The Novel $CD4^+CD25^+$ Regulatory T Cell Effector Molecule Fibrinogen-like Protein 2 Contributes to the Outcome of Murine Fulminant Viral Hepatitis

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Fulminant viral hepatitis (FH) remains an important clinical problem in which the underlying pathogenesis is not well understood. Here, we present insight into the immunological mechanisms involved in FH caused by murine hepatitis virus strain 3 (MHV-3), indicating a critical role for $CD4^+CD25^+$ regulatory T cells (Tregs) and production of the novel Treg effector molecule FGL2. Before infection with MHV-3, susceptible BALB/cJ mice had increased numbers of Tregs and expression of *fgl2* messenger RNA (mRNA) and FGL2 protein compared with resistant A/J mice. After MHV-3 infection, plasma levels of FGL2 in BALB/cJ mice were significantly increased, correlating with increased percentage of Tregs. Treatment with anti-FGL2 antibody completely inhibited Treg activity and protected susceptible BALB/cJ mice against MHV-3-liver injury and mortality. Adoptive transfer of wild-type Tregs into resistant $fgl2^{-/-}$ mice increased their mortality caused by MHV-3 infection, whereas transfer of peritoneal exudate macrophages had no adverse effect. *Conclusion:* This study demonstrates that FGL2 is an important effector cytokine of Tregs that contributes to susceptibility to MHV-3–induced FH. The results further suggest that targeting FGL2 may lead to the development of novel treatment approaches for acute viral hepatitis infection. (HEPATOLOGY 2009;49:387-397.)

Naturally occurring $CD4^+CD25^+$ regulatory T cells (Tregs) have been demonstrated to play an important role in maintenance of peripheral self-tolerance.[1] Depletion or functional alteration of Tregs in mice results in the development of autoimmune disease.[1] In athymic nude mice, transfer of syngeneic splenic cells depleted of Tregs produces autoimmune disease that is preventable by the co-transfer of small numbers of Tregs.[1] In addition to their role in the control of self-tolerance, Tregs are also involved in regulation of T cell homeostasis, modulation of immune responses to cancer, pathogens, and alloantigens, as well as the prevention of allograft rejection.[1]

Tregs have been implicated in suppressing T cell immune responses in viral and bacterial infections.[2] Patients with chronic hepatitis B (HBV) and hepatitis C virus infection have increased numbers of Tregs, which impair immune responses against HBV and hepatitis C virus, thus leading to viral persistence and chronic infection.[3,4] It has been shown that circulating and liver-resident Tregs actively influence the antiviral immune response and disease progression in patients with HBV.[5] Moreover, depletion of Tregs in mice results in enhancement of HBV-specific $CD8^+$ T cell responses primed by DNA immunization.[6] The role of Tregs in acute viral hepatitis, however, has not been reported.

Fibrinogen-like protein 2 (FGL2), a member of the fibrinogen-related superfamily of proteins known to be

Abbreviations: ANOVA, analysis of variance; APC, antigen-presenting cell; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; FGL2, fibrinogen-like protein 2; FH, fulminant viral hepatitis; HBV, hepatitis B virus; HE, hematoxylin-eosin; IgG, immunoglobulin G; IL, interleukin; MHV-3, murine hepatitis virus strain 3; mRNA, messenger RNA; PEM, peritoneal-exudate macrophages; PFU, plaque-forming units; SEM, standard error of the mean; TGF-β, transforming growth factor beta; Tregs, $CD4^+CD25^+$ regulatory T cells;

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secreted by T cells,[7,8] has recently been reported by a number of groups to be highly expressed by Tregs and has been proposed to have a role in Treg effector function.[9-11] In this study, we addressed the contribution of Treg-expressed FGL2 to the pathogenesis of murine hepatitis virus strain 3 (MHV-3)–induced murine fulminant viral hepatitis (FH). The results show that the frequency of Tregs and Tregs expression of *fgl2* messenger RNA (mRNA) and FGL2 protein are higher in lymphoid tissues of uninfected BALB/cJ mice compared with uninfected A/J mice. After MHV-3 infection, Tregs were increased, correlating with elevated levels of FGL2 in the plasma of susceptible BALB/cJ mice. Treatment with FGL2 antibody blocked Treg activity *in vitro* and protected against MHV-3–induced liver injury and mortality *in vivo*. Adoptive transfer of wild-type Tregs to resistant $fgl2^{-/-}$ mice resulted in increased mortality after MHV-3 infection. Collectively, the results support the concept that Tregs and the effector cytokine FGL2 are logical targets for molecular manipulation in the development of novel treatment approaches for patients with viral hepatitis.

Materials and Methods

Mice. Female BALB/cJ and A/J mice aged 6 to 8 weeks (Jackson Laboratories) were maintained in micro isolator cages and housed in the animal colony at the Toronto General Hospital, University of Toronto, and fed standard laboratory chow diet and water *ad libitum*. The Animal Welfare Committee approved all protocols.

Virus. MHV-3 was obtained from the American Type Culture Collection, Manassas, VA. It was first plaque-purified and then expanded in murine 17CL1 cells to a concentration of 1×10^7/mL. Virus-containing supernatants were collected and subsequently stored at −80°C until use. Mice were infected with 100 plaque-forming units (PFU) by the intraperitoneal route.

Viral Infection and Treatment with Anti-FGL2 Antibody. Mice received an intraperitoneal injection of 100 PFU MHV-3 and were monitored daily for symptoms of disease, including ruffled fur, tremors, and lack of activity. Additionally, BALB/cJ mice were treated with 25, 50, or 100 μg of an immunoglobulin G (IgG) 2a monoclonal anti-FGL2 antibody (1F4.2) or an isotype control antibody daily for 7 days pre-MHV-3 infection and 7 days post-MHV-3 infection by tail vein injection.

Viral Titers. Livers were removed aseptically, cut into small pieces, and homogenized in 10% ice-cold Dulbecco's modified Eagle's medium, using a Polytron homogenizer (Fisher Scientific, Whitby, Ontario, Canada). Viral titers were determined as described previously.[12]

Isolation of Tregs. $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ T cells were purified from spleens and lymph nodes with either the EasySep Mouse $CD4^+$ T Cell Enrichment Kit (StemCell Technologies Inc.) or with the magnetic-activated cell sorting system (Miltenyi Biotech Inc., Auburn, CA) according to the manufacturers' instructions followed by fluorescence-activated cell sorting using a FACSAria cell sorter (Becton Dickinson, CA). Purity of cell populations was greater than 95% as assessed by flow cytometry.

Tissue Isolation. Mice were sacrificed on days 0, 1, 2, 3, and 8 (only A/J mice) post-MHV-3 infection. Blood was collected via cardiac puncture, and serum was stored at −80°C. Livers were collected for histology and immunohistochemistry.[13] To quantify the effect of anti-FGL2 antibody on liver histology, a digitalized image analysis system (HP-88; Hewlett Packard Co. Ltd., Mississauga, Ontario) was used.[14] The areas of necrosis were encircled, yielding a percentage representing the proportion of diseased liver in that particular section. For each animal, three random sections were assayed in this fashion and the mean ± standard error of the mean (SEM) was calculated. Frozen tissue was embedded in optimal cutting temperature (Fisher)-filled cryomolds (TissueTek), snap-frozen in liquid nitrogen and stored at −80°C for immunohistochemistry.

