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Role of regulatory T cells in coronavirus-induced acute encephalitis ☆

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ABSTRACT

C57BL/6 mice infected with mouse hepatitis virus, strain JHM (JHMV) develop a rapidly fatal acute encephalitis. Previously, we showed that this disease is partially CD4 T cell-mediated since infection with a recombinant JHMV (rJ) mutated in only a single immunodominant CD4 T cell epitope (epitope M133, rJ.M_{Y135Q}) results in a nonlethal disease. Increased mortality correlated with a greater number of JHMV-specific CD4 T cells in the brains of rJ compared to rJ.M_{Y135Q}-infected mice. Here, we extend these results to show that the diminished number of virus-specific T cells correlates with a reduced cytokine/chemokine response in the infected brain. We also show that regulatory CD4 T cells (Tregs) are critical for mild disease in rJ.M_{Y135Q}-infected mice because their depletion results in increased mortality. Further, a relative paucity of Tregs characterizes lethal infection because adoptive transfer of Tregs into rJ-infected mice increases survival from 0% to 50%. These results support the notion that clinical disease in coronavirus-induced acute encephalitis results from a balance between factors critical for virus clearance, such as virus-specific effector T cells and anti-inflammatory elements, such as Tregs. These findings also show that unlike chronic infections, in which an excessive number of Tregs contributes to pathogen persistence, Tregs in the setting of acute encephalitis may help to limit immunopathological disease without delaying virus clearance.

Introduction

The outcome of viral infections is dependent upon complex interactions between the pathogen and host pro- and anti-inflammatory immune responses. A dysregulated and overexuberant innate immune response has been implicated in fatal H5N1 influenza (de Jong et al., 2006) and may also be involved in the pathogenesis of SARS (Severe Acute Respiratory Syndrome), caused by a novel coronavirus (Perlman and Dandekar, 2005). Excessive T cell responses have also been implicated in disease in mice infected with lymphocytic choriomeningitis virus (LCMV), herpes simplex virus or respiratory syncytial virus, with tissue damage occurring during the process of virus clearance (Hussell et al., 2001; Oldstone, 2002; Suvas et al., 2004). To minimize excessive pro-inflammatory responses, cells with anti-inflammatory activity, such as regulatory T cells and Tr1 cells, which express IL-10 (Roncarolo et al., 2006), are also induced during a normal immune response. An appropriate anti-inflammatory response will prevent immunopathological disease without adversely affecting virus clearance. Regulatory T cells, which suppress the pro-inflammatory response, encompass several subsets including thymically derived $CD4^+CD25^+$ cells that express the transcription factor Foxp3 (natural Tregs, abbreviated Tregs herein) (Belkaid, 2007; Roncarolo and Battaglia, 2007). Many studies have assessed the role of Tregs in autoimmunity, where a relative deficiency of these cells occurs, and cancer, where they inhibit anti-tumor T cell responses (Nomura and Sakaguchi, 2005; Sakaguchi, 2005; Sakaguchi et al., 2006). These cells have also been implicated in chronic viral diseases. This was first demonstrated in mice infected with Friend's virus or herpes simplex virus and subsequently in humans infected with viruses such as hepatitis C virus (HCV) and HIV (Belkaid, 2007; Belkaid and Rouse, 2005; Iwashiro et al., 2001; MacDonald et al., 2002; Robertson and Hasenkrug, 2006; Suvas et al., 2004). In infectious settings, Tregs can dampen the immune response, thus minimizing tissue damage but at the cost of inhibiting virus clearance (reviewed in Belkaid, 2007; Belkaid and Rouse, 2005; Robertson and Hasenkrug, 2006).

Tregs might be predicted to have a lesser role in acute viral diseases, since these infections are often characterized by uncontrolled virus replication and rapid disease progression. In murine coronavirus-induced acute encephalitis, mice infected with the neurovirulent JHM strain (JHMV) of mouse hepatitis virus (MHV) develop a fatal disease with death occurring at 7–8 days p.i. (Stohlman et al., 1998). Virus-specific T cells are first detected in the brain at 6 days p.i., 24–48 h prior to the death of the animals. In C57BL/6 (B6) mice, the majority of brain-derived CD8 T cells recognize two epitopes in the surface (S) glycoprotein (S510 and S598), and 20–25% of CD4 T cells respond to a single epitope, (M133, spanning residues 133–147 of the transmembrane (M) protein) (Haring et al., 2001).

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Table 1
Levels of cytokines and chemokines in the brains of rJ and rJ.M_{Y135Q}-infected mice

Virus	IL-1β[a]	IL-6[a]	IFN-γ[a]	TNF-α[a]	CCL2[a]	CCL5[a]
Day 5						
rJ	50.8±5.2	94.9±8.9**	36.1±8.5*	738.3±266.8	2827.2±329.1**	417.9±12.5**
rJ.M_{Y135Q}	37.4±2.8	46.7±16.2	13.6±3.2	390.3±66.3	598.0±204.9	171.1±49.8
Day 7						
rJ	11.5±1.4	13.0±4.4	16.9±3.3	39.8±2.5	1019.4±293.1	449.9±16.5
rJ.M_{Y135Q}	12.8±1.0	5.0±0.6	13.4±3.9	37.5±1.6	476.6±189.1	311.4±112.6

[a] pg/ml.
* $p<0.05$.
** $p<0.02$, when rJ and rJ.M_{Y135Q}-infected mice are compared.

While disease is generally attributed to the extensive neuronal damage observed in these mice (Dubois-Dalcq et al., 1982; Kyuwa and Stohlman, 1990), we recently showed that the JHMV-specific CD4 T cell response contributed to a fatal outcome (Anghelina et al., 2006). Infecting mice with a recombinant JHMV (rJ) in which M133 was mutated (rJ.M_{Y135Q}) so that it was no longer recognized by CD4 T cells resulted in a change in mortality from 100% to 0% without changes in the virus-specific CD8 T cell response, in the Th2 CD4 T cell response or in the

