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ORIGINAL ARTICLE

Listerial invasion protein internalin B promotes entry into ileal Peyer's patches *in vivo*

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ABSTRACT

Listeria monocytogenes **(Lm) invades the host intestine using listerial invasion proteins, internalins. The *in vivo* role of internalin A (InlA) and internalin B (InlB) is reported here. Intragastric (i.g.) administration and ligated loop assays with Δ*inlB*-Lm demonstrated that a lack of InlB significantly attenuates the invasive ability of Lm into various organs. On the other hand, *InlA*^{m}-Lm expressing a mutant InlA with two substitutions, S192N and Y369S, which has been reported to increase the affinity of InlA to mouse E-cadherin, resulted in little increase in intestinal infection according to both ligated loop and i.g. infection assays. Lm preferentially enters ileal Peyer's patch (PP) via M cells and Δ*inlB*-Lm showed severely reduced ability to invade though these cells. The present results reveal the importance of InlB, which accelerates listerial invasion into M cells on ileal PPs *in vivo*.**

Key words internalin B, *Listeria monocytogenes*, M cell, Peyer's patches.

Listeria monocytogenes (Lm) is a gram-positive intracellular pathogen that causes severe disease in both humans and animals. Lm mainly infects immunocompromised individuals, pregnant women, neonates, and elderly people via the intestine, causing listeriosis which results in bacteremia, sepsis, meningitis, encephalitis, and abortion (1).

Lm infects both phagocytes and a wide range of non-phagocytic cells. Entry into non-phagocytic cells is mediated by the Lm invading proteins, InlA and InlB in Lm. InlA promotes listerial uptake into human IECs, which express an InlA receptor, E-cad (2). InlB has been shown to promote invasion into hepatocytes expressing a major InlB receptor, c-Met, which is a receptor for hepatocyte growth factor. InlB activates c-Met, and facilitates entry into cells by inducing actin polymerization (3). In addition, gC1qR, a complement receptor, and glycosaminoglycans also bind InlB and promote invasion. Recent studies have shown involvement of other proteins such as Septin 2, Septin 11 and CD44v6 (3–5).

Although various previous studies have revealed the role of InlA and InlB *in vitro*, listerial invasion mechanisms *in vivo* are still largely unclear except for a few

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List of Abbreviations: BHI, brain heart infusion; cfu, ycolony forming units; DC, dendritic cell; E-cad, E-cadherin; FITC, fluorescein isothiocyanate; hE-cad, human E-cad; IEC, intestinal epithelial cell; i.g., intragastric; ILF, isolated lymphoid follicle; InlA, internalin A; InlB, internalin B; Lm, *Listeria monocytogenes*; mLN, mesenteric lymph node; PP, Peyer's patch; *S. enteric, Salmonella enteric*; Tg, transgenic; TRITC, tetramethyl rhodamine isothiocyanate; wt, wild type; *Y. enterocolitica, Yersinia enterocolitica*

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reports demonstrating *in vivo* listerial invasion into IECs in the guinea pig and gerbil and its entry via PPs in mouse intestine (1, 6).

Most *in vivo* studies with Lm and mice have employed non-natural routes, such as intravenous or intraperitoneal inoculation, to infect the mice because of the poor invasive efficiency of Lm via the mouse intestine. This poor efficiency is believed to be due to differences between humans and mice in the structure of E-cad (7). To overcome this problem, hE-cad Tg mice expressing hE-cad on their IECs have been developed (8). However, a relatively large number of bacteria are still required for establishment of infection, possibly due to the fact that hE-cad is expressed only on the IECs in hE-cad-Tg mice. In 2007, it was reported that a structural biological approach employing two substitutions of Ser192 and Tyr369 of InlA with Asn and Ser, respectively, increased the affinity of InlA to mouse E-cad, and that Lm expressing this mutated *inlA* (*inlA*^{m}-Lm) was able to infect mice *in vivo* (9).

In the present study, we constructed *inlA*^{m}-Lm and *inlB* deleted-Lm (Δ*inlB*-Lm) to further examine the role of InlA and InlB in invading the mouse intestine *in vivo*. Our results show that Lm enters ileal PPs mainly via M cells depending on InlB.

MATERIALS AND METHODS

Mice

BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). All mice were kept in our specific pathogen-free animal facility. For all experiments, sex and age matched mice were used and maintained under specific pathogen free conditions before infection. Experiments were performed in accordance with our institutional guidelines. PP-null mice were obtained by intravenous administration of IL-7Rα Ab into pregnant mice as previously described (10, 11).

Listeria

A streptomycin resistant *Listeria monocytogenes* strain 10403s was obtained from T. Chakraborty (University of Manitoba, Winnipeg, MB, Canada). Bacteria were cultured at 37°C, dissolved in sterile PBS, and prepared for freeze stock as previously described (12). For immunohistochemistry, Lm were labeled with FITC or TRITC as previously described (13).

Construction of mutated Lm

Full length DNA encoding *inlA* and *inlB* was amplified from genomic DNA with the primers 5′-GAGCTCACCA ACGAGCCAACCGTGGTAA-3′ and 5′-GGATCCAT TTAATGCGTAGCCTCCAAGTCC-3′, and inserted into the vector pBluescript KS(-). To make *inlA*^{m}-Lm, site-direct mutations in S192N and Y369S were introduced using a Quikchange Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) as previously described (9). To make Δ*inlB*-Lm, AccIII sites were introduced between *inlA* and *inlB* loci and in the *inlB* locus using a Quikchange Mutagenesis kit (Agilent Technologies) with the primer 5′- AGCTAGATGTGGTTTCCGGACTATATCTAGC-3′ and 5′-GCTAGATATAGTCCGGAAACCACATCTAGCT-3′. The vector containing the newly introduced AccIII sites was digested with AccIII, and the longer fragment was religated. The plasmid containing the *inlA* mutation or *inlB* deletion was digested and the fragment introduced into a pAUL-A shuttle vector (14). The plasmids were introduced into Lm by electroporation. The transformants were screened on BHI agar plates supplemented with 5 μg/mL of erythromycin, and recombinants selected by temperature sensitivity. Mutations were confirmed by sequencing on a Perkin Elmer ABI Prism-310 (Boston, MA, USA).