Flow Cytometry. Cell suspensions obtained from spleen, lymph nodes (inguinal, axillary, mesenteric, and cervical), and thymus were washed and suspended in phosphate-buffered saline containing 5% mouse serum (Cedarlane) at a final concentration of 1×10^7cells/mL to block Fc receptors. After staining of the cell membrane with anti-CD4 fluorescein isothiocyanate, cells were fixed and permeabilized for intracellular staining with anti-Foxp3 phycoerythrin and anti-rat IgG2a-phycoerythrin isotype control (eBiosciences) according to the manufacturer's instructions. Cell staining was assessed using a Coulter FC500 flow cytometer, and data were analyzed using CXP/RXP software (Beckman Coulter). Live cells were gated according to forward and side scatter parameters.

Sandwich Enzyme-Linked Immunosorbent Assay. Plates were coated and incubated overnight with 2 μg/mL monoclonal anti-FGL2 (6H12) (IgG1) as a capture antibody. Plasma samples (50 μL) were added to each well, and after a 1-hour incubation at 37°C and three washes with Tris-Tween buffered saline, the wells were incubated with 2 μg/mL polyclonal rabbit anti-FGL2 antibody[15] for 2 hours at 37°C. The plate was washed again, and polyclonal anti-FGL2 binding was detected with a secondary horseradish peroxidase–conjugated anti-rabbit antibody. Tetramethylbenzidine was then added and ab-

sorbance was measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader.

Real-time Polymerase Chain Reaction. After the purification of splenic $CD4^+CD25^+$ Tregs from either A/J or BALB/cJ mice by fluorescence-activated cell sorting, total RNA was extracted from the cells, and the levels of *fgl2* mRNA expression were measured by real-time polymerase chain reaction. The levels of *fgl2* were normalized to the housekeeping genes hypoxanthine-guanine phosphoribosyl transferase, glyceraldehyde 3-phosphate dehydrogenase, and ribosomal protein L13. Expression of *fgl2* mRNA in $CD4^+CD25^+$ Tregs from BALB/cJ mice relative to $CD4^+CD25^+$ Tregs from A/J mice was calculated by the $2^{-\Delta\Delta CT}$ method and corrected for the absolute numbers of $CD4^+CD25^+$ Tregs in each strain.

Immunohistochemical Staining for Infiltrating Tregs in Liver Tissue. Infiltrating cells in liver tissue of A/J and BALB/cJ mice were characterized both before - and after MHV-3 infection by indirect immunohistochemistry using purified anti-Foxp3 antibody or isotype negative control antibody and a secondary horseradish peroxidase–conjugated anti-rat antibody according to the manufacturer's instructions (eBioscience).

Suppression Assay. Cultures were set up consisting of 4×10^4/well $CD4^+CD25^-$ T cells (responder) and $CD4^+CD25^+$ Tregs (suppressor) at different suppressor: responder ratios in the presence of 2×10^5/well irradiated syngeneic antigen-presenting cells (APC) and anti-CD3 antibody (0.5 μg/mL). Monoclonal antibody to FGL2 (10 μg/mL) (Abnova, Taiwan) or isotype-negative control antibody (10 μg/mL) were added to these cultures, which were then cultured for 96 hours and proliferation determined by incorporation of ^{3}H-thymidine.

Adoptive Transfer Studies. $Fgl2^{-/-}$ mice (n = 15/group) were infused with 120×10^3 to 300×10^3 purified wild-type Tregs by intravascular injection 1 hour before MHV-3 infection. $Fgl2^{-/-}$ mice (n = 10/group) were injected intraperitoneally with 5×10^6 wild-type peritoneal-exudate macrophages (PEMs) or 5×10^6 wild-type splenocytes. $Fgl2^{-/-}$ mice (n = 20/group) that did not receive infusions of Tregs and $fgl2^{+/+}$ mice (n = 10/group) were used as controls. Survival rate and hematoxylin-eosin (HE) staining of livers from infected mice were analyzed to assess the effect of Treg-expressed FGL2 on the survival and severity of MHV-3 disease.

Statistical Analysis. Results are reported as mean ± SEM unless otherwise specified. One-way or two-way analysis of variance (ANOVA) followed by the Bonferroni test for post-hoc analysis were used for group comparison. Rates of animal survival were calculated using the Kaplan-Meier method and compared between groups with the log rank test. Differences with $P \leq 0.05$ were considered significant.

Results

Increased Numbers and Percentage of Tregs in Uninfected BALB/cJ Mice Compared with A/J Mice. The percentages of Tregs in the lymphoid organs of uninfected BALB/cJ and A/J mice were compared based on cells co-expressing CD4 and Foxp3 identified by flow cytometry. Spleens of BALB/cJ mice contained a 1.6-fold increase in the proportion of Tregs compared with A/J mice (3.3% compared with 2%, respectively). The proportions of Tregs (5.9%) found in inguinal, axillary, mesenteric, and cervical lymph nodes of uninfected BALB/cJ mice were higher than that of A/J mice (5.1%). Naturally occurring Tregs composed 0.71% of the total thymic cell population in BALB/cJ mice, whereas only 0.29% Tregs were present in A/J thymus (Fig. 1A, B). The increased proportion of Tregs found in lymphoid tissues of BALB/cJ mice reflected an increase in the absolute numbers of Tregs in BALB/cJ mice compared with A/J mice (Fig. 1C).

Increased Levels of FGL2 by Tregs in the Plasma of Uninfected BALB/cJ Mice Compared with A/J Mice. The expression of *fgl2* mRNA and FGL2 protein by Tregs and plasma FGL2 levels in BALB/cJ and A/J mice were first compared. Tregs from BALB/cJ mice had a 1.6-fold higher level of *fgl2* mRNA compared with A/J mice after normalization to reference gene expression (Fig. 2A). By ELISA, a fourfold increase in levels of FGL2 was detected in cell culture media of Tregs isolated from BALB/cJ mice compared with Tregs isolated from A/J mice (Fig. 2B).

Consistent with the higher *fgl2* mRNA and FGL2 protein expression by BALB/cJ Tregs, we detected 124 ± 36 ng/mL FGL2 in the plasma of BALB/cJ mice compared with 79 ± 17 ng/mL in A/J mice at baseline before infection with MHV-3 (Fig. 2C).