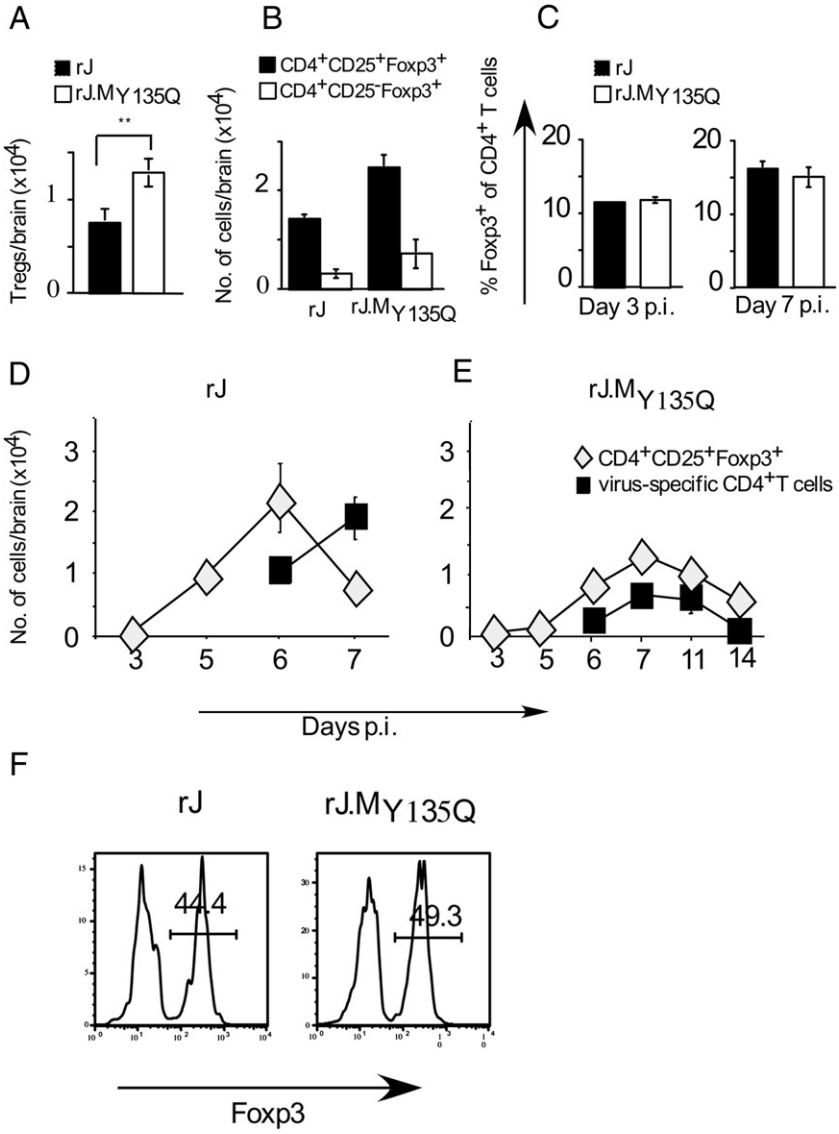

Fig. 1. Regulatory T cells in the brains of mice inoculated with rJ or rJ.M_{Y135Q}. (A) Cells were harvested at 7 days p.i. from the brains of mice infected with rJ or rJ.M_{Y135Q} and stained for CD4, CD25 and Foxp3. The number of $CD4^+CD25^+$ cells that express Foxp3 is shown ($n=8$ for each virus). **$p<0.02$. (B) Numbers of CD4 T cells that are $CD25^+Foxp3^+$ or $CD25^-Foxp3^+$ in brains of mice infected with rJ or rJ.M_{Y135Q} at 7 days p.i. ($n=5$ for each virus). (C) Frequency of CD4 T cells that are $Foxp3^+$ in CLN of rJ and rJ.M_{Y135Q}-infected mice ($n=3$–5 mice for each virus at each time point). (D, E) Cells were harvested from the brains of mice infected with rJ or rJ.M_{Y135Q} and analyzed for numbers of Tregs and virus-specific CD4 T cells (epitopes M133, S358, S333-specific) by IFN-γ intracellular staining at the indicated times. Numbers of virus-specific CD4 T cells and Tregs are shown. Three to eight mice per virus were analyzed at each time point. (F) Cells were harvested from the brains of mice infected with rJ or rJ.M_{Y135Q} at 3 days p.i. and stained for CD4, CD25 and Foxp3. Percentage of $CD4^+CD25^+$ T cells expressing Foxp3 is indicated. Representative histograms are shown. Four mice infected with each virus were analyzed. Of note, very few CD4 T cells (approximately 2000) were detected in infected mice at day 3 p.i.

kinetics of virus clearance (Anghelina et al., 2006). Severe disease correlated with a greater number of virus-specific Th1 CD4 T cells in the brain. These results, showing that the virus-specific CD4 T cell response is partly pathogenic, raise the possibility that a more potent anti-inflammatory response would minimize the collateral effects of the anti-viral immune response, ideally without delaying virus clearance. Here we substantiate the notion that the inflammatory response is diminished in rJ.M_{Y135Q}-infected mice by showing that levels of pro-

