nature medicine

Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke

Arthur Liesz[1], Elisabeth Suri-Payer[2], Claudia Veltkamp[3], Henrike Doerr[4], Clemens Sommer[4], Serge Rivest[5], Thomas Giese[6] & Roland Veltkamp[1]

Systemic and local inflammatory processes have a key, mainly detrimental role in the pathophysiology of ischemic stroke. Currently, little is known about endogenous counterregulatory immune mechanisms. We examined the role of the key immunomodulators $CD4^+CD25^+$ forkhead box P3 $(Foxp3)^+$ regulatory T lymphocytes (T_{reg} cells), after experimental brain ischemia. Depletion of T_{reg} cells profoundly increased delayed brain damage and deteriorated functional outcome. Absence of T_{reg} cells augmented postischemic activation of resident and invading inflammatory cells including microglia and T cells, the main sources of deleterious cerebral tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), respectively. Early antagonization of TNF-α and delayed neutralization of IFN-γ prevented infarct growth in T_{reg} cell–depleted mice. Intracerebral interleukin-10 (IL-10) substitution abrogated the cytokine overexpression after T_{reg} cell depletion and prevented secondary infarct growth, whereas transfer of IL-10–deficient T_{reg} cells in an adoptive transfer model was ineffective. In conclusion, T_{reg} cells are major cerebroprotective modulators of postischemic inflammatory brain damage targeting multiple inflammatory pathways. IL-10 signaling is essential for their immunomodulatory effect.

Stroke is the third leading cause of death and the most frequent cause of permanent disability in adults worldwide[1]. Despite considerable advances in the understanding of the pathophysiology of cerebral ischemia[2], therapeutic options for acute stroke are still limited[1]. Inflammatory mechanisms that are activated within hours after brain ischemia represent a key target of current translational cerebrovascular research[3]. Upregulation of cerebral proinflammatory cytokines, activation of local microglia and systemic lymphocytes and invasion of leukocytes in the brain contribute substantially to ischemic brain damage[4–6]. The interaction between the brain and immune cells after ischemia, however, is multifaceted. Severe brain ischemia also perturbs innate and adaptive immune cells, resulting in systemic immunodepression that predisposes patients after stroke to life-threatening infections[7]. Postischemic alterations in the immune system might also represent a useful immunomodulatory adaptation, preventing autoimmune reactions against exposed central nervous system antigens after stroke. It is difficult to generate an integrated concept of these crucial pathophysiological processes at present because the fundamental endogenous counterregulatory immune mechanisms limiting inflammatory damage after ischemic stroke are poorly understood[7,8].

Thymus-derived $CD4^+CD25^+Foxp3^+$ T_{reg} cells play a key part in controlling immune responses under physiological conditions and in various systemic and central nervous system inflammatory diseases[9–11]. They are capable of modulating the function of effector T cells, they alter the activity of antigen-presenting cells via direct interaction and they secrete anti-inflammatory molecules, including IL-10 and TGF-β[10,12,13]. Indeed, these potent mechanisms enable T_{reg} cells to be pivotal players in the fields of self tolerance, immunologic homeostasis and damage control at the site of inflammation[14]. Previous studies have reported that IL-10 has a beneficial effect in cerebral ischemia[15], that neuroprotective mucosal immunization is mediated by inducible regulatory lymphocytes[16] and that T_{reg} cells show relative resistance to cerebral ischemia[17]. However, the functional role of T_{reg} cells in cerebral ischemia has not been investigated so far.

Here we characterize the profound impact of endogenous T_{reg} cells on outcome after ischemic stroke, and we identify their cellular targets and protective signaling pathways.

RESULTS

Regulatory T cells are cerebroprotective after brain ischemia

We first investigated the effect of $CD4^+CD25^+Foxp3^+$ lymphocytes on infarct volume after focal brain ischemia in mice by *in vivo* depletion of this T cell subpopulation with a CD25-specific antibody. PBS injection was used as the control treatment in all experiments after observing that infarct sizes were the same in mice receiving intraperitoneal (i.p.) PBS or IgG1 isotype control (**Supplementary Fig. 1a** online). Consistent with previous data[18], the injection of antibodies to

[1]Department of Neurology, University Heidelberg, Im Neuenheimer Feld 400, 69120 Heidelberg, Germany. [2]Department of Immunogenetics, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany. [3]Department of Internal Medicine IV, University Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany. [4]Department of Neuropathology, University Mainz, Langenbeckstrasse 1, 55131 Mainz, Germany. [5]Laboratory of Molecular Endocrinology, Laval University, Centre Hospitalier de l'Université Laval Research Center, 2705 Boulevard Laurier, G1V 4G2 Quebec, Canada. [6]Department of Immunology, University Heidelberg, Im Neuenheimer Feld 305, 69120 Heidelberg, Germany. Correspondence should be addressed to R.V. (Roland.Veltkamp@med.uni-heidelberg.de).

Received 21 October 2008; accepted 2 January 2009; published online 25 January 2009; doi:10.1038/nm.1927

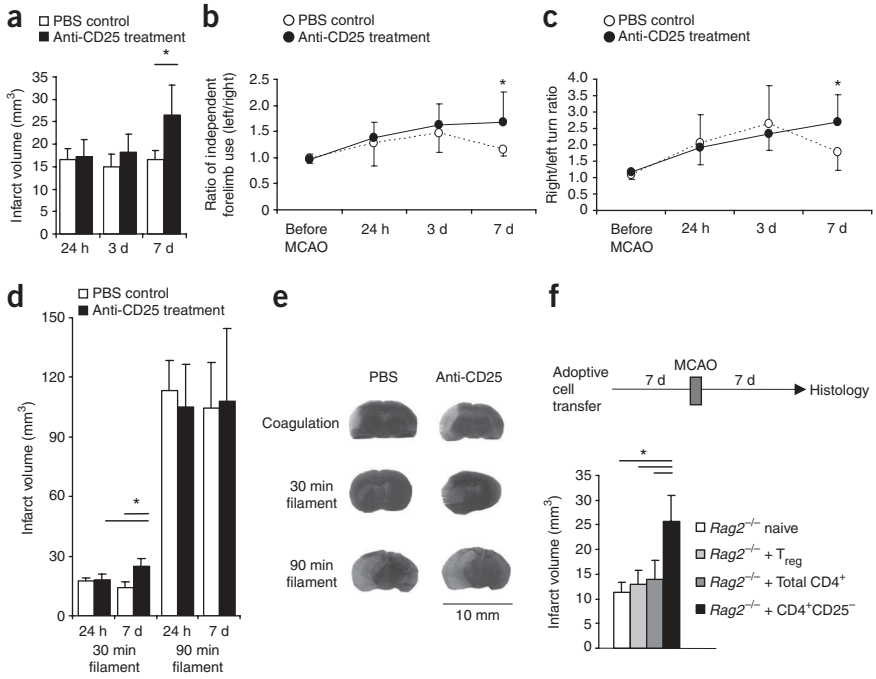

Figure 1 T_{reg} cell depletion exacerbates ischemic lesion size and behavioral outcome. (**a**) Infarct volume at various time points after transtemporal MCAO coagulation 48 h after i.p. injection of either PBS (control) or 300 μg of CD25-specific antibody (anti-CD25). (**b**,**c**) Sensorimotor dysfunction after stroke was assessed by the forelimb use asymmetry test (**b**) and the corner test (**c**). Consistent with infarct volume progression, T_{reg} cell–depleted mice had also a significantly worsened functional outcome. (**d**) Infarct volumes, as measured 24 h and 7 d after ischemia induction. T_{reg} cell depletion and infarct determination was reproduced in two ischemia-reperfusion models with occlusion of the MCA for 30 min or 90 min, respectively. (**e**) Representative silver-stained coronal sections of the three MCAO models used to analyze infarct size of control (PBS) and T_{reg} cell–depleted mice (anti-CD25). Localization of the ischemic lesion differed between models. (**f**) Either 8×10^6 $CD4^+$ cells (including $CD4^+CD25^+$ T_{reg} cells; control), 8×10^6 purified T_{reg} cells or 8×10^6 $CD4^+CD25^-$ cells were transferred into *Rag2*$^{-/-}$ mice 7 d before permanent MCAO, and infarct sizes were determined 7 d after MCAO. $^*P < 0.05$ between the indicated groups.

CD25 resulted in depletion of at least half of the $Foxp3^+$ cells and a 90% reduction of $CD25^+$ cells in lymphatic tissue (data not shown) but did not affect other leukocytes (**Supplementary Fig. 2** online). Permanent middle cerebral artery occlusion (MCAO) distal to the lenticulostriate arteries was induced 48 h after injecting either CD25-specific monoclonal antibody (mAb) or PBS (control) and mainly affected the cerebral cortex. Twenty-four hours after MCAO, infarct sizes did not differ significantly ($P = 0.74$, **Fig. 1a**). Between 3 d and 7 d after MCAO, the infarct size in the PBS group remained almost unchanged, whereas infarcts enlarged significantly (at 3 d, $n = 8$, $P = 0.058$; at 7 d, $n = 11$, $P < 0.001$) in T_{reg} cell–depleted mice (**Fig. 1a**).

Functional neurological deficit was assessed by the forelimb use asymmetry test[19] and the corner test[20], which sensitively detect sensorimotor dysfunction. In the forelimb use asymmetry test, mice receiving CD25-specific antibodies had a significantly ($n = 10$,

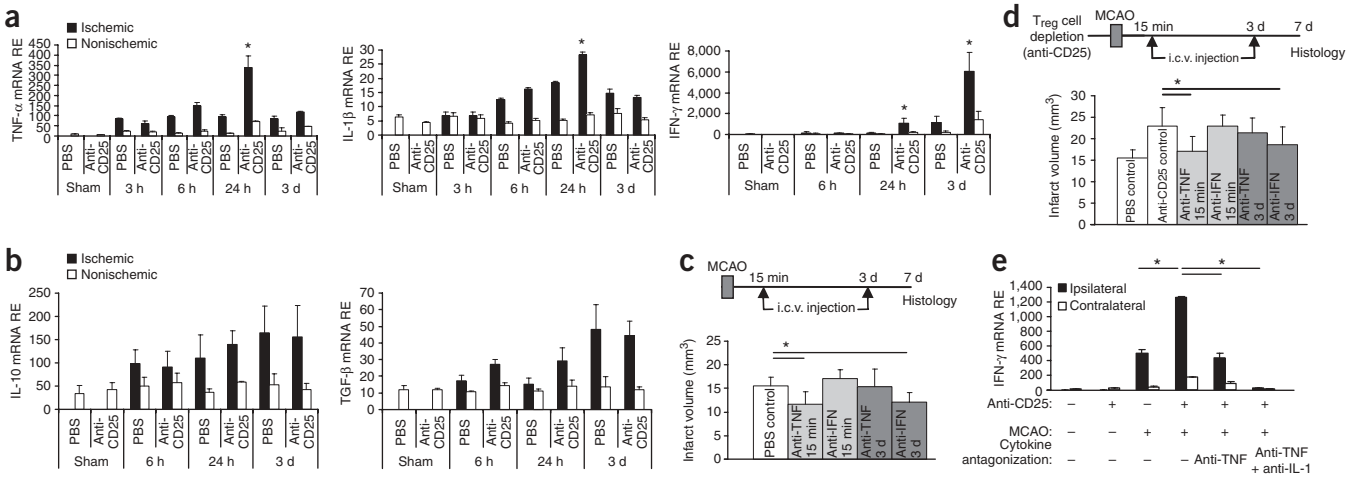

Figure 2 Proinflammatory cytokine expression is elevated in the ischemic brain after T_{reg} cell depletion, and their intracerebral antagonization reduces lesion size. (**a**) Cytokine mRNA levels of proinflammatory cytokines TNF-α, IL-1β and IFN-γ, as measured by RT-PCR in the ipsilateral (ischemic) and contralateral (nonischemic) hemispheres of control (PBS) and T_{reg} cell–depleted (anti-CD25) mice. (**b**) Analysis of the expression of the anti-inflammatory cytokines IL-10 and TGF-β. Values are indicated as relative mRNA expression (RE; mean ± s.d.; $^*P < 0.05$ between respective PBS- and anti-CD25–treated group of the ischemic hemisphere). (**c**) Analysis of the effect of early (15 min after MCAO) and late (3 d after MCAO) antagonization of TNF-α and IFN-γ on infarct volume, respectively. Infarct volumes were determined 7 d after ischemia induction. (**d**) A similar experimental protocol was applied as in **c**, but with T_{reg} cell depletion 48 h before MCAO. TNF-α or IFN-γ were antagonized at 15 min or 3 d after MCAO and infarct volumes measured at day 7. (**e**) IFN-γ mRNA levels measured 3 d after MCAO and the indicated treatment. Separate ischemic mice were treated with anti–TNF-α i.c.v. or combined anti–TNF-α and anti–IL-1β i.c.v. 15 min after MCAO induction; control mice received only control vehicle i.c.v. (BSA in artificial cerebrospinal fluid). $^*P < 0.05$ between the indicated groups.

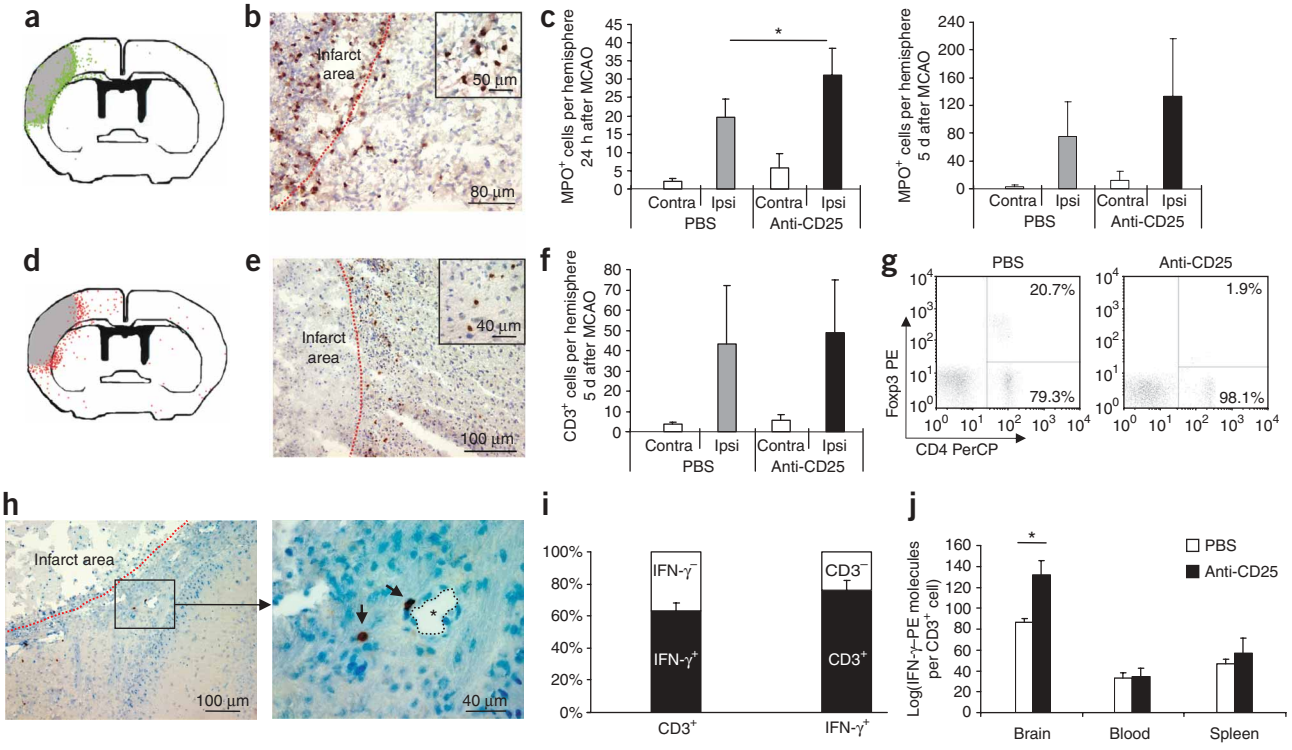

Figure 3 T_{reg} cells reduce the early invasion of neutrophils into the brain and the activation of invading T cells. (**a**,**b**) Topographic map of five accumulated brain sections and representative histological section 24 h after MCAO showing myeloperoxidase-positive (MPO^+) neutrophilic granulocytes predominantly located in the necrotic area and the border of the ischemic zone. (**c**) Analysis of MPO^+ cells counted in the ischemic (ipsi) and nonischemic (contra) hemispheres. Left, 24 h after MCAO; right, 5 d after MCAO. (**d**,**e**) Topographic map and representative histological section of $CD3^+$ T lymphocytes invading the ischemic brain 5 d after MCAO. (**f**) Analysis of $CD3^+$ cell counts in the ischemic and nonischemic hemispheres of control and T_{reg} cell–depleted mice 5 d after MCAO. (**g**) Invasion of T_{reg} cells into the ischemic brain, as analyzed by flow cytometry 5 d after MCAO (representative plots of five experiments). PE, phycoerythrin; PerCP, peridin-chlorophyll-protein complex. (**h**) Immunohistochemical sections showing T_{reg} cells predominantly in the peri-infarct area. Arrows indicate $Foxp3^+$ T_{reg} cells; the asterisk marks a blood vessel. (**i**) $CD3^+$ cells isolated from ischemic brain hemispheres were stimulated *in vitro* and analyzed for the number of IFN-γ–expressing cells and vice versa ($n = 12$). (**j**) The quantity of intracellular IFN-γ molecules per $CD3^+$ T cell, as measured by quantitative FACS in brain, spleen and blood. $^*P < 0.05$.

$P = 0.02$) worse functional outcome 7 d after MCAO than did control mice (**Fig. 1b**). Similarly, in the corner test, mice in both groups deviated from the normal ratio of 1.0 for right to left turns to an increased number of right turns at 24 h and 3 d after MCAO (**Fig. 1c**). Mice in the PBS group recovered toward a balanced ratio at day 7, whereas the right to left turn ratio in the CD25-specific antibody–treated group had actually increased ($n = 10$, $P = 0.01$; **Fig. 1c**).

Exacerbation of infarct volume after T_{reg} cell depletion was confirmed in a second experimental stroke model of reversible filament-induced MCAO with reperfusion after 30 min (**Fig. 1d**). Corresponding to the permanent MCAO model, infarct volumes did not differ between groups 24 h after MCAO, but 7 d after MCAO controls showed only small lesions in the striatum whereas treatment with antibody to CD25 significantly extended the infarct ($n = 7$, $P = 0.006$) (**Fig. 1d**). In contrast, 90-min transient filament-MCAO induced extensive ischemic lesions that extended through approximately 70% of the ischemic cerebral hemisphere, encompassing cortical and subcortical structures 24 h after ischemia in control mice (**Fig. 1e**). Subsequently, T_{reg} cell depletion did not augment infarct volume beyond this extensive damage (**Fig. 1d**).

To confirm that secondary infarct growth after MCAO coagulation was indeed the specific consequence of T_{reg} cell depletion, we validated our findings in a model of adoptive T cell transfer into lymphocyte-deficient $Rag2^{-/-}$ mice. Consistent with the antibody depletion experiments, mice receiving only $CD4^+CD25^-$ cells showed markedly larger infarcts than mice receiving either total $CD4^+$ cells or only T_{reg} cells or untreated $Rag2^{-/-}$ mice ($n = 10$, $P < 0.001$; **Fig. 1f**). The mean infarct size of naive $Rag2^{-/-}$ mice did not significantly change at days 1, 3 and 7 after MCAO (**Supplementary Fig. 1b**).

T_{reg} cell depletion elevates brain cytokine expression

We examined the relative expression of cytokine messenger RNA in the ischemic and nonischemic hemispheres. In T_{reg} cell–depleted mice, TNF-α, INF-γ and IL-1β expression was substantially upregulated after ischemia (**Fig. 2a**). The temporal pattern of this upregulation differed among cytokines. TNF-α mRNA expression in T_{reg} cell–depleted mice began to increase already 3 h after MCAO as compared to baseline levels; at 24 h its expression significantly differed from control values ($n = 9$, $P = 0.027$). After 3 d, TNF-α mRNA levels in T_{reg} cell–depleted mice returned to baseline levels similar to control group values (**Fig. 2a**). The transcription of IL-1β showed a similar temporal pattern, but was less pronounced (**Fig. 2a**). Cerebral IFN-γ mRNA expression increased later and was more sustained than TNF-α and IL-1β mRNA expression, and it was significantly augmented in ischemic hemispheres of T_{reg} cell–depleted mice at 24 h and 3 d ($n = 9$, $P < 0.001$). T_{reg} cell depletion had a comparatively minor effect on postischemic cerebral expression of the anti-inflammatory cytokines IL-10 and TGF-β (**Fig. 2b**).

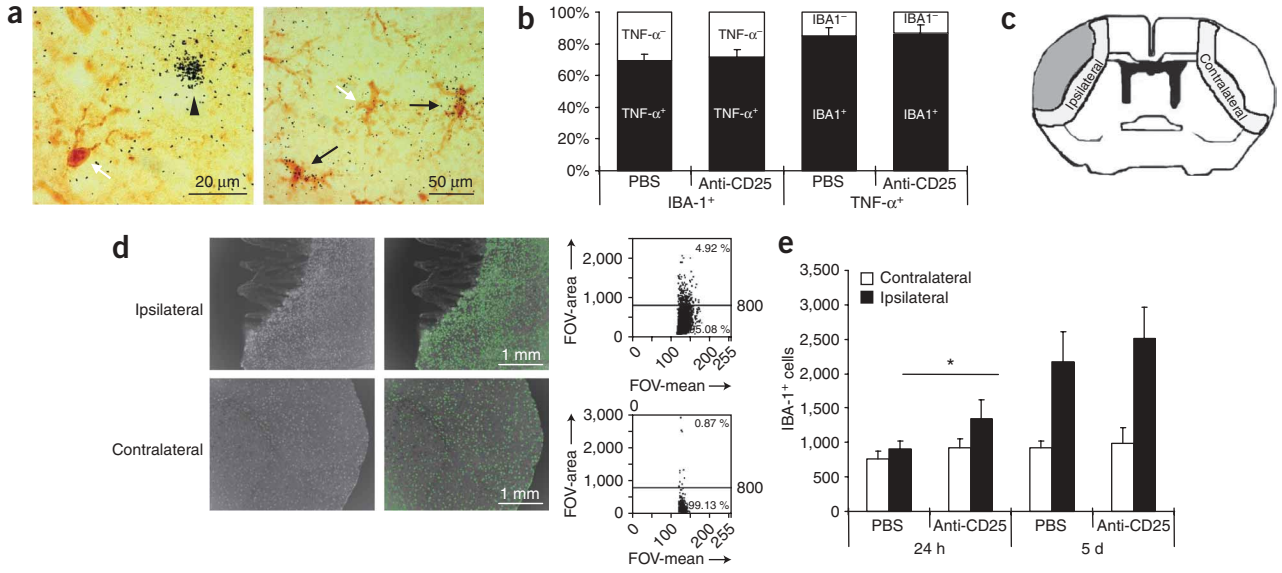

Figure 4 Microglia are activated after ischemia, are the main source of cerebral TNF-α and are more abundant in brains of T_{reg} cell–depleted mice. (**a**) TNF-α production in IBA-1$^+$ microglial cells, as assessed by a combination of *in situ* hybridization and immunohistology (black arrowhead, TNF-α–positive cells (IBA-1$^-$TNF-α$^+$); white arrows, TNF-α–negative microglia (IBA-1$^+$TNF-α$^-$); black arrows, double-positive cells (IBA-1$^+$TNF-α$^+$)). (**b**) Percentage of TNF-α expression in the IBA1$^+$ population, and vice versa, as analyzed in four predefined regions in the peri-infarct zone of ischemic hemispheres of T_{reg} cell–depleted and control mice ($n = 10$). (**c**,**d**) The absolute number of positive cells, as determined in the peri-infarct area and the corresponding area of same size in the unaffected contralateral side on coronal sections (**c**) and counted by cell-based scattergram analysis (**d**). Left images, microphotographs of IBA-1$^+$ stained sections; middle images, automated cell-based cytoplasm identification (positive cells marked green by the software); right images, scattergrams with exclusion thresholds. (**e**) Statistical analysis of counted IBA-1$^+$ cells in ipsilateral and contralateral regions in control (PBS) and T_{reg} cell–depleted mice (anti-CD25) at 24 h and 5 d after MCAO induction. $^*P < 0.01$.

Proinflammatory cytokine antagonization reduces brain damage

Proinflammatory mediators such as TNF-α and IFN-γ contribute to the detrimental postischemic brain inflammation[21–23]. To validate the role of these cytokines in the present MCAO model, we antagonized TNF-α and IFN-γ by intracerebroventricular (i.c.v.) injection of the respective neutralizing mAb and analyzed the infarct volume 7 d after MCAO (**Fig. 2c**). Early antagonization of TNF-α, but not IFN-γ, 15 min after MCAO significantly reduced infarct size ($n = 10$, $P = 0.01$). In contrast, injection of the antibodies 3 d after MCAO produced an inverse efficacy pattern, with significant infarct reduction only after i.c.v. IFN-γ antagonization ($n = 10$, $P = 0.017$). Similarly, early TNF-α and late IFN-γ antagonization significantly prevented secondary infarct growth in T_{reg} cell–depleted mice (**Fig. 2d**).

To detect a potential relationship between early TNF-α and delayed IFN-γ upregulation, we measured IFN-γ mRNA levels 3 d after MCAO in mice receiving i.c.v. antibodies to TNF-α alone or combined with IL-1β–specific antibodies 15 min after MCAO (**Fig. 2e**). Increased IFN-γ expression in T_{reg} cell–depleted mice 3 d after MCAO was significantly reduced by i.c.v. TNF-α neutralization. The combined neutralization of TNF-α and IL-1β significantly reduced IFN-γ expression even below values from control mice, indicating their importance for the delayed induction of overshooting IFN-γ production.

T_{reg} cell depletion alters cerebral leukocyte invasion

Leukocytes are major effectors of inflammatory damage after experimental brain ischemia[4,8]. We analyzed leukocyte subsets in the brain to determine the effect of T_{reg} cell depletion on leukocyte invasion and to elucidate the source of enhanced cerebral proinflammatory

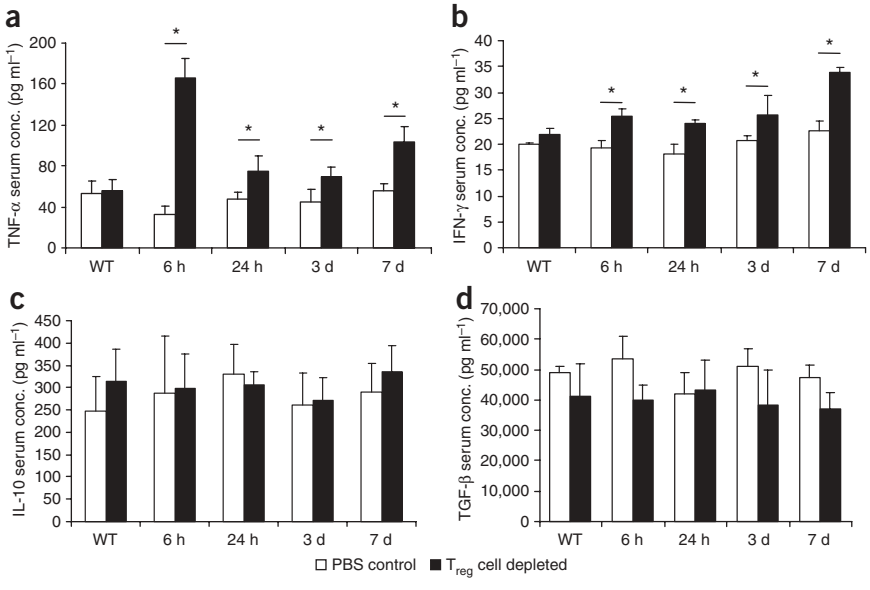

Figure 5 Blood cytokine concentrations after MCAO. (**a**–**d**) Blood concentrations of key cytokines, as measured by ELISA at various time points after MCAO and in nonischemic mice (WT) after T_{reg} cell depletion or PBS control injection. We analyzed the serum concentrations of the proinflammatory cytokines TNF-α (**a**) and IFN-γ (**b**) as well as the anti-inflammatory cytokines IL-10 (**c**) and TGF-β ($n = 10$ per group, $^*P < 0.05$).

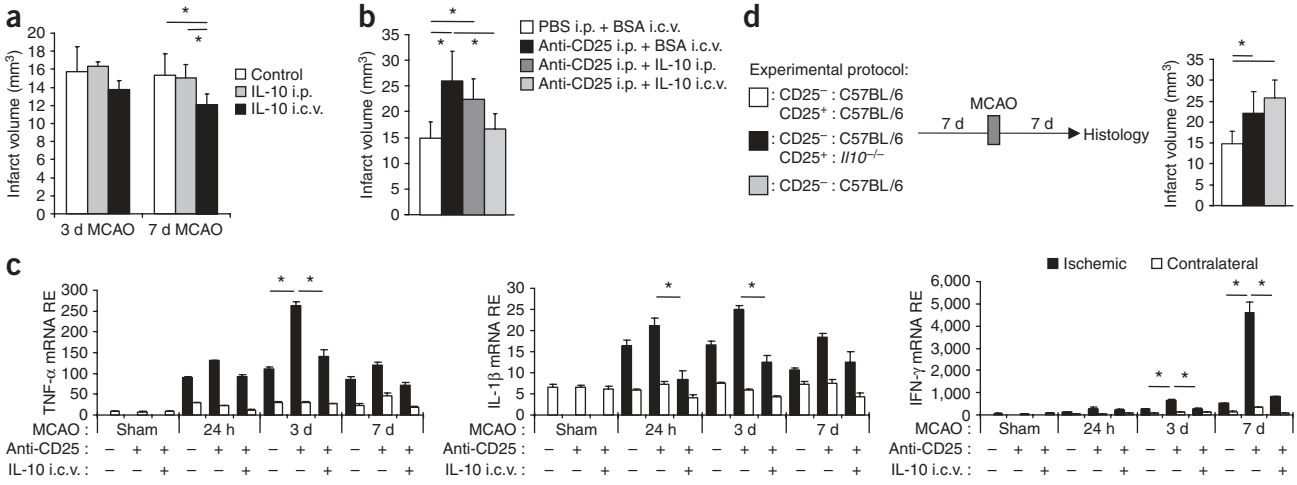

Figure 6 T_{reg} cell–derived IL-10 is the main mediator of the T_{reg} cells' cerebroprotective effect. (**a**) Quantification of infarct volume 3 d and 7 d after mice were injected with a single dose of recombinant mouse IL-10 either i.c.v. or i.p. 15 min after MCAO ($n = 5$ per group, $^*P < 0.05$). (**b**) Quantification of infarct volume 7 d after T_{reg} cell–depleted mice (anti-CD25 i.p.) received a single IL-10 injection either i.c.v. or i.p. 15 min after MCAO ($n = 8$ per group, $^*P < 0.05$ between the indicated groups). (**c**) Cerebral cytokine expression of TNF-α, IL-1β and IFN-γ, as analyzed in control mice (PBS), T_{reg} cell–depleted mice (anti-CD25) and T_{reg} cell–depleted mice receiving IL-10 i.c.v. 15 min after MCAO induction (anti-CD25 + IL-10) in the ischemic hemisphere and contralateral hemisphere ($n = 5$ per group, $^*P < 0.05$). (**d**) Right, quantification of infarct volume 7 d after lymphocyte-deficient *Rag2*$^{-/-}$ mice received donor lymphocyte injections as shown in the experimental protocol (left) and as described in the Methods section. Each mouse received 8×10^6 cells in total ($n = 8$ per group, $^*P < 0.05$).

cytokine production. The location of invading neutrophilic granulocytes was determined immunohistochemically by using myeloperoxidase, which is abundantly expressed in neutrophils. Myeloperoxidase-positive cells were predominantly detected in the ischemic core and the peri-infarct region (**Fig. 3a**,**b**). Neutrophil invasion in the infarcted hemisphere differed significantly between groups at 24 h ($n = 10$, $P = 0.036$) but no longer at day 5 (**Fig. 3c**). Brain invasion of $CD3^+$ T cells on immunohistochemical sections was negligible at 24 h and 3 d (data not shown) but was apparent 5 d after MCAO (**Fig. 3d**,**e**). In contrast to myeloperoxidase-positive cells, T cells did not invade the necrotic area but were mainly located in the peri-infarct zone (**Fig. 3d**,**e**). Invasion of $CD3^+$ cells in the ischemic hemisphere did not differ between treatment groups (**Fig. 3f**).

As postischemic T_{reg} cell brain invasion had not been studied before, we additionally analyzed this at various time points after ischemia. Flow cytometric analysis of $Foxp3^+$ expression revealed a well defined T_{reg} cell population from 3 d on after MCAO within the $CD4^+$ population of the ipsilateral hemisphere (**Fig. 3g**). Some $Foxp3^+$ cells could also be detected in the contralateral hemisphere (see **Supplementary Fig. 3** online for summary). On immunohistochemically stained sections, $Foxp3^+$ cells became visible 5 d after MCAO and were restricted to the peri-infarct zone (**Fig. 3h**), consistent with the topography of $CD3^+$ cells (compare to **Fig. 3d**).

Lymphocytes and microglia are the major cytokine sources

To elucidate the main source of delayed IFN-γ production in the ischemic brain, we stimulated lymphocytes and analyzed them by intracellular FACS (**Supplementary Fig. 4a** online). Of all brain-invading T cells, 63.2 ± 6.0% were positive for IFN-γ, and 76.0 ± 5.2% of all IFN-γ–producing cells in the ischemic hemisphere were $CD3^+$ T cells (**Fig. 3i**). Thus, T cells were the major source of IFN-γ in the ischemic hemisphere. Next, we quantified IFN-γ expression by determining the number of IFN-γ–phycoerythrin molecules per cell of systemic and brain-invading T cells (**Supplementary Fig. 4b**). In blood and spleen, the IFN-γ expression of T cells did not differ between T_{reg} cell–depleted and control mice (**Fig. 3j**). In contrast, T_{reg} cell depletion resulted in highly elevated IFN-γ production in brain-invading T cells 5 d after MCAO as compared to those of PBS-treated control mice ($n = 7$, $P = 0.015$), suggesting a restrictive effect of T_{reg} cells on activation and cytokine production of cerebral T cells (**Fig. 3j**).

To clarify the attenuating effect of T_{reg} cells on early postischemic proinflammatory cytokine production, we focused on microglia, the main source of cerebral TNF-α and IL-1β production[24,25]. For colocalization studies, we performed TNF-α *in situ* hybridization and immunohistochemistry for ionized calcium-binding adaptor molecule-1 (IBA-1) as a microglial marker on brain sections 24 h after MCAO and analyzed expression in four predefined regions in the peri-infarct area (**Fig. 4a**). Some 71 ± 8% of IBA-1^+ cells were colocalized with silver grain clusters, indicating TNF-α mRNA expression in these cells (**Fig. 4b**). In turn, 86 ± 7% of all TNF-α mRNA clusters were localized in IBA-1^+ cells, in accordance with previous reports marking microglia as the main source of TNF-α[26]. Furthermore, we examined immunohistochemical IBA-1 expression as an established marker of microglia activation[27] (**Fig. 4c**,**d**). Significantly more IBA-1^+ cells were present in the ischemic hemisphere of T_{reg} cell–depleted mice than in that of control mice 24 h after MCAO ($n = 8$, $P = 0.002$; **Fig. 4e**).

T_{reg} cells suppress enhanced systemic cytokine expression

As cerebral ischemia also induces alterations of the systemic immune system[28], we repeatedly measured postischemic blood cytokine concentrations (**Fig. 5**). The concentrations of the proinflammatory cytokines TNF-α (**Fig. 5a**) and IFN-γ (**Fig. 5b**) were significantly higher in T_{reg} cell–depleted mice than in control mice at all measured time points up to 7 d after MCAO. At 6 h after MCAO, TNF-α serum levels were highly elevated after T_{reg} cell depletion as compared to controls ($n = 10$, $P = 0.002$). In contrast, postischemic serum concentrations of IL-10 and TGF-β were not altered compared to wild-type mice and did not differ between treatment groups either (**Fig. 5c**,**d**).

IL-10 is the main mediator of the T_{reg} cell cerebroprotective effect

The immunosuppressive effect of T_{reg} cells at the site of inflammation is mediated by diverse mechanisms, including direct cell-to-cell contact–dependent pathways and secretion of anti-inflammatory cytokines[13,14]. Owing to the delayed kinetics of T_{reg} cell brain invasion, intracerebral cell-to-cell contact–dependent mechanisms could not explain the modification of early postischemic cytokine expression. IL-10 is a key mediator of T_{reg} cells in various experimental systems and showed beneficial effects in previous brain ischemia experiments[15,29]. Therefore, we focused on IL-10 and tested the effect of i.p. (10 μg) or i.c.v. (100 ng) IL-10 injection in our ischemia model (**Fig. 6a**). Mimicking the kinetics of our previous experiments, 7 d after MCAO, infarct size was significantly reduced by IL-10 i.c.v. treatment compared to i.p. administration ($n = 5$, $P = 0.016$) and compared to control mice ($n = 5$, $P = 0.036$), whereas infarct volume did not differ 3 d after MCAO (**Fig. 6a**). Furthermore, i.c.v., but not i.p., IL-10 administration prevented infarct enlargement in T_{reg} cell–depleted mice (**Fig. 6b**). Intracerebroventricular IL-10 also lowered the cerebral expression of proinflammatory cytokines. A single i.c.v. injection early after MCAO induction prevented the postischemic upregulation of TNF-α and IL-1β in T_{reg} cell–depleted mice. Notably, i.c.v. IL-10 also abrogated the strong IFN-γ mRNA upregulation 3 d after MCAO (**Fig. 6c**).

To elucidate whether T_{reg} cells are the essential source of cerebroprotective IL-10 signaling after ischemia, we performed a cotransfer experiment with $CD4^+CD25^-$ and $CD4^+CD25^+$ lymphocytes harvested from either C57BL/6 or *Il10*$^{-/-}$ mice (**Fig. 6d**). *Rag2*$^{-/-}$ mice receiving $CD4^+CD25^+$ cells from *Il10*$^{-/-}$ donors had significantly larger infarct volumes than did control mice ($n = 8$, $P = 0.005$). Thus, despite the lack of T_{reg} cell–derived IL-10 being the only difference between groups, infarct volumes detected 7 d after MCAO were similar to those observed after physical T_{reg} cell depletion, supporting a key role of T_{reg} cell–derived IL-10.

DISCUSSION

Inflammatory processes contribute substantially to ischemic brain damage. Although the search for counteractive therapies is a focus of current translational cerebrovascular research, the pathophysiology of *intrinsic* immune system modulation is barely understood[3,4,30]. To our knowledge, our study is the first to examine the role of T_{reg} cells in acute cerebral ischemia. Our major new findings are that T_{reg} cells prevent secondary infarct growth by counteracting excessive production of proinflammatory cytokines and by modulating invasion and/or activation of lymphocytes and microglia in the ischemic brain. We have found that T_{reg} cells antagonize enhanced TNF-α and IFN-γ production, which induce delayed inflammatory brain damage, and that T_{reg} cell–derived secretion of IL-10 is the key mediator of the cerebroprotective effect via suppression of proinflammatory cytokine production.

Most of our experiments were performed in a well established permanent MCAO model inducing cortical infarction that reflects many embolic human strokes. In our study, the endogenous protective effect of T_{reg} cells was verified in two independent experimental paradigms—antibody-mediated depletion and adoptive cell transfer. Of note, experimental T_{reg} cell depletion was equally deleterious in another model of brief, reversible filament-MCAO for inducing subcortical infarcts, whereas it did not augment the already extensive ischemic damage caused by prolonged filament-MCAO. This discrepancy is well explained by manifest infarction of the entire MCA territory 24 h after prolonged filament-MCAO, which precludes secondary infarct growth due to exacerbated inflammation. However, although early deleterious mechanisms such as energy deprivation, depolarization and neuroexcitotoxicity are more likely to determine the kinetics of infarct development in this model[31], it is also associated with severe immune dysregulation, including simultaneous activation of cerebral inflammatory signaling[4,32] and systemic immunodepression[17,33,34].

Cytokine-dependent pathways after brain ischemia are complex[35], interact in multiple signaling networks and have diverse sources[22,36,37]. Proinflammatory cytokines, which are substantially upregulated in large infarcts[24,28], were elevated after circumscribed cortical ischemia only in T_{reg} cell–depleted mice. TNF-α expression was elevated early after ischemia both in the brain, where it was predominantly generated by microglia and systemically in T_{reg} cell–depleted mice. Furthermore, early, but not delayed, i.c.v. antagonization effectively reduced the lesion size. These findings are in line with early involvement of TNF-α in the inflammatory cascade and its role in the induction of inflammatory pathways, cell death signaling, chemoattraction of leukocytes to the site of inflammation[38] and augmentation of subsequent IFN-γ production. IFN-γ maintains inflammatory reactions and may influence cerebral tissue necrosis, vascular adhesion molecule expression and microglial activation[22,23,37,39]. Whereas IFN-γ is almost absent in normal brain tissue, its expression increased at a later time point after MCAO than did TNF-α expression, and its expression was strongly induced after T_{reg} cell depletion. Remarkably, IFN-γ antagonization substantially reduced infarct size in our study even when injected as late as 3 d after MCAO, consistent with previous ischemia studies[22] and its late interference with the inflammatory cascade. Invading T cells represented the major source of cerebral IFN-γ and, after T_{reg} cell depletion, were strongly activated 5 d after ischemia. As lymphocytes were detected at earlier time points only by FACS but not by immunohistochemistry, other cells may also have contributed to cerebral IFN-γ production. T cell activation occurred in response to stimulation by TNF-α secreted by other cells[24,40], as early TNF-α antagonization abrogated the late increase in IFN-γ expression.

Various cellular targets of T_{reg} cells after ischemia were identified. In addition to modifying the postischemic activation of microglia and cerebral cytokine secretion by T cells, early granulocyte invasion was attenuated. The very early impact of T_{reg} cell depletion on both innate and adaptive immune pathways suggests an antigen-nonspecific T_{reg} cell function in this model, a T_{reg} cell–typical function described as 'bystander suppression'[13,14]. Early suppression of TNF-α and IL-1β release as well as a reduction in microglial activation in the presence of steady-state T_{reg} cells are probably due to the continuous activation of a large part of autoreactive T_{reg} cells[41]. By the time T cells are activated and IFN-γ production is inhibited, additional antigen-dependent T_{reg} cell activation may also have a role. Indeed, previous ischemia experiments have indicated that tolerizing lymphocytes to specific brain antigens by mucosal exposure before ischemia induction provides effective protection[8,16].

Although we cannot rule out that other T_{reg} cells mediators, including TGF-β and cytotoxic T lymphocyte antigen-4, or their direct cellular interactions contribute to T_{reg} cell–induced protection, we concentrated on IL-10 as a universal signaling mechanism used by T_{reg} cells[42–44]. IL-10 potently reduced infarct size in normal mice in a previous study[45] and in our present one and prevented delayed lesion growth after T_{reg} cell depletion when injected i.c.v. This effect resulted from the suppression of overshooting proinflammatory cytokine expression in the absence of T_{reg} cells. While various brain cells can produce IL-10, T_{reg} cells were the crucial source as the cotransfer of T_{reg} cells derived from IL-10–deficient mice failed to be protective.

In conclusion, our study provides new insights into the endogenous adaptive immune response after acute brain ischemia. Specifically, we have described a previously unknown role of T_{reg} cells as cerebroprotective immunomodulators after stroke, a function that affects diverse cytokine-dependent and cellular inflammatory targets via IL-10 signaling.

METHODS

Mice. The study was conducted in accordance with national guidelines for the use of experimental animals, and the protocols were approved by the governmental committees (Animal care committee, Regierungspraesidium Karlsruhe, Referat 35, Germany). We used age-matched, mature male mice (C57BL/6, Charles River Laboratories) unless stated otherwise. For transfer experiments, we used lymphocyte-deficient *Rag2*$^{-/-}$ (C57BL/6 background; a gift from the German Cancer Research Center, Heidelberg) mice as recipients. T cell donors were C57BL/6 or IL-10 knockout mice (*Il10*$^{-/-}$ on C57BL/6 background, Jackson Laboratories).

Ischemia model. We induced MCAO by transtemporal coagulation. We induced reversible ischemia (occlusion-reperfusion), used only for the experiments depicted in **Figure 1d**, by advancing a filament to the MCA origin for 30 min or 90 min. Surgical procedures and intracerebroventricular drug administration are described in the **Supplementary Methods** online. We injected 300 μg of CD25-specific mAb (clone PC61) intraperitoneally 48 h before ischemia induction for *in vivo* depletion of regulatory T cells. We determined the infarct volume on cryosections stained with the high-contrast silver staining technique[46] (**Supplementary Methods**).

Functional outcome tests. We performed two different functional tests 24 h before MCAO and on days 1, 3 and 7 after MCAO. For the forelimb use asymmetry test[47], we placed mice in a transparent glass cylinder and analyzed the independent use of forelimbs. We additionally used the 'corner test' to measure sensorimotor dysfunction as previously described[20] (see **Supplementary Methods** for details).

Cell sorting and transfer. We purified lymphocyte subpopulations for transfer experiments by magnetic cell sorting (Miltenyi Biotec), with C57BL/6 mice as donors. We injected a total of 8×10^6 $CD4^+$ T cells for all cell transfer experiments into *Rag2*$^{-/-}$ mice. In the cotransfer experiment, one group of lymphocyte-deficient *Rag2*$^{-/-}$ mice received all 8×10^6 cells from normal C57BL/6 mice (after magnetic cell sorting for 7.2×10^6 $CD4^+CD25^-$ cells and 0.8×10^6 $CD4^+CD25^+$ cells), whereas the other group received 7.2×10^6 $CD4^+CD25^-$ cells from C57BL/6 mice and 0.8×10^6 $CD4^+CD25^+$ cells from *Il10*$^{-/-}$ mice. The purity of T_{reg} cells was >90% (**Supplementary Fig. 1d**). After transfer into mice, we allowed the cells to expand for 7 d before MCAO.

FACS analysis. We collected organs (spleen, blood and lymph nodes) after transcardial perfusion with saline at various time points after MCAO for flow cytometric analysis. We employed a modified version of a published protocol[40] for FACS analysis of lymphocytes infiltrating the brain. We then used the isolated cells for cell staining following the manufacturers' protocols (see **Supplementary Methods** for a description of the procedures and a list of the antibodies used).

Cytokine enzyme-linked immunosorbent assay. We collected serum and froze it immediately until analysis of cytokine protein concentrations with commercial kits for the quantitative assay of TNF-α, IFN-γ, TGF-β (all from R&D Systems), and IL-10 (eBioscience); we performed each in duplicate. The detection limits were 5.1 pg ml^{-1} for TNF-α, 2.0 pg ml^{-1} for IFN-γ, 7.0 pg ml^{-1} for TGF-β and 4.0 pg ml^{-1} for IL-10.

RNA isolation and reverse transcription PCR. We isolated RNA from separated cerebral hemispheres with RNApure (Peqlab) and performed reverse transcription with the High Capacity cDNA Archive Kit (Applied Biosystems) and real-time PCR with SYBR-Green assays (Applied Biosystems) on a GeneAmp 5700 SDS from Applied Biosystems. We purchased primers as ready-to-use primer sets for each gene (Super Array). We ran all assays in duplicate. We normalized the results for each individual gene to the level of the housekeeping gene encoding peptidylprolyl isomerase A (cyclophilin), whose expression was not influenced by the anti-CD25 treatment (**Supplementary Fig. 1c**).

Immunohistology. Staining protocols for regulatory T cells, lymphocytes, granulocytes and microglia are described in the **Supplementary Methods**. We processed all images for the analysis of microglial cell number with TissueQuest software (TissueGnostics) for cell-based counting of automatically recognized IBA-1$^+$ cells in a FACS-like manner of scattergram analysis as described previously[48]. We excluded unspecifically stained structures and too-large objects from the analysis (see **Fig. 4d**, right images; less than 5% of counted cells were excluded).

Combination of immunocytochemistry and *in situ* hybridization. We used an IBA-1–specific antibody to stain microglial cells. Additionally, we performed *in situ* hybridization with an antisense riboprobe for TNF-α followed by a protocol previously described in detail[49,50] (**Supplementary Methods**). We detected the presence of TNF-α transcripts by silver grain agglomerations. We identified microglial cells by brown staining of the cell surface with typical morphology. We analyzed 811 cells in total ($n = 10$ brains).

Statistical analyses. All values are expressed as mean ± s.d. We performed analysis of infarct volumes and functional outcome tests by two-tailed Student's *t*-test between two groups and analysis of variance for multiple comparisons with *post hoc* Tukey's test, respectively, after validating the normal distribution of these datasets (Kolmogorov-Smirnov test). For the remaining data, we used two-tailed Wilcoxon rank-sum test for comparison between two groups; for three and more groups we applied the Kruskal-Wallis *H* test with *post hoc* paired Mann-Whitney testing and Holm's sequential Bonferroni adjustment for *P* values, using SPSS analysis software. A $P < 0.05$ was considered statistically significant.

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

ACKNOWLEDGMENTS

This study was supported by grants from the Else-Kröner Fresenius Stiftung and the Ministerium für Wissenschaft und Kultur Baden-Württemberg to R.V. We would like to thank H. Bürgers and D. Stefan for excellent technical assistance and I. Galani (German Cancer Research Center, Heidelberg) for providing the *Rag2*$^{-/-}$ mice.

AUTHOR CONTRIBUTIONS

A.L. designed and performed experiments, analyzed data and wrote the manuscript; H.D. performed experiments and analyzed data; E.S.-P. provided crucial input on T_{reg} cell function and contributed to experimental design and manuscript writing; C.V. designed experiments and contributed to manuscript writing; C.S. designed experiments and analyzed data; S.R. performed experiments and analyzed data; T.G. provided specific input to flow cytometric analysis and contributed to manuscript writing; R.V. initiated and directed the entire study, designed experiments, analyzed data and wrote the manuscript.

Published online at http://www.nature.com/naturemedicine/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Donnan, G.A., Fisher, M., Macleod, M. & Davis, S.M. Stroke. *Lancet* **371**, 1612–1623 (2008).
2. Lo, E.H., Dalkara, T. & Moskowitz, M.A. Mechanisms, challenges and opportunities in stroke. *Nat. Rev. Neurosci.* **4**, 399–415 (2003).
3. Dirnagl, U. Inflammation in stroke: the good, the bad, and the unknown. *Ernst Schering Res. Found. Workshop.* **47**, 87–99 (2004).
4. Wang, Q., Tang, X.N. & Yenari, M.A. The inflammatory response in stroke. *J. Neuroimmunol.* **184**, 53–68 (2007).
5. Arumugam, T.V., Granger, D.N. & Mattson, M.P. Stroke and T cells. *Neuromolecular Med.* **7**, 229–242 (2005).
6. Perera, M.N. *et al.* Inflammation following stroke. *J. Clin. Neurosci.* **13**, 1–8 (2006).
7. Meisel, C., Schwab, J.M., Prass, K., Meisel, A. & Dirnagl, U. Central nervous system injury-induced immune deficiency syndrome. *Nat. Rev. Neurosci.* **6**, 775–786 (2005).
8. Gee, J.M., Kalil, A., Shea, C. & Becker, K.J. Lymphocytes: potential mediators of postischemic injury and neuroprotection. *Stroke* **38**, 783–788 (2007).

9. McGeachy, M.J., Stephens, L.A. & Anderton, S.M. Natural recovery and protection from autoimmune encephalomyelitis: contribution of $CD4^+CD25^+$ regulatory cells within the central nervous system. *J. Immunol.* **175**, 3025–3032 (2005).
10. Suri-Payer, E. & Fritzsching, B. Regulatory T cells in experimental autoimmune disease. *Springer Semin. Immunopathol.* **28**, 3–16 (2006).
11. Sakaguchi, S. *et al.* $Foxp3^+$ $CD25^+$ $CD4^+$ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol. Rev.* **212**, 8–27 (2006).
12. O'Connor, R.A. & Anderton, S.M. $Foxp3^+$ regulatory T cells in the control of experimental CNS autoimmune disease. *J. Neuroimmunol.* **193**, 1–11 (2008).
13. O'Garra, A. & Vieira, P. Regulatory T cells and mechanisms of immune system control. *Nat. Med.* **10**, 801–805 (2004).
14. Tang, Q. & Bluestone, J.A. The $Foxp3^+$ regulatory T cell: a jack of all trades, master of regulation. *Nat. Immunol.* **9**, 239–244 (2008).
15. Spera, P.A., Ellison, J.A., Feuerstein, G.Z. & Barone, F.C. IL-10 reduces rat brain injury following focal stroke. *Neurosci. Lett.* **251**, 189–192 (1998).
16. Gee, J.M., Kalil, A., Thullbery, M. & Becker, K.J. Induction of immunologic tolerance to myelin basic protein prevents central nervous system autoimmunity and improves outcome after stroke. *Stroke* **39**, 1575–1582 (2008).
17. Offner, H. *et al.* Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. *J. Immunol.* **176**, 6523–6531 (2006).
18. McNeill, A., Spittle, E. & Backstrom, B.T. Partial depletion of CD69-expressing natural regulatory T cells with the anti-CD25 monoclonal antibody PC61. *Scand. J. Immunol.* **65**, 63–69 (2007).
19. Schallert, T., Fleming, S.M., Leasure, J.L., Tillerson, J.L. & Bland, S.T. CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology* **39**, 777–787 (2000).
20. Zhang, L. *et al.* A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. *J. Neurosci. Methods* **117**, 207–214 (2002).
21. Barone, F.C. *et al.* Tumor necrosis factor-α. A mediator of focal ischemic brain injury. *Stroke* **28**, 1233–1244 (1997).
22. Yilmaz, G., Arumugam, T.V., Stokes, K.Y. & Granger, D.N. Role of T lymphocytes and interferon-γ in ischemic stroke. *Circulation* **113**, 2105–2112 (2006).
23. Lambertsen, K.L. *et al.* A role for interferon-γ in focal cerebral ischemia in mice. *J. Neuropathol. Exp. Neurol.* **63**, 942–955 (2004).
24. Lambertsen, K.L., Meldgaard, M., Ladeby, R. & Finsen, B. A quantitative study of microglial-macrophage synthesis of tumor necrosis factor during acute and late focal cerebral ischemia in mice. *J. Cereb. Blood Flow Metab.* **25**, 119–135 (2005).
25. Rothwell, N.J. & Luheshi, G.N. Interleukin 1 in the brain: biology, pathology and therapeutic target. *Trends Neurosci.* **23**, 618–625 (2000).
26. Gregersen, R., Lambertsen, K. & Finsen, B. Microglia and macrophages are the major source of tumor necrosis factor in permanent middle cerebral artery occlusion in mice. *J. Cereb. Blood Flow Metab.* **20**, 53–65 (2000).
27. Ito, D., Tanaka, K., Suzuki, S., Dembo, T. & Fukuuchi, Y. Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain. *Stroke* **32**, 1208–1215 (2001).
28. Offner, H. *et al.* Experimental stroke induces massive, rapid activation of the peripheral immune system. *J. Cereb. Blood Flow Metab.* **26**, 654–665 (2006).
29. Grilli, M. *et al.* Interleukin-10 modulates neuronal threshold of vulnerability to ischaemic damage. *Eur. J. Neurosci.* **12**, 2265–2272 (2000).
30. del Zoppo, G.J., Becker, K.J. & Hallenbeck, J.M. Inflammation after stroke: is it harmful? *Arch. Neurol.* **58**, 669–672 (2001).
31. Dirnagl, U., Iadecola, C. & Moskowitz, M.A. Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci.* **22**, 391–397 (1999).
32. del Zoppo, G. *et al.* Inflammation and stroke: putative role for cytokines, adhesion molecules and iNOS in brain response to ischemia. *Brain Pathol.* **10**, 95–112 (2000).
33. Chamorro, A., Urra, X. & Planas, A.M. Infection after acute ischemic stroke: a manifestation of brain-induced immunodepression. *Stroke* **38**, 1097–1103 (2007).
34. Prass, K. *et al.* Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1–like immunostimulation. *J. Exp. Med.* **198**, 725–736 (2003).
35. Planas, A.M., Gorina, R. & Chamorro, A. Signalling pathways mediating inflammatory responses in brain ischaemia. *Biochem. Soc. Trans.* **34**, 1267–1270 (2006).
36. Hurn, P.D. *et al.* T- and B-cell-deficient mice with experimental stroke have reduced lesion size and inflammation. *J. Cereb. Blood Flow Metab.* **27**, 1798–1805 (2007).
37. Schroeter, M. & Jander, S. T-cell cytokines in injury-induced neural damage and repair. *Neuromolecular Med.* **7**, 183–195 (2005).
38. Wajant, H., Pfizenmaier, K. & Scheurich, P. Tumor necrosis factor signaling. *Cell Death Differ.* **10**, 45–65 (2003).
39. Baird, A.E. The forgotten lymphocyte: immunity and stroke. *Circulation* **113**, 2035–2036 (2006).
40. Campanella, M., Sciorati, C., Tarozzo, G. & Beltramo, M. Flow cytometric analysis of inflammatory cells in ischemic rat brain. *Stroke* **33**, 586–592 (2002).
41. Fisson, S. *et al.* Continuous activation of autoreactive $CD4^+$ $CD25^+$ regulatory T cells in the steady state. *J. Exp. Med.* **198**, 737–746 (2003).
42. Frenkel, D. *et al.* Neuroprotection by IL-10–producing MOG $CD4^+$ T cells following ischemic stroke. *J. Neurol. Sci.* **233**, 125–132 (2005).
43. Strle, K. *et al.* Interleukin-10 in the brain. *Crit. Rev. Immunol.* **21**, 427–449 (2001).
44. Vignali, D.A., Collison, L.W. & Workman, C.J. How regulatory T cells work. *Nat. Rev. Immunol.* **8**, 523–532 (2008).
45. O'Garra, A., Vieira, P.L., Vieira, P. & Goldfeld, A.E. IL-10–producing and naturally occurring $CD4^+$ Tregs: limiting collateral damage. *J. Clin. Invest.* **114**, 1372–1378 (2004).
46. Vogel, J., Mobius, C. & Kuschinsky, W. Early delineation of ischemic tissue in rat brain cryosections by high-contrast staining. *Stroke* **30**, 1134–1141 (1999).
47. Schallert, T., Hernandez, T.D. & Barth, T.M. Recovery of function after brain damage: severe and chronic disruption by diazepam. *Brain Res.* **379**, 104–111 (1986).
48. Steiner, G.E., Ecker, R.C., Kramer, G., Stockenhuber, F. & Marberger, M.J. Automated data acquisition by confocal laser scanning microscopy and image analysis of triple stained immunofluorescent leukocytes in tissue. *J. Immunol. Methods* **237**, 39–50 (2000).
49. Blais, V. & Rivest, S. Effects of TNF-α and IFN-γ on nitric oxide–induced neurotoxicity in the mouse brain. *J. Immunol.* **172**, 7043–7052 (2004).
50. Nadeau, S. & Rivest, S. Role of microglial-derived tumor necrosis factor in mediating CD14 transcription and nuclear factor κ B activity in the brain during endotoxemia. *J. Neurosci.* **20**, 3456–3468 (2000).