

SCIENTIFIC REPORTS



OPEN

The role of macrophages in the susceptibility of Fc gamma receptor IIb deficient mice to *Cryptococcus neoformans*

Received: 06 June 2016
Accepted: 30 November 2016
Published: 11 January 2017

Saowapha Surawut^{1,*}, Thunnicha Ondee^{2,*}, Sujittra Taratummarat¹, Tanapat Palaga^{3,4}, Prapaporn Pisitkun⁵, Ariya Chindamporn³ & Asada Leelahavanichkul^{3,6}

Dysfunctional polymorphisms of Fc γ RIIb, an inhibitory receptor, are associated with Systemic Lupus Erythaematosus (SLE). Cryptococcosis is an invasive fungal infection in SLE, perhaps due to the de novo immune defect. We investigated cryptococcosis in the Fc γ RIIb $-/-$ mouse-lupus-model. Mortality, after intravenous *C. neoformans*-induced cryptococcosis, in young (8-week-old) and older (24-week-old) Fc γ RIIb $-/-$ mice, was higher than in age-matched wild-types. Severe cryptococcosis in the Fc γ RIIb $-/-$ mice was demonstrated by high fungal burdens in the internal organs with histological cryptococcoma-like lesions and high levels of TNF- α and IL-6, but not IL-10. Interestingly, Fc γ RIIb $-/-$ macrophages demonstrated more prominent phagocytosis but did not differ in killing activity *in vitro* and the striking TNF- α , IL-6 and IL-10 levels, compared to wild-type cells. Indeed, *in vivo* macrophage depletion with liposomal clodronate attenuated the fungal burdens in Fc γ RIIb $-/-$ mice, but not wild-type mice. When administered to wild-type mice, Fc γ RIIb $-/-$ macrophages with phagocytosed *Cryptococcus* resulted in higher fungal burdens than Fc γ RIIb $+/+$ macrophages with phagocytosed *Cryptococcus*. These results support, at least in part, a model whereby, in Fc γ RIIb $-/-$ mice, enhanced *C. neoformans* transmigration occurs through infected macrophages. In summary, prominent phagocytosis, with limited effective killing activity, and high pro-inflammatory cytokine production by Fc γ RIIb $-/-$ macrophages were correlated with more severe cryptococcosis in Fc γ RIIb $-/-$ mice.

Systemic Lupus Erythaematosus (SLE) is an autoimmune disease with multi-factorial pathogenesis, including genetic and environmental factors^{1,2}. Fc gamma receptors (Fc γ Rs) recognize the Fc portion of immunoglobulin G. This recognition results in immune activation or inhibition³⁻⁵, depending upon integrated signalling through different Fc γ Rs. Interestingly, the Fc gamma receptor IIb (Fc γ RIIb) is the only inhibitory signalling receptor in the Fc γ R family in either the mouse or human^{5,6}. *In vitro*, hyper-immune responses, due to Fc γ RIIb defects, have been demonstrated in several immune cells, including dendritic cells, macrophages and B cells⁷. Fc γ RIIb-deficient (Fc γ RIIb $-/-$) mice develop lupus nephritis spontaneously at 6 months and are currently used as a lupus mouse model⁵. In spite of the deficiency, Fc γ RIIb $-/-$ mice show highly effective immune responses against several types of pathogens, including bacteria, plasmodia and mycobacteria⁷⁻⁹. The fungal immune responses of Fc γ RIIb $-/-$ mice have been inadequately tested, despite the high susceptibility to several fungal infections in patients with SLE¹⁰. Indeed, a high prevalence of cryptococcosis among Chinese patients with SLE has been reported¹¹, and Fc γ RIIb loss-of-function polymorphisms are commonly associated with SLE in Asian populations¹²⁻¹⁵. Because cryptococcosis is an important invasive fungal infection in SLE, we examined the immune responses of Fc γ RIIb $-/-$ mice to *Cryptococcus neoformans*, *in vivo* and *in vitro*.

¹Medical Microbiology, Interdisciplinary Program, Graduate School, Chulalongkorn University, Bangkok, Thailand. ²Medical Sciences Program, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. ³Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. ⁴Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. ⁵Division of Allergy, Immunology, and Rheumatology, Department of Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. ⁶Center of Excellence in Immunology and Immune-mediated Diseases, Department of Microbiology, Faculty of Medicine, Bangkok, Thailand. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to A.L. (email: aleelahavanit@gmail.com)

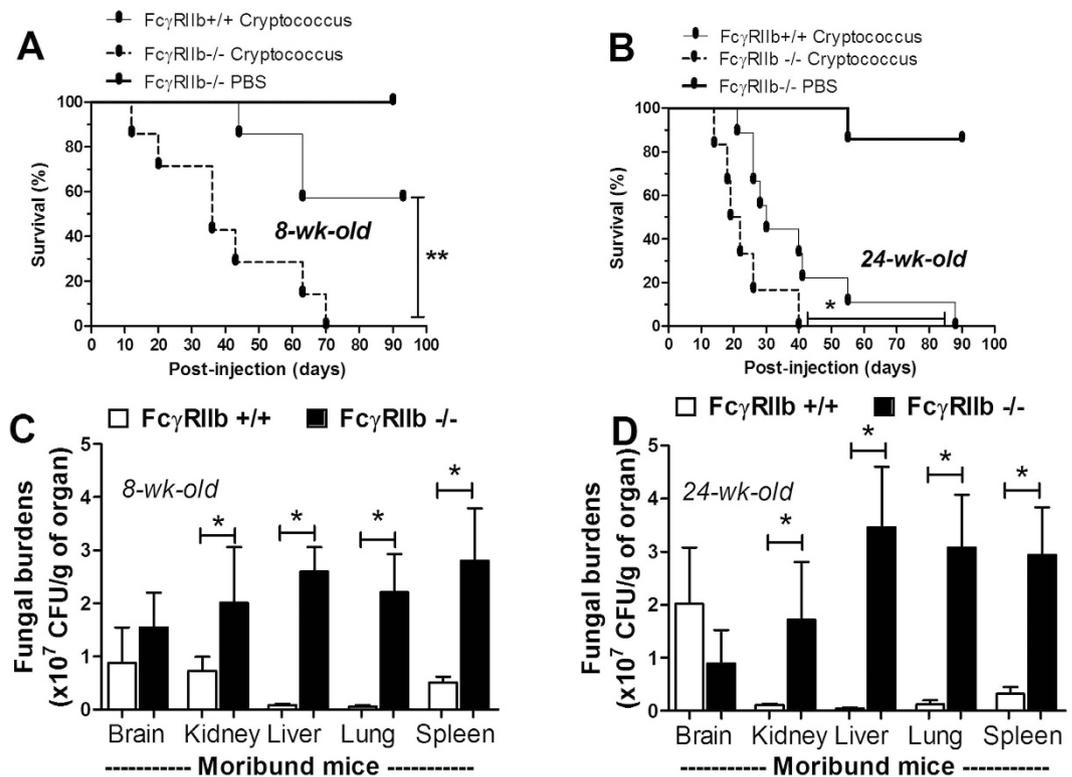


Figure 1. Survival analysis after *C. neoformans* administration in 8-week-old ($n = 7$ /group: **A**) and 24-week-old mice ($n = 7$ –9/group: **B**) are shown. In parallel, the fungal burdens in several internal organs of 8-week-old ($n = 4$ –6/group: **C**) and 24-week-old mice ($n = 5$ –6/group: **D**) are shown. The data are shown as the mean \pm SE, and the presented values are combined from 2–3 independent experiments. $*p < 0.05$, $**p < 0.01$.

Results

Increased severity of cryptococcosis in Fc gamma receptor IIb deficient mice. Lupus nephritis in Fc γ RIIb^{-/-} mice developed spontaneously, as determined by high urine protein (urine protein creatinine index: UPCI) and anti-dsDNA antibodies at 24 weeks (Supplementary Fig. S1B,C). Age-related lupus manifestations in Fc γ RIIb^{-/-} mice thus allowed the exploration of 2 SLE phases, asymptomatic and symptomatic, as determined by proteinuria and anti-dsDNA antibodies, despite normal serum creatinine (Scr) in both age groups (Supplementary Fig. S1A).

After *C. neoformans* administration, Fc γ RIIb^{-/-} mice demonstrated higher mortality than the age-matched wild-type control mice in both the asymptomatic and symptomatic lupus groups (Fig. 1A–D). In the young mouse group, all of the Fc γ RIIb^{-/-} mice, but only 57% of the wild-type mice, died or became moribund within 90 days of challenge (Fig. 1A). In contrast, all of the older mice with cryptococcosis died or became moribund by 40 and 90 days in the Fc γ RIIb^{-/-} and wild-type groups, respectively (Fig. 1B). Interestingly, moribund Fc γ RIIb^{-/-} mice, from both the young and old age groups, demonstrated higher fungal burdens and cryptococcoma-like-lesions in several internal organs, namely brain, kidney, liver, lung and spleen (Fig. 1C,D and Fig. 2). Conversely, in wild-type mice of both age groups, such lesions were found in only the brain, a major organ of infection, at the moribund stage (Fig. 2, Supplementary Fig. S2; only older mice shown).