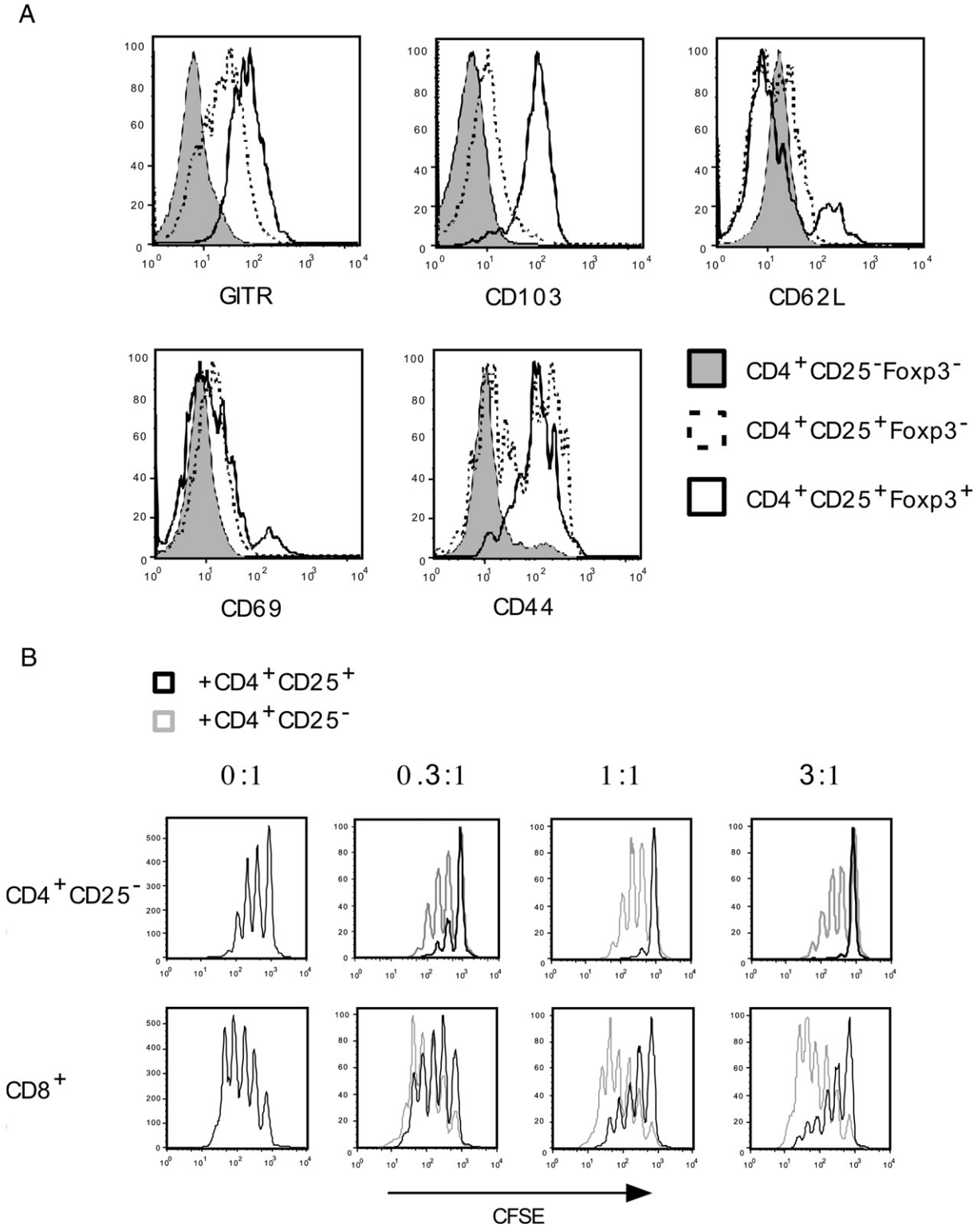

Fig. 2. Functional and phenotypic characterization of Tregs harvested from infected mice. (A) Mononuclear cells harvested at 7 days p.i. from the brains of B6 mice infected with rJ or rJ.$_{MY135Q}$ were stained directly *ex vivo* for CD4, CD25, Foxp3 and GITR, CD103, CD69, CD62L or CD44. Cells harvested from a rJ.$_{MY135Q}$-infected mouse are shown in the figure. (B) $CD4^+CD25^+$ (Tregs) and $CD4^+CD25^-$ (non-Tregs) T cells were sorted from the CLN of rJ.M_{Y135Q}-infected B6 mice (7 days p.i.) as described in Materials and methods. $CD4^+CD25^-$ and $CD8^+$ cells were prepared from the lymph nodes of naive mice, labeled with CFSE, stimulated with 1 μg/ml anti-CD3 and incubated for 72 h with the sorted Tregs or non-Tregs and irradiated splenocytes at the indicated ratios (Treg or non-Treg: CD8 or $CD4^+CD25^-$). Cells were analyzed for CFSE dilution (proliferation) by flow cytometry as described in Materials and methods.

inflammatory cytokine/chemokines are lower in the brains of these mice compared to those infected with rJ. Further, since Tregs are believed to contribute to the anti-inflammatory response, we reasoned that a relative deficiency of these cells might contribute to rJ-mediated encephalitis. Here we demonstrate that this is indeed the case and that Tregs have a protective role in the context of a rapidly fatal acute infectious disease.

Results

Levels of proinflammatory cytokines and chemokines are greater in the rJ relative to the rJ.M$_{Y135Q}$-infected CNS

rJ-infected mice develop fatal acute encephalitis, whereas mice infected with rJ.M$_{Y135Q}$ develop a nonlethal mild encephalitis and these differences correlate with numbers of JHMV-specific CD4 T cells in the brain (Anghelina et al., 2006). Since the host response to infection is determined by relative contributions of pro and anti-inflammatory factors, we reasoned that increased clinical disease in rJ-infected mice, when compared to those infected with rJ.M$_{Y135Q}$, might result from not only an increased number of virus-specific CD4 Th1 cells, but also higher levels of pro-inflammatory cytokines and chemokines. To investigate this possibility, we measured protein levels of several molecules known to be upregulated in the brains of JHMV-infected mice (IL-1β, IL-6, IFN-γ, TNFα, CCL2, CCL5) (Bergmann et al., 2006) at days 5 and 7 p.i. (Table 1). Significantly higher levels of IL-6, CCL2, CCL5 and IFN-γ , but not IL-1β or TNF-α were measured in rJ compared to rJ.M$_{Y135Q}$-infected mice at day 5 p.i. In general, levels of cytokines were lower at day 7 when compared to day 5, in agreement with previous studies (Rempel et al., 2004), but levels of IL-6, CCL2 and CCL5 remained increased in rJ compared to rJ.M$_{Y135Q}$-infected brains. Elevated levels of pro-inflammatory cytokines, particularly IL-6 and TNF-α, have been associated with decreased Treg function (Korn et al., 2007) and autoimmunity is diminished in IL6$^{-/-}$ mice, in part because Tregs are fully suppressive in the absence of this cytokine (Pasare and Medzhitov, 2003). IL-6 was significantly lower and disease was milder in rJ.M$_{Y135Q}$ compared to rJ-infected mice, prompting us to assess levels and function of Tregs in infected mice.

Rates of accumulation and numbers of Tregs are different in rJ and rJ.M$_{Y135Q}$-infected brains

To determine whether Tregs also contributed to the more modest inflammatory milieu detected in rJ.M$_{Y135Q}$-infected mice, we measured the numbers of CD4$^+$CD25$^+$Foxp3$^+$ cells in the brains of infected mice at day 7 p.i. by flow cytometry, the time when most rJ-infected mice die from acute encephalitis. As shown in Fig. 1A, a greater number of Tregs were detected in the brains of rJ.M$_{Y135Q}$ compared to rJ-infected mice. Other studies have shown that CD4$^+$CD25$^-$Foxp3$^+$ cells are as immunosuppressive as CD4$^+$CD25$^+$Foxp3$^+$ cells (Kim and Rudensky, 2006). In subsequent experiments, we showed that the majority of Foxp3$^+$ cells in the brains of rJ and rJ.M$_{Y135Q}$-infected mice expressed CD25 (Fig. 1B). Thus, total numbers of Foxp3$^+$ cells in the brains of mice infected with rJ.M$_{Y135Q}$ were greater than in rJ-infected brains even when CD25$^-$Foxp3$^+$ cells were included in the analyses. These differences were confined to the brain because nearly identical percentages of Tregs were detected in the draining (cervical) lymph nodes (CLN) of rJ and rJ.M$_{Y135Q}$-infected mice (Fig. 1C), suggesting that Tregs attenuated the immune response at the site of inflammation (brain) in rJ.M$_{Y135Q}$-infected mice.

We next determined the temporal relationship between the numbers of Tregs (CD4$^+$CD25$^+$Foxp3$^+$) and virus-specific effector CD4 T cells in the brains of mice infected with rJ or rJ.M$_{Y135Q}$ (Figs. 1D,E). Of note, we previously showed that, at each time point, similar numbers of total CD4 T lymphocytes were detected in rJ and rJ.M$_{Y135Q}$-infected brains (Anghelina et al., 2006). We used IFN-γ expression as a marker for effector Th1 CD4 T cells since virtually no Th2 CD4 T cells are present in infected brains as assayed by expression of IL-5 (Anghelina et al., 2006). In addition to epitope M133, at least two additional CD4 T cell epitopes, encompassing residues 333–347 and 358–372, are recognized in JHMV-infected B6 mice (Xue and Perlman, 1997). We were consistently able to detect CD4$^+$CD25$^+$Foxp3$^+$ T cells in the brain as early as day 3 after infection with either virus (Fig. 1F, equivalent to approximately 50–100 Tregs/brain) but could not reliably identify JHMV-specific CD4 T cells using an intracellular IFN-γ assay until day 6 p.i. In the rJ-infected brain, the numbers of Tregs decreased and virus-specific effector CD4 T cells greatly increased between 6 and 7 days p.i. These changes coincided with the time when mice become moribund. In contrast, numbers of Tregs predominated in the rJ.M$_{Y135Q}$-infected brain at all time points up to 14 days p.i.; these mice developed minimal clinical disease with no mortality. Of note, it is striking that the numbers of Tregs in the rJ-infected brain was much higher than in the brains of mice infected with rJ.M$_{Y135Q}$ at day 6 p.i. (Figs. 1D,E). However, these cells were unable to protect mice from lethal disease and their number decreased considerably over the next 24 h. While these results may reflect the greater number of virus-specific CD4 T cells present in the rJ-infected brain, it is also possible that the elevated levels of IL-6 present in the brains of rJ-infected mice resulted in a loss of functionality, as previously described in mice with EAE (acute autoimmune encephalomyelitis) (Korn et al., 2007).

