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IFN-β Facilitates Neuroantigen-Dependent Induction of CD25$^+$ FOXP3$^+$ Regulatory T Cells That Suppress Experimental Autoimmune Encephalomyelitis

Duncheng Wang,*,[1] Debjani Ghosh,* S. M. Touhidul Islam,* Cody D. Moorman,* Ashton E. Thomason,* Daniel S. Wilkinson,* and Mark D. Mannie*,†

This study introduces a flexible format for tolerogenic vaccination that incorporates IFN-β and neuroantigen (NAg) in the Alum adjuvant. Tolerogenic vaccination required all three components, IFN-β, NAg, and Alum, for inhibition of experimental autoimmune encephalomyelitis (EAE) and induction of tolerance. Vaccination with IFN-β + NAg in Alum ameliorated NAg-specific sensitization and inhibited EAE in C57BL/6 mice in pretreatment and therapeutic regimens. Tolerance induction was specific for the tolerogenic vaccine Ag PLP178-191 or myelin oligodendrocyte glycoprotein (MOG)35–55 in proteolipid protein– and MOG-induced models of EAE, respectively, and was abrogated by pretreatment with a depleting anti-CD25 mAb. IFN-β/Alum–based vaccination exhibited hallmarks of infectious tolerance, because IFN-β + OVA in Alum–specific vaccination inhibited EAE elicited by OVA + MOG in CFA but not EAE elicited by MOG in CFA. IFN-β + NAg in Alum vaccination elicited elevated numbers and percentages of FOXP3$^+$ T cells in blood and secondary lymphoid organs in 2D2 MOG-specific transgenic mice, and repeated boosters facilitated generation of activated CD44^{high} CD25$^+$ regulatory T cell (Treg) populations. IFN-β and MOG35–55 elicited suppressive FOXP3$^+$ Tregs in vitro in the absence of Alum via a mechanism that was neutralized by anti–TGF-β and that resulted in the induction of an effector CD69$^+$ CTLA-4$^+$ IFNAR$^+$ FOXP3$^+$ Treg subset. In vitro IFN-β + MOG–induced Tregs inhibited EAE when transferred into actively challenged recipients. Unlike IFN-β + NAg in Alum vaccines, vaccination with TGF-β + MOG35-55 in Alum did not increase Treg percentages in vivo. Overall, this study indicates that IFN-β + NAg in Alum vaccination elicits NAg-specific, suppressive CD25$^+$ Tregs that inhibit CNS autoimmune disease. Thus, IFN-β has the activity spectrum that drives selective responses of suppressive FOXP3$^+$ Tregs. ***The Journal of Immunology*****, 2016, 197: 2992–3007.**

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the CNS with a presumed autoimmune etiology (1–3). MS is a leading cause of nontraumatic neurologic disability of young adults in the western world, including an estimated 350,000 individuals in the United States. Cell types that infiltrate the CNS in MS include CD4$^+$ T cells, which are thought to be the primary instigators of disease, together with CD8$^+$ T cells and monocytes/macrophages. B cells may also mediate important effector functions in MS, as reflected by the presence of ectopic intrathecal B cell follicles in the CNS of some MS patients and the presence of oligoclonal Ig species in the cerebrospinal fluid. First-line therapeutics for MS include the immunomodulatory IFN-β drugs, which have been used since 1993 (4–9). Disadvantages of contemporary IFN-β therapy for MS include a requirement for chronic treatment, limited efficacy, and a loss of treatment benefit upon discontinuation of therapy. IFN-β efficacy is marked by substantial interpatient variability, with the implication that IFN-β therapy is optimal when MS pathogenesis is mediated by a spectrum of Th1-like effector cells (10, 11). The underlying mechanisms of IFN-β therapy are unresolved and are thought to involve rebalancing of proinflammatory versus anti-inflammatory cytokines, such as IL-12 and IL-10, and a reset of effector and regulatory CD4$^+$ T cell subsets. New insight into the underlying mechanisms of IFN-β action in T cell regulation and autoimmune disease may reveal new approaches to exploit the beneficial actions of IFN-β in MS.

Experimental autoimmune encephalomyelitis (EAE) is widely used as an animal model of MS, in part because of their commonalities in T cell regulatory strategies (12, 13). In rodent models of EAE, CD4$^+$ Th cells, particularly T cells of the Th1 IFN-γ–producing subset and the Th17 IL-17–producing subset, are the primary instigators of disease. In select models of EAE, B cells and Ab mediate important pathogenic roles in CNS inflammation (14, 15). EAE has been successfully controlled by different strategies of tolerogenic vaccination (16–19), and several of these strategies have been advanced in MS as a means to specifically target pathogenic myelin-specific T cells. Tolerogenic vaccine approaches that evolved from studies of EAE into clinical testing in MS are based on s.c. injection (20) or oral delivery (21) of

*Department of Microbiology and Immunology, Brody School of Medicine, East Carolina University, Greenville, NC 27834; and †The Harriet and John Wooten Laboratory for Alzheimer's and Neurodegenerative Disease Research, Brody School of Medicine, East Carolina University, Greenville, NC 27834

[1]Current Address: Department of Pediatrics, East Carolina University, Greenville, NC.

ORCIDs: 0000-0002-2085-9512 (D.W.); 0000-0003-2720-4102 (S.M.T.I.); 0000-0003-0475-8516 (M.D.M.).

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Address correspondence and reprint requests to Dr. Mark D. Mannie, Department of Microbiology and Immunology, Brody School of Medicine, East Carolina University, 600 Moye Boulevard, Greenville, NC 27834. E-mail address: manniem@ecu.edu

Abbreviations used in this article: CTV, CellTrace Violet; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NAg, neuroantigen; PLP, proteolipid protein; Treg, regulatory T cell.

various myelin basic protein (MBP) preparations, direct administration of MBP peptide (MBP8298; Dirucotide) (22–24), or administration of a fusion protein containing MBP and proteolipid protein (PLP) epitopes (MP4) (25, 26). Although these approaches had success in EAE, attempts to translate these myelin-specific vaccine strategies in MS did not show robust clinical efficacy. Indeed, use of altered peptide variants of the MBP83–99 peptide resulted in treatment-induced exacerbations of MS (27). Additional tolerogenic vaccine strategies that advanced from EAE to MS include the use of a pooled set of naked myelin peptide Ags (28), DNA vaccines that encode MBP (29), transdermal application of myelin peptides (30, 31), leukocyte-coupled peptides (32–34), and fusion proteins incorporating myelin peptides linked to a dendritic cell (DC)-targeting domain (35, 36).

A novel tolerogenic vaccine strategy designed to improve therapeutic efficacy is based on the use of cytokine–Ag fusion proteins that incorporate a regulatory cytokine, such as IFN-β, GM-CSF, IL-2, or IL-16, with a dominant encephalitogenic epitope of a myelin autoantigen within a single-chain recombinant protein (37–44). Two previous studies (38, 41) provided evidence that IFN-β could be repurposed from an inhibitory cytokine to a tolerogenic vaccine as a single-chain IFN-β–neuroantigen (NAg) fusion protein and, thereby, used to enable immunological tolerance to a myelin NAg. The IFN-β–NAg vaccine immunotherapy has particular promise given the widespread use of IFN-β as a well-tolerated effective therapy for MS. A tolerogenic vaccine–induced memory would decrease the clinical need for high-frequency high-dose administration of IFN-β and, thereby, would mitigate the antigenic stimulus responsible for generation of neutralizing anti–IFN-β Ab. Overall, an IFN-β–based tolerogenic vaccine may have qualitative advantages compared with IFN-β monotherapy or tolerogenic vaccination with naked myelin peptides.

Tolerogenic vaccination with cytokine–NAg fusion proteins generally required physical linkage of cytokine and NAg for optimal tolerance induction (37–40, 43, 44). This study is based on the concept that physical linkage of IFN-β and NAg could also be achieved in a substantially more flexible format by the use of an intermediate that facilitated indirect noncovalent bonds between IFN-β and NAg. The solution was provided by the Alum adjuvant, which is in common clinical use as an adjuvant in vaccine formulations and is well-known to bind and immobilize proteins onto a common matrix. IFN-β and NAg peptides mixed in the Alum adjuvant were predicted to have the required physical linkage in that both IFN-β and NAg would noncovalently bind a common substrate. This study provides evidence that IFN-β and NAg, when mixed and administered in the Alum adjuvant, elicited active tolerogenic responses that inhibited EAE in C57BL/6 (B6) mice by a mechanism dependent upon IFN-β, NAg, and Alum. The tolerogenic mechanisms reflected induction of a major FOXP3$^+$ regulatory T cell (Treg) population because culture with IFN-β and myelin oligodendrocyte glycoprotein (MOG)35–55 induced MOG-specific FOXP3$^+$ T cells in vitro, and vaccination of mice with IFN-β + MOG35–55 in Alum directly elicited FOXP3$^+$ Tregs in vivo. Tolerogenic vaccination with IFN-β + MOG35–55 in Alum inhibited MOG-induced EAE but not PLP-induced EAE, and vice versa, vaccination with IFN-β + PLP178–191 in Alum inhibited PLP-induced EAE but not MOG-induced EAE. In both cases, Ab-mediated depletion of CD25$^+$ Tregs in vivo blocked tolerogenic vaccination. These data reveal IFN-β as a key cytokine controlling specification of a FOXP3$^+$ Treg lineage in vitro and in vivo. IFN-β–based tolerogenic vaccines represent a new class of tolerogenic vaccines that could be exploited as a therapy for MS.

Materials and Methods

Animals and reagents

B6 mice, Foxp3-IRES-GFP–knock-in (FIG) mice (B6.Cg-*Foxp3*^{tm2Tch}/J; stock no. 006772), and MOG35–55–specific TCR-transgenic 2D2 mice [B6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J]; stock no. 006912) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were housed and bred in the Department of Comparative Medicine at East Carolina University Brody School of Medicine. 2D2-FIG mice were obtained through intercross breeding. 2D2 mice have a MOG-specific self-reactive T cell repertoire. Routine screening of 2D2 mice was performed by FACS analysis of PBMCs using Abs specific for TCR Vβ11 and/or Vα3.2. The FIG genotype was screened using forward (5′-CAC CTA TGC CAC CCT TAT CC-3′) and reverse (5′-ATT GTG GGT CAA GGG GAA G-3′) primers. The FIG product was 390 bp, and the wild-type product was 341 bp. Animal care and use were performed in accordance with approved animal use protocols and guidelines of the East Carolina University Institutional Animal Care and Use Committee.

Ags and IFN-β–NAg fusion proteins

Synthetic MOG35–55 (M-E-V-G-W-Y-R-S-P-F-S-R-V-V-H-L-Y-R-N-G-K) or PLP178–191 (N-T-W-T-T-C-Q-S-I-A-F-P-S-K) was obtained from Genscript (Piscataway, NJ). The initial preparation of rTGF-β1 used in this project was a generous gift from Dr. Peter Sun (National Institutes of Health). Subsequently, a rat TGF-β1 sequence was cloned into the pIRES2-AcGFP1 vector (Clontech, Mountain View, CA) and expressed via stable transfection of human embryonic kidney cells. TGF-β1 was designed as described (45). This expression vector encoded a rat serum albumin leader sequence, an eight-histidine purification tag, the latency-associated peptide, the native RHRR cleavage site, and the C-terminal TGF-β1 sequence. A C32S substitution in the latency-associated peptide domain enabled high-level expression. The protein was expressed in human embryonic kidney cell supernatants, purified on Ni-NTA affinity columns, and activated by 10 min of exposure to 70°C. The quantitative bioactivity of each TGF-β1 preparation was verified by induction of FOXP3 in cultures of MOG-stimulated 2D2-FIG splenocytes.

Derivation, expression, purification, and bioassay of the murine cytokine–NAg fusion proteins were described in previous studies (38, 42). Murine IFN-β and the N-terminal domain of IFN-β–MOG consist of the murine IFN-β sequence (accession number NP_034640, http://www.ncbi.nlm.nih.gov/gene), with the exception that a nonnative alanine residue was added as the second amino acid to encode an optimal Kozak translation-initiation site (5′-GCCGCCACC-ATG-GCC-3′). The C terminus of the IFN-β–MOG fusion protein included an enterokinase linker cleavage site, the MOG35–55 sequence, and an eight-histidine affinity chromatography purification tag. IFN-β also had a C-terminal enterokinase linker and an eight-histidine purification tag. Expression supernatants were concentrated on YM10 ultrafiltration membranes and directly applied to consecutive Ni-NTA Agarose columns (QIAGEN, Chatsworth, CA) followed by extensive washing of the resin (50 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazole [pH 8]). IFN-β–MOG or IFN-β was eluted by acid elution (pH 4) or with 250 mM imidazole (pH 8) and was concentrated and diafiltrated in Amicon Ultra-15 centrifugal filter devices (EMD Millipore, Billerica, MA). Protein quantity was assessed by absorbance at 280 nm, and purity was assessed by SDS-PAGE. The bioactivities of murine IFN-β recombinant proteins were confirmed in vitro by inhibition of IL-2–dependent T cell proliferation, as shown by inhibitory constants (K_i/IC_{50}) in the low picomolar range (i.e., half-maximal inhibition in the 1–10 pM range), and by induction of FOXP3$^+$ T cells in cultures of MOG-stimulated 2D2-FIG splenocytes.

