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Trefoil factor 2 rapidly induces interleukin 33 to promote type 2 immunity during allergic asthma and hookworm infection

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The molecular mechanisms that drive mucosal T helper type 2 (TH2) responses against parasitic helminths and allergens remain unclear. In this study, we demonstrate in mice that TFF2 (trefoil factor 2), an epithelial cell–derived repair molecule, is needed for the control of lung injury caused by the hookworm parasite Nippostrongylus brasiliensis and for type 2 immunity after infection. TFF2 is also necessary for the rapid production of IL-33, a TH2-promoting cytokine, by lung epithelia, alveolar macrophages, and inflammatory dendritic cells in infected mice. TFF2 also increases the severity of allergic lung disease caused by house dust mite antigens or IL-13. Moreover, TFF2 messenger RNA expression is significantly increased in nasal mucosal brushings during asthma exacerbations in children. These experiments extend the biological functions of TFF2 from tissue repair to the initiation and maintenance of mucosal TH2 responses.

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The mechanisms that regulate IL-33 release and whether IL-33 functions as a cytokine or nuclear factor remain unclear (Carriere et al., 2007; Ohno et al., 2009). Although epithelial cell death by necrosis may serve as one mechanism for IL-33 release, recent evidence shows that viral infection can result in IL-33 production from alveolar macrophages (Chang et al., 2011). Furthermore, IL-33 administration to mice infected with the GI nematode *Trichuris muris* promotes worm expulsion (Humphreys et al., 2008), but the endogenous mechanisms that regulate IL-33 production during worm infection are currently unknown.

Trefoil factor family peptides (TFF1–3) are protease-resistant mediators that serve critical roles in mucosal barrier function and repair (Podolsky, 1997; Paulsen et al., 2008). These proteins are constitutively released within the mucus gel layer that covers ocular, urinary, respiratory, and GI surfaces (Taupin and Podolsky, 2003). Furthermore, the trefoil motif, which resembles a three-leaf clover, is highly conserved among TFF proteins from frogs to humans (Taupin and Podolsky, 2003). TFFs are produced from epithelia, fibroblasts, and tissue macrophages within hours of injury to promote epithelial cell restitution through mechanisms that may require CXCR4, the putative TFF2 receptor (Dubeykovskaya et al., 2009). As such, TFF2 promotes organ regeneration and healing in several injury models (Farrell et al., 2002; Poulsen et al., 2005; Fox et al., 2007; Kurt-Jones et al., 2007). TFF2 expression is up-regulated during tissue injury associated with allergic lung inflammation in mice through STAT-6–dependent and –independent mechanisms (Nikolaidis et al., 2003, 2006). However, its absence did not affect lung inflammation or mucus metaplasia (Nikolaidis et al., 2006). Thus, its biological importance in the context of type 2 immunopathologies is not well understood.

This study investigated whether TFF2 served any role in the development of allergic lung disease or after infection with the hookworm *Nippostrongylus brasiliensis*. Our results indicate that TFF2 (a) promotes type 2 lung immunopathology induced by allergen or IL-13, (b) controls the extent of lung injury caused by migratory infectious stage larvae (L3), and (c) modulates early Th2 development. This mechanism involves rapid, coordinated IL-33 production from lung epithelia, alveolar macrophages, and inflammatory DCs. IL-33 induction requires the putative TFF2 receptor, CXCR4, but occurs independently of the TLR adaptor molecule MyD88. Collectively, these experiments demonstrate an important role for TFF2 in the regulation of IL-33 release at mucosal surfaces and the development of type 2 immune responses.

### RESULTS

#### TFF2 expression is a central feature of asthma in humans and mice

TFF2 expression is induced within airway epithelial cells of mice challenged with allergen or type 2 cytokines (Nikolaidis et al., 2003; Follettie et al., 2006). To determine whether increased TFF2 expression was a feature of human asthma, we obtained nasal mucosa biopsies from pediatric patients experiencing asthma exacerbations and compared their TFF2 messenger RNA (mRNA) levels to nonasthmatic control subjects. TFF2 was significantly elevated in asthma patients over controls (Fig. 1 A), which prompted us to evaluate the biological role of TFF2 in the pathogenesis of murine asthma driven by house dust mite (HDM) antigens or IL-13 administration. Exposure of TFF2−/− mice to HDM antigens caused significantly less AHR (Fig. 1 B) and reduced the numbers of eosinophils and neutrophils recruited into the bronchoalveolar lavage (BAL), as compared with HDM-sensitized WT mice (Fig. 1 C). There were no differences between naive WT and TFF2−/− mice in the numbers of eosinophil or neutrophil precursors within the BM (not depicted). This may suggest that TFF2−/− mice have the...
normal potential to produce granulocytes but show impaired allergic airway inflammation in response to HDM antigens.

We also investigated whether the TFF2 deficiency modulated the development of type 2 allergic lung disease in an IL-13–driven model of murine asthma (Wills-Karp et al., 1998). rIL-13–treated WT C57BL/6 mice developed robust AHR, goblet cell metaplasia, and eosinophilic airway inflammation; however, these responses were significantly attenuated in TFF2−/− mice (Fig. 2 A–C). Moreover, IL-13–treated TFF2−/− mice showed impaired induction of lung mRNA transcripts for the goblet cell–specific genes MUC5AC, CLCA3, and RETNLB (Fig. 2 D), as well as the alternatively activated macrophage marker RETNLA (Fig. 2 D). Collectively, these findings suggest that TFF2 deficiency protects mice against experimental asthma induced by allergen or IL-13 and that increased TFF2 expression in asthma patients is associated with disease exacerbation.