To verify that these CD4$^+$CD25$^+$Foxp3$^+$ cells were phenotypically Tregs, we stained brain-derived cells for GITR, CD103, CD62L, CD69 and CD44 (Fig. 2A). No differences were observed in marker expression when Tregs from rJ and rJ.M$_{Y135Q}$-infected brains were compared. Most importantly, both CD103 (α_ε integrin), associated with targeting Tregs to sites of inflammation, and GITR (glucocorticoid-induced tumor necrosis factor receptor family-related gene) were expressed at higher levels on CD4$^+$CD25$^+$Foxp3$^+$ cells than on either CD4$^+$CD25$^+$Foxp3$^-$ or CD4$^+$CD25$^-$Foxp3$^-$ cells. Most Tregs were CD62L$^-$. Thus, these cells had the phenotype of "effector/memory"

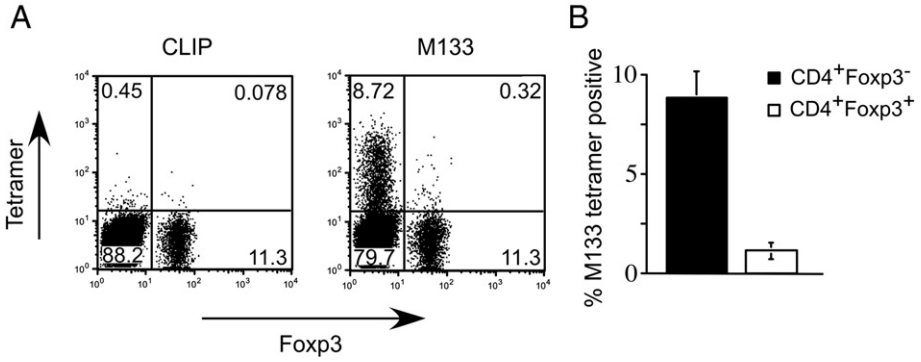

Fig. 3. Identification of epitope M133-specific Tregs in rJ-infected mice. (A) Cells were harvested from the brains of infected *Foxp3-GFP* mice and assayed for epitope M133-specific CD4 T cells by staining with specific and non-specific (human CLIP-specific) MHC class II tetramers and anti-CD4 antibody. Cells shown in the figure were gated for CD4 expression. (B) Frequencies of M133-specific Tregs (CD4$^+$Foxp3$^+$) and M133-specific non-Tregs (CD4$^+$Foxp3$^-$) within total CD4 T cells in the brain are shown. Ten mice were analyzed.

Tregs ($CD44^+GITR^+CD103^+CD62L^-$) consistent with their isolation from a site of inflammation (Huehn and Hamann, 2005).

To show that these $CD4^+CD25^+Foxp3^+$ cells were functionally Tregs, we analyzed their suppressive capability in *in vitro* proliferation assays. For these experiments, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were harvested from the CLN of rJ.M_{Y135Q}-infected mice at day 7 p.i. and incubated with CFSE-labeled $CD4^+CD25^-$ or CD8 T cells, obtained from naive mice, in the presence of anti-CD3 antibody and irradiated splenocytes. We used Tregs from CLN in these assays because only small numbers of Tregs could be harvested from the rJ.M_{Y135Q}-infected brain (maximum of about 10,000 Tregs/brain, assuming complete recovery (Fig. 1E)) and brain-derived lymphocytes do not generally survive in *in vitro* cultures (Irani et al., 1997). These $CD4^+CD25^+$ cells but not $CD4^+CD25^-$ T cells potently suppressed CD4 and CD8 T cell proliferation (Fig. 2B). Thus, Tregs were present in higher numbers than JHMV-specific effector T cells in the brains of rJ.M_{Y135Q}-infected mice at all time points and were phenotypically and, by extrapolation from these studies of CLN-derived Tregs, functionally identical to Tregs described in other settings (Nomura and Sakaguchi, 2005; Sakaguchi, 2005; Sakaguchi et al., 2006).

Virus-specificity of Tregs in the brains of infected mice

In some instances, Tregs recognizing an antigen present at the site of inflammation are more potently immunosuppressive than natural Tregs (Tang and Bluestone, 2006; Tarbell et al., 2007). Therefore, to determine whether Tregs in the brain are virus-specific, we used MHC class II/peptide M133 tetramers to detect M133-specific Tregs in rJ-infected mice. Because intracellular staining for Foxp3 requires permeabilization, which diminishes tetramer binding, we infected *Foxp3gfp* knock-in mice (Fontenot et al., 2005), in which Tregs are identified by GFP expression without a requirement for permeabilization. We found that 8.8±0.9% (n=10) of $Foxp3^-$ CD4 T cells in the brain were M133-specific (Fig. 3) by tetramer staining. Only 1.2%±0.3% (n=10) of $Foxp3^+(GFP^+)$ CD4 T cells bound tetramer M133. Although this likely represents a minimal estimate of the number of virus-specific Tregs in the brain, we did not further investigate their protective role because they were present in such low numbers.

Morbidity and mortality were increased in rJ.M_{Y135Q}-infected mice after Treg depletion

While these results demonstrate the presence of Tregs in the infected CNS, they do not show whether these cells are immunosuppressive *in vivo*. If Tregs have a significant role in protection, they should be critical for limiting disease in rJ.M_{Y135Q}-infected mice. Consequently, their depletion should result in enhanced disease. Therefore, we depleted Tregs by treatment with anti-CD25 mAb (mAb PC61) three days prior to infection with rJ.M_{Y135Q}; this treatment resulted in an approximate 90% decrease in Tregs in the infected brain at 5 days p.i. (Fig. 4A). Anti-CD25-treated mice developed significant weight loss and a mortality of 50% whereas control mice, treated with rat IgG, developed only mild disease and uniformly survived the infection (Figs. 4B,D). By decreasing the anti-inflammatory environment, Treg depletion might facilitate virus clearance. However, depletion of these cells did not change virus clearance in rJ.M_{Y135Q}-infected mice, as similar levels of infectious virus were measured at day 5 p.i. in control and Treg-depleted mice (Fig. 4C). Of note, we measured levels of a single representative cytokine, IL-6, at day 5 p.i. to determine whether mAb PC61 treatment resulted in increased cytokine/chemokine levels. Day 5 p.i. was chosen because levels diminish greatly by day 7 p.i. (Fig. 1). However, we saw no statistically significant differences in IL-6 levels when IgG and anti-CD25-treated mice were compared. This lack of