In vitro invasion assay

Caco2 (ATCC HTB-37) and CMT-93 (ATCC CCL-223) cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) with 10% FCS, and 1% nonessential amino acids at 37°C/5% CO_2. A few days prior to *in vitro* infection, 3×10^5 Caco2 or CMT-93 cells were seeded on 24 well plates. Lm were added to monolayers of cells in each well at a multiplicity of infection of 4, centrifuged for 5 min at 500 *g*, and incubated at 37°C for 1 hr. The cells were then washed three times with wash buffer solution (sterile PBS containing 50 μg/mL gentamicin), and cultured with media containing 100 μg/mL gentamicin to kill any extracellular bacteria. After 4 hr, the cells were washed five times with wash buffer solution, and lysed with lysis buffer solution (sterile water containing 0.2% Triton X-100). Cell lysates were diluted ad libitum and plated onto BHI agar plate containing 50 μg/mL streptomycin. After incubation at 37°C for 24 hr, the colonies were counted.

Ligated loop assay

Before being subject to surgery, the mice were fasted overnight to reduce their gastrointestinal contents. After anesthetization with 40 mg/kg sodium pentobarbital, a midline laparotomy incision of approximately 7 mm was made to expose the cecum and small intestine. The intestine was ligated at the medial jejunum and distal ileum with silk thread, isolating approximately 23 cm of small intestine, and 1×10^9 cfu Lm in 700 μL of sterile PBS were injected

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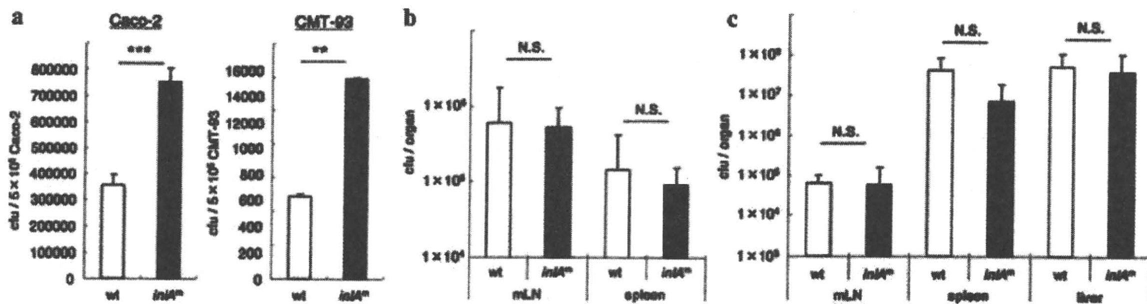

Fig. 1. The efficiency of intracellular growth in cell lines and bacterial burdens in mouse with wt-Lm and *inlA*^{m}-Lm. (a) Intracellular growth curve of wt-Lm and *inlA*^{m}-Lm in Caco-2 and CMT-93 cells. (b) The number of Lm in mLN and spleen in ligated loop assay ($n = 8$ for each Lm). (c) The number of Lm in mLN, spleen, and liver on day 3 after i.g.-inoculation ($n = 13$ for each Lm). **, $P < 0.01$; ***, $P < 0.001$; N.S., not significant.

into the loop with a 30G needle. The intestines were then returned to the abdominal cavity and the incision closed. After 30 min, the abdomen was reopened, and the mLN and spleen were harvested. All PPs, and 2 cm of intestine without PPs in the jejunum or ileum, were excised from the ligated loop region. The villi and PPs were washed three times in sterile PBS containing 50 μg/mL gentamicin, and weighed. Lm which had invaded the various organs were homogenized in lysis buffer solution containing 100 μg/mL streptomycin and plated as in the *in vitro* "invasion" assay.

Intragastric inoculation

Mice were infected by gavage with 1×10^7 cfu of Lm. After three days, the spleen, liver, and mLN were harvested. Bacterial count was performed as in the "ligated loop" assay.

Immunohistochemistry

After ligated loop assay with an equal number of FITC-labeled wt-Lm and TRITC-labeled Δ*inlB*-Lm, the small intestines were dissected. Each sample was mounted, blocked, and fixed as previously described (12). Sections were stained with biotin-conjugated anti-mouse GP2 antibody (15) and PE-conjugated *Ulex europaeus* agglutinin-1 for M cells, Alexa Fluor Phalloidin (Invitrogen) to visualize actin filaments, and 4′,6-diamidino-2-phenylindole for the nuclei. Slides were examined under a confocal fluorescent microscopy Carl Zeiss LSM 510 (Jena, Germany).

Statistical analysis

Statistical analysis was performed using the Mann-Whitney U-test for *in vitro* assay, F-test for *in vivo* assay, Kaplan-Meier method for survival rate, and Bonferroni method for multiple sample comparisons. *P*-values < 0.05 were considered significant. Error bars denote the SD, unless otherwise stated.

RESULTS

Role of *inlA* and *inlA*^{m} *in vivo*

To assess the function of InlA in mouse, we first aimed to establish a mouse model of i.g.-infection using *inlA*^{m}-Lm and the procedure described in a previous study (9). We employed an Lm 10403s strain as a parental strain; this enabled us to distinguish Lm from other intestinal bacteria by selection with streptomycin. Internalization of *inlA*^{m}-Lm into a human IEC line, Caco-2, was increased twofold compared to wt-Lm (Fig. 1a). Similarly, entry of *inlA*^{m}-Lm into a mouse IEC line, CMT-93, was also increased twofold (Fig. 1a). We noted, however, that the efficiency of entry of *inlA*^{m}-Lm into CMT-93 was still 50-fold lower than that into Caco-2.

Next, we performed a ligated loop assay and i.g.-infection to investigate the invasive capacity of *inlA*^{m}-Lm into mouse intestine *in vivo*. No significant differences between wt-Lm and *inlA*^{m}-Lm were observed in bacterial burdens in either mLN or spleen (Fig. 1b, c), indicating that the mutations in *inlA*^{m} were insufficient to make the Lm capable of invading mouse intestine.

