

# Effects of Mycobacteria Major Secretion Protein, Ag85B, on Allergic Inflammation in the Lung

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## Abstract

Many epidemiological studies have suggested that the recent increase in prevalence and severity of allergic diseases such as asthma is inversely correlated with *Mycobacterium bovis* bacillus Calmette Guerin (BCG) vaccination. However, the underlying mechanisms by which mycobacterial components suppress allergic diseases are not yet fully understood. Here we showed the inhibitory mechanisms for development of allergic airway inflammation by using highly purified recombinant Ag85B (rAg85B), which is one of the major protein antigens secreted from *M. tuberculosis*. Ag85B is thought to be a single immunogenic protein that can elicit a strong Th1-type immune response in hosts infected with mycobacteria, including individuals vaccinated with BCG. Administration of rAg85B showed a strong inhibitory effect on the development of allergic airway inflammation with induction of Th1-response and IL-17 and IL-22 production. Both cytokines induced by rAg85B were involved in the induction of Th17-related cytokine-production innate immune cells in the lung. Administration of neutralizing antibodies to IL-17 or IL-22 in rAg85B-treated mice revealed that IL-17 induced the infiltration of neutrophils in BAL fluid and that allergen-induced bronchial eosinophilia was inhibited by IL-22. Furthermore, enhancement of the expression of genes associated with tissue homeostasis and wound healing was observed in bronchial tissues after rAg85B administration in a Th17-related cytokine dependent manner. The results of this study provide evidence for the potential usefulness of rAg85B as a novel approach for anti-allergic effect and tissue repair other than the role as a conventional TB vaccine.

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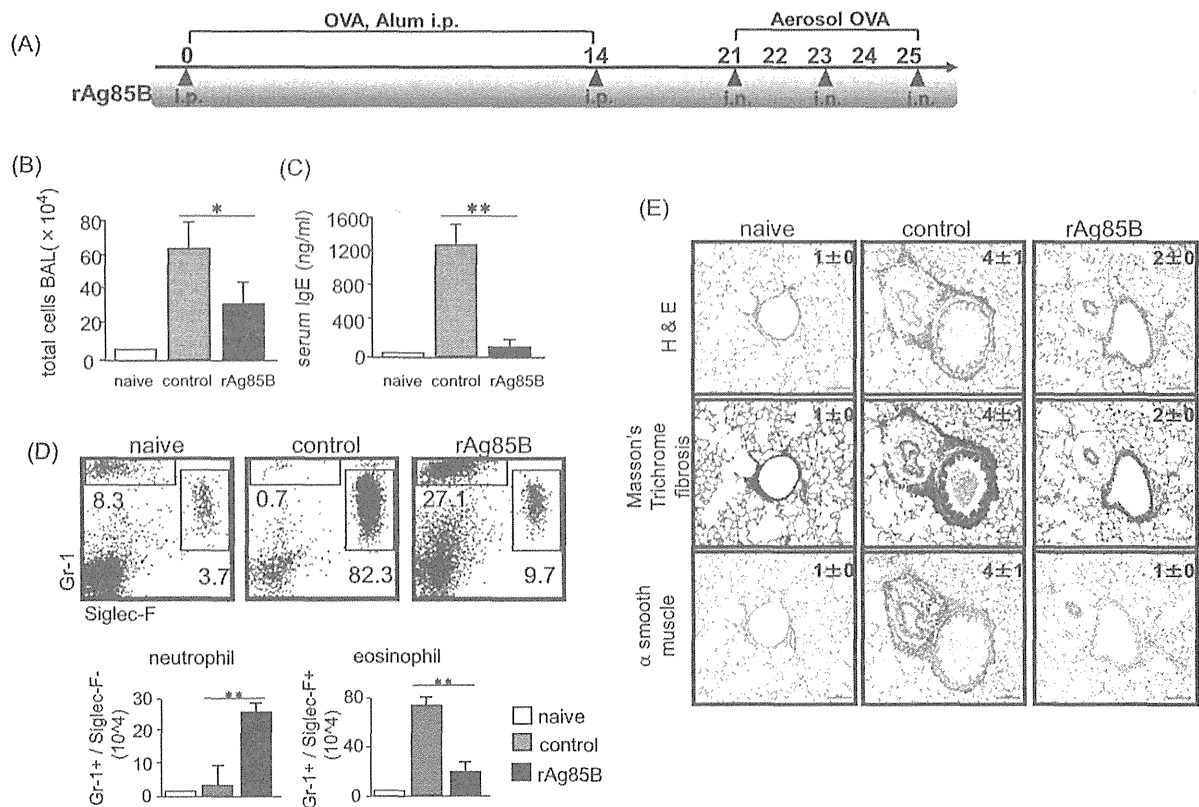
## Introduction

Epidemiological studies showed that treatments with bacterial and viral products might be effective therapeutic strategies for suppressing the development of allergic responses [1–3]. Administration of mycobacteria, including *Mycobacterium bovis*-Bacillus Calmette Guerin (BCG), has been thought to be effective for preventing the development of asthma by induction of Th1-type immune responses [4], regulatory T (Treg) cells [5,6] and NKT cells [7,8]. On the other hand, recent data have revealed that *Mycobacterium tuberculosis* infection induced not only IFN- $\gamma$  but also IL-17, which promotes granuloma organization followed by neutrophil recruitment, and IL-22, which promotes regeneration and protects against tissue damage [9]. In addition, vaccination with the mycobacteria-secreted immunogenic protein Ag85A had important links with Th1/Th17 cell induction and Treg cell

reduction [10]. However, the role of mycobacteria-mediated Th17-related cytokines in allergic asthma remains unknown.

The airway epithelium and innate immune cells are considered to be essential controllers of inflammatory, immune and regenerative responses to allergens that contribute to asthma pathogenesis [11]. Dysfunction of the epithelium leading to chronic injury was suggested to be a consequence of sustained airway inflammation that is associated with Th2-driven adaptive immunity [12]. Tissue homeostasis at exposed surfaces of the lung is regulated by Th17-related cytokines, especially IL-22, in the innate immune system [13]. Therefore, the functional and structural maintenance of tissue might be necessary to induce both innate and adaptive immunity.

One immunogenic protein that can induce a strong Th1-type immune response in hosts sensitized by BCG is thought to be Ag85B. Ag85B is one of the most dominant protein antigens secreted from all mycobacterial species and has been shown to



**Figure 1. Functions of rAg85B in allergic inflammation.** Experimental design used to investigate the effects of rAg85B on OVA-induced allergic lung inflammation (A). BALB/c mice were intraperitoneally immunized with OVA on days 0 and 14. On days 21 to 25 after the first immunization, mice were exposed to aerosolized 5% OVA for 20 min. Three hours prior to OVA inhalation, the mice were i.p. (100  $\mu$ g; days 0 and 14) and i.n. (20  $\mu$ g; days 21, 23, and 25) administered rAg85B. One day after the last challenge, the BAL cells were counted (B) and OVA-specific serum IgE concentrations were determined by ELISA (C). Flow cytometry of BAL cells from naïve or OVA sensitized BALB/c mice treated with PBS or rAg85B, stained with anti-Gr-1 and anti-Siglec-F. Numbers adjacent to outlined area indicate percent of eosinophils (Gr-1<sup>dim</sup>, Siglec-F<sup>+</sup>), and neutrophils (Gr-1<sup>+</sup>, Siglec-F<sup>neg</sup>) (D). Formalin-fixed tissue sections were stained with hematoxylin and eosin to visualize cell recruitment (upper row, scale bar, 100  $\mu$ m), Masson's trichrome (center row, scale bar, 100  $\mu$ m), and  $\alpha$ -smooth muscle actin (lower row, scale bar, 50  $\mu$ m). Numbers in quadrants indicate the score scale from 0 to 5 in each (E). Data are representative of at least three independent experiments. (\*P<0.05, \*\*P<0.01 compared with OVA control. error bars, s.d.; n=6 mice).

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induce substantial Th cell proliferation and vigorous Th1 cytokine production in humans and mice [14]. In addition, we have reported the possibility of using Ag85B DNA as an immunological strategic tool to induce both Th1 and Treg cells in immunotherapy for atopic dermatitis and allergic asthma [15,16].

In the present study, we found that highly purified recombinant Ag85B protein (rAg85B) had suppressive effects depending on induction of Th1 immune responses in a mouse model of allergic lung inflammation. Remarkably, rAg85B administration also promoted IL-17 and IL-22 production in both Th17 cells in lymph nodes (LNs) and various innate immune cells such as gamma delta T ( $\gamma\delta$ T) cells, Nkp46<sup>+</sup> cells, lymphoid tissue inducer (LTi)-like cells, and CD11c<sup>+</sup> cells in BAL fluid. More interestingly, Th17-related cytokines induced by rAg85B were involved in enhancement of the expression of genes related to maintenance of tissue homeostasis. This is the first report demonstrating that mycobacteria major secretory protein Ag85B plays an important role in the regulation of allergic airway inflammation by inducing not only a Th1-response but also recruitment of an IL-17 and/or IL-22-producing Th cell subset in LNs and innate immune BAL

cells in a manner dependent on Th17-related cytokines in order to retain tissue integrity.

## Materials and Methods

### Animal and Ethic Statement

Specific pathogen-free BALB/c mice (six-week-old, female) were purchased from CLEA Japan. All of the experiments in this study were performed in accordance with the Guidelines for Animal Use and Experimentation, as set out by the National Institute of Biomedical Innovation. The protocol was approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation (Permit Number: DS23-8R2). All animal procedures were used to minimize animal pain and suffering.

### Experimental protocol

BALB/c mice were intraperitoneally immunized with 10  $\mu$ g ovalbumin (OVA) with 1 mg aluminum hydroxide on days 0 and 14. On days 21 to 25 after the first immunization, mice were exposed to aerosolized 5% OVA for 20 min. Three hours prior to

OVA inhalation, the mice were intraperitoneally (i.p.) (100 µg; days 0 and 14) and intranasally (i.n.) (20 µg; days 21, 23, and 25) administered rAg85B. OVA-sensitized Balb/c mice were challenged intranasally with PBS, rAg85B, rAg85B plus 5 µg anti-IL-17 Abs and/or 10 µg anti-IL-22 Abs (R&D Systems) with the same time course as that of rAg85B i.n. administration. The isotype-matched control antibody for neutralization experiments was set using normal goat IgG control (R&D systems).

### Recombinant protein Ag85B production

Plasmids containing the Ag85B gene were transformed into *E. coli* TG1. The expressed inclusion body (IB) was harvested from the disrupted cell pellet by a homogenizer with lysis buffer (30 mM sodium phosphatase, 100 mM NaCl, 5 mM EDTA and 0.5% Triton X-100). This IB of Ag85B was unfolded in 8 M urea and refolded by dilution to 0.4 M urea. The urea in the refolding buffer was removed by anion exchange chromatography using 20 mM Tris buffer and 20 mM Tris buffer with 1 M NaCl (pH 8.5). The refolded Ag85B was loaded on a cation exchange column, and crude Ag85B was passed through the resin using 50 mM NaOAc buffer and 50 mM NaOAc buffer with 1 M NaCl (pH 6.0). Finally, Ag85B was purified by anion exchange chromatography using 20 mM Tris buffer and 20 mM Tris buffer with 1 M NaCl (pH 7.6).

### Endotoxin test

The endotoxin value of Ag85B was measured by Kinetic turbidimetric LAL assay kit (Lonza). Test was carried out according to the manufacturer's instruction. The endotoxin value was measured kinetically on ELISA after mixing sample and LAL reagent and was calculated automatically according to standard curve. Purified Ag85B had a purity of >95% analyzed by SDS-PAGE and contaminated less than 0.02 EU/mg of endotoxin. Protein quantitation was carried out by UV spectroscopy at 280 nm.

### Isolation and analysis of lymph node and BAL cells

BAL cells were prepared according to a published protocol [16]. Single cell suspensions from BAL fluid and mediastinal lymph nodes (MLNs) were obtained by crushing through cell strainers. Cells were stained with antibodies to the following markers: CD3, CD4, CD8, CD19, CD11b, CD11c, CD25, γδ TCR, NKp46, Gr-1, Siglec-F, CD127, IFN-γ, IL-4, Foxp3, IL-17 and IL-22 (BD).

For analysis of intracellular cytokine production, cells were stimulated directly by incubation for 5 h with 50 ng/ml PMA and 750 ng/ml ionomycin (Sigma-Aldrich) at 37°C and with 10 µg/ml brefeldin A (eBioscience) added in the last 3 h. Flow cytometry data collection was performed on a FACS Calibur (BD). Files were analyzed using CellQuest Software (BD).

### Quantification of cytokines and chemokines

Concentrations of cytokines and chemokines in BAL fluid and culture supernatants of OVA-restimulated lymph node cells were determined by ELISA using commercial kits from R&D Systems. Twenty-four hours after the last OVA sensitization, MLNs and BAL fluid were harvested. MLNs were cultured with 50 µg/ml OVA, and cytokines in the culture supernatant were determined 48 h after incubation. The BAL fluid were measured directly.

### Lung histology

The organs were removed and placed in 4% buffered paraformaldehyde (PFA) overnight. Excess paraformaldehyde was removed by incubation in fresh PBS. Fixed tissues were incubated at 4°C in 70% ethanol. PFA-fixed lung sections were stained with hematoxylin and eosin, Masson's trichrome, and α-smooth muscle actin. Peribronchial infiltrates, fibrosis, and smooth muscle hyperplasia were assessed by a semiquantitative score (0–5) by a pathologist.

### Quantitative real-time PCR

RNA was isolated from whole lung tissue using mechanical homogenization and TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentrations were measured with a Nanodrop ND 1000 (Nucliber). Omniscript reverse transcriptase was used according to the protocol of the manufacturer (QIAGEN) for the production of cDNA in a reaction volume of 20 µl. Primers for quantitative real-time RT-PCR were designed with the Universal ProbeLibrary Assay Design Center (Roche Applied Science). Reactions were run on an RT-PCR system (LightCycler 480; Roche Applied Science) Samples were normalized to b-actin and displayed as fold induction over naïve or untreated controls unless otherwise stated.

