Interplay between the immune response and environmental factors during multiple sclerosis

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"Interplay between the immune response and environmental factors during multiple sclerosis"

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1. SUMMARY

Multiple sclerosis (MS) is a common neurologic and autoimmune disorder affecting young patients. MS and its animal model, the experimental autoimmune encephalomyelitis (EAE), are characterized by inflammatory cell infiltrates and demyelination of the central nervous system (CNS). The development of this disease is under the control of both genetic and environmental factors. In addition to several genetic predisposing factors, environmental elements such as sun exposure, viral exposition or smoking contribute to MS occurrence.

This thesis is based on a compilation of a selection of my publications. We first assessed signaling molecular pathways of $CD4^+$ T cells and could identify elements, which in addition to their link with environment, shape the immune response during EAE. For examples the aryl hydrocarbon receptor (AhR) controls cytokines secretion and is identified as the receptor of dioxin, a toxin that is potentially lethal; metallothioneins (MTs) negatively regulate Interleukin-10 production but are suited as sensors for heavy metals. I further oriented my research toward immunometabolism and studied the role of the lipid metabolites and T cell migration to the CNS during EAE. My future perspectives are to study the link between lipid metabolism and gut immunology during neuroinflammatory diseases.

SCIENTIFIC PUBLICATIONS AND REVIEWS DISCUSSED IN THIS THESIS

- **Pot C**, Jin H, Awasthi A, Liu S, Lai C, Madan R, Sharpe A.H, Karp C.L, Miaw S, Ho I.C, and Kuchroo V.K. ***Cutting edge****: IL-27 Induces the Transcription Factor c-Maf, Cytokine IL-21, and the Costimulatory Receptor ICOS That Coordinately Act Together to Promote Differentiation of IL-10-Producing Tr1 Cells*. **J Immunol**. 2009 July 15; 183(2): 797-801.

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***These authors equally contributed to the work**

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2. INTRODUCTION

2.1 Multiple sclerosis

Multiple sclerosis (MS) is a common neurologic and autoimmune disorder affecting young patients. MS is one of the most debilitating acquired neurologic diseases by virtue of its frequency, chronicity, and tendency to attack young adults. It affects more than 2 millions individuals in the world, and approximately 10'000 patients in Switzerland.

In order to efficiently treat MS, it is important to better characterize the disease and to understand its underlying physiopathology. MS is an autoimmune disease characterized by inflammation and demyelination of the central nervous system (CNS) with localized areas of inflammation and demyelination. It is a heterogeneous disease with different clinical phenotypes: relapsing-remitting (RR), primary progressive (PP) and secondary progressive (SP). Over the last two decades several disease-modifying drugs (DMF) have been commercialized to treat RRMS, and shown to dampen the disease inflammatory activity and to decreases relapses rate of the RRMS. The mechanisms of action and administration routes of those DMF are summarized below (Table 1). Those drugs are long-term treatments and no cure for MS has been unravelled yet. Discoveries of neuroprotective, neuroregenerative and remyelination drugs remain an important field of research that will not be detailed here.

Another challenge lies in preventing MS. Identifying environmental risk factors and lifestyle habits that could be changed would be an approach to prevent MS and to contribute, together with DMF, to harness disease activity in MS patients. We identified several candidates factors involved in shaping the immune response that are related to environmental factors. Aryl hydrocarbon receptor (AhR), a ligand for the toxin dioxin, the heavy metal scavengers metallothioneins are examples of the identified factors that will be detailed in the following sections. The possibility to modulate the disease course

by changing lifestyle/environmental factors remains an important question for MS patients and a challenge for MS doctors.

Table 1: Disease modifying drugs (DMF) available in Switzerland in January 2016

Drug	Mechanism of action	Route of administration
Interferon beta-1a	Exact mechanism of action unknown, Possibly inhibit the T-lymphocytes (T cells) from being activated.	IM or SC
Interferon beta-1b	Exact mechanism of action unknown, Possibly inhibit the T-lymphocytes (T cells) from being activated.	SC
Glatiramer acetate	Exact mechanism of action unknown, Possibly induces regulatory T cells and a TH1-TH2 shift with increased secretion of anti-inflammatory cytokines	SC
Fingolimod	Sphingosine 1-phosphate receptor modulator that sequesters lymphocytes within Lymph nodes	Oral
Teriflunamide	Slows the division of rapidly dividing cells (including lymphocytes)	Oral
Dimethyl fumarate	Exact mechanism of action unknown, Anti-inflammatory and anti-oxidant effects by interacting with Nrf2 pathway.	Oral
Natalizumab	Humanized monoclonal antibody which inhibits lymphocytes migration to the CNS by inhibiting the binds to α4β1-integrin	IV
Alemtuzumab	Humanized monoclonal antibody against CD52, an antigen found on the surface of lymphocytes	IV

Personal contribution

2.2 From genetic predisposition to environment risk factors

It is increasingly recognized that MS is a heterogeneous disease with multiple underlying predisposing factors. The development of MS is under the control of both genetic and environmental factors.

Great efforts have been conducted to understand the underlying pathology of this disease. The immune system plays a crucial role in driving the disease. At the genetic level, association studies have shown a strong link between MS susceptibility and human leukocyte antigen (HLA) in particular within HLA-*DRB1* family with HLA-DRB1*1501 gene having the strongest association with MS (Figure 1). In addition variation in HLA-A gene confirms an independent protective effect attributable to class

I region, and for example HLA-A*02 has been associated with a lower risk to develop MS (Figure 1). Those associations were confirmed by a genome-wide association studies (GWAS) study that have examined biological samples from 9772 patients suffering of MS compared to 17376 controls world-wide (International Multiple Sclerosis Genetics et al., 2011).

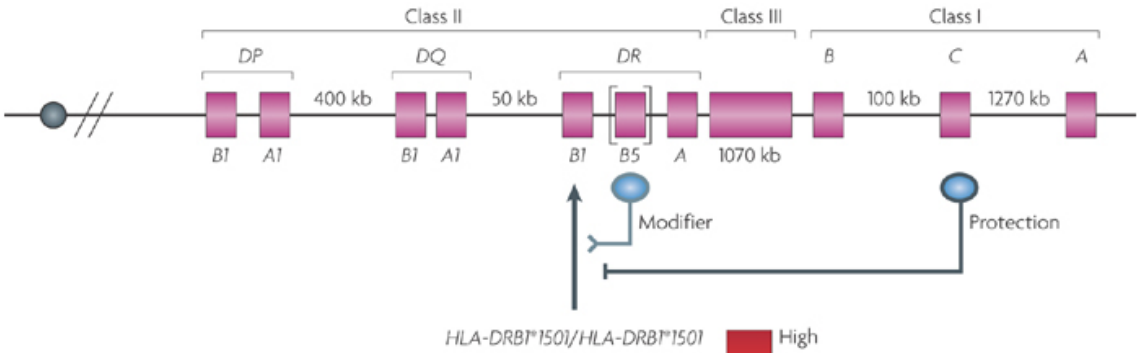

Figure 1. The human leukocyte antigen (HLA) gene complex is located on the short arm of chromosome 6. Two major classes of HLA-encoding genes are involved in antigen presentation: HLA-class I genes, contained in the telomeric stretch, and HLA-class II genes in the centromere proximal region. HLA class I and class II encode molecules are cell-surface glycoproteins, which present short antigenic peptide fragments to specific T cells during an immune response. HLA-DRB1 allelic copy number were detected and HLA-DRB1*1501 homozygotes showed the strongest association with MS. Adapted from the Nature review (Oksenberg et al., 2008).

Interestingly, epidemiological studies have shown a cumulative risk between HLA-DRB1*1501 and specific environmental factors. Professor L. Alfredsson and his team described, using Swedish and American case–control studies, an enhanced risk to develop MS in patients that smoke (Hedstrom et al., 2014a) or are obese (Hedstrom et al., 2014b) if they carry the HLA-DRB1*1501 gene. For example, while obesity increases the risk to develop MS by a factor of 5 in patients that are HLA-DRB1*1501

negative but HLA-A*02 positive, the combination of obesity in individuals carrying the HLA-DRB1*1501 but not the protective HLA-A*02 increases the risk of developing MS by more than 15 (Figure 2).

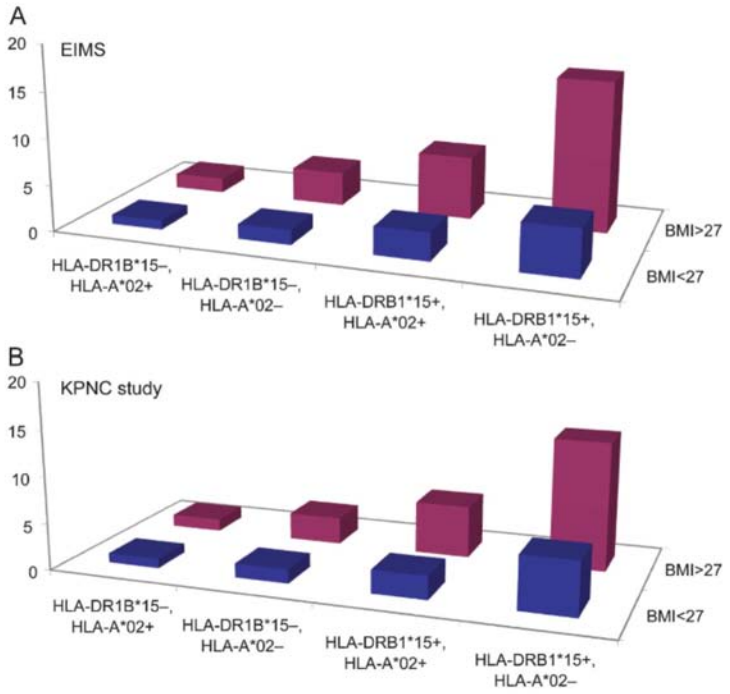

Figure 2. Professor L. Alfredsson and his team took advantage of the analysis of two existing cohorts: the *Epidemiological Investigation of MS* (EIMS), a study base comprising the Swedish population aged 16 to 70 years (panel A) and the American case-control study assessing a population of white non-Hispanic people identified among members of *Kaiser Permanente Medical Care Plan, Northern California Region* (KPN) (panel B). They showed a different risk for developing MS if an obese individual caries the HLA-DRB1*15 and/or the HLA-A*02 genes. Adapted from J. of Neurology (Hedstrom et al., 2014b).

The incidence of MS is highest in countries farthest from the equator, both in the north and south ends of the world strongly pointing towards environmental factors promoting MS. Sun exposure, vitamin D and recently melatonin levels have been proposed as key

environmental risk factors for MS (Farez et al., 2015; Lysandropoulos et al., 2011). Interestingly, genes linked with Vitamin D metabolism have been associated with MS in the above-mentioned GWAS study (International Multiple Sclerosis Genetics et al., 2011).

In addition to the association with HLA subsets and MS, detailed genetic analyses have highlighted the association of genes related to the immune system and MS (Figure 3). Strong evidence for association with MS was noted with genes involved in the T helper (T_H) cell differentiation pathway, particularly those acting as cell surface receptors. Several genes were linked to $CD4^+$ T cell subset, that produce distinct patterns of cytokines, including T_H1 and T_H17 cell that will be detailed in the section below *2.4 From Immune response to cytokine signaling*. Interestingly several Signal Transducers and Activators of Transcription family of transcription factors (STAT), including STAT-1 and STAT-3 have been associated with MS (Figure 3). In this thesis, we have shown that both STAT-1 and STAT-3 are implicated in driving the effect of MTs in regulatory subset of T cells and that the balance between those two factors shapes the development of T_H17 versus T_R1 cells.

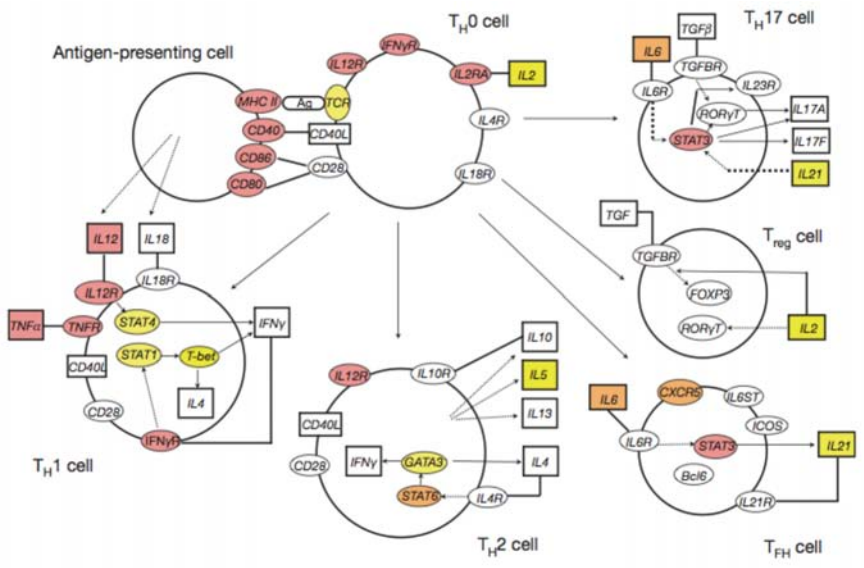

Figure 3. Several genes associated with MS were identified as key regulatCor of the adaptive immune response, in particular in CD4+ T cell subsets. Adapted from (International Multiple Sclerosis Genetics et al., 2011).

2.3 Mouse model for MS

Experimental autoimmune encephalomyelitis (EAE) is an animal model that recapitulates many clinical and histological features of MS (Alvord et al., 1984). EAE is typically characterized by lymphocytic and mononuclear cell inflammatory infiltrates and demyelination throughout the CNS. It can be induced by direct immunization of susceptible animals with myelin antigens such as myelin basic protein (MBP), myelin proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG). Immunization of a number of animal species with myelin proteins induces T cells that traffic to the CNS where they induce inflammation and demyelination resulting in the induction of a paralytic disease, which is characterized histologically by perivascular mononuclear cell infiltration and demyelination. PLP and MOG are major encephalitogens but their propensity to induce EAE depends on the mouse strains. In SJL mice two major epitopes, $PLP_{139-151}$ and $_{178-191}$ have been identified to induce EAE, with the strongest immune response to $PLP_{139-151}$ peptide. Epitopes of MOG were identified particularly MOG_{35-55} which was shown to induce EAE in C57Bl/6 mice (Table 2)

Mouse strain	Antigen specificity	Donor immunization period (days)	In vitro antigen concentration (μg/ml)	In vitro IL-12 (ng/ml)	In vitro culture Time (hr)	Number of blasts transferred ($\times 10^6$)	Disease type	Disease severity
SJL	PLP	7-14	50-100	—	72-96	5-10	Relapsing-remitting	Severe
	$PLP_{139-151}$	7-14	20	—	72-96	1-5	Relapsing-remitting	Severe
	MBP	7-14	50-100	—	72-96	40-60	Relapsing-remitting	Moderate
	MBP_{84-104}	7-14	50	—	72-96	10-20	Relapsing-remitting	Moderate
C57BL/6	MBP	10-14	50-100	—	72-96	50	Monophasic/ chronic	Mild
	MBP_{84-104}	10-14	50	—	72-96	50	Monophasic/ chronic	Mild
	MOG	10-14	50	25	72-96	20	Chronic	Moderate
	MOG_{35-55}	10-14	10	25	72-96	20	Chronic	Moderate
B10.PL[b]	MBP_{Ac1-11}	—	50	10	72	1	Monophasic	Moderate
B10.S	MBP	10-11	25	20	96	35	Monophasic	Moderate
	MBP_{87-106}	10-11	50	20	96	35	Monophasic	Moderate

Table 2. Summary of general antigens and general parameters for EAE mouse Models. In this thesis, we have used the C57BL/6 model with immunization using the MOG_{35-55} peptide. Adapted from Current protocols in Immunology (2007) 15.1.1-15.1.18.

After preparation of an emulsion with the peptide, MOG_{35-55} for C57Bl6 mice, in complete Freund's adjuvant, the emulsion is injected sub-cutaneous in the flanks of the mice. In addition pertussis toxin is injected, as it possibly opens the blood-brain-barrier. Mice are observed daily for development of clinical signs characteristics of myelitis by the following system: 0, no clinical deficits; 1, tail weakness; 2, hind limb paresis; 2.5, partial hind limb paralysis; 3, full hind limb paralysis; 4, full hind limb paralysis and forelimb paresis; 5, premorbid or dead (Figure 4).

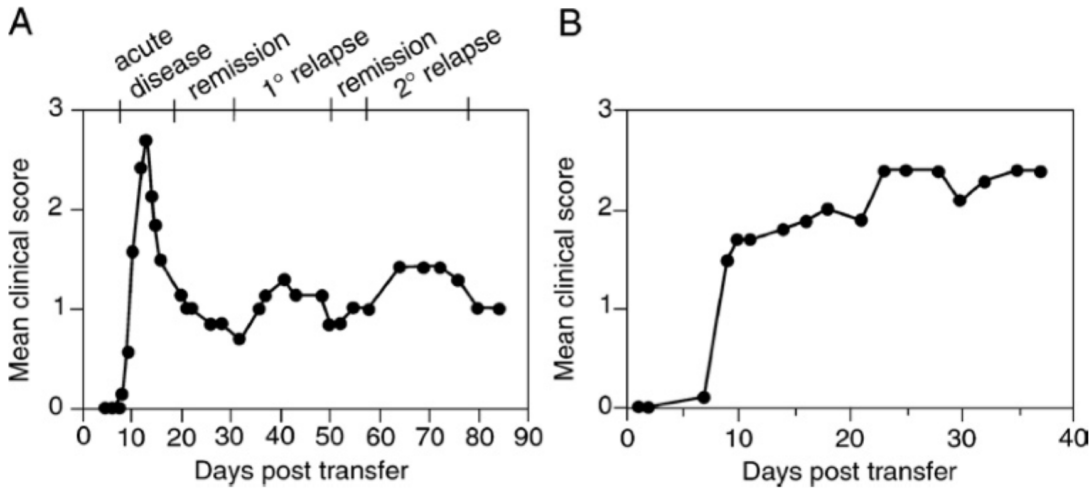

Figure 4 EAE clinical diseases. Typical course of EAE in SJL (panel A) or C57Bl6 with MOG_{35-55} peptide (panel B). After immunization each mouse is scored for clinical signs by the following system: 0, no clinical deficits; 1, tail weakness; 2, hind limb paresis; 2.5, partial hind limb paralysis; 3, full hind limb paralysis; 4, full hind limb paralysis and forelimb paresis; 5, premorbid or dead. Adapted from Current protocols in Immunology (2007) 15.1.1-15.1.18.

2.4 From Immune response to cytokine signaling

The development and progression of EAE, like other autoimmune diseases, results from the balance between pathogenicity of effector cells and the negative regulation imposed by regulatory cells. Subpopulations of $CD4^+$ T cells (T_H) produce distinct patterns of cytokines, and this has led to the concept of functional heterogeneity among T_H cells (Mosmann and Coffman, 1989). T_H1 produce interleukin 2 (IL-2) and/or interferon gamma (IFN-γ), elicit delayed type hypersensitivity (DTH) responses and activate macrophages. T_H2, on the other hand, produce IL-4, IL-5 and IL-10 and are especially important for IgE production and eosinophilic inflammation, and may suppress cell-mediated immunity. The potential role of these two subsets of T_H cells in various immunopathological conditions like Leprosy, Leishmaniasis and Schistosomiasis, has been demonstrated (Heinzel et al., 1989; Romagnani, 1994). Since the original classification by Mosmann and Coffman of $CD4^+$ T lymphocytes into T_H1 and T_H2, the repertoire of effector $CD4^+$ T cell subsets has recently expanded to include additional effector T cell subsets like T_H17 and T_H9 cells (Dardalhon et al., 2008; Veldhoen et al., 2008). In addition, a number of regulatory T cell subsets have been identified that suppress effector T cells, tissue inflammation and autoimmunity. Two important classes of regulatory T cells within the $CD4^+$ subset are the $Foxp3^+$ regulatory T-cells (Tregs) and IL-10-producing regulatory type I cells (T_R1) cells that do not express Foxp-3 **(Table 3)**. $Foxp3^+$ Tregs play an important regulatory role during EAE but they are not able to counterpart alone the very highly inflammatory milieu of the brain. $T_R1$cells have been described to maintain peripheral tolerance, control autoimmunity, prevent allograft rejection and graft versus host disease, and have been implicated in mediating recovery during EAE. Furthermore, the generation of T_R1 cells has been shown to be

impaired in patients suffering from MS compared to healthy volunteers, suggesting that T_R1 cells may have a protective role during MS.

	nTregs	**iTregs**	**T_R1**
Place of generation	Thymus	Peripheral lymphoid organs	Peripheral lymphoid organs
Differentiating cytokine	-	TGF-β	IL-27
Surface markers	CD25 (IL-2R), CTLA4, GITR	CD25 (IL-2R), CTLA4, GITR	Unknown
Transcription factors	Foxp3	Foxp3, AhR	AhR, c-Maf, Tbx21
Growth promoting cytokine	IL-2	IL-2	IL-21
Mode of suppression	Multiple	Multiple	Multiple
Contact dependent	CD39	CD39	Granzyme B
Contact independent	TGF-β, IL-35, IL-10	TGF-β, IL-35, IL-10	IL-10

Table 3. Characteristic of murine regulatory T cells. CD4 regulatory T cells can be divided in natural Foxp3+ Tregs that are generated in the thymus (nTregs) or in the periphery that comprise iFoxp3+ Tregs and T_R1 cells. CTL4, Cytotoxic Antigen 4; GITR, glucocorticoid-induces TNF receptor family-regulated gene; Tbx21, T-box transcription factor TBX21. Adapted from (Pot, 2012)

While mouse studies have demonstrated that increasing the numbers and/or function of T_R1 cells could improve the course of autoimmune diseases, the lack of protocols consensus and the inability to generate T_R1 cell *in vitro* in large numbers has hampered identification of the molecular mechanisms responsible for their differentiation. The identification of IL-27 as a differentiating factor for T_R1 cell generation provided a mean by which T_R1 cells facilitated their functional analysis. The anti-inflammatory role of IL-27 was exemplified both in the mouse EAE model, where daily injection of IL-27 drastically reduces the severity of the disease course and in humans where IFN-β, a

therapy approved for MS, induces IL-27 which is thought to contribute to its therapeutic effect. In this thesis, we studied the downstream signaling molecules involved in IL-27 signaling and observed that the transcription factors c-maf, AhR as well as the MTs were fine regulators of IL-27-induced T_R1 cells and could be new target to fine-tune the immune response during EAE and MS.

2.5 Metabolic fine-tuner

In the second part of my work, I studied the role of lipid metabolites in shaping the immune response during EAE. It is recognized that steroid metabolism influences the immune system but the underlying mechanisms are largely unraveled. Cholesterol can be converted into the soluble oxysterol form by endoplasmic reticulum membrane-associated enzymes and transported to the liver where they are converted into bile acids that are critical for normal vitamin and lipid metabolism. In addition to their contributions to basic metabolic processes, oxysterols have been ascribed a number of regulatory roles in signaling pathways that regulate inflammation through their influence on the activation status and function of macrophages, mast cells, T cells, and B cells. Several forms of oxysterols are potent inhibitors of immune cell function including lymphocyte protein kinase C activity and NK cell activity. Furthermore, serum oxysterols levels could be suitable candidate biomarkers for neurological diseases, and could help differentiate different phases and/or severity of MS. Serum levels of the oxysterol 24-hydroxycholesterol are decreased in MS patients, most profoundly in primary progressive (PP). Furthermore, 15-oxysterol 15α-hydroxycholestene has been reported to be increased in patients with secondary progressive multiple sclerosis and in mice with secondary progressive EAE. Interestingly, the MS approved oral immunosuppressive drug Fingolimod (Table 1) stimulates the production of the

oxysterol 27-hydroxycholesterol when applied in vitro on human macrophages. Finally, oxysterols have been reported to modulate inflammatory cytokines secretion and the oxysterol 7-ketocholesterol was described mainly to enhance interleukin (IL)-1β secretion in vascular endothelial cells, while the oxysterol 25-hydroxycholesterol was reported to modulate the secretion of IL-8 (a proatherogenic cytokine involved in firm adhesion of monocytes to vascular endothelial cells). Together, these studies suggest that oxysterols are immune modulators however the precise mechanisms of their effects on immune cell function remain unclear. In this work, we assessed the role of oxysterols during EAE using mice deficient for the enzyme enzyme cholesterol 25 hydroxylase (Ch25h), the rate limiting step to synthetize the oxysterol 7a,25-dihydroxycholesterol (7a,25-OHC) from cholesterol. We reported that Ch25h deletion significantly attenuated EAE disease course by limiting trafficking of pathogenic $CD4^+$ T lymphocytes to the central nervous system (CNS). This opens a new domain of research on metabolism and autoimmunity.

3. SCIENTIFIC CONTRIBUTIONS

3.1 IL-27 Induces c-Maf, IL-21, and ICOS That Promote Differentiation of T_R1

Cells Ref: J Immunol. 2009 July 15; 183(2): 797-801

IL-27 is a cytokine of the IL-12 family. It is mainly produced by antigen-presenting cells but also by other cell types including cells in the central nervous system such as astrocytes and microglia. The molecular mechanisms by which IL-27-mediated generation and/or expansion of T_R1 cell remained elusive. To understand the mechanisms leading to T_R1 cell differentiation generation, we identified genes upregulated by IL-27. In the following article, our analysis revealed that IL-27 is a potent inducer of three essential elements: the transcription factor c-Maf, cytokine IL-21 and co-stimulatory receptor ICOS (Figure 1). We further demonstrated that those elements coordinately work together to promote differentiation of T_R1 cells. The depletion of any of those components indeed impairs the development of T_R1 cells.

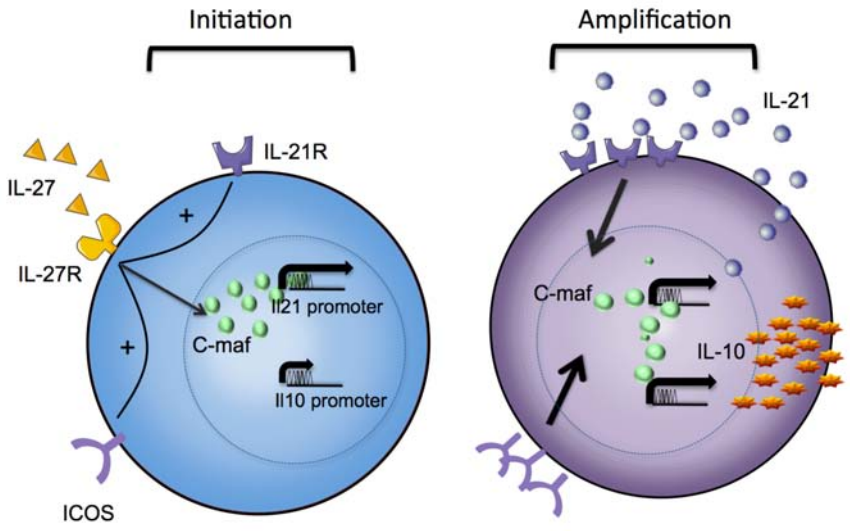

Figure 1. Molecular events leading to the generation of IL-27 induced T_R1 cells

Treatment of naïve CD4+ T cells with IL-27 enhances the expression of the transcription factor c-Maf, IL-21 receptor and ICOS that are all essential for the differentiation of T_R1 cells. Upon induction of T_R1 cell differentiation (initiation phase), c-Maf transactivates the *il21* and *il10* promoters (left panel). During T_R1 cell expansion (amplification phase), IL-10 and IL-21 expression become independent of IL-27/IL-27R signaling as ICOS and IL-21 signaling maintain c-Maf expression (right panel). (adapted from Pot C. et al, J Interferon Cytokine Res. 2010 June; 30 (6): 381-387)

Cutting Edge

THE JOURNAL OF IMMUNOLOGY

Cutting Edge: IL-27 Induces the Transcription Factor c-Maf, Cytokine IL-21, and the Costimulatory Receptor ICOS that Coordinately Act Together to Promote Differentiation of IL-10-Producing Tr1 Cells[1]

Caroline Pot,* Hulin Jin,* Amit Awasthi,* Sue Min Liu,* Chen-Yen Lai,§ Rajat Madan,¶ Arlene H. Sharpe,‡ Christopher L. Karp,¶ Shi-Chuen Miaw,§ I-Cheng Ho,† and Vijay K. Kuchroo[2]*

IL-27 has recently been identified as a differentiation factor for the generation of IL-10-producing regulatory type 1 (Tr1) T cells. However, how IL-27 induces the expansion of Tr1 cells has not been elucidated. In this study we demonstrate that IL-27 drives the expansion and differentiation of IL-10-producing murine Tr1 cells by inducing three key elements: the transcription factor c-Maf, the cytokine IL-21, and the costimulatory receptor ICOS. IL-27-driven c-Maf expression transactivates IL-21 production, which acts as an autocrine growth factor for the expansion and/or maintenance of IL-27-induced Tr1 cells. ICOS further promotes IL-27-driven Tr1 cells. Each of those elements is essential, because loss of c-Maf, IL-21-signaling, or ICOS decreases the frequency of IL-27-induced differentiation of IL-10-producing Tr1 cells. ***The Journal of Immunology,*** **2009, 183: 797–801.**

Interleukin-27, a member of the IL-12/IL-23 heterodimeric family of cytokines produced by APCs, is composed of two chains, IL-27p28 and EBV-induced gene 3 (1). Activated T cells and NK cells have the highest expression of IL-27R, which is composed of two chains, a specific IL-27Ra chain (WSX-1 or TCCR) and a signaling chain, gp130, that it shares with IL-6R (1). Initial studies have suggested that, similarly as IL-12, IL-27 induces the expansion of proinflammatory Th1 cells by activating the STAT-1-mediated T-bet pathway (1). However, analysis of the IL-27Ra$^{-/-}$ (WSX-1$^{-/-}$) mice infected with various pathogens resulted in clearance of the parasites with exaggerated T cell responses and enhanced proinflammatory cytokine production (1). Furthermore, IL-27Ra$^{-/-}$ mice developed severe experimental autoimmune encephalomyelitis with enhanced Th17 responses (1), and treatment with rIL-27 suppressed disease and decreased the frequency of Th17 cells (1). These paradoxical observations led to the hypothesis that IL-27 may not be necessary for the generation of proinflammatory T cells (Th1 or Th17), but rather play a crucial role in regulating T cell responses. Subsequently, three groups, including ours, reported that IL-27 not only inhibited the generation of Th17 cells but also induced differentiation of IL-10-producing regulatory T cell type 1 (Tr1)[3] from naive T cells (2–4).

Tr1 cells are a subset of T cells that have strong immunosuppressive properties, predominantly produce IL-10 with variable amounts of IFN-γ, but do not express Forkhead box 3 (Foxp3) (5). Adoptive transfer of Tr1 cells has been shown to suppress autoimmunity, colitis, graft-vs-host disease, and tissue inflammation (6). Although initial studies suggested that Tr1 cells are induced by repetitive antigenic stimulation of T cells in the presence of IL-10 (6), T cells differentiated in the presence of IL-10 could not be propagated long term in culture. The identification of IL-27 as a differentiating factor for the generation of Tr1 cells provided a means by which they could be grown in large numbers and facilitated their functional analysis. However, the molecular mechanisms by which IL-27 mediates the generation and/or expansion of Tr1 cells are not well understood. Thus, we analyzed the expression of various key cytokines and transcription factors induced by IL-27. Our results show that IL-27 is a potent inducer of three essential elements: the transcription factor c-Maf, the cytokine IL-21, and the costimulatory

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[2] Address correspondence and reprint requests to Dr. Vijay K. Kuchroo, Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, Harvard Institute of Medicine, Room 786, 77 Avenue Louis Pasteur, Boston, MA 02115. E-mail address: vkuchroo@rics.bwh.harvard.edu

[3] Abbreviations used in this paper: Tr1, regulatory T cell type 1; Foxp3, Forkhead box P3; TFh, T follicular helper cell; WT, wild type.

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receptor ICOS, which coordinately work together to promote differentiation of Tr1 cells.

Materials and Methods

Mice and reagents

IL-10-enhanced GFP reporter mice (Vert-X), *Foxp3.gfp* "knock-in" mice, ICOS$^{-/-}$ mice on C57BL/6 background, were generated as described (7, 8). WSX-1$^{-/-}$ mice on C57BL/6 background were obtained from C. Saris (Amgen, Thousand Oaks, CA), and IL-21R-deficient mice on NOD background (9) from N. Sarvetnick (Scripps Research Institute, La Jolla, CA). c-Maf$^{-/-}$ mice on an N5 BALB/c background have been described (10). Mice were housed in conventional, pathogen-free facilities at the Harvard Institute of Medicine (Boston, MA). All experiments were undertaken in accordance with guidelines from the Committee on Animals at Harvard Medical School (Boston, MA).

T cell differentiation and proliferation in vitro

Naive CD4$^+$ T cells (CD4$^+$CD62L^{high}CD25$^-$), pooled from both spleen and lymph nodes, or memory cells (CD4$^+$CD44$^+$), obtained from lymph nodes, were purified by flow cytometry and stimulated with plate-bound Ab against CD3 (145-2C11; 1 μg ml^{-1}) and CD28 (PV-1; 1 μg ml^{-1}). Cells were cultured as previously described (8). Monoclonal anti-TGF-β1 Ab (10 μg ml^{-1}), mouse IL-21 (80 ng ml^{-1}), mouse IL-27 (25 ng ml^{-1}), and anti-mouse IL-21 Ab (25 μg ml^{-1}) were all purchased from R&D Systems. Proliferation assay was performed as previously described (8).

Measurement of cytokines

Secreted cytokines were measured after 48 h by cytometric bead array (BD Biosciences) or ELISA. Intracellular cytokine staining was performed by as previously described (8).

Quantitative real-time PCR

RNA was extracted with RNeasy mini kits (Qiagen) and analyzed by real-time PCR according to the manufacturer's instructions (Applied Biosystems). The following primers/probe mixtures were purchased from Applied Biosystems: IL-10 (catalog no. Mm 00439615_g1); ICOS (catalog no Mm004497600_m1); c-Maf (catalog no Mm 02581355_S1); IL-21 (catalog no. Mm00517640_m1); and IL-21R (catalog no. Mm00600319_M1).

Luciferase assay

HEK 293T cells (10^5) were cotransfected with pGL3-IL-21-Luc reporter plasmid and *Renilla* luciferase reporter plasmid (pRL-TK) and vector pcDNA3.1, pcDNA3.1(hemagglutinin-c-Maf), pcDNA3.1(T-bet), or pcDNA3.1(human GATA3). Cells were collected 24 h post-transfection and IL-21 promoter activities were analyzed using Dual-Glo luciferase assay system (Promega) according to the manufacturer's instructions. The luciferase activities were normalized against the *Renilla* luciferase activity.

Fetal thymic organ culture

Thymi from c-Maf$^{-/-}$ fetuses were removed on embryonic day 16.5 and individual lobes were cultured for 7 days. Genotyping was performed using DNA isolated from the fetal limbs. Thymocytes were recovered on day 7 of culture after collagenase digestion. CD4$^+$CD8$^-$CD25$^-$ cells were sorted and cultured for 4 days on anti-CD3 (2 μg ml^{-1})- and anti-CD28 (2 μg ml^{-1})-coated plates.

Statistics

Statistical analysis was performed using an unpaired Student's *t* test.

Results and Discussion

Our previous studies showed that TGF-β and IL-27 acted synergistically to generate Tr1 cells. However, under such culture conditions low Foxp3 expression was also induced by TGF-β. Using IL-10-enhanced GFP reporter mice (Vert-X), we developed culture conditions under which IL-27 alone could induce Tr1 cells. These in vitro derived Tr1 cells were as suppressive as natural Foxp3$^+$ regulatory T (Treg) cells in inhibiting T cell proliferation in vitro (supplemental Fig. 1).[4]

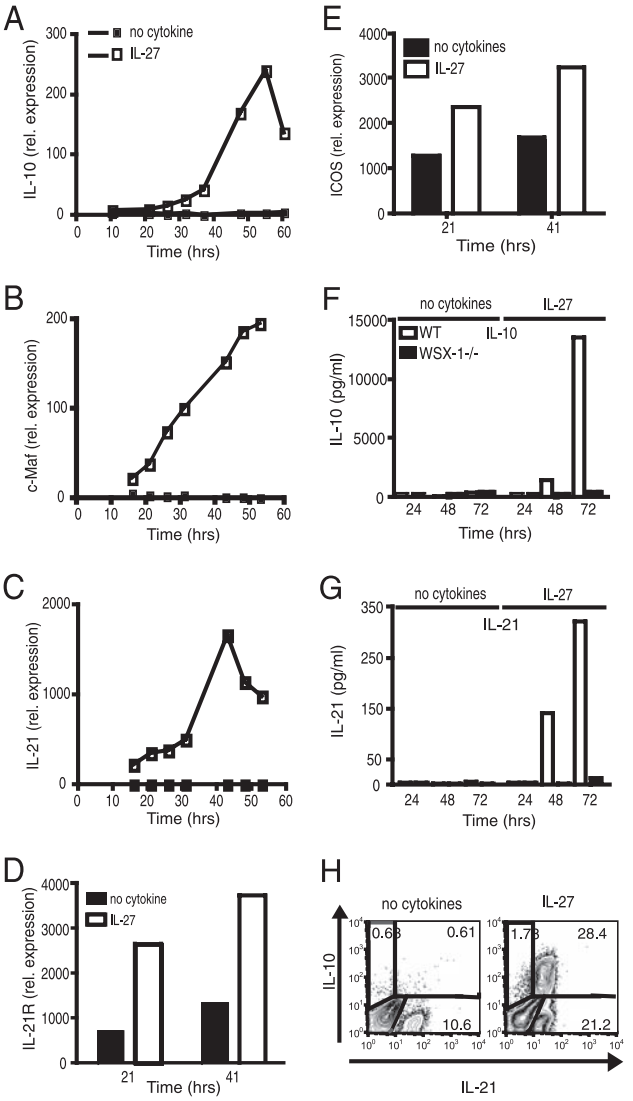

FIGURE 1. IL-27 induces c-Maf, IL-21, IL-21R, and ICOS. *A–E*, RNA isolated from naive CD4$^+$CD62L^{high}CD25$^-$ cells cultured with IL-27 (open squares) or without IL-27 (closed squares) were subjected to real-time PCR relative to the expression (rel. expression) of mRNA encoding β-actin ($2^{-\Delta C_T} \times 100{,}000$; where C_T is cycle threshold) to examine the expression of cytokines at different time points following activation. Real-time PCR of IL-10 (*A*), c-Maf (*B*), IL-21 (*C*), IL-21R (*D*), and ICOS (*E*) induction by IL-27 is shown. *F* and *G*, IL-10 and IL-21 production was measured by cytokine bead array as induced by IL-27 in WT (open bars) and IL-27Ra$^{-/-}$ (WSX-1$^{-/-}$) (filled bars) CD4$^+$CD62L^{high}CD25$^-$ cells. *H*, Intracellular cytokine staining of IL-10 and IL-21 by T cells following activation in the presence of IL-27.

To understand the molecular mechanisms by which IL-27 induces and expands Tr1 cells, we analyzed the expression of genes up-regulated by IL-27 at multiple time points following T cell activation in the presence of IL-27. As expected, IL-27 induced IL-10 mRNA expression that peaked 48 h postactivation (Fig. 1*A*). Interestingly, IL-27 induced the expression of the transcription factor c-Maf, at early time points, and this expression progressively increased over time (Fig. 1*B*). Consistent

[4] The online version of this article contains supplemental material.

with our previous observation that c-Maf regulates IL-21 expression in Th17 cells (8), we observed that IL-27 also induced IL-21 mRNA and that IL-10 and IL-21 showed similar kinetics of mRNA expression (Fig. 1, *A* and *C*). Although IL-21R expression was low in unactivated T and B cells, TCR-driven activation up-regulated this expression and IL-27 further up-regulated IL-21R expression in activated T cells (Fig. 1*D*). We (8) and others (11) have shown that the c-Maf transcription factor is downstream of ICOS, and because IL-10-producing T cells were first shown to be preferentially costimulated by ICOS (12), we examined the expression of ICOS mRNA and observed that the addition of IL-27 indeed induced higher ICOS expression than T cell activation without IL-27 (Fig. 1*E*). Thus, IL-27, in addition to inducing IL-10 production, induced c-Maf, IL-21, IL-21R, and ICOS expression. At the protein level, T cells activated in the presence of IL-27 produced both IL-10 and IL-21, thus confirming mRNA expression (Fig. 1, *F* and *G*). Besides the mRNA expression, we also observed an increase in ICOS expression induced by IL-27 at the protein level (data not shown). Furthermore, IL-27Ra$^{-/-}$ (WSX-1$^{-/-}$) mice produced no detectable IL-10 or IL-21 and did not show an increased ICOS expression upon activation, indicating that IL-10 and IL-21 production and increased ICOS expression were specifically induced by IL-27 (Fig. 1, *F* and *G*, and data not shown). To analyze the cells that produce both IL-10 and IL-21, we undertook intracellular cytokine staining for IL-10 and IL-21 after 3 days of culture in vitro with IL-27 and found that the IL-10-producing cells also produced IL-21 (Fig. 1*H*).

Because IL-21 belongs to the IL-2 cytokine family and uses the common γ-chain receptor, we hypothesized that IL-27-driven IL-21 production from T cells may be an autocrine growth factor for the generation of Tr1 cells. To test this, we first added a neutralizing IL-21 Ab in the presence of IL-27 and found that blocking IL-21 reduced the frequency of IL-10-producing T cells significantly by >75% (Fig. 2*A*) and IL-10 cytokine production in the culture supernatants by >50% (Fig. 2*B*). Further addition of IL-21 together with IL-27 increased IL-10 production, but this increase with exogenous IL-21 was modest (Fig. 2*B*). These data raised the issue of whether IL-21 could directly expand IL-10-producing T cells. However, activation of T cells from mice lacking IL-27Ra (WSX-1) signaling in the presence of IL-21 and IL-27 did not expand Tr1 cells (supplemental Fig. 2). We further confirmed the role of IL-21 in the expansion of Tr1 cells using CD4$^+$ T cells from IL-21R-deficient mice. Loss of IL-21 signaling resulted in the inhibition of IL-27-driven generation of IL-10-producing T cells by >75% (Fig. 2*C*) and IL-10 cytokine production in the culture supernatants by over 90% (Fig. 2*D*). However, loss of IL-21 signaling had no effect on IL-27-driven IFN-γ production (data not shown). Furthermore, IL-21R-deficient CD4$^+$ T cells stimulated with IL-27 expressed lower levels of c-Maf and IL-21, as determined by real-time PCR (Fig. 2*E*). These data suggest that IL-21 may be an important growth factor induced by IL-27 to expand Tr1 cells without affecting the expansion of IFN-γ-producing cells. Because IL-27 not only induces IL-21 production but also induces IL-21R expression, these data suggest that IL-27-mediated IL-21R up-regulation might be required for IL-21 to expand Tr1 cells.

To study the relevance of IL-21 in expanding Tr1 cells in vivo, we examined the frequency of IL-10-producing Tr1 cells generated in vivo in IL-21R$^{-/-}$ mice. We found that the frac-

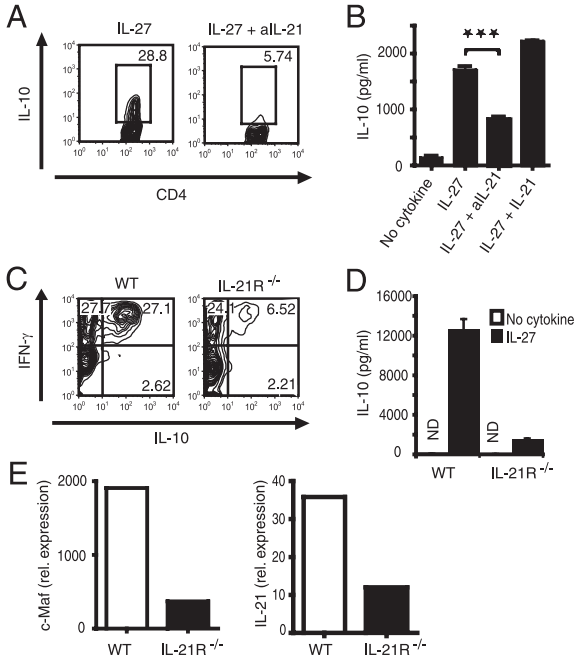

FIGURE 2. IL-21 is necessary for IL-10 production in Tr1 cells. *A*, IL-10.GFP expression as analyzed by flow cytometry in naive T cells activated in the presence of IL-27 for 72 h with or without the addition of neutralizing anti-IL-21 (aIL-21) Ab. *B*, ELISA to detect IL-10 production in the supernatant of naive T cells differentiated with IL-27 and anti-IL-21 or IL-21 (mean and SD; ***, $p = 0.0003$). *C*, IL-10 and IFN-γ production by WT and IL-21R$^{-/-}$ naive T cells activated in the presence of IL-27 for 72 h as determined by intracellular cytokine staining and analysis by flow cytometry. *D*, Supernatants from IL-27 differentiated naive T cells from WT and IL-21R$^{-/-}$ mice analyzed by IL-10 cytokine ELISA (mean and SD); ND, Not detected. *E*, Real-time PCR analysis of c-Maf and IL-21 in WT and IL-21R$^{-/-}$ CD4$^+$ cells stimulated with IL-27; rel. expression, Relative expression.

tion of IL-10-producing CD4$^+$CD44$^+$ memory T cells was significantly reduced in IL-21R$^{-/-}$ mice, which showed only 10% as many IL-10-producing T cells compared with wild-type (WT) mice (Fig. 3*A*). In contrast, the frequency of IFN-γ producers was similar in WT and IL-21R$^{-/-}$ cells. To further determine whether IL-27 could correct the defect in Tr1 cell development, CD4$^+$CD44$^+$ memory T cells were activated in the presence of IL-27, but IL-21R$^{-/-}$ mice continued to show a significant reduction in IL-10-producing T cells (data not shown). CD4$^+$CD44$^+$ T cells purified from IL-21R$^{-/-}$ mice also showed a lower expression of c-Maf and IL-21 mRNA (Fig. 3*B*), highlighting the importance of this amplification loop in generating Tr1 cells. In addition to the induction of c-Maf, IL-21, and IL-21R, IL-27 also enhanced the expression of ICOS. Because IL-10-producing T cells were first shown to be preferentially costimulated by ICOS (12), we analyzed the effect of a lack of ICOS signaling on the induction of Tr1 cells by IL-27. In vitro differentiation of T cells from ICOS-deficient mice demonstrated that IL-10 production by ICOS$^{-/-}$ T cells was similar to that by WT T cells at 48 h, but by 72 h there was a significant defect in IL-10 production induced by IL-27, as determined by cytometric bead array (Fig. 3*C*) and by intracellular staining (data not shown). In the plate-bound Ab system used here, the ICOS ligand (ICOS-L) is most likely provided by the CD4$^+$ T cells, because T cells can express ICOS-L when activated (13). When Tr1 cells were differentiated from

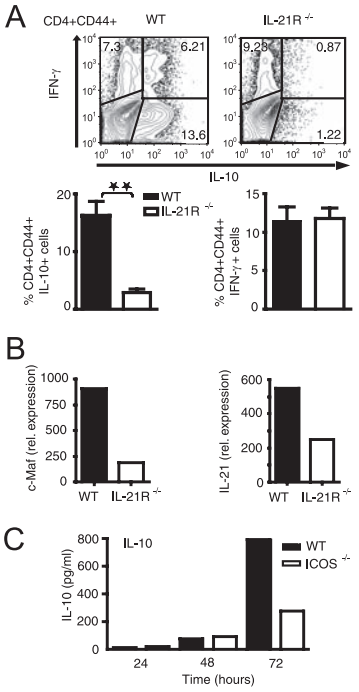

FIGURE 3. Memory $CD4^+CD44^+$ cells from IL-$21R^{-/-}$ mice are defective in IL-10 production in vivo. *A*, IL-10 and IFN-γ production as detected by intracellular cytokine staining in memory $CD4^+CD44^+$ T cells after 72 h of in vitro activation ($n = 5$ mice per group). IL-$21R^{-/-}$ $CD4^+CD44^+$ T cells from lymph nodes are defective in IL-10 production (**, $p = 0.0077$) but not for IFN-γ as compared with WT controls. *B*, Real-time PCR analysis of IL-21 and c-Maf in WT and IL-$21R^{-/-}$ $CD4^+CD44^+$ T cells cultured in vitro for 48 h; rel. expression, Relative expression. *C*, IL-10 production induced by IL-27 in WT (filled bars) and $ICOS^{-/-}$ (open bars) $CD4^+CD62L^+$ $CD25^-$ T cells as measured by cytokine bead array.

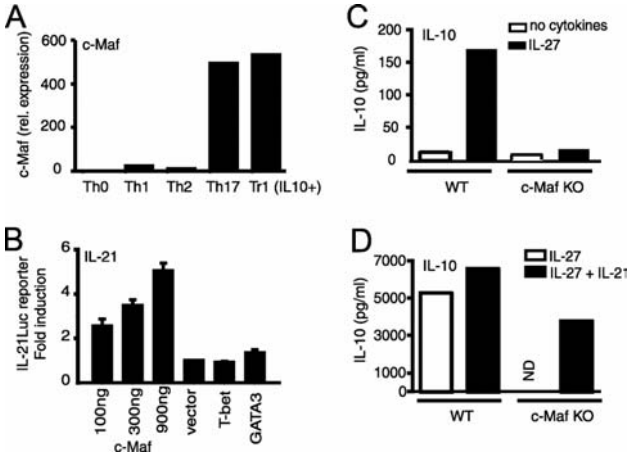

FIGURE 4. IL-27 induces c-Maf, which transactivates IL-21. *A*, Relative expression (rel. expression) of c-Maf in Th0, Th1, Th2, and Th17 cells as detected by real-time PCR. *B*, c-Maf transactivates the IL-21 promoter in HEK293 T cells as detected by cotransfection of the IL-21-luciferase reporter plasmid (IL-21Luc reporter) with c-Maf expression vector (c-Maf) transfected at three different plasmid concentrations (100, 300 or 900 ng), the control empty expression vector (vector; 900 ng), the T-bet vector (900 ng), or the GATA3 (900 ng) vector. Promoter activity was quantified by a luciferase assay 24 h post-transfection. The promoter-luciferase activity observed by transfection of the empty expression vector was normalized to 1. *C* and *D*, IL-10 production induced by IL-27 (*C*) or IL-27 plus IL-21 (*D*) in WT and c-$Maf^{-/-}$ $CD4^+CD8^-CD25^-$ T cells after 4 days in culture, as detected by cytokine bead array. ND, Not detectable; KO, knockout.

$ICOS^{-/-}$ mice, it was clear that $ICOS^{-/-}$ mice had a defect in sustaining growth/expansion of Tr1 cells in vitro.

Our results clearly demonstrated that IL-27 induces expression of c-Maf and that expressions of c-Maf and IL-21 mRNA appeared to be coexpressed in differentiating Tr1 cells under various differentiation conditions (Figs. 1, 2*E*, and 3*B*). Therefore, we compared c-Maf expression in T cell subsets (Th0, Th1, Th2, Th17, and Tr1), and observed that Tr1 cells had ~500-fold higher expression than Th1 or Th2 cells (Fig. 4*A*). We have observed that c-Maf and IL-21 mRNA were coexpressed by Tr1 cells, suggesting that c-Maf may be a transcription factor for IL-21, which in turn expands Tr1 cells. Our analysis of the IL-21 promoter revealed four putative conserved binding sites located 1070 bp (half MARE), 370 bp (v-MARE), 260 bp (half MARE) and 200 bp (v-MARE) upstream of the transcriptional start site (where MARE is Maf recognition element and v-MARE is v-Maf recognition element). To test this, an IL-21 promoter-luciferase reporter construct was cotransfected with a c-Maf expression plasmid into HEK 293T cells. Interestingly, c-Maf could transactivate IL-21 promoter-luciferase in a dose-dependent manner, but the transcription factors T-bet and GATA3, which are involved in Th1 and Th2 differentiation, could not (Fig. 4*B*), suggesting that c-Maf may expand Tr1 cells by inducing IL-21 production. Indeed, we have previously shown that c-Maf-deficient mice have a defect in IL-21 production (8). To address whether c-Maf-deficient mice have a defect in the IL-27-mediated IL-10 and IFN-γ production, we activated c-Maf-deficient T cells in the presence of IL-27 and analyzed the expression of IL-10 and IFN-γ. IL-27 was not able to induce either IL-10 or IL-21 production in c-Maf-deficient $CD4^+$ T cells (Fig. 4*C* and data not shown), but the IL-27-mediated IFN-γ response was not affected (data not shown). As c-Maf has been described to directly transactivate the IL-10 promoter (14, 15), we further added IL-21 to IL-27-activated c-Maf-deficient $CD4^+$ T cells and showed that exogenous IL-21 can partially rescue IL-10 production in c-$Maf^{-/-}$ T cells (Fig. 4*D*), highlighting the importance of IL-21 transactivation by c-Maf.

These data clearly show that IL-27 induces c-Maf, a transcription factor previously identified in Th2 cells, to transactivate IL-21, which then drives the expansion of Tr1 cells. Therefore, similar to Th17 and T follicular helper (TFh) cells, Tr1 cells express c-Maf and IL-21 and use IL-21 for autocrine growth and expansion. It is interesting to note that three different T cell subsets, Tr1, Th17, and TFh cells, which express c-Maf and IL-21, also produce IL-10, albeit at different levels (8, 16, 17). IL-21 acts as an autocrine growth/differentiation factor for all three subsets of T cells. It stands to reason that IL-21, which belongs to the family of IL-2 growth factors and uses the common γ-chain for signaling, may act as an expansion/growth factor for cells that do not produce IL-2. Consistent with this idea, loss of IL-21 or IL-21 signaling results in a defect in all the three T cell subsets, Th17, TFh, and Tr1 (8, 16). Our data are consistent with a recent study showing that IL-21 mediates its inhibitory effects by inducing IL-10 production (18). Similar to IL-6, which induces IL-21 by inducing phospho-STAT-3, IL-27 also induces phospho-STAT-3 and IL-21, most likely due to the fact that IL-6 and IL-27 both share the gp130 chain for signaling.