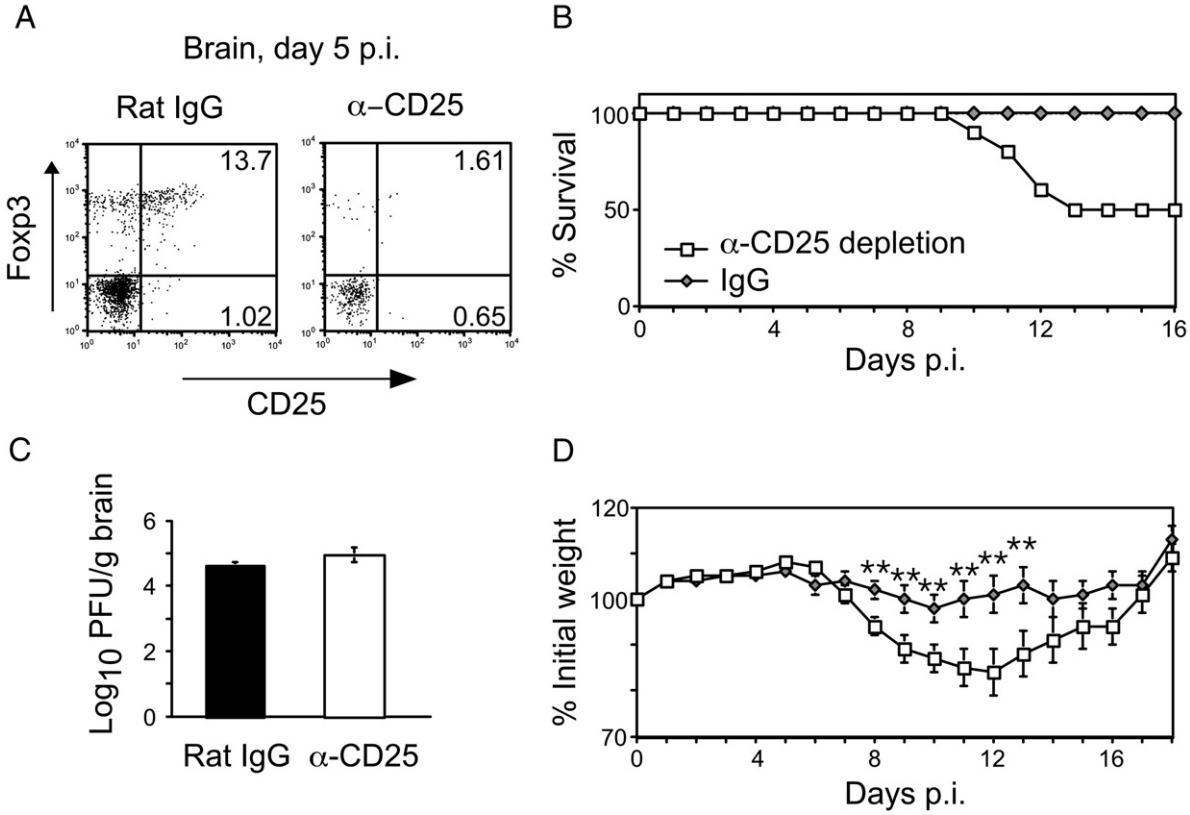

Fig. 4. Increased mortality and weight loss in rJ.M_{Y135Q}-infected mice after Treg depletion. (A) Mice were treated with 0.5 mg anti-CD25 antibody (mAb PC61) or rat IgG 3 days prior to infection with rJ.M_{Y135Q}. Percentage of $Foxp3^+CD25^+$ of total CD4 T cells in the brain at day 5 p.i. (8 days post treatment) is shown. (B, D) B6 mice were infected with rJ.M_{Y135Q} three days after treatment with anti-CD25 antibody (n=10) or rat IgG (n=15) and monitored for survival (B) and weight loss (D). Results from three independent experiments are shown. Mortality ($p<0.05$) and weight loss ($p<0.05$ at days 8–13) were significantly increased in anti-CD25 mAb-treated mice. (C) Viral titers in the brain at day 5 p.i after treatment with mAb PC61 or rat IgG (n=5 for each antibody).

difference may have occurred because only 50% of mice died after PC61 treatment and it was not possible to identify these mice at day 5 p.i.

Enhanced survival in rJ-infected recipients of transferred Tregs

Conversely, a relative deficiency of Tregs might contribute to the increased mortality observed in rJ-infected mice. To assess this possibility, we adoptively transferred $CD4^+CD25^+$ Tregs from naive B6 spleens into infected animals. The purity of the transferred cells was consistently 95–98% (Fig. 5A). These cells were functional in *in vitro* proliferation assays, performed as described in Fig. 2A (data not shown). In preliminary experiments, we observed optimal protection when cells were transferred one day p.i. so transfers were performed at this time in subsequent experiments.

