



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.



phd
medicine

Thesis 11010

The Role of Innate Immune Cells in the Pathogenesis of Experimental Autoimmune Encephalomyelitis



2015

Niamh Mc Guinness

B.A. (Mod) Neuroscience

A thesis submitted to the University of Dublin, Trinity College for the
Degree of Doctor of Philosophy

Supervisors: Prof. Marina Lynch and Prof. Kingston Mills

Trinity College Institute of Neuroscience,
Department of Physiology,
School of Medicine

TRINITY LIBRARY
27 JUL 2016
DUBLIN

For Mam and Dad, who believed I could do it, so I did.

I. Declaration

This thesis is submitted by the undersigned for the degree of Doctor of Philosophy at the University of Dublin. I declare that this thesis, with the exception of Figure 4.8 A and B, which belong to Dr. Lara Dungan, is entirely my own work. This work has not been submitted in whole or part to this or any other university for any other degree. The author gives permission to the library to lend or copy this work upon request.

II. Abstract

Experimental autoimmune encephalomyelitis (EAE) is a widely exploited animal model of multiple sclerosis (MS) and is a valuable tool in understanding the cellular and molecular basis of autoimmunity and inflammation in the central nervous system (CNS). Like MS, the primary pathogenic event in EAE is the infiltration of encephalitogenic CD4⁺ T cells into the brain and spinal cord, particularly autoantigen-specific, IL-17-producing Th17 cells, and IFN- γ -producing Th1 cells. Once these cells enter the CNS, they mediate damage to axons through targeted destruction of the myelin sheath, and thus contribute to neuronal dysfunction. Recently it has come to light that cells of the innate immune system, such as natural killer (NK) cells, and macrophages, may also play a critical role in the induction of EAE through modulation of the encephalitogenicity of CD4⁺ T cells.

It was demonstrated that IFN- γ -producing NK cells infiltrated the CNS before the onset of EAE, and significantly, before the entry of Th1 and Th17 cells. The infiltration of IFN- γ -producing NK cells was associated with an increase in production of the M1-related cytokine, TNF- α , by microglia. *In vitro* studies revealed that NK cells were potent activators of microglia, and could simultaneously enhance microglial mRNA expression of the M1 markers iNOS, TNF- α and MHC II, and reduce the expression of the M2 markers MRC1 and ARG1.

While depletion of NK cells continuously throughout, or at the effector stage of EAE had no effect on clinical scores, depletion of NK cells at the induction of disease significantly diminished the severity of EAE. This attenuation was associated with a significant reduction in the infiltration of Th1 and Th17 cells into the brain. In contrast, depletion of NK cells at the induction of EAE had no effect on the progression of disease in IFN- γ ^{-/-} mice, confirming that the observed effects of NK cells early in EAE are mediated by IFN- γ .

Very late antigen-4 (VLA-4) is a critical mediator of the encephalitogenicity of CD4⁺ T cells in EAE and is required for the infiltration of these cells into the CNS. Blockade of VLA-4 using a monoclonal antibody against the α 4 subunit, CD49d, was effective in reducing clinical scores in EAE when administered at the effector phase of active

disease and completely preventing the development of EAE when administered at the time of adoptive transfer of MOG-specific cells. Co-incubation experiments revealed that NK cells, through polarisation of macrophages to the M1 phenotype, could enhance VLA-4 expression on CD4⁺ T cells, thus providing a mechanism for the observed pathogenicity of IFN- γ -producing NK cells early in EAE.

IL-4 is the signature cytokine of Th2 cells and is required for M2 polarisation of macrophages, which are instrumental in the resolution of inflammation. This study identified a suppressive role for IL-4 in EAE. Culturing MOG-specific cells with exogenous IL-4 enhanced the mRNA expression of the M2 markers MRC1 and ARG1, and reduced expression of the M1 marker iNOS. IL-4 also reduced expression of VLA-4 by CD4⁺ T cells in the culture. *In vivo*, MOG-specific cells re-stimulated with MOG, IL-1 β and IL-23 with exogenous IL-4 failed to transfer EAE, and recipient mice displayed diminished expression of MHC II by microglia. This was associated with a reduced infiltration of Th1 and Th17 cells into the brain. Further *in vitro* assays revealed that IL-4 could reduce VLA-4 expression by CD4⁺ T cells in two ways; directly, or through induction of M2 macrophages, which suppressed the expression of this integrin by CD4⁺ T cells in culture.

Collectively, the results from this thesis establish a crucial role for cells of the innate immune system in the induction of EAE. NK cells, by virtue of innate IFN- γ production, can polarise microglia to the M1 phenotype and contribute to neuroinflammation. In the periphery, IFN- γ -producing NK cells can induce the M1 state in macrophages, which in turn enhance VLA-4 expression by CD4⁺ T cells. In contrast, the induction of the M2 state in macrophages by IL-4 can reduce expression of this integrin and thus prevent disease.

III. Lay Abstract

EAE is an animal model for MS, and has been widely used to gain an understanding of the biological events which are responsible for the illness. A commonality between EAE and MS is the infiltration of CD4⁺ T cells into the brain and spinal cord. These cells, which can produce inflammatory proteins, called cytokines, like IFN- γ (Th1 cells) or IL-17 (Th17 cells) target and destroy myelin, a fatty substance which surrounds neurons and is crucial for their function. Destruction of myelin can lead to neuronal death which correlates with the motor, sensory and autonomic deficits in MS.

CD4⁺ T cells constitute part of the adaptive immune system, which is so-called due to its ability to recognise and remember specific antigens or foreign substances and mount a tailored response against the offending agent. MS and EAE are autoimmune diseases, in which the adaptive immune system erroneously targets self-antigens and causes damage to the host. In MS and EAE, the self-antigens in question are components of the myelin sheath, for example myelin oligodendrocyte glycoprotein (MOG). For many years, the autoantigen-specific, adaptive, CD4⁺ T cells have been considered the primary disease-causing cells. However, more recent evidence suggests that cells of the innate immune system, which is the first line of defence against foreign agents, may also have an important role to play. Of particular interest are two cell types, called NK cells and macrophages. This study has highlighted a role for these cells in modulating the disease-causing ability, or encephalitogenicity, of CD4⁺ T cells.

Flow cytometric analysis of the brains of mice with EAE revealed that IFN- γ -producing NK cells infiltrated the CNS before the onset of EAE, and significantly, before the entry of Th1 and Th17 cells. The infiltration of IFN- γ -producing NK cells was associated with an increase in production of an inflammatory cytokine, TNF- α , by microglia, the resident immune cells of the CNS. Microglia and macrophages, while distinct in their origin, share an important characteristic, namely the capacity to adopt distinct "activation states" in response to their environment. Exposure to IFN- γ or the bacterial component LPS induces the classical or M1 activation state, which is associated with the production of pro-inflammatory cytokines and nitric oxide, which are required for pathogen destruction. Conversely, the M2 or alternative activation state is induced by

exposure to the anti-inflammatory cytokine IL-4, and is associated with resolution of inflammation and wound healing. As TNF- α is the signature cytokine of the M1 activation state, the correlation between IFN- γ -producing NK cell infiltration and TNF- α production by microglia suggested that NK cells may play a role in microglial activation. *In vitro* studies revealed that NK cells were potent activators of microglia, and could simultaneously enhance microglial mRNA expression of the M1 markers iNOS, TNF- α and MHC II, and reduce the expression of the M2 markers MRC1 and ARG1.

To further investigate the role of NK cells in the pathogenesis of EAE, these cells were depleted from mice with EAE using an antibody against asialo GM1, which induces NK cell death *in vivo*. While depletion of NK cells continuously throughout, or at the effector stage of EAE had no effect on clinical scores, depletion of NK cells at the induction of disease significantly diminished the severity of EAE. This attenuation was associated with a significant reduction in the infiltration of Th1 and Th17 cells into the brain. In contrast, depletion of NK cells at the induction of EAE had no effect on the progression of disease in genetically modified mice deficient in IFN- γ , confirming that the observed effects of NK cells early in EAE are mediated by IFN- γ .

The integrin very late antigen-4 (VLA-4) is a critical mediator of the encephalitogenicity of CD4⁺ T cells in EAE and is required for the infiltration of these cells into the CNS. Blockade of VLA-4 using a monoclonal antibody against the α 4 subunit, CD49d, was effective in reducing clinical scores in EAE when administered at the effector phase of active disease and completely preventing the development of EAE when administered at the time of adoptive transfer of MOG-specific cells. Co-incubation experiments revealed that NK cells, through polarisation of macrophages to the M1 phenotype, could enhance VLA-4 expression on CD4⁺ T cells, thus providing a mechanism for the observed pathogenicity of IFN- γ -producing NK cells early in EAE.

IL-4 is required for M2 polarisation of macrophages, which are instrumental in the resolution of inflammation. This study identified a suppressive role for IL-4 in EAE. Culturing MOG-specific cells with exogenous IL-4 enhanced the mRNA expression of the M2 markers MRC1 and ARG1, and reduced expression of the M1 marker iNOS. IL-4

also reduced expression of VLA-4 by CD4⁺ T cells in the culture. *In vivo*, MOG-specific cells re-stimulated with MOG and exogenous IL-4 failed to transfer EAE, and recipient mice displayed diminished expression of the M1 marker MHC II by microglia. This was associated with a reduced infiltration of Th1 and Th17 cells into the brain. Further *in vitro* assays revealed that IL-4 could reduce VLA-4 expression by CD4⁺ T cells in two ways; directly, or through induction of M2 macrophages, which suppressed the expression of this integrin by CD4⁺ T cells in culture.

Collectively, the results from this thesis establish a crucial role for cells of the innate immune system in the induction of EAE. NK cells, by virtue of innate IFN- γ production, can polarise microglia to the M1 phenotype and contribute to neuroinflammation. In the periphery, IFN- γ -producing NK cells can induce the M1 state in macrophages, which in turn enhance VLA-4 expression by CD4⁺ T cells. In contrast, the induction of the M2 state in macrophages by IL-4 can reduce expression of this integrin and thus prevent disease.

IV. Acknowledgements

First and foremost, I would like to thank my wonderful supervisors, Professor Kingston Mills and Professor Marina Lynch for their unwavering support over the last three years. They were a constant source of guidance and encouragement and I count myself extremely lucky have had the chance to work in the lab of two such incredible scientists.

I would like to also thank the incomparable Dr. Lara Dungan for her help with the early EAE experiments, for her patience, for her infectious enthusiasm and for her friendship. She gave me the best possible introduction to the field of immunology and I am eternally grateful for that.

Thanks to Dr. Sarah Edwards and Aoife McGinley for all of their help with the termination of EAE experiments.

Thanks to Barry Moran for his invaluable expertise in all things FACS-related.

To Fintan, Simon and Deborah, thank you all so much for your patience in helping me to unravel the mysteries of Seahorse.

Many of the figures were created using the open source Servier Slide Kit, © 2015 Les Laboratoires Servier.

Finally, thank you to PRTL, who funded this project and made it all possible.

V. Thanks to Friends

I was in the rather privileged position of not only having two excellent supervisors, but also being a member of two stellar lab groups. With regards to the members of MAL lab, I would like to thank Aedín, Niamh, Karen, Edel, Raasay, Eric, Claire, Derek, Roisin, James, Rob, Colin and Hannah for all their support and patience. Despite the fact my appearances in KM lab were less frequent, I was always made feel entirely welcome and I never had a question that went unanswered, so I owe massive thanks to Caroline, Kevin, Paddy, Anna, Conor, Ania (Stefanska and Malara), Mieszko, Lydia, Aideen, Aoife, Alicja, Mathilde, Rob, Sarah (Higgins and Edwards), Lara, Jan and Heather.

To my Room 3.61 office buddies, you have no idea how grateful I am for your friendship over the last few years. Colin, Hannah, Roisín and Rob, thank you for putting up with my almost constant nattering, singing, and emotional outbursts. I am entirely certain I could not have gotten through it without you. I owe you all so much.

To Colin, Steven, Justin, Eimear, Catherine, and Niamh – thank you for being my family in all but name, and for being there always. I only wish I could keep you all close forever.

To the Purtles, Sarah, Jade, Deborah, Brian, Simon, Donal, Fintan and David, it was an absolute pleasure to share the journey with you all.

To my Rouge Ladies, Sarah and Aoife, I barely know how to even go about thanking you both. Thank you for the help with experiments, thank you for listening, and most of all thank you for being two of my favourite people. I'm honoured to share a publication with you two.

To Orla, Vivienne and Heather, thanks to all of you for the Trojan work in keeping KM lab running smoothly and dealing with my frantic last-minute orders!

To Sarah, Raasay, Aedin and Eric, thank you all so much for your excellent work in making the PRTL program about so much more than just research.

To Kieran, Aidan and Alice, and all the staff in Physiology, thanks for everything (including the biscuits!).

To all of the staff in TCIN past and present, Ciarán, Barbara, Lisa, Laura, Denise, Susan and Katy- thank you for making the 3rd floor such a lovely place to work.

To the wonderful Eimear, thank you for being the best housemate a girl could ask for. You're some kind of angel.

To Ben, despite the fact you were an ocean away for the last few months, thank you for the support, for listening to my increasingly erratic phonecalls and for not losing patience with me when anyone else would have. I can't wait for our Canadian adventure.

And finally, to my Mam and Dad – it's very difficult to put into words how much your support has meant to me over the last four years. Thank you for listening, for encouraging and supporting me. There is no way I could have done it without you.

VI. Aims and Hypothesis

Aims I

- 1) To investigate the effect of IFN- γ and IL-4 on mRNA expression of M1 and M2 markers in macrophages, microglia and astrocytes.
- 2) To elucidate the metabolic profiles of IFN- γ and IL-4-polarised cells.

Hypothesis I

- 1) The mRNA expression of M1 and M2 markers in response to treatment with IFN- γ and IL-4 respectively will be similar in macrophages, microglia and astrocytes.
- 2) The similarities in mRNA expression profiles will be mirrored in the metabolic phenotypes in these cell types.

Aims II

- 1) To investigate the kinetics of NK cell infiltration into the CNS of mice with EAE.
- 2) To examine IFN- γ production by NK cells in the CNS of mice with in EAE.
- 3) To determine the effect of NK cell depletion at different time points on the severity of EAE.

Hypothesis II

- 1) The temporal profile of NK cell infiltration into the CNS will mirror that of CD4⁺ T cells, and will be present in highest numbers in the peak of EAE.
- 2) IFN- γ production by NK cells in the CNS of mice with in EAE will be high at the peak of disease.
- 3) NK cell depletion at all timepoints in EAE will result in reduction in disease severity.

Aims III

- 1) To establish whether the pattern of CNS NK cell infiltration and IFN- γ production correlated with an upregulation in the M1 state in microglia.

- 2) To investigate the effect of NK cell co-incubation with glia and macrophages on cell phenotype.
- 3) To examine the effect of NK cell co-incubation with macrophages on cell phenotype and consequently VLA-4 expression on CD4⁺ T cells.
- 4) To determine the effect of VLA-4 blockade at the induction and effector phases of EAE.

Hypothesis III

- 1) NK cell infiltration and IFN- γ in the CNS will positively correlate with an increase in the expression of M1 markers in microglia.
- 2) Incubation of glia and macrophages with NK cells will result in polarisation of the former to the M1 phenotype.
- 3) Incubation of glia and macrophages with NK cells will result in polarisation of the former to the M1 phenotype and will therefore elicit an increase in VLA-4 expression by CD4⁺ T cells.
- 4) VLA-4 blockade will diminish EAE severity.

Aims IV

- 1) To assess the effect of IL-4 addition on the M1/M2 polarisation status of a MOG-specific culture.
- 2) To examine the effect of IL-4 addition to a culture of MOG-specific cells on encephalitogenic function of CD4⁺ T cells through assessment of VLA-4 expression and cytokine production.
- 3) To assess the effect of IL-4 addition to a culture of MOG-specific cells on their ability to induce EAE in recipient mice.
- 4) To investigate microglial activation/infiltration of CD4⁺ T cells in the brains of recipient mice that received MOG-specific cells treated with IL-4.

5) To elucidate the direct effect of IL-4 on VLA-4 expression on CD4⁺ T cells, as well as indirectly through induction of M2-polarised innate cells.

Hypothesis IV

1) IL-4 addition will induce the M2 state in a MOG-specific culture.

2) IL-4 addition to a culture of MOG-specific cells will reduce expression of VLA-4 and pathogenic cytokine production.

3) IL-4 addition to a culture of MOG-specific cells will reduce their ability to induce EAE in recipient mice.

4) Microglial activation/infiltration of CD4⁺ T cells in the brains of recipient mice that received MOG-specific cells treated with IL-4 will be reduced.

5) Direct application of IL-4 to CD4⁺ T cells will reduce VLA-4 expression, as will incubation with M2-polarised innate cells.

VII. Value of Research

Despite the fact that MS was first characterised in 1868, and that its animal model EAE was developed in the early 1930s, the etiology of MS remains unclear. While compounds that prevent T cell egress from the lymph nodes (Fingolimod) or infiltration into the CNS (Natalizumab) have been successful in reducing MS symptoms in a subset of patients, neither have been capable of curing the disease, nor even halting its progression in a large number of cases. This highlights a need to understand the early events underlying MS pathogenesis, and identify the cellular interactions responsible for the observed encephalitogenicity of CD4⁺ T cells. Here a role for innate cells in the initiation of EAE has been presented. Prior to the appearance of CD4⁺ T cells in the CNS, NK cells infiltrate and produce IFN- γ , thus contributing to neuroinflammation through induction of the inflammatory M1 state in glia. In the periphery, these IFN- γ producing cells also elicit an enhancement in the M1 state in macrophages, which can enhance CD4⁺ T cell encephalitogenicity through induction of VLA-4 expression by these cells. In contrast, EAE can be prevented through polarisation of innate cells to the M2 phenotype (using IL-4), which reverses the VLA-4 induction observed with exposure to IFN- γ from NK cells.

Taken together, these data uncover a novel role for innate immune cells in the initiation of EAE. It is yet to be elucidated whether or not these early events also occur in MS. If so, the work in this thesis further highlight the need for the development of prophylactic therapies which will target the early M1 polarisation of innate immune cells, and thus prevent the enhancement of VLA-4 expression of CD4⁺ T cells prior to their migration to the CNS, which ultimately results in irreversible neurodegeneration.

VIII. Outputs

Publications:

Tanveer, R., **McGuinness, N.**, Daniel, S., Gowran, A., Campbell, V.A. (2012) Cannabinoid receptors and neurodegenerative diseases. *WIREs Membrane Transport and Signalling*, 1(5): 633-639

Dungan, L.S.*, **McGuinness, N.C.***, Boon, L., Lynch, M.A., Mills, K.H.G (2014) Innate IFN- γ promotes development of experimental autoimmune encephalomyelitis: a role for NK cells and M1 macrophages. *European Journal of Immunology*, 44 (10): 2903-2917

*both authors contributed equally to this work

Edwards, S.C., McGinley, A.M., **McGuinness, N.C.**, Mills, K.H.G (2015) $\gamma\delta$ T cells and NK cells – distinct pathogenic roles as innate-like immune cells in CNS autoimmunity. *Frontiers in Immunology*, 6 (455): 1-6

Oral Presentations:

“NK cells in animal models of MS”- Frontiers in Neurology, Trinity College Dublin, 15th November 2013.

“Natural killer cells promote the induction of EAE but are protective at the acute stage of disease” - Postgraduate Research Day 18th November 2013, St. James' Hospital.

“IFN- γ -producing natural killer cells are pathogenic in experimental autoimmune encephalomyelitis by promoting M1 macrophage activation and VLA-4 expression on CD4⁺ T cells” - International Society of Neuroimmunology (ISNI) 12th Congress - 9th-13th November 2014, Mainz, Germany.

“A role for innate IFN- γ in the pathogenesis of experimental autoimmune encephalomyelitis”- Trinity College Institute of Neuroscience symposium - November 4th 2014.

Posters:

“Natural killer cells promote the induction of EAE but are protective at the acute stage of disease” - International Congress of Immunology, Milan, August 22-27 2013

“Natural killer cells promote the induction of EAE but are protective at the acute stage of disease” - Young Life Scientists Symposium - Cell Signalling, University College Cork, 11th September 2013

“Natural killer cells promote the induction of EAE but are protective at the acute stage of disease” - Neuroscience Ireland, University College Cork, 12-13th September 2013: Prize Winner

“Natural killer cells promote the induction of EAE but are protective at the acute stage of disease” - Frontiers in Neurology, Trinity College Dublin, 15th November 2013

Abstracts:

“Natural killer cells promote the induction of EAE but are protective at the acute stage of disease” - International Congress of Immunology, Milan, August 22-27 2013

“Natural killer cells promote the induction of EAE but are protective at the acute stage of disease” - Young Life Scientists Symposium - Cell Signalling, University College Cork, 11th September 2013

“Natural killer cells promote the induction of EAE but are protective at the acute stage of disease” - Neuroscience Ireland, University College Cork, 12-13th September 2013

“Natural killer cells promote the induction of EAE but are protective at the acute stage of disease” - Frontiers in Neurology, Trinity College Dublin, 15th November 2013

“IFN- γ -producing natural killer cells are pathogenic in experimental autoimmune encephalomyelitis by promoting M1 macrophage activation and VLA-4 expression on CD4⁺ T cells” - International Society of Neuroimmunology (ISNI) 12th Congress - 9th-13th November 2014, Mainz, Germany.

“A role for innate IFN- γ in the pathogenesis of experimental autoimmune encephalomyelitis” - Trinity College Institute of Neuroscience symposium - November 4th 2014.

IX. Table of Contents

Contents

I. Declaration	i
II. Abstract	iii
III. Lay Abstract	v
IV. Acknowledgements	viii
V. Thanks to friends	ix
VI. Aims and Hypothesis	xi
VII. Value of Research	xiv
VIII. Outputs	xv
IX. Table of Contents	xvii
X. List of Figures	xxiv
XI. List of Tables	xxvii
XII. List of Abbreviations	xxviii
Chapter 1: Introduction	1
1.1 Multiple Sclerosis	3
Figure 1.1 Types of multiple sclerosis	3
1.1.1 MS therapies	4
1.2 Murine Experimental Autoimmune Encephalomyelitis	5
1.3 The role of T cells in MS/EAE	8
1.3.1 Type 1 T helper (Th1) cells	8
1.3.2 Th17 cells	9
Figure 1.2 The differentiation of T helper cell subsets	10
1.3.3 CD8 ⁺ T cells	11
1.3.4 $\gamma\delta$ T cells	11
1.4 The role of integrins in T cell pathogenicity in MS/EAE	11
1.4.1 VLA-4 ($\alpha 4\beta 1$ integrin)	12
Figure 1.3 Membrane topology and signalling of VCAM-1	13
1.4.2 LFA-1 ($\alpha L\beta 2$ integrin)	13
1.5 Natural killer Cells	14
1.5.1 NK cell maturation	14
1.5.2 NK cell distribution	16

1.5.3 NK cell function	16
Figure 1.4 NK cell tolerance	18
Figure 1.5 NK cell killing	18
1.6 The role of NK cells in EAE/MS.....	19
1.7 An introduction to IFN- γ	22
Figure 1.6 Signalling induced by ligation of Type 1 and Type 2 interferons with their cognate receptors	23
1.8 The role of IFN- γ in MS/EAE.....	24
1.9 An introduction to IL-4.....	24
Figure 1.7 IL-4 signalling.....	25
1.10 The role of IL-4 in EAE/MS	26
1.11 Microglia	27
1.12 Macrophages.....	27
1.13 Activation States	29
Figure 1.8 An outline of the M1 and M2 activation states in microglia and macrophages	29
1.13.1 Classical/ M1 Activation.....	30
1.13.2 Alternative/ M2 activation.....	30
1.13.3 Activation-induced changes in cellular morphology.....	32
1.14 The role of microglia in EAE/MS	32
1.15 The role of macrophages in EAE/MS	34
1.16 Astrocytes	35
1.16.1 Can astrocytes adopt M1/M2-like activation states?.....	36
1.17 The role of astrocytes in EAE/MS.....	36
1.18 Metabolic Signatures of Polarised Cells.....	38
1.18.1 Glucose metabolism.....	38
Figure 1.9 The conversion of glucose to pyruvate in glycolysis	38
1.18.2 Glucose metabolism and CD4 ⁺ T cell function	39
1.18.3 Glucose metabolism and macrophage function	40
Figure 1.10 Macrophage polarisation and glucose metabolism.....	42
1.18.4 Glucose metabolism and microglial function	42
1.18.5 Glucose metabolism and astrocyte function	43
1.18.6 Glucose metabolism and EAE	43
Chapter 2: Materials and Methods	45
2.1 Animals.....	47

2.2 Cell Counting	47
2.3 Isolation and culture of murine mixed glia	47
2.4 Isolation and culture of primary murine astrocytes	48
2.5 Isolation and culture of primary murine microglia	48
2.6 Maintenance of L929 cell line	49
2.7 Isolation and culture of murine bone marrow-derived macrophages (BMDMs)	49
2.8 <i>In vitro</i> M1/M2 polarisation of cells	50
2.9 Isolation of peritoneal macrophages (PMacs) from murine peritoneal exudate cells (PEC)	50
2.10 Removal of spleen and lymph nodes	52
Figure 2.1 Location of murine lymph nodes	52
2.11 Isolation of murine CD3 ⁺ T cells	52
2.12 Co-incubation of PMacs and CD3 ⁺ T cells	53
2.13 Treatment of stimulated CD3 ⁺ T cells with IL-4	53
2.14 Isolation and culture of murine natural killer (NK) cells	53
Figure 2.2 Representative dot plot of typical NK cell purity following culture period	55
2.15 Co-culture of murine BMDMs, mixed glia or microglia with NK cells	55
Figure 2.3 Experimental design of NK cell co-culture with BMDMS, mixed glia or microglia	55
2.16 Generation of MOG-specific CD4 ⁺ T cells	56
2.17 Co-incubation of MOG-specific CD4 ⁺ T cells, BMDMs and NK cells, and assessment of VLA-4 expression	56
2.18 Cell Harvesting and Supernatant Collection	57
2.19 ELISA (Enzyme-Linked Immunosorbent Assay – R&D Systems, UK)	57
2.20 LDH Assay (Lactate dehydrogenase CytoTox 96 [®] assay (Promega, USA))	58
2.21 RNA Extraction	58
2.22 Quantification of RNA Concentration	59
2.23 Reverse transcription for cDNA Synthesis	60
2.24 cDNA amplification by RT-PCR	60
2.25 PCR Quantification	61
2.26 Investigation of metabolic signatures of polarised cells: Using the Seahorse Extracellular Flux (XF24) Analyser (Seahorse Bioscience, USA)	61
Figure 2.4 Seahorse cell plate layout and inhibitor concentrations	62
Figure 2.5 Reference ECAR and OCR graphs provided by Seahorse Bioscience	63
2.27 Emulsification of MOG for EAE induction	63

2.28 Subcutaneous Injection.....	64
2.29 Intraperitoneal Injection.....	64
Figure 2.6 Schematic outlining the active and passive EAE protocols.....	65
Figure 2.7 Timeline of NK cell depletion.....	66
2.31 Adoptive Transfer/Passive EAE.....	66
2.31.1 Addition of IL-4 to cultures.....	67
2.32 Blockade of VLA-4.....	67
2.33 Disease monitoring and clinical score regime.....	67
2.35 Removal of brain and spinal cord.....	68
Figure 2.8 Appearance of cells after density-dependent centrifugation in Percoll.....	70
2.38 Flow Cytometry: Extracellular Staining and Fixation.....	70
2.39 Flow Cytometry: Permeabilisation and Intracellular Staining.....	71
2.40 Flow Cytometry: Compensation and Fluorescence Minus One (FMO) Controls....	71
2.41 Flow Cytometric Analysis.....	72
2.42 Statistical Analysis.....	72
Figure 2.9 Gating strategies for flow cytometric analysis of CNS cell populations.....	73
Figure 2.10 Cytokine production by CD4 ⁺ T cells used to induce EAE by passive transfer .	74
Chapter 3: Characterisation of the M1 and M2 phenotypes and corresponding metabolic profiles of BMDMs, microglia, and astrocytes.....	77
3.1 Introduction.....	79
3.2 Results.....	82
Figure 3.1 IFN- γ enhances mRNA expression of the M1 markers iNOS, TNF- α and MHC II, and IL-4 enhances mRNA expression of the M2 markers MRC1, ARG1 and CHI3L3 in macrophages.....	85
Figure 3.2 IFN- γ enhances mRNA expression of the M1 markers iNOS, TNF- α and MHC II, and IL-4 enhances mRNA expression of the M2 markers MRC1, ARG1 and CHI3L3 in microglia.....	86
Figure 3.3 IFN- γ enhances mRNA expression of the M1 markers iNOS, TNF- α and MHC II, and IL-4 enhances mRNA expression of the M2 markers MRC1, ARG1 and CHI3L3 in astrocytes.....	87
Figure 3.4 IFN- γ significantly enhances baseline ECAR and reduces maximal respiration in macrophages.....	88
Figure 3.5 IFN- γ increases baseline ECAR and IL-4 increases maximal respiration in microglia.....	89
Figure 3.6 Neither IFN- γ nor IL-4 treatment affects ECAR or OCR in astrocytes.....	90

Figure 3.7 IFN- γ increases ECAR/OCR and reduces OCR/ECAR ratio in macrophages, IL-4 increases OCR/ECAR and reduces ECAR/OCR in microglia and astrocyte ratios are unaffected by either treatment	91
3.3 Discussion.....	92
Figure 3.8 The effect of treatment with IFN- γ or IL-4 on the polarisation and metabolic profiles of macrophages, microglia and astrocytes.	96
Chapter 4: NK cells infiltrate the CNS prior to disease onset in EAE and provide an early source of innate IFN-γ	97
4.1 Introduction	99
4.2 Results.....	101
Figure 4.1 CD3 ⁺ NK1.1 ⁺ cells infiltrate the brain prior to the onset of clinical signs of EAE	104
Figure 4.2 CD3 ⁺ NK1.1 ⁺ cells infiltrate the spinal cord prior to the onset of clinical signs of EAE	105
Figure 4.3 The number of CD3 ⁺ NK1.1 ⁺ IFN- γ ⁺ cell increases in the brain early in EAE	106
Figure 4.4 CD3 ⁺ CD4 ⁺ IFN- γ ⁺ cells infiltrate the brain at the peak of disease.....	107
Figure 4.5 CD3 ⁺ CD4 ⁺ IL-17 ⁺ cells infiltrate the brain at the peak of disease	108
Figure 4.6 Neither continuous nor late depletion of NK cells affects the clinical course of EAE , while early depletion of NK cells delays onset and rreduces severity of EAE	109
Figure 4.7 Early NK cell depletion reduces the number of CD11b ⁺ CD45 ^{low} cells and the infiltration of CD3 ⁺ CD4 ⁺ IFN- γ ⁺ and CD3 ⁺ CD4 ⁺ IL-17 ⁺ in the brains of mice with EAE	110
Figure 4.8 IFN- γ ^{-/-} mice exhibit a delayed onset and increased severity of active EAE relative to WT mice, however EAE clinical scores in IFN- γ ^{-/-} mice are unaffected by NK cell depletion	111
4.3 Discussion.....	112
Figure 4.9 A role for NK cells early in EAE	115
Chapter 5: Induction of the M1 phenotype and CD4⁺ T cell encephalitogenicity by NK cells	117
5.1 Introduction	119
5.2 Results.....	122
Figure 5.1 Production of TNF- α by CD11b ⁺ CD45 ^{low} cells increases in the murine brain early in EAE.....	127
Figure 5.2 Addition of IL-12 significantly enhances IFN- γ production by NK cells	128
Figure 5.3 Incubation with NK cells, but not conditioned medium from NK cells, induces microglial cell death	129

Figure 5.4 Incubation with conditioned medium from NK cells increases mRNA expression of the M1 markers iNOS, TNF- α and MHC II and reduces mRNA expression of the M2 markers MRC1 and ARG1 in microglia	130
Figure 5.5 NK cells are not cytotoxic towards mixed glia	131
Figure 5.6 NK cells or NK cell-conditioned medium increases mRNA expression of the M1 markers iNOS, MHC II and TNF- α in mixed glia.....	132
Figure 5.7 NK cells are not cytotoxic towards BMDMs.....	133
Figure 5.8 NK cells or NK cell-conditioned medium increases mRNA expression of the M1 markers iNOS and MHC II in BMDMs and reduces mRNA expression of the M2 markers MRC1 and CHI3L3 in BMDMs.....	134
Figure 5.9 Treatment of NK cells with IL-12 enhances IFN- γ production and upregulates expression of the M1 marker MHC II by BMDMs	135
Figure 5.10 NK cell-derived IFN- γ promotes M1 macrophage polarisation which increases VLA-4 expression on MOG-specific CD4 ⁺ T cells	136
Figure 5.11 Blockade of VLA-4 in active EAE from day 7 significantly attenuates EAE, while blockade of VLA-4 in recipient mice up until day 12 completely attenuates EAE induced by cell transfer	137
5.3 Discussion.....	138
Figure 5.12 A role for NK cells in modulating glial and macrophage polarisation and CD4 ⁺ T cell encephalitogenicity in EAE	143
Chapter 6: The role of IL-4 in EAE.....	145
6.1 Introduction	147
6.2 Results.....	149
Figure 6.1 IL-4 increases mRNA expression of M2 markers MRC1 and ARG1 and reduces mRNA expression of the M1 marker iNOS and IL-17A production in a MOG-specific culture	152
Figure 6.2 IL-4 reduces expression of VLA-4 by CD4 ⁺ T cells in a MOG-specific culture ...	153
Figure 6.3 IL-4 prevents the ability of MOG-specific cells to transfer EAE to recipient mice	154
Figure 6.4 Adoptive transfer of IL-4-treated MOG-specific cells reduces MHC II expression by CD11b ⁺ CD45 ^{low} cells, and the number of infiltrating CD3 ⁺ CD4 ⁺ IFN- γ ⁺ and CD3 ⁺ CD4 ⁺ IL-17 ⁺ cells in the brains of recipient mice	155
Figure 6.5 IL-4 reduces expression of CD49d, and mRNA expression of IFN- γ and IL-17A by anti-CD3 and anti-CD28-stimulated CD3 ⁺ T cells	156
Figure 6.6 Incubation with M2 peritoneal macrophages reduces VLA-4 expression on CD3 ⁺ T cells.....	157
6.3 Discussion.....	158

Figure 6.7 IL-4 can prevent the transfer of EAE through induction of M2 macrophages and reduction of VLA-4 expression by CD4 ⁺ T cells.....	161
Chapter 7: General Discussion.....	163
Figure 7.1 Schematic detailing the proposed mechanism of action of NK-derived IFN- γ and recombinant IL-4 in modulating CD4 ⁺ T cell encephalitogenicity in EAE	173
Chapter 8: Bibliography	175
Appendix I: List of Publications	196
Appendix II: List of Materials	197
Appendix III: List of Solutions.....	200

X. List of Figures

Figure 1.1 Types of multiple sclerosis (Lublin and Reingold, 1996)	3
Figure 1.2 The differentiation of T helper cell subsets	10
Figure 1.3 Membrane topology and signalling of VCAM-1	13
Figure 1.4 NK cell tolerance	18
Figure 1.5 NK cell killing	18
Figure 1.6 Signalling induced by ligation of Type 1 and Type 2 interferons with their cognate receptors	23
Figure 1.7 IL-4 signalling	25
Figure 1.8 An outline of the M1 and M2 activation states in microglia and macrophages	29
Figure 1.9 The conversion of glucose to pyruvate in glycolysis	38
Figure 1.10 Macrophage polarisation and glucose metabolism	42
Figure 2.1 Location of murine lymph nodes	52
Figure 2.2 Representative dot plot of typical NK cell purity following culture period	55
Figure 2.3 Experimental design of NK cell co-culture with BMDMS, mixed glia or microglia	55
Figure 2.4 Seahorse cell plate layout and inhibitor concentrations	62
Figure 2.5 Reference ECAR and OCR graphs provided by Seahorse Bioscience	63
Figure 2.6 Schematic outlining the active and passive EAE protocols	65
Figure 2.7 Timeline of NK cell depletion	66
Figure 2.8 Appearance of cells after density-dependent centrifugation in Percoll	70
Figure 2.9 Gating strategies for flow cytometric analysis of CNS cell populations.....	73
Figure 2.10 Cytokine production by CD4 ⁺ T cells used to induce EAE by passive transfer	74
Figure 3.1 IFN- γ enhances mRNA expression of the M1 markers iNOS, TNF- α and MHC II, and IL-4 enhances mRNA expression of the M2 markers MRC1, ARG1 and CHI3L3 in macrophages	85
Figure 3.2 IFN- γ enhances mRNA expression of the M1 markers iNOS, TNF- α and MHC II, and IL-4 enhances mRNA expression of the M2 markers MRC1, ARG1 and CHI3L3 in microglia	86
Figure 3.3 IFN- γ enhances mRNA expression of the M1 markers iNOS, TNF- α and MHC II, and IL-4 enhances mRNA expression of the M2 markers MRC1, ARG1 and CHI3L3 in astrocytes ...	87

Figure 3.4 IFN- γ significantly enhances baseline ECAR and reduces maximal respiration in macrophages	88
Figure 3.5 IFN- γ increases baseline ECAR and IL-4 increases maximal respiration in microglia	89
Figure 3.6 Neither IFN- γ nor IL-4 treatment affects ECAR or OCR in astrocytes	90
Figure 3.7 IFN- γ increases ECAR/OCR and reduces OCR/ECAR ratio in macrophages, IL-4 increases OCR/ECAR and reduces ECAR/OCR in microglia and astrocyte ratios are unaffected by either treatment	91
Figure 3.8 The effect of treatment with IFN- γ or IL-4 on the polarisation and metabolic profiles of macrophages, microglia and astrocytes	96
Figure 4.1 CD3 ⁻ NK1.1 ⁺ cells infiltrate the brain prior to the onset of clinical signs of EAE	104
Figure 4.2 CD3 ⁻ NK1.1 ⁺ cells infiltrate the spinal cord prior to the onset of clinical signs of EAE	105
Figure 4.3 The number of CD3 ⁻ NK1.1 ⁺ IFN- γ ⁺ cell increases in the brain early in EAE	106
Figure 4.4 CD3 ⁺ CD4 ⁺ IFN- γ ⁺ cells infiltrate the brain at the peak of disease	107
Figure 4.5 CD3 ⁺ CD4 ⁺ IL-17 ⁺ cells infiltrate the brain at the peak of disease	108
Figure 4.6 Neither continuous nor late depletion of NK cells affects the clinical course of EAE , while early depletion of NK cells delays onset and reduces severity of EAE.....	109
Figure 4.7 Early NK cell depletion reduces the number of CD11b ⁺ CD45 ^{low} cells and the infiltration of CD3 ⁺ CD4 ⁺ IFN- γ ⁺ and CD3 ⁺ CD4 ⁺ IL-17 ⁺ in the brains of mice with EAE	110
Figure 4.8 IFN- γ ^{-/-} mice exhibit a delayed onset and increased severity of active EAE relative to WT mice, however EAE clinical scores in IFN- γ ^{-/-} mice are unaffected by NK cell depletion	111
Figure 4.9 A role for NK cells early in EAE	115
Figure 5.1 Production of TNF- α by CD11b ⁺ CD45 ^{low} cells increases in the murine brain early in EAE	127
Figure 5.2 Addition of IL-12 significantly enhances IFN- γ production by NK cells	128
Figure 5.3 Incubation with NK cells, but not conditioned medium from NK cells, induces microglial cell death	129
Figure 5.4 Incubation with conditioned medium from NK cells increases mRNA expression of the M1 markers iNOS, TNF- α and MHC II and reduces mRNA expression of the M2 markers MRC1 and ARG1 in microglia	130
Figure 5.5 NK cells are not cytotoxic towards mixed glia	131

Figure 5.6 NK cells or NK cell-conditioned medium increases mRNA expression of the M1 markers iNOS, MHC II and TNF- α in mixed glia	132
Figure 5.7 NK cells are not cytotoxic towards BMDMs	133
Figure 5.8 NK cells or NK cell-conditioned medium increases mRNA expression of the M1 markers iNOS and MHC II in BMDMs and reduces mRNA expression of the M2 markers MRC1 and CHI3L3 in BMDMs	134
Figure 5.9 Treatment of NK cells with IL-12 enhances IFN- γ production and upregulates expression of the M1 marker MHC II by BMDMs.....	135
Figure 5.10 NK cell-derived IFN- γ promotes M1 macrophage polarisation which increases VLA-4 expression on MOG-specific CD4 ⁺ T cells	136
Figure 5.11 Blockade of VLA-4 in active EAE from day 7 significantly attenuates EAE, while blockade of VLA-4 in recipient mice up until day 12 completely attenuates EAE induced by cell transfer.....	137
Figure 5.12 A role for NK cells in modulating glial and macrophage polarisation and CD4 ⁺ T cell encephalitogenicity in EAE	138
Figure 6.1 IL-4 increases mRNA expression of M2 markers MRC1 and ARG1 and reduces mRNA expression of the M1 marker iNOS and IL-17A production in a MOG-specific culture	152
Figure 6.2 IL-4 reduces expression of VLA-4 by CD4 ⁺ T cells in a MOG-specific culture	153
Figure 6.3 IL-4 prevents the ability of MOG-specific cells to transfer EAE to recipient mice ...	154
Figure 6.4 Adoptive transfer of IL-4-treated MOG-specific cells reduces MHC II expression by CD11b ⁺ CD45 ^{low} cells, and the number of infiltrating CD3 ⁺ CD4 ⁺ IFN- γ ⁺ and CD3 ⁺ CD4 ⁺ IL-17 ⁺ cells in the brains of recipient mice	155
Figure 6.5 IL-4 reduces expression of CD49d, and mRNA expression of IFN- γ and IL-17A by anti-CD3 and anti-CD28-stimulated CD3 ⁺ T cells	156
Figure 6.6 Incubation with M2 peritoneal macrophages reduces VLA-4 expression on CD3 ⁺ T cells	167
Figure 6.7 IL-4 can prevent the transfer of EAE through induction of M2 macrophages and reduction of VLA-4 expression by CD4 ⁺ T cells	161
Figure 7.1 Schematic detailing the proposed mechanism of action of NK-derived IFN- γ and recombinant IL-4 in modulating CD4 ⁺ T cell encephalitogenicity in EAE	173

XI. List of Tables

Table 1.1 The most common types of EAE	7
Table 1.2 Reported effects of NK cell depletion in EAE	21
Table 2.1 Components of Master Mix (for 25 μ l sample)	60
Table 2.2 Gene Names and Gene Expression Assay Numbers of primers used for PCR	61
Table 2.3 FACS antibodies	75

XII. List of Abbreviations

AD	Alzheimer's Disease
A β	Amyloid Beta
ADP	Adenosine Diphosphate
ALS	Amyotrophic Lateral Sclerosis
APC	Antigen Presenting Cell
APP/PS1	Amyloid Precursor Protein/Presinilin 1 Double Mutation
ARG1	Arginase
Asialo GM1	Asialo Ganglio-N-Tetraosylceramide 1
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
BMDM	Bone Marrow-Derived Macrophage
BSA	Bovine Serum Albumin
C3	Complement Component 3
CARKL	Carbohydrate Kinase-Like Protein
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFA	Complete Freund's Adjuvant
CHI3L3	Chitinase 3-like 3
CLP	Common Lymphoid Progenitor
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CT	Computerised Tomography
CTL	Cytotoxic T Lymphocyte
DAMP	Damage-Associated Molecular Pattern
DC	Dendritic Cell

DLN	Draining Lymph Node
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EAE	Experimental Autoimmune Encephalomyelitis
EAU	Experimental Autoimmune Uveitis
EBAO	Ethidium Bromide Acridine Orange
ECAR	Extracellular Acidification Rate
EDTA	Ethylenediaminetetraacetic Acid
FIZZ1	Found In Inflammatory Zone Protein 1
FMO	Fluorescence Minus One
FSC-A	Forward Scatter Area
FSC-W	Forward Scatter Width
GA	Glatiramer Acetate
GLUT-1	Glucose Transporter 1
GFAP	Glial Fibrillary Acidic Protein
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HIF-1 α	Hypoxia-Inducible Factor 1-alpha
HSC	Haematopoietic Stem Cell
IBA-1	Ionized Calcium Binding Adaptor Molecule 1
ICAM-1	Intercellular Adhesion Molecule 1
IFN- γ	Interferon Gamma
IFNGR	Interferon Gamma Receptor
IGIF	Interferon-Gamma-Inducing-Factor
iNOS	Inducible Nitric Oxide Synthase
IL	Interleukin
iNK	Immature Natural Killer

JAK	Janus Kinase
KIR	Killer Immunoglobulin Receptor
LDH	Lactate Dehydrogenase
LFA-1	Lymphocyte Function-Associated Antigen 1
LN	Lymph Node
LPKF2	Liver Phosphofructokinase 2
LPS	Lipopolysaccharide
MBP	Myelin Basic Protein
MCMV	Murine Cytomegalovirus
MCT4	Monocarboxylate Transporter 4
MHC	Major Histocompatibility Complex
MIF	Macrophage Migration Inhibitory Factor
MOG	Myelin Oligodendrocyte Glycoprotein
MRC1	Mannose Receptor
mRNA	Messenger Ribonucleic Acid
mSOD1	Mutant Superoxide Dismutase 1
mTOR	Mechanistic Target of Rapamycin,
MS	Multiple Sclerosis
NAWM	Normal-Appearing White Matter
NK	Natural Killer
NKP	Natural Killer Precursor
NMO	Neuromyelitis Optica
NO	Nitric Oxide
NOD	Nucleotide-Binding Oligomerization Domain-Containing Protein
NOS2	Nitric Oxide Synthase 2
OCR	Oxygen Consumption Rate

RA	Rheumatoid Arthritis
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RRMS	Relapsing-Remitting Multiple Sclerosis
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PEC	Peritoneal Exudate Cells
PLP	Proteolipid Protein
PMac	Peritoneal Macrophage
PML	Progressive Multifocal Leukoencephalopathy
PPMS	Primary- Progressive Multiple Sclerosis
PPP	Pentose Phosphate Pathway
PRMS	Progressive-Relapsing Multiple Sclerosis
PT	Pertussis Toxin
RA	Rheumatoid Arthritis
S1P1R	Sphingosine 1-Phosphate 1 Receptor
SEM	Standard Error of the Mean
SIP	Stock Isotonic Percoll
SLE	Systemic Lupus Erythematosus
SPF	Specific Pathogen Free
SPMS	Secondary-Progressive Multiple Sclerosis
SSC-A	Side Scatter Area
STAT	Signal Transducer and Activator of Transcription
T1DM	Type 1 Diabetes Mellitus

TCA	Tricarboxylic Acid
TCR	T cell receptor
TGF- β	Transforming Growth Factor Beta
Th	T helper
TLR	Toll-like Receptor
TNF- α	Tumour Necrosis Factor Alpha
Treg	Regulatory T cell
u-PKF2	Ubiquitous Phosphofructokinase 2
WT	Wild-Type
VCAM-1	Vascular Cell Adhesion Molecule-1
VLA-4	Very Late Antigen-4

Chapter 1: Introduction

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic, debilitating inflammatory disorder of the central nervous system (CNS) which was first described by Jean-Martin Charcot in 1868 (Murray, 2009). MS is a demyelinating disease, which is thought to be caused by infiltration of CD4⁺ T cells into the CNS which mediate damage to the myelin sheath (Lassmann et al., 2001). It affects an estimated 2 million people worldwide and most commonly occurs in persons aged 20 to 50 years (Milo and Kahana, 2010). The CNS location of the demyelinating “lesions” determines the presentation of symptoms, which range from sensory (chronic pain) to motor (spasticity, rigidity and ambulatory deficits) to autonomic (micturition deficits, sexual dysfunction) to emotional (depression, fatigue) (Thompson et al., 2010). MS disease course varies from patient to patient. **Figure 1.1** outlines the four main types of MS, and their progression in descending order of incidence.

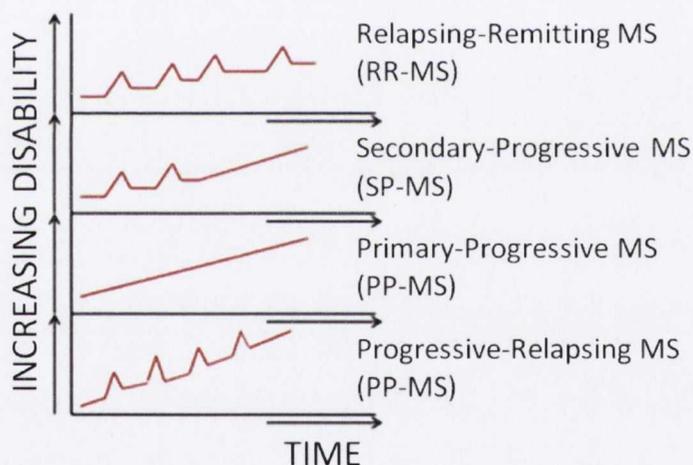


Figure 1.1 Types of multiple sclerosis (Lublin and Reingold, 1996)

Relapsing-remitting MS (RRMS) accounts for 85% of cases, and is characterised by episodic relapses that are punctuated by periods of remission, which may be partial or complete. After a number of years, a proportion of these patients may enter a phase of progressively worsening MS, with or without attacks. This type of MS has been called secondary-progressive MS (SPMS). Approximately 15% of patients experience primary progressive MS (PPMS) which is associated with a slow, gradual worsening of symptoms over time. A small number of these patients may relapse, and this type of MS is termed progressive-relapsing MS (PRMS). PRMS is the least common form of the disease. (Murray, 2006).

Demyelination and inflammatory events that occur in the immediate vicinity of demyelinating lesions are thought to account for periods of relapse, and conversely, resolution of inflammation and limited remyelination by oligodendrocyte precursor cells are thought to be responsible for the intermittent periods of remission. The irreversibility of disability in patients with progressive disease has been found to correlate with axonal loss, which can be substantial over a lifetime with MS (Bjartmar et al., 2003).

1.1.1 MS therapies

MS is presently an incurable disease; however a number of therapies have been developed with the aim of managing symptoms, with varying degrees of success. Interferon beta (IFN- β), which received FDA approval in 1993 (Paty and Li, 1993) is thought to lessen the severity of disease by exerting anti-inflammatory effects on the damaged CNS and also by reducing blood-brain barrier (BBB) permeability (Comabella et al., 2009). Glatiramer acetate (GA) received FDA approval 3 years later in 1996 (Comi et al., 2001). GA is thought to act by binding to MHC molecules, thereby preventing the T cell response to several myelin antigens (Schrempf and Ziemssen, 2007).

Two more recent therapies act by preventing the infiltration of encephalitogenic cells into the CNS. Natalizumab (Tysabri[®]) is a monoclonal antibody against the α 4 subunit of very late antigen 4 (VLA-4 - a dimer of α 4 and β 1 integrins), which is expressed on almost all activated leukocytes and facilitates travel across the blood-brain barrier. Natalizumab received FDA approval in 2004, but was later withdrawn from the market when it was linked with a small number of cases of a rare, fatal neurological disorder called progressive multifocal leukoencephalopathy (PML). Despite the high risk (1:100) of developing the condition after 2 years of use, the benefits are regarded to outweigh the risk and so natalizumab re-entered the market in 2009 (Clifford et al., 2010).

Fingolimod (FTY720, Gilenya), which received FDA approval in 2010 is the first oral drug for MS (all aforementioned therapies are administered through subcutaneous or intramuscular injection). Fingolimod is a functional antagonist of the sphingosine-1

phosphate receptor 1 (S1PR1), inhibits S1P1-mediated egress of lymphocytes from the secondary lymphoid organs, and thus prevents their trafficking into the CNS. The route of administration and (on average) 50% reduction in relapse frequency have made fingolimod a promising therapy for MS (Kappos et al., 2010).

As RRMS is the most common form of MS, these disease modifying drugs are only effective in ameliorating this type of disease, and unfortunately there are very few treatments for the progressive forms of MS. One is mitoxantrone, which received FDA approval as a therapy for SPMS in 2000. It was originally developed as an anti-cancer drug, used in the treatment of acute myeloid leukaemia and symptomatic hormone-refractory prostate cancer. Its efficacy in MS is due to its ability to inhibit antigen presentation and the secretion of pro-inflammatory cytokines, as well as its downregulation of macrophage-mediated myelin degradation. Some of the side effects (cardiotoxicity) mean that the life-time dosage of this drug is limited, however, in many cases it is the only option available to patients with SPMS (Fox, 2004).

Despite these advances in MS treatment, the fact still remains that MS is an incurable disease, and once patients reach the phase of severe disability, as precipitated by irreversible neurodegeneration, treatment is no longer possible. As the damaging events leading to the development of MS can occur prior to the onset of clinical symptoms, it would seem that the best strategy for the management of MS is probably a preventative one. Furthermore, recent evidence has challenged the simple paradigm that MS is caused by dysregulation of the adaptive immune system, indeed innate immune cells such as macrophages, microglia and NK cells may also play an important role in the pathogenesis of MS and should be considered in the development of future therapies (Haghikia et al., 2013).

1.2 Murine Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is a widely-exploited animal model of autoimmunity and is most commonly used in the study of MS (Baxter, 2007). The model was first described in primates by Thomas M. Rivers (Rivers and Schwentker, 1935). The condition is associated with a progressive ascending paralysis, and a clinical

score, usually between 0 and 5, ranging from a limp tail (1) to tetra paralysis (5)) (Lalor et al., 2011).

The disease can be induced either actively or by adoptive transfer of autoantigen-specific T cells. In active induction, the animal is immunised subcutaneously with a myelin antigen, with a concomitant intraperitoneal injection with the microbial product pertussis toxin (PT). The use of PT became more widespread in the 1970s; prior to that the disease incidence was low and followed an unpredictable course, even in a genetically-homogeneous population. The exacerbative effect of pertussis toxin is thought to be due to a reduction in the integrity of the blood-brain barrier and greater ease of infiltration of encephalitogenic T cells into the CNS (Hofstetter et al., 2002). In adoptive transfer (also referred to as “passive EAE”), an experimentally naïve “donor” mouse is immunised subcutaneously with myelin antigen without PT. Encephalitogenic T cells are isolated from these animals, restimulated with the myelin antigen *in vitro* for 72 h, and injected intraperitoneally or intravenously into a “recipient” mouse (Stromnes and Goverman, 2006).

The disease course depends upon the strain of mouse and also the particular antigen used. The two most commonly-used mouse models are a chronic progressive form of the disease induced by immunisation of C57BL/6 mice with myelin oligodendrocyte glycoprotein (MOG) in complete Freund’s adjuvant (CFA) and a relapsing remitting EAE induced in the SJL/J strain using proteolipid protein (PLP) in CFA (Gold et al., 2006). The most common forms of EAE in the literature are outlined in **Table 1.1**

Induction Agent	Species/strain	Disease course	Pathology
MOG₃₅₋₅₅ /immunodominant peptide* in CFA with Ptx or transfer of MOG-specific cell lines	Mouse (C57BL/6, Biozzi ABH, SJL, B.10 PL) Rat (Dark Agouti, Lewis)	Monophasic, chronic in C57BL/6 mice. Relapsing-remitting EAE in SJL mice. *MOG ₁₋₂₁ can induce chronic-relapsing EAE in Biozzi ABH mice. *MOG ₁₋₁₂₅ can induce chronic-relapsing EAE in Dark Agouti and Lewis rats.	Multifocal areas of inflammatory cell infiltration, demyelination in cervical spinal cord white matter.
MBP₈₄₋₁₀₄ /immunodominant peptide in CFA with Ptx or transfer of MBP-specific cell lines	Mouse (C57BL/6, PLJ, SJL, B.10 PL) Rat (Dark Agouti, Lewis)	Monophasic, chronic. Relapsing-remitting in SJL mice and Lewis rats.	Severe inflammation particularly in lumbar regions of spinal cord, little demyelination.
PLP₁₃₉₋₁₅₁, PLP₁₇₈₋₁₉₁ /immunodominant peptide in CFA	Mouse (SJL)	Relapsing-remitting	Inflammation in optic nerve, brainstem, spinal cord, cerebellum and cortex, followed by resolution of the inflammation with concurrent progression of white matter demyelination.

Table 1.1 The most common types of EAE

Demyelination in EAE usually occurs perivenously in the spinal cord and brain. Localisation of inflammatory lesions depends on which autoantigen is selected for induction of the disease. In MBP and PLP-mediated EAE, inflammation is predominant in lumbar regions, whereas in the MOG-mediated disease, inflammation is more clearly seen in the cervical region of the spinal cord (Sriram and Steiner, 2005).

EAE is certainly not a flawless model of MS. While a number of transgenic models facilitate the study of spontaneously developed EAE, most forms of EAE require active immunisation with CNS antigens in the presence of strong adjuvants. The immunostimulatory effects of such adjuvants are unlikely to occur under normal physiological conditions, even in infection. Furthermore, MS is an extremely heterogeneous disease, with patterns of demyelination and axonal degeneration varying widely from patient to patient. This is in stark contrast with the predictable and homogeneous disease course observed in an inbred mouse strain (Gold et al., 2006). Despite the fact that EAE does not faithfully recapitulate all aspects of MS, it has been an invaluable tool in the understanding of CNS inflammation and the development of a number of therapies for MS (natalizumab, for example), it reflects the increased female susceptibility to MS, and is characterised by similar white and grey matter pathology to the human disease. The examination of a number of disease models should continue to provide new insights into CNS autoimmunity and prove instrumental in the ongoing development of new therapies (Steinman and Zamvil, 2005).

1.3 The role of T cells in MS/EAE

1.3.1 Type 1 T helper (Th1) cells

The development of EAE and MS is primarily dependent on the action of T cells positive for the cluster of differentiation 4 (CD4⁺) immunophenotype. T helper cell differentiation is outlined in **Figure**. Induction of EAE preferentially promotes the activation of T cells that express major histocompatibility complex (MHC) Class II, which can bind one or more myelin peptide-derived antigens. Within this grouping, it was originally thought that a further subtype, the T helper 1 (Th1) effector T cells were

the primary effector T cells responsible for EAE pathology. This theory was supported by a number of observations. Adoptive transfer of myelin-specific Th1 cells was sufficient to induce disease (Lovett-Racke et al., 2011). Interferon gamma (IFN- γ), the primary Th1 cytokine, was found to enhance disease severity when it underwent clinical trials as an MS treatment in the 1980s (Panitch et al., 1987). Th1 differentiation requires interleukin-12 (IL-12), which is a heterodimer of p35 and p40, and mice deficient in IL-12p40 are resistant to EAE (Gran et al., 2002). However, this paradigm was challenged by the finding that both mice deficient in IFN- γ and IL-12p35 were still susceptible to EAE (Ferber et al., 1996, Gran et al., 2002). The latter result was particularly surprising as both IL-12p35^{-/-} and IL-12p40^{-/-} mice fail to produce the IL-12 p70 heterodimer, and in both of these knockouts, differentiation of Th1 cells during a primary immune response cannot occur (Becher et al., 2002). This apparent paradox was resolved by the discovery of IL-23, a heterodimer of p19 and IL-12p40 (Oppmann et al., 2000).

1.3.2 Th17 cells

The newly-discovered IL-23 was found to be vital for the differentiation of a distinct CD4⁺ T cell subset, which could produce IL-17A, IL-17F and IL-6, as well as contribute to autoimmune inflammation. These Th17 cells were found to surpass Th1 cells in encephalitogenicity – PLP-specific draining lymph node (DLN) cells cultured with IL-23 induced severe EAE, which was absent in mice that received the same number of antigen-specific DLN cells cultured with IL-12. Furthermore, IL-23p19^{-/-} mice were resistant to EAE, in spite of the fact that these mice had an intact Th1 response, as well as detectable Th1 cells in the CNS (Langrish et al., 2005). Since their discovery, Th17 cells have also been found to play a role in the pathogenesis of a number of autoimmune diseases such as psoriasis (Lowe et al., 2008), rheumatoid arthritis (Shen et al., 2009) and of course, MS (Fletcher et al., 2009).

For almost a decade after their identification, the factor that was responsible for Th17 encephalitogenicity was debated. IL-17A seemed like a likely candidate, until the discovery that Th17 cells, which produced abundant levels of IL-17A and IL-10 in response to stimulation with transforming growth factor beta (TGF- β) and IL-6, were

not pathogenic. The addition of anti-IL-10 to these cells did not restore pathogenicity, suggesting that IL-17A production is not sufficient to cause EAE (McGeachy et al., 2007). A study by El-Behi and colleagues proposed that the elusive encephalitogenicity factor was granulocyte macrophage colony-stimulating factor (GM-CSF), which is produced by Th17 cells in response to IL-1 β and IL-23. It had already been demonstrated that GM-CSF deficient mice were resistant to EAE (McQualter et al., 2001) and that GM-CSF deficient CD4⁺ T cells were incapable of transferring EAE to naïve hosts, even in the presence of GM-CSF from other sources (Ponomarev et al., 2007c). In this study, it was also shown that GM-CSF production by CD4⁺ T cells can induce IL-23 expression by APCs, resulting in maintenance of the Th17 phenotype and amplification of the inflammatory response. Interestingly, Th1 cells were also found to produce GM-CSF in response to IL-1 β , however it is possible that as a large proportion of Th1 cells in the CNS originated from Th17 cells, that both the Th17 and “ex-Th17” cells are responsible for GM-CSF production and pathogenicity, with the classical Th1 cells assuming a less important role (El-Behi et al., 2011a).

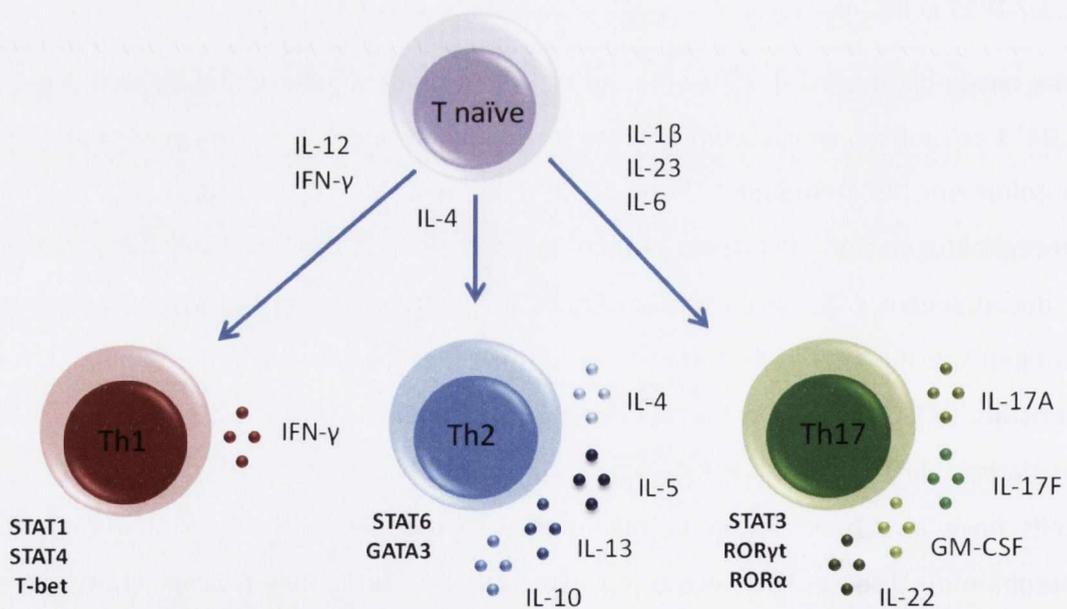


Figure 1.2 The differentiation of T helper cell subsets

Antigen-activated naïve CD4⁺ T cells (Tnaive) adopt distinct phenotypes as directed by cytokine cues within the local microenvironment and by transcription factors (in bold).

