Dissecting the Inflammatory Twitch in Allergically Inflamed Mice

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ABSTRACT

We have previously advanced the hypothesis that the allergic inflammatory response in the lungs occurs as a self-limited sequence of events that begins with the onset of inflammation and then resolves back to baseline over a predetermined time course (*J Immunol* 190: 3510, 2013). In the present study we tested a key prediction of this hypothesis, which is that the instigation of the allergic inflammatory response should be accompanied by a later refractory period during which the response cannot be reinitiated. We challenged groups of ovalbumin-sensitized BALB/c mice for 3, 14, 21 and 31 consecutive days with aerosolized ovalbumin. We measured airways responsiveness as well as cell counts and cytokines in bronchoalveolar lavage fluid after the final challenge in subgroups from each group. In other subgroups we performed the same measurements following rest periods and after a final single recall challenge with antigen. We determined that the refractory periods for GM-CSF, KC and IL-5 are no longer than 10 days, while those for IFNγ and IL-10 are no longer than 28 days. The refractory periods for total leukocytes and neutrophils were no greater than 28 days, while that for eosinophils was more than 28 days. The refractory period for airways resistance was less than 17, while for lung elastance it was longer than 28 days. Our results thus demonstrate that the components of the allergic inflammatory response in the lung have finite refractory periods, with the refractory period of the entire response being in the order of a month.

Key words: lung function, cytokines, bronchoalveolar lavage, refractory period
INTRODUCTION

The inflammatory response to a noxious stimulus is crucial for survival, and is initiated by a cascade of events beginning with the recognition of the stimulus by sentinel cells. Equally important for health, however, is the resolution of the inflammatory response when it is no longer needed. Indeed, failure of the inflammatory response to resolve may underlie a variety of chronic diseases. This raises the fundamental question of how the duration of the inflammatory response is controlled. In particular, it is difficult to imagine the existence of some agent in the body with the capacity to decide when inflammation is no longer necessary, so how is the inflammatory response turned off?

As a possible response to this question, we recently postulated that inflammation is subject to the same control strategy as skeletal muscle, namely through the repetitive invocation of self-limiting unitary events that, in the case of muscle, are known as twitches (16). We hypothesized that the inflammatory equivalent, which we term the inflammatory twitch, continues to be invoked so long as the allergic stimulus is present. This allows an ongoing inflammatory response stream to be maintained while it is needed, but ensures that the response will automatically dissipate when the stimulus disappears. Of course, a skeletal muscle twitch lasts in the order of 100 ms while an inflammatory response would have to last for much longer, but the two systems may still have strong formal similarities in control structure despite their huge differences in timescale.

We previously used an agent-based computational model in a proof-of-concept study to show that a twitch-like event can, in principle, arise with respect to allergic inflammation in the lung (16). We then used the model to perform a series of in silico experiments that explored how the
inflammatory twitch could potentially fail to resolve (15), the notion being that such failure might explain the chronic inflammation characteristic of allergic asthma. Nevertheless, experimental data in support of the inflammatory twitch hypothesis remain somewhat sparse. In particular, while there is some evidence that the normal allergic inflammatory response is indeed self-limited (18, 20), there has yet to be a comprehensive test of another key prediction of the hypothesis, namely that the response should be accompanied by a refractory period during which it cannot be reinitiated by continued antigen stimulation. Accordingly, the goal of the present study was to perform a detailed examination of the time-course of the components of the allergic inflammatory response in the lung and to determine if any of these components exhibit periods of refractoriness. We performed this investigation in ovalbumin-sensitized mice that were challenged with aerosolized ovalbumin for varying durations and then re-challenged after varying rest periods.

MATERIALS AND METHODS

Our studies conformed to the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the University of Vermont’s Institutional Animal Care and Use Committee (IACUC).

