Accepted Manuscript

Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis

Áine C. Murphy, Stephen J. Lalor, Marina A. Lynch, Kingston H.G. Mills

PII: S0889-1591(10)00041-3
DOI: [10.1016/j.bbi.2010.01.014](https://doi.org/10.1016/j.bbi.2010.01.014)
Reference: YBRBI 1522

To appear in: *Brain, Behavior, and Immunity*

Received Date: 7 December 2009
Revised Date: 13 January 2010
Accepted Date: 24 January 2010

Please cite this article as: Murphy, Á., Lalor, Á.J., Lynch, Á.A., Mills, K.H.G., Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis, *Brain, Behavior, and Immunity* (2010), doi: [10.1016/j.bbi.2010.01.014](https://doi.org/10.1016/j.bbi.2010.01.014)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the
course of experimental autoimmune encephalomyelitis**

Áine C. Murphy^{a,b}, Stephen J. Lalor^b, Marina A. Lynch^{a*}, and Kingston H.G. Mills^{b*}

^a*Trinity College Institute for Neuroscience, Trinity College, Dublin 2, Ireland*

^b*School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland*

* These authors contributed equally.

Corresponding authors: Prof. Marina Lynch, Trinity College Institute for Neuroscience,
Trinity College Dublin 2, Ireland. Email: marina.lynch@tcd.ie or Prof. Kingston Mills,
School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland. Email:
kingston.mills@tcd.ie; Fax +353-1-6772986; phone +353-128963573

21 **ABSTRACT**

22 Experimental autoimmune encephalomyelitis (EAE) is a mouse model for multiple sclerosis,
23 where disease is mediated by autoantigen-specific T cells. Although there is evidence linking
24 CD4⁺ T cells that secrete IL-17, termed Th17 cells, and IFN- γ -secreting Th1 cells with the
25 pathogenesis of EAE, the precise contribution of these T cell subtypes or their associated
26 cytokines is still unclear. We have investigated the infiltration of CD4⁺ T cells that secrete
27 IFN- γ , IL-17 or both cytokines into CNS during development of EAE and have examined the
28 role of T cells in microglial activation. Our findings demonstrate that Th17 cells and CD4⁺ T
29 cells that produce both IFN- γ and IL-17, which we have called Th1/Th17 cells, infiltrate the
30 brain prior to the development of clinical symptoms of EAE and that this coincides with
31 activation of CD11b⁺ microglia and local production of IL-1 β , TNF- α and IL-6 in the CNS.
32 In contrast, significant infiltration of Th1 cells was only detected after the development of
33 clinical disease. Co-culture experiments, using mixed glia and MOG-specific T cells,
34 revealed that T cells that secreted IFN- γ and IL-17 were potent activators of pro-inflammatory
35 cytokines but T cells that secrete IFN- γ , but not IL-17, were less effective. In contrast both
36 Th1 and Th1/Th17 cells enhanced MHC class II and co-stimulatory molecule expression on
37 microglia. Our findings suggest that T cells which secrete IL-17 or IL-17 and IFN- γ infiltrate
38 the CNS prior to the onset of clinical symptoms of EAE, where they may mediate CNS
39 inflammation, in part, through microglial activation.

40

41 **Key words:** Experimental autoimmune encephalomyelitis, Th1 cells, Th17 cells, IFN- γ , IL-
42 17, microglia, inflammatory cytokine, multiple sclerosis.

1. Introduction

Multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), an animal model for MS, are chronic demyelinating diseases of the central nervous system (CNS). During active disease, demyelination within the CNS is associated with inflammatory responses that are orchestrated by infiltrating T cells and endogenous glia. Both IFN- γ -secreting Th1 cells and IL-17-secreting Th17 cells has been shown to have a pathogenic role in EAE (Kroenke et al., 2008; Stromnes et al., 2008). Adoptive transfer of MOG-specific Th1 cells can induce EAE, which is characterized by infiltration of a predominantly macrophage population into the CNS (Kroenke et al., 2008). In addition, Th1 cells promote inflammation within the CNS and facilitate Th17 cell infiltration into EAE lesions (O'Connor et al., 2008). Autoantigen-specific Th17 cells have been shown to transmigrate across the blood brain barrier (BBB) and induce EAE. The inflammation induced by Th17 cells in the CNS, is characterized by neutrophil recruitment into the CNS (Kroenke et al., 2008) and killing of neurons (Kebir et al., 2007).

Encephalitogenic T cells that invade the CNS during disease interact with antigen presenting cells (APC), resulting in reactivation of the T cells and activation of the APC (Shrikant and Benveniste, 1996). A number of cell types including resident astrocytes and microglia, and infiltrating macrophages and dendritic cells (DC), can function as APC in the CNS (Carson et al., 2006). Microglia constitutively express CD45, CD86 and CD40 at low levels, (Carson et al., 1998), but expression of CD45, MHC class II, CD80, CD86 and CD40 is upregulated in response to inflammatory stimuli, thus enhancing their ability to function as APC (Shrikant and Benveniste, 1996). Indeed the low level of expression of CD45 has been used to distinguish between microglia and macrophages, on which CD45 expression is high (Carson et al., 1998). It is noteworthy that more recently CD11c expression, which is low on microglia (Fischer and Reichmann, 2001) but high on bone marrow-derived macrophages

(Simard and Rivest, 2004), has also been used to distinguish between these cells. Moreover, it has also been shown that T cells can induce pro-inflammatory cytokine production from microglia (Dasgupta et al., 2005)

Activation of microglia and macrophages during CNS disease leads to production of proinflammatory cytokines, such as IL-1 β , TNF- α , and IL-6, as well as chemokines, which contribute to inflammation and myelin damage within the CNS (Benveniste, 1997). IL-1, together with IL-23, plays a crucial role in the expansion of memory Th17 cells (Sutton et al., 2006) and also promotes innate IL-17 production by $\gamma\delta$ T cells (Sutton et al., 2009) and, significantly, IL-1RI-defective mice are resistant to the induction of EAE (Sutton et al., 2006). IL-1 β can cause BBB breakdown, microglia and astrocyte activation and demyelination (Ferrari et al., 2004). Production of TNF- α within the CNS potently induces inflammation by increasing antigen presentation, promoting astrocytic proliferation and altering chemokine and adhesion molecule expression, thus regulating cell trafficking into the CNS (Sedgwick et al., 2000; Selmaj, 2000). IL-6 is necessary for the induction of cerebrovascular adhesion molecules, such as VCAM-1, which are essential for leukocyte trafficking to the CNS during EAE (Eugster et al., 1998). Furthermore, IL-6 increases the expression of proinflammatory genes and activates astrogliosis and microgliosis (Quintana et al., 2009). Decreased expression of TNF- α and IL-6 in the CNS is associated with attenuated EAE (Penkowa and Hidalgo, 2001), confirming the importance of these cytokines in CNS pathology.

