Recombinant Ag85B vaccine by taking advantage of characteristics of human parainfluenza type 2 virus vector showed Mycobacteria-specific immune responses by intranasal immunization.

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31 Abbreviations used in this article: BAL, bronchoalveolar lavage; BCG, *Mycobacterium*
32 *bovis* bacille Calmette-Guérin; BEAS cells, bronchial epithelial cells; hPIV2, human
33 parainfluenza type 2 virus; pLN, pulmonary lymph node; Mtb, *Mycobacterium*
34 *tuberculosis*; NHBE, normal human bronchial epithelial; rhPIV2-Ag85B, recombinant
35 hPIV2 expressing Ag85B; TB, tuberculosis.

36

ABSTRACT

Viral vectors are promising vaccine candidates for eliciting suitable Ag-specific immune response. Since *Mycobacterium tuberculosis* (Mtb) normally enters hosts via the mucosal surface of the lung, the best defense against Mtb is mucosal vaccines that are capable of inducing both systemic and mucosal immunity. Although *Mycobacterium bovis* bacille Calmette-Guérin is the only licensed tuberculosis (TB) vaccine, its efficacy against adult pulmonary forms of TB is variable. In this study, we assessed the effectiveness of a novel mucosal TB vaccine using recombinant human parainfluenza type 2 virus (rhPIV2) as a vaccine vector in BALB/c mice. Replication-incompetent rhPIV2 (M gene-eliminated) expressing Ag85B (rhPIV2-Ag85B) was constructed by reverse genetics technology. Intranasal administration of rhPIV2-Ag85B induced Mtb-specific immune responses, and the vaccinated mice showed a substantial reduction in the number of CFU of Mtb in lungs and spleens. Unlike other viral vaccine vectors, the immune responses against Ag85B induced by rhPIV2-Ag85B immunization had an advantage over that against the viral vector. In addition, it was revealed that rhPIV2-Ag85B in itself has an adjuvant activity through the retinoic

acid-inducible gene I receptor. These findings provide further evidence for the possibility of rhPIV2-Ag85B as a novel TB vaccine.

1. INTRODUCTION

Recombinant viral vector vaccines have several advantages for preventing infection with pathogens [1]. The vaccines induce a full spectrum of immune responses including humoral and cellular immune responses. These immune responses can be initially induced at the viral vector infection site such as mucosal immune responses [2]. Moreover, the viral vector itself has adjuvant activities through the innate immune systems [3]. Pre-existing or post-priming immune responses against the vaccine vector itself, however, could be an obstacle to effective immune responses to recombinant Ag [4]. Negligible immune responses against vector viruses compared with recombinant vaccine Ags after immunization is considered most desirable for recombinant viral vaccines.

Mycobacterium bovis bacille Calmette-Guérin (BCG) has substantially contributed to the control of tuberculosis (TB) for more than 80 years and affords about 80% protection against tuberculosis meningitis and miliary tuberculosis in infant and young children. However, it is well known that the protective efficacy of BCG against pulmonary TB in adults is variable and partial [5, 6]. Therefore, development of new

vaccines is urgently needed for the elimination of TB as a public health threat and should be a major global public health priority.

Many infectious diseases, including TB, initially establish infection on mucosal surfaces. Therefore, the best defense against these predominantly mucosal pathogens is mucosal vaccines that are capable of inducing both systemic and mucosal immunity. However, the mucosal immune system is quite unique and is different from systemic immune responses [7, 8]. Mucosal immunization provides mucosal immune responses in all mucosal effector tissues in the concept of a common mucosal immune system [9].

Human parainfluenza type 2 virus (hPIV2) is a member of the genus *Rubulavirus* of the family *Paramyxoviridae* and possesses a single-stranded, nonsegmented and negative-stranded RNA genome. This virus does not have a DNA phase during its life cycle and can avoid genetic modifications. Additionally, this virus becomes replication-incompetent by elimination of some viral genes [10]. Moreover, it is likely to lead to elicit stronger inserted antigen-specific immune responses than vector-specific responses unlike other viral vaccine vectors using inserted antigen expression mechanisms of hPIV2. In the present study, we evaluated the effectiveness of intranasal administration of Ag85B-expressed non-replicating human parainfluenza type

2 virus (rhPIV2-Ag85B), which induces weak immune responses against a viral vector, as a novel mucosal TB vaccine.

2. MATERIALS AND METHODS

2.1. Immunization

Six-week-old BALB/c female mice were immunized with rhPIV2-Ag85B or rhPIV2 control vector 3 or 4 times at 2-week intervals by intranasal inoculation of 1×10^8 TCID50 virus in 20 µl PBS. Another group of mice was intramuscularly immunized twice with Ag85B DNA vaccine [11] and intranasally immunized twice with rhPIV2-Ag85B. As a control group, a group of mice was vaccinated using 1×10^7 CFU of BCG Tokyo by subcutaneous injection.

2.2. Infection assay

Two weeks (rhPIV2-Ag85B-immunized mice) or 6 weeks (BCG-immunized mice) after the final immunization, mice were challenged with *Mycobacterium tuberculosis* (Mtb) Kurono strain by inhalation. This bacterial preparation and infection assay were performed as previously described [12]. In brief, the mice were infected via the airborne route by placing them into the exposure chamber of a Glas-Col aerosol generator. The nebulizer compartment was filled with 5 ml of a suspension containing 10^6 CFU of Kurono strain so that approximately 50 bacteria would be deposited in the

110 lungs of each animal. Eight weeks after Mtb infection, mice were sacrificed and the
111 preventive effects of the vaccine were assessed.
112
113 *2.3. Cell culture*
114 Human bronchial epithelial cells (BEAS cells) and primary cultured normal human
115 bronchial epithelial (NHBE) cells were obtained from the American Type Culture
116 Collection (Manassas, VA) and Lonza (Walkersville, MD). These cells were grown in
117 bronchial epithelial growth medium containing supplements (Lonza). These cells were
118 infected with rhPIV2 or rhPIV2-Ag85B (MOI of 10) or treated with recombinant
119 Ag85B (10 μg/ml) for 6 to 48 h in a 37°C incubator with a 5% CO_2 atmosphere.
120
121 *2.4. FACS analysis*
122 Spleen, pulmonary lymph node (pLN), and bronchoalveolar lavage (BAL) cells were
123 obtained from immunized mice, and single-cell suspensions were prepared. The cells
124 were incubated with recombinant Ag85B protein (10 μg/ml final concentration) for 4 h
125 in the presence of Brefeldin A at 37°C with 5% CO_2. The cells were stained for surface
126 markers with anti-CD3 and anti-CD4 (BD Biosciences, San Joes, CA) for 30 min at 4°C,

followed by fixation for 30 min at 4°C in 2% paraformaldehyde. IFN-γ was detected by staining with anti-IFN-γ (BD Biosciences) for 30 min at 4°C. Flow cytometry data collection was performed on a FACS Canto II (BD Biosciences). Files were analyzed using FACSDiva Software (BD Biosciences). BEAS cells infected with rhPIV2-Ag85B were stained with anti-ICAM-1 (BioLegend, San Diego, CA) and analyzed as described above.

2.5. Evaluation of Ag85B-specific immune responses by ELISPOT assay

The number of Ag85B-specific, IFN-γ-secreting cells was determined by the ELISPOT assay according to the method reported previously [11]. Triplicate samples of whole, $CD4^+$, and $CD8^+$ T cells (separated by a MACS system) (Miltenyi Biotec, Bergisch Gladbach, Germany) collected from the spleen, pLN, and BAL were plated at 1×10^6 cells/well. These cells were stimulated by addition of 2×10^5 mitomycin C (Sigma-Aldrich, Saint Louis, MO)-treated syngeneic spleen cells infected with recombinant vaccinia virus expressing Ag85B or rhPIV2-Ag85B.

2.6. Statistical analysis

144 Data are presented as means ± SD. Statistical analyses were performed using the
145 Mann-Whitney *U* test. Statistically significant differences compared with the control
146 are indicated by asterisks.
147

3. RESULTS

3.1. Characteristics of rhPIV2-Ag85B

A construction of rhPIV2-Ag85B was shown in Fig. 1A. To examine gene expression levels of the inserted Ag85B, BEAS cells were infected with rhPIV2-Ag85B. Abundant and rapid expression of mRNA of Ag85B was observed in BEAS cells infected with rhPIV2-Ag85B compared with the expression of NP mRNA (Fig. 1B). These results were also confirmed by analysis of protein expression (Fig. 1C). The production of Ag85B was earlier than that of NP, which is usually the earliest synthesized protein in hPIV2 infection.

These responses were considered to be advantageous effects in cellular immune response to inserted Ag85B versus rhPIV2 vector. To confirm this advantageous response, cells from immunized mice were re-stimulated *in vitro* with syngeneic spleen cells infected with rhPIV2 or rhPIV2-Ag85B. Although responses to both Ag85B and rhPIV2 vector were observed, Ag85B-specific responses were clearly seen, especially in pLN and BAL cells after single immunization (Fig. 1D). After performing immunization twice, Ag85B-specific responses were also seen in spleen cells as booster effects more than responses to the vector virus (Fig. 1E). These results indicated that

166 rhPIV2-Ag85B immunization elicited inserted Ag85B-specific immune responses
167 without being hidden by vector responses.
168
169 *3.2. Intranasal administration of rhPIV2-Ag85B prevents infection with Mtb in mice.*
170 To investigate the ability of intranasal administration of rhPIV2-Ag85B to elicit a
171 protective effect against pulmonary TB, rhPIV2-Ag85B-immunized mice were
172 aerosol-infected with highly pathogenic Mtb kurono strain [13]. One group of mice
173 were intranasally immunized with rhPIV2-Ag85B 4 times at 2-week intervals, and
174 another group of mice were intranasally immunized with rhPIV2-Ag85B twice
175 following intramuscular immunization with Ag85B DNA twice (Fig. 2A). Intranasal
176 administration of rhPIV2-Ag85B resulted in a decreases in granulomatous lesions and
177 inflammatory area. However, there were no apparent histopathological differences,
178 such as infiltrating cell types, between the each group of mice, and these results are
179 similar to the results of another study focusing on TB vaccine [14]. On the other hand,
180 these vaccine effects were clearly seen by staining for acid-fast bacillus. Mice
181 immunized with rhPIV2-Ag85B showed a substantial reduction in the infiltration of
182 bacteria, and this inhibitory effect on bacterial expansion was correlated with the
183 number of rhPIV2-Ag85B intranasal administrations (Fig. 2B). CFU of Mtb in

184 spleens from both groups of immunized mice were also significantly lower than those in
185 mice immunized with the control vector (Fig. 2C). As for a preventive effect on Mtb
186 infection in the lung, the mice immunized with rhPIV2-Ag85B clearly showed a
187 substantial reduction in CFU.
188
189 *3.3. Ag85B-specific immune response is elicited by rhPIV2-Ag85B administration.*
190 The capacity of rhPIV2-Ag85B intranasal immunization to elicit effector cells that
191 recognize endogenously expressed Ag85B was assessed. Spleen, pLN, and BAL cells
192 obtained from immunized mice were re-stimulated *in vitro* with syngeneic spleen cells
193 infected with the recombinant vaccinia virus expressing Ag85B, and endogenously
194 expressed Ag85B-specific cellular immune response was examined by ELISPOT assays.
195 Both $CD4^+$ and $CD8^+$ splenocytes exhibited Ag85B-specific responses, and $CD8^+$ T
196 cells showed much stronger responses than those of $CD4^+$ T cells in splenocytes from
197 mice immunized with rhPIV2-Ag85B (Fig. 3A). Ag85B-specific responses were also
198 seen in both $CD4^+$ and $CD8^+$ T cells at almost the same levels in pLN and BAL cells
199 (Fig. 3 B and C).
200

3.4. Analysis of Ag-specific effector cells and immune responses in pLN cells and the lung

Delayed initial activation of effector cells in lungs has been reported in the case of Mtb infection [15]. To control bacterial expansion in the early phase of infection, rapid Mtb Ag-specific $CD4^+$ T cell responses are required. Thus, we next analyzed recruitment of Ag85B-specific IFN-γ^+ $CD4^+$ T cells in pLN and BAL cells in mice immunized with rhPIV2-Ag85B. Mice were intranasally immunized with rhPIV2-Ag85B or the control vector virus 3 times at 2-week intervals. Another group of mice were immunized with BCG by subcutaneous injection. Two weeks (rhPIV2-Ag85B-immunized mice) or 6 weeks (BCG-immunized mice) after the final immunization, all mice were challenged with Mtb Kurono strain by inhalation (Fig. 4A). At each time point after immunization or Mtb challenge, the percentage and absolute number of Ag85B-specific IFN-γ^+ $CD4^+$ cells were determined by flow cytometry. Before Mtb challenge, the percentage of IFN-γ^+ $CD4^+$ cells in pLN cells was increased by immunization with rhPIV2-Ag85B but not by BCG immunization (Fig. 4 B and C, top). However, a significant increase in IFN-γ^+ $CD4^+$ cells was not detected in BAL cells (Fig. 4 B and C, bottom). Interestingly, expansion of IFN-γ^+ $CD4^+$ cells occurred after Mtb challenge in BAL cells more dramatically than that in pLN cells in terms of

absolute number (Fig. 4C). These responses induced by rhPIV2-Ag85B immunization were much stronger than those induced by BCG immunization.

Similarly, an increase in Ag85B-specific responses was observed by the ELISPOT assay (Fig. 4D). The number of Ag85B-specific IFN-γ secreting cells increased in pLN cells from mice immunized with rhPIV2-Ag85B in a number of immunizations-dependent manner. Furthermore, strong Ag85B-specific responses were detected after Mtb challenge in pLN and BAL cells, and the responses were much stronger than those in BCG immunized mice.

3.5. rhPIV2-Ag85B induces innate immune responses.

We explored innate immune responses induced by rhPIV2-Ag85B infection. We confirmed that Ag85B did not affect the viability of rhPIV2-Ag85B infected cells. (Supplemental Fig. 1). Type I IFNs were assessed after infection with rhPIV2-Ag85B in NHBE and BEAS cells as an indication of innate immune responses. Both types of cells showed mRNA expression of type I IFNs after infection with rhPIV2-Ag85B but not after addition of recombinant Ag85B protein (Fig. 5A). Production of IFN-β was also detected in the culture supernatant by ELISA (Fig. 5B). The mRNA expression of intracellular receptors, RIG-I, MDA5, and TLR3, and the induction of cytokines, IL-6

and IL-15, were also enhanced by infection with rhPIV2-Ag85B, whereas these effects were not observed with the addition of recombinant Ag85B protein (Fig. 5 C and D). Furthermore, the expression of ICAM-1 was induced by infection with rhPIV2-Ag85B (Fig. 5E). Similar results were obtained after infection with rhPIV2 vector alone or rhPIV2-GFP (Supplemental Fig. 2). Other co-stimulation molecules, CD80, CD86, ICAM-2 and selectin, were not detected (data not shown).

To further investigate the participation of these receptors in innate immune activation induced by rhPIV2-Ag85B infection, expression of these receptors was knocked down by transfecting siRNA. At 48 h after transfection with siRNA, expression levels of these receptors were reduced by approximately 90% or expression was no longer detectable (Fig. 5F). IFN-β production induced by rhPIV2-Ag85B infection was inhibited when the cells were treated with RIG-I siRNA. For other receptors, MDA5 and TLR3, siRNA treatment did not result in inhibition of IFN-β production induced by rhPIV2-Ag85B infection (Fig. 5G). This result was confirmed by phosphorylation of IRF3, which is a downstream molecule of RIG-I in epithelial cells. The phosphorylation of IRF3 induced by rhPIV2-Ag85B infection was inhibited when epithelial cells were treated with siRNA of RIG-I (Fig. 5H).

4. DISCUSSION

In the present study, we demonstrated the effectiveness of hPIV2 vectors for TB vaccines to induce systemic and mucosal immune responses. The rhPIV2 vector is a weak immunogenic; however, intranasal immunization with rhPIV2-Ag85B showed more potent protection against pulmonary TB in BALB/c mice than did conventional BCG vaccination. The rhPIV2-Ag85B shows a vaccine effect by itself alone, and this effect is more useful than the effects of other vectors for TB vaccines.

Viral vectors are promising vaccine candidates for eliciting Ag-specific immune responses [16, 17]. Pre-existing anti-vector antibodies, however, constitute an obstacle for use in humans [18-20]. Although antibodies against hPIV are known to cross-react with Sendai virus, Sendai virus vector is considered to be effective for human use by intranasal administration [21]. Additionally, Sendai virus vector is not affected by antibodies against Sendai virus for induction of T cell responses, especially when it is administered intranasally [4]. From these findings, intranasal administration of the hPIV2 vector is also considered to be effective for human use. In fact, multiple administrations with rhPIV2-Ag85B also showed preventive effects more clearly than did immunization 2 times with rhPIV2-Ag85B (Fig. 2).

Many viral vectors have been tested as recombinant viral vaccines eliciting suitable recombinant Ag-specific immune responses, and many of these vaccine vectors are not vaccine viruses such as vaccinia virus Ankara (MVA), adenovirus, Sendai virus, and CMV. These viral vectors have also been used in several vaccine trials in TB or HIV vaccine [22-24]. Experience in the HIV vaccine field has emphasized the importance of avoiding anti-vector immune responses when developing a vectored vaccine [25]. Immune responses to vaccine vectors prevent the induction of aimed immune responses against recombinant Ag. From these findings, elimination of the immunogenicity of a vaccine vector is critical for a recombinant viral vaccine. The immunogenicity of viral vectors depends on the amount of vector viral proteins. Approximately 80 poxvirus proteins are encoded by its over 130 - 300 kbp and the adenovirus genome sizes are 26 - 45 kbp. The genome sizes of these two viral vectors are much larger than that of hPIV2 (15.65 kbp), and induction of immune responses to hPIV2 vector might be lower than other viral vectors. In TB vaccines, recombinant vaccinia virus and adenovirus, which are immunogenic viruses, did not show clear vaccine effects against TB infection by immunization with themselves alone. These two recombinant TB vaccines, adenovirus and MVA, were utilized as boost immunization after BCG priming [26, 27]. These heterologous prime-boost strategies diminish immune responses to the vector

290 virus and indicate the possibility of a practical and efficient strategy for prevention of
291 TB [28, 29]. On the other hand, the most common method for obtaining an attenuated
292 virus is gene elimination of the viral construct protein to make a replication-deficient
293 virus *in vivo*. The rhPIV2 vector is a weak immunogenicity by elimination of
294 structural protein (M) gene; however, the rhPIV2-Ag85B shows a vaccine effect by
295 immunization with itself alone, and this effect is more useful than the effects of other
296 vectors for a recombinant TB vaccine.

297 The hPIV2 vector has an additional advantage over other viral vectors. The
298 inserted Ag85B gene, which is only 978 bp, is a minor component of rhPIV2-Ag85B.
299 Despite that, the cellular immune response against Ag85B had an advantage over that
300 against the virus vector in mice. This advantageous effect is thought to depend on
301 Ag85B expression mechanisms. The frequency with which viral RNA polymerase
302 reinitiates the next mRNA at gene junctions is imperfect, and this leads to a gradient of
303 mRNA abundance that decreases according to distance from the genome 3' end [30].
304 Insertion of the Ag85B gene into the 3' proximal first locus between the leader sequence
305 and the NP gene results in the highest level of gene expression. Ag85B is transcribed
306 earlier and more abundantly than other viral products (Fig. 1 B and C). This property
307 of rhPIV2-Ag85B leads to elicit stronger Ag85B-specific immune responses than

308 vector-specific responses in our system (Fig. 1 D and E), although recombinant virus
309 vaccine immunization usually induces overwhelming viral-specific immune responses
310 compared with an inserted gene product [31, 32]. We also demonstrated that intranasal
311 administration of the rhPIV2 vector had no adverse effects and provided sufficient
312 immunogenicity and a sufficient vaccine effect against Mtb in mice. These results
313 suggest that intranasal administration of rhPIV2-Ag85B does not cause functional
314 failure as a vaccine by multiple administrations, and these features of the rhPIV2 vector
315 are definitely advantages for clinical use.

316 Another major feature of rhPIV2-Ag85B is effective prevention of TB by intranasal
317 administration. Vaccination in the respiratory tract may enhance protection against
318 Mtb infection, since Mtb initially establishes infection on mucosal surfaces of the
319 respiratory tract. Indeed, a number of recombinant TB vaccines have been developed
320 and evaluated for respiratory mucosal immunization [33-35]. It is important to note
321 that lack of Ag-specific effector cells persists even up to about 21 days after pulmonary
322 Mtb infection caused by a bacterial component [15, 36]. In the present study, the
323 arrival of Ag-specific T cells was detected in lung and pLN by rhPIV2-Ag85B
324 immunization, and this arrival of effector cells was recognized faster than BCG
325 immunization after Mtb challenge (Fig. 4 B and C). We were able to establish a novel

intranasal vaccine, rhPIV2-Ag85B, against TB by utilizing various advantages of intranasal administration. Nasal administration of a vaccine to induce mucosal and systemic immune responses has several advantages other than the induction of effective immune responses. It is even possible that intranasal administration of replication-incompetent rhPIV2-Ag85B limits the areas of infection in respiratory organs and induces a respiratory tract mucosal immune response in addition to a systemic immune response against TB. Our study suggested that intranasal administration of rhPIV2-Ag85B, which can induce both mucosal and systemic immune responses against Mtb, has a great advantage as a TB vaccine.

Attempts have been made to use various types of adjuvants for enhancing an immune responses to vaccines, including vaccines against TB [37]. In fact, a protein-based TB vaccine required the addition of an adjuvant to induce effective immune responses [38-41]. For the generation of adaptive immune responses, induction of innate immunity is crucial for vaccines to elicit potent Ag-specific immune responses. Pattern recognition receptors have been studied as potential targets for an adjuvant. dsRNA is a dominant activator of innate immunity because viral dsRNA is recognized by TLR3, RIG-I, and MDA5 [42, 43]. As a result, it was demonstrated that the rhPIV2 vector had a potent adjuvant activity as dsRNA recognized by the RIG-I

receptor and enhanced not only local innate immunity but also systemic adaptive immunity. It is possible that no extra addition of an adjuvant is required to prevent TB by vaccination with rhPIV2-Ag85B. Furthermore, the inhibitory effects on the growth of rhPIV2-Ag85B *in vivo* by IFN through the innate receptor are not required to consider since the rhPIV2 vector is replication-incompetent *in vivo* by elimination of the M gene (Fig. 1A).

In summary, our results provide evidence for the possibility of rhPIV2-Ag85B as a novel intranasal vaccine for eliciting Mtb-specific mucosal immunity. Immunization with rhPIV2-Ag85B showed significant protection against TB without any prime vaccine or addition of an adjuvant in mice. Further studies will contribute to the ultimate goal of establishing a new vaccine strategy that can definitely prevent Mtb infection.

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FIGURE LEGENDS

Figure 1. Expression of Ag85B and advantageous effects in cellular immune response against Ag85B versus virus vector in immunized mice.

(A) Construction of rhPIV2-Ag85B. (B) Expression of Ag85B (left panel) and NP (right panel) gene in BEAS cells infected with rhPIV2 or rhPIV2-Ag85B at each time point was determined by real-time PCR. Total RNA was extracted at 6, 24, and 48 h after infection. Fold increase of each target gene was normalized to β-actin, and the expression levels are represented as relative values to naïve cells. Error bars represent standard deviation. ND indicates non-detected. (C) Expression of Ag85B and NP proteins was detected by anti-Ag85B and anti-NP antibodies at 6 and 24 h after infection, respectively. (D,E) Mice were immunized 1 (D) or 2 (E) times with rhPIV2 or rhPIV2-Ag85B at a 2-week interval by intranasal inoculation (n = 5 per group). Spleen, pLN, and BAL cells were collected from immunized mice (n = 5 per group) 2 weeks after the final immunization for examination by an ELISPOT assay. These isolated cells were stimulated *in vitro* with syngeneic spleen cells infected with control rhPIV2, rhPIV2-Ag85B, or recombinant Ag85B protein (rAg85B) (10 μg/ml final concentration) for 24 h. Error bars represent standard deviations. Statistically

significant differences are indicated by asterisks (*, $P < 0.05$ compared to the group stimulated with rhPIV2).

Figure 2. Repeated immunization with rhPIV2-Ag85B results in protection from TB.

(A) Groups of mice were vaccinated in this schedule. (B) Histological images of the lungs of Mtb-infected mice. Groups of mice ($n = 10$) immunized 4 times with rhPIV2 (left panel), 2 times with Ag85B DNA vaccine and 2 times with rhPIV2-Ag85B (middle panel) or 4 times with rhPIV2-Ag85B (right panel) were challenged by Mtb infection. Arrows point to tubercles. Lower panels in (B) show magnified images of images in the middle panels. (C) Inhibition of bacterial growth by immunization with rhPIV2-Ag85B in the lung and spleen. Groups of mice immunized 2 times with Ag85B DNA vaccine and 2 times with rhPIV2-Ag85B or immunized 4 times with rhPIV2-Ag85B or BCG were challenged by Mtb infection. The numbers of Mtb CFU in the lung and spleen were determined by a colony enumeration assay. The bacterial load is represented as mean $\log_{10}$ CFU per organ. Error bars represent standard deviations. Statistically significant differences are indicated by asterisks (*, $P < 0.05$, **, $P < 0.005$)

Figure 3. Induction of Ag85B-specific cellular immune responses in rhPIV2-Ag85B-immunized mice.

Mice were immunized with rhPIV2, rhPIV2-Ag85B, or BCG (*n* = 5 per group) according to the schedule shown in Fig. 2A. Two (rhPIV2 or rhPIV2-Ag85B) or 4 weeks (BCG) after the final immunization, the spleen, pLN, and BAL were collected. Isolated cells from the spleen (A), pLN (B), or BAL (C) were separated into whole (left panels), $CD4^+$ (middle panels), and $CD8^+$ (right panels) T cells and examined for IFN-γ production in an ELISPOT assay. These cells were stimulated *in vitro* with syngeneic spleen cells infected with control vaccinia virus (Vac) or recombinant vaccinia virus carrying the Ag85B gene (Vac-Ag85B) for 24 h. Error bars represent standard deviations. Statistically significant differences are indicated by asterisks (*, $P < 0.01$ compared to the group stimulated with Vac).

Figure 4. Analysis of Ag-specific effector cells and these immune responses in pLN and BAL.

561 (A) Groups of mice were immunized with rhPIV2, rhPIV2-Ag85B, or BCG (*n* = 10 per
562 group) and challenged by Mtb infection in this schedule. (B) Representative flow
563 cytometry plots of IFN-γ^+ cells on gated $CD4^+$ cells from pLN (top panels) and BAL
564 (bottom panels) are shown. Numbers shown beside the gates represent the percentages
565 within $CD4^+$ T cells. (C) Kinetics of recruitment of Ag85B-specific IFN-γ^+ cells in
566 pLN (top panel) and BAL (bottom panel). Absolute numbers of IFN-γ^+ $CD4^+$ cell
567 populations at each time points are shown. Error bars represent standard deviations.
568 (D) Isolated cells from the pLN and BAL at each time point were examined for IFN-γ
569 production in an ELISPOT assay. These cells were stimulated *in vitro* with syngeneic
570 spleen cells infected with control vaccinia virus (Vac) or recombinant vaccinia virus
571 carrying the Ag85B gene (Vac-Ag85B) for 24 h. Error bars represent standard
572 deviations.
573
574 **Figure 5. Evaluation of adjuvant activity of rhPIV2-Ag85B in vitro.**
575 NHBE and BEAS cells were treated with rAg85B protein (10 μg/ml) or infected with
576 rhPIV2-Ag85B (MOI of 10) for 24 h, and the increases in mRNA levels of IFN-α,
577 IFN-β (A), RIG-I, MDA5, TLR3 (C), IL-6, IL-15 (D), and ICAM-1 (E, left panel) were
578 determined by real-time PCR. Fold increase of each target gene was normalized to

β-actin, and the expression levels are represented as relative values to the control. Culture supernatants were also collected, and amounts of secreted IFN-α and IFN-β were measured by ELISA (B). Expression of ICAM-1 was also confirmed by FACS analysis in BEAS cells (E, right panel). Data are averages of triplicate samples from three identical experiments, and error bars represent standard deviations. Statistically significant differences between control cells and rhPIV2-Ag85B-infected cells are indicated by asterisks (*, $P < 0.01$). BEAS cells were treated with siRNA targeting RIG-I, MDA5, TLR3, or the negative control siRNA (NC) for 48 h. Depletion of them was examined by immunoblotting (F). Those cells were stimulated by rAg85B protein (10 μg/ml) or infected with rhPIV2-Ag85B (MOI of 10) and then production of IFN-β was measured by ELISA (G). Data are averages of triplicate samples from three identical experiments, and error bars represent standard deviations. Statistically significant differences are indicated by asterisks (*, $P < 0.01$ compared to NC). The effects of depletion of RIG-I on IRF3 phosphorylation were tested. BEAS cells treated with NC or siRNA targeting RIG-I (ΔR) for 48 h were infected with rhPIV2-Ag85B or not infected (control). Whole IRF3 and phosphorylated IRF3 (pIRF3) were detected by immunoblotting 6 h after infection (H).

Supplementary Materials

Construction and preparation of rhPIV2-Ag85B

rhPIV2-Ag85B was constructed according to the method reported previously [10]. In brief, the gene of Ag85B from *Mycobacterium kansasii* was cloned into cDNA encoding the rhPIV2 genome. rhPIV2-Ag85B was recovered by co-transfection of an antigenomic plasmid and plasmids expressing NP, P and L into BSRT7/5 cells expressing T7 polymerase [44] and then co-cultured with Cos7 cells transfected with a plasmid encoding the M gene.

Construction of recombinant vaccinia virus

pBMSF plasmid provided by Dr. Kohara (The Tokyo Metropolitan Institute of Medical Science, Japan) was used to construct a complementary transfer vector for homologous recombination into the hemagglutinin locus of vaccinia virus LC16m8. This plasmid possesses an ATI/p7.5 synthetic hybrid promoter [45]. The *E. coli* guanine phosphoribosyltransferase (gpt) gene and p7.5 promoter gene were inserted into pBMSF (pBMSF/gpt). pBMSF/gpt could obtain the recombinant vaccinia virus by hypoxanthineguanine phoshoribosyltransferase selection [46]. A DNA fragment

615 encoding the Ag85B gene of *M. bovis* was amplified by PCR and inserted into
616 pBMSF/gpt. This plasmid was transfected by an Electric Square Porater (BTX) into
617 chick embryo fibroblasts infected with vaccinia virus Lister strain at an MOI of 10.
618 Recombinant vaccinia virus Lister strain expressing Ag85B was isolated and purified by
619 hypoxanthineguanine phoshoribosyltransferase selection.
620
621 *Recombinant protein Ag85B production*
622 Plasmids containing the Ag85B gene of *M. bovis* were transferred into *E. coli* TG1.
623 The expressed IB was harvested from a disrupted cell pellet by a homogenizer. The
624 IB of Ag85B was unfolded in 8 M urea solution and refolded by dilution. The urea
625 in the refolding buffer was removed by anion exchange chromatography. The
626 refolded Ag85B was loaded on a cation exchange column and crude Ag85B was
627 passed through the resin. Finally, Ag85B was purified by anion exchange
628 chromatography.
629
630 *siRNA experiment*
631 siRNA duplexes used for silencing human RIG-I (target sequence:
632 TTCTACAGATTTGCTCTACTA), MDA5 (target sequence:

633 CAGAACTGACATAAGAATCAA), and TLR3 (target sequence:
634 AAGAACTGGATATCTTTGCCA) were purchased from QIAGEN (Tokyo, Japan).
635 NHBE and BEAS cells were transiently transfected using HiPerFect Transfection
636 Reagent (QIAGEN) with or without siRNA at a final concentration of 10 nM for 48 h.
637 Treatment with the siRNA and HiPerFect Transfection Reagent had little effect on cell
638 viability.
639
640 *Quantitative real-time PCR*
641 Total RNA was isolated from infected cells using TRIzol reagent (Invitrogen, San
642 Diego, CA) and then reverse-transcribed to cDNAs using an Omuniscript system
643 (QIAGEN). The cDNA was subjected to real-time PCR for RIG-I, MDA5, TLR3,
644 β-actin, Ag85B, NP, IFN-α, IFN-β, IL-6, IL-15, CD80, CD86, ICAM-1, ICAM-2, and
645 selectin using a LightCycler (Roche Applied Science, Tokyo, Japan). The specific
646 primers for each target (listed below) and probes were designed by Universal
647 ProbeLibrary Assay Design Center (Roche Applied Science). Primers used in this
648 study were
649 5’-TGGACCCTACCTACATCCTGA 5’-GGCCCTTGTTGTTTTTCTCA for RIG-I,
650 5’-AGGCACCATGGGAAGTGAT 5’-GGTAAGGCCTGAGCTGGAG for MDA5,

5’-AGAGTTGTCATCGAATCAAATTAAAG 5’-AATCTTCCAATTGCGTGAAAA for TLR3, 5’-CCAACCGCGAGAAGATGA 5’-CCAGAGGCGTACAGGGATAG for β-actin, 5’-CTTCATGGCGTTGAGCTG 5’-ACGCCGTGTTCAATTTGG for Ag85B, 5’-GAGAGGTGCTGGCTTTTGAA 5’-TTTGGTGATTAAGGGTATCAGGA for NP, 5’-CCCTCTCTTTATCAACAAACTTGC 5’-TTGTTTTCATGTTGGACCAGA for IFN-α, 5’-CGACACTGTTCGTGTTGTCA 5’-GAAGCACAACAGGAGGAGCAA for IFN-β, 5’-GATGAGTACAAAAGTCCTGATCCA 5’-CTGCAGCCACTGGTTCTGT for IL-6, 5’-CAGATAGCCAGCCAATACAAG 5’-GGCTATGGCAAGGGGTTT for IL-15, 5’-TCCTGGGCCATTACCTTAATC 5’-CATCTTGGGGCAAAGCAG for CD80,5’-CAGAAGCAGCCAAAATGGAT 5’-GAATCTTCAGAGGAGCAGCAC for CD86, 5’-CCTTCCTCACCGTGTACTGG 5’-AGCGTAGGGTAAGGTTCTTGC for ICAM-1, 5’-CAATGAATTCCAACGTCAGC 5’-ACCAAAGTGGGTTGCAGTGT for ICAM-2, and 5’-ACCAGCCCAGGTTGAATG 5’-GGTTGGACAAGGCTGTGC for Selectin.

Immunoblotting

Cells were lysed with RIPA buffer (Thermo, Waltham, MA) containing protease and phosphatase inhibitor cocktail (Thermo). They were separated on 12% polyacrylamide

669 gels (BIO-RAD, Hercules, CA) and transferred to a PVDF membrane (Millipore),
670 which was incubated overnight at 4°C with primary antibodies anti-RIG-I (Enzo Life
671 Sciences, Farmingdale, NY), anti-MDA5 (Santa Cruz Biotechnology, Inc., Santa Cruz,
672 CA), anti-TLR3 (Epitomics, Burlingame, CA), anti-β-actin (AnaSpec, Fremont, CA),
673 anti-IRF3 (OriGene Technologies, Rockville, MD), anti-phosphorylated IRF3
674 (Epitomics), anti-Ag85B (provided by Japan BCG Laboratory), and anti-NP (Toshiba
675 Japan, Tokyo, Japan) at a dilution of 1:5000 in 5% bovine serum albumin. The
676 membrane was then washed three times in Tris-buffered saline with 0.01% Tween 20,
677 incubated for 30 min with HRP-conjugated secondary antibody (R&D Systems or
678 Epitomics) at 0.01 μg/ml, and then washed again. Immunoreactions were visualized
679 using an enhanced chemiluminescence detection system (GE Healthcare Life Science,
680 Piscataway, NJ).

Figure(s)

A

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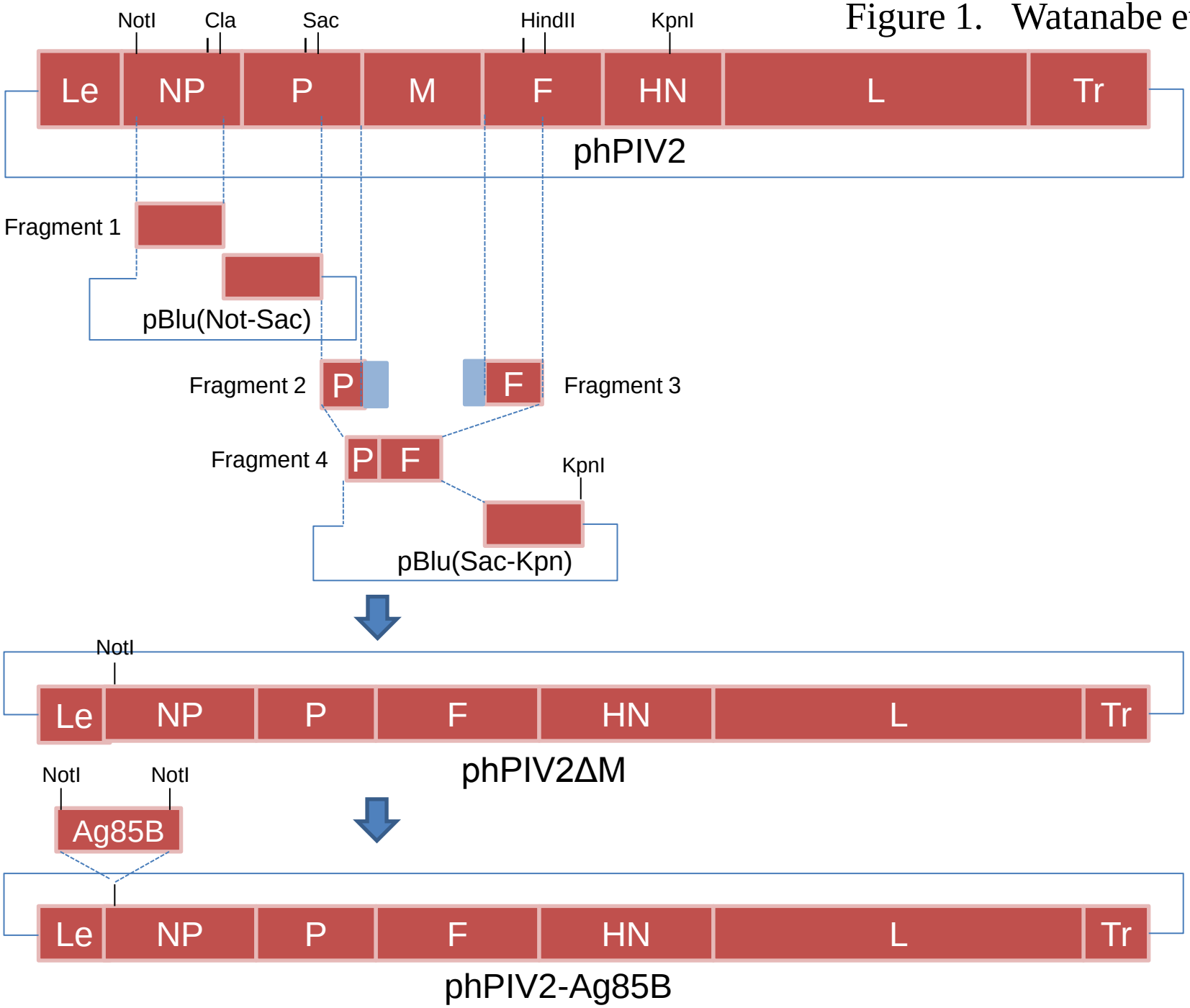

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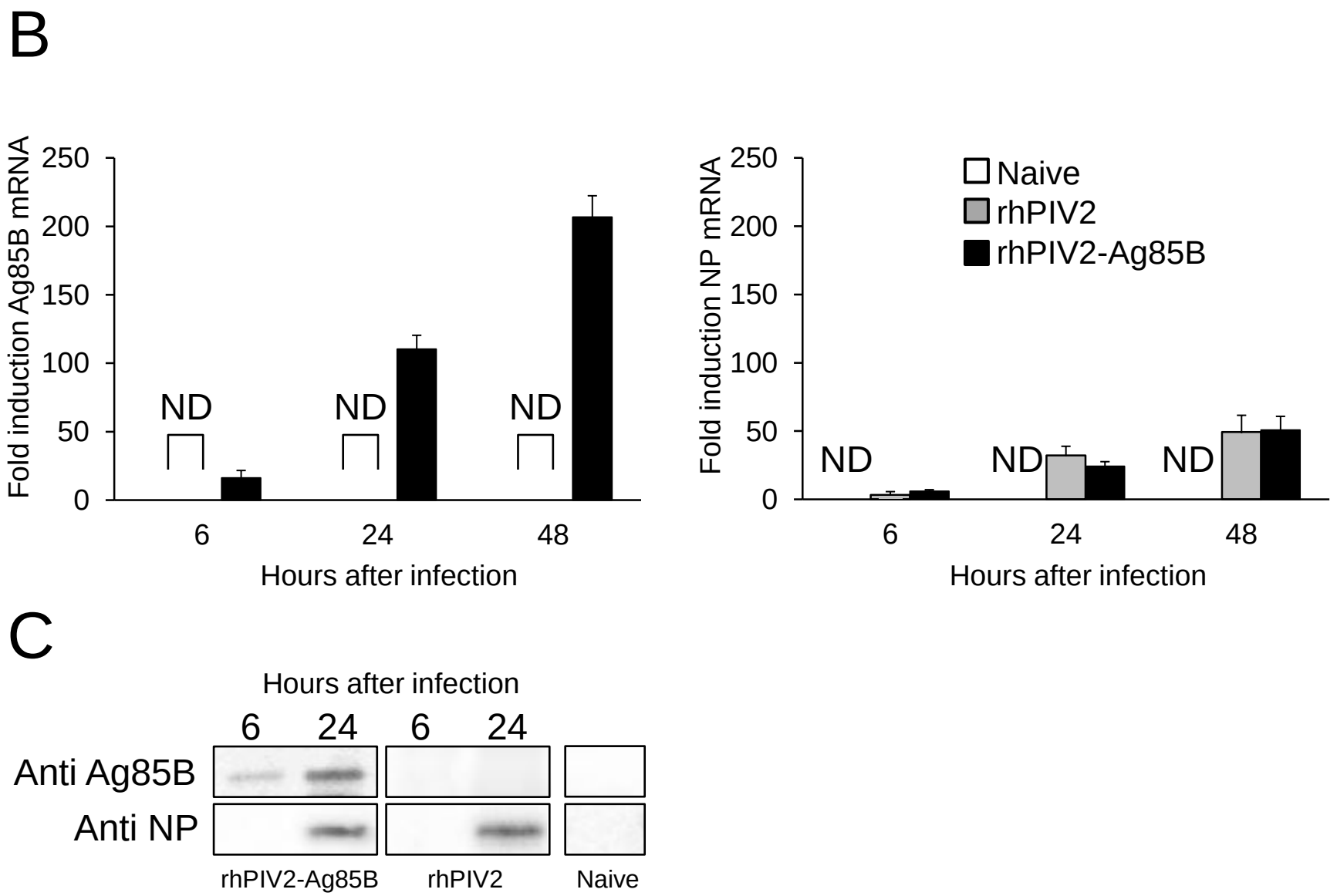

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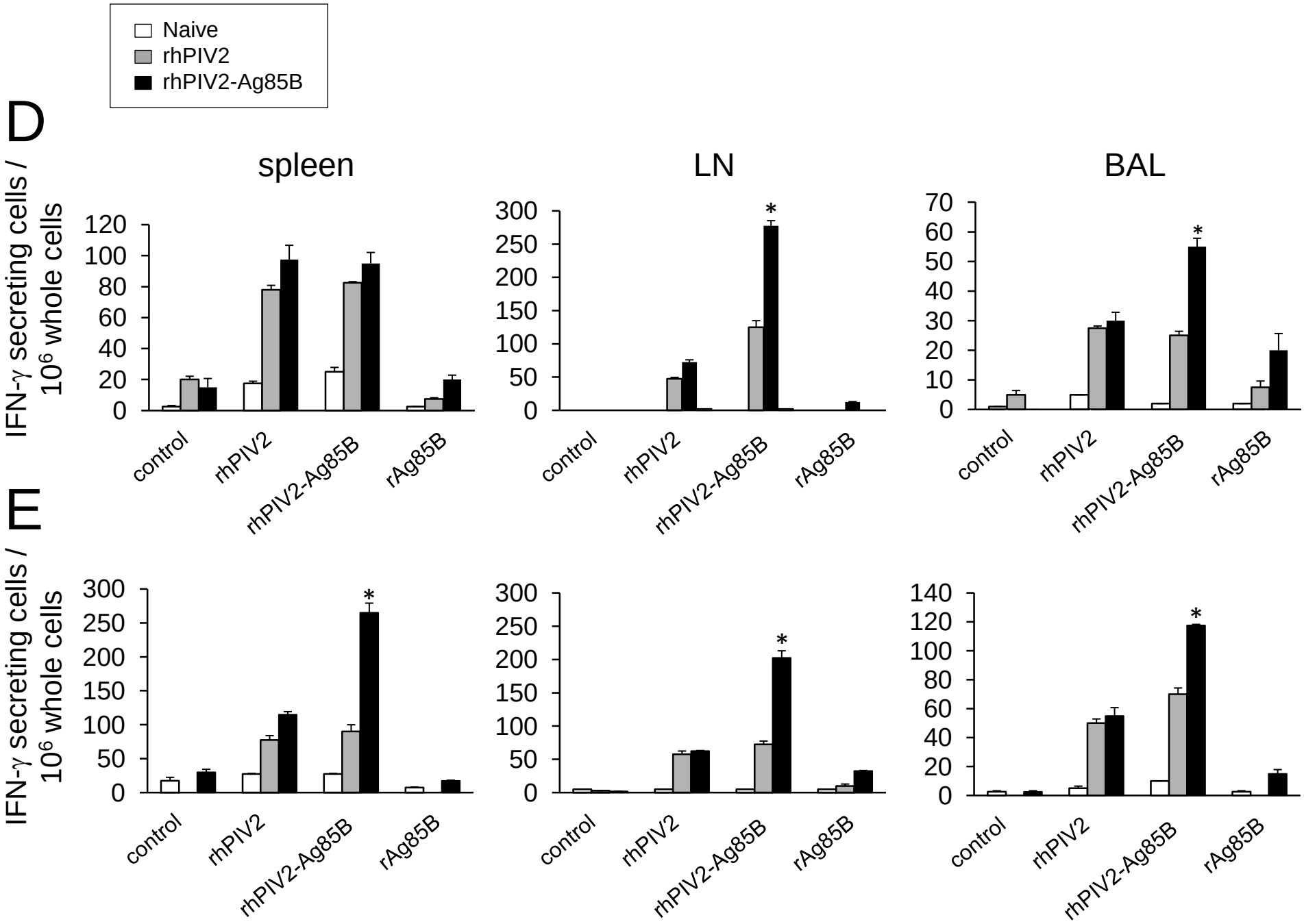

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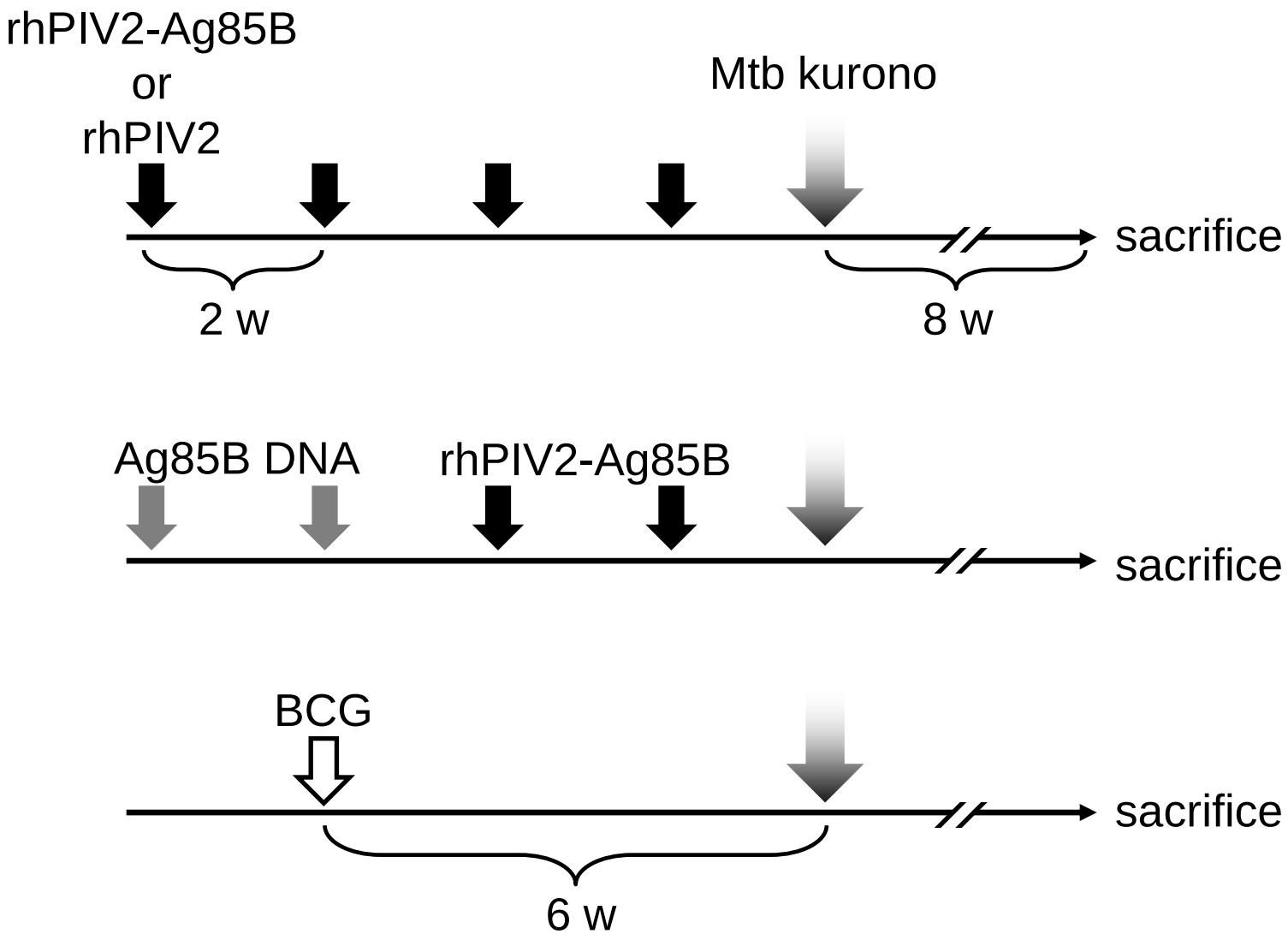

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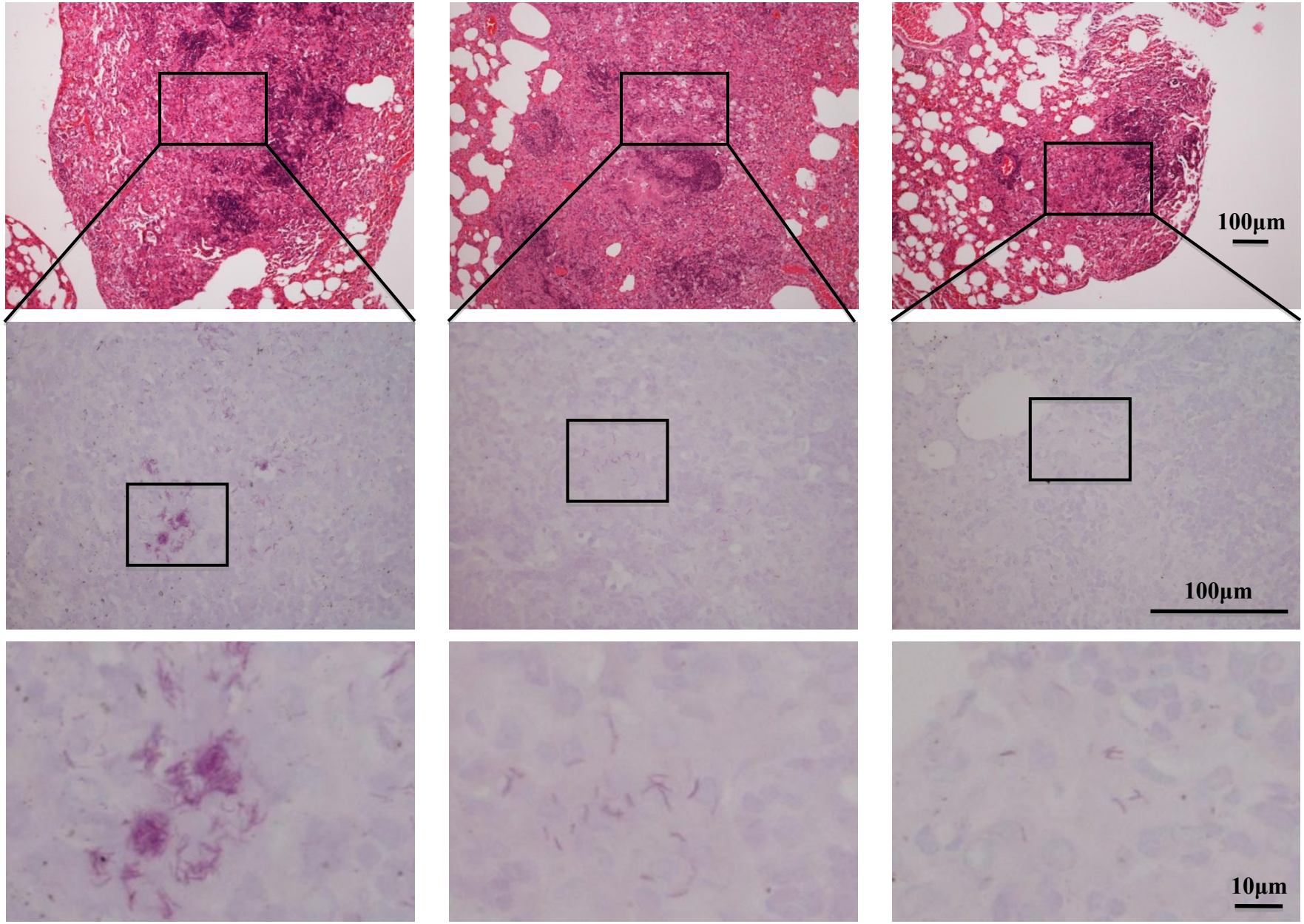

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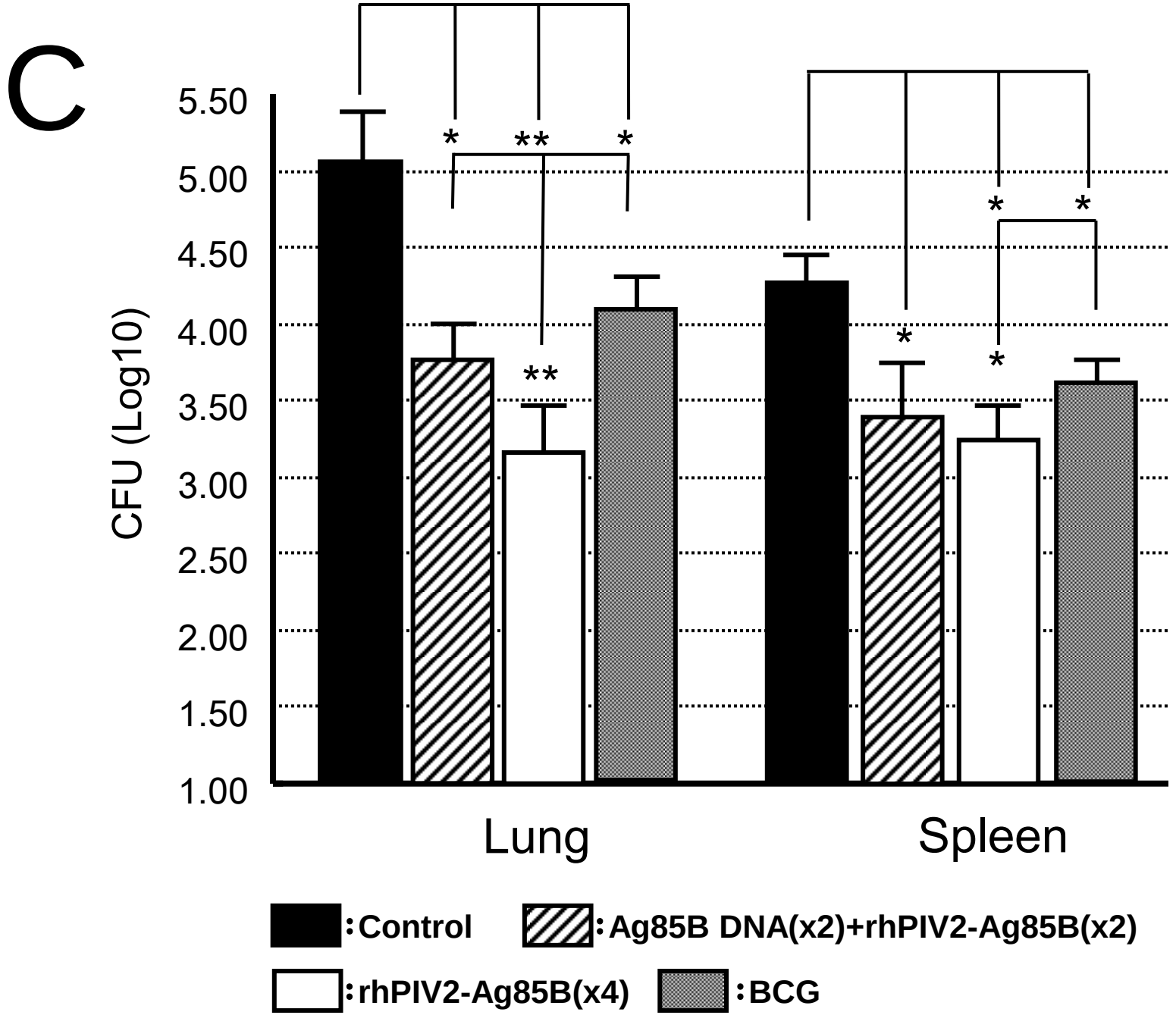

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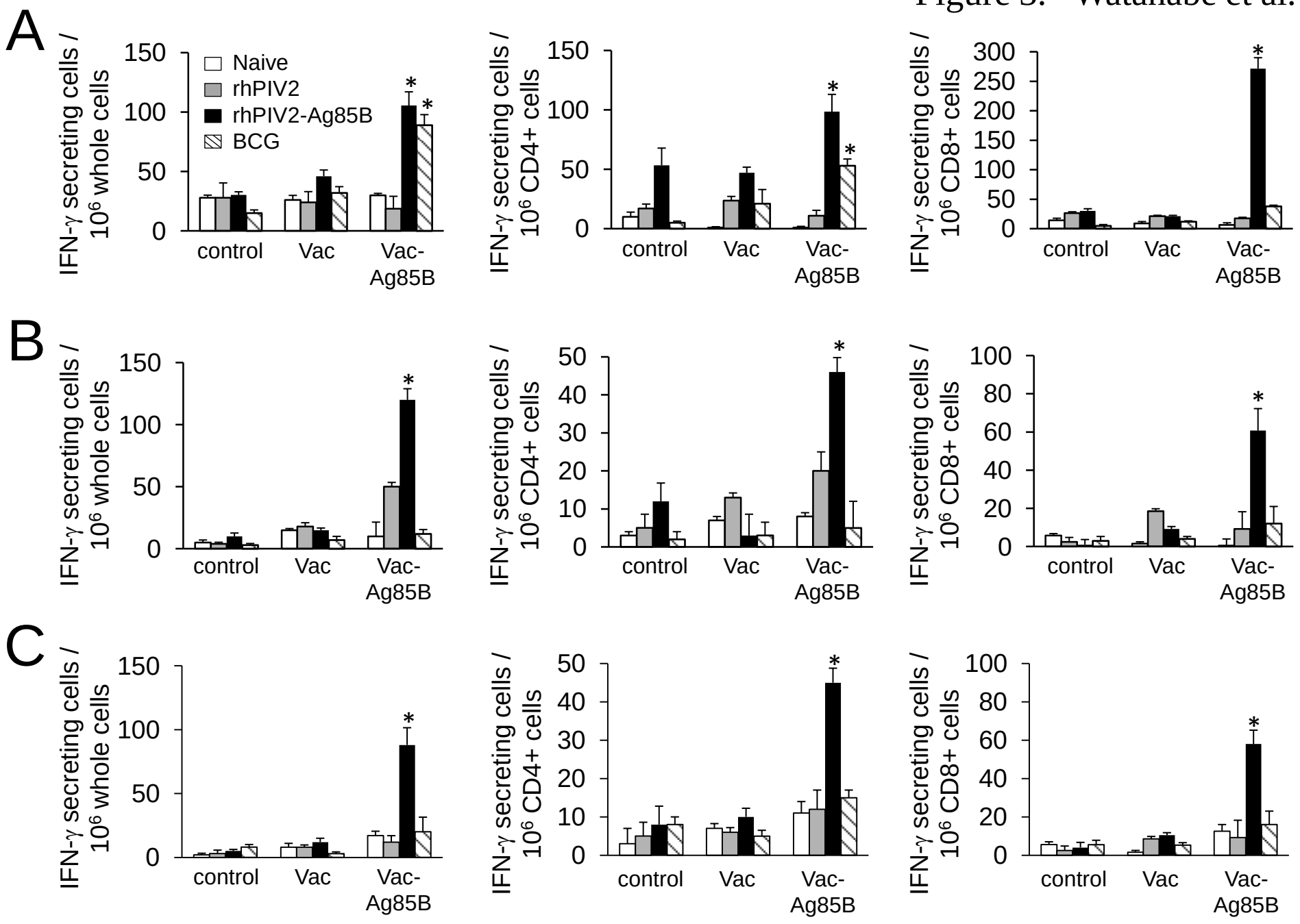

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A

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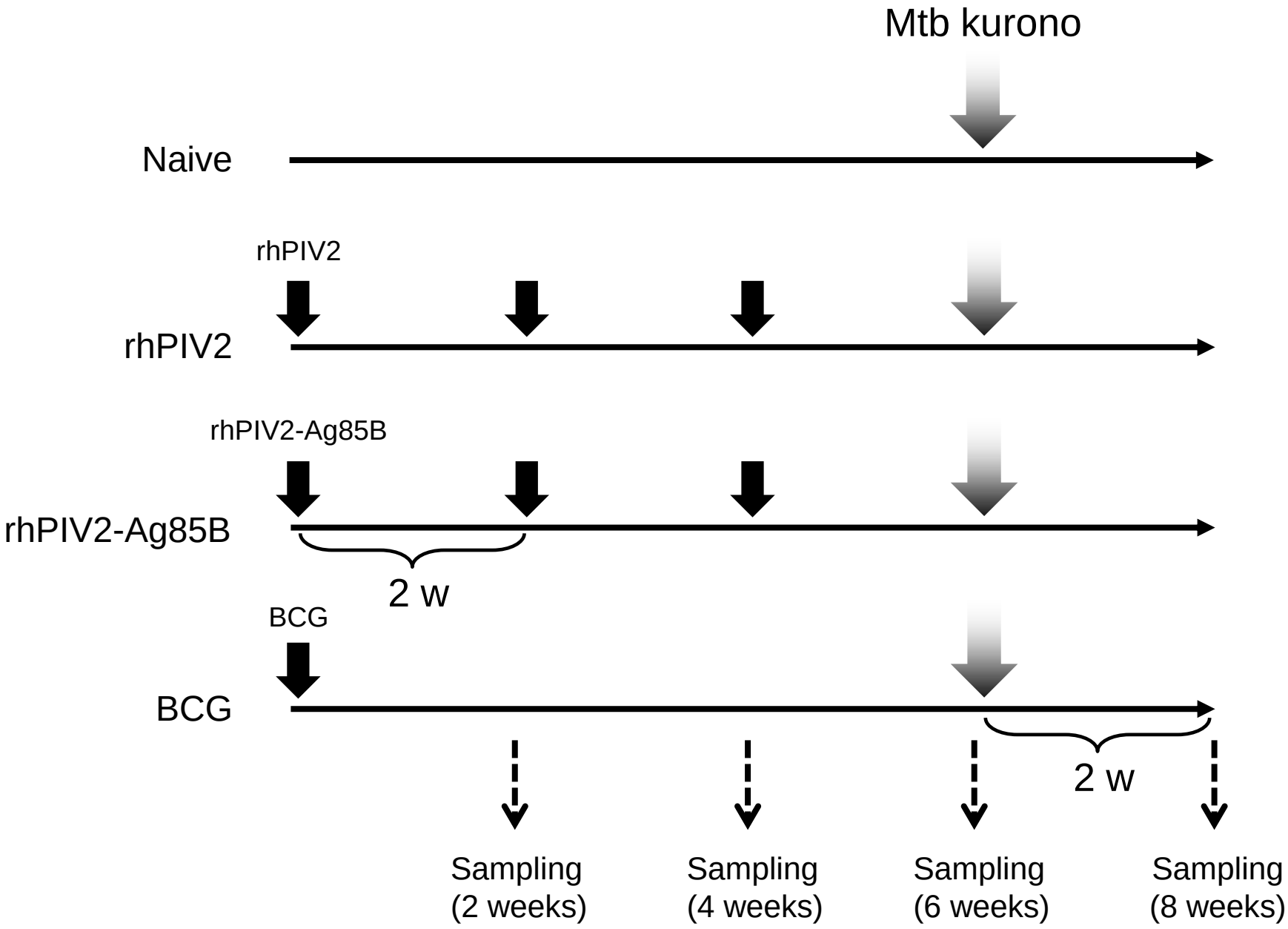

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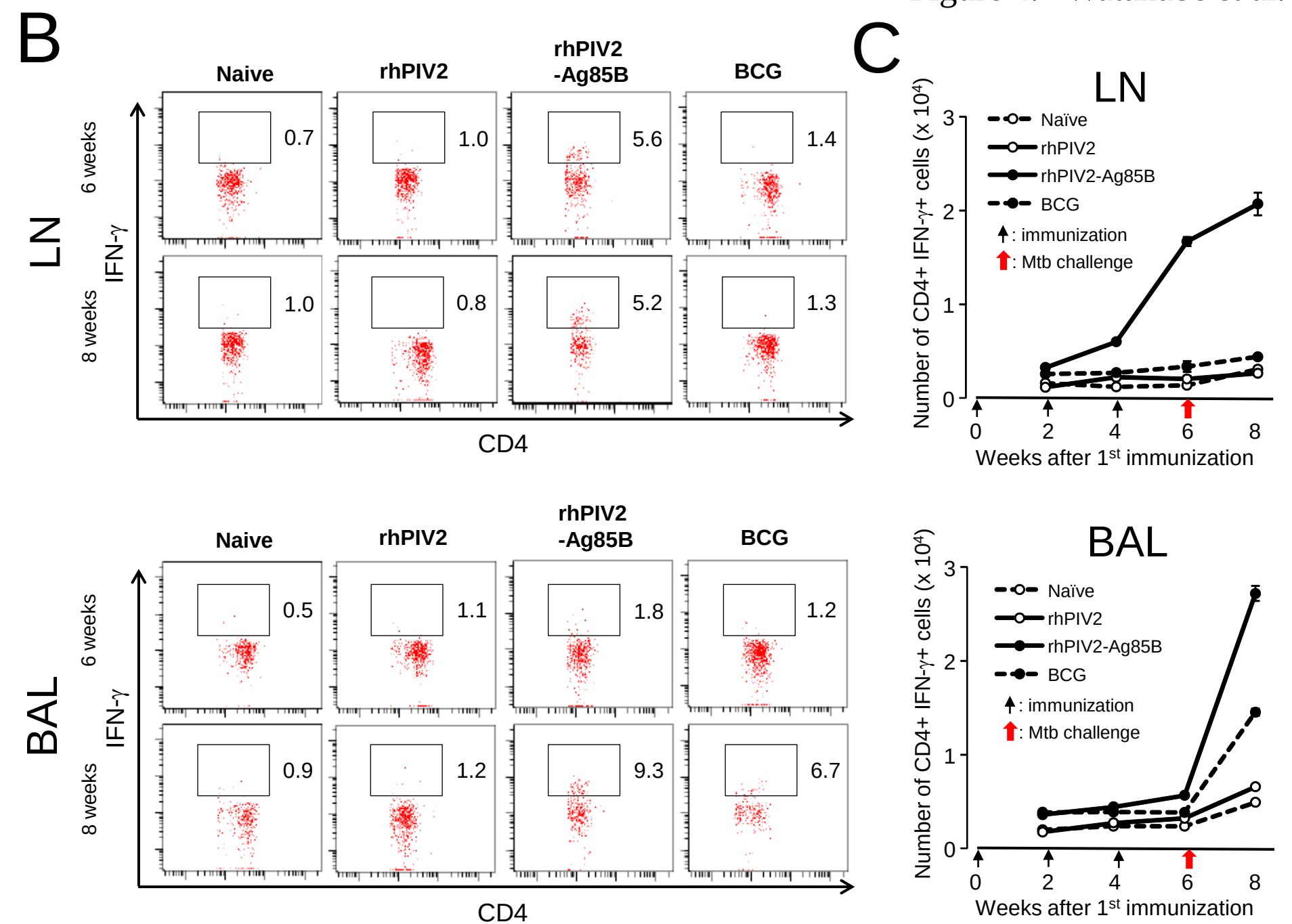

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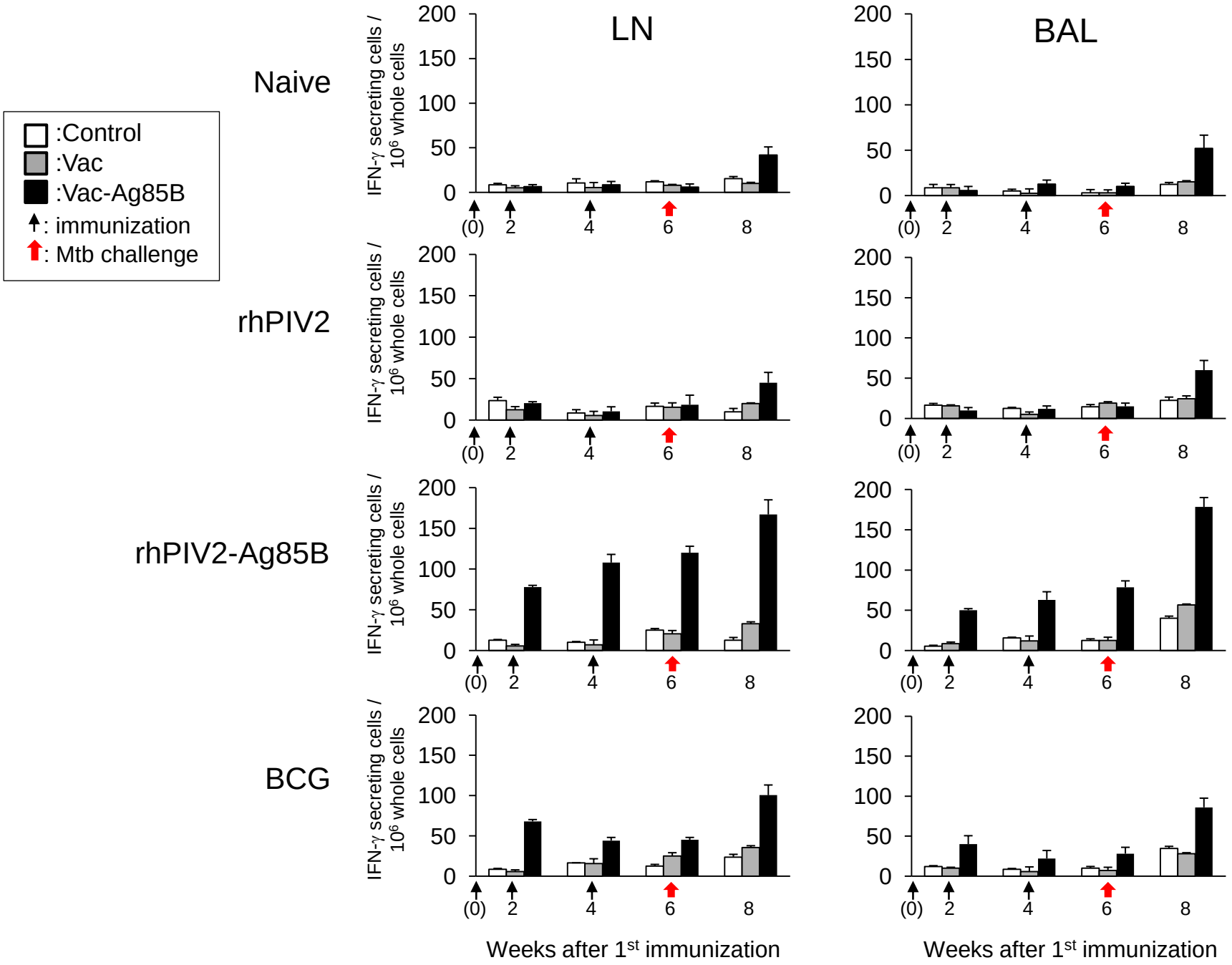

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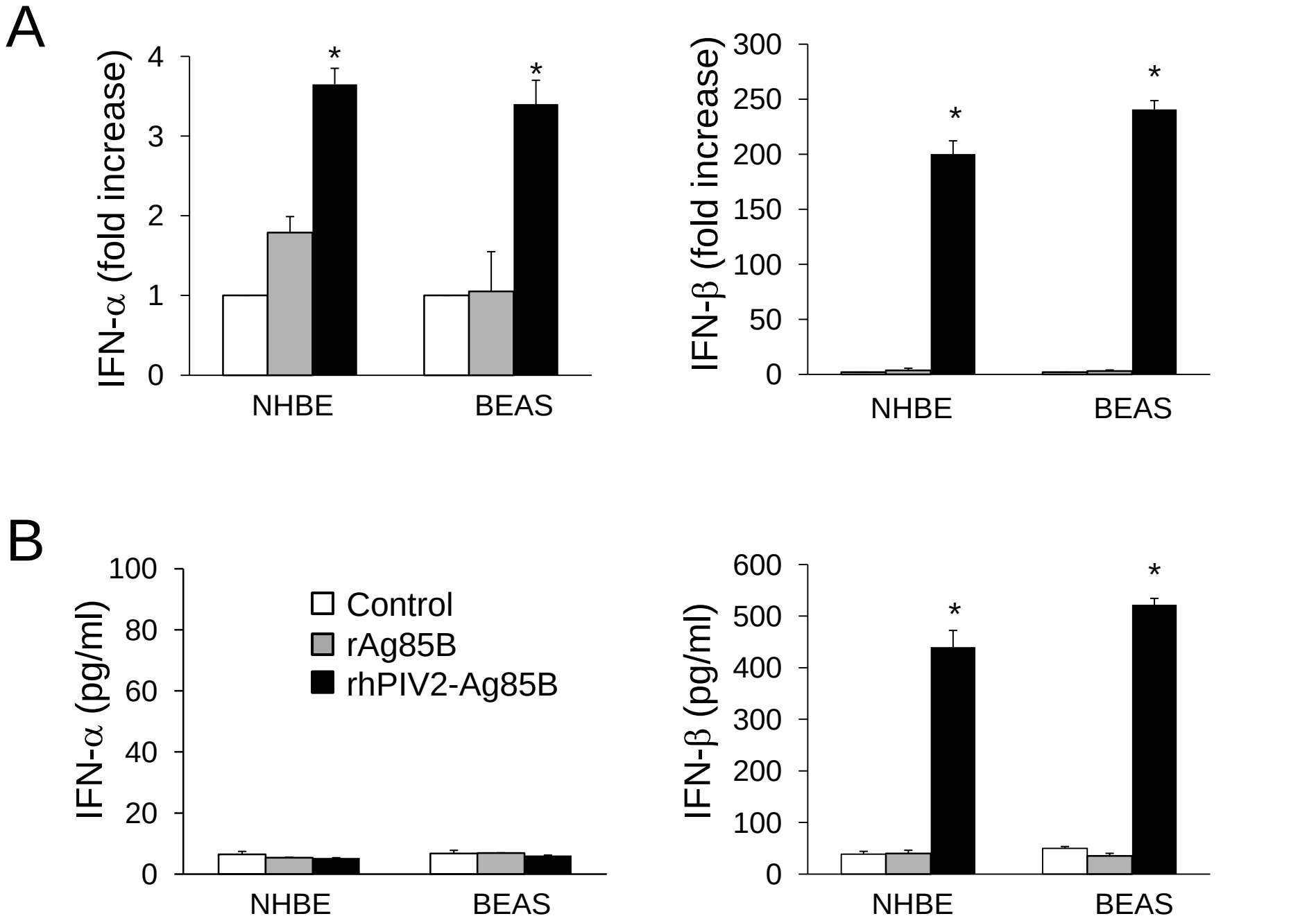

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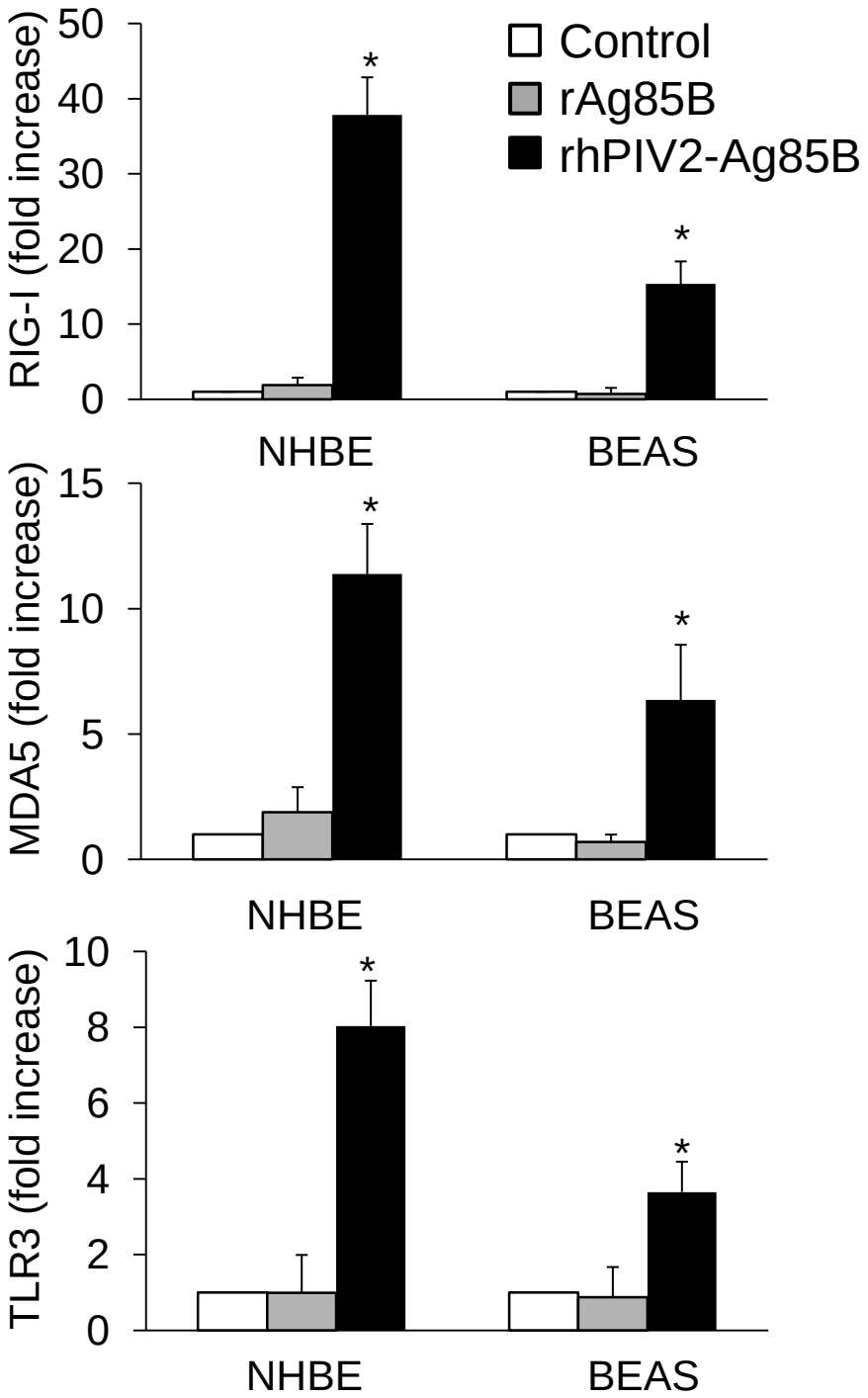

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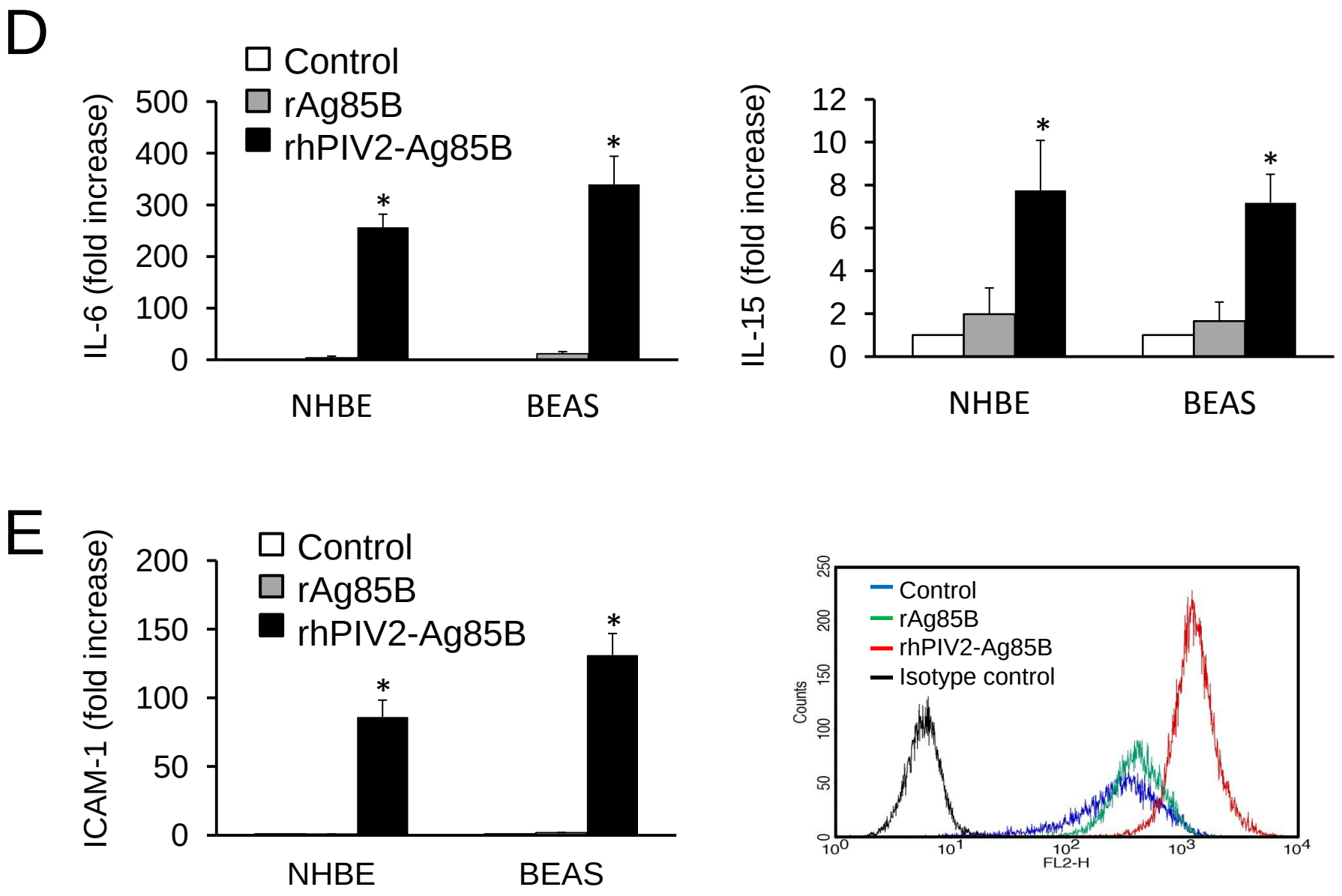

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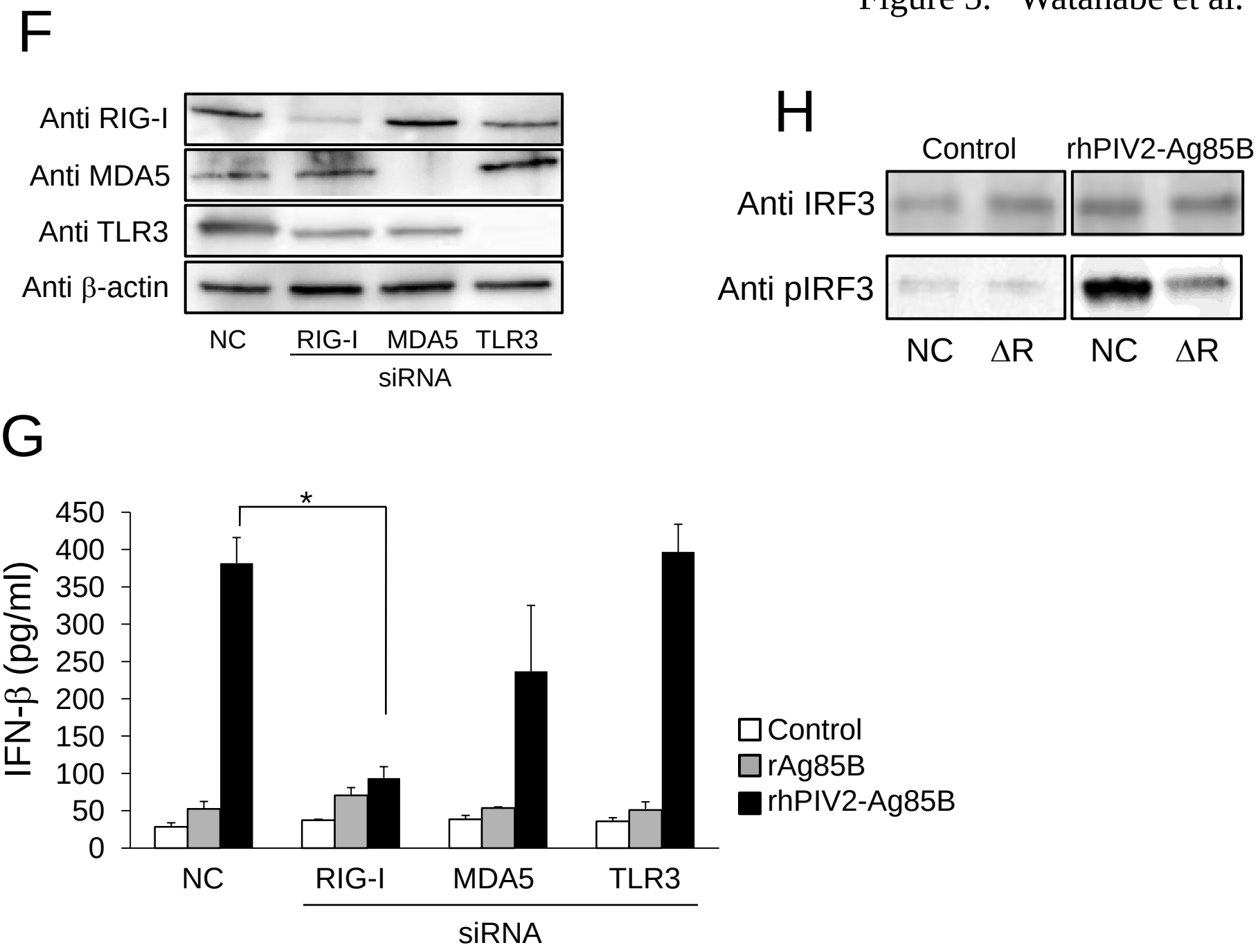
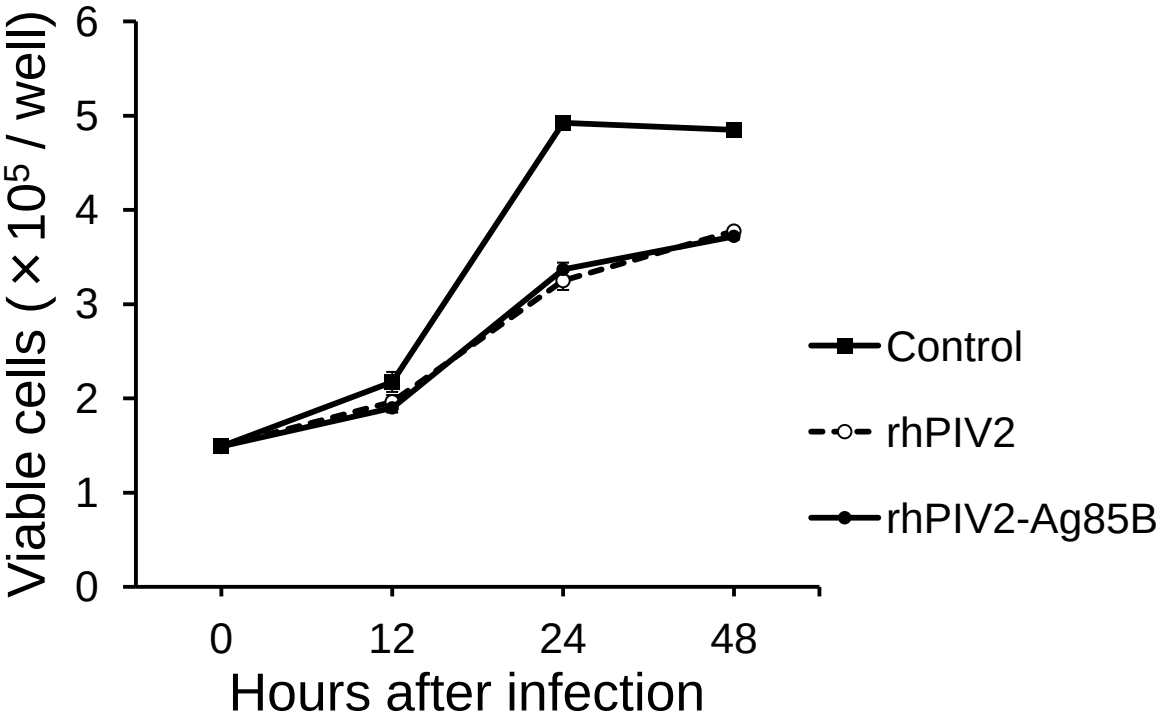
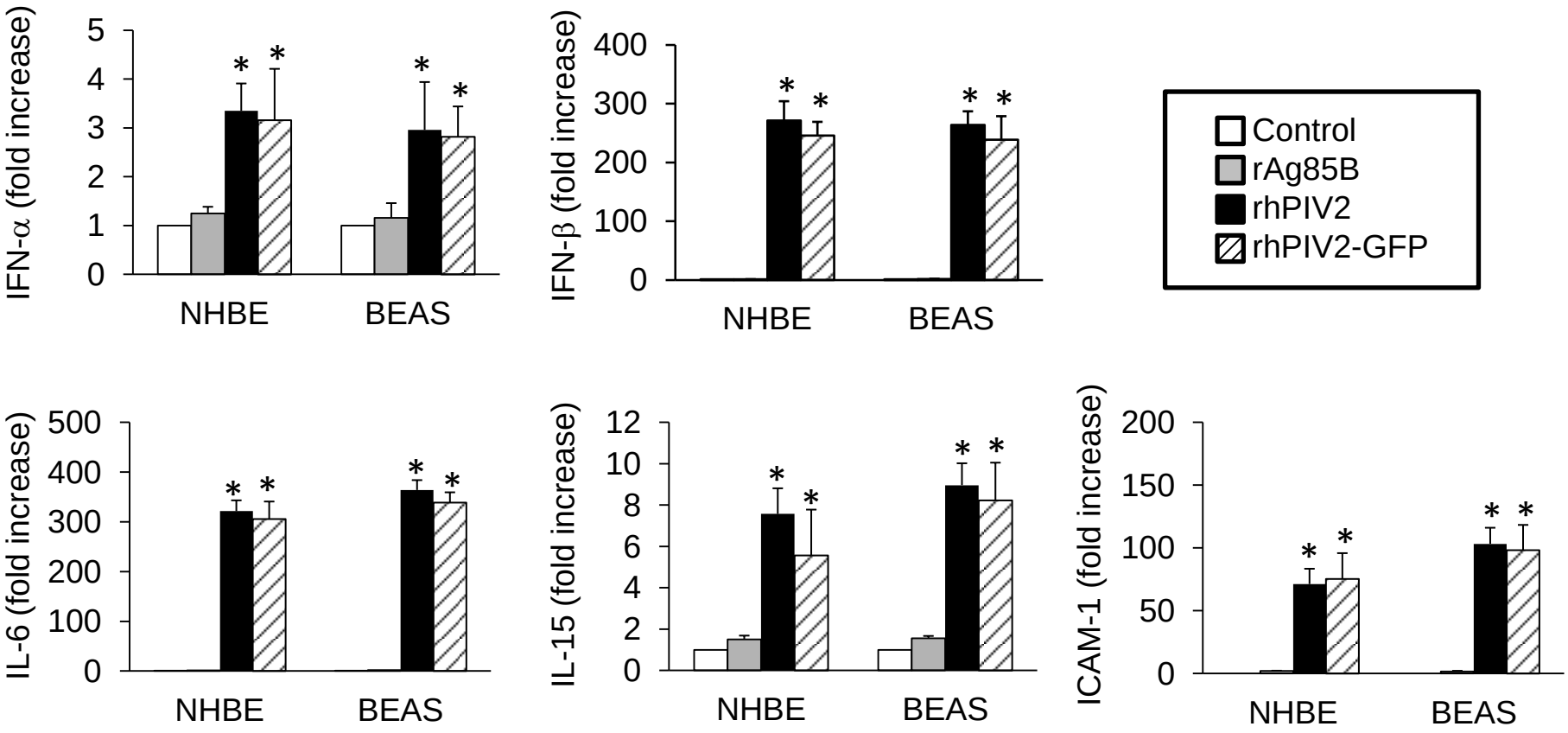




Supplemental figure 1. ***Expression of Ag85B has no toxic effect in BEAS cells infected with rhPIV2-Ag85B.***

BEAS cells were cultured in 12 wells plate, and infected with rhPIV2 or rhPIV2-Ag85B (MOI of 10). Number of viable cells were determined at each time points. Data are averages of triplicate samples from three identical experiments, and error bars represent standard deviations.



Supplemental figure 2. ***rhPIV2 vector has a potent adjuvant activity.***
NHBE and BEAS cells were treated with rAg85B protein (10 μg/ml) or infected with rhPIV2 or rhPIV2 expressing GFP (rhPIV2-GFP) (MOI of 10) for 24 h, and the increases in mRNA levels of IFN-α, IFN-β, IL-6, IL-15, and ICAM-1 were determined by real-time PCR. Fold increase of each target gene was normalized to β-actin, and the expression levels are represented as relative values to the control. Data are averages of triplicate samples from three identical experiments, and error bars represent standard deviations. Statistically significant differences are indicated by asterisks (*, $P < 0.01$ compared to Control).