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A mucosal vaccine formulation against tuberculosis by exploiting the adjuvant activity of S100A4—a damage-associated molecular pattern molecule

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ABSTRACT

Mycobacterium tuberculosis (Mtb), the causative agent of tuberculosis (TB), remains one of the top three causes of death. Currently, the only licensed vaccine against TB is the bacillus Calmette-Guerin (BCG), which lacks efficacy in preventing and controlling pulmonary TB in adults. We aimed to evaluate a nasal TB vaccine formulation composed of the Mtb-specific vaccine antigen ESAT-6, an Mtb-associated protein that can trigger protective immune responses, and S100A4, a recently characterized novel mucosal adjuvant. Mice were intranasally given recombinant ESAT-6 in the presence or absence of S100A4 as an adjuvant. We have provided experimental evidence demonstrating that S100A4 admixed to ESAT-6 could induce Mtb-specific adaptive immune responses after intranasal immunization. S100A4 remarkably augmented the levels of anti-ESAT-6 IgG in serum and IgA in mucosal sites, including lung exudates, bronchoalveolar lavage fluid (BALF) and nasal lavage. Furthermore, in both lung and spleen tissues, S100A4 strongly promoted ESAT-6-specific expansion of CD4 T cells. Both CD4 and CD8 T cells from these tissues expressed increased levels of IFN-γ, TNF-α, and IL-17, cytokines critical for antimicrobial activity. Antigen-reencounter-induced T cell proliferative responses, a key vaccine performance indicator, were augmented in the spleen of S100A4-adjuvanted mice. Furthermore, CD8 T cells from the spleen and lung tissues of these mice expressed higher levels of granzyme B upon antigen re-stimulation. S100A4-adjuvanted immunization may predict good mucosal protection against TB.

Keywords: Tuberculosis; Mucosal vaccine; Adjuvant; ESAT-6; S100A4

1. INTRODUCTION

Mycobacterium tuberculosis (Mtb), the causative agent of tuberculosis (TB), is the world's leading cause of death by a single infectious agent [1]. TB is estimated to have infected a quarter of the world's population [2]. Despite advances in diagnosis and treatment, TB continues to cause enormous human suffering by presenting a significant disease with a substantial economic burden.

Vaccination is the most cost-effective strategy for preventing infectious diseases. Thus, effective vaccination strategies against this dreadful infectious disease are desperately needed. The only vaccine approved for human use against TB is the bacillus Calmette-Guerin (BCG), which has a protective efficacy against extrapulmonary forms of TB in children. However, BCG is notoriously variable in its efficacy for protecting adolescents and adults, who account for the majority of pulmonary TB transmission [3]. Due to BCG's lack of protection against pulmonary TB and its ineffectiveness in adults, other alternative TB vaccine candidates have been explored and tested, but with disappointing clinical trial results [4]. The currently available vaccination strategies are insufficient to eradicate TB. Therefore, new TB vaccine formulations that elicit improved protective immunity, especially for adults against the pulmonary form of TB, are warranted [5].

The Mtb virulence is closely correlated to the ability of the mycobacteria to produce and secrete a number of virulence factors. ESAT-6 is a well-known virulence factor that is important for Mtb to evade the immune system and persist in the host by various pathogenic mechanisms, including preventing the phagolysosome fusion and promoting phagosomal membrane rupture, processes that facilitate mycobacterial survival and escape from the phagosome [6]. ESAT-6 is essential for MTB to adhere to and cross the lung epithelial barrier [7, 8]. Since its discovery almost three decades ago, ESAT-6 has been explored in various platforms as a promising vaccine antigen [9, 10]. The absence of the virulence factor ESAT-6 is regarded as at least one of the factors accounting for the low efficacy of BCG in protecting against pulmonary TB [6]. As one of a few most immunodominant Mtb antigens, ESAT-6 can trigger a potent T cell response [11-13]. Engineered BCG expressing ESAT-6 and CFP-10 conveyed better protection against Mtb infection in mice and guinea pigs [14]. However, to enhance the immune response to ESAT-6, an

effective mucosal adjuvant is required, as vaccination with ESAT-6 alone is not sufficient to induce robust immune responses [9]. Unfortunately, only a limited number of adjuvants, including multi-component formulations and bacteria-derived toxins, have been evaluated for their use in TB vaccine development [15, 16].

The calcium-binding protein S100A4 is originally characterized for its endogenous role during oncogenesis by promoting cancer metastasis [17]. However, S100A4 has later been found to exhibit biological functions in many normal cell types [17, 18]. S100A4 interacts with cellular targets via at least the receptor for advanced glycation endproducts (RAGE) and TLR4, resulting in inflammatory responses [19, 20]. The immune regulatory function of S100A4 was first revealed when the role of this protein in allergic inflammation was identified using bioinformatics approaches and experimental validation [21]. Consistently, we have recently reported that S100A4 is required for mast cell activation [22]. The potential role of S100A4 in immunization was hinted when this protein was found to be required for maintaining the functionality of dendritic cells [23]. The possible contribution of this molecule to mucosal immunization is supported by the observation that S100A4 is critical for the maturation and development of microfold cells (M cells) [24], which play a key role in the antigen capture at mucosal surfaces and initiation of mucosal immune responses. We recently demonstrated that intranasal administration of S100A4 admixed to ovalbumin remarkably enhanced antigen-specific adaptive immune responses both at the mucosal compartment and circulation [25].

In this work, we designed a TB vaccine formulation by combining the well-characterized Mtb vaccine candidate ESAT-6 with the novel mucosal adjuvant S100A4. We have provided experimental evidence showing that this formulation could remarkably potentiate ESAT-6-specific humoral and cell-mediated immune responses in both respiratory mucosal tissues and systemic circulation after intranasal immunization of mice. Our work has broadened the knowledge base for further preclinical evaluation of S100A4 as a robust mucosal adjuvant in augmenting vaccine responses for TB and other disease models.

2. Materials and Methods

2.1. Experimental animals

Female C57BL/6 mice (6-8 weeks old) were used for this study. Animals were bred in-house at the Centralized Animal Facilities at the Hong Kong Polytechnic University with unrestricted access to food and water. The animal research was approved by the Animal Subject Ethics Sub-Committee of the Research Committee at The Hong Kong Polytechnic University. All animal procedures were carried out in accordance with institutional animal and bio-safety guidelines.

2.2. Immunization and collection of specimens

Mice were anesthetized with isoflurane before receiving the vaccine preparation for intranasal immunization. A 20-µl phosphate-buffered saline (PBS) solution containing the Mtb antigen ESAT-6 (5 µg; MyBioSource; MBS204541) in the presence or absence of S100A4 (10 µg; Gentaur Molecular Products; 01-2081A4M; with His-tag) was administered dropwise to the external nares of the mice (10 µl per nostril). Some mice were immunized with ESAT-6 (5 µg) admixed to cholera toxin (CT; 1 µg; List Biological Labs; 100B) as a control adjuvant. Ten days after the last intranasal immunization, various tissues and samples were collected and analyzed, including blood, bronchoalveolar lavage fluid (BALF), nasal lavage, lungs, and spleen.

2.3. Antigen recall responses

Lung and spleen cells (2×10^6 cells/ml) were seeded in a 96-well round-bottom plate in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4 mmol/l L-glutamine, 50 µmol/l 2-mercaptoethanol, 100 µg/ml penicillin/streptomycin (all from Sigma-Aldrich). ESAT-6 (2 µg/ml) was added to stimulate antigen-specific lymphocytes. Brefeldin A (eBioscience; 00−4506−51) was added to block the protein transport to facilitate intracellular protein measurement according to the manufacturer's instructions. After 6 h of antigen re-stimulation, the cells were harvested and washed with PBS to measure the intracellular production of cytokines and other cellular products. In some experiments, splenocytes were incubated with the ESAT-6 antigen for 72 h to measure cell activation, proliferation, and cytokine release.

2.4. Preparation and stimulation of mouse bone marrow-derived dendritic cells (BMDCs)

BMDCs were obtained by culturing bone marrow cells in complete RPMI 1640 (Thermo Fisher Scientific; 61870127) media in the presence of 200 ng/ml recombinant FLT3L (PeproTech; 250-31L) for nine days. For BMDC activation, cells were treated in vitro with ESAT-6 (1 μg/ml) alone or in the presence of S100A4 (1 μg/ml) at 37°C in 5% CO_2. CT (1 μg/ml) was used as a positive control.

2.5. Measurement of antigen-specific antibody

ESAT-6-specific antibody levels were measured using the enzyme-linked immunosorbent assay (ELISA). A 96-well flat-bottom ELISA microtiter plate (Nunc-Thermo Fisher Scientific; 467320) was coated overnight at 4°C with 100 µl PBS containing ESAT-6 (1.5 µg/ml). After overnight incubation, the ELISA plate was washed twice with a washing buffer containing 0.01% Tween-20. The plate was blocked by a blocking buffer (100 µl/well) containing 1% fetal bovine serum, followed by incubation for 1 h at 37°C. After washing, an appropriate volume of the sample was added, and the plate was incubated for 2 h at 37°C. The unbound primary antibody was removed by vigorous washing. Next, goat anti-mouse secondary antibodies for IgG (Southern Biotech; 1030-05), IgG1 (Southern Biotech; 1070-05), IgG2c (Southern Biotech; 1079-05) or IgA (Southern Biotech; 1040-05) conjugated with horseradish peroxidase was added, followed by standard color development. Absorbance measurement and titer calculation were carried out using a BMG SPECTROStar Nano microplate reader.

2.6. Measurement of released cytokines and chemokines

For the measurement of the production of cytokines and chemokines, cell-free culture supernatants were analyzed using the Bio-Plex multiplex system (Bio Plex™, Bio-Rad Laboratories; M60009RDPD) according to the manufacturer's protocol. The target cytokines and chemokines included TNF-α, IFN-γ, IL-4, IL-5, IL-10, IL-13, IL-6, IL-17, IL-1β, GM-CSF, G-CSF, RANTES, MIP-1β, MIP-1α, MCP-1 (MCAF), eotaxin, CXCL1 (KC), and IL-9. The data were acquired using a Bio-Plex 200 reader (Bio-Rad). The concentrations of each of the cytokines and chemokines were determined by comparing them with a standard curve, and the results were analyzed using Bio-Plex Manager software (Bio-Rad). Cytokines including IFN-γ, IL-12, IL-1β, IL-4, IL-5, and IL-6 in the blood circulation were measured using ProcartaPlex™

Multiplex Immunoassay (Thermo Fisher Scientific; EPX110-20820-901) according to the manufacturer's instructions.

