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IL-21 initiates an alternative pathway to induce proinflammatory T_H17 cells

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Abstract

On activation, naive T cells differentiate into effector T-cell subsets with specific cytokine phenotypes and specialized effector functions[1]. Recently a subset of T cells, distinct from T helper (T_H)1 and T_H2 cells, producing interleukin (IL)-17 (T_H17) was defined and seems to have a crucial role in mediating autoimmunity and inducing tissue inflammation[2–5]. We and others have shown that transforming growth factor (TGF)-β and IL-6 together induce the differentiation of T_H17 cells, in which IL-6 has a pivotal function in dictating whether T cells differentiate into $Foxp3^+$ regulatory T cells (T_{reg} cells) or T_H17 cells[6–9]. Whereas TGF-β induces Foxp3 and generates T_{reg} cells, IL-6 inhibits the generation of T_{reg} cells and induces the production of IL-17, suggesting a reciprocal developmental pathway for T_H17 and T_{reg} cells. Here we show that IL-6-deficient ($Il6^{-/-}$) mice do not develop a T_H17 response and their peripheral repertoire is dominated by $Foxp3^+$ T_{reg} cells. However, deletion of T_{reg} cells leads to the reappearance of T_H17 cells in $Il6^{-/-}$ mice, suggesting an additional pathway by which T_H17 cells might be generated *in vivo*. We show that an IL-2 cytokine family member, IL-21, cooperates with TGF-β to induce T_H17 cells in naive $Il6^{-/-}$ T cells and that IL-21-receptor-deficient T cells are defective in generating a T_H17 response.

We previously proposed a reciprocal relationship between T_H17 and T_{reg} cells and suggested that IL-6 is pivotal in determining whether an immune response is dominated by T_H17 or T_{reg} cells[9,10]. We predicted that $Il6^{-/-}$ mice would not develop a T_H17 response and should have high numbers of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the peripheral repertoire. Thus, we crossed $Il6^{-/-}$ mice with *Foxp3gfp* 'knock-in' (*Foxp3gfp*.KI) mice[9,11] and analysed the presence of T_{reg} versus T_H17 cells in $Il6^{-/-}$ × *Foxp3gfp*.KI mice after immunization with the encephalitogenic myelin oligodendrocyte glycoprotein 35–55

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($MOG_{35–55}$) peptide emulsified in complete Freund's adjuvant (CFA). We and others have shown previously that *Il6*$^{-/-}$ mice are resistant to the development of experimental autoimmune encephalomyelitis (EAE)[9,12–15]. However, the cellular or molecular basis for this resistance was unclear although some studies suggested that *Il6*$^{-/-}$ mice might have a defect in T-cell priming[12,13,16]. On immunization, *Il6*$^{-/-}$ × *Foxp3gfp*.KI mice mounted an attenuated T-cell response with a defect in the generation of T_H17 cells (Fig. 1a). We found that *Il6*$^{-/-}$ × *Foxp3gfp*.KI mice had a significantly elevated fraction of Foxp3$^+$ T_{reg} cells in the draining lymph nodes (Fig. 1b). Thus, the 'impaired priming' of antigen-specific T-cell responses reported in *Il6*$^{-/-}$ mice could simply be due to the expansion of T_{reg} cells at the expense of effector T cells in an environment that is devoid of IL-6. We therefore used $MOG_{35–55}/IA^b$ (MHC class II) tetramers to analyse the frequency of antigen-specific (tetramer$^+$) effector T cells (Foxp3/GFP$^-$) and T_{reg} cells (Foxp3/GFP$^+$). In contrast with wild-type mice, *Il6*$^{-/-}$ mice had more MOG-specific T_{reg} cells than effector T cells and the ratio of antigen-specific T_{reg} to effector T cells was reversed (Fig. 1c, d). Thus, the lack of IL-6 favoured the generation or expansion of antigen-specific T_{reg} cells and inhibited the development of effector T-cell responses. These data are consistent with our hypothesis that IL-6 is crucial in dictating the balance between effector T cells and T_{reg} cells.