1.3.3 CD8⁺ T cells

CD8⁺ T cells (also referred to as cytotoxic T-lymphocytes (CTLs)) recognise antigens presented on MHC class I, and kill the antigen-bearing cells through the perforin and Fas pathways (Mosmann et al., 1997). These cells have been identified in MS lesions, those that are myelin-reactive are present in higher numbers in MS patients (McFarland and Martin, 2007) and a model of EAE induced by adoptive transfer of CD8⁺ T cells alone has been developed (Ji and Goverman, 2007).

1.3.4 $\gamma\delta$ T cells

Whereas the vast majority of T cells express a T cell receptor (TCR) made up of an $\alpha\beta$ heterodimer, a small subset express a $\gamma\delta$ TCR. These cells can act as an innate source of IFN- γ and IL-17, thus providing the first line of defence against infection (Ribot et al., 2010). Although the role of $\gamma\delta$ T cells in EAE/MS is contentious, the majority of the data would suggest a pathogenic role for these cells, especially early in disease. $\gamma\delta$ T cells have been found in the CSF of recently-diagnosed MS patients (Burns et al., 1995). $\gamma\delta$ T cells have also been detected in the CNS of mice with EAE, and the proportion of these cells rose and fell with corresponding peaks and troughs in disease activity (Rajan et al., 1996). In agreement with this, EAE is less severe in a transgenic model which renders the $\gamma\delta$ TCR unresponsive (TCRd^{-/-}). $\gamma\delta$ T cell pathogenicity may be attributed to the fact that IL-17 from these cells can also augment further IL-17 production by CD4⁺ T cells (Sutton et al., 2009).

1.4 The role of integrins in T cell pathogenicity in MS/EAE

Under physiological conditions, the CNS is protected from the aberrant entry of large molecules and peripheral immune cells by the blood-brain barrier (BBB). This barrier has a number of unique features that contribute to its formidable integrity; (i) the CNS microvessels are continuous and unfenestrated, (ii), they are surrounded by endothelial cells which are connected by “tight junctions”, (iii) the abluminal faces of these endothelial cells are protected by a “basement membrane, containing mural cells and pericytes and (iv) astrocytes extend processes called “end-feet” that in turn ensheath these cells (Daneman and Prat, 2015)

However, in EAE and MS, the potency of the BBB is compromised. Specifically, the tight junction proteins claudin 1 and 3 are lost, with downregulation of laminin in the basal membrane (Abbott et al., 2006). An early event in both EAE and MS is the infiltration of peripheral leukocytes into the CNS compartment. Whether or not this is a cause or effect of BBB permeability is yet to be determined. Entry of peripheral leukocytes into the CNS is sequential; first the cells loosely tether to the endothelial cells which can then be activated and enhance their expression of cell adhesion molecules and chemokines. This in turn strengthens the association between the endothelial cells and leukocytes, which now undergo arrest and adhesion. The leukocytes can then extravasate in either a para- or trans-cellular manner (Sallusto et al., 2012).

1.4.1 VLA-4 ($\alpha 4\beta 1$ integrin)

The processes of arrest and adhesion have been of particular interest to those engaged in a search for promising therapeutic targets in MS. In a seminal paper published in the early 1990s, Yednock and colleagues described complete prevention of EAE in Lewis rats by the administration of an antibody against $\alpha 4\beta 1$ integrin (also known as very late antigen 4 or VLA-4) (Yednock et al., 1992a). $\alpha 4\beta 1$ integrin is expressed on most leukocytes, including activated CD4⁺ T cells. The ligand for $\alpha 4\beta 1$ integrin is vascular cell adhesion molecule-1 (VCAM-1), which is upregulated by activated endothelial cells. The signalling induced by interaction of VLA-4 with VCAM-1 on endothelial cells is outlined in **Figure 1.3**. VLA-4 is a heterodimer, composed of CD49d ($\alpha 4$ integrin) and CD29 ($\beta 1$ integrin). The blockade of either subunit has proven effective in the reduction of EAE severity in a number of species, including mice, rats and guinea pigs. This is due to the fact that functional blocking of these molecules leads to instantaneous detachment of disease-causing intraluminally attached T cells, and this provides the scientific basis for the development of natalizumab (Engelhardt and Ransohoff, 2012).

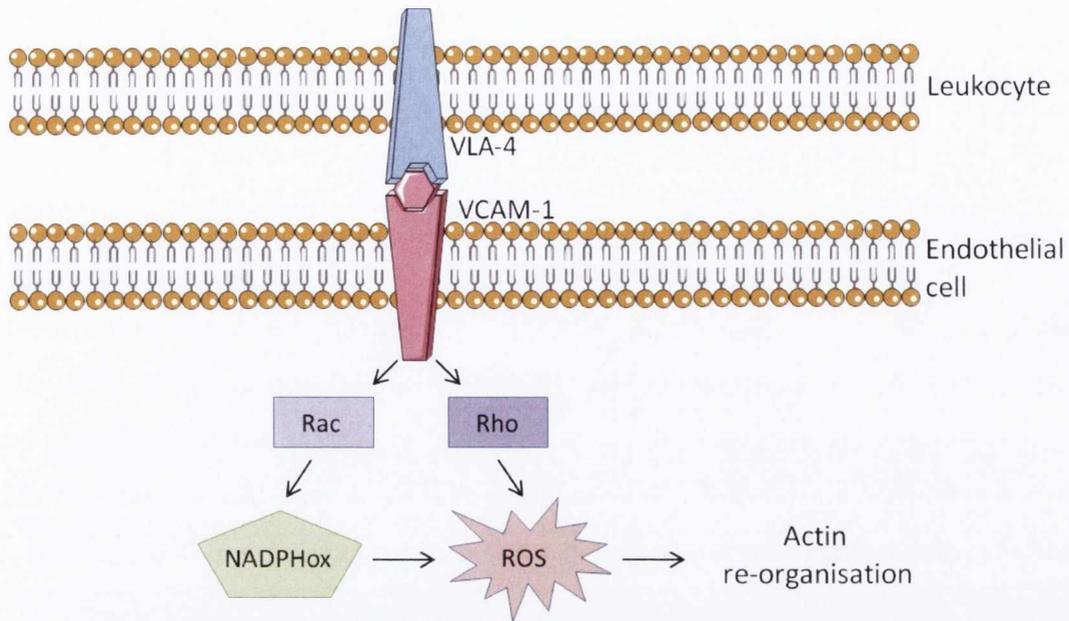


Figure 1.3 Membrane topology and signalling of VCAM-1

VCAM-1 expression is upregulated on activated endothelial cells. Binding with VLA-4 on leukocytes triggers downstream signalling which culminates in actin reorganisation, allowing for leukocyte transmigration.

1.4.2 LFA-1 (α L β 2 integrin)

Another adhesion protein implicated in the pathogenesis of both MS and EAE is α L β 2 integrin, also known as lymphocyte function-associated antigen 1 (LFA-1). LFA-1 is a heterodimer, composed of CD11a (α L integrin) and CD18 (β 2 integrin). LFA-1 binds to intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, and high immunoreactivity for both of these molecules has been reported in MS brain tissue, particularly in chronic active lesions (Cannella and Raine, 1995).

Interference with LFA-1 in EAE has yielded conflicting results. In one study, it was reported that actively-induced EAE was reduced in CD11a^{-/-} mice. Interestingly, while transfer of MOG specific cells from CD11a^{-/-} mice to WT mice resulted in a similar reduction in disease severity was reported with active induction, transfer of WT cells to CD11a^{-/-} mice resulted in more pronounced disability (Dugger et al., 2009). A possible explanation for this anomaly was provided by a study the following year in which the authors demonstrated that these knockout mice have a deficit in thymic

regulatory T (Treg) cell generation. Thus, the enhanced disease in the CD11a^{-/-} mice may thus be due to enhanced expansion and activation of encephalitogenic cells in the absence of a full Treg cell cohort (Gültner et al., 2010).

More recently it has been proposed that encephalitogenic Th17 cells preferentially require LFA-1 to enter the spinal cord parenchyma, unlike Th1 cells, which have been conclusively proven to depend upon VLA-4 for this process. In α 4 integrin-deficient mice, Th17 cells can enter supraspinal regions of the CNS and cause an “atypical” ataxic syndrome. The treatment of these mice with anti-CD11a completely abolished Th17 cells entry into the CNS and reversed clinical symptoms (Rothhammer et al., 2011).

1.5 Natural killer Cells

Natural killer (NK) cells were identified in 1975 as a subset of lymphoid cells that were cytolytically reactive towards allogeneic and syngeneic tumour cells (Kiessling et al., 1975, Herberman et al., 1975). They were identified as lymphocytes on the basis of their morphology and presence of lymphoid markers. The speculation that they were a subpopulation of T cells was abandoned when it was discovered that they did not express T cell antigen receptors or the CD3 complex. NK cells were henceforth regarded as effector cells of the innate immune system. However, recent evidence has suggested that NK cells are in fact endowed with a certain degree of immunological adaptivity and have a type of antigen-specific memory (Vivier et al., 2011).

1.5.1 NK cell maturation

Despite the wealth of knowledge in the literature regarding NK cell function, the sequence of events leading to NK cell maturation has not yet been wholly defined, however, the basic mechanism seems to be largely conserved between rodents and primates. First, the haematopoietic stem cell (HSC) becomes committed to the lymphoid versus the myeloid lineage, forming a common lymphoid progenitor (CLP). This cell can give rise to T cells, B cells, dendritic cells (DCs) and NK cells. The first step in the generation of NK cells appears to be related to the acquisition of the IL-15 receptor complex, which is comprised of the IL-15 receptor α (IL-15R α), IL-2R β (CD122)

and IL-2 receptor common γ (IL-2R γ c) chains which allow the NK precursor (NKP) cell to become responsive to IL-15. IL-15 is required for subsequent NK cell development and survival (Kim et al., 2002). In the mouse, these cells can progress to the immature NK cell (iNK) stage, which is characterised by the acquisition of NK1.1 and activating receptor NKG2D expression. A fully functional, mature NK cell gains cell surface expression of receptors of the Ly49 family, which recognise major histocompatibility complex I (MHC I) proteins and are similar to the killer cell immunoglobulin-like receptors (KIRs) in humans. Mature NK cells also express members of the DX5 family, such as CD49b, the α 2 subunit of VLA-2 (Fathman et al., 2011).

Human NK precursors become immature NK cells with the acquisition of CD161, which is the human equivalent of the murine NK1.1 marker. However, progression to a fully mature state differs between mice and humans, due to the existence of two distinct subsets of human NK cells, the separation of which is based on degrees of expression of CD16 and CD56 (Caligiuri, 2008). These were first identified in the early 1980s (Lanier et al., 1983) and have since been well characterised. CD16⁺CD56^{dim} cells are considered to be the more “mature” cell, and the remainder are the immature CD16⁻CD56^{bright} subset. Both cell types express the activating receptor NKp46, but CD56^{dim} cells are considered the more mature cell type by virtue of their expression of KIRs, which are absent on the CD56^{bright} subset. They also differ in their expression of the inhibitory receptor CD94/NKG2A, which is more highly expressed on CD56^{bright} cells (Poli et al., 2009).

Recent evidence suggests that the bone marrow is the primary site of NK cell development and maturation in mice (Fathman et al., 2011). However, a small population of CD127-expressing NK cells have been found to develop in the thymus, and a population of immature NK cells also reside in the liver (Sun and Lanier, 2011). It is also likely that NK cell development does not occur solely in the bone marrow in humans. The first challenge to this simple paradigm was the discovery of a population of CD56^{bright} cells in the lymph nodes and tonsils (Caligiuri, 2008). It is still unclear whether or not these populations are distinct NK cell lineages in mouse and human, or are merely less mature cells that originated in the bone marrow and migrated to these sites early in development.

1.5.2 NK cell distribution

NK cells are widely distributed in mammals in both lymphoid and non-lymphoid organs, although whether this is due to their recirculation or their development *in situ* is not yet clear. The following murine organs have appreciable NK cell populations, ordered according to NK cell population size: spleen > lung > bone marrow > peripheral blood mononuclear cells (PBMCs) > lymph nodes > liver > thymus (Grégoire et al., 2007). There is also some evidence to suggest the presence of NK cells in healthy epithelial tissue such as the skin in humans (Ebert et al., 2006) and the gut in mice (Reynders et al., 2011). NK cells have also been detected in the uterus during pregnancy in both humans and mice. They can develop *in situ* or migrate from the periphery to the decidua (uterine lining during gestation) in response to trophoblast chemokine secretion. Their role in the uterus is unclear, although it has been suggested that they may contribute to vascular modifications during pregnancy (Shi et al., 2011a).

1.5.3 NK cell function

NK cells in both rodents and primates display a striking functional heterogeneity, reflected by differential expression of inhibitory receptors across the NK cell population (Colucci et al., 2002). CD16⁺CD56^{low} cells contain more intracellular perforin and are more efficient cytotoxic cells, whereas the CD16⁻CD56^{high} subset produce greater amounts and a wider variety of cytokines, however they can also become cytotoxic after prolonged stimulation (Strowig et al., 2008). The most common NK cell-produced cytokines are IFN- γ , tumour necrosis factor alpha (TNF- α), GM-CSF, IL-13 and IL-10 (Huntington et al., 2007).

NK cells can rapidly mobilise and migrate to the site of infection or trauma. For example, these cells have been shown to migrate in large numbers to the synovial fluid surrounding the damaged joint in human patients with rheumatoid arthritis and in mice with collagen-induced arthritis, where they are thought to exacerbate the inflammatory status of the site (Söderström et al., 2010).

NK cells, as well as being competent cytokine producers, also express a large array of cytokine receptors on their cell surface. IL-2 enhances the proliferation and cytotoxicity of NK cells *in vitro* and *in vivo*, and can also enhance responsiveness to IL-12 by (i) upregulating cell surface expression of the IL-12 receptor and (ii) enhancing IL-12-mediated IFN- γ production (Wang et al., 2000). The cytokines TNF- α , IL-1 β , IL-15 and IL-18 (previously referred to as interferon- γ -inducing factor or IGIF) can also synergise with IL-12 to enhance IFN- γ production which is crucial in modulating the adaptive immune response to viral invasion and other insults (Biron et al., 1999).

The direct cytolytic activity of NK cells is mediated by perforin, a protein which induces the formation of pores in the target cell, an action which ultimately results in apoptosis of that cell. After the formation of an immune synapse, NK cell secretory granules migrate to the point of contact with the target cell, fuse with the NK cell membrane, and release their contents into the synapse (Stinchcombe and Griffiths, 2007). The potential for self-destruction is tightly controlled by a tolerance mechanism mediated by inhibitory receptors for major histocompatibility complex (MHC) class 1 molecules, which are present on healthy cells (**Figure 1.4**). NK cells express a suite of such inhibitory receptors. The NKG2A/CD94 receptor is conserved in rodents and primates. This heterodimeric receptor recognises HLA-E in humans and the murine homologue QA1^b. The Ly49 family of receptors, expressed only in mice, recognise polymorphic epitopes on H-2D and H-2K class 1 molecules. The killer cell immunoglobulin-like receptors (KIR, also collectively known as CD158), expressed only in primates, recognize polymorphic epitopes on human leukocyte antigen classes HLA-A, HLA-B, and HLA-C (Ravetch and Lanier, 2000). Infected cells or tumour cells lose expression of MHC1 and this loss of self (along with potential cell-surface expression of viral haemagglutinin (HA) which ligates with an activating receptor in the example of a virally infected cell) leads to NK cell activation and targeted cell killing (**Figure 1.5 (A) and (B)**). The activating NKp46 receptor is expressed in both primates and rodents (Kim et al., 2005).

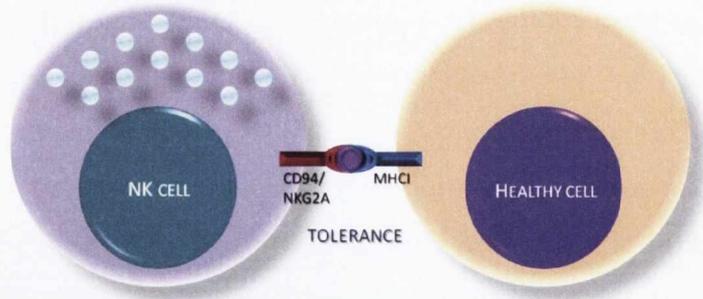


Figure 1.4 NK cell tolerance

Healthy cells express self antigens through MHC I, which ligate with the inhibitory receptor CD94/NKG2A on the surface of the NK cell. This leads to tolerance.

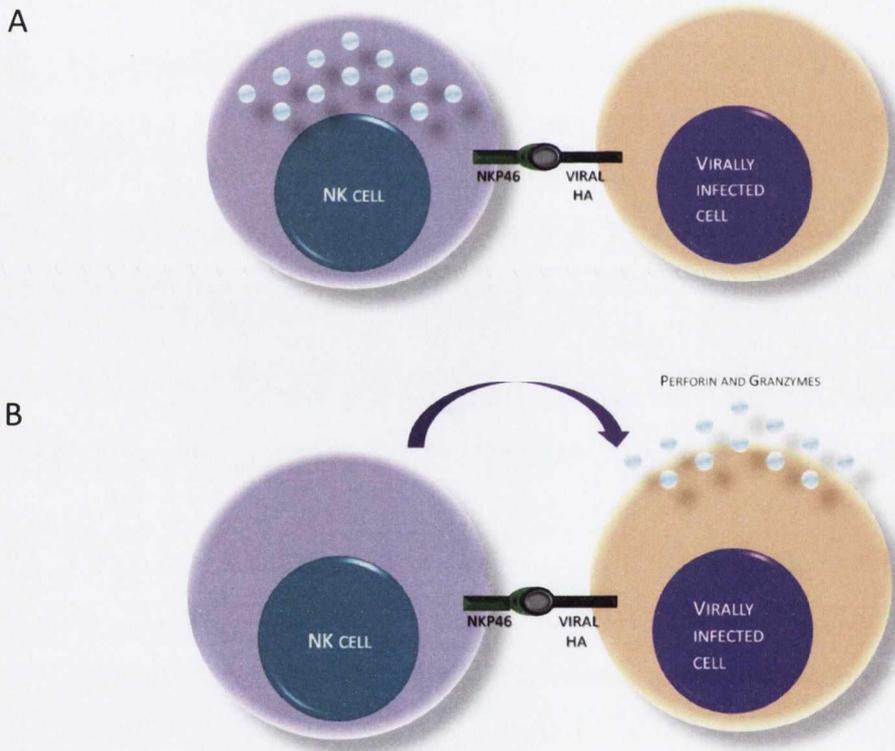


Figure 1.5 NK cell killing

In the case of viral infection, the host cell loses self antigen expression. This lack of binding to the NK cell inhibitory receptor, coupled with the ligation of viral HA with the NK cell activating receptor, NKP46 results in a loss of tolerance, activation of the NK cell, release of lytic granules and subsequently death of the target cell.

1.6 The role of NK cells in EAE/MS

One of the first observations that NK cells might be implicated in the pathogenesis of MS was made by Benczur and colleagues in the late 1970s, who found that the cytolytic activity, reactivity to interferon and viral infection (as replicated with Poly I:C stimulation) of NK cells taken from MS patients was greatly reduced relative to healthy age-matched controls. This was particularly evident in the male subjects with progressive, rather than relapsing, disease (Benczur et al., 1980). The advent and widespread use of flow cytometry allowed further characterisation of this apparent MS-related NK cell deficit, and it was later found that the number of circulating NK cells was in fact lower in active relapsing MS patients than controls (Munschauer et al., 1995). Subsequent studies elaborated on this finding, discovering that this reduction in NK cell number and functional activity seemed to reflect increased susceptibility to the formation of active lesions and clinical attack or relapse (Kastrukoff et al., 1998, Kastrukoff et al., 2003).

The general consensus in the literature to date is that the role of NK cells in MS is a protective one, and that NK cell killing activity dampens autoimmunity. Recently, the focus has shifted to the immature CD56^{high} subset, as it has been found that a number of disease modifying therapies, like IFN- β and natalizumab, as well as those currently in clinical trials, such as daclizumab and alemtuzumab, can elicit an expansion of this subset of NK cells in the peripheral blood (Chanvillard et al., 2013). Interestingly, this population of NK cells is the predominant one in the cerebrospinal fluid (CSF) of patients with neuroinflammatory disorders, including MS (Hamann et al., 2013).

The specific targets of CD56^{high} immunoregulatory NK cells have recently been identified. Nielsen and colleagues recently demonstrated that both CD56^{high} NK cells kill activated, but not resting, autologous CD4⁺ T cells by inducing apoptosis through degranulation (Nielsen et al., 2012). More specifically, these cells seem to preferentially transfer the less well-known Granzyme K, which causes mitochondrial dysfunction and subsequent, caspase-independent apoptosis in activated T cells (Jiang et al., 2011).

The role of NK cells in the pathogenesis of EAE is considerably more controversial. There are two dominant schools of thought regarding the role of NK cells in EAE. One is that NK cells are inherently protective in the disease state, and modulate the magnitude of autoimmunity through direct cytolytic activity against encephalitogenic T cells (Xu et al., 2005). A potential mechanism may involve CD94–NKG2A inhibitory receptors expressed by NK cells interacting with MHC molecule Qa-1–Qdm on activated T cells; disruption of the inhibitory Qa-1–NKG2A interaction may allow NK cell-dependent elimination of activated autoreactive cells (Lu et al., 2007b). A more recent study has demonstrated that NK cells may also kill aberrantly-activated microglia through this CD94–NKG2A: Qa-1–Qdm interaction (Hao et al., 2010b). It has been suggested that antibody-mediated targeting of this interaction may prove clinically useful; anti-NKG2A treatment was shown to diminish progression of both actively induced and adoptively-transferred EAE progression by skewing the proportion of IL-17 and IFN- γ producing CD4⁺ T cells toward the more protective IL-4 and IL-10-secreting CD4⁺ subpopulations (Leavenworth et al., 2010b). The other school of thought suggests that NK cells promote EAE by secreting IFN- γ , thus directing differentiation of naïve CD4⁺ T cells and Th1 polarisation (Tian et al., 2012).

Considering the relative ease with which NK cells can be depleted from mice, thus offering indirect insight into the role they may play in EAE, there is still huge variation in the reported outcomes of depletion experiments. These are outlined in **Table 1.2**. The two antibodies most commonly used in the depletion of NK cells are anti-NK1.1 and anti-asialo GM1. Administration of the monoclonal anti-NK1.1 *in vivo* induces apoptosis in NK 1.1-expressing cells (NK and NKT cells) (Asea and Stein-Streilein, 1998). This alloantigen is not expressed in certain mouse strains, like the BALB/C or SJL strain and so is most commonly used in C57BL/6 mouse models. Asialo GM1 is the more concise name for asialo ganglio-N-tetraosylceramide, which is a glycolipid, similar in structure to a ganglioside, but without a sialic acid group. Asialo GM1 is expressed on murine, human, bovine and hamster NK cells (Naiki et al., 1974) and administration of the polyclonal antibody anti-asialo GM1 causes complement-dependent lysis of these cells (Shimada and Iwata, 1987).

Reference	Antibody	Administration	Effect
(Zhang et al., 1997b)	Anti-NK1.1	-1d PI	Increase in MOG-induced EAE in C57BL/6 mice
(Hao et al., 2010b)	Anti-NK1.1	-2 d PI, every 5d thereafter	Increase in MOG-induced EAE in C57BL/6 mice
(Xu et al., 2005)	Anti-NK1.1	-1d , 14d PI	Increase in PLP-induced EAE in SJL mice
(Winkler-Pickett et al., 2008)	Anti-NK1.1	-3d to -1d PI	Decrease in MOG-induced EAE in C57BL/6 mice
(Shi et al., 2000)	Anti-NK1.1	-2 d PI, every 5d thereafter	Decrease in MOG-induced EAE in C57BL/6 mice
(Matsumoto et al., 1998b)	Anti-asialo GM1	-7, -3, 0, 7, 14d PI	Increase in MBP-induced EAE in Lewis rats
(Winkler-Pickett et al., 2008)	Anti-asialo GM1	-3d to -1d PI	Decrease in MOG-induced EAE in C57BL/6 mice

Table 1.2 Reported effects of NK cell depletion in EAE

The discrepancies regarding the reported effect of NK cell depletion on EAE in the literature are most likely due to a difference in the administration regimen, species as well as differing EAE induction and clinical score regimes. Anti NK1.1-mediated depletion is effective for up to 7 days (Carroll et al., 2001) after that the efficacy may

begin to wane, confounding the interpretation of some of the results. Similarly, according to the guidelines provided by Wako Chemicals, the distributor of the anti-asialo GM1 antibody used in these studies, one should expect a “gradual diminution” 4 days after the initial injection. As such, these data should be viewed in light of such facts and interpreted carefully. The role of NK cells in the pathogenesis of EAE still remains elusive despite almost two decades of research.

1.7 An introduction to IFN- γ

The interferons (IFNs) were originally identified as molecules that could inhibit viral replication (Isaacs and Lindenmann, 1957). Where once they were characterised according to the secretory cell type, now they are classified according to structure and receptor specificity. The type 1 IFNs (IFN- α , IFN- β , IFN- ω and IFN- τ) and bind to a heterodimeric receptor comprised of IFNAR1 and IFNAR2 chains. IFN- γ on the other hand, is the only type 2 IFN and binds to the IFN- γ receptor (IFNGR) which is made up of two ligand-binding IFNGR1 chains and two signal-transducing IFNGR2 chains (Pestka et al., 2004). Type 1 and 2 IFN signalling is outlined in **Figure 1.6**. IFN- γ is produced by a host of immune cell types, including Th1 cells, CD8⁺ cytotoxic T cells, NK cells, NKT cells and some professional antigen presenting cells (APCs), like macrophages and dendritic cells. The production of IFN- γ is induced by exposure of these cells to cytokines produced by APCs, IL-12 and IL-18 in particular. When IFN- γ binds to its receptor, the janus kinase/ signal transducer and activator of transcription (JAK/STAT) signalling pathway is initiated, with eventual activation of the transcription factor STAT1, and transcription of immune effector genes (Schroder et al., 2004).

IFN- γ signalling is immunostimulatory. Not only can it enhance antigen presenting capacity and thus CD4⁺ T cell activation by upregulating expression of MHC I and II in APCs, it can also act upon naïve CD4⁺ cells themselves, influencing Th1 differentiation (Boehm et al., 1997). IFN- γ as well as certain Toll-like receptor (TLR) agonists are capable of inducing classically-activated, M1-type macrophages and microglia (discussed in Section 1.13.1). The ability of IFN- γ to orchestrate such an array of cellular programs means has made it a subject of intense research in the field of inflammatory disease. Of particular interest in this context is its role in MS and EAE.

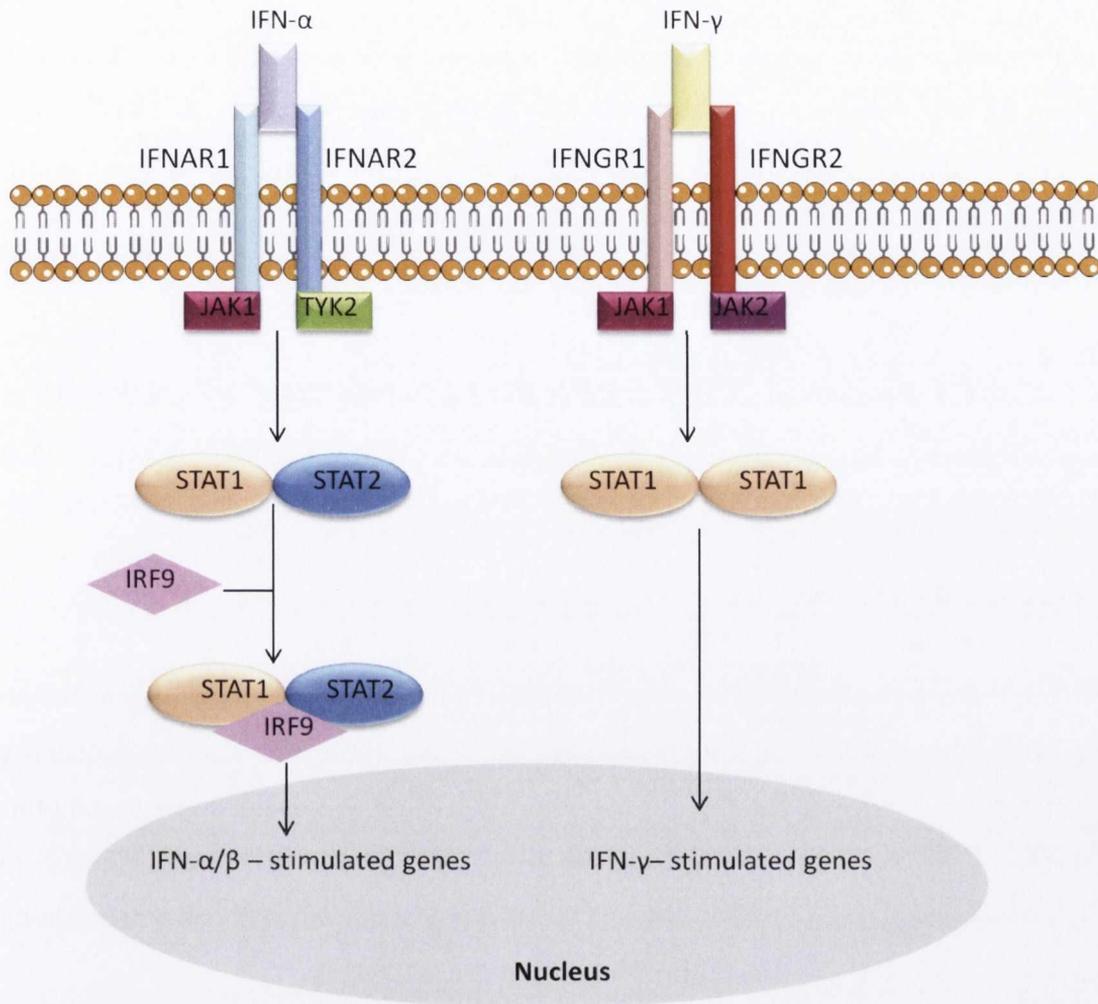


Figure 1.6 Signalling induced by ligation of Type 1 and Type 2 interferons with their cognate receptors

Binding IFN- α with its receptor (composed of IFNAR1 and IFNAR2 subunits), leads to activation of JAK1 and TYK2 recruitment and phosphorylation of STAT1 and STAT2. STAT1 and STAT2 form a heterodimer, to which IRF9 binds, and which translocates to the nucleus and induces transcription of Type 1 IFN-stimulated genes. Binding of IFN- γ with its receptor (composed of IFNGR1 and IFNGR2 subunits) leads to activation of JAK1 and JAK2, recruitment and phosphorylation of STAT 1, which forms a homodimer and translocates to the nucleus and induces transcription of IFN- γ -responsive genes.

1.8 The role of IFN- γ in MS/EAE

The role of IFN- γ in both MS and EAE is controversial, and it is still unclear whether it is pathogenic or protective in either disease state (Arellano et al., 2015). As IFN- β was found to have a beneficial therapeutic effect in the treatment of MS, and there were some reports of a deficient IFN- γ production in patients affected by the disease (Neighbour et al., 1981), administration of the cytokine as a therapy for MS underwent clinical trials in the mid-1980s. This treatment caused a significant increase in clinical severity in these patients, with enhanced MHC II expression in circulating monocytes, proliferation in peripheral blood leukocytes and augmented NK cell activity (Panitch et al., 1987). Microarray analysis of MS lesions revealed increased transcription of a number of genes that code for pro-inflammatory cytokines, notably IL-6, IL-17 and IFN- γ (Lock et al., 2002).

In the relapsing-remitting EAE model, transgenic expression of IFN- γ in murine oligodendrocytes prevented remission and resulted in chronic demyelination (Renno et al., 1998). However, the discovery that EAE could occur in the absence of IFN- γ (Ferber et al., 1996) or the IFNGR (Willenborg et al., 1996), suggested that while this cytokine certainly plays a role in the pathogenesis of EAE, it is not essential. In agreement with these studies, it was reported that administration of a neutralising antibody against IFN- γ exacerbated EAE (Billiau et al., 1988).

1.9 An introduction to IL-4

IL-4 is a pleiotropic cytokine with a diverse array of effects on the immune response. IL-4 is produced by activated T cells, eosinophils and basophils, and shares many features with IL-13, namely sequence homology, cell surface receptors and signalling induced by receptor binding (Mueller et al., 2002). IL-4 receptor (IL-4R) complexes themselves lack intrinsic kinase activity, but dimerisation of the receptor complexes following ligand binding leads to activation of the JAKs and eventual tyrosine phosphorylation of STAT-6 (Mikita et al., 1996). An outline of the signalling cascade can be seen in **Figure 1.7**. IL-4 signalling is perhaps most well known for its vital role in regulating Th2 differentiation (Swain et al., 1990) which is instrumental in the orchestration of an immune response to allergy and parasitic infection (Yazdanbakhsh

et al., 2002) and also in inducing the “alternative” or “M2” activation state in macrophages (Gordon, 2003) and microglia (Ponomarev et al., 2007a). A reciprocal inhibition can occur between the archetypal Th1 cytokine IFN- γ and the IL-4, the primary Th2 cytokine whereby IL-4 can inhibit Th1 cells and IFN- γ can inhibit Th2 cells (Maggi et al., 1992). This antagonism also occurs within the M1/classical and M2/alternative states, which is discussed in detail in Sections and 1.13.1 and 1.13.2.

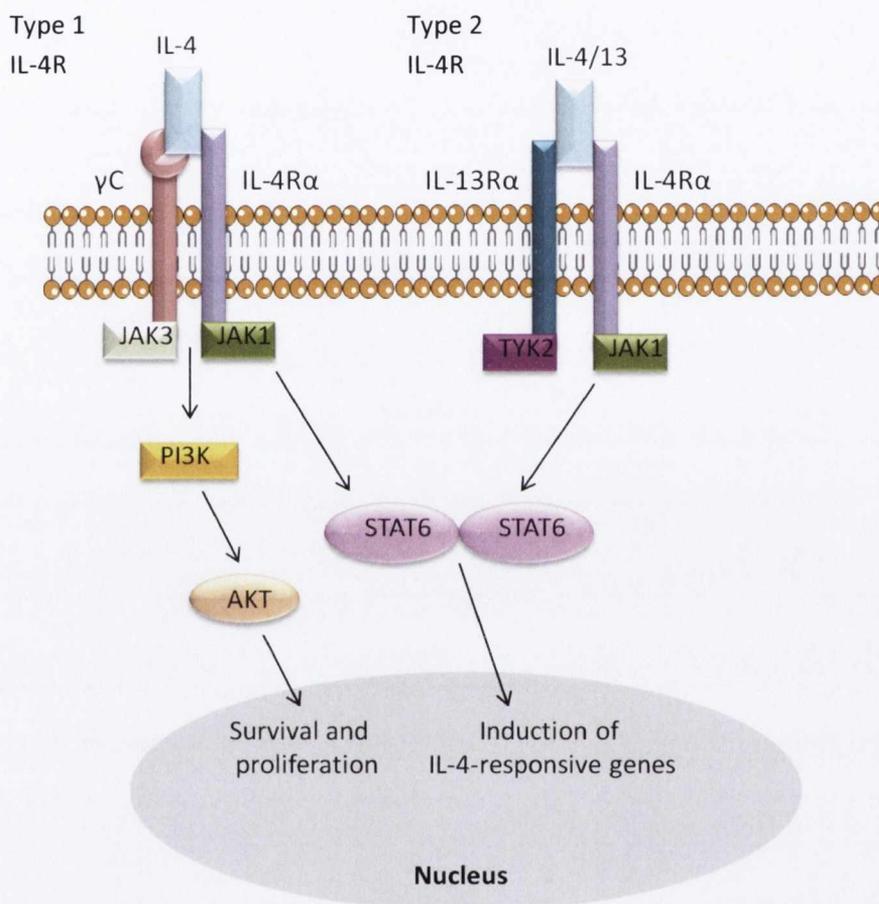


Figure 1.7 IL-4 signalling

The Type 1 IL-4 receptor is composed of the common γ C and the IL-4R α subunits, and the Type 2 IL-4 receptor is composed of the IL-4R α and the IL-13R α subunits. The Type 1 IL-4R mediates its effects through JAK1 and JAK2, while the Type 2 IL-4R associates with TYK2 and JAK1. On both cases, ligation of the receptor leads to phosphorylation of STAT6, which forms a homodimer and translocates to the nucleus where it induces gene transcription. Binding of IL-4 to the Type 1 receptor also induces activation of the PI3K/AKT pathway which is important in the regulation of the cell cycle.

1.10 The role of IL-4 in EAE/MS

The literature concerning a direct role for IL-4 in modulating neuroinflammation is sparse; however, on the whole, it seems that IL-4 may be protective in MS and EAE (Singh et al., 1999). It has been reported that administration of the MS-modifying therapy GA enhanced serum levels of IL-4 in patients (Oreja-Guevara et al., 2012). Circulating numbers of a T cell subset that co-express surface B7-1 and intracellular IL-4 were also found to be decreased in MS patients (Kipp et al., 2000).

The data on the role of IL-4 in EAE, while similarly scant, are certainly more convincing. Firstly, IL-4-deficient C57BL/6 mice exhibit significantly enhanced EAE scores. This exacerbation is associated with an increase in perivascular inflammation, demyelination and mRNA expression of IFN- γ , IL-1 and TNF- α (Falcone et al., 1998). This was further investigated in a study in which chimera mice were generated by transplanting WT bone marrow into IL-4^{-/-} mice. The IL-4 deficiency was thus CNS-specific and induction of EAE in these mice, in agreement with the previous study, was more severe than WT chimeras, with an earlier onset. On analysis of the IL-4-deficient CNS, this enhancement in EAE severity corresponded with an increase in infiltrating peripheral lymphocytes and macrophages (Ponomarev et al., 2007a).

Consistent with these studies was the observation that administration of IL-4 for 5 days before immunisation of Lewis rats with MBP significantly reduced EAE clinical scores (Der Meide, 1999), as did IL-4 administration following transfer of MBP-specific lymph node cells to these animals (Racke et al., 1994). Furthermore, incubation of PLP-stimulated spleen cells with IL-4 failed to transfer EAE to SJL/J mice (Young et al., 2000). Taken together these data suggest that IL-4 is protective in CNS autoimmunity, although the principal cellular source of this cytokine, as well as the mechanism underlying this protection, have yet to be determined.

1.11 Microglia

“By reason of its difference in characteristics and origin from nerve cells (first element) and neuroglia (second element), the microglia constitute the true “third element” of the CNS”.

Pio del Rio-Hortega, 1932 (Rezaie and Male, 2002)

Microglia are a class of CNS-resident mononuclear phagocytes which were named and characterised by Pio del Rio-Hortega in 1932. Microglia account for 10 to 15% of all glial cells, and despite ontogenic distinction, are often referred to as the “tissue specific macrophages of the CNS” (Nayak et al., 2014). The origin of microglia has been a subject of debate for decades. It has been agreed that microglia are of mesodermal origin, unlike neurons, oligodendrocytes and astrocytes, all of which originate in the neuroectoderm. Whereas macrophages are derived from “classic” haematopoietic stem cells that persist in the bone marrow and reach their eventual destination through the bloodstream, microglial precursors infiltrate the future brain from the yolk sac very early in embryonic development (before embryonic day 8 in the mouse) (Ginhoux et al., 2010). However, until recently, microglial ancestry was not completely defined. A recent study, using transgenic mice, has suggested that the elusive microglial progenitor is in fact an erythromyeloid precursor that exists solely in the embryo and whose developmental fate is restricted to macrophages and erythrocyte lineages. On the other hand, the haematopoietic stem cells from which most tissue macrophages (including perivascular brain macrophages) arise are multipotent and located in the bone marrow (Kierdorf et al., 2013).

1.12 Macrophages

Macrophages are highly plastic, phagocytic cells of the innate immune system which are found in all tissues and display striking functional diversity (Wynn et al., 2013). The discovery of these cells is credited to the Russian zoologist Ilya Mechnikov (commonly anglicised to Elie Metchnikoff). In the early 1880s, he made an observation that the insertion of thorns into starfish larvae induced recruitment of phagocytic cells from

the haemolymph to the site of entry. He published these findings in 1883 in a journal edited by Carl Claus, who coined the term “phagocytosis” the following year. Metchnikoff won a Nobel Prize for this work in 1908 (Cavaillon, 2011).

Recent work has contributed to the hitherto incomplete understanding of macrophage origin and homeostasis. Until recently, the widely-accepted dogma, advocated by van Furth’s mononuclear phagocyte system (1968), was that tissue macrophage numbers are constantly replenished from blood monocytes. While under certain conditions, such as inflammation, and in certain tissues, like the skin and gut, monocytes can in fact contribute to the tissue-resident macrophage pool, they can self-renew in many tissues in the adult mammal under steady-state conditions. This suggests that these cells may have a different ontogeny, and indeed recent studies have shown that many tissue macrophages (with the above exceptions) arise from an embryonic progenitor which seeds developing tissue prenatally. Unlike bone marrow-derived macrophages, which enter a monocytic intermediate stage during differentiation, these cells use an entirely different pathway to become mature macrophages in embryonic tissue. However, even within the macrophage populations of embryonic origin there is some ontogenic distinction; microglia arise from the yolk sac, whereas it is believed that Langerhans cells arise from the foetal liver. It has been suggested that most other macrophages of embryonic origin are derived from the foetal liver with some small contribution from the yolk sac (Ginhoux and Jung, 2014).

Macrophages engage in a diverse array of functions, not all of which are immune-related. Macrophages are extremely competent phagocytes, and exploit this ability not only in the removal of pathogens, but also in the clearance of cellular debris, apoptotic cells, as well as approximately 10^{11} erythrocytes/day. A suite of receptors on their cell surface, such as scavenger, phosphatidylserine, thrombospondin, integrin and complement receptors mediate these crucial homeostatic mechanisms (Mosser and Edwards, 2008).

1.13 Activation States

Microglia and macrophages, while ontogenically distinct, share a number of functional characteristics; they are both competent phagocytes and both can adopt phenotypically distinct “activation states” in response to their particular microenvironment. Recent evidence suggests that macrophages and microglia exhibit a spectrum of activation, and the extremes of this spectrum are broadly outlined in **Figure 1.8**.

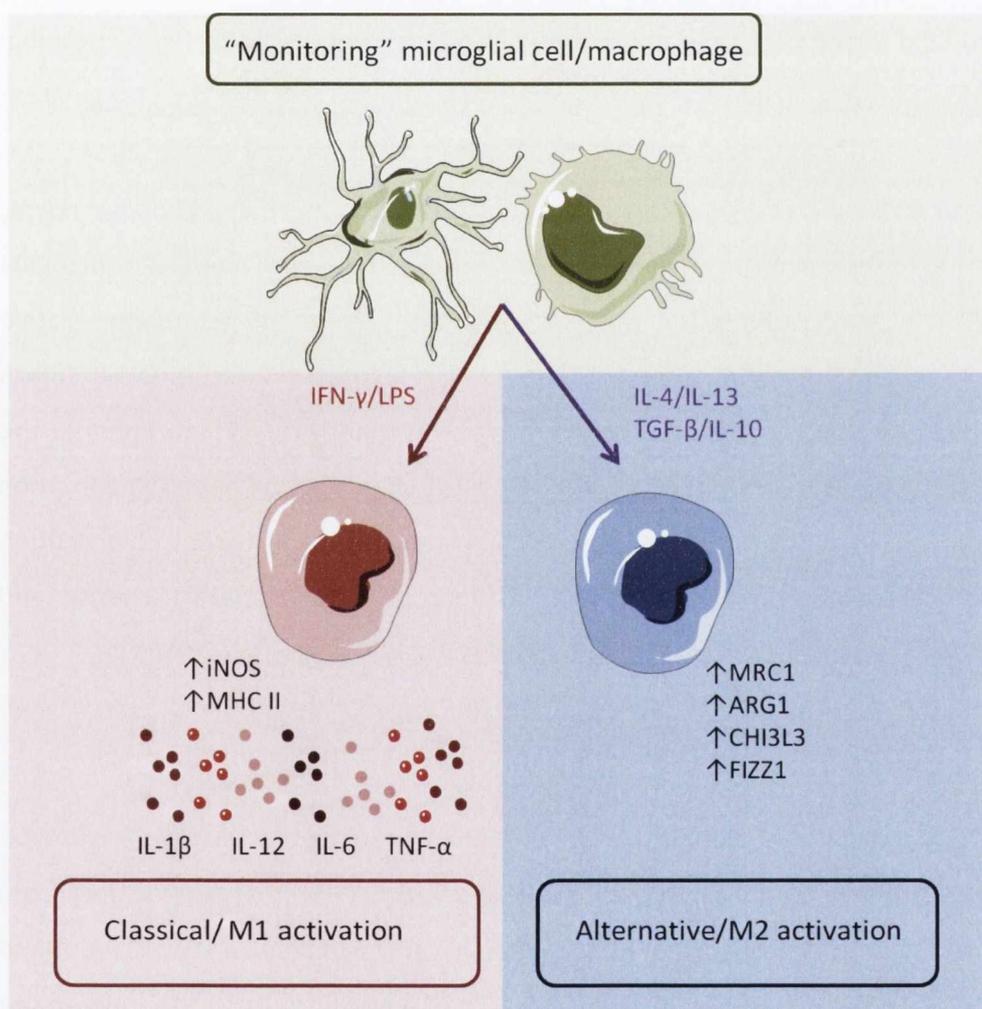


Figure 1.8 An outline of the M1 and M2 activation states in microglia and macrophages

Microglia and macrophages can adopt phenotypically distinct activation states in response to their microenvironment. In response to exposure to IFN- γ or LPS, cells can adopt the pro-inflammatory M1 or classical activation state which is required for pathogen elimination. In contrast, stimulation with the anti-inflammatory cytokines IL-4 or IL-13 induces the M2 or alternative activation state which is associated with wound healing and repair.

1.13.1 Classical/ M1 Activation

Microglia and macrophages have a number of cell surface pattern recognition receptors (PRRs) which recognise pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), which activate the cells to initiate a defensive response, which usually takes the form of the M1 or classical activation state. IFN- γ and lipopolysaccharide (LPS), a component of Gram negative bacteria and a TLR4 agonist, can induce this activation state. This state is characterised by the production of pro-inflammatory cytokines such TNF- α , IL-1 β , IL-6 and IL-12 as well as upregulation of the production of nitric oxide (NO) (as evidenced by the transcriptional upregulation of the inducible nitric oxide synthase gene (NOS2)), and the upregulation of the cell surface expression of MHC II (Colton, 2009) (Murray et al., 2014). This cytotoxic phenotype is instrumental in the destruction of the offending agent, however its dysregulation can be damaging to neurons and aberrant microglial activation has been implicated to some degree in a number of neurodegenerative conditions, such as Alzheimer's disease (AD), Parkinson's disease (PD) and MS (Block and Hong, 2005). Macrophages that reside in, or infiltrate the CNS and undergo the switch to M1 can also contribute to the damage that occurs in neurodegeneration (Amor et al., 2010). In the periphery, macrophage activation syndrome, a complication of paediatric rheumatic diseases such as systemic juvenile idiopathic arthritis and systemic juvenile lupus erythematosus can prove fatal (Bennett et al., 2012).

1.13.2 Alternative/ M2 activation

In the early 1990s, Siamon Gordon and colleagues identified a macrophage phenotype induced by exposure to the Th2-type cytokines IL-4 and IL-13 that was distinct from the classically activated state (Stein et al., 1992). Later TGF- β and IL-10 were identified as two further induction agents for this "alternative" activation state that antagonised the classical state by engaging in processes required for parasitic containment, resolution of inflammation and tissue repair. Unsurprisingly, microglia can also adopt this phenotype, however in this case, the anti-inflammatory cytokines responsible for the induction of this "M2" state come not from Th2 cells as in the periphery, but primarily from other microglia, astrocytes and even neurons (Colton, 2009). Like the

classical activation state, the alternative activation state has a number of defining markers. One is the upregulation of arginase (ARG1), an enzyme that competes with the archetypal M1 enzyme iNOS for substrate, namely L-arginine. Whereas iNOS converts L-arginine to L-hydroxyarginine which can be further broken down to yield destructive NO free radicals, ARG1 converts it to L-ornithine which can give rise to polyamines and proline, involved in cell proliferation and collagen production respectively. Alternative activation is also associated with alterations in the pattern of expression of cell membrane receptors involved in both phagocytosis and trafficking to the membrane. One such receptor is the mannose receptor (MRC1), which is involved in the uptake of a number of mannosylated glycoproteins and particulates (Varin and Gordon, 2009). Two other commonly cited markers of the alternative activation state are found in inflammatory zone 1 (FIZZ1) and chitinase 3-like 3 (CHI3L3). Although both markers are induced by IL-4 and IL-13, their roles in the alternative response are not well known. FIZZ1 (also known as resistin-like molecule alpha (RELM- α)) has recently been implicated in a model of murine pulmonary fibrosis, and has exhibited chemoattractive activity toward bone marrow derived cells (Liu et al., 2014). CHI3L3 (also called YM1) is a catalytically inactive member of the mammalian chitinase family and has been recently shown to be an important regulator of Th17 responses to helminth infection. Overexpression of this protein in the lungs increased IL-17 production and neutrophil recruitment, thus controlling local anti-worm immunity (Sutherland et al., 2014).

It is thought that the M2 state can be further subdivided into a number of related phenotypes although there is a lack of consensus on the exact categorisation of these subtypes. It is almost universally accepted however, that on exposure to factors such as TGF- β or IL-10 that microglia and macrophages begin to wind down their classical inflammatory phenotype and return to their original "monitoring state". This phenomenon is often referred to as "acquired deactivation" (Saijo and Glass, 2011). A question that still remains unanswered is which factors act as the molecular switch between the activation states, and until this is addressed, modulation of microglial/macrophage activation as a therapy for (neuro)inflammation will be impracticable.

1.13.3 Activation-induced changes in cellular morphology

Microglia in a “monitoring state” (the term “resting state” is misleading as microglia by their very nature are never really at rest), adopt a ramified morphology, maintain homeostasis and engage in immunosurveillance as their processes sample their surroundings. Pio del Rio-Hortega’s original drawings show that microglia abandon this branched morphology when activated and adopt a more rounded, amoeboid shape. The simple paradigm that microglial activation state can be determined based on morphology is probably untrue; it is likely that a number of intermediate, transitional states also exist that share morphological and phenotypic features with other states (Lynch, 2009). Although traditionally the morphological change with activation was associated with microglia rather than macrophages, recent work has shown that they also alter their shape in response to activating stimuli *in vitro* – they become more round with IFN- γ and LPS treatment and more elongated with IL-4 and IL-13 treatment (McWhorter et al., 2013)

1.14 The role of microglia in EAE/MS

Aberrant microglial activation is observed in the CNS in both MS and EAE. Clusters of microglia, referred to in the literature as “microglial nodules” appear in the normal-appearing white matter (NAWM) very early in MS lesion development, even before the observation of demyelination, astrogliosis and demyelination (van Horssen et al., 2012).

Microglia begin to proliferate and become activated before the onset of clinical signs in EAE (Ponomarev et al., 2005). These cells upregulate the cell surface markers MHC II, CD40, and CD86 at this time, and at the second stage of disease activation, a further upregulation in these markers occurs, with a reduction in proliferation. CD40 seems to play a key role in the ability of activated microglia to exert their detrimental effects in EAE, as CD40-deficient microglia were shown to be unable to reach a level of full activation, and this was associated with a reduction in myelin-specific T cell expansion, as well as a reduction in leukocyte infiltration into the CNS. This microglial

CD40 deficiency resulted in an improvement in clinical symptoms. (Ponomarev et al., 2006).

It is thought that “priming” of microglia occurs in MS and EAE, a preconditioning event where a disease-causing stimulus “primes” microglia, with a second stimulus provoking over-activation and thus a more severe response. Recent evidence suggests the priming mechanism may be complement component 3 (C3)-dependent (Ramaglia et al., 2012). Selective inhibition of microglial release of nitrite, pro-inflammatory cytokines and chemokines has been shown to both ameliorate the disease and limit axonal and myelin damage in EAE (Heppner et al., 2005). Dipyridamole, a drug which has been shown to attenuate LPS-induced increase in TNF- α production in adult human microglia, as well as preventing the switch to an amoeboid morphology was effective in significantly reducing clinical signs in EAE. This amelioration was associated with reduced immunoreactivity for ionized calcium binding adaptor molecule 1 (Iba-1), indicative of a decline in microglial activation (Sloka et al., 2013).

Microglia act as antigen presenting cells (APCs) as well as mediating CNS damage through the production of pro-inflammatory cytokines such as TNF- α and cytotoxic factors such as NO (Benveniste, 1997). Recent work suggests that microglial activation is necessary for EAE onset, whereas infiltration of peripheral monocytes correlates with progression to the paralytic stage of the disease, without contributing to the resident microglial pool (Ajami et al., 2011b). Microglial phagocytosis of neuronal debris in MS has been proposed as a mechanism through which microglia may present neuronal antigens, thus contributing further to exacerbation of autoimmune processes and worsening of the disease state (Huizinga et al., 2012). However, a more recent study, which allowed discrimination between monocyte-derived macrophages and resident microglia, suggested that while macrophages are highly phagocytic and inflammatory, microglia remain quite inert during the demyelination stage of EAE and rather engage in clearance of debris (Yamasaki et al., 2014).

A putative trigger of microglial activation with T cell infiltration, as well as IFN- γ , may be GM-CSF. This cytokine is secreted by Th17 cells (El-Behi et al., 2011b) and Th1 cells

and has been shown to be critical for development of T cell encephalitogenicity. GM-CSF-deficient mice are resistant to the onset of the EAE, and in the disease state, it has been shown that microglia require this cytokine for their activation. This provides further evidence for the importance of microglial activation in the onset of EAE (Ponomarev et al., 2007b).

It seems however, that microglia may also adopt the more anti-inflammatory, reparatory M2 phenotype during remyelination. In a murine model of demyelination achieved through stereotaxic injection of lysolethicin into the corpus callosum, microglial phenotype was examined at days 3, 10 and 21 post-lesion which correspond with key steps in the remyelination process. At day 10, the initiation of remyelination, vastly more cells were positive for the M2 marker ARG1 than the M1 marker iNOS. Correlative analysis was also performed on post-mortem human brain tissue. M2 microglia were found to promote oligodendrocyte proliferation, and depletion of these cells ablated this effect. A blockade of oligodendrocyte differentiation is a hallmark of chronic MS lesions (Miron et al., 2013). A similar study demonstrated that administration of *ex-vivo* M2-polarised monocytes suppressed severe EAE (Mikita et al., 2011). Taken together, these findings suggest that a therapy aimed at manipulating M2 polarisation might enhance not only neuroprotection, but regeneration in the CNS, thus encouraging recovery in multiple sclerosis. This further highlights the importance of fully understanding the intricacies of the multi-faceted phenomenon of microglial activation, as these cells undeniably present a promising target for therapeutic intervention not only in MS, but other neurodegenerative conditions.

1.15 The role of macrophages in EAE/MS

Macrophages are typically found in early and late “active demyelinating” MS lesions, and have been found to contain myelin degradation products. Uptake of myelin gives these cells a so-called “foamy” appearance. (Lucchinetti et al., 2011). The activation status of these foamy macrophages is not clear-cut. It has been suggested that their degree of activation is dependent on micro-location within the lesion - cells on the outer rim are more reactive for MOG, whereas those located in the inner rim as well as perivascular cells have high expression of a number of anti-inflammatory markers,

such as mannose receptor, IL-1 receptor antagonist (IL-1ra) as well as the pleiotropic cytokine IL-6. Interestingly, when foamy macrophages were artificially generated *in vitro* through the incubation of macrophages isolated from human PBMCs with pure myelin, these cells were found to mount an entirely different response to LPS stimulation than cells treated with LPS alone. Not only did the myelin uptake reduce LPS-induced IL-12p40 and TNF- α mRNA expression, but incubation of control cells with myelin induced an upregulation in the mRNA expression of the M2 associated chemokine CCL18 (Boven et al., 2006). However, more recent data has suggested that the modulation of cytokine release that had been previously observed in macrophages incubated with myelin was in fact due to LPS insensitivity which was induced by LPS contamination in the myelin preparations (Glim et al., 2010).

1.16 Astrocytes

Astrocytes are the most numerous cell type in the brain and engage in a diverse array of functions, they are a vital element in the maintenance of the BBB, they maintain homeostasis of fluids, ions, pH and neurotransmitter tone in the synaptic interstitial fluid, they regulate local CNS blood flow, and can store glycogen to sustain neuronal activity during periods of hypoglycaemia and high neuronal function (Sofroniew and Vinters, 2010). Astrocytes also exhibit a degree of functional and structural heterogeneity and can be broadly classified on the basis of their location in the brain; protoplasmic astrocytes reside in the grey matter, and fibrous astrocytes can be found in the white matter (Miller and Raff, 1984).

Astrocytes are reactive and a commonality in a number of CNS insults is “reactive astrogliosis”, the degree of which correlates with the severity of the trauma. Mild-to-moderate astrogliosis, as can occur following a mild trauma, is characterised by upregulation of glial fibrillary acidic protein (GFAP), with preservation of individual domains. Severe astrogliosis, as occurs at lesion sites or in the case of neurodegeneration is also associated with increased GFAP expression, with enhanced proliferation and a resulting disruption of individual cellular domains. In the case of overt tissue damage and inflammation, the severe astrogliosis can be accompanied by

the formation of a glial scar, which is thought to act as a neuroprotective barrier from infiltrating cells and infectious agents (Pekny et al., 2014).

While astrocytes were classically regarded as supportive, damage-limiting cells, more recent evidence has suggested they might also have a role in innate CNS immunity. Astrocytes express a number of TLRs, the NOD-like receptors NOD1 and 2, scavenger receptors as well as components of the complement system. Furthermore, treatment of cultured astrocytes with poly I:C, which is structurally similar to double-stranded viral RNA, induces production of the cytokines TNF- α , IL-6, IFN- β , GM-CSF and TGF- β as well as secretion of the chemokines CCL2, CCL5, CCL20, CXCL8 and CXCL10 (Farina et al., 2007).

1.16.1 Can astrocytes adopt M1/M2-like activation states?

Interestingly, apart from the reactive astrogliosis that is observed in CNS trauma, astrocytes have also been demonstrated to adopt some of the phenotypic characteristics of the M1/M2 activation states described in microglia and macrophages. In response to exposure to archetypal inducers of the classical activation state, LPS and IFN- γ , astrocytes were found to upregulate mRNA expression of the M1 markers IL-1 β , TNF- α , and iNOS. Furthermore, the astrocytes were also found to respond in a similar manner to microglia and macrophages to IL-4 stimulation, with increases in expression of the M2/alternative markers ARG1, MRC1, FIZZ1, Ym1 and IL-10 (Jang et al., 2013). The roles of these differentially polarised astrocytes *in vivo* have yet to be determined, although there is some evidence that M2-like astrocytes can upregulate ARG1 expression in a model of spinal cord injury (Ahn et al., 2012a) and EAE (Ahn et al., 2012b) which may help to regulate neurodegeneration.

1.17 The role of astrocytes in EAE/MS

During MS, astrogliosis and glial scar formation causes the “sclerosis” for which the disease is named. However, despite the fact that astrocytes are one of the constituent cell types in the MS plaque, astrocytes have often been overlooked as a potential

participant in disease pathogenesis (Brosnan and Raine, 2013). However, recent developments in the field of the neuromyelitis optica (NMO) research have led to the re-evaluation of the putative role of astrocytes in neurodegenerative conditions. NMO, like MS is an inflammatory demyelinating disease which attacks the optic nerve and spinal cord. Unlike MS, NMO is most commonly observed in Asian populations. A key finding in NMO research was the identification of antibodies against the water channel aquaporin-4 (AQP4) in the circulation of NMO patients as a biomarker for the disease (Lennon et al., 2004). In the CNS, expression of AQP4 can be found on astrocytic end-feet at the BBB, and binding of antibodies to these sites lead to activation of the complement cascade, destruction of perivascular astrocytes with eventual apoptosis of oligodendrocytes and demyelination (Parratt and Prineas, 2010).

Astrocyte activation in autoimmune neurodegeneration is not restricted to glial scar or direct modulation of demyelination, however. Astrocytes can also respond to inflammatory mediators like IFN- γ , and upregulate expression of MHC II. It has been proposed that astrocytes may act as non-traditional antigen-presenting cells (APCs) and thus reactivate, and induce proliferation of, myelin-specific T cells (Carpentier et al., 2005). However, there has been some controversy surrounding these findings, as astrocytes are weak APCs, and these *in vitro* data have not been successfully recapitulated *in vivo*. What is more likely is that astrocytic modulation of the adaptive immune response in the damaged CNS is more to do with chemoattraction of peripheral leukocytes, as astrocytes have been shown to upregulate expression of ICAM-1 in response to IFN- γ (Satoh et al., 1991) and in active MS lesions (Brosnan et al., 1995). Furthermore, astrocytes can mount a differential response to IL-17 exposure, characterised by production of the chemokines CCL2, CCL12, CXCL1, and CXCL2 (Sarma et al., 2009). CCL2-positive astrocytes have also been detected in the CNS of EAE mice, and peaks in production of this chemokine corresponding with periods of relapse (Glabinski et al., 1997). Taken together these data suggest that apart from mediating the formation of a damage-limiting glial scar, astrocytes may also serve to recruit more effective APCs to the site of CNS lesions, with a less important role in restimulation of myelin-specific cells.

1.18 Metabolic Signatures of Polarised Cells

Recent evidence suggests that immune cell activation is also accompanied by fundamental changes in their metabolic profiles.

1.18.1 Glucose metabolism

Cellular energy is largely derived from the metabolism of glucose, which is metabolised to pyruvate through glycolysis (see **Figure 1.9**).

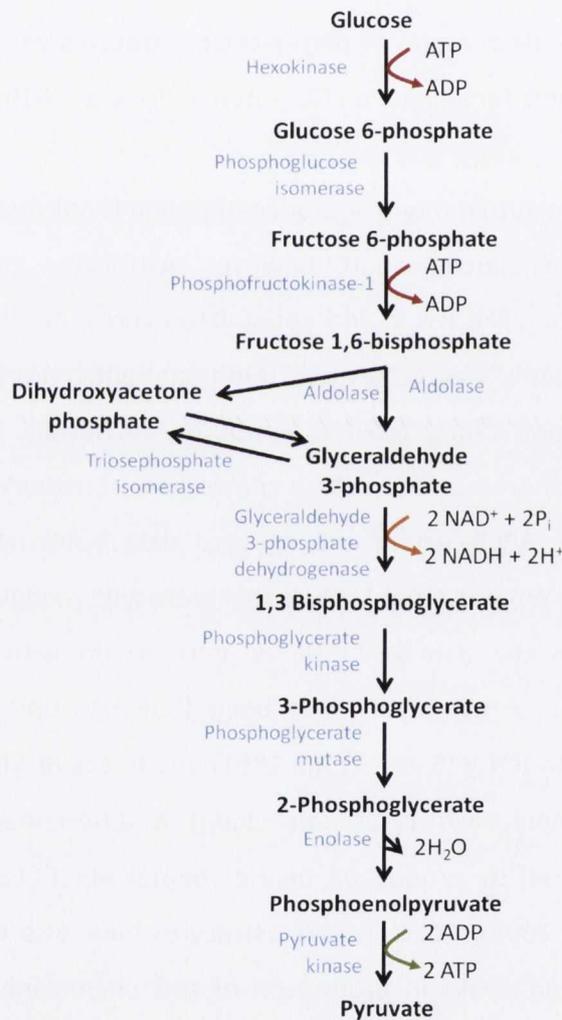


Figure 1.9 The conversion of glucose to pyruvate in glycolysis

Glycolysis involves a series of nine distinct enzyme-catalysed reactions resulting in the conversion of glucose to pyruvate, with production of the high energy molecules ATP and NADH.