Generation, purification, and administration of mAb

The PC61-5.3 anti-CD25 rat IgG1(λ) hybridoma (46), the Y13-259 anti–v-H-Ras rat IgG1(κ) hybridoma (47), and the 1D11.16.8 anti-mouse–TGF-β1/2/3 mouse IgG1 hybridoma (48, 49) were obtained from American Type Culture Collection and were subcloned twice to ensure stability. The LRTC1 anti-rat LFA-1 mouse IgG1 hybridoma was originally derived in our laboratory (50–52) and had no cross-reactivity with mouse LFA-1. PC61 and Y13 were used as sources of CD25$^+$ Treg-depleting mAb and the isotype-control mAb, respectively. 1D11 and LRTC1 were used as sources of anti–TGF-β mAb and the isotype-control mAb, respectively. For all four hybridomas, cells were cultured in supplemented DMEM in C2011 hollow fiber cartridges (FiberCell Systems, Frederick, MD). Hybridoma supernatants were clarified at 7200 × *g*, precipitated with 50% ammonium sulfate, and dissolved in PBS. mAb preparations were purified on protein G agarose columns. Ab was eluted with 200 mM glycine (pH 3) and immediately neutralized by 1 M Tris buffer (pH 9). The purity of these mAbs was verified by SDS-PAGE. Specific activities of all PC61

preparations were determined by staining of murine CD25$^+$ T cells with serial 1/2-log dilutions of the mAb. After washing, PC61-stained T cells were labeled with a PE-conjugated goat anti-rat IgG(H+L) secondary Ab, followed by flow cytometric analysis. As designated for pretreatment experiments, purified mAbs were administered i.p. at a dose of 250 μg per injection to mice on days −5 and −3 (or days −4 and −2), unless designated otherwise. Depletion of specific lymphoid subsets was confirmed by flow cytometric analysis of PBMCs on day −1 or 0. Active immunization with MOG35–55 in CFA was initiated on day 0.

Flow cytometric analyses of splenocytes and PBMCs

Cells were washed in HBSS with 2% heat-inactivated FBS and stained with designated cocktails of fluorochrome-conjugated Abs for 1 h at 4°C in the dark. After staining PBMCs, erythrocytes were lysed with 1:10 HBSS for 20 s at 4°C, followed by the addition of 2× PBS. Cells were washed three times with HBSS/2% FBS. Data were collected using an LSR II flow cytometer (Becton Dickinson, San Jose, CA) and analyzed with FlowJo software. In designated experiments, reference counting beads (AccuCheck Counting Beads; Life Technologies, Frederick, MD or APC-conjugated CaliBRITE beads; BD Biosciences, San Jose, CA) were added to samples immediately before flow cytometric analysis. The use of reference beads enabled comparisons of cell yield or absolute cell numbers. Pairwise comparisons were analyzed by two-tailed *t* tests for data that passed normality (Shapiro–Wilk) and equal variance (Brown–Forsythe) tests. Otherwise, data were assessed with a Mann–Whitney rank-sum test. Fluorochrome-conjugated mAb were obtained from BioLegend or BD Biosciences and included CD19 (6D5), CD25 (PC61 or 3C7), CD28 (E18), CD3 (17A2 or 145-2C11), CD4 (GK1.5), CD44 (IM7), CD45 (30-F11), CD5 (53-7.3), CD69 (H1.2F3), CD8 (53-6.7), CD80 (16-10A1), CD86 (GL-1), CTLA-4 (UC10-4B9), GARP (F011-5), I-A^b (AF6-120.1), IFNAR-1 (MAR1-5A3), Ly-6G (Gr1) (1A8), PD1 (CD279) (29F.1A12), programmed cell death ligand-1 (PDL1; CD274) (10F.9G2), PDL2 (CD273) (TY25), TCR-Va3.2 (RR3-16), and TCR-Vβ11 (KT11). Multicolor panels were designated in the figure legends.

Preparation of tolerogenic vaccines

A PBS solution was prepared that contained designated doses of IFN-β, TGF-β, or peptide NAg. Aluminum Hydroxide Gel colloidal suspension (A8222 Alum; Sigma Aldrich or Alhydrogel adjuvant catalog no. vac-alu-250; InvivoGen) was mixed thoroughly. Equal volumes of Alum and a solution containing IFN-β and NAg were combined for a total injection volume of 100 μl per mouse. IFN-β and NAg were given in matched equimolar doses (e.g., a 2-nmol vaccine dose included 2 nmol IFN-β and 2 nmol NAg or a 5-nmol vaccine dose included 5 nmol IFN-β and 5 nmol NAg), unless designated otherwise. The mixture was incubated for 1 h on ice with continuous agitation to allow the protein/peptide to attach to the Alum gel. The vaccine was administered s.c. by two injections of 50 μl each. No signs of inflammation were noted at the injection site.

Induction and assessment of EAE

CFA (IFA with 4 mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra; BD Biosciences) was mixed 1:1 with MOG35–55 or PLP178–191 in PBS. The CFA/Ag mixture was emulsified by sonication. EAE was elicited in B6 mice by injection of 200 μg MOG35–55 or PLP178–191 in a total volume of 100 μl emulsion via three s.c. injections of 33 μl across the lower back. Each mouse received separate injections (200 ng i.p.) of pertussis toxin on days 0 and 2. All immunizations were performed under isoflurane anesthesia (Abbott Laboratories, Chicago, IL). Mice were assessed daily for clinical score and body weight. The following scale was used to score the clinical signs of EAE: 0, no disease; 0.5, partial paralysis of tail without ataxia; 1, flaccid paralysis of tail or ataxia but not both; 2, flaccid paralysis of tail with ataxia or impaired righting reflex; 3, partial hind limb paralysis marked by inability to walk upright but with ambulatory rhythm in both legs; 3.5, same as above but with full paralysis of one leg; 4, full hindlimb paralysis; and 5, total hindlimb paralysis with forelimb involvement or moribund. A score of 5 was a humane end point for euthanasia.

The incidence of EAE reflected the number of mice afflicted with EAE compared with the total group size. Cumulative EAE scores were calculated by summing daily scores for each mouse across the designated time course of disease. Maximal scores were calculated as the most severe EAE score for each mouse. Mice that did not exhibit EAE had a score of 0 for the cumulative and maximal scores, and these scores were included in the group average. Attrition reflected the number of mice that reached clinical end points (e.g., score of 5). Mice that reached humane end points, as assessed by body weight loss, body score, or clinical score of 5, were subjected to humane euthanasia and were omitted from scoring thereafter. Thus, groups of mice exhibiting substantial attrition had artificially depressed mean cumulative scores, but attrition did not affect mean maximal scores. Time-course graphs portrayed daily mean maximal scores. Cumulative and maximal EAE scores were converted to ranked scores and analyzed by nonparametric ANOVA. To calculate the percentage maximal weight loss, 100% body weight was assigned as the maximal body weight obtained from day 1 through day 10, and daily body weights were calculated for each day after normalization to this 100% value. The minimum body weight was defined as the lowest body weight after normalization to the 100% value from day 11 until the end of the experiment. Maximal weight loss was calculated by subtraction of the normalized minimum value from the 100% value. Negative weight loss values represented weight gain. Weight loss was analyzed by parametric ANOVA. Nonparametric and parametric ANOVA were assessed with a Bonferroni post hoc test, unless noted otherwise. Incidence of EAE was analyzed in a pairwise fashion using the Fisher exact test. Mean EAE and weight loss data are shown with the SEM.

Results

Vaccination with an IFN-β–NAg fusion protein was a therapeutic intervention in EAE

Previous studies revealed that covalent linkage of murine IFN-β and PLP139–151 was essential for tolerance induction in the SJL relapsing-remitting model of EAE (38). To assess the generality of these findings, experiments were performed to assess whether covalent linkage was needed for tolerance induction in the B6 model of chronic EAE (Fig. 1A, 1B). A treatment model in B6 mice was chosen because chronic EAE in this model is typically resistant to Ag-specific interventions. To address the requirement of cytokine–NAg covalent linkage, B6 mice were immunized to elicit paralytic EAE on day 0 (Fig. 1A, 1B, Table I). On day 13, mice were matched for severity of EAE and were treated with 2 nmol of murine IFN-β–MOG fusion protein, a mixture of IFN-β and MOG, MOG alone, or saline. Treatments were given in saline on day 13 and again on days 15 and 17, with a final treatment on day 19. Mice treated with the IFN-β–MOG fusion protein or the combination of IFN-β and MOG35–55 in saline exhibited a partial recovery marked by a nadir in paralytic signs from days 22 to 24 (Fig. 1A). Mice treated with IFN-β–MOG remained stable, with mild EAE thereafter and did not relapse, whereas mice treated with the combination of IFN-β and MOG relapsed by day 30 and exhibited severe paralytic EAE during the remainder of the experiment. Mice treated with MOG35–55 or saline exhibited a sustained course of paralytic EAE. These data revealed that the covalent linkage of IFN-β and MOG35–55 is critical for a prolonged beneficial activity of IFN-β–MOG in the B6 model of EAE.

IFN-β + MOG in Alum was therapeutic and tolerogenic in EAE

Given that physical linkage is needed for optimal tolerogenic activity, we predicted that such linkage could be noncovalent and indirect rather than covalent and direct. We reasoned that the widely used Alum adjuvant may fulfill the requirement for indirect noncovalent linkage because the aluminum hydroxide gel matrix is well known to bind protein and peptide Ags. The prediction was that IFN-β and NAg peptides would be bound, immobilized, and cross-linked by the Alum adjuvant matrix and, thereby, achieve the requisite linkage needed for IFN-β–mediated tolerogenic activity. To test this prediction, vaccines containing IFN-β + MOG35–55 in Alum, MOG35–55 in Alum, and saline in Alum were administered once after the onset of paralytic EAE on day 15 in groups that had been matched for mean cumulative and maximal disease scores (Fig. 1C, 1D, Table I). A single administration of 5 nmol IFN-β + 5 nmol MOG35–55 in Alum reversed the course of paralytic EAE, facilitated clinical recovery, and ameliorated EAE-associated weight loss. The vaccines MOG35–55 in Alum and saline in Alum had no effect, in that the respective mice

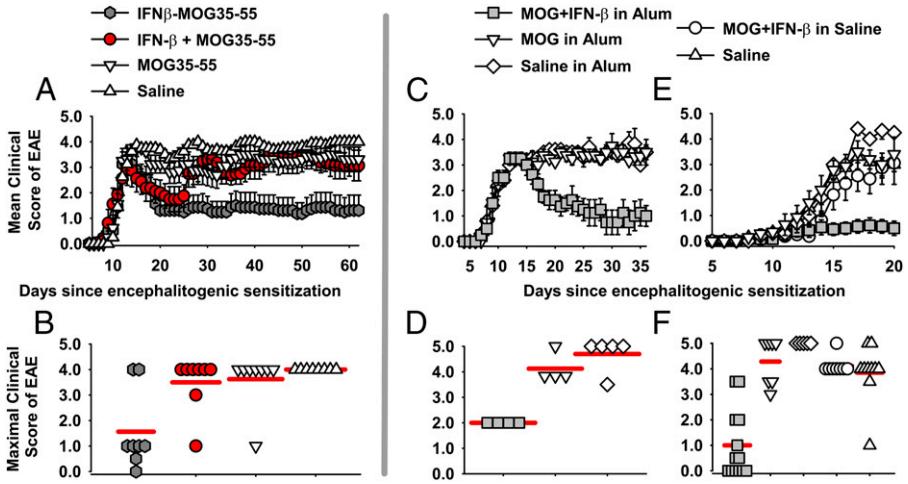

FIGURE 1. IFN-β is a tolerogenic vaccine adjuvant in EAE. (**A**–**F**) EAE was induced in B6 mice on day 0 by injection of 200 μg of MOG35–55 in CFA with i.p. injections of pertussis toxin on days 0 and 2. EAE time courses (A, C, and E) and density dot plots (B, D, and F) for treatment (A–D) and pretreatment (E and F) experiments. (A and B) On day 13, groups were matched for severity of EAE (mean maximal scores of 3.1–3.3, mean cumulative scores of 25.0–26.5). MOG/CFA-immunized mice were treated (2 nmol each in saline) with IFN-β–MOG, a combination of IFN-β and MOG35–55, MOG35–55, or saline by s.c. injections in saline on days 13, 15, 17, and 19. (B) Maximal EAE scores during days 20–62. (C and D) MOG/CFA-immunized mice were matched for severity of EAE on day 14 (mean maximal scores of 3.2–3.4, mean cumulative scores of 16.5–17.0). Matched groups were given one s.c. injection (5 nmol) of saline in Alum, MOG35–55 in Alum, or IFN-β + MOG in Alum on day 15. (D) Maximal scores of EAE during days 18–36. (E and F) Designated vaccines were given at a dose of 2 nmol on days −21, −14, and −7. The EAE time course is shown through day 20, and the mean maximal scores are shown based on data collected through day 32. Error bars represent SEM. Red horizontal lines represent the average of the values in each group. Tabular data and statistical analyses for these experiments are shown in Table I.

continued to exhibit severe paralytic EAE throughout the experiment, and several mice progressed to a score of 5 (humane end point, Table I). These data indicated that the IFN-β + MOG35–55 in Alum vaccine had robust efficacy as a therapeutic intervention.