**TFF2 regulates hookworm-mediated lung injury**

TFF2 production can be rapidly induced, within minutes of mucosal injury (Taupin et al., 1999), as well as during allergen-induced inflammation (Taupin et al., 1999; Nikolaidis et al., 2003). We investigated whether TFF2 regulated tissue repair and/or T12-dependent host immunity against the parasitic helminth *N. brasiliensis*. In this model, migratory L3 enter the lung tissues between 0 and 2 d after infection and cause hemorrhagic tissue injury that resolves over several weeks around migrating parasites at day 1 after inoculation, followed by a progressive closure of the damaged areas of lung tissue at days 3 and 7 after inoculation (Fig. 3 B). In contrast, WT mice developed focal hemorrhagic lesions around migrating parasites at day 1 after inoculation, followed by a progressive closure of the damaged areas of lung tissue at days 3 and 7 after inoculation (Fig. 3 B). To quantitatively measure lung injury, the numbers of erythrocytes within the BAL fluid were counted from cohorts of WT and TFF2−/− mice between days 0 and 7 after infection (Meyer et al., 1998). Fig. 3 C shows that from as early as day 1 after infection, TFF2−/− lungs had significantly greater numbers of erythrocytes than the WT strain and remained elevated over the course of observation. This was consistent with the histological evidence of excessive lung hemorrhage in the absence of TFF2.

TFF2 was also necessary for chronic lung repair after *N. brasiliensis* infection–induced injury. At day 28 after infection, when parasites were no longer present (not depicted), TFF2−/− animals developed excessive lung hemorrhage at day 1 and enlarged distal airspaces from alveolar wall destruction at days 3 and 7 after infection (Fig. 3 B). Subsequently, by day 3 after infection, >90% of worms have exited the lung and enter the small intestine (Herbert et al., 2009). As shown in Fig. 3 A, TFF2 mRNA levels increased over threefold within the lung at day 1 and within the jejunum at day 3 after infection of WT C57BL/6 mice with 500 *N. brasiliensis* L3. This expression pattern for TFF2 was consistent with the kinetics of parasite migration through the host and suggested a potential role for this molecule in each mucosal compartment.

To investigate whether TFF2 served a critical function within hookworm-infected lungs, we compared lung histology between infected WT and TFF2−/− strains. Although naive TFF2−/− lung tissue showed moderately enlarged alveolar space at baseline, infected TFF2−/− animals developed excessive lung hemorrhage at day 1 and enlarged distal airspaces from alveolar wall destruction at days 3 and 7 after infection (Fig. 3 B). In contrast, WT mice developed focal hemorrhagic lesions around migrating parasites at day 1 after inoculation, followed by a progressive closure of the damaged areas of lung tissue at days 3 and 7 after inoculation (Fig. 3 B). To quantitatively measure lung injury, the numbers of erythrocytes within the BAL fluid were counted from cohorts of WT and TFF2−/− mice between days 0 and 7 after infection (Meyer et al., 1998). Fig. 3 C shows that from as early as day 1 after infection, TFF2−/− lungs had significantly greater numbers of erythrocytes than the WT strain and remained elevated over the course of observation. This was consistent with the histological evidence of excessive lung hemorrhage in the absence of TFF2.

**Figure 2. TFF2 is required for IL-13–driven allergic airway inflammation and airway hyperresponsiveness.** (A) Airway pressure time index (APTI) of WT and TFF2−/− mice was measured 8 d after administration of rIL-13 (5 µg/mouse) or vehicle (1× PBS). Data represent mean ± SE of eight mice/group. (B) Quantification of PAS-positive cells within airway epithelium at day 8 after injection of rIL-13 or PBS. Data represent mean ± SE of eight mice/group. (C) Numbers of epithelial, macrophages, eosinophils, and lymphocytes in BAL fluid at day 8 after injection of rIL-13 or PBS. Data represent mean ± SE of eight mice/group. (D) Quantification of mRNA transcripts encoding RETNLB, MUC5AC, CLCA3, and RETNLA in the lung at day 8 after injection of rIL-13 or PBS. Data represent mean ± SE of four to six mice/group. Data are representative of four independent experiments [***, P < 0.001].
Thus, immunity against *N. brasiliensis* adult worms in the intestine is accompanied by increased TFF2 production. In the absence of TFF2, more worm eggs are passed into the environment and parasites reside within their hosts for longer periods of time.

TFF2 promotes an early Th2 response during *N. brasiliensis* infection
IL-4 and IL-13 are both necessary and sufficient for host protection against most GI nematodes (Finkelman et al., 2004) and most allergic lung immunopathologies (Wills-Karp et al., 1998). Given that TFF2−/− mice had significant defects in both worm clearance and allergic lung pathology, we postulated that TFF2 might promote type 2 cytokine production. Consistent with this hypothesis, TFF2−/− mice displayed defects in both the early (lung) and late (intestinal) stages of *N. brasiliensis* infection. In the early phase, TFF2−/− mice expressed fewer IL-4 and IL-13 lung mRNA transcripts than WT mice at day 3 after inoculation (Fig. 5 A). Intracellular IL-13 staining of TCR-β+CD4+ cells within the lung draining LNs (Fig. 5 B and Fig. S1) revealed that at day 3 after infection, TFF2−/− mice produced three- to fourfold less IL-13 than infected WT animals (Fig. 5 B and C), yet there were no obvious differences in T cell recruitment to the LN between these strains.