The role of InlB *in vivo*

We next focused on InlB. Although InlB is considered to be important in the invasion of Lm into hepatocytes, the *in vivo* function of InlB has thus far been unclear. We therefore constructed Δ*inlB*-Lm using the 10403s strain. In an *in vitro* invasion assay, although the number of Lm entering Caco-2 cells was similar in wt-Lm and Δ*inlB*-Lm, internalization of Δ*inlB*-Lm declined to 1% of wt-Lm in CMT-93 (Fig. 2a). In addition, Δ*inlB*-Lm showed

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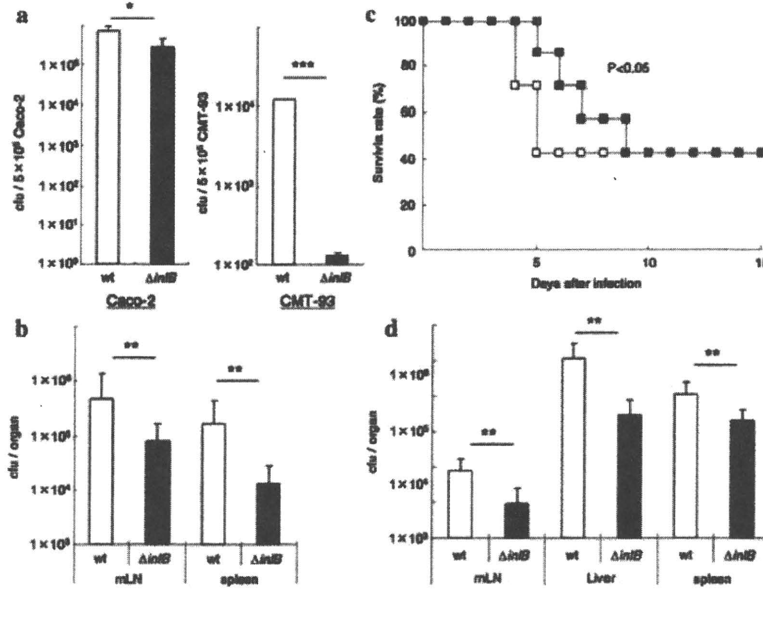

Fig. 2. The efficiency of intracellular growth in cell lines and bacterial burdens in mouse infected with wt-Lm and Δ*inlB*-Lm. (a) Intracellular growth in Caco-2 and CMT-93 cells. (b) The number of Lm in mLN and spleen in ligated loop assay ($n = 11$ for each Lm). (c) Survival curves of mice i.g.-infected with wt-Lm or Δ*inlB*-Lm ($n = 7$ for each Lm). The longer survival of Δ*inlB*-Lm infected mice is significant as assessed by the Kaplan-Meier method ($P < 0.05$). Closed squares, Δ*inlB*-Lm infected mice, open squares, wt-Lm infected mice. (d) The effect of bacterial burdens in mLN, liver and spleen of mouse infected with wt-Lm and Δ*inlB*-Lm on day 3 after i.g.-inoculation ($n = 15$ for each Lm). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

less efficiency in invading mLN and spleen in the ligated loop assay, in which the efficiency was approximately 10% of wt-Lm (Fig. 2b). These results collectively suggest the importance of InlB in invasion of mouse intestine.

Based on the findings that InlB might accelerate the entry of Lm into the intestine, we examined the role of InlB on the survival of mice *in vivo*. In a mortality assay, mice infected with Δ*inlB*-Lm showed a significantly longer survival relative to wt-Lm (Fig. 2c). On day 3 after i.g.-infection, the loads of Δ*inlB*-Lm in various organs were significantly smaller than were those of wt-Lm. The bacterial loads of Δ*inlB*-Lm showed significant decreases in the mLN (90%), spleen (90%), and liver (99%) as compared to wt-Lm (Fig. 2d). These results collectively indicate that InlB promotes intestinal invasion and growth in various organs *in vivo*.

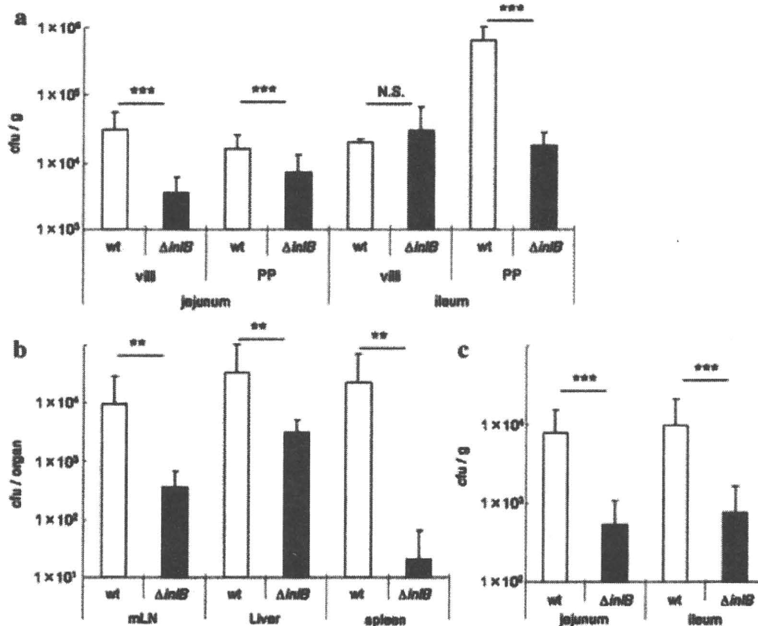

Fig. 3. Entry of wt-Lm and Δ*inlB*-Lm into mouse IECs and PPs. (a) The number of Lm in villi and PPs of jejunum or ileum in ligated loop assay ($n = 9$ for each Lm). Multiple comparisons of the numbers of bacteria in different tissues for wt-Lm or Δ*inlB*-Lm were performed by the Bonferroni method. Ileal PPs had significant difference in samples inoculated with wt-Lm ($P < 0.01$), whereas no significant difference was observed in samples inoculated with Δ*inlB*-Lm. Significant differences in invasion of each portion of intestine by *inlB* deficiency are shown by asterisks. (b,c) The efficiency of bacterial burdens in PP-null mouse at ligated loop assay (b) in each organ and (c) small intestine ($n = 8$ in each Lm). **, $P < 0.01$; ***, $P < 0.001$; N.S., not significant.

