cell deficiency impairs control of Epstein--Barr virus and worsens with age in multiple sclerosis. *J Neurol Neurosurg Psychiatry* **83**, 353-354, doi:10.1136/jnnp-2011-300213 (2012).

93 Bar-Or, A. *et al.* Epstein-Barr Virus in Multiple Sclerosis: Theory and Emerging Immunotherapies. *Trends Mol Med*, doi:10.1016/j.molmed.2019.11.003 (2019).

94 Casiraghi, C. *et al.* Gammaherpesvirus latency accentuates EAE pathogenesis: relevance to Epstein-Barr virus and multiple sclerosis. *PLoS Pathog* **8**, e1002715, doi:10.1371/journal.ppat.1002715 (2012).

95 Lassmann, H. & Bradl, M. Multiple sclerosis: experimental models and reality. *Acta Neuropathol* **133**, 223-244, doi:10.1007/s00401-016-1631-4 (2017).

96 Kabat, E. A., Wolf, A. & Bezer, A. E. The Rapid Production of Acute Disseminated Encephalomyelitis in Rhesus Monkeys by Injection of Heterologous and Homologous Brain Tissue with Adjuvants. *J Exp Med* **85**, 117-130, doi:10.1084/jem.85.1.117 (1947).

97 Rivers, T. M. & Schwentker, F. F. Encephalomyelitis Accompanied by Myelin Destruction Experimentally Produced in Monkeys. *J Exp Med* **61**, 689-702, doi:10.1084/jem.61.5.689 (1935).

98 Paterson, P. Y. Transfer of allergic encephalomyelitis in rats by means of lymph node cells. *J Exp Med* **111**, 119-136, doi:10.1084/jem.111.1.119 (1960).

99 Arnason, B. G., Jankovic, B. D., Waksman, B. H. & Wennersten, C. Role of the thymus in immune reactions in rats. II. Suppressive effect of thymectomy at birth on reactions of delayed (cellular) hypersensitivity and the circulating small lymphocyte. *J Exp Med* **116**, 177-186, doi:10.1084/jem.116.2.177 (1962).

100 Jankovic, B. D., Waksman, B. H. & Arnason, B. G. Role of the thymus in immune ractions in rats. I. The immunologic response to bovine serum albumin (antibody formation, Arthus reactivity, and delayed hypersensitivity) in rats thymectomized or splenectomized at various times after birth. *J Exp Med* **116**, 159-176, doi:10.1084/jem.116.2.159 (1962).

101 Waksman, B. H., Arnason, B. G. & Jankovic, B. D. Role of the thymus in immune reactions in rats. III. Changes in the lymphoid organs of thymectomized rats. *J Exp Med* **116**, 187-206, doi:10.1084/jem.116.2.187 (1962).
102 Ben-Nun, A., Wekerle, H. & Cohen, I. R. The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. *Eur J Immunol* **11**, 195-199, doi:10.1002/eji.1830110307 (1981).
103 Pettinelli, C. B. & McFarlin, D. E. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1+ 2- T lymphocytes. *J Immunol* **127**, 1420-1423 (1981).
104 Sallusto, F. Heterogeneity of Human CD4(+) T Cells Against Microbes. *Annu Rev Immunol* **34**, 317-334, doi:10.1146/annurev-immunol-032414-112056 (2016).
105 O'Shea, J. J. & Paul, W. E. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science* **327**, 1098-1102, doi:10.1126/science.1178334 (2010).
106 Mosmann, T. R. & Coffman, R. L. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* **7**, 145-173, doi:10.1146/annurev.iy.07.040189.001045 (1989).
107 Bettelli, E. *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441**, 235-238, doi:10.1038/nature04753 (2006).
108 Mangan, P. R. *et al.* Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* **441**, 231-234, doi:10.1038/nature04754 (2006).
109 Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M. & Stockinger, B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* **24**, 179-189, doi:10.1016/j.immuni.2006.01.001 (2006).
110 Manel, N., Unutmaz, D. & Littman, D. R. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol* **9**, 641-649, doi:10.1038/ni.1610 (2008).
111 Volpe, E. *et al.* A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. *Nat Immunol* **9**, 650-657, doi:10.1038/ni.1613 (2008).
112 Acosta-Rodriguez, E. V., Napolitani, G., Lanzavecchia, A. & Sallusto, F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* **8**, 942-949, doi:10.1038/ni1496 (2007).
113 Korn, T. *et al.* IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* **448**, 484-487, doi:10.1038/nature05970 (2007).
114 Zhou, L. *et al.* IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* **8**, 967-974, doi:10.1038/ni1488 (2007).
115 Nurieva, R. *et al.* Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* **448**, 480-483, doi:10.1038/nature05969 (2007).
116 Ciofani, M. *et al.* A validated regulatory network for Th17 cell specification. *Cell* **151**, 289-303, doi:10.1016/j.cell.2012.09.016 (2012).

117 DuPage, M. & Bluestone, J. A. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease. *Nat Rev Immunol* **16**, 149-163, doi:10.1038/nri.2015.18 (2016).
118 Brucklacher-Waldert, V. *et al.* Phenotypical characterization of human Th17 cells unambiguously identified by surface IL-17A expression. *J Immunol* **183**, 5494-5501, doi:10.4049/jimmunol.0901000 (2009).
119 Brucklacher-Waldert, V., Stuerner, K., Kolster, M., Wolthausen, J. & Tolosa, E. Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis. *Brain* **132**, 3329-3341, doi:10.1093/brain/awp289 (2009).
120 Acosta-Rodriguez, E. V. *et al.* Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* **8**, 639-646, doi:10.1038/ni1467 (2007).
121 Reboldi, A. *et al.* C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat Immunol* **10**, 514-523, doi:10.1038/ni.1716 (2009).
122 Sato, W., Aranami, T. & Yamamura, T. Cutting edge: Human Th17 cells are identified as bearing CCR2+CCR5- phenotype. *J Immunol* **178**, 7525-7529, doi:10.4049/jimmunol.178.12.7525 (2007).
123 Singh, S. P., Zhang, H. H., Foley, J. F., Hedrick, M. N. & Farber, J. M. Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. *J Immunol* **180**, 214-221, doi:10.4049/jimmunol.180.1.214 (2008).
124 Annunziato, F. *et al.* Phenotypic and functional features of human Th17 cells. *J Exp Med* **204**, 1849-1861, doi:10.1084/jem.20070663 (2007).
125 Kleinschek, M. A. *et al.* Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation. *J Exp Med* **206**, 525-534, doi:10.1084/jem.20081712 (2009).
126 Larochelle, C. *et al.* Melanoma cell adhesion molecule identifies encephalitogenic T lymphocytes and promotes their recruitment to the central nervous system. *Brain* **135**, 2906-2924, doi:10.1093/brain/aws212 (2012).
127 Lock, C. *et al.* Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med* **8**, 500-508, doi:10.1038/nm0502-500 (2002).
128 Tzartos, J. S. *et al.* Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol* **172**, 146-155, doi:10.2353/ajpath.2008.070690 (2008).
129 Kebir, H. *et al.* Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. *Ann Neurol* **66**, 390-402, doi:10.1002/ana.21748 (2009).
130 Kebir, H. *et al.* Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med* **13**, 1173-1175, doi:10.1038/nm1651 (2007).
131 Setiadi, A. F. *et al.* IL-17A is associated with the breakdown of the blood-brain barrier in relapsing-remitting multiple sclerosis. *J Neuroimmunol* **332**, 147-154, doi:10.1016/j.jneuroim.2019.04.011 (2019).

132 Elain, G., Jeanneau, K., Rutkowska, A., Mir, A. K. & Dev, K. K. The selective anti-IL17A monoclonal antibody secukinumab (AIN457) attenuates IL17A-induced levels of IL6 in human astrocytes. *Glia* **62**, 725-735, doi:10.1002/glia.22637 (2014).
133 Kostic, M. *et al.* IL-17 and glutamate excitotoxicity in the pathogenesis of multiple sclerosis. *Scand J Immunol* **79**, 181-186, doi:10.1111/sji.12147 (2014).
134 Frisullo, G. *et al.* IL17 and IFNgamma production by peripheral blood mononuclear cells from clinically isolated syndrome to secondary progressive multiple sclerosis. *Cytokine* **44**, 22-25, doi:10.1016/j.cyto.2008.08.007 (2008).
135 Matusevicius, D. *et al.* Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult Scler* **5**, 101-104, doi:10.1177/135245859900500206 (1999).
136 Hu, D. *et al.* Transcriptional signature of human pro-inflammatory TH17 cells identifies reduced IL10 gene expression in multiple sclerosis. *Nat Commun* **8**, 1600, doi:10.1038/s41467-017-01571-8 (2017).
137 van Langelaar, J. *et al.* T helper 17.1 cells associate with multiple sclerosis disease activity: perspectives for early intervention. *Brain* **141**, 1334-1349, doi:10.1093/brain/awy069 (2018).
138 McQualter, J. L. *et al.* Granulocyte macrophage colony-stimulating factor: a new putative therapeutic target in multiple sclerosis. *J Exp Med* **194**, 873-882, doi:10.1084/jem.194.7.873 (2001).
139 El-Behi, M. *et al.* The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol* **12**, 568-575, doi:10.1038/ni.2031 (2011).
140 Codarri, L. *et al.* RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol* **12**, 560-567, doi:10.1038/ni.2027 (2011).
141 Croxford, A. L. *et al.* The Cytokine GM-CSF Drives the Inflammatory Signature of CCR2+ Monocytes and Licenses Autoimmunity. *Immunity* **43**, 502-514, doi:10.1016/j.immuni.2015.08.010 (2015).
142 Spath, S. *et al.* Dysregulation of the Cytokine GM-CSF Induces Spontaneous Phagocyte Invasion and Immunopathology in the Central Nervous System. *Immunity* **46**, 245-260, doi:10.1016/j.immuni.2017.01.007 (2017).
143 Komuczki, J. *et al.* Fate-Mapping of GM-CSF Expression Identifies a Discrete Subset of Inflammation-Driving T Helper Cells Regulated by Cytokines IL-23 and IL-1beta. *Immunity* **50**, 1289-1304 e1286, doi:10.1016/j.immuni.2019.04.006 (2019).
144 Noster, R. *et al.* IL-17 and GM-CSF expression are antagonistically regulated by human T helper cells. *Sci Transl Med* **6**, 241ra280, doi:10.1126/scitranslmed.3008706 (2014).
145 Restorick, S. M. *et al.* CCR6(+) Th cells in the cerebrospinal fluid of persons with multiple sclerosis are dominated by pathogenic non-classic Th1 cells and GM-CSF-only-secreting Th cells. *Brain Behav Immun* **64**, 71-79, doi:10.1016/j.bbi.2017.03.008 (2017).
146 Rasouli, J. *et al.* Expression of GM-CSF in T Cells Is Increased in Multiple Sclerosis and Suppressed by IFN-beta Therapy. *J Immunol* **194**, 5085-5093, doi:10.4049/jimmunol.1403243 (2015).

147 Hartmann, F. J. *et al.* Multiple sclerosis-associated IL2RA polymorphism controls GM-CSF production in human TH cells. *Nat Commun* **5**, 5056, doi:10.1038/ncomms6056 (2014).
148 Imitola, J. *et al.* Elevated expression of granulocyte-macrophage colony-stimulating factor receptor in multiple sclerosis lesions. *J Neuroimmunol* **317**, 45-54, doi:10.1016/j.jneuroim.2017.12.017 (2018).
149 Galli, E. *et al.* GM-CSF and CXCR4 define a T helper cell signature in multiple sclerosis. *Nat Med*, doi:10.1038/s41591-019-0521-4 (2019).
150 Mexhitaj, I. *et al.* Abnormal effector and regulatory T cell subsets in paediatric-onset multiple sclerosis. *Brain* **142**, 617-632, doi:10.1093/brain/awz017 (2019).
151 Martin, R. *et al.* A myelin basic protein peptide is recognized by cytotoxic T cells in the context of four HLA-DR types associated with multiple sclerosis. *J Exp Med* **173**, 19-24, doi:10.1084/jem.173.1.19 (1991).
152 Ota, K. *et al.* T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature* **346**, 183-187, doi:10.1038/346183a0 (1990).
153 Meinl, E. *et al.* Myelin basic protein-specific T lymphocyte repertoire in multiple sclerosis. Complexity of the response and dominance of nested epitopes due to recruitment of multiple T cell clones. *J Clin Invest* **92**, 2633-2643, doi:10.1172/JCI116879 (1993).
154 Sun, J. B. *et al.* Autoreactive T and B cells responding to myelin proteolipid protein in multiple sclerosis and controls. *Eur J Immunol* **21**, 1461-1468, doi:10.1002/eji.1830210620 (1991).
155 Olsson, T. *et al.* Autoreactive T lymphocytes in multiple sclerosis determined by antigen-induced secretion of interferon-gamma. *J Clin Invest* **86**, 981-985, doi:10.1172/JCI114800 (1990).
156 Zhang, J. *et al.* Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J Exp Med* **179**, 973-984, doi:10.1084/jem.179.3.973 (1994).
157 Burns, J., Rosenzweig, A., Zweiman, B. & Lisak, R. P. Isolation of myelin basic protein-reactive T-cell lines from normal human blood. *Cell Immunol* **81**, 435-440 (1983).
158 Raddassi, K. *et al.* Increased frequencies of myelin oligodendrocyte glycoprotein/MHC class II-binding CD4 cells in patients with multiple sclerosis. *J Immunol* **187**, 1039-1046, doi:10.4049/jimmunol.1001543 (2011).
159 Allegretta, M., Nicklas, J. A., Sriram, S. & Albertini, R. J. T cells responsive to myelin basic protein in patients with multiple sclerosis. *Science* **247**, 718-721 (1990).
160 Johnson, M. C. *et al.* Distinct T cell signatures define subsets of patients with multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm* **3**, e278, doi:10.1212/NXI.0000000000000278 (2016).
161 Banwell, B. *et al.* Abnormal T-cell reactivities in childhood inflammatory demyelinating disease and type 1 diabetes. *Ann Neurol* **63**, 98-111, doi:10.1002/ana.21244 (2008).
162 Scholz, C., Patton, K. T., Anderson, D. E., Freeman, G. J. & Hafler, D. A. Expansion of autoreactive T cells in multiple sclerosis is independent of exogenous B7 costimulation. *J Immunol* **160**, 1532-1538 (1998).