IL-27-enhanced expression of ICOS is of interest because ICOS was initially shown to costimulate IL-10-producing T cells (12). We and others have shown that ICOS/ICOS ligand interaction induces c-Maf expression (8, 11) which may further enhance stable IL-21 production from developing Tr1 cells. Like Th17 and TFh cells, ICOS appears to be crucial in maintaining IL-27-driven Tr1 cells. These results are supported by the observation that $ICOS^{-/-}$ mice indeed have a defect in IL-10-producing T cells and therefore develop more severe autoimmunity (8, 19). IL-27 also up-regulates ICOS for maintenance and survival of Tr1 cells.

In summary, we have demonstrated that IL-27 drives the expansion and differentiation of Tr1 cells by inducing expression of three key elements: the transcription factor c-Maf, the growth factor IL-21, and the costimulatory receptor ICOS, which coordinately act to mediate Tr1 differentiation (supplemental Fig. 3). Loss of any of these factors results in a defective IL-27-driven, IL-10-producing Tr1 cells.

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Disclosures

The authors have no financial conflict of interest.

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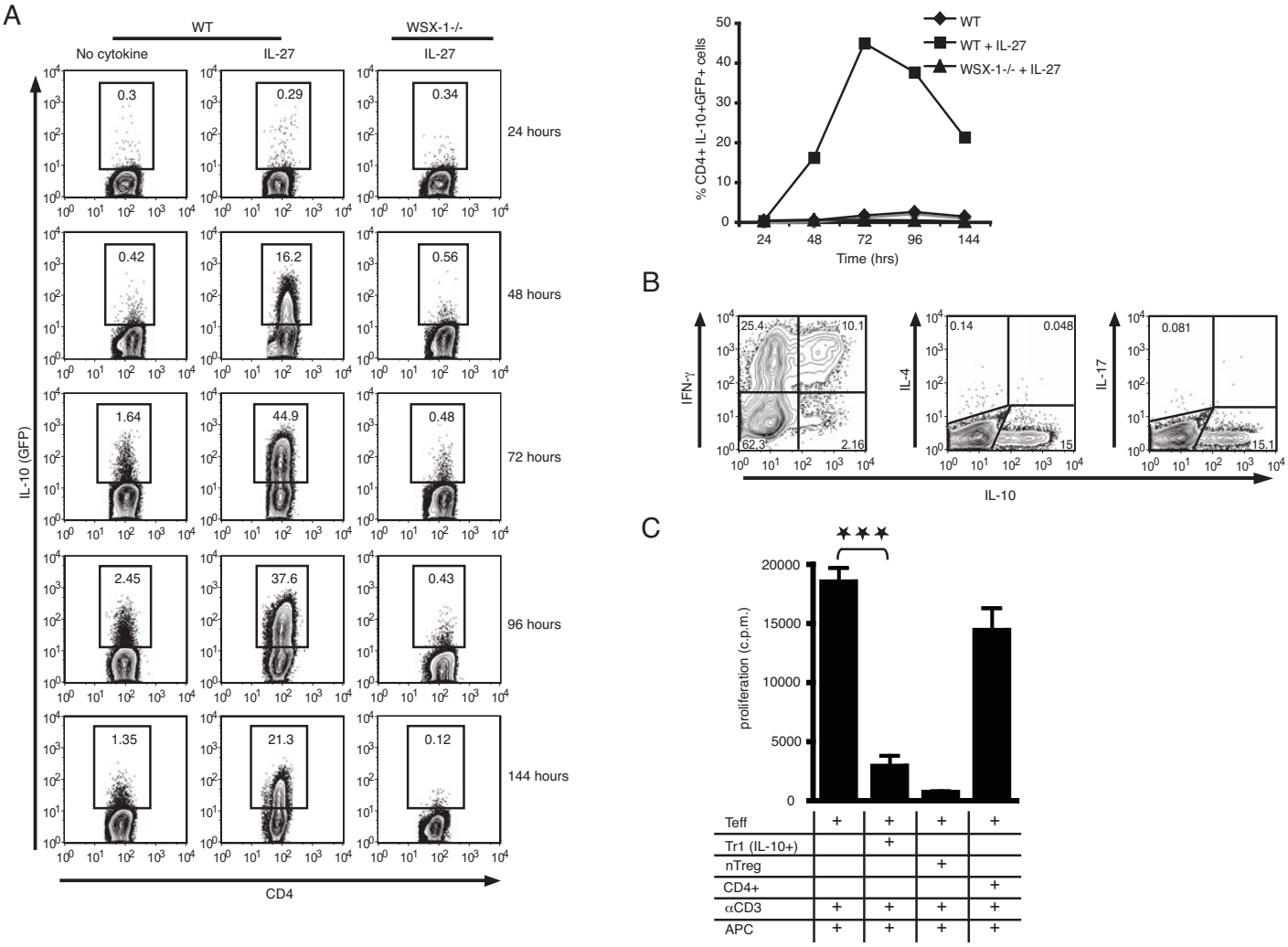
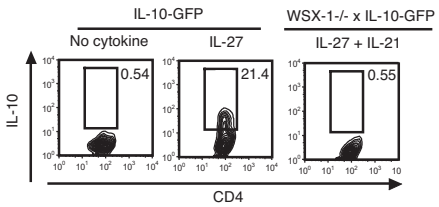


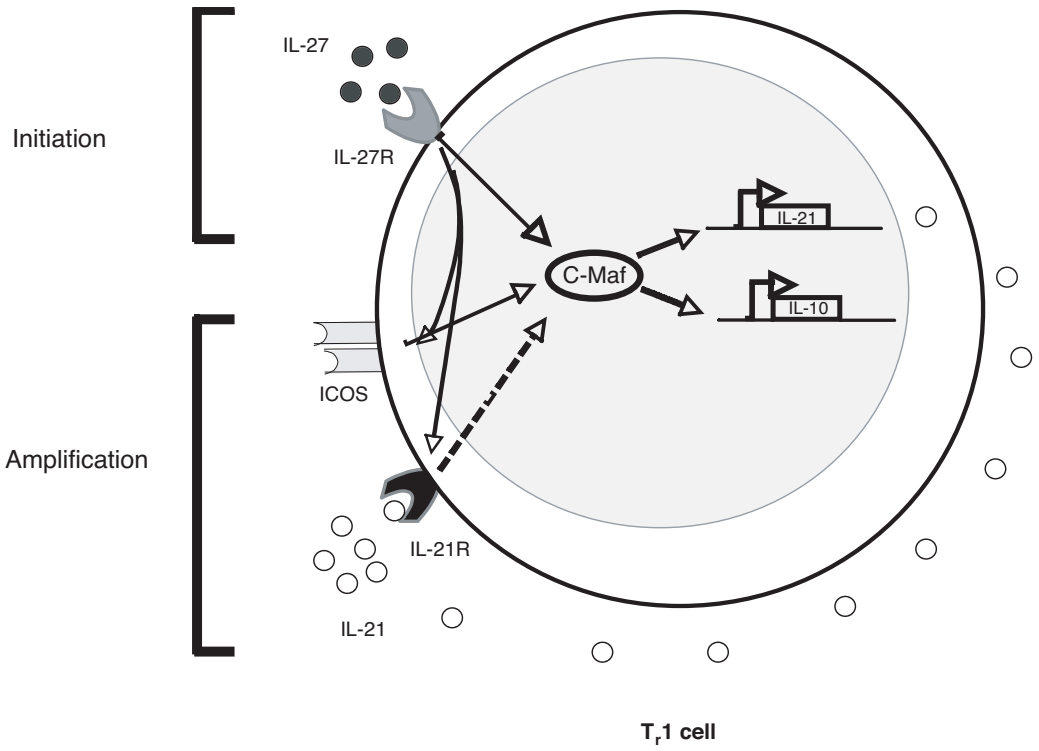
Supplementary Figure 1. **IL-27 induces IL-10-producing Tr1 cells that suppress T cell responses.** (A) Naïve CD4+CD62LhiCD25- T cells obtained from wild type IL-10-eGFP reporter ("Vert-X") or IL-27 ra-/- (WSX-1-/-) x Vert-X mice were differentiated in the presence of IL-27 and anti-TGF-β and examined at different time points following activation for the expression of IL-10.GFP+ T cells by flow cytometry. (B) Coexpression of IL-10 with IFN-γ, IL-4 or IL-17 was assessed by intracellular cytokine staining in T cells differentiated with IL-27 after 48 h; (C) Tr1 cells generated in the presence of IL-27 were tested for their suppressive capacity in an in vitro proliferation assay. A fixed number of naïve responder T cells (Teff, CD4+Foxp3- from Foxp3.gfp.KI mice) were cultured with Tr1 cells (CD4+IL-10.GFP+ from Vert-X mice) in the presence of irradiated syngenic splenocytes as APC and anti-CD3. Mean [3H] thymidine incorporation indicated as c.p.m. (+s.d.) in triplicate wells is shown. A positive control, nTreg (naïve CD4+Foxp3+ from Foxp3.gfp.KI mice) and a negative control, naïve CD4+ cells (CD4+) from Vert-X mice cultured without IL-27, were included. Tr1 cells significantly suppressed the proliferation of responder T cells (Teff) ($P<0.0005$).

Supplementary Figure-2



Supplementary Figure 2. IL-21 cannot induce IL-10 in the absence of IL-27 signaling.
IL-10.GFP expression as analyzed by flow cytometry in naïve T cells activated for 72 h, in the absence or presence of IL-27 from wild type IL-10-eGFP reporter ("Vert-X") mice and in the presence of IL-27 and IL-21 from IL-27R-/- (WSX-1) x Vert-X reporter mice.

Supplementary Figure-3



Supplementary Figure 3. Induction of Tr1 cells by IL-27. IL-27 / IL-27R signaling induces c-Maf that directly transactivates IL-21 promoter. IL-21 amplifies Tr1 by acting as a growth factor for them. ICOS costimulatory molecule induced by IL-27 further increases c-Maf expression, IL-21 production and IL-10 transactivation.

3.2 AhR interacts with c-Maf to drive IL-27-induced Tr1 cell generation

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Another IL-27 induced gene that we identified was the ligand–activated transcription factor aryl hydrocarbon receptor (AhR). AhR had been described to control Foxp3^{+}Treg and T_H17 cells differentiation and a single injection of TCDD, an agonist to AhR, protects against EAE. We showed that the AhR agonists TCDD or FICZ could also potentiate IL-27-induced T_R1 cells *in vitro* by increasing their IL-10 production. We further demonstrated that under T_R1-skewing conditions, AhR binds to c-Maf and promotes the transactivation of both *il10* and *il21* promoters, resulting in the generation of T_R1 cells (Figure 2). Finally, AhR controls the IL-10-dependent immunosuppressive activity of IL-27 in an *in vivo* adoptive transfer model of Experimental Autoimmune Encephalomyelitis. Together, those findings suggest that the manipulation of AhR signaling could be beneficial in the resolution of excessive inflammatory responses.

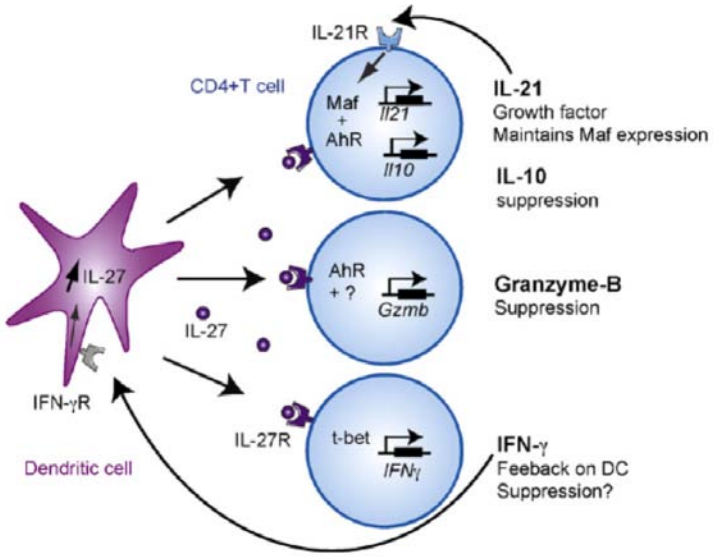

Figure 2. Molecular mechanisms governing the induction of IL-27-induced Tr1 cells

Three different pathways are induced by IL-27. IL-27 drives the expression of the transcription factors Maf and AhR, which bind together to transactivate the *Il21* and *Il10* promoters. IL-21 maintains Maf and AhR expression, while IL-10 is essential for the suppressive function of Tr1 cells (Upper panel). IL-27-induced AhR, alone or with an uncharacterized cofactor, promotes Granzyme-B expression that mediates the contact-dependent suppressive activity of Tr1 cells (middle panel). Finally IL-27 promotes t-bet expression that resulting in IFN-γ secretion. IFN-γ then acts on DCs to enhance IL-27 expression and further support Tr1 cell differentiation (lower panel) (adapted from Pot C. et al, Semin Immunol. 2011 Jun;23(3):202-8).

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The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27

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Type 1 regulatory T cells (Tr1 cells) that produce interleukin 10 (IL-10) are instrumental in the prevention of tissue inflammation, autoimmunity and graft-versus-host disease. The transcription factor c-Maf is essential for the induction of IL-10 by Tr1 cells, but the molecular mechanisms that lead to the development of these cells remain unclear. Here we show that the ligand-activated transcription factor aryl hydrocarbon receptor (AhR), which was induced by IL-27, acted in synergy with c-Maf to promote the development of Tr1 cells. After T cell activation under Tr1-skewing conditions, the AhR bound to c-Maf and promoted transactivation of the *Il10* and *Il21* promoters, which resulted in the generation of Tr1 cells and the amelioration of experimental autoimmune encephalomyelitis. Manipulating AhR signaling could therefore be beneficial in the resolution of excessive inflammatory responses.

Type 1 regulatory T cells (Tr1 cells) have emerged as an important subset of CD4$^+$ T cells that help to control excessive inflammatory responses[1]. The anti-inflammatory effects of Tr1 cells rely on their secretion of interleukin 10 (IL-10), which dampens the function of antigen-presenting cells and antigen-specific effector T cells to suppress tissue inflammation and autoimmunity. However, progress in the molecular analysis and understanding of the biological functions of Tr1 cells has been hampered by the lack of appropriate methods for generating large numbers of IL-10-producing T cells *in vitro*.

IL-27, a heterodimeric cytokine of the IL-12 family, was initially suggested to induce the expansion of proinflammatory T helper type 1 (T_H1) cells by activating the transcription factors STAT1 and T-bet, similarly to IL-12 (ref. 2). However, mice that lack the IL-27 receptor (*Il27ra*$^{-/-}$ mice) develop exaggerated proinflammatory T cell responses[3] and autoimmunity[4], which suggests that IL-27 might be directly involved in the inhibition of tissue inflammation. Indeed, IL-27 has been shown to be a growth and differentiation factor for Tr1 cells[5–7]. The activation of naive CD4$^+$ cells in the presence of IL-27 or transforming growth factor-β (TGF-β) plus IL-27 results in the differentiation of IL-10-producing Tr1 cells that can potently suppress inflammation and autoimmunity. In addition to its effects on the differentiation of Tr1 cells, IL-27 directly inhibits the differentiation of IL-17-producing helper T cells (T_H17 cells)[4,8] and TGF-β-induced regulatory T cells (T_{reg} cells) positive for the transcription factor Foxp3 (ref. 5).

IL-27 drives the population expansion of Tr1 cells by inducing the expression of IL-21, a member of the IL-2 family of cytokines, which acts as an autocrine growth factor for Tr1 cells[9–11]. As with IL-10, IL-21 expression was initially reported to be specific for T_H2 cells[12], but later studies showed that IL-21 is also produced by Tr1 cells[9], T_H17 cells[13] and follicular helper T cells[14]. All these cell types produce IL-10, albeit in different amounts, which suggests that the production of IL-21 and of IL-10 might be linked. Even though IL-21 promotes the population expansion of T_H17 cells[15], *Il21r*$^{-/-}$ mice, like *Il27ra*$^{-/-}$ mice, show greater susceptibility to experimental autoimmune encephalomyelitis (EAE), which indicates that IL-21 might have an important regulatory role *in vivo*[16].

In an effort to delineate the molecular mechanisms by which IL-27 induces Tr1 cells, IL-27 was found to directly induce the transcription factor c-Maf, which is crucial for Tr1 cell differentiation[9]. In the absence of c-Maf, the generation and population expansion of Tr1 cells are compromised. Indeed, c-Maf directly transactivates the *Il10* and *Il21* promoters[9,17]. Although these findings highlight the importance of c-Maf and IL-21 for the biology of Tr1 cells, the addition of recombinant IL-21 to naive CD4$^+$ T cells alone fails to generate Tr1 cells, which suggests that additional IL-27-driven molecular signals contribute to Tr1 cell differentiation.

To explore the molecular mechanisms that account for the effects of IL-27 in Tr1 cell differentiation, we have analyzed the gene expression of IL-27-induced Tr1 cells and found that the ligand-activated transcription factor aryl hydrocarbon receptor (AhR) was induced by IL-27 in Tr1 cells. Furthermore, during Tr1 cell differentiation, AhR physically associated with c-Maf and transactivated the *Il10* and *Il21*

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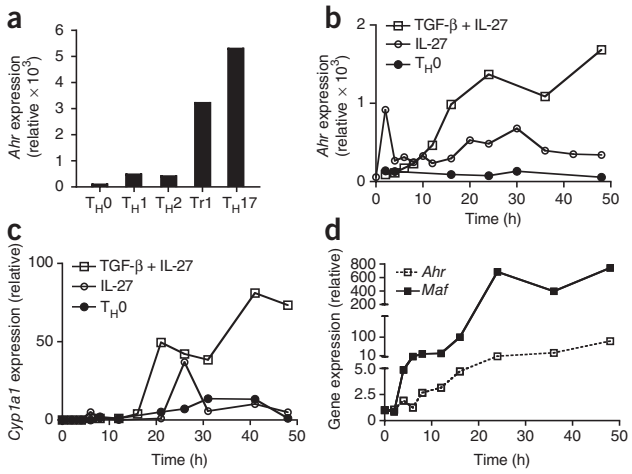

Figure 1 IL-27 upregulates AhR in Tr1 cells. (**a**) RT-PCR analysis of *Ahr* expression at 48 h in T_H0, T_H1, T_H2, T_H17 or Tr1 cells differentiated from naive $CD4^+CD44^{lo}CD62L^{hi}CD25^-$ cells with no cytokines, IL-12, IL-4, TGF-β plus IL-6, or TGF-β plus IL-27, respectively, in the presence of anti-CD3 and anti-CD28; results are presented relative to the expression of *Actb* mRNA (encoding β-actin). (**b**,**c**) RT-PCR kinetic analysis of the expression of *Ahr* (**b**) and *Cyp1a1* (**c**) in T_H0 or Tr1 cells differentiated with IL-27 or TGF-β plus IL-27 in the presence of anti-CD3 and anti-CD28; results are presented relative to *Actb* mRNA expression. (**d**) RT-PCR kinetic analysis of the expression of *Ahr* and *Maf* in Tr1 cells, differentiated with TGF-β plus IL-27, presented relative to expression in T_H0 cells. Data are representative of three experiments.

promoters. Mice with impaired AhR signaling showed less production of IL-10 and resistance to IL-27-mediated inhibition of EAE. Together our results show that AhR and c-Maf act in synergy to induce IL-27-mediated Tr1 cell differentiation.

RESULTS

High expression of *Ahr* in IL-27-induced-Tr1 cells

We first studied *Ahr* expression by RT-PCR in various subsets of $CD4^+$ T cells. Whereas the expression of *Ahr* was modest in T_H1 or T_H2 cells differentiated from naive $CD4^+CD25^-CD62L^+CD44^{lo}$ T cells, *Ahr* expression was very high in Tr1 cells induced with TGF-β and IL-27 (**Fig. 1a**). The expression of *Ahr* in Tr1 cells was similar to that found in T_H17 cells, in which AhR controls IL-22 production[18,19].

We investigated the kinetics of *Ahr* expression during the differentiation of Tr1 cells with TGF-β and IL-27 and found that *Ahr* expression was substantially upregulated 12 h after the initiation of the culture and high expression was sustained throughout Tr1 cell differentiation (**Fig. 1b**). Given that Tr1 cells can also be differentiated by IL-27 without TGF-β (ref. 9), we analyzed the kinetics of *Ahr* expression during the differentiation of Tr1 cells with IL-27 alone. *Ahr* expression was also induced by treatment with IL-27 alone, albeit at lower expression than that in cells induced by both IL-27 and TGF-β (**Fig. 1b**). T cells activated without any polarizing cytokines (T_H0 condition) had only marginal expression of *Ahr*.

The expression of the xenobiotic metabolizing enzyme cytochrome P450, encoded by *Cyp1a1*, is directly controlled by the AhR, which transactivates the *Cyp1a1* promoter[20]. To test whether the AhR is activated during Tr1 cell differentiation, we analyzed *Cyp1a1* expression in naive $CD4^+$ T cells treated with IL-27 with or without TGF-β. *Cyp1a1* was expressed in $CD4^+$ cells as early as 20 h after activation (**Fig. 1c**). Tr1 cells that had been differentiated with IL-27 alone expressed *Cyp1a1* transiently, whereas high *Cyp1a1* expression was sustained in Tr1 cells that had been differentiated with TGF-β and IL-27 (**Fig. 1c**).

The transcription factor c-Maf is important for Tr1 cell differentiation[9]. Therefore, we analyzed the expression of *Ahr* and *Maf* during the differentiation of Tr1 cells with TGF-β and IL-27. We detected *Maf* expression 6 h after the initiation of differentiation, whereas we first detected *Ahr* expression 8 h after differentiation and found it was lower than that of *Maf*. High expression of both *Ahr* and *Maf* was sustained after 24 h (**Fig. 1d**). Overall, these studies show that *Ahr*, like *Maf*, is highly expressed and active during IL-27-triggered differentiation of Tr1 cells.

AhR controls the development of Tr1 cells

Various AhR ligands, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the prototypic environmental AhR agonist, or the putative endogenous ligand 6-formylindolo[3,2-b]carbazole (FICZ), regulate the differentiation of $Foxp3^+$ T_{reg} cells and T_H17 cells[18,19]. The high expression of *Ahr* during Tr1 cell differentiation led us to hypothesize that AhR ligands might also affect Tr1 cell differentiation. To investigate the effect of AhR signaling on Tr1 cell development, we differentiated naive $CD4^+$ cells from Vert-X reporter mice expressing IL-10 linked to enhanced green fluorescent protein (eGFP)[21] using TGF-β and IL-27 in the presence of either of the AhR ligands TCDD and FICZ. Both TCDD and FICZ doubled the number of IL-10–producing T cells (**Fig. 2a**) and increased the secretion of IL-10 more than threefold over controls (**Fig. 2b**), which suggested that activation of AhR promotes Tr1 cell differentiation. We obtained similar results when we induced differentiation with IL-27 alone (**Supplementary Fig. 1**). Although TCDD has been proposed to support the development of $Foxp3^+$ T_{reg} cells[19,22], the addition of AhR ligands together with IL-27 during Tr1 cell differentiation did not induce Foxp3 expression (**Supplementary Fig. 2** and data not shown), which rules out any possible involvement of $Foxp3^+$ T_{reg} cells in the enhanced IL-10 expression. These results are consistent with those of a study showing that FoxP3 is not induced in T_{reg} cells generated by AhR ligands during graft-versus-host responses[23]. To further characterize the contribution of AhR to Tr1 cell differentiation, we knocked down *Ahr* expression using small inhibitory RNA (siRNA). We used TGF-β and IL-27 to differentiate Tr1 cells in the presence of siRNA specific for AhR or control siRNA and analyzed IL-10 expression by RT-PCR and flow cytometry. We found that naive T cells in which *Ahr* expression had been downregulated by siRNA were less able to produce IL-10 than were cells treated with control siRNA (**Fig. 2c**). We obtained similar results when we knocked down *Maf* as a positive control. In agreement with our previous results, addition of the AhR antagonist CH-223191 during the differentiation of Tr1 cells with TGF-β plus IL-27 led to lower IL-10 production than that of cells differentiated without CH-223191 (**Supplementary Fig. 3a**). Similarly, the ability of AhR-deficient T cells to differentiate into Tr1 cells in the presence of TGF-β plus IL-27 was impaired (**Supplementary Fig. 3b**), which confirmed that AhR is essential for the differentiation of Tr1 cells. In summary, modulation of AhR signaling with the agonists FICZ or TCDD, the antagonist CH-223191, by siRNA-mediated downregulation or genetic deletion of *Ahr* profoundly affects the development of IL-10-producing Tr1 cells.

To confirm that AhR is involved in IL-10 expression, we retrovirally transduced primary $CD4^+$ T cells with a bicistronic overexpression vector expressing *Ahr* and green fluorescent protein (*Ahr*-GFP) or an empty GFP-encoding control vector and monitored expression of IL-10 on GFP^+ cells. The expression of IL-10, but not of interferon-γ (IFN-γ), was substantially induced in primary $CD4^+$ T cells that overexpressed

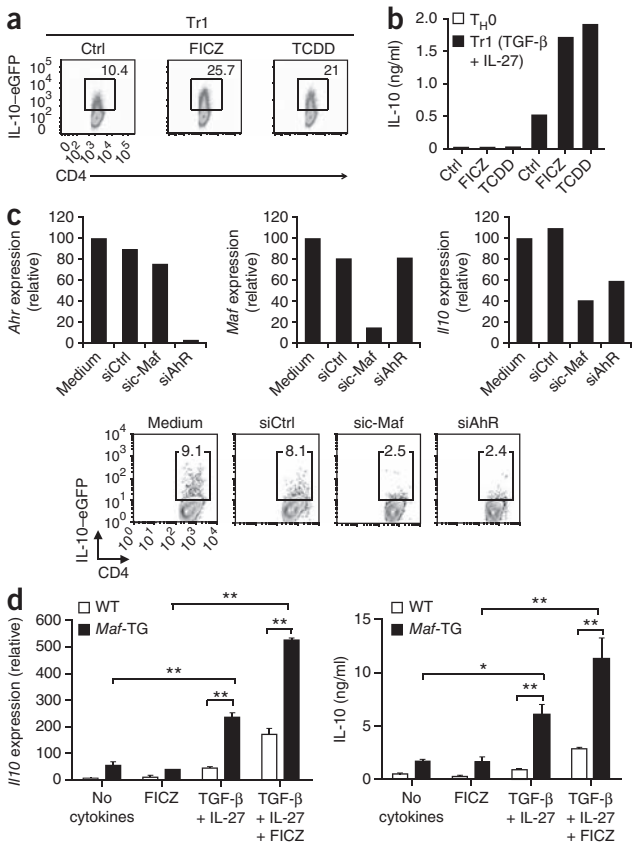

Figure 2 AhR regulates IL-10 production in Tr1 cells induced by TGF-β and IL-27. (**a**) Flow cytometry analysis of IL-10–eGFP expression by naive $CD4^+$ T-cells obtained from Vert-X IL-10 reporter mice and cultured for 72 h with IL-27 and TGF-β in the presence or absence (control (Ctrl)) of the AhR agonists FICZ (100 nM) or TCDD (100 nM). Numbers adjacent to outlined areas indicate percent IL-10–eGFP$^+$CD4$^+$ cells. (**b**) Cytokine bead-array analysis of IL-10 protein after 48 h of culture as in **a**. (**c**) Quantitative PCR analysis of the expression of *Ahr*, *Maf* and *Il10* mRNA (top) and flow cytometry analysis of IL-10–eGFP expression (bottom) in Tr1 cells derived from naive cells obtained from Vert-X IL-10 reporter mice, cultured with IL-27 plus TGF-β and transfected with irrelevant control siRNA (siCtrl) or siRNA specific for c-Maf (sic-Maf) or AhR (siAhR), assessed after 24 h (top) or 48 h (bottom) in culture. Expression of mRNA (top) is presented relative to expression in cells treated with medium alone, set as 100; numbers adjacent to outlined areas (bottom) indicate percent IL-10–eGFP$^+$CD4$^+$ cells. (**d**) Quantitative PCR analysis of *Il10* mRNA expression (left) and enzyme-linked immunosorbent assay of IL-10 secretion (right) by naive T cells isolated from wild-type (WT) or *Maf*-transgenic (*Maf*-TG) mice and differentiated into T_H0 or Tr1 cells with TGF-β and IL-27 in the presence or absence of FICZ (100 nM), analyzed at 48 h (left) or 72 h (right). *Il10* expression (left) is presented relative to *Actb* mRNA expression. $^*P < 0.05$ and $^{**}P < 0.01$ (Student's *t*-test). Data are representative of four (**a**,**b**) or three (**c**,**d**) experiments (error bars (**d**), s.e.m.).

AhR (**Supplementary Fig. 4**). These results suggest that the IL-27-driven expression of AhR is responsible for the enhanced secretion of IL-10 during Tr1 cell differentiation. Given that c-Maf is essential for the generation of Tr1 cells[9], we evaluated the relative contributions of c-Maf and AhR to the development of Tr1 cells. We differentiated naive T cells from mice overexpressing a *Maf* transgene under the control of the *Cd4* promoter without any cytokine or with TGF-β plus IL-27. In addition, to activate AhR signaling, we added FICZ to *Maf*-transgenic T cells. In line with published reports[17], overexpression of c-Maf was sufficient to drive IL-10 secretion by T_H0 cells (**Fig. 2d**). As both TGF-β and IL-27 are required for AhR expression, FICZ alone did not alter the secretion of IL-10 from *Maf*-transgenic cells in the absence of TGF-β and IL-27. However, we found that T cells that overexpressed c-Maf secreted considerably more IL-10 after differentiation with TGF-β plus IL-27 and FICZ than did T cells without the transgene (**Fig. 2d**), which suggested that the two transcription factors AhR and c-Maf act together to enhance IL-10 secretion from Tr1 cells.

AhR regulates IL-21 expression in Tr1 cells

IL-27 acts on Tr1 cells to trigger the production of IL-21, which acts as an autocrine growth factor for Tr1 cells[9]. Therefore, we examined the effect of AhR signaling on the production of IL-21 by Tr1 cells. We first differentiated naive $CD4^+$ T cells with TGF-β plus IL-27 in the presence of FICZ and found that treatment with FICZ led to fourfold more IL-21 production by Tr1 cells than that of cells differentiated without FICZ (**Fig. 3a**). Another AhR ligand, TCDD, had a similar effect (**Supplementary Fig. 5**).

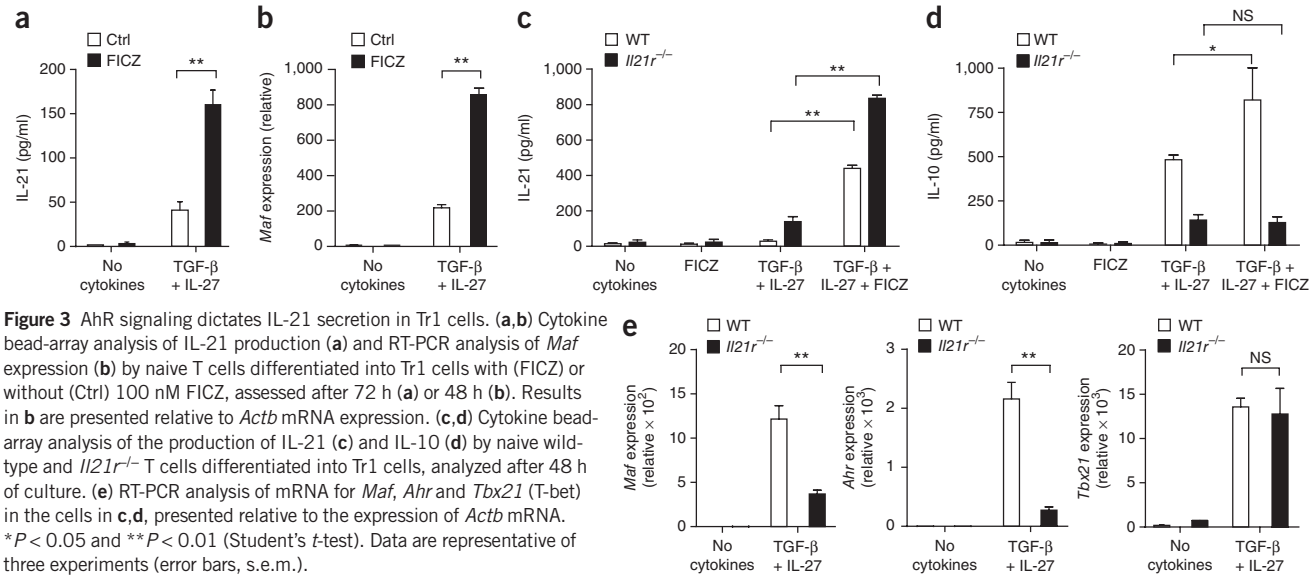

Figure 3 AhR signaling dictates IL-21 secretion in Tr1 cells. (**a**,**b**) Cytokine bead-array analysis of IL-21 production (**a**) and RT-PCR analysis of *Maf* expression (**b**) by naive T cells differentiated into Tr1 cells with (FICZ) or without (Ctrl) 100 nM FICZ, assessed after 72 h (**a**) or 48 h (**b**). Results in **b** are presented relative to *Actb* mRNA expression. (**c**,**d**) Cytokine bead-array analysis of the production of IL-21 (**c**) and IL-10 (**d**) by naive wild-type and *Il21r*$^{-/-}$ T cells differentiated into Tr1 cells, analyzed after 48 h of culture. (**e**) RT-PCR analysis of mRNA for *Maf*, *Ahr* and *Tbx21* (T-bet) in the cells in **c**,**d**, presented relative to the expression of *Actb* mRNA. $^*P < 0.05$ and $^{**}P < 0.01$ (Student's *t*-test). Data are representative of three experiments (error bars, s.e.m.).

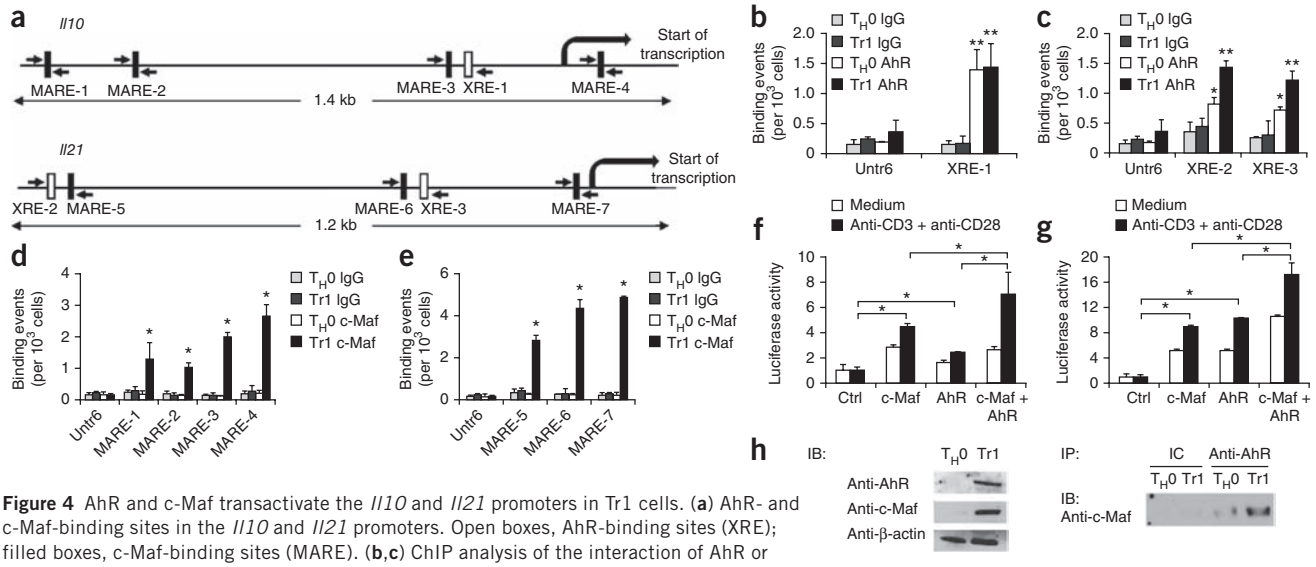

Figure 4 AhR and c-Maf transactivate the *Il10* and *Il21* promoters in Tr1 cells. (**a**) AhR- and c-Maf-binding sites in the *Il10* and *Il21* promoters. Open boxes, AhR-binding sites (XRE); filled boxes, c-Maf-binding sites (MARE). (**b**,**c**) ChIP analysis of the interaction of AhR or isotype-matched control antibody (IgG) with the control Untr6 site or the XRE sites in the *Il10* promoter (**b**) and *Il21* promoter (**c**) in *in vitro*–differentiated Tr1 cells or control T_H0 cells. $^*P < 0.01$ and $^{**}P < 0.001$, AhR versus IgG (two-way analysis of variance (ANOVA)). (**d**,**e**) ChIP analysis of the interaction of c-Maf or isotype-matched control antibody (IgG) with the control Untr6 site or the MARE sites in the *Il10* promoter (**d**) and *Il21* promoter (**e**) in *in vitro*–differentiated Tr1 cells or control T_H0 cells. $^*P < 0.001$, c-Maf versus IgG (two-way ANOVA). (**f**,**g**) Luciferase activity of EL4 mouse lymphoma T cells transfected with a luciferase reporter construct for the *Il10* promoter (**f**) or *Il21* promoter (**g**) along with vectors encoding AhR and/or c-Maf; firefly luciferase activity measured 24 h later is presented relative to the renilla luciferase activity of a cotransfected control. $^*P < 0.001$ (two-way ANOVA). (**h**) Immunoblot analysis (IB) of the expression of AhR and c-Maf in *in vitro*–differentiated T_H0 or Tr1 cells (left) and immunoblot analysis of c-Maf and AhR complexes after immunoprecipitation (IP) of AhR from nuclear extracts with an isotope-matched control antibody (IC) or a specific antibody (right), probed anti-c-Maf. Data are representative of two experiments (error bars (**b**–**g**), s.e.m. of triplicates).

Because c-Maf regulates IL-21 production[9,14], we examined *Maf* expression in Tr1 cells differentiated in the presence of AhR ligands. As well as enhancing IL-21 expression, AhR activation by FICZ during Tr1 cell differentiation led to the upregulation of *Maf* (**Fig. 3b**). Treatment with FICZ or TCDD in the absence of differentiating cytokines had no effect on *Maf* expression (**Fig. 3b** and **Supplementary Fig. 5**), which indicated that AhR activation is not sufficient to upregulate *Maf*. Thus, AhR activation potentiates the expression of c-Maf and IL-21 during the differentiation of Tr1 cells triggered by IL-27.

Naive $CD4^+$ T cells from IL-21R–deficient mice have an impaired capacity to differentiate into Tr1 cells[9]. To test whether the effects of AhR activation on Tr1 cell differentiation were mediated by IL-21, we differentiated naive $CD4^+$ T cells from *Il21r*$^{-/-}$ mice with TGF-β plus IL-27, with or without FICZ. IL-21 secretion was enhanced in FICZ-treated Tr1 cells from either wild-type or *Il21r*$^{-/-}$ mice (**Fig. 3c**). However, IL-10 production was substantially impaired in Tr1 cells derived from *Il21r*$^{-/-}$ mice (**Fig. 3d**). These results suggest that the effects of AhR activation on Tr1 cell differentiation are at least partly mediated by IL-21.

IL-21 is an autocrine Tr1 cell growth factor that enhances IL-10 and c-Maf expression in Tr1 cells[9]. Therefore, we investigated the effect of IL-21 on the expression of *Maf* and *Ahr* mRNA during Tr1 cell differentiation. In agreement with our previous findings, *Maf* expression in Tr1 cells was controlled by IL-21 signaling (**Fig. 3e**). Strikingly, there was much lower *Ahr* expression in Tr1 cells derived from *Il21r*$^{-/-}$ mice than in cells from wild-type mice, whereas expression of mRNA for the transcription factor T-bet, which is induced by IL-27 during Tr1 cell differentiation[24], was unaffected (**Fig. 3e**). This suggests that as well as controlling c-Maf, IL-21 also modulates Tr1 cell development by inducing and/or maintaining AhR expression. Collectively, these results show that AhR signaling controls Tr1 cell differentiation in part by regulating IL-21 production, which in turn contributes to Tr1 cell development as a positive feedback mechanism, probably enhancing the expression of AhR mRNA and the production of IL-21 and IL-10.

AhR and c-Maf transactivate *Il10* and *Il21*

It has been shown that c-Maf transactivates the *Il10* and *Il21* promoters[9,17]. On the basis of our findings that the AhR has a key role in the regulation of IL-10 and IL-21 during Tr1 cell differentiation, we hypothesized that the AhR might transactivate the *Il10* and *Il21* promoters in Tr1 cells as well. We first searched the *Il10* and *Il21* promoters for AhR- and c-Maf-binding sites. We found one putative AhR-binding site (xenobiotic response element (XRE-1)) and four putative c-Maf-binding sites (Maf-recognition elements MARE-1, MARE-2, MARE-3 and MARE-4) in the *Il10* promoter (**Fig. 4a** and **Supplementary Fig. 6**). Similarly, we identified two putative AhR-binding sites (XRE-2 and XRE-3) and three putative c-Maf-binding sites (MARE-5, MARE-6 and MARE-7) in the *Il21* promoter (**Fig. 4a** and **Supplementary Fig. 6**).

To investigate whether AhR binds to XRE sequences in the *Il10* and *Il21* promoters, we monitored whether AhR translated *in vitro* would interact with an oligonucleotide containing the putative AhR-binding site located in the *Il10* or *Il21* promoter. As binding of AhR with AhR nuclear translocator (Arnt) transforms AhR into its high-affinity DNA-binding form[25], we studied the binding of AhR in complex with Arnt to XRE-1, XRE-2 and XRE-3. We incubated the AhR-Arnt complex with a radiolabeled oligomer containing the putative AhR-binding site from the *Il10* or *Il21* promoter and visualized the AhR-Arnt-DNA protein complex by electrophoretic mobility-shift assay (**Supplementary Fig. 7**). Binding of AhR to XRE sequences from the *Il10* or *Il21* promoter was inhibited by the inclusion of a competitor oligonucleotide containing the *Cyp1a1* XRE3 AhR DNA-binding

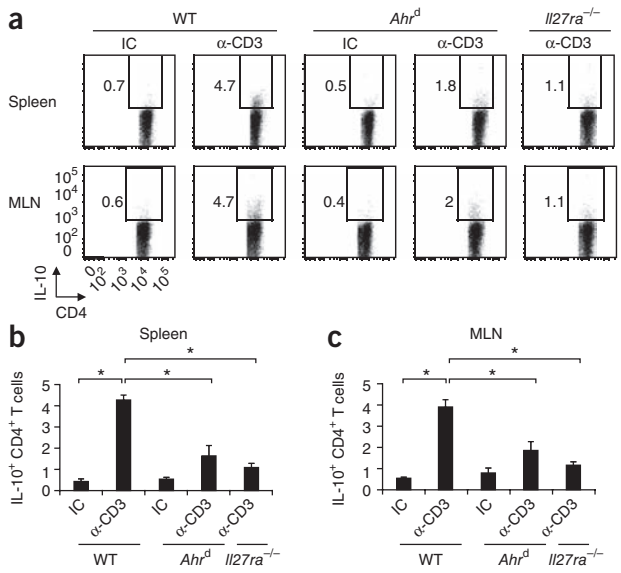

Figure 5 AhR controls the generation of Tr1 cells *in vivo*. (**a**) Flow cytometry analysis of IL-10 expression in CD4$^+$ cells from the spleens and MLNs of *Ahr*^{d}, wild-type or *Il27ra*$^{-/-}$ mice injected intraperitoneally with 20 μg anti-CD3 (α-CD3) or isotype-matched control antibody (IC) once every 3 d for a total of three injections. Numbers adjacent to outlined areas indicate percent IL-10$^+$CD4$^+$ cells. (**b**,**c**) Frequency of IL-10$^+$CD4$^+$ T cells in the spleen (**b**) or MLNs (**c**) of the mice in **a**. *$P < 0.01$ (one-way ANOVA). Data are representative of two experiments (mean and s.d. of three to five mice in **b**,**c**).

site[26] (**Supplementary Fig. 7**). To confirm that AhR can also interact with its target sequences in the *Il10* and *Il21* promoters under physiological conditions, we carried out chromatin immunoprecipitation (ChIP) assays using Tr1 cells differentiated *in vitro* with IL-27 and TGF-β. AhR interacted with XRE-1 in the *Il10* promoter in both Tr1 and T_H0 cells and with XRE-2 and XRE-3 in the *Il21* promoter in Tr1 cells (**Fig. 4b**,**c** and **Supplementary Fig. 6**). Similarly, ChIP assays showed that c-Maf interacted with MARE-1–MARE-4 and MARE-5–MARE-7 on the *Il10* and *Il21* promoters, respectively, but only in Tr1 cells (**Fig. 4d**,**e** and **Supplementary Fig. 6**). We found no interaction with the XRE or MARE sequences in either the *Il10* or *Il21* promoter when we used isotype-matched control antibodies (immunoglobulin G (IgG)), and we found no significant binding of AhR or c-Maf in the control sequence Untr6 (**Fig. 4b–e**). These results suggest that c-Maf controls the cell specificity of the transcription of *Il10* and *Il21*.

To determine the relevance of the binding of AhR and c-Maf to their target sequences in *Il10* and *Il21*, we studied the ability of AhR and c-Maf to transactivate the *Il10* and *Il21* promoters in reporter assays. We used reporter constructs containing the firefly luciferase gene under the control of the *Il10* promoter or the *Il21* promoter. Cotransfection of the *Il10* or *Il21* luciferase reporter construct with a construct encoding mouse c-Maf resulted in slight upregulation of the transcription of *Il10* and *Il21* (**Fig. 4f**,**g**). Transcription was similarly upregulated when we cotransfected the *Il10* or *Il21* luciferase reporter construct with a construct encoding mouse AhR (**Fig. 4f**,**g**). Notably, cotransfection of c-Maf and AhR resulted in additive transactivation of the expression of both *Il10* and *Il21* (**Fig. 4f**,**g**), which suggested that AhR and c-Maf act together to control the transcriptional activity of both promoters.

The concomitant upregulation of c-Maf and AhR during Tr1 cell differentiation (**Fig. 1d**), their ability to bind to *Il10* promoter elements to induce IL-10 secretion (**Fig. 4**) and the proximity of the putative binding sites for c-Maf and AhR on the *Il10* and *Il21* promoters led us to test whether c-Maf and AhR physically interact with each other. AhR interacts with diverse transcription factors, including NF-κB[27] and the estrogen receptor[28]. To address this issue, we differentiated naive CD4$^+$ T cells into either T_H0 or Tr1 cells and immunoprecipitated proteins followed by immunoblot analysis. AhR and c-Maf were upregulated in Tr1 cells (**Fig. 4h**, left). Moreover, we were able to precipitate c-Maf and AhR together in a Tr1 cell nuclear extract by using antibody to AhR (anti-AhR) to immunoprecipitate the complex, followed by immunoblot analysis with anti-c-Maf (**Fig. 4h**, right), which suggested that AhR physically interacts with c-Maf. Together our results show that AhR and c-Maf interact in Tr1 cells to transactivate the *Il10* and *Il21* promoters.

AhR controls Tr1 cell generation *in vivo*

Repeated *in vivo* treatment with anti-CD3 induces IL-10$^+$ T_{reg} cells[29]. Given that we have shown that *Ahr* and *Maf* were induced after *in vitro* differentiation of Tr1 cells (**Fig. 1**), we assumed that they might be similarly induced *in vivo* in IL-10$^+$ T cells induced by treatment with anti-CD3. To test this, we repeatedly administered anti-CD3 or an isotype-matched control antibody to Vert-X reporter mice (expressing IL-10 linked to eGFP) and assessed *Ahr* and *Maf* expression in eGFP$^+$ IL-10-producing T cells in the spleen and mesenteric lymph nodes (MLNs) 4 h after the final injection. In line with our *in vitro* findings, both *Ahr* and *Maf* were substantially induced in IL-10$^+$ T cells (**Supplementary Fig. 8**). Thus, we used this model to analyze the role of AhR in the generation of Tr1 cells *in vivo*. We administered anti-CD3 or an isotype-matched control antibody to wild-type mice, mice with a mutant AhR (*Ahr*^{d}) and *Il27ra*$^{-/-}$ mice and studied the frequency of IL-10$^+$ T cells in the spleen and MLNs. *Ahr*^{d} mice have point mutations in sequence encoding the AhR ligand-binding pocket and therefore show defective AhR-mediated responses *in vivo*[30]. As IL-10 is produced by T_H17 cells[17], Foxp3$^+$ T_{reg} cells[29] and Tr1 cells, we analyzed the production of IL-10 by Foxp3$^-$IL-17$^-$CD4$^+$CD3$^+$TCRαβ$^+$ T cells (**Supplementary Fig. 9**). The administration of anti-CD3 to wild-type mice resulted in significant induction of IL-10$^+$ T cells in the spleen and the MLNs (**Fig. 5**). The induction of IL-10-Dproducing cells in this setting was mediated by IL-27, as it was not present in anti-CD3-treated *Il27ra*$^{-/-}$ mice (**Fig. 5**). In addition, *Ahr*^{d} mice also showed a significant impairment in their ability to produce IL-10$^+$ T cells, both in the spleen and the MLNs, after being treated with anti-CD3 (**Fig. 5**). Thus, AhR controls the generation of IL-10$^+$ T cells *in vivo*.

AhR controls the IL-27-mediated inhibition of EAE

To address the *in vivo* relevance of AhR in inducing IL-27-driven Tr1 cells and its effect on regulating autoimmunity and tissue inflammation, we exploited an adoptive-transfer model of EAE in which IL-10, induced by IL-27, regulates EAE induced by adoptive transfer of T cells[7]. We used this model system to investigate the role of AhR in the *in vivo* suppressive activity of IL-27-induced IL-10 production. This model allowed us to exclude the effects of AhR on non-T_R1 T cells, as IL-27, when given *in vivo*, can also inhibit T_H17 and Foxp3$^+$ T_{reg} cell differentiation[5,31]. We first immunized wild-type or *Ahr*^{d} mice with the myelin oligodendrocyte peptide of amino acids 35–55 (MOG(35–55)) emulsified in complete Freund's adjuvant and tested the secretion of IL-10 by CD4$^+$ T cells reactivated with MOG(35–55) and IL-27. We observed significantly less production of IL-10 by CD4$^+$ T cells from *Ahr*^{d} mice treated with IL-27 (**Fig. 6a**).

In line with published findings[7], wild-type CD4$^+$ T cells reactivated solely with MOG(35–55) were poor inducers of EAE (data

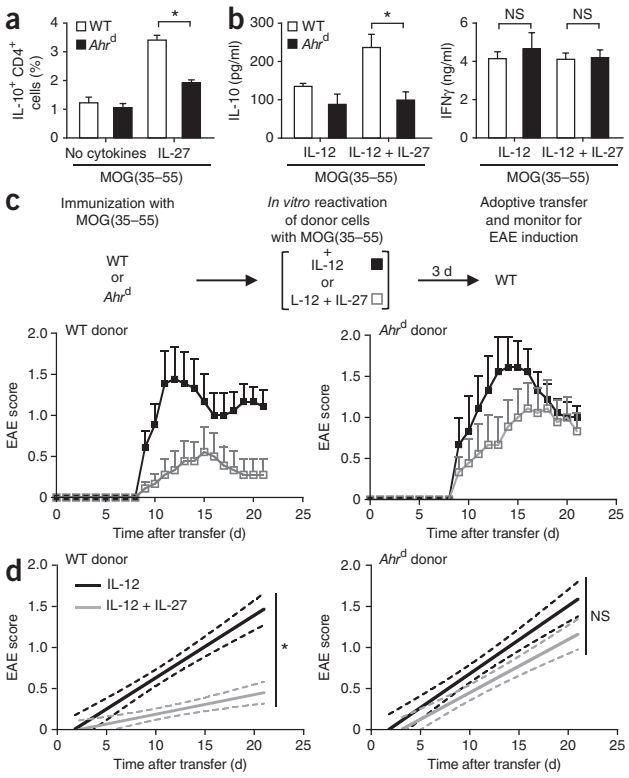

Figure 6 AhR controls the IL-27-mediated inhibition of EAE. (**a**) Flow cytometry analysis of IL-10-producing CD4$^+$ T cells obtained from the spleen and lymph nodes of MOG(35–55)-immunized wild-type or *Ahr*^{d} mice and cultured for 5 d with MOG(35–55) in the presence or absence of IL-27. *$P < 0.05$ (Student's *t*-test). (**b**) Cytokine bead-array analysis of the secretion of IL-10 and IFN-γ by cells restimulated for 3 d as in **a** *in vitro* in the presence of IL-12 with or without IL-27. NS, not significant; *$P < 0.05$ (Student's *t*-test). (**c**) EAE development in wild-type recipient mice given adoptive transfer of wild-type (left) or *Ahr*^{d} (right) MOG-specific cells prepared as in **b**. (**d**) Linear-regression curves of the disease for each group in **c** (dashed lines, 95% confidence intervals). *$P < 0.0001$, IL-12 versus IL-12 plus IL-27 (Student's *t*-test). Data are representative of three experiments (error bars (**a**–**c**), s.e.m.).

not shown). However, reactivation of T cells in the presence of IL-12 before adoptive transfer generated MOG(35–55)-specific donor cells that induce EAE with high incidence[7]. We compared the secretion of IL-10 by donor cells obtained from MOG(35–55)-immunized wild-type or *Ahr*^{d} mice that had been restimulated *in vitro* with IL-12 with or without IL-27. We found that IL-27 enhanced IL-10 production in wild-type T cells but not *Ahr*^{d} T cells (**Fig. 6b**). IL-27 did not affect the production of IFN-γ triggered by IL-12 (**Fig. 6b**), and we did not detect notable amounts of IL-17 after stimulating donor cells with IL-12 and IL-27 (data not shown). We then adoptively transferred these cells into wild-type mice to induce EAE and found that wild-type effector cells reactivated in the presence of IL-12 induced disease in most recipient mice. The incidence of EAE was significantly lower for donor cells reactivated with IL-12 and IL-27 *in vitro* (**Fig. 6c**,**d** and **Table 1**). *Ahr*^{d} donor T cells activated in the presence of IL-12 also induced EAE after adoptive transfer. However, the activation of *Ahr*^{d} T cells in the presence of IL-27 resulted in a significantly greater incidence of disease than did wild-type T cells treated similarly (**Fig. 6c**,**d** and **Table 1**), which suggests that AhR is essential for the IL-27-mediated inhibition of EAE *in vivo*. These data show that IL-27 controls the adoptive induction of EAE by wild-type but not *Ahr*^{d} T cells and that this disease inhibition by IL-27 correlates with the AhR-dependent induction of IL-10 production by IL-27.