TFF2 promotes early Th2 response during *N. brasiliensis* infection
During the later stages of *N. brasiliensis* infection, TFF2−/− mice secreted less IL-4 than WT animals at day 7 after infection (Fig. 5 D). Also, compared with WT controls, TFF2−/− mice had significantly fewer intestinal mRNA transcripts for RELM-β (Resistin-like molecule β), an IL-4/IL-13–dependent goblet cell–derived cytokine which is both necessary and sufficient for the normal expulsion of *N. brasiliensis* adult worms from the GI tract (Fig. 5 E; Herbert et al., 2009). However, these defects were transient, as TFF2−/− mice produced IL-4 and expressed RELM-β transcripts at levels equivalent to infected WT mice by day 12 after infection (Fig. 5 E), which was closely associated with the ability of TFF2−/− mice to eventually clear worm infection by day 14 after infection.

Based on these observations, we hypothesized that the delay in IL-4 secretion, RELM-β production, and worm clearance in TFF2−/− mice resulted from a proximal defect in type 2 cytokine production. To directly test this possibility, we administered rIL-4 to mice in the form of IL-4/anti–IL-4 mAb complexes (IL-4C; Finkelman et al., 1993) to infected WT or TFF2−/− mice on days 0 and 4 followed by quantitation of adult worm numbers at day 8 after infection. Means ± SE of 8–10 mice/group are shown. n.d., not detected.

**Figure 4.** TFF2 promotes spontaneous expulsion and termination of egg production during *N. brasiliensis* infection. (A) *N. brasiliensis* intestinal worm numbers recovered from WT versus TFF2−/− mice at the indicated time points after a primary infection. Means ± SE of 8–10 mice/group are shown. n.d., not detected. (B) *N. brasiliensis* fecal egg numbers in WT versus TFF2−/− mice after a primary infection. Means ± SE of 8–10 mice/group are shown. (C) Immunostaining for Tff2 protein (magenta precipitate indicated by arrows) in paraffin-embedded sections of jejunum from *N. brasiliensis*-infected WT (top) or TFF2−/− (bottom) mice at day 7 after infection. Bars, 70 µm. Data are representative of four independent experiments (*, P < 0.05; **, P < 0.01).
Figure 5. Early defect Th2 expansion and RELM-β expression in TFF2−/− mice is responsible for delayed immunity against N. brasiliensis infection. (A) IL-4 and IL13 mRNA transcripts in naive WT and TFF2−/− mice or 3 d after N. brasiliensis infection. Means ± SE of five to six mice/group are shown. (B) Representative dot plot showing IL-13–positive CD4+ lymphocytes within the LN of naive WT and TFF2−/− mice or 3 d after N. brasiliensis infection. Four individual mice/group were analyzed for infected samples, whereas naive LNs were pooled from two to three mice. (C) Mean percentage ± SE of IL-13–positive CD4+ lymphocytes from the experiment described in B. (D) Serum IL-4 abundance, as determined by in vivo cytokine capture assay at 0, 7, and 12 d after infection. Means ± SE of five to six mice/group are shown. (E) Intestinal RELM-β mRNA transcript levels at 0, 7, and 12 d after infection. Means ± SE of five to six mice/group are shown. (F) N. brasiliensis intestinal worms recovered from intestinal lumen on day 8 from WT versus TFF2−/− mice that were injected with vehicle or rIL-4/anti–IL-4 complexes (IL-4C) at days 0 and 4. Means ± SE of five to six mice/group are shown. n.d., not detected. (G) Immunofluorescence staining for RELM-β within the jejunum of mice from the experiment described in F. Cell nuclei stained with DAPI. Bars, 50 µm. Data are representative of three to four independent experiments (*, P < 0.05; **, P < 0.01).
macrophages also up-regulated IL-33 production at both days 1 and 3 after infection. In contrast, these IL-33+ alveolar macrophages were significantly less abundant within TFF2−/− lung tissues at both time points (Fig. 6, J and K). Overall, this indicates that TFF2 promotes IL-33 mRNA and protein expression in lung epithelia and alveolar macrophages within the first 3 d after hookworm infection.

Given that TFF2 regulated IL-33 from lung epithelia, we investigated whether it was also important for IL-33 production from alveolar macrophages. Our gating strategy defined alveolar macrophages as CD11b−, CD11c+, CD317−/+, Ly6G/C−, and CD103− (Fig. 6 H; Lambrecht and Hammad, 2009). Results shown in Fig. 6 I demonstrate that WT macrophages also up-regulated IL-33 production at both days 1 and 3 after infection. In contrast, these IL-33+ alveolar macrophages were significantly less abundant within TFF2−/− lung tissues at both time points (Fig. 6, J and K). Overall, this indicates that TFF2 promotes IL-33 mRNA and protein expression in lung epithelia and alveolar macrophages within the first 3 d after hookworm infection.

TFF2 deficiency impairs IL-33 production from inflammatory DCs, and IL-33 is necessary for host immunity against N. brasiliensis

Inflammatory DCs have been shown as critical drivers for the in vivo differentiation of Th2 cells (Hammad et al., 2010; Phythian-Adams et al., 2010). Therefore, it was important to
determine whether TFF2 also regulated IL-33 production in this important cell population. Inflammatory DCs within mediastinal LNs (MLNs) were defined as CD11b+CD11c+MHC class II+ and FcεR1+ (Fig. 7 A; Hammad et al., 2010). Inflammatory DCs that coexpressed ST2 and IL-33 increased five-fold in percentage by day 3 after infection within WT mice, but this population was distinctly IL-33neg within TFF2−/− MLNs (Fig. 7, B and C). We did not detect these differences in naive mice (Fig. 7 B) or at day 1 after infection (not depicted).