Treatment regimens are used to measure the clinically significant modality of therapeutic efficacy. Conversely, pretreatment regimens are used to measure tolerogenic activity because vaccine-mediated inhibitory activity must be remembered by the immune

Table I. IFN-β is a tolerogenic adjuvant

Figure[a]	Group	Incidence	Attrition	Cumulative Score (Mean ± SE)	*p* Value	Maximal Score (Mean (± SE)	*p* Value	Maximal Weight Loss (%)	*p* Value
1A, 1B	IFN-β-MOG	7/8	0/8	57.9 ± 21.6	*	1.6 ± 0.5	*	17.0 ± 3.5	*
1A, 1B	IFN-β + MOG	8/8	1/8	125.9 ± 15.2	ns	3.5 ± 0.4	0.023	22.0 ± 3.5	ns
1A, 1B	MOG35–55	8/8	0/8	138.3 ± 19.9	0.024	3.6 ± 0.4	0.006	23.9 ± 2.4	ns
1A, 1B	Saline	8/8	0/8	163.3 ± 3.8	0.001	4.0 ± 0.0	0.001	27.7 ± 1.6	ns
1C, 1D	IFN-β + MOG in Alum	4/4	0/4	23.6 ± 7.8	*	2.0 ± 0.0	*	15.3 ± 3.4	*
1C, 1D	MOG in Alum	4/4	1/4	61.8 ± 3.9	0.014	4.1 ± 0.3	0.010	30.6 ± 4.3	0.037
1C, 1D	Saline in Alum	5/5	4/5	57.9 ± 5.3	0.034	4.7 ± 0.3	0.001	28.7 ± 3.1	0.054
1E, 1F	IFN-β + MOG in Alum	7/13	0/13	9.1 ± 3.9	*	1.0 ± 0.4	*	8.5 ± 1.6	*
1E, 1F	IFN-β + MOG in saline	8/8	5/8	31.0 ± 5.1	0.022	4.1 ± 0.1	<0.001	20.3 ± 1.5	0.002
1E, 1F	MOG in Alum	7/7	4/7	30.5 ± 3.5	0.013	4.3 ± 0.3	<0.001	16.4 ± 2.7	ns
1E, 1F	Saline in Alum	5/5	5/5	23.1 ± 3.0	ns	5.0 ± 0.0	<0.001	21.2 ± 1.5	0.005
1E, 1F	Saline	10/10	8/10	21.7 ± 5.5	ns	3.9 ± 0.4	<0.001	22.2 ± 2.6	<0.001
3A, 3B	MOG-MOG	6/7	0/7	16.5 ± 4.8	*	2.4 ± 0.6	*	4.7 ± 2.9	*
3A, 3B	MOG-MOG (anti-CD25)	7/7	3/7	46.9 ± 2.5	0.002	4.1 ± 0.2	0.001	19.2 ± 2.3	0.001
3A, 3B	PLP-MOG	5/5	3/5	45.3 ± 5.4	0.002	4.2 ± 0.2	<0.001	21.1 ± 3.6	0.001
3A, 3B	PLP-MOG (anti-CD25)	6/6	3/6	46.9 ± 2.7	0.003	4.1 ± 0.2	0.001	20.9 ± 1.5	0.001
3C, 3D	MOG-PLP	5/5	2/5	81.1 ± 2.5	0.000	4.3 ± 0.3	0.004	17.0 ± 1.8	0.009
3C, 3D	MOG-PLP (anti-CD25)	5/5	3/5	65.0 ± 4.1	ns	4.4 ± 0.4	0.003	17.1 ± 3.3	0.009
3C, 3D	PLP-PLP	3/5	0/5	11.1 ± 10.6	*	0.8 ± 0.6	*	3.8 ± 0.6	*
3C, 3D	PLP-PLP (anti-CD25)	5/5	1/5	68.2 ± 7.8	0.016	3.9 ± 0.3	0.028	15.3 ± 3.1	0.026
5A, 5B	IFN-β–Tregs	2/8	0/8	1.8 ± 1.4	*	0.2 ± 0.1	*	1.0 ± 0.8	*
5A, 5B	Control T cells	10/10	0/10	30.0 ± 4.6	<0.001	2.7 ± 0.2	<0.001	11.3 ± 2.5	0.030
5A, 5B	Saline	11/11	1/11	24.4 ± 3.5	<0.001	2.9 ± 0.4	<0.001	11.9 ± 2.5	0.016

[a]The data are portrayed graphically in Figs. 1, 3, 5A, and 5B. The experimental approach is described in the respective figure legend. For Fig. 3, group designations MOG-MOG, PLP-MOG, MOG-PLP, and PLP-PLP refer first to the peptide (MOG35–55 or PLP178–191) in the Alum + IFN-β tolerogenic vaccine and second to the peptide in the CFA challenge.

Nonparametric ANOVA based on ranked scores was used to assess group differences in mean cumulative scores and mean maximal scores, and parametric ANOVA was used to assess group differences with regard to maximal weight loss relative to the comparator group (*Bonferroni post hoc test).

ns, not significant.

system to impact a subsequent encephalitogenic challenge. To test pretreatment efficacy, the IFN-β + MOG35–55 in Alum vaccine, along with control vaccine formulations, was administered at a dose of 2 nmol on days −21, −14, and −7, followed by an encephalitogenic challenge on day 0 (Fig. 1E, 1F, Table I). The IFN-β + MOG35–55 in Alum vaccine attenuated the subsequent induction of EAE, whereas MOG in Alum, saline in Alum, MOG + IFN-β in saline, and saline vaccines had no impact on the course of severe chronic EAE. The EAE time course is shown through day 20 and not beyond because of attrition of mice that reached a score of 5 in the four control groups (Table I). These data support the hypothesis that NAg in the context of an IFN-β in Alum adjuvant specifies a strong NAg-specific tolerogenic response.

Pretreatment with the IFN-β + MOG in Alum vaccine altered MOG-specific sensitization in MOG/CFA sensitized mice

To address whether this vaccine approach modulated MOG/CFA-induced sensitization, B6 mice were vaccinated with 5 nmol of IFN-β + MOG in Alum, MOG in Alum, or saline in Alum on day −7 and were immunized with 200 μg of MOG in CFA on day 0. After 13 d, draining lymph node cells were labeled with CellTrace Violet (CTV) and cultured or not with 1 μM MOG35–55 for 4 d. Lymph node cells from mice vaccinated with MOG in Alum or saline in Alum exhibited robust, MOG-specific proliferative responses. Responsive cell types included CD4$^+$ T cells (Fig. 2A, 2B), CD8$^+$ T cells (Fig. 2C, 2D), and B cells (Fig. 2E, 2F). In contrast, mice vaccinated with IFN-β + MOG in Alum did not exhibit MOG-specific responses and instead exhibited proliferative responses that were autologous (i.e., independent of exogenous MOG35–55). Mice vaccinated with IFN-β + MOG in Alum lacked MOG-specific proliferative responses and instead exhibited autologous proliferative responses when assayed 10 or 18 d after MOG/CFA sensitization (data not shown). These data indicate that the IFN-β adjuvant qualitatively alters MOG-specific sensitization in the draining lymph nodes.

Depletion of CD25$^+$ Tregs impairs tolerogenic vaccination

Vaccine-induced tolerogenic memory, as shown in Fig. 1, suggested the potential involvement of MOG-specific CD25$^+$ Tregs as an underlying mechanism. To test this possibility, B6 mice were vaccinated with two distinct vaccines (IFN-β + MOG35–55 in Alum versus IFN-β + PLP178–191 in Alum) on days −21, −14, and −7 (Fig. 3, Table I). Vaccines were administered s.c. at a dose of 2 nmol (Fig. 3A, 3B) or 5 nmol (Fig. 3C, 3D). Mice were treated with the anti-CD25 PC61 mAb (rat IgG1, 250 μg i.p.) or an isotype control (Y13-259 rat IgG1) on days −4 and −2 to deplete CD25$^+$ Tregs. These mice were then subjected to induction of EAE with MOG35–55/CFA (Fig. 3A, 3B) or PLP178–191/CFA (Fig. 3C, 3D) on day 0. The results showed that tolerogenic vaccination was NAg specific. The MOG-specific vaccine inhibited MOG-induced EAE but lacked suppressive activity in PLP/CFA-challenged mice. In contrast, the PLP-specific vaccine inhibited PLP-induced EAE but lacked suppressive activity in MOG/CFA-challenged mice. Pretreatment with the anti-CD25 PC61 mAb, but not the isotype-control Ab, eliminated circulating CD25$^+$ Tregs (data not shown). Pretreatment with the anti-CD25 PC61 mAb reversed the suppressive action of the respective tolerogenic vaccine, such that the PC61-treated mice showed a chronic course of paralytic EAE (Fig. 3A, 3C) and weight loss (Fig. 3B, 3D) equivalent to those of the control groups (i.e., PC61 pretreatment restored full EAE susceptibility in MOG-vaccinated mice challenged with MOG/CFA). Likewise, PC61 pretreatment restored full EAE susceptibility in PLP-vaccinated mice challenged with PLP/CFA. Notably, PC61-mediated depletion of Tregs had no impact on EAE in groups not subjected to NAg-specific tolerance induction. In conclusion, these data indicate that IFN-β + NAg in Alum tolerogenic vaccination elicited CD25$^+$ NAg-specific Tregs that inhibited EAE via a mechanism of active NAg-specific tolerance.

IFN-β and MOG elicited FOXP3 expression in vitro

PC61-mediated reversal of tolerance suggested that IFN-β may support induction of CD25$^+$ FOXP3$^+$ Tregs. Hence, 2D2-FIG mice were used to test whether IFN-β induced FOXP3 during T cell Ag recognition of MOG35–55 in vitro (Fig. 4A). 2D2-FIG mice have a transgenic T cell repertoire specific for MOG35–55 and express a GFP reporter of FOXP3 expression. Naive 2D2-FIG splenic T cells were cultured in duplicate for 7 d with or without 1 μM MOG35–55 in the presence or absence of 1 μM IFN-β. TGF-β (1 nM) was used as a positive control for the Ag-dependent induction of FOXP3. IFN-β (1 μM) elicited FOXP3 expression

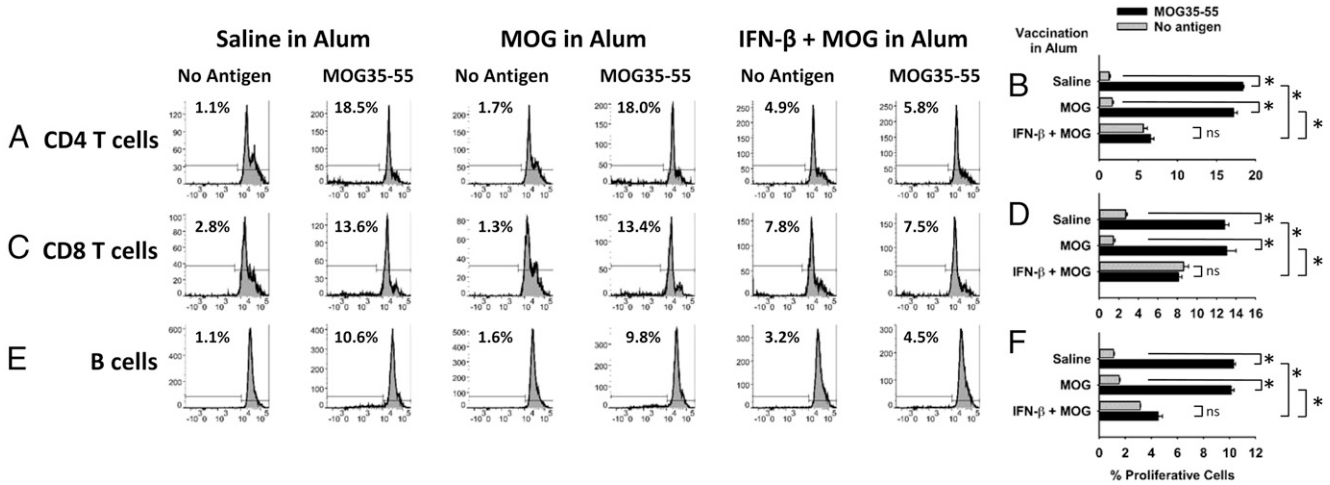

FIGURE 2. Pretreatment with the IFN-β + MOG in Alum vaccine impairs MOG-specific sensitization in MOG/CFA-sensitized mice. B6 mice were vaccinated with 5 nmol of IFN-β + MOG in Alum, MOG in Alum, or saline in Alum on day −7 and were immunized with 200 μg MOG in CFA on day 0. Draining lymph nodes were isolated on day 13, labeled with CTV, and cultured in triplicate wells, with or without 1 μM MOG35–55, for 4 d. Cells were analyzed for CD45-BV785, CD3-PE, CD4-allophycocyanin, CD8-FITC, and CTV. Alternatively, T cells were analyzed for CD45-BV785, CD3-PE.594, CD19-PE, I-A^b–allophycocyanin, and CTV. Proliferative CD4$^+$ T cells (**A** and **B**), CD8$^+$ T cells (**C** and **D**), and B cells (**E** and **F**) were measured by CTV dye dilution and defined by the threshold shown in the graphs. These data are representative of four experiments. *$p \leq 0.001$. ns, not significant.