163 Lovett-Racke, A. E. *et al.* Decreased dependence of myelin basic protein-reactive T cells on CD28-mediated costimulation in multiple sclerosis patients. A marker of activated/memory T cells. *J Clin Invest* **101**, 725-730, doi:10.1172/JCI1528 (1998).
164 Hedegaard, C. J. *et al.* T helper cell type 1 (Th1), Th2 and Th17 responses to myelin basic protein and disease activity in multiple sclerosis. *Immunology* **125**, 161-169, doi:10.1111/j.1365-2567.2008.02837.x (2008).
165 Cao, Y. *et al.* Functional inflammatory profiles distinguish myelin-reactive T cells from patients with multiple sclerosis. *Sci Transl Med* **7**, 287ra274, doi:10.1126/scitranslmed.aaa8038 (2015).
166 Dhaunchak, A. S. *et al.* Implication of perturbed axoglial apparatus in early pediatric multiple sclerosis. *Ann Neurol* **71**, 601-613, doi:10.1002/ana.22693 (2012).
167 Planas, R. *et al.* GDP-l-fucose synthase is a CD4(+) T cell-specific autoantigen in DRB3*02:02 patients with multiple sclerosis. *Sci Transl Med* **10**, doi:10.1126/scitranslmed.aat4301 (2018).
168 Ruhl, G. *et al.* Multiple sclerosis: Molecular mimicry of an antimyelin HLA class I restricted T-cell receptor. *Neurol Neuroimmunol Neuroinflamm* **3**, e241, doi:10.1212/NXI.0000000000000241 (2016).
169 Ito, Y. *et al.* Detection of T cell responses to a ubiquitous cellular protein in autoimmune disease. *Science* **346**, 363-368, doi:10.1126/science.1259077 (2014).
170 Wing, J. B., Tanaka, A. & Sakaguchi, S. Human FOXP3(+) Regulatory T Cell Heterogeneity and Function in Autoimmunity and Cancer. *Immunity* **50**, 302-316, doi:10.1016/j.immuni.2019.01.020 (2019).
171 Yamaguchi, T., Wing, J. B. & Sakaguchi, S. Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions. *Semin Immunol* **23**, 424-430, doi:10.1016/j.smim.2011.10.002 (2011).
172 Josefowicz, S. Z., Lu, L. F. & Rudensky, A. Y. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* **30**, 531-564, doi:10.1146/annurev.immunol.25.022106.141623 (2012).
173 Bennett, C. L. *et al.* The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* **27**, 20-21, doi:10.1038/83713 (2001).
174 Brunkow, M. E. *et al.* Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* **27**, 68-73, doi:10.1038/83784 (2001).
175 Wildin, R. S. *et al.* X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* **27**, 18-20, doi:10.1038/83707 (2001).
176 Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* **4**, 330-336, doi:10.1038/ni904 (2003).
177 Hori, S., Nomura, T. & Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057-1061, doi:10.1126/science.1079490 (2003).
178 Khattri, R., Cox, T., Yasayko, S. A. & Ramsdell, F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* **4**, 337-342, doi:10.1038/ni909 (2003).

179 Kim, J. M., Rasmussen, J. P. & Rudensky, A. Y. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol* **8**, 191-197, doi:10.1038/ni1428 (2007).
180 Itoh, M. *et al.* Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* **162**, 5317-5326 (1999).
181 Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* **155**, 1151-1164 (1995).
182 Asano, M., Toda, M., Sakaguchi, N. & Sakaguchi, S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* **184**, 387-396, doi:10.1084/jem.184.2.387 (1996).
183 Chinen, T., Volchkov, P. Y., Chervonsky, A. V. & Rudensky, A. Y. A critical role for regulatory T cell-mediated control of inflammation in the absence of commensal microbiota. *J Exp Med* **207**, 2323-2330, doi:10.1084/jem.20101235 (2010).
184 Kim, H. J. *et al.* Stable inhibitory activity of regulatory T cells requires the transcription factor Helios. *Science* **350**, 334-339, doi:10.1126/science.aad0616 (2015).
185 Yi, J. *et al.* Unregulated antigen-presenting cell activation by T cells breaks self tolerance. *Proc Natl Acad Sci U S A* **116**, 1007-1016, doi:10.1073/pnas.1818624116 (2019).
186 Hsieh, C. S., Zheng, Y., Liang, Y., Fontenot, J. D. & Rudensky, A. Y. An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat Immunol* **7**, 401-410, doi:10.1038/ni1318 (2006).
187 Levine, A. G. *et al.* Suppression of lethal autoimmunity by regulatory T cells with a single TCR specificity. *J Exp Med* **214**, 609-622, doi:10.1084/jem.20161318 (2017).
188 Sun, C. M. *et al.* Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* **204**, 1775-1785, doi:10.1084/jem.20070602 (2007).
189 Maynard, C. L. *et al.* Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nat Immunol* **8**, 931-941, doi:10.1038/ni1504 (2007).
190 Levings, M. K., Sangregorio, R. & Roncarolo, M. G. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* **193**, 1295-1302, doi:10.1084/jem.193.11.1295 (2001).
191 Dieckmann, D., Plottner, H., Berchtold, S., Berger, T. & Schuler, G. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J Exp Med* **193**, 1303-1310, doi:10.1084/jem.193.11.1303 (2001).
192 Baecher-Allan, C., Brown, J. A., Freeman, G. J. & Hafler, D. A. CD4+CD25high regulatory cells in human peripheral blood. *J Immunol* **167**, 1245-1253, doi:10.4049/jimmunol.167.3.1245 (2001).
193 Ng, W. F. *et al.* Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood* **98**, 2736-2744 (2001).
194 Lombardi, G., Sidhu, S., Batchelor, R. & Lechler, R. Anergic T cells as suppressor cells in vitro. *Science* **264**, 1587-1589 (1994).

195 Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. & Muller, W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**, 263-274 (1993).
196 Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. & Powrie, F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* **190**, 995-1004, doi:10.1084/jem.190.7.995 (1999).
197 Rubtsov, Y. P. *et al.* Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* **28**, 546-558, doi:10.1016/j.immuni.2008.02.017 (2008).
198 Chaudhry, A. *et al.* Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity* **34**, 566-578, doi:10.1016/j.immuni.2011.03.018 (2011).
199 Huber, S. *et al.* Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. *Immunity* **34**, 554-565, doi:10.1016/j.immuni.2011.01.020 (2011).
200 Bettelli, E. *et al.* IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J Immunol* **161**, 3299-3306 (1998).
201 Cua, D. J., Groux, H., Hinton, D. R., Stohlman, S. A. & Coffman, R. L. Transgenic interleukin 10 prevents induction of experimental autoimmune encephalomyelitis. *J Exp Med* **189**, 1005-1010, doi:10.1084/jem.189.6.1005 (1999).
202 Zhang, X. *et al.* IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+CD4+ regulatory T cells. *Int Immunol* **16**, 249-256, doi:10.1093/intimm/dxh029 (2004).
203 Chaudhry, A. *et al.* CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science* **326**, 986-991, doi:10.1126/science.1172702 (2009).
204 Esplugues, E. *et al.* Control of TH17 cells occurs in the small intestine. *Nature* **475**, 514-518, doi:10.1038/nature10228 (2011).
205 Atarashi, K. *et al.* Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* **331**, 337-341, doi:10.1126/science.1198469 (2011).
206 Liu, W. *et al.* CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* **203**, 1701-1711, doi:10.1084/jem.20060772 (2006).
207 Seddiki, N. *et al.* Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* **203**, 1693-1700, doi:10.1084/jem.20060468 (2006).
208 Mason, G. M. *et al.* Phenotypic Complexity of the Human Regulatory T Cell Compartment Revealed by Mass Cytometry. *J Immunol* **195**, 2030-2037, doi:10.4049/jimmunol.1500703 (2015).
209 Kunicki, M. A., Amaya Hernandez, L. C., Davis, K. L., Bacchetta, R. & Roncarolo, M. G. Identity and Diversity of Human Peripheral Th and T Regulatory Cells Defined by Single-Cell Mass Cytometry. *J Immunol* **200**, 336-346, doi:10.4049/jimmunol.1701025 (2018).
210 Zemmour, D. *et al.* Single-cell gene expression reveals a landscape of regulatory T cell phenotypes shaped by the TCR. *Nat Immunol* **19**, 291-301, doi:10.1038/s41590-018-0051-0 (2018).

211 Miyara, M. *et al.* Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* **30**, 899-911, doi:10.1016/j.immuni.2009.03.019 (2009).
212 Ito, T. *et al.* Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. *Immunity* **28**, 870-880, doi:10.1016/j.immuni.2008.03.018 (2008).
213 Dong, S. *et al.* Multiparameter single-cell profiling of human CD4+FOXP3+ regulatory T-cell populations in homeostatic conditions and during graft-versus-host disease. *Blood* **122**, 1802-1812, doi:10.1182/blood-2013-02-482539 (2013).
214 Bin Dhuban, K. *et al.* Coexpression of TIGIT and FCRL3 identifies Helios+ human memory regulatory T cells. *J Immunol* **194**, 3687-3696, doi:10.4049/jimmunol.1401803 (2015).
215 Voo, K. S. *et al.* Identification of IL-17-producing FOXP3+ regulatory T cells in humans. *Proc Natl Acad Sci U S A* **106**, 4793-4798, doi:10.1073/pnas.0900408106 (2009).
216 Beriou, G. *et al.* IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood* **113**, 4240-4249, doi:10.1182/blood-2008-10-183251 (2009).
217 Duhen, T., Duhen, R., Lanzavecchia, A., Sallusto, F. & Campbell, D. J. Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells. *Blood* **119**, 4430-4440, doi:10.1182/blood-2011-11-392324 (2012).
218 Halim, L. *et al.* An Atlas of Human Regulatory T Helper-like Cells Reveals Features of Th2-like Tregs that Support a Tumorigenic Environment. *Cell Rep* **20**, 757-770, doi:10.1016/j.celrep.2017.06.079 (2017).
219 Cuadrado, E. *et al.* Proteomic Analyses of Human Regulatory T Cells Reveal Adaptations in Signaling Pathways that Protect Cellular Identity. *Immunity* **48**, 1046-1059 e1046, doi:10.1016/j.immuni.2018.04.008 (2018).
220 Koch, M. A. *et al.* The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* **10**, 595-602, doi:10.1038/ni.1731 (2009).
221 Koch, M. A. *et al.* T-bet(+) Treg cells undergo abortive Th1 cell differentiation due to impaired expression of IL-12 receptor beta2. *Immunity* **37**, 501-510, doi:10.1016/j.immuni.2012.05.031 (2012).
222 Levine, A. G. *et al.* Stability and function of regulatory T cells expressing the transcription factor T-bet. *Nature* **546**, 421-425, doi:10.1038/nature22360 (2017).
223 Ohnmacht, C. *et al.* MUCOSAL IMMUNOLOGY. The microbiota regulates type 2 immunity through RORgammat(+) T cells. *Science* **349**, 989-993, doi:10.1126/science.aac4263 (2015).
224 Sefik, E. *et al.* MUCOSAL IMMUNOLOGY. Individual intestinal symbionts induce a distinct population of RORgamma(+) regulatory T cells. *Science* **349**, 993-997, doi:10.1126/science.aaa9420 (2015).
225 Garg, G. *et al.* Blimp1 Prevents Methylation of Foxp3 and Loss of Regulatory T Cell Identity at Sites of Inflammation. *Cell Rep* **26**, 1854-1868 e1855, doi:10.1016/j.celrep.2019.01.070 (2019).
226 Overacre-Delgoffe, A. E. *et al.* Interferon-gamma Drives Treg Fragility to Promote Anti-tumor Immunity. *Cell* **169**, 1130-1141 e1111, doi:10.1016/j.cell.2017.05.005 (2017).
227 Dominguez-Villar, M. & Hafler, D. A. Regulatory T cells in autoimmune disease. *Nat Immunol* **19**, 665-673, doi:10.1038/s41590-018-0120-4 (2018).

228 Balint, B. *et al.* T-cell homeostasis in pediatric multiple sclerosis: old cells in young patients. *Neurology* **81**, 784-792, doi:10.1212/WNL.0b013e3182a2ce0e (2013).
229 Hug, A. *et al.* Thymic export function and T cell homeostasis in patients with relapsing remitting multiple sclerosis. *J Immunol* **171**, 432-437, doi:10.4049/jimmunol.171.1.432 (2003).
230 Haas, J. *et al.* Prevalence of newly generated naive regulatory T cells (Treg) is critical for Treg suppressive function and determines Treg dysfunction in multiple sclerosis. *J Immunol* **179**, 1322-1330, doi:10.4049/jimmunol.179.2.1322 (2007).
231 Haas, J. *et al.* Fingolimod does not impair T-cell release from the thymus and beneficially affects Treg function in patients with multiple sclerosis. *Mult Scler* **21**, 1521-1532, doi:10.1177/1352458514564589 (2015).
232 Haas, J., Korporal, M., Schwarz, A., Balint, B. & Wildemann, B. The interleukin-7 receptor alpha chain contributes to altered homeostasis of regulatory T cells in multiple sclerosis. *Eur J Immunol* **41**, 845-853, doi:10.1002/eji.201041139 (2011).
233 Venken, K. *et al.* Natural naive CD4+CD25+CD127low regulatory T cell (Treg) development and function are disturbed in multiple sclerosis patients: recovery of memory Treg homeostasis during disease progression. *J Immunol* **180**, 6411-6420, doi:10.4049/jimmunol.180.9.6411 (2008).
234 Haas, J. *et al.* Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. *Eur J Immunol* **35**, 3343-3352, doi:10.1002/eji.200526065 (2005).
235 Feger, U. *et al.* Increased frequency of CD4+ CD25+ regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients. *Clin Exp Immunol* **147**, 412-418, doi:10.1111/j.1365-2249.2006.03271.x (2007).
236 Venken, K. *et al.* Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. *Immunology* **123**, 79-89, doi:10.1111/j.1365-2567.2007.02690.x (2008).
237 Frisullo, G. *et al.* Regulatory T cells fail to suppress CD4T+-bet+ T cells in relapsing multiple sclerosis patients. *Immunology* **127**, 418-428, doi:10.1111/j.1365-2567.2008.02963.x (2009).
238 Kumar, M. *et al.* CD4+CD25+FoxP3+ T lymphocytes fail to suppress myelin basic protein-induced proliferation in patients with multiple sclerosis. *J Neuroimmunol* **180**, 178-184, doi:10.1016/j.jneuroim.2006.08.003 (2006).
239 Viglietta, V., Baecher-Allan, C., Weiner, H. L. & Hafler, D. A. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med* **199**, 971-979, doi:10.1084/jem.20031579 (2004).
240 Huan, J. *et al.* Decreased FOXP3 levels in multiple sclerosis patients. *J Neurosci Res* **81**, 45-52, doi:10.1002/jnr.20522 (2005).
241 Hill, J. A. *et al.* Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* **27**, 786-800, doi:10.1016/j.immuni.2007.09.010 (2007).
242 Gavin, M. A. *et al.* Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* **445**, 771-775, doi:10.1038/nature05543 (2007).

