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Short Article

Gut Dysbiosis Promotes M2 Macrophage Polarization and Allergic Airway Inflammation via Fungi-Induced PGE_2

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SUMMARY

Although imbalances in gut microbiota composition, or "dysbiosis," are associated with many diseases, the effects of gut dysbiosis on host systemic physiology are less well characterized. We report that gut dysbiosis induced by antibiotic (Abx) treatment promotes allergic airway inflammation by shifting macrophage polarization in the lung toward the alternatively activated M2 phenotype. Adoptive transfer of alveolar macrophages derived from Abx-treated mice was sufficient to increase allergic airway inflammation. Abx treatment resulted in the overgrowth of a commensal fungal *Candida* species in the gut and increased plasma concentrations of prostaglandin E_2 (PGE_2), which induced M2 macrophage polarization in the lung. Suppression of PGE2 synthesis by the cyclooxygenase inhibitors aspirin and celecoxib suppressed M2 macrophage polarization and decreased allergic airway inflammatory cell infiltration in Abx-treated mice. Thus, Abx treatment can cause overgrowth of particular fungal species in the gut and promote M2 macrophage activation at distant sites to influence systemic responses including allergic inflammation.

INTRODUCTION

Imbalances in gut microbiota composition, described as "dysbiosis," are caused by many factors, including host genetics, lifestyle, and exposure to microorganisms or various medical procedures (Round and Mazmanian, 2009). Dysbiosis has been associated not only with intestinal inflammation (Elinav et al., 2011; Mazmanian et al., 2008) but also with many diseases outside the gut, such as atopic dermatitis, allergy, obesity, and diabetes (Arumugam et al., 2011; Henao-Mejia et al., 2012; Penders et al., 2007; Vijay-Kumar et al., 2010). However, how the gut dysbiosis influences host immunity outside the gastrointestinal tract is largely unknown.

Several examples of the systemic influence of the commensal bacteria on peripheral immune responses have recently been provided. Peptidoglycan from orally inoculated *E. coli* enhanced killing of *Streptococcus pneumoniae* and *Staphcylococcus aureus* by bone-marrow-derived neutrophils in a Nod1-dependent manner (Clarke et al., 2010). Short-chain fatty acids (SCFAs), which were produced by fermentable dietary fiber induced by commensal bacteria, protect against the development of inflammatory diseases including colitis, arthritis, and allergy (Maslowski et al., 2009). However, the vast majority of these studies on interplay between commensal microbiota and systemic immune responses have focused on gut bacteria but not other microbes such as fungi or viruses.

Although more than 99% of microbiota consists of bacteria, fungi, most of which are *Candida* species, are also detectable in gastrointestinal sections of about 70% of healthy human adults (Cohen et al., 1969). Dysbiosis can result from a loss of beneficial commensal bacteria and an overgrowth of fungi (Giuliano et al., 1987; Samonis et al., 1990). *Candida* infection can induce production of inflammatory mediators by host cells. *Candida* also produces ligands for pattern recognition receptors (PRRs), including β-glucans, chitin, mannans, β-(1,2)-linked oligomannosides, and fungal nucleic acids, which stimulate innate immune responses. In addition, *Candida* produces proinflammatory substances such as alcohol (Santelmann and Howard, 2005) and prostaglandin (PGE_2) (Noverr et al., 2001). Several studies have suggested that gut fungi can influence inflammatory disorders such as inflammatory bowel disease (Iliev et al., 2012; Ott et al., 2008) or allergic airway inflammation (Noverr et al., 2004). However, although the study of the fungal microbiota is a rapidly emerging field, the mechanisms by which gut dysbiosis-driven fungal overgrowth in the gut affects host immune responses remain poorly understood.

Here we provide the evidence that *Candida* overgrowth promotes M2 macrophage polarization via PGE_2, which plays a critical role in the increased allergic airway inflammatory cell infiltration.

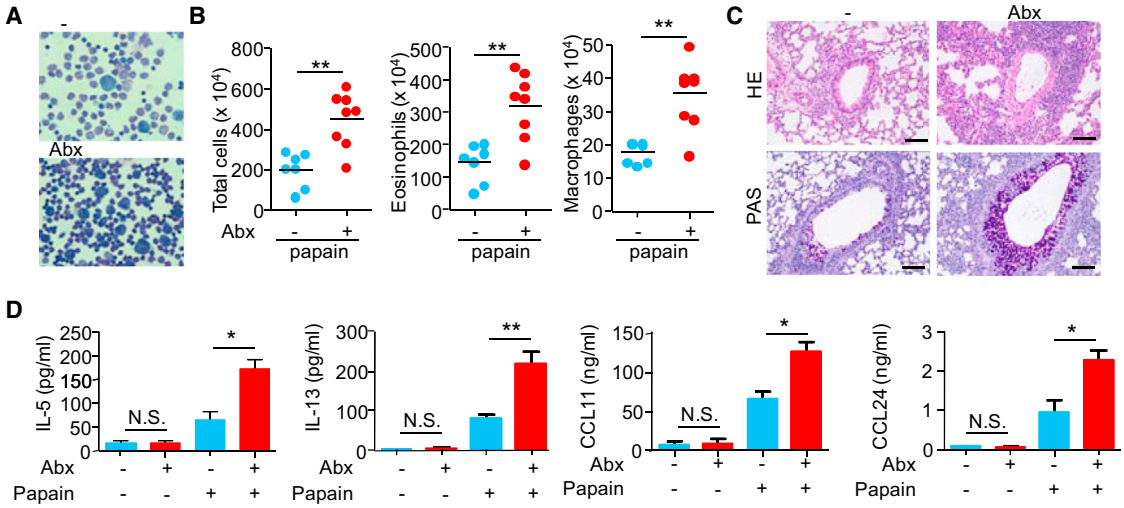

Figure 1. Antibiotic Treatment Exacerbates Allergic Airway Inflammation

(A–D) Antibiotic (clindamycin + cefoperazone) (Abx)-treated or untreated (−) mice were challenged intranasally with papain every 3 days a total of five times. One day after the final challenge, mice were analyzed for morphology of BAL cells (Wright's stain, original magnification 1,000×) (A), total and differentiated cell counts in BAL (B), lung histology (HE or PAS stain) (scale bar, 100 μm) (C), and cytokine concentration in BAL fluids (D). Representative results in each group of A (n = 7 or 8) and C (n = 4∼6) are shown. Error bars indicate SD (D) (n = 7). Results are representative of three independent experiments (B and D). *$p < 0.05$; **$p < 0.01$. N.S., not significant. See also Figure S1.

RESULTS

Antibiotic Treatment Promotes Allergic Airway Inflammation

Treatment with antibiotics induces profound changes in the numbers and composition of gut microbiota (Round and Mazmanian, 2009). To directly assess the impact of dysbiosis on host immunity outside the gut, we induced allergic airway inflammation by intranasal inhalation of the protease allergen papain in control mice and mice treated with the antibiotics clindamycin and cefoperazone (Abx-treated mice). Mice pretreated with antibiotics and challenged with papain had significantly greater numbers of total cells, eosinophils, and macrophages in the airways than did control mice (Figures 1A and 1B). We also observed similar results when mice were challenged with house dust mite extract (Figures S1A and S1B available online). Papain-challenged Abx-treated mice also exhibited greater goblet cell hyperplasia and peribronchial inflammatory cell infiltration than did control mice (Figure 1C). We also found that the production of IL-5, IL-13, CCL11, and CCL24, which are critical molecules for type 2 immune responses and eosinophil recruitment and in the pathogenesis of asthma, were significantly higher in Abx-treated mice than in control mice after papain inhalation (Figure 1D). Together, these findings suggest that antibiotic treatment promotes allergen-induced airway inflammatory cell infiltration.

