Final Report

Project Title "The polymicrobial sepsis and fungal sepsis severity of Fc gamma receptors IIb decicient induced Systemic Lupus Erythematosus mice with sepsis and monocyte/macrophage signaling"

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June/2016 (2)

Month/ Year (finish date)

Contract No. TRG5780207

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Researcher Institute
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This project granted by the Thailand Research Fund

Abstract

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Project Period : 2 years

Abstract:

Because FcγRIIb de-funntioning polymorphism is the common genetic association with Systemic Lupus erythematosus (SLE) in Thai population, we explored the immune response to infections of FcγRIIb deficient mice, a model mimic FcγRIIb de-funntioning polymorphism, for a more understanding in the infectious immune response of SLE patients. Defunctioning-polymorphisms of FcγRIIb, an inhibitory receptor, results in the hyper immune response and increase the possibility of spontaneous auto-antibody production. Then FcγRIIb deficient mice have spontaneous anti-dsDNA at 8-wk-old and proteinuria, a manifestation of SLE, at 24-wk-old. We test the response to polymicrobial sepsis with cecal ligation and puncture (CLP) and fungal sepsis with *Cryptococcus neoforman* injection.

Surprisingly, FcγRIIb deficient mice were susceptible to Cryptococcosis (a fungal infection) but not to polymicrobial bacterial infection. Then we focus on the immune response to Cryptococcosis. We found that the unique properties of FcγRIIb deficient macrophage, apparent phagocytosis with prominent pro-inflammatory cytokines production, with the intracellular ability of *C. neoforman* leaded to the cryptococcosis susceptibility.

We proposed that the test for FcγRIIb polymorphisms should be done in patients with SLE or patients with the family history of immune-competent cryptococcosis, especially in the endemic area. These patients might be highly susceptible to cryptococcosis and need a special concern and ealier clinical recognition of disseminate cryptococcosis.

Keywords : 3-5 words FcγRIIb deficient, Polymicrobial sepsis, Cryptococcosis

Final report content:

Abstract

Defunctioning-polymorphisms of FcγRIIb, an inhibitory receptor, frequently associated with Systemic Lupus Erythematosus (SLE), especially among Asian. In parallel, Cryptococcosis is also a common invasive fungal infection of patients with SLE in Asia, perhaps due to the de novo immune defect. Then we tested cryptococcosis in a lupus model of FcγRIIb-/- mice. *C. neoformans* was administered intravenously then determined the disease severity. The mortality rate of cryptococcosis in either young asymptomatic lupus (8-wks-old without proteinuria) or symptomatic lupus (24-wks-old with proteinuria) group was higher than age-matched wild type mice. Additionally, severe cryptococcosis in FcγRIIb-/- mice was also demonstrated by high fungal burdens in internal organs with histological cryptococcoma-like lesions and high serum pro-inflammatory cytokines (TNF-α and IL-6), but not anti-inflammatory cytokine (IL-10). Interestingly, FcγRIIb-/- macrophages demonstrated the prominent phagocytosis but non-different in the killing activity compared with wild type. Indeed, *in vivo* macrophage deletion with liposomal clodronate attenuated fungal burdens in liver, lung and spleen of FcγRIIb-/- mice. As such, fungi-phagocytosed FcγRIIb-/- macrophages administered into wild type mice resulted in a higher fungal burdens in brain and liver in comparison with phagocytosed FcγRIIb+/+ macrophage injection. These results supported the enhanced fungi-dissemination through macrophages by the “Trojan horse mechanism”, organism transmigration through infected macrophages. Moreover, FcγRIIb-/- macrophages incubated *in vitro* with *C. neoformans* produced high TNF-α and IL-6 but low IL-10. This was comparable with the *in vivo* prominent cytokines response. In conclusion, the prominent phagocytosis with limited effective killing activity and high pro-inflammatory cytokines production of FcγRIIb-/- macrophage were responsible for the more severe cryptococcosis in FcγRIIb-/- mice.

Executive summary

Objective: To understand the immune response to organisms of FcγRIIb-/- mice as a model of FcγRIIb de-functioning polymorphisms which are commonly found in Asian and Thai.

Research methodology: Animal experiments

Animal, cecal ligation and puncture (CLP) and *Cryptococcus neoformans* injection model

FcγRIIb-/- mice on C57BL/6 background were kindly provided by Dr. Sylvia M. Bolland (NIAID, NIH, Maryland, USA). Female 8- and 24-wks-old C57BL/6 wild type mice were purchased from the National Laboratory Animal Center, Nakornpathom, Thailand. The animal protocols were approved by Faculty of Medicine, Chulalongkorn University followed the NIH criteria. For cecal ligation and puncture (CLP) sepsis model, cecum was ligate at 1.5 Cms from tips and needle number 21 were puncture through and through. For *C. neoforman* fungal infection model, *C. neoformans* were

isolated from patient samples (Mycology Unit, King Chulalongkorn Memorial Hospital), identified by morphology together with urease production and melanin synthesis (L-3,4-dihydroxyphenylalanine or DOPA test) and stored in Sabouraud dextrose Broth (SDB) (Thermo Scientific, Hampshire, UK) at -80°C. Before conducting all of the experiments, *C. neoformans* was subcultured on SDA at 37°C for 24h. For the individual mouse, *C. neoformans* at the dose of $1x10^5$ yeast cells counted by hemocytometer (Bright-Line, Denver, CO, USA) diluted in 200 μl of NSS were injected through tail vein. For survival analysis mice were sacrificed at 90 days after fungal administration or at the moribund stage as determined by an inability to walk after touch stimulation. In another set of experiments, mice were sacrifice at 2 wks after fungal administration. At the time of euthanasia, blood was collected through cardiac puncture under isoflurane anesthesia and internal organs (brain, heart, lung, kidney, liver and spleen) were fixed with 10% formalin for histology or processed for fungal burdens experiments (details later).

In vivo macrophage depletion

To determine the role of macrophage in cryptococcosis, macrophage depletion with liposomal-clodronate injection was performed following the previous protocol[22]. Female 8-wks-old mice of FcγRIIb-/- and wild type was administered C. neoformans through tail vein. Then liposomal clodronate (Encapsula Nanoscience, Nashville, TN, USA) at 200 μl/mouse, or control liposome, was daily injected through tail vein for 4 consecutive days, started from the 3rd days of fungi administration, to induce sustained monocyte depletion. At 7 days after fungi administration, mice were sacrificed and internal organs were processed for fungal burdens and also fixed in 10% formalin to confirm macrophage depletion by immunohistochemical staining (see below).

Transfer of fungi internalized-macrophage *in vivo*

BM macrophage from FcγRIIb-/- and wild type were allowed for phagocytosis before administered into wild type mice as previous described[25, 26]. Briefly, BM macrophage cultured in 96-well plate at 2.5 $x10^4$ cell/well with 20% mouse serum were incubated with *C. neoformans* at multiplicity of infection (MOI) ratio of 5:1 (yeast : cell) for 2 h. The un-ingested fungi were washed out 3-5 times with DMEM media. Then fungi internalized-macrophages were detached by cold-PBS for 3-5 times until no macrophage on well, centrifuged at 1,000 rpm 4°C for 10 min and re-suspended cell pellet with DMEM media. The macrophage were counted and stained with trypan blue. Either internalized fungi-FcγRIIb-/- or wild type macrophage at $2.5x10^4$ cell were intravenously administered to wild type mice through tail-vein. Mice were sacrificed at 24h later to determine fungal burdens.

