Defective immunoregulation in RSV vaccine-augmented viral lung disease restored by selective chemoattraction of regulatory T cells

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Short title: **Chemoattraction of Tregs in augmented RSV disease**

ABSTRACT

Human trials of formaldehyde-inactivated RSV (FI-RSV) vaccine in 1966-67 caused disastrous worsening of disease and death in infants during subsequent natural RSV infection. The reasons behind vaccine-induced augmentation are only partially understood, and fear of augmentation continues to hold back vaccine development. We now show that mice vaccinated with FI-RSV show enhanced local recruitment of conventional $CD4^+$ T cells accompanied by a profound loss of regulatory T cells (Tregs) in the airways. This loss of Tregs was so complete that additional depletion of Tregs (in transgenic DEREG mice) produced no additional disease enhancement. Transfer of conventional $CD4^+$ T cells from FI-RSV vaccinated mice into naïve RSV infected recipients also caused a reduction in airway Treg responses, and boosting Tregs with IL-2 immune complexes failed to restore normal levels of Tregs or to ameliorate disease. However, delivery of CCL17/22 *via* the airway selectively recruited airway Tregs and attenuated vaccine-augmented disease, reducing weight loss and inhibiting local recruitment of pathogenic $CD4^+$ T cells. These findings reveal a novel and unexpected mechanism of vaccine-induced disease augmentation, and indicate that selective chemoattraction of Tregs into diseased sites may offer a novel approach to the modulation of tissue-specific inflammation.

Introduction

Respiratory syncytial virus (RSV) causes common colds in adults but is the major cause of infantile bronchiolitis (1), characterized by an intense local inflammatory response to infection. RSV is estimated to cause 34 million cases of lung infection annually, some 3.4 million hospitalizations and the deaths approximately 199 000 children under 5 years of age worldwide (2). Between 25 and 40% of previously healthy RSV infected infants develop signs of lower respiratory tract infection which may develop into viral bronchitis, bronchiolitis or pneumonia (2). Those recovering from severe disease are at high risk of recurrent wheeze in later childhood and RSV is increasingly recognized as an important cause of winter deaths in the elderly. Despite this enormous disease burden, there is still no vaccine for human use.

Trials of formaldehyde-inactivated RSV (FI-RSV) vaccine in 1966-67 caused disastrous worsening of disease and deaths in infants during subsequent natural RSV infection (3). The mechanisms that cause disease augmentation are incompletely understood, but include aberrant Th2-mediated disease triggered by generation of carbonyl groups (4), defective Toll-like receptor signalling (5) and induction of poorly neutralising antibody (6). Although it is clear that CD4 T cells play a major pathogenic role, little is known how these responses are regulated.

$CD4^{+}CD25^{hi}$forkhead box p3 $(Foxp3)^{+}$ regulatory T cells (Treg) play a crucial role in controlling immune responses. Human genetic Treg defects causes multi-organ inflammatory disease (7, 8), and depletion of Treg in mice can lead to an analogous inflammatory syndrome mainly affecting the gut and skin (9, 10). Tregs are widely distributed in lymphoid and non-lymphoid sites and express a variety of chemoattractant receptors and adhesion molecules that determine their migration in

and out of tissue compartments (11). Amongst the various homing and migration receptors CCR4/8 expression on human Tregs seems particularly important (12), suggesting that the ligands CCL1, CCL17 and CCL22 may guide the migration of Tregs to specific areas and thus suppress local inflammation (13-15). Most remarkably, mice lacking CCR4 expression on Tregs develop lymphocytic infiltration and severe inflammatory disease of the skin and lungs (16), and CCL22-directed Treg recruitment has been used to prevent murine autoimmune diabetes (17).

We now show that mice vaccinated with FI-RSV exhibit an almost complete absence of Tregs in the airways during RSV infection. Moreover, selective recruitment of Tregs by chemokine administration of CCL17/22 re-established a local airway Treg population, so attenuating the pathogenic effects of vaccine-induced CD4 T cells. The use of appropriate chemokines to draw regulatory cells into inflamed tissues offers a novel approach to the treatment of inflammatory disorders.

Results

FI-RSV vaccination attenuates airway Treg responses to RSV infection

Mice vaccinated with formalin inactivated RSV (FI-RSV) showed augmented disease and weight loss after RSV infection (Fig. 1A). This was accompanied by elevated total cell numbers on day 4 post RSV infection in BAL and lung (Fig. 1B). Flow cytometric analysis of the BAL cells showed a significant increase of CD4 T cells ($CD3^+CD4^+Foxp3^-$) but a remarkable loss (both frequency and numbers) of Tregs in FI-RSV vaccinated mice, compared to unvaccinated control mice infected with RSV. The loss of Tregs in FI-RSV vaccinated mice was evident as early as day 2, but was

virtually complete by day 4 (Fig. 1D and E). RSV infected mice vaccinated with formalin treated mock infected Hep-2 cell material (FI-Mock) showed a significant but less pronounced increase of CD4 T cells and a slight drop in Treg frequencies on day 4 post infection compared to unvaccinated controls (Fig. 1C-E); such effects have previously been noted, and ascribed to anti-cell responses since both the virus and the vaccine stocks were grown on Hep-2 cells (18). However, the ratio of Tregs to CD4 T cells was most markedly reduced in the airways of FI-RSV vaccinated mice (Fig. 1 F). Uninfected mice showed no cell efflux into the airways, regardless of vaccination with FI-RSV or FI-Mock. The effects were most obvious in the BAL cells, but similar changes in Treg proportions and numbers were detected in the lung cells (Fig.S1 A and B). However, there was no significant loss of Tregs in local draining lymph nodes (Fig.S1 C), indicating that the effect is tissue-specific and confined to the site of infection.

Since disease enhancement was associated with reduced numbers of Treg cells, we attempted to reduce disease severity by administration of immune complexes of IL-2/anti-IL-2 (IL-2 Cx) prior to RSV infection. This procedure has been shown to selectively activate and expand Tregs without significantly affecting conventional effector CD4 T cells (19, 20), causing active suppression of immune activation and disease *in vitro* and *in vivo* and reducing lung inflammation in RSV disease (20). These IL-2 immune complexes increased the proportion of Tregs in the airways at day 2 of FI-RSV vaccinated, RSV infected mice (e.g. Fig. S1D and E cf. 1 D), but the benefits were only transient and Tregs showed a marked decline by day 4 (Fig. S1 D and E). Despite this treatment, the proportion of disease-associated conventional CD4 T cells increased during this period, just as it did in mice without IL-2 Cx injections (e.g. Figs. S1E cf. 1 C).

Although it was previously demonstrated that Tregs can be starved of IL-2 produced by effector T cells during acute infection (21) our results suggest that IL-2 withdrawal is not the cause of the observed ‘crash’ in Tregs.

Selective Treg depletion does not lead to additional enhancement of disease

Bacterial artificial chromosome-transgenic "depletion of regulatory T cell" (DEREG) mice express diphtheria toxin (DT) receptor and enhanced green fluorescent fusion protein under the control of the *foxp3* gene locus, allowing selective depletion of $Foxp3^+$ Treg cells by DT injection (22). Non-depleted FI-RSV vaccinated DEREG mice respond to RSV infection similarly to WT mice. We have shown that two consecutive injections of DT into DEREG mice causes virtually complete Treg depletion, resulting in considerable disease enhancement after RSV infection (20). However, depletion of Tregs from FI-RSV vaccinated, RSV infected DEREG mice did not produce any additional enhancement of disease (Fig.S2 A, B and C) and failed to increase the numbers or CD4 T cells in the lung or BAL on day 2 or 4 post RSV infection (Fig. S2B and C). Thus, the functional deficit of Tregs caused by FI-RSV vaccination is effectively complete, since depletion of Tregs had no significant additional effect.

Vaccine-primed CD4 T cells attenuate airway Treg responses

To determine the role of CD4 T cells in causing the loss of airway Tregs after RSV infection of primed mice, naive mice were injected i.v. with purified CD4 T cells from spleen and mesenteric lymph nodes derived from either FI-RSV vaccinated or naïve

mice. Mice that received CD4 T cells from FI-RSV vaccinated mice displayed enhanced weight loss and increased cellular influx into the airways on day 4 post RSV infection (Fig. 2 A and B). Flow cytometric analysis of the BAL showed a significant increase of CD4 T cells but a remarkable loss of Treg frequencies on day 4 post infection in RSV infected mice which received CD4 T cells from FI-RSV vaccinated mice compared to controls (Fig. 2 C and D). This also led to a significant decrease of the Treg/CD4 T cells ratio (Fig. 2E). Adoptive transfer of naive CD4 T cells also led to increased cellular influx into the airways on day 4 post infection (Fig.2 B and D; probably due to large numbers of partially activated CD4 T cells reaching the lungs after i.v. injection), but these effects were significantly less marked than those of CD4 cells from FI-RSV vaccinated mice. Therefore, primed and activated CD4 T cells (especially those induced by FI-RSV vaccination) are responsible for the attenuation of airway Treg responses after RSV challenge.

CCR4 expression by Tregs and chemokine levels in the airways.

To further examine the causes of Treg deficiency in the airways of mice with augmented disease, we studied chemokine receptor expression and chemokine production by the infected airways. CCR4 plays a pivotal role in Treg migration in both man and mouse (15, 16, 23), and expression of CCR4 on Tregs is required for appropriate migration to the skin or respiratory tract. Mice lacking CCR4 on Tregs develop lymphocytic infiltration and inflammation of the lung and skin with local influx of CD4 T cells and granulocytes (16). This pattern of pathology is reminiscent of the inflammation seen in the lungs of FI-RSV vaccinated children or mice infected with RSV (24).

We therefore analysed the expression of CCR4 on airway Tregs. After RSV infection, airway Tregs of all experimental groups showed greater expression of CCR4 than conventional CD4 T cells, either by flow cytometry (Fig. S3A and B) or by quantitative PCR analysis of gene expression on sorted cells from BAL (Fig. S3C). or from lung (Fig. S3D). Therefore, the observed defect in Treg recruitment cannot be explained by reduced chemokine receptor expression on Tregs.