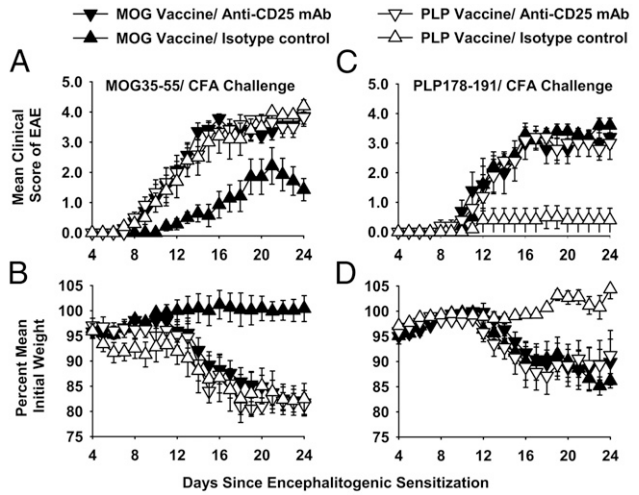

FIGURE 3. Depletion of CD25$^+$ Tregs reversed the suppressive action of tolerogenic vaccination. B6 mice were vaccinated with IFN-β + MOG35–55 in Alum or IFN-β + PLP178–191 in Alum on days −21, −14, and −7 [2 nmol in (**A**) and (**B**); 5 nmol in (**C**) and (**D**)]. Mice were treated with the anti-CD25 PC61 mAb or the Y13 rat IgG1 isotype control (250 μg i.p.) on days −4 and −2 and challenged with 200 μg MOG35–55 in CFA (A and B) or 200 μg PLP178–191 in CFA (C and D) on day 0 (pertussis toxin was given on days 0 and 2). Time course data through day 24 for EAE (A and C) and weight loss (B and D). Tabular data and statistical analyses are shown in Table I.

in ~30–40% of all 2D2-FIG T cells in the presence of MOG35–55. Induction of FOXP3 was NAg dependent because only 1–2% of T cells expressed FOXP3 in the absence of MOG35–55, despite the presence or absence of IFN-β, TGF-β, or both cytokines. These data showed that IFN-β facilitated induction of FOXP3 in MOG-stimulated naive T cells. Given that Alum was absent from this in vitro system, one can conclude that IFN-β has Treg-biasing activities that are independent from Alum.

However, the ability of IFN-β to induce FOXP3 (30–40% FOXP3$^+$ Tregs) was less than that achieved with TGF-β (50–60% FOXP3$^+$ Tregs) (Fig. 4A, 4B). IFN-β and TGF-β were not synergistic or additive. Rather, the interaction was nonadditive or antagonistic, in that the induction of FOXP3 by TGF-β was reduced in the presence of IFN-β to that observed in cultures with IFN-β alone (Fig. 4B). To standardize culture-to-culture comparisons, 50,000 fluorochrome-conjugated reference CaliBRITE beads (BD Biosciences) were added to each well to enable assessment of T cell numbers relative to control numbers of reference beads (Fig. 4C). In the absence of IFN-β, activation of T cells with MOG35–55 increased T cell numbers by ~4-fold. IFN-β concentrations in the range of 10 pM to 1 nM progressively reduced T cell numbers, consistent with the known proapoptotic action of this cytokine. However, the number of viable T cells increased in the range of 1 nM to 1 μM IFN-β to the maximal levels obtained in activation cultures without IFN-β, resulting in a U-shaped concentration curve (Fig. 4C, MOG35–55 viable T cells). This paradoxical increase in T cell numbers at high IFN-β concentrations was driven by selective increases in the percentages of FOXP3$^+$ Tregs rather than FOXP3^{null} conventional T cells (Tconvs). Indeed, frequencies of Tconvs remained essentially equal in the high IFN-β concentration range. The FOXP3$^+$ T cell population had higher levels of CD25 (data not shown) and size (mean forward scatter values, Fig. 4D) than Tconvs or total viable T cells. However, at high concentrations of IFN-β (100 nM to 1 μM), the size of FOXP3$^+$ Tregs decreased and was comparable to that of

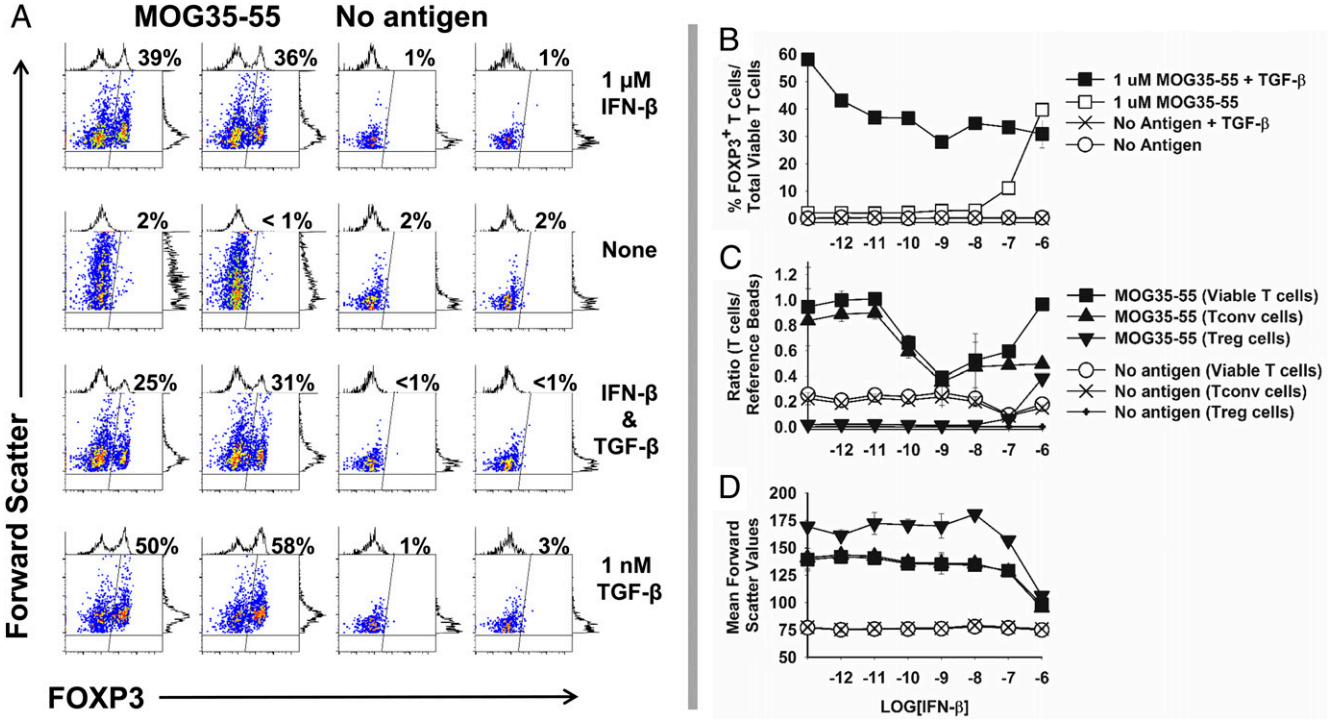

FIGURE 4. IFN-β elicited MOG-dependent induction of FOXP3 in naive 2D2-FIG T cells. (**A**) Naive 2D2-FIG splenocytes (100,000 per well) were cultured or not in duplicate with 1 μM MOG35–55 in the presence or absence of 1 μM IFN-β and/or 1 nM TGF-β. After 7 d of culture, CD4$^+$ T cells were assessed for expression of GFP as an indicator of FOXP3, which is indicated as a percentage in the upper right of each dot plot. Shown are duplicate samples for each condition. (**B**–**D**) 2D2-FIG splenocytes were cultured in the presence or absence of 1 μM MOG35–55, 1 nM TGF-β, or IFN-β (*x*-axis, 1 pM to 1 μM) for 7 d. (B) FOXP3$^+$ T cells as a percentage of total viable cells. When cultured without TGF-β, total viable T cells, FOXP3^{neg} Tconvs, and FOXP3$^+$ Tregs were assessed after a 7-d culture as a ratio relative to a fixed number of fluorescent reference beads (50,000 beads per well) (C) or as a function of cell size (forward scatter) (D). Error bars represent SD. These data are representative of four independent experiments.

Tconvs. Overall, these data provided evidence that high IFN-β concentrations (100 nM to 1 μM) selectively favored the emergence of a FOXP3$^+$ Treg subset.

Function and phenotype of IFN-β–induced Tregs

A central question was whether IFN-β–dependent expression of FOXP3 in MOG-specific T cells was associated with the induction of Treg-mediated suppressive activity. To address this question, splenic 2D2-FIG IFN-β–induced Tregs were assessed for suppressive activity in adoptive-transfer experiments (Fig. 5A, 5B, Table I). Donor IFN-β–Tregs were compared with control FOXP3^{null} 2D2 Tconvs that were not cultured with IFN-β. T cells were washed extensively and injected 4 d after encephalitogenic challenge. Recipients of IFN-β Tregs were protected from EAE, as measured by clinical grade or weight loss (Fig. 5A, 5B), compared with recipients of control T cells or saline-treated mice. These data indicated that IFN-β supported the MOG-dependent expression of FOXP3 (Fig. 4) and elicited the acquisition of suppressive activity in adoptive-transfer models (Fig. 5A, 5B). The implication was that IFN-β represented a gateway for the differentiation of suppressive MOG-specific FOXP3$^+$ Tregs. IFN-β–induced Tregs were also more heterogeneous than TGF-β–induced Tregs (Fig. 5C). For example, IFN-β–Tregs had a CD28^{high} population, a CD5^{low} GARP$^+$ population, a PD-1^{high} population, a PD-1^{neg} PDL2$^+$ population, and a CD69$^+$ CTLA-4$^+$ population that were largely absent in the TGF-β–Treg population. The CD69$^+$ CTLA-4$^+$ IFN-β Treg population also had high levels of the type I IFNR (IFNAR)-1 (Fig. 5E). The IFN-β–mediated induction of CTLA-4 and IFNAR was enriched in the FOXP3$^+$ subset but was not exclusive to FOXP3$^+$ Tregs because IFN-β also induced a CTLA-4$^+$ IFNAR$^+$ Tconv subset (data not shown). Thus, IFN-β induced the expression of these markers on IFN-β–cultivated Tconv and Treg subsets. In the absence of IFN-β, T cells cultured with MOG alone or with MOG and TGF-β did not exhibit CTLA-4, CD69, or IFNAR (Fig. 5D, 5F). These data revealed a potential IFN-β regulatory loop in which culture with IFN-β elicited expression of IFNAR on a subset of IFN-β–induced T cells.

IFN-β and MOG elicited FOXP3 expression in vivo

Just as the combination of IFN-β and MOG elicited differentiation of Tregs in vitro (Figs. 4, 5), immunization of 2D2-FIG mice with IFN-β + MOG in Alum elicited FOXP3$^+$ Tregs in 2D2-FIG mice in vivo (Fig. 6). On days 0 and 7, 2D2-FIG mice were injected s.c. (5 nmol dose) with IFN-β + MOG in Alum or IFN-β + MOG in saline (data shown) or with IFN-β in saline, MOG35–55 in saline, or saline or were left untreated (data not shown). On day 26, booster injections were repeated for all groups, but Alum rather than saline was used in all five groups. On days 7, 14, 29, 33, and 40, mice were bled via the submandibular vein, and circulating CD45$^+$ CD3$^+$ T cells were assessed for FOXP3$^+$ expression. In mice vaccinated with IFN-β + MOG in Alum, >17% of all circulating 2D2-FIG T cells expressed FOXP3 on day 7 (Fig. 6). In mice vaccinated with IFN-β + MOG in saline, only 0.2–0.4% of T cells expressed FOXP3. Mice that were vaccinated with IFN-β + MOG in Alum three times (days 0, 7, and 26) maintained a steady population of circulating FOXP3$^+$ T cells through the last day of analysis (day 40) (Fig. 6). In contrast, mice that received IFN-β + MOG in saline on days 0 and 7 plus a boost of IFN-β + MOG in Alum on day 26 exhibited FOXP3$^+$ T cells on day 29 but not thereafter. Like mice that were left untreated, mice that received IFN-β in saline, MOG35–55 in saline, or saline along with a booster of the same in Alum on day 26 did not express FOXP3 beyond background levels (<1%) at any point during the experiment. These data indicate that IFN-β, when combined with NAg and the Alum adjuvant, constitutes a vehicle for tolerogenic vaccination via induction of NAg-specific CD25$^+$ FOXP3$^+$ T cells.

The IFN-β + NAg in Alum vaccine elicited tolerance in 2D2-FIG mice

Given that one can use an IFN-β–based vaccine to elicit Treg responses (Fig. 6), an important question was whether one could

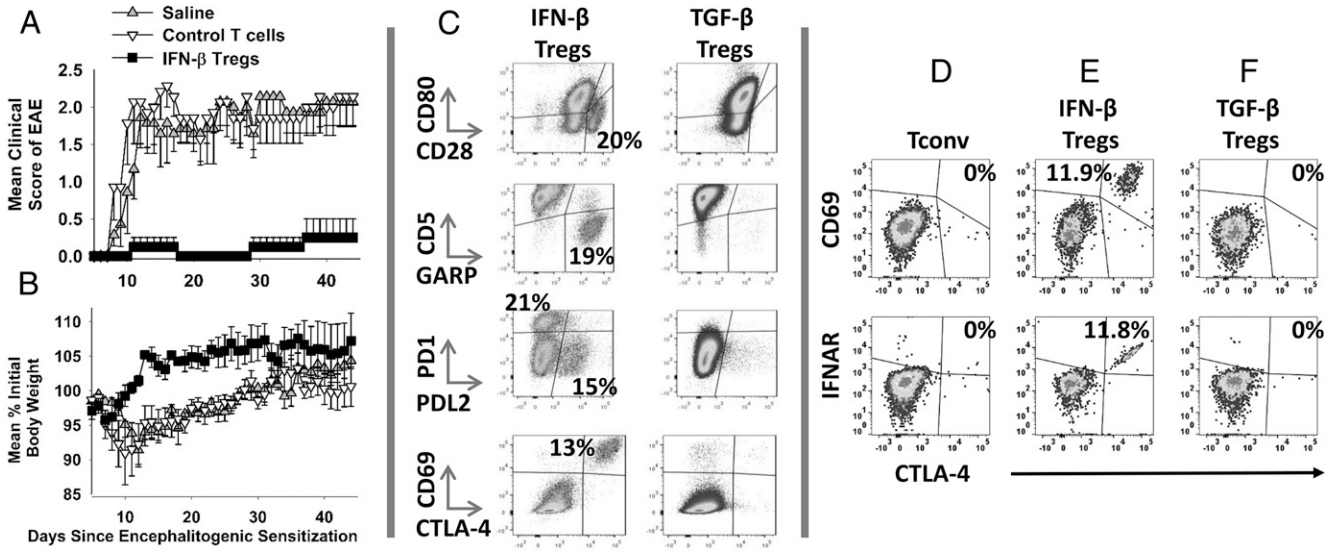

FIGURE 5. Function and phenotype of IFN-β–induced Tregs. 2D2-FIG splenocytes were cultured with 1 μM MOG35–55 and IL-2 in the presence (IFN-β–Tregs) or absence (Control T cells) of 1 μM IFN-β for 7 d. (**A** and **B**) Donor IFN-β–Tregs and control T cells were extensively washed after the 7-d culture and injected at a dose of 10^7 total T cells on day 4 into recipients that were previously challenged with MOG35–55/CFA (day 0) and pertussis toxin (days 0 and 2). Error bars represent SEM. Shown are one of two experiments that were compiled in Table I. (**C**–**F**) 2D2-FIG T cells were activated with 1 μM IFN-β + MOG35–55 [(C) and (E), FOXP3$^+$ Treg gate], 1 nM TGF-β + 1 μM MOG35–55 [(C) and (F), FOXP3$^+$ Treg gate], or 1 μM MOG35–55 alone [(D), Tconv cell gate] for 7 d. T cells were analyzed for the designated surface markers. These data are representative of three independent experiments. Cells were stained with CD45-BV785 and CD3-PE.CF594 within different panels that included [CD86-BV421, CD80-PE, and CD28-allophycocyanin], [CD69-BV421, CD5-PE, GARP–Alexa Fluor647, and PDL1-PE.Cy7], [PD1-BV421, PDL2-PE, and PDL1-allophycocyanin], and [CD69-BV421, CTLA4-PE, and IFNAR1-allophycocyanin].

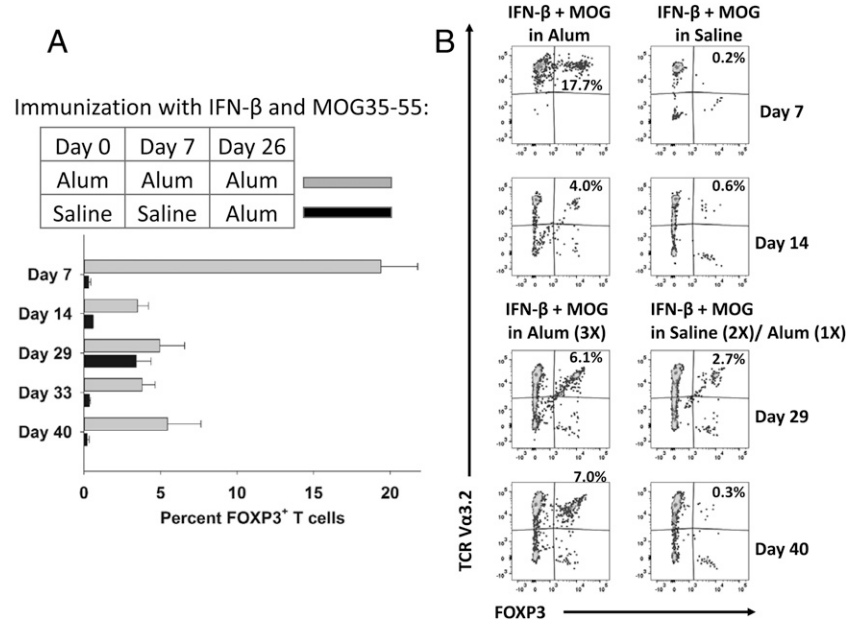

FIGURE 6. The IFN-β + MOG in Alum vaccine elicited FOXP3$^+$ Tregs in vivo. (**A** and **B**) On days 0, 7, and 26, 2D2-FIG mice were injected s.c. (5 nmol dose) with IFN-β + MOG in Alum. Another group was injected with 5 nmol of IFN-β + MOG in saline on days 0 and 7, followed by one injection of IFN-β + MOG in Alum on day 26 ($n = 2$). On days 7, 14, 29, 33, and 40, mice were bled via the submandibular vein, and circulating CD45$^+$ CD3$^+$ T cells were assessed for expression of the 2D2 TCR-transgenic Vα3.2 receptor and GFP (FOXP3$^+$) expression. (B) The total combined percentages of transgenic Vα3.2$^+$ and nontransgenic Vα3.2$^-$ FOXP3$^+$ Tregs is given in the upper right corner of each dot plot. Analysis panels included CD25-BV421, TCR–Vα3.2–PE, CD3-PE.CF594, TCR–Vβ11–Alexa Fluor 647, and CD45-BV785.

use repeated booster vaccinations to amplify effector/memory Treg responses. 2D2-FIG mice were given three (3×) injections (days 0, 7, and 14), two (2×) injections (days 7 and 14), or one (1×) injection (day 14) of 5 nmol of the IFN-β + MOG in Alum vaccine (1×, 2×, 3× vaccine, Fig. 7). Control groups were given three (3×) injections (days 0, 7, and 14) of 5 nmol of MOG in Alum or saline in Alum. Tolerogenic vaccination with IFN-β + MOG in Alum (3×, 2×, and 1×) elicited FOXP3$^+$ Tregs in PBMCs when assayed on days 17, 21, 28, and 35 (Fig. 7A, 7B). Repeated boosters generally resulted in higher FOXP3$^+$ Treg percentages (Fig. 7A) and numbers (Fig. 7B). By day 28, mice that received three vaccinations also had circulating Tregs that expressed high levels of CD44, whereas Tregs from the 2×, 1×, or control groups expressed intermediate levels of CD44 (Fig. 7C–E). These data indicated that multiple tolerogenic boosters increased the abundance and memory phenotype of circulating FOXP3$^+$ Tregs.

By day 35, the percentages of Tregs waned in that only low levels of FOXP3$^+$ Tregs remained in circulation. An important question was whether waning Treg levels in the blood correlated with

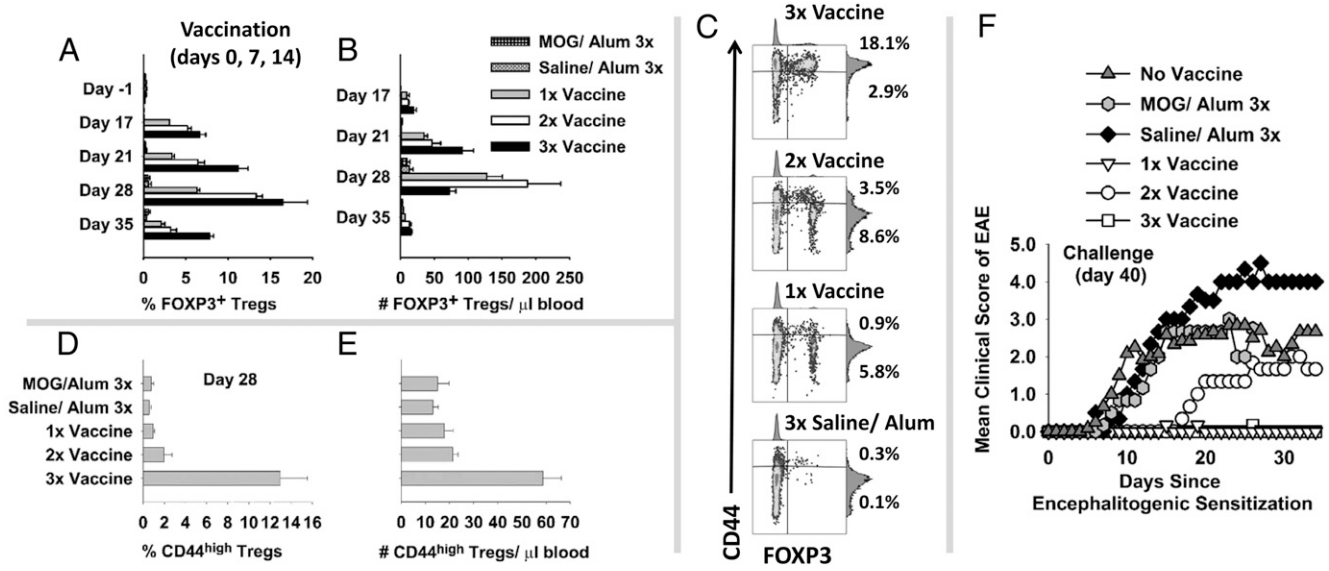

FIGURE 7. The IFN-β + MOG in Alum vaccine elicited FOXP3$^+$ Tregs and tolerance in 2D2 TCR-transgenic mice. 2D2-FIG mice were given three (3×) injections (days 0, 7, and 14), two (2×) injections (days 7 and 14), or one (1×) injection (day 14) of 5 nmol of the IFN-β + MOG in Alum vaccine ($n = 3$). When not receiving tolerogenic vaccination, mice received a control injection of saline in Alum. Control mice were given three (3×) injections (days 0, 7, and 14) of saline in Alum or 5 nmol of MOG in Alum ($n = 3$). Mice were assessed for percentages (**A**) (relative to 2D2 T cell population) and absolute numbers (**B**) (cells per μl blood) of circulating FOXP3$^+$ Tregs on days 17, 21, 28, and 35. (A) $p < 0.010$, 3× or 2× versus MOG or saline (days 17–35); $p < 0.05$, 1× versus MOG or saline (days 17–28). (B) $p < 0.006$, 3× versus MOG or saline (days 17–35); $p < 0.006$, 2× versus MOG or saline (days 17–28). (**C**) On day 28, CD3$^+$ T cells were analyzed for expression of CD44 (*y*-axis) and FOXP3$^+$ (*x*-axis). Percentages of CD44^{high} Tregs and CD44^{low} Tregs (upper and lower right quadrants) are given for each representative dot plot. Mean percentages (**D**) and numbers (**E**) of CD44^{high} FOXP3$^+$ Tregs. $p \leq 0.001$, 3× versus the other four groups. Data in (A), (B), (D), and (E) are mean and SEM. Analysis included CD25-BV421, TCR–Vα3.2–PE, CD3-PE.CF594, CD44-allophycocyanin, and CD45-BV785. (**F**) Mice were challenged with 100 μg MOG35–55 in CFA on day 40, given pertussis toxin on days 40 and 42, and weighed/scored for EAE daily for the next 34 d. The compiled clinical data and statistical analysis of EAE are provided in Table II.

Table II. Tolerogenic vaccination with IFN-β + MOG in Alum elicits tolerance in 2D2 TCR-transgenic mice

Figure	Group[a]	Incidence	Attrition	Cumulative EAE	*p* Value	*p* Value	Maximal EAE	*p* Value	*p* Value	Maximum Weight Loss (%)	*p* Value
7F	(a) IFN-β + MOG in Alum (3×)	1/3	0/3	0.2 ± 0.2	0.017		0.2 ± 0.2	0.020		1.2 ± 1.3	
7F	(b) IFN-β + MOG in Alum (2×)	2/3	0/3	25.8 ± 13.1	ns	0.000	2.2 ± 1.1	ns	0.000	13.3 ± 4.5	0.019
7F	(c) IFN-β + MOG in Alum (1×)	1/3	0/3	0.3 ± 0.3	0.022		0.2 ± 0.2	0.020		2.6 ± 1.1	
7F	(d) Saline in Alum (3×)	3/3	2/3	64.5 ± 11.9	ns		4.7 ± 0.3	*		25.6 ± 1.6	
7F	(e) MOG in Alum (3×)	3/3	2/3	41.2 ± 17.4	ns	*	3.7 ± 1.3	ns	*	20.4 ± 6.7	*
7F	(f) Naive	6/6	3/6	56.3 ± 11.9	*		3.5 ± 0.6	ns		10.0 ± 5.0	
8	(g) IFN-β + MOG in Alum (3×)	8/8	0/8	2.9 ± 0.9	0.002	0.005	0.6 ± 0.1	0.003	0.003	4.5 ± 1.2	0.000
8	(h) IFN-β + OVA in Alum (3×)	8/8	2/8	31.8 ± 5.4		*	3.9 ± 0.3		*	24.7 ± 3.5	*
8	(i) MOG in Alum (3×)	6/6	1/6	38.2 ± 4.3	*		4.3 ± 0.3	*		29.9 ± 2.7	*

Nonparametric ANOVA based on ranked scores was used to assess group differences in mean cumulative scores and mean maximal scores relative to the comparator group (*), whereas parametric ANOVA was used to assess weight loss (Bonferroni post hoc test; a–e versus f; g versus h and i). An independent samples *t* test was used to analyze differences in data compiled for tolerogenic vaccination groups (a–c) versus control groups (d–f).