At the moribund stage, cryptococcosis was more severe in both the young and old groups of Fc γ RIIb^{-/-} mice. The levels of Scr, ALT and cytokines (TNF- α , IL-6 and IL-10) in wild-type were 0.33 ± 0.02 mg/dl, 49 ± 9 U/L and 99 ± 10 , 89 ± 14 and 389 ± 142 pg/ml and in Fc γ RIIb^{-/-} young mice were 0.32 ± 0.04 mg/dl, 77 ± 7 U/L, and 147 ± 8 , 257 ± 48 and 446 ± 199 pg/ml (Fig. 3A–E). In parallel, these parameters in the old group of wild-type were 0.43 ± 0.04 mg/dl, 39 ± 7 U/L, and 82 ± 2 , 47 ± 16 and 327 ± 50 pg/ml and in Fc γ RIIb^{-/-} old mice were 0.58 ± 0.08 mg/dl, 87 ± 9 U/L and 123 ± 7 , 307 ± 227 and 403 ± 59 pg/ml (Fig. 3F–J). After 2 weeks of cryptococcosis, the fungal burdens in the internal organs were higher in Fc γ RIIb^{-/-} mice at the moribund stage (Fig. 4A,E). Cryptococcoma-like lesions were found in most organs in Fc γ RIIb^{-/-} mice, but only in the brain and kidney in wild-type mice, in both age groups (Fig. 5, Supplementary Fig. S3; only 8-week mice shown). Anti-dsDNA antibody titer was slightly elevated in 2 out of 4 of 8-week-old Fc γ RIIb^{-/-} mice (Supplementary Fig. S1), implying the beginning of SLE symptoms (incipient SLE) in this age group. The high susceptibility to fungi in these mice might due to ongoing SLE disease. When fungi were administered to younger Fc γ RIIb^{-/-} mice, 4 weeks of age, none showed elevated anti-dsDNA antibodies, suggesting the absence of incipient SLE (Supplementary Fig. S6). Fungal burdens in brain, lung and spleen, but not kidney and liver, were higher in 4-week-old Fc γ RIIb^{-/-} mice compared with age-matched wild-type controls (Supplementary Fig. S6).

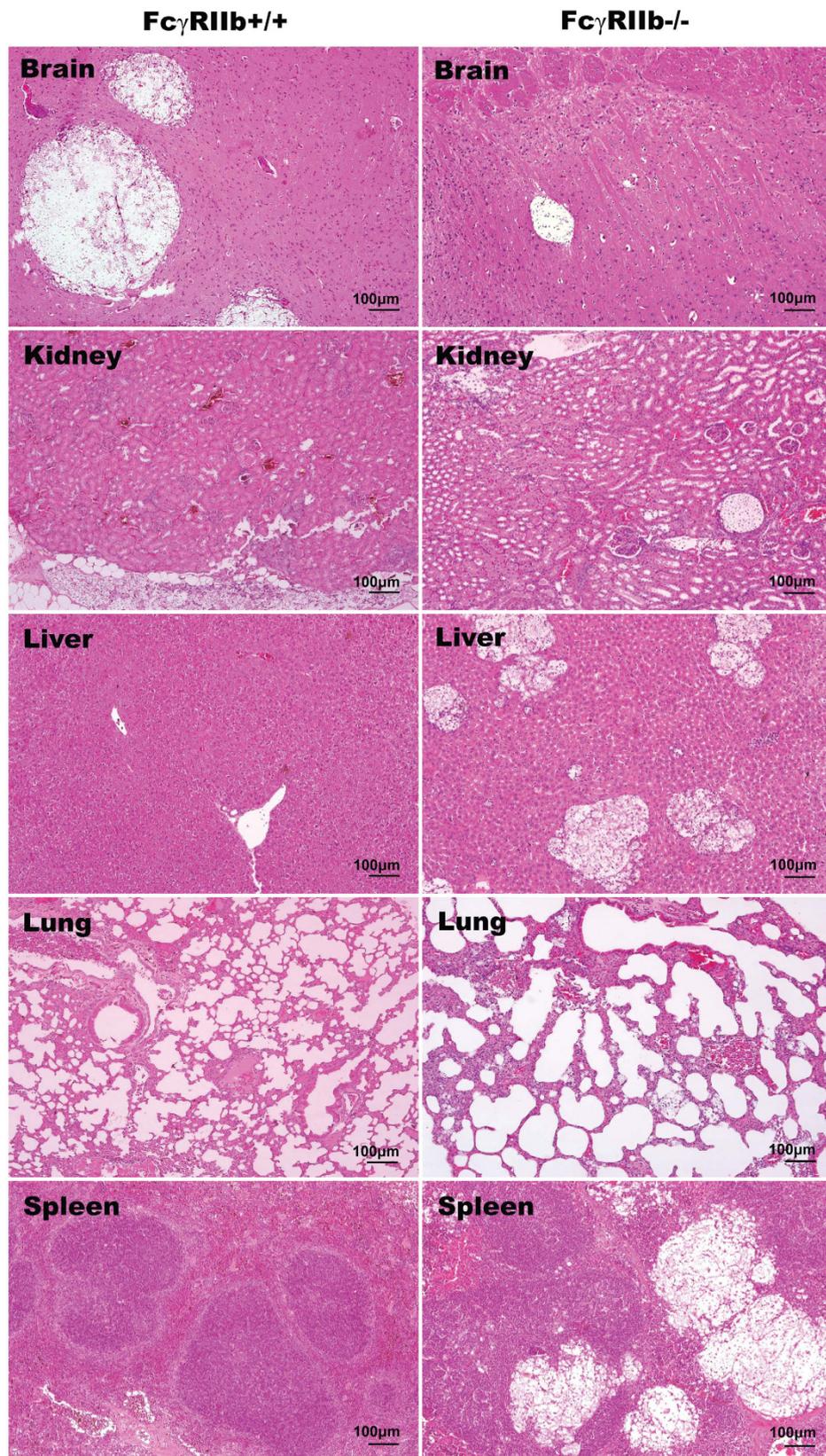


Figure 2. Representative histology with H&E (hematoxylin and eosin staining) at 100x magnification from 24-week-old mice in the moribund stage after *C. neoformans* administration. Cryptococcoma-like lesions were observed only in the brains of $Fc\gamma RIIB^{+/+}$ mice (left column). In $Fc\gamma RIIB^{-/-}$ mice (right column), several internal organs had cryptococcoma-like lesions (brain, kidneys, liver, lung and spleen).