Increased Percentage of Tregs in BALB/cJ Mice Compared with A/J Mice After MHV-3 Infection. BALB/cJ and A/J mice were infected with 100 PFU MHV-3 intraperitoneally, sacrificed on days 1, 2, or 3 after infection and on day 8 after infection. All remaining A/J mice were sacrificed, and the proportion of Tregs in their lymphoid organs was assessed by flow cytometry. At all time points after infection, the percentage of Tregs was higher in spleens and lymph nodes of BALB/cJ mice compared with A/J mice (Fig. 3A). However, the percentage of Tregs in spleens and lymph nodes of both strains decreased after MHV-3 infection. After MHV-3 infection, a marked increase in the percentage of Tregs in the thymus

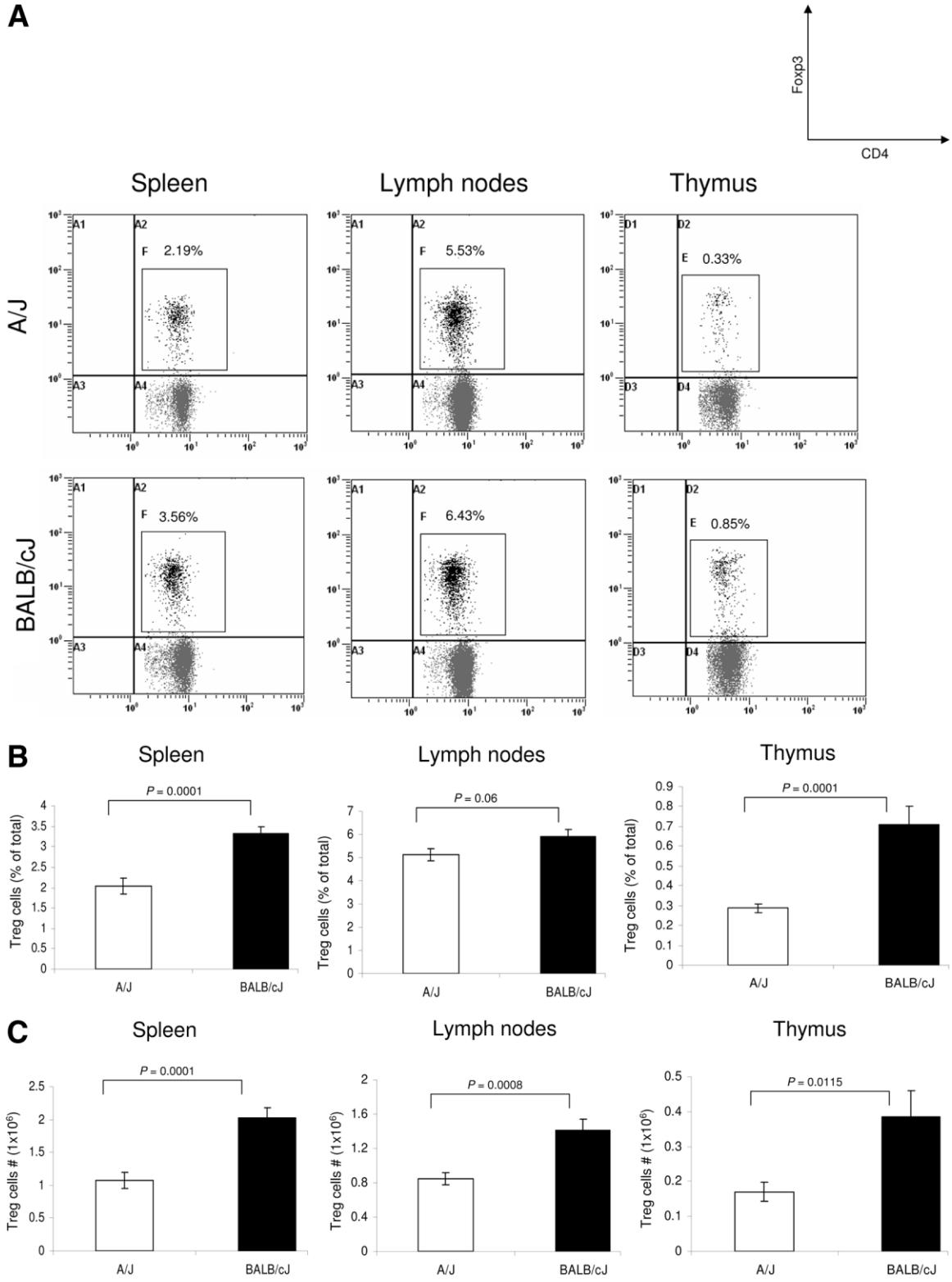

Fig. 1. Increased numbers and percentage of Tregs in uninfected BALB/cJ mice compared with A/J mice. (A) Representative flow cytometry plots displaying Treg proportions in the lymphoid tissues of uninfected BALB/cJ (bottom) and A/J mice (top) based on CD4 and Foxp3 co-expression. For thymic cells, the Treg population was analyzed by gating on the $CD4^+CD8^-$ population. (B) Graphs show the % mean $\pm$ SEM of Treg proportions in the lymphoid tissues of uninfected BALB/cJ and A/J mice. (C) Graphs show the mean $\pm$ SEM of absolute numbers of Tregs in the lymphoid tissues of uninfected BALB/cJ and A/J mice. Data were collected from three independent experiments of three to four mice in each group. Comparison between groups was performed using a one-way ANOVA for statistical analysis.