Antigen Sensitization and Challenge Protocol

We studied 104 female BALB/cJ mice at 7-8 weeks of age (Jackson Laboratories) separated into five groups: Saline-3 (16 mice), Saline-31 (8 mice), OVA-3 (24 mice), OVA-31 (8 mice), OVA-14 (24 mice) and OVA-21 (24 mice). Numbers after Saline or OVA correspond to the number of consecutive days for which the mice were challenged. The experimental protocol is outlined in Table. 1. All mice were initially sensitized with intraperitoneal (IP) injections of 0.1 mL
containing 20 µg ovalbumin (OVA) in phosphate-buffered saline suspended in 2 mg of alum (ThermoFisher Scientific) on Day -20 and Day -6. The different animal groups were then subjected to different regimens of daily OVA challenge, each regimen beginning on Day 1 as indicated in Table 1. A daily challenge consisted of placing the animals in a closed chamber for 30 minutes within which they were exposed to aerosols of either 1% OVA in phosphate-buffered saline or to phosphate-buffered saline, as indicated in Table 1.

The Saline-3 and OVA-3 groups were challenged for the shortest duration of 3 consecutive days. Mice in Saline-3 were exposed to nebulized saline and then divided into two subgroups each containing 6-8 mice. One subgroup was studied (see below) one day after the 3 challenges (i.e. on Day 4). The other subgroup was maintained without further challenge for another 28 days, subjected to a saline recall exposure on day 31, and then studied a day later. Mice in OVA-3 were treated identically to the Saline-3 animals except that they were exposed to nebulized OVA instead of saline, with a subgroup characterized immediately prior to the recall challenge on Day 31.

The Saline-31 and OVA-31 groups were challenged for the longest duration of 31 days. Mice in the Saline-31 group underwent daily exposure to nebulized saline (Day 1 to Day 31) and were studied a day later (i.e. on Day 32). Mice in OVA-31 were treated identically to Saline-31 animals except that they were exposed to OVA instead of saline.

The OVA-14 and OVA-21 groups were challenged with OVA for the intermediate durations of 14 and 21 days, respectively. Mice in OVA-14 were divided into three subgroups. One subgroup was studied one day after the final challenge (i.e. on Day 15). The other subgroups were maintained without challenge until Day 31 at which point one subgroup was characterized while
the other received a recall challenge with OVA and then was studied a day later (i.e. on Day 32). Mice in OVA-21 were treated similarly except that their initial sequence of daily challenges with OVA ended on Day 21.

Study Procedures

Lung Physiology: Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (90 mg/kg), tracheostomized, and cannulized. They were then attached to a computer-controlled mechanical ventilator (Flexivent, Scireq, Montreal, Quebec, Canada), paralyzed with an IP injection of pancuronium bromide (0.8 mg/kg), and administered regular ventilation at 200 breaths/min against a positive-end-expiratory pressure (PEEP) of 3 cmH₂O while EKG was monitored to ensure depth of anesthesia. The forced oscillation technique was used to measure respiratory impedance ($Z_{rs}$) over the frequency range from 1 to 20.5 Hz using a 2 s broadband perturbation in volume applied by the Flexivent, exactly as we have done in a number of prior studies (1, 22, 23). Each measurement of $Z_{rs}$ was fit to the constant-phase model (5, 22). We used two of the best-fit parameter values of the constant-phase model as our outcomes variables: 1) the Newtonian resistance ($R_n$) of the lung, which accurately estimates airway resistance (21), and 2) lung stiffness ($H$), which is essentially equivalent to lung elastance (22). Two baseline values of $R_n$ and $H$ were obtained in this way. We then exposed each animal to an aerosol of saline, delivered for 10 s via the inspiratory line of the Flexivent, and then determined $R_n$ and $H$ every 10 s for 3 minutes for a total of 18 measurements of each parameter. This sequence was then repeated replacing the saline aerosol with aerosolized methacholine at a concentration of 12.5 mg/ml. Airway responsiveness to saline and to methacholine was determined from the averages of the 18 measurements of $R_n$ and $H$ made following each challenge.
Broncho-alveolar lavage: Following the end of the AHR protocol, bronchoalveolar lavage fluid (BALF) was gathered by lavaging the lungs with 1 mL of saline. This lavage fluid was centrifuged at 600 g at 10ºC for 10 minutes and the resulting cell pellet was re-suspended in saline. Half of this sample underwent cell counting using an Advia 120 Hematology System (Bayer Healthcare, Tarrytown, NY) from which total cell numbers were counted. The other half of the sample was used to make cytospins that were stained and from which differential cell types were identified.