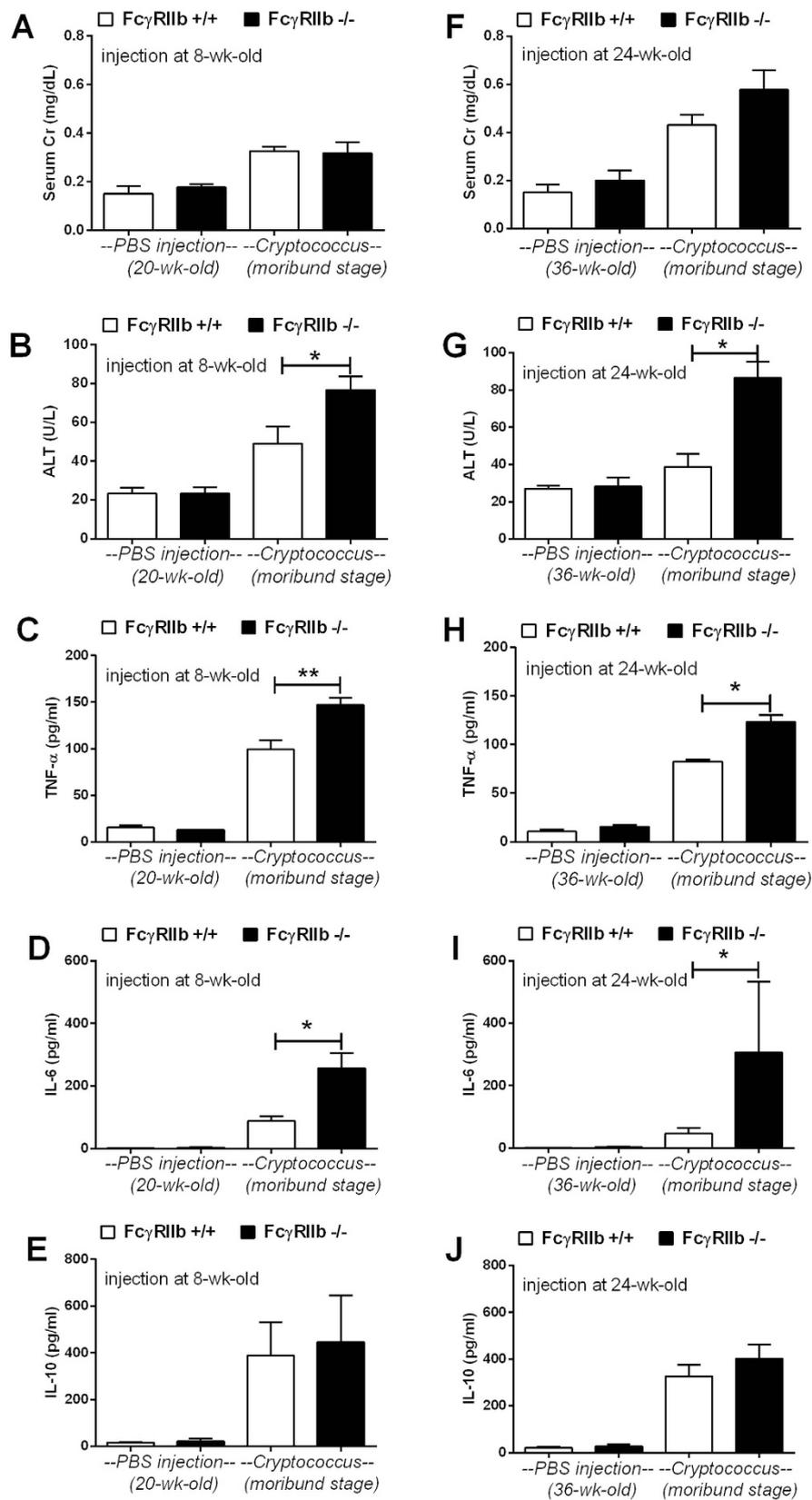


Figure 3. Organ injury and inflammatory cytokines at the moribund stage in 8-week-old (left column) (A–E) and 24-week-old mice (right column) (F–J), as demonstrated by serum creatinine (Scr), alanine transaminase (ALT), TNF- α , IL-6 and IL-10 levels. The data are shown as the mean \pm SE, and the presented values are combined from 2 independent experiments ($n = 4\text{--}5/\text{group}$). $*p < 0.05$.

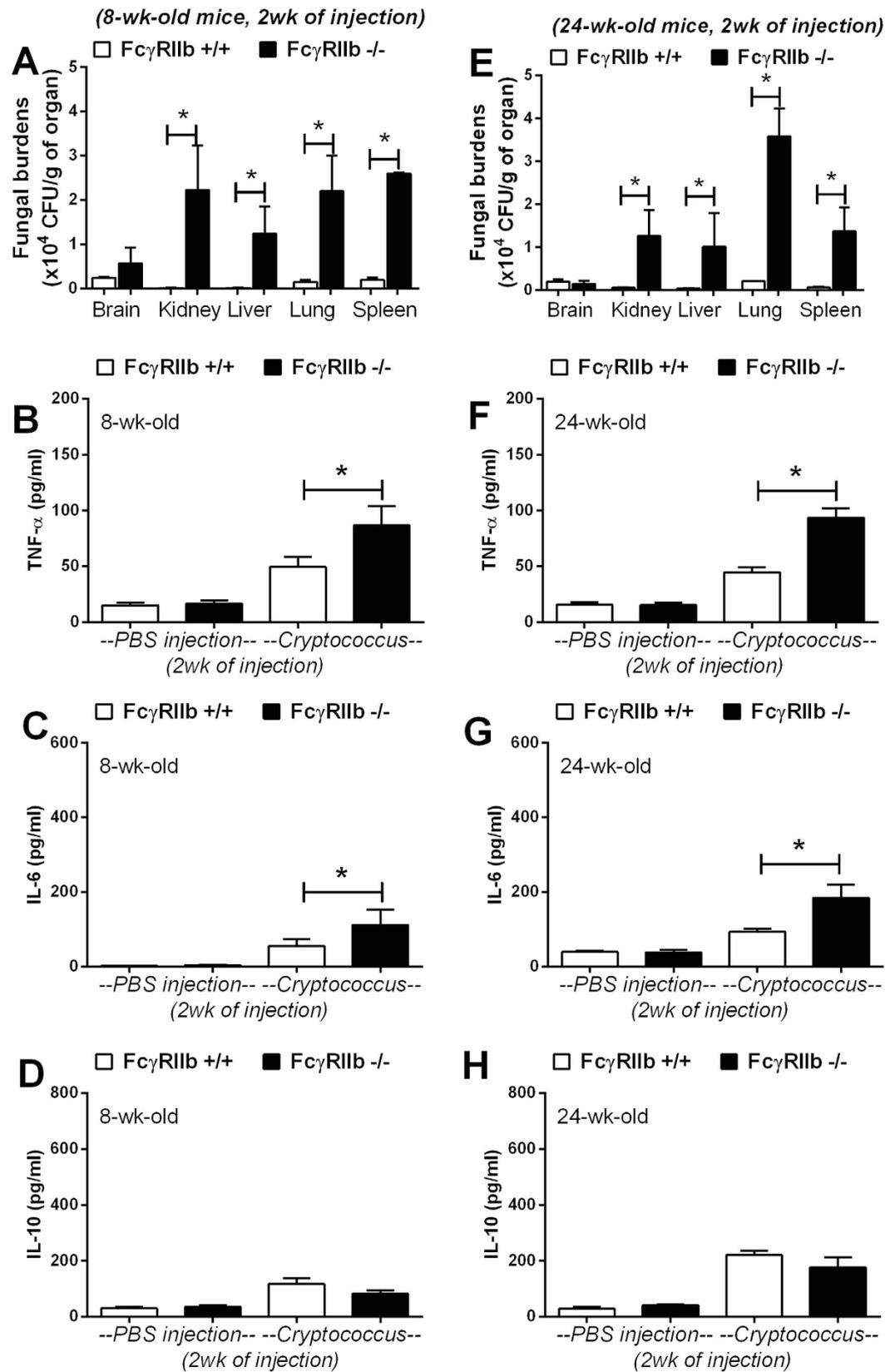


Figure 4. Fungal burdens in the internal organs of 8-week-old (left column) and 24-week-old mice (right column) at 2 weeks post-*C. neoformans* administration (A,E). Serum cytokines measured include TNF- α (B,F), IL-6 (C,G) and IL-10 (D,H). The data are shown as the mean \pm SE, and the presented values are the combined from 2 independent experiments (n = 4–5/group). * $p < 0.05$.

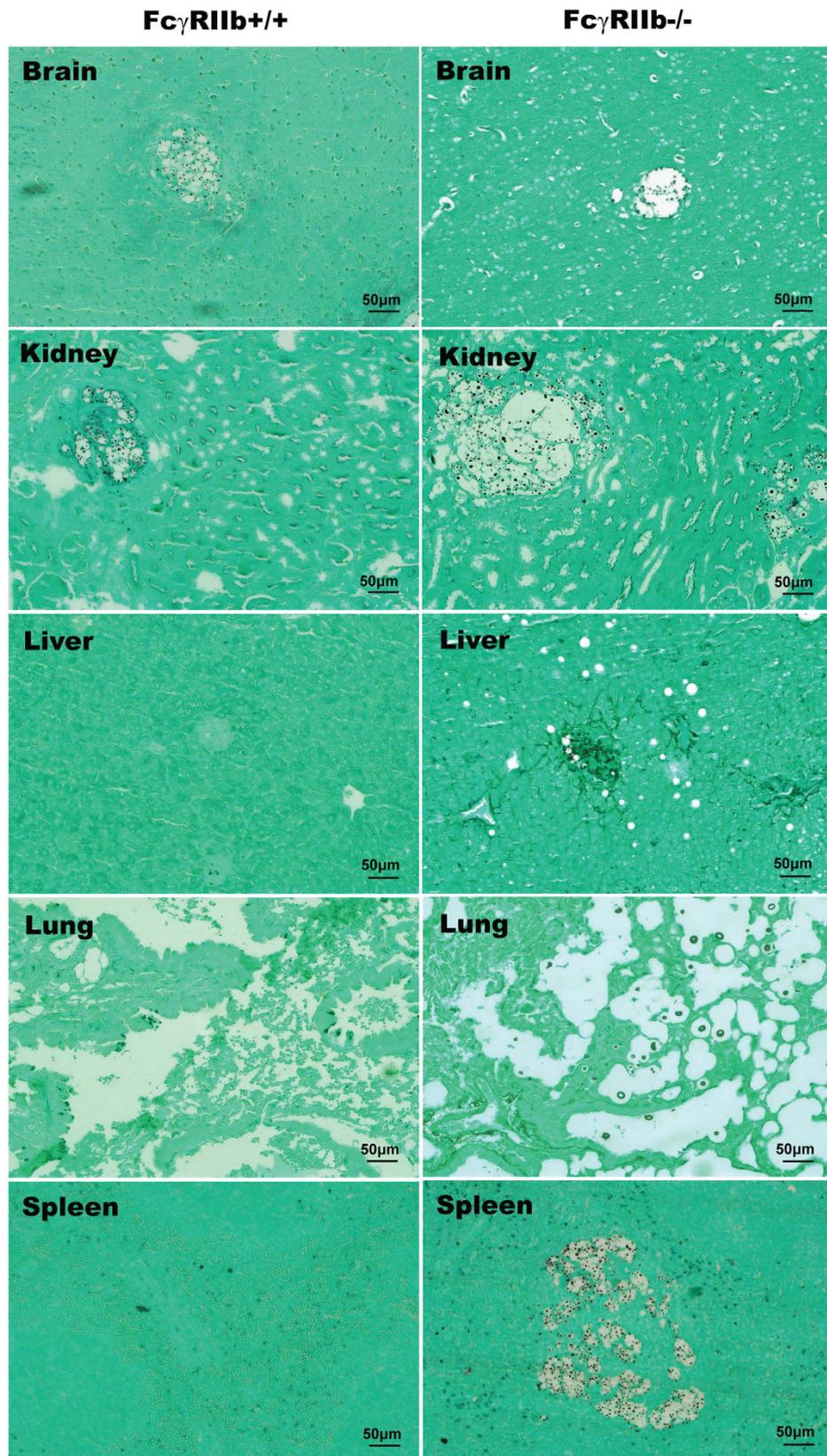


Figure 5. Organ histology with Grocott's silver staining (GMS) at 200x magnification from 8 week-old mice at 2 weeks post-*C. neoformans* infusion, demonstrating cryptococcoma-like lesions in the brain and kidney of Fc γ RIIb^{+/+} mice (left column), and in several internal organs (brain, kidneys, liver, lung and spleen) of Fc γ RIIb^{-/-} mice (right column).

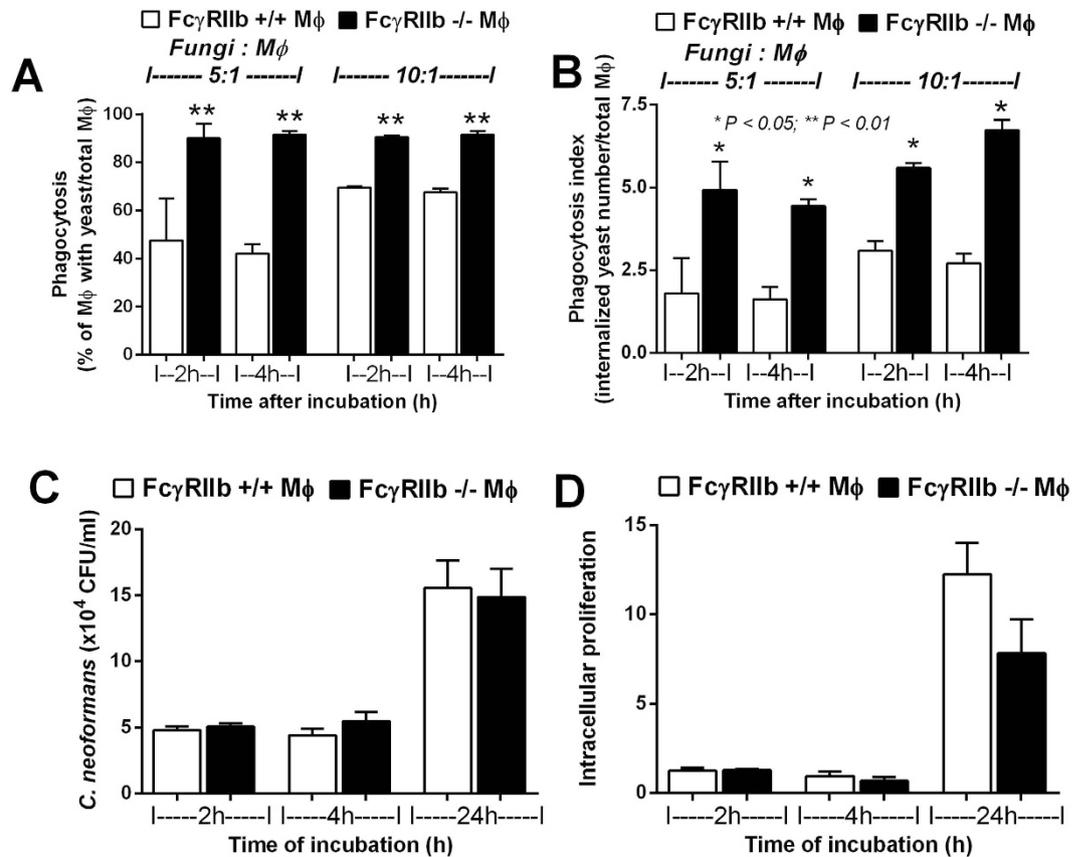


Figure 6. Percentage of macrophages (MΦ) demonstrating phagocytosis (A) and the average number of phagocytosed fungi per MΦ (total number of phagocytosed fungi/total MΦ) after incubation with *C. neoformans* at ratios of fungi: MΦ of 5:1 and 10:1 (B) are shown. The total killing ability (extruded and intracellular yeast) (C) and intracellular proliferation (concerning only intracellular yeast; see methods) (D) of MΦ determined by the number of *C. neoformans* colonies after incubation with MΦ from wild-type and FcγRIIb^{-/-} mice are shown. * $p < 0.05$, ** $p < 0.01$ (experiments were performed in triplicate).

Accordingly, the high susceptibility to cryptococcosis in the FcγRIIb^{-/-} strain appeared to be due to the gene defect and less likely a result of autoantibody stimulation or ongoing SLE.

Additionally, the levels of pro-inflammatory serum cytokines, TNF-α and IL-6, but not an anti-inflammatory cytokine, IL-10, were higher in FcγRIIb^{-/-} mice (Fig. 4B–D, F–H). Moreover, infected FcγRIIb^{-/-} mice showed a tendency toward higher levels of total immunoglobulin (measured as total gamma globulin protein from protein electrophoresis) but this did not reach a statistically significant level (Supplementary Fig. S4). Therefore, cryptococcosis, either at 2 weeks or when moribund, was more severe in FcγRIIb^{-/-} than wild-type mice, as demonstrated by higher fungal burdens in most internal organs, higher liver enzyme levels, and higher pro-inflammatory cytokine levels but not higher anti-inflammatory cytokine levels.

FcγRIIb^{-/-} macrophage responses to *C. neoformans*: prominent phagocytosis and pro-inflammatory cytokine production.

Due to the importance of macrophages in fungal infection response and the presence of FcγRIIb in macrophages, we explored macrophage responses to *C. neoformans*. Interestingly, FcγRIIb^{-/-} macrophages showed higher phagocytosis of *C. neoformans*. Macrophages were incubated with heat-killed *C. neoformans* in ratios of 5:1 and 10:1 (fungal cells to macrophages) for 2 h and 4 h (Fig. 6A). With the ratio of fungal cells to macrophages of 5:1, the percentages of macrophages with phagocytosed *C. neoformans* in wild-type and FcγRIIb^{-/-} after 2 h incubation were $47.5 \pm 17.5\%$ and $90.1 \pm 6.2\%$, respectively, and after 4 h incubation were $42.1 \pm 4.2\%$ and $91.5 \pm 1.5\%$, respectively. At the ratio of 10:1, the percentages of phagocytosed macrophages in wild-type and FcγRIIb^{-/-} after 2 h incubation were $69.5 \pm 0.5\%$ and $90.5 \pm 0.5\%$, respectively, and after 4 h incubation were $67.5 \pm 1.5\%$ and $91.5 \pm 1.5\%$, respectively.