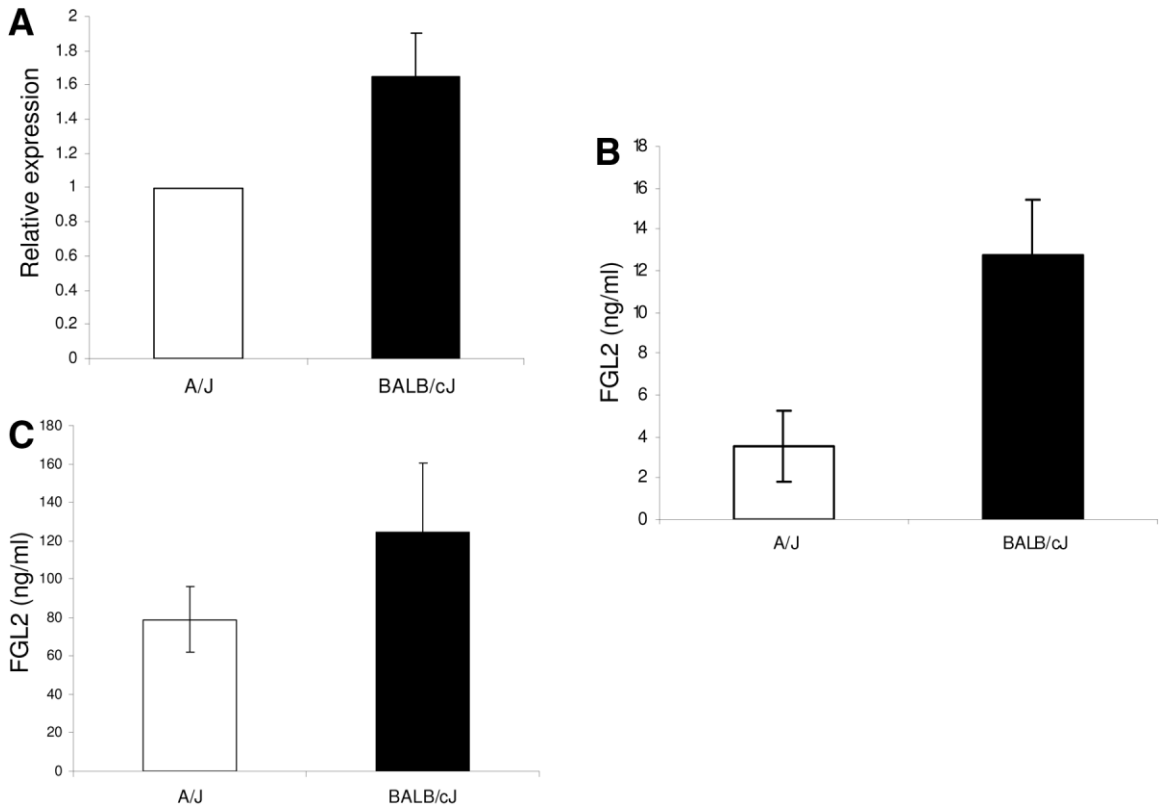

Fig. 2. Increased levels of FGL2 by Tregs in the plasma of uninfected BALB/cJ mice compared with A/J mice. (A) Calculated levels of *fgl2* mRNA expression in $CD4^+CD25^+$ Tregs of BALB/cJ mice relative to $CD4^+CD25^+$ Tregs of A/J mice as measured by real-time polymerase chain reaction; n = 3. (B) FGL2 levels in culture supernatants of freshly isolated Tregs from BALB/cJ or A/J mice that were cultured for 4 days as measured by ELISA. Data show the mean ± SEM of three independent experiments of five mice in each group. (C) Graph shows the mean ± SEM of the levels of plasma FGL2 in A/J and BALB/cJ mice as determined by ELISA; n = 15. Comparison between groups was performed using a one-way ANOVA for statistical analysis.

of BALB/cJ mice compared with A/J mice was observed (Fig. 3B).

Increased Tregs Infiltration in Livers of BALB/cJ Mice After Viral Infection. After infection, Tregs were undetectable in both mouse strains. On day 1 after MHV-3 infection, livers of BALB/cJ mice had high numbers of $Foxp3^+$ Tregs, whereas Foxp3-staining was negative in livers of MHV-3–infected A/J mice (Fig. 3C, D). However, on days 2 and 3 after infection, small numbers of Tregs were detected in livers of A/J mice, whereas at these time points the livers of BALB/cJ mice were largely necrotic, making it difficult to assess Foxp3 staining (data not shown). MHV-3 was not detected in livers from BALB/cJ mice at 1 day after infection, whereas low viral titers were found in A/J mice [1.3×10^2 (PFU/g)]. On days 2 and 3 after infection, viral titers increased in BALB/cJ mice [day 2, $5.3 \times 10^5 \pm 1.32$ (PFU/g); day 3, $7.2 \times 10^6 \pm 2.63$ (PFU/g)] and in A/J mice [day 2, 2.6×10^3 (PFU/g); day 3, $3 \times 10^3 \pm 1.23$ (PFU/g)].

Levels of FGL2 in the Plasma of BALB/cJ and A/J Mice After Viral Infection. By ELISA, levels of FGL2 in BALB/cJ mice rose significantly after infection, correlating with disease progression. Within 2 days after infection, levels of FGL2 rose from a baseline of 124 ± 36 ng/mL to 443 ± 166 ng/mL, reaching a maximal level of 1589 ± 75 ng/mL on day 3 after infection. MHV-3-resistant A/J mice showed low basal levels of FGL2, which rose to 138 ± 80 ng/mL by day 3 after infection but returned to normal levels by day 8 (Fig. 4).

FGL2 Is an Effector of Treg Function. To evaluate whether FGL2 accounts for the immunosuppressive activity of Tregs as has been suggested,[9,10] a standard *in vitro* suppression assay was performed. Monoclonal antibody to FGL2 (10 μg/mL) or isotype negative control (10 μg/mL) were added to co-cultures of $CD4^+CD25^-$ T cells (responder) and $CD4^+CD25^+$ Tregs (suppressor) at different suppressor:responder ratios in the presence of syngeneic APC and anti-CD3 antibody (0.5 μg/mL). Addition of monoclonal anti-FGL2 completely inhibited Treg activity at all suppressor:responder ratios assessed, whereas the isotype control antibody had no inhibitory effect on Treg function (Fig. 5).

