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Innate IFN-γ promotes development of experimental autoimmune encephalomyelitis: a role for NK cells and M1 macrophages

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Abbreviations: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis, MOG, myelin oligodendrocyte glycoprotein; VLA-4, very late antigen-4, BBB, blood brain barrier; rIFN-γ, recombinant IFN-γ.

Abstract

The role of IFN-γ in the pathogenesis of autoimmune diseases is controversial. Although Th1 cells can induce EAE, IFN-γ can suppress Th17 cells that are pathogenic in EAE. Here we show that NK cells provide an early source of IFN-γ during development of EAE. Depletion of NK cells or neutralization of IFN-γ delayed the onset of EAE and was associated with reduced infiltration of IL-17^{+} and GM-CSF^{+} T cells into the CNS. In the passive transfer model, immune cells from myelin oligodendrocyte glycoprotein (MOG)-immunized IFN-γ$^{-/-}$ mice failed to induce EAE, despite producing IL-17 and GM-CSF. The macrophages expressed markers of M2 activation and the T cells had low very late antigen-4 (VLA-4) expression and failed to infiltrate the CNS. Addition of recombinant IFN-γ to immune cells from the IFN-γ$^{-/-}$ mice activated M1 macrophages and restored VLA-4 expression, migratory and encephalitogenic activity of T cells. Furthermore, treatment of recipient mice with anti-VLA-4 neutralizing antibody abrogated EAE induced by transfer of T cells from WT mice. Our findings demonstrate IFN-γ-producing T cells are not required for development of EAE, but NK cell-derived IFN-γ has a key role in promoting M1 macrophage expansion and VLA-4-mediated migration of encephalitogenic T cells into the CNS.

Introduction

IFN-γ was considered to be the key pathogenic cytokine in many autoimmune diseases. Early studies in relapsing remitting MS patients and EAE, a mouse model for MS, showed that administration of IFN-γ exacerbated symptoms of disease [1, 2]. The differentiation of IFN-γ-secreting Th1 cells is driven by IL-12, which is composed of IL-12p35 and IL-12p40 subunits, while expansion of Th17 cells requires IL-23, which also includes IL-24p40 as well as an IL-23p19 subunit [3]. Myelin-specific T cells stimulated with IL-12 induced EAE when transferred into naive hosts [4]. While IL-12-p40$^{-/-}$ mice were resistance to EAE, IL-12p35$^{-/-}$ mice were completely susceptible to disease [5, 6] and IL-12Rβ2$^{-/-}$ mice developed earlier and more severe EAE, with extensive demyelination and CNS inflammation [7]. It was subsequently demonstrated that IL-23p19$^{-/-}$ mice were resistant to EAE and that IL-23 could expand a population of myelin-specific IL-17-producing T cells that could transfer EAE to naive mice, whereas IL-12-expanded cells failed to induce EAE [8]. Although IFN-γ was considered to be the key pathogenic cytokine in relapsing remitting MS patients and EAE (REFS 1,2), these studies suggested that Th17 and not Th1 cells were pathogenic in EAE and are consistent with earlier reports showing that mice deficient in IFN-γ or IFN-γR are susceptible to EAE [9, 10]. Furthermore, injection of neutralising antibodies to IFN-γ exacerbated actively- and passively- induced EAE [11, 12]. Finally, Th17 cells from T-bet$^{-/-}$ mice that produced low concentration of IFN-γ in vivo induced mild EAE following transfer to naive hosts [13].

Recent studies have suggested that both Th1 and Th17 cells may play distinct pathogenic roles in EAE. It has been demonstrated that myelin-specific Th1 cells, devoid of contaminating Th17 cells, were capable of inducing EAE and the evidence suggested that Th1 cells play a key role in the entry of pathogenic Th17 cells into the non-inflamed CNS [14]. It appears that Th1 cells use the adhesion molecule VLA-4 to gain access to the spinal cord during EAE. VLA-4 is a heterodimeric integrin composed of α4 (CD49d) and β1 (CD29) subunits [15] and is expressed on the surface of T cells. It interacts with vascular cell adhesion molecule 1 expressed by endothelial cells lining the microvasculature of the CNS and slows the movement of T cells, allowing them to transmigrate across the blood brain barrier (BBB) and emerge in the perivascular space of the CNS [16]. A monoclonal antibody against the α4β1 integrin VLA-4 attenuates development of EAE by preventing leukocyte traffic across the BBB [17]. Furthermore, natalizumab, a humanised monoclonal antibody that targets the α4-chain of VLA-4 is an effective therapy for relapsing MS [18]. It has been

reported that blockade of VLA-4 prevents EAE by inhibiting migration of Th1 cells into the CNS [19]. In the absence of Th1 cell infiltration, Th17 cells induced an ataxic form of EAE, characterised by supraspinal LFA-1-mediated infiltration of Th17 cells into the brain parenchyma but not the spinal cord [19].

Cells of the innate immune system also play a role in many autoimmune diseases. γδ T cells activated by IL-1β and IL-23, without TCR engagement, have a clear pathogenic role in early IL-17 production which drives Th17 responses that mediate EAE and other autoimmune diseases [20, 21]. The role of NK cells is less clear in EAE, with some studies suggesting a protective function [22, 23] and others a pathogenic role for IFN-γ-secreting NK cells [24]. The balance between classically-activated or type 1 (M1) macrophage and alternatively-activated or type 2 (M2) macrophages has also been shown to affect the severity of EAE, with M1 macrophages associated with more severe disease and M2 macrophages suppressing disease [25].

In this study we have examined the role of IFN-γ in the pathogenesis of EAE. Our findings suggest that IFN-γ from innate cells, including NK cells, plays a role at the induction phase of EAE by polarizing M1 macrophages that promote encephalitogenic activity, at least in part by enhancing VLA-4 expression on pathogenic T cells, required for their migration into the CNS.

Results

IFN-γ is protective during acute disease but has a pathogenic role at the induction of EAE

We analysed the role of IFN-γ in EAE induced by active immunization of WT C57BL/6 or IFN-$\gamma^{-/-}$ mice with MOG in CFA. We observed a delay in onset of clinical signs of EAE in IFN-$\gamma^{-/-}$ compared with WT mice; the mean day of onset for WT mice was day 6 post-immunization, compared with day 10 for IFN-$\gamma^{-/-}$ mice (Fig. 1A). However, the peak of disease was more severe in the IFN-$\gamma^{-/-}$ mice. WT mice lost weight up to day 15 then began to recover. In contrast, IFN-$\gamma^{-/-}$ mice lost a similar amount of weight in the early phase of disease but, unlike WT mice, did not recover and lost weight until the termination of the experiment on day 25 after immunization (Fig. 1B).

Confirmation of these findings was provided by the demonstration that the onset of EAE was significantly delayed in mice treated with anti-IFN-γ (Fig. 1C). The mean day of onset of EAE in mice treated with anti-IFN-γ was day 12 after immunization, compared with day 7 for control mice (Fig. 1C). Mice treated with anti-IFN-γ gained weight in the early phase of disease, whereas untreated mice with EAE lost weight (Fig. 1D). Conversely, from day 12 to day 18 after immunization, blocking IFN-γ exacerbated the clinical signs of EAE; clinical scores were significantly higher than control mice by day 15 (Fig. 1C). Furthermore, anti-IFN-γ treated mice lost weight, whereas PBS-treated mice gained body weight from day 13 (Fig. 1D).

Since it has been suggested that Th1 cells may promote the entry of Th17 cells into the CNS [14], the delay in the onset of EAE in anti-IFN-γ-treated mice may be due to a failure of T cells to gain entry into the CNS. To test this hypothesis, we examined infiltration of cytokine secreting T cells on day 12, when mice treated with anti-IFN-γ had milder disease, and at day 18, when mice treated with anti-IFN-γ had more severe disease. On day 12, anti-IFN-γ treated mice had less IFN-γ-, IL-17A- and GM-CSF-producing CD4 cells in the brain and spinal cord than untreated mice with EAE (Fig. 1E). This reduction was associated with fewer macrophages and neutrophils in both the brain and spinal cord (Fig. 1F).

On day 18 after immunization, anti-IFN-γ treated mice had increased numbers of IFN-γ-, IL-17A- and GM-CSF-producing CD4 T cells in the spinal cord, but similar numbers of these cells in the brain when compared with untreated mice with EAE (Fig. 1G). In addition, there were more macrophages in the brain and spinal cord of mice treated with anti-

IFN-γ at this time point (Fig. 1H). Collectively these data indicate that blocking IFN-γ at the induction of active EAE in C57BL/6 mice delays the onset of clinical symptoms, which may result from a constraint on cellular infiltration into the CNS, but leads to more severe disease once the inflammatory response has been established.

NK cells provide an early source of innate IFN-γ that promotes development of EAE

Our findings indicate that IFN-γ has a pathogenic role in the early stages of EAE, suggesting that innate sources of IFN-γ may be involved in the development of EAE. In a kinetic study designed to assess the appearance of IFN-γ-secreting cells in the CNS of mice with EAE, we found that there were significant numbers of IFN-γ-secreting NK cells in the brains of mice 7 days post induction of EAE, whereas significant numbers of IFN-γ-secreting CD4 T cells were detected at day 17 post induction (Fig. 2).

Depletion of NK cells with anti-asialo GM1 during development of EAE (days -1, 1 and 4) significantly delayed the onset of disease; control mice had clear clinical symptoms of EAE 9-10 days after induction, whereas NK cell-depleted mice had no symptoms at this time point (Fig. 3A). In contrast, the course of EAE was not affected by early depletion of NK cells in IFN-$\gamma^{-/-}$ mice (Fig 3B) or late depletion (from day 8) in WT mice (data not shown). An examination of the T cells in the brain of WT mice at day 12 revealed that depletion of NK cells at induction of disease resulted in a significant reduction in the frequency and absolute numbers of infiltrating $CD3^+$ T cells, IFN-γ^+ CD4 T cells and IL-17^+ CD4 T cells (Fig. 3C and D). These findings suggest that NK cells provide an important early source of IFN-γ for the development of EAE, which appears to be necessary for migration of pathogenic T cells into the CNS.

Treatment of donor mice with anti-IFN-γ prevents EAE induced by cell transfer

Our data suggest that IFN-γ is required for induction but not effector function of EAE. In order to confirm these findings and to examine the mechanism involved, we used the passive transfer EAE model, where we examined the effect of neutralizing IFN-γ antibody at induction in donor mice or in recipient mice following transfer of MOG-specific T cells. Treatment of donor mice with an anti-IFN-γ antibody during immunization with MOG and CFA significantly reduced the pathogenicity of the cells transferred from these mice; recipients had reduced clinical scores (Fig. 4A). In contrast, when anti-IFN-γ antibody was administered to the recipient mice at the time of cell transfer, these mice developed severe EAE with clinical scores indistinguishable from untreated mice (Fig. 4A). Treatment of donor

mice with anti-IFN-γ did not affect the induction of MOG-specific cells that secrete IFN-γ, IL-17A or GM-CSF (Fig. 4B). An examination of T cells in the brain of mice on day 14 post transfer revealed low numbers of GM-CSF, IL-17A and IFN-γ-secreting T cells in recipients of cells from WT donor mice treated with anti-IFN-γ, but not in recipient mice that had been treated with the anti-IFN-γ antibody (Fig. 4C, D). These data indicate that IFN-γ has a key pathogenic role during the induction phase, but it is not essential for pathogenicity of the autoantigen-specific T cells in the recipient mouse in the adoptive transfer model of EAE

MOG-specific T cells from WT but not IFN-$\gamma^{-/-}$ mice induce EAE in WT recipient mice

Our findings that EAE is reduced following treatment of donor, but not recipient, mice with anti-IFN-γ suggest a pathogenic role for IFN-γ during active induction of EAE. In order to confirm the finding, we used lymph node and spleen cells from WT or IFN-$\gamma^{-/-}$ mice immunized with MOG and CFA and re-stimulated with either MOG and IL-1β + IL-23 or MOG and IL-12. We found that cells from WT mice stimulated with antigen in the presence of IL-12 or IL-1β and IL-23, which generated predominantly, but not exclusively, IFN-γ-secreting or IL-17-secreting CD4 T cells respectively (Fig. S1), induced EAE with severe clinical scores (Fig. 5A). In contrast, the majority (82%) of mice injected with IFN-$\gamma^{-/-}$ cells stimulated with IL-1β and IL-23 either developed no clinical symptoms or only very mild EAE (Fig. 5A). Furthermore, mice injected with IFN-$\gamma^{-/-}$ cells stimulated with IL-12 did not develop typical EAE, but a minority developed an atypical form of EAE characterised by axial rotation, balance impairment and spinning (Fig. 5A and B). The failure of T cells from IFN-$\gamma^{-/-}$ mice to induce EAE is unlikely to be due to cell death since we recovered similar numbers of viable T cells from cultures of lymph node and spleen cells from IFN-$\gamma^{-/-}$ and WT mice.

An examination of lymphocyte infiltration and demyelination in the spinal cords revealed that transfer of MOG-activated cells from WT mice had marked lymphocyte infiltration into the meninges, perivascular space and parenchyma of the white matter tracts of the spinal cord (Fig. 5C). This inflammation was associated with moderate to severe myelin degeneration. In contrast, mice injected with IFN-$\gamma^{-/-}$ cells had little or no inflammatory changes and no myelin degeneration in the spinal cord (Fig. 5C). Furthermore, the numbers of GM-CSF, IL-17A or IFN-γ-secreting $CD4^+$ T cells was lower in the brains of mice injected with IFN-$\gamma^{-/-}$ cells compared with WT cells (Fig. 5D and E). Collectively these data indicate that MOG-specific T cells from MOG-immunized WT mice are capable of inducing severe symptoms of EAE associated with CNS infiltration of $CD4^+$ T cells secreting

pro-inflammatory cytokines, whereas MOG-specific T cells from IFN-$\gamma^{-/-}$ mice induced only very mild EAE in a minority of recipients accompanied by little cellular infiltration into the CNS.

Enhanced M2 macrophages and reduced VLA-4 in cells from IFN-$\gamma^{-/-}$ mice that fail to induce EAE

We next focused on the difference between the cultured cells derived from WT and IFN-$\gamma^{-/-}$ mice that did or did not induce EAE following transfer to naive mice. Following 72 h stimulation with MOG, IL-1β and IL-23, cells from both WT and IFN-$\gamma^{-/-}$ mice secreted comparable levels of IL-17A and IL-17F while, predictably, cells from WT, but not IFN-$\gamma^{-/-}$, mice secreted high concentrations of IFN-γ (Fig. 6A). Interestingly, immune cells from IFN-$\gamma^{-/-}$ mice secreted significantly higher concentrations of GM-CSF and the Th2 cytokines IL-13 and IL-5 than cells from WT mice (Fig. 6A). We also observed enhanced expression of M2 macrophages markers, *chi3l3* (YM1), *arg-1* and *retnla* (Fizz1) in cells from IFN-$\gamma^{-/-}$ mice (Fig. 6B). FACS analysis confirmed an enhancement of dectin-1^{+} M2 macrophages in cells from IFN-$\gamma^{-/-}$ mice (Fig. 6C). These findings suggest that the absence of IFN-γ allows the activation or expansion of Th2 cells and M2 macrophages.

Since transferred T cells from IFN-$\gamma^{-/-}$ mice do not appear to be capable of infiltrating the CNS of mice following adoptive transfer, we examined the possible role of VLA-4, an adhesion molecule known to be essential for the entry of pathogenic T cells into the CNS. CD3 cells were purified from the cultures after 72 h and analysed for expression of VLA-4 by FACS and RT-PCR. We found significantly enhanced expression of VLA-4 on $CD3^{+}$ T cells from WT mice immunized with MOG and CFA following culture with MOG, IL-1β and IL-23 for 72 h, which was significantly reduced in cells from IFN-$\gamma^{-/-}$ mice (Fig. 6D, E). Furthermore, *cd49d* (VLA-4) mRNA expression was significantly lower on MOG-activated $CD3^{+}$ cells from IFN-$\gamma^{-/-}$ compared with WT mice (Fig. 6F). These data indicate that, in the absence of IFN-γ, MOG-specific T cells have significantly reduced VLA-4 expression and this may explain their failure to infiltrate the CNS and cause EAE.

M2 macrophages suppress encephalitogenic activity of MOG-specific cells

We have shown that the failure of MOG-stimulated cells from IFN-$\gamma^{-/-}$ mice to induce EAE following adoptive transfer correlated with higher expression of M2 macrophage markers, lower M1 macrophage markers and lower VLA-4 expression on T cells. Therefore, we examined the possibility that M2 macrophages may suppress the encephalitogenic activity

of the T cells by suppressing VLA-4. First, we examined the effect of innate (T-depleted) cells from IFN-$\gamma^{-/-}$ mice on the induction of EAE by T cells from WT mice. Purified CD3 T cells from MOG- and CFA- immunized WT mice, stimulated in culture with MOG, IL-1β and IL-23 in the presence of non-T cells from WT mice, induced EAE following adoptive transfer to naive mice (Fig. 5A). In contrast, purified CD3 T cells from MOG and CFA immunized WT mice stimulated in culture with MOG, IL-1β and IL-23 in the presence of non-T cells from IFN-$\gamma^{-/-}$ mice failed to induce EAE following adoptive transfer to naive mice (Fig. 7A). The T cells cultured with non-T cells from IFN-$\gamma^{-/-}$ mice that failed to induce EAE were not compromised in their ability to produce IL-17A or GM-CSF following in vitro stimulation with MOG (Fig. 7B). However the non-T cells from IFN-$\gamma^{-/-}$ mice had a significantly higher proportion of dectin-1^{+} M2 macrophages than non-T cells from WT mice (Fig. 7C).

We confirmed the suppressive influence of M2 macrophages on encephalitogenic activity of T cells by adding type 2 polarizing cytokines IL-4 and IL-13 to the cultures of MOG-stimulated cells prior to transfer in vivo; this also suppressed their ability to induce EAE following transfer to naive mice (Fig. 7D). Assessment of cytokine production prior to cell transfer showed that the production of IFN-γ and GM-CSF was reduced, but the production of MOG-specific IL-17A and IL-17F was not affected by addition of IL-4 and IL-13 to the cultures (Fig. 7E). The macrophages in the cultures had significantly elevated M2 markers *chi3l3, arg-1* and *retnla* detected by RT-PCR (Fig. 7F), and a higher frequency of dectin-1^{+} M2 macrophages was detected by FACS (Fig. 7G). These findings suggest that the failure of lymph node and spleen cells from MOG and CFA-immunized IFN-$\gamma^{-/-}$ mice to induce EAE in recipient mice after culture with MOG and IL-1β and IL-23 may, at least in part, reflect an increase in M2 macrophages in the absence of IFN-γ signalling, which is known to promote M1 macrophages.

rIFN-γ confers encephalitogenic function on MOG-specific T cells from IFN-$\gamma^{-/-}$ mice

Our data so far suggest that immune cells from IFN-$\gamma^{-/-}$ mice failed to induce EAE not because of an absence of IFN-γ-secreting T cells, but because of an absence of innate IFN-γ, which leads to an expansion of M2 over M1 macrophages; this results in a failure of MOG-specific T cells to express VLA-4 and migrate into the CNS. To confirm this hypothesis, we added recombinant IFN-γ (rIFN-γ) in vitro to the cells from IFN-$\gamma^{-/-}$ mice to see if it would restore their encephalitogenic potential. Addition of rIFN-γ to MOG, IL-1β and IL-23 cultured cells derived from MOG-immunized IFN-$\gamma^{-/-}$ mice restored the ability of these cells

to induce EAE following transfer to naive mice, with earlier onset EAE and weight loss than seen with cells from WT mice (Fig. 8A).

An examination of the cytokine production profiles of CNS-infiltrating T cells 16 d after T cell transfer showed that the absolute number of IL-17A and GM-CSF-secreting CD4 T cells was considerably greater in the brains and spinal cords of mice injected with cultured cells from WT compared with cells from IFN-$\gamma^{-/-}$ mice (Fig. 8B-E). Addition of rIFN-γ to the culture reversed the defect in IL-17A and GM-CSF-secreting CD4 T cells infiltrating the brain and to a lesser extent the spinal cord. However, fewer IFN-γ-secreting CD4 cells were found in the brains and spinal cords of mice injected with IFN-$\gamma^{-/-}$ cells treated with rIFN-γ despite the high clinical scores of this group of mice (Fig. 8B-E). These data demonstrated that CNS-infiltrating T cells induced robust disease in naive recipient mice in the absence of IFN-γ production.

Prior to the cell transfer, the cultured cells were examined for expression of markers of M2 and M1 macrophages. Cells from IFN-$\gamma^{-/-}$ mice had significantly elevated levels of *retnla* (Fizz1) and *chi3l3* (YM1) which are markers of M2 macrophages and significantly reduced expression of *nos2*, an M1 macrophage marker (Fig. 8F). Addition of recombinant IFN-γ to the MOG-stimulated cells reversed the expression of M2 macrophage markers *retnla* and *chi3l3* and enhanced expression of the M1 macrophage marker, *nos2*. The expansion of M2 macrophages in cells from IFN-$\gamma^{-/-}$ mice and the reversal by adding IFN-γ to the cultures was confirmed by FACS analysis of dectin-1 expression, a marker of M2 macrophages (Fig. 8G). Finally, we examined VLA-4 expression on T cells by flow cytometry and found that the reduced VLA-4 expression on cells from IFN-$\gamma^{-/-}$ mice was restored to that of WT mice by addition of recombinant IFN-γ (Fig. 8H).

These data indicate that, in the absence of IFN-γ, macrophages are committed to an alternatively activated and suppressive phenotype and consequently T cells fail to express VLA-4. Collectively our finding demonstrated that innate IFN-γ plays a critical role in the induction of EAE by promoting M1 macrophage polarization which enhances encephalitogenic activity of MOG-specific T cells by promoting their migration into the CNS.

NK cell-derived IFN-γ promote MI macrophage expansion and encephalitogenic activity of T cells

Our data demonstrated that IFN-γ from NK cells may be involved in the early stages of development of EAE and that IFN-γ may promote the encephalitogenic activity of T cells by

enhancing polarization of macrophages to the M1 phenotype and VLA-4 expression on T cells. In order to confirm this, we first examined the effect of M1 and M2 macrophages on VLA-4 expression by T cells. Peritoneal macrophages were stimulated in vitro with the M1 and M2 polarizing molecules IFN-γ and LPS or IL-4 and IL-13 respectively. After 72 h of culture, macrophages stimulated with IL-4 and IL-23 had significantly higher expression of M2 markers, *chi3l3* and *arg-1* detected by RT-PCR, whereas macrophages stimulated with IFN-γ and LPS had higher expression of *nos2* (Fig. 9A). Addition of M1 but not M2 macrophages enhanced VLA-4 expression on anti-CD3 stimulated T cells (Fig. 9B). These finding suggest that IFN-γ has a positive influence on VLA-4 expression on T cells indirectly by polarizing macrophages to adopt the M1 phenotype.

In order to confirm these findings and to examine the effect of NK cell-derived IFN-γ, we carried out co-culture expression with IL-12-activated NK cells, macrophages and MOG-specific CD4 T cells from mice immunized with MOG and CFA. We found that culture of bone marrow-derived macrophages with purified NK cells that had been stimulated with IL-12 to promote IFN-γ production (Fig. 9C), enhanced expression of the M1 marker *nos2*, and reduced expression of the M2 marker *chi313* (Fig. 9D). Furthermore, addition of macrophages activated with IL-12-stimulated NK cells to MOG-specific $CD4^+$ T cells significantly enhanced VLA4 expression on the T cells and this exceeded the effect observed with IFN-γ-activated macrophages (Fig. 9 E and F).

Having shown that NK cell-derived IFN-γ enhances VLA-4 expression on $CD4^+$ T cells by activating type 1 macrophages, we next examined if neutralization of VLA-4 could prevent EAE induced by transfer of MOG-specific T cells. Treatment of recipient mice with anti-VLA-4 up to day 12 completely attenuated disease at least until day 30 post transfer (Fig. 9G). Furthermore, VLA-4 blockade in mice with actively induced EAE from day 7, when MOG-specific effector T cells had been generated, also significantly attenuated EAE (Fig 9H). These findings suggest that NK cell-derived IFN-γ promotes M1 macrophages and encephalitogenic activity by promoting VLA4 expression on T cells.

Discussion

The significant new finding of our study is that T cells that are pathogenic in EAE do not need to produce IFN-γ to induce disease, but IFN-γ from innate sources induces M1 macrophage activation that confers encephalitogenic activity on T cells by promoting VLA4 expression and migration into the CNS.

The role of IFN-γ in the pathogenesis of autoimmune diseases has been the subject of debate. Studies in EAE, a mouse model for MS, have shown that IFN-γ-secreting CD4 T cells can induce disease, while others have demonstrated a protective role for IFN-γ in suppressing Th17 cells. The present study provides an explanation for these anomalies. We demonstrate that IFN-γ has a protective role at the effector phase of disease, but has a pathogenic role in the induction of EAE, and that this IFN-γ may come from innate sources, especially NK cells. The onset of actively-induced EAE was significantly delayed in IFN-$\gamma^{-/-}$ mice, mice treated with anti-IFN-γ or in mice depleted of NK cells, but not in IFN-$\gamma^{-/-}$ mice depleted of NK cells. Furthermore, the failure of MOG-specific T cells from IFN-$\gamma^{-/-}$ mice to induce EAE in WT micewas reversed by in vitro culture with IFN-γ which enhanced M1 macrophages and VLA-4 expression on T cells. However, it has previously been reported that EAE can be induced by passive transfer of T cells from IFN-$\gamma R^{-/-}$ mice on a 129/Sv background [10]. The difference with the present study may reflect the differences in the receptor versus cytokine knockout or mouse strain; unlike C57BL/6 mice, WT 129 mice are resistant to EAE.

The literature on the role of Th1 versus Th17 and their secreted cytokines, IFN-γ, IL-17 and GM-CSF, in autoimmune disease is conflicting. The results of clinical trials have shown that antibodies that target the IL-17 cytokine family are effective against psoriasis, multiple sclerosis and other autoimmune disorders in humans [26]. Anti-IL-17A is also protective against EAE, whereas antibodies that block IFN-γ exacerbate disease [8]. Our data are consistent with these findings and with previous studies in animal models demonstrating that IFN-γ can inhibit production of IL-17 and other cytokines by Th17 cells. We found that, at the peak of disease, EAE was more severe in IFN-$\gamma^{-/-}$ or anti-IFN-γ-treated mice and this was associated with enhanced IL-17A and GM-CSF production.

The demonstration that IFN-γ is protective in EAE through inhibition of Th17 cells is consistent with the suggestion that IL-17-secreting Th17 cells expanded with IL-23 are dramatically more encephalitogenic than IFN-γ-secreting Th1 cells expanded with IL-12 [8]. However, more recent studies have shown that Th1 cells or Th17 cells can induce EAE. It was reported that spleen and lymph nodes cells from mice immunized with PLP and IFA and cultured with PLP in vitro in the presence of either Th1 or Th17 polarizing cocktails induced

EAE in recipient mice; IL-12-and IL-23-polarized cells induced EAE with distinct CNS cellular infiltrates dominated by macrophages and neutrophils respectively [4]. It has been reported that Th1 cells have an essential role in facilitating entry of Th17 cells into the CNS, whereas highly polarized autoantigen-specific Th17 cells failed to induce EAE and pointed to a role for TNF-α, since both Th1 and Th17 cells produced this cytokine [14]. Others have suggested that GM-CSF, which is produced by both Th1 and Th17 cells, is the critical cytokine in the encephalitogenicity of CD4 T cells [27, 28]. Our data are consistent with the study by O'Connor and colleagues [14]. We found that transfer of MOG-specific cells polarized in vitro with either IL-12 or IL-23 and IL-1β could induce EAE; both cell populations produced GM-CSF, but also include substantial numbers of IFN-γ-secreting CD4 T cells. In contrast, transfer of T cells defective in IFN-γ production from MOG and CFA immunized IFN-$\gamma^{-/-}$ mice and stimulated in vitro with either IL-12 or IL-1β and IL-23 were virtually incapable of inducing any symptoms of EAE in recipient mice, despite the fact that they produced significant concentrations of IL-17A, IL-17F and GM-CSF. Furthermore, IL-17A, GM-CSF and IFN-γ-secreting CD4 T cells were detectable in the CNS of mice that received MOG-specific WT cells but not in recipients of MOG-specific cells from IFN-$\gamma^{-/-}$ mice. These findings provide evidence that IFN-γ has a critical role in the encephalitogenic function of auto-antigen specific T cells and are consistent with the hypothesis that Th17 cells lacking early production of IFN-γ are incapable of entry into the CNS. However, MOG-specific T cells from IFN-$\gamma^{-/-}$ mice stimulated in vitro with rIFN-γ could induce EAE, suggesting that T cell expression of IFN-γ is not essential.

One explanation for the failure of T cells from IFN-$\gamma^{-/-}$ mice to induce EAE by T cell transfer is that Th2 or regulatory cells expand in vitro in the absence of the inhibitory effects of IFN-γ. Indeed we found enhanced IL-5 and IL-13 production by MOG-specific cells from MOG and CFA immunized IFN-$\gamma^{-/-}$ mice, but interestingly IL-17A and IL-17F were similar and GM-CSF enhanced when compared with cultured cells from WT mice, suggesting that the failure to induce EAE was not due to switch from a Th1/Th17 to a Th2 subtype. M2 macrophages are also known to have a regulatory role in the development of EAE [25]. Interestingly, we observed significant augmentation in M2 macrophages and a decrease in M1 macrophages in lymph node and spleen cells from MOG and CFA immunized IFN-$\gamma^{-/-}$ mice stimulated with MOG, IL-23 and IL-1β. Addition of rIFN-γ to these cultures reversed the expansion of M2 and increased M1 macrophages. This is consistent with the established role of IFN-γ in polarizing macrophages to the M1 phenotype [29]. We could mimic the effect observed with cells from IFN-$\gamma^{-/-}$ mice by polarizing M2 macrophages in the culture;

this abrogated the ability of the T cells in these cultures to induce EAE following adoptive transfer to WT mice.

It has previously been reported that Th1 cells utilize VLA-4 for entry into the CNS. Blockade of VLA-4 with an antibody to α4 integrin (CD49d) prevented adoptively transferred Th1 cells from entering the brain and spinal cord and inducing EAE [19]. Antibody treatment also prevented migration of Th17 cells into the spinal cord, but did not prevent Th17 cells entering the brain, where they induced an atypical form of EAE characterized by gait ataxia [19]. Blockade of VLA-4 with natalizumab, a humanised monoclonal antibody that targets the α4-chain of α4β1 integrin is an effective therapy for patients with relapsing MS [18]. We found that VLA-4 expression was significantly reduced on T cells from MOG and CFA-immunized IFN-$\gamma^{-/-}$ mice that failed to induce EAE by adoptive transfer. Furthermore, addition of rIFN-γ to cultured cells with MOG and polarizing cytokines augmented VLA-4 expression and restored the ability of T cells from IFN-$\gamma^{-/-}$ mice to induce EAE. Finally, we showed that VLA-4 blockade abrogated EAE induced by cell transfer and significantly attenuated actively induced EAE when the antibody was administered after induction of effector T cells. These findings suggest that IFN-γ need not be produced by T cells to induce EAE, but innate IFN-γ, which is produced early in EAE by NK cells, does have a crucial role in conferring encephalitogenic activity at least in part by promoting VLA-4 expression on T cells. Collectively, our findings suggest that innate IFN-γ plays an important role in the induction of EAE by expanding M1 over M2 macrophages, thereby prompting pathogenic activity on T cells by enhancing their ability to migrate into the CNS.

Materials and Methods

Mice

C57BL/6 and IFN-$\gamma^{-/-}$ on a C57BL/6 background and age and sex matched (provided by Rachel McLoughlin from a colony established from breeding pairs from Jackson laboratories) mice were bred under SPF conditions. All mice were maintained according to European Union regulations, and experiments were performed under license (BI00/2412) from The Irish Medicine Board and with approval from the Trinity College Dublin BioResources Ethics Committee.

Induction and assessment of actively induced EAE (Fig. S2A)

C57BL/6 and IFN-$\gamma^{-/-}$ mice were immunized s.c. with 100 μg $MOG_{35–55}$ peptide (GenScript) emulsified in CFA containing 4 mg/ml (0.4 mg/mouse) of heat-killed *M. tuberculosis* (Chondrex). Mice were injected i.p. with 500 ng of pertussis toxin (Kaketsuken) on day 0 and 2. In certain experiments mice were treated with anti-IFN-γ antibody (500 μg/mouse) administered i.p. on day 0, 7 and 14 post-induction or depleted of NK cells by i.p administration of anti-asialo GM1 antiserum (Cedarlane; 10 μl/ mouse) on days -1, 1, and 4. Control mice were administered vehicle (PBS) at the same time points. Disease severity was assessed according to percentage weight change and typical clinical scores as follows: no clinical signs, 0; limp tail, 1; ataxic gait, 2; hind limb weakness, 3; hind limb paralysis, 4; tetra paralysis/moribund, 5.

Induction and assessment of EAE by adoptive transfer (Fig. S2B)

C57BL/6 and IFN-$\gamma^{-/-}$ mice were immunized with MOG and CFA as described above. On day 10 post-induction mice were sacrificed and their brachial lymph nodes (LNs), axillary LNs, inguinal LNs and spleens were removed and single cell suspensions prepared. Cells were stimulated as indicated in figure legends with combinations of MOG (100 μg/ml) and IL-23 (10 ng/ml) + IL-1β (10 ng/ml) or IL-12 (10 ng/ml) in the presence or absence of recombinant IFN-γ (rIFN-γ; 10 ng/ml) or IL-4 (20 ng/ml) and IL-13 (20 ng/ml) for 72 h in vitro in cRPMI at 10 X 10^6 cells/ml in 75 ml tissue culture flasks. All cyokines used were of mouse origin. After 72 h, cells were washed and cytokine production examined by intracellular staining and FACS or in supernatants by ELISA. 15 X 10^6 viable cells were injected i.p. into wild type C57BL/6 recipient mice. In certain experiments WT recipient mice also received 500 μg/mouse of IFN-γ neutralising antibody i.p. or a PBS control. Animals were monitored daily

for signs of clinical disease and their weights were recorded. Disease severity was assessed as above for typical symptoms of EAE or on an atypical scale as follows: no clinical signs, 0; slight listing/difficulty righting, 1; obvious imbalance but able to ambulate, 2; severely impaired balance/ambulation, 3; incapacitated due to inability to maintain upright posture, 4; spinning, 4.5; moribund, 5.

Effect of M1 and M2 polarized macrophages on VLA-4 expression by T cells

Peritoneal exudate cells (PEC) were isolated from WT mice and stimulated *in vitro* with M1 or M2 macrophage polarizing cytokines IFN-γ (100 ng/ml) and LPS (500 ng/ml) or IL-4 (20 ng/ml) and IL-13 (20 ng/ml) respectively. $CD3^+$ T cells were purified from naive mice. M1- or M2-polarized PEC were cultured at a ratio of 1:2 with purified CD3 T cells with anti-CD3 (5 μg/ml). After 6 h cells were surface stained for CD4 and VLA-4 and FACS analysis performed.

Effect of NK cells on M1 and M2 polarization and VLA4 expression by T cells

NK cells were separated from spleen cells using the NK cell isolation kit II (Miltenyi Biotec) and expanded *in vitro* for 6 days with rIL-2 (100 ng/ml). Bone marrow-derived macrophages (BMDM) were generated by culture of bone marrow from WT mice with supernatants of M-CSF-secreting L929 cells for 7 days. MOG-specific $CD4^+$ T cells were purified using a CD4 T cell Isolation Kit (Miltenyi) from spleen and lymph node cells derived from MOG and CFA immunized mice and re-stimulated in vitro for 3 days with MOG (100 μg/ml). Purified $CD4^+$ T cells were incubated with, NK cells pre-treated for 24 h with IL-12 (20 ng/ml) or IL-2 (100 ng/ml) or in the presence or absence of BMDMs (all cells at 2.5 x 10^5/ml), with or without IFN-γ (50 ng/ml). After a 24 h culture, supernatants were collected for ELISA and cells harvested to determine CD4 and VLA-4 expression by FACS analysis or for PCR to assess expression of M1 or M2 macrophage markers.

FACS analysis

Mononuclear cells were isolated from the brains and spinal cords of mice following perfusion with PBS and purification of mononuclear cells by percoll density centrifugation. Cells were stained with CD11b, F4/80, Ly-6G or NK1.1, and FACS analysis was performed to determine the frequency of macrophages, neutrophils and NK cells. Alternatively, mononuclear cells were stimulated for 5 h with PMA (10 ng/ml) and ionomycin (1 mg/ml) in the presence of brefeldin A (5 μg/ml). Cells were washed and stained for surface CD3 and

CD4 (eBioscience). Cells were then fixed and permeabilized (Fix and Perm cell permeabilization kit; Caltag Laboratories) and stained for intracellular IL-17A, GM-CSF and IFN-γ (eBioscience). M2 macrophages in cultures of lymph node and spleen cells from MOG and CFA-immunized C57BL/6 mice were determined by surface staining for F4/80, CD11b, Ly-6G, dectin-1 and MHC Class II. VLA-4 expression on CD4 T cells was determined using an antibody specific for CD49d, a subunit of VLA-4. Flow cytometric analysis was performed using a CyANADP Flow Cytometer (DakoCytomation) and analyzed with FloJo software.

Histology

Mice were euthanised with pentobarbital sodium and intracardiacally perfused with ice-cold PBS. Brains and whole spinal columns were isolated and transferred into paraformaldehyde. Coronal sections (3 mm) of the brain and spinal cord were placed into cassettes and processed to paraffin embedded tissue. Sections from each paraffin block cut at 4 μm were stained with haematoxylin and eosin and Luxol fast. Nine spinal cord sections and four brain sections were examined for each mouse. Demyelination was assessed as mild, moderate and severe on Luxol-fast stained sections.

Real-time PCR

Cells were processed and mRNA was isolated using the TRIzol (Invitrogen)/chloroform method or the Nucleospin RNA II extraction kit from Machery-Nagel. mRNA expression was evaluated by real-time PCR following reverse transcription into cDNA using a QuantiTect Reverse transcription kit (Qiagen). Real-time PCR for the detection of *arg1* (Mm00475988_m1), *retnla* (Mm00445109_m1), *chi3l3* (Mm00657889_mH), *nos2* (Mm00440502_m1) and *itga4* (cd49d; Mm01277951_m1) mRNA was performed using predesigned TaqMan gene expression assays (Applied Biosystems). 18s rRNA was used as an endogenous control. Samples were assayed on an Applied Biosystems 7500 Fast Real-Time PCR machine.

Statistical analysis

Statistical analyses were performed using GraphPad Prism statistical analysis software. Group differences were analyzed by unpaired Student *t* test or two-way ANOVA with multiple comparisons, followed by Bonferroni post-test comparisons, for three or more groups. Differences between groups for clinical scores in EAE were analyzed by two-way

ANOVA with repeated measures. The p values ≤ 0.05 were considered significant.

Acknowledgments

We are grateful to Rachel McLoughlin for supplying IFN-$\gamma^{-/-}$ mice, and to Caroline Sutton and Jean Fletcher for helpful discussions. This work was supported by grants from Science Foundation Ireland (PI grant 11/PI/1036 to KHGM), an IRC EMBARK Postgraduate Fellowship (to LSD.) and The Irish Higher Education Authority (HEA) Programme for Research in Third-Level Institutions (PRTLI) (to NCMcG, KHGM. and MAL).

Conflict of Interest

Kingston Mills is co-founder and shareholder of Opsona Therapeutics Ltd, and TriMod Therapeutics Ltd, University spin-out companies involved in the development of immunotherapeutics. Other authors declare no financial or commercial conflict of interest.

References

1 **Panitch, H. S., Hirsch, R. L., Haley, A. S. and Johnson, K. P.,** Exacerbations of multiple sclerosis in patients treated with gamma interferon. *Lancet* 1987. **1**: 893-895.

2 **Renno, T., Taupin, V., Bourbonniere, L., Verge, G., Tran, E., De Simone, R., Krakowski, M., Rodriguez, M., Peterson, A. and Owens, T.,** Interferon-gamma in progression to chronic demyelination and neurological deficit following acute EAE. *Mol Cell Neurosci* 1998. **12**: 376-389.

3 **Mills, K. H.,** Induction, function and regulation of IL-17-producing T cells. *Eur J Immunol* 2008. **38**: 2636-2649.

4 **Kroenke, M. A., Carlson, T. J., Andjelkovic, A. V. and Segal, B. M.,** 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* 2008. **205**: 1535-1541.

5 **Gran, B., Zhang, G. X., Yu, S., Li, J., Chen, X. H., Ventura, E. S., Kamoun, M. and Rostami, A.,** 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. *J Immunol* 2002. **169**: 7104-7110.

6 **Becher, B., Durell, B. G. and Noelle, R. J.,** Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. *J Clin Invest* 2002. **110**: 493-497.

7 **Zhang, G. X., Gran, B., Yu, S., Li, J., Siglienti, I., Chen, X., Kamoun, M. and Rostami, A.,** Induction of experimental autoimmune encephalomyelitis in IL-12 receptor-beta 2-deficient mice: IL-12 responsiveness is not required in the pathogenesis of inflammatory demyelination in the central nervous system. *J Immunol* 2003. **170**: 2153-2160.

8 **Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A. and Cua, D. J.,** IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 2005. **201**: 233-240.

9 **Ferber, I. A., Brocke, S., Taylor-Edwards, C., Ridgway, W., Dinisco, C., Steinman, L., Dalton, D. and Fathman, C. G.,** Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J Immunol* 1996. **156**: 5-7.

10 **Willenborg, D. O., Fordham, S., Bernard, C. C., Cowden, W. B. and Ramshaw, I. A.,** IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *J Immunol* 1996. **157**: 3223-3227.

11 **Billiau, A., Heremans, H., Vandekerckhove, F., Dijkmans, R., Sobis, H., Meulepas, E. and Carton, H.,** Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma. *J Immunol* 1988. **140**: 1506-1510.

12 **Duong, T. T., St Louis, J., Gilbert, J. J., Finkelman, F. D. and Strejan, G. H.,** Effect of anti-interferon-gamma and anti-interleukin-2 monoclonal antibody treatment on the development of actively and passively induced experimental allergic encephalomyelitis in the SJL/J mouse. *J Neuroimmunol* 1992. **36**: 105-115.

13 **Grifka-Walk, H. M., Lalor, S. J. and Segal, B. M.,** Highly polarized Th17 cells induce EAE via a T-bet independent mechanism. *Eur J Immunol* 2013. **43**: 2842-2831.

14 **O'Connor, R. A., Prendergast, C. T., Sabatos, C. A., Lau, C. W., Leech, M. D., Wraith, D. C. and Anderton, S. M.,** Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *J Immunol* 2008. **181**: 3750-3754.

15 **Pribila, J. T., Quale, A. C., Mueller, K. L. and Shimizu, Y.,** Integrins and T cell-mediated immunity. *Annu Rev Immunol* 2004. **22**: 157-180.

16 **Larochelle, C., Alvarez, J. I. and Prat, A.,** How do immune cells overcome the blood-brain barrier in multiple sclerosis? *FEBS Lett* 2011. **585**: 3770-3780.

17 **Yednock, T. A., Cannon, C., Fritz, L. C., Sanchez-Madrid, F., Steinman, L. and Karin, N.,** Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. *Nature* 1992. **356**: 63-66.

18 **Polman, C. H., O'Connor, P. W., Havrdova, E., Hutchinson, M., Kappos, L., Miller, D. H., Phillips, J. T., Lublin, F. D., Giovannoni, G., Wajgt, A., Toal, M., Lynn, F., Panzara, M. A. and Sandrock, A. W.,** A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med* 2006. **354**: 899-910.

19 **Rothhammer, V., Heink, S., Petermann, F., Srivastava, R., Claussen, M. C., Hemmer, B. and Korn, T.,** Th17 lymphocytes traffic to the central nervous system independently of alpha4 integrin expression during EAE. *J Exp Med* 2011. **208**: 2465-2476.

20 **Sutton, C. E., Lalor, S. J., Sweeney, C. M., Brereton, C. F., Lavelle, E. C. and Mills, K. H.,** Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 2009. **31**: 331-341.

21 **Sutton, C. E., Mielke, L. A. and Mills, K. H.,** IL-17-producing gammadelta T cells and innate lymphoid cells. *Eur J Immunol* 2012. **42**: 2221-2231.

22 **Zhang, B., Yamamura, T., Kondo, T., Fujiwara, M. and Tabira, T.,** Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. *J Exp Med* 1997. **186**: 1677-1687.

23 **Lu, L., Ikizawa, K., Hu, D., Werneck, M. B., Wucherpfennig, K. W. and Cantor, H.,** Regulation of activated CD4+ T cells by NK cells via the Qa-1-NKG2A inhibitory pathway. *Immunity* 2007. **26**: 593-604.

24 **Shi, F. D., Takeda, K., Akira, S., Sarvetnick, N. and Ljunggren, H. G.,** IL-18 directs autoreactive T cells and promotes autodestruction in the central nervous system via induction of IFN-gamma by NK cells. *J Immunol* 2000. **165**: 3099-3104.

25 **Mikita, J., Dubourdieu-Cassagno, N., Deloire, M. S., Vekris, A., Biran, M., Raffard, G., Brochet, B., Canron, M. H., Franconi, J. M., Boiziau, C. and Petry, K. G.,** 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. *Mult Scler* 2011. **17**: 2-15.

26 **Jones, S. A., Sutton, C. E., Cua, D. and Mills, K. H.,** Therapeutic potential of targeting IL-17. *Nat Immunol* 2012. **13**: 1022-1025.

27 **El-Behi, M., Ciric, B., Dai, H., Yan, Y., Cullimore, M., Safavi, F., Zhang, G. X., Dittel, B. N. and Rostami, A.,** The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol* 2011. **12**: 568-575.

28 **Codarri, L., Gyulveszi, G., Tosevski, V., Hesske, L., Fontana, A., Magnenat, L., Suter, T. and Becher, B.,** RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol* 2011. **12**: 560-567.

29 **Biswas, S. K. and Mantovani, A.,** Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol* 2010. **11**: 889-896.

FIGURE LEGENDS

Figure 1. IFN-γ is pathogenic early but plays a protective role at later stages of EAE. (A,B) EAE was induced in C57BL/6 and IFN-$\gamma^{-/-}$ mice by immunization with MOG emulsified in CFA. Mice were assessed for the development of EAE by clinical score (A) and % weight change (B). ***p < 0.001 versus WT control. (C-H) EAE was induced in C57BL/6 mice and mice treated by i.p. injection with 500 μg of anti-IFN-γ (αIFN-γ) neutralising antibody or control on day 0, 7 and 14 post-induction. Mice were assessed for the development of EAE by clinical score (C) and % weight change (D). *p < 0.05, **p < 0.01, ***p < 0.001 versus control. (E-H) Mice were sacrificed either on day 12 post-induction or on day 18 post-induction and mononuclear cells were isolated from the brain and pooled spinal cords. Cells were stained for surface CD4 and intracellular IFN-γ, IL-17A and GM-CSF or CD11b, Ly6G and F4/80 and analysed by flow cytometry. Results are absolute number of IFN-γ^{+}, IL-$17A^{+}$ and GM-CSF^{+} CD4 T cells (E,G) or $CD11b^{+}$ $Ly6G^{+}$ neutrophils (NEU) and $CD11b^{+}$ $F4/80^{+}$ macrophages (Mθ) (F, H) for anti-IFN-γ treated mice versus control. *p < 0.05, **p < 0.01 versus control. Data are representative of 2 or 3 independent experiments, with 5 or 6 mice in each experimental group in each experiment.

Figure 2. NK cells provide an early source of IFN-γ in EAE. (A) Frequency of infiltrating NK cells, IFN-γ-secreting NK cells and IFN-γ-secreting CD4 T cells in brains of mice with EAE induced by immunization with MOG in CFA after staining for intracellular IFN-γ and surface CD45, CD3, CD4 and NK1.1. (B) Mean absolute numbers of cells in brain (n=4 per group). ***p < 0.001 versus day 3.

Figure 3. Depletion of NK cells attenuates Th1 and Th17 migration into CNS and delays onset of EAE.. EAE clinical scores in C57BL/6 WT (A) or IFN-$\gamma^{-/-}$ (B) mice injected i.p. with anti-asialo GM1 antiserum (10 μl/ mouse) or control serum on day -1, 1, and 4 of EAE. *p < 0.05, versus control (n=6). (C) Absolute numbers of infiltrating $CD3^{+}$ T cells, IFN-γ- or IL-17A-secreting CD4 cells on day 18 in brain mice described in A. *p < 0.05, versus control (n=6 per group). (D) Representative dot plots from B.

Figure 4. Treatment of donor mice with anti-IFN-γ attenuates EAE induced by cell transfer. C57BL/6 mice were immunized with MOG and CFA and injected with 500 μg anti-IFN-γ neutralizing antibody (anti-IFN-γ donor) or PBS on day 0. Mice were sacrificed 10 days post-

induction and their lymph node and spleen cells were stimulated in vitro with MOG (100 μg/ml), IL-23 (10 ng/ml) and IL-1β (10 ng/ml). Cells were harvested washed and injected i.p. into naïve C57BL/6 recipient mice. Recipient mice were injected on the day of cell transfer with 500 μg IFN-γ neutralizing antibody (anti-IFN-γ recipient) or PBS. Mice were assessed for the development of EAE by clinical score (A) $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus WT cells (B) Prior to cell transfer and after 72 h culture IL-17A, GM-CSF and IFN-γ concentrations in the supernatants were quantified by ELISA. (C-D) Recipient mice were sacrificed on day 14 post-transfer and mononuclear cells were isolated from the brain. Cells were stained for surface CD4 and intracellular IL-17A, GM-CSF and IFN-γ and analyzed by flow cytometry. Results are absolute numbers of CNS-infiltrating IL-17A$^+$, GM-CSF$^+$ and IFN-γ$^+$ CD4 T cells (C) and sample FACS plots (D). (n=6 per group).

Figure 5. MOG-specific cells from IFN-γ$^{-/-}$ mice fail to induce EAE following transfer to WT recipient mice. WT or IFN-γ$^{-/-}$ mice were immunized with MOG and CFA. Mice were sacrificed 10 days post-induction and their lymph node and spleen cells were stimulated in vitro with MOG (100 μg/ml) and IL-12 (10 ng/ml) or MOG, IL-23 (10 ng/ml) and IL-1β (10 ng/ml). After 72 h of culture, cells were harvested, washed and injected i.p. into naïve C57BL/6 recipients (15 X 10^6 cells per mouse). Mice were assessed for the development of EAE by typical clinical score (A) or atypical EAE clinical score (B). Data are combined from five independent experiments (n=25 per group). (C) On day 14 post-induction of EAE by cell transfer, coronal sections of the spinal cord were taken for histological analysis and stained with haematoxylin and eosin to assess lymphocyte infiltration and luxol fast blue to assess demyelination. (D-E) Mononuclear cells from brains of perfused mice (day 14), transferred with cells from MOG-immunized WT or IFN-γ$^{-/-}$ mice stimulated with MOG, IL-1β and IL-23, were stained for surface CD4 and intracellular IL-17A, GM-CSF and IFN-γ and analyzed by flow cytometry. Results are absolute numbers of CNS-infiltrating IL-17A$^+$, GM-CSF$^+$ and IFN-γ$^+$ CD4 T cells (D) with representative FACS plots (E). $**p < 0.01$, $***p < 0.001$ versus WT cells.

Figure 6. IFN-γ$^{-/-}$ cells that fail to induce EAE have enhanced M2 macrophages and reduced VLA-4 expression on T cells. WT or IFN-γ$^{-/-}$ mice were immunized with MOG and CFA and on day 10 their lymph node and spleen cells were stimulated for 72 h with MOG, IL-23 and IL-1β as described in Fig 4. (A) IL-17A, IL-17F, IFN-γ, GM-CSF, IL-13 and IL-5 concentrations in the supernatants were quantified by ELISA. (B) Cells were assessed for the

expression of M2 macrophage markers *retnla*, *arg1* and *chi3l3* (YM1) by real-time PCR. (C) Cells were surface stained for F4/80, CD11b, Ly6G, dectin-1 and FACS analysis performed; results are shown for dectin-1 versus CD11b following gating on $Ly6G^{-}F4/80^{+}$cells. (D-E) Before (0 h) and 72 h after culture, cells were also surface stained for CD4 and VLA-4 (CD49d). Results are representative FACS plots (D) and mean percentage $CD4^{+}$ $VLA\text{-}4^{+}$ cells (E). (F) After 72 h culture, CD3 T cells were MACS purified (>95% pure) and *itga4/cd49d* (VLA-4) mRNA expression was evaluated by real-time PCR normalised to 18s rRNA. $^{**}p < 0.01$, $^{***}p < 0.001$ versus WT control. (n=6 per group; representative of 5 experiments).

Figure 7. Macrophages from IFN-$\gamma^{-/-}$ mice have an alternatively-activated phenotype and suppress the pathogenicity of MOG-specific T cells. (A) T cells from MOG and CFA immunized WT mice were co-cultured with non-T cells from MOG and CFA immunized WT or IFN-$\gamma^{-/-}$ mice and injected i.p. into naïve C57BL/6 recipients as described in Fig. 4. Mice were assessed for the development of EAE by clinical score. (B) The supernatants from the 72 h cultured cells described in A prior to transfer were assessed for IL-17A, IFN-γ and GM-CSF concentrations by ELISA (C) Macrophages in the cultures were assessed for CD11b, F4/80 and dectin-1 expression as a measure of M2 macrophages. (D) Spleen and lymph node cells from MOG-immunized mice were stimulated in vitro with MOG, IL-23 and IL-1β, with and without recombinant IL-4 (20 ng/ml) and IL-13 (20 ng/ml). After 72 h, cultured cells were harvested, washed and injected i.p. into naïve C57BL/6 recipients. Mice were assessed for the development of EAE by clinical score. (E) The supernatants from the 72 h cultured cells described in D prior to transfer were assessed for IL-17A, IL-17F, IFN-γ and GM-CSF concentrations by ELISA. (F) Cells were assessed for expression of *retnla*, *arg1* and *chi3l3* by real-time PCR. (G) $F4/80^{+}$ macrophages in the cultures assessed for dectin-1 expression as a measure of M2 macrophages. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus WT. (n=6 per group; representative of 3 experiments).

Figure 8. rIFN-γ suppresses M2 macrophages, enhances VLA4 expression and restores the defect in the ability of cells from IFN-$\gamma^{-/-}$ mice to induced EAE. (A) Lymph node and spleen cells from MOG-immunized IFN-$\gamma^{-/-}$ mice were stimulated in vitro with MOG, IL-23 and IL-1β, with and without rIFN-γ (100 ng/ml). After 72 h of culture, cells were harvested, washed and injected i.p. into naïve C57BL/6 recipients. Mice were assessed for the development of EAE by clinical score and % weight change. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus mice

injected with WT cells (n=6). Absolute numbers of infiltrating IL-17A$^+$, GM-CSF$^+$ or IFN-γ$^+$ CD4 cells in brain (B) and spinal cord (C) on day 15 of mice in A, with representative dot plots (D) and (E) respectively. *p < 0.05 versus, **p < 0.01 versus IFN-γ$^{-/-}$ mice. (F) CD3-depleted cells from cultures described in A were evaluated for *retnla/fizz1*, *chi3l3* (*ym1*) and *Nos2* mRNA expression by real-time PCR normalised to 18s rRNA. (G) Absolute numbers of CD11b$^+$ Ly6G$^-$ F4/80$^+$ Dectin-1 cells in culture described in A. (H) Frequency of CD4$^+$VLA-4$^+$ cells in culture described in A. ***p < 0.001 as indicated. Representative of 3 experiments (n=6-7 per group)

Figure 9. NK cells promoted M1 macrophages, which enhance VLA-4 expression on T cells required for their encephalitogenic activity. (A) Peritoneal macrophages were isolated from WT mice and stimulated *in vitro* with M1 or M2 macrophage polarizing cytokines IFN-γ (100 ng/ml) and LPS (500 ng/ml) or IL-4 (20 ng/ml) and IL-13 (20 ng/ml) respectively. (A) After 6 h cells were assessed for the expression of *Nos2*, *arg1* and *chi3l3* (YM1) by real-time PCR. ***p < 0.001 WT vs IFN-γ$^{-/-}$ by ANOVA. (B) M1- or M2-polarized macrophages were cultured at a ratio of 1:2 with purified CD3 T cells in the presence of anti-CD3. After and 72 h cells were surface-stained for CD4 and VLA-4. (C-E) MOG-specific CD4$^+$ T cells were cultured with BMDMs (with or without 50 ng/ml IFN-γ) with or without NK cells (pre-incubated for 24 h with 20 ng/ml IL-12 or 100 ng/ml IL-2). After 24 h, supernatants were removed and IFN-γ production was quantified by ELISA (C). mRNA was prepared from cell pellets and expression of *nos2* and *chi313* were quantified by real time PCR (D). Cells were surface-stained for CD4 and VLA-4. Representative FACS plots (E) and mean+/- SD values for 5 samples (F). **p < 0.01 versus IFN-γ, ^{+++}p < 0.001 versus NK cells + IL-12 (without macrophages). (G) EAE was induced by cell transfer from WT mice; mice were injected with a VLA-4 neutralizing antibody or an isotype control antibody (200 μg/mouse on day 0, 3, 6, 9 and 12) or (H) EAE was induced by active immunization and mice were treated with anti-VLA4 or control antibody on days 7, 10, 13, 16 and 19. Mice were assessed for the development of EAE by clinical score. Data are representative of 2 independent experiments (n=6 per group).

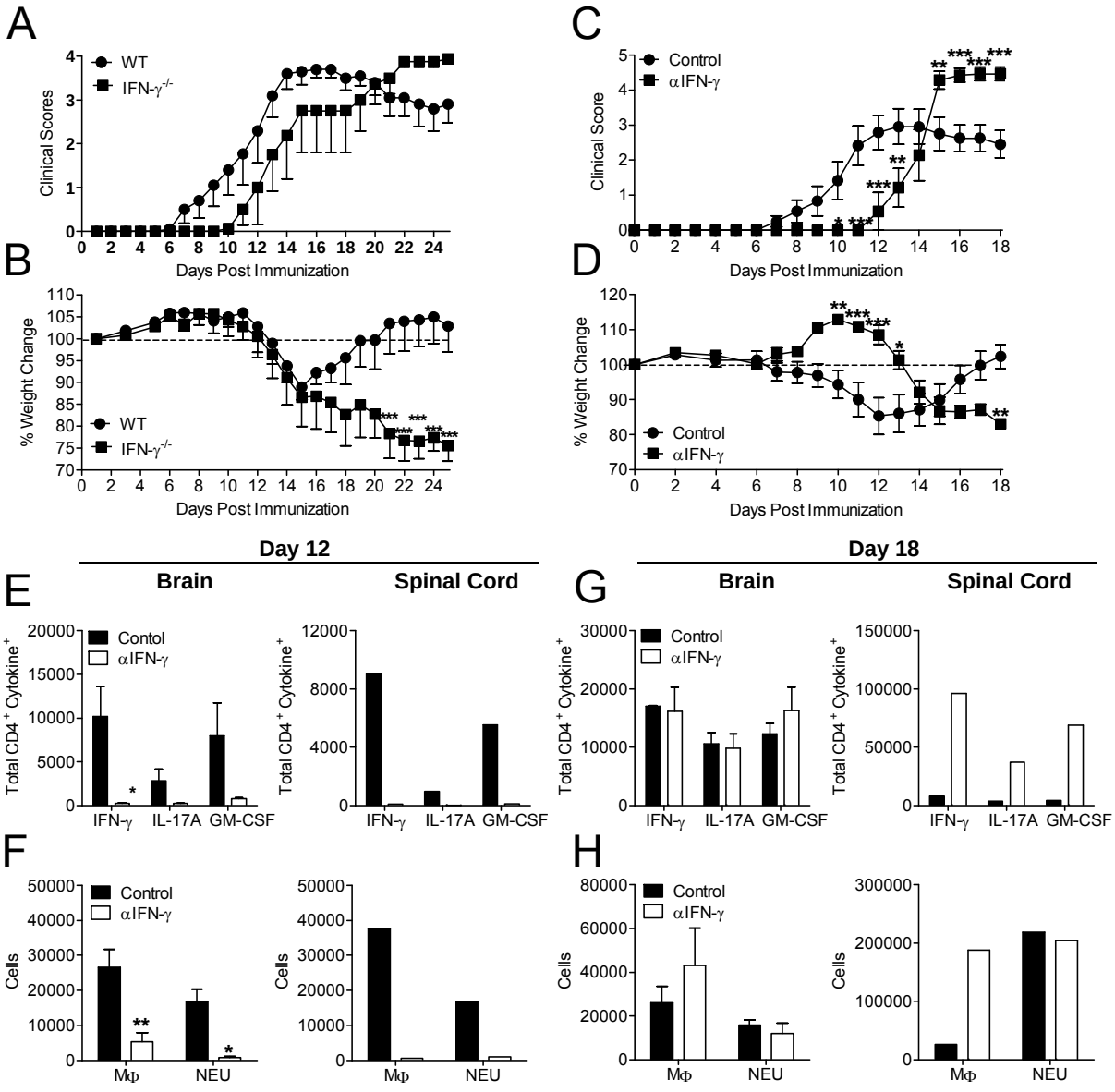
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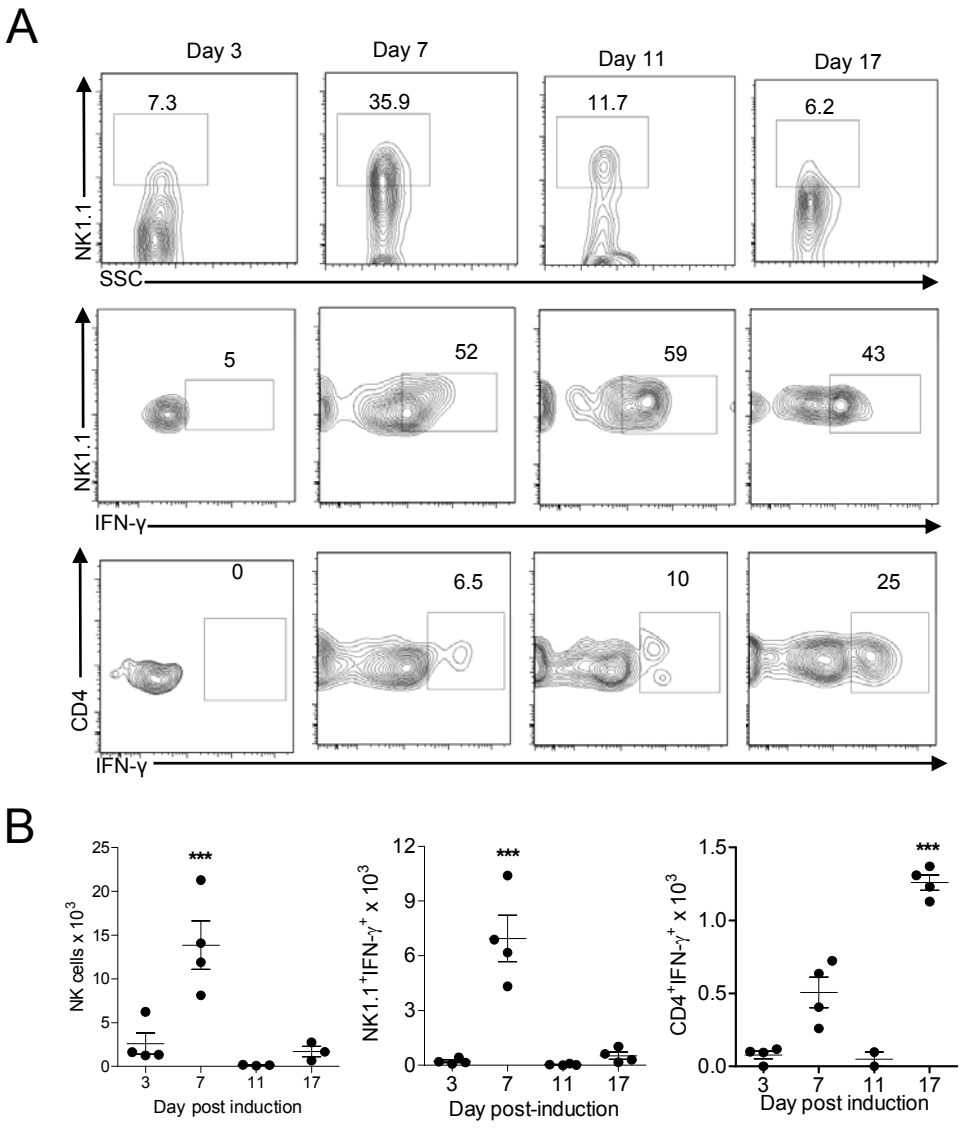
Figure 1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Day post induction

Figure 2

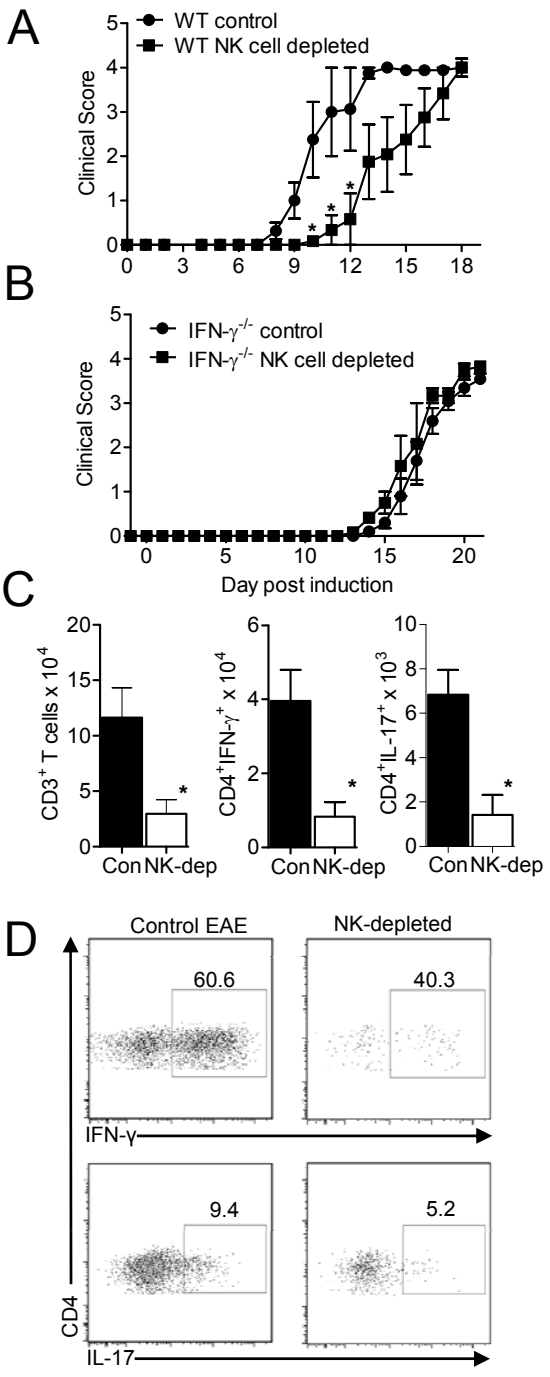
A
WT control
WT NK cell depleted
Clinical Score
B
IFN-γ-/- control
IFN-γ-/- NK cell depleted
Clinical Score
Day post induction
C
CD3+ T cells x 10^4
CD4+IFN-γ+ x 10^4
CD4+IL-17+ x 10^3
Con
NK-dep
D
Control EAE
NK-depleted
60.6
40.3
IFN-γ
9.4
5.2
CD4
IL-17

Figure 3

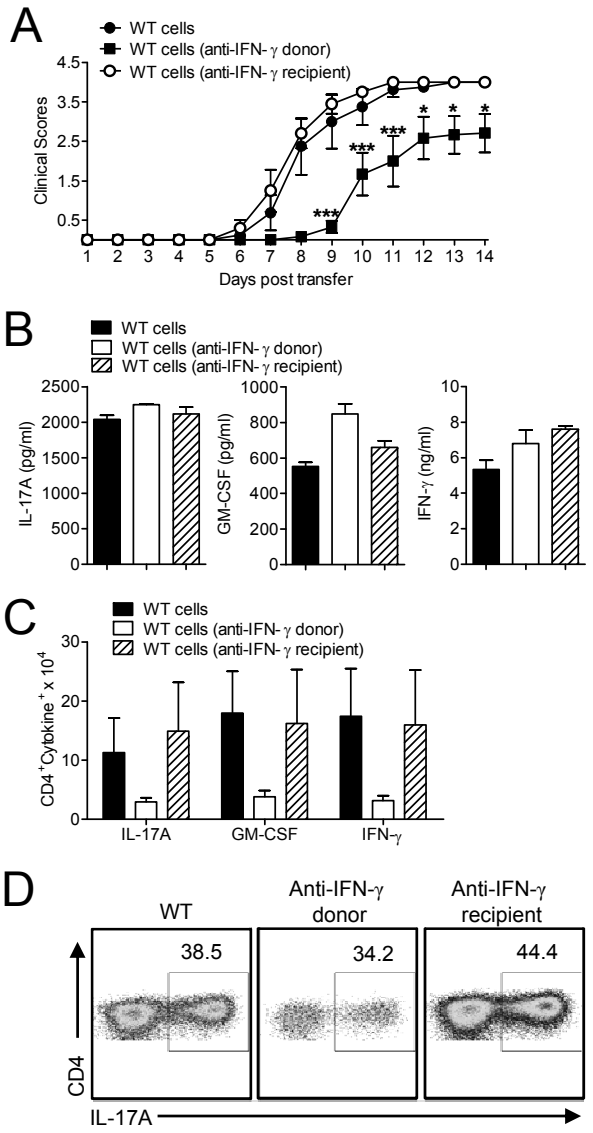
A
WT cells
WT cells (anti-IFN-γ donor)
WT cells (anti-IFN-γ recipient)
Clinical Scores
Days post transfer
B
WT cells
WT cells (anti-IFN-γ donor)
WT cells (anti-IFN-γ recipient)
IL-17A (pg/ml)
GM-CSF (pg/ml)
IFN-γ (ng/ml)
C
WT cells
WT cells (anti-IFN-γ donor)
WT cells (anti-IFN-γ recipient)
CD4+Cytokine+ x 10^4
IL-17A
GM-CSF
IFN-γ
D
WT
Anti-IFN-γ donor
Anti-IFN-γ recipient
38.5
34.2
44.4
CD4
IL-17A

Figure 4

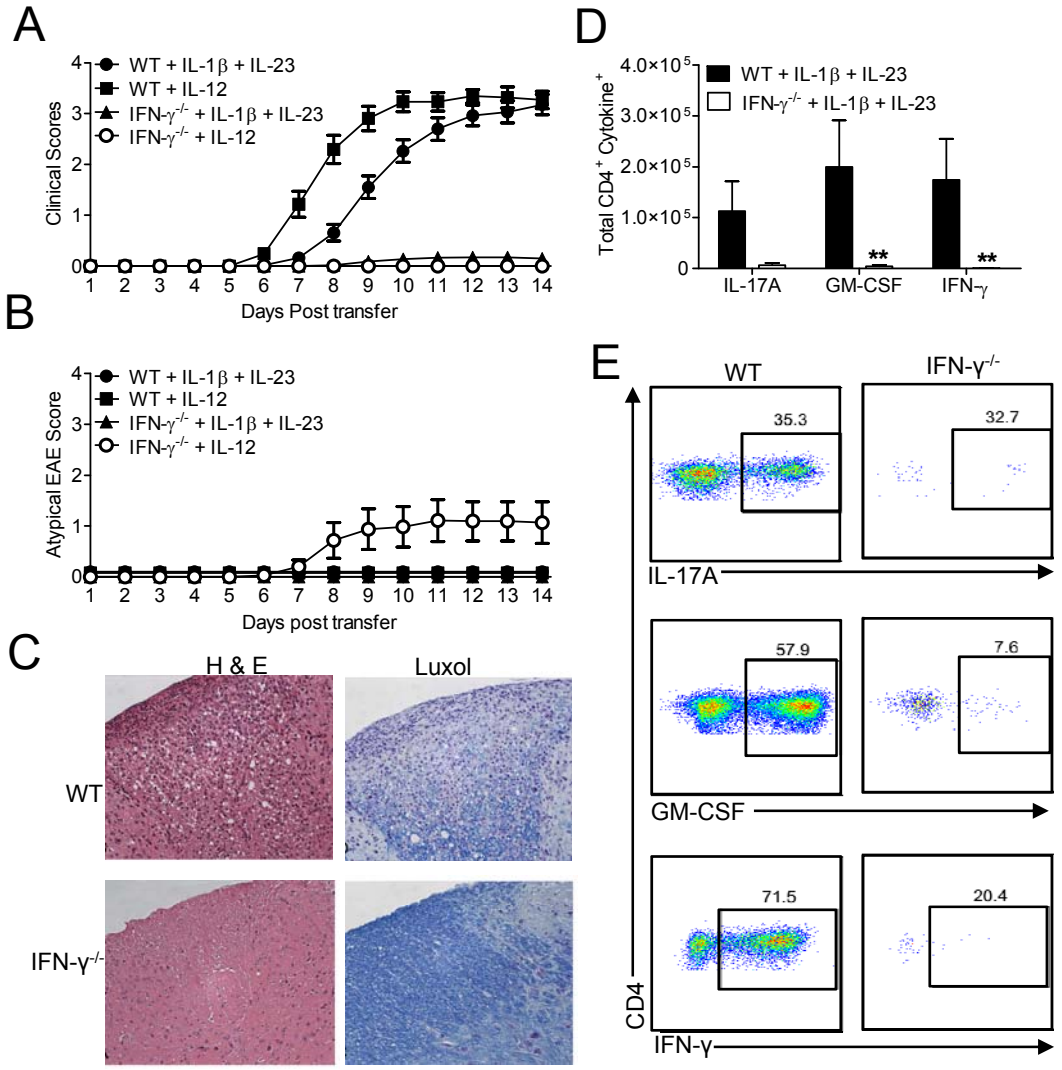
A
WT + IL-1β + IL-23
WT + IL-12
IFN-γ-/- + IL-1β + IL-23
IFN-γ-/- + IL-12
Clinical Scores
Days Post transfer
B
Atypical EAE Score
Days post transfer
C
H & E
Luxol
WT
IFN-γ-/-
D
Total CD4+ Cytokine+
IL-17A
GM-CSF
IFN-γ
**
E
WT
IFN-γ-/-
35.3
32.7
IL-17A
57.9
7.6
GM-CSF
71.5
20.4
CD4
IFN-γ

Figure 5

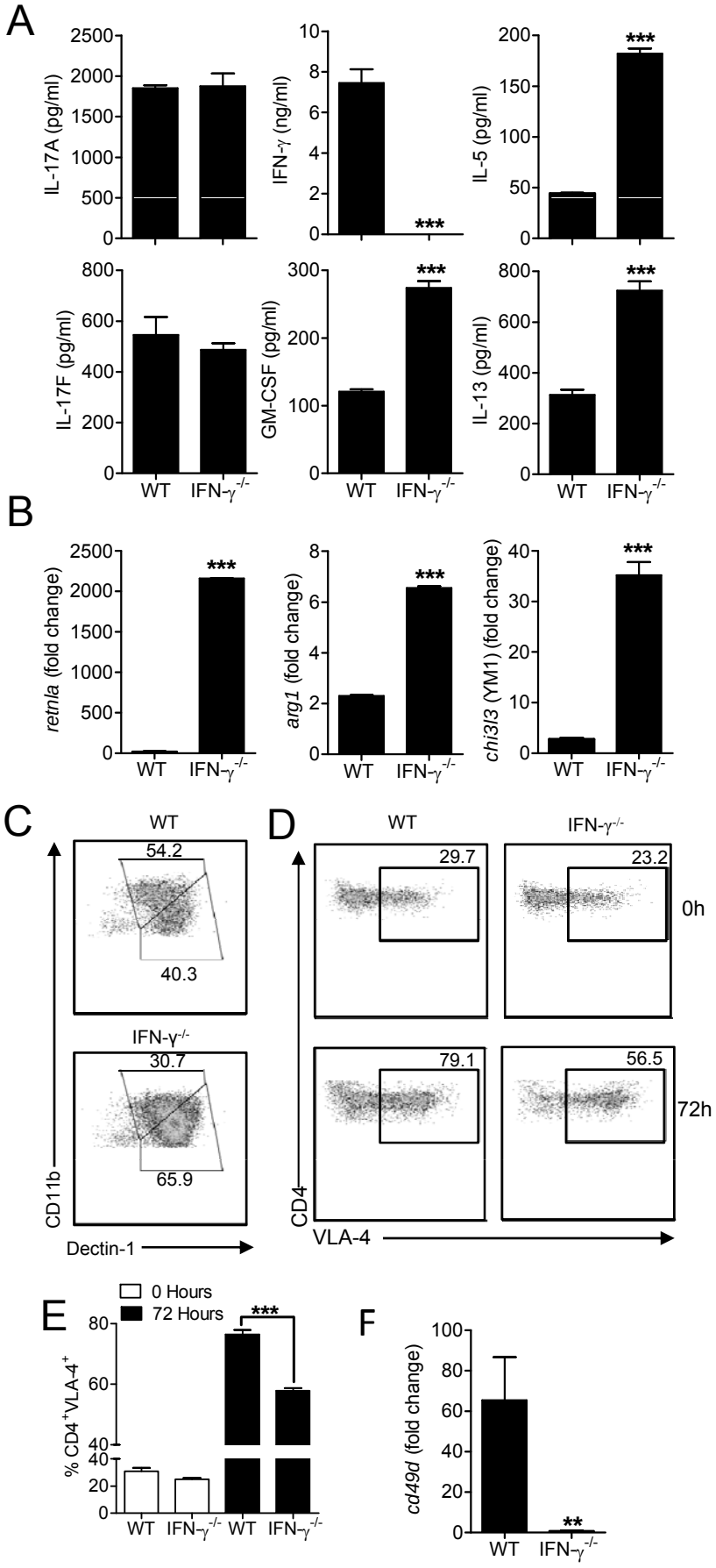
A
IL-17A (pg/ml)
IFN-γ (ng/ml)
IL-5 (pg/ml)
IL-17F (pg/ml)
GM-CSF (pg/ml)
IL-13 (pg/ml)
WT
IFN-γ-/-
B
retnla (fold change)
arg1 (fold change)
chi3l3 (YM1) (fold change)
C
WT
54.2
40.3
IFN-γ-/-
30.7
65.9
CD11b
Dectin-1
D
WT
IFN-γ-/-
29.7
23.2
0h
79.1
56.5
72h
CD4
VLA-4
E
0 Hours
72 Hours
% CD4+VLA-4+
F
cd49d (fold change)

Figure 6

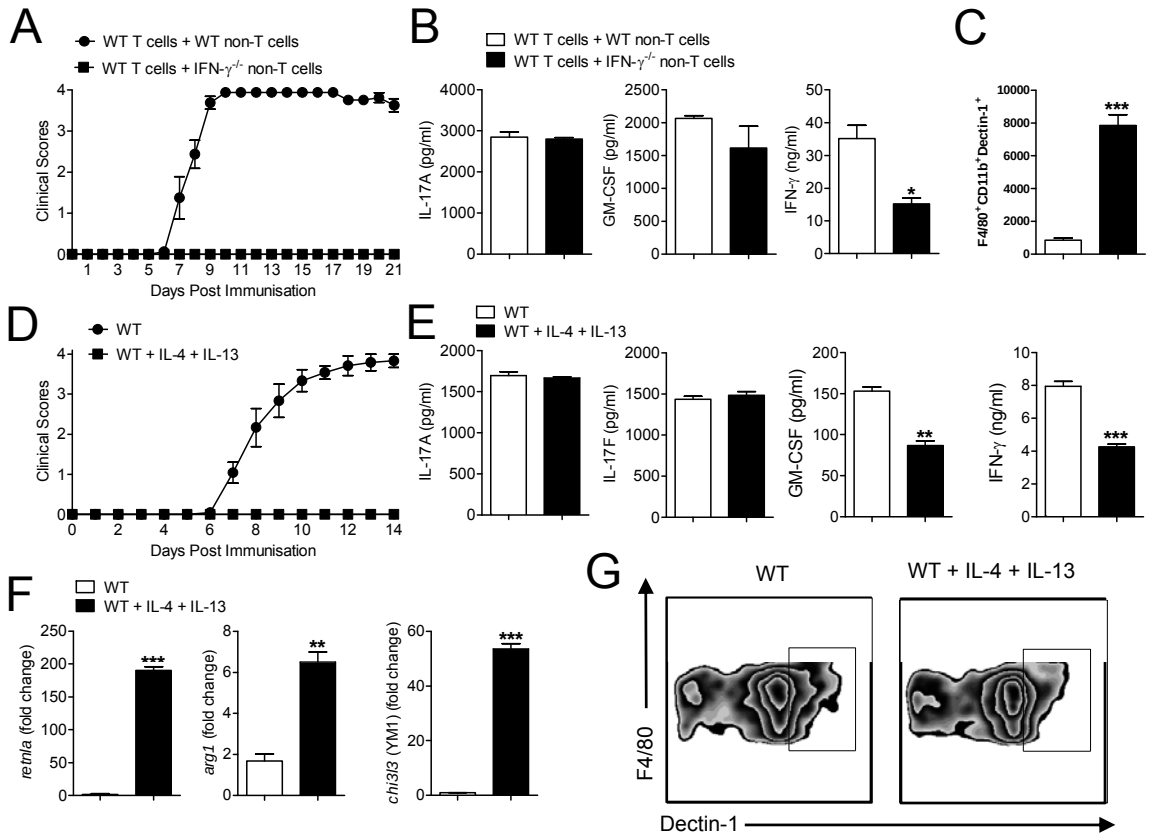
A
WT T cells + WT non-T cells
WT T cells + IFN-γ-/- non-T cells
Clinical Scores
Days Post Immunisation
B
WT T cells + WT non-T cells
WT T cells + IFN-γ-/- non-T cells
IL-17A (pg/ml)
GM-CSF (pg/ml)
IFN-γ (ng/ml)
C
F4/80+CD11b+Dectin-1+
D
WT
WT + IL-4 + IL-13
Clinical Scores
Days Post Immunisation
E
WT
WT + IL-4 + IL-13
IL-17A (pg/ml)
IL-17F (pg/ml)
GM-CSF (pg/ml)
IFN-γ (ng/ml)
F
WT
WT + IL-4 + IL-13
retnla (fold change)
arg1 (fold change)
chi3l3 (YM1) (fold change)
G
WT
WT + IL-4 + IL-13
F4/80
Dectin-1

Figure 7

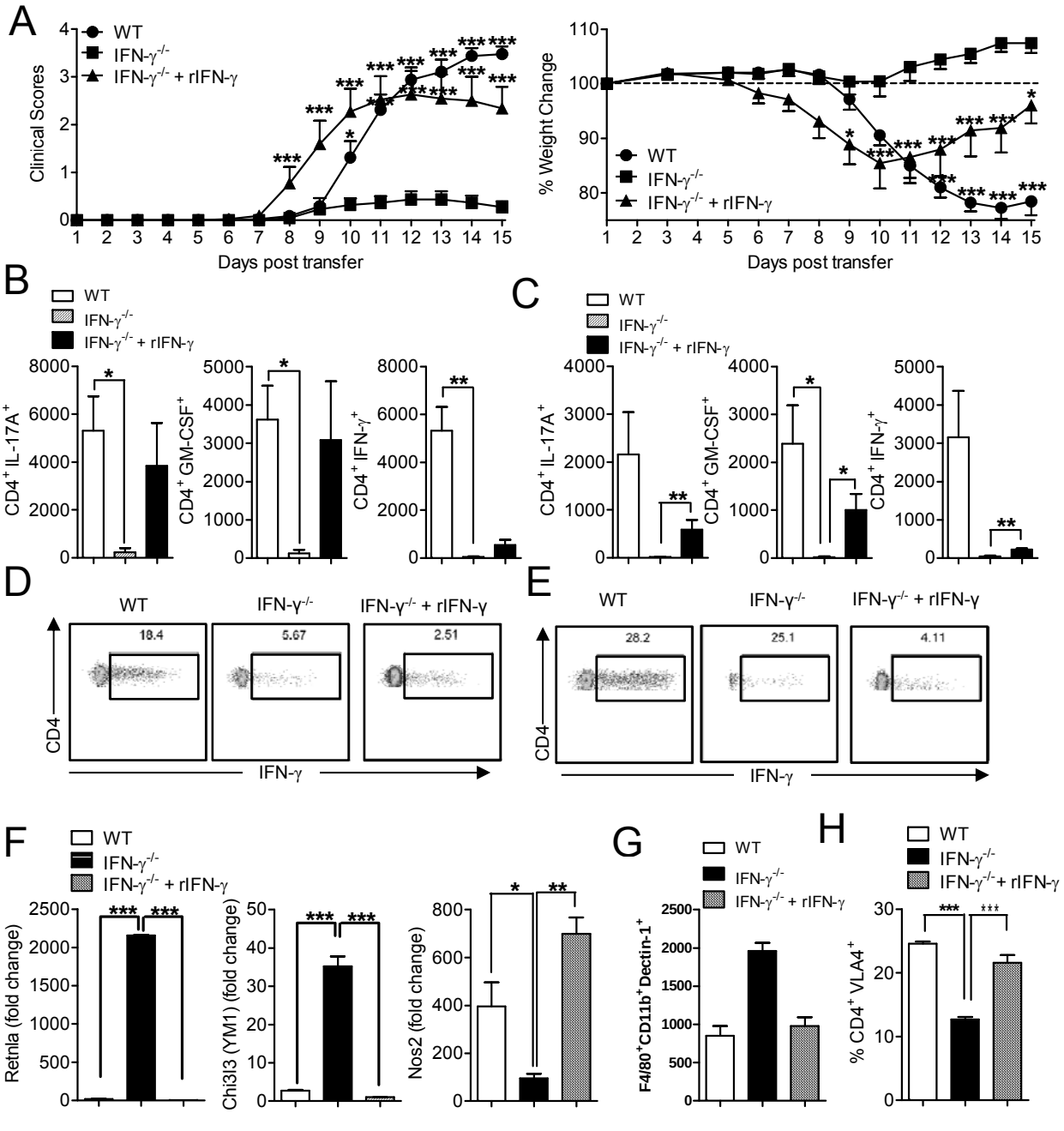
A
WT
IFN-γ-/-
IFN-γ-/- + rIFN-γ
Clinical Scores
Days post transfer
% Weight Change
B
C
CD4+ IL-17A+
CD4+ GM-CSF+
CD4+ IFN-γ+
D
E
18.4
5.67
2.51
28.2
25.1
4.11
CD4
IFN-γ
F
G
H
Retnla (fold change)
Chi3l3 (YM1) (fold change)
Nos2 (fold change)
F4/80+CD11b+Dectin-1+
% CD4+ VLA4+

Figure 8

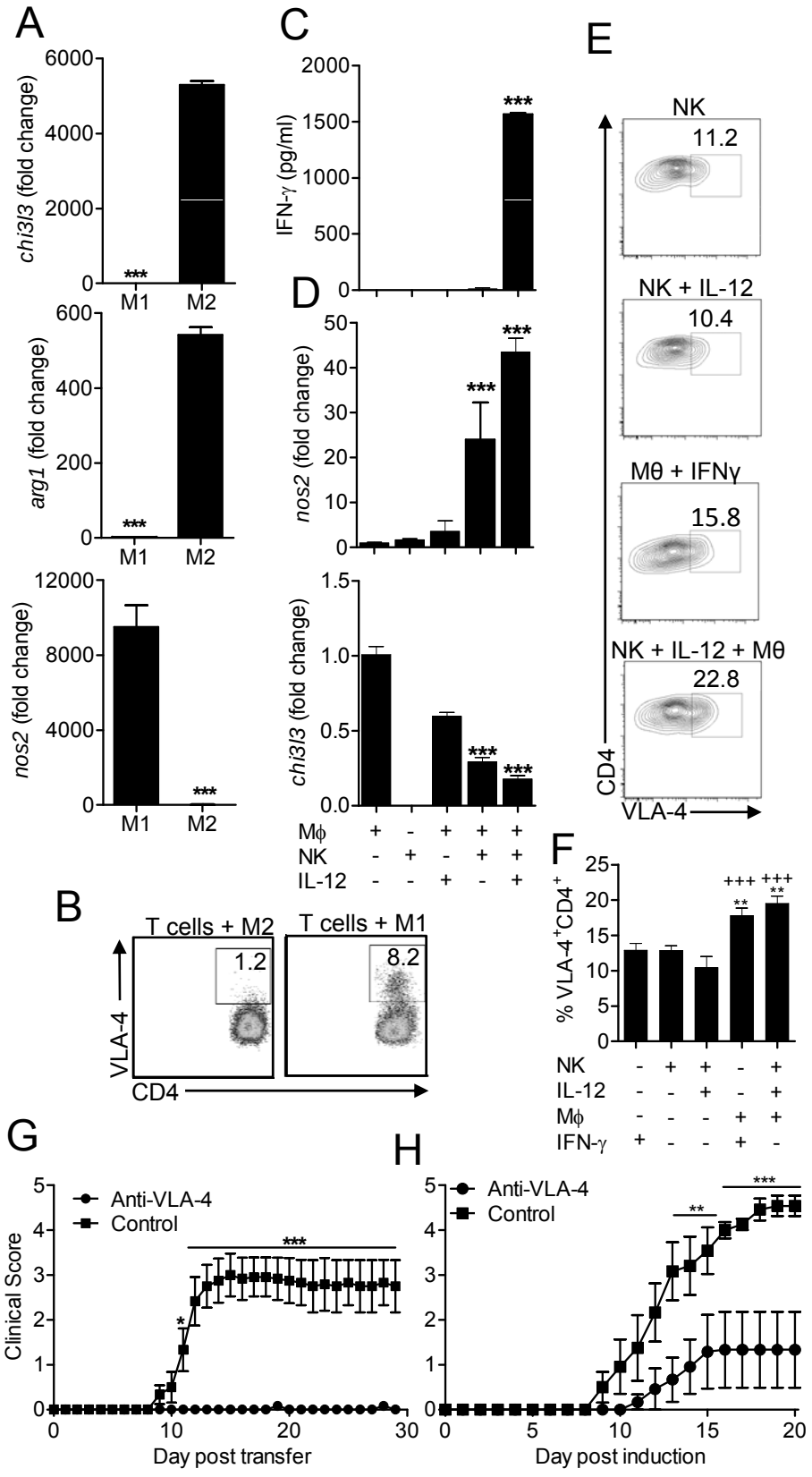
A
chi3l3 (fold change)
arg1 (fold change)
nos2 (fold change)
M1
M2
C
IFN-γ (pg/ml)
D
nos2 (fold change)
chi3l3 (fold change)
Mϕ
NK
IL-12
E
NK
11.2
NK + IL-12
10.4
Mθ + IFNγ
15.8
NK + IL-12 + Mθ
22.8
CD4
VLA-4
B
T cells + M2
T cells + M1
1.2
8.2
VLA-4
CD4
F
% VLA-4+CD4+
NK
IL-12
Mϕ
IFN-γ
G
Anti-VLA-4
Control
Clinical Score
Day post transfer
H
Anti-VLA-4
Control
Day post induction

Figure 9

Supporting Information
for

Innate IFN-γ promotes development of experimental autoimmune encephalomyelitis: a role for NK cells and M1 macrophages

Lara S. Dungan, Niamh C. McGuinness, Louis Boon, Marina A. Lynch and Kingston H. G. Mills

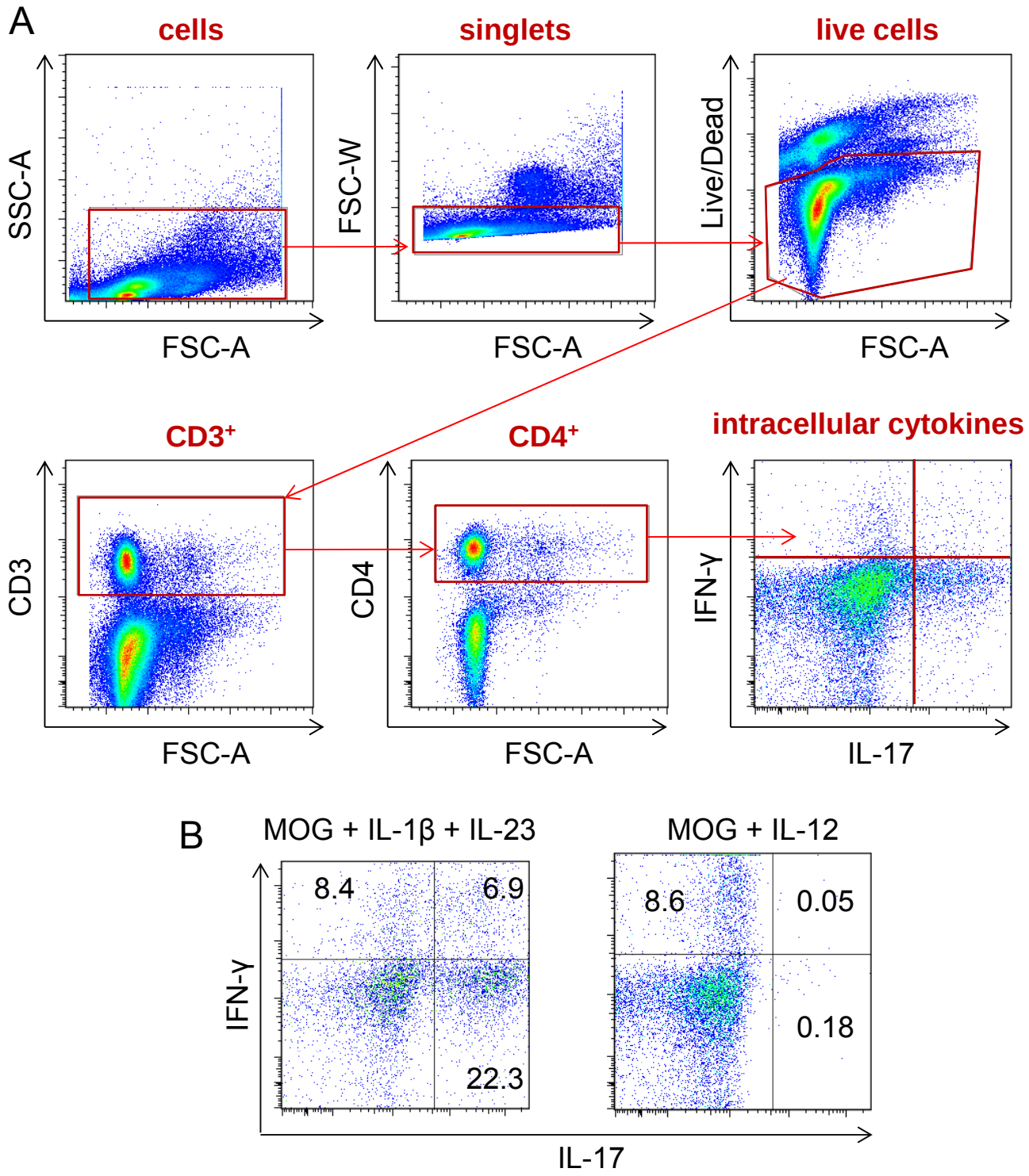

Figure S1. Cyokine production by CD4 T cells used to induce EAE by passive transfer. Mice were immunized with MOG and CFA and sacrificed 10 days later. Lymph node and spleen cells were cultured with MOG (100 µg/ml) and IL-12 or IL-23 + IL-1β (10 ng/ml). After 72 h, cells were washed and stained for surface CD3 and CD4 and for intracellular IL-17 and IFN-γ. A) Gating strategy and B) sample dot plots from intercellular cytokine staining.

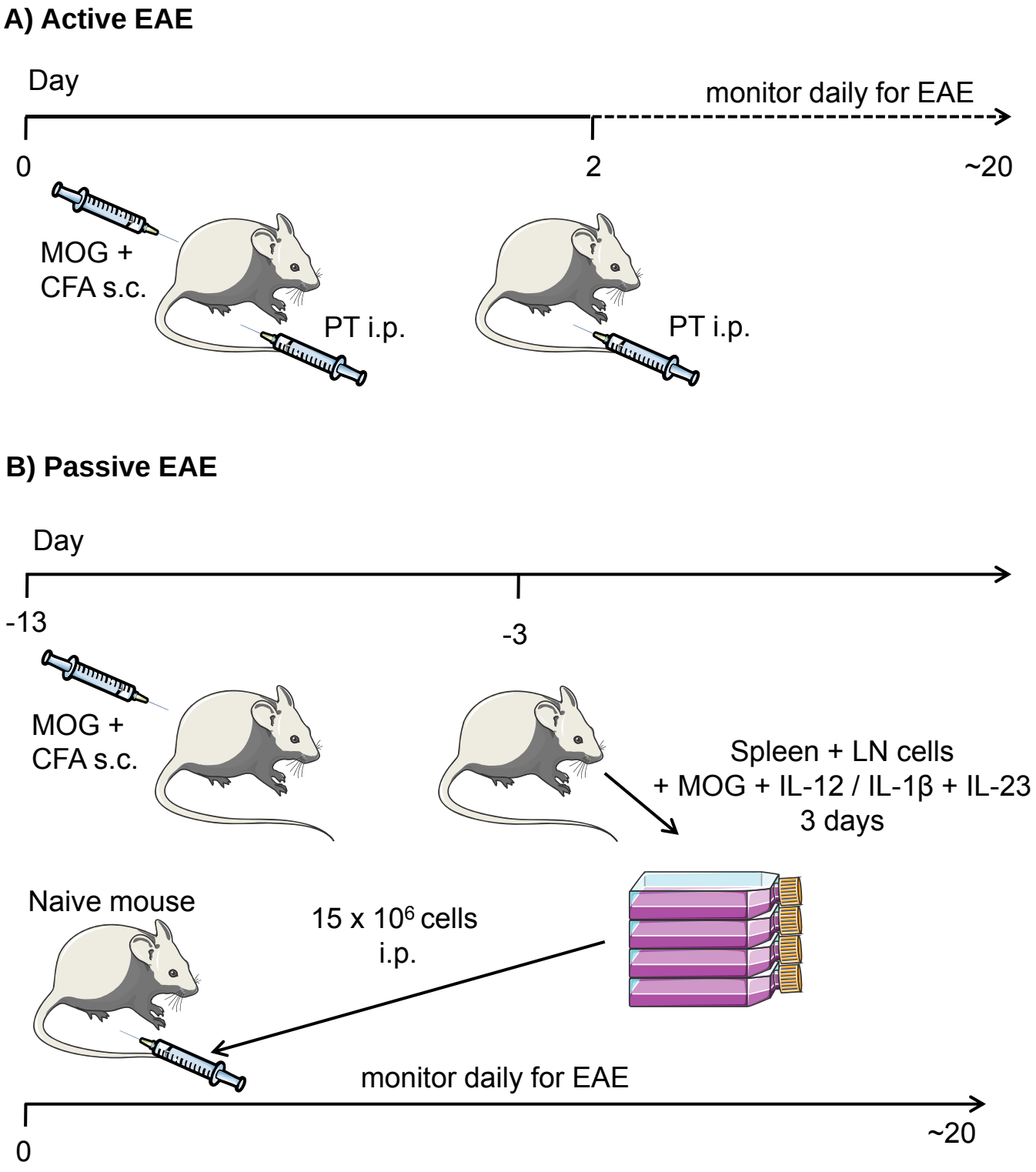

Figure S2. Scheme for induction of EAE by A) active immunization or B) passive cell transfer.