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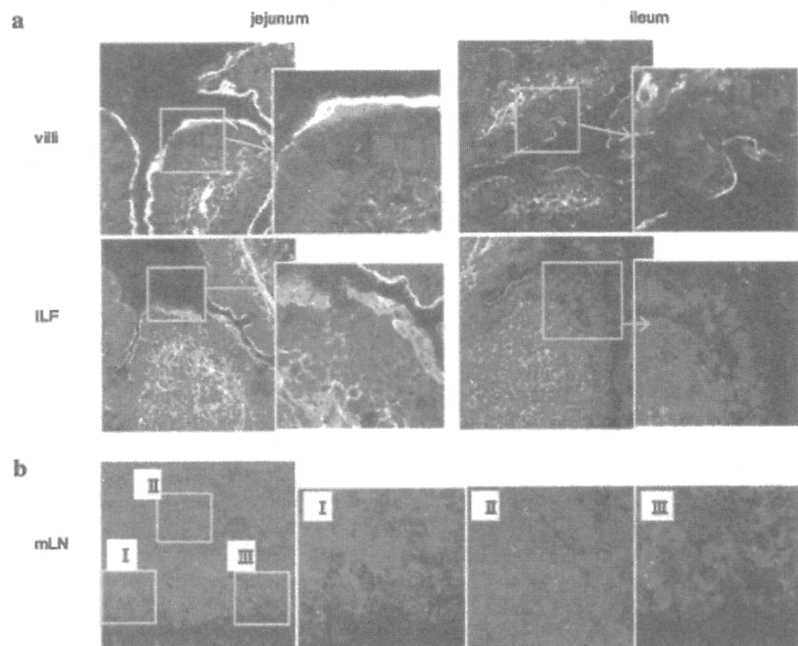

Fig. 4. Fluorescence staining of wt-Lm and Δ*inlB*-Lm in villi or ILF in (a) jejunum and ileum, and (b) mLN. FITC conjugated wt-Lm (green) and TRITC conjugated Δ*inlB*-Lm (red) were administered into a ligated loop. Nuclei (blue) and actin filaments (white) were counterstained. Right panels are enlarged images of portions of the left panel. The roman numerals in the right panels denote their sites of origin in the left panel.

InlB promotes entry into ileal M cells on PPs

The main invasive pathway of Lm remains undetermined, even though IECs or PPs are the most commonly recognized invasive routes. To determine which of these two is critical, and whether InlB participates in entry, we performed a ligated loop assay. We counted the number of Lm in PPs and a 2 cm portion of jejunum or ileum without PPs. The results showed that ileal PPs were the main invasion site of Lm (Fig. 3). The invasion of wt-Lm into ileal PPs was more than 10-fold greater than into other sites (Fig. 3a, open bars). Invasion into ileal PPs was much less efficient in the absence of InlB (Fig. 3a, closed bars). In addition, the loads of Δ*inlB*-Lm in jejunal villi and jejunal PPs were also reduced compared to wt-Lm (Fig. 3a).

Based on the above results, we next carried out a ligated loop assay in PP-null mouse to examine the importance of PPs in InlB dependent entry. The bacterial burdens in each organ of PP-null mice (Fig. 3b, open bars) were significantly reduced compared to those in normal PP-sufficient mice inoculated with wt-Lm (Fig. 2b, open bars). The listerial counts in the jejunum and ileum were not significantly reduced in PP-null mice compared to normal mice (Fig. 3a, c, open bars). Interestingly, deficiency in *inlB* reduced the invasive number in each organ even in PP-null mice (Fig. 3b, c, closed bars).

We further compare the localization of wt-Lm and Δ*inlB*-Lm using a ligated loop assay and fluorescence microscopic analysis with a equal mixture of FITC-labeled wt-Lm and TRITC-labeled Δ*inlB*-Lm (Fig. 4). A small number of Lm were observed in villi and ILF in both jejunum and ileum (Fig. 4a). In addition, numerous Lm had invaded ileal PPs, whereas only modest numbers of Lm were detected in jejunal PPs (Fig. 5a). Moreover, many Lm were observed in M cells in PPs (Fig. 5b). As shown in Figure 5c, most of the Lm were observed in or next to M cells. Consistent with the results shown in Figure 2b, the uptake of Δ*inlB*-Lm in PPs and mLN was less than that of wt-Lm (Figs 4b, 5a). In mLN, bacteria were observed on the edges of the mLN, and there was no difference in the localization of wt-Lm and Δ*inlB*-Lm.

DISCUSSION

In this study we have examined the *in vivo* role of InlA and InlB in listerial infection. Based on previous studies demonstrating the involvement of E-cad and InlA (8, 9), we first focused on InlA.

We generated *inlA*^{m}-Lm as described previously (9) in a 10403s strain in order to distinguish Lm from other intestinal bacteria by using their differences in streptomycin resistance. The present results show that the two amino acid substitutions of *inlA* were not sufficient to achieve effective *in vivo* infection. Inoculation of 10^7 bacteria was needed to detect organ invasion with *inlA*^{m}-Lm. In addition, the loads of *inlA*^{m}-Lm in various organs were similar

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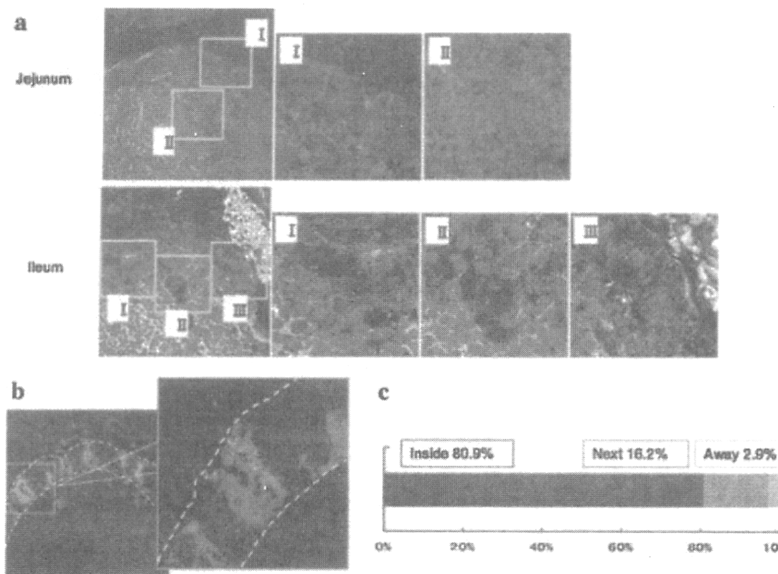

