

-Running Title:

Inheritance of Immune Polarization Patterns to *C. neoformans* Infection

**Inheritance of Immune Polarization Patterns is linked to Resistance versus Susceptibility to
Cryptococcus neoformans in a Mouse Model.**

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Abstract

Genetic background variation between inbred strains accounts for different levels of susceptibility to *C. neoformans* in the mouse infection model. To elucidate the inheritance of immunophenotypic traits and their associations with clearance outcomes during cryptococcal infection, we compared C57BL/6, BALB/c and their first generation hybrid, CB6F1 (F1) mice. Mice from each group were infected with *C. neoformans* (10^4 CFU) and analyzed at weekly intervals over a 6-week period. BALB/c mice progressively cleared the cryptococcal infection in the lungs and showed Th1-skewed immune response: a Th1 shifted cytokine profile, modest lung pathology, and no significant elevation in systemic IgE level. In contrast, C57BL/6 mice developed a chronic infection with a Th2-skewed immune response: a Th2 shifted cytokine profile, pulmonary eosinophilia, severe lung pathology, elevated serum IgE, fungemia, and cryptococcal CNS dissemination. F1 mice demonstrated intermediate resistance to *C. neoformans*, with a stronger resemblance to the immunophenotype of the resistant (BALB/c) mice. F1 mice also demonstrated enhanced pulmonary recruitment of lymphocytes, especially CD8⁺ T cells in comparison to both parental strains, suggesting positive heterosis. We conclude that the inheritance of traits responsible for early cytokine induction in the infected lungs and DC maturation/activation status in draining nodes is responsible for an intermediate polarization and clearance outcomes observed initially in the lungs of F1 mice. The enhanced pulmonary lymphocyte recruitment could be responsible for a gradual shutdown of undesirable Th2 arm of the immune response and subsequently improved anticryptococcal resistance in F1 mice.

Introduction

Cryptococcus neoformans is one of the major opportunistic fungal pathogens worldwide and is a leading cause of fatal mycosis in immunocompromised individuals (3). Clearance of *C. neoformans* infection requires the development of a protective T-cell mediated immune response. Lacking this T-cell mediated response, as is seen in HIV-positive patients or patients undergoing aggressive immunosuppressive therapies, is a major risk factor for clinical *C. neoformans* infections (7, 24, 36). Invasive cryptococcosis has been also reported in non-compromised patients, indicating that *C. neoformans* can evade the host's immune responses even in the absence of the apparent defects in the immune system (14, 15, 40). Recently, Marroni *et al* (31) suggested that specific genetic defects in the natural immune system could be responsible for pulmonary cryptococcosis in an apparent immunocompetent patient. Therefore, genetic predisposition is one of the possible factors that could account for the increased susceptibility to *C. neoformans* infection in non-compromised patients.

In mice, the host's genetic background has significant effects on the pulmonary immune responses to cryptococcal infections. Inbred mouse strains, CBA/J, BALB/c, and C.B-17 are able to clear *C. neoformans* infection, while C57BL/6 mice develop chronic pulmonary infections (13, 16-19). In addition, host factors such as gender and age have influence on susceptibility to cryptococcosis (28). Experimental *C. neoformans* infections in knock out (KO) mice lacking specific cytokine or immunological receptor genes have shown a wide spectrum of effects including: complete lack of clearance (2, 5), diminished clearance (33), and in some instances enhanced clearance rates (11) when compared to their WT counterparts. These studies indicate that a single defect in the immune response can greatly alter the clearance phenotype from resistant to susceptible and vice versa.

The different resistance/susceptibility patterns to cryptococcal infection in various strains of mice have been linked to differential phenotypes of the inflammatory responses (13). Successful clearance of *C. neoformans* relies on the development of a T1 immune response, while a T2-polarized immune response elicits enhanced susceptibility in a number of mice models (12, 20, 22, 34, 38). Many of the observed differences strongly suggest that susceptibility/resistance are linked with differential polarization of the immune responses in specific strains of mice (13, 16). However, very little is known about the inheritance of the resistance/susceptibility patterns and how these inherited elements may be important in the resistant phenotype. Investigating the patterns of inheritance of a number of immunological factors required for clearance in resistant versus susceptible strains could reveal how genetic differences affect clearance.

Apart from the well established role of T cells in host defenses to *C. neoformans*, the role of pulmonary macrophages and their activation profile has been increasingly appreciated. Alveolar macrophages are involved in the initial recognition of *C. neoformans* and secretion of cytokines/chemokines which aid the development of the adaptive immune response (2, 39). When properly activated, macrophages play an important role in cryptococcal clearance as an effector cell that destroys *C. neoformans* following phagocytosis. The secretion of cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-12 by macrophages support the development of protective immunity and is crucial to the host's ability to clear fungal infections (12, 17, 21, 22). Alternatively, macrophages can harbor live *C. neoformans* without induction of proper pro-inflammatory cytokine signals. In this case, protective immunity does not develop and macrophages become a reservoir of *C. neoformans* in the infected host (2, 39). It is likely that the ability of alveolar macrophages to rapidly recognize *C. neoformans* and deliver robust

danger signals, such as TNF- α , is an important component of the inherited resistance to this pathogen. Furthermore, the critical role of alveolar macrophages as a central effector cell of host innate immune system has been attributed to the differences in susceptibility to pulmonary cryptococcal infection in rats and mice (23, 35).

Although work analyzing susceptibility and resistance patterns in wild type mouse strains has been performed, the patterns of inheritance of host factors that affect the clearance process remain largely unknown. Our studies focus on the inheritance of a wide variety of immune factors that are expressed during cryptococcal infection in mice. In order to elucidate the immunological phenotypic components caused by differential host genetic background, parallel analysis of *C. neoformans* infection in the widely used susceptible C57BL/6, and resistant BALB/c, and the first generation hybrid of these two mice (CB6F1/J, denoted as F1 mice) was performed.

Materials and Methods

Mice

These studies were approved by both VA and UM Committees for Animal Use and Care. Female wild-type C57BL/6 and BALB/c mice and their first generation hybrid CB6F1/J (F1) used in these studies were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were raised in specific pathogen-free conditions at the Ann Arbor VA Medical Center using sterilized cages covered with a filter top and were fed sterile food/water *ad libitum*. Mice were aged to 6-8 weeks at the time of infection.

Cultures of C. neoformans

C. neoformans strain 52D (ATCC 24067) was obtained from the American Type Culture Collection (Manassas, VA). For infection, yeast were grown to stationary phase after an incubation period of 48-72 hours at 37°C in Sabouraud dextrose broth (1% neopeptone and 2% dextrose; Difco, Detroit, MI) on a shaker. Cultures were washed twice with saline, counted on a hemocytometer and diluted to 3.3×10^5 Colony Forming Units (CFU)/ml in nonpyrogenic saline.

Intratracheal Inoculation of C. neoformans

Mice were anesthetized by an intraperitoneal injection of Ketamine/Xylazine solution and restrained on a surgical board. A small incision was made through the skin covering the trachea. The underlying salivary glands and muscle were separated. A 1-ml syringe filled with the appropriate concentration of *C. neoformans* was attached to a 30-gauge needle and inserted to the trachea. The delivery of 30 μ l of inoculum (10^4 CFUs) into the trachea was visibly confirmed. The needle was removed and the skin closed with a cyanoacrylate adhesive. Mice recovered with minimal visible trauma.

Organ CFU Assay

For lung CFU, small aliquots were collected from lung digests. For spleen and brain CFU, the respective organs were excised, placed in 2 ml of sterile water, and homogenized. Series of 10-fold dilutions of the lungs, spleen, and brain samples were plated on Sabouraud dextrose agar plates in duplicates of ten-microliter aliquots, and incubated at room temperature. *C. neoformans* colonies were counted 3 days later, and the number of CFUs was determined on a per organ basis.

Preparation and enumeration of Lung Leukocytes

Mice were sacrificed by CO₂ asphyxiation. To isolate pulmonary leukocytes, the abdominal vena cava of the mice was severed to remove red blood cells from the lungs prior to excision. Individual lungs were excised from sacrificed mice, minced with scissors and enzymatically digested for 30 minutes in 15 ml of digestion buffer (RPMI, 5% FCS, Penicillin and Streptomycin, 1mg/ml collagenase, 0.25 ml/mouse of DNase) at 37°C. The resulting cell suspension was further dispersed through the bore of a 10-ml syringe 20 times and centrifuged. Following enzymatic digestion, the erythrocyte pellets were lysed by the addition of 3 ml of NH₄Cl buffer for 3 minutes. 10 ml excess medium was added to return the solution to isotonicity. Resuspended cells were filtered through a 70 micron sterile nylon screen and centrifuged for 25 minutes at 3000 rpm in the presence of 20% Percoll to separate leukocytes from cell debris and epithelial cells. Total lung leukocyte numbers were enumerated in a hemocytometer using a trypan blue exclusion assay.

Leukocyte Subset Analysis

Macrophages, neutrophils, eosinophils, monocytes and lymphocytes were visually counted in Wright-Giemsa-stained samples of lung cell suspensions cytospun onto glass slides. Slides were stained by fixing for two minutes with a one-step methanol based Wright-Giemsa stain followed by steps two and three of the Diff-Quik whole blood stain kit. 200-300 cells from each sample were then counted from a randomly chosen field using a high-powered microscope. The percentage of a leukocyte subset was multiplied by the total number of leukocytes to give the absolute number of the specific leukocyte subset in the sample.

Lung Leukocyte Culture

Isolated lung leukocytes were diluted to 5×10^6 cells/ml and were cultured in 24-well plates with 2 ml of complete RPMI media at 37°C and 5 % CO₂ for 24 hours. Plates were centrifuged to remove leukocytes and supernatants were collected for subsequent ELISA tests.

Antibody staining and flow cytometric analysis

All staining reactions were performed on ice. Cells were incubated with 2.4G2 Fc receptor Ab to minimize nonspecific binding. Data was collected on a FACS LSR2 flow cytometer using DIVA Software and analyzed using FlowJo software (Tree Star Inc., San Carlos, CA). A minimum of 50,000 cells was analyzed per sample. Initial gates were set based on light scatter characteristics to exclude debris, red cells, and cell clusters. To detect DC in the lung associate lymph node (LALN), cell were isolated and stained with APC-labeled anti-CD11c (HL3), PE-labeled anti-MHC Class II (Ia^{d/b}) Abs. The phenotype of CD11c^{hi} MHC II^{hi} cells was determined by using either PerCPCy5.5-labeled anti-CD86 or FITC-labeled anti-CD80 and CD40 Abs. To detect lung lymphocyte subsets, single cells were stained with PerCPCy5.5-labeled anti-CD45. The lymphocyte subsets of CD45⁺ cells were determined by using APC-labeled anti-CD8, and PE-labeled anti-CD19 (for B cells), and FITC-labeled anti-CD4 Abs. All mAb reagents were purchased from PharMingen, San Diego, CA. The absolute number of each lymphocyte subset in the sample was obtained by the percentage of that type of lymphocyte subset multiplied by the total number of leukocytes.

Bronchoalveolar lavage (BAL)

Euthanized mice were lavaged after cannulation of the trachea with polyethylene tubing (PE50), which was attached to a 25-gauge needle on a tuberculin syringe. The lungs were lavaged twice with 0.8 ml of PBS containing 5 mM EDTA. The recovered fluid (1.3–1.4 ml total) was spun at 1500 rpm, and the supernatant was removed and stored at -20°C until analyzed for cytokines by ELISA (OptEIA; PharMingen).