Since CCR4 is present and expressed on Tregs regardless of priming, we investigated the possibility that a lack of CCR4's specific chemokines (CCL17/22) might explain the reduced Treg recruitment in vaccinated RSV infected mice. We attempted to measure levels of CCL17 and CCL22 in the airway fluid in the different groups (Fig S3E). In most of the groups CCL17 was under the detection limit. However airway fluid from IL-2 Cx injected mice contained higher levels of CCL17 and CCL22 on day 2 post infection. Levels of both these chemokines decreased by day 4 post infection at the time of declining Treg numbers, compatible with the possibility that the increased Treg frequencies in the airways of IL-2 Cx treated mice on day 2 post infection were due to CCL17/22-mediated chemoattraction of Treg trafficking into the inflamed airways (16). There was no significant difference in levels of CCL22 between the other groups (Fig S3E). These findings suggest that CCL17/22 may play a role in attracting Treg to the airway and modulating disease, but that our methods of detecting these chemokines may not be sufficiently sensitive to fully elucidate their role.

Administration of CCL17/22 via the airways re-establishes Treg population

Since Tregs displayed CCR4 and we were unable to show whether there was a defect in chemokine production, we next tested the effect of delivering CCL17/22 via

the airway. Since topical vaginal application of CXCL9/10 recruits herpes virus-specific tissue-resident primed memory T cells to the genital mucosa (25), we reasoned that delivering CCL17/22 via the airway might recruit Tregs and thus reduce disease severity. We gave a single intranasal dose of CCL17/22 on day 2 after RSV challenge, and found that this greatly reduced weight loss up to day 8 post infection (Fig. 3A), while increasing Treg recruitment into the BAL (Fig 3 B and C). This was accompanied by a marked reduction in conventional CD4 T cells compared to PBS control mice (Fig.3B and C). Administration of either CCL17 or CCL22 alone was much less effective (Fig.S4A), suggesting that these 2 chemokines act synergistically. CCL17/22 administration also reduced the proportion of CD4 T cells that produced IFN-γ and/or TNF-α (Fig. 3D and Fig. S4B) and significantly increased the ratio of Tregs to CD4 T cells (Fig. 3E) but did not affect total cell numbers in the lung or BAL or the recruitment of granulocytes to the airway (Fig. S4C). We could not detect IL-17 or IL-4 producing CD4 T cells in the lung or airways at any time by flow cytometry, suggesting that Tregs predominantly modulate Th1, rather than the Th2 or Th17 CD4 T cell subsets in this situation. Although the Treg recruitment to the airways in FI-RSV vaccinated mice was inhibited for at least 8 days post infection the increase of Tregs in the airways after chemokine treatment was sustained at least until day 8 post infection (Fig. S5A to C). Notably, the weight loss recovery already started by day 5 post RSV infection, as soon as the number of FI-RSV specific CD4 T cells declined. There was no significant difference between levels or total numbers of CD4 T cells between CCL17/22 treated and untreated mice on day 8 post infection (Fig. S5B and C). However, CCL17/22 administration did not affect mice undergoing primary RSV infection, in which case the balance of regulatory and disease-causing cells is already under appropriate control. These results show

clearly that selective chemoattraction of Tregs to the airway by administration of CCL17/22 reduces the recruitment and activity of conventional CD4 T cells and reverses the enhanced lung inflammation resulting from vaccination with FI-RSV.

We next examined whether neutralization of CCL17/ 22, by i.p. injection of anti-chemokine antibodies on day 1 post RSV infection (with or without chemokine instillation on day 2 post infection), was able to prevent Treg recruitment into the airways and the consequent effects (Fig. S6). Anti-CCL17/ 22 (but not goat IgG isotype control) prevented the effects of CCL17/22 treatment, reducing Treg and increasing CD4 T cell frequencies on day 4 post infection. Importantly, anti-CCL17/22 treatment further decreased Treg frequencies in the airways in vaccinated mice compared to vaccinated control mice, emphasizing the important role of CCL17/22 in Treg recruitment even under these conditions.

In order to demonstrate that additional recruited Tregs in the airways of FI-RSV vaccinated mice treated with CCL17/22 are directly responsible for the decrease in CD4 T cells after RSV infection, FI-RSV vaccinated BALB/c DEREG mice were depleted of Tregs by injections of DT and infected with RSV. Treg depleted, FI-RSV vaccinated DEREG or untreated and non-depleted vaccinated BALB/c mice, CCL17/22 treated or untreated mice served as controls. We found that only non-depleted mice given CCL17/22 showed significant reduced weight loss after RSV infection accompanied by increased Treg recruitment and by a marked reduction in CD4 T cells and Treg/CD4 T cells ratio in the BAL (Fig.4A, B and C). Therefore, Treg recruitment is necessary for the beneficial effects of administration of CCL17/22 *via* the airway.

Discussion

Studying vaccine-augmented disease, our results show that RSV infected mice suffering the effects of exuberant RSV-specific CD4 T cell memory responses show a marked reduction in airway Treg cells, with a virtually complete loss of immune regulatory function. The 'crash' in Tregs can be partially reproduced by adoptive transfer of activated CD4 T cells. Most remarkably, selective recruitment of disease-attenuating Tregs by the administration of CCL17/22 into the airways rapidly restored local immunoregulation and caused a sustained reduction in disease severity, even if the chemokines were delivered after virus-induced inflammation was at its peak. Together, these results show that Tregs play a key role in determining the balance between the beneficial and disease-enhancing components of cellular immunity.

Consideration of the site at which different components of the immune response act, is essential in understanding this complex but informative disease model. It is known that generalized depletion of Tregs (either with anti-CD25 antibody or in genetically modified mice) enhances RSV-induced disease and increases T cell activation at multiple sites (20, 26-28). We now show that an almost complete local depletion of Tregs occurs in the airways of vaccine-sensitised RSV infected mice, and that this crash in local Tregs allows the effect of disease-causing T cells to become dominant. Whereas Treg depletion during primary RSV infection causes disease enhancement, depletion of Tregs in FI-RSV vaccinated DEREG mice caused no further disease enhancement during RSV infection. Thus, the functional deficit of Tregs in the airways was virtually complete under these conditions and additional depletion of Tregs had no significant additional effect. The disappearance of Tregs, which was most evident in the airways, was seen also in the lung but not in the spleen or

draining nodes. The loss of Tregs in FI-RSV vaccinated mice was evident as early as day 2, but was virtually complete by day 4 continued to at least day 8 after RSV infection. Thus, attenuation of Treg responses is focussed at the site of enhanced disease.

To explain our observations, we suggest that primed conventional CD4 T cells cause a loss of chemoattraction of Tregs (29), probably by an action on the cells that make CCL17 (e.g. airway epithelial cells and DCs) and CCL22 (DCs and macrophages; illustrated diagrammatically in Fig. S7). This explanation is supported by our finding that passive transfer of CD4 T cells from vaccinated mice led to reduced Treg recruitment, and is compatible with the known effects of CD4 T cells on innate immune responses (30). Although T cell subsets that do not express Foxp3 can have suppressive functions (31) and might play an important role in controlling lung inflammation, our studies indicate that variations in $FoxP3^{+}$ cells rather than additional regulatory subsets sufficiently explain the observed changes in disease in FI-RSV vaccinated mice. However, it is clearly important to investigate the role of additional suppressive mechanism in future studies.

Recent studies suggested an important role for CCR4 in human and mouse Treg migration (15, 16, 23). Notably, Sather *et al* showed that CCR4 expression on Tregs is required for appropriate migration to the skin or respiratory tract, and that mice lacking CCR4 on Tregs develop lymphocytic infiltration and inflammation of the lung and skin with local influx of CD4 T cells and granulocytes (16). This chronic and progressive disease bears close similarity to the acute inflammatory disease that we observe in FI-RSV vaccinated mice after infection with RSV (24). However, we found that FI-RSV vaccinated mice had similar levels of CCR4 expression on lung and airway Tregs to those seen in mice undergoing primary infection with RSV, in which

no crash in Tregs occurs. Thus, we do not believe that downregulation of CCR4 on Tregs is responsible for their failure to accumulate in enhanced disease.

Since we could not detect CCL17 in the BAL or lung homogenates and found only low levels of CCL22 in the lungs, we were unable to show directly whether a lack chemoattraction was responsible for the crash or Tregs. However, soluble mediators can be difficult to detect, especially if they are rapidly taken up, destroyed or bound to cells. It has recently been found that adenovirus recombinant expression of CCL22 in pancreatic islets recruits Tregs and causes long-term protection from autoimmune diabetes in NOD mice (17) and that local administration of chemokines can 'pull' activated virus-specific cells into the vaginal mucosa (25). In order to test a similar approach we administered the chemokines CCL17/ 22 *via* the airway in the hope of attracting Tregs into the site of infection and attenuating disease. A single dose of this chemokine cocktail was indeed effective, even if administered on day 2 after RSV challenge (a time at which enhanced disease is already well established in FI-RSV vaccinated mice), showing that administration of additional CCL17/22 *via* the airway can re-establish a local Treg population and attenuate vaccine-enhanced disease.

The implication of these findings is that selective recruitment of Tregs by appropriate chemokine administration might rapidly reduce the severity of inflammation and disease, even when inflammation is well established, offering a novel therapeutic avenue in the treatment of tissue-specific inflammation. Although the lung is unique in allowing chemokine delivery via the airway, it is possible that disease at other sites (e.g. the skin, joints and vagina) might also respond favourably to the local administration of chemokines that selectively recruit regulatory T cells to the site of inflammation.

MATERIAL AND METHODS

Mice, virus stocks and infection and FI-RSV vaccination

Plaque-purified human RSV (type A2 strain, ATCC) was grown in HEp-2 cells. Formalin-inactivated RSV vaccine (FI-RSV) was prepared as described before (4). In brief, RSV was grown in HEp-2 cells, flasks were frozen and thawed, cells harvested and pooled. The cell suspension was sonicated for 3 min on ice and spun at 1,000 *g* for 10 min at 4 °C. A 40 % formalin solution was added to the supernatant to give a final concentration of 1:4000 (2.5 µl of formalin per each 4 ml of virus stock) and incubated for 72 h at 37 °C, 5 % CO_2. After, the supernatant was centrifuged at 50,000 *g* for 1h at 4 °C and the pellet diluted (1:25 of the starting volume) in serum-free medium. Aluminium Hydroxide (12 µl per 1 ml of supernatant) was added and the suspension shaken for 30 min at RT before centrifugation at 1,000 *g* for 30 min. The final pellet was resuspended 1:4 in PBS (i.e. 1:100 of the starting volume) and stored frozen at -80°C.

Six to ten week old BALB/c mice (Harlan, UK) or DEREG mice (22) on BALB/c background were maintained in pathogen-free conditions under UK Home Office guidelines. Age- and sex-matched mice were lightly anesthetized and infected intranasally (i.n.) with 10^6 focus-forming units (FFU) RSV in 100 µl. For FI-RSV vaccination, BALB/c mice were injected intramuscularly (i.m.) with 50 µl FI-RSV (3 mg/ml protein). Three weeks later mice were infected with RSV as described above.

IL-2 Complex injections

IL-2 complexes (IL-2 Cx) were obtained as described (19) by mixing 1 µg rmIL-2 (Peprotech) and 5 µg anti-IL-2 (Clone JES6-1A12; eBioscience) and incubating at 37

326 ˚C for 30 min. Age- and sex-matched BALB/c mice received daily i.p. injections of IL-
327 2 Cx or PBS for three consecutive days (-3,-2 and -1) before RSV infection (20).

328 **Diphtheria toxin injections**

329 DEREG mice (22) were injected with 0.75 µg of DT (Merck) in PBS i.p. on days -2
330 and -1, days 2 and 5 after RSV infection to induce and maintain $Foxp3^+$ T cell
331 depletion as previously described (20).

332 **Chemokine and antibody administration**

333 Chemokine administration was performed by intranasal instillation of 500 ng of
334 CCL17 and 22 (R&D Systems) in 100 µl PBS under light anaesthesia, ensuring deep
335 lung inhalation on day 2 post infection. For neutralization of CCL17 and 22, mice
336 were injected with one dose i.p. of 20 µg of anti CCL17 and anti-CCL22 or IgG
337 isotype control (goat anti- mouse antibodies, R&D Systems) in 200 µl PBS on day 1
338 post RSV infection.

339 **Adoptive cell transfer**

340 BALB/c mice were injected intramuscularly (i.m.) with 50 µl FI-RSV. Three weeks
341 later isolation of CD4 T cells from spleen and mesenteric lymph nodes was
342 performed using a negative CD4 T cell isolation kit II (Miltenyi) and the Auto MACS
343 pro (Miltenyi). Purity was confirmed by flow cytometry and was ≥ 90 %. Purified CD4
344 T cells ($27x10^6$/mouse) were transferred intravenously into naïve recipients. These
345 mice were infected with RSV i.n. 3 days later.

346 **Real-time PCR**

347 Lung and BAL CD4 T cells ($CD4^+GFP^-$) and Tregs ($CD4^+GFP^+$) from FI-RSV
348 vaccinated and RSV infected DEREG mice were sorted on a FACS Aria II (BD).

Total RNA was isolated from purified T cells using the Qiagen RNeasy Micro Kit with on-column DNase digestion using the RNase-Free DNase set (according to the manufacturer's protocol). cDNA was generated using the Superscript III FirstStrand Synthesis SuperMix for RT-PCR and oligodT primers (Invitrogen), according to the manufacturer's protocol. cDNA was used as a template for quantitative real-time PCR using TaqMan Gene Expression Assay (Applied Biosystems) for mouse CCR4. PCR and analysis was performed using a 7500 Fast Realtime PCR System (Applied Biosystems). Gene expression was calculated relative to GAPDH by the formular $1/2^{\Delta\Delta CT}$.

Cell collection and preparation

Bronchoalevolar lavage (BAL) was carried out using 1 ml PBS containing 12 mM lidocaine flushing the lungs 3 times. In order to obtain a single cell suspension, LNs were mashed through a cell strainer and lungs were processed with the gentleMax dissociator (Miltenyi Biotech) according to the manufacturer's protocol using Collagenase D (50 µg/ml, Sigma). Total cell counts were determined by flow cytometry using Count Bright counting beads (Invitrogen) and dead cells were excluded by staining for 7-amino-actinomycin D (7-AAD, Sigma). For determination of cellular composition in the BAL, cells were transferred onto a microscope slides (Cytospin, Thermo Scientific, UK) and stained with hematoxylin and eosin (H&E) (Reagena, Gamidor, UK).

Flow cytometry

For flow analysis the LIVE/DEAD Fixable Red Dead cell stain kit (Invitrogen) was used to exclude dead cells. Cells were incubated with FcγIII/II receptor antibody (BD Biosciences) diluted in PBS containing 1% BSA and 5 mM EDTA and were

subsequently labelled with the following antibodies (from BD Biosciences unless otherwise stated): PE-Cy7 or V500 conjugated anti-CD3 (145-2C11), Pacific Blue conjugated anti-CD4 (RM4-5), PE conjugated anti-CCR4 (2G12; Biolegend). For intracellular staining for Foxp3, the Foxp3 staining kit (eBioscience) using APC or FITC conjugated anti-Foxp3 (FJK-16s;eBioscience) was used following manufacturer's recommendations. In order to detect intracellular IFN-γ production, cells were stimulated with 100 ng/ml PMA and 1 µg/ml Ionomycin in complete RPMI. After 1 h incubation monensin (Golgi Stop, BD) was added. Following 2 additional hours of incubation, cell surface staining was followed by intracellular staining with Percp Cy 5.5 anti-IFN-γ (XMG1), PE anti-TNF-α (MP6-XT22), APC anti-IL-4 (11B1) or Alexa 700 anti-IL-17 (TC11-18H1) using the Foxp3 staining kit (eBioscience). Cells were acquired on a LSR II (BD, United Kingdom) with data analyzed using Flow Jo software. Cells were gated for live cells, singlets and lymphocytes before analysis of indicated markers.

Chemokine detection

CCL17 and CCL22 levels in the BAL were measured by ELISA following manufacturer's recommendations (R&D). The concentration of cytokines in each sample was determined according to the standard curve. The detection limit for CCL17 was 31.2 pg/ml and for CCL22 7.8 pg/ml.

Statistical analysis

Results are presented as means ± SEM. The significance of results between the groups was analyzed by two-tailed, unpaired Student's t test (*p< 0.05, **p < 0.01, ***p< 0.001) (Prism software; Graph-Pad Software Inc.). p values of <0.05 were considered significant.*p < 0.05, **p < 0.01, ***p < 0.001 was used to compare

different groups on one time point and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ was used for comparing one experimental group at different time points.

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Reference List

1. Hall, CB, Weinberg, GA, Iwane, MK, Blumkin, AK, Edwards, KM, Staat, MA, Auinger, P, Griffin, MR, Poehling, KA, Erdman, D, Grijalva, CG, Zhu, Y, & Szilagyi, P (2009) The burden of respiratory syncytial virus infection in young children. *N. Engl. J. Med.* 360:588-598.

2. Collins, PL & Graham, BS (2008) Viral and host factors in human respiratory syncytial virus pathogenesis. *J. Virol.* 82:2040-2055.

3. Smyth, RL & Openshaw, PJ (2006) Bronchiolitis. *Lancet* 368:312-322.

4. Moghaddam, A, Olszewska, W, Wang, B, Tregoning, JS, Helson, R, Sattentau, QJ, & Openshaw, PJ (2006) A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines. *Nat. Med.* 12:905-907.

5. Delgado, MF, Coviello, S, Monsalvo, AC, Melendi, GA, Hernandez, JZ, Batalle, JP, Diaz, L, Trento, A, Chang, HY, Mitzner, W, Ravetch, J, Melero, JA, Irusta, PM, & Polack, FP (2009) Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. *Nat. Med.* 15:34-41.

6. Murphy, BR, Prince, GA, Walsh, EE, Kim, HW, Parrott, RH, Hemming, VG, Rodriguez, WJ, & Chanock, RM (1986) Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. *J Clin. Microbiol.* 24:197-202.

7. Bennett, CL, Christie, J, Ramsdell, F, Brunkow, ME, Ferguson, PJ, Whitesell, L, Kelly, TE, Saulsbury, FT, Chance, PF, & Ochs, HD (2001) The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* 27:20-21.

8. Littman, DR & Rudensky, AY (2010) Th17 and regulatory T cells in mediating and restraining inflammation. *Cell* 140:845-858.

9. Kim, J, Lahl, K, Hori, S, Loddenkemper, C, Chaudhry, A, deRoos, P, Rudensky, A, & Sparwasser, T (2009) Cutting edge: depletion of Foxp3+ cells leads to induction of autoimmunity by specific ablation of regulatory T cells in genetically targeted mice. *J. Immunol.* 183:7631-7634.

10. Kim, JM, Rasmussen, JP, & Rudensky, AY (2007) Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat. Immunol.* 8:191-197.

11. Campbell, DJ & Koch, MA (2011) Phenotypical and functional specialization of FOXP3(+) regulatory T cells. *Nat. Rev. Immunol.* 11:119-130.

12. Iellem, A, Mariani, M, Lang, R, Recalde, H, Panina-Bordignon, P, Sinigaglia, F, & D'Ambrosio, D (2001) Unique chemotactic response profile and specific

expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. *J. Exp. Med.* 194:847-853.

13. Haas, J, Schopp, L, Storch-Hagenlocher, B, Fritzsching, B, Jacobi, C, Milkova, L, Fritz, B, Schwarz, A, Suri-Payer, E, Hensel, M, & Wildemann, B (2008) Specific recruitment of regulatory T cells into the CSF in lymphomatous and carcinomatous meningitis. *Blood* 111:761-766.

14. Soler, D, Chapman, TR, Poisson, LR, Wang, L, Cote-Sierra, J, Ryan, M, McDonald, A, Badola, S, Fedyk, E, Coyle, AJ, Hodge, MR, & Kolbeck, R (2006) CCR8 expression identifies CD4 memory T cells enriched for FOXP3+ regulatory and Th2 effector lymphocytes. *J. Immunol.* 177:6940-6951.

15. Hirahara, K, Liu, L, Clark, RA, Yamanaka, K, Fuhlbrigge, RC, & Kupper, TS (2006) The majority of human peripheral blood CD4+CD25highFoxp3+ regulatory T cells bear functional skin-homing receptors. *J. Immunol.* 177:4488-4494.

16. Sather, BD, Treuting, P, Perdue, N, Miazgowicz, M, Fontenot, JD, Rudensky, AY, & Campbell, DJ (2007) Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific inflammatory disease. *J. Exp. Med.* 204:1335-1347.

17. Montane, J, Bischoff, L, Soukhatcheva, G, Dai, DL, Hardenberg, G, Levings, MK, Orban, PC, Kieffer, TJ, Tan, R, & Verchere, CB (2011) Prevention of murine autoimmune diabetes by CCL22-mediated Treg recruitment to the pancreatic islets. *J. Clin. Invest* 121:3024-3028.

18. Boelen, A, Andeweg, A, Kwakkel, J, Lokhorst, W, Bestebroer, T, Dormans, J, & Kimman, T (2000) Both immunisation with a formalin-inactivated respiratory syncytial virus (RSV) vaccine and a mock antigen vaccine induce severe lung pathology and a Th2 cytokine profile in RSV-challenged mice. *Vaccine* 19:982-991.

19. Webster, KE, Walters, S, Kohler, RE, Mrkvan, T, Boyman, O, Surh, CD, Grey, ST, & Sprent, J (2009) In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. *J. Exp. Med.* 206:751-760.

20. Loebbermann, J, Thornton, H, Durant, L, Sparwasser, T, Webster, KE, Sprent, J, Culley, FJ, Johansson, C, & Openshaw, PJ (2012) Regulatory T cells expressing granzyme B play a critical role in controlling lung inflammation during acute viral infection. *Mucosal. Immunol.* 5:161-172.

21. Oldenhove, G, Bouladoux, N, Wohlfert, EA, Hall, JA, Chou, D, Dos, SL, O'Brien, S, Blank, R, Lamb, E, Natarajan, S, Kastenmayer, R, Hunter, C, Grigg, ME, & Belkaid, Y (2009) Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity.* 31:772-786.

22. Lahl, K, Loddenkemper, C, Drouin, C, Freyer, J, Arnason, J, Eberl, G, Hamann, A, Wagner, H, Huehn, J, & Sparwasser, T (2007) Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J. Exp. Med.* 204:57-63.

23. Yuan, Q, Bromley, SK, Means, TK, Jones, KJ, Hayashi, F, Bhan, AK, & Luster, AD (2007) CCR4-dependent regulatory T cell function in inflammatory bowel disease. *J. Exp. Med.* 204:1327-1334.

24. Openshaw, PJ & Tregoning, JS (2005) Immune responses and disease enhancement during respiratory syncytial virus infection. *Clin. Microbiol. Rev.* 18:541-555.

25. Shin, H & Iwasaki, A (2012) A vaccine strategy that protects against genital herpes by establishing local memory T cells. *Nature* doi:10.1038/nature11522.

26. Ruckwardt, TJ, Bonaparte, KL, Nason, MC, & Graham, BS (2009) Regulatory T cells promote early influx of CD8+ T cells in the lungs of respiratory syncytial virus-infected mice and diminish immunodominance disparities. *J. Virol.* 83:3019-3028.

27. Fulton, RB, Meyerholz, DK, & Varga, SM (2010) Foxp3+ CD4 regulatory T cells limit pulmonary immunopathology by modulating the CD8 T cell response during respiratory syncytial virus infection. *J. Immunol.* 185:2382-2392.

28. Lee, DC, Harker, JA, Tregoning, JS, Atabani, SF, Johansson, C, Schwarze, J, & Openshaw, PJ (2010) CD25+ natural regulatory T cells are critical in limiting innate and adaptive immunity and resolving disease following respiratory syncytial virus infection. *J. Virol.* 84:8790-8798.

29. Pease, JE (2011) Targeting chemokine receptors in allergic disease. *Biochem. J.* 434:11-24.

30. Strutt, TM, McKinstry, KK, & Swain, SL (2011) Control of innate immunity by memory CD4 T cells. *Adv. Exp. Med. Biol.* 780:57-68.

31. Loebbermann, J, Schnoeller, C, Thornton, H, Durant, L, Sweeney, NP, Schuijs, M, O'Garra, A, Johansson, C, & Openshaw, PJ (2012) IL-10 regulates viral lung immunopathology during acute respiratory syncytial virus infection in mice. *PLoS. ONE.* 7:e32371.

Figure Legends

Figure 1. FI-RSV vaccination attenuates BAL Treg responses during RSV infection.

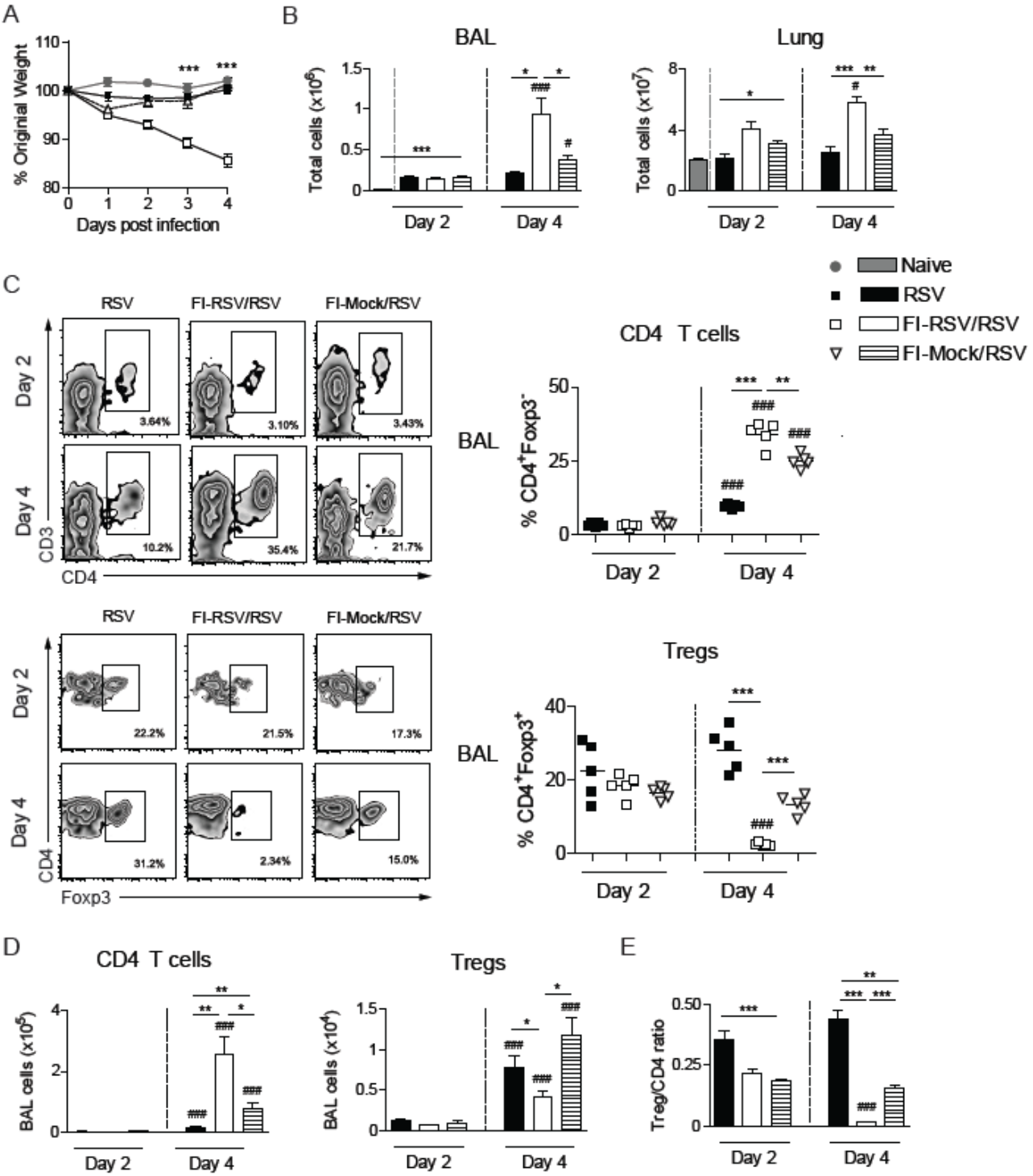


Figure 1 Loebbermann et al.

Naive, FI-RSV or FI-Mock vaccinated mice were infected intranasally with RSV (day 0). (A) Illness was monitored daily by weight for 4 days after RSV infection. On day 4, BAL fluid was obtained and cells extracted from the disrupted lungs. (B) Total numbers of cells in the BAL and lung were enumerated on day 2 and 4. (C) Frequencies of $Foxp3^-$ gated $CD3^+CD4^+$ T cells (CD4 T cells) and (D) $CD3^+CD4^+$ gated $Foxp3^+$ T cells (Tregs) were quantified using flow cytometry on day 2 and 4 post RSV infection. (E) Total cell numbers of CD4 T cells and Tregs on day 2 and 4 post RSV infection. (F) Ratio of total number of Tregs to total number of CD4 T cells in the BAL on day 2 and 4 post RSV infection. Graphs show data from one of two independent experiments with 5 mice per group. Results are presented as means ± SEM. The significance of results between the groups was analyzed by two-tailed, unpaired Student's t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ was used to compare different groups on one daypoint, and #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ for comparing one group at different daypoints.

537 **Figure 2. Transferred CD4 T cells attenuate airway Treg responses**.

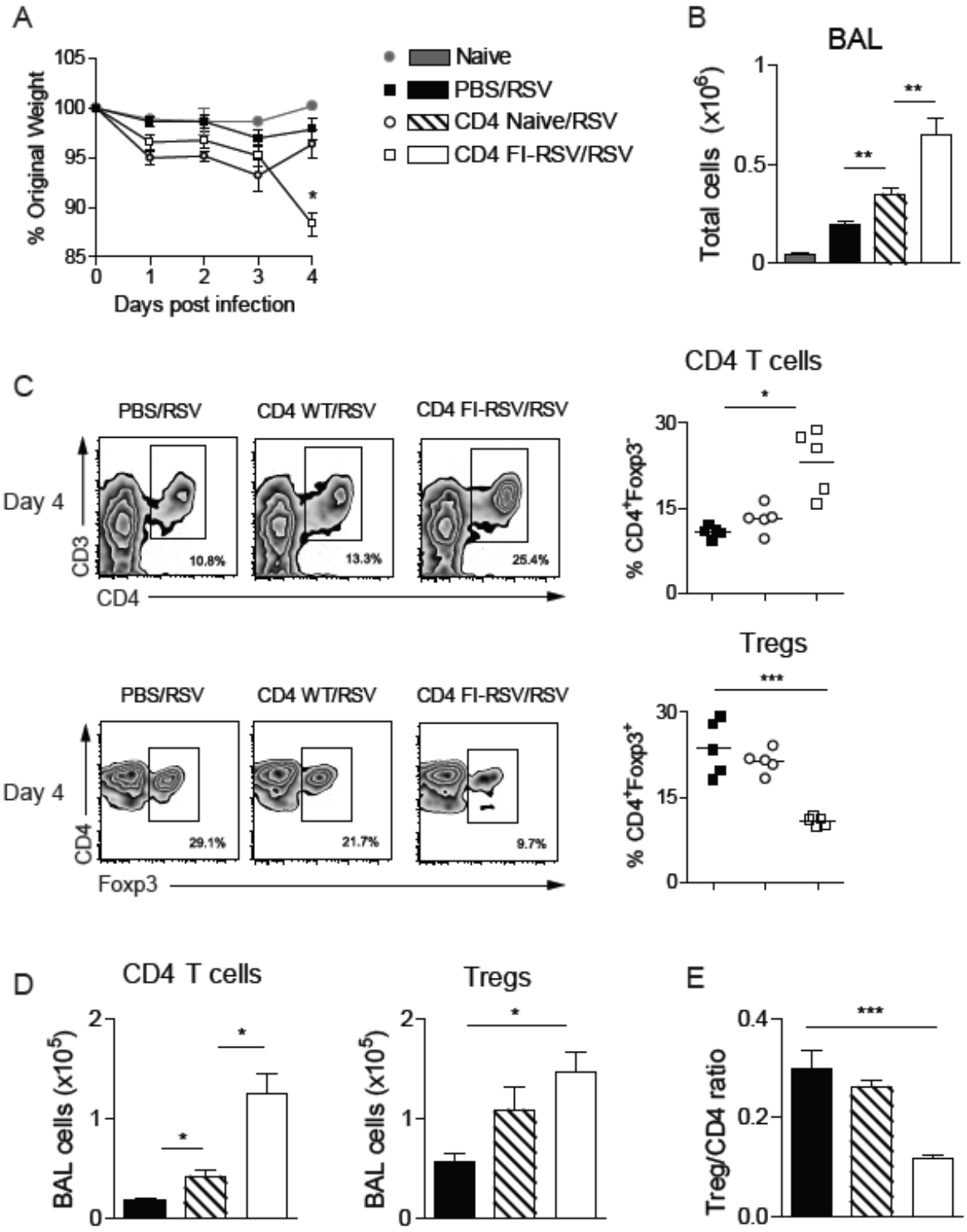


Figure 2 Loebbermann et al.

538

539 CD4 T cells from naïve or FI-RSV vaccinated mice were transferred intravenously
540 into naïve recipients on day -3, with RSV infection on day 0 and BAL harvest on day
541 4. (A) Illness was monitored daily by weight, displayed as percentage of original
542 weight. (B) Total numbers of BAL cells. (C) Frequencies of $Foxp3^-$ gated $CD3^+CD4^+$
543 T cells (CD4 T cells) and Tregs quantified using flow cytometer.(D) Total calculated
544 numbers of CD4 T cells and Tregs. (E) Ratio of Tregs to CD4 T cells. One
545 representative study of two independent experiments with 5 mice per group is
546 shown, presented as means ± SEM. The significance of results between the groups
547 was analyzed by two-tailed, unpaired Student's t test. *$p < 0.05$, **$p < 0.01$, ***$p <$
548 0.001.

549

Figure 3. Effects of chemoattraction of Tregs by CCL17/22 administration via the airway.

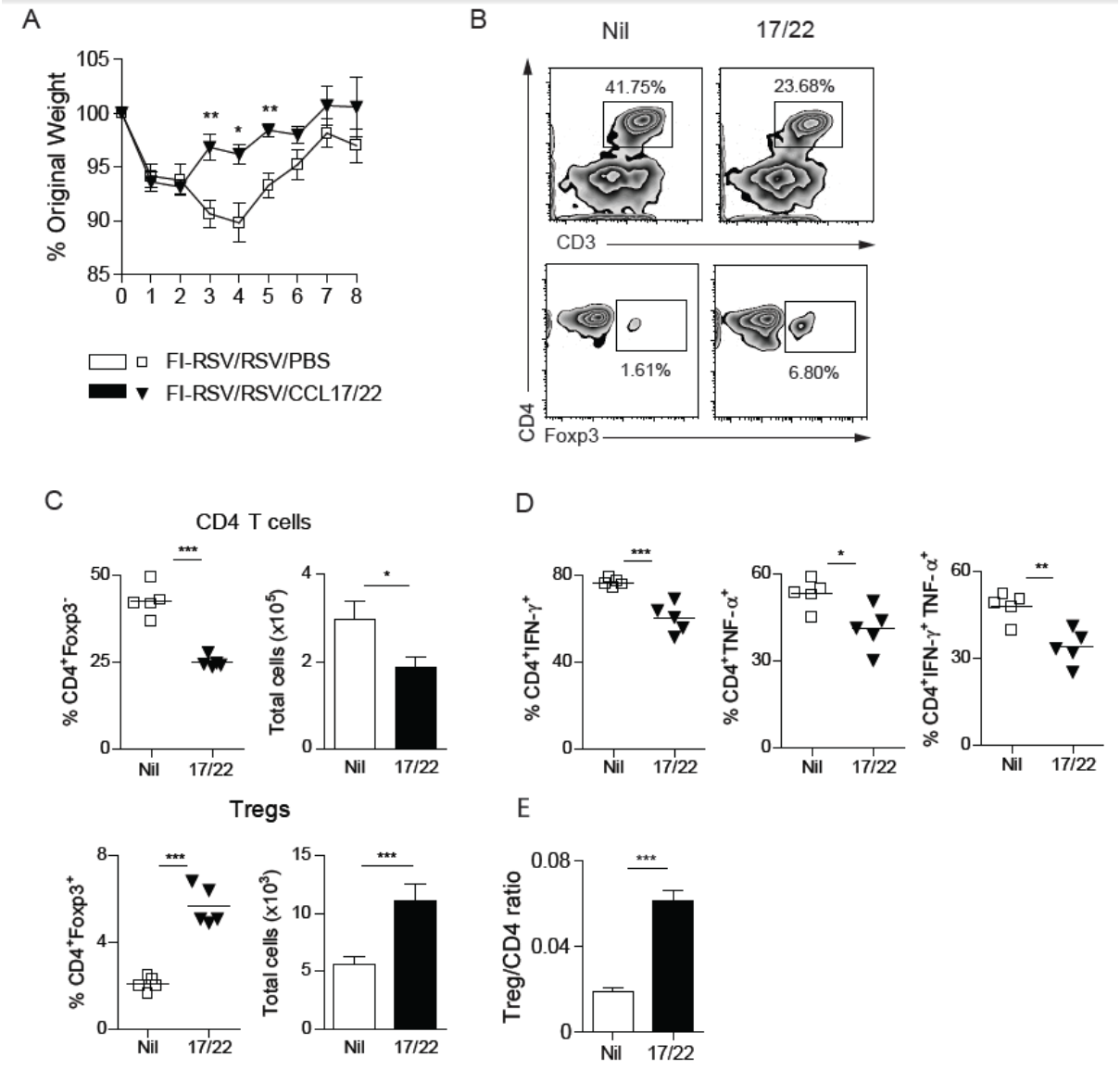


Figure 3 Loebbermann et al.

BALB/c mice were vaccinated with FI-RSV and infected three weeks later with RSV i.n. (day 0). Mice were given a mixture of 0.5 µg CCL17 and 0.5 µg of CCL22, or PBS intranasally on day 2, and BAL cells were harvested from 5 mice per group on day 4 and weight monitored to day 8 in the remainder. (A) Daily weight after RSV infection as percentage of original weight. (B) Frequencies of Tregs and Foxp3$^-$

gated CD4 T cells by flow cytometry in mice with (17/22) or without ('nil') intranasal CCL17/22. (C) Frequencies (left) and total numbers (right) of Tregs and CD4 T cells. (D) 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 post vaccination and RSV infection. (E) Ratio of total number of Tregs to total number of CD4 T cells in the BAL on day 4 post RSV infection. One representative study of two independent experiments with 5 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's t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 4. Recruited Tregs are necessary to control CD4 T cells response in the airways.

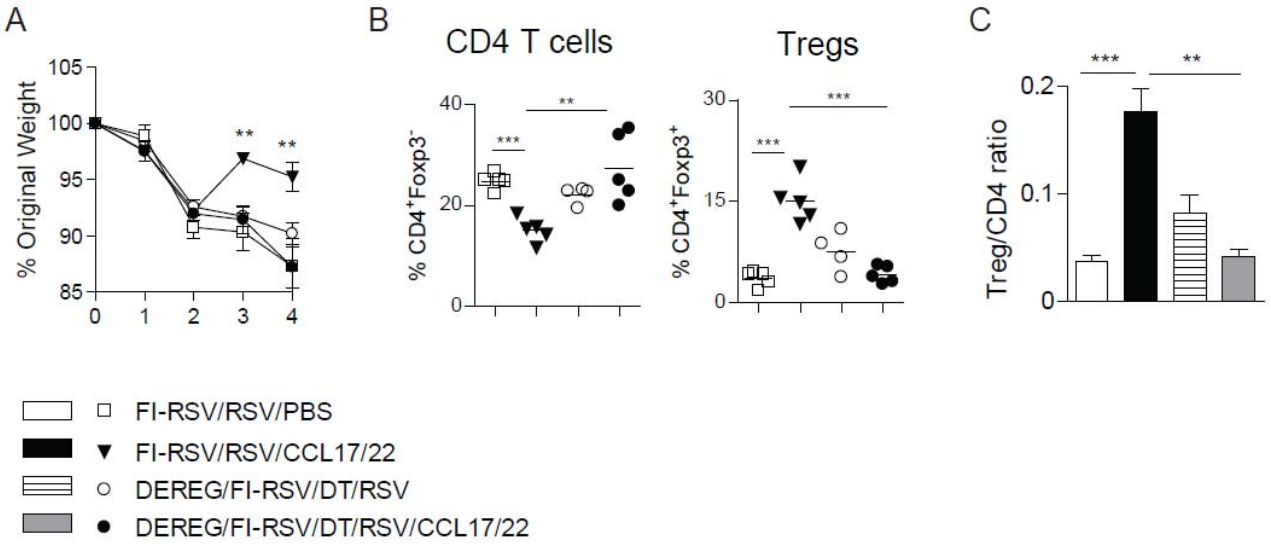


Figure 4 Loebbermann et al.

BALB/c WT or DEREG mice or were vaccinated with FI-RSV and infected three weeks later with RSV i.n. (day 0). When indicated, mice were treated i.n. with a mixture of 0.5 µg both CCL17 and 22, or PBS on day 2 post RSV infection or/ and depleted of Tregs by diphtheria toxin injections on day -2,-1 and 2. All mice were sacrificed on day 4 and BAL cells obtained. (A) Daily weights as percentage of original weight. (B) Frequencies of CD4 T cells and Tregs by flow cytometry. (C) Ratio of numbers of Tregs to CD4 T cells. One representative study of two independent experiments with 5 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's t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Supplemental Figure Legends

582 **Supplemental Figure S1. FI-RSV vaccination leads to a reduction of Tregs in**

583 **the lung but not in the draining LN after RSV infection**.

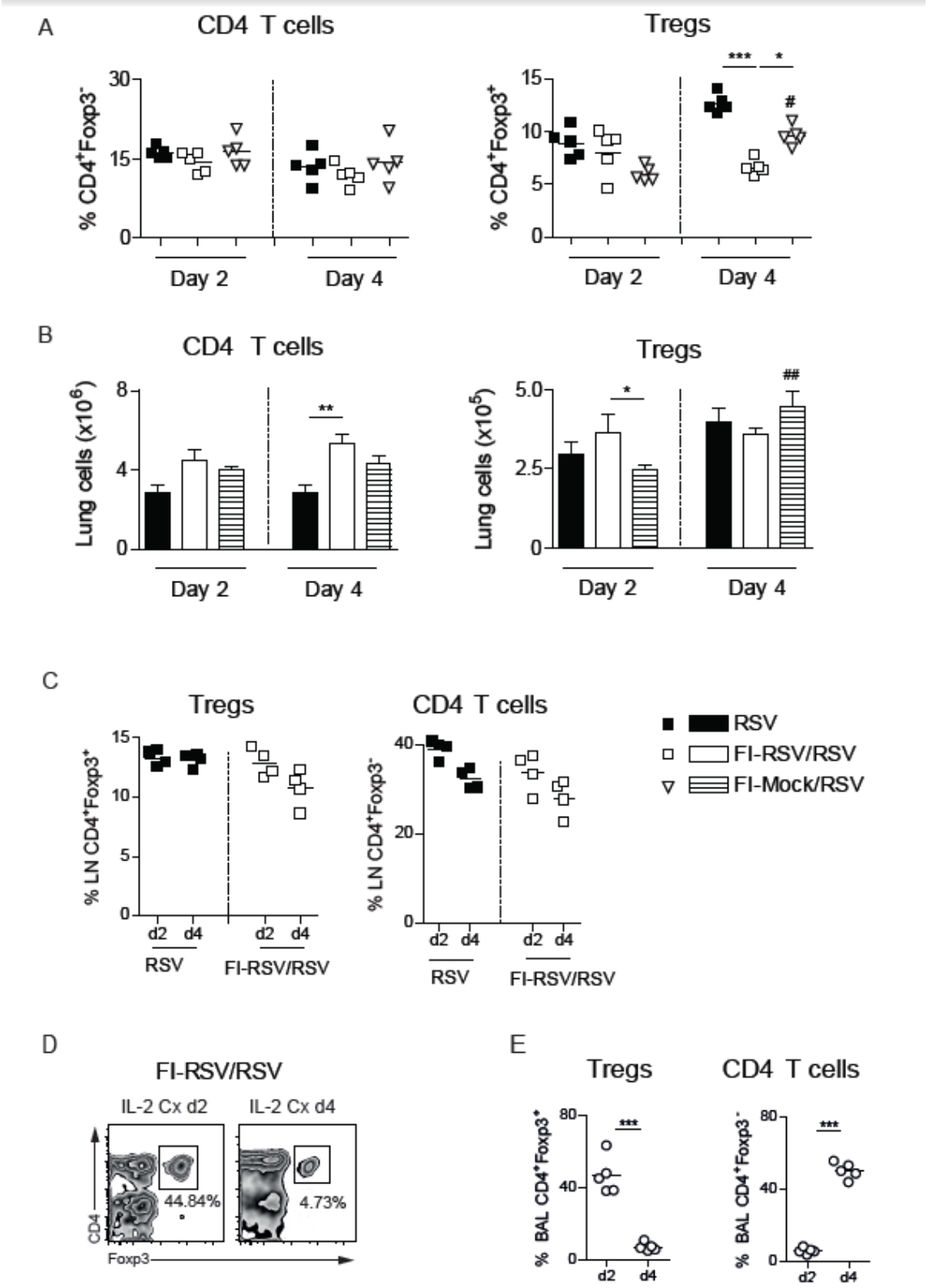


Figure S1 Loebbermann et al.

584

585 FI-RSV or FI-Mock vaccinated mice were infected with RSV on day 0. Lungs were
586 harvested on day 2 and day 4 post infection. (A) Quantification of frequencies of
587 Tregs and Foxp3^{-} gated CD3^{+}CD4^{+} T cells (CD4 T cells) in the lung on day 2 and 4
588 post RSV infection. (B) Total cell numbers of Tregs and CD4 T cells in the lung on
589 day 2 and 4 post RSV infection. (C) Frequencies of Tregs and CD4^{+} T cells in the
590 mediastinal LNs on day 2 and 4 post RSV infection. (D) Frequencies of
591 CD3^{+}CD4^{+}gated Tregs in the BAL of FI-RSV vaccinated, IL-2 Cx injected mice on
592 day 2 and 4 post RSV infection. (E) Quantification of frequencies of Tregs and CD4
593 T cells on day 2 and 4 post RSV infection in FI-RSV vaccinated, IL-2 Cx injected
594 mice. One representative study of two independent experiments with 5 mice per
595 group is shown. Results are presented as means ± SEM. The significance of results
596 between the groups was analyzed by two-tailed, unpaired Student's t test. *p < 0.05,
597 **p < 0.01, ***p < 0.001 was used to compare different groups on one daypoint and
598 #p < 0.05, ##p < 0.01, ###p < 0.001 was used for comparing one group at different
599 daypoints. *p<0.05, **p<0.01, ***p<0.001.

600

Supplemental Figure S2. Depletion of Foxp3^{+} cells in FI-RSV vaccinated DEREG mice does not increase weight loss or cellular influx into the lungs after RSV infection.

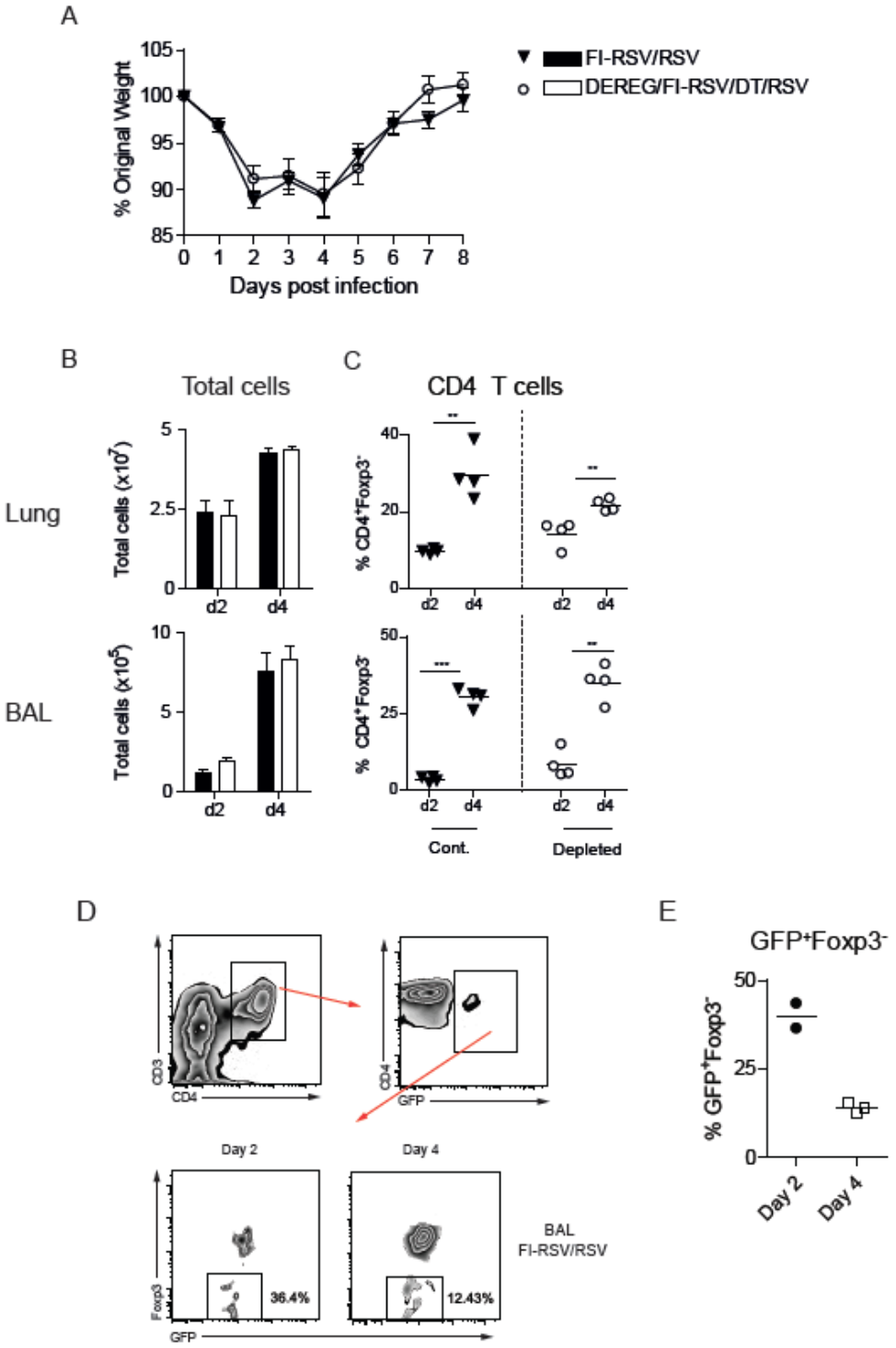


Figure S2 Loebbermann et al.

(A) Illness was monitored daily by weight for 8 days after RSV infection, displayed as percentage of original weight. (B) Total numbers of cells in the lung (top panel) and BAL (bottom panel) was enumerated on day 2 and 4 from RSV infected FI-RSV vaccinated mice, with and without Treg depletion. (C) Frequencies of CD4 T cells in the lung (top panel) and BAL (bottom panel) quantified by flow cytometry on day 2 and 4 post RSV infection. Graphs show data from one representative out of three independent experiments with 5 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's t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

615 **Supplemental Figure S3. Tregs in the lung express CCR4.**

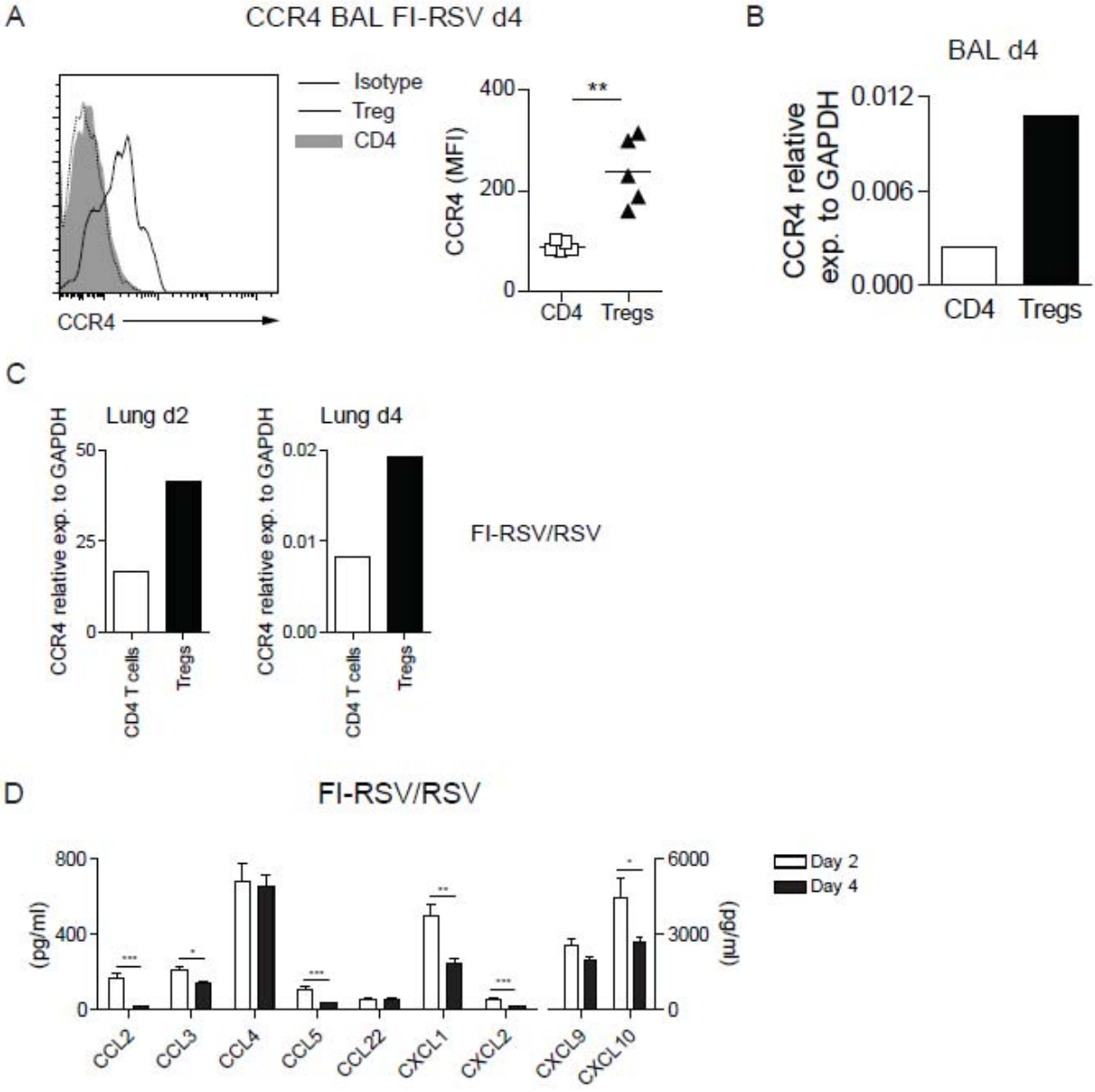


Figure S3 Loebbermann et al.

616

617 FI-Mock or FI-RSV vaccinated mice were infected with RSV on day 0 and
618 lymphocytes recovered by BAL on day 4. When indicated mice were injected with IL-
619 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 post infection. (B) Quantification of CCR4 expression on Tregs and CD4 T cells in the BAL on days 2 and 4 post infection measured as mean fluorescent intensity (MFI). (C) Relative expression of CCR4 to GAPDH of BAL CD4 T cells and Tregs on day 4 post infection 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 day 2 and day 4 post infection 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 day 2 and 4 post RSV infection. Data is pooled from two independent experiments, 4-5 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's t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Supplemental Figure S4. Effects of instillation of CCL17 and/or CCL22 in the airways of FI-RSV vaccinated mice after RSV infection.

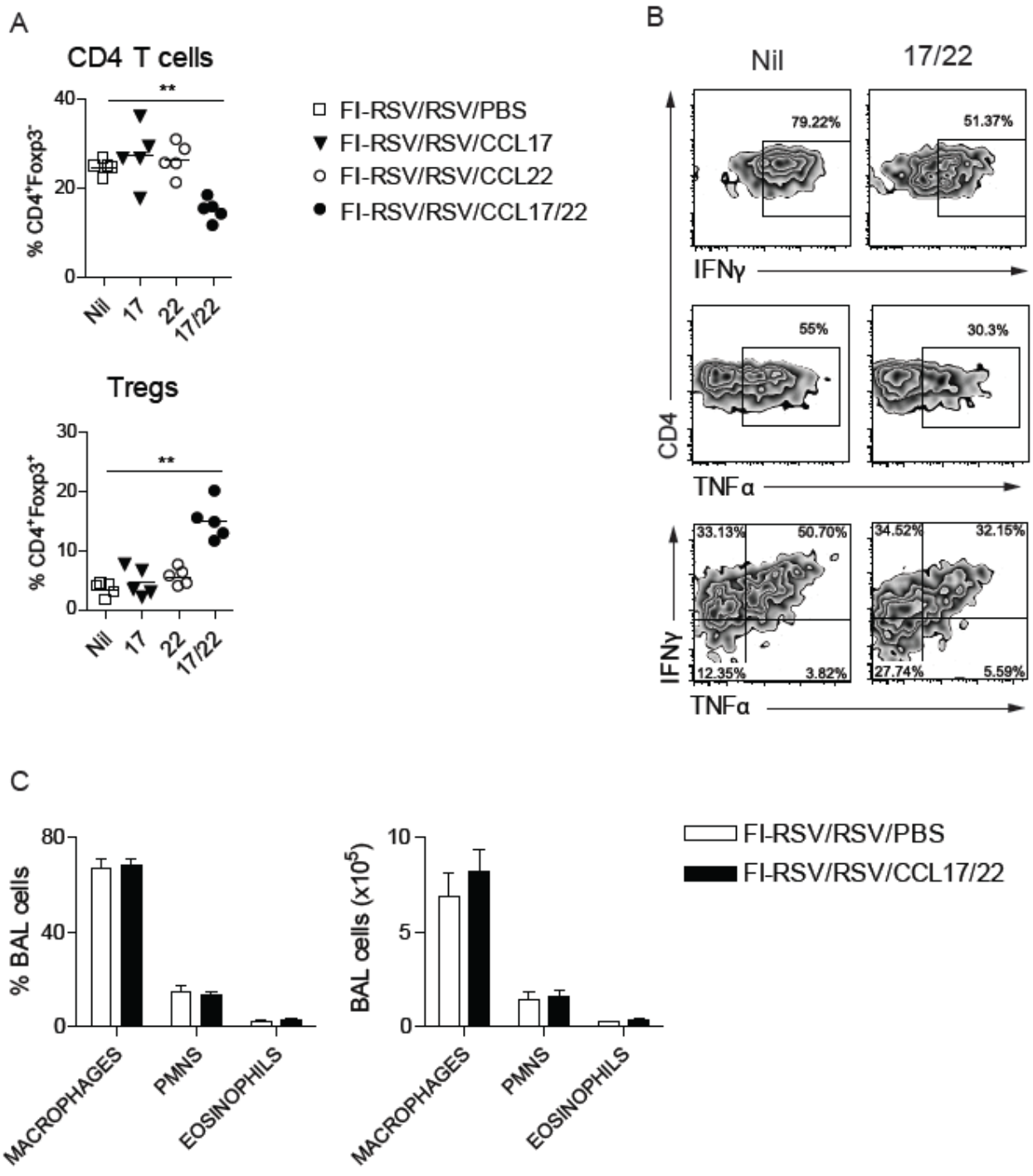


Figure S4 Loebbermann et al.

(A) Quantification of frequencies of Tregs and CD4 T cells on day 2 and 4 post 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 post RSV infection were quantified using flow cytometry on day 2 and 4 post 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 post infection. Graphs show data from one representative out of two independent experiments with 5 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's t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

650 **Supplemental Figure S5. Late effects of chemoattraction of Tregs into the**

651 **airways by CCL17/22.**

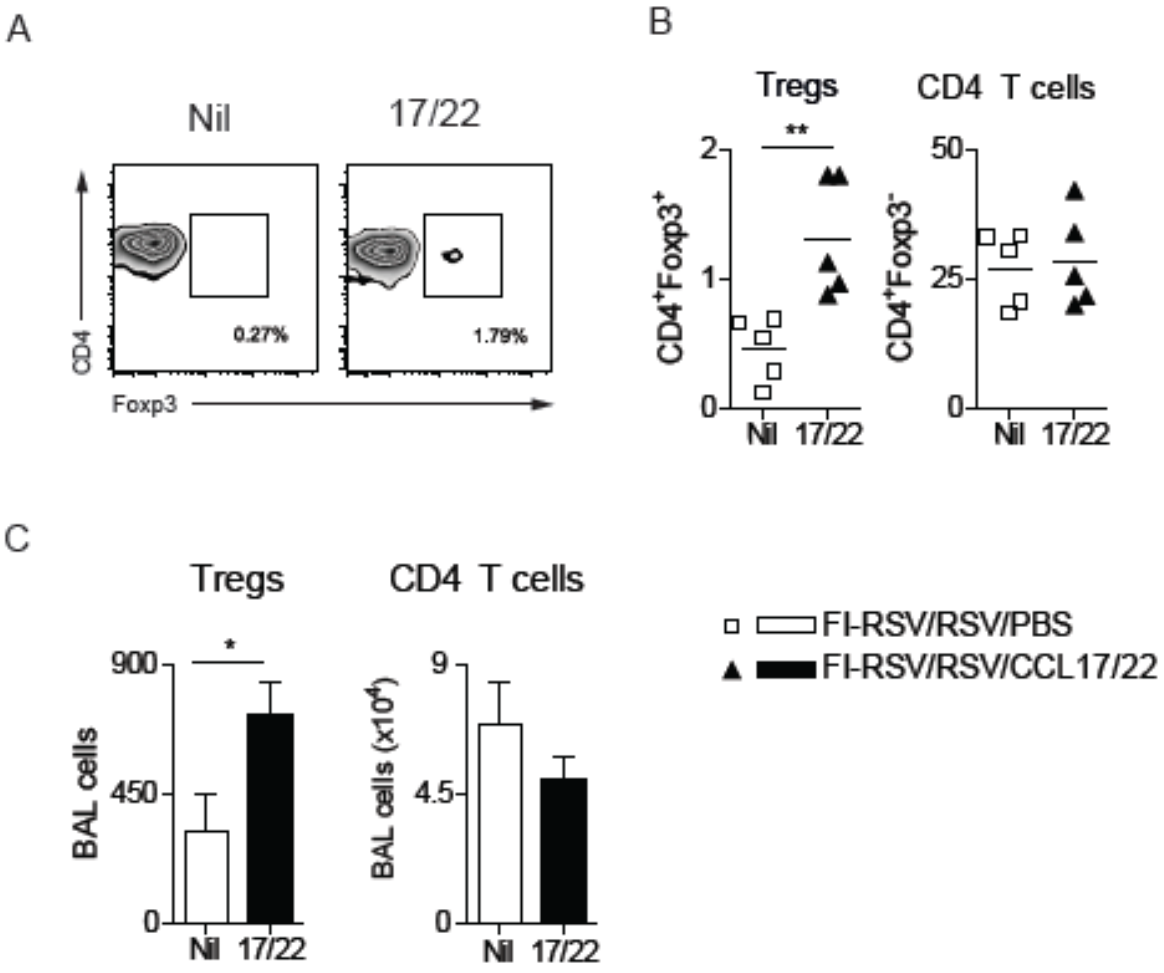
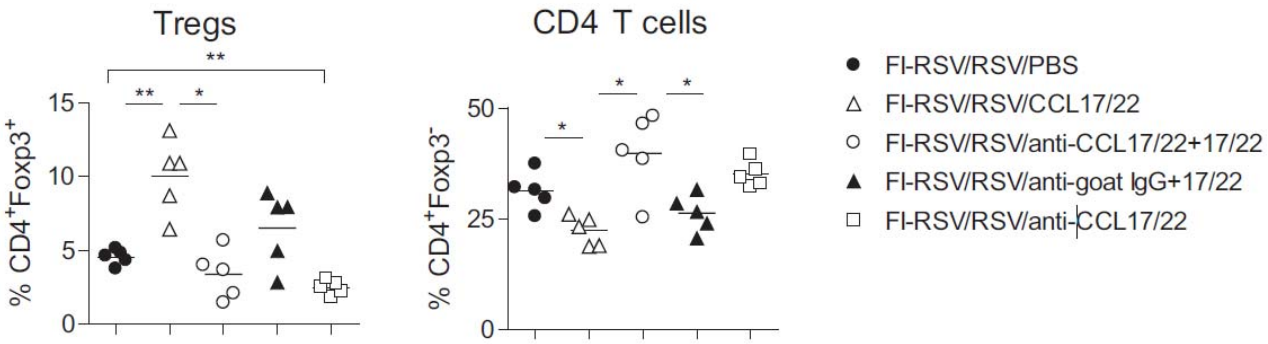


Figure S5 Loebbermann et al.

652

BALB/c mice were vaccinated with FI-RSV and infected three weeks later with RSV 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 post RSV infection. (A) Frequencies of Tregs in the BAL were quantified using flow cytometry on day 8 post RSV infection with ('17/22') or without ('nil') intranasal administration of CCL17/22. (B) Quantification of Tregs and CD4 T cells frequencies in the BAL on day 8 post RSV infection. (C) Quantification of total numbers of Tregs and CD4 T cells in the BAL on day 8 post RSV infection. Graphs show data from one representative out of two independent experiments with 5 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's t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Supplemental Figure 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 post RSV infection into FI-RSV vaccinated mice prior to instillation of CCL17/22 on day 2 post infection. Quantification of Tregs (left) and CD4 T cells (right) frequencies in the BAL on day 4 post RSV infection. Graphs show data from one representative out of two independent experiments with 5 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's t test. *p < 0.05, **p < 0.01, ***p < 0.001.

Supplemental Figure S7. The balance of regulatory and disease-causing T cells in RSV infection.

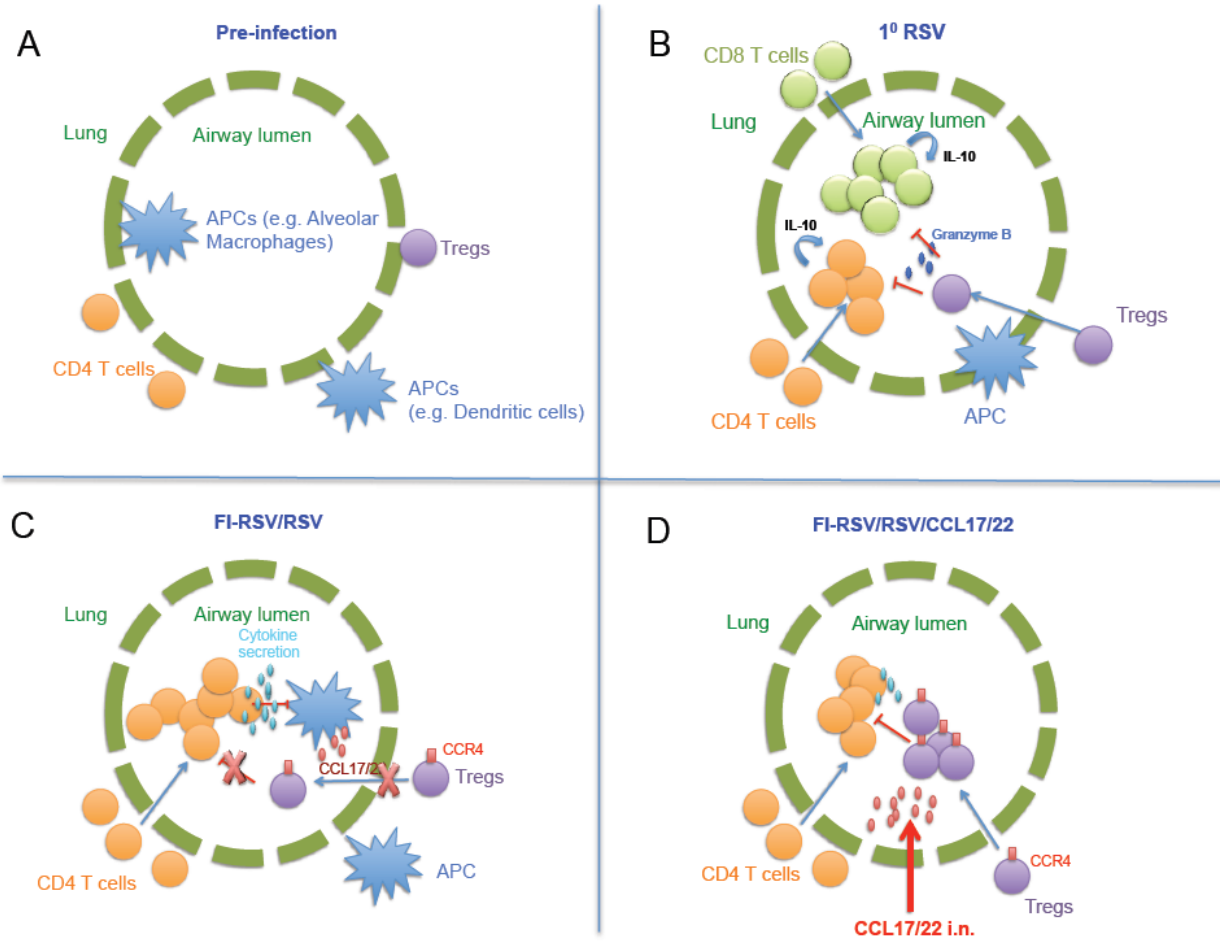


Figure S6 Loebbermann et al

(A) The airways of clean 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 (20); both CD4 and CD8 T cells produce IL-10, which assists in modulation of the immune response (31). (C) RSV infection of mice previously vaccinated with FI-RSV show a rapid and exuberant CD4 T cell response, leading to mediator release into the local environment and shut-down 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)

687 Administration of CCL17/22 intranasally bypasses the shut-down of Treg recruitment
688 and attenuates vaccine-augmented disease, reducing disease severity.

689