In this study we investigated T cell infiltration and microglial activation in the CNS during the progression of EAE. We report that IL-17⁺ and IL-17⁺IFN- γ ⁺ T cells infiltrate the brain before the onset of clinical symptoms and that this is associated with enhanced expression of IL-1 β in brain tissue. At the onset of clinical symptoms, there is evidence of significant infiltration of IFN- γ -secreting T cells and macrophages into the brain and upregulation of MHC class II, CD40, CD80 and CD86 expression on activated microglia and

93 macrophages. We also provide evidence that MOG-specific T cells polarized *in vitro* with IL-
94 12 to secrete IFN- γ , or polarized with IL-1 β and IL-23 to secrete IFN- γ and IL-17, can
95 activate CD11b⁺ microglia, inducing expression of MHC class II and co-stimulatory
96 molecules. Critically, MOG-specific T cell lines that secrete IL-17 and IFN- γ , but not T cells
97 that secrete IFN- γ , promoted production of the proinflammatory cytokines IL-1 β , TNF- α and
98 IL-6 by glial cells.

99 2. Materials and Methods

100

101 2.1. MOG-induced EAE

102 C57BL/6 mice (Harlan UK) were maintained in the BioResources unit at Trinity College,
 103 Dublin, Ireland under veterinary supervision throughout the study, and experiments were
 104 performed under a licence issued by the Department of Health (Ireland) and in accordance
 105 with the guidelines laid down by the university ethical committee. C57BL/6 mice (8-10
 106 weeks) were injected subcutaneously (s.c.) with 150 µg MOG₃₅₋₅₅ peptide (Cambridge
 107 Biosciences, UK) in complete Freund's adjuvant (CFA) containing 4 mg/ml H37RA
 108 (*Mycobacterium tuberculosis*, Chondrex). Mice were injected intraperitoneally (i.p.) with 500
 109 ng pertussis toxin (PT; Kaketsukin) on day 0 and day 2. EAE was scored according to a 0-5
 110 scale as follows: no clinical symptoms 0; limp tail, 1; wobbly gait, 2; hind limb weakness, 3;
 111 hind limb paralysis, 4; tetra paralysis, 5.

112

113 2.2. Preparation of primary glial cell cultures

114 Primary mixed glia were isolated from brains of postnatal 1-day old C57BL/6 mice as
 115 previously described (Downer et al., 2008). Mixed glia (1×10^6 cells/ml) were added to 24-
 116 well plates (100 µl per well) and allowed to adhere for 2 h at 37°C in a humidified 5% CO₂
 117 environment. Wells were then flooded with 300 µl of pre-warmed DMEM. The medium was
 118 changed every 2-3 days and glia cultured for 14 days.

119

120 2.3. Generation of MOG-specific T cell lines

121 At 7 days post immunization with MOG and CFA, spleen cells and lymph node cells (2×10^6
 122 cells/ml) were re-stimulated *ex vivo* with MOG (25 µg/ml) in the presence IL-23 (10 ng/ml,
 123 R&D Systems) and IL-1β (20 ng/ml, R&D systems) or IL-12 (1 ng/ml, R&D Systems). IL-2

(5 ng/ml, R&D Systems) was added after 4 days. After 7 days of culture, surviving T cells were washed and co-cultured with glia and MOG (25 µg/ml).

2.4. Co-culture of T cells and glia

MOG₃₅₋₅₅-specific T cells were added to mixed glial cultures at a ratio of 1:2 (0.5×10^5 T cells: 1×10^5 glia) in the presence of MOG (25 µg/ml). After 24 h of culture, supernatants were removed for analysis of cytokine concentrations, and cells recovered for analysis cell surface marker expression by flow cytometry.

2.5. Real-time PCR

RNA was isolated from brain tissue obtained from control mice, and mice with EAE, using Nucleospin® RNAII kit, (Macherey-Nagel, Duren, Germany); this process was followed by reverse transcription into cDNA using High-Capacity cDNA RT kit (Applied Biosystems, Warrington, UK). Real-time PCR for the detection of *Il1b* (Mm00434228_m1), *tnfa* (Mm00443258_m1) and *Il6* (Mm00446191_m1) mRNA was performed with predesigned Taqman gene expression assays (Applied Biosystems). mRNA concentrations were normalised to the endogenous control, 18S ribosomal RNA. Samples were assayed on an Applied Biosystems 7500 Fast Real Time PCR machine

2.6. Cytokine quantification by ELISA

The concentrations of IL-1β, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α in T cell and glial supernatants or in brain homogenates were measured by ELISA. Cytokine concentrations in the test samples were evaluated with reference to the standard curve prepared using recombinant cytokines of known concentration.

148

149 *2.7. Flow cytometry*

150 Cultured mixed glial cells and mononuclear cells isolated by percoll density centrifugation
151 from the brains or spinal cords of PBS-perfused control mice and mice with EAE, were
152 washed and blocked with Fc γ R blocker (1 μ g/ml; BD Biosciences, USA). Cultured glial cells
153 were stained with anti-CD11b, anti-MHC class II, anti-CD80, anti-CD86, or anti-CD40.
154 Mononuclear cells were stained with anti-CD45, anti CD11b, anti-MHC class II, anti-CD80,
155 anti-CD86, or anti-CD40 antibody, or alternatively with anti-CD3 and anti-CD4, these cells
156 were then fixed and permeabilized (Fix and Perm Cell Permeabilization Kit; Caltag) and
157 stained intracellularly for IL-17 or IFN- γ . Flow cytometric analysis was performed using a
158 CyAN_{ADP} Flow Cytometer (Dako Cytomation) with FloJo software, with gating set on FMOs.