Pyruvate can then be metabolised to CO_2 in the tricarboxylic acid (TCA) cycle through the process of oxidative phosphorylation. This yields a large amount of energy in the form of adenosine triphosphate (ATP); up to 36 ATP molecules per one molecule of glucose. Pyruvate can also be converted to organic acids or alcohols (such as lactate or ethanol) in a reductive process known as fermentation. Fermentation can occur anaerobically but is less efficient than the TCA cycle/oxidative phosphorylation, and yields only two molecules of ATP for every glucose molecule (Lunt and Vander Heiden, 2011). Despite this compromise in ATP load, glucose fermentation can occur even in the presence of oxygen. This was first observed in cancer cells in 1924 by Otto Warburg (Warburg et al., 1924).

It seems that this metabolic reprogramming from oxidative phosphorylation to aerobic glycolysis occurs when cells are engaged in processes which require a rapid and large energy supply, such as activation and proliferation. Interestingly, this phenomenon is not unique to transformed cells. In a series of experiments carried out by Karl Brand and colleagues, it came to light that rat thymocytes, when stimulated with the mitogen concanavalin A, not only exhibited a 53-fold increase in glucose uptake, but also converted 90% of this glucose to lactate. Approximately 1% was oxidised to CO_2 . This was in stark contrast to resting cells, which metabolised only 56% to lactate and oxidised 27% to CO_2 (Brand, 1985). Mitogen stimulation was also unsurprisingly associated with an 8- to 10-fold increase in the induction of a number of glycolytic enzymes, namely hexokinase, phosphofructokinase-1, pyruvate kinase and lactate dehydrogenase (Greiner et al., 1994).

1.18.2 Glucose metabolism and CD4^+ T cell function

More recently, it has been shown that not only is cell differentiation/polarisation characterised by a distinct metabolic profile, but that alteration of metabolic parameters can in turn impact upon polarisation and differentiation itself. CD4^+ T cells can be broadly categorised as effector or regulatory cells, the balance between which can influence the magnitude of the immune response. It has been found that naïve CD4^+ T cells that have been treated such that they adopt the effector Th1, Th17 and

Th2 phenotypes exhibited high glucose transporter 1 (GLUT 1) expression as well as high rates of glycolysis. Additionally, it was found that effector T cells were enriched in transgenic mice in which GLUT1 was overexpressed, and that the cytokine secretion from these cells was more prolific than those from wild-type counterparts. Conversely, the Treg cell subset exhibited significantly lower levels of both glycolysis and GLUT1 expression than their effector counterparts, as well as more fatty acid oxidation. Furthermore, inhibition of the protein kinase mechanistic target of rapamycin (mTOR), which can result in the increased oxidation of fatty acids, promoted Treg differentiation, and the addition of fatty acids to the culture medium during early effector T cell generation strongly inhibited Th1 differentiation (by reducing Tbet expression). This was also associated with reduced production of the T helper cytokines IFN- γ , IL-17 and IL-4 (Michalek et al., 2011). Consistent with these findings is the observation that hypoxia-inducible factor 1- α , (HIF-1 α) which is a vital transcription factor in the expression of glycolytic genes, is required to promote Th17 (versus Treg) differentiation (Shi et al., 2011b).

1.18.3 Glucose metabolism and macrophage function

Distinct metabolic profiles have also been observed in differentially-polarised macrophages. Macrophages that upregulate the M1 state upregulate glycolysis. This process can speedily fulfil the considerable energy requirements of engaging in microbicidal activity and cytokine production. Conversely, the M2-polarised macrophages display a low glycolytic rate and instead rely upon the more efficient, if slower, oxidative glucose metabolism pathway. This provides the sustained energy required for the resolution of inflammation, tissue remodelling and repair (Biswas and Mantovani, 2012).

A number of recent studies have investigated, in depth, the processes that underlie these metabolic changes with activation. Rodriguez-Prados and colleagues reported a large increase in peritoneal macrophage glucose uptake and lactate production with M1 activation, neither of which were seen in response to M2-inducing stimuli. This was accompanied by a corresponding increase in both the amount of fructose 2,6-

bisphosphate detected and a switch from the liver type phosphofructokinase 2 (L-PFK2) to the more active and ubiquitous form of the enzyme (uPFK2). Interestingly there was a reciprocal interaction between the M1 state and this enzyme - interfering with uPFK2 using siRNA led to a reduction of the protein levels of the M1 markers iNOS and COX-2. The M1-associated increase in glycolytic flux and corresponding change in enzymatic activity was absent in cells deficient in HIF-1 α (Rodríguez-Prados et al., 2010). HIF-1 α promotes the expression of a number of glycolytic enzymes as well as the expression of the glucose transporters GLUT 1 and 3, and the monocarboxylate transporter 4 (MCT4) which transports lactate out of the cell (Porporato et al., 2011).

The interplay between polarisation and metabolism was further investigated in a more recent study by Haschemi and colleagues, in which a sedoheptulose kinase called carbohydrate kinase-like protein (CARKL) was proposed as a suppressor of M1 macrophage polarisation through control of glucose metabolism. CARKL is responsible for the production of sedoheptulose-7 phosphate, which is an intermediate of the pentose phosphate pathway (PPP). Overexpression of this protein in macrophages led to a blunted pro-inflammatory cytokine response and reduced MHCII expression in response to LPS stimulation in tandem with an enhanced IL-10, macrophage migration inhibitory factor (MIF) and IL-4R expression. The authors also investigated the metabolic profiles of these cells by examining extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) as proxy markers of glycolysis and oxidative phosphorylation respectively. In cells which expressed normal CARKL levels, LPS induced a large increase in ECAR relative to both the control and IL-4 treated cells, and while IL-4 treated cells displayed similar OCR to the control cells, it was reduced in LPS-treated cells. The overexpression of CARKL, however, reduced the robust increase in ECAR with LPS treatment and also increased OCR relative to the cells with normal CARKL expression (Haschemi et al., 2012) Consistent with these findings was the observation that experimental knockdown of this kinase primed macrophages to adopt a state similar to the M1 state prior to LPS stimulation, with increased glycolysis and reduced OCR. Taken together these data provide further evidence for the metabolic reprogramming of macrophages in response to polarising stimuli (Porporato

et al., 2011). The reported effects of macrophage polarisation on the subsequent metabolic phenotype are summarised in **Figure 1.8**.

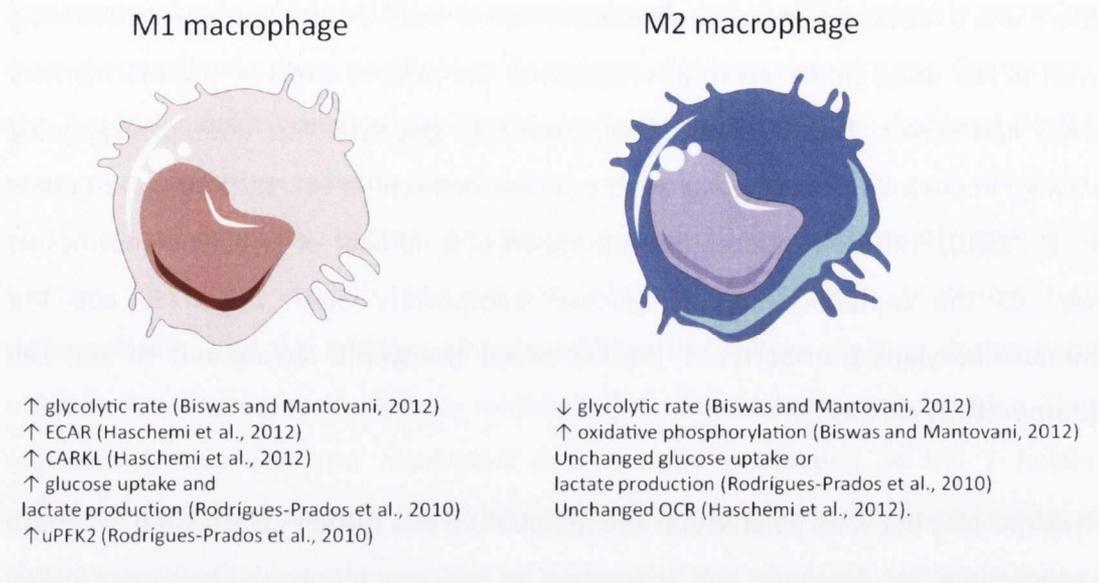


Figure 1.10 Macrophage polarisation and glucose metabolism

While the pro-inflammatory M1 or classical activation state is associated with an increase in glycolysis, the M2 state is associated with a reduced glycolytic rate and preferential utilisation of oxidative metabolism.

1.18.4 Glucose metabolism and microglial function

In spite of the many similarities between microglial and macrophage activation states, much less is known about the correlation between microglial activation and any putative changes in metabolic profile. It has been reported, however, that the immortalised BV2 microglial cell line exhibits an increase in ECAR and a decrease in OCR with LPS treatment, which is consistent with the macrophage data (Voloboueva et al., 2013). Another more recent paper, reported greater uptake of glucose in BV2 cells with LPS and IFN- γ treatment, as well as enhanced activity of hexokinase, which catalyses the conversion of glucose to glucose 6-phosphate, the first step in glycolysis. The authors also reported an increase in lactate production with the application of the M1-polarising stimuli. Taken together these data indirectly suggest an increase in glycolysis with LPS and IFN- γ treatment (Gimeno-Bayón et al., 2014). It should be noted that while microglial cell lines which have a number of advantages over primary

microglia, namely higher yields, shorter processing time and lower cost, the BV2 microglial line, in particular, has exhibited an often markedly different inflammatory profile to primary cultures and thus may not be an ideal tool in this context (Stansley et al., 2012).

1.18.5 Glucose metabolism and astrocyte function

While it has been suggested that astrocytes may adopt M1/M2-like states in response to the polarising stimuli IFN- γ and IL-4 respectively, it has not yet been investigated whether these interventions could affect astrocytic glucose metabolism. That is not to say that astrocytic governance of CNS metabolism has not been extensively researched. In response to periods of intense neuronal activity, astrocytes have been found to undergo glycolysis, convert glucose to lactate, which is subsequently taken up by neurons to fulfil their energy demands (Pellerin and Magistretti, 1994). Interestingly, the M1 product NO can act upon astrocytes to upregulate glycolysis (Almeida et al., 2001). This finding was elaborated upon by a later study, in which the endothelial cell was proposed as a cellular source of NO. Synthesis of glycolytic enzymes in the astrocytes in response to NO was found to be under the control of HIF-1 α (Brix et al., 2012).

1.18.6 Glucose metabolism and EAE

There has also been some investigation into changes in glucose metabolism in neurodegenerative disease states. In general, it seems activated immune cells that either cause, or migrate to the site of neuroinflammation, display enhanced glycolysis. Radu and colleagues used 2-[18F] Fluoro-2-deoxy-D-glucose positron emission tomography ([18F]FDG PET) in tandem with computed tomography (CT) to visualise inflammatory infiltrates in the spinal cord and show that they exhibited increased glycolytic rates in EAE (Radu et al., 2007). HIF-1 α is important for generation of Th17 cells. Unsurprisingly, cells prepared from HIF-1 α knockout mice were significantly less capable of transferring EAE to WT recipients than cells from WT mice. These authors also showed that treatment of cells from WT mice with the glycolytic inhibitor 2-DG significantly reduced their capacity to transfer EAE to WT recipient mice (Shi et al.,

2011b). The metabolic profiles of encephalitogenic cells in MS are more difficult to characterise and thus not much is known about the relative contributions of aerobic glycolysis or oxidative phosphorylation to glucose metabolism in the CNS of affected individuals. The only consistent observation is that glucose metabolism is perturbed in the brains of patients, and that elevated pyruvate levels in the blood have been tentatively associated with relapse. However these studies have focused on neuronal metabolism, rather than the profiles of infiltrating cells (Mathur et al., 2014). Taken together, these data suggest that cells that are pathogenic in EAE preferentially depend upon aerobic glycolysis as a means of glucose metabolism and further investigation into this process in MS pathogenesis may well present a hitherto unexploited route for therapeutic intervention.

Chapter 2: Materials and Methods

2.1 Animals

C57BL/6 mice were purchased from Harlan, with the exception of those from which glia were isolated, which were obtained from an inbred strain of C57BL/6 mice from the BioResources Unit, Trinity College Dublin. IFN- γ ^{-/-} mice were provided by Rachel McLoughlin from a colony established from breeding pairs from Jackson laboratories. All mice were housed in groups in a specific pathogen free (SPF) environment. A 12 h light/dark cycle was employed. Temperature was maintained at 20 °C to 22 °C. Water and irradiated laboratory food were provided *ad libitum*. All mice were maintained according to European Union regulations, and experiments were performed under license from the Department of Health and Children and with approval from the Trinity College Dublin BioResources Ethics Committee. All tests were conducted under national law and European Union directives on animal-based experiments.

2.2 Cell Counting

Cell counts were obtained by diluting cells (1: 10) with Trypan Blue or ethidium bromide acridine orange EBAO (Sigma-Aldrich, UK). An aliquot (10 μ l) of the cell suspension was loaded onto a disposable haemocytometer (Hycor Biomedical, UK). Viable cells, which exclude the Trypan Blue dye, appear white under a light microscope. The live cells were counted. When stained with EBAO, viable cells appear green under a fluorescent microscope, whereas the dead cells appear red. The live cells were counted.

In both cases, the cells in the top left hand section and bottom right hand section (both of which are large squares made up of 16 smaller squares) were counted and the average was calculated. The number of cells in 1 ml was calculated by multiplying the value by 10⁴.

2.3 Isolation and culture of murine mixed glia

Neonatal C57BL/6 mice were decapitated under sterile conditions in a laminar flow hood. Brains were removed and were mechanically dissociated by vigorous chopping with a sterile scalpel (Swann-Morton, UK) in pre-warmed complete DMEM (cDMEM, see appendix, GIBCO, UK). The chopped tissue in medium was transferred to a 15 ml

Falcon tube (Sarstedt Ltd., UK) and triturated using a sterile Pasteur pipette (Fisher Scientific, UK). A single cell suspension was obtained by applying the triturated material through 40 µm cell strainers (BD Plastipak, USA) placed on top of 50 ml Falcon tubes (Sarstedt Ltd., UK) and flushing through with pre-warmed cDMEM. The cells were centrifuged (1250 rpm; 5 min). The supernatant was then discarded; cells were resuspended in cDMEM (250 µl) and seeded onto 6-well tissue culture plates (Fisher Scientific, UK) at a density of 1 brain per plate. The wells were topped up with medium (750 µl) 2 h later. The medium was changed if necessary and cells were used between days 10 and 12 (or at which time they had reached > 80% confluence).

2.4 Isolation and culture of primary murine astrocytes

For primary astrocyte culture, brains were harvested from neonatal C57BL/6 mice as per Section 2.3. Cells were then seeded (1 ml) onto the centre of T25 cm² tissue culture flasks (Sigma-Aldrich, UK). The flasks were topped up with cDMEM (8 ml) 2 h later. The medium was changed if necessary and on day 14 (or the point at which the microglia had begun to detach from the base), flasks were placed on a shaker for 2 h to dislodge any remaining cells. The supernatants containing the microglial cells were discarded. The remaining astrocytes were detached from the flasks by application of trypsin-EDTA (2 ml; Hyclone, USA) for up to 5 min. The trypsin was deactivated by the addition of cDMEM (5 ml). The supernatants containing the cells were poured off and centrifuged (1250 rpm; 5 min). The cells were counted using Trypan Blue (1:10) as per Section 2.2.1 and seeded onto 6 well tissue culture plates at a density of 2×10^5 cells/250µl/well. The wells were topped up with medium (750 µl) 2 h later, and left to incubate for 48 h before treatment. The cells were cultured in a humidified atmosphere of 95 % air and 5 % CO₂ at 37 °C. Astrocytic cultures were typically >90% pure.

2.5 Isolation and culture of primary murine microglia

For primary microglial culture, brains were harvested from neonatal mice as per Section 2.3. Cells were then seeded (1 ml) onto the centre of T25 cm² tissue culture flasks. The flasks were topped up with cDMEM (8 ml) 2 h later. The following day, cDMEM (1 ml) containing 10x growth factors (M-CSF (100 ng/ml) and GM-CSF

(100ng/ml), R&D Systems, UK) was added to each flask. The media was changed 5 to 6 d later without further addition of growth factors. On day 14 (or the point at which the microglia had begun to detach from the base), flasks were placed on a shaker for 2 h to dislodge any remaining cells. The supernatants containing the cells were poured off and centrifuged (1250 rpm; 5 min). The cells were counted using Trypan Blue (1:10), resuspended, and seeded onto 6-well tissue culture plates at a density of 2×10^5 cells/250 μ l/well. The wells were topped up with medium (750 μ l) 2 h later, and left to incubate for 2 d before treatment or co-incubation. The cells were cultured in a humidified atmosphere of 95 % air and 5 % CO₂ at 37 °C. Astrocytic cultures were typically >80% pure.

2.6 Maintenance of L929 cell line

L929 cells were kindly donated by Professor Luke O'Neill. L929 cells are a murine fibroblast cell line which produces M-CSF, used for the *in vitro* differentiation of bone marrow derived-macrophages (BMDMs). L929 cells were cultured in T175 cm² flasks (Fisher Scientific, UK) until confluent and harvested using heat-activated trypsin-EDTA. The cells were centrifuged (850 x g; 4 min). The resulting pellet was resuspended in cDMEM and the cells were counted using Trypan Blue. The cells were seeded in cDMEM (40 ml) in T175 cm² flasks at a density of 0.5×10^6 cells/ml and maintained in culture for 1 week, at which time the L929 conditioned media was removed and the cells reseeded at 0.5×10^6 cells/ml in a fresh T175 cm² flask. To remove any remaining cells, the conditioned media was centrifuged (850 x g; 4 min). The upper 80 % of supernatant was collected and filter-sterilised using a syringe filter (0.22 μ M, Millipore, Germany). This medium was stored at -20 °C for future use in BMDM culture. L929-conditioned medium is routinely used as an alternative source of M-CSF to avoid the costs associated with the use of recombinant growth factors.

2.7 Isolation and culture of murine bone marrow-derived macrophages (BMDMs)

Following CO₂ asphyxiation, hind limbs were removed from adult C57BL/6 mice. The skin and muscle were removed to expose the femur and tibia (whole, still joined at the patella). The femur and tibia were separated through mechanical dislocation at the patella. Both bones were snipped at either end to create a continuous "tube" of bone.

A 23 gauge needle (BD Microlance, USA) was attached to a 20 ml syringe (BD Plastipak, USA) containing cDMEM. The media containing the flushed bone marrow was collected in a 50 ml Falcon tube. The cell suspension was then passed through a 40 µm cell strainer. The cells were centrifuged (1250 rpm; 5 min). The supernatant was discarded and the pellet was resuspended in sterile Red Blood Cell Lysis Buffer (1.5 ml; see appendix) for 3 min. and centrifuged at (1250 rpm; 5 min). The pellet was resuspended in medium (10 ml) and counted by diluting 1:10 with EBAO as per Section 2.2.2. The cells were then seeded out at a density of 0.5×10^6 /ml in 30 ml medium supplemented with 20 % L929-conditioned medium. On day 3, L929-supplemented cDMEM (20 ml) was added to the flasks and on day 6, the medium was poured off the cells and replaced with L929-supplemented cDMEM (25 ml). On day 8 (or the time at which the cells were 100% confluent), the cells were gently scraped in the medium from the base of the flask. The cell suspension was poured into a 50 ml Falcon tube and centrifuged (1250 rpm; 5 min). The supernatant was discarded and the pellet was resuspended medium (10 ml). The cells were counted by diluting 1 : 10 with EBAO, and then plated on a 6 well plate at a density of 2.5×10^5 / ml in cDMEM with 10 % L929 conditioned medium and incubated for 48 h a humidified atmosphere of 95 % air and 5 % CO₂ at 37 °C.

2.8 *In vitro* M1/M2 polarisation of cells

After the 48 h plating period, medium was removed from microglia, astrocytes and BMDMs replaced with cDMEM alone, cDMEM containing IFN-γ (50 ng/ml, R&D Systems, UK) or IL-4 (20 ng/ml, R&D Systems, UK). Cells were incubated with these treatments for 24 h before harvesting as per Section 2.18.

2.9 Isolation of peritoneal macrophages (PMacs) from murine peritoneal exudate cells (PEC)

Following CO₂ asphyxiation, adult C57BL/6 mice were placed on their backs on a polystyrene board and the paws were fastened down. The abdomens were sprayed down with alcohol. Using a forceps, the abdominal skin was tented and a small incision was made, taking care not to pierce the peritoneal sac. A 27 gauge needle was attached to a 10ml syringe containing ice-cold sterile PBS (Sigma-Aldrich, UK) and

inserted into the peritoneal cavity. The PBS was injected into the peritoneal cavity, and then the mouse was gently agitated to dislodge any cells. A 19 gauge needle was then attached to another 10ml syringe and the PBS was recovered, placed into a 15ml Falcon tube and put on ice immediately to reduce cell adhesion to the plastic. The cell suspension was then centrifuged (1250 rpm; 5 min). The pellet was resuspended in complete RPMI (1 ml; cRPMI, see appendix) and cell number was determined by counting the cells in EBAO (1: 10). The cells were plated out a density of $1 \times 10^5/200\mu\text{l}$ /well in cRPMI a 96-well flat bottomed plate (Fisher Scientific, UK). The cells were incubated at 37°C for 2 h. The typical proportion of adherent cells that remained after this time was determined empirically through counting 6 wells reserved for this purpose. The non-adherent cells were aspirated and the adherent cells were rinsed twice with cRPMI (GIBCO, UK) before treatment for 6 h with medium alone or IL-4 (20 ng/ml).

2.10 Removal of spleen and lymph nodes

The murine spleen and brachial, axillary, mesenteric and inguinal lymph nodes were located according to **Figure 2.1** and stored in ice-cold medium until further processing.

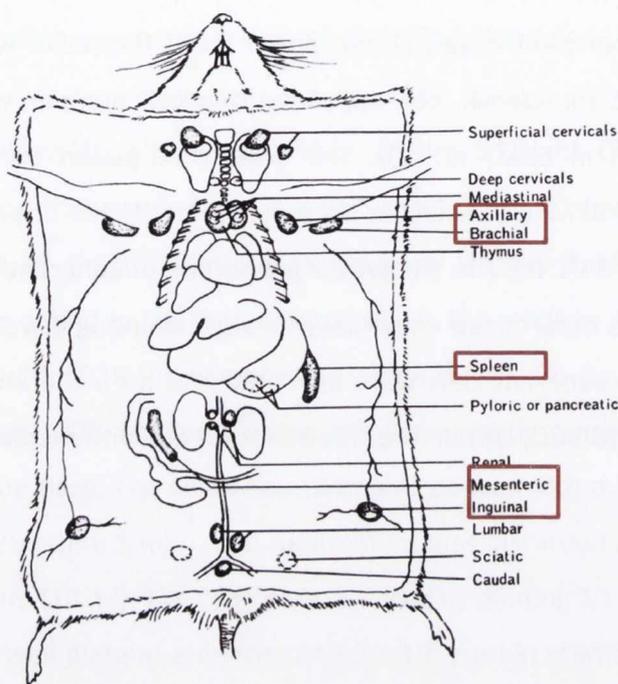


Figure 2.1 Location of murine lymph nodes (Adapted from Dunn, T.B. 1954. Normal and pathologic anatomy of the reticular tissue in laboratory mice, with a classification and discussion of neoplasms. *J. Nat. Cancer Inst.* 14: 1281-1434)

2.11 Isolation of murine CD3⁺ T cells

Spleens were removed from adult C57BL/6 mice as per Section 2.10. The tissue was mechanically dissociated, applied to a 40 μm cell strainer and flushed through with cRPMI for T cell culture. The resulting suspension was centrifuged (1250 rpm; 5 min) and the supernatant was discarded. The supernatant was aspirated and the cells were resuspended in pre-warmed medium (1 ml) and counted. CD3⁺ T cells were isolated using the CD3⁺ T Cell Isolation Kit (Miltenyi Biotec, Germany) as per the manufacturer's instructions. Briefly, the cells were centrifuged at (1250 rpm; 5 min). The resulting pellet was resuspended in MACS buffer (see appendix, 40 μl per 10^7 cells). Biotin-Antibody Cocktail (10 μl per 10^7 cells) was added, and the cells incubated at 4 °C for 5 min. MACS buffer (80 μl per 10^7 cells) and Anti-Biotin Microbeads (20 μl per 10^7 cells)

were added and cells were incubated for another 10 min at 4 °C. The suspension was then applied the MS MACS column (Miltenyi Biotec, Germany), which had been previously rinsed through with MACS buffer (3 ml). The effluent was collected, and this fraction represented the enriched CD3⁺ T cells. The cells in MACS buffer collected from the column were centrifuged (1250 rpm; 5 min). The pellet was resuspended in cRPMI for T cell culture (1 ml) and counted using EBAO (1: 10).

2.12 Co-incubation of PMacs and CD3⁺ T cells

After the 6 h treatment with medium or IL-4 referred to in Section 2.9, the supernatants were removed from PMacs and cells were harvested for RNA isolation as per Section 2.18. The remaining cells were incubated with CD3⁺ T cells (0.5 x 10⁵ PMac: 1 x 10⁵ CD3⁺ T cells) in cRPMI for T cell culture in the presence of anti-CD3e (5 µg/ml, EBioscience, USA) for 72 h. At the end of the incubation period cells were extracellularly stained for CD4 and CD49d as per Section 2.38 before undergoing flow cytometric analysis as per Section 2.41.

2.13 Treatment of stimulated CD3⁺ T cells with IL-4

A 96-round bottomed tissue culture plate (Fisher Scientific, UK) was coated with anti-CD3e (1 µg/ml) and incubated at 37°C for 2h. After this time, the plate was washed twice with PBS. CD3⁺ T cells were isolated as per Section 2.11 and added to the plate at a density of 2.5 x 10⁵/200µl/well and treated for 24 h with cRPMI for T cell culture with anti-CD28 (5µg/ml; EBioscience, USA) with or without IL-4 (20 ng/ml). After this time cells were harvested for RNA isolation as per Section 2.18 or stained extracellularly for CD4 and CD49d as per Section 2.38 before undergoing flow cytometric analysis as per Section 2.41.

2.14 Isolation and culture of murine natural killer (NK) cells

Spleens were removed from adult C57BL/6 mice as per Section 2.10. The tissue was mechanically dissociated, applied to a 40 µm cell strainer and flushed through with DMEM for NK cell culture. The resulting suspension was centrifuged (1250 rpm; 5 min) and the supernatant was discarded. The pellet was resuspended in sterile Red Blood

Cell Lysis Buffer (1.5 ml) and incubated for 3 min. This mixture was then centrifuged (1250 rpm; 5 min). The supernatant was aspirated and the cells were resuspended in pre-warmed DMEM for NK cell culture (1 ml) and counted. NK cells were isolated using the NK cell Isolation Kit II (Miltenyi Biotec, Germany) as per the manufacturer's instructions. Briefly, the cells were centrifuged (1250 rpm; 5 min). The resulting pellet was resuspended in MACS buffer (40 μ l per 10^7 cells). Biotin-Antibody Cocktail (10 μ l per 10^7 cells) was added, and the cells incubated at 4 °C for 5 min. MACS buffer (80 μ l per 10^7 cells) and Anti-Biotin Microbeads (20 μ l per 10^7 cells) were added and cells were incubated for another 10 min at 4 °C. The suspension was then applied to the MS MACS column (Miltenyi Biotec), which had been previously rinsed through with MACS buffer (3 ml). The effluent was collected, and this fraction represented the enriched NK cells.

The cells in MACS buffer collected from the column were centrifuged (1250 rpm; 5 min). The supernatant was discarded and the cells were resuspended in DMEM for NK cell culture with recombinant human IL-2 (100 ng/ml; Immunotools, Germany) at a density of 1×10^6 /ml. The cells were then plated out at a density of 2×10^5 /200 μ l/well on a 96-well round-bottomed tissue culture plate. The cells were split every 2 d with addition of fresh medium with IL-2, and cultured for 6 d in a humidified atmosphere of 95 % air and 5 % CO₂ at 37 °C.

On the day of co-incubation, cells (2×10^5) were taken to assess purity using flow cytometry. The cells were stained extracellularly for CD3 and NK1.1 as per Section 2.38, before undergoing flow cytometric analysis as per Section 2.41. A representative dot plot is shown in **Figure 2.2**.

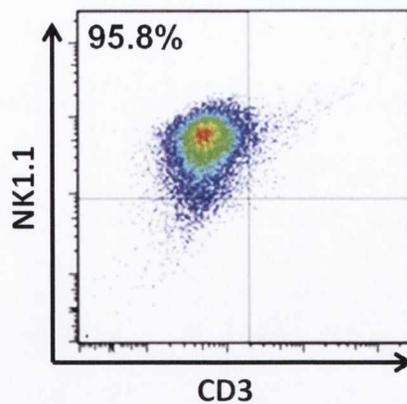


Figure 2.2 Representative dot plot of typical NK cell purity following culture period

At the end of the 7-day culture period, NK cell (Live/Dead Aqua⁻CD3⁻NK1.1⁺) purity was typically >95%.

2.15 Co-culture of murine BMDMs, mixed glia or microglia with NK cells

Co-culture experiments were performed as outlined in **Figure 2.3**.

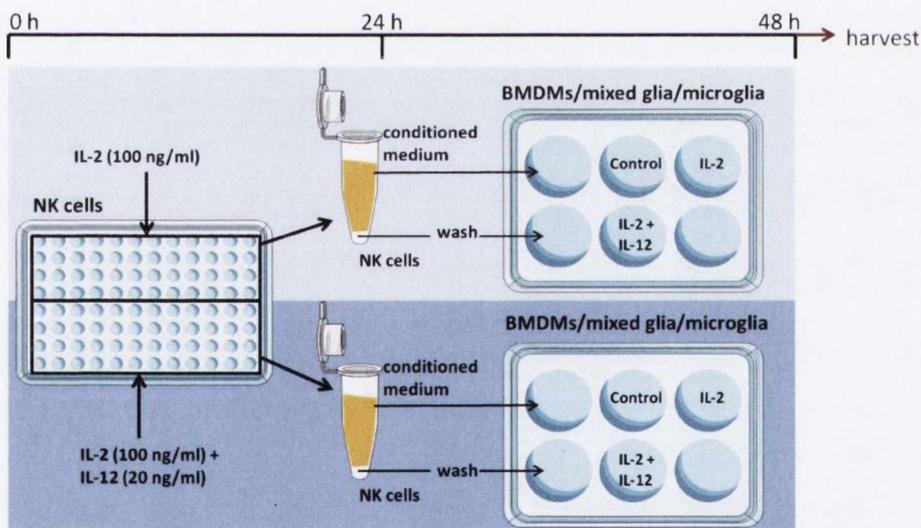


Figure 2.3 Experimental design of NK cell co-culture with BMDMS, mixed glia or microglia

NK cells were cultured as per Section 2.14. On day 7, the cells were treated with IL-2 (100 ng/ml) with or without additional IL-12 (20 ng/ml) for 24 h. The NK cells were then centrifuged (1250 rpm; 5 min), and the conditioned medium was collected from both treatment groups. The NK cells were washed and resuspended in fresh cDMEM for NK cell culture. The BMDMs/mixed glia/microglia were then incubated with either medium alone, IL-2 (100 ng/ml) with or without IL-12 (20 ng/ml), conditioned medium from either NK cell treatment groups,

or the NK cells themselves. Cells were incubated for 24 h in a humidified atmosphere of 95 % air and 5 % CO₂ at 37 °C.

The medium/NK cells were removed after 24 h and the BMDMs/mixed glia/microglia were harvested as per Section 2.18 for RNA isolation.

2.16 Generation of MOG-specific CD4⁺ T cells

MOG₃₅₋₅₅ (100 µg; Genscript, USA) was emulsified in CFA (Chondrex, USA) and emulsified as per Section 2.27. Adult female C57BL/6 mice were immunised with the emulsion as per in Section 2.28. After 10 d, the spleens inguinal, mesenteric, axial and brachial lymph nodes were removed as per Section 2.10. The organs were mechanically dissociated using a 40 µm cell strainer and resuspended in cRPMI at a density of 10 x 10⁶/ml. The cells were stimulated with MOG (100 µg/ml) for 72 h. The CD4⁺ T cells were then purified from these cultures using the CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec, Germany) as per the manufacturer's guidelines. Briefly, the cells were centrifuged (1250 rpm; 5 min). The resulting pellet was resuspended in MACS buffer (40 µl per 10⁷ cells). Biotin-Antibody Cocktail (10 µl per 10⁷ cells) was added, and the cells incubated at 4 °C for 10 min. MACS buffer (80 µl per 10⁷ cells) and Anti-Biotin Microbeads (20 µl per 10⁷ cells) was added and cells were incubated for another 15 min at 4 °C. The suspension was then applied to the MS MACS column (Miltenyi Biotec), which had been previously rinsed through with MACS buffer (3 ml). The effluent was collected, and this fraction represented the enriched CD4⁺ cells.

2.17 Co-incubation of MOG-specific CD4⁺ T cells, BMDMs and NK cells, and assessment of VLA-4 expression

The MOG-specific CD4⁺ T cells were resuspended at a density of 2.5 x 10⁵ cells/ml and co-incubated at a 1:1 ratio for 24 h with either (a) medium alone, (b) IFN-γ (50ng/ml), (c) NK cells (pre-treated with 100 ng/ml IL-2), (d) NK cells (pre-treated with 20ng/ml IL-12), (e) IFN-γ -pre-treated BMDMs (50ng/ml), (f) BMDMs with IL-2 pre-treated NK cells or (g) BMDMs with IL-12 pre-treated NK cells. The cells were surface stained for CD4 and VLA-4 as per Section 2.38 and underwent flow cytometric analysis as per Section 2.41.

2.18 Cell Harvesting and Supernatant Collection

In the case of adherent cells, supernatants were aspirated and transferred into 1.5 ml Eppendorf tubes (Sarstedt Ltd., UK). A master mix of RA1 buffer (Machery-Nagel, Germany) and β -mercaptoethanol (Sigma-Aldrich, UK) was made up at a ratio of 100:1 and 350 μ l was added to each well. The cells were dislodged from the base of the plate using a cell scraper (Sarstedt Ltd., UK) and collected into separate labelled RNase-free microcentrifuge tubes (1.5 ml; Eppendorf, Germany). In the case of non-adherent cells, cells were dislodged from the base of the plate using a cell scraper. The cells in the supernatants were then transferred to RNase-free microcentrifuge tubes and centrifuges (11,000 $\times g$; 5 min). The supernatants were aspirated and transferred to 1.5 ml Eppendorf tubes. The RA1/ β -mercaptoethanol mix (350 μ l) was added to each pellet and the cells were resuspended in this lysis buffer. All cells and supernatants were stored at -20 °C until further analysis.

2.19 ELISA (Enzyme-Linked Immunosorbent Assay – R&D Systems, UK)

The concentrations of the cytokines IFN- γ , IL-17A and GM-CSF were measured using commercially available ELISA kits (R&D Systems). A 96-well microplate (Sigma-Aldrich/NUNC, UK) was coated with Capture Antibody (50 μ l) at the appropriate dilution in PBS. The plate was covered with a microplate sealer (Cruinn Diagnostics, Ireland) and left to incubate overnight at room temperature. The Capture Antibody was then aspirated from all wells and the plate was washed with Wash Buffer (Tween-20 in 1L PBS; 500 μ l) 3 times. After the last wash, any remaining Wash Buffer left in the wells was removed by inverting the plate and blotting it on clean paper towels. The plates were then blocked by adding Blocking Buffer (1% BSA in PBS; 150 μ l) to all wells. The plate was sealed and then left to incubate for 1 h at room temperature. The plate was washed again, as before. The standards and samples were diluted accordingly and 50 μ l aliquots were added to the plate in duplicate. The plate was sealed and left to incubate at room temperature for 2 h. The plate was washed again. At this point, 50 μ l of Detection Antibody diluted appropriately in Reagent Diluent (in this case the type of medium in which the cells were originally cultured) was added to all wells. The plate was sealed again and left to incubate at room temperature for a further 2 h. The plate

was then washed and 50µl of Streptavidin-HRP (1:2000 in Blocking Buffer; 50 µl) was added to each well. The plate was covered in tin foil and left to incubate for 20 minutes at room temperature. The plate was washed and Substrate Solution (50 µl) was added. The plate was placed in the dark and periodically observed until the colour change had progressed to an appropriate degree. At this point, Stop Solution (1M Sulphuric Acid; 50 µl) was added to each well. The plate was read on a microplate reader set to an absorbance of 450nm. The standard curve was constructed in Microsoft Excel using standard values from which the blank had been subtracted. The concentration of protein (in pg/ml) in each sample was thus calculated and graphed using Graphpad Prism Version 5.

2.20 LDH Assay (Lactate dehydrogenase CytoTox 96® assay (Promega, USA))

Cell viability was assessed by indirect, relative measure of lactate dehydrogenase (LDH) concentration in the supernatants. LDH, which catalyses the conversion of pyruvate to lactate, and vice versa, is released upon cell lysis and so detection of this enzyme in the supernatant indicates the occurrence of cell death. Supernatants (50 µl) were transferred to a 96-well protein assay plate (Sarstedt) and incubated with LDH substrate mix (50µl) for 30 min in the dark. Stop solution (50 µl) was added to each well and absorbance was read at 490nm using a BioTek ELx800 Microtitre Plate Reader. Optical density (OD) values for each sample were graphed using Graphpad Prism.

2.21 RNA Extraction

The Nucleospin® RNA II Kit (Macherey-Nagel, Germany) was used to extract RNA from the harvested cells. The cells (in RA1 buffer and β-mercaptoethanol) were applied to the NucleoSpin® Filter units (violet). The filtrate was collected and centrifuged (11000 x *g*; 1 min). The NucleoSpin® Filter units were discarded and 70 % molecular grade ethanol (Sigma-Aldrich) diluted in RNase-free water (Macherey-Nagel, Germany) was applied to the filtrate to adjust RNA binding conditions. NucleoSpin® column units (light blue), which bind the RNA, were placed in new 2 ml collection tubes. The filtrate was loaded and the tubes (containing the filter columns) were centrifuged (8000 x *g*; 30 sec). The columns were placed in new collecting tubes and Membrane Desalting Buffer (350 µl) was loaded onto the columns to remove residual salt from the column's

silica membrane. The samples were centrifuged again (11000 x *g*; 1 min). DNase Reaction Mixture (10:1 ratio of rDNase Reaction Buffer and rDNase; 95 μ l) was applied directly to the centre of the silica membrane of the column and left at room temperature for 15 min. The purpose of DNase application was the digestion of DNA content in the samples. The silica membrane was then washed using RA2 buffer (200 μ l) and centrifuged (8000 x *g*; 30 sec). The column was transferred to a new collecting tube and washed again, this time with RA3 buffer (600 μ l). It was then centrifuged again (8000 x *g*; 30 sec). The filtrate was discarded and the column was placed back into the same collecting tube. The column was washed once more with RA3 buffer (250 μ l) and centrifuged (11000 x *g*; 2 min). The column was placed in a new (pre-labelled) RNase-free 1.5 ml microcentrifuge tube, and the RNA was eluted using RNase-free water (40 μ l). The tubes were centrifuged (11000 x *g*; 1 min). The elute was reapplied to the column and the centrifugation step was repeated to maximise purity and yield. The columns were discarded, and the RNA elute was stored at -80 °C until quantification of RNA concentration.

2.22 Quantification of RNA Concentration

The RNA concentrations of each sample were determined using the Nanodrop Spectrophotometer ND-1000 (USA). Once the values were obtained, the concentrations of RNA in each sample (in μ g/ μ l) were calculated and were equalised with the addition of appropriate amounts of RNase-free water.

2.23 Reverse transcription for cDNA Synthesis

RNA was reverse transcribed into cDNA using high-capacity cDNA archive kit (Applied Biosystems, USA). The Master Mix was prepared according to Table 2.1 and stored on ice.

Solution	Volume per sample (μ l)
10x reverse transcription buffer	5
25x dNTPs	2
10x random primers	5
Multiscribe™ Reverse Transcriptase	2.5
RNase-free water	10.5

Table 2.1 Components of Master Mix (for 25 μ l sample)

RNA sample (25 μ l) and Mastermix (25 μ l) were added to labelled PCR tubes (Sarstedt). The contents were mixed by aspirating and dispensing using a pipette and bubbles were removed using the mini-centrifuge. The PCR tubes were placed in the thermal cycler (PTC-200, Peltier Thermal Cycler, USA) which subjected the samples to a 10 min 25 °C cycle and a 2 h 37 °C cycle. The resulting cDNA was stored at -20 °C until amplification.

2.24 cDNA amplification by RT-PCR

The cDNA samples were diluted with RNase-free water (1:4) and aliquots (8 μ l) were added to each well of a 96-well PCR plate (Thermo Scientific, USA). Master Mix (12 μ l) was added to each well. The Master Mix contained Sensimix Probe II (10 μ l; Bioline, UK) composed of a heat-inactivated DNA-polymerase, pure dNTPs and MgCl₂ (6 mM), an endogenous control (1 μ l; β -actin) and the appropriate primer per well (1 μ l). These real-time PCR dye-labelled primers were delivered as Taqman® Gene Expression Assays (Applied Biosystems, USA) for the mouse genes listed in Table 2.2. Real-time PCR was performed using Applied Biosystems ABI Prism 7300 Fast Track Sequence Detection System v1.4.1. The plate was subjected to 40 cycles of the following

temperature variations: Stage 1: 50 °C for 2 minutes, Stage 2: 95 °C for 10 minutes, Stage 3: 95 °C for 15 secs, Stage 4: 60 °C for 1 minute.

Gene Name	TaqMan Gene Expression Assay Number
Nos2 (iNOS)	Mm00440502_m1
Tnf (TNF- α)	Mm00443258_m1
Ciita (MHC II)	Mm00482914_m1
Mrc1	Mm00485148_m1
Arg1	Mm00475988_m1
Chi3l3	Mm00657889_mH
Ifng (IFN- γ)	Mm01168134_m1
Il17a (IL-17A)	Mm00439618_m1
β -actin	Mm00607939_s1

Table 2.2 Gene Names and Gene Expression Assay Numbers of primers used for PCR

2.25 PCR Quantification

The expression of each target gene was determined using the efficiency-corrected comparative CT method. Target genes in different samples were compared to a reference gene (β -actin). These values were normalised to the control sample and the relative differences in expression of the target gene between the samples were expressed as a ratio.

2.26 Investigation of metabolic signatures of polarised cells: Using the Seahorse Extracellular Flux (XF24) Analyser (Seahorse Bioscience, USA)

The optimal cell numbers and concentration of inhibitors were determined empirically before the commencement of experiments:

BMDMs: 6×10^5 /well

Microglia: 1×10^5 /well

Astrocytes: 1×10^5 /well

BMDMs, microglia and astrocytes were isolated and cultured as per Sections 2.7, 2.5 and 2.4 respectively. On day 8/14 the cells were removed from their flasks and seeded in cDMEM (100 μ l, with 10 % L929 conditioned medium in the case of the BMDMs) in specialised XF24 cell culture microplates (Seahorse Bioscience, USA). Cells were topped up with medium 2 h later. The background correction wells were left free of cells (as per Figure 2.4). The plate was left to incubate overnight at 37°C. The following day, the medium was removed and the cells were treated for 24 h with medium alone (500 μ l), IFN- γ (50 ng/ml) or IL-4 (20 ng/ml) in cDMEM (with 10 % L929 conditioned medium in the case of BMDMs). The sensor cartridge was hydrated by adding Seahorse XF Calibrant solution (1 ml; Seahorse Bioscience, USA) to each well of the utility plate and was left overnight in a no-CO₂ incubator at 37°C. The cell plate was removed from the incubator, the medium was removed and replaced with Seahorse XF Assay medium (supplemented with glucose; 25nM). The cell plate was then placed into the no-CO₂ incubator for 30 min. Oligomycin (Abcam, UK), FCCP (Sigma-Aldrich, UK) and antimycin A (Sigma-Aldrich, UK) were diluted to the previously determined concentrations in glucose-supplemented Seahorse medium, and were injected into the appropriate wells of the sensor cartridge according to **Figure 2.4**.

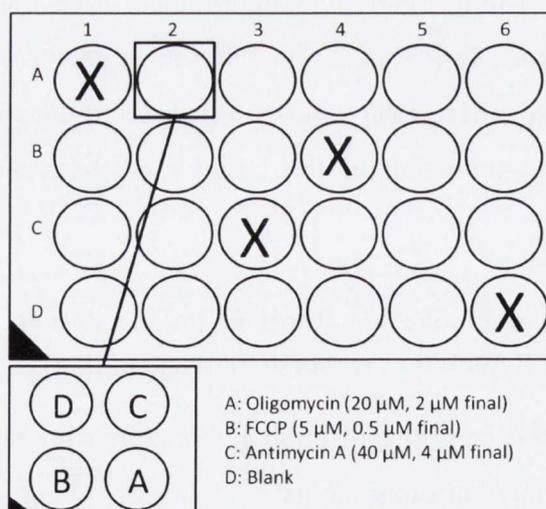


Figure 2.4 Seahorse cell plate layout and inhibitor concentrations

The plate was loaded according to the above diagram, with the omission of cells from the background correction wells, and the injection of the relevant compounds into the relevant ports.

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were subsequently measured using the XF24 extracellular analyzer (Seahorse Bioscience, USA). 3 baseline measurements were taken before and after sequential injection of mitochondrial inhibitors and antioxidants. 3 readings were also taken subsequent to the injection of each compound. ECAR and OCR were automatically calculated by the Seahorse XF24 software. Every point represents an average of 3–6 different wells. Sample ECAR and OCR plots can be seen in **Figure 2.5**.

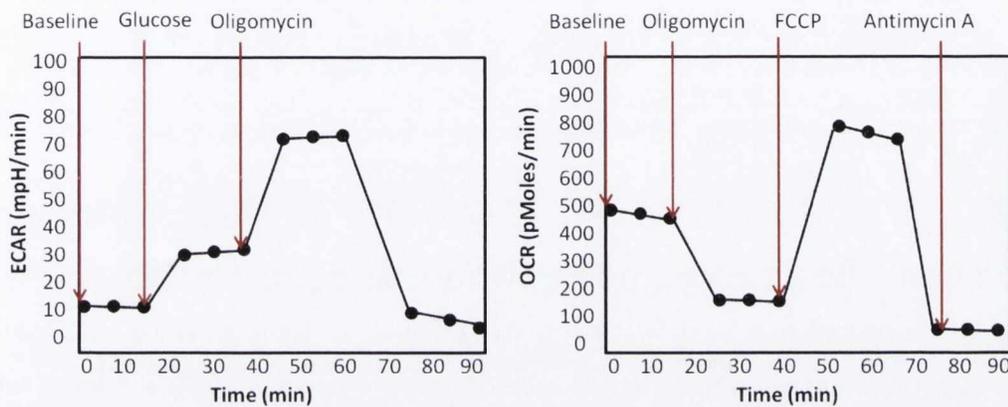


Figure 2.5 Reference ECAR and OCR graphs provided by Seahorse Biosciences

ECAR is measured at baseline and subsequent to the addition of glucose and oligomycin, an ATP synthase inhibitor. OCR is measured at baseline and following the injection of oligomycin, FCCP (an uncoupling agent) and antimycin A (inhibitor of Complex III of the electron transport chain).

2.27 Emulsification of MOG for EAE induction

MOG₃₅₋₅₅ peptide (at a quantity of 100 µg per mouse; GenScript, USA) was emulsified in CFA (containing 4 mg/ml heat killed *Mycobacterium tuberculosis*; Chondrex, USA) and sterile PBS, such that 1/2 of the overall volume was CFA, 1/10 was MOG and the remainder was PBS. The aqueous layer (PBS and MOG) was placed into a 30 ml specimen tube first, on top of which the lipid layer (CFA) was applied. A tissue homogeniser (Kinematica Polytron PT 2100, Switzerland) was used to emulsify the MOG and CFA. The homogeniser tip was similarly inserted into the aqueous layer and the mixture was homogenised for 20 to 30 min. Emulsion of the peptide is hastened by low temperatures and therefore the specimen tube was kept on ice throughout the whole procedure. The suitability of the MOG emulsion was verified by dropping a

small amount from the syringe into a Petri dish of PBS; when formed a solid, floating bolus that did not seep at the edges, it was deemed ready to inject.

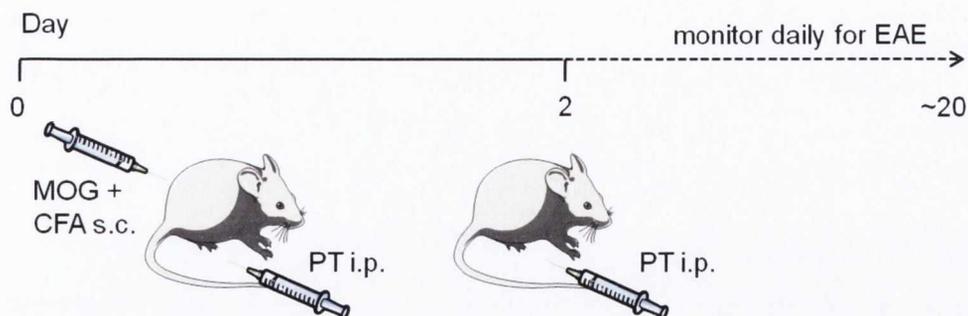
2.28 Subcutaneous Injection

The emulsion (200 μ l/mouse) was injected subcutaneously into WT or IFN- γ ^{-/-} mice. Mice were restrained in a 50 ml Falcon tube with a section cut out to allow access to the loose skin of the back, which was grasped between the thumb and forefinger. The needle was inserted bevelled edge up into the fold of skin and the emulsification was administered.

2.29 Intraperitoneal Injection

On the same day, and on day 2, the animals were injected intraperitoneally with pertussis toxin (500 ng/mouse; 1:40 dilution in sterile PBS; Kaketsuken, Japan). Intraperitoneal injection was achieved by employing the “scruffing” restraint technique. The mouse was tilted downwards to expose the abdomen. The needle was inserted into the abdomen just right of the midline at a 30 ° angle, the abdominal skin was tented to avoid damage to the viscera, and the toxin was injected. The induction protocol for active EAE is outlined schematically in **Figure 2.6 (A)**.

A) Active EAE



B) Passive EAE

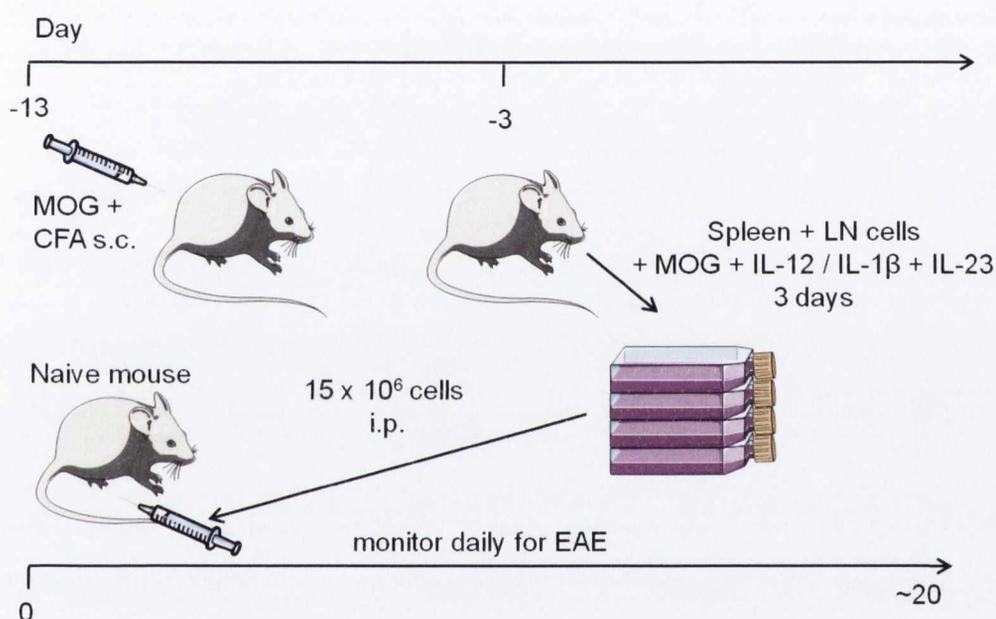


Figure 2.6 Schematic outlining the active and passive EAE protocols

EAE was induced either actively through subcutaneous administration of MOG emulsified in CFA with a concomitant intraperitoneal injection of pertussis toxin, or through adoptive transfer of cells taken from MOG-immunised donors.

2.30 NK cell depletion

NK cells were depleted from WT/IFN- γ ^{-/-} mice using a rabbit anti-mouse asialo GM1 polyclonal antibody (Cedarlane, Canada). A preliminary check of the efficacy of the antibody revealed a 72 % depletion of NK cells in the spleen 1 d post-administration, with optimal efficacy for up to 4 d. This considered, the frequency of administration was set at every 2 to 3 d. The timing of the administration was dependent on the

group to which the mice were assigned (**outlined in Figure 2.7**). Mice received an intraperitoneal injection of a 1:10 dilution of antibody in sterile PBS (200 μ l), while control mice received sterile rabbit serum (1:10 dilution in sterile PBS; 200 μ l; Sigma-Aldrich, UK).

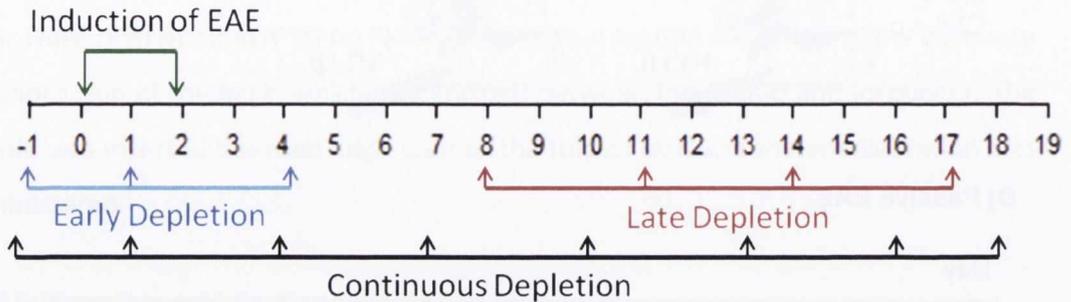


Figure 2.7 Timeline of NK cell depletion

NK cells were depleted through administration of anti-asialo GM1 early in EAE (on days -1, 1 and 4), continuously throughout EAE (on days -1, 1, 4 and every 3 d thereafter) or late in disease (on day 8 and every 3 d thereafter).

2.31 Adoptive Transfer/Passive EAE

Donor mice received MOG subcutaneously as in Section 2.28. After 10 d, the mice were sacrificed and the spleens and inguinal, mesenteric, axial and brachial lymph nodes were removed (as per Section 2.10) and placed in ice-cold cRPMI for T cell culture. Splenic and lymphoid tissues were separately mechanically dissociated and passed through 40 μ m nylon strainers to obtain single cell suspensions. The cells were then centrifuged at 1250 rpm for 5 min, resuspended in medium (1 ml) and counted using EBAO (1: 10). Spleen and lymph node cells (at a ratio of 70:30) were cultured in cRPMI for T cell culture in the presence of MOG (100 μ g/ml), IL-1 β (10 ng/ml) and IL-23 (10 ng/ml) for 72 h in a humidified atmosphere of 95 % air and 5 % CO₂ at 37 °C. After the culture period the cells were washed twice with PBS and injected intraperitoneally into naïve recipient mice at a density of 15 x 10⁶ cells/mouse. The adoptive transfer method of inducing EAE is outlined schematically in **Figure 2.6**.

2.31.1 Addition of IL-4 to cultures

In one experiment, the cells prepared as above were split into two groups, one cultured in the presence of MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) and one with MOG (100 µg/ml), IL-1β (10 ng/ml), IL-23 (10 ng/ml) and additional IL-4 (20 ng/ml).

2.32 Blockade of VLA-4

VLA-4 ($\alpha_4\beta_1$ integrin) was neutralised using a monoclonal antibody against CD49d (α_4 subunit, clone PS/2; Bio X Cell, USA). The antibody/ appropriate isotype control (rat IgG2b, Louis Boon) was injected (200 µg/mouse in PBS) intraperitoneally. In the active EAE experiment, the antibody was administered on day 7 post induction and every 3 d thereafter. In the adoptive transfer experiment the antibody was administered at the time of cell transfer and every 3 d thereafter.

2.33 Disease monitoring and clinical score regime

The animals were scored and weighed daily. Disease severity was scored as follows:

0: no clinical signs, 1: limp tail, 2: ataxic gait, 3: hind limb weakness, 4: hind limb paralysis, 5: moribund.

Any animals who reached a clinical score of greater than 4 or that lost over 20 % of their original body weight were euthanized by CO₂ asphyxiation. The onset of symptoms was usually observed between days 8 and 10. The mean clinical scores and body weights were calculated and graphed. The experiments were terminated and the tissue harvested when at least one group had reached a uniform score of 4, or in the case of the NK cell infiltration timecourse experiment, at days 0, 3, 7, 11 and 17.

2.34 Intracardial saline perfusion

Once the mice had progressed to a uniform clinical score of 4, they underwent CO₂ asphyxiation. They were placed on their backs on a polystyrene board and the paws were fastened down. The abdomens were sprayed down with alcohol. Using a forceps, the abdominal skin was tented and a small incision was made under the diaphragm.

The connective tissue at the base of the diaphragm was cut and thus disconnected from the ribcage. Two end vertical cuts were made on either side of the ribcage, which was pinned back to allow access to the thoracic cavity. The right atrium was cut, and a 23 gauge needle attached to a 20 ml syringe filled with PBS was inserted directly into the protrusion of the left ventricle to extend straight up about 5 mm. The PBS was gently introduced into the circulatory system. Perfusion was deemed complete when the liver had paled in colour.

2.35 Removal of brain and spinal cord

The animal was placed on its front. The ears were pinched together and the mouse was decapitated with one clean incision, angling the scissors from behind the ears towards the throat. Care was taken to avoid transection of the cervical vertebrae with the head severed at the spinomedullary junction. The ears were removed and the skin around the skull was cut away. The cortex was partially visible through the skull and had a white appearance as a consequence of the perfusion. The head was held steady with a forceps and the skull was cut along the medial longitudinal fissure. The brain, exposed by pulling the skull away on either side using another forceps, was then removed from the cranial cavity anterior to the olfactory bulbs, and was stored in cold RPMI media on ice until dissociation.

The skin was pinched and tented just above the tail and a horizontal incision was made. The skin was cut away on either side of the spine (taking care to avoid piercing the bolus formed by the previously injected emulsified MOG peptide). The spinal cord was severed roughly at the level of the cauda equina. All connected tissue and the ribs were cut away, leaving behind just the spinal column itself. The spinal column was placed with the dorsal side facing up. The tip of the scissors was inserted into the hollow in the cervical vertebrae and the column was cut all the way along the midline up to the sacral vertebrae. Using a forceps, the spinal cord tissue was harvested and stored in cRPMI on ice until later dissociation.

2.36 CNS tissue lysis

The brains and spinal cords were lysed using the Tissue Lyser II (Qiagen, Netherlands). Briefly, the organs were placed into 2 ml eppendorf tubes with cRPMI (1 ml) and a 5 mm stainless steel bead. The tubes were placed into the 2 x 48 Tissue Lyser Adapter. The tissue was lysed for 3 min at 30 Hz, disassembled and rotated such that the tubes nearest the centre were placed in the outermost position. The 3 min lysing step was repeated and the samples were removed from the machine.

2.37 Percoll Gradients

The lysed samples were transferred to 15 ml Falcon tubes, which were topped up to 10 ml with cRPMI and centrifuged (1250 rpm; 5 min). The supernatants were aspirated and the pellets were resuspended in 40 % Percoll (5 ml; GE Healthcare, UK). 70 % Percoll (5 ml) was placed into separate 15 ml Falcon tubes. The 70 % layer was then overlaid with the 40 % Percoll/lysed tissue suspension. The tubes were then centrifuged at (1600 rpm; 20 min) with the centrifuge brake off. The tubes were removed from the centrifuge. The result of this density-dependent centrifugation is the separation of the myelin (which floats to the top) from the mononuclear cell fraction, which can be visualised as a cloudy collection of cells between the 40%:70 % interface (**Figure 2.9**). The myelin was removed in one Pasteur aspiration and disposed of. The cloudy collection of cells was then aspirated and transferred to another set of 15 ml Falcons, which were topped up to 14 ml with cRPMI and centrifuged (1250 rpm; 5 min). The supernatants were aspirated and the pellets were resuspended in PBS (10 ml) and centrifuged again (1250 rpm; 5 min) to wash off any residual Percoll. The supernatant was discarded once more and the pellets were resuspended in cRPMI (1 ml). When two separate staining panels were required, this was split into 2 x 500 μ l aliquots and transferred to sealed polystyrene FACS tubes (BD Bioscience, USA) to facilitate the use of 2 different staining panels. For intracellular cytokine staining, the cells were stimulated with a mixture of PMA (10 ng/ml; Sigma-Aldrich, UK), ionomycin (1 μ g/ml; Sigma-Aldrich, UK) and Brefeldin A (5 μ g/ml; Sigma-Aldrich, UK) for 5h at 37 °C. an aliquot was taken from each sample for cell counts.

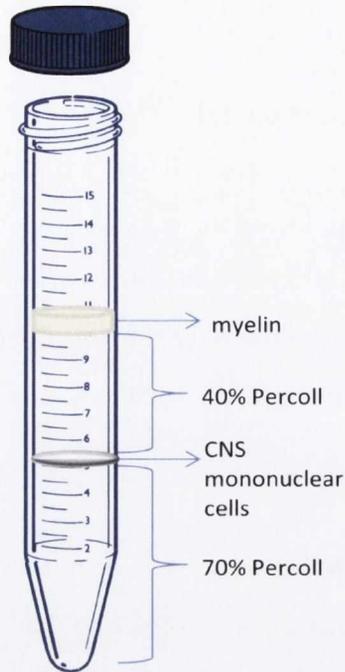


Figure 2.8 Appearance of cells after density-dependent centrifugation in Percoll

Myelin, which is fatty, rises to the top and can be easily removed. Mononuclear cells appear in the interface between 40 % and 70 % Percoll.

2.38 Flow Cytometry: Extracellular Staining and Fixation

The FACS tubes were filled with PBS and centrifuged (1250 rpm; 5 min). The PBS was discarded and the necks of the tubes were dabbed on a pad of clean tissue paper. Live/Dead stain (50 μ l; diluted 1:1000 with PBS, Life Technologies, USA) was added to all tubes, which were vortexed thoroughly and left in the dark for 30 min. The tubes were half-filled with PBS and an aliquot of the suspension was taken from one of the tubes for the Live/Dead compensation. The tubes were centrifuged again (1250 rpm; 5 min). The PBS was poured off again and the tubes were dabbed as before. Extracellular Master Mix (50 μ l; a mixture of the appropriate antibodies diluted in PBS) was added to all tubes, which were vortexed and left in the dark for 15 min. PBS (2 ml) was added to each tube, and they were centrifuged (1250 rpm; 5 min). The PBS was poured off and the tubes were dabbed as before. The cells were then fixed with 2 % paraformaldehyde (Thermo Scientific - diluted in PBS; 50 μ l). The cells were washed and dabbed.

2.39 Flow Cytometry: Permeabilisation and Intracellular Staining

Intracellular Master Mix (50 μ l; a mixture of the appropriate antibodies diluted in 0.5 % saponin in PBS) was added to each tube and vortexed, then left in the dark to incubate for 15 min. The cells were washed, but not dabbled in order to retain a small amount of liquid in the base of the tube for reading.

