IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

The Development of Mixed Cryoglobulinemia in *Capillaria hepatica*—Infected Mice Is Associated with the *Capillaria* Antigen—Induced Selective Proliferation of Splenic B-1a Cells in Response to Interleukin-5 Stimulation

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Chronic infection by pathogens such as hepatitis C virus induces monoclonal or oligoclonal proliferation of B cells, which produce IgM rheumatoid factor, leading to the development of mixed cryoglobulinemia (MC). Antigen-driven lymphoproliferation is essential to the onset of MC; however, the underlying mechanism is largely unknown. Herein, we show that type II MC is induced by *Capillaria hepatica* infection through a mechanism in which splenic B-1a cells reacting to *C. hepatica*-specific antigen selectively proliferate, producing IgM rheumatoid factor under co-stimulation of the specific worm antigen and IL-5. *In vitro* assays using B-1a cells from infected mice showed that stimulation by *C. hepatica* soluble fraction promoted the proliferation of B-1a cells and the secretion of IgM, which reacted with the 75-kDa antigen in the soluble fraction. The severity of MC was correlated with the increase in serum IL-5 levels in the infected mice. Furthermore, i.p. injection of the soluble worm fraction caused MC without an inflammatory response in IL-5 transgenic mice, indicating that IL-5 is critical for the development of MC. These results indicate that the selective proliferation of IgM rheumatoid factor-secreting B-1a cells is induced by co-stimulation by the specific pathogen antigen and IL-5 in the development of MC in *C. hepatica*-infected mice. (*Am J Pathol* 2015, 185: 172–184; <http://dx.doi.org/10.1016/j.ajpath.2014.09.017>)

Cryoglobulins are Igs that precipitate at low temperatures.¹ Cryoglobulinemia is subdivided into three types according to the components of cryoglobulins, namely, single (type I) or mixed (types II and III).² Mixed cryoglobulinemia (MC) is characterized by monoclonal (type II) or oligoclonal (type III) expansion of B-cell clones that produce IgM rheumatoid factor (IgM-RF).³ The development of MC is associated with several infectious diseases, such as hepatitis C virus (HCV) infection.⁴ Chronic infection induces monoclonal or oligoclonal proliferation of B cells that produce IgM-RF, and the selective proliferation of IgM-RF-secreting B cells is essential for the onset of MC; however, the underlying mechanism is largely unknown. The clonal expansion of the IgM-RF-secreting cells

suggests that specific antigenic stimulation is responsible for B-cell proliferation in MC; however, the effect of the specific antigen is not sufficient to explain the mechanism of clonal proliferation.

MC in HCV-infected patients has been studied extensively, and the pathogenesis of MC via selective lymphoproliferation includes multiple stages; an inciting factor induces polyclonal B-cell activation and, under certain immune conditions, oligoclonal B-cell activation. If a single clone is subsequently

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activated, monoclonal B-cell proliferation is induced.^{3,5} The conditions that promote selective lymphoproliferation during the early stages of MC have not been defined because of the difficulty associated with the collection of samples from early-stage MC patients. Studies have shown the importance of activation of T helper (Th) 1 immunity in the pathogenesis of MC-associated vasculitis in HCV-infected patients.^{6–9} However, these studies were based on samples from MC patients in the chronic stage, in which the Th1/Th2 immune condition is not suitable for the correct evaluation of the selective lymphoproliferation process. To resolve this issue, the pathology of MC should be investigated at the early stages of the disease. An alternative animal model showing selective lymphoproliferation induced by infection would be of value to investigate the pathogenesis of MC at the early stage.

Recently, we reported a novel type II MC mouse model induced by *Capillaria hepatica* infection, which is the first experimental animal model of MC induced by infection.¹⁰ *C. hepatica*, a nematode parasitizing the liver, consistently induces type II MC in infected mice. Elevation of IgM-RF levels in the serum and clonal expansion of IgM⁺κ⁺ B cells in the spleen were observed 20 to 30 days post-infection (DPI). The *C. hepatica*—induced MC model allows the analysis of selective lymphoproliferation during the early stage of MC. To clarify the mechanism of selective lymphoproliferation, the following factors are important: identification of the antigen triggering the process, the phenotype of IgM-RF—secreting cells, and the adequate immune status supporting MC progression.

B cells proliferating in HCV infection—related lymphoproliferative disorders, such as MC and B-cell non-Hodgkin lymphoma, express highly restricted Ig variable gene subfamilies encoded by the *VH1-69* variable gene. Analysis of the Ig sequences of *VH1-69*—expressing B cells showed high homology with the antibody specific for the HCV structural protein E2.^{11,12} HCV E2 activates polyclonal B cells by binding to the tetraspanin CD81,¹³ although the direct role of the HCV E2 protein in selective B-cell proliferation has not been demonstrated. Nevertheless, B-cell proliferation induced by stimulation with a specific antigen is a key event in the development of MC. Identification of the specific antigen in this model and comparative analysis of antigen structure between HCV and *C. hepatica* can be helpful to clarify the mechanism of MC development.

The phenotype of IgM-RF—secreting B cells in MC remains controversial. Despite the conflicting data, clonal expansion of CD5⁺ B cells has been observed in MC induced by HCV infection and regarded as an IgM-RF—secreting cell population.¹⁴ Identification of the secreting cell will contribute to the identification of factors essential for MC development. IL-5 plays a critical role in the activation and proliferation of CD5⁺ B cells^{15,16}; however, whether IL-5 contributes to MC development is not clear in HCV-induced MC patients.¹⁷ To clarify the phenotype of IgM-RF—secreting cells in this model, we classified proliferated B cells according to CD5 expression and examined the effect of the Th1/Th2 immune response and the role of IL-5 in MC development.

Herein, we first identified the IgM-RF—secreting cells and the specific antigen triggering the clonal expansion of IgM-RF—secreting cells and IgM-RF production in this model. Next, we defined the suitable host Th1/Th2 immune response for MC development. Finally, to identify the essential cytokine for MC development, we focused on the involvement of IL-5.

