

***Mycobacterium tuberculosis* infection prevents asthma and abrogates eosinophilopoiesis in an experimental model**

To the Editor,

Tuberculosis (TB) is one of the top 10 causes of death worldwide, with 10 million new cases and 1.5 million associated deaths according to WHO TB Report 2018. TB in humans is mostly caused by *Mycobacterium tuberculosis* (Mtb). Following Mtb entry within the host by the respiratory airways, the pathogen colonizes and multiplies inside lung macrophages. In 90% of the cases, infection is controlled, although not eliminated, when TB-specific T cells reach the lungs and produce proinflammatory cytokines that trigger macrophage activation. TB control is associated with a Th1 response, and indeed, individuals with polymorphisms in Th1-associated genes such as *IFNG*, *IL12A/B*, or *STAT1* present a higher susceptibility to develop active TB. Equilibrium between human immune system and bacterial virulence factors drives to formation of granulomas, structures conformed by a nucleus of infected macrophages surrounded by a barrier of lymphocytes that restrict bacterial spread. At this state, Mtb infection is in a latent stage (LTBI), and it is asymptomatic¹.

Since many types of allergies, including asthma, have been typically linked with a Th2 response, it has been speculated for years that mycobacterial infections could confer unspecific protection against this type of pathologies. This has been widely revised in the context of BCG vaccination, with controversial studies showing opposite conclusions². With respect to TB disease, some epidemiological studies have identified a significant inverse correlation between asthma prevalence and TB notification cases in countries where standardized data are available³. Regarding LTBI, a study performed in South Africa demonstrated a strong association between tuberculosis skin test (TST) positivity and lower prevalence of different types of allergy. Interestingly, in the case of asthma, authors found a substantial reduction in asthma incidence in the segment of population with the highest TST value analyzed in the study (>20 mm), suggesting a correlation between magnitude of the TB-specific immune response and degree of unspecific protection⁴. Beyond these observational studies, to our knowledge correlation between virulent Mtb infection and unspecific asthma protection has never been tested in animal models. In the present study, we evaluated whether presence of pulmonary Mtb affects allergen-induced airway eosinophilia in an ovalbumin-driven asthma mouse model.

Inbred mice C57BL/6 are considered resistant to TB⁵. They tolerate high lung bacterial burdens during long periods of time without presenting any symptomatology. We intranasally challenged OVA-sensitized C57BL/6 mice with a low dose (150 CFU) of the Mtb reference strain H37Rv. Four weeks later, lung eosinophilia was

induced by intranasal OVA administration during three consecutive days (Figure 1A). At this time point after infection, lung bacterial replication is efficiently controlled by host immune system in immunocompetent mice (Figure S1), and therefore, this scenario would be comparable to a latent infection. Massive lung eosinophil infiltration was observed by hematoxylin-eosin staining (Figure 1B). Eosinophils were quantified in bronchoalveolar lavages (BAL) and lungs by flow cytometry (Figures 1C, S2). Our results demonstrated that OVA-driven eosinophilia was abrogated following Mtb infection, both in BAL and in lungs (Figures 1C, 1D, S3). Moreover, Mtb infection specifically prevented eosinophil infiltration, without reducing significantly other myeloid populations as neutrophils, alveolar macrophages, or T lymphocytes (Figure S3).

We next assessed Th1 and Th2 cytokine profiles. Our data demonstrated a substantial reduction of Th2 cytokines IL-5, IL-4, and IL-13 in BAL and lungs from OVA-challenged mice following H37Rv infection, concomitantly with an increase of IFN γ (Figure 1E, 1F). We also evaluated allergen-specific response following ex vivo OVA stimulation of mediastinal lymph node cells. We showed a substantial production of OVA-induced IL-5 and IL-13 in the OVA-challenged group, in the absence of IFN γ response. Conversely, Mtb infection drove to an inversion of this profile, with a reduction of IL-5 and IL-13 levels, and a dramatic increase of OVA-specific IFN γ response (Figure 1G).

Previous studies indicated that newly produced eosinophils in the bone marrow substantially contribute to lung eosinophilia in OVA-induced mouse asthma⁶. In addition, another study demonstrated that T cell-derived IFN γ abrogated eosinophil production in the bone marrow⁷. Based on these results, we next evaluated whether eosinophilopoiesis was affected by Mtb infection under our experimental settings (Figure S4). Our results indicated an increase of eosinophil precursors in the bone marrow following OVA challenge, as it had been previously described⁶. Remarkably, eosinophil progenitors were dramatically reduced in the H37Rv-infected mice, suggesting that inflammatory response induced by TB infection profoundly affects eosinophilopoiesis (Figure 2).

Our data suggest that T cell-driven IFN γ production in Mtb-infected OVA-challenged mice is not only generated by expanded lymphocytes that recognize Mtb antigens, but also by allergen-specific T cells, that seem to be re-educated from a Th2 to a Th1 profile due to the inflammatory environment associated with Mtb infection. In this regard, switch from Th2 to Th1 profile has been previously described, and it is mediated by IL-12 and interferons⁸, cytokines classically associated with TB infection.

To our knowledge, our results provide the first evidence about the capacity of Mtb infection to impair eosinophil production at a

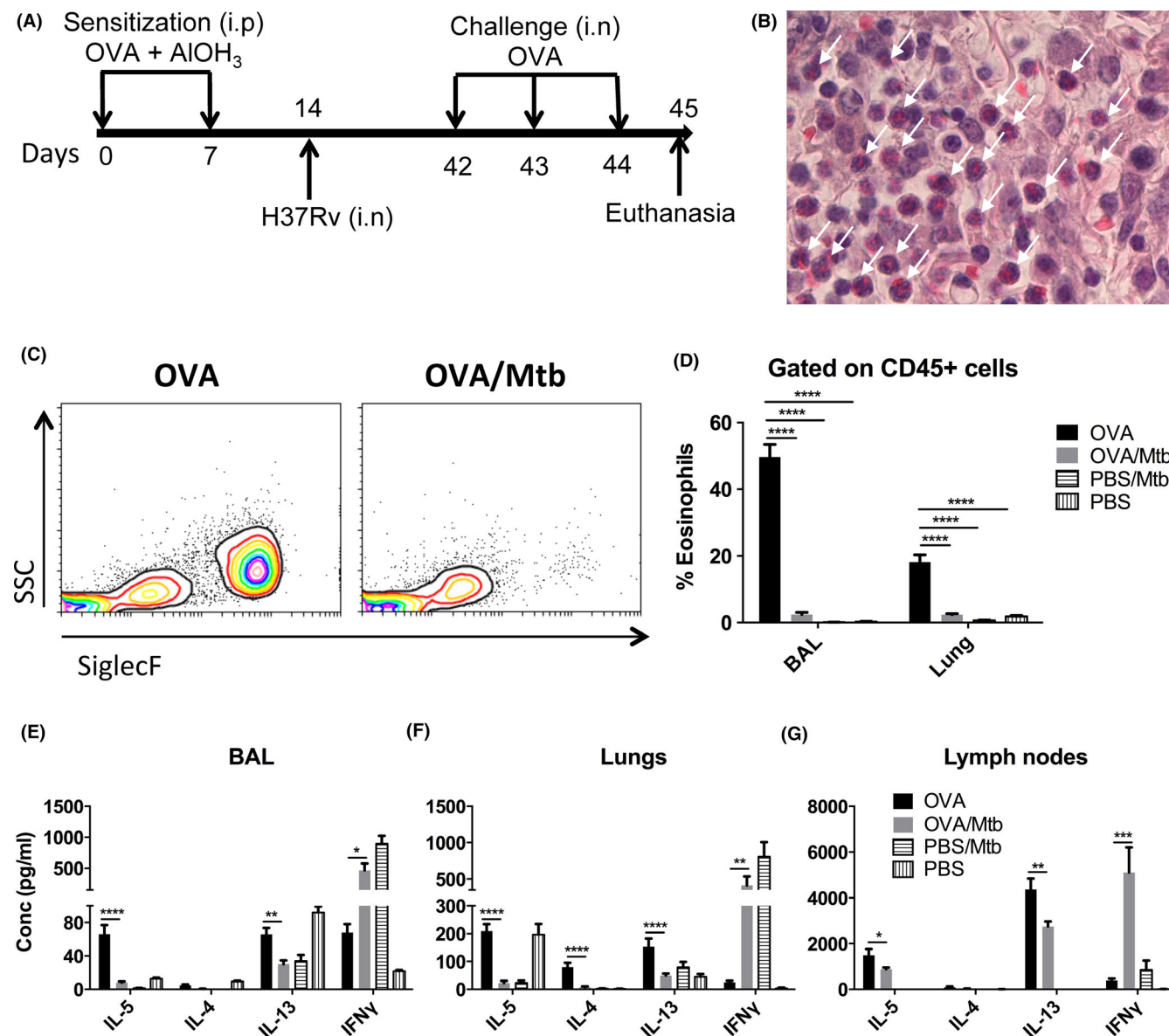


FIGURE 1 *Mycobacterium tuberculosis* (Mtb) infection prevents airway eosinophilia in an OVA-driven asthma mouse model. A, Model of allergic asthma. ip: intraperitoneal; in: intranasal. B, Representative hematoxylin-eosin image (amplified 40×) of a lung from an OVA-treated mouse. White arrows indicate eosinophils. C, Eosinophils detection in bronchoalveolar lavage (BAL) samples by flow cytometry. D, Percentage of eosinophils in BAL and lungs at day 45. E–G, Cytokine concentration in BAL (E), lungs (F) or in supernatants from mediastinal lymph node cells incubated with ovalbumin OVA (data are shown after subtracting baseline values obtained from unstimulated controls) (E). Graphs represent mean $\pm$ SEM from pooled data of two experiments ($n = 12$ mice for OVA-challenged and 6 for PBS groups). D, * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$, by two-way ANOVA and Bonferroni post-test. E–G, For each cytokine, * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$, by one-way ANOVA and Bonferroni post-test

bone marrow level. Studies in the literature indicate that Mtb can colonize bone marrow stem cells during LTBI⁹. Thus, we could speculate that Mtb could alter local inflammatory response in the host bone marrow, enhancing IFN γ production, and impairing IL-5, which would have an impact on generation of eosinophil progenitors and therefore in restricting production of mature eosinophils that could be recruited to peripheral tissues upon allergen exposure in asthmatic individuals.

The global niche of LTBI individuals is currently estimated in around 1.7 billion people¹, with only a small proportion (5%–10%)

expected to develop active TB over their lifetime. In this sense, Mtb could be considered both as a pathogen in active TB, or as a symbiont in 90% of individuals with LTBI. We could speculate that selection of tolerance to latent TB throughout human evolution could have been favored due to unspecific protection conferred by Mtb-mediated Th1 response against certain Th2 cytokine-driven allergies, including asthma. Mtb and humans have coevolved for more than 70 000 years, long before human migrations out of Africa, when societies were rudimentarily composed of small groups of people dedicated to hunt and gathering¹⁰. In this regard,

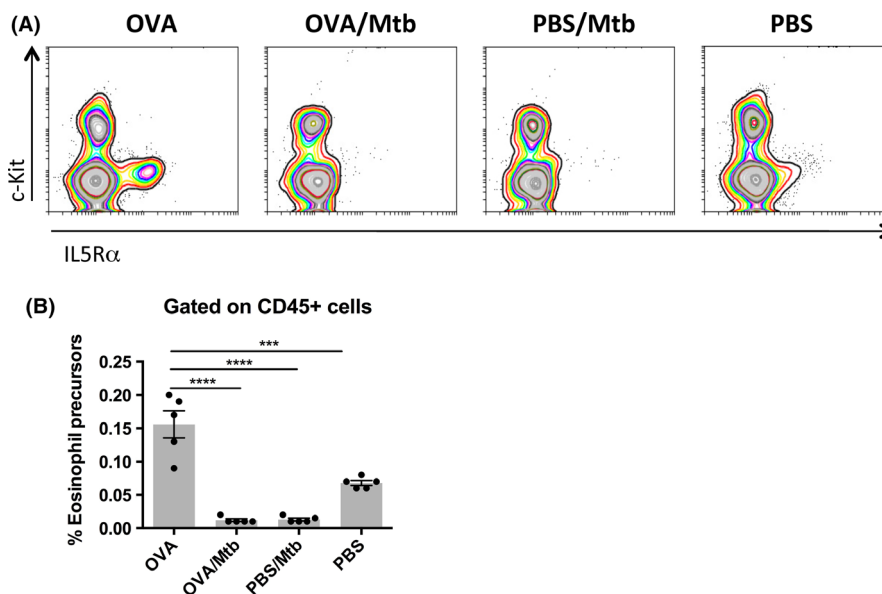


FIGURE 2 *Mycobacterium tuberculosis* (Mtb) infection abrogates bone marrow eosinophil precursors. A, Eosinophil precursor detection in bone marrow samples by flow cytometry, defined as Lin[−]Sca1[−]CD34⁺IL5Rα⁺cKit^{low} cells. B, Percentage of eosinophil precursors in bone marrow at day 45. Data are mean ± SEM from one experiment (n = 5 mice). ***P < 0.01; ****P < 0.0001, by one-way ANOVA and Bonferroni post-test

considering lifestyle of these ancient societies, we can speculate that asthma protection conferred by asymptomatic LTBI could have resulted advantageous to humans, particularly if we consider asthma-associated symptoms, which should be especially detrimental in individuals whose survival crucially depended on physical activity.

At present, multiple clinical trials in TB endemic countries are on course to develop novel tools against this disease. Based on our results and the observational studies available, we consider that the systematic inclusion of asthma detection as an additional exploratory endpoint in TB clinical trials could provide highly valuable information. These trials represent an excellent opportunity to study in depth the relationship between asthma and LTBI and could help to elucidate the immunological bases of asthma protection induced by Mtb infection.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Serum CXCL9 as a potential marker of Type 1 inflammation in the context of eosinophilic asthma

To the Editor,

CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (i-TAC) are considered Th1-type-chemokines (T1 chemokines), as they are produced by airway epithelial cells with the stimulation of IFN- γ .¹ However, in vitro studies indicated that T1-chemokines are involved in bronchial asthma which is generally considered a T2 disease, as they strongly enhance adhesion and release of granule proteins by eosinophils.² Moreover, in vivo study using CXCR3-knockout (KO) mice indicated that T1-chemokines pathway is significantly involved in eosinophilic inflammation and airway hyper-responsiveness (AHR).³ We investigated potential usefulness of serum CXCL9 as a biomarker of eosinophilic asthma.

We collected serum samples from 74 doctor-diagnosed asthma patients and 19 healthy controls (HC) (Table S1), and measured CXCL9 and IL-25, as previously described,⁴ using the DuoSet ELISA Development Systems (R&D Systems). Patients with asthma displayed either AHR evaluated by serial inhalation of acetylcholine and histamine or bronchial reversibility evaluated by inhalation of salbutamol. Patients with asthma were classified into eosinophilic (EA) or noneosinophilic asthmatic (NEA) groups, based on the presence or absence of eosinophils in the sputum stained by Hansel's method.

Next, patients with asthma were classified into AHR-positive and AHR-negative patients, based on the inhalation challenge of serial dilution of acetylcholine or histamine. Moreover, patients with asthma were classified into atopic and nonatopic asthma groups, based on the existence of IgE antibodies against at least one perennial allergens. The study was approved by the research and ethics committees of Sagami-hara National Hospital and Sysmex Corporation. All the study procedures were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Serum CXCL9 concentrations were significantly higher in patients with asthma than in healthy controls (Figure 1A), as well as in EA than in NEA (Figure 1B). On the other hand, no significant difference was observed between HC and NEA. CXCL9 concentrations of AHR-positive patients were significantly higher than those of AHR-negative patients (Figure 1C). In EA, acute exacerbation was significantly more common in patients whose CXCL9 concentrations were higher than the HC distribution (Figure 1D). Moreover, CXCL9 concentrations were negatively correlated with %FEV₁ values (Figure 1E). These results collectively suggest that CXCL9 is involved in the pathogenesis of asthma.

To further characterize EA and NEA groups, T2 markers such as pro-Th2 cytokine IL-25, blood eosinophil ratio, and serum total IgE were compared. These markers of EA were significantly higher than those of NEA, indicating that our EA population represented T2 phenotype (Figure S1). CXCL9 concentrations were significantly

Abbreviations: AHR, airway hyper-responsiveness; BALF, Bronchoalveolar lavage fluid; CS, corticosteroids; DHBC, diseased human bronchial epithelial cells; EA, eosinophilic asthma; ELISA, enzyme-linked immunosorbent assay; FEV, forced expiratory volume; HC, healthy control; NEA, noneosinophilic asthma; NHBC, normal human bronchial epithelial cells.

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LogCFUs



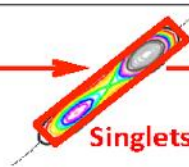
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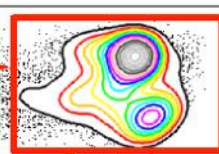
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Singlets

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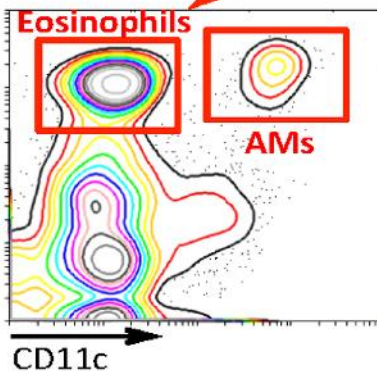
CD45



Eosinophils

SiglecF

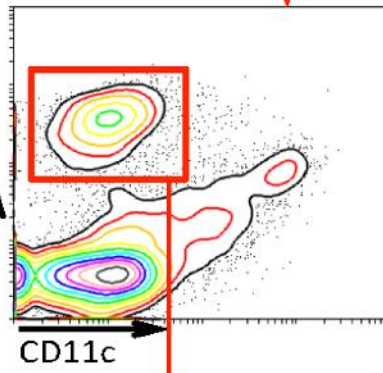
CD11c



AMs

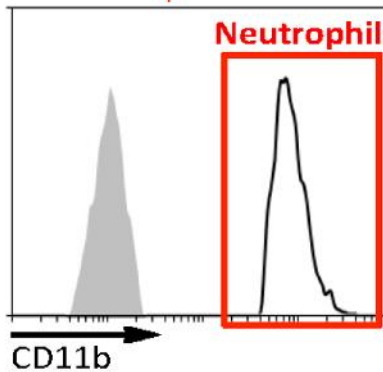
Ly6G

CD11c

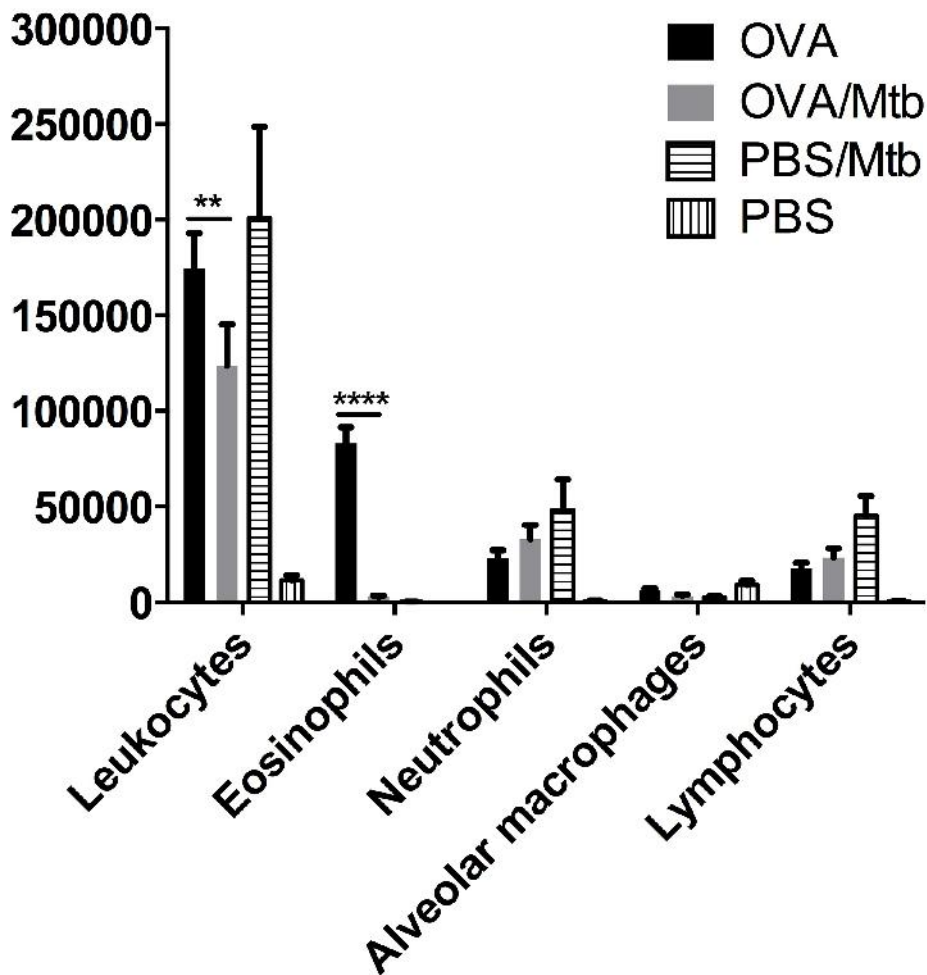


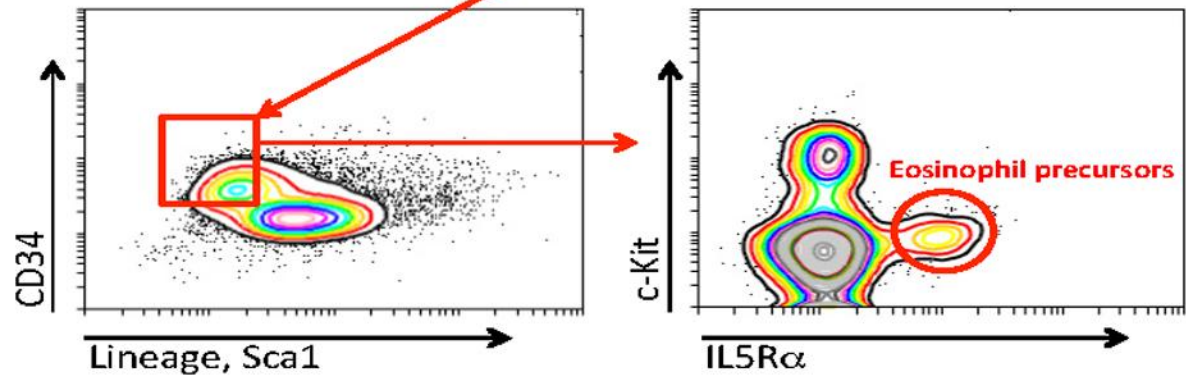
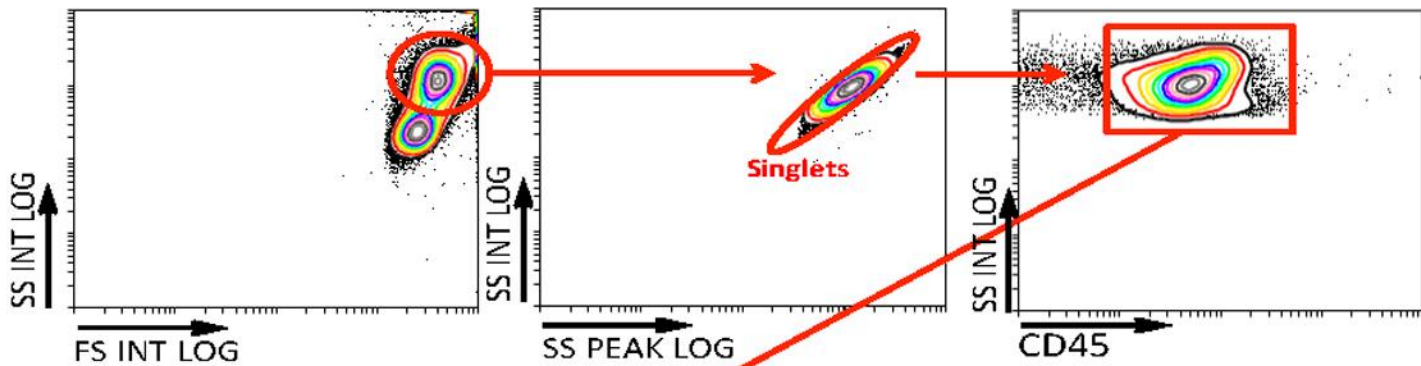
Neutrophils

CD11b



No. cells





SUPPLEMENTARY MATERIAL

Methodology

Animal studies

All mice were kept under controlled conditions and observed for any sign of disease. Experimental work was conducted in agreement with European and national directives for protection of experimental animals and with approval from the Ethics Committee from University of Zaragoza (protocol PI22/15).

For induction of OVA-specific asthma, 8-to-10 weeks old female C57BL/6 (Janvier Biolabs) were sensitized with intraperitoneal injections of 50 µg chicken egg ovalbumin (lyophilized powder, ≥98% (Sigma)) with 2 mg aluminium hydroxide (Sigma, St. Louis, MO) twice one week apart. One week later mice were intranasally infected with a standard low-dose challenge (150 CFU) of the Mtb reference strain H37Rv. Four weeks later animals were intranasally challenged with 100 µg OVA in sterile PBS for 3 consecutive days, and the day after they were humanely sacrificed. In the control groups, OVA was replaced by PBS.

For bronchoalveolar lavage (BAL) collection, trachea was cannulated and BAL performed with 0.8 ml of ice-cold PBS. Supernatant was separated from cells by centrifugation 5 min at 4500 xg.

In some experiments, left lung was fixed in formol for subsequent processing and hematoxylin-eosin staining. Images were obtained with a microscope Leica DM5000B.

Lungs for cellular suspensions were removed aseptically and added to HEPES buffer (HEPES 10 mM; NaCl 0,15 M; KCl 5 mM; MgCl₂ 1 mM; CaCl₂ 1,8 mM pH 7,4) containing collagenase D 100 mg/ml (Roche) and DNase I 400 IU (AppliChem), incubated at 37°C for 30 minutes, and homogenised using GentleMACS system. Afterwards, residual red blood cells were lysed using Red Blood Cells Lysing Buffer (Sigma).

Lungs for cytokine analysis were cut into small pieces with sterile scissors. Then, they were cultured overnight in 0.5 ml of complete cell culture medium (10%FCS, L-glutamine and antibiotics). Supernatant was recovered for subsequent analysis.

Bone marrow cells were harvested from femurs and tibias by flushing cell culture media with a syringe. Red blood cells were lysed using Red Blood Cells Lysing Buffer (Sigma).

Flow cytometry analysis

Cells were acquired with a Gallios flow cytometer (Beckman Coulter) and analyzed with the Weasel software. The laser configuration used was the provided by the manufacturer. Three laser acquisition protocols were used in all cases: violet (405nm), blue (488nm) and red (638nm). Flow panels were previously set up using isotype controls to assess fluorescent basal levels and compensation.

10⁶ lung or BAL cells were incubated for 15 minutes at 4°C with Fc receptor blocking reagent (Miltenyi Biotech). Then, eosinophils, neutrophils, alveolar macrophages and T lymphocytes were determined by extracellular staining with the following antibodies: CD45-FITC (Clone REA737), siglecF-APC (Clone ES22-10D8),

CD11c-PE (Clone N418), CD11b-PerCP/Cy5.5 (Clone REA592), Ly-6G-Vioblue (Clone REA526), CD3-PerCP/Cy5.5 (Clone REA641) from Miltenyi Biotech; and CD4-FITC (Clone RMA4-5), CD8-PE (Clone 52-6.7) from BD Biosciences. Analysis was performed as shown in supplementary Figure 2. Percentages are referred in all cases to CD45⁺ cells. Eosinophils were defined as CD11b⁺SiglecF⁺CD11c⁻; neutrophils as Ly6G⁺CD11b⁺CD11c⁻; and Alveolar Macrophages as SiglecF⁺CD11c⁺CD11b^{dim} cells.

Eosinophil precursors at the bone marrow were characterized with the following antibodies: CD45-Vioblue (Clone REA737), CD34-FITC (Clone RAM34), c-Kit-APC (Clone REA791), IL5R α -PerCP-Vio700 (Clone REA343), Sca1-PE (Clone D7), CD3-PE (Clone REA641), NKP46-PE (Clone REA815), B220-PE (Clone REA755), GR1-PE (Clone RB6-8C5), TER119-PE (Clone REA847), Fc γ R1alpha-PE (Clone MAR-1), from Miltenyi Biotech; and CD4-PE (Clone H120.19), CD8-PE (Clone 52-6.7) from BD Biosciences. Analysis was performed as shown in supplementary Figure 4. Eosinophil precursors were defined as Lineage⁻Sca1⁻CD34⁺c-Kit^{low}IL5R α ⁺ cells, using CD3, CD4, CD8, NKP46, B220, GR1, TER119 and Fc γ R1alpha markers to define Lineage cells.

Cytokine analysis

Mediastinal lymph nodes were removed aseptically and mechanically disrupted for cell collection. 2x10⁶ cells were incubated in complete cell culture media with or without OVA 10 μ g/ml for 96 hours. Then, supernatant was collected to determine cytokine concentration. OVA-specific response for each cytokine was determined subtracting the baseline values from unstimulated control to the cytokine concentration obtained following OVA stimulation. Quantification of IL-5, IL-4, IL-13 and IFN- γ was performed in all cases using specific commercial ELISA kits following manufacturer instructions (Mabtech Biotech).

Statistics

Commercial mice were randomly distributed in groups of 6 animals per cage prior to start experimental procedures. Results were not blinded for analysis. No statistical method was used to calculate sample size in animal experiments. GraphPrism 6 software (Version 6.0) was used for statistical analysis. Statistical tests used for each experiment are indicated in the figure legends. All statistical tests used were two-tailed. Outlier values were determined applying the Grubb's test to all data sets, and discarded from the final statistical analysis. Differences were considered significant at p<0.05.

Supplementary Figure Legends

Supplementary Figure 1. Replication curve of Mtb in mouse lungs after low-dose intranasal challenge. Groups of mice (n=3 per timepoint) were intranasally challenged with 150 CFU, and lung Mtb bacteria determined at the indicated weeks. Graph represent mean from one representative experiment.

Supplementary Figure 2. Gating strategy for determination of eosinophils, neutrophils and alveolar macrophages in BAL and lung cellular suspensions. AMs: alveolar macrophages. In the histogram, the solid grey peak represents the isotype control.

Supplementary Figure 3. Total number of BAL cells. Number of leukocytes, eosinophils, neutrophils, alveolar macrophages (AM) and T lymphocytes in BAL at day 45, determined by flow cytometry. Graphs represent mean±SEM from pooled data from two experiments (n=12 mice for OVA-challenged and 6 for PBS groups). **p<0.01; ****p<0.0001, by two-way ANOVA and Bonferroni post-test.

Supplementary Figure 4. Gating strategy for determination of bone marrow eosinophil progenitors by flow cytometry.