[a]These data are portrayed graphically in Figs. 7F, 8A, and 8B. The experimental approach is described in the figure legends.

ns, not significant.

waning resistance to EAE, but this was not the case (Fig. 7F, Table II). When challenged to induce EAE on day 40, 2D2-FIG mice previously vaccinated with IFN-β + MOG in Alum once (1×) or three (3×) times exhibited profound resistance to EAE compared with controls. Two mice in the 2× vaccine group exhibited late-breaking paralytic EAE, whereas one mouse remained free of disease throughout the observation period. EAE breakthrough in the 2× group most likely represented stochastic events. Therefore, we refrain from making any conclusions regarding comparison of the 2× group with the 1× or 3× groups of mice. When the 1×, 2×, and 3× groups were analyzed in aggregate, mice that received tolerogenic vaccination (Table II, groups a–c) were significantly more resistant to EAE than were mice in the three pooled control groups (Table II, groups d–f). These data indicate that tolerogenic vaccination with IFN-β + MOG in Alum elicits an enduring tolerance in 2D2 TCR-transgenic mice. Induction of tolerance in TCR-transgenic mice is a stringent test of tolerogenic vaccine efficacy because the vast majority of T cells bear the transgenic MOG-specific TCR, and the vaccine must control this expanded MOG-specific T cell population. The persistence of tolerance despite the gradual disappearance of FOXP3+ Tregs from the blood is consistent with the possibility that circulating Tregs emigrate from the blood into the peripheral tissues to maintain tolerance. To confirm induction of tolerance in the 2D2-FIG model, vaccines consisting of IFN-β + MOG35–55 in Alum, IFN-β + OVA323–339 in Alum, or MOG35–55 in Alum (5 nmol) were given on days −21, −14, and −7, followed by active challenge with MOG35–55/CFA on day 0. As shown in Fig. 8 and Table II, IFN-β + MOG in Alum prevented the subsequent induction of EAE and EAE-associated weight loss, whereas the control vaccines had no effect on induction of severe paralytic EAE.

Does IFN-β adjuvant promote infectious tolerance?

An important question is whether the IFN-β in Alum adjuvant fosters infectious tolerance via the spread to Ags beyond those epitopes directly included in the vaccine formulation. To address this question (Fig. 9, Table III), B6 mice were vaccinated with OVA in Alum, IFN-β + OVA + MOG in Alum, or IFN-β + OVA in Alum on day −8 and were challenged with OVA + MOG in CFA or MOG in CFA on day 0. The results showed that the OVA in Alum vaccine had no activity, whereas IFN-β + OVA + MOG in Alum inhibited the subsequent induction of EAE (Fig. 9A, 9B). The key observation was that the IFN-β + OVA in Alum vaccine was or was not effective, depending on whether OVA was or was not included in the MOG/CFA emulsion, respectively (Fig. 9C, 9D) (i.e., the IFN-β + OVA in Alum vaccine inhibited EAE upon immunization with OVA +MOG/CFA but did not impact EAE upon immunization with MOG/CFA). These data were consistent with the possibility that vaccine-induced OVA-specific Tregs modulated EAE only when OVA was included in the encephalitogenic emulsion. These data revealed the potential cross-regulation of encephalitogenic T cell precursors by OVA-specific Tregs when the respective epitopes were presented in the same localized environment or on the same APC.

Interplay of TGF-β and IFN-β in mechanisms of Treg induction

IFN-β and TGF-β shared overlapping functionality for Ag-dependent induction of CD25+ FOXP3+ Tregs. However, IFN-β was less efficient and required substantially higher concentrations than TGF-β (e.g., 1 μM IFN-β versus 1 nM TGF-β, Fig. 4). This observation suggested the possibility that IFN-β–mediated Treg induction is TGF-β dependent. To assess this issue, naive 2D2-FIG

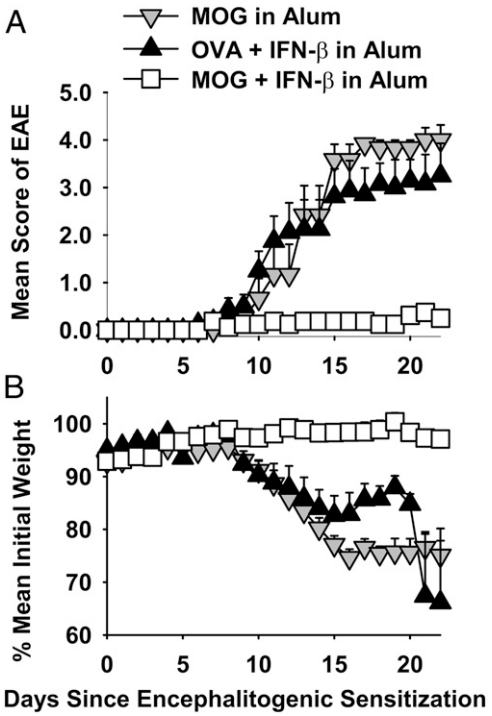

FIGURE 8. Pretreatment with the IFN-β + MOG in Alum vaccine inhibited the subsequent induction of EAE in MOG35–55 TCR-transgenic mice. Vaccines containing MOG35–55 in Alum, IFN-β + OVA323–339 in Alum, or IFN-β + MOG35–55 in Alum were given on days −21, −14, and −7 to 2D2-FIG mice at dosages of 5 nmol each for IFN-β and Ag. Mice were challenged with 100 μg MOG35–55 in CFA on day 0 and were given pertussis toxin on days 0 and 2. Time courses for EAE (**A**) and body weight (**B**) through day 22. Error bars represent SEM. The statistical analysis is given in Table II.

splenocytes were cultured in the presence or absence of 1 μM IFN-β, 100 pM TGF-β, 31.6 μg/ml anti-mouse TGF-β (1D11.16.8, mouse IgG1), or an isotype-control anti-rat LFA-1 (LRTC 1, mouse IgG1) with 1 μM MOG35–55 (Fig. 10) or without Ag (data not shown). The results showed that induction of IFN-β–Tregs was fully blocked by the anti–TGF-β mAb (Fig. 10A–C, 10E). Control cultures showed that TGF-β activity was also blocked by anti–TGF-β (Fig. 10A, 10D, 10E). IFN-β–mediated induction of Tregs, as measured by T cell percentage or absolute numbers, was inhibited by anti–TGF-β but not by the isotype-control mAb (Fig. 10B, 10E). The inhibitory activity of the 1D11 anti–TGF-β mAb reflected a competitive interaction (Fig. 10D). In cultures induced with 1 μM IFN-β or 100-316 pM TGF-β, 50% inhibition was evident at 1D11 concentrations ~ 1 μg/ml (Fig. 10C, 10D). Overall, these data indicate that IFN-β elicits FOXP3$^+$ Treg differentiation in vitro, at least in part, through the action of TGF-β. Although IFN-β elicited FOXP3$^+$ Tregs via a TGF-β–dependent mechanism, these data did not preclude the possibility that unique IFN-β activities other than TGF-β shaped Treg differentiation and function.

Given the possibility that IFN-β may act indirectly via the induction of TGF-β, a relevant question was whether TGF-β, like IFN-β, exhibits activity as a tolerogenic adjuvant in Alum. Thus, we assessed the relative activity of IFN-β versus TGF-β for induction of FOXP3$^+$ T cells upon tolerogenic vaccination of 2D2-FIG mice (Fig. 11). The full biological potency of IFN-β and TGF-β preparations was confirmed by in vitro bioassay. Administration of IFN-β + MOG35–55 in Alum elicited increased percentages (Fig. 11A, 11B) and numbers (Fig. 11C) of Tregs in PBMCs compared with vaccination with saline in Alum. The IFN-β + MOG35–55 in Alum vaccine also increased the percentages (Fig. 11D) and numbers (data not shown) of circulating granulocytes. The latter observation may reflect IFN-β–mediated activation of an innate immune response. Administration of TGF-β at a dose of 5 or 2 nmol with MOG35–55 (5 nmol) in Alum also increased the total number of FOXP3$^+$ Tregs per μl microliter of blood (Fig. 11C), but this increase reflected a proportional increase in the number of CD3$^+$ T cells (Fig. 11E) and total leukocytes (data not shown). Overall, TGF-β–based vaccination did not elicit increased FOXP3$^+$ Tregs as a percentage of the CD4$^+$ T cell pool (Fig. 11A, 11B) or the total leukocyte pool. Cytokine adsorption to Alum was evident for IFN-β and TGF-β because both were completely bound during incubation with Alum (data not shown). These data revealed differential activities of IFN-β and TGF-β for Treg induction in vivo, with the implication that IFN-β may have a more dedicated alignment with the FOXP3 T cell lineage compared with TGF-β.

Discussion

IFN-β is a gateway cytokine for the immunosuppressive FOXP3$^+$ Treg lineage

IFN-β has been a front-line therapeutic for MS for nearly two decades and is uniformly inhibitory in wild-type models of EAE (11, 53–72), but underlying suppressive mechanisms of IFN-β remain unresolved. This study provides evidence that IFN-β is a gateway cytokine that polarizes T cell differentiation toward the immunosuppressive FOXP3$^+$ Treg lineage. Furthermore, this study reveals that the regulatory activities of IFN-β can be exploited to impose tolerogenic memory for specific myelin NAg.

Several lines of evidence support a role for type I IFNs in Treg development and homeostasis. In MS therapy, IFN-β treatment is associated with the restoration of Treg frequency and suppressive function (73–79). Indeed, 3–12 mo of IFN-β therapy increased the frequencies and suppressive activity of peripheral CD4$^+$ CD25$^+$ FOXP3$^+$ T cells ex vivo. Collectively, these studies support the idea that IFN-β alleviates MS, in part, via the induction of Treg numbers and function. A similar relationship between IFN-β treatment and Treg induction was noted in EAE: IFN-β administration induced the expression of various costimulatory ligands on DCs that, in turn, enhanced Treg expansion and function (78). Type I IFN administration was also required for maintenance of FOXP3 expression and suppressive Treg activity in a mouse model of colitis by a mechanism dependent upon the expression of IFN-α/β receptors (80). In competitive environments, IFNAR signaling in T cells, and particularly in FOXP3$^+$ Tregs, was needed for homeostasis and functional differentiation (81). Type I IFNs also facilitated alternative Treg lineages. Type I IFNs were implicated as pivotal cytokines for the differentiation of the IL-10–producing Tr1 regulatory T cell subset (82–85) and the recently discovered FOXA1$^+$ FOXP3^{neg} regulatory T cell subset (86). FOXA1 drives the *Pdl1* promoter in this Treg subset to confer expression of PDL1 to enable Treg killing of activated conventional effector T cells. Adoptive-transfer experiments showed that FOXA1$^+$ Tregs suppressed EAE by a mechanism that required T cell–intrinsic IFN-α/β receptor signaling. In relapsing-remitting MS, IFN-β treatment increased the frequencies of suppressive FOXA1$^+$ Tregs in blood. In summary, type I IFNs are implicated as potential lineage-specification/polarization factors for three unique Treg subsets, including FOXP3$^+$, Tr1, and FOXA1$^+$ T cells.