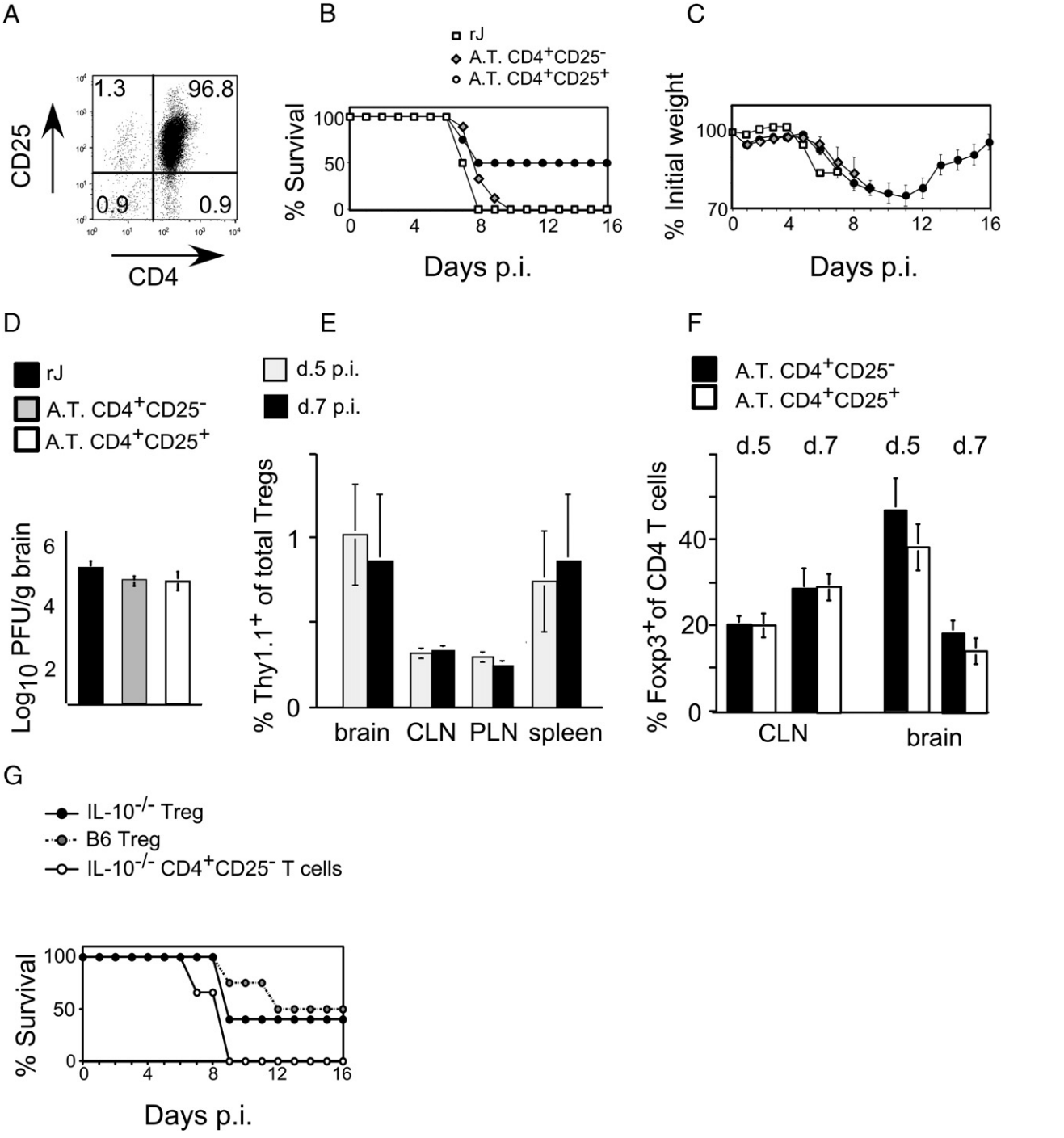

Fig. 5. Increased survival after adoptive transfer of Tregs into rJ-infected mice. (A) $CD4^+CD25^+$ T cells isolated from naive B6 spleens as described in Materials and methods were 95–98% pure as assessed by flow cytometry. (B) 3.7×10^5 $CD4^+CD25^+$ T cells were adoptively transferred into mice at day 1 after infection with rJ. As a control, rJ-infected mice received $CD4^+CD25^-$ cells or PBS. Mice were monitored for survival (B) and weight loss (C). Three independent experiments were performed, $n=10$ mice/group. Mice receiving $CD4^+CD25^+$ T cells survived at a higher frequency than those receiving $CD4^+CD25^-$ cells or PBS ($p<0.05$). (D) Virus titers were the same in rJ-infected recipients of $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells or PBS at day 7 p.i. Four mice were analyzed in each group. (E) Tregs were purified from the spleens of naive Thy1.1 mice and transferred into Thy1.2 B6 mice 1 day after infection with rJ. Mice were sacrificed at day 5 or 7 p.i. and Tregs from brains, spleens, CLN and peripheral (PLN) lymph nodes were analyzed for Thy1.1/1.2 expression. A total of five mice were analyzed at each day p.i. in two independent experiments. (F) Brains and CLN were harvested at days 5 and 7 p.i. from rJ-infected mice that received $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells. $Foxp3^+$ CD4 T cells were determined by flow cytometry. (G) IL-$10^{-/-}$ ($n=5$) or B6 Tregs ($n=4$) or $CD4^+$ $CD25^-$IL-$10^{-/-}$ T cells ($n=3$) were transferred into B6 mice at day 1 p.i. Two independent experiments were performed. Mice were monitored for survival. A.T.—adoptive transfer.

Adoptive transfer of 3.7×10^5 Tregs increased survival of rJ-infected mice from 0% to 50%, but had no significant effect on weight loss (Figs. 5B,C). Transfer of the same number of $CD4^+CD25^-$ T cells did not prolong survival and had no effect on weight loss (Figs. 5B,C). In other experiments, we observed that transfer of as few as 10^5 Tregs to rJ-infected mice also resulted in protection (50% survival) but recovery was prolonged. In some instances, such as in mice with EAE (Izcue et al., 2006; Zhang et al., 2004), Treg mediate immunosuppression via expression of IL-10. However, adoptively transferred IL-$10^{-/-}$ Tregs were as effective in protecting mice from death in rJ-infected mice as were B6 Tregs (Fig. 5G).

Although these results showed that transferred Tregs prolonged survival, we were unable to demonstrate any differences in virus titers or in the virus-specific T cell immune response when we compared recipients of Tregs and $CD4^+CD25^-$ T cells. Initially, we measured virus titers in the brain at day 7 p.i. in mice that received Tregs, $CD4^+CD25^-$ T cells or no transferred cells, to determine if Tregs delayed virus clearance. However, as shown in Fig. 5D, adoptive transfer of Tregs did not change virus titers at day 7 p.i. Mice that did not receive Tregs died by days 7–8 p.i. precluding measurement of titers at later times. Of note, infectious virus was completely cleared by day 18 p.i. from surviving Treg-treated mice.

Next, to begin to assess whether the transferred Tregs suppressed the anti-virus T cell response, we determined their site of localization. Preferential accumulation in the CLN would suggest an effect during priming or accumulation in the brain would be consistent with a role in modulating T cell function during the effector stage. To distinguish transferred Tregs from endogenous cells, we transferred Thy1.1 Tregs into B6 (Thy1.2) mice. Unexpectedly, given the clinical effect of the transferred Tregs, only a low percentage of Tregs at any site examined (brain, CLN, peripheral lymph nodes, spleen) were donor in origin (Fig. 5E). This percentage was not increased in the brain or CLN compared to the spleen or more distant lymph nodes. In other studies, transferred Tregs, even if present in low numbers, were able to induce the proliferation or retention of endogenous Tregs (Tarbell et al., 2007). However, we detected no difference in the percentage of Tregs in the brain or CLN of mice that received Tregs when compared to those that received $CD4^+CD25^-$ T cells (Fig. 5F).

As these initial assays did not provide insight into the mode of action of the transferred Tregs, we next assessed whether these cells modified the virus-specific T cell response in the infected brain. However we detected only marginally lower numbers of S510-specific CD8 and M133-specific CD4 T cells in recipients of $CD4^+CD25^+$ cells (Table 2); the difference in percentage of S510-specific CD8 T cells in Treg recipients (7.7 ± 1.4) compared to those receiving $CD4^+CD25^-$ T cells (12.2 ± 1.6) nearly reached statistical significance ($p=0.06$) while there were no differences in percentages of M133-specific CD4 T cells. Only very low frequencies of JHMV-specific CD8 and CD4 T cells are detectable in the CLN (Haring et al., 2001) and we detected no differences in these numbers when mice that received Tregs or $CD4^+CD25^-$ cells were compared (data not shown).