Although ST2+IL-33+ inflammatory DCs were a relatively small population within the MLNs of N. brasiliensis–infected WT mice, intact ST2 signaling in the host served a critical role for the development of host immunity. Data shown in Fig. 8 indicate that treatment of WT C57BL/6 mice with two 750-µg doses of neutralizing anti–ST2 mAb significantly increased both parasite egg production (Fig. 8 A) and the numbers of adult hookworms recovered from the intestine at day 9 after infection (Fig. 8 B). Thus, TFF2 promotes IL-33 production from inflammatory DCs during the early stages of hookworm infection, and neutralization of ST2 (IL-33 receptor) impairs the normal expulsion of N. brasiliensis adult worms from infected hosts.

Given this novel role for TFF2 in the rapid production of IL-33 from a variety of cellular sources, we sought to determine whether the administration of rTFF2 was alone sufficient to induce a type 2 response. As eosinophils are a hallmark of type 2 inflammation and are constitutively fluorescent within IL-4 reporter mice (4Get; Voehringer et al., 2006), we inoculated naive 4Get animals i.p. with 4 µg rTFF2 over 4 d and evaluated whether these cells were recruited to the peritoneal cavity on day 5. rTFF2 administration selectively increased the percentage of eosinophils as compared with PBS treatment but did not increase the percentage of macrophages, PMNs, or lymphocytes (Fig. 9 A). rTFF2 administration did not increase the percentage of mast cells or basophils (not depicted). Importantly, ST2 was required for this effect because the increase of peritoneal eosinophils (FSCmed SSChigh Siglec-F+, GFP+) was abrogated by the treatment of mice with anti–ST2 mAb during rTFF2 administration (Fig. 9 B). This is consistent with evidence that rIL-33 administration to

Figure 7. TFF2 is required for inflammatory DCs to express IL-33 within the lung draining LNs after N. brasiliensis infection. (A) Gating strategy for the identification of inflammatory DCs as CD11b+, CD11c+, FcεR1+, and MHC class II+ within the MLNs. (B) Plots are gated on inflammatory DCs in MLNs of naive WT and TFF2−/− mice or day 3 after infection with N. brasiliensis. Numbers indicate the percentage in each quadrant. (C) Percentage of IL-33+, TI/ST2+ inflammatory DCs from the experiment described in B. TI/ST2 (also known as IL-1RL1) is a critical subunit of the murine IL-33 receptor. Means ± SE of four mice/group are shown. Data are representative of three independent experiments (** P < 0.01).

Figure 8. ST2 is necessary for host immunity against N. brasiliensis. (A) N. brasiliensis fecal egg numbers in WT C57BL/6 mice after treatment with anti-T1/ST2 mAb or isotype control on days 0 and 4. Means ± SE of six mice/group are shown. (B) N. brasiliensis intestinal worm numbers at day 9 after infection from the experiment described in A. Means ± SE of six mice/group are shown. Data are representative of two independent experiments (*, P < 0.05; **, P < 0.01; and ***, P < 0.001).
naive mice results in the rapid and selective recruitment of eosinophils (Schmitz et al., 2005). Collectively, these data show that increased levels of TFF2 are sufficient to rapidly induce eosinophil recruitment through a mechanism that is dependent on the IL-33 receptor ST2.

TFF2 drives antigen-specific TH2 differentiation through IL-33

Although our data indicated that TFF2 was required for IL-33 production, it remained unclear whether TFF2 could directly stimulate IL-33 release. As macrophages are known to produce more IL-33 than DCs (Ohno et al., 2009), we generated WT BM-derived macrophages (BMDMs) and treated these cells with rTFF2 and/or bacterial LPS followed by quantitation of mRNA transcripts for the canonical T12-promoting cytokines, IL-33, IL-25, and thymic stromal lymphopoietin (TSLP). Surprisingly, TFF2 itself was a selective stimulus for IL-33, whereas TFF2 alone did not induce expression of IL-25 or TSLP (Fig. 10 A). As expected, LPS alone induced a moderate increase of IL-33, but co-administration of TFF2 and LPS actually reduced IL-33 levels compared with TFF2 alone (Fig. 10 A). In contrast, co-administration of TFF2 and LPS stimulated a significant increase of IL-25, which suggested a possible synergistic mechanism regarding the production of this type 2–promoting cytokine (Fig. 10 A).

To directly test whether TFF2–induced IL-33 production alone was sufficient to drive T12 cell differentiation from naive T cell precursors, rTFF2-exposed BMDMs were used to stimulate naive OVA-specific CD4+ T cells (OTII). T cell lineage commitment was determined by mRNA quantification for levels of the following transcription factors: TBET/TBX21 (TH1), GATA3 (TH2), RORC (TH17), and FOXP3 (T reg). Within 96 h of co-culture, rTFF2-treated, OVA-pulsed BMDMs increased GATA3 expression nearly 10-fold, whereas co-cultures containing naive OTII cells with mock-treated, OVA-pulsed BMDMs did not up-regulate GATA3 (Fig. 10 B). Interestingly, rTFF2 treatment moderately suppressed TBET, RORC, and FOXP3 expression compared with mock-treated, OVA-pulsed BMDMs (Fig. 10 B). Targeted knockdown of IL-33 in BMDMs blocked TFF2-driven GATA3 expression and IL-13 production but increased TBET expression and IFN-γ production (Fig. 10, C and D). Moreover, rTFF2-treatment of OVA-pulsed BM-derived DCs (BMDCs) also caused a significant up-regulation of GATA3 expression within the OVA-specific OTII cells (Fig. 10 E). Overall, these findings demonstrated that TFF2 functioned as a specific stimulus for IL-33 production, which, at least in this in vitro co-culture system, functioned to selectively drive antigen-specific T12 differentiation from naive T cell precursors.