243 Fletcher, J. M. *et al.* CD39+Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis. *J Immunol* **183**, 7602-7610, doi:10.4049/jimmunol.0901881 (2009).
244 Bopp, T. *et al.* Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* **204**, 1303-1310, doi:10.1084/jem.20062129 (2007).
245 Deaglio, S. *et al.* Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* **204**, 1257-1265, doi:10.1084/jem.20062512 (2007).
246 Cekic, C. & Linden, J. Purinergic regulation of the immune system. *Nat Rev Immunol* **16**, 177-192, doi:10.1038/nri.2016.4 (2016).
247 Borsellino, G. *et al.* Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* **110**, 1225-1232, doi:10.1182/blood-2006-12-064527 (2007).
248 Astier, A. L., Meiffren, G., Freeman, S. & Hafler, D. A. Alterations in CD46-mediated Tr1 regulatory T cells in patients with multiple sclerosis. *J Clin Invest* **116**, 3252-3257, doi:10.1172/JCI29251 (2006).
249 Martinez-Forero, I. *et al.* IL-10 suppressor activity and ex vivo Tr1 cell function are impaired in multiple sclerosis. *Eur J Immunol* **38**, 576-586, doi:10.1002/eji.200737271 (2008).
250 Dominguez-Villar, M., Baecher-Allan, C. M. & Hafler, D. A. Identification of T helper type 1-like, Foxp3+ regulatory T cells in human autoimmune disease. *Nat Med* **17**, 673-675, doi:10.1038/nm.2389 (2011).
251 Sumida, T. *et al.* Activated beta-catenin in Foxp3(+) regulatory T cells links inflammatory environments to autoimmunity. *Nat Immunol* **19**, 1391-1402, doi:10.1038/s41590-018-0236-6 (2018).
252 Kitz, A. *et al.* AKT isoforms modulate Th1-like Treg generation and function in human autoimmune disease. *EMBO Rep* **17**, 1169-1183, doi:10.15252/embr.201541905 (2016).
253 Lucca, L. E. *et al.* TIGIT signaling restores suppressor function of Th1 Tregs. *JCI Insight* **4**, doi:10.1172/jci.insight.124427 (2019).
254 Kleinewietfeld, M. *et al.* Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. *Nature* **496**, 518-522, doi:10.1038/nature11868 (2013).
255 Hernandez, A. L. *et al.* Sodium chloride inhibits the suppressive function of FOXP3+ regulatory T cells. *J Clin Invest* **125**, 4212-4222, doi:10.1172/JCI81151 (2015).
256 Wu, C. *et al.* Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. *Nature* **496**, 513-517, doi:10.1038/nature11984 (2013).
257 Fitzgerald, K. C. *et al.* Sodium intake and multiple sclerosis activity and progression in BENEFIT. *Ann Neurol* **82**, 20-29, doi:10.1002/ana.24965 (2017).
258 Cortese, M., Yuan, C., Chitnis, T., Ascherio, A. & Munger, K. L. No association between dietary sodium intake and the risk of multiple sclerosis. *Neurology* **89**, 1322-1329, doi:10.1212/WNL.0000000000004417 (2017).
259 Farez, M. F. *et al.* Melatonin Contributes to the Seasonality of Multiple Sclerosis Relapses. *Cell* **162**, 1338-1352, doi:10.1016/j.cell.2015.08.025 (2015).
260 Jangi, S. *et al.* Alterations of the human gut microbiome in multiple sclerosis. *Nat Commun* **7**, 12015, doi:10.1038/ncomms12015 (2016).

261 Berer, K. *et al.* Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. *Proc Natl Acad Sci U S A* **114**, 10719-10724, doi:10.1073/pnas.1711233114 (2017).
262 Chen, J. *et al.* Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls. *Sci Rep* **6**, 28484, doi:10.1038/srep28484 (2016).
263 Cekanaviciute, E. *et al.* Gut bacteria from multiple sclerosis patients modulate human T cells and exacerbate symptoms in mouse models. *Proc Natl Acad Sci U S A* **114**, 10713-10718, doi:10.1073/pnas.1711235114 (2017).
264 Berer, K. *et al.* Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* **479**, 538-541, doi:10.1038/nature10554 (2011).
265 Lee, Y. K., Menezes, J. S., Umesaki, Y. & Mazmanian, S. K. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* **108 Suppl 1**, 4615-4622, doi:10.1073/pnas.1000082107 (2011).
266 Cosorich, I. *et al.* High frequency of intestinal TH17 cells correlates with microbiota alterations and disease activity in multiple sclerosis. *Sci Adv* **3**, e1700492, doi:10.1126/sciadv.1700492 (2017).
267 Mangalam, A. *et al.* Human Gut-Derived Commensal Bacteria Suppress CNS Inflammatory and Demyelinating Disease. *Cell Rep* **20**, 1269-1277, doi:10.1016/j.celrep.2017.07.031 (2017).
268 Tremlett, H. *et al.* Associations between the gut microbiota and host immune markers in pediatric multiple sclerosis and controls. *BMC Neurol* **16**, 182, doi:10.1186/s12883-016-0703-3 (2016).
269 Tremlett, H. *et al.* Gut microbiota composition and relapse risk in pediatric MS: A pilot study. *J Neurol Sci* **363**, 153-157, doi:10.1016/j.jns.2016.02.042 (2016).
270 Tremlett, H. *et al.* Gut microbiota in early pediatric multiple sclerosis: a case-control study. *Eur J Neurol* **23**, 1308-1321, doi:10.1111/ene.13026 (2016).
271 Li, R., Patterson, K. R. & Bar-Or, A. Reassessing B cell contributions in multiple sclerosis. *Nat Immunol* **19**, 696-707, doi:10.1038/s41590-018-0135-x (2018).
272 Bar-Or, A. *et al.* Rituximab in relapsing-remitting multiple sclerosis: a 72-week, open-label, phase I trial. *Ann Neurol* **63**, 395-400, doi:10.1002/ana.21363 (2008).
273 Hauser, S. L. *et al.* B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med* **358**, 676-688, doi:10.1056/NEJMoa0706383 (2008).
274 Hauser, S. L. *et al.* Ocrelizumab versus Interferon Beta-1a in Relapsing Multiple Sclerosis. *N Engl J Med* **376**, 221-234, doi:10.1056/NEJMoa1601277 (2017).
275 Montalban, X. *et al.* Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis. *N Engl J Med* **376**, 209-220, doi:10.1056/NEJMoa1606468 (2017).
276 Bar-Or, A. *et al.* Subcutaneous ofatumumab in patients with relapsing-remitting multiple sclerosis: The MIRROR study. *Neurology* **90**, e1805-e1814, doi:10.1212/WNL.0000000000005516 (2018).
277 Kappos, L. *et al.* Atacicept in multiple sclerosis (ATAMS): a randomised, placebo-controlled, double-blind, phase 2 trial. *Lancet Neurol* **13**, 353-363, doi:10.1016/S1474-4422(14)70028-6 (2014).

278 Cross, A. H., Stark, J. L., Lauber, J., Ramsbottom, M. J. & Lyons, J. A. Rituximab reduces B cells and T cells in cerebrospinal fluid of multiple sclerosis patients. *J Neuroimmunol* **180**, 63-70, doi:10.1016/j.jneuroim.2006.06.029 (2006).
279 Piccio, L. *et al.* Changes in B- and T-lymphocyte and chemokine levels with rituximab treatment in multiple sclerosis. *Arch Neurol* **67**, 707-714, doi:10.1001/archneurol.2010.99 (2010).
280 Stuve, O. *et al.* Long-term B-lymphocyte depletion with rituximab in patients with relapsing-remitting multiple sclerosis. *Arch Neurol* **66**, 259-261, doi:10.1001/archneurol.2008.551 (2009).
281 Li, R. *et al.* Proinflammatory GM-CSF-producing B cells in multiple sclerosis and B cell depletion therapy. *Sci Transl Med* **7**, 310ra166, doi:10.1126/scitranslmed.aab4176 (2015).
282 Bar-Or, A. *et al.* Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? *Ann Neurol* **67**, 452-461, doi:10.1002/ana.21939 (2010).
283 Duddy, M. *et al.* Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. *J Immunol* **178**, 6092-6099, doi:10.4049/jimmunol.178.10.6092 (2007).
284 Pieper, K., Grimbacher, B. & Eibel, H. B-cell biology and development. *J Allergy Clin Immunol* **131**, 959-971, doi:10.1016/j.jaci.2013.01.046 (2013).
285 Rickert, R. C., Jellusova, J. & Miletic, A. V. Signaling by the tumor necrosis factor receptor superfamily in B-cell biology and disease. *Immunol Rev* **244**, 115-133, doi:10.1111/j.1600-065X.2011.01067.x (2011).
286 Dall'Era, M. *et al.* Reduced B lymphocyte and immunoglobulin levels after atacicept treatment in patients with systemic lupus erythematosus: results of a multicenter, phase Ib, double-blind, placebo-controlled, dose-escalating trial. *Arthritis Rheum* **56**, 4142-4150, doi:10.1002/art.23047 (2007).
287 Hartung, H. P. & Kieseier, B. C. Atacicept: targeting B cells in multiple sclerosis. *Ther Adv Neurol Disord* **3**, 205-216, doi:10.1177/1756285610371146 (2010).
288 Meffre, E. The establishment of early B cell tolerance in humans: lessons from primary immunodeficiency diseases. *Ann N Y Acad Sci* **1246**, 1-10, doi:10.1111/j.1749-6632.2011.06347.x (2011).
289 Herve, M. *et al.* CD40 ligand and MHC class II expression are essential for human peripheral B cell tolerance. *J Exp Med* **204**, 1583-1593, doi:10.1084/jem.20062287 (2007).
290 Sng, J. *et al.* AIRE expression controls the peripheral selection of autoreactive B cells. *Sci Immunol* **4**, doi:10.1126/sciimmunol.aav6778 (2019).
291 Kinnunen, T. *et al.* Accumulation of peripheral autoreactive B cells in the absence of functional human regulatory T cells. *Blood* **121**, 1595-1603, doi:10.1182/blood-2012-09-457465 (2013).
292 Kinnunen, T. *et al.* Specific peripheral B cell tolerance defects in patients with multiple sclerosis. *J Clin Invest* **123**, 2737-2741, doi:10.1172/JCI68775 (2013).
293 Dhaeze, T. *et al.* Circulating Follicular Regulatory T Cells Are Defective in Multiple Sclerosis. *J Immunol* **195**, 832-840, doi:10.4049/jimmunol.1500759 (2015).
294 Lassmann, H. Multiple Sclerosis Pathology. *Cold Spring Harb Perspect Med* **8**, doi:10.1101/cshperspect.a028936 (2018).

295 Prineas, J. W. & Graham, J. S. Multiple sclerosis: capping of surface immunoglobulin G on macrophages engaged in myelin breakdown. *Ann Neurol* **10**, 149-158, doi:10.1002/ana.410100205 (1981).
296 Genain, C. P., Cannella, B., Hauser, S. L. & Raine, C. S. Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nat Med* **5**, 170-175, doi:10.1038/5532 (1999).
297 Lucchinetti, C. *et al.* Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol* **47**, 707-717 (2000).
298 Popescu, B. F. & Lucchinetti, C. F. Pathology of demyelinating diseases. *Annu Rev Pathol* **7**, 185-217, doi:10.1146/annurev-pathol-011811-132443 (2012).
299 Colombo, M. *et al.* Accumulation of clonally related B lymphocytes in the cerebrospinal fluid of multiple sclerosis patients. *J Immunol* **164**, 2782-2789, doi:10.4049/jimmunol.164.5.2782 (2000).
300 Villar, L. M. *et al.* Early differential diagnosis of multiple sclerosis using a new oligoclonal band test. *Arch Neurol* **62**, 574-577, doi:10.1001/archneur.62.4.574 (2005).
301 Villar, L. M. *et al.* Intrathecal synthesis of oligoclonal IgM against myelin lipids predicts an aggressive disease course in MS. *J Clin Invest* **115**, 187-194, doi:10.1172/JCI22833 (2005).
302 Villar, L. M. *et al.* Lipid-specific immunoglobulin M bands in cerebrospinal fluid are associated with a reduced risk of developing progressive multifocal leukoencephalopathy during treatment with natalizumab. *Ann Neurol* **77**, 447-457, doi:10.1002/ana.24345 (2015).
303 Villar, L. M. *et al.* Immunoglobulin M oligoclonal bands: biomarker of targetable inflammation in primary progressive multiple sclerosis. *Ann Neurol* **76**, 231-240, doi:10.1002/ana.24190 (2014).
304 Villar, L. M. *et al.* Immunological markers of optimal response to natalizumab in multiple sclerosis. *Arch Neurol* **69**, 191-197, doi:10.1001/archneurol.2011.971 (2012).
305 Joseph, F. G. *et al.* CSF oligoclonal band status informs prognosis in multiple sclerosis: a case control study of 100 patients. *J Neurol Neurosurg Psychiatry* **80**, 292-296, doi:10.1136/jnnp.2008.150896 (2009).
306 Obermeier, B. *et al.* Matching of oligoclonal immunoglobulin transcriptomes and proteomes of cerebrospinal fluid in multiple sclerosis. *Nat Med* **14**, 688-693, doi:10.1038/nm1714 (2008).
307 von Budingen, H. C., Harrer, M. D., Kuenzle, S., Meier, M. & Goebels, N. Clonally expanded plasma cells in the cerebrospinal fluid of MS patients produce myelin-specific antibodies. *Eur J Immunol* **38**, 2014-2023, doi:10.1002/eji.200737784 (2008).
308 von Budingen, H. C. *et al.* Clonally expanded plasma cells in the cerebrospinal fluid of patients with central nervous system autoimmune demyelination produce "oligoclonal bands". *J Neuroimmunol* **218**, 134-139, doi:10.1016/j.jneuroim.2009.10.005 (2010).
309 Blauth, K. *et al.* Antibodies produced by clonally expanded plasma cells in multiple sclerosis cerebrospinal fluid cause demyelination of spinal cord explants. *Acta Neuropathol* **130**, 765-781, doi:10.1007/s00401-015-1500-6 (2015).
310 Brandle, S. M. *et al.* Distinct oligoclonal band antibodies in multiple sclerosis recognize ubiquitous self-proteins. *Proc Natl Acad Sci U S A* **113**, 7864-7869, doi:10.1073/pnas.1522730113 (2016).