2.40 Flow Cytometry: Compensation and Fluorescence Minus One (FMO) Controls

Within a flow cytometer, the appropriate ranges of excitation and emission wavelengths are selected by bandpass filters. However, when emission spectra overlap, fluorescence from more than one fluorochrome may be detected. To correct for this spectral overlap, a process of fluorescence compensation is used. This ensures that the fluorescence detected in a particular detector derives is solely from the fluorochrome that is being measured. One compensation control per colour must be used (the antibody selected does not necessarily have to mirror that used in the stain, provided it is the right colour). With the exception of the Live/Dead compensation (which uses cells that have been stained with the Live/Dead antibody), compensation beads are used (BD Bioscience, USA). The beads are polystyrene microspheres that are coated with IgG (positive compensation beads) that react with all mouse or rat/hamster isotypes. Compensation beads (3 drops) were added to the unstained control tube, and diluted with enough PBS to ensure that there would be enough for 50 μ l in each tube (each tube representing a different fluorochrome). Antibody was added to each tube at the same concentration used in the stain.

FMO controls are used in multicolour experiments to allow for ease of identification of gating boundaries. The use of multiple fluorochromes in a panel can result in data spread, which can complicate the interpretation of results. For each fluorochrome, a tube containing cells was stained with every fluorochrome (at the same concentration as in the samples) except for the one under investigation.

2.41 Flow Cytometric Analysis

Flow cytometric analysis was performed using a FACSCanto II flow cytometer (Becton-Dickinson, USA) and analysed with FlowJo software (Tree Star Inc., USA). Absolute cell numbers were determined by multiplying the total cell counts (taken after cell isolation) by the frequency of each cell type. The gating strategies are outlined in Figure 2.10.

2.42 Statistical Analysis

Statistical analysis was carried out using Graphpad Prism. Data were expressed as the mean \pm standard error of the mean (SEM). The statistical test used was determined by the number of groups in a particular experiment. For two groups, a Student's t-test was used. For three or more groups, a one-way ANOVA with a subsequent Newman Keuls post-hoc test was used. For analysis of EAE clinical scores, which had been split on two independent variables, namely day post-immunisation and treatment, a two-way ANOVA with a subsequent Bonferroni post-hoc test. Post-hoc tests were used to identify which means were significantly different. A p value of less than 0.05 was deemed significant.

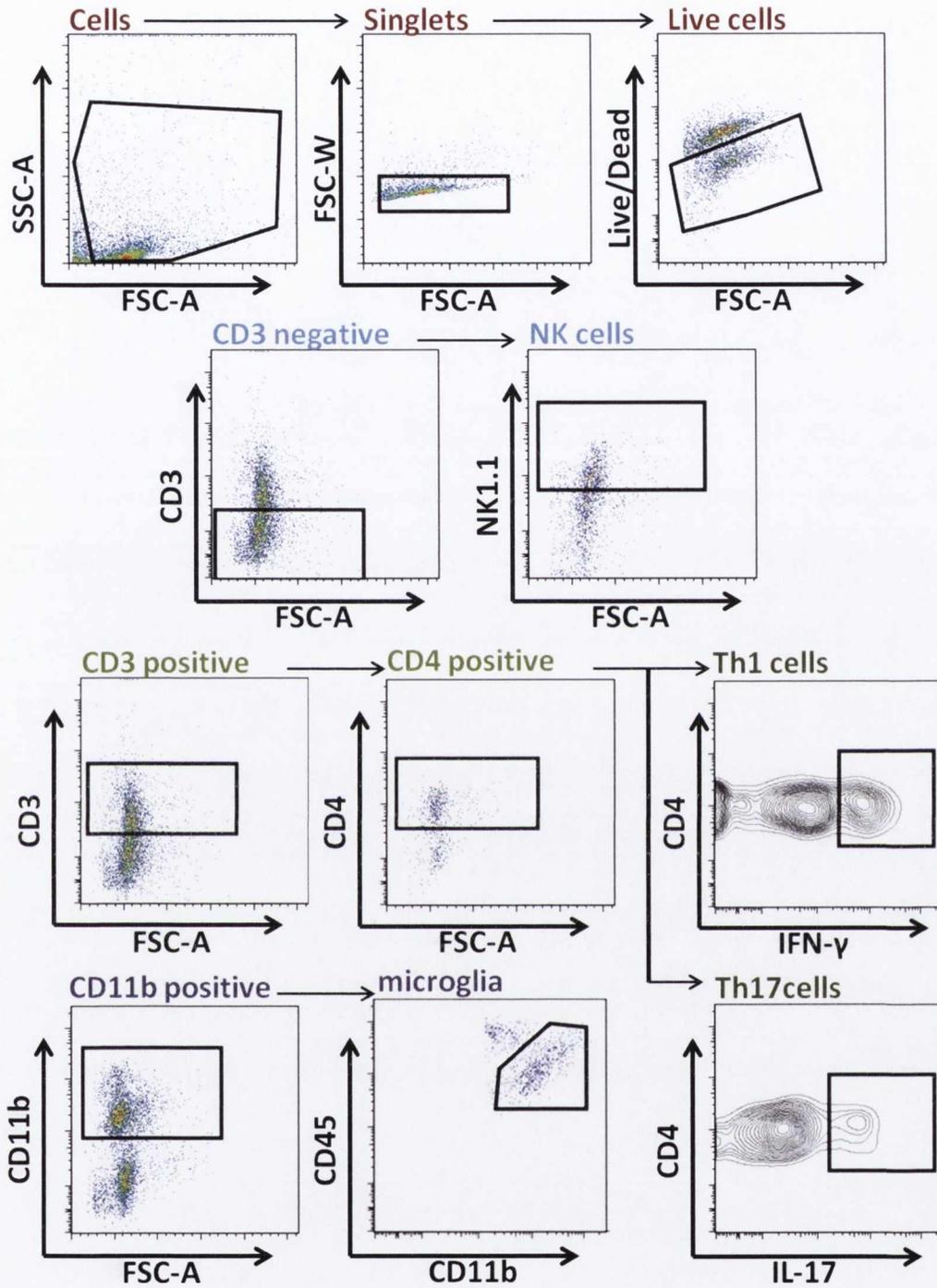


Figure 2.9 Gating strategies for flow cytometric analysis of CNS cell populations

This gating strategy allows for the hierarchical selection of singlets, live cells, NK cells, microglia, Th1 and Th17 cells.

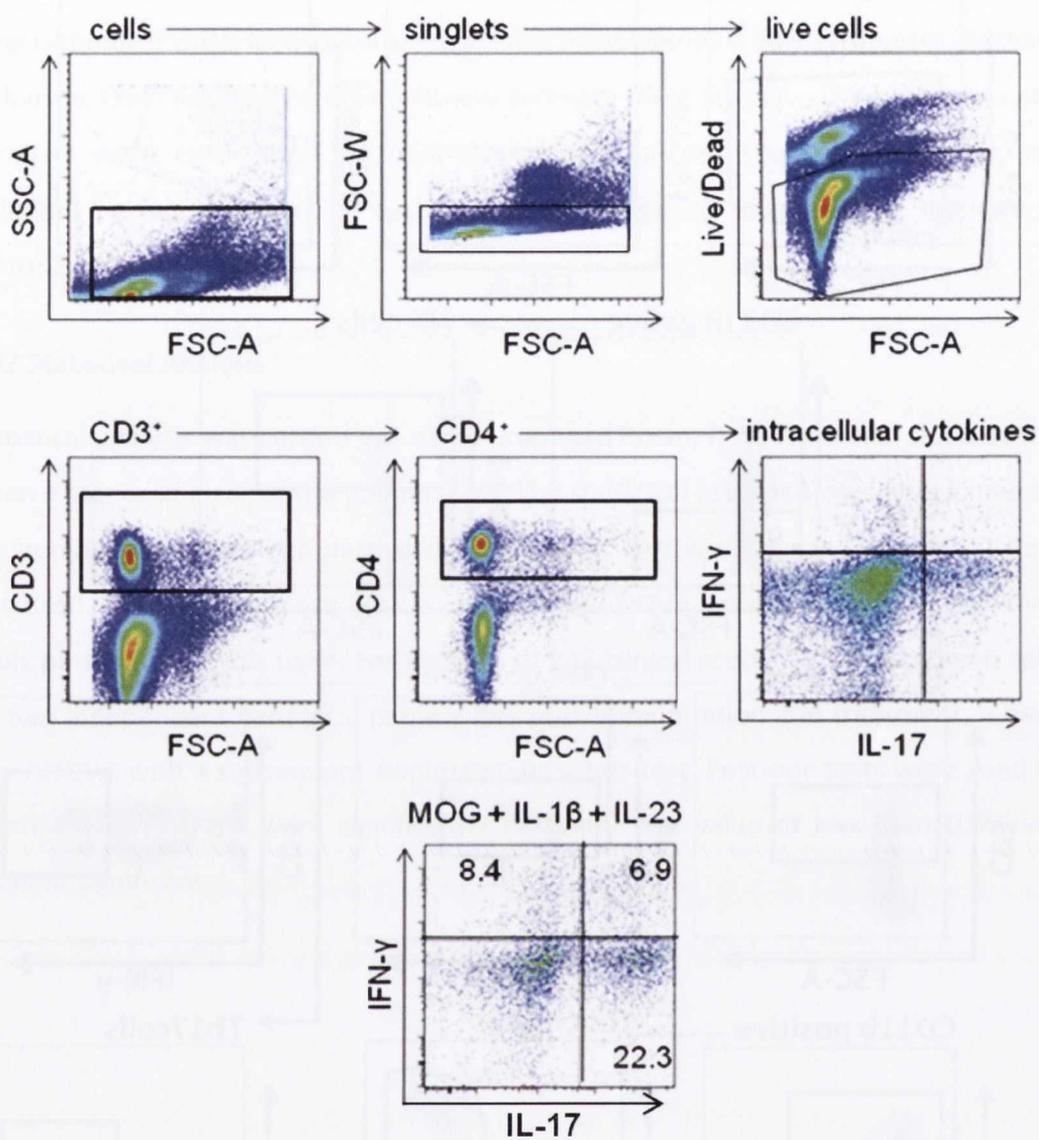


Figure 2.10 Cytokine production by CD4⁺ T cells used to induce EAE by passive transfer

After 3 d in culture, MOG-specific CD4⁺ T cells are typically positive for the pathogenic cytokines IFN-γ and IL-17 in the proportions outlined above.

FACS Antibody	Fluorescent Label	Dilution	Supplier
Live/Dead	Aqua Live/Dead	1/1000	Life Technologies
CD3	PE/APC/FITC	1/100	BD Biosciences
CD4	PE/APC/FITC	1/100	BD Biosciences
CD11b	A780/FITC	1/100	BD Biosciences
CD45	PE	1/100	BD Biosciences
IFN- γ	PE/Pe-Cy7/FITC	1/50	BD Biosciences
IL-17	PE/Pe-Cy7	1/50	BD Biosciences
MHC II	V450	1/100	eBioscience
NK1.1	PerCPCy5.5	1/100	eBioscience
TNF- α	PeCy7	1/50	BD Biosciences
CD49d	PerCPeFluor7.10	1/50	BD Biosciences

Table 2.3 FACS antibodies

**Chapter 3: Characterisation of the M1 and
M2 phenotypes and corresponding
metabolic profiles of BMDMs, microglia,
and astrocytes**

3.1 Introduction

Cells of the innate immune system, such as macrophages and microglia, can adopt phenotypically distinct activation states in response to their microenvironment. The classical or M1 activation state can be induced by exposure to IFN- γ , a cytokine primarily released by Th1 and NK cells, or to LPS, a component of Gram negative bacteria (Mosser and Edwards, 2008). This state is characterised by the upregulation of iNOS expression, enhancement of the expression of antigen presentation molecules such as MHC II (Schroder et al., 2004) and production of pro-inflammatory cytokines such as TNF- α , IL-6, IL-1 β and IL-12. The products of the M1 state enable the activated cells to engage in anti-microbial activity and mount a speedy response against insult or infection (Sica and Mantovani, 2012). In contrast, the alternative or M2 activation state is induced by exposure to the anti-inflammatory cytokines IL-4 or IL-13, which are primarily released by Th2 cells, basophils, eosinophils (Martinez and Gordon, 2014) and Type-2 innate lymphoid cells (ILC2) (Zhu, 2015). M2-polarised cells display enhanced expression of ARG1, which competes with the M1-associated enzyme iNOS for the substrate ornithine, as well as MRC1 and chitinase-like proteins such as CHI3L3 (Mantovani et al., 2007). The M2 state is associated with parasite encapsulation, tissue repair and remodelling (Mantovani et al., 2005).

Persistent M1 activation is a hallmark of a number of chronic, inflammatory, neurodegenerative diseases such as AD (Heneka et al., 2015), PD (Lee et al., 2010), ALS (Murdock et al., 2015), MS (Peferoen et al., 2015) and its animal model EAE (Miron and Franklin, 2014). On the other hand, the M2 state is associated with the resolution of inflammation. Harnessing microglial activation and orchestrating a switch from the inflammatory M1 state to the modulatory M2 state has proven effective in reducing pathology in a number of diseases, including EAE (Yu et al., 2015) and the APP/PS1 model of AD (Kawahara et al., 2012).

The majority of a cell's energy is derived from the metabolism of glucose. Glucose is first broken down to pyruvate, which can then either enter the TCA cycle to undergo oxidative phosphorylation and yield up to 36 molecules of ATP for every molecule of glucose. Alternatively, in the absence of oxygen, glucose can undergo the

comparatively speedy process of glycolysis to yield lactic acid or ethanol, resulting in the production of only 2 molecules of ATP per molecule of glucose (Vander Heiden et al., 2009). Despite this considerable reduction in ATP load, it has been demonstrated that even in the presence of oxygen, cells can undergo “aerobic glycolysis”, a phenomenon that was first described in cancer cells in the 1920s by Otto van Warburg (Warburg et al., 1924).

Aerobic glycolysis is not confined to transformed cells, however. There is also a bias toward lactate production in proliferating cells; this has been observed in mitogen-stimulated, healthy human lymphocytes (Hedekov, 1968), mouse lymphocytes (Wang et al., 1976) and rat thymocytes (Brand, 1985). It is still unclear why proliferating cells use the less efficient process of glycolysis to produce ATP. However, the argument has been made that despite the reduction in the ratio of ATP:glucose, the speed at which glycolysis occurs translates to greater ATP production overall, thus facilitating the high-energy process of proliferation (Pfeiffer et al., 2001).

Recently it has come to light that cells of the innate immune system can adopt differential metabolic profiles, depending upon their polarisation status. This has perhaps been best established in macrophages. M1 macrophages display increased cytokine production and depend upon glycolysis to quickly fulfil the considerable energy demands of microbicidal activity, another feature of the M1 state. On the other hand, M2 macrophages display low glycolytic activity and preferentially utilise oxidative metabolism, which provides the sustained energy required for the resolution of inflammation, tissue remodelling and repair (Biswas and Mantovani, 2012).

Despite the many similarities between the activation states of macrophages and microglia, little is known about how polarisation may affect microglial metabolism. In the BV2 microglial cell line, treatment with M1 polarising stimuli IFN- γ and LPS elicited an increase in glycolysis, as evidenced by increased lactate production, as well as increased activity of hexokinase, the enzyme that catalyses the first step in glycolysis (Gimeno-Bayón et al., 2014).

Astrocytes may also adopt M1 and M2-like states that have similar characteristics to those found in microglia and macrophages. In response to LPS and IFN- γ , astrocytes

can produce the archetypal M1 cytokines IL-1 β , IL-6 and TNF- α . Furthermore, IL-4 treatment can upregulate mRNA expression of the M2 markers MRC1 and CHI3L3 (Jang et al., 2013). It remains unclear whether astrocyte polarisation translates to a change in metabolic profile.

The simple M1 versus M2 paradigm (originally named for the polarisation induced by Th1 and Th2 cytokines respectively) and the means by which these states are characterised have come under increasing criticism in recent years. As research into these states intensified, a greater number of intermediate states were identified, thus blurring the boundaries between the M1 and M2 phenotypes (Heppner et al., 2015). Moving forward, the use of metabolic profiling may prove a novel method in the definition of innate cell phenotypes.

The advent of new technologies has refined the study of cellular metabolism. One of the most advantageous developments has been the introduction of the Seahorse Extracellular Flux (XF) Analyzer. This instrument measures, in real time, the excretion of metabolic products. It can simultaneously measure pH and oxygen tension in a culture. Extracellular acidification rate (ECAR) is an indirect measure of glycolysis, as lactic acid is a by-product of this process. Conversely, oxygen consumption rate (OCR) is an indicator of oxidative metabolism. This allows for the characterisation of metabolic profile in a cell population, and investigation of the impact of polarising stimuli on glycolysis and oxidative metabolism (Wu et al., 2007).

Study aims

- 1) To investigate the effect of IFN- γ and IL-4 on mRNA expression of M1 and M2 markers in macrophages, microglia and astrocytes.
- 2) To elucidate the metabolic profiles of IFN- γ and IL-4-polarised cells.

3.2 Results

3.2.1 IFN- γ and IL-4 enhance mRNA expression of M1 and M2 markers respectively in BMDMs

In order to characterise the M1/M2 mRNA expression profiles of BMDMs, cells were treated with IFN- γ or IL-4 respectively for 24 h. Treatment with IFN- γ significantly enhanced mRNA expression of the M1 markers iNOS, TNF- α and MHC II in BMDMs relative to controls and IL-4-treated cells (Figure 3.1 A-C; $p < 0.001$; ANOVA). Treatment with IL-4 significantly enhanced mRNA expression of the M2 markers MRC1, ARG1 and CHI3L3 relative to controls and IFN- γ -treated cells (Figure 3.1 D-F; $p < 0.001$; ANOVA).

3.2.2 IFN- γ and IL-4 enhance mRNA expression of M1 and M2 markers respectively in microglia

Similar to the data on BMDM polarisation, IFN- γ significantly enhanced mRNA expression of the M1 markers iNOS, TNF- α and MHC II in microglia relative to controls and IL-4-treated cells (Figure 3.2 A-C; $p < 0.01$, $p < 0.001$; ANOVA). Treatment with IL-4 significantly enhanced mRNA expression of the M2 markers MRC1, ARG1 and CHI3L3 relative to controls and IFN- γ -treated cells (Figure 3.2 D-F; $p < 0.001$; ANOVA).

3.2.3 IFN- γ and IL-4 enhance mRNA expression of M1 and M2 markers respectively in astrocytes

Having characterised the profile of M1 and M2 marker expression in BMDMs and microglia, the effect of IFN- γ and IL-4 treatment on astrocytes was also examined. Treatment with IFN- γ significantly enhanced mRNA expression of the M1 markers iNOS, TNF- α and MHC II in astrocytes relative to controls and IL-4-treated cells (Figure 3.3 A-C; $p < 0.001$; ANOVA). Treatment with IL-4 significantly enhanced mRNA expression of the M2 markers MRC1, ARG1 and CHI3L3 relative to controls and IFN- γ -treated cells (Figure 3.3 D-F; $p < 0.001$; ANOVA).

3.2.4 IFN- γ treatment significantly enhances baseline ECAR and reduces maximal respiration in BMDMS

Having verified that IFN- γ and IL-4 induced the M1 and M2 states respectively in BMDMS, the corresponding ECAR and OCR profiles in response to these cytokines were examined. IFN- γ treatment significantly enhanced baseline ECAR relative to control and IL-4-treated cells (Figure 3.4 C; $p < 0.05$; ANOVA). Neither IFN- γ nor IL-4 treatment affected basal respiration (Figure 3.4 D; $p > 0.05$; ANOVA), however maximal respiration was significantly reduced in IFN- γ -treated BMDMS relative to control and IL-4-treated cells (Figure 3.4 E; $p < 0.01$; ANOVA).

3.2.5 Microglia upregulate baseline ECAR in response to IFN- γ and both basal and maximal respiration in response to IL-4, while neither treatment affects these parameters in astrocytes

In order to investigate whether the similarities in M1 and M2 marker expression between BMDMs, microglia and astrocytes were reflected in their metabolic profiles, ECAR and OCR were also investigated in microglia and astrocytes. IFN- γ treatment enhanced baseline ECAR in microglia relative to control and IL-4-treated cells (Figure 3.5 C; $p < 0.05$; ANOVA). IL-4 treatment enhanced basal and maximal respiration in microglia relative to control and IFN- γ -treated cells (Figure 3.5 D-E; $p < 0.01$, $p < 0.001$; ANOVA). Baseline ECAR, basal respiration and maximal respiration in astrocytes were unaffected by either IFN- γ or IL-4 treatment (Figure 3.6 C-E; $p > 0.05$; ANOVA).

3.2.6 IFN- γ increases ECAR/OCR and reduces OCR/ECAR ratio in macrophages, IL-4 increases OCR/ECAR and reduces ECAR/OCR in microglia and astrocyte ratios are unaffected by either treatment

IFN- γ treatment significantly increased ECAR/OCR ratio in BMDMs relative to control and IL-4 treated cells (Figure 3.7 A; $p < 0.01$; ANOVA). IFN- γ also significantly reduced OCR/ECAR ratio in BMDMs relative to control and IL-4-treated cells (Figure 3.7 B; $p < 0.05$; ANOVA). Treatment with IL-4 significantly reduced the ECAR/OCR ratio in microglia relative to controls and IFN- γ -treated cells (Figure 3.7 C; $p < 0.001$; ANOVA). ECAR/OCR and OCR/ECAR ratios were unaltered by treatment with IFN- γ or IL-4 in

astrocytes (Figure 3.7 D-E; $p < 0.05$; ANOVA). Treatment with IL-4 also significantly increased the OCR/ECAR ratio relative to controls and IFN- γ -treated cells (Figure 3.7 D; $p < 0.001$; ANOVA).

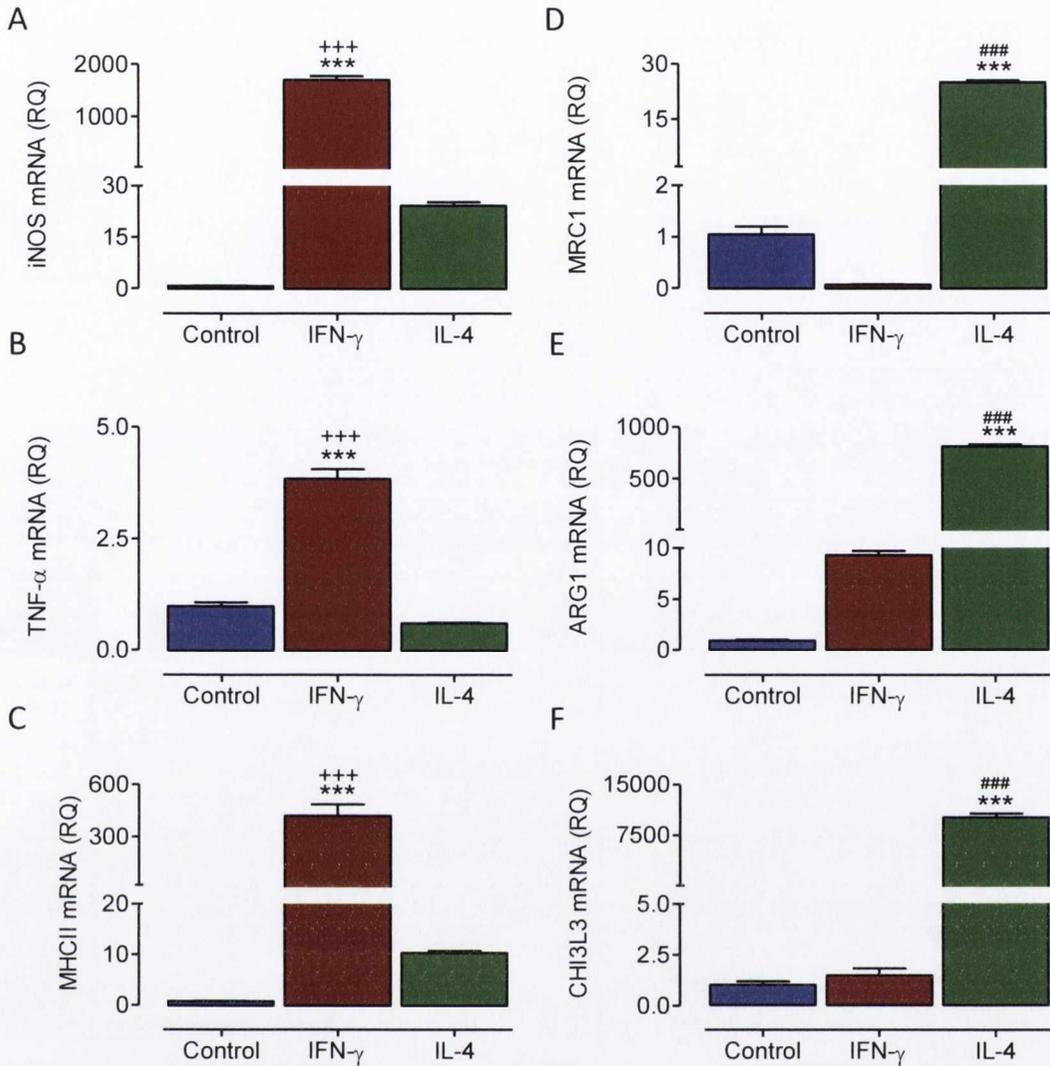


Figure 3.1 IFN- γ enhances mRNA expression of the M1 markers iNOS, TNF- α and MHC II, and IL-4 enhances mRNA expression of the M2 markers MRC1, ARG1 and CHI3L3 in macrophages

BMDMs were incubated for 24 h with medium alone, IFN- γ (50 ng/ml) or IL-4 (20 ng/ml). Cells were harvested for assessment of mRNA expression of iNOS (A), TNF- α (B), MHC II (C), MRC1 (D), ARG1 (E) and CHI3L3 (F). *** p <0.001 versus control, +++ p <0.001 versus IL-4, ### p <0.001 versus IFN- γ ; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the mean \pm SEM (n=4)

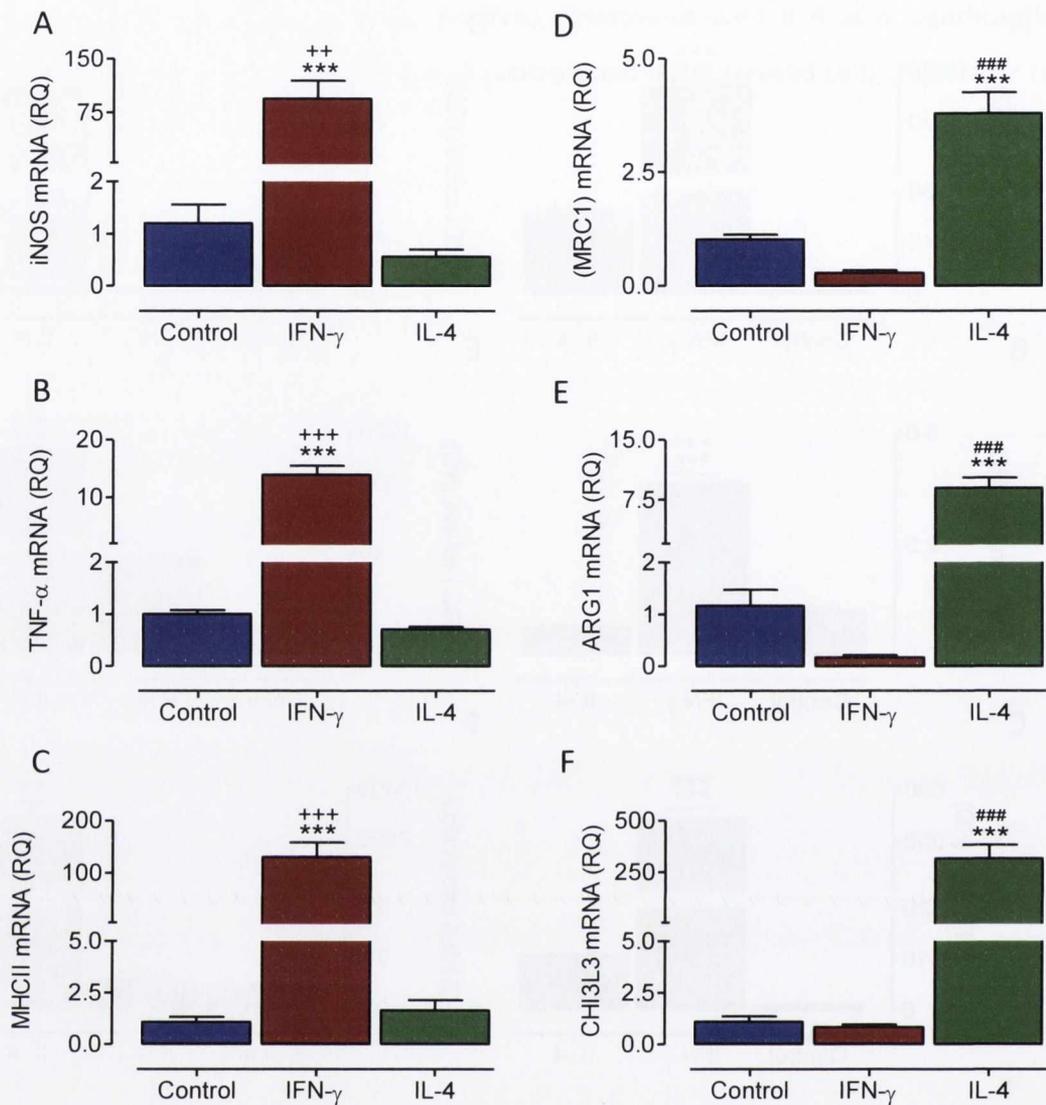


Figure 3.2 IFN- γ enhances mRNA expression of the M1 markers iNOS, TNF- α and MHC II, and IL-4 enhances mRNA expression of the M2 markers MRC1, ARG1 and CHI3L3 in microglia

Microglia were incubated for 24 h with medium alone, IFN- γ (50 ng/ml) or IL-4 (20 ng/ml). Cells were harvested for assessment of mRNA expression of iNOS (A), TNF- α (B), MHC II (C), MRC1 (D), ARG1 (E) and CHI3L3 (F) *** p <0.001 versus control, ** p <0.01, *** p <0.001 versus IL-4, ### p <0.001 versus IFN- γ ; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the mean \pm SEM (n=4)

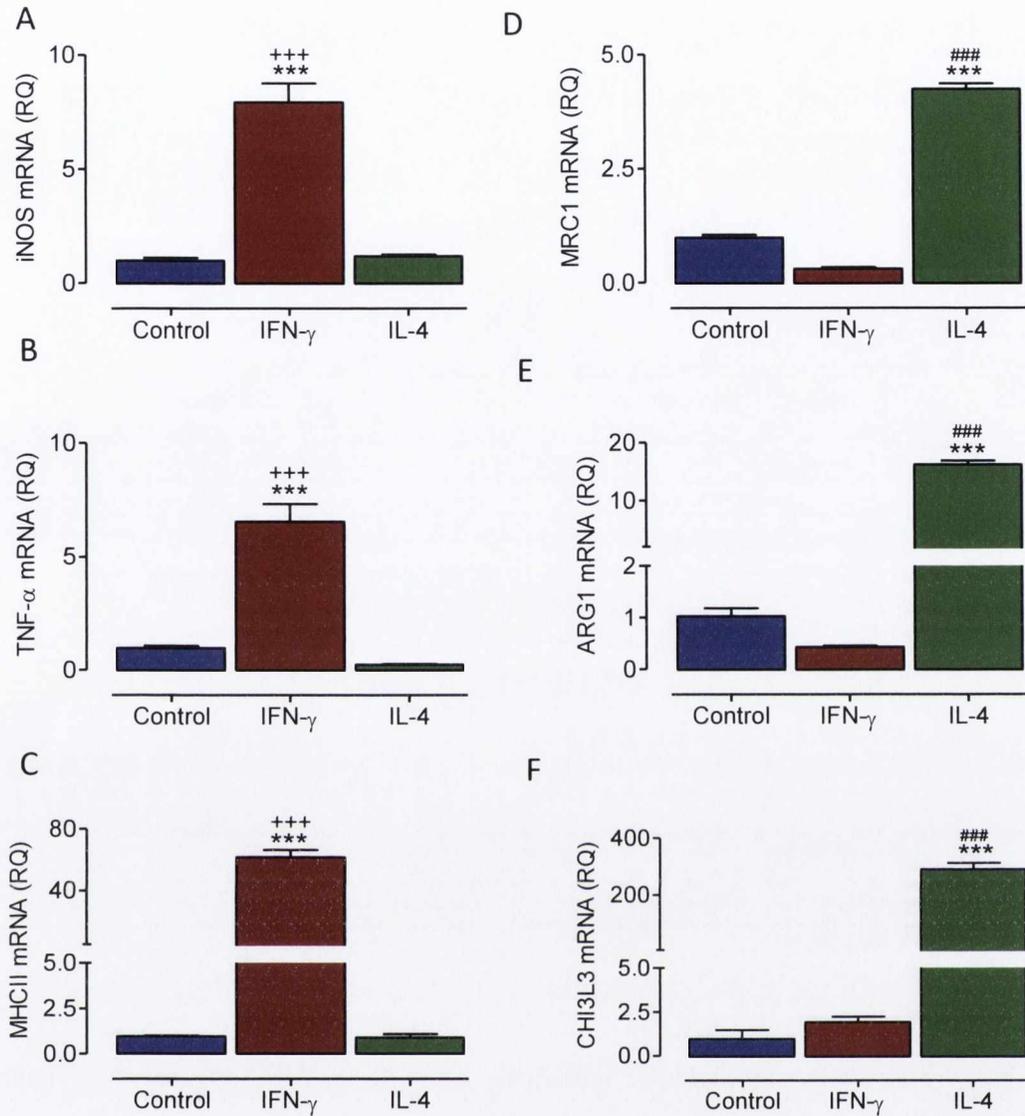


Figure 3.3 IFN- γ enhances mRNA expression of the M1 markers iNOS, TNF- α and MHC II, and IL-4 enhances mRNA expression of the M2 markers MRC1, ARG1 and CHI3L3 in astrocytes

Astrocytes were incubated for 24 h with medium alone, IFN- γ (50 ng/ml) or IL-4 (20 ng/ml). Cells were harvested for assessment of mRNA expression of iNOS (A), TNF- α (B), MHC II (C), MRC1 (D), ARG1 (E) and CHI3L3 (F) ***p < 0.001 versus control, +++p < 0.001 versus IL-4, ###p < 0.001 versus IFN- γ ; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the mean \pm SEM (n=4)

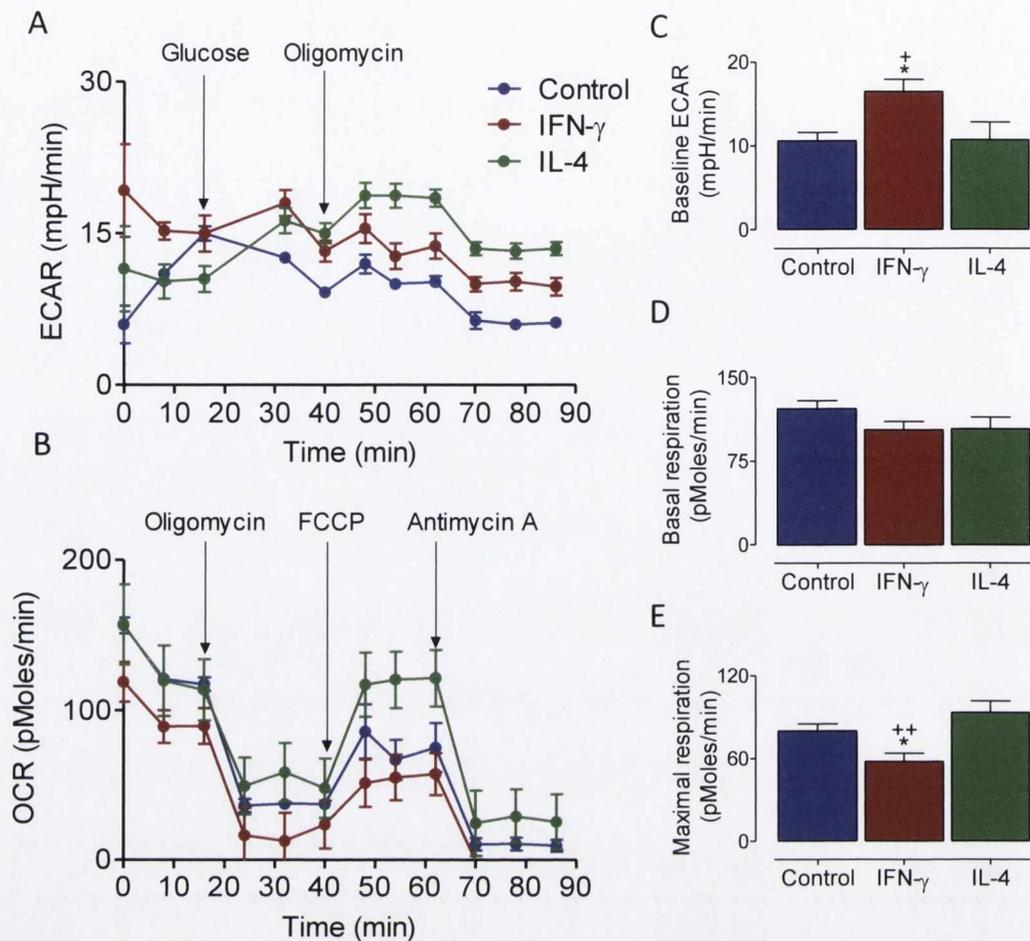


Figure 3.4 IFN- γ significantly enhances baseline ECAR and reduces maximal respiration in macrophages

BMDMs were seeded onto 24-well XF24 microplates and incubated for 24 h with medium alone, IFN- γ (50 ng/ml) or IL-4 (20 ng/ml). ECAR (A) and OCR (B) were assessed using the Seahorse XF24 Analyser. ECAR measurements were taken every 8 min, with addition of glucose (25nM) after 16 min and oligomycin (2 μ M) after 40 min. ECAR was plotted at baseline (C). OCR measurements were also taken every 8 min, with addition of oligomycin (2 μ M) after 16 min, FCCP (0.5 μ M) after 40 min and antimycin A (4 μ M) after 64 min. Basal respiration was calculated by taking the final 3 measurements from the initial 3 and obtaining the average per well (D). Maximal respiration was calculated by taking the final 3 measurements from those made after the addition of FCCP and obtaining the average per well (E). * p <0.05 versus control, ⁺ p <0.05, ⁺⁺ p <0.01 versus IL-4; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the mean \pm SEM (n=4).

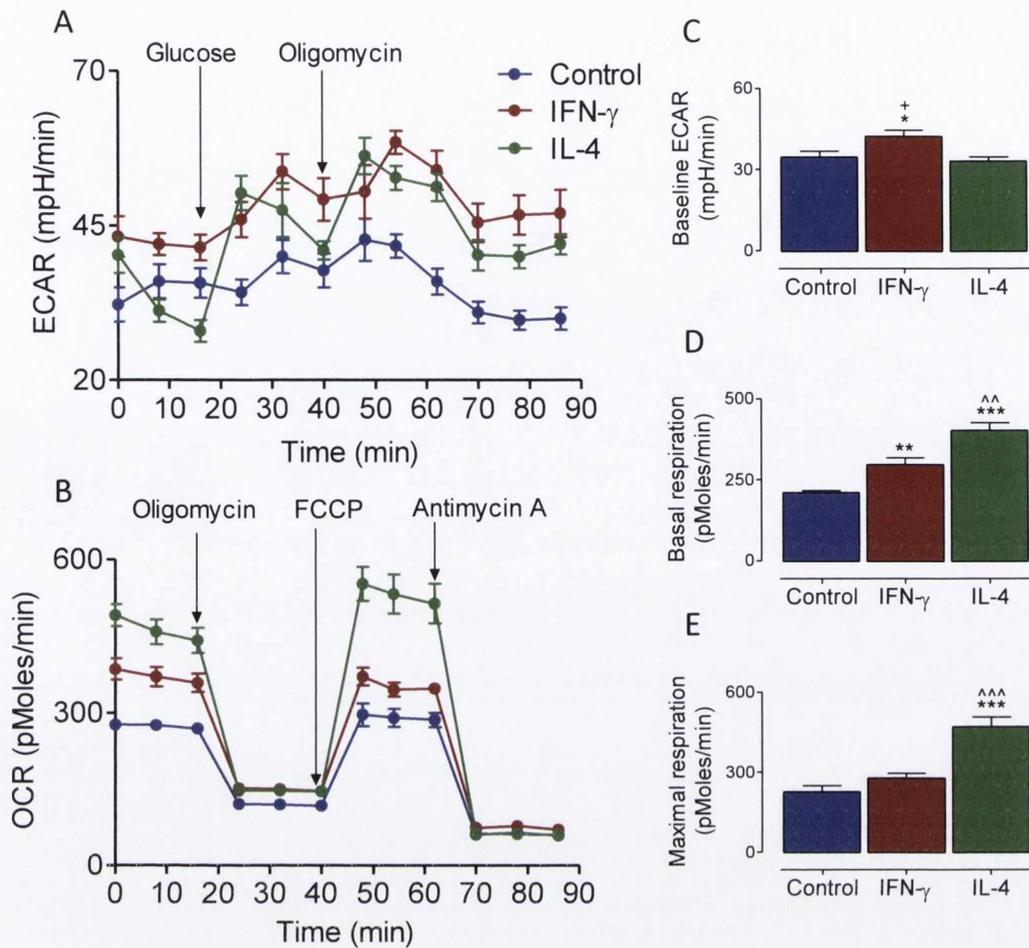


Figure 3.5 IFN- γ increases baseline ECAR and IL-4 increases maximal respiration in microglia

Microglia were seeded onto 24-well XF24 microplates and incubated for 24 h with medium alone, IFN- γ (50 ng/ml) or IL-4 (20 ng/ml). ECAR (A) and OCR (B) were assessed using the Seahorse XF24 Analyser. Microglial baseline ECAR (C), basal respiration (D) and maximal respiration (E) were calculated as per Figure 3.7. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus ECAR of control microglia, + $p < 0.05$, versus ECAR of IL-4-treated microglia, ^^ $p < 0.01$, ^^ $p < 0.001$ versus OCR of IFN- γ -treated microglia; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the mean \pm SEM (n=4).

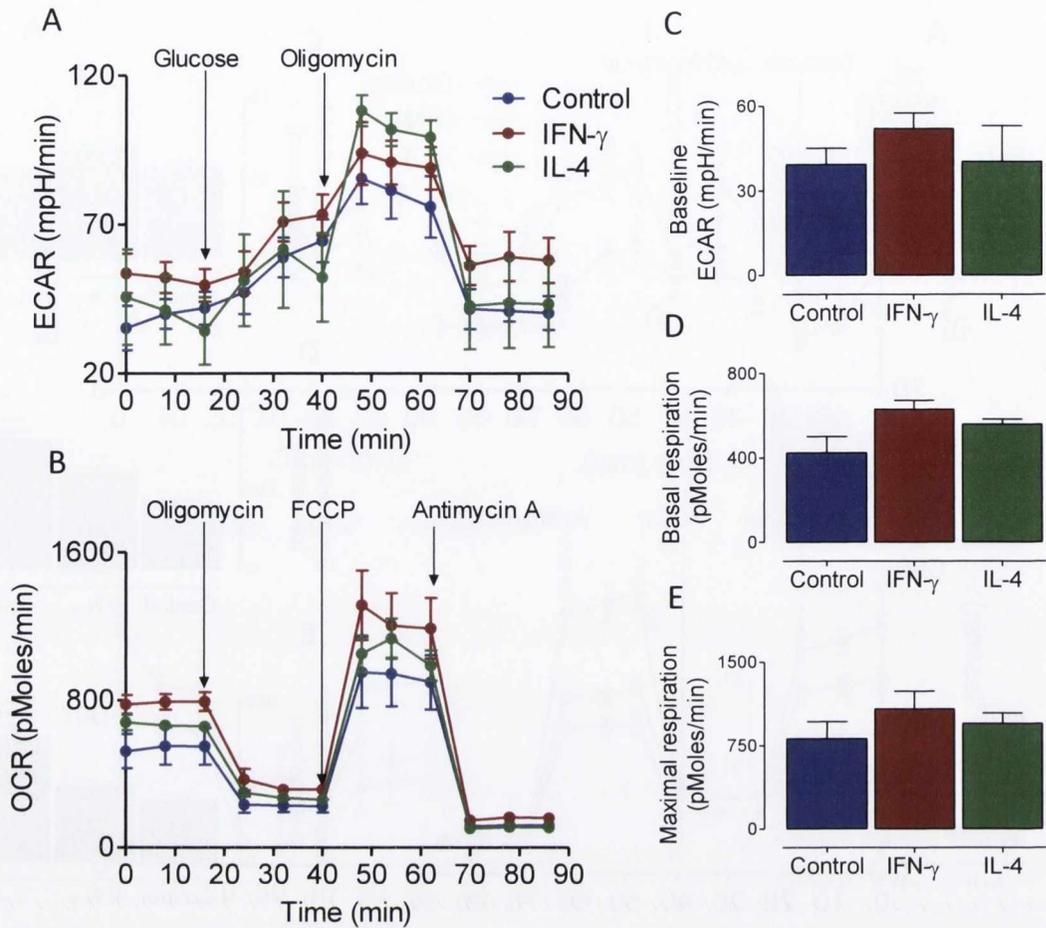


Figure 3.6 Neither IFN- γ nor IL-4 treatment affects ECAR or OCR in astrocytes

Astrocytes were seeded onto 24-well XF24 microplates and incubated for 24 h with medium alone, IFN- γ (50 ng/ml) or IL-4 (20 ng/ml). Astrocyte ECAR (A), and OCR (B) were assessed using the Seahorse XF24 Analyser. Astrocyte baseline ECAR (C), basal respiration (D) and maximal respiration (E) were calculated as per Figure 3.7. There were no significant differences between any the parameters in control, IFN- γ -treated or IL-4-treated astrocytes; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the mean \pm SEM (n=4).

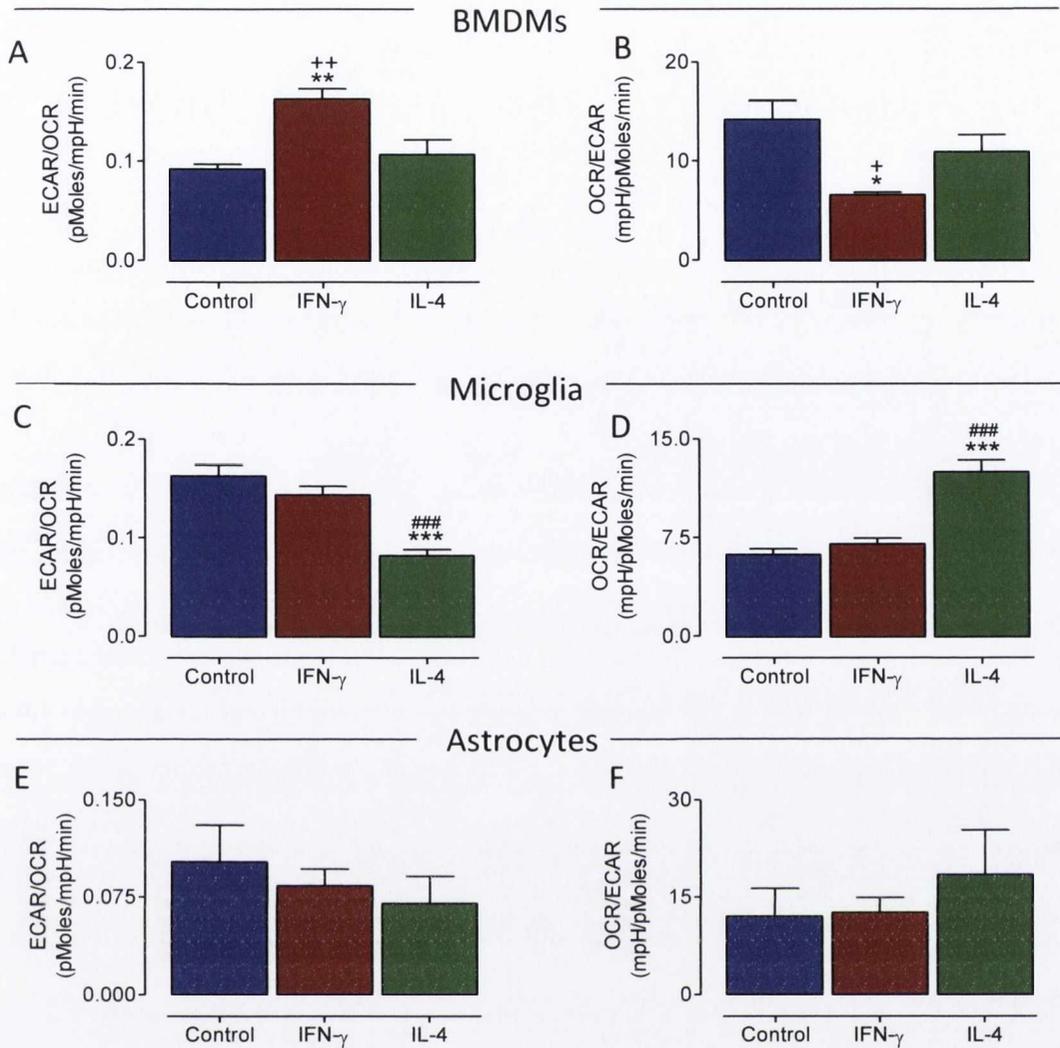


Figure 3.7 IFN- γ increases ECAR/OCR and reduces OCR/ECAR ratio in macrophages, IL-4 increases OCR/ECAR and reduces ECAR/OCR in microglia and astrocyte ratios are unaffected by either treatment

Baseline ECAR and basal OCR values for each well were used to calculate the ECAR/OCR and OCR/ECAR ratios for BMDMs (A,B), microglia (C,D) and astrocytes (E,F). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus controls, + $p < 0.05$, ++ $p < 0.01$ versus IL-4, ### $p < 0.001$ versus IFN- γ ; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the mean \pm SEM ($n=4$).

3.3 Discussion

Cells of the innate immune system display striking functional plasticity, and can be polarised to adopt distinct activation states. Recent evidence suggests that this plasticity is governed by metabolic adaptation, and that activation of innate cells may also be associated with distinct profiles in glucose metabolism. The present study has demonstrated for the first time that macrophages, microglia and astrocytes exhibit similar mRNA expression profiles in response to exposure to IFN- γ and IL-4. IFN- γ treatment enhanced expression of the M1 markers iNOS, TNF- α and MHC II, and IL-4 treatment increased expression of the M2 markers MRC1, ARG1 and CHI3L3 in all cell types. Despite these similarities, macrophages, microglia and astrocytes displayed different metabolic profiles in response to IFN- γ and IL-4. While IFN- γ enhanced ECAR and reduced OCR in macrophages, IL-4 did not affect OCR. Microglia exhibited a similar increase in ECAR with IFN- γ treatment; however IL-4 drove a further increase in OCR in these cells. Astrocytes did not display any changes in metabolic profile under these polarising conditions. Taken together these data suggest that while macrophages, microglia and astrocytes display similar patterns of M1 and M2 marker expression, each cell type exhibits a different metabolic profile in response to exposure to IFN- γ and IL-4.

It has been known for some time that macrophage and microglial phenotypes are intrinsically connected to metabolic processes; indeed the chief distinction between the M1 and M2 states is the manner in which arginine is catabolised. Exposure to the M1-polarising stimulus IFN- γ induces transcription of iNOS, which converts arginine to NO (Förstermann and Sessa, 2012). NO is involved in controlling microbial infections such as *Mycobacterium tuberculosis* (Nicholson et al., 1996) and parasitic infections caused by *Leishmania major* (Stenger et al., 1994), as well as modulating both acute and chronic inflammation (Wong and Billiar, 1994). On the other hand, IL-4 enhances transcription of ARG1, which converts arginine to the polyamine, ornithine. ARG1-expressing and ornithine-producing macrophages are required for wound healing (Albina et al., 1990).

IFN- γ -treated BMDMs exhibited increased mRNA expression of the M1 markers iNOS, TNF- α and MHC II as well as enhanced baseline ECAR, relative to control and IL-4-treated cells. This is in agreement with number of studies that have provided evidence showing that M1 macrophages upregulate glycolysis. For example, in response to IFN- γ and LPS, peritoneal macrophages have been reported to increase both lactate output and the expression of two key regulators of glycolysis, HIF-1 α and uPFK2 (Rodríguez-Prados et al., 2010). More recently, other groups have investigated the link between macrophage polarisation and ECAR. In line with the current data, an M1-related increase in ECAR has been reported both relative to M2-polarised (Huang et al., 2014) and control BMDMs (Van den Bossche et al., 2014). However, while a combination of LPS and IFN- γ was used to induce the M1 state in these papers, the present study has shown that IFN- γ alone is sufficient to drive glycolysis in macrophages.

IFN- γ -treated microglia exhibited a similar increase in mRNA expression of the archetypal M1 markers iNOS, TNF- α and MHC II to that observed in IFN- γ -treated macrophages. Interestingly, the similarities between polarised microglia and macrophages were also reflected with regard to modulation of glycolysis. Baseline ECAR was also enhanced in IFN- γ -treated microglia relative to control and IL-4-treated cells. The metabolic profile of polarised microglia is less well established, however, a very recent paper reported an LPS-induced increase the ECAR of BV2 cells relative to controls (Orihuela et al., 2015). Here, it has been shown for the first time that IFN- γ can upregulate this parameter in primary microglia. Taken together these data suggest that, despite the ontogenic distinction between these two cell types, they exhibit comparable M1 activation profiles, in terms of both marker expression and glucose metabolism.

IL-4-treated microglia exhibited enhanced expression of the M2 markers MRC1, ARG1 and CHI3L3, as well as a robust increase in both basal and maximal respiration relative to both control and IFN- γ treated cells. At the time of writing, the only other available data on OCR in primary microglia was that from the previously cited paper by Orihuela and colleagues, who reported that a combination of IL-4 and IL-13 did not enhance basal OCR in either primary microglia or BV2 cells (Orihuela et al., 2015). The discrepancy between these observations may be accounted for by differences in both

the M2-polarising stimuli (IL-4 and IL-13 versus IL-4 alone in the current study) and also the choice of mouse strain; the authors obtained microglia from CD1 mice, which have been found to exhibit greater inflammatory responses than microglia from C57BL/6 mice (Nikodemova and Watters, 2011).

In contrast to the similarities in glycolytic response between microglia and macrophages, these cell types displayed distinct OCR profiles. While IL-4 enhanced mRNA expression of MRC1, ARG1 and CHI3L3 in both BMDMs and microglia, basal respiration in BMDMs was unchanged by treatment with IL-4. Maximal respiration was however lower in IFN- γ -treated cells relative to controls and IL-4-treated cells. This is in agreement with a study by Haschemi and colleagues, who failed to detect an IL-4-induced change in OCR, but did observe an IFN- γ -related decrease in OCR in peritoneal macrophages (Haschemi et al., 2012).

The role of IFN- γ in skewing macrophages toward a glycolytic phenotype was reflected in the ECAR:OCR ratio, which was significantly higher in IFN- γ -treated BMDMs than controls or IL-4-treated cells. Consequently, the OCR:ECAR ratio was also significantly decreased in these cells. Taken together, these data suggest that in macrophages, it is not that IFN- γ and IL-4-polarised cells display distinct bioenergetic profiles, rather that IFN- γ treatment has opposing effects on metabolism, by driving glycolysis and thus inhibiting oxidative phosphorylation.

On the other hand, microglia respond to IL-4 treatment with a robust increase in maximal respiration, a parameter that is unaffected by IFN- γ treatment. This is reflected in the OCR:ECAR ratio, which is significantly higher in IL-4-treated cells than controls or IFN- γ -treated cells. Taken together, these data suggest that both glycolysis and oxidative metabolism in microglia are modulated independently by IFN- γ and IL-4 respectively.

There is increasing evidence to suggest that the M1/M2 dichotomy is not unique to macrophages and microglia; astrocytes can also respond to IFN- γ /IL-4 and upregulate some of the common markers of both activation states (Hua et al., 2002, Jang et al., 2013). Here it has been shown that, as previously demonstrated in microglia and macrophages, IFN- γ treatment can enhance mRNA expression of iNOS, TNF- α and

MHCII, and IL-4 can increase mRNA expression of MRC1, ARG1 and CHI3L3 in astrocytes.

However, these similarities in marker expression between these cell types were not reflected in the bioenergetic status of polarised astrocytes; neither IFN- γ nor IL-4 treatment had any effect on either the ECAR or OCR profile in these cells. This may reflect a fundamental difference between the mechanisms of glucose utilisation between astrocytes and microglia/macrophages. In the brain, glucose metabolism is chiefly under the control of astrocytes, which are constitutively highly glycolytic (Bittner et al., 2010). It has been known for over twenty years that glutamate is a key mediator of glycolysis and lactate production in astrocytes, and that this astrocyte-derived lactate is integral to neuronal energy metabolism (Pellerin and Magistretti, 1994). The literature surrounding the effect of cytokine exposure on astrocyte metabolism is contradictory; one study reported that while IFN- γ and IL-4 treatment significantly increased and decreased glucose uptake respectively in astrocytes, neither treatment had any effect on lactate release (Bélanger et al., 2011). In contrast, it has also been shown that treatment of primary murine astrocytes with LPS and IFN- γ (Pellerin and Magistretti, 1994) as well as IFN- γ alone (Garg et al., 2009), can enhance lactate production in astrocytes; the latter study also demonstrated that IL-4 could also elicit this increase. Prior to the current study, the role of IFN- γ or IL-4 on the modulation of astrocyte oxygen consumption has not been addressed. However, it has been shown that at a high concentration (1 $\mu\text{g}/\text{ml}$) a combination of LPS and IFN- γ can significantly reduce astrocyte OCR relative to controls after 8h but not 24 h of treatment (Ribeiro et al., 2015). It has also been demonstrated that astrocytic OCR increases with age (Jiang and Cadenas, 2014).

It is difficult to draw a conclusion on the role of IFN- γ and IL-4 on modulating astrocytic metabolism. It seems that control of glycolysis is not differentially regulated by M1 or M2 cytokines. This is not to say that astrocytic glycolysis is not modulated in some way by cytokine tone in the brain, merely that astrocytic polarisation to the M1 or M2 phenotype is not associated with a distinct metabolic profile, as demonstrated in microglia and macrophages.

Taken together these data suggest that while microglia and macrophages share similar glycolytic signatures in response to IFN- γ , oxidative phosphorylation is differentially modulated in these cells, with IL-4 inducing a boost in microglial OCR relative to controls, an effect that is absent in M2 macrophages. Instead, macrophage glycolysis and oxidative metabolism are differentially regulated by IFN- γ alone. Furthermore, the bioenergetic profile of astrocytes was entirely unaffected by either manipulation. This suggests that these cell types undergo distinct fundamental changes in metabolism in response to M1 and M2-polarising stimuli, despite displaying similar profiles of marker expression. The effects of IFN- γ and IL-4 on the polarisation and metabolic profiles of macrophages, microglia and astrocytes are summarised in **Figure 3.8**.

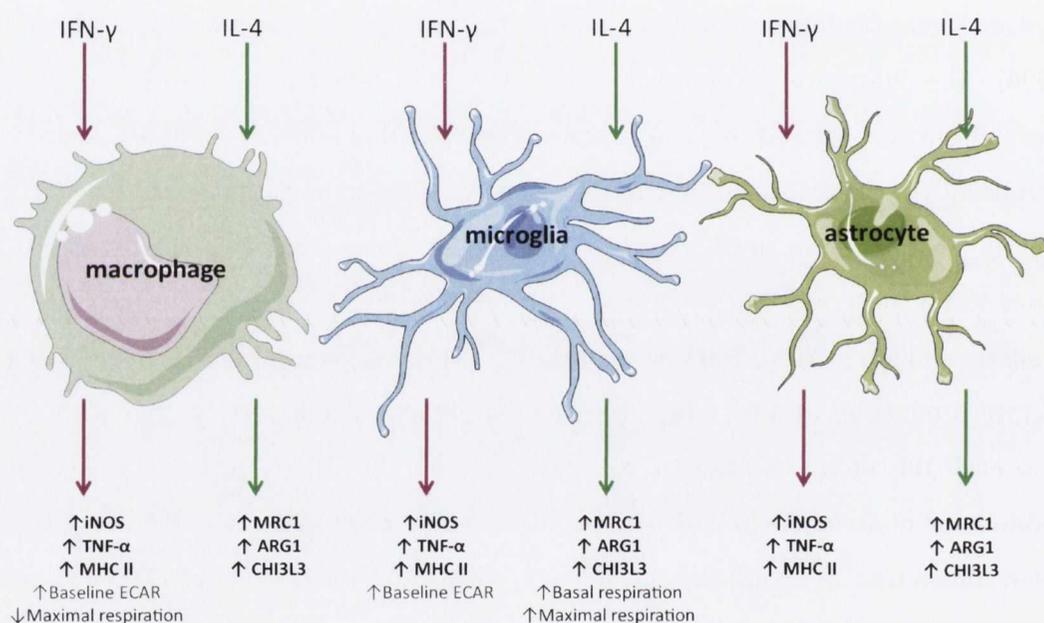


Figure 3.8 The effect of treatment with IFN- γ or IL-4 on the polarisation and metabolic profiles of macrophages, microglia and astrocytes.

IFN- γ significantly enhanced mRNA expression of the M1 markers iNOS, TNF- α and MHC II, and IL-4 significantly upregulated mRNA expression of the M2 markers MRC1, ARG1 and CHI3L3 in macrophages, microglia and astrocytes. IFN- γ increased baseline ECAR and reduced maximal respiration in macrophages, however IL-4 had no effect on OCR. While IFN- γ also enhanced baseline ECAR in microglia, it did not affect OCR. In contrast, IL-4 increased both basal and maximal respiration in microglia. Neither ECAR nor OCR in astrocytes were altered by either treatment.

Chapter 4: NK cells infiltrate the CNS prior to disease onset in EAE and provide an early source of innate IFN- γ

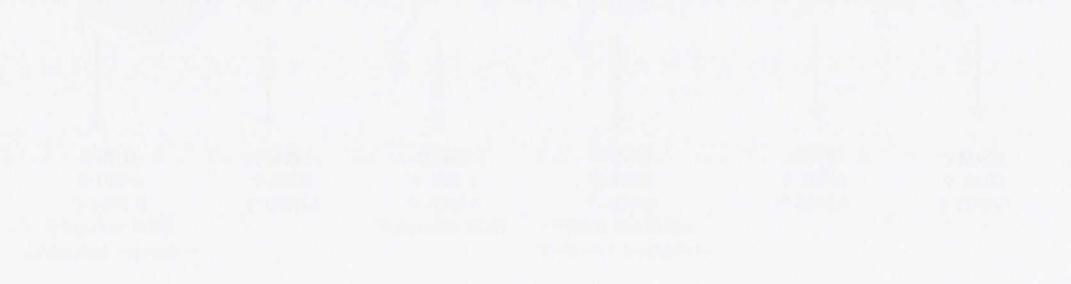
The first step in the process of identifying a source of contamination is to determine the type of contamination. This can be done by looking at the physical characteristics of the contaminant, such as its color, odor, and taste. Once the type of contamination has been identified, the next step is to determine the source. This can be done by looking at the location of the contamination and the activities that are taking place in the area.

Once the source of contamination has been identified, the next step is to determine the extent of the contamination. This can be done by looking at the distribution of the contaminant in the environment. Once the extent of the contamination has been determined, the next step is to determine the cause of the contamination. This can be done by looking at the activities that are taking place in the area and the potential sources of contamination.

Once the cause of the contamination has been identified, the next step is to determine the appropriate remediation measures. This can be done by looking at the type of contamination and the extent of the contamination. Once the appropriate remediation measures have been determined, the next step is to implement the measures.

