



Mabtech IRIS™
SPOT ANALYSIS REINVENTED

Read how >

MABTECH
Capture | Detect | Discover



Aeroallergens Exacerbate *Histoplasma capsulatum* Infection

Chelsea L. Bueter and George S. Deepe, Jr.

This information is current as of November 26, 2018.

J Immunol 2018; 201:3352-3361; Prepublished online 22 October 2018;
doi: 10.4049/jimmunol.1800644
<http://www.jimmunol.org/content/201/11/3352>

Supplementary Material <http://www.jimmunol.org/content/suppl/2018/10/19/jimmunol.1800644.DCSupplemental>

References This article **cites 34 articles**, 12 of which you can access for free at:
<http://www.jimmunol.org/content/201/11/3352.full#ref-list-1>

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>



Aeroallergens Exacerbate *Histoplasma capsulatum* Infection

Chelsea L. Bueter* and George S. Deepe, Jr.*[†]

Allergens such as house dust mites (HDM) and papain induce strong Th2 responses, including elevated IL-4, IL-5, and IL-13 and marked eosinophilia in the airways. *Histoplasma capsulatum* is a dimorphic fungal pathogen that induces a strong Th1 response marked by IFN- γ and TNF- α production, leading to rapid clearance in nonimmunocompromised hosts. Th1 responses are generally dominant and overwhelm the Th2 response when stimuli for both are present, although there are instances when Th2 stimuli downregulate a Th1 response. We determined if the Th2 response to allergens prevents the host from mounting a Th1 response to *H. capsulatum* in vivo. C57BL/6 mice exposed to HDM or papain and infected with *H. capsulatum* exhibited a dominant Th2 response early, characterized by enhanced eosinophilia and elevated Th2 cytokines in lungs. These mice manifested exacerbated fungal burdens, suggesting that animals skewed toward a Th2 response by an allergen are less able to clear the *H. capsulatum* infection despite an intact Th1 response. In contrast, secondary infection is not exacerbated by allergen exposure, indicating that the memory response may suppress the Th2 response to HDM and quickly clear the infection. In conclusion, an in vivo skewing toward Th2 by allergens exacerbates fungal infection, even though there is a concurrent and unimpaired Th1 response to *H. capsulatum*. *The Journal of Immunology*, 2018, 201: 3352–3361.

Asthma is a common chronic disease of the airways characterized by episodes of reversible airflow obstruction. In the United States there has been a steady increase in the prevalence of asthma, especially among children and adult women (1). Sensitivities to allergic stimuli, such as house dust mites (HDM), air pollutants, or fungal spores, induce Th2 responses marked by production of IL-4 and IL-13, influx of eosinophils into the lungs, and AHR. Mouse models of allergic asthma often use HDM. The active component of HDM is Der p 1 (2), which has been shown to disrupt the tight junctions between epithelial cells, contributing to allergic disorders (3). Papain is structurally similar to Der p 1 and can be used to induce a strong allergic response in model systems (4).

Inhalation of environmental fungal spores is known to produce an allergic response. In fact, admission to intensive care for asthma is strongly associated with skin test reactivity to one or more fungi (5, 6). *Alternaria* species are the most common fungi to induce an allergic response, along with *Cladosporium* and *Coprinus* (7–9). Among severe asthmatics, sensitivity to fungi ranges from 25 to >70% (10). The soil-based fungus *Histoplasma capsulatum*, however, does not induce an allergic phenotype. It is a dimorphic fungus found worldwide, and in the United States it is most prominent in the Ohio and Mississippi River Valleys. Exposure to *H. capsulatum* spores is common, and most individuals are able to clear an infection readily; yet there are ~25,000 life-threatening infections in the United States

every year, chiefly in immunocompromised patients, such as those with AIDS (11). Infection with *H. capsulatum* induces a strong Th1 response highlighted by production of TNF- α and IFN- γ , and this is critical to fungal clearance. Conversely, in models with a Th2-skewed response there is a significantly worse infection (12, 13).

Although Th1 and Th2 responses are not mutually exclusive, one generally governs. The initial cytokine environment is significant in establishing the development of Th1 or Th2 responses, and in most cases of competing Th1 and Th2 stimuli, the former are dominant. The Th1 cytokine IL-12 induces IFN- γ and thereby prevents development of a Th2 response (14). Additionally, a strong skewing of the cytokine environment toward Th2 in filarial patients in vivo does not influence the Th1-type immune response to purified protein derivative of *Mycobacterium tuberculosis* or the ability of macrophages to limit *M. tuberculosis* growth (15, 16). In vitro, when Th1 and Th2 stimuli are added to dendritic cell (DC) cultures simultaneously, only IFN- γ -producing cells are generated (14). In contrast, there is also evidence that Th2 conditioning by helminth extracts downregulates Th1 responses in mice and suppresses DC function (14, 17). Differentiated Th1 cells fail to express IL-4 even when they are restimulated under Th2 conditions, although partially differentiated cells retain their capacity to produce IL-4 (18). Conversely, even stably committed Th2 cells are plastic and can switch to produce IFN- γ upon stimulation with a Th1 inducer (19).

In this paper we evaluated a system stimulated with the Th2-promoting HDM and Th1-promoting *H. capsulatum*. Mice treated with HDM and infected with *H. capsulatum* mount concurrent Th1 and Th2 responses, with the Th2 response to the HDM remaining strong initially in the presence of a mounting Th1 response to *H. capsulatum* before the lung environment switches fully to Th1. However, animals with a memory response to *H. capsulatum* are unable to mount a Th2 response to HDM during a secondary infection, suggesting the order of exposure and the generation of memory responses are very important to determining the outcome.

Materials and Methods

Mice

Male wild-type (WT) C57BL/6 and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). IL-33^{-/-} mice were house

*Division of Infectious Diseases, University of Cincinnati College of Medicine, Cincinnati, OH 45267; and [†]Veterans Affairs Hospital, Cincinnati, OH 45220

Received for publication May 4, 2018. Accepted for publication September 20, 2018.

This work was supported by Grants AI-126818, AI-106269, and T32 HL 007752-23 from the National Institutes of Health and Grant I01 BX000717 from the Department of Veterans Affairs.

Address correspondence and reprint requests to Dr. George S. Deepe, Jr., Division of Infectious Diseases, University of Cincinnati College of Medicine, Room 7168 Medical Sciences Building, ML0560, 231 Albert Sabin Way, Cincinnati, OH 45267-0560. E-mail address: george.deepe@uc.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; HDM, house dust mite; PAS, periodic acid–Schiff; p.i., postinfection; WT, wild-type.

Copyright © 2018 by The American Association of Immunologists, Inc. 0022-1767/18/\$37.50

bred. Animals were housed in isolator cages and were maintained by the Department of Laboratory Animal Medicine, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experiments were performed in accordance with the Animal Welfare Act guidelines of the National Institutes of Health, and all protocols were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Allergen administration, preparation of H. capsulatum, and infection

HDM (Greer Laboratories, Lenoir, NC), papain (*Carica papaya*; MilliporeSigma, Billerica, MA), or HDM combined with papain was administered intranasally to mice anesthetized with isoflurane. On day 0 they were sensitized with 100 μ g of allergen, then they were challenged on days 7–11 with the same dose (Supplemental Fig. 1). Mice were rested 2 d before infection with *H. capsulatum*.

Preparation of H. capsulatum and infection of mice

H. capsulatum yeast strain G217B was grown for 72 h at 37°C as previously described (12). Naive or allergen-sensitized mice were infected intranasally with 2×10^6 yeast cells in ~ 20 μ l of HBSS (HyClone, Logan, UT). Mice receiving allergen received 100 μ g/d throughout the course of infection.

Organ culture

Lungs were homogenized in 5 ml of sterile HBSS and serially diluted before being plated upon Mycosel agar (Becton Dickinson, Franklin Lakes, NJ) plates containing 5% sheep blood agar and 5% glucose. Plates were incubated for 8 d at 30°C.

Survival

Mice were sensitized and challenged with allergen and infected as described above. When mice appeared moribund they were euthanized.

RNA isolation, cDNA synthesis, and quantitative real-time RT-PCR

Total RNA from whole lungs of mice was isolated using TRIzol (Invitrogen, Carlsbad, CA). Oligo(dT)-primed cDNA was prepared using the reverse transcription system (Promega, Madison, WI) according to the manufacturer's instructions. Quantitative real-time PCR for analysis of gene transcription was performed using TaqMan Master Mix and primers obtained from Applied Biosystems (Foster City, CA). Samples were analyzed with ABI Prism 7500 (Applied Biosystems). In each experiment, the hypoxanthine phosphoribosyl transferase housekeeping gene was used as an internal control, and samples are compared with uninfected WT controls. The conditions used for amplification were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Flow cytometry

Lungs were homogenized with 10 mg of collagenase and 200 U of DNase I for 30 min at 37°C in a 5% CO₂ incubator before being filtered and then fixed in 4% paraformaldehyde. The phenotype of cells was determined by incubating lung homogenates with the indicated Abs and CD16/32 to limit nonspecific binding. Cells were stained at 4°C for 30 min in PBS containing 1% BSA and 0.01% sodium azide. For intracellular staining, mice were given 250 μ g of brefeldin A i.p. After 6 h, lungs were collected and processed as described above (20). Cells were stained with combinations of the following Abs: PE-Cy7-conjugated CD11c, Alexa Fluor 700-conjugated Ly6G and Ly6C, PE-conjugated CD11b, allophycocyanin-conjugated CD4, allophycocyanin-Cy7-conjugated Ly6C and CD4, Horizon PE-CF594-conjugated SiglecF, Horizon V500-conjugated MHC class II and CD8, allophycocyanin-conjugated CD3, PE-conjugated IFN- γ , and PE-conjugated IL-4 from BD Biosciences (San Diego, CA); eFluor 450-conjugated CD11b and CD45 and FITC-conjugated CD45 from eBioscience (Thermo Fisher Scientific, San Diego, CA); Pacific Blue-conjugated CD11c and PE-conjugated CD64 from BioLegend (San Diego, CA); and PE-conjugated ST2 from R&D Systems (Minneapolis, MN). Cells were washed and resuspended in PBS containing 1% BSA and 0.01% sodium azide. Appropriate isotype controls were performed in parallel. Data were acquired using a BD LSR II flow cytometer (BD Biosciences) in the FACS core at Cincinnati Children's Hospital and were analyzed using FCS Express 6.02 (DeNovo Software, Glendale, CA). See Supplemental Fig. 2 for gating strategy and naive controls. Eosinophils were defined as CD45⁺CD11b⁺CD11c[−]SiglecF⁺. Alveolar macrophages were defined as CD45⁺CD11b⁺CD11c[−]SiglecF⁺CD64⁺.