311 Vermeulen, N. *et al.* Identification of a novel autoantigen in inflammatory bowel disease by protein microarray. *Inflamm Bowel Dis* **17**, 1291-1300, doi:10.1002/ibd.21508 (2011).
312 Ho, P. P. *et al.* Identification of naturally occurring fatty acids of the myelin sheath that resolve neuroinflammation. *Sci Transl Med* **4**, 137ra173, doi:10.1126/scitranslmed.3003831 (2012).
313 Spadaro, M. *et al.* Autoantibodies to MOG in a distinct subgroup of adult multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm* **3**, e257, doi:10.1212/NXI.0000000000000257 (2016).
314 Link, H. *et al.* Persistent anti-myelin basic protein IgG antibody response in multiple sclerosis cerebrospinal fluid. *J Neuroimmunol* **28**, 237-248 (1990).
315 Xiao, B. G., Linington, C. & Link, H. Antibodies to myelin-oligodendrocyte glycoprotein in cerebrospinal fluid from patients with multiple sclerosis and controls. *J Neuroimmunol* **31**, 91-96 (1991).
316 Srivastava, R. *et al.* Potassium channel KIR4.1 as an immune target in multiple sclerosis. *N Engl J Med* **367**, 115-123, doi:10.1056/NEJMoa1110740 (2012).
317 Brickshawana, A. *et al.* Investigation of the KIR4.1 potassium channel as a putative antigen in patients with multiple sclerosis: a comparative study. *Lancet Neurol* **13**, 795-806, doi:10.1016/S1474-4422(14)70141-3 (2014).
318 Nerrant, E. *et al.* Lack of confirmation of anti-inward rectifying potassium channel 4.1 antibodies as reliable markers of multiple sclerosis. *Mult Scler* **20**, 1699-1703, doi:10.1177/1352458514531086 (2014).
319 Colombo, M. *et al.* Maintenance of B lymphocyte-related clones in the cerebrospinal fluid of multiple sclerosis patients. *Eur J Immunol* **33**, 3433-3438, doi:10.1002/eji.200324144 (2003).
320 Qin, Y. *et al.* Intrathecal B-cell clonal expansion, an early sign of humoral immunity, in the cerebrospinal fluid of patients with clinically isolated syndrome suggestive of multiple sclerosis. *Lab Invest* **83**, 1081-1088 (2003).
321 Qin, Y. *et al.* Clonal expansion and somatic hypermutation of V(H) genes of B cells from cerebrospinal fluid in multiple sclerosis. *J Clin Invest* **102**, 1045-1050, doi:10.1172/JCI3568 (1998).
322 Baranzini, S. E. *et al.* B cell repertoire diversity and clonal expansion in multiple sclerosis brain lesions. *J Immunol* **163**, 5133-5144 (1999).
323 Lovato, L. *et al.* Related B cell clones populate the meninges and parenchyma of patients with multiple sclerosis. *Brain* **134**, 534-541, doi:10.1093/brain/awq350 (2011).
324 Eggers, E. L. *et al.* Clonal relationships of CSF B cells in treatment-naive multiple sclerosis patients. *JCI Insight* **2**, doi:10.1172/jci.insight.92724 (2017).
325 von Budingen, H. C. *et al.* B cell exchange across the blood-brain barrier in multiple sclerosis. *J Clin Invest* **122**, 4533-4543, doi:10.1172/JCI63842 (2012).
326 Owens, G. P. *et al.* Single-cell repertoire analysis demonstrates that clonal expansion is a prominent feature of the B cell response in multiple sclerosis cerebrospinal fluid. *J Immunol* **171**, 2725-2733, doi:10.4049/jimmunol.171.5.2725 (2003).
327 Palanichamy, A. *et al.* Immunoglobulin class-switched B cells form an active immune axis between CNS and periphery in multiple sclerosis. *Sci Transl Med* **6**, 248ra106, doi:10.1126/scitranslmed.3008930 (2014).

328 Stern, J. N. *et al.* B cells populating the multiple sclerosis brain mature in the draining cervical lymph nodes. *Sci Transl Med* **6**, 248ra107, doi:10.1126/scitranslmed.3008879 (2014).
329 Bankoti, J. *et al.* In multiple sclerosis, oligoclonal bands connect to peripheral B-cell responses. *Ann Neurol* **75**, 266-276, doi:10.1002/ana.24088 (2014).
330 von Budingen, H. C., Bar-Or, A. & Zamvil, S. S. B cells in multiple sclerosis: connecting the dots. *Curr Opin Immunol* **23**, 713-720, doi:10.1016/j.coi.2011.09.003 (2011).
331 Kwak, K., Akkaya, M. & Pierce, S. K. B cell signaling in context. *Nat Immunol* **20**, 963-969, doi:10.1038/s41590-019-0427-9 (2019).
332 Cyster, J. G. & Allen, C. D. C. B Cell Responses: Cell Interaction Dynamics and Decisions. *Cell* **177**, 524-540, doi:10.1016/j.cell.2019.03.016 (2019).
333 Pollinger, B. *et al.* Spontaneous relapsing-remitting EAE in the SJL/J mouse: MOG-reactive transgenic T cells recruit endogenous MOG-specific B cells. *J Exp Med* **206**, 1303-1316, doi:10.1084/jem.20090299 (2009).
334 Krishnamoorthy, G., Lassmann, H., Wekerle, H. & Holz, A. Spontaneous opticospinal encephalomyelitis in a double-transgenic mouse model of autoimmune T cell/B cell cooperation. *J Clin Invest* **116**, 2385-2392, doi:10.1172/JCI28330 (2006).
335 Molnarfi, N. *et al.* MHC class II-dependent B cell APC function is required for induction of CNS autoimmunity independent of myelin-specific antibodies. *J Exp Med* **210**, 2921-2937, doi:10.1084/jem.20130699 (2013).
336 Genc, K., Dona, D. L. & Reder, A. T. Increased CD80(+) B cells in active multiple sclerosis and reversal by interferon beta-1b therapy. *J Clin Invest* **99**, 2664-2671, doi:10.1172/JCI119455 (1997).
337 Mathias, A. *et al.* Increased ex vivo antigen presentation profile of B cells in multiple sclerosis. *Mult Scler* **23**, 802-809, doi:10.1177/1352458516664210 (2017).
338 Opelz, G., Kiuchi, M., Takasugi, M. & Terasaki, P. I. Autologous stimulation of human lymphocyte subpopulation. *J Exp Med* **142**, 1327-1333, doi:10.1084/jem.142.5.1327 (1975).
339 Steinman, R. M. & Witmer, M. D. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Natl Acad Sci U S A* **75**, 5132-5136, doi:10.1073/pnas.75.10.5132 (1978).
340 Lam, T. S., van de Meent, M., Falkenburg, J. F. & Jedema, I. Monocyte-derived dendritic cells can induce autoreactive CD4(+) T cells showing myeloid lineage directed reactivity in healthy individuals. *Eur J Immunol* **45**, 1030-1042, doi:10.1002/eji.201444819 (2015).
341 Scheinecker, C. *et al.* Initiation of the autologous mixed lymphocyte reaction requires the expression of costimulatory molecules B7-1 and B7-2 on human peripheral blood dendritic cells. *J Immunol* **161**, 3966-3973 (1998).
342 Jelcic, I. *et al.* Memory B Cells Activate Brain-Homing, Autoreactive CD4(+) T Cells in Multiple Sclerosis. *Cell* **175**, 85-100 e123, doi:10.1016/j.cell.2018.08.011 (2018).
343 Shen, P. & Fillatreau, S. Antibody-independent functions of B cells: a focus on cytokines. *Nat Rev Immunol* **15**, 441-451, doi:10.1038/nri3857 (2015).
344 Harris, D. P., Goodrich, S., Gerth, A. J., Peng, S. L. & Lund, F. E. Regulation of IFN-gamma production by B effector 1 cells: essential roles for T-bet and the IFN-gamma receptor. *J Immunol* **174**, 6781-6790, doi:10.4049/jimmunol.174.11.6781 (2005).

345 Harris, D. P. *et al.* Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat Immunol* **1**, 475-482, doi:10.1038/82717 (2000).
346 Bao, Y. *et al.* Identification of IFN-gamma-producing innate B cells. *Cell Res* **24**, 161-176, doi:10.1038/cr.2013.155 (2014).
347 Olalekan, S. A., Cao, Y., Hamel, K. M. & Finnegan, A. B cells expressing IFN-gamma suppress Treg-cell differentiation and promote autoimmune experimental arthritis. *Eur J Immunol* **45**, 988-998, doi:10.1002/eji.201445036 (2015).
348 Harris, D. P., Goodrich, S., Mohrs, K., Mohrs, M. & Lund, F. E. Cutting edge: the development of IL-4-producing B cells (B effector 2 cells) is controlled by IL-4, IL-4 receptor alpha, and Th2 cells. *J Immunol* **175**, 7103-7107, doi:10.4049/jimmunol.175.11.7103 (2005).
349 Hurdayal, R. *et al.* IL-4-producing B cells regulate T helper cell dichotomy in type 1- and type 2-controlled diseases. *Proc Natl Acad Sci U S A* **114**, E8430-E8439, doi:10.1073/pnas.1708125114 (2017).
350 Bermejo, D. A. *et al.* Trypanosoma cruzi trans-sialidase initiates a program independent of the transcription factors RORgammat and Ahr that leads to IL-17 production by activated B cells. *Nat Immunol* **14**, 514-522, doi:10.1038/ni.2569 (2013).
351 Vazquez-Tello, A. *et al.* IL-17A and IL-17F expression in B lymphocytes. *Int Arch Allergy Immunol* **157**, 406-416, doi:10.1159/000329527 (2012).
352 Duddy, M. E., Alter, A. & Bar-Or, A. Distinct profiles of human B cell effector cytokines: a role in immune regulation? *J Immunol* **172**, 3422-3427, doi:10.4049/jimmunol.172.6.3422 (2004).
353 Guerrier, T. *et al.* Proinflammatory B-cell profile in the early phases of MS predicts an active disease. *Neurol Neuroimmunol Neuroinflamm* **5**, e431, doi:10.1212/NXI.0000000000000431 (2018).
354 Miyazaki, Y. *et al.* A novel microRNA-132-sirtuin-1 axis underlies aberrant B-cell cytokine regulation in patients with relapsing-remitting multiple sclerosis [corrected]. *PLoS One* **9**, e105421, doi:10.1371/journal.pone.0105421 (2014).
355 Barr, T. A. *et al.* B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. *J Exp Med* **209**, 1001-1010, doi:10.1084/jem.20111675 (2012).
356 Rauch, P. J. *et al.* Innate response activator B cells protect against microbial sepsis. *Science* **335**, 597-601, doi:10.1126/science.1215173 (2012).
357 Weber, G. F. *et al.* Pleural innate response activator B cells protect against pneumonia via a GM-CSF-IgM axis. *J Exp Med* **211**, 1243-1256, doi:10.1084/jem.20131471 (2014).
358 Knier, B. *et al.* Myeloid-derived suppressor cells control B cell accumulation in the central nervous system during autoimmunity. *Nat Immunol* **19**, 1341-1351, doi:10.1038/s41590-018-0237-5 (2018).
359 Weber, G. F. *et al.* Interleukin-3 amplifies acute inflammation and is a potential therapeutic target in sepsis. *Science* **347**, 1260-1265, doi:10.1126/science.aaa4268 (2015).
360 Li, R. *et al.* Antibody-Independent Function of Human B Cells Contributes to Antifungal T Cell Responses. *J Immunol* **198**, 3245-3254, doi:10.4049/jimmunol.1601572 (2017).

361 Katz, S. I., Parker, D. & Turk, J. L. B-cell suppression of delayed hypersensitivity reactions. *Nature* **251**, 550-551 (1974).
362 Neta, R. & Salvin, S. B. Specific suppression of delayed hypersensitivity: the possible presence of a suppressor B cell in the regulation of delayed hypersensitivity. *J Immunol* **113**, 1716-1725 (1974).
363 Wolf, S. D., Dittel, B. N., Hardardottir, F. & Janeway, C. A., Jr. Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. *J Exp Med* **184**, 2271-2278, doi:10.1084/jem.184.6.2271 (1996).
364 Fillatreau, S., Sweenie, C. H., McGeachy, M. J., Gray, D. & Anderton, S. M. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* **3**, 944-950, doi:10.1038/ni833 (2002).
365 Matsushita, T., Yanaba, K., Bouaziz, J. D., Fujimoto, M. & Tedder, T. F. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J Clin Invest* **118**, 3420-3430, doi:10.1172/JCI36030 (2008).
366 Lampropoulou, V. *et al.* TLR-activated B cells suppress T cell-mediated autoimmunity. *J Immunol* **180**, 4763-4773, doi:10.4049/jimmunol.180.7.4763 (2008).
367 Rafei, M. *et al.* A granulocyte-macrophage colony-stimulating factor and interleukin-15 fusokine induces a regulatory B cell population with immune suppressive properties. *Nat Med* **15**, 1038-1045, doi:10.1038/nm.2003 (2009).
368 Matsushita, T., Horikawa, M., Iwata, Y. & Tedder, T. F. Regulatory B cells (B10 cells) and regulatory T cells have independent roles in controlling experimental autoimmune encephalomyelitis initiation and late-phase immunopathogenesis. *J Immunol* **185**, 2240-2252, doi:10.4049/jimmunol.1001307 (2010).
369 Matsumoto, M. *et al.* The calcium sensors STIM1 and STIM2 control B cell regulatory function through interleukin-10 production. *Immunity* **34**, 703-714, doi:10.1016/j.immuni.2011.03.016 (2011).
370 Miles, K. *et al.* A tolerogenic role for Toll-like receptor 9 is revealed by B-cell interaction with DNA complexes expressed on apoptotic cells. *Proc Natl Acad Sci U S A* **109**, 887-892, doi:10.1073/pnas.1109173109 (2012).
371 Yoshizaki, A. *et al.* Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. *Nature* **491**, 264-268, doi:10.1038/nature11501 (2012).
372 Shen, P. *et al.* IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature* **507**, 366-370, doi:10.1038/nature12979 (2014).
373 Mauri, C., Gray, D., Mushtaq, N. & Londei, M. Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med* **197**, 489-501, doi:10.1084/jem.20021293 (2003).
374 Mizoguchi, A., Mizoguchi, E., Takedatsu, H., Blumberg, R. S. & Bhan, A. K. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* **16**, 219-230 (2002).
375 Carter, N. A. *et al.* Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells. *J Immunol* **186**, 5569-5579, doi:10.4049/jimmunol.1100284 (2011).
376 Blair, P. A. *et al.* Selective targeting of B cells with agonistic anti-CD40 is an efficacious strategy for the generation of induced regulatory T2-like B cells and for the suppression

of lupus in MRL/lpr mice. *J Immunol* **182**, 3492-3502, doi:10.4049/jimmunol.0803052 (2009).

377 Blair, P. A. *et al.* CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity* **32**, 129-140, doi:10.1016/j.immuni.2009.11.009 (2010).

378 Gray, M., Miles, K., Salter, D., Gray, D. & Savill, J. Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. *Proc Natl Acad Sci U S A* **104**, 14080-14085, doi:10.1073/pnas.0700326104 (2007).

379 Horikawa, M. *et al.* Regulatory B cell (B10 Cell) expansion during Listeria infection governs innate and cellular immune responses in mice. *J Immunol* **190**, 1158-1168, doi:10.4049/jimmunol.1201427 (2013).

380 Bankoti, R., Gupta, K., Levchenko, A. & Stager, S. Marginal zone B cells regulate antigen-specific T cell responses during infection. *J Immunol* **188**, 3961-3971, doi:10.4049/jimmunol.1102880 (2012).

381 Schioppa, T. *et al.* B regulatory cells and the tumor-promoting actions of TNF-alpha during squamous carcinogenesis. *Proc Natl Acad Sci U S A* **108**, 10662-10667, doi:10.1073/pnas.1100994108 (2011).

382 Neves, P. *et al.* Signaling via the MyD88 adaptor protein in B cells suppresses protective immunity during Salmonella typhimurium infection. *Immunity* **33**, 777-790, doi:10.1016/j.immuni.2010.10.016 (2010).