Cytokine Protein Measurement

Cytokines TNF- α , IFN- γ , IL-12, IL-4 and IL-10 were quantified by ELISA using DuoSet Kits (R&D Systems, Minneapolis, MN) and OPT-EIA kits (BD Biosciences, San Jose, CA) following manufacturer's specifications. All plates were read on a Versamax plate reader (Molecular Devices, Sunnyvale, CA).

Total Serum Immunoglobulins

Serum was obtained from the blood samples collected by severing the vena cava of the mice during lung excision. Samples were then allowed to clot and after approximately two hours were spun to obtain the serum. Serum samples were diluted as needed and assayed for specific immunoglobulin (IgE) levels following standard ELISA protocol (*see above*) using an IgE sandwich ELISA.

Histological Analysis

Lungs were fixed by inflation with 1 ml of 10% neutral buffered formalin. After paraffin embedding, 5 μ m sections were cut and stained with hematoxylin and eosin. Sections were analyzed using light microscopy.

Calculations and Statistics

Statistical significance was calculated using One-way ANOVA with Student-Newman-Keuls test post-hoc, and Kruskal Wallis test was used, whenever data did not follow normal distribution. Two-way ANOVA (mouse strain and time as between subject factors) was used to analyze overall strain effect when multiple time points were compared. Means with P values <0.05 were considered significantly different. All values are reported as mean $\pm$ standard error (SEM).

Results

F1 mice inherit the ability to clear C. neoformans infection from their lungs.

Our first objective was to assess the clearance phenotype in the first generation hybrid (F1) cross between a resistant (BALB/c) and susceptible (C57BL/6) mouse strains. The F1 mice and the parental strains were infected with *C. neoformans* and evaluated at weeks 1 through 6 post infection (Fig. 1). Rapid pulmonary growth of *C. neoformans* (~1000 fold) was observed at week 1 post infection in all three strains of mice. Cryptococcal load reached a plateau at week 2 post infection and subsequently followed differential clearance patterns between the three strains of mice (weeks 3-6). C57BL/6 mice developed a chronic infection i.e. a constant fungal burden averaging above 10^7 CFU, for the duration of the study. In contrast, BALB/c mice demonstrated gradual clearance of *C. neoformans* beginning at week 2, marked by a 1,000-fold decrease in pulmonary fungal load between weeks 2 and 6 post infection. F1 mice maintained a constant level of infection throughout week 3; however, at the later time points (weeks 4 to 6 post

infection) F1 mice exhibited a progressive decrease in pulmonary load. The pulmonary microbial burden in F1 mice was lower than in C57BL/6 mice from week 3 on, throughout the remaining course of infection, but it was also significantly higher than in BALB/c mice at weeks 3 and 6 post-infection (Fig. 1). Although the difference in clearance between C57BL/6 and F1 mice was more pronounced, the factorial analysis showed significant overall CFU difference between F1 and BALB/c strains ($p=0.001$). Thus, F1 mice did not demonstrate the clearance of either parental strain; F1 mice could clear *C. neoformans* from their lungs in contrast with C57BL/6 mice, but with less efficiency than the resistant BALB/c mice.

F1 mice show intermediate susceptibility to fungemia and CNS dissemination.

Dissemination from the local site of infection (lung) into the blood and into the brain is another measure of susceptibility to *C. neoformans* infection. Therefore spleen and brain cryptococcal loads were compared at week 3 post infection. All of the susceptible C57BL/6 mice developed fungemia, illustrated by 100%-positive spleen CFU (Fig. 2A). In contrast, only 67% of resistant BALB/c mice and 83% of F1 mice demonstrated detectable spleen dissemination. The logarithmic spleen CFU values were significantly different between all 3 strains ($n=6$ animals per group). Consistently with the observed propensity for fungemia, the highest frequency of brain dissemination was observed in C57BL/6 mice (50%), while none of the BALB/c mice had detectable brain CFU (Fig. 2B). F1 mice demonstrated modest degree of brain dissemination with 33% of positive brain cultures. While brain CFU loads were not significantly different between the groups because of low numbers of positive animals (3/6 mice in C57BL/6 group and 2/6 in F1 group), this data distribution indicates that F1 mice were not entirely protected from cryptococcal dissemination into the brain. Thus F1 mice show intermediate pattern of

susceptibility/resistance to systemic *C. neoformans* dissemination compared to parental C57BL/6 and BALB/c mice.

Increased cellular inflammatory response in C. neoformans-infected lungs of F1 mice does not correlate with pulmonary clearance

To determine if the fungal clearance correlated with the magnitude and/or timing of the development of inflammatory responses in the lungs of infected F1, C57BL/6 and BALB/c mice, pulmonary leukocytes were enumerated. At week 1 post infection, all three strains of mice demonstrated a similar, mild increase in pulmonary leukocyte numbers consistent with the development of an inflammatory immune response (Fig. 3). Between weeks 1 and 2 post infection, the leukocyte numbers increased significantly and maintain elevation through week 4 in all three strains. Interestingly, both F1 and C57BL/6 strains demonstrated greater inflammation than BALB/c mice (Fig. 3). At week 6, all three strains exhibited significant differences in the number of pulmonary leukocytes; C57BL/6 mice maintained high numbers of leukocytes while BALB/c and F1 mice showed gradual resolution of the inflammatory response. Thus F1 mice initially exhibit the highest accumulation of pulmonary leukocytes; however the magnitude of inflammatory response does not correlate directly with the rate of *C. neoformans* clearance.

F1 mice exhibit “first generation hybrid effect” on the pulmonary lymphocyte recruitment in response to C. neoformans infection

Clearance to *C. neoformans* is T-cell dependent. Our next objective was to compare lymphocyte populations the lungs of infected F1, C57BL/6 and BALB/c mice. No differences in relative

frequencies of pulmonary lymphocytes were observed between the parental strains at any given timepoints post infection. However, F1 mice showed higher percentages of pulmonary lymphocytes compared to both parental strains at week 1 and to C57BL/6 mice at weeks 2 and 3 (Table 1). These increases in lymphocyte percentages resulted from significantly greater numbers of lymphocytes that F1 mice recruited into the lungs from week 1 on in comparison to both parental strains (20.0 ± 2.0 millions/lung versus 12.8 ± 1.8 millions/lung and 16.2 ± 2.8 millions/lung in C57BL/6 and BALB/c, respectively). These differences prompted subsequent flow cytometry study to enumerate specific lymphocyte subsets at weeks 0, 1, and 3 post-infection in all three strains of mice (Fig. 4). All three strains exhibited significant differences in the frequency of CD4⁺ cells, with BALB/c mice showing highest, F1 intermediate and C57BL/6 lowest CD4⁺ frequencies prior to the infection. However, the absolute numbers of pulmonary CD4⁺ cells in uninfected mice were not significantly different between all three strains. BALB/c mice continued to show significantly higher CD4⁺ cell frequencies at weeks 1 and 3 post infection compared to F1 and C57BL/6 mice. However, C57BL/6 mice showed lower absolute numbers of CD4⁺ cells in the lungs at weeks 1 and 3 compared to both BALB/c and F1 mice (Fig 4). In terms of CD8⁺ and B220⁺ cells there was no difference in their frequencies or absolute numbers between any of the uninfected mouse strains. There was also no difference in the recruitment of these cells into the lungs in the infected parental C57BL/6 and BALB/c mouse strains. However, F1 mice recruited higher numbers of CD8⁺ cells into their lungs (week 1-3) in comparison to either parental strain. Additionally, strong trends of elevated B220⁺ cells were found in the lungs of F1 mice with a significant increase over C57BL/6 mice at week 1. Taken together, these results demonstrate that F1 mice had increased recruitment of lymphocytes

(enhanced CD8⁺ and to some degree B220⁺ cell numbers) compared to parental strains, and increased total CD4⁺ cell number (but not the frequency) compared to C57BL/6 mice.

Chronic pulmonary eosinophilia observed in C. neoformans infected C57BL/6 mice was not inherited by F1 mice.

Since, the differences in pulmonary lymphocyte numbers could not explain the intermediate *C. neoformans* clearance pattern in F1 mice, we analyzed eosinophil populations (hallmark of non-protective Th2 response in the lungs) as well as the remaining subsets of pulmonary leukocytes. Table 1 shows percentages of each leukocyte subset evaluated microscopically in enzymatically digested lungs. No differences in relative frequencies of pulmonary leukocyte subsets were observed between the strains prior to the infection. From week one C57BL/6 mice demonstrated elevated eosinophil percentages compared with both F1 and BALB/c mice. This elevated frequency and number of pulmonary eosinophils in C57BL/6 mice was observed consistently throughout the entire course of infection (Fig. 5A). At week 2 post infection, F1 mice had significantly increased recruitment of eosinophils compared to BALB/c mice and showed total numbers similar to those in C57BL/6 mice (Fig. 5A) and an intermediate frequency of eosinophils (Table 1). However, except for week 2, both F1 and BALB/c mice demonstrated similar frequencies and total numbers of eosinophil throughout the course of the infection (Table 1 and Fig. 5A). Thus, only C57BL/6 mice developed persistent pulmonary eosinophilia, while F1 exhibited transient elevation in lung eosinophil numbers at week 2 post-infection comparable to that found in C57BL/6 mice.

F1 mice show no evidence of systemic Th2 polarization during the infection with C. neoformans

Since we observed transient but significant pulmonary eosinophilia in F1 mice, we suspected the possibility of temporal shift to the Th2 immune response in these mice. To address this concern we compared serum IgE levels (a systemic hallmark of Th2 response) between *C. neoformans*-infected F1, C57BL/6, and BALB/c mice. All three mouse strains exhibited only minimal IgE production during the initial 2 weeks of infection. As expected, C57BL/6 demonstrated significant increase of serum IgE over time (Fig 5B, ANOVA, $p=0.001$). Furthermore, C57BL/6 showed significant increases in serum IgE antibody levels compared to F1 mice and BALB/c mice at week 5 post infection, (Fig. 5B). Surprisingly, BALB/c mice demonstrated a small elevation of serum IgE compared to C57BL/6 and F1 mice that reach statistical significance at week 2 (Fig 5B). However, BALB/c IgE levels remained constant throughout the subsequent time points. Although, F1 mice showed some modest increase in serum IgE at week 5 compared with all the other time points ($p<0.05$), this was not significantly higher compared to IgE level in BALB/c mice (Fig 5B). Thus, unlike C57BL/6 mice, the F1 mice did not appear to develop the systemic Th2 response.

Host genetics of F1 mice affects the development of lung pathology in C. neoformans-infection

To determine if *C. neoformans* infection in F1 mice resulted in the development of lung pathology that resembled either of the paternal strains, infected lungs of F1, C57BL/6 and BALB/c mice, were examined histologically 6 weeks post infection.

As previously reported, C57BL/6 mice developed significant lung pathology with overwhelming features of lesions driven by type 2 immunity (2, 5). Microscopic examination revealed consolidation of large portions of lung and loss of functional lung tissue in C57BL/6 mice (Fig. 6A). The mixed inflammatory infiltrate filling the airspace was marked by numerous

eosinophils and multinucleated giant macrophages, many of which contained intracellular *C. neoformans* (Fig. 7A). Moreover, infected-lungs contained both intracellular and extracellular eosinophilic YM1/YM2 crystals (2) consistent with the presence of Alternatively Activated Macrophages (AAMs).