TFF2-induced IL-33 production requires CXCR4 but not ST2 or MyD88

To investigate the mechanisms required for TFF2-mediated IL-33 production, naive mice results in the rapid and selective recruitment of eosinophils (Schmitz et al., 2005). Collectively, these data show that increased levels of TFF2 are sufficient to rapidly induce eosinophil recruitment through a mechanism that is dependent on the IL-33 receptor ST2.

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TFF2-induced IL-33 production requires CXCR4 but not ST2 or MyD88

To investigate the mechanisms required for TFF2-mediated IL-33 production,
with the CXCR4 antagonist AMD3100 completely blocked TFF2-mediated IL-33 mRNA expression and protein release (Fig. 10 G). Collectively, these data indicate that TFF2 functions through CXCR4, not MyD88, for the induction of IL-33 production by BMDMs.

Lastly, to determine whether BMDMs required autocrine IL-33 signaling to promote T\(_\text{H}2\) differentiation, we generated BMDMs from ST2\(^{-/-}\) mice. Data show that TFF2-treated, OVA-pulsed BMDMs from ST2\(^{-/-}\) mice induced significantly more GATA3 expression within OVA-specific OTII cells as compared with untreated ST2\(^{-/-}\) BMDMs (Fig. 10 H). Thus, TFF2-mediated effects on macrophages did not require MyD88 or autocrine IL-33 signaling but were dependent on CXCR4, the putative TFF2 receptor.

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**Figure 10.** TFF2-treated antigen-presenting cells selectively drive T\(_\text{H}2\) differentiation through an IL-33-dependent mechanism. (A) BMDMs from WT mice were evaluated for mRNA expression levels of IL33, IL25, and TSLP after exposure to media (MED), 100 ng/ml LPS, 40 ng/ml rhTFF2 (TFF2), or LPS and TFF2 for 48 h. Mean ± SE of quadruplicate wells is shown. (B) Quantification of mRNA transcripts for GATA3, TBX21, RORc, and FOXP3 in co-cultures of OTII CD4+ T cells and BMDMs that were left untreated (MED), treated with 40 ng/ml rhTFF2, pulsed with 50 µg/ml OVA, or pretreated with rhTFF2 and pulsed with 50 µg/ml OVA (TFF2 + OVA). Cells were analyzed at 96 h. Data show mean ± SE of triplicate wells. (C) BMDMs were transfected with siRNA specific for IL-33 (IL-33 siRNA) or scrambled control (control) 48 h before the co-culture experiment described in B. IL-13 and IFN-γ protein levels were determined by ELISA, and mRNA levels of GATA3 and TBX21 were determined by quantitative RT-PCR. Mean ± SE of triplicate wells is shown. (D) BMDMs transfected with scrambled control or IL-33 siRNA were tested for IL-33 induction 96 h after treatment with OVA and 40 ng/ml rhTFF2. Mean ± SE of triplicate wells is shown. (E) GM-CSF differentiated BMDCs from naive WT C57BL/6 mice were stimulated as in B. Error bars indicate SE. (F) IL-33 protein levels in supernatants of WT versus MyD88\(^{-/-}\) BMDMs that were subjected to the co-culture conditions described in B. The experiment was performed two times. Data show mean ± SE of triplicate wells. (G) IL33 mRNA expression and IL-33 protein levels from WT BMDMs that were exposed to 1 µg/ml AMD3100 (CXCR4 antagonist) 24 h before exposure to stimulation with 40 ng/ml TFF2 or media for 48 h. Data show mean ± SE of triplicate wells. (H) GATA3 expression levels from co-cultures of WT naive CD4+ OTII cells and TI/ST2\(^{-/-}\) BMDMs that were treated with 40 ng/ml TFF2 or left untreated before pulse with OVA (endotoxin <0.2 ng/ml free) and co-cultured with OTII cells for 96 h. Data show mean ± SE from triplicate wells. Data are representative of two to four independent experiments (*, P < 0.05; **, P < 0.01; and ***, P < 0.001).
DISCUSSION

The molecular and cellular mechanisms that initiate type 2 immunity remain topics of considerable debate and controversy. Although mechanically unclear, most of the evidence suggests that epithelial cells make important contributions toward Th2 differentiation (Artis and Grenchik, 2008; Paul and Zhu, 2010). Herein, we demonstrate that the epithelial-derived repair molecule TFF2 promotes the release of IL-33, an established regulator of type 2 inflammation. In mice, this mechanism is critical for allergic lung immunopathologies and the development of host immunity against hookworms. Moreover, we demonstrate that pediatric asthma is associated with an increase of mucosal TFF2 expression.

As type 2 cytokines have previously been shown to induce TFF2 expression in the mouse lung (Nikolaidis et al., 2003; Follottie et al., 2006), we investigated its functional role in both the HDM- and IL-13–induced models of allergic asthma. HDM-induced Th2 responses may result from the injurious effects of protease-containing allergens upon airway epithelia. Indeed, HDM antigens are a major cause of clinical asthma and Th2-driven allergic inflammation in humans (Karp, 2010). Similar to HDM antigens, rIL-13 administration in WT mice normally drives mucus hypersecretion, eosinophil recruitment, and AHR (Wills-Karp et al., 1998). TFF2−/− mice were significantly protected against most of the allergic features induced by either HDMs or IL-13. This suggests that TFF2 may function as an effector molecule of type 2 immunity during murine asthma, which may partially explain how IL-13 causes airway epithelia to promote AHR, goblet cell activation, and mucus hypersecretion (Kuperman et al., 2002). Consistent with this hypothesis, TFF2 is known to increase mucus viscosity, which is a contributing factor to airflow obstruction during asthma pathogenesis (Thim et al., 2002; Agrawal et al., 2007). In addition, we show that children experiencing acute asthma exacerbations expressed high levels of TFF2 mRNA in the nasal mucosa. Collectively, these results suggest that TFF2 release, perhaps in response to epithelial injury caused by allergens, serves an important role in the subsequent expression of type 2 cytokine–mediated immune responses in the allergic airway mucosa.