Cytokine Analysis: Following the gathering of BALF, left lobes of the mouse lungs were dissected and ground to a fine powder using a liquid nitrogen-chilled mortar and pestle, and subsequently resuspended and vortexed in 400 µL of saline. The suspensions were transferred into separate QiaShredder spin columns (Qiagen) and centrifuged at 11,000 rpm at 10ºC for 10 minutes. Flow-throughs were then transferred to clean microcentrifuge tubes, while avoiding the pellet on the bottom, and were stored at -80ºC until analysis. Following thawing on ice, total protein concentrations were assessed using a Bio-Rad (Bradford) assay, with relative cytokine concentrations assessed using a custom R&D Systems mouse cytokine panel and a Luminex instrument, according to manufacturer’s instructions.

Statistics: Inter-group comparisons were made using paired t-tests or ANOVA, with post-hoc Bonferroni corrections for multiple comparisons as appropriate. Statistical significance was taken as p < 0.05.

RESULTS

Twitch Time Course
Figure 1 shows that the temporal patterns of the lung cytokines over the 31 day study period could be separated into two groups. GM-CSF, KC and IL-5 levels in the OVA-challenged animals peaked transiently at Day 3 where they were significantly different from control (Figs. 1A-C). Afterwards, GM-CSF and IL-5 levels fell below at least one of the groups challenged with saline (control), KC levels also dropped after Day 3 but remained higher than both control measurements, and by Day 31 IL-5 was at a statistically significant but physiologically meaningless difference from baseline. In contrast, IFNγ and IL-10 levels in the challenged animals peaked transiently at Day 21 but were statistically indistinguishable from control at Day 3 and Day 31 (Figs. 1D and E). IL-4, IL-13 and IL-17 did not rise significantly above control in response to OVA (Figs. 2A-C), while IL-6 levels fell to control levels at Day 14 before rising again at Day 21 and Day 31 (Fig. 2D).

BALF total leukocytes and eosinophils for the OVA-treated mice were significantly higher than control mice at all time points measured from Day 3 to Day 31, both peaking at Day 14 (Figs. 3A and B). In contrast, neutrophil levels at Day 3 were not significantly different between OVA-treated and control animals but rose thereafter in the treated group to become significantly different from at least one control out to Day 31 (Fig. 3C).

The lung mechanics parameters \( R_n \) and \( H \) in response to methacholine were significantly elevated in the OVA-treated mice compared to controls at all measured time points from days 3 to 31. \( R_n \) in the treated animals peaked at day 21 (Fig. 4A) while \( H \) peaked at day 14 (Fig. 4B).

### Refractory Period

The existence of a refractory period in allergic inflammation is indicated by the need to wait for a certain duration of time following cessation of OVA challenge before inflammation can be re-
instigated by a recall challenge. Figures 5A-C show that GM-CSF, KC and IL-5 all exhibited significant increases in their levels with wait periods of 10, 17 and 28 days after the end of the initial period of challenge, although the biological significance of the increase in GM-CSF at 28 days appears minimal. These findings suggest that the refractory period for these cytokines is no longer than 10 days. In contrast, IFNγ and IL-10 only increased significantly following a wait period of 28 days (Figs. 5D and E). Interestingly, for GM-CSF, IFNγ and IL-10 the gain in cytokine levels decreased as the wait period increased, whereas for IL-5 the gain increased with the wait period duration.

Variability also occurred with respect to the cellularity of the BALF. The number of total leukocytes and neutrophils were only increased by a recall challenge when it was given 28 days after the end of the initial challenge sequence, but not when the wait time was 10 or 17 days (Figs. 6A and C), indicating that their refractory periods are greater than 17 days. In contrast, eosinophils showed no significant increase in response to recall challenges at any of the three wait periods (Fig. 6B), indicating that the eosinophil refractory period is more than 28 days in length.

