Inhibition of ERK MAP kinase suppresses IL-23 and IL-1 driven IL-17 production and attenuates autoimmune disease[1]

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Running title: ERK mediates IL-23-driven Th17 responses

Keywords: suppression, Th17 cells, autoimmunity, EAE, ERK MAPK

Abstract

IL-17 producing $CD4^+$ T (Th17) cells are pathogenic in many autoimmune diseases. The induction and expansion of Th17 cells is directed by cytokines, including IL-23 and IL-1β, produced by innate immune cells through activation of pathogen recognition receptors (PRRs). The NFκB and interferon regulatory factor (IRF) families of transcriptional factors mediate IL-12 production; however, distinct signalling pathways appear to be required for IL-23 production. Here we show that inhibition of ERK MAP kinase suppressed IL-23 and IL-1β production by dendritic cells (DC) stimulated with TLR or dectin-1 agonists, but did not affect IL-12p70 production. Furthermore, an ERK inhibitor suppressed the ability of antigen-pulsed TLR-activated DC to induce antigen-specific Th17 cells in vivo, but interestingly also inhibited the induction of Th1 cells. Treatment with an ERK inhibitor attenuated experimental autoimmune encephalomyelitis (EAE), when administered either at the induction phase of acute EAE or during remission in the relapsing-remitting EAE model. This was associated with significant suppression of autoantigen-specific Th17 and Th1 responses. The suppressive effect of the ERK inhibitor on attenuation of EAE was reversed by administration of IL-1β and IL-23. Our findings suggest that ERK MAP kinase plays a critical and hitherto un-described role in activating innate production of IL-23 and IL-1β, which promote pathogenic T cell responses, and therefore represents an important target for therapeutic intervention against autoimmune diseases.

Introduction

IL-17-producing T cells (Th17 cells) and IFN-γ-secreting Th1 cells are discrete populations of inflammatory T cells that have distinct and possibly complementary roles in the pathogenesis of autoimmune diseases and in protection against infection (1-4). The differentiation of Th1 cells is mediated by IL-12, while the IL-12 family member, IL-23, especially in synergy with IL-1, plays an essential role in the induction or expansion of murine and human Th17 cells (1, 5-7). IL-6 or IL-21 with TGF-β can also promote the differentiation of Th17 cells from naïve $CD4^+$ T cells (8, 9). IL-1RI and IL-23 defective mice are resistant to the development of experimental autoimmune encephalomyelitis (EAE) (5, 10). Furthermore, anti-IL-23 therapy reduced IL-17 concentrations in serum and in the CNS, and prevented disease relapse in relapsing-remitting EAE (11). Consequently, IL-23 and IL-17 are now considered to be important new targets for treating autoimmune diseases in humans (12). In contrast, the roles of IL-12 and IFN-γ are less clear, with evidence for suppression of Th17 development by IFN-γ (2, 3) and exacerbated autoimmunity in IL-12 or IFN-γ defective mice (10, 13), but also reports that Th1 cells do have pathogenic roles in certain autoimmune diseases (14-16).

IL-12 and IL-23 are produced by cells of the innate immune system, such as dendritic cells (DC), following binding of pathogen associated molecular patterns (PAMPs) or endogenous danger signals to TLR, NOD-like receptor (NLR) or other pathogen-recognition receptors (PRRs), such as dectin-1 (6, 7). However, the induction of IL-12 and IL-23 are differentially regulated by TLR and NLR-activated DC (7). NFκB, interferon regulatory factor (IRF)1 and IRF8 signalling pathways play important roles in the expression of IL-12p35 and IL-12p40 and in driving Th1 responses (17-20). In contrast, it has been demonstrated that IL-6 and IL-23 production in DC by agonists of dectin-1, and consequent development of Th17 cells, is mediated by signalling via Syk and CARD9 (21). However, the signalling molecules involved in promoting the IL-23 production are still unclear. A study on the role of IRF-1

demonstrated that this transcriptional factor was essential for IL-12-driven Th1 responses, but was not required for IL-23-dependent IL-17 production (20).

In this study we examined the role of ERK MAP kinase in TLR-induced IL-23 and IL-1β production by DC and the subsequent expansion of Th17 cells. We found that inhibition of MEK1/2, the kinase upstream of ERK, suppressed IL-23 and IL-1β production by DC stimulated with the TLR4 agonist, LPS, but also with the dectin-1 agonist, curdlan and with heat killed *Mycobacteria tuberculosis*. In contrast, IL-12p70 production was not affected by inhibition of ERK activation. Adoptive transfer experiments with antigen-pulsed and TLR agonist-activated DC revealed that ERK activation played a major role in their ability to promote Th17 responses in vivo. Furthermore, inhibition of ERK suppressed autoantigen-specific Th17 and Th1 responses and attenuated EAE, when administered at the induction of acute EAE or therapeutically in a relapsing-remitting model. These findings provide new evidence that IL-12 and IL-23 production employ distinct signalling pathways in DC and demonstrate that the ERK signalling pathway may be an important target for new therapeutic interventions against T-cell mediated autoimmune diseases.

Materials and Methods

Mice

C57BL/6 and SJL/J mice were purchased from Harlan UK. All mice were maintained according to EU regulations and experiments were performed under licence from the Department of Health and Children and with approval from the Trinity College Dublin Bioresources Ethics Committee.

Dendritic cells

Bone marrow-derived immature dendritic cells (DC) were prepared by culturing bone marrow cells from C57BL/6 mice in medium containing 10 % foetal calf serum supplemented with 20 ng/ml GM-CSF. On day 3 fresh medium containing 20 ng/ml GM-CSF was added. On day 6 semi-adherent cells were removed using 0.02 % EDTA (Sigma Aldrich). Cells were re-cultured in medium supplemented with 20 ng/ml GM-CSF. On day 8 fresh medium containing 20 ng/ml GM-CSF was added. On day 10 loosely adherent cells were collected, washed and used for assays. DC were cultured with LPS (100 ng/ml; Alexis) heat-killed *M. tuberculosis* (MTB; 2-50 μg/ml; Chondrex Inc.) or curdlan (100 μg/ml; Wako) with or without the MEK1/2 inhibitors, U0126 (Calbiochem), PD98059 (Calbiochem) or the Calbiochem MEK1/2 inhibitor (1-10 μM). Supernatants were recovered after 24 h and IL-1β (R&D Systems), IL-23 (eBiosciences), IL-12p70 (R&D Systems) and IL-12p40 (BD Pharmingen) concentrations were determined by ELISA.

Western blotting

Murine bone marrow-derived DC were incubated with medium or LPS (100 ng/ml) alone or in the presence of increasing concentrations of the MEK1/2 inhibitor, U0126. Cell lysates were prepared and resolved on 12% SDS-PAGE gels and transferred to nitrocellulose membrane.

Blots were incubated with anti-phospho (p)-ERK, or anti-ERK (Santa Cruz, Germany) and with peroxidase conjugated secondary antibodies and developed using enhanced chemiluminescence.

Real time RT-PCR

RNA was harvested from the DC using the Trizol (Invitrogen) - chloroform method followed by reverse transcription into cDNA using a QuantiTect Reverse Transcription kit (Qiagen). Real time RT-PCR for the detection of IL-12p35, IL-23p19 and IL-1β mRNA was performed using pre-designed Taqman gene expression assays (Applied Biosystems). 18s ribosomal RNA was used as an endogenous control. Samples were assayed on an Applied Biosystems 7500 Fast Real Time PCR machine.

DC transfer

DC were stimulated for 24 h with medium only, KLH (20 μg/ml) or KLH and LPS (100 ng/ml) with or without U0126 (1 μM). Cells were washed and transferred (5 x 10^5) s.c. to naïve mice. After 7 d lymph node cells (1 X 10^6 / ml) were re-stimulated in vitro with KLH (2-50 μg/ml) or medium only. Supernatants were recovered after 72 h and IL-17, IFN-γ, IL-10 and IL-13 concentrations quantified by ELISA.