Materials and Methods

Isolation of *C. hepatica* Eggs

C. hepatica was maintained by serial passage in Fischer 344 rats, and *C. hepatica* eggs were obtained from the livers of the infected rats. To obtain embryonated eggs, we used previously described methods¹⁰ with listed modifications. The homogenate of infected livers was sieved through a wire mesh (aperture diameter, 500 μm), followed by several washing and sedimentation steps using distilled water. The homogenate was then suspended in phosphate-buffered saline (PBS) containing 0.5% formalin in a Petri dish and incubated at 25°C for 30 days. The embryonated eggs were sieved through a nylon mesh (aperture diameter, 100 μm), and the number of embryonated eggs was counted under a microscope.

Mice

Eight-week-old female BALB/c and C57BL/6 (C57BL) mice were purchased from Japan SLC (Hamamatsu, Japan). IL-5—overexpressing transgenic mice on a C57BL background (IL-5tgC57BL) were purchased from Riken Bio-Resource Center (Tsukuba, Japan). The serum level of IL-5 and the number of B-1 cells were constitutively elevated without stimulation in IL-5tgC57BL mice.¹⁵ Mice were maintained under specific pathogen—free conditions. All the animal procedures were performed at the animal experiment facilities of the Research Institute of Bioscience, Azabu University (Sagamihara, Japan).

Antibodies

Monoclonal antibodies (mAbs) for mouse CD5 (53-7.3, allophycocyanin conjugated), CD45R/B220 (RA3-6B2, Alexa Fluor 700 conjugated), κ light chain [187. 1, phycoerythrin (PE)-cyanine dye (Cy)7 conjugated], IL-5 receptor (T21, PE conjugated), CD4 (RM4-5, PE-Cy5 conjugated), interferon γ (IFN-γ) [XMG1.2, fluorescein isothiocyanate (FITC) conjugated], and IL-4 (11B11, PE conjugated) were purchased from BD Pharmingen (San Diego, CA). mAbs for mouse CD5 (53-7.3, FITC conjugated) and CD45R/B220 (RA3-6B2, PE conjugated) were purchased from eBioscience Inc. (San Diego, CA). mAb against mouse macrophage receptor with collagenous structure (ED31) was purchased from AbD Serotec (Oxford, UK). Goat anti-mouse μ chain (FITC conjugated) was purchased from Cappel Laboratories Inc. (Cochranville, PA). Goat anti-mouse κ light chain and λ light chain were purchased from Bethyl Laboratories Inc.

Figure 1 Cryoglobulin and IgM rheumatoid factor (IgM-RF) in the sera of *Capillaria hepatica*-infected mice. **A:** After cold incubation, a visible precipitate formed in the sera of infected mice at 24 days post-infection (DPI) (arrow). **B:** Quantitative analysis of IgM-RF by enzyme-linked immunosorbent assay showed a significant increase at 24 DPI. **C:** Immunofluorescence analysis of the reaction to *C. hepatica* worms. The IgM of sera from 24 DPI mice reacted with the whole worm, whereas the IgM of the cryoglobulin solution reacted only with the *C. hepatica* digestive tract (arrows). **D:** Electrophoretic patterns of *C. hepatica* soluble fraction (ChSF) were presented by staining with Coomassie Brilliant Blue (CBB). Western blot analysis showed no signal against μ chain in the ChSF sample. **E:** The IgM of infected mice sera reacted with various *C. hepatica* antigens. **F:** The IgM in cryoglobulin solutions reacted with an antigen of approximately 75 kDa (arrow). Data are expressed as means $\pm$ SD (**A** and **B**). * $P < 0.05$. Original magnification: $\times 400$ (**C**). CG, cryoglobulin solution; H&E, hematoxylin and eosin; I, intestine; ND, not detectable; R, reproductive duct. **Black line**, muscle layer; **red line**, bacillary band (**C**).

(Montgomery, TX). Alexa Fluor 488-conjugated donkey anti-goat IgG, Alexa Fluor 568-conjugated rabbit anti-goat IgG, and Alexa Fluor 568-conjugated goat anti-rat IgG were purchased from Life Technologies Inc. (Gaithersburg, MD). Horseradish peroxidase-conjugated rabbit anti-goat antibody was purchased from Dako (Glostrup, Denmark).

Experimental Infection of *C. hepatica*

Eight-week-old mice (BALB/c, C57BL, and IL-5tgC57BL) were orally infected with 1000 or 5000 embryonated *C. hepatica* eggs in 0.2 mL of PBS. For all the experiments except eosinophil counting, samples from the 1000 egg-infected mice were used. Eosinophil counting was performed using 5000 egg-infected mice. Uninfected control mice were orally administered 0.2 mL of PBS. BALB/c and C57BL mice were sacrificed at 12, 16, 20, and 24 DPI and IL-5tgC57BL mice at 20 and 24 DPI. The blood, kidney, liver, spleen, and bone marrow were harvested at necropsy.

Cryoglobulin

Blood samples were coagulated at 37°C, and the sera were separated by centrifugation. The sera were stored for 7 days at

4°C, and the precipitate was confirmed by visual observation.¹⁸ After centrifugation, the precipitate was resuspended in 100 μ L of 0.1% SDS, as previously described,¹⁰ and used as cryoglobulin solution. The protein content was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Isolation of *C. hepatica* Adult Worms

Adult *C. hepatica* worms were isolated using the digestion technique,^{19,20} with some modifications. Livers of infected mice and rats at 24 DPI were digested in a hydrochloride-pepsin solution (0.37% HCl and 1% pepsin in distilled water) at 37°C under constant stirring in a 500-mL glass Erlenmeyer flask. The supernatant containing adult worms was filtered through a wire mesh (aperture diameter, 250 μ m) every 15 minutes, and worms on the mesh were collected, followed by several washing and sedimentation steps using distilled water (Supplemental Figure S1).

