Supporting Information

Loebbermann et al. 10.1073/pnas.1217580110

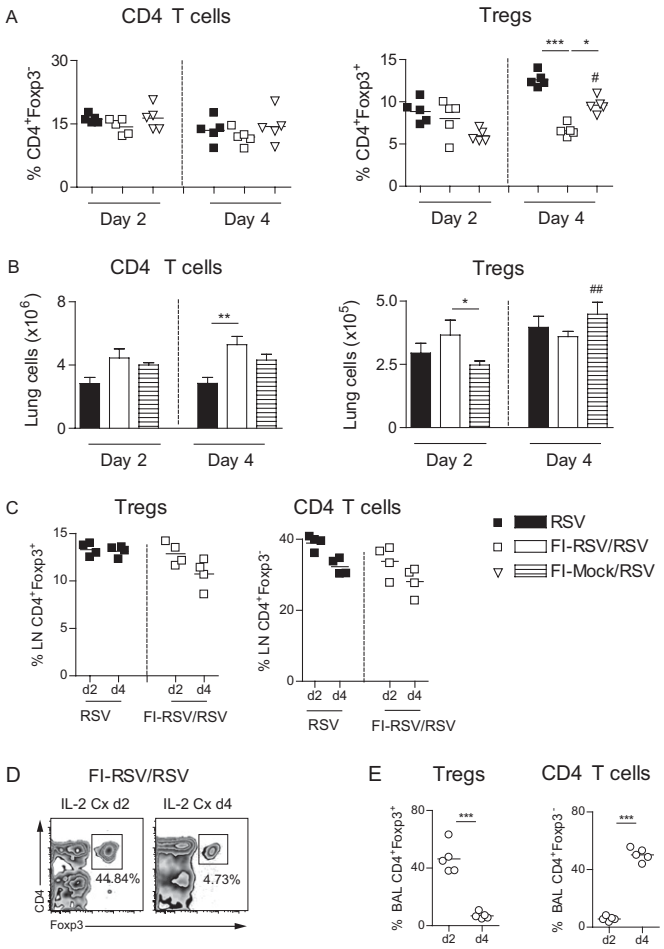

Fig. S1. Formaldehyde-inactivated respiratory syncytial virus (FI-RSV) vaccination leads to a reduction of regulatory T cells (Tregs) in the lung but not in the draining lymph node (LN) after RSV infection. FI-RSV– or mock-infected Hep-2 cell material (FI-Mock)-vaccinated mice were infected with RSV on day 0. Lungs were harvested on days 2 and 4 postinfection. (*A*) Quantification of frequencies of Tregs and forkhead box p3 (Foxp3)$^-$ gated CD3$^+$CD4$^+$ T cells (CD4 T cells) in the lung on days 2 and 4 after RSV infection. (*B*) Total cell numbers of Tregs and CD4 T cells in the lung on days 2 and 4 after RSV infection. (*C*) Frequencies of Tregs and CD4$^+$ T cells in the mediastinal LNs on days 2 and 4 after RSV infection. (*D*) Frequencies of CD3$^+$CD4$^+$gated Tregs in bronchoalveolar lavage (BAL) of FI-RSV–vaccinated, immune complexes of IL-2/anti–IL-2 (IL-2 Cx)-injected mice on days 2 and 4 after RSV infection. (*E*) Quantification of frequencies of Tregs and CD4 T cells on days 2 and 4 after RSV infection in FI-RSV–vaccinated, IL-2 Cx–injected mice. One representative study of two independent experiments with five mice per group is shown. Results are presented as means ± SEM. The significance of results between the groups was analyzed by two-tailed, unpaired Student *t* test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ was used to compare different groups on one day point and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ was used for comparing one group at different day points. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

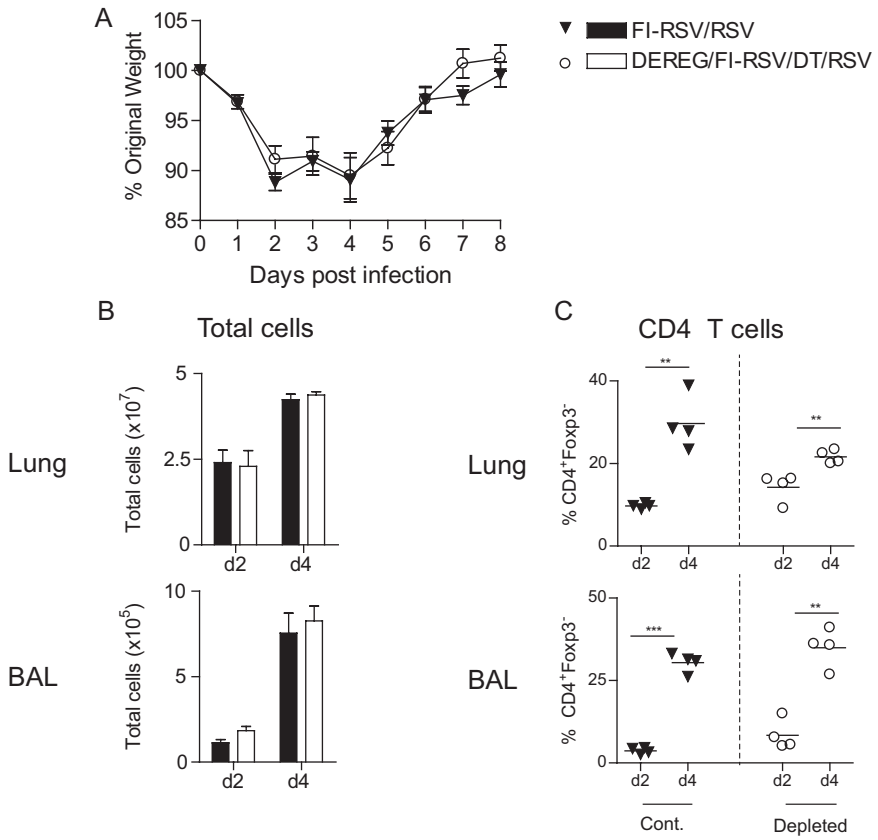

Fig. S2. Depletion of Foxp3$^+$ cells in FI-RSV–vaccinated depletion of regulatory T-cell (DEREG) mice does not increase weight loss or cellular influx into the lungs after RSV infection. (*A*) Illness was monitored daily by weight for 8 d after RSV infection, displayed as percentage of original weight. (*B*) Total numbers of cells in the lung (*Upper*) and BAL (*Lower*) was enumerated on days 2 and 4 from RSV-infected FI-RSV–vaccinated mice, with and without Treg depletion. (*C*) Frequencies of CD4 T cells in the lung (*Upper*) and BAL (*Lower*) quantified by flow cytometry on days 2 and 4 after RSV infection. Graphs show data from one representative out of three independent experiments with five mice per group in each case. Results are presented as means ± SEM. The significance of results between the groups was analyzed by two-tailed, unpaired Student *t* test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

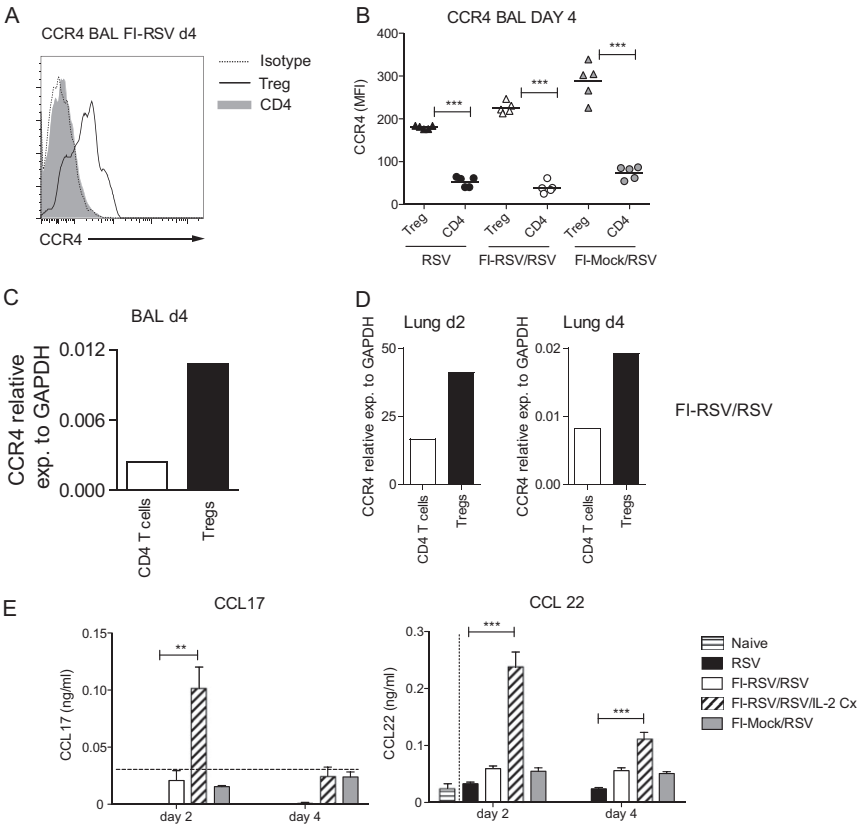

Fig. S3. Tregs in the lung express CCR4. FI-Mock– or FI-RSV–vaccinated mice were infected with RSV on day 0, and lymphocytes recovered by BAL on day 4. When indicated, mice were injected with IL-2 Cx on days −3, −2, and −1. (*A*) Expression of CCR4 on Tregs and CD4 T cells in the BAL measured by flow cytometry on day 4 postinfection. (*B*) Quantification of CCR4 expression on Tregs and CD4 T cells in the BAL on days 2 and 4 postinfection measured as mean fluorescent intensity (MFI). (*C*) Relative expression of CCR4 to GAPDH of BAL CD4 T cells and Tregs on day 4 postinfection determined by real-time PCR of FI-RSV–vaccinated and RSV-infected mice. PCR was performed on pooled FACS-sorted cells. (*D*) Relative expression of CCR4 to GAPDH of CD4 T cells and Tregs in the lung on days 2 and day 4 postinfection determined by real-time PCR of pooled FACS-sorted cells. (*E*) Levels of chemokines CCL17 and CCL22 in the BAL of FI-RSV vaccinated mice measured by ELISA on days 2 and 4 after RSV infection. Data are pooled from two independent experiments, four to five mice per group in each graph. Results are presented as means ± SEM. The significance of results between the groups was analyzed by two-tailed, unpaired Student *t* test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

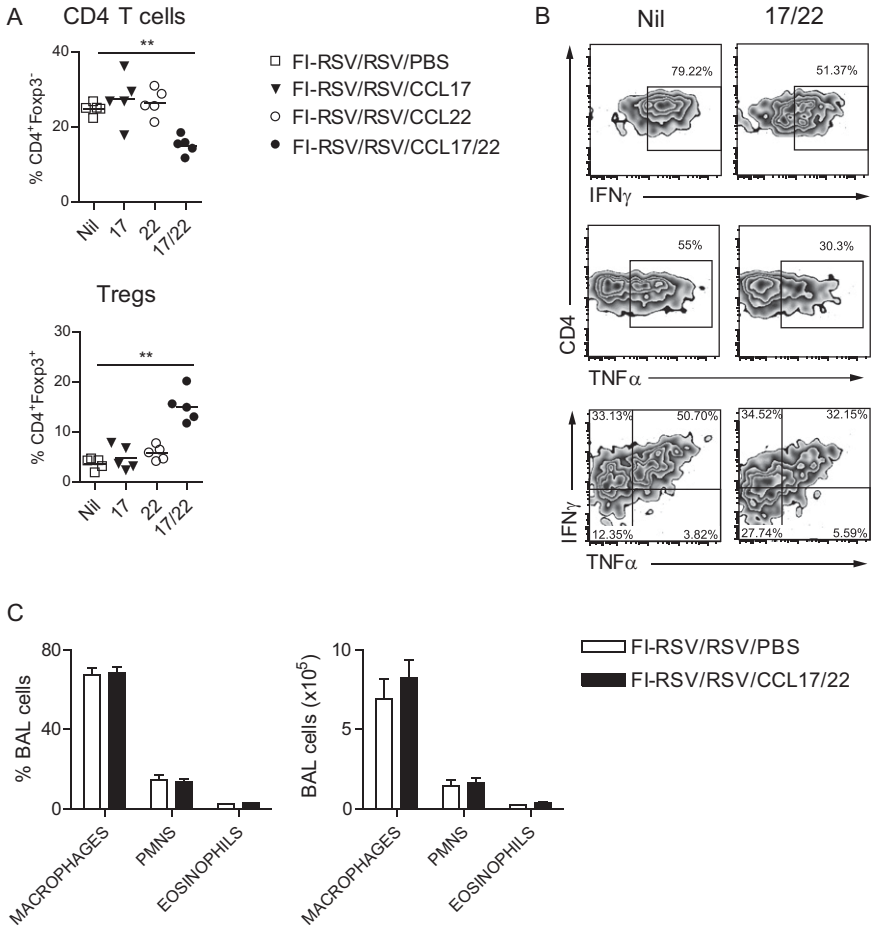

Fig. S4. Effects of instillation of CCL17 and/or CCL22 in the airways of FI-RSV–vaccinated mice after RSV infection. (*A*) Quantification of frequencies of Tregs and CD4 T cells on days 2 and 4 after RSV infection in FI-RSV–vaccinated and either CCL17- or CCL22-treated mice. (*B*) Frequencies of $CD4^+IFN\text{-}\gamma^+$ T cells, $CD4^+TNF\text{-}\alpha^+$ T cells, and $CD4^+TNF\text{-}\alpha^+IFN\text{-}\gamma^+$ T cells in the BAL on day 4 after RSV infection were quantified using flow cytometry on days 2 and 4 after RSV infection. (*C*) Frequencies (*Right*) and total numbers (*Left*) of macrophages, neutrophils (PMNS), and eosinophils in the BAL were quantified using differential cell counting of H&E-stained cytospins slides on day 4 postinfection. Graphs show data from one representative out of two independent experiments with five mice per group in each case. Results are presented as means ± SEM. The significance of results between the groups was analyzed by two-tailed, unpaired Student *t* test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

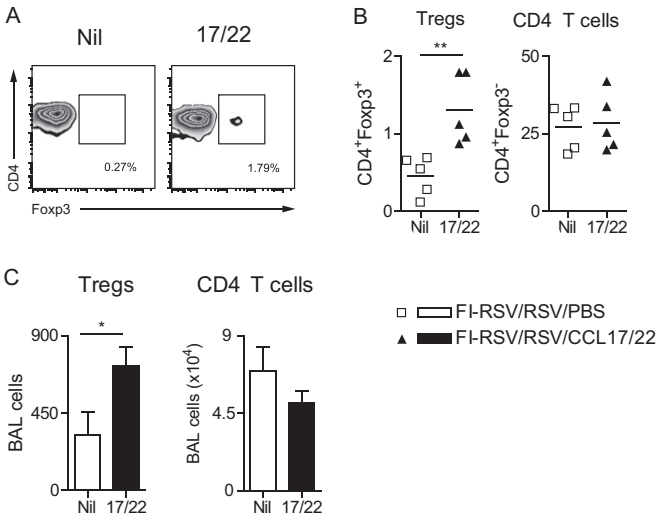

Fig. S5. Late effects of chemoattraction of Tregs into the airways by CCL17/22. BALB/c mice were vaccinated with FI-RSV and infected 3 wk later with RSV intranasally (i.n.) (day 0). When indicated, mice were treated i.n. with a mixture of 0.5 μg of both CCL17 and 22, or PBS on day 2 after RSV infection. (*A*) Frequencies of Tregs in the BAL were quantified using flow cytometry on day 8 after RSV infection with (17/22) or without (nil) i.n. administration of CCL17/22. (*B*) Quantification of Tregs and CD4 T-cell frequencies in the BAL on day 8 after RSV infection. (*C*) Quantification of total numbers of Tregs and CD4 T cells in the BAL on day 8 after RSV infection. Graphs show data from one representative out of two independent experiments with five mice per group in each case. Results are presented as means ± SEM. The significance of results between the groups was analyzed by two-tailed, unpaired Student *t* test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

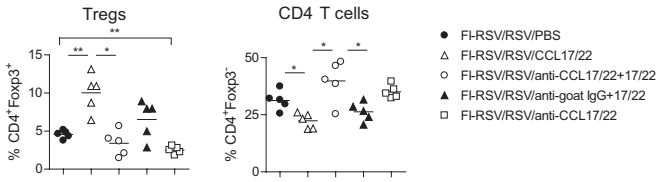

Fig. S6. Anti-CCL17/22 treatment decreases Treg frequencies. Mice were injected i.p. with anti-CCL17 and 22 antibodies or goat IgG control on day 1 after RSV infection into FI-RSV–vaccinated mice before instillation of CCL17/22 on day 2 postinfection. Quantification of Tregs (*Left*) and CD4 T-cell (*Right*) frequencies in the BAL on day 4 after RSV infection. Graphs show data from one representative out of two independent experiments with five mice per group in each case. Results are presented as means ± SEM. The significance of results between the groups was analyzed by two-tailed, unpaired Student *t* test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

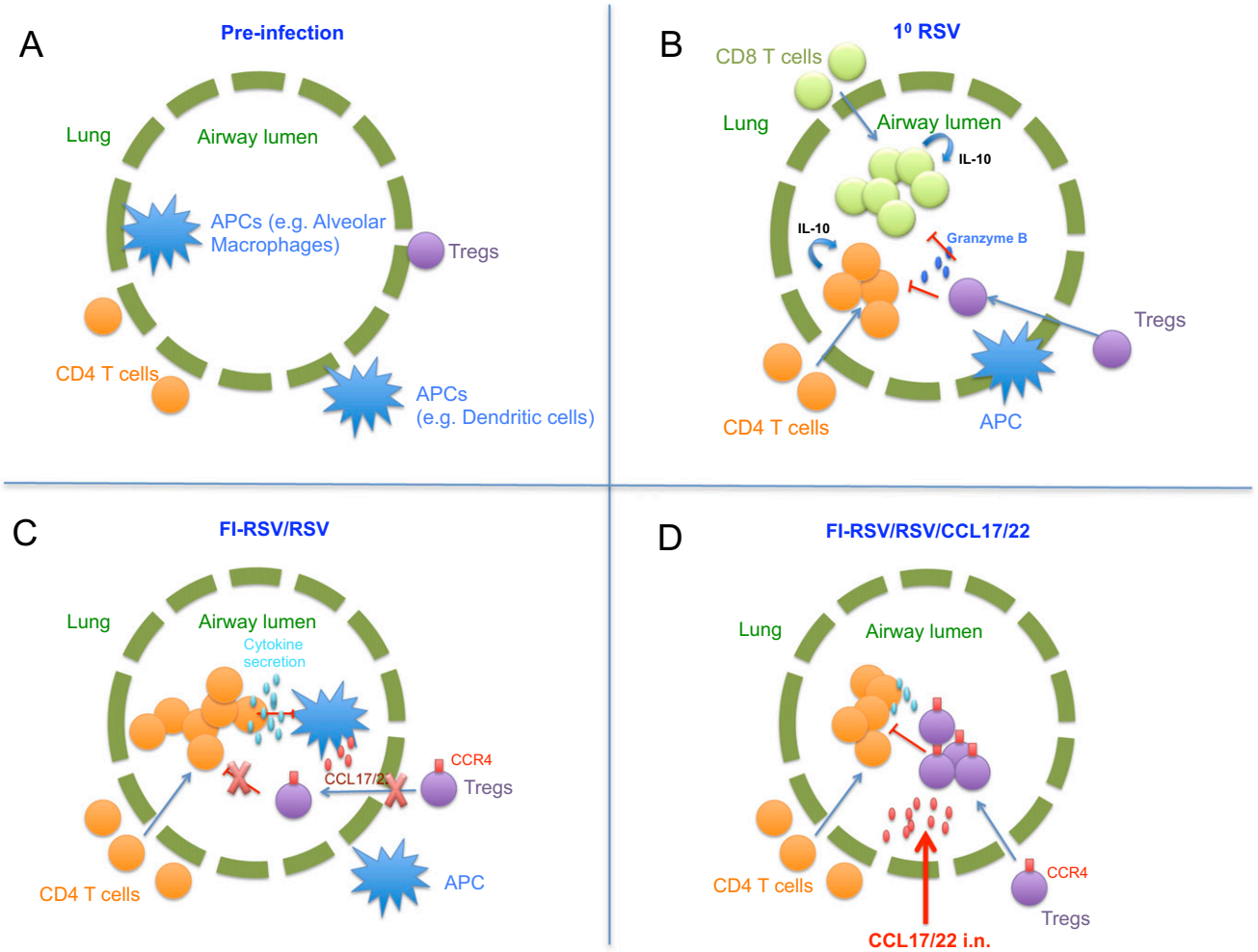

Fig. S7. The balance of regulatory and disease-causing T cells in RSV infection. (*A*) The airways of naive mice contain alveolar macrophages but no other immune cells. (*B*) In RSV primary infection, macrophages become activated and CD4 and CD8 T cells migrate into the lung and airways. These are kept in check by Tregs, which require granzyme B to function (1); both CD4 and CD8 T cells produce IL-10, which assists in modulation of the immune response (2). (*C*) RSV infection of mice previously vaccinated with FI-RSV shows a rapid and exuberant CD4 T-cell response, leading to mediator release into the local environment and shutdown of the production of CCL17/22 by resident cells. These chemokines normally recruit Tregs into the airways, so a decline in chemoattraction of Treg enhances disease. (*D*) Administration of CCL17/22 intranasally bypasses the shutdown of Treg recruitment and attenuates vaccine-augmented disease, reducing disease severity. APC, Antigen presenting cell.

1. Loebbermann J, et al. (2012) Regulatory T cells expressing granzyme B play a critical role in controlling lung inflammation during acute viral infection. *Mucosal Immunol* 5(2):161–172.
2. Loebbermann J, et al. (2012) IL-10 regulates viral lung immunopathology during acute respiratory syncytial virus infection in mice. *PLoS ONE* 7(2):e32371.