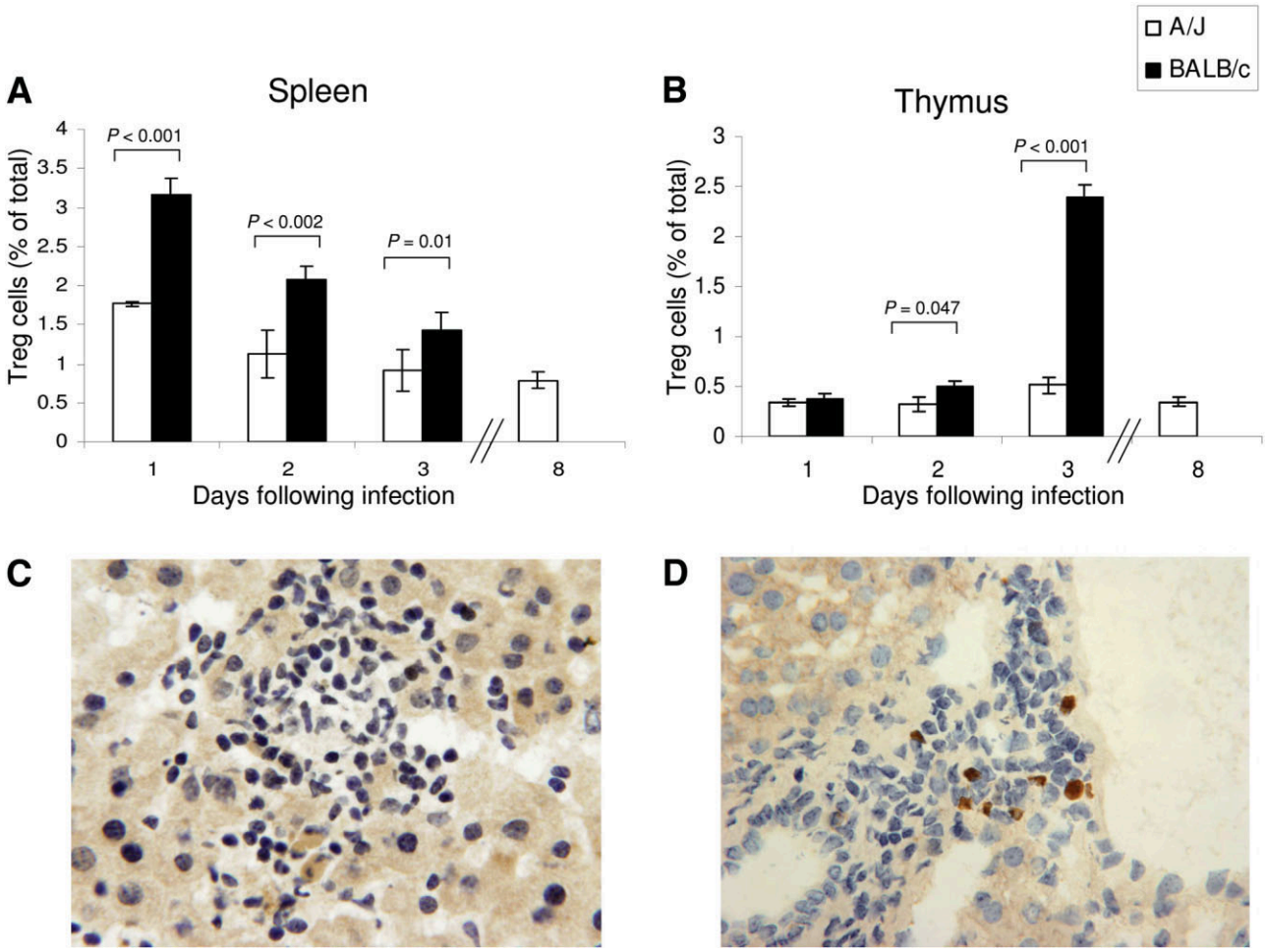

Fig. 3. Increased percentage of Tregs in BALB/cJ mice compared with A/J mice after MHV-3 infection. The percentages of Tregs in the spleen (A) or thymus (B) of BALB/cJ and A/J mice are shown at different time points after viral infection. Graphs represent the mean ± standard deviation from two independent experiments of three to four mice in each group. Increased Treg infiltration in livers of BALB/cJ mice was observed after viral infection. Representative Foxp3$^+$ Treg staining in liver of A/J (C) or BALB/cJ mice (D) at 1 day post-MHV-3 infection (magnification × 400). A two-way ANOVA with a Bonferroni test for post hoc analysis were used to compare means.

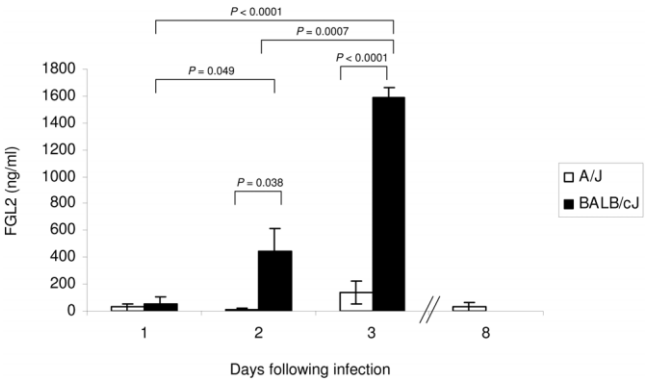

Fig. 4. Levels of FGL2 in the plasma of BALB/cJ and A/J mice after viral infection. Levels of FGL2 protein in the plasma of BALB/cJ or A/J mice are shown at different time points after MHV-3 infection. Data represent mean ± SEM of four to six mice in each group. A two-way ANOVA with a Bonferroni test for post hoc analysis were used to compare means.

Anti-FGL2 Antibody Treatment Prolongs the Survival of BALB/cJ Mice After MHV-3 Infection. To further evaluate the contribution of FGL2 to the pathogenesis of MHV-3–induced liver disease, BALB/cJ mice were infected with MHV-3 and treated with an IgG2a monoclonal antibody to FGL2.[16] Treatment with the monoclonal antibody 1F4.2 daily for 7 days pre-infection and 7 days after infection by tail vein injection markedly reduced hepatic necrosis, inhibited viral replication, and prolonged the survival of MHV-3–infected BALB/cJ mice in a dose-dependent manner. All animals that received 100 μg IF4.2/day survived, whereas untreated mice and animals treated with isotype-negative control antibody died within 2 to 5 days after infection (Fig. 6A). Untreated MHV-3–infected BALB/cJ mice developed histological evidence of liver disease by day 3 after infection (Figs. 6, 7). In contrast, mice infected with MHV-3 but treated with antibody to FGL2 showed a marked

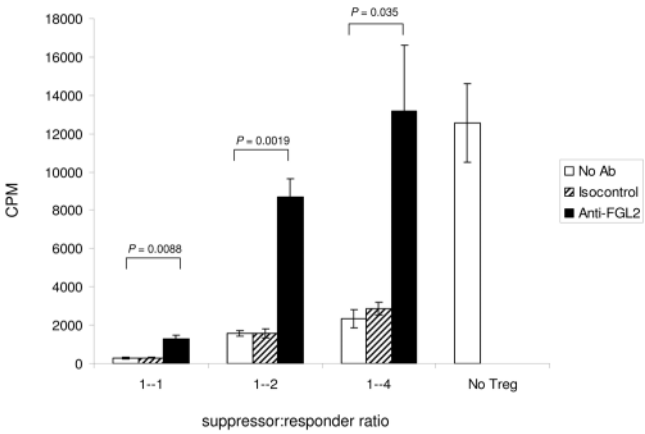

Fig. 5. FGL2 is an effector of Treg function. Monoclonal antibody to FGL2 (10 μg/mL) or isotype-negative control (10 μg/mL) were added to co-cultures of $CD4^+CD25^-$T cells (responder) and $CD4^+CD25^+$ Tregs (suppressor) at different suppressor:responder ratios in the presence of syngeneic APC and anti-CD3 antibody (0.5 μg/mL). Anti-FGL2 antibody significantly blocked the suppressive activity of Tregs at all suppressor: responder ratios. Data represent mean ± SEM of two independent experiments. A two-way ANOVA with a Bonferroni test for post hoc analysis were used to compare means.

reduction in liver necrosis in a dose-dependent fashion (Fig. 7). Morphometric image analysis revealed that the proportion of necrotic liver tissue was significantly different between anti-FGL2–treated and untreated mice after MHV-3 infection (Fig. 6B). Liver histology from all survivors at 10 and 14 days after infection was normal. Coincident with reduced liver necrosis and increased survival, viral titers from livers of infected, IF4.2-treated mice were markedly reduced compared with infected, untreated mice at all time points (Fig. 6C).