Table 1 EAE incidence after cell transfer

Group	Disease incidence	Maximum score	Time of onset (d)
WT, IL-12	8/9 (89%)	2.1 ± 0.2	11.5 ± 1.5
WT, IL-12 + IL-27	3/9 (33%)*	1.8 ± 0.5	10.3 ± 1.3
Ahr^d, IL-12	8/9 (89%)	2.4 ± 0.2	11.0 ± 0.9
Ahr^d, IL-12 + IL-27	8/9 (89%)	2.0 ± 0.2	12.8 ± 1.4

EAE in wild type and mice treated as in **Figure 6c**. Disease incidence is presented as diseased mice/total mice; time of onset is presented as mean ± s.e.m.
*$P < 0.05$ (Fisher's exact test).

DISCUSSION

IL-27 promotes the differentiation of Tr1 cells that are instrumental in controlling autoimmunity and tissue inflammation[5]. Here we have reported that *Ahr*, like the proto-oncogene *Maf*, was strongly induced during Tr1 cell differentiation and that its expression was as high in Tr1 cells as it was in T_H17 cells. In addition to increasing IL-10 production, activation of AhR by the putative endogenous ligand FICZ also increased IL-21 production in Tr1 cells, and this supported their development. Furthermore, the two transcription factors (AhR and c-Maf) associated with each other to transactivate the *Il10* and *Il21* promoters. The relevance of these findings is underscored by the ability of AhR signaling to control IL-27-driven IL-10-producing T cells *in vivo*.

Tr1 cells are an important T_{reg} cell type that produce mainly IL-10 and do not express Foxp3 but suppress tissue inflammation, graft-versus-host disease and autoimmunity in an IL-10-dependent manner. Although IL-10 was initially described as a differentiation factor for Tr1 cells, IL-27 also generates IL-10-producing Tr1 cells[5,8]. IL-27 induces both IL-10 and IFN-γ in T cells. These T cells, which produce both IL-10 and IFN-γ, have been reported to be generated *in vivo* after treatment with altered peptide ligands[32] and regulate autoimmune tissue inflammation. Whether IL-10 and IFN-γ both contribute to the immunosuppressive function is not clear, but initial data suggest that IFN-γ produced by Tr1 cells is a potent inhibitor of T_H17 cells. This supports the view that both IFN-γ and IL-10 might contribute to the immunoregulatory properties of Tr1 cells.

Although the ability of c-Maf to transactivate the *Il10* promoter has been described[17], published findings have shown that the ability of c-Maf to transactivate *Il10* in hepatocytes stimulated by fatty acids requires additional cofactors essential for inducing *Il10* expression[33]. In addition, it has been proposed that c-Maf and AhR act together to induce the transcription of genes encoding β_7 integrins[34]. Here we have shown that IL-27 induced AhR, which associated with c-Maf to generate IL-10-producing Tr1 cells. Our results have demonstrated that AhR and c-Maf bound to proximal regions in both the *Il10* promoter (XRE-1 and MARE-3) and *Il21* promoter (XRE-2 and MARE-5; XRE-3 and MARE-6). This, along with our observation that AhR and c-Maf bound to each other to transactivate the *Il10* and *Il21* promoters, supports the idea that these two transcription factors have a key role in the development of Tr1 cells. In addition, our ChIP results have shown that whereas AhR bound to the *Il10* and *Il21* promoters in both Tr1 and T_H0 cells, c-Maf associated with *Il10* and *Il21* promoters only in Tr1 cells, which suggests that c-Maf controls the tissue specificity of the transcription of *Il10* and *Il21*.

IL-21 acts as a growth factor for both Tr1 and T_H17 cells. IL-21 has been reported to support IL-17 secretion from T_H17 cells through a self-amplifying loop[35]. Similarly, our results suggest that during Tr1 cell differentiation, AhR and c-Maf participate in a self-amplifying

feed-forward loop driven by IL-21 signaling, which is essential for the amplification and maintenance of the phenotype of differentiated Tr1 cells. However, the mechanism by which IL-21 induces and/or maintains expression of AhR and c-Maf remains to be determined. IL-21 could mediate this effect by increasing the frequency of IL-10-producing Tr1 cells and could also strengthen the expression of both c-Maf and AhR in a cell-intrinsic manner. Overall, we propose that during the differentiation of Tr1 cells with IL-27, AhR is essential for supporting c-Maf in its ability to transactivate the *Il10* and *Il21* promoters and thereby enhances the differentiation of Tr1 cells.

AhR is essential for production of IL-22 as well as of IL-10 (ref. 18). IL-22, a T_H17-specific cytokine, promotes acanthosis in psoriasis but also protects mice from dextran sulfate–mediated colitis and concanavalin A–induced liver damage[36]. IL-22 is a member of the IL-10-related cytokine family[36] and might be regulated similarly. Therefore, our results raise the possibility that the interaction between c-Maf and AhR might control the production of not only IL-10 but also IL-22. Indeed, motif analysis has shown that the *Il22* promoter, like the *Il10* promoter, contains c-Maf- and AhR-binding sites in close proximity, which suggests that c-Maf and AhR might also act together to induce *Il22* transcription.

Environmentally ubiquitous polycyclic aromatic and planar halogenated hydrocarbons, for which AhR is a cellular receptor, represent two important classes of environmental pollutants to which humans are regularly exposed. Endogenous ligands, although incompletely classified[37], represent an additional category of AhR activators. Notably, exposure to polycyclic aromatic and planar halogenated hydrocarbons can result in contrasting AhR-dependent effects on the immune response. For example, TCDD-driven AhR activation enhances inflammation in rheumatoid arthritis[38], and endogenous ligand-driven AhR activation induces production of inflammatory cytokines by T_H17 cells[18,19]. However, prototypic polycyclic aromatic and planar halogenated hydrocarbons can impair B cell and T cell proliferative responses, alter antibody isotype switching, block plasma cell differentiation, compromise antibody production, induce apoptosis in developing lymphocytes, inhibit natural killer cell activity, modulate cytokine production, decrease cytotoxic T lymphocyte activity and promote tumor growth[39–46]. In this context, our report, together with results by Gandhi *et al.* published in this issue of *Nature Immunology*[47], provides evidence that the interaction of AhR with c-Maf is essential for the generation of mouse and human IL-10-secreting Tr1 cells that suppress inflammatory responses. These contrasting outcomes suggest that the *in vivo* immunological effects of AhR activation are tissue specific, ligand specific or both. In the context of autoimmunity, the outcome probably depends on the type of T cell–differentiation pathway activated by a given AhR ligand. As AhR and c-Maf are expressed in T_H17 cells[17], Foxp3$^+$ T_{reg} cells and Tr1 cells[9], it is unlikely that AhR alone or in combination with c-Maf acts as a specific 'lineage-specification' transcription factor for Tr1 cells. We instead postulate that AhR, in combination with c-Maf, controls parts of the Tr1 cell transcriptional and differentiation program. Thus, in response to different environmental ligands, AhR can induce opposing subsets of T cells in different ways, resulting in either tissue inflammation or immunosuppression. Therefore, although the molecular basis for the differences in the differentiation pathways favored by an AhR ligand (or more AhR ligands) is not clear, we predict that AhR ligands direct the nature of downstream signaling and thus provide specificity and dictate T cell subset dominance (Tr1 versus T_H17) during an immune response. In summary, we have shown that AhR, together with c-Maf, regulates the generation of Tr1 cells induced by IL-27. In addition to delineating the molecular mechanisms that account for the generation of Tr1 cells, our findings, together with other studies[18,19], suggest that AhR is not only a receptor for environmental pollutants but also an important target for regulating T cell differentiation and the quality of immune responses *in vivo*.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

L.A., F.J.Q. and C.P. did *in vitro* and *in vivo* experiments and wrote the manuscript; N.J. did *in vivo* experiments; S.X., D.K. and E.J.B. did *in vitro* experiments; D.H.S. provided reagents and advice; and H.L.W. and V.K.K. supervised the study, edited the manuscript and contributed equally to this manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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3.3 Metallothioneins negatively regulate IL-27-induced T_R1-cell differentiation.

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IL-27 induces T_R1 cells that produce IL-10. STAT1 and STAT3 have been described as key transcription factors that promote IL-10 secretion from T_R1 cells induced by IL-27. However, the molecular pathways for negatively regulating T_R1 cell differentiation remain elusive. Here, we show that IL-27 induces metallothioneins (MTs) that in turn prevent T_R1 cell development. MTs had been previously studied as heavy metal scavenger. Environmental stressors as well as tumor-promoting agents have been shown to induce MTs as exemplified by the high levels of MTs expression at sites of inflammation and in breast and lung cancers (McGee et al.). In this regard, MTs were reported to mediate neuroprotection during pathological conditions by preventing tissue damage due to oxidative stress (Pedersen et al., 2009). We observed that MT expression leads to the reduction of STAT1 and STAT3 phosphorylation under T_R1 differentiation condition, resulting in impaired IL-10 production. Accordingly, T_R1 cells derived from MT-deficient mice showed an increased ability to produce IL-10 and potently suppress Experimental Autoimmune Encephalomyelitis (EAE) upon adoptive transfer. Moreover, activation of STAT1 and/or STAT3 can overcome the suppression of IL-10 by MTs, indicating a dynamic balance between STATs and MTs in regulating IL-10 during T_R1 cell differentiation.

Metallothioneins negatively regulate IL-27–induced type 1 regulatory T-cell differentiation

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IL-27–induced type 1 regulatory T (Tr1) cells suppress autoimmunity by producing IL-10. Signal transducer and activator of transcription (STAT) 1 and STAT3 have been described as key transcription factors that promote IL-10 secretion from Tr1 cells induced by IL-27. However, the molecular pathways for negatively regulating Tr1 cell differentiation remain elusive. Here, we show that IL-27 induces metallothioneins (MTs) that in turn prevent Tr1 cell development. MT expression leads to the reduction of STAT1 and STAT3 phosphorylation under Tr1 differentiation condition, resulting in impaired IL-10 production. Accordingly, Tr1 cells derived from MT-deficient mice showed an increased ability to produce IL-10 and potently suppress experimental autoimmune encephalomyelitis upon adoptive transfer. Moreover, activation of STAT1 and/or STAT3 can overcome the suppression of IL-10 by MTs, indicating a dynamic balance between STATs and MTs in regulating IL-10 during Tr1 cell differentiation.

IL-10–producing type 1 regulatory (Tr1) cells are an emerging regulatory T-cell subset, which was shown by us (1) and others (2), to be induced by IL-27. They have been proposed to control autoimmunity and tissue inflammation in mouse models of human autoimmune diseases including multiple sclerosis, inflammatory bowel disease and graft-versus-host disease (3). In addition, Tr1 cells were reported to suppress the induction of cytotoxic effector T cell (CTL) responses and inhibit antitumor immunity (4). Tr1 cells produce both IFN-γ and IL-10, without expressing the regulatory T-cell (Treg)-specific transcription factor, forkhead box P3 (Foxp3) (5). Transcriptional analysis of Tr1 cells showed that Tr1 cells differentiated from *Tbx21*$^{-/-}$ cells exhibited severely compromised IFN-γ but not IL-10 production, which was in contrast to *Stat1*$^{-/-}$ Tr1 cells that showed reduction in both IFN-γ and IL-10 production (6). More recently, we have discovered that the transcription factors c-Maf and aryl hydrocarbon receptor (AhR), both of which are induced by IL-27, bind to the *Il10* promoter, and are essential for the induction of IL-10 in Tr1 cells (1, 7). In addition, IL-27–induced protooncogene c-Maf (*Maf*) and *Ahr* cooperatively bind to the promoter and transactivate the *Il21* gene, which acts as a growth factor for the generation of Tr1 cells. Although the molecular landscape for the generation of Tr1 cells is being identified, very little is known about the negative regulation of Tr1 cell development.

To identify candidate molecules that can control Tr1 cell differentiation, we have performed a comparative gene microarray analysis of Tr1 cells generated with IL-27 and identified that isoforms 1 and 2 of metallothionein (MT) were strongly induced in Tr1 cells by IL-27. MT1 and MT2 are low-molecular-weight proteins involved in the detoxification of heavy metals and in the regulation of oxidative stress (8). There are four different MT genes constitutively expressed in the liver, of which MT1 and MT2 are the most abundantly expressed (9). MT genes are highly induced under different stresses such as inflammation (9) and are specifically induced by proinflammatory cytokines like TNF-α, IL-1, and IL-6 (10). However, the role of MTs in IL-27–induced Tr1 cell differentiation and IL-10 production is not known.

Here, we show that MTs control IL-10 production as Tr1 cells from MT-deficient mice exhibit increased IL-10 production both in vitro and in vivo. At the mechanistic level, we found that, in the absence of MTs, IL-27 induces increased phosphorylation of STAT1 and STAT3 but not STAT4, resulting in enhanced IL-10 production. Furthermore, compared with WT Tr1 cells, *Mt*$^{-/-}$ Tr1 cells were more efficient in their ability to suppress effector T cell proliferation and inhibit the development of experimental autoimmune encephalomyelitis (EAE). Taken together, our data suggest that MTs act as negative regulators for IL-27–induced Tr1 cells.

Results

Late Expression of MTs in IL-27–Induced Tr1 Cells. To gain insight into the differentiation of IL-27–induced Tr1 cells, we performed a comparative microarray analysis of developing Tr1 cells at 72 h after stimulation with IL-27. We found that MT1 and -2 were highly expressed in IL-27–induced Tr1 cells generated from naïve $CD4^+CD25^-CD62L^+CD44^{low}$ T cells compared with T cells similarly activated without the presence of differentiating cytokines (Th0) (Fig. S1*A*). We then analyzed kinetics of MT1 and MT2 expression during the differentiation of Tr1 cells with IL-27. In contrast to Th0 cells, which express only marginal levels of MT1 and -2, both MT isoforms were highly expressed in IL-27–induced Tr1 cells (Fig. S2). Although MT expression was dramatically enhanced in Tr1 cells after 72 h, we failed to detect any significant MT expression before 48 h. Interestingly, this delayed induction of MTs coincided with the induction of IL-10 in developing Tr1 cells (Fig. S2). We also examined the expression levels of MT1 and MT2 in different subsets of $CD4^+$ T cells. We found that both MTs were highly expressed in Tr1 and T helper 17 cells (Th17), whereas Th1 or Th2 cells exhibited modest expression of MT1 and MT2 (Fig. S1*B*).

MTs Impair IL-10 Expression in IL-27–Induced Tr1 Cells. We then investigated the role of MTs on IL-27–induced Tr1 cells by differentiating naïve $CD4^+$ T cells from WT or *Mt*$^{-/-}$ mice. Although IFN-γ production from Tr1 cells was unaffected in the absence of MTs, the frequency of IL-10–producing cells and the secretion of IL-10 were notably enhanced in Tr1 cells derived from *Mt*$^{-/-}$ mice (Fig. 1 *A* and *B*). We and others have previously shown that TGF-β1 and IL-6-derived Th17 cells also express IL-10 (11, 12). MTs are expressed at high levels in Th17 cells differentiated with TGF-β1 and IL-6, whereas MT-deficient Th17 cells did not show a difference in IL-10 or other Th17 cytokines (Fig. S3*A*). Addition of recombinant MT1 or MT2 during differentiation of naïve $CD4^+$ T

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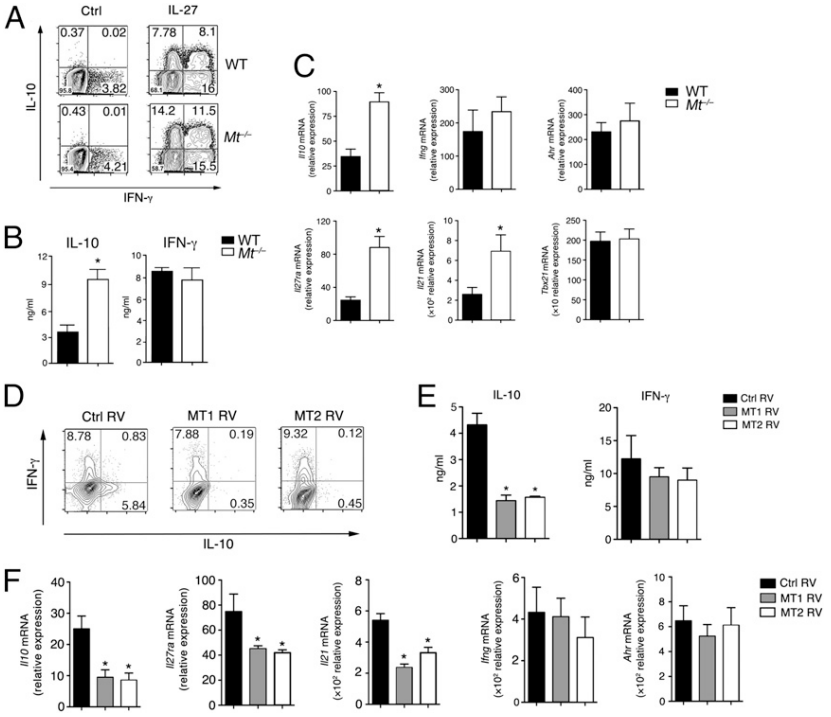

Fig. 1. MTs impair *Il10* expression by IL-27–induced Tr1 cells. (*A* and *B*) Naïve CD4$^+$ T-cells from WT or *Mt*$^{-/-}$ mice were differentiated without (Ctrl) or with IL-27, and the frequencies of IL-10 and IFN-γ expressing cells were determined by flow cytometry after 4 d of stimulation (*A*) and IL-10 and IFN-γ secretion were measured by ELISA at 72 h (*B*). (*C*) The expression levels of Tr1 cell signature genes were determined by RT-PCR 72 h after stimulation and are displayed as values normalized to mRNA expression of β-actin. (*D* and *E*) WT Naïve CD4$^+$ T cells differentiated with IL-27 were transduced with control, MT1-, or MT2-expressing retrovirus, and IFN-γ and IL-10 expression was determined by flow cytometry (*D*) and ELISA (*E*). (*F*) Three days after stimulation, retrovirus-transduced T cells were sorted for GFP$^+$ cells and RT-PCR was subsequently performed to examine the expression levels of the indicated genes. The data are representative of three independent experiments. *$P < 0.05$ (Student *t* test, error bars show SD).

cells with IL-27 severely impaired IL-10 secretion, but it did not affect IFN-γ production. IL-17 secretion from differentiating Th17 cells was not impaired, whereas IL-10 secretion was modestly decreased in cells treated with MT1 or MT2 (Fig. S3*B*). Analysis of the signature Tr1 cytokines from WT and *Mt*$^{-/-}$ Tr1 cell cultures showed increased IL-10 and unchanged IFN-γ production at mRNA level as well. Additionally, we found that both *Il21* and *Il27r*, which are critical for Tr1 cell development (1, 2), were also up-regulated in the absence of MTs (Fig. 1*C*). However, other genes that are also expressed in Tr1 cells, like *Ahr* and *Tbx21*, are unchanged in the absence of MTs (Fig. 1*C*). To assess whether MTs play a role in Tr1 cells generated with other stimuli than IL-27, we differentiated naïve sorted CD4$^+$ T cells using vitamin D3 and dexamethasone (13). We observed that the *Mt*$^{-/-}$ Tr1 cells exhibited elevated IL-10 production under both vitamin D3 and dexamethasone stimulation after 72 h. The enhancement of IL-10 became more profound when these two were combined (Fig. S3*C*). Altogether, these data indicate that MTs impede IL-10 secretion from Tr1 cells.

We then determined whether endogenous overexpression of MTs can reverse the phenotype we observed in the *Mt*$^{-/-}$ Tr1 cells. Retroviral overexpression of GFP-tagged MT1 or MT2 in WT T cells under IL-27 stimulation resulted in reduced expression of IL-10 and had no affect on IFN-γ, as detected by intracellular staining and ELISA (Fig. 1 *D* and *E*). Furthermore, we sorted out the GFP$^+$ cells from the MT1 or MT2 retroviral transduced Tr1 cells and examined the expression of other key genes expressed in Tr1 cells. Consistent with MT-deficient T cells, the expression of *Il10*, *Il27r*, and *Il21* genes was down-regulated, whereas *Ahr* and IFN-g were unchanged by MT1 or MT2 overexpression (Fig. 1*F*). Thus, our data demonstrate that MTs negatively regulate Tr1 differentiation by inhibiting IL-10 production and impairing the IL-27 signaling pathway by repressing *Il27r* and *Il21* expression.

To test whether MTs are also relevant for human Tr1 cell biology, we differentiated human Tr1 cell in vitro to analyze MT expression and the function of MTs in these cells. More than 10 isoforms of MT have been identified in the human genome, compared with only 3 isoforms in the mouse genome. According to the National Center for Biotechnology Information HomoloGene database, human MT1E and MT1H are the closest homologs to murine MT1 and MT2, respectively. Therefore, we decided to test the mRNA expression level of these two isoforms in human Tr1 cells. We observed that *Mt1e* and *Mt1h* were highly expressed in human Tr1 cells compared with Th0 cells (Fig. S4*A*). To understand the relevance of MTs in human Tr1 cells, we differentiated human Tr1 cells in the presence of recombinant MT1 and MT2 proteins. We observed that both MT1 and MT2 significantly suppressed the IL-10 production of human Tr1 cells (Fig. S4 *B* and *C*). Moreover, we observed that the supernatants from cultured Tr1 cells inhibited proliferation of bystander CD4$^+$ T cells, whereas the supernatants from the Tr1 cells cultured with additional MT1 or MT2 exhibited reduced ability to suppress proliferation, which was abrogated by anti–IL-10 antibody (Fig. S4*D*). These data suggest that the inhibitory functions of human Tr1 cells are also negatively regulated by MTs.

MTs Negatively Regulate STAT1 and STAT3 Activation. Previous studies have shown that STAT1 and STAT3 are both critical for the induction of IL-10 production in Tr1 cells (6, 14). To assess whether MTs regulate Tr1 differentiation by influencing the activation of STATs, we examined the activation level of STAT1 and STAT3 after IL-27 stimulation. To this end, naïve CD4$^+$ T cells from WT and *Mt*$^{-/-}$ mice were activated with IL-27 and phosphorylation of STAT1 and STAT3 was analyzed. We found that both STAT1 and STAT3 were hyperphosphorylated upon IL-27 stimulation in *Mt*$^{-/-}$ compared with WT T cells (Fig. 2 *A* and *C*). Besides STAT1 and STAT3, STAT4 is also required for IL-10 production (15); however, there was no difference in the levels of pSTAT4 in WT and *Mt*$^{-/-}$ T cells (Fig. 2 *B* and *C*). These results indicate that MTs may inhibit IL-10 production in Tr1 cells by regulating the activation of STAT1 and STAT3.

To identify a potential relationship between STAT1/3 and MTs during Tr1 cell differentiation, we analyzed T cells from *Stat1*$^{-/-}$ and *Stat3*$^{-/-}$ mice. Consistent with previous findings, both STAT1- and STAT3-deficient Tr1 cells exhibited reduced *Il10* expression. Additionally, both *Stat1*$^{-/-}$ and *Stat3*$^{-/-}$ Tr1 cells displayed reduced mRNA expression levels of *Mt1* and *Mt2* (Fig. 2*D*). We next coinfected naïve T cells with retroviruses encoding MT1 or MT2 (MIT vector, Thy1.1 reporter) and STAT1 or

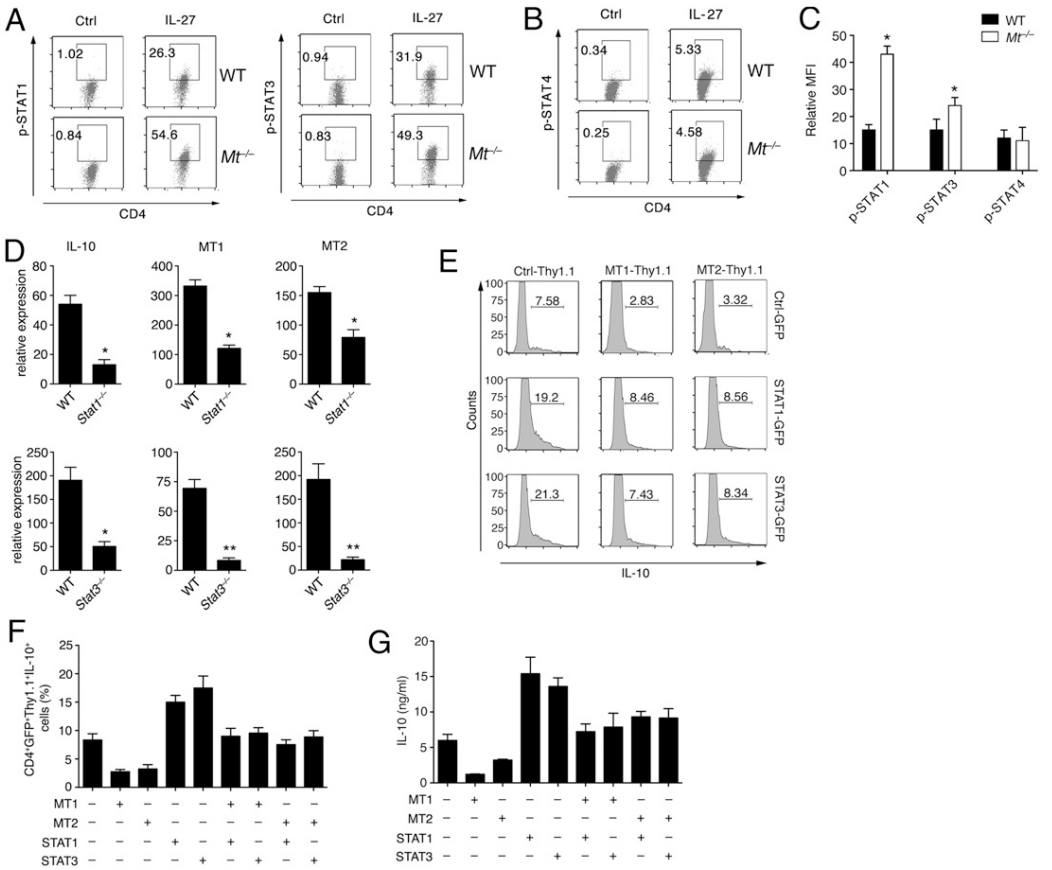

Fig. 2. STAT1/3 and MTs form a kinetic balance to control IL-10 production in Tr1 cells. (*A* and *B*) Levels of intracellular phosphorylated STAT1 (p-STAT1), STAT3 (p-STAT3) (*A*) or STAT4 (p-STAT4) (*B*) were determined by flow cytometry 20 min after stimulation of naïve $CD4^+$ T cells from WT or $Mt^{-/-}$ mice either unstimulated (Ctrl) or treated with IL-27. (*C*) MFI quantification of p-STAT1, p-STAT3, and p-STAT4 of IL-27–stimulated $CD4^+$ T-cell obtained from WT or $Mt^{-/-}$ mice. (*D*) After 72 h, expression levels of indicated genes in IL-27–induced Tr1 cells from WT and $Stat1^{-/-}$ or $Stat3^{-/-}$ mice were determined by RT-PCR. (*E*) Twenty-four hours after activation, $CD4^+$ T cells were transduced with the indicated combinations of retroviruses and subsequently cultured in the presence of IL-27 for 3 d before stimulation with PMA and ionomycin for 4 h. Expression levels of IL-10 were determined by intracellular cytokine staining (data shown are gated on $GFP^+Thy1.1^+$ cells). (*F*) Quantification of $CD4^+GFP^+Thy1.1^+IL\text{-}10^+$ T cells transduced with the indicated retroviruses as in *D*. (*G*) Flow cytometry-sorted $CD4^+GFP^+Thy1.1^+$ cells were stimulated for 4 h with PMA and ionomycin. Levels of secreted IL-10 in the culture media were measured by ELISA. The data are representative of three independent experiments. $^*P < 0.05$ (Student *t* test, error bars show SD).

STAT3 [MSCV-IRES-GFP (MIG) vector, GFP reporter] and stimulated the cells with IL-27. Overexpression of either STAT1 or STAT3 could reverse the suppressive effect of MT1 or MT2 on IL-10 production (Fig. 2 *E*–*G*). These results imply that MTs inhibit Tr1 cell differentiation and potentially compete with positive regulators of IL-10 such as STATs, subsequently defining the STAT-driven threshold for the induction of IL-10. However, our results also suggest that both STAT1 and STAT3 are dominant over MTs in the regulation of IL-10 during Tr1 cell polarization. Thus, expression levels and activation status of STAT1/3 and MTs may form a kinetic balance to control IL-10 production in Tr1 cells.

MTs Control the Induction of Tr1 Cells in Vivo. To study the relevance of MTs in expanding Tr1 cells in vivo, we examined IL-10–producing Tr1 cells generated from $CD4^+CD25^-CD44^{hi}CD62L^-$ memory T cells from WT versus $Mt^{-/-}$ mice. We found that IL-10 secretion from memory T cells from $Mt^{-/-}$ mice was increased by over 50% compared with WT mice (Fig. 3*A*). It has previously been shown that repeated in vivo treatment with anti-CD3 antibody induces Tr1 cells, which are dependent on IL-27 for their generation (1, 16). Because MTs are expressed in other tissues, such as liver, it is not clear whether the in vivo increase in IL-10 is due to a direct effect on T cells. To exclude any effects from the nonhematopoietic cell-derived MTs in our system, we generated bone marrow (BM) chimeras in which the WT host were reconstituted with either WT or $Mt^{-/-}$ BM. Eight weeks after reconstitution, we repeatedly administered anti-CD3 or an isotype control antibody to the WT and $Mt^{-/-}$ BM chimera mice. After treatment, we analyzed the Tr1 cell frequency in peyer's patches (PP) and lamina propria (LP). To rule out the confounding effects by other IL-10–producing T-cell subsets and specifically examine Tr1 cells, we analyzed IL-10 production by gating on $CD4^+IL\text{-}17^-Foxp3^-$ cells. We found a significant increase in Tr1 cells in $Mt^{-/-}$ BM chimera's PP and LP compared with WT chimeras (Fig. 3 *B* and *C*). These results further emphasize that MTs regulate the generation of $IL\text{-}10^+$ Tr1 cells by specifically acting on the hematopoietic compartment in vivo.

Enhanced Suppressive Capacity of IL-10 Producing T Cells in the Absence of MT in Vivo. To further understand the role of MTs and their relevance to the function of Tr1 cells in vivo, we studied the impact of MT-deficient Tr1 cells in an adoptive transfer model of EAE. We first immunized WT or $Mt^{-/-}$ mice with myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide with Freund's Complete Adjuvant (CFA). Ten days following immunization we isolated lymphocytes from immunized WT or $Mt^{-/-}$ mice and reactivated them under various conditions. The response was antigen specific as depicted by the proliferation with MOG but not with OVA peptide (Fig. 4 *A*–*D*). When these cells were restimulated by MOG_{35-55} and

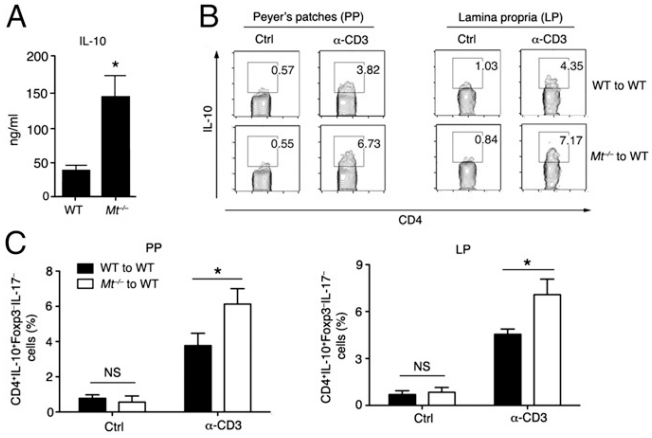

Fig. 3. MTs control IL-10–producing T cells in vivo. (*A*) Memory $CD4^+$ $CD25^-CD44^{hi}CD62L^-$ cells purified by FACS-sorting from WT or $Mt^{-/-}$ mice were activated in vitro with anti-CD3 and anti-CD28 and IL-10 production was assessed by ELISA after 4 d of culture. (*B*) WT or $Mt^{-/-}$ chimera mice ($n = 5$) were injected i.p. with 20 μg of anti-CD3 antibody or PBS once every 3 d, for a total of four times. Four hours after the last injection, mice were killed and IL-10 production of $CD4^+$ T cells from peyer's patches (PP) and lamina propria (LP) was analyzed by flow cytometry. (*C*) Quantification of the percentage of $CD4^+IL\text{-}10^+Foxp3^-IL17^-$ T cells from PP and LP of WT or $Mt^{-/-}$ chimera mice. The data are representative of three independent experiments. $^*P < 0.05$ (Student *t* test, error bars show SD).

IL-23, which has been reported to induce and reactivate Th17 cells (17), IL-17 production from $CD4^+$ T cells was not altered (Fig. 4 *A* and *B*). Although we did not see a significant change in IL-10 production with specific antigen $MOG_{35–55}$ by flow cytometry, we indeed observed a higher production of IL-10 in $CD4^+$ T cells from $Mt^{-/-}$ mice than WT mice when T cells were reactivated in the presence of $MOG_{35–55}$ in the cultured supernatant, which was further amplified in the presence IL-27 (Fig. 4 *C* and *D*). Moreover, IFN-γ production by cells from WT and $Mt^{-/-}$ mice was comparable following either stimulation condition (Fig. 4 *A*–*D*). Consistent with our in vitro data, these results confirm the specific role MTs play in the regulation of IL-10 in IL-27–stimulated Tr1 cells.

Next, we tested whether $Mt^{-/-}$ Tr1 cells display enhanced ability to suppress autoimmunity because IL-10 production has been used as a criterion for Tr1 cell anti-inflammatory activity. We first generated pathogenic effector T cells from MOG-immunized WT mice. Simultaneously, WT or $Mt^{-/-}$ T cells were cultured in the presence of MOG and IL-27 to generate antigen specific Tr1 cells. Because $Mt^{-/-}$ mice are on the SV129 background and this strain is not optimal for EAE induction by $MOG_{35–55}$ (18), we used an alternative recipient strain (SV129 × B6) F1, which were genetically compatible with SV129 and were susceptible to EAE following adoptive transfer (19). We then adoptively transferred WT effector T cells with or without differentiated Tr1 cells from either WT or $Mt^{-/-}$ mice at a ratio of 3:1. As expected, WT Tr1 cells significantly suppressed EAE development. However, $Mt^{-/-}$ Tr1 cells suppressed disease more efficiently than WT Tr1 cells both in terms of disease severity and incidence (Fig. 4*E*). We further tested the ability of Tr1 cells to suppress EAE by titrating the Tr1 cells. $Mt^{-/-}$ Tr1 cells exhibited the suppressive capacity on the disease process, whereas the suppressive effect of WT Tr1 cells was no longer dominant when the ratio of the effector T cells (Teff)-to-Tr1 was 5:1 (Fig. S5). Using the same adoptive transfer system, we labeled WT effector cells with carboxyfluorescein diacetate succinimidyl ester (CFSE) before transfer. On day 4 after transfer, lymphocytes were isolated from lymph nodes of the recipient mice and $CFSE^+$ effector cell proliferation was analyzed. We found significant reduction of effector cell proliferation if they had been transferred together with Tr1 cells. Moreover, $Mt^{-/-}$ Tr1 cells displayed a superior suppressive capacity, inhibiting effector T cell proliferation more profoundly compared with WT Tr1 cells (Fig. 4*F*).

Discussion

As one of the suppressive T cell subsets, Tr1 cells have been described to regulate inflammation, graft-versus-host disease, and autoimmunity by producing IL-10 (3). The results presented in this study show that the nonenzymatic proteins MT1 and MT2 can negatively regulate the production of IL-10 but not IFN-γ during Tr1 cells' development and thus regulate the anti-inflammatory properties of those cells. Loss of MT1 and MT2 enhanced IL-10 production within Tr1 cells upon IL-27 or vitamin D3/dexamethasone stimulation without affecting IFN-γ. Furthermore, the enhancement of IL-10 increases the capacity of MT-deficient Tr1 cells to suppress effector T cells, in turn leading to the abatement of autoimmunity. Our results reveal that MTs blunt IL-10 secretion from Tr1 cells by preventing the activation of the transcription factor STAT1 and STAT3. Consistent with these data, anti-CD3–induced generation of Tr1 cells was increased in the absence of MTs in vivo, underscoring the key role of MTs in regulating IL-10 production in Tr1 cells.

Although the key roles of MTs in mediating heavy metal detoxification have been abundantly documented (20), their implication in the control of gene transcription is still unclear. It has been proposed that MT could bind to the p50 subunit of the NF-κB complex, thereby increasing its ability to act as a transcriptional activator (21). So far, there is no evidence showing that MTs have any DNA-binding sites, making it unlikely that they directly regulate gene expression. However, it has been shown that MTs can control the binding of the estrogen receptor to its DNA-binding site by modulating zinc levels (22). Our data suggest that MTs regulate induction of pSTAT1 and pSTAT3, both of which are required for induction of many important cytokine-driven functions in T cells. Whether MTs directly bind to STAT1 or STAT3 and interfere with their phosphorylation or indirectly by interfering with Jak-mediated phosphorylation is not clear at this stage. However, because it was reported that Zinc binding disrupts the association of STAT3 with Jak2 kinase (23), we can speculate that zinc might be involved in the MT-driven inhibition of phosphorylation of STAT1/3. Nonetheless loss of MTs resulted in enhanced phosphorylation and transcription of STAT1 and STAT3, suggesting STAT1/3 but not STAT4 as one of the targets of MTs. Although MTs are also expressed in Th17 cells, MT expression does not regulate the expression of proinflammatory proteins such as IL-17. We cannot rule out the effect of MTs in IL-10 production by Th17 cells, because IL-6 is one of the cytokines that induces IL-10 and activation of STAT3 in Th17 cells (24).

Proinflammatory cytokines such as IL-6 induce MTs by activating the transcription factor STAT1 and STAT3 (10). Endotoxin (LPS) produced during bacterial infection has also been shown to elevate the MT expression level (10). Both LPS and IL-6 can initiate STAT1 and STAT3 expression and activation. pSTAT1 and pSTAT3 in turn can directly bind to the promoter of MTs and potentiate its transcription (10), forming a feedback inhibitory loop whereby STAT1/3 induction of MTs results in decreased induction and/or activation of STAT1/3. This mechanism of action would be reminiscent of the action of the suppressors of cytokine signaling 3 (SOCS3), which is induced by STAT3 and limits STAT3 phosphorylation, thereby dampening the secretion of proinflammatory cytokines like IL-17 (25). Here, the induction of MTs by IL-27 would limit the induction of Tr1 cells to prevent excessive immune regulation that might favor the emergence of viral infections or cancers.

Both STAT1 and STAT3 are phosphorylated upon IL-27 signaling (6), leading to transactivation of IL-10. The absence of MTs results in hyperphosphorylation of both STAT1 and STAT3 under the stimulation of IL-27. On the other hand, either STAT1- or STAT3-deficient Tr1 cells exhibit reduced MT1 and MT2 expression. We thus hypothesize that MTs and STATs compete with each other during Tr1 cell development to control IL-10 production. Although MT1/2 overexpression can lead to IL-10 suppression from Tr1 cells, coregulation of STAT1/3 results in restoration of IL-10. This result indicates that STAT1 or STAT3 can override MT-dependent suppression of IL-10 during Tr1

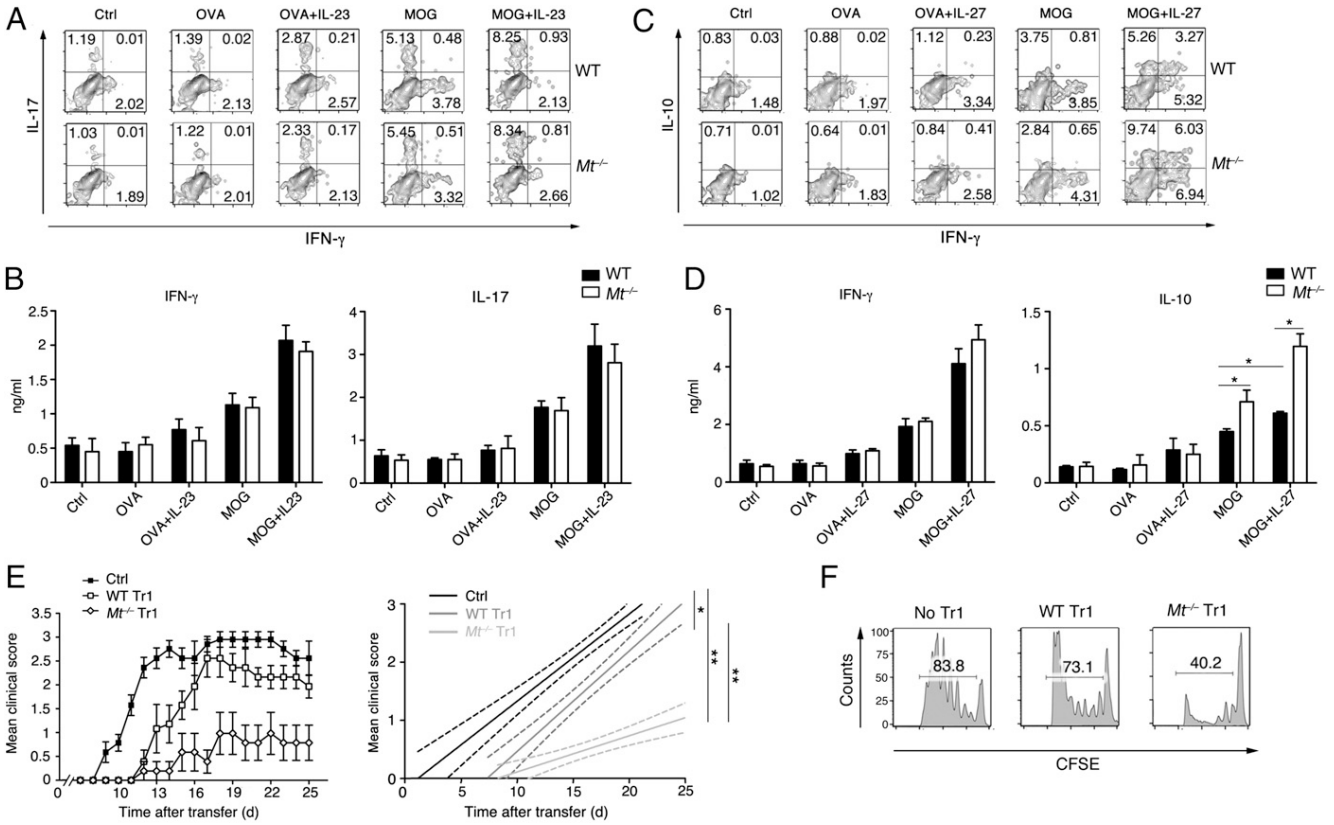

Fig. 4. MTs regulate suppressive capacity of Tr1 cells in vivo. Lymphocytes were isolated from lymph nodes of WT and $Mt^{-/-}$ mice 10 d after immunization with MOG_{35-55} and were restimulated with MOG_{35-55} or $OVA_{323-339}$ in combination with IL-23 (*A* and *B*) or IL-27 (*C* and *D*) for 3 d. (*A* and *C*) The frequency of IL-10–, IL-17–, and IFN-γ–expressing cells was determined by flow cytometry. (*B* and *D*) IL-10, IL-17, and IFN-γ secretion was measured by ELISA. (*E*) $CD4^+$ T cells were isolated from lymph nodes of MOG_{35-55}–immunized WT and $Mt^{-/-}$ mice, and subsequently restimulated in vitro with MOG_{35-55} and IL-23 and IL-12 (effector cells) or IL-27 (Tr1 cells). (*Left*) EAE development in WT (129/Sv B6 F1) recipients adoptively transfered with WT MOG_{35-55}–specific effector T cells with (3:1) or without WT or $Mt^{-/-}$ Tr1 cells. (*Right*) Data are expressed as a linear-regression curve of the disease score over time. (*F*) Experimental setup as *E*, except for labeling WT effector T cells with CFSE before adoptive transfer. Four days after transfer, the percentage of proliferating CFSE-labeled $CD4^+$ T cells in the lymph nodes was determined by flow cytometry. The data are representative of three independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$ (Student *t* test, error bars show SD).

differentiation. MTs possibly function at the later stage of Tr1 cell development by their temporal expression profile in Tr1 cells.

It has been described that MT proteins play significant roles during different inflammatory conditions, such as collagen-induced arthritis (CIA) (26) or EAE (27), but the function of MTs within different cell compartments varies. During CNS inflammation, it has been reported that MTs play an important role for EAE recovery, because MT proteins were found to be elevated within the CNS, specifically in astrocytes and activated macrophages (27). Together with the Zn-MT2 treatment resulting in EAE reduction, this result implicates that MTs have a strong neuroprotective effect within the CNS (28). Moreover, in the absence of MTs, mice exhibited enhanced production of proinflammatory cytokines such as IL-1 and TNF-α, which in turn can lead to inhibition of leukocyte recruitment and ameliorate EAE (29, 30). In this study, we established the correlation between MTs and IL-10 production within Tr1 cells and their proinflammatory role in the EAE model. Passive transfer EAE experiments allowed us to dissect the function of MTs specifically in T cells, excluding any effects due to the neuroprotective role of MTs. We and others have shown that the repetitive administration of anti-CD3 antibody can induce Tr1 cells in the gut (1, 16). Repetitive administration of anti-CD3 induced Tr1 cells in the gut more efficiently when we used $Mt^{-/-}$ BM chimera compared with $Mt^{-/-}$ mice, further emphasizing the importance of the cellular environment in the control of MT responses. These data further suggest that MTs have divergent functions in the immune response, depending on tissue and cells. It is also important to evaluate distinct effects of MTs in specific circumstances to identify all of their various and sometimes contradicting functions.

Altogether, we have identified MT1 and MT2 as negative regulators of Tr1 cell differentiation and IL-10 production. We also illustrate the balance between MT and STAT signaling in controlling Tr1 cell development. Our work emphasizes that MTs, beyond their essential role in the regulation of metal homeostasis, also shape the quality of immune responses in vivo. By identifying a specific function for MTs in Tr1 cells, our study also provides a target for development of selective therapeutic strategies for regulating Tr1 cell expansion and autoimmunity.

Materials and Methods

Animals. $Mt^{-/-}$ mice (129/Sv-MT1MT2$^{tml\ bri}$), 129/Sv (control), 129/Sv B6 F1, 2D2, $Stat1^{-/-}$ and C57BL/6 mice were purchased from Jackson Laboratory. $Stat3^{-/-}$ mice were a kind gift from John O'Shea (National Institutes of Health, Bethesda). Mice were housed in conventional, pathogen-free facilities at the Harvard Institute of Medicine. All experiments were undertaken in accordance with guidelines from the Committee on Animals at Harvard Medical School.

In Vitro T-Cell Differentiation. Naïve ($CD44^{lo}CD62L^+CD25^-$) or memory ($CD44^{hi}CD62L^{lo}CD25^-$) $CD4^+$ T cells from spleens and lymph nodes of WT or $Mt^{-/-}$ mice were purified by fluorescence-activated cell sorting (FACS). The purity of isolated T-cell populations routinely exceeded 98%. Naïve $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (145-2C11, 1 μg/mL) and anti-CD28 (PV-1, 1 μg/mL) in the presence of the following reagents: mouse IL-27 (50 ng/mL) or vitamin D3 (50 pg/mL; Sigma), and/or dexamethasone

(30 pg/mL; Sigma) for Tr1 cells; human TGF-β1 (2 ng/mL) and mouse IL-6 (20 ng/mL) for Th17 differentiation; mouse IL-12 (5 ng/mL) and anti-mouse IL-4 (10 μg/mL; 11B11) for Th1 differentiation; or mouse IL-4 (10 ng/mL) and anti-mouse IL-12 (10 μg/mL; C17.8) for Th2 differentiation; or human TGF-β1 (2 ng/mL) for iTregs. All recombinant cytokines were purchased from R&D Systems.

Statistical Analysis. Statistical analysis was performed using Prism software (GraphPad). P values < 0.05 were considered significant.

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3.4 T_H17 Differentiation in the Absence of STAT1 Signaling.

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Cytokines play a pivotal role in Multiple sclerosis (MS) pathogenesis, a common neurological disease. Interleukin-12 (IL-12) family of cytokines, which includes IL-6 and IL-27, are important mediators of the immune response. While IL-6 has pro-inflammatory properties and contributes to the generation of IL-17-producing $CD4^+$ (T_H17) cells that play a major role during MS, IL-27 has predominant anti-inflammatory properties. Despite opposite functions, IL-6 and IL-27 signal through a common chain receptor and drive the activation of the signal transducers and activators of transcription (STAT)-1 and 3. In the absence of STAT1 signaling, IL-27 function as IL-6 and leads to encephalitogenic T_H17 cells generation. In the absence of STAT1, IL-27 induces IL-23R expression that is crucial for T_H17 cells proliferation. While pharmacological manipulation of IL-27 signaling could be exploited therapeutically to control autoimmune disease development, precise understanding of its downstream signaling is needed to avoid uncontrolled pro-inflammatory response.

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IL-27 Induces Th17 Differentiation in the Absence of STAT1 Signaling

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It is known that differentiation of Th17 cells is promoted by activation of STAT3 and inhibited by activation of STAT1. Although both transcription factors are activated by several cytokines, including IL-6, IL-21, and IL-27, each of these cytokines has a very different effect on Th17 differentiation, ranging from strong induction (IL-6) to strong inhibition (IL-27). To determine the molecular basis for these differences, we measured STAT3 and STAT1 activation profiles for IL-6, IL-21, and IL-27, as well as for cytokine pairs over time. We found that the ratio of activated STAT3/activated STAT1 is crucial in determining whether cytokines promote or inhibit Th17 differentiation. IL-6 and IL-21 induced p-STAT3/p-STAT1 ratios > 1, leading to the promotion of Th17 differentiation, whereas IL-27 or IL-6+IL-27 induced p-STAT3/p-STAT1 ratios < 1, resulting in inhibition of Th17 differentiation. Consistent with these findings, we show that IL-27 induces sufficient p-STAT3 to promote Th17 differentiation in the absence of STAT1. Furthermore, IL-27–induced STAT1-deficient T cells were indistinguishable from bona fide highly proinflammatory Th17 cells because they induced severe experimental autoimmune encephalomyelitis upon adoptive transfer. Our results suggest that the ratio of p-STAT3/p-STAT1 induced by a cytokine or cytokine pairs can be used to predict whether they induce a competent Th17-differentiation program. ***The Journal of Immunology*****, 2015, 195: 4144–4153.**

CD4$^+$ Th cells are key orchestrators of the adaptive immune system. Depending on activation conditions and the cytokine milieu, these cells can differentiate into a variety of effector T cell subsets that are characterized by the cytokines they secrete. It was originally believed that there were two types of effector Th cells: Th1 and Th2 cells (1, 2). However, more recently, another lineage of effector T cells, called Th17 cells, was discovered in the context of autoimmune inflammation (3–5) and was shown to induce CNS inflammation during experimental autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis (6). These proinflammatory effector cells can be induced in vitro by culturing naive CD4$^+$ T cells under activating conditions in the presence of IL-6 and TGF-β (7–9).

IL-6 signals primarily through the JAK/STAT pathway (10), whereas TGF-β signals primarily through the SMAD pathway (11). IL-6 binds to IL-6R, causing JAKs to activate members of the STAT family by phosphorylation. Activated STATs (p-STATs) form dimers, revealing a nuclear-targeting sequence that allows them to translocate to the nucleus and initiate transcriptional programs. IL-6 is thought to primarily activate STAT3 (12), and STAT3 is the primary input to the genetic network governing Th17 differentiation (13, 14). It was demonstrated that STAT3 is crucial for the induction of Th17 cells, as evidenced by a nearly complete loss of IL-17 secretion in its absence (15). However, IL-6 is just one of several cytokines that activates STAT3, but it remains the most potent inducer of IL-17–producing cells. Other cytokines that activate STAT3 include IL-21 (16), which induces weaker IL-17 production in the presence of TGF-β than does IL-6 (17), and the anti-inflammatory cytokines IL-10 (18, 19), which does not induce Th17 differentiation, and IL-27 (20–23), a potent inhibitor of Th17 differentiation (24).

How these different STAT3-inducing cytokines cause such a large range of effects on Th17 differentiation is an unresolved question in the field (25). Part of the answer may be found in another STAT protein induced by these cytokines: p-STAT1 (25). Three forms of p-STAT1 and p-STAT3 can exist in the nucleus as a result of JAK/STAT signaling: these two particular p-STATs can form homodimers (p-STAT1–p-STAT1, p-STAT3–p-STAT3) or a heterodimer (p-STAT1–p-STAT3) (26–28). Importantly, each dimer binds to different DNA sequences and, thus, has different effects on transcription. It was shown that IL-27 completely loses its ability to inhibit Th17 differentiation in STAT1-knockout (S1KO) mice (25, 29), suggesting that p-STAT1 is an inhibitor of the Th17-differentiation pathway. A simple theory to account for the different effects of these cytokines on Th17 differentiation is that each cytokine induces different amounts of activated STAT3 and STAT1, with IL-6 inducing primarily p-STAT3, IL-27 inducing primarily p-STAT1, and IL-21 inducing a combination of

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Abbreviations used in this article: EAE, experimental autoimmune encephalomyelitis; MFI, mean fluorescence intensity; S1KO, STAT1 knockout; WT, wild-type.

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the two. One way to test this theory is to comparatively measure STAT1 and STAT3 activation among the cytokines. Previous studies used Western blots or flow cytometry to measure STAT activation by IL-21 (30), IL-6 and IL-10 (18, 31), and IL-6 and IL-27 (22) in a variety of cell types, but not in the same study or same cell type, so it is impossible to compare the kinetics and amplitude of STAT activation for all cytokines.

In this study, we investigated cytokine specificity in the context of Th17 differentiation by measuring the STAT1 and STAT3 activation profiles for three cytokines (IL-6, IL-21, and IL-27) in a systematic way. We show that, among the three cytokines, IL-6 activates the most STAT3, as well as the most STAT1, raising the question of how it can be such a potent inducer of Th17 differentiation when it activates the highest amount of the Th17-inhibitory transcription factor, STAT1. Our experiments suggest that it is actually the ratio of p-STAT3/p-STAT1, rather than the total amount of either p-STAT3 or p-STAT1, which determines the ability of a cytokine to induce Th17 differentiation. This led to the finding that, in the absence of STAT1, IL-27 becomes a potent inducer of Th17 differentiation and that IL-27–induced S1KO Th17 cells are indistinguishable from bona fide Th17 cells with regard to their expression of key Th17-associated factors and their encephalitogenic properties.