Given the central role of TFF family proteins in epithelial cell restitution after mucosal injury (Taupin and Podolsky, 2003), we examined the effects of TFF2 deficiency on hookworm-induced lung pathology. Migration of N. brasiliensis L3 through lung tissue causes extensive hemorrhage during acute infection (Marsland et al., 2008). Pulmonary repair mechanisms subsequent to the exit of L3 from the lung result in progressive structural changes that resemble chronic obstructive pulmonary disorder and emphysema, in addition to the IL-13–dependent processes that drive increased AHR (Marsland et al., 2008). Our study demonstrates that N. brasiliensis infection causes a rapid up-regulation of lung TFF2 expression (24 h), which functionally limits the extent of tissue injury caused by migratory L3, as TFF2−/− mice show excessive hemorrhage and accelerated development of emphysematous changes. This is consistent with TFF2’s known role in accelerating tissue repair in several other systems (Farrell et al., 2002; Poulsen et al., 2005).

Although TFF2 may induce tissue repair directly, our data show that TFF2 was also a prerequisite for the initial production of type 2 cytokines in the lung, as TFF2−/− mice had impaired IL-4 and IL-13 responses at day 3 after inoculation compared with WT mice. This initial Th2 cell expansion at day 3 after infection was STAT-6 independent (unpublished data), as previously demonstrated by others (Jankovic et al., 2000; van Panhuys et al., 2008). Although TFF2 played an essential role in the early phase of type 2 immunity against hookworms, during later stages of the response, the TFF2 deficiency did not prevent the host from eventually producing sufficient levels of IL-4 to drive RELM-β–dependent worm expulsion (Herbert et al., 2009). Furthermore, an early administration of rIL-4 to N. brasiliensis–infected TFF2−/− mice rapidly increased RELM-β levels and facilitated more rapid worm clearance. This demonstrated that TFF2−/− mice had impaired immunity to N. brasiliensis infection because of an early defect in type 2 cytokines and that TFF2 functioned in a redundant manner with other Th2 effector molecules during the later stages of infection. This is not unexpected, as there are multiple redundant mechanisms that promote Th2 responses (Neill et al., 2010; Paul and Zhu, 2010). Consistent with this hypothesis, repeated immunization of TFF2−/− mice with chicken egg OVA over 3–4 wk resulted in only a moderate reduction of eosinophilic inflammation (Nikolaidis et al., 2006). Thus, TFF2 may primarily function to rapidly respond to injurious agents and provide early instruction toward Th2 development.

The epithelial-derived cytokines IL-25, IL-33, and TSLP are demonstrated regulators of type 2 immune responses (Fallon et al., 2006; Zaph et al., 2007; Humphreys et al., 2008). Thus, we postulated that TFF2 might induce type 2 immunity through inducing the production of one or more of these molecules. Surprisingly, TFF2 promoted IL-33 release from lung epithelia and alveolar macrophages within 24 h after infection. Even more striking was that TFF2 was required for IL-33 production within inflammatory DCs recruited to the lung draining LNs by day 3 after infection. Given the recent demonstration that inflammatory DCs are central initiators of type 2 immunity during allergic responses and worm infection (Hammad et al., 2010; Phylthian-Adams et al., 2010), our data provide one potential mechanism underlying this capability. Our coculture experiments demonstrated that rTFF2 was sufficient to confer Th2–promoting abilities upon BMDMs and BMDCs. However, it remains possible that TFF2 might also induce TSLP and/or IL-25 under certain conditions, something which is supported by our demonstration that BMDMs produced IL-25 when exposed to a combination of LPS and TFF2. Collectively, our demonstration that TFF2 regulates IL-33 production from both hematopoietic and nonhematopoietic cell lineages suggests that we have uncovered an important mechanism of mucosal immunoregulation.

IL-33 is a well-established regulator of allergic lung inflammation, anaphylactic shock, and colitis (Kurowska-Stolarska
et al., 2008; Kearley et al., 2009; Pushparaj et al., 2009; Oboki et al., 2010; Rani et al., 2011). To date however, the biologically relevant signals for IL-33 release in the context of type 2 pathologies have been unclear. Our study provides the following insights: (a) TFF2 regulates IL-33 production, (b) hookworm infection results in IL-33 release into the lung tissue, and (c) pulmonary epithelia, alveolar macrophages, and inflammatory DCs are important sources of IL-33. Chang et al. (2011) recently demonstrated that influenza-infected rodents have IL-33+ alveolar macrophages and develop IL-13–driven AHR. These data are remarkably consistent with the central conclusions of our study, which are that infection-induced lung injury causes TFF2 release that promotes IL-33 from innate sources for the rapid development of type 2 immunity.

IL-33 has been shown to regulate the immunobiology of DCs (Rank et al., 2009), basophils (Pecaric-Petkovic et al., 2009), mast cells (Hsu et al., 2010), and T lymphocytes (Guo et al., 2009) and promote the development of alternatively activated macrophages (Kurowska-Stolarska et al., 2009; Zais et al., 2011). We found that ST2 expression on macrophages was not essential in order for TFF2 to instruct these cells to drive antigen-specific GATA3 expression within the CD4+ T cells, which implies that ST2 expression on T cells is sufficient for Th2 differentiation. Indeed, ST2 was necessary for host protection against N. brasiliensis. This finding is consistent with evidence that mice deficient in IL-1RL1 (subunit of IL-33 receptor) show increased susceptibility to N. brasiliensis infection and that rIL-33 administration promotes the expulsion of the whipworm T. musis (Humphreys et al., 2008; Neill et al., 2010).