Chapter 4: How cells indicate the CNS prior to disease onset in AD and prionosis

Early sources of markers if AD



The diagram illustrates the early sources of markers for Alzheimer's Disease (AD). It is organized into five main categories: Cognitive decline, Behavioral changes, Physical changes, Biochemical changes, and Genetic changes. Each category lists specific markers and associated symptoms or findings.

Cognitive decline: Memory loss, Disorientation, Impaired judgment.

Behavioral changes: Apathy, Irritability, Agitation.

Physical changes: Weight loss, Depression, Anxiety.

Biochemical changes: Abnormal CSF levels, Abnormal blood levels.

Genetic changes: APOE4, APP, PSEN1.

4.1 Introduction

NK cells are innate lymphocytes named for their cytolytic activity, which can control tumour growth and microbial infection. NK cells can produce the pro-inflammatory cytokines IFN- γ and TNF- α , as well as the immunosuppressive cytokine IL-10 and the growth factor GM-CSF in response to IL-12, IL-15 or IL-18 (Vivier et al., 2011). NK cells can mobilise effector cytokines, in particular IFN- γ , rapidly after immunisation or viral, bacterial and parasitic infection, which is thought to be due to constitutive cytokine mRNA expression (Stetson et al., 2003).

NK cells possess a suite of inhibitory and activating receptors and integration of signals from these receptors governs NK cell effector function. NK cell inhibitory receptors recognise MHC I molecules, and these interactions are vital in the orchestration of self-tolerance. For example, the inhibitory NKG2A/CD94 receptor, which is conserved in rodents and primates, recognises HLA-E in humans and the murine homologue QA1^b. Loss of MHC I molecule expression occurs in tumour cells, virally-infected and autoreactive cells. Disturbance of this interaction causes NK cell activation, the release of cytotoxic perforin and granzymes, and death of the target cell (Anfossi et al., 2006).

Human NK cells differ from murine NK cells in that their function is determined by differential expression of CD56 and CD16. CD56^{dim} NK cells account for almost 90% of circulating peripheral NK cells in humans and express high levels of CD16, inhibitory killer immunoglobulin-like receptors (KIRs), and perforin. By virtue of these characteristics, CD56^{dim} are more cytotoxic than CD56^{bright} cells. These cells, in contrast, express low levels of CD16, KIRs, and perforin, with higher expression levels of a number of cytokine receptors and CD94/NKG2A. As a result CD56^{bright} cells are less efficient killers, but are more effective cytokine producers, and assume a more immunoregulatory role (Béziat et al., 2011).

A deficit in NK cell function or NK cell number in the peripheral blood of patients is common in many human autoimmune disorders, such as MS, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren's syndrome, and type I diabetes mellitus (T1DM) (Fogel et al., 2013). The reduction in circulating NK cell number has been conclusively associated with relapse in MS in particular (Kastrukoff et al., 2003).

Further weight to the theory that NK cells are protective in this disease has been provided by the observation that immunoregulatory CD56^{bright} NK cell subtype are expanded in the peripheral blood of patients that have received the disease-modifying drugs IFN- β (Saraste et al., 2007), daclizumab (Bielekova et al., 2006) and natalizumab (Putzki et al., 2010).

The greater flexibility to investigate the potential role of NK cells in EAE, an animal model of autoimmunity, has generated both considerably more data and more controversy. A common approach has been to deplete NK cells using anti-NK1.1 or anti-asialo GM1 antibodies, which induce apoptosis (Asea and Stein-Streilein, 1998) and complement-dependent lysis (Shimada and Iwata, 1987) of NK cells respectively. Interestingly, NK cell depletion has generated conflicting reports of both exacerbation (Zhang et al., 1997a, Hao et al., 2010a, Xu et al., 2005, Matsumoto et al., 1998a) and amelioration (Winkler-Pickett et al., 2008, Shi et al., 2000) of clinical signs of EAE. These discrepancies may reflect differences in the antibodies used, inconsistencies in depletion regimen, and a focus on the peak of disease.

While much of the focus has been on the role of NK cells in modulating the adaptive immune response in the periphery, there is evidence that NK cells can also home to the CNS in EAE (Gan et al., 2012). The neuronal chemokine fractalkine (CX3CL1) is thought to be responsible for recruitment of NK cells to the inflamed CNS (Gan et al., 2012). What is yet to be conclusively determined is the timing of NK cell infiltration into the CNS, and the contribution of these cells in modulating neuroinflammation.

Study aims

- 1) To investigate the kinetics of NK cell infiltration into the CNS of mice with EAE
- 2) To examine IFN- γ production by NK cells in the CNS of mice with in EAE
- 3) To determine the effect of NK cell depletion at different time points on the severity of EAE

4.2 Results

4.2.1 CD3⁻NK1.1⁺ cells infiltrate the brain and spinal cord and produce significant quantities of IFN- γ prior to the onset of clinical signs of EAE

In order to determine the kinetics of NK cell infiltration into the CNS during EAE, FACS analysis was used to identify the CD3⁻NK1.1⁺ population among mononuclear cells from the brains and spinal cords of mice with active EAE (Figure 4.1 A and Figure 4.2 A). The frequency and absolute number of CD3⁻NK1.1⁺ cells in the brains of mice was higher at day 7 post induction than days 0, 3, 11 or 17 (Figure 4.1 B, C; $p < 0.001$; ANOVA). The frequency of CD3⁻NK1.1⁺ cells in the spinal cords of mice was also significantly higher at day 7 than days 0, 3, 11 or 17 (Figure 4.2 B; $p < 0.001$; ANOVA). The absolute number of CD3⁻NK1.1⁺ cells in the spinal cords of mice was higher at day 17 than days 0, 3 or 11 (Figure 4.2 C; $p < 0.05$; ANOVA).

The frequency of IFN- γ -positive NK cells (CD3⁻NK1.1⁺IFN- γ ⁺) was significantly higher in the brains of mice on days 7, 11 and 17 than at days 0 or 3 (Figure 4.3 B; $p < 0.05$, $p < 0.01$; ANOVA). The absolute number of IFN- γ -producing NK cells was significantly higher in the brains of mice on day 7 than days 0, 3, 11, or 17 (Figure 4.3 C; $p < 0.001$; ANOVA). These data indicate that NK cells infiltrate the CNS before the onset of disease and provide an early source of IFN- γ .

4.2.2 CD3⁺CD4⁺IFN- γ ⁺ and CD3⁺CD4⁺IL-17⁺ cells infiltrate the brain at the peak of disease

Infiltration of Th1 (CD3⁺CD4⁺IFN- γ ⁺) cells and Th17 (CD3⁺CD4⁺IL-17⁺) cells during the development of EAE was analysed in the brain using FACS (Figure 4.4 A and Figure 4.5 A). The frequency and absolute number of CD3⁺CD4⁺IFN- γ ⁺ cells and CD3⁺CD4⁺IL-17⁺ cells were significantly higher in the murine brains at day 17 than days 0, 3, 7 or 11 (Figure 4.4 and Figure 4.5 B, C; $p < 0.001$; ANOVA). Taken together, these data indicate that CD4⁺ T cells infiltrate the brain later in EAE than NK cells.

4.2.3 Neither continuous nor late depletion of NK cells delays the onset of EAE

In order to elucidate the role of NK cells in the development of EAE, anti-asialo GM1 antibody was used, which depletes NK cells through complement-mediated lysis.

Continuous NK cell depletion delayed the onset of EAE by 3 d with a corresponding increase in weights relative to control mice though this did not reach statistical significance (Figure 4.6 A, B; $p < 0.05$; ANOVA). Late depletion of NK cells did not affect the clinical course of EAE (Figure 4.6 E, F; $p < 0.05$; ANOVA).

4.2.4 Early NK cell depletion reduces the severity of EAE, and is associated with a reduction in the number of CD4⁺ T cells and microglia in the brain

As NK cells were found to infiltrate the CNS early in EAE, the effect of NK cell depletion at this time was examined. The clinical scores of mice that were depleted of NK cells at the time of EAE induction were significantly lower than control mice on days 10-14 (Figure 4.6 C; $p < 0.05$, $p < 0.01$, $p < 0.001$; ANOVA), and their percentage weight change was significantly higher than control mice on day 11 (Figure 4.6 D; $p < 0.05$; ANOVA).

FACS analysis was used to compare the number of CD4⁺ T cells and microglia in the brains of control and NK-depleted mice with EAE. The absolute numbers of CD3⁺CD4⁺IFN- γ ⁺ cells, CD3⁺CD4⁺IL-17⁺ cells and microglia (CD11b⁺CD45^{low}) were significantly lower in the brains of mice that were depleted of NK cells at the time of EAE induction relative to control mice (Figure 4.7 B, D, F; $p < 0.05$, $p < 0.01$; t-test). These data provide indirect evidence of a role for NK cells in promoting CD4⁺ T cell infiltration and microglial proliferation in the brains of mice with EAE.

4.2.5 The onset of active EAE is delayed, with enhanced severity, in IFN- γ ^{-/-} mice

Having demonstrated that IFN- γ -secreting NK cells infiltrate the CNS early in EAE and that depletion of NK cells at this time reduces disease severity, the role of IFN- γ in EAE was examined using transgenic mice. The onset of EAE was delayed in IFN- γ ^{-/-} mice; the mean day of onset in these mice was day 10 post induction versus day 7 in the control mice, however this was not statistically significant (Figure 4.8 A; $p < 0.05$; ANOVA). The percentage weight change was lower in the IFN- γ ^{-/-} mice relative to control mice between days 21 and 25 (Figure 4.8 B; $p < 0.001$; ANOVA). These data suggest that IFN- γ may be pathogenic in the induction of EAE, but protective at the effector stage of disease.

4.2.6 Early NK cell depletion does not affect the clinical course of actively induced EAE in IFN- γ ^{-/-} mice

As NK cells were shown to produce IFN- γ early in EAE, the hypothesis that IFN- γ is responsible for NK-mediated pathogenicity at this time was tested through depletion of NK cells from IFN- γ ^{-/-} mice with EAE at the induction of disease. Disease severity was compared with untreated IFN- γ ^{-/-} mice. Early NK cell depletion did not alter the clinical scores or percentage weight changes in IFN- γ ^{-/-} mice (Figure 4.8 C, D; $p > 0.05$; ANOVA). This suggests that NK cell pathogenicity at the induction of disease is mediated, at least in part, by IFN- γ .

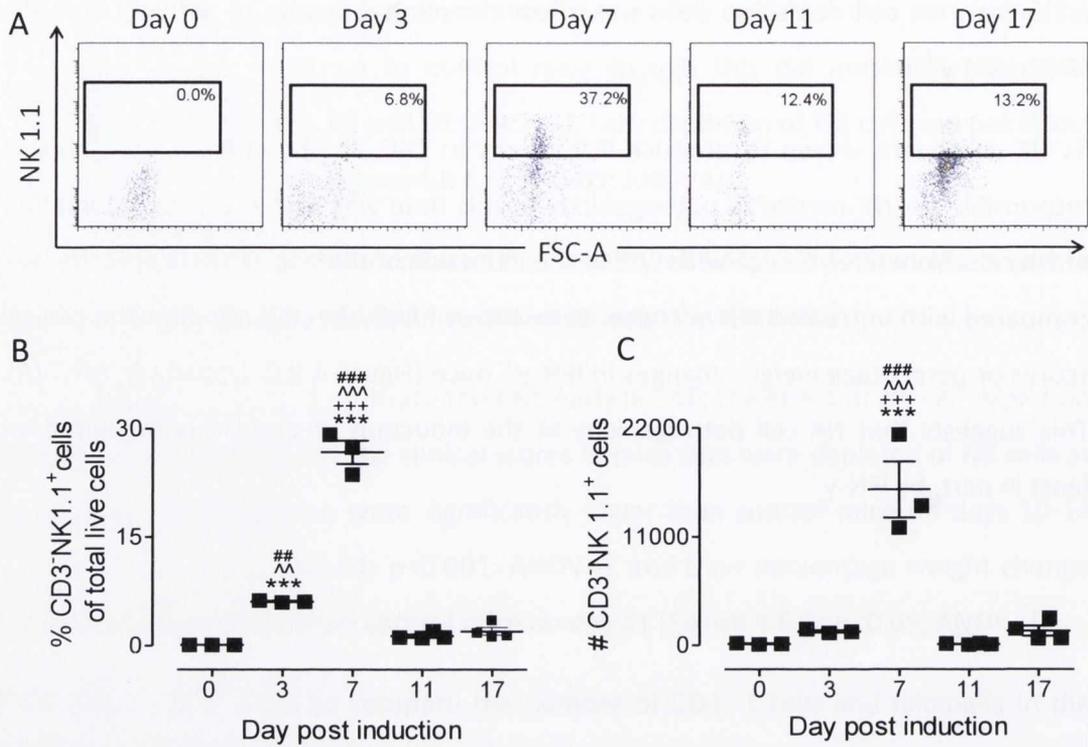


Figure 4.1 CD3⁻NK1.1⁺ cells infiltrate the brain prior to the onset of clinical signs of EAE

EAE was induced in C57BL/6 mice by immunization with MOG (100 µg/mouse) emulsified in CFA. Groups of 4 mice were sacrificed on days 0, 3, 7, 11 and 17. Mononuclear cells were isolated from the brains. Results are presented as representative FACS plots (A), mean frequency (B) or absolute numbers (C) of CD3⁻NK1.1⁺ cells in the brain. ***p<0.001 versus day 0, +++p<0.001 versus day 3, ^^p<0.01, ^^p<0.001 versus day 11, ##p<0.01, ###p<0.001 day 17; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the means ± SEM (n=4).

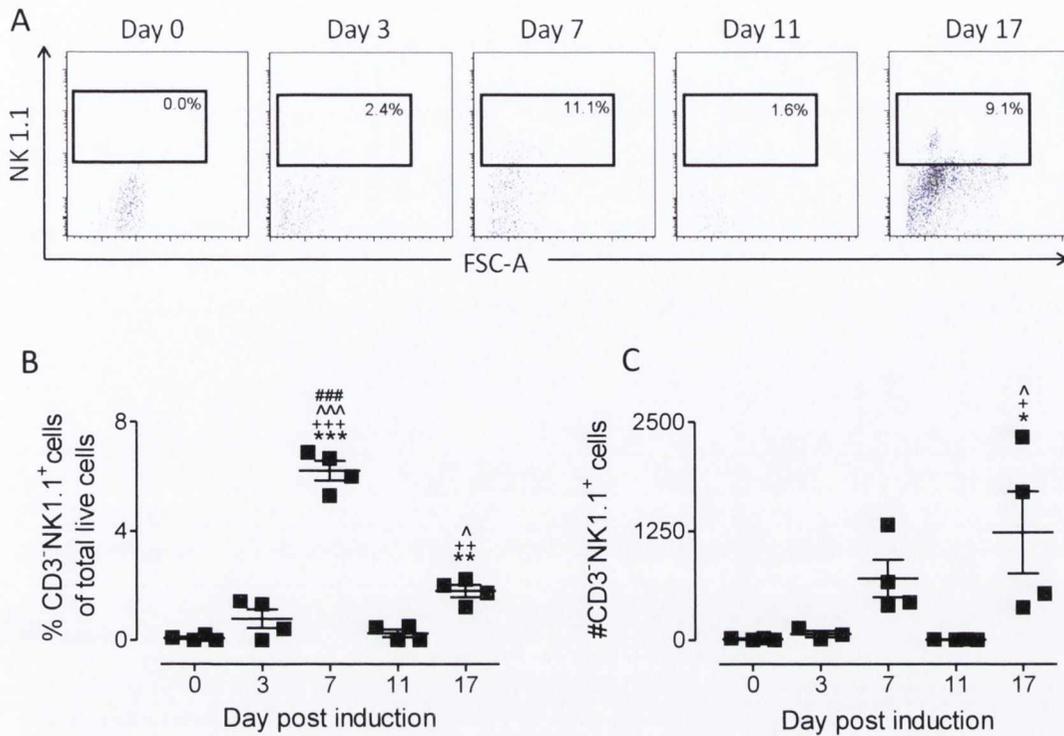


Figure 4.2 CD3⁻NK1.1⁺ cells infiltrate the spinal cord prior to the onset of clinical signs of EAE

EAE was induced in C57BL/6 mice by immunization with MOG (100 µg/mouse) emulsified in CFA. Groups of 4 mice were sacrificed on days 0, 3, 7, 11 and 17. Mononuclear cells were isolated from the spinal cords. Results are presented as representative FACS plots (A), mean frequency (B) or absolute numbers (C) of CD3⁻NK1.1⁺ cells in the spinal cord. *p<0.05, **p<0.01, ***p<0.001 versus day 0, ⁺p<0.05, ⁺⁺p<0.01, ⁺⁺⁺p<0.001 versus day 3, [^]p<0.05, ^{^^}p<0.001 versus day 11, ^{###}p<0.001 versus day 17; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the means ± SEM (n=4)

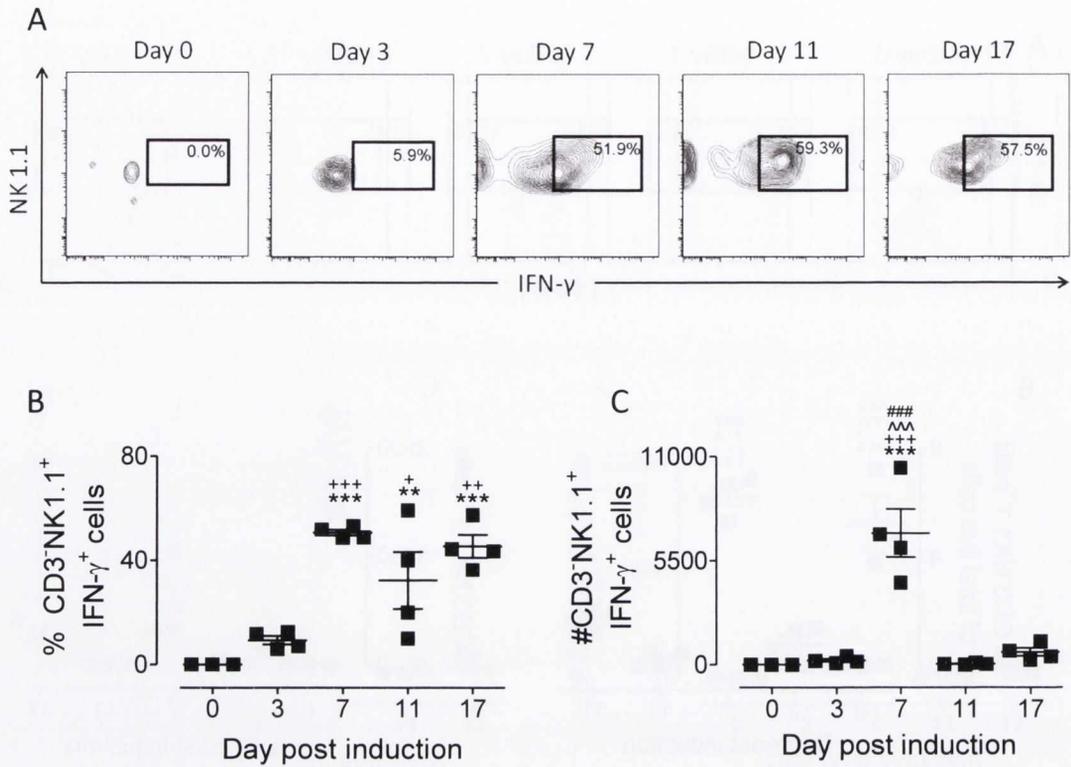


Figure 4.3 The number of CD3⁺NK1.1⁺IFN- γ ⁺ cell increases in the brain early in EAE

EAE was induced in C57BL/6 mice by immunization with MOG (100 μ g/mouse) emulsified in CFA. Groups of 4 mice were sacrificed on days 0, 3, 7, 11 and 17. Mononuclear cells were isolated from the brains and stimulated with PMA (10 ng/ml), ionomycin (1 μ g/ml) and brefeldin A (5 μ g/ml) for 5 h. Results are presented as representative FACS plots (A), mean frequency (B) or absolute numbers (C) of CD3⁺NK1.1⁺IFN- γ ⁺ cells in the brain. **p<0.01, ***p<0.001 versus day 0, +p<0.05, ++p<0.01, +++p<0.001 versus day 3, ^^p<0.01 versus day 11, ###p<0.001 versus day 17; one-way ANOVA with Newman-Keuls post hoc test. Data are presented as the means \pm SEM (n=4).

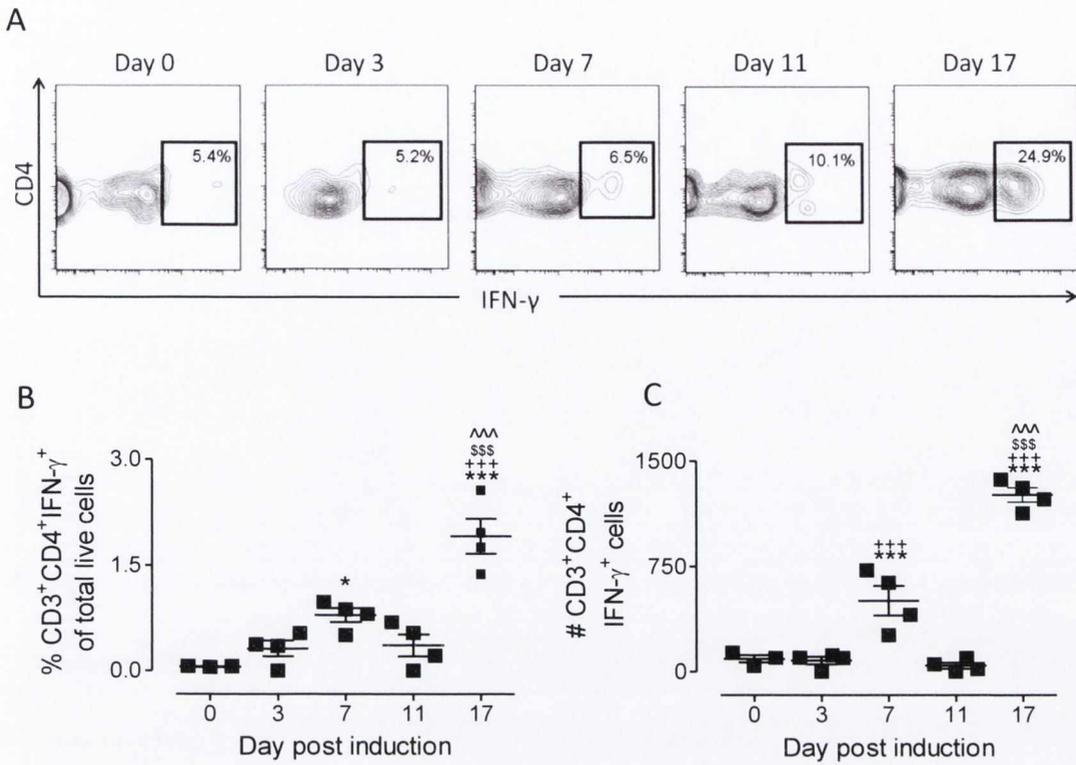


Figure 4.4 CD3⁺CD4⁺IFN- γ ⁺ cells infiltrate the brain at the peak of disease

EAE was induced in C57BL/6 mice by immunization with MOG (100 μ g/mouse) emulsified in CFA. Groups of 4 mice were sacrificed on days 0, 3, 7, 11 and 17. Mononuclear cells were isolated from the brains and stimulated with PMA (10 ng/ml), ionomycin (1 μ g/ml) and brefeldin A (5 μ g/ml) for 5 h. Results are presented as representative FACS plots (A), mean frequency (B) or absolute numbers (C) of CD3⁺CD4⁺IFN- γ ⁺ cells in the brain. * p <0.05, *** p <0.001 versus day 0, +++ p <0.001 versus day 3, \$\$\$ p <0.001 versus day 7, ^^ p <0.001 versus day 11; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the means \pm SEM (n=4).

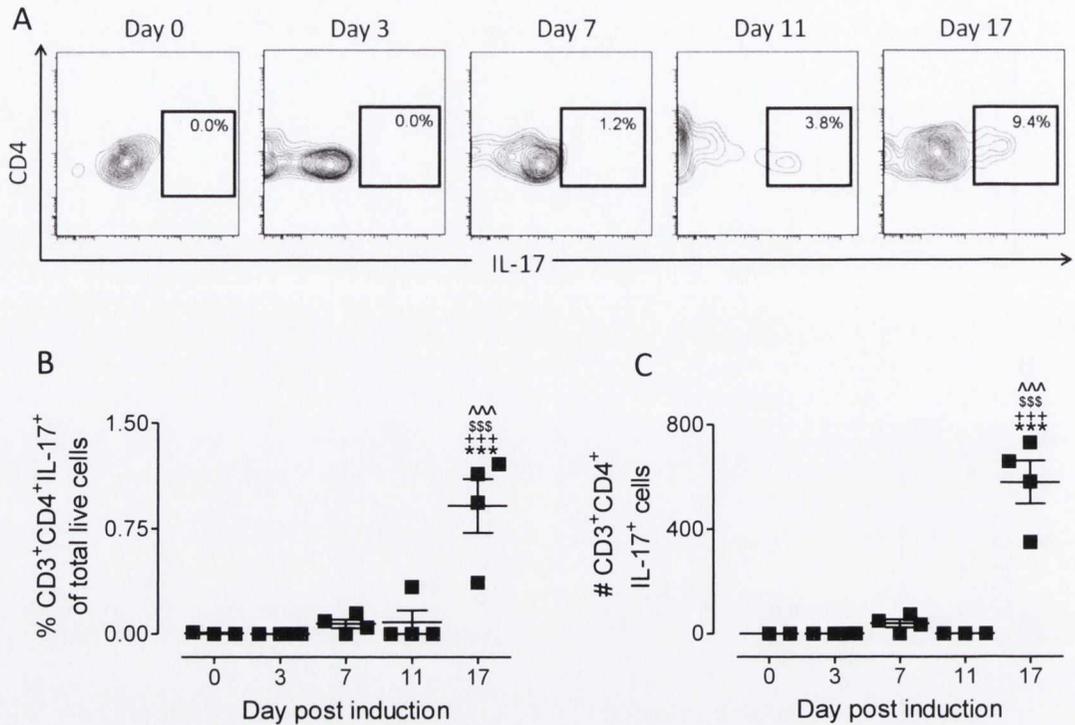


Figure 4.5 CD3⁺CD4⁺IL-17⁺ cells infiltrate the brain at the peak of disease

EAE was induced in C57BL/6 mice by immunization with MOG (100 µg/mouse) emulsified in CFA. Groups of 4 mice were sacrificed on days 0, 3, 7, 11 and 17. Mononuclear cells were isolated from the brains and stimulated with PMA (10 ng/ml), ionomycin (1 µg/ml) and brefeldin A (5 µg/ml) for 5 h. Results are presented as representative FACS plots (A), mean frequency (B) or absolute numbers (C) of CD3⁺CD4⁺IL-17⁺ cells in the brain. ***p<0.001 versus day 0, +++p<0.001 versus day 3, \$\$\$p<0.001 versus day 7, ###p<0.001 versus day 11; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the means ± SEM (n=4).

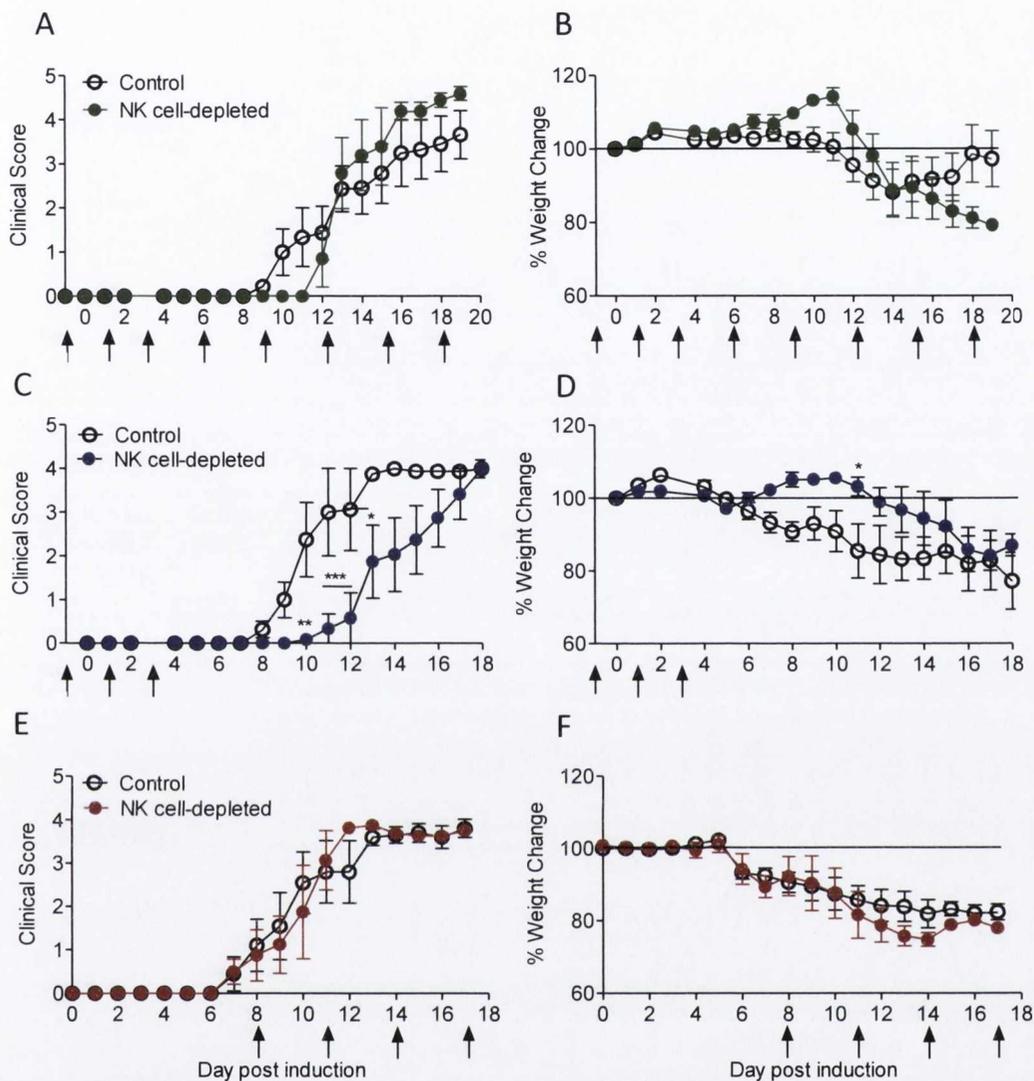


Figure 4.6 Neither continuous nor late depletion of NK cells affects the clinical course of EAE, while early depletion of NK cells delays the onset and reduces the severity of EAE

C57BL/6 mice were immunised with MOG (100 µg/mouse) emulsified in CFA and injected with anti-asialo GM1 antiserum (10 µl/mouse) or rabbit serum continuously (on day -1, 1, 3 and every 3 d thereafter), early (on day -1, 1 and 3) or late (on day 8 and every 3 d thereafter; arrows denote timing of injections). Mice were assessed for the development of EAE by clinical score (A, C, E) and % weight change (B, D, E). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control mice; two-way ANOVA with Bonferroni post-hoc test. Data are presented as the mean \pm SEM (n=6)

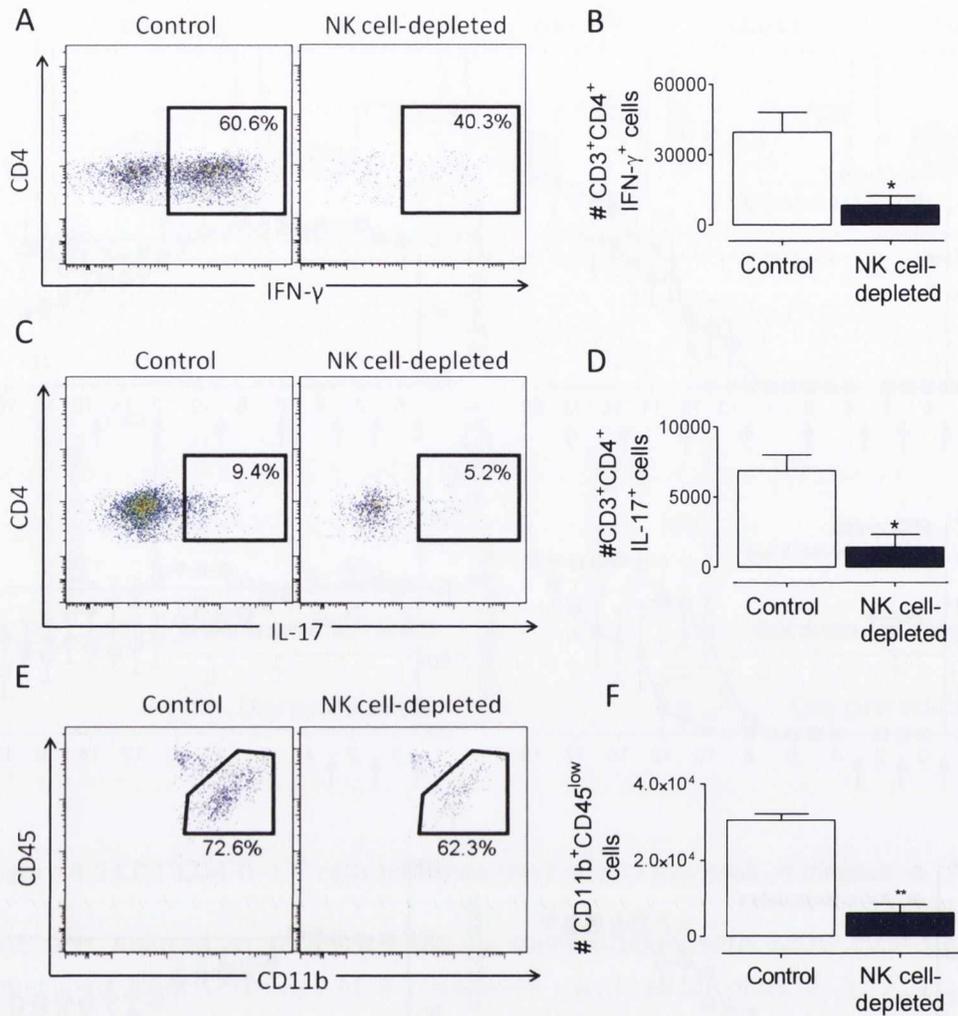


Figure 4.7 Early NK cell depletion reduces the number of CD11b⁺CD45^{low} cells and the infiltration of CD3⁺CD4⁺IFN- γ ⁺ and CD3⁺CD4⁺IL-17⁺ in the brains of mice with EAE

C57BL/6 mice were immunised with MOG (100 μ g/mouse) emulsified in CFA and injected with anti-asialo GM1 antiserum (10 μ l/mouse) or rabbit serum on day -1, 1 and 3. Animals were sacrificed on day 13. Mononuclear cells were isolated from the brains and stimulated with PMA (10 ng/ml), ionomycin (1 μ g/ml) and brefeldin A (5 μ g/ml) for 5 h. Th1 cells (CD3⁺CD4⁺IFN- γ ⁺), Th17 cells (CD3⁺CD4⁺IL-17⁺) and microglia (CD11b⁺CD45^{low}) were identified using FACS (A, C, E). Absolute numbers of CD3⁺CD4⁺IFN- γ ⁺ cells (B) CD3⁺CD4⁺IL-17⁺ cells (D) and CD11b⁺CD45^{low} cells (F) were calculated from original mononuclear cell counts. *p<0.05, **p<0.01 versus control mice; t-test. Data are presented as the means \pm SEM (n=3).

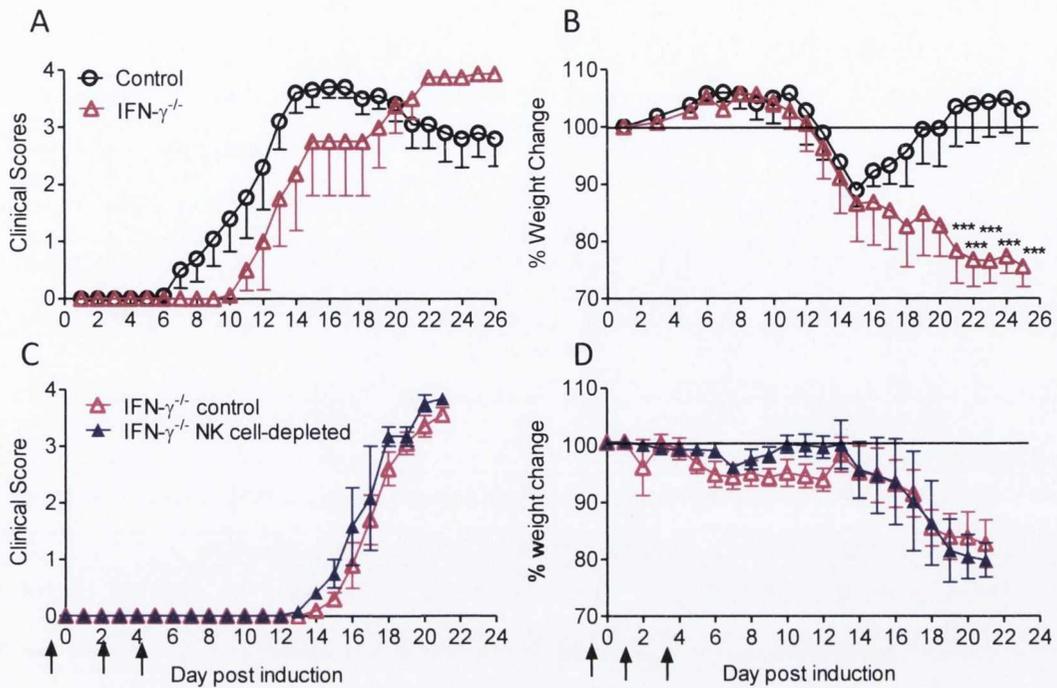


Figure 4.8 $\text{IFN-}\gamma^{-/-}$ mice exhibit a delayed onset and increased severity of active EAE relative to WT mice, however EAE clinical scores in $\text{IFN-}\gamma^{-/-}$ mice are unaffected by NK cell depletion

WT and $\text{IFN-}\gamma^{-/-}$ C57BL/6 mice were immunised with MOG (100 $\mu\text{g}/\text{mouse}$) emulsified in CFA. Mice were assessed for the development of EAE by clinical score (A) and % weight change (B). $\text{IFN-}\gamma^{-/-}$ C57BL/6 mice were immunised with MOG (100 $\mu\text{g}/\text{mouse}$) emulsified in CFA and injected with anti-asialo GM1 antiserum (10 $\mu\text{l}/\text{mouse}$) or rabbit serum on day -1, 1, and 3 (arrows denote timing of injections). Mice were assessed for the development of EAE by clinical score (C) and % weight change (D). *** $p < 0.001$ versus control (WT) mice; two-way ANOVA with Bonferroni post-hoc test. Data are presented as the mean \pm SEM ($n=4$). There were no significant differences in the clinical scores or percentage weight changes between the NK cell-depleted and control $\text{IFN-}\gamma^{-/-}$ mice, $p > 0.05$; ANOVA. Data are presented as the mean \pm SEM ($n=5$).

4.3 Discussion

Cells of the adaptive immune system, in particular CD4⁺ T cells, have long been considered the primary disease-causing lymphocytes in MS, and the animal model, EAE. However, in recent years, this dogma has been increasingly challenged by mounting evidence that cells of the innate immune system may also play an important role in CNS inflammation. One such candidate is the NK cell, an innate cytotoxic lymphocyte that has been presented in this study as having a crucial pathogenic role early in EAE.

4.3.1 NK cell infiltration into the CNS in EAE

Previous studies have reported detection of NK cells in the CNS of mice with EAE at the peak of disease (Huang et al., 2006). However until now, the kinetics of NK cell infiltration from disease induction to development of clinical signs has not been examined. The present study revealed that NK cell numbers peaked in the brain and spinal cord at day 7 post-induction, which preceded the onset of clinical signs of EAE. At this time NK cells accounted for almost 30% of all live mononuclear cells isolated from the brain. In contrast, NK cells only accounted for only 5% of cells detected in the brain at the peak of disease. This highlights some of the deficiencies of previous studies that focused on NK cell infiltration into the CNS at the peak of disease.

Rapid NK cell activation is a feature of many inflammatory responses. For example, in the well-established murine cytomegalovirus model (MCMV), there is rapid proliferation of NK cells, peaking only a few days after infection, and a speedy contraction when the cells undergo apoptosis (Robbins et al., 2004). This phenomenon is not confined to peripheral insults. In a mouse model of ischemic stroke, NK cells were found to rapidly infiltrate the brain following experimental occlusion of the middle cerebral artery, where they enhanced lesion size by killing ischemic neurons, and through release of IFN- γ , a strong inducer of neuroinflammation (Gan et al., 2014). The present study demonstrated a pathogenic role for brain-infiltrating NK cells early in EAE. A high frequency of these cells secreted IFN- γ by day 7, which persisted at least until day 17. Early in the course of EAE, NK cells seemed to be the primary source of IFN- γ . In contrast, the maximum infiltration of IFN- γ -secreting CD4⁺ T cells (Th1 cells)

did not occur until the peak of disease at day 17. A similar gradual increase in infiltration of Th17 cells was also observed, with maximum numbers detected on day 17. These data are in agreement with previous work from our laboratory, which demonstrated that Th1 and Th17 cells were found to be present in the CNS in highest numbers later in EAE (Murphy et al., 2010). Taken together, these data present NK cells as an important, early source of IFN- γ in the CNS in EAE.

4.3.2 The effect of NK cell depletion on the pathogenesis of EAE

A role for NK cells in the pathogenesis of EAE was further established through the use of anti-asialo GM1, a polyclonal antibody that depletes NK cells through complement-mediated lysis (Shimada and Iwata, 1987). Previous studies have used antibodies against NK1.1 to examine the role of NK cells in EAE, however this antigen is also expressed on NKT cells and a small subset of $\gamma\delta$ T cells (Haas et al., 2009), which complicated interpretation of results. The present study revealed that administration of anti-asialo GM1 *in vivo* resulted in depletion of NK cells that persisted for up to 4 days, with a gradual diminution in effect after this time. Administration of the antibody on days -1, 1 and 3 ensured depletion of NK cells up until their time of maximum observed CNS infiltration and IFN- γ production at day 7.

Crucially, the results revealed that early depletion of NK cells delayed the onset of EAE by almost 3 days and significantly reduced the severity of disease until day 14. These data are in agreement with the observations made by Winkler-Pickett and colleagues, who reported that NK cell depletion by anti-asialo GM1 reduced the severity of MOG-induced EAE (Winkler-Pickett et al., 2008). In contrast, Matsumoto and colleagues reported that a similar method of early NK cell depletion led to enhanced disease severity; however, this discrepancy may be accounted for by the fact that the experimental animals the authors used were Lewis rats, not mice, the induction agent was MBP, not MOG, and the clinical course of EAE was transient, with complete recovery by day 21 (Matsumoto et al., 1998a).

Interestingly, removal of NK cells (and consequently a source of IFN- γ) early in disease had an impact on the cellular environment in CNS of these animals. On day 13 of EAE, the total number of cells in the brains of the NK cell-depleted animals was significantly

lower than in the brains of untreated mice. This translated to a striking reduction in number of infiltrating Th1 and Th17 cells into the brains of depleted mice. Furthermore, the number of microglia was also reduced in the brains of these mice. It is possible that this reduction in microglial number with NK cell depletion was due to a consequential reduction in IFN- γ in the CNS, as microglia have been found to proliferate in response to exposure to IFN- γ (Grau et al., 1997). Microglial proliferation and activation are hallmarks of a number of neuroinflammatory disorders and can contribute to pathology in the CNS (Kreutzberg, 1996). The anti-viral drug ganciclovir, which is capable of abrogating microglial proliferation, has been successful in reducing disease severity in EAE (Ding et al., 2014). The combined reduction in CD4⁺ T cell infiltration and attenuation of aberrant microglial proliferation may provide an explanation as to why NK cell depletion was efficacious in reducing EAE severity.

4.3.3 The role of IFN- γ in the pathogenesis of EAE

The role of IFN- γ in EAE is contentious. Before the identification of Th17 cells as the primary pathogenic lymphocytes in EAE (Leavenworth et al., 2010a, Langrish et al., 2005), IFN- γ -producing Th1 cells were thought to be the primary pathogenic mediator in EAE. Indeed, MOG-specific Th1 cells can transfer disease (O'Connor et al., 2008). However, EAE can still be induced in mice with a disrupted IFN- γ gene (Ferber et al., 1996), and administration of a monoclonal antibody against IFN- γ can exacerbate EAE severity (Billiau et al., 1988). In this study, the absence of IFN- γ at the induction of EAE translated to delayed onset of disease, however, in the effector stage the transgenic mice exhibited enhanced disease severity. Taken together, these observations suggest that IFN- γ may play a pathogenic role at the induction stage, and a protective one at the effector stage of EAE.

NK cells have thus far been presented as a vital early source of IFN- γ in EAE. It is therefore possible that the reduced EAE severity and attenuation in neuroinflammation observed with depletion of NK cells at the induction of disease was related to a reduction in IFN- γ , which was previously observed to be pathogenic early in EAE. Indeed, in contrast to wild-type mice, NK cell depletion at the induction of EAE

had no effect on the clinical course in IFN- γ ^{-/-} mice, confirming that NK cells are pathogenic early in EAE by virtue of their production of IFN- γ .

Depletion of NK cells throughout the course of EAE did not have a significant effect on clinical scores, although mean EAE onset was delayed by 3 days, and tended toward a slight increase in severity later on, similar to the pattern seen in the IFN- γ ^{-/-} mice. This suggests that IFN- γ from NK cells may be protective later in EAE and removal of NK cells at this time may in turn lessen the beneficial effects of early depletion. The fact that the effect of continuous NK cell depletion is less profound than the absence of IFN- γ is likely to be due to compensatory production of this cytokine by other cell types, for example CD4⁺ T cells.

Taken together, these data uncover an important role for NK cells in the induction of EAE. IFN- γ -producing NK cells infiltrate the CNS before the onset of disease. Removal of NK cells early in EAE leads to an attenuation of clinical signs, and reduced numbers of CD4⁺ T cells and microglia in the CNS. The novel finding that this effect is lost in IFN- γ knockout mice provides strong evidence that NK cells are an early, innate, source of IFN- γ , a factor which likely contributes to their observed pathogenicity in the induction phase of EAE.

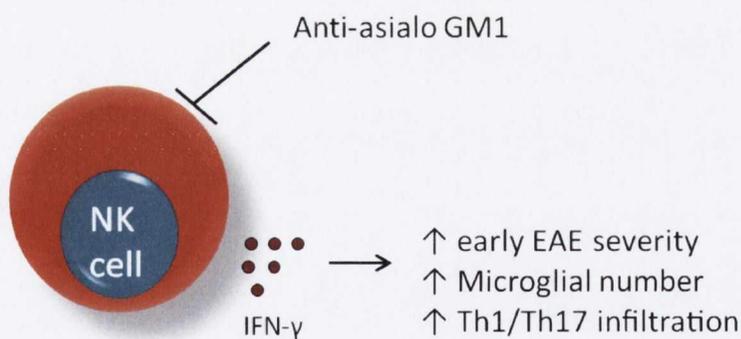


Figure 4.9 A role for NK cells early in EAE

IFN- γ from NK cells contributes to severity early in EAE through enhancement of microglial number and CD4⁺ T cell infiltration in the brain. Depletion of NK cells early in EAE using a polyclonal antibody against asialo GM1 therefore reduces disease severity

**Chapter 5: Induction of the M1 phenotype
and CD4⁺ T cell encephalitogenicity by NK
cells**

Chapter 2: Induction of the M1 phenotype
and CD4⁺ T cell encephalitogenicity by NK

cells

5.1 Introduction

Microglia are the principal CNS-resident immune cells, and have long been a subject of interest in the context of neurodegenerative and autoimmune conditions, such as MS and EAE. Like peripheral macrophages, microglia can adopt phenotypically distinct activation states in response to their particular environment. Broadly, microglial activation can be categorised as M1 or M2. The classical or M1 state is induced by exposure to LPS or IFN- γ , and is characterised by the production of NO, the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6 and IL-12, and upregulation of MHC II expression. The alternative or M2 state is induced by exposure to IL-4 and IL-13, and is associated with an increase in expression of ARG1, MRC1, and chitinase-like proteins (Colton, 2009). There is much evidence to suggest that microglial activation is an important feature of MS, with clusters of activated microglia, referred to as “nodules” appearing in the CNS before the onset of demyelination (van Horssen et al., 2012). Early microglial activation is also a feature of the animal model EAE, with appearance of M1 microglia in the CNS before the onset of disease (Ponomarev et al., 2005). In the absence of substantial inflammatory infiltrates composed of CD4⁺ T cells, which are not detected in the CNS until later in disease, it is still unclear what is responsible for this early glial activation.

The scope of NK cell interaction with other facets of the immune system extends beyond cytotoxicity. NK cell cytokine production has been found to modulate both the innate and adaptive arms of the immune response to insult. NK cell-derived IFN- γ plays an important role in Th1 differentiation in *Leishmania major* infection (Scharton and Scott, 1993). Depletion of NK cells in mice infected with *Toxoplasma gondii* proved fatal, as the reduction in IFN- γ impaired the generation of an adequate Th1 response (Goldszmid et al., 2007). NK cells can also modulate the Th1 response indirectly through activation of DCs. Secretion of IFN- γ by NK cells contributed to DC maturation and production of IL-12, a cytokine which is also required for Th1 differentiation (Gerosa et al., 2002).

IFN- γ is also potent inducer of the M1 macrophage phenotype. The theory that production of IFN- γ by NK cells could induce this pro-inflammatory state in

macrophages persisted for some time before direct evidence was provided by Prajeeth and colleagues. They demonstrated that co-incubation of NK cells with *Leishmania*-infected macrophages led to an increase in the expression of the archetypal M1 marker iNOS, an effect that did not require cell-cell contact, and which was lost if the NK cells were obtained from IFN- γ -deficient mice (Prajeeth et al., 2011). However, the specifics of non-cytotoxic NK cell interactions with other immune cells in the context of autoimmunity have not yet been established.

VLA-4 was first identified as an important player in the homing of CD4⁺ T cells to the inflamed CNS in 1992. This integrin interacts with VCAM-1, which allows transmigration of inflammatory cells across the BBB. It was found that antibody-mediated blockade of this molecule prevented both leukocyte infiltration and clinical paralysis in the EAE model (Yednock et al., 1992b). VLA-4 is a dimer of CD29 and CD49d and a monoclonal antibody against the latter, called natalizumab, is currently used as a treatment for relapsing-remitting MS. Despite withdrawal of the drug from the market following the development of a fatal progressive multifocal leukoencephalopathy (PML) in a number of patients, natalizumab was returned to the market in 2006 and still remains one of the leading treatments for MS (Goodin, 2006).

On resting CD4⁺ T cells, integrins such as VLA-4 are locked in the low-affinity state. Only when the cell is activated, for example by exposure to growth factors or chemokines, or with ligation of the T cell receptor, will the integrin adopt the high-affinity state that facilitates ligand binding and ultimately, cell adhesion (Sixt et al., 2006). Exposure to cytokines can also determine integrin expression; naïve CD4⁺ T cells cultured under Th1 polarising conditions (with IL-2, IL-12, IFN- γ , anti-IL-4) have higher VLA-4 expression than those cultured under Th2 polarising conditions (with IL-2, IL-4, anti-IL-12 and anti-IFN- γ) (Sasaki et al., 2009). While the detection of VLA-4⁺ CD4⁺ T cells in the CNS is a hallmark of both EAE (Soilu-Hänninen et al., 1997) and MS (Brosnan et al., 1995), little is known about how VLA-4 expression is modulated to enhance encephalitogenicity in this context.

Study aims

- 1) To establish whether the pattern of CNS NK cell infiltration and IFN- γ production correlated with an upregulation in the M1 state in microglia.
- 2) To investigate the effect of NK cell co-incubation with glia and macrophages on cell phenotype.
- 3) To examine the effect of NK cell co-incubation with macrophages on cell phenotype and consequently VLA-4 expression on CD4⁺ T cells.
- 4) To determine the effect of VLA-4 blockade at the induction and effector phases of EAE.

5.2 Results

5.2.1 Production of TNF- α by microglia increases in the brain at the time of enhanced NK cell infiltration on day 7

In order to examine the time-course of M1 microglial activation in the brains of mice with EAE, FACS analysis was used to assess expression of the M1-related cytokine TNF- α in microglia (CD11b⁺CD45^{low} cells, Figure 5.1 A). Interestingly, the frequency of CD11b⁺CD45^{low}TNF- α ⁺ cells was significantly higher at day 7 than days 0, 3, 11 or 17 (Figure 5.1 B; $p < 0.001$; ANOVA). The absolute number of CD11b⁺CD45^{low}TNF- α ⁺ cells was significantly higher at day 7 than days 3 or 11 (Figure 5.1 C; $p < 0.01$; ANOVA). This correlated with the increase in NK cell infiltration into the CNS described in Figure 4.1.

5.2.2 Treatment of NK cells with recombinant IL-12 significantly enhances IFN- γ production

Having demonstrated that NK cell infiltration into the CNS in EAE corresponded with an increase in microglial activation, *in vitro* assays were used to examine the effect of NK cell co-incubation on microglial phenotype. A preliminary experiment investigated IFN- γ production by NK cells in response to IL-2, which is required for their expansion and purification in culture with or without additional IL-12, which has been found to stimulate IFN- γ production by NK cells. NK cells treated with additional IL-12 produced significantly more IFN- γ than those treated with IL-2 alone (Figure 5.2, $p < 0.001$; ANOVA).

5.2.3 Incubation with NK cells induces microglial cell death

As NK cells are by their very nature cytotoxic, the levels of microglial cell death in response to NK cell co-incubation were examined using an LDH assay. The LDH OD was significantly higher in the supernatants taken from microglia that had been co-incubated with NK cells treated with IL-2 with or without additional IL-12 than in those from any other treatment group (Figure 5.3, $p < 0.001$; ANOVA). Interestingly, there was no increase in LDH OD in the supernatants taken from microglia incubated with conditioned medium taken from NK cells ($p > 0.05$; ANOVA) suggesting that NK cells kill microglia in a contact-dependent manner.

5.2.4 Conditioned medium from NK cells enhances the M1 state and reduces the M2 state in microglia

In order to circumvent the NK cell-mediated microglial cytotoxicity observed with direct co-incubation, the effect of NK-conditioned medium on microglial phenotype was examined. Conditioned medium taken from NK cells treated with IL-2 and IL-12 significantly enhanced microglial mRNA expression of the M1 markers iNOS, TNF- α and MHC II (Figure 5.4 A-C), and reduced expression of the M2 markers MRC1 and ARG1 (Figure 5.4 D, E) relative to all other treatment groups, including those cells that were incubated with conditioned medium taken from IL-2-treated NK cells ($p < 0.001$; ANOVA). Conditioned medium from IL-2-treated NK cells also increased mRNA expression of TNF- α relative to control microglia and those treated with IL-2 with or without additional IL-12 ($p < 0.001$; ANOVA). Taken together these data present a role for NK cells in enhancing the M1, and reducing the M2 phenotype in microglia.

5.2.5 NK cells or NK cell-conditioned medium enhance the M1 state and reduce in the M2 state in mixed glia

NK cells were found to kill microglia in a contact-dependent manner; however it was unclear if this cytotoxicity would be preserved in the presence of astrocytes. As such the co-incubation was repeated, this time using a mixed glial preparation. Mixed glial preparations are usually composed primarily of astrocytes, with about 20% microglia. Addition of NK cells did not induce any changes in LDH OD in supernatants taken from mixed glia (Figure 5.5; $p > 0.05$; ANOVA). Instead, the addition of NK cells treated with IL-2 with/without IL-12 enhanced the mRNA expression of the M1 markers iNOS, TNF- α and MHC II relative to the other treatment groups (Figure 5.6 B, D, F; $p < 0.05$, $p < 0.01$, $p < 0.001$; ANOVA). The expression of MHC II was also significantly higher in the mixed glia that were incubated with NK cells treated with IL-2 and IL-12 than those incubated with IL-2-treated NK cells ($p < 0.01$; ANOVA).

Only conditioned medium from NK cells treated with both IL-2 and IL-12 increased mRNA expression of iNOS, TNF- α and MHC II relative to control mixed glia, and cells treated with IL-2 with or without IL-12 (Figure 5.6 A, C, E; $p < 0.001$; ANOVA). iNOS mRNA expression was also higher in mixed glia incubated with medium from NK cells

treated with IL-2 and IL-12 than in mixed glia incubated with medium from IL-2-treated NK cells ($p < 0.001$; ANOVA).

On analysis of the mRNA expression of the M2 marker MRC1, it was found that both NK cells treated with IL-2 alone and those treated with additional IL-12 significantly reduced mRNA expression of MRC1 in mixed glia relative to all other treatment groups (Figure 5.6 H; $p < 0.05$; ANOVA). Conditioned medium from IL-2 and IL-12 treated NK cells significantly downregulated MRC1 expression relative to all other groups, including the cells that were incubated with conditioned medium from NK cells treated with IL-2 alone (Figure 5.6 G; $p < 0.05$; ANOVA). These data suggest that, similar to the observations made in primary microglia, NK cells can enhance the M1 phenotype and reduce the M2 phenotype in mixed glia.

5.2.6 NK cells or NK cell-conditioned medium enhances the M1 state and reduces the M2 state in BMDMs

Having demonstrated that NK cells can drive the M1 phenotype and reduce the M2 phenotype in both microglia and mixed glia, the effect of NK cell co-incubation on peripheral macrophages was examined. Addition of NK cells did not induce any changes in LDH OD in supernatants taken from BMDMs (Figure 5.7, $p > 0.05$; ANOVA). Instead, the addition of NK cells treated with IL-2 alone or with additional IL-12 increased expression of iNOS (Figure 5.8 B) relative to all the other treatment groups, whereas only the addition of NK cells treated with both cytokines elicited an upregulation in expression of MHC II in BMDMs, which was significantly higher than that detected in all other treatment groups (Figure 5.8 D; $p < 0.01$, $p < 0.001$; ANOVA). The addition of NK cells also elicited a significant reduction the mRNA expression of the M2 markers MRC1 (Figure 5.8 F) and CHI3L3 (Figure 5.8 H) relative to all other control groups ($p < 0.001$; ANOVA).

Incubation with conditioned medium from NK cells treated with IL-2 and IL-12 increased the mRNA expression of iNOS (Figure 5.8 A) and MHC II (Figure 5.8 C) in BMDMs relative to all the other treatment groups, including BMDMs incubated with conditioned medium from NK cells that were treated with IL-2 alone ($p < 0.001$; ANOVA). Only conditioned medium from NK cells treated with both IL-2 and IL-12

significantly reduced mRNA expression MRC1 in BMDMs relative to all other treatment groups (Figure 5.8 E; $p < 0.001$; ANOVA). NK cell conditioned medium had no effect on expression of CHI3L3 in BMDMs (Figure 5.8 G; $p > 0.05$; ANOVA). These findings demonstrate that NK cells are also capable of driving M1 polarisation and reducing M2 polarisation of macrophages.

5.2.7 IFN- γ from NK cells can drive M1 polarisation of macrophages and enhance expression of VLA-4 by MOG-specific CD4⁺ T cells

Thus far it has been shown that NK cells are most numerous in the brain early in EAE, and that depletion of these cells at the induction of disease leads to a reduction in EAE severity. NK cells can induce the pro-inflammatory M1 state in glia, which may partially account for the reduction in clinical scores with NK cell depletion, but does not entirely explain the corresponding reduction in CD4⁺ T cell number in the brain. Having found that NK cells can also polarise peripheral macrophages to the M1 state, the possibility that NK cells may have a role in the periphery that directly or indirectly influences the encephalitogenic capacity of CD4⁺ T cells was investigated.

In agreement with the ELISA data in Figure 5.2, the frequency of CD3⁻NK1.1⁺IFN- γ ⁺ NK cells was significantly enhanced with IL-12 treatment (Figure 5.9 A, B; $p < 0.001$; ANOVA). MHC II was significantly upregulated in BMDMs that had been treated with IFN- γ or co-incubated with NK cells treated with IL-2 with or without IL-12, relative to control BMDMs (Figure 5.9 C, D; $p < 0.001$; ANOVA).

Having verified that NK cells were producing IFN- γ and that BMDMs had adopted the M1 state, the effect of these elements on expression of the integrin VLA-4 on CD4⁺ T cells was investigated using FACS (Figure 5.10 A). Interestingly, VLA-4 expression was significantly higher in CD4⁺ T cells cultured with BMDMs in the presence of NK cells treated with IL-2 with or without additional IL-12 relative to all other groups (Figure 5.10 B; $p < 0.05$, $p < 0.01$, $p < 0.001$; ANOVA). VLA-4 expression was also significantly higher in CD4⁺ T cells co-incubated with IFN- γ -treated BMDMs than those co-incubated with IL-2 and IL-12-treated NK cells ($p < 0.01$; ANOVA). These data reveal a role for NK cells in polarising M1 macrophages and thus enhancing CD4⁺ T cell VLA-4 expression.

5.2.8 Neutralisation of VLA-4 at the induction and effector phases of EAE significantly attenuates disease

In order to examine the role of VLA-4 in the pathogenesis of EAE, a monoclonal antibody against the $\alpha 4$ subunit of the integrin was administered during both active and passive EAE. Administration of anti-CD49d antibody led to a significant attenuation of EAE in the active model from day 13 onwards (Figure 5.11 A, $p < 0.01$, $p < 0.001$; ANOVA). This was reflected in the percentage weight change, which was significantly higher in the antibody-treated mice than controls from day 15 onwards (Figure 5.11 B, $p < 0.05$, $p < 0.01$, $p < 0.001$; ANOVA). Administration of the antibody in the adoptive transfer EAE model completely prevented the development of disease, an effect which persisted for 17 d after the last dose of anti-CD49d. The clinical scores of the control mice were significantly higher than the mice that received the antibody from day 11 onwards (Figure 5.11 C; $p < 0.05$, $p < 0.001$; ANOVA). The percentage weight changes were significantly lower in control mice than the antibody-treated mice, also from day 11 onwards (Figure 5.11 D, $p < 0.05$, $p < 0.01$, $p < 0.001$; ANOVA). These data suggest that VLA-4 blockade is most beneficial at the induction stage of disease.