In BALB/c mice, infected lungs had fewer areas of inflammatory infiltrates as well as fewer intra- and extra-cellular cryptococci compared to the lungs of C57BL/6 and F1 mice (Fig. 6). The airspaces of BALB/c lungs were largely free of microbes and inflammation. Very few eosinophils and no evidence of AAMs (YM1/YM2 crystals) were observed in *C. neoformans* infected lungs of BALB/c mice (Fig. 7C). The tight mononuclear infiltrates of predominantly small mononuclear cells surrounding infected lung areas are consistent with the effective cryptococcal clearance and the absence/resolution of pathological lesions.

In F1 mice, large areas of lung tissue were replaced by dense inflammatory infiltrates, much as in C57BL/6 mice, but F1 mice also exhibited areas of tight mononuclear infiltrates as seen in BALB/c mice (Fig. 6B). Some granulocytes were observed in F1 mice, however, they were much fewer in number than in C57BL/6 mice. Although F1 mice exhibited areas of lung pathology filled with multinucleated giant macrophages, fewer macrophages with an morphological AAM phenotype were found when compared to those in C57BL/6 mice and no areas with YM1/YM2 crystal deposition were observed (Fig. 7B). Thus, F1 mice inherited an immune response that resulted in a mixed phenotype of pathological findings compared to Th2 driven pathology in parental C57BL/6 mice and findings considered with Th1-driven clearance in BALB/c mice.

F1 mice generate an intermediate Th1/Th2 cytokine profile during early infection that evolves towards a weaker Th1 response.

To determine if F1 mice inherited a preference for either Th1 or Th2 immune polarization of the response, the levels of TNF- α , IL-12, IFN- γ , IL-10, and IL-4 were analyzed at day 7 post infections in BAL fluid. Compared with C57BL/6 and F1 mice, BALB/c mice produced significantly more pro-Th1 cytokines TNF- α and IL-12 (Fig. 8A). BALB/c mice had significantly greater levels of early IFN- γ compared with C57BL/6 and strong trend towards increase of IFN- γ level compared with F1 mice. Although IFN- γ levels at week 1 were significantly different between C57BL/6 and F1 mice but not F1 and BALB/c mice, IL-12 levels in F1 mice were similar to those of C57BL/6 but different from BALB/c mice. F1 mice showed an intermediate level of IL-4 in BAL fluid that was significantly lower than in C57BL/6 mice but greater than in BALB/c mice (Fig. 8A) and no difference between the parental strains and the F1 mice were found in IL-10 BAL level (not shown). To assess the overall Th1/Th2 cytokine balance at the time of immune response generation, the ratios between driving Th2 (IL-4) versus driving Th1 (IFN- γ) cytokines were calculated. F1 mice demonstrated an intermediate IL-4/IFN- γ ratio compared to their parental strains by week 1 of infection (Fig. 8B), while cytokine production was strongly Th1-polarized in BALB/c and Th2-polarized in C57BL/6 mice (Fig. 8B). No differences were found between the F1 mice and the parental strains when uninfected lung leukocyte cytokine ratios were compared (data not shown). Thus, at an early stage of infection, the signals leading to Th1/Th2 cytokine polarization in F1 mice showed intermediate trends.

From week 2 on we monitored changes in the cytokine profile by analysis of cytokine production from isolated lung leukocytes in cultures. As at week 1, BALB/c mice demonstrated

overall highest level of IFN- γ compared to C57BL/6 or F1 mice (Fig. 9); levels of IFN- γ production were consistent with the observed differential rates of cryptococcal clearance. In contrast, only leukocytes from C57BL/6 mice produced a considerable amount of IL-4, while both BALB/c and F1 mice showed similar near baseline levels of IL-4 production at weeks 2 and 3 (Fig. 9). No differences in IL-10 cytokine production were observed between parental strains and F1 mice. Similarly to what was observed at week 1, BALB/c mice produced higher levels of TNF- α than C57BL/6 throughout the remaining time of infection. However, TNF- α levels of F1 mice fluctuated, reaching similar levels as in BALB/c mice at week 2 and decreasing again at week 3 to the level observed in C57BL/6 mice. These cytokine profiles are consistent with F1 mice developing a weaker Th1 response in contrast with the robust Th1 response in BALB/c mice and Th2 biased response in C57BL/6 mice.

F1 mice exhibit intermediate DC-maturation phenotype in lung draining lymph nodes

Pulmonary cytokine milieu strongly affects the maturation status of DC that migrate from the lungs into the LALN to subsequently induce T-cell responses. To determine the maturation status of DC in LALN in each of the infected mouse strains, flow cytometry analysis of isolated LALN cells was performed. CD11c⁺MHCII⁺ double positive DC populations (consistent with conventional DCs) were gated to analyze the surface expression of co-stimulatory molecules (Fig. 10A). Consistent with DC migration from the infected lungs into the nodes, the LALN from infected mice yielded significantly higher numbers and percentages of CD11c⁺MHCII⁺ DC than the LALN from uninfected mice (Fig. 10A). No differences in frequencies or total numbers of DC were observed in the nodes from the infected F1, BALB/c, and C57BL/6 mice (data not shown). Up-regulation of co-stimulatory molecules CD40, CD80, and CD86 was used as a surrogate of DC maturity, and these cells were denoted as CD40^{hi}, CD80^{hi}, and CD86^{hi}

respectively. The plots (Fig. 10B) illustrate the differences in peak fluorescence intensity and panel 10 C summarizes frequencies of CD40^{hi}, CD80^{hi}, and CD86^{hi} in the gated CD11c⁺MHCII⁺-cell population. Consistent with the robust Th1 response, BALB/c mice had the highest levels of all three maturation markers and significantly higher percentages of DC with strong co-stimulatory molecules expression compared to C57BL/6 mice. However, F1 mice exhibited an intermediate level of fluorescence intensity and percentage of all three markers, showing no significant difference from either parental strain. Thus, level of costimulatory molecule expression by DC in LALN correlated with differences in Th1 and Th2 immune polarization exhibited in all three strains of mice with F1 mice expressing an intermediate DC phenotype.

Discussion

This study analyzed the inheritance pattern of susceptibility versus resistance to cryptococcal infection by the first generation hybrid between resistant (BALB/c) and susceptible (C57BL/6) mice. Our data show that: 1) F1 mice inherit intermediate resistance to *C. neoformans*, but show a stronger resemblance to the immunophenotype of the resistant (BALB/c) mice; 2) some of the Th1/Th2 polarization traits are inherited independently from each other in F1 generation, but the clearance and dissemination patterns shows good correlation with overall Th1/Th2 balance; 3) early cytokine production in the lungs and DC maturation/activation status in LALN play significant roles in predicting Th1/Th2 bias of the immune response and the subsequent cryptococcal clearance; 4) apart from DC in LALN, other cell types in the lungs, such as CD8 cells, may influence the immune bias and contribute to the inherited immunophenotype.

Differential patterns of resistance and susceptibility to *C. neoformans* infection in different mouse strains have been described previously (16, 41). Our parallel experiments

carefully outlined the differences between C57BL/6 and BALB/c immunophenotypes over time, and analyzed their inheritance by their first generation progeny, F1 mice. C57BL/6 mice exhibited a susceptible phenotype. They developed a non-protective immune response with ineffective clearance, expressed numerous hallmarks of Th2 response and severe Th2-driven lung pathology, and were prone to systemic *C. neoformans* dissemination. In contrast, BALB/c mice progressively cleared *C. neoformans* 52D infection, did not develop severe lung pathology, demonstrated numerous hallmarks of a protective Th1 response and were protected from CNS dissemination. The first generation hybrid, F1 mice, unlike the C57BL/6 mice, maintained the ability to clear the infection from the lungs, but their clearance was slower compared to BALB/c mice. F1 mice also developed intermediate lung pathology, intermediate phenotype in regards to early expression of Th1/Th2 immunity hallmarks in the lungs and intermediate systemic dissemination of *C. neoformans*. However, some of the observed traits in the immune response of F1 mice were not uniform in terms of similarities and differences from the parental strains during the course of the infection. For example, in terms of *C. neoformans* clearance from the lungs, F1 mice separated from C57BL/6 mice and BALB/c at the same time (week 3) and showed significantly different cryptococcal lung burden from both parental strains at week 6. However, at weeks 4-5 pulmonary clearance was not significantly different from BALB/c mice. These outcomes suggest that clearance of the infection is not a linear process and is most likely driven by multiple factors over time.

C. neoformans affects mostly immunocompromised hosts and differential recruitment of leukocytes to the lungs could explain differential clearance phenotype. Interestingly, there was no positive correlation between the total numbers of pulmonary leukocytes and the rate of clearance exhibited by the three strains of mice. The F1 mice recruited on average the highest

numbers of leukocytes at week 2-4 post infection, but exhibited an intermediate rate of clearance. On the other hand, BALB/c mice demonstrated the fastest clearance rate while having the lowest number of inflammatory cells in their lungs throughout the study. Finally, C57BL/6 mice did not clear *C. neoformans* while recruiting intermediate numbers of leukocytes into their lungs (Fig. 1 and Fig. 3).

Subsequent analysis of the leukocyte subsets demonstrated 2 major differences between C57BL/6 mice and BALB/c. Firstly, BALB/c mice recruited greater numbers of CD4⁺ cells into the lungs post-infection. Secondly, the cellular composition (pulmonary eosinophils and macrophage phenotype in the infected lungs) reflected differential Th1/Th2 immune bias in the parental strains. With respect to CD4⁺ cell numbers, F1 mice recruited similar numbers of CD4⁺ cells as did BALB/c mice. However, CD4⁺ cell frequencies were similar between F1 and C57BL/6 mice, and differed from BALB/c mice. Although, we cannot exclude that the lower CD4⁺ cell recruitment in C57BL/6 mice could contribute to the higher sensitivity of these mice to *C. neoformans* infection, the immune polarization plays critical role in the outcome of clearance in this otherwise immunocompetent mouse strain. This notion is supported by our previous report, that IL-4 gene deletion from C57BL/6 results in the absence of Th2 response and the improved clearance of cryptococcal infection (2).

Eosinophilia in the lungs is a marker of aTh2 response and is associated with the lack of *C. neoformans* clearance/pathology (4, 16, 33). We demonstrate that pulmonary eosinophilia in C57BL/6 mice is persistent throughout the course of infection (Table 1 and Fig 5A). In contrast, BALB/c had the fewest eosinophils and demonstrated the most effective cryptococcal clearance in the lungs. F1 mice did not show the persistent eosinophilia, however they showed initial

eosinophil recruitment at week 2 similar to that found in C57BL/6 mice. From week 3 on eosinophilia in F1 mice subsided, concurrently with the beginning of clearance process.

Another clue regarding the inheritance of the anticytotoxic resistance and its linkage with the immune bias comes from morphological appearance of macrophages and the deposition of YM1/YM2 crystals (Figs 6-7). Our group reported previously that these distinct phenotypes of classically and alternatively activated macrophage (CAM and AAM) in the Th1 and Th2 biased immune responses in *C. neoformans* infected lungs (2, 29, 30). Consistent with these reports, an AAM phenotype predominated in C57BL/6 mice along with the development of Th2-driven lung pathology (Fig. 7). In contrast there was no morphological evidence of AAM in the lungs of infected BALB/c mice which showed tight mononuclear cell infiltrates, characteristic for Th1 granulomas with classically activated macrophages. In the infected lungs of F1 mice, both types of macrophage morphologies were present (Fig. 7). This mixed macrophage phenotype in F1 mice corroborated with the development of the immune response that was protective, but not as effective as the response in BALB/c mice.