159

160 *2.8. Statistical analysis*

161 Statistical analysis was performed using the computer-based statistical package GraphPad
162 Prism. One-way ANOVA, using the Newman Keuls post-hoc test, was used to determine
163 statistically significant differences.

3. Results

3.1 Infiltration of $CD3^+$ T cells and $CD11b^+CD45^{hi}$ cells into the brain of C57BL/6 mice with EAE

EAE was induced in C57BL/6 mice by s.c. injection of MOG₃₅₋₅₅ emulsified in CFA, with PT injected i.p. on days 0 and 2. Control animals were injected with PBS (200 μ l) i.p. Animals were monitored daily for signs of clinical disease. 97% of mice had developed EAE by day 13 post immunization (Figure 1A). Clinical symptoms began with loss of tail tone (stage 1), and progressed through varying degrees of hind limb weakness and lead ultimately to hind limb paralysis (stage 4). Mice were sacrificed 7, 10, 14 and 21 days post-immunization and the brains and spinal cords were removed to assess immune cell infiltration and microglial and macrophage activation.

To assess the level of T cell infiltration into the brain during EAE, mononuclear cells isolated from the brains of control mice and mice with EAE were stained with anti-CD3 and the total number of T cells was calculated. There was a significant increase in $CD3^+$ T cell infiltration into the brain throughout the course of the disease ($F_{(4,11)} = 4.98$, $*p < 0.05$; Fig. 1B and C). The numbers of $CD3^+$ cells increased steadily from less than 1×10^4 in control mice to a peak of over 2×10^5 14 days after induction of EAE.

Microglia can be distinguished from macrophages on the basis of CD45 expression. In normal and pathological CNS tissue, CD45 expression has been correlated with the ability to present antigen to naïve T cells (Carson et al., 1998); activated microglia are known to upregulate their expression of CD45 to intermediate or high levels, thus improving their antigen presenting capabilities (Carson et al., 1998). Mononuclear cells isolated from the brains of control mice and mice with EAE were stained with anti-CD11b and anti-CD45 to identify a resting microglial population ($CD11b^+CD45^{lo}$) and activated microglia and brain

infiltrating macrophage population ($CD11b^{+}CD45^{hi}$). The number of resting microglia in the brain was not significantly altered throughout the course of disease. However, the numbers of activated microglia and activated infiltrating macrophages into the brain was markedly elevated at the onset of clinical symptoms (day 10) ($F_{(4,14)} = 6.32$, $**p < 0.01$; Fig. 1D). Representative dotplots of $CD11b^{+}CD45^{+}$ cells in the brains of control mice and mice with EAE at the onset of clinical symptoms are shown in Figure 1E.

3.2 Increased frequency of $CD4^{+}$ T cells that express IL-17, IFN- γ or both cytokines in the brain of mice with EAE

There has been some debate in the literature concerning the possible contribution of Th1 and Th17 cells to the pathogenesis of EAE. Here we examined the induction of IL-17-secreting and IFN- γ -secreting $CD4^{+}$ T cells in the periphery and their infiltration into the brain and spinal cord during the development of EAE. Intracellular cytokine staining was performed on spleen cells and mononuclear cells isolated from the brain and spinal cord of control mice or mice with EAE. Cells were stained with anti-CD4, fixed and permeabilized, stained intracellularly with anti-IFN γ and anti-IL-17 and analysed by flow cytometry. There was a small and transient increase in the frequency of $CD4^{+}$ T cells that secreted IL-17 (Th17 cells) in the spleen 7 days after immunization (Fig. 2A). The frequency of $CD4^{+}$ T cells that secreted IL-17 and IFN- γ (IL-17 $^{+}$ IFN- γ^{+} $CD4^{+}$ T cells, termed Th1/Th17 cells) was also increased approximately 3 fold compared with control mice 7 days after immunization (Fig. 2A). The percentage of $CD4^{+}$ T cells that secreted IFN- γ , but not IL-17 (Th1 cells) was marginally, though significantly greater than in control mice 14 days post immunization (Fig. 2A). In contrast with the periphery, an examination of $CD4^{+}$ T cells in the brain revealed substantial infiltration or activation of $CD4^{+}$ T cells that secrete IL-17, IFN- γ or both cytokines, with different kinetics for different populations. There was a significant increase in

the percentage of IL-17-secreting CD4⁺ T cells in the brain at day 7, before the onset of clinical symptoms ($F_{(4,15)}=21.97$, $*p<0.05$, $***p<0.001$) and this increased further by day 10 and remained higher than control levels on day 14 and 21 (Fig. 2B and D). The frequency of IL-17⁺IFN- γ ⁺ CD4⁺ T cells was also significantly increased 7, 10 and 14 days after immunization, but the peak infiltration was observed a little later than the IL-17⁺IFN- γ ⁻ CD4⁺ T cells at 14 days post immunization (Fig. 2B). There was also a significant increase in the percentage of IFN- γ ⁺IL-17⁻ CD4⁺ T cells in the brain of mice with EAE compared with control mice, but this was not observed until 14 days after immunization ($F_{(4,15)}=75.08$, $***p<0.001$; Fig. 2B). In the spinal cord there was also a significant increase in the percentage of IL-17-secreting CD4⁺ T cells at day 7, before the onset of clinical symptoms ($F_{(4,15)}=24.16$, $*p<0.05$; Fig 2C). There was also a modest and non-significant increased frequency of IL-17⁺IFN- γ ⁺ CD4⁺ T cells in the spinal cord 7, 10 and 14 days after immunization. Finally, the frequency of IFN- γ ⁺IL-17⁻ CD4⁺ T cells was significant enhanced in the spinal cord of mice 14 days after induction of EAE ($F_{(4,15)}=26.64$, $*p<0.05$; Fig. 2C).