Additionally, the average number of fungi in each macrophage was higher in FcγRIIb^{-/-} cells, as determined by the phagocytosis index (number of internalized fungi/total macrophages) (Fig. 6B). In contrast, the macrophage killing activity, as determined by fungal viability after incubation with macrophages for 2, 4 and 24 h, was not different between wild-type and FcγRIIb^{-/-} cells. The numbers of viable fungi in the macrophages with total killing ability (both extruded and intracellular yeasts were determined; see methods), *in vitro*, at 2, 4, and 24 h of fungal incubation with wild-type cells versus FcγRIIb^{-/-} macrophages were 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 ($\times 10^4$) CFU/ml, respectively (Fig. 6C). No difference in macrophage killing activity was observed using the intracellular proliferation assay. The intracellular proliferation

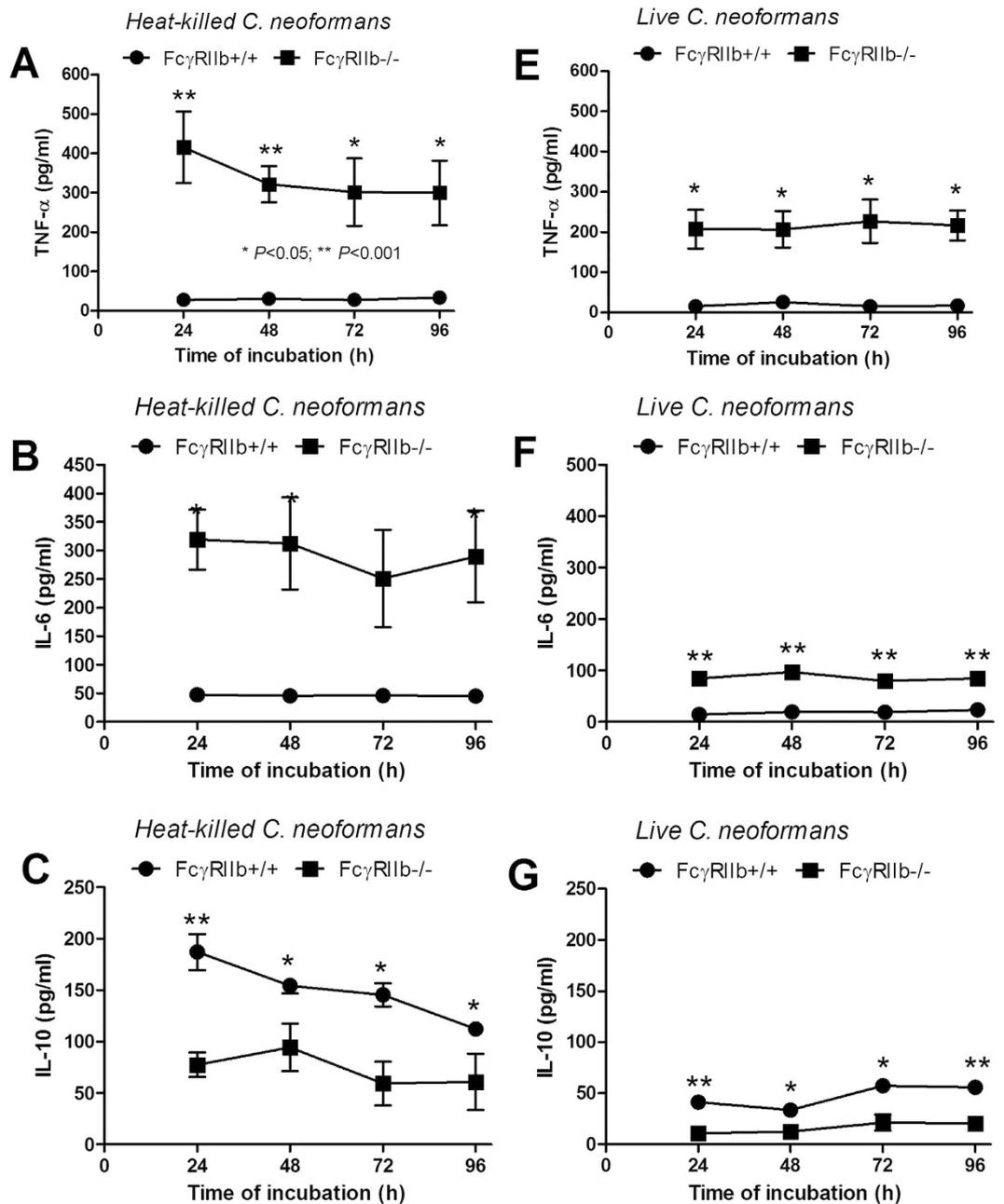


Figure 7. Cytokine levels (TNF- α , IL-6 and IL-10) in the supernatant media from macrophages of Fc γ RIIb^{+/+} or Fc γ RIIb^{-/-} mice after activation with heat-killed (A–C) or live *C. neoformans* (E–G) are shown. * $p < 0.05$, ** $p < 0.01$ (experiments were performed in triplicate).

assay that determined the viability of only intracellular yeasts also performed (see methods). Indeed, the intracellular proliferation amounts at 2, 4, and 24 h of fungi incubated with wild-type cells versus fungi incubated with Fc γ RIIb^{-/-} macrophages were 1.3 ± 0.2 , 1.0 ± 0.2 and 12.3 ± 1.8 vs. 1.3 ± 0.1 , 0.7 ± 0.2 and 8.8 ± 1.9 units, respectively (Fig. 6D). Although a trend toward greater intracellular-killing by Fc γ RIIb^{-/-} macrophages was observed, it was not statistically significant. The presence of macrophages did not reduce the colony count of fungi in the fungicidal activity assay, implying that cryptococci were viable, intracellularly.

In addition, the cytokine responses of Fc γ RIIb^{-/-} macrophages to fungi were tested. Fc γ RIIb^{-/-} macrophages, in response to heat-killed or live *C. neoformans*, produced higher levels of TNF- α and IL-6 but lower IL-10 levels (Fig. 7A–G), recapitulating the *in vivo* results (Figs 3 and 4).

Fc γ RIIb^{-/-} macrophages enhance the dissemination of cryptococci. *C. neoformans* is a facultative intracellular pathogen that demonstrates intracellular viability. Host phagocytes are used for fungal dissemination, and this is referred to as a “Trojan horse” mechanism¹⁶. Observing enhanced phagocytosis capacity but limited killing activity of Fc γ RIIb^{-/-} macrophages, we hypothesized that Fc γ RIIb^{-/-} macrophages may