2.7. Flow cytometric analysis

Cultured cells or single-cell suspensions prepared from various tissues were collected for flow cytometric analysis. CD4 T cells that could recognize the ESAT-6 peptide (QQWNFAGIEAAASA) presented by MHC class II were detected using a BV421-conjugated I-A^b/ESAT-6 tetramer (NIH tetramer core facility, Atlanta, USA). Cells were stained for 30 min at 37°C in darkness with the I-A^b/ESAT-6 tetramer. For staining cell surface markers, cells were incubated for 30 min on ice in darkness with fluorescent antibodies to mouse B220, CD3, CD4, CD8, CD11c, CD44, CD25, CD40, CD69, CD80, CD86, CD103, MHC class II, TLR2 and TLR4 (BD Biosciences or eBioscience). A dye (BD Horizon™ Fixable Viability 620) for discriminating live and dead cells was used to facilitate live cell gating. For the measurement of intracellular molecules, cells were first stained with antibodies against surface markers as described above, followed by fixation and permeabilization using the fixation buffer set (eBioscience™; 00-5523-00; or Thermo Fisher Scientific; 88882400) according to the manufacturer's instructions. Cells were then incubated for 30 min at room temperature in darkness with fluorescent antibodies to mouse IL-1β, IL-6, IFN-γ, TNF-α, IL-17, Ki-67, T-bet, and granzyme B (BD Biosciences or eBioscience). Cells were analyzed using a flow cytometer (BD FACSAria III), and data were analyzed using FlowJo software (Tree Star).

2.8. Extraction of total RNA, cDNA synthesis and RT-qPCR

Total RNA was extracted according to the manufacturer's instructions using the RNeasy Mini Kit (Qiagen; 74106). cDNA was synthesized using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific; K1622). The synthesized first-strand cDNA was used as a DNA template for RT-qPCR, which was carried out using the PowerTrack™ SYBR™ Green Master Mix (Applied Biosystems) in the ViiA7 Real-Time PCR System (Thermo Fisher Scientific; A25776). Briefly, the PCR reaction (10 µl), including the template and relevant primers, was started with incubation for 2 min at 50°C and another 2 min at 95°C, followed by 40 cycles of 95°C for 15 sec for denaturation and 60°C for 1 min for annealing/extension. Melt curve analysis was employed to confirm the specificity of the PCR reaction. The double delta Ct ($2^{-\Delta\Delta Ct}$) method was used to calculate the gene

expression levels of various samples. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference control. The forward and reverse primers for GAPDH were 5'-GGTGAAGGTCGGTGTGAACGGA-3' and 5'-TGTTAGTGGGGTCTCGCTCCTG-3'; for IL-1β 5'-GGAGAACCAAGCAACGACAAAATA-3' and 5'-TGGGGAACTCTGCAGACTCAAAC-3'; for IL-6 5'-CCACTTCACAAGTCGGAGGCTTA-3' and 5'-CCAGTTTGGTAGCATCCATCATTTC-3'; for IL-10 5'-GCCAGAGCCACATGCTCCTA-3' and 5'-GATAAGGCTTGGCAACCCAAGTAA -3'; for CD86 5'-TCCTGTAGACGTGTTCCAGA-3' and 5'-TGCTTAGACGTGCAGGTCAA-3'; for CD80 5'-GGTATTGCTGCCTTGCCGTT-3' and 5'-TCCTCTGACACGTGAGCATC-3'; for CD40 5'-GTTTAAAGTCCCGGATGCGA-3' and 5'-CTCAAGGCTATGCTGTCTGT-3'; for IL-12 5'-TGGGAGTACCCTGACTCCTG-3' and 5'-GGAACGCACCTTTCTGGTTA-3'; for IL-4 5'-ACGGGAGAAGGGACGCCA-3' and 5'-GAAGCCCTACAGACGAGCTCA-3'; for IFN-γ 5'-TAGCCAAGACTGTGATTGCGG -3' and 5'-AGACATCTCCTCCCATCAGCAG-3'; for IL-23 5'-TGCCCAGCCTGAGTTCTAGT-3' and 5'-AGACAGAGTTGCTGCTCCGT-3'; for TNF-α 5'-AAGCCTGTAGCCCACGTCGTA-3' and 5'-AGGTACAACCCATCGGCTGG-3'; for TLR2 5'-GCTGGAGGACTCCTAGGCT-3' and 5'-GTCAGAAGGAAACAGTCCGC -3'; and for TLR4 5'-GCTTTCACCTCTGCCTTCAC-3' and 5'-GAAACTGCCATGTTTGAGCA -3'

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software. The *P*-value was determined by Student's *t*-test, Mann-Whitney *U* test, or ANOVA with a multiple comparison test.

3. RESULTS

3.1. S100A4 as an adjuvant potentiates antigen-specific antibody production after intranasal immunization with the Mtb-derived antigen ESAT-6

Although cell-mediated immune responses are believed to be critical in controlling infection by the intracellular pathogen Mtb, there is evidence that humoral immunity also contributes to protection against TB [26, 27]. To confirm the adjuvant activity of S100A4 in enhancing antibody production, we immunized mice with ESAT-6, a candidate vaccine antigen derived from Mtb, alone or admixed to S100A4 or CT, three times at an interval of 10 days. Various mucosal tissue secretions and serum were collected to determine the levels of antibody production 10 days after the last immunization (Fig. 1A). ESAT-6-specific total IgG, IgG1, and IgG2c antibody levels were robustly augmented in the blood circulation of mice that received S100A4 admixed to ESAT-6 (Fig. 1B). Of note, S100A4 enhanced the production of ESAT-6-specific IgA and IgG antibody in the lung tissue exudate to a greater extent than the performance of CT for comparison as a benchmark adjuvant (Fig. 1C). Furthermore, intranasal administration of S100A4 substantially enhanced ESAT-6-specific IgA antibody production in BALF and nasal mucosa (Fig. 1D and E). The neutralizing role of IgA for blocking pathogens from breaching the epithelial barrier has been well defined [28]. IgA is predicted to be superior to IgG in preventing MTB infection [29]. Taken together, these findings demonstrate that ESAT-6-specific antibody production was profoundly elevated in both systemic and mucosal compartments, implying that S100A4 is a potent mucosal adjuvant with respect to antigen-specific humoral immune responses.

3.2. S100A4 as an adjuvant expands antigen-specific CD4 T cell responses after intranasal immunization with the Mtb-derived antigen ESAT-6

To determine whether S100A4 could expand CD4 T cells that recognized the immunizing Mtb antigen, we used a tetramer that is composed of an ESAT-6 peptide (QQWNFAGIEAAASA) in association with the MHC class II molecule on the C57BL/6 background (I-A^b/ESAT-6 tetramer) for the measurement of ESAT-6 peptide-specific CD4 T cells in the lung and spleen after immunization using flow cytometry (Fig. 1F). Administration of the immunizing antigen ESAT-6 admixed to S100A4 overwhelmingly enhanced the expansion of CD4 T cells that could recognize the MHC class II-restricted ESAT-6 peptide in both the lung and spleen tissues (Fig. 1G). The adjuvant quality

of S100A4 was superior to CT in light of T cell activation (Fig. 1G). In addition to profiling the expansion of ESAT-6-specific $CD4^+$ T cells, we measured the expansion of lung tissue-resident memory T cells in the lungs. S100A4 enhanced ESAT-6-specific $CD4^+$ tissue-resident memory T cell responses in the lungs (Supplementary Fig. 1). Of note, antigen-specific T cell accumulation in the lung tissue as a result of the use of S100A4 was at a much greater level than in the spleen. We have previously observed the much-augmented pulmonary antigen-specific T cell responses compared with the spleen reaction following nasal immunization with ovalbumin as an experimental vaccine antigen adjuvanted with S100A4 [25]. Vaccine delivery through the nasal route, in the presence of a robust mucosal adjuvant, has been recognized as an effective immune potentiation method to mobilize mucosal defence [30].

3.3. Immunization adjuvanted with S100A4 increases cytokine secretion in the blood circulation in response to intranasal immunization with the Mtb-derived antigen ESAT-6

The importance of cytokines in TB protection has been well established [31]. We thus analyzed whether S100A4-adjuvanted immunization could augment cytokine levels in the blood circulation. Immunization adjuvanted with S100A4 consistently increased the levels of a panel of cytokines, including IFN-γ, IL-1β, and IL-6 (Fig. 2). IL-12, IL-4, and IL-5 also demonstrated a trend of enhancement although not reaching a statistical level of significance (Fig. 2). These cytokines have various immune potentiating roles in adaptive immune responses. Of note, S100A4-mediated increases in cytokine levels were quite modest, probably due to a time lag of 10 days between the last immunization and the measurement. Furthermore, lung tissues were collected and processed to determine the mRNA expression of various cytokines. While the expression of TNF-α was remarkably enhanced, there was a strong trend of augmented expression of IFN-γ, IL-4, and IL-23 in the lung tissue of mice that received S100A4 (Supplementary Fig. 2).

3.4. Immunization adjuvanted with S100A4 enhances antigen reencounter-mediated T cell activation and proliferation

Given the above observed antigen-specific cellular immune responses by analyzing T cells directly taken from the mice after immunization (Fig. 1G), we next would like to demonstrate that these antigen-specific T cells were functional. To this end, primed splenocytes, which contained both T cells and antigen-presenting cells, were harvested from mice that had been

immunized. Splenocytes were re-stimulated ex vivo with the immunizing Mtb antigen ESAT-6 (Fig. 3A). Next, T cell activation and proliferation were analyzed using the gating strategies defined in Fig. 3B. The addition of ESAT-6 induced greater levels of splenic T cell proliferation, evidenced by increased expression of the proliferation marker Ki-67 in both CD8 and CD4 T cells if mice had received S100A4 as an adjuvant (Fig. 3C and D). Similarly, S100A4-adjuvanted immunization consistently increased the expression of the cell activation marker CD69 in splenic CD4 and CD8 T cells upon antigen reencounter (Fig. 3E and F). Next, cell activation and proliferation were simultaneously measured by gating on CD69 and Ki-67 double-positive cells (Fig. 3B). S100A4-mediated increase of $CD69^{+}Ki\text{-}67^{+}$ double positive CD4 and CD8 cells after antigen re-stimulation was observed (Fig. 3G and H). Furthermore, immunization of mice in the presence of S100A4 as adjuvant led to the generation of B cells with higher expression levels of Ki-67 and CD69 (Fig. 3I and J), indicating enhanced proliferation and activation of these cells upon reencounter with the immunizing antigen ESAT-6.