Adoptive Transfer of Wild-Type Tregs into Resistant fgl2$^{-/-}$ Mice Increases Mortality to MHV-3 Infection. To directly investigate the contribution of Treg-expressed FGL2 to the outcome of MHV-3 infection, resistant *fgl2*$^{-/-}$ mice were infused with wild-type Tregs and their survival and liver histology examined (Fig. 8). *Fgl2*$^{-/-}$ mice not receiving wild-type Tregs nearly all survived MHV-3 infection (Fig. 8A). However, adoptive transfer of Tregs from *fgl2*$^{+/+}$ mice into *fgl2*$^{-/-}$ mice resulted in increased mortality to MHV-3 infection, at rates

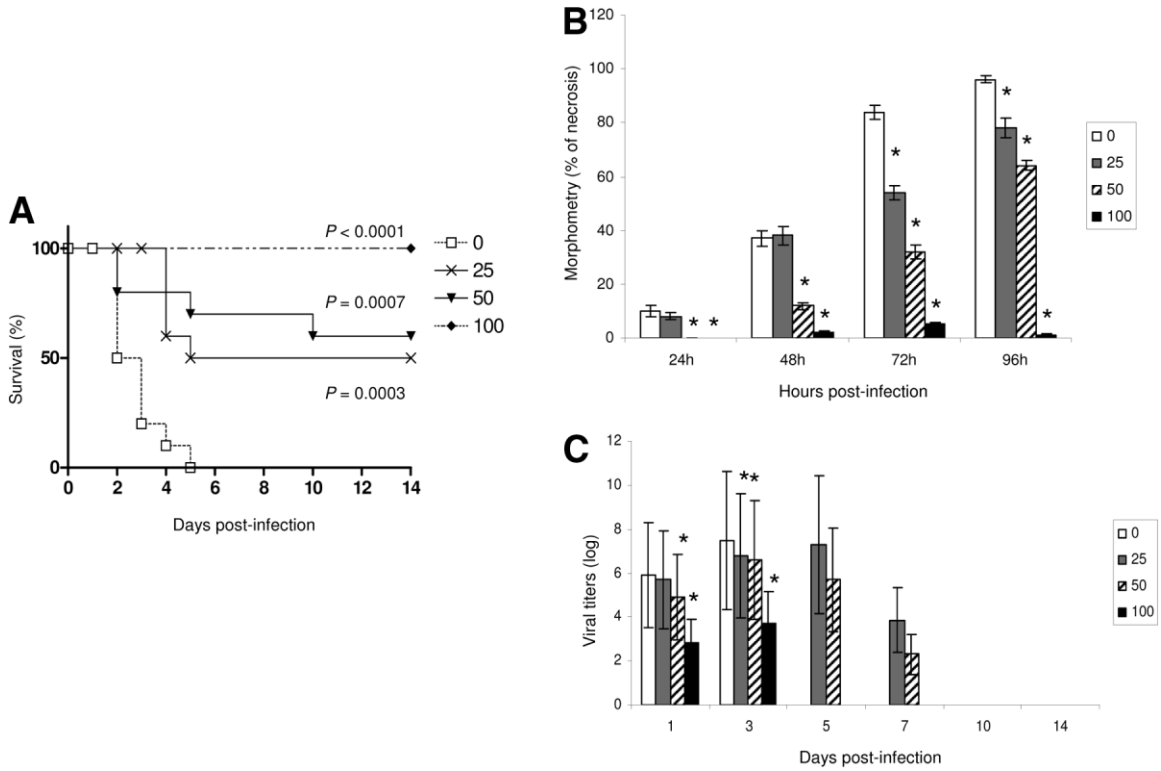

Fig. 6. Effect of monoclonal anti-FGL2 antibody treatment on the course of MHV-3 infection. (A) BALB/cJ mice were either untreated (0) or pretreated with 25, 50, or 100 μg monoclonal anti-FGL2 antibody (1F4.2) for 7 days before infection with 100 PFU MHV-3. Antibody treatment was continued in treated animals for 7 days after infection, and survival was studied (n = 10 mice/group). The graph represents Kaplan-Meier cumulative survival. There was a significant difference in animal survival between the untreated and FGL2-treated groups (log-rank test). (B) Morphometric analysis of liver necrosis after MHV-3 infection. At 24, 48, 72, and 96 hours after infection, a marked difference in the proportion of liver necrosis was seen between untreated and monoclonal antibody IF4.2-treated groups. (C) The effect of treatment with monoclonal antibody on viral replication. High titers of virus were recovered from untreated, MHV-3-infected animals at all time points. In contrast, monoclonal antibody treatment at doses of 25, 50, and 100 μg attenuated the titers of virus recovered from the liver in a dose-dependent manner. Graphs show the mean ± SEM of five mice in each group. The stars in panels B and C indicate the statistical significance of each treatment group compared with untreated MHV-3–infected control mice.

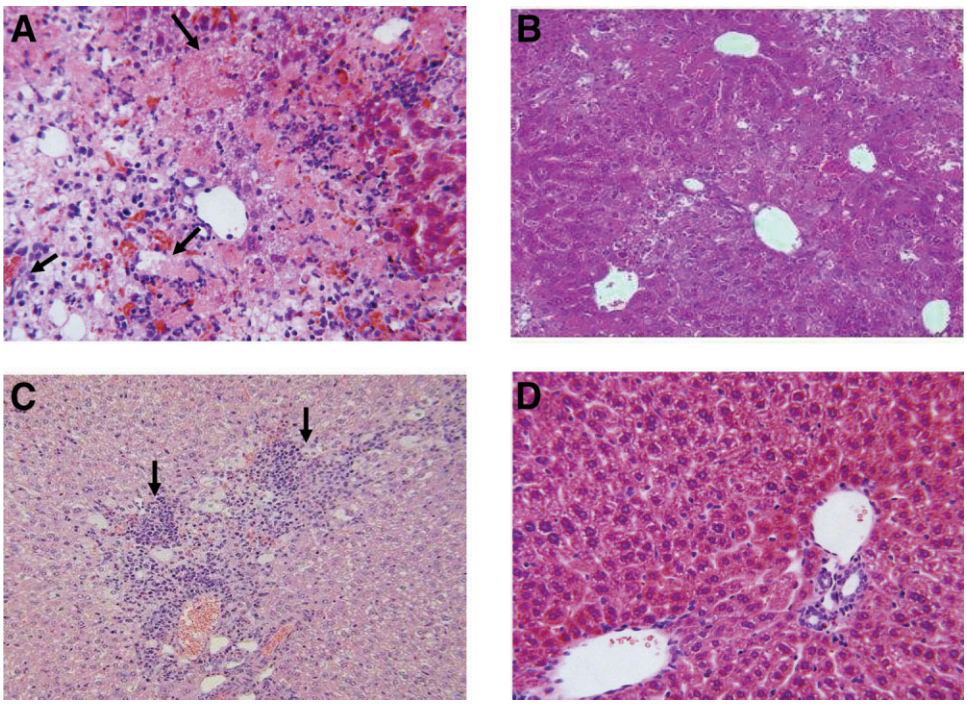

Fig. 7. The effect of monoclonal anti-FGL2 antibody on liver histology after MHV-3 infection. Liver sections from MHV-3–infected and (A) untreated mice showed massive hepatic necrosis with hemorrhage and fibrin deposition on day 4 after infection (arrows). In contrast, in mice that received pre-infection and after-infection treatment (B) with 25 μg antibody, necrosis was markedly reduced; (C) with 50 μg antibody, liver was near normal with only portal inflammatory infiltrates (arrows) and minimal necrosis; and (D) with 100 μg antibody, there was near-normal histology (HE stain: original magnification $\times$225).