Neutrophils were defined as CD45⁺CD11b⁺Ly6G⁺. Inflammatory monocytes were defined as CD45⁺CD11c[−]CD11b⁺Ly6C^{hi}. Monocyte-derived DCs were defined as CD45⁺CD11c⁺CD11b⁺CD64⁺. T cells were defined as CD45⁺CD3⁺ and CD4⁺ or CD8⁺.

Measurement of cytokines and chemokines by ELISA

Eotaxin-1, IL-4, IL-12, and IFN- γ protein concentration was quantified in lung homogenates using ELISA kits from R&D Systems.

Histologic analysis

Lungs were inflated with 3 ml of formalin before paraffin-embedded sections were stained by periodic acid–Schiff (PAS) and H&E at the Histology core at Cincinnati Children's Hospital. Images were acquired on a Zeiss Imager A.2 with an AxioCam MRc 5. Images were analyzed using AxioVs40 $\times$ 60 version 4.9.1.0.

Statistics

Statistical analyses were performed using the Student *t* test or ANOVA with Bonferroni correction. A *p* value <0.05 was considered statistically significant. For all graphs, **p* < 0.05, ***p* < 0.01, and ****p* < 0.005.

Results

HDM exacerbate H. capsulatum infection

C57BL/6 mice were sensitized to HDM before being infected with *H. capsulatum* to skew the animals toward a Th2 response, and throughout the course of infection, mice were given HDM daily to maintain a Th2 response. Mice exposed to HDM had significantly elevated fungal burdens at late intervals during infection compared with infected controls (Fig. 1A). This finding was also true for the more Th2-skewed BALB/c mouse background (Supplemental Fig. 3A). Although the fungal burden plateaued around day 7, animals treated with HDM began to die by day 13, and by day 16, 50% had died (Fig. 1B).

Eosinophil influx to the lungs is induced early in response to HDM, and at day 3 there was no difference in eosinophil percentage between animals given HDM and those animals sensitized to HDM and infected with *H. capsulatum* (Fig. 1C). Throughout the course of infection, the animals treated with HDM and infected exhibited significant decreases in eosinophil numbers compared with the HDM-alone group. Alveolar macrophages were increased in response to HDM; however, in infected animals this population disappears by day 10 postinfection (p.i.) (Fig. 1C). Neutrophils, inflammatory monocytes, and monocyte-derived DCs did not vary greatly between conditions, although there were significantly more inflammatory monocytes at day 10 in the HDM-treated and infected group compared with the other two groups (Fig. 1E–G). Additionally, both IFN- γ ⁺ and IL-4⁺ CD4⁺ T cells were increased in the animals treated with HDM; however, upon infection, HDM-treated and infected animals showed no differences compared with infected controls (Fig. 1H).

HDM-induced Th2 response persists throughout infection

The influx of eosinophils associated with HDM seen in Fig. 1 corresponded to elevated levels of the eosinophil chemoattractants eotaxin 1 and eotaxin 2 throughout the course of the experiment in uninfected mice (Fig. 2A, 2B). In infected animals treated with HDM, the eotaxins were significantly elevated early before declining thereafter (Fig. 2A, 2B). The canonical Th2 cytokines IL-4 and IL-13 followed a similar profile, suggesting the *H. capsulatum* infection may suppress the Th2 response late in infection (Fig. 2C, 2D). Protein analysis of lung homogenate supernatants at day 7 p.i. for eotaxin 1 and IL-4 exhibits a similar pattern to the RNA expression data (Fig. 2E, 2F).

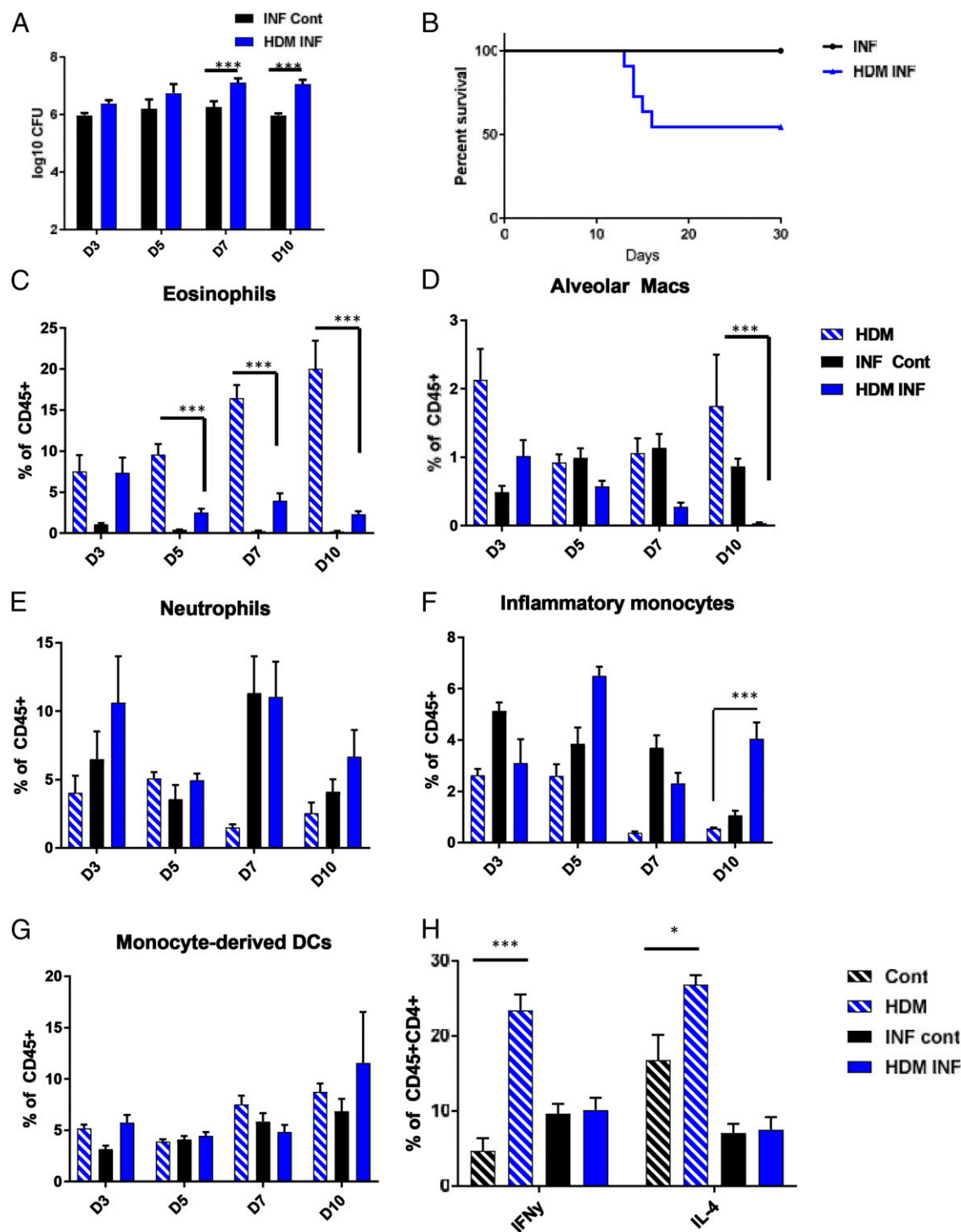


FIGURE 1. Pretreatment with HDM exacerbates fungal infection. **(A)** Pulmonary fungal burden represented as log₁₀ CFU in control or HDM-treated mice at days 3, 5, 7, and 10 p.i. **(B)** Survival curve for control and HDM-treated mice infected with 2×10^6 CFU. **(C–G)** Cell populations presented as percentage of CD45⁺ leukocytes in lung homogenates from HDM-treated, infected, and HDM-treated and infected mice at days 3, 5, 7, and 10 p.i. **(H)** Percentage of CD45⁺CD4⁺ T cells that are IFN-γ⁺ or IL-4⁺ in uninfected mice and at day 7 p.i. Data are represented as mean ± SEM, two to three independent experiments, $n = 8$ –12 mice. * $p < 0.05$, *** $p < 0.005$.

Th1 response to *H. capsulatum* is intact

The strong Th2 response to HDM was established before the animals were infected and could therefore suppress the Th1 response to the infection. However, both IL-12 and IFN-γ expression (Fig. 3A, 3B) and protein concentration (Fig. 3C, 3D) were similar between the infected controls and the infected and HDM-treated groups, suggesting an intact Th1 response. Similar trends were

observed in the BALB/c background for Th1 and Th2 cytokines (Supplemental Fig. 3B–D).