Variable refractory periods were also observed for the parameters of lung mechanics. Waiting 17 days before initiating recall challenge resulted in a statistically significant increase in $R_n$, although it was not until 28 days that this increase became biologically significant (Fig. 7A). In contrast, $H$ did not increase significantly with recall challenge after any of the wait times investigated (Fig. 7B). These findings thus suggest that the refractory period for lung hyperresponsiveness in terms of tissue elastance is longer than 28 days while in terms of airway resistance it is less than or equal to 17 days.
DISCUSSION

The findings of the present study show that each of the players involved in the allergic inflammatory response is part of a team that together produces a self-limited dynamic inflammatory response. The individual cell types (Fig. 3) and their cytokine products (Figs. 1 and 2) exhibit varying dynamics over the course of a month from the beginning of continual antigen challenge, but collectively they are responsible for the dynamics of the physiologic phenotype evident in Fig. 4. Our results go further than simply dissecting the components of the transient response to antigen, however, by demonstrating clear evidence of a refractory period in the dynamic phenotype (Fig. 7) as well as in some of its components (Figs. 5 and 6). A refractory period is key to any form of self-limited response because re-initiation of the response must be precluded for at least some period of time to allow it to be forced to resolve back to baseline. Evidence for the existence of such a refractory period in the allergic inflammatory response is seen in previous studies that showed both that continual stimulation with antigen for 30 days eventually leads to non-responsiveness (2) and that re-stimulation with antigen after a rest period elicits another vigorous response (18). In fact, the dissipation of an allergic response in the face of continued antigen challenge is well-known to the immunology community as the phenomenon of local inhalational tolerance (8). The inflammatory twitch hypothesis provides a teleological basis for the phenomenon of tolerance by showing how it potentially solves the thorny control problem presented by the need to automatically turn off a response that would be harmful if it persisted beyond when it is needed, but for which there is no obvious “off switch”.

We note that while there are clear signs of twitch-like behavior in our data, this does not apply to every relevant player in the allergic inflammatory response. An exception is the response pattern exhibited by neutrophils, which showed no signs of resolving to baseline (Fig. 3C). Also, total
cell numbers (Fig. 3A), $Rn$ (Fig. 4A), and KC (Fig. 1B) all peaked in their responses but remained at elevated plateaus out to Day 31, and some of the cytokines did not differ in their time courses relative to control (Fig. 2). There are several possible ways to interpret these findings. One is that those quantities that did not return baseline eventually would have if we had continued the experiment for long enough, which would mean that the inflammatory twitch lasts longer than the one month period over which we investigated it. Another possibility is that there are aspects of the response to continual antigen stimulation that are maintained indefinitely, and which would thus not conform to the twitch hypothesis. Indeed, such persistence is what one would expect of anything related to immune memory, so while those aspects of the allergic inflammatory response that need to be transient might be controlled in a twitch-like fashion, there are other aspects that need to remain active for the health of the organism. Also, it is possible that some components of the inflammatory response are capable of summation in the same way that force can summate in skeletal muscle. Thus, the refractory period in the allergic inflammatory twitch might be defined by events that resolve more quickly than the physiological phenotype shown in Fig. 4, such as the group of early cytokines that appear to have a refractory period of 10 days or less (Fig. 1A-C), which would potentially allow the methacholine responsiveness phenotype to summate.

Putting these results together generates an overall picture of the allergic inflammatory response. While the complete response may take more than 31 days to manifest fully, within the 31 day window that we examined in the present study there are a number of distinct events that wax and wane over different time scales. First, there is an initial transient phase of early cytokines that peaks at Day 3 followed by responses in eosinophils and lung tissue stiffness that peaks at Day 14. Then there is a leukocyte peak between Day 14 and Day 21 that is followed by both the later
cytokines and airway resistance, both peaking at Day 21. Drawing on our analogy with the skeletal muscle twitch, these various events are similar to the components of the muscle twitch that include the early action potential followed soon after by calcium influx and then later by force development. In other words, the muscle twitch and inflammatory twitch are both composed of a sequence of events in which the early events trigger the later events until there is eventual resolution back to baseline. It should be noted, however, that this may paint a somewhat oversimplified picture of the inflammatory twitch because its various cell and cytokine components do not function in isolation but rather operate as part of networks. The time-course of a particular component may thus reflect its changing environment as much as, or even more than, its own intrinsic dynamics.