Although our data also support the notion that IL-33/ST2 signaling promotes primary Th2 development (Townsend et al., 2000), there is controversy regarding whether ST2 signaling promotes Th2 responses (Hoshino et al., 1999). Thus, we cannot rule out the possibility that IL-33 only functions to expand naive Th2 precursors (Guo et al., 2009). However, our data are consistent with reports that ST2 deletion or blockade impairs type 2 responses in vivo (Löhning et al., 1998; Coyle et al., 1999; Townsend et al., 2000). Thus, we propose that a TFF2/IL-33 axis exists, which serves an important function in the development of Th2 responses and host immunity against hookworm infection.

Several lines of evidence suggest that TFF2 preferentially drives type 2 inflammation and antigen-specific Th2 differentiation through its ability to induce IL-33 production. First, rTFF2 could rapidly induce the selective recruitment of eosinophils in 4Get mice through a mechanism that was blocked by inhibition of ST2, the critical subunit of the IL-33 receptor. Second, TFF2 treatment of BMDMs or BMDCs co-cultured with OVA-specific CD4+ T cells (OTII) resulted in the specific induction of GATA3, the Th2 lineage–specific transcription factor (Ouyang et al., 2000; Seki et al., 2004), as well as IL-13 production in culture supernatants. These cultures also contained IL-5 but no IL-10, IFN-γ, or IL-17A (unpublished data). Lastly, IL-33 production from BMDMs was necessary to promote T cell differentiation toward a Th2 phenotype and for the concomitant suppression of Th1 development. IL-33 most likely works directly on the T cells and not through autocrine effects on BMDMs, as Th2 cytokines also induced GATA3 expression after TFF2 treatment.

TFF2-mediated IL-33 production required the CXCR4 receptor, which is a chemokine receptor used by T cell tropic strains of the human immunodeficiency virus (Grivel and Margolis, 1999). The natural ligand for CXCR4 is SDF-1 (stromal cell–derived factor 1; Allen et al., 2004). Consistent with evidence that the TFF2 receptor is CXCR4, we demonstrate that AMD3100 (a specific antagonist of CXCR4) blocks TFF2-induced IL-33 production. This is in concordance with evidence that the CXCR4/SDF-1 axis is required for Th2-mediated allergic airway disease (Gonzalo et al., 2000) and that AMD3100 abolishes cockroach-induced murine asthma (Lukacs et al., 2002). Moreover, we have preliminary evidence that AMD3100 treatment also exacerbates N. brasiliensis–induced lung injury (unpublished data).

Our model predicts that TFF2 initiates a rapid, coordinated type 2 immune response in the lung after allergen exposure or worm infection–induced mucosal injury. We propose the following: (a) within hours of insult, TFF2 secretion drives epithelial cell restitution, which prevents excessive hemorrhage and also starts the tissue remodeling process; (b) within 24 h, TFF2 promotes IL-33 release from lung epithelia and alveolar macrophages, but although IL-33 production in epithelia is rapidly lost, IL-33 production in myeloid cells is sustained for several days; (c) within 3 d, TFF2 promotes IL-33 expression within ST2+ inflammatory DCs that migrate to draining LNs; (d) this results in the initial clonal burst of antigen-specific Th2 cells within the LN; and (e) once sufficient amounts of IL-4 and IL-13 are produced, these cytokines drive a myriad of effector functions within epithelia and macrophages (including TFF2 production) that promote worm expulsion and asthma pathogenesis (Kuperman et al., 2002; Herbert et al., 2009).

In the context of parasitic infections, the induction of type 2 immunity and tissue remodeling protects the host against organ damage and pathogen persistence. However, in the context of asthma, excess production of type 2 cytokines and tissue repair molecules is pathogenic. It is possible that the TFF2/IL-33 axis may have evolved under selective pressure from parasitic helminths for the initiation of IL-4+ and IL-13–dependent host defense mechanisms, while simultaneously mediating the repair of damaged mucosa. This is consistent with the view that allergies and asthma represent maladaptive Th2 responses intended for worm parasites and suggests that TFF2 may be a possible target for therapeutic intervention in a broad array of allergic and parasitic human diseases.

**MATERIALS AND METHODS**

**Mice, parasites, and asthma.** All experiments used male WT C57BL/6 or TFF2−/− mice (C57BL/6 × 129) that have been backcrossed to the C57BL/6 background for more than six generations (Farrell et al., 2002). Although pure WT C57BL/6 B6 mice were used as controls throughout the paper, all infection data were additionally confirmed with +/+ and +/− littermate controls. 4Get mice on a BALB/c background have been described previously (Mohrs et al., 2001). Worm burdens were assessed by opening...
mouse intestines longitudinally and incubating them in PBS at 37°C for 3 h in a modified Baermann apparatus in which tissues were placed in a sterile stop a 250-ml beaker. Parasites that collected at the bottom were counted. For fecal egg counts, feces were collected, weighed, and incubated in saturated NaCl solution, and eggs were counted using McMaster slides (Herbert et al., 2009). Allergic airway responses induced by HDMs or 5 µg/mlIL-13 have been described previously (Wills-Karp et al., 1998) The Institutional Animal Care and Use Committee at the Cincinnati Children’s Hospital Medical Center (CCHMC) approved all protocols and procedures.