Fungal burdens and organ histology

For measuring internal organs fungal burdens, the organs were weighed, homogenized, mixed well and plated in a serial volume onto Sabouraud dextrose agar (SDA) (Thermo Scientific, Waltham, MA, USA) at 37°C and counted for fungal colonies at 48h later. For the histology, tissue samples were fixed in 10% formalin and embedded in paraffin; 4 μm sections were stained with hematoxylin and eosin color (H&E) and Grocott's silver stain (GMS) for the *C. neoformans* identification. To confirm the condition of macrophage depletion, immunohistochemistry staining was performed with F4/80 antibody (Biolegend, San Diego, CA, USA) and counted under 400x magnification. Macrophages were not detectable in organs of liposomal clodronate injection either in FcGRIIb-/- mice or wild type (data not showed).

Blood and urine chemistry and supernatant media cytokines analysis

Organs injury was determined by serum creatinine (Scr) (QuantiChrom Creatinine Assay, DICT-500, BioAssay, Hayward, CA, USA) and alanine transaminase (ALT) (EnzyChrom ALT assay, EALT-100, BioAssay). Urine protein and urine creatinine were measured by Bradford protein analysis and QuantiChrom Creatinine assay (BioAssay), respectively. Urine protein creatinine index (UPCI), a representative of 24h urine protein, determined by the following equation; UPCI = spot urine protein/spot urine creatinine. Cytokines measurement (TNF-α, IL-6 and IL-10) in serum and supernatant media were measured by ELISA assays (ReproTech, Princeton, NJ, USA).

Anti-dsDNA antibodies detection

Calf DNA (Invitrogen, Carlsbad, CA, USA) coated on 96-well plates were used for measuring anti-dsDNA antibodies followed the previous protocol[27]. In short, calf DNA at100 μg/well were prepared and incubated overnight at 4 °C. Then the plate was dried, filled with 100 μl of blocking solution per well at room temperature for 1.5h and washed. Subsequently, mouse serum sample at 100 μl/well was added and incubated for 1h. Then the plate was washed, incubated with peroxidase conjugated goat anti-mouse antibodies (BioLegend, San Diago, CA, USA) at 100 μl/well at room temperature for 1h, washed and developed with ABTS Peroxidase Substrate Solution (TMB Substrate Set; BioLegend) for 10 min in dark. Finally, the stop solution (2N H_2SO_4) was added and read with microplate photometers at a wavelength of 450 nm.

Bone marrow derived macrophages preparation

Bone marrow (BM) derived macrophages were produced follows the established protocol[28]. In short, BM cells from femurs were centrifuged at 1,000 rpm in 4 C° for 10 min. Then cells were incubated in the high glucose DMEM supplement with 10% fetal bovine serum, 1%

penicillin/streptomycin, HEPES with sodium pyruvate, 5% horse serum and 20% L929-conditioned media in humidified 5% CO_2 incubator at 37 °C for 7 days. Subsequently, cells were harvested at the end of the culture period using very cold PBS and confirmed macrophage phenotype with anti-F4/80 and anti-CD11c antibodies (BioLegend).

In vitro Cryptococcus neoformans induced macrophage cytokines production

Heat-killed *C. neoformans*, by immersion in 60°C water bath for 1h , or live *C. neoformans* at the dose of $5x10^5$ yeast cells were incubated with macrophage $1x10^5$ cells/well in 96 well polystyrene tissue culture plate[29]. The culture supernatant was collected at various time points and stored at -80 °C until used. After the incubation, cell viability was measured by MTS cell proliferation assay (One Solution Cell Proliferation Assay, Promega Corporation, WI, USA) according to the manufacturer's instruction[30]. In short, 20 µl of MTS was added to the culture plates for 2h at 37°C in 5% CO_2 incubator then read with microplate photometers with a wavelength at 490 nm. All experiments showed cell viability more than 95% (data not showed).

Phagocytosis and intracellular macrophage killing assays

The phagocytosis and intracellular killing were performed followed the previous protocol with slightly modification[25, 26]. In short, BM derived macrophages were put into 96 well-tissue culture plate with $2.5x10^4$ cell/well in DMEM complete containing L929 culture supernatant or BMM media and 500 ng LPS. After incubation in 5% CO_2 at 37 ^{o}C for 24h, media were removed then DMEM complete at 100 µl with 20% normal mouse serum, a source of opsonin, were added with the heat-killed *C. neoformans* at the ratio of macrophage: yeast at 5:1 and 10:1 and incubated for various duration. After the incubation, media were removed and wash with 200 µl of PBS at least 3 times to remove un-ingested yeast cell and then detach macrophage with 200µl of cold-PBS. Then macrophages were transferred to CytoSpin chamber (Thermo Sciencetific), centrifuged at 600 rpm for 5 min to isolate into the single cell layer for the easier visualization and stained with Diff-Quick staining (Life Science Dynamic Division, Nonthaburi, Thailand). Macrophages with the close proximity to blastospores were count as the macrophages with phagocytosis. At least 100 macrophages per well were counted. In parallel, the ingestion ability of each individual macrophage was determined by an average number of fungi in each macrophage (phagocytosis index) calculated by the total number of ingested fungi divided by the total number of macrophage. The phagocytosis activity was determined by the percentage of macrophage with phagocytosis and phagocytosis index. All experiments performed in triplicate.

For Killing activity, BM derived macrophages at $1x10^5$ cell/well were co-cultured with live *C. neoformans* with the ratio of macrophage: yeast at 1:1 for 2, 4 and 24 h. After incubation,

culture supernatant was discarded and a lysis medium (distilled water containing 0.01% bovine serum albumin and 0.01% Tween-80) was added to rupture macrophage cell membrane. Serial dilutions of the lysates were plated on SDA for yeast viability colony forming unit (CFU) count. Macrophage killing activity is reverse correlated with the number of yeast colonies.

Statistical analysis

The mean ± SE was used for the data presentation and the differences between groups were examined for statistical significance using the unpaired Student t-test or one-way analysis of variance (ANOVA) with Tukey's comparison test for the analysis of experiments with 2 and 3 groups, respectively. Survival analyses were evaluated with the log-rank test. *P* values < 0.05 were considered statistically significant. SPSS 11.5 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis.

Result

Lupus nephritis in FcγRIIb-/- mice developed spontaneously; Scr, urine protein (determined by urine protein creatinine index: UPCI) and anti-dsDNA of mice at 8-wks and 24-wks-old were 0.19±0.04 mg/dl, 16.25±3.38 mg/mg and 0.58±0.33 unit versus 0.24±0.05 mg/dl, 54.75±4.03 mg/mg and 2.28±0.43 unit, respectively (fig 1A-C). Age-related lupus manifestations in FcγRIIb-/- mice allowed to exploring 2 phases of the disease; asymptomatic lupus (8-wks-old mice) and symptomatic lupus (24-wks-old mice) as determined by proteinuria and anti-dsDNA. However, renal function determined by Scr was not different between these groups (fig 1A-C). Scr at 8-wks old and 24-wks old in FcγRIIb-/- mice were 0.19±0.04 versus 0.24±0.05 mg/dl, respectively. We selected young (8 weeks old) and old mice (24 weeks old) to challenge with bacterial sepsis (cecal ligation and puncture) and fungal sepsis (cryptococcosis). In sepsis model, we found that young mice did not show different mortality rate but there was a significant different in an old mice (data not showed). However, it is not a surprise that old mice with preconditioning by the kidney injury as demonstrate by proteinuria showed higher mortality rate. But the equal mortality rate of sepsis in young mice regardless of the Fcgr genotype demonstrated that the innate immune response to an acute bacterial infection is intact in Fcgr -/- mice. Further investigation in the part of innate immune response to bacterial infection might not worth following. Then we focus on fungal sepsis.