An interesting question raised by our results is why some cytokines peak at Day 3 (GM-CSF, KC and IL-5 in Figs. 1A-C) while others peak at Day 21 (IL-10 and IFNγ in Figs. 1D and E). IFNγ and IL-10 are known to be associated with Th1 and T-regulatory cells, respectively (6, 9, 12), while IL-5 is associated with Th2 cells (3). GM-CSF and KC are also known to be associated with the activation of dendritic cells and the recruitment of neutrophils (4, 10), cells also involved in the early phase of the allergic response. One possibility, therefore, is that the cytokine picture in Fig. 1 indicates a switch from an early Th2-mediated inflammatory phase to a later Th1-mediated resolution phase that involves T-regulatory cells. This is consistent with other studies showing that in mice allergic to *Aspergillus fumigatus*, interventions that target the Th2-mediated phase of the response result in decreased levels of not just IL-5, but also IFNγ, IL-4, IL-13 and lymphocytes (specifically CD4+ T-cells) (14). Interestingly, this also reduced airways hyperresponsiveness, although others have shown in mice allergic to OVA that Th1 cytokines do not induce airway hyperresponsiveness (11).
The inflammatory twitch hypothesis raises the possibility that pathologic alterations in some of the molecular and/or cellular events involved in the allergic inflammatory twitch could transform it into a non-resolving event. Such a transformation might correspond to the chronic inflammation characteristic of allergic asthma. We investigated this possibility theoretically in a previous study by manipulating our computational model in ways corresponding to plausible biological abnormalities (15). Many of these manipulations, such as altering the amounts of chemical signals released by cells, knocking out certain cell types, and changing the speed of movement of cells within the tissue environment, did relatively little to change inflammatory twitch dynamics. However, increasing the life span (i.e. delaying apoptosis) of pro-inflammatory cells in the model had a powerful effect on extending the magnitude and duration of simulated inflammation (15). Interestingly, inflammatory cells have been documented to have longer lifespans in individuals with airway disease (7, 13, 17, 19). The results of the present study suggest that this might apply particularly to eosinophils (Fig. 3).

Finally, we must view the findings of our study within the context of its limitations. Perhaps most significant is the limitation that placed by our study design on our ability to resolve the various features expected of a twitch-like response. We chose a design aimed at obtaining as much information as possible about the inflammatory twitch from a limited number of animals, but this gave far from complete coverage of all the possibilities. For example, we are not able to distinguish between the inflammatory effects due to length of antigen challenge from those of a rest period prior to recall challenge because challenge duration and rest duration varied oppositely in our study design (Table 1). We also employed only a single day of recall challenge, allowing us to study only the most rapid response features inflammatory twitch that may differ from those of a more extended recall challenge. Fully characterizing the inflammatory twitch in
all its aspects would require that our experiments be repeated over a significantly longer time scale than one month, and that many more configurations of challenge and rest be examined. This would have required a large number of additional mice, and we studied 104 mice over a total time of a month and a half as it was. Questions about the dynamics of the allergic inflammatory twitch thus remain. Nevertheless, we were able in the present study to add significantly to our understanding of these dynamics, and to place this understanding within the framework of the inflammatory twitch hypothesis.

In conclusion, we have provided experimental evidence consistent with our allergic inflammatory twitch hypothesis. Specifically, we have demonstrated transient behavior in many of the cells and cytokines involved in the response as well as its phenotypic manifestation in terms of lung function. These findings support the notion that the allergic inflammatory twitch in normal mice lasts in the order of one month. We have also demonstrated clear evidence of refractory periods in many of these components of the response, some as short as only a few days. These findings corroborate the notion that control of the host response employs frequency modulation in the same way that control is exerted over muscle force, neural signaling, and indeed a host of other biological processes that are initiated by single instigating events. The inflammatory twitch hypothesis thus provides a general mechanism to explain how inflammation is controlled in a normal healthy individual, and also suggests how it might become aberrant in individuals who develop chronic inflammatory diseases such as allergic asthma.