Induction and assessment of EAE and treatment with a MEK1/2 inhibitor

C57BL/6 mice were immunised s.c. with 150 μg of MOG peptide 35-55 (GenScript) emulsified in CFA (Chondrex Inc,) supplemented with 4 mg/ml heat killed MTB (Chondrex Inc). Alternatively, SJL/J mice were immunised s.c. with 150 μg of PLP peptide 139-151 (GenScript) emulsified in CFA, supplemented with 4 mg/ml heat killed MTB. All mice were injected i.p. with 500 ng pertussis toxin (PT; Kaketsuken) on days 0 and 2. In the MOG-

induced EAE model, the MEK1/2 inhibitor U0126 (50 μg/mouse) was administered s.c. to C57BL/6 mice with the MOG and CFA on day 0 and again on days 1 and 2 or on days 5, 7, 9, 11, 13, 15 and 17. In the PLP-induced relapsing remitting EAE model, the MEK1/2 inhibitor U0126 (50 μg/mouse) was administered s.c. to SJL/J mice every 2 d from day 18 post immunization. EAE was also induced by adoptive transfer of MOG-specific T cells. Lymph node and spleen cells from C57BL/6 mice with EAE were cultured in vitro for 3 d with MOG, MOG and IL-1β + IL-23 or IL-1β + IL-23 in the presence of a neutralizing anti-IFN-γ antibody or with MOG and IL-12. Cells were washed and transferred i.v. to naïve C57BL/6 mice (5 x 10^6 per mouse). In certain experiments mice were treated with U0126 (50 μg/mouse) by s.c. administration at the time of T cell transfer and again 1 and 2 days later. Alternatively DC were stimulated with MOG (50 μg/ml) and heat killed MTB (10 μg/ml), with or without U0126 (5 μM). Cells were washed and transferred s.c. to naïve C57BL/6 mice (1 x 10^6 cells per mouse). Disease severity was recorded as follows: grade 0, normal; grade 1, limp tail; grade 2, wobbly gait; grade 3, hind limb weakness; grade 4, hind limb paralysis; grade 5, tetraparalysis/death.

Antigen-specific proliferation and IL-17and IFN-γ production

Lymph node cells (1 x 10^6 cells/ml) were stimulated with MOG or PLP peptides (1-50 μg/ml). After 3 d of culture, IL-17 (R&D Systems) and IFN-γ (BD Pharmingen) concentrations in the supernatants were determined by ELISA. Proliferation was determined on day 4 by ^{3}H-thymidine incorporation as described (5).

Intracellular cytokine staining

Spleen or lymph node cells (2 x 10^6 cells/ml) were stimulated for 4 h with PMA (10 ng/ml) and ionomycin (1 μg/ml) with brefeldin A (5 ng/ml) for the last 2 h. Cells were washed,

blocked with Fcγ block (BD Pharmingen 1 μg/ml) before extracellular staining for surface CD3 or CD4 (BD Pharmingen). Cells were then fixed and permeabilized (Fix and Perm cell permeabilization kit, Caltag) and stained for intracellular IL-17 or IFN-γ. Flow cytometric analysis was performed using a Cyan ADP Flow Cytometer (Dako Cytomation).

Statistical analysis

Data were compared by unpaired *t*-test or by one- or two-way ANOVA. Where significant differences were found, the Tukey-Kramer multiple comparisons test was used to identify differences between individual groups.

Results

ERK MAP kinase is required for IL-23, but not IL-12, production by DC

Previous studies had suggested that distinct signalling pathways were required for production of IL-12 and IL-23 and the development of Th1 and Th17 cells (18). Here we examined the role of ERK MAP kinase in IL-12 and IL-23 production. Since we and others have shown that IL-1 is required for the induction or expansion of Th17 cells, we also determined the role of ERK in IL-1 production. We first demonstrated that DC expressed endogenous phosphorylated ERK, which was enhanced following stimulation with LPS and suppressed by culture with the MEK1/2 inhibitor, U0126 (Fig. 1A). We next examined the effect of inhibiting ERK on IL-12, IL-23 and IL-1β production and found that U0126 inhibited IL-23 and IL-1β, but not IL-12p70 production by LPS-activated DC (Fig. 1B). Furthermore, LPS-induced IL-23p19 and IL-1β, but not IL-12p35, mRNA expression in DC was suppressed by the ERK inhibitor (Fig. 1C).

The suppressive effect of the MEK1/2 inhibitor on IL-23 and IL-1β production was not confined to TLR4 activation, as U0126 also suppressed IL-23 and IL-1β production by DC in response to the dectin-1 agonist, curdlan (Fig. 2). Furthermore, suppression of LPS- or curdlan-induced IL-23 and IL-1β was observed with 3 distinct inhibitors of MEK1/2, UO126, PD98059 and a commercially available (Calbiochem) MEK1/2 inhibitor over a range of inhibitor concentrations (Fig 2).

Since *M. tuberculosis* includes a range of PAMPs and is used as the adjuvant to induce many experimental autoimmune diseases in mice, including EAE, we examined the effect of inhibiting ERK signalling on cytokine production by DC stimulated with heat killed *M. tuberculosis*. Incubation of DC with U0126 suppressed IL-1β and IL-23 production but had little effect on IL-12p40 production in response to heat-killed *M. tuberculosis* (Fig. 3). These

findings demonstrate that distinct signalling pathways in DC mediate IL-23 and bioactive IL-12 production.

Inhibition of ERK in DC suppresses their ability to induce Th17 responses in vivo

Since IL-1 and IL-23 production by innate immune cells promotes IL-17 production by T cells, we examined the effect of inhibiting ERK on the ability of TLR-activated DC to drive Th17 responses. We also examined the effect on Th1 and Th2 responses. DC were pulsed in vitro with the antigen, KLH, alone or with LPS, in the presence or absence of U0126 and transferred s.c. to naïve mice. Assessment of KLH-specific cytokine production by lymph node cells ex vivo 7 d after transfer, revealed that antigen-specific IL-17 and IFN-γ production was significantly suppressed in mice injected with DC that had been pulsed with antigen and LPS in the presence of the MEK1/2 inhibitor (Fig. 4A). In contrast, IL-10 and IL-13 production, induced by transfer of DC pulsed with KLH was not suppressed by U0126 (Fig. 4B). In order to provide further evidence that the inhibitory effect on Th17 responses did not result from a non-specific effect on DC function or viability, we also examined the effect of the ERK inhibitor on LPS-induced DC maturation and viability. Incubation of DC with LPS for 24 h significantly enhanced MHC Class II and CD40 expression. Co-incubation with the ERK inhibitor did not affect class II or CD40 expression or reduce cell viability, as determined by PI staining (Fig. 4C). Our findings suggest that ERK activation in DC plays a major role in IL-23 and IL-1β production and in promoting the induction of Th17 and Th1 cells in vivo.

Treatment with an ERK inhibitor suppresses autoantigen-specific Th17 and Th1 cells in vivo and attenuates EAE

It has been reported that IL-23, IL-1 and Th17 cells have a pathogenic role in many experimental autoimmune diseases including EAE (1, 5, 10), collagen-induced arthritis (CIA) (22) and experimental autoimmune uveitis (EAU) (14). Furthermore, inhibition of ERK can attenuate arthritis in mice, although the mechanism of suppression was not clear and its effect on Th17 responses were not examined (23). Here we examined if blocking ERK activation might attenuate the symptoms of EAE by suppressing the induction or expansion of autoantigen-specific T cells. We found that the incidence and severity of EAE were significantly reduced in mice treated with U0126 on days 0, 1 and 2 during the induction phase of EAE (Fig. 5A). Furthermore, myelin oligodendrocyte glycoprotein (MOG)-specific IL-17 and IFN-γ production was significantly reduced in the lymph nodes of mice treated with the ERK inhibitor (Fig. 5B). In contrast, IL-17 and IFN-γ production induced by polyclonal activation with anti-CD3 and PMA was unaffected. MOG-induced proliferation of lymph node cells was also suppressed in mice treated with the ERK inhibitor, though this was only significant at the higher concentration of MOG employed for in vitro re-stimulation (Fig. 5C). Proliferation induced by anti-CD3 and PMA was unaffected, providing further evidence that the suppressive effect of the ERK inhibitor on IL-17 and IFN-γ production is not due to some non-specific effect on T cells. Intracellular cytokine staining on lymph node cells demonstrated that the frequency of $CD3^+$ and $CD4^+$ T cells secreting IL-17 or IFN-γ was suppressed by treatment with the ERK inhibitor (Fig. 5D), suggesting that the ERK inhibitor suppressed the induction or expansion of Th1 and Th17 cells in vivo (Fig. 5D). The percentage of $CD4^+$ T cells secreting IL-17 was consistently lower than that observed for $CD3^+$ T cells. This can be accounted for by a population of IL-17-secreting γδ T cells, which are also suppressed by the ERK inhibitor (data not shown). We have found that IL-1 and IL-

23, as well as promoting IL-17 production by memory Th17 cells also promote IL-17 by γδ T cells. Furthermore, γδ T cells and $CD4^+$ T cells both contribute to IL-17 production in mice with EAE. (Sutton et al, manuscript in review).

Administration of the ERK inhibitor on days 5-18 after the induction of EAE had a less significant effect on clinical scores (Fig. 5E), suggesting that once autoantigen-specific T cells are induced, inhibition of IL-1 and IL-23 production was not as effective at modulating the course of disease in this model.

We next determined the efficacy of ERK inhibition in proteolipid protein (PLP)-induced relapsing-remitting EAE in SJL/J mice, which more closely resembles the course of disease in multiple sclerosis patients. Administration of U0126 after the acute phase of disease resulted in significant attenuation of clinical symptoms compared with mice treated with vehicle alone (Fig. 6A). This was associated with significant suppression of PLP-specific IL-17 production (Fig. 6B). These findings demonstrate that inhibition of ERK attenuates the development of acute EAE when administered during the induction phase and also prevents relapse in the relapsing-remitting model of EAE. Furthermore, the attenuation is associated with significant suppression of autoantigen-specific IL-17 production.

MOG-specific T cells expanded with antigen in the presence of IL-1 and IL-23 induce EAE following adoptive transfer in vivo

We have previously shown that IL-1 and IL-23 promote IL-17 production by $CD3^+$ T cells from naïve mice (5). The findings of the present study demonstrate that ERK plays an important role in the production of IL-23 and IL-1β, but not IL-12. Surprisingly, inhibition of ERK suppressed both IL-17 and IFN-γ production in vivo. Therefore, we examined the influence of IL-1β and IL-23 on activation of MOG-specific Th1 and Th17 cells in vitro. Mice were immunized with MOG and CFA and sacrificed at day 10. Lymph node cells were re-

stimulated in vitro with MOG alone or in combination with IL-23, IL-1β or IL-1 plus IL-23. The results showed that IL-23 significantly enhanced MOG-induced IL-17 production and that this was further augmented by addition of IL-1β (Fig 7A). In contrast, IFN-γ production was enhanced by culture with MOG in the presence of IL-1β, but was this was not further augmented by addition of IL-23 (Fig. 7A).

We next examined the ability of MOG-specific T cells activated in vitro with MOG, IL-1β and IL-23 to transfer EAE to naive mice. Lymph node and spleen cells from mice with EAE cultured in vitro with MOG alone, which produced IFN-γ but low levels of IL-17 (Fig 7B), did not induce EAE following adoptive transfer to naïve mice (Fig 7C). In contrast, transfer of MOG-specific T cells cultured *in vitro* in the presence of IL-1 and IL-23, which expanded a mixed population of Th1 and Th17 cells (Fig. 7B), induced EAE in naïve mice (Fig. 7C). Transfer of MOG-specific T cells cultured with MOG, IL-1 and IL-23 in the presence of anti-IFN-γ, which were predominantly IL-17-secreting T cells (Fig. 7B), also induced EAE, but with a milder clinical course than that observed with the mixed population of Th1 and Th17 cells (Fig. 7C). Furthermore transfer of MOG-specific Th1 cells, expanded by culture with MOG in the presence of IL-12 (Fig 7B), also induced EAE, but with milder and more transient symptoms compared with mice that received a combination of Th1 and Th17 cells (Fig. 7C). Taken together, these findings provide further evidence of a role for IL-1 and IL-23 in the activation of antigen-specific memory Th17, but also suggest that Th1 and Th17 cells may co-operate in the development of disease in the MOG-induced EAE model.

We next examined the effect of the ERK inhibitor on EAE induced by transfer of MOG-specific T cells or by DC pulsed in vitro with MOG and MTB. Consistent with the data in Fig 7C, transfer of lymph node and spleen cells stimulated with MOG only failed to induce EAE, whereas T cells cultured with MOG and IL-1β and IL-23 induced symptoms of EAE (Fig 7D). Treatment with the ERK inhibitor at the time of T cell transfer resulted in a modest,

but statistically non-significant, attenuation of EAE (Fig 7D). In contrast, transfer of DC pulsed in vitro with MOG and MTB induced mild EAE, which was completely suppressed when the cells were cultured with MOG and MTB in the presence of the ERK inhibitor (Fig. 7E). These findings suggest that in our system, a major effect of the ERK inhibitor is to suppress MTB-induced IL-1 and IL-23 production by DC, which promote induction of IL-17-producing T cells, but that the ERK inhibitor may also suppress endogenous IL-1 and IL-23 production in vivo, required for expansion or survival of Th17 cells.

The attenuating effect of the ERK inhibitor on Th17 and Th1 responses and EAE is mediated via suppression of IL-1 and IL-23 production

In order to confirm that the suppressive effect of the ERK inhibitor on Th17 responses and on the clinical course of EAE was mediated by inhibition of IL-23 and IL-1β, we added back these cytokines to see if we could reverse the suppression. EAE was induced in C57BL/6 mice by immunization with MOG and CFA and the ERK inhibitor was administered on day 0, 1 and 2 with or without IL-23 or IL-1β and IL-23. Assessment of IL-17 production in the supernatants of MOG-stimulated lymph node cells by ELISA demonstrated that treatment with the ERK inhibitor during immunization with MOG and CFA completely suppressed the induction of IL-17-producing T cells (Fig. 8A). This was partially reversed by co-administration of IL-23 and completely reversed by co-administration of IL-1β and IL-23. MOG-specific IL-17 production was also enhanced, but not significantly when mice were immunized with MOG and CFA in the presence of IL-1β and IL-23, without the ERK inhibitor (Fig. 8A). Intracellular cytokine staining on lymph node cells from mice immunized with MOG and CFA revealed that the frequency of IL-17 producing by $CD3^+$ T cells was reduced by administration of the ERK inhibitor, and this was reversed by co-administration of IL-23 (Fig. 8B). Assessment of IL-17 production by $CD4^+$ T cells confirmed that treatment

with UO126 suppressed the development of Th17 cells, which was reversed by administration of IL-23 in vivo (Fig. 8B).

We also examined the effect of IL-1β and IL-23 on the suppressive effect of the ERK inhibitor on the course of disease in the EAE model. Consistent with our earlier data, treatment with the ERK inhibitor significantly reduced the symptoms of EAE (Fig. 8C). The attenuating effect of the ERK inhibitor on the development of EAE was reversed by co-administration of IL-1β and IL-23 (Fig. 8C). Treatment with IL-1β and IL-23 in the absence of the ERK inhibitor did not alter the course of disease. These findings suggest that the ERK inhibitor attenuates the clinical signs of EAE by suppressing IL-1β and IL-23 production.

Discussion

It has previously been reported that MyD88-mediated activation of NFκB, IRF-1 and IRF-8 are required for IL-12p35 and IL-12p40 gene expression and driving Th1 responses (17-20). However, these studies failed to find a role for these transcriptional factors in IL-23-driven Th17 responses and suggested that alternative signalling molecules may be involved (18, 20). Our study demonstrates that ERK MAPK is an essential component of an alternative pathway required for IL-23-dependent IL-17 production.

The findings demonstrate the TLR and dectin-1-mediated activation of IL-23 and IL-1β production by DC is mediated through activation of ERK and that this plays a critical role in promoting Th17 responses in vivo. While IL-6 and TGF-β have a clearly established role in the differentiation of naïve T cells into Th17 cells (8, 9, 24), and IL-21 is involved in an autocrine amplification loop (25-27), the present study supports previous reports that IL-23 and IL-1 play a major role in the induction and expansion of Th17 cells. Studies in EAE and EAU models have demonstrated that IL-23p19$^{-/-}$ mice produce less IL-17 and are resistant to induction of autoimmunity (10, 14). Furthermore, treatment with anti-IL-23 can suppress IL-17 production and attenuate clinical signs of autoimmunity in EAE and EAU models (11, 14). In the EAE model, adoptive transfer of T cells from WT into IL-23p19$^{-/-}$ mice or IL-1RI$^{-/-}$ mice, demonstrated that IL-23 and IL-1 play a critical role in the development of Th17 cells (5, 28).

This study has shown that inhibition of MEK1/2 suppresses the clinical signs of disease in two EAE models by suppressing autoantigen-specific T cell responses. It has previously been reported that ERK1$^{-/-}$ mice have increased susceptibility to EAE (29). While these findings appear to be at variance with our study, they are not directly comparable, as the MEK1/2 inhibitors used in our study block ERK1 and ERK2, whereas the knock out mice are only defective in ERK1. Furthermore, the ERK inhibitor was only administered during or after

the induction of EAE, whereas the knockout mice lack ERK1 through the course of their development. Interestingly, we previously reported that inhibition of ERK enhanced IL-17 production by T cells in vitro (5), whereas the present study shows that inhibition of ERK suppresses IL-23 and IL-1β production by DC. Furthermore, *in vitro* treatment of DC with an ERK inhibitor prior to pulsing with antigen and a TLR agonist suppressed their ability to promote the induction of antigen-specific IL-17 production *in vivo*. Moreover, addition of an ERK inhibitor during in vitro pulsing of DC with MOG and MTB, suppressed their ability to induce clinical signs of EAE in naïve mice. Treatment with the ERK inhibitor also suppressed, though not significantly, the induction of EAE by transfer of MOG-specific Th1 and Th17 cells, but not to as great an extent. These findings suggest that the dominant effect of the ERK inhibitor in vivo is suppression rather than enhancement of Th17 responses and this reflects its ability to inhibit innate IL-1 and IL-23, which promote the development and expansion of Th17 cells. We have recently demonstrated that IL-1 and IL-23, as well as activating memory Th17 cells also promotes IL-17 production by γδ cells and that both populations are a source of IL-17 in mice with EAE (Sutton et al, manuscript in review). Here we found that attenuation of EAE by treatment with the ERK inhibitor was associated with suppression of IL-17 production by γδ T cell as well as $CD4^+$ T cells. Therefore the effect of the ERK inhibitor suppresses innate cytokines that stimulate unconventional as well as conventional T cells.

Our data clearly demonstrate that treatment with an ERK inhibitor reduces the severity of autoimmune disease in two EAE models and this is consistent with its protective efficacy against CIA (23). The ERK inhibitor prevented relapse when administered during remission in the relapsing-remitting model, but interestingly only had a significant protective effect in the acute MOG-induced model when administered during induction of EAE, but not after development of disease. This correlated with suppression of the induction of IL-17-

producing T cells. It has previously been shown that anti-IL-23 therapy reduced IL-17 concentrations in serum and in the CNS, and prevented disease relapse in relapsing-remitting EAE (11). Furthermore, treatment with anti-IL-23 reduced IL-17 production and attenuated EAU, when administered immediately before and after induction of disease, but not at the effector stage of the disease (14).

Surprisingly, inhibition of ERK suppressed IFN-γ as well as IL-17 production. This effect was independent of IL-12, as the ERK inhibitors did not suppress TLR-induced IL-12 production by DC, but inhibited IL-23 and IL-1β production. Culture of MOG-specific T cells with antigen in the presence of recombinant IL-23 and IL-1β enhanced IFN-γ and IL-17 production. Furthermore, adoptive transfer of a mixed population of MOG-specific Th1 and Th17 cells cultured in vitro in the presence of IL-1 and IL-23 induced EAE. In contrast, transfer of polarized Th1 cells, expanded by culture with MOG and IL-12, or Th17 cells, expanded by culture with IL-1β and IL-23 in the presence of neutralizing IFN-γ, induced only transient symptoms of EAE, which were significantly milder than those induced by the combination of Th1 and Th17 cells. These findings are consistent with a pathogenic role for Th1 as well as Th17 cells in EAE (15, 16) and uveitis (14) and suggest that a combination of the two populations may be optimum for induction of EAE, with each having different functions in the disease process. Indeed it has been demonstrated IL-23-driven T cells promote neutrophil infiltration into white matter lesions, whereas macrophages were more prominent in the inflammatory CNS infiltrate driven by Th1 cells (15). Our findings suggest that blocking IL-1 and IL-23 by targeting the ERK signalling pathway in innate immune cells is an effective means of suppressing the induction or expansion of Th1, Th17 and IL-17-secreting γδ T cells and may be a promising approach in the design of new immunotherapeutic drugs against autoimmune and chronic inflammatory diseases.

Footnotes

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[3]Abbreviations used in this paper: Th17 cell, IL-17 producing T cell; PRR, pathogen recognition receptor; IRF, interferon regulatory factor; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; PAMP, pathogen associated molecular pattern; TLR, Toll-like receptor; NLR, NOD-like receptor; CIA, collagen induce arthritis; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein.

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Figure legends

FIGURE 1. Inhibition of MEK1/2 signalling in DC suppresses TLR4-induced IL-1β and IL-23 but not IL-12 production by DC. *A,* Murine bone marrow-derived DC were incubated with medium or LPS (100 ng/ml) alone or in the presence of increasing concentrations of the MEK1/2 inhibitor, U0126. Cell lysates were prepared and resolved on SDS-PAGE gels and transferred to nitrocellulose membrane. Blots were probed with anti-pERK, or anti-ERK. *B and C,* DC were stimulated with LPS (100 ng/ml) with or without the MEK1/2 inhibitor, U0126 (1 or 5 μM). Supernatants were collected after 24 h and IL-12p70, IL-23 and IL-1β concentrations quantified by ELISA (B). IL-12p35, IL-23p19 and IL-1β mRNA expression was evaluated by real-time RT-PCR normalized to 18S rRNA (C). Data are shown as fold induction over DC cultured in medium only. Results are means and SEM of triplicate samples from duplicate cultures and are representative of 4 experiments. *** $P < 0.001$, LPS + U0126 versus LPS.

FIGURE 2. Three different ERK inhibitors suppress IL-23 and IL-1β production by DC in response to LPS or curdlan. Mouse bone marrow-derived DC were stimulated with LPS (100 ng/ml) or curdlan (100 μg/ml) in the presence of increasing concentrations of the MEK1/2 inhibitors, U0126, PD98059 or the Calbiochem MEK1/2 inhibitor. Supernatants were recovered after 24 h and IL-23 and IL-1β concentrations quantified by ELISA. Supernatants were recovered after 24 h and IL-23 and IL-1β concentrations quantified by ELISA. Results are means and SEM of triplicate cultures and are representative of 3 experiments.

FIGURE 3. Inhibition of MEK1/2 suppresses IL-23 and IL-1β production by DC in response to *M. tuberculosis.* DC were stimulated with heat-killed *M. tuberculosis* (MTB; 2, 10 or 50 μg/ml), LPS (100 ng/ml) or with medium in the presence or absence of the MEK1/2 inhibitor, U0126 (5 μM). Supernatants were recovered after 24 h and IL-12p40, IL-23 and IL-1β concentrations quantified by ELISA. Results are means and SEM for triplicate cultures and are representative of 2 experiments. ** $P < 0.01$, *** $P < 0.001$ with versus without U0126, by one way ANOVA.

FIGURE 4. Inhibition of ERK in antigen-pulsed DC suppresses their ability to induce Th17 and Th1 cells in vivo, but does not affect their maturation or ability to induce Th2 responses. *A,* Murine DC were stimulated for 24 h with medium only (Med), KLH and LPS (100 ng/ml) with or without U0126 (1 μM). *B,* Murine DC were stimulated for 24 h with medium only (Med), KLH (20 μg/ml) with or without U0126 (1 μM). Cells were washed and transferred s.c. to naïve mice. After 7 d draining lymph node cells were removed and re-stimulated in vitro with KLH (2-50 μg/ml) or medium only. Supernatants were removed after 72 h and IL-17, IFN-γ, IL-10 and IL-13 concentration quantified by ELISA. ** $P < 0.01$, KLH + LPS + U0126 versus KLH + LPS by ANOVA. Results are means and SEM of triplicate cultures and are representative of 2 experiments. *C,* Murine DC were stimulated for 24 h with medium only (grey histrogram), 100 ng/ml LPS (black line) or LPS and 1 μM U0126 (dotted line) and cells were labelled with anti-CD40, anti-MHC class II or PI and FACS analysis performed.

FIGURE 5. Treatment with an ERK inhibitor suppresses autoantigen-specific Th17 cells and attenuates EAE. *A-C,* EAE was induced in C57BL/6 mice by immunization with MOG in CFA. Mice were treated s.c. with the MEK1/2 inhibitor U0126 (50 μg/mouse) or vehicle (PBS with DMSO) as a control as part of the MOG and CFA emulsion and again on days 1 and 2.

A, Mice were observed for clinical signs of EAE. Data are mean score ± SEM for 8 mice per group and are representative of two independent experiments. Numbers in parentheses represent disease incidence. * $P < 0.05$; ** $P < 0.01$, EAE + U0126 versus untreated EAE on individual days, by unpaired *t*-test. *** $P < 0.001$, with versus without UO126, curves compared by two-way repeated measures ANOVA. *B-C,* Mice were sacrificed on day 22 and lymph node cells were stimulated with medium, MOG or PMA and anti-CD3. After 3 days of culture the concentrations of IL-17 and IFN-γ in supernatants was quantified by ELISA (*B*) and proliferation determined on day 4 by ^{3}H-thymidine incorporation (*C*). Data are mean ± SEM for triplicate assays performed on individual mice (n = 8 per group) * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, EAE Control versus EAE + U0126. *D,* Intracellular cytokine staining was performed for IL-17 and IFN-γ, with cells gated on CD3 or CD4. *E*, EAE was induced in C57BL/6 mice by immunization with MOG in CFA. Mice were treated s.c. with the MEK1/2 inhibitor U0126 (50 μg/mouse) or vehicle (PBS with DMSO) as a control on days 5, 7, 9, 11, 13, 15 and 17. Arrows indicate treatment days. Mice were observed for clinical signs of EAE. * $P < 0.05$, ** $P < 0.01$, EAE + U0126 versus EAE control by unpaired *t*-test. * $P < 0.05$, with versus without UO126, curves compared by two-way repeated measures ANOVA. Results are means and SEM for 7 mice per group. Numbers in parentheses represent disease incidence.

FIGURE 6. Treatment with a MEK1/2 inhibitor suppresses Th17 responses and prevents relapse in the relapsing-remitting EAE model. *A-B,* EAE was induced in SJL mice by immunization with PLP in CFA followed by PT. Mice were treated with PBS (control) or U0126 (50 μg/mouse) every 2 d from day 18. Arrows indicate treatment days. Data are means ± SEM (n = 9 per group). *** $P < 0.001$, EAE + U0126 versus EAE control, curves compared by two-way repeated measures ANOVA (A). *B,* Mice were sacrificed on day 36 and lymph

node cells re-stimulated in vitro with PLP (1-25 μg/ml). After 3 d IL-17 production in supernatants was quantified by ELISA. * $P < 0.05$, EAE + U0126 versus EAE control by ANOVA. Data are mean ± SEM for triplicate stimulations of lymph node cells from individual mice (n = 9 per group) and are representative of 2 experiments.

FIGURE 7. IL-1β and IL-23 expand IFN-γ and IL-17 producing T cells that induce EAE following transfer to naïve mice. *A*, Lymph node cells from mice with EAE (21 d after immunization with MOG and CFA) were stimulated in vitro with MOG (2-50 μg/ml) and IL-1β (10 ng/ml), IL-23 (10 ng/ml) or IL-1β and IL-23. After 3 d supernatants were recovered and IFN-γ and IL-17 production quantified by ELISA. *B-C,* Lymph node and spleen cells from C57BL/6 mice with EAE were cultured in vitro for 3 d with MOG, MOG and IL-1β + IL-23 or IL-1β + IL-23 in the presence of a neutralizing anti-IFN-γ antibody (aIFN-γ), or with MOG and IL-12. Supernatants were recovered and IL-17 and IFN-γ production quantified by ELISA (*B*). Cells were washed and transferred to naïve C57BL/6 mice. Mice were observed daily for clinical signs of EAE (*C*). *D,* Lymph node and spleen cells from C57BL/6 mice with EAE were cultured in vitro for 3 d with MOG or MOG and IL-1β + IL-23. Cells were washed and transferred to naïve C57BL/6 mice (5 X 10^6/ mouse). Mice were left untreated or treated with U0126 (50 μg/mouse) on the day of transfer and on days 1 and 2. Mice were observed daily for clinical signs of EAE. ns, not significant, with versus without UO126, curves compared by two-way repeated measures ANOVA. *E*, DC were pulsed with MOG (50 μg/ml), MTB (10 μg/ml) with or without U0126 (5 μM) for 24 h. Control DC were cultured with medium only. Cells were washed and transferred to naïve C57BL/6 mice (1 X 10^6/ mouse). Mice were observed daily for clinical signs of EAE. Data are means ± SEM for 5 mice per group. ns, not significant, *** $P < 0.001$, with versus without UO126, curves

compared by two-way repeated measures ANOVA. Numbers in parentheses represent disease incidence.

FIGURE 8. IL-1β and IL-23 reverse the suppressive effect of the ERK inhibitor on IL-17 production and on the clinical course of EAE. EAE was induced in C57BL/6 mice by immunization with MOG in CFA followed 2 d later with PT. Mice were treated with the MEK 1/2 inhibitor U0126 (50 μg/mouse) alone or with IL-1β (100 ng) and IL-23 (100 ng) or with IL-1β and IL-23 alone on days 0, 1 and 2. *A,* Mice were sacrificed on day 12 and MOG-specific IL-17 production by lymph node cells from individual mice was quantified by ELISA. ** $P < 0.01$, U0126 versus untreated control; ++ $P < 0.01$, +++ $P < 0.001$ U0126 + IL-1β + IL-23 versus U0126 by ANOVA. *B,* Intracellular cytokine staining was performed for IL-17, with cells gated on CD3 or CD4. *C,* Mice were observed for clinical signs of EAE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, U0126 versus untreated control by ANOVA. Results are mean score ± SEM for 5 mice per group or means ± SEM for triplicate cultures and are representative of 2 experiments.

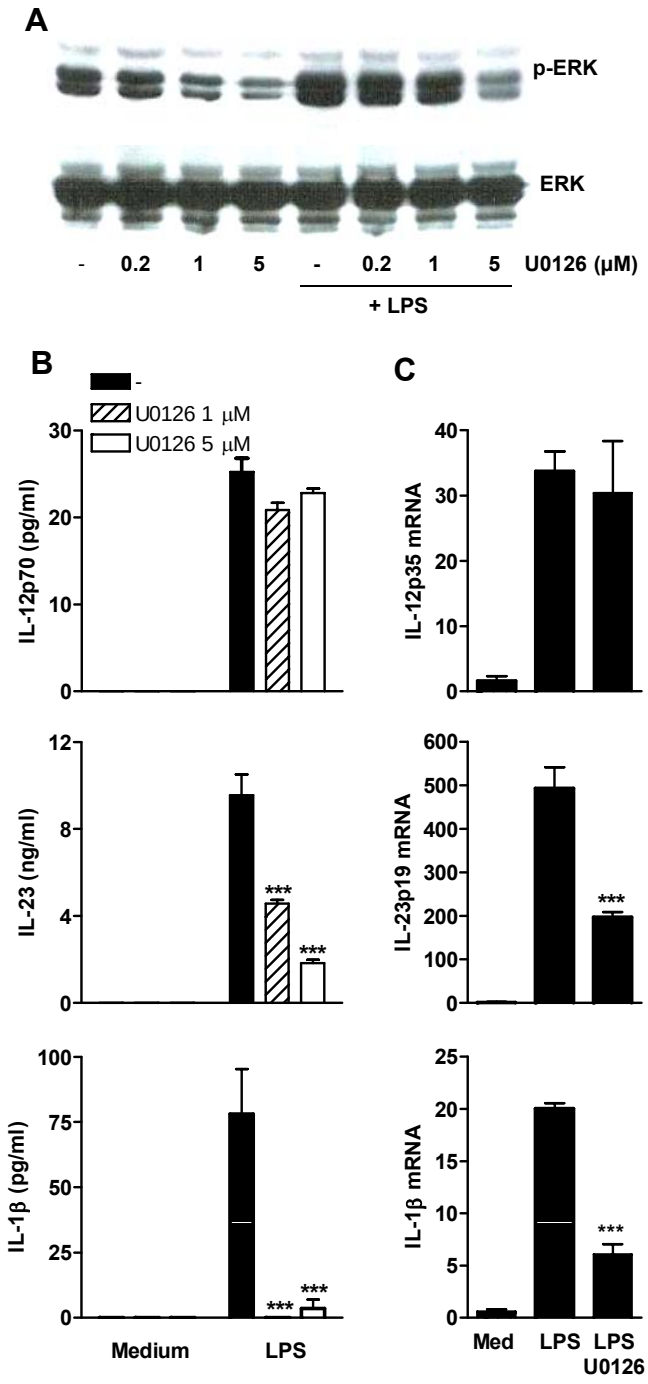
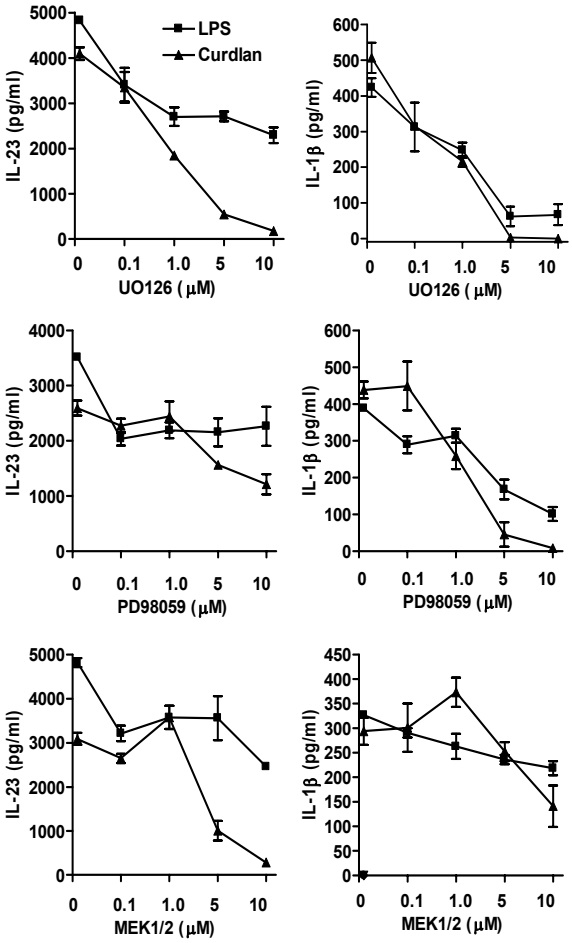

A
p-ERK
ERK
- 0.2 1 5 - 0.2 1 5 U0126 (μM)
+ LPS
B
C
-
U0126 1 μM
U0126 5 μM
IL-12p70 (pg/ml)
IL-12p35 mRNA
IL-23 (ng/ml)
IL-23p19 mRNA
IL-1β (pg/ml)
IL-1β mRNA
***
Medium
LPS
Med
LPS
LPS U0126

Figure 1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Curdlan
IL-23 (pg/ml)
IL-1β (pg/ml)
UO126 (μM)
PD98059 (μM)
MEK1/2 (μM)
0
0.1
1.0
5
10

Figure 2

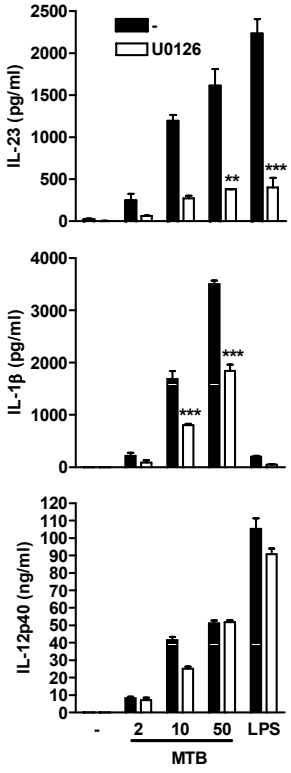
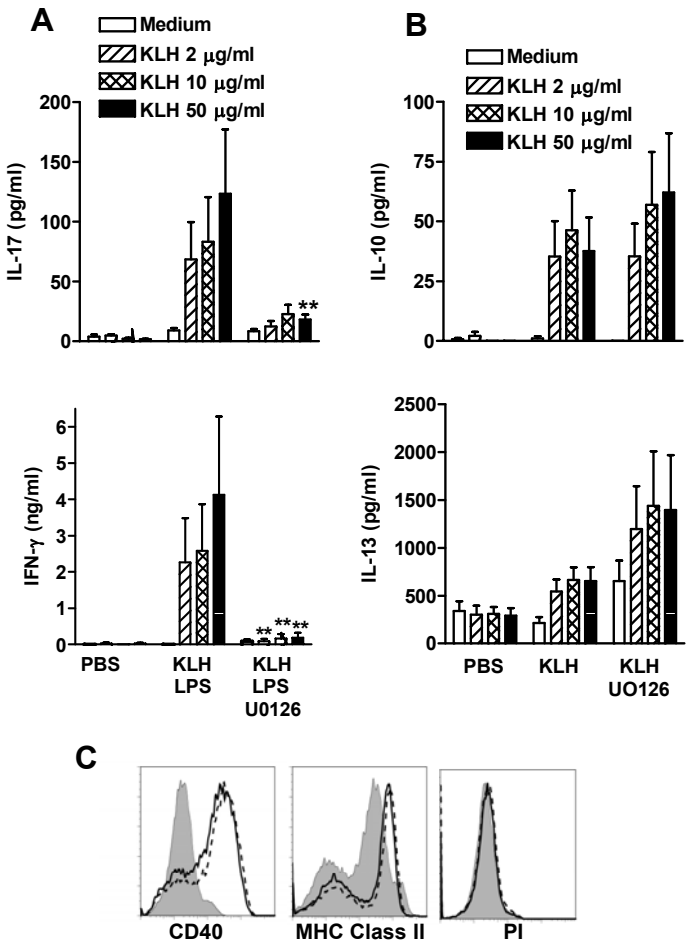

2500
2000
1500
1000
500
0
-
U0126
IL-23 (pg/ml)
**
***
4000
3000
2000
1000
0
IL-1β (pg/ml)
***
***
120
110
100
90
80
70
60
50
40
30
20
10
0
IL-12p40 (ng/ml)
-
2
10
50
LPS
MTB

Figure 3


A
Medium
KLH 2 μg/ml
KLH 10 μg/ml
KLH 50 μg/ml
IL-17 (pg/ml)
200
150
100
50
0
**
B
Medium
KLH 2 μg/ml
KLH 10 μg/ml
KLH 50 μg/ml
IL-10 (pg/ml)
100
75
50
25
0
IFN-γ (ng/ml)
6
5
4
3
2
1
0
**
**
**
PBS
KLH
KLH LPS
KLH LPS U0126
IL-13 (pg/ml)
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2000
1500
1000
500
0
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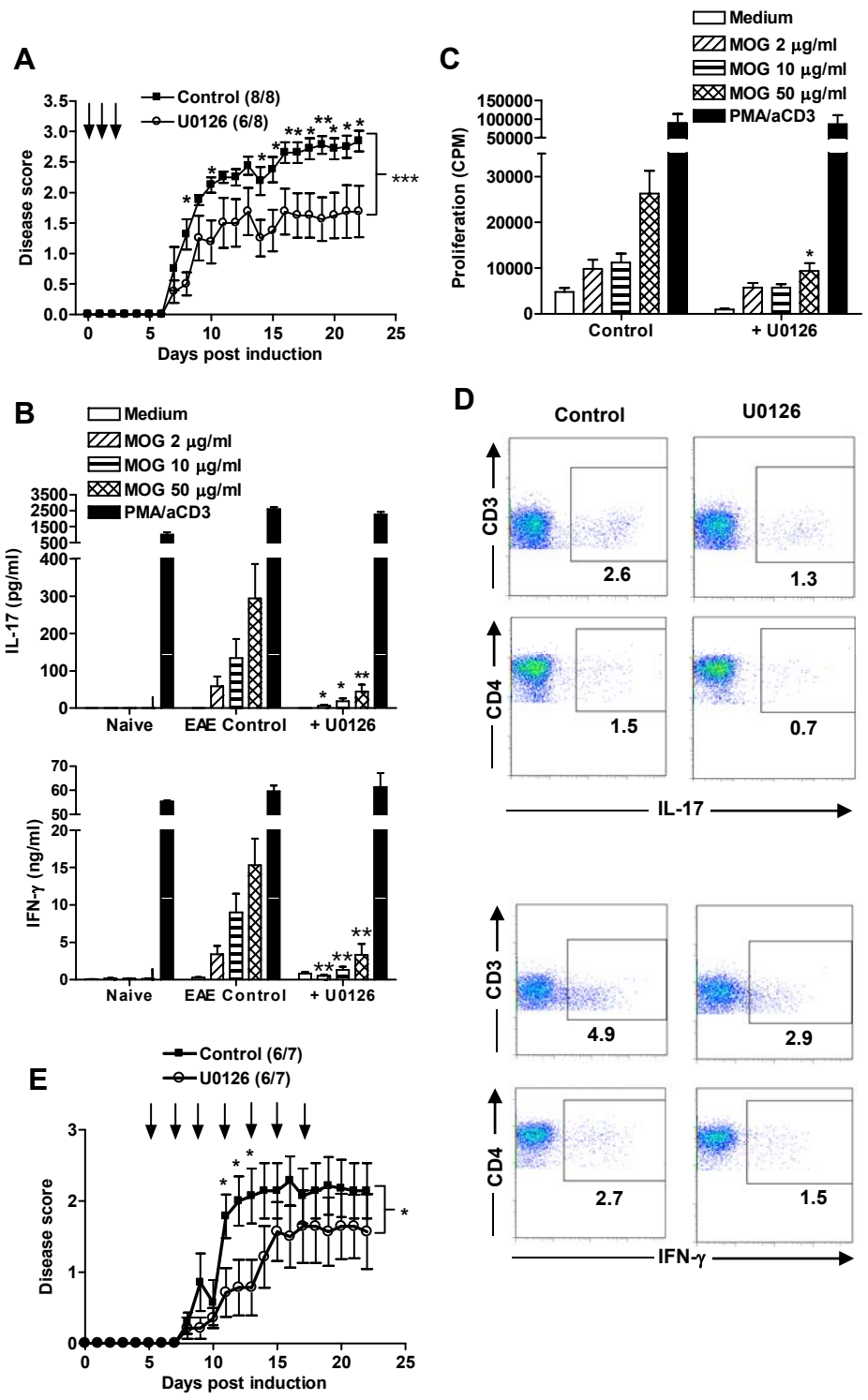
KLH
KLH UO126
C
CD40
MHC Class II
PI

Figure 4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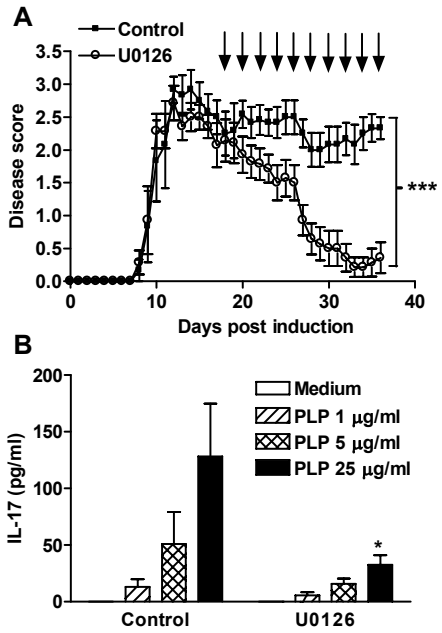
Control (8/8)
U0126 (6/8)
Disease score
Days post induction
***
B
Medium
MOG 2 µg/ml
MOG 10 µg/ml
MOG 50 µg/ml
PMA/aCD3
IL-17 (pg/ml)
IFN-γ (ng/ml)
Naive
EAE Control
+ U0126
C
Proliferation (CPM)
Control
+ U0126
D
Control
U0126
CD3
CD4
IL-17
IFN-γ
2.6
1.3
1.5
0.7
4.9
2.9
2.7
1.5
E
Control (6/7)
U0126 (6/7)
Disease score
Days post induction

Figure 5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Control
U0126
Disease score
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3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
Days post induction
***
B
IL-17 (pg/ml)
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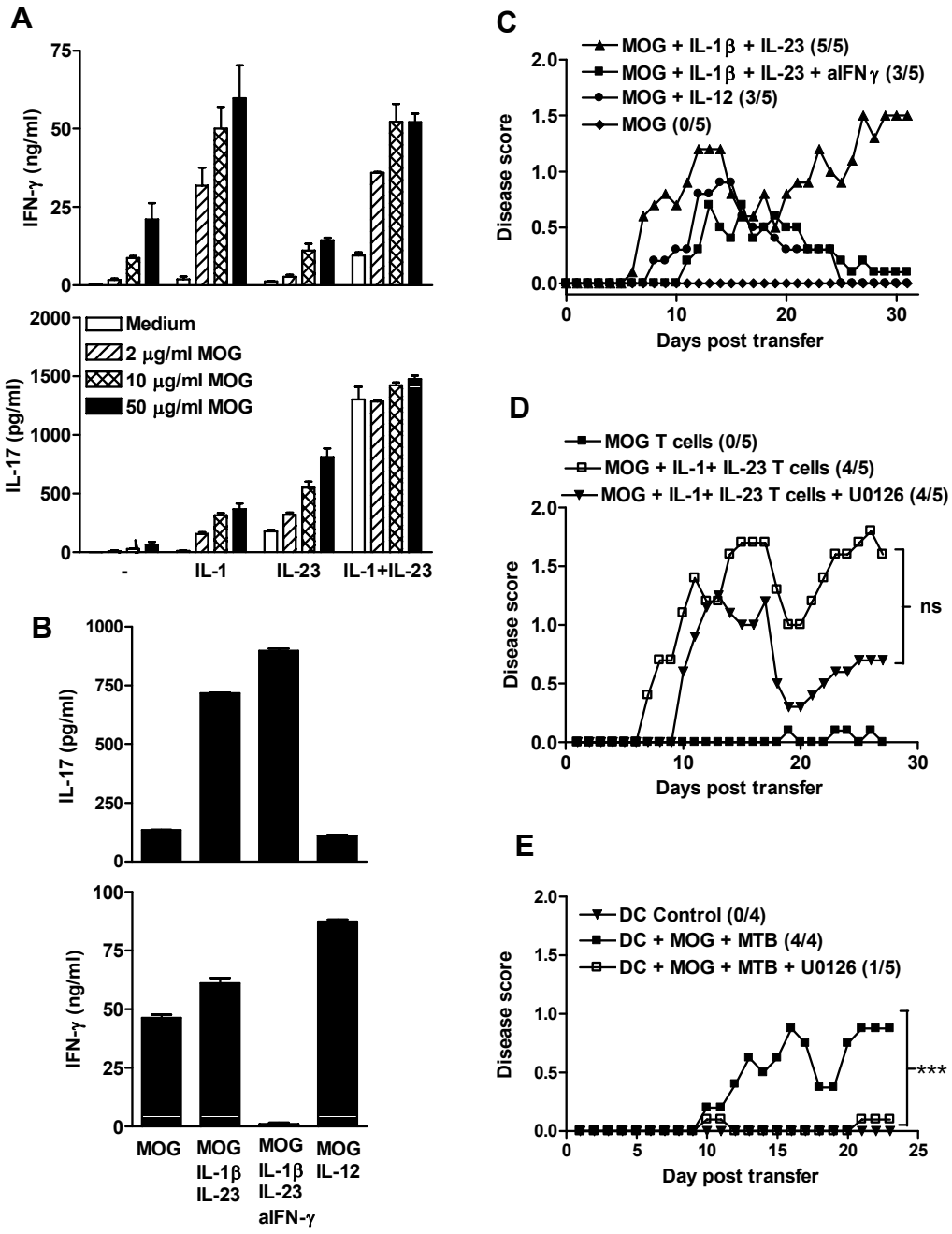
150
100
50
0
Medium
PLP 1 µg/ml
PLP 5 µg/ml
PLP 25 µg/ml
*
Control
U0126

Figure 6


A
IFN-γ (ng/ml)
75
50
25
0
IL-17 (pg/ml)
2000
1500
1000
500
0
Medium
2 μg/ml MOG
10 μg/ml MOG
50 μg/ml MOG
-
IL-1
IL-23
IL-1+IL-23
B
IL-17 (pg/ml)
1000
750
500
250
0
IFN-γ (ng/ml)
100
75
50
25
0
MOG
MOG IL-1β IL-23
MOG IL-1β IL-23 aIFN-γ
MOG IL-12
C
MOG + IL-1β + IL-23 (5/5)
MOG + IL-1β + IL-23 + aIFNγ (3/5)
MOG + IL-12 (3/5)
MOG (0/5)
Disease score
2.0
1.5
1.0
0.5
0.0
0
10
20
30
Days post transfer
D
MOG T cells (0/5)
MOG + IL-1+ IL-23 T cells (4/5)
MOG + IL-1+ IL-23 T cells + U0126 (4/5)
Disease score
2.0
1.5
1.0
0.5
0.0
0
10
20
30
Days post transfer
ns
E
DC Control (0/4)
DC + MOG + MTB (4/4)
DC + MOG + MTB + U0126 (1/5)
Disease score
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1.5
1.0
0.5
0.0
0
5
10
15
20
25
Day post transfer
***

Figure 7

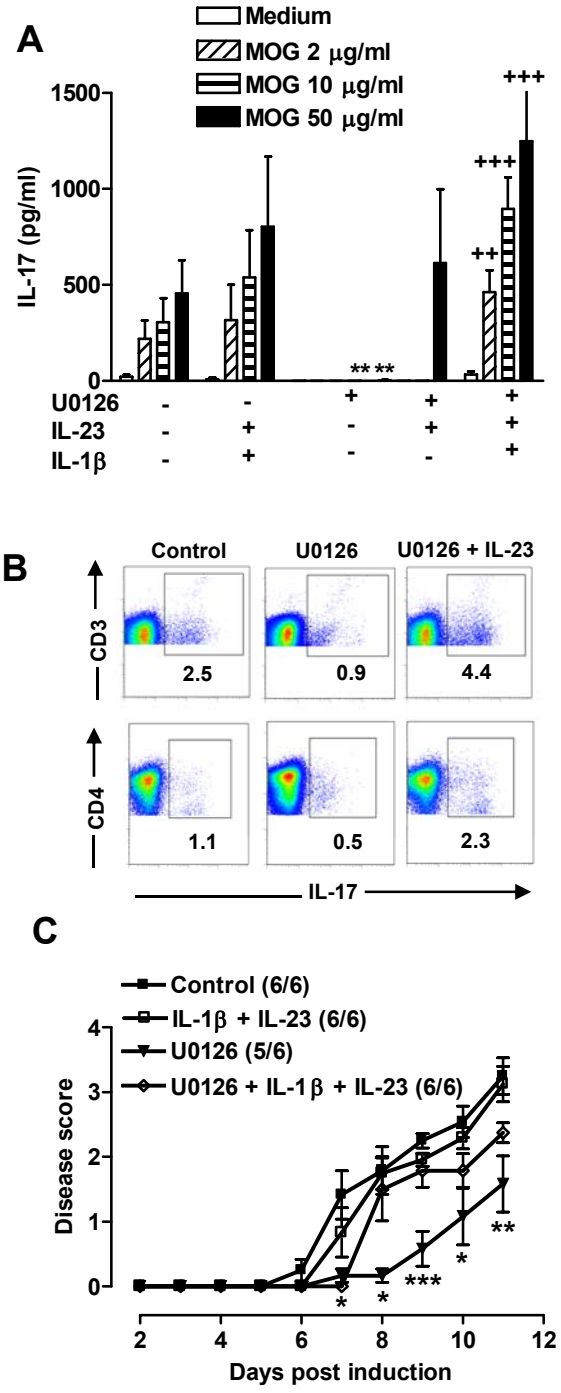
A
Medium
MOG 2 μg/ml
MOG 10 μg/ml
MOG 50 μg/ml
IL-17 (pg/ml)
1500
1000
500
0
U0126
IL-23
IL-1β
B
Control
U0126
U0126 + IL-23
CD3
2.5
0.9
4.4
CD4
1.1
0.5
2.3
IL-17
C
Control (6/6)
IL-1β + IL-23 (6/6)
U0126 (5/6)
U0126 + IL-1β + IL-23 (6/6)
Disease score
Days post induction

Figure 8