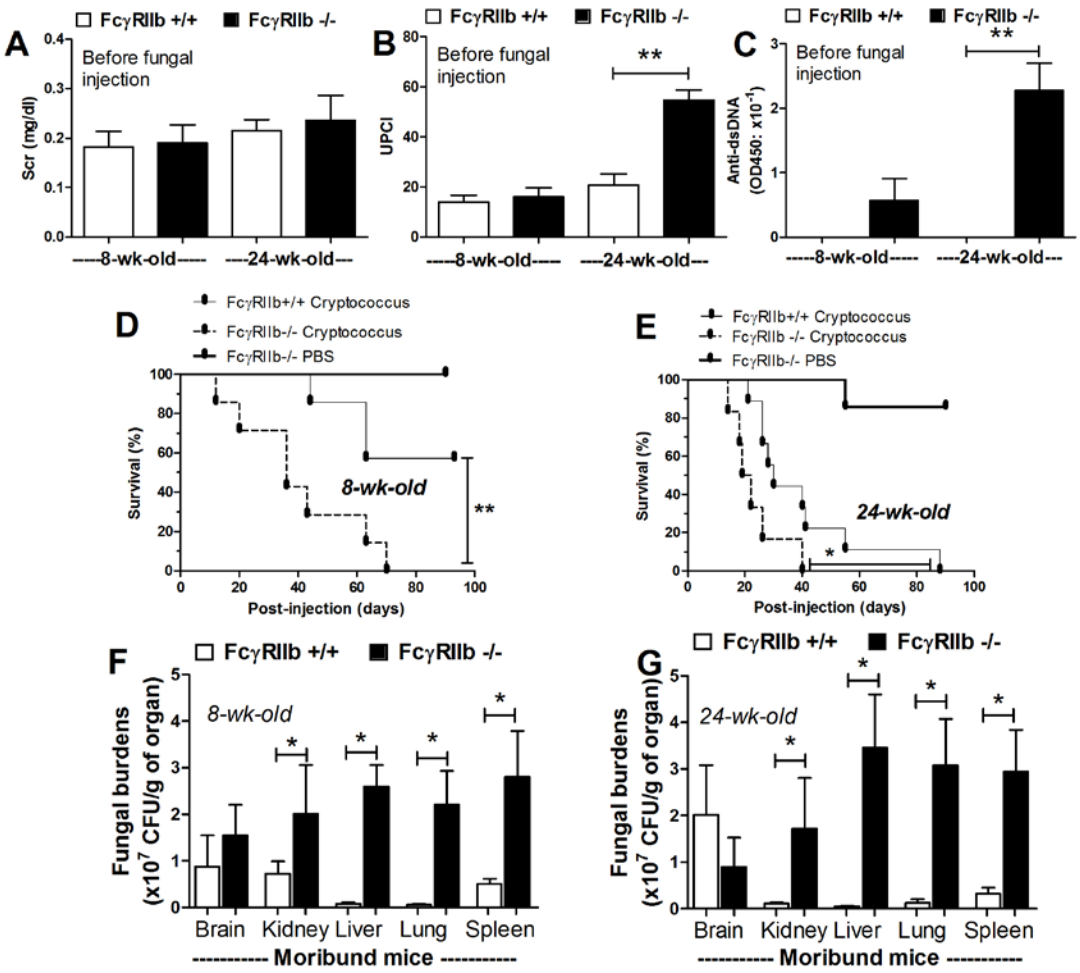

Figure 1. The characteristics of FcγRIIb-/-mice in younger and older age, at 8 and 24 wks-old, respectively, as demonstrated by serum creatinine (Scr) (A), proteinuria by urine protein creatinine index (UPCI) (B) and anti-dsDNA (C), (n=4-5/group). The survival analysis after *C. neoformans* administration in 8 wks-old (n=7/group: D) and 24 wks-old mice (n=7-9/ group: E) were showed. In parallel, the fungal burdens in several internal organs of younger (n = 4-6/group: F) and older mice (n = 5-6/group: G) demonstrated. ** $p<0.05$, ** $p<0.01$*

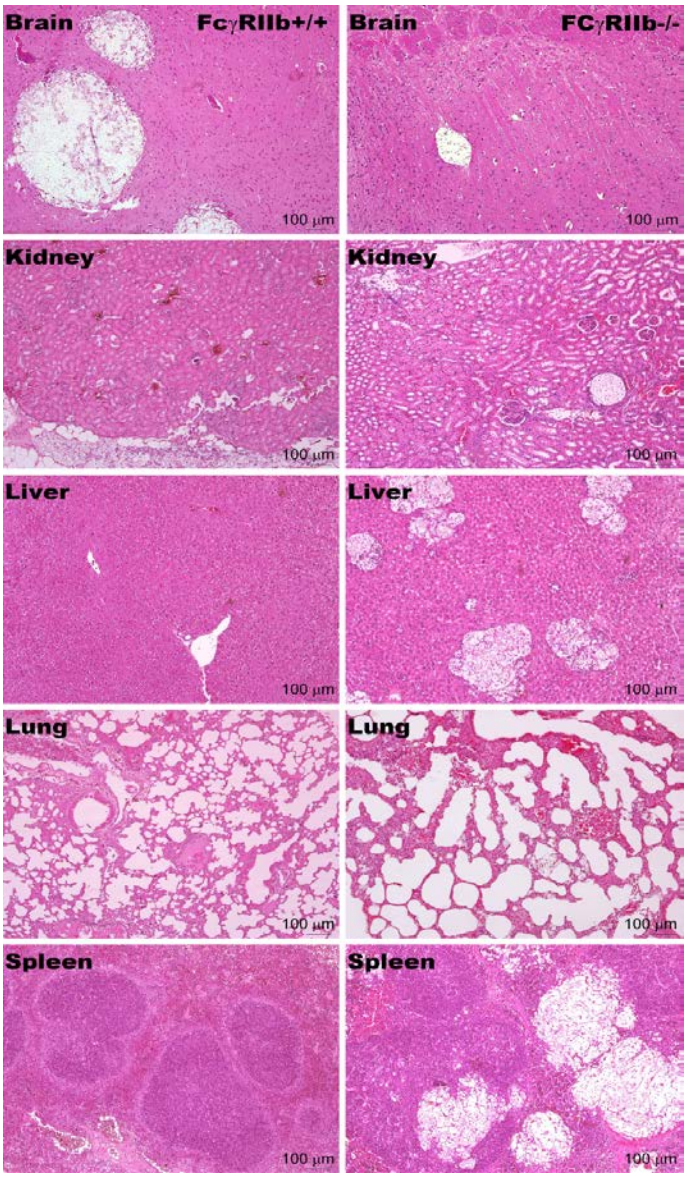

Figure 2. Representative histology with H&E (Hematoxylin and eosin staining) at x100 original magnification from 24 wks-old mice in the moribund stage after *C. neoformans* administration demonstrated the cryptococcoma-liked lesions only in brain of FcγRIIb+/+ (left column) but in several internal organs (brain, kidneys, liver, lung and spleen) of FcγRIIb-/- mice (right column).

High severity of cryptococcosis demonstrated in Fc gamma receptor IIb deficient mice either 8-wks-old, asymptomatic lupus, or 24-wks-old, symptomatic lupus

After *C. neoformans* administration, FcγRIIb-/- mice demonstrated the higher mortality rate than age-matched wild type control either in asymptomatic or symptomatic lupus group (fig 1D-F). In young mice group, all FcγRIIb-/- mice but only 57% of wild type mice reached moribund stage within 90 days of the observation (fig 1D). In parallel, in older age group, all mice with cryptococcosis reached the moribund stage within 40 and 90 days in FcγRIIb-/- and wild type, respectively (fig 1E). Interestingly, moribund FcγRIIb-/- mice demonstrated the higher fungal burdens and cryptococcoma-liked-lesions in several internal organs; brain, kidney, liver, lung and spleen in both young and old age group (fig 1F, G and 2). On the other hand, such lesions in wild type mice were found only in brain, a major organ of infection, in moribund stage of both age groups (fig 2, showed only old mice).