The deletion of T_{reg} cells from the peripheral repertoire of *Il6*$^{-/-}$ mice should therefore result in T-cell priming and induction of autoimmunity dominated by interferon (IFN)-γ-producing T_H1 cells but not T_H17 cells. We depleted T_{reg} cells in *Il6*$^{-/-}$ mice by using a monoclonal antibody against CD25 (which led to more than 50% depletion of Foxp3$^+$ T_{reg} cells; Supplementary Fig. 1) and then immunized with $MOG_{35–55}$/CFA. As predicted and in contrast with untreated *Il6*$^{-/-}$ mice, T_{reg}-depleted *Il6*$^{-/-}$ mice became susceptible to EAE (Fig. 2a, b and Table 1). However, cytokine analysis by enzyme-linked immunosorbent assay (ELISA) and intracellular cytokine staining of CD4$^+$ T cells from T_{reg}-depleted *Il6*$^{-/-}$ mice yielded a reappearance of T_H17 cells (Fig. 2c, d). IL-17 production in T_{reg}-depleted *Il6*$^{-/-}$ mice reached almost wild-type levels, suggesting that T_H17 cells can be generated *in vivo* in the absence of IL-6. After confirming that *Il6*$^{-/-}$ mice did not produce residual amounts of IL-6 (Supplementary Fig. 2), we reasoned that there might be an alternative pathway that induces T_H17 cells in the absence of IL-6.

Our previous studies suggested a reciprocal relationship between T_{reg} and T_H17 cells[9]. Using this logic, we screened several cytokines to identify possible candidates that could substitute for IL-6. We first tested those cytokines that were able to suppress the TGF-β-mediated induction of Foxp3 in sorted naive Foxp3/GFP$^-$ T cells derived from *Foxp3gfp*.KI mice. As well as IL-6, the IL-12 family member IL-27 and the IL-2 family member IL-21 were most efficient in inhibiting TGF-β-driven Foxp3 induction (Fig. 3a, b, and Supplementary Fig. 3). Our analysis confirmed previous results[17,18] showing that IL-27 did not induce the differentiation of T_H17 cells when combined with TGF-β. In contrast, IL-21 in combination with TGF-β not only suppressed Foxp3 expression but also induced IL-17 production from naive T cells, although not as strongly as TGF-β plus IL-6 (Fig. 3b, c). Retinoic acid receptor-related orphan receptor (ROR)-γt has been shown to be a transcription factor involved in the differentiation of T_H17 cells[19], and TGF-β plus IL-21 also induced ROR-γt expression in differentiating T_H17 cells (Fig. 3d). Thus, IL-21 could substitute for IL-6 in the induction of ROR-γt and IL-17, indicating that in addition to IL-6, IL-21 might cooperate with TGF-β to drive the differentiation of T_H17 cells.

To test whether IL-21 was responsible for the induction of IL-17 from naive T cells that do not produce IL-6, we compared the differentiation of naive wild-type and *Il6*$^{-/-}$ T cells *in vitro* in the presence of TGF-β plus IL-21 or TGF-β plus IL-6. TGF-β plus IL-21 was sufficient to drive *Il6*$^{-/-}$ T cells into the T_H17 pathway, although less efficiently than for wild-type T cells (Fig. 4a). Thus, in an IL-6-deficient environment, TGF-β plus IL-21 could

act independently of IL-6 to induce T_H17 cells. However, the frequency of T_H17 cells induced by TGF-β plus IL-21 was consistently lower than that of the induction of T_H17 cells driven by TGF-β plus IL-6.

Next we evaluated whether IL-21 was involved in the induction of T_H17 differentiation under 'standard' differentiation conditions of IL-6 plus TGF-β and whether the two cytokines (IL-6 and IL-21)cooperated to induce T_H17 cells. T cells activated in the presence of IL-6 alone or in combination with TGF-β expressed IL-21 (Fig. 4b). Furthermore, of all $CD4^+$ T-cell subsets, T_H17 cells were the highest producers of IL-21 (Fig. 4b). This suggested that there might be an amplification loop in which IL-21 produced by T_H17 cells participates in enhancing further differentiation of T_H17 cells. We therefore compared the induction of T_H17 cells by TGF-β plus IL-6 in wild-type and *Il21r*$^{-/-}$ T cells[20]. When IL-6 and TGF-β were used to drive the differentiation of T_H17 cells, the frequency of T_H17 cells induced in *Il21r*$^{-/-}$ $CD4^+$ T cells was half that of wild-type T cells (Fig. 4c), suggesting that IL-21 might normally contribute to T_H17 differentiation mediated by TGF-β plus IL-6. If these in vitro data were accurate, the T_H17 response in *Il21r*$^{-/-}$ mice should be deficient. We found that the fraction of $CD4^+CD44^+IL\text{-}17^+$ T cells was significantly reduced in *Il21r*$^{-/-}$ mice in comparison with wild-type controls (data not shown), and this difference was also observed when $CD44^{hi}$ T cells were activated *in vitro* (Fig. 4d, e). Notably, the addition of recombinant IL-23 could not compensate for the defective T_H17 response observed in $CD44^+$*Il21r*$^{-/-}$ T cells (Fig. 4d, e). On immunization, *Il21r*$^{-/-}$ mice showed no appreciable defect in T-cell proliferation but a marked decrease in their ability to generate a T_H17 response, whereas the production of IFN-γ seemed to be increased (Fig. 4f). These data suggest that IL-21 produced by differentiated T_H17 cells must have a function in amplifying T_H17 differentiation such that in the absence of IL-21 signalling, the T_H17 response is compromised. Whereas IL-6 is predominantly produced by cells of the innate immune system, IL-21 is produced mainly by the adaptive immune system, and activated T cells are the main source of IL-21 (Supplementary Fig. 4). We therefore speculate that TGF-β and IL-6 together initiate the differentiation of T_H17 cells, which in turn can produce IL-21 and thus further amplify the T_H17 differentiation process. However, in the absence of IL-6, TGF-β together with IL-21 can drive T_H17 differentiation on their own. IL-6 and IL-21 cooperate in T_H17 differentiation, which is further supported by the observation that *in vivo* neutralization of IL-21 in T_{reg}-depleted *Il6*$^{-/-}$ mice essentially abrogated the generation of T_H17 cells (Supplementary Fig. 5). This suggests that IL-6 and IL-21 independently and together must cooperate with TGF-β to induce T_H17 differentiation.

In summary, together with TGF-β, IL-21 constitutes an additional pathway for the generation of pathogenic T_H17 cells. The induction of IL-17 in naive T cells by a combination of TGF-β and IL-21 also suppresses Foxp3 expression, suggesting that similarly to IL-6, IL-21 is able to regulate the reciprocal developmental pathway of generation of T_{reg} and T_H17 cells. The importance of the Idd3 genetic locus, which controls susceptibility to both EAE and type 1 diabetes and regulates the function of T_{reg} cells[21], might in part be due to the presence of *Il21* in addition to *Il2* in the *Idd3* interval. IL-21 binds to the IL-21 receptor, which consists of a unique IL-21 receptor subunit and the common cytokine receptor γ-chain shared by other cytokine receptors including the receptors for IL-2, IL-4, IL-7, IL-9 and IL-15 (ref. 22). In contrast to these cytokines, binding of IL-21 to its receptor preferentially activates the downstream signalling molecules STAT1 and STAT3 (ref. 23). Because STAT3 is an important signalling molecule in T_H17 differentiation[24], it is likely that IL-21-induced activation of STAT3, like IL-6-induced activation of STAT3, cooperates with TGF-β signalling to induce the transcription factor ROR-γt and drive T_H17 differentiation.