### TLR/NLR ligand screening

The presence of TLR and NLR ligands were tested on recombinant human embryonic kidney 293 (HEK293) cell lines

**Table 1.** Effects of rAg85B to Toll-Like and NOD-Like Receptor.

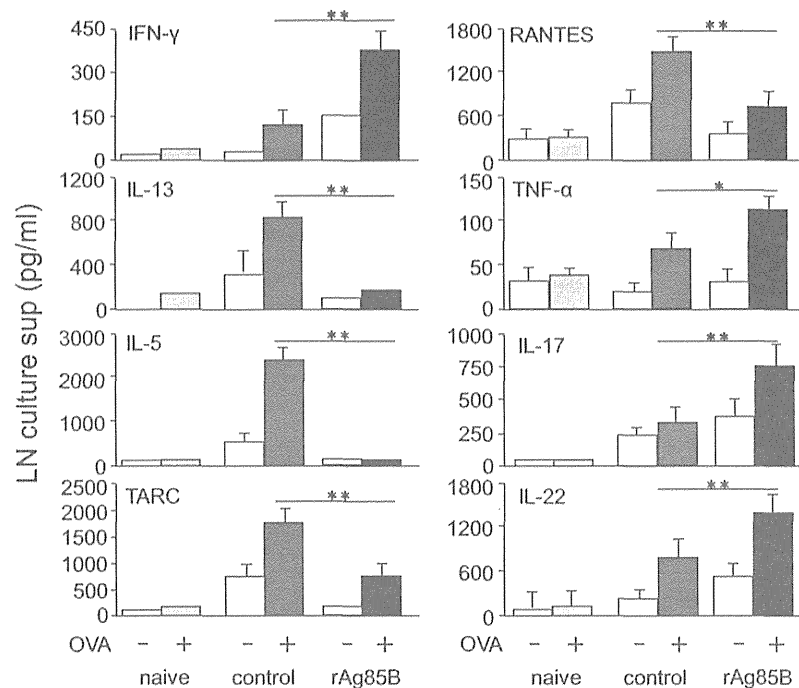
receptor	No ligand	rAg85B	control (+)
mTLR2	0.1±0.0	0.1±0.0	2.3±0.1
mTLR3	0.1±0.0	0.1±0.0	2.4±0.2
mTLR4	0.1±0.0	0.1±0.0	2.7±0.1
mTLR5	0.1±0.0	0.1±0.0	2.7±0.1
mTLR7	0.1±0.0	0.1±0.0	2.1±0.0
mTLR8	0.1±0.0	0.1±0.0	2.3±0.1
mTLR9	0.1±0.0	0.1±0.0	2.6±0.1
mNOD1	0.1±0.0	0.1±0.0	1.7±0.1
mNOD2	0.2±0.0	0.1±0.0	1.6±0.1

The results are provided as optical density values (650 nm).

The values represent the means and standard deviations of three screenings.

TLR/NLR ligand screening were performed by InvivoGen, as described in Methods.

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**Figure 2. Administration of rAg85B induced immune deviation from a Th2-response towards a Th1, Th17-related response in OVA-stimulated LN cells.** OVA-immunized (i.p., day 0 and 14) and sensitized (5% aerosolized-OVA, day 21 to 25) BALB/c mice were challenged with PBS or rAg85B protein (i.p. (100  $\mu$ g; days 0 and 14) and i.n. (20  $\mu$ g; days 21, 23, and 25)). At 24 h after the last OVA sensitization, mediastinal lymph nodes (MLNs) from naive or OVA sensitized BALB/c mice treated with PBS or rAg85B, were harvested. MLNs were cultured with OVA (50  $\mu$ g/ml), and cytokines in the culture supernatant were determined 48 h after incubation by ELISA. Data are representative of at least three independent experiments (\*P < 0.05, \*\*P < 0.01 compared with OVA control. error bars, s.d.; n = 6 mice). doi:10.1371/journal.pone.0106807.g002

which utilize a nuclear factor- $\kappa$ B inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene as the read-out. These HEK293-derived cells are functionally expressing a given TLR or NOD gene from human or mouse. A recombinant HEK293 cell line for the reporter gene only was used as negative control. Positive control ligands are heat-killed *Listeria monocytogenes* (HKLM) for TLR2, Poly(I:C) for TLR3, Lipopolysaccharide (LPS); K12 for TLR4, Flagellin for TLR5, CL097 for TLR7, CL075 and poly(dT) for TLR8, CpG ODN for TLR9, C12-iEDAP for NOD1, and L18-MDP for NOD2. rAg85B (10  $\mu$ g/mL) was added to the reaction volume. TLR/NLR ligand screening were performed by InvivoGen.

#### Statistical analysis

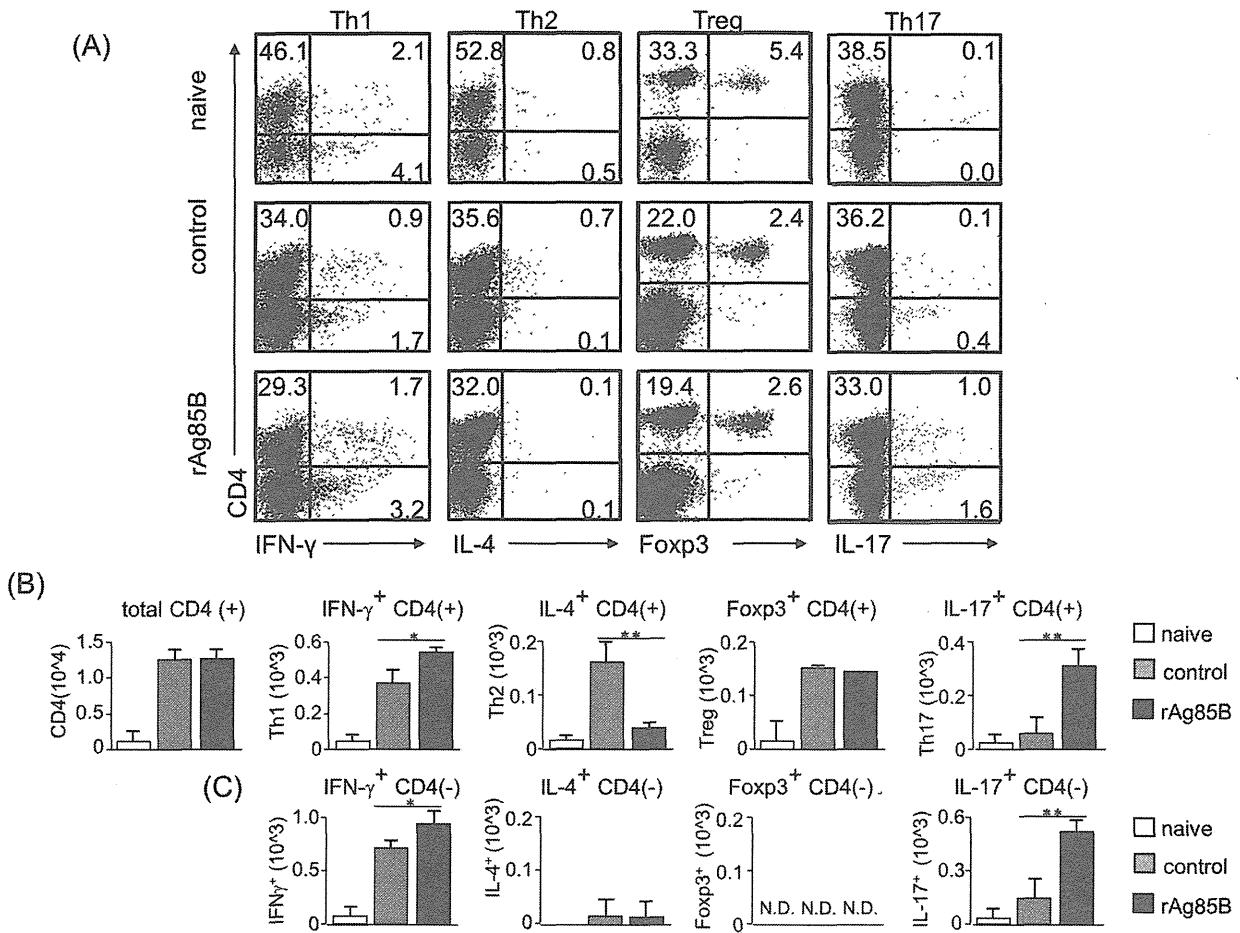
Data are shown as means  $\pm$  SD. Statistical significance of differences between the OVA-control group and rAg85B-treated group was assessed by the non-parametric Mann-Whitney U-test. Statistical comparisons between groups of rAg85B+isotype control and rAg85B+neutralization antibody were performed using the non-parametric Kruskal-Wallis H-test.

## Results

### Effects on allergic inflammation by administration of rAg85B

To investigate the role of rAg85B in pulmonary allergic inflammation, we examined the frequently used mouse model of ovalbumin (OVA)-induced allergic lung inflammation. The mice were intraperitoneally (i.p.) (days 0 and 14) and intranasally (i.n.)

(days 21, 23, and 25) administered with rAg85B (Fig. 1A). The purity of rAg85B was evaluated by silver staining of SDS-PAGE gel (Fig. S1) and the Limulus Amebocyte Lysate (LAL) assay (less than 0.02 EU (endotoxin units)/ml). Furthermore rAg85B was not contaminated with any TLR/NLR binding immune stimulants (Table 1). Twenty-four hours after the final OVA challenge, inflammatory cell recruitment into the lungs was analyzed. The OVA-induced allergic manifestation was suppressed with a decrease in the total number of bronchoalveolar lavage (BAL) cells and serum IgE level in the rAg85B-administered mice (Fig. 1B, 1C). A marked reduction in eosinophil (Gr-1(+)/Siglec-F(+)) infiltration was observed by flow cytometric (FACS) analysis of BAL in rAg85B-administered mice (Fig. 1D). In association with decreased eosinophilia, neutrophil (Gr-1(+)/Siglec-F(-)) recruitment was seen in rAg85B-administered mice (Fig. 1D). These results were confirmed by histopathological observation of hematoxylin and eosin (H&E) staining (Fig. 1E). Mice administered rAg85B showed inhibition of infiltration of cells (Fig. 1E). Lung sections were also stained with Masson's trichrome to evaluate fibrosis, and stained with  $\alpha$ -smooth muscle actin. Sizes of both the peribronchial smooth muscle area and lung fibrosis area were increased in OVA-sensitized control mice; however, mice administered rAg85B showed strong suppression of both fibrosis and  $\alpha$ -smooth muscle actin expression as well as reduction in inflammation severity assessed by H&E staining (Fig. 1E). These observations indicated that rAg85B has a critical function of regulating airway inflammation in a mouse model of allergen-induced asthma. Moreover, rAg85B i.n. administration induced wound repair including suppression of both fibrosis and  $\alpha$ -smooth



**Figure 3. IFN- $\gamma$  and IL-17-producing CD4 T cell subsets proliferated in lymph nodes after rAg85B administration.** OVA-immunized (i.p., day0 and 14) and sensitized (5% aerosolized-OVA, day21 to 25) BALB/c mice were challenged with PBS or rAg85B protein (i.p. (100  $\mu$ g; days 0 and 14) and i.n. (20  $\mu$ g; days 21, 23, and 25)). At 24 h after the last OVA sensitization, mediastinal lymph nodes (MLNs) from naive or OVA sensitized BALB/c mice treated with PBS or rAg85B, were harvested. MLNs were stimulated with ionomycin and PMA for 5 h, and with brefeldin A added in the last 3 h. Flow cytometry of stimulated MLNs from naive (upper), PBS-treated (middle) and rAg85B protein-treated (lower) OVA-sensitized mice stained with specific antibodies indicated marker. Numbers in quadrants indicate percent of cells in each (A). Absolute numbers of various cell populations (above graphs) in lymph nodes (B, C). Data are representative of three independent experiments (\*P<0.05, \*\*P<0.01 compared with OVA control. error bars, s.d.; n=6 mice). doi:10.1371/journal.pone.0106807.g003

muscle actin expression. Incidentally, previous data showed that either i.p. (days 0, 14) or i.n. (days 21, 23, and 25) challenge with rAg85B did not induce strong suppression of Th2-response in OVA-sensitized mice (data not shown).

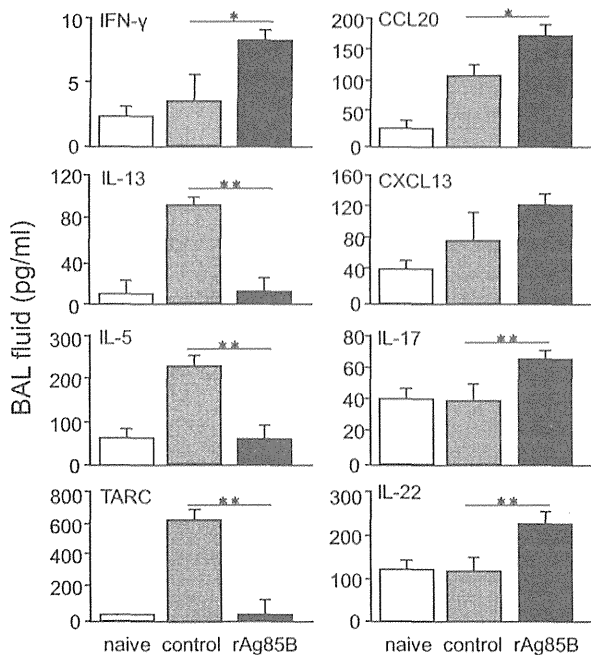
**Immune deviation from a Th2-response towards a Th1, Th17-related response by rAg85B administration**

We next assessed the production of OVA-specific cytokines in lymph node cells after *in vitro* stimulation with OVA (Fig. 2). Cells from mediastinal lymph nodes (mLNs) were stimulated *in vitro* with OVA and the production of various types of cytokines was assessed. The level of the Th1 cytokine IFN- $\gamma$  in culture supernatants of cells from rAg85B-administered mice was increased. On the other hand, the levels of Th2 cytokines IL-5 and IL-13 in culture supernatants of cells from rAg85B-administered mice were lower than those in culture supernatants of cells from control mice. Similarly, mice administered rAg85B showed inhibition of production of the CCL5 (RANTES) and the

thymus- and activation-regulated chemokine CCL17 (TARC), which contribute to allergic inflammation. Production of IL-17, IL-22 and TNF- $\alpha$  was also enhanced in culture supernatants of OVA-stimulated mLN cells from rAg85B-administered mice. These results suggested that Th1 and Th17 cytokines are crucial factors in the suppressive effect of rAg85B on airway inflammation.

**CD4<sup>+</sup> T cells producing IFN- $\gamma$  and IL-17 were increased in mediastinal lymph nodes by rAg85B administration**

We next examined Th cell responses in the mouse asthma model by intracellular staining analysis. mLN cells were stimulated with or without PMA and ionomycin, and cell fractions were analyzed by intracellular cytokine staining. Stained CD4<sup>+</sup> T cells producing IFN- $\gamma$  or IL-17 were increased in mice administered rAg85B, whereas IL-4-secreting cells were decreased in those mice (Fig. 3A, 3B). On the other hand, rAg85B administration was not associated with the induction of Treg cells, which express Foxp3



**Figure 4. Administration of rAg85B resulted in the reduction of Th2 cytokine and chemokine levels and in the enhancement of Th1 and Th17 cytokine levels in BAL fluid.** OVA-immunized (i.p., day0 and 14) and sensitized (5% aerosolized-OVA, day21 to 25) BALB/c mice were challenged with PBS or rAg85B protein (i.p. (100 µg; days 0 and 14) and i.n. (20 µg; days 21, 23, and 25)). At 24 h after the last OVA sensitization, BAL fluid from naïve or OVA sensitized BALB/c mice treated with PBS or rAg85B, were harvested. Levels of cytokines in the BAL fluid were measured directly by ELISA. Data are representative of at least three independent experiments (\* $P < 0.05$ , \*\* $P < 0.01$  compared with OVA control. error bars, s.d.;  $n = 6$  mice). doi:10.1371/journal.pone.0106807.g004

and CD25, in LNs (Fig. 3A, 3B, S2). These results were the same for not only the fraction of CD4<sup>+</sup> T cells but also the fraction of CD4<sup>-</sup> cells producing cytokines in mLNs (Fig. 3C). These results suggested that rAg85B administration was involved in the induction of IFN- $\gamma$  or IL-17-producing CD4<sup>+</sup> T cells and CD4<sup>-</sup> cells in LNs.

#### Mice administered rAg85B showed reduction in levels of Th2 cytokines and chemokines levels and increase in levels of Th1 and Th17 cytokines in BAL

The pathogenesis of asthma is associated with many cell types and several molecular/cellular pathways in the lung. Therefore, we investigated whether rAg85B administration regulates various cytokines associated with the pathogenesis of allergic inflammation in BAL fluid. Control mice in which allergic inflammation developed showed increased production of Th2 cytokines and chemokines in BAL fluid, such as IL-13, IL-5 and TARC. Mice administered rAg85B showed inhibition of the induction of IL-13, IL-5 and TARC (Fig. 4). Furthermore, enhancement of IFN- $\gamma$ , IL-17 and IL-22 production was observed in BAL fluid from mice administered rAg85B. Production of chemokines secreted from non-T cells, CCL20 and CXCL13, was also increased in BAL fluid from rAg85B-administered mice. The chemokine CCL20 is thought to be associated with the recruitment of Th17 lymphocytes and LTi-like or NK-like cells [17,18], and CXCL13 is a chemokine ligand of C-X-C motif receptor 5 (CXCR5) that is

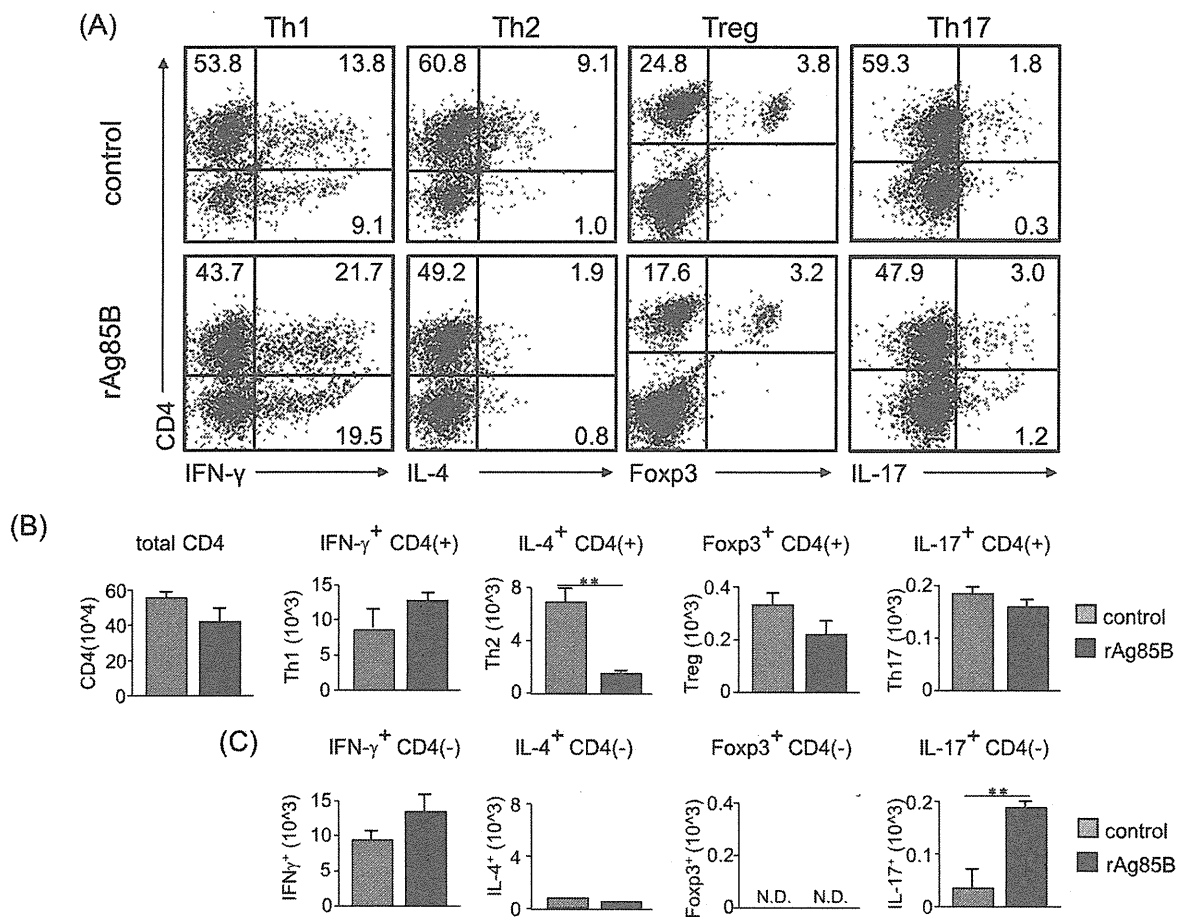
expressed on LTi-like cells. These findings suggested that rAg85B administration was involved in induction of immune responses from both CD4<sup>+</sup> T cells and other innate cells in BAL fluid of mice in which allergic inflammation has developed.

rAg85B administration elicits IL-17-producing CD4<sup>-</sup> negative cells rather than CD4<sup>+</sup> T cell subsets in BAL fluid

To determine the peripheral Th cell population in the lungs of rAg85B-treated mice, BAL cells from experimental mice were analyzed by intracellular cytokine staining. The percentages of IFN- $\gamma$  and IL-17-positive cells from rAg85B-administered mice were higher than those from control cells in agreement with the results of FACS analysis of mLN cells (Fig. 5A), and Treg cells in BAL fluid from rAg85B-administered mice were also the same as the results for mLN cells (Fig. 5A). The absolute number of CD4<sup>+</sup> T cells stained for IL-17 was not increased in BAL cells from rAg85B-administered mice, unlike the results for mLN cells; however, total IL-17-secreting cells, CD4<sup>-</sup> IL-17<sup>+</sup> cells, were increased in rAg85B-administered mice compared with those in control mice (Fig. 5B, 5C). CD4<sup>-</sup> IFN- $\gamma$ -producing cells were observed in BAL fluid from rAg85B-administered mice as same to CD4<sup>+</sup> cells. In addition, IL-4-secreting CD4<sup>+</sup> cells were decreased in rAg85B-treated mice, whereas IL-4-producing CD4<sup>-</sup> cells were not observed. These observations indicated that IL-17 was produced by CD4<sup>-</sup> cells rather than by CD4<sup>+</sup> T cells in BAL, unlike IFN- $\gamma$  and IL-4. Furthermore, the types of BAL cells greatly changed after rAg85B treatment (Figure S3).

rAg85B administration was involved in recruitment of innate immune cells that secrete IL-17-related cytokines in BAL fluid

Recent studies have demonstrated that IL-17 was not only secreted by Th17 cells and the source of Th17-related cytokines was modified in various environmental conditions [19]. Mice administered rAg85B showed infiltration of CD4<sup>-</sup> negative immune cells, which secreted IL-17 cytokine in BAL fluid (Fig. 4, Fig. 5). From these findings, we next investigated the proportions of infiltrating CD4<sup>-</sup> cells that produce IL-17, including  $\gamma\delta$ T cells, IL-7R<sup>+</sup> Lin<sup>-</sup> cells (LTi-like cells), CD3<sup>-</sup> NKp46<sup>+</sup> cells and CD11c<sup>+</sup> cells, in BAL fluid from experimental mice. OVA-sensitized BALB/c mice administered rAg85B, but not mice administered PBS, showed an increased number of innate immune cells in BAL fluid (Fig. 6A). The percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in BAL fluid from rAg85B-administered mice were similar to those in BAL fluid from control mice. However, the percentages of  $\gamma\delta$ T cells, LTi-like cells, NKp46<sup>+</sup> cells, and CD11c<sup>+</sup> cells in BAL fluid from rAg85B-administered mice were higher than those in BAL fluid from control mice (Fig. 6A). Since innate immune cells, which secrete IL-17 and related cytokines, IL-22, were thought to be induced by rAg85B administration, we next explored the source of IL-17-related cytokines in BAL fluid. Small numbers of IL-17-producing  $\gamma\delta$ T cells, LTi-like cells and CD11c<sup>+</sup> cells were observed (Fig. 6D, 6F, 6G), while production of IL-17 from CD8<sup>+</sup> T cells and NKp46<sup>+</sup> cells was not detected (Fig. 6C, 6E). In the present study, a Th17-related cytokine, IL-22, was also detected in BAL fluid from mice administered rAg85B (Fig. 4). All of the cells from BAL secreting Th17-related cytokines, including CD4<sup>+</sup> T cells,  $\gamma\delta$ T cells, NKp46<sup>+</sup> cells, LTi-like cells and CD11c<sup>+</sup> cells, that were examined in this study showed IL-22 production in mice administered rAg85B (Fig. 6B, 6D, 6E, 6F, 6G). On the other hand, production of IL-17 from NKp46<sup>+</sup> cells and CD11c<sup>+</sup> cells were not detected (Fig. 6E, 6G). Although it is now known that NKT cells, alveolar macrophages and neutrophils might also



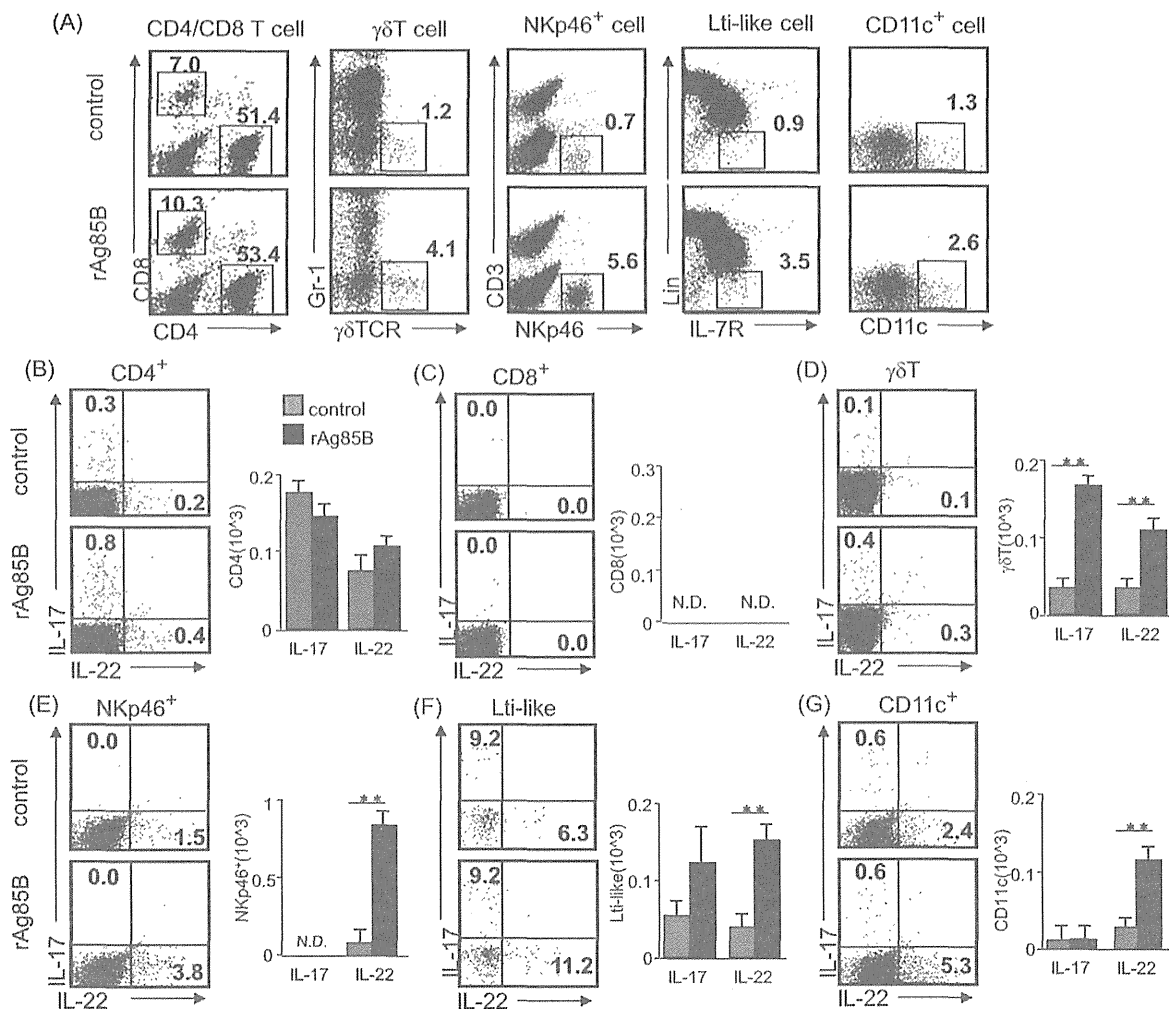
**Figure 5. IFN- $\gamma$  and IL-17-producing CD4-negative cell subsets proliferated in BAL fluid after rAg85B administration.** OVA-immunized (i.p., day0 and 14) and sensitized (5% aerosolized-OVA, day21 to 25) BALB/c mice were challenged with PBS or rAg85B protein (i.p. (100  $\mu$ g; days 0 and 14) and i.n. (20  $\mu$ g; days 21, 23, and 25)). At 24 h after the last OVA sensitization, BAL fluid from naïve or OVA sensitized BALB/c mice treated with PBS or rAg85B, were harvested. BAL cells were stimulated with ionomycin and PMA for 5 h, and with brefeldin A added in the last 3 h. Flow cytometry of stimulated BAL cells from PBS-treated (upper) and rAg85B protein-treated (lower) OVA-sensitized mice stained with specific antibodies indicated marker. Numbers in quadrants indicate percent of cells in each (A). Absolute numbers of various cell populations (above graphs) in BAL fluid (B, C). Data are representative of three independent experiments (\*\* $P < 0.01$  compared with OVA control. error bars, s.d.;  $n = 6$  mice). doi:10.1371/journal.pone.0106807.g005

produce IL-17 in certain conditions, the numbers of these IL-17-secreting cells in BAL fluid from rAg85B-administered mice showed little or no change compared with those in the control group in our experimental setting. Remarkably, these responses induced by rAg85B were observed in allergic animals but not in naïve ones (data not shown).

#### Functions of IL-17 and IL-22 in rAg85B-administered mice

We next investigated the importance of Th17-related cytokines by using neutralizing antibodies (Abs) to IL-17 and IL-22 in rAg85B-administered experimental mice. Administration of neutralizing Abs to IL-17 and IL-22 did not show any systemic inhibitory effects induced by rAg85B as a result of IgE production (Fig. 7A). Furthermore, neutralization of IL-17 and IL-22 did not restore the functions of rAg85B with immune deviation from a disease-promoting Th2 response towards a Th1 response, whereas inhibition of TARC production regulated by rAg85B was reversed by neutralizing IL-22 Abs treatment (Fig. 7B). These results suggested that IL-17 and IL-22 induced by rAg85B have little or

no systemic inhibitory effect on the development of allergic inflammation in the lung. Neutralization of IFN- $\gamma$  at the challenge phase also had little or no suppressive effect on serum IgE expressions and eosinophilia induced by rAg85B treatment. (data not shown). The number of infiltrating cells in BAL fluid were also not changed in mice administered neutralizing Abs to IL-17 and IL-22 (Fig. 7C); however, fractions of infiltrating cells in BAL fluid were different. Neutralization of IL-17 by IL-17-specific Abs prevented neutrophil infiltration by rAg85B administration in the airway, and this preventive effect on infiltration of neutrophils was partial in IL-22-specific Abs administered mice (Fig. 7D). Eosinophilia suppression by rAg85B administration was reversed by neutralizing IL-22 Abs treatment (Fig. 7D). These results parallel previous observations of the specificity of IL-17 and IL-22 effects [20]. Enhancement of innate immune cell recruitment induced by rAg85B was fully reversed by neutralizing IL-17 Abs treatment, and this rAg85B effect was partially reversed by administration of neutralizing IL-22 Abs in  $\gamma\delta$ T cells (Fig. 7D). These results showed that Th17-related cytokines induced by rAg85B have pivotal roles in innate immune cell recruitment in BAL fluid and in severity of



**Figure 6. Innate immune cells that secrete Th17-related cytokines are induced by rAg85B administration in BAL fluid.** OVA-immunized (i.p., day0 and 14) and sensitized (5% aerosolized-OVA, day21 to 25) BALB/c mice were challenged with PBS or rAg85B protein (i.p. (100 μg; days 0 and 14) and i.n. (20 μg; days 21, 23, and 25)). At 24 h after the last OVA sensitization, BAL fluid from naïve or OVA sensitized BALB/c mice treated with PBS or rAg85B, were harvested. BAL cells were stimulated with Ionomycin and PMA for 5 h, and with brefeldin A added in the last 3 h. Flow cytometry of BAL cells from PBS-treated (upper) and rAg85B protein-treated (lower) OVA-sensitized mice stained with anti-CD3, anti-CD4, anti-CD8, anti-Gr-1, anti-γδ TCR, anti-NKp46, anti-CD11c, anti-CD127 (IL-7R) and Lineage specific marker (CD3, CD19, Gr-1, CD11b, CD11c). Numbers in quadrants indicate percent of cells in each (A). Intracellular IL-17 and IL-22 staining in indicated cells by flow cytometry (dot plots) and absolute numbers of those cell populations (side graphs) in the BAL fluid (B, C, D, E, F, G). Data are representative of at least two independent experiments (\*\*P<0.01 compared with OVA control. error bars, s.d.; n=6 mice). doi:10.1371/journal.pone.0106807.g006

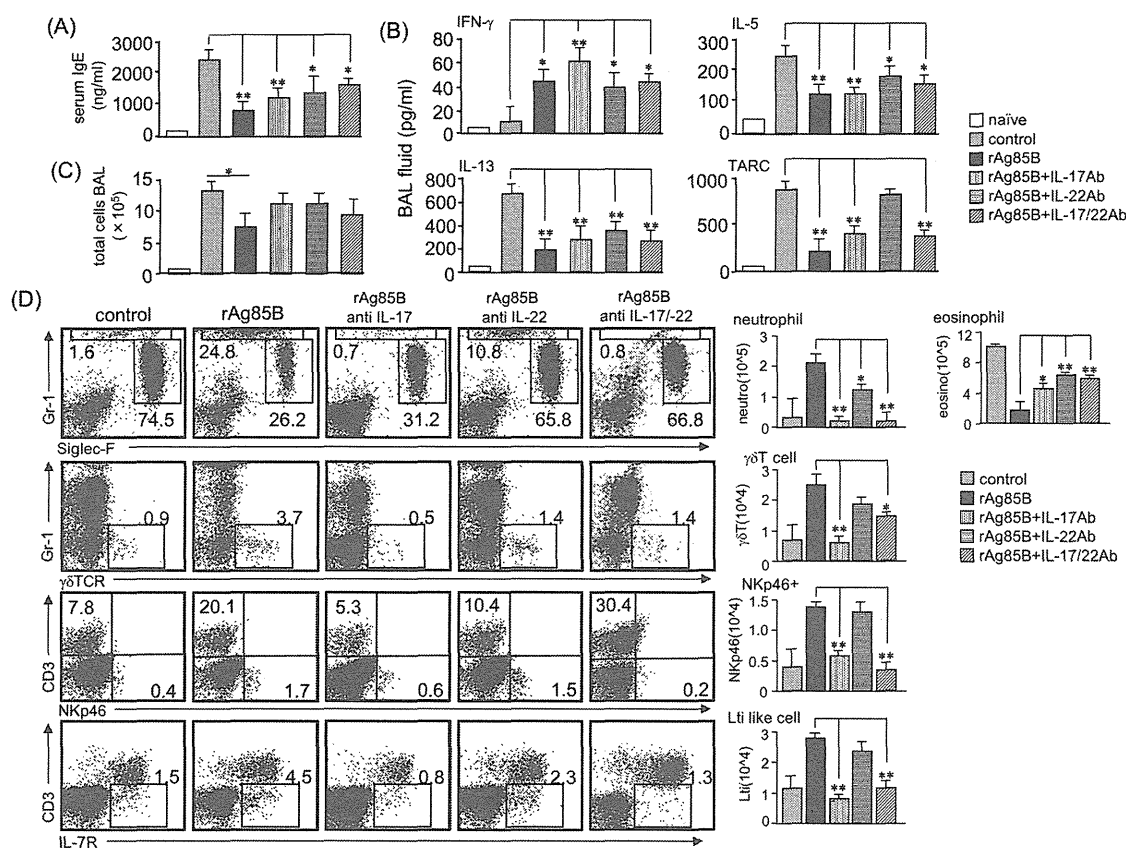
lung inflammation but not in regulated systemic allergic inflammation involving Th responses.

#### Administration of rAg85B promoted Th17-related innate responses in the lung

Our data suggested an important link between rAg85B and airway innate immune cells producing IL-17 and IL-22 that contributed to the homeostasis expression of Th17-related cytokine response genes. However, these two cytokines induced by rAg85B administration did not clearly show inhibitory effects on systemic allergy responses (Fig. 7A, 7B). From these findings, we next explored the relationship between the roles of airway innate immune cells and wound repair in mice that received i.n. administration of rAg85B. Mice that received IL-17 or IL-22 or

both neutralizing antibodies showed a marked induction of fibrosis and actin staining but incomplete cancellation of rAg85B suppressive effects at the same levels as those in OVA control mice (Fig. 8A). Histological findings suggested that IL-17 and IL-22 induced by rAg85B i.n. administration were partially involved in regulation of local tissue allergic inflammation. The inhibition of rAg85B effects by neutralizing Abs of IL-17 and IL-22 to allergic inflammation was partial; however, tissue repair in lungs was seen in rAg85B-administered mice by histopathological examination. These results led us to hypothesize that IL-17 and IL-22 induced by rAg85B induced local tissue remodeling/repair molecules. To confirm this, the induction of tissue homeostasis-related gene expression in rAg85B-administered mice was examined by real-time RT-PCR. Rb2, Cyclin D1 and c-Myc are associated with





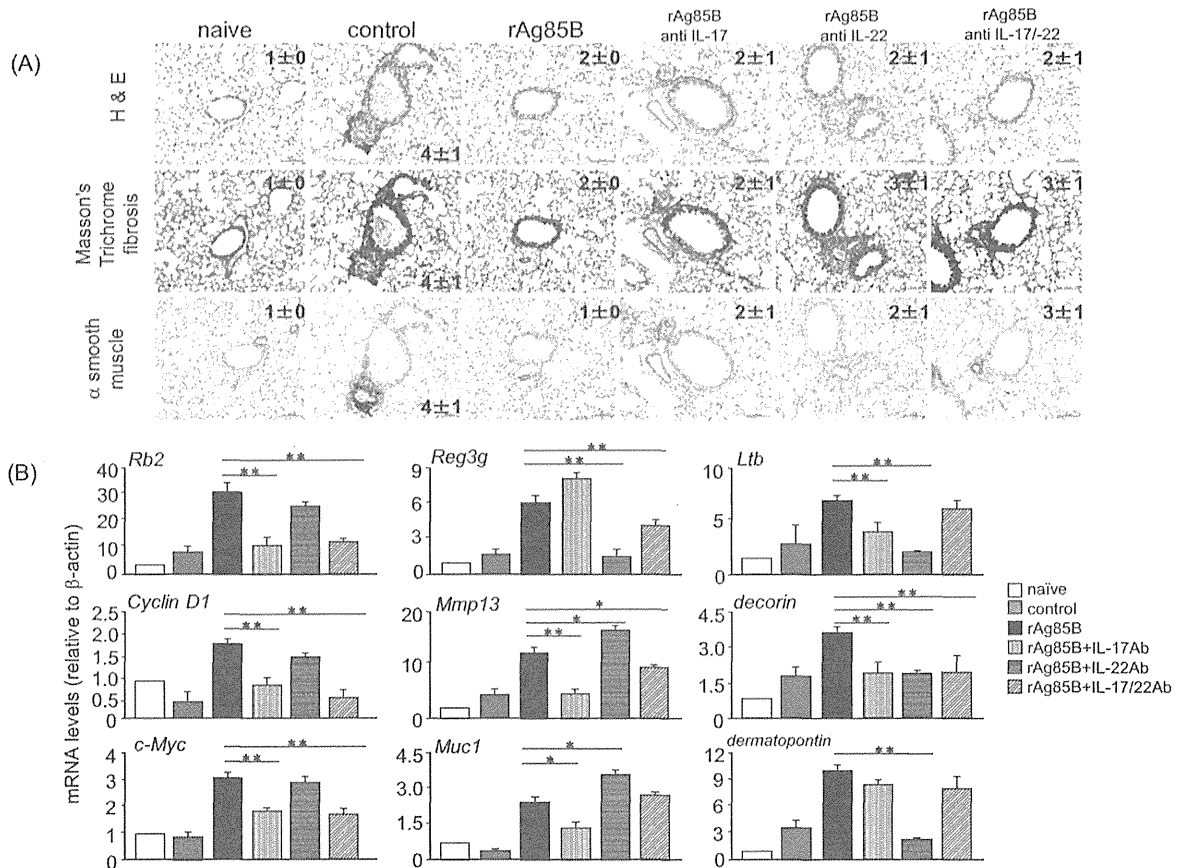
**Figure 7. Neutralization of Th17-related cytokines inhibits cell recruitment to the lung but does not change cytokine and chemokine production.** OVA-sensitized BALB/c mice (5% aerosolized-OVA, day 21 to 25) were challenged intranasally with PBS (control), rAg85B (rAg85B + isotype-matched control antibody (Ab)), rAg85B plus neutralizing IL-17 (rAg85B + IL-17Ab) or IL-22 (rAg85B + IL-22Ab), or a combination of both antibodies (rAg85B + IL-17/22Ab) on days 21, 23, and 25. The isotype control was treated with the same time course as neutralization Ab i.n. administration. One day after the last challenge, OVA-specific serum IgE concentration and levels of cytokines and chemokines in BAL fluid were determined by ELISA (A, B). BAL cells from naive or OVA sensitized BALB/c mice treated with PBS or rAg85B with/without neutralization Ab were counted (C), and were stained with anti-Gr-1, anti-Siglec-F, anti- $\gamma\delta$  TCR, anti-CD3, anti-NKp46, and anti-CD127 for flow cytometric analysis. Numbers adjacent to outlined area indicate percent of eosinophils (Gr-1<sup>dim</sup>, Siglec-F<sup>+</sup>), neutrophils (Gr-1<sup>+</sup>, Siglec-F<sup>neg</sup>),  $\gamma\delta$ T cells (Gr-1<sup>neg</sup>,  $\gamma\delta$ TCR<sup>+</sup>), NKp46<sup>+</sup> cells (CD3<sup>neg</sup>, NKp46<sup>+</sup>), LTI like cells (CD3<sup>neg</sup>, L-7R<sup>+</sup>), and absolute numbers of those cell populations (side graphs) in BAL fluid (D). Data are representative of at least two independent experiments (\* $P < 0.05$ , \*\* $P < 0.01$  compared with rAg85B+isotype control challenged group. error bars, s.d.; n=6 mice). doi:10.1371/journal.pone.0106807.g007

wound healing, tissue repair and remodeling including proliferative molecules [21]. Mucl [22,23], matrix metalloproteinase 13 (MMP13) [24], and the extracellular matrix proteins decorin and dermatopontin [25] produce protective mucus. Lymphotoxin-beta (Ltb) is a molecule related to signaling in stromal cells to produce factors that organize lymphoid cells into lymph nodes [26]. The transcription of Reg3 $\gamma$  is involved in tissue repair and antimicrobial responses [27]. The expression of these genes involved in innate immune response-mediated signaling was significantly enhanced in the lungs of rAg85B-administered mice (Fig. 8B). The increases in mRNA levels of all molecules other than Reg3 $\gamma$  and dermatopontin were inhibited by treatment with neutralizing Abs of IL-17 (Fig. 8B). On the other hand, the expression of mRNA of molecules enhanced by rAg85B administration was decreased after treatment with IL-22 neutralizing Abs except for Rb2, Cyclin D1, c-Myc, Mmp13 and Mucl (Fig. 8B). These results suggested that IL-17 and IL-22 induced by rAg85B administration affected induction of pulmonary innate response. In conclusion, IL-17 induced by rAg85B administration induced the expression of various types of wound healing, tissue repair and

remodeling molecules. Interestingly, IL-22 in rAg85B-immunized mice induces the expression of molecules mainly associated antimicrobial responses such as Reg3 $\gamma$ , decorin, dermatopontin and Ltb. In summary, Th1 and Th17 cells are induced in regional lymph nodes by administration of rAg85B; however, Th17 cells are not induced in BAL unlike in Th1 cells. IL-17 is produced by innate immune cells with IL-22 production. IL-17 and IL-22 are important in not only anti-allergic effects, such as eosinophil inhibition, but also wound healing and tissue repair in the lung (Fig. 9).

## Discussion

Results of several experimental studies on mycobacteria involving mycobacterial antigens in mouse models of allergic airway inflammation have been reported. In murine asthma models, intranasal administration of BCG suppressed asthma manifestations probably through Th1 response [2,4], Treg cells [5,6,28], or NKT cells [7,8,29]. In our experimental setting using rAg85B protein, we did not find any detectable effect or



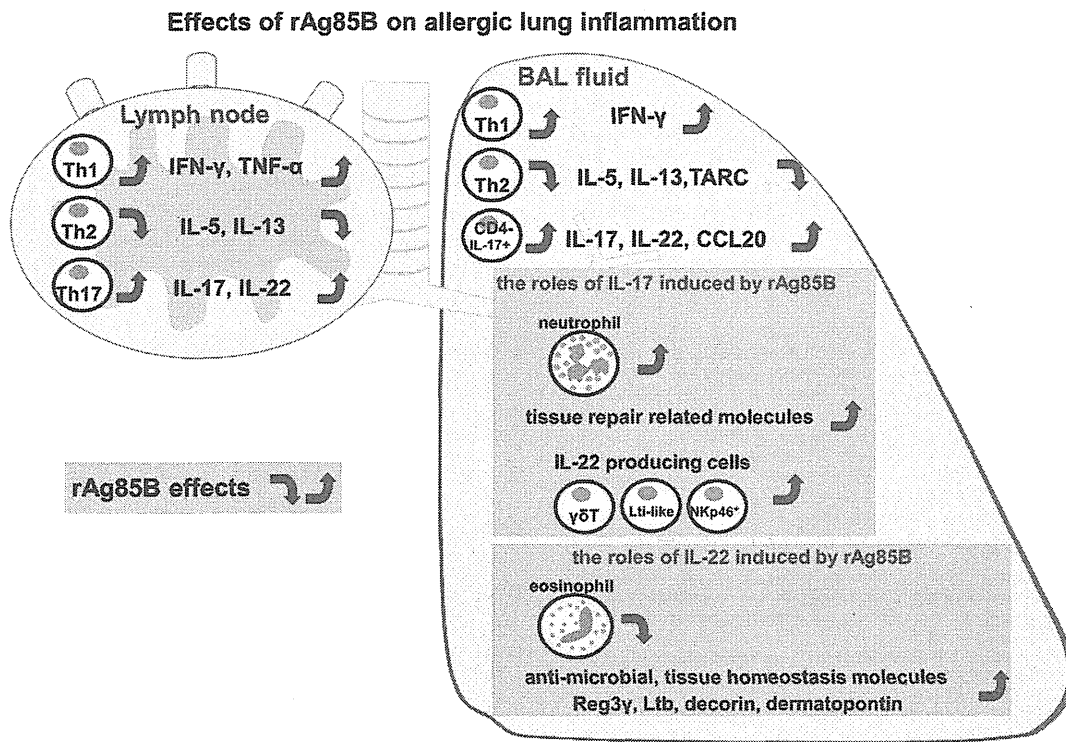
**Figure 8. Ag85B administration promotes Th17-related innate responses in the lung.** OVA-sensitized BALB/c mice (5% aerosolized-OVA, day21 to 25) were challenged intranasally with PBS (control), rAg85B (rAg85B + isotype control), rAg85B plus neutralizing IL-17 (rAg85B + IL-17Ab) or IL-22 (rAg85B + IL-22Ab), or a combination of both antibodies (rAg85B +IL-17/22Ab) on days 21, 23, and 25. The isotype-matched control antibody was treated with the same time course as neutralization Ab i.n. administration. Lungs from naïve or OVA sensitized BALB/c mice treated with PBS or rAg85B with/without neutralization Ab were sampled one day after the last challenge for histological analysis and quantification of mRNA levels. Lung sections were stained with hematoxylin and eosin (left row, scale bar, 100 mm), Masson's trichrome (center row, scale bar, 100 mm), α-smooth muscle actin (right row, scale bar, 50 mm). Numbers in quadrants indicate the score scale from 0 to 5 in each (A). Real-time RT-PCR was performed for the indicated molecules expression on RNA isolated from individual mice lungs (B). Data are representative of at least two independent experiments (\*P<0.05, \*\*P<0.01 compared with rAg85B+isotype control challenged group. error bars, s.d.; n=6 mice). doi:10.1371/journal.pone.0106807.g008

substantial change in numbers of both NKT cells and Treg cells in BAL fluid from rAg85B-administered mice. Moreover, microarray analysis revealed that the gene expression pattern of splenocytes stimulated with rAg85B and that of splenocytes stimulated with BCG were very different (data not shown). This discrepancy in the effects of vaccination with BCG and vaccination with rAg85B might be related to the factors affecting immune responses. BCG contains many essential components to induce early immune response such as glycolipid and DNA, whereas rAg85B is a single immunogenic protein.

The present study indicated that Th17-related immune responses induced by rAg85B administration had a suppressive effect on allergic airway inflammation, and we attributed this suppressive effect to the larger proportion of Th17-related cytokine-producing innate immune cells in BAL fluid. It has been reported that Mycobacterium antigens increased the number of  $\gamma\delta$ T cells that express IFN- $\gamma$  [30] or IL-17 [31], and these responses induce healing to epithelial surfaces [32]. Given the integral role of  $\gamma\delta$ T cells in innate immunity,  $\gamma\delta$ T cells are one of the crucial factors in the rAg85B immune regulatory functions.

Interestingly, our study also showed that IL-22-producing cells in lungs of rAg85B-administered mice were NKp46<sup>+</sup> cells, LTI-like cells,  $\gamma\delta$ T cells and CD11c<sup>+</sup> cells. This is the first time demonstration of an important link between the mycobacterium antigen rAg85B and IL-22-producing cells. Although we could not rule out the possibility of Th17 cytokine-producing cells other than those described here, NKp46<sup>+</sup> cells, LTI-like cells,  $\gamma\delta$ T cells and CD11c<sup>+</sup> cells were thought to be rapid innate sources of IL-22, which is required in the early stage to maintain epithelial cell integrity and to suppress eosinophilia. Moreover, IL-22 can act synergistically or additively with other cytokines, including IL-17 or TNF, to promote gene expression for antimicrobial peptides, chemokines, matrix metalloproteinases, cytokines, and acute-phase proteins from epithelial cells in the lung [33]. These findings also support our results showing that simultaneous induction of these cytokines and expression of many genes may be beneficial functions of rAg85B treatment in local allergic pathology.

Pulmonary infection of mycobacteria induced not only a neutrophil-mediated response but also T cell-mediated IFN- $\gamma$



**Figure 9. Schematic illustration of the proposed effects of rAg85B in a mouse model of allergic inflammation.** IFN- $\gamma$  and IL-17-producing Th cells are induced in regional lymph nodes by rAg85B challenge, however, Th17 cells do not enter the lung unlike Th1 cells. Th17-related cytokine-secreting cells in lungs from rAg85B-administered mice are innate immune cells including  $\gamma\delta$ T cells, IL-7R<sup>+</sup> Lin<sup>-</sup> cells, CD3<sup>-</sup> Nkp46<sup>+</sup> cells and CD11c<sup>+</sup> cells. IL-17 and IL-22 induced by rAg85B in an allergic environment have crucial roles in not only anti-allergic effects but also regulation of tissue homeostatic reactions.

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production and granuloma formation depending on IL-17 from especially  $\gamma\delta$ T cells [34]. The hallmark of mycobacterial infection in the lung is granuloma formation with infiltrating neutrophils, which creates an immune microenvironment in which the infection can be controlled. On the other hand, it also provides the mycobacterium with a niche in which it can survive, modulating the immune response to ensure its survival without damage over a long period of time [35,36]. Mature granulomas include fibroblasts and extracellular matrix, which surround and separate the granulomas from the normal environment. Administration of anti-IL-17 Abs during the inhalatory challenge phase abolished the bronchial neutrophilia and the upregulation of genes related to tissue repair and homeostasis observed in rAg85B-administered mice in the present study. Neutrophils may also promote epithelial healing [37] and are now known to be rich sources of prestored and expressible proteins [38] that may directly promote wound healing [39,40]. In the present study, induction of neutrophilia and upregulation of described genes related to wound healing with suppression of tissue injury might be the mechanisms of granuloma formation induced by mycobacteria infection.

In the present study, IFN- $\gamma$  and Th17-related cytokines were key factors to regulate allergic severity in our experimental setting. Although infiltration of Th1 and Th17 cells elicited by rAg85B was induced in pulmonary lymph nodes, such effector cells were not increased in BAL fluid of mice showing anti-allergic effects of Ag85B administration. Moreover, our results suggested that the accumulation of neutrophils and IL-17 and/or IL-22-producing innate immune cells contributed to the homeostatic functions in

the Th1-balanced environment induced by rAg85B administration. These findings provide a new insight into the regulatory effects of various innate immune factors induced by the mycobacteria major secretion protein rAg85B in allergic inflammation.

### Supporting Information

**Figure S1 SDS-PAGE separation and silver staining of rAg85B.** The recombinant purified Ag85B was solubilized in sample buffer to the desired concentration, and boiled for 5 min. 15  $\mu$ l/well from each samples were separated on 10% SDS gel using mini-PROTEAN electrophoresis instrument (Bio-Rad Laboratories). Silver staining of the gel was performed according to the standard protocol of EzStain Silver (ATTO). Various concentration of rAg85B on the gel (12.5, 25, 50, 100, 200, and 400 ng). (TIF)

**Figure S2 CD4<sup>+</sup> Foxp3<sup>+</sup> T cells were almost expressing CD25.** OVA-immunized (i.p., day0 and 14) and sensitized (5% aerosolized-OVA, day21 to 25) BALB/c mice were challenged with PBS or rAg85B protein (i.p. (100  $\mu$ g; days 0 and 14) and i.n. (20  $\mu$ g; days 21, 23, and 25)). At 24 h after the last OVA sensitization, mediastinal lymph nodes (MLNs) from naive or OVA sensitized BALB/c mice treated with PBS or rAg85B, were harvested. MLNs cells from naive (upper), PBS-treated (middle) and rAg85B protein-treated (lower) OVA-sensitized mice were

stained with anti-CD4 and anti-Foxp3. Cells in R1-R4 were analyzed for the expression of CD25. (TIF)

**Figure S3 The composition of BAL cells in rAg85B administered mice.** OVA-immunized (i.p., day0 and 14) and sensitized (5% aerosolized-OVA, day21 to 25) BALB/c mice were challenged with PBS (control) or rAg85B (i.p. (100 µg; days 0 and 14) and i.n. (20 µg; days 21, 23, and 25)). One day after the last challenge, BAL cells from OVA sensitized BALB/c mice treated with PBS or rAg85B were counted. Different BAL cells

populations were measured by surface staining. Flow cytometry of BAL cells stained with anti-CD3, anti-CD4, anti-CD8, anti-Gr-1, anti-γδ TCR, anti-NKp46, anti-CD11c, anti-CD127 (IL-7R) and Lineage specific marker (CD3, CD19, Gr-1, CD11b, CD11c). (TIF)

## Author Contributions

Conceived and designed the experiments: YY. Performed the experiments: YT HI MY. Analyzed the data: YY KM. Contributed reagents/materials/analysis tools: TF. Contributed to the writing of the manuscript: YT YY.

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# CD4<sup>+</sup> T Cells Modified by the Endoribonuclease MazF Are Safe and Can Persist in SHIV-infected Rhesus Macaques

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MazF, an endoribonuclease encoded by *Escherichia coli*, specifically cleaves the ACA (adenine–cytosine–adenine) sequence of single-stranded RNAs. Conditional expression of MazF under the control of the HIV-1 LTR promoter rendered CD4<sup>+</sup> T cells resistant to HIV-1 replication without affecting cell growth. To investigate the safety, persistence and efficacy of MazF-modified CD4<sup>+</sup> T cells in a nonhuman primate model *in vivo*, rhesus macaques were infected with a pathogenic simian/human immunodeficiency virus (SHIV) and transplanted with autologous MazF-modified CD4<sup>+</sup> T cells. MazF-modified CD4<sup>+</sup> T cells were clearly detected throughout the experimental period of more than 6 months. The CD4<sup>+</sup> T cell count values increased in all four rhesus macaques. Moreover, the transplantation of the MazF-modified CD4<sup>+</sup> T cells was not immunogenic, and did not elicit cellular or humoral immune responses. These data suggest that the autologous transplantation of MazF-modified CD4<sup>+</sup> T cells in the presence of SHIV is effective, safe and not immunogenic, indicating that this is an attractive strategy for HIV-1 gene therapy.

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**Subject Category:** Therapeutic proof-of-concept Gene insertion, deletion & modification

## Introduction

Antiretroviral therapy (ART), which is based on a combination of different classes of inhibitors, is widely used for the treatment of human immunodeficiency virus type 1 (HIV-1) infection and effectively suppresses HIV-1 replication to low or undetectable levels, corresponding with a recovery of CD4<sup>+</sup> T cell counts.<sup>1–3</sup> ART treatment dramatically improves the survival rate of HIV-1-infected individuals and has transformed HIV-1 infection into a controllable illness. However, the need for lifelong therapy and difficulties in adherence to medication regimes are likely to lead to the emergence of drug-resistant HIV-1 strains. Long-term side effects, such as cardiovascular disease, hepatotoxicity and dementia, have been reported in association with HIV-1 infection.<sup>4–6</sup> The most serious deficiency of ART is that it cannot eradicate latent virus.<sup>7</sup> Once the treatment is interrupted, a replicable HIV-1 reemerges. Thus, the benefits of current ARTs are limited. Therefore, it remains necessary to discover and develop novel approaches for the management of HIV-1 infection, including treatment options used in combination with the ART.<sup>8</sup>

The report of the “Berlin Patient,” who appears to have been cured of HIV-1 infection by stem cell transplantation with HIV-1 resistant CCR5Δ32/Δ32 cells<sup>9</sup> has had a major impact. This patient developed acute myelogenous leukemia and received bone marrow transplantation with cells bearing a homozygous Δ32 mutation in the CCR5 gene. No HIV-1 was detectable in this patient, even in the absence of ART.<sup>10</sup> However, allogeneic bone marrow transplantation as a cure for HIV-1 infection is not a realistic strategy because there is a risk of death, and the long-term effects are unclear. In contrast, gene therapy for HIV-1 has steadily progressed as an alternative to antiretroviral drug regimens.<sup>11,12</sup> A number of

strategies have been developed, including strategies involving dominant negative inhibitory proteins, fusion inhibitors, antisense RNA, aptamers, RNA decoys, ribozymes, RNA interference, and HIV-1 entry inhibition.<sup>13–18</sup> These protocols target autologous T cells or hematopoietic stem cells for gene modification using retrovirus, lentivirus, or adenovirus vectors to deliver anti-HIV-1-payloads. Some of these methods have progressed to clinical trials.<sup>19,20</sup>

Recently, we proposed a new approach for gene therapy for HIV-1 using the endoribonuclease MazF.<sup>21</sup> MazF is encoded by *Escherichia coli* and specifically cleaves the ACA sequence of single-stranded RNAs. MazF does not interfere with ribosomal RNAs. When overexpressed in mammalian cells, MazF preferentially cleaves mRNA but not rRNA.<sup>22</sup> Previous studies have demonstrated that the expression of MazF under the control of the HIV-1 LTR promoter was successfully induced upon HIV-1 replication and rendered CD4<sup>+</sup> T cells resistant to HIV-1 and simian/human immunodeficiency virus (SHIV) without affecting cellular mRNAs.<sup>21,23</sup> Because HIV-1 RNA has more than 240 ACA sequences, viral RNA is assumed to be highly susceptible to MazF. A key regulator of MazF expression in this system is the HIV-1 Tat protein, which activates transcription from the HIV-1 LTR.<sup>24</sup> In this system, the Tat protein induces HIV-1 replication and MazF expression. Furthermore, the autologous transplantation of MazF-modified CD4<sup>+</sup> T cells in cynomolgus macaques has been shown to be safe, and the modified cells showed little or no immunogenicity.<sup>25</sup> These results suggest that the conditional expression of MazF is an attractive strategy for anti-HIV-1 gene therapy.

To investigate the safety, persistence and efficacy of MazF-modified CD4<sup>+</sup> T cells in a nonhuman primate model *in vivo* in the presence of viral infection, six rhesus macaques were

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infected with a SHIV 89.6P.<sup>26</sup> Four rhesus macaques were transplanted with MazF-modified CD4<sup>+</sup> T (MazF-Tmac) cells, and two were transplanted with control ZsGreen1-modified CD4<sup>+</sup> T (ZsG-Tmac) cells. After transplantation of the gene-modified cells, changes in the CD4<sup>+</sup> T cell count values, changes in plasma SHIV viral loads, and the persistence of gene-modified cells were monitored throughout the experimental period. The humoral and cellular immune responses elicited by MazF were assessed. At necropsy, distributions of the transplanted MazF-Tmac cells in the distal lymphoid tissues, including several lymph nodes and the spleen, were analyzed.

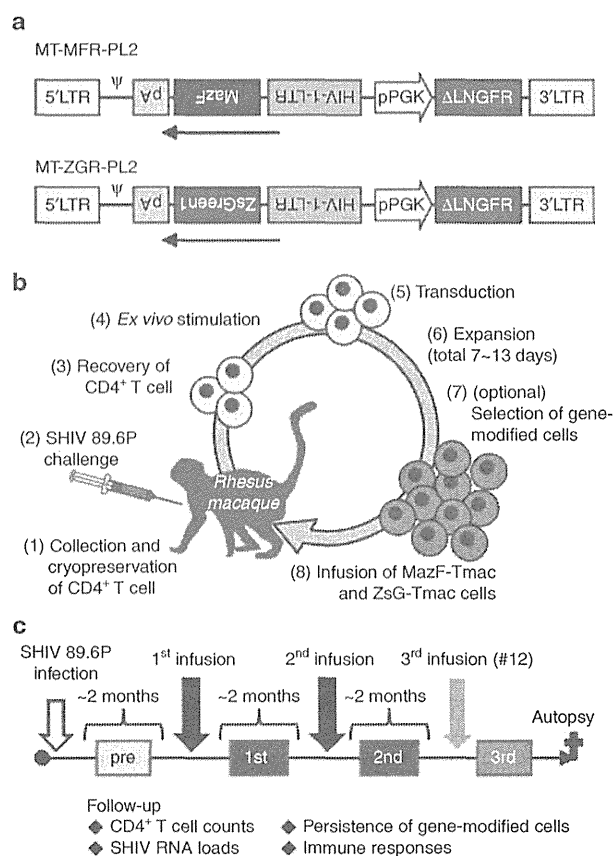
## Results

### Study protocol and SHIV challenge

Six rhesus macaques (#12, #13, #14, #15, #16, and #17) were used for this experiment. Each rhesus macaque was challenged with SHIV 89.6P, followed by transplantation with autologous CD4<sup>+</sup> T cells transduced with the MazF retroviral vector MT-MFR-PL2 (#12, #13, #14, and #15) or the control vector MT-ZGR-PL2 (#16 and #17) (Figure 1a). The rhesus macaques were monitored over 6 months for changes in CD4<sup>+</sup> T cell counts, changes in SHIV viral loads in the plasma, persistence of the gene-modified cells, and the immune responses elicited by the gene-modified cells. An outline of the experiment is shown in Figure 1b,c. The dose of SHIV, day of infusion and experimental period are summarized in Supplementary Table S1. Initially, rhesus macaque #15 was challenged with (5000) 50% tissue culture infective dose (TCID<sub>50</sub>), which we speculated would be a proper dose based on our previous experience; however, the viral loads declined to the limit of detection. We, therefore, increased the dose of TCID<sub>50</sub> for the other experiments. However, #12, #16, and #17 showed high viral loads, whereas #13 and #14 showed low viral loads. Such differences might have been due to individual variation in the sensitivity of the rhesus macaques used in this experiment.

### Gene-modified T cell manufacturing and transplantation

MazF- or ZsGreen1-modified cells were manufactured from previously collected CD4<sup>+</sup> T cells and transplanted into each rhesus macaque 2 months after SHIV 89.6P infection. Repeated transplantations were performed at 2-month intervals. To transplant more than 10<sup>9</sup> MazF-Tmac or ZsG-Tmac cells, 1–2 × 10<sup>7</sup> primary CD4<sup>+</sup> T cells were recovered, stimulated, and transduced either with the MT-MFR-PL2 vector or the MT-ZGR-PL2 vector, and expanded as described in Supplementary Materials and Methods. The numbers and characteristics of the gene-modified CD4<sup>+</sup> T cells for each transplantation are summarized in Table 1. The transduction efficiencies of the MazF and ZsGreen1 vectors were 52.0–69.5% and 53.8–75.7%, respectively. The gene-modified cells marked with a truncated form of the human low-affinity nerve growth factor receptor (LNGFR/CD271) were concentrated with an anti-CD271 monoclonal antibody for the first transplantation of rhesus macaques #12 and #14, and the second transplantation of rhesus macaques #13 and #15. The gene-modified cells that were positively selected were over 97% pure. There was no selection of control ZsG-Tmac cells for transplantation. The majority of the expanded cells were CD3<sup>+</sup> CD4<sup>+</sup> T cells



**Figure 1** Diagram of autologous CD4<sup>+</sup> T cell transplantation in a primate model. **(a)** Structures of the gamma-retroviral vectors MT-MFR-PL2 and MT-ZGR-PL2. LTR, long terminal repeat; MoMLV, Moloney murine leukemia virus; LNGFR, low-affinity nerve growth factor receptor gene; pPGK, phosphoglycerate kinase promoter. **(b)** Flow diagram of gene therapy in the rhesus macaques. <sup>1</sup> Peripheral blood was collected by apheresis; the CD4<sup>+</sup> T cells were isolated and cryopreserved. <sup>2</sup> The rhesus macaques were challenged *ex vivo* with SHIV 89.6P.<sup>3,4</sup> The CD4<sup>+</sup> T cells were recovered and stimulated *ex vivo* with anti-CD3/CD28 beads.<sup>5</sup> The stimulated cells were transduced twice with retroviral vectors on days 3 and 4.<sup>6</sup> The transduced cells were expanded for an additional 3–9 days.<sup>7</sup> The ΔLNGFR-positive cells were selected at the second transplantation for rhesus macaques #13 and #15 and at the first transplantation for rhesus macaques #12 and #14.<sup>8</sup> On days 7–13, the expanded autologous cells were collected, washed and transplanted into the rhesus macaques intravenously. **(c)** Four rhesus macaques were transplanted with MazF-Tmac cells, and two were transplanted with control ZsG-Tmac cells. The rhesus macaques were followed over six months.

(>98%). More than 90% of these cells expressed CD95 and CD28, which are known markers of the central memory phenotype;<sup>27</sup> central memory cells generally have a longer lifespan than effector memory cells.<sup>28</sup> The expression levels of CXCR4, which is a known coreceptor for X4-tropic HIV-1 and SHIV entry, varied among animals and expansion periods.

### Body weight and hematological data

There was no significant change in body weight throughout the experiment in the MazF-Tmac-transplanted rhesus macaques (see Supplementary Figure S1a). A gradual

**Table 1** The summary of characteristics of gene-modified CD4<sup>+</sup> T cells for each transplantation

	#12	#13	#14	#15	#16	#17
<b>First transplantation</b>						
Culture period (days)	13	9	13	9	9	9
Number of infused cells	2.1 × 10 <sup>9</sup>	3.0 × 10 <sup>9</sup>	2.3 × 10 <sup>9</sup>	1.6 × 10 <sup>9</sup>	1.6 × 10 <sup>9</sup>	1.1 × 10 <sup>9</sup>
CD271 <sup>+</sup> (gene-modified) (%)	97.2 <sup>a</sup>	52.0	99.0 <sup>a</sup>	54.1	53.8	75.0
CD3 <sup>+</sup> CD4 <sup>+</sup> (%)	98.3	99.9	99.0	99.8	99.2	99.8
CD28 <sup>+</sup> CD95 <sup>+</sup> CM (%)	93.4	97.5	95.4	98.7	N.D.	N.D.
CD28 <sup>-</sup> CD95 <sup>+</sup> EM (%)	6.3	2.3	4.5	0.9	N.D.	N.D.
CXCR4 <sup>+</sup> (%)	36.3	63.2	8.9	65.5	67.9	52.6
<b>Second transplantation</b>						
Culture period (days)	9	13	9	13	10	10
Number of infused cells	2.7 × 10 <sup>9</sup>	3.3 × 10 <sup>9</sup>	1.8 × 10 <sup>9</sup>	2.7 × 10 <sup>9</sup>	3.8 × 10 <sup>9</sup>	2.8 × 10 <sup>9</sup>
Gene-modified (%)	69.5	96.3 <sup>a</sup>	57.0	99.8 <sup>a</sup>	55.2	75.7
CD3 <sup>+</sup> CD4 <sup>+</sup> (%)	99.0	99.9	98.7	99.8	99.5	99.6
CD28 <sup>+</sup> CD95 <sup>+</sup> CM (%)	96.9	90.8	97.7	97.6	N.D.	N.D.
CD28 <sup>-</sup> CD95 <sup>+</sup> EM (%)	2.7	9.0	2.1	2.2	N.D.	N.D.
CXCR4 <sup>+</sup> (%)	55.4	47.8	35.7	84.0	63.5	63.4
<b>Third transplantation</b>						
Culture period (days)	7					
Number of infused cells	0.55 × 10 <sup>9</sup>					
Gene-modified (%)	65.4					
CD3 <sup>+</sup> CD4 <sup>+</sup> (%)	99.5					
CD28 <sup>+</sup> CD95 <sup>+</sup> CM (%)	96.9					
CD28 <sup>-</sup> CD95 <sup>+</sup> EM (%)	3.1					
CXCR4 <sup>+</sup> (%)	77.0					

The autologous MazF-Tmac cells and the ZsG-Tmac cells were manufactured from the cryopreserved CD4<sup>+</sup> T cells by stimulating the cells with anti-CD3/CD28 beads, and transducing them with the MT-MFR-PL2 and MT-ZGR-PL2 retroviral vectors. The CD3, CD4, CD28, CD95, CD271, and CXCR4 expression in the gene-modified cells at the time of transplantation were analyzed by flow cytometry.

<sup>a</sup>Gene-modified cells were concentrated based on their expression of the ΔLNGFR surface marker. CM, central memory; EM, effector memory; N.D., not determined.

decrease in body weight was observed in one ZsG-Tmac-transplanted rhesus macaque #16 by the end of the experiment (data not shown). Hematological data, including white blood cell count, hemoglobin concentration and platelets, were also analyzed, and significant changes were observed in rhesus macaque #16 at the end of the experiment (data not shown). Rhesus macaque #16 was sacrificed prior to the scheduled autopsy because of worsening symptoms. No significant changes were observed in any of the MazF-Tmac-transplanted rhesus macaques (see **Supplementary Figure S1b–d**). Thus, MazF-Tmac cells are considered safe based on the clinical observations.

#### CD4<sup>+</sup> T cell counts in peripheral blood

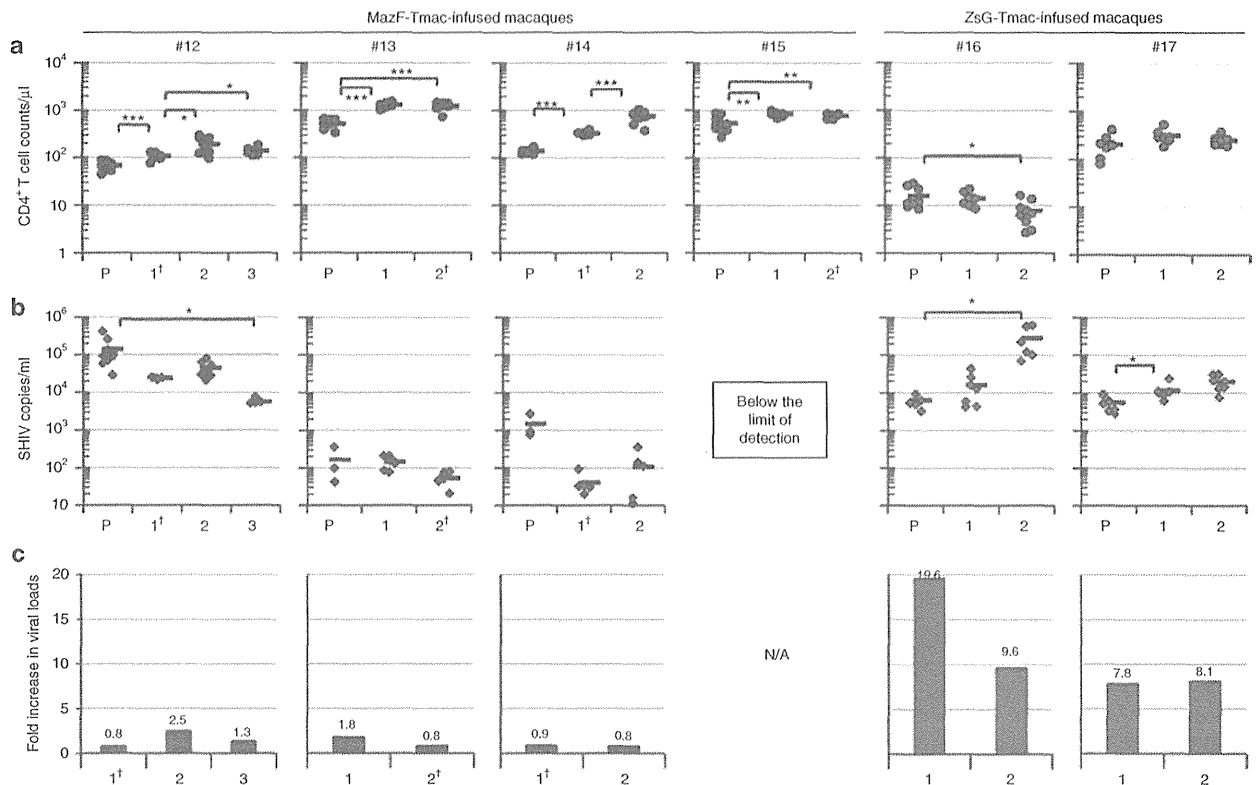
To evaluate the impact of the autologous transplantation of gene-modified CD4<sup>+</sup> T cells, the posttransplantation CD4<sup>+</sup> T cell count values were compared with the values measured prior to transplantation. As shown in **Figure 2a**, the increases in the CD4<sup>+</sup> T cell counts after transplantation of MazF-Tmac cells were significant in all four rhesus macaques, while no significant increases were observed in the two rhesus macaques transplanted with ZsG-Tmac cells. In the case of rhesus macaque #12, which was robustly infected with SHIV and had high viral loads (>10<sup>5</sup> copies/ml), the average CD4<sup>+</sup> T cell count was 68 cells/μl before the gene therapy treatment, and increased to 107 cells/μl and 193 cells/μl after the first and second transplantation, respectively. Unfortunately,

the mean CD4<sup>+</sup> T cell count after the third transplantation decreased slightly to 139 cells/μl; this value was still higher than the baseline values.

The effectiveness was most clearly shown in the rhesus macaque #14, whose average CD4<sup>+</sup> T cell count was 137 cells/μl before the gene therapy treatment, and subsequently increased to 329 cells/μl and 754 cells/μl after the first and second transplantations, respectively. To investigate the long-term safety of the treatment, this rhesus macaque was followed up for one and a half years. The other rhesus macaques (#13 and #15) had average CD4<sup>+</sup> T cell counts of approximately 530 cells/μl before the gene therapy treatment that also increased to 1296 cells/μl and 856 cells/μl after the first transplantation, respectively, and retained these levels after the second transplantation. Thus, the transplantation of MazF-Tmac cells has the potential to increase CD4<sup>+</sup> T cell counts.

#### Plasma SHIV viral loads

To investigate the influence of the transplantation of gene-modified cells, SHIV viral loads in the plasma were measured using quantitative PCR (qPCR). In rhesus macaque #15, the viral loads were below the detection limit at the time of gene therapy treatment. In rhesus macaques #13 and #14, the available data were limited to three time points due to a relatively short window in which they exhibited a stable set point before the infusion of MazF-Tmac cells. For this reason, we did not



**Figure 2** Changes in CD4<sup>+</sup> T cell counts and viral loads. (a) The CD4<sup>+</sup> T cell counts of MazF-Tmac- and ZsG-Tmac-transplanted rhesus macaques. The CD4<sup>+</sup> T cell counts in the area of the viral load set point were averaged by multiple time point sampling 20 to 80 days after SHIV infection or transplantation. (b) Plasma SHIV viral RNA loads. The plasma SHIV viral RNA loads in the area of the set point were averaged by multiple time point sampling 20–80 days after SHIV infection or transplantation of gene-modified cells. (c) The fold increase of the plasma viral loads of MazF-Tmac- and ZsG-Tmac-transplanted rhesus macaques. The fold increase values were calculated by dividing [the average viral loads one week after transplantation of gene-modified cells] by [the average viral loads three weeks before transplantation of gene-modified cells]. P, pretransplantation of gene-modified cells; 1, after the first transplantation; 2, after the second transplantation; 3, after the third transplantation. Statistical significance indicated by Student's *t*-test (\**P* < 0.05; \*\**P* < 0.01; and \*\*\**P* < 0.001). †Gene-modified cells were concentrated using the  $\Delta$ LNGFR marker. N/A, not applicable due to undetectable viral loads.

employ statistical analyses between pre and postinfusion for these two macaques. However, there was no increase and tendency toward decrease in the viral loads after transplantation of MazF-Tmac cells (Figure 2b). For rhesus macaques #16 and #17, significant increases in the set point of SHIV viral loads were observed upon transplantation of ZsG-Tmac cells (Figure 2b). A dramatic rebound was detected immediately after transplantation (Figure 2c). These data indicate that the transplantation of ZsG-Tmac cells, which have no protective payload for SHIV, significantly impacted the viral loads. No remarkable rebound was observed after transplantation of MazF-Tmac cells (Figure 2c).

#### Longitudinal persistence of MazF-Tmac cells and ZsG-Tmac cells

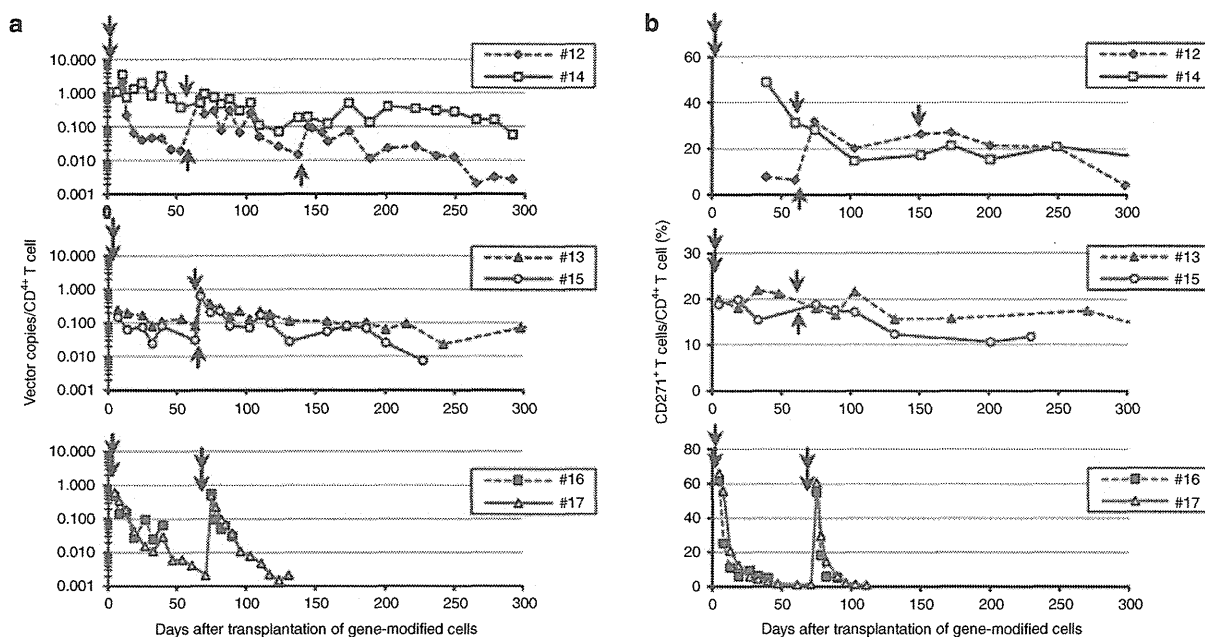
To examine the *in vivo* persistence of transplanted MazF-Tmac cells and ZsG-Tmac cells, peripheral blood samples were collected to monitor the presence of gene-modified cells. The proviral copy number of the transduced retroviral vector was monitored by the qPCR method throughout the experiment. MazF-Tmac cells persisted for more than 6 months in the presence of SHIV. In particular, persistence

for longer than one and a half years was observed in rhesus macaque #14. The ZsG-Tmac cells had nearly disappeared within two months after transplantation (Figure 3a). Similar results were obtained in flow cytometry analyses to detect the surface marker  $\Delta$ LNGFR in the CD4<sup>+</sup> T cells (Figure 3b). The calculated half-lives measured within 2 months of transplantation of the MazF-Tmac cells were much longer than that of the ZsG-Tmac cells (Table 2). The half-lives of the MazF-Tmac cells became even longer in the late phase of the experiment. Notably, in rhesus macaque #14, the half-life of the MazF-Tmac cells was 128.6 days when the period of analysis was extended to 9 months posttransplantation.

#### MazF antigen-specific interferon gamma (IFN- $\gamma$ ) enzyme-linked immunospot (ELISPOT) assay

To assess whether a cellular immune response was elicited by MazF-Tmac cells, an IFN- $\gamma$  ELISPOT assay was performed. Peripheral blood mononuclear cells (PBMCs) from the MazF-Tmac-transplanted rhesus macaques were stimulated with a cocktail of MazF-overlapping peptides (see **Supplementary Figure S2**). As a negative control, PBMCs prepared from a normal (*i.e.*, untransplanted) rhesus





**Figure 3** *In vivo* persistence of MazF-Tmac and ZsG-Tmac cells. (a) The persistence of MazF-Tmac cells and ZsG-Tmac cells was quantified using qPCR. The percentage of CD4<sup>+</sup> T cells was analyzed using flow cytometry, and the proviral vector copy number was analyzed using qPCR. Using these data, the copy number of the transgene in CD4<sup>+</sup> T cells was calculated. The arrows indicate the time point at which the gene-modified cells were transplanted. (b) The persistence of MazF-Tmac cells and ZsG-Tmac cells was quantified using flow cytometry. The percentage of gene-modified cells in the CD4<sup>+</sup> T-cell population was determined after expanding the PBMCs with anti-CD3/CD28 beads stimulation for seven days, followed by antibody staining for CD4 and CD271 surface markers. The arrows indicate the time points at which the gene-modified cells were transplanted.

**Table 2** The half-lives of gene-modified CD4<sup>+</sup> T cells in each transplantation

	MazF-Tmac transplantation				ZsG-Tmac transplantation	
	#12	#13	#14	#15	#16	#17
First transplantation	7.7	42.8	35.6	32.4	3.9	4.6
Second transplantation	14.9	57.6	33.8	51.3	4.2	6.9
Third transplantation	29.0	N/A	N/A	N/A	N/A	N/A

The *in vivo* half-lives of the gene-modified cells in each rhesus macaque within a period of two months after transplantation was calculated by linear regression analyses using Microsoft Excel software.

N/A, not applicable.

macaque were stimulated. As shown in Figure 4a, no significant cellular immune responses related to the MazF-specific antigens were observed, indicating that the infused MazF-Tmac cells did not elicit a cellular immune response in the rhesus macaques in the presence of SHIV infection.

#### Detection of antibodies against MazF or ZsGreen1 in rhesus macaque blood

The evidence of longitudinal persistence of the MazF-Tmac cells supports the idea that these cells are not highly immunogenic; however, it is still important to assess the production of antibodies against MazF. As shown in Figure 4b, no significant production of antiMazF antibodies was detected in the blood samples from any of the rhesus macaques after transplantation with the MazF-Tmac cells. For the ZsG-Tmac cells, significant production of antibodies against

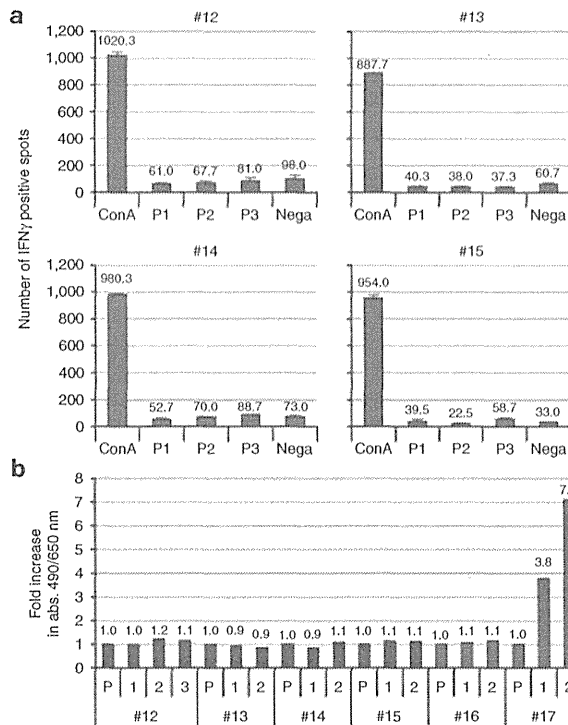
ZsGreen1 was detected in rhesus macaque #17, while no antibody production was detected in rhesus macaque #16. The MazF-Tmac cells persisted for an extended period *in vivo*, and the MazF-Tmac-transplanted rhesus macaques produced no antibodies against MazF, indicating that the infused MazF-Tmac cells can be considered safe and not immunogenic in rhesus macaques in the presence of SHIV infection.

#### Distribution of gene-modified cells

At autopsy, lymphocytes isolated from several organs were analyzed for the distribution of the gene-modified cells using flow cytometry and qPCR. As shown in Table 3, ΔLNGFR-positive cells were detected by flow cytometry in the CD4<sup>+</sup> T cells isolated from several lymph nodes, the spleen and the peripheral blood of the MazF-Tmac-transplanted rhesus macaques. A similar trend was observed in the qPCR analysis. The bone marrow, liver, and small intestine were also analyzed, but there were no detectable signs of gene-modified cells (data not shown). In the ZsG-Tmac-transplanted rhesus macaques, no gene-modified cells were detected in the organs or peripheral blood. These data strongly suggest that transplanted MazF-Tmac cells could circulate to the peripheral blood and the secondary lymphoid organs.

#### Histopathological analyses

It is advantageous to use primate models to investigate the safety of gene-modified cells because these animals can



**Figure 4** Detection of MazF-specific cellular and humoral immune responses. **(a)** IFN- $\gamma$  enzyme-linked immunospot assay. The number of IFN- $\gamma$  positive spots was measured under each stimulation condition. The PBMCs were prepared two months after the final MazF-Tmac cell transplantation. ConA: PBMCs from a MazF-Tmac-transplanted rhesus macaque were stimulated with concanavalin A. P1-3, PBMCs from a MazF-Tmac-transplanted rhesus macaque were stimulated with MazF peptide pools 1-3. Nega, nonstimulated PBMCs. Error bars represent the mean  $\pm$  SD. **(b)** Detection of MazF or ZsGreen1 specific humoral immune response using an enzyme-linked immunosorbent assay (ELISA). Plasma samples taken before transplantation (P), 4 weeks after the first transplantation, (1) 4 weeks after the second transplantation, (2) and 4 weeks after the third transplantation (3) of gene-modified cells were tested for the presence of antibodies against MazF in rhesus macaques #12, #13, #14, and #15 and for ZsGreen1 in rhesus macaques #16 and #17. The relative fold increases in the absorbance values compared with the pretransplantation values are shown.

be used for surgical pathological analyses. We performed experimental autopsies at the end of the experiment. The histopathological findings of the specimens are summarized in **Supplementary Table S2**. Severe involution of the thymus was observed in rhesus macaque #12. This involution appeared to be the result of a physiological factor, such as aging, and was considered to be unrelated to the treatment of transplantation of MazF-Tmac cells. No serious adverse events related to MazF-Tmac cell transplantation were observed in any of the rhesus macaques in this treatment group.

Rhesus macaques #16 and #17 were affected by SHIV infection or transplantation of ZsG-Tmac cells, which have no SHIV resistance payload. As shown in **Supplementary Figure S3**, several disorders were observed in the axillary lymph nodes of the ZsG-Tmac-transplanted macaques,

**Table 3** Distribution of gene-modified cells in lymphoid tissues

	MazF-Tmac transplantation							
	#12		#13		#14		#15	
	FCM (%) <sup>a</sup>	qPCR <sup>b</sup>	FCM (%) <sup>a</sup>	qPCR <sup>b</sup>	FCM (%) <sup>a</sup>	qPCR <sup>b</sup>	FCM (%) <sup>a</sup>	qPCR <sup>b</sup>
PBMC	4.1	2.9	15.4	18.0	4.8	11.1	11.8	26.4
Inguinal LN	N/A	3.0	13.2	16.8	4.2	8.1	7.3	17.1
Axillary LN	N/A	3.1	11.9	17.7	2.7	6.5	6.7	17.3
Mesenteric LN	N/A	0.54	13.0	18.2	4.0	10.1	5.6	14.3
Spleen	N/A	0.28	13.3	7.4	2.7	3.9	7.2	14.7

The lymphocytes isolated from several organs at autopsy were analyzed by flow cytometry and qPCR to determine the distribution of gene-modified cells.

FCM, flow cytometry; LN, lymph nodes; N/A, not applicable; PBMC, peripheral blood mononuclear cell.

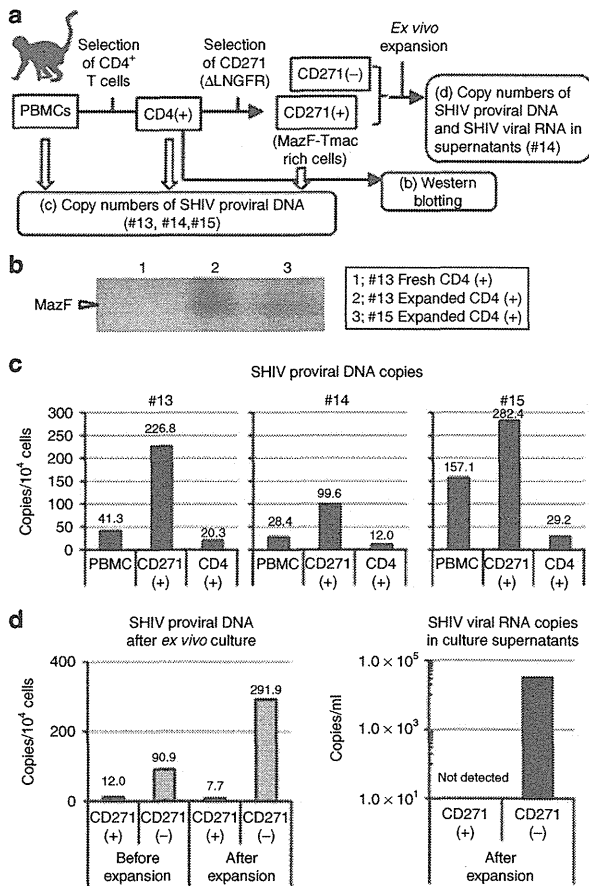
<sup>a</sup>The percentage of  $\Delta$ LNGFR-positive CD4<sup>+</sup> T cells was determined by flow cytometry. qPCR, <sup>b</sup>Proviral vector copy numbers per 10<sup>2</sup> CD4<sup>+</sup> T cells.

including a decrease in size, destruction of the structures, (see **Supplementary Figure S3a**) and a decrease in lymphoid cells (see **Supplementary Figure S3b**). These disorders were not observed in the MazF-Tmac-transplanted rhesus macaques. Rhesus macaque #16 was seriously affected by atrophy of the thymus, a decrease in lymphocytes in the inguinal and mesenteric lymph nodes, atrophy and lymphoid necrosis in the splenic marginal zone and a decrease in lymphoid cells in the periarterial lymphatic sheath and red pulp (see **Supplementary Table S2**). These observations were considered to be representative of changes relevant to the SHIV infection or transplantation of ZsG-Tmac cells. In rhesus macaque #17, atrophy of the thymus and a decrease in the lymphocytes in the inguinal lymph nodes were considered to be related to SHIV infection or the transplantation of ZsG-Tmac cells.

#### Function of persisting MazF-Tmac cells

To examine the Tat-dependent expression of MazF and its antiviral efficacy maintained in the rhesus macaques, CD4<sup>+</sup> T cells were isolated from the peripheral blood at 5 and 18 months after the first transplantation in rhesus macaques #13 and #15 and rhesus macaque #14, respectively. The expression of the MazF protein, the proviral copy numbers of SHIV and the production of SHIV in *ex vivo* culture were analyzed (**Figure 5a**).

A qPCR assay and Western blotting analysis were employed in PBMCs and isolated CD4<sup>+</sup> T cell samples from rhesus macaques #13 and #15 to detect the intact expression of MazF *in vivo*; however, there was no signal with either method. We speculate that the specific detection of MazF expression *in vivo* is challenging due to a limited number of MazF-Tmac cells in PBMCs and the low infectivity of SHIV *in vivo* compared with *in vitro* conditions. Next, the isolated CD4<sup>+</sup> T cells were expanded *ex vivo* for seven days, during which SHIV actively replicated, and Tat-dependent expression was expected to be induced in the MazF-Tmac cells in the CD4<sup>+</sup> T-cell population. The expression of MazF was observed in the expanded cells by Western blotting analysis (**Figure 5b**). Although the intact expression of MazF *in vivo* was below the detection limit by qPCR and Western blotting, the Tat-dependent conditional expression system was maintained long term after transplantation.



**Figure 5 Function of long-term persisting MazF-Tmac cells.** (a) Outline of the experiment. (b) CD4<sup>+</sup> T cells were isolated from the peripheral blood of MazF-Tmac-transplanted rhesus macaques five months after the first transplantation. The isolated CD4<sup>+</sup> T cells were expanded for seven days *ex vivo* and used for Western blotting analysis to detect the expression of MazF. (c) The CD271-positive and -negative cells were separated from the isolated CD4<sup>+</sup> T cells of rhesus macaques #13, #14, and #15. DNA samples were collected from the PBMCs, the CD4<sup>+</sup> T cells and the CD271<sup>+</sup>-MazF-Tmac-enriched population to analyze the proviral copy number of SHIV. (d) The CD271<sup>+</sup>-MazF-Tmac-enriched and CD271<sup>-</sup>-MazF-Tmac-negative populations isolated from rhesus macaque #14 were expanded *ex vivo* for 7 days. Changes in the SHIV proviral DNA levels in the cells and the SHIV RNA levels in the supernatants were analyzed in the CD271<sup>+</sup>-MazF-Tmac-enriched and CD271<sup>-</sup>-MazF-Tmac-negative populations.

The persisted MazF-Tmac cells, which expressed the surface marker  $\Delta$ LNGFR, were concentrated from a blood sample taken from rhesus macaques #13, #14, and #15 using an anti-CD271 monoclonal antibody. The number of proviral DNA copies of SHIV was measured in the PBMC population, CD4<sup>+</sup> T-cell population and CD271<sup>+</sup>-MazF-Tmac-enriched population. As shown in Figure 5c, the number of proviral SHIV copies in the CD271<sup>+</sup>-MazF-Tmac-enriched population was one log lower than that in the CD4<sup>+</sup> T-cell population in all three MazF-Tmac-transplanted rhesus macaques. To assess the function of the MazF-Tmac cells after transplantation, the CD271<sup>+</sup>-MazF-Tmac-enriched population

and CD271<sup>-</sup>-MazF-Tmac-negative population isolated from rhesus macaque #14 at 18 months after transplantation were expanded *ex vivo* for 7 days in the absence of anti-viral drugs. Changes in the SHIV proviral DNA level in the cells and the SHIV RNA level in the supernatants were analyzed. As shown in Figure 5d, the SHIV proviral DNA of the CD271<sup>-</sup>-MazF-Tmac-negative population increased by more than threefold after the expansion, indicating that SHIV was replicated in the CD271<sup>-</sup>-MazF-Tmac-negative population. The SHIV proviral DNA of the CD271<sup>+</sup>-MazF-Tmac-enriched population decreased slightly, indicating that replication of SHIV *ex vivo* was suppressed in the CD271<sup>+</sup>-MazF-Tmac-enriched population. The SHIV RNA copies accumulated in the supernatant of the CD271<sup>-</sup>-MazF-Tmac-negative population in the absence of antiviral drugs, while there were no detectable SHIV RNA copies in the culture supernatants of the CD271<sup>+</sup>-MazF-Tmac-enriched population. These data indicate that the MazF-Tmac cells (CD271<sup>+</sup> cells) are functional and possess the capability to suppress SHIV replication even one and a half years after transplantation.

## Discussion

MazF is an endoribonuclease that specifically cleaves ACA sequences in RNA.<sup>29</sup> Because there are more than 240 ACA sequences in HIV-1 RNA, HIV-1 should have almost no chance to gain a MazF-related escape mutation. Therefore, anti-HIV-1 gene therapy using MazF is an attractive strategy to suppress a broad spectrum of HIV-1. HIV-1 Tat-dependent conditional expression of MazF in CD4<sup>+</sup> T cells suppresses the replication of HIV-1 and SHIV 89.6P without affecting cellular mRNAs.<sup>21,23</sup> Because MazF is a bacterial protein and has never been tested in humans, it is important to assess the safety of the MazF-system *in vivo* using a nonhuman primate model. In a previous report, we showed the long-term persistence and safety of autologous transplantation of MazF-Tmac cells using cynomolgus macaques<sup>25</sup> that were not infected with a pathogenic virus. To obtain a better understanding of the MazF-modified CD4<sup>+</sup> T cells in the presence of a viral infection *in vivo*, rhesus macaques were infected with pathogenic SHIV 89.6P and transplanted with autologous MazF-Tmac cells.

An engraftment of 1–2% of gene-modified cells in the peripheral circulation has been reported after the transplantation of approximately 10<sup>10</sup> cells in adoptive T cell transfer gene therapy for humans,<sup>13</sup> and higher cell doses result in higher measurable engraftment levels.<sup>30</sup> We decided to transplant more than 10<sup>9</sup> cells in the primate model, reflecting one-tenth of the scale of the human gene therapy trials. To investigate the influence of repeated transplantations, transplantations were performed two or three times in this study.

The CD4<sup>+</sup> T cell count values of the SHIV-infected rhesus macaques increased after the transplantation of the MazF-Tmac cells with statistical significance, while such increases were not observed in the ZsG-Tmac-transplanted rhesus macaques. The infused MazF-Tmac cells persisted for a long period *in vivo*, with half-lives ranging between 7.7 and 58 days. It is also possible that some of the MazF-Tmac cells were vigorously infected by SHIV and killed. To gain a longer

therapeutic benefit from the infusion of gene-modified T cells, gene-modified T cells are expected to expand *in vivo*; however, MazF-Tmac cells did not preferentially expand *in vivo* in this experiment. We are currently addressing this issue and attempting to confer self-expansion capability on MazF-Tmac cells in the presence of viral infections.

The viral load of MazF-Tmac-infused macaques did not decrease dramatically. In general, the major roles of CD4<sup>+</sup> helper T cell are activation and regulation of the immune system. The helper T cell does not directly affect the viral load or infected cells as like cytotoxic T-lymphocytes or antibodies, so we speculate that the dramatic decrease of SHIV viral load was not observed though CD4<sup>+</sup> T cell counts increased.

We used six rhesus macaques, which were divided in two arms—four rhesus macaques for MazF-modified T cell-treated arm and two macaques for ZsGreen1-modified T cell-treated arm. Only one rhesus macaque in the MazF-modified T cell-treated arm was highly infected with SHIV; the other three were weakly infected. However, SHIV proviral DNA was detected in the PBMCs of all of the macaques, and SHIV reproduced in culture medium when collected PBMCs were expanded *ex vivo* (Figure 5). The primary purpose of this experiment was to confirm the safety and persistence of MazF-Tmac cells in the presence of SHIV infection; thus, we used not only high viral load macaque but also weakly infected macaques for this study. No immune responses related to MazF were observed, and half-lives were extended after the repeated transplantation. The evidence of longitudinal persistence of MazF-Tmac cells suggests that MazF-modified T cells are not highly immunogenic. Because MazF remains at a constant low level upon viral infection,<sup>23</sup> MazF-Tmac cells are unlikely to activate an immune response. To obtain more safety information on MazF-modified CD4<sup>+</sup> T cells in the presence of HIV infection, further investigations are needed, and a clinical trial entitled “A phase I, open label, dual cohort, single center study to evaluate the safety, tolerability and immunogenicity of autologous CD4 T cells modified with a retroviral vector expressing the *mazF* endoribonuclease gene in patients with HIV” is now ongoing in the United States (clinicaltrials.gov, identifier NCT01787994). Antiviral effect would also be assessed in the cohort 2 arm of this clinical trial.

In contrast, the infused ZsG-Tmac cells did not persist for an extended period *in vivo*, and the half-lives were not extended after repeated transplantation. The transient rebound of SHIV and marked decrease in the number of vector copies occurred simultaneously within 2 weeks after the transplantation of ZsG-Tmac cells; antiZsGreen1 antibodies developed gradually and reached their maximum level 40 days after the transplantation (see **Supplementary Figure S4**). We hypothesize that the ZsG-Tmac cells, which have no payload related to SHIV resistance, secreted a large amount of SHIV particles after infection and were destroyed by SHIV *in vivo*. The internalization of ZsGreen1 protein and antigen presentation by antigen-presenting cells induced the immune responses. In the case of rhesus macaque #17, ZsGreen1 protein might have been highly expressed by vigorous infection and triggered antibody production. In the case of rhesus macaque #16, ZsGreen1 protein was most likely expressed abundantly as in #17. Because this rhesus macaque exhibited

severely reduced CD4<sup>+</sup> T cell counts, we speculate that the B cells were not stimulated by Th2 effector cells due to a lack of CD4<sup>+</sup> helper T cells; thus, no antibodies against ZsGreen1 were detected in rhesus macaque #16.

To address whether MazF-modified CD4<sup>+</sup> T cells are associated with carcinogenicity *in vivo*, clonal expansion of the gene-modified cells was assessed using the linear amplification-mediated-PCR (LAM-PCR) method,<sup>31</sup> which traces the progeny of transduced cells by detecting the random insertion of the retrovirus or lentivirus vector. Although the preliminary data were collected from four macaques during a limited experimental period, there were no specific clonal expansions in any of the MazF-Tmac-transplanted rhesus macaques (see **Supplementary Figure S5**).

The histopathological analyses of the major organs, as well as the secondary lymphoid tissues, revealed that the transplantation of the MazF-Tmac cells was not associated with any carcinogenicity during the study period (see **Supplementary Figure S3** and **Supplementary Table S2**). A decrease in lymphocyte number was observed in the inguinal and axillary lymph nodes of the ZsG-Tmac-transplanted rhesus macaques (see **Supplementary Figure S3** and **Supplementary Table S2**). Although the transplanted ZsG-Tmac cells were not detected at the time of autopsy, the ZsG-Tmac cells might have migrated to the lymph nodes after transplantation, and the SHIV might have vigorously replicated in the migrated ZsG-Tmac cells, leading to cell death and, ultimately, bystander apoptosis of the neighboring uninfected cells and damage to the lymph nodes.<sup>32</sup> There was no such damage to the lymph nodes in rhesus macaque #12, which exhibited the highest SHIV viral loads and had undergone transplantation of MazF-Tmac cells three times.

Because gene therapy for HIV-1 should aim to reconstitute an HIV-1-resistant immune system, it is important for the gene-modified cells to not only inhibit viral replication but also maintain their distribution for long periods *in vivo*. Although the long-term persistence of gene-modified T cells or hematopoietic stem cells has been reported in the context of human gene therapy, it is difficult to obtain information about the distribution of these cells throughout the body. The use of primate models is advantageous for investigating the distribution pattern. At the time of the experimental autopsy, lymphocytes were isolated from the principal organs. MazF-Tmac cells were detected in the secondary lymphoid tissues, including several lymph nodes and the spleen, as well as the peripheral blood. A similar tendency was observed in previous data from cynomolgus macaques.<sup>25</sup> In contrast, ZsG-Tmac cells were not detected in any of the organs at the time of autopsy. The MazF-Tmac cells tend to persist primarily in the peripheral blood and secondary lymphoid tissues, regardless of the SHIV infection status.

The number of proviral SHIV DNA copies in the harvested CD271<sup>+</sup>-MazF-Tmac-enriched population was significantly lower than that in the CD4<sup>+</sup> T-cell population (Figure 5c). The exact mechanism of low SHIV copies in MazF-Tmac cells *in vivo* remains unclear. We examined the expression of coreceptor CXCR4 in gene-modified cells at the time of infusion, and no difference in expression levels was observed between the gene-modified and unmodified cells. One possible hypothesis is that the majority of the SHIV-infected CD4<sup>+</sup>