At the moribund stage of cryptococcosis in younger age group; Scr, ALT and cytokines (TNF-α, IL-6 and IL-10) in wild type vs. FcγRIIb-/- mice were 0.33±0.02 mg/dl, 49±9 U/L and 99±10, 89±14 and 389±142 pg/ml vs. 0.32±0.04 mg/dl, 77±7 U/L, and 147±8, 257±48 and 446±199 pg/ml, respectively (fig 3A-E). In parallel, these parameters of the older age group were 0.43±0.04 mg/dl, 39±7 U/L, 82±2, 47±16 and 327±50 pg/ml vs. 0.58±0.08 mg/dl, 87±9 U/L and 123±7, 307±227 and 403±59 pg/ml, respectively (fig 3F-J). At 2 wks of cryptococcosis, fungal burdens in the internal organs were higher in FcγRIIb-/- mice in parallel with the moribund stage (fig 4A, E) and cryptococcoma-liked-lesions found in most organs in FcγRIIb-/- mice but only in brain and kidney in wild type in both age groups (fig 5, showed only young mice). In addition, after 2 wks of fungi administration, TNF-α, IL-6 and IL-10 in wild type vs. FcγRIIb-/- mice in the younger age group were 50±9, 55±19 and 117±21 vs. 87±17, 112±41 and 82±11 pg/ml, respectively (fig 4A-D). In parallel, TNF-α, IL-6 and IL-10 in wild type vs. FcγRIIb-/- mice of the older age group were 45±5, 93±8 and 220±16 vs. 94±9, 184±36 and 177±34 pg/ml, respectively (fig 4E-H).

In brief, cryptococcosis either at moribund or at 2 wks, FcγRIIb-/- mice demonstrated the more severe fungal burdens in most internal organs (except for brain), higher liver enzyme and pro-inflammatory cytokines (but not anti-inflammatory cytokine), in comparison with wild type.

FcγRIIb-/- macrophage in responses to *C. neoformans*; a prominent phagocytosis and an apparent pro-inflammatory cytokine production

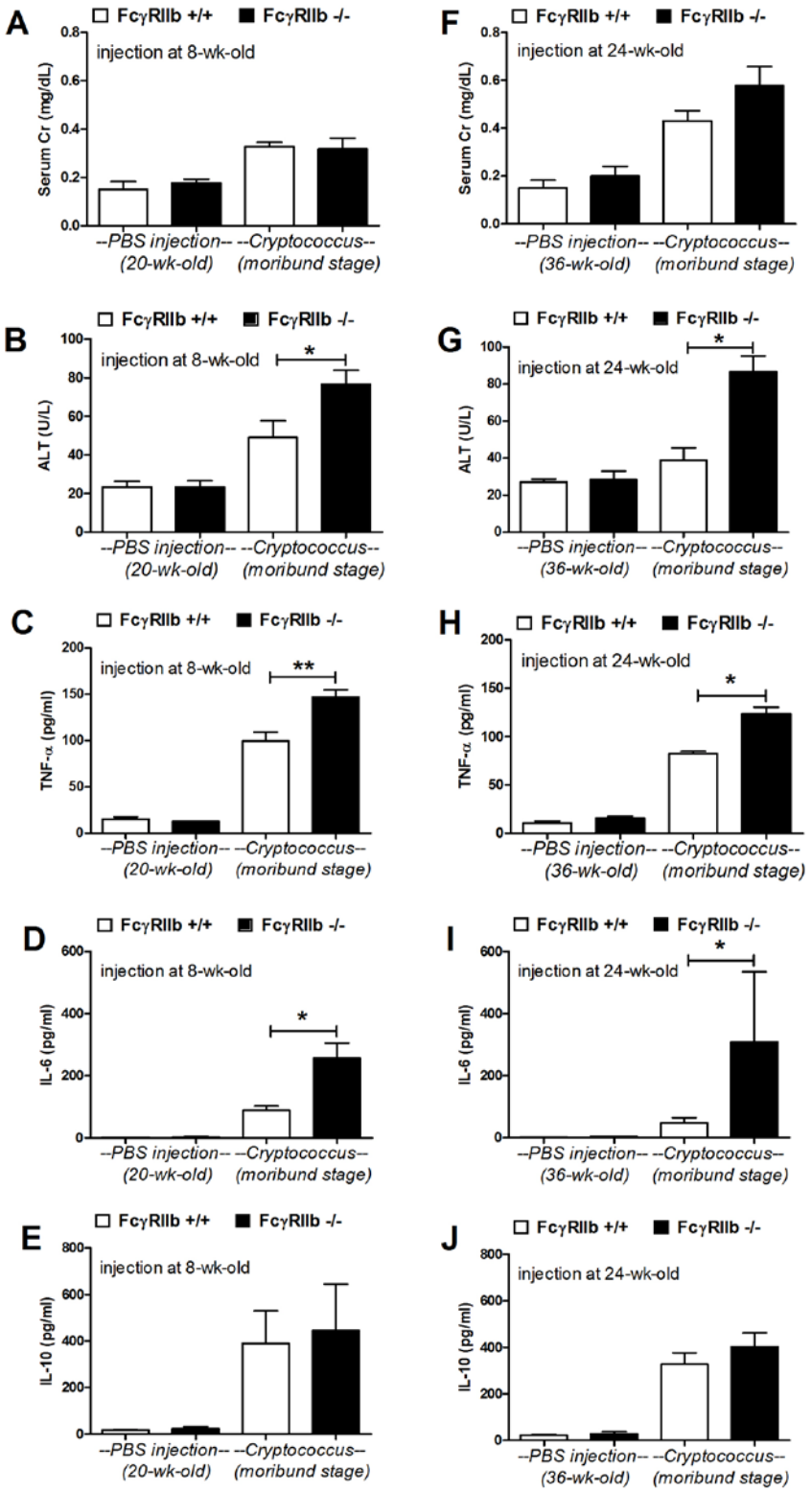

Figure 3. The organs injury and inflammatory cytokines at the moribund stage in 8 wks-old (left column) (A-E) and 24 wks-old mice (right column) (F-J) as demonstrated by serum creatinine (Scr), alanine transaminase (ALT), TNF-α, IL-6 and IL-10 were showed. (n = 4-5/group), * $p<0.05$