Materials and Methods

Mice

Six- to eight-week-old C57BL/6J mice were obtained from The Jackson Laboratory, and 6–8-wk-old 129S6/SvEvTac mice (wild-type [WT]) and 129S6/SvEv-Stat1(tm1Rds) (S1KO) mice (32) were obtained from Taconic. S1KO mice on the C57BL/6 background were a kind gift from Prof. Dr. M. Mueller (Institute of Animal Breeding and Genetics, University of Veterinary Medicine, Vienna, Austria) (33). 2D2 mice were described previously (34) and were crossed with C57BL/6 S1KO mice. Mice were housed in a specific pathogen–free, viral Ab–free animal facility at the Harvard Institutes of Medicine or at the University of Geneva. All breeding and experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Harvard Medical School or by the local veterinary office (Geneva, Switzerland), according to Swiss ethical regulations.

Intracellular staining of p-STAT1, p-STAT3, and p-STAT5

T cells were isolated from spleen and lymph nodes of WT or S1KO mice. $CD4^+$ T cells were purified using magnetic beads coated with anti-CD4 Ab, according to the manufacturer's instructions (Miltenyi Biotec). $CD4^+$ cells were cultured for various times at a concentration of 1.0–2.0 × 10^6/ml with IL-6 (10 ng/ml), IL-21 (20 ng/ml), IL-27 (25 ng/ml), or IL-7 (10 ng/ml) in the presence of plate-bound anti-CD3 Ab (2 μg/ml) (clone 145-2C11) and anti-CD28 Ab (2 μg/ml) (clone PV-1; both from Bio X Cell). All cytokines were purchased from R&D Systems. After the desired incubation times, the cells were collected and fixed with 4% paraformaldehyde (EM Grade) for 12 min at 37°C. The cells were then rinsed and permeabilized in 90% methanol for 30 min on ice. Subsequently, the cells were rinsed with Permeabilization Buffer (eBioscience) and stained with anti–p-STAT1 and anti–p-STAT3 Abs. Anti-STAT1 (pY701; clone 4a), anti-STAT3 (pY705; clone 4/P-STAT3), and anti-STAT5 (pY694; clone 47/STAT5) Abs were purchased from BD Biosciences. All flow cytometry data were acquired on a BD FACSCalibur and analyzed with FlowJo software (TreeStar).

Th17 differentiation in vitro

T cells were isolated from spleen and lymph nodes of WT or S1KO mice. $CD4^+$ T cells were purified using magnetic beads coated with anti-CD4 Ab, according to the manufacturer's instructions (Miltenyi Biotec). Subsequently, $CD4^+$ cells were stained with anti-CD4, anti-CD25, and anti-CD62L Abs and sorted into naive $CD4^+CD25^-CD62L^{hi}$ T cells with a BD FACSAria. Naive cells were cultured at a concentration of 1.0–1.5 × 10^6/ml. Cells were stimulated in the presence of 5–7.5 × 10^6/ml irradiated splenocytes and 1–2 μg/ml anti-CD3 Ab (clone 145-2C11) or in the presence of plate bound anti-CD3 Ab (1–3 μg/ml) and anti-CD28 Ab (1–2 μg/ml) (clone PV-1; all from Bio X Cell). For the generation of Th17 cells, naive T cells were cultured with IL-6 (30 ng/ml), TGF-β (3 ng/ml), and IL-27 (50 ng/ml). In some experiments, Th17 cells were supplemented with 10 ng/ml IL-23 after 48 h. Cytokines were purchased from R&D Systems or eBioscience. After 4 d, production of IL-17 and IFN-γ was

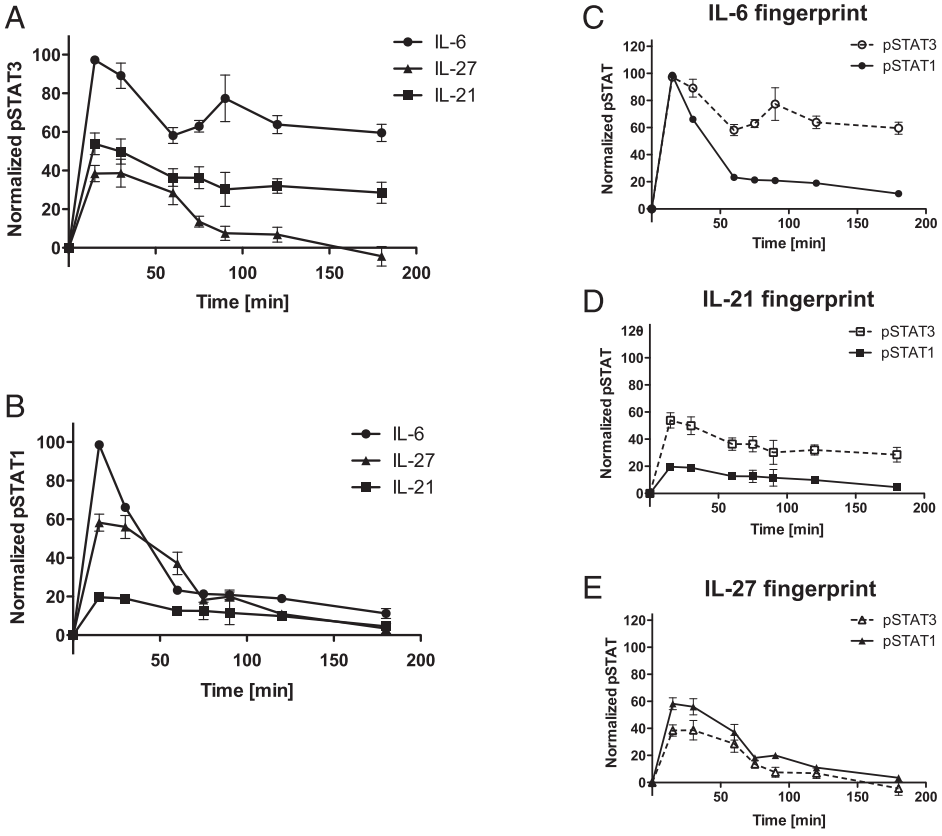

FIGURE 1. p-STAT1 and p-STAT3 expression over time in response to different cytokines. Flow cytometry measurement of p-STAT3 and p-STAT1 at different time points in $CD4^+$ T cells ($CD4^+CD25^-CD62L^{hi}$) cultured with anti-CD3/anti-CD28 for 1 h and then IL-6, IL-21, or IL-27 for up to 4 h. p-STAT3 (**A**) and p-STAT1 (**B**) levels for all cytokines over time. Results are the averages of 4–13 MFI measurements at a given time point and a given condition, normalized using Eq. 1. Error bars represent SEM. Curves were significantly different ($p < 0.0001$), as determined by two-way ANOVA with a Bonferroni post test. (**C**–**E**) The cytokine fingerprints illustrate how the relative amounts of p-STAT1 and p-STAT3 develop over time.

measured by intracellular cytokine staining and subsequent flow cytometry. After 1 or 2 d, cells were collected for RNA extraction, and mRNA levels were analyzed by quantitative PCR, as described below.

Quantitative real-time PCR

RNA was extracted with TRIzol reagent (Invitrogen). cDNA was synthesized with random hexamers and Superscript II reverse transcriptase (Invitrogen) and was used as the template for quantitative PCR. PCR was performed with the Real-Time PCR Detection System (Applied Biosystems StepOne plus) and SYBR Green Supermix (KAPA SYBR FAST Universal; Labgene). Expression of Tbet (FW: 5′-AGGGAACCGCTTATATGTCC-3′ and Rev: 5′-TCTCCATCATTCACCTCCAC-3′), Rorc (FW: 5′-CCTCTACACGGCCCTGGTT-3′ and Rev: 5′-GATGTTCCACTCTC CTCTTCTCTTG-3′), IL-23R (FW: 5′-TGGAACACTGGGAAGCCTAC-3′ and Rev: 5′-GGAATTGACAGCTTGGACCC-3′), IL-12Rβ1 (FW: 5′-CGCTGCGAGGCTGAAGAC-3′ and Rev: 5′-CGCAGTCCGTCAAG TGTCAC-3′), IL12Rβ2 (FW: 5′-CGCTTCTGCACCCACTCAC-3′ and Rev: 5′-TGCCAGGTCACTAGAATGTTGTC-3′), and actin (FW: 5′-CCTGTATGCCTCTGGTCGTA-3′ and Rev: 5′-CCATCTCCTGCTCGAA GTCT-3′) were assessed with specific primers and probes. Gene expression was normalized to expression of the housekeeping gene β-actin.

Measurement of cytokines

Secreted cytokines were measured after 48 h by ELISA (purchased at eBioscience) if not otherwise specified.

In vitro T cell proliferation

Proliferation was measured by incorporation of [^{3}H]thymidine (PerkinElmer) for the last 16 h of a 3 d culture. After 48 h, cultures were supplemented with IL-23. Analysis of incorporated [^{3}H]thymidine was performed using a beta counter (1450 Microbeta; Trilux; PerkinElmer).

Experimental autoimmune encephalomyelitis

For passive induction of EAE, naive 2D2 T cells were purified as described above and differentiated by stimulation with soluble anti-CD3 (1 μg/ml) and irradiated syngeneic splenocytes in the presence of the indicated cytokine cocktails. Five million cells were adoptively transferred i.p. Animals were monitored daily for the development of classical and atypical signs of EAE, as previously described (6, 35), according to the following criteria: 0, no disease; 1, decreased tail tone or mild balance defects; 2, hind limb weakness, partial paralysis, or severe balance defects that cause spontaneous falling over; 3, complete hind limb paralysis or very severe balance defects that prevent walking; 4, front and hind limb paralysis or inability to move body weight into a different position; and 5, moribund state.

Histology

For analysis of CNS inflammation, mice were sacrificed 20 days after immunization and perfused with cold PBS, followed by 4% paraformaldehyde fixation. Brain and spinal cord tissue were embedded in paraffin. Sections were stained with H&E for light microscopy. Images of tissue sections were scanned using a Mirax slide scanner (Zeiss). Sections were stained with H&E, and consecutive sections were examined by immunohistochemistry. Immunostaining was performed to assess the numbers of activated macrophages/microglia (Mac3, clone M3/84; BP Pharmingen) and T cells (CD3, clone CD3-12; AbD Serotec). The avidin-biotin technique with 3,3-diaminobenzidine was used for the visualization of bound primary Abs.

Statistics

Statistical evaluations of gene expression data were performed with the unpaired Student t test. p-STAT profiles of different cytokines were evaluated using two-way ANOVA with the Bonferroni post test. The p values < 0.05 were considered significant.

Results

Normalization method allows for cytokine comparison

We sought to find a method that would allow us to reproducibly and reliably characterize the signaling pathways of the three

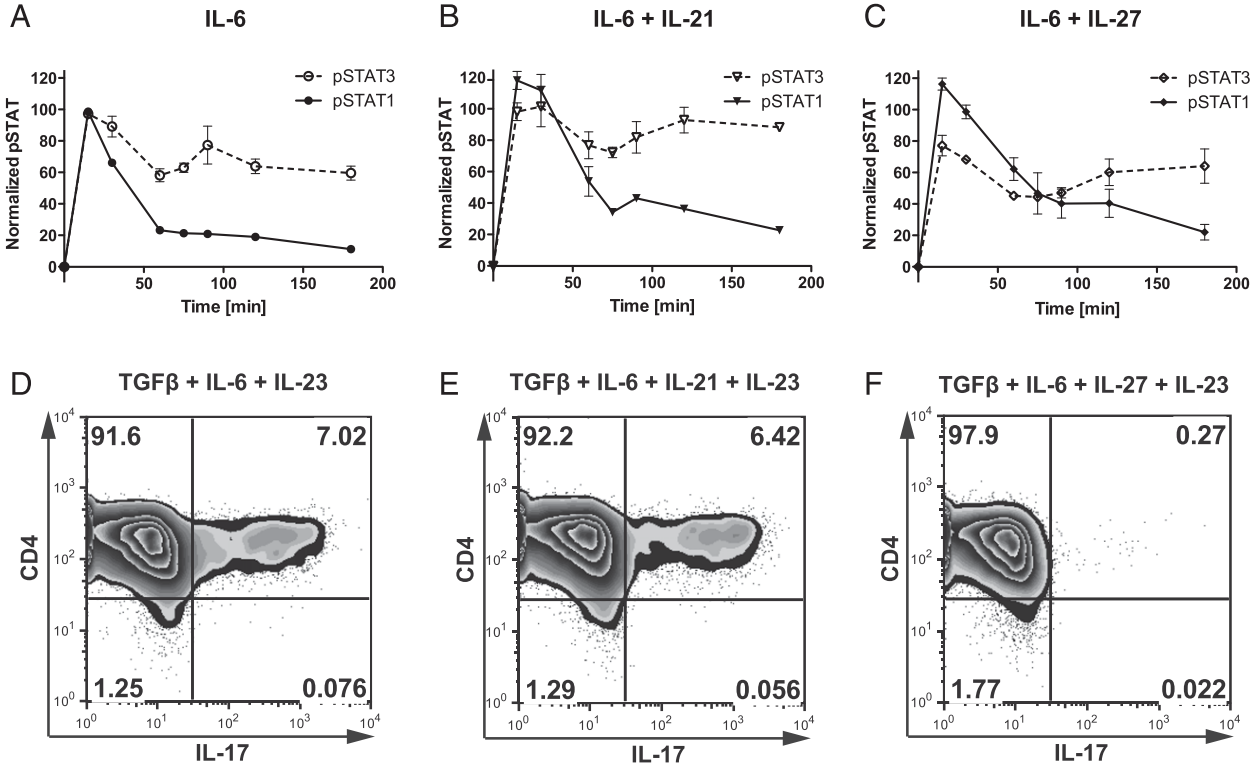

FIGURE 2. p-STAT1/p-STAT3 ratio during Th17 differentiation. Flow cytometry measurement of p-STAT3 and p-STAT1 at different time points in CD4$^+$ T cells cultured with anti-CD3/anti-CD28 for 1 h and then with IL-6 (**A**), IL-6 and IL-21 (**B**), or IL-6 and IL-27 (**C**) for up to 4 h. Results are the averages of 3 to 13 MFI measurements at a given time point and a given condition, normalized using Eq. 1. Error bars represent SEM. Flow cytometry of naive CD4$^+$ T cells cultured with IL-6 (**D**), IL-6 and IL-21 (**E**), or IL-6 and IL-27 (**F**) in the presence of TGF-β, anti–IL-4, and anti–IFN-γ. After 48 h, all cultures were supplemented with IL-23; after 4 d, cultures were stimulated for 4 h with PMA and ionomycin, followed by intracellular cytokine staining with anti–IL-17A and anti-CD4.

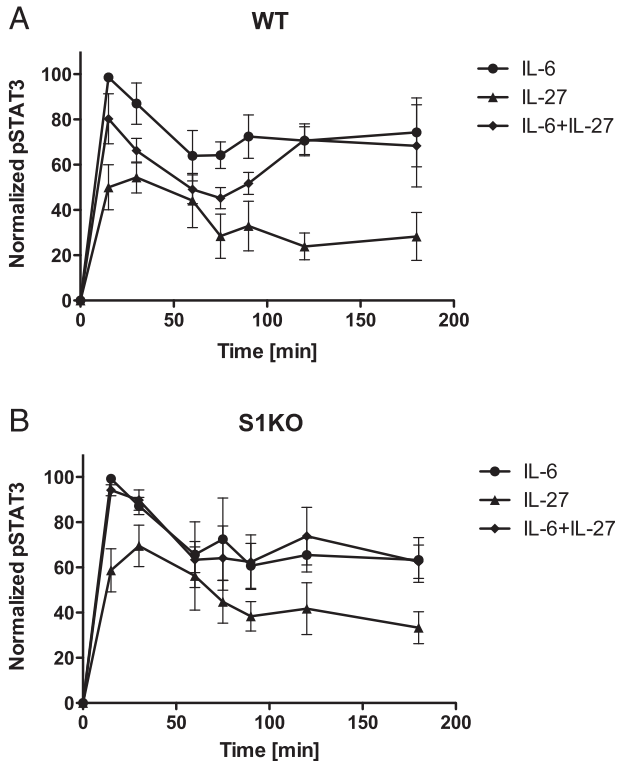

FIGURE 3. IL-6, IL-6+IL-27, and IL-27 induce similar p-STAT3 profiles in S1KO T cells. Flow cytometry measurement of p-STAT3 and p-STAT1 at different time points in naive $CD4^+$ T cells from WT (**A**) and S1KO (**B**) mice after culture with anti-CD3/anti-CD28 for 1 h and then IL-6, IL-27, or IL-6+IL-27 for up to 3 h. Results are MFI measured at a given time point and a given condition, normalized using Eq. 1 and the MFI of p-STAT3 induced by IL-6 at 15 min. Graphs show cumulative data of three independent experiments. Error bars represent SEM. Curves were significantly different in WT T cells (A) ($p = 0.0139$) but not in S1KO T cells (B) ($p = 0.0812$), as determined by two-way ANOVA with a Bonferroni post test.

cytokines of interest (IL-6, IL-21, and IL-27) early during T cell differentiation to determine why they behave differently despite activating the same two transcription factors: STAT1 and STAT3. To measure the expression of p-STAT1 and p-STAT3, $CD4^+$ T cells were activated with anti-CD3/anti-CD28 Abs in the presence of the cytokines of interest. Measurements of p-STAT1 and p-STAT3 levels were made only during the first 3–4 h after T cell activation to ensure that the STAT activation levels were entirely attributable to the cytokine of interest and not due to secondary effects of other cytokines secreted by the T cells over time.

We compared different techniques to measure differences in STAT activation. Given the number of conditions that we sought to measure, it became clear that flow cytometry was the best technique because of the ease of measurement and the availability of a quantitative comparable result. Because it was not feasible to do all of the flow cytometry measurements in 1 d, we devised a way to display all of our data collected over several months of separate experiments in one graph. Using the mean fluorescence intensity (MFI), we were able to extract just one number from each FACS measurement that could be plotted and compared with other samples. Daily variations in FACS measurements and mouse-to-mouse variations could be corrected for by determining the conditions resulting in the highest and the lowest signal in every experiment. By testing numerous conditions and time points, we determined that the maximum signal obtained for both p-STAT1 and p-STAT3 was always measured in cells that received 15 min of IL-6 stimulation, whereas the minimum signal for both p-STATs was always measured in unstimulated cells (Supplemental Fig. 1). Therefore, these two conditions were included in every experiment and used to normalize that day's measurements to all other measurements using Eq. 1:

$$Normalized\ p\text{-}STAT = 100 * \frac{MFI_{Sample} - MFI_{Untreated}}{MFI_{IL6,15min} - MFI_{Untreated}} \quad (1)$$

In each experiment, IL-6 at 15 min was assigned a value of 100 for p-STAT1 and for p-STAT3, whereas a value of 0 indicated a basal level of STAT activation measured in cells cultured without cytokines. It is important to note that p-STAT1 and p-STAT3 were normalized using different MFI values; thus, a value of 100 for p-STAT1 and a value of 100 for p-STAT3 do not indicate that there are equivalent amounts of the two transcription factors at the molecular level. The values observed for $MFI_{IL6,15min}/MFI_{Untreated}$ were typically 1.5–2.0 for p-STAT3 and 8.0–15.0 for p-STAT1. As a result, because of the greater separation between the maximum signal and the background, less variation was observed in the normalized p-STAT1 measurements compared with the p-STAT3 measurements.

Cytokines induce different STAT activation profiles

We measured STAT3 and STAT1 activation profiles in $CD4^+$ T cells treated with the three cytokines for different time periods and normalized the profiles using the method described above. This enabled us to compare the activation profiles of STAT1 and STAT3 among the different cytokines (Fig. 1).

IL-6 clearly induced the most p-STAT3 of the three cytokines at all time points measured (Fig. 1A). Comparatively, IL-21 activated roughly half the amount of STAT3 during that time period. IL-27 had peak STAT3 activation at 15–30 min, reaching ~33% of the p-STAT3 levels induced by IL-6 at those time points. Activation of STAT3 by IL-27 then steadily decreased to basal levels during the remainder of the first 2 h. Given that STAT3 is vital to Th17 differentiation (15), it is logical that IL-6 is the best inducer of Th17 cells (in combination with TGF-β), because it induced the most p-STAT3. IL-21 is a weak inducer of Th17 cells, and it induces roughly half the amount of p-STAT3 as IL-6. IL-27 does not induce Th17 cells and showed the lowest induction of p-STAT3. These data prompted the question whether there is a threshold of p-STAT3 induction below that of IL-21 and above that of IL-27 that needs to be reached for a cytokine to induce Th17 cells.

It was generally believed that IL-27 inhibits Th17 differentiation by inducing high amounts of p-STAT1, because IL-27 fails to inhibit Th17 differentiation in S1KO mice (25, 29). Therefore, we expected IL-27 to induce higher levels of p-STAT1 than either of the other cytokines. Indeed, IL-21 induced relatively small amounts of p-STAT1 compared with IL-27. However, we observed that IL-6 induced more p-STAT1 than IL-27 at almost all time points in the first 4 h (Fig. 1B). At its peak induction of p-STAT1 at 15 min, IL-27 only induced ~60% of the amount induced by IL-6. These data raised the question of how IL-6 can induce the most p-STAT1, which is known to inhibit Th17 differentiation, and still be the strongest inducer of Th17 cells.

Visualizing the data in the form of cytokine "fingerprints" might assist in addressing this question. To create a "fingerprint" of a given cytokine's signaling pattern, the time courses of p-STAT1 and p-STAT3 levels are combined in the same graph. This allows for a comparison of the relative amounts of p-STAT3 and p-STAT1 induced at all of the time points for a given cytokine

(Fig. 1C–E). As mentioned previously, the values for p-STAT1 and p-STAT3 cannot be compared directly; however, regardless of the absolute number of STAT1 or STAT3 molecules, it is still valid to compare the ratio of p-STAT3/p-STAT1 over time among the different cytokines, because all of the data were normalized the same way. The fingerprint for IL-6 showed that T cells treated with IL-6 for 15 min had a p-STAT3/p-STAT1 ratio of 1. At 3 h, the ratio of p-STAT3/p-STAT1 increased to 5 (Fig. 1C). Similarly, IL-21 induced a p-STAT3/p-STAT1 ratio of 2–3 in T cells during the first 3 h (Fig. 1D). In contrast, IL-27 was the only cytokine to induce a higher relative amount of p-STAT1 than p-STAT3. In fact, at all time points, T cells treated with IL-27 had more p-STAT1 than p-STAT3 (Fig. 1E). These observations suggest that the ratio of p-STAT3/p-STAT1 is an important parameter in determining whether a cytokine will induce Th17 differentiation: cytokines, such as IL-6 and IL-21, which induce a p-STAT3/p-STAT1 ratio $>$ 1 at most or all time points promote Th17 differentiation, whereas cytokines, such as IL-27, which induce a p-STAT3/p-STAT1 ratio $<$ 1 at all time points inhibit Th17 differentiation.

p-STAT1/p-STAT3 ratio determines whether a cytokine pair inhibits or induces Th17 cells

To further investigate the importance of the p-STAT3/p-STAT1 ratio for the ability of cytokines to induce Th17 differentiation, we began looking at STAT activation profiles for pairs of cytokines. We were interested in the STAT-activation profiles for pairs of cytokines for three reasons. First, in experiments showing that IL-27 inhibits Th17 differentiation, IL-27 was added in addition to IL-6 and TGF-β at time 0 (25, 29). Therefore, the cell is exposed to both IL-6 and IL-27 at the same time, so their individual profiles are not directly applicable. Second, we may gain new mechanistic insight by determining how individual profiles combine. If the sum of the individual profiles does not equal that of the cytokine pair's profile, then there are saturating or inhibitory effects. Finally, the profiles of cytokine pairs will help us to define which profiles lead to Th17 cell differentiation.

We measured the fingerprints of IL-6 (Fig. 2A), IL-6 with IL-21 (Fig. 2B), and IL-6 with IL-27 (Fig. 2C). All measurements were normalized as before using the MFI from untreated T cells as the baseline and the MFI from T cells treated with IL-6 for 15 min as the maximum. Assuming that the profiles are additive, it was now possible for the cytokine pairs to induce p-STAT values $>$ 100, because IL-6 was present in all pairs, and all measurements were normalized to IL-6 at 15 min. Thus, the addition of either IL-21 or IL-27 to IL-6 increased the value of p-STAT1 to $>$100 at 15 min and shifted the p-STAT1 curve upward in value at all time points while maintaining the same shape as the p-STAT1 curve in the IL-6 fingerprint (Fig. 2A–C). Interestingly, the p-STAT1 profiles in the IL-6+IL-21 fingerprint and the IL-6+IL-27 fingerprint were nearly identical. This similarity, together with the fact that IL-27 inhibits Th17 differentiation via STAT1, suggests that the addition

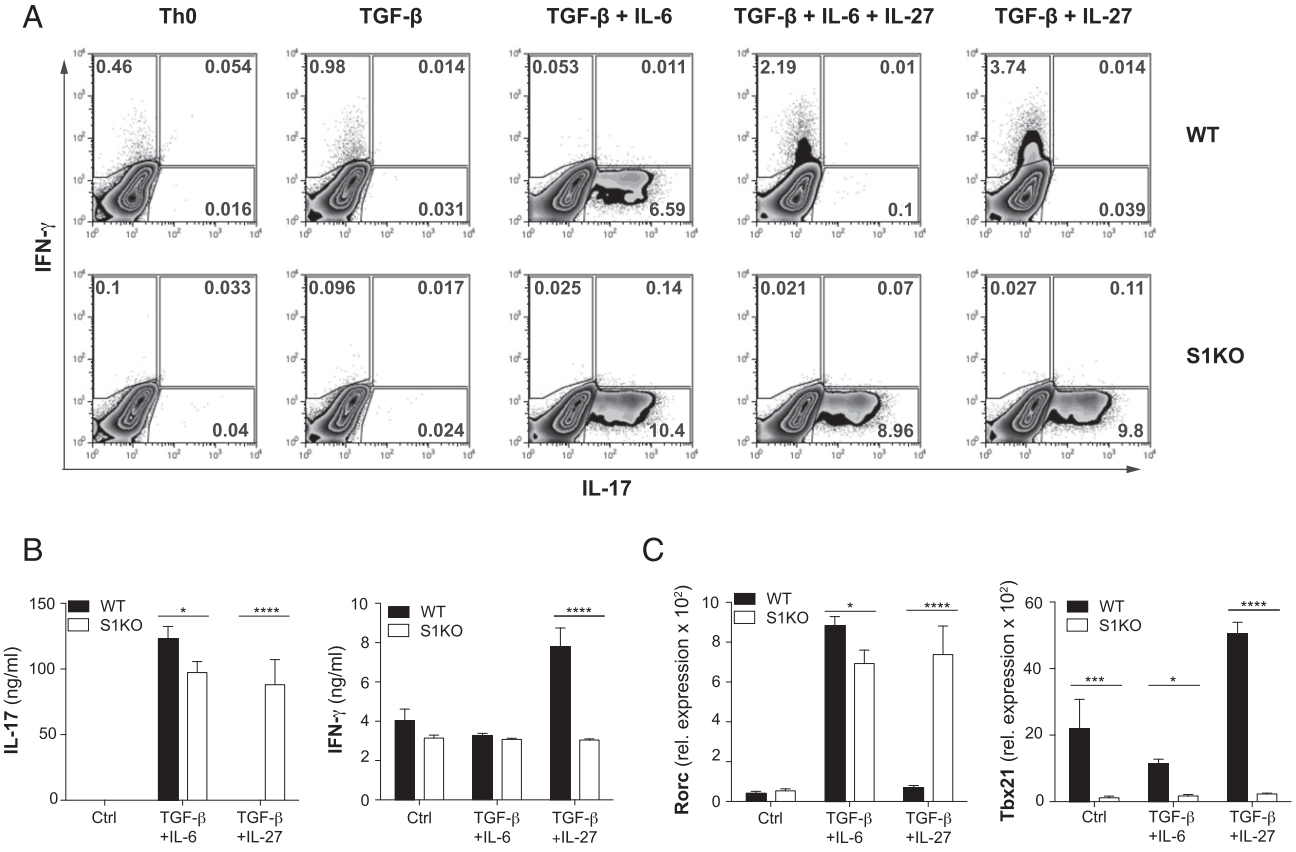

FIGURE 4. IL-27 induces Th17 cells in the absence of STAT1. (**A**) Sorted naive $CD4^+$ T cells from WT or S1KO mice were cultured for 4 d in the presence of IL-6+TGF-β, IL-27+TGF-β, IL-6+IL-27+TGF-β, TGF-β alone, or no cytokines (Th0) and then stimulated for 3.5 h with PMA and ionomycin. Cytokine production was measured by intracellular cytokine staining with anti–IL-17A and anti–IFN-γ and analyzed by flow cytometry. Data are representative of four independent experiments. (B and C) FACS-sorted naive $CD4^+CD62L^+CD44^-CD25^-$ T cells from WT or S1KO mice were cultured with plate-bound anti-CD3 and anti-CD28 in the presence of the indicated cytokines. (**B**) IL-17 and IFN-γ release was detected by ELISA after 48 h of culture. (**C**) RNA was isolated after 24 h of culture. mRNA levels of Rorc and Tbx-21 relative to mRNA levels of β-actin were measured by real-time PCR. Error bars indicate SD of triplicates. These data are representative of at least three separate experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

of IL-21 at time 0 should also inhibit Th17 differentiation. To test this hypothesis, we measured Th17 differentiation in the presence of TGF-β+IL-6 (Fig. 2D), TGF-β+IL-6+IL-21 (Fig. 2E), and TGF-β+IL-6+IL-27 (Fig. 2F) by flow cytometry. We measured a similar frequency of IL-17$^+$ T cells in the presence of TGF-β+IL-6 and TGF-β+IL-6+IL-21, whereas the presence of IL-27 almost completely inhibited Th17 differentiation. These data raise the question of why IL-21 does not inhibit Th17 differentiation although it induces an almost identical p-STAT1 profile as IL-27. Again, the answer can be obtained by considering the relative p-STAT1 and p-STAT3 profiles. Addition of IL-21 to IL-6 caused an upward shift in both the p-STAT1 and p-STAT3 curves in the IL-21+IL-6 fingerprint, resulting in a similar p-STAT3/p-STAT1 ratio as observed in the IL-6 fingerprint (Fig. 2A–C). However, when IL-27 was added to IL-6, the p-STAT1 curve was shifted upward, and the p-STAT3 curve was shifted downward, resulting in a significant change in the p-STAT3/p-STAT1 ratio compared with the IL-6 fingerprint (Fig. 2A–C). Therefore, considering just the p-STAT1 profiles alone is not sufficient for making conclusions about what effect a cytokine has on Th17 differentiation, because one would infer that IL-21 should be inhibitory to Th17 differentiation. Instead, these data suggest that determining the ratio of p-STAT3/p-STAT1 induced by a cytokine is superior to individual profiles for predicting the effect that the cytokine may have on Th17 differentiation.

IL-6 and IL-27 induce similar p-STAT3 profiles in S1KO T cells

It was shown that inhibition of Th17 differentiation via IL-27 is dependent on STAT1, because S1KO T cells stimulated with TGF-β, IL-6, and IL-27 generate a similar percentage of IL-17–producing cells as S1KO T cells stimulated with TGF-β and IL-6 alone (25, 29). Based on the relatively low amount of p-STAT3 induced by IL-27 compared with IL-6 (Fig. 1), and given the similarity in Th17 induction, we expected IL-6 with and without IL-27 to induce similar p-STAT3 profiles in S1KO T cells. To test this, we measured the p-STAT3 profiles over time in WT (Fig. 3A) and S1KO (Fig. 3B) T cells after stimulation with IL-6, IL-27, or IL-6 and IL-27. In WT T cells, IL-6 induced the most p-STAT3 at all times, and IL-27 induced the least amount of p-STAT3. As early as 15 min, a decrease in p-STAT3 was observed in WT T cells upon addition of IL-27 to IL-6, so that the p-STAT3 profile of WT T cells stimulated with IL-6+IL-27 fell in between the p-STAT3 profiles of IL-6– and IL-27–treated WT cells (Fig. 3A). In contrast, in S1KO cells, the STAT3-activation profiles for IL-6 and IL-6+IL-27 were almost identical, implying that the presence of STAT1 is somehow able to reduce the level of activation of STAT3. In addition, we observed that IL-27 induced only slightly less p-STAT3 in S1KO T cells compared with the other two conditions. Statistical analysis supported that p-STAT3 profiles induced by IL-6 and IL-27 in WT cells were significantly different ($p = 0.0139$), whereas p-STAT3 profiles induced by IL-6 and IL-27 in S1KO cells were not significantly different ($p = 0.0812$). IL-27 also was described to activate STAT5 (36). Thus, we measured STAT5-activation profiles in response to IL-6, IL-27, and IL-6+IL-27 in WT and S1KO CD4 T cells. In addition, we included the cytokine IL-7, a strong activator of STAT5, as a positive control. As expected, IL-7 induced high levels of p-STAT5 at all times, peaking at 15 min in WT and S1KO T cells. In contrast, the other cytokines induced only negligible amounts of p-STAT5 at all times (Supplemental Fig. 2), indicating that activation of STAT5 in response to IL-27 is not relevant in our experimental system.

IL-27 induces Th17-like cells in S1KO T cells

Given the similarity between the p-STAT3 profiles induced by IL-6 and IL-27 in S1KO T cells, we predicted that, in the absence of STAT1, IL-27 induces enough p-STAT3 to drive Th17 differentiation when combined with TGF-β. To test our prediction, we cultured naive CD4$^+$ T cells (CD4$^+$CD25$^-$CD62L^{hi}) from either WT or S1KO mice with anti-CD3 and anti-CD28 Abs and with no cytokines (Th0), TGF-β, TGF-β+IL-6, TGF-β+IL-6+IL-27, or TGF-β+IL-27. After 4 d, we measured IL-17 production (Fig. 4A). Consistent with previous studies, IL-27 potently inhibited Th17 differentiation in WT cells treated with IL-6+IL-27+TGF-β, but it did not inhibit Th17 differentiation in S1KO cells treated with IL-6+IL-27+TGF-β (25, 29). As expected, IL-27 and TGF-β did not induce any Th17 differentiation in WT cells. In contrast, treatment of S1KO T cells with IL-27 and TGF-β generated a significant percentage of IL-17$^+$ cells (Fig. 4A), as we predicted based on the p-STAT3 profiles. Accordingly, S1KO T cells treated with IL-27+TGF-β displayed a Th17-like phenotype: they produced and secreted similar amounts of IL-17 and expressed similar RORγt (*Rorc*) mRNA levels as did WT Th17 cells generated via IL-6+TGF-β. In addition, IL-27+TGF-β–treated S1KO T cells did not express Th1-associated markers [i.e., they did not produce IFN-γ nor did they express the Th1 transcription factor T-bet (*Tbx21*)] (Fig. 4B, 4C).

IL-27 induces IL-23R expression in S1KO CD4 T cells

IL-23 was shown to be critical for the expansion and maintenance of Th17 cells (37). To determine whether IL-27–induced S1KO Th17 cells also respond to IL-23, we evaluated the expression of IL-23R. The specific subunit IL-23R pairs with IL-12Rβ1 to form IL-23R (38). In contrast, IL-27 was described to increase the expression of IL-12Rβ2, which pairs with IL-12Rβ1 to form the IL-12R. Thus, we determined expression of all three subunits in our WT and S1KO Th17 cells. As expected, IL-6 induced high

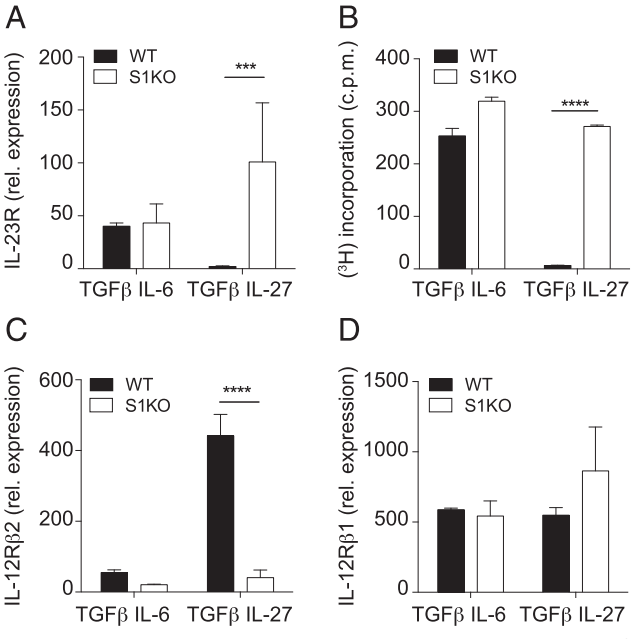

FIGURE 5. IL-27–induced S1KO Th17 cells respond to IL-23. CD4$^+$ T cells from WT or S1KO mice were cultured with plate-bound anti-CD3 and anti-CD28 in the presence of the indicated cytokines. RNA was isolated after 24 h of culture, and mRNA levels of IL-23R (**A**), IL-12Rβ2 (**C**), and IL-12Rβ1 (**D**) relative to β-actin were measured by real-time PCR. (**B**) Sorted naive 2D2 CD4$^+$ T cells from WT or S1KO mice were stimulated with irradiated APCs and anti-CD3 in the presence of the indicated polarizing cytokines and supplemented with IL-23 after 48 h. Proliferation was measured by [^{3}H]thymidine incorporation (cpm ± SD). Data are representative of at least three separate experiments. ***$p \leq 0.001$, ****$p \leq 0.0001$.

levels of IL-23R mRNA, whereas IL-27 did not induce IL-23R expression in WT T cells. In contrast, IL-27 and IL-6 both induced high levels of IL-23R mRNA in S1KO CD4 T cells (Fig. 5A). These data suggest that IL-27–induced S1KO Th17 cells can form a functional IL-23R and, thus, respond to IL-23. To test this, we measured proliferation of IL-27–induced S1KO Th17 cells and WT Th17 cells in response to IL-23 using a radiolabeled thymidine assay. Importantly, S1KO $CD4^+$ T cells stimulated with IL-27 proliferated just as well as S1KO and WT T cells stimulated with IL-6, whereas WT T cells cultured with IL-27 did not proliferate in the presence of IL-23 (Fig. 5B). In addition, expression of IL-12Rβ2 was increased by IL-27 in WT cells but not in S1KO cells (Fig. 5C), indicating that IL-27 acts via STAT1 to induce IL-12Rβ2 expression. The IL-12Rβ1 subunit was constitutively expressed in all $CD4^+$ T cells subsets (Fig. 5D).

IL-27–induced S1KO Th17 cells are encephalitogenic upon adoptive transfer

In the absence of STAT1, IL-27 induces $CD4^+$ T cells that resemble Th17 cells because they express the lineage-specific transcription factor RORγt, the cytokine IL-17A, and IL-23R. However, we and other investigators showed that Th17 cells come in different flavors that do not necessarily harbor identical pathogenicity (39). To test the pathogenic potential of IL-27–induced S1KO Th17 cells, we performed adoptive-transfer experiments using $CD4^+$ T cells from either 2D2 mice (which genetically express a TCR specific for myelin oligodendrocyte glycoprotein) or S1KO 2D2 mice. Naive $CD4^+$ T cells were differentiated in vitro into Th17 cells, as previously described (6) and as represented in Fig. 6A. Confirming our previous result, IL-27 induced IL-17 production in S1KO CD4 T cells to the same extent as did IL-6 in WT and S1KO T cells (Fig. 6B). Upon adoptive transfer, we observed that S1KO $CD4^+$ T cells differentiated with IL-27 induced EAE disease similar to WT Th17 cells or S1KO T cells differentiated with IL-6 (Fig. 6C). Although Th1 cell transfer is known to induce classical EAE, which is characterized by an ascending paralysis, Th17 cell recipients were described to also develop atypical EAE signs characterized by an unbalanced gait and severe axial and barrel rotatory defects (40). When the mice were scored over 20 d, recipients of WT Th17 cells, IL-27–induced S1KO Th17 cells, and IL-6–induced S1KO Th17 cells displayed a similar disease course, with similar disease severity and onset (Fig. 6D, Supplemental Table I). As a result of the limited proliferative capacity shown in Fig. 5B, we were not able to sufficiently

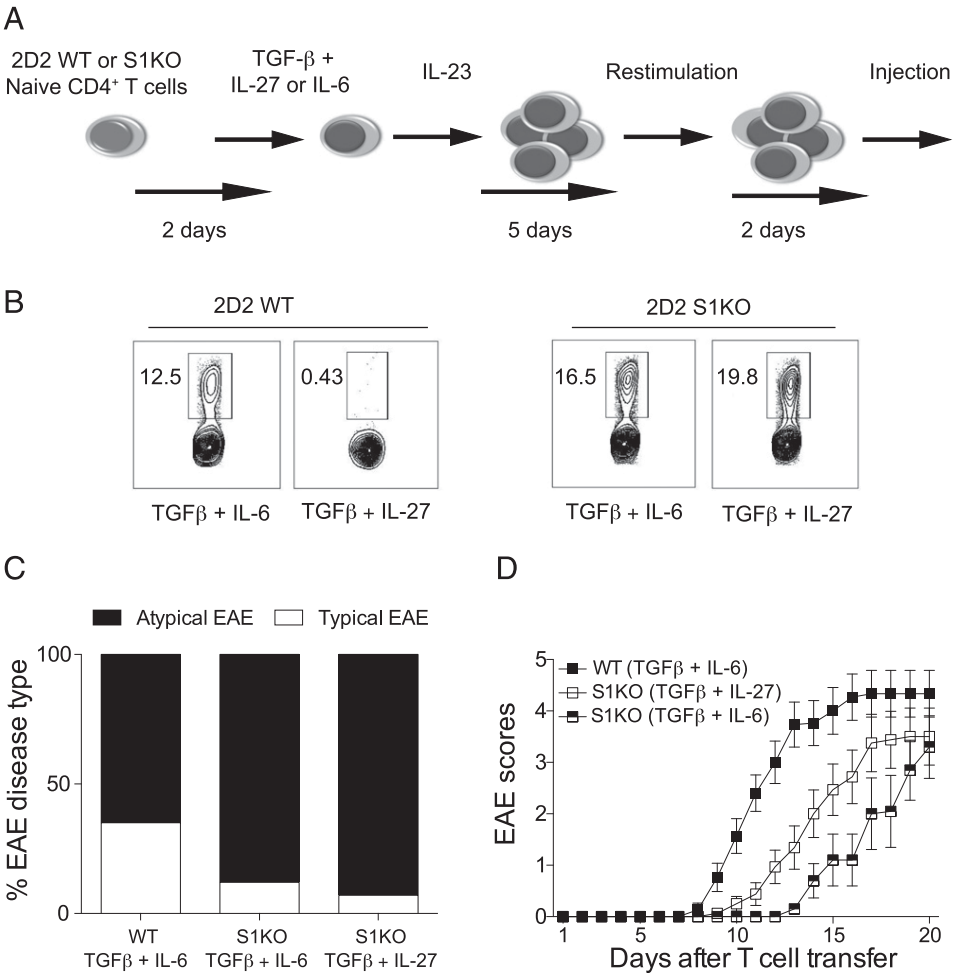

FIGURE 6. IL-27–induced S1KO Th17 cells cause EAE upon adoptive transfer. (**A**) Naive myelin oligodendrocyte glycoprotein–specific $CD4^+$ T cells from WT or S1KO mice were stimulated with irradiated APCs and anti-CD3 in the presence of the indicated cytokines; IL-23 was added after 48 h of culture. After 7 d, $CD4^+$ T cells were restimulated in the presence of anti-CD3 and anti-CD28 Abs for 48 h before in vivo injection. (**B**) Intracellular IL-17 cytokine profile of in vitro–differentiated T cell subsets, as analyzed by FACS before injection. A total of 3×10^6 $CD4^+$ T cells was injected i.p. into C57BL/6 WT recipient mice that were observed for the development of clinical signs of typical and atypical EAE. (**C**) Percentages of classical and atypical EAE incidence in the different recipient mice. (**D**) Mean EAE scores over time in recipient mice. Graph shows cumulative data from two independent experiments. Error bars represent SEM.

expand WT CD4 T cells differentiated with IL-27 for adoptive transfer. In summary, IL-27–induced S1KO Th17 cells are highly pathogenic and capable of inducing EAE with similar severity as IL-6–induced Th17 cells.

IL-27–induced S1KO Th17 cells lead to cerebellum and brain stem lesions

Atypical EAE is associated with lesions in the brain, in particular in the cerebellum and the brainstem. Because Th17 cell recipients exhibited an atypical neurologic disease with severe ataxia, we compared the localization of CNS lesions in sick mice from the different groups (Fig. 7A). Histological and immunohistochemistry analysis revealed that recipients of IL-27–induced S1KO Th17 cells exhibited immune cell infiltration that was as severe as in recipients of classically differentiated Th17 cells (Fig. 7B). Cellular infiltrates were particularly abundant in the cerebellum and brainstem. Anti-CD3 and anti–Mac3 staining further showed marked infiltration of T cells and myeloid cells. Our histologic analysis showed that WT Th17 cells and S1KO Th17 cells induced by IL-27 or IL-6 caused a very similar pattern and degree of inflammation, cellular infiltration, and tissue destruction.

Discussion

IL-6 (18, 22, 31), IL-21 (30), and IL-27 (22) all activate STAT3 but have very different effects on Th17 differentiation. In this study, we systematically measured and compared the p-STAT3 and p-STAT1 profiles in T cells in response to all three cytokines separately and in response to cytokine pairs. In these analyses, we made the assumption that STAT activation in the first 4 h is relevant in determining a cytokine's ability to induce Th17 differentiation, a process spanning several days. However, we observed that STAT3 reaches its peak expression within 2 h during Th17 differentiation and quickly drops off to low levels for the remainder of the differentiation process (K.D. Fowler and V.K. Kuchroo, unpublished observations), confirming that it is relevant to measure STAT activation at early times.

IL-6 activated the most STAT3 of the three cytokines, followed by IL-21 and IL-27, suggesting that the amount of p-STAT3 induced by a cytokine may directly correlate with its relative ability to induce Th17 differentiation. Surprisingly, we observed that, of the three cytokines, IL-6 also induced the most p-STAT1, which is known to inhibit Th17 differentiation. Because IL-6 is the best inducer of Th17 cells, and STAT1 is entirely responsible for IL-27's ability to potently inhibit Th17 differentiation (25, 29), we expected IL-27 to induce the most p-STAT1 among these cytokines. These data indicate that considering the total amounts of p-STAT1 or p-STAT3 induced by a cytokine separately is not sufficient to predict its effect on Th17 differentiation. Measuring both p-STAT3 and p-STAT1 profiles in response to stimulation with cytokine pairs revealed that the p-STAT3/p-STAT1 ratio is important in determining whether cytokines will induce or inhibit Th17 differentiation. Thus, T cells treated with IL-21+IL-6 had a high p-STAT3/p-STAT1 ratio, similar to T cells treated with IL-6 alone, and they showed a similar degree of Th17 differentiation. In contrast, the p-STAT3/p-STAT1 ratio was significantly lowered in T cells treated with IL-27+IL-6 compared with IL-6 alone,

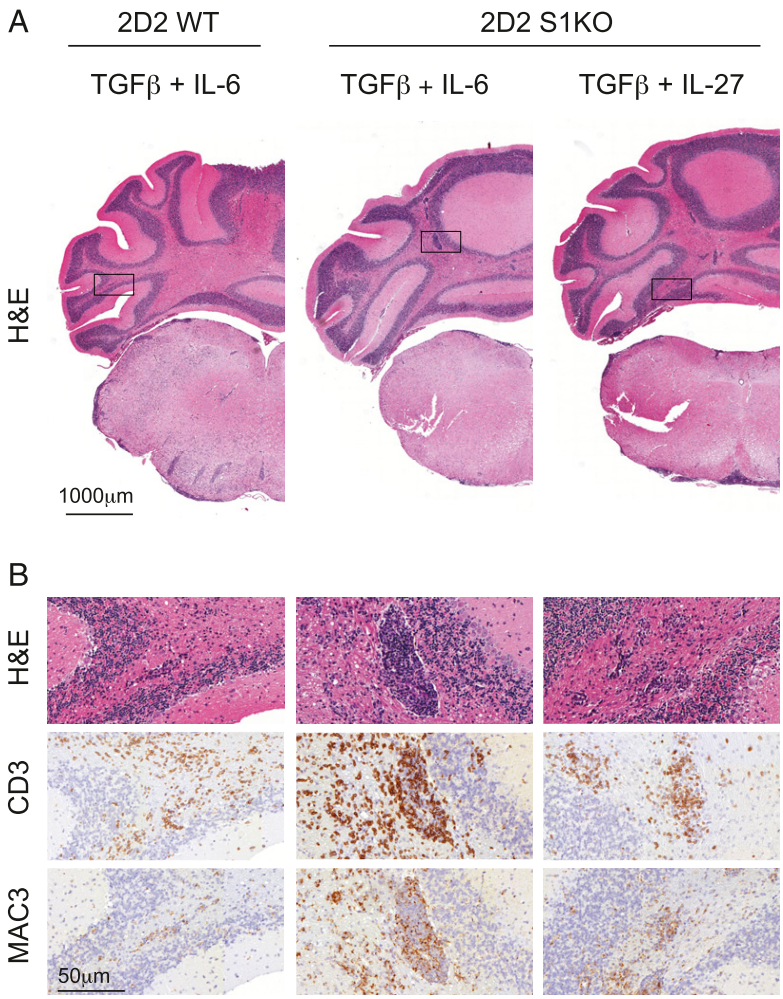

FIGURE 7. IL-27–induced Th17 cells drive infiltration of the brainstem and cerebellum. Histological analysis of brainstem and cerebellum of the three recipient mice groups showing severe cellular infiltration, as visualized at low magnification by H&E staining (**A**) and high magnification with H&E staining and immunohistochemistry using anti-CD3 and anti-Mac3 Abs (**B**) of the representative regions in (A) indicated by the rectangles in the cerebellum.

resulting in inhibition of Th17 differentiation. Consistent with this concept, IL-27 was the only cytokine that induced a higher amount of p-STAT1 compared with p-STAT3 on the normalized scale, leading to a p-STAT3/p-STAT1 ratio < 1 and inhibition of Th17 differentiation.

Because IL-6 and IL-27 both require the receptor subunit gp130, paired with the specific subunits IL-6R and WSX-1, respectively, to signal through their receptors, competition for gp130 could be an issue. Thus, it is possible that the IL-6+IL-27 fingerprint reflects the action of the dominant cytokine rather than equal action of both cytokines. Several arguments point against this hypothesis. First, we could not drive Th17 cell differentiation with IL-27 in the absence of WSX-1 (data not shown). Furthermore, although IL-6R is highly expressed on naive T cells, it is downregulated upon T cell activation (41), whereas WSX-1 is upregulated upon activation of naive T cells (23). Thus, by activating our T cells before adding cytokines, we reduced the competition for gp130. Considering also that IL-6+IL-27 generates a different fingerprint than IL-6 or IL-27 alone, it is reasonable to assume that the IL-6+IL-27 fingerprint reflects the action of both cytokines. Thus, we believe that our observations directly reflect the differences in p-STAT profiles and that these observations cannot be explained by competition for gp130.

The p-STAT3/p-STAT1 ratio affects the distribution of the three dimeric forms: conceivably the highest relative amount of heterodimer is formed when p-STAT1 and p-STAT3 are present in similar quantities (1:1 ratio). The relative amount of heterodimer decreases as one of the STATs starts dominating in numbers over the other, and the homodimeric form of the dominant STAT will be present in the higher relative amount. Thus, a high p-STAT3/p-STAT1 ratio leading to Th17 differentiation is characterized by the dominance of p-STAT3–p-STAT3 homodimers, whereas a low p-STAT3/p-STAT1 ratio is characterized by the abundance of p-STAT1–p-STAT1 homodimers, leading to inhibition of Th17 differentiation. We attempted to determine the relative amounts of homo- and heterodimers in response to different cytokines more precisely using an EMSA. However, we found that in our hands, EMSA lacked both precision and reproducibility to allow for reliable quantification of the different dimers.

Based on the similar p-STAT3 profiles induced by IL-6 and IL-27 in S1KO $CD4^+$ T cells, we predicted that IL-27 would induce Th17 differentiation in S1KO T cells just like IL-6. Indeed, it was shown previously that IL-27 could promote IL-17 production in the absence of STAT1 (42); however, the degree of differentiation, lineage, stability, and pathogenic properties of these IL-17–producing cells were not investigated. We demonstrated in this study that IL-27 not only promoted transient IL-17 production, but, in fact, induced the complete Th17 program. Thus, IL-27–induced S1KO Th17 cells expressed RORγt but not Tbet, responded to IL-23, and were long-lived and pathogenic, as demonstrated in our adoptive-transfer EAE experiments. The atypical clinical disease and increased lesion frequency in the brainstem and cerebellum further underline the "Th17-likeness" of the IL-27–induced S1KO T cells, because these are characteristic features of Th17-associated CNS inflammation (6, 35, 40, 43). Intriguingly, Hirahara et al. (44) recently described the actions of STAT1 and STAT3 in response to cytokines on a genomic scale and found that many genes that are usually uniquely regulated by IL-6 or IL-27 become common genes in the absence of STAT1, indicating that STAT1 is essential for cytokine specificity, whereas STAT3 controls the transcriptomic output. Although they did not look at ratios, their findings are consistent with our data showing that, in S1KO T cells, IL-27 and IL-6 are equally able to drive the Th17-differentiation process.

A built-in assumption in our analysis is that no other relevant pathways are activated or inhibited in S1KO T cells that may account for the differences. Thus, one could argue that IL-27 only induces Th17 differentiation in S1KO cells because they are more prone to Th17 differentiation as a result of the significant reduction in IFN-γ in these cells, which is known to inhibit Th17 cells. However, treatment of IFN-$\gamma^{-/-}$ T cells with IL-27 and TGF-β did not induce any Th17 differentiation (data not shown), confirming that the Th17 differentiation seen in S1KO cells in response to IL-27 is not due to the lack of IFN-γ. Thus, we believe that our results and verified predictions can be largely explained by considering p-STAT1 and p-STAT3 profiles and the resulting ratio.