Fig. 5. Lm invades into PP via M cells. FITC conjugated wt-Lm (green) and TRITC conjugated Δ*inlB*-Lm (red) were administered into a ligated loop. (a) Fluorescence staining of PPs. Counterstaining was performed as described in Fig. 4. (b) Fluorescence staining of M cells on ileal PPs. Frozen sections were stained with anti-GP2 (blue) and UEA1 (red). (c) The proportions of Lm invading inside, next to, or away from M cells were calculated. Right panels are enlarged images of portions of the left panel. The roman numerals in the right panels denote their sites of origin in the left panel of the same row.

to those of wt-Lm. If *inlA*^{m}-Lm is very efficient at invasion, as reported previously (9), listerial numbers in each organ in *inlA*^{m}-Lm infected mice would have increased compared to wt-Lm at early time points after infection, such as on day 1 or 2 post infection. However, it was not until day 4 that a relative increase in the number of *inlA*^{m}-Lm in internal organs compared to wt-Lm was observed (9), suggesting that *inlA*^{m}-Lm has an *in vivo* invasive ability similar to that of wt-Lm. We used the 10403s strain as a parental strain, in contrast to the previously used strain, EGD. It is possible, although unlikely, that the discrepancy between our results and previously reported results is due to the difference in parental strains.

It is also possible that the binding of InlA and E-cad alone is insufficient for *in vivo* invasion. Other protein(s) may play an important role as a co-receptor(s) for InlA-mediated invasion and such protein(s) is not functional in mice.

There are few studies demonstrating the *in vivo* role of InlB, except for one that shows that InlB is involved in crossing the placental barrier during pregnancy in the gerbil (6). We have revealed here the importance of InlB in *in vivo* invasion of Lm, as systemic spread of Lm via i.g.-infection was impaired more than 10-fold in the absence of InlB. Our results also show that the main invasive route of Lm is ileal PPs, and this route is dependent on InlB (Fig. 3).

We confirmed by fluorescent staining analysis that Lm is indeed localized within the M cells of ileal PPs. Entry through PPs has been demonstrated for various enteroinvasive pathogens. The bacterial protein of *Yersinia pseudotuberculosis*, invasin, promotes invasion into M cells (16). *S. enterica* serovar Typhimurium also enters M cells on PPs (17). However, the precise role of PPs in listerial uptake *in vivo* has been unclear (18). Although entry of Lm into PPs has been observed in a previous study, the study reported that listerial entry is independent of InlA or InlB (19). This apparent discrepancy may be ascribed to the *in vitro* system used in that study. Our present results clearly demonstrate the importance of InlB-dependent entry through M cells of ileal PPs *in vivo*.

Besides PPs, it has been demonstrated that InlB is involved in entry at the murine villous tip (20). We also noted that entry into IECs was reduced by a lack of InlB (Fig. 3a, c). Based on the large decrease in invasion into PPs when InlB is lacking (Fig. 3a), we speculate that the contribution of InlB to invasion into PP is greater than that into IECs. Furthermore, it has been suggested that lamina propria DCs expressing the chemokine receptor CX3CR1, as well as villous M cells, can directly sample luminal antigen (21, 22). It is possible that InlB contributes to listerial uptake by villous M cells and/or DCs.

With only a few exceptions, the molecular mechanisms by which bacteria are transported through M cells have been unclear. GP2 of M cells bind the FimH gene product of gram-negative bacteria such as *Escherichia coli*, *S. enterica* serovar Typhimurium, *Salmonella enteriditis* and play a pivotal role of transcytosis of these bacteria (15). Many other enteroinvasive pathogens including Lm, *Pseudomonas aeruginosa*, and *Y. enterocolitica* do not express FimH, and no difference in invasion into PPs has been

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shown in GP2 knockout mice infected with *Y. enterocolitica* (15). It is presently unknown whether c-Met is expressed on M cells and the molecular target of InlB-dependent Lm entry into PPs remains to be elucidated in future studies.

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CHAPTER 2

Natural Helper Cells: A New Player in the Innate Immune Response against Helminth Infection

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Abstract

The Th2-type immune response, characterized by the production of IL-4, IL-5, and IL-13, is a critical immune response against helminths invading cutaneous or mucosal sites. Th2 cytokines are induced soon after helminth infection, even before a pathogen-specific adaptive immune response is established. Although the expulsion and clearance of helminths usually requires pathogen-specific Th2-mediated immunity, early induction of Th2 cytokines during the innate immune phase is important for host protection from helminth invasion. Recent studies have shed light on such Th2 cytokine production by formerly uncharacterized innate immune cells such as a newly identified natural helper cell. We discuss here the mechanisms of innate production of Th2 cytokines in host immune responses against helminth infection.

1. INTRODUCTION: HELMINTH INFECTION AND TH2 IMMUNITY

The World Health Organization reported that more than 1 billion people suffer from neglected tropical diseases such as helminthiasis, which is a major health problem throughout developing countries and a food safety issue worldwide (Albonico *et al.*, 1999; Savioli, 2009). A major concern of helminth infection is that these metazoan parasites may also impair effective immune responses against other microbial pathogens, including *Mycobacterium tuberculosis* and human immunodeficiency virus (HIV; Hotez and Kamath, 2009; Willyard, 2009).

A wide variety of lumen/tissue-dwelling helminths infect humans including *Anisakis* spp. (*Anisakis physeteris*, *Anisakis simplex*, *Pseudoterranova decipiens*), guinea worms (*Dracunculus medinensis*), hookworms (*Ancylostoma duodenale* and *Necator americanus*), lymphatic filaria (*Brugia malayi*, *Brugia timori*, *Wuchereria bancrofti*), pinworms (*Enterobius vermicularis*), roundworms (*Ascaris lumbricoides*), subcutaneous filaria (*Mansonella streptocerca*, *Oncocerca volvulus*), threadworms (*Strongyloides stercoralis*), trichina worms (*Trichinella* spp.), and whipworms (*Trichuris trichiura*) (Albonico *et al.*, 1999). Helminth infections are also a major concern in veterinary medicine (Urban *et al.*, 2007).

Each type of helminth has a unique strategy for infecting and taking up residence in a distinct microenvironment in the host. Conversely, the host employs a variety of immune cells to expel those invading helminths. The Th2-type immune response characterized by the production of IL-4, IL-5, and IL-13 is induced against helminths invading cutaneous or mucosal sites and functions as protective immunity against those pathogens. Interestingly, Th2 cytokines are induced soon after helminth infection and before pathogen-specific Th2 cells are established. Although

the final expulsion of helminths usually requires Th2-mediated immunity, an early Th2-type innate immune response is important in the restriction of helminth invasion before the adaptive immune response initiates. Recent studies shed light on formerly uncharacterized innate immune cells that carry out such innate Th2 cytokine production.