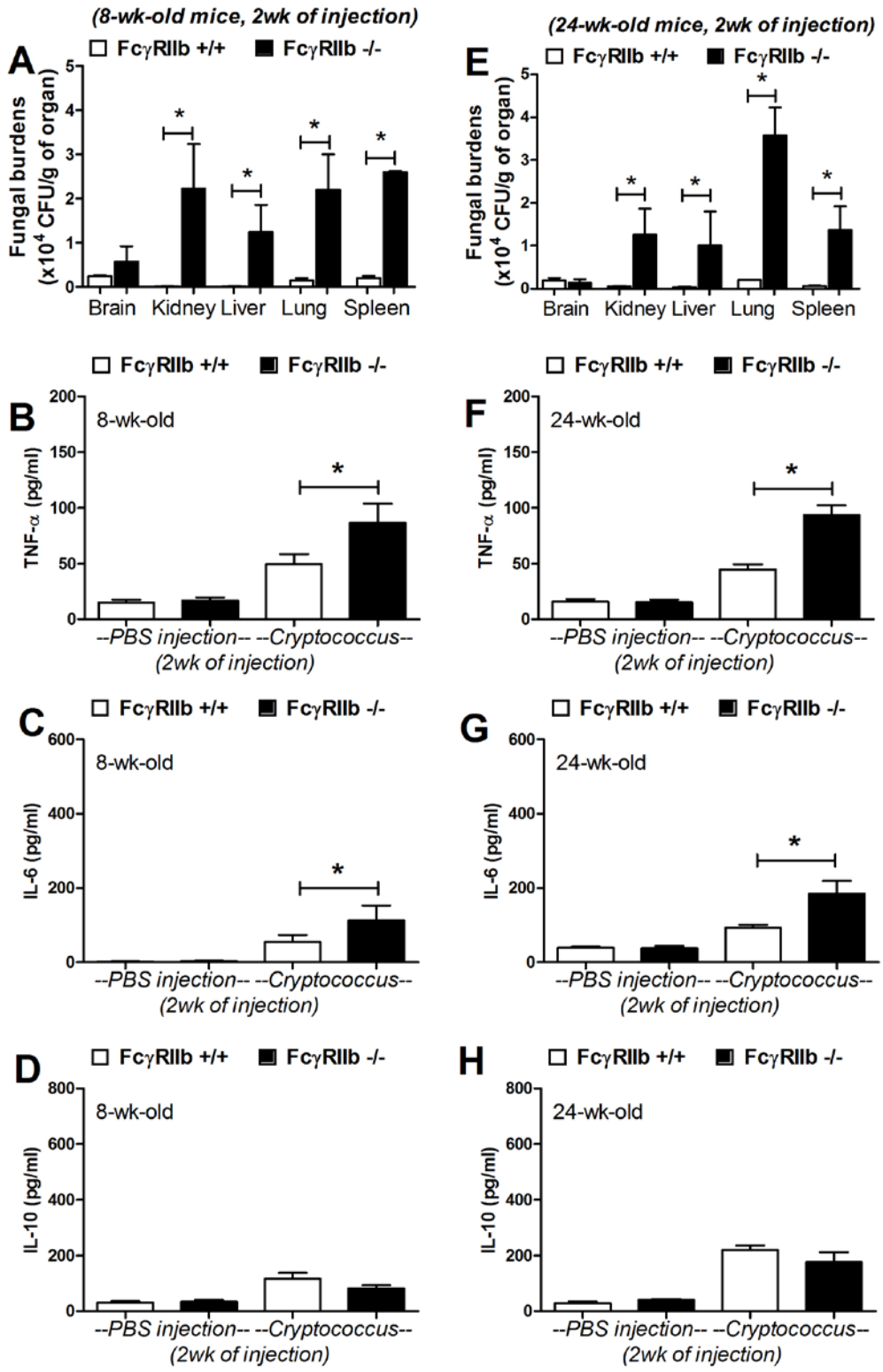

Figure 4. The fungal burdens in the internal organs of 8 wks-old (left column) and 24 wks-old mice (right column) at 2 wks after C. neoformans administration (A, B) and serum cytokines as demonstrated by TNF-α (C, D), IL-6 (E, F) and IL-10 (G, H) were showed. (n = 4-5/group), *$p<0.05$

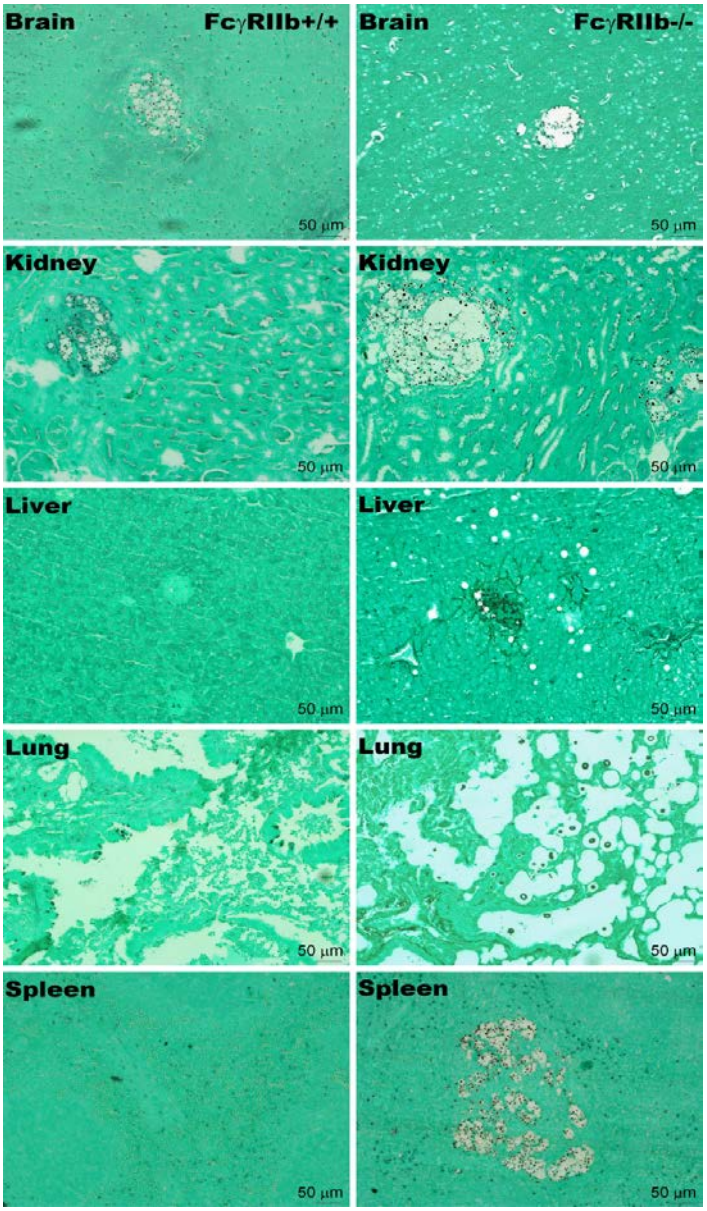

Figure 5. Organs histology with Grocott's silver staining (GMS) at x50 original magnification from 8 wks-old mice after 2 wkss of *C. neoformans* administration demonstrated the cryptococcoma-liked lesions in brain and kidney of FcγRIIb+/+ (left column) but in several internal organs (brain, kidneys, liver, lung and spleen) of FcγRIIb-/- mice (right column).

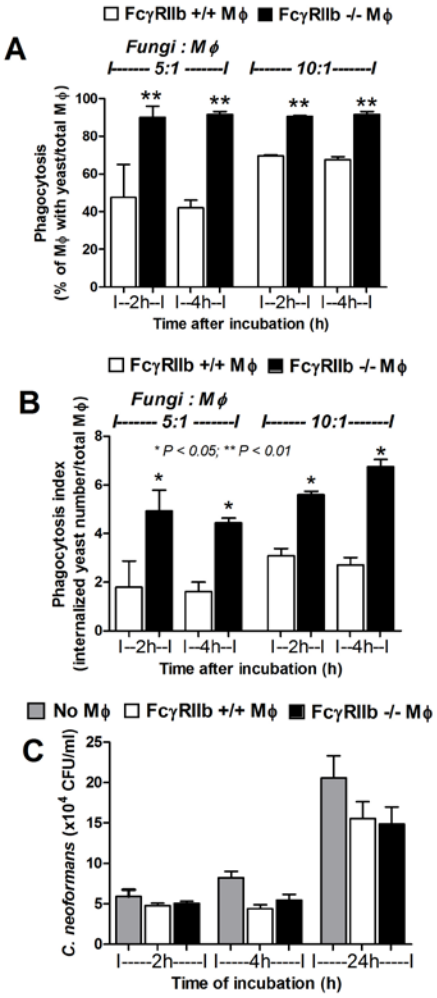

Figure 6. The percentage of macrophage (MΦ) undergoing phagocytosis, demonstrated by the presentation of fungi in a close proximity, (A) and the average number of phagocytosed fungi per MΦ, calculated by total number of phagocytosed fungi/ total MΦ, (B) after incubated with *C. neoformans* at the ratio of fungi: MΦ at 5:1 and 10:1 for 2 and 4h in FcγRIIb-/- and wild type macrophage was showed. In parallel, the killing activity of MΦ determined by the number of *C. neoformans* after 2, 4 and 24h incubation with MΦ of FcγRIIb-/- or wild type in comparison with no MΦ groups (C) were demonstrated. * *p<0.05,* ** *p<0.01* (Separated experiments were done in triplicate)