IL-4, but not IL-33, is responsible for HDM phenotype

IL-4 was elevated in response to HDM (Fig. 2C), therefore we ascertained if neutralization of this cytokine would prevent the HDM-associated increase in fungal burden (Supplemental

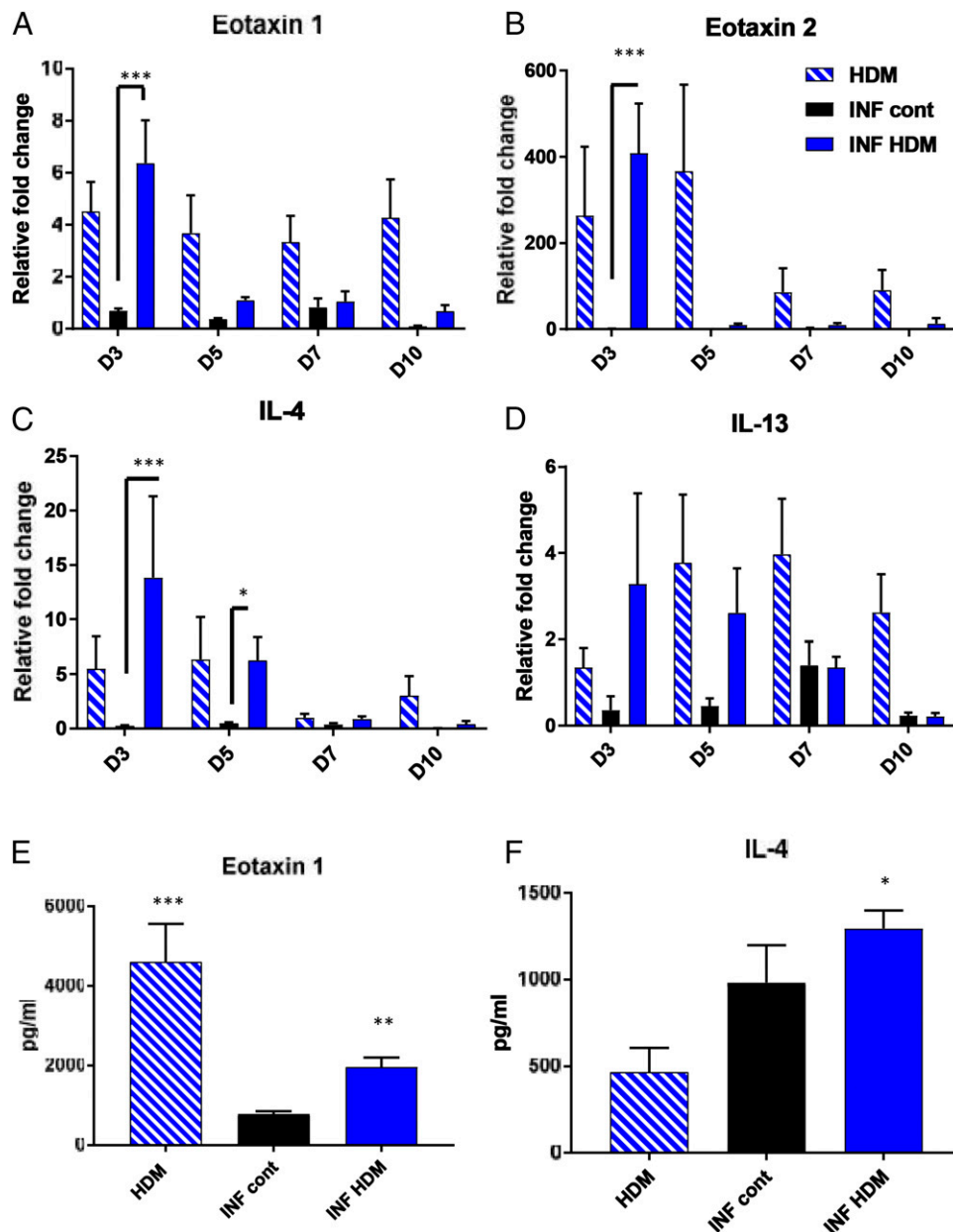


FIGURE 2. HDM induces a Th2 response early, but this response is eventually suppressed by the infection. (A–D) Quantitative real-time RT-PCR analysis of Th2 chemokine and cytokine genes in response to HDM, infection, or HDM and infection at days 3, 5, 7, and 10 p.i. (E and F) ELISA quantification of eotaxin 1 and IL-4 protein levels at day 7 p.i. Data are represented as mean $\pm$ SEM, $n = 8$ –12 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Fig. 1B for method schematic). Animals treated with α IL-4 Ab have significantly reduced fungal burden when exposed to HDM than that seen in the IgG control animals (Fig. 4A). Additionally, α IL-4- and HDM-treated animals did not exhibit as much eosinophil influx as controls (Fig. 4B). α IL-4 reduced eotaxin 2 while having no effect on IFN- γ (Fig. 4C, 4D). We next asked if IL-33 influenced the exaggerated fungal burden. IL-33 $^{-/-}$ animals sensitized with HDM exhibited elevated fungal burdens similar to those of WT mice treated with HDM and IL-4, and IFN- γ levels were also similar (Supplemental Fig. 4A, 4B). Thus, the elevated fungal burdens associated with HDM were not caused by IL-33.

Papain treatment also exacerbates infection

Papain is a protease allergen with similar activity to the active component of HDM, Der P 1 (4). When administered using the

protocol established for HDM, papain exacerbated fungal infection more severely than HDM (Fig. 5A). Administration of both to mice produced a similar fungal burden to that of papain alone. Papain also induced slightly more death than HDM (Fig. 5B). Similar to HDM, animals sensitized with papain or papain plus HDM have an intact Th1 response and produce IL-12b and IFN- γ in response to infection but also displayed elevated levels of Th2 cytokines *Arg1*, IL-4, eotaxin 1, and eotaxin 2 (Fig. 5C–H). Similar results were seen in BALB/c mice, a more Th2-prone mouse strain (Supplemental Fig. 3).

Mucus accumulates in the airways of the lungs when responding to an allergen challenge (21). *Gob-5* production is elevated in response to allergen challenge, but upon infection disappears (Fig. 5I). PAS-stained lung histology sections of day 7 show significant mucus staining surrounding the airways of

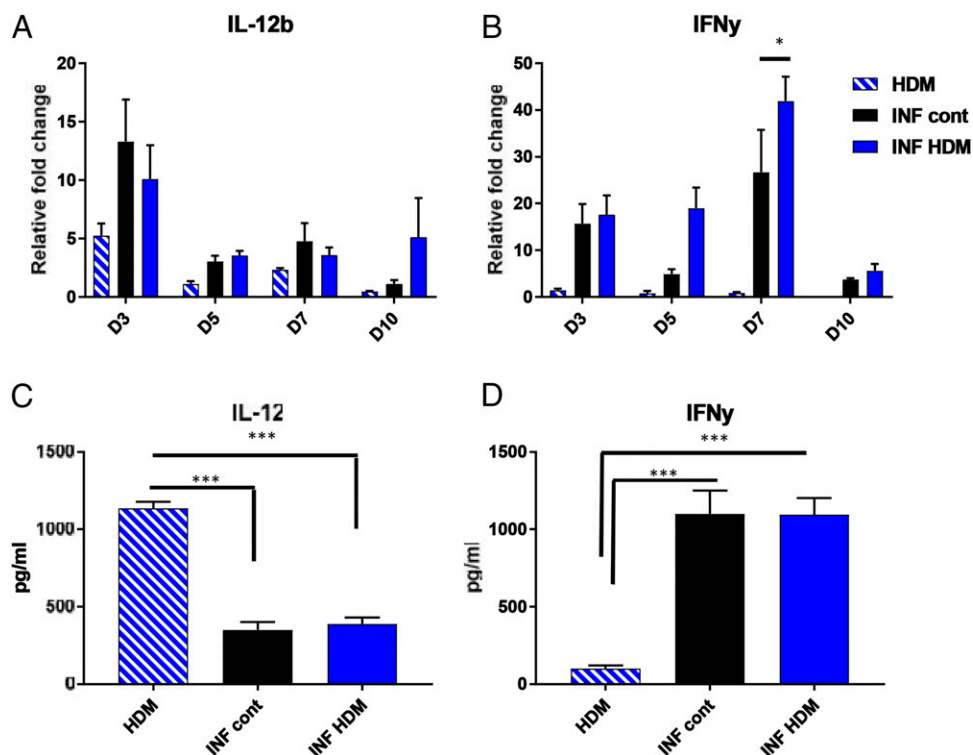


FIGURE 3. HDM-induced Th2 response does not suppress *H. capsulatum* Th1 response. (A and B) Quantitative real-time RT-PCR analysis of Th1 cytokine genes in response to HDM, infection, or HDM and infection at days 3, 5, 7, and 10 p.i. (C and D) ELISA of IL-12 and IFN- γ at day 7 p.i. Data are represented as mean $\pm$ SEM, two to three independent experiments, $n = 8$ –12 mice. * $p < 0.05$, *** $p < 0.005$.

the allergen-treated animals (arrows, Fig. 6A). In contrast, massive cell infiltrates are present in the infected and allergen-sensitized animals (Fig. 6A, 6B). Papain induced severe

inflammation that is further exacerbated by infection and is a possible explanation for the worse infection in the papain-treated animals compared with the HDM-treated animals.