2. HELMINTHS' INVASION AND HOST IMMUNE RESPONSES

Host immune responses to several species of helminths have been studied using animal models. Helminths have adapted to reside in the hosts during evolution and their unique life cycles have been established likely through the fierce battles raging between the invading helminths and the host's immune responses. As a result, each helminth has a unique, characteristic life cycle and the host immune system deals with each type of helminth in a distinct way (Patel *et al.*, 2009; Fig. 2.1).

2.1. Helminth life cycles

2.1.1. *Trichinella spiralis* (Fig. 2.1A)

The encysted first stage larva excyst in the host stomach a few hours after ingestion of contaminated meat and invade duodenal or jejunal epithelium. Larvae rapidly mature into adult worms and adult worms mate during the next 36 h. Adult worms reside in the intestine by forming syncytia within the epithelial cell layer. One week after infection, female worms begin to release larvae. Larvae enter intestinal lymphatics or mesenteric venules and migrate throughout host body, settling most heavily in host striated muscle. This invasion induces differentiation of muscle cells into nurse cells and encapsulation begins 17–21 days after infection. In the rodent intestine, adult worms can be expelled in less than 2 weeks while they remain in the human gut for several weeks (Capó and Despommier, 1996; Murrell, 1985).

2.1.2. *Trichuris muris* (Fig. 2.1B)

The first stage larvae hatch from environmentally resistant eggs that are orally ingested by the host. Larvae immediately invade duodenal or jejunal mucosa and reside there for a few days. The developed larvae then migrate to the cecum and proximal colon where they invade the mucosal epithelial cells at the crest of the crypt, such that the worm's head and part of its filamentous anterior portion are embedded in host mucosal epithelia. Thus, the worms survive and grow to egg-laying adult worms in an isolated tunnel-like environment (Mahida, 2003).

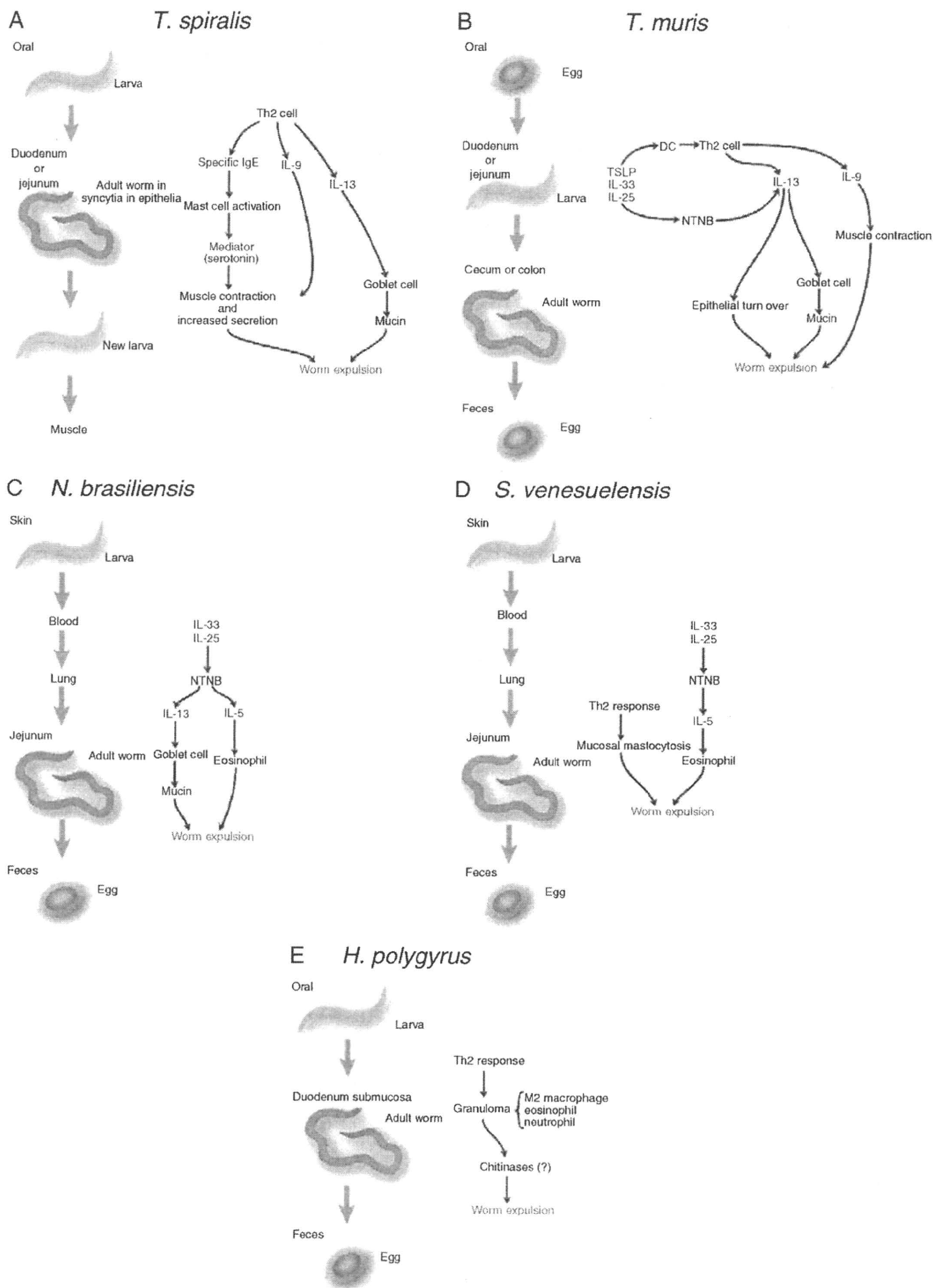

FIGURE 2.1 Schematic models of the course of helminths' infection and the path of host immunity for the expulsion of helminths. (A) *T. spiralis*, (B) *T. muris*, (C) *N. brasiliensis*, (D) *S. venezuelensis*, and (E) *H. polygyrus*.