Interestingly and consistent with our results, STAT1 and STAT3 imbalance also affects Th17 responses in humans. Thus, patients with STAT1 gain-of-function mutations, who presumably have a low p-STAT3/p-STAT1 ratio, suffer from chronic mucocutaneous candidiasis due to an impaired Th17 response (45, 46). Similarly, patients with STAT3 loss-of-function mutations also suffer from recurrent *Candida* and *Staphylococcus* infections (47, 48). In contrast, patients with STAT3 gain-of-function mutations, who presumably have a very high p-STAT3/p-STAT1 ratio, develop multiorgan autoimmune disease (49). Although the underlying mechanisms are still unclear and likely involve defects in several cell types, including regulatory T cells, increased Th17 responses may contribute to this immune dysregulation, because one patient showed elevated IL-17 levels and responded well to anti–IL-6R therapy (50). These results imply that the p-STAT3/p-STAT1 ratio could play an important role for both beneficial and pathogenic Th17 differentiation in mice, as well as in humans. In conclusion, the current study demonstrates the relevance and predictive value of the p-STAT3/p-STAT1 ratio for the induction of pathogenic Th17 cells. Modulation of the STAT activation balance could be foreseen as a novel therapeutic approach to fine-tune the immune response during infections or autoimmune disorders.

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Disclosures

The authors have no financial conflicts of interest.

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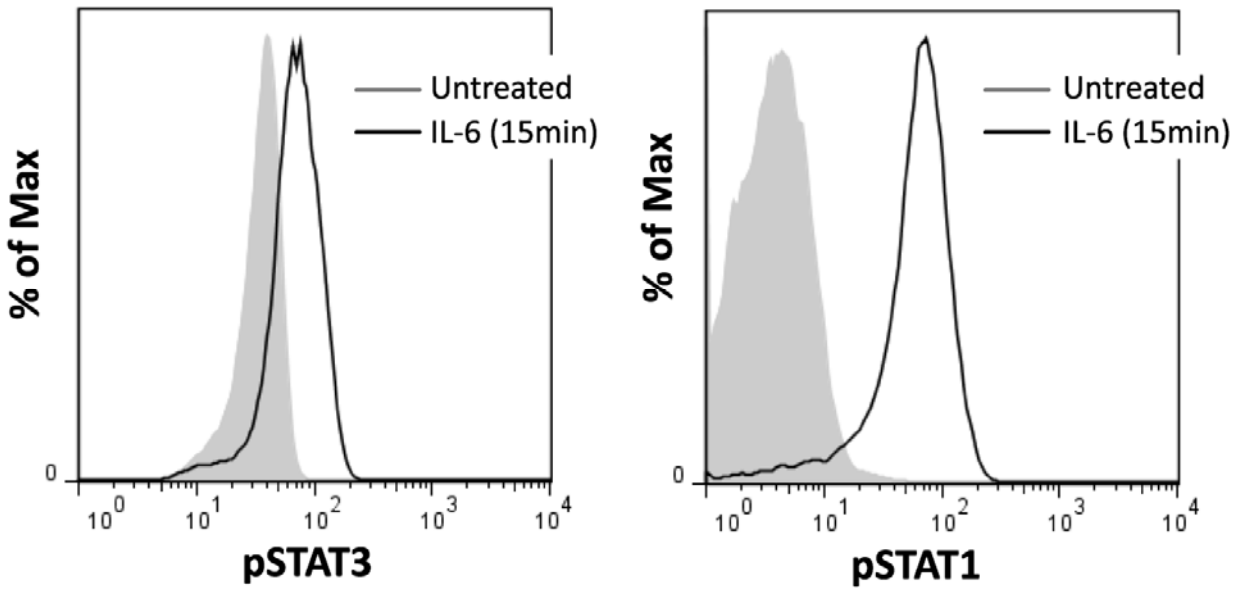
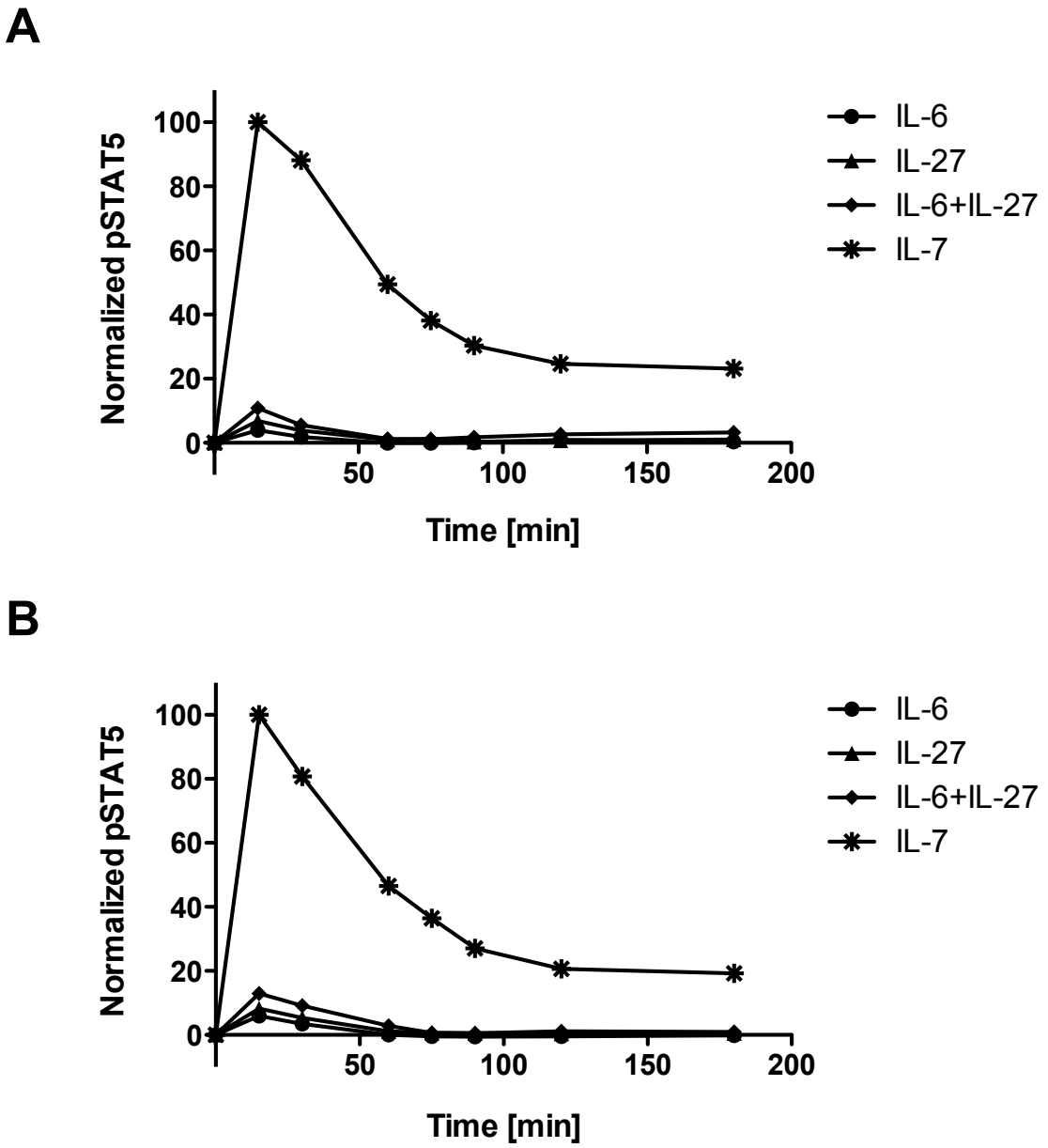


Supplementary Figure 1
Maximum observed signal to noise ratios for pSTAT1 and pSTAT3. Flow cytometry measurement of pSTAT3 (left) and pSTAT1 (right) in $CD4^+$ T cells cultured with α-CD3 / α-CD28 for one hour and then IL-6 (black line) or no cytokine (solid gray) for 15 minutes.



Supplementary Figure 2
Flow cytometry measurement of pSTAT5 in naïve $CD4^+$ T cells from (**A**) WT or (**B**) S1KO mice after being cultured with α-CD3 / α-CD28 for 1h and then IL-7, IL-6, IL-27, or IL-6 and IL-27 at different time points for up to 3h. Results are the MFIs measured at a given time point and a given condition, and normalized using Equation 1 using the MFI of pSTAT5 induced by IL-7 at 15 minutes. Graphs are representative of two independent experiments.

Supplementary Table 1. EAE in recipients of different in vitro generated Th17 cells

Transferred T cells	Incidence	Mean max. score (± SD)	Mean day of onset (± SD)
WT: TGF-β +IL-6	86% (13/15)	5 ± 0	11.6 ± 2
S1KO: TGF-β +IL-6	80% (8/10)	4.5 ± 1	15.5 ± 2.5
S1KO: TGF-β +IL-27	75% (12/16)	4 ± 1	13.8 ± 2.2

3.5 Oxysterols regulate encephalitogenic $CD4^+$ T cell trafficking during EAE

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Perturbation of steroids pathways is linked to inflammation and chronic diseases, however the underlying mechanism remains unclear. Oxysterols, oxidised forms of cholesterol, are not only essential for bile synthesis and sterol transportation but have recently been shown to contribute to the immune response. In addition, serum oxysterols levels have been proposed as suitable candidate biomarkers for neurological diseases such as multiple sclerosis (MS). However how oxysterols modulate adaptive immunity is unknown and their functions in autoimmunity have not been investigated. The enzyme cholesterol 25 hydroxylase (Ch25h) is the rate limiting step to synthetize the oxysterol 7a,25-dihydroxycholesterol (7a,25-OHC) from cholesterol. We here report, using the MS murine model experimental autoimmune encephalomyelitis (EAE), that Ch25h deletion significantly attenuated EAE disease course by limiting trafficking of pathogenic $CD4^+$ T lymphocytes to the central nervous system (CNS). Mechanistically, we show a critical involvement for oxysterols in recruiting leukocytes into inflamed tissues and propose that 7a,25-OHC preferentially promotes the migration of IL-17A-producing $CD44^+CD4^+$ T helper (T_H17) cells by binding the G protein-coupled receptor called Epstein-Barr virus induced gene 2 (Ebi2). Collectively, our results support a pro-inflammatory role for oxysterols during EAE and identify oxysterols as a potential therapeutic target to treat autoimmune diseases.

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Oxysterols regulate encephalitogenic CD4$^+$ T cell trafficking during central nervous system autoimmunity

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ABSTRACT

Perturbation of steroids pathways is linked to inflammation and chronic diseases, however the underlying mechanism remains unclear. Oxysterols, oxidized forms of cholesterol, are not only essential for bile synthesis and sterol transportation but have recently been shown to contribute to the immune response. In addition, serum oxysterols levels have been proposed as suitable candidate biomarkers for neurological diseases such as multiple sclerosis (MS). However how oxysterols modulate adaptive immunity is unknown and their functions in autoimmunity have not been investigated. The enzyme cholesterol 25 hydroxylase (Ch25h) is the rate limiting step to synthesize the oxysterol 7α,25-dihydroxycholesterol (7α,25-OHC) from cholesterol. We here report, using the MS murine model experimental autoimmune encephalomyelitis (EAE), that Ch25h deletion significantly attenuated EAE disease course by limiting trafficking of pathogenic CD4$^+$ T lymphocytes to the central nervous system (CNS). Mechanistically, we show a critical involvement for oxysterols in recruiting leukocytes into inflamed tissues and propose that 7α,25-OHC preferentially promotes the migration of activated CD44$^+$CD4$^+$ T cells by binding the G protein-coupled receptor called Epstein-Barr virus induced gene 2 (EBI2). Collectively, our results support a pro-inflammatory role for oxysterols during EAE and identify oxysterols as a potential therapeutic target to treat autoimmune diseases.

1. Introduction

The role of cholesterol metabolism during multiple sclerosis (MS) is debated and the underlying physiopathology largely unknown. Dyslipidaemia is associated with worsening inflammatory activity and MS disease progression [1–3]. In the mouse model experimental autoimmune encephalomyelitis (EAE), the cholesterol lowering drugs statins (HMG-CoA reductase inhibitors) induced a significant reduction in disease severity [4] and an initial clinical study confirmed those results in patients by showing significant reduction of inflammation by statins during MS [5]. However, subsequent trials that evaluated the role of statins during MS have generated conflicting results [6,7]. Therefore the contribution of cholesterol metabolism during MS remains unclear and may not be limited to sole dyslipidemia.

Cholesterol is converted by endoplasmic reticulum membrane-associated enzymes into its soluble forms oxysterols that are further transported to the liver and transformed into bile acids. In addition to their contributions to basic metabolic processes, oxysterols have recently been implicated in immune cell biology [8,9]. Furthermore, serum oxysterols levels have been proposed as suitable candidate biomarkers for neurological diseases such as multiple sclerosis (MS) [10]. Cholesterol 25-hydroxylase (Ch25h) converts cholesterol into 25-hydroxycholesterol (25-OHC) which is further metabolized into 7 alpha, 25-dihydroxycholesterol (7α,25-OHC). The human and mouse Ch25h enzymes are endoplasmic reticulum-associated glycoproteins but do not belong to the cytochrome P-450 family like the other enzymes converting cholesterol into oxysterol [11]. This suggested an additional role for Ch25h signaling pathway besides regulating lipid homeostasis. Indeed Ch25h deficient mice normally regulate fatty acid and cholesterol

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metabolism [12]. Furthermore, immune cells such as macrophages [12] and stromal cells [13] are a rich source of Ch25h. Both 25-OHC and 7α,25-OHC are implicated in the immune response: 25-OHC suppresses IgA production by B cells [12] and has broad anti-viral properties [14,15] while 7α,25-OHC guides B cell, dendritic cells and macrophages within the germinal follicles of the spleen and lymph nodes [13,16–18]. However, how oxysterols modulate adaptive immunity and participate to T lymphocytes biology is largely unknown and their role in autoimmunity has not been explored.

In the present study, we investigated the role of Ch25h and its downstream oxysterols during EAE. We demonstrated that Ch25h deletion attenuates EAE development by controlling the trafficking of encephalitogenic $CD4^+$ T cell subsets. We further described molecular mechanisms allowing specific oxysterols to promote T cell migration.

2. Material and methods

2.1. Animals

$Ch25h^{-/-}$ mice, backcrossed on C57Bl6J background at least for 11 generations were purchased from Jackson laboratories [12]. $EBI2^{-/-}$ mice, backcrossed on C57Bl6J background at least for 10 generations were provided by Novartis Institutes for BioMedical Research [16]. All mice were maintained under specific-pathogen-free conditions. Animal experiments were approved by the local veterinary office (Geneva, Switzerland) according to Swiss ethical regulations.

2.2. Bone marrow chimeras

Bone marrow (BM) was recovered from tibias and femurs by flushing with PBS-EDTA, dissociated by repeated passages through a 20-gauge needle. A total of 10×10^6 BM cells were injected intravenously into irradiated mice recipients (2 irradiations with 500 cGy using a Gammacell 40 exactor system at 4 h interval). Reconstitution was assessed by analyzing blood cells by flow cytometry after 6 weeks. Eight weeks after reconstitution, EAE experiments were performed.

2.3. Induction of EAE and clinical evaluation

Mice were immunized subcutaneously with 100 μg myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 (MOG_{35-55}) (Anawa) in complete Freund's adjuvant (CFA) (Difco) containing 5 mg ml^{-1} *Mycobacterium tuberculosis* (H37Ra, Difco). 300 ng pertussis toxin (Sigma) was injected intravenously on the day of immunization and 2 days later. Littermate mice were sex- and age-matched (6–10 weeks), and were scored daily for clinical signs by the following system: 0, no clinical deficits; 1, tail weakness; 2, hind limb paresis; 2.5, partial hind limb paralysis; 3, full hind limb paralysis; 4, full hind limb paralysis and forelimb paresis; 5, premorbid or dead. Mice were euthanized if they reached a score > 3. For disease incidence, a sick mouse was scored at 1 as soon as she reached an EAE score of 1, if a mice stayed asymptomatic a score 0 was given.

2.4. Histology

Mice were perfused at the end of the EAE disease with cold PBS followed by 4% paraformaldehyde fixation. Brain and spinal cord tissue were embedded in paraffin. For light microscopy, sections were stained with Hematoxylin and Eosin (HE) or Luxol Fast Blue and Periodic Acid Schiff (LFB/PAS) (Sigma–Aldrich). Images of tissue sections were scanned using a Mirax slide scanner (Zeiss). Inflammatory foci per spinal cord were quantified on HE–stained cross-sections. LFB/PAS staining was performed to assess demyelination, and the demyelinated area of spinal cross-sections (expressed as percentage of total white matter) was measured using the Mirax viewer (Zeiss). Average values of at least five cross-sections per animal were calculated.

2.5. Antigen-specific proliferative and cytokine responses

Mice were immunized with MOG_{35-55}-peptide in CFA as described above. Single-cell suspensions were prepared from draining lymph nodes (dLN) or spleens 12 days after immunization. Single cell suspensions were prepared and cells were restimulated with MOG_{35-55} for 48 h in DMEM with 10% (vol/vol) FCS supplemented with 50 M mercaptoethanol, 1 mM sodium pyruvate, nonessential amino acids, L-glutamine, and 100 U/ml penicillin-streptomycin. For proliferation assays, cells were pulsed with 1 Ci [3H]thymidine (PerkinElmer) during the final 16 h and analysis of incorporated [3H]thymidine was performed in a β-counter (1450 Microbeta; Trilux; PerkinElmer). Secreted cytokines were measured after 48 h of culture with MOG_{35-55} by ELISA (ebiosciences).

For intracellular cytokine staining, cells were obtained directly from the dLN as described above and stimulated for 4 h at 37 °C in culture medium containing PMA (20 ng/ml; Sigma), ionomycin (1 μg/ml; Sigma) and brefeldin (10 μg/ml; Sigma). After staining for surface markers, cells were fixed and permeabilized according to the manufacturer's instructions (ebiosciences), then stained for intracellular products.

2.6. Serological assays

Serum levels of total IgG autoantibodies against MOG_{35-55} antibodies were quantified by an ELISA. For autoantibodies, MOG_{35-55} was coated to ELISA plates pre-coated with poly-L-lysine (Sigma–Aldrich). The plates were then incubated with 1/100 diluted serum samples, and the assay was developed with alkaline phosphatase-labeled goat anti-mouse IgG. For lipid profile, mice were fasted for 16 h overnight, blood collected and serum separated by centrifugation and stored at −80 °C until lipid analysis. Lipid profile parameters were measured using a Roche Diagnostics (Basel, Switzerland) Cobas c111 clinical chemistry analyzer by services of the Mouse Metabolic Evaluation Facility (MEF), Center for Integrative Genomics, University of Lausanne.

2.7. Tissue preparation

For CNS preparation, mice were perfused through the left ventricle with cold PBS. Brains were dissected and spinal cords extruded by flushing the vertebral canal with cold PBS. CNS tissue was cut into pieces and digested for 45 min, in a DMEM containing collagenase D (2.5 mg/ml Sigma) and Dnase 1 (1 mg/ml Sigma) to give a single-cell suspension. Mononuclear cells were isolated by passage of the tissue through a cell strainer (70 μm), followed by Percoll gradient centrifugation (70%/37%). Mononuclear cells were removed from the interphase, washed and resuspended in culture medium for further analysis.

2.8. Flow cytometry

Antibodies directed against mouse CD3, CD4, CD44, IFNγ, IL-17A, CD11b, Ly6C, Ly6G, $MHCII^+$, CD45, CD8, B220, TCRγδ, TCRβ were purchased from ebioscience and CD11c from BD biosciences. Infiltrating leukocyte subsets were identified as $CD45^{int}CD11b^+$(microglia),

$CD45^{high}Ly6G^{+}$(neutrophils), $CD45^{high}Ly6G^{-}CD11b^{+}CD11c^{-}$ (macrophages), $CD45^{hi}CD3^{-}CD11c^{int}CD11b^{+}Ly6C^{+}MHCII^{+}$ (moDCs), $CD45^{hi}CD11c^{hi}CD11b^{-}Ly6C^{-}MHCII^{+}$ (cDCs), $CD45^{high}Ly6G^{-}CD11b^{-}CD11c^{-}CD3^{+}$ ($CD3^{+}$ T cells), $CD45^{high}Ly6G^{-}CD11b^{-}CD11c^{-}CD3^{+}CD4^{+}$ ($CD4^{+}$ T cells), $CD45^{high}Ly6G^{-}CD11b^{-}CD11c^{-}CD3^{+}CD8^{+}$ ($CD8^{+}$T cells), $CD45^{high}$ $Ly6G^{-}CD11b^{-}CD11c^{-}B220^{+}$ (B cells). All acquisitions were performed with a CyAn™ (Beckman Coulter) or Gallios3 (Beckman Coulter) equipped with Submitt and Kaluza Software. Sorting was done with a BD FACSAria as described previously [19]. All data were analyzed with FlowJo software (Tree Star, Ashland, Oregon).

2.9. Real-time PCR

RNA was extracted with the Trizol (Invitrogen). cDNA was synthesized with random hexamers and Superscript II reverse transcriptase (Invitrogen) and was used as template for quantitative PCR. PCR was performed with the Real-Time PCR Detection System (Applied Biosystems® StepOne plus) and SYBR green Supermix (KAPA SYBR® FAST Universal, Labgene). Expression of Il17a (FW TGAGCTTCCCAGATCACAGA Rev TCCAGAAGGCCCTCAGACTA), Rorc (FW CCTCTACACGGCCCTGGTT Rev GATGTTCCACTCTCCTCTTCTCT TG), Ebi2 (FW ACAACGGAGGTCCTAGCCA Rev GCTGTGGTGGGCATAGAGA), Ch25h (FW CCAGCTCCTAAGTCACGTC Rev CACGTCGAAGAAGGTCAG), Cyp7b1 (FW TTCCTCCACTCATACACAATG Rev CGTGCTTTTCTTCTTACCATG), Hsd3b7 (FW AAGAGGCCAGCAATACCCAG Rev ACCATCCACAAAGTCAACG) and actin (FW CCTGTATG CCTCTGGTCGTA, Rev CCATCTCCTGCTCGAAGTCT) were assessed with specific primers and probes. Gene expression was normalized to expression of the housekeeping gene actin.

2.10. T cell differentiation

Total or naïve $CD4^{+}$ T cells ($CD4^{+}CD62L^{hi}$) were obtained from spleens and lymph nodes of C57BL6 wildtype using MACS purification. $CD4^{+}$ T cells were cultured in DMEM complemented with MEM vitamins (100×) (SIGMA), MEM Non-essential Amino Acid Solution (100×) (SIGMA), Sodium Pyruvate 1.5 mM (SIGMA), folic acid 14mM (SIGMA), L-Asparagine 0.3 mM (SIGMA), L-Arginine 0.7 mM (SIGMA), L-Glutamine 2 mM (SIGMA), Penicillin-streptomycin 100 U/ml, β-Mercaptoethanol 14.3 mM, inactivated FCS 10% and stimulated with plate-bound antibodies against CD3 (145-2C11, 2 mg/ml) and CD28 (PV-1, 2 mg/ml) (BioXcell) in the presence of IL-12 and anti-IL-4 (T_H1); TGF-β, IL-6, IL-23 and anti-IFNγ (T_H17). IL-6 (25 ng/ml), IL-12 (20 ng/ml), IL-23 (10 ng/ml) and TGF-β1 (2.5 ng/ml) were purchased from ebiosciences. Anti-IL-4 (clone 1B11) and anti-IFNγ (clone C17.8) (10 μg/ml) antibodies were purchased from BioXcell (West Lebanon, NH, USA).

2.11. In vitro migration assay

Migration assays were performed using 96-well transwell plate (Corning, 3488). *In vitro* polarized into T_H17 or T_H1 cell differentiated as described above were washed and resuspended at 1.5x106 cells/ml in DMEM 1% BSA. 75 μl of the suspension was added to a top chamber of the 96-well transwell plate. 7α25-OHC or 25-OHC (purchased at Avanti polar lipids) were prepared at the indicated concentration in a volume of 240 μl DMEM 1% BSA and added to the bottom chamber. Cells were incubated for 3 h at 37 °C. Migrated cells in the bottom chamber were enumerated by flow cytometry using a CyAn™ ADP Analyzer (Beckman Coulter). Migration index was expressed as the ratio of number of migrating cells to compound to number of migrating cells to medium only.

2.12. Human cell isolation

Peripheral blood mononucleated cell (PBMCs) were obtained by density gradient centrifugation of human peripheral blood from four healthy donors. For analysis of EBI2 expression on lymphocytes, cells were stained with antibodies against CD4, CD45RA and EBI2. EBI2 antibody was kindly provided by Novartis (clone mouse anti-human 57C9B5C9). For each sample, the geometric mean fluorescence intensity (MFI) of specific EBI2−positive cells was measured and compared to the isotype (mouse anti-human IgG2a) matched-control cells.

2.13. Statistical analysis

Statistical evaluations were performed with the unpaired Student's *t* test. EAE scores were evaluated with two-way ANOVA and Bonferroni's post-testing, and percentage of EAE free mice with log-rank Mantel−Cox test. Two-tailed *p*-values <0.05 were considered significant.

3. Results

3.1. Ch25h deficiency attenuates EAE disease scores

We investigated the role of Ch25h in EAE, a murine model of MS characterized by mononuclear cell inflammatory infiltrates and demyelination throughout the central nervous system (CNS) [20]. At steady state, we confirmed that mice deficient for Ch25h ($Ch25h^{-/-}$) have similar lipid profile (Supplementary Fig. 1A) and immune cell composition in blood, lymph nodes (Supplementary Fig. 1B and C) and spleen (data not shown) compared to wild-type mice as previously published [12]. We further immunized $Ch25h^{-/-}$ and wild-type mice with MOG_{35-55} peptide emulsified in CFA. $Ch25h^{-/-}$ mice displayed a less severe disease course and decreased cumulative disease scores as measured with the area under the curve (AUC) (Fig. 1A and Table 1). Furthermore they presented decreased weight loss compared to wild-type littermates (Fig. 1A). Moreover, $Ch25h^{-/-}$ mice showed a delay of 5 days for the disease onset compared to wild-type mice (Fig. 1A and Table 1). Indeed 100% of the wild-type but only 15% of $Ch25h^{-/-}$ mice exhibited EAE disease signs 16 days after immunization and 43% of $Ch25h^{-/-}$ mice still remained free of disease 20 days after immunization (Fig. 1A and Table 1). Overall, $Ch25h^{-/-}$ mice developed a milder disease with delayed onset.

While the disease severity was attenuated and its onset delayed in $Ch25h^{-/-}$ mice, the maximum score reached by sick $Ch25h^{-/-}$ mice was identical to the maximum scores of sick wild-type mice (Table 1). We further evaluated the extent of spinal cord infiltrates and demyelination in EAE diseased animals at the end of the experiment. No significant differences were observed between sick $Ch25h^{-/-}$ and sick wild-type mice with regard to the numbers of inflammatory lesions and to the extent of demyelination (LFB and PAS) staining 27 days after immunization (Fig. 1B). Non-diseased $Ch25h^{-/-}$ mice showed few inflammatory and demyelinated foci compared to sick mice of both genotypes (data not shown).

Aberrant oxysterol synthesis has been associated with neurodegenerative diseases such as Alzheimer's disease [21,22] and oxysterols, such as 7-ketocholesterol have cytotoxic potential on neurons [23]. To evaluate the possible role for neuroprotection in mice deficient for Ch25h, we assessed the respective contribution of both hematopoietic and non-hematopoietic cells using bone marrow (BM) chimeras. Wild-type mice reconstituted with $Ch25h^{-/-}$ BM (Ch25h → wild-type) but not $Ch25h^{-/-}$ mice reconstituted with wild-type BM (wild-type → Ch25h) showed a significant attenuated EAE disease course that was associated with

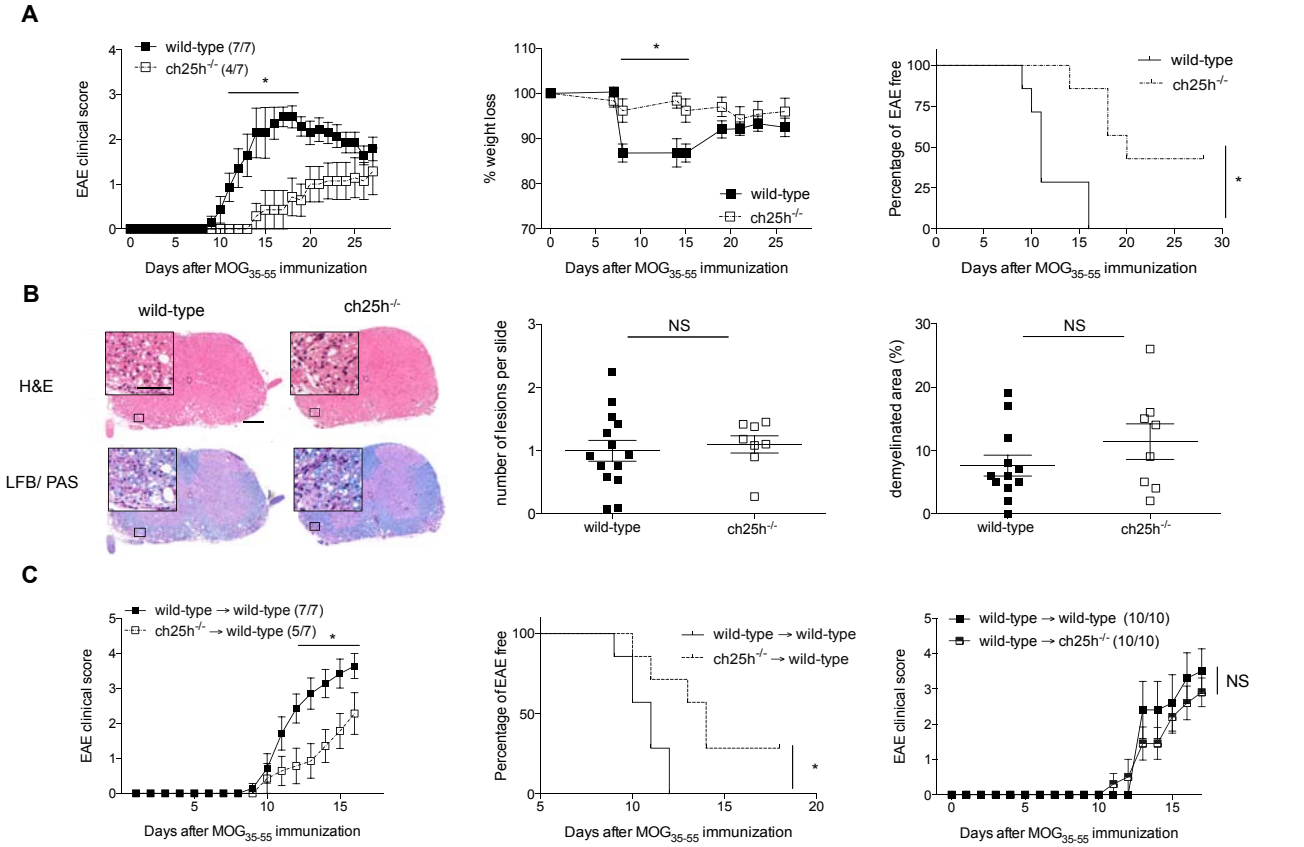


Fig. 1. Ch25h deletion attenuates disease course in EAE mice. (A) EAE was induced in Ch25h$^{-/-}$ mice and wild-type mice by immunization with MOG_{35-55} plus CFA, PTX was injected i.v. at day 0 and 2. EAE clinical scores shown as mean clinical score and percentage body weight loss (*$p < 0.001$ two-way ANOVA with Bonferroni's post-test) (±s.e.m., $n = 7$) were recorded over a period of 27 days. Percentage of EAE free mice from both strains (*p value < 0.001 analyzed with log-rank Mantel–Cox test). Data shown are representatives of at least three independent experiments. (B) Histopathological stainings and quantifications of spinal cord sections of diseased mice 27 days after immunization for cellular infiltration (H&E) and myelin (LFB and PAS); scale bars 200 μm in main image and 20 μm in inset. Shown are results from mice pooled from two independent experiments. (C) Sub-lethally irradiated wild-type mice received bone marrow transplantations from either genotype (wild-type → wild-type, Ch25h$^{-/-}$ → wild-type or wild-type → Ch25h$^{-/-}$, $n = 7$ to 10 in each group). After 8 weeks of reconstitution, EAE was induced. EAE clinical scores shown as mean clinical score and EAE disease free mice (±s.e.m. $n = 7$) were recorded.

decreased disease incidence (Fig. 1C) as compared to littermates reconstituted with wild-type BM (wild-type → wild-type). Ch25h$^{-/-}$ mice reconstituted with Ch25h$^{-/-}$ BM (Ch25h → Ch25h) showed significant attenuated EAE disease course as compared to littermates reconstituted with wild-type BM (wild-type → wild-type) (Supplementary Fig. 2). Those results indicate a contribution of the hematopoietic system and are not in favor of a neuroprotective effect in the absence of Ch25h.

3.2. Ch25h-deficient mice do not show altered systemic immune responses

We then questioned whether the above observations could be explained by altered immune cell activation in the periphery. We compared the antigen specific responses of wild-type and Ch25h$^{-/-}$ CD4$^+$ T cells to MOG_{35-55} peptide. For this, we immunized wild-type and Ch25h$^{-/-}$ mice with MOG_{35-55} peptide emulsified in CFA. Ten days after active immunization, lymphocytes from dLN and spleen of Ch25h$^{-/-}$ or wild-type mice were harvested and cultured with MOG peptide for 48 h *in vitro*. The repartition of leukocytes in both organs were comparable between Ch25h$^{-/-}$ and wild-type mice (data not shown). Deletion of Ch25h did not alter proliferation of peripheral MOG_{35-55}–specific T cells analyzed by [3H]thymidine incorporation after MOG_{35-55} immunization and recall stimulation with MOG_{35-55} peptide neither in dLN (Fig. 2A) nor in the spleen (Fig. 2B). Furthermore, the amounts of IL-17A, IFNγ and GM-CSF produced by MOG_{35-55}-activated CD4$^+$ T cells were equivalent in wild-type and Ch25h$^{-/-}$ mice (Fig. 2C). These data indicate that antigen-specific sensitization with MOG_{35-55} was not impaired in the absence of Ch25h. Furthermore, we found that the generation of IgG MOG specific antibodies was similar between wild-type and Ch25h$^{-/-}$ mice (Fig. 2D), indicating that B cell immune response is not defective in the absence of Ch25h$^{-/-}$ during EAE.

Table 1
EAE in wild-type and Ch25h$^{-/-}$ mice. AUC: area under the curve, EAE incidence, maximum scores and mean day of onset (±SD) are shown *$p < 0.05$ (Mann–Whitney test).

Group	Disease incidence	Maximum score	Mean day of onset	AUC
Wild-type	100% (7/7)	2.85 ± 0.24	12.17 ± 1.25	33.39 ± 4.74
Ch25h$^{-/-}$	57% (4/7)	2.25 ± 0.25	17.50 ± 1.26*	11 ± 4.76*

3.3. Ch25h deletion delays CNS inflammatory cell infiltration and impairs activated CD44$^+$CD4$^+$ T cell trafficking

Trafficking and infiltration of myelin antigen-specific T cells from the periphery into the CNS plays an important role in the

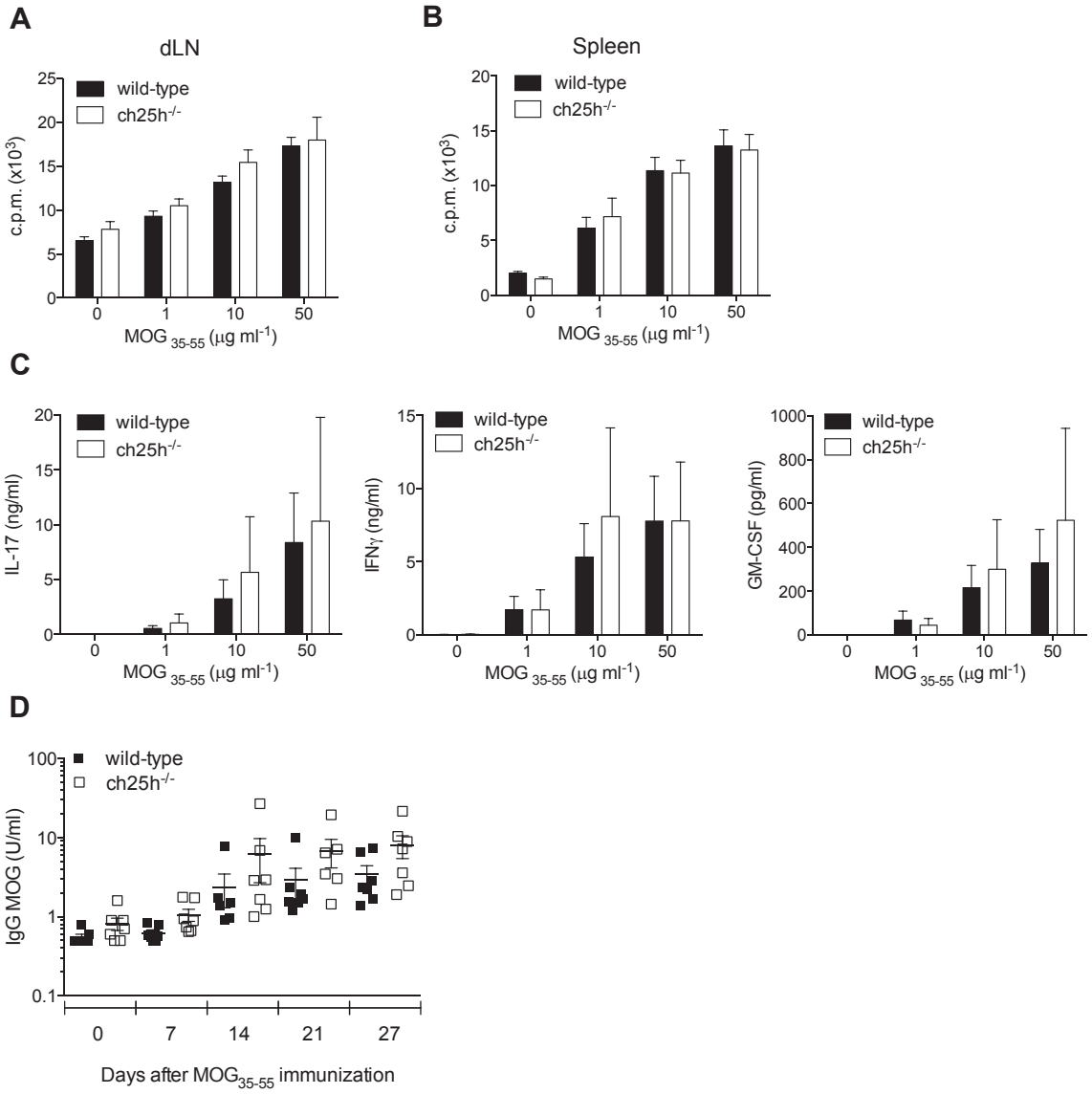


Fig. 2. Ch25h deficiency does not alter T or B cell subset ability to mount a systemic immune response. Ch25h$^{-/-}$ mice and wild-type littermates were immunized with MOG_{35-55} in CFA. (A and B) On day 12 after immunization, leucocytes extracted from the dLN and splenocytes were restimulated with MOG_{35-55} *in vitro*. After 48 h, the antigen-specific proliferative response was determined by ^{3}H-thymidine incorporation during 16 h (A and B, means + SEM, $n = 4$). (C) IL-17A, IFNγ and GM-CSF cytokines were measured by ELISA after 48 h of culture with the indicated concentration of MOG_{35-55}. (D) Serum levels of anti-MOG_{35-55} IgG antibodies were determined by ELISA in EAE wild-type or Ch25h$^{-/-}$ mice ($n = 7$) at given time after immunization. Each symbol represents an individual animal. Shown is one representative experiment of at least two independent experiments.

development and disease progression during EAE. Having established that peripheral immune response to MOG peptide was preserved in Ch25h$^{-/-}$ mice, we next asked if Ch25h could control T lymphocyte trafficking from the periphery to the CNS. This hypothesis is supported by the fact that 7,α25-OHC, the main metabolite downstream of Ch25h, has known chemotactic properties on immune cells. To characterize the relevance of Ch25h and to evaluate its impact on EAE disease onset, we dissected the dLN and the CNS (brain and spinal cord) at EAE disease onset when wild-type mice were sick but Ch25h$^{-/-}$ mice still disease free (Fig. 3A). While cell counts of lymphocytes in the dLN revealed similar or slightly increased cell counts in Ch25h$^{-/-}$ compared to wild-type mice (Fig. 3B), cell counts and flow cytometry analysis of CNS infiltrating mononuclear cells revealed significantly less numbers of CNS-infiltrating mononuclear cells as assessed by total cell counts and CD45^{hi} staining in the absence of Ch25h (Fig. 3C). Unimmunized mice did not show CNS cellular infiltrates (data not shown). The percentages of CD3^{+}CD4^{+} T cells in the dLN and infiltrating CNS were similar between wild-type and Ch25h$^{-/-}$ mice (Fig. 3D and E). However CD3^{+}CD4^{+} T cells from the dLN of Ch25h$^{-/-}$ mice showed a significant increase in CD44^{+} expression while Ch25h$^{-/-}$ CD3^{+}CD4^{+} T cells from the CNS depicted a decreased in CD44^{+} expression compared to wild-type (Fig. 3D and E). Other cell subsets both in percentage and absolute number were similar in the dLN of Ch25h$^{-/-}$ mice compared to wild-type (Supplementary Fig. 3A). We did not observe differences in percentage in other cell subsets of the CNS infiltrate despite reduction in absolute numbers of all cell subsets (Supplementary Fig. 3B). At the peak of EAE disease, when CNS cell infiltrates were compared at different time points but at similar EAE disease scores, nearly 100%

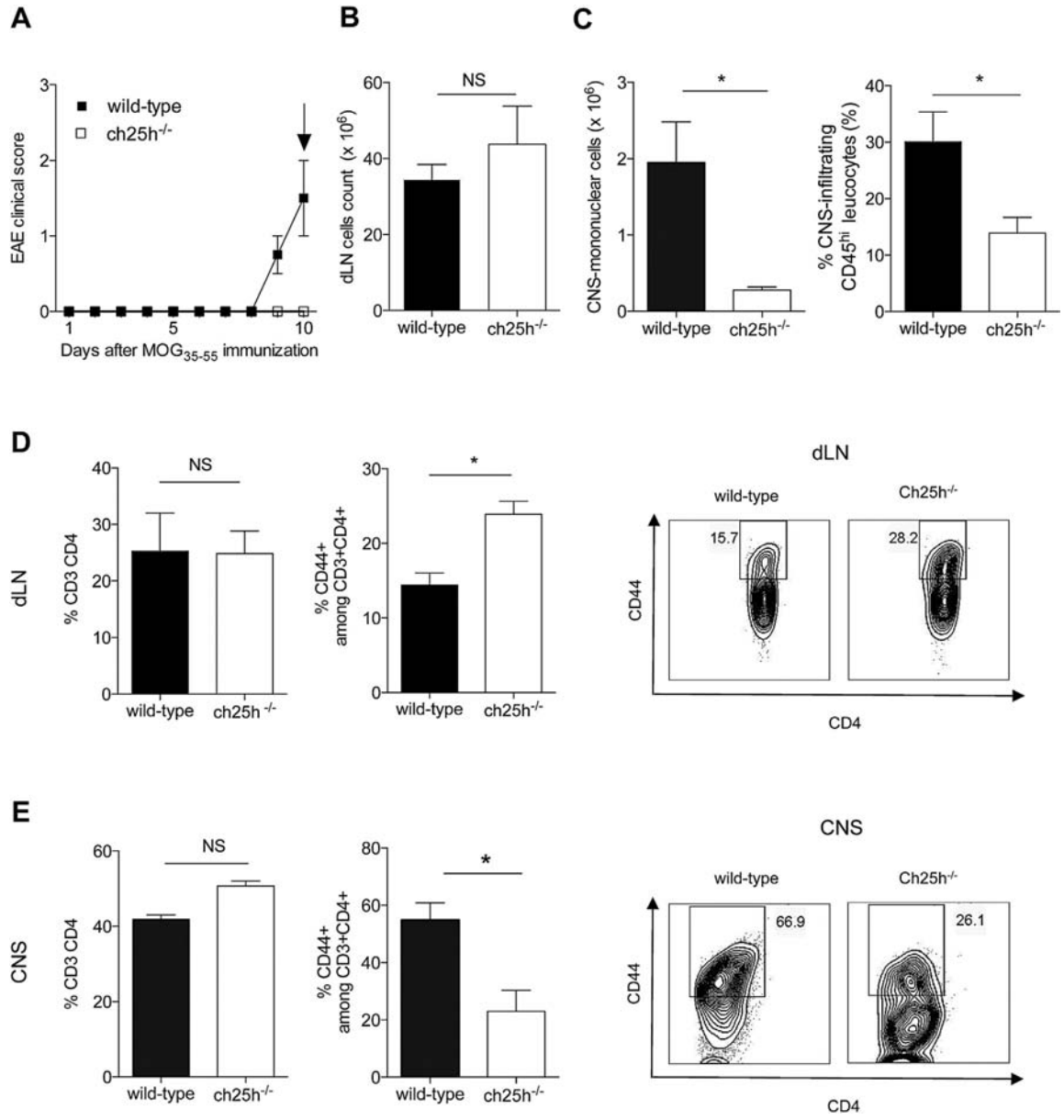


Fig. 3. $CD4^+CD44^+$ T cells accumulate in the dLN and depict a delayed CNS infiltration in the absence of Ch25h. Wild-type and $Ch25h^{-/-}$ mice were immunized with MOG_{35-55} plus CFA. (A) Clinical EAE scores were recorded, dLN and CNS cells were obtained at the initiation of the EAE disease as the time indicated with the arrow (B) Total count of lymphocytes in the dLN of wild-type and $Ch25h^{-/-}$ mice (C) Total count of infiltrative mononuclear cells and of $CD45^{hi}$ cells CNS-infiltrating in MOG_{35-55}-immunized wild-type versus $Ch25h^{-/-}$ EAE mice as assessed by flow cytometric analysis. Fraction profile of lymphocytes from (D) dLN and (E) CNS-infiltrating $CD3^+CD4^+$ T cells, $CD4^+CD44^+$ and representative flow cytometric analysis showing the percentage of $CD3^+CD4^+$ T cells expressing $CD44^+$. Data shown are representatives of two independent experiments.

of $CD4^+$ cells were expressing $CD44^+$ both in wild-type and $Ch25h^{-/-}$ mice (data not shown), emphasizing a delayed recruitment of activated T cells in the CNS at the disease initiation. Recruitment of encephalitogenic $CD4^+CD44^+$ T cells into the CNS is crucial to EAE development. Our results suggested that the delayed disease course observed in $Ch25h^{-/-}$ mice is related to the accumulation of $CD44^+$ T cells in the periphery and retarded pathogenic lymphocytes infiltration into the CNS.

3.4. Activated IL-17A-producing $CD4^+$ T cells accumulate in the dLN

We then assessed by flow cytometry intracellular pro-inflammatory IL-17A and IFNγ cytokines secretion directly *ex-vivo* in $CD4^+$ T cells obtained from the CNS and dLN after stimulation with PMA-ionomycin for 4 h. We focused our attention on pro-inflammatory cytokines IL-17A and IFNγ that are produced by $CD4^+$ T cells during autoimmunity. While absolute numbers of IL-17-producing T cells are decreased in the CNS of $Ch25h^{-/-}$ mice, they are increased in the dLN of the same mice (Fig. 4A). $Ch25h^{-/-}$ $CD4^+$ T cells showed a higher percentage of IL-17A compared to wild-type mice (5.6% versus 2.9%) (Fig. 4B) while the percentage of IFNγ producing $CD4^+$ T cells were not significantly different between the two strains. To confirm those results, RNA was isolated from the dLN and the expression of Il17a as well as the T_H17 "master-regulator" transcription factor nuclear receptor ROR-γt (Rorc) was assessed by real-time PCR analysis. In concordance with the above

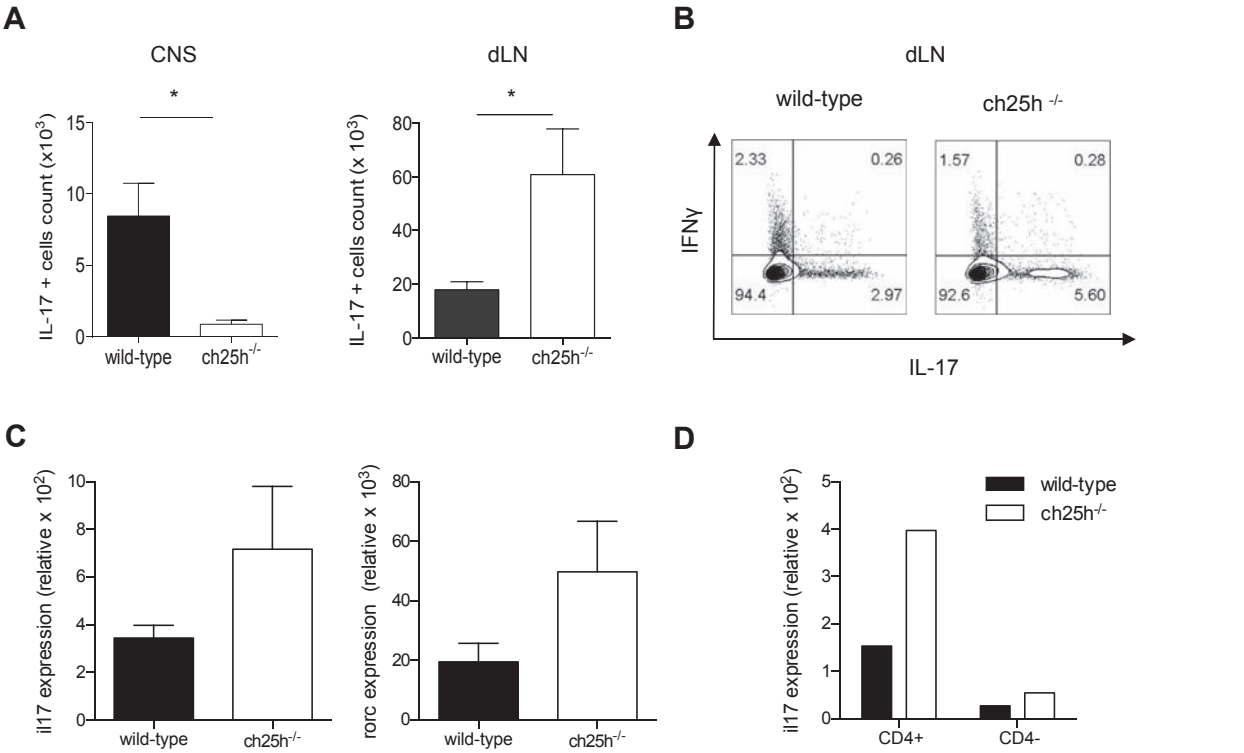


Fig. 4. Altered $CD4^+$ T cell subset distribution within CNS and dLN in the absence of Ch25h. Wild-type or $Ch25h^{-/-}$ littermates were immunized with MOG_{35-55} in CFA. (A) Absolute number of IL-17A-producing $CD4^+$ T cells quantified from flow cytometry analysis from CNS and dLN. (B) Representative Flow cytometry profile for intracellular cytokine IFNγ and IL-17A secretion in $CD4^+$ T cells at day 10 obtained from mononuclear cells isolated dLN and activated with PMA-ionomycin (C) Total RNA was extracted from the dLN and RT-PCR for Il17a and Rorc expression (relative to actin) was performed. (D) RT-PCR for expression of Il17a on MACS-sorted $CD4^+$ and $CD4^-$ cells. Shown are representatives of three independent experiments.

results, IL17a and Rorc expression were higher in the dLN of $Ch25^{-/-}$ mice compared to wild-type mice (Fig. 4C). To confirm that IL17a was expressed by $CD4^+$ T cells, we isolated $CD4^+$ T cells and non $CD4^+$ T cells by MACS purification from dLN of immunized mice and compared IL17a expression in these cell fractions. IL17a expression was detected in $CD4^+$ T cells but not in $CD4^-$ cells and higher IL17a expression was measured in $Ch25h^{-/-}$ compared to wild-type $CD4^+$ T cells (Fig. 4D). In our model, we observed a delayed EAE onset in $Ch25h^{-/-}$ mice that correlates with an accumulation of T_H17 cells within the dLN at the early stage of EAE disease.

3.5. 7α,25-OHC promotes $CD4^+$ T lymphocytes trafficking through EBI2 signaling

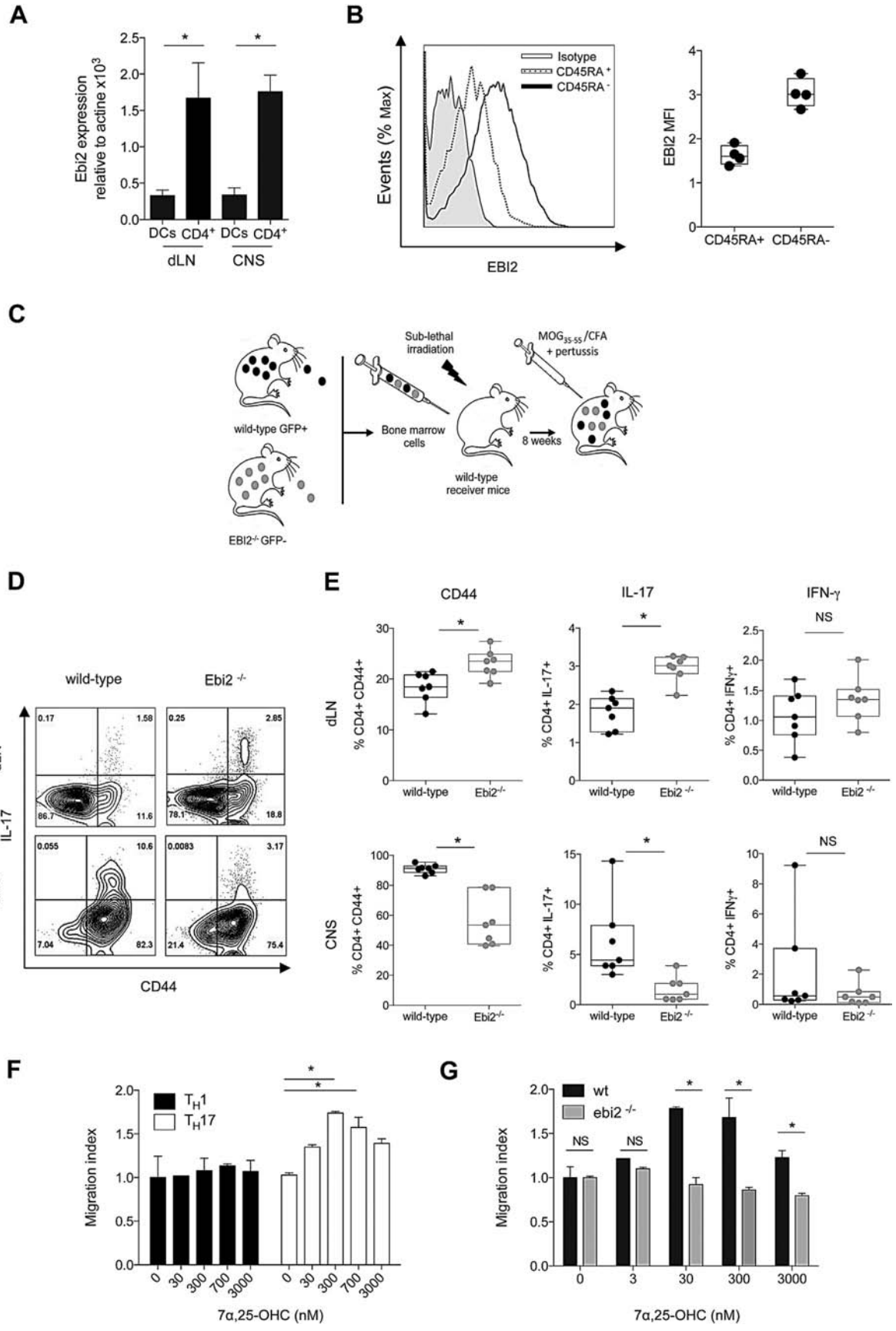
As the loss of Ch25h led to an accumulation of IL-17A-producing T cells in the dLN of MOG-immunized mice, we further asked whether oxysterol could impact $CD4^+$ T cell chemotaxis. 25-OHC is synthesized from cholesterol by the enzyme Ch25h and further converted into 7α,25-OHC by the enzyme Cyp7b1. 7α,25-OHC is a strong ligand for the G-protein coupled receptor Epstein–Barr virus-gene 2 (EBI2) [16,24] that drives B lymphocytes [13] and macrophages migration [25].

We first investigated the role of EBI2 on T cell trafficking properties *in vivo* in the EAE mouse model and examined EBI2 expression on immune cells during EAE. RNA was obtained from $CD4^+$ T lymphocytes and dendritic cells (DCs) sorted by flow cytometry from the dLN and CNS 13 days after immunization with MOG_{35-55}. EBI2 expression was assessed by real-time PCR. EBI2 was highly expressed by $CD4^+$ lymphocytes compared to DCs in both organs during EAE (Fig. 5A). Those results suggested a possible role for oxysterols in driving $CD4^+$ T cells migration into inflamed tissues.

To further assess if EBI2 may play a role in human $CD4^+$ T lymphocytes, we tested EBI2 expression on PBMC of healthy donors by flow cytometry. We found that EBI2 was expressed on $CD4^+$ T lymphocytes at a higher level in memory $CD45RA^-$ $CD4^+$ T cells (the equivalent of $CD44^+CD4^+$ in mouse) compared to $CD45RA^+CD4^+$ T cells (naïve T cells) (Fig. 5B). Those results suggest that the Ch25h-EBI2 pathway impacts $CD4^+$ T lymphocytes biology in mouse and in human.

To further test the role of EBI2 in driving T cells migration, we generated co-chimeric mice reconstituted with 50% BM from wild-type Ubiquitin-GFP^+ mice and 50% BM from $EBI2^{-/-}$ mice. After reconstitution, mice were immunized with MOG_{35-55} peptide (Fig. 5C). At the first EAE disease signs, mice were sacrificed and cells from the dLN and CNS harvested. Expression of CD44, IL-17A and IFNγ in $CD4^+$ T cells from both wild-type (GFP^+ cells) or EBI2-deficient cells (GFP^- cells) was assessed by flow-cytometry. We observed a significantly increased proportion of both $CD44^+CD4^+$ T cells and IL-$17A^+CD4^+$ T cells in the dLN and reduced proportion of both populations in the CNS from $EBI2^{-/-}$ cells compared to wild-type cells (Fig. 5D and E). IL-17A is secreted exclusively by $CD44^+CD4^+$ T cells (Fig. 5D). There was no statistical difference in IFNγ producing T cells neither in the dLN nor in the CNS between GFP^+ and GFP^- population (Fig. 5E). Collectively, these results indicate that 7α,25-OHC preferential induced recruitment of $CD44^+CD4^+$ T cells that secrete the pro-inflammatory cytokine IL-17A via EBI2 signaling.

Although it has been proposed that oxysterols impact T lymphocyte migration [24], the chemotactic role of oxysterols has not been addressed in $CD4^+$ T subsets. We further tested the migrational properties of $CD4^+$ T cells toward 7α,25-OHC *in vitro* using a transwell assay. As we observed an accumulation of T_H17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Ebi2 expression relative to actine x10^3
DCs CD4+ DCs CD4+
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C
wild-type GFP+
EBI2-/- GFP-
Bone marrow cells
Sub-lethal irradiation
MOG35-55/CFA + pertussis
8 weeks
wild-type receiver mice
D
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Ebi2 -/-
dLN
CNS
IL-17
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E
CD44
IL-17
IFN-γ
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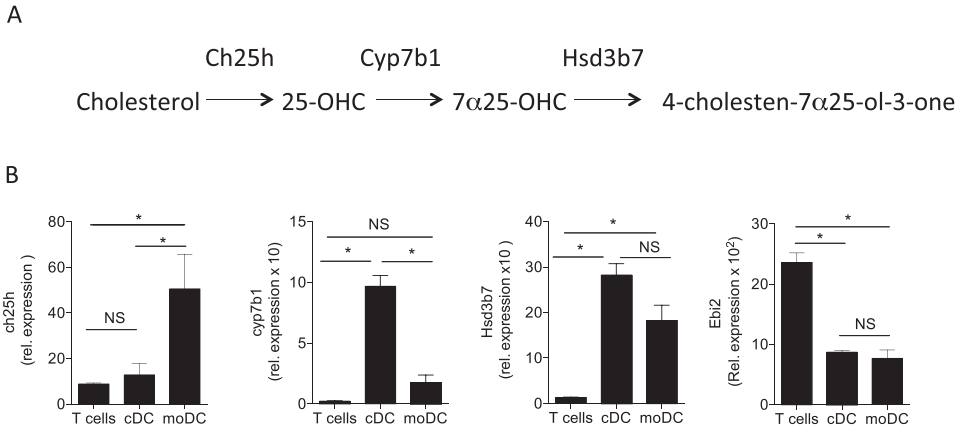

Fig. 6. MoDCs express ch25h *in vivo* (A) schematic representation of oxysterol pathway (B) Wild-type mice were immunized with MOG_{35-55} in CFA. After 8 days, dLN were dissected and T cells ($CD45^{hi}CD3^{+}CD4^{+}$), MoDCs ($CD45^{hi}CD3^{-}CD11c^{int}CD11b^{+}Ly6C^{+}MHCII^{+}$) and cDCs ($CD45^{hi}CD11c^{hi}CD11b^{-}ly6C^{-}MHCII^{+}$) were purified by FACS-sorting. RNA was extracted and Ch25h, Cyp7b1, Hsd3b7 and EBI2 expression were assessed by real-time PCR.

but not T_H1 cells in the dLN during EAE, we tested the capacities of T_H17 and T_H1 cells to migrate towards 7α,25-OHC. Naive T cells were purified from the peripheral immune compartment of wild-type mice and differentiated into T_H1 or T_H17 cells *in vitro*. After 3 days of culture, T_H1 and T_H17 cells were tested for their properties to migrate toward 7α,25-OHC. T_H17 but to a lesser extent T_H1 cells migrated toward 7α,25-OHC (Fig. 5F). T_H17 cells migrated toward 7α,25-OHC but not toward Cholesterol nor 25-OHC (Supplementary Fig. 4A). 7α,25-OHC binds the receptor EBI2 with high affinity. Therefore, we differentiated T_H17 cells from either wild-type or $EBI2^{-/-}$ mice. T_H17 cells differentiated from wild-type or $EBI2^{-/-}$ mice showed no defects during differentiation and produced similar level of IL-17A compared to wild-type cells (Supplementary Fig. 4B). However, T_H17 cells deficient for EBI2 could not migrate toward 7α,25-OHC (Fig. 5G). $Ch25h^{-/-}$ deficient T_H17 did not show defect in migration towards 7α,25-OHC (Supplementary Fig. 4C). These data support the idea that 7α,25-OHC preferentially drives activated IL-17A-producing $CD4^{+}$ T cell trafficking by binding EBI2.

3.6. Monocyte-derived DCs express ch25h during EAE

We finally asked which cell express Ch25h and promote 7α,25-OHC production during EAE. Cholesterol is converted to 25-OHC and then to 7α,25-OHC by the enzymes Ch25h and Cyp7b1 respectively [13]. 7α,25-OHC is degraded by the enzyme Hsd3b7 (Fig. 6A). Ch25h, Cyp7b1 and Hsd3b7 have been shown to be expressed by different cell subsets [13] and Ch25h to be induced under inflammatory conditions in macrophages and dendritic cells [26]. Moreover monocyte-derived DCs (moDCs), characterized by the markers $CD11b^{+}Ly6C^{+}MHCII^{+}CD11c^{int}$ but not conventional DCs (cDCs: $CD11b^{+/-}Ly6C^{-}MHCII^{+}CD11c^{high}$) have been proposed to be critical in T_H17-mediated diseases [19]. MoDCs are not detected under steady state condition but are seen in the dLN 8 days after $MOG\text{-}_{35-55}$ immunization. They are critical to mediate inflammation [19]. As MoDCs are derived from the monocytes lineage, we hypothesized that they may express high levels of Ch25h. To test our hypothesis, we isolated by FACS sorting moDCs, cDCs and $CD4^{+}$ T cells from the dLN 8 days after MOG_{35-55} immunization (Supplementary Fig. 5A) and assessed Ch25h, Cyp7b1, Hsd3b7 and EBI2 expression by real-time PCR in the different cell subsets. MoDCs were expressing the highest level of Ch25h compared to T cells and cDCs. The enzyme Cyp7b1 that was solely expressed by cDCs, and Hsd3b7 was expressed both by MoDCs and cDCs. EBI2 was merely expressed in $CD4^{+}$ T lymphocytes but not in MoDCs nor cDCs (Fig. 6B). Recently it has been proposed that, during septic shock, 25-OHC inhibited IL-1β production, a key cytokine involved with IL-23 in T_H17 cell generation [27]. While IL-1β and IL-23 were produced by MoDCs during EAE, $Ch25h^{-/-}$ moDCs expressed similar levels of IL-1β and IL-23 expression compared to wild-type moDCs (Supplementary Fig. 5B) suggesting that $Ch25h^{-/-}$ moDCs do not impair T_H17 cell differentiation.

4. Discussion

Oxysterols have been recently ascribed new roles in modulating the immune response, however, their role in autoimmunity has not been assessed. Our studies indicate that Ch25h-induced oxysterols control the immune response by promoting encephalitogenic T cells trafficking to the CNS during EAE and thus enhancing a pro-inflammatory response. This is reminiscent of recent commercialized drugs used to treat MS such as the monoclonal antibody Natalizumab that inhibits leucocytes migration to the CNS or the drug FTY-720 that controls $CD4^{+}$ T cell egression from the peripheral LN.

Cholesterol homeostasis is essential for the CNS and most of brain cholesterol is synthesized endogenously [28]. Oxysterols are lipophilic metabolites that can cross the blood–brain-barrier and mediate cholesterol elimination [29]. Aberrant oxysterol synthesis

Fig. 5. 7α,25-OHC controls T_H17 cell migration in an EBI2-dependant manner (A) EBI2 expression assessed by real-time PCR from FACS sorted $CD3^{+}CD4^{+}$ or DCs (pooled cDCs and MoDCs) extracted from the dLN and CNS 13 days after MOG_{35-55} immunization. (B) Flow cytometry for EBI2 expression performed on human PBMC and gated on $CD4^{+}$ T Lymphocytes that were either $CD45RA^{-}$ (memory) or $CD54RA^{+}$ (naïve). Data show representative histogram overlays of isotype (filled histogram) and EBI2–stained cells gated on $CD4^{+}CD45RA^{-}$ (memory T cells) and $CD4^{+}CD45RA^{+}$ (naïve T cells). Differences in MFI median levels were examined by a two-way Anova test $^{*}p < 0.05$. (C) Co-chimeric mice for wild-type and $EBI2^{-/-}$ were generated. After reconstitution, mice were immunized with MOG_{35-55}. At the first sign of disease, mice were killed and dLN and CNS tissue extracted and analyzed by flow cytometry. Percentages of different populations were assessed by flow cytometry. (D) Representative staining of CD44 and IL-17A expression gated on both GFP^{+} (wild-type) or GFP^{-} ($EBI2^{-/-}$) $CD4^{+}$ T cells isolated from dLN and CNS of cells. (E) Fraction profile showing the percentage of $CD4^{+}CD44^{+}$, $IL\text{-}17A^{+}CD4^{+}$ and $IFN\gamma^{+}CD4^{+}$ T cell populations. (F) Naive $CD4^{+}$ T cells were isolated using MACS purification and further differentiated into T_H1 or T_H17 cells *in vitro*. After 3 days of culture, T_H1 and T_H17 cells were tested for migration toward 7α,25-OHC using a transwell assay. (G) T_H17 cells from wild-type or $EBI2^{-/-}$ mice were differentiated *in vitro* and tested for migration.

has been associated with neurodegenerative diseases and Ch25h polymorphism is linked to Alzheimer's disease [21,22]. In addition, oxysterols, such as 7-ketocholesterol have been proposed to have a cytotoxic potential on neuronal cells [23]. Using chimeric mice, we demonstrated that in our model oxysterols do not promote neurodegeneration during EAE. Indeed, our results attribute a pro-inflammatory function to Ch25h-induced oxysterols during autoimmune diseases. Our findings are in line with recent publications that ascribed 25-OHC with a pro-inflammatory role in atherosclerosis [30,31]. Furthermore, in addition to driving antiviral properties [14,15], Ch25h pathway can amplify inflammatory signaling and increase inflammation during viral infections [30]. Recently, 25-OHC has been proposed to exert anti-inflammatory functions in a septic shock animal model by dampening IL-1β signaling [27]. In line with our results, a dichotomy between IL-17-producing cells and IFNγ-producing cells was observed. However, in the aforementioned study, 25-OHC was suggested to exert a protective function during EAE. Further studies are therefore needed to dissect the underlying mechanisms explaining the apparently divergent findings of Ch25h during neuroinflammation.

Our data furthermore suggest that Ch25h-induced oxysterols could play an important role in modulating the immune cell migration. Mechanistically, we observed impaired trafficking of $CD44^+CD4^+$ T cells in mice deficient for Ch25h. $CD44^+CD4^+$ T cells lacking EBI2, the receptor of 7α-25OHC, were delayed in reaching the CNS during EAE. CD44 molecule is a marker of memory T cells in mice and is expressed on antigen-activated T cells. CD44 is further involved in the homing of primed lymphocytes to the CNS [32]. Recruitment of encephalitogenic $CD4^+CD44^+$ T cells into the CNS is crucial for EAE development. A meta-analysis of EBI2 expression by real-time PCR on human peripheral blood cells suggested that EBI2 is expressed on $CD4^+$ T lymphocytes at higher level in memory $CD45RA^-$ $CD4^+$ T cells (the equivalent of $CD44^+CD4^+$ in mouse) compared to naïve T cells [16]. We confirmed that EBI2 expression is higher on human memory $CD4^+$ T cells compared to naïve $CD4^+$ T cells at the protein level. This suggests an important role for EBI2 on $CD4^+$ T cells migration not only in murine but also in human T lymphocytes.

IL-17A producing T cells participate in the induction of EAE [33] [34]. IL-17A expression reaches its highest CNS level at disease initiation while it decreases at the peak of disease. On the other hand IFNγ achieves its highest value at the peak of disease [35]. Lymphocytes that produce IL-17A in the brain are expressing the activation marker $CD44^+$. Those data suggest that activated $CD44^+$ T cells that produce IL-17A reach the CNS at an early stage of the disease. In our model, we observed a delayed EAE onset in $Ch25h^{-/-}$ mice, which correlates with an accumulation of IL-17A producing $CD4^+$ T cells within the dLN at the early stage of EAE disease. Those results are in accordance with recent studies showing that T_H1 and T_H17 cells harbor distinct migratory properties that drive their recruitment into the CNS [36]. We propose that different chemotactic responses to oxysterols could be considered as a new molecular mechanism distinguishing $CD4^+$ T cells subset in regards to their migratory properties.

Finally, we show that MoDCs are a rich source of Ch25h during EAE, corroborating previous notions of Ch25h expression in monocytes and macrophages [12]. MoDCs are detected under inflammatory states and reach the dLN during EAE [19]. As MoDCs can drive T_H17 cells differentiation *in situ* by secretion of IL-1β, we assessed the expression of IL-1β and IL-23 from MoDCs of both wild-type and $Ch25h^{-/-}$ but we did not observe any differences in both cytokines expression. This suggests that oxysterols produced by MoDCs impact T_H17 cell migration rather than their differentiation. MoDCs did not express Cyp7b1, the enzyme converting 25-OHC into the high-affinity ligand for EBI2, 7-α25-OHC. Moreover, we observed that cDCs, which are constitutively detected in the LN [19], express Cyp7b1. Thus, we propose that cDCs are responsible for the conversion of 25-OHC into 7α,25-OHC. This is in line with other reports which showed that Ch25h and Cyp7b1 enzymes were not expressed by the same cells during immune response [13].

5. Conclusion

We here describe for the first time a role for oxidized cholesterol metabolites, oxysterols, in promoting autoimmunity. Our data show that Ch25h-induced oxysterols drive a pro-inflammatory response during EAE by promoting encephalitogenic $CD4^+$ T cells trafficking to the CNS. Impaired activated lymphocytes trafficking in the absence the oxysterol receptor EBI2 further results in delayed inflammatory cells recruitment into the CNS during EAE. The discovery of oxysterols as new mediators of different subsets of $CD4^+$ T cell trafficking during EAE may lead to new targets to harness encephalitogenic immune cells not only during multiple sclerosis but also in other autoimmune diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2014.10.001.

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4. FINAL CONCLUSION AND PERSPECTIVES

MS is a heterogeneous disease which development is under the control of both genetic and environmental factors. Among environmental factors that contribute to the development of MS, exposure to smoking or toxins have been identified. The evolution and progression of MS and its animal model EAE results from the balance between pathogenicity of effector cells and the negative regulation imposed by regulatory cells. As we were studying the signaling pathways of the cytokine IL-27, that drives the generation of regulatory T cells, we identified factors known for their link with environmental pollutant such as AhR with dioxin or MTs with heavy metals. Those discoveries further oriented my research toward immunometabolism studies, in particular on assessing the role of lipid metabolites on autoimmunity. I am currently pursuing my research on MS to evaluate the putative link between nutrition, lipid metabolism, gut immunology, including microbiota analysis and neuroinflammation.

The major focus of my research exposed in the first part of my thesis was based on the study of the cytokine IL-27. We conducted functional analysis and characterization of IL-27-induced type I regulatory T cells (T_R1) in order to analyze their role in autoimmune diseases, principally in EAE. The initial analysis revealed that IL-27 is a potent inducer of three essential elements: the transcription factor c-Maf, cytokine IL-21 and co-stimulatory receptor ICOS. We further demonstrated that those elements coordinately work together to promote differentiation of T_R1 cells. The depletion of any of those components indeed impairs the development of T_R1 cells. Another IL-27 induced gene detected was the ligand–activated transcription factor AhR. AhR controls $Foxp3^+$Treg and T_H17 cell differentiation and a single injection of TCDD, an agonist to AhR, protects against EAE (Quintana et al., 2008). We further showed that the AhR agonists TCDD or FICZ could also potentiate IL-27-induced T_R1 cells *in vitro* by

increasing IL-10 production. Under T_R1-skewing conditions, AhR binds to c-Maf and promotes the transactivation of both *il10* and *il21* promoters, resulting in the generation of T_R1 cells. Finally, AhR controls the IL-10-dependent immunosuppressive activity of IL-27 in an *in vivo* adoptive transfer model of EAE. Together, those findings suggest that the manipulation of AhR signaling could be beneficial in the resolution of excessive inflammatory responses (Apetoh, 2010).

AhR has been studied for many decades in toxicology as the ligand for the environmental contaminant dioxin. Exposure to high levels of dioxins causes chloracne, a severe skin disease and damages to liver and to the nervous system. Environmental toxins modulate immune responses and can both impair response to infection or trigger autoimmunity. AhR has emerged as a critical regulator of the immune system. Besides environmental contaminants, natural AhR ligands are present in the diet or can be generated in the gastrointestinal tract from dietary compounds such as indirubin, a component in a traditional Chinese herbal medicine (Adachi et al., 2001; Wang et al., 2008), curcumin, a common spice used in Indian cuisine or indole-3-carbinol found in cruciferous vegetables. Interests in natural AhR ligands and gut immunology are rising. A recent study pointed out that exposure to AhR ligands through the diet already in the first weeks of life is critical for the development of immune responses as they control the maturation of innate lymphoid cells (ILC) (Kiss et al., 2011). ILC drive immune responses against intestinal infections and their generation is impaired in AhR-deficient mice. Mice fed with diets lacking natural AhR ligands suffer from deficient ILC generation and are prone to intestinal infection. The sole addition of the natural AhR ligand indole-3-carbinol in the diet restores both the generation of ILC and the immune response in an AhR-dependent manner. This highlights the importance of exposure to

AhR agonists through food intake and their role in the maintenance of intestinal homeostasis. To corroborate those results, the group of L. Zhou similarly showed that AhR is necessary for the development of a subset of ILC (that are RORgt^{+}) in mice (Qiu et al., 2012). Adult AhR-deficient mice have reduced numbers of ILC in both small and large intestine and are more susceptible to gut infections. Strikingly the deficits in ILC became evident when AhR-deficient mice were close to weaning age pointing toward the implication of exogenous factors such as food intake in the development of the immune response. The authors proposed that IL-22 production induced by AhR signaling is an important mediator for AhR protective effects on the gut as IL-22 expression could rescue AhR-deficient mice from succumbing to severe gut infection. In addition, AhR drives the development of ICL22 and postnatal lymphoid tissues that are critical for responding to gut infections (Lee et al., 2011a). AhR signaling could then regulate IL-22 partially through Notch pathways. Similarly, intraepithelial lymphocytes (IELs), that are important for the first line defense against intestinal infection or skin defense, depend on AhR activation by dietary-derived ligands to maintain their generation and to control microbial load and composition in the gut (Li et al., 2011). Taken together, the results of those studies suggest that AhR signaling is important for the maintance of gut immunity and further that exogeneous dietary AhR ligands, are crucial to shape our immune response.

In another study, we identified MTs as negative regulators of IL-27-induced T_R1 cells. Environmental stressors as well as tumor-promoting agents have been shown to induce MTs at sites of inflammation and in breast and lung cancers (McGee et al.). MTs induce splenocytes proliferation, act synergistically with T cell- and B cell-specific mitogens (Lynes et al., 1990) and enhance the capacity of naive B lymphocytes to differentiate

into plasma cells (Borghesi et al., 1996). In contrast, MTs decrease humoral immune responses (Crowthers et al., 2000; Lynes et al., 1993) and suppress the function of cytotoxic T lymphocytes, suggesting that MTs can differentially affect immune responses depending on the environmental context (Youn and Lynes, 1999). This contention is further supported by the ability of MTs to interact with the transcription factor NF-kB, which shapes immunity to infections and tumors (Abdel-Mageed and Agrawal, 1998). MTs were reported to mediate neuroprotection during pathological conditions by preventing tissue damage due to oxidative stress (Pedersen et al., 2009). We have unraveled an unexpected function for MTs in the differentiation of IL-27-induced T_R1 cells. Interestingly MTs, first described to bind heavy metals and to play a role in detoxification, are also recognized as binding essential metal such as Zinc. Zinc modulates the immune response through its availability, which is regulated by several transporters including MTs. If zinc availability is reduced or if its transport is impaired, altered differentiation and proliferation of cells in particular, immune cells, have been reported (reviewed in (Bonaventura et al., 2015). Metal metabolism, in particular zinc, may participate to the effects of MTs observed on T_R1 cells. This question would require additional studies to better characterize the role of metals during autoimmunity.

Epidemiological data show a link between obesity during childhood and increased risk of developing MS (Langer-Gould et al., 2013; Munger et al., 2009). Dysregulation of lipid metabolism is observed during obesity; furthermore cholesterol metabolites, among which oxysterols, are altered in animal models for obesity (Guillemot-Legris et al., 2016) and in obese patients (Tremblay-Franco et al., 2015). Although cholesterol and markers of its turnover are associated with changes in MS disease outcome, the underlying mechanisms are still largely disputed (Zhornitsky et al., 2016). Obesity

further promotes low-grade inflammation and increases pro-inflammatory cytokines production, such as IL-6 that drives the generation of pathogenic lymphocytes, such as IL-17-producing CD4+ T helper (T_H17) cells. The balance between pro-inflammatory CD4+ T cells (T_H1 or T_H17) and the negative regulation imposed by regulatory T cells (Foxp3+ Tregs or type-regulatory T (T_R1) cells) largely contribute to the development of EAE and MS. Physiologically T_H17 cells first serve in mucosal host defense before promoting autoimmunity and a substantial proportion of T_H17 cells are found in the lamina propria of the intestine (Atarashi et al., 2008). However how oxysterols modulate immunity was until recently largely unknown. We therefore focused our research on the metabolic pathway of the enzyme cholesterol 25 hydroxylase (Ch25h) that convert cholesterol into 25-hydroxycholesterol (25-OHC). We discovered that Ch25h pathway contribute to the inflammatory immune response by controlling T_H17 cell migration during autoimmune diseases (Chalmin et al., 2015). Altogether our results points toward new roles for 25-OHC during adaptive immune response. We are currently assessing the role of oxysterols and their receptors on biological samples obtained from MS patients. We think it is of great interest to translate the mouse EAE results in the human setting, as we believe that oxysterols control both immune cell migration and transcriptional signaling, which may be altered in MS patients.

To further study the impact of lipid metabolism and its links to environmental factors such as alimentary habits, we plan to assess the interaction between gut immunity, microbiota and lipid metabolism. Indeed, obesity impacts the composition of gut flora, which has been recognized as critical in the development of inflammatory diseases (Hooper et al., 2012). Obese versus lean humans are colonized by different gut flora (Ley et al., 2006; Turnbaugh et al., 2006) and genetically obese (ob/ob) mice display

altered gut flora compared with lean controls (Ley et al., 2005). Gut flora has a potential activity of its own to transfer disease as obese gut microbiota flora can cause lean mice to gain weight (Turnbaugh et al., 2006). Likewise modulating commensal microbiota can directly impact EAE development (Berer et al., 2011; Lee et al., 2011b). For example, mouse segmented filamentous bacteria (SFBs) can drive T_H17 cell generation in the digestive track (Ivanov et al., 2009) and encephalitogenic T_H17 cells can be generated in the gut with SFBs monocolonization (Lee et al., 2011b). Similarly, transfer of gut microbiota can restore EAE disease susceptibility in mice that are resistant to EAE development (Berer et al., 2011). As future perspectives, we will examine the role of oxysterols on gut immunity during EAE as we hypothesize that oxysterols will enhance the pathogenicity of T_H17 cells within the gut.

On the long run, I plan to delineate the implication of cholesterol metabolism, diet, gut immunology and microbiota that are with no doubts implicated in MS development. I believe that carrying out this research is needed for scientific reasons exposed above but also for socio-economic reasons. Indeed, given the potential importance of our results for human health, our research work may contribute to development of novel therapeutic protocols and to a scientific-based reevaluation of dietary approaches in our daily fight against MS and other autoimmune diseases.

Our findings may further be translated to other autoimmune diseases. AhR signaling pathway participate in addition to EAE to the development of several experimental models of autoimmune diseases, such as colitis, chronic obstructive pulmonary inflammation, type 1 diabetes or skin inflammation (reviewed in a dedicated issue of Seminars in Immunopathology (Pot et al., 2013). However, the role of AHR in

modulating autoimmune disorders in humans and in particular in dioxin-exposed people is still largely unknown. Research in this direction should be followed in the future. Similarly obesity drives several autoimmune disorders in addition to MS both on murine models and in human diseases (Versini et al., 2014). It is of interest to highlight that obesity and autoimmune diseases have both experienced a dramatic increase in Western countries. While the link between those two entities is strongly supported by epidemiological studies, understanding the underlying mechanisms has become of major challenge, especially with the current "outbreak" of children's obesity. In conclusion understanding how environmental factors and lifestyle shapes susceptibility to autoimmunity may lead to a personalized medical approach not only for MS but also for several autoimmune diseases.

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6. ADJUNCTS: REVIEWS

6.1 Aryl hydrocarbon receptor controls regulatory $CD4^+$T cell function.

Ref: Swiss Med Wkly. 2012 May 31;142:0.

6.2 Induction of regulatory T_R1 cells and inhibition of T_H17 cells by IL-27.

Ref: Semin Immunol. 2011 Dec; 23(6):438-45.

6.3 Type 1 Regulatory T cells (T_R1) in autoimmunity

Ref: Semin Immunol. 2011 Jun;23(3):202-8.

6.4 Molecular Pathways inducing Interleukin-27-Driven Regulatory T_R1 cells.

Ref: J Interferon Cytokine Res. 2010 June; 30 (6): 381-387.

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Aryl hydrocarbon receptor controls regulatory CD4$^+$ T cell function

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Summary

The ligand activated transcription factor aryl hydrocarbon receptor (AhR) has been studied for many decades in toxicology as the ligand for the environmental contaminant dioxin. However, AhR has recently emerged as a critical physiological regulator of immune responses affecting both innate and adaptive systems, and several AhR ligands with different pharmacological profiles have recently been studied. The current review discusses new insights into the role of AhR signalling and AhR ligands on the regulation of the immune system, with a focus on regulatory T cells which maintain immune tolerance. Notably, AhR is expressed and modulates the development of two induced regulatory CD4$^+$ T cell subsets, the forkhead box P3-positive (Foxp3$^+$) regulatory T cells (iTreg) and the IL-10-secreting type 1 regulatory T (T_R1) cells, through different signalling pathways. We will finally discuss how AhR ligands could be exploited to alleviate human autoimmune diseases. Clearly, drugs targeted against AhR should promote the development of new strategies to fight against autoimmune diseases.

Key words: *regulatory type 1 T cells; Foxp3$^+$ regulatory T cells; aryl hydrocarbon receptor; aryl hydrocarbon receptor ligands*

Introduction

The aryl hydrocarbon receptor (AhR) has been studied for its role in mediating toxicity of environmental contaminants such as dioxin. It is increasingly recognised that environmental toxins modulate immune responses and can both impair response to infection or trigger autoimmunity. AhR has emerged over the last decade as a critical regulator of the immune system. AhR is expressed both in cells involved in the innate immune response such as the dendritic cells (DCs) [1] or the innate lymphoid cells (ILC) [2], and in CD4$^+$ T cells which are crucial for adaptive immunity. Besides T_H1 and T_H2 subsets described by Mosmann and Coffman in 1986 [3], CD4$^+$ T cells can differentiate in the periphery into additional subsets including pro-inflammatory effector T cells (T_H17, T_H22) and regulatory T cells (forkhead box P3 transcription factor (Foxp3)$^+$ iTregs, type 1 regulatory T (T_R1) cells or T_H3) (reviewed in [4, 5]). AhR is expressed at high levels in two subsets of regulatory T cells (Tregs), the iFoxp3$^+$Tregs and T_R1 cells, as wells in T_H17 [6–10]. This expression profile suggests that AhR plays an important role in regulatory T cells.

The immune system has evolved to protect organisms from infection but needs to avoid harmful responses to itself. In this regard, regulatory CD4$^+$ T cells are crucial to maintain tolerance. Although suppressor T cells were described in the early 1970s, interest in regulatory T cells has been revived thirty years later with the discovery of the "lineage specific" transcription factor Foxp3 [11, 12]. Foxp3$^+$CD4$^+$Treg cells have emerged as critical regulators of the immune system as illustrated by the severe autoimmune inflammation observed in mice deficient in Foxp3 [13] or in patients with dysfunctional FOXP3 protein [14]. Foxp3$^+$Tregs can be divided into thymus-derived natural Tregs (nTregs) that express the α chain of the IL-2 receptor CD25 and into inducible CD4$^+$Tregs cells which are generated from CD25$^-$ precursors in the peripheral lymphoid organs (iTregs) (reviewed in [15]). Aside from iTregs, additional inducible regulatory CD4$^+$T cells have been described including TGF-β-secreting T_H3 [16] and type-1 regulatory T-cells (T_R1) [17]. Despite their common role in the regulation of immune responses, induced regulatory T-cell subsets feature differences in their biology, such as the cytokines that induce them or the mechanisms by which they mediate suppression (table 1). Both iTregs and T_R1 cells express AhR at a high level. The role of AhR signalling and the molecular basis downstream AhR activation differ between these two cell types, which will be discussed in more detail.

AhR ligands modulate immune response

AhR is a transcription factor that necessitates activation by a ligand to mediate its transcriptional activity. AhR reside in an inactive form in the cytosol in a complex composed of several proteins (Hsp90 or ARA9). Upon activation with a ligand, AhR undergoes conformational changes that enable its translocation to the nucleus. AhR can then initiate the transcription of promoters containing a dioxin-responsive element (DRE) consensus sequence. DRE are found in multiple gene promoters involved in the immune system.

AhR ligands can be divided into compounds of (1.) exogenous origin from environmental contaminants (man-made aromatic environmental pollutants or pharmaceutical components) or from natural origin (ligands synthetised by microbes or plants that can be found for example in food) and of (2.) endogenous ligands present in the human body (table 2). The ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most studied exogenous ligand and the greatest understanding of AhR biology are based on its effect. *In vivo* treatment with TCDD attenuates the disease course of several murine models of autoimmune diseases including multiple sclerosis, [7, 10] colitis [18] and uveoretinitis [19]. However, because of the unfavourable biological profile of TCDD (high affinity for AhR, long half-life) and implications in embryogenesis and tumourigenesis, its implementation in clinics is limited [20].

Besides environmental contaminants, natural AhR ligands are present in the diet or can be generated in the gastrointestinal tract from dietary compounds such as indirubin, a component found in traditional Chinese herbal medicine [21, 22], curcumin, a common spice used in Indian cuisine, or indole-3-carbinol found in cruciferous vegetables (table 2). Interests in natural AhR ligands and gut immunology are rising as illustrated by several recent publications. A first study pointed out that exposure to AhR ligands through the diet even in the first weeks of life is critical for the development of immune responses as they control the maturation of innate lymphoid cells (ILC) [2]. ILC drive immune responses against intestinal infections and their generation is impaired in AhR-deficient mice. Mice fed with diets lacking natural AhR ligands suffer from deficient ILC generation and are prone to intestinal infection. The sole addition of the natural AhR ligand indole-3-carbinol (table 2) in the diet restores both the generation of ILC and the immune response in an AhR-dependent manner. This highlights the importance of exposure to AhR agonists through food intake and their role in the maintenance of intestinal homeostasis. To corroborate those results, the group of Zhou similarly showed that AhR is necessary for the development of a subset of ILC (that are RORγt$^+$) in mice [23]. Adult AhR-deficient mice have reduced numbers of ILC in both the small and large intestine, and are more susceptible to gut infections. Strikingly the deficits in ILC became evident when AhR-deficient mice were close to weaning age pointing towards the implication of exogenous factors such as food intake in the development of the immune response. The authors proposed that IL-22 production induced by AhR signalling is an important mediator for AhR protective effects in the gut, as IL-22 expression could rescue AhR-deficient mice from succumbing to severe gut infection. The group of Colonna obtained similar results and showed that AhR drives the development of ICL22 and postnatal lymphoid tissues which are critical for responding to gut infections [24]. They propose that AhR signalling regulates IL-22 partially through Notch pathways. Similarly, the group of Veldhoen showed that intra-epithelial lymphocytes (IELs), which are important in the first line defence against intestinal infection or skin defence, depend on AhR activation by dietary-derived ligands to maintain their generation and to control microbial load and composition in the gut [25]. Taken together, the results of those studies suggest that AhR signalling is important for the maintenance of gut immunity and also that exogeneous dietary AhR ligands are crucial to shape our immune response.

The recognition of endogenous ligands confers AhR a physiological role in fine-tuning the immune response and does not restrain AhR to be solely a receptor for environmental compounds. The ligand 6-formylindolo[3,2-b]carbazole (FICZ) was the first described endogenous AhR ligand (table 2). FICZ is formed when its precursor tryptophan is exposed to light in the skin. Similarly to TCDD, FICZ has immunoregulatory properties and administration of FICZ decreases the severity of colitis in different mouse models of inflammatory bowel disease (IBD) by reducing inflammatory responses and promoting the production of IL-22, an IL-10 family cytokine [18]. Another putative endogenous AhR ligand, the 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), induces functional Tregs that suppress EAE similarly to TCDD [26]. Recently, focus has been driven on the ligand kynurenine (Kyn), the first breakdown product in the indoleamine-2,3-dioxygenase (IDO)-dependent tryptophan, which is now increasingly recognised as an endogenous AhR ligand [9]. Indeed, Kyn is generated by dendritic cells (DCs) at the site of inflammation and promotes the generation of regulatory T cells akin to TCDD.

AhR and regulatory T cells

Funatake et al. first described the importance of AhR signalling in the generation of Tregs *in vivo* [27]. Using a mouse model of acute graft-versus-host response, they showed that donor T cells led to the generation of a subset

Table 1: Characteristics regulatory T cells.
CD4$^+$ regulatory T cells can be divided in natural Foxp3$^+$ Tregs which are generated in the thymus (nTregs) or Tregs generated in the periphery that comprise iFoxp3$^+$Tregs, T_R1 and T_H3 cells.
CTLA4, Cytotoxic T-Lymphocyte Antigen 4; GITR, glucocorticoid-induced TNF receptor family-regulated gene; Tbx21, T-box transcription factor TBX21; LAP, latency-associated peptide.

	nTregs	**iTregs**	**T_R1**	**T_H3**
Place of generation	Thymus	Peripheral lymphoid organs	Peripheral lymphoid organs	Peripheral lymphoid organs
Differentiating cytokine	–	TGF-β	IL-27	Anti-CD3 (oral tolerance)
Surface markers	CD25 (IL-2R), CTLA4, GITR	CD25 (IL-2R), CTLA4, GITR	Unknown	LAP CD25-
Transcription factors	Foxp3	Foxp3, AhR	AhR, c-Maf, Tbx21	?
Growth promoting cytokine	IL-2	IL-2	IL-21	?
Mode of suppression	Multiple	Multiple	Multiple	Multiple
Contact dependant	CD39	CD39	Granzyme B	LAP
Contact independent	TGF-β, IL-35, IL-10	TGF-β, IL-35, IL-10	IL-10	TGF-β, IL-10

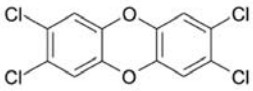
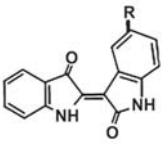
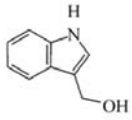
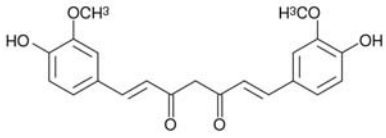
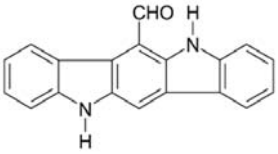
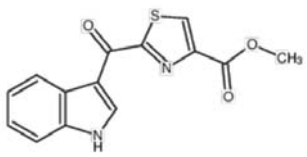
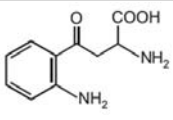
of regulatory $CD4^+$ T cells when transferred into mice treated *in vivo* with TCDD. The induction of those regulatory T cells was AhR dependant and was abolished if recipient mice were deficient for AhR. The generation of those TCDD-induced Tregs did not results in an expansion of already circulating nTregs as TCDD-induced Tregs could be generated in a system devoid of nTregs. The authors pursued the characterisation of those TCDD-induced Tregs and performed an *ex vivo* characterisation of TCDD-induced $CD4^+$ cells by comparing TCDD-induced Tregs with n-Tregs. They showed that TCDD-induced regulatory T cells were different from nTregs, expressed the surface markers CD25, CTLA4 and GITR and produced high levels of IL-10 and Granzyme B [6]. These pioneer *in vivo* studies provided the impetus to further study differentiation of T cells *in vitro* with purified T cells.

AhR and Foxp3$^+$Tregs

As mentioned above, Foxp3$^+$Tregs can be divided in thymus-derived nTregs and adaptive iTregs generated in the periphery. It has been proposed that *in vivo* TCDD do not enlarge nTregs population but promote the generation of iTregs in the periphery [27]. As early immuno-toxicological studies showed that exposure to TCDD contributes to thymic involution [28], one might think that exposure to TCDD impairs nTregs generation. The effect of TCDD or other AhR ligands on nTregs development in the thymus has not been formally studied as the analyses of TCDD-induced Tregs have been performed in adult mice.

TGF-β is crucial for the development of iTregs both *in vitro* and *in vivo* [29]. iTregs express high levels of CD25, the receptor of IL-2, and are dependent on an external IL-2 source to promote their growth (table 1). To study the role of AhR activation on Treg biology, numerous studies have been performed with the high affinity AhR agonist TCDD. Interestingly it was described many years ago that TCDD dampens effector T-cell function and promotes the development of infections. Over the last decade, it was discovered that TCDD further induces Tregs which are beneficial in fighting autoimmune diseases such as type I diabetes [30], multiple sclerosis [7] and colitis [31], and also in reducing the severity of graft versus host disease

Table 2: AhR ligands.
Structures and origins of exogeneous, environmental pollutants and dietary, and endogeneous AhR ligands discussed in this review.

AhR Ligands	Structure	Origin
Exogeneous ligands		
Environmental pollutants		
– 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)		Toxic formed during combustion of organic compound
Dietary		
– Indirubin		Component of Chinese medicine
– Indol-3-carbinol		Cruciferous vegetables (broccoli)
– Curcumin		Indian spice
Endogenous		
– 6-formylindolo[3,2-b]carbazole (FICZ)		Tryptophan photoproduct
– 1'H-indole-3'-carbonyl)-thiazole-4 carboxylic acid methyl ester (ITE)		Compound derived from indirubin
– Kynurenine (Kyn)		Tryptophan product

[27]. Doubts about the impact of TCDD on Tregs have been raised, as it has been postulated that an increased number of Tregs could result from an indirect toxic effect of TCDD. It has been proposed that TCDD leads to the death of conventional T cells, while Tregs are believed to be more resistant to apoptosis [32] (reviewed in [33]). Although the subject is still debated, this hypothesis has never been proven experimentally. Indeed, TCDD does not alter the initial expansion of activated $CD4^{+}$T cells in the spleen of antigen-challenged mice but promotes a premature decline in their number before effector cell development. Furthermore, TCDD did not show a direct toxic effect when tested on multiple cell lines [34]. On the other hand, accumulating evidence indicates that AhR signalling has a specific impact on Treg's generation. First, naïve T cells isolated from AhR null mice inefficiently generated Tregs *in vitro* [35]. Secondly, independent groups have shown that natural AhR ligands such as the dietary AhR ligands derived from indirubin, indole-3-carbinol and indirubin-3′-oxime [36] as well as the endogenous AhR ligands ITE and Kyn promote the differentiation of Tregs that suppress autoimmunity [26, 37].

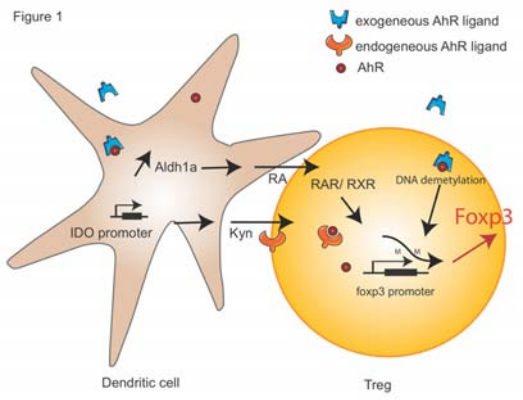


Figure 1

AhR signalling in Tregs.
The molecular mechanisms by which AhR promotes $Foxp3^{+}$Tregs cell are shown. Exogenous AhR ligand induces tolerogenic DCs by multiple pathways. Firstly, the activation of AhR enhances the expression of Aldh1a1 which drives RA secretion. In T cells, RA binds to RAR and RXRs, which in turn bind to the *Foxp3* promoter. Secondly, the activation of AhR induces the formation of Kyn in an IDO-dependant manner in DCs. Kyn further acts as an endogenously AhR ligand and promotes Foxp3 expression. In addition, the activation of AhR mediates partial demethylation of *Foxp3* promoter in $CD4^{+}$ T cells.
RA, retinoic acid; RAR, retinoic acid receptor; RXR, Retinoid X Receptor; Kyn, Kynurenine; IDO, Indoleamine 2,3-dioxygenase.

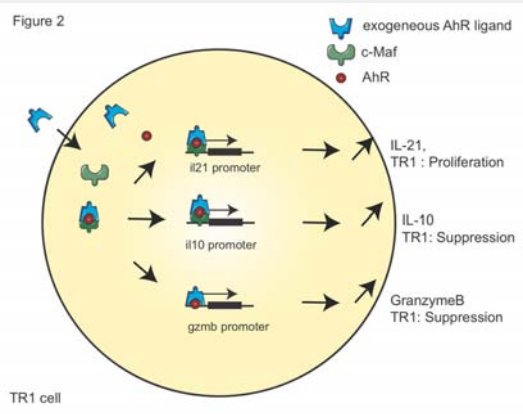


Figure 2

AhR signalling in TR1 cells.
The molecular mechanisms by which AhR promotes TR1 cell differentiation are shown. Firstly, AhR ligands bind to AhR and transactivates *il21* promoter with c-Maf that promotes TR1 cell proliferation. Secondly, the same complex of AhR and c-Maf transactivates *il10* promoter and mediates TR1 cell suppressive function. Thirdly, AhR drives the transactivation of *Gzmb* promoter that promotes granzyme B expression, which contributes to the suppressive activity of TR1 cells.

The molecular basis by which AhR signalling modulates Treg's biology is beginning to be understood. AhR signalling directly shapes Treg differentiation by dictating the state of *Foxp3* promoter methylation (fig. 1). DNA methylation in a gene promoter region is associated with loss of that gene's expression. TCDD mediates partial demethylation of *Foxp3* promoter and hence enhances Foxp3 expression, while it mediates methylation of *Il17* promoter and decreases the expression of IL-17, a pro-inflammatory cytokine secreted by T_H17 cells [31].

Furthermore, AhR induces tolerogenic DCs that promote the generation of Tregs. Different mechanisms by which AhR tolerise DCs have been proposed. First, AhR activation promotes the induction of IDO by binding the *Ido* promoter that contain putative DRE consensus sequences [38]. Accumulating evidence indicate that IDO plays a pivotal role in the induction of tolerogenic DCs (reviewed in [39]). IDO depletes tryptophan in local tissue microenvironments and generates immunoregulatory catabolites, such as Kyn. Both tryptophan starvation and the presence of Kyn promote the generation of Tregs. *In vivo* AhR activation with TCDD induces IDO1 and IDO-like protein IDO2 which further drive Foxp3 expression [40]. In addition, a recent publication explored the link between AhR activation by Kyn and the generation of Tregs *in vitro* [41]. These authors showed that IDO generated by DCs leads to an increased Kyn formation that directly promotes the generation of Tregs (fig. 1).

Generation of retinoic acid (RA), the active form of vitamin A, is another mechanism by which AhR ligand induces tolerogenic DCs that support Treg's differentiation [26]. Treatment of DCs with the AhR ligand ITE promotes the expression of the retinal dehydrogenases Aldh1a1 that enhances RA secretion (fig. 1). Notably, RA forms a complex with the nuclear receptors, RA receptors (RARs) and retinoid X receptors (RXRs) that controls the transactivation of *foxp3* promoter in coordination with other transcription factor such as MAD homolog 3 (Smad3), thereby promoting Treg differentiation.

AhR signalling not only controls mouse but also human Treg's generation. In contrary to murine Tregs, human naïve T cells differentiated with TGF-β in vitro express Foxp3 but do not acquire suppressive properties. On the other hand, addition of an AhR ligand onto naïve T cells differentiated in the presence of TGF-β induces suppressive Foxp3+ Tregs. AhR activation does not lead to increased levels of Foxp3 expression but promotes the expression of CD39, an ectonucleotidase that hydrolyses ATP

and mediates suppressive activity of Treg [8, 42]. CD39 deficiency is linked with exacerbation of autoimmune diseases in murine colitis, and human polymorphism in the CD39 gene is associated with higher susceptibility to inflammatory bowel diseases [43]. In conclusion, AhR modulates the development of Tregs both in mouse and human by multiple mechanisms.

AhR and T_R1 cells

TR1 cells are an important subset among IL-10-secreting regulatory T cells and are characterised by a unique profile of cytokine production: high levels of IL-10, IL-21, some IFN-γ, low levels of IL-2 and no IL-4 (table 1) (reviewed in [17]). Interestingly, TR1 cells were first described in severe combined immuno-deficient (SCID) patients who had developed long-term tolerance to stem cell allografts, suggesting that these cells suppressed immune responses in humans [44]. Indeed, during the course of human inflammatory diseases such as multiple sclerosis, the frequency and the functionality of TR1 cells are impaired [45, 46]. Studies of TR1 cells have been difficult due to the lack of known specific lineage transcription factor or surface markers which would facilitate their tracking in vivo, and the difficulty to grow them in vitro due to their low proliferative properties. Interest in TR1 cells has recently been revived by the discovery that the cytokine IL-27 is essential for their development [47]. Exogenous and endogenous AhR ligands (TCDD, FICZ or IDE) have been shown to promote TR1 cell function as they enhance the expression of key proteins involved in their biology, which are IL-10, IL-21 [9] and Granzyme B [8]. Murine naïve T cells cultivated in the presence of AhR ligands alone are not able to convert to TR1 cells in the absence of IL-27. Indeed, IL-27 induces the expression of the transcription factors AhR and of the proto-oncogene c-Maf that enables the activation of AhR signalling. IL-27 inefficiently drives TR1 cell generation in the absence of AhR or c-Maf [9]. Upon activation AhR forms a complex with c-Maf that promotes the transactivation of il10 and il21 promoters (fig. 2) [9, 48, 49]. IL-10 and IL-21 are two essential cytokines involved in TR1 cell biology: IL-10 is crucial for the suppressive characteristics of TR1 cells and IL-21 for the expansion and maintenance of TR1 cells [48] (reviewed in [49, 50]). Interestingly, it has been recently shown that the complex c-Maf and AhR also plays a role during TH17 differentiation by controlling the expression of IL-22 [51]. Mice injected with the AhR ligand FICZ are protected against the development of colitis and have marked down-regulation of inflammatory cytokines but induction of IL-22 by TH17 cells [18]. It is noteworthy that AhR and c-Maf are expressed in TR1 cells but do not drive IL-22 expression in this cell type. This promotes the idea that while AhR and c-Maf are crucial in driving the expression of cytokines involved in the differentiation of different T cell subsets, additional yet unknown IL-27-induced triggers are critical to control the transcriptional regulation of TR1 cell.

Akin to mouse TR1 cells, AhR signalling is essential for TR1 cell differentiation in humans. Consistent with results obtained in mouse TR1 cells, the human transcription factor AhR interacts with c-Maf, ultimately resulting in an enhanced IL-10 secretion from TR1 cells [9]. While these observations are in line with the role of AhR in mouse TR1 cell differentiation, activation of human CD4+ T cells with AhR ligands in the absence of IL-27 was able to drive TR1 cell differentiation. It is noteworthy that c-Maf which is essential for mouse TR1 cell differentiation is detectable in human T cells activated without IL-27. As c-Maf expression is strictly dependent on IL-27 in mice, the observation that TCDD or FICZ alone drives the expansion of human Tr1 cells could be due to their expression of c-Maf upon sole activation. Indeed, the over-expression of c-Maf induces marginal expression of IL-10 from human CD4+ T cells, while the TCDD-driven activation of AhR combined with c-Maf over-expression leads to significant IL-10 expression [8]. IL-10 is necessary but not sufficient for the function of human TR1 cells as their capacity to suppress immune responses also relies on granzyme B expression [52, 53]. Interestingly, besides increasing IL-10 expression, AhR transactivates Gzmb promoter and drives the expression of granzyme B, which contributes to the suppressive activity of TR1 cells (fig. 2) [54]. In conclusion, akin to Foxp3+ Tregs, AhR modulates the development of TR1 cells both in mouse and human using different signalling pathways.

AhR and IL-22

While evidence generated from the studies discussed above support the contention that AhR is involved in the generation of regulatory cells, ex vivo study of T cells obtained from a man intoxicated with very high levels of TCDD did not show the expansion of Foxp3+Treg cells nor of IL-10-producing T cells [55]. Interestingly, T cells from this patient exhibited an IL-22 phenotype. As discussed previously, IL-22 is a member of the IL-10-cytokine family (reviewed in [5]) that plays a crucial role in skin and intestinal mucosa [51]. To strengthen the link between AhR and IL-22 production, it has been shown that mice injected with FICZ have enhanced IL-22 production and were protected against several murine colitis models [18]. IL-10 and IL-22 belong to the same family, suggesting that those two cytokines could be regulated by similar mechanisms [5]. New insights have been obtained in two recent studies showing that AhR signalling enhances IL-22 production by directly either binding to il22 promoter in co-ordination with the transcription factor RORγt [23] or via Notch signalling [56]. While the precise molecular mechanism behind the Notch-mediated IL-22 induction remains unknown, it has been proposed that the Notch effect could be indirect by promoting the production of endogenous ligands for AhR [56].