Antibiotic Treatment Induces M2 Macrophage Polarization

We next examined whether the increase in papain-induced airway inflammatory cell infiltration by antibiotics requires the adaptive immune system. To address this question, we treated *Rag-1*$^{-/-}$ mice, which were deficient in mature B and T lymphocytes due to disruption of their differentiations, with antibiotics and then challenged them with papain. Papain-induced airway inflammatory cell infiltration was also significantly increased in antibiotic-treated *Rag-1*$^{-/-}$ mice than in control mice, suggesting that antibiotic treatment can affect host immune responses in the absence of adaptive immunity (Figure S1C). Treatment with clodronate liposomes (liposome-encapsulated dichloromethylene bisphosphonate, Cl_2MBP), which deplete macrophages (Mϕs) and, in some cases, granulocytes and dendritic cells in vivo, decreased the total number of cells and the numbers of eosinophils and Mϕs in the BAL fluid after papain inhalation in mice that were either left untreated or treated with Abx and resulted in the comparable numbers of BAL fluid cells between both groups (Figure S2A), suggesting that alveolar Mϕs (AMϕs) are involved in Abx-induced allergic airway inflammatory cell infiltration. Moreover, mice that received AMϕs from Abx-treated mice exhibited significantly greater numbers of BAL fluid cells than those that received AMϕs from control mice (Figure 2A). These results suggest that Abx treatment altered macrophage function in some way that promotes inflammation in the lung.

Mϕs acquire distinct functional phenotypes by undergoing classical activation as M1 macrophages or alternative activation as M2 macrophages in response to various environmental factors. We found that, compared with AMϕs purified from control mice, AMϕs purified from Abx-treated mice exhibited significantly increased expression of *arg1*, *chi3l3*, and *retnla*, which are markers of alternatively activated M2 macrophages (Figures 2B and S2B). Intranasal treatment with serum amyloid P (SAP), which attenuates M2 macrophage activation (Moreira et al., 2010), inhibited M2 macrophage activation in the lung, but not in the peripheral blood, and suppressed papain-induced airway inflammation, as evidenced by significant decrease in the total

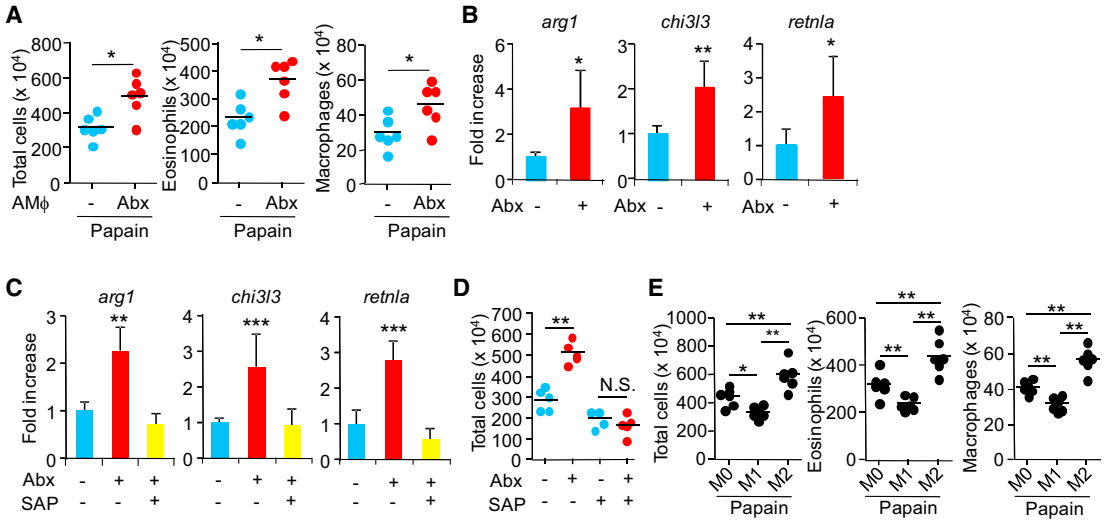

Figure 2. Antibiotic Treatment Induces M2 Macrophage Polarization

(A) Mice were adoptively transferred i.n. with alveolar macrophages (AMϕs) purified from untreated (–) or Abx-treated mice and challenged with papain and analyzed for BAL cells, as described in Figure 1.

(B) AMϕs purified from untreated (–) or Abx-treated (+) mice were subjected to real-time RT-PCR (n = 5).

(C) Abx-treated (+) or untreated (–) mice were administered i.n. with SAP (+) or PBS (–) every 3 days a total of five times. One day after the final SAP treatment, mice were analyzed for expression of M2 macrophage marker genes in AMϕs (n = 5).

(D) Mice were left untreated (–) or treated with Abx and were challenged with papain every 3 days a total of five times. The mice were also injected i.n. with serum amyloid P (SAP) or PBS 1 day before each papain challenge every 3 days a total of five times. One day after the final challenge with papain, mice were analyzed for BAL cells, as described in Figure 1.

(E) Mice were adoptively transferred i.n. with bone-marrow-derived cultured macrophages (M0), M1 macrophages, or M2 macrophages and then challenged i.n. with papain and analyzed for BAL cells, as described in Figure 1. Results are representative of three independent experiments.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. N.S., not significant. Error bars, SD. See also Figure S2.

numbers of cells in the BAL fluids of mice treated with both Abx and SAP compared with those treated with Abx and PBS (Figures 2C, 2D, and S2C). Adoptive transfer of M2, but not M1, macrophages generated by culture of bone-marrow-derived macrophages (M0 macrophages) into the nasal cavity significantly increased papain-induced airway inflammatory cell infiltration compared with transfer of M0 macrophages (Figure 2E), consistent with previous observations that M2 macrophages increase allergic airway inflammatory cell infiltration (Ford et al., 2012; Moreira et al., 2010). These results suggest that alveolar M2 Mϕs are involved in the increased papain-induced allergic airway inflammation.

Antibiotic Treatment Facilitates Fungal Overgrowth in the Gut, which Induces M2 Macrophage Polarization

Antibiotics disrupt healthy microbial communities, allowing the overgrowth of fungi (Giuliano et al., 1987; Samonis et al., 1990). Furthermore, previous reports demonstrated that administration of oral antibiotics followed by oral inoculation of *Candida* facilitated fungal overgrowth in the gut and promoted allergic inflammation (Noverr et al., 2004). Therefore, we next examined the effect of antibiotic treatment on fungal colonization of the intestine. Treatment with cefoperazone, ampicillin, or streptomycin as well as combination with cefoperazone and clindamycin, but not with clindamycin, or metronidazole, significantly promoted fungal overgrowth in the gut (Figure 3A). We therefore compared the effect of combination with cefoperazone and clindamycin with that of metronidazole on the microbiota in the feces to analyze how Abx (cefoperazone and clindamycin) treatment induced fungal overgrowth. Quantitative RT-PCR analyses demonstrated that treatment with clindamycin and cefoperazone markedly decreased the copy numbers of several species of microbiota, including *Clostridium* clusters IV and XIVab, *Lactobacillus* genus and *Bacteroides* genus (Figure S3A). In contrast, these species of microbiota were not affected by the treatment with metronidazole that did not induce the fungal overgrowth in the gut (Figure S3A), suggesting that these microbiota might be involved in inhibition of fungal overgrowth in the gut. However, we did not observe fungal overgrowth in the lung and oral cavity of Abx-treated mice (Figures 3A and S3B). Interestingly, the level of intestinal fungal overgrowth was correlated with the papain-induced enhancement of inflammatory cell influx into the BAL fluid (Figures 3B and 3C). We obtained a fungal isolate from the feces of Abx-treated mice and identified it as *Candida parapsilosis* (*C. parapsilosis*) by 16S rDNA sequencing (data not shown). Histological analyses of intestinal tissue sections of mice treated with Abx showed that *Candida* infiltration was common in the lamina propria of cecal tissues but was uncommon in the small intestine or colon (Figures 3D, 3E, and S3C). Notably, Abx-treated mice did not show obvious histological signs of inflammation throughout the gastrointestinal tract (Figure S3C).