We next evaluated the absolute number of cytokine-secreting CD4⁺ T cells in the brain and spinal cord during the course of EAE and these were generally consistent with the data presented as percentage of total CD4⁺ T cells, except for the later time points (partly due to an overall increase in the total number of immune cells in the brain), with evidence of significant infiltration of Th17, Th1/Th17 and Th1 cells into the brain at different stages of disease. The total number of IL-17⁺IFN- γ ⁻ and IL-17⁺IFN- γ ⁺ CD4⁺ T cells was significantly increased at or before the onset of disease (days 7 and 10) and was maintained at high levels at least until day 21 post immunization (Fig 3A). The increase in the number of IFN- γ ⁺IL-17⁻ CD4⁺ T cells, although greater at the peak, did not occur until a little later in disease (Fig 3A). Our findings demonstrate that frequency of IL-17, IFN- γ or double positive CD4⁺ T cells is significantly greater in the brain than in the periphery. In the spinal cord the absolute numbers of

infiltrating T cells were substantially lower and the variability greater due to the low and variable recovery of cells from this tissue. Nevertheless, we did see a non-significant increase in T cell expressing IL-17, IFN- γ or both cytokines on days 10-21 at and after onset of symptoms of disease (Fig 3B). Our findings demonstrate that Th1, Th17 and T cell secreting IL-17 and IFN- γ infiltrated the brain and spinal cord during development of EAE. Th17 and Th1/Th17 first infiltrate the brain before the onset of disease, whereas the Th1 cells are not detected at significant numbers until after the onset of clinical symptoms. In general there were more Th1 relative to Th17 cells in the spinal cord when compared with the brain. In particular at day 10, the number of Th17 cells exceeded that of Th1 cells in the brain but not in the spinal cord.

3.3 Enhanced expression of MHC class II, CD40, CD80 and CD86 on CD11b⁺CD45^{hi} cells in the brain of mice with EAE

It has been shown that CD11b⁺CD45^{hi} cells purified from the inflamed CNS are more efficient than CD11b⁺CD45^{lo} cells in presenting antigens to T cells (Miller et al., 2007). Here, we examined MHC class II, CD40, CD80 and CD86 expression on CD11b⁺CD45^{lo} and CD11b⁺CD45^{hi} cells prepared from the brain over the course of EAE. There was a non-significant increase in the expression of MHC class II on resting microglia (CD11b⁺CD45^{lo}) and a small, but significant, increase in the frequency of CD11b⁺CD45^{lo} cells expressing CD40 ($F_{(4,15)}=34.52$, *** $p<0.001$) and CD80 ($F_{(4,15)}=66.07$, *** $p<0.001$), in the brain at the onset of clinical symptoms, 10 days post immunization (Fig. 4). However, the expression of CD86 on resting microglia in the brain was not significantly elevated throughout the course of disease (Fig. 4).

In comparison with CD11b⁺CD45^{lo} microglia, CD11b⁺CD45^{hi} cells showed a significantly greater degree of activation over the course of EAE. The percentage of CD11b⁺CD45^{hi} cells

expressing MHC class II was significantly enhanced in the brain 10, 14 and 21 days post-immunization ($F_{(4,15)}=7.71$, $*p<0.05$; $***p<0.001$, Fig. 4A). In addition, the number of CD11b⁺CD45^{hi} cells expressing CD40 was significantly elevated in the brain on days 7, 10 and 21 days after immunization ($F_{(4,15)}=226.1$, $*p<0.05$, $**p<0.01$, $***p<0.001$; Fig. 5). The frequency of CD80 expressing CD11b⁺CD45^{hi} cells was significantly increased in the brain before and at disease onset (day 7 and day 10) ($F_{(4,15)}=53.85$, $*p<0.05$, $***p<0.001$; Fig. 5). The expression of CD86 was also significantly elevated on CD11b⁺CD45^{hi} cells in the brain on days 10, 14 and 21 after immunization ($F_{(4,15)}=46.66$, $*p<0.05$, $***p<0.001$; Fig. 5). Collectively the data reveal significant activation of CD11b⁺CD45^{hi} cells at the onset of clinical symptoms of EAE; because expression of CD45 increases when the cells are activated, these cells may be either macrophages or activated microglia.

3.4 Expression of IL-1 β , TNF- α and IL-6 is significantly increased in the brain during development of EAE

Since IL-1 β , IL-6 and TNF- α play a role in CNS inflammation, we examined their expression in the brain during the course of EAE. The concentration of IL-1 β was significantly elevated 7 days after immunization before the onset of clinical symptoms of EAE, reached a peak at day 10 and remained greater than background on day 21 (Fig. 6A). Consistent with this, IL-1 β mRNA expression in the brain was significantly increased on day 7 and was further elevated at the onset of clinical symptoms ($F_{(4,15)}=12.85$, $**p<0.01$; $***p<0.001$, Fig. 6B). TNF- α mRNA expression was significantly augmented in the brain at the onset of clinical symptoms ($F_{(4,15)}=2.72$, $**p<0.01$; Fig. 6B). IL-6 mRNA expression was enhanced in the brain of mice with EAE 7 and 10 days post immunization ($F_{(4,15)}=5.20$, $*p<0.05$; Fig. 6B). Although close to the detection limit of the assay, TNF- α and IL-6 protein

concentrations were also significantly increased TNF- α on day 10 and IL-6 on day 7 and 10 post immunization (data not shown).