similar to those seen in *fgl2*$^{+/+}$ mice. Similarly, infected *fgl2*$^{-/-}$ mice that were reconstituted with splenocytes from *fgl2*$^{+/+}$ mice, which contained Tregs, all died within 3 to 5 days after infection (Fig. 8A). In contrast, *fgl2*$^{-/-}$ mice that were infused with peritoneal-exudate macrophages (PEMs) from *fgl2*$^{+/+}$ mice nearly all survived MHV-3 infection (Fig. 8A). These results support a role for Treg-expressed *fgl2*/FGL2 in the pathogenesis of MHV-3–induced FH. Livers harvested from infected mice before death demonstrated widespread hepatic necrosis associated with marked fibrin deposition in both *fgl2*$^{+/+}$ and *fgl2*$^{-/-}$ mice that had received Tregs, whereas livers were near normal in *fgl2*$^{-/-}$ mice (Fig. 8B). FGL2 was detected in the plasma of *fgl2*$^{-/-}$ mice, which received Tregs from *fgl2*$^{+/+}$ mice (22.26 $\pm$ 2.5 ng/mL), whereas it was undetectable in *fgl2*$^{-/-}$ mice. Levels of alanine transaminase (ALT) increased in the serum of infected-*fgl2*$^{-/-}$ mice that were reconstituted with *fgl2*$^{+/+}$ Tregs compared with *fgl2*$^{-/-}$ mice that were not infused with *fgl2*$^{+/+}$ Tregs (Fig. 8C) and correlated with development of liver disease.

Discussion

In the current study, the contribution of Treg-expressed FGL2 to the pathogenesis of MHV-3-induced FH was examined. Uninfected, susceptible BALB/cJ mice had increased percentage/number of Tregs in lymphoid organs accompanied by increased FGL2 expression compared with resistant A/J mice. After MHV-3 infection, BALB/cJ mice had a significant increase in Tregs and plasma levels of FGL2 versus low levels of plasma FGL2 and Tregs detected in A/J mice. Treatment with antibody to FGL2 increased survival of susceptible mice and adoptive transfer of Tregs from *fgl2*$^{+/+}$ mice into *fgl2*$^{-/-}$ mice resulted in increased mortality to MHV-3 infection. Collectively, these results support the hypothesis that Treg-expressed FGL2 contributes to the pathogenesis of MHV-3–induced FH.

It has been reported that Tregs overexpress a subset of Th2 response genes including interleukin (IL)-10 and transforming growth factor beta (TGF-β),[17] suppressing Th1 responses and resulting in Th2 polarization.[18,19] BALB/cJ mice that are known to exhibit a Th2-phenotype[20] have increased numbers of Tregs compared with A/J mice that predominantly mount Th1 immune responses.[21] Others have shown that BALB/cJ mice display nonprotective Th2 responses and these mice are susceptible to infection with other pathogens, including the intracellular parasite *Leishmania major*, whereas mice that develop a Th1 immune response are protected from infection.[22]

After MHV-3 infection, Tregs increased in the thymus of susceptible BALB/cJ mice, whereas they were reduced in the spleens of both resistant and susceptible mice. We propose that the increase in Tregs in the thymus of BALB/cJ mice is an attempt of susceptible mice to suppress the pro-inflammatory cytokine storm that is known to occur after MHV-3 infection.[21,23] It has been proposed that inflammatory cytokines, including tumor necrosis factor alpha and IL-6, can reduce the numbers of Treg and inhibit Treg function, providing a potential explanation for the findings in the current study.[24,25]

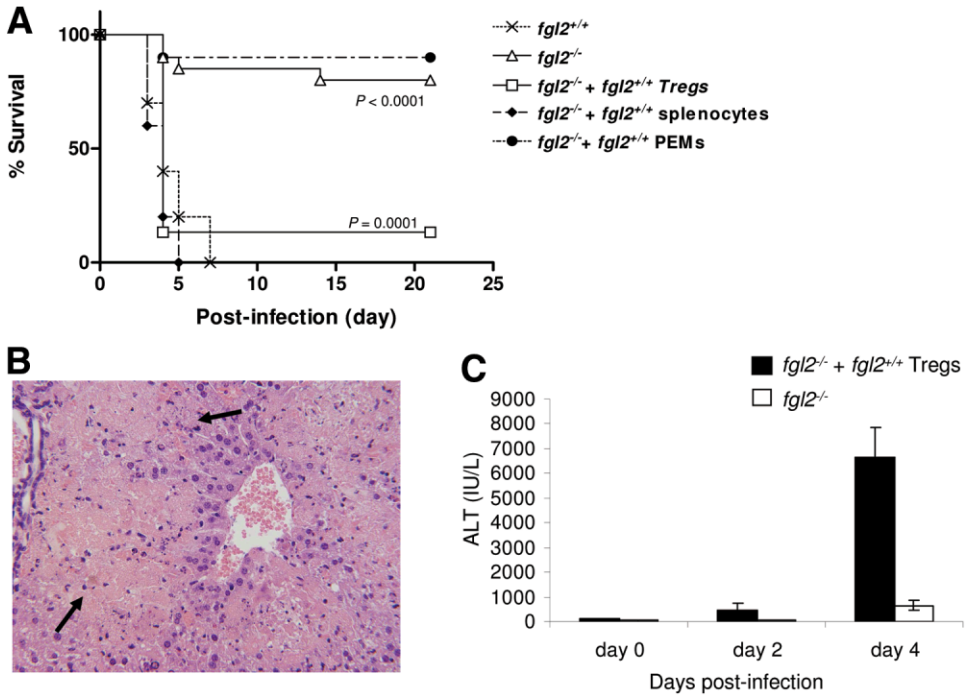

Fig. 8. Adoptive transfer of wild-type Tregs into resistant $fgl2^{-/-}$ mice increases mortality to MHV-3 infection. (A) $Fgl2^{+/+}$ mice (n = 10), $fgl2^{-/-}$ mice (n = 20), $fgl2^{-/-}$ mice infused with $fgl2^{+/+}$ Tregs (n = 15), $fgl2^{-/-}$ mice infused with $fgl2^{+/+}$ PEMs (n = 10), and $fgl2^{-/-}$ mice infused with $fgl2^{+/+}$ splenocytes (n = 10) were infected with MHV-3 (100 PFU), and survival was studied. $Fgl2^{-/-}$ mice infused with $fgl2^{+/+}$ Tregs had increased mortality after MHV-3 infection compared with resistant $fgl2^{-/-}$ mice, which did not receive $fgl2^{+/+}$ Tregs (log-rank test). (B) Representative HE staining of liver harvested 4 days after MHV-3 infection. A liver lobule is shown from $fgl2^{-/-}$ mice infused with $fgl2^{+/+}$ Tregs. A terminal hepatic vein (central vein) is present in the center of the micrograph and on the far left is part of a portal tract with a bile duct (*). There is complete acute necrosis involving the entire lobular parenchyma aside from a narrow rim of surviving hepatocytes in zone 3, the area immediately surrounding the hepatic vein (arrows). The diffuse pink staining material that replaces normal hepatocytes throughout the lobule represents the residua of totally necrotic liver parenchymal cells. There is over 90% necrosis of liver parenchyma. The pathology is that of massive acute hepatic necrosis of the liver. The extent of the necrosis is estimated as over 90% necrosis (HE stain: original magnification ×300). (C) Alanine aminotransferase levels in the serum of $fgl2^{-/-}$ mice that had been infused with or without $fgl2^{+/+}$ Tregs. Graph shows mean ± SEM of three mice in each group.