Preparation of *C. hepatica* Soluble Fraction

The isolated *C. hepatica* adult worms were sonicated in PBS on ice for 10 minutes at 1-minute intervals. The final solution was

centrifuged at $15,000 \times g$ at 4°C for 15 minutes, and the supernatant was filtered through a $0.45\text{-}\mu\text{m}$ (pore-size) cellulose acetate membrane filter (Advantec Dismic-25cs; Toyo Roshi Kaisha, Tokyo, Japan). The supernatant was used as a *C. hepatica* soluble fraction (ChSF). The protein content of the ChSF was determined using the Bio-Rad protein assay kit and a spectrophotometer (model 680 microplate reader; Bio-Rad Laboratories).

Histologic Analysis

The kidney, liver, spleen, and bone marrow samples were collected at necropsy. Small pieces of each tissue were fixed for 24 hours in 10% neutral buffered formalin and embedded in paraffin blocks or were embedded in OCT compound (Sakura FineTek, Tokyo, Japan) and quickly frozen in liquid nitrogen for frozen sections. Histologic sections ($4\text{ }\mu\text{m}$ thick) were stained with hematoxylin and eosin, periodic acid–Schiff, and Congo red.

Immunofluorescence Analysis

The reactivity of sera of infected mice or cryoglobulin solutions to *C. hepatica* worms was tested using formalin-fixed, paraffin-embedded sections of the infected rats' livers at 24 DPI. The $4\text{-}\mu\text{m}$ -thick sections were reacted with serum samples or cryoglobulin samples, and the binding of mouse μ chain in these samples was detected using FITC-conjugated anti-mouse μ chain. For the detection of IgM deposition in the glomeruli, formalin-fixed, paraffin-embedded kidney sections were stained with FITC-conjugated anti-mouse μ chain. The frozen spleen sections were stained with anti-mouse κ light chain, λ light chain, or macrophage receptor with collagenous structure antibodies; were visualized using Alexa Fluor 568–conjugated secondary antibodies; and then were stained with FITC-conjugated anti-mouse μ chain. Fluorescence signals in the sections were observed using an FSX100 fluorescence microscope (Olympus, Tokyo, Japan). Triple staining of spleen sections using allophycocyanin-conjugated CD5 antibody, PE-conjugated IL-5 receptor antibody, and FITC-conjugated anti-mouse μ chain was observed using a Leica SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Sections with the primary antibody omitted were used as negative controls.

Flow Cytometric Analysis of Splenic Lymphocytes

Spleens were cut into small fragments using scissors in RPMI 1640 medium (Gibco Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal calf serum and 1% antibiotic-antimycotic (Gibco Invitrogen). The suspensions were filtered through a nylon mesh (aperture diameter, $70\text{ }\mu\text{m}$). After red blood cells were depleted with ammonium chloride, splenic lymphocytes were separated by centrifugation at $300 \times g$ for 30 minutes using Percoll gradients. For three- to four-color immunofluorescence analysis, single-cell suspensions (10^6

Figure 2 Characterization of proliferating B cells in the spleens of *Capillaria hepatica*–infected mice. **A**: Representative appearance of spleens. The spleen was significantly enlarged at 24 days post-infection. Spleen/body weight ratios of infected mice were significantly larger than those of uninfected mice. **B**: Periodic acid–Schiff–positive Mott cells increased in the splenic red pulp of 24 DPI mice (arrowheads). **C**: Immunofluorescence staining showed numerous μ chain [fluorescein isothiocyanate (FITC)]–positive cells in the splenic red pulp of infected mice at 24 days post-infection. **D**: Most μ chain (FITC)–positive cells in the splenic red pulp of infected mice were κ light chain positive instead of λ light chain positive. **E**: Flow cytometric analysis of splenic B cells. IgM $^{+}$ κ^{+} cells were classified into three groups according to CD5 and CD45R/B220 expression. B-1a cells significantly increased in 24 DPI mice. **F**: B-1a cells (IgM $^{+}$ CD5 $^{+}$ B220 $^{+}$ cells) were observed in the flow cytometric analysis sample by immunofluorescence analysis. Data are expressed as means $\pm$ SD (**A** and **E**). $^{*}P < 0.005$, $^{***}P < 0.0005$. Scale bars: 10 mm (**A**); 20 μm (**B**); 500 μm (**C**). Original magnification: $\times 400$ (**D**). MARCO, macrophage receptor with collagenous structure.

Figure 3 Stimulation of splenic B-1a cells from infected mice by *Capillaria hepatica* soluble fraction (ChSF). **A:** IgM production increased by ChSF stimulation in a dose-dependent manner. **B:** The IgM produced in the medium reacted with the 75-kDa antigen in the ChSF. **C:** ChSF stimulation significantly promoted cell proliferation. **D:** Amplification of IgM variable region (V_HDJ_H -Cmu) transcripts using RT-PCR. A single band of approximately 1100 bp was observed in ChSF-stimulated samples incubated for 2 days. **E:** The common band of 1100 bp was detected in splenic lymphocytes from infected mice but not in those from uninfected mice. Data are expressed as means $\pm$ SD (**C**). * $P < 0.05$. CFSE, carboxyfluorescein diacetate succinimidyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; M, molecular size marker.

cells) were stained at room temperature using optimal concentrations of mAbs for 30 minutes and were analyzed using the ec800 cell analyzer (Sony Biosciences, Tokyo, Japan). Cells were gated through a gate defined by forward and side scatter.

Purification of B-1a Cell Subsets

Splenic lymphocytes from BALB/c mice at 24 DPI or uninfected BALB/c mice were separated by the same method used in flow cytometric analysis. The splenic lymphocytes were first enriched using CD19 mAb-coated microbeads (Miltenyi Biotec Inc., Auburn, CA). The enriched CD19⁺ cells were stained with FITC-conjugated CD5 and PE-conjugated CD45R/B220 antibodies. CD5⁺CD45R/B220⁺ cells (B-1a cell subset) were isolated using a BD FACSARIA III cell sorter (BD Biosciences, San Jose, CA). The purified B-1a cell population was then used for the *in vitro* assay or RT-PCR as described immediately below and in *RT-PCR of μ Heavy Chain Variable Region (V_HDJ_H -Cmu)*.