A hallmark feature of T cell activation is the active production of cytokines by T cells. We thus measured the intracellular production of cytokines, including TNF-α, IL-17, and IFN-γ, by T cells in the antigen recall response as described above, but with a 6-hour incubation after the addition of the immunizing antigen ESAT-6 (Fig. 4A). $CD4^{+}CD44^{+}$ and $CD8^{+}CD44^{+}$ memory cells were gated for analysis (Fig. 4B). Antigen-specific memory CD4 T cell-associated production of IFN-γ, TNF-α and IL-17 was enhanced in lung cells and splenocytes derived from mice that received ESAT-6 admixed to S100A4 (Fig. 4C). Expansion of antigen-specific polyfunctional CD4 T cells that simultaneously produced the pro-inflammatory cytokines IFN-γ, TNF-α, and IL-17 in the S100A4-adjuvanted mice was also observed (Supplementary Fig. 3). Next, we assessed ESAT-6-specific cytotoxic CD8 T cell-associated expression of pro-inflammatory cytokines. Increased production of IL-17, IFN-γ, and TNF-α was observed in CD8 T cells in both the lung and spleen tissues derived from mice immunized with ESAT-6 in the presence of S100A4 (Fig. 4D). Moreover, S100A4 enhanced the antigen reencounter-mediated production of granzyme B by memory cytotoxic T cells in both the lung and spleen tissues (Fig. 4D). The adjuvant activity of S100A4 was superior or at least comparable to CT (Fig. 4C and D). Production of IFN-γ and TNF-α in T cell responses is a strong predictor of effective T cell-mediated protection against intracellular pathogens including Mtb [32].

Furthermore, IFN-γ promotes the differentiation of cytotoxic T cells and induces antibody class switching of B cells.

Taken together, these findings suggest that S100A4 is a robust mucosal adjuvant that promotes the development of Th1 (IFN-γ and TNF-α), Th17 (IL-17), and CD8 (IFN-γ, TNF-α, IL-17 and granzyme B) T cells, which is fundamentally essential in controlling Mtb infection.

In addition to the measurement of intracellular cytokine production by flow cytometry (Fig. 4), we also undertook to confirm these findings by measuring the release of cytokines into the supernatants using a multiplex assay (Supplementary Fig. 4). Consistent with the intracellular cytokine assay, antigen reencounter-mediated production of Th1-associated cytokines, including TNF-α (Supplementary Fig. 4A) and IFN-γ (Supplementary Fig. 4B), was potentiated at a greater level if the mice had previously been immunized with ESAT-6 admixed to S100A4. Co-immunization with S100A4 elicited consistently increased production of Th2-associated cytokines, including IL-4, IL-5, IL-10, IL-13, IL-6, and IL-9 (Supplementary Fig. 4C to H). These cytokines are essential for enhancing Th2 effector functions, including B cell activation and antibody responses.

IL-1β production was also observed to be enhanced in antigen recall responses if mice had been immunized with S100A4 admixed to ESAT-6 (Supplementary Fig. 4I). IL-1β is a prominent pro-inflammatory cytokine required for the full-blown adaptive immune responses [33]. Moreover, S100A4 markedly increased antigen recall-induced production of GM-CSF (Supplementary Fig. 4J), a critical cytokine for restricting Mtb growth during infection [34]. Although there was a trend of antigen recall-mediated augmentation of G-CSF and IL-17 (Supplementary Fig. 4K and L), the increases have not reached statistically significant levels. S100A4 also modestly augmented the production of a number of chemokines in the antigen recall response, including MIP-1β, MIP-1α, RANTES, and MCP-1 (Supplementary Fig. 4M to P). Chemokines are crucial molecules in orchestrating the localization of T lymphocytes at the infection site following infection with Mtb [31]. Chemokines are also required for controlling granuloma formation during Mtb infection by regulating the activity of various innate and adaptive immune cells [35].

3.5. Immunization adjuvanted with S100A4 enhances activation of antigen-specific Th1 memory cells after re-stimulation ex vivo with the immunizing antigen ESAT-6 derived from Mtb

Both Th1 and Th17 cells are critical to the clearance of intracellular pathogens including Mtb [36]. Elicitation of the Th1 subset of T cells with strong IFN-γ responses to Mtb is a rational vaccine strategy to prevent clinical tuberculosis. To investigate whether S100A4 could promote the expansion of Th1 cells, we measured the expression of T-bet, the transcription factor for Th1 lineage differentiation, after immunization adjuvanted with S100A4. We stimulated splenocytes and lung cells isolated from immunized mice with the Mtb antigen as described in Fig. 4A. Antigen-specific $CD4^+$ Th1 cell-associated T-bet expression was determined by flow cytometry using the gating strategy defined in Fig. 5A and B. The frequencies of ESAT-6-specific T-bet-expressing $CD4^+$ Th1 cells were increased in the lung tissue isolated from immunized mice adjuvanted with S100A4 (Fig. 5C), although this increase was not observed in spleen T cells (Fig. 5D). Co-immunization with CT failed to promote the expression of T-bet by $CD4^+$ T cells in the lung tissue (Fig. 5C).

3.6. S100A4 potentiates dendritic cell activation in the presence of ESAT-6

The cross-talk between antigen-specific T cells and antigen-presenting cells is critical to the successful induction of adaptive immunity. Previously, we have demonstrated the potential of S100A4 in activating dendritic cells, the most important antigen-presenting cell type, in a model system without the presence of any pathogen-derived molecules [25]. The currently proposed vaccination model uses a pathogen-derived vaccine antigen (i.e., ESAT-6) to target Mtb. As ESAT-6, which is capable of interacting with TLR2 and TLR4 [37], might itself be capable of activating dendritic cells, which constitutively express both TLR2 and TLR [38], we would like to investigate whether S100A4 could further activate dendritic cells in the presence of ESAT-6. We first tried to determine whether S100A4 could upregulate the expression of these two receptors. For this purpose, $CD11c^+$ BMDCs were cultured to examine the expression of TLR2 and TLR4 (Fig. 6A). S100A4 augmented the expression of TLR2, but not TLR4, at the protein level (Fig. 6B). For the mRNA transcript expression, we could consistently show that S100A4 augmented the expression of TLR2, but not TLR4 (Fig. 6C).

Next, we investigated the effect of S100A4 in the presence of ESAT-6 on the activation of cultured BMDCs. While ESAT-6 modestly enhanced the expression of MHC class II and costimulatory

molecules, including CD86, CD80, and CD40, the addition of S100A4 further augmented the expression of these molecules (Fig. 7A). The expression of the pro-inflammatory cytokine IL-1β was also enhanced after treatment with S100A4 (Fig. 7A). A trend of IL-6 increase was also noted. Furthermore, the expression of costimulatory molecules, including CD80, CD86 and CD40, and the expression of a group of pro-inflammatory cytokines, including IL-12, TNF-α, IL-6, and IL-1β, were significantly upregulated at the mRNA level following incubation with S100A4 (Fig. 7B). The potency of S100A4 was either comparable or superior to CT (Fig. 7). Taken together, these data confirm that S100A4 was potent in further activating dendritic cells determined by the augmented expression of costimulatory molecules and cytokines in the presence of a pathogen-derived vaccine antigen.

4. DISCUSSION

The search for novel vaccine formulations with reliable protective efficacy to replace the currently licensed BCG vaccine for tackling TB remains one of the major challenges for the vaccinology community. In fact, vaccination with the BCG vaccine, which is available globally, can effectively control the extrapulmonary form of TB in children [39]. However, despite the widespread use of the BCG vaccine, TB remains one of the most devastating infectious diseases. This is assumed to be due to the ineffectiveness of BCG in protecting adults, especially against pulmonary infection. Over the last two decades, numerous vaccine candidates have been vigorously evaluated using various vaccine development platforms. Unfortunately, the results of recent clinical trials of several TB vaccine candidates are discouraging [4].

T cells are crucial in protecting against intracellular pathogens, such as Mtb. Therefore, T cell responses are an important measure for evaluating TB vaccine efficacy. For example, immunization with M72/AS01E improves protection efficacy against TB in clinical trials determined by the IFN-γ release assay, a measure of antigen-specific T cell responses [40]. Mucosal immunization with H56/CAF01, which contains Ag85B, ESAT-6, and Rv2660c as antigen together with the CAF01 adjuvant, induces polyfunctional CD4 T cells that localize to the lung parenchyma [41]. Pulmonary delivery of peptide nanofibers bearing an Ag85B CD4 T cell epitope increased the frequency of antigen-specific T cells in BCG-primed mice [42].

Furthermore, for achieving effective protection and long-lasting immune memory against intracellular pathogens such as Mtb, adjuvants that can modulate the host response toward Th1 cell-dependent immunity are demanded [16]. Therefore, many of the adjuvants currently under exploitation for augmenting the efficacy of anti-Mtb vaccines, including AS01E, IC31, GLA-SE, and CAF01, are being tested for their capacity in potentiating Th1-polarized effector cytokine responses [16]. Another formulation, H1/IC31, which contains the Mtb antigens Ag85B and ESAT-6, together with the adjuvant IC31, induces Th1-mediated immune responses with the Th1-associated effector cytokines [43]. Therefore, our data, which revealed a strong Th1-associated activity of S100A4, further support the relevance of S100A4 as a mucosal adjuvant in the vaccine formulation effective in protecting against Mtb infection.

In addition to Th1, Th17 cells have emerged as key players in protecting against mycobacterial infection [44]. Synergistically, Th1 and Th17 cells play a critical role in vaccine-mediated immunity by establishing anti-mycobacterial phagosomal activity to enhance host control against Mtb infection [45]. Various adjuvanted subunit vaccine formulations containing relevant Mtb antigens are shown to promote strong Th1/Th17 immune responses, conferring protection against Mtb infection [46, 47]. Our work has also revealed that S100A4 promoted Th17 cell responses, with increased levels of IL-17 expression in both systemic and respiratory mucosal compartments.

Cytotoxic T cells are central effector cells that directly combat intracellular pathogens. Activated cytotoxic T cells release a variety of effector molecules, including perforin and granzymes. Granzyme B is a toxic molecule essential for the potent killing of target cells by cytotoxic T cells [48]. Proinflammatory cytokines, particularly IFN-γ and TNF-α, are expressed by cytotoxic T cells, facilitating their killing capacity [49]. In this study, the fact that S100A4 could enhance antigen-specific cytotoxic T cell-associated expression of granzyme B and proinflammatory cytokines supports the capacity of S100A4 in driving the cytotoxic T cell-mediated clearance of intracellular pathogens such as Mtb.

Although the primary immunologic axis in developing TB vaccines points to cell-mediated immune responses, mounting evidence suggests that antibody responses also play a critical role

in controlling intracellular infection [50]. A number of studies have recently reported evidence of the protective potential of Mtb-specific IgG antibodies [51-53]. A variety of mechanisms underlying antibody-mediated protection against Mtb have been described, including antibody-dependent cell-mediated cytotoxicity (ADCC), enhancement of phagocytosis, neutralization, and inflammasome activation [54]. Antigen-antibody interaction can promote a rapid uptake, processing, and presentation of pathogen-derived antigens by antigen-presenting cells through Fc receptors [55]. In this study, we show that S100A4-adjuvanted mice displayed robust production of ESAT-6-specific IgG antibodies in the blood circulation and ESAT-6-specific IgA and IgG antibodies in mucosal tissues. These findings, having confirmed the role of S100A4 in augmenting robust humoral immune responses, also support the relevance of an S100A4-containing TB vaccine formulation. Furthermore, our data demonstrate that S100A4-adjuvanted immunization increased the production of antigen-specific Th1-dependent IgG2c and Th2-dependent IgG1 antibody subclasses, indicating effective activation of both Th1 and Th2 arms of immunity.

In conclusion, our work demonstrates that S100A4 is an effective mucosal adjuvant that augments robust antigen-specific humoral and cellular immune responses. The adjuvant effect size of S100A4 was comparable to or even better than that of CT, the gold standard experimental mucosal adjuvant, in most cases. Our data support the inclusion of S100A4 in a clinically applicable vaccine formulation, exemplified by robust S100A4-mediated adaptive immune responses against the immunizing Mtb antigen both systemically and in mucosal tissues.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Author contributions

OZ and ZX designed the research. OZ, SL, Y-WY, YW, ASC, NSL, and CD performed the experiment; all the authors contributed to data analysis. OZ and ZX wrote the paper. SL contributed substantially to the manuscript revision. All authors approved the final version of the paper.

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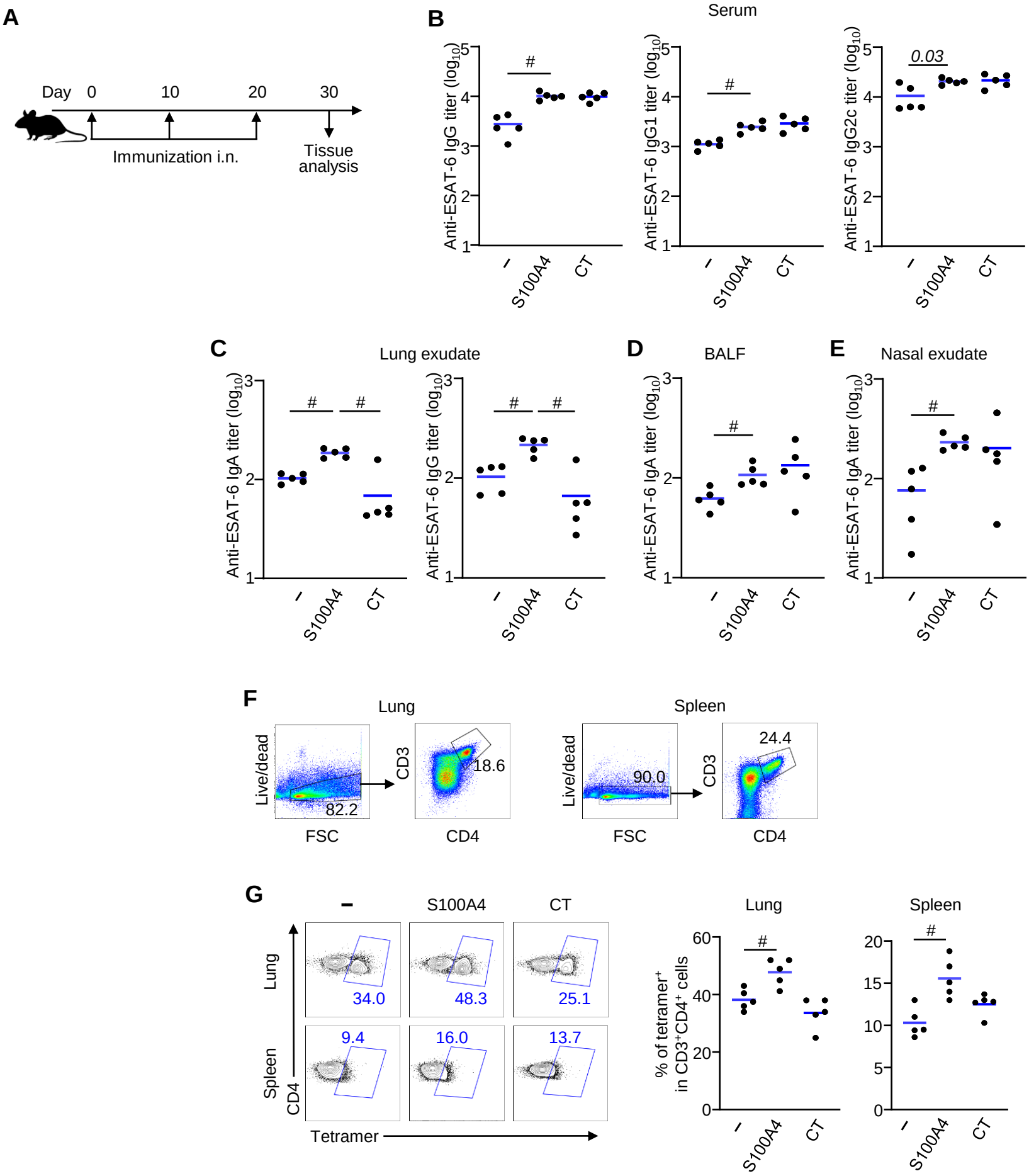
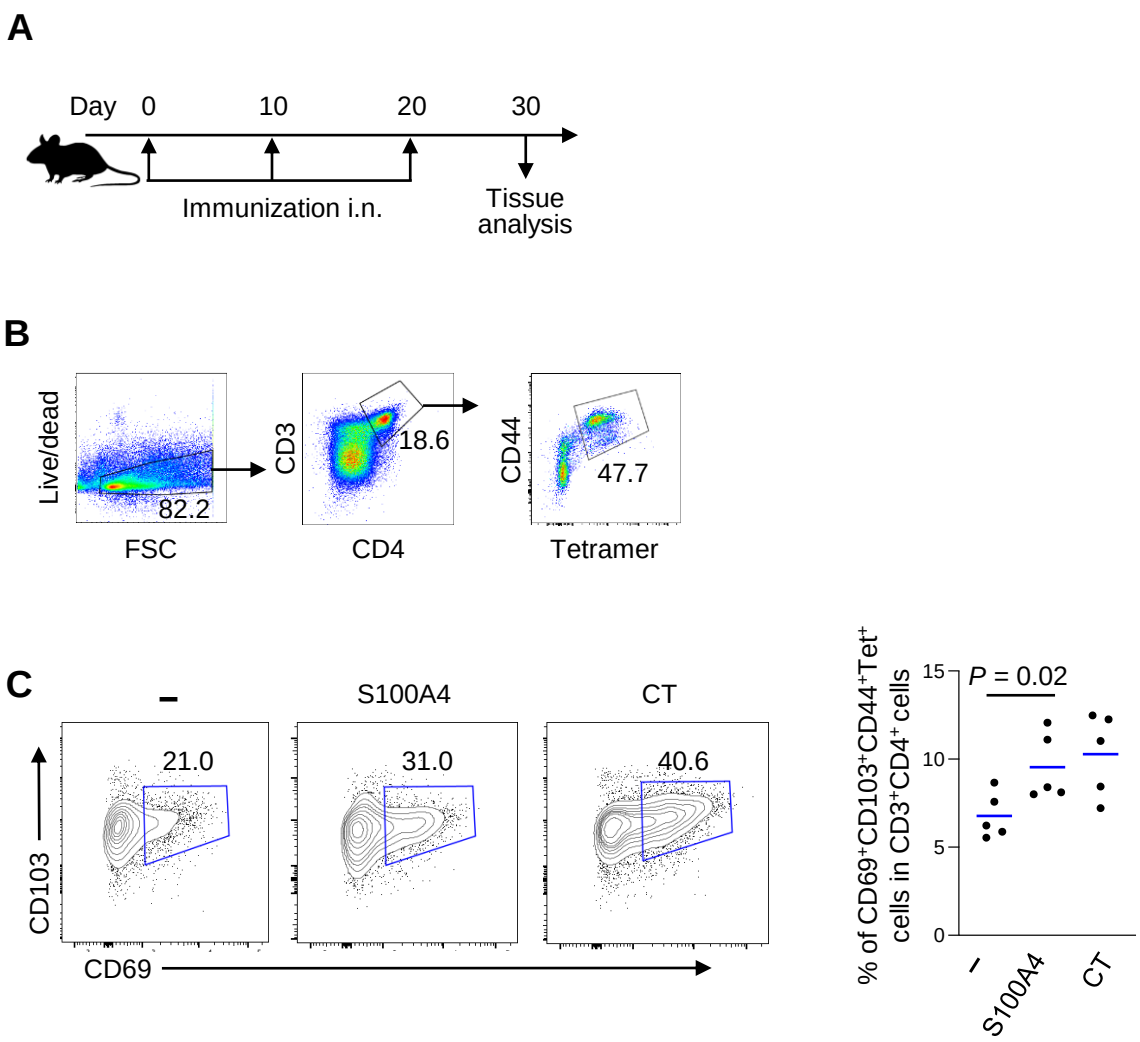


Fig. 1. S100A4 potentiates humoral and cellular immune responses after intranasal immunization with the Mtb-derived antigen ESAT-6. Mice were intranasally (i.n.) immunized with ESAT-6 (5 μg) alone or admixed to S100A4 (10 μg) or cholera toxin (CT; 1 μg) three times at an interval of 10 days. Various samples were harvested 10 days after the last immunization for analysis (A). Levels of ESAT-6-specific total IgG, IgG1 and IgG2c in serum (B), ESAT-6-specific IgA and total IgG in lung exudates (C), and ESAT-6-specific IgA in bronchoalveolar lavage fluid (BALF) (D) and nasal exudates (E) were measured using ELISA. Mouse lungs and spleen were harvested for flow cytometric analysis. Gating strategies for identifying CD4 T cells from the lung and spleen tissues are indicated (F). Frequencies of I-A^b/ESAT-6-specific $CD4^+$ T cells were examined, and representative contour plots indicate results from a single mouse in each treatment condition (G; left panels). Pooled results from all the mice are plotted (G; right panels). Numbers adjacent to outlined areas indicate percent cells in each gate. Each dot represents measurement from a single mouse, and blue lines indicate the average values. #*P* = 0.007 or the exact *P*-value (italic number) is determined by Mann-Whitney *U* test.



Supplementary Fig. 1. S100A4 enhances tissue-resident memory (TRM) T cell responses in the lung after intranasal immunization with the Mtb-derived antigen ESAT-6. Mice were intranasally (i.n.) immunized with ESAT-6 (5 μg) alone or admixed to S100A4 (10 μg) or cholera toxin (CT; 1 μg) three times at an interval of 10 days. Lung tissues were harvested 10 days after the last immunization for analysis using flow cytometry (A). Expansion of the antigen-specific memory cells (tetramer$^+$CD44$^+$) was first gated (B), followed by further gating based on CD69 and CD103 for analyzing TRM T cells (CD44$^+$CD69$^+$CD103$^+$) and representative contour plots from a single mouse in each treatment condition are shown (C; left panels). Pooled results from all the mice are plotted for showing the frequencies of tetramer (Tet)$^+$CD44$^+$CD69$^+$CD103$^+$ cells (C; right panel). Numbers adjacent to outlined areas indicate percent cells in each gate. Each dot represents measurement from an individual mouse, and blue lines indicate the average values. The *P*-value is determined by Student's *t*-test.

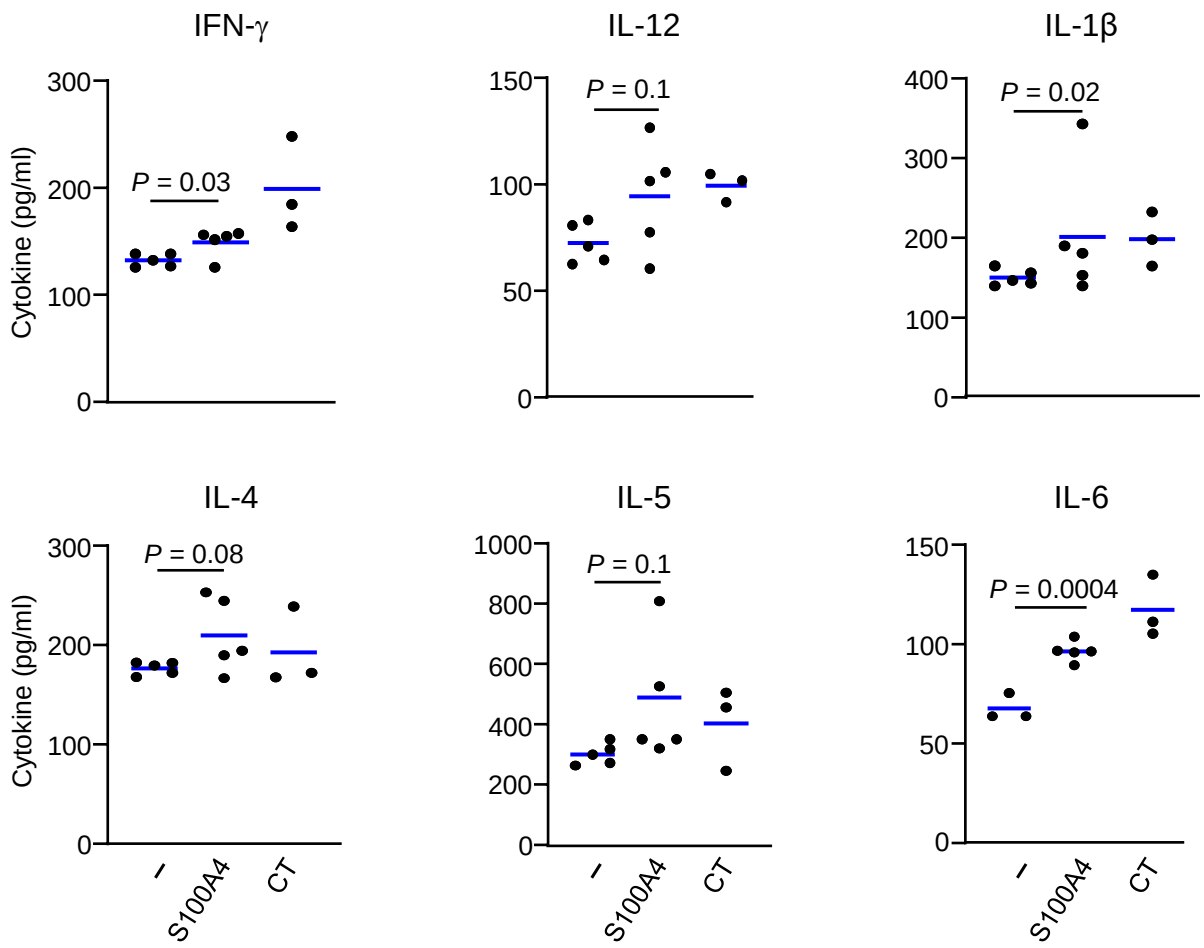
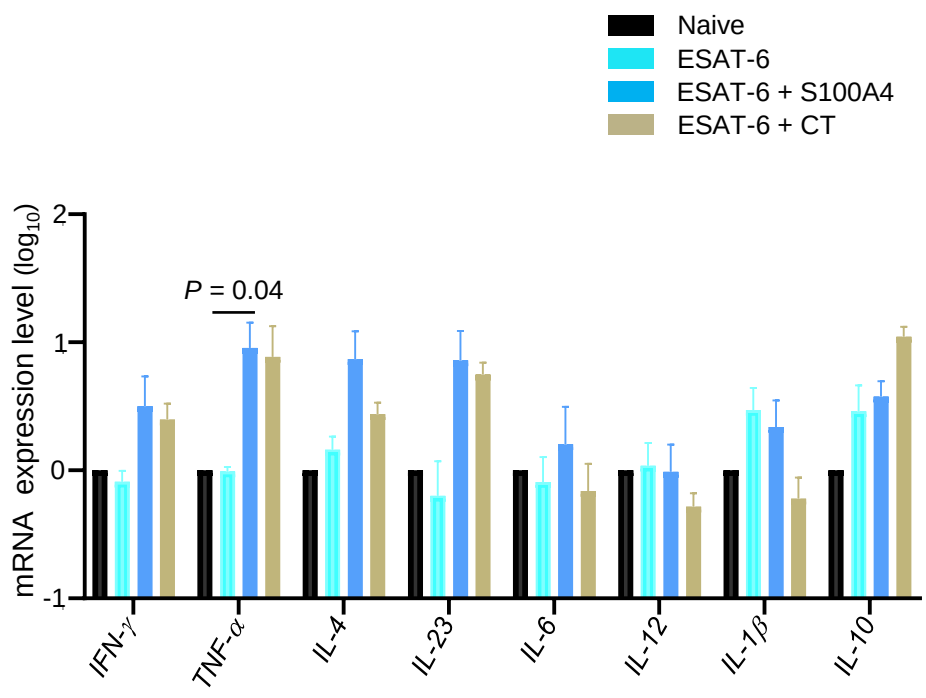


Fig. 2. S100A4 upregulates cytokine secretion in the blood circulation in response to intranasal immunization with the Mtb-derived antigen ESAT-6. Mice were intranasally (i.n.) immunized three times as described in Fig. 1A. Mouse blood was collected 10 days after the last immunization for analysis. Serum levels of various cytokines as indicated were determined using the Bio-Plex multiplex assay. Data were analyzed using Bio-Plex Manager software. The average values are represented by blue lines, and each dot indicates measurement from a single mouse. The *P*-values are determined by Student's *t*-test.



Supplementary Fig. 2. Immunization adjuvanted with S100A4 induces the expression of cytokines in lung tissues after immunization with the Mtb-derived antigen ESAT-6. Mice were intranasally (i.n.) immunized three times as described in Fig. 1A. Mouse lung tissues were harvested and processed for the measurement of mRNA transcript expression of relevant cytokines as indicated. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the calibrator gene to normalize the expression of genes of interest. Data are expressed as mean + SEM. The *P*-value is determined by ANOVA with a multiple comparison test.

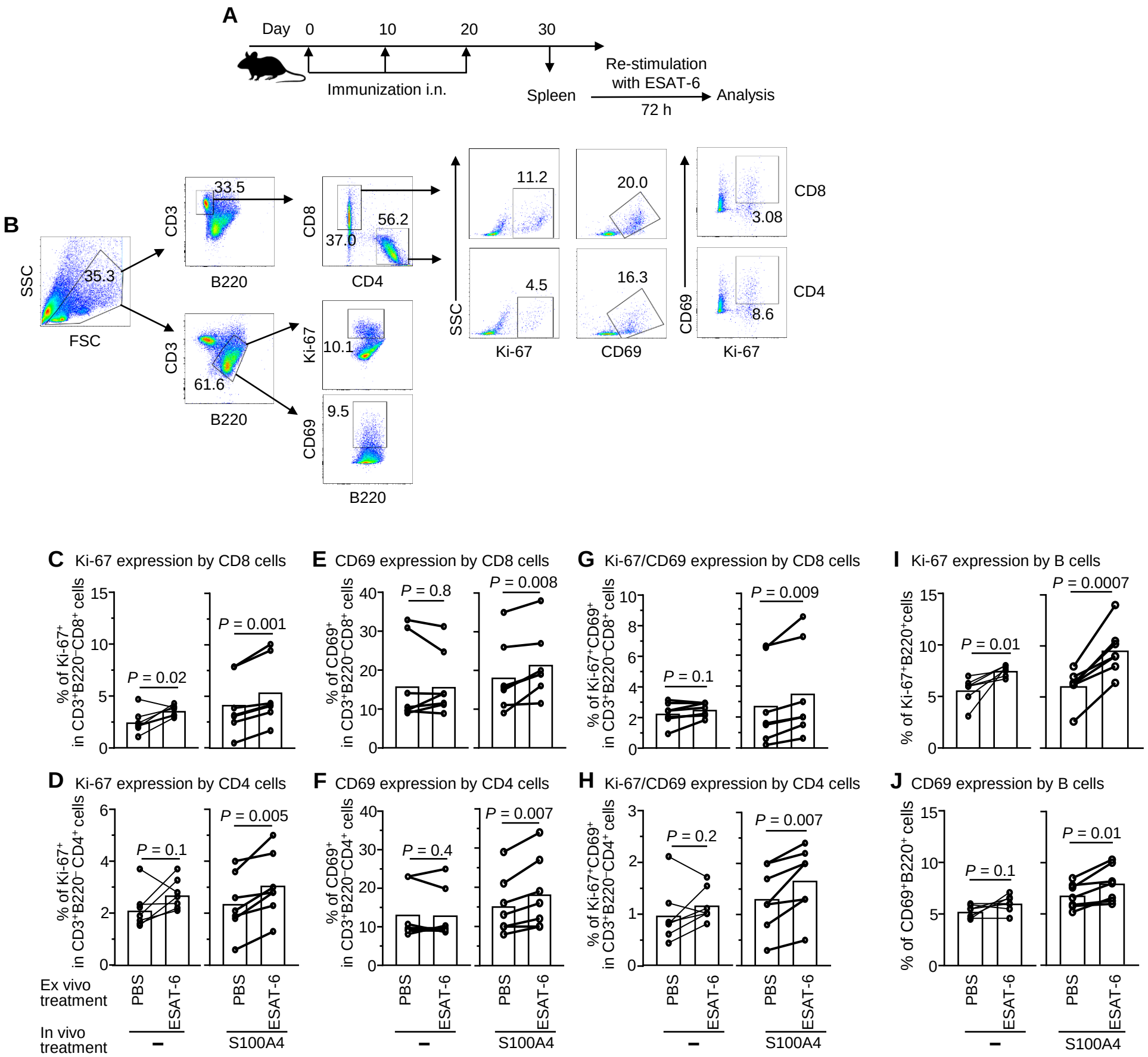


Fig. 3. Immunization adjuvanted with S100A4 enhances the proliferation and activation of spleen T cells after re-stimulation ex vivo with the immunizing Mtb-derived antigen ESAT-6. Mice were intranasally (i.n.) administered with ESAT-6 (5 μg) in the absence or presence of S100A4 (10 μg) three times at an interval of 10 days. Splenocytes were treated with or without ESAT-6 (2 μg/ml) for 72 h (A). $CD8^+$ and $CD4^+$ T cells and $B220^+$ B cells were gated for assessing their expression of Ki-67, a cell proliferation marker, as well as CD69, a cell activation marker; representative flow cytometry gating strategies are shown (B). The expression levels of various markers in different cell types are indicated (C to J). Numbers in or adjacent to outlined areas indicate percent cells in each gate. Each line represents measurement of a single mouse, and columns indicate the average values (C to J). The *P*-values are determined by Student's *t*-test.

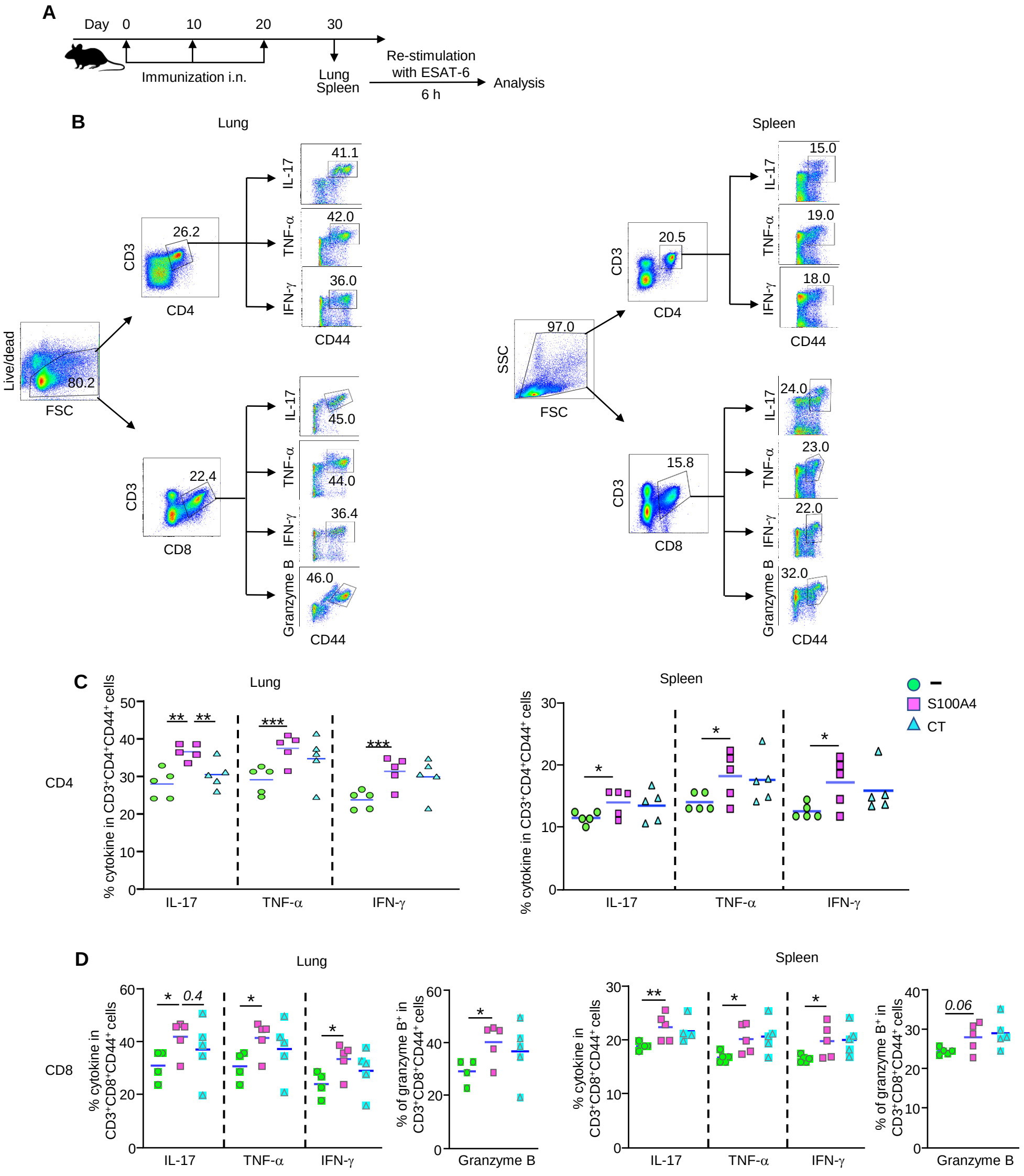
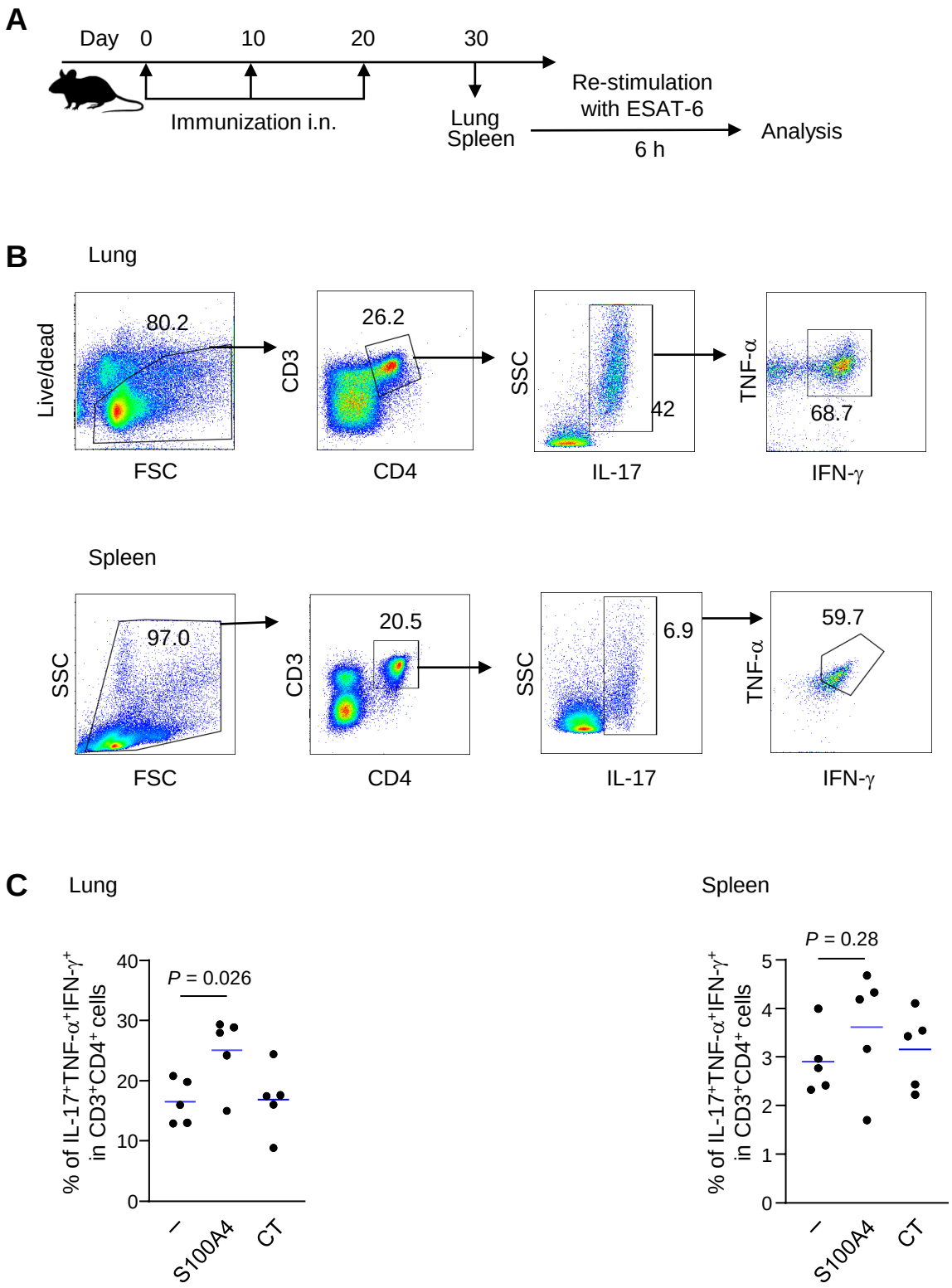
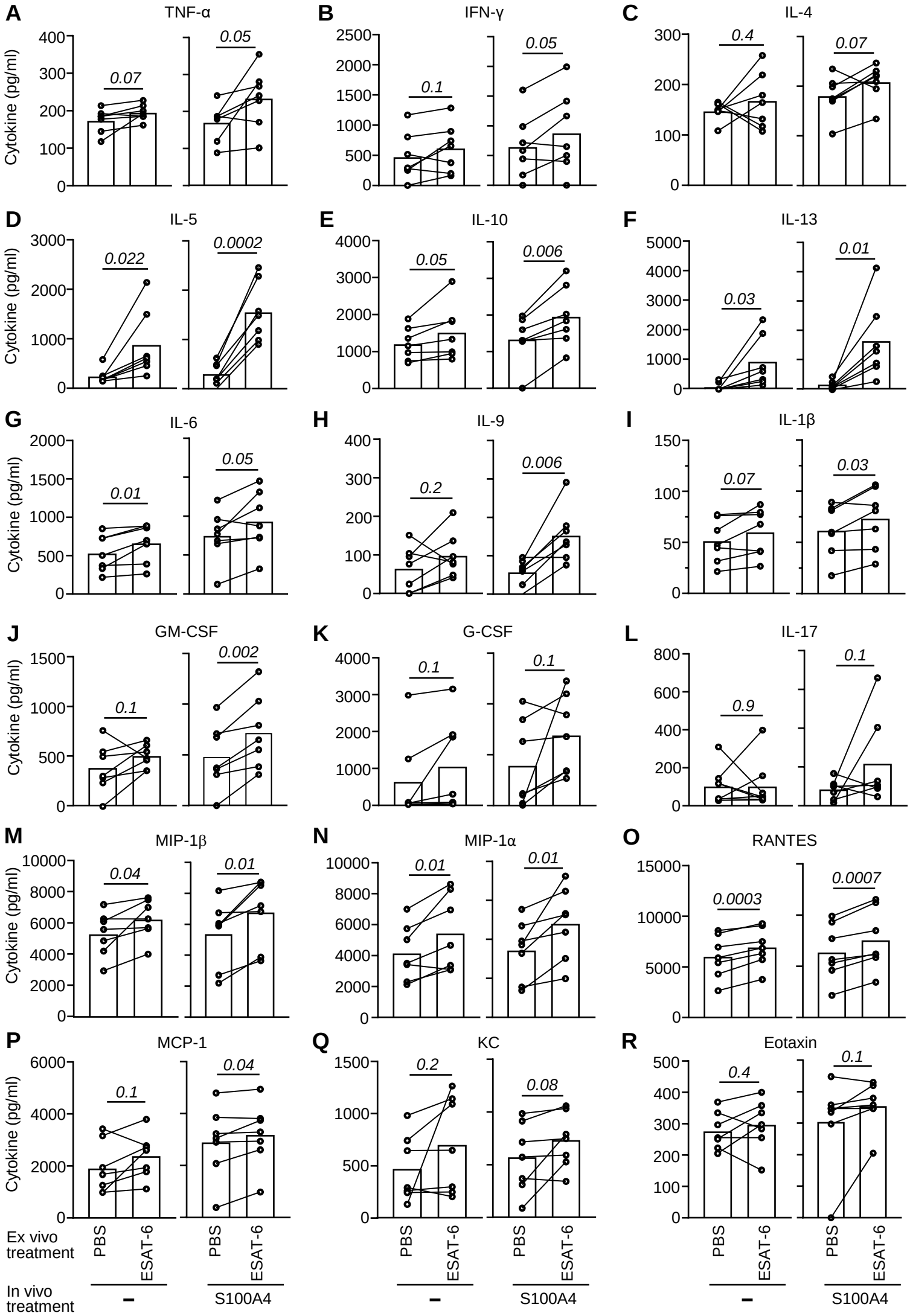


Fig. 4. Immunization adjuvanted with S100A4 promotes T cell cytokine production upon re-stimulation with the immunizing Mtb-derived antigen ESAT-6. Mice were intranasally (i.n.) administered with ESAT-6 (5 μg), or ESAT-6 admixed with S100A4 (10 μg) or cholera toxin (1 μg) three times at an interval of 10 days. Mouse lungs and spleens were harvested 10 days after the last immunization for single-cell preparation. Lung and spleen cells were treated with ESAT-6 (2 μg/ml) for 6 h (A). Frequencies of T cells that produced various types of cytokines and granzyme B were measured using flow cytometry (B). Pooled results from all the mice are shown (C and D). Numbers in or adjacent to outlined areas indicate percent cells in each gate. Each symbol represents data from an individual mouse and blue lines indicate the average values. *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$ or the exact P-values (italic numbers) are determined by Student's t-test.



Supplementary Fig. 3. Immunization adjuvanted with S100A4 promotes cytokine production by polyfunctional CD4 T cells upon re-stimulation with the immunizing Mtb-derived antigen ESAT-6. Mice were intranasally (i.n.) administered with ESAT-6 (5 μg), or ESAT-6 admixed with S100A4 (10 μg) or cholera toxin (1 μg) three times at an interval of 10 days. Mouse lungs and spleens were harvested 10 days after the last immunization for single-cell preparation. Lung and spleen cells were treated with ESAT-6 (2 μg/ml) for 6 h (A). Frequencies of T cells that simultaneously produced IL-17, TNF-α and IFN-γ were determined using flow cytometry with gating strategies shown (B). Pooled results from all the mice are shown (C). Numbers in or adjacent to outlined areas indicate percent cells in each gate. Each symbol represents data from an individual mouse and blue lines indicate the average values. Statistical examination was conducted using Student's t-test.



Supplementary Fig. 4. S100A4 augments cytokine and chemokine secretion by splenocytes after re-stimulation ex vivo with the immunizing Mtb-derived antigen ESAT-6. Mice were intranasally (i.n.) immunized three times and spleen tissues were treated as described in Fig. 3A. Concentrations of secreted cytokines and chemokines as indicated in the splenocyte culture supernatants were determined using the Bio-Plex multiplex assay (A to R). Each line represents data from a single mouse, and each column indicates the average values. The *P*-values (italic numbers) are determined by Student's *t*-test

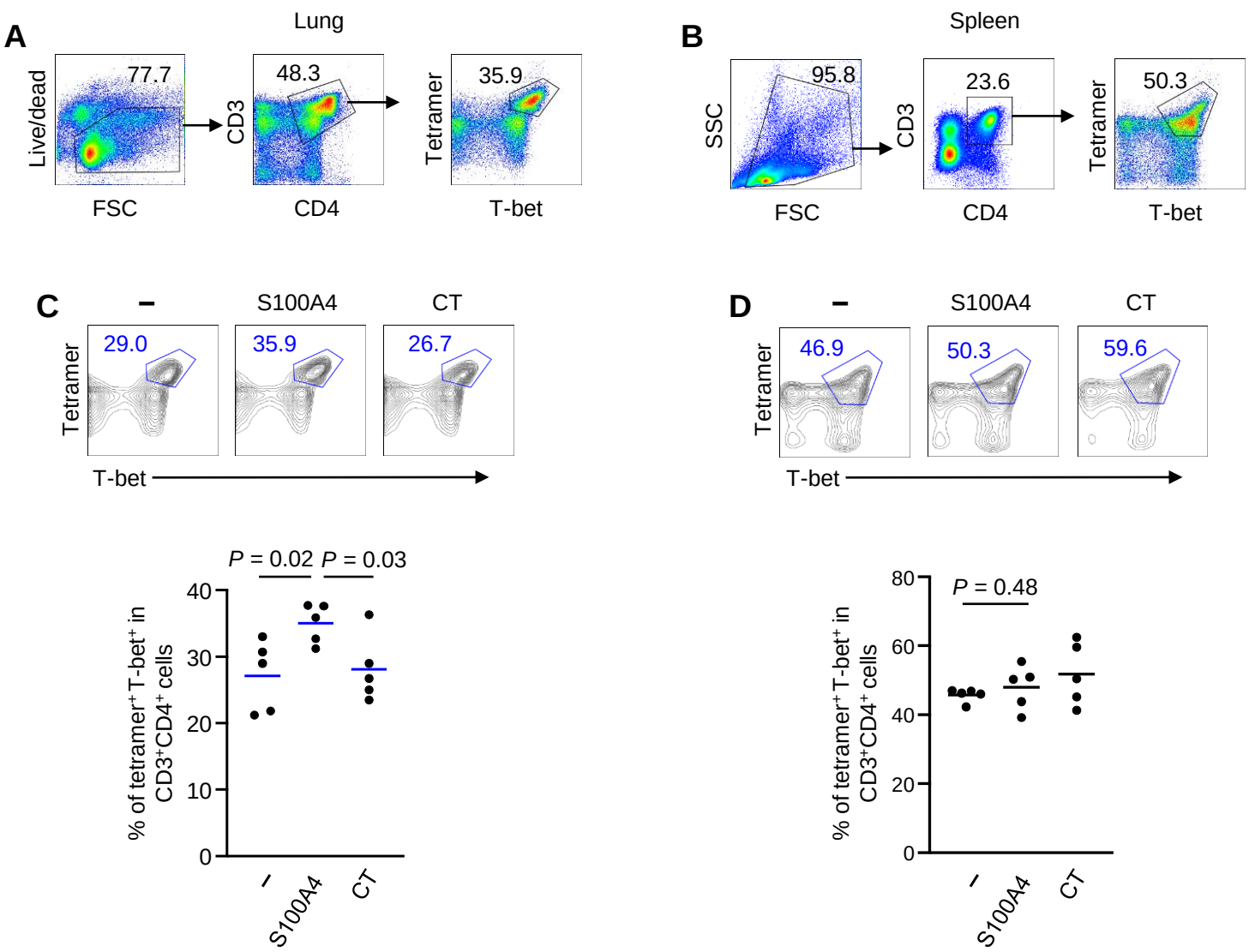


Fig. 5. S100A4 enhances activation of antigen-specific Th1 cells after re-stimulation ex vivo with the Mtb-derived antigen ESAT-6. Mice were intranasally (i.n.) immunized three times and tissues were treated as described in Fig. 4A. T cell recognition of the ESAT-6 peptide (QQWNFAGIEAAASA) was determined by using an I-A^b/ESAT-6 peptide tetramer. Frequencies of I-A^b/ESAT-6 peptide-specific $CD4^+$ T cells that expressed the Th1 cell transcription factor T-bet from the lung (A) and spleen (B) cultures were gated using flow cytometry. Representative contour plots indicate analysis of a single mouse in each treatment condition (upper panels) and pooled results from all the mice (lower panels) in the lung (C) and spleen (D) are shown. Numbers adjacent to outlined areas indicate percent cells in each gate. Each dot represents measurement from a single mouse, and blue lines indicate the average values. The *P*-values are determined by Student's *t*-test.

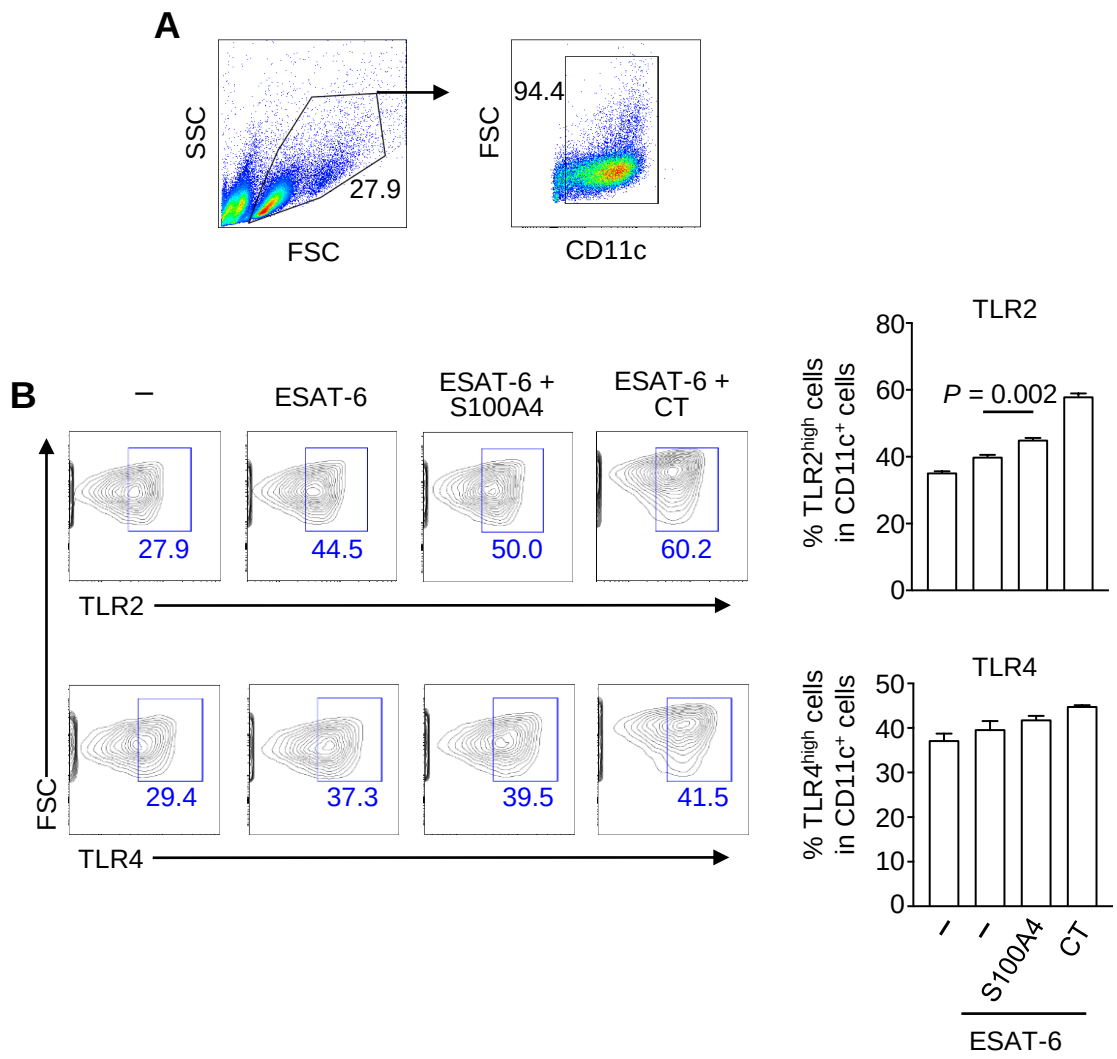


Fig. 6. S100A4 augments the expression of TLR2 on dendritic cells. Mouse bone marrow cells were cultured in the presence of Flt3-L for nine days to obtain bone marrow-derived dendritic cells (BMDCs) confirmed by CD11c expression using flow cytometry (A). (B and C) BMDCs were treated overnight (B) or for 3 h (C) with or without ESAT-6 (1 µg/ml), or with ESAT together with S100A4 (1 µg/ml) or cholera toxin (CT; 1 µg/ml). The expression of TLR2 and TLR4 by dendritic cells was analyzed using flow cytometry (B). Representative contour plots based on one experiment (left panels) and pooled results from three separate experiments (right panels) are shown, and numbers adjacent to outlined areas indicate percent cells in each gate (B). Total RNA was extracted to measure the mRNA expression of TLR2 and TLR4 using RT-qPCR (C). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a calibrator gene to normalize gene expression (C). Data are presented as mean + SEM of three separate experiments. The *P*-values are determined by Student's *t*-test.

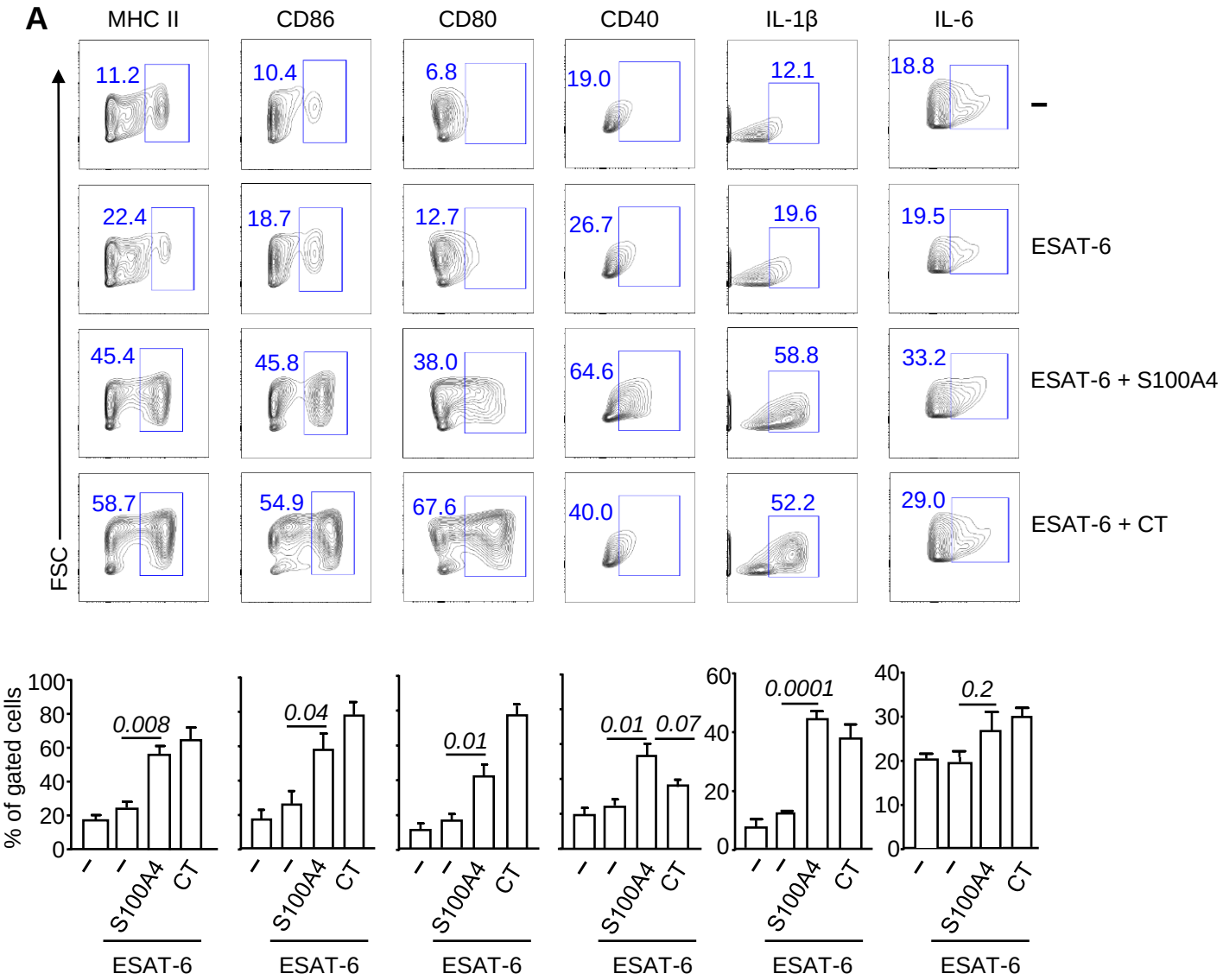


Fig. 7. S100A4 augments the expression of costimulatory molecules and pro-inflammatory cytokines by dendritic cells. Mouse bone marrow cells were cultured as described in Fig. 6 for obtaining bone marrow-derived dendritic cells (BMDCs). (A) BMDCs were treated overnight with or without ESAT-6 (1 μg/ml), or with ESAT-6 together with S100A4 (1 μg/ml) or cholera toxin (CT; 1 μg/ml). Dendritic cell activation was determined by measuring the expression of MHC class II (MHC II), costimulatory molecules including CD86, CD80 and CD40, and pro-inflammatory cytokines, including IL-1β and IL-6, using flow cytometry. Representative contour plots based on one experiment (upper panels) and pooled results from three biological replicates (lower panels) are shown. Numbers adjacent to outlined areas indicate percent cells in each gate. (B) BMDCs were treated for 3 h with ESAT-6 (1 μg/ml) alone or together with S100A4 (1 μg/ml) or CT (1 μg/ml). mRNA transcript expression of various costimulatory molecules and pro-inflammatory cytokines as indicated were measured using RT-qPCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the calibrator gene to normalize gene expression. Data are presented as mean + SEM of three biological replicates. The *P*-values (italic numbers) are determined by Student's *t*-test.