Discussion

This study supports the notion that the outcome in coronavirus-induced encephalitis results from a balance between pro-inflammatory modalities required for virus clearance and anti-inflammatory factors, such as Tregs, necessary to prevent an excessive, deleterious host immune response. This response does not necessitate a numerical superiority of Tregs to virus-specific effector T cells, but rather the presence of sufficient numbers of both cell types and of a balanced cytokine environment, which cooperate to clear virus and minimize tissue destruction. The importance of this balance was illustrated when rJ and rJ.M_{Y135Q}-infected mice were compared. Both rJ and rJ.M_{Y135Q} grew equally well in tissue culture cells and to similar titers in B6 mice, with equivalent kinetics of clearance (Anghelina et al., 2006). However, differences in the balance between virus-specific effector CD4 T cells and Tregs correlated with dramatic differences in survival and depletion or transfer of Tregs also influenced the outcome. Greater numbers of virus-specific effector CD4 T cells (Fig. 2), associated with increased levels of several pro-inflammatory mediators (Fig. 1), were detected in the brains of rJ compared to rJ.M_{Y135Q}-infected mice (Anghelina et al., 2006) and likely contributed to more severe disease and a uniformly fatal outcome. IL-6 may be especially important in contributing to a fatal outcome because this cytokine, in conjunction with TNF, is able to inhibit Treg function even when Tregs are present in substantial numbers in the inflamed brain (Korn et al., 2007). In the presence of fewer virus-specific CD4 T cells, as occurred in rJ.M_{Y135Q}-mice, the effect of anti-inflammatory factors such as Tregs was dominant. Thus, a nearly ideal balance is present in rJ.M_{Y135Q}-infected mice, in which clearance of a virulent virus occurs with minimal clinical disease.

Whether Tregs function primarily during T cell priming or in the infected brain during the effector stage of the inflammatory process requires further clarification. Differences in numbers of Tregs in the brains but not the CLN of rJ and rJ.M_{Y135Q}-infected mice suggest that these cells function during the effector stage in ameliorating disease. At the peak of the infection, there were approximately 10,000 and 20,000 Tregs in the rJ.M_{Y135Q} and rJ-infected brain, respectively. While this represents only about 5–15% of all CD4 T cells, these percentages are consistent with those observed in other inflammatory settings in the brain (Korn et al., 2007; O'Connor et al., 2007). The mechanism of Treg-mediated immunosuppression in the rJ.M_{Y135Q}-infected brain is not known, but may involve production of molecules such as TGF-β or IL-35 as occurs in other experimental settings (Vignali et al., 2008); our data suggest that Tregs do not function via IL-10 expression (Fig. 5G).

We could not determine whether adoptively transferred Tregs functioned in the brain or CLN. While these transferred cells increased survival, they did not delay virus clearance or significantly modify the anti-virus T cell response, as measured by IFN-γ expression. Several explanations are possible for this inability to demonstrate a mechanism of action of the transferred Tregs. First, since mice that do not receive transferred Tregs uniformly die by days 7–8 p.i., all assays must be performed at this time. However, at this time p.i., mice that will survive after Treg transfer cannot be distinguished from those that will not, thus potentially obscuring any effects of the transferred Tregs. Second, as shown in Fig. 4, only 50% of Treg recipients survive and there are no significant differences in weight loss between recipients of Tregs as opposed to those mice that receive $CD4^+CD25^-$ cells. Thus the Tregs play an important role in enhancing survival, but all mice, whether they survive or not, have severe disease. Other factors with anti-inflammatory activity, such as IL-10, may also function sub-optimally in rJ-infected mice and enhancing their function, in conjunction with the transfer of Tregs, may contribute to an improved clinical course and more obvious changes in the

Table 2
Ag specificity of CD4 and CD8 T cells in rJ-infected recipients of transferred cells

Donor cells	No. analyzed	CD8			CD4		
		% CD8	% S510/CD8 (% irrelevant peptide)	No. S510	% CD4	% M133/CD4 (% no peptide)	No. M133
$CD4^+CD25^+$	7	12.7 ± 2.7	$7.7\pm1.4(2.4\pm0.3)$	$1.4\times10^4\pm0.5\times10^4$	1.7 ± 0.2	$19.1\pm2.4(4.4\pm0.7)$	$4.2\times10^3\pm0.8\times10^3$
$CD4^+CD25^-$	9	11.1 ± 1.5	$12.2\pm1.6(3.6\pm0.5)$	$1.8\times10^4\pm0.4\times10^4$	1.9 ± 0.2	$18.7\pm1.3(1.9\pm0.2)$	$5.5\times10^3\pm1.1\times10^3$

immune response. Consistent with this possibility, infection of IL-10$^{-/-}$ mice with an attenuated variant of JHMV (Lin et al., 1998) or rJ.M$_{Y135Q}$ (data not shown) resulted in increased mortality and weight loss, showing that IL-10 is required for optimal disease outcome. Our results show, however, that Treg expression of IL-10 is not required for the protective effects of transferred cells in rJ-infected mice (Fig. 5G), suggesting that IL-10 must be produced by another type of cell in infected mice. In other infections, macrophages, dendritic cells and CD4$^+$Foxp3$^-$ T cells have all been shown to express IL-10 (reviewed in Couper et al., 2008). Third, Tregs function by cell-to-cell contact with either dendritic cells at sites of priming or with effector T cells or other cells in sites of inflammation (Tadokoro et al., 2006; Tang et al., 2006; Vignali et al., 2008). It is possible that the transferred Tregs function to subtly change the immune response in a localized manner without globally affecting total numbers of virus-specific T cells.

The importance of virus-specificity of Tregs in JHMV-infected mice is not yet known. Antigen-specific Tregs more effectively suppress the response of T effector cells to cognate antigen than non-antigen-specific Tregs (Tang and Bluestone, 2006; Tarbell et al., 2007), but our results show that very few virus-specific Tregs are present in the infected brain (Fig. 3). It is also likely that the effects mediated by transferred natural Tregs in rJ-infected mice do not require virus-specificity, since transfer of a relatively low number (3.7×10^5 cells, Fig. 5) reduced mortality. Only a few JHMV-specific naive Tregs should be present in this number of cells, suggesting that if antigen specificity is required for Treg function, virus-specific Tregs would need to expand rapidly in order to dampen the pro-inflammatory immune response. Treg numbers could also be increased by peripheral conversion of CD25$^-$CD4$^+$ T cells to Tregs but the extent to which peripheral conversion occurs within the brain is controversial. While Liu et al. reported that neurons are able to induce conversion of effector CD4 T cells to CD25$^+$TGFβ1$^+$CTL4$^+$Foxp3$^+$ cells in the context of EAE (Liu et al., 2006), others have found no evidence for such conversion in the same setting (Korn et al., 2007; O'Connor et al., 2007). It will be critical in future studies to determine whether JHMV-specific Tregs are derived from virus-specific effector CD4 T cells.

Our study is one of only a few that address the role of Tregs in acute infectious disease (Luhn et al., 2007; Lund et al., 2008). Similar to our results, a relative deficiency of these cells is present in patients with severe acute dengue infection, when compared to those with mild disease (Luhn et al., 2007). Our data are concordant with studies of SIV-infected African green monkeys (AGM) and macaques. In infected AGMs, Tregs are detected early after infection and dampen the initial pro-inflammatory immune response. Furthermore, AGMs do not develop the chronic T cell activation that is characteristic of the progression to AIDS (Kornfeld et al., 2005). In macaques, which develop AIDS, Tregs appear later and may actually inhibit an effective anti-viral CD8 T cell response (Estes et al., 2006). Collectively, these results suggest that optimization of Treg numbers and function at very early times during the infectious process will diminish the likelihood of an excessive immune response; our results show that this effect may be sufficiently profound to prevent death in an otherwise lethal disease.