In Vitro Assessment of B-1a Cell Stimulation by ChSF

The purified splenic B-1a cells were labeled with 5 μ M carboxyfluorescein diacetate succinimidyl ester (Invitrogen) at 37°C for 10 minutes and were washed three times. Then,

2×10^5 carboxyfluorescein diacetate succinimidyl ester-labeled B-1a cells were cultured in 200 μ L of culture medium containing 0, 1, 10, or 100 μ g/mL of ChSF at 37°C in a 5% CO₂ atmosphere for 1 to 3 days on 96-well plates. After incubation, half of the cells were used for analysis of IgM variable region transcripts by RT-PCR, and the remaining cells were used to analyze carboxyfluorescein diacetate succinimidyl ester dilution by flow cytometry. The culture medium was analyzed for the presence of mouse IgM and its reactivity to *C. hepatica* antigens by Western blot analysis.

Western Blot Analysis Using ChSF or Culture Media

For SDS-PAGE of ChSF, a 35- μ g sample of the ChSF protein was loaded into a lane, separated on a 10% polyacrylamide gel, and stained with Coomassie Brilliant Blue. To evaluate the contamination of the endogenous μ chain that was derived from infected murine tissue in ChSF, the μ chains in the ChSF sample were analyzed by Western blot analyses using goat anti-mouse μ -chain antibodies. The ChSF protein sample (8 μ g) was separated on a 10% polyacrylamide gel and then was electrophoretically transferred onto a polyvinylidene difluoride membrane. The membranes were subsequently treated with goat anti-mouse μ -chain antibodies that were diluted 1:4000

using 5% skim milk in PBS for 1 hour at room temperature and then treated with horseradish peroxidase—conjugated rabbit anti-goat antibodies that were diluted 1:2000 with 5% skim milk in PBS for 1 hour at room temperature. Immunoreactivity was visualized using Luminata Forte Western horseradish peroxidase substrate (Millipore, Temecula, CA).

To test the reactivity of the IgM for *C. hepatica* antigens in serum, cryoglobulin, and culture medium of B-1a cells, Western blot analyses against ChSF were performed. In all the Western blot analyses of ChSF, ChSF samples were supplemented with an equal amount of Laemmli sample buffer in a tube and boiled for 10 minutes. The protein in the boiled sample (8 μ g) was loaded into each lane, separated on a 10% polyacrylamide gel, and electrophoretically transferred onto a polyvinylidene difluoride membrane. The membrane was incubated at 4°C overnight in 5% skim milk in PBS with 5 μ g/mL infected or control murine sera, 1 μ g/mL cryoglobulin solutions, and 10 μ L/mL culture medium of B-1a cells or medium without cells. The membranes were treated with goat anti-mouse μ -chain antibodies as the primary antibodies, and immunoreactivity was visualized by the previously mentioned method. The primary antibodies were omitted in the negative controls of the Western blot analyses.

For detecting IgM in the culture media of B-1a cells, 10 μ L of each culture medium was used as loading samples. The samples were analyzed using the previously mentioned methods using goat anti-mouse μ -chain antibodies and the horseradish peroxidase—conjugated rabbit anti-goat antibody.

RT-PCR of μ Heavy Chain Variable Region (V_HDJH -Cmu)

Total RNA was extracted from splenic lymphocyte or B-1a cell samples using the RNeasy Plus micro kit (Qiagen, Hilden, Germany). Oligo dT primers (Promega Corp., Madison, WI) and Sensiscript reverse transcription (Qiagen) were used to generate cDNA. All components of the PCR mix (TaKaRa Ex Taq) were purchased from TaKaRa Bio Inc. (Shiga, Japan) and were used according to the manufacturer's instructions. After incubation at 94°C for 10 minutes, cDNAs were amplified by 40 cycles at 94°C for 1 minute, 55°C for 1 minute, 72°C for 1.5 minutes, and then 72°C for 10 minutes. The amplicons were resolved by electrophoresis in a nondenaturing 1.0% agarose gel and then were stained with ethidium bromide. The sense mouse *IgM* variable region primer (V_H FR1) was 5'-SARGTNMAGCTGSAGSAGTC-3', and the antisense mouse *IgM* variable region primer (Cmu) was 5'-CTTAT-CAGACAGGGGGCTCT-3'.^{21,22} The sense mouse *GAPDH* primer was 5'-TGTCCTACCCCCAATGTGT-3', and the antisense mouse *GAPDH* primer was 5'-TGGGTGCAGC-GAACTTTATT-3'.²³

Subtyping of the Th1/Th2 Subset by Intracellular Cytokine Production

Splenic lymphocytes from BALB/c or C57BL mice at 24 DPI were separated by the same method used in flow cytometric

analysis. For the detection of intracellular cytokines, splenic lymphocytes were incubated in RPMI 1640 medium with 50 μ g/mL PHA-M (Cosmo Bio Co. Ltd., Tokyo, Japan) for 18 hours. Six hours before harvesting, 5 μ g/mL brefeldin A (Sigma-Aldrich, St. Louis, MO) was added to cultures to retain cytokines in the cytoplasm. For analysis of cytokine expression, splenic lymphocytes (1×10^6 cells per mouse) were stained with PE-Cy5—conjugated anti-CD4 mAb, PE-conjugated anti-IL-4 mAb, and FITC-conjugated anti-IFN- γ mAb. The samples were analyzed using the ec800 cell analyzer. Lymphocytes were gated through a gate defined by

Figure 4 T helper (Th) 1/Th2 immune response after infection in BALB/c and C57BL/6 (C57BL) mice. **A:** Subtyping of Th1/Th2 subsets was performed using flow cytometric analysis. The percentage of IL-4⁺CD4⁺ (Th2) cells was significantly increased in infected BALB/c mice. **B:** Eosinophil infiltration was severe in infected BALB/c mice. Hematoxylin and eosin (H&E) and Congo red staining are shown. **C:** The number of eosinophils in the peripheral blood was significantly increased in infected BALB/c mice. Data are expressed as means $\pm$ SD (**A** and **C**) ** P < 0.005, *** P < 0.0005. Scale bar = 50 μ m.

forward and side scatter, and CD4⁺ lymphocytes were deemed to be Th cells. Intracellular IL-4 and IFN- γ were stained; IFN- γ ⁺CD4⁺ cells were deemed to be Th1 cells, and IL-4⁺CD4⁺ cells were deemed to be Th2 cells.