Due to the importance of macrophages against fungal infection and the availability of FcγRIIb in macrophage, we explored macrophage responses to *C. neoformans*. Interestingly, percentage of macrophages with phagocytosis process after incubated with live *C. neoformans* in the ratio of fungi:

cell at 5:1 and 10:1 for 2h in wild type versus FcγRIIb-/- cell were 47.5±17.5% and 69.5±0.5% vs. 90±6% and 90.5±0.5 %, respectively, and for 4h incubation were 42±4% and 67.5±1.5% vs. 91.5±1.5% and 91.5±1.5 %, respectively (fig 6A). FcγRIIb-/- macrophages were easier triggered into the phagocytosis process. In parallel, with the ratio at 5:1 and 10:1, the average fungi in each macrophage of wild type and FcγRIIb-/- mice for 2h incubation were 1.8±1.1 and 3.1±0.3 (at ratio 5:1) and 4.9±0.9 and 5.6±0.1 (at ratio 10:1), respectively. In parallel, the parameters for 4h incubation were 1.6±0.4 and 2.7±0.3 (at ratio 5:1) vs. 4.5±0.2 and 6.7±0.3 (at ratio 10:1), respectively (fig 6B). In contrast, macrophage killing activity determined by fungal viability after incubated with macrophage for 2, 4 and 24h was not different between wild type and FcγRIIb-/- cell (fig 6C). The number of viable fungi *in vitro* at 2, 4, and 24h of fungi alone versus fungi with wild type cells versus fungi with FcγRIIb-/- macrophages were 5.9±0.8, 4.8±0.3 and 5.1±0.3 vs. 8.2±0.8, 4.4±0.5 and 5.5±0.7 vs. 20.6±2.8, 15.6±2.1 and 14.9±2.1 ($x10^4$) CFU/ml, respectively (fig 6C).

In addition, cytokine responses of FcγRIIb-/- macrophage to fungi were tested. Indeed, FcγRIIb-/- macrophages in response to heat-killed or live *C. neoformans* produced a higher TNF-α and IL-6 but lower IL-10 (fig 7) resemble to the *in vivo* results (fig 3, 4).

FcγRIIb-/- macrophage enhance Trojan horse mechanism induced cryptococcal dissemination *C. neoformans* is a facultative intracellular pathogen that could be viable intracellularly and could utilized host phagocytes for fungi transmigration as refer to “Trojan horse mechanism”[16]. Together with the prominent phagocytosis capacity but limited killing activity of FcγRIIb-/- macrophages, we hypothesized that FcγRIIb-/- macrophages are responsible for the more severe cryptococcosis *in vivo*. Then we tested cryptococcosis in FcγRIIb-/- and wild type mice with liposomal clodronate induced macrophage depletion. Interestingly, macrophage depletion attenuated fungal burdens in liver, lung and spleen in FcγRIIb-/-mice, but not in wild type (fig 8). Fungal burdens at 7 day after fungi administration in brain, kidney, liver, lung and spleen of FcγRIIb-/- mice with control liposome versus liposomal clodronate were 2.8±1.2, 2.8±0.9, 3.7±1.0, 4.4±1.4, 5.5±0.1 vs. 1.9±1.4, 1.8±0.6, 1.0±0.2, 0.9±0.3 and 0.7±0.2 ($x10^4$) CFU per organ weight (g), respectively (fig 8). Subsequently, to see if a high phagocytosis capacity of FcγRIIb-/- macrophages possibly enhanced cryptococcal dissemination, we incubated fungi with wild type and knock-out macrophages and administered phagocytosed cells into wild type mice. Indeed, fungal burdens in brain, a major infected organ of cryptococcosis, and in liver was higher in mice with phagocytosed FcγRIIb-/- cells injection (fig 9). Fungal burdens in brain, kidney, liver, lung and spleen at 1 day after the administration of macrophages from wild type versus from FcγRIIb-/- cells were 1.1±0.2, 8.3±2.5, 15.1±3.4, 16.1±5.3, 20±7.7 vs. 2.9±0.4, 31.4±9.5, 44.8±5.2, 70.7±31.2 and 53.3±15.7 ($x10^2$) CFU per organ weight (g), respectively (fig 9).

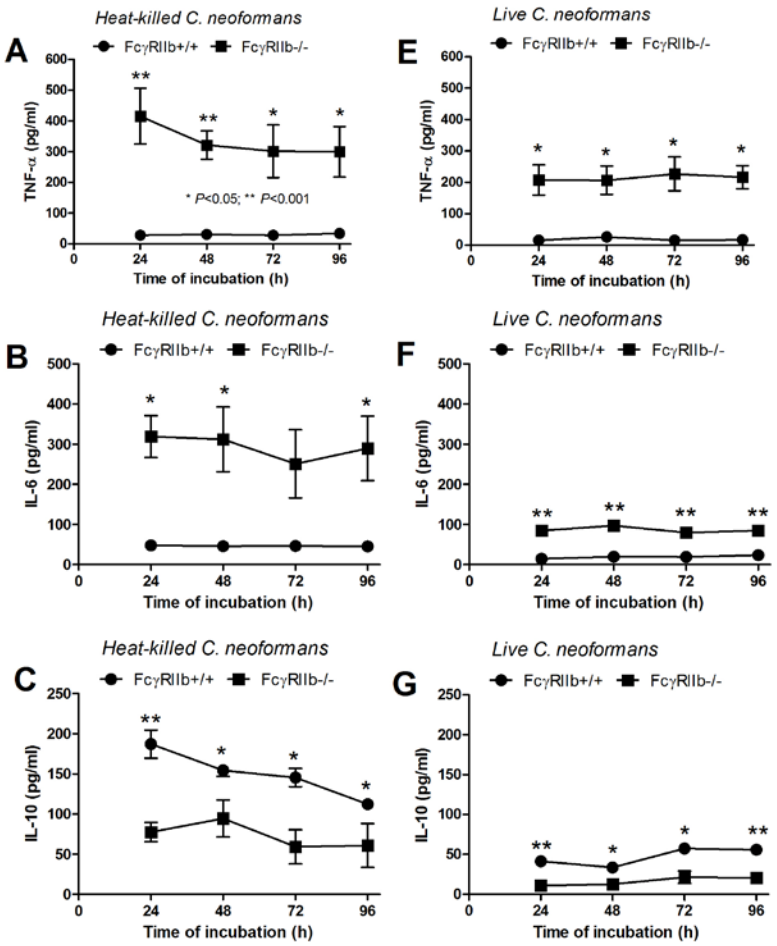

Figure 7. The cytokine responses in supernatant media from macrophages of wild type (FcγRIIb+/+) or FcγRIIb-/- after activated with heat-killed (left column) and live *C. neoformans* (right column) as demonstrated by TNF-α (A, B), IL-6 (C, D) and IL-10 (E, F). * *p<0.05,* ** *p<0.01* (Separated experiments were done in triplicate)

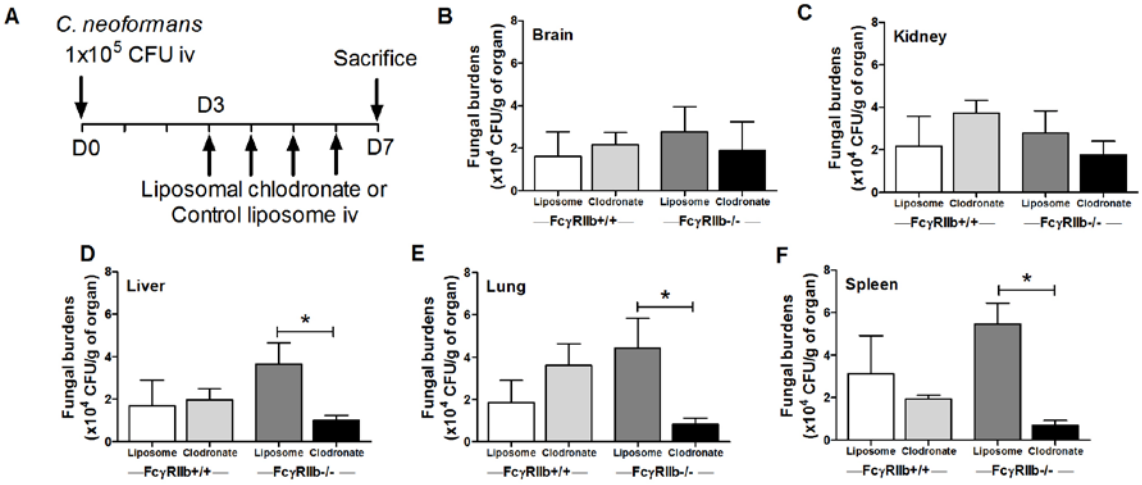

Figure 8. Timeline of a model of cryptococcosis in liposomal clodronate induced macrophage depletion in 8-wks-old mice of FcγRIIb+/+ and FcγRIIb-/- (A) and fungal burdens on brain (B), kidney (C), liver (D), lung (E) and spleen (F) were showed (n = 4/group). * *p<0.05*