The major hallmark of the polarized systemic Th2 response is antibody class switch resulting in the enhanced production of IgE (2, 6). As expected, C57BL/6 mice showed the most pronounced elevation in serum IgE (Fig. 5B). The IgE levels in infected F1 mice were also significantly elevated by week 5 post infection compared with the uninfected and week1-3 IgE levels. However, they were not significantly higher in F1 mice than in BALB/c mice, and were significantly lower than the IgE levels in C57BL/6 mice sera. This suggests that unlike C57BL/6 mice, F1 mice did not exhibit a significant shift to a systemic Th2 response.

Inheritance of the propensity for Th1 versus Th2 bias of the immune responses was further demonstrated by the cytokine profile elicited by lung leukocytes in the parental strains

and in the F1 mice. At week 1 post infection the pulmonary cytokine profiles in the parental strains were skewed in the directions of polarization subsequently seen in these mice. BALB/c mice had higher levels of Th1 driving cytokines (IFN- γ , IL-12 and TNF- α) compared to C57BL/6 mice and lower IL-4 level (Fig 8). F1 mice demonstrated an intermediate Th1 cytokine profile with IL-12 lower than in BALB/c mice, intermediate TNF- α , and IFN- γ that was higher in F1 than in C57BL/6 and a strong decreasing trend compared to BALB/c mice. IL-4 level was also intermediate in F1 mice, different from both C57BL/6 and BALB/c mice. The ratio between driving IL-4 and IFN- γ shows that the balance between Th1/Th2 responses was significantly different and strongly in line with the resistance level demonstrated by all 3 strains of mice (Figs 1-2 and 8). Interestingly, the proportions in the Th1/Th2 polarizing cytokines levels did not stay the same during the course of infection in F1 mice. IL-4 levels in F1 and BALB/c mice at week 2 and 3 were no longer different, which suggests that F1 mice did not become Th2 biased (Fig 9). The decline in IL-4 production in F1 mice from week 2 post-infection explains the subsequent resolution of pulmonary eosinophilia, the lack of obvious hallmarks of AAM, and the absence of significant elevation in serum IgE. On the other hand, the substantially lower IFN- γ and fluctuations in TNF- α levels can explain slower and variable clearance rate in F1 mice. These data combined with previous reports further support the notion that the ability to induce early cytokines in response to *C. neoformans* is engendered by genetic factors (12, 17). Secondly, the expression of these early cytokine genes results in differential outcomes of the immune response polarization and the anti-cryptococcal resistance in our model.

It remains unknown which underlying genetic differences are responsible for differential expression of early cytokines between BALB/c, C57BL/6 and their F1 progeny. We speculate that the cells of the innate immune system in C57BL/6 mice do not efficiently recognize some of

the molecular patterns of *C. neoformans* and therefore induce insufficient amount of early pro-inflammatory cytokines. These quantitative traits are most likely inherited by F1 mice in such way that their early response to *C. neoformans* is intermediate.

Dendritic cells are the main antigen sensing and antigen presenting cells of the immune system. Our previous studies have shown that the early cytokine signals, especially the TNF- α priming, is required for optimal DC maturation and activation (12, 20, 22, 34, 38). These studies demonstrated that injection of immature DC pulsed with cryptococcal mannoprotein inhibits development of Th1 immunity and *C. neoformans* clearance in resistant mice (12, 20, 22, 34, 38) and that the overexpression of adenoviral TNF can prevent the development of Th2 and promote clearance in C57BL/6 mice (32). The present study further illustrates this relationship by showing that maturation status of DC in draining lymph nodes correlates with the early cytokine profile and the effectiveness of cryptococcal clearance in all three strains of mice. F1 mice demonstrate an intermediate DC maturation phenotype in the regional nodes, which is consistent with early cytokine profile – in particular intermediate TNF- α production in F1 mice (Figs 9 and 10). These data suggest a scenario of the intermediate inheritance of early signals leading to a polarization in F1 mice and intermediate activation of DC that migrated into LALN. It is likely that this intermediate DC activation phenotype still present at week 3 post-infection in F1 mice was responsible for longer period of uncommitted Th1/Th2 bias in these mice. The decrease in IL-4 production observed at week 3 post infection occurs concurrently with the decline in eosinophil numbers in the lungs of F1 mice (Fig 5A and 9). This drift in the F1 mice polarization towards a weak Th1 rather than developing an intermediate Th0-like response could be a result of local interactions between the T-cells recruited into the lungs from LALN within the local lung microenvironment. These effects were reported to play a role in the final

development of T-cell effector functions during a pulmonary *C. neoformans* infection (26). Interestingly we found a 2-fold increase in pulmonary CD8⁺ cells in the F1 mice compared to either parental strain (Fig. 4), attributed to first generation hybrid effect (heterosis) (8-10). CD8⁺ cells can play a supportive role in generation of Th1 milieu in the lungs and benefit cryptococcal clearance (1, 25, 27, 37). Thus, the increased numbers of CD8⁺ cells, could contribute to the gradual shift towards Th1 phenotype in F1 mice and/or contributed to the improved clearance after week 3 post-infection.

In summary, we found that the susceptibility versus resistance to *C. neoformans* infection was inherited in a complex fashion, and that it was linked to immune response polarization. Although the data presented in this study apply specifically to BALB/c and C57BL/6 parental mouse strains and their first generation progeny, the mechanisms that contribute to these outcomes may be universally important for the inheritance of traits important for anticytotoxic immunity.

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TABLE 1

		BALB/c	F1	C57BL/6
Uninfected	Monocyte	36.3% ± 3.9	39.5% ± 6.7	39.0% ± 6.7
	Macrophage	28.9% ± 6.7	30.9% ± 6.6	29.0% ± 7.9
	Eosinophil	1.0% ± 0.3	1.0% ± 0.2	0.7% ± 0.3
	Neutrophil	2.6% ± 0.4	1.9% ± 0.6	3.4% ± 0.5
	Lymphocyte	31.2% ± 4.5	26.8% ± 5.2	28.0% ± 3.9
Week 1	Monocyte	32.5% ± 2.8	33.8% ± 1.6	28.9% ± 1.6*
	Macrophage	22.8% ± 2.4	18.7% ± 1.2	23.1% ± 2.5
	Eosinophil	5.0% ± 1.1	6.4% ± 1.3	13.3% ± 2.0*, **
	Neutrophil	18.1% ± 1.8*	13.2% ± 1.6	12.6% ± 1.7**
	Lymphocyte	21.7% ± 2.1*	27.0% ± 1.7	22.3% ± 1.7*
Week 2	Monocyte	35.2% ± 1.6*	28.3% ± 1.5	29.4% ± 1.8**
	Macrophage	24.1% ± 1.0	24.9% ± 1.7	23.7% ± 3.6
	Eosinophil	8.4% ± 1.1*	13.1% ± 2.3	23.1% ± 3.7*, **
	Neutrophil	11.3% ± 1.6	10.7% ± 1.8	8.1% ± 0.9
	Lymphocyte	19.4% ± 2.3	22.9% ± 2.1	15.7% ± 3.3*
Week 3	Monocyte	38.9% ± 2.0*	31.6% ± 1.5	32.3% ± 2.1**
	Macrophage	28.2% ± 2.6*	22.3% ± 1.9	21.6% ± 3.1
	Eosinophil	4.7% ± 1.3	6.4% ± 1.2	19.5% ± 2.5*, **
	Neutrophil	10.4% ± 1.7*	18.1% ± 2.3	8.7% ± 1.3*
	Lymphocyte	17.7% ± 2.1	21.6% ± 1.9	17.4% ± 1.5*
Week 5	Monocyte	44.4% ± 2.4*	32.5% ± 4.2	36.9% ± 2.4**
	Macrophage	25.5% ± 3.3	32.3% ± 2.4	27.1% ± 2.4
	Eosinophil	2.1% ± 0.8	1.5% ± 0.4	8.5% ± 3.5*
	Neutrophil	7.8% ± 1.2	13.9% ± 4.5	11.6% ± 2.3
	Lymphocyte	20.0% ± 2.1	20.1% ± 2.4	17.6% ± 2.5

* = Significantly different from F1 mice

** = Significantly different from BALB/c

TABLE 1. Relative leukocyte subset frequencies in *C. neoformans*-infected BALB/c, F1, and C57BL/6 mice at weeks 0,1,2,3, and 5 post infection. Leukocyte frequencies were evaluated using high-powered microscope counts of Wright-Giemsa-stained lung cell suspensions on cytopun slides. Leukocyte subsets are expressed as a percent of the total leukocytes present in the isolate to illustrate alterations in the cellular composition in the infected lungs. Values represent means $\pm$ SEM, n = 6-20 mice/group/timepoint. * indicates a significant difference from F1 mice; ** indicates a significant difference from BALB/c mice. Data pooled from five parallel experiments.

FIGURE LEGENDS

FIGURE 1. Pulmonary clearance of *C. neoformans* infection in BALB/c, F1 and C57BL/6 mice. Mice were inoculated intratracheally with 10^4 CFU of *C. neoformans* 52D and pulmonary *C. neoformans* burden was evaluated at week 1-6 post-infection. Values represent means $\pm$ SEM. n = 6-20 mice/group/timepoint. * indicates a significant difference from F1 mice; ** indicates a significant difference from BALB/c mice. Data pooled from three parallel experiments.

FIGURE 2. Systemic dissemination of *C. neoformans* from the lung in BALB/c, F1 and C57BL/6 mice. Mice were inoculated intratracheally with 10^4 CFU of *C. neoformans* 52D and spleen (A) and brain (B) *C. neoformans* burden were evaluated at week 3 post-infection. Values represent means $\pm$ SEM. n = 6 mice/group/timepoint. * indicates a significant difference from F1 mice; ** indicates a significant difference from BALB/c mice. Data pooled from three parallel experiments.

FIGURE 3. Leukocyte recruitment in *C. neoformans*-infected BALB/c, F1, and C57BL/6 mice. Lung leukocytes were isolated from individual mice and enumerated at weeks 0, 1, 2, 4, and 6 post infection. Values represent means $\pm$ SEM, n = 6-20 mice/group/timepoint. * indicates a significant difference from F1 mice; ** indicates a significant difference from BALB/c mice. Data pooled from five parallel experiments.

FIGURE 4. Lymphocyte subset recruitment following pulmonary *C. neoformans* 52D infection. Samples of leukocyte suspensions from infected mice were stained with fluorochrome-labeled antibodies specific for CD4⁺, CD8⁺, and B220⁺ lymphocytes and analyzed by flow cytometry as described in Materials and Methods. Results are illustrated as % and absolute numbers of each lymphocyte subset in the total lung leukocyte isolate. Values represent means $\pm$ SEM. n = 4-6 mice/group/timepoint. * indicates a significant difference from F1 mice; ** indicates a significant difference from BALB/c mice. Data pooled from two parallel experiments.

FIGURE 5. T2 hallmarks: Pulmonary eosinophilia and elevated levels of serum IgE.

A) Pulmonary eosinophils following *C. neoformans* 52D infection. Aliquots of leukocyte suspension were cytopun onto slides and cell subsets enumerated at weeks 0,1,2,3, and 5 post infection. Results are illustrated as number of eosinophils in the total lung leukocyte isolate. Values represent means $\pm$ SEM. n = 6-20 mice/group/timepoint. **B)** Total serum IgE levels were evaluated using serum samples from BALB/c, F1, and C57BL/6 mice. Samples were evaluated

for concentration of total IgE levels using the ELISA. Values represent means $\pm$ SEM, n = 6-10 mice/group/timepoint. * indicates a significant difference from F1 mice. ** indicates a significant difference from BALB/c mice. Data pooled from three parallel experiments.