Eosinophil Counting in Peripheral Blood

The total number of leukocytes in peripheral blood was counted using a hemocytometer (Erma Inc., Tokyo, Japan). Blood smears were stained with Giemsa stain. At least 200 leukocytes were identified, and the percentage of eosinophils was calculated. Then, the total number of eosinophils per microliter of peripheral blood was calculated.

Enzyme-Linked Immunosorbent Assay for IgM-RF and IL-5

Serum levels of IgM-RF and IL-5 were measured using a mouse IgM-RF enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi Co. Ltd., Shibukawa, Japan) and a mouse IL-5 ELISA Ready-Set-Go! kit (eBioscience Inc.). All analyses were performed according to the manufacturer's instructions.

I.P. Injection of ChSF Test

Female IL-5tgC57BL mice ($n = 12$, 12 weeks old) and female BALB/c mice ($n = 12$, 8 weeks old) were divided into four groups for each strain: ChSF 3 μ g per mouse injected, 30 μ g per mouse injected, 300 μ g per mouse injected, and untreated. At 0, 3, and 6 days, ChSF was injected i.p. at doses of 1, 10, and 100 μ g per mouse at every injection time. Ten days after the first injection, the mice were sacrificed and samples were collected.

SDS-PAGE of Cryoglobulin Solution

The cryoprecipitate from the sera of ChSF-injected mice was resuspended in 100 μ L of 0.1% SDS, and 10 μ L of each sample was supplemented with an equal amount of Laemmli sample buffer and boiled for 10 minutes. Each sample was loaded into a lane, separated on 10% polyacrylamide gel, and stained with Coomassie Brilliant Blue.

Statistical Analysis

All values are expressed as means $\pm$ SD. Statistical analysis was performed using one-way analysis of variance with a Tukey-Kramer posttest or Student's t -test using GraphPad Prism software version 5 (GraphPad Software Inc., San Diego, CA).

Results

Characterization of Serum Cryoglobulin in *C. hepatica*-Infected Mice

The sera of uninfected and 24 DPI mice were examined for the formation of cryoglobulins. After cold incubation, visible

precipitates formed only in the serum samples collected at 24 DPI (Figure 1A). IgM-RF in the serum was quantified in *C. hepatica*-infected and uninfected BALB/c mice by ELISA. The serum levels of IgM-RF in *C. hepatica*-infected mice increased over time. The IgM-RF level at 24 DPI was significantly different from that of uninfected mice ($P < 0.05$) (Figure 1B). To examine the reactivity of the cryoglobulin to *C. hepatica* antigens, we performed immunofluorescence analysis using serum samples or cryoglobulin solution samples as primary antibody against the sections of rat liver collected at 24 DPI. Whereas the IgM in the serum from 24 DPI mice strongly reacted with the whole *C. hepatica* worm (Figure 1C), the IgM in the cryoglobulin solution from 24 DPI mice reacted specifically with the *C. hepatica* digestive tract. The surface of the *C. hepatica* worm was reacted with the IgM in the serum and the cryoglobulin solution from 24 DPI and was weakly reacted with the IgM in the serum of uninfected mice. This represents nonspecific binding of IgM to the surface of the worm. To examine the reactivity of cryoglobulin against ChSF, we performed Western blot analysis. Electrophoretic patterns of the ChSF sample are presented in Figure 1D. To eliminate the possibility of contamination with the endogenous μ chain, the μ chains in ChSF were detected using a goat anti-mouse μ -chain antibody, and no signal was observed in this lane (Figure 1D). Therefore, we eliminated the possibility of contamination with the endogenous μ chain that was co-purified with the worms in ChSF. Whole serum samples of infected mice at 24 DPI strongly reacted against various worm antigens, although no specific signal was observed

Figure 5 Serum IL-5 in BALB/c, C57BL/6 (C57BL), and IL-5—overexpressing transgenic mice on a C57BL background (IL-5tgC57BL) mice. Quantitative analysis of IL-5 by enzyme-linked immunosorbent assay showed a significant increase at 20 days post-infection (DPI) in BALB/c mice. C57BL mice did not show a significant elevation in serum IL-5 levels. The level of serum IL-5 in uninfected IL-5tgC57BL mice was 40.5 pg/mL and was significantly increased by the infection. Horizontal lines show means and SD. ** $P < 0.005$, *** $P < 0.0005$.

Figure 6 Cryoglobulinemia in BALB/c, C57BL/6 (C57BL), and IL-5—overexpressing transgenic mice on a C57BL background (IL-5tgC57BL) mice. **A:** A visible cryoprecipitate (arrows) formed in the serum samples from infected BALB/c and IL-5tgC57BL mice. **B:** IgM deposition in the glomerulus was severe in BALB/c and IL-5tgC57BL mice and mild in C57BL mice. **C:** Quantitative analysis of IgM rheumatoid factor by enzyme-linked immunosorbent assay showed significantly high values in BALB/c and IL-5tgC57BL mice compared with C57BL mice. **D:** The spleen/body weight ratios of BALB/c and IL-5tgC57BL mice were significantly higher than those of C57BL mice. Data are expressed as means $\pm$ SD (**A**, **C**, and **D**). * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$. Original magnification: $\times 600$ (**B**). DPI, days post-infection; ND, not detectable.

(Figure 1E). In contrast, cryoglobulin solution samples from several 24 DPI mice reacted with a specific antigen of approximately 75 kDa (Figure 1F). Little signal in the uninfected mouse serum lane was observed.

In this model, the specific binding of IgM in cryoglobulin to the digestive tract of the *C. hepatica* adult worm and to a 75-kDa antigen in the ChSF was common to all infected mice. Therefore, we considered that the cryoglobulin was composed of the monoclonal or oligoclonal IgM raised against the specific antigen in the ChSF.