3.5 IFN- γ or IFN- γ and IL-17-secreting MOG-specific T cells enhances proinflammatory cytokine production and MHC class II and co-stimulatory molecule expression on microglia

Having demonstrated that IFN- γ^+ , IL-17 $^+$ and IFN- γ^+ IL-17 $^+$ CD4 $^+$ T cells infiltrate the brain during development of EAE, and that this is temporally associated with activation of CD11b $^+$ CD45 hi cells in the brain, we examined the influence of the MOG-specific T cells on microglial activation *in vitro*. MOG₃₅₋₅₅-specific T cell lines were generated from mice with EAE by culture of spleen and lymph node cells with MOG₃₅₋₅₅ (25 μ g/ml) and IL-12 (1 ng/ml) or MOG, IL-23 (10 ng/ml) and IL-1 β (10 ng/ml). We had previously shown that MOG-specific T cells cultured under either of these conditions induced EAE following transfer into naïve mice (Sutton et al., 2006). An examination of cytokine secretion by these short-term MOG-specific T cell lines revealed that IL-12-polarized cells secreted high levels of IFN- γ and no IL-17 or IL-4, a clear Th1-type profile. In contrast, MOG-specific T cells polarized with IL-1 β and IL-23 produced high levels of IL-17 as well as IFN- γ , but no IL-4 (Fig. 7A). These cells are referred to as Th1/Th17 cells hereafter.

We examined the ability of MOG-specific T cells to induce microglia activation *in vitro*. Co-cultures of glia and Th1/Th17 cells significantly increased IL-1 β , TNF- α and IL-6 production when compared with glia co-cultured with Th1 cells or medium only ($F_{(4,10)}=539.8$, *** $p<0.001$; $F_{(4,10)}=3872$, *** $p<0.001$; $F_{(4,10)}=77.19$, *** $p<0.001$ respectively, *** $p<0.001$ versus glia co-cultured with Th1 cells, Fig. 7B). Low concentrations of IL-1 β , TNF- α and IL-6 were detected in cultures of MOG-stimulated Th1/Th17 cells alone, but this substantially enhanced following co-culture with glia. In contrast any pro-inflammatory cytokines produced by Th1 cells alone was not enhanced by addition of glia (Fig. 7B).

The effect of the MOG₃₅₋₅₅-specific T cell subsets on the expression of MHC-class II and co-stimulatory molecules on the surface of CD11b⁺ microglia was examined by flow cytometry. Co-culture of glia with Th1 cells or with Th1/Th17 cells in the presence of MOG₃₅₋₅₅ (25µg/ml) significantly enhanced expression of MHC class II on the surface of microglia ($F_{(2,6)}=32.07$, ** $p<0.01$, *** $p<0.001$; Fig. 7C and D). Expression of CD40, CD80 and CD86 on microglia was also enhanced, although not significantly, following co-culture with Th1 cells and Th1/Th17 cells (Fig. 7). These findings demonstrate that interaction with Th1 cells enhanced surface marker expression on microglia, whereas MOG-specific T cells that secrete IFN- γ and IL-17 enhance pro-inflammatory cytokine as well as surface marker expression of CD11b⁺ microglia.

4. Discussion

The significant findings of this study are that IL-17⁺ and IFN- γ ⁺IL-17⁺ CD4⁺ T cells infiltrate the CNS during EAE prior to the onset of clinical symptoms and this is associated with activation of microglia and elevated expression of IL-1 β and IL-6 in the brain. The numbers of CD11b⁺CD45^{hi} microglia and infiltrating macrophages with an activated phenotype, characterized by enhanced expression of MHC class II and the co-stimulatory molecules CD80, CD86 and CD40, peaked at the onset of clinical symptoms. In contrast, we found that the numbers of resting microglial cells (CD11b⁺CD45^{lo}) was not significantly altered and MHC class II and co-stimulatory molecule expression was only modestly increased on CD11b⁺CD45^{lo} cells during the course of EAE. Microglia in active MS lesions exhibit an activated morphology with increased expression of cell surface MHC class II and the adhesion/costimulatory molecules CD54, CD40, CD80 and CD86 (Cannella et al., 1995; Cannella and Raine, 1995). It has also been shown that CD11b⁺CD45^{lo} microglia purified from the inflamed CNS are largely incapable of activating either naïve or effector T cells, whereas CD11b⁺CD45^{hi} cells can efficiently present endogenous myelin antigens to pre-activated effector myelin-specific T cells and naïve T cells (Miller et al., 2007). Collectively these findings suggest that the CD11b⁺CD45^{hi} cells are more likely to have antigen presentation capabilities in the CNS during EAE.

IL-1 β , IL-6 and TNF- α play a role in CNS inflammation, either directly through activation of microglia and astrocytes (Ferrari et al., 2004) and alteration of chemokine and adhesion molecule expression and antigen presentation within the CNS (Sedgwick et al., 2000; Selmaj, 2000), or indirectly by promoting the differentiation and expansion of Th17 cells. We found that expression of pro-inflammatory cytokines, IL-1 β , TNF- α and IL-6 was significantly increased in the brain before the onset of clinical symptoms of EAE. Although expression of these cytokines was also enhanced in the spleen 7 days after immunization, it

was substantially greater in brain and spinal cord (Lalor et al, unpublished observations). It has previously been reported that activated microglia are the major cellular source of IL-1 β in the brain (Minogue et al., 2003), but we found that the enhanced expression of IL-1 β preceded the activation of microglia, as assessed by surface marker expression. IL-1 β , TNF- α and IL-6 are known to be involved in the pathology of EAE and are considered to be downstream inflammatory mediators induced by Th17 cells. However, these cytokines are also involved upstream of Th17 cell activation. IL-1 β is involved in the induction of Th17 cells (Sutton et al., 2006) and activation of $\gamma\delta$ T cells (Sutton et al., 2009). Furthermore, IL-6, together with TGF- β , supports Th17 cell differentiation, a process which is then amplified by IL-1 β and TNF- α (Veldhoen et al., 2006). (Lalor et al unpublished data). Therefore it is possible that early IL-1 β and IL-6 production by microglia may promote the re-activation or expansion of Th17 and $\gamma\delta$ T cells in the brain.