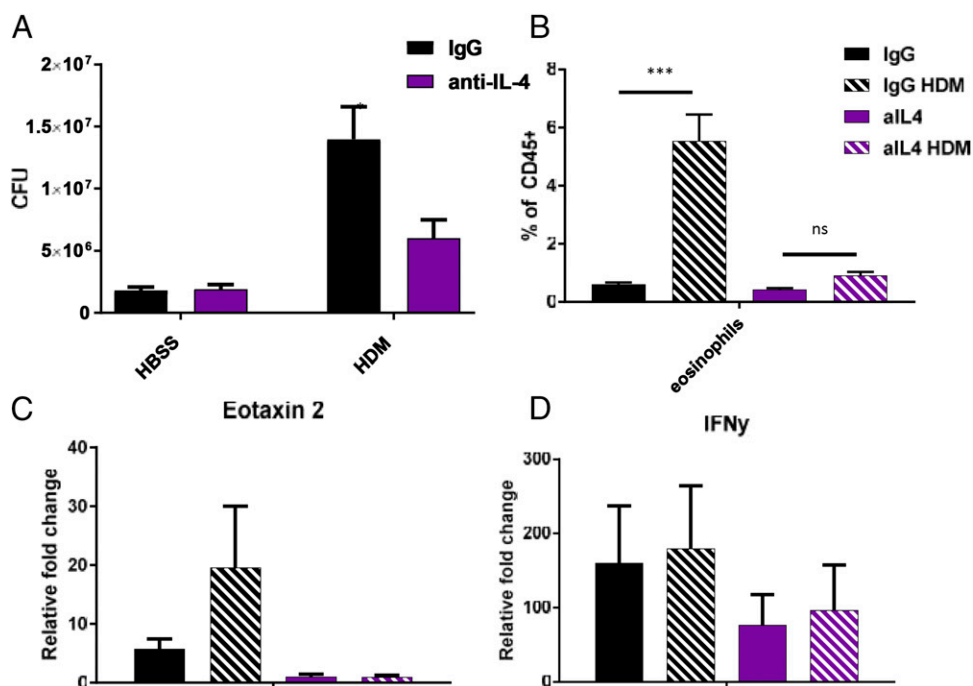


FIGURE 4. IL-4 is responsible for part but not all of the HDM phenotype. (A) Day 7 p.i. pulmonary fungal burdens represented as CFUs in control and HDM-treated IgG- and α IL-4-treated mice. (B) Eosinophil populations presented as percentage of CD45⁺ leukocytes in lung homogenates from HDM-treated, control, and HDM-treated and infected mice for day 7 p.i. Quantitative real-time RT-PCR analysis of whole-lung homogenates from IgG- and α IL-4-treated mice for eotaxin 2 (C) and IFN- γ (D), following infection and HDM treatment. Data are represented as mean $\pm$ SEM, three independent experiments, $n = 12$ mice. *** $p < 0.005$.

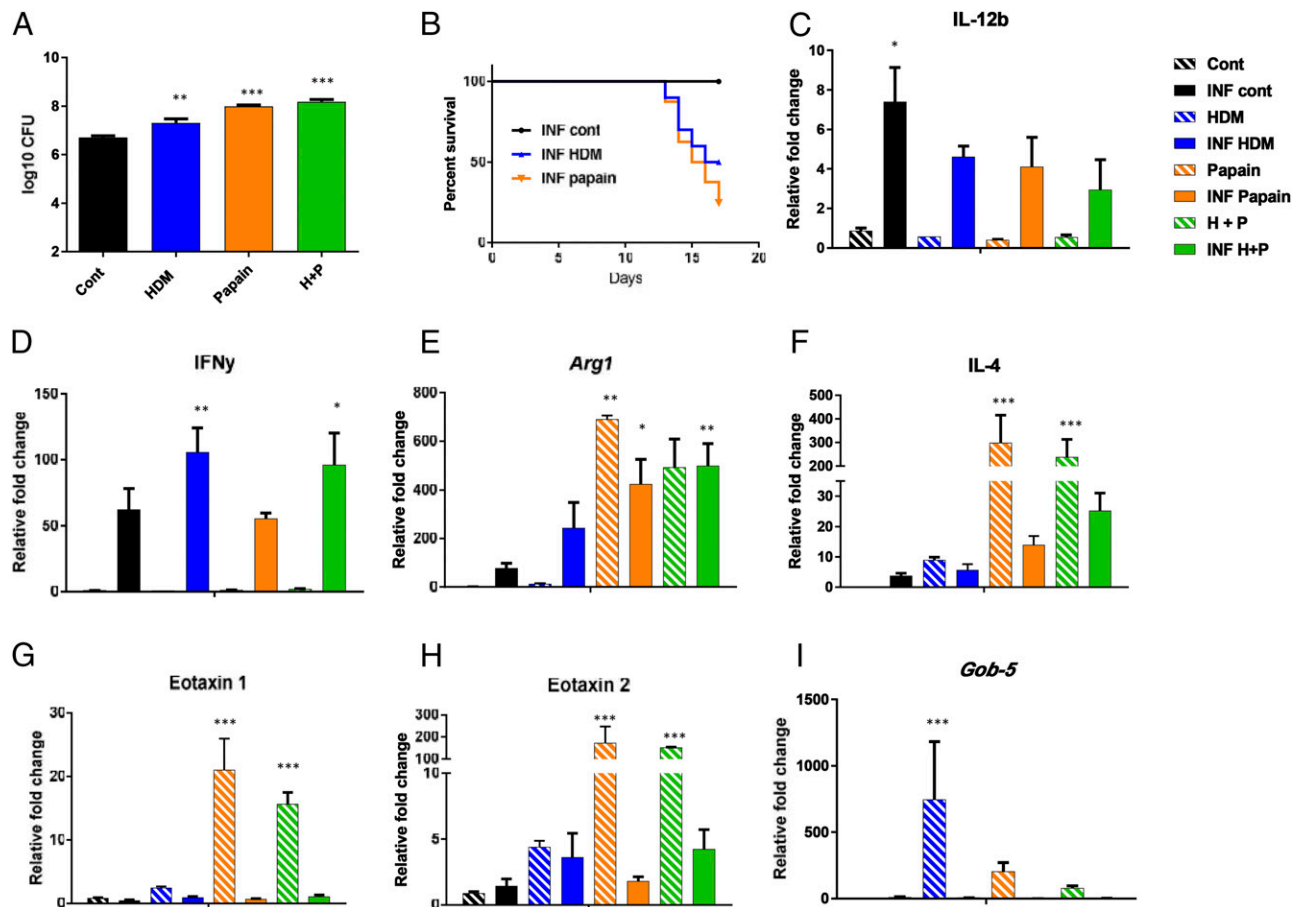


FIGURE 5. Papain similarly exacerbates infection. (A) Pulmonary fungal burden represented as log₁₀ CFU in control, HDM-, papain-, or HDM- and papain-treated mice at day 7 p.i. (B) Survival curve for control infected, HDM-treated and infected, and papain-treated and infected mice infected with 2×10^6 CFU. (C and D) Quantitative real-time RT-PCR analysis of whole-lung homogenates for Th1 cytokine genes at day 7 p.i. (E–I) Quantitative real-time RT-PCR analysis of whole-lung homogenates for Th2 cytokine and chemokine genes, significance indicated when compared with control. Data are represented as mean $\pm$ SEM, two to three independent experiments, $n = 8$ –12 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Primary infection does not suppress Th2 response to HDM

Mice were given one sensitization dose of allergen before infection and then challenged with allergen during infection. These mice still exhibited increased fungal burdens compared with control animals, despite not being fully skewed toward a Th2 response before being infected (Fig. 7A). In fact, one sensitization dose of papain given a week before infection, with no additional allergen challenges, was enough to significantly increase fungal burden. IFN- γ was unchanged between the conditions (Fig. 8B). The mice that were challenged with allergen p.i. still had elevated levels of IL-4, eotaxins 1 and 2, and *Gob-5* (Fig. 7C–F).

Memory response to *H. capsulatum* suppresses Th2 response to HDM

During acute infections, HDM causes elevated fungal burdens. To determine what transpires in a secondary infection, mice were infected with *H. capsulatum* before being allowed to recover and then treated with the same protocol as before to ascertain the effect of a memory response to *H. capsulatum* (Supplemental Fig. 1C). This memory response resulted in similar fungal burdens between control animals and those treated with HDM (Fig. 8A). IFN- γ was also similar between the infected groups (Fig. 8B). Unlike the elevation of Th2 cytokines seen in response to HDM in a primary infection,

IL-4, eotaxin 1, and eotaxin 2 were absent during secondary infection in animals treated with HDM. (Fig. 8C–E). However, uninfected age-matched animals did exhibit an elevated Th2 response to HDM. IFN- γ protein levels are slightly elevated in infected mice treated with HDM; however, all Th2 cytokines are similar between the groups (Fig. 8F).

Discussion

In this study, we sought to understand the interplay between Th2 stimuli and Th1 stimuli in vivo. HDM establishes a strong Th2 phenotype, with influx of eosinophils to the lungs, elevated levels of Th2 cytokines and chemokines, and an increase in mucus production. The Th2 response induced by HDM was able to persist through the initial *H. capsulatum* infection before fading after about a week, with eosinophil and alveolar macrophage influx receding. However, by the time the Th1 response appeared to be suppressing the Th2 response, animals had already incurred so much lung inflammation and fungal burden that half of them had died. Although in vitro results suggest a quick switch from Th2 to Th1, in vivo is more complicated and appears to allow concurrent responses to form (19).

HDM sensitization creates a distinct Th2 phenotype; however, skewing to a Th2 phenotype does not appear to hinder a Th1 response to the *H. capsulatum* infection. IL-12b and IFN- γ