2.1.3. *Nippostrongylus brasiliensis* and *Strongyloides venezuelensis* (Fig. 2.1C and D)

Infective third stage larvae penetrate the host skin, travel to the blood stream via subcutaneous lymphatics and eventually migrate to the lung 24–48 h later. Larvae are then coughed up and swallowed, and mature into adults in the jejunum. Adult worms begin to produce eggs 5–6 days after infection. Adult worms are usually expelled from the gut less than 2 weeks after infection (Camberis *et al.*, 2003; Negrão-Corrêa, 2001; Vadlamudi *et al.*, 2006).

2.1.4. *Heligmosomoides polygyrus* (Fig. 2.1E)

This nematode generally establishes a chronic infection and lives in the gut of mammalian hosts. Infective third stage larvae enter the wall of the anterior small intestine within 24–72 h of oral infection, reside in the duodenal mucosa and migrate to the submucosa. One week after infection, they return to the gut lumen and rapidly mature into adults. Adult worms reside in host intestinal mucosa and survive for several months (Camberis *et al.*, 2003; Negrão-Corrêa, 2001; Robinson *et al.*, 1989).

2.2. Host's immune responses

2.2.1. *Trichinella spiralis* (Fig. 2.1A)

Worm expulsion is dependent on Th2 immune responses. The Th2 cytokines, IL-4 and IL-13, are critical for worm expulsion and the inhibition of both cytokines significantly delays worm expulsion (Finkelman *et al.*, 2004). Eosinophils are able to kill larvae *in vitro* but IL-5 and eosinophilia are dispensable for *T. spiralis* expulsion (Dixon *et al.*, 2006; Gurish *et al.*, 2002).

Mast cells and *T. spiralis*-specific IgE play important roles in protective immunity (Gurish *et al.*, 2004; Knight *et al.*, 2002; McDermott *et al.*, 2003) although one report showed that *T. spiralis* can be expelled in B cell deficient mice (Finkelman *et al.*, 2004). It has been suggested that gut smooth muscle contraction induced by mast cell-derived serotonin is important for the worm expulsion (Vermillion and Collins, 1988) and that a mast cell-mediated chloride ion-dependent increase in short circuit current induces fluid secretion, which also plays a role in the expulsion of this helminth (Finkelman *et al.*, 2004; Harari *et al.*, 1987). Capture of helminth antigens by IgE bound to a high affinity Fc receptor for IgE (FcεRI) on mast cells induces mast cell degranulation, releasing various chemical mediators including histamine and serotonin. Goblet cell hyperplasia is also involved in protection from mucosal stage *T. spiralis* as mucins produced by IL-13-stimulated goblet cells block adhesion of worms on the epithelial layer (Knight *et al.*, 2008).

2.2.2. *Trichuris muris* (Fig. 2.1B)

Comparison of resistant and susceptible strains suggests that the Th2 response is also critical for the expulsion of *T. muris*. In fact, Th2 cells accumulate in the epithelium at the time of worm expulsion and macrophages accumulate in the lamina propria (Little *et al.*, 2005). Thymic stroma lymphopoietin (TSLP) derived from epithelial cells may play a role in supporting Th2 cells in the epithelium as blockade of TSLP function by deletion of TSLP receptor, suppression of TSLP production by IKKβ inhibition reduces Th2 cells and enhances Th1 and Th17 cells in the epithelia (Zaph *et al.*, 2007). IL-33 is also reported to be involved in the expulsion of *T. muris* (Humphreys *et al.*, 2008). In addition to goblet cell hyperplasia, IL-13 accelerates the migration and turnover of epithelial cells. Epithelial cell turnover dislodges *T. muris* from its niche in the lumen. Blocking of CXCL10 also enhances epithelial cell turnover and such forced epithelial cell turnover was sufficient to expel the worm in SCID mice (Cliffe *et al.*, 2005), indicating that accelerated epithelial cell turnover is an important expulsion mechanism for *T. muris*. IL-9 is also reported to be involved in the expulsion of *T. muris* and induces colonic muscle hypercontractility in a mast cell-independent manner; such muscle contraction helps the clearance of *T. muris* but not *T. spiralis* (Khan *et al.*, 2003; Richard *et al.*, 2000).

2.2.3. *Nippostrongylus brasiliensis* and *Strongyloides venezuelensis* (Fig. 2.1C and D)

Migration of these helminths to the lung induces an inflammatory response characterized by pulmonary eosinophilic infiltration. IL-5 and eosinophilia play important roles in the protective immunity against *N. brasiliensis* and *S. venezuelensis* in the lung stage as eosinophils effectively kill larvae of *N. brasiliensis* and *S. venezuelensis* (Dent *et al.*, 1999; Korenaga *et al.*, 1994). During the intestinal stages, the Th2 immune response is also critical for the expulsion of *N. brasiliensis* and *S. venezuelensis*. Mast cells are not essential in the expulsion of *N. brasiliensis* in the gut stage as *N. brasiliensis* can be expelled from W/W^v mice. Although the expulsion is slower in W/W^v mice than in wild-type mice, the slow expulsion in W/W^v mice was not corrected by wild-type bone marrow transfer, suggesting that the slow expulsion is in part due to the impaired development of the c-Kit$^+$ interstitial cell of Cajal, a pacemaker cell in the intestine (Ishikawa *et al.*, 1994). Goblet cell hyperplasia followed by mucin secretion is an important mechanism of *N. brasiliensis* expulsion (Fallon *et al.*, 2006; Ishikawa *et al.*, 1993; McKenzie *et al.*, 1998).

In contrast to *N. brasiliensis* infection, *S. venezuelensis* infection resolves significantly more slowly in W/W^v mice compared to *N. brasiliensis* infection in W/W^v mice and bone marrow transplantation significantly

corrected the protection against *S. venezuelensis* (Khan *et al.*, 1993). Mast cells of the mucosal type are important in this process and chondroitin sulfate secreted by mucosal mast cells is involved in the expulsion of *S. venezuelensis* (Maruyama *et al.*, 2000). Expulsion of *S. venezuelensis* was slow in mice lacking gastrointestinal mast cells but adoptive transfer of bone marrow-derived cultured mast cells (BMMCs) was sufficient to correct the protective immunity (Fukao *et al.*, 2002). It is of note that incubation of BMMCs with Th2 cytokines such as IL-4 and IL-10 was critical for the expulsion of *S. venezuelensis*, underscoring the importance of Th2 cytokines for arming mucosal mast cells (Fukao *et al.*, 2002; Ghildyal *et al.*, 1992). Mucosal mast cells also secrete β-chymase and mouse mast cell protease (mMCP)-1 for increasing the permeability of the epithelial layer by degrading tight junction proteins (McDermott *et al.*, 2003). One of the molecules secreted by goblet cells, resistin-like molecule (RELM)-β, binds to *S. stercoralis* and impairs the chemosensory mechanisms of the worm (Artis *et al.*, 2004).