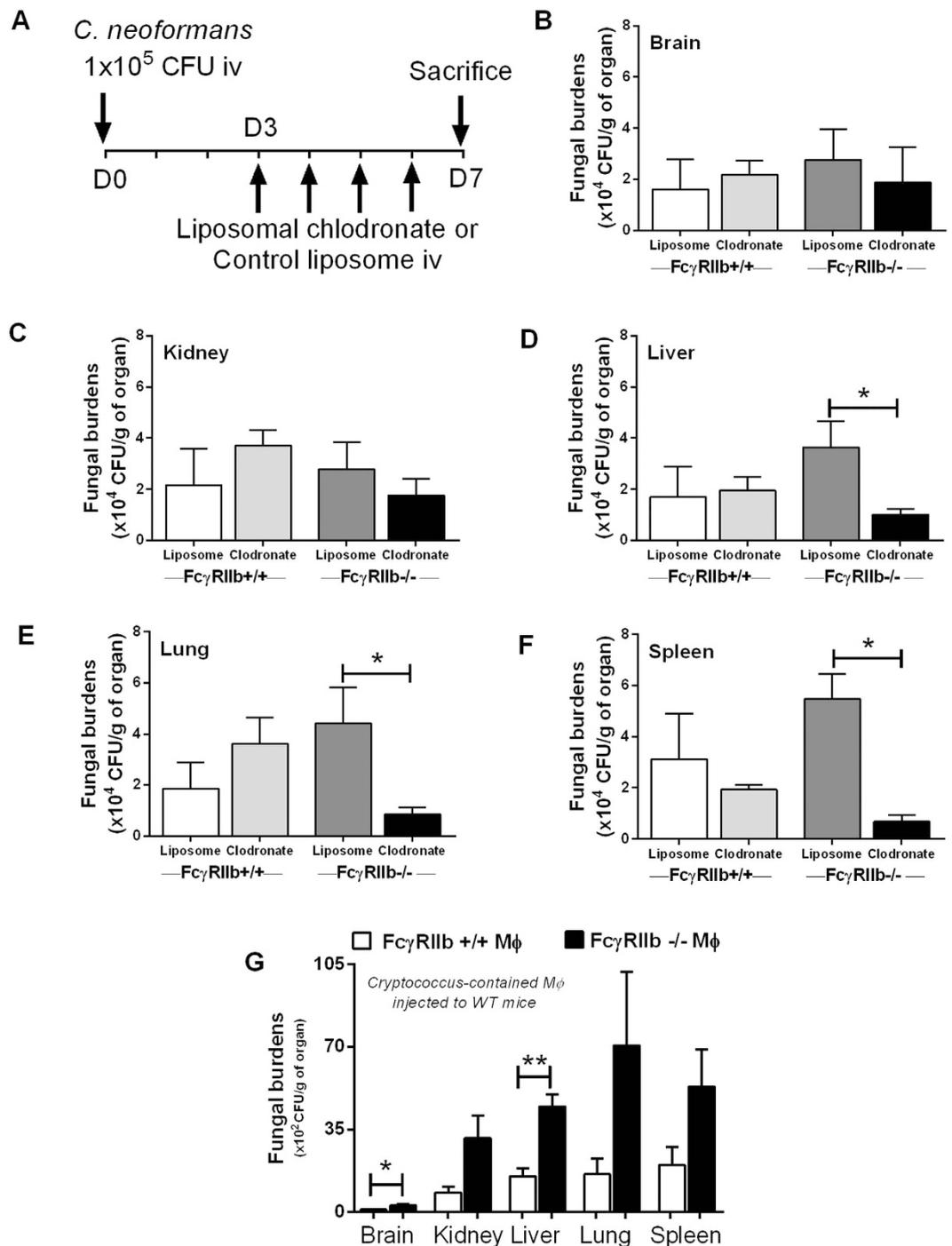


Figure 8. Timeline of a model for cryptococcosis in liposomal clodronate-induced macrophage depletion in 8-week-old Fc γ RIIb+/+ and Fc γ RIIb-/- mice (A) and fungal burdens in brain (B), kidney (C), liver (D), lung (E) and spleen (F) are shown (n = 4–5/group). Organ fungal burdens of Fc γ RIIb+/+ mice at 24 h after infusion of *Cryptococcus*-containing macrophages (infected macrophages) from Fc γ RIIb+/+ and Fc γ RIIb-/- mice are shown (G). (n = 4–5/group). Data are shown as the mean \pm SE, and the presented values are combined from 2 independent experiments. * $p < 0.05$, ** $p < 0.01$.

be responsible for more severe cryptococcosis *in vivo*. Accordingly, we tested cryptococcosis in Fc γ RIIb-/- and wild-type mice with liposomal clodronate-induced macrophage depletion (Fig. 8). Interestingly, macrophage depletion attenuated the fungal burdens in liver, lung and spleen in Fc γ RIIb-/- mice, but not in wild-type mice (Fig. 8). Fungal burdens at 7 days after fungal administration in the brain, kidney, liver, lung and spleen of Fc γ RIIb-/- mice with control (PBS) liposomes versus liposomal clodronate were 2.8 ± 1.2 , 2.8 ± 0.9 , 3.7 ± 1.0 , 4.4 ± 1.4 and 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 ($\times 10^4$) CFU per organ weight (g), respectively (Fig. 8B–F). Subsequently, to determine if the high phagocytosis capacity of

Fc γ RIIb $^{-/-}$ macrophages enhanced cryptococcal dissemination, we incubated yeast with wild-type and Fc γ RIIb $^{-/-}$ macrophages and administered the cells, with the phagocytosed cryptococcal cells, into wild-type mice. Indeed, the fungal burdens both in the brain, a major target organ of cryptococcosis, and in the liver, were higher in mice receiving *Cryptococcus*-containing Fc γ RIIb $^{-/-}$ cells (Fig. 8G). The fungal burdens in brain, kidney, liver, lung and spleen at 1 day after the administration of wild-type or Fc γ RIIb $^{-/-}$ macrophages were 1.1 ± 0.2 , 8.3 ± 2.5 , 15.1 ± 3.4 , 16.1 ± 5.3 and 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 ($\times 10^2$) CFU per gram organ weight, respectively (Fig. 8G). Subsequently, the fungal burdens in the internal organs decreased due to the immunocompetence of wild-type mice (data not shown).

Discussion

The Fc γ RIIb loss-of-function polymorphism is one of the important genetic associations of SLE, particularly in Asian populations^{12–15}. Accordingly, Fc γ RIIb $^{-/-}$ mice, a lupus nephritis model, may be a useful mouse model of SLE in Asians. Our results suggest the possibility that the increased severity of cryptococcosis in Fc γ RIIb $^{-/-}$ mice is, at least in part, due to the unique properties of Fc γ RIIb $^{-/-}$ macrophages including enhanced phagocytosis and elevated pro-inflammatory cytokine responses.

Although intravenous administration is not the natural route of cryptococcal infection, which is transmission through pulmonary system, an intravenous model is adequate for a proof of concept of the difference between the two mouse strains. Indeed, Fc γ RIIb $^{-/-}$ mice showed more severe cryptococcosis than wild-type mice. The immune response against fungal infection is predominantly dependent on cell-mediated immune responses. The defect in Fc γ RIIb, the only inhibitory receptor in the Fc γ R family, induces an enhanced immune response and effectively controls several organisms^{7–9}. Fc γ RIIb $^{-/-}$ mice were more susceptible to cryptococcosis, either in symptomatic or asymptomatic lupus, compared to the age-matched wild-type controls. There was also an age-dependent increase in the mortality rate for cryptococcosis. The mortality rate in wild-type mice at 8-weeks-old vs. 24-weeks-old was different ($p = 0.015$ by log-rank test, Fig. 1A,B), but there was no difference between the Fc γ RIIb $^{-/-}$ groups. The severity of cryptococcosis in Fc γ RIIb $^{-/-}$ mice was independent of the manifestation of lupus symptoms and age. These results support the susceptibility to cryptococcosis of patients with Fc γ RIIb polymorphisms, with either SLE or non-SLE^{10–12}.

Subsequently, the severity of cryptococcosis was evaluated with different parameters at 2 weeks, the earlier stage of infection, and in the moribund stage. The 2 age groups, as expected, showed similar results. Disseminated cryptococcosis, including fungal organisms found in several internal organs, which was observed in Fc γ RIIb $^{-/-}$ mice, was similar to the disseminated cryptococcosis observed in patients with compromised immune systems¹⁷. However, the lesions were limited mostly to the brain in wild-type mice, a major target organ¹⁸, as usually observed in the immuno-competent human host. Surprisingly, despite the absence of generalized lesions in wild-type mice, the brain lesions were larger than in Fc γ RIIb $^{-/-}$ mice. This difference remains to be investigated. It seems that the neurotropism characteristic of *C. neoformans* is not apparent in the infection in Fc γ RIIb $^{-/-}$ mice because the organism could survive in any organ. However, in wild-type mice, *C. neoformans* may prefer to grow in the brain because of more suitable nutritional conditions^{19–21}. Enhanced pathogenesis due to disseminated disease was also indicated by more severe liver injury in Fc γ RIIb $^{-/-}$ mice.

Enhanced cryptococcal dissemination in the Fc γ RIIb $^{-/-}$ mice underscores the role of this genetic lesion in pathogenesis. The inflammatory cytokines, TNF- α and IL-6, but not IL-10, were higher in Fc γ RIIb $^{-/-}$ than wild-type mice. This is a response to the higher fungal burden, in addition to the anti-inflammatory defect. Perhaps serum IL-10 is too low to balance out the pro-inflammatory immune responses, leading to more severe organ histopathology and symptoms. However, total immunoglobulin levels did not differ between the Fc γ RIIb $^{-/-}$ and wild-type mice after cryptococcal infection, despite the previous report of immunoglobulin hyperproduction in bacterial infection⁸. This inconsistency is possibly due to the difference in adaptive immune-response activated by these two organisms. Nevertheless, the beneficial effect of humoral immune response in controlling *C. neoformans* was demonstrated^{22–24}.

Macrophages and T helper cells are the main immune cells responsible for the immune response to cryptococcosis^{25,26}. Fc γ RIIb presents in macrophages but not in T cells²⁷. Therefore, the higher fungal burdens in Fc γ RIIb $^{-/-}$ mice may be due to the primary defect in macrophages. Accordingly, we evaluated the phagocytosis activity, killing activity and cytokine responses of macrophages after exposure to *C. neoformans*. Interestingly, the phagocytosis of Fc γ RIIb $^{-/-}$ macrophages in response to cryptococci was elevated compared with wild-type cells, as reported for other organisms^{8,9}. In Fc γ RIIb $^{-/-}$ mice, nearly all of the macrophages incubated with heat-killed *C. neoformans* phagocytosed approximately 6–7 yeasts per cell at 4 h (phagocytosis index). In contrast, approximately only 50–60% of wild-type macrophages phagocytosed yeast, and they did so with a reduced activity of 2–3 yeasts per cell. On the other hand, the total killing capacity and the intracellular proliferation activity of Fc γ RIIb $^{-/-}$ macrophages was not different from wild-type macrophages, unlike the responses to other organisms⁸, perhaps due to the immune evasion properties of *C. neoformans*²⁶.

Cryptococcus is a facultative intracellular pathogen, which can utilize host macrophages to spread within the body, via the Trojan horse mechanism¹⁶. Cryptococci typically escape extracellular immune responses, survive and replicate intracellularly, transfer laterally between macrophages, and eventually invade tissue and organs¹⁶. They can use macrophages as trafficking vehicles for dissemination, particularly to pass through the blood-brain barrier into the central nervous system²⁸. Interestingly, the depletion of macrophages, at least in certain situations, is associated with less severe pathogenesis²⁹. We hypothesized that the elevated phagocytosis of Fc γ RIIb $^{-/-}$ macrophages and the immune evasion properties of *C. neoformans* enhanced the Trojan horse mechanism, resulting in more severe cryptococcosis *in vivo*. We tested cryptococcosis severity in a macrophage depletion model with daily liposomal clodronate injection in Fc γ RIIb $^{-/-}$ and wild-type mice. As expected, at 1 week after fungal administration, macrophage depletion led to lower fungal burdens in the liver, lung and spleen of Fc γ RIIb $^{-/-}$ mice, but not of wild-type mice. Nevertheless, liposomal clodronate not only depleted monocytes/macrophages

but also reduced the numbers of dendritic cells and regulatory T cells^{30,31}. Although loss of dendritic cells after liposomal clodronate injection might be responsible for the less severe cryptococcosis, the enhanced cryptococcosis severity after *Cryptococcus*-infected macrophage injection supports the greater pathogenic role of macrophages compared to dendritic cells. The inoculation of fungi-containing Fc γ RIIb $^{-/-}$ macrophages increased the fungal burdens in the brains and livers of wild-type mice at 24 h after administration. These results support the high phagocytosis capacity of Fc γ RIIb $^{-/-}$ macrophages and the enhancement of fungal transmission by macrophages, particularly through the blood-brain barrier. Together, these results support the importance of macrophages in cryptococcosis pathogenesis in Fc γ RIIb $^{-/-}$ mice and in patients with Fc γ RIIb loss-of-function polymorphisms.

In addition, the prominent pro-inflammatory cytokine response (TNF- α and IL-6), but not the anti-inflammatory cytokine (IL-10) response, was demonstrated in the Fc γ RIIb $^{-/-}$ groups, both *in vivo* and *in vitro*. This might be because glucuronoxylomannan (GXM), an important cryptococcal capsular polysaccharide, induces potent immunosuppression by direct engagement of Fc γ RIIb, an immunoinhibitory receptor, and stimulates greater IL-10 production^{32,33}. In Fc γ RIIb $^{-/-}$ mice, perhaps GXM was unable to induce IL-10, resulting in the more severe pro-inflammatory cytokine storm and fungal sepsis. More studies on this topic are needed to explain the underlying mechanism.

Taken together, we conclude that more severe cryptococcosis in Fc γ RIIb $^{-/-}$ mice was due to enhanced dissemination, possibly through the Trojan horse mechanism, and the hyper-responsiveness of pro-inflammatory cytokine production during sepsis. This is the first report of the disadvantage of the prominent macrophage function of Fc γ RIIb $^{-/-}$ mice in cryptococcosis. In clinical translation, we propose Fc γ RIIb loss-of-function-polymorphisms as a new risk factor for cryptococcosis. Screening for Fc γ RIIb polymorphisms in patients with SLE, particularly in areas of endemic cryptococcosis, might be beneficial for patient management. Our results also implied the importance of the genetic-background differences among patients with SLE to micro-organism responses. The examination of the genetic background of individual patients with SLE might be clinically beneficial.

Methods

Animal models and *Cryptococcus neoformans* injection method. Fc γ RIIb $^{-/-}$ mice on the C57BL/6 background were kindly provided by Dr. Silvia Bolland (NIAID, NIH, Maryland, USA). The mice were originally constructed in a 129Sv/B6-hybrid background and were backcrossed onto the C57BL/6 background for 12 generations. Female C57BL/6 wild-type mice, age-matched to Fc γ RIIb $^{-/-}$ mice, were purchased from the National Laboratory Animal Center in Nakornpathom, Thailand. The animal protocols were approved by the Faculty of Medicine of Chulalongkorn University and followed NIH criteria. *C. neoformans* was isolated from a patient sample (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) at -80°C . The sample accession process was approved by the Ethical Institutional Review Board, faculty of Medicine, Chulalongkorn University according to the declaration of Helsinki, with written informed consent. The same strain of *C. neoformans* was used in all of the experiments. Before use in experiments, *C. neoformans* was sub-cultured on Sabouraud dextrose agar (SDA) at 37°C for 24 h. Asymptomatic (8-week-old without proteinuria) or symptomatic lupus (24-week-old with proteinuria) mice or age-matched wild-type control groups were injected, via tail vein, with 1×10^5 yeast cells *C. neoformans* diluted in 200 μl of PBS. For survival analysis, the mice were observed for 90 days after fungal administration. The mice were sacrificed at the moribund stage, as determined by an inability to walk after touch stimulation. In other experiments, the mice were sacrificed at 2 weeks after fungal administration. At the time of euthanasia, blood was collected via cardiac puncture under isoflurane anaesthesia, and the internal organs (brain, lung, kidney, liver and spleen) were fixed with 10% formalin for histology or processed for fungal burden experiments (details below). In addition, to further investigate the importance of Fc γ R deficiency relative to autoantibody and incipient SLE status, 4-week-old Fc γ RIIb $^{-/-}$ mice and age-matched wild-type (non-significantly different anti-dsDNA antibody titers between both groups; Supplementary Fig. S6) were intravenously injected with *C. neoformans* at 1×10^5 yeast cells and internal organ fungal burdens were determined at 2 weeks.

***In vivo* macrophage depletion.** Macrophage depletion with liposomal clodronate injection was performed in order to determine the role of macrophages in cryptococcosis, following a previously published protocol²⁸. Female 8-week-old Fc γ RIIb $^{-/-}$ and wild-type mice were administered *C. neoformans* (yeast form) via the tail vein. Then, 200 μl /mouse liposomal clodronate (Encapsula Nanoscience, Nashville, TN, USA) (5 mg/ml) or control liposomes were injected to induce sustained monocyte depletion (Fig. 8A). The daily injections began on the third day of fungal administration and continued for 4 consecutive days. At 7 days post-inoculation, the mice were sacrificed and the internal organs were processed for fungal burdens and fixed in 10% formalin to confirm macrophage depletion (by immunohistochemical staining with an F4/80 antibody; Biolegend, San Diego, CA, USA). Macrophages were not detectable in organs after liposomal clodronate treatment in either Fc γ RIIb $^{-/-}$ or wild-type mice (Supplementary Fig. S5).

Transfer of *Cryptococcus*-containing macrophages *in vivo*. Bone marrow (BM)-derived macrophages from Fc γ RIIb $^{-/-}$ and wild-type mice were allowed to phagocytose yeast cells and then they were infused into wild-type mice, as previously described^{34,35}. Briefly, BM macrophages cultured in a 96-well plate at 2.5×10^4 cells/well with 20% mouse serum were incubated with *C. neoformans* in the ratio of 5:1 (fungal cells to macrophages) for 2 h. The un-phagocytosed fungi were washed out with DMEM (3–5 washes). Subsequently, the macrophages were detached with cold-PBS washing (3–5 times) and centrifuged at 1,000 rpm for 10 min at 4°C . Cell pellets were then resuspended with DMEM. The macrophages were counted and stained with trypan

blue. Either Fc γ RIIb $-/-$ or wild-type macrophages with internalized *Cryptococcus*, at 2.5×10^4 cells, were intravenously administered to wild-type mice through the tail vein. The mice were sacrificed at 24 h and the fungal burdens determined.

Fungal burdens and organ histology. To measure internal organ fungal burdens, the organs were weighed, homogenized, plated onto SDA and incubated at 37 °C for fungal colony enumeration at 48 h. For histology, the tissue samples were fixed in 10% formalin and embedded in paraffin; 4- μ m sections were stained with haematoxylin and eosin colour (H&E) and Grocott's silver stain (GMS) for *C. neoformans* identification. Quantitative measurement of the fungal infection area was performed by 2 blinded observers. Fields (10 selected randomly) were examined at 200x magnification, with the following criteria: 0, no fungi; 1, area of fungal infection <25%; 2, infected area involving 25–50% of the field; and 3, infected area \geq 50% of the field.