IL-17-secreting CD4⁺, CD8⁺ and $\gamma\delta$ T cells have been shown to produce IL-1 β (McCandless et al., 2009). Therefore, the IL-17-secreting CD4⁺ T cells may also contribute to the production of IL-1 β in the brain during disease. CD4⁺ T cells that secrete IL-17 or IL-17 and IFN- γ first infiltrate the brain around day 7 prior to the onset of clinical symptoms of EAE and this correlates with IL-1 β production in the brain. The frequency of these cells peaks at day 10-14 and declines thereafter. CD4⁺ T cells that secrete IFN- γ but not IL-17 also infiltrate the brain during development of EAE, but these cells are not detected in significant numbers until day 10-14, which follows the onset of clinical symptoms, and the numbers of these cells continues to increase at least until day 21. We found comparatively greater infiltration of Th17 cells in the brain than in the spinal cord. Indeed the number of Th17 cells was greater than Th1 cells in the brain at day 10 post immunization, whereas there were 4 fold more Th1 than Th17 cells in the spinal cord at the same time point. This is consistent with a report that Th17 cells homed preferentially to the brain, while Th1 cells homed preferentially

to the spinal cord (Stromnes et al., 2008). However, we found that at later time points during active disease that Th1 cells were the dominant cell type in the brain and spinal cord. An early report had suggested that EAE can be induced by transfer of T cells that secrete IL-17, but not by T cells that secrete IFN- γ , although both types of cells can cross the blood-brain barrier and infiltrate the CNS (Langrish et al., 2005). More recent studies have suggested that both Th1 and Th17 cells mediate pathology in EAE by distinct mechanisms. O'Connor and colleagues (2008) reported that adoptively transferred Th1 cells infiltrate the non-inflamed CNS and facilitate the entry of Th17 cells into established lesions. Conversely, Kroenke and colleagues (2008) found that adoptive transfer of IL-12p70- or IL-23-polarised T cells resulted in clinically indistinguishable disease. However, IL-12p70-driven disease was characterised by macrophage-rich infiltrates and enhanced iNOS expression, whereas neutrophils and GM-CSF were prominent in IL-23-driven lesions. A possible protective function for IFN- γ has been suggested following the demonstration that IFN- γ can inhibit the development of Th17 cells *in vitro*, possibly by down-regulating expression of the IL-23 receptor (Harrington et al., 2005). Consequently, whilst CD4⁺ Th1 cells may contribute to inflammation in CNS inflammatory disease, they may also have a regulatory role by suppressing expansion of activated of IL-17-secreting T cells. This is consistent with our demonstration that the decreased frequency of IL-17-producing T cells later in disease corresponds with an increase in Th1 cells.

In order to examine the possible role of IL-17 or IFN- γ -secreting T cells in microglial activation, we established MOG-specific T cell lines from mice with EAE. MOG-specific T cells polarized with IL-12 or IL-1 β and IL-23 secreted IFN- γ and not IL-17 or IFN- γ and IL-17, respectively. It was possible to generate Th17-type cells that secrete IL-17, but not IFN- γ , by culture with IL-1 β and IL-23 in the presence of anti-IFN- γ , but this phenotype was unstable and the cells reverted to double producers after 2 rounds of antigen stimulation

(unpublished observations). Furthermore, short term highly polarized MOG-specific Th17 cell lines were less effective than the double producers at inducing EAE following transfer into naive mice (Brereton et al., 2009). Therefore we confined our experiments to a study of Th1 or IL-17 and IFN- γ -producing cells, which we have called Th1/Th17 cells. Indeed, consistent with a previous report (Suryani and Sutton, 2007), we found that IL-17⁺IFN- γ ⁺ CD4⁺ T cells represent a major subtype in the brain throughout the course of EAE. Co-culturing of glia with Th1 or Th1/Th17 cells significantly enhanced expression of MHC class II and co-stimulatory molecules on CD11b⁺ microglia. In contrast, MOG-specific Th1/Th17 cells consistently produced more TNF- α , IL-6 and IL-1 β than Th1 cells. This may account for their greater encephalitogenic potential in the brain during EAE (Brereton et al., 2009).

This study provides new insights into the infiltration of T cell subsets, expression of MHC class II and co-stimulatory molecules on activated microglia and infiltrating macrophages and production of proinflammatory cytokines in the brain during EAE. Our study has assessed for the first time the comparative infiltration of Th1, Th17 and double producing Th1/Th17 cells in the brain, spinal cord and periphery and reveals that each of these cell types infiltrates the brain and spinal cord with different kinetics. Infiltration of Th17 cells into the brain precedes the onset of clinical symptoms and corresponds to an increase in expression of IL-1 β and IL-6. Activation of microglia and infiltration of macrophages occurs at the onset of clinical symptoms while Th1 cells infiltrate the brain later in disease. Th1 and Th1/Th17 cells activated microglia towards an inflammatory phenotype, characterised by co-stimulatory molecule expression, and crucially only the interaction of glia with Th1/Th17 cells induced significant production of proinflammatory cytokines. Specifically targeting the interactions of glia and Th1/Th17 cells may be an important therapeutic approach to modulate inflammatory responses in the CNS during disease.