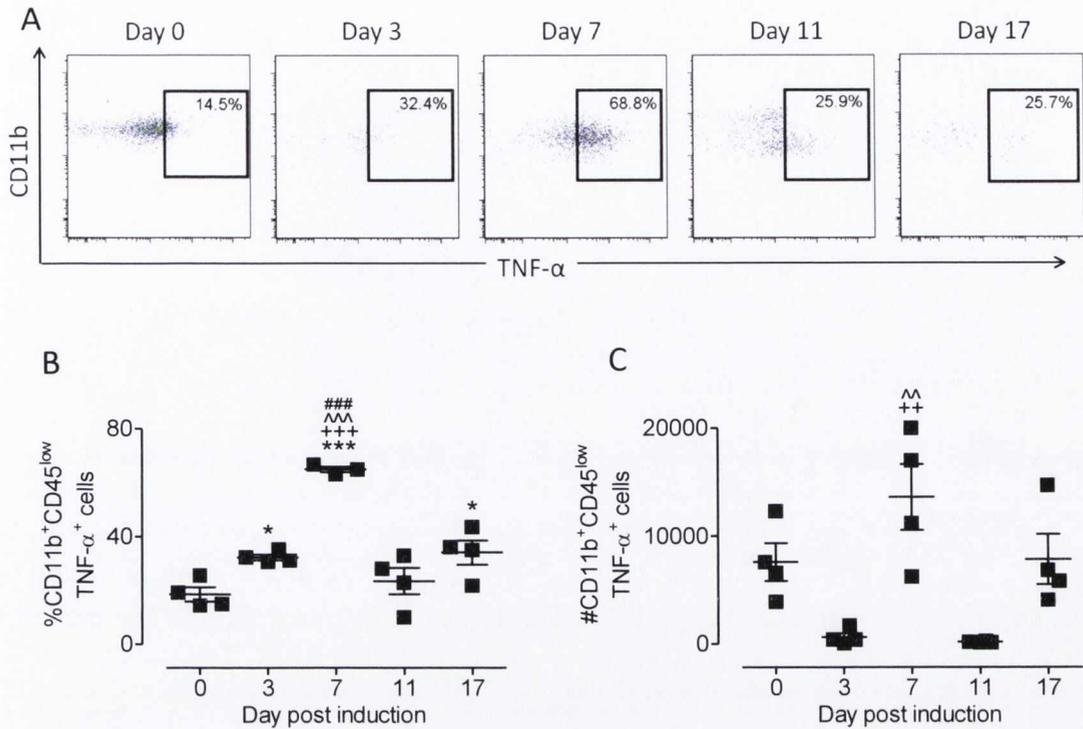


Figure 5.1 Production of TNF- α by CD11b⁺CD45^{low} cells increases in the murine brain early in EAE

EAE was induced in C57BL/6 mice by immunization with MOG (100 μ g/mouse) emulsified in CFA. Groups of 4 mice were sacrificed on days 0, 3, 7, 11 and 17. Mononuclear cells were isolated from the brains and stimulated with PMA (10 ng/ml), ionomycin (1 μ g/ml) or brefeldin A (5 μ g/ml) for 5 h. Results are presented as representative FACS plots (A), mean frequency (B) or absolute numbers (C) of CD11b⁺CD45^{low}TNF- α ⁺ cells in the brain. *p<0.05, ***p<0.001 versus day 0, **p<0.01, +++p<0.001 versus day 3, ^^p<0.01, ^^p<0.001 versus day 11, ###p<0.001 versus day 17; one way ANOVA with Newman-Keuls post-hoc test. Data are presented as the means \pm SEM (n=4).

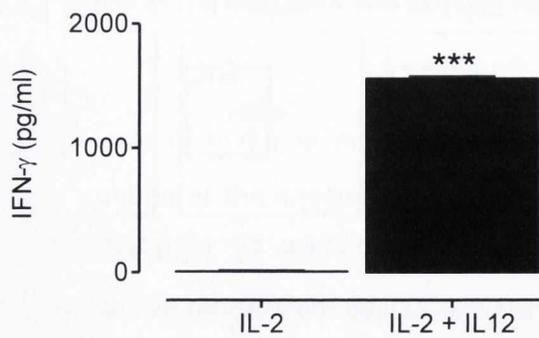


Figure 5.2 Addition of IL-12 significantly enhances IFN- γ production by NK cells

NK cells were treated for 24 h with IL-2 (100 ng/ml) with or without additional IL-12 (20 ng/ml). The supernatants were collected for assessment of IFN- γ concentration by ELISA. *** $p < 0.001$ versus IL-2-treated NK cells. Data are presented as the means \pm SEM (n=3).

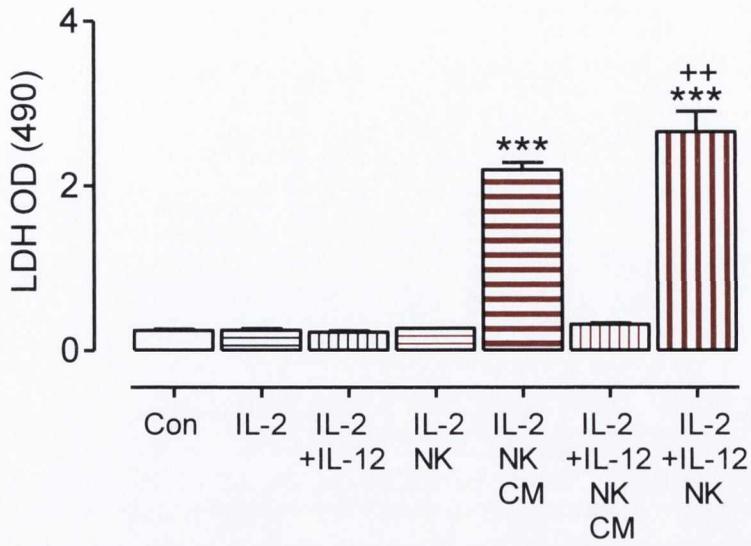


Figure 5.3 Incubation with NK cells, but not conditioned medium from NK cells, induces microglial cell death

Microglia were incubated for 24 h with medium alone, IL-2 (100 ng/ml) with or without IL-12 (20 ng/ml), NK cells treated with IL-2 with or without IL-12, or conditioned medium from NK cells. The supernatants were collected for assessment of LDH concentration by colorimetric assay. *** $p < 0.001$ versus controls, IL-2 with or without IL-12, conditioned medium taken from either NK cell group, ++ $p < 0.001$ versus IL-2-treated NK cells; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the mean \pm SEM ($n=4$).

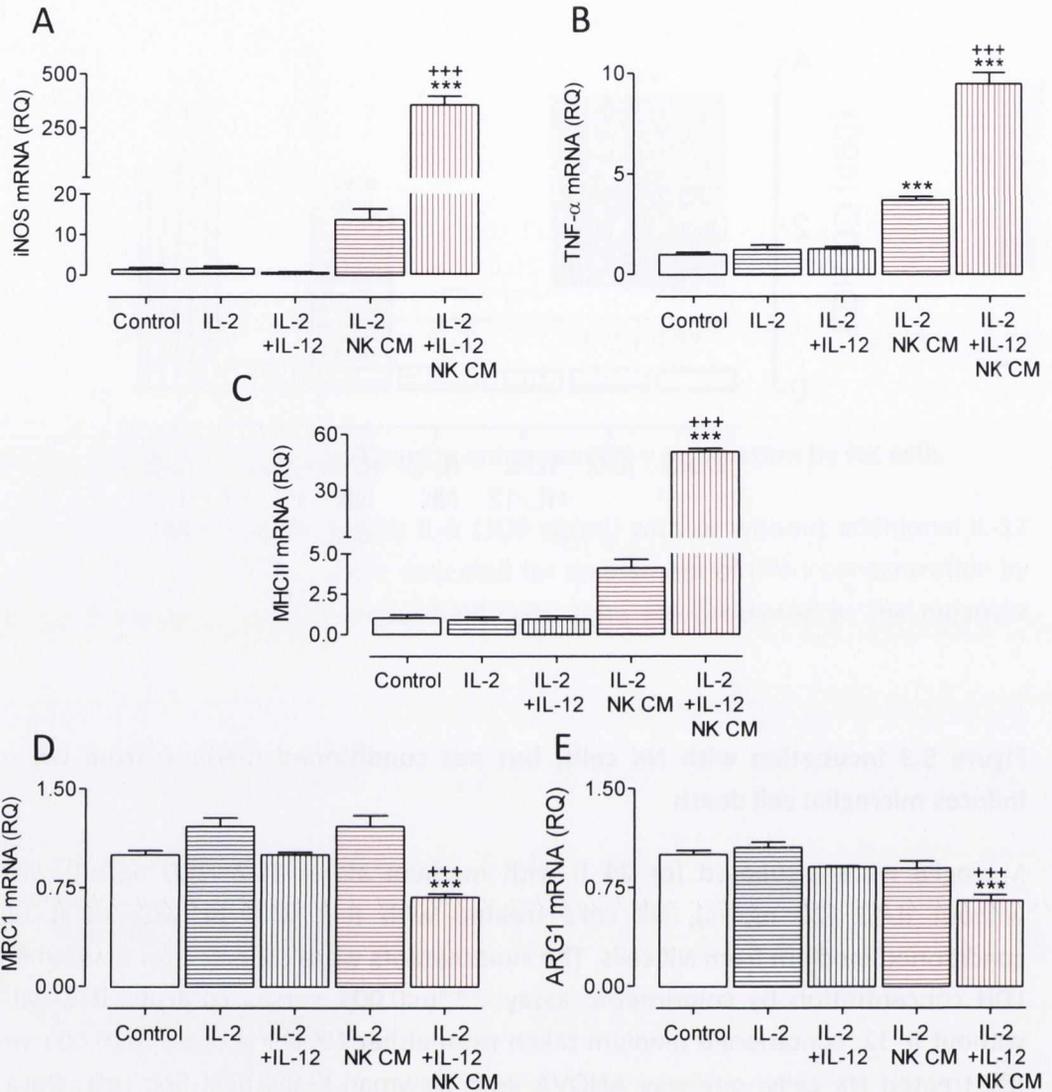


Figure 5.4 Incubation with conditioned medium from NK cells increases mRNA expression of the M1 markers iNOS, TNF- α and MHC II and reduces mRNA expression of the M2 markers MRC1 and ARG1 in microglia

Microglia were incubated for 24 h with medium alone, IL-2 (100 ng/ml) with or without IL-12 (20 ng/ml), or conditioned medium collected from NK cells treated with IL-2 with or without IL-12. Cells were harvested for assessment of mRNA expression of iNOS (A), TNF- α (B), MHC II (C) MRC1 (D) and ARG1 (E) by PCR. *** $p < 0.001$ versus control, IL-2 with or without IL-12, **** $p < 0.001$ versus conditioned medium from IL-2 treated NK cells; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the mean \pm SEM (n=4).

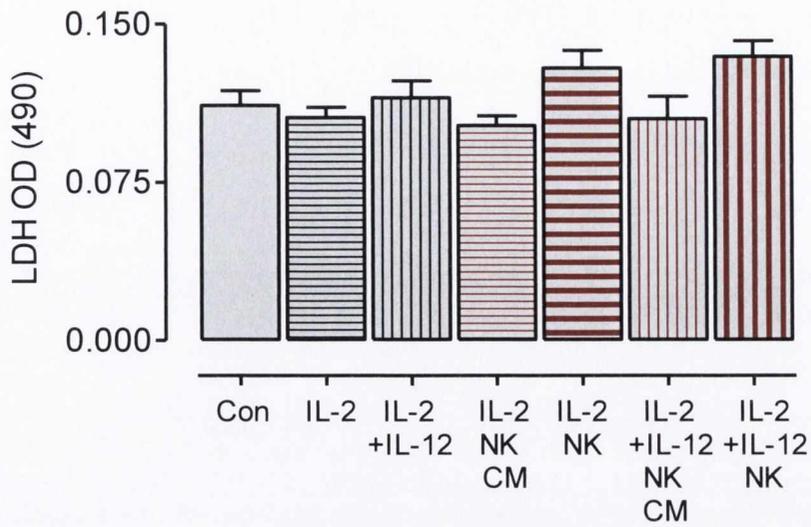


Figure 5.5 NK cells are not cytotoxic towards mixed glia

Mixed glia were incubated for 24 h with medium alone, IL-2 (100 ng/ml) with or without IL-12 (20 ng/ml), NK cells treated with IL-2 with or without IL-12, or conditioned medium from NK cells. The supernatants were collected for assessment of LDH by colorimetric assay. There were no significant differences between the LDH OD detected in the supernatants from any of the treatment groups; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the mean \pm SEM (n=4).

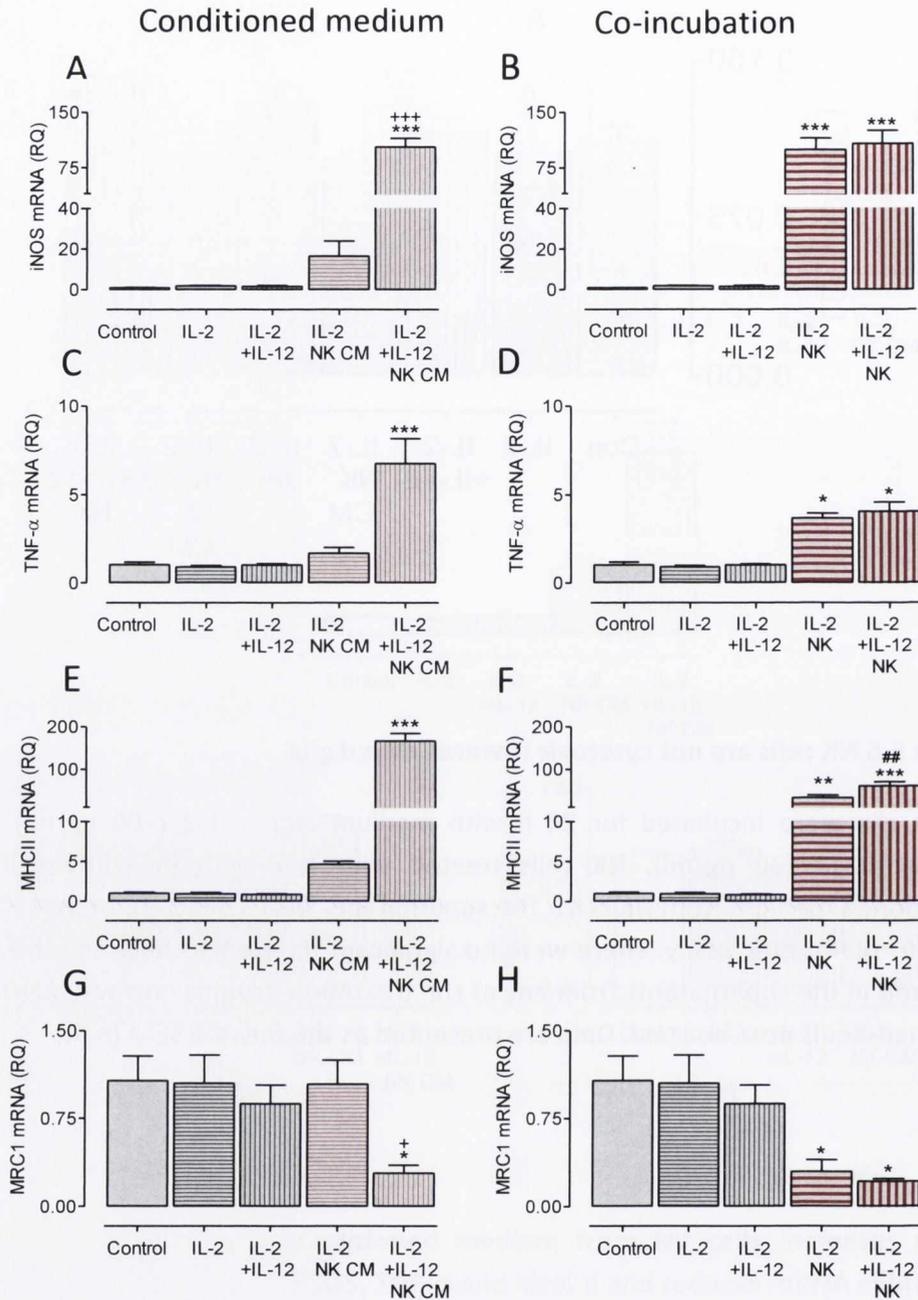


Figure 5.6 NK cells or NK cell-conditioned medium increases mRNA expression of the M1 markers iNOS, MHC II and TNF- α in mixed glia

Mixed glia were treated/incubated with NK cells as described in Figure 5.5. Cells were harvested for assessment of mRNA expression of iNOS (A, B), TNF- α (C, D) MHC II (E, F) or MRC1 (G, H) by PCR. * $p < 0.05$, *** $p < 0.001$ versus control, IL-2 with or without IL-12, + $p < 0.05$, +++ $p < 0.001$ versus conditioned medium from IL-2 and IL-12 treated NK cells, ## $p < 0.001$ versus IL-2-treated NK cells; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the mean \pm SEM ($n = 4$).

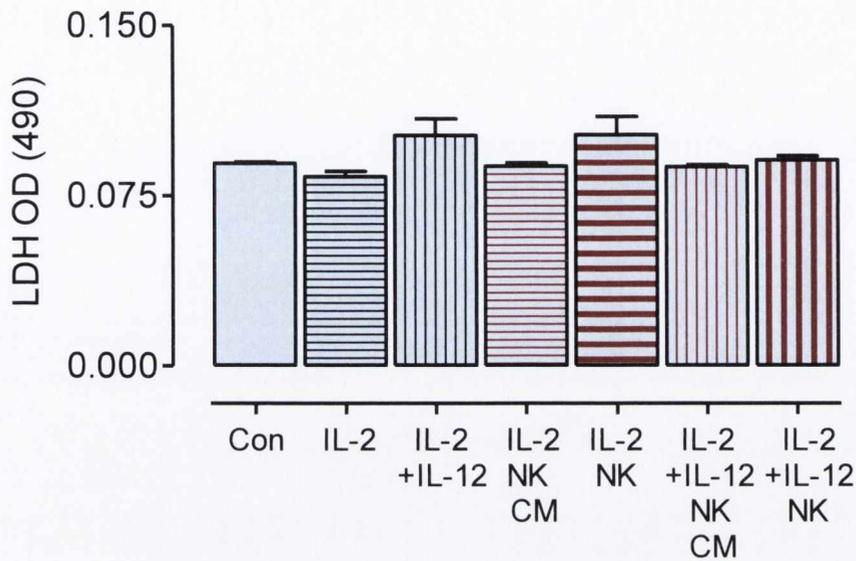


Figure 5.7 NK cells are not cytotoxic towards BMDMs

BMDMs were incubated for 24 h with medium alone, IL-2 (100 ng/ml) with or without IL-12 (20 ng/ml), NK cells treated with IL-2 with or without IL-12, or conditioned medium from NK cells. The supernatants were collected for assessment of LDH concentration by colorimetric assay. There were no significant differences between the LDH OD detected in the supernatants from any of the treatment groups; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the mean \pm SEM (n=4).

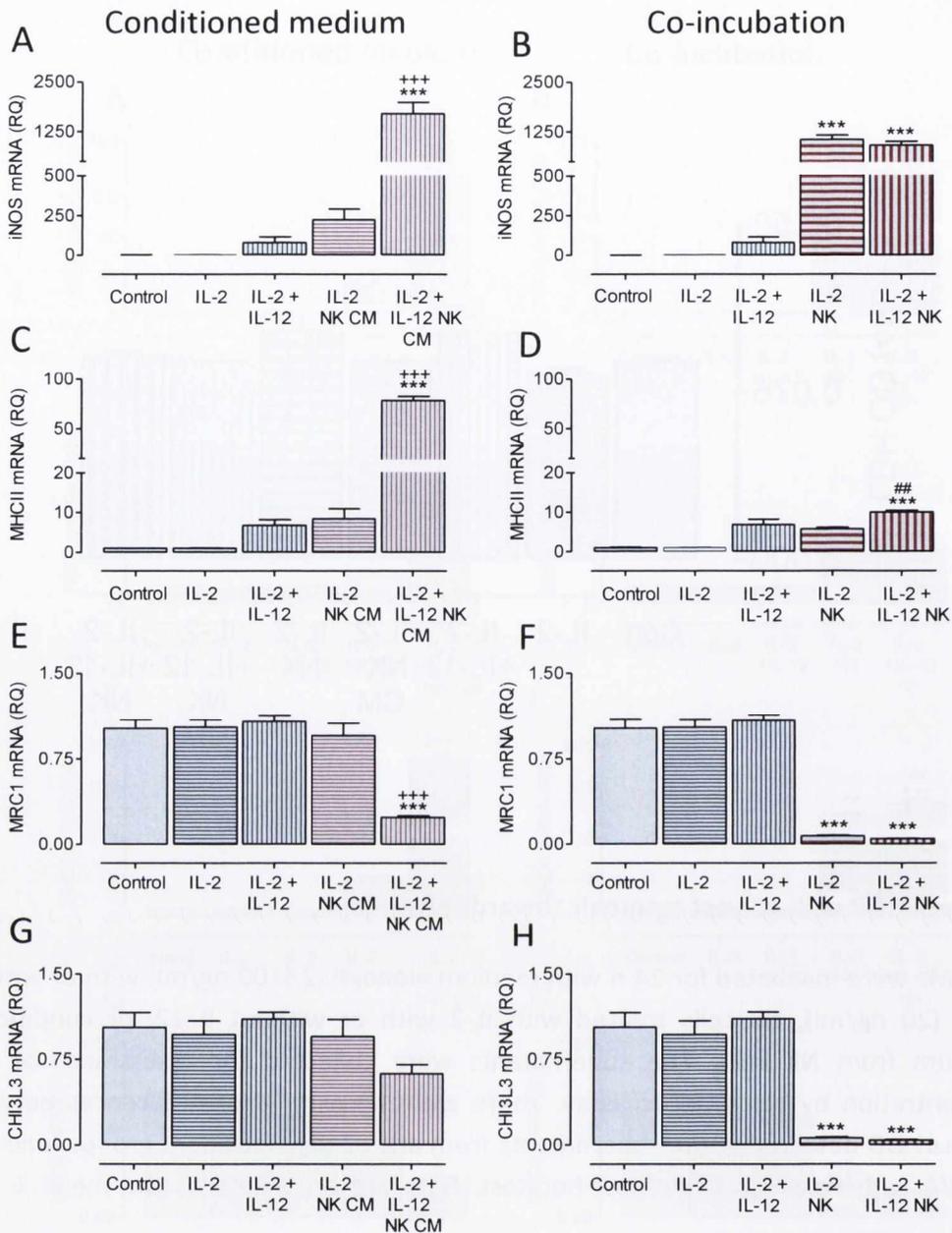


Figure 5.8 NK cells or NK cell-conditioned medium increases mRNA expression of the M1 markers iNOS and MHC II in BMDMs and reduces mRNA expression of the M2 markers MRC1 and CHI3L3 in BMDMs

BMDMs were treated/incubated with NK cells as described in Figure 5.9. Cells were harvested for assessment of mRNA expression of iNOS (A, B), MHC II (C, D) MRC1 (E, F) or CHI3L3 (G, H) by PCR. *** $p < 0.001$ versus control, IL-2 with or without IL-12, +++ $p < 0.001$ versus conditioned medium from IL-2-treated NK cells, ## $p < 0.001$ versus IL-2-treated NK cells; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the mean \pm SEM ($n=4$).

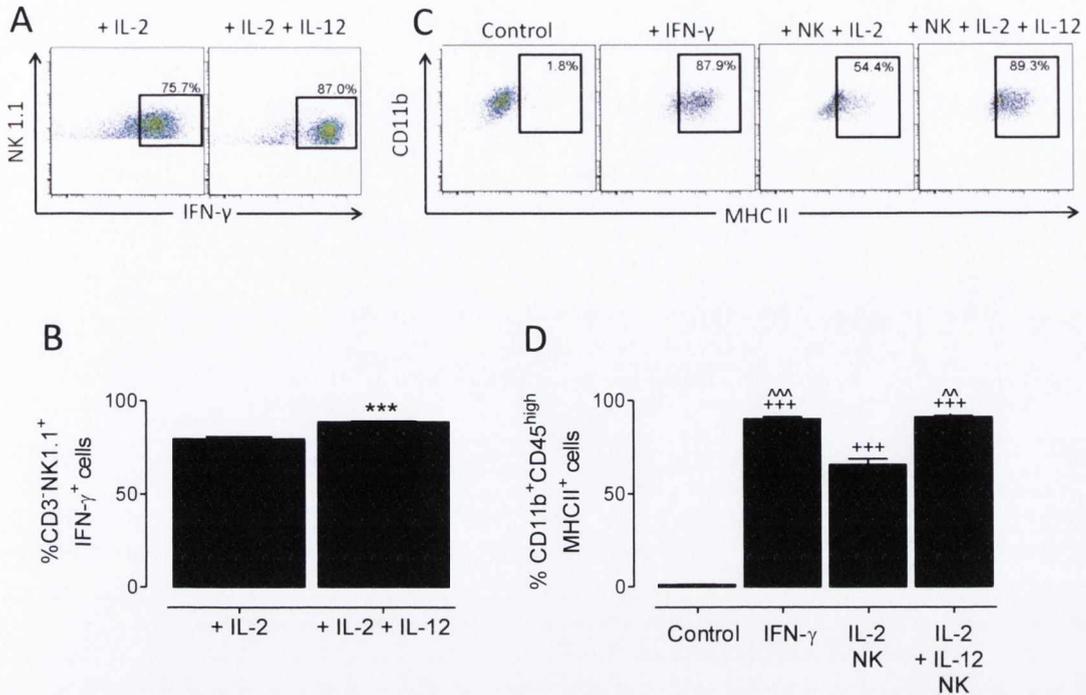


Figure 5.9 Treatment of NK cells with IL-12 enhances IFN- γ production, and upregulates expression of the M1 marker MHC II by BMDMs

NK cells were treated for 24 h with IL-2 (100 ng/ml) with or without additional IL-12 (20 ng/ml) after which time IFN- γ ⁺ NK cells (CD3⁺ NK1.1⁺ IFN- γ ⁺) were identified using FACS (A) and expressed as a frequency of the total CD3⁺ NK1.1⁺ population (B). BMDMs were incubated with medium alone, IFN- γ (50 ng/ml) or NK cells that had been pre-treated with IL-2 with or without additional IL-12 for 24 h, after which time they were assessed for MHC II expression using FACS (C). MHC II-expressing BMDMs (CD11b⁺ CD45^{high} MHC II⁺) were expressed as a percentage of the total CD11b⁺ CD45^{high} population (D). ***p<0.001 versus IL-2, +++p<0.001 versus controls, ^^p<0.001 versus IL-2-pre-treated NK cells. Data are presented as the mean \pm SEM (n=4)

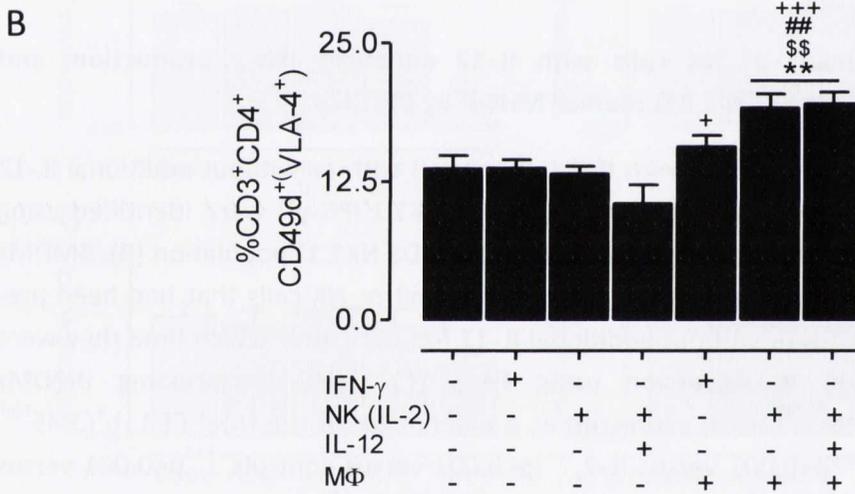
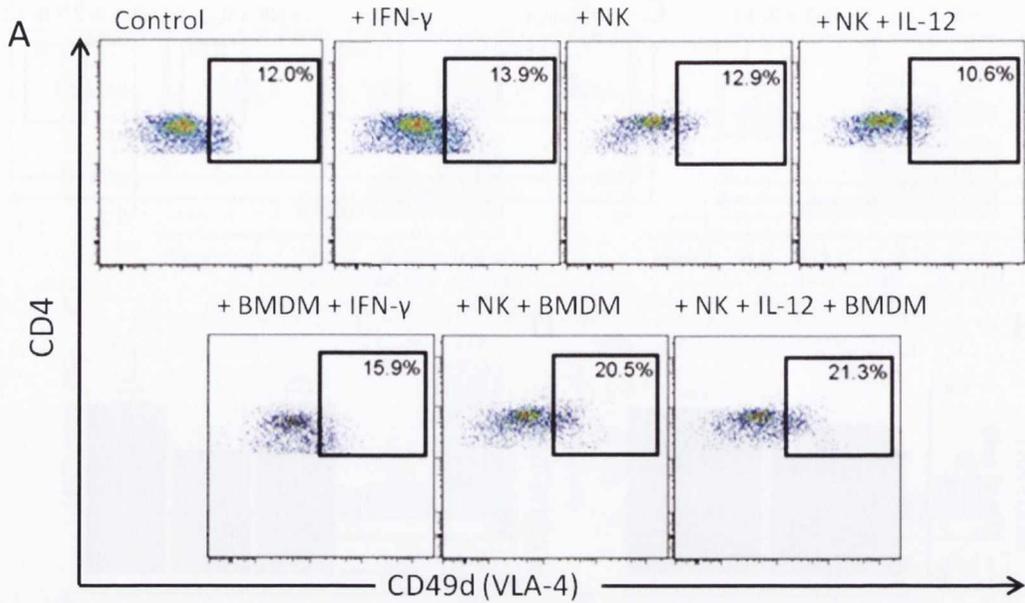


Figure 5.10 NK cell-derived IFN- γ promotes M1 macrophage polarisation which increases VLA-4 expression on MOG-specific CD4⁺ T cells

MOG-specific CD4⁺ T cells were incubated for 24 h with medium alone, IFN- γ (50 ng/ml), NK cells treated with IL-2 (100 ng/ml) with or without additional IL-12 (20 ng/ml), IFN- γ -pre-treated BMDMs or BMDMs with NK cells pre-treated with IL-2 with or without additional IL-12. VLA-4-expressing CD4⁺ T cells (CD3⁺CD4⁺CD49d (VLA-4)⁺) were identified using FACS (A) and VLA-4-expressing CD4⁺ T cells were expressed as a percentage of the total CD3⁺CD4⁺ cell population (B). ** $p < 0.01$ versus control, ^{\$\$} $p < 0.01$ versus IFN- γ , ^{###} $p < 0.01$ versus IL-2 pre-treated NK cells, ⁺ $p < 0.05$, ⁺⁺⁺ $p < 0.001$ versus IL-12 pre-treated NK cells, [^] $p < 0.05$ versus IFN- γ -treated BMDMs; one-way ANOVA with Newman-Keuls post-hoc test. Data are presented as the mean \pm SEM (n=4)

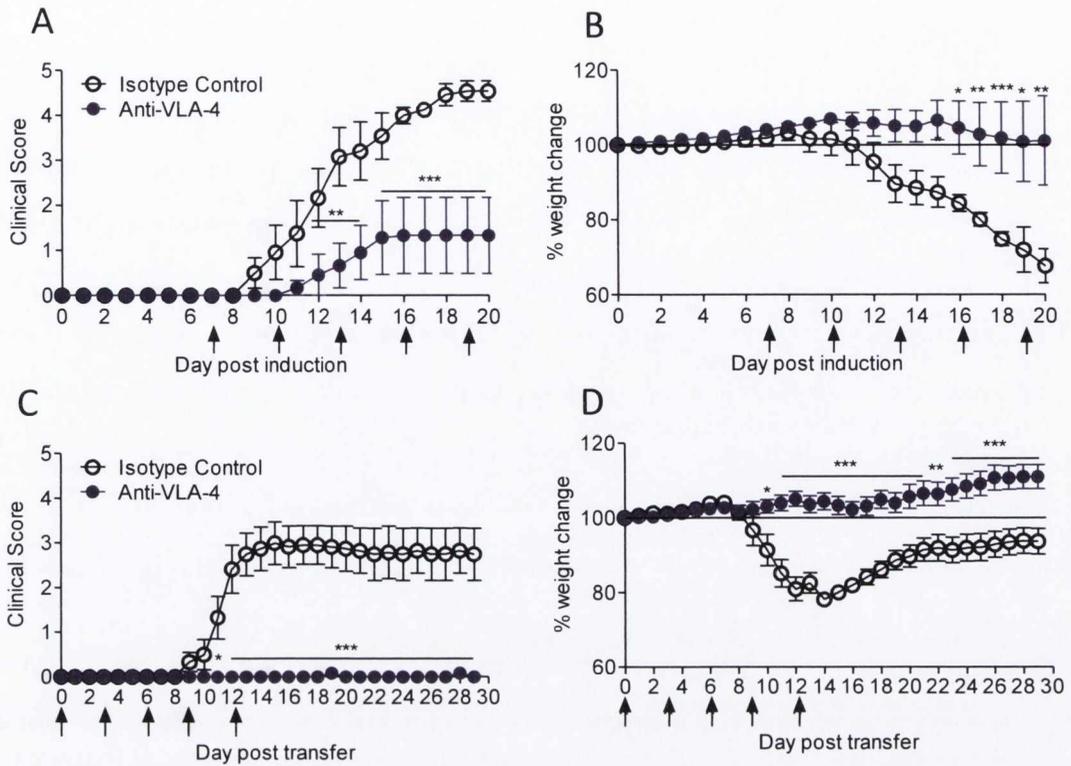


Figure 5.11 Blockade of VLA-4 in active EAE from day 7 significantly attenuates EAE, while blockade of VLA-4 in recipient mice up until day 12 completely attenuates EAE induced by cell transfer

C57BL/6 mice were immunised with MOG (100 $\mu\text{g}/\text{mouse}$) emulsified in CFA and injected with anti-VLA-4 (200 $\mu\text{g}/\text{mouse}$) or isotype control on day 7 and every 3 d thereafter (arrows denote timing of injections). Donor mice were immunised with MOG (100 $\mu\text{g}/\text{mouse}$) and CFA. After 10 d, spleens and LNs were taken and cultured in a 70:30 ratio in the presence of MOG (100 $\mu\text{g}/\text{ml}$), IL-1 β (10 ng/ml) and IL-23 (10 ng/ml) for 72 h. Cells were washed and injected into recipient mice at a density of $15 \times 10^6/\text{mouse}$. Mice were injected with anti-VLA-4 (200 $\mu\text{g}/\text{mouse}$) or isotype control on the day of transfer and every 3 d thereafter (arrows denote timing of injections). Mice were assessed for the development of EAE by clinical score (A, C) and % weight change (B, D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus clinical scores or % weight change of control mice with EAE. Data are presented as the mean \pm SEM ($n=6$).

5.3 Discussion

The data in Chapter 4 showed that NK cells were an important early innate source of IFN- γ in the development of EAE. Depletion of NK cells at the induction of EAE significantly reduced disease severity, which correlated with a reduction in the number of CD4⁺ T cells and microglia detected in the CNS. The results presented in this chapter provide a potential mechanism for the pathogenicity of NK cells in EAE. The findings demonstrate that NK cells can polarise both glia and macrophages to the pro-inflammatory M1 phenotype. Crucially, NK cell-mediated induction of the M1 phenotype in macrophages was found to upregulate VLA-4 expression on CD4⁺ T cells.

5.3.1 A role for NK cells in enhancing the M1 activation state in microglia

The study on NK cell kinetics during the course of EAE described in Chapter 4 revealed that production of TNF- α by microglia increased on day 7, before the onset of disease. TNF- α is a signature cytokine of the classical or M1 activation state in microglia, and its production can be induced by exposure to LPS or IFN- γ (Saijo and Glass, 2011). An increase in TNF- α production in the CNS is a characteristic of many neurodegenerative conditions, such as PD (Mogi et al., 1994), AD (Fillit et al., 1991) and MS (Beck et al., 1988). TNF- α has also been shown to play an important role in the pathogenesis of EAE, and its production in the CNS has largely been attributed to microglia and infiltrating macrophages (Renno et al., 1995). In agreement with the present study, TNF- α has shown to have a role in EAE; mice deficient in TNF- α display delayed onset of disease relative to wild type controls (Körner et al., 1997). It is possible that the upregulation in TNF- α production by microglia was induced by NK cells, which infiltrated the CNS and produced IFN- γ by day 7. Further support for this theory is provided by the observation that Th1 cells, which have traditionally been considered as the main source of IFN- γ in the CNS in EAE, did not infiltrate the brain in significant numbers until later in disease. While it has been known for some time that microglial activation is an early event in EAE (Ponomarev et al., 2005), the present study has identified a role for NK cells in this activation.

To date there has been little published data on putative interactions between microglia and NK cells, however the results on NK cell infiltration into the CNS

suggested a possible role for NK cells in polarising microglia to the M1 state. In agreement with this, NK-conditioned medium, containing IFN- γ , was found to increase mRNA expression on the M1 markers iNOS, TNF- α and MHC II by primary microglia. Furthermore, the addition of NK cell-conditioned medium also reduced mRNA expression of the M2 markers MRC1 and ARG1. Interestingly, direct co-incubation of NK cells with microglia was highly cytotoxic. The observation that NK cells can kill microglia *in vitro* has also been reported by Lünemann and colleagues, who used a similar co-incubation system to demonstrate the cytotoxic effect of human NK cells on human microglia in culture (Lünemann et al., 2008). However, while these authors used a NK: microglia ratio of 10:1, cytotoxic activity in the present study was observed with a cell ratio of 1:1.

5.3.2 A role for NK cells in enhancing the M1 activation state in mixed glia

In contrast to the observations made in primary microglia, incubation of NK cells with a mixed glial culture (containing microglia and astrocytes) did not induce cell death. This suggests a potential role for astrocytes in protecting microglia from NK cell-induced cytotoxicity. It has been demonstrated that astrocytes may play a role in suppressing deleterious microglial responses; astrocytes can reduce microglial production of pro-apoptotic factors and NO in response to exposure to amyloid- β (A β) (von Bernhardi and Eugenin, 2004). TGF- β from astrocytes can also dampen aberrant microglial activation (Herrera-Molina and von Bernhardi, 2005). While there have been very few studies on NK cell-astrocyte interactions, a recent study which used real-time technology to profile human NK killing of NT2A cells (an astrocyte-like cell line). Here it was revealed that while NK cells could kill NT2A cells, it took almost 5 days to achieve maximum cytotoxicity. Furthermore, killing activity was dampened if the NK cells were activated while in culture with NT2A cells, suggesting that astrocytes may suppress NK cell killing (Moodley et al., 2011).

The incubation of NK cells with the mixed glial culture elicited an increase in mRNA expression of the M1 markers iNOS, TNF- α and MHC II as well as a reduction in expression of the M2 marker MRC1. In agreement with the microglial data, conditioned medium from NK cells enhanced mRNA expression of the M1 markers

iNOS, TNF- α and MHC II, and reduced expression of the M2 marker MRC1 in mixed glia. Taken together, these data provide strong evidence for a novel role for NK cells in preferentially enhancing the pro-inflammatory M1 state in glia.

5.3.3 A role for NK cells in enhancing the M1 activation state in macrophages and VLA-4 expression by CD4⁺ T cells

Although ontogenically distinct, microglia share many characteristics with their peripheral equivalent, the macrophage, among which is the ability to become activated and assume the M1 phenotype in response to IFN- γ or LPS. While it has been widely assumed that NK cells, by virtue of their IFN- γ production, may be capable of polarising M1 macrophages, there is little direct evidence of this in the literature. In agreement with the observations made in both primary microglia and mixed glia, NK cells were found to polarise BMDMs to the M1 phenotype, as demonstrated by increased mRNA expression of iNOS and MHC II. NK cells also had the complementary effect of downregulating the M2 state, specifically expression of MRC1 and CHI3L3.

It is possible that the observed pathogenicity of NK cells early in EAE is due in part to polarisation of glia and macrophages to the pro-inflammatory M1 phenotype, with downregulation of the immunomodulatory M2 phenotype. Indeed, it has been conclusively shown that M1 microglia and macrophages can contribute to the pathogenesis of EAE. Targeted deletion of activated microglia has proven efficacious in reducing the severity of EAE (Heppner et al., 2005), as has administration of minocycline, which selectively inhibits M1 polarisation of microglia (Popovic et al., 2002). M1 microglia can secrete a number of chemotactic factors, including CCL2, also known as monocyte chemoattractant protein-1 (MCP-1), which can disrupt BBB integrity and enhance infiltration of peripheral inflammatory macrophages (Dogan et al., 2008). Infiltration of M1 macrophages into the CNS can contribute to axonal loss (Hendriks et al., 2005). Conversely, it has been shown that the therapeutic efficacy of IL-33 administration in EAE is due in part to M2 macrophage polarisation (Jiang et al., 2012). M1 microglia can also provide a CNS-resident source of the p40 subunit common to the cytokines IL-12 and IL-23, thus enhancing T cell encephalitogenicity in EAE (Becher et al., 2003). Finally, M1 microglia and macrophages can present antigen

through upregulated expression of MHC molecules, as well as the co-stimulatory signal via CD40, CD80/86, thus re-stimulating T cells that have entered the CNS and enhancing disease via epitope spreading (McMahon et al., 2005).

In Chapter 4, the results demonstrated that depletion of NK cells early in EAE was associated with reduced numbers of CD4⁺ T cells in the brain. The results from the current chapter suggest that NK cells may enhance CD4⁺ T cell encephalitogenicity in EAE via induction of M1 macrophages, as NK-polarised BMDMs were capable of enhancing expression of the integrin VLA-4 on MOG-specific CD4⁺ T cells.

5.3.4 The role of the integrin VLA-4 in the pathogenesis of EAE

VLA-4 ($\alpha 4\beta 1$ integrin) is a member of the integrin-like cell adhesion molecule family and is expressed by a range of haematopoietic cells, T and B lymphocytes, monocytes, NK cells, eosinophils and neutrophils (Smith, 2008). VLA-4 has two known natural ligands, VCAM-1 and fibronectin, and is involved in leukocyte trafficking and extravasation to inflammatory sites (Lin and Castro, 1998). Of particular interest in the context of this study is the interaction of VLA-4 with VCAM-1 on endothelial cells, which is required for CD4⁺ T cells to gain entry to the CNS in MS and EAE. Blockade of this interaction using a monoclonal antibody against $\alpha 4$ integrin (CD49d) can prevent EAE (Yednock et al., 1992b) and a humanised form of this antibody, natalizumab, is a current therapy for MS (Goodin, 2006). VLA-4 expression has been found to be of particular importance in facilitating Th1 cell entry to the CNS. Th17 cells also utilise VLA-4 to gain access to the spinal cord, but can still enter the brain and cause an ataxic form of EAE following administration of anti-VLA-4. Th17 infiltration is only completely prevented with concurrent blockade of LFA-1 ($\alpha L\beta 2$ integrin) (Rothhammer et al., 2011).

In agreement with the early observations made by Yednock and colleagues, administration of an antibody against VLA-4 at the time of cell transfer and every 3 days thereafter was effective in completely preventing EAE in the present study. This abrogation of clinical signs persisted for almost 20 days after the last dose of anti-VLA-4. While the administration of the antibody from day 7 (2 days prior to onset) onwards successfully reduced the mean clinical scores of mice with active EAE, the attenuation

was not complete. These data were consistent with a recent study by Mindur and colleagues who also reported that treatment with anti-VLA-4 2 days before onset reduced, but did not abolish, clinical signs in active EAE. The later the antibody was administered, the less effectively it suppressed disease (Mindur et al., 2014). Taken together, these data suggest that treatment with anti-VLA-4 would be most efficacious if administered early in disease.

For VLA-4 to assume its high affinity state, “inside-out” signalling must be triggered by exposure to growth factors, chemokines (for example, those secreted by activated endothelial cells) or ligation of the T cell receptor (Sixt et al., 2006). Although it has been consistently shown that CD4⁺ T cell encephalitogenic potential is determined by the expression of VLA-4, the mechanisms through which its expression is modulated in the context of EAE are still unclear.

5.3.5 Conclusions

In the present study, it was demonstrated that VLA-4 expression was significantly enhanced on CD4⁺ T cells cultured with M1 macrophages, particularly if NK cells were present to induce M1 polarisation. In Chapter 4, NK cells were presented as an important mediator of pathogenicity in the induction phase of EAE, by virtue of early IFN- γ production. In this chapter, two distinct mechanisms through which IFN- γ -producing NK cells are pathogenic early in EAE have been presented; through enhancement of the pro-inflammatory M1 state in glia, and through induction of M1 macrophages with consequential enhancement of CD4⁺ T cell encephalitogenicity (**Figure 5.12**). Collectively, these findings suggest a hitherto unidentified, crucial role for NK cells in the induction of EAE.

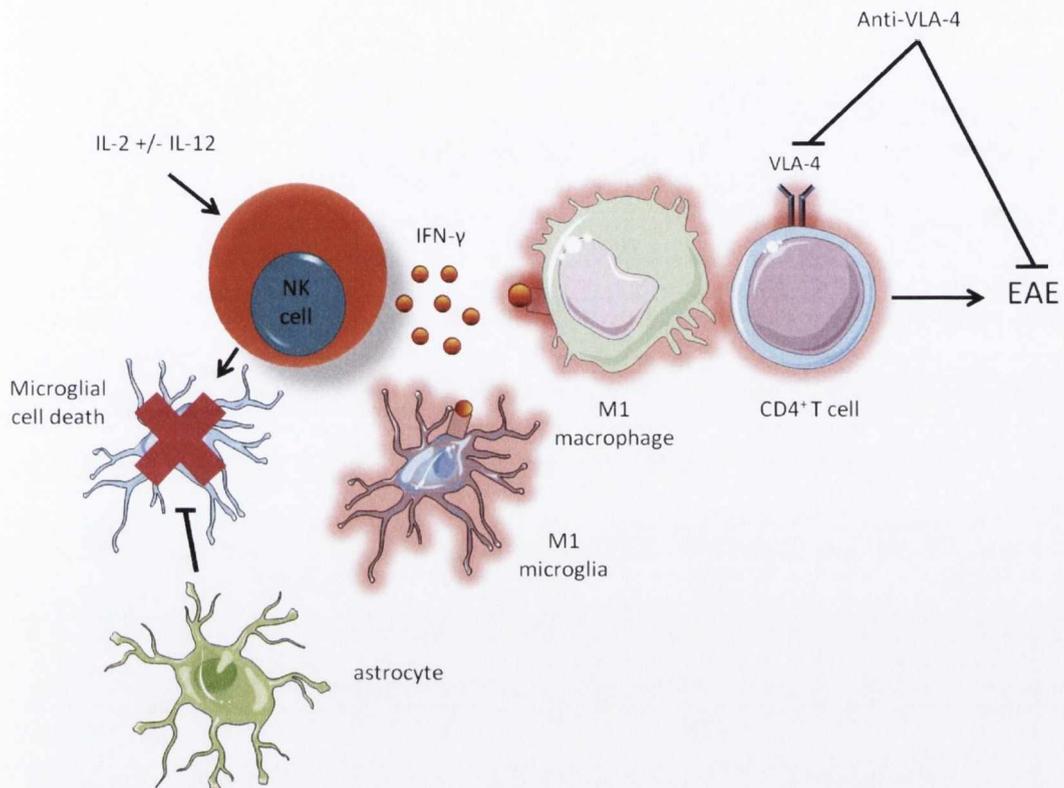


Figure 5.12 A role for NK cells in modulating glial and macrophage polarisation and CD4⁺ T cell encephalitogenicity in EAE

Treatment of NK cells with IL-2 additional IL-12 enhances IFN- γ production, which enhances M1 polarisation of macrophages and thus VLA-4 expression by CD4⁺ T cells, which contributes to EAE pathogenesis. Blockade of VLA-4 can prevent EAE. NK cells kill microglia in a contact-dependent manner, which is prevented by the presence of astrocytes. However, IFN- γ secreted by NK cells can increase M1 polarisation of glia without affecting cell viability.

Chapter 6: The role of IL-4 in EAE

6.1 Introduction

IL-4 is a pleiotropic cytokine with a diverse array of roles in regulation of the immune response. IL-4 is produced by Th2-polarised CD4⁺ T cells, granulocytes, monocytes, basophils and eosinophils (Luzina et al., 2012). IL-4 is structurally and functionally similar to IL-13, and the type II IL-4 receptor is composed of IL-4R α and IL-13R α subunits. The type I IL-4 receptor, on the other hand, is composed of IL-4R α and the common γ C subunit. The availability of each chain on the particular cell surface determines the signalling pathway. The γ C subunit is preferentially expressed on haematopoietic immune cells and signalling through the type I receptor complex is mediated by STAT6 (Kaplan et al., 1996).

IL-4 is required for the generation of Th2 cells (Swain et al., 1990). Development of these cells can be inhibited by disruption of either the IL-4 (Kopf et al., 1993) or STAT6 gene (Kaplan et al., 1996). Like IFN- γ from Th1 cells, production of IL-4 by Th2 cells can act as an autocrine growth factor for further expansion of these cells. Furthermore, Th1-produced IFN- γ and Th2-produced IL-4 can act as reciprocal inhibitory cytokines on the opposing cell type (Fernandez-Botran et al., 1988).

The Th1/Th2 nomenclature is mirrored in macrophages, which can adopt the M1 state in response to stimulation with the archetypal Th1 cytokine IFN- γ , and the M2 state in response to stimulation with the primary Th2 cytokine, IL-4 (Gordon, 2003). M1 macrophages engage in Type 1-polarised responses such as production of effector molecules like ROS and RNS, as well as pro-inflammatory cytokines, which rapidly eliminate pathogens. In contrast, M2 macrophages participate in Type 2-polarised functions, are immunoregulatory, required for parasite encapsulation, tissue repair and remodelling (Mantovani et al., 2005).

Like IFN- γ , the role of IL-4 in EAE is controversial. Studies using IL-4-deficient mice have generated conflicting reports of increased (Falcone et al., 1998), and unchanged (Liblau et al., 1997) clinical severity relative to control mice. This may reflect differences in the induction agents and also inconsistencies in the scoring protocols used. The paper by Falcone and colleagues reported a very weak EAE in the control C57BL/6 mice in response to immunisation with a preparation of guinea pig myelin in

CFA. Susceptibility to EAE was enhanced in the BALB/C strain, which exhibited similar EAE clinical scores to their IL-4^{-/-} littermates. Liblau and colleagues reported a more robust EAE in the PL/J strain, which was not significantly altered in the IL-4 deficient mice. This was in agreement with a paper published in the same year that reported that IL-4^{-/-} mice on a C57BL/6 background exhibited comparable MOG-induced EAE scores to their non-transgenic littermates (Bettelli et al., 1998).

The observation that IL-4 deficiency did not greatly affect the clinical course of EAE is somewhat surprising, as IL-4 has traditionally been associated with the generation of Th2 cells, which do not transfer EAE, and can even abrogate the development of established EAE (Kuchroo et al., 1995). Furthermore, IL-4 polarises M2 macrophages, which have been found to be protective in EAE (Liu et al., 2013, Mikita et al., 2011, Denney et al., 2012). However, while Th2 responses are diminished in the absence of IL-4, Th2-like lineages can still be isolated from these animals (Brewer et al., 1996). This is likely due to the preservation of IL-13, which also plays an important role in generation of Th2 responses (McKenzie et al., 1998) and can also induce M2 macrophages in the absence of IL-4 (Gordon, 2003).

Study aims

- 1) To assess the effect of IL-4 addition on the M1/M2 polarisation status of a MOG-specific culture.
- 2) To examine the effect of IL-4 addition to a culture of MOG-specific cells on encephalitogenic function of CD4⁺ T cells through assessment of VLA-4 expression and cytokine production.
- 3) To assess the effect of IL-4 addition to a culture of MOG-specific cells on their ability to induce EAE in recipient mice.
- 4) To investigate microglial activation/infiltration of CD4⁺ T cells in the brains of recipient mice that received MOG-specific cells treated with IL-4.
- 5) To elucidate the direct effect of IL-4 on VLA-4 expression on CD4⁺ T cells, as well as indirectly through induction of M2-polarised innate cells.

6.2 Results

6.2.1 Addition of IL-4 increases M2 and reduces M1 polarisation in a culture of MOG-specific cells

Having described a role for IFN- γ production by NK cells in the induction of EAE through polarisation of M1 macrophages in Chapter 5, the role of the M2-polarising cytokine IL-4 in EAE was next examined. Addition of IL-4 to a culture of spleen and lymph node cells from mice immunised with MOG and CFA, and re-stimulated with MOG, IL-1 β and IL-23, reduced the mRNA expression of the M1 marker iNOS relative to control cells (Figure 6.1 A; $p < 0.01$; t-test) and increased the mRNA expression of the M2 markers MRC1 and ARG1 relative to control cells (Figure 6.1 B, C; $p < 0.05$, $p < 0.001$; t-test).

A common readout of the pathogenic capacity of a MOG-specific culture is the production of pro-inflammatory cytokines, such as IL-17A, IFN- γ and GM-CSF. The addition of IL-4 to the culture reduced MOG-specific IL-17A production relative to control cells (Figure 6.1 D; $p < 0.001$; t-test). The production of IFN- γ and GM-CSF was unaffected by addition of IL-4 (Figure 6.1 E, F; $p > 0.05$; t-test).

6.2.2 Addition of IL-4 reduces the expression of VLA-4 on MOG-specific CD4⁺ T cells

It was demonstrated in Chapter 5 that NK cell-derived IFN- γ contributed to pathogenicity in EAE through induction of M1 macrophages and enhancement of VLA-4 expression on CD4⁺ T cells. In Figure 6.1, it was shown that IL-4 increased M2 polarisation and reduced expression of the M1 marker iNOS in the MOG-specific culture. Here, FACS analysis revealed that the addition of IL-4 to the culture also reduced VLA-4 expression on CD4⁺ T cells relative to control cells (Figure 6.2; $p < 0.001$; t-test), thus providing a potential mechanism through which IL-4 can suppress pathogenicity in passive EAE.

6.2.3 Addition of IL-4 to a culture of MOG-specific cells inhibits their ability to transfer EAE

Addition of IL-4 to a culture of spleen and lymph node cells from mice immunised with MOG and CFA, and re-stimulated with MOG, IL-1 β and IL-23, prevented their ability to

induce EAE following transfer to naïve mice. This was reflected in the clinical scores, which were significantly lower than those of mice that received control cells (Figure 6.3 A; $p < 0.001$; ANOVA), and also in the percentage weight change, which was significantly higher in the mice that received IL-4-treated cells than those that received control cells from day 9 onwards (Figure 6.3 B; $p < 0.001$; ANOVA).

6.2.4 MHC II expression by microglia is reduced in the brains of mice that received MOG-specific cells cultured with IL-4

The effect of IL-4 addition to the culture on the neuroinflammatory status of recipient mice was compared with mice that received cells cultured with MOG, IL-1 β and IL-23 in the absence of IL-4. The frequency and number of MHC II-expressing microglia (CD11b⁺CD45^{low}MHCII⁺) were reduced in the brains of mice that received IL-4-treated MOG-specific cells relative to those that received control cells (Figure 6.4 B, C; $p < 0.01$, $p < 0.001$; t-test).

6.2.5 The infiltration of CD3⁺CD4⁺IFN- γ ⁺ and CD3⁺CD4⁺IL-17⁺ cells is reduced in the brains of mice that received MOG-specific cells cultured with IL-4

The infiltration of Th1 (CD3⁺CD4⁺IFN- γ ⁺) cells and Th17 (CD3⁺CD4⁺IL-17⁺) cells into the brains of recipient mice was analysed using FACS. The frequency and number of CD3⁺CD4⁺IFN- γ ⁺ cells and CD3⁺CD4⁺IL-17⁺ cells were reduced in the brains of mice that received MOG-specific cells cultured with IL-4 relative to those that received MOG-specific cells cultured in the absence of IL-4 (Figure 6.4 E, F, Figure 6.4 H, I; $p < 0.05$, $p < 0.001$; t-test). This suggests that IL-4 reduces infiltration of CD4⁺ T cells into the brain in EAE, which is in agreement with the data in Figure 6.2 showing that addition of IL-4 can reduce VLA-4 expression on CD4⁺ T cells in a MOG-specific culture.

6.2.6 IL-4 reduces VLA-4 expression and the mRNA expression of IFN- γ and IL-17 in stimulated CD3⁺ T cells

As the MOG-specific cultures used in adoptive transfer EAE are a heterogeneous population of spleen and lymph node cells, the cellular target of IL-4 was still unknown. In order to determine the mechanism through which IL-4 suppresses EAE, CD3⁺ T cells were isolated and stimulated with anti-CD3 and anti-CD28 in the presence

or absence of IL-4. Treatment of anti-CD3 and anti-CD28-stimulated CD3⁺ T cells with IL-4 reduced VLA-4 expression, as assessed by FACS (Figure 6.5 B; $p < 0.001$; t-test) and mRNA expression of IFN- γ (Figure 6.5 C) and IL-17 as assessed by PCR, relative to control cells (Figure 6.5 D, $p < 0.05$, $p < 0.01$; t-test). This suggests that IL-4 could act directly on pathogenic T cells and reduce their encephalitogenicity through reduction of cytokine production and integrin expression.

6.2.7 Incubation of stimulated CD3⁺ T cells with M2 macrophages reduces expression of VLA-4

Having demonstrated in Chapter 5 that M1 macrophages can enhance expression of VLA-4 expression on CD4⁺ T cells, a potential role for IL-4 in polarising M2 macrophages and thus reducing VLA-4 expression on CD4⁺ T cells was examined. Peritoneal macrophages were treated with medium alone or IL-4 for 6 h, then washed and incubated with CD3⁺ T cells in the presence of anti-CD3. M2 polarisation of peritoneal macrophages was verified by analysis of mRNA expression of the M1 marker iNOS, and the M2 markers MRC1, ARG1, and CHI3L3. IL-4 treatment significantly enhanced expression of MRC1, ARG1 and CHI3L3 and reduced expression of iNOS relative to controls (Figure 6.6 A-D; $p < 0.05$, $p < 0.01$, $p < 0.001$; t-test). Incubation of stimulated CD3⁺ T cells with IL-4-polarised peritoneal macrophages reduced expression of VLA-4 on CD4⁺ T cells relative to controls and those incubated with untreated peritoneal macrophages (Figure 6.6 F; $p < 0.001$; ANOVA). These findings provide an alternative mechanism through which IL-4 suppresses CD4⁺ T cell encephalitogenicity in EAE; indirectly through M2 macrophages.

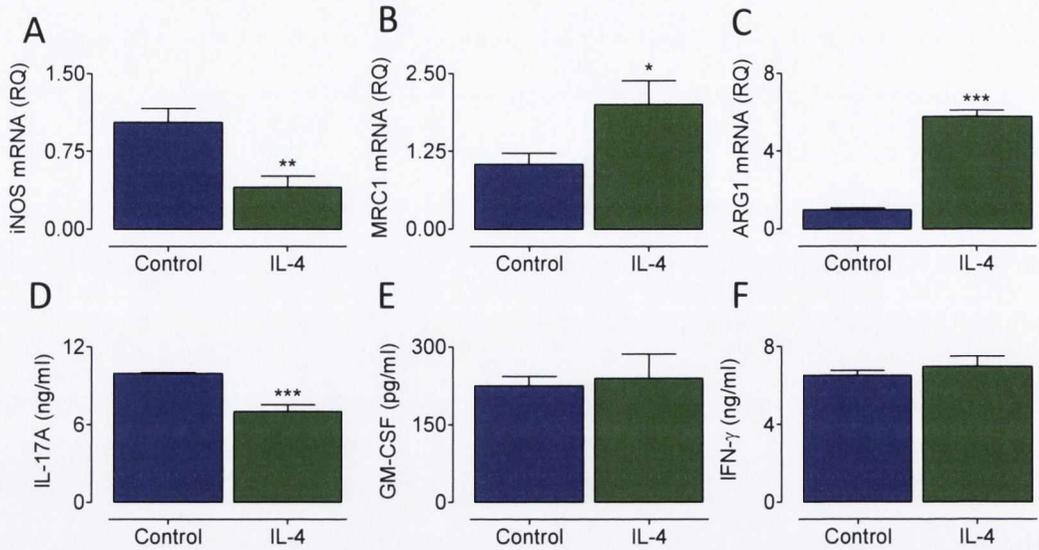


Figure 6.1 IL-4 increases mRNA expression of M2 markers MRC1 and ARG1 and reduces mRNA expression of the M1 marker iNOS and IL-17A production in a MOG-specific culture

Donor mice were immunised with MOG and CFA. After 10 d, spleen and LN cells were cultured for 72 h in a 70:30 ratio with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of IL-4 (20 ng/ml). Cells were harvested for assessment of mRNA expression of (A) iNOS (B) MRC1 and (C) ARG1 by PCR. Supernatants were harvested for assessment of IL-17A (D), IFN-γ (E) and GM-CSF (F) concentrations by ELISA *p<0.05, **p<0.01, ***p<0.001 versus control; t-test. Data are presented as the mean ± SEM (n=3).

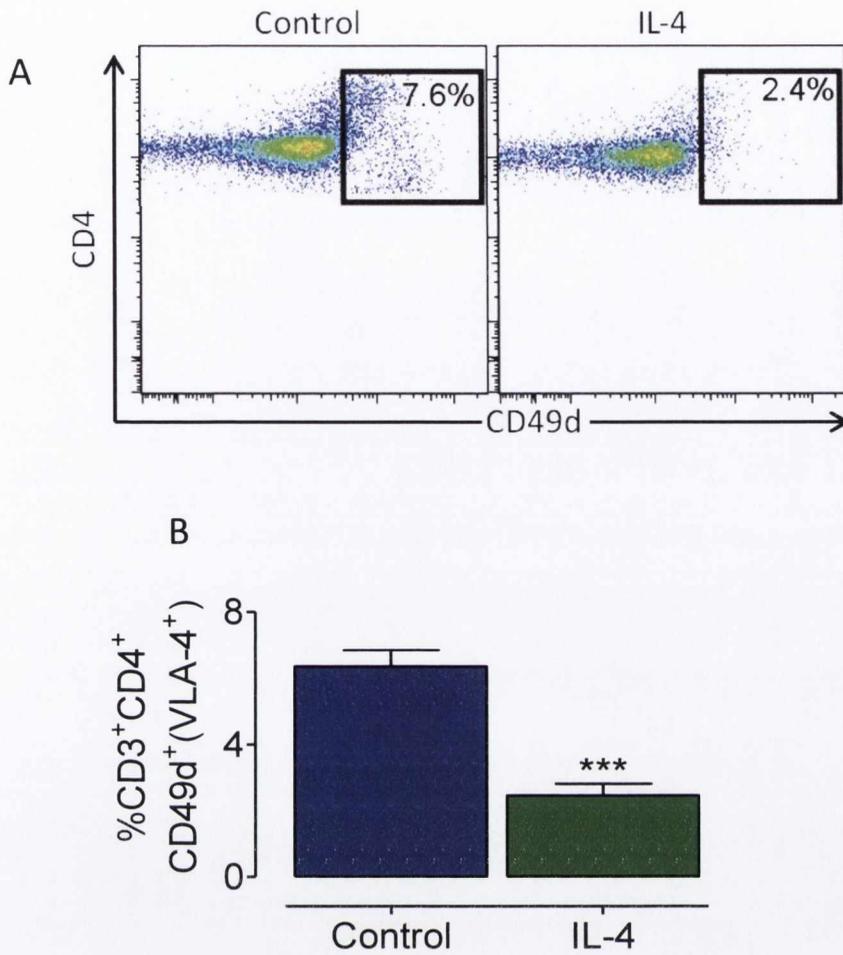


Figure 6.2 IL-4 reduces expression of VLA-4 by CD4⁺ T cells in a MOG-specific culture

Donor mice were immunised with MOG and CFA. After 10 d, spleen and LN cells were cultured for 72 h in a 70:30 ratio with MOG (100 µg/ml), IL-1β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of IL-4 (20 ng/ml). VLA-4-expressing cells were identified using FACS (A) and expressed as a frequency of the total CD3⁺CD4⁺ population (B). ***p<0.001 versus control; t-test. Data are presented as the mean ± SEM (n=3).

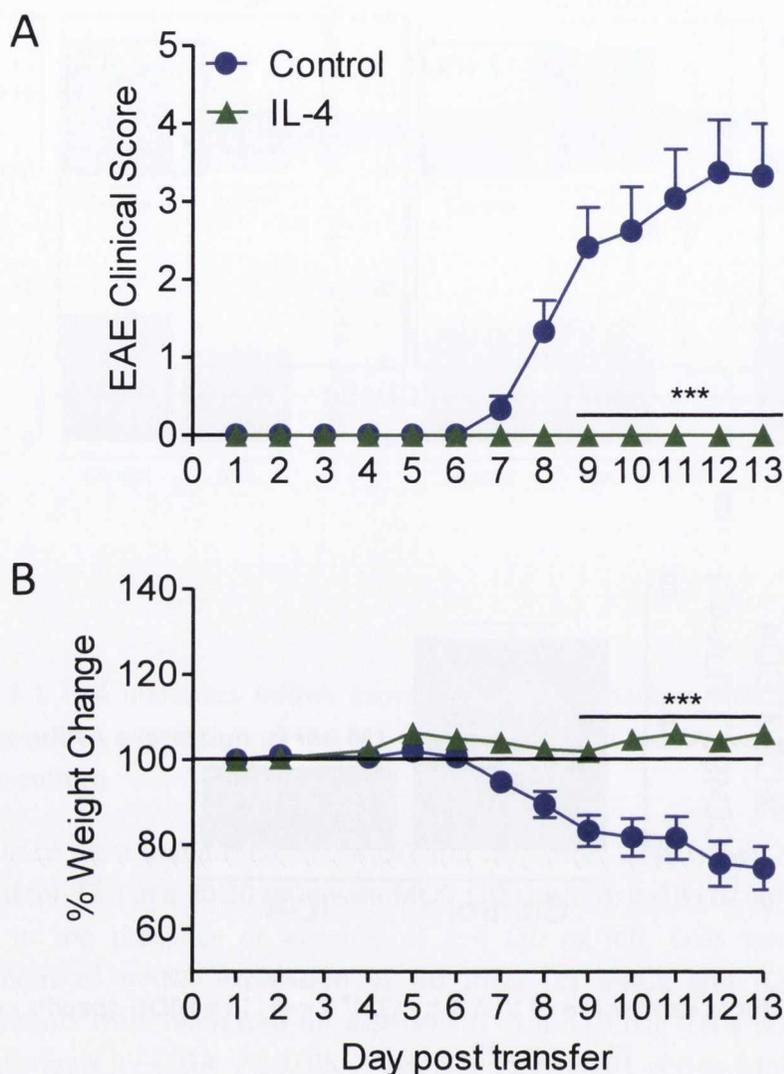


Figure 6.3 IL-4 prevents the ability of MOG-specific cells to transfer EAE to recipient mice

Donor mice were immunised with MOG and CFA. After 10 d, spleen and LN cells were cultured for 72 h in a 70:30 ratio with MOG (100 $\mu\text{g/ml}$), IL-1 β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of IL-4 (20 ng/ml). Cells were washed and injected into recipient mice (15×10^6 /mouse). Mice were assessed for the development of EAE by (A) clinical score and (B) % weight change. *** $p < 0.001$ versus controls; two-way ANOVA with Bonferroni post-hoc test. Data are presented as the mean \pm SEM ($n=6$)

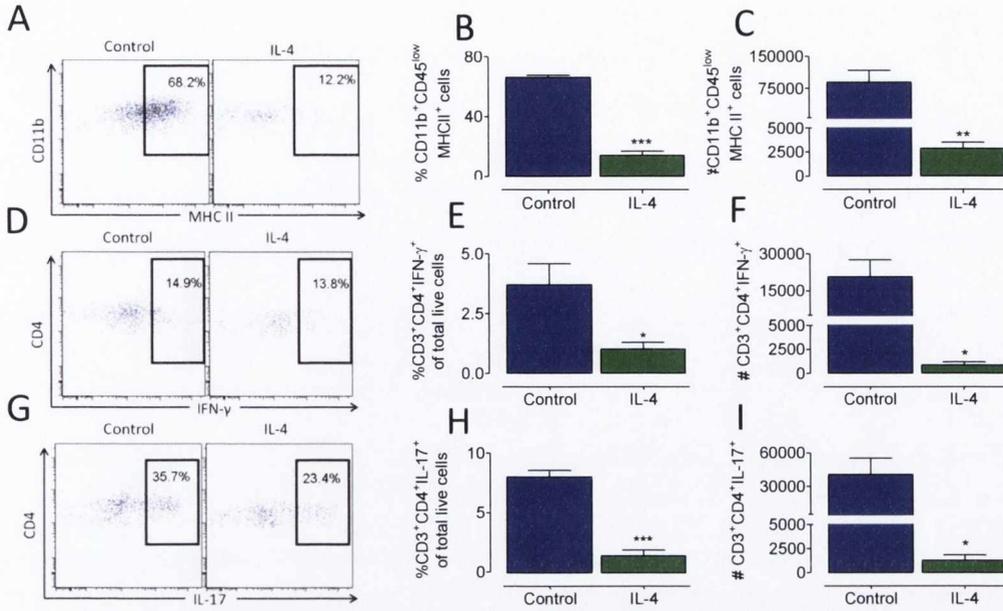


Figure 6.4 Adoptive transfer of IL-4-treated MOG-specific cells reduces MHC II expression by CD11b⁺CD45^{low} cells, and the number of infiltrating CD3⁺CD4⁺IFN- γ ⁺ and CD3⁺CD4⁺IL-17⁺ cells in the brains of recipient mice

Donor mice were immunised with MOG and CFA. After 10 d, spleens and LN cells were cultured for 72 h in a 70:30 ratio in the presence of MOG (100 μ g/ml), IL-1 β (10 ng/ml) and IL-23 (10 ng/ml) in the presence or absence of IL-4 (20 ng/ml). Cells were washed and injected into recipient mice (15 \times 10⁶/mouse). Mice were sacrificed on day 13. CD11b⁺CD45^{low}MHC II⁺, CD3⁺CD4⁺IFN- γ ⁺ and CD3⁺CD4⁺IL-17⁺ cells were identified in the brains using FACS (A, D, G) and expressed as a frequency (B, E, H) of the total CD11b⁺CD45^{low}/ live cell population. The absolute number of CD11b⁺CD45^{low}MHC II⁺, CD3⁺CD4⁺IFN- γ ⁺ and CD3⁺CD4⁺IL-17⁺ cells were calculated from total mononuclear cell counts in the brains (C, F, I). *p<0.05, **p<0.01, ***p< 0.001 versus controls; t-test. Data are presented as the mean \pm SEM (n=6).

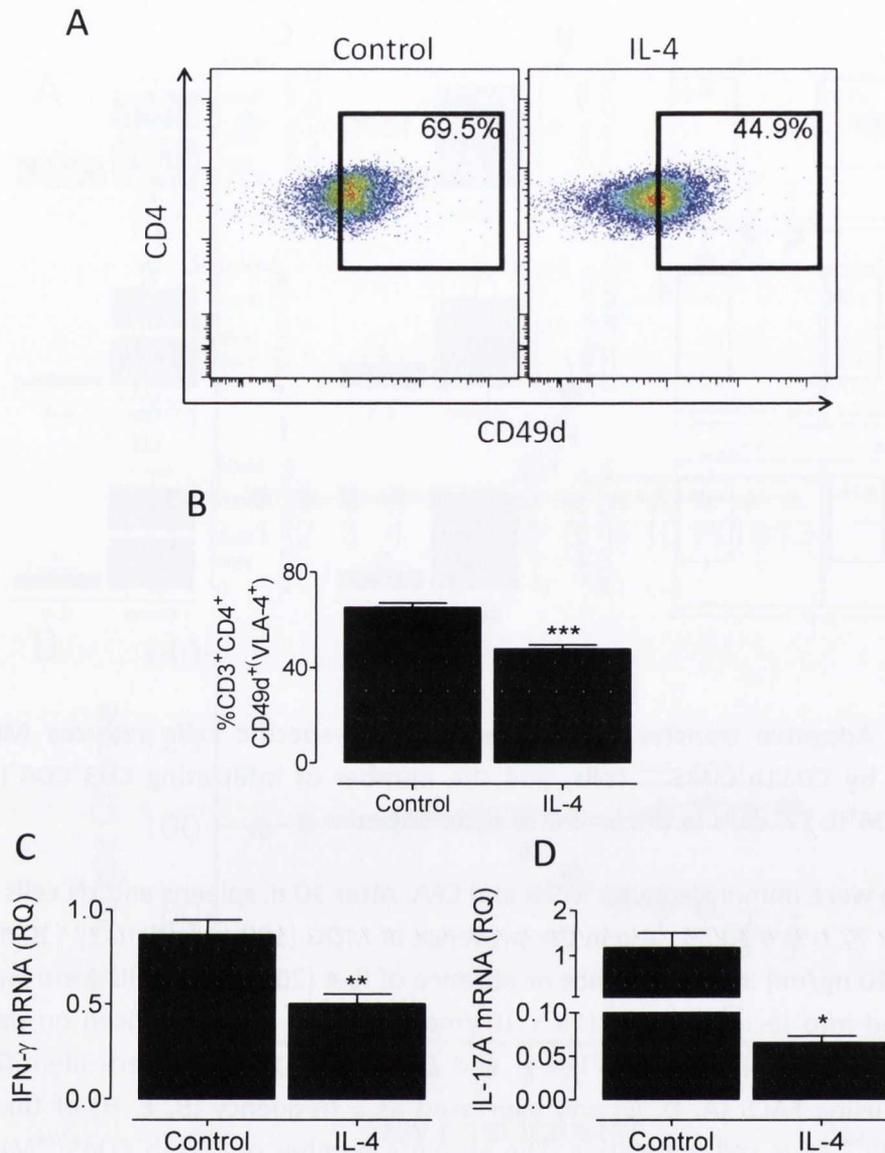


Figure 6.5 IL-4 reduces expression of CD49d, and mRNA expression of IFN- γ and IL-17A by anti-CD3 and anti-CD28-stimulated CD3⁺ T cells

CD3⁺ T cells were isolated from murine spleens and stimulated for 24 h with anti-CD3 (1 μ g/ml) and anti-CD28 (5 μ g/ml) in the presence or absence of IL-4 (20 ng/ml). VLA-4-expressing CD4⁺ T cells (CD3⁺CD4⁺CD49d⁺) were identified using FACS (A) and expressed as a frequency of the total CD4⁺ population (B) or harvested for assessment of mRNA expression of IFN- γ (C) and IL-17A (D) by PCR. * p <0.05, ** p <0.01, *** p <0.001 versus control; t-test. Data are presented as the mean \pm SEM (n =3).

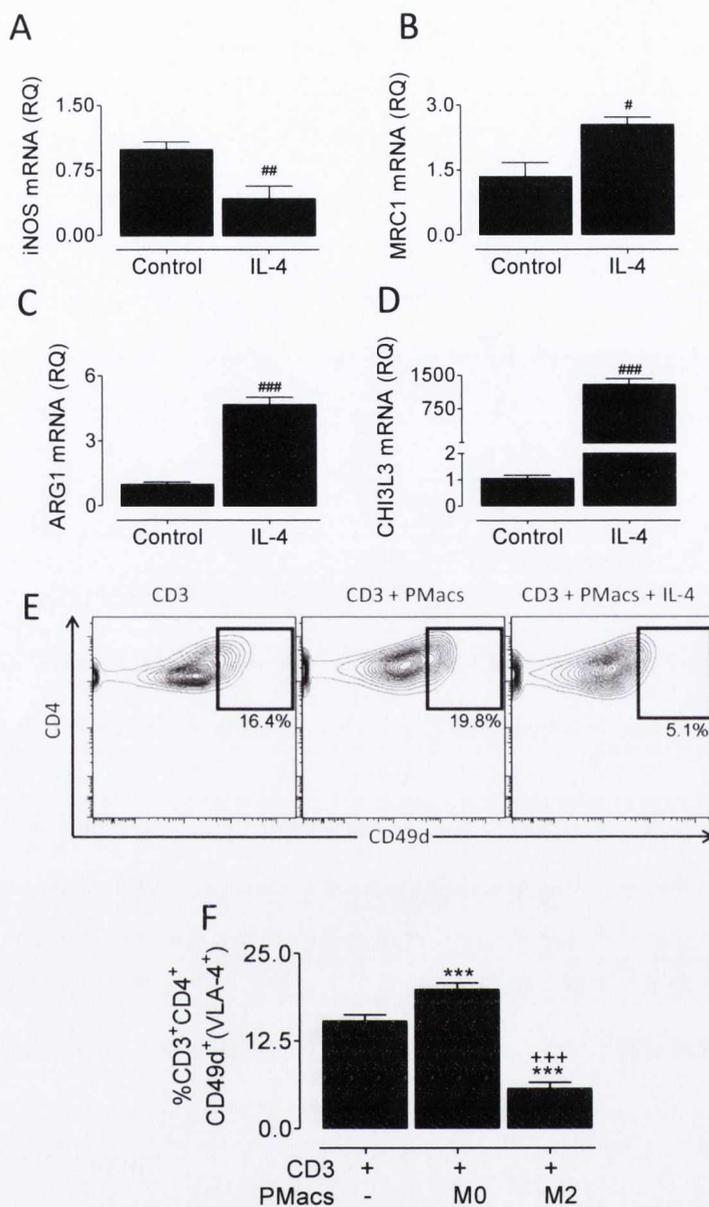


Figure 6.6 Incubation with M2 peritoneal macrophages reduces VLA-4 expression on CD3⁺ T cells

Peritoneal macrophages (PMacs) were incubated with medium alone (M0) or IL-4 (20 ng/ml; M2). After 6 h cells were harvested and assessed for mRNA expression of iNOS (A), MRC1 (B), ARG1 (C) and CHI3L3 (D) by PCR. PMacs were washed twice with PBS and incubated for 72 h with CD3⁺ T cells in the presence of anti-CD3 (5 µg/ml). VLA-4-expressing CD4⁺ T cells (CD3⁺CD4⁺CD49d⁺) were identified using FACS (E) and expressed as a frequency of the total CD4⁺ population (F). [#]p<0.05, ^{##}p<0.01, ^{###}p<0.001 versus controls; t-test. ^{***}p<0.001 versus CD3 alone, ⁺⁺⁺p<0.001 versus CD3 and PMacs; one-way ANOVA with Neuman-Keuls post-hoc test. Data are presented as the mean ± SEM (n=3)

6.3 Discussion

The data in Chapters 4 and 5 demonstrated that NK cells are pathogenic in EAE through production of IFN- γ , which polarises M1 macrophages and enhances VLA-4 expression on CD4⁺ T cells. This chapter focused on assessing the modulatory role of the M2-polarising cytokine IL-4 in the pathogenesis of EAE. The data show that IL-4 can confer protection against passive EAE through the induction of M2 macrophages and a reduction in VLA-4 expression on CD4⁺ T cells. Crucially, this highlights an important role for M2 macrophages in the control of EAE, through suppression of pathogenic T cells.

Addition of IL-4 to a culture of MOG-specific cells completely prevented the transfer of disease in the passive EAE model. IL-4, even in the presence of MOG, IL-1 β and IL-23, was successful in polarising cells to the M2 phenotype, as reflected by the enhanced mRNA expression of MRC1 and ARG1, and reduced mRNA expression of iNOS in the culture. Analysis of the cytokine production by MOG-specific cells at the end of the culture period revealed that the addition of IL-4 also significantly reduced IL-17A production. While IL-17A has an important role in the encephalitogenicity of CD4⁺ T cells in EAE (Langrish et al., 2005), IL-4-treated cells still produced 60% of that detected in the supernatants from cells cultured with MOG, IL-1 β and IL-23 in the absence of IL-4, suggesting that the complete prevention of disease by cell transfer was unlikely to be entirely due to this observed reduction in IL-17A. Furthermore, the production of GM-CSF, which has more recently emerged as a critical factor in the encephalitogenicity of IL-23-polarised Th17 cells (El-Behi et al., 2011a), was not reduced in the supernatants taken from cells cultured with additional IL-4.

In Chapter 5, it was shown that M1 macrophages enhanced VLA-4 expression by MOG-specific CD4⁺ T cells. It was also demonstrated that VLA-4 plays an important role in the pathogenesis of EAE; blockade of the integrin using a monoclonal antibody against CD49d significantly reduced EAE severity in both the active and passive models. Vitally, neutralisation of VLA-4 at the time of cell transfer completely prevented EAE for almost 20 days after the last dose of antibody. Here it was hypothesised that IL-4-mediated enhancement of the M2 state in these cultures may oppose the stimulatory

effects of M1 macrophages on CD4⁺ T cell VLA-4 expression, and help to explain the failure of the IL-4-treated cultures to transfer disease. Indeed, on analysis of the CD4⁺ population in the culture, VLA-4 expression was indeed reduced by addition of IL-4.

The absence of clinical signs in mice that received MOG-specific cells cultured with IL-4 also corresponded with an alteration in some of the neuroinflammatory readouts of disease. Firstly, these mice exhibited diminished expression of the M1 marker MHCII on microglia relative to mice that received cells cultured with MOG, IL-1 β and IL-23 in the absence of IL-4. Microglial activation is an important contributor to the pathogenesis of EAE, and interventions that prevent microglial activation are efficacious in reducing disease severity (Bhasin et al., 2007). Crucially, the recipient mice that received IL-4-treated cells also displayed a striking reduction in the infiltration of both Th1 and Th17 cells into the brain. This is in agreement with the previous observation that the expression of VLA-4 by CD4⁺ T cells in the culture was significantly reduced by the addition of IL-4. VLA-4 expression is required for CD4⁺ T cells to gain access to the brain in EAE (Yednock et al., 1992b).

So far, it has been demonstrated that addition of IL-4 to a culture of MOG-specific cells prevents the transfer of EAE. IL-4 induced the cells to adopt an M2-like phenotype and reduced VLA-4 expression by CD4⁺ T cells. As the MOG-specific culture is a heterogeneous population of splenic and lymph node cells, it was still unclear whether IL-4 was acting directly on CD4⁺ T cells to reduce VLA-4 expression, or whether this occurred indirectly through M2 macrophages. Interestingly, the addition of IL-4 directly to stimulated CD3⁺ T cells was sufficient to reduce VLA-4 expression, as well as mRNA expression of both IFN- γ and IL-17. This is in agreement with a study by Sasaki and colleagues, who reported that murine CD4⁺ T cells cultured in Th2 conditions (with IL-4) displayed significantly reduced VLA-4 expression relative to those cultured in Th1-polarising conditions (with IL-12 and IFN- γ). Furthermore, this reduction in VLA-4 expression in the Th2-polarised cells could be prevented by the inclusion of anti-IL-4 to the culture (Sasaki et al., 2009). This suggests that IL-4 may act directly on CD4⁺ T cells in the culture to attenuate encephalitogenicity via reduction of VLA-4 expression.

The observation that the IL-4-treated MOG-specific culture still maintained an M2 bias even in the presence of MOG, IL-1 β and IL-23 suggested a potential role for M2-polarised innate cells in preventing EAE by cell transfer. In order to address this, peritoneal macrophages were treated with IL-4, and then incubated with CD3⁺ T cells in the presence of anti-CD3. Peritoneal macrophages which had been treated with IL-4 successfully adopted the M2 state, as shown by significant upregulation in expression of the M2 markers MRC1, ARG1 and CHI3L3, as well as reduced mRNA expression of the M1 marker iNOS. Crucially, interaction with these M2 macrophages significantly reduced VLA-4 expression by CD4⁺ T cells. These data are consistent with those presented in Chapter 5, which demonstrated a role for M1 macrophages in enhancing VLA-4 expression on CD4⁺ T cells.

Taken together, the results in this chapter indicate a suppressive role for IL-4 in the induction of EAE, through induction of M2 macrophages and a reduction of VLA-4 expression by CD4⁺ T cells.

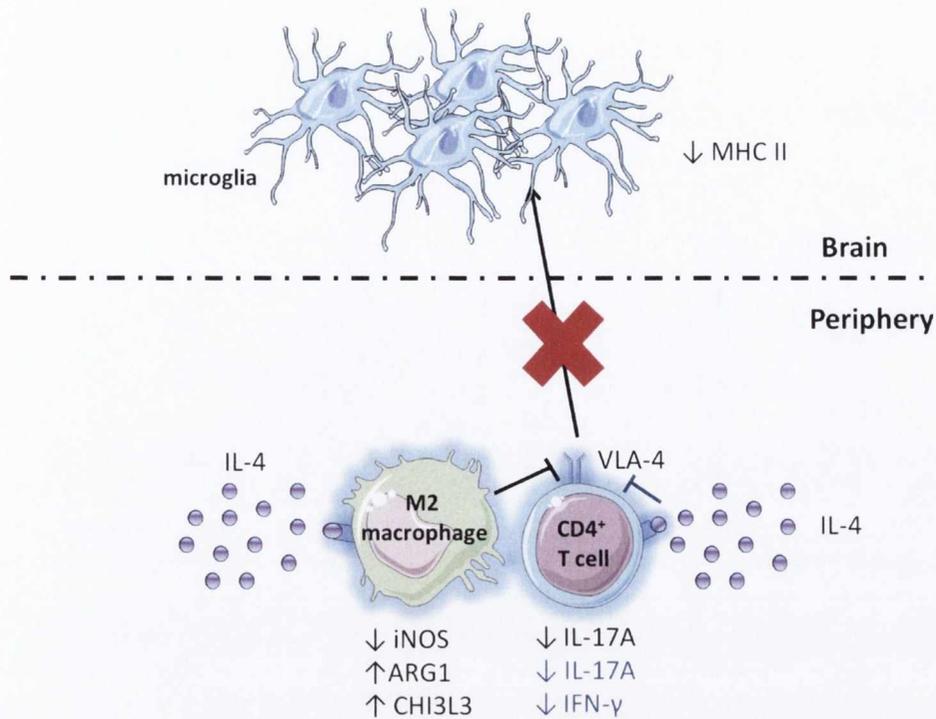


Figure 6.7 IL-4 can prevent the transfer of EAE through induction of M2 macrophages and reduction of VLA-4 expression by CD4⁺ T cells

IL-4 reduces mRNA expression of the M1 marker iNOS with a concurrent reduction in the M2 markers MRC1, ARG1 and CHI3L3 in macrophages. M2 macrophages can impede CD4⁺ T cell encephalitogenicity through a reduction in VLA-4 expression. This prevents EAE induced by cell transfer. As such, the sequelae of EAE, such as infiltration of Th1 and Th17 cells, as well as microglial activation (MHC II expression), are also reduced.

Chapter 7: General Discussion

Chapter 7: General Discussion

While it is widely accepted that the vital pathogenic event of both EAE and MS is the infiltration of myelin-reactive CD4⁺ T cells into the CNS, the contribution of the innate immune system to the generation of T cell encephalitogenicity must not be neglected. This study has identified a role for NK cells, macrophages and microglia in mediating the pathogenic events preceding the CNS invasion of CD4⁺ T cells. The findings revealed that NK cells infiltrated the brain and spinal cord before the onset of EAE, and produced IFN- γ , which enhanced M1 activation of microglia. In the periphery, NK cell-produced IFN- γ polarised macrophages to the M1 phenotype, which in turn enhanced CD4⁺ T cell expression of VLA-4, an integrin which is required for the infiltration of these cells into CNS. In contrast, IL-4-mediated M2 polarisation of macrophages downregulated expression of VLA-4 on CD4⁺ T cells and thus prevented the development of EAE. Taken together, these findings highlight the potential of targeting both the innate and adaptive arms of autoimmunity in the development of future therapies for MS.

7.1 The role of NK cells in the pathogenesis of EAE

The role of NK cells in the pathogenesis of EAE is controversial, as evidenced by the lack of consensus in the literature regarding their contribution to disease. Most of the current opinions are derived from studies on NK cell depletion using antibodies against NK1.1 or asialo GM1. Administration of anti-NK1.1 induces apoptosis of NK1.1-expressing cells, such as NK and NKT cells (Smyth et al., 2000) and anti-asialo GM1 induces complement-mediated lysis of NK cells (Shimada and Iwata, 1987). Prior to the present study, the proposed role of NK cells in EAE could be divided into two opposing schools of thought. The first suggests that NK cells protect against EAE either through killing of autoreactive T cells (Xu et al., 2005, Lu et al., 2007a) and microglia (Hao et al., 2010a) or prevention of CD4⁺ T cell proliferation (Zhang et al., 1997a). In these studies, the authors observed that administration of anti-NK1.1 enhanced the severity of EAE. The second school of thought is governed by the underlying principle that NK cells may contribute to EAE pathogenicity by producing IFN- γ , thus promoting auto-aggressive Th1 cells (Shi et al., 2000, Vollmer et al., 2005, Winkler-Pickett et al., 2008). In these studies, the depletion of NK cells using either anti-asialo GM1 or anti-NK1.1 was found to be ameliorative, thus reducing EAE clinical scores.

It is likely that the lack of consensus regarding the effect of NK depletion on the clinical course of EAE is due to discrepancies in the species, depleting antibodies and administration regimen used in these studies. The findings of the present study favour the latter theory that NK cells are pathogenic in EAE, specifically early in disease. Administration of anti-asialo GM1 significantly reduced the clinical severity of disease when administered before induction, and subsequently on days 1 and 3 post-induction. However, administration of the antibody continuously throughout disease or at the effector stage of disease (from day 7 onwards) had no significant effect on disease outcome. This is in agreement with the previously cited studies by Shi and colleagues, and Winkler-Pickett and colleagues, who both reported that the depletion of NK cells did not alter the clinical severity of EAE if administered after disease induction.

In agreement with the observation that NK cells were pathogenic early in EAE, the present study found that NK cells infiltrated the brain and spinal cord in significant numbers before the onset of disease. On day 7, NK cells accounted for almost 30% of live cells detected in the brains of mice with EAE. At this time around half of the CNS-infiltrating NK cells were IFN- γ -positive. Interestingly, the appearance of IFN- γ -producing NK cells in the brain corresponded with a substantial increase in the frequency of TNF- α -positive microglia. TNF- α is a signature cytokine of the IFN- γ -induced M1 state and can contribute to EAE pathology through induction of apoptosis in oligodendrocytes, as well as enhancing demyelination. Furthermore, TNF- α levels in the CSF have been found to positively correlate with disease severity in MS patients (Finsen et al., 2001). Further investigation revealed that Th1 cells, which have classically been perceived as the dominant source of IFN- γ in the CNS of mice with EAE (Wheeler et al., 2006), were not detected in significant numbers in the brain until the peak of disease. This suggests that NK cells may be responsible for the early activation of microglia in EAE which has been reported here and in previous studies (Ponomarev et al., 2005, Ajami et al., 2011a).

7.2 The role of NK cells in enhancing the M1 phenotype in glia

The novel finding that early NK cell infiltration into the CNS corresponded with an increase in the production of the M1 cytokine TNF- α by microglia led to further investigation of the role of NK cells in glial polarisation. In agreement with the kinetics study, incubation of microglia with NK cell-conditioned medium, which was found to contain IFN- γ , elicited an upregulation in mRNA expression of TNF- α and MHC II as well as the archetypal M1 marker iNOS. Furthermore, this conditioned medium elicited a corresponding decrease in the mRNA expression of M2 markers MRC1 and ARG1. Interestingly, the co-incubation of microglia and NK cells induced high levels of microglial cytotoxicity, as evidenced by the significant enhancement in the detection of LDH in the co-culture supernatants. That NK cells can kill microglia *in vitro* has been demonstrated before in human cells (Lünemann et al., 2008) albeit with a much higher ratio of NK cells to microglia than used in the current study.

In contrast, NK cells did not prove cytotoxic when incubated with a mixed glial culture (containing microglia and astrocytes). Instead, similar to the observations made in primary microglia, the addition of NK cells or NK cell-conditioned medium enhanced mRNA expression of the M1 markers iNOS, MHC II and TNF- α , with a corresponding reduction in the mRNA expression of the M2 marker MRC1. This suggests that isolated primary microglia may be particularly vulnerable to the cytotoxic effects of NK cells, but protected in the presence of astrocytes. Under physiological conditions, microglial function is likely to be influenced by adjacent astrocytes, as evidenced by the fact that conditioned medium from astrocytes can diminish aberrant microglial activation and restore a ramified morphology to activated cells (Hailer et al., 2001). The presence of astrocytes in the mixed glial cultures may therefore explain why there was no significant increase in the detection of LDH in the co-culture supernatants after the addition of NK cells. Taken together, these data suggest that NK cells may play a role in enhancing M1 activation in microglia, which is a pathological hallmark of a number of neurodegenerative diseases and can contribute to neuronal loss in AD (Lynch, 2014), PD (Moehle and West, 2014), ALS (Frakes et al., 2014) MS (Strachan-Whaley et al., 2014) and EAE (Prinz and Priller, 2014). Harnessing microglial activation and orchestrating a switch from the inflammatory M1 state to the modulatory M2 state

has proven effective in reducing EAE severity. Administration of galectin-1 or intracerebroventricular administration of galectin-1-treated microglia significantly reduced EAE clinical scores, which was attributed to the ability of galectin-1 to deactivate M1 microglia (Starossom et al., 2012). More recently, a small molecule inhibitor of microglial activation, inflachromene, has also been found to suppress EAE (Lee et al., 2014). Finally, adoptive transfer of M2-polarised microglia can alleviate disease severity in ongoing EAE (Zhang et al., 2014).

NK cell depletion reduced the number of microglia in the brain, and as microglia have been found to proliferate in times of inflammatory stress such as EAE (Ding et al., 2014), this suggests that the pathogenic role of NK cells early in disease may be mediated through effects on microglia. However, this was unlikely to account for the reduced number of infiltrating CD4⁺ T cells in the brains of NK-depleted mice.

7.3 The role of NK cells in inducing the M1 phenotype in macrophages and enhancing VLA-4 expression by CD4⁺ T cells

It has been suggested that NK cells may play a role in polarisation of macrophages to the M1 phenotype in the literature, although little direct evidence to support this hypothesis has been provided. Most of the available studies on the interplay between macrophages and NK cells have focused on how macrophage phenotypes impact upon NK cell activation rather than the effect of NK cells on macrophage phenotype. For example, during infection with *Salmonella*, macrophages become polarised to the M1 state and produce IL-12, and thus enhance IFN- γ production by NK cells (Lapaque et al., 2009). The current study provided evidence of a definitive role for NK cells in polarising M1 macrophages. Incubation of BMDMs with either NK cells or NK cell-conditioned medium enhanced mRNA expression of the M1 markers iNOS and MHC II, and reduced expression of the M2 markers MRC1 and CHI3L3. Like microglia, M1 macrophages have also been implicated in the pathology of EAE (Hu et al., 2015), although most of the focus has been placed on brain-resident or brain-infiltrating macrophages, which can be difficult to distinguish from microglia (Greter et al., 2015).

7.4 A crucial role for VLA-4 in CD4⁺ T cell encephalitogenicity in EAE

VLA-4 ($\alpha 2\beta 1$ integrin) is a critical mediator of the encephalitogenicity of CD4⁺ T cells in EAE and is required for the infiltration of these cells into the CNS (Baron et al., 1993). Such is the importance of VLA-4 in T cell invasion of the CNS, a humanised monoclonal antibody against $\alpha 2$ integrin (CD49d), also called natalizumab, is one of the leading treatments for MS (Castro-Borrero et al., 2012), and it has been well accepted for over 20 years that blockade of this integrin can abolish clinical signs of EAE (Yednock et al., 1992b). Crucially, expression of CD49d was highest in CD4⁺ T cells that were incubated with NK cells and macrophages (which had adopted the M1 state as a result). Taken together, these data suggest that NK cells are pathogenic early in EAE through M1 polarisation of glia and macrophages, the latter of which can enhance expression of CD49d on CD4⁺ T cells.

The role of CD49d in mediating pathogenicity in EAE was also confirmed in this study through administration of anti-CD49d in both the active and passive models of EAE. Administration of the antibody after the induction of active EAE (from day 7 onwards) significantly reduced the clinical scores. However, it is important to note that even with administration of the antibody before the onset of disease, an EAE incidence of 30 % was still observed. This was in agreement with a recent study by Mindur and colleagues, who reported reduced efficacy against EAE when anti-VLA-4 was administered later in disease (Mindur et al., 2014). In contrast, the current study showed that administration of the antibody at the time of adoptive transfer of MOG-specific cells and every 2 days until day 12 completely prevented EAE for almost 20 days after the final administration of antibody. This suggests that prevention of T cell infiltration is most efficacious at the induction of disease and explains some of the shortcomings of current therapies for MS that can only be administered once disease has begun to manifest.

7.5 NK cells: an early source of IFN- γ in EAE

NK cell pathogenicity was found to be due to early IFN- γ production, as evidenced by the fact that early NK cell depletion did not affect the course of active EAE in IFN- γ ^{-/-} mice. The suggestion that the role of NK cells in EAE was primarily mediated by

cytokine production rather than cytotoxicity was also made by Winkler-Pickett and colleagues, who reported that studies in perforin knockout mice revealed no regulatory role in primary EAE induction (Winkler-Pickett et al., 2008).

NK cells can rapidly mobilise IFN- γ after immunisation or infection, which is thought to be due to constitutive expression of IFN- γ mRNA transcripts (Stetson et al., 2003). The role of IFN- γ in EAE is contentious, however. A pathogenic role is consistent with the observations that administration of IFN- γ to MS patients worsened disease severity (Panitch et al., 1987), and transgenic expression of IFN- γ in murine oligodendrocytes prevented remission from EAE (Renno et al., 1998). However, EAE can still occur in mice with a disrupted IFN- γ gene (Ferber et al., 1996), and administration of anti-IFN- γ exacerbated disease in both the active (Billiau et al., 1988) and passive (Duong et al., 1992) forms of EAE. This suggests a complex role for IFN- γ in the pathogenesis of EAE. Data from the current study have demonstrated that EAE onset is delayed in IFN- γ ^{-/-} mice, with increased severity later in disease. This suggests that IFN- γ is pathogenic early in EAE, but protective later. In a recent publication from our laboratory, it was demonstrated that MOG-specific cells from IFN- γ ^{-/-} mice failed to transfer disease to WT mice (Dungan et al., 2014). Cultures of spleen and lymph node cells from IFN- γ ^{-/-} mice immunised with MOG and CFA were found to have a reduction in M1 macrophages and VLA-4 expression on CD4⁺ T cells, suggesting that IFN- γ is required for the induction of disease through M1 polarisation and enhancement of the encephalitogenicity of CD4⁺ T cells. The current study suggests that NK cells provide an early innate source of IFN- γ . This is in agreement with a recent study in which NK cells have been proposed as the principal early innate source of IFN- γ in experimental autoimmune uveitis (EAU) (Chong et al., 2015).

Thus far, a pathogenic role for NK cells early in EAE has been presented, which was attributed to production of IFN- γ , a cytokine whose role in M1 polarisation of innate immune cells was extensively characterised in Chapter 3. A bias toward the M1 state is a hallmark of a number of inflammatory pathologies, and in this study the M1 macrophage has been highlighted as a critical mediator of T cell encephalitogenicity. IL-4 on the other hand, is capable of inducing the M2 state, which is associated with resolution of inflammation and tissue repair, and which was also characterised in

innate immune cells in Chapter 3. The M1 and M2 states are two extremes of what is often referred to as a “continuum” of activation (Martinez and Gordon, 2014), and interventions which skew the immune response toward the latter have been successful in reducing pathology in a number of animal models of autoimmune disorders such as the APP/PS1 model of AD, (Heneka et al., 2013), the mutant superoxide dismutase (mSOD1) model of ALS (Liao et al., 2012) and EAE (Liu et al., 2013).

7.6 A role for IL-4 in reducing the encephalitogenicity of CD4⁺ T cells

The addition of IL-4 to a culture of MOG-specific cells re-stimulated with MOG, IL-1 β and IL-23 induced an M2 bias in the culture, as demonstrated by increased expression of MRC1 and ARG1, and reduced expression of iNOS. Interestingly, the addition of IL-4 to the culture reduced VLA-4 expression by MOG-specific CD4⁺ T cells. The detection of the M2 state in combination with reduced VLA-4 expression by CD4⁺ T cells is in agreement with data from Chapter 5, in which M1 macrophages were conversely shown to enhance VLA-4 expression on MOG-specific CD4⁺ T cells. Vitrally, culture of MOG-specific cells with exogenous IL-4 suppressed their ability to transfer EAE to naïve recipient mice. Analysis of the brains of recipient mice on day 13 post transfer revealed that the mice that received MOG-specific cells cultured with MOG, IL-1 β and IL-23 with exogenous IL-4 displayed reduced expression of the M1 marker MHC II on microglia. This was associated with a marked attenuation of the infiltration of Th1 and Th17 cells relative to mice that received cells that were cultured with MOG, IL-1 β and IL-23 without IL-4. The latter observation of a diminished infiltration of CD4⁺ T cells into the brain is likely due to the observed reduction in expression of VLA-4, which has a crucial role in enhancing CD4⁺ T cell entry to the CNS.

As the MOG-specific culture used in adoptive transfer model of EAE was a heterogeneous population of spleen and lymph node cells, it was initially unclear whether IL-4 was acting directly on CD4⁺ T cells to reduce VLA-4 expression, or whether this effect was mediated by M2-polarised innate cells. Subsequent *in vitro* assays revealed that IL-4 could act directly upon CD4⁺ T cells to elicit a reduction in VLA-4 expression, which is in agreement with a previous study which detailed the

suppressive effect of IL-4 on VLA-4 expression by committed Th1 cells (Sasaki et al., 2009) and type I CD8⁺ T cells (Sasaki et al., 2008). However, incubation of CD4⁺ T cells with M2-polarised macrophages also elicited a decrease in expression of VLA-4. Taken together, these data suggest that the observed loss of encephalitogenicity by the MOG-specific cultures with addition of IL-4 is due to either a direct suppression of VLA-4 expression on CD4⁺ T cells, indirectly through induction of M2 macrophages, or a combination of the two.

7.7 Conclusions

Overall, the findings of this study highlight a crucial role for cells of the innate immune system in the induction of EAE (**Figure 7.1**). NK cells infiltrate the CNS before disease onset and contribute to local neuroinflammation by virtue of innate IFN- γ production, which can polarise glia to the M1 state. In the periphery, IFN- γ -producing NK cells also mediate EAE pathogenesis through polarisation of macrophages to the M1 phenotype and enhancement of VLA-4 expression by CD4⁺ T cells. Conversely, the induction of the M2 phenotype in macrophages by IL-4 can attenuate expression of this integrin and thus prevent the development of disease.

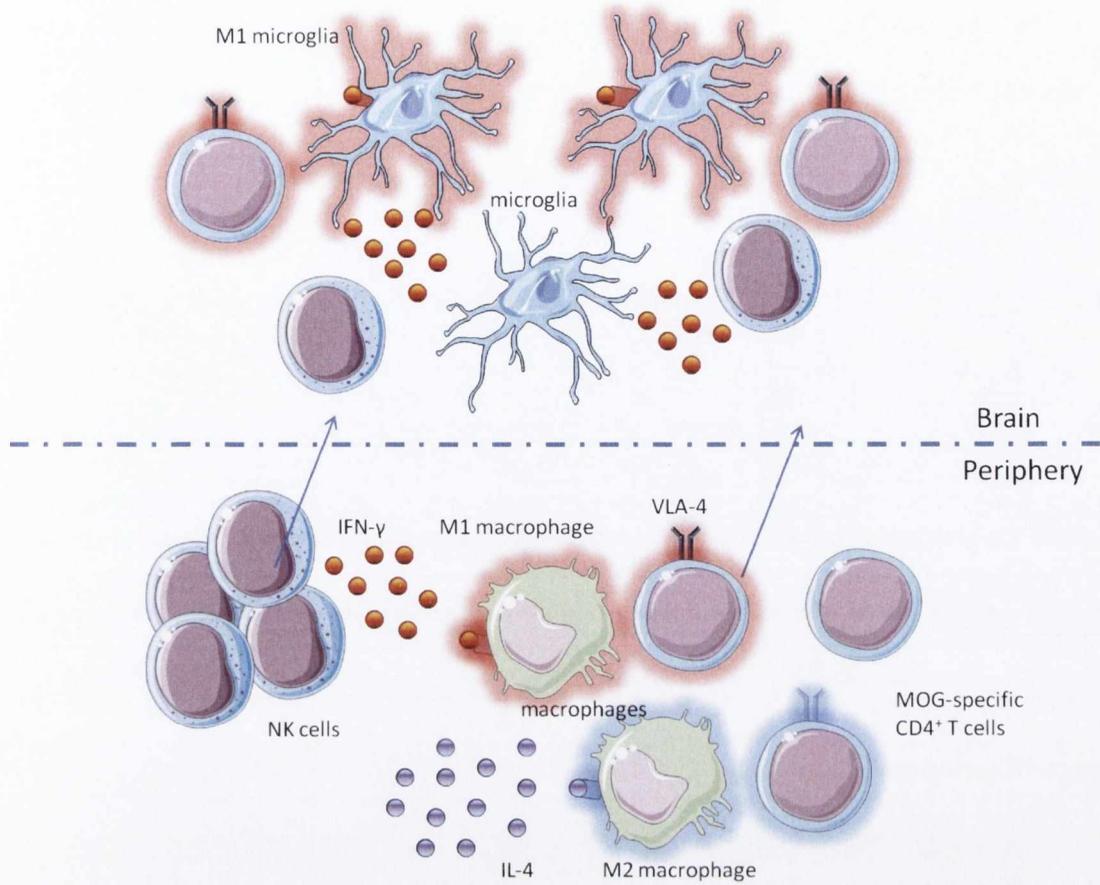


Figure 7.1 Schematic detailing the proposed mechanism of action of NK-derived IFN- γ and recombinant IL-4 in modulating CD4⁺ T cell encephalitogenicity in EAE

NK cells enter the CNS early in EAE and produce IFN- γ , which polarises microglia to the M1 phenotype. In the periphery, IFN- γ from NK cells polarises macrophages to the M1 phenotype, which interact with CD4⁺ T cells and enhance VLA-4 expression, thus allowing pathogenic CD4⁺ T cells to infiltrate the brain. In contrast, IL-4 can induce M2 activation of macrophages, which suppress VLA-4 expression by CD4⁺ T cells, thus preventing their infiltration into the brain.

Chapter 8: Bibliography

Chapter 8: Bibliography

- ABBOTT, N. J., RÖNNBÄCK, L. & HANSSON, E. 2006. Astrocyte–endothelial interactions at the blood–brain barrier. *Nature Reviews Neuroscience*, 7, 41-53.
- AHN, M., LEE, C., JUNG, K., KIM, H., MOON, C., SIM, K.-B. & SHIN, T. 2012a. Immunohistochemical study of arginase-1 in the spinal cords of rats with clip compression injury. *Brain research*, 1445, 11-19.
- AHN, M., YANG, W., KIM, H., JIN, J.-K., MOON, C. & SHIN, T. 2012b. Immunohistochemical study of arginase-1 in the spinal cords of Lewis rats with experimental autoimmune encephalomyelitis. *Brain research*, 1453, 77-86.
- AJAMI, B., BENNETT, J. L., KRIEGER, C., MCNAGNY, K. M. & ROSSI, F. M. 2011a. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nature neuroscience*, 14, 1142-1149.
- AJAMI, B., BENNETT, J. L., KRIEGER, C., MCNAGNY, K. M. & ROSSI, F. M. V. 2011b. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nature neuroscience*, 14, 1142-1149.
- ALBINA, J. E., MILLS, C., HENRY, W. & CALDWELL, M. 1990. Temporal expression of different pathways of 1-arginine metabolism in healing wounds. *The Journal of Immunology*, 144, 3877-3880.
- ALMEIDA, A., ALMEIDA, J., BOLAÑOS, J. P. & MONCADA, S. 2001. Different responses of astrocytes and neurons to nitric oxide: the role of glycolytically generated ATP in astrocyte protection. *Proceedings of the National Academy of Sciences*, 98, 15294-15299.
- AMOR, S., PUENTES, F., BAKER, D. & VAN DER VALK, P. 2010. Inflammation in neurodegenerative diseases. *Immunology*, 129, 154-169.
- ANFOSSI, N., ANDRÉ, P., GUIA, S., FALK, C. S., ROETYNCK, S., STEWART, C. A., BRESO, V., FRASSATI, C., REVIRON, D., MIDDLETON, D., ROMAGNÉ, F., UGOLINI, S. & VIVIER, E. 2006. Human NK Cell Education by Inhibitory Receptors for MHC Class I. *Immunity*, 25, 331-342.
- ARELLANO, G., OTTUM, P. A., REYES, L. I., BURGOS, P. I. & NAVES, R. 2015. Stage-specific role of interferon-gamma in experimental autoimmune encephalomyelitis and multiple sclerosis. *Frontiers in immunology*, 6.
- ASEA, A. & STEIN-STREILEIN, J. 1998. Signalling through NK1. 1 triggers NK cells to die but induces NK T cells to produce interleukin-4. *Immunology*, 93, 296.
- BARON, J. L., MADRI, J. A., RUDDLE, N. H., HASHIM, G. & JANEWAY, C. 1993. Surface expression of alpha 4 integrin by CD4 T cells is required for their entry into brain parenchyma. *The Journal of experimental medicine*, 177, 57-68.
- BAXTER, A. G. 2007. The origin and application of experimental autoimmune encephalomyelitis. *Nature Reviews Immunology*, 7, 904-912.
- BECHER, B., DURELL, B. G. & NOELLE, R. J. 2002. Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. *The Journal of clinical investigation*, 110, 493-497.
- BECHER, B., DURELL, B. G. & NOELLE, R. J. 2003. IL-23 produced by CNS-resident cells controls T cell encephalitogenicity during the effector phase of experimental autoimmune encephalomyelitis. *Journal of Clinical Investigation*, 112, 1186.
- BECK, J., RONDOT, P., CATINOT, L., FALCOFF, E., KIRCHNER, H. & WIETZERBIN, J. 1988. Increased production of interferon gamma and tumor necrosis factor precedes clinical manifestation in multiple sclerosis: do cytokines trigger off exacerbations? *Acta Neurologica Scandinavica*, 78, 318-323.
- BÉLANGER, M., ALLAMAN, I. & MAGISTRETTI, P. J. 2011. Differential effects of pro-and anti-inflammatory cytokines alone or in combinations on the metabolic profile of astrocytes. *Journal of neurochemistry*, 116, 564-576.

- BENCZUR, M., PETRANYL, G., PALFFY, G., VARGA, M., TALAS, M., KOTSY, B., FÖLDES, I. & HOLLAN, S. 1980. Dysfunction of natural killer cells in multiple sclerosis: a possible pathogenetic factor. *Clinical and experimental immunology*, 39, 657.
- BENNETT, T. D., FLUCHEL, M., HERSH, A. O., HAYWARD, K. N., HERSH, A. L., BROGAN, T. V., SRIVASTAVA, R., STONE, B. L., KORGENSKI, E. K. & MUNDORFF, M. B. 2012. Macrophage activation syndrome in children with systemic lupus erythematosus and children with juvenile idiopathic arthritis. *Arthritis & Rheumatism*, 64, 4135-4142.
- BENVENISTE, E. N. 1997. Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis. *Journal of molecular medicine*, 75, 165-173.
- BETTELLI, E., DAS, M. P., HOWARD, E. D., WEINER, H. L., SOBEL, R. A. & KUCHROO, V. K. 1998. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10-and IL-4-deficient and transgenic mice. *The Journal of Immunology*, 161, 3299-3306.
- BÉZIAT, V., DUFFY, D., QUOC, S. N., LE GARFF-TAVERNIER, M., DECOCQ, J., COMBADIÈRE, B., DEBRÉ, P. & VIEILLARD, V. 2011. CD56brightCD16+ NK cells: a functional intermediate stage of NK cell differentiation. *The Journal of Immunology*, 186, 6753-6761.
- BHASIN, M., WU, M. & TSIRKA, S. E. 2007. Modulation of microglial/macrophage activation by macrophage inhibitory factor (TKP) or tuftsin (TKPR) attenuates the disease course of experimental autoimmune encephalomyelitis. *BMC immunology*, 8, 10.
- BIELEKOVA, B., CATALFAMO, M., REICHERT-SCRIVNER, S., PACKER, A., CERNA, M., WALDMANN, T. A., MCFARLAND, H., HENKART, P. A. & MARTIN, R. 2006. Regulatory CD56bright natural killer cells mediate immunomodulatory effects of IL-2R α -targeted therapy (daclizumab) in multiple sclerosis. *Proceedings of the National Academy of Sciences*, 103, 5941-5946.
- BILLIAU, A., HEREMANS, H., VANDEKERCKHOVE, F., DIJKMANS, R., SOBIS, H., MEULEPAS, E. & CARTON, H. 1988. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN- γ . *The Journal of Immunology*, 140, 1506-1510.
- BIRON, C. A., NGUYEN, K. B., PIEN, G. C., COUSENS, L. P. & SALAZAR-MATHER, T. P. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annual review of immunology*, 17, 189-220.
- BISWAS, S. K. & MANTOVANI, A. 2012. Orchestration of metabolism by macrophages. *Cell metabolism*, 15, 432-437.
- BITTNER, C. X., LOAIZA, A., RUMINOT, I., LARENAS, V., SOTELO-HITSCHFELD, T., GUTIÉRREZ, R., CÓRDOVA, A., VALDEBENITO, R., FROMMER, W. B. & BARROS, L. F. 2010. High resolution measurement of the glycolytic rate. *Frontiers in neuroenergetics*, 2.
- BJARTMAR, C., WUJEK, J. & TRAPP, B. 2003. Axonal loss in the pathology of MS: consequences for understanding the progressive phase of the disease. *Journal of the neurological sciences*, 206, 165-171.
- BLOCK, M. L. & HONG, J. S. 2005. Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. *Progress in neurobiology*, 76, 77-98.
- BOEHM, U., KLAMP, T., GROOT, M. & HOWARD, J. 1997. Cellular responses to interferon- γ . *Annual review of immunology*, 15, 749-795.
- BOVEN, L. A., VAN MEURS, M., VAN ZWAM, M., WIERENGA-WOLF, A., HINTZEN, R. Q., BOOT, R. G., AERTS, J. M., AMOR, S., NIEUWENHUIS, E. E. & LAMAN, J. D. 2006. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis.
- BRAND, K. 1985. Glutamine and glucose metabolism during thymocyte proliferation. Pathways of glutamine and glutamate metabolism. *Biochem. J*, 228, 353-361.
- BREWER, J. M., CONACHER, M., SATOSKAR, A., BLUETHMANN, H. & ALEXANDER, J. 1996. In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to Freund's complete adjuvant, but continues to induce T helper 2 cytokine production. *European journal of immunology*, 26, 2062-2066.

- BRIX, B., MESTERS, J. R., PELLERIN, L. & JÖHREN, O. 2012. Endothelial cell-derived nitric oxide enhances aerobic glycolysis in astrocytes via HIF-1 α -mediated target gene activation. *The Journal of neuroscience*, 32, 9727-9735.
- BROSNAN, C. F., CANNELLA, B., BATTISTINI, L. & RAINE, C. S. 1995. Cytokine localization in multiple sclerosis lesions Correlation with adhesion molecule expression and reactive nitrogen species. *Neurology*, 45, S16-S21.
- BROSNAN, C. F. & RAINE, C. S. 2013. The astrocyte in multiple sclerosis revisited. *Glia*, 61, 453-465.
- BURNS, J., BARTHOLOMEW, B. & LITTLEFIELD, K. 1995. γ δ T cells participate in the immune response against activated, myelin basic protein-specific, human T cells. *Journal of neuroimmunology*, 58, 177-182.
- CALIGIURI, M. A. 2008. Human natural killer cells. *Blood*, 112, 461-469.
- CANNELLA, B. & RAINE, C. S. 1995. The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Annals of neurology*, 37, 424-435.
- CARPENTIER, P. A., BEGOLKA, W. S., OLSON, J. K., ELHOFY, A., KARPUS, W. J. & MILLER, S. D. 2005. Differential activation of astrocytes by innate and adaptive immune stimuli. *Glia*, 49, 360-374.
- CARROLL, J. L., NIELSEN, L. L., PRUETT, S. B. & MATHIS, J. M. 2001. The Role of Natural Killer Cells in Adenovirus-mediated p53 Gene Therapy¹. *Molecular cancer therapeutics*, 1, 49-60.
- CASTRO-BORRERO, W., GRAVES, D., FROHMAN, T. C., FLORES, A. B., HARDEMAN, P., LOGAN, D., ORCHARD, M., GREENBERG, B. & FROHMAN, E. M. 2012. Current and emerging therapies in multiple sclerosis: a systematic review. *Therapeutic advances in neurological disorders*, 5, 205-220.
- CAVAILLON, J.-M. 2011. The historical milestones in the understanding of leukocyte biology initiated by Elie Metchnikoff. *Journal of leukocyte biology*, 90, 413-424.
- CHANVILLARD, C., JACOLIK, R. F., INFANTE-DUARTE, C. & NAYAK, R. C. 2013. The role of natural killer cells in multiple sclerosis and their therapeutic implications. *Frontiers in immunology*, 4.
- CHONG, W. P., VAN PANHUYS, N., CHEN, J., SILVER, P. B., JITTAYASOTHORN, Y., MATTAPALLIL, M. J., GERMAIN, R. N. & CASPI, R. R. 2015. NK-DC crosstalk controls the autopathogenic Th17 response through an innate IFN- γ -IL-27 axis. *The Journal of experimental medicine*, jem. 20141678.
- CLIFFORD, D. B., DELUCA, A., SIMPSON, D. M., ARENDT, G., GIOVANNONI, G. & NATH, A. 2010. Natalizumab-associated progressive multifocal leukoencephalopathy in patients with multiple sclerosis: lessons from 28 cases. *The Lancet Neurology*, 9, 438-446.
- COLTON, C. A. 2009. Heterogeneity of microglial activation in the innate immune response in the brain. *Journal of Neuroimmune Pharmacology*, 4, 399-418.
- COLUCCI, F., DI SANTO, J. P. & LEIBSON, P. J. 2002. Natural killer cell activation in mice and men: different triggers for similar weapons? *Nature immunology*, 3, 807-813.
- COMABELLA, M., RÍO, J., ESPEJO, C., RUIZ DE VILLA, M., AL-ZAYAT, H., NOS, C., DEISENHAMMER, F., BARANZINI, S. E., NONELL, L. & LÓPEZ, C. 2009. Changes in matrix metalloproteinases and their inhibitors during interferon-beta treatment in multiple sclerosis. *Clinical Immunology*, 130, 145-150.
- COMI, G., FILIPPI, M. & WOLINSKY, J. S. 2001. European/Canadian multicenter, double-blind, randomized, placebo-controlled study of the effects of glatiramer acetate on magnetic resonance imaging-measured disease activity and burden in patients with relapsing multiple sclerosis. *Annals of neurology*, 49, 290-297.
- DANEMAN, R. & PRAT, A. 2015. The Blood-Brain Barrier. *Cold Spring Harbor perspectives in biology*, 7, a020412.

- DENNEY, L., KOK, W. L., COLE, S. L., SANDERSON, S., MCMICHAEL, A. J. & HO, L.-P. 2012. Activation of invariant NKT cells in early phase of experimental autoimmune encephalomyelitis results in differentiation of Ly6Chi inflammatory monocyte to M2 macrophages and improved outcome. *The Journal of Immunology*, 189, 551-557.
- DER MEIDE, V. 1999. Dendritic cell-derived nitric oxide is involved in IL-4-induced suppression of experimental allergic encephalomyelitis (EAE) in Lewis rats. *Clinical & Experimental Immunology*, 118, 115-121.
- DING, Z., MATHUR, V., HO, P. P., JAMES, M. L., LUCIN, K. M., HOEHNE, A., ALABSI, H., GAMBHIR, S. S., STEINMAN, L. & LUO, J. 2014. Antiviral drug ganciclovir is a potent inhibitor of microglial proliferation and neuroinflammation. *The Journal of experimental medicine*, 211, 189-198.
- DOGAN, R.-N. E., ELHOFY, A. & KARPUS, W. J. 2008. Production of CCL2 by central nervous system cells regulates development of murine experimental autoimmune encephalomyelitis through the recruitment of TNF- and iNOS-expressing macrophages and myeloid dendritic cells. *The Journal of Immunology*, 180, 7376-7384.
- DUGGER, K. J., ZINN, K. R., WEAVER, C., BULLARD, D. C. & BARNUM, S. R. 2009. Effector and suppressor roles for LFA-1 during the development of experimental autoimmune encephalomyelitis. *Journal of neuroimmunology*, 206, 22-27.
- DUNGAN, L. S., MCGUINNESS, N. C., BOON, L., LYNCH, M. A. & MILLS, K. H. 2014. Innate IFN- γ promotes development of experimental autoimmune encephalomyelitis: A role for NK cells and M1 macrophages. *European journal of immunology*, 44, 2903-2917.
- DUONG, T. T., LOUIS, J. S., GILBERT, J. J., FINKELMAN, F. D. & STREJAN, G. H. 1992. Effect of anti-interferon- γ and anti-interleukin-2 monoclonal antibody treatment on the development of actively and passively induced experimental allergic encephalomyelitis in the SJL/J mouse. *Journal of neuroimmunology*, 36, 105-115.
- EBERT, L. M., MEUTER, S. & MOSER, B. 2006. Homing and function of human skin $\gamma\delta$ T cells and NK cells: relevance for tumor surveillance. *The Journal of Immunology*, 176, 4331-4336.
- EL-BEHI, M., CIRIC, B., DAI, H., YAN, Y., CULLIMORE, M., SAFAVI, F., ZHANG, G.-X., DITTEL, B. N. & ROSTAMI, A. 2011a. The encephalitogenicity of TH17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nature immunology*, 12, 568-575.
- EL-BEHI, M., CIRIC, B., DAI, H., YAN, Y., CULLIMORE, M., SAFAVI, F., ZHANG, G. X., DITTEL, B. N. & ROSTAMI, A. 2011b. The encephalitogenicity of TH17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nature immunology*, 12, 568-575.
- ENGELHARDT, B. & RANSOHOFF, R. M. 2012. Capture, crawl, cross: the T cell code to breach the blood-brain barriers. *Trends in immunology*, 33, 579-589.
- FALCONE, M., RAJAN, A. J., BLOOM, B. R. & BROSNAN, C. F. 1998. A critical role for IL-4 in regulating disease severity in experimental allergic encephalomyelitis as demonstrated in IL-4-deficient C57BL/6 mice and BALB/c mice. *The Journal of Immunology*, 160, 4822-4830.
- FARINA, C., ALOISI, F. & MEINL, E. 2007. Astrocytes are active players in cerebral innate immunity. *Trends in immunology*, 28, 138-145.
- FATHMAN, J. W., BHATTACHARYA, D., INLAY, M. A., SEITA, J., KARSUNKY, H. & WEISSMAN, I. L. 2011. Identification of the earliest natural killer cell-committed progenitor in murine bone marrow. *Blood*, 118, 5439-5447.
- FERBER, I. A., BROCKE, S., TAYLOR-EDWARDS, C., RIDGWAY, W., DINISCO, C., STEINMAN, L., DALTON, D. & FATHMAN, C. G. 1996. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *The Journal of Immunology*, 156, 5-7.

- FERNANDEZ-BOTRAN, R., SANDERS, V., MOSMANN, T. & VITETTA, E. 1988. Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells. *The Journal of experimental medicine*, 168, 543-558.
- FILLIT, H., DING, W., BUEE, L., KALMAN, J., ALTSTIEL, L., LAWLOR, B. & WOLF-KLEIN, G. 1991. Elevated circulating tumor necrosis factor levels in Alzheimer's disease. *Neuroscience letters*, 129, 318-320.
- FINSEN, B., ANTEL, J. & OWENS, T. 2001. TNFalpha: kill or cure for demyelinating disease? *Molecular psychiatry*, 7, 820-821.
- FLETCHER, J. M., LONERGAN, R., COSTELLOE, L., KINSELLA, K., MORAN, B., O'FARRELLY, C., TUBRIDY, N. & MILLS, K. H. 2009. CD39+ Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis. *The Journal of Immunology*, 183, 7602-7610.
- FOGEL, L. A., YOKOYAMA, W. M. & FRENCH, A. R. 2013. Natural killer cells in human autoimmune disorders. *Arthritis Res Ther*, 15, 216.
- FÖRSTERMANN, U. & SESSA, W. C. 2012. Nitric oxide synthases: regulation and function. *European heart journal*, 33, 829-837.
- FOX, E. J. 2004. Mechanism of action of mitoxantrone. *Neurology*, 63, S15-S18.
- FRAKES, A. E., FERRAIUOLO, L., HAIDET-PHILLIPS, A. M., SCHMELZER, L., BRAUN, L., MIRANDA, C. J., LADNER, K. J., BEVAN, A. K., FOUST, K. D. & GODBOUT, J. P. 2014. Microglia induce motor neuron death via the classical NF- κ B pathway in amyotrophic lateral sclerosis. *Neuron*, 81, 1009-1023.
- GAN, Y., LIU, Q., WU, W., YIN, J.-X., BAI, X.-F., SHEN, R., WANG, Y., CHEN, J., LA CAVA, A., POURSIINE-LAURENT, J., YOKOYAMA, W. & SHI, F.-D. 2014. Ischemic neurons recruit natural killer cells that accelerate brain infarction. *Proceedings of the National Academy of Sciences*, 111, 2704-2709.
- GAN, Y., LIU, R., WU, W., BOMPRESZI, R. & SHI, F.-D. 2012. Antibody to α 4 integrin suppresses natural killer cells infiltration in central nervous system in experimental autoimmune encephalomyelitis. *Journal of neuroimmunology*, 247, 9-15.
- GARG, S. K., KIPNIS, J. & BANERJEE, R. 2009. IFN- γ and IL-4 differentially shape metabolic responses and neuroprotective phenotype of astrocytes. *Journal of neurochemistry*, 108, 1155-1166.
- GEROSA, F., BALDANI-GUERRA, B., NISII, C., MARCHESINI, V., CARRA, G. & TRINCHIERI, G. 2002. Reciprocal activating interaction between natural killer cells and dendritic cells. *The Journal of experimental medicine*, 195, 327-333.
- GIMENO-BAYÓN, J., LÓPEZ-LÓPEZ, A., RODRÍGUEZ, M. & MAHY, N. 2014. Glucose pathways adaptation supports acquisition of activated microglia phenotype. *Journal of neuroscience research*, 92, 723-731.
- GINHOUX, F., GRETER, M., LEOEUF, M., NANDI, S., SEE, P., GOKHAN, S., MEHLER, M. F., CONWAY, S. J., NG, L. G. & STANLEY, E. R. 2010. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science*, 330, 841-845.
- GINHOUX, F. & JUNG, S. 2014. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat Rev Immunol*, 14, 392-404.
- GLABINSKI, A. R., TANI, M., STRIETER, R. M., TUOHY, V. K. & RANSOHOFF, R. M. 1997. Synchronous synthesis of alpha- and beta-chemokines by cells of diverse lineage in the central nervous system of mice with relapses of chronic experimental autoimmune encephalomyelitis. *The American journal of pathology*, 150, 617.
- GLIM, J., VEREYKEN, E., HEIJNEN, D., VALLEJO, J. & DIJKSTRA, C. 2010. The release of cytokines by macrophages is not affected by myelin ingestion. *Glia*, 58, 1928-1936.
- GOLD, R., LININGTON, C. & LASSMANN, H. 2006. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain*, 129, 1953-1971.