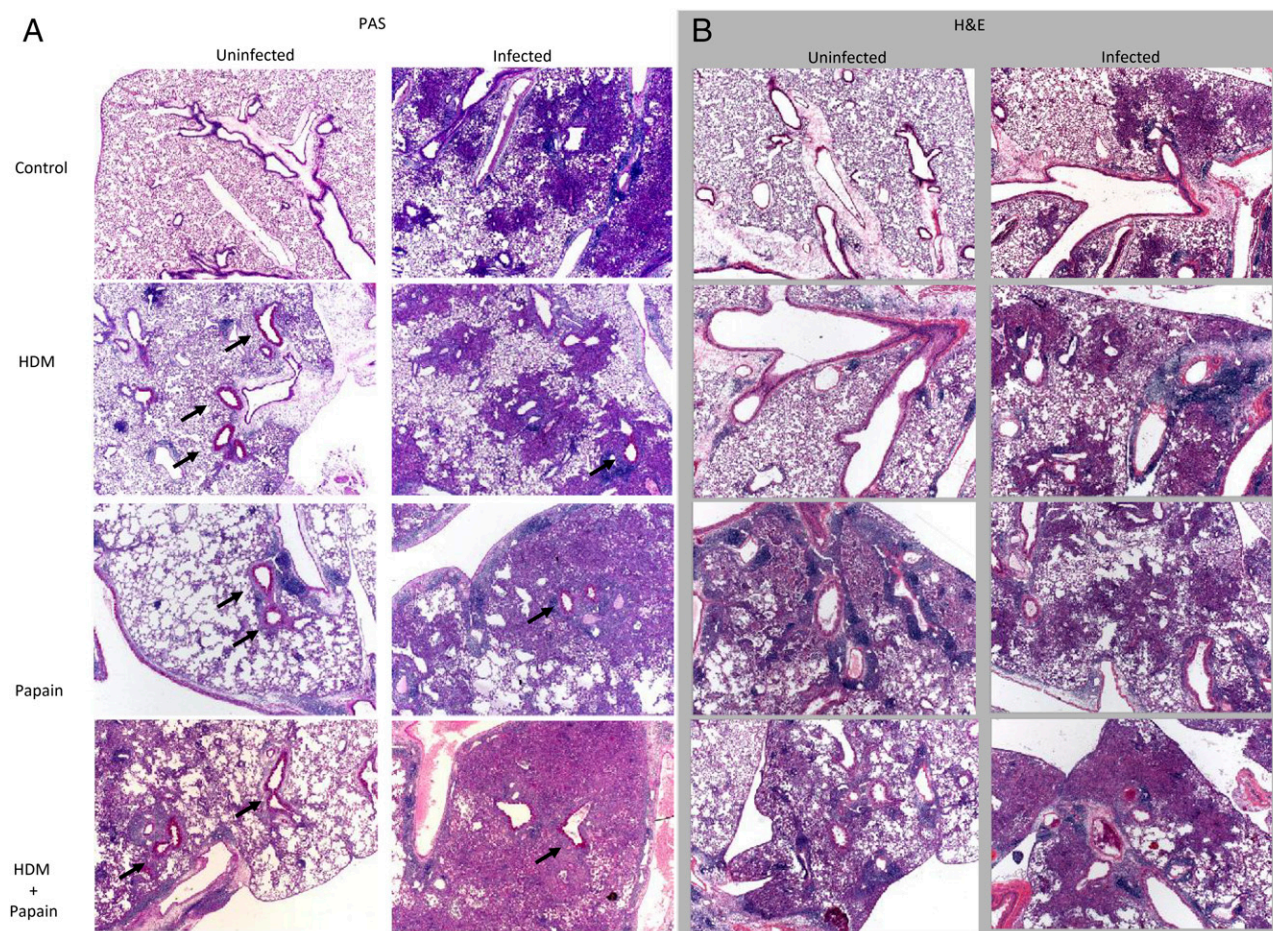


FIGURE 6. Allergens induce mucus accumulation in the airways. Day 7 p.i. histology of PAS- (**A**) and H&E-stained (**B**) lung sections from control mice and mice treated with HDM, papain, or HDM and papain, both uninfected and infected. Arrows indicate mucin⁺ airways in the PAS-stained sections. Original magnification $\times 25$. Micrographs are representative of $n = 4$.

expression are similar between infected controls and those mice first treated with HDM. The expression of these cytokines demonstrates that the established Th2 environment in the lungs does not suppress a Th1 response from forming. Peng et al. found that mice stimulated with goat anti-mouse IgD antiserum (G α M δ) and then infected with *H. capsulatum* showed an initial Th2 response that resulted in increased fungal burden and that this initial response was quickly skewed to Th1 in response to the infection. However, unlike in our study, this phenotype was dependent upon IL-4 and IL-10 (22); infected mice treated with HDM or not showed no difference in IL-10 expression levels (Supplemental Fig. 4C). In our study, the response to the infection appears to gradually switch the lung environment from Th2 to Th1, and within a week of infection, the animals no longer exhibit an influx of eosinophils in the lungs, Th2 cytokines or chemokines, or many mucin⁺ cells. IFN- γ is known to block the accumulation of eosinophils in the airway by binding its receptor and possibly inducing apoptosis or recirculation to the lymph nodes and may be a reason for their disappearance (23). Despite the ability to switch the lung environment to one more favorable to resolving infection, initial contact in a Th2 environment is sufficient to result in the death of 50% of the mice pretreated with HDM and 75% of the mice pretreated with papain. This is a more severe phenotype than seen in other model systems; mice do not exhibit a more severe *M. tuberculosis* infection in a Th2-skewed filarial infection environment (15).

IL-4 is not solely responsible for the exacerbated *H. capsulatum* infection exhibited with HDM pretreatment. α IL-4 reduced the fungal burden but not quite to the burden of control mice. Another possible mediator is IL-33. It has been shown to mediate inflammatory cell influx because of HDM and papain (24–26). Despite multiple reports signifying a key role for IL-33, it does not appear to contribute (Supplemental Fig. 3) in our system. IL-13 is a possible candidate responsible for mediating the HDM exacerbation of fungal infection, although there are no data regarding the influence of IL-13 on host defenses to this pathogen. It is another canonical Th2 cytokine known to be elevated in response to HDM and induce AHR independently of IgE and eosinophils responding to IL-4 (27). IL-13 plays an important role in the effector phase of asthma by inducing airway remodeling, AHR, and mucus hyperproduction (26). In OVA-induced AHR, IL-4 deficiency was not enough to prevent the onset of Th2 immune responses, but deficiency in IL-13 was (28). HDM-treated mice exhibited elevated IL-13, which persisted through early infection and may have contributed to the elevated fungal burdens in these mice.

Although most studies investigating asthma or allergy in mice use BALB/c, which is the more Th2-inclined biased mouse strain, we used C57BL/6, which is considered to be more Th1 prone (29). Despite this predisposition, the C57BL/6 mice responded to HDM similarly to the BALB/c mice, with a robust Th2 response that persisted for almost a week before switching to a Th1 phenotype

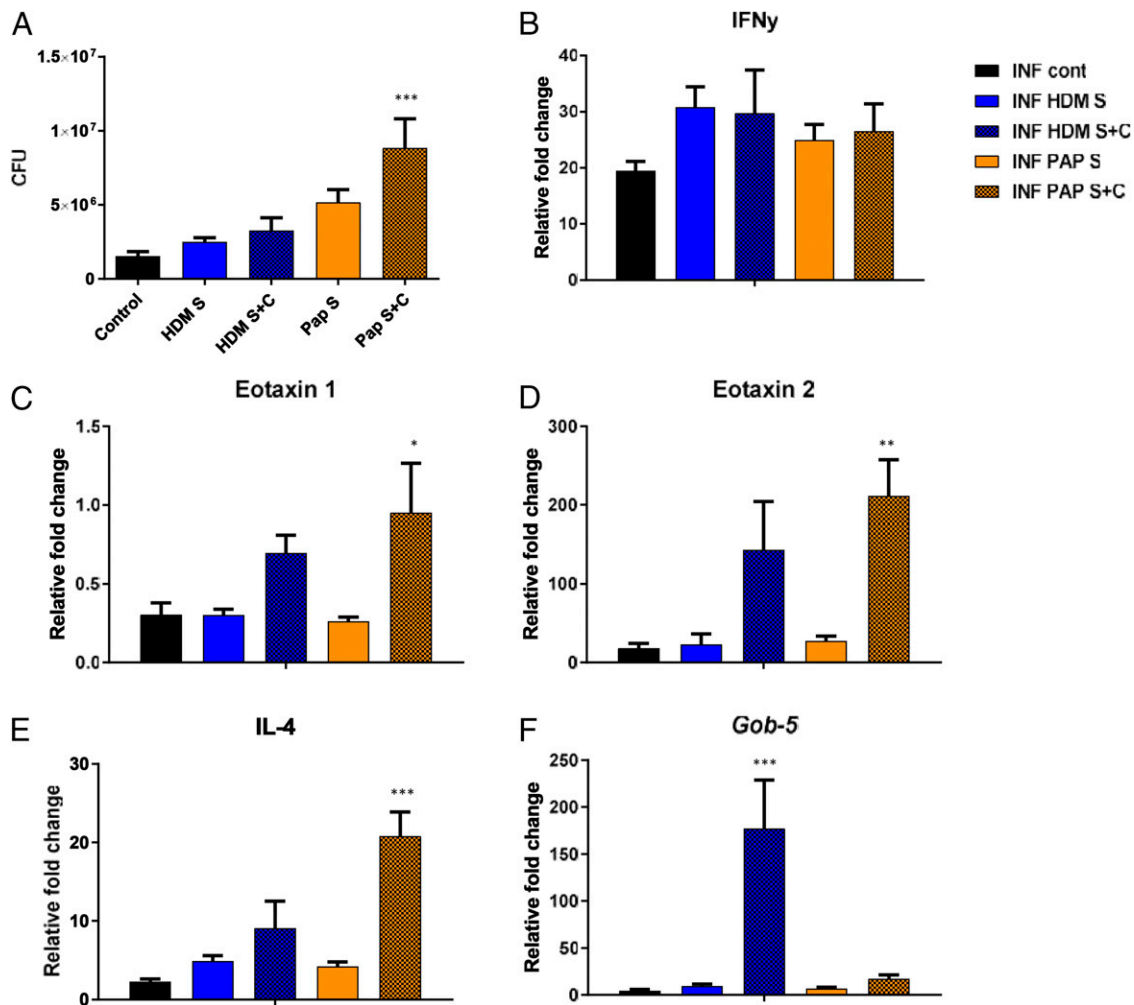


FIGURE 7. Primary infection does not overwhelm allergic response. (A) Pulmonary fungal burden represented as log₁₀ CFU in control, HDM-, and papain-treated mice at 7 d p.i. Allergen-treated mice were sensitized to the allergen 7 d before infection. During infection, mice were either left unchallenged (S) or challenged (S+C) with daily doses of 100 μ g of HDM or papain throughout the course of infection. Quantitative real-time RT-PCR analysis of whole-lung homogenates for Th1 cytokine genes (B) and Th2 cytokine and chemokine genes (C–F), significance indicated when compared with control infected mice. Data are represented as mean $\pm$ SEM, two independent experiments, $n = 8$ mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

induced by *H. capsulatum* infection. BALB/c mice are often chosen for allergy studies because C57BL/6 mice are more apt to become tolerant (30) and in some model systems do not show persistence of AHR or inflammation (31). This does not appear to be the case in our model, as the mice continue to have robust Th2 responses throughout the course of the experiment in uninfected animals.