We did not observe overgrowth of any fungal species, including *C. albicans*, *C. glabrata*, and *C. tropicalis*, as well as *C. parapsilosis*, in the intestines of mice of the same strain obtained from a commercial supplier (Clea Japan, Tokyo) after

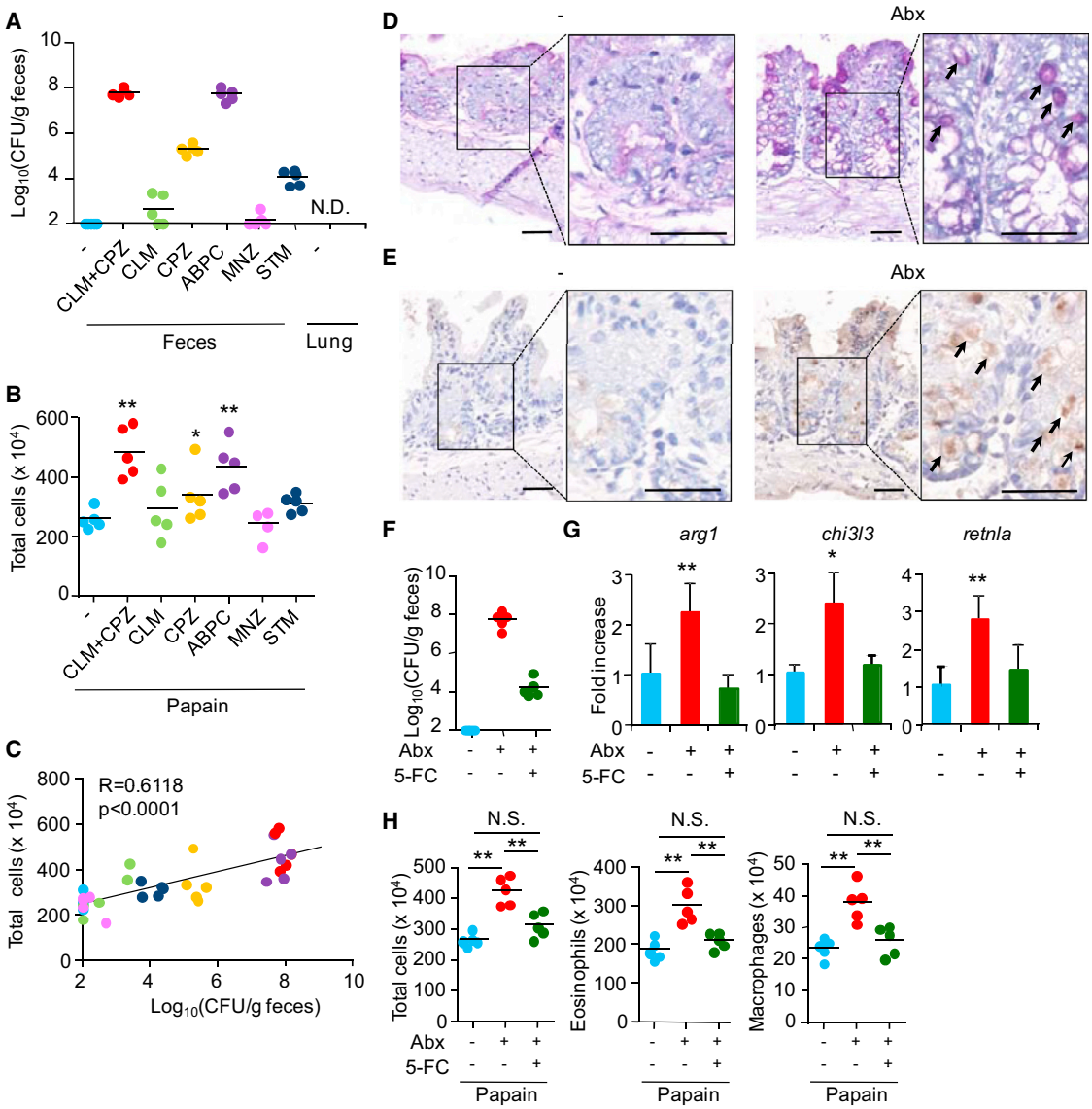

Figure 3. Antibiotic Treatment Facilitates Fungal Overgrowth in the Gut, which Induces M2 Macrophage Polarization

(A–C) Mice were left untreated or treated orally with Abx (CLM, clindamycin; CPZ, cefoperazone; ABPC, ampicillin; MNZ, metronidazole; STM, streptomycin) for 2 weeks and analyzed for fungal colonization in the feces (A). Mice were then challenged i.n. with papain and analyzed for BAL cells, as described in Figure 1 (B). Correlation between fungal colonization and total cell number in BAL was analyzed (C).

(D and E) Cecum sections in mice untreated (−) or treated with Abx (clindamycin + cefoperazone) for 2 weeks were stained with PAS (D) or DAB (E), as described in the Supplemental Information. Arrows indicate *Candida* in the lamina propria. Scale bar, 10 μm.

(F–H) Mice were left untreated or treated orally with either Abx (clindamycin + cefoperazone) or Abx plus 5-FC for 2 weeks and analyzed for fungal colonization in the feces (F). AMϕs were subjected to real-time RT-PCR (G) (n = 5). Mice were then challenged i.n. with papain and analyzed for BAL cell number, as described in Figure 1 (H). Results are representative of three independent experiments. *$p < 0.05$; **$p < 0.01$. N.S., not significant. See also Figure S3.

treatment with Abx, suggesting that fungal overgrowth induced by antibiotics was dependent on the mouse colony. Nevertheless, inoculation of commercial mice from Clea Japan into the gut with exogenous *C. parapsilosis* enhanced the allergic airway inflammatory cell infiltration induced by papain (Figure S3D, data not shown). Furthermore, oral treatment of the commercial mice with human-isolated *Candida* species (*C. albicans*, *C. glabrata*, or *C. tropicalis*) led to fungal overgrowth in the gut (Figure S3D) and significantly exacerbated airway inflammation induced by papain (Figure S3D). Administration of 5-FC, an antifungal drug, significantly reduced the fecal counts of fungi in Abx-treated mice (Figure 3F). Furthermore, 5-FC treatment suppressed the upregulation of M2 macrophage markers expression in AMϕs from Abx-treated mice (Figure 3G) and significantly decreased the BAL fluid cells of Abx-treated mice in our mouse colony (positive for *C. parapsilosis*), but not in Abx-treated commercial mice (negative for *C. parapsilosis*) after challenge with papain (Figures 3H and S3E). These results indicated that

Abx-induced fungal overgrowth exacerbated papain-induced airway inflammation by shifting macrophage polarization toward the M2 phenotype.

Increased PGE_2 Levels Associated with Intestinal Fungal Overgrowth Contribute to M2 Polarization and Increase in Allergic Airway Inflammatory Cell Infiltration

Because commensal-derived signals can stimulate immune cells systemically (Abt et al., 2012), we investigated whether fungal overgrowth in the intestine would affect the function of peripheral blood monocytes. In Abx-treated mice, peripheral blood monocytes also exhibited increased expression of M2 markers, *arg1*, *chi3l3*, and *retnla* (Figure 4A). Moreover, culture of naive AMϕs in the presence of sera obtained from mice after, but not before, Abx treatment upregulated expression of M2 markers (Figure S4A). These results suggest that fungal components act systemically to alter macrophage function by regulating the M2 phenotype in the lung. However, we did not detect β-D-glucan and mannan, two major cell components of *Candida*, in the sera of Abx-treated mice (data not shown).

Several evidences demonstrated that many fungi secrete prostaglandins de novo or via conversion of exogenous arachidonic acid (Noverr et al., 2001, 2002). In addition, *Candida* can activate host cells to produce prostaglandins (Gagliardi et al., 2010; Lee et al., 2009). Prostaglandins such as prostaglandin E_2 (PGE_2) enhance allergic inflammation (Church et al., 2012), suggesting a possibility that overgrowth of gut fungal microbiota may alter immune responses via PGE_2. In fact, we found that *C. parapsilosis* converted exogenous arachidonic acid into prostaglandins (Figure 4B). Furthermore, PGE_2 metabolite (PGEM) levels were significantly greater in both the sera and BAL fluid of Abx-treated mice than in control mice (Figures 4C and 4D), whereas the levels of PGEM in the sera were decreased in mice treated with both Abx and 5-FC (Figure 4C). Papain inhalation further increased PGEM levels in the BAL fluid of Abx-treated mice, which were significantly decreased by the additional treatment with 5-FC (Figure 4D). Innoculation of Abx-treated commercial mice with human-isolated *Candida* species also increased plasma PGE_2 levels (Figure S4B). These results suggest that fungal overgrowth in the gut increases the levels of systemic and local PGE_2.

Ptges$^{-/-}$ mice are deficient in microsomal prostaglandin E synthase-1 (mPGES-1) responsible for elevation of host-derived PGE_2 in inflamed tissues (Boulet et al., 2004; Church et al., 2012; Claveau et al., 2003). Since fungal overgrowth was not observed in both Abx-treated *Ptges*$^{+/+}$ and *Ptges*$^{-/-}$ mice, exogenous *C. parapsilosis* was orally inoculated to those mice. As a result, fungal colonization in the gut was similarly observed in both Abx-treated *Ptges*$^{+/+}$ and *Ptges*$^{-/-}$ mice (Figure S4C). Abx-treated WT mice showed significantly higher levels of plasma PGEM than did control WT mice (Figures 4C and 4E). Consistent with a previous report (Church et al., 2012), the basal levels of the plasma PGEM were lower in *Ptges*$^{-/-}$ mice than in WT mice (Figure 4E). Importantly, however, Abx treatment also increased the PGEM levels in *Ptges*$^{-/-}$ mice as well as in *Ptges*$^{+/+}$ mice significantly higher than in respective control mice, although the increment of PGEM by the treatment with Abx was smaller in *Ptges*$^{-/-}$ mice than in *Ptges*$^{+/+}$ mice (Figure 4E). Nonetheless, these results suggest that the increased PGEM might not be derived from host cells. Interestingly, treatment with aspirin, an inhibitor of cyclooxygenase (Cox) that converts arachidonic acid into PGG_2 and thus inhibits PGE_2 synthesis, decreased PGE_2 levels in both Abx-treated WT and *Ptges*$^{-/-}$ mice (Figure 4E). Aspirin treatment did not affect fungal overgrowth in both mice (Figure S4C). Accordingly, aspirin also downregulated M2 macrophage marker expression in AMϕs from both Abx-treated *Ptges*$^{+/+}$ and *Ptges*$^{-/-}$ mice (Figures 4F and S4D). Furthermore, treatment with aspirin or celecoxib, a selective Cox-2 inhibitor, decreased papain-induced airway inflammatory cell infiltration in both Abx-treated WT mice and *Ptges*$^{-/-}$ mice (Figures 4G, 4H, and S4E). Collectively, these results suggest that inhibition of PGE_2 synthesis is sufficient to prevent increase in allergic inflammatory cell infiltration in Abx-treated mice.

Intraperitoneal injection of PGE_2 significantly enhanced expressions of M2 macrophage markers in AMϕs (Figure 4I) and increased papain-induced airway inflammatory cell infiltration (Figure 4J). Finally, adoptive transfer of AMϕs stimulated with PGE_2 in vitro into the nasal cavity of naive mice significantly increased papain-induced airway inflammatory cell infiltration compared with the transfer of untreated AMϕs (Figure 4K). Although papain-induced airway inflammatory cell infiltration was suppressed by the treatment with 5-FC in Abx-treated mice that transferred i.n. with untreated AMϕs, Abx-treated mice transferred with PGE_2-stimulated AMϕs showed enhanced papain-induced allergic airway responses regardless of the additional treatment with 5-FC (Figure S4F). Taken together, these results suggest that PGE_2 produced by gut fungi exacerbates eosinophil-mediated airway inflammation by altering the AMϕ phenotype toward M2 in Abx-treated mice.

DISCUSSION

Previous reports showed that treatment with antibiotics promotes overgrowth of fungi in the gut and T cell-dependent airway inflammation by an undetermined mechanism (Noverr et al., 2005). The present study demonstrates that gut fungi alter the macrophage phenotype toward M2 via PGE_2, which can modulate macrophage responses, resulting in enhancement of allergic airway inflammation. A recent report also demonstrated that PGE_2 promotes lung inflammation (Church et al., 2012). We provided evidence that the elevated PGE_2 level induced by gut fungal overgrowth is involved in airway inflammation via macrophage polarization into alternative M2 type. In vitro studies suggested that *Candida* not only produces PGE_2 in itself (Noverr et al., 2001, 2002) but also induces PGE_2 production from host cells (Gagliardi et al., 2010; Lee et al., 2009). We showed that Abx treatment significantly increased the PGEM levels in *Ptges*$^{-/-}$ mice (Figure 4E), suggesting that the increased PGE_2 might not be derived from host cells. We also showed that the elevated plasma PGEM was decreased by treatment with aspirin. Cox inhibitors such as aspirin and celecoxib are able to suppress the PGE_2 synthesis by host cells. However, they are also able to affect the biofilm formation of *Candida*, resulting in the decreased PGE_2 production by *Candida* (Alem and Douglas, 2004). Thus, our data suggest that *C. parapsilosis* in the gut, rather than in host cells, elevated the plasma PGE_2