383 Lino, A. C. *et al.* LAG-3 Inhibitory Receptor Expression Identifies Immunosuppressive Natural Regulatory Plasma Cells. *Immunity* **49**, 120-133 e129, doi:10.1016/j.immuni.2018.06.007 (2018).

384 Shalapour, S. & Karin, M. Pas de Deux: Control of Anti-tumor Immunity by Cancer-Associated Inflammation. *Immunity* **51**, 15-26, doi:10.1016/j.immuni.2019.06.021 (2019).

385 Mauri, C. & Menon, M. The expanding family of regulatory B cells. *Int Immunol* **27**, 479-486, doi:10.1093/intimm/dxv038 (2015).

386 Ouyang, W. & O'Garra, A. IL-10 Family Cytokines IL-10 and IL-22: from Basic Science to Clinical Translation. *Immunity* **50**, 871-891, doi:10.1016/j.immuni.2019.03.020 (2019).

387 O'Shea, J. J. *et al.* The JAK-STAT pathway: impact on human disease and therapeutic intervention. *Annu Rev Med* **66**, 311-328, doi:10.1146/annurev-med-051113-024537 (2015).

388 Evans, J. G. *et al.* Novel suppressive function of transitional 2 B cells in experimental arthritis. *J Immunol* **178**, 7868-7878, doi:10.4049/jimmunol.178.12.7868 (2007).

389 Yanaba, K. *et al.* A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity* **28**, 639-650, doi:10.1016/j.immuni.2008.03.017 (2008).

390 Yanaba, K., Bouaziz, J. D., Matsushita, T., Tsubata, T. & Tedder, T. F. The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals. *J Immunol* **182**, 7459-7472, doi:10.4049/jimmunol.0900270 (2009).

391 Rojas, O. L. *et al.* Recirculating Intestinal IgA-Producing Cells Regulate Neuroinflammation via IL-10. *Cell* **177**, 492-493, doi:10.1016/j.cell.2019.03.037 (2019).

392 Matsumoto, M. *et al.* Interleukin-10-producing plasmablasts exert regulatory function in autoimmune inflammation. *Immunity* **41**, 1040-1051, doi:10.1016/j.immuni.2014.10.016 (2014).
393 Iwata, Y. *et al.* Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. *Blood* **117**, 530-541, doi:10.1182/blood-2010-07-294249 (2011).
394 Flores-Borja, F. *et al.* CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. *Sci Transl Med* **5**, 173ra123, doi:10.1126/scitranslmed.3005407 (2013).
395 van de Veen, W. *et al.* IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. *J Allergy Clin Immunol* **131**, 1204-1212, doi:10.1016/j.jaci.2013.01.014 (2013).
396 Menon, M., Blair, P. A., Isenberg, D. A. & Mauri, C. A Regulatory Feedback between Plasmacytoid Dendritic Cells and Regulatory B Cells Is Aberrant in Systemic Lupus Erythematosus. *Immunity* **44**, 683-697, doi:10.1016/j.immuni.2016.02.012 (2016).
397 Kleffel, S. *et al.* Interleukin-10+ regulatory B cells arise within antigen-experienced CD40+ B cells to maintain tolerance to islet autoantigens. *Diabetes* **64**, 158-171, doi:10.2337/db13-1639 (2015).
398 Sun, C. M., Deriaud, E., Leclerc, C. & Lo-Man, R. Upon TLR9 signaling, CD5+ B cells control the IL-12-dependent Th1-priming capacity of neonatal DCs. *Immunity* **22**, 467-477, doi:10.1016/j.immuni.2005.02.008 (2005).
399 Xiao, S. *et al.* Defect in regulatory B-cell function and development of systemic autoimmunity in T-cell Ig mucin 1 (Tim-1) mucin domain-mutant mice. *Proc Natl Acad Sci U S A* **109**, 12105-12110, doi:10.1073/pnas.1120914109 (2012).
400 Yu, J. *et al.* Surface receptor Toso controls B cell-mediated regulation of T cell immunity. *J Clin Invest* **128**, 1820-1836, doi:10.1172/JCI97280 (2018).
401 Rosser, E. C. *et al.* Regulatory B cells are induced by gut microbiota-driven interleukin-1beta and interleukin-6 production. *Nat Med* **20**, 1334-1339, doi:10.1038/nm.3680 (2014).
402 Liu, B. S., Cao, Y., Huizinga, T. W., Hafler, D. A. & Toes, R. E. TLR-mediated STAT3 and ERK activation controls IL-10 secretion by human B cells. *Eur J Immunol* **44**, 2121-2129, doi:10.1002/eji.201344341 (2014).
403 de Masson, A. *et al.* CD24(hi)CD27(+) and plasmablast-like regulatory B cells in human chronic graft-versus-host disease. *Blood* **125**, 1830-1839, doi:10.1182/blood-2014-09-599159 (2015).
404 Meng, X. *et al.* Hypoxia-inducible factor-1alpha is a critical transcription factor for IL-10-producing B cells in autoimmune disease. *Nat Commun* **9**, 251, doi:10.1038/s41467-017-02683-x (2018).
405 Wang, R. X. *et al.* Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med* **20**, 633-641, doi:10.1038/nm.3554 (2014).
406 Dambuza, I. M. *et al.* IL-12p35 induces expansion of IL-10 and IL-35-expressing regulatory B cells and ameliorates autoimmune disease. *Nat Commun* **8**, 719, doi:10.1038/s41467-017-00838-4 (2017).

407 Hauser, S. L. *et al.* Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions. *Ann Neurol* **19**, 578-587, doi:10.1002/ana.410190610 (1986).
408 McCallum, K., Esiri, M. M., Tourtellotte, W. W. & Booss, J. T cell subsets in multiple sclerosis. Gradients at plaque borders and differences in nonplaque regions. *Brain* **110 (Pt 5)**, 1297-1308, doi:10.1093/brain/110.5.1297 (1987).
409 Booss, J., Esiri, M. M., Tourtellotte, W. W. & Mason, D. Y. Immunohistological analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis. *J Neurol Sci* **62**, 219-232 (1983).
410 Babbe, H. *et al.* Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J Exp Med* **192**, 393-404, doi:10.1084/jem.192.3.393 (2000).
411 Hoftberger, R. *et al.* Expression of major histocompatibility complex class I molecules on the different cell types in multiple sclerosis lesions. *Brain Pathol* **14**, 43-50 (2004).
412 Frischer, J. M. *et al.* The relation between inflammation and neurodegeneration in multiple sclerosis brains. *Brain* **132**, 1175-1189, doi:10.1093/brain/awp070 (2009).
413 Machado-Santos, J. *et al.* The compartmentalized inflammatory response in the multiple sclerosis brain is composed of tissue-resident CD8+ T lymphocytes and B cells. *Brain* **141**, 2066-2082, doi:10.1093/brain/awy151 (2018).
414 Smolders, J. *et al.* Tissue-resident memory T cells populate the human brain. *Nat Commun* **9**, 4593, doi:10.1038/s41467-018-07053-9 (2018).
415 Ifergan, I. *et al.* Central nervous system recruitment of effector memory CD8+ T lymphocytes during neuroinflammation is dependent on alpha4 integrin. *Brain* **134**, 3560-3577, doi:10.1093/brain/awr268 (2011).
416 Larochelle, C. *et al.* Melanoma cell adhesion molecule-positive CD8 T lymphocytes mediate central nervous system inflammation. *Ann Neurol* **78**, 39-53, doi:10.1002/ana.24415 (2015).
417 Bitsch, A., Schuchardt, J., Bunkowski, S., Kuhlmann, T. & Bruck, W. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. *Brain* **123 (Pt 6)**, 1174-1183, doi:10.1093/brain/123.6.1174 (2000).
418 Kuhlmann, T., Lingfeld, G., Bitsch, A., Schuchardt, J. & Bruck, W. Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. *Brain* **125**, 2202-2212, doi:10.1093/brain/awf235 (2002).
419 Abrahamsson, S. V. *et al.* Non-myeloablative autologous haematopoietic stem cell transplantation expands regulatory cells and depletes IL-17 producing mucosal-associated invariant T cells in multiple sclerosis. *Brain* **136**, 2888-2903, doi:10.1093/brain/awt182 (2013).
420 Annibali, V. *et al.* CD161(high)CD8+T cells bear pathogenetic potential in multiple sclerosis. *Brain* **134**, 542-554, doi:10.1093/brain/awq354 (2011).
421 Willing, A. *et al.* CD8(+) MAIT cells infiltrate into the CNS and alterations in their blood frequencies correlate with IL-18 serum levels in multiple sclerosis. *Eur J Immunol* **44**, 3119-3128, doi:10.1002/eji.201344160 (2014).
422 Salou, M. *et al.* Neuropathologic, phenotypic and functional analyses of Mucosal Associated Invariant T cells in Multiple Sclerosis. *Clin Immunol* **166-167**, 1-11, doi:10.1016/j.clim.2016.03.014 (2016).

423 Held, K. *et al.* alphabeta T-cell receptors from multiple sclerosis brain lesions show MAIT cell-related features. *Neurol Neuroimmunol Neuroinflamm* **2**, e107, doi:10.1212/NXI.0000000000000107 (2015).
424 Nicol, B. *et al.* An intermediate level of CD161 expression defines a novel activated, inflammatory, and pathogenic subset of CD8(+) T cells involved in multiple sclerosis. *J Autoimmun* **88**, 61-74, doi:10.1016/j.jaut.2017.10.005 (2018).
425 Jacobsen, M. *et al.* Oligoclonal expansion of memory CD8+ T cells in cerebrospinal fluid from multiple sclerosis patients. *Brain* **125**, 538-550, doi:10.1093/brain/awf059 (2002).
426 Junker, A. *et al.* Multiple sclerosis: T-cell receptor expression in distinct brain regions. *Brain* **130**, 2789-2799, doi:10.1093/brain/awm214 (2007).
427 Skulina, C. *et al.* Multiple sclerosis: brain-infiltrating CD8+ T cells persist as clonal expansions in the cerebrospinal fluid and blood. *Proc Natl Acad Sci U S A* **101**, 2428-2433, doi:10.1073/pnas.0308689100 (2004).
428 Planas, R. *et al.* Central role of Th2/Tc2 lymphocytes in pattern II multiple sclerosis lesions. *Ann Clin Transl Neurol* **2**, 875-893, doi:10.1002/acn3.218 (2015).
429 Salou, M. *et al.* Expanded CD8 T-cell sharing between periphery and CNS in multiple sclerosis. *Ann Clin Transl Neurol* **2**, 609-622, doi:10.1002/acn3.199 (2015).
430 de Paula Alves Sousa, A. *et al.* Intrathecal T-cell clonal expansions in patients with multiple sclerosis. *Ann Clin Transl Neurol* **3**, 422-433, doi:10.1002/acn3.310 (2016).
431 Alves Sousa, A. P. *et al.* Comprehensive Analysis of TCR-beta Repertoire in Patients with Neurological Immune-mediated Disorders. *Sci Rep* **9**, 344, doi:10.1038/s41598-018-36274-7 (2019).
432 Hafler, D. A. *et al.* Oligoclonal T lymphocytes in the cerebrospinal fluid of patients with multiple sclerosis. *J Exp Med* **167**, 1313-1322, doi:10.1084/jem.167.4.1313 (1988).
433 Laplaud, D. A. *et al.* Blood T-cell receptor beta chain transcriptome in multiple sclerosis. Characterization of the T cells with altered CDR3 length distribution. *Brain* **127**, 981-995, doi:10.1093/brain/awh119 (2004).
434 Laplaud, D. A. *et al.* Serial blood T cell repertoire alterations in multiple sclerosis patients; correlation with clinical and MRI parameters. *J Neuroimmunol* **177**, 151-160, doi:10.1016/j.jneuroim.2006.05.006 (2006).
435 van Nierop, G. P. *et al.* Intrathecal CD4(+) and CD8(+) T-cell responses to endogenously synthesized candidate disease-associated human autoantigens in multiple sclerosis patients. *Eur J Immunol* **46**, 347-353, doi:10.1002/eji.201545921 (2016).
436 van Nierop, G. P., Mautner, J., Mitterreiter, J. G., Hintzen, R. Q. & Verjans, G. M. Intrathecal CD8 T-cells of multiple sclerosis patients recognize lytic Epstein-Barr virus proteins. *Mult Scler* **22**, 279-291, doi:10.1177/1352458515588581 (2016).
437 Tsuchida, T. *et al.* Autoreactive CD8+ T-cell responses to human myelin protein-derived peptides. *Proc Natl Acad Sci U S A* **91**, 10859-10863, doi:10.1073/pnas.91.23.10859 (1994).
438 Biddison, W. E. *et al.* Chemokine and matrix metalloproteinase secretion by myelin proteolipid protein-specific CD8+ T cells: potential roles in inflammation. *J Immunol* **158**, 3046-3053 (1997).

439 Honma, K. *et al.* Identification of an epitope derived from human proteolipid protein that can induce autoreactive CD8+ cytotoxic T lymphocytes restricted by HLA-A3: evidence for cross-reactivity with an environmental microorganism. *J Neuroimmunol* **73**, 7-14 (1997).
440 Jurewicz, A., Biddison, W. E. & Antel, J. P. MHC class I-restricted lysis of human oligodendrocytes by myelin basic protein peptide-specific CD8 T lymphocytes. *J Immunol* **160**, 3056-3059 (1998).
441 Zang, Y. C. *et al.* Increased CD8+ cytotoxic T cell responses to myelin basic protein in multiple sclerosis. *J Immunol* **172**, 5120-5127, doi:10.4049/jimmunol.172.8.5120 (2004).
442 Niland, B., Banki, K., Biddison, W. E. & Perl, A. CD8+ T cell-mediated HLA-A*0201-restricted cytotoxicity to transaldolase peptide 168-176 in patients with multiple sclerosis. *J Immunol* **175**, 8365-8378, doi:10.4049/jimmunol.175.12.8365 (2005).
443 Berthelot, L. *et al.* Blood CD8+ T cell responses against myelin determinants in multiple sclerosis and healthy individuals. *Eur J Immunol* **38**, 1889-1899, doi:10.1002/eji.200838023 (2008).
444 Medana, I., Martinic, M. A., Wekerle, H. & Neumann, H. Transection of major histocompatibility complex class I-induced neurites by cytotoxic T lymphocytes. *Am J Pathol* **159**, 809-815, doi:10.1016/S0002-9440(10)61755-5 (2001).
445 Schneider, R. *et al.* B cell-derived IL-15 enhances CD8 T cell cytotoxicity and is increased in multiple sclerosis patients. *J Immunol* **187**, 4119-4128, doi:10.4049/jimmunol.1100885 (2011).
446 Saikali, P., Antel, J. P., Pittet, C. L., Newcombe, J. & Arbour, N. Contribution of astrocyte-derived IL-15 to CD8 T cell effector functions in multiple sclerosis. *J Immunol* **185**, 5693-5703, doi:10.4049/jimmunol.1002188 (2010).
447 Ma, N. *et al.* BAFF suppresses IL-15 expression in B cells. *J Immunol* **192**, 4192-4201, doi:10.4049/jimmunol.1302132 (2014).
448 Huseby, E. S. *et al.* A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis. *J Exp Med* **194**, 669-676, doi:10.1084/jem.194.5.669 (2001).
449 Sun, D. *et al.* Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice. *J Immunol* **166**, 7579-7587, doi:10.4049/jimmunol.166.12.7579 (2001).
450 Anderson, A. C. *et al.* A transgenic model of central nervous system autoimmunity mediated by CD4+ and CD8+ T and B cells. *J Immunol* **188**, 2084-2092, doi:10.4049/jimmunol.1102186 (2012).
451 Sasaki, K. *et al.* Relapsing-remitting central nervous system autoimmunity mediated by GFAP-specific CD8 T cells. *J Immunol* **192**, 3029-3042, doi:10.4049/jimmunol.1302911 (2014).
452 Ji, Q., Perchellet, A. & Goverman, J. M. Viral infection triggers central nervous system autoimmunity via activation of CD8+ T cells expressing dual TCRs. *Nat Immunol* **11**, 628-634, doi:10.1038/ni.1888 (2010).
453 Page, N. *et al.* Expression of the DNA-Binding Factor TOX Promotes the Encephalitogenic Potential of Microbe-Induced Autoreactive CD8(+) T Cells. *Immunity* **48**, 937-950 e938, doi:10.1016/j.immuni.2018.04.005 (2018).