- GOLDSZMID, R. S., BAFICA, A., JANKOVIC, D., FENG, C. G., CASPAR, P., WINKLER-PICKETT, R., TRINCHIERI, G. & SHER, A. 2007. TAP-1 indirectly regulates CD4+ T cell priming in *Toxoplasma gondii* infection by controlling NK cell IFN- γ production. *The Journal of experimental medicine*, 204, 2591-2602.
- GOODIN, D. 2006. The return of natalizumab: weighing benefit against risk. *The Lancet Neurology*, 5, 375-377.
- GORDON, S. 2003. Alternative activation of macrophages. *Nature Reviews Immunology*, 3, 23-35.
- GRAN, B., ZHANG, G.-X., YU, S., LI, J., CHEN, X.-H., VENTURA, E. S., KAMOUN, M. & ROSTAMI, A. 2002. IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination. *The Journal of Immunology*, 169, 7104-7110.
- GRAU, V., HERBST, B., VAN DER MEIDE, P. H. & STEINIGER, B. 1997. Activation of microglial and endothelial cells in the rat brain after treatment with interferon-gamma in vivo. *Glia*, 19, 181-189.
- GRÉGOIRE, C., CHASSON, L., LUCI, C., TOMASELLO, E., GEISSMANN, F., VIVIER, E. & WALZER, T. 2007. The trafficking of natural killer cells. *Immunological reviews*, 220, 169-182.
- GREINER, E. F., GUPPY, M. & BRAND, K. 1994. Glucose is essential for proliferation and the glycolytic enzyme induction that provokes a transition to glycolytic energy production. *Journal of Biological Chemistry*, 269, 31484-31490.
- GRETER, M., LELIOS, I. & CROXFORD, A. L. 2015. Microglia versus Myeloid Cell Nomenclature During Brain Inflammation. *Frontiers in Immunology*, 6, 249.
- GÜLTNER, S., KUHLMANN, T., HESSE, A., WEBER, J. P., RIEMER, C., BAIER, M. & HUTLOFF, A. 2010. Reduced Treg frequency in LFA-1-deficient mice allows enhanced T effector differentiation and pathology in EAE. *European journal of immunology*, 40, 3403-3412.
- HAAS, J. D., GONZÁLEZ, F. H. M., SCHMITZ, S., CHENNUPATI, V., FÖHSE, L., KREMMER, E., FÖRSTER, R. & PRINZ, I. 2009. CCR6 and NK1. 1 distinguish between IL-17A and IFN- γ -producing $\gamma\delta$ effector T cells. *European journal of immunology*, 39, 3488-3497.
- HAGHIKIA, A., HOHLFELD, R., GOLD, R. & FUGGER, L. 2013. Therapies for multiple sclerosis: translational achievements and outstanding needs. *Trends in molecular medicine*, 19, 309-319.
- HAILER, N. P., WIRJATIJASA, F., ROSER, N., HISCHBETH, G. T. & KORF, H. W. 2001. Astrocytic factors protect neuronal integrity and reduce microglial activation in an in vitro model of N-methyl-d-aspartate-induced excitotoxic injury in organotypic hippocampal slice cultures. *European Journal of Neuroscience*, 14, 315-326.
- HAMANN, I., DÖRR, J., GLUMM, R., CHANVILLARD, C., JANSSEN, A., MILLWARD, J. M., PAUL, F., RANSOHOFF, R. M. & INFANTE-DUARTE, C. 2013. Characterization of natural killer cells in paired CSF and blood samples during neuroinflammation. *Journal of neuroimmunology*, 254, 165-169.
- HAO, J., LIU, R., PIAO, W., ZHOU, Q., VOLLMER, T. L., CAMPAGNOLO, D. I., XIANG, R., LA CAVA, A., VAN KAER, L. & SHI, F.-D. 2010a. Central nervous system (CNS)-resident natural killer cells suppress Th17 responses and CNS autoimmune pathology. *The Journal of experimental medicine*, 207, 1907-1921.
- HAO, J., LIU, R., PIAO, W., ZHOU, Q., VOLLMER, T. L., CAMPAGNOLO, D. I., XIANG, R., LA CAVA, A., VAN KAER, L. & SHI, F. D. 2010b. Central nervous system (CNS)-resident natural killer cells suppress Th17 responses and CNS autoimmune pathology. *The Journal of experimental medicine*, 207, 1907-1921.
- HASCHEMI, A., KOSMA, P., GILLE, L., EVANS, C. R., BURANT, C. F., STARKL, P., KNAPP, B., HAAS, R., SCHMID, J. A. & JANDL, C. 2012. The sedoheptulose kinase CARKL directs

- macrophage polarization through control of glucose metabolism. *Cell metabolism*, 15, 813-826.
- HEDESKOV, C. 1968. Early effects of phytohaemagglutinin on glucose metabolism of normal human lymphocytes. *Biochem. J.*, 110, 373-380.
- HENDRIKS, J. J., TEUNISSEN, C. E., DE VRIES, H. E. & DIJKSTRA, C. D. 2005. Macrophages and neurodegeneration. *Brain Research Reviews*, 48, 185-195.
- HENEKA, M. T., CARSON, M. J., EL KHOURY, J., LANDRETH, G. E., BROSSERON, F., FEINSTEIN, D. L., JACOBS, A. H., WYSS-CORAY, T., VITORICA, J. & RANSOHOFF, R. M. 2015. Neuroinflammation in Alzheimer's disease. *The Lancet Neurology*, 14, 388-405.
- HENEKA, M. T., KUMMER, M. P., STUTZ, A., DELEKATE, A., SCHWARTZ, S., VIEIRA-SAECKER, A., GRIEP, A., AXT, D., REMUS, A. & TZENG, T.-C. 2013. NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature*, 493, 674-678.
- HEPPNER, F. L., GRETER, M., MARINO, D., FALSIG, J., RAIVICH, G., HÖVELMEYER, N., WAISMAN, A., RÜLCKE, T., PRINZ, M. & PRILLER, J. 2005. Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nature medicine*, 11, 146-152.
- HEPPNER, F. L., RANSOHOFF, R. M. & BECHER, B. 2015. Immune attack: the role of inflammation in Alzheimer disease. *Nature Reviews Neuroscience*, 16, 358-372.
- HERBERMAN, R. B., NUNN, M. E. & LAVRIN, D. H. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. *International journal of cancer*, 16, 216-229.
- HERRERA-MOLINA, R. & VON BERNHARDI, R. 2005. Transforming growth factor- β 1 produced by hippocampal cells modulates microglial reactivity in culture. *Neurobiology of disease*, 19, 229-236.
- HOFSTETTER, H. H., SHIVE, C. L. & FORSTHUBER, T. G. 2002. Pertussis toxin modulates the immune response to neuroantigens injected in incomplete Freund's adjuvant: induction of Th1 cells and experimental autoimmune encephalomyelitis in the presence of high frequencies of Th2 cells. *The Journal of Immunology*, 169, 117-125.
- HU, X., LEAK, R. K., SHI, Y., SUENAGA, J., GAO, Y., ZHENG, P. & CHEN, J. 2015. Microglial and macrophage polarization [mdash] new prospects for brain repair. *Nature Reviews Neurology*, 11, 56-64.
- HUA, L. L., KIM, M. O., BROSNAN, C. F. & LEE, S. C. 2002. Modulation of astrocyte inducible nitric oxide synthase and cytokine expression by interferon β is associated with induction and inhibition of interferon γ -activated sequence binding activity. *Journal of neurochemistry*, 83, 1120-1128.
- HUANG, D., SHI, F.-D., JUNG, S., PIEN, G. C., WANG, J., SALAZAR-MATHER, T. P., HE, T. T., WEAVER, J. T., LJUNGGREN, H.-G. & BIRON, C. A. 2006. The neuronal chemokine CX3CL1/fractalkine selectively recruits NK cells that modify experimental autoimmune encephalomyelitis within the central nervous system. *The FASEB Journal*, 20, 896-905.
- HUANG, S. C.-C., EVERTS, B., IVANOVA, Y., O'SULLIVAN, D., NASCIMENTO, M., SMITH, A. M., BEATTY, W., LOVE-GREGORY, L., LAM, W. Y. & O'NEILL, C. M. 2014. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nature immunology*, 15, 846-855.
- HUIZINGA, R., VAN DER STAR, B. J., KIPP, M., JONG, R., GERRITSEN, W., CLARNER, T., PUENTES, F., DIJKSTRA, C. D., VALK, P. & AMOR, S. 2012. Phagocytosis of neuronal debris by microglia is associated with neuronal damage in multiple sclerosis. *Glia*.
- HUNTINGTON, N. D., VOSSHENRICH, C. A. & DI SANTO, J. P. 2007. Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nature Reviews Immunology*, 7, 703-714.
- ISAACS, A. & LINDENMANN, J. 1957. *Virus Interference. I. The Interferon*.
- JANG, E., KIM, J.-H., LEE, S., KIM, J.-H., SEO, J.-W., JIN, M., LEE, M.-G., JANG, I.-S., LEE, W.-H. & SUK, K. 2013. Phenotypic polarization of activated astrocytes: the critical role of

- lipocalin-2 in the classical inflammatory activation of astrocytes. *The Journal of Immunology*, 191, 5204-5219.
- JI, Q. & GOVERMAN, J. 2007. Experimental autoimmune encephalomyelitis mediated by CD8+ T cells. *Annals of the New York Academy of Sciences*, 1103, 157-166.
- JIANG, H. R., MILOVANOVIĆ, M., ALLAN, D., NIEBALA, W., BESNARD, A. G., FUKADA, S. Y., ALVES-FILHO, J. C., TOGBE, D., GOODYEAR, C. S. & LININGTON, C. 2012. IL-33 attenuates EAE by suppressing IL-17 and IFN- γ production and inducing alternatively activated macrophages. *European journal of immunology*, 42, 1804-1814.
- JIANG, T. & CADENAS, E. 2014. Astrocytic metabolic and inflammatory changes as a function of age. *Aging cell*, 13, 1059-1067.
- JIANG, W., CHAI, N. R., MARIC, D. & BIELEKOVA, B. 2011. Unexpected role for granzyme K in CD56bright NK cell-mediated immunoregulation of multiple sclerosis. *The Journal of Immunology*, 187, 781-790.
- KAPLAN, M. H., SCHINDLER, U., SMILEY, S. T. & GRUSBY, M. J. 1996. Stat6 is required for mediating responses to IL-4 and for the development of Th2 cells. *Immunity*, 4, 313-319.
- KAPPOS, L., RADUE, E.-W., O'CONNOR, P., POLMAN, C., HOHLFELD, R., CALABRESI, P., SELMAJ, K., AGOROPOULOU, C., LEYK, M. & ZHANG-AUBERSON, L. 2010. A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. *New England Journal of Medicine*, 362, 387-401.
- KASTRUKOFF, L. F., LAU, A., WEE, R., ZECCHINI, D., WHITE, R. & PATY, D. W. 2003. Clinical relapses of multiple sclerosis are associated with 'novel' valleys in natural killer cell functional activity. *Journal of neuroimmunology*, 145, 103-114.
- KASTRUKOFF, L. F., MORGAN, N. G., ZECCHINI, D., WHITE, R., PETKAU, A. J., SATOH, J.-I. & PATY, D. W. 1998. A role for natural killer cells in the immunopathogenesis of multiple sclerosis. *Journal of neuroimmunology*, 86, 123-133.
- KAWAHARA, K., SUENOBU, M., YOSHIDA, A., KOGA, K., HYODO, A., OHTSUKA, H., KUNIYASU, A., TAMAMAKI, N., SUGIMOTO, Y. & NAKAYAMA, H. 2012. Intracerebral microinjection of interleukin-4/interleukin-13 reduces β -amyloid accumulation in the ipsilateral side and improves cognitive deficits in young amyloid precursor protein 23 mice. *Neuroscience*, 207, 243-260.
- KIERDORF, K., ERNY, D., GOLDMANN, T., SANDER, V., SCHULZ, C., PERDIGUERO, E. G., WIEGHOFER, P., HEINRICH, A., RIEMKE, P. & HÖLSCHER, C. 2013. Microglia emerge from erythromyeloid precursors via Pu. 1-and Irf8-dependent pathways. *Nature neuroscience*, 16, 273-280.
- KIESSLING, R., KLEIN, E. & WIGZELL, H. 1975. „Natural“ \square killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *European journal of immunology*, 5, 112-117.
- KIM, S., IIZUKA, K., KANG, H.-S. P., DOKUN, A., FRENCH, A. R., GRECO, S. & YOKOYAMA, W. M. 2002. In vivo developmental stages in murine natural killer cell maturation. *Nature immunology*, 3, 523-528.
- KIM, S., POURSIANE-LAURENT, J., TRUSCOTT, S. M., LYBARGER, L., SONG, Y.-J., YANG, L., FRENCH, A. R., SUNWOO, J. B., LEMIEUX, S. & HANSEN, T. H. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature*, 436, 709-713.
- KIPP, B., BAR-OR, A., GAUSLING, R., OLIVEIRA, E. M., FRUHAN, S. A., STUART, W. H. & HAFNER, D. A. 2000. A novel population of B7-1+ T cells producing intracellular IL-4 is decreased in patients with multiple sclerosis. *European journal of immunology*, 30, 2092-2100.
- KOPF, M., LE GROS, G., BACHMANN, M., LAMERS, M. C., BLUETHMANN, H. & KÖHLER, G. 1993. Disruption of the murine IL-4 gene blocks Th2 cytokine responses.

- KÖRNER, H., RIMINTON, D. S., STRICKLAND, D. H., LEMCKERT, F. A., POLLARD, J. D. & SEDGWICK, J. D. 1997. Critical points of tumor necrosis factor action in central nervous system autoimmune inflammation defined by gene targeting. *The Journal of experimental medicine*, 186, 1585-1590.
- KREUTZBERG, G. W. 1996. Microglia: a sensor for pathological events in the CNS. *Trends in neurosciences*, 19, 312-318.
- KUCHROO, V. K., DAS, M. P., BROWN, J. A., RANGER, A. M., ZAMVIL, S. S., SOBEL, R. A., WEINER, H. L., NABAVI, N. & GLIMCHER, L. H. 1995. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell*, 80, 707-718.
- LALOR, S. J., DUNGAN, L. S., SUTTON, C. E., BASDEO, S. A., FLETCHER, J. M. & MILLS, K. H. G. 2011. Caspase-1-Processed Cytokines IL-1 β and IL-18 Promote IL-17 Production by $\gamma\delta$ and CD4 T Cells That Mediate Autoimmunity. *The Journal of Immunology*, 186, 5738.
- LANGRISH, C. L., CHEN, Y., BLUMENSCHIN, W. M., MATTSON, J., BASHAM, B., SEDGWICK, J. D., MCCLANAHAN, T., KASTELEIN, R. A. & CUA, D. J. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *The Journal of experimental medicine*, 201, 233-240.
- LANIER, L. L., LE, A. M., PHILLIPS, J. H., WARNER, N. L. & BABCOCK, G. F. 1983. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. *The Journal of Immunology*, 131, 1789-1796.
- LAPAQUE, N., WALZER, T., MÉRESSE, S., VIVIER, E. & TROWSDALE, J. 2009. Interactions between human NK cells and macrophages in response to Salmonella infection. *The Journal of Immunology*, 182, 4339-4348.
- LASSMANN, H., BRÜCK, W. & LUCCHINETTI, C. 2001. Heterogeneity of multiple sclerosis pathogenesis: implications for diagnosis and therapy. *Trends in molecular medicine*, 7, 115-121.
- LEAVENWORTH, J. W., SCHELLACK, C., KIM, H.-J., LU, L., SPEE, P. & CANTOR, H. 2010a. Analysis of the cellular mechanism underlying inhibition of EAE after treatment with anti-NKG2A F(ab')₂. *Proceedings of the National Academy of Sciences*, 107, 2562-2567.
- LEAVENWORTH, J. W., SCHELLACK, C., KIM, H. J., LU, L., SPEE, P. & CANTOR, H. 2010b. Analysis of the cellular mechanism underlying inhibition of EAE after treatment with anti-NKG2A F(ab')₂. *Proceedings of the National Academy of Sciences*, 107, 2562-2567.
- LEE, E.-J., WOO, M.-S., MOON, P.-G., BAEK, M.-C., CHOI, I.-Y., KIM, W.-K., JUNN, E. & KIM, H.-S. 2010. α -Synuclein activates microglia by inducing the expressions of matrix metalloproteinases and the subsequent activation of protease-activated receptor-1. *The Journal of Immunology*, 185, 615-623.
- LEE, S., NAM, Y., KOO, J. Y., LIM, D., PARK, J., OCK, J., KIM, J., SUK, K. & PARK, S. B. 2014. A small molecule binding HMGB1 and HMGB2 inhibits microglia-mediated neuroinflammation. *Nature chemical biology*, 10, 1055-1060.
- LENNON, V. A., WINGERCHUK, D. M., KRYZER, T. J., PITTOCK, S. J., LUCCHINETTI, C. F., FUJIHARA, K., NAKASHIMA, I. & WEINSHENKER, B. G. 2004. A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. *The Lancet*, 364, 2106-2112.
- LIAO, B., ZHAO, W., BEERS, D. R., HENKEL, J. S. & APPEL, S. H. 2012. Transformation from a neuroprotective to a neurotoxic microglial phenotype in a mouse model of ALS. *Experimental neurology*, 237, 147-152.
- LIBLAU, R., STEINMAN, L. & BROCKE, S. 1997. Experimental autoimmune encephalomyelitis in IL-4-deficient mice. *International immunology*, 9, 799-803.
- LIN, K.-C. & CASTRO, A. C. 1998. Very late antigen 4 (VLA4) antagonists as anti-inflammatory agents. *Current opinion in chemical biology*, 2, 453-457.

- LIU, C., LI, Y., YU, J., FENG, L., HOU, S., LIU, Y., GUO, M., XIE, Y., MENG, J. & ZHANG, H. 2013. Targeting the shift from M1 to M2 macrophages in experimental autoimmune encephalomyelitis mice treated with fasudil. *PLoS One*, 8, e54841.
- LIU, T., YU, H., ULLENBRUCH, M., JIN, H., ITO, T., WU, Z., LIU, J. & PHAN, S. H. 2014. The in vivo fibrotic role of FIZZ1 in pulmonary fibrosis. *PloS one*, 9, e88362.
- LOCK, C., HERMANS, G., PEDOTTI, R., BRENDOLAN, A., SCHADT, E., GARREN, H., LANGER-GOULD, A., STROBER, S., CANNELLA, B. & ALLARD, J. 2002. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nature medicine*, 8, 500-508.
- LOVETT-RACKE, A. E., YANG, Y. & RACKE, M. K. 2011. Th1 versus Th17: are T cell cytokines relevant in multiple sclerosis? *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1812, 246-251.
- LOWES, M. A., KIKUCHI, T., FUENTES-DUCULAN, J., CARDINALE, I., ZABA, L. C., HAIDER, A. S., BOWMAN, E. P. & KRUEGER, J. G. 2008. Psoriasis vulgaris lesions contain discrete populations of Th1 and Th17 T cells. *Journal of Investigative Dermatology*, 128, 1207-1211.
- LU, L., IKIZAWA, K., HU, D., WERNECK, M. B., WUCHERPFENNIG, K. W. & CANTOR, H. 2007a. Regulation of activated CD4+ T cells by NK cells via the Qa-1-NKG2A inhibitory pathway. *Immunity*, 26, 593-604.
- LU, L., IKIZAWA, K., HU, D., WERNECK, M. B. F., WUCHERPFENNIG, K. W. & CANTOR, H. 2007b. Regulation of activated CD4+ T cells by NK cells via the Qa-1-NKG2A inhibitory pathway. *Immunity*, 26, 593-604.
- LUBLIN, F. D. & REINGOLD, S. C. 1996. Defining the clinical course of multiple sclerosis results of an international survey. *Neurology*, 46, 907-911.
- LUCCHINETTI, C. F., POPESCU, B. F. G., BUNYAN, R. F., MOLL, N. M., ROEMER, S. F., LASSMANN, H., BRÜCK, W., PARISI, J. E., SCHEITHAUER, B. W., GIANNINI, C., WEIGAND, S. D., MANDREKAR, J. & RANSOHOFF, R. M. 2011. Inflammatory Cortical Demyelination in Early Multiple Sclerosis. *New England Journal of Medicine*, 365, 2188-2197.
- LÜNEMANN, A., LÜNEMANN, J. D., ROBERTS, S., MESSMER, B., DA SILVA, R. B., RAINE, C. S. & MÜNZ, C. 2008. Human NK cells kill resting but not activated microglia via NKG2D-and NKp46-mediated recognition. *The Journal of Immunology*, 181, 6170-6177.
- LUNT, S. Y. & VANDER HEIDEN, M. G. 2011. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annual review of cell and developmental biology*, 27, 441-464.
- LUZINA, I. G., KEEGAN, A. D., HELLER, N. M., ROOK, G. A., SHEA-DONOHUE, T. & ATAMAS, S. P. 2012. Regulation of inflammation by interleukin-4: a review of "alternatives". *Journal of leukocyte biology*, 92, 753-764.
- LYNCH, M. A. 2009. The multifaceted profile of activated microglia. *Molecular neurobiology*, 40, 139-156.
- LYNCH, M. A. 2014. The impact of neuroimmune changes on development of amyloid pathology; relevance to Alzheimer's disease. *Immunology*, 141, 292-301.
- MAGGI, E., PARRONCHI, P., MANETTI, R., SIMONELLI, C., PICCINNI, M., RUGIU, F. S., DE CARLI, M., RICCI, M. & ROMAGNANI, S. 1992. Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human Th1 and Th2 clones. *The Journal of Immunology*, 148, 2142-2147.
- MANTOVANI, A., SICA, A. & LOCATI, M. 2005. Macrophage polarization comes of age. *Immunity*, 23, 344-346.
- MANTOVANI, A., SICA, A. & LOCATI, M. 2007. New vistas on macrophage differentiation and activation. *European journal of immunology*, 37, 14-16.
- MARTINEZ, F. O. & GORDON, S. 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000prime reports*, 6.

- MATHUR, D., LÓPEZ-RODAS, G., CASANOVA, B. & MARTI, M. B. 2014. Perturbed glucose metabolism: insights into multiple sclerosis pathogenesis. *Frontiers in neurology*, 5.
- MATSUMOTO, Y., KOHYAMA, K., AIKAWA, Y., SHIN, T., KAWAZOE, Y., SUZUKI, Y. & TANUMA, N. 1998a. Role of natural killer cells and TCR $\alpha\beta$ T cells in acute autoimmune encephalomyelitis. *Eur. J. Immunol*, 28, 1681-1688.
- MATSUMOTO, Y., KOHYAMA, K., AIKAWA, Y., SHIN, T., KAWAZOE, Y., SUZUKI, Y. & TANUMA, N. 1998b. Role of natural killer cells and TCR $\gamma\delta$ T cells in acute autoimmune encephalomyelitis. *European journal of immunology*, 28, 1681-1688.
- MCFARLAND, H. F. & MARTIN, R. 2007. Multiple sclerosis: a complicated picture of autoimmunity. *Nature immunology*, 8, 913-919.
- MCGEACHY, M. J., BAK-JENSEN, K. S., CHEN, Y., TATO, C. M., BLUMENSCHNEIN, W., MCCLANAHAN, T. & CUA, D. J. 2007. TGF- β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell-mediated pathology. *Nature immunology*, 8, 1390-1397.
- MCKENZIE, G. J., EMSON, C. L., BELL, S. E., ANDERSON, S., FALLON, P., ZURAWSKI, G., MURRAY, R., GRENCIS, R. & MCKENZIE, A. N. 1998. Impaired development of Th2 cells in IL-13-deficient mice. *Immunity*, 9, 423-432.
- MCCMAHON, E. J., BAILEY, S. L., CASTENADA, C. V., WALDNER, H. & MILLER, S. D. 2005. Epitope spreading initiates in the CNS in two mouse models of multiple sclerosis. *Nature medicine*, 11, 335-339.
- MCQUALTER, J. L., DARWICHE, R., EWING, C., ONUKI, M., KAY, T. W., HAMILTON, J. A., REID, H. H. & BERNARD, C. C. 2001. Granulocyte Macrophage Colony-Stimulating Factor A New Putative Therapeutic Target in Multiple Sclerosis. *The Journal of experimental medicine*, 194, 873-882.
- MCWHORTER, F. Y., WANG, T., NGUYEN, P., CHUNG, T. & LIU, W. F. 2013. Modulation of macrophage phenotype by cell shape. *Proceedings of the National Academy of Sciences*, 110, 17253-17258.
- MICHALEK, R. D., GERRIETS, V. A., JACOBS, S. R., MACINTYRE, A. N., MACIVER, N. J., MASON, E. F., SULLIVAN, S. A., NICHOLS, A. G. & RATHMELL, J. C. 2011. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4⁺ T cell subsets. *The Journal of Immunology*, 186, 3299-3303.
- MIKITA, J., DUBOURDIEU-CASSAGNO, N., DELOIRE, M. S., VEKRIS, A., BIRAN, M., RAFFARD, G., BROCHET, B., CANRON, M.-H., FRANCONI, J.-M. & BOIZIAU, C. 2011. Altered M1/M2 activation patterns of monocytes in severe relapsing experimental rat model of multiple sclerosis. Amelioration of clinical status by M2 activated monocyte administration. *Multiple Sclerosis Journal*, 17, 2-15.
- MIKITA, T., CAMPBELL, D., WU, P., WILLIAMSON, K. & SCHINDLER, U. 1996. Requirements for interleukin-4-induced gene expression and functional characterization of Stat6. *Molecular and Cellular Biology*, 16, 5811-5820.
- MILLER, R. H. & RAFF, M. C. 1984. Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct. *The Journal of neuroscience*, 4, 585-592.
- MILO, R. & KAHANA, E. 2010. Multiple sclerosis: geoepidemiology, genetics and the environment. *Autoimmunity reviews*, 9, A387-A394.
- MINDUR, J. E., ITO, N., DHIB-JALBUT, S. & ITO, K. 2014. Early Treatment with Anti-VLA-4 mAb Can Prevent the Infiltration and/or Development of Pathogenic CD11b⁺ CD4⁺ T Cells in the CNS during Progressive EAE.
- MIRON, V. E., BOYD, A., ZHAO, J.-W., YUEN, T. J., RUCKH, J. M., SHADRACH, J. L., VAN WIJNGAARDEN, P., WAGERS, A. J., WILLIAMS, A. & FRANKLIN, R. J. 2013. M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. *Nature neuroscience*, 16, 1211-1218.

- MIRON, V. E. & FRANKLIN, R. J. 2014. Macrophages and CNS remyelination. *Journal of neurochemistry*, 130, 165-171.
- MOEHLE, M. & WEST, A. 2014. M1 and M2 immune activation in Parkinson's disease: foe and ally? *Neuroscience*.
- MOGI, M., HARADA, M., RIEDERER, P., NARABAYASHI, H., FUJITA, K. & NAGATSU, T. 1994. Tumor necrosis factor- α (TNF- α) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients. *Neuroscience letters*, 165, 208-210.
- MOODLEY, K., ANGEL, C. E., GLASS, M. & GRAHAM, E. S. 2011. Real-time profiling of NK cell killing of human astrocytes using xCELLigence technology. *Journal of neuroscience methods*, 200, 173-180.
- MOSMANN, T. R., LI, L. & SAD, S. Functions of CD8 T-cell subsets secreting different cytokine patterns. *Seminars in immunology*, 1997. Elsevier, 87-92.
- MOSSER, D. M. & EDWARDS, J. P. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*, 8, 958-969.
- MUELLER, T. D., ZHANG, J.-L., SEBALD, W. & DUSCHL, A. 2002. Structure, binding, and antagonists in the IL-4/IL-13 receptor system. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1592, 237-250.
- MUNSCHAUER, F., HARTRICH, L., STEWART, C. & JACOBS, L. 1995. Circulating natural killer cells but not cytotoxic T lymphocytes are reduced in patients with active relapsing multiple sclerosis and little clinical disability as compared to controls. *Journal of neuroimmunology*, 62, 177-181.
- MURDOCK, B. J., BENDER, D. E., SEGAL, B. M. & FELDMAN, E. L. 2015. The dual roles of immunity in ALS: injury overrides protection. *Neurobiology of disease*, 77, 1-12.
- MURPHY, Á. C., LALOR, S. J., LYNCH, M. A. & MILLS, K. H. 2010. Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis. *Brain, behavior, and immunity*, 24, 641-651.
- MURRAY, P. J., ALLEN, J. E., BISWAS, S. K., FISHER, E. A., GILROY, D. W., GOERDT, S., GORDON, S., HAMILTON, J. A., IVASHKIV, L. B. & LAWRENCE, T. 2014. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*, 41, 14-20.
- MURRAY, T. 2006. Diagnosis and treatment of multiple sclerosis. *BMJ: British Medical Journal*, 332, 525.
- MURRAY, T. J. 2009. The history of multiple sclerosis: the changing frame of the disease over the centuries. *Journal of the neurological sciences*, 277, S3-S8.
- NAIKI, M., MARCUS, D. M. & LEDEEN, R. 1974. Properties of antisera to ganglioside GM1 and asialo GM1. *The Journal of Immunology*, 113, 84-93.
- NAYAK, D., ROTH, T. L. & MCGAVERN, D. B. 2014. Microglia Development and Function. *Annual review of immunology*.
- NEIGHBOUR, P. A., MILLER, A. E. & BLOOM, B. R. 1981. Interferon responses of leukocytes in multiple sclerosis. *Neurology*, 31, 561-561.
- NICHOLSON, S., DA G BONECINI-ALMEIDA, M., E SILVA, J. L., NATHAN, C., XIE, Q., MUMFORD, R., WEIDNER, J. R., CALAYCAY, J., GENG, J. & BOECHAT, N. 1996. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *The Journal of experimental medicine*, 183, 2293-2302.
- NIELSEN, N., ØDUM, N., URSØ, B., LANIER, L. L. & SPEE, P. 2012. Cytotoxicity of CD56bright NK cells towards autologous activated CD4+ T cells is mediated through NKG2D, LFA-1 and TRAIL and dampened via CD94/NKG2A. *PloS one*, 7, e31959.
- NIKODEMOVA, M. & WATTERS, J. J. 2011. Outbred ICR/CD1 mice display more severe neuroinflammation mediated by microglial TLR4/CD14 activation than inbred C57Bl/6 mice. *Neuroscience*, 190, 67-74.
- O'CONNOR, R. A., PRENDERGAST, C. T., SABATOS, C. A., LAU, C. W., LEECH, M. D., WRAITH, D. C. & ANDERTON, S. M. 2008. Cutting edge: Th1 cells facilitate the entry of Th17 cells to

- the central nervous system during experimental autoimmune encephalomyelitis. *The Journal of Immunology*, 181, 3750-3754.
- OPPMANN, B., LESLEY, R., BLOM, B., TIMANS, J. C., XU, Y., HUNTE, B., VEGA, F., YU, N., WANG, J. & SINGH, K. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity*, 13, 715-725.
- OREJA-GUEVARA, C., RAMOS-CEJUDO, J., AROEIRA, L. S., CHAMORRO, B. & DIEZ-TEJEDOR, E. 2012. TH1/TH2 Cytokine profile in relapsing-remitting multiple sclerosis patients treated with Glatiramer acetate or Natalizumab. *BMC neurology*, 12, 95.
- ORIHUELA, R., MCPHERSON, C. A. & HARRY, G. J. 2015. Microglial M1/M2 polarization and metabolic states. *British journal of pharmacology*.
- PANITCH, H., HALEY, A., HIRSCH, R. & JOHNSON, K. 1987. Exacerbations of multiple sclerosis in patients treated with gamma interferon. *The Lancet*, 329, 893-895.
- PARRATT, J. D. & PRINEAS, J. W. 2010. Neuromyelitis optica: a demyelinating disease characterized by acute destruction and regeneration of perivascular astrocytes. *Multiple Sclerosis*, 16, 1156-1172.
- PATY, D. & LI, D. B. 1993. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis II. MRI analysis results of a multicenter, randomized, double-blind, placebo-controlled trial. *Neurology*, 43, 662-662.
- PEFEROEN, L. A., VOGEL, D. Y., UMMENTHUM, K., BREUR, M., HEIJNEN, P. D., GERRITSEN, W. H., PEFEROEN-BAERT, R. M., VAN DER VALK, P., DIJKSTRA, C. D. & AMOR, S. 2015. Activation status of human microglia is dependent on lesion formation stage and remyelination in multiple sclerosis. *Journal of Neuropathology & Experimental Neurology*, 74, 48-63.
- PEKNY, M., WILHELMSSON, U. & PEKNA, M. 2014. The dual role of astrocyte activation and reactive gliosis. *Neuroscience letters*, 565, 30-38.
- PELLERIN, L. & MAGISTRETTI, P. J. 1994. Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proceedings of the National Academy of Sciences*, 91, 10625-10629.
- PESTKA, S., KRAUSE, C. D. & WALTER, M. R. 2004. Interferons, interferon-like cytokines, and their receptors. *Immunological reviews*, 202, 8-32.
- PFEIFFER, T., SCHUSTER, S. & BONHOEFFER, S. 2001. Cooperation and competition in the evolution of ATP-producing pathways. *science*, 292, 504-507.
- POLI, A., MICHEL, T., THÉRÉSINE, M., ANDRÈS, E., HENTGES, F. & ZIMMER, J. 2009. CD56bright natural killer (NK) cells: an important NK cell subset. *Immunology*, 126, 458-465.
- PONOMAREV, E. D., MARESZ, K., TAN, Y. & DITTEL, B. N. 2007a. CNS-derived interleukin-4 is essential for the regulation of autoimmune inflammation and induces a state of alternative activation in microglial cells. *The Journal of neuroscience*, 27, 10714-10721.
- PONOMAREV, E. D., SHRIVER, L. P. & DITTEL, B. N. 2006. CD40 expression by microglial cells is required for their completion of a two-step activation process during central nervous system autoimmune inflammation. *The Journal of Immunology*, 176, 1402-1410.
- PONOMAREV, E. D., SHRIVER, L. P., MARESZ, K. & DITTEL, B. N. 2005. Microglial cell activation and proliferation precedes the onset of CNS autoimmunity. *Journal of neuroscience research*, 81, 374-389.
- PONOMAREV, E. D., SHRIVER, L. P., MARESZ, K., PEDRAS-VASCONCELOS, J., VERTHELYI, D. & DITTEL, B. N. 2007b. GM-CSF production by autoreactive T cells is required for the activation of microglial cells and the onset of experimental autoimmune encephalomyelitis. *The Journal of Immunology*, 178, 39.
- PONOMAREV, E. D., SHRIVER, L. P., MARESZ, K., PEDRAS-VASCONCELOS, J., VERTHELYI, D. & DITTEL, B. N. 2007c. GM-CSF production by autoreactive T cells is required for the activation of microglial cells and the onset of experimental autoimmune encephalomyelitis. *The Journal of Immunology*, 178, 39-48.

- POPOVIC, N., SCHUBART, A., GOETZ, B. D., ZHANG, S. C., LININGTON, C. & DUNCAN, I. D. 2002. Inhibition of autoimmune encephalomyelitis by a tetracycline. *Annals of neurology*, 51, 215-223.
- PORPORATO, P. E., DADHICH, R. K., DHUP, S., COPETTI, T. & SONVEAUX, P. 2011. Anticancer targets in the glycolytic metabolism of tumors: a comprehensive review. *Frontiers in Pharmacology*, 2.
- PRAJEETH, C. K., HAEBERLEIN, S., SEBALD, H., SCHLEICHER, U. & BOGDAN, C. 2011. Leishmania-infected macrophages are targets of NK cell-derived cytokines but not of NK cell cytotoxicity. *Infection and immunity*, 79, 2699-2708.
- PRINZ, M. & PRILLER, J. 2014. Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease. *Nature Reviews Neuroscience*, 15, 300-312.
- PUTZKI, N., BARANWAL, M. K., TETTENBORN, B., LIMMROTH, V. & KREUZFELDER, E. 2010. Effects of natalizumab on circulating B cells, T regulatory cells and natural killer cells. *European neurology*, 63, 311-317.
- RACKE, M. K., BONOMO, A., SCOTT, D. E., CANNELLA, B., LEVINE, A., RAINE, C. S., SHEVACH, E. M. & RÖCKEN, M. 1994. Cytokine-induced immune deviation as a therapy for inflammatory autoimmune disease. *The Journal of experimental medicine*, 180, 1961-1966.
- RADU, C. G., SHU, C. J., SHELLY, S. M., PHELPS, M. E. & WITTE, O. N. 2007. Positron emission tomography with computed tomography imaging of neuroinflammation in experimental autoimmune encephalomyelitis. *Proceedings of the National Academy of Sciences*, 104, 1937-1942.
- RAJAN, A. J., GAO, Y.-L., RAINE, C. S. & BROSNAN, C. F. 1996. A pathogenic role for gamma delta T cells in relapsing-remitting experimental allergic encephalomyelitis in the SJL mouse. *The Journal of Immunology*, 157, 941-949.
- RAMAGLIA, V., HUGHES, T. R., DONEV, R. M., RUSEVA, M. M., WU, X., HUITINGA, I., BAAS, F., NEAL, J. W. & MORGAN, B. P. 2012. C3-dependent mechanism of microglial priming relevant to multiple sclerosis. *Proceedings of the National Academy of Sciences*, 109, 965-970.
- RAVETCH, J. V. & LANIER, L. L. 2000. Immune inhibitory receptors. *Science*, 290, 84-89.
- RENNO, T., KRAKOWSKI, M., PICCIRILLO, C., LIN, J. & OWENS, T. 1995. TNF-alpha expression by resident microglia and infiltrating leukocytes in the central nervous system of mice with experimental allergic encephalomyelitis. Regulation by Th1 cytokines. *The Journal of Immunology*, 154, 944-953.
- RENNO, T., TAUPIN, V., BOURBONNIÈRE, L., VERGE, G., TRAN, E., DE SIMONE, R., KRAKOWSKI, M., RODRIGUEZ, M., PETERSON, A. & OWENS, T. 1998. Interferon- γ in progression to chronic demyelination and neurological deficit following acute EAE. *Molecular and Cellular Neuroscience*, 12, 376-389.
- REYNDERS, A., YESSAAD, N., VU MANH, T. P., DALOD, M., FENIS, A., AUBRY, C., NIKITAS, G., ESCALIÈRE, B., RENAULD, J. C. & DUSSURGET, O. 2011. Identity, regulation and in vivo function of gut NKp46+ ROR γ t+ and NKp46+ ROR γ t- lymphoid cells. *The EMBO journal*, 30, 2934-2947.
- REZAIE, P. & MALE, D. 2002. Mesoglia & microglia—a historical review of the concept of mononuclear phagocytes within the central nervous system. *Journal of the History of the Neurosciences*, 11, 325-374.
- RIBEIRO, S. M., GIMÉNEZ-CASSINA, A. & DANIAL, N. N. 2015. Measurement of Mitochondrial Oxygen Consumption Rates in Mouse Primary Neurons and Astrocytes. *Mitochondrial Regulation*. Springer.
- RIBOT, J. C., CHAVES-FERREIRA, M., D'OREY, F., WENCKER, M., GONÇALVES-SOUSA, N., DECALF, J., SIMAS, J. P., HAYDAY, A. C. & SILVA-SANTOS, B. 2010. Cutting Edge: Adaptive Versus Innate Receptor Signals Selectively Control the Pool Sizes of Murine

- IFN- γ -or IL-17-Producing $\gamma\delta$ T Cells upon Infection. *The Journal of Immunology*, 185, 6421-6425.
- RIVERS, T. M. & SCHWENTKER, F. F. 1935. Encephalomyelitis accompanied by myelin destruction experimentally produced in monkeys. *The Journal of experimental medicine*, 61, 689-702.
- ROBBINS, S. H., TESSMER, M. S., MIKAYAMA, T. & BROSSAY, L. 2004. Expansion and contraction of the NK cell compartment in response to murine cytomegalovirus infection. *The Journal of Immunology*, 173, 259-266.
- RODRÍGUEZ-PRADOS, J.-C., TRAVÉS, P. G., CUENCA, J., RICO, D., ARAGONÉS, J., MARTÍN-SANZ, P., CASCANTE, M. & BOSCA, L. 2010. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. *The Journal of Immunology*, 185, 605-614.
- ROTHHAMMER, V., HEINK, S., PETERMANN, F., SRIVASTAVA, R., CLAUSSEN, M. C., HEMMER, B. & KORN, T. 2011. Th17 lymphocytes traffic to the central nervous system independently of $\alpha 4$ integrin expression during EAE. *The Journal of experimental medicine*, 208, 2465-2476.
- SAIJO, K. & GLASS, C. K. 2011. Microglial cell origin and phenotypes in health and disease. *Nature Reviews Immunology*, 11, 775-787.
- SALLUSTO, F., IMPELLIZZIERI, D., BASSO, C., LARONI, A., UCCELLI, A., LANZAVECCHIA, A. & ENGELHARDT, B. 2012. T-cell trafficking in the central nervous system. *Immunological reviews*, 248, 216-227.
- SARASTE, M., IRJALA, H. & AIRAS, L. 2007. Expansion of CD56Bright natural killer cells in the peripheral blood of multiple sclerosis patients treated with interferon-beta. *Neurological Sciences*, 28, 121-126.
- SARMA, J. D., CIRIC, B., MAREK, R., SADHUKHAN, S., CARUSO, M. L., SHAFAGH, J., FITZGERALD, D. C., SHINDLER, K. S. & ROSTAMI, A. 2009. Functional interleukin-17 receptor A is expressed in central nervous system glia and upregulated in experimental autoimmune encephalomyelitis. *Journal of Neuroinflammation*, 6, 14.
- SASAKI, K., PARDEE, A. D., OKADA, H. & STORKUS, W. J. 2008. IL-4 inhibits VLA-4 expression on Tc1 cells resulting in poor tumor infiltration and reduced therapy benefit. *European journal of immunology*, 38, 2865-2873.
- SASAKI, K., PARDEE, A. D., QU, Y., ZHAO, X., UEDA, R., KOHANBASH, G., BAILEY, L. M., OKADA, H., MUTHUSWAMY, R. & KALINSKI, P. 2009. IL-4 suppresses Very Late Antigen-4 expression which is required for therapeutic Th1 T cell trafficking into tumors. *Journal of immunotherapy (Hagerstown, Md.: 1997)*, 32, 793.
- SATO, J.-I., KASTRUKOFF, L. F. & KIM, S. U. 1991. Cytokine-Induced Expression of Intercellular Adhesion Molecule-1 (ICAM-1) in Cultured Human Oligodendrocytes and Astrocytes. *Journal of Neuropathology & Experimental Neurology*, 50, 215-226.
- SCHARTON, T. M. & SCOTT, P. 1993. Natural killer cells are a source of interferon gamma that drives differentiation of CD4+ T cell subsets and induces early resistance to Leishmania major in mice. *The Journal of experimental medicine*, 178, 567-577.
- SCHREMPF, W. & ZIEMSEN, T. 2007. Glatiramer acetate: mechanisms of action in multiple sclerosis. *Autoimmunity reviews*, 6, 469-475.
- SCHRODER, K., HERTZOG, P. J., RAVASI, T. & HUME, D. A. 2004. Interferon- γ : an overview of signals, mechanisms and functions. *Journal of leukocyte biology*, 75, 163-189.
- SHEN, H., GOODALL, J. C. & HILL GASTON, J. 2009. Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. *Arthritis & Rheumatism*, 60, 1647-1656.
- SHI, F.-D., LJUNGGREN, H.-G., LA CAVA, A. & VAN KAER, L. 2011a. Organ-specific features of natural killer cells. *Nature Reviews Immunology*, 11, 658-671.

- SHI, F.-D., TAKEDA, K., AKIRA, S., SARVETNICK, N. & LJUNGGREN, H.-G. 2000. IL-18 directs autoreactive T cells and promotes autodestruction in the central nervous system via induction of IFN- γ by NK cells. *The Journal of Immunology*, 165, 3099-3104.
- SHI, L. Z., WANG, R., HUANG, G., VOGEL, P., NEALE, G., GREEN, D. R. & CHI, H. 2011b. HIF1 α -dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *The Journal of experimental medicine*, 208, 1367-1376.
- SHIMADA, S. & IWATA, D. 1987. Preparation of monoclonal antibodies against a glycolipid asialo GM1. *Microbiology and immunology*, 31, 923-933.
- SICA, A. & MANTOVANI, A. 2012. Macrophage plasticity and polarization: in vivo veritas. *The Journal of clinical investigation*, 122, 787-795.
- SINGH, V., MEHROTRA, S. & AGARWAL, S. 1999. The paradigm of Th1 and Th2 cytokines. *Immunologic research*, 20, 147-161.
- SIXT, M., BAUER, M., LÄMMERMANN, T. & FÄSSLER, R. 2006. β 1 integrins: zip codes and signaling relay for blood cells. *Current opinion in cell biology*, 18, 482-490.
- SLOKA, S., METZ, L. M., HADER, W., STARREVELD, Y. & YONG, V. W. 2013. Reduction of microglial activity in a model of multiple sclerosis by dipyridamole. *Journal of neuroinflammation*, 10, 89.
- SMITH, C. W. 2008. 3. Adhesion molecules and receptors. *Journal of Allergy and Clinical Immunology*, 121, S375-S379.
- SMYTH, M. J., THIA, K. Y., STREET, S. E., CRETNEY, E., TRAPANI, J. A., TANIGUCHI, M., KAWANO, T., PELIKAN, S. B., CROWE, N. Y. & GODFREY, D. I. 2000. Differential tumor surveillance by natural killer (NK) and NKT cells. *The Journal of experimental medicine*, 191, 661-668.
- SÖDERSTRÖM, K., STEIN, E., COLMENERO, P., PURATH, U., MÜLLER-LADNER, U., DE MATOS, C. T., TARNER, I. H., ROBINSON, W. H. & ENGLEMAN, E. G. 2010. Natural killer cells trigger osteoclastogenesis and bone destruction in arthritis. *Proceedings of the National Academy of Sciences*, 107, 13028-13033.
- SOFRONIEW, M. V. & VINTERS, H. V. 2010. Astrocytes: biology and pathology. *Acta neuropathologica*, 119, 7-35.
- SOILU-HÄNNINEN, M., RÖYTTÄ, M., SALMI, A. & SALONEN, R. 1997. Therapy with antibody against leukocyte integrin VLA-4 (CD49d) is effective and safe in virus-facilitated experimental allergic encephalomyelitis. *Journal of neuroimmunology*, 72, 95-105.
- SRIRAM, S. & STEINER, I. 2005. Experimental allergic encephalomyelitis: a misleading model of multiple sclerosis. *Annals of neurology*, 58, 939-945.
- STANLEY, B., POST, J. & HENSLEY, K. 2012. A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease. *Journal of Neuroinflammation*, 9, 115.
- STAROSSOM, S. C., MASCANFRONI, I. D., IMITOLA, J., CAO, L., RADDASSI, K., HERNANDEZ, S. F., BASSIL, R., CROCI, D. O., CERLIANI, J. P. & DELACOUR, D. 2012. Galectin-1 deactivates classically activated microglia and protects from inflammation-induced neurodegeneration. *Immunity*, 37, 249-263.
- STEIN, M., KESHAV, S., HARRIS, N. & GORDON, S. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *The Journal of experimental medicine*, 176, 287-292.
- STEINMAN, L. & ZAMVIL, S. S. 2005. Virtues and pitfalls of EAE for the development of therapies for multiple sclerosis. *Trends in immunology*, 26, 565-571.
- STENGER, S., THÜRING, H., RÖLLINGHOFF, M. & BOGDAN, C. 1994. Tissue expression of inducible nitric oxide synthase is closely associated with resistance to *Leishmania major*. *The Journal of experimental medicine*, 180, 783-793.

- STETSON, D. B., MOHRS, M., REINHARDT, R. L., BARON, J. L., WANG, Z.-E., GAPIN, L., KRONENBERG, M. & LOCKSLEY, R. M. 2003. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *The Journal of experimental medicine*, 198, 1069-1076.
- STINCHCOMBE, J. C. & GRIFFITHS, G. M. 2007. Secretory mechanisms in cell-mediated cytotoxicity. *Annu. Rev. Cell Dev. Biol.*, 23, 495-517.
- STRACHAN-WHALEY, M., RIVEST, S. & YONG, V. W. 2014. Interactions between microglia and T cells in multiple sclerosis pathobiology. *Journal of Interferon & Cytokine Research*, 34, 615-622.
- STROMNES, I. M. & GOVERMAN, J. M. 2006. Active induction of experimental allergic encephalomyelitis. *Nature protocols*, 1, 1810-1819.
- STROWIG, T., BRILOT, F. & MÜNZ, C. 2008. Noncytotoxic functions of NK cells: direct pathogen restriction and assistance to adaptive immunity. *The Journal of Immunology*, 180, 7785-7791.
- SUN, J. C. & LANIER, L. L. 2011. NK cell development, homeostasis and function: parallels with CD8+ T cells. *Nature Reviews Immunology*, 11, 645-657.
- SUTHERLAND, T. E., LOGAN, N., RÜCKERL, D., HUMBLE, A. A., ALLAN, S. M., PAPAYANNOPOULOS, V., STOCKINGER, B., MAIZELS, R. M. & ALLEN, J. E. 2014. Chitinase-like proteins promote IL-17-mediated neutrophilia in a tradeoff between nematode killing and host damage. *Nature immunology*.
- SUTTON, C. E., LALOR, S. J., SWEENEY, C. M., BRERETON, C. F., LAVELLE, E. C. & MILLS, K. H. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from $\gamma\delta$ T cells, amplifying Th17 responses and autoimmunity. *Immunity*, 31, 331-341.
- SWAIN, S. L., WEINBERG, A. D., ENGLISH, M. & HUSTON, G. 1990. IL-4 directs the development of Th2-like helper effectors. *The Journal of Immunology*, 145, 3796-3806.
- THOMPSON, A. J., TOOSY, A. T. & CICCARELLI, O. 2010. Pharmacological management of symptoms in multiple sclerosis: current approaches and future directions. *The Lancet Neurology*, 9, 1182-1199.
- TIAN, Z., GERSHWIN, M. E. & ZHANG, C. 2012. Regulatory NK cells in autoimmune disease. *Journal of autoimmunity*, 39, 206-215.
- VAN DEN BOSSCHE, J., NEELE, A. E., HOEKSEMA, M. A., DE HEIJ, F., BOSHUIZEN, M. C., VAN DER VELDEN, S., DE BOER, V. C., REEDQUIST, K. A. & DE WINTHER, M. P. 2014. Inhibiting epigenetic enzymes to improve atherogenic macrophage functions. *Biochemical and biophysical research communications*, 455, 396-402.
- VAN HORSSSEN, J., SINGH, S., VAN DER POL, S., KIPP, M., LIM, J. L., PEFFEROEN, L., GERRITSEN, W., KOOI, E.-J., WITTE, M. E. & GEURTS, J. 2012. Clusters of activated microglia in normal-appearing white matter show signs of innate immune activation. *J Neuroinflammation*, 9, 156.
- VANDER HEIDEN, M. G., CANTLEY, L. C. & THOMPSON, C. B. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *science*, 324, 1029-1033.
- VARIN, A. & GORDON, S. 2009. Alternative activation of macrophages: immune function and cellular biology. *Immunobiology*, 214, 630-641.
- VIVIER, E., RAULET, D. H., MORETTA, A., CALIGIURI, M. A., ZITVOGEL, L., LANIER, L. L., YOKOYAMA, W. M. & UGOLINI, S. 2011. Innate or adaptive immunity? The example of natural killer cells. *Science*, 331, 44.
- VOLLMER, T. L., LIU, R., PRICE, M., RHODES, S., LA CAVA, A. & SHI, F.-D. 2005. Differential effects of IL-21 during initiation and progression of autoimmunity against neuroantigen. *The Journal of Immunology*, 174, 2696-2701.
- VOLOBOUEVA, L. A., EMERY, J. F., SUN, X. & GIFFARD, R. G. 2013. Inflammatory response of microglial BV-2 cells includes a glycolytic shift and is modulated by mitochondrial glucose-regulated protein 75/mortalin. *FEBS Letters*, 587, 756-762.

- VON BERNHARDI, R. & EUGENÍN, J. 2004. Microglial reactivity to β -amyloid is modulated by astrocytes and proinflammatory factors. *Brain research*, 1025, 186-193.
- WANG, K. S., FRANK, D. A. & RITZ, J. 2000. Interleukin-2 enhances the response of natural killer cells to interleukin-12 through up-regulation of the interleukin-12 receptor and STAT4. *Blood*, 95, 3183-3190.
- WANG, T., MARQUARDT, C. & FOKER, J. 1976. Aerobic glycolysis during lymphocyte proliferation.
- WARBURG, O., POSENER, K. & NEGELEIN, E. 1924. Ueber den stoffwechsel der tumoren. *Biochemische Zeitschrift*, 152, 319-344.
- WHEELER, R. D., ZEHNTNER, S. P., KELLY, L. M., BOURBONNIÈRE, L. & OWENS, T. 2006. Elevated interferon gamma expression in the central nervous system of tumour necrosis factor receptor 1-deficient mice with experimental autoimmune encephalomyelitis. *Immunology*, 118, 527-538.
- WILLENBORG, D. O., FORDHAM, S., BERNARD, C., COWDEN, W. B. & RAMSHAW, I. A. 1996. IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *The Journal of Immunology*, 157, 3223-3227.
- WINKLER-PICKETT, R., YOUNG, H. A., CHERRY, J. M., DIEHL, J., WINE, J., BACK, T., BERE, W. E., MASON, A. T. & ORTALDO, J. R. 2008. In vivo regulation of experimental autoimmune encephalomyelitis by NK cells: alteration of primary adaptive responses. *The Journal of Immunology*, 180, 4495-4506.
- WONG, J. M. & BILLIAR, T. R. 1994. Regulation and function of inducible nitric oxide synthase during sepsis and acute inflammation. *Advances in pharmacology (San Diego, Calif.)*, 34, 155-170.
- WU, M., NEILSON, A., SWIFT, A. L., MORAN, R., TAMAGNINE, J., PARSLOW, D., ARMISTEAD, S., LEMIRE, K., ORRELL, J. & TEICH, J. 2007. Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *American Journal of Physiology-Cell Physiology*, 292, C125-C136.
- WYNN, T. A., CHAWLA, A. & POLLARD, J. W. 2013. Macrophage biology in development, homeostasis and disease. *Nature*, 496, 445-455.
- XU, W., FAZEKAS, G., HARA, H. & TABIRA, T. 2005. Mechanism of natural killer (NK) cell regulatory role in experimental autoimmune encephalomyelitis. *Journal of neuroimmunology*, 163, 24-30.
- YAMASAKI, R., LU, H., BUTOVSKY, O., OHNO, N., RIETSCH, A. M., CIALIC, R., WU, P. M., DOYKAN, C. E., LIN, J. & COTLEUR, A. C. 2014. Differential roles of microglia and monocytes in the inflamed central nervous system. *The Journal of experimental medicine*, 211, 1533-1549.
- YAZDANBAKHSH, M., KREMSNER, P. G. & VAN REE, R. 2002. Allergy, parasites, and the hygiene hypothesis. *Science*, 296, 490-494.
- YEDNOCK, T. A., CANNON, C., FRITZ, L. C., SANCHEZ-MADRID, F., STEINMAN, L. & KARIN, N. 1992a. Prevention of experimental autoimmune encephalomyelitis by antibodies against $\alpha 4\beta 1$ integrin. *Nature*, 356, 63-66.
- YEDNOCK, T. A., CANNON, C., FRITZ, L. C., SANCHEZ-MADRID, F., STEINMAN, L. & KARIN, N. 1992b. Prevention of experimental autoimmune encephalomyelitis by antibodies against $\alpha 4\beta 1$ integrin.
- YOUNG, D. A., LOWE, L. D., BOOTH, S. S., WHITTERS, M. J., NICHOLSON, L., KUCHROO, V. K. & COLLINS, M. 2000. IL-4, IL-10, IL-13, and TGF- β from an altered peptide ligand-specific Th2 cell clone down-regulate adoptive transfer of experimental autoimmune encephalomyelitis. *The Journal of Immunology*, 164, 3563-3572.

- YU, Z., SUN, D., FENG, J., TAN, W., FANG, X., ZHAO, M., ZHAO, X., PU, Y., HUANG, A. & XIANG, Z. 2015. MSX3 Switches Microglia Polarization and Protects from Inflammation-Induced Demyelination. *The Journal of Neuroscience*, 35, 6350-6365.
- ZHANG, B.-N., YAMAMURA, T., KONDO, T., FUJIWARA, M. & TABIRA, T. 1997a. Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. *The Journal of experimental medicine*, 186, 1677-1687.
- ZHANG, B., YAMAMURA, T., KONDO, T., FUJIWARA, M. & TABIRA, T. 1997b. Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. *The Journal of experimental medicine*, 186, 1677.
- ZHANG, X. M., LUND, H., MIA, S., PARSA, R. & HARRIS, R. A. 2014. Adoptive transfer of cytokine-induced immunomodulatory adult microglia attenuates experimental autoimmune encephalomyelitis in DBA/1 mice. *Glia*, 62, 804-817.
- ZHU, J. 2015. T helper 2 (Th2) cell differentiation, type 2 innate lymphoid cell (ILC2) development and regulation of interleukin-4 (IL-4) and IL-13 production. *Cytokine*, 75, 14-24.

Appendix I: List of Publications

Tanveer, R., **McGuinness, N.**, Daniel, S., Gowran, A., Campbell, V.A. (2012) Cannabinoid receptors and neurodegenerative diseases. *WIREs Membrane Transport and Signalling*, 1(5): 633-639

Dungan, L.S.*, **McGuinness, N.C.***, Boon, L., Lynch, M.A., Mills, K.H.G (2014) Innate IFN- γ promotes development of experimental autoimmune encephalomyelitis: a role for NK cells and M1 macrophages. *European Journal of Immunology*, 44 (10): 2903-2917

*both authors contributed equally to this work

Edwards, S.C., McGinley, A.M., **McGuinness, N.C.**, Mills, K.H.G (2015) $\gamma\delta$ T cells and NK cells – distinct pathogenic roles as innate-like immune cells in CNS autoimmunity. *Frontiers in Immunology*, 6 (455): 1-6

Appendix II: List of Materials

2-ME	Sigma-Aldrich
Ammonium chloride	Sigma-Aldrich
Anti-asialo GM1	Cedarlane
Anti-CD3	EBioscience
Anti-CD28	EBioscience
Anti-CD49d (Clone PS/2)	Bio X Cell
Antimycin A	Sigma-Aldrich
BFA	Sigma-Aldrich
BSA	Sigma-Aldrich
CD3+ T cell Isolation Kit	Miltenyi Biotec
CD4 ⁺ T cell Isolation Kit	Miltenyi Biotec
cDNA Archive Kits	Applied Biosystems
Cell scrapers	Sarstedt
Cell strainers (40 μ m, 70 μ m)	Fisherbrand
CFA	Chondrex
Compensation Beads	BD Bioscience
DMEM	Gibco
EBAO	Sigma-Aldrich
ELISA kits	R&D Systems
ELISA plates	Sigma Aldrich/NUNC
Ethanol (molecular grade)	Sigma Aldrich
FACS antibodies	BD Bioscience/EBioscience
Falcon tubes (15 ml, 50 ml)	Fisher Scientific
FBS	Gibco

FCCP	Sigma-Aldrich
Haemocytometer	Hycor Biomedical
Ionomycin	Sigma-Aldrich
LDH Assay Kit	Promega
L-glutamine	Sigma-Aldrich
Live/Dead Stain	Life Technologies
Microplate Sealers	Cruinn Diagnostics/Greiner
Microcentrifuge tubes (1.5 ml, RNase free)	Eppendorf
MOG	Genscript
Needles (19, 23 and 27 gauge)	BD Microlance
NK cell Isolation Kit	Miltenyi Biotec
Non-essential amino acids	Hyclone
Nucleospin RNA Isolation Kits	Machery-Nagel
Oligomycin	Abcam
Pasteur pipettes	Fisher Scientific
PBS (sterile)	Sigma-Aldrich
PBS tablets	Sigma-Aldrich
PCR plates	Fisher Scientific
PCR tubes	Sarstedt
Penicillin/Streptomycin	Hyclone
Percoll Plus	GE Healthcare Life Sciences
Petri dish	Gibco
PFA	Thermo Scientific
PMA	Sigma-Aldrich
Potassium bicarbonate	Sigma-Aldrich
Protein Assay Plates (96-well, flat-bottomed)	Sarstedt

PT	Kaketsuken
RA1 buffer	Machery-Nagel
Rabbit serum (sterile)	Sigma-Aldrich
Recombinant human IL-2	Immunotools
Recombinant mouse GM-CSF	R&D Systems
Recombinant mouse IFN- γ	R&D Systems
Recombinant mouse IL-1 β	R&D Systems
Recombinant mouse IL-4	R&D Systems
Recombinant mouse IL-12	R&D Systems
Recombinant mouse IL-23	R&D Systems
Recombinant mouse M-CSF	R&D Systems
RPMI	Sigma-Aldrich
Saponin	Sigma-Aldrich
Scalpels	Swann-Morton
Syringes (hypodermic; 1 ml, 5 ml, 10 ml, 20 ml)	BD Plastipak
Sodium pyruvate	Hyclone
Syringe filter (0.22 μ m)	Millipore
Taqman Gene Expression Assays	Applied Biosystems
Tissue culture flasks (T25, T75, T175)	Fisher Scientific
Tissue culture plates	Fisher Scientific
Trypan Blue	Sigma-Aldrich
Trypsin-EDTA	Hyclone
XF24 calibration buffer	Seahorse Bioscience
XF24 cartridges	Seahorse Bioscience
XF24 cell culture plates	Seahorse Bioscience
XF24 assay medium	Seahorse Bioscience

Appendix III: List of Solutions

Buffers

Blocking Buffer (ELISA)

100ml PBS

1 g BSA

Lysis Buffer for PCR

100:1 ratio of RA1 buffer (Machery-Nagel) and β -mercaptoethanol (Sigma-Aldrich)

MACS Buffer

Sterile PBS (500 ml, Sigma-Aldrich)

FCS (0.05 %/2.5 ml) GIBCO)

EDTA (2 mM, Sigma-Aldrich)

PBS

5 tablets (Sigma-Aldrich) per 1 L dH₂O

Red Blood Cell Lysis Buffer

8.3 g ammonium hydrochloride (NH₄Cl; Sigma-Aldrich)

1 g potassium bicarbonate (KHCO₃; Sigma-Aldrich)

0.037 g EDTA (Sigma-Aldrich)

1 L dH₂O

Filter sterilised before use

Stop Solution (ELISA)

1M H₂SO₄

Substrate Solution (ELISA)

1:1 mixture of hydrogen peroxide and tetramethylbenzidine (Sigma-Aldrich)

Wash buffer (ELISA)

1 L PBS

500 µl Tween-20

Medium**Complete DMEM**

Dulbecco's Modified Eagle Medium (with 2.5 mM L-glutamine and 15 mM HEPES, 3.15 g/L glucose, GIBCO)

FBS (10%, heat activated (56°C, 60 min); Hyclone)

Penicillin/streptomycin (100 µg/ml; Hyclone)

DMEM for NK cell culture

Dulbecco's Modified Eagle Medium (with 2.5 mM L-glutamine and 15 mM HEPES, 3.15 g/L glucose, GIBCO)

Penicillin/streptomycin (10000 U/ ml, Hyclone)

Foetal bovine serum (FBS, 10 %, GIBCO)

2-mercaptoethanol (50 µM, Sigma-Aldrich)

Non-essential amino acids (0.1 mM, Hyclone)

Sodium pyruvate (2 mM, Hyclone)

Complete RPMI (cRPMI)

Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich)

L-glutamine (2.5 mM, Sigma-Aldrich)

Foetal bovine serum (FBS, 10 %, GIBCO)

Penicillin/streptomycin (10000 U/ ml, Hyclone)

Complete RPMI (cRPMI) for T cell culture

Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich)

L-glutamine (2.5 mM, Sigma-Aldrich)

Foetal bovine serum (FBS, 10 %, GIBCO)

Penicillin/streptomycin (10000 U/ ml, Hyclone)

2-mercaptoethanol (50 μ M, Sigma-Aldrich)

Percoll Gradients

Percoll PLUS (GE Healthcare Life Sciences)

Dulbecco's Phosphate Buffered Saline (PBS) (Sigma-Aldrich)

10 x Dulbecco's Phosphate Buffered Saline (PBS) (Sigma-Aldrich)

For 100 ml

40% Percoll – 40 ml Stock Isotonic Percoll (36 ml Percoll PLUS + 4 ml 10x PBS (Sigma-Aldrich)) + 60 ml PBS

70% Percoll – 70 ml Stock Isotonic Percoll (63 ml Percoll PLUS + 7 ml 10x PBS (Sigma-Aldrich)) + 30 ml PBS.