Various molecular and cellular events have been proposed to explain the mechanism by which Tregs suppress immune responses. These include cell-to-cell contact-dependent suppression, cytotoxicity, and immunosuppressive cytokine secretion.[24] Some studies have suggested that anti-inflammatory cytokines, such as IL-10 and TGF-β, are important mediators of Treg activity *in vivo*.[24] However, the importance of these cytokines remains controversial, as several reports have demonstrated that antibodies against IL-10 and TGF-β fail to block Treg suppressive function.[24] Also, Tregs from TGF-β–deficient mice have normal suppressive activity *in vitro* and can prevent development of autoimmune disease.[24]

Recently, it has been reported that Tregs have increased expression of *fgl2* mRNA, and it has been suggested that FGL2 might be an important Treg effector molecule.[9-11] The *fgl2* gene was first cloned by Koyama et al. from cytotoxic T lymphocytes, and the encoded protein has been classified as a member of the fibrinogen-related superfamily based on its homology to the β and γ chains of fibrinogen.[26] Previously we reported that in addition to its role in innate immunity as a membrane-associated prothrombinase, FGL2 also may have an important role in adaptive immune responses, similar to other members of the fibrinogen-like family of proteins, which include tenascin and angiopoietin.[27] Recombinant FGL2 was shown previously to suppress T cell proliferation to alloantigens, anti-CD3/CD28 antibodies, and Con A.[27] FGL2 also has been shown to inhibit maturation of bone marrow–derived dendritic cells (DCs) and polarized an allogeneic immune response toward a Th2 cytokine profile.[27] In *fgl2*-deficient mice, Th1 cytokine levels and activity of DC, B, and T cells were all increased.[28]

Levels of FGL2 in the plasma correlated with numbers of Tregs in both resistant and susceptible strains of mice before and after MHV-3 infection (Figs. 1-3), suggesting that Tregs are a major source of FGL2. The development of a Th2 immune response in BALB/cJ mice after MHV-3 infection[21] fits with the demonstrated effect of FGL2 to promote production of Th2 cytokines and subsequent inhibition of Th1 immunity.[27]

To evaluate the importance of FGL2 for Treg function, we assessed the effect of a monoclonal antibody against FGL2 on Treg activity *in vitro*. Antibody to FGL2 completely inhibited Treg function, consistent with the hypothesis that FGL2 is an important effector molecule for Treg activity. Further evidence for the importance of FGL2 to Treg function is the observation that Tregs from *fgl2*-deficient mice have impaired function and that *fgl2*-deficient mice develop autoimmune glomerulonephritis with increased age.[28] Based on these findings, we proceeded to investigate the contribution of FGL2 as a putative effector cytokine of Tregs to the pathogenesis of MHV-3 infection *in vivo*. The data presented here demonstrated that antibody directed to the C terminal domain of FGL2, which is known to account for its immunosuppressive activity,[27] protected mice from the lethality of MHV-3 infection. Others have reported that treatment with antibody against TGF-β, a known important Treg effector molecule, also resulted in prolonged survival of susceptible strains to MHV-3 infection, further supporting our hypothesis that Tregs are important in the pathogenesis of MHV-3–induced FH.[29] In addition, $fgl2^{+/+}$ Tregs that were transferred to $fgl2^{-/-}$ mice but not PEMs increased mortality to MHV-3 infection, further supporting a role for Treg-expressed FGL2 in the outcome of the infection.

Although the transfer of PEMs, in numbers comparable to frequency of macrophages in the spleen, did not adversely affect the survival of MHV-3-infected-$fgl2^{-/-}$ mice, this does not negate the importance of membrane-associated FGL2 prothrombinase production by reticuloendothelial cells as previously reported.[30] The lack of effect of macrophages might be attributable to the fact that insufficient cell numbers of $fgl2^{+/+}$ PEMs were transferred into $fgl2^{-/-}$ mice to recapitulate the endogenous production of FGL2 by liver-resident reticuloendothelial cells. It should be noted that macrophages from A/J mice can produce FGL2 to interferon gamma *in vitro*, yet do not generate FGL2 to MHV-3 *in vivo*.[31-33] Taken together, the data demonstrate that production of FGL2 is tightly regulated and supports the important role of Treg-expressed FGL2 in the pathogenesis of MHV-3–induced FH.

The mechanism by which FGL2 mediates its immunosuppressive activity is currently under intensive investigation. Recent data from our group have demonstrated that FGL2 binds to the inhibitory FcγRIIB receptor expressed primarily on APCs.[28,34] This FGL2-FcγRIIB interaction was shown to induce B cell apoptosis and inhibit DC maturation.[28,34] We have shown that Tregs isolated from A/J mice have equipotent immunosuppressive activity to Tregs from BALB/cJ mice (Shalev et al, unpublished data), indicating that differences in resistance and susceptibility between BALB/cJ and A/J mice cannot be explained by the lack of or reduced suppressive function of Tregs in resistant mice. However, in contrast to BALB/cJ mice, FGL2 does not bind to the inhibitory FcγRIIB receptor on DC and B cells from A/J mice because of an allelic polymorphism of the FcγRIIB receptor, but binds rather to the activating FcγRIII expressed on APCs.[34] We postulate that the reduced production of FGL2 *in vivo* and binding of FGL2 only to the FcγRIII may account for the protective Th1 response in A/J mice and resistance of A/J mice to MHV-3 infection.

Other factors that may reflect differences in the genetic background of the host, including the increased production of pro-inflammatory cytokines, such as interferon gamma and tumor necrosis factor, in BALB/cJ mice compared with A/J mice, could also contribute to resistance/susceptibility to MHV-3 infection of these two strains of mice, as has been reported. Both interferon gamma and tumor necrosis factor can induce *fgl2* linking these observations to the pathogenesis of MHV-3.[21,35,36]

The results of this study clearly demonstrate that FGL2 is an important effector cytokine of Tregs that contributes to host susceptibility to MHV3-induced FH. Because we have shown that patients with FH and chronic HBV and hepatitis C virus infection have increased levels of FGL2,[30,37] the data presented here in concert with human studies suggest that measurement of FGL2 levels in the plasma may be useful in predicting the outcome of both experimental and human viral hepatitis and may provide a rationale for targeting FGL2 for the treatment of patients with acute and chronic viral hepatitis.

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