454 Na, S. Y. *et al.* Oligodendrocytes enforce immune tolerance of the uninfected brain by purging the peripheral repertoire of autoreactive CD8+ T cells. *Immunity* **37**, 134-146, doi:10.1016/j.immuni.2012.04.009 (2012).
455 Friese, M. A. *et al.* Opposing effects of HLA class I molecules in tuning autoreactive CD8+ T cells in multiple sclerosis. *Nat Med* **14**, 1227-1235, doi:10.1038/nm.1881 (2008).
456 Willis, C. M. *et al.* Extracellular vesicle fibrinogen induces encephalitogenic CD8+ T cells in a mouse model of multiple sclerosis. *Proc Natl Acad Sci U S A* **116**, 10488-10493, doi:10.1073/pnas.1816911116 (2019).
457 Petersen, M. A., Ryu, J. K. & Akassoglou, K. Fibrinogen in neurological diseases: mechanisms, imaging and therapeutics. *Nat Rev Neurosci* **19**, 283-301, doi:10.1038/nrn.2018.13 (2018).
458 Ryu, J. K. *et al.* Blood coagulation protein fibrinogen promotes autoimmunity and demyelination via chemokine release and antigen presentation. *Nat Commun* **6**, 8164, doi:10.1038/ncomms9164 (2015).
459 Ji, Q., Castelli, L. & Goverman, J. M. MHC class I-restricted myelin epitopes are cross-presented by Tip-DCs that promote determinant spreading to CD8(+) T cells. *Nat Immunol* **14**, 254-261, doi:10.1038/ni.2513 (2013).
460 Huber, M. *et al.* IL-17A secretion by CD8+ T cells supports Th17-mediated autoimmune encephalomyelitis. *J Clin Invest* **123**, 247-260, doi:10.1172/JCI63681 (2013).
461 Wang, H. H. *et al.* Interleukin-17-secreting T cells in neuromyelitis optica and multiple sclerosis during relapse. *J Clin Neurosci* **18**, 1313-1317, doi:10.1016/j.jocn.2011.01.031 (2011).
462 Sun, D., Qin, Y., Chluba, J., Epplen, J. T. & Wekerle, H. Suppression of experimentally induced autoimmune encephalomyelitis by cytolytic T-T cell interactions. *Nature* **332**, 843-845, doi:10.1038/332843a0 (1988).
463 Jiang, H. *et al.* Regulatory CD8+ T cells fine-tune the myelin basic protein-reactive T cell receptor V beta repertoire during experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* **100**, 8378-8383, doi:10.1073/pnas.1432871100 (2003).
464 Jiang, H., Zhang, S. I. & Pernis, B. Role of CD8+ T cells in murine experimental allergic encephalomyelitis. *Science* **256**, 1213-1215 (1992).
465 Koh, D. R. *et al.* Less mortality but more relapses in experimental allergic encephalomyelitis in CD8-/- mice. *Science* **256**, 1210-1213 (1992).
466 Miller, A., Lider, O., Roberts, A. B., Sporn, M. B. & Weiner, H. L. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering. *Proc Natl Acad Sci U S A* **89**, 421-425, doi:10.1073/pnas.89.1.421 (1992).
467 Khoury, S. J., Hancock, W. W. & Weiner, H. L. Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with downregulation of inflammatory cytokines and differential upregulation of transforming growth factor beta, interleukin 4, and prostaglandin E expression in the brain. *J Exp Med* **176**, 1355-1364, doi:10.1084/jem.176.5.1355 (1992).
468 Najafian, N. *et al.* Regulatory functions of CD8+CD28- T cells in an autoimmune disease model. *J Clin Invest* **112**, 1037-1048, doi:10.1172/JCI17935 (2003).

469 Jiang, H., Braunstein, N. S., Yu, B., Winchester, R. & Chess, L. CD8+ T cells control the TH phenotype of MBP-reactive CD4+ T cells in EAE mice. *Proc Natl Acad Sci U S A* **98**, 6301-6306, doi:10.1073/pnas.101123098 (2001).

470 Lu, L., Kim, H. J., Werneck, M. B. & Cantor, H. Regulation of CD8+ regulatory T cells: Interruption of the NKG2A-Qa-1 interaction allows robust suppressive activity and resolution of autoimmune disease. *Proc Natl Acad Sci U S A* **105**, 19420-19425, doi:10.1073/pnas.0810383105 (2008).

471 York, N. R. *et al.* Immune regulatory CNS-reactive CD8+T cells in experimental autoimmune encephalomyelitis. *J Autoimmun* **35**, 33-44, doi:10.1016/j.jaut.2010.01.003 (2010).

472 Ortega, S. B. *et al.* The disease-ameliorating function of autoregulatory CD8 T cells is mediated by targeting of encephalitogenic CD4 T cells in experimental autoimmune encephalomyelitis. *J Immunol* **191**, 117-126, doi:10.4049/jimmunol.1300452 (2013).

473 Ortega, S. B., Kashi, V. P., Cunnusamy, K., Franco, J. & Karandikar, N. J. Autoregulatory CD8 T cells depend on cognate antigen recognition and CD4/CD8 myelin determinants. *Neurol Neuroimmunol Neuroinflamm* **2**, e170, doi:10.1212/NXI.0000000000000170 (2015).

474 Saligrama, N. *et al.* Opposing T cell responses in experimental autoimmune encephalomyelitis. *Nature*, doi:10.1038/s41586-019-1467-x (2019).

475 Nakagawa, H., Wang, L., Cantor, H. & Kim, H. J. New Insights Into the Biology of CD8 Regulatory T Cells. *Adv Immunol* **140**, 1-20, doi:10.1016/bs.ai.2018.09.001 (2018).

476 Kim, H. J., Verbinnen, B., Tang, X., Lu, L. & Cantor, H. Inhibition of follicular T-helper cells by CD8(+) regulatory T cells is essential for self tolerance. *Nature* **467**, 328-332, doi:10.1038/nature09370 (2010).

477 Kim, H. J. *et al.* CD8+ T regulatory cells express the Ly49 Class I MHC receptor and are defective in autoimmune prone B6-Yaa mice. *Proc Natl Acad Sci U S A* **108**, 2010-2015, doi:10.1073/pnas.1018974108 (2011).

478 Joosten, S. A. *et al.* Identification of a human CD8+ regulatory T cell subset that mediates suppression through the chemokine CC chemokine ligand 4. *Proc Natl Acad Sci U S A* **104**, 8029-8034, doi:10.1073/pnas.0702257104 (2007).

479 Correale, J. & Villa, A. Isolation and characterization of CD8+ regulatory T cells in multiple sclerosis. *J Neuroimmunol* **195**, 121-134, doi:10.1016/j.jneuroim.2007.12.004 (2008).

480 Legoux, F., Salou, M. & Lantz, O. Unconventional or Preset alphabeta T Cells: Evolutionarily Conserved Tissue-Resident T Cells Recognizing Nonpeptidic Ligands. *Annu Rev Cell Dev Biol* **33**, 511-535, doi:10.1146/annurev-cellbio-100616-060725 (2017).

481 Gherardin, N. A., McCluskey, J., Rossjohn, J. & Godfrey, D. I. The Diverse Family of MR1-Restricted T Cells. *J Immunol* **201**, 2862-2871, doi:10.4049/jimmunol.1801091 (2018).

482 Mori, L., Lepore, M. & De Libero, G. The Immunology of CD1- and MR1-Restricted T Cells. *Annu Rev Immunol* **34**, 479-510, doi:10.1146/annurev-immunol-032414-112008 (2016).

483 Porcelli, S., Yockey, C. E., Brenner, M. B. & Balk, S. P. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. *J Exp Med* **178**, 1-16, doi:10.1084/jem.178.1.1 (1993).

484 Tilloy, F. *et al.* An invariant T cell receptor alpha chain defines a novel TAP-independent major histocompatibility complex class Ib-restricted alpha/beta T cell subpopulation in mammals. *J Exp Med* **189**, 1907-1921, doi:10.1084/jem.189.12.1907 (1999).
485 Treiner, E. *et al.* Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature* **422**, 164-169, doi:10.1038/nature01433 (2003).
486 Boudinot, P. *et al.* Restricting nonclassical MHC genes coevolve with TRAV genes used by innate-like T cells in mammals. *Proc Natl Acad Sci U S A* **113**, E2983-2992, doi:10.1073/pnas.1600674113 (2016).
487 Huang, S. *et al.* MR1 antigen presentation to mucosal-associated invariant T cells was highly conserved in evolution. *Proc Natl Acad Sci U S A* **106**, 8290-8295, doi:10.1073/pnas.0903196106 (2009).
488 Tsukamoto, K., Deakin, J. E., Graves, J. A. & Hashimoto, K. Exceptionally high conservation of the MHC class I-related gene, MR1, among mammals. *Immunogenetics* **65**, 115-124, doi:10.1007/s00251-012-0666-5 (2013).
489 Le Bourhis, L. *et al.* Antimicrobial activity of mucosal-associated invariant T cells. *Nat Immunol* **11**, 701-708, doi:10.1038/ni.1890 (2010).
490 Gold, M. C. *et al.* Human mucosal associated invariant T cells detect bacterially infected cells. *PLoS Biol* **8**, e1000407, doi:10.1371/journal.pbio.1000407 (2010).
491 Kurioka, A. *et al.* MAIT cells are licensed through granzyme exchange to kill bacterially sensitized targets. *Mucosal Immunol* **8**, 429-440, doi:10.1038/mi.2014.81 (2015).
492 Kjer-Nielsen, L. *et al.* MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature* **491**, 717-723, doi:10.1038/nature11605 (2012).
493 Corbett, A. J. *et al.* T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature* **509**, 361-365, doi:10.1038/nature13160 (2014).
494 Kurioka, A. *et al.* Diverse Streptococcus pneumoniae Strains Drive a Mucosal-Associated Invariant T-Cell Response Through Major Histocompatibility Complex class I-Related Molecule-Dependent and Cytokine-Driven Pathways. *J Infect Dis* **217**, 988-999, doi:10.1093/infdis/jix647 (2018).
495 Lepore, M. *et al.* Parallel T-cell cloning and deep sequencing of human MAIT cells reveal stable oligoclonal TCRbeta repertoire. *Nat Commun* **5**, 3866, doi:10.1038/ncomms4866 (2014).
496 Howson, L. J. *et al.* MAIT cell clonal expansion and TCR repertoire shaping in human volunteers challenged with Salmonella Paratyphi A. *Nat Commun* **9**, 253, doi:10.1038/s41467-017-02540-x (2018).
497 Dias, J., Leeansyah, E. & Sandberg, J. K. Multiple layers of heterogeneity and subset diversity in human MAIT cell responses to distinct microorganisms and to innate cytokines. *Proc Natl Acad Sci U S A* **114**, E5434-E5443, doi:10.1073/pnas.1705759114 (2017).
498 Keller, A. N. *et al.* Drugs and drug-like molecules can modulate the function of mucosal-associated invariant T cells. *Nat Immunol* **18**, 402-411, doi:10.1038/ni.3679 (2017).
499 Gold, M. C. *et al.* Human thymic MR1-restricted MAIT cells are innate pathogen-reactive effectors that adapt following thymic egress. *Mucosal Immunol* **6**, 35-44, doi:10.1038/mi.2012.45 (2013).

500 Chua, W. J. *et al.* Endogenous MHC-related protein 1 is transiently expressed on the plasma membrane in a conformation that activates mucosal-associated invariant T cells. *J Immunol* **186**, 4744-4750, doi:10.4049/jimmunol.1003254 (2011).
501 Koay, H. F. *et al.* A three-stage intrathymic development pathway for the mucosal-associated invariant T cell lineage. *Nat Immunol* **17**, 1300-1311, doi:10.1038/ni.3565 (2016).
502 Martin, E. *et al.* Stepwise development of MAIT cells in mouse and human. *PLoS Biol* **7**, e54, doi:10.1371/journal.pbio.1000054 (2009).
503 Leeansyah, E., Loh, L., Nixon, D. F. & Sandberg, J. K. Acquisition of innate-like microbial reactivity in mucosal tissues during human fetal MAIT-cell development. *Nat Commun* **5**, 3143, doi:10.1038/ncomms4143 (2014).
504 Koay, H. F., Godfrey, D. I. & Pellicci, D. G. Development of mucosal-associated invariant T cells. *Immunol Cell Biol* **96**, 598-606, doi:10.1111/imcb.12039 (2018).
505 Gutierrez-Arcelus, M. *et al.* Lymphocyte innateness defined by transcriptional states reflects a balance between proliferation and effector functions. *Nat Commun* **10**, 687, doi:10.1038/s41467-019-08604-4 (2019).
506 Ben Youssef, G. *et al.* Ontogeny of human mucosal-associated invariant T cells and related T cell subsets. *J Exp Med* **215**, 459-479, doi:10.1084/jem.20171739 (2018).
507 Salou, M. *et al.* A common transcriptomic program acquired in the thymus defines tissue residency of MAIT and NKT subsets. *J Exp Med* **216**, 133-151, doi:10.1084/jem.20181483 (2019).
508 Dias, J. *et al.* The CD4(-)CD8(-) MAIT cell subpopulation is a functionally distinct subset developmentally related to the main CD8(+) MAIT cell pool. *Proc Natl Acad Sci U S A* **115**, E11513-E11522, doi:10.1073/pnas.1812273115 (2018).
509 Dusseaux, M. *et al.* Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood* **117**, 1250-1259, doi:10.1182/blood-2010-08-303339 (2011).
510 Wong, M. T. *et al.* A High-Dimensional Atlas of Human T Cell Diversity Reveals Tissue-Specific Trafficking and Cytokine Signatures. *Immunity* **45**, 442-456, doi:10.1016/j.immuni.2016.07.007 (2016).
511 Fergusson, J. R. *et al.* CD161 defines a transcriptional and functional phenotype across distinct human T cell lineages. *Cell Rep* **9**, 1075-1088, doi:10.1016/j.celrep.2014.09.045 (2014).
512 Voillet, V. *et al.* Human MAIT cells exit peripheral tissues and recirculate via lymph in steady state conditions. *JCI Insight* **3**, doi:10.1172/jci.insight.98487 (2018).
513 Godfrey, D. I., Koay, H. F., McCluskey, J. & Gherardin, N. A. The biology and functional importance of MAIT cells. *Nat Immunol*, doi:10.1038/s41590-019-0444-8 (2019).
514 Brozova, J., Karlova, I. & Novak, J. Analysis of the Phenotype and Function of the Subpopulations of Mucosal-Associated Invariant T Cells. *Scand J Immunol* **84**, 245-251, doi:10.1111/sji.12467 (2016).
515 Walker, L. J. *et al.* Human MAIT and CD8alphaalpha cells develop from a pool of type-17 precommitted CD8+ T cells. *Blood* **119**, 422-433, doi:10.1182/blood-2011-05-353789 (2012).

516 Patin, E. *et al.* Natural variation in the parameters of innate immune cells is preferentially driven by genetic factors. *Nat Immunol* **19**, 302-314, doi:10.1038/s41590-018-0049-7 (2018).
517 Walker, L. J., Tharmalingam, H. & Klenerman, P. The rise and fall of MAIT cells with age. *Scand J Immunol* **80**, 462-463, doi:10.1111/sji.12237 (2014).
518 Gherardin, N. A. *et al.* Human blood MAIT cell subsets defined using MR1 tetramers. *Immunol Cell Biol* **96**, 507-525, doi:10.1111/imcb.12021 (2018).
519 Tang, X. Z. *et al.* IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells. *J Immunol* **190**, 3142-3152, doi:10.4049/jimmunol.1203218 (2013).
520 Eberhard, J. M. *et al.* Reduced CD161(+) MAIT cell frequencies in HCV and HIV/HCV co-infection: Is the liver the heart of the matter? *J Hepatol* **65**, 1261-1263, doi:10.1016/j.jhep.2016.07.031 (2016).
521 Wilson, R. P. *et al.* STAT3 is a critical cell-intrinsic regulator of human unconventional T cell numbers and function. *J Exp Med* **212**, 855-864, doi:10.1084/jem.20141992 (2015).
522 Beziat, V. *et al.* A recessive form of hyper-IgE syndrome by disruption of ZNF341-dependent STAT3 transcription and activity. *Sci Immunol* **3**, doi:10.1126/sciimmunol.aat4956 (2018).
523 Tangye, S. G., Pelham, S. J., Deenick, E. K. & Ma, C. S. Cytokine-Mediated Regulation of Human Lymphocyte Development and Function: Insights from Primary Immunodeficiencies. *J Immunol* **199**, 1949-1958, doi:10.4049/jimmunol.1700842 (2017).
524 Okada, S. *et al.* IMMUNODEFICIENCIES. Impairment of immunity to Candida and Mycobacterium in humans with bi-allelic RORC mutations. *Science* **349**, 606-613, doi:10.1126/science.aaa4282 (2015).
525 Martinez-Barricarte, R. *et al.* Human IFN-gamma immunity to mycobacteria is governed by both IL-12 and IL-23. *Sci Immunol* **3**, doi:10.1126/sciimmunol.aau6759 (2018).
526 Gerart, S. *et al.* Human iNKT and MAIT cells exhibit a PLZF-dependent proapoptotic propensity that is counterbalanced by XIAP. *Blood* **121**, 614-623, doi:10.1182/blood-2012-09-456095 (2013).
527 Lee, O. J. *et al.* Circulating mucosal-associated invariant T cell levels and their cytokine levels in healthy adults. *Exp Gerontol* **49**, 47-54, doi:10.1016/j.exger.2013.11.003 (2014).
528 Novak, J., Dobrovolny, J., Novakova, L. & Kozak, T. The decrease in number and change in phenotype of mucosal-associated invariant T cells in the elderly and differences in men and women of reproductive age. *Scand J Immunol* **80**, 271-275, doi:10.1111/sji.12193 (2014).
529 Meermeier, E. W., Harriff, M. J., Karamooz, E. & Lewinsohn, D. M. MAIT cells and microbial immunity. *Immunol Cell Biol* **96**, 607-617, doi:10.1111/imcb.12022 (2018).
530 Condotta, S. A. & Richer, M. J. The immune battlefield: The impact of inflammatory cytokines on CD8+ T-cell immunity. *PLoS Pathog* **13**, e1006618, doi:10.1371/journal.ppat.1006618 (2017).
531 Wang, H. *et al.* IL-23 costimulates antigen-specific MAIT cell activation and enables vaccination against bacterial infection. *Sci Immunol* **4**, doi:10.1126/sciimmunol.aaw0402 (2019).

532 Berkson, J. D. & Prlic, M. The MAIT conundrum - how human MAIT cells distinguish bacterial colonization from infection in mucosal barrier tissues. *Immunol Lett* **192**, 7-11, doi:10.1016/j.imlet.2017.09.013 (2017).
533 Ussher, J. E., Willberg, C. B. & Klenerman, P. MAIT cells and viruses. *Immunol Cell Biol* **96**, 630-641, doi:10.1111/imcb.12008 (2018).
534 Wang, H. *et al.* MAIT cells protect against pulmonary Legionella longbeachae infection. *Nat Commun* **9**, 3350, doi:10.1038/s41467-018-05202-8 (2018).
535 Loh, L. *et al.* Human mucosal-associated invariant T cells contribute to antiviral influenza immunity via IL-18-dependent activation. *Proc Natl Acad Sci U S A* **113**, 10133-10138, doi:10.1073/pnas.1610750113 (2016).
536 Wilgenburg, B. V. *et al.* MAIT cells contribute to protection against lethal influenza infection in vivo. *Nat Commun* **9**, 4706, doi:10.1038/s41467-018-07207-9 (2018).
537 Chen, Z. *et al.* Mucosal-associated invariant T-cell activation and accumulation after in vivo infection depends on microbial riboflavin synthesis and co-stimulatory signals. *Mucosal Immunol* **10**, 58-68, doi:10.1038/mi.2016.39 (2017).
538 Jeffery, H. C. *et al.* Biliary epithelium and liver B cells exposed to bacteria activate intrahepatic MAIT cells through MR1. *J Hepatol* **64**, 1118-1127, doi:10.1016/j.jhep.2015.12.017 (2016).
539 Meierovics, A. I. & Cowley, S. C. MAIT cells promote inflammatory monocyte differentiation into dendritic cells during pulmonary intracellular infection. *J Exp Med* **213**, 2793-2809, doi:10.1084/jem.20160637 (2016).
540 Gibbs, A. *et al.* MAIT cells reside in the female genital mucosa and are biased towards IL-17 and IL-22 production in response to bacterial stimulation. *Mucosal Immunol* **10**, 35-45, doi:10.1038/mi.2016.30 (2017).
541 Schmaler, M. *et al.* Modulation of bacterial metabolism by the microenvironment controls MAIT cell stimulation. *Mucosal Immunol* **11**, 1060-1070, doi:10.1038/s41385-018-0020-9 (2018).
542 Reantragoon, R. *et al.* Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in mucosal-associated invariant T cells. *J Exp Med* **210**, 2305-2320, doi:10.1084/jem.20130958 (2013).
543 Rahimpour, A. *et al.* Identification of phenotypically and functionally heterogeneous mouse mucosal-associated invariant T cells using MR1 tetramers. *J Exp Med* **212**, 1095-1108, doi:10.1084/jem.20142110 (2015).
544 Leeansyah, E. *et al.* Activation, exhaustion, and persistent decline of the antimicrobial MR1-restricted MAIT-cell population in chronic HIV-1 infection. *Blood* **121**, 1124-1135, doi:10.1182/blood-2012-07-445429 (2013).
545 Varelias, A. *et al.* Recipient mucosal-associated invariant T cells control GVHD within the colon. *J Clin Invest* **128**, 1919-1936, doi:10.1172/JCI91646 (2018).
546 Fan, X. & Rudensky, A. Y. Hallmarks of Tissue-Resident Lymphocytes. *Cell* **164**, 1198-1211, doi:10.1016/j.cell.2016.02.048 (2016).
547 Constantinides, M. G. *et al.* MAIT cells are imprinted by the microbiota in early life and promote tissue repair. *Science* **366**, doi:10.1126/science.aax6624 (2019).
548 Legoux, F. *et al.* Microbial metabolites control the thymic development of mucosal-associated invariant T cells. *Science* **366**, 494-499, doi:10.1126/science.aaw2719 (2019).

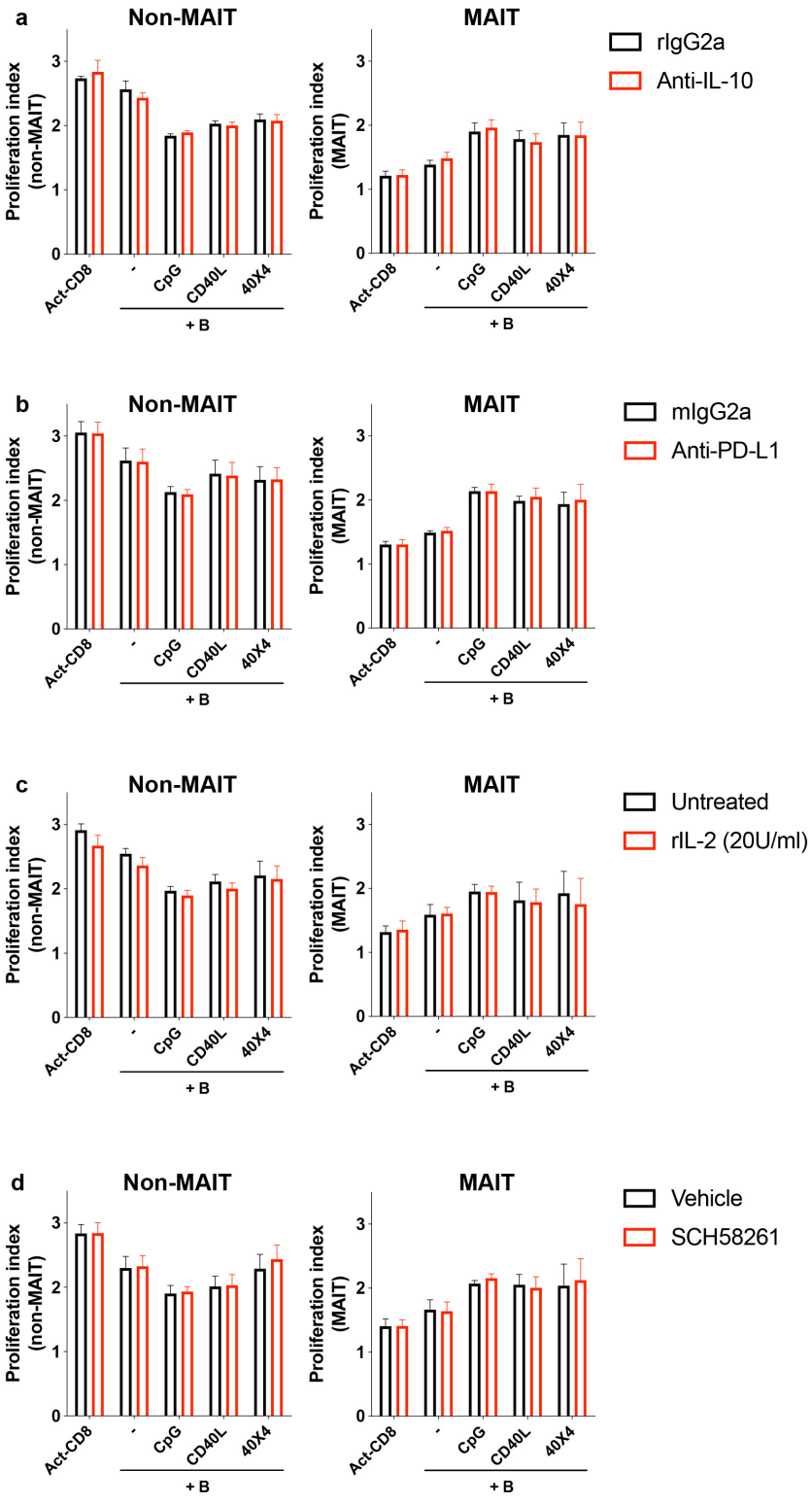
549 Gherardin, N. A. *et al.* Diversity of T Cells Restricted by the MHC Class I-Related Molecule MR1 Facilitates Differential Antigen Recognition. *Immunity* **44**, 32-45, doi:10.1016/j.immuni.2015.12.005 (2016).
550 Lepore, M. *et al.* Functionally diverse human T cells recognize non-microbial antigens presented by MR1. *Elife* **6**, doi:10.7554/eLife.24476 (2017).
551 Toubal, A., Nel, I., Lotersztajn, S. & Lehuen, A. Mucosal-associated invariant T cells and disease. *Nat Rev Immunol*, doi:10.1038/s41577-019-0191-y (2019).
552 Rouxel, O. *et al.* Cytotoxic and regulatory roles of mucosal-associated invariant T cells in type 1 diabetes. *Nat Immunol* **18**, 1321-1331, doi:10.1038/ni.3854 (2017).
553 Kuric, E. *et al.* No Evidence for Presence of Mucosal-Associated Invariant T Cells in the Insulitic Lesions in Patients Recently Diagnosed with Type 1 Diabetes. *Am J Pathol* **188**, 1744-1748, doi:10.1016/j.ajpath.2018.04.009 (2018).
554 Serriari, N. E. *et al.* Innate mucosal-associated invariant T (MAIT) cells are activated in inflammatory bowel diseases. *Clin Exp Immunol* **176**, 266-274, doi:10.1111/cei.12277 (2014).
555 Dunne, M. R. *et al.* Persistent changes in circulating and intestinal gammadelta T cell subsets, invariant natural killer T cells and mucosal-associated invariant T cells in children and adults with coeliac disease. *PLoS One* **8**, e76008, doi:10.1371/journal.pone.0076008 (2013).
556 Magalhaes, I. *et al.* Mucosal-associated invariant T cell alterations in obese and type 2 diabetic patients. *J Clin Invest* **125**, 1752-1762, doi:10.1172/JCI78941 (2015).
557 O'Brien, A. *et al.* Obesity Reduces mTORC1 Activity in Mucosal-Associated Invariant T Cells, Driving Defective Metabolic and Functional Responses. *J Immunol*, doi:10.4049/jimmunol.1801600 (2019).
558 Carolan, E. *et al.* Altered distribution and increased IL-17 production by mucosal-associated invariant T cells in adult and childhood obesity. *J Immunol* **194**, 5775-5780, doi:10.4049/jimmunol.1402945 (2015).
559 Chiba, A. *et al.* Activation status of mucosal-associated invariant T cells reflects disease activity and pathology of systemic lupus erythematosus. *Arthritis Res Ther* **19**, 58, doi:10.1186/s13075-017-1257-5 (2017).
560 Cho, Y. N. *et al.* Mucosal-associated invariant T cell deficiency in systemic lupus erythematosus. *J Immunol* **193**, 3891-3901, doi:10.4049/jimmunol.1302701 (2014).
561 Miyazaki, Y., Miyake, S., Chiba, A., Lantz, O. & Yamamura, T. Mucosal-associated invariant T cells regulate Th1 response in multiple sclerosis. *Int Immunol* **23**, 529-535, doi:10.1093/intimm/dxr047 (2011).
562 Williams, J. W., Huang, L. H. & Randolph, G. J. Cytokine Circuits in Cardiovascular Disease. *Immunity* **50**, 941-954, doi:10.1016/j.immuni.2019.03.007 (2019).
563 Friedrich, M., Pohin, M. & Powrie, F. Cytokine Networks in the Pathophysiology of Inflammatory Bowel Disease. *Immunity* **50**, 992-1006, doi:10.1016/j.immuni.2019.03.017 (2019).
564 Hammond, T. R., Marsh, S. E. & Stevens, B. Immune Signaling in Neurodegeneration. *Immunity* **50**, 955-974, doi:10.1016/j.immuni.2019.03.016 (2019).
565 Lamoreaux, L., Roederer, M. & Koup, R. Intracellular cytokine optimization and standard operating procedure. *Nat Protoc* **1**, 1507-1516, doi:10.1038/nprot.2006.268 (2006).

566 Assenmacher, M., Lohning, M. & Radbruch, A. Detection and isolation of cytokine secreting cells using the cytometric cytokine secretion assay. *Curr Protoc Immunol* **Chapter 6**, Unit 6 27, doi:10.1002/0471142735.im0627s46 (2002).
567 Campbell, J. D. *et al.* Rapid detection, enrichment and propagation of specific T cell subsets based on cytokine secretion. *Clin Exp Immunol* **163**, 1-10, doi:10.1111/j.1365-2249.2010.04261.x (2011).
568 Chatila, T., Silverman, L., Miller, R. & Geha, R. Mechanisms of T cell activation by the calcium ionophore ionomycin. *J Immunol* **143**, 1283-1289 (1989).
569 Brignall, R. *et al.* Integration of Kinase and Calcium Signaling at the Level of Chromatin Underlies Inducible Gene Activation in T Cells. *J Immunol* **199**, 2652-2667, doi:10.4049/jimmunol.1602033 (2017).
570 Feske, S., Giltnane, J., Dolmetsch, R., Staudt, L. M. & Rao, A. Gene regulation mediated by calcium signals in T lymphocytes. *Nat Immunol* **2**, 316-324, doi:10.1038/86318 (2001).
571 Macian, F. *et al.* Transcriptional mechanisms underlying lymphocyte tolerance. *Cell* **109**, 719-731, doi:10.1016/s0092-8674(02)00767-5 (2002).
572 Pinder, C. L. *et al.* Isolation and Characterization of Antigen-Specific Plasmablasts Using a Novel Flow Cytometry-Based Ig Capture Assay. *J Immunol* **199**, 4180-4188, doi:10.4049/jimmunol.1701253 (2017).
573 Oleinika, K. *et al.* CD1d-dependent immune suppression mediated by regulatory B cells through modulations of iNKT cells. *Nat Commun* **9**, 684, doi:10.1038/s41467-018-02911-y (2018).
574 Tan, A. H. *et al.* Aberrant presentation of self-lipids by autoimmune B cells depletes peripheral iNKT cells. *Cell Rep* **9**, 24-31, doi:10.1016/j.celrep.2014.08.043 (2014).
575 DiLillo, D. J., Yanaba, K. & Tedder, T. F. B cells are required for optimal CD4+ and CD8+ T cell tumor immunity: therapeutic B cell depletion enhances B16 melanoma growth in mice. *J Immunol* **184**, 4006-4016, doi:10.4049/jimmunol.0903009 (2010).
576 Shalapour, S. *et al.* Immunosuppressive plasma cells impede T-cell-dependent immunogenic chemotherapy. *Nature* **521**, 94-98, doi:10.1038/nature14395 (2015).
577 Shalapour, S. *et al.* Inflammation-induced IgA+ cells dismantle anti-liver cancer immunity. *Nature* **551**, 340-345, doi:10.1038/nature24302 (2017).
578 Winer, D. A., Winer, S., Shen, L., Chng, M. H. & Engleman, E. G. B lymphocytes as emerging mediators of insulin resistance. *Int J Obes Suppl* **2**, S4-S7, doi:10.1038/ijosup.2012.2 (2012).
579 Gaya, M. *et al.* Initiation of Antiviral B Cell Immunity Relies on Innate Signals from Spatially Positioned NKT Cells. *Cell* **172**, 517-533 e520, doi:10.1016/j.cell.2017.11.036 (2018).
580 King, I. L. *et al.* Invariant natural killer T cells direct B cell responses to cognate lipid antigen in an IL-21-dependent manner. *Nat Immunol* **13**, 44-50, doi:10.1038/ni.2172 (2011).
581 Bosma, A., Abdel-Gadir, A., Isenberg, D. A., Jury, E. C. & Mauri, C. Lipid-antigen presentation by CD1d(+) B cells is essential for the maintenance of invariant natural killer T cells. *Immunity* **36**, 477-490, doi:10.1016/j.immuni.2012.02.008 (2012).
582 Wermeling, F., Lind, S. M., Jordo, E. D., Cardell, S. L. & Karlsson, M. C. Invariant NKT cells limit activation of autoreactive CD1d-positive B cells. *J Exp Med* **207**, 943-952, doi:10.1084/jem.20091314 (2010).

583 Vomhof-DeKrey, E. E. *et al.* Cognate interaction with iNKT cells expands IL-10-producing B regulatory cells. *Proc Natl Acad Sci U S A* **112**, 12474-12479, doi:10.1073/pnas.1504790112 (2015).
584 Turtle, C. J. *et al.* Innate signals overcome acquired TCR signaling pathway regulation and govern the fate of human CD161(hi) CD8alpha(+) semi-invariant T cells. *Blood* **118**, 2752-2762, doi:10.1182/blood-2011-02-334698 (2011).
585 Slichter, C. K. *et al.* Distinct activation thresholds of human conventional and innate-like memory T cells. *JCI Insight* **1**, doi:10.1172/jci.insight.86292 (2016).
586 Bhattacharyya, A. *et al.* Graft-Derived Reconstitution of Mucosal-Associated Invariant T Cells after Allogeneic Hematopoietic Cell Transplantation. *Biol Blood Marrow Transplant* **24**, 242-251, doi:10.1016/j.bbmt.2017.10.003 (2018).
587 Cosgrove, C. *et al.* Early and nonreversible decrease of CD161++ /MAIT cells in HIV infection. *Blood* **121**, 951-961, doi:10.1182/blood-2012-06-436436 (2013).
588 Nishimura, S. *et al.* Adipose Natural Regulatory B Cells Negatively Control Adipose Tissue Inflammation. *Cell Metab* **18**, 759-766, doi:10.1016/j.cmet.2013.09.017 (2013).
589 Geginat, J., Sallusto, F. & Lanzavecchia, A. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. *J Exp Med* **194**, 1711-1719, doi:10.1084/jem.194.12.1711 (2001).
590 Geginat, J., Lanzavecchia, A. & Sallusto, F. Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood* **101**, 4260-4266, doi:10.1182/blood-2002-11-3577 (2003).
591 Gao, Y. *et al.* Mucosal-Associated Invariant T (MAIT) Cells Are Impaired in Th17 Associated Primary and Secondary Immunodeficiencies. *PLoS One* **11**, e0155059, doi:10.1371/journal.pone.0155059 (2016).
592 Gozalbo-Lopez, B. *et al.* The MHC-related protein 1 (MR1) is expressed by a subpopulation of CD38+, IgA+ cells in the human intestinal mucosa. *Histol Histopathol* **24**, 1439-1449, doi:10.14670/HH-24.1439 (2009).
593 Buck, M. D., Sowell, R. T., Kaech, S. M. & Pearce, E. L. Metabolic Instruction of Immunity. *Cell* **169**, 570-586, doi:10.1016/j.cell.2017.04.004 (2017).
594 Kornberg, M. D. *et al.* Dimethyl fumarate targets GAPDH and aerobic glycolysis to modulate immunity. *Science* **360**, 449-453, doi:10.1126/science.aan4665 (2018).
595 Klotz, L. *et al.* Teriflunomide treatment for multiple sclerosis modulates T cell mitochondrial respiration with affinity-dependent effects. *Sci Transl Med* **11**, doi:10.1126/scitranslmed.aao5563 (2019).
596 Palanichamy, A. *et al.* Rituximab efficiently depletes increased CD20-expressing T cells in multiple sclerosis patients. *J Immunol* **193**, 580-586, doi:10.4049/jimmunol.1400118 (2014).
597 Schuh, E. *et al.* Features of Human CD3+CD20+ T Cells. *J Immunol* **197**, 1111-1117, doi:10.4049/jimmunol.1600089 (2016).
598 von Essen, M. R. *et al.* Proinflammatory CD20+ T cells in the pathogenesis of multiple sclerosis. *Brain* **142**, 120-132, doi:10.1093/brain/awy301 (2019).
599 Sabatino, J. J., Jr. *et al.* Anti-CD20 therapy depletes activated myelin-specific CD8(+) T cells in multiple sclerosis. *Proc Natl Acad Sci U S A* **116**, 25800-25807, doi:10.1073/pnas.1915309116 (2019).

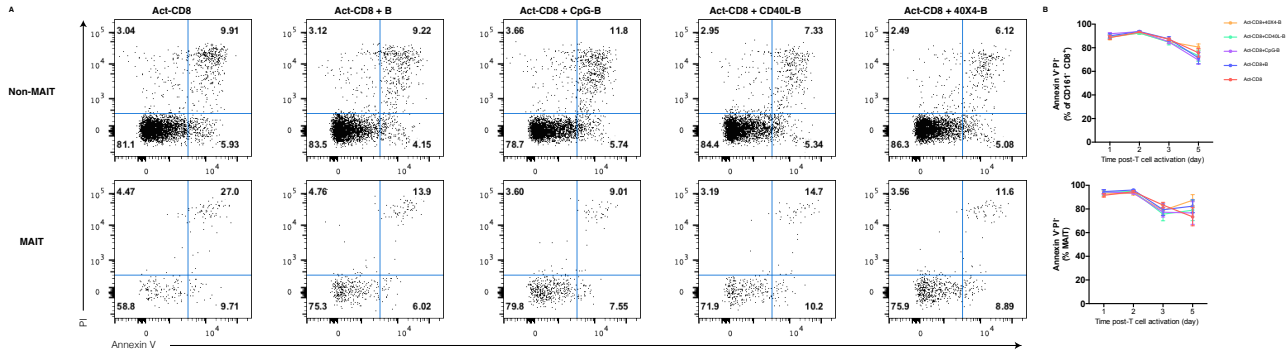
600 Gilli, F., Li, L., Campbell, S. J., Anthony, D. C. & Pachner, A. R. The effect of B-cell depletion in the Theiler's model of multiple sclerosis. *J Neurol Sci* **359**, 40-47, doi:10.1016/j.jns.2015.10.012 (2015).
601 Croxford, J. L., Miyake, S., Huang, Y. Y., Shimamura, M. & Yamamura, T. Invariant V(alpha)19i T cells regulate autoimmune inflammation. *Nat Immunol* **7**, 987-994, doi:10.1038/ni1370 (2006).
602 Sundstrom, P. *et al.* Tumor-infiltrating mucosal-associated invariant T (MAIT) cells retain expression of cytotoxic effector molecules. *Oncotarget* **10**, 2810-2823, doi:10.18632/oncotarget.26866 (2019).
603 Melo, A. M. *et al.* Mucosal-Associated Invariant T Cells Display Diminished Effector Capacity in Oesophageal Adenocarcinoma. *Front Immunol* **10**, 1580, doi:10.3389/fimmu.2019.01580 (2019).
604 Cabrita, R. *et al.* Tertiary lymphoid structures improve immunotherapy and survival in melanoma. *Nature*, doi:10.1038/s41586-019-1914-8 (2020).
605 Helmink, B. A. *et al.* B cells and tertiary lymphoid structures promote immunotherapy response. *Nature*, doi:10.1038/s41586-019-1922-8 (2020).
606 Petitprez, F. *et al.* B cells are associated with survival and immunotherapy response in sarcoma. *Nature*, doi:10.1038/s41586-019-1906-8 (2020).
607 Germain, C. *et al.* Presence of B cells in tertiary lymphoid structures is associated with a protective immunity in patients with lung cancer. *Am J Respir Crit Care Med* **189**, 832-844, doi:10.1164/rccm.201309-1611OC (2014).
608 Sautes-Fridman, C., Petitprez, F., Calderaro, J. & Fridman, W. H. Tertiary lymphoid structures in the era of cancer immunotherapy. *Nat Rev Cancer* **19**, 307-325, doi:10.1038/s41568-019-0144-6 (2019).

Appendix figure 1



Appendix figure 1. B cells regulate non-MAIT cell proliferation independent of IL-10, PD-1, adenosine, IL-2 signaling. MACS-sorted CD8+ T cells were activated with beads coated with anti-CD2, anti-CD3 and anti-CD28 and co-cultured with autologous MACS-sorted B cells (unstimulated or pre-activated with CpG, CD40L or CD40L + anti-IgM + IL-4) for 5 days before assessing T cell proliferation by flow cytometry. Anti-IL-10 antibody, anti-PD-L1 antibody, SCH58261, and IL-2 or appropriate controls were added at the start of cell co-culture. B cells maintained their suppressive effect on non-MAIT cells and maintained their helper effect on MAIT cells independent of IL-10 (A), PD-1 (B), IL-2 (C) and adenosine (D), signaling (n=3-5). All values are represented as mean ± SEM.

Appendix figure 2



Appendix figure 2. B cells do not impact CD8$^+$ T cell subset survival. MACS-sorted CD8+ T cells were activated with beads coated with anti-CD2, anti-CD3 and anti-CD28 and co-cultured with autologous MACS-sorted B cells (unstimulated or pre-activated with CpG, CD40L or CD40L + anti-IgM + IL-4) for up to 5 days before assessing T cell survival by flow cytometry. The presence of B cells in co-culture with T cells does not induce significant changes in cell survival quantified using Annexin V and PI, as shown in (A) a representative experiment, and (B) summarized for n=5 independent experiments. All values are represented as mean ± SEM.