Infection with *H. capsulatum* in an established Th2 lung environment exacerbated the fungal burden. Likewise, exposure to HDM or papain in mice with ongoing infection impaired anti-fungal immunity. These mice were given 2 d to establish a Th1 environment before being given a Th2 stimulus, and, therefore, should have been skewed toward Th1 (14, 18). However, the lungs of these mice exhibited elevated levels of eotaxins 1 and 2 and IL-4 while maintaining IFN- γ levels, indicating that the Th2 response was developing despite a preexisting Th1 environment. This suggests that despite the dogma of a Th1 environment suppressing the development of a Th2 response, the mice were still able to develop a Th2 response, maintaining both Th1 and Th2 concurrently.

In a secondary infection, HDM treatment did not exacerbate infection, and there was no Th2 response. These results suggest

that memory T cells against *H. capsulatum* that were likely Th1 skewed were quickly dominant and able to suppress T cells that may have otherwise been skewed toward Th2. However, passive transfer of Th1 cells before OVA-induced airway inflammation resulted in exacerbated inflammation (32), not the lack of Th2 response we saw in our secondary infection and HDM-treated animals. This observation supports the hygiene hypothesis that theorizes that increased cleanliness has led to an increase in asthma prevalence. And although there is debate over the legitimacy of the hypothesis (33), at least for our model it appears that a memory response to *H. capsulatum* is able to suppress HDM-induced Th2 responses during secondary infection.

Individuals from areas of endemic *H. capsulatum* will have been exposed early in life and therefore likely have a memory response to *Histoplasma*. However, nonendemic asthmatics visiting an endemic region may be more susceptible to developing histoplasmosis, as a case study of a young asthmatic who developed pulmonary histoplasmosis after visiting an endemic region shows (34).

In summary, primary *H. capsulatum* infection is exacerbated when initiated in a Th2 environment. Although the host is able to relatively quickly skew the lungs toward a Th1 response to

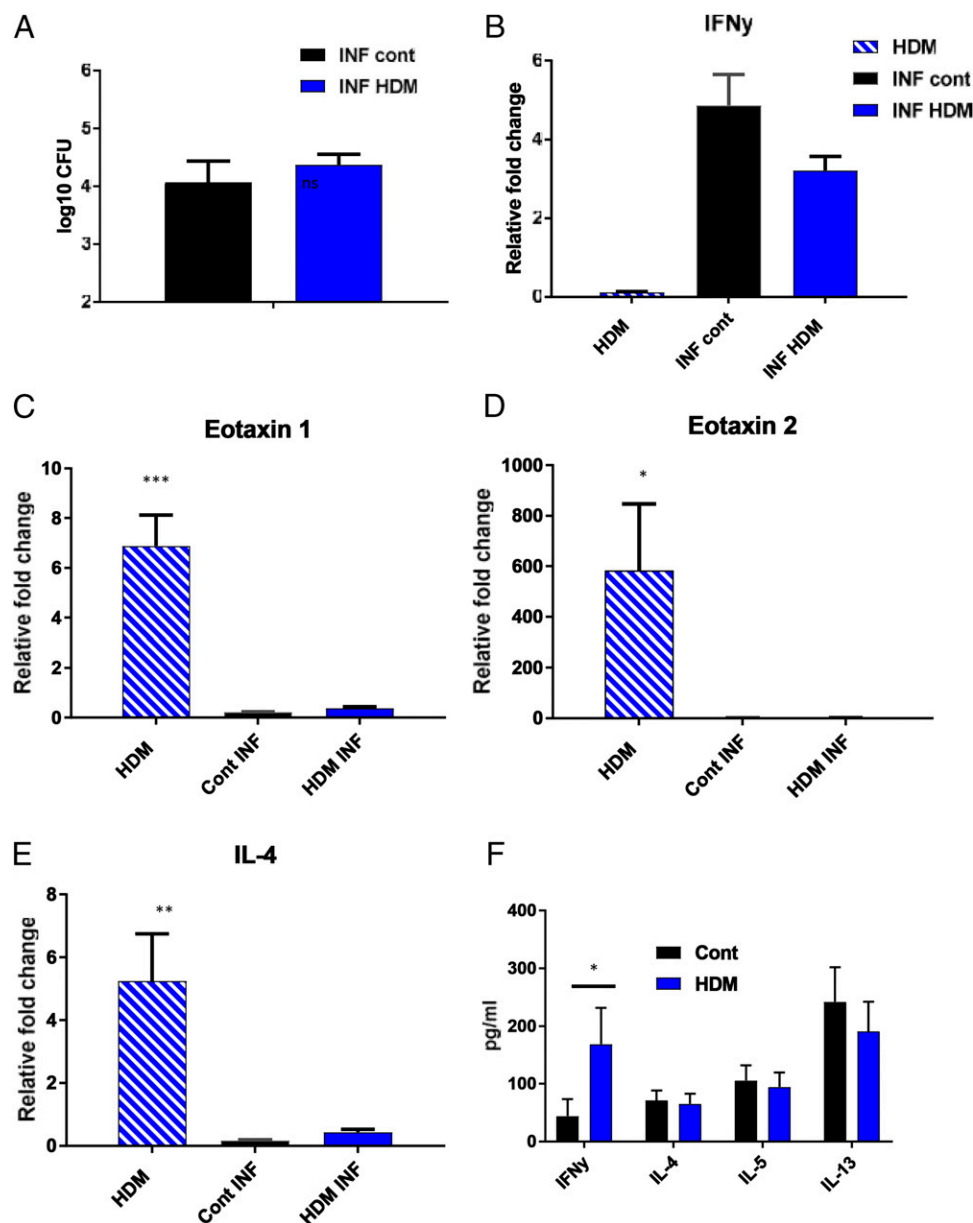


FIGURE 8. Secondary *H. capsulatum* infection suppresses HDM-induced Th2 phenotype. **(A)** Pulmonary fungal burden represented as log₁₀ CFU in control and HDM-treated mice at 7 d postsecondary infection. **(B)** Quantitative real-time RT-PCR analysis of whole-lung homogenates of Th1 cytokine genes. **(C–E)** Quantitative real-time RT-PCR analysis of whole-lung homogenates of Th2 cytokine genes. **(F)** ELISA of IFN-γ, IL-4, IL-5, and IL-13 at day 7 p.i. Data are represented as mean ± SEM, two independent experiments, *n* = 8 mice. **p* < 0.05, ***p* < 0.01, ****p* < 0.005.

fight the infection, it is not always enough to resolve the infection. However, a secondary infection with an intact memory response results in quick clearance and quick suppression of the allergen-induced Th2 phenotype. Susceptible populations from nonendemic regions may wish to exhibit caution if their symptoms are not well controlled.

Disclosures

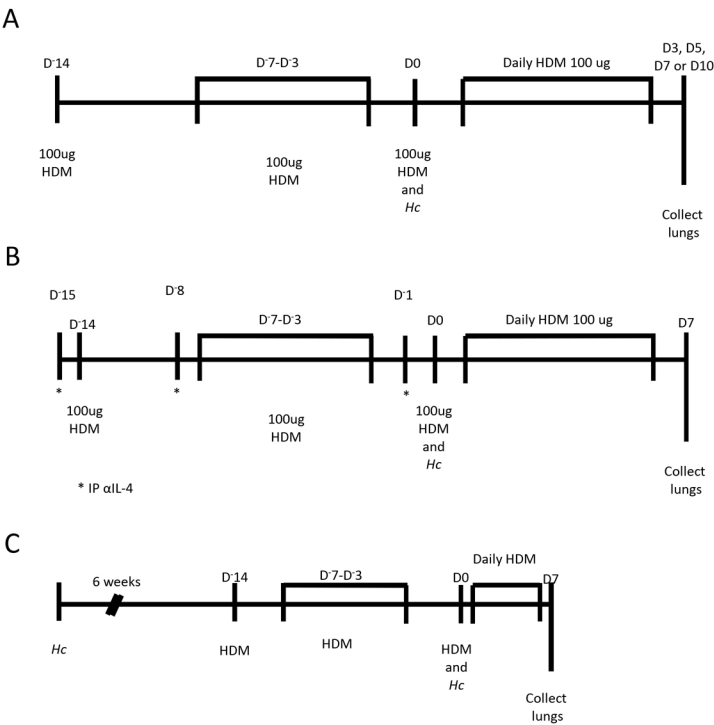
The authors have no financial conflicts of interest.

References

- Moorman, J. E., L. J. Akinbami, C. M. Bailey, H. S. Zahran, M. E. King, C. A. Johnson, and X. Liu. 2012. National surveillance of asthma: United States, 2001–2010. *Vital Health Stat.* 3 35: 1751–58.
- King, C., S. Brennan, P. J. Thompson, and G. A. Stewart. 1998. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J. Immunol.* 161: 3645–3651.
- Morita, H., K. Arae, H. Unno, K. Miyauchi, S. Toyama, A. Nambu, K. Oboki, T. Ohno, K. Motomura, A. Matsuda, et al. 2015. An interleukin-33-mast cell-interleukin-2 axis suppresses papain-induced allergic inflammation by promoting regulatory T cell numbers. *Immunity* 43: 175–186.
- Agoro, R., J. Piotet-Morin, J. Palomo, C. Michaudel, S. Vigne, I. Maillat, P. Chenuet, N. Guillou, J. Le Bérichel, M. Kisielow, et al. 2016. IL-1R1-MyD88 axis elicits papain-induced lung inflammation. *Eur. J. Immunol.* 46: 2531–2541.
- Denning, D. W., B. R. O'Driscoll, C. M. Hogaboam, P. Bowyer, and R. M. Niven. 2006. The link between fungi and severe asthma: a summary of the evidence. *Eur. Respir. J.* 27: 615–626.
- Porter, P., S. C. Susarla, S. Polikepahad, Y. Qian, J. Hampton, A. Kiss, S. Vaidya, S. Sur, V. Onger, T. Yang, et al. 2009. Link between allergic asthma and airway mucosal infection suggested by proteinase-secreting household fungi. *Mucosal Immunol.* 2: 504–517.
- Tham, R., C. H. Katelaris, D. Vicendese, S. C. Dharmage, A. J. Lowe, G. Bowatte, P. Taylor, P. Burton, M. J. Abramson, and B. Erbas. 2017. The role of outdoor fungi on asthma hospital admissions in children and adolescents: a 5-year time stratified case-crossover analysis. *Environ. Res.* 154: 42–49.
- Tham, R., D. Vicendese, S. C. Dharmage, R. J. Hyndman, E. Newbiggin, E. Lewis, M. O'Sullivan, A. J. Lowe, P. Taylor, P. Bardin, et al. 2017. Associations between outdoor fungal spores and childhood and adolescent asthma hospitalizations. *J. Allergy Clin. Immunol.* 139: 1140–1147.e4.

9. Yang, I. V., C. A. Lozupone, and D. A. Schwartz. 2017. The environment, epigenome, and asthma. *J. Allergy Clin. Immunol.* 140: 14–23.
10. Lilly, L. M., M. A. Gessner, C. W. Dunaway, A. E. Metz, L. Schwiebert, C. T. Weaver, G. D. Brown, and C. Steele. 2012. The β -glucan receptor dectin-1 promotes lung immunopathology during fungal allergy via IL-22. *J. Immunol.* 189: 3653–3660.
11. Brown, G. D., D. W. Denning, N. A. Gow, S. M. Levitz, M. G. Netea, and T. C. White. 2012. Hidden killers: human fungal infections. *Sci. Transl. Med.* 4: 165rv13.
12. Szymczak, W. A., and G. S. Deepe, Jr. 2009. The CCL7-CCL2-CCR2 axis regulates IL-4 production in lungs and fungal immunity. *J. Immunol.* 183: 1964–1974.
13. Verma, A., D. N. Kretz, J. L. Tweedle, and G. S. Deepe, Jr. 2015. Type II cytokines impair host defense against an intracellular fungal pathogen by amplifying macrophage generation of IL-33. *Mucosal Immunol.* 8: 380–389.
14. Jankovic, D., M. C. Kullberg, P. Caspar, and A. Sher. 2004. Parasite-induced Th2 polarization is associated with down-regulated dendritic cell responsiveness to Th1 stimuli and a transient delay in T lymphocyte cycling. *J. Immunol.* 173: 2419–2427.
15. Sartono, E., Y. C. Kruize, A. Kurniawan, R. M. Maizels, and M. Yazdanbakhsh. 1996. In Th2-biased lymphatic filarial patients, responses to purified protein derivative of *Mycobacterium tuberculosis* remain Th1. *Eur. J. Immunol.* 26: 501–504.
16. Monin, L., K. L. Griffiths, W. Y. Lam, R. Gopal, D. D. Kang, M. Ahmed, A. Rajamanickam, A. Cruz-Lagunas, J. Zúñiga, S. Babu, et al. 2015. Helminth-induced arginase-1 exacerbates lung inflammation and disease severity in tuberculosis. *J. Clin. Invest.* 125: 4699–4713.
17. Pearce, E. J., P. Caspar, J. M. Grzych, F. A. Lewis, and A. Sher. 1991. Down-regulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, *Schistosoma mansoni*. *J. Exp. Med.* 173: 159–166.
18. Zhu, J., and W. E. Paul. 2010. Heterogeneity and plasticity of T helper cells. *Cell Res.* 20: 4–12.
19. Murphy, K. M., and B. Stockinger. 2010. Effector T cell plasticity: flexibility in the face of changing circumstances. *Nat. Immunol.* 11: 674–680.
20. Foster, B., C. Prussin, F. Liu, J. K. Whitmire, and J. L. Whitton. 2007. Detection of intracellular cytokines by flow cytometry. *Curr. Protoc. Immunol.* Chapter 6: Unit 6.24.
21. Lambrecht, B. N., and H. Hammad. 2012. The airway epithelium in asthma. *Nat. Med.* 18: 684–692.
22. Peng, J. K., J. S. Lin, J. T. Kung, F. D. Finkelman, and B. A. Wu-Hsieh. 2005. The combined effect of IL-4 and IL-10 suppresses the generation of, but does not change the polarity of, type-1 T cells in histoplasma infection. *Int. Immunol.* 17: 193–205.
23. Cohn, L., C. Herrick, N. Niu, R. Homer, and K. Bottomly. 2001. IL-4 promotes airway eosinophilia by suppressing IFN-gamma production: defining a novel role for IFN-gamma in the regulation of allergic airway inflammation. *J. Immunol.* 166: 2760–2767.
24. Oboki, K., T. Ohno, N. Kajiwara, K. Arae, H. Morita, A. Ishii, A. Nambu, T. Abe, H. Kiyonari, K. Matsumoto, et al. 2010. IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proc. Natl. Acad. Sci. USA* 107: 18581–18586.
25. Kamijo, S., H. Takeda, T. Tokura, M. Suzuki, K. Inui, M. Hara, H. Matsuda, A. Matsuda, K. Oboki, T. Ohno, et al. 2013. IL-33-mediated innate response and adaptive immune cells contribute to maximum responses of protease allergen-induced allergic airway inflammation. *J. Immunol.* 190: 4489–4499.
26. Endo, Y., K. Hirahara, T. Iinuma, K. Shinoda, D. J. Tumes, H. K. Asou, N. Matsugae, K. Obata-Ninomiya, H. Yamamoto, S. Motohashi, et al. 2015. The interleukin-33-p38 kinase axis confers memory T helper 2 cell pathogenicity in the airway. *Immunity* 42: 294–308.
27. Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282: 2258–2261.
28. Kirstein, F., N. E. Nieuwenhuizen, J. Jayakumar, W. G. Horsnell, and F. Brombacher. 2016. Role of IL-4 receptor α of IL-CD4(+) T cells in chronic airway hyperresponsiveness. *Allergy Clin. Immunol.* 137: 1852–1862.e9.
29. Kearley, J., K. F. Buckland, S. A. Mathie, and C. M. Lloyd. 2009. Resolution of allergic inflammation and airway hyperreactivity is dependent upon disruption of the T1/ST2-IL-33 pathway. *Am. J. Respir. Crit. Care Med.* 179: 772–781.
30. Van Gramberg, J. L., M. J. de Veer, R. E. O’Hehir, E. N. Meeusen, and R. J. Bischof. 2013. Use of animal models to investigate major allergens associated with food allergy. *J. Allergy (Cairo)* 2013: 635695.
31. Kariyawasam, H. H., and D. S. Robinson. 2005. Airway remodelling in asthma: models and supermodels? *Clin. Exp. Allergy* 35: 117–121.
32. Randolph, D. A., C. J. Carruthers, S. J. Szabo, K. M. Murphy, and D. D. Chaplin. 1999. Modulation of airway inflammation by passive transfer of allergen-specific Th1 and Th2 cells in a mouse model of asthma. *J. Immunol.* 162: 2375–2383.
33. Brooks, C., N. Pearce, and J. Douwes. 2013. The hygiene hypothesis in allergy and asthma: an update. *Curr. Opin. Allergy Clin. Immunol.* 13: 70–77.
34. Gudala, M., and S. Hegde. 2015. Delayed diagnosis of pulmonary histoplasmosis in an immunocompetent young asthmatic female from Florida, a non-endemic area for *Histoplasma capsulatum*. *Med. Mycol. Open Access* 1: 1.