Materials and methods

Mice

Specific pathogen-free 5–6 week old B6 mice were purchased from the National Cancer Institute, Bethesda, MD. IL-10$^{-/-}$ and Foxp3-GFP mice were kindly provided by Drs. D. Elliot (University of Iowa) and A. Rudensky (University of Washington), respectively. Mice were inoculated intranasally (i.n.) with $4–8\times10^4$ plaque forming units (PFU) of recombinant JHMV in 12 μL of DMEM and were examined and weighed daily. In all experiments, surviving mice were euthanized 18 days post infection (p.i.). Virus was harvested from the infected brain and titered by plaque assay on HeLa cells expressing the cellular receptor for mouse hepatitis virus (HeLa-MHVR) (Perlman et al., 1987). All animal studies were approved by the University of Iowa Animal Care and Use Committee.

Viruses and cells

Recombinant JHMV was grown in mouse 17Cl-1 cells (a BALB/c-derived fibroblast cell line) and titered on HeLa-MHVR cells as described previously (Perlman et al., 1987).

Multiplex assays

Brain homogenates were prepared in 1.5 ml cell lysis buffer (Bio-Rad, Hercules, CA) containing protease inhibitors. Total protein concentration was determined by Bradford assay. All tissue samples were diluted with cell lysis buffer to a final protein concentration of 500 μg/ml, irradiated and aliquoted. Concentrations of IL-6, IL-1β, IFN-γ, TNF-α, RANTES (CCL5), MCP-1 (CCL2) were determined using a Bio-Plex cytometric bead array, as described (Hulse et al., 2004).

Antibodies and surface and intracellular staining

All antibodies were purchased from BD-Pharmingen (San Diego, CA) unless indicated below. Lymphocytes were prepared from brains as described previously (Anghelina et al., 2006). Briefly, brains were harvested from mice after PBS perfusion and were mechanically homogenized using frosted glass slides. Cells were suspended in 30% Percoll (Pharmacia, Piscataway, NJ) and centrifuged at 800 ×g at 4 °C for 30 min. Percoll and lipid layers were aspirated and the cell pellet was resuspended and counted. The number of lymphocytes harvested from each infected brain ranged from $1–3\times10^6$. For detection of intracellular cytokines, CD8 T cells were stimulated with peptide S510 (spanning residues 510–518 of the S glycoprotein, 1.2 μM) or irrelevant peptide (Ova 257–264). CD4 T cells were stimulated with peptides corresponding to epitopes M133, S358 or S333 used at final concentrations of 5 μM, respectively. Intracellular expression of IFN-γ was detected as previously described (Anghelina et al., 2006).

To detect Tregs, cells harvested from brains, spleens or lymph nodes were stained for the transcription factor Foxp3 as per the manufacturer. Briefly, cells were stained with anti-CD4-FITC or -PerCP (clone GK1.5) and anti-CD25-PE-Cy7 (clone PC61) mAbs. After permeabilization and fixation, cells were stained with either anti-Foxp3-PE or FITC (clone FJK-16s) or isotype control Rat IgG2a-PE or FITC (clone eBR2a) mAb, both purchased from eBiosciences (San Diego, CA). For phenotypic analysis of Tregs, cells were surface stained with the following mAbs: anti-GITR-FITC (clone DTA-1), anti-CD103-PE (clone 2E7), anti-CD44-PE (clone IM7), anti-CD69-PE (clone H1.2F3), anti-CD62L-FITC (clone MEL-14) or anti-CD25-PE (clone 7D4). After staining, washing and fixation, cells were analyzed using a FACScan or LSR Flow Cytometer (BD Biosciences, Mountain View, CA).

In some experiments, epitope M133-specific cells were detected using MHC class II/peptide tetramers, obtained from the N.I.H. Tetramer Facility, Atlanta, GA. Cells were stained with 8 μg/ml tetramer for 2–3 h at 37 °C.

Purification of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells

CD4$^+$CD25$^+$ Tregs were purified using an autoMACS Separator and a CD4$^+$CD25$^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. Briefly, lymphocytes from spleens or lymph nodes were enriched for CD4 T cells by depletion with a cocktail of lineage-specific biotin-conjugated antibodies against CD8 (Ly-2), CD11b (Mac-1), CD45R (B220), CD49b (DX5), Ter-119 and anti-biotin microbeads, followed by positive selection with anti-CD25-PE mAb and anti-PE microbeads.

The $CD4^+CD25^-$ T cell population was subsequently depleted of residual $CD4^+CD25^+$ cells using anti-CD25-PE mAb and anti-PE microbeads. Cells were examined for purity by flow cytometry and were routinely found to be 96–98% pure.

In vitro CD4 and CD8 T cell proliferation assays

$CD4^+CD25^-$ and CD8 T cells were purified from naive mouse lymph nodes, labeled with freshly prepared CFSE (2 μM, Molecular Probes, Carlsbad, CA) for 10 min at 37 °C, washed 3 times with RP10 media and cultured in 96-well U-bottom plates (5×10^4 cells/well) in the presence of 1 μg/ml anti-CD3 (clone 145-2C11; eBioscience) and 2×10^5 irradiated splenocytes (2500 Gy). Bead-purified $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells from naive or $rJ.M_{Y135Q}$-infected B6 cervical lymph nodes (CLN) were added at ratios of 0.3:1, 1:1 and 3:1 in triplicate. Plates were incubated at 37 °C for 72 h. Cells were harvested and analyzed on a FACScan Flow Cytometer. Data were processed using FlowJo software version 6.3.4 (Tree Star, Ashland, OR).

Adoptive transfer of $CD4^+CD25^+$ T cells

$CD4^+CD25^+$ T cells (1 or 3.75×10^5 cells in 0.5 ml PBS/mouse) were purified from naive spleens and adoptively transferred by intravenous inoculation into 5–6 week old B6 mice one day after infection with rJ. Control rJ-infected mice received the same numbers of $CD4^+CD25^-$ T cells or PBS. Mice were monitored for mortality and weight loss. T cell responses and virus titers were analyzed at 5 and 7 days p.i.

In vivo depletions

To deplete $CD25^+$ cells, a single intraperitoneal injection of 0.5 mg of rat mAb PC61 (American Type Culture Collection, Manassas, VA, prepared by the University of Iowa hybridoma facility) or control rat IgG (Jackson Immunoresearch, West Grove, PA) was administered three days prior to infection with $rJ.M_{Y135Q}$.

Statistics

Two-tailed unpaired Student's *t* tests were used to analyze differences in mean values between groups. All results are expressed as means ± standard errors of the means (SEM). *p* values of <0.05 were considered statistically significant.

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