Characterization of the B-Cell Phenotype in the Spleen of *C. hepatica*—Infected Mice

At 24 DPI, *C. hepatica*—infected BALB/c mice showed significant splenomegaly. Spleen/body weight ratios of infected mice were more than five times those of uninfected mice ($P < 0.0005$) (Figure 2A). Histologically, lymphocytes increased in white and red pulp, and hypertrophy of lymphoid follicles was observed in the infected mice at 24 DPI. A large number of plasma cells accompanying periodic acid—Schiff —positive Mott cells were observed in the splenic red pulp of 24 DPI mice (Figure 2B). Because cryoglobulins in the infected mice were mainly composed of IgM, we postulated that IgM⁺ B cells proliferated in the spleen. Then, to analyze the phenotype and localization of the proliferated B cells, we performed double immunofluorescence staining for μ heavy chain and macrophage receptor with collagenous structure, a marker of marginal zone macrophages,

or μ heavy chain and κ or λ light chains in the spleen sections. Numerous IgM-positive cells were detected in the splenic red pulp of infected mice compared with the spleen of uninfected mice (Figure 2C). Most of the IgM-positive cells in the splenic red pulp possessed κ light chains (Figure 2D). To characterize the IgM⁺ κ ⁺ cells proliferating in the spleen of infected mice, we divided the IgM⁺ κ ⁺ cells into three groups (B-2, B-1a, and B-1b) according to the expression of CD5 and CD45R/B220 using flow cytometric analysis (Figure 2E). Splenic lymphocytes were gated through a gate defined by forward and side scatter, and then IgM⁺ κ ⁺ cells were selected. CD5-positive B-1a cells were rare in the spleen of uninfected mice and significantly increased in the spleen of *C. hepatica*—infected mice. The expression of IgM, κ , and CD45R/B220 on B-1a cells in flow cytometric analysis samples was also confirmed by immunofluorescence analysis (Figure 2F). These findings suggested that IgM⁺ κ ⁺ B-1a cells proliferated principally in the splenic red pulp of infected mice. We postulated that the B-1a cells that proliferated in the spleen might produce cryoglobulin; therefore, the response of splenic B-1a cells from infected mice against ChSF was analyzed by assessing cell proliferation and IgM production.

Effect of ChSF on Splenic B-1a Cells from *C. hepatica*—Infected Mice

We used an *in vitro* assay to examine the effect of the ChSF on splenic B-1a cells proliferating in the spleens of *C. hepatica*—infected mice. The concentration of IgM in the

Figure 7 The i.p. injection of *Capillaria hepatica* soluble fraction (ChSF) in BALB/c and IL-5—overexpressing transgenic mice on a C57BL background (IL-5tgC57BL) mice. **A:** Serum IL-5 levels in BALB/c and IL-5tgC57BL mice were not affected by injection of ChSF. The horizontal lines show means and SD. **B:** A visible cryoprecipitate formed only in the serum samples from IL-5tgC57BL mice injected with 300 µg of ChSF (arrow). **C:** SDS-PAGE of cryoprecipitate solutions from each group. The cryoprecipitate was mainly composed of μ heavy chain (75 kDa). **D:** IgM deposition in the glomerulus was detected only in IL-5tgC57BL mice injected with 300 µg of ChSF. Data are expressed as means $\pm$ SD (**B**). ND, not detectable; PBS, phosphate-buffered saline.

culture medium increased in response to stimulation with ChSF in a dose-dependent manner (Figure 3A). The IgM that was produced reacted specifically with the 75-kDa antigen in the ChSF (Figure 3B). The reactivity of the IgM against 75-kDa proteins was weakly detected in the culture media that were nonstimulated or stimulated with 1 µg/mL ChSF, and it was strongly detected in the medium stimulated with 10 µg/mL ChSF (Figure 3B). The medium supplied with ChSF, which was a negative control sample, showed a weak signal that was lower than that of 75-kDa bands.

Assessment of cell proliferation by carboxyfluorescein diacetate succinimidyl ester dilution showed that the rate of proliferation was higher in cells stimulated by ChSF than in unstimulated cells (Figure 3C). To examine the clonality of the proliferating cells, IgM variable region transcripts were analyzed by RT-PCR using *in vitro* assay samples. A single band of approximately 1100 bp was observed in ChSF-stimulated samples, especially in samples incubated for 2 days, whereas multiple bands were detected in the B-1a sample after sorting purification (Figure 3D). B-1a cells isolated from uninfected mice showed multiple band patterns immediately after purification, and the IgM variable region mRNA was reduced by culture with ChSF. This suggested that specific B-1a clones expressing a restricted IgM variable region repertoire proliferate in response to stimulation by ChSF. We then performed RT-PCR of IgM variable region

transcripts using the spleens of infected and uninfected mice. The common band of 1100 bp was detected in the spleens of infected mice but not in those of uninfected mice (Figure 3E).

The present results indicated the robust expansion of splenic B-1a cells in infected mice. Furthermore, *in vitro* assays using splenic B-1a cells from infected mice demonstrated that stimulation by ChSF induced B-1a cells to proliferate and produce IgM. The produced IgM bound to the 75-kDa antigen similar to cryoglobulin, and B-1a cells expressing a restricted repertoire of μ -chain variable regions selectively proliferated. These results suggested that antigen-specific splenic B-1a clones are cryoglobulin-secreting cells.

