Muc5ac: a critical component mediating the rejection of enteric nematodes

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De novo expression of Muc5ac, a mucin not normally expressed in the intestinal tract, is induced in the cecum of mice resistant to *Trichuris muris* infection. In this study, we investigated the role of Muc5ac, which is detected shortly before worm expulsion and is associated with the production of interleukin-13 (IL-13), in resistance to this nematode. Muc5ac-deficient mice were incapable of expelling *T. muris* from the intestine and harbored long-term chronic infections, despite developing strong T_H2 responses. Muc5ac-deficient mice had elevated levels of IL-13 and, surprisingly, an increase in the T_H1 cytokine IFN-γ. Because T_H1 inflammation is thought to favor chronic nematode infection, IFN-γ was neutralized in vivo, resulting in an even stronger T_H2-type immune response. Nevertheless, despite a more robust T_H2 effector response, the Muc5ac-deficient mice remained highly susceptible to chronic *T. muris* infection. Importantly, human MUC5AC had a direct detrimental effect on nematode vitality. Moreover, the absence of Muc5ac caused a significant delay in the expulsion of two other gut-dwelling nematodes (*Trichinella spiralis* and *Nippostrongylus brasiliensis*). Thus, for the first time, we identify a single mucin, Muc5ac, as a direct and critical mediator of resistance during intestinal nematode infection.

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Abbreviations used: EC, epithelial cell; mLN, mesenteric LN; PAS, periodic acid-Schiff; p.i., postinfection.

The intestinal epithelium is covered by a thick layer of mucus that enables the host to inhibit the access of pathogens to the underlying mucosa (Deplancke and Gaskins, 2001). Specialized epithelial secretory cells termed goblet cells secrete gel-forming mucins, the major macromolecular components of the mucus barrier which are responsible for its viscoelastic properties (Thornton et al., 2008). In the adult intestine, MUC2/Muc2 is the major gel-forming mucin stored within the granules of goblet cells (Thornton et al., 2008). Aberrant expression of MUC2 accompanies many human gastrointestinal diseases such as ulcerative colitis (Longman et al., 2006), colorectal carcinomas (Andrianifahanana et al., 2001), and parasitic infections (Lidell et al., 2006). MUC5AC, a mucin normally expressed in nonintestinal mucosa, has been reported to be expressed in the intestine, along with MUC2, during inflammation in diseases such as ulcerative colitis and adenocarcinoma (Forgue-Lafitte et al., 2007). A host-protective role for this mucin in the intestine after parasitic infection has not been demonstrated.

Trichuriasis, which is caused by the nematode *Trichuris trichiura*, is one of the most prevalent nematode infections causing significant morbidity and mortality in humans (Artis and Grencis, 2008). The nematode *Trichuris muris* is closely related to *T. trichiura* at a morphological and antigenic level and is thus used as a well-established mouse model of human Trichuriasis

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(Cliffe and Grencis, 2004). It is well established that the expulsion of the *T. muris* nematode is critically dependent on T_H2-associated cytokines, whereas a T_H1-type immune response is beneficial for the nematode, resulting in a chronic infection. Our recent studies have highlighted that the mucus barrier is a significant component of the well-coordinated response initiated against the nematode, influenced by the T_H2-type cytokines (Hasnain et al., 2010, 2011), and we have recently demonstrated that in the absence of the major intestinal mucin, Muc2, a significant delay in worm expulsion occurs (Hasnain et al., 2010). Interestingly, MUC5AC/Muc5ac, a mucin predominantly expressed in the adult airway, stomach, and ocular epithelia (Inatomi et al., 1996; Buisine et al., 1998; Bara et al., 2003), was expressed in the cecum of resistant mice and associated with worm expulsion. We hypothesize that Muc5ac may function as a pharmacological regulator to damage the nematodes directly or indirectly via associated protective molecules. In addition, the incorporation of Muc5ac within the mucus layer may influence the biochemical properties of the mucus gel and thus facilitate worm expulsion (Hasnain et al., 2010). To investigate the role of Muc5ac in enteric parasitic infection, we examined the etiology of *T. muris* infection in the targeted Muc5ac-deficient mice and the interaction of MUC5AC with *T. muris* in vitro.

In this study, we demonstrate, for the first time, that Muc5ac induced by IL-13 is critical for worm expulsion. Loss of Muc5ac resulted in susceptibility to chronic *T. muris* infection compared with WT mice, which expelled their worm burden. These findings are particularly important as previously described effector mechanisms were activated, including a T_H2-type immune response (Grencis, 1993), goblet cell hyperplasia, up-regulation of Muc2 (Hasnain et al., 2010), and the activation of the epithelial cell (EC) escalator (Cliffe et al., 2005), without parasite expulsion. Additionally, the absence of Muc5ac caused a significant delay in the expulsion of *Trichinella spiralis* and *Nippostrongylus brasiliensis* from the intestines of infected mice. Together, these results identify a unique functional role for the Muc5ac mucin during intestinal infection.