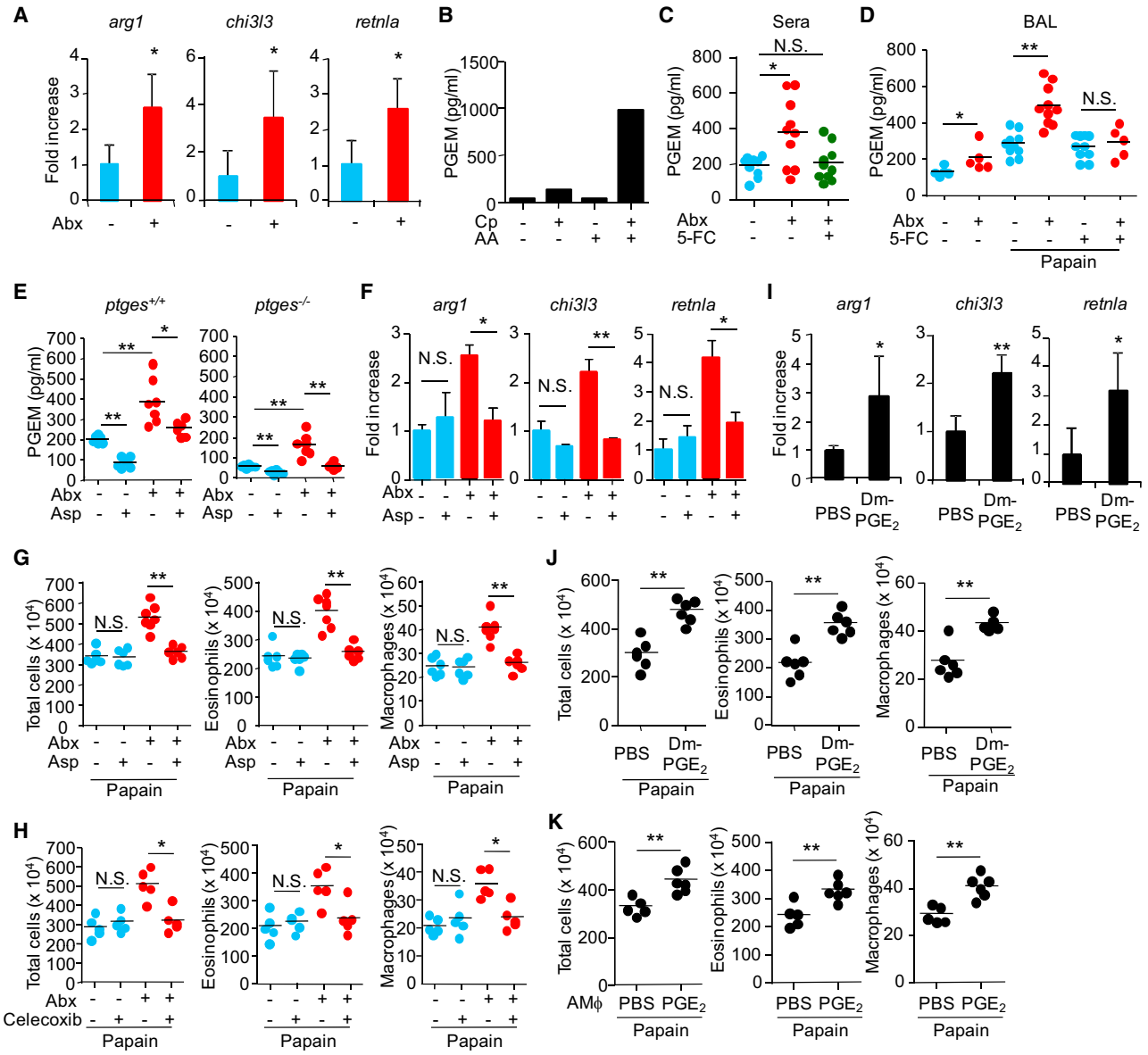

Figure 4. Elevated PGE_2 Levels Associated with Intestinal Fungal Overgrowth Contribute to M2 Polarization and Increase of Allergic Airway Inflammatory Cell Infiltration

(A) Peripheral blood monocytes purified from untreated (–) or Abx (clindamycin + cefoperazone)-treated mice were subjected to real-time RT-PCR (n = 5).

(B) *Candida parapsilosis* (Cp) isolated from mouse feces was cultured or not for 48 hr in the presence or absence of 500 μM arachidonic acid (AA) and assayed for prostaglandin E_2 (PGE_2) metabolite (PGEM) in the culture supernatant.

(C) Mice were left untreated or treated orally with either Abx, or Abx plus 5-FC for 2 weeks and analyzed for serum PGEM levels.

(D) Abx-treated or untreated (–) mice were challenged i.n. with PBS or papain every 3 days a total of five times. One day after the final challenge, mice were analyzed for PGEM concentration in BAL fluids.

(E–G) *Ptges*$^{+/+}$ (E–G) or *Ptges*$^{-/-}$ (E) mice were left untreated or treated orally with either Abx, aspirin (Asp), or both for 2 weeks and analyzed for serum PGEM levels (E) and expression of M2 macrophage marker genes in AMϕs by real-time RT-PCR (F) (n = 5). The mice were then challenged i.n. with papain and analyzed, as described in Figure 1 (G).

(H) Mice were left untreated or treated orally with either Abx, celecoxib, or both for 2 weeks and then challenged i.n. with papain and analyzed, as described in Figure 1.

(I) Mice were intraperitoneally injected with dimethyl PGE_2 (Dm-PGE_2) or PBS every day a total of five times. One day after the final challenge, mice were analyzed for expression of M2 macrophage marker genes in AMϕs (n = 5).

(J) Mice were challenged i.n. with papain every 3 days a total of five times. The mice were also injected i.p. with dimethyl PGE_2 (Dm-PGE_2) or PBS 1 day before each papain challenge a total of five times. One day after the final challenge with papain, mice were analyzed for BAL cells, as described in Figure 1.

(K) AMϕs purified from naive mice were cultured in the presence or absence of PGE_2 for 24 hr and transferred i.n. to naive mice. The mice were then challenged i.n. with papain and analyzed for BAL cells, as described in Figure 1. Results are representative of three independent experiments. *$p < 0.05$; **$p < 0.01$. Error bars, SD. N.S., not significant. See also Figure S4.

level in Abx-treated mice, which induced the polarization of macrophage toward M2 type and then exacerbated allergic airway inflammation.

Candida overgrowth in the intestine can be caused by excessive dietary intake of sugar or carbohydrates (Vargas et al., 1993), as well as by antibiotic treatment (Giuliano et al., 1987; Samonis et al., 1990). In our present study, only certain antibiotics could promote fungal overgrowth in the gut (Figure 3A), suggesting that specific commensal bacteria have an ability to prevent fungal colonization. *Lactobacillus* can prevent colonization of *Candida* through displacement of the fungi from the epithelial layer of stomach (Savage, 1969). Furthermore, penicillin treatment reduces *Lactobacillus* populations and promotes fungal colonization of the gastric epithelium (Savage, 1969). Indeed, we observed that treatment with the antibiotics clindamycin and cefoperazone significantly reduced several commensal bacteria, including *Lactobacillus*. In contrast, treatment with metronidazole, which did not induce the gut fungal overgrowth, did not affect the number of *Lactobacillus* (Figure S3). Further work is needed to reveal adaptation of fungi to the host environment during antibiotic treatment. Several reports demonstrated that patients with allergic diseases exhibited fungal overgrowth in the intestine (Buslau et al., 1990; Pawlik et al., 2002). Therefore, control of fungal overgrowth associated with gut dysbiosis may be important for the treatment of, or protection against, allergic inflammatory disorders.

EXPERIMENTAL PROCEDURES

Mice

We used C57BL/6J mice derived from a colony maintained at the University of Tsukuba Animal Research Center or, in some experiments, from Clea Japan. *Rag-1*$^{-/-}$ mice were purchased from Jackson Laboratories. *Ptges* $^{\pm}$ mice on the C57BL/6 background were kindly provided by Leslie Crofford (Vanderbilt University). *Ptges*$^{+/+}$ and *Ptges*$^{-/-}$ mice derived from the *Ptges*$^{+/-}$ parents were bred at the University of Michigan (Mason et al., 2013). All experiments were performed in accordance with the guidelines of the animal ethics committee of the University of Tsukuba Animal Research Center and the University of Michigan.

Antibiotics, Aspirin, Celecoxib, and SAP

Mice were provided with autoclaved drinking water supplemented with clindamycin (0.5 mg/mL, Tokyo Chemical), cefoperazon (0.4 mg/mL, Toronto Research Chemicals), ampicillin (1 mg/mL, Sigma-Aldrich), metronidazole (1 mg/mL, Sigma-Aldrich), streptomycin (5 mg/mL, Wako Pure Chemical), 5-fluorocytosine (2 mg/mL, Tokyo Chemical), aspirin (50 μg/mL, Tokyo Chemical), or celecoxib (1 μg/mL, Tokyo Chemical). Antibiotic treatment was started 2–4 weeks before inhalation of allergens and continued for the duration of the experiment. Mice were intranasally treated with a drop of SAP (100 μg in PBS per mouse, Calbiochem) solution every 3 days a total of five times.

Papain-Induced Airway Inflammation

Every 3 days, mice were subjected to intranasal inhalation of papain (150 μg in PBS per mouse, Wako Pure Chemical, >0.5 units/g) by placing a drop in the nasal cavity of the mouse, a total of five challenges. BAL was performed three times by instillation of 1 ml of PBS through a tracheal cannula. Differential cell counts were performed on cytospins stained with Wright's stain solution (Muto Pure Chemicals).

Fungal Numbers in Feces or Lungs

Fecal pellets or lungs were collected from individual mice, homogenized in cold PBS, and plated at serial dilutions onto Sabouraud dextrose agar containing 50 μg/ml gentamycin and 50 μg/ml chloramphenicol, and the number of CFUs was determined after overnight incubation at 37°C. Detection limit was 10^2 CFU/g feces.

Candida Inoculation

Candida species were derived from a single colony picked from Sabouraud dextrose GC agar plate streaked with mouse or human samples. *Candida parapsilosis* that we isolated from mouse feces or *C. albicans*, *C. glabrata*, and *C. tropicalis* isolated from patients were grown to stationary phase (48 hr) at 37°C in Sabouraud dextrose broth with shaking. For infection of mice, the cultures were washed and diluted to 5×10^7 CFU/mL in sterile PBS. Mice obtained from Clea Japan or *Ptges*$^{+/+}$ and *Ptges*$^{-/-}$ mice were inoculated with *Candida* (10^7 CFU in 200 μL) by oral gavage using mouse feeding needles on day 1 of antibiotic treatment.

Cytokine, Chemokine, and Prostaglandin E_2 Levels

Cytokine and chemokine were measured with enzyme-linked immunosorbent assay kits (R&D Systems). Serum levels of 13,14-dihydro-15-keto PGE_2 (prostaglandin E_2 metabolite, PGEM) were measured with Prostaglandin E Metabolite EIA Kit (Cayman Chemicals) in accordance with the manufacturer's instructions.

cDNA Synthesis and Real-Time RT-PCR

Total RNA was extracted with a Total RNA Kit I (OMEGA Bio-Tek. Inc.), and cDNA was synthesized by using a High Capacity RNA-to-cDNA Kit (Applied Biosystems). Real-time PCR was performed with SYBR Green master mix (Applied Biosystems). Expression of each target gene was normalized against that of β-actin.

PGE_2 and Treatment

Dimethyl PGE_2 (dm-PGE_2, Calbiochem) (50 μg/mL per mouse) was given intraperitoneally once a day for 5 days, and alveolar macrophages were purified 1 day after final dm-PGE_2 treatment for real-time RT-PCR analysis. Dm-PGE_2 (50 μg/mL in PBS per mouse) was also given intraperitoneally 1 day before each papain treatment every 3 days a total of five challenges. For adoptive transfer, alveolar macrophages purified from naive mice were incubated with PGE_2 (50 ng/mL, Tokyo Chemical) for 24 hr.

Adoptive Transfer of Macrophages

Alveolar macrophages were obtained from naive or antibiotic-treated mice after BAL with PBS. BBMs, generated as described (Celada et al., 1984), were cultured for 24 hr in the presence of either IFN-γ (20 ng/mL) or IL-4 (10 ng/mL), IL-13 (10 ng/mL), and IL-10 (20 ng/mL; all from R&D Systems) for differentiation into M1 or M2 macrophages, respectively. Macrophages (2×10^5 cells) were transferred intranasally into naive mice 8 hr before the first papain inhalation.

Statistical Analysis

Statistical significance between groups was determined by Mann-Whitney test. In vivo and in vitro mRNA expression was analyzed with two-tailed t test with unequal variance (Aspin-Welch's t test). Differences were considered significant when p values were <0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.12.010.

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Supplemental Information

Gut Dysbiosis Promotes M2 Macrophage Polarization and Allergic Airway Inflammation via Fungi-Induced PGE_2

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SUPPLEMENTARY FIGURES

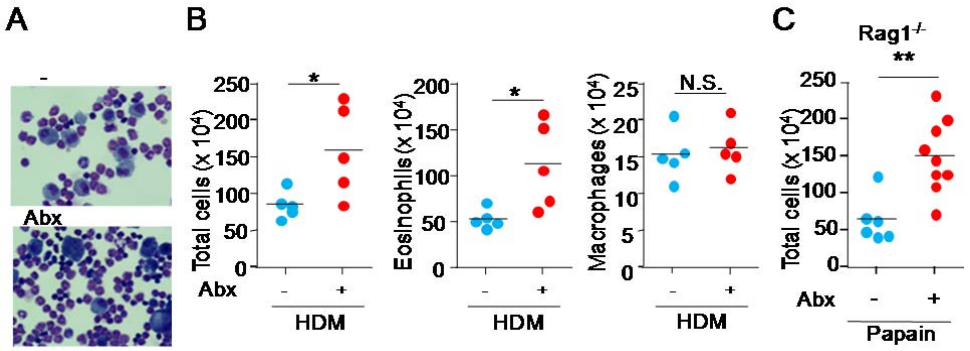

Figure S1. Antibiotics treatment exacerbates allergic airway inflammatory cell infiltration. Related to Figure 1.

Wild type (A, B) or Rag-1$^{-/-}$ mice (C) were left untreated (-) or treated with antibiotics (clindamycin + cefoperazone) for 2 weeks and then challenged i.n. with house dust mite (HDM) extract five times per week for 4 consecutive weeks or papain every 3 days for a total of five times for analysis of BAL cells. One day after the final challenge, mice were analyzed for morphology of BAL cells (Wright's stain, original magnification x 1000) (A), total and differentiated cell counts in BAL (B, C). Means ± SD are shown. *$P < 0.05$. **$P < 0.01$. N.S. denotes not significant.

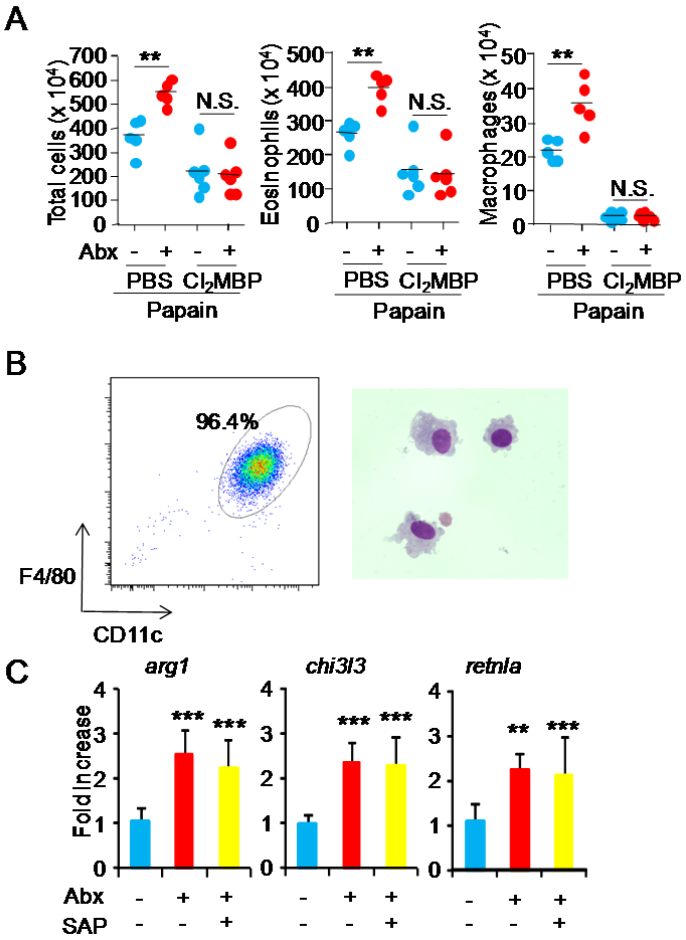

Figure S2. Alveolar M2 Mϕs are involved in Abx-induced allergic airway inflammation. Related to Figure 2.

(A) Abx-treated (+) or untreated (-) mice were administered i.n. with clodronate-liposomes (Cl_2MBP) or PBS-liposomes one day before each papain challenge every 3 days for a total of five times, and then analyzed for BAL cells one day after the final challenge, as described in Fig 1.

(B) Adherent cells isolated from BAL fluid were stained with anti-CD11c and F4/80 mAbs and analyzed by flow cytometry. The alveolar macrophage population ($CD11c^{+}F4/80^{+}$) was determined and analyzed for morphology (Wright's stain, original magnification x 1000).

(C) Mice were left untreated (-) or treated with antibiotics (clindamycin + cefoperazone) (+) and administered i.n. with SAP (+) or PBS (-) every 3 days for a total of five challenges. One day after the final SAP or PBS treatment, peripheral blood monocytes were subjected to real-time RT-PCR (n=5). Results are representative of three independent experiments. Means ± SD are shown.

$^{**}P < 0.01$, $^{***}P < 0.001$. N.S. denotes not significant.

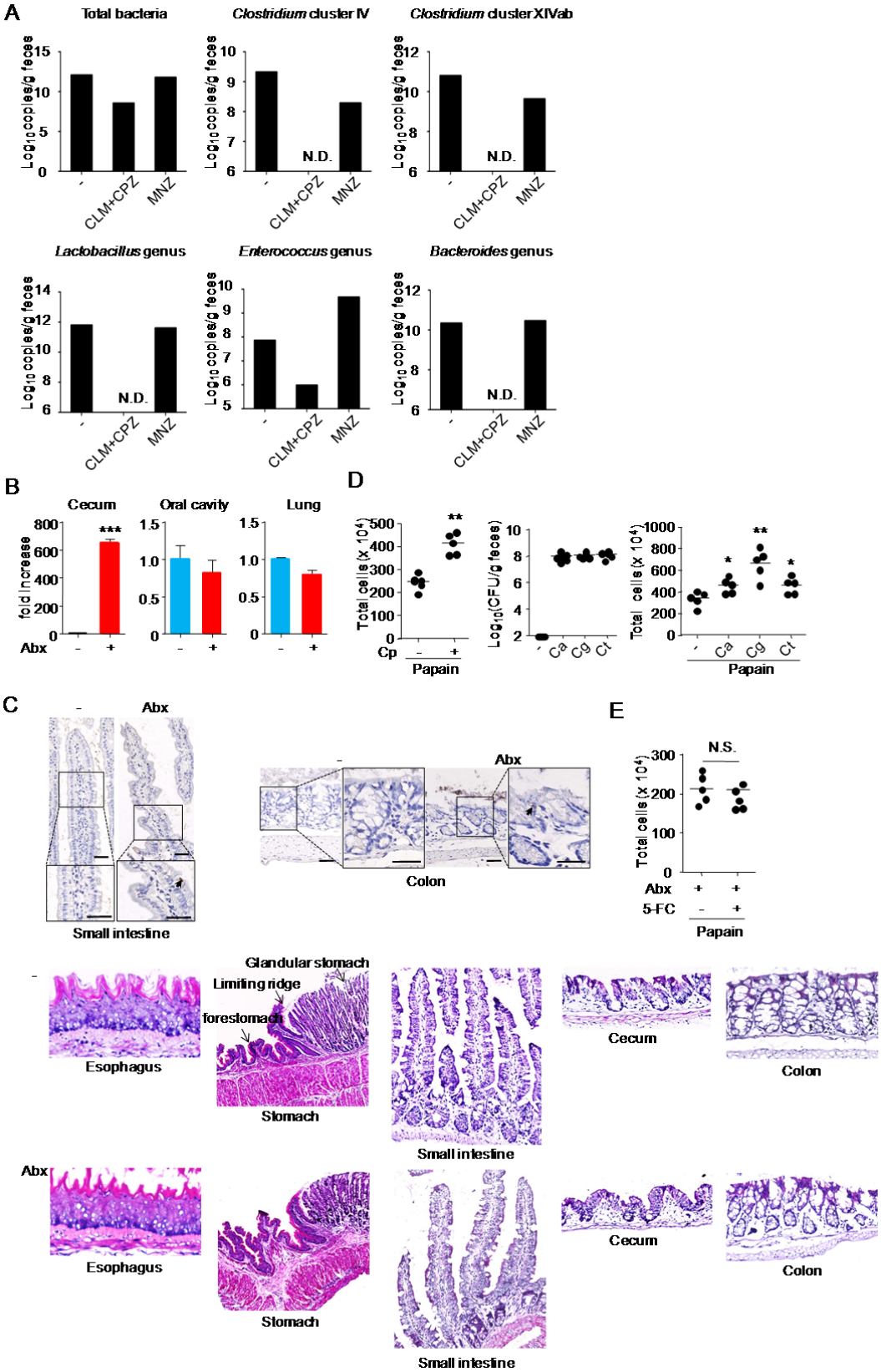
A
Total bacteria
Clostridium cluster IV
Clostridium cluster XIVab
Lactobacillus genus
Enterococcus genus
Bacteroides genus
Log10 copies/g feces
N.D.
-
CLM+CPZ
MNZ
B
Cecum
Oral cavity
Lung
fold increase
Abx
C
Small intestine
Colon
D
Total cells (x 10⁴)
Log10(CFU/g feces)
Cp
Papain
Ca
Cg
Ct
E
N.S.
5-FC
Esophagus
Glandular stomach
Limiting ridge
forestomach
Stomach
Small intestine
Cecum
Colon

Figure S3. Fungal overgrowth in the gut is involved in Abx-induced allergic airway inflammation. Related to Figure 3.

(A) Bacterial DNA in the feces were extracted from mice untreated (-) or treated with clindamycin + cefoperazone (CLM +CPZ) or metronidazole (MNZ) for 2 weeks and subjected to real-time PCR to analyze the copy number of total bacteria and each bacterial species by using specific primers to each 16S rDNA.

(B, C) Mice were left untreated (-) or treated with antibiotics (clindamycin + cefoperazone) for 2 weeks. Mice were then analyzed for prevalence of fungi in cecum, oral cavity, and lung by quantitative RT-PCR for ITS1-2 rDNA (B). Means ± SD are shown (B). Mice were also analyzed for histology of the organs indicated with DAB or Hematoxylin & Eosin staining (C). Arrows indicate *Candida* in the lamina propria of the small intestine and the colon. Scale bar, 10 μm.