Because differentiated T_H17 cells may be the most robust producers of IL-21, we propose that the ability of T_H17 cells to produce IL-21 further amplifies the T_H17 response. This reverberating pathway is similar to the mechanism in which IL-4 produced by T_H2 cells promotes T_H2 differentiation[25,26]. However, T_H17 responses also seem to be tightly counter-regulated in that IL-27, an IL-12 family member produced by cells of the innate immune system, and IL-25, an IL-17 family member produced by the innate immune system and activated T cells, can dampen and eventually shut off T_H17 responses[17,18,27]. We suggest that enhanced autoimmunity observed in mice injected with exogenous IL-21 (ref. 28) might be partly due to increased differentiation of T_H17 cells and suppression of T_{reg} generation *in vivo*. Targeting IL-21 in autoimmune diseases may therefore readjust the balance between pathogenic T_H17 and Foxp3$^+$ T_{reg} cells, which is believed to be defective in human autoimmune diseases.

In vitro T-cell proliferation and measurement of cytokines

Proliferation was determined by incorporation of [^{3}H]thymidine. Cytokines were measured by ELISA, cytometric bead array (BD Biosciences) or fluorescent bead assay (Luminex). Intracellular cytokine staining and isolation of mRNA for determination of cytokine expression by real-time polymerase chain reaction (PCR) were performed after stimulation with 12-*O*-tetradecanoylphorbol-13-acetate/ionomycin (see Methods).

T-cell differentiation *in vitro*

Naive CD4$^+$ T cells (CD4$^+$CD62L^{hi} or CD4$^+$CD62L^{hi}Foxp3/GFP$^-$) were purified by fluorescence-activated cell sorting and stimulated for three days with plate-bound antibody against CD3 (145-2C11, 4 μg ml^{-1}) plus soluble antibody against CD28 (PV-1, 2 μg ml^{-1}) or by soluble anti-CD3 (2 μg ml^{-1}) plus irradiated syngeneic splenocytes as antigen-presenting cells and recombinant cytokines: human TGF-β1 (3 ng ml^{-1}), mouse IL-6 (30 ng ml^{-1}), mouse IL-21 (100 ng ml^{-1}) or mouse IL-27 (25 ng ml^{-1}; all from R&D Systems). Blocking of IL-21 activity *in vitro* was performed by the addition of a goat anti-mouse IL-21 antibody (25 μg ml^{-1}; R&D Systems) or by IL-21R-Ig (100 μg ml^{-1}). Polarization of T cells into T_H1, T_H2 or T_H17 cells was performed by solid-phase anti-CD3 (4 μg ml^{-1}) and soluble anti-CD28 (2 μg ml^{-1}) in the presence of recombinant mouse IL-12 (10 ng ml^{-1}; R&D Systems) plus anti-IL-4 (11B.11; 10 μg ml^{-1}) for T_H1, mouse IL-4 (10 ng ml^{-1}; R&D Systems) plus anti-IL-12 (C17.8; 10 μg ml^{-1}) for T_H2, and TGF-β plus IL-6 for T_H17. Monoclonal antibodies against mouse CD3, mouse CD28, mouse IL-4 and mouse IL-12 were purified from the supernatants of hybridomas obtained from the American Type Culture Collection (ATCC).

METHODS

Induction of EAE

EAE was induced by subcutaneous immunization of mice with 100 μl of an emulsion containing 100 μg of $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) and 250 μg of *M. tuberculosis* H37 Ra (Difco) in incomplete Freund's adjuvant oil plus an intraperitoneal injection of 200 ng of pertussis toxin (List Biological Laboratories) on days 0 and 2. For T_{reg} depletion, animals were injected intraperitoneally with 500 μg of monoclonal antibody against CD25 (PC61) on days −5 and −3 before immunization. Clinical signs of EAE were assessed as reported[11]. Linear regression analysis of individual EAE scores was performed for the acute disease phase (until the mean maximum score was reached in each group) and the chronic disease phase (over the complete disease course). Animals were kept in a conventional, pathogen-free facility at the Harvard Institutes of Medicine, and all

experiments were performed in accordance with the guidelines prescribed by the standing committee of animals at Harvard Medical School, Boston, Massachusetts.