Th1/Th2 Immune Response after Infection in BALB/c and C57BL Mice

To verify the hypothesis that the Th1/Th2 immune response plays a role in the development of cryoglobulinemia, we used two mouse strains with different Th immune responses. Th cell polarization and histologic findings were examined in *C. hepatica*—infected BALB/c and C57BL mice. The percentage of IL-4⁺CD4⁺ cells (Th2 cells) in infected BALB/c mice was significantly higher than that in uninfected BALB/c mice, whereas Th2 polarization did not occur in infected C57BL mice (Figure 4A). The percentage of IFN- γ ⁺CD4⁺ cells (Th1 cells) did not change

Figure 8 Schematic illustration of the pathogenesis of cryoglobulinemia in the *Capillaria hepatica* mouse model. If *C. hepatica* infection causes a T helper 2 (Th2)—biased immunoresponse, it will increase serum IL-5 levels. Coincidentally, *C. hepatica* worms parasitizing the liver release several soluble antigens, including a specific antigen. In response to co-stimulation with the specific antigen and IL-5, splenic B-1a cell clones reacting to the specific antigen may selectively proliferate and produce IgM rheumatoid factor, resulting in the induction of cryoglobulinemia. CG, cryoglobulin.

significantly compared with infected and uninfected mice in both strains. In addition, the systemic inflammatory response was different between the two strains. Severe eosinophil infiltration in the liver, spleen, and bone marrow was observed in infected BALB/c mice but not in infected C57BL mice (Figure 4B), and eosinophil counts in the peripheral blood were significantly elevated by infection in BALB/c mice but not in C57BL mice (Figure 4C). Therefore, *C. hepatica* infection induced Th2 polarization accompanied by systemic eosinophilia in BALB/c mice but not in C57BL mice.

Serum IL-5 in BALB/c, C57BL, and IL-5tgC57BL Mice

Eosinophil proliferation and activation are mediated by IL-5, which is a Th2-type cytokine.²⁴ IL-5 is also known as a critical growth factor for B-1 cells.²⁵ Splenic B-1a cells in infected mice expressed the IL-5 receptor (Supplemental Figure S2); therefore, we postulated that IL-5 is the essential promoting factor of cryoglobulinemia and measured the serum IL-5 level in BALB/c, C57BL, and IL-5tgC57BL mice. Serum IL-5 levels at 20 DPI were significantly higher in infected BALB/c mice than in uninfected BALB/c mice and infected C57BL mice ($P < 0.0005$) (Figure 5). The serum IL-5 level in uninfected IL-5tgC57BL mice was 40.5 pg/mL and increased significantly in response to infection at 20 and 24 DPI.

Cryoglobulinemia in BALB/c, C57BL, and IL-5tgC57BL Mice

Next, we examined the development of cryoglobulinemia in BALB/c, C57BL, and IL-5tgC57BL mice. At 24 DPI, cryoprecipitates were observed in the sera of infected BALB/c and IL-5tgC57BL mice (Figure 6A). Severe IgM deposition in the glomeruli was observed in infected BALB/c mice, whereas the deposition was slight in infected C57BL mice (Figure 6B). The level of IgM-RF in the serum was significantly higher in infected BALB/c mice than in infected C57BL mice (Figure 6C). The degree of splenomegaly was also significantly different between BALB/c and C57BL mice (Figure 6D). IgM deposition in the glomeruli, serum level of IgM-RF, and spleen size in infected IL-5tgC57BL mice were more severe than those in infected C57BL mice

and were comparable with those in infected BALB/c mice. These findings showed that cryoglobulinemia was exacerbated in the Th2-biased immune condition and that IL-5 was a critical promoting factor of cryoglobulinemia.

I.P. Injection of ChSF in BALB/c and IL-5tgC57BL Mice

To verify the involvement of IL-5 and ChSF in cryoglobulinemia development, we performed i.p. injection of ChSF into IL-5tgC57BL and BALB/c mice. Serum IL-5 levels were higher in IL-5tgC57BL mice than in BALB/c mice, and i.p. injection of ChSF did not affect IL-5 levels (Figure 7A). Histologically, injection of ChSF did not induce an inflammatory response in the liver, spleen, or kidney. As a result, a visible cryoprecipitate was observed only in the sera of IL-5tgC57BL mice injected with 300 μ g per mouse of ChSF ($n = 3$) (Figure 7B). The cryoprecipitate was mainly composed of μ heavy chain by Western blot analysis, and the μ heavy chain band was distinctly observed in IL-5tgC57BL mice injected with 300 μ g of ChSF and slightly observed in BALB/c mice injected with 300 μ g of ChSF on SDS-PAGE (Figure 7C). IgM deposition in the glomeruli was observed only in 300- μ g ChSF-injected IL-5tgC57BL mice (Figure 7D). Therefore, cryoglobulinemia can develop by co-stimulation with ChSF and IL-5 without infection *in vivo*. The other inflammatory factors induced by *C. hepatica* infection were not considered essential for MC development because i.p. injection of ChSF did not induce any inflammatory responses that could be detected histologically.

Discussion

Herein, we identified the following three factors essential for the pathogenesis of cryoglobulinemia after selective lymphoproliferation: i) splenic B-1a cells as IgM-RF-secreting cells, ii) *C. hepatica*-specific antigens as a trigger of B-1a cell proliferation and IgM production, and iii) IL-5 as an essential promoting factor of the selective lymphoproliferative stage of MC. A brief overview of the pathogenesis of cryoglobulinemia in *C. hepatica*-infected mice is shown in Figure 8. If *C. hepatica* infection causes a Th2-biased immunoresponse, it will increase serum IL-5 levels. Coincidentally with these events, *C. hepatica* worms parasitizing

the liver release soluble antigens, including a specific antigen. In response to co-stimulation with the specific antigen and IL-5, splenic B-1a cell clones reacting to the specific antigen may selectively proliferate and produce IgM-RF, resulting in the induction of cryoglobulinemia. Other inflammatory factors induced by *C. hepatica* infection are not essential for MC development.

Murine B-cell lineages are classified into two distinct types, namely, B-1 and B-2 cells, and B-1 cells are subdivided into CD5⁺ B-1a cells and CD5⁻ B-1b cells.^{26,27} In humans, CD5⁺ B cells are considered equivalent to murine B-1a cells. B-1a cells are a major source of natural IgM, which is both polyreactive and weakly autoreactive, displaying RF activity.^{28,29} On the other hand, B-1a cells also produce specific antibodies to certain antigens, such as phosphorylcholine,^{30–32} α 1-3 dextran,^{33,34} and *Francisella tularensis* lipopolysaccharide.^{35–37} *C. hepatica* infection will induce clonal expansion of selected B-1 cell clones by stimulation with the specific antigen in ChSF.