Blood chemistry, urine chemistry and cytokine analysis. Kidney injury was determined by serum creatinine (Scr) (QuantiChrom Creatinine Assay, DICT-500, BioAssay, Hayward, CA, USA), and liver injury was assessed via alanine transaminase levels (ALT) (EnzyChrom ALT assay, EALT-100, BioAssay). For the evaluation of antibody responses, mouse serum was analysed for total immunoglobulin³⁶ by capillary protein electrophoresis (MINICAP-2 Sebia, Evry Cedex, France). The percentage of protein in the gamma zone of protein electrophoresis was converted into total immunoglobulin level by multiplying the ratio of protein at the gamma zone by the serum total protein. Serum total protein and urine protein were measured by Bradford protein assay. Urine protein creatinine index (UPCI), a representative of 24 h urine protein, was determined by the following equation; UPCI = spot urine protein/spot urine creatinine. Cytokine measurement (TNF- α , IL-6 and IL-10) in serum and supernatant media were measured using ELISA assays (eBioscience, San Diego, CA, USA).

Anti-dsDNA antibody detection. Calf DNA (Invitrogen, Carlsbad, CA, USA) coated on 96-well plates was used for measuring anti-dsDNA antibodies, following a previously published protocol³⁷. In brief, the plates were coated with calf DNA at 100 μ g/well and incubated overnight at 4 °C. The plates were dried, filled with 100 μ l/well of blocking solution, incubated at room temperature for 1.5 h and washed. Subsequently, mouse serum samples at 100 μ l/well were added and incubated for 1 h. Then, the plate was washed, incubated with peroxidase-conjugated goat anti-mouse antibodies (BioLegend, USA) at 100 μ l/well at room temperature for 1 h, washed and developed with ABTS peroxidase substrate solution (TMB Substrate Set; BioLegend) for 10 min in the dark. Finally, the stop solution (2 N H₂SO₄) was added, and the plate was read with a microplate photometer at a wavelength of 450 nm.

Bone marrow-derived macrophage preparation. Bone marrow (BM)-derived macrophages were produced following an established protocol³⁸. In brief, BM cells from femurs were centrifuged at 1,000 rpm in 4 °C for 10 min. Then, the cells were incubated in BMM or DMEM complete (DMEM supplement with 10% fetal bovine serum, 1% penicillin/streptomycin, HEPES and sodium pyruvate) plus 5% horse serum (HyCloneTM donor horse serum, Thermo Scientific, Waltham, MA, USA) and 20% L929-conditioned media in a humidified 5% CO₂ incubator at 37 °C for 7 days. The cells were harvested at the end of the culture period using cold PBS, and the macrophage phenotype was confirmed by flow cytometry with anti-F4/80 and anti-CD11b antibodies (BioLegend, USA).

In vitro *Cryptococcus neoformans*-induced macrophage cytokine production. Heat-killed *C. neoformans*, (immersion in a 60 °C water bath for 1 h) or live *C. neoformans*, at a dose of 5×10^5 yeast cells/ml, were incubated with macrophages (1×10^5 cells/well) in 96-well polystyrene tissue culture plates³⁹. The culture supernatants were collected at various time points and stored at -80 °C until use. After the incubation, the cell viability was measured by the MTS cell proliferation assay (One Solution Cell Proliferation Assay, Promega Corporation, WI, USA) according to the manufacturer's instructions⁴⁰. Briefly, 20 μ l of MTS were added to the culture plates, incubated for 2 h at 37 °C in a 5% CO₂ incubator, and then read with a microplate photometer at a wavelength of 490 nm. All of the experiments showed cell viability of greater than 95% (data not shown).

Phagocytosis, macrophage total killing activity and intracellular proliferation assays. The phagocytosis and macrophage killing activity assessments were performed according to a previous protocol, with slight modifications^{34,35}. Briefly, BM-derived macrophages were added to 96-well plates at 2.5×10^4 cells/well in DMEM complete and incubated overnight. After the incubation, LPS from *Escherichia coli* 026:B6 (Sigma-Aldrich, St. Louis, USA) was added at final concentration of 100 ng/ml and the plates were incubated in 5% CO₂ at 37 °C for 24 h. The medium was then removed and 100 μ l of complete DMEM with 20% normal mouse serum, as a source of opsonin, were added with heat-killed *C. neoformans* at various ratios of fungal cells to macrophages. These were incubated for various periods. After incubation, the wells were washed with 200 μ l of PBS at least 3 times to remove un-ingested yeast and then the macrophages were detached with 200 μ l of cold PBS. Then, the macrophages were transferred to a CytoSpin chamber (Thermo Scientific) and centrifuged at 600 rpm for 5 min to concentrate the cells into a single cell-layer for easier visualization. The macrophages were stained with Diff-Quick stain (Life Science Dynamic Division, Nonthaburi, Thailand). Macrophages containing yeast were counted as showing phagocytosis. At least 100 macrophages per well were counted. In parallel, the ingestion ability of each individual macrophage was determined as the average number of fungal cells in each macrophage (phagocytosis index), calculated as the total number of ingested fungi divided by the total number of macrophages. The phagocytosis activity was determined as the percentage of macrophages with phagocytosed cryptococci. All of the experiments were performed in triplicate.

Total cryptococcal cell killing activity was assessed using a previously published method which both extruded and intracellular yeasts were determined³⁴. Briefly, BM-derived macrophages at 1×10^5 cells/well were

co-cultured with live *C. neoformans* at a ratio of 1:1 for 2, 4 and 24 h. LPS and mouse serum were used as mentioned above. After incubation, the culture supernatant was separated, and a lysis medium (distilled water containing 0.01% bovine serum albumin and 0.01% Tween-80) was added to the wells, for 20 min at 37 °C, to rupture the macrophage cell membranes. Then, the supernatant and the lysate were well mixed. Serial dilutions of the mixed lysates were plated on SDA for viable yeast colony forming unit (CFU) counts. Control cultures consisted of incubation medium alone plus *C. neoformans*. Macrophage total killing activity is inversely correlated with the number of yeast colonies. The intracellular and extracellular killing activity of macrophages was evaluated with this method.

Moreover, to determine only the intracellular killing activity, the intracellular proliferation assay was performed with methods that were slightly modified from those previously published^{41,42}. Briefly, BM-derived macrophages, at 2.5×10^4 cells/well, were incubated for 17 h with IFN- γ (10 ng/ml final concentration; BioLegend, USA). Then, LPS (100 ng/ml final concentration; Sigma-Aldrich) was added and incubation continued for 24 h. Cells were then washed with PBS. After that, live *C. neoformans*, at a ratio of 5:1, were added and incubated for 2 h in 20% normal mouse serum containing media to promote phagocytosis. After 2 h, the wells were extensively washed (4–5 times) to remove extracellular fungi. This was set as the 0 h time-point. For some of the culture wells at this time-point, macrophage cell lysis were induced by lysis medium and plated on SDA for the visualization of intracellular fungal viability for “phagocytosis activity at the 0 h time-point” for the further calculation. The remaining culture wells from the 0 h time-point were maintained in DMEM media at 37 °C, and the cells were subsequently lysed at 2, 4 and 24 h, for the determination intracellular fungal viability, as mentioned above. Because the difference in intracellular fungi might be due to the difference in phagocytosis activity at the 0 h time-point, the phagocytosis activity at the 0 h time-point was used for the normalization with the following equation: intracellular proliferation at specific time points = CFU of fungi at 2, 4 or 24 h after the 0 h time-point/CFU of fungi after phagocytosis at the 0 h time-point. Macrophage intracellular killing activity is inversely correlated with the number of yeast colonies (intracellular proliferation).

Statistical analysis. The mean \pm SE was used for data presentation, and the differences among groups were examined for statistical significance using the unpaired Student’s 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. The repeated measures ANOVA with Bonferroni post hoc analysis was used for the analysis of the data with several time-points. 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.

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Acknowledgements

This work was conducted at the Center of Excellence in Immunology and Immune-Mediated Diseases and supported by the Research Grant for New Scholars, Thailand Research fund (TRG5780207) and grants supported by Chulalongkorn University, including the Grant for Development of New Faculty Staff, the 90th Anniversary of Chulalongkorn University, Rachadapisek Sompote Fund, the Graduate School Thesis Grant of Chulalongkorn University and Grant for International Research Integration, Chula Research Scholar, Ratchadaphiseksomphot Endowment Fund. The authors thank Dr. Malcolm Finkelman for his help in the final editing of the English language of this paper.

Author Contributions

S.S., T.O., A.C. and A.L. designed experiments. S.S., T.O., S.T., and A.L. performed experiments. S.S., T.P., P.P. and A.L. prepared the manuscript. All authors discussed the results and commented on the manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Surawut, S. *et al.* The role of macrophages in the susceptibility of Fc gamma receptor IIb deficient mice to *Cryptococcus neoformans*. *Sci. Rep.* **7**, 40006; doi: 10.1038/srep40006 (2017).

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