Generation of IL-21 receptor-Ig (IL-21R-Ig)

The complementary DNA fragment encoding the extracellular domain (amino acids 20–236) of mouse IL-21R (GenBank accession number NM_021887) was amplified by PCR from a mouse splenocyte cDNA library. A second cDNA fragment encoding the Fc portion of human IgG4 was cloned by PCR from a human cDNA library from peripheral blood mononuclear cells. The two cDNA fragments were joined in frame by overlapping PCR and subsequently cloned into the mammalian expression vector pSecTag/FRT/V5-His-TOPO (Invitrogen). The ligated construct (1.0 μg) was co-transfected with pOG44 (10.0 μg; Invitrogen) into the Flp-In CHO cell line (Invitrogen), using GeneJammer transfection reagent (Stratagene), in accordance with the manufacturer's instructions. Transfectants were selected in 800 μg ml^{-1} hygromycin B, and maintained in UltraCHO (BioWhittaker). IL-21R-Ig was purified from the culture supernatant by passage through a Protein G–Sepharose column. Bound protein was eluted with 100 mM glycine-HCl pH 3.0 and immediately neutralized with 1.25 M Tris-HCl pH 8.8. The eluted protein was concentrated with an UltraFree-4 centrifugal device (Millipore), and the concentration was determined spectrophotometrically. The reagent was tested *in vitro* for its ability to neutralize the activity of IL-21-mediated T_H17 differentiation.

Preparation of mononuclear cells from the central nervous system

After perfusion through the left cardiac ventricle with cold PBS, forebrain and cerebellum were dissected and spinal cords were flushed out with PBS by hydrostatic pressure followed by digestion with collagenase D (2.5 mg ml^{-1}; Roche Diagnostics) and DNase I (1 mg ml^{-1}; Sigma) at 37 °C for 45 min. Mononuclear cells were isolated by passing the tissue through a cell strainer (70 μm mesh) and Percoll gradient (70%–37%) centrifugation. Mononuclear cells were removed from the interphase, washed and resuspended in culture medium for further analysis.

In vitro T-cell proliferation

Draining lymph-node cells or splenocytes were cultured in DMEM/10% FCS supplemented with 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, non-essential amino acids, L-glutamine and 100 U ml^{-1} penicillin/100 μg ml^{-1} streptomycin. For antigen-specific recall cultures, 2.5×10^6 lymph-node cells or 5×10^6 ml^{-1} splenocytes were cultured for three days in the presence of $MOG_{35–55}$ or $OVA_{323–339}$. During the last 16 h, cells were pulsed with 1 μCi of [^{3}H]thymidine (PerkinElmer). [^{3}H]Thymidine incorporation in triplicate wells was measured with a β-counter (1450 MicroBeta Trilux; PerkinElmer).

Measurement of cytokines

Cell culture supernatants were collected after 48 h and the secreted cytokines were determined by ELISA (antibodies for IL-17 from BD Biosciences), by cytometric bead array (BD Biosciences) or by fluorescent bead assay (Luminex) for the indicated cytokines, in accordance with the manufacturers' instructions. For quantitative PCR, RNA was extracted from FACS-sorted cells by using RNAeasy columns (Qiagen) after 48 h of stimulation *in vitro*. cDNA was transcribed as recommended (Applied Biosystems) and used as a template for quantitative PCR. Primer/probe mixtures for mouse ROR-γt and mouse IL-21 were obtained from Applied Biosystems. The Taqman analysis was performed on the AB 7500 Fast System (Applied Biosystems). Gene expression was normalized to the expression of β-actin.

Staining with $MOG_{35–55}/IA^b$ tetramers and intracellular cytokine staining

$MOG_{35–55}/IA^b$ tetramers were generated as reported[11]. The procedure for staining *ex vivo* with $MOG_{35–55}/IA^b$ tetramers has been described in detail previously[29]. In brief, single-cell suspensions were incubated at a density of 10^7 cells ml^{-1} with the IA^b multimers (30 μg ml^{-1}) in DMEM supplemented with 5 μM IL-2 and 2% FCS (pH 8.0) at room temperature for 2.5 h. After being washed, cells were stained with 7-AAD (Molecular Probes) and CD4 (RM4-5; BD Biosciences). The percentage of $tetramer^+$ cells was determined in the $CD4^+$ gate of live ($7\text{-}AAD^-$) cells. To control for non-specific binding, IA^s control tetramers were used[29]. For intracellular cytokine staining, cells were stimulated for 4 h in culture medium containing 12-O-tetradecanoylphorbol-13-acetate (50 ng ml^{-1}; Sigma), ionomycin (1 μg ml^{-1}; Sigma), and monensin (GolgiStop, 1 μl ml^{-1}; BD Biosciences) at 37 °C under 10% CO_2. After staining of surface markers, cells were fixed and permeabilized (Cytofix/Cytoperm and Perm/Wash buffer, BD Biosciences) followed by staining with monoclonal antibodies against mouse IL-17 and IFN-γ (BD Biosciences) and fluorocytometric analysis (FACSCalibur).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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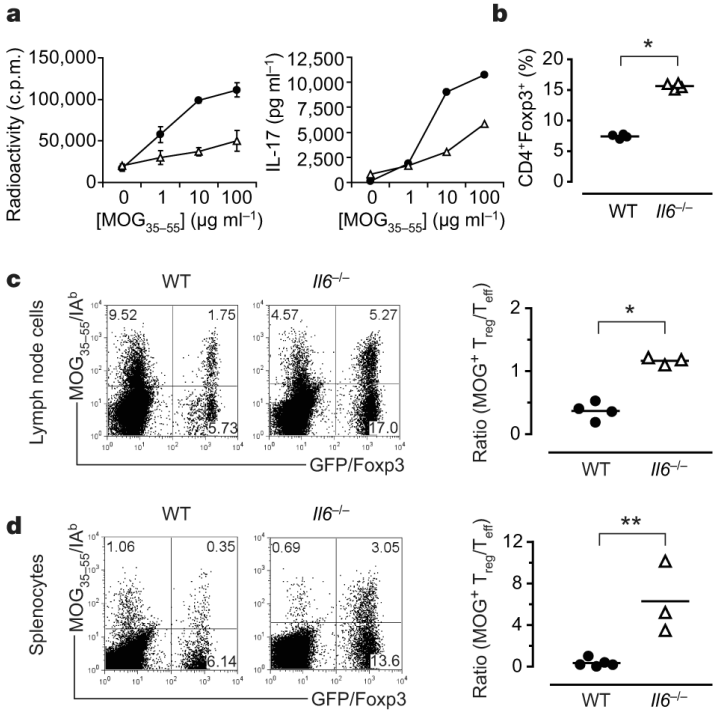

Figure 1. In the absence of IL-6, antigen-specific Foxp3[1] T_{reg} cells expand at the expense of effector T cells (T_{eff} cells)*in vivo*

Foxp3gfp.KI (filled circles)or *Il6*$^{-/-}$ × *Foxp3gfp*.KI (*Il6*$^{-/-}$; open triangles) mice were immunized with $MOG_{35–55}$/CFA. **a**, Draining lymph-node cells were tested for MOG-specific proliferation and IL-17 production (means ± s.d. for triplicate determinations). **b**, The fraction of Foxp3/GFP^{+} T cells was determined *ex vivo* by flow cytometry (asterisk, $P < 6 \times 10^{-7}$; *t*-test). WT, wild type. **c, d**, Lymphnode cells (**c**) and splenocytes (**d**) from $MOG_{35–55}$/CFA-immunized WT and *Il6*$^{-/-}$ mice were cultured for four days in the presence of $MOG_{35–55}$ and stained with a $MOG_{35–55}/IA^{b}$ tetramer. The ratios of antigen-specific T_{reg} cells (MOG tetramer^{+}CD4^{+}Foxp3^{+}) to T_{eff} (MOG tetramer^{+}CD4^{+}Foxp3^{-}) cells are presented (asterisk, $P < 0.0003$; two asterisks, $P < 0.05$; *t*-test).

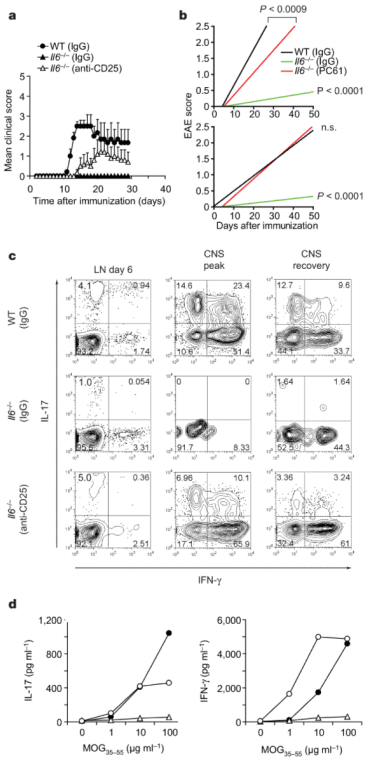

Figure 2. Depletion of T_{reg} cells in *Il6*$^{-/-}$ mice restores the development of T_H17 cells and susceptibility to EAE

Il6$^{-/-}$ × *Foxp3gfp*.KI mice were treated with antibody against CD25 to deplete T_{reg} cells or with a control immunoglobulin (rat IgG1) and then immunized with $MOG_{35–55}$/CFA. **a, b**, Clinical EAE scores (means and s.e.m.) (**a**) and linear regression analysis in acute (**b**, top) and chronic (**b**, bottom) stages of the disease for wild-type (WT), control *Il6*$^{-/-}$ and T_{reg}-depleted *Il6*$^{-/-}$ mice (n.s., not significant). **c**, Lymph-node (LN) cells and mononuclear cells from the central nervous system (CNS) were recovered on days 6, 14–17 (peak disease) and 29 (recovery) and stained for CD4 and intracellular IL-17 and IFN-γ. The numbers in the quadrants show percentages. **d**, Splenocytes (day 10) were stimulated with $MOG_{35–55}$*in vitro*. Culture supernatants were collected after 48 h, and IL-17 and IFN-γ concentrations were determined. Filled circles, WT (IgG); open triangles, *Il6*$^{-/-}$ (IgG); open circles, *Il6*$^{-/-}$ (anti-CD25).

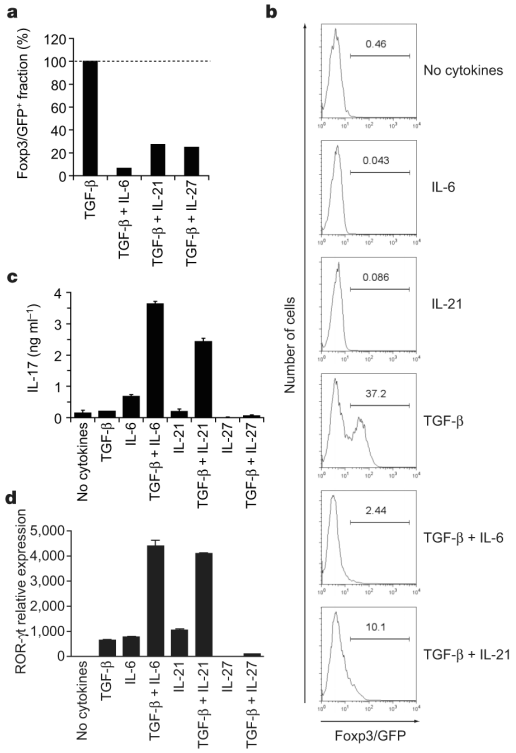

Figure 3. Inhibition of induction of T_{reg} cells and generation of T_H17 cells by IL-21

$CD4^+CD62L^{hi}$Foxp3/GFP^- T cells from *Foxp3gfp*.KI mice were stimulated with anti-CD3 and anti-CD28 for three days in the presence of the indicated cytokines. **a**, The expression of Foxp3/GFP was measured and the fraction of $Foxp3^+$ cells induced by TGF-β was normalized to 100%. **b**, Individual histograms showing Foxp3/GFP expression. The numbers above the histogram regions (horizontal lines) represent the percentages of Foxp3/GFP^+ cells. **c**, IL-17 production in these cultures after 48 h as measured by ELISA. **d**, ROR-γt expression as determined by quantitative RT–PCR in naive 2D2 (ref. 30) T cells activated for 48 h with anti-CD3 in the presence of irradiated syngeneic antigen-presenting cells and the indicated cytokines. ROR-γt expression is shown as mean and s.e.m. for duplicate determinations, relative to β-actin.

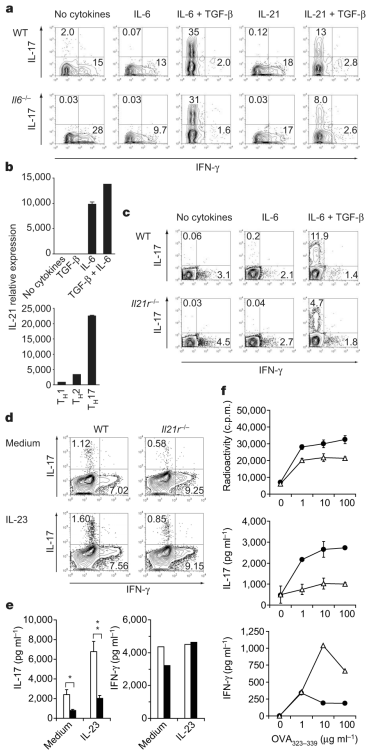

Figure 4. IL-21-driven T_H17 differentiation is independent of IL-6

Naive (**a, b**), total (**c**) or $CD44^+CD4^+$ T cells (**d, e**) were cultured with anti-CD3 plus corresponding irradiated antigen-presenting cells (**a, c**) or anti-CD3 plus anti-CD28 (**b, d, e**) and the indicated cytokines. **a, c**, Percentages of IL-17^+ and IFN-γ^+ cells in T cells from wild-type (WT) or *Il6*$^{-/-}$ mice (**a**) and WT or *Il21r*$^{-/-}$ mice (**c**) after four days of culture. **b**, IL-21 mRNA was determined by quantitative RT–PCR (means and s.e.m. for duplicate determinations). **d, e**, $CD4^+CD44^+$ T cells from WT or *Il21r*$^{-/-}$ mice were stimulated with or without recombinant IL-23 for 48 h. IL-17 and IFN-γ production were determined by intracellular cytokine staining (**d**) and ELISA (**e**; open columns, WT; filled columns, *Il21r*$^{-/-}$) (asterisk, $P < 0.0005$; two asterisks, $P < 0.003$; *t*-test). **f**, WT (filled circles) and *Il21r*$^{-/-}$ (open triangles) mice were immunized with ovalbumin 323–339 peptide ($OVA_{323–339}$)/CFA. Draining lymph-node cells were assayed for antigen-specific proliferation and cytokine production (means and s.d. for triplicate cultures).

Table 1

EAE in wild-type and *Il6*$^{-/-}$ mice

Group	Incidence	Mean day of onset (mean±s.d.)	Mean maximum score (mean±s.d.)
Wild type (IgG)	11 of 13 (85%)	12.4±1.0 [*]	2.5±0.7 [‡]
Il6$^{-/-}$ (IgG)	2 of 7 (29%)	16.5±3.5 [†]	1.0±0 [§]
Il6$^{-/-}$ (anti-CD25)	6 of 11 (55%)	14.5±1.9 [*†]	2.9±0.5 [‡§]

Mice treated with rat IgG1 (control) or monoclonal antibody against CD25 (PC61) were immunized with $MOG_{35–55}$ peptide emulsified in complete Freund's adjuvant. The animals were monitored for EAE development. Statistical analysis was performed by comparing groups using one-way analysis of variance.

[*] $P<0.008$.

[†] Not significant.

[‡] Not significant (mean maximum score of wild-type (IgG) versus *Il6*$^{-/-}$ (anti-CD25)).

[§] $P<0.002$ (mean maximum score of *Il6*$^{-/-}$ (IgG) versus *Il6*$^{-/-}$ (anti-CD25)).