The selective proliferation of specific B-1a cells in *C. hepatica*-infected mice could be attributed to the properties of B-1 cells. B cells that play a role in the early defense from infection, such as B-1 cells and marginal zone B cells, are known to be intrinsically more prone to clonal expansion than are conventional B-2 cells.^{38–40} Among pathogen-driven CD5⁺ B-cell lymphoproliferative disorders, not only HCV-induced MC^{41,42} but also *Helicobacter pylori*-induced mucosa-associated lymphoid tissue lymphomas^{43,44} or fungal antigen β -(1, 6)-glucan-associated B-cell chronic lymphocytic leukemia⁴⁵ have been reported. Although the mechanism of clonal proliferation of CD5⁺ B cells in these disorders remains unknown, a common pathogenesis or structural similarity of specific antigens may exist among these pathogen-induced CD5⁺ B-cell lymphoproliferative disorders in humans and mice.

Herein, the reactivity of IgM, which is produced by B-1a cells stimulated with ChSF, and cryoglobulin in the infected mice against 75-kDa antigen of the worm suggest that the worm-specific antigen works as a trigger for the development of cryoglobulinemia in this model. Although the signal at approximately 75 kDa of the cryoglobulin sample was less clear than that of the culture media observed during the *in vitro* assay, our previous report suggests that IgM in the cryoprecipitate coprecipitated with various Igs. Therefore, the obscurity of the signal may be due to contamination of various Igs in the cryoprecipitate of the serum. The present results suggest that the specific antigen is soluble and located in the digestive tract of the worms. The date of onset of MC at 20 to 24 DPI was in accord with the date when adult worms parasitized the liver most severely. Therefore, the antigen released into the blood from the worms would be prone to entrapment by B-1a cells. In the *in vitro* assay, the selective proliferation of specific B-1a cells and the secretion of IgM were accelerated by stimulation with ChSF, and these responses occurred in a T-cell-independent manner. This suggests that the specific antigen would directly

stimulate B-1a cells depending on their specific B-cell receptor and in a T-cell-independent manner. This is supported by the finding that i.p. injection of ChSF induced cryoglobulinemia in IL-5tgC57BL mice despite the fact that parasitism or inflammatory response was not induced. Overall, the present findings suggest that the specific antigen stimulates B-1a cells in a T-cell-independent manner and that continuous exposure to the specific antigen contributes to clonal expansion of B-1a cells. However, the antigen that specifically stimulates IgM-RF-secreting B-1a cells could not be identified in this study. Identification of the specific antigen in this model will contribute to clarifying the mechanism of antigen-driven B-cell clonal expansion.

In adult mice, B-1a cells are the main B-cell population in the peritoneal and pleural cavities and are rare in the spleen.⁴⁶ Whether the B-1a cells that proliferated in the spleens of infected mice were derived from the spleen or peritoneal cavity was not clear in the present study. B-1 cells activated in the peritoneal cavity have been reported to migrate rapidly to the spleen, and they proliferate and differentiate into IgM-secreting cells.^{26,47} Identifying the origin of the B-1a cells will provide new insights into the role of the spleen in the development of cryoglobulinemia.

The finding that cryoglobulinemia was significantly exacerbated in IL-5-overexpressing transgenic mice compared with wild-type (C57BL) mice indicated that IL-5 is a critical factor for the development of cryoglobulinemia in *C. hepatica*-infected mice. In addition, the finding that i.p. injection of ChSF induced cryoglobulinemia without an inflammatory response, including hepatitis in IL-5 transgenic mice, clearly indicates that the selective proliferation of IgM-RF-secreting cells is regulated by one cytokine, IL-5, among the various factors associated with the inflammatory condition induced by the infection. Although the role of IL-5 in cryoglobulinemia in humans is largely unknown, it has been reported that HCV-infected MC patients had increased serum levels of IL-5 compared with noncryoglobulinemic HCV-infected patients⁴⁸; therefore, the contribution of IL-5 may be common to MCs in humans.

The IL-5-secreting cells in this model were Th2 cells, as indicated by the fact that increased IL-5 levels were induced only in the infected BALB/c mice showing a Th2 polarized immune response against the infection but not in the infected C57BL mice, which did not show this response. This suggests that differences in the host immune response against infection affect the development of cryoglobulinemia, whereas Th2 cells contribute indirectly to the development of MC by secreting IL-5. Although BALB/c mice had the potential to develop severe cryoglobulinemia by *C. hepatica* infection, i.p. injection of ChSF did not induce cryoglobulinemia in these mice. This may be because the inflammation caused by the worms parasitizing the liver is important for IL-5 production in Th2 cells, although the mechanism by which the Th2-polarized immune response was induced by the infection only in BALB/c mice could not be elucidated.

Herein, although the elevation in serum IL-5 level was temporary at 20 DPI in the infected BALB/c mice, it was associated with a dramatic increase in the severity of MC. This finding suggests that IL-5 acts at specific short periods during the selective lymphoproliferation process. These findings suggest that identification of the critical factor for MC development should be performed at a specific period during which selective lymphoproliferation occurs.

We were unable to determine whether the Th2-polarized immune response contributed only to elevating IL-5 levels or had other assisted roles in the development of MC. The MC of infected BALB/c mice was more severe than that of IL-5tgC57BL mice, which were injected with ChSF; however, whether the difference was attributed to differences in IL-5 level or the dose of the specific antigens or to other assisted roles of the Th2-polarized immune condition remains unclear. Studies have shown that CD5⁺ B cells are more resistant to apoptosis than are CD5⁻ B cells in patients with chronic HCV infection, and certain Th2 cytokines, such as IL-2, IL-4, and IL-10, which suppress apoptosis of CD5⁺ B cells, are significantly increased in HCV-infected patients.¹⁷ The role of other Th2 cytokines should be investigated more precisely in this model.

Herein, we investigated type II MC in *C. hepatica*-infected mice and showed the selective proliferation of splenic B-1a cells by co-stimulation with *C. hepatica*-specific antigen and IL-5. MC is accompanied by chronic infection in humans, and various inflammatory factors are essential for the development of MC. However, these results indicate that the development of MC in this model may be regulated by a specific cytokine, IL-5.

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Supplemental Data

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