RESULTS AND DISCUSSION

Our recent study has shown that Muc5ac is up-regulated in the cecum during *T. muris* expulsion in a strong T_H2-type environment and therefore may be a critical component of the immune-regulated response (Hasnain et al., 2010). In this study, we addressed this functionally by measuring Muc5ac levels in IL-4– and IL-4R–deficient mice during *T. muris*

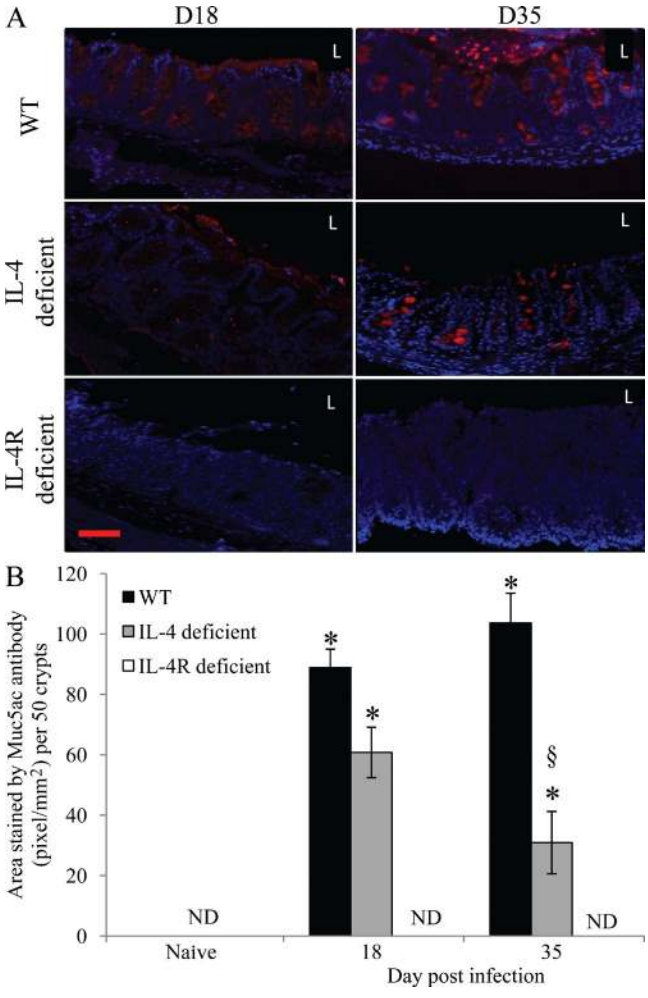

Figure 1. IL-13 induces Muc5ac as part of the normal response to worm expulsion. (A) Levels of Muc5ac were determined using immunofluorescence microscopy with the 45M1 antibody in WT and IL-4– and IL-4R–deficient mice (BALB/c background) at days 18 and 35 p.i. with *T. muris*. L marks the luminal side of the cecum. Bar, 10 µm. (B) Area stained with 45M1 was quantified. Results are representative of three individual experiments and are presented as the mean value of five mice per group ± SEM. ND, none detected. *, $P < 0.05$ compared with naive controls; §, $P < 0.05$ compared with WT.

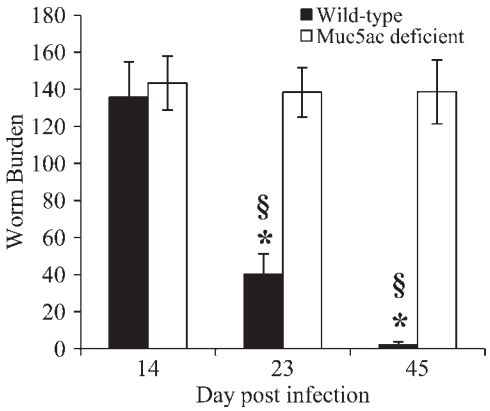

Figure 2. Muc5ac deficiency renders resistant mice susceptible to chronic *T. muris* infection. Muc5ac-deficient mice and the WT C57BL/6 mice were infected with a high dose of *T. muris* eggs, and worm burdens were assessed on days 14, 23, and 45 p.i. Results are representative of three individual experiments and are presented as the mean value of three to five mice per group ± SEM. *, $P < 0.01$ compared with day 14; §, $P < 0.01$ compared with Muc5ac-deficient mice.

infection to determine whether the induction of Muc5ac was regulated by IL-4 and IL-4–IL-13 pathways, respectively. The IL-4–deficient mice had a delay in worm expulsion and were able to mount a goblet cell response, whereas the IL-4R–deficient mice (which have defective IL-4 and IL-13 signaling) were susceptible to chronic infection and did not exhibit goblet cell hyperplasia (Hasnain et al., 2011). Expression of Muc5ac was assessed using immunofluorescence microscopy. De novo Muc5ac expression was observed in IL-4–deficient mice, on days 18 and 35 postinfection (p.i.), correlating with worm expulsion. However, Muc5ac was undetectable in cecal crypts during infection in IL-4R–deficient mice, which manifest defects in both IL-4 and IL-13 signaling (Fig. 1), indicating that Muc5ac in the cecum was regulated by IL-13 and to a lesser extent IL-4, which previously has been reported in vitro (Rose et al., 2000).

Muc5ac expression correlates with worm expulsion in resistant mice even in the absence of the predominant baseline intestinal secreted mucin Muc2 (Hasnain et al., 2010). Therefore, we investigated the role of Muc5ac during nematode expulsion. Muc5ac-deficient mice on the resistant C57BL/6 background (Fig. S1) were infected with a high dose of *T. muris* eggs. Infection became established similarly in the WT and Muc5ac-deficient mice as seen on day 14 p.i. (Fig. 2). As expected, WT mice cleared 70% of their worms by day 23 p.i. and were essentially parasite free by day 45 p.i. (Fig. 2). However, there was no significant reduction in worm burdens in the Muc5ac-deficient mice at any time point (day 14, 23, and 45 p.i.), demonstrating that these mice were susceptible to chronic infection (Fig. 2). Therefore, the inability of mice to express Muc5ac renders them susceptible to chronic infection.

The EC escalator (Cliffe et al., 2005) and goblet cell hyperplasia (Hasnain et al., 2010) are immune-mediated effector mechanisms that contribute to worm expulsion. The rate of EC turnover was determined by measuring BrdU incorporation into ECs and measuring the length of longitudinal cecal crypts during infection (Cliffe et al., 2005). On day 23 p.i., a marked increase in EC turnover was observed in Muc5ac-deficient mice compared with uninfected controls (Fig. S2 A). Furthermore, this was accompanied by an increase in cecal crypt length, which is indicative of increased proliferation of

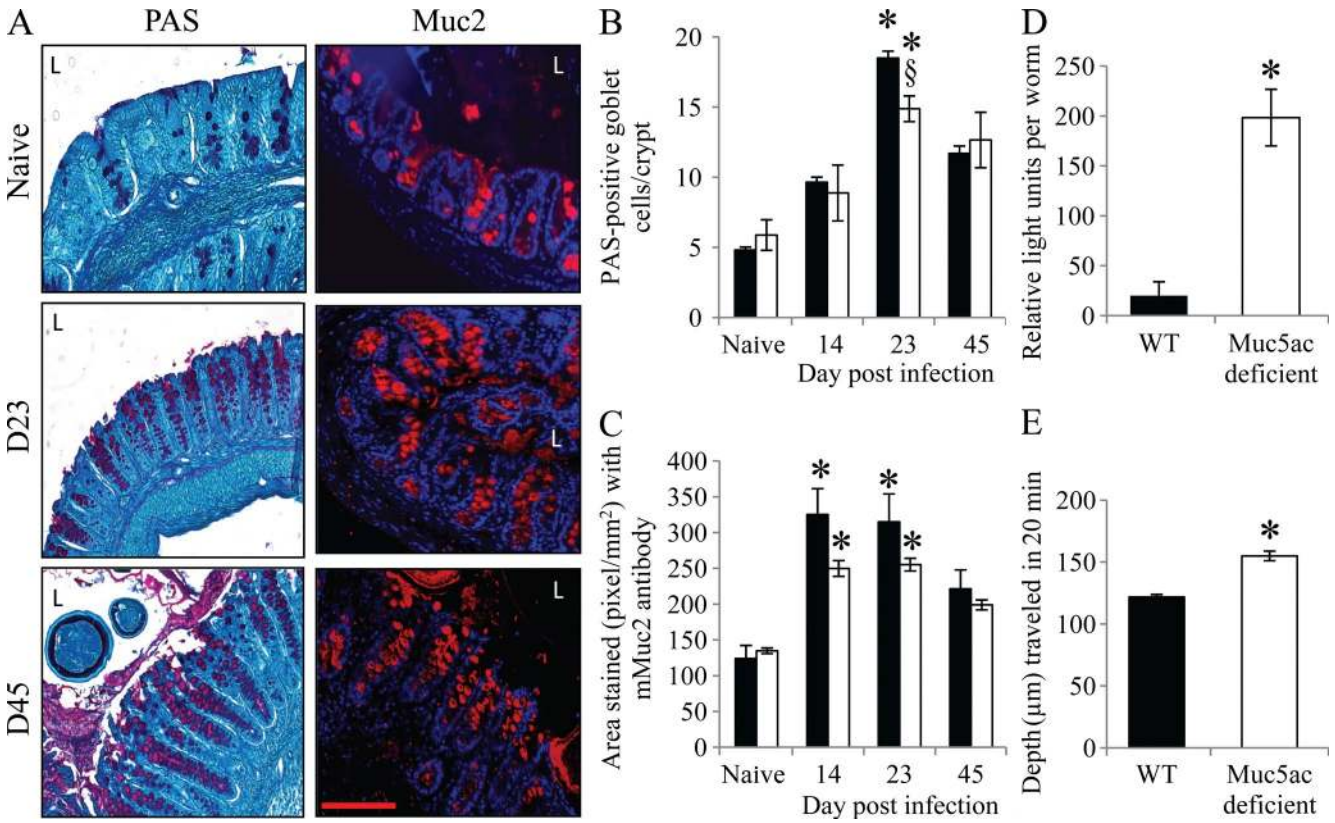

Figure 3. Goblet cell hyperplasia in the susceptible Muc5ac-deficient mice. (A) PAS staining and immunofluorescence staining with mMuc2 in cecal tissue from naive mice or Muc5ac-deficient mice infected for 23 or 45 d with *T. muris*. L marks the luminal side of the cecum. Bar, 10 µm. (B and C) Quantification of goblet cell numbers (B) and mMuc2 antibody staining (represented as area stained in pixels per square millimeter; C) in the cecum of WT (closed bars) and Muc5ac-deficient (open bars) mice at the indicated times after infection. (D and E) Energy levels of worms (presented as relative light units per worm; D) and mucus permeability (using fluorescent beads; E) were measured in the WT and Muc5ac-deficient mice on day 23 p.i. Results are representative of three individual experiments and are presented as the mean value of three to five mice per group ± SEM. *, $P < 0.01$ compared with naive mice (B and C) or WT mice (D and E); §, $P < 0.05$ compared with WT mice.

ECs at the base of the crypt unit (Fig. S2 B). Despite the increase in EC turnover, the worms were not expelled from the intestine in the absence of Muc5ac.

During infection, an increase in goblet cell numbers and Muc2 levels was apparent in Muc5ac-deficient mice infected with *T. muris* (day 23 and 45 p.i.) compared with naive mice (Fig. 3, A–C). However, the total number of periodic acid-Schiff (PAS)–positive goblet cells was significantly lower in the Muc5ac-deficient mice compared with infected WT mice (Fig. 3, A and B) during *T. muris* infection. The absence of Muc5ac in naive and infected Muc5ac-deficient mice was confirmed with immunofluorescence microscopy with 45M1 (MUC5AC/Muc5ac) antibody (Fig. S3). Furthermore, it is important to note that Muc2 and Muc5ac could be produced by the same goblet cells (Fig. S4).

We have previously shown that during the development of resistance to chronic parasitic infection, the mucin network is less porous and the overall changes in the environmental niche can be detrimental to the worm's vitality (Hasnain et al., 2010). To address whether changes in worm viability are affected by the absence of Muc5ac, we measured worm ATP levels. Interestingly, the worms extracted from Muc5ac-deficient mice on day 23 p.i. have significantly higher levels of ATP compared with worms isolated from the WT mice (Fig. 3 D), which are consistent with worms isolated from mouse strains that develop chronic infections (Hasnain et al., 2010). Furthermore, we demonstrated that fluorescent beads placed at the top of the mucus traveled a greater distance through the mucus barrier in Muc5ac-deficient mice as compared with WT mice (Fig. 3 E), suggesting that the absence of Muc5ac resulted in a more porous network. Whether this is the result of the lower mucin content of the mucus or different organization of the barrier caused by the lack of Muc5ac remains to be elucidated. Nevertheless, these data clearly show that the absence of Muc5ac increases the viability of the worm and increases the porosity of the mucus network. We have also previously shown that Muc2 plays a significant but partial role in protection against *T. muris* infection (Hasnain et al., 2010), in which the effect of the loss of Muc2 was delayed with worm elimination by 1 wk (Hasnain et al., 2010). In contrast, Muc5ac deficiency prevented parasite expulsion, with little or no worm expulsion detected as late as 45 d p.i., demonstrating a greater requirement for Muc5ac. Therefore, these data suggest that although Muc2 and Muc5ac act in concert to promote worm expulsion, Muc5ac is playing the dominant role in IL-13–driven immunity.

It is well established that a strong T_H2-type immune response mediates worm expulsion; in particular, IL-13 is

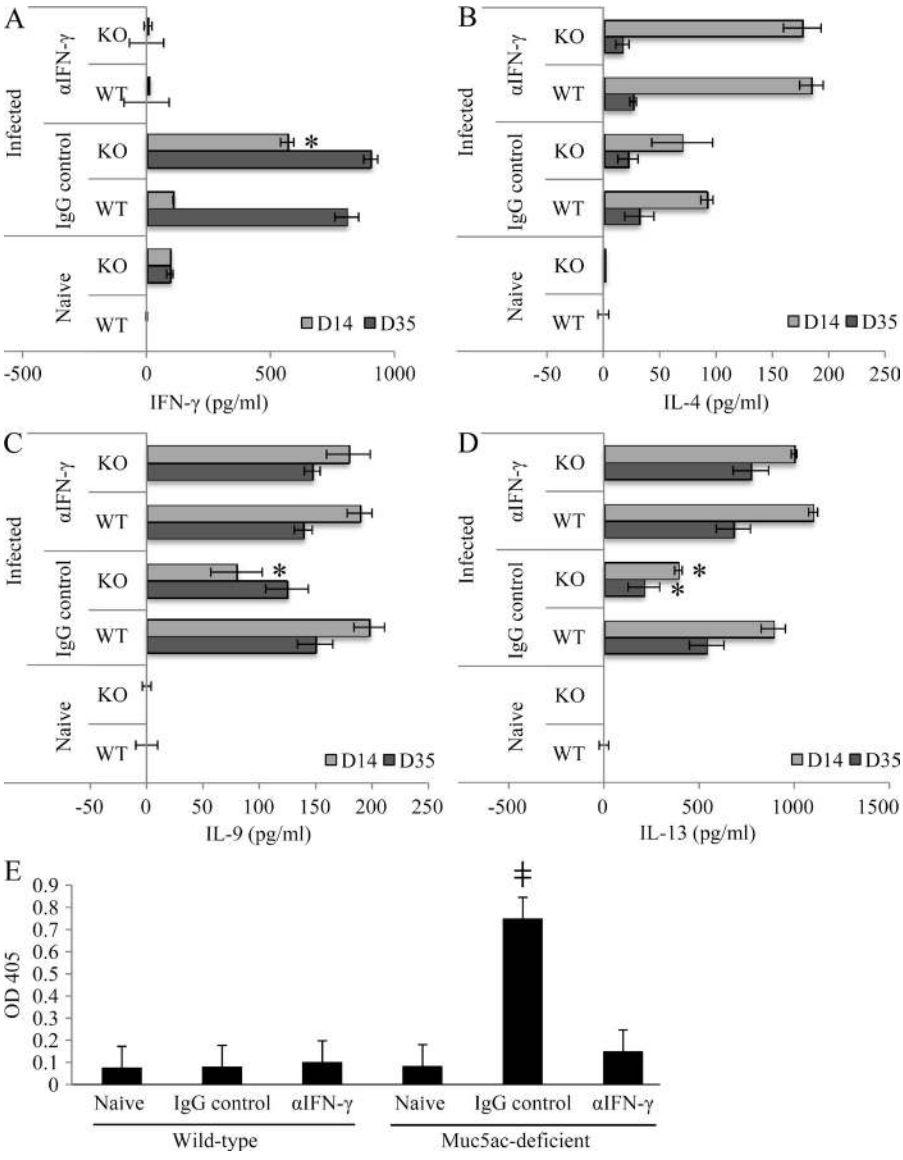

Figure 4. Chronic *T. muris* infection despite a T_H2-type immune response. Muc5ac-deficient and WT mice were infected with a high dose of *T. muris* eggs. Infected mice received either anti–IFN-γ or a control IgG antibody at 0.5mg/injection. (A–E) On day 14 and 35 p.i., the levels of IFN-γ (A), IL-4 (B), IL-9 (C), and IL-13 (D; pg/ml) were determined by *T. muris* excretory/secretory antigen stimulation of mLNs. (E) Antibody IgG2a levels were determined using sandwich ELISA on intestinal homogenates. Results are representative of two individual experiments and are presented as the mean value of four to five mice per group ± SEM. *, $P < 0.05$ compared with WT mice; ǂ, $P < 0.02$ compared with all groups.

critical for resistance to *T. muris* infection (Cliffe and Grencis, 2004; Artis and Grencis, 2008). The unimpaired T_H2 response (elevated levels of IL-4, IL-13, and IL-9; Fig. 4) seen after infection was surprising given the susceptible phenotype of the Muc5ac-deficient mice; however, this likely explains the increases in goblet cell numbers, Muc2 expression, and EC turnover observed in the Muc5ac-deficient mice (Fig. S2). IFN-γ was also significantly elevated in the Muc5ac-deficient mice after infection. There was also a slight increase in IFN-γ in uninfected Muc5ac-deficient mice (Fig. 4 A). Increased levels of IFN-γ may skew the immune response toward a T_H1-dominant environment that is known to promote chronic infection. Therefore, we sought to determine whether skewing the immune response of the Muc5ac-deficient mice to a more polarized T_H2-type immune response would return them to a more resistant phenotype. To this end, mice were treated with anti–IFN-γ antibody or an IgG control antibody, both before and during infection (every 2 d starting on 2 d before infection until day 26 p.i.). Expression of IFN-γ, IL-4, IL-9, and IL-13 was measured after stimulation of mesenteric LNs (mLNs) with *T. muris* excretory/secretory antigen (Fig. 4). Treatment with anti–IFN-γ antibody abolished IFN-γ expression and induced a much stronger T_H2-type immune response, characterized by increased production of IL-4, IL-9, and IL-13 and lower IgG2a expression (Fig. 4). The Muc5ac-deficient mice treated with anti–IFN-γ remained highly susceptible to chronic *T. muris* infection, despite developing strong T_H2-type immune responses; indeed, similar numbers of worms were observed in both control IgG– and anti–IFN-γ–treated mice (Fig. S5). In addition, we investigated whether deletion of Muc5ac altered the goblet cell lineage and affected the expression of goblet cell bioactive factors that may contribute to the expulsion of *T. muris*. Immunohistochemical and immunofluorescence microscopy were used to determine the expression of intestinal trefoil factor (Tff3) and Relm-β (Resistin-like molecule-β), respectively. Staining for Tff3 and Relm-β were similar within the cecum of the WT and Muc5ac-deficient mice on day 35 after the neutralization of IFN-γ (Fig. S6). These data clearly establish an important and indispensable role for Muc5ac in worm expulsion, even during chronic infection. Furthermore, they imply that Muc5ac functions as the key immune effector molecule during the development of T_H2-dependent immunity.

The question that arises in light of this evidence is how does the absence of Muc5ac result in chronic *T. muris* infection despite an on-going T_H2-type immune response? To explore whether Muc5ac has a direct effect on the nematode, we used human colonic cell lines producing either MUC5AC (HT-29) or MUC2 (LS174T). Worms were extracted from highly susceptible athymic nude mice and therefore were in a low-stress state. Worms were then placed on either MUC2-producing LS174T cells or a MUC5AC-producing subclone of HT-29 cells (Kirkham et al., 2002) for 24 h (Fig. 5). Interestingly, the data showed that the nematodes placed on MUC2-producing cells produced significantly higher levels of ATP (used as a measure of worm viability) compared with those placed on the MUC5AC-producing cells, suggesting that MUC5AC may have a direct effect on the vitality of the nematode. However, the levels of ATP of worms extracted from a resistant environment were significantly lower than those observed in vitro (HT-29 cells; Fig. 5). The differences observed in vitro compared with in vivo might be caused by the differences in concentration or chemical composition between human MUC5AC and mouse Muc5ac, and in particular, the glycosylation pattern in vivo may differ from that in vitro. Nevertheless, MUC5AC had a deleterious effect on the vitality of the nematode, as reflected by ATP levels, whereas MUC2 did not (Fig. 5 B). The exact mechanism by which Muc5ac/MUC5AC is exerting its effects is not yet known. An interesting possibility by which Muc5ac/MUC5AC may affect the nematode is via factors that are either produced from the same goblet cells and/or are specifically associated

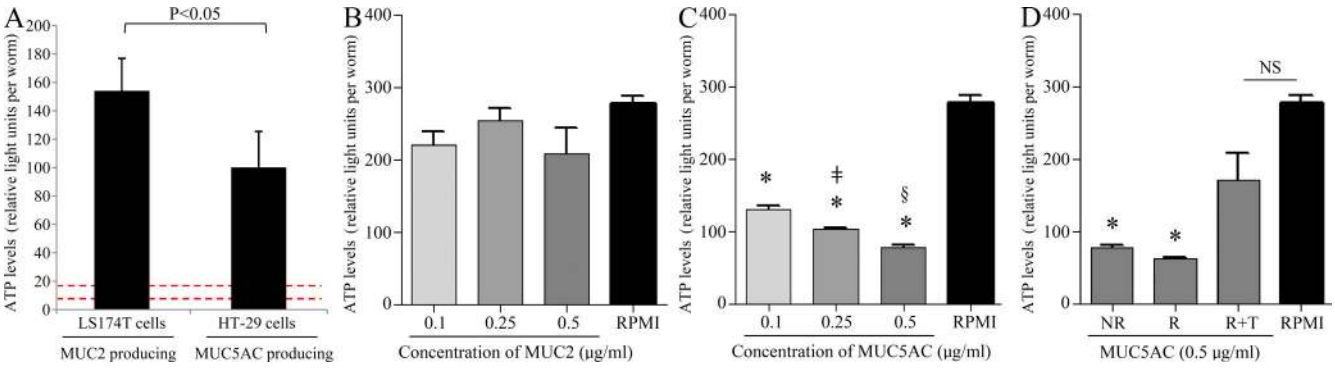

Figure 5. MUC5AC can directly affect nematode vitality. (A) Live worms extracted from a nude mouse on day 26 p.i. were placed on HT-29 (MUC5AC producing) cells or LS174T (MUC2 producing) cells for 24 h before measuring ATP levels. Red dashed lines indicate worms extracted from resistant mice. (B and C) Worms were treated with 0.1, 0.25, or 0.5 μg/ml MUC2 or MUC5AC or RPMI media only for 24 h before measuring ATP levels. (D) Worms were treated with 0.5 μg/ml nonreduced (NR), reduced and carboxymethylated (R), or reduced, carboxymethylated, and trypsin-digested (R + T) MUC5AC or RPMI media for 24 h before measuring ATP levels. ATP levels are presented as relative light units per worm. Results represent the mean value of 100 worms per group ± SEM. Results are representative of three individual experiments. *, $P < 0.05$ compared with RPMI control; ǂ, $P < 0.05$ compared with 0.1 μg/ml MUC5AC; §, $P < 0.001$ compared with 0.1 μg/ml MUC5AC.

with this mucin in mucus. However, because Relm-β and Tff3, which are induced during nematode infection (Herbert et al., 2009), were still expressed within the goblet cells of the Muc5ac-deficient mice (Fig. S6), our findings suggest that Muc5ac itself plays a key role in protection.

To address whether MUC5AC was directly having a detrimental effect on the vitality of the nematode, we altered the concentration and structure of polymeric MUC5AC produced by HT-29 cells in culture (Fig. 5, C and D). A dose-dependent decrease in the nematodes' ATP levels was observed when they were treated with an increasing concentration of MUC5AC. To test whether the intact polymer was required for the direct effect on the nematode, the MUC5AC preparation was depolymerized by treatment with dithiothreitol. Incubation of the nematodes with the monomeric, unfolded mucins did not have a significant effect on the glycoprotein's function in terms of reducing nematode vitality (Fig. 5 D). Subsequently, the monomeric MUC5AC mucins were treated with trypsin, which degrades the protein-rich N- and C-terminal domains and the internal cys domains but leaves intact the highly glycosylated mucin domains. Importantly, the digested MUC5AC mucin did not reduce the worm vitality, highlighting the specific role of MUC5AC as an effector that acts to damage the nematode as part of the coordinated worm expulsion process.

Interestingly, *Muc5ac* messenger RNA has also been shown to be up-regulated in the intestines during *Trichuris suis* infection in pigs (Kringel et al., 2006), but its contribution to immunity was not determined. Another recent study has also reported the up-regulation of Muc5ac in intestinal ECs in vitro with exposure to the intestinal nematode *N. brasiliensis* (Takeda et al., 2010). Additionally, we now present data that show that Muc5ac was up-regulated during infection of mice with *T. spiralis* (Fig. 6, A and B). Moreover, the expulsion of *T. spiralis* from Muc5ac-deficient mice was significantly delayed in comparison with WT mice (Fig. 6 C). In a similar vein, the expulsion of *N. brasiliensis* from the intestines of Muc5ac-deficient mice was also significantly compromised (Fig. 6 D). In contrast, Muc5ac expression was not observed in WT mice after a primary infection with *Heligmosomides polygyrus bakeri* (Fig. 6 A). These parasites are not expelled from the intestine and persist for many months in most strains of mouse. Therefore, we propose that de novo expression of Muc5ac observed as part of the IL-13–mediated immune response serves as a universal protective mechanism during expulsion of gut-dwelling nematodes. Moreover, it highlights a novel role for Muc5ac not just as a structural component of the mucus barrier but also as a crucial effector molecule during infections of the intestine.

MATERIALS AND METHODS

Animals. The WT (C57BL/6) mice were commercially bought from Harlan Laboratories, and IL-4–deficient and their WT (BALB/c) littermates were originally obtained from A. McKenzie (University of Cambridge, Cambridge, England, UK; McKenzie et al., 1998). IL-4R–deficient mice were obtained from F. Brombacher (University of Cape Town, Cape Town, South Africa; Mohrs et al., 1999). Muc5ac-deficient mice were produced by targeted gene mutation at the University of Texas MD Anderson Cancer Center. The Muc5ac locus was targeted by inserting LoxP sites into the 5′ flanking region and intron 1 in CJ7 embryonic stem cells (Fig. S1 A). Global knockout mice were then produced by mating founder animals with Zp3-Cre transgenic

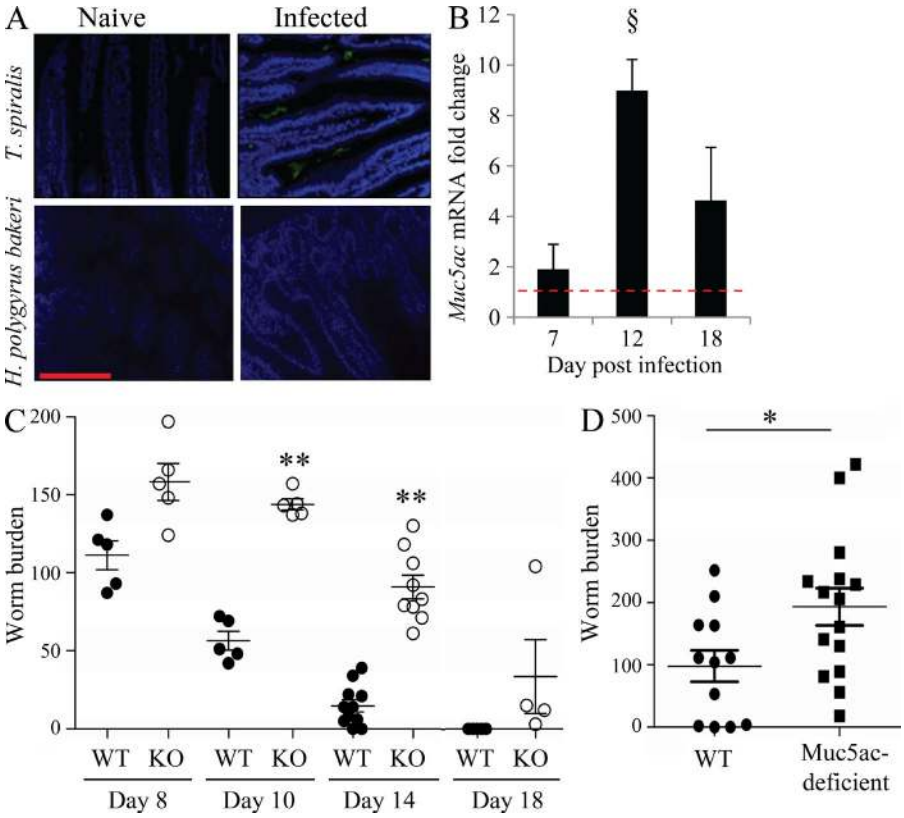

Figure 6. Absence of Muc5ac results in delayed expulsion of *T. spiralis* and *N. brasiliensis*. (A and B) Immunofluorescence microscopy (A) and RT-PCR (B) were used to assess the expression of Muc5ac in WT mice infected with *T. spiralis* and *H. polygyrus bakeri*. No Muc5ac messenger RNA (mRNA) was detected in *H. polygyrus bakeri*–infected mice. The red dashed line indicates naive controls. (C) Worm burdens were assessed in Muc5ac-deficient mice and WT mice on days 10, 14, and 18 p.i. with *T. spiralis*. (D) Worm burdens were assessed in Muc5ac-deficient mice and WT mice on day 8 p.i. with *N. brasiliensis* larvae. Results are representative of combined data from two individual experiments for *T. spiralis* and three individual experiments for *N. brasiliensis* and presented as the mean ± SEM value of four to five mice per group. §, $P < 0.05$ compared with naive controls; *, $P < 0.0264$; and **, $P < 0.001$ compared with WT mice.

(C57BL/6-Tg(Zp3-cre)93Knw/J) and subsequently crossing progeny with C57BL/6J mice. Mice were backcrossed onto a C57BL/6J lineage for eight generations, and saturation of the C57BL/6J genome was confirmed using microsatellite marker–assisted congenic analysis at the University of Texas MD Anderson Cancer Center Genetic Services Facility. Quantitative RT-PCR was used to confirm loss of Muc5ac expression in the stomachs of knockout animals (Fig. S1 E) using methods described previously (Young et al., 2007). All of the animals for the experiments with *T. muris*, *T. spiralis*, and *H. polygyrus bakeri* were maintained in the Biological Services Unit at the University of Manchester. The protocols used were in accordance with guidelines by the Home Office Scientific Procedures Act (1986). All mice were kept in sterilized, filter-topped cages and fed autoclaved food in the animal facilities. Only 6–12-wk-old male mice were used. WT C57BL/6 mice were purchased from the National Institute of Allergy and Infectious Diseases animal facilities at Taconic Farms. 20-wk-old female Muc5ac-deficient and WT mice were used for *N. brasiliensis* infections and were housed under specific pathogen-free conditions at the National Institutes of Health in an American Association for the Accreditation of Laboratory Animal Care–approved facility. The National Institute of Allergy and Infectious Diseases animal care and use committee approved all of the experimental procedures.

Parasitological techniques. The techniques used for *T. muris* maintenance and infection were described previously (Wakelin, 1967). Mice were orally infected with ∼100–300 *T. muris* eggs for a high-dose infection. Worm burdens were assessed by counting the number of worms present in the cecum as described previously (Wakelin, 1967). Techniques used for the maintenance of *T. spiralis* (Wakelin and Lloyd, 1976) and *H. polygyrus bakeri* (Behnke et al., 1993) and the methods used for preparation of infective larvae and assessment of worm burdens have been described previously. Third-stage (L3) infective *N. brasiliensis* larvae were originally obtained from J.F. Urban Jr. (United States Department of Agriculture, Beltsville, MD). Maintenance of mouse-adapted *N. brasiliensis* and infection were performed as previously described at the National Institutes of Health (Camberis et al., 2003). Mice were infected via subcutaneous injection of 500 L3 larvae. Worm burdens were assessed by counting adult worms collected from the small intestine on day 8 p.i. as previously described (Camberis et al., 2003).

Histology, immunohistochemistry, and immunofluorescence microscopy. A 1-cm segment or the whole cecum or small intestine (rolled) was fixed in 95% ethanol and processed by using standard histological techniques. Sections were treated with 0.1 M KOH for 30 min before staining with PAS reaction. Slides were counterstained with either hematoxylin and eosin or 1% fast green. Standard immunohistochemical and immunofluorescent staining methods were used to determine the levels of Muc2 and Muc5ac.

Antibodies. Immunodetection was performed using a polyclonal antibody raised against a murine Muc2 (mMuc2; Heazlewood et al., 2008). Commercially available 45M1 antibody was used for the detection of mouse Muc5ac (Hasnain et al., 2010). Detection of BrdU incorporated into nuclei was performed using a monoclonal anti-BrdU antibody (AbD Serotec). Commercially available mRelm-β (Abcam) and mITF (Santa Cruz Biotechnology, Inc.) antibodies were used to detect Relm-β and Tff3, respectively. The rat immunoglobulin G1 monoclonal antibody XMG1.6 and GL113 antibody (for isotype control) were purified from supernatant by cell culture passage and protein G–Sepharose column and concentrated using a Centricon Centriprep tube. Antibody was administered at 0.5 mg per 200 µl PBS by intraperitoneal injection. Injections were given every 2 d starting from day −2 to day 26 p.i.

Analysis of mucus network properties. Cecal tissue isolated from mice was cut longitudinally, washed with PBS, and kept hydrated in a 6-well plate. 0.1-µm blue fluorescently labeled polymer microspheres (Duke Scientific) were placed on top of the luminal surface of the cecum (set as a reference), and their position was analyzed using the upright confocal microscope (model C1; Nikon). 3D optical stacks were taken every 5 µm and combined to obtain a z stack at the time points stated (Hasnain et al., 2010).

Worm isolation for ATP analysis. The cecum was longitudinally cut and segmented before incubation with 0.1 M NaCl for 2 h at 37°C with frequent shaking. Worms were counted after separation from debris and ECs using a 0.7-µm filter and kept in RPMI 1640 supplemented with 10% FCS. Alive worms were subsequently homogenized using the FastPrep homogenizer (MP Biomedicals).

Energy status of worms. The CellTiter-Glo luminescent cell viability assay (Promega) was performed according to the manufacturer's instructions. Relative light units (RLUs) were calculated per worm: RLU = (sample light units − blank light units)/number of worms. Substrate only was used as a blank control, whereas worms were boiled before homogenization for negative controls. To determine recovery of energy status, worms recovered on day 19 p.i. were washed extensively in DMEM and added to 6-well plates with LS174T cells (maintained as previously described) for 24 h before measuring ATP levels (Hasnain et al., 2010). MUC5AC and MUC2 were purified as previously described (Sheehan et al., 2000). Mucins were reduced, carboxymethylated, and treated with trypsin as previously described (Carlstedt et al., 1983).

Rate of EC turnover. The rate of intestinal EC turnover was assessed by visualizing BrdU incorporated into nuclei after mice were injected with 10 mg BrdU 16 h before sacrifice as described previously (Cliffe et al., 2005).

In vitro cytokine analysis. mLNs were removed, and cells were isolated and resuspended at 5×10^6 cells/ml in RPMI 1640 with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cultures were stimulated with 50 µg/ml *T. muris* excretory/secretory antigen for 24 h at 37°C and 5% CO_2. Cell-free supernatants were stored at −80°C; cytokine analysis was performed by cytometric bead assay (BD; Hayes et al., 2007). The analysis of *T. muris*–specific antibodies (IgG2a) was performed by sandwich ELISA using homogenized intestinal tissue.

Quantification of histological staining. The numbers of goblet cells expressed per crypt were counted in 50 longitudinally sectioned crypt units. The area stained (pixel/square millimeter) per 100 crypts was determined by using ImageJ software version 1.39a (National Institutes of Health).

Statistical analysis. All results are expressed as the mean ± SEM. Statistical analysis was performed using Prism version 3.2 (GraphPad Software). Statistical significance of different groups was assessed by using parametric tests (one-way analysis of variance with posttest according to statistical standards or paired Student's *t* test). $P < 0.05$ was considered statistically significant.

Online supplemental material. Fig. S1 shows the generation of Muc5ac-deficient mice. Fig. S2 shows that the EC escalator is active in the Muc5ac-deficient mice. Fig. S3 shows the absence of Muc5ac in Muc5ac-deficient mice. Fig. S4 shows that the same goblet cells can produce Muc2 and Muc5ac. Fig. S5 shows that neutralization of IFN-γ induces a T_H2 immune response without altering the outcome of infection. Fig. S6 shows chronicity despite the presence of goblet cell bioactive factors. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20102057/DC1.

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