422 **Acknowledgments**

423 This work was supported by grants from the Health Research Board and Science Foundation

424 Ireland

ACCEPTED MANUSCRIPT

References

- Benveniste, E.N., 1997. Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis. *J Mol Med* 75, 165-173.
- Brereton, C.F., Sutton, C.E., Lalor, S.J., Lavelle, E.C., Mills, K.H., 2009. Inhibition of ERK MAPK suppresses IL-23- and IL-1-driven IL-17 production and attenuates autoimmune disease. *J Immunol* 183, 1715-1723.
- Cannella, B., Aquino, D.A., Raine, C.S., 1995. MHC II expression in the CNS after long-term demyelination. *J Neuropathol Exp Neurol* 54, 521-530.
- Cannella, B., Raine, C.S., 1995. The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Ann Neurol* 37, 424-435.
- Carson, M.J., Doose, J.M., Melchior, B., Schmid, C.D., Ploix, C.C., 2006. CNS immune privilege: hiding in plain sight. *Immunol Rev* 213, 48-65.
- Carson, M.J., Reilly, C.R., Sutcliffe, J.G., Lo, D., 1998. Mature microglia resemble immature antigen-presenting cells. *Glia* 22, 72-85.
- Dasgupta, S., Jana, M., Liu, X., Pahan, K., 2005. Myelin basic protein-primed T cells of female but not male mice induce nitric-oxide synthase and proinflammatory cytokines in microglia: implications for gender bias in multiple sclerosis. *J Biol Chem* 280, 32609-32617.
- Downer, E.J., Cowley, T.R., Lyons, A., Mills, K.H., Berezin, V., Bock, E., Lynch, M.A., 2008. A novel anti-inflammatory role of NCAM-derived mimetic peptide, FGL. *Neurobiol Aging*.
- Eugster, H.P., Frei, K., Kopf, M., Lassmann, H., Fontana, A., 1998. IL-6-deficient mice resist myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *Eur J Immunol* 28, 2178-2187.
- Ferrari, C.C., Depino, A.M., Prada, F., Muraro, N., Campbell, S., Podhajcer, O., Perry, V.H., Anthony, D.C., Pitossi, F.J., 2004. Reversible demyelination, blood-brain barrier breakdown,

and pronounced neutrophil recruitment induced by chronic IL-1 expression in the brain. *Am J Pathol* 165, 1827-1837.

Fischer, H.G., Reichmann, G., 2001. Brain dendritic cells and macrophages/microglia in central nervous system inflammation. *J Immunol* 166, 2717-2726.

Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M., Weaver, C.T., 2005. Interleukin 17-producing CD4⁺ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6, 1123-1132.

Kebir, H., Kreymborg, K., Ifergan, I., Dodelet-Devillers, A., Cayrol, R., Bernard, M., Giuliani, F., Arbour, N., Becher, B., Prat, A., 2007. Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med* 13, 1173-1175.

Kennedy, M.K., Torrance, D.S., Picha, K.S., Mohler, K.M., 1992. Analysis of cytokine mRNA expression in the central nervous system of mice with experimental autoimmune encephalomyelitis reveals that IL-10 mRNA expression correlates with recovery. *J Immunol* 149, 2496-2505.

Kroenke, M.A., Carlson, T.J., Andjelkovic, A.V., Segal, B.M., 2008. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. *J Exp Med* 205, 1535-1541.

Langrish, C.L., Chen, Y., Blumenschein, 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. *J Exp Med* 201, 233-240.

McCandless, E.E., Budde, M., Lees, J.R., Dorsey, D., Lyng, E., Klein, R.S., 2009. IL-1R signaling within the central nervous system regulates CXCL12 expression at the blood-brain barrier and disease severity during experimental autoimmune encephalomyelitis. *J Immunol* 183, 613-620.

- Miller, S.D., McMahon, E.J., Schreiner, B., Bailey, S.L., 2007. Antigen presentation in the CNS by myeloid dendritic cells drives progression of relapsing experimental autoimmune encephalomyelitis. *Ann N Y Acad Sci* 1103, 179-191.
- Minogue, A.M., Schmid, A.W., Fogarty, M.P., Moore, A.C., Campbell, V.A., Herron, C.E., Lynch, M.A., 2003. Activation of the c-Jun N-terminal kinase signaling cascade mediates the effect of amyloid-beta on long term potentiation and cell death in hippocampus: a role for interleukin-1beta? *J Biol Chem* 278, 27971-27980.
- 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. *J Immunol* 181, 3750-3754.
- Penkowa, M., Hidalgo, J., 2001. Metallothionein treatment reduces proinflammatory cytokines IL-6 and TNF-alpha and apoptotic cell death during experimental autoimmune encephalomyelitis (EAE). *Exp Neurol* 170, 1-14.
- Quintana, A., Muller, M., Frausto, R.F., Ramos, R., Getts, D.R., Sanz, E., Hofer, M.J., Krauthausen, M., King, N.J., Hidalgo, J., Campbell, I.L., 2009. Site-specific production of IL-6 in the central nervous system retargets and enhances the inflammatory response in experimental autoimmune encephalomyelitis. *J Immunol* 183, 2079-2088.
- Sedgwick, J.D., Riminton, D.S., Cyster, J.G., Korner, H., 2000. Tumor necrosis factor: a master-regulator of leukocyte movement. *Immunol Today* 21, 110-113.
- Selmaj, K.W., 2000. Tumour necrosis factor and anti-tumour necrosis factor approach to inflammatory demyelinating diseases of the central nervous system. *Ann Rheum Dis* 59 Suppl 1, i94-102.
- Shrikant, P., Benveniste, E.N., 1996. The central nervous system as an immunocompetent organ: role of glial cells in antigen presentation. *J Immunol* 157, 1819-1822.

- Simard, A.R., Rivest, S., 2004. Bone marrow stem cells have the ability to populate the entire central nervous system into fully differentiated parenchymal microglia. *FASEB J* 18, 998-1000.
- Stromnes, I.M., Cerretti, L.M., Liggitt, D., Harris, R.A., Gorman, J.M., 2008. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat Med* 14, 337-342.
- Suryani, S., Sutton, I., 2007. An interferon-gamma-producing Th1 subset is the major source of IL-17 in experimental autoimmune encephalitis. *J Neuroimmunol* 183, 96-103.
- Sutton, C., Brereton, C., Keogh, B., Mills, K.H., Lavelle, E.C., 2006. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J Exp Med* 203, 1685-1691.
- 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 gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 31, 331-341.
- Veldhoen, M., Hocking, R.J., Atkins, C.J., Locksley, R.M., Stockinger, B., 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24, 179-189.