The requirement for linked recognition of IFN-β and NAg

Previous studies of cytokine–NAg fusion proteins established the general concept that cytokine action and T cell Ag recognition must be tightly linked to achieve tolerance induction. In this

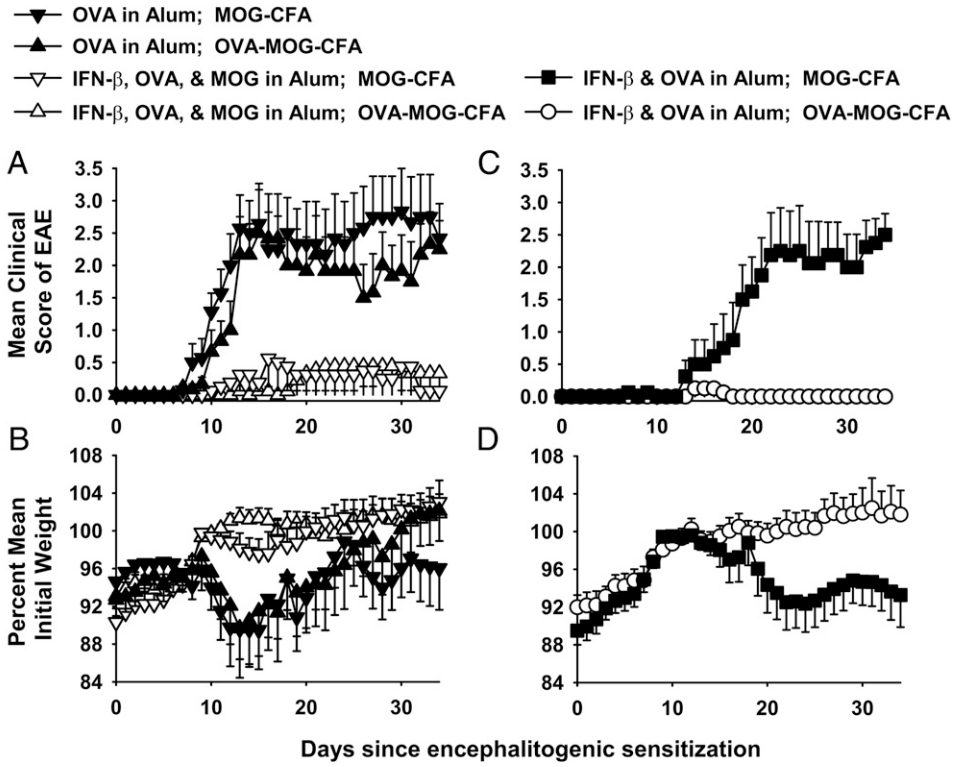

FIGURE 9. Does IFN-β adjuvant promote infectious tolerance? Mice were vaccinated once with three different vaccines, including IFN-β + OVA + MOG in Alum, IFN-β + OVA in Alum, and OVA in Alum (5 nmol dose for all reagents) on day −8. Mice were challenged with two different emulsions to induce EAE, including OVA + MOG in CFA or MOG in CFA (100 μg dose for each peptide) on day 0. Pertussis toxin was given on days 0 and 2. Time course data through day 34 for EAE (**A** and **C**) and weight loss (**B** and **D**). Statistical analyses are given in Table III.

regard, the covalent linkage of cytokine and NAg imposed tight linkage of cytokine and NAg recognition, and this linkage was required for tolerance induction. The only exception was noted in studies of the rat IFN-β–NAg tolerogenic vaccine that consisted of the rat IFN-β fused to the dominant 73–87 encephalitogenic epitope of MBP (41). Unlike all other fusion proteins (37–40, 42–44, 87), the covalent attachment of IFN-β and NAg was not necessary, because separate injections of IFN-β and NAg at adjacent sites were as effective as injection of the IFN-β–NAg fusion protein for prevention of disease. When treatment was initiated after disease onset, the rat IFN-β–NAg fusion protein blunted disease progression and elicited an accelerated recovery. Although not as effective as the fusion protein, treatment with the mixture of IFN-β plus NAg had suppressive efficacy that was superior to IFN-β or NAg alone. These findings presented the unexpected prospect that IFN-β and NAg could induce tolerance without the constraints of engineering single-chain fusion proteins.

Murine models of EAE, including the B6 chronic model and the SJL relapsing-remitting model, were used to confirm that IFN-β–NAg fusion proteins had tolerogenic activity and to test the requirement for covalent cytokine-NAg linkage (38). However, unlike the rat EAE system, covalent linkage of the murine IFN-β domain with PLP139–151 was necessary for tolerance induction, because equimolar doses of IFN-β and PLP139–151 as a mix of separate molecules had limited tolerogenic activity (38). Likewise, the IFN-β–MOG fusion protein facilitated clinical recovery in the chronic B6 model of EAE, whereas the combination of IFN-β and MOG had limited beneficial action (Fig. 1A, 1B). Thus, in two murine EAE systems, attachment of IFN-β to the NAg was required for long-lasting tolerance induction.

Why the rat and murine IFN-β–NAg had differential requirements for cytokine-NAg linkage is not known. One possibility is that the rat and mouse EAE systems had intrinsic differences in regulatory mechanisms, given that Lewis rats exhibited a brief monophasic disease, whereas both mouse models exhibited chronic EAE. Perhaps mixtures of IFN-β + NAg exhibit a temporary inhibitory action that might be sufficient to suppress EAE in Lewis rats but might not be sufficient to control the sustained inflammatory insult in chronic murine EAE. For example, IFN-β + MOG resulted in a temporary suppression of EAE in B6 mice from approximately day 20 to day 25 (Fig. 1A), but this inhibitory action was not sustained beyond day 30. Perhaps such a temporary

Table III. IFN-β is an adjuvant that fosters infectious tolerance

Vaccine in Alum[a]	Challenge in CFA	Incidence	Mean (± SE) Cumulative Score	*p* Value	Maximal Score (Mean (± SE)	*p* Value	Maximal Weight Loss (%)	*p* Value
IFN-β + OVA + MOG	OVA + MOG	4/9	6.5 ± 6.2	ns	0.6 ± 0.4	ns	2.7 ± 1.5	ns
IFN-β + OVA + MOG	MOG	3/8	7.2 ± 5.2	ns	0.8 ± 0.5	ns	5.6 ± 1.9	ns
IFN-β + OVA	OVA + MOG	2/8	0.4 ± 0.4	*	0.2 ± 0.1	*	2.9 ± 1.1	*
IFN-β + OVA	MOG	8/8	37.3 ± 9.9	<0.001	3.0 ± 0.4	≤0.001	11.7 ± 2.9	ns
OVA	OVA + MOG	6/6	47.6 ± 11.2	<0.001	3.0 ± 0.5	≤0.001	13.1 ± 4.8	ns
OVA	MOG	7/7	54.5 ± 13.2	<0.001	3.4 ± 0.5	≤0.001	15.7 ± 3.7	0.027

Mice were vaccinated once with three different vaccines, including IFN-β + OVA + MOG in Alum, IFN-β + OVA in Alum, and OVA in Alum (5-nmol dose for all reagents) on day −8. Mice were then challenged with two different emulsions to induce EAE, including OVA + MOG in CFA and MOG in CFA (100-μg dose for each peptide) on day 0. Pertussis toxin was given on days 0 and 2. Nonparametric ANOVA based on ranked scores was used to assess group differences in mean cumulative scores and mean maximal scores, and parametric ANOVA was used to assess group differences in percentage of maximal weight loss relative to the comparator group (*Bonferroni post hoc test).

[a]These data are portrayed graphically in Fig. 9.

ns, not significant.

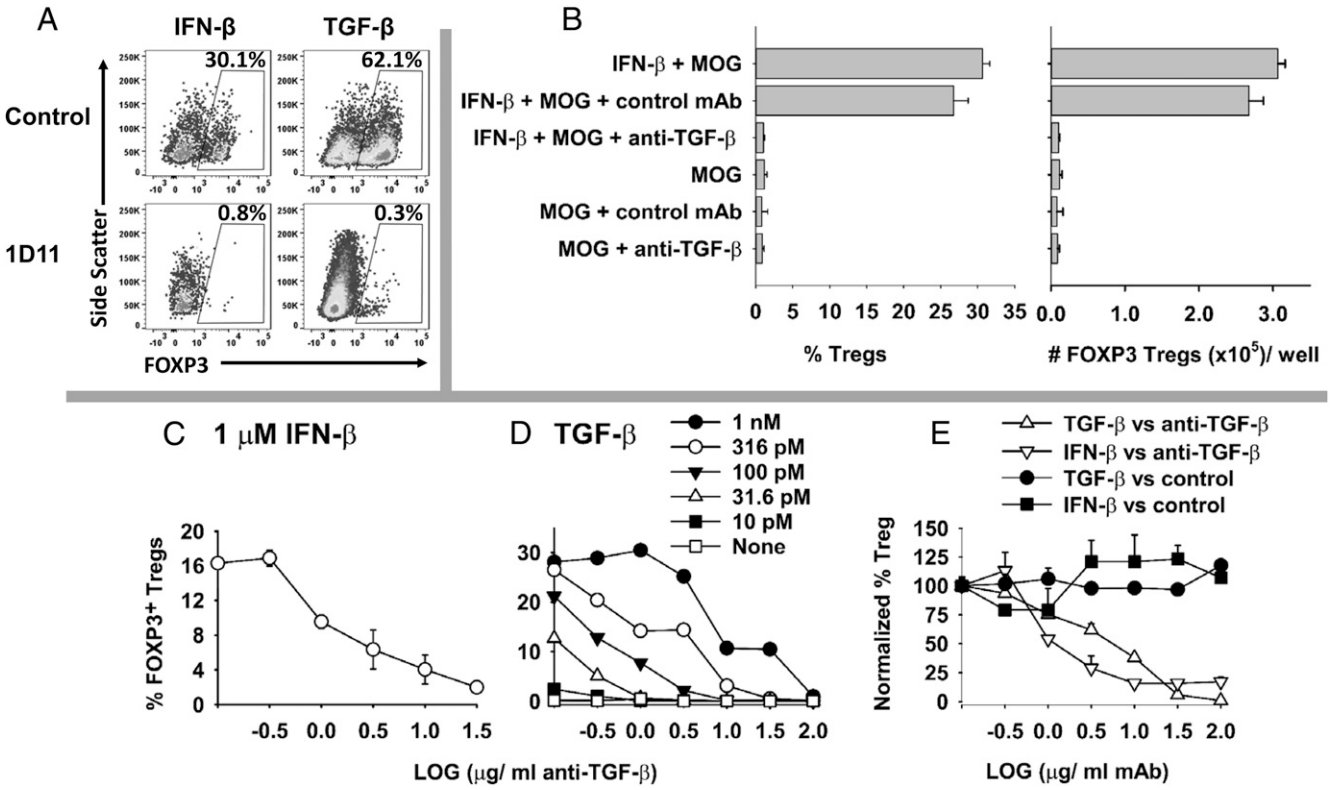

FIGURE 10. The anti–TGF-β mAb 1D11 inhibits IFN-β–dependent induction of FOXP3$^+$ Tregs in vitro. (**A**–**E**) Naive 2D2-FIG splenocytes (200,000 per well) were cultured in triplicate with 1 μM MOG35–55 plus 1 μM IFN-β or 100 pM TGF-β [or as designated in (D)]. Cultures also included anti-mouse TGF-β (1D11) or the isotype-control mAb (LRTC 1; 31.6 μg/ml) (A and B) or as designated on the *x*-axis (C–E). After 7 d of culture, CD4$^+$ T cells were assessed for GFP expression. Representative dot plots of side scatter (*y*-axis) and FOXP3 expression (*x*-axis) (A) together with percentages and absolute numbers of FOXP3$^+$ Tregs (B) after culture with MOG35–55 in the presence or absence of IFN-β and mAb. Quantitative neutralization profiles for the anti–TGF-β 1D11 mAb in cultures of IFN-β-induced Tregs (C and E) and TGF-β–induced Tregs (D and E). Error bars represent SEM. These data are representative of three independent experiments.

suppressive activity is sufficient to control a monophasic course of disease, because the rodents would exhibit spontaneous recovery before the erosion of vaccine-mediated suppression. Thus, the short-term inhibitory action of IFN-β + NAg and the long-term tolerogenic activity of IFN-β–NAg simply may not be distinguished in monophasic rat EAE. As another possibility to explain the lack of requirement for covalent linkage in the rat model, rat IFN-β and the MBP peptide may exhibit a direct or indirect

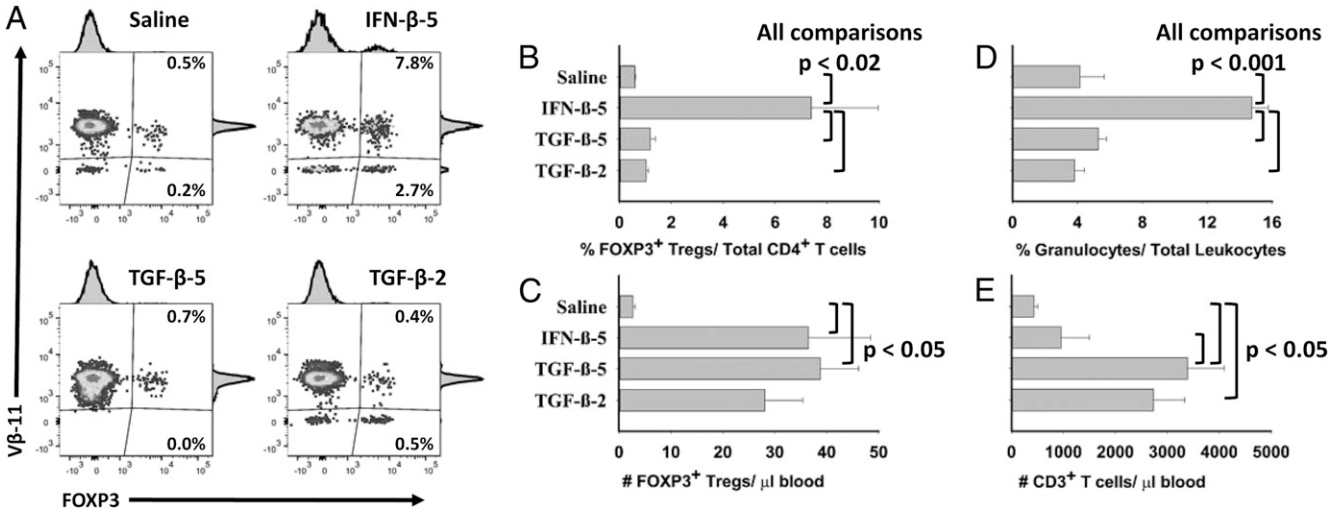

FIGURE 11. When combined with Alum and MOG35–55, IFN-β uniquely increased the percentages of FOXP3$^+$ Tregs relative to the total pool of CD4$^+$ T cells. On day 0, 2D2-FIG mice (n = 5) were injected with saline in Alum, 5 nmol IFN-β + 5 nmol MOG35–55 in Alum (IFN-β–5), 5 nmol TGF-β + 5 nmol MOG35–55 in Alum (TGF-β–5), or 2 nmol TGF-β + 5 nmol MOG35–55 in Alum (TGF-β–2). On day 13, PBMCs were assessed for FOXP3$^+$ T cells as a percentage of the CD45$^+$ CD3$^+$ CD4$^+$ T cell population (**A** and **B**), the total numbers of FOXP3$^+$ Tregs per μl of blood (**C**), the percentages of granulocytes as a percentage of total leukocytes (**D**), and the total numbers of CD3$^+$ T cells per μl of blood (**E**). Analysis panels included CD3-BV421, CD4-PE, Vβ11–Alexa Fluor 647, and CD45-BV785.

binding interaction (perhaps via a third-party molecule) by which the IFN-β became noncovalently associated with NAg peptide. If so, then the requirement for physical linkage of IFN-β and NAg may be due to APC targeting (i.e., the IFN-β domain of IFN-β–NAg complex may interact with type I IFNRs [IFNAR] on DCs to evoke DC-mediated tolerogenic activity while simultaneously targeting the physically associated NAg into the MHC class II Ag-processing pathway). The net effect would be the concentrated presentation of NAg on tolerogenic IFN-β–conditioned DCs and the consequent induction of NAg-specific Tregs.

The prospect of an effective tolerogenic vaccine containing nonlinked IFN-β and NAg moieties would have many advantages. Multivalent tolerogenic vaccines could be formulated by including selected NAg peptides in the IFN-β tolerogenic vaccine. This advantage would accommodate the prospect that MS may be mediated by a polyclonal repertoire of myelin-reactive T cells. Such vaccines could be adapted to include NAg epitopes to match myelin specificities on a per-patient basis and could be adjusted based on emerging pathogenic epitopes due to epitope spread and disease evolution. A major advantage would also be realized in manufacturing and quality control. Such vaccines would obviate the potential difficulty that attachment of some NAg peptides to the IFN-β C terminus might impair the biological activity of IFN-β or create immunogenic neoantigens. Given the development and use of IFN-β over the past two decades as a front-line MS therapeutic, the main manufacturing challenge would encompass synthetic NAg peptides rather than recombinant fusion proteins.

Although physical linkage is needed for optimal tolerogenic activity in SJL and B6 models of EAE, one would predict that such linkage could be noncovalent and indirect rather than covalent and direct. To test this possibility, we reasoned that the Alum adjuvant may fulfill the requirement for indirect noncovalent linkage because the aluminum hydroxide gel is well known to bind protein and peptide Ags. The prediction is that IFN-β and NAg peptides would be cross-linked and immobilized by the Alum gel and, thereby, achieve stable physical linkage of IFN-β and NAg that is requisite for APC targeting and IFN-β–mediated tolerogenic activity. Aluminum-based adjuvants have been extensively used in vaccines over the past century, remain the only adjuvant approved and licensed for routine use in human vaccines, and represent the most common adjuvants used in veterinary vaccines. These Alum adjuvants are composed of aluminum phosphate ($AlPO_4$) or aluminum hydroxide [$Al(OH)_3$]. Despite intensive research into alternative adjuvants, Alum adjuvants continue to dominate the adjuvant landscape as a result of their significant advantages in safety, manufacturing, quality control, and adjuvanticity for humoral (but not cellular) vaccine responses. Alum is thought to bind vaccine Ags via electrostatic interactions to form a concentrated tissue depot that promotes recognition, phagocytosis, Ag processing, and presentation by DCs. In this regard, the prediction is that the IFN-β + MOG in Alum vaccine forms a concentrated depot at the inoculation site and within the associated lymphatic drainage. This antigenic depot may attract DCs that engage high concentrations of IFN-β and NAg to provide the stimuli for NAg-specific FOXP3$^+$ Treg responses.

IFN-β–based tolerogenic vaccines and the directed specification of FOXP3$^+$ Tregs. The tolerogenic vaccine had three components necessary for inhibition of EAE, including IFN-β as the key Treg-specification adjuvant, NAg as the clonotypic-specification Ag, and Alum as the requisite cohesive adjuvant (Figs. 1, 3, 7–9). The IFN-β + NAg in Alum vaccine also inhibited MOG-induced sensitization (Fig. 2). This is an effective and practical approach for tolerogenic vaccination based on inductive suppressive responses by NAg-specific FOXP3$^+$ Tregs. Although tolerogenic vaccination in vivo was entirely contingent upon Alum, IFN-β and NAg elicited Treg differentiation in vitro in the absence of Alum (Figs. 4, 5). These observations indicate that IFN-β was key for the specification of the Treg lineage, whereas Alum was a passive adjuvant that was compatible, but not directive, for Treg differentiation. Alum appeared to have three main attributes. We postulate that Alum provided a stable Ag depot required for persistent antigenic exposure, provided the physical matrix that enabled IFN-β/NAg cross-linking requisite for effective vaccination, and had the compatibility needed for induction of tolerogenic FOXP3$^+$ Treg responses, because the adjuvant's intrinsic activities on innate immunity did not interfere or pre-empt Treg responses.

Tolerogenic vaccination with IFN-β + MOG in Alum appeared contingent upon CD25$^+$ FOXP3$^+$ Tregs. Pretreatment with the anti-CD25 PC61 mAb purged circulating CD25$^+$ FOXP3$^+$ Tregs (88) and fully reversed the suppressive activity of the IFN-β + MOG in Alum vaccine (Fig. 3). The suppressive activity of the Tregs was NAg-specific because the IFN-β + MOG in Alum vaccine inhibited MOG-induced EAE but not PLP-induced EAE. Conversely, the IFN-β + PLP in Alum vaccine inhibited PLP-induced EAE but not MOG-induced EAE. Thus, prophylactic vaccination induced Treg-mediated suppressive activity by a mechanism restricted to the TCR-specific Treg clonotype. Although strict clonotypic specificity was noted in this paradigm, tolerogenic clonotypes nonetheless controlled disparate specificities if both epitopes were included in the encephalitogenic emulsion (i.e., an IFN-β + OVA in Alum vaccine inhibited EAE when mice were subsequently challenged with MOG + OVA in CFA emulsion, whereas the same vaccine had no impact on mice challenged with MOG in CFA) (Fig. 9). These data revealed that clonotypic Tregs have the capacity to impact clonotypically distinct Tconvs if the two clonotypes are bridged by proximal APC-recognition events. Thus, IFN-β + NAg in Alum vaccines may be useful for establishing broad CNS-based tolerogenic coverage of epitopes that are unknown and not directly included in the vaccine.

Repeated tolerogenic vaccinations with IFN-β + MOG in Alum appeared to boost Treg abundance and elicit high-level expression of the CD44 glycoprotein on FOXP3$^+$ Tregs (Fig. 7). Given that CD44 is a marker of effector or memory status, repeated vaccine boosters may elicit more efficient functional responses. Three repeated vaccinations with IFN-β + MOG in Alum uniquely resulted in accumulation of the CD44^{high} Treg subset. In contrast, one or two vaccinations elicited Tregs that mostly expressed intermediate levels of CD44. These findings provided evidence that repeated boosters of tolerogenic vaccines may not simply elicit more Tregs but may also drive memory/effector Treg populations. In support, IFN-β–induced Tregs exhibited diverse phenotypic subsets not observed among TGF-β–induced Tregs (Fig. 5C–F). Of interest, IFN-β uniquely evoked a FOXP3$^+$ T cell subset that expressed high levels of CD69, CTLA-4, and IFNAR. Overall, these findings support the concept that IFN-β facilitates nascent NAg-specific Treg differentiation and maturation of Treg effector function.

A central question pertained to the potential interplay between IFN-β and TGF-β in that both cytokines had overlapping activities that fostered expression of FOXP3 and tolerogenic Treg-mediated pathways. TGF-β was more effective than IFN-β in vitro (Fig. 4) in that low concentrations of TGF-β (1 nM) stimulated FOXP3 expression in >50% of MOG-stimulated 2D2-FIG T cells, whereas relatively high concentrations of IFN-β (1 μM) were required to approximate this response. Importantly, the 1D11 anti–TGF-β mAb blocked the ability of IFN-β to elicit FOXP3$^+$ Tregs in vitro (Fig. 10). These data indicate that the

Treg-inductive properties of IFN-β stem, at least in part, from an IFN-β–induced TGF-β–dependent response pathway possibly involving enhanced production of TGF-β during culture. However, the interaction of IFN-β and TGF-β was nonadditive or antagonistic (Fig. 4), but this observation may simply reflect a bell-shaped concentration curve because increasing TGF-β concentrations above the optimal 1-nM concentration diminished Treg induction (data not shown). The paradox was that IFN-β was more efficient than TGF-β as a vaccine for the selective induction of FOXP3$^+$ Tregs in vivo (Fig. 11). Unlike IFN-β, vaccination with TGF-β + MOG in Alum did not result in increased percentages of FOXP3$^+$ Tregs in the circulation. However, TGF-β–based vaccination was not inert, as evidenced by increased numbers of T cells and total leukocytes in the blood but without alteration of Treg/CD3$^+$ T cell ratios (i.e., TGF-β–based vaccines increased total Treg numbers along with proportional increases in CD3$^+$ T cells and total leukocytes, such that the frequencies of Tregs did not differ from control mice). One possibility is that TGF-β is pluripotent and supports differentiation of multiple T cells subsets, including Th17 T cells, among others. TGF-β may not be strictly dedicated or restricted to the specification of the FOXP3 lineage, such that a TGF-β–based vaccination strategy may lack the singular focus needed to drive this Treg subset. Based on this argument, the efficacious activity of IFN-β–based vaccination in vivo may reflect a close alignment of IFN-β activity with a focused action on FOXP3$^+$ T cell differentiation and bystander suppression of alternative T cell subsets.

What is the physiological rationale for IFN-β involvement as a differentiation factor for the FOXP3$^+$ Treg subset?

The paradox is that IFN-β has a critical role in innate immune responses against pathogenic viruses and other infectious pathogens yet facilitates tolerance during adaptive T cell responses. Why would IFN-β serve as a linchpin for innate immunity but function as an inhibitor of adaptive immunity? A potential answer dovetails with the strategic role of IFN-β in viral immunity. IFN-β is a major impediment to viral spread by ensuring that the neighboring uninfected cells lack the metabolic capacity necessary to support viral replication, propagation, and transmission to adjacent cells. This containment strategy provides time for B cells to mount antiviral Ab responses that prevent further viral dissemination; however, it may be thwarted by infiltrating antiviral T cells if these T cells themselves become infected and spread the viral infection by migration throughout the lymphatics and other peripheral tissues. A conceivable solution is that IFN-β may coordinate with IFN-β–induced Tregs to inhibit the activity and migration of highly mobile conventional CD4$^+$ T cells as a strategy to limit viral spread. The subset of IFN-β–induced Tregs may be relatively immobile and may be specialized to be the dominant T cell subset in areas replete with high concentrations of IFN-β. As shown in this study, FOXP3$^+$ Tregs exhibited dominant survival and growth compared with Tconv subsets in cultures supplemented with high concentrations of IFN-β (Fig. 4C). In this regard, the regulatory activities of Tregs may reflect inhibitory cross-regulatory circuits that pre-empt other conventional subsets of effector T cells (89). In support of this, a subset of IFN-β–induced Tregs expressed high levels of CD69, IFNAR, and CTLA-4 (Fig. 5). In addition to enforcing regional tolerance and suppression of Tconv subsets, IFN-β–induced Tregs may mediate specialized antiviral cytotoxic activity and, thereby, may directly resolve viral infection. Although Tregs are often considered to be exclusively immunosuppressive, one must consider the possibility that Tregs instead have specialized effector activities (89), particularly because Tregs have a phenotype most consistent with activated effector T cells. Accordingly, previous studies showed that Treg ablation during mucosal HSV infection in mice resulted in an accelerated fatal infection marked by elevated viral loads and deficient type I IFN production at the site of infection (90). These data denote a direct effector action of Tregs in antiviral immunity and a connection with local protective IFN-β production. Thus, IFN-β–induced Tregs may be an extension of the IFN-β–based strategy of viral containment and may mediate T cell–mediated antiviral immunity, together with robust cross-inhibitory suppression of competing Tconv subsets.

In conclusion, this study shows that IFN-β and a myelin NAg synergistically caused the emergence of a suppressive myelin-specific FOXP3$^+$ Treg subset. Thus, these data revealed the existence of an IFN-β/FOXP3 Treg axis and implicated this axis as an important mechanism of suppression in EAE. This IFN-β/FOXP3 Treg axis may serve as the experimental foundation for effective tolerogenic vaccination in CNS autoimmune disease. Thus, this study revealed a novel and exquisitely simple approach by which IFN-β and a myelin NAg can be formulated in Alum as an effective tolerogenic vaccine.

Disclosures

The authors have no financial conflicts of interest.

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