(D) (Left panel) Mice obtained from a commercial supplier (Clea Japan) were left untreated (-) or innoculated p.o. with *C. parapsilosis* (Cp) (+) after Abx treatment (clindamycin + cefoperazone). Mice were then subjected to intranasal inhalation of papain (150 μg/mouse) every 3 days for a total of five challenges. One day after the final challenge, total cell numbers in the BAL fluid were counted . (Center and right panels) Mice obtained from a commercial supplier (Clea Japan) were left untreated (-)

or innoculated p.o. with human-isolated *Candida* species (Ca: *C. albicans*; Cg: *C. glabrata*; Ct*: C. tropicalis*) after Abx-treatment (clindamycin + cefoperazone). *Candida* colonization was measured as CFU/g of feces (center panel). Mice were then treated with intranasal inhalation of papain and analyzed for total cell numbers in the BAL fluid, as described above (right panel).

(E) Mice obtained from a commercial supplier (Clea Japan) were treated with either Abx (clindamycin + cefoperazone) or Abx plus 5-FC for 2 weeks and then challenged i.n. with papain and analyzed for BAL cell number, as described above. Results are representative of three independent experiments.

*P < 0.05, **P < 0.01, ***P < 0.001. N.S. denotes not significant.

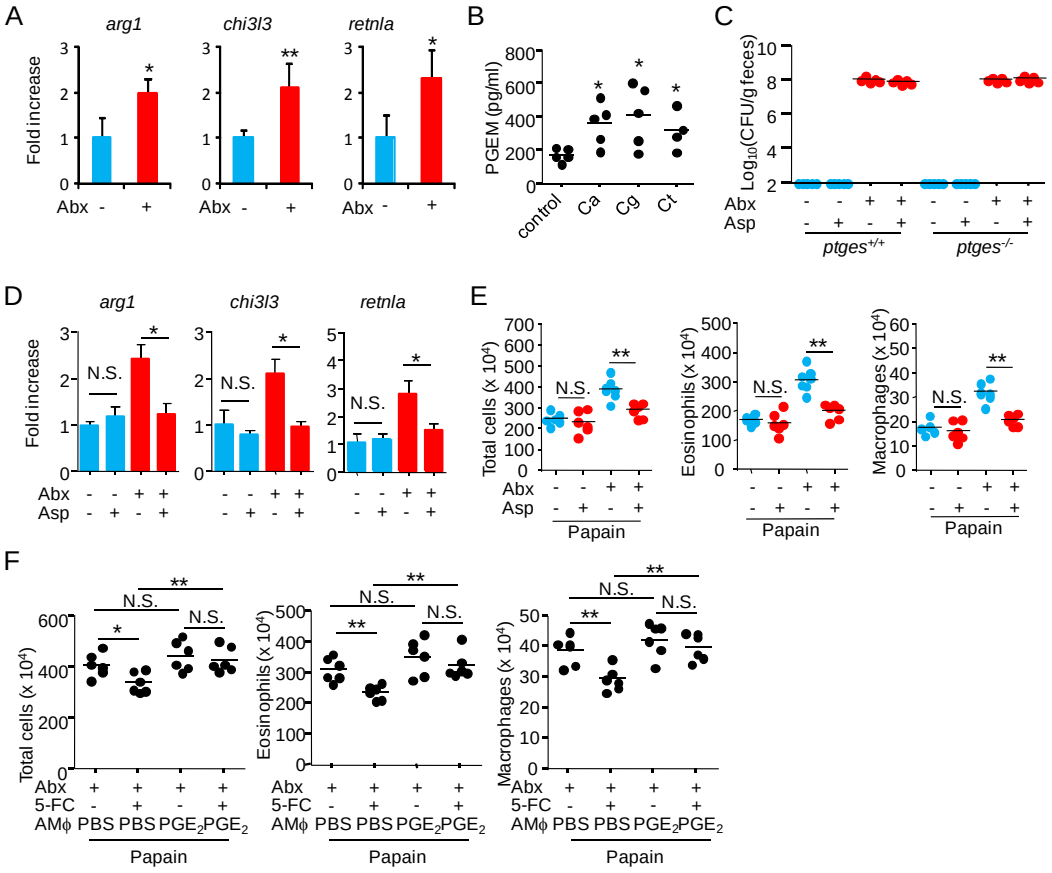

Figure S4. PGE_2 associated with fungal overgrowth is involved in Abx-induced allergic airway inflammation. Related to Figure 4.

(A) Alveolar macrophages isolated from naïve mice were cultured for 3 days in the presence of 40 % serum obtained from mice before (-) or 14 days after (+) treatment with Abx (clindamycin + cefoperazone) and subjected to real-time RT-PCR (n=4). Means ± SD are shown.

(B) Mice obtained from a commercial supplier (Clea Japan) were left untreated (control) or innoculated p.o. with human-isolated *Candida* species (Ca: *C. albicans*; Cg: *C. glabrata*; Ct*: C. tropicalis*) after Abx treatment (clindamycin + cefoperazone). Two weeks after the fungal innoculation, the mice were analyzed for serum PGEM levels.

(C-E) *Ptges*$^{+/+}$ or *Ptges*$^{-/-}$ mice were left untreated or treated orally with Abx (clindamycin + cefoperazone) or Abx plus aspirin for 2 weeks. Mice were then analyzed for fungal colonization in the feces (C) and for expression of M2 macrophage marker genes in AMϕs from the *Ptges*$^{-/-}$ by real-time RT-PCR (D) (n=5 in each). The *Ptges*$^{-/-}$ mice were then challenged i.n. with papain and analyzed for BAL cell numbers, as described above (E). Means ± SD are shown.

(F) AMϕs purified from naive mice were cultured in the presence of PBS or PGE_2 for 24 h and transferred i.n. into mice that had been treated with Abx (clindamycin + cefoperazone) alone or Abx plus 5-FC for 2 weeks. The mice were then challenged i.n. with papain and analyzed for BAL cells, as described above. Results are representative of three independent experiments.

*$P < 0.05$, **$P < 0.01$. N.S. denotes not significant.

Supplemental Experimental Procedures

House dust mite–induced airway inflammation

Mice were treated intranasally with HDM extract (10 μg in PBS per mouse, LSL, Tokyo, Japan) in saline, or with saline alone, five times per week for 4 consecutive weeks.

In vivo depletion of alveolar macrophages

For alveolar macrophage depletion, clodronate liposomes (liposome-encapsulated dichloromethylene bisphosphonate: Cl_2MBP) or control PBS liposomes (Encapsula NanoSciences, Nashville, TN) were prepared as previously described (Van Rooijen, 1989). Mice were treated intranasally with 30 μL liposomes 1 day before each papain treatment every 3 days for a total of five times.

Fungal rDNA gene analysis

Cecum, oral cavity (tongue and cheek pouches), and lung samples were incubated in 50 mM Tris buffer (pH7.5) containing 1 mM EDTA, 0.2% β-mercaptoethanol (Sigma) and 1000 U/ml of lyticase (Sigma) at 37 °C for 30 min and tissue and fungal genomic DNA

were isolated using QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions. For detection of tissue-associated fungi, quantitative PCR was performed on DNA isolated from tissues using fungal-specific primers (Iliev et al., 2012). Relative quantity was calculated by the ΔCt method and normalized to the amount of β-actin (Iliev et al., 2012).

Bacterial 16S rDNA gene analysis

Bacterial DNA in the feces was extracted by using Magtration® System 12GC (Techno Suruga Laboratory Co., Ltd., Shizuoka, Japan). Real-time PCR was performed to analyze the copy number of total bacteria, *Clostridium clusters IV* and *XIVab*, *Lactobacillus genus, Enterococcus genus, Bacteroides,* and *Enterobacteriaceae,* by using specific primers to each 16S rDNA (Techno Suruga Laboratory Co., Ltd.).

In vitro serum treatment

Alveolar macrophages obtained from naïve mice were cultured for 3 days in the presence of 40% serum collected from mice before or 14 days after treatment with antibiotics (clindamycin + cefoperazone).