Figure legends

Figure 1. Infiltration of CD3⁺ T cells and CD11b⁺CD45^{hi} cells into the brain of C57BL/6 mice with EAE. (A) EAE was induced in C57BL/6 mice by immunization of MOG₃₅₋₅₅ in CFA followed by PT. Control animals were injected i.p. with PBS (200µl). Mice were observed for clinical signs of EAE. Data are mean clinical score ± SEM. (B-D) Mononuclear cells were isolated from the brain of control mice and mice 7, 10, 14 and 21 days post induction of EAE. Cells were stained with anti-CD3 to quantify the number of CD3⁺ T cells infiltrating the brain (B; with representative dot plot in C) and with anti-CD11b and anti-CD45 to identify the total numbers of CD11b⁺CD45^{lo} cells and CD11b⁺CD45^{hi} cells in the brain during EAE (D; with representative dot plot in E). *p<0.05, **p<0.01; versus control mice, by one-way ANOVA, Student Newman-Keuls. Results are representatives of two experiments with 4 or 5 mice per group at each time point. Numbers in gates are percentage of total mononuclear cells.

Figure 2. Increased frequency of CD4⁺T cells that secrete IL-17, IFN-γ or both cytokines in the brain and spinal cord of mice with EAE. Spleen cells (A) and mononuclear cells from the brain (B) or spinal cord (C) were prepared from control mice or mice day 7, 10, 14 and 21 induction of EAE (n=4 per timepoint). Cells were stimulated overnight with PMA (10 ng/ml) and ionomycin (1 µg/ml), and incubated with Brefeldin A (5 µg/ml). Cells were stained with anti-CD45, anti-CD3 and anti-CD4, fixed and permeabilized, stained intracellularly with anti-IL-17 and anti-IFN-γ and analysed by flow cytometry. Results are presented as the mean frequency of CD4⁺ cells in the CD3⁺ population ± SEM for spleen (A), brain (B) and spinal cord (C), with representative dot plots for the brain (D). Results are representatives of two experiments with 4 or 5 mice per group at each time point.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus control mice, by one-way ANOVA, Student Newman-Keuls.

Figure 3. Absolute numbers of CD4⁺T cells that secrete IL-17, IFN- γ or both cytokines in the brain and spinal cord of mice with EAE. Mononuclear cells were prepared from the brain (A) or spinal cord (B) and intracellular cytokine staining performed as described in the legend to Fig 2. Results are presented as the absolute numbers of CD4⁺ cells expressing IL-17, IFN- γ or both cytokines, and are representatives of two experiments with 4 or 5 mice per group at each time point. * $p < 0.05$, versus control mice, by one-way ANOVA, Student Newman-Keuls.

Figure 4. Increased frequency of CD11b⁺CD45^{lo} cells expressing CD40 and CD80 in the brain of mice with EAE. Mononuclear cells were isolated from the brain of control mice and mice 7, 10, 14 and 21 days after induction of EAE. The cells were gated on CD11b⁺ and CD45^{lo} expression, the percentage of MHC class II, CD40, CD80 and CD86 expressing CD11b⁺CD45^{lo} cells are shown in the scatter plots (A). Representative histograms for control mice (gray histogram) and mice with EAE on day 10 (solid line) (B). Results are representatives of 3 experiments with 4 mice per group at each time point. *** $p < 0.001$; versus control mice, by one-way ANOVA, Student Newman-Keuls.

Figure 5. Enhanced expression of MHC class II, CD40, CD80 and CD86 on CD11b⁺CD45^{hi} cells in the brain of mice with EAE. Mononuclear cells were isolated from the brain of control mice and mice 7, 10, 14 and 21 days post induction of EAE (n=4 per timepoint). The cells were gated on CD11b and CD45^{hi} expression and the percentage of CD11b⁺CD45^{hi} cells expressing MHC class II, CD40, CD80 and CD86 are shown in the

scatter plots (A). Representative histograms for control mice (gray histogram) and mice with EAE on day 10 (solid line) (B). Results are representatives of 3 experiments with 4 mice per group at each time point. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; versus control mice, by one-way ANOVA, Student Newman-Keuls.

Figure 6. Expression of IL-1 β , TNF- α and IL-6 mRNA is significantly increased in the brain during EAE. Brains from control mice or mice with EAE were dissected at 7, 10, 14 and 21 days after the induction of EAE. (A) The concentration of IL-1 β was quantified in brain homogenate by ELISA. (B) RNA was extracted to assess IL-1 β mRNA, TNF- α mRNA and IL-6 mRNA. Values are expressed as relative quantities (RQ) normalized to the endogenous control gene, 18S. Results are representatives of 3 experiments with 4 or 5 mice per group at each time point * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$; versus control mice, by one-way ANOVA, Student Newman-Keuls. Data presented as mean $\pm$ SEM.

Figure 7. Addition of MOG-specific Th1/Th17 cells enhances proinflammatory cytokine production in co-cultures with glia and upregulates the expression of MHC class II, CD40, CD80 and CD86 on microglia (A) EAE was induced in C57BL/6 mice by immunization with MOG in CFA followed by PT. After 7 days, spleen and inguinal nodes were removed, the cells were stimulated with MOG₃₅₋₅₅ (25 μ g/ml) and Th1 polarising cytokine IL-12 (1 ng/ml) and Th1/Th17 polarising cytokines IL-23 (10 ng/ml) and IL-1 β (10 ng/ml). After 7 days, supernatants were removed and IFN- γ , IL-17 and IL-4 concentrations were quantified by ELISA. (B-D) MOG-specific Th1 cells and Th1/Th17 cells were added to cultured glial cells for 24 h at a ratio of 0.5:1. (B) Supernatant was removed and the concentration of IL-1 β , TNF- α and IL-6 was quantified by ELISA. ** $p < 0.01$; *** $p < 0.001$; versus medium treated glia, +++ $p < 0.001$; versus Th1/Th17 cells alone and glia treated with

Th1 cells, by one-way ANOVA, Student Newman-Keuls. Data presented as mean $\pm$ SEM. Representative of 3 independent experiments. (C) Cells were harvested and stained for flow cytometry. Cells were gated on CD11b expression and the percentage of these cells expressing MHC class II, CD40, CD80 and CD86 was assessed. (D) Representative histograms of CD11b⁺ cells expressing MHC class II, CD40, CD80 and CD86 after treatment for 24 h with medium only (gray histogram), Th1 cells (solid line) and Th1/Th17 cells (dotted line). Representative of 3 independent experiments.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7