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ORIGINAL ARTICLE

Retinoic acid suppresses IL-17 production and pathogenic activity of γδ T cells in CNS autoimmunity

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Retinoic acid (RA) in the steady state enhances induction of Foxp3$^+$ regulatory T (Treg) cells and inhibits differentiation of Th1 and Th17 cells, thereby maintaining tolerance, but can in inflammatory conditions promote effector Th1 and Th17 cells that mediate inflammation. IL-17-producing γδ T cells have recently been shown to have a major pathogenic role in autoimmune diseases. Here, we examined the immunomodulatory effects of RA on γδ T cells. We found that RA had a dramatic suppressive effect on IL-17A and IL-17F production by γδ T cells stimulated with IL-1β and IL-23. RA suppressed RORγt, IL-1R and IL-23R expression in γδ T cells. Treatment of mice with RA suppressed IL-17 production by γδ T cells *in vivo*. Furthermore, treatment of T cells with RA attenuated their ability to induce disease in experimental autoimmune encephalomyelitis (EAE), a murine model for multiple sclerosis. This was associated with a reduction in the number of central nervous system-infiltrating γδ T cells, but also CD4$^+$ T cells that produced IL-17A, IL-17F or GM-CSF. Interestingly, treatment of γδ T cells with RA or removal of γδ T cells from a bulk population of T cells significantly reduced their capacity to induce EAE, demonstrating a critical role for γδ T cells in promoting pathogenic Th17 cells. Our findings demonstrate that the anti-inflammatory properties of RA are mediated in part by suppressing STAT3-mediated activation of cytokine production and cytokine receptor expression in γδ T cells, which suppresses their ability to activate Th17 cells.
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At the border between innate and adaptive immunity, γδ T cells express T-cell receptor (TCR) that recognize non-major histocompatibility complex-restricted antigens, but also express receptors for cytokines and Toll-like receptors. These cells are rapidly activated upon inflammation, stress or infection, provide early sources of cytokines such as IFN-γ, IL-17 and IL-21, and enhance immune responses, for instance by influencing antigen-specific CD4$^+$ T cells.[1–3] In experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis (MS), the role of γδ T cells, and particularly of IL-17-producing γδ T cells has been the subject of debate, although the consensus view is that they have a pathogenic role in this disease.[1,4–6] γδ T cells secrete IL-17 in response to stimulation with IL-23 and IL-1β, IL-1α or IL-18, without TCR engagement.[3,7] During development of EAE, IL-17$^+$ γδ T cells and in particular the Vγ4 subtype of γδ T cells are the first cells to produce IL-17 and are found at high numbers in the brains of the mice early in the course of disease.[2,3,8] Moreover, mice lacking functional γδ T cells (TCRδ$^{-/-}$ mice) have delayed and attenuated EAE and the production of IL-17A by autoantigen-specific Th17 cells is reduced in these mice, indicating that γδ T cells enhance the pathogenicity of CD4$^+$ T cells in this model.[3,9] Activated γδ T cells also antagonize the anti-inflammatory activity of regulatory T (Treg) cells and inhibit their differentiation, thereby enhancing the activity of effector T cells that mediate the development of EAE.[2]

Retinoic acid (RA), the active metabolite of vitamin A, is a powerful immunomodulator, which, in the steady state, promotes immune tolerance by enhancing Foxp3$^+$ Treg cells and inhibiting the differentiation of Th17 cells.[10] However, in inflammatory conditions, RA can promote effector Th1 and Th17 and thereby enhance inflammation.[11,12] AM80, a selective agonist of the RA nuclear receptor-α (RARα) has been shown to have a protective effect in the early stages of EAE by inhibiting the differentiation of pathogenic Th17 cells.[12–15] Administered at later stages of the disease, AM80 delays the recovery from EAE by inducing the apoptosis and differentiation of immature myeloid-derived suppressor cells, a cell population that exerts immunosuppressive effects on effector T cells.[16] RA has also been shown to protect mice from EAE by inhibiting the maturation of dendritic cells and monocytes thereby impairing their antigen presentation function.[17]

We have previously reported that RA has a protective effect in a mouse model of colitis by promoting IL-22 production by γδ T cells and type 3 innate lymphoid cells (ILC3).[18] Given the important role of IL-17A-producing γδ T cells in the development of EAE, we addressed the hypothesis that RA may be protective in EAE through modulating the function of γδ T cells. We found that RA significantly inhibited

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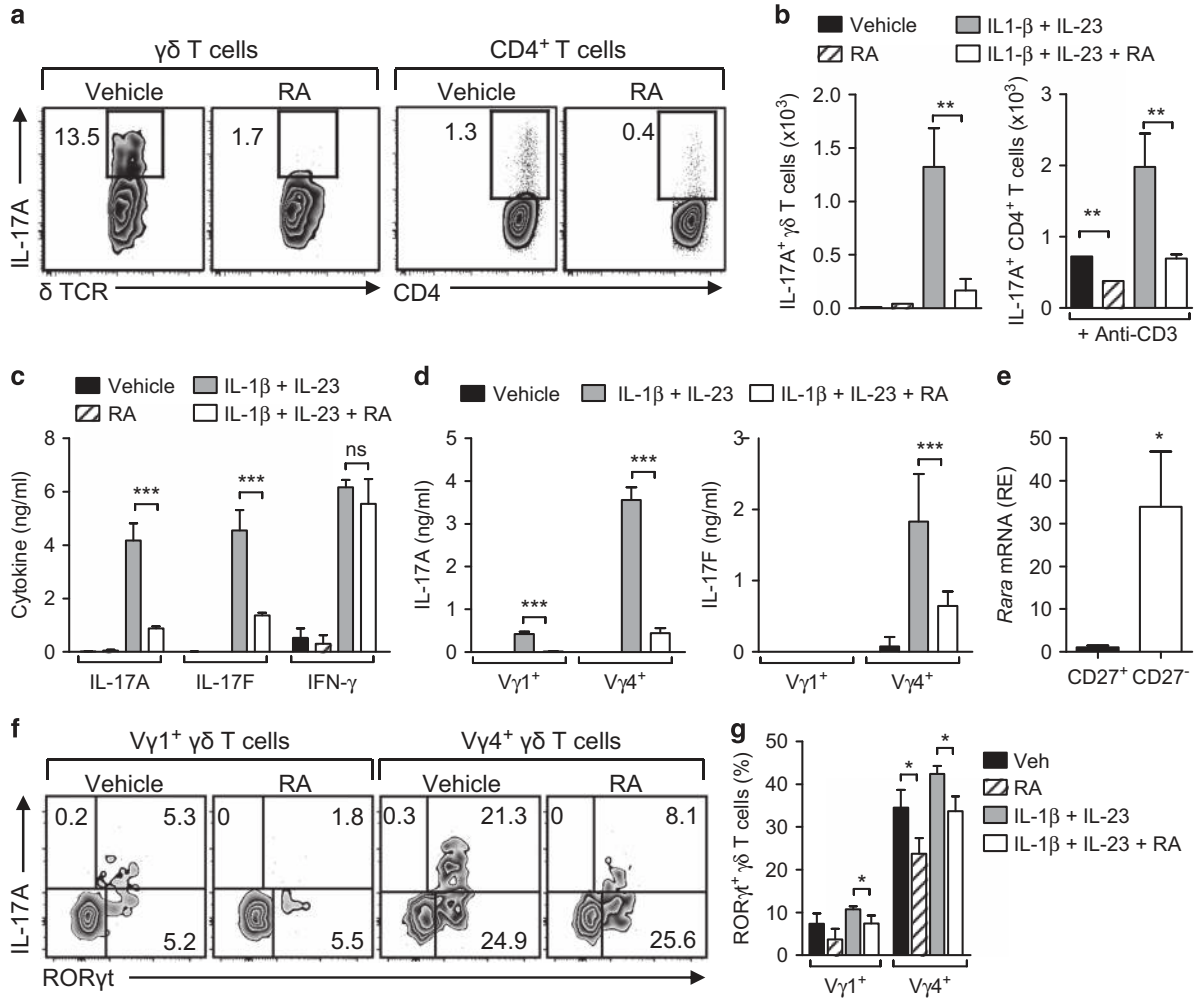