2.2.4. *Heligmosomoides polygyrus* (Fig. 2.1E)

The inoculation of *H. polygyrus* results in chronic infection. At 4 days after infection, third stage larvae in the duodenum are surrounded by innate immune cells, neutrophils, and macrophages. Treatment of infected mice with antihelminth drugs clears the helminth and such mice can expel the worm upon subsequent secondary infection, indicating the establishment of adaptive immunity and immunological memory. After secondary infection, larvae in the duodenum are again surrounded by granuloma with neutrophils and M2-type or oxidative macrophages induced by Th2 cytokines (Fairweather and Ciháková, 2009; Murata *et al.*, 2002). These are distinct from Th1-type granulomas consisting of M1-type or reductive macrophages induced by *M. tuberculosis*. Furthermore, dendritic cells (DCs), Th2 cells, and eosinophils are also observed around the granuloma structure (Morimoto *et al.*, 2004). Although there is no concrete evidence that such Th2-type granulomas directly function in the expulsion of worms, M2-type macrophages may be involved in the expulsion by secreting chitinase and related family members including RELM-α and -β (Anthony *et al.*, 2006).

3. INDUCTION OF TH2 IMMUNE RESPONSES

Although the final effector mechanisms that expel helminths are distinct for each helminth likely due to the different invasion strategy of each helminth, Th2 immunity is key for protective immunity to all helminths.

Naïve CD4 T cells differentiate into several different types of T helper (Th) cells, namely Th1, Th2, Th9, Th17, follicular helper T cell (Tfh), Tr1, and inducible regulatory T cell (iTreg) subsets depending upon the invading

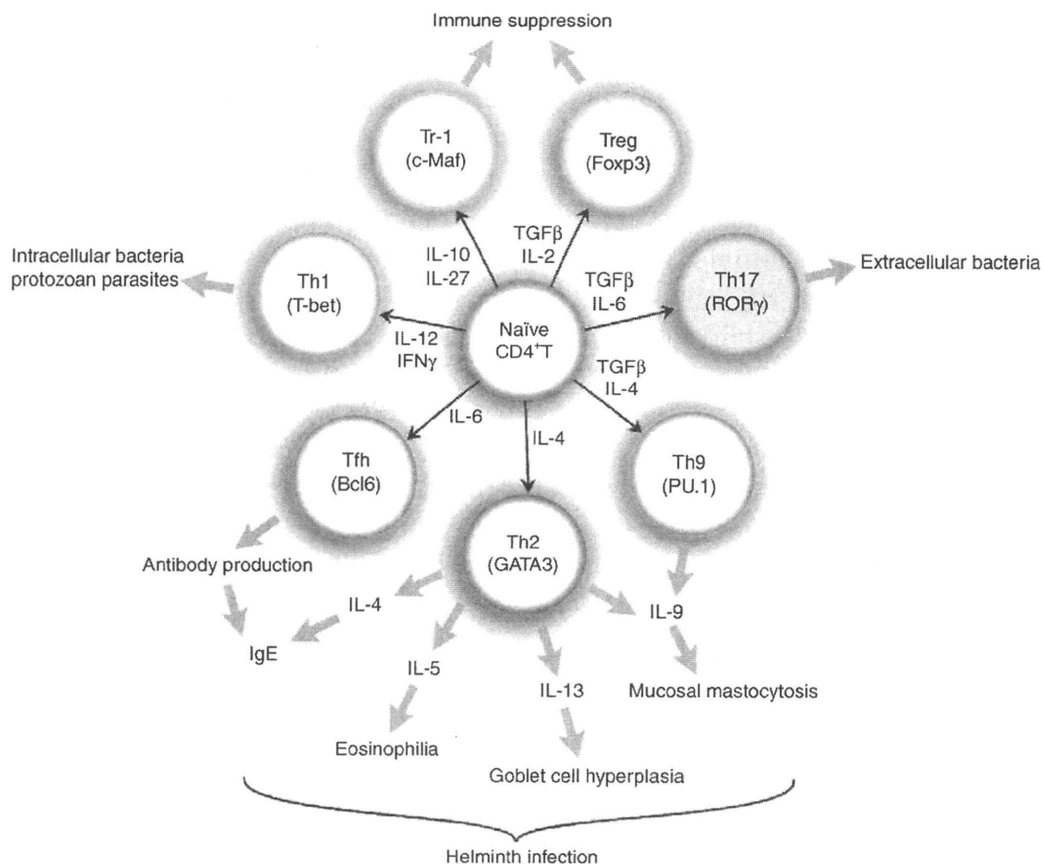

FIGURE 2.2 Differentiation and function of Th cells. Transcription factors characterizing each Th subset are shown in parentheses.

pathogen(s). Innate immune responses against invading pathogens result in the production of various cytokines and distinct cytokine milieus induce different Th cells (Zhu *et al.*, 2010; Fig. 2.2). Each cell type is induced by a specific combination of cytokines and characterized by the expression of a unique transcription factor responsible for the expression of distinct sets of cytokines upon antigen stimulation of Th cells. Among them, Th2 cells producing IL-4, IL-5, IL-9, and IL-13 are induced by IL-4, at least *in vitro*. IL-4 upregulates the expression of GATA-3, a master transcription factor characterizing Th2 cells (Zhang *et al.*, 1997; Zheng and Flavell, 1997).

Activation of adaptive immunity requires the activation of innate immunity (Novak *et al.*, 2010; Steinman and Hemmi, 2006). Invading pathogens are usually sensed by innate immune cells such as macrophages and DCs, which induce innate cytokine production. Upon bacterial and/or protozoan infection, proinflammatory cytokines such as TNFα, IFNγ and IL-1β are induced by these innate cells. DCs that engulf pathogens and are activated by Toll-like receptors (TLRs) mature and migrate to the draