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Intranasal vaccination against *Acinetobacter baumannii*–associated pneumonia in mice

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Short title: Intranasal vaccination against *A. baumannii*-associated pneumonia

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ABSTRACT

We recently demonstrated that a mouse model of respiratory *A. baumannii* infection using the strain LAC-4 results in disease progression that is similar to that observed in humans. In the current study, we used this model in conjunction with an inactivated whole cell vaccine, to evaluate the feasibility of developing protective mucosal vaccines against respiratory *A. baumannii* and the potential mechanism of protection of such vaccines. Most experimental studies to date have evaluated vaccines against systemic *A. baumannii* infections following systemic immunization. Our study showed that intransal immunization with formalin killed whole cells of the LAC-4 strain elicited mucosal and systemic antigen-specific immune responses, and protected the mice against lethal intranasal or intraperitoneal challenge. The pathogen burden in the lungs of the immunized mice was significantly lower, and the pathogen was barely detectable in blood and spleens at 24 h post intranasal challenge, indicating the ability of the immunized mice in controlling extrapulmonary dissemination of the pathogen. Further, it was shown that neutrophils and B cells but not FcRγ had a potential role in the protection against respiratory *A. baumannii* challenge of intranasally immunized mice. The study demonstrates that it should be possible to develop intranasal vaccines to protect against respiratory *A. baumannii* infection.

Keywords: *Acinetobacter baumannii;* pneumonia; mucosal vaccination; B cells; neutrophils; mice

INTRODUCTION

Acinetobacter baumannii is a significant causative agent of healthcare associated infections (hospital- and community-acquired) worldwide {Hartzell, 2007 #825}, with the host respiratory tract being an important entry portal {Kuo, 2007 #495}. Mortality rates of 35 – 70% have been reported for *A. baumannii* pneumonia {Vila, 2008 #971}, and in 2003 the pathogen was implicated in about 7% of nosocomial cases of pneumonia in intensive care units in the USA {Gaynes, 2005 #543}. In addition to the increasing incidence of *A. baumannii* infections, the rapid emergence of antibiotic-resistant strains, including multi-drug and pan-resistant strains {Fournier, 2006 #460;Abbo, 2007 #973;Valencia, 2009 #974;Taccone, 2006 #975;McConnell, 2012 #832} has made it increasingly difficult to treat these infections. Therefore, there is an urgent need for, and a great deal of interest in, the development of novel, effective, nonantibiotic based intervention strategies to combat *A. baumannii* {Garcia-Quintanilla, 2013 #976}.

Vaccination is one of the most effective measures for infectious disease control, and its efficacy is less likely to be affected by the drug resistance of the pathogen. Indeed, several laboratories have recently initiated efforts to develop *A. baumannii* vaccines for active and passive immunization of targeted populations {Bentancor, 2012 #828} {Luo, 2012 #985;McConnell, 2010 #796}. Vaccines based on inactivated whole cells {McConnell, 2010 #796} as well as a number of potential protective antigens such as biofilm associated protein

{Fattahian, 2011 #986}, K1 capsular polysaccharide {Russo, 2010 #738}, trimeric autotransporter protein Ata {Bentancor, 2012 #828}, surface-associated polysaccharide poly-*N*-acetyl-β-(1-)-glucosamine or PNAG {Bentancor, 2012 #987}, outer membrane complexes {McConnell, 2011 #817}, and outer membrane protein A (OmpA) {Luo, 2012 #985;Lin, 2013 #988}, are being evaluated. Due to the low virulence of most *A. baumannii* strains in mice, many vaccine researchers have used neutropenic or diabetic mice to increase their susceptibility to the infection {McConnell, 2012 #832;Luo, 2012 #985}. However, considering that neutrophils play a crucial role in the early onset stage of *A. baumannii* infection in mice {van Faassen, 2007 #575;Breslow, 2011 #811}, use of neutropenic murine models may not be representative of the disease progression in immunocompetent hosts {McConnell, 2012 #832}. Further, although the vaccine studies to date have substantially advanced the field, the majority, if not all, of these efforts have so far been limited to evaluating protection against systemic infections, and hence may not be indicative of potential protection against *A. baumannii* pneumonia observed under clinical settings {Garcia-Quintanilla, 2013 #976} wherein the infection is initiated via the respiratory route. For more predictive evaluation of the efficacy of vaccines against *A. baumannii* pneumonia, an intranasal challenge model would be useful.

We recently demonstrated that LAC-4, a clinical isolate of *A. baumannii*, caused 100% mortality in conventional, immunocompetent BALB/c and C57BL/6 mice

within 48 hours of administration of an intranasal (i.n.) dose of 10^8 CFU {Harris, 2013 #964}. Most importantly, unlike the other mouse models which involved systemic infections, i.n. infection using LAC-4 resulted in the development of acute *A. baumannii* pneumonia that more closely resembled the course of infection (e.g., high mortality, extrapulmonary dissemination, high tissue burdens, severe pulmonary pathology) observed in human pulmonary disease {Harris, 2013 #964}. Therefore, in the current study we utilized the i.n. LAC-4 challenge model to evaluate the potential for developing mucosal vaccines against *A. baumannii* pneumonia and to understand the potential mechanism of mucosal protection, using a vaccine based on inactivated (formalin killed) whole cells. Inactivated whole cell vaccine was selected for evaluation since it would potentially involve several different protective antigens, and because such a vaccine has previously shown protective efficacy in a systemic immunization/systemic challenge mouse model {McConnell, 2010 #910}.

MATERIALS AND METHODS

Mice. Eight- to 12-wk-old, specific-pathogen-free BALB/c and C57BL/6 mice were purchased from Charles River Laboratories (St. Constant, Quebec). *Igh-J*^{tm1Dhu} (B cell-/-) and *Fcer1g*^{tm1Rav} (FcRγ-/-) mice on BALB/c background, and their age- and sex-matched control BALB/c (wild-type, WT) mice were purchased from Taconic Farms, Inc. (Hudson, New York). The animals were maintained and used in accordance with the recommendations of the Canadian Council on

Animal Care Guide to the Care and Use of Experimental Animals, and all experimental procedures were approved by the institutional animal care committee.

Intranasal immunization with live or inactivated *A. baumannii* cells.

For live *A. baumannii* immunization, mice were anesthetized by i.p. injection of xylazine and ketamine, and each mouse was then administered once with 10^5 – 10^7 CFU of freshly grown *A. baumannii* LAC-4 inoculum (50 µl) prepared from frozen stock culture as described earlier {van Faassen, 2007 #575}. For immunization with inactivated *A. baumannii*, mice were intranasally administered, under isoflurane anesthesia, at 0, 14 and 21 d with varying numbers of formalin-fixed LAC-4 (ffLAC-4) cells in 50 µl saline. The ffLAC-4 cells were prepared as described previously {Harris, 2013 #964}. The dosage of the live inoculum was verified by plating of 10-fold serial dilutions on brain-heart infusion agar plates. The dosage of the ff-LAC-4 preparation was based on the CFU before formalin treatment and further confirmed by direct microscoping counting after formalin treatment. No viable cells were recovered in the ffLAC-4 preparation. At 28 d, fecal pellets and sera from sub-mandibular bleed samples were collected from individual mice for ELISA assays to determine *A. baumannii*-specific antibody responses.

ELISA assays for *A. baumannii* whole cell-specific IgA and IgG antibodies. Levels of *A. baumannii* whole cell-specific antibodies in sera and mucosal samples were determined by ELISA assays as previously described {Yan, 2009 #727}. Briefly, 96-well microplates were pre-coated with 10^6 ffLAC-4 cells/well, in 100 μl of sodium bicarbonate buffer (pH 9.6). Samples were pre-diluted (1:2 for fecal, and 1:100 for serum IgG and IgA) for the assays. Pooled samples collected from a previous experiment where mice that had been intranasally immunized with ffLAC-4, served as positive controls for the ELISA assays. Similar samples collected from naïve mice served as the negative ELISA assay controls.

***A. baumannii* challenge and post-challenge sample collection.** Naïve and immunized mice were challenged intranasally or intraperitoneally at day 42 with freshly grown inocula prepared from frozen stocks of *A. baumannii*, as previously described {van Faassen, 2007 #575}. For i.n. challenge, mice were anesthetized by i.p. injection of xylazine and ketamine, and each mouse was then administered 10^8 CFU (approximately 5 x LD_{100}) of *A. baumannii* LAC-4 in 50 μl volume. For the i.p. challenge, 10^6 CFU (approximately 5 x LD_{100}) of the fresh inoculum (100 μl volume) was injected into the peritoneal cavity of each mouse. The actual challenge dose in each experiment was determined by plating 10-fold serial dilutions on brain-heart infusion agar plates. The body weight, clinical scores, and the survival of the mice were monitored for up to 10 days as

described previously {van Faassen, 2007 #575}. In some experiments, groups of five mice were sacrificed just before challenge (0 h), or at 4 and 24 h post-challenge, and various tissues were aseptically removed and used for quantitative bacteriology (lung and spleen) or histopathology (lung, spleen, liver and kidneys). In addition, blood samples were collected for bacteriology or serum separation and lungs were lavaged to collect bronchoalveolar lavage (BAL) fluid, as described below.

Bronchoalveolar lavage (BAL). One milliliter of saline containing 3 mM EDTA and 1% fetal bovine serum was used to lavage the lungs 5 times, as previously described {Chen, 1992 #46}. The total number of cell in the BAL fluid were quantitated using a hemacytometer, and differential cell counts were determined by examining 200 cells on cytospin slides (Cytospin 3, Shandon, Pittsburgh, PA) stained with Hema-3® (Fisher Scientific, Kalamazoo, MI). The remaining BAL fluid was centrifuged (3,200 *xg*, 7 min), and the supernatant was collected and stored at -80°C.

Quantitative bacteriology and histopathology. Aerosol-proof homogenizers were used to homogenize lungs and spleens in sterile saline. The number of viable *A. baumannii* cells in the respective organs were determined by plating 100 µl aliquots of 10-fold serial dilutions of the homogenates on brain-heart infusion agar plates as described earlier {van Faassen, 2007 #575}. Serial

dilutions of blood samples were similarly cultured for assessing the bacterial burden. For histopathology, the organs were immediately immersed in 10% neutral buffered formalin, and processed by standard paraffin embedding methods {van Faassen, 2007 #575}. Sections were cut 4-μm thick, stained with haematoxylin-eosin (Department of Laboratory Medicine, University of Ottawa, Ottawa, ON), and examined under light microscope.

Cytokine and chemokine assays. The amounts of selected cytokines and chemokines in sera and BAL fluid were measured with 21-plex Milliplex MAP® mouse cytokine/chemokine kits (Millipore, Ltd. Billerica, MA) using a Luminex® 100IS system (Luminex, Austin, TX), as specified by the kit manufacturer. Samples were assayed in duplicate, and the cytokine/chemokine concentrations were calculated against the standards, using Beadview® software (ver 1.03, Upstate){van Faassen, 2007 #575}.

Depletion of neutrophils in vaccinated mice with monoclonal antibody RB6-8C5. Groups of five immunized BALB/c mice were treated intraperitoneally, with either 25 μg rat anti-mouse monoclonal antibody RB6-8C5 (RB6, injection volume of 0.25 mL) for *in vivo* depletion of neutrophils or equivalent amount of purified rat immunoglobulin G (IgG)(Sigma-Aldrich, St Louis, MA) as a control, as described previously {van Faassen, 2007 #575}. The treatment was given 18 hours before and at the time (0 h) of i.n. LAC-4 challenge. The specificity and

efficacy of the RB6-8C5 antibody have been previously confirmed by us and others {van Faassen, 2007 #575}{Marks, 2007 #556}{KuoLee, 2011 #818}.

Passive serum transfer protection. To assay the potential role of antibodies in the protection against i.n. challenge with LAC-4, various mouse sera preparations were transferred, by i.p. injection, to groups of naïve BALB/c mice. The hyperimmune sera were separated from blood collected at day 48 from BALB/c mice that had been intranasally immunized with 10^7 ffLAC-4 cells at day 0, 14 and 21. The convalescent sera were collected at 7 days after BALB/c mice were infected intranasally with a sub-lethal dose (~5 x 10^6 CFU) of LAC-4. All infected mice showed clinical signs but recovered from infection. Naïve sera were collected from age- and sex-matched BALB/c mice. Sera obtained from individual mice in each group was pooled for use. Serum transfer was administered 1 day before (500 μl per mouse) and at the time (0 h, 250 μl per mouse) of i.n. challenge with 1.6 x 10^8 CFU of LAC-4.

Statistical analysis. Unless indicated otherwise, the data are presented as means ± SD for each group. The survival rates were analyzed by log-rank test. Differences in quantitative measurements were assessed by Student's *t* test, one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* multiple comparison tests, when appropriate. Differences were considered significant when $P<0.05$.

RESULTS AND DISCUSSION

Intranasal immunization with live A. *baumannii* LAC-4 induces robust protection against a lethal i.n. LAC-4 challenge in mice. Although several studies have convincingly demonstrated that prophylactic vaccination can afford protection against a systemic *A. baumannii* challenge in several animal models {McConnell, 2011 #817;McConnell, 2010 #910}, the potential of these vaccines for protection against the respiratory challenge has not been reported. As the first step in investigating the feasibility of mucosal immunization in inducing protection against a lethal respiratory infection by *A. baumannii*, groups of BALB/c mice were intranasally immunized with 10^5 to 10^7 CFU of live *A. baumannii* LAC-4 at day 0, and intranasally challenged with a lethal dose (1.5 x 10^8 CFU) of LAC-4 at day 42. Compared to the naïve group where 100% of the mice succumbed to the challenge by day 2, 100% of the mice in the 10^6 and 10^7 CFU immunized groups survived and showed only mild, transient clinical signs and loss of body weight over the first 4 days post-challenge (Fig. 1). The mice in the 10^5 CFU immunized group demonstrated more body weight loss than the other two groups, but 80% still survived the challenge (Fig. 1). These results indicate that intranasal immunization with live LAC-4 induces robust protection against a subsequent lethal intranasal challenge, demonstrating the feasibility of using the mucosal route for vaccinating against *A. baumannii* respiratory infection.

Intranasal immunization of mice with inactivated whole LAC-4 cells induces antigen-specific mucosal and systemic antibody responses, and protects against lethal mucosal or systemic LAC-4 challenge. Since immunization with live *A. baumannii* is not an ideal approach because of its potential side effects, further studies on understanding the potential of mucosal vaccination in protection against *A. baumannii* pneumonia was undertaken using inactivated (formalin killed) whole cells (ffLAC-4) as the prototype vaccine. Groups of BALB/c and C57BL/6 mice were intranasally immunized with 5×10^3 (low dose), 5×10^5 (medium dose) or 5×10^7 (high dose) ffLAC-4 cells. At 28 d, sera and fecal samples were collected for antibody assays. At 42 d the mice were lethally challenged either intranasally (7×10^7 CFU) or intraperitoneally (8.4×10^5 CFU) with LAC-4. As seen in Figure 2A, both BALB/c and C57BL/c mice immunized with 5×10^7 ffLAC-4 cells developed high levels of ffLAC-4-specific serum IgG1 and IgG2a responses. This vaccination dose also induced moderate serum and fecal (an indication of mucosal response) ffLAC-4-specific IgA responses. Mucosal and systemic antibody responses in mice vaccinated with 5×10^5 ffLAC-4 cells were also observed in BALB/c mice, although at a lower magnitude, but not at all in the C57BL/6 mice. Mice vaccinated with 5×10^3 ffLAC-4 cells failed to develop detectable serum or fecal antibody responses (Fig. 2A).

More significantly, all mice immunized with the high dose (10^7 CFU) of ffLAC-4 survived the i.n. and the i.p. challenge, irrespective of the mouse strain (Fig. 2B). From the mice immunized at the medium dose (10^5 CFU) of ffLAC-4, only the BALB/c mice survived both the i.n., and the i.p. challenge. At this immunization dose 100% of the C57BL/6 mice survived the i.n. challenge, but only 20% survived the i.p. challenge (Fig. 2B). Immunization with the low dose (10^3 CFU) of ffLAC-4 provided little protection against i.n. or i.p. challenge, based on the 80 – 100 % observed mortality. All the naïve BALB/c and C57BL/6 mice died from the challenge at 1 day (i.p. challenge) or 2 day (i.n. challenge) post-challenge, respectively (Fig. 2B). These results demonstrate that intranasal immunization of BALB/c mice with 10^5 to 10^7 CFU equivalent of ffLAC-4 can elicit robust protection against lethal respiratory or systemic challenge with LAC-4. The C57BL/6 mice were protected against i.n. or i.p. challenge when immunized at the high dose, but were more susceptible to the i.p. challenge when immunized at the two lower ffLAC-4 doses. Therefore, unless indicated to the contrary, further studies were done with BALB/c mice intranasally immunized with 10^7 ffLAC-4 cells.

The results of this study extends the previous observations by McConnell and colleagues that intramuscular immunization of mice with an inactivated whole cell vaccine elicits a good protective immunity against i.p. challenge with multiple *A. baumannii* strains {McConnell, 2010 #796}. More recently, we have shown

that i.n. immunization with ffLAC-4 resulted in the development of LAC-4-specific systemic (serum) IgG and mucosal (vaginal wash) IgA) responses in mice and the partial protection against a 100 x LD_{100} i.p. challenge with the hypervirulent LAC-4 {Harris, 2013 #964}. Taken together, those results suggest that mucosal immunization might be effective in affording protection against both systemic and respiratory infections with *A. baumannii*.

Quantitative bacteriology and histopathology. To determine if i.n. ffLAC-4 immunization promotes the pulmonary clearance of *A. baumannii* and limits systemic dissemination of the pathogen, groups of immunized or naive mice were challenged intranasally with 1.6 x 10^8 CFU of LAC-4 at day 42. The mice were euthanized at 4 and 24 hrs post-challenge for quantitative bacteriology (lung, spleen and blood) and histopathology (lung, spleen, liver and kidneys). At 4 h post-challenge, the bacterial burdens in the lung (Fig. 3A) and spleen (Fig. 3B) of naïve and ffLAC-4-immunized mice were similar, with the burdens in the spleen being much lower than in the lung. At this time point, pathogen recovery from the blood of both immunized and naïve mice was barely above the detection limit (Fig. 3C). At 24 h post-challenge, the burdens of LAC-4 in the lungs of the ffLAC-4 immunized mice were significantly lower (>10-fold) than those in the lungs of the naive mice (Fig. 3A). In addition, significant numbers of LAC-4 were recovered from the spleens and blood of the naïve mice at this time point, indicating extrapulmonary dissemination. In contrast, only small numbers

of LAC-4 were recovered from the spleens of the immunized mice (Fig. 3B), and the bacterial numbers in the blood (Fig. 3C) were barely above the detectable limit.

Histologically, the lungs from naïve mice at 24 hpi showed moderate dilation and infiltration of mixed inflammatory cells in the perivascular and peribronchial space in the lung (Fig. 4A) with the occasional presence of moderate numbers of mixed neutrophils and mononuclear cells in the airway lumen (Fig. 4A). The histopathological changes in the lungs of immunized mice killed at this time point were much milder and limited largely to the alveolar space where small to moderate numbers of neutrophils and macrophages are presented (Fig. 4B). The histological changes in the spleens between the naïve and immunized mice killed at 24 hpi were relatively mild and comparable although the naïve mice showed more congestion in the medullar region than the immunized mice (Fig. 4, C and D). The liver from both groups of the mice showed only mild increase in the number and size of Kupffer cells and there were no abnormal changes in the kidneys of any infected mice (data not shown). These results (Figs. 3 and 4) suggest that the protective immunity induced by intranasal ffLAC-4 immunization is likely due to the induction of both pulmonary and systemic protective immunity, which controls the otherwise rapid, local bacterial replication in the lungs and also restricts the extrapulmonary dissemination of this hypervirulent strain,

rather than prevent the attachment and initial colonization of the pathogen at the site of entry.

Cytokine and chemokine responses. In an effort to further understand the protective mechanism induced by intranasal ffLAC-4 immunization, we next determined the levels of certain pro-inflammatory cytokines and chemokines, which had been previously implicated in the pathogenesis of and host defense against *A. baumannii* infection {Qiu, 2009 #687;Qiu, 2009 #688;van Faassen, 2007 #575}{Russo, 2008 #616;Russo, 2009 #739}{Renckens, 2006 #463}{Breslow, 2011 #811}, in the sera and BAL fluid. Mice immunized with ffLAC-4 were intranasally challenged with LAC-4, and sera and BAL fluid collected at 4 and 24 h post-challenge were assayed (Fig. 5). At 4 h post-challenge, the amounts of IL-6, KC, MIP-2, and TNF-α detected in the BAL fluid from the immunized and naive mice were comparable (Fig. 5A). The levels of IL-1β, MCP-1 or RANTES were generally below the detection limit (<10 pg/ml) in the BAL fluid at 4 h post-challenge, but these levels increased dramatically at 24 h post-challenge. At 24 h post-challenge, compared to the naïve mice, the levels of IL-6, KC and MCP-1 in the immunized mice were significantly lower whereas the levels of RANTES and TNF-α were significantly higher (Fig. 5A). However, there were no significant differences in the levels of IL-1β or MIP-2 between the two groups of mice.

Similar to the observations in BAL fluid, the majority of cytokines/chemokines were low or below the detection limit in the serum samples at 4 h post-challenge, with the exception of moderate and comparable amounts of IL-6 and KC detected in the naïve and immunized groups of mice (Fig. 5B). Compared to at 4 h, the serum levels of all the cytokines and chemokines assayed were generally substantially higher at 24 h post-challenge, with levels in the naïve mice being significantly higher ($p < 0.01$ to 0.001) than those in the immunized mice (Fig. 5B).

Role of B cells and antibodies in the ffLAC-4 vaccination-induced protection. Since *A. baumannii* is an extracellular, Gram-negative bacterium, we hypothesized that B cells and antibodies play an important role in the host defense against this pathogen. To test this hypothesis, groups of B cell KO, FcRγ KO or WT BALB/c mice were intranasally immunized with ffLAC-4 and intranasally challenged with 10^7 (for bacteriology) or 10^8 (for clinical outcome) CFU of LAC-4. In the mice challenged with 10^7 CFU of LAC-4, the bacterial burdens in the lungs of the B cell KO mice were nearly two logs higher than those in WT or FcRγ KO mice at 24 h post-challenge (Fig. 6A). In addition, extrapulmonary bacterial dissemination (as surmised from the pathogen burdens in blood and spleen) was observed only in B cell KO mice (Fig. 6A). More significantly, all B cell KO mice succumbed to the 10^8 CFU LAC-4 challenge by day 2, whereas all of the FcRγ KO mice and 80% of WT mice survived from the challenge (Fig. 6B). These results

indicate that B cells, but not FcRγ, are important in the ffLAC-4 induced protection against respiratory *A. baumannii* infection, and imply that the B cell-mediated protection does not function through FcRγ.

Since FcRγ plays an important role in the IgG function, the comparable protection between FcRγ KO and WT mice suggests that antigen-specific IgG is not indispensable in host defense against i.n. *A. baumannii* infection. To examine this possibility, groups of BALB/c mice were transferred by i.p. injection with hyperimmune serum, convalescent serum, naive serum or saline before they were intranasally challenged with 10^8 CFU of LAC-4. The mice that had received the hyperimmune serum showed a prolonged mean time to death, whereas mice in all of the other groups died from the infection by day 2 (Fig. 7). These results suggest that passive transfer of hyperimmune serum only provides marginal protection against a lethal intranasal challenge.

Neutrophils are essential in the ffLAC-4 vaccine-induced protection. It has been previously shown by us {van Faassen, 2007 #575} and others {Breslow, 2011 #811} that neutrophils play an important role in the host resistance against primary *A. baumannii* infection. In this study, we determined whether the neutrophils are also crucial in the vaccine-induced protection against i.n. *A. baumannii* infection. Groups of BALB/c mice that were previously intranasally immunized with ffLAC-4 were treated by i.p. with 25 μg of RB6 or rat

IgG at 18 and 0 h prior to an intranasal LAC-4 challenge (10^8 CFU). As can be seen in Fig. 8, all of the RB6-treated, ffLAC-4 immunized mice died of the challenge by day 2, similar to the saline immunized/saline treated group. However, 100% of the rat IgG-treated, ffLAC-4 immunized mice survived the challenge, suggesting that neutrophils are essential in the vaccine-induced protection against i.n. challenge in this model.

In summary, this study demonstrates the feasibility of developing a mucosal vaccine to protect the host against respiratory *A. baumannii* infection. The results also suggested the potential role of neutrophils and B cells (but not FcRγ) in affording protection against respiratory *A. baumannii* infection.

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REFERENCES

FIG. 1 Intranasal immunization of mice with live *A. baumannii* induces robust protection against a lethal i.n. challenge with LAC-4. Groups of male BALB/c mice (n=5) were intranasally immunized with 10^5 to 10^7 CFU of LAC-4 at day 0, and subsequently intranasally challenged with 1.5×10^8 CFU of LAC-4 at day 42. The body weight, clinical score, and survival rate were monitored daily for up to 10 days. The overall clinical sign for each mouse was scored as 0 (normal, active, healthy), -1.0 (slightly sick, slightly ruffled fur, otherwise normal), -2.0 (sick, ruffled fur, slow movement, hunching), -3.0 (very sick, ruffled fur, very slow movement, hunched, eyes shut), -4.0 (moribund), or -5.0 (dead). Clinical scores and body weights are expressed as mean ± SD.

Fig. 2 Intranasal immunization with inactivated *A. baumannii* LAC-4 cells induces robust protection against a lethal i.n. or i.p. challenge with LAC-4. Groups (n = 5) of female BALB/c and C57BL/6 mice were intranasally immunized with 5×10^3, 5×10^5 or 5×10^7 CFU equivalent of ffLAC-4 at day 0, 14 and 21. Blood and fecal pellets were collected at day 28 for the measurement of specific IgA and IgG (IgG1 and IgG2a) antibodies by ELISA assays (A). The mice were subsequently challenged at day 42 with LAC-4 via the i.n. (7×10^7 CFU) or the i.p. (8.4×10^5 CFU) route and their survival rates were monitored daily for up to 10 days (B).

FIG. 3 Intranasal immunization with inactivated LAC-4 cells reduces the lung bacterial burdens and limits the extrapulmonary dissemination and bacteremia in vaccinated mice. Groups of female BALB/c mice (n = 5) were intranasally immunized with 10^7 ffLAC-4 cells at day 0, 14 and 21, and intranasally challenged with 1.6 x 10^8 CFU of LAC-4 at day 42. *A. baumannii* burdens in the lungs (A), spleen (B) and blood (C) in the naïve and immunized mice were determined by quantitative bacteriology at 4 and 24 hours post-challenge. The data are presented as mean $\log_{10}$ CFU ± SD, and represent one of at least two experiments with similar results. The detection limits (dotted lines) for bacterial burdens were 1.3 $\log_{10}$ CFU/organ for lungs and spleen, and 1.0 $\log_{10}$ CFU/ml blood, respectively. ***, $p<0.001$.

Fig. 4 Photomicrographs of lung (A and B) and spleen (C and D) histopathology following i.n. challenge with LAC-4 in naïve (A and C) and ffLAC-4-immunized (B and D) mice. Groups of female BALB/c mice (n = 5) were intranasally immunized with 10^7 ffLAC-4 cells at day 0, 14 and 21, and intranasally challenged with 1.6 x 10^8 CFU of LAC-4 at day 42. The mice were sacrificed at 24 hours post-challenge and tissues collected for histopathology. (A) The lung from a naïve, challenged mouse showing moderate inflammatory cell infiltration in the perivascular and peribronchial areas (arrowheads), and within the

airway lumen (*). (B) The lung from an immunized, challenged mouse showing the mild inflammatory cell infiltration in the alveolar spaces. (C) The spleen from a naïve, challenged mouse showing the moderate congestion of the medullar region. (D) The spleen from an immunized, challenged mouse showing normal tissue architecture. H&E, Bar = 100 μm in A and B and 50 μm in C and D.

FIG. 5 Cytokine and chemokine levels in the bronchoalveolar lavage (BAL) fluid (A) and sera (B) of immunized and naïve mice following a lethal i.n. challenge with LAC-4. Groups of female BALB/c mice (n = 5) were intranasally immunized with 10^7 ffLAC-4 cells at day 0, 14 and 21. The mice were intranasally challenged with 1.6×10^8 CFU of LAC-4 at day 42, and sacrificed at 4 and 24 hours post-challenge. Cytokine and chemokine levels were determined in the BAL fluid and sera using the Milliplex® mouse cytokine/chemokine kits on a Luminex® 100IS system. The detection limit for all cytokines and chemokines is <10 pg/ml. All values are mean ± SD. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Fig. 6 Role of B cells and FcRγ in ffLAC-4 vaccine-induced protection against lethal intranasal challenge with LAC-4. Groups of female B cell KO, FcRγ KO or WT (BALB/c) mice (n = 5) were intranasally immunized with 10^7 ffLAC-4 cells at day 0, 14 and 21. The mice were intranasally challenged

at day 42 with 1.1 x 10^7 CFU of LAC-4 for determining tissue and blood bacterial burdens at 24 h post-challenge (A), or with 0.7 x 10^8 CFU of LAC-4 for determining survival rates (B). The bacterial burden data are presented as mean $\log_{10}$ CFU ± SD. **, $p<0.01$; ***, $p<0.001$ vs WT mice.

Fig. 7 Passive serum transfer provides limited protection against a lethal intranasal challenge with LAC-4. Groups of naïve, female BALB/c mice (n = 5) were intraperitoneally injected with hyperimmune, convalescent, naïve sera, or saline at 24 h (0.5 ml) and 0 h (0.25 ml) before intranasal challenge with 1.6 x 10^8 CFU of LAC-4. Their survival rates were monitored for 5 days.

Fig. 8 Role of neutrophils in ffLAC-4 induced protection against i.n. LAC-4 challenge. Groups of female, BALB/c mice (n = 5) were intranasally immunized with 10^7 ffLAC-4 cells on day 0, 14 and 21, or left unimmunized (naïve). At day 42, the immunized mice were treated by i.p. with 25 μg RB6-8C5 for neutrophil depletion, or equivalent amount of rat IgG as control at 18 h and immediately (0 h) before intranasal challenge with 1.6 x 10^8 CFU of freshly grown LAC-4. The survival rates of the challenged mice were recorded.

Figure 1

GH081

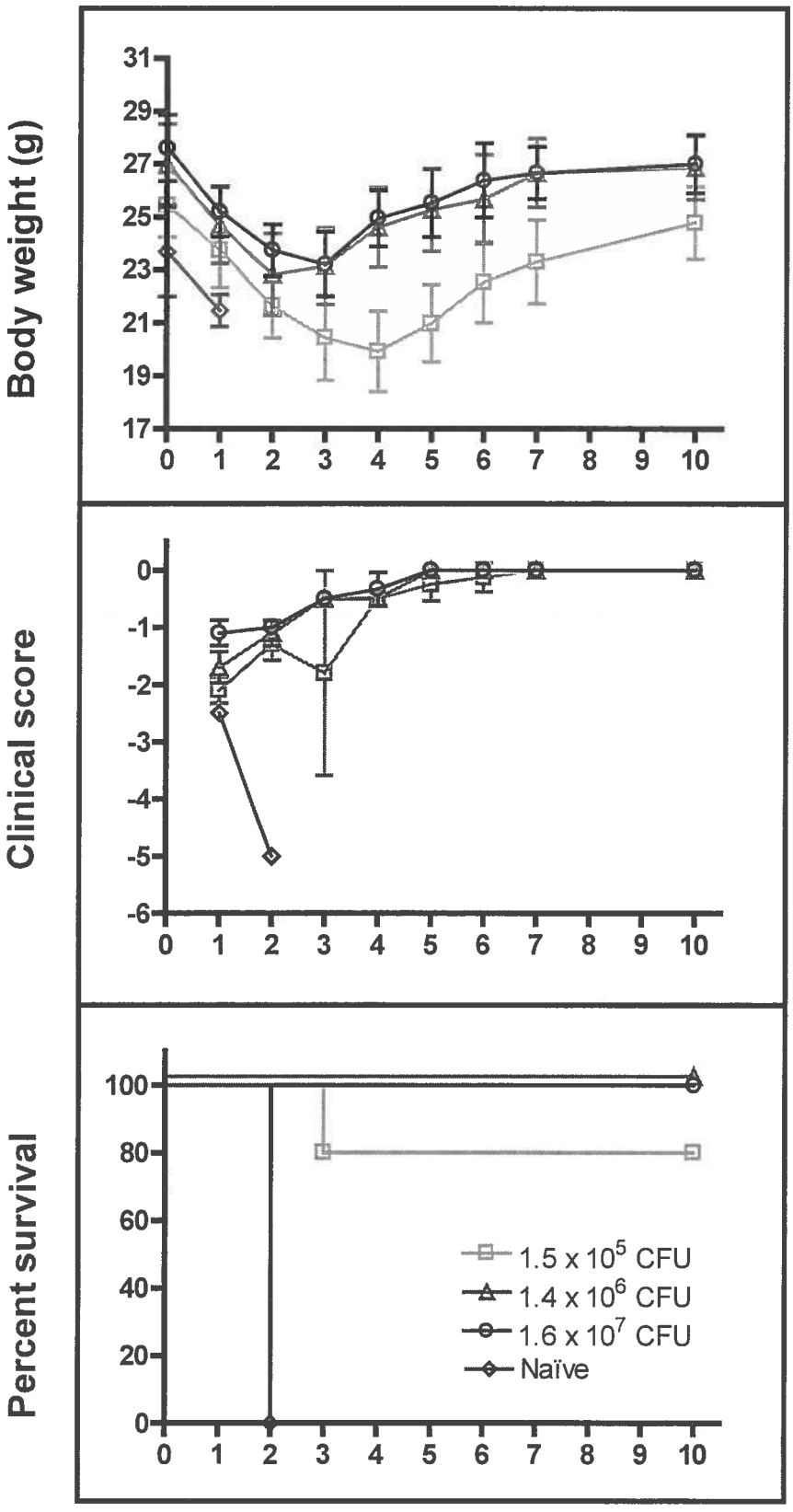
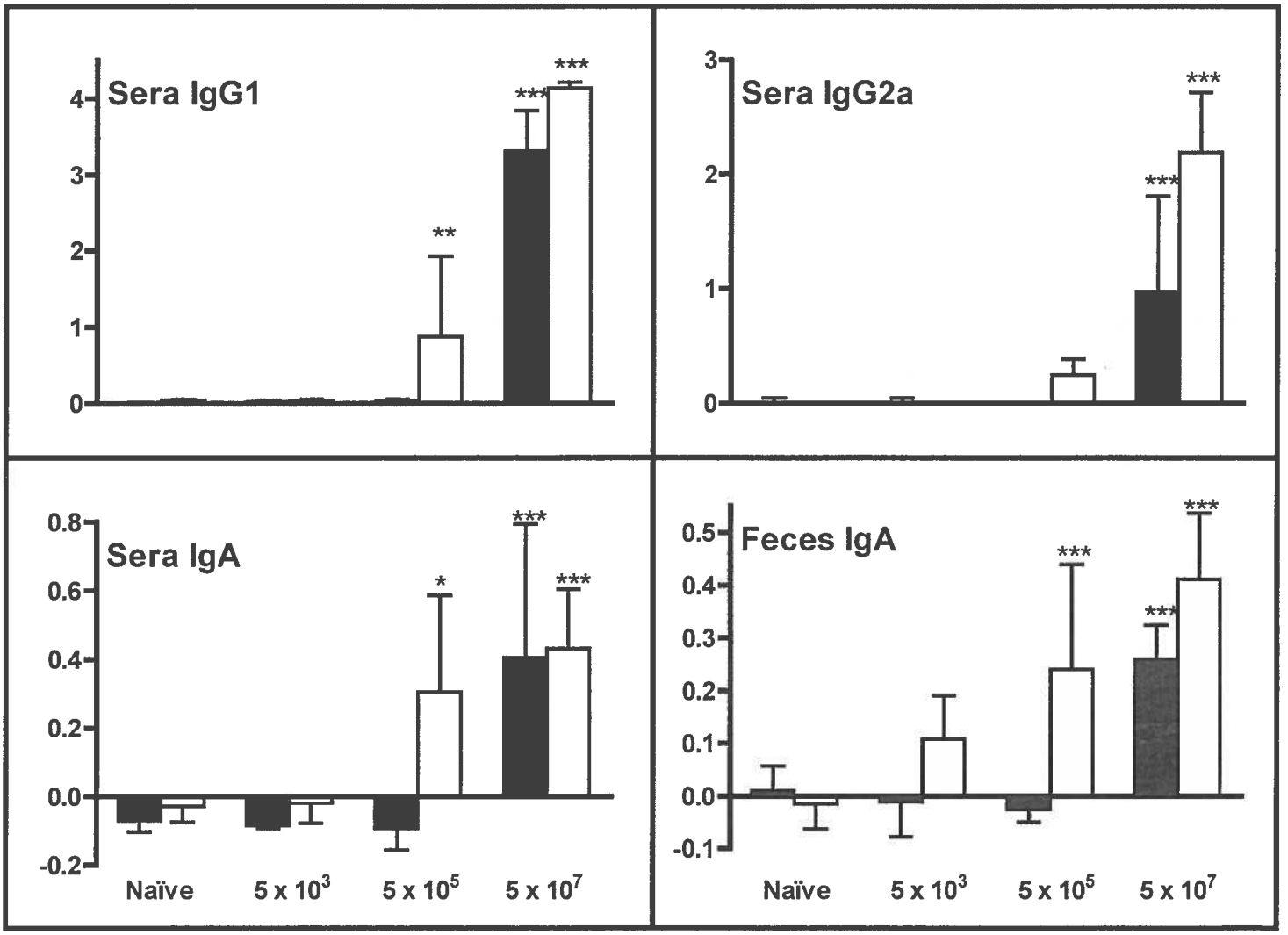


Figure 2A

RK202



Stats are 2-way ANOVA by the way

Figure 2B

RK202

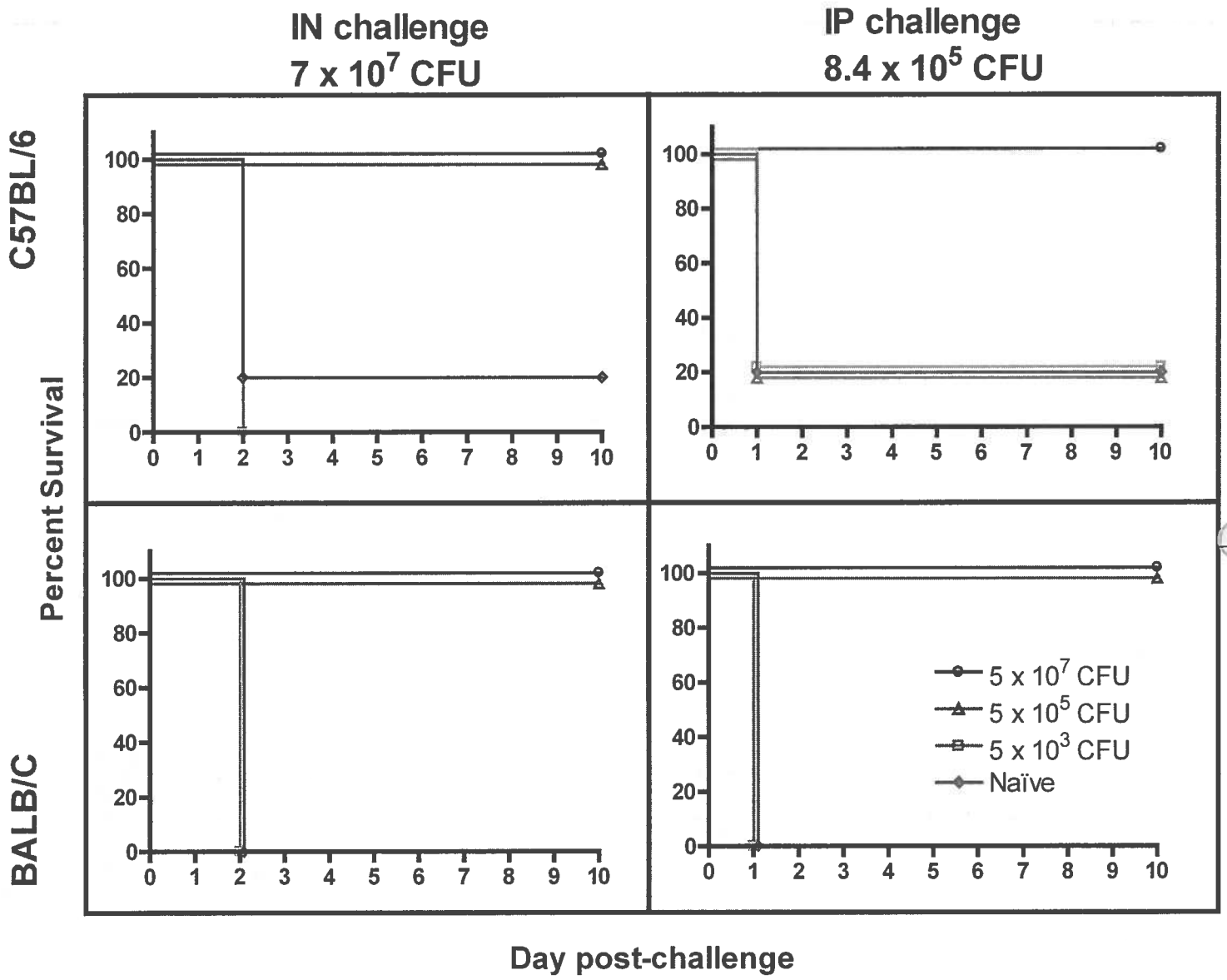


Figure 3

GH082

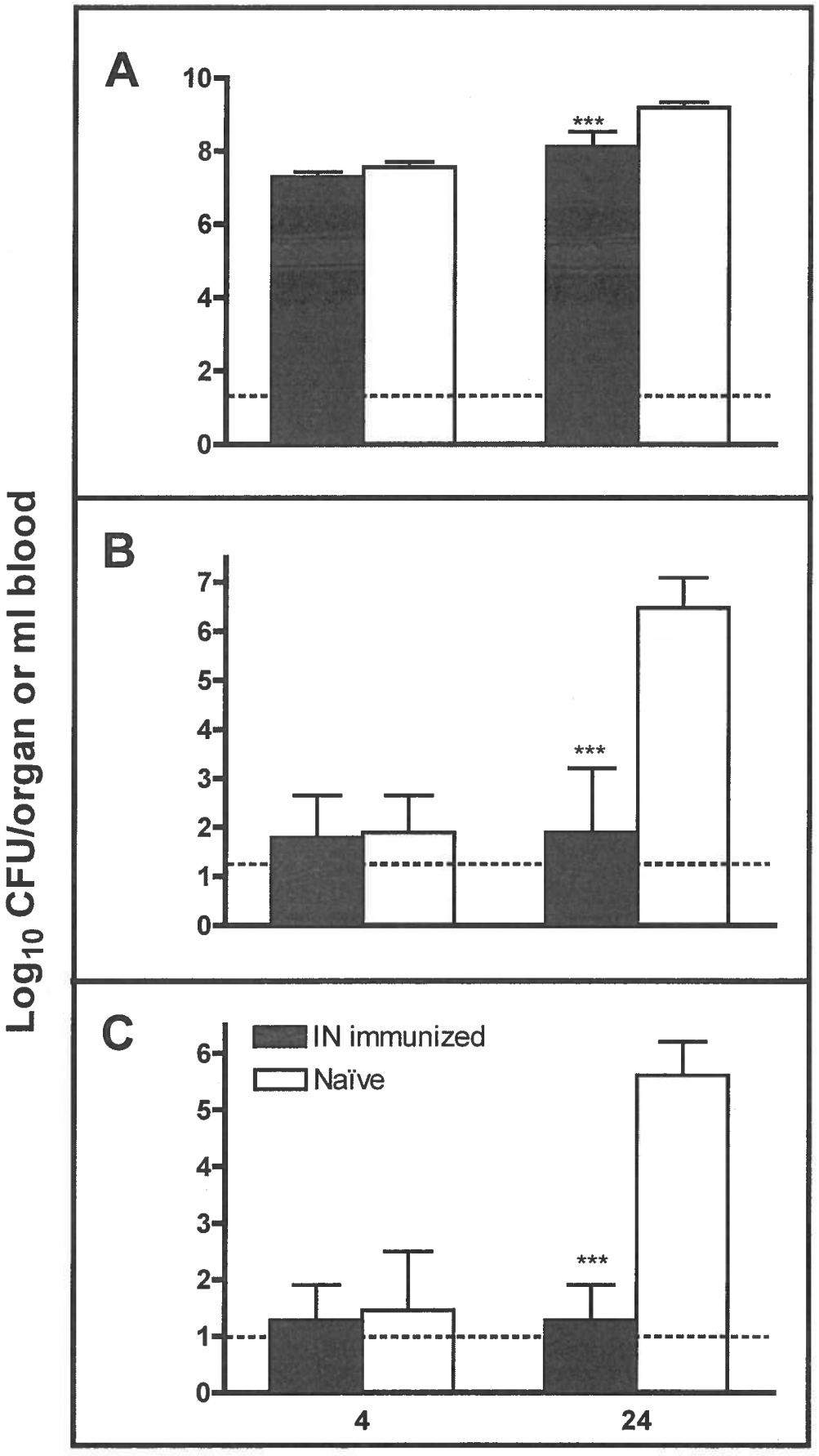


Figure 4

GH082

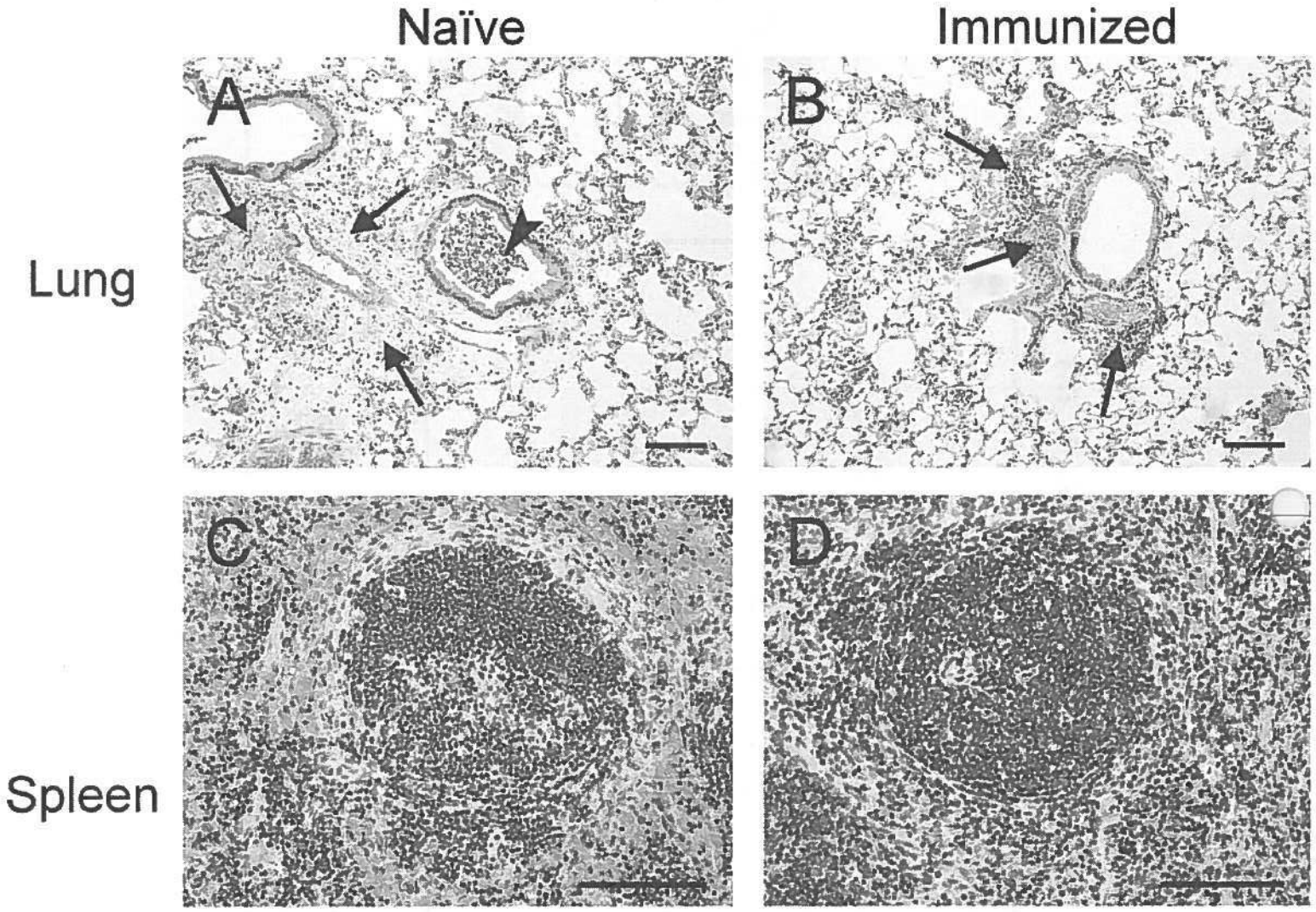


Figure 5

GH082

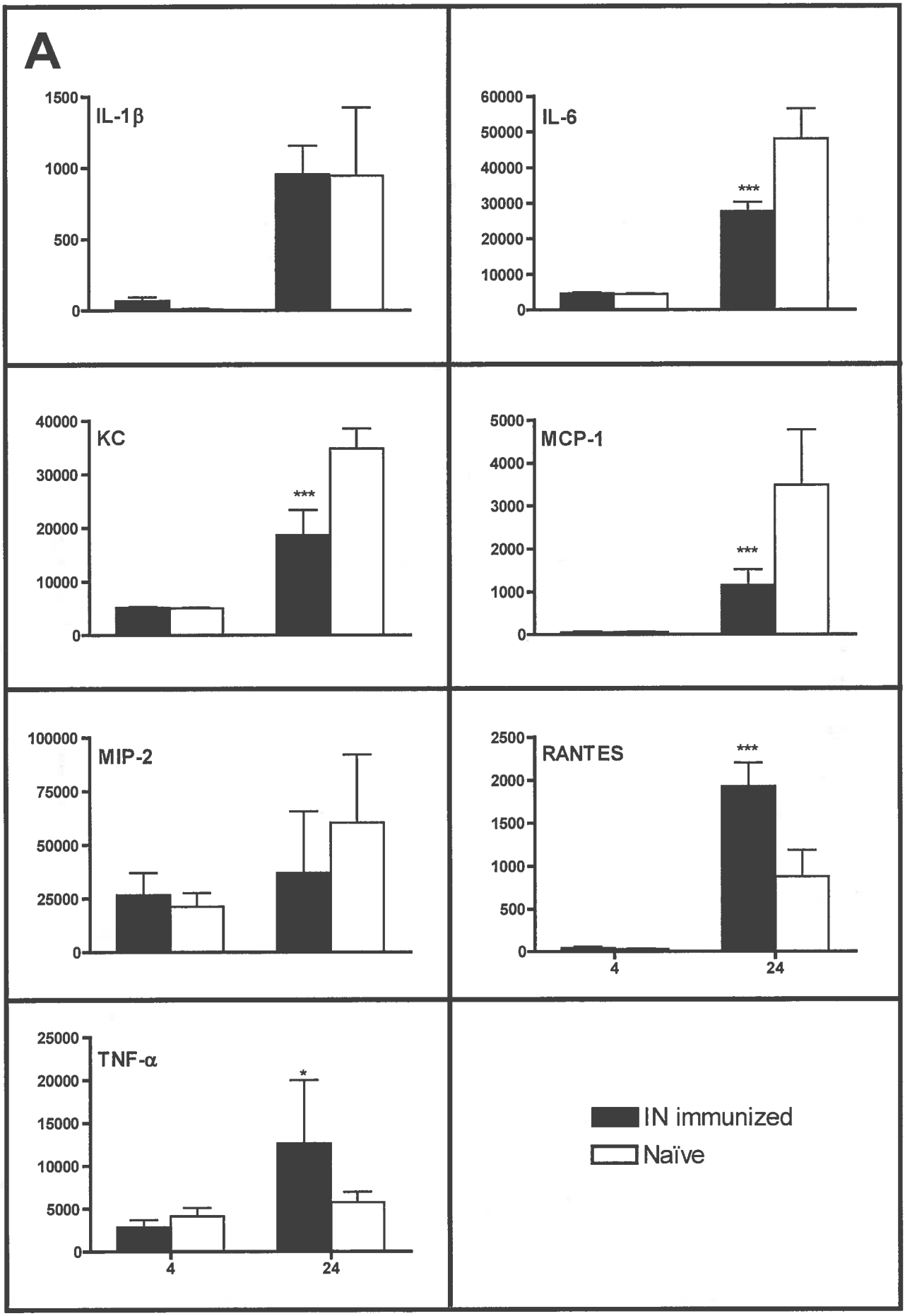


Figure 5

GH082

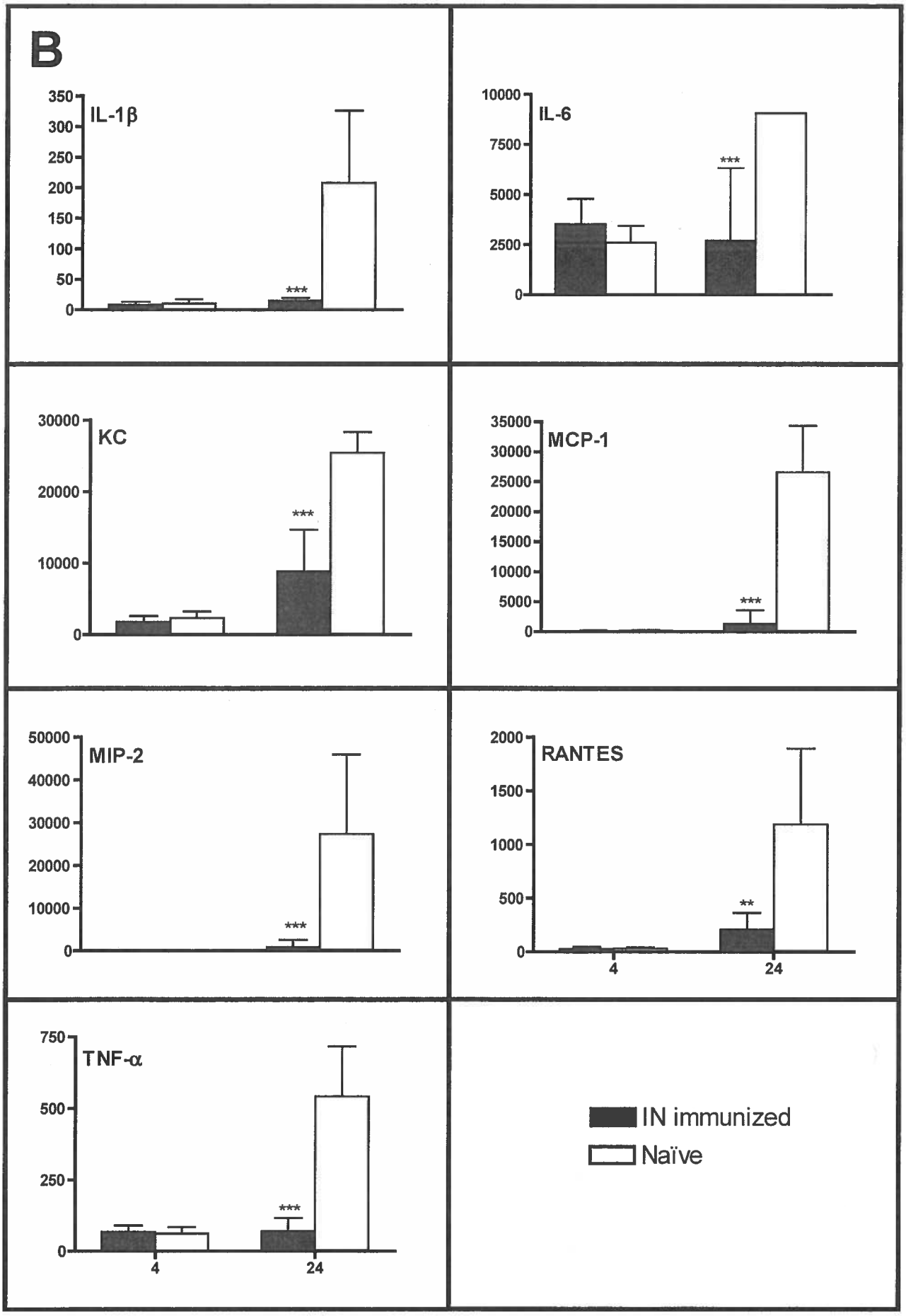
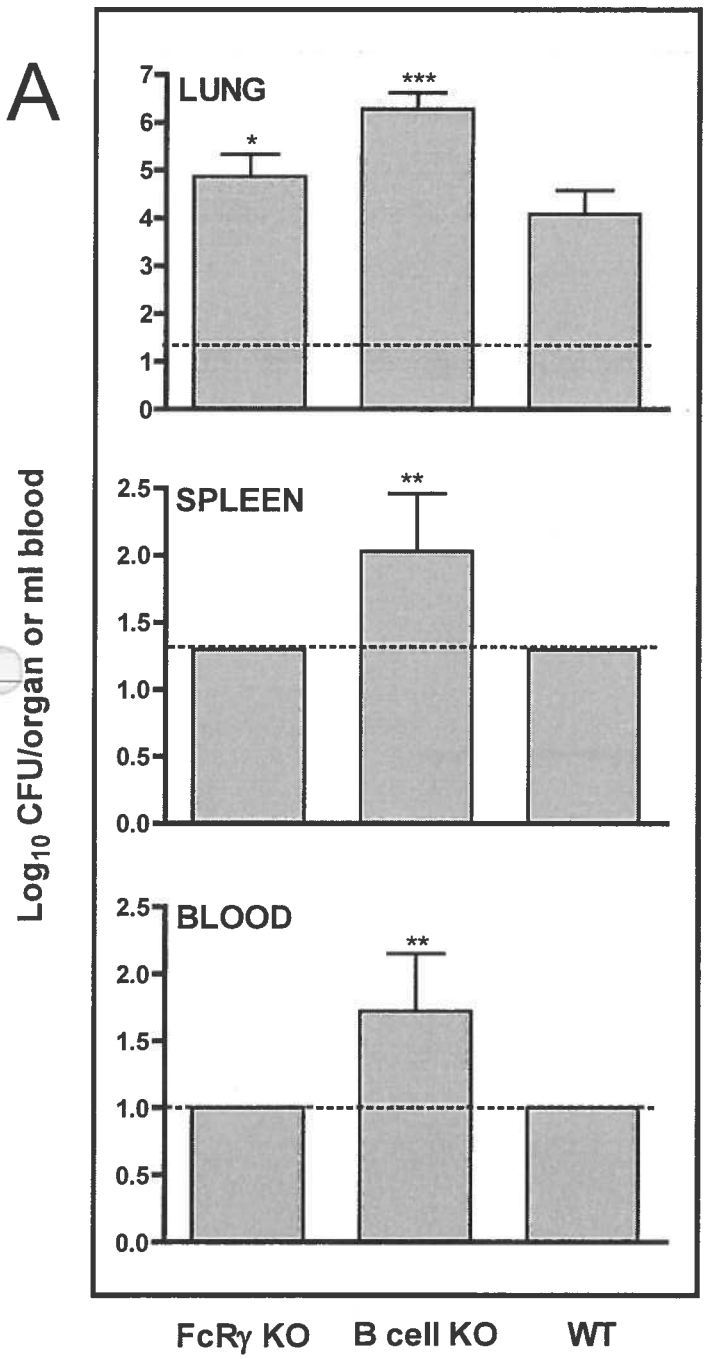
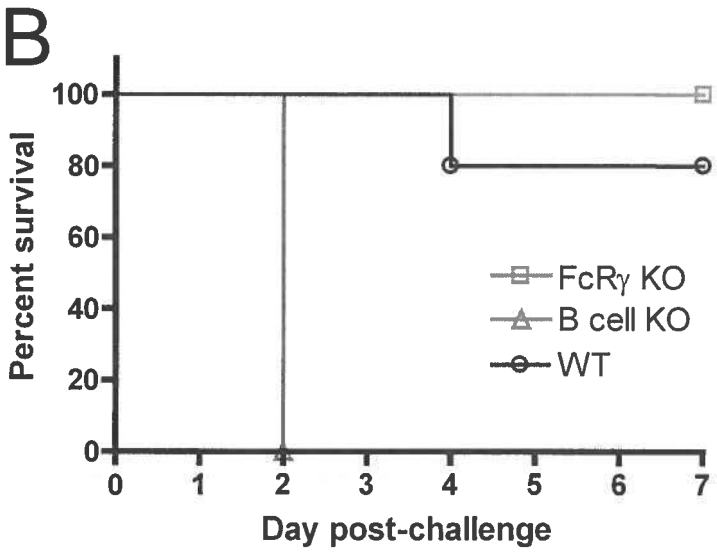


Figure 6

RK213
RK223





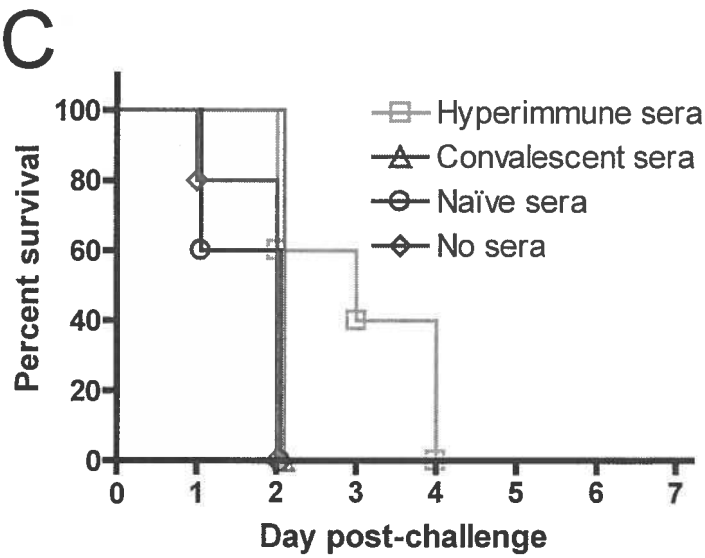


Figure 7

RK213