IL-22 expression can be associated with IL-17 and is expressed in TH17 cells [57] where AhR is expressed at a high level [7, 10]. It has been proposed that AhR signalling could promote TH17 cell development. It is important to note that TCDD or ITE injections in vivo favour the development of regulatory T cells and reduce the severity of EAE disease but treatment with FICZ aggravates the disease course of EAE. While FICZ promotes TH17 cell generation in vitro, the AhR dependent induction of TH17 cells has yet to be formally confirmed in vivo. The results of those studies have, however, led to the idea that activation of AhR by different ligands could differentially influ-

ence CD4+ T cell differentiation. However, this concept needs to be formally proven. The dosis of AhR ligands used in different studies could also be critical to the impact of AhR treatment of autoimmune diseases. Finally, the new concept that TH17 cells represent a heterogeneous population could help understanding the apparently contradictory role of FICZ on TH17 cell development. TH17 cell generated in the absence of TGF-β are highly pathogenic and do not express either AhR or IL-10, while they produce both IFN-γ and IL-17. In contrast, TH17 generated in the presence of TGF-β (TH17β) express both AhR and c-Maf, produce IL-10 and are less pathogenic [58]. Indeed, c-Maf promotes the expression of the anti-inflammatory cytokine IL-10 expression in TH17 [59] and inhibits IL-22 production [60] thereby reducing the pathogenicity of TH17 cells. The putative role of AhR signalling in different subtypes of TH17 differentiated needs to be formally addressed.

Concluding remarks

A precise balance is necessary to maintain immune surveillance but at the same time prevent the development of autoimmunity. AhR ligands have been assigned valuable immunomodulatory properties and can fine-tune the immune response. As we discussed in the first part of this review, the importance of AhR signalling in gut immunity and the availability of AhR ligands through food intake has been highlighted by numerous recent publications [2, 23–25]. AhR signalling properties could be further exploited in the clinical practice, not only in the field of infectiology but also in autoimmunity. However, several points have to be resolved before their implementation in the clinical practice. First, there is a need to design AhR ligands with safe pharmacological profiles. Indeed, as we discussed previously, the most studied AhR ligand TCDD, has valuable immunomodulatory properties but is not applicable in clinical practice because of its pharmacological properties. Furthermore, the impact of distinct AhR ligands on the immune system has to be better understood. For example, while TCDD and FICZ have opposite effects on iFoxp3^{+}Tregs or on T_H17 cells, they both promote T_R1 cell or can induce IL-22 secretion depending on the experimental setting. Therefore the specificity, the pharmacology and the dose-effect of each ligand will have to be carefully assessed in different diseases models. Finally, the effect of AhR ligands needs to be cautiously monitored to avoid excessive responses. For example, the endogeneous AhR ligand Kyn has been recently shown to be secreted by human tumours via the tryptophan-2,3-dioxygenase and promote the generation of Tregs *in vivo* [61]. While Tregs are beneficial in preventing autoimmune diseases, they are regarded as inhibitors of anti-tumour immunity and impair the development of successful immunotherapy [62].

In conclusion, AhR ligands are promising compounds for pharmaceutical drugs. However, further studies should aim at designing a new generation of AhR ligands which could specifically target different cell types of the immune system with limited side effects. More research is required to evaluate the potential of AhR targeting for the treatment of autoimmune and inflammatory diseases in humans.

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Figures (large format)

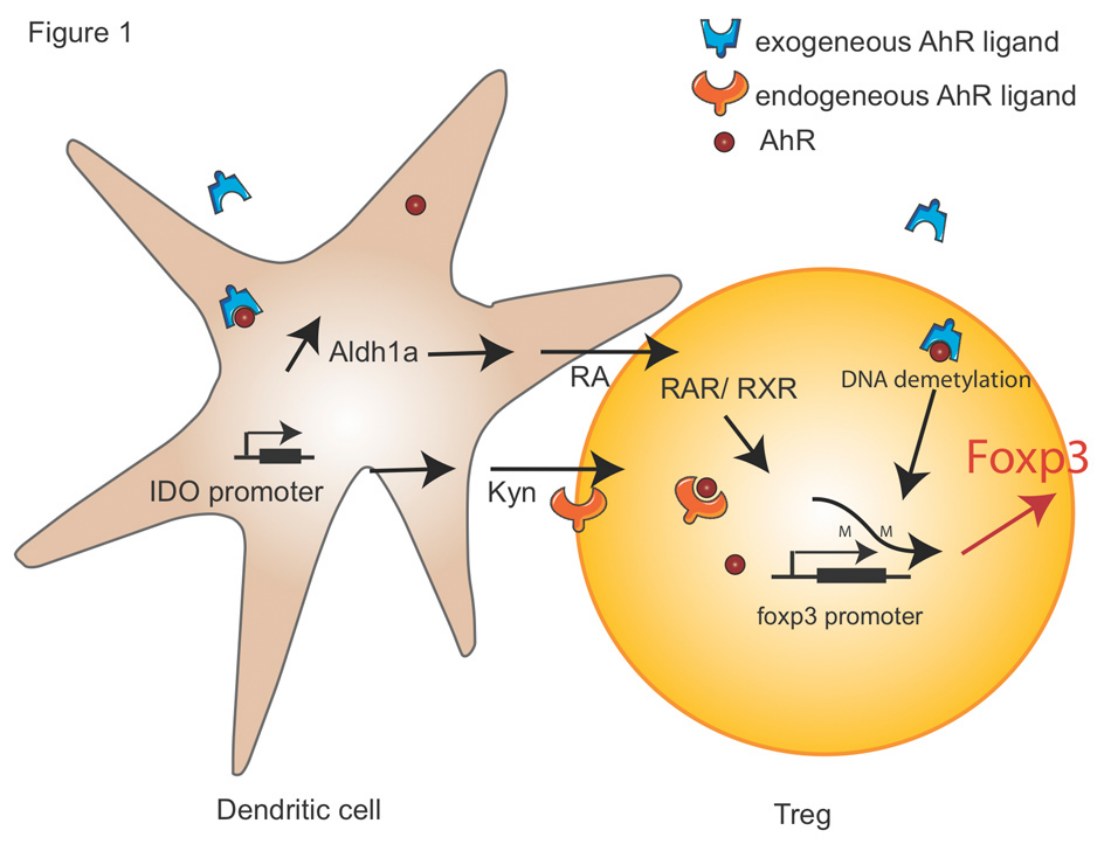

Figure 1

AhR signalling in Tregs.

The molecular mechanisms by which AhR promotes Foxp3^{+}Tregs cell are shown. Exogenous AhR ligand induces tolerogenic DCs by multiple pathways. Firstly, the activation of AhR enhances the expression of Aldh1a1 which drives RA secretion. In T cells, RA binds to RAR and RXRs, which in turn bind to the *Foxp3* promoter. Secondly, the activation of AhR induces the formation of Kyn in an IDO-dependant manner in DCs. Kyn further acts as an endogenously AhR ligand and promotes Foxp3 expression. In addition, the activation of AhR mediates partial demethylation of *Foxp3* promoter in CD4^{+} T cells.

RA, retinoic acid; RAR, retinoic acid receptor; RXR, Retinoid X Receptor; Kyn, Kynurenine; IDO, Indoleamine 2,3-dioxygenase.

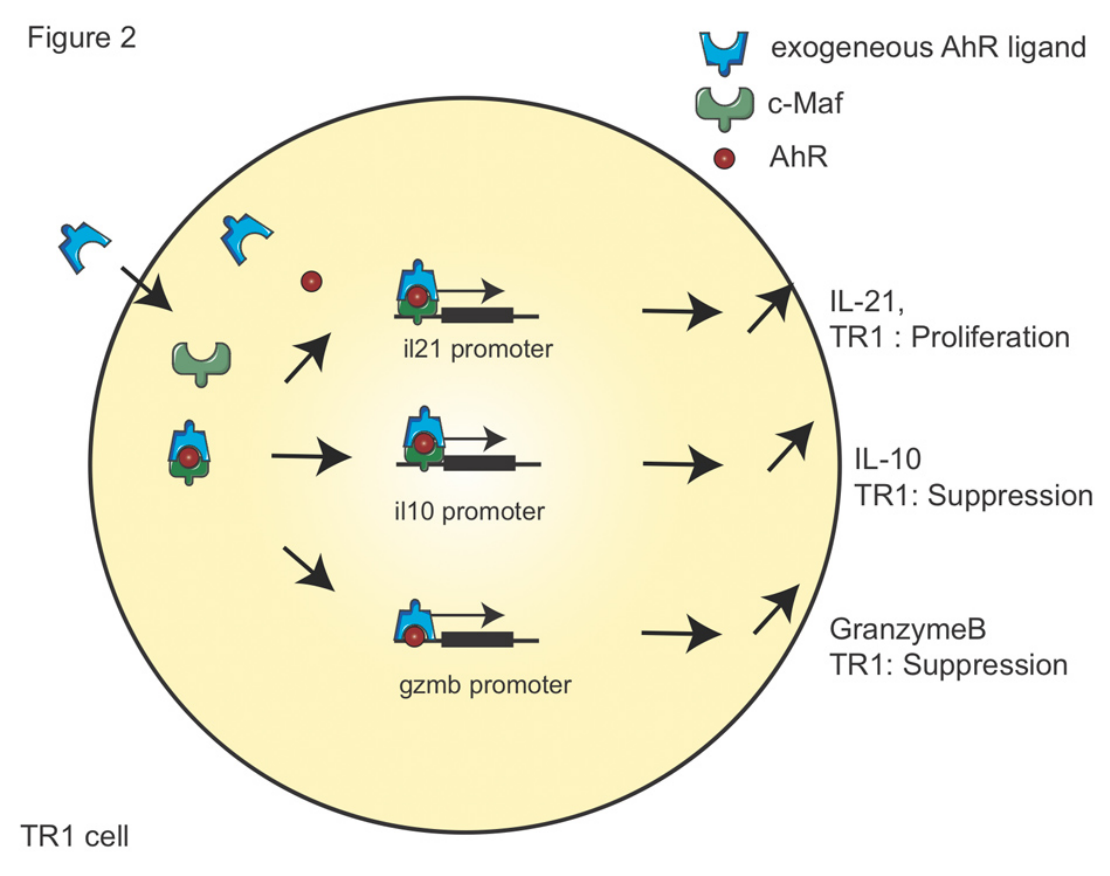

Figure 2

AhR signalling in Tr1 cells.

The molecular mechanisms by which AhR promotes Tr1 cell differentiation are shown. Firstly, AhR ligands bind to AhR and transactivates *il21* promoter with c-Maf that promotes Tr1 cell proliferation. Secondly, the same complex of AhR and c-Maf transactivates *il10* promoter and mediates Tr1 cell suppressive function. Thirdly, AhR drives the transactivation of *Gzmb* promoter that promotes granzyme B expression, which contributes to the suppressive activity of Tr1 cells.

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Review

Induction of regulatory Tr1 cells and inhibition of T_H17 cells by IL-27

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ABSTRACT

Accumulating evidence indicates that IL-27, a member of the IL-12 family of cytokines, alleviates the severity of autoimmune diseases in both mice and men. The IL-27-induced activation of signal transducer and activator of transcription (Stat)1 and Stat3 promotes the generation of IL-10- producing type 1 regulatory T (Tr1) cells that inhibit effector T cells. In addition, IL-27 also suppresses the development of pathogenic IL-17-producing CD4$^+$ T cells (T_H17) cells suggesting that pharmacological manipulations of IL-27 signaling pathway could be exploited therapeutically in regulating tissue inflammation. Here, we review how IL-27 controls inflammation through the regulation of Tr1 and T_H17 responses.

1. Introduction

Since the original classification by Mosmann and Coffman of CD4$^+$ helper T (T_H) lymphocytes into T_H1 and T_H2 subsets [1], the repertoire of T_H subsets has expanded to include additional effector and regulatory T cell subsets such as T_H17 cells and regulatory T cells (Foxp3$^+$Tregs and Tr1 cells). T_H1 cells, which predominantly produce interferon (IFN)-γ and lymphotoxin, are essential for eliminating intracellular pathogens, but were also regarded as the major effector T cells in inducing tissue inflammation in organ-specific autoimmunity. However, mice lacking the component of T_H1-IFN-γ pathway (*Il12*$^{-/-}$, *Ifng*$^{-/-}$, *Ifngr1*$^{-/-}$, *Il12rb2*$^{-/-}$) were not protected but overly susceptible to autoimmune diseases including Experimental Autoimmune Encephalomyelitis (EAE) [2], Experimental Autoimmune Uveitis (EAU) [3] and collagen-induced arthritis (CIA) [4]. Subsequent studies revealed that T_H17 cells, instead of T_H1 cells, induce tissue inflammation in autoimmune diseases. Although T_H17 cells are essential for eliminating extracellular pathogens [5,6], exaggerated T_H17 response promotes autoimmunity. Elevated amounts of IL-17A and IL-17F are detected in several autoimmune diseases including multiple sclerosis (MS) [7], rheumatoid arthritis (RA) [8] and psoriasis [9]. The involvement of T_H17 cells in tissue inflammation was confirmed in mouse models such as EAE where IL-17-neutralizing antibodies ameliorate clinical scores [10] or CIA where IL-17-deficient animals develop attenuated disease [11]. The differentiation factors for both mouse and human T_H17 cells were found to be a combination of TGF-β1 and IL-6 or TGF-β1 and IL-21 [12]. The activation of signal transducer and activator of transcription (Stat)3 by IL-6 or IL-21 is critical for inducing the expression of the T_H17 cell master transcription factors retinoid-related orphan receptor (ROR)γt, encoded by the gene Rorc, and RORα (Rora) [13–15]. Rorc$^{-/-}$ and Rora$^{-/-}$ mice show defective T_H17 cell generation [15]. In addition, Chip-Sequencing analysis revealed Stat3 binding sites in the promoters regions of il17a and il17f genes [12]. Furthermore RORγt drives the expression of GM-CSF that is essential for inducing pathogenic T_H17 cells, and mice deficient in making GM-CSF are resistant to develop EAE [16]. These observations indicate that RORγt is essential for the development of T_H17 cells. Indeed T_H17 cell generation can be inhibited by directly targeting RORγt using small chemical compounds such as digoxin and SR1001 [17]. While IL-23 is not required for the induction of T_H17 cell differentiation, IL-23 has a prominent role in expansion and stabilization of pathogenic T_H17 cells [18–20]. Both IL-12p19$^{-/-}$ and IL-23R$^{-/-}$ mice are resistant to EAE, and few T_H17 cells are found in the central nervous system (CNS) of those mice [21–23]. The IL-23-T_H17 pathway has been shown to be critical in many autoimmune diseases, which is consistent with the fact that IL-23R polymorphisms have been genetically associated with a number of human autoimmune diseases including psoriasis, inflammatory bowel diseases (IBD) and ankylosing spondylitis [24]. More recent studies suggested that T_H17 cells could also be induced with the combination of IL-1β, IL-6

Abbreviations: Tr1 cells, type 1 regulatory T cells; T_H17, T helper 17; Stat, signal transducer and activator of transcription; Maf, transcription factor Maf; Ahr, Aryl hydrocarbon receptor.

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and IL-23 in the absence of TGF-β1, suggesting that T_H17 cells might actually represent a heterogeneous population of proinflammatory cells that are highly pathogenic and can be induced by multiple different ways.

Exaggerated inflammatory responses are prevented by regulatory T cell subsets that suppress activation of effector T cells. $CD4^+$ regulatory T cells comprise $Foxp3^+$ regulatory T-cells (Tregs) and IL-10-producing regulatory type I (Tr1) cells [25]. $Foxp3^+$Tregs are important to maintain self-tolerance as illustrated by the severe autoimmune inflammation observed in mice deficient in Foxp3 [26] or in patients with dysfunctional FOXP3 protein [27]. Although $Foxp3^+$Tregs inhibit effector T cell responses, they lose their suppressive functions in inflammatory conditions [28]. Therefore, IL-10-producing Tr1 cells might be crucial in controlling tissue inflammation. In humans, Tr1 cells were first described in severe combined immunodeficient (SCID) patients who had developed long-term tolerance to stem cell allografts, supporting the existence of these cells in humans and suggesting that they may play a role in mediating T cell tolerance [29]. Tr1 cells mediate immune suppression by secreting the suppressive cytokine IL-10 and by killing effector cells via Granzyme-B and Perforin [30,31]. While IL-10 was initially described to be the differentiation factor for Tr1 cells, these T cells could not expand in the presence of IL-10. Therefore there was an emphasis on identifying growth/differentiation factors for Tr1 cells. Recent identification of IL-27 as a differentiation/growth factor for Tr1 cells has revived the interest in examining their role in tissue inflammation [32–34].

2. IL-27 dampens autoimmune inflammation

IL-27, an heterodimeric cytokine composed by the subunit p28 (IL-27p28) and the Epstein–Barr virus-induced gene 3 (EBI3), is mainly produced by activated antigen-presenting cells APCs [35]. IL-27 signals through a receptor complex consisting of the common IL-6 receptor chain, gp130, and the unique IL-27 receptor alpha chain (IL-27Ra or WSX-1) that is homologous to IL-12Rβ2 of IL-12 receptor [35,36]. Based on the structural homology between IL-12 and IL-27 and their receptors, IL-27 was initially described as a proinflammatory cytokine that could induce T_H1 differentiation, which was consistent with the ability of IL-27 to induce T-bet (Tbx21), the master transcription factor for the generation of T_H1 cells. Subsequent work, using both T_H1 and T_H2 associated pathogens, established that IL-27 suppresses T_H cells (T_H1, T_H2 and T_H17 cells) functions *in vivo*, as *Il27ra*$^{-/-}$ mice showed enhanced T cell functions (reviewed in [37]). However, the mechanism by which IL-27-induced inhibition of T cell functions was not understood until the discovery that IL-27 can induce IL-10 production from $CD4^+$ T cells.

3. IL-27 controls T cell responses

3.1. Regulation of T_H1 and T_H2 differentiation

While IL-27 induces T-bet and expression of IL-12Rβ2 in naïve $CD4^+$ T cells, IL-27 signaling is not mandatory for T_H1 differentiation as illustrated by mice lacking the IL-27R subunit (*Il27ra*$^{-/-}$) that can mount adequate T_H1 responses to eliminate intracellular pathogens [38–40]. Moreover, *Il27ra*$^{-/-}$ mice die due to uncontrolled immunopathology and severe tissue inflammation associated with exaggerated T cell responses and enhanced production of IFN-γ and TNF-α [38–40]. IL-27 was also reported to control the generation of T_H2 cells. IL-27 treatment during *Strongyloides venezuelensis* infection decreases T_H2 responses against the parasite and treated mice failed to develop intestinal mastocytosis and exhibited a marked delay in parasite expulsion [41]. Furthermore,

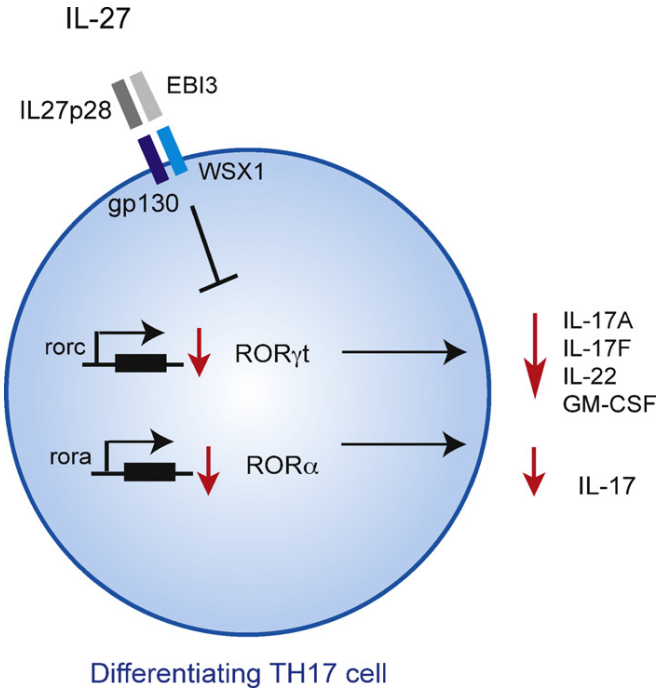

Fig. 1. IL-27 inhibition of differentiating T_H17 cells. On differentiating T_H17 cells, IL-27 inhibits the expression of transcription factors Rorγt and Rorα, thereby impairing the secretion the T_H17-related cytokines, IL-17A, IL-17F, IL-22 and GM-CSF.

intranasal administration of IL-27 inhibits OVA-induced airway hyperresponsiveness and inflammation in OVA-sensitized animals [41]. At the transcriptional level, IL-27 has been shown to suppress the master T_H2 transcription factor GATA-3 [41]. Recently, genome-wide association study (GWAS) has shown that a single nucleotide polymorphism (SNP) in the *IL-27p28* gene was associated with an increased susceptibility to asthma [42] or COPD [43] and IL-27 has been proposed as a potential treatment for bronchial asthma.

3.2. Inhibition of T_H17 cell differentiation

In addition to inhibiting both T_H1 and T_H2 development, IL-27 prevents the development of T_H17 cells *in vitro* and *in vivo*. *Il27ra*$^{-/-}$ mice are overly susceptible to EAE compared to wild-type mice and present an increased accumulation of T_H17 cells in the draining lymph nodes and in the CNS [44]. In this model, neutralization of IL-17 in *Il27ra*$^{-/-}$ mice during EAE disease course attenuated their disease phenotype [44]. Accordingly, recombinant IL-27 treatment decreases the disease incidence and severity in EAE with the inhibition of development of T_H17 cells [45]. Similarly, *Il27ra*$^{-/-}$ mice chronically infected with *Toxoplasma gondii* developed severe neuropathology mediated by $CD4^+$ T cells, associated with increased T_H17 cell development. IL-27 inhibits the production of IL-17 by BMNCs from chronically infected mice stimulated with IL-23 [46]. Finally in the absence of IL-27 during murine flu infection, flu-specific T cell responses are skewed towards T_H17 [47].

Above observations clearly indicated that IL-27 is negative regulator of development of T_H17 cells. However, the mechanism by which IL-27 inhibits the development of T_H17 cells is not clearly understood. Accumulating data suggest that IL-27 utilizes multiple mechanisms to inhibit the development of T_H17 cells (Figs. 1 and 2). During T_H17 cell differentiation, IL-27 directly suppresses the expression of both RORγt, the master transcription factor of T_H17 cells [48] and RORα [49] (Fig. 1). IL-27 inhibits expression of RORγt in T_H17 cells both in mouse and man [48]. Interestingly, IL-27 decreases the expression of GM-CSF and thereby dampens the

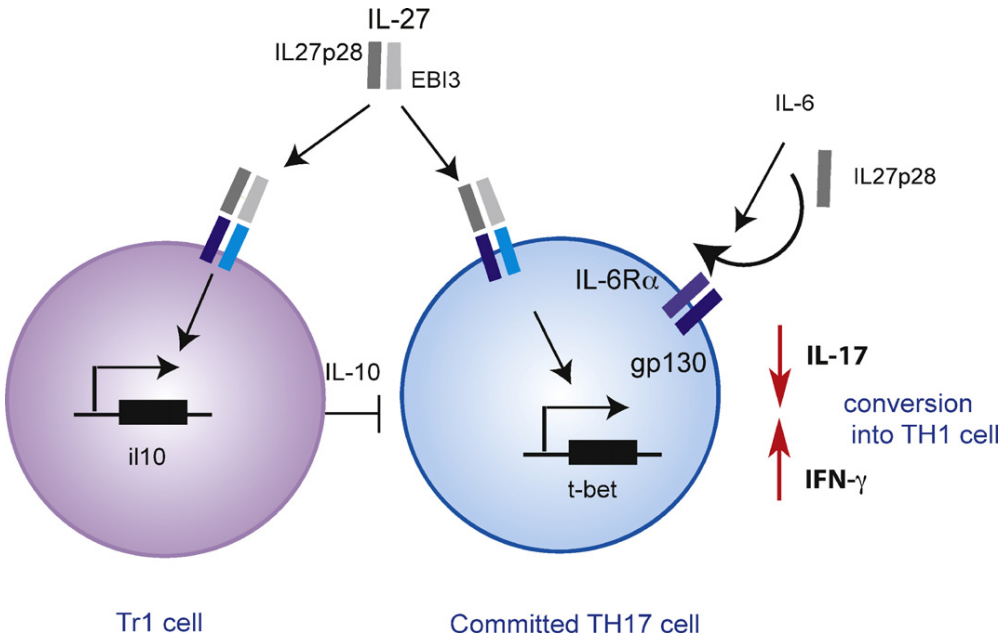

Fig. 2. IL-27 inhibition of committed T_H17 cells. IL-27 induces the differentiation of Tr1 cells that inhibit T_H17 cells in an IL-10-dependent manner. IL-27p28 monomers interfere with IL-6 cytokine signaling through gp130 and thereby inhibit the maintenance of T_H17 cells and their IL-17 secretion. IL-27 further induces T-bet expression that drives IFN-γ production and promotes the conversion of T_H17 cells into T_H1 cells.

pathogenicity of T_H17 cells [16]. By blocking GM-CSF secretion and inhibiting both RORα and RORγt expression, IL-27 interferes with T_H17 cell differentiation at several levels, explaining its potent ability to suppress the induction of T_H17 cells.

Whether IL-27 can directly suppress effector/memory T_H17 cells or fully differentiated T_H17 cells is still debated. Indeed, T_H17 maintained in culture for at least two rounds become unresponsive to IL-27 as IL-27 fails to inhibit the expression of RORα and RORγt in these cells [49]. However, IL-27 could modulate effector/memory T_H17 cells using different strategies. Among the two IL-27 cytokine subunits, EBI3 is constitutively expressed but IL-27p28 secretion is transcriptionally regulated. IL-27p28 monomers can interfere with the IL-6-mediated production of IL-17 by preventing IL-6 signaling through gp130, suggesting that IL-27p28 monomers could also be exploited in regulating T cell responses [50]. IL-27p28 thus limits the generation and maintenance of T_H17 cells *in vivo* without directly interfering with T_H17 transcriptional program (Fig. 2). Furthermore, it has been proposed that T_H17 could be converted into T_H1 cells that are presumably less pathogenic [51,52]. One putative mechanism by which IL-27 could converts T_H17 into T_H1 cells may be by inducing the expression of T-bet that drives IFN-γ expression and reduces the expression of IL-17 (Fig. 2). However, this hypothesis by which IL-27 may increase T_H17 plasticity has not been proven experimentally.

3.3. Induction of Tr1 cells

IL-27, while inhibiting TGF-β-induced Foxp-3$^+$ Tregs, induces IL-10$^+$, IFNγ$^+$ T cells that are immunosuppressive, a phenotype in line with the previously described Tr1 cells [32–34,53,54]. The role of IL-27 in generation of IL-10-producing Tr1 cells was further emphasized *in vivo*. IL-27 treated MOG-specific splenocytes lose their ability to transfer EAE in an IL-10 dependent manner [33]. Furthermore, during flu infection, IL-27 generates regulatory T cells that inhibit T_H17 cells by secreting IL-10 and IFN-γ. In the absence of IL-10, flu-specific T cell responses developed a stronger T_H17 component [47]. Furthermore, it has been shown that Tr1 cells can inhibit T_H17 cells *in vivo* in an IL-10 dependent manner during murine colitis [55] (Fig. 2). Akin to what has been observed in murine T cells, activation of naïve human T cells in the presence of IL-27 similarly induces Tr1 cells that produce both IFN-γ and IL-10 [56].

4. Molecular pathways involved in IL-27 biology

Similar to other type 1 cytokine receptors, IL-27 also induces the activation of Janus kinase/Stat pathway. IL-27 predominantly induces the phosphorylation of Stat1 and Stat3. Here we will discuss the IL-27-induced signaling events following the activation of the Stats and analyze their roles in inhibiting T_H17 cell and in inducing Tr1 cell differentiation.

4.1. IL-27 and Stat1 activation

4.1.1. Stat1 activation by IL-27 represses T_H17 differentiation and induces Tr1 cells

The activation of the IL-27 specific subunit WSX-1 drives the tyrosine phosphorylation of JAK1 that further activates Stat1. Indeed, JAK1, but not other JAKs, coprecipitates with the WSX1 subunit [57].

The Stat1 signaling pathway is necessary for IL-27-induced T-bet expression [58]. T-bet not only drives the expression of IFN-γ but also plays an important role in the inhibition of T_H17 cytokines, independently of IFN-γ. T-bet can reprogram committed T_H17 cells by repressing T_H17 gene program, which results in fewer transcripts of *Rorc*, *il17a*, *il17f*, *il23r* [59]. These finding were supported by studies showing that T-bet utilizes Runt-related transcription factor 1 (Runx1), a transcriptional activator that sequesters *Rorc* away from the regulatory regions on *Rorc* promoter [59]. Indeed Runx1 binding site is located upstream of T-bet binding site on *Rorc* promoter. By sequestering Runx1, T-bet inhibits the expression of RORγt, resulting impaired development of T_H17 cell [59] (Fig. 3).

Stat1$^{-/-}$ and T-bet$^{-/-}$ mice exhibit an increased number of T_H17 cells both during systemic inflammation *in vivo* or during T_H17 cells differentiation *in vitro*. IL-17 production is greater in the absence of T-bet compared to the absence of Stat1 [60]. This

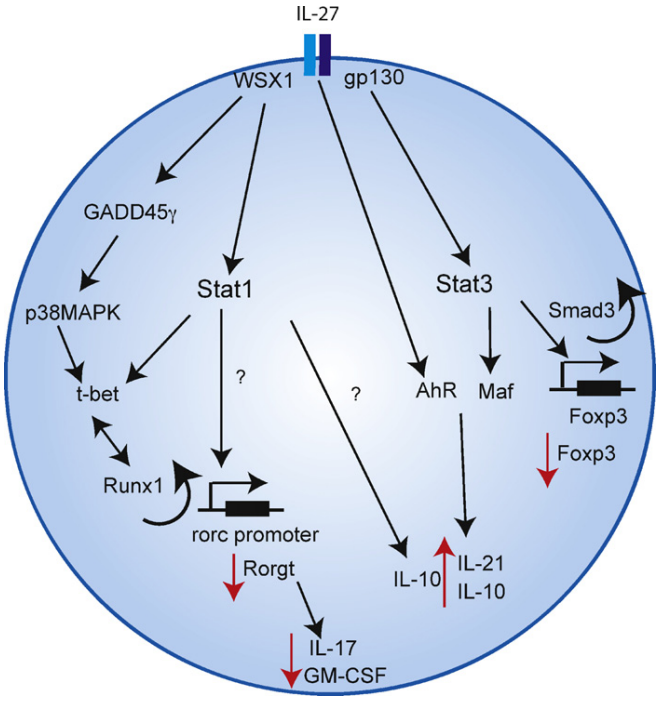

Fig. 3. Reciprocal regulation of T_H17 and Tr1 cells by IL-27. The molecular mechanisms by which IL-27 promotes Foxp3$^-$IL-10$^+$Tr1 cell differentiation and represses T_H17 cell development through activation of Stat1 and Stat3 activation are shown. IL-27 activates Stat1 through the subunit WSX1 that inhibits Rorγt expression through T-bet-dependent as well as T-bet-independent pathways. Alternatively, IL-27 can promote T-bet expression in a Stat1 independent pathway via GADD45γ. In addition, IL-27 activates Stat3 signaling through gp130. Stat3 induction then drives Maf transcription. Maf together with Ahr transactivates *il21* and *il10* promoters. On the other hand, IL-27 inhibits Foxp3 transcription in a Stat3/Smad3 dependent manner.

may be related to the fact that T-bet might also be induced in a Stat1 independent manner. In this vein, Owaki et al. have shown that IL-27 induces a Stat1 independent T-bet expression [61]. Indeed IL-27 induces the expression of GADD45γ that further drives the phosphorylation of p38 MAPK leading to T-bet expression (Fig. 3).

It has been further proposed that Stat1 could inhibit RORα and RORγt expression in differentiating T_H17 cells in a T-bet independent manner (Fig. 3). While a direct inhibitory effect of Stat1 on RORα and RORγt expression has not been ruled out, Stats could also indirectly affect T_H17 responses by promoting the function of auxiliary inhibitory T_H17 factors. Different repressors of T_H17 cells differentiation have been identified, including Ets-1, which negatively regulates T_H17 cell differentiation [62]. Stat1 and Ets-1 have been shown to bind together [63] and might cooperate to inhibit T_H17 cell differentiation by directly or indirectly interfering with RORγt function in T_H17 cells.

IL-27 has been shown to induce IL-10 expression from CD4$^+$ T cells using both Stat1 and Stat3 pathways (Fig. 3). Indeed, in the absence of Stat1 signaling, IL-27 driven IL-10 production is decreased. While it is clear that the Stat1 driven IL-10 secretion is independent of T-bet signaling, the underlying mechanisms still remain unclear [34].

4.2. IL-27 and Stat3 activation

4.2.1. Stat3 activation by IL-27 does not enhance T_H17 cell differentiation

IL-27 utilizes gp130 subunit of IL-6 receptor complex, which results in activation of Stat3 signaling. A genetic defect in Stat3 signaling in humans, in hyperIgE syndrome, results in defective T_H17 cells and in unrelenting fungal infections, supporting the critical role of Stat3 in the generation of T_H17 cells [64]. At the first glance, it is puzzling that IL-6 and IL-27, which both activate Stat3 pathways, have antagonistic properties. It has been proposed that IL-6 leads to a faster and more persistent pattern of Stat3 phosphorylation that is crucial to drive pro-inflammatory signals downstream Stat3. pStat-3 directly binds to *il17a* and *il17f* promoters and transactivate these genes by collaborating with other transcription factors like IRF-4 and RORγt. Furthermore, the formation of Stat1–Stat3 heterodimers in response to IL-27 rather than the formation of mainly Stat3 homodimers in response to IL-6 or IL-21 may play a role in the difference between IL-6 and IL-27 signaling. Indeed preliminary data from our laboratory supports this hypothesis. In addition, IL-6 activation rapidly induces Stat3 repressor SOCS3 [65]. SOCS3 is an essential negative regulator of Stat3 phosphorylation and constrains T_H17 cell differentiation [66,67]. While IL-27 induces expression of SOCS3, IL-27-mediated inhibition of IL-17 production is independent of SOCS3 [46]. It therefore seems unlikely that IL-27-induced SOCS3 contributes to the inhibition of T_H17 cells. Instead, the inhibition of T_H17 differentiation might mainly be mediated through Stat1 and T-bet as discussed above.

4.2.2. Stat3 activation by IL-27 promotes Tr1 cell differentiation

IL-27-induced Stat3 phosphorylation is essential for the anti-inflammatory role of IL-27, as it triggers IL-10 secretion from CD4$^+$ T cells [34] (Fig. 3). Sustained activation of Stat3 leads to the induction of the transcription factor Maf [68]. We and others have recently shown that Maf is essential for IL-10 production induced by IL-27 [53]. Similarly to Stat3 deficient CD4$^+$ T cells, Maf deficient CD4$^+$ T cells cannot produce IL-10 in response to IL-27. It has been further shown that Maf directly transactivates *il10* and *il21* promoters [53]. In addition to Maf, IL-10 production by IL-27 is regulated by the ligand activated transcription factor Aryl hydrocarbon receptor (AhR) that binds to Maf resulting in a complex that induces both *il10* and *il21* transcription [69]. The finding of AhR involvement in IL-10 production is significant as it provides impetus to design AhR ligands that can modulate the anti-inflammatory properties of Tr1 cells both *in vitro* and *in vivo* (reviewed in [31]). The expression of the cytokine IL-21 is further essential for IL-27-induced-IL-10 production [53] (reviewed in [37]). In the absence of IL-21, IL-10 production is reduced in Tr1 cells. IL-21 secretion can be further amplified by AhR activation [69].

4.2.3. Stat3 activation by IL-27 and inhibition of Foxp3

IL-27 inhibits the generation of Foxp3$^+$Tregs [70]. The fact that Foxp3$^+$Tregs express IL-27R strongly suggested that IL-27 might block the development of those regulatory cells *in vitro* [71]. IL-27 indeed leads to a decreased expression of Foxp3 through a mechanism that is at least partially dependent on Stat3 [70]. Smad3 binding to Foxp3 promoter is implicated in Foxp3 transcription. It has been proposed that IL-27-induced pStat3 binds to a gene silencer region (enhancer II) in a conserved region of Foxp3 gene that reduces the acetylation in the region of Smad3 binding site and decreases the binding of pSmad3 to Foxp3 promoter [72]. This results in a decreased accessibility and binding of Smad3 to Foxp3 promoter and thereby decreases *Foxp3* transcription (Fig. 3). IL-27 impacts Foxp3$^+$Treg development and function *in vivo*. Indeed mice that overexpress both IL-27 subunits, IL-27p28 and EBI3, have decreased number of Foxp3$^+$Tregs and developed spontaneous inflammation similar to mice that lack Foxp3$^+$Tregs such as the scurfy Foxp3 mutant mice or IL-2$^{-/-}$ mice [73]. Interestingly, IL-27 transgenic mice are deficient in IL-2. Those results are in accordance with another recent study showing that IL-27 inhibits Foxp3$^+$Treg *in vivo* in a murine T cell transfer colitis model.

Il27ra$^{-/-}$ deficient T cells transferred an attenuated disease due to a larger percentage of transferred cells expressing Foxp3 compared to wild-type T cells [74].

5. Therapeutic implications

5.1. IL-27 confers protection against multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease affecting the central nervous system resulting in inflammation, demyelization and axonal loss. It is a common neurological disorder, which attacks young adults. T_H17 cells were shown to contribute to MS development [75]. By contrast, IL-27 protects against autoimmune inflammation in the mouse model EAE as exemplified by *Il27ra*$^{-/-}$ mice which develop an accelerated EAE disease course compared to WT controls and show increased levels of T_H17 cells in the CNS [44]. Furthermore, daily intrathecal treatment with IL-27 during EAE alleviates the disease and decreases both the inflammation in the brain and the number of infiltrating T_H17 cells [45]. Similarly in a T cell adoptive transfer model, pre-treatment of autoreactive CD4$^+$ T cells with IL-27 leads to a reduction of their pathogenicity in an IL-10 dependent manner [33]. Interestingly, IL-27 was also shown to mediate the protective effect of Bone marrow stromal cells (BMSCs) that prevent EAE in mice and suppress IL-17 production [76].

Support for IL-27 in regulating autoimmune tissue inflammation has also been provided in humans. The immunomodulatory drug IFN-β, used in the first line of treatment for MS, has been shown to induce IL-27 production from dendritic cells (DCs). Interferon (IFN)-β, a member of the type I interferon family, is an approved treatment for relapsing remitting MS (RRMS) that reduces the rate of relapses by 30%. While the therapeutic mechanisms of IFN-β remain poorly understood, recent studies indicate that IL-27 contributes to its regulatory properties both in mouse [77] and human [78,79]. One limitation of IFN-β treatment is that 20–50% of patients fail to respond to therapy thus delaying a change in the treatment strategy of those patients. While the presence of neutralizing antibodies (Nabs) against IFN-β in the blood has been proposed to correlate with treatment failure [80], a proportion of non-responder patients do not develop Nabs, limiting the use of Nabs to predict the response to IFN-β therapy [81]. IL-27 secretion from PBMC from RRMS patients has been proposed as a predictive factor of clinical response to IFN-β treatment. Indeed, PBMC isolated from RRMS patients that respond to IFN-β treatment secrete more IL-27 when exposed *in vitro* to IFN-β than PBMC isolated from "non-responder" patients [78]. Finally, other therapies proposed for treating MS, such as Statins, which in addition to their cholesterol-lowering activity have anti-inflammatory properties, were shown to increase *in vitro* IL-27 secretion from human monocytes of MS patients [82].

5.2. IL-27 protects against rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic inflammatory disorder that principally attacks synovial joints. T_H17 cells and IL-17 expression is elevated in RA synovial tissue and fluid macrophages compared to controls [83,84]. Elevated levels of IL-17 have been reported in the animal model of RA, collagen-induced arthritis (CIA), and IL-17 neutralization prevents bone destruction suggesting a pathological role of T_H17 cells in the development of RA [85]. Administration of IL-27 in mice suffering from CIA reduces the severity of the disease, as shown by reduced cellular infiltration in the joints, synovial hyperplasia, and joint erosion [84]. IL-27 treatment further decreases serum levels of IL-6. In addition, lymphocytes isolated from spleen and lymph node of IL-27-treated mice produce significantly reduced amounts of IFN-γ and IL-17 when cultured with type II collagen *in vitro* compared with lymphocytes from control mice. Similar results were obtained when IL-27 was ectopically expressed in the joints [86]. These studies highlight in the therapeutic potential of IL-27 in RA, especially with the feasibility of local, intra-articular, administration of recombinant IL-27.

5.3. Controversial role of IL-27 in inflammatory bowel disease

IL-27 is implicated in the pathogenesis of IBD, Crohn's disease and ulcerative colitis. Genome wide studies have identified SNPs in the gene encoding p28 subunit associated with a lower expression of IL-27 and early onset inflammatory bowel disease, which would be consistent with a protective role of IL-27 in IBD [87]. Two other studies have found transcripts for IL-27p28 [88] and Ebi3 [89] to be overexpressed in biopsy samples from IBD patients. The function of IL-27 has been assessed using different murine models of IBD. In the mouse IBD model of acute inflammation, which relies on the presence of dextran sulfate sodium (DSS) to induce inflammation, *Il27ra*$^{-/-}$ mice receiving 5–10% DSS in drinking water were more susceptible to disease [90]. *Il27ra*$^{-/-}$ deficient mice showed a reduction in T_H1 IFNγ-producing cells and an increase in T_H17 cells in gut-associated lymphoid tissue pointing towards an important regulatory role of IL-27 in dampening T_H17 cell function [90]. In the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced mouse acute colitis model, subcutaneous scIL-27 (EBI3 and p28 subunits generated as a single-chain human IL-27) treatment significantly improved in a dose-dependent manner the extent of the lesions as well as necrosis, ulceration and thickening of mucosal epithelium. scIL-27 suppressed several inflammatory cytokines in inflamed colon, including IL-17 [91]. However, in a T cell transfer colitis model, IL-27 was shown to exert proinflammatory effects as it suppressed induced Treg development *in vivo* [74]. In contrast, in the DSS model, *Il27ra*$^{-/-}$ mice treated with lower doses of DSS (0.5% in drinking water), were protected compared to WT controls [92]. The implication of different pathogenic or regulatory subsets and the heterogenicity of the models may explain the different responses to IL-27 treatment in murine models of colitis. However, in models where T_H17 cells are implicated in the development of the disease, the anti-inflammatory role of IL-27 appears to be dominant. Indeed, T_H17 cells have been shown to be crucial for the development of TNBS-induced colitis as IL-17 receptor A (IL-17RA) knockout mice do not develop TNBS colitis [93] and IL-17F-deficient mice develop more severe DSS colitis than controls [94]. A better understanding of the pathogenesis of IBD should provide additional insight into the role of IL-27 in colitis.

6. Open questions and concluding remarks

While IL-27 promotes Tr1 cells, it inhibits CD4$^+$Foxp3$^+$Tregs induced by TGF-β. These observations are reminiscent of the action of AhR ligands such as FICZ that promotes Tr1 cells but inhibits Foxp3$^+$Tregs. This paradoxical effect on regulatory T cells might stem from different and/or complementary roles of regulatory T cells. Tr1 cells but not Foxp3$^+$Tregs may develop *in situ* in the inflamed tissue as IL-27 can be secreted by resident cells in the target organ, such as in the brain during EAE and MS. Foxp3$^+$Tregs cannot inhibit highly pathogenic effector T cells in the target organ [95] but they induce tolerogenic plasmacytoid dendritic cell (DC) that secrete IL-27 thus promoting Tr1 cell generation [32]. Under inflammatory settings, Foxp3$^+$Tregs can produce cytokines that belong to other lineages [96,97] and we propose that Tr1 cells could be more stable and thereby regulate tissue inflammation at the target site.

IL-27 controls inflammation by inhibiting T_H17 cells and by promoting the development of IL-10-producing regulatory Tr1 cells. Despite their opposite *in vivo* functions, Tr1 and T_H17 cells harbor striking similarities. First, they rely on the transcription factors Maf and AhR for their generation. Second, they require IL-21 for their growth. Third, they produce IL-10. In this regard, Ghoreschi et al. showed that T_H17 differentiated with TGF-β and IL-6 ($T_H17(\beta)$) produced IL-10 and were poorly pathogenic *in vivo* in contrast to T_H17 cells induced by IL-6, IL-1β and IL-23 (T_H17) (23) that did not produce IL-10 and were highly pathogenic. In addition, TGF-β induced T_H17 expressed higher levels of Maf and AhR compared to T_H17 induced with IL-1, IL-6 and IL-23 (23). This observation would thus be in line with a previous work suggesting that the Maf-driven induction of IL-10 in T_H17 cells reduced their pathogenicity [98]. Since we have shown that the expression of Maf and AhR is required for the production of IL-10 and IL-21 in Tr1 cells, it might be interesting to explore whether IL-27 could actually be converting T_H17 to Tr1 cells. We are currently conducting a functional transcriptional analysis of Tr1 (differentiated with IL-27) and T_H17 (IL-6 and TGF-β) cells using a computational approach and a whole genome microarray analysis to address this question.

In the same line, IL-21 has been ascribed a functional role in promoting both T_H17 [99,100] and Tr1 cells [53]. The role of IL-21 during autoimmune disease such as EAE is controversial. While initial studies have proposed that IL-21R$^{-/-}$ mice presented a less severe EAE disease [100], longer observation of EAE disease course showed that IL-21R$^{-/-}$ mice developed a more severe disease [101,102]. Besides being a growth factor for T_H17 cells [103], IL-21 may behave as an anti-inflammatory effect by promoting IL-10 secretion from different T cell subtypes. It remains to be seen whether IL-27 and its downstream cytokine IL-21 can modulate the pathogenicity and stability of different subtypes of T_H17 cells that have been further treated with IL-23. In conclusion, IL-27 not only induces the generation of anti-inflammatory Tr1 cells but broadly controls autoimmune responses by inhibiting effector T cells in various target organs.

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Review

Type 1 regulatory T cells (Tr1) in autoimmunity

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ABSTRACT

The ability of IL-10 producing Type 1 regulatory T cells (Tr1) to restrain the activation of effector immune cells during autoimmune responses underscores their essential role in maintaining immune tolerance. While mouse studies have demonstrated that increasing the numbers and/or function of Tr1 cells could improve the course of autoimmune diseases, the inability to generate Tr1 cells *in vitro* in large numbers has hampered identification of the molecular mechanisms responsible for their differentiation. Interleukin-27 (IL-27), a member of the IL-12 heterodimeric cytokine family, was identified as an important cytokine that suppresses effector T_H17 cells and promotes the generation of Tr1 cells. Tr1 cells dampen autoimmunity and tissue inflammation partly through their secretion of the immunosuppressive cytokine IL-10. Here we review the molecular mechanisms involved in IL-27-induced Tr1 cell differentiation, with a focus on the role of two transcription factors, the aryl hydrocarbon receptor (AhR) and c-Maf. We also discuss how ligands that bind to AhR and affect the biology of IL-27-induced Tr1 cells can be exploited as a therapeutic approach to alleviate human autoimmune diseases.

1. Introduction

Mosmann and Coffman originally classified $CD4^+$ T lymphocytes into T_H1 and T_H2 subsets [1]. However, the repertoire of effector $CD4^+$ T cell subsets has recently expanded to include additional effector T cell subsets like T_H17 cells [2–5] and T_H9 cells [6,7]. In addition, a number of regulatory T cell subsets have been identified that suppress effector T cells, tissue inflammation and autoimmunity. Interest in regulatory T (Tregs) cells was revived with the discovery of FoxP3, a transcription factor exclusively expressed in Tregs cells, and by the evidence that a genetic loss of FoxP3 and Tregs results in loss of immunological tolerance and induction of multi-organ autoimmune disease in both mice ("scurfy", an X-linked recessive mouse mutant) [8] and humans (immunodysregulation, polyendocrinopathy, and enteropathy, X-linked syndrome IPEX) [9]. Two major classes of $CD4^+$ regulatory T cells have been described: $CD4^+$ $FoxP3^+$ Tregs [10] and type 1 regulatory T cells (Tr1 cells) that secrete IL-10 and lack FoxP3 expression. Tr1 cells are additionally characterized by their high levels secretion of TGF-β and, like $FoxP3^+$ Tregs, by their ability to suppress immune and autoimmune responses [11].

Although extensive studies have been performed to differentiate Tr1 cells from other Tregs, the lack of consensus in their phenotype and differentiation factors have complicated their study. The original protocols designed to generate Tr1 cells *in vitro* involved the activation of naïve T cells in an antigen-specific or TCR-mediated manner in the presence of IL-10 alone, IL-10 together with Vitamin D3, IFN-α or other immunosuppressive drugs such as dexamethasone or Rapamycin [11–14]. In humans, Tr1 cells have also been induced *in vitro* with anti-CD46 antibodies in the presence of IL-2 [15], however mice do not express CD46 and therefore the biological role of CD46 in differentiation of Tr1 cells could not be undertaken in mice. While many of these protocols resulted in the differentiation of IL-10-secreting cells that shared immunosuppressive functions, the inability to expand Tr1 cells in large quantities *in vitro* has hampered progress in understanding the biology of these cells. In this respect, different groups have tried to unravel a specific Tr1 signature gene. Cobbold et al. have compared gene expression between murine Tr1 cells clones with $CD4^+CD25^+Foxp3^+$ Tregs and found that the repressor of GATA-3 (ROG) was expressed specifically in Tr1 cells but not in natural Tregs [16]. However, the *in vivo* relevance of ROG in the biology of Tr1 cells was not further explored as ROG is also expressed in other T_H cells stimulated with anti-CD3 [17]. Overall, Tr1 cells are important for the suppressing tissue inflammation and inhibiting autoimmunity, which underscores a need to identify reliable protocols

Abbreviations: c-Maf, avian musculoaponeurotic fibrosarcoma v-maf; Tr1 cell, type 1 regulatory T cell; AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; FICZ, 6-formylindolo[3,2-b]carbazole.

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to grow them in large numbers for testing their *in vivo* biology and identify molecular mechanisms for their generation and function.

2. Tr1 cells in transplantation and autoimmune diseases

Given the immunosuppressive functions of Tr1 cells, research has focused on developing ways to utilize Tr1 cells to alleviate inflammatory pathologies in a wide range of contexts, in particular in transplantation and autoimmune diseases [11,14,18]. Tr1 cells were first described in severe combined immunodeficient (SCID) patients who had developed long-term tolerance to stem cell allografts, suggesting that these cells might naturally regulate immune responses in humans [19]. Administration of IL-10 in combination with Rapamycin *in vivo* was also shown to induce Tr1 cells that mediated tolerance in type 1 diabetic mice after pancreatic islet transplantation [14]. Furthermore, cell therapy with alloantigen-specific Tr1 cells could promote an IL-10-dependent graft-specific tolerance in a mouse model of islet transplant, eliminating the need of an immunosuppressive treatment [20]. In humans, Tr1 cells may also play an important role in inducing transplantation tolerance, as peripheral blood mononuclear cells (PBMC) isolated from patients who underwent islet transplant and became insulin independent produced significantly higher IL-10 when compared with transplant subjects that continued to be insulin-dependent [21]. Induction of Tr1 cells was also described in patients who spontaneously developed tolerance to kidney or liver allografts [22]. Taken together, these data indicate that IL-10-producing Tr1 cells can be induced under different states of transplantation tolerance and may be naturally involved in inducing tolerance to allotransplants.

Tr1 cells have also been identified in various tissues during autoimmune inflammation. They are, for instance, found in large numbers in the intestine where they are proposed to have a protective role during colitis. Maynard et al. have suggested that Tr1 may play a major role in maintaining immune homeostasis to the intestinal microbiota [23], which is consistent with the finding that loss of IL-10 results in the development spontaneous enterocolitis in IL-10 deficient mice. The role of Tr1 cells in the prevention of autoinflammation was also demonstrated in another colitis model where the pathogenicity of transferred $CD45RB^{high}CD4^+$ T cells into SCID mice could be mediated by co-transfer of murine Tr1 clones derived from $CD4^+$ T cells expressing a transgenic T cell receptor specific for an ovalbumin (OVA) peptide. These cells inhibited colitis only in recipients that received specific antigen (OVA) in their drinking water thus demonstrating that the immune suppression relied on the antigen specific activation of Tr1 cells *in vivo* [11]. Similarly, in mice suffering from experimental autoimmune encephalomyelitis (EAE), the rodent model of human multiple sclerosis (MS), transfer of *in vitro* generated OVA specific Tr1 cells could prevent the development of neurological symptoms when OVA peptide was injected intracranially [12]. Tr1 cells have also been induced *in vivo* using the soluble peptide Myelin Basic Protein (MBP) p87-99, which could reverse ongoing EAE disease in rats immunized with MBP [24]. In addition, Meiron et al. reported that stromal cell-derived factor 1α (CXCL12), redirects the polarization of effector Th1 cells into $CD4^+CD25^-Foxp3^-IL\text{-}10^{high}$ antigen-specific regulatory T cells that suppressed autoimmune inflammation in mice undergoing EAE [25]. Finally, the generation of IL-10-producing T cells was impaired in patients suffering from MS compared to healthy volunteers, suggesting that Tr1 cells may have a protective role during MS [26]. These studies suggest that Tr1 cells may play a crucial role in suppressing autoimmunity, not only in experimental autoimmune disease models, but also in many human autoimmune diseases by suppressing tissue inflammation and maintaining self-tolerance.

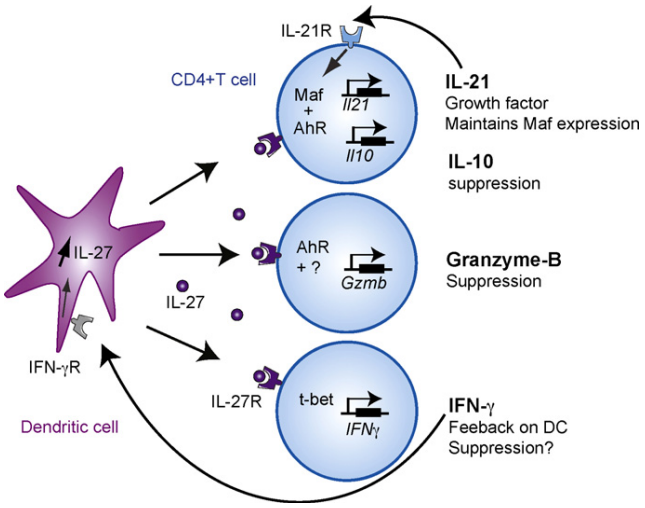

Fig. 1. Molecular mechanisms governing the induction of IL-27-induced Tr1 cells. Three different pathways are induced by IL-27 during Tr1 cell differentiation. IL-27 drives the expression of the transcription factors c-Maf and AhR, which bind together to transactivate the *Il21* and *Il10* promoters. IL-21 maintains *Maf* and *AhR* expression, while IL-10 is essential for the suppressive function of Tr1 cells (upper panel). IL-27-induced AhR, alone or with an unknown cofactor, promotes Granzyme-B expression that mediates the contact-dependent suppressive activity of Tr1 cells (middle panel). Finally IL-27 promotes T-bet expression that results in IFN-γ secretion. IFN-γ then acts on DCs to enhance IL-27 expression and further supports Tr1 cell differentiation (lower panel).