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REFERENCES


### Table 1: Experimental design.

All groups of mice were given IP injections of OVA and alum on day -14 and Day -7 relative to the beginning of the challenge period. All groups were given a single recall challenge on Day 31. Open circles represent challenge with saline. Closed circles represent challenge with 1% OVA.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Days of challenge starting on Day 1</th>
<th>Recall challenge on Day 31</th>
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<tbody>
<tr>
<td>Saline-3</td>
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<tr>
<td>OVA-3</td>
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| OVA-14             | ●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●
Figure Captions

Figure 1: Closed circles show concentrations of cytokines measured in the left lobes of lungs of mice, characterized 24 hours following the last day of daily antigen challenge, and on day 31 immediately preceding and following recall challenge with OVA: A) GM-CSF, B) KC, C) IL-5, D) IFN-γ, and E) IL-10. Open circles show corresponding control measurements made in control mouse lungs 24 hours after 3 days of saline and a recall challenge with saline on day 31. All points are reported as means ± standard errors. * indicates a value statistically higher than Control 1, ** indicates a value statistically higher than Control 2, and *** indicates a value statistically higher than the other three values in OVA-challenged animals.

Figure 2: Closed circles show concentrations of cytokines measured in the left lobes of mouse lungs 24 hours following the final daily OVA challenge, and on day 31 immediately following recall challenge: A) IL-4, B) IL-17, C) IL-3, and D) IL-6. Open circles show corresponding control measurements made 24 hours after Day 3 of saline challenge (Control 1: left-hand point) and one day after saline recall challenge (Control 2: right-hand point). Points represent mean ± standard error. * indicates statistically higher than Control 1. ** indicates statistically higher than Control 2.

Figure 3: Closed circles show various cell types measured in BALF from mouse lungs 24 hours after 3, 14, 21 and 31 days of daily OVA challenge: A) total leukocytes, B)
eosinophils, and C) neutrophils. Open circles show corresponding control
measurements made 24 hours after 3 days (Control 1: left-hand point) and 31 days
(Control 2: right-hand point) of saline challenge. Points represent mean ± standard
error. * indicates statistically higher than Control 1. ** indicates statistically
higher than Control 2.

Figure 4: Closed circles show lung mechanics parameters measured in mice measured 24
hours after 3, 14, 21 and 31 days of daily OVA challenge: A) $R_n$, and B) $H$. Open
circles show corresponding control measurements made 24 hours after 3 days
(Control 1: left-hand point) and 31 days (Control 2: right-hand point) of saline
challenge. Points represent mean ± standard error. * indicates statistically greater
than Control 1. ** indicates statistically greater than Control 2. *** indicates
statistically distinct from the other three measurements in the OVA-challenged
animals.

Figure 5: Concentrations of cytokines in the left lobes of lungs of mice measured 24 hours
following 3, 14 and 21 days of OVA challenge, and both immediately preceding
and following recall challenge: A) GM-CSF, B) KC, C) IL-5, D) IFN-γ, and E)
IL-10. Control measurements were made 24 hours after 3 days of saline
challenge and after a recall challenge with saline on day 31. Points represent mean
± standard error. * indicates significant increase following the recall challenge. **
and *** indicate significant increases following removal of an outlier from the
post-recall group and pre-recall groups, respectively.
Figure 6: Numbers of cells measured in BALF from mouse lungs 24 hours following 3, 14, and 21 days of OVA challenge, and both immediately preceding and following recall challenge: A) total leukocytes, B) eosinophils, and C) neutrophils. Control measurements were made 24 hours after 3 days of saline challenge and after a recall challenge with saline on day 31. Points represent mean ± standard error. * indicates significant increase following the recall challenge.

Figure 7: Measurements of lung mechanics parameters in mice 24 hours following 3, 14, and 21 days of OVA challenge, and both immediately preceding and following recall challenge: A) $R_n$, and B) $H$. Control measurements were made 24 hours after 3 days of saline challenge and after a recall challenge with saline on day 31. Points represent mean ± standard error. * indicates significant increase following the recall challenge.