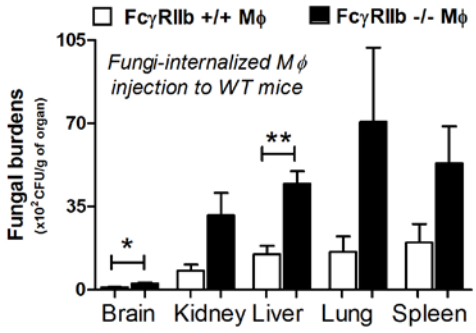

Figure 9. Organs fungal burdens of wild type mice at 24h after administration of fungi-internalized macrophages from FcγRIIb+/+ and FcγRIIb-/- mice was showed. (n = 3/group). * *p<0.05, ** p<0.01*

Conclusion and Discussion

FcγRIIb defunctioning polymorphism is one of the important genetic associations of SLE, especially among Asian[12-15]. Likewise, studying in FcγRIIb-/- mice, a lupus nephritis model, should be a good representative mouse model of SLE in Asian. We demonstrated the high severity of cryptococcosis in FcγRIIb-/- mice due to the unique properties of FcγRIIb-/- macrophage including prominent phagocytosis and apparent pro-inflammatory cytokines responses. These characteristics lead to a more severe dissemination as refer to the “Trojan horse mechanism” and a severe sepsis cytokine storm in cryptococcosis.

FcγRIIb-/- mice showed a higher severity of cryptococcosis compared with wild type Immune response against fungal infection is predominantly depended on cell mediated immune responses. The defect of FcγRIIb, an only inhibitory receptor in FcγR family, induces hyper-responsive immune responses and effectively controls several organisms[7-9]. We demonstrated that FcγRIIb-/- mice was susceptible to cryptococcosis, either in symptomatic or asymptomatic lupus, in comparison to the age-matched wild type control. Indeed, the higher mortality rate of cryptococcosis was age-dependent. The mortality rate in wild type mice at 8-wks-old vs. 24-wks-old was different (p = 0.015 by log-rank test, fig 1A and E; no direct comparison demonstrated), but not different between FcγRIIb-/- groups. The severity of cryptococcosis in FcγRIIb-/- mice was independent to lupus manifestations and mouse age, perhaps due to the same *de novo* immune defect in both age-groups. Nevertheless, these results supported the susceptibility of cryptococcosis in patients with FcγRIIb polymorphisms either with SLE or non-SLE[10-12].

Subsequently, the severity of cryptococcosis was determined with different parameters in the moribund stage and at the earlier stage of infection (at 2 wks) in 2 age-groups which, as expected, showed the corresponding results. The generalized cryptococcosis, fungi found in several internal organs, demonstrated in FcGRIIb-/- mice compatible with the generalized cryptococcosis of patients with compromised immune system[17]. But the lesion limited mostly in brain, a major target organ[18], in wild type as found in immune-competent host. As expected, the lesion could worsen organs function as demonstrated by a more liver injury in FcγRIIb-/- mice. However, there were also cryptococcoma-like lesion in wild type kidneys at 2 wks of infection (fig 5) but not at the moribund stage (fig 2) implied the kinetics of this lesion. The generalized cryptococcosis implied the synergistic immunological conditions of FcGRIIb-/- mice toward the cryptococci dissemination.

Moreover, inflammatory cytokines, TNF-α and IL-6, but not IL-10 were higher in FcγRIIb-/- mice over wild type implied the higher inflammatory responses probably to the higher fungal burdens or the *de novo* prominent pro-inflammatory stage. Perhaps, IL-10 is relatively too low to balance with the pro-inflammatory immune responses lead to the more severe sepsis.

Immune responses of FcγRIIb-/- macrophage to *C. neoformans*; Trojan horse mechanism enhancement and inadequate IL-10 production Macrophages and T helper cells are the main immune cells responsible for the immune responses to cryptococcosis[19, 20]. Due to the availability of FcγRIIb in macrophages but not in T cells[21], we hypothesized that the higher fungal burdens in FcγRIIb-/- mice was due to the primary defect in macrophages. Then we tested the phagocytosis, killing activity and cytokines response of macropahges to *C. neoformans*.

Interestingly, the phagocytosis of FcγRIIb-/- macrophages in response to cryptococci was very prominent compared with wild type cells resemble to reports with other organisms[8, 9]. In FcγRIIb-/-, nearly all of the macrophages incubated with lived *C. neoformans* underwent phagocytosis with a higher phagocytosis capacity, approximately 6-7 yeasts per cell at 4h (phagocytosis index). In contrast, only about 50-60% of wild type macrophage showed phagocytosis with a less capacity, 2-3 yeast per cell. In contrast, the killing activity of FcGRIIb-/- macrophages was not different with wild type, unlike the responses to other organisms mentioned[8], perhaps due to the immune evasion property of *C. neoformans*[20].

Indeed, cryptococcus is a facultative intracellular pathogen which could utilize host macrophages to spread within the body as refer to Trojan horse mechanism[16]. Cryptococci intend to escape extracellular immune responses, be survive and replicate intracellular, lateral transfer between macrophages and eventually expulse into the target organs[16]. They could use macrophages as trafficking vehicles for the dissemination, especially to pass through blood brain barrier into the central nervous system[22]. Interestingly, the depletion of macrophage, at least in certain situations, associated with the less severe injury[23]. We hypothesized that the prominent phagocytosis of FcγRIIb-/- macrophage and the immune evasion properties of *C. neoformans* enhanced the Trojan horse mechanism resulted in the more severe cryptococcosis *in vivo*. Then we tested cryptococcosis severity in macrophage depletion model with daily liposomal clodronate injection in FcγRIIb-/- and wild type mice. Indeed, at 1 wk after fungi administration, depletion of macrophage resulted in the lower fungal burdens in liver, lung and spleen of FcγRIIb-/- mice but not in wild type mice. In addition, to further support the importance of macrophage to cryptococcosis pathogenesis, FcγRIIb-/- and wild type macrophages after internalization of fungi was administered into wild type mice. As expected, the inoculation of fungi internalized-FcγRIIb-/- macrophages increased fungal burdens in brain and liver of wild type mice at 24h after fungi administration. This supported a high phagocytosis capacity of FcγRIIb-/- macrophage and the enhance transmigration of fungi by macrophage, especially, through blood brain barrier. All in all, these results, at least in part, supported the importance of macrophages in cryptococcosis pathogenesis in FcγRIIb-/- mice and perhaps in patients with FcγRIIb defunctioning polymorphisms.