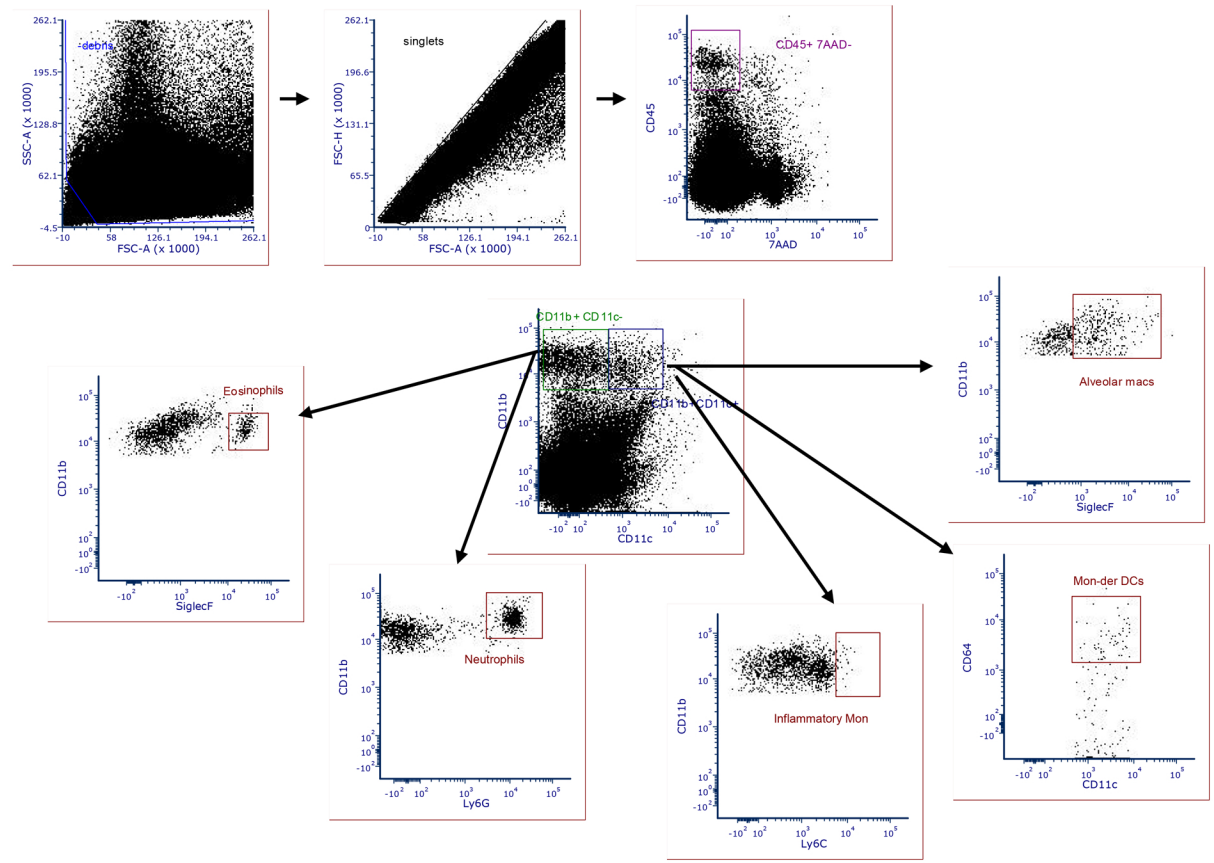
Supplementary Figure 1



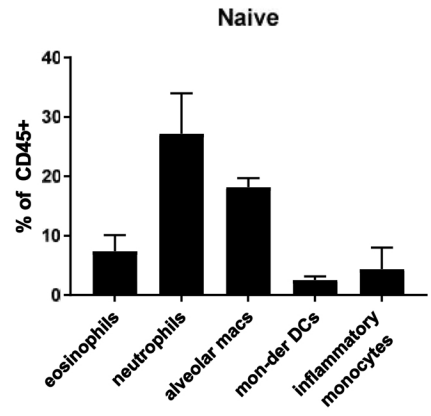
Supplemental Figure 1: Methods schematics. A) Schematic diagram of standard protocol used in the majority of experiments. B) Protocol for α IL-4 experiments. C) Diagram of the memory response protocol.

Supplementary Figure 2

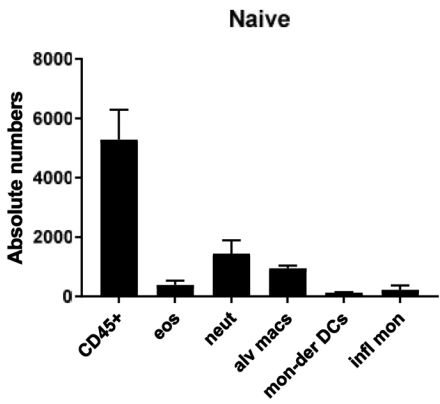
A



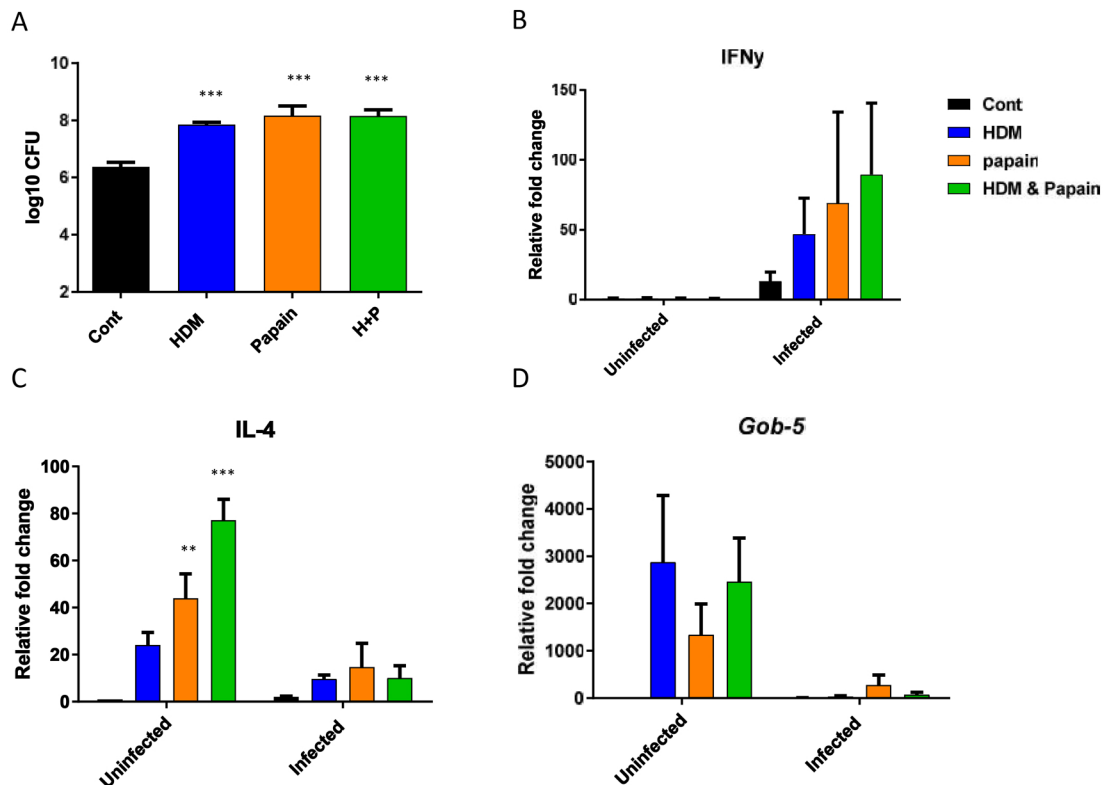
B



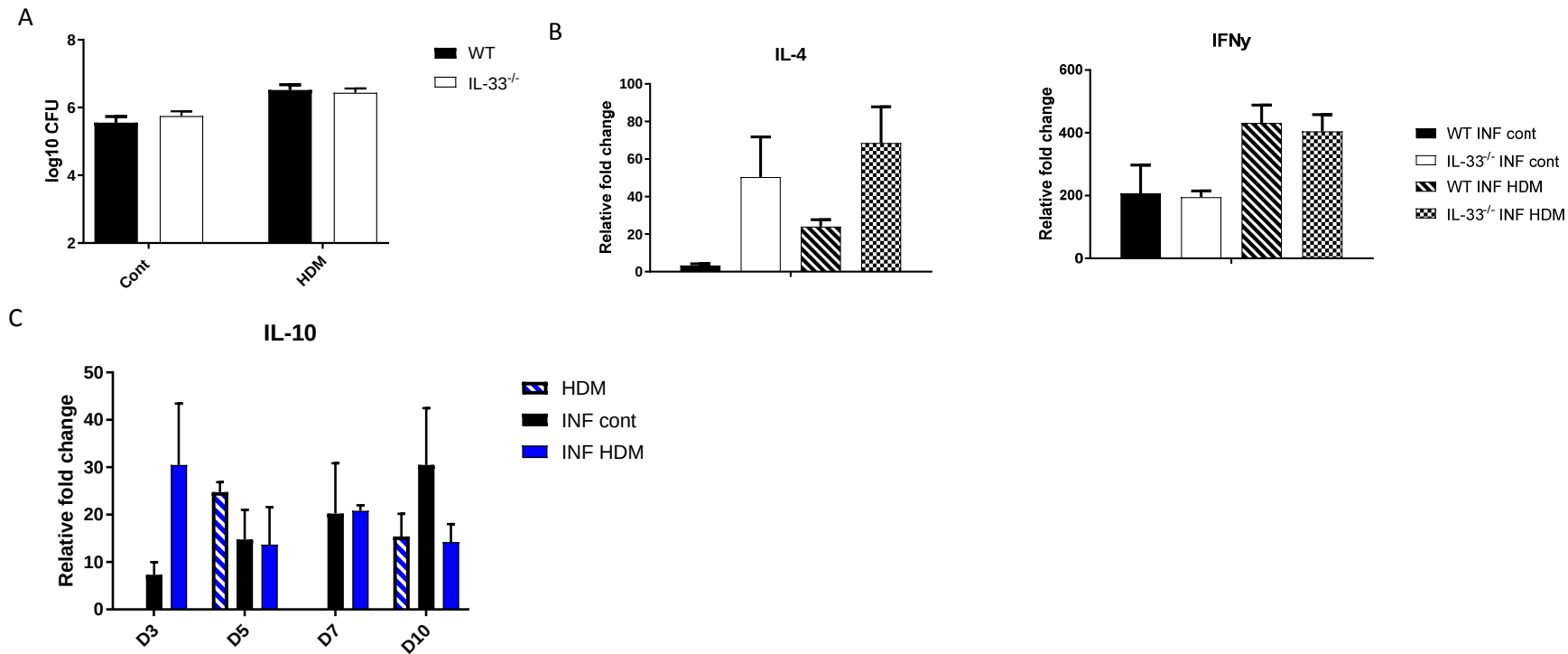
C



Supplemental Figure 2: Gating strategy. A) Flow gating strategy. Naïve mice cell population proportions (B) and absolute numbers (C).



Supplemental Figure 3: BALB/c mice respond similarly to C57BL/6 mice. A) Day 7 p.i. pulmonary fungal burdens represented as log₁₀ CFU in control, HDM-, papain-, and HDM and papain-treated mice. qRT-PCR analysis of whole lung homogenates for IFN γ (B) and IL-4 (C) and *Gob-5* (D) following infection and allergen treatment. Data are represented as mean $\pm$ SEM, 2 independent experiments, n= 8 mice.



Supplemental Figure 4: IL-33 is not required for HDM-induced exacerbation of *H. capsulatum* infection and IL-10 expression is unchanged in infected animals with or without HDM treatment. A) Day 7 p.i. pulmonary fungal burdens represented as log₁₀ CFU in control and HDM-treated WT and IL-33^{-/-} mice. B) qRT-PCR analysis of whole lung homogenates for IL-4 and IFN γ (B) following infection and HDM treatment. Data are represented as mean $\pm$ SEM, 2 independent experiments, n= 7-11 mice. C) qRT-PCR analysis of whole lung homogenates for IL-10. Data are represented as mean $\pm$ SEM, n= 8-12 mice. Data are represented as mean $\pm$ SEM, 2 independent experiments, n= 8 mice