3. IL-27 induces IL-10 producing Tr1 cells

IL-27, a member of the IL-12 cytokine family, which consists of two subunits p28 and the Epstein-Barr virus-induced gene 3 (EBI3), has recently been identified as a differentiation factor for the generation of IL-10-producing Tr1 cells [27–29]. IL-27 is secreted by tolerogenic dendritic cells (DCs) that were conditioned *in vitro* or *in vivo* by $FoxP3^+$ Treg cells. These Tregs-modified DC express plasmacytoid-like markers, similar to what has been previously described for tolerogenic DCs [30].

Based on the structural homology between IL-12 and IL-27, IL-27 was initially described as a proinflammatory cytokine that could act primarily act on naïve $CD4^+$ T-cells to induce T_H1 response [31–33]. This interpretation was consistent with the ability of IL-27 to induce the transcription factor T-bet, the master T_H1 transcription factor that transactivates IFN-γ and IL-12Rβ2 genes (Fig. 1) [31,34,35]. Subsequent work from Chris Hunter's laboratory established that IL-27 was actually not a proinflammatory cytokine but in fact might suppresses excessive immune responses *in vivo* (reviewed in [36]) as IL-27R-deficient mice did not have a defect in inducing T_H1 cells but died due to excessive immune response following *Leishmania* infection [37,38]. Subsequently, three independent laboratories including ours discovered that addition of IL-27 to naïve T cells induced expansion and differentiation of $CD4^+$ T cells that secreted high levels of IL-10 (Fig. 1). Those IL-27-driven Tr1 cells proliferate poorly following TCR-mediated activation and suppress effector T cells *in vitro* partly through IL-10 production [39]. Additional studies revealed that Tr1 cells also suppress effector T-cells responses in a contact dependent manner, possibly through Granzyme-B-mediated lysis, since we found that in addition to IL-10, Granzyme B is induced by IL-27 during Tr1 differentiation (Fig. 2). Finally, IL-27 can also induce suppressive IL-10-producing Tr1 cells in humans from naïve $CD4^+$ T cells [40]. Taken together, these findings provide impetus to evaluate the potential of IL-27-induced Tr1 cells in autoimmune diseases.

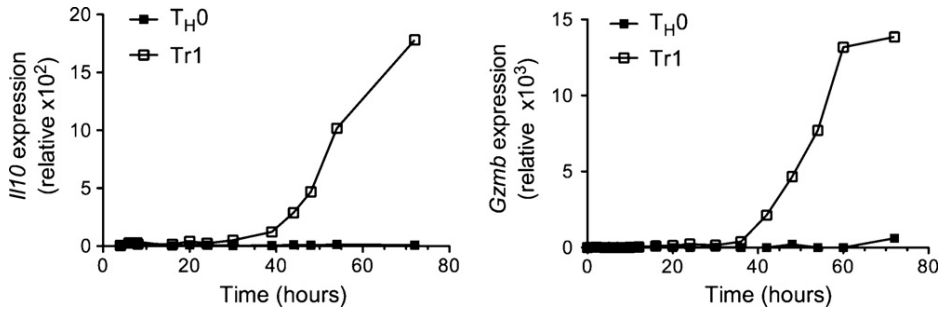

Fig. 2. IL-27 induces Granzyme-B expression. RNA isolated from naïve $CD4^+CD44^{lo}CD62L^{hi}CD25^-$ cells differentiated with IL-27 (25 ng/ml) (Tr1) or without (T_H0) in the presence of anti-CD3 and anti-CD28 antibodies (2 μg/ml) was subjected to quantitative real-time PCR amplification relative to the expression of mRNA encoding β-actin to examine expression of *Il10* (left panel) and *Gzmb* (right panel) *Il10* at different time points following activation.

4. Transcriptional regulation of IL-27-induced Tr1 cells

4.1. Maf and AhR in Tr1 cell differentiation

The molecular mechanisms involved in IL-27-induced Tr1 cell differentiation are progressively being unraveled. We and others have identified an essential role of IL-21, a member of the IL-2 cytokine family that signals through the common gamma-chain receptor, in Tr1 cell differentiation [39,41]. IL-21 secretion was induced by IL-27 and was indispensable for the development of Tr1 cells, as demonstrated by the defective generation of Tr1 cells in IL-21R deficient T cells [39]. Since the expression of IL-21 in T_H17 cells is controlled by the transcription factor c-Maf [42], we have tested *Maf* expression during Tr1 cell differentiation and found that *Maf* indeed was induced at high levels in Tr1 cells (Fig. 1). C-Maf indeed binds *il21* promoter and transactivates *il21* gene. Additional studies of the role of c-Maf during Tr1 cell differentiation using *Maf* deficient T cells revealed that c-Maf was absolutely critical for IL-10 secretion since IL-27-induced Tr1 differentiation was defective in c-$Maf^{-/-}$ mice [39]. Interestingly, IL-21 induced by IL-27 during Tr1 cell differentiation in turn maintains *Maf* expression, suggesting that IL-21 signaling acts as a feed-forward loop to induce c-Maf which further acts as a growth factor for Tr1 cell development (Fig. 1) (reviewed in [36]). It is noteworthy that these findings are reminiscent of results obtained in T_H17 cells where IL-21 was shown to support IL-17 secretion from T_H17 cells through a self-amplifying feed-forward loop [43].

Despite its crucial role as a growth factor for Tr1 cells, IL-21 on its own failed to promote Tr1 cell differentiation from naïve T cells, suggesting the existence of additional factors that are required for Tr1 cell differentiation. By undertaking gene expression profiling of mouse IL-27-induced Tr1 cells, we found that T cell activation in the presence of IL-27 not only induces *Maf* but also the arylhydrocarbon receptor (*Ahr*), a ligand-activated transcription factor that mediates cellular responses to environmental pollutants. While the involvement of AhR in host responses against the prototypical AhR ligand TCDD (Dioxin) have been well-characterized, recent findings regarding the function of the AhR in the immune system have led immunologists to investigate its role in the differentiation of $CD4^+$ T cells. AhR was found to be expressed in both T_H17 and $Foxp3^+$ Treg cells. The activation of AhR signaling by FICZ, a putative endogenous AhR ligand, specifically induced the expression of IL-22, and supported T_H17 cell differentiation [44]. Conversely, the activation of AhR signaling by TCDD enhanced the induction of $Foxp3^+$Tregs and suppressed T_H17 cells [45]. These studies support the contention that AhR, rather than being a lineage specific transcription factor, may promote $CD4^+$ T cell differentiation based on which ligand activates it.

We have found that during Tr1 cell differentiation, AhR is activated as shown by the transcription of its target gene *cyp1a1*. IL-27-induced AhR expression enhanced both IL-21 and IL-10 secretion from developing Tr1 cells [46]. In the absence of AhR signaling, the ability of murine naïve $CD4^+$ T cells activated in the presence of IL-27 to produce IL-10 and IL-21 was severely compromised. We have additionally identified AhR binding sites (xenobiotic response element, XRE) within the *il10* and *il21* promoters and showed that AhR could directly transactivate both promoters in Tr1 cells (Fig. 1). The findings that c-Maf and AhR were concomitantly induced by IL-27 and the reported ability of the AhR to interact directly with other proteins including nuclear factors such as NF-kb [47,48], retinoblastoma protein [49] and estrogen receptor [50] led us test whether AhR can associate with c-Maf. Our results revealed that AhR binds to c-Maf in Tr1 cells resulting in an enhanced transcriptional activity of the *il10* and *il21* promoters. Since AhR signaling controlled Tr1 cells generation and IL-10 secretion, we hypothesized that the immunoregulatory action of IL-27 would be abrogated in the absence of AhR signaling *in vivo*. Our findings indeed revealed that $CD4^+$ T cells isolated from MOG-immunized AhR^d mice, which present defective AhR signaling *in vivo*, showed decreased IL-10 production when treated with MOG and IL-27. While wild-type MOG specific $CD4^+$ T cells treated with IL-27 protected mice against EAE, IL-27 treated $CD4^+$ T cells from AhR^d mice failed to do so.

4.2. Modulation of AhR signaling in Tr1 cell differentiation

We have shown that mouse Tr1 cell induction by IL-27 can be affected by AhR signaling. Naïve $CD4^+$ T cells differentiated *in vitro* with IL-27 in the presence of the AhR ligand TCDD had enhanced secretion of both IL-10 and IL-21. Those results were in accordance with the work of Marshall et al., who showed that regulatory T cells generated upon TCDD administration, prevented graft-versus-host disease in a transplantation model [51]. Interestingly, *in vivo* TCDD-induced regulatory T cells were not only suppressive but also secreted significant amounts of IL-10 in response to polyclonal and alloantigen stimuli. In addition, these TCDD-induced $CD4^+$ T cells expressed Granzyme B but not Foxp3, a phenotype that shares striking similarities with IL-27-induced Tr1 cells (Fig. 1). TCDD is highly stable *in vivo* and thus chronically activates AhR [52,53]. The systemic toxicity resulting from sustained AhR activation has raised concerns regarding chronic engagement of AhR *in vivo* [54]. 6-formylindolo[3,2-b]carbazole (FICZ), a tryptophan derivative, is a photoproduct that is generated in response to UVB stress and leads to the transcriptional induction of cytochrome P450 through AhR activation. UVB irradiation has indeed been shown to generate FICZ in the cytosol of living cells, supporting the idea that FICZ is an endogenous ligand for AhR [55]. Further studies have shown that FICZ, unlike TCDD, is sensitive to xenobiotic-metabolizing enzymes (XMEs) and therefore only transiently activates AhR. Despite their similarity in binding to AhR, TCDD supports $Foxp3^+$ Treg cell dif-

ferentiation while FICZ enhances IL-17 and IL-22 secretion from T_H17 cells [44,56] and inhibits TGF-β-induced Treg development [45]. It has been proposed that the promotion of Treg and T_H17 differentiation by AhR may result from its ability to interact with different transcriptional partners in different cellular contexts [45]. In this regard, it is noteworthy that the addition of FICZ during Tr1 cell differentiation increased both IL-10 and IL-21 from Tr1 cells in a similar manner to TCDD. While the intracellular signaling pathways accounting for these observations remain elusive, the ability of both endogenous and exogenous AhR ligands to enhance IL-27-induced Tr1 cell generation clearly underscores the major role of AhR in the Tr1 transcriptional program.

Tr1 cells may have a role in regulating autoimmune responses in humans. A number of studies have revealed that during the course of inflammatory diseases such as MS, the frequency and the functionality of Tr1 cells are severely impaired [26,57]. In order to contemplate the use of Tr1 cells for cellular therapy of human diseases, it is thus essential to understand the pathways controlling their generation. Like in mouse, AHR was also shown to be essential for Tr1 cell differentiation in humans. The activation of human $CD4^+$T cells with TCDD or FICZ selectively enhanced their IL-10 secretion [58]. Accordingly, functional analyses further confirmed that AHR transactivated the *il10* promoter during human Tr1 cell differentiation, following addition of TCDD. Furthermore, human TCDD-treated T cells exhibited an AHR-dependent suppressive activity, indicating that TCDD-treated T cells harbor not only phenotypic but also functional properties of Tr1 cells. Consistent with results obtained in mouse Tr1 cells, the human AHR was found to interact with the transcription factor c-MAF ultimately resulting in an enhanced IL-10 secretion from Tr1 cells. While these observations are in line with the recently proposed role of AHR in mouse Tr1 cell differentiation, it is noteworthy that the activation of murine $CD4^+$ T cells with TCDD in the absence of any differentiating cytokines was unable to drive Tr1 cell differentiation [46]. Since mouse AhR has at least a 10-fold higher sensitivity for TCDD than human AhR [59], it is unlikely that these phenotypic differences can be attributable to AhR-mediated signaling alone. In this regard, it is notable that the transcription factor c-Maf that is essential for mouse Tr1 cell differentiation is detectable in human T cells activated without IL-27. Since *Maf* expression is strictly dependent on IL-27 in mice, we would postulate that the observation that TCDD mediated expansion of human Tr1 cells is due to their endogenous expression of c-MAF. Indeed, overexpression of c-MAF alone only induces marginal expression of IL-10 from human $CD4^+$ T cells, while the TCDD-driven activation of AhR combined with c-MAF led to significant IL-10 expression [58].

It has been previously shown that IL-10 was necessary but not sufficient for the differentiation and function of human Tr1 cells, and that their capacity to suppress immune responses also relied on Granzyme B and perforin [15,60]. Interestingly, besides increasing IL-10 expression, AHR activation with TCDD induced Granzyme B in human Tr1 cells [58]. Granzyme B was indeed required for the suppressive activity of TCDD-treated T cells as illustrated by the ability of a Granzyme B inhibitor to abrogate the suppressive activity TCDD-induced human Tr1 cells. Overall, results obtained from both mouse and human studies support a major role of AhR in dictating Tr1 cell differentiation and effector functions, thereby opening avenues for therapeutic interventions.

5. Therapeutic use of Tr1 cells

Over the last few years, great progress has been made to differentiate and expand murine and human Tr1 cells *ex vivo*. A subset of IL-10-producing human dendritic cells (DCs), termed DC-10, were recently characterized *in vivo* and can be induced *in vitro* with IL-10. DC-10 express $CD14^+$, $CD16^+$, $CD11c^+$, $CD11b^+$ and $HLA\text{-}DR^+$ as well as the costimulatory molecules CD40, CD80 and CD86. DC-10 have been shown to support antigen-specific IL-10-producing Tr1 cell differentiation. Importantly, monocyte-derived DC-10 could induce IL-10-producing antigen-specific Tr1 cells from naïve T cells after a single round of stimulation [61]. While these results support the development of cell therapy strategies relying on Tr1 cells (reviewed in [62]), the implementation of cell therapy protocols requires a clinical cell therapy unit with strong expertise in good manufactory practice along with highly skilled and trained personnel.

Thus, while the generation of Tr1 cells using IL-27 could be hindered by its short *in vivo* half-life, strategies designed to enhance IL-27 induction from DC and/or to target downstream mediators of the IL-27R pathway in Tr1 cells could enhance immunosuppressive Tr1 cell immunosuppressive functions, thereby providing an attractive alternative for the treatment of autoimmune disorders.

5.1. Affecting DC biology to support Tr1 differentiation

Immunosuppressive drugs have been shown to increase IL-27 production from DCs. Among the therapies implemented for treating MS, statins, beyond their cholesterol-lowering activity, were indeed recently ascribed to have anti-inflammatory properties. Simvastatin was for instance shown to increase IL-27 secretion from human monocytes of MS patients (Fig. 3) [63], suggesting that IL-27 might contribute to the anti-inflammatory effects of this treatment. Similarly, IFN-β, a member of the type I interferon family that is approved for the treatment of MS, promotes IL-27 from DC in humans (Fig. 3) [64]. The molecular mechanisms by which IFN-β increases IL-27 production have been studied using murine macrophages. It was demonstrated that IFN-β increased IL-27 production through Toll-IL-1 receptor domain-containing adaptor using IFN-β (TRIF) as illustrated by a reduced IL-27 secretion from TRIF-deficient macrophages in response to LPS-induced type I IFN. Additionally, type I interferon receptor $IFNAR^{-/-}$ mice were shown to be highly sensitive to EAE and this was associated with a decreased IL-27 expression in central nervous system (CNS) tissues compared to wild-type mice. Importantly, the severity of the disease in $IFNAR^{-/-}$ mice was alleviated by administration of recombinant IL-27 protein, suggesting that IFN-β-mediated IL-27 secretion protects the CNS from autoimmune injury. Since IFN-β-mediated IL-27 production by innate immune cells was critical for the immunoregulatory role of IFN-β in this mouse model of CNS autoimmune disease [65], it is tempting to speculate that drugs that selectively target the TRIF or type I IFN signaling pathways could increase IL-27 production (Fig. 3) from innate immune cells and promote Tr1 cell differentiation.

DC maturation is an essential step in the induction of T-cell responses as it leads to the upregulation of costimulatory molecules that provide essential second signals for T cell activation and differentiation [66]. Alterations in the glycan structure during DC maturation led to the hypothesis that protein–glycan interactions may affect the DC/T cell crosstalk. Ilarregui et al. have reported that galectin 1, an endogenous glycan-binding protein, could support DC ability to promote immunosuppression. DC exposed to galectin-1 secreted IL-27 (Fig. 3), induced T cell tolerance and abrogated neuroinflammation in an IL-10-dependent manner. In line with these findings, in DC galectin-1 expression was the highest during the peak and the resolution of EAE. While the molecular mechanisms that control the ability of galectin-1 to enhance IL-27 secretion from DC remain unclear, these findings suggest that the manipulation of this immunoregulatory circuit between galectin-1, IL-27-secreting DC and IL-10-secreting Treg/Tr1 cells could provide therapeutic tools for the treatment of autoimmune disorders [67].

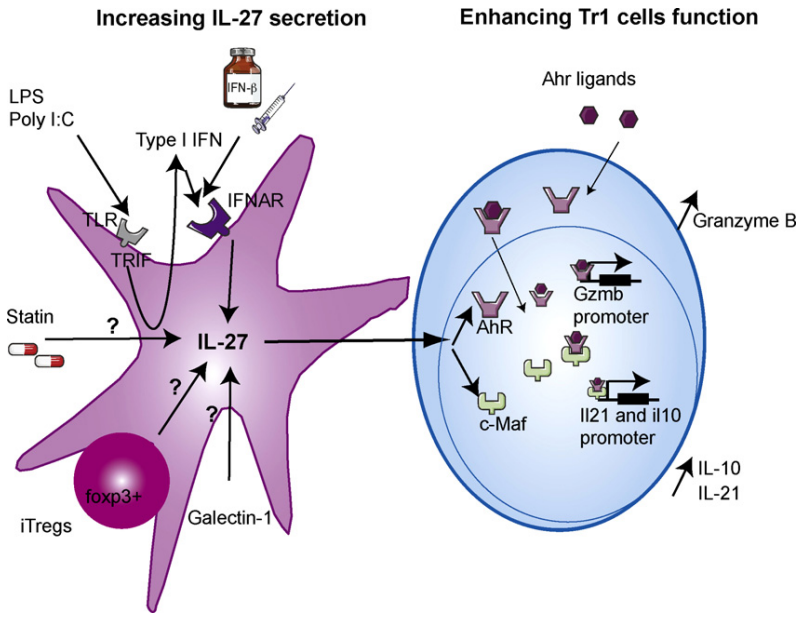

Fig. 3. Pharmacologic tools to support the differentiation of IL-27-induced Tr1 cells. Different treatments induce IL-27 secretion from dendritic cells (left panel) that will in turn enhance the development of Tr1 cells, resulting in the resolution of autoimmune inflammation. TLR stimulation with Poly I:C or LPS will activate TRIF, leading to endogenous type I IFN secretion. Endogenous type I IFN or exogenous treatment with IFN-β signals through their receptor IFNAR and enhance IL-27 expression. Statins, Galectin-1 as well as Foxp3$^+$ induced Tregs promote IL-27 secretion through yet unknown mechanisms. IL-27 induces c-Maf and AhR expression in Tr1 cells (right panel). AhR ligands bind to the cytoplasmic AhR and both translocate into the nucleus. These newly formed complexes enhance the expression of Granzyme B and bind to c-Maf to transactivate *Il10* and *Il21* promoters.

5.2. Modulating the intrinsic signaling of Tr1 cells

We have recently identified the role of the AhR during Tr1 cell differentiation. Different AhR ligands have been shown to promote either T cell inflammation or tolerance [45] as illustrated by the ability of FICZ or TCDD to induce T_H17 and Treg differentiation *in vitro*, respectively. AhR ligands, such as the polycyclic aromatic hydrocarbons, are rapidly metabolized by AhR-inducible enzymes to generate active metabolites that could produce diverse effects on the immune system. There would therefore be a strong rationale for identifying AhR ligands that differentially induce Tr1, Foxp3$^+$Tregs or T_H17 cells in order to regulate T-cell differentiation and the quality of immune responses *in vivo*.

In vivo immunologic effects of AhR activation are tissue- and/or ligand-specific. Indeed, AhR ligands can modulate immune response differently depending on the cytokine milieu. For example, in an inflammatory environment where IL-6 is a predominant cytokine, FICZ promotes and supports T_H17 cell differentiation, as in the presence of the anti-inflammatory cytokines IL-27 and TGF-β, it promotes Tr1 cells. In this regard, AhR transcription factor can thus be regarded as a stabilizer of different CD4$^+$ T cell phenotypes depending on the environmental context and the transcription factors it can interact with to promote T cell differentiation.

TCDD is highly toxic and can lead to hepatocellular damage, epithelial changes, cancer, birth defects, thymic involution [68], ruling out its clinical application. There is therefore a need to design non-toxic AhR ligands that could constitute potential new drugs for the therapeutic induction or inhibition of Tr1 cells (Fig. 3). In this regard, it is notable that we are exposed to numerous naturally occurring AHR ligands both through endogenous biological processes and diet. Some of these compounds are converted in the gut to high-affinity AHR ligands. Indole-3-carbinol, a metabolite of glucobrassicin found in cruciferous vegetables, is a weak AHR ligand that is converted to its acid condensation product indolo-(3,2-b)-carbazole and 3,3′-diindolylmethane that binds and activates AHR with high affinity. Another AhR ligand provided through diet is resveratrol, a compound found in a large number of plant products including mulberries, peanuts, and red grapes. Resveratrol is already commercialized as a nutritional supplement in the market because of its anti-oxidant properties. Resveratrol treatment decreased the clinical signs and inflammatory responses in experimental allergic encephalomyelitis (EAE)-induced mice [69]. The authors noted an up-regulation of Foxp3 gene in splenocytes of mice treated with resveratrol and decreased production of IL-17 in the sera, compared with control mice. In a separate study, resveratrol fed orally during EAE has been shown to increase IL-10 production by T cells in the brain of EAE mice [70].

AhR ligands are also produced during different endogenous biological processes. The essential amino acid tryptophan (Trp) is metabolized and photo-oxidized into multiple AHR ligands. Kynurenine, the first breakdown product in the IDO-dependent tryptophan degradation pathway, binds and activates AhR to promote the generation of CD25$^+$Foxp3$^+$ Tregs but not T_H17 cells [71]. Absence of the AhR in T cells prevented these ligands from inducing Foxp3$^+$ T cells. Furthermore, the antiallergic drug Tranilast, approved for bronchial asthma, is a derivative of the tryptophan metabolite 3-hydroxyanthranilic acid. It has been shown to suppress EAE through the induction of regulatory cells [72]. Furthermore, it has been proposed that the binding and activation of AHR by Tranilast could be responsible for the generation of those Tregs [73].

6. Open questions and concluding remarks

As no lineage specific transcription factor or specific surface marker for Tr1 cells has been identified [74], the identity of Tr1 cells as a unique T cell subset is still debated. IL-10-secreting regulatory T cells could be generated from different T_H cells that have undergone chronic stimulation, resulting in the disappearance of effector T-cell cytokines but the maintenance of IL-10 levels. Indeed, "regulatory IL-10-producing T_H1 cells" have been described which are similar in their phenotype to IL-27 induced Tr1 cells in that IL-27

also induces IL-10 together with the transcription factor T-bet that induces IFN-γ production (Fig. 1). Whereas IL-10 can suppress T cells and DC function, IFN-γ produced by IL-27 induced Tr1 cells is also critical in inhibiting immune responses. In a study by Murugaiyan et al., it was shown that it was not IL-10 but IFN-γ produced by Tr1 cells that suppressed T_H17 responses [75].

While there is still ongoing debate regarding the origin of Tr1 cells, it is now established that those cell play a major role in controlling tissue inflammation and autoimmune responses. Modulation of their function and number using IL-27-promoting drugs or different AhR ligands could suppress immune inflammation through the induction of Tr1 cells. The efficacy of aminoflavones (AFP464; Tigris Pharmaceuticals Industry), that have been reported to target the AhR, are being evaluated in a phase II clinical trial in breast cancer patients (ClinicalTrials.gov: NCT01015521), suggesting that AhR ligands devoid of toxicity can be effectively utilized to promote Tr1 differentiation. Further research is now warranted to evaluate the potential of AhR targeting for the treatment of autoimmune diseases.

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Molecular Pathways in the Induction of Interleukin-27-Driven Regulatory Type 1 Cells

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Type 1 regulatory (Tr1) cells have emerged as key players in the prevention of autoimmunity. They produce high levels of the immunosuppressive cytokine interleukin (IL)-10 and confer protection against a wide panel of autoimmune diseases. However, the molecular pathways leading to their generation have long remained elusive. We have recently identified IL-27, a member of the IL-12 cytokine family, as a novel cytokine that induces Tr1 cells. Further analysis of IL-27-driven Tr1 cells have identified a critical role of the transcription factor avian musculoaponeurotic fibrosarcoma v-maf and of IL-21 in the generation of IL-27-induced Tr1 cells. Importantly, IL-27 also induces Tr1 cells in humans, suggesting that IL-27 administration may dampen tissue inflammation in humans as well. Here, we review the role of IL-27 in the generation of Tr1 cells and discuss its potential to alleviate autoimmune diseases.

Introduction

Type 1 regulatory cells in autoimmune diseases

Regulatory T cells (Tregs) have a fundamental role in the establishment and maintenance of tolerance. Deficits in the numbers and/or function of different types of Tregs were shown to contribute to the development of autoimmunity, allergy, and graft rejection (Wing and Sakaguchi 2010). Conversely, an overabundance of Tregs can inhibit immune response to tumors and infections (Zou 2006). Two important classes of Tregs within the $CD4^+$ subset have been identified: $FoxP3^+$ Tregs and interleukin (IL)-10-producing type 1 regulatory (Tr1) cells. Despite their common role in the regulation of immune responses, these 2 Treg subsets feature major differences in their biology, including the cytokines that induce them and the mechanisms by which they mediate their suppressor function (Roncarolo and others 2006). Whereas both regulatory populations produce IL-10, Tr1 cells do not express the master Treg transcription factor Foxp3, a forkhead box family transcription factor associated with the generation of natural Tregs (Batten and others 2008).

Tr1 cells were first described in severe combined immunodeficient (SCID) patients who had developed long-term tolerance to stem cell allografts, suggesting that these cells suppressed immune responses in humans (Bacchetta and others 1994). The regulatory properties of Tr1 cells were further exemplified in another study that demonstrated that *ex vivo* activation of human or mouse $CD4^+$ T cells with high doses of IL-10 induced T cell clones with a cytokine secretion profile distinct from that of T helper 1 (Th1) or Th2 cells but similar to that of host-reactive T cell clones isolated from successfully transplanted SCID patients (Groux and others 1997).

$CD4^+$ Tr1 cells are characterized by their low proliferative capacity and their high levels of IL-10 secretion. The ability of Tr1 cells to downmodulate effector T cell responses has been ascribed to their high IL-10 production (Groux and others 1997). Although the suppressive activity of Tr1 cells can be reversed by the neutralization of IL-10, additional mechanisms such as the secretion of transforming growth factor (TGF)-β (Groux and others 1997) and cytotoxicity (Grossman and others 2004) also contribute to their regulatory function.

IL-10 has been known to exert an immunosuppressive activity for many years as it was first identified by its ability to inhibit T cell activation and effector functions *in vitro* (Moore and others 1990; de Waal Malefyt and others 1991). The importance of antiinflammatory properties of IL-10 was confirmed in IL-10-deficient animals. It was indeed shown that IL-10 deficient mice develop spontaneous colitis in their early age (Kuhn and others 1993). Similarly, immunization of IL-10 deficient mice with myelin antigens showed enhanced neuroinflammation with loss of recovery in experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis (MS) (Bettelli and others 1998). Altogether, these findings pointed to a key role of IL-10 in suppressing immune responses and maintaining tolerance. Many cell types have been described to produce IL-10 such as dendritic cells (DCs), macrophages, and Th1 and Th2 cells (Moore and others 2001). In contrast to Th2 cells, Tr1 cells produce TGF-β but very low levels of IL-2 and no IL-4 (Groux and others 1997). However, one striking feature of Tr1 cells is their

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ability to secrete particularly high levels of IL-10. It is because of their ability to produce overwhelming amounts of IL-10 that Tr1 cells have been shown to represent one of the main T-cell mediators of cytokine-dependent immune regulation in both mice and humans.

These pioneering studies provided impetus to study the potential of Tr1 cells to dampen tissue inflammation *in vivo*. Subsequent analysis performed using experimental models have demonstrated that manipulating the numbers and/or function of Tr1 cells alleviates pathology in a wide range of contexts, including transplantation and autoimmune diseases (Groux and others 1997; Battaglia and others 2006; Ahangarani and others 2009). Tr1 cells were indeed shown to be abundant in the intestine, and it has been proposed that their major function was to maintain immune homeostasis to the intestinal microbiota (Maynard and others 2007). The *in vivo* immunosuppressive activity of Tr1 cells in colitis was exemplified by studies from Groux et al., who showed that colitis induced in SCID mice by transfer of CD45RBhigh $CD4^+$ T cells could be prevented by cotransfer of murine Tr1 clones derived from $CD4^+$ T cells that expressed a transgenic T cell receptor specific for a peptide of ovalbumin (OVA). Importantly, the immune suppression relied on the antigen-specific activation of Tr1 cells *in vivo* as these cells inhibited colitis only in recipients that received specific antigen (OVA) in their drinking water. The regulatory properties of Tr1 cells have also been examined in human patients suffering from MS. In an elegant study, Astier and others (2006) showed that the generation of Tr1 cells from peripheral $CD4^+$ T cells was greatly impaired in MS patients in comparison to healthy volunteers.

Given the strong immunosuppressive functions exhibited by Tr1 cells, several groups have attempted to generate large numbers of Tr1 *in vitro*, with the long-term goal to transfer Tr1 cells into humans suffering from autoimmune and inflammatory diseases. Tr1 cells have so far been generated *in vitro* by activating naïve $CD4^+$ T cells in the presence of either IL-10 alone, IL-4 and IL-10 (Groux and others 1997), IL-10 and interferon (IFN)-α (Levings and others 2001), or 1,25 (OH) 2-vitamine D3 and dexamethasone (Barrat and others 2002; Roncarolo and others 2006). Moreover, tolerogenic DCs or DCs treated with IL-10 have also been shown to favor the generation/expansion of Tr1 cells (Mahnke and others 2003). Although these reports have increased our understanding on the biology of Tr1 cells, it has been difficult to expand Tr1 cells *in vitro* because of the immunosuppressive nature of IL-10 that prevents further proliferation of the differentiated Tr1 cells. We and others therefore investigated the role of other cytokines for their ability to promote the differentiation of naïve $CD4^+$ T cells into Tr1 cells and found that IL-27, a cytokine of the IL-12 family, promotes the generation of Tr1 cells (Awasthi and others 2007; Fitzgerald and others 2007b; Stumhofer and others 2007).

The Biology of IL-27-Induced Tr1 Cells

IL-27 as an immunoregulatory cytokine

IL-27 is a heterodimeric cytokine that consists of Epstein-Barr virus–induced gene 3, an IL-12p40-related protein, and p28, a newly discovered IL-12p35-related polypeptide, which signals through a receptor containing the common IL-6 receptor chain gp130 and the unique IL-27 receptor α chain also called WSX-1 (Pflanz and others 2002, 2004). WSX-1 is expressed on T and B cells, natural killing cells, mast cells, monocytes/macrophages, DCs, and endothelial cells (Pflanz and others 2004). IL-27 was initially described as a proinflammatory cytokine. On the basis of the structural homology between IL-12 and IL-27, it was first proposed that IL-27 could sensitize lymphocytes to IL-12 and thus promote Th1 responses. Initial studies indeed showed that recombinant IL-27 could increase naïve $CD4^+$ T cell proliferation and mediate IFN-γ production. Furthermore, this interpretation was consistent with the ability of IL-27 to induce the transcription factor T-bet, a master transcription factor whose target genes IL-12Rβ2 and IFN-γ are essential components of Th1 responses (Hibbert and others 2003; Lucas and others 2003; Takeda and others 2003). Consistent with this hypothesis, naïve WSX-1-deficient $CD4^+$ T cells produced less IFN-γ than wild-type cells when cultured under non-polarizing condition (Chen and others 2000; Yoshida and others 2001).

However, the aforementioned findings were later questioned by studies where WSX-1-deficient mice were challenged with intracellular pathogens. The analysis of WSX-1-deficient mice infected with *Toxoplasma gondii* unexpectedly revealed that, although the mice efficiently controlled parasitic inflammation, they developed severe immunopathology and died because of T-cell-mediated tissue inflammation (Villarino and others 2003). Similarly, infection of WSX-1-deficient mice with *Leishmania donovani* resulted in clearance of parasites with enhanced proinflammatory cytokines production and exaggerated T cell responses, resulting in severe immunopathology (Artis and others 2004; Rosas and others 2006), and infection with *Trypanosom cruzi* led to the development of immune-mediated liver necrosis (Hamano and others 2003). This phenotype was not only restricted to parasitic infections as WSX-1-deficient mice displayed more severe lung pathology after *Mycobacterium tuberculosis* and ultimately succumbed because of overwhelming inflammation (Pearl and others 2004; Holscher and others 2005).

These studies highlighted the important regulatory functions of IL-27, and this was further strengthened by the direct effects that IL-27 has on the development of other effector T cells like Th17 cells. Th17 cells that produce cytokine IL-17A, IL-17F, IL-21, and IL-22 are highly proinflammatory effector T cells that play an important role in the development of tissue inflammation and autoimmunity. In this regard, WSX-1-deficient mice showed enhanced central nervous system (CNS) inflammation in mice infected with *Toxoplasma Gondii* (Stumhofer and others 2006) and were highly susceptible to development of EAE with enhanced Th17 responses (Batten and others 2006). Further, administration of recombinant IL-27 was able to reduce the frequency of proinflammatory Th17 cells, resulting in a decreased severity in autoimmunity (Fitzgerald and others 2007b). These results led to hypothesis that IL-27 may not be necessary for the generation of proinflammatory T cells but may instead play a crucial role in regulating proinflammatory T cell responses.

The identification of IL-27 as an inhibitor of Th1 and Th17 responses suggested that IL-27 may have a broader role in controlling T cell responses, raising the possibility that IL-27 may induce regulatory T cells that control effector T cell responses and prevent development of tissue inflammation.

IL-27 in the promotion of Tr1 cells

IL-27 has recently been identified as a differentiation factor for the generation of IL-10-producing Tr1 cells that lack Foxp3 expression and regulate T cell functions in an IL-10-dependent manner (Awasthi and others 2007; Fitzgerald and others 2007b; Stumhofer and others 2007). DCs primed *in vivo* by induced Foxp3$^+$ Tregs have been reported to secrete IL-27 and suppress immune responses. These Tregs-modified DCs express plasmacytoid-like markers, similar to what has been previously described for tolerogenic DCs (Ochando and others 2006), which in turn induced generation of IL-10-producing Tr1-like cells by an IL-27-dependent mechanism (Awasthi and others 2007). These findings were recapitulated *in vitro* as the addition of IL-27 to naïve T cells was able to drive the expansion of Tr1 cells, thus underscoring that IL-27 is indeed an essential differentiation/growth factor for Tr1 cell generation both *in vitro* and *in vivo* (Awasthi and others 2007). These findings provided a means by which Tr1 cells could be grown in large numbers *in vitro* and facilitated their functional analysis. However, the molecular mechanism by which IL-27 induces generation and/or expansion of Tr1 cells is just beginning to be understood (see below).

IL-21 is essential for the IL-27-induced expansion of Tr1 cells

Although IL-10 secretion is crucial for the effector functions of Tr1 cells, early studies have ruled out IL-10 as a potential growth factor for these cells. In an attempt to identify essential factors for Tr1 cell expansion, we have analyzed different cytokines secreted during the course of Tr1 cell differentiation and made the unexpected finding that IL-27-driven Tr1 cells secrete IL-21. IL-21, a member of IL-2 cytokine family, was initially identified as a soluble molecule produced by activated human T cells (Parrish-Novak and others 2000). IL-21 signals through a heterodimeric receptor containing IL-21R and the common cytokine receptor γ chain, which is also shared by other receptors like IL-2, IL-4, IL-7, IL-9, and IL-15 (Asao and others 2001). IL-21 has been reported to enhance humoral responses and to synergize with IL-7 or IL-15 to promote CD8$^+$ T-cell expansion. On the other hand, IL-21 has been reported to dampen innate and adaptive immune responses by inducing B cell apoptosis and inhibiting DC maturation and function, thus suggesting that the effects of IL-21 may vary depending on the biological context (Leonard and Spolski 2005). In a recent report using BXSB-Yaa mice with systemic lupus erythematosus, Spolski and colleagues reported that IL-21 overexpression decreases specific antibody production after immunization in an IL-10-dependent fashion and that IL-21 signaling is required for maximal induction of IL-10 by IL-6 or IL-27 (Spolski and others 2009). This report therefore suggested that IL-21 may exert its immunosuppressive activity by inducing IL-10 production.

Recent studies have shown that Th1, Th2, and Th17 CD4$^+$ T cell subsets also secrete IL-21. IL-21 has been proposed as a growth factor for Th17 cells (Korn and others 2007a; Nurieva and others 2007; Wei and others 2007). Given the strong links with the expression of IL-21 and the promotion of IL-10 secretion *in vivo*, we hypothesized that IL-27-driven IL-21 production from T cells may similarly act as an autocrine growth factor for the generation of Tr1 cells. To test this, we first isolated naïve T cells and differentiated them with IL-27 and assessed their ability to secrete IL-21. We found that IL-27-induced Tr1 cells, like Th17 cells, secrete substantial amounts of IL-21 (Pot and others 2009). To test the relevance of IL-21 secretion on the biology of IL-27-induced Tr1 cells, we then generated Tr1 cells with IL-27 but in the presence of a neutralizing IL-21 antibody. Consistent with our hypothesis, blockade of IL-21 significantly reduced the frequency of IL-10-producing T cells. We further confirmed the critical role of IL-21 in the expansion of Tr1 cells using naïve CD4$^+$ T cells from IL-21R-deficient mice as the loss of IL-21 signaling also abrogated the generation of Tr1 cells even in the presence of IL-27. In contrast to recent studies using total CD4 splenocytes (Spolski and others 2009), it was noteworthy that the addition of recombinant IL-21 alone (without IL-27) on cell-sorted naïve CD4$^+$ T cells was unable to generate Tr1 cells. This shows that IL-21 is necessary but not sufficient to induce the generation of Tr1 cells and suggested that additional molecular signals triggered by IL-27 are required for Tr1 differentiation.

Transcriptional Regulation of Tr1 Cells Generated with IL-27

Avian musculoaponeurotic fibrosarcoma v-maf

To identify the molecular signals that coordinately act together with IL-21 for the differentiation of Tr1 cells, we performed a microarray analysis of naïve T cells generated in the presence of IL-27 and found that the transcription factor avian musculoaponeurotic fibrosarcoma v-maf (c-Maf) was induced by IL-27 in Tr1 cells. c-Maf belongs to a growing family of basic leucine zipper transcription factors (Blank and others 1997) and was first proposed to be essential for IL-4 production and differentiation of Th2 cells (Ho and others 1998; Hwang and others 2002). Subsequent studies have shown that the engagement of the Stat3 pathway by IL-6 leads to increased c-Maf expression as Stat3 transactivates the c-Maf promoter (Yang and others 2005). Our previous work had revealed that c-Maf was strongly upregulated in naïve T-cells differentiated into Th17 cells with TGF-β and IL-6 (Bauquet and others 2009). The role of c-Maf in different population of CD4$^+$ T cells has been recently reassessed, and it has been proposed that c-Maf expression, in addition to inducing IL-4 in Th2 cells, was necessary for IL-10 expression in multiple CD4$^+$ T helper subsets (Saraiva and others 2009). These results are supported by the study of Xu and others (2009), who showed that c-Maf directly transactivates the *il10* promoter through binding to a conserved binding site (half Maf recognition element [MARE] located 500 bp upstream of the transcription initiation site in the *il10* promoter).

Tr1 cells differentiated for 4 days in the presence of IL-27 had 500-fold higher levels of c-Maf expression than Th1, Th2, or Th0 cells reaching similar levels to Th17 cells. Upon monitoring the kinetics of c-Maf expression during the differentiation of Tr1 cells with IL-27, we noted that c-Maf expression could be detected as early as 12 h after the initiation of the differentiation. To investigate the role of c-Maf in the induction of Tr1 cells, we differentiated either wild-type or c-Maf-deficient naïve CD4$^+$ T cells into Tr1 cells with IL-27. Strikingly, we noted that the absence of c-Maf greatly impaired the generation of IL-10-producing Tr1 cells,

suggesting that c-Maf is critically involved in the generation of Tr1 cells (Pot and others 2009).

Given that we had previously shown that c-Maf is controlling IL-21 expression in Th17 cells, we hypothesized that c-Maf might induce Tr1 cells by transactivating *il21* promoter. This contention was further supported by our observations, indicating that c-Maf and IL-21 were concomitantly expressed during Tr1 cell differentiation, suggesting that c-Maf could be a transcription factor for inducing IL-21 gene transcription. We thus investigated whether c-Maf could possibly bind to the *il21* promoter and we identified 4 putative conserved binding sites located 1070 bp (half MARE), 370 bp (v-MARE), 260 bp (half MARE), and 200 bp (v-MARE) upstream of the transcriptional start site (where MARE is Maf recognition element and v-MARE is v-Maf recognition element). The functional relevance of those sites for the binding of c-Maf was then verified by cotransfecting of HEK 293T cells with a c-Maf expression plasmid and an *il21* promoter-luciferase reporter. In this setting, we demonstrated that c-Maf could transactivate the *il21* promoter-luciferase in a dose-dependent manner, supporting the hypothesis that c-Maf expands Tr1 cells by inducing IL-21 production. We finally showed that while c-Maf-deficient T cells activated in the presence of IL-27 featured a sustained production of IFNγ, the cells were unable to secrete IL-21 and IL-10. Collectively, these results established that c-Maf, by transactivating *il21* and *il10* promoters, is dictating the generation of IL-27-induced IL-10-producing Tr1 cells.

Inducible T-cell Costimulatory Molecule

The inducible costimulatory molecule (ICOS), a member of the CD28/CTLA4 family, was initially reported to be a costimulatory molecule responsible for the induction of IL-10 (Hutloff and others 1999). However, later ICOS was found to be expressed on activated T cells and has been shown to play an essential role in the expansion of all T cells in that T cell activation, growth, and survival are defective in the absence of ICOS. In addition, the finding that ICOS-deficient mice showed greatly enhanced susceptibility to EAE indicated that ICOS has a protective role in inflammatory autoimmune diseases (Dong and others 2001). Expression of ICOS *in vivo* by $CD4^+$ T cells has been associated to their high secretion of IL-10 (Lohning and others 2003).

We have recently identified a role for ICOS in generation of Th17 cells. While ICOS was not essential for the differentiation of Th17 cells, we showed that ICOS-deficient mice demonstrated a defect in sustained expansion of Th17 cells after IL-23 stimulation. We could ascribe this defect as an essential function of ICOS to maintain a sustained level of c-Maf and thus continued IL-21 expression during Th17 differentiation (Bauquet and others 2009). We later found that ICOS also played an important role in stabilizing the phenotype of Tr1 cells. We first noticed that IL-27 upregulated ICOS expression in Tr1 cells (Pot and others 2009). Using ICOS-deficient T cells, we showed that the generation of IL-10-producing T cells was impaired at late stages of differentiation compared to wild-type T cells. Altogether, these findings led us to propose that ICOS, like in Th17 cells, in fact maintains c-Maf expression and promotes Tr1 cell differentiation. Our results would therefore suggest that ICOS signaling stabilizes IL-10 and IL-21 expression by maintaining c-Maf expression independently of IL-27 signaling. On the basis of our data we propose a model of self-sustainable generation of Tr1 cells in which an innate cytokine like IL-27 secreted by DCs first induces differentiation of naïve T cells into Tr1 cells. Subsequent expression of ICOS and IL-21 by differentiated Tr1 cells enables them to maintain their phenotype in the absence of IL-27. Overall, the differentiation of IL-27-induced Tr1 cells is therefore dependent on the concerted action of three different molecules, c-Maf, ICOS, and IL-21 (Pot and others 2009) (Fig. 1).

Clinical Implications of IL-27- and IL-27-Induced Tr1 Cells

Four single-nucleotide polymorphisms within the human IL-27 gene have been associated with an enhanced susceptibility to asthma (Chae and others 2007) and inflammatory bowel disease (Li and others 2009), suggesting an important role of IL-27 in human autoimmune diseases. However, the *in vivo* role of IL-27 in humans is poorly understood. In a recent study, Murugaiyan and others (2009) showed that IL-27 can induce IL-10-producing Tr1 cells from human's PBMC that can inhibit the proliferation of $CD4^+$ T cells in an IL-10-dependent manner. Similar to murine T cells, IL-27 was shown to regulate various molecules associated with the function and maintenance of Th17 cells. Indeed, IL-27 1) substantially reduced expression of the Th17 lineage-specific transcription factor Rorc; 2) decreased the production of the signature Th17 cytokines IL-17A, IL-17F, and IL-22; 3) reduced expression of IL-23R, which is responsible for the maintenance of Th17 lineage; and 4) modulated expression of the chemokine CCL20 and its receptor CCR6. IL-27 was also shown to inhibit IL-1β, IL-6, and IL-23 secretion from DCs and thereby impair the generation of Th17 cells (Murugaiyan and others 2009). These results confirm previous findings obtained in rodents that indicate that IL-27 not only induces Tr1 cells but also decreases the pathogenicity of Th17 cells (Fitzgerald and others 2007a). In conclusion, IL-27 appears to be a very potent suppressive cytokine that inhibits generation of Th17 cells and concomitantly induce Tr1 cells both in humans and in rodents.

Another interesting aspect of IL-27 lies in the ability of this cytokine to be produced directly at the site of inflammation. Li and others (2005) reported that IL-27 is secreted by antigen-presenting cells (APCs) at the inflammatory interface between pathogenic cells and vulnerable neurons in the CNS of mice undergoing EAE. Further, microglia cells, resident brain APCs, have also been shown to secrete IL-27 (Sonobe and others 2005). While FoxP3+ Tregs fail to control autoimmune inflammation in the brain (Korn and others 2007b), this observation raises the possibility that IL-27 may be produced at the inflammation site and inhibit tissue damage by concomitantly dampening the proinflammatory functions of Th17 cells and promoting *in situ* differentiation of Tr1 cells (Fig. 2). This is further supported by the work from our laboratory that showed that tolerogenic, IL-27-secreting DCs could be induced by $FoxP3^+$ Tregs. $FoxP3^+$ Tregs are not able to readily suppress the activated effector cells from the brain during CNS inflammation, but the effector T cells eventually decrease with commensurate increase in $FoxP3^-$ IL-10-producing T cells that are phenotypically similar to Tr1 cells, resulting in resolution of inflammation. Tr1 cells may therefore play a crucial role in resolving inflammation and inducing recovery in an autoimmune disease.

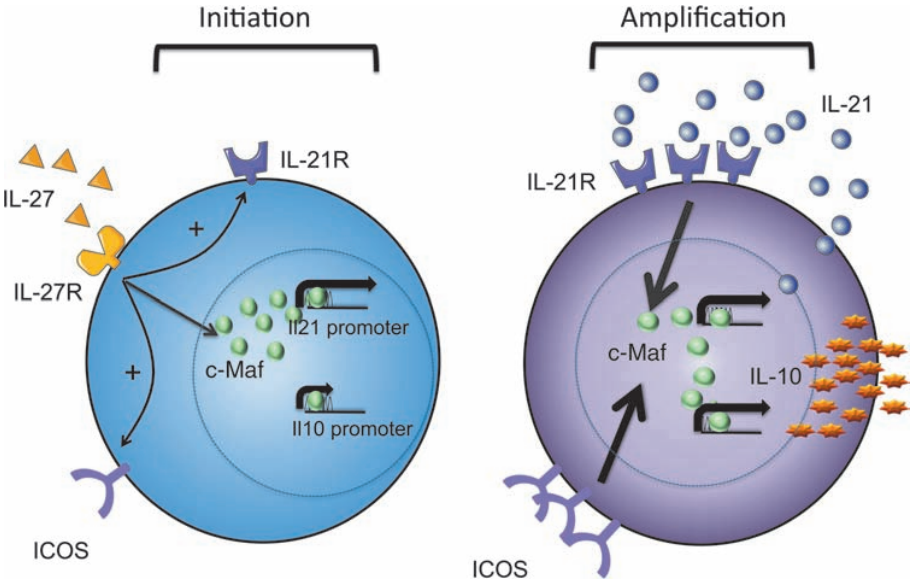

FIG. 1. Molecular events leading to the generation of interleukin (IL)-27-induced type 1 regulatory (Tr1) cells. Treatment of naïve $CD4^+$ T cells with IL-27 enhances expression of the transcription factor avian musculoaponeurotic fibrosarcoma v-maf (c-Maf), IL-21 receptor, and inducible costimulatory molecule (ICOS), which are all essential for the differentiation of Tr1 cells. Upon induction of Tr1 cell differentiation (initiation phase), c-Maf transactivates the *il21* and *il10* promoters (*left panel*). During Tr1 cell expansion (amplification phase), IL-10 and IL-21 expression become independent of IL-27/IL-27R signaling as ICOS and IL-21 signaling maintain c-Maf expression in differentiating Tr1 cells (*right panel*).

The induction of IL-27 from DC is further exemplified in humans where IFN-β, a therapy approved for MS, increases induction of IL-27 from human DCs (Fig. 2) (Zhang and others 2009). This phenomenon has also been observed with other MS treatments such as statins, which are extensively used as cholesterol-lowering agents and were recently attributed with antiinflammatory properties (Fig. 2). Indeed, Zhang and others (2008) have shown that one of the statins, simvastatin, has the ability to increase IL-27 secretion from human monocytes of MS patients, suggesting that IL-27 may play a key role in mediating the antiinflammatory effects after this treatment. This raises the possibility that IL-27 may be an effective target that could be exploited for treatment of many immune-mediated diseases.

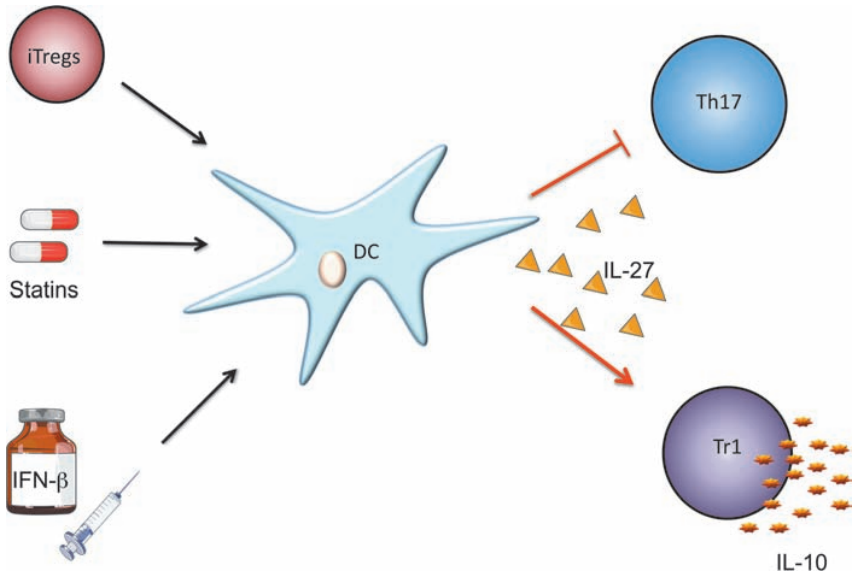

FIG. 2. Immunomodulation of *in vivo* inflammatory responses by interleukin (IL)-27. Induced $Foxp3^+$ Tregs, interferon (IFN)-β, or statins induce the secretion of IL-27 from dendritic cells (DCs) that will in turn inhibit proinflammatory T helper 17 (Th17) cells and enhance the development of type 1 regulatory (Tr1) cells, resulting in the resolution of autoimmune inflammation.

Conclusions and Perspectives

IL-27 was initially characterized in 2002 as a cytokine that induces the proliferation of naïve $CD4^+$ cells. Although IL-27 was initially categorized as a proinflammatory cytokine that induces Th1 differentiation, pioneering work from Chris Hunter's laboratory later established that this cytokine in fact suppresses excessive immune responses. Over the last 3 years, mechanisms by which IL-27 mediates immunosuppression have been identified. First, IL-27 is a critical mediator secreted by tolerogenic DC that suppresses effector Th17 cells and supports the *in vivo* generation of regulatory IL-10-producing Tr1 cells. Second, molecular studies of IL-27-induced Tr1 cells revealed that the transcription factor c-Maf, ICOS, and IL-21 are critical for the generation of Tr1 cells *in vitro* and *in vivo*.

The lineage-specific transcription factors T-bet, Gata-3, Rorc, and Foxp3 have been associated with differentiation of Th1, Th2, Th17, and Treg cells, respectively. However, to the best of our knowledge, no lineage-specification transcription factor has been identified for Tr1 cells. Therefore, in contrast to $FoxP3^+$ Tregs, which can be induced by TGF-β and then characterized by expression of the FoxP3 protein, the analysis of molecular mechanisms of Tr1 cells is hampered by the numerous variations between the experimental procedures and inability to produce Tr1 cells in large quantities. In this regard, the finding that IL-27 induces IL-10-producing Tr1 cells provides a simple method to obtain Tr1 cells for both molecular and functional analysis. This procedure would be advantageous over currently used conditions that involve the addition of feeder cells and multiple cytokines to generate Tr1 cells (Brun and others 2009). In conclusion, there is a substantial amount of evidence showing that cytokine-mediated therapy has an important place in the treatment of autoimmune diseases, including MS, and we believe that IL-27 may be exploited in the treatment of many autoimmune disorders based on its strong antiinflammatory properties. IL-27 can thus be considered as an attractive target, but the short *in vivo* half-life of this cytokine might hinder its clinical application. Therefore, a detailed understanding of the different IL-27-induced pathways will permit the discovery of new potential therapeutic targets downstream of IL-27.

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Author Disclosure Statement

No competing financial interests exist.

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