In addition, the prominent pro-inflammatory cytokines (TNF-α and IL-6) but not anti-inflammatory cytokine (IL-10) responses demonstrated in FcγRIIb-/- groups either *in vivo* or *in vitro*. Because glucuronyloxylomannan (GMX), a molecule shed from *C. neoformans* could directly bind to FcγRIIb receptor[24], the relatively low IL-10 in FcγRIIb-/- groups might due to the absence of the receptor. Nevertheless, the very high TNF-α and IL-6 but relatively low IL-10 could lead to an easier septic shock in FcγRIIb-/- mice with cryptococcosis.

Taken together, we concluded that a more severe cryptococcosis in FcγRIIb-/- mice was due to enhanced dissemination through the Trojan horse mechanism and hyper-responsiveness of pro-inflammatory cytokines production during sepsis. The screening for FcγRIIb polymorphisms in patients with SLE, especially in endemic area of cryptococcosis, might be beneficial for the prevention strategies.

Reference

1. Tsokos, G.C. Systemic lupus erythematosus. *N Engl J Med* 365, 2110-21 (2011).

2. Crispin, J.C., Hedrich, C.M. & Tsokos, G.C. Gene-function studies in systemic lupus erythematosus. *Nat Rev Rheumatol* 9, 476-84 (2013).

3. Radaev, S. & Sun, P. Recognition of immunoglobulins by Fcgamma receptors. *Mol Immunol* 38, 1073-83 (2002).

4. Bajtay, Z., Csomor, E., Sandor, N. & Erdei, A. Expression and role of Fc- and complement-receptors on human dendritic cells. *Immunol Lett* 104, 46-52 (2006).

5. Bolland, S. & Ravetch, J.V. Spontaneous autoimmune disease in Fc(gamma)RIIB-deficient mice results from strain-specific epistasis. *Immunity* 13, 277-85 (2000).

6. Nimmerjahn, F. & Ravetch, J.V. Fcgamma receptors: old friends and new family members. *Immunity* 24, 19-28 (2006).

7. Maglione, P.J., Xu, J., Casadevall, A. & Chan, J. Fc gamma receptors regulate immune activation and susceptibility during Mycobacterium tuberculosis infection. *J Immunol* 180, 3329-38 (2008).

8. Clatworthy, M.R. & Smith, K.G. FcgammaRIIb balances efficient pathogen clearance and the cytokine-mediated consequences of sepsis. *J Exp Med* 199, 717-23 (2004).

9. Clatworthy, M.R. et al. Systemic lupus erythematosus-associated defects in the inhibitory receptor FcgammaRIIb reduce susceptibility to malaria. *Proc Natl Acad Sci U S A* 104, 7169-74 (2007).

10. Chen, H.S., Tsai, W.P., Leu, H.S., Ho, H.H. & Liou, L.B. Invasive fungal infection in systemic lupus erythematosus: an analysis of 15 cases and a literature review. *Rheumatology (Oxford)* 46, 539-44 (2007).

11. Hu, X.P. et al. Association of Fcgamma receptor IIB polymorphism with cryptococcal meningitis in HIV-uninfected Chinese patients. *PLoS One* 7, e42439 (2012).

12. Chu, Z.T. et al. Association of Fcgamma receptor IIb polymorphism with susceptibility to systemic lupus erythematosus in Chinese: a common susceptibility gene in the Asian populations. *Tissue Antigens* 63, 21-7 (2004).

13. Jakes, R.W. et al. Systematic review of the epidemiology of systemic lupus erythematosus in the Asia-Pacific region: prevalence, incidence, clinical features, and mortality. *Arthritis Care Res (Hoboken)* 64, 159-68 (2012).

14. Siriboonrit, U. et al. Association of Fcgamma receptor IIb and IIIb polymorphisms with susceptibility to systemic lupus erythematosus in Thais. *Tissue Antigens* 61, 374-83 (2003).

15. Tsuchiya, N. & Kyogoku, C. Role of Fc gamma receptor IIb polymorphism in the genetic background of systemic lupus erythematosus: insights from Asia. *Autoimmunity* 38, 347-52 (2005).

16. Voelz, K. & May, R.C. Cryptococcal interactions with the host immune system. *Eukaryot Cell* 9, 835-46 (2010).

17. Meesing, A. et al. Disseminated cryptococcosis in an HIV-seronegative pregnant woman with transient T-lymphocytopenia: a case report and review of the literature. *Southeast Asian J Trop Med Public Health* 45, 647-53 (2014).

18. Srikanta, D., Santiago-Tirado, F.H. & Doering, T.L. Cryptococcus neoformans: historical curiosity to modern pathogen. *Yeast* 31, 47-60 (2014).

19. Koguchi, Y. & Kawakami, K. Cryptococcal infection and Th1-Th2 cytokine balance. *Int Rev Immunol* 21, 423-38 (2002).

20. Garcia-Rodas, R. & Zaragoza, O. Catch me if you can: phagocytosis and killing avoidance by Cryptococcus neoformans. *FEMS Immunol Med Microbiol* 64, 147-61 (2012).

21. Ravetch, J.V. & Bolland, S. IgG Fc receptors. *Annu Rev Immunol* 19, 275-90 (2001).

22. Charlier, C. et al. Evidence of a role for monocytes in dissemination and brain invasion by Cryptococcus neoformans. *Infect Immun* 77, 120-7 (2009).

23. Kechichian, T.B., Shea, J. & Del Poeta, M. Depletion of alveolar macrophages decreases the dissemination of a glucosylceramide-deficient mutant of Cryptococcus neoformans in immunodeficient mice. *Infect Immun* 75, 4792-8 (2007).

24. Monari, C. et al. Microbial immune suppression mediated by direct engagement of inhibitory Fc receptor. *J Immunol* 177, 6842-51 (2006).

25. Nicola, A.M. & Casadevall, A. In vitro measurement of phagocytosis and killing of Cryptococcus neoformans by macrophages. *Methods Mol Biol* 844, 189-97 (2012).

26. Vonk, A.G., Wieland, C.W., Netea, M.G. & Kullberg, B.J. Phagocytosis and intracellular killing of Candida albicans blastoconidia by neutrophils and macrophages: a comparison of different microbiological test systems. *J Microbiol Methods* 49, 55-62 (2002).

27. Mihara, M. et al. CTLA4Ig inhibits T cell-dependent B-cell maturation in murine systemic lupus erythematosus. *J Clin Invest* 106, 91-101 (2000).

28. Boonyatecha, N., Sangphech, N., Wongchana, W., Kueanjinda, P. & Palaga, T. Involvement of Notch signaling pathway in regulating IL-12 expression via c-Rel in activated macrophages. *Mol Immunol* 51, 255-62 (2012).

29. Guillot, L., Carroll, S.F., Homer, R. & Qureshi, S.T. Enhanced innate immune responsiveness to pulmonary Cryptococcus neoformans infection is associated with resistance to progressive infection. *Infect Immun* 76, 4745-56 (2008).

30. Lee, H.S. et al. Anti-oxidizing effect of the dichloromethane and hexane fractions from Orostachys japonicus in LPS-stimulated RAW 264.7 cells via upregulation of Nrf2 expression and activation of MAPK signaling pathway. *BMB Rep* 47, 98-103 (2014).

7. Appendix; None

8. Output (Acknowledge the Thailand Research Fund);

This work was conducted at the Center of Excellence in Immunology and Immune-Mediated Diseases and supported by the Research Grant for New Scholar, Thailand Research fund (TRG5780207) and the grants supported from Chulalongkorn University including Ratchadapiseksompotch Fund from the Faculty of Medicine; Grant for Development of New Faculty Staff.

International Journal Publication; submitted to “Journal of infectious disease”

Application; Our study is a basic research supported that people with de-functioning polymorphisms of FcγRIIb might susceptible to *C. neoformans*, especially in the endemic area. The people with a possible risk of having de-functioning polymorphisms of FcγRIIb such as patients with SLE or patients with immune-competent cryptococcosis and their close relatives might have benefit on screening for polymorphisms of FcγRIIb. Further studies in patients needed.

Others e.g. national journal publication, proceeding, international conference, book chapter, patent