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Microglial Activation Milieu Controls Regulatory T Cell Responses

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Although mechanisms leading to brain-specific inflammation and T cell activation have been widely investigated, regulatory mechanisms of local innate immune cells in the brain are only poorly understood. In this study, to our knowledge we show for the first time that MHC class II⁺CD40^{dim}CD86^{dim}IL-10⁺ microglia are potent inducers of Ag-specific CD4⁺Foxp3⁺ regulatory T cells (Tregs) *in vitro*. Microglia differentially regulated MHC class II expression, costimulatory molecules, and IL-10 depending on the amount of IFN- γ challenge and Ag dose, promoting either effector T cell or Treg induction. Microglia-induced Tregs were functionally active *in vitro* by inhibiting Ag-specific proliferation of effector T cells, and *in vivo* by attenuating experimental autoimmune encephalomyelitis disease course after adoptive transfer. These results indicate that MHC class II⁺CD40^{dim}CD86^{dim}IL-10⁺ microglia have regulatory properties potentially influencing local immune responses in the CNS. *The Journal of Immunology*, 2013, 191: 5594–5602.

During pathological conditions such as CNS infections, brain ischemia, or neuroinflammatory diseases such as multiple sclerosis or experimental autoimmune encephalomyelitis (EAE), leukocytes are recruited into the CNS parenchyma (1), mediating destructive inflammatory processes (2). Microglia, the immune sentinels of the brain, present the first line of immune defense in the CNS whenever there is injury or disease (3). Under steady-state, CNS microglia display a low APC phenotype. In EAE experiments, activated microglia are present in demyelinating lesions and express molecules of APC function (MHC class II [MHC II], CD40, CD80, CD86) (4–6) necessary to interact with brain-invading CD4⁺ immune cells (7). It is generally thought that Th1 and also Th17 cells, directed against myelin Ags, enter the brain where they re-encounter their Ags presented by microglia and/or perivascular macrophages and initiate a destructive inflammatory cascade (2).

Activated resident microglia also accumulate in lesion sites of acute brain ischemia (8) and noninflammatory entorhinal cortex lesion (ECL) (9). In case of ECL, which results in axonal degeneration, cell death, and massive release of myelin Ags, microglia are activated, increase in number (10), phagocytose neuronal debris, and present Ags in an MHC II–dependent way. Additionally, a certain number of myelin-specific lymphocytes expand in draining lymph nodes, invade regions of degeneration (9, 11), and mediate secondary white matter damage (12). However, traumatic ECL does not elicit autoimmune encephalomyelitis-like white matter damage. Thus, the initial expansion of myelin-specific Th1 cells in regions of degeneration are tightly regulated and kept under control, implying active maintenance of tolerance.

In the periphery, CD4⁺ regulatory T cells (Tregs) ensure immune homeostasis and maintain immunological self-tolerance (13). Natural thymus-derived Foxp3⁺ Tregs and inducible Ag-specific Foxp3⁺ Tregs secrete anti-inflammatory cytokines, for example, IL-10 and TGF- β , and they alter the activity of APCs (14). The induction of Ag-specific tolerance is essential to control autoreactive effector T cells and thus prevent autoimmune diseases (15). Along with invading Th1/Th17 cells during EAE disease course, several groups have now identified CD4⁺CD25⁺ Foxp3⁺ Tregs directly in the inflamed CNS (16, 17).

Professional, MHC II–expressing APCs favoring Foxp3⁺ Treg induction, such as suboptimal primed dendritic cells (DCs) or anti-inflammatory type II monocytes, are missing in the healthy CNS, but brain-resident microglia have the potential to migrate to regions of tissue damage, undergo proliferation, increase phagocytic activity, secrete cytokines and chemokines, and upregulate molecules for effective Ag presentation. The activation status of microglia thereby contributes to mechanisms of immune surveillance and homeostasis in the CNS (18). Moreover, microglia properties for limiting CNS inflammation or regulating immune responses have been discussed (19). We hypothesized that microglia can do both, that is, act as an inducer or amplifier of inflammatory events as well as an inducer of regulatory mechanisms. To study whether microglia cells can have bifunctional effects we investigated APC properties of microglia under varying inflammatory signals. We investigated the potential of microglia to induce Foxp3⁺ Tregs

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The online version of this article contains supplemental material.

Abbreviations used in this article: CD40L, CD40 ligand; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; ECL, entorhinal cortex lesion; MBP, myelin basic protein; mgTreg, microglia-induced regulatory T cell; MHC II, MHC class II; MOG, myelin oligodendrocyte glycoprotein; nTreg, natural regulatory T cell; Treg, regulatory T cell; WT, wild-type.

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in vitro and the regulatory potential of microglia-induced Tregs (mgTregs) in vitro and in vivo. Our data show that depending on the inflammatory signal strength, IFN- γ concentration, and Ag dose, and thus resulting activation status, microglia modulate T cell responses by either enhancing effector or Treg generation.

Materials and Methods

Mice

C57BL/6J wild-type mice were purchased from Elevage Janvier. C57BL/6J-*Foxp3^{EGFP}* reporter mice were provided by B. Malissen (20). TCR transgenic C57BL/6-Tg (Tcr α 2D2, Tcr β 2D2)1Kuch/J mice, known as 2D2 mice, and MHC II-deficient mice (B6.129S2-*H2^{dAb1-Ea}*/J) were purchased from The Jackson Laboratory. 2D2 mice, reactive to myelin oligodendrocyte glycoprotein (MOG)_{35–55}, were intercrossed with *Foxp3^{EGFP}* reporter mice (2D2.*Foxp3^{EGFP}*). All mice were bred and maintained under specific pathogen-free conditions and all experiments were performed according to institutional guidelines with approval of the Ethical Review Board at the animal facility of Charité–University Medicine Berlin.

EAE immunization

One day before EAE immunization, female C57BL/6J mice received either 500,000 CD4⁺CD25⁺ Tregs i.v. in 200 μ l PBS or PBS alone. Mice were immunized s.c. in the flanks with 250 μ g MOG_{35–55} peptide (MEVG-WYRSPFSRVVHLYRNGK, Pepceuticals) emulsified in CFA containing H37 Ra (BD Biosciences). Pertussis toxin (List Biological Laboratories) was administered (400 ng/mouse) i.p. on the day of immunization and day 2. Individual animals were observed for signs of EAE for up to 30 d and scored using the following scale: 0, no disease; 1, limp tail and/or hindlimb ataxia; 2, hindlimb paresis; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; 5, death.

T cell suppression assay

SNARF-1-labeled 2D2 CD4⁺ T cells were seeded at 3×10^5 cells/well in a 96-well plate in RPMI 1640 media (10% FCS, 1% penicillin/streptomycin, 0.5 μ M 2-ME) together with irradiated splenic cells (APCs) from the same animals (5×10^5 cells/well). Sorted mgTregs (CD4⁺CD25⁺Foxp3⁺) were cocultured with APCs and SNARF-1-labeled T cells at various ratios for 4 d (37°C in 5% CO₂) in the presence of 10 μ g/ml TCR peptide (MOG_{35–55}). As positive control, SNARF-1-labeled cells and APCs were cocultured with microglia-induced CD4⁺CD25⁺Foxp3⁻ T cells or alone in DMEM containing 10 μ g/ml MOG_{35–55}. Proliferation was then assessed by SNARF-1 dilution using a FACSCanto II analyzer (BD Biosciences) at day 5.

Primary microglia culture and T cell coculture assay

Mixed glial cell cultures were prepared from cerebral cortex of newborn (P0–2) C57BL/6J mice as described previously (21). Cortices and brain stem were isolated and the blood vessels and meninges were carefully removed. Following dissection, the tissues were subjected to enzymatic dissociation (0.05% trypsin-EDTA [Invitrogen] and 25 μ g/ml DNase I [Roche]), washed in FCS containing HBSS, and resuspended in DMEM (10% FCS, 1% penicillin/streptomycin, 0.5 μ M 2-ME). Mixed glial cells were seeded into poly-D-lysine-coated flasks and grown at 37°C and 5% CO₂. After 10–12 d microglia were separated from the underlying astrocytic layer by gentle shaking of the flask. Cultures usually contained >95% microglial (CD11b⁺) cells. For coculture experiments microglia were seeded in 96-well plates (8×10^4 cells/well) and stimulated with varying amounts of rIFN- γ (PeproTech) for 24 h (low dose, 10 U/ml; high dose, 100 U/ml) and then pulsed with synthesized MOG_{35–55} peptide (Pepceuticals) for another 24 h. The treatment media was completely removed and the preactivated and pulsed microglia were washed and cocultured with 2D2.*Foxp3^{EGFP}* CD4⁺ T cells. After 7 d of coculture, T cells were harvested from the microglial monolayer and analyzed for CD4, CD25, and Foxp3^{EGFP} by flow cytometry (FACSCanto II). For kinetic experiments, CD4⁺ T cells were analyzed daily between days 1 and 7. For studying the role of CD40/CD40 ligand (CD40L) interaction, microglia were high dose-stimulated and cocultured with CD4⁺ T cells in the presence of CD40L Ab (anti-CD154, clone MR1, 40 μ g/ml).

Cell preparation and cell sorting

For coculture experiments, CD4⁺ T cells from 2D2.*Foxp3^{EGFP}* mice were purified by negative selection using a CD4⁺ T cell isolation kit (Miltenyi Biotec). When indicated, CD25⁺ cells were further depleted by positive

selection with PE-conjugated anti-CD25 and anti-PE magnetic microbeads (Miltenyi Biotec). The purity of CD4⁺CD25⁻ or CD4⁺ cells was routinely >95% as determined by flow cytometry (FACSCanto II). For adoptive transfer experiments, CD4⁺ T cells were further enriched for CD25⁺ with PE-anti-CD25 and anti-PE magnetic beads. For functional analysis, mgTregs (CD4⁺CD25⁺Foxp3^{EGFP+}) or CD4⁺CD25⁻Foxp3^{EGFP-} T cells were sorted on a FACSAria cell sorter (BD Biosciences).

Cytokine and chemokine detection

The concentrations of IL-13, IL-23, IL-6, IL-10, IL-1 α , MCP-1, IL-4, TNF- α , and IL-17 in coculture supernatants were determined with bead-based multiple analyte detection (FlowCytomix; Bender MedSystems) according to the manufacturer's instructions. GM-CSF and TGF- β concentrations were analyzed using a multiplex protein assay (Milliplex; Millipore).

Flow cytometry analysis

Cells were acquired using a FACSCanto II analyzer (Diva software) and postacquisition data analysis was carried out using FlowJo software (Tree Star). The following mAbs were used: CD11b (M1/70), CD25 (PC61), CD4 (RM4-5), MHC II (M5/114.15.2), V β 11 (RR3-15), and TCR β -chain (H57-597) from BD Biosciences. The following cell proliferation dyes were used: eFluor 670 (eBioscience) and SNARF-1 (Molecular Probes).

TaqMan PCR

Predeveloped TaqMan gene expression assays and TaqMan Fast Mastermix (Applied Biosystems) were used for amplification of mRNA transcripts of *Cd40* (Mm00441891_m1), *Cd80* (Mm00711662_m1), *Cd86* (Mm00444543_m1), *MhcII* (Mm01271198_m1), *Ilio* (Mm00439614_m1), *Ii2* (Mm00434256_m1), *Tgfb* (Mm00441724_m1), and *Hprt* (Mm01545399_m1). *Hprt* was used as housekeeping gene. The results for the relative gene expression were calculated using the 2^{- $\Delta\Delta C_t$} method. All amplification reactions were performed on an ABI Prism 7500 Fast real-time PCR System (Applied Biosystems).

Epigenetic

Genomic DNA was analyzed by Epiontis (Berlin, Germany) as previously described (22). Genomic DNA was isolated from mgTregs using a DNeasy blood kit (Qiagen) following the manufacturer's protocol. For analyzing the degree of methylation of individual CpG motifs within the murine *Foxp3* locus, two overlapping amplicons were selected. DNA methylation analysis was performed by bisulfite sequencing by Epiontis. Cells harvested for bisulfite sequencing from microglia cocultures and lymph nodes were enriched for CD4⁺CD25⁺, but not Foxp3, resulting in a CD4⁺Foxp3⁺/CD4⁺Foxp3⁻ ratio of 1:1 (mgTregs/effector T cells).

Statistical analysis

Kolmogorov–Smirnov test was used to analyze Gaussian distribution. In the case of normal distribution, a Student *t* test or paired *t* test was used. When data failed a normality test, a Mann–Whitney *U* test or Wilcoxon signed rank test for paired observations was performed. For analysis, we used GraphPad Prism software, version 5.0a.

Results

CD4⁺Foxp3⁺ Tregs are induced by microglia

We set up an in vitro microglia/CD4⁺ T cell coculture system mimicking different microglial activation states by using rIFN- γ in combination with the neuronal Ag MOG. CD4⁺ T cells were isolated from 2D2 mice intercrossed with *Foxp3^{EGFP}* reporter mice (2D2.*Foxp3^{EGFP}*), providing us with MOG-specific CD4⁺ cells that express GFP in Foxp3⁺ Tregs. We observed a differential effect of microglia activation on CD4⁺ T cell responses when stimulated with either low-dose IFN- γ /MOG (10 U/ml/1 μ g/ml) or high-dose IFN- γ /MOG (100 U/ml/10 μ g/ml). When interacting with low dose-primed microglia, CD4⁺ T cells preferentially differentiated into Foxp3⁺CD4⁺CD25⁺ Tregs (mgTregs; Fig. 1A, 1B), whereas high dose-activated microglia induced the expansion of CD25⁺Foxp3⁻ T effector cells (Fig. 1A, 1B). Initial numbers of Foxp3⁺ mgTregs (mean cell number of 12,000 per well) at the beginning of a coculture did thereby increase on average 2.5-fold with low-dose IFN- γ /MOG treatment and yielded a recovery of 30,000 Foxp3⁺ mgTregs per well. In contrast, from cocultures

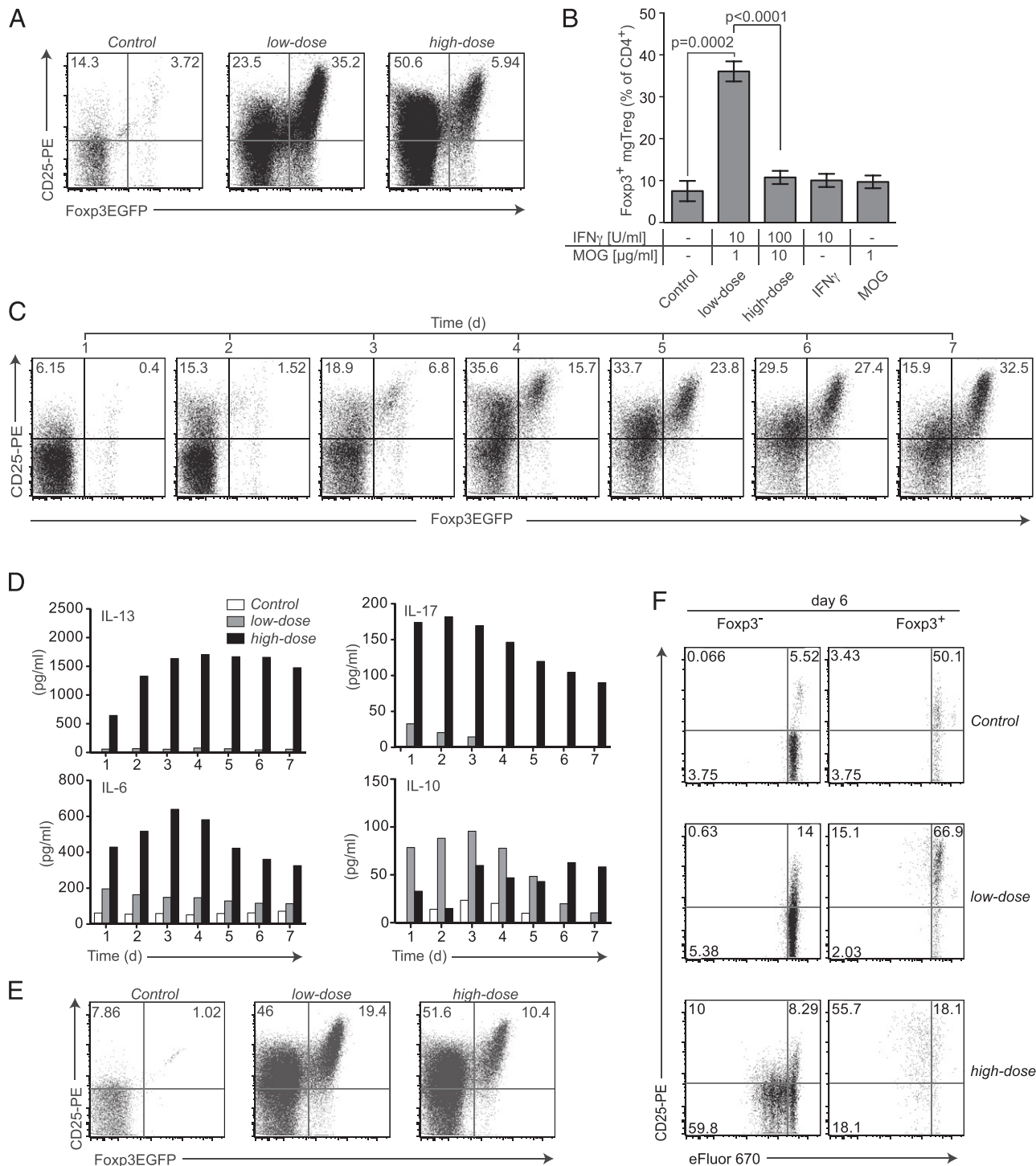


FIGURE 1. Low dose IFN- γ /MOG-stimulated microglia induce predominantly Foxp3⁺ Tregs in vitro. Differentially stimulated microglia were cocultured for 7 d with CD4⁺ T cells derived from 2d2.Foxp3^{EGFP} mice. **(A)** At day 7, T cells were analyzed by flow cytometry. Plots are gated for live T cells, CD11b⁻ T cells, and CD4⁺ T cells. Data are representative of four separate experiments. **(B)** Comparison of mgTreg frequencies in cocultures with differentially stimulated microglia ($n = 4-6$, Student t test). **(C)** Kinetic analysis of Foxp3⁺ mgTregs in low dose-stimulated microglia cocultures during 7 d. Data are representative of four separate experiments. **(D)** Kinetic analysis of coculture supernatants from control microglia, low-dose, and high-dose conditions for IL-13, IL-17, IL-10, and IL-6. Data are representative of three individual experiments. Exemplarily, mean x -fold increases \pm SEM of high dose- versus low dose-stimulated supernatants on day 2 were: IL-13, 24.2 ± 2.5 ; IL-17, 11.74 ± 3.8 ; IL-6, 3.96 ± 0.7 . IL-10 increase in low-dose versus high-dose supernatants was 3.66 ± 1.2 -fold (mean \pm SEM). **(E)** Flow cytometric analysis of Treg (de novo) induction from CD4⁺ T cells depleted of CD25⁺ and Foxp3⁺ cells (CD4⁺CD25⁻Foxp3⁻) after 7 d of coculture with control-, low dose-, and high dose-stimulated microglia. Data are representative of three separate experiments (mean \pm SEM: low dose [$16.8 \pm 2.3\%$] versus high dose [$9.3 \pm 1.3\%$]). **(F)** Flow cytometric proliferation analysis of Foxp3⁻ and Foxp3⁺ T cells labeled with eFluor 670 at day 6 of coculture. Data are representative of two separate experiments (mean \pm SEM of Foxp3⁺ Tregs at day 6 low dose [$14.05 \pm 1.1\%$] versus high dose [$56.55 \pm 0.9\%$]).

of high-dose IFN- γ /MOG-stimulated microglia cells, we could only recover 8500 Foxp3⁺ cells per well. Furthermore, in cocultures of unstimulated microglia cells or microglia cells, which were stimulated with either IFN- γ or MOG alone, we did not detect an increase in Foxp3⁺ mgTregs. That elevated mgTreg frequencies were strictly dependent on low-dose pretreatment of both, MOG peptide and IFN- γ , which was further proven by titrating MOG peptide concentrations in the presence of either low- or high-dose IFN- γ (Supplemental Fig. 1A).

Kinetic experiments in low dose-activated microglia/T cell cocultures revealed an increase of Foxp3⁺ Tregs starting on day 3 (Fig. 1C). The frequency of Foxp3⁺ mgTregs in low-dose cocultures constantly increased during 7 d. In contrast, the population of CD25⁺Foxp3⁻ T cells increased on day 1 and started to decrease on day 4 (Fig. 1C). Within that time frame, we determined levels of IL-13, IL-17, IL-6, IL-10, TNF- α , MCP-1, and IFN- γ in coculture supernatants (Fig. 1D, Supplemental Fig. 1B). Levels of IL-13, IL-17, and IL-6 were significantly elevated only in high dose-treated cocultures. Low-dose pretreatment inhibited permanent production of IL-17 and reduced IL-6 and IL-13 levels. TNF- α and MCP-1 concentrations were increased in high- and low-dose IFN- γ /MOG cocultures. In contrast, only low-dose stimulation of microglia resulted in a strong increase in the production of anti-inflammatory IL-10, especially during the first 4 d of coculture. Notably, we could not detect IL-4, IL-1 α , IL-12, GM-CSF, or IL-23. Thus, depending on the IFN- γ -induced activation of microglia, CD4⁺ T cells were directed into a more regulatory or effector state. Next, we deter-

mined whether microglia-induced Tregs derive from pre-existing Foxp3⁺ cells or are induced de novo using FACS-sorted CD4⁺CD25⁻Foxp3⁻ cells. Coculturing these Foxp3⁻ cells with unstimulated (control) low dose- and high dose-activated microglia resulted in a significantly higher induction of Foxp3^{EGFP} expressing mgTregs under low-dose conditions 16.8 versus 9.3% in high dose-activated microglia cocultures (Fig. 1E). In complementary experiments, we labeled CD4⁺ T cells with a proliferation dye (eFluor 670) before coculturing them with microglia and assayed proliferation of Foxp3⁻ and Foxp3⁺ cells on day 6 of coculture. Whereas under control conditions T cells did not divide, low dose-activated microglia induced only proliferation of Foxp3⁺ cells (14%, Fig. 1F). In contrast, high dose-stimulated microglia induced proliferation of Foxp3⁻ and Foxp3⁺ cells to the same extent (Fig. 1F). Thus, we conclude that low dose-activated microglia promote de novo induction of Foxp3⁺ Tregs, as well as promoting proliferation of pre-existing CD4⁺Foxp3⁺ Tregs.

mgTregs suppress the proliferation of encephalogenic T cells in vitro and reduce EAE severity in vivo

To prove suppressive function of mgTregs, we generated MOG-specific Tregs in vitro by coculturing CD4⁺ T cells with low dose-activated microglia. Purified FACS-sorted Foxp3⁺CD25⁺ Tregs efficiently suppressed the proliferation of effector T cells in vitro up to a ratio of 1:50 (Fig. 2A, 2B, upper panel). In the control, Foxp3⁻CD25⁺ cells (effector T cells) from the same cultures did not inhibit responder T cell proliferation (Fig. 2B, lower panel). We further

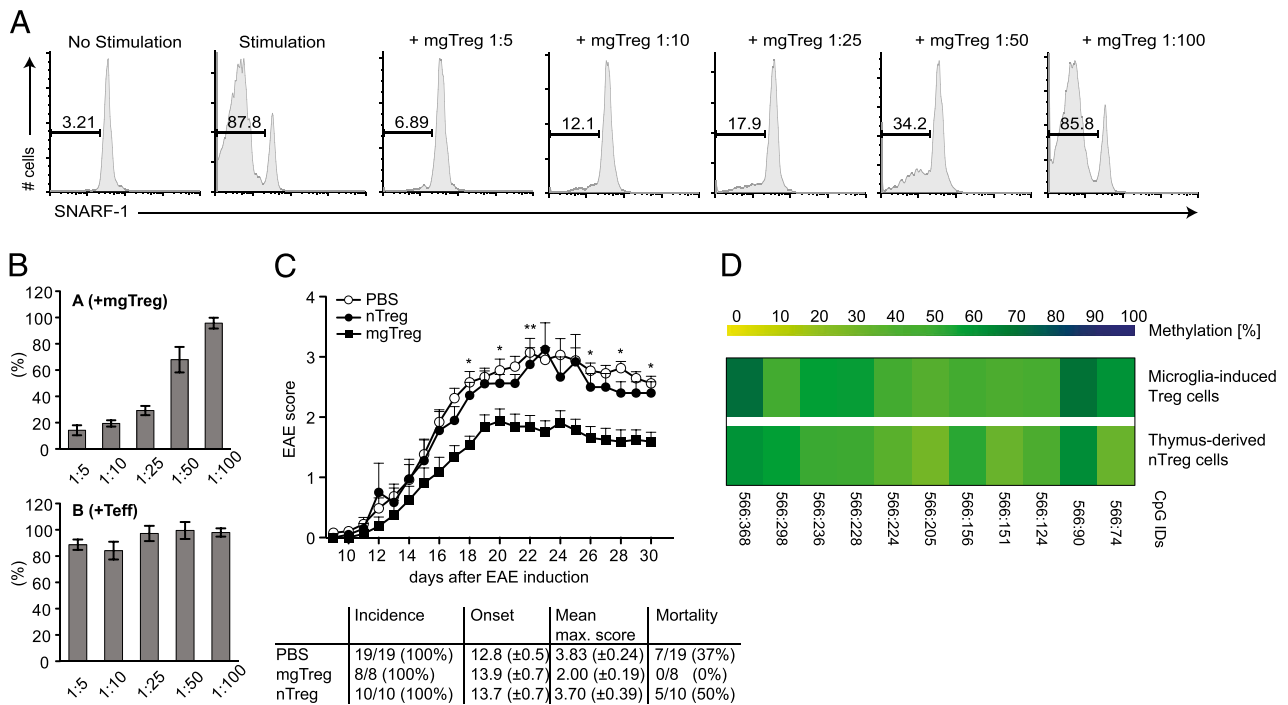


FIGURE 2. Microglia-induced Tregs are functionally active in vitro and in vivo and are epigenetically stable. Microglia-induced T cells were sorted for Foxp3⁺ Tregs and Foxp3⁻ effector T cells after 7 d of coculture. (A) Proliferation of SNARF-1-labeled responder T cells incubated without MOG Ag and irradiated splenocytes (no stimulation) or with irradiated splenocytes and MOG in the absence (stimulation) or presence of Foxp3⁺ mgTregs. The ratio of MOG-specific mgTreg/responder T cells is indicated above plots. Numbers above bracketed lines refer to percentage of proliferating cells. Data are representative of three separate experiments, summarized in proliferation index [(B) upper panel, mean ± SEM]. Proliferation index of Foxp3⁻ microglia-induced effector T cells is shown as control (B, lower panel, mean ± SEM). Microglia-induced, MOG-specific Tregs (mgTregs) or thymus-derived, MOG-specific nTregs were adoptively transferred in C57BL/6J recipients (500,000 cells/mouse) 1 d before immunizing mice with MOG₃₅₋₅₅ peptide. (C) EAE disease course of PBS-treated mice (n = 19, ○), mgTreg-treated mice (n = 8, ■), and nTreg-treated mice (n = 10, ●) in mean clinical score ± SEM (*p < 0.05, **p < 0.01, Mann-Whitney U test comparing mgTregs with nTregs). Table shows descriptive statistics for incidence, onset (mean days ± SEM), mean maximum score (±SEM), and mortality (total animals and percentage). (D) DNA methylation status of two overlapping amplicons within mouse foxp3 locus. The upper panel depicts the microglia-induced Treg pool in comparison with thymus-derived nTregs (lower panel). Single CpG motifs are separated by horizontal lines resulting in boxes with methylation rates being color-coded (yellow, 0%; blue, 100% methylation).

purified microglia-induced, MOG-specific CD25⁺ Tregs from cocultures and as a control isolated natural Treg cells (nTregs) from MOG TCR transgenic animals and adoptively transferred them into C57BL/6J recipients (500,000 cells/mouse) 1 d before induction of active EAE (MOG₃₅₋₅₅). Transfer of mgTregs resulted in a constantly reduced clinical disease severity (Fig. 2C) whereas transfer of MOG-specific nTregs showed no change in clinical scores when compared with PBS-treated mice (Fig. 2C). Mortality rates were 37% for the PBS control group, 50% for the nTreg group, and 0% when mgTregs were transferred (Fig. 2C). To investigate signs of stability of in vitro-generated mgTregs we analyzed the conserved CpG-rich Treg-specific demethylated region (23) from Tregs induced by microglia and compared them to CD4⁺CD25⁺ nTregs. Treg-specific demethylated region demethylation in the pool of microglia-induced CD4⁺CD25⁺ Tregs was comparable to that of thymus-derived CD4⁺CD25⁺ nTregs (Fig. 2D), indicating a stable *Foxp3* expression. Taken together, these data demonstrate that microglia-induced Tregs are potentially stable and highly suppressive in vitro and in vivo.

Microglia-mediated Treg induction is MHC class II-dependent and Ag-specific

In the steady-state brain, microglia do not express MHC II, but rapidly upregulate MHC II in response to injuries or inflammation

and to IFN- γ exposition derived from brain-infiltrating-activated effector T cells or NK cells (24). We detected a clear IFN- γ dose-dependent increase of MHC II expression of primary microglia cells, with a 3.5-fold higher increase in MHC II mRNA expression in high dose-stimulated microglia compared with low dose-stimulated microglia after 48 h treatment (Fig. 3A). Low dose-activated cultures resulted on average in 24.38% ($\pm 1.08\%$) MHC II⁺ microglia cells, whereas in high dose-stimulated cultures up to 37.8% ($\pm 1.92\%$) cells were MHC II⁺ (Fig. 3B).

To test whether Treg induction is MHC II-dependent, we cocultured MOG-specific CD4⁺ cells with primary microglia cells derived from MHC II-deficient (MHC II^{-/-}) and wild-type (WT) mice (Fig. 3C). MHC II^{-/-} microglia did not induce increased Foxp3⁺ Treg frequencies regardless of IFN- γ /MOG stimulation (Fig. 3D). In a second set of experiments, we pulsed IFN- γ -activated microglia with a different peptide, which is not recognized by the 2D2 transgenic TCR, such as myelin basic protein (MBP). Coculture of MOG-specific CD4⁺ T cells and low dose (IFN- γ /MBP)-activated microglia did not result in induction of Foxp3⁺ Tregs (5.9%, Fig. 3E). In complementary experiments, V β 11 and V α 3.2 staining confirmed the MOG-specific TCR of microglia-induced Foxp3⁺ Tregs excluding non-Ag-specific induction of Tregs (data not shown). These results clearly illustrate that induction of mgTregs depends on MHC II-mediated presentation of cognate Ag.

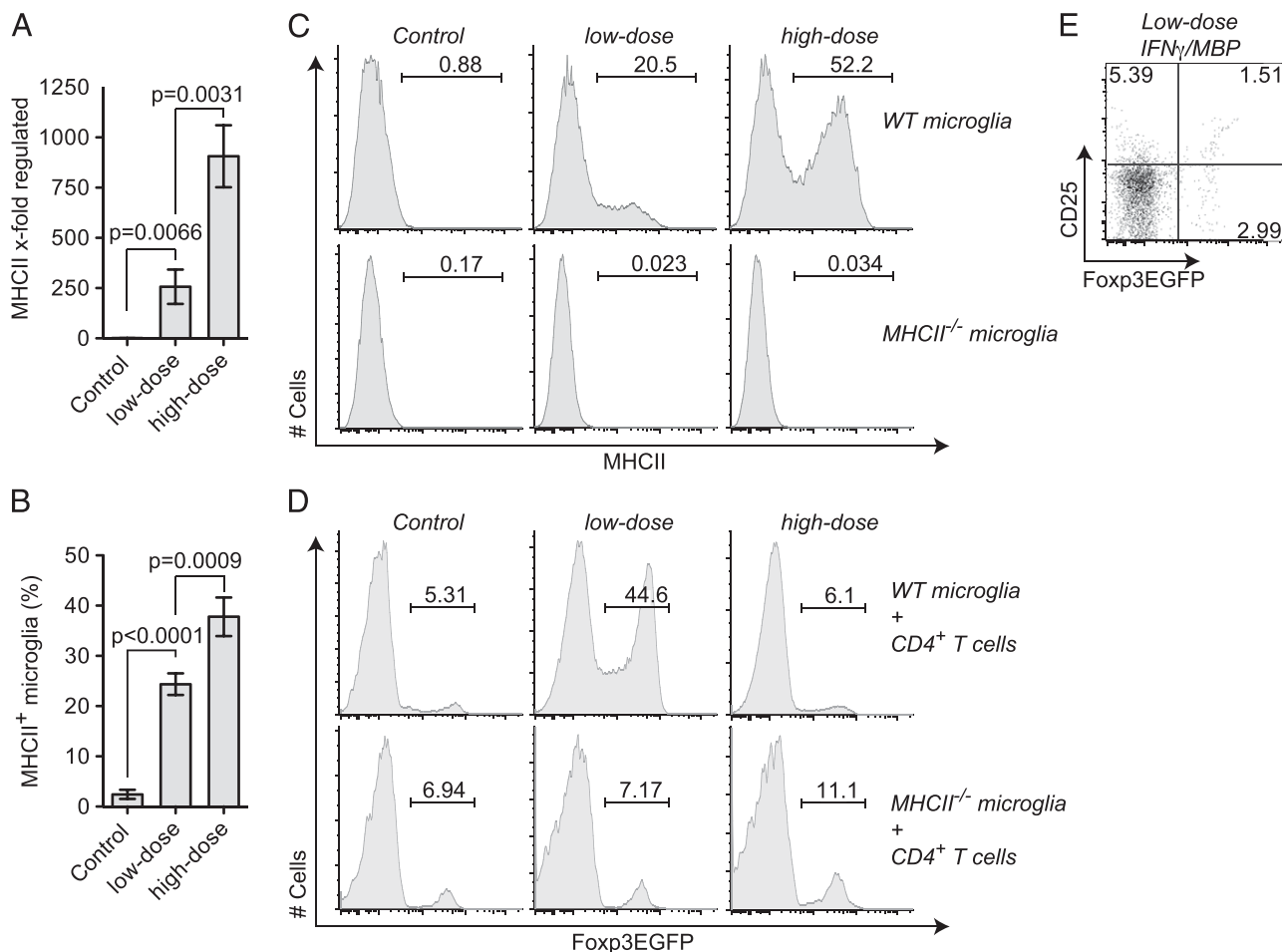


FIGURE 3. Microglia-mediated Treg induction depends on MHC class II. Microglia cultures were analyzed 48 h after IFN- γ /MOG treatment for (A) x-fold increase of MHC II mRNA ($n = 3$) and (B) percentage of MHC II⁺ microglia in cultures ($n = 4$) (mean \pm SEM, Student *t* test). (C) Analysis of microglial MHC II expression of WT and MHC II^{-/-} mice 48 h after IFN- γ /MOG treatment (gated on live CD11b⁺ cells). (D) Analysis of Foxp3⁺ Treg frequencies after 7 d of coculture with microglia from WT and MHC II^{-/-} mice (gated on live CD11b⁻CD4⁺ cells). (E) Analysis of Foxp3⁺ Treg frequency in coculture with IFN- γ /MBP pretreatment (gated on live CD11b⁻CD4⁺ cells; data are representative for $n = 3$ separate experiments, mean \pm SEM of 5.9 \pm 0.8%).

Treg-inducing microglia are MHC II⁺CD40^{dim}CD86^{dim}IL-10⁺

Tolerogenic DCs express a distinct pattern of costimulatory molecules, cytokines, and tolerogenic markers (25). We therefore examined the expression of CD40, CD80, CD86, and inducible NO synthase in microglia 48 h after IFN- γ /MOG pretreatments. Stimulation significantly increased CD40 (Fig. 4A), CD86 (Fig. 4B), and inducible NO synthase (Supplemental Fig. 2D) in a dose-dependent manner but had no effect on CD80 (Fig. 4B). The detected differences in CD86 mRNA expression were further supported by data from FACS analysis. High dose-stimulated microglia cells showed a significantly higher increase of CD86 surface expression per cell in comparison with low dose-stimulated microglia cells (Supplemental Fig. 2A). To investigate the functional impact of variable CD40 expression levels and CD40/CD40L interaction during microglia/T cell cocultures, we performed neutralization experiments. We cocultured high dose-activated microglia with CD4⁺ T cells in the presence of a neutralizing anti-CD154 (CD40L) Ab. Indeed, blocking of CD40/CD40L interaction resulted in a marked decrease of CD25⁺Foxp3⁻ effector cells, but did not effect generation of CD25⁺Foxp3⁺ cells (Fig. 4C). These experiments demonstrate that high CD40 expression and CD40/CD40L interaction is important for T effector cell but not mgTreg generation during cocultures.

Interestingly, low-level stimulation enhanced IL-10 mRNA expression by microglia (Fig. 4D), whereas high-dose IFN- γ stimulation resulted in only a minor increase in IL-10 expression (Fig. 4D). Because IL-10 is known to reduce Ag presentation via, for example, modulation of MHC II expression, we analyzed MHC II expression after combined treatment of high-dose IFN- γ /MOG and different doses of IL-10 (Fig. 5A). Increasing levels of IL-10

resulted in a dose-dependent suppression of MHC II expression on microglia. Notably, we found the combination of high-dose IFN- γ /MOG and 2 ng/ml IL-10 corresponding to low-dose levels of MHC II expression (21.6%, Fig. 5A). Additionally, we tested whether TGF- β signaling is important for microglia-mediated induction of Tregs. We analyzed supernatants of control-, low dose-, and high dose-treated microglia before T cell coculture after 48 h of stimulation for the presence of TGF- β protein by multiplex protein assay. We were able to detect TGF- β protein, but without statistical difference between unstimulated, low dose-, and high dose-treated microglia, suggesting that the secretion level of TGF- β was not affected by IFN- γ /MOG stimulation (Supplemental Fig. 2C). This was further supported by analysis of microglial TGF- β expression on mRNA level. In line with the protein level, we could not detect TGF- β expression differences between control, low-dose-, and high dose-stimulated microglia (data not shown).

To test whether there is a direct link between IL-10 production and the regulatory microglia phenotype inducing Tregs, we combined high-dose IFN- γ /MOG and IL-10 (2 ng/ml) treatment before coculture. Surprisingly, this combination of high-dose IFN- γ /MOG and IL-10 pretreatment prevented the differentiation of effector T cells and strongly induced Foxp3⁺ Treg comparable to that of low-dose IFN- γ /MOG stimulation (30.5%, Fig. 5B). Kinetic analysis of high dose- compared with high dose plus IL-10 (2 ng/ml)-stimulated cocultures did also show IL-10-mediated suppression of effector T cells (Fig. 5C) and coculture kinetics comparable to those of low-dose pretreatment (Figs. 1C, 5C). Because addition of IL-10 to high dose-stimulated microglia did reverse the "high-dose" effect of effector T cell induction/accumulation and led to high frequencies of Foxp3⁺ Tregs instead (Fig. 5B), we wondered whether we could detect expression differences besides those of MHC II between high dose- and high dose plus IL-10-treated microglia (Supplemental Fig. 2E). Although the regulation of the costimulatory molecules CD40, CD80, and CD86 was not significantly affected, there was a trend to lower CD40 transcription upon IL-10 addition. Furthermore, exogenous IL-10 resulted in a significant downregulation of the chemokine CXCL10 and IFN- γ inducible NO synthase, which is in line with findings from other groups (26–28).

Thus, IL-10 is crucial for the regulatory phenotype of microglia in an inflammatory environment such as high doses of IFN- γ /MOG. Collectively, these data demonstrate that dose-dependent activation of microglia leads to different phenotypes of microglia comparable to tolerogenic/inflammatory DCs or anti-inflammatory type II monocytes in the periphery.

Discussion

In this study, we identified MHC II⁺CD40^{dim}CD86^{dim}IL-10⁺ microglia cells as a potent inducer of Foxp3⁺ Tregs. The capacity of microglia to induce Tregs depends on the amount of IFN- γ and cognate Ag used to stimulate microglia. High dose-stimulated microglia with high MHC II and costimulatory molecule expression induced predominantly effector T cells, whereas low dose-stimulated microglia with low MHC II and costimulatory molecule expression induced preferentially Foxp3⁺ Tregs. We suggest a fine-tuned mechanism by which microglia activation is controlled by a threshold of local IFN- γ danger signals and Ag load (Fig. 6). Below that threshold, low IFN- γ signals induce a regulatory microglia phenotype. Above that threshold, high IFN- γ induces an inflammatory phenotype resulting in an effector T cell response.

We assume that particularly in weak inflammatory CNS injuries low IFN- γ levels from the initial invasion of T and NK cells give rise to microglia with a regulatory phenotype. In these settings,

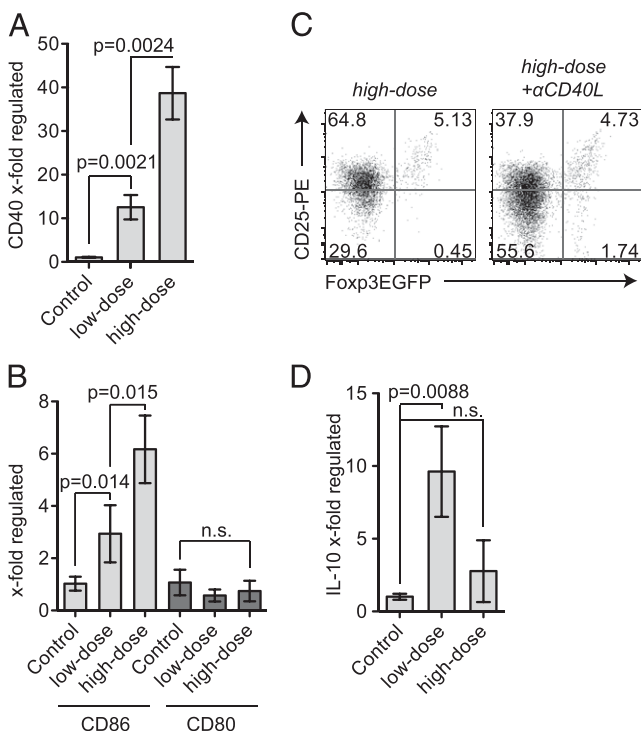


FIGURE 4. Foxp3⁺ Treg-inducing microglia are MHC II⁺CD40^{dim}CD86^{dim}IL-10⁺. Microglia cultures were analyzed 48 h after IFN- γ /MOG treatment for (A) x-fold increase of CD40 mRNA ($n = 3$, mean \pm SEM, Student t test) and (B) x-fold increase of CD80 and CD86 mRNA ($n = 3$ –4, mean \pm SEM, Student t test). (C) High dose-stimulated microglia cocultured with CD4⁺ T cells in the presence of CD40L Ab (anti-CD154, 40 μ g/ml). (D) x-Fold increase of IL-10 mRNA ($n = 3$, mean \pm SEM, Student t test).

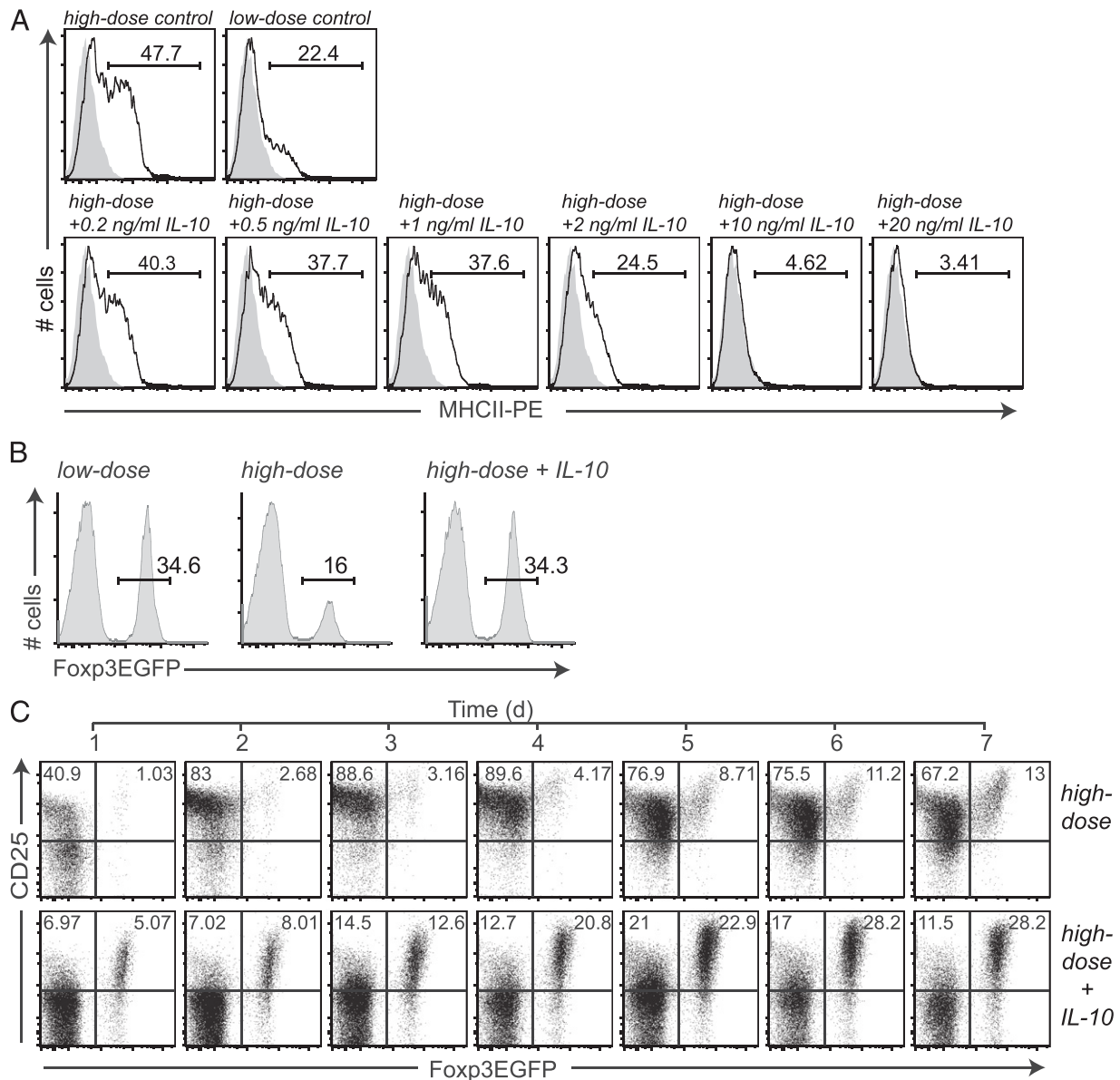


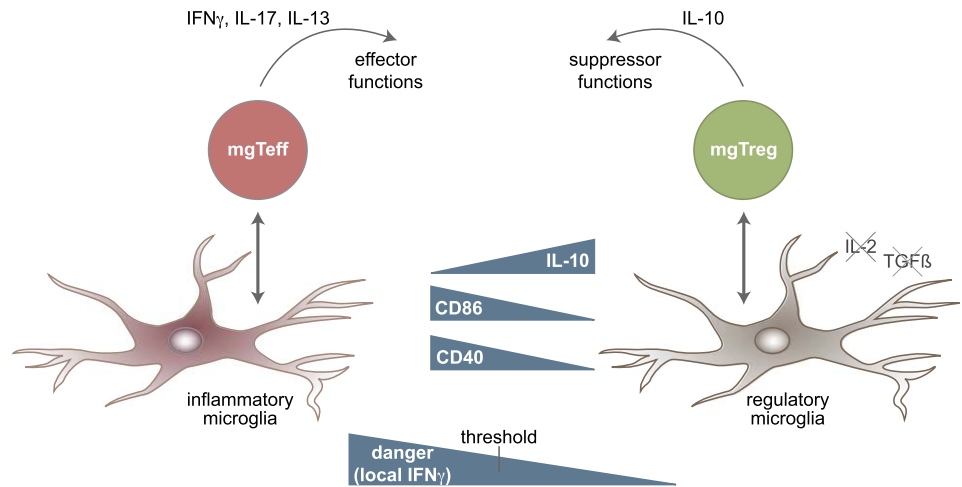
FIGURE 5. IL-10 rescues microglia-mediated Treg induction. **(A)** Dose response curve for high-dose IFN- γ /MOG treatment in combination with rIL-10. Numbers above bracketed lines indicate percentage of MHC II⁺ cells in comparison with unstimulated control (gray-filled histograms). Data are representative of $n = 4$ separate experiments (mean \pm SEM of $21.6 \pm 2.8\%$ MHC II⁺ cells following high-dose plus 2 ng/ml IL-10 stimulation). **(B)** Analysis of Foxp3⁺ Treg frequencies in cocultures pretreated with a combination of high-dose IFN- γ /MOG and rIL-10 (2 ng/ml) in comparison with low-dose and high-dose treatment alone. Numbers above bracketed lines indicate percentage of Foxp3^{EGFP+} cells. Data are representative of three separate experiments (mean \pm SEM of $30.5 \pm 2.1\%$ Foxp3⁺ Tregs following high-dose plus IL-10 stimulation). **(C)** Kinetic analysis of Foxp3⁺ expression in high dose- and high dose plus IL-10 (2 ng/ml)-stimulated cocultures. Data are representative of three separate experiments.

newly recruited T cells, which require Ag recognition, would then encounter microglia directing them toward a regulatory phenotype. This might also explain the late and long-lasting accumulation of Tregs after experimental stroke (middle cerebral artery occlusion) as demonstrated by our group (29), which is correlated with a significant accumulation of MHC II expressing microglia (CD45^{dim}CD11b⁺ cells) in the ipsilateral hemispheres. Within this study we further demonstrated that Foxp3⁺ Tregs contact MHC II-expressing Iba1⁺ cells (e.g., microglia) in the ischemic hemisphere and that infiltrating Foxp3⁺ Tregs can proliferate locally (29). In this study, we show that in vitro microglia have the potential to mediate de novo induction and proliferation of Foxp3⁺ Tregs. Therefore, subimmunogenic activation of microglia by low-level IFN- γ could be a possible regulatory mechanism that limits the expansion of autoantigen-specific effector T cells in the CNS.

Such mechanisms could also explain why ECL brain injury, despite the presence of myelin-specific T cells in injured regions, does not lead to destructive autoimmunity (30).

In the periphery, mature DCs are enhancing T cell immunity, whereas immature DCs are involved in the induction of peripheral T cell tolerance to self-Ag (31) or to alloantigens (32). Immature DCs are characterized by low expression of MHC II and adhesion and costimulatory molecules, leading to differentiation of Tregs rather than T effector cells (33). Other groups showed that type II monocytes have anti-inflammatory properties promoting either Th2 or Treg responses in the periphery (34). In this study, to our knowledge we show for the first time that brain-resident microglial cells activated by low-dose IFN- γ and Ag induce Ag-specific, Foxp3⁺ Tregs in vitro (Fig. 1), similar to type II monocytes. The regulatory potential of microglia-induced Tregs was validated

FIGURE 6. Model of how microglia activation can be specifically controlled by local IFN- γ signals. IFN- γ danger signals below a certain threshold result in a regulatory microglia phenotype inducing Tregs, whereas IFN- γ signals above this threshold induce an inflammatory phenotype with proinflammatory responses of the adaptive immune system (Th cells).



using a proliferation assay *in vitro* (Fig. 2). Moreover, diminished EAE severity after adoptive Treg transfer reflects the suppressive function of microglia-induced Tregs *in vivo*. The administration of mgTregs during priming did not delay onset or peak of disease, but significantly decreased disease severity and mortality rate, suggesting that mgTregs interfere with disease progression. Because Korn et al. (17) found that CNS-derived autoantigen-specific effector T cells isolated at the peak of disease cannot be suppressed *in vivo* and *in vitro* by either CNS-derived or peripheral Ag-specific Tregs, disease amelioration mediated by transfer of microglia-induced Treg might already take place in the periphery during priming of T effector cells (e.g., draining lymph nodes). Surprisingly, in our study nTregs isolated from naive MOG TCR transgenic animals failed to suppress EAE disease scores. This is in apparent contrast to findings of Kohm et al. (35) who reported significant protection from the development of clinical EAE in BL6/J mice when transferring CD4⁺CD25⁺ nTregs from WT animals (35). However, the authors transferred 2×10^6 cells, a considerably greater number of cells than used in our experimental set-up (500,000 cells/mouse).

mgTregs are epigenetically in a stable state, comparable to that of nTregs. We demonstrate in this study that microglia molecules required for effective Ag presentation or costimulation are tightly regulated by IFN- γ levels, leading to opposite T cell responses (Figs. 3, 4). The primary sources of IFN- γ in the inflamed brain are Th1 and NK cells, DCs, and macrophages, but it has also been shown that low levels of IFN- γ can be produced by astrocytes, fibroblasts, cerebrovascular endothelial cells, and microglia itself in the healthy CNS (36–38). Microglia-mediated Treg induction is cell–cell contact-dependent, Ag-specific (dependent on MHC II and cognate peptide), and requires intermediate levels of CD40 and CD86 on the microglia site. These findings are further supported by a study from Ponomarev et al. (39) showing two populations of MHC II-expressing microglia during the peak of EAE: CD45^{high}-activated microglia expressing also high levels of costimulatory CD40 and CD86, and resting CD45^{low} microglia expressing very low levels of CD40 and CD86. They speculated that low level-activated microglia downmodulate CNS inflammation by mediating tolerance. Also, our results support that reducing the degree of CD40/CD40L interaction in microglia/T cell cocultures affects T effector cell generation (Fig. 3C).

Performing ECL surgery on rats, CD80 and CD86 regulation on microglia in response to mechanically induced axonal degeneration (ECL) was studied by the Bechmann et al. (9). They showed that after ECL, myelin is presented in a MHC II/CD86 rather than in a MHC II/CD80 context, leading to potential autoimmunity (9).

This is in line with our *in vitro* findings showing that the different IFN- γ /MOG stimulations applied to microglial cells did not lead to changes in CD80 mRNA levels.

Moreover, our data suggest a crucial role of IL-10 in modulating distinct microglia phenotypes. The pretreatment with IL-10 rescues the Treg response in cocultures with inflammatory (high dose)-activated microglia (Fig. 5). Because IL-10 is known to downregulate MHC II, CD86, and CD80 on APCs, this can explain the suppressive phenotype of intermediately activated microglia. Also in our experiments, addition of IL-10 could downregulate MHC II but also CXCL10 expression of high dose-stimulated microglia (Fig. 5B). Interestingly, it has been shown that addition of IP-10 can promote T effector cell proliferation and cytokine production (40).

Moreover, DCs exposed to IL-10 proved to be in a tolerogenic rather than an inflammatory phenotype (41). Because inducible Tregs can be generated in the presence of TGF- β (42) and/or IL-2, we analyzed TGF- β and IL-2 expression on mRNA level and found neither TGF- β being regulated nor IL-2 being expressed in microglia. Additionally, we analyzed TGF- β protein levels after 48 h stimulation, but found no significant difference between control-, low dose-, and high dose-stimulated microglia (Supplemental Fig. 2C). Because TGF- β levels were reported to increase in lesioned brains (43) and microglia are known to secrete TGF- β under pathological conditions (44), the contribution of TGF- β cannot be ruled out, but it seems to be IFN- γ -independent.

Consistent with other findings, we could further show that microglia-induced activation of T cells leads to a time-dependent plasticity reflected by a mixture of cytokines released (45). High-dose cocultures resulted in a massive release of IL-17, IL-13, IL-6, and TNF- α (Fig. 1D, Supplemental Fig. 1B), whereas low dose-primed microglia T cell cocultures showed only intermediate levels of IL-6 and TNF- α and very small amounts of IL-13 or IL-17. These findings are in line with other studies describing a suppressive role for IL-6 in TGF- β -induced Foxp3⁺ Treg induction, and moreover the induction of pathogenic Th17 cells instead (46, 47).

Taken together, our results identify MHC II⁺CD40^{dim}CD86^{dim}IL-10⁺ microglia as a potential inducer of Tregs *in vitro*. The expression of the anti-inflammatory molecule IL-10 seems to be responsible for the immature/regulatory phenotype of the microglia. The response of microglia cells to different amounts of local IFN- γ and Ag load results in a fine-tuned immune response and contributes to either microglia-induced effector T cell or mgTreg generation. Microglia, as inducible APCs, have the ability to balance between beneficial and inflammatory T cell responses

depending on activation state and microenvironment. Our findings enhance the understanding of how microglia may contribute to the control of adaptive immune responses in the brain.

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Disclosures

The authors have no financial conflicts of interest.

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