FIGURE 6. Photomicrographs of lung histology from: **A)** C57BL/6 mice; **B)** F1 mice; and **C)** BALB/c mice. **LEFT)** uninfected lung sections **RIGHT)** *C. neoformans*-infected lung sections at week 6 post infection. Note: the multinucleated giant macrophages composing a large portion of the mixed leukocyte infiltrate consolidating the infected lungs in C57BL/6 mice; less diffuse inflammatory response and a mixture of giant cells and tightly packed mononuclear cell infiltrate in F1 mice; tightly packed mononuclear cell infiltrate and largely normal lung morphology in BALB/c mice. Pictures taken from H & E-stained lung sections at the objective power of 20X.

FIGURE 7. High power photomicrographs of lung pathologies from **A)** C57BL/6 mice, **B)** F1 mice, and **C)** BALB/c mice at week 6 post-infection. Note: the multinucleated giant macrophages containing both intracellular YM1/YM2 crystals (YM) and cryptococcal organisms (*) consistent with the presence of Alternatively Activated Macrophages (AAMs), as well as small red granulocytes consistent with eosinophils (Eo) C57BL/6 mice; the tight mononuclear cell infiltrate, the absence of AAMs and eosinophils in BALB/c mice consistent with Th1 granulomatous inflammation; intermediate lung pathology shown in the lungs of F1 mice. Pictures taken from H & E-stained lung sections at the objective power of 40X.

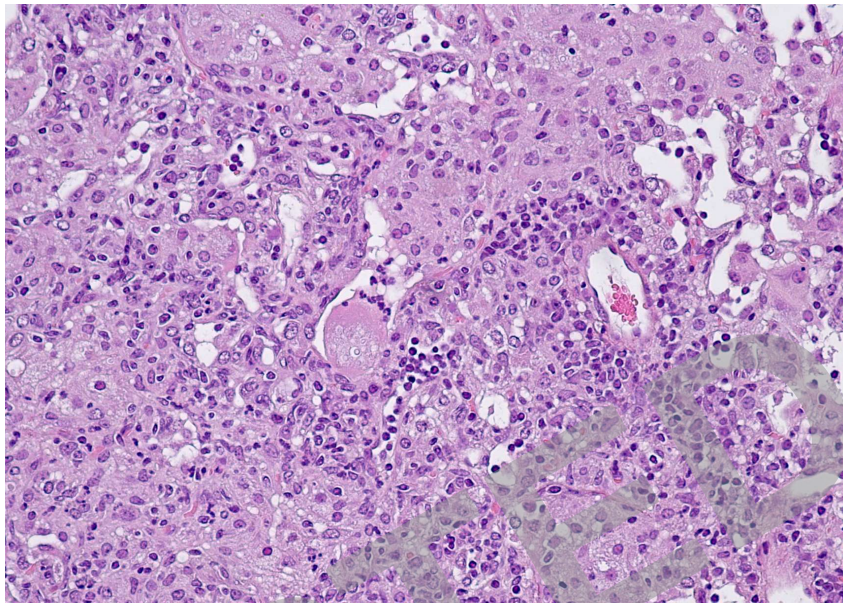
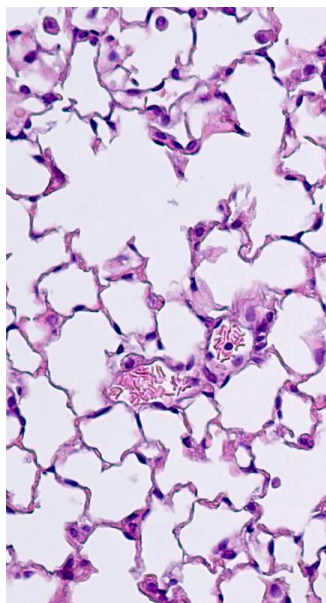
FIGURE 8. Cytokine production in BAL fluid from *C. neoformans*-infected BALB/c, F1, and C57BL/6 mice. **A)** BAL samples were collected at 7 days post infection and analyzed for cytokines by ELISA. **B)** The IFN- γ / IL-4 ratio was shown to illustrate a general trend in the immune response polarization. Data pooled from two parallel experiments. n = 4-6. Values represent means $\pm$ SEM in pg/ml supernatant. * indicates a significant difference from F1 mice; ** indicates a significant difference from BALB/c mice.

FIGURE 9. Cytokine production in pulmonary leukocyte culture supernatants from *C. neoformans*-infected BALB/c, F1, and C57BL/6 mice at weeks 0, 2 and 3 post infection. Isolated pulmonary leukocytes (5×10^6 /ml) were cultured for 24 hours post-harvest and cytokines were evaluated by ELISA. Values represent means $\pm$ SEM in pg/ml supernatant with n=6 and above for each of the analyzed time points. * indicates a significant difference from F1 mice; ** indicates a significant difference from BALB/c mice.

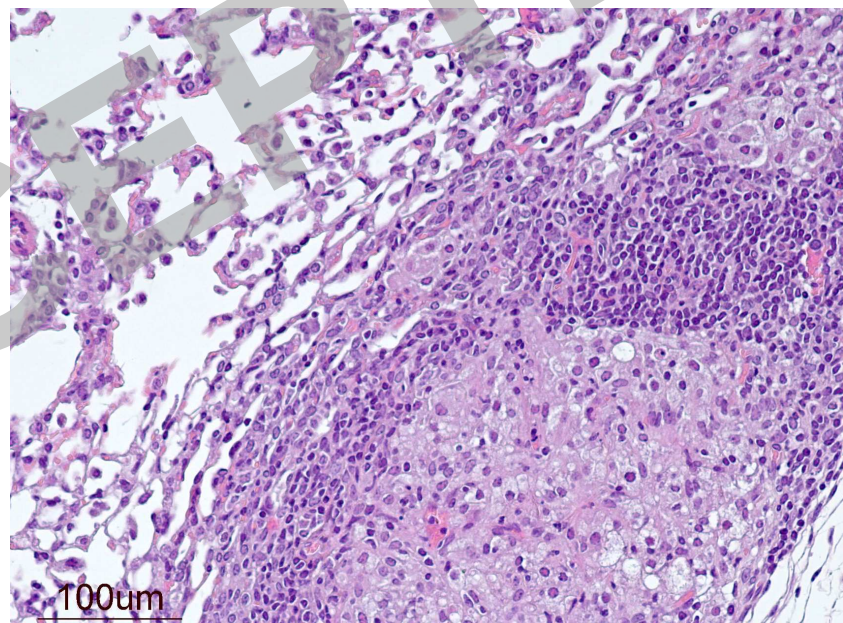
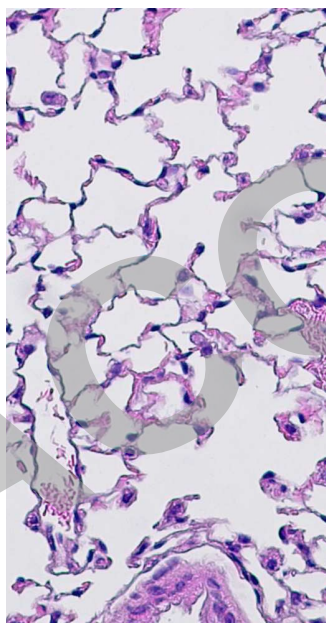
FIGURE 10. Phenotypical analysis of DC in lung draining lymph nodes isolated from BALB/c, F1, and C57BL/6 mice. **A)** Gating strategy for DC in the lung draining lymph nodes. CD11c⁺MHCII⁺ populations were gated to analyze proportion of cells with a high co-stimulatory molecule expression. Note, increased density of DC (CD11c⁺MHCII⁺ population) in the nodes in response to *C. neoformans* infection. **B)** Overlaying histograms of BALB/c (light gray line), F1 (black line), C57BL/6 (solid black area) and uninfected F1 mice (solid gray area). Percentages of DC with high expression of CD40, DC80 and CD86 molecules are listed below each respective histogram. Values represent means $\pm$ SEM, n = 6 mice/group. ** indicates a significant difference from BALB/c mice. Data pooled from two parallel experiments.

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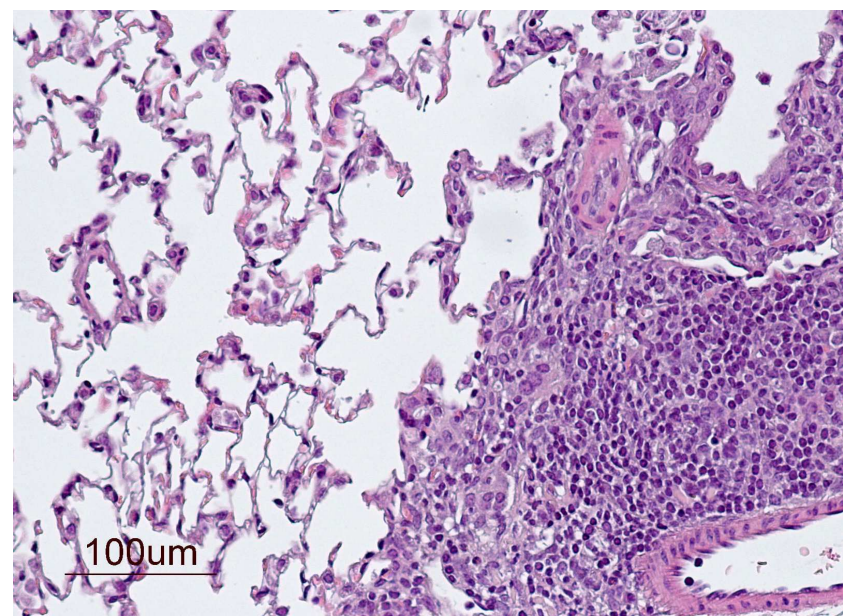
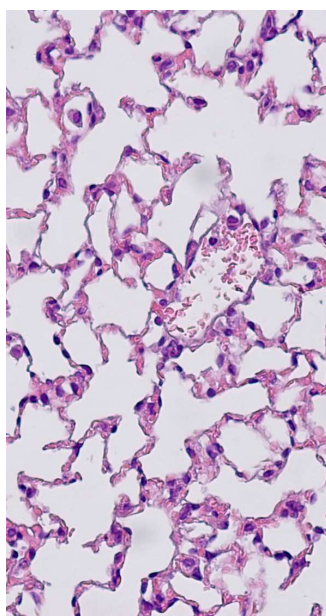
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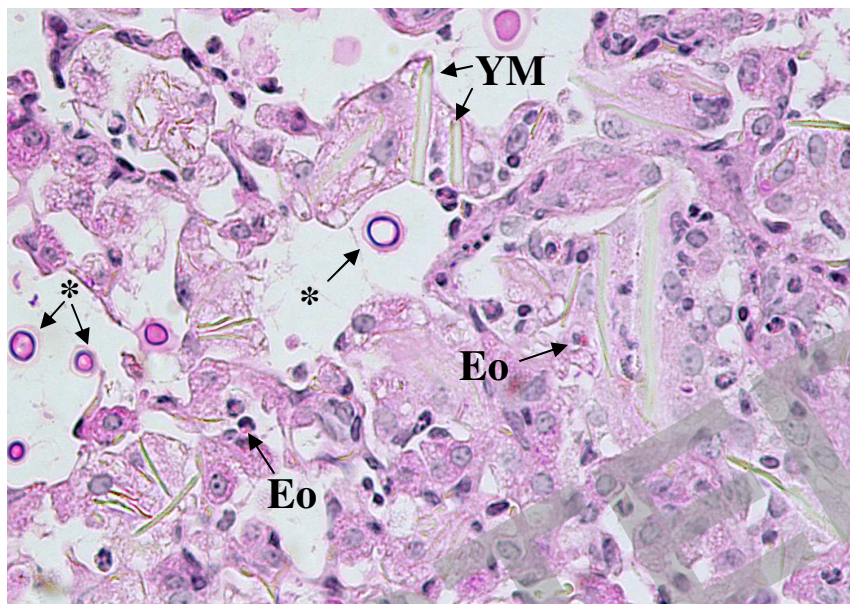
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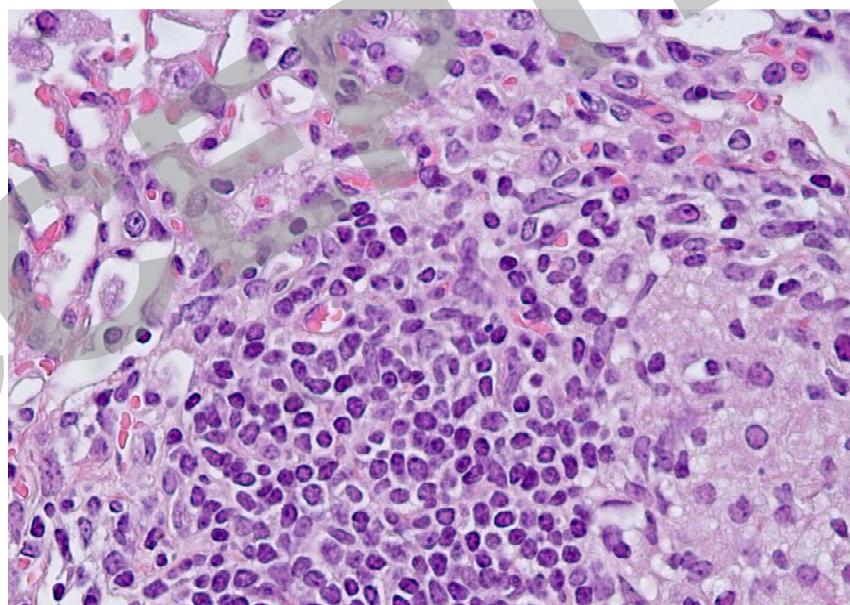
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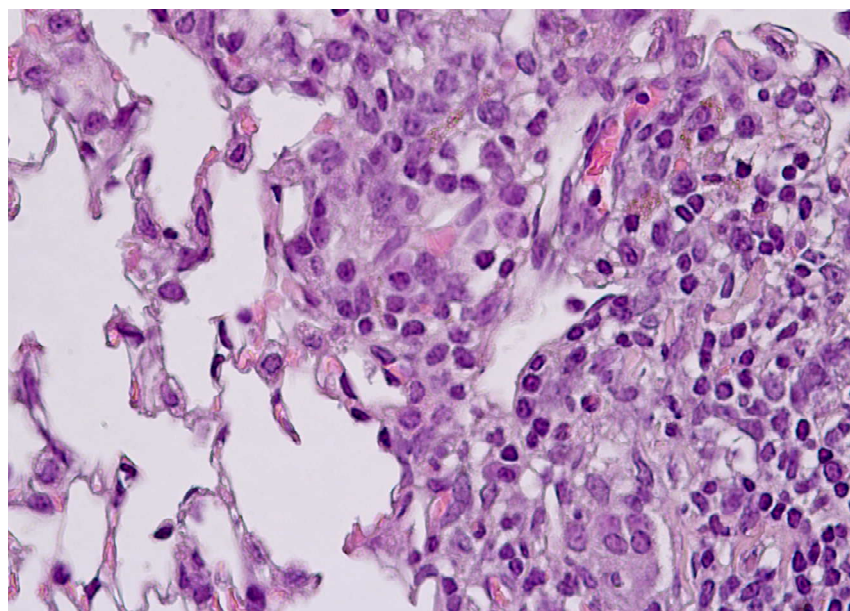
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C



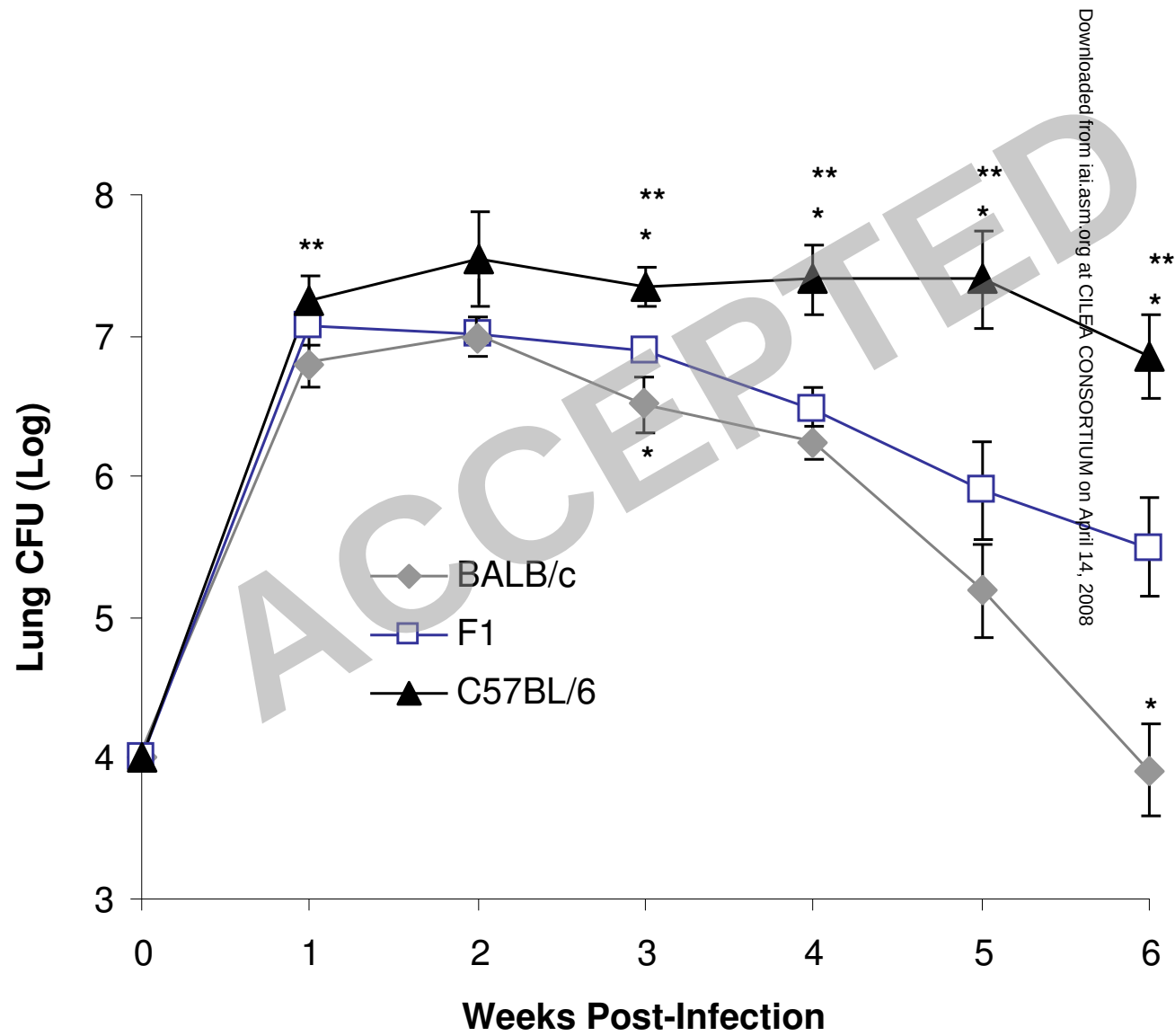


Fig. 1

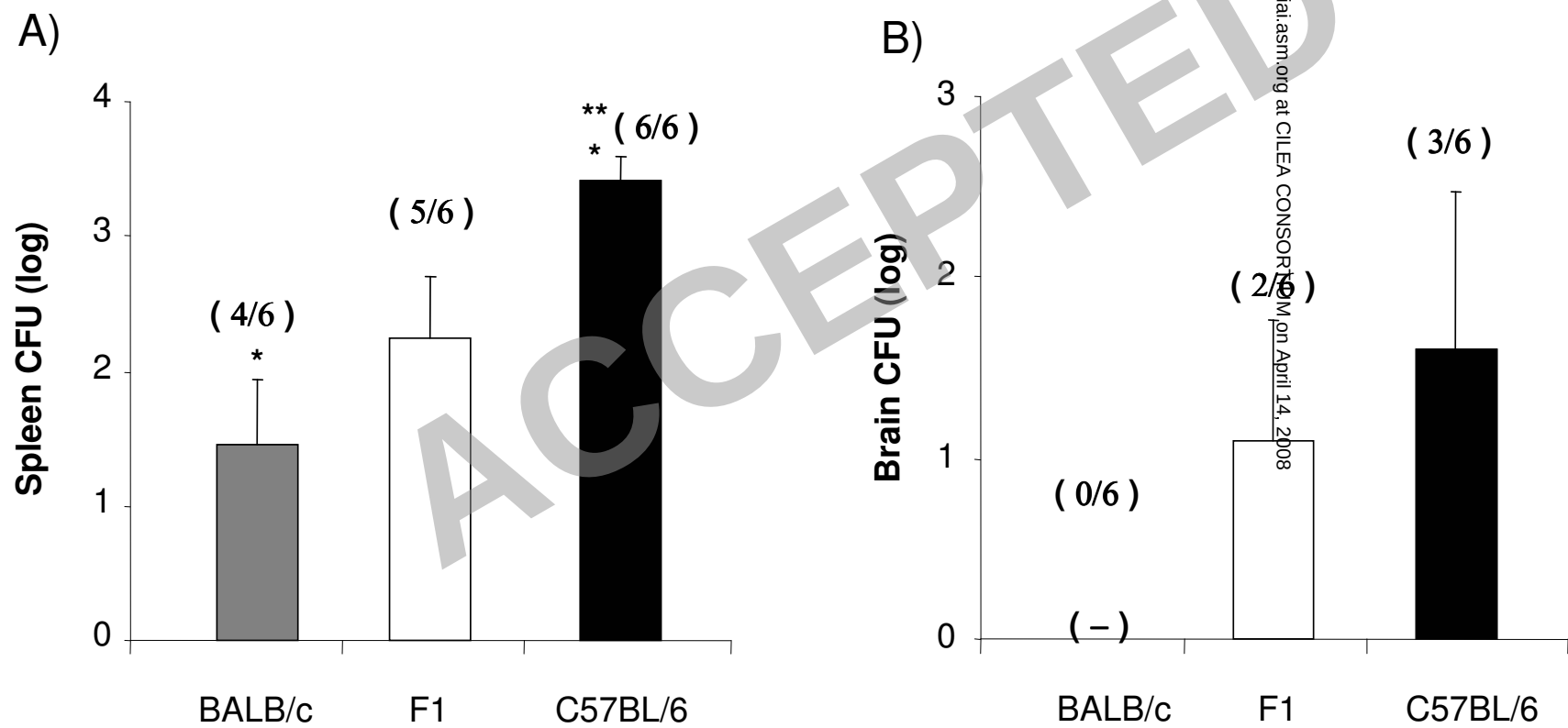


Fig. 2

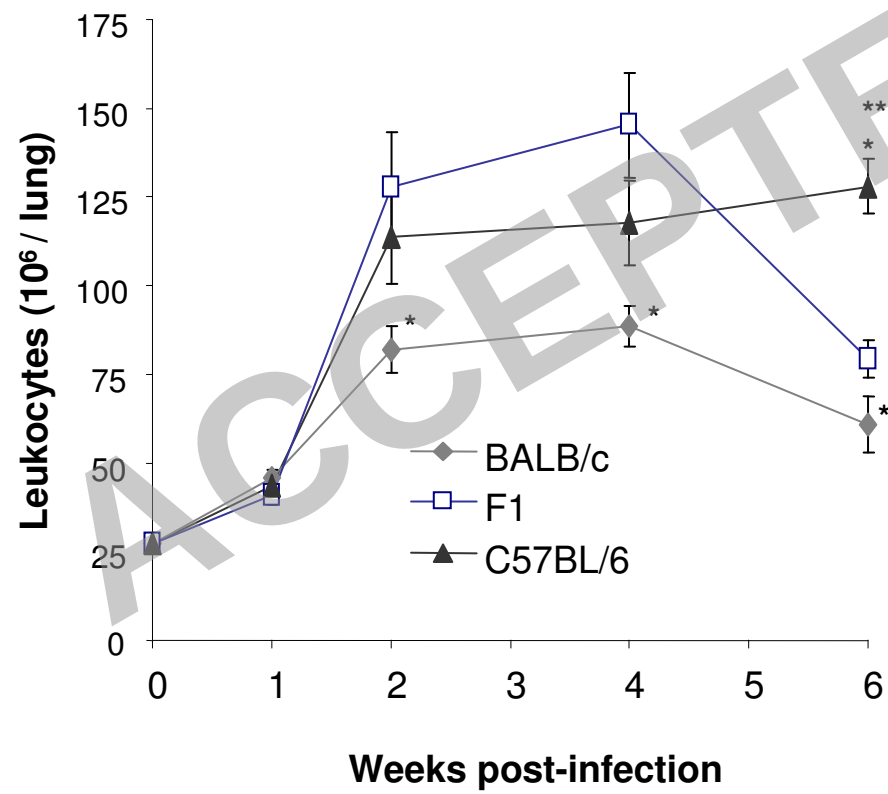


Fig. 3

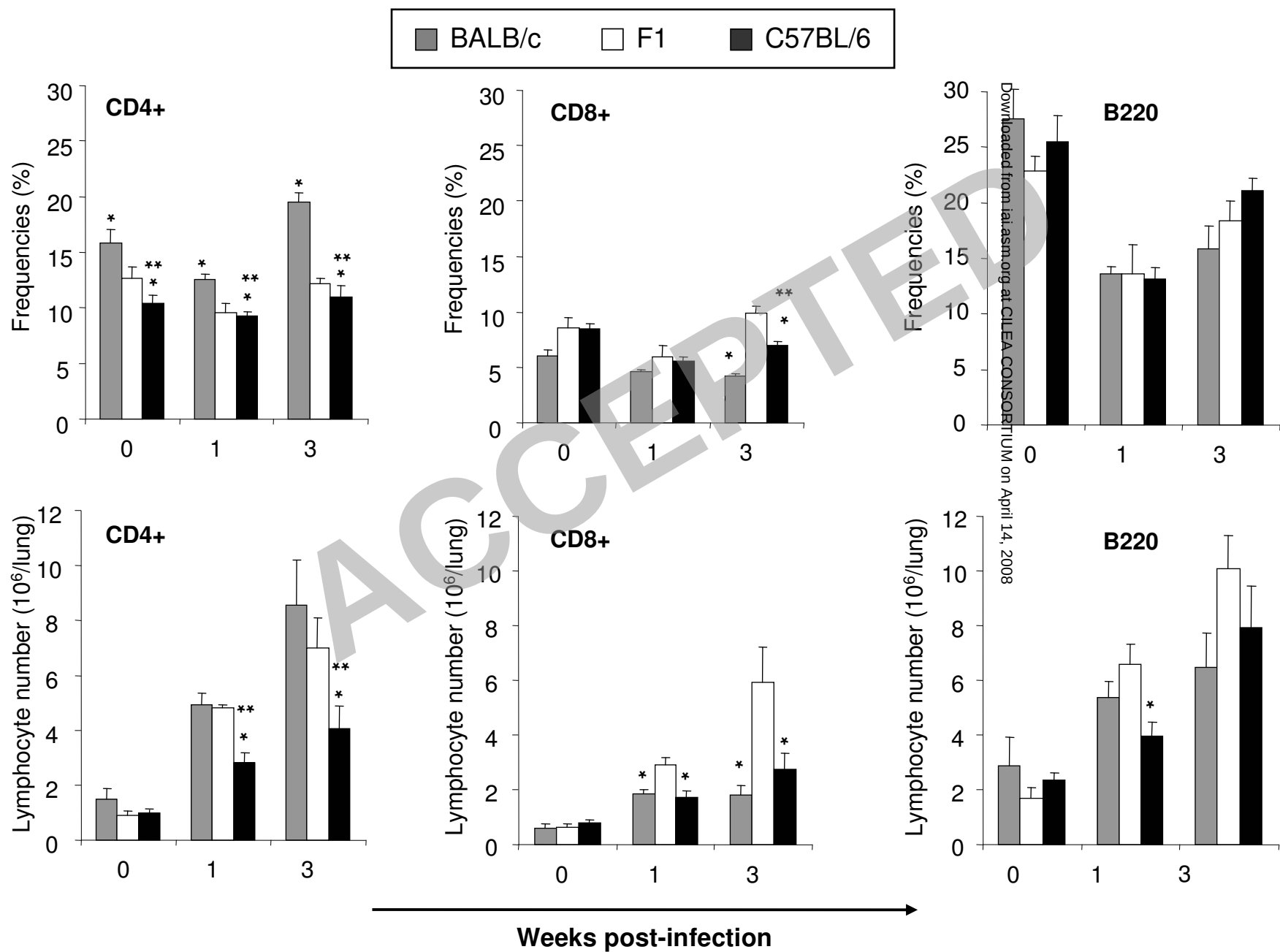


Fig. 4

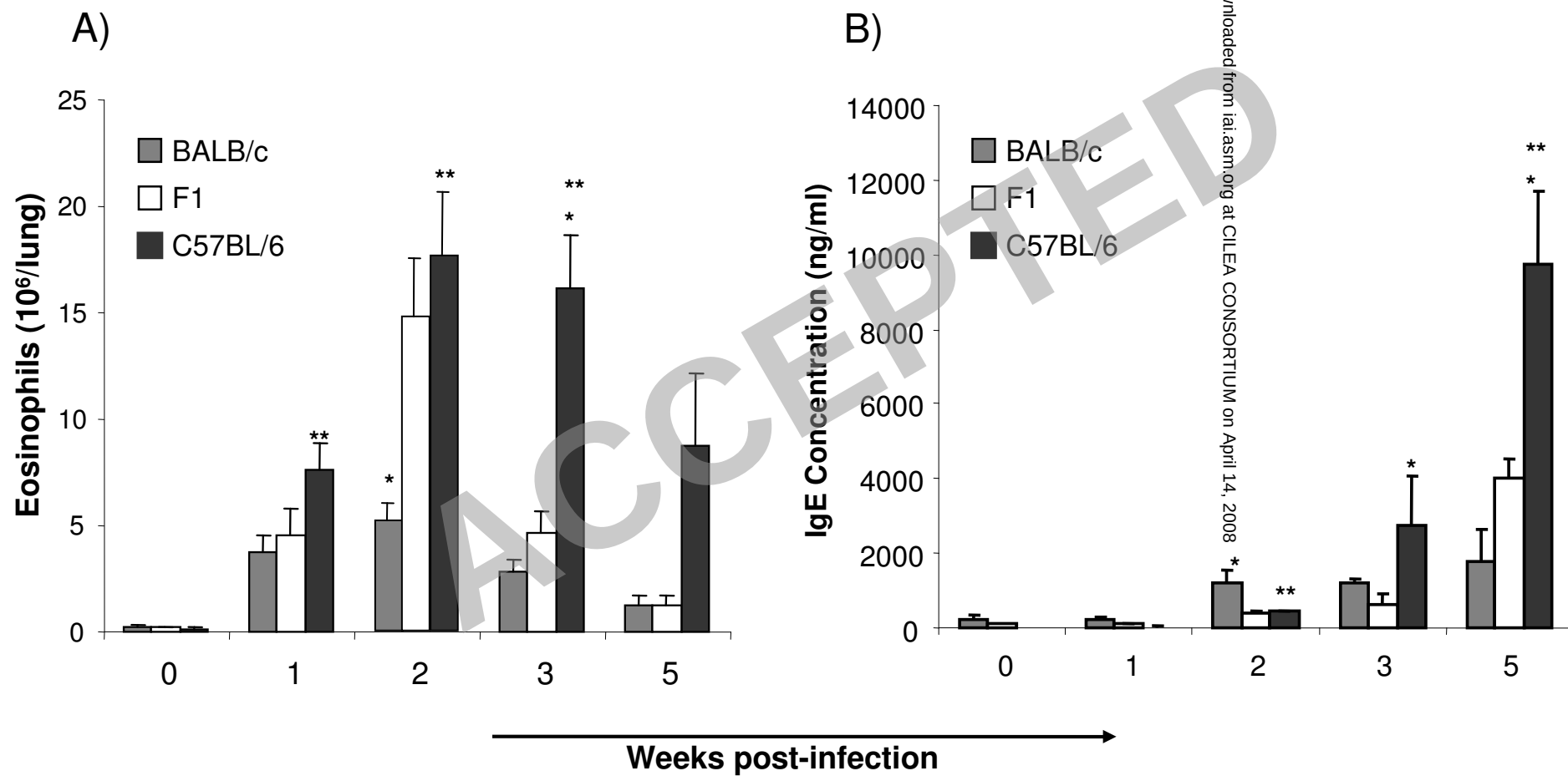


Fig. 5

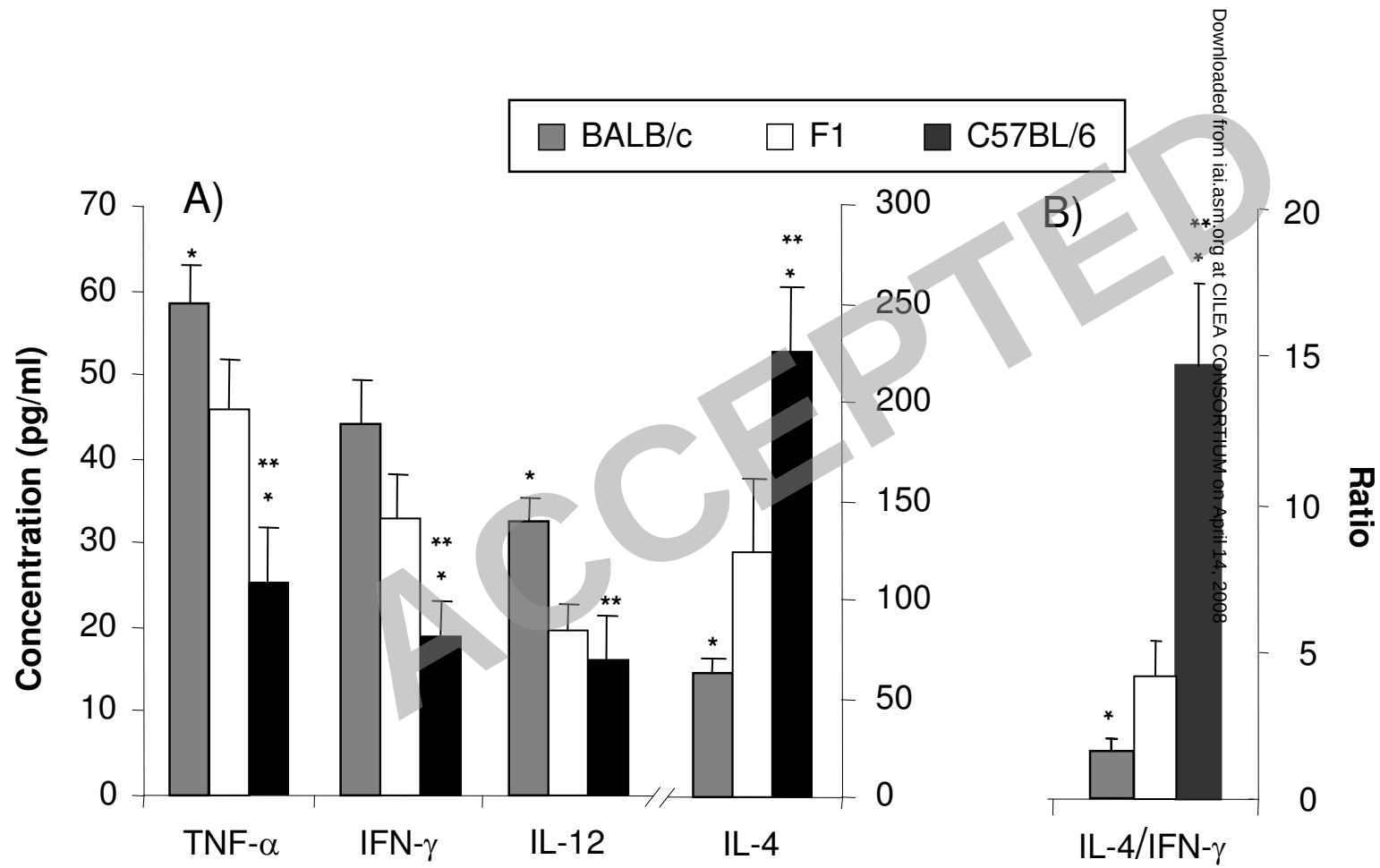


Fig. 8

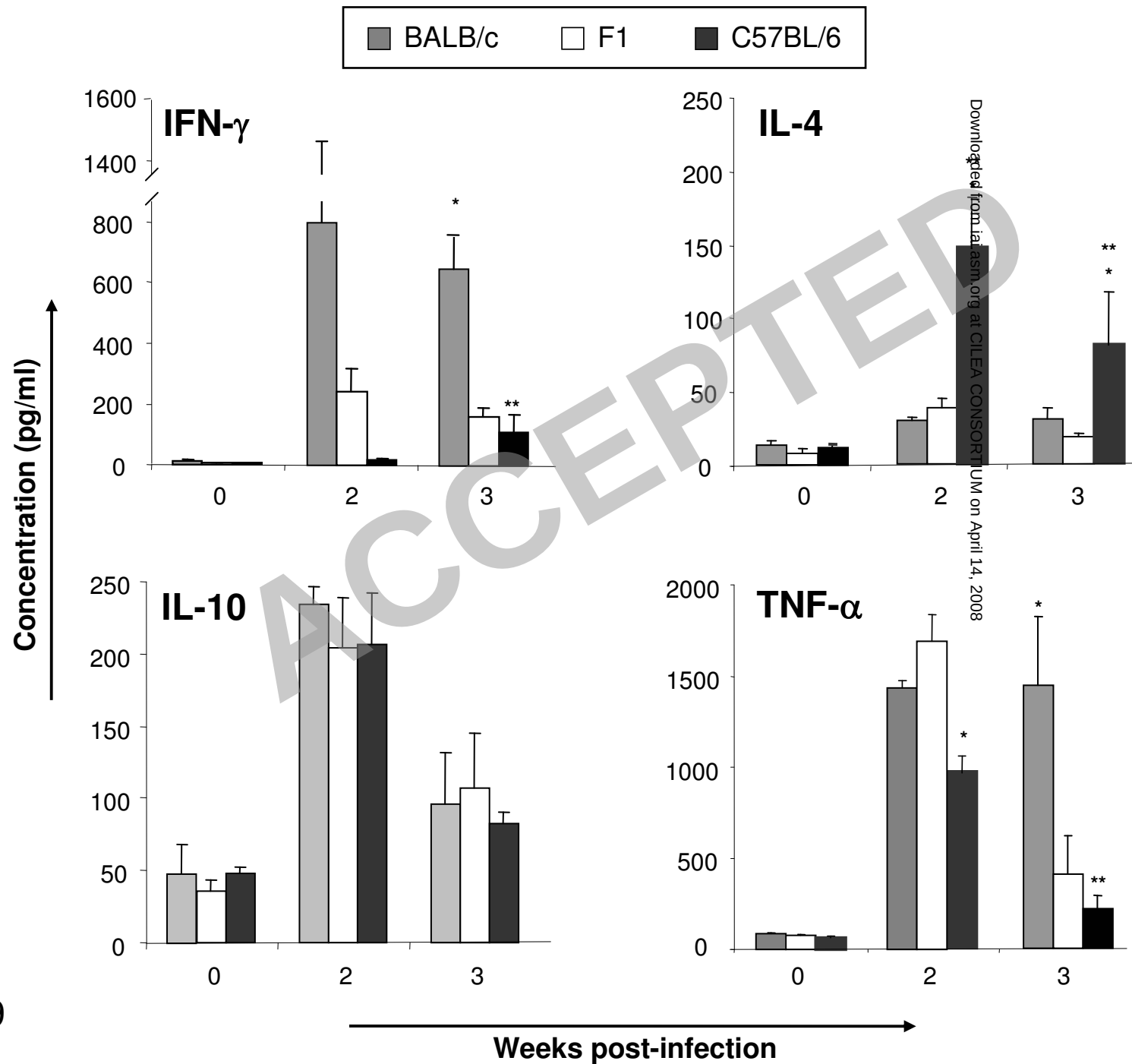
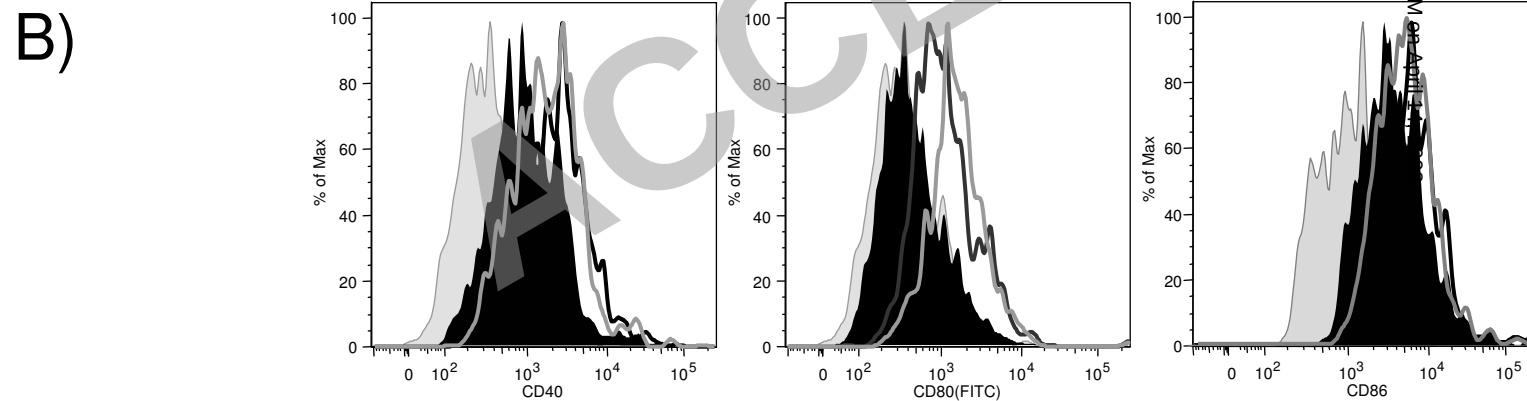
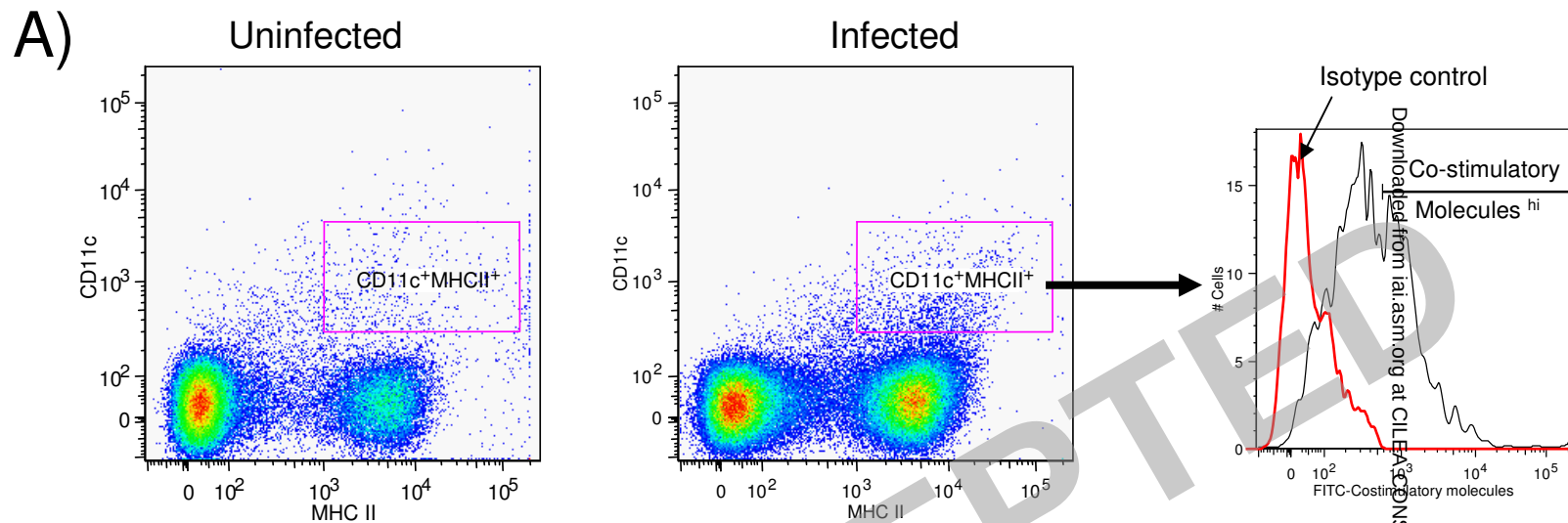


Fig. 9



Group	Percentages of CD11c ⁺ MHCII ⁺		
	CD40 ^{hi}	CD80 ^{hi}	CD86 ^{hi}
BALB/c	54.8±2.1	59.9±6.8	75.2±2.9
F1	46.1±2.6	50.8±17.9	73.0±2.3
C57BL/6	44.5±1.0**	34.3±3.1**	65.8±3.3**