**Subjects.** Nasal epithelial samples were obtained from healthy and asthmatic children at CCHMC and processed for RNA extraction and cDNA synthesis as previously described (Guajardo et al., 2005). Asthmatic children and their parents attending the hospital clinics and emergency department in the CCHMC were invited to participate in the study. Participants were included in exacerbation of asthma (asthma-E group, n = 23) or healthy nonatopic children (control group, n = 9), as previously described (Guajardo et al., 2005).

**Assessment of airway responsiveness and BAL cellularity.** To evaluate airway responses, mice were anaesthetized, intubated, and respired at a rate of 120 breaths per minute with a constant tidal volume (0.2 ml) and paralyzed with 25 mg/kg decamethonium bromide 24 h after the final HDM or IL-13 challenge. 50 µg/kg acetylcholine was injected into the inferior vena cava, and dynamic airway pressure (cm H2O × sec) was followed for 5 min. To collect BAL fluid, lungs were lavaged three times with a 1.0-ml aliquot of cold Hanks’ balanced salt solution (Invitrogen). Recovered lavage fluid (70–80%) was centrifuged (300 g for 10 min), and the cell pellet was resuspended in 1.0 ml of 10% PBS in PBS. Total cells were counted with a hemocytometer. Slides were prepared by cytocentrifugation (Cytospin 3; Shandon Instruments) and stained with Diff-Quik (Dade Behring). BAL cell differential counts were determined using morphological criteria under a light microscope (BX40; Olympus) with evaluation of ≥500 cells/slide.

**Lung histopathology.** To assess airway inflammation and mucous cell content in the airway wall, lungs were excised and stained with hematoxylin and eosin and periodic acid Schiff (PAS). The left lung was removed and fixed in 10% formalin, washed in methanol, dehydrated and embedded in paraffin, and cut into 5-µm sections. Sections were mounted on slides and stained with hematoxylin and eosin and periodic acid Schiff (PAS). The left lung was removed and fixed in 10% neutral buffered formalin. Lungs were bisected and oriented cut side down in a paraffin block such that sections would reveal cross sections of consistent airways for comparison. Two, 5-µm sections, 30 µm apart, were PAS stained and examined for mucin production. Examination included assessment of the percentage of airways with PAS-positive cells (number of airways containing any PAS cells/number of total airways) as well as total number of goblet cells per section.

**Microscopy/histology.** 5-mm formalin-fixed, paraffin-embedded sections of lung or jejunum were prepared by the CCHMC Morphology core. A microscope (Eclipse E600; Nikon) fitted with a 40× objective oil-immersion lens (Plan Apochromat; Nikon) was used for image acquisition, and photos were captured with a SPOT Diagnostics RT slider digital color camera using a SPOT Diagnostics imaging system.

**ELISA and real-time PCR.** RNA was DNase I treated and cDNA prepared using SuperScript II reverse transcription (Invitrogen). Real-time PCR was performed on a Gene Amp 7500 instrument (PE Biosystems) with the Syber Green detection reagent. Cycle threshold (CT) values for genes evaluated were determined and expressed using the 1/ΔΔCt method (Table S1), as described previously (Herbert et al., 2010). Mouse cytokine ELISA kits specific for IL-13, IFN-γ, and IL-33 were obtained from ebioscience.

**Isolation of lung tissue cells and BAL collection.** Lungs were perfused with 1× PBS and minced with scissors followed by digestion in serum-free RPMI containing 0.5 mg/ml Liberase Cl (Roche) and 0.5 mg/ml DNase I (Sigma-Aldrich) RPMI for 30 min at 37°C with shaking. Samples were further dissociated by repeated passage through a 18-g needle and finally passed through a 70-µm cell strainer to obtain a single cell suspension. For cytokine measurements, BAL fluid was collected using established protocols (Levkowich et al., 2005) followed by concentration with Amicon Ultra centrifugal filter units with 3K cut-off (Millipore). Magnetic bead isolation of EpCAM- and CD11c-positive cells was performed according to the manufacturer’s protocol (Miltenyi Biotec).

**Flow cytometric analyses.** Single cell suspensions of lung or MLN tissue were stained with one or more of the following fluorescently labeled mAbs: ST2L/IL-1R4 (clone 245707) and anti–IL-33 (R&D Systems), CD11c (clone N418), CD11b (clone M1/70), anti-CD326/EpCAM (clone G8.8), I-Ag (clone Af6-120.1), Ly6G/C (clone RB6-8C5), FcεRI (clone MAR-1), CD103 (clone 2E7), CD45 (clone 30-F11), CD317 (clone 120g8), and isotype control (MOPC-173; ebioscience). Before intracellular cytokine staining, cells were either stimulated with PMA/Ionomycin/Golgi-stop (BD) or 1 µg/ml anti-CD3/anti-CD28 mAb for 16 h, with Golgi-plug added the last 4 h before staining. Acquisition was performed with an LSR II flow cytometer (BD) or FACS Calibur (BD), and data were analyzed with FlowJo software (version 8.8; Tree Star). Rat anti-mouse ST2 (clone M955) and control IgG antibodies were provided by Agen.
Statistical analysis. Statistical significance was assessed by either the two-tailed Student’s t test (two groups) or analysis of variance for multiple groups with a post-hoc Tukey test to determine significance, all performed using Prism 4.0 software (GraphPad. * P < 0.05; ** P < 0.01; and *** P < 0.001).

Online supplemental material. Fig. S1 shows the gating strategy for the identification of CD4+TCR-β T lymphocytes within the MLNs. Table S1 lists the primer sequences used for this study. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110079/DC1.

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