Figure 1 RA impairs IL-17A but not IFN-γ production by γδ T cells and CD4+ T cells. (**a**, **b**) Spleen cells from naive mice were cultured for 3 days with medium or anti-CD3 and in combination with IL-1β and IL-23 and in the presence or absence of RA. (**a**) Representative FACS plots of IL-17A expression by γδ T cells and CD4+ T cells. (**b**) Mean absolute numbers of IL-17A+ and IFN-γ+ γδ T cells and CD4+ T cells in the cultures. (**c–f**) γδ T cells purified from lymph nodes of naive mice were cultured for 3 days with IL-1β+IL-23, with or without RA. (**c**) IL-17A, IL-17F and IFN-γ concentration measured by ELISA in the supernatants of purified γδ T cells cultured for 3 days with IL-1β+IL-23, with or without RA. (**d**) IL-17A and IL-17F concentration measured by ELISA in the supernatants of purified Vγ1+ or Vγ4+ γδ T cells cultured for 3 days with IL-1β+IL-23, with or without RA. (**e**) Relative expression of *rara* in CD27+ and CD27− γδ T cells purified by FACS. (**f**, **g**) Representative FACS plots (**f**) and mean frequencies (**g**) of Vγ1+ and Vγ4+ T cells expressing IL-17A and RORγt after 3 days of culture with IL-1β+IL-23, with or without RA. Data are representative of three independent experiments. Bars are mean+s.d.; $*P<0.05$, $**P<0.01$ and $***P<0.001$, two-tailed unpaired t-test.

IL-17A and IL-17F production by γδ T cells and CD4+ T cells stimulated with IL-1β and IL-23, but the effect on γδ T cells was more dramatic. Furthermore, treatment of γδ T cells with RA significantly inhibited the capacity of T cells from myelin oligodendrocyte glycoprotein (MOG)-immunized mice to induce EAE by adoptive transfer. In addition, RA impaired the differentiation of IL-17A-expressing γδ T cells by inhibiting RORγt expression and downregulating expression of receptors for IL-1β and IL-23.

RESULTS

RA inhibits IL-17 production by γδ T cells *in vitro* and *in vivo*

We have previously demonstrated that γδ T cells secrete IL-17 and IFN-γ following stimulation with IL-1β and IL-23 without TCR activation, whereas CD4+ memory T cells secrete IL-17 following stimulation with IL-1β and IL-23 in the presence of anti-CD3.[3,19] Here, we examined the effect of RA on cytokine production by γδ T cells. We found that RA suppressed IL-17 production by both γδ T cells within a bulk population of spleen cells stimulated with IL-1β and IL-23 (Figures 1a and b). IL-1β and IL-23 also induced IL-17 production by CD4+ T cells but only when co-stimulated with anti-CD3 and this was also inhibited by co-culture with RA (Figures 1a and b). We then examined the effect of RA on purified γδ T cells or Vγ1+ or Vγ4+ γδ T cells from lymph nodes of naive mice. Stimulation of γδ T cells with RA alone did not change basal cytokine production (Figure 1c). Stimulation of γδ T cells with IL-1β and IL-23 induced high concentrations of IL-17A and IL-17F, mainly by Vγ4+ T cells (Figures 1c–e), as well as IFN-γ (Figure 1c). The addition of RA significantly reduced the production of IL-17A and IL-17F (Figures 1c, d and f). In contrast, RA did not affect the production of IFN-γ by γδ T cells (Figure 1c and data not shown). The expression of the nuclear receptor for RA, RARα, was 34 times higher in the IL-17-producing γδ T cells (CD27−) when compared with γδ T cells that express IFN-γ (CD27+) (Figure 1e). Interestingly, the expression of the transcription factor RORγt, required for the production of IL-17, was impaired by

RA in γδ T cells stimulated or not with IL-1β and IL-23, and particularly in Vγ4$^+$ T cells (Figures 1f and g).

We next assessed the effect of RA on cytokine production by γδ T cells and CD4$^+$ T cells *in vivo*. The production of IL-17A by γδ T cells was strongly inhibited in mice injected with RA when compared with mice injected with vehicle (Figures 2a and b). The production of IL-17A by CD4$^+$ T cells was also affected in the mice injected with RA but to a lesser extent when compared with the effect on γδ T cells (Figures 2a and b). In contrast, RA did not affect the production of IFN-γ by γδ T cells or CD4$^+$ T cells (Figure 2b).

RA inhibits IL-17 production by CD4 and γδ T cells from mice with EAE

IL-17A-producing γδ T cells and CD4$^+$ T cells (Th17 cells) have been shown to have pathogenic roles in a range of autoimmune diseases, including MS and EAE where they promote inflammation. It has previously been reported that retinoids can attenuate development of EAE.[15,17] However, these studies did not examine the effect of RA on γδ T cells. Having shown that RA impairs IL-17A production mainly by γδ T cells but also by CD4$^+$ T cells, we examined its effect on γδ T cells and CD4$^+$ T cells from mice with EAE. Mice were immunized with the myelin antigen MOG emulsified in complete Freund's adjuvant and 10 days later their spleens and draining lymph nodes cells were isolated and cultured with MOG, IL-1β and IL-23 to drive the differentiation of IL-17-producing γδ T cells and Th17 cells, and in the presence of RA or vehicle control. The production of IL-17A, IL-17F and IFN-γ measured in the supernatant of the cultures by ELISA was strongly attenuated by addition of RA (Figure 3a). We next examined more closely the effect of RA on cytokine production by γδ T cells and CD4$^+$ T cells using intracellular cytokine staining and fluorescence-activated cell sorting (FACS) analysis (Figures 3b and c) or by RT-PCR performed on γδ T cells and CD4$^+$ T cells sorted at the end of the culture (Figure 3d). We found that RA inhibited IL-17A principally in γδ T cells and particularly in the Vγ4$^+$ γδ T cells, but also in CD4$^+$ T cells (Figures 3b–d). The expression of IL-17F was also strongly reduced by RA in both γδ T cells and CD4$^+$ T cells (Figure 3d). Interestingly, the number of IFN-γ$^+$ γδ T cells was unchanged and the number of IFN-γ$^+$ CD4$^+$ T cells was increased by RA (Figures 3b and c). However, assessment of other IFN-γ-producing cell types by FACS indicated that RA impaired IFN-γ expression in CD8$^+$ T cells (data not shown). This may explain the overall reduction in IFN-γ production detected by ELISA on cultures of mixed LN and spleen cells stimulated with MOG, IL-1β and IL-23 (Figure 3a).

We also assessed the effect of RA on expression of the gene coding for RORγt by RT-PCR in γδ T cells and CD4$^+$ T cells purified from the 3-day cultures and found that the expression of *rorc* was strongly reduced by RA in both cell types (Figure 3e). We also found that the proliferation of γδ T cells (but not CD4$^+$ T cells) and more specifically of the Vγ4$^+$ γδ T cells was impaired by treatment with RA leading to a reduced number of Vγ4$^+$ γδ T cells in the culture (Figures 3f and g and data not shown). Collectively, these findings demonstrated that RA suppresses IL-17 production by γδ T cells and Th17 cells and affects the polarization of these cells. Our data also show that RA has the most pronounced inhibitory effect on activation and proliferation of IL-17-secreting Vγ4$^+$ γδ T cells.

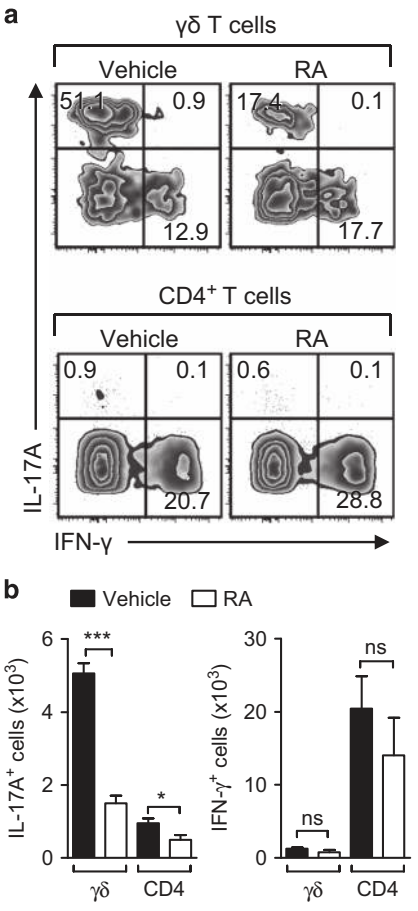

Figure 2 RA inhibits IL-17 production by γδ T cells and CD4$^+$ T cells *in vivo*. (**a**, **b**) Representative FACS plots (**a**) and absolute numbers (**b**) of IL-17A$^+$ and IFN-γ$^+$ γδ T cells and CD4$^+$ T cells isolated from peritoneal cavity 18 h after injection of RA or vehicle only (dimethyl sulfoxide) and re-stimulation with phorbol 12-myristate 13-acetate, ionomycin and brefeldin A. Bars are mean +s.e.m.; $^*P<0.05$ and $^{***}P<0.001$, two-tailed unpaired *t*-test.

RA suppresses the pathogenic function of γδ T cells and CD4$^+$ T cells in the EAE model

Having shown that RA inhibits IL-17A and IL-17F production principally by γδ T cell but also by CD4$^+$ T cells from MOG-immunized mice, we examined the effect of RA on the effector and pathogenic function of these cells in EAE by adoptive transfer to naive mice. Spleen and lymph nodes cells from mice immunized with MOG and complete Freund's adjuvant were cultured under Th17-polarizing conditions (MOG, IL-1β and IL-23) in the presence of RA or a vehicle control. After 3 days, the cells were washed thoroughly and transferred to naive recipient mice (15×10^6 cells per mouse). The mice injected with the vehicle-treated cells developed signs of EAE by day 7 (Figure 4a). In contrast, the onset of EAE was delayed by several days and the clinical signs of disease were strongly attenuated in mice injected with the RA-treated cells (Figure 4a). An examination of infiltrating lymphocytes in the brains of the mice 16 days after the cell transfer revealed that the number of CD3$^+$ T cells infiltrating the brains was greatly reduced in the recipients of the RA-treated cells when compared with the mice injected with the control cells (Figure 4b). Moreover, the number of CD4$^+$ T cells and γδ T cells and the production of the cytokines IL-17A, IL-17F, IFN-γ and GM-CSF by these cells were significantly lower in the brains of the animals injected with the RA-treated cells when compared with the control group (Figures 4c and d). Thus, RA decreased the production of IFN-γ, IL-17A and IL-17F induced by IL-1β and IL-23 and particularly the production of IL-17A and IL-17F by γδ T cells and Th17 (Figure 3), which resulted in a reduced number of pathogenic γδ T cells and CD4$^+$

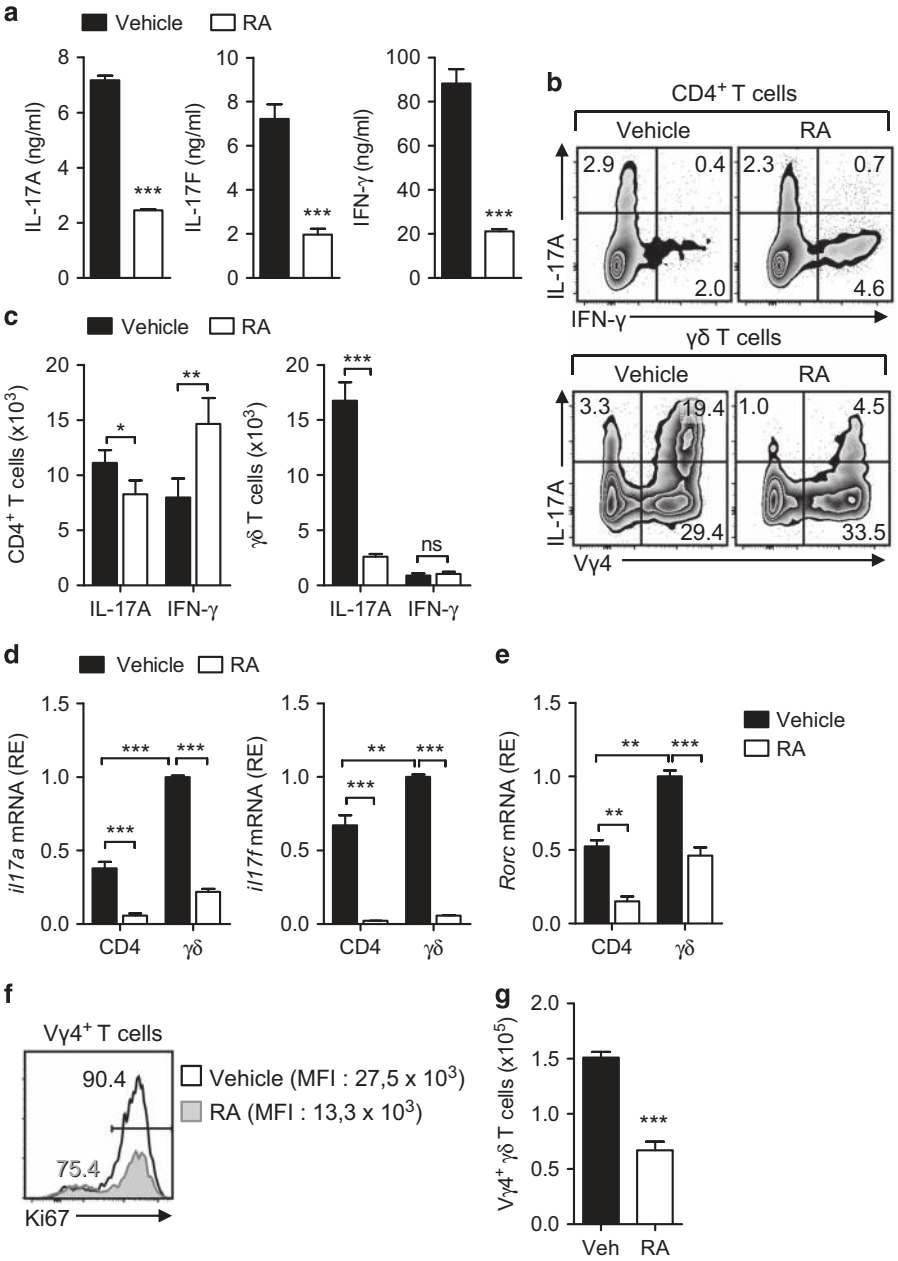

Figure 3 RA suppresses IL-17 production and RORγt expression in CD4+ T cell and γδ T cell and impairs Vγ4+ T-cell proliferation under inflammatory conditions. Mice were immunized subcutaneously with MOG emulsified in complete Freund's adjuvant. Ten days after immunization, lymph node and spleen cells were cultured together for 3 days with MOG, IL-1β and IL-23 and either RA or vehicle. (**a**) IL-17A, IL-17F and IFN-γ production was quantified in supernatants by ELISA at the end of the 3-day culture. (**b**) Representative FACS plots of the IL-17A+ and IFN-γ+ CD4+ T cells and IL-17A+ Vγ4+ γδ T cells at the end of the 3-day culture. (**c**) Mean absolute numbers of IL-17A+ and IFN-γ+ CD4+ T cells and γδ T cells at the end of the 3-day culture. (**d**, **e**) Relative expression of *il17a*, *il17f* (**d**) and *rorc* (**e**) mRNA measured by RT-PCR on CD4+ T cells and γδ T cells after 3 days of culture. (**f**) Representative histogram, mean frequencies and mean fluorescence intensity quantifying Ki67 expression in Vγ4+ T cells at the end of the 3-day culture. (**g**) Absolute number of Vγ4+ γδ T cells after 3 days of culture. Bars are mean+s.d. $*P<0.05$, $**P<0.01$ and $***P<0.001$ (two-tailed unpaired *t*-test).

T cells infiltrating the brains and a consequent attenuation of the clinical signs of EAE (Figure 4).

RA mediates immunomodulatory activity largely through its effect on γδ T cells

As the impact of RA seemed particularly striking on γδ T cells (Figures 1, 2 and 3), we examined more closely the effect of RA on the pathogenic function of these cells. We first evaluated the importance of γδ T cells in our EAE model by performing an adoptive transfer of cells from MOG-immunized mice depleted of γδ T cells. Spleen and lymph nodes cells from mice immunized with MOG and complete Freund's adjuvant were depleted of γδ T cells, or left un-separated, before being stimulated for 3 days in Th17-polarizing conditions and then transferred to recipient animals. Depletion of γδ T cells prior to culture reduced the production of IL-17A, IL-17F and IFN-γ by the spleen and lymph node cells (Figure 5a). Intracellular cytokine staining and FACS showed that depletion of γδ T cells prior to culture impaired the expansion of Tbet+ and RORγt+ CD4+ T cells (Th1 and

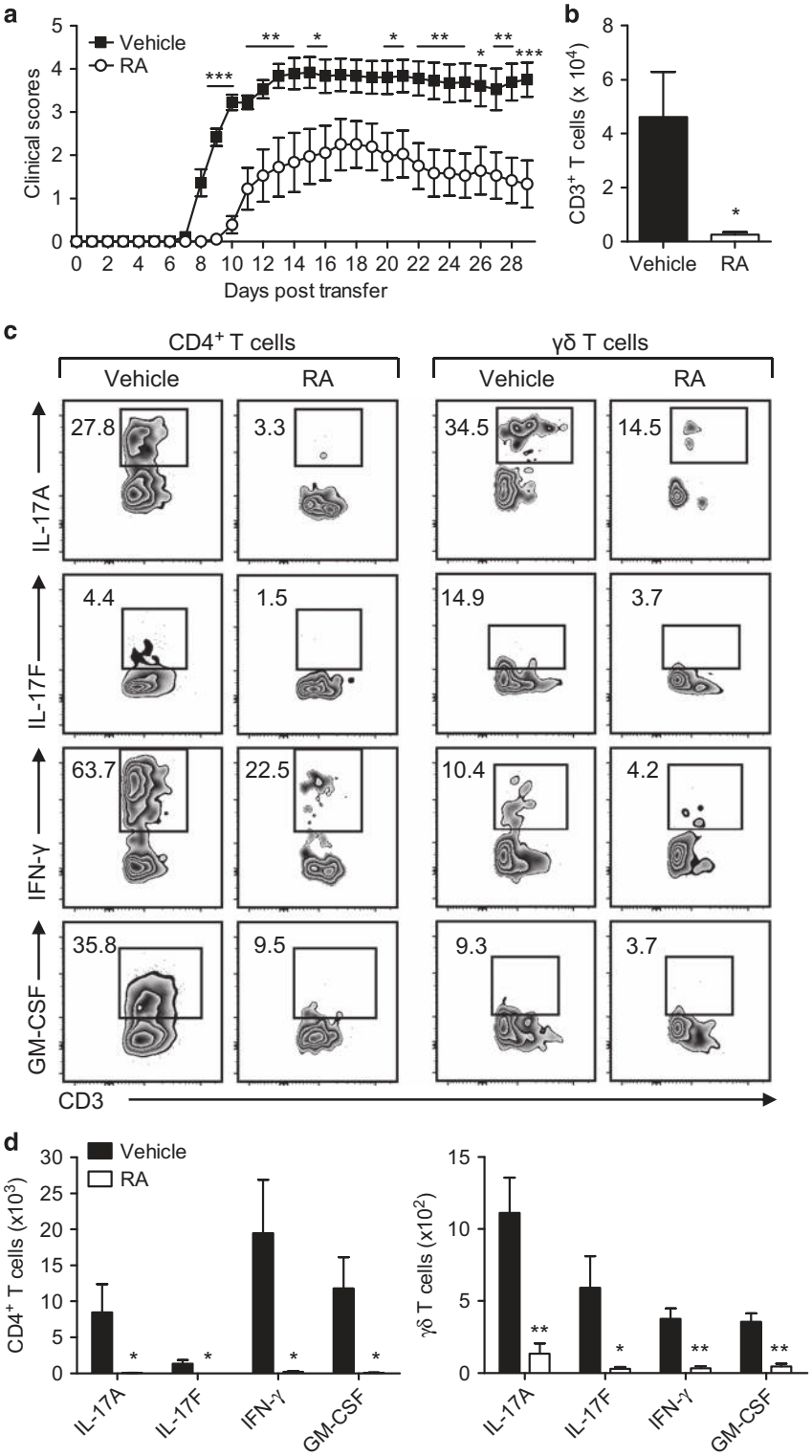

Figure 4 Treatment of T cells from MOG-immunized mice with RA suppresses their ability to induce EAE by adoptive transfer. Donor mice were immunized with MOG+complete Freund's adjuvant and after 10 days, spleen and lymph node cells were cultured for 3 days with MOG, IL-1β+IL-23 and RA or vehicle and then 15×10^6 cells were transferred to naive mice. (**a**) EAE clinical scores in recipient mice. (**b**) Absolute number of $CD3^+$ T cells detected in the brains of the recipient mice 16 days post transfer. Representative FACS plots (**c**) and mean absolute numbers (**d**) of infiltrating IL-17A$^+$, IFN-γ$^+$, IL-17F$^+$ and GM-CSF$^+$ CD4$^+$ and γδ T cells in the brains of the recipient mice 16 days post transfer and re-stimulated *ex vivo* for 5 h with phorbol 12-myristate 13-acetate, ionomycin and brefeldin A. Data are representative of three independent experiments. Data shown as mean ± s.e.m. (**a**) or mean+s.e.m. (**b**, **d**) and statistics were calculated using a two-way analysis of variance with Bonferroni post-test (**a**) or two-tailed unpaired *t*-test (**b**–**d**). $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

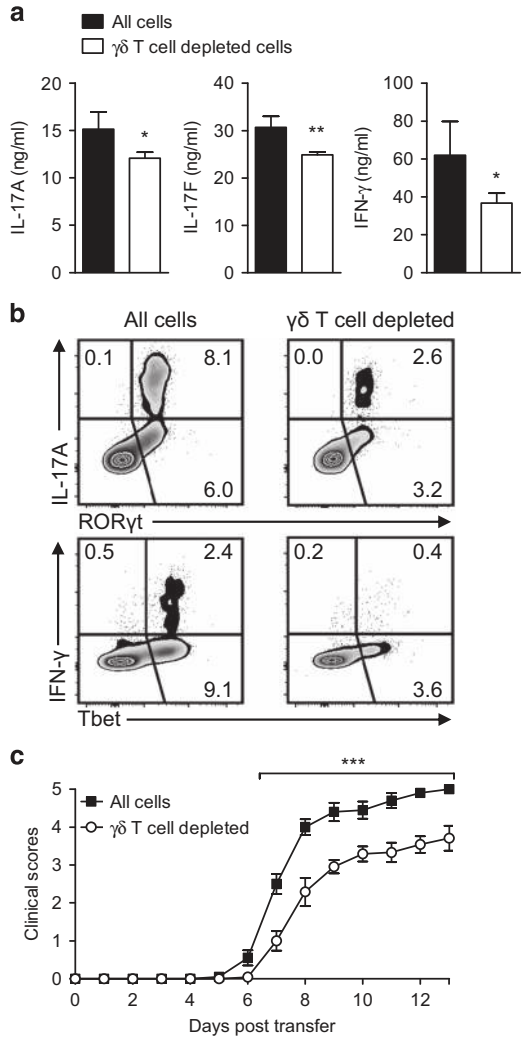

Figure 5 Depletion of γδ T cells attenuates EAE induced by T-cell transfer. Spleen and lymph node cells from MOG-immunized mice were depleted of γδ T cells or un-depleted. Cells were cultured for 3 days with MOG, IL-1β +IL-23, and then 15×10^6 cells were transferred to naive mice. (**a**) IL-17A, IL-17F and IFN-γ concentration measured by ELISA in supernatants the cultures after 3 days. (**b**) Representative FACS plots of IL-17A, RORγt, IFN-γ and Tbet expression in T helper cells at the end of the 3-day culture. (**c**) EAE clinical scores of recipient mice. Data are representative of two independent experiments and are shown as mean+s.d. (**a**) or mean ± s.e.m. (**c**). Statistics were calculated using two-tailed unpaired *t*-test (**a**) or a two-way analysis of variance with Bonferroni post-test (**c**). $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

Th17 cells, respectively) and attenuated their ability to produce IFN-γ and IL-17A, respectively (Figure 5b). Finally, we found that removal of γδ T cells prior to *in vitro* re-stimulation of MOG-primed T cells significantly impaired the ability of these T cells to induce EAE when transferred to naive mice (Figure 5c). These results indicate that γδ T cells contribute to the polarization of both Th1 and Th17 cells, reflected in their reduced pathogenic activity in the EAE model.

We next examined whether the attenuating effects of RA on induction of EAE by cell transfer could be mediated directly through its effect on γδ T cells. Thus, we isolated spleen and draining lymph node cells from MOG-immunized mice and separated them into γδ T cells and γδ T-cell-depleted fractions. The γδ T cells were stimulated for 6 h with IL-1β and IL-23, in the presence of RA or its vehicle, before being washed thoroughly and cultured with the γδ T-cell-depleted fraction. The combined cells were then cultured with IL-1β, IL-23 and MOG for 3 days and transferred to recipient mice. Prior to transfer, we examined the cytokine profile in the supernatants at the end of the 3-day culture. Consistent with the treatment of all the cells with RA, treatment of only the γδ T cells with RA resulted in a significant reduction of IL-17A and IL-17F production (Figure 6a). FACS analysis of the cells at the end of the 3-day culture revealed that IL-17A expression was significantly impaired in γδ T cells and principally in the $V\gamma4^+$ γδ T cells, which constituted the main IL-17A producers among γδ T cells in our model (Figures 6c and d). Interestingly, the production of IFN-γ was not affected by the RA-treated γδ T cells (Figure 6c and data not shown).

We next examined the effect of RA-treated γδ T cells on the capacity of MOG-specific T cell to induce EAE by adoptive transfer. Transfer of cells cultured with the vehicle-treated γδ T cells to naive mice induced EAE by day 6 in these mice (Figure 6d). In contrast, the onset of the disease was delayed and the severity of the symptoms was significantly reduced in recipients of the MOG-specific cells containing the RA-treated γδ T cells (Figure 6d). The frequency and absolute number of $CD4^+$ T cells producing IL-17A, IFN-γ and GM-CSF was reduced in the brains of the mice injected with the cells containing RA-treated γδ T cells (Figures 6e and f and data not shown). Taken together, our findings provide further evidence of a pathogenic role for γδ T cells in central nervous system autoimmunity, and demonstrate that treatment of these cells with RA impairs their IL-17A production, especially by the $V\gamma4^+$ subtype, which results in significant attenuation of their pathogenic function *in vivo*.

RA inhibits the IL-1β and IL-23 pathway in γδ T cells

We investigated the mechanisms by which RA modulates activation of γδ T cells. We first assessed the effect of RA on γδ T cells purified from naive mice. We found that, within 3 h of culture with IL-1β and IL-23, RA inhibited the expression of the receptors subunits for IL-1β and IL-23, which transduce the signals to promote IL-17A and IL-17F expression in γδ T cells (Figure 7a). Interestingly, stimulation of purified γδ T cells with IL-12 and IL-18, which are more potent stimuli for IFN-γ production, induced low concentration of IL-17A, which was significantly enhanced by RA, while the production of IL-17A induced by IL-1β and IL-23 was inhibited by RA (Figures 7b and c). In contrast, RA did not affect IFN-γ production by purified γδ T cells stimulated with IL-12 and IL-18 or IL-1β and IL-23 (Figure 7b). Moreover, RA did not impair IL-17 production by $CD4^+$ T cells and γδ T cells in cultures of spleen and LN cells stimulated with MOG alone (Figure 7d). We then examined the effect of RA on expression of *il1r1* and *il23r* on purified γδ T cells from MOG-immunized mice. The results revealed that RA inhibited the expression of *il1r1* and *il23r* in γδ T cells and principally in the $V\gamma4^+$ γδ T-cell subset (Figures 7e and f) and dramatically reduced the number of $V\gamma4^+$ γδ T cells expressing cell surface receptors for IL-1β and IL-23 (Figure 7g). The activation of the IL-1β/IL-23 pathway is known to involve the phosphorylation of STAT3, which induces Th17-type cytokine in IL-17-producing cells.[20] We found that treatment of cells from MOG-immunized mice with RA inhibited STAT3 phosphorylation (Figure 7h). Taken together, our data demonstrate that RA affects the expression of the receptors for IL-1β and IL-23 at the surface of γδ T cells, resulting in an impaired response of these cells to IL-1β and IL-23.

DISCUSSION

In this study, we demonstrate that RA significantly inhibits the function of IL-17A-producing γδ T cells, impairing their proliferation, cytokine production and their pathogenic activity *in vivo* in the EAE model. In addition, we show that under steady state conditions and during inflammation, RA also inhibits cytokine production by Th17 cells, which are considered to be the primary pathogenic T cells in EAE and other autoimmune diseases. Our findings suggest that a significant component of the attenuating effect of RA in immune-mediated disease is mediated through its effect on γδ T cells and in particular on the Vγ4$^+$ subtype. Finally, we show that RA has a selective suppressive effect on IL-17A and IL-17F production by γδ T cells, with little effect on IFN-γ, which is explained by its inhibitory effect on the IL-1β/IL-23 signaling pathway.

Following the discovery of Th17 cells, these cells have become the main focus of attention as mediators of pathology in many autoimmune diseases, including EAE–a mouse model for MS.[21,22] Indeed, IL-17A$^{-/-}$ mice are resistant to the development of EAE and the administration of anti-IL-17A-neutralizing antibody during the early stages of EAE delays the onset of the symptoms.[23,24] However, γδ T cells have also been shown to be an important source of IL-17A and to have a key pathogenic role in EAE.[3] Previous studies on the effect of RA on IL-17-producing T cells have focused on CD4$^+$ T cells; it has been reported that AM80, a RARα agonist, inhibits IL-17 production by Th17 cells.[15] However, this and other studies on the effect of RA or vitamin A deficiency did not examine the effect on γδ T cells. We report that RA has a potent inhibitory effect on IL-17 production by γδ T cells and in particular on the Vγ4$^+$ subtype that is pathogenic in EAE. Vγ4Vδ4$^+$ cells are the main IL-17-producing-γδ T-cell subtype in a model of collagen-induced arthritis.[25] Although we did not examine Vγ4$^+$Vδ4$^+$, we did examine the effect of RA on purified Vγ4$^+$ and Vγ1$^+$ cells and while Vγ4$^+$ were the predominant IL-17 producing cells, IL-17 production by both subtypes was inhibited by RA. Importantly, we demonstrated that RA had a greater effect on IL-17-producing γδ T cells than Th17 cells. One explanation for this observation may be that when compared with Th17 cells, IL-17-producing γδ T cells respond very quickly and strongly to the inflammatory cytokines IL-1β and IL-23 (inhibited by RA), without a need of TCR stimulation. It is also possible that IL-17-producing γδ T cells may express higher levels of nuclear receptors for RA.

We and others have previously reported that γδ T cells are an important source of IL-17 and related cytokines in the development of EAE and have demonstrated that γδ T cells as well as producing early IL-17 also promote activation of Th17 cells.[3,5,8] Consistent with these findings, the present study demonstrates that the differentiation of Th17 and Th1 cells and the production of their associated cytokines IL-17A, IL-17F and IFN-γ are impaired when γδ T cells are depleted from spleen and lymph node cells from MOG-immunized mice and stimulated *in vitro* with MOG, IL-1β and IL-23. Thus, the reduction in

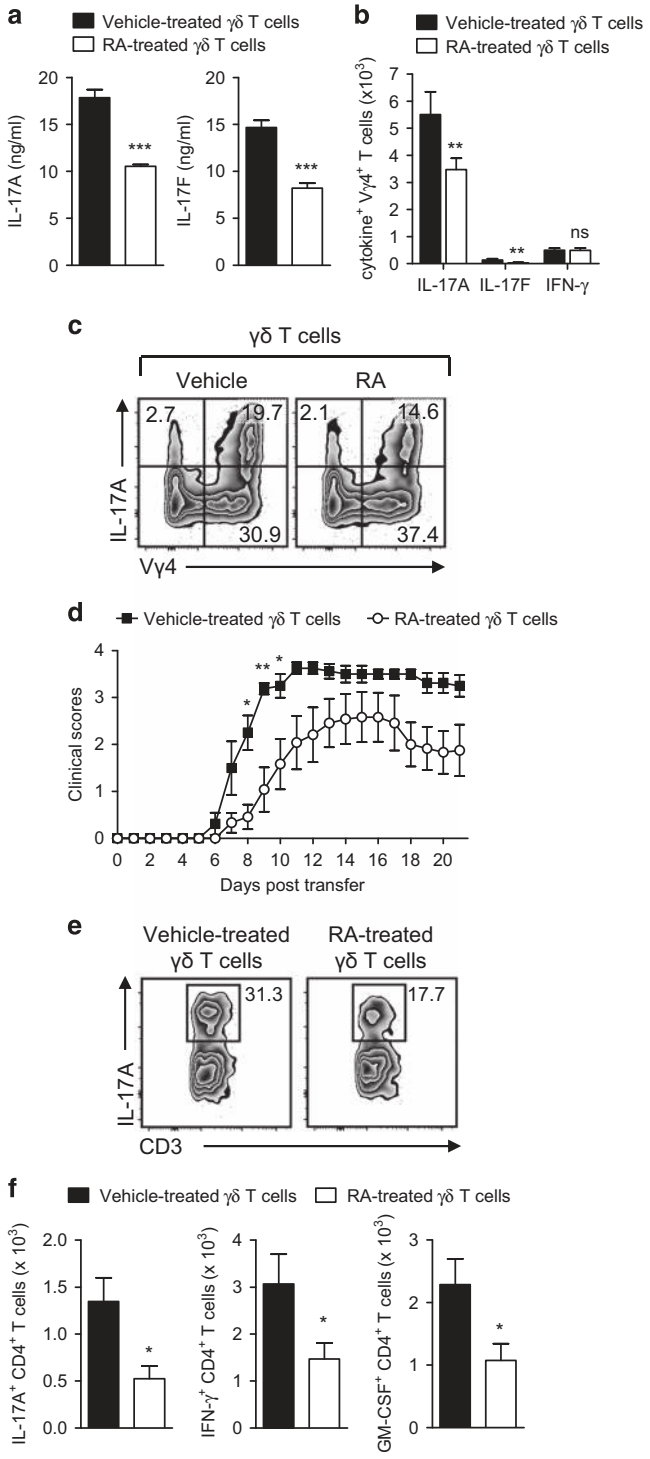

Figure 6 Selective treatment of γδ T cells with RA decreases the production of IL-17A and IL-17F during EAE and alters the capacity of T cells from MOG-immunized mice to induce EAE by cell transfer. Spleen and lymph node cells from MOG-immunized mice were isolated and the γδ T cells purified and cultured separately with RA or its vehicle for 6 h before being put back in culture with the rest of the cells. The total cells containing either the vehicle- or the RA-treated γδ T cells were then cultured with MOG, IL-1β and IL-23. After 3 days of culture, 10×10^6 cells were transferred to naive mice. (**a**) IL-17A and IL-17F expression as measured by ELISA in the supernatants at the end of the 3-day culture. (**b**) Mean absolute numbers of IL-17A$^+$, IL-17F$^+$ and IFN-γ$^+$ Vγ4$^+$ γδ T cells at the end of the 3-day culture. (**c**) Representative FACS plots indicating the proportion of IL-17A$^+$ Vγ4$^+$ and IL-17A$^+$ Vγ4$^-$ γδ T cells at the end of the 3-day culture. (**d**) EAE clinical scores of recipient mice. (**e**) Representative FACS plots indicating the proportion of IL-17A$^+$ CD4$^+$ T cells in the brains of the recipient mice on day 25 post transfer. (**f**) Absolute numbers of infiltrating IL-17A$^+$, IFN-γ$^+$ and GM-CSF$^+$ CD4$^+$ T cells in the brains of the recipient mice on day 25 post transfer. Data are representative of two independent experiments. Bars are mean+s.d. (**a**, **b**), mean ± s.e.m. (**d**) or mean+s.e.m. (**f**). Statistics are performed as and two-tailed unpaired *t*-test (**a**–**c** and **f**) or two-way analysis of variance with Bonferroni post-test (**d**); $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

pathogenic cytokines observed with RA treatment can be attributed to a combination of a direct effect of RA on IL-17 production by γδ T cells and an indirect effect of impaired IL-17 production by γδ T cells on Th1- and Th17-cell differentiation. Furthermore, the reduced IFN-γ production observed after depletion of γδ T cells reflect a removal of IFN-γ-secreting γδ T cells and impaired differentiation of Th1 cells. This is consistent with our demonstration that depletion of γδ T cells from the cultures impaired the ability of T cells to induce EAE when transferred to naive recipient mice. We have previously shown that RA protects against the development of colitis by inducing IL-22 production by both γδ T cells and ILC3.[18] Here, we show that in the adoptive transfer model of EAE, while RA reduces cytokine production by Th17 cells, it has a more dramatic effect on γδ T cells, impairing their proliferation, RORγt expression and production of IL-17A and IL-17F. Recipient mice injected with RA-treated T cells developed very mild EAE with delayed onset when compared with that seen after transfer of untreated cells. Furthermore, central nervous system infiltration of $CD4^+$ and γδ T cells producing IL-17A, IL-17F, GM-CSF and IFN-γ was strongly impaired in mice injected with RA-treated cells. Moreover, we demonstrate that the treatment of γδ T cells only with RA conferred significant protection against the development of EAE in the recipient mice, and this was associated with a significant decrease in the number of IL-17A, GM-CSF and IFN-γ producing $CD4^+$ T cells infiltrating the brain.

IL-1β and IL-23 are key pathogenic cytokines, which drive the development of many autoimmune diseases.[19,21] Mice deficient for the IL-1β receptor IL-1R1 or mice lacking the IL-23 subunit IL-23p19 are resistant to induction of EAE.[3,26,27] Interestingly, we demonstrate that RA directly inhibits the expression of the receptors for IL-1β and IL-23, IL-1R1 and IL-23R respectively, on activated γδ T cells *in vitro*. Finally, we demonstrate that RA impairs the IL-23 pathway by inhibiting phosphorylation of the transcriptional activator of *il17a* and *il17f,* STAT3.[28]

There is convincing evidence from animal models that Th17 cells and more recently IL-17-secreting γδ T cells have crucial pathogenic roles in EAE and other autoimmune diseases. However, the role of IFN-γ, produced by Th1 cells, $CD8^+$ T cells and NK cells is less clear.[29–31] Interestingly, we find little effect of RA on IFN-γ production by $CD4^+$ T cells or even when γδ T cells are cultured with IL-12 and IL-18, the classical stimuli for IFN-γ production.

It has been reported that RA synergizes with TGF-β to induce Treg cells, especially in the gut in a steady state.[10] Moreover, in the pro-inflammatory context of collagen-induced arthritis, a mouse model of rheumatoid arthritis, RA has a protective effect against the development of the disease by promoting $Foxp3^+$ Treg cells, as well as inhibiting the differentiation of autoreactive Th17 cells.[32] In the EAE model, TGF-β protects against disease by inducing $Foxp3^+$ Treg cells, which migrate into the central nervous system and counterbalance the ratio of effector to Treg cells.[33] Moreover, this effect is mediated through IL-10.[33] We found that while RA induces the expression of TGF-β during EAE, the production of IL-10 is decreased (unpublished observations), indicating that RA does not protect against disease by inducing Treg cells. Interestingly, Klemann and colleagues[15] demonstrated that a RARα agonist inhibited EAE induced by active immunization. Collectively, these findings suggest that in the context of inflammatory responses, RA can exert immunosuppressive effects on effector T cells.[11]

In this study, we provide further evidence that γδ T cells have a significant pathogenic role in EAE, directly or through the activation of Th17 cells. Furthermore, we show that RA can suppress IL-17A and IL-17F production by γδ T cells, thereby attenuating their pathogenic activity *in vivo* in central nervous system autoimmunity. Our results provide new insight into the mechanisms of action of RA and demonstrate that RA selectively inhibits the production of pathogenic cytokines in autoimmune disease. The findings also provide further evidence for the potential of targeting γδ T cells in the treatment of autoimmune diseases.

METHODS

Mice

C57BL/6 mice (Harlan, Bicester, UK) were bred under specific pathogen free conditions and maintained according to European Union regulations. All experiments were performed under license from the Health Products Regulatory Authority and with approval from the Trinity College Dublin Animal Research Ethics Committee. The mice were co-housed for every EAE experiment.

Stimulation and FACS analysis of peritoneal exudate cells

Mice were injected intraperitoneally with 250 μg of RA or vehicle (dimethyl sulfoxide) in phosphate-buffered saline. After 18 h, the mice were killed and the peritoneal exudate cells collected and cultured for 2 h with phorbol 12-myristate 13-acetate (Sigma-Aldrich, St Louis, MO, USA, 10 ng ml^{-1}), ionomycin (Sigma-Aldrich, 1 μg ml^{-1}) in the presence of brefeldin A (5 μg ml^{-1}). Cytokine production was assessed by intracellular cytokine staining and FACS analysis of the cells.

Induction and assessment of EAE by adoptive transfer

Donor mice were immunized subcutaneously with 100 μg of MOG_{35-55} peptide (GenScript, Piscataway, NJ, USA) emulsified in complete Freund's adjuvant containing 4 mg ml^{-1} (0.4 mg per mouse) heat-killed *Mycobacterium tuberculosis* (Chondrex, Redmond, WA, USA). After 10 days, the mice were killed, their brachial, axillary and inguinal lymph nodes and spleens were collected and a single cell suspension was prepared. The cells were then stimulated for 3 days at a concentration of 10×10^6 cells per ml with medium, MOG (100 μg ml^{-1}) or with MOG, IL-1β (Immunotools, Friesoythe, Germany, 10 ng ml^{-1}) and IL-23 (R&D Systems, 10 ng ml^{-1}) and with RA (Enzo, Farmingdale, NY, USA, 10^{-6} M) or its vehicle (ethanol). In some experiments, γδ T cells were purified by magnetic-activated cell sorting (Miltenyi, Bergisch Gladbach, Germany, purity >90%; Supplementary Figure 1) prior to the culture and either depleted from the culture or added back to the rest of the cells (control). The cells were then stimulated for 3 days with MOG, IL-1β and IL-23. In other experiments, γδ T cells were magnetic-activated cell sorting-purified prior to the culture and stimulated separately with IL-1β, IL-23 and RA or its vehicle for 6 h. γδ T cells were washed thoroughly, put back with the rest of the cells and the total cells were then stimulated with MOG, IL-1β and IL-23 for 3 days. Cytokine production was assessed by intracellular cytokine staining and FACS analysis of the cells or by ELISA (R&D Systems, Minneapolis, MN, USA and BD Biosciences, San Jose, CA, USA) on the supernatants. For transfer to recipient animals, the cells were thoroughly washed and $10–15 \times 10^6$ live cells were injected intraperitoneally into co-housed recipient mice. The animals were weighed and monitored for signs of EAE daily. The disease severity was assessed as follows: 0, no clinical sign; 1, limp tail; 2, ataxic gait; 3, hind limb weakness; 4, hind limb paralysis; 5, tetraplegia/moribund.

Isolation and FACS analysis of brain cells

Mice were killed and perfused with phosphate-buffered saline, and their brains were isolated. Brain mononuclear cells were then purified by density gradient centrifugation in Percoll and stimulated for 5 h with phorbol 12-myristate 13-acetate (Sigma-Aldrich, 10 ng ml^{-1}), ionomycin (Sigma-Aldrich, 1 μg ml^{-1}) in the presence of brefeldin A (5 μg ml^{-1}) and then stained for intracellular cytokines and analyzed by FACS.

Purification and culture of γδ T cells

γδ T cells were purified from lymph node cells by magnetic-activated cell sorting (purity >90%; Supplementary Figure 1) and $CD27^+$ and $CD27^-$ or $V\gamma1^+$ and $V\gamma4^+$ γδ T cells were sorted by FACS (Dako Moflo, Beckman Coulter, Indianapolis, IN, USA). The cells were stimulated with IL-1β and IL-23

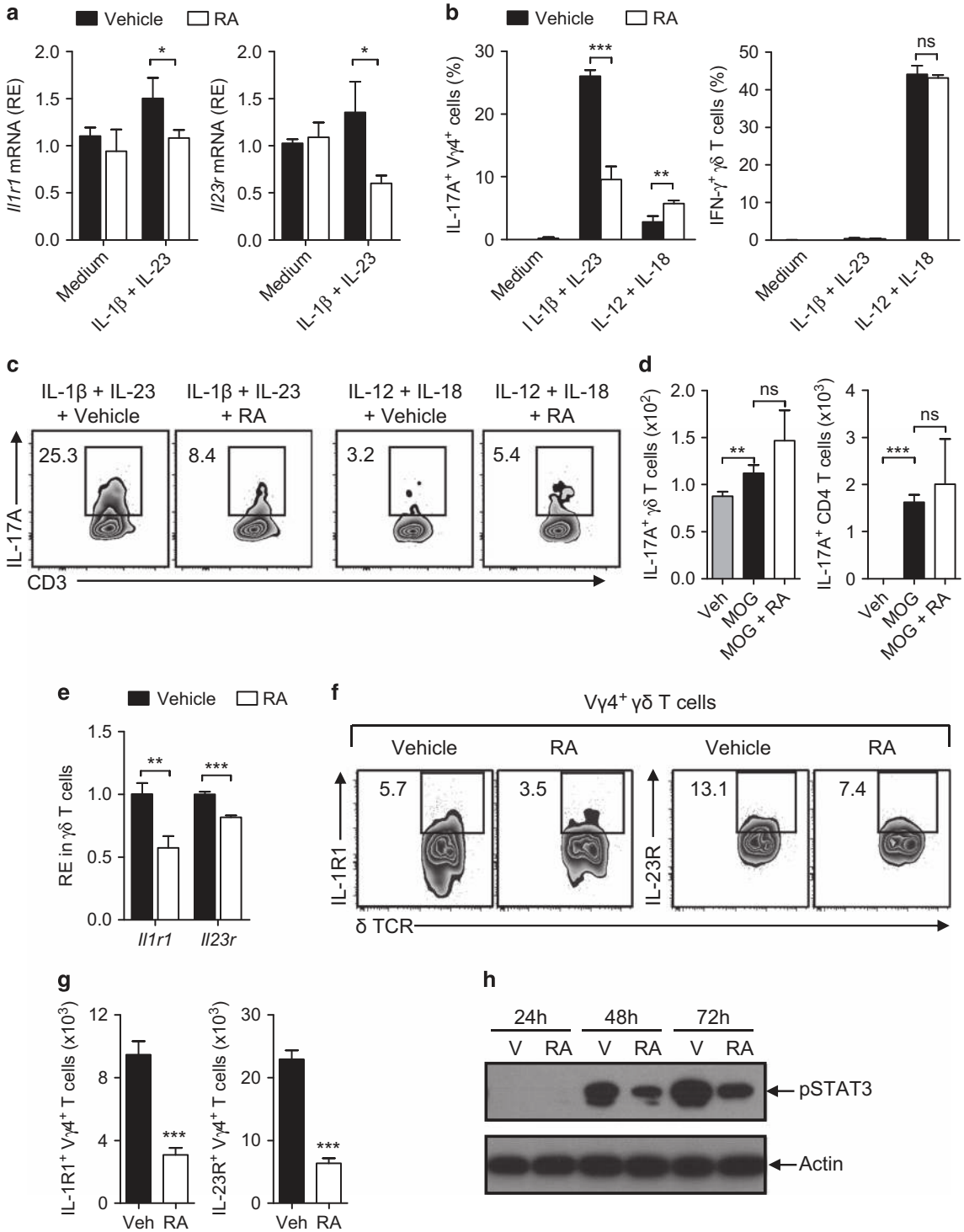

Figure 7 RA impairs the IL-1β and IL-23 pathway in γδ T cells but does not alter cytokine production induced by IL-12 and IL-18. (**a**) Relative expression of *il1r1* and *il23r* mRNA measured by RT-PCR in γδ T cells purified from naive mice and cultured for 3 h with IL-1β+IL-23, with or without RA. (**b**, **c**) γδ T cells purified from lymph nodes of naive mice were cultured for 24 h with IL-1β+IL-23 or IL-12+IL-18, and with or without RA. Mean frequencies (**b**) and corresponding FACS plots (**c**) of IL-17A$^+$ Vγ4$^+$ CCR6$^+$ γδ T cells and IFN-γ$^+$ γδ T cells in the culture. (**d**) Cells from MOG-immunized mice were cultured with vehicle, MOG or MOG+RA for 24 h and absolute numbers of IL-17A$^+$ γδ T cells and CD4 T cells were determined by FACS. (**e–g**) Cells from MOG-immunized mice were cultured for 3 days with MOG, IL-1β and IL-23 and either RA or its vehicle. (**e**) Relative expression of *il1r1* and *il23r* mRNA measured by RT-PCR on γδ T cells sorted at the end of the 3-day culture. (**f**, **g**) Representative FACS plots (**f**) and mean frequencies (**g**) of IL-1R1 and IL-23R expression on Vγ4$^+$ T cells after 3 days of culture. (**h**) Western blots showing the effect of RA on STAT3 phosphorylation (pSTAT3) with actin as a control after 24, 48 and 72 h of culture (V: vehicle). Bars are mean+s.d.; $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, two-tailed unpaired *t*-test.

(γδ T cells, Vγ1$^+$ and Vγ4$^+$ γδ T cells), or with IL-12 and IL-18 (γδ T cells), together with RA (10^{-6} M) or its vehicle (ethanol) for 3 h (RT-PCR), 24 h (flow cytometry) or 72 h (ELISA). The concentrations of the cytokines IL-17A, IL-17F and IFN-γ in culture supernatants were quantified by ELISA.

Flow cytometry

Brefeldin A (Sigma-Aldrich, 5 μg ml^{-1}) was added to the cell cultures for 4 h. Cells were washed before being incubated with a live/dead stain and then surface-stained with antibodies specific for CD3, CD4, CD8, δTCR, Vγ1, Vγ4, IL-1R1, IL-23R and CCR6. The cells were washed, fixed and permeabilized using 2% PFA (Pierce, Walthman, MA, USA) or the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA), which allow the staining of intranuclear proteins. The cells were stained in 0.5% saponin (Sigma-Aldrich) or permeabilization buffer (eBioscience) containing antibodies directed against IL-17A, IL-17F, IFN-γ, GM-CSF, RORγt, Tbet and Ki67. Cells were analyzed using a flow cytometer LSRFortessa (BD) and the data were analyzed with FloJo software. The FACS profile for the different intracellular and surface proteins analyzed were established by gating on single, then live cells and further on CD3$^+$ TCRδ$^+$ for γδ T cells; CD3$^+$TCδ$^+$ Vγ1$^+$ for Vγ1$^+$ γδ T cells; CD3$^+$TCRδ$^+$Vγ4$^+$ for Vγ4$^+$ γδ T cells; CD3$^+$ TCRδ$^+$Vγ4$^+$CCR6$^+$ for the IL-17A-producing Vγ4$^+$ γδ T cells; CD3$^+$ TCRδ$^-$CD8$^-$CD4$^+$ for CD4$^+$ T cells.

Reverse transcription-PCR

RNA was extracted from the cells using TRIzol (Invitrogen, Grand Island, NY, USA) and reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). *Rara*, *il17a*, *il17f*, *rorc*, *il1r1* and *il23r* expression were quantified by RT-PCR using commercially available primers and an ABI PRISM7500 Sequence Detection System (Applied Biosystems). The amount of each cytokine was determined by normalization to 18S rRNA.

Western blots

Cells were resuspended in ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 1.5 mM Na_3VO_4 and 1 mM EDTA), supplemented with SIGMAFAST protease inhibitors (Sigma-Aldrich) and benzonase (Merck Millipore, Billerica, MA, USA, 25 U per cell pellet), and lysed for 30 min at 4 °C with rotation. Samples were then centrifuged at 17 000 *g* for 20 min at 4 °C. Supernatants were assayed for protein concentration using the Pierce 660 nm protein assay and diluted to the same protein concentration with lysis buffer. Aliquots corresponding to 10 μg of cell protein were diluted with 5× SDS-PAGE sample buffer, heated to 95 °C for 10 min and centrifuged at 12 000 *g* for 5 min at room temperature. Samples were run on a 10% SDS-PAGE gel, transferred to polyvinylidene difluoride and analyzed by immunoblot using polyclonal rabbit antibody raised against mouse phospho-STAT3 (Cell Signaling, Danvers, MA, USA), mouse monoclonal antibody raised against beta actin (Abcam, Cambridge, MA, USA), anti-mouse IgG, HRP conjugate (Promega, Fitchburg, WI, USA) and anti-rabbit IgG, HRP conjugate (Dako, Carpinteria, CA, USA).

Statistics

Statistical analysis was performed using GraphPad Prism 5. Analysis of variance with Bonferroni post-test or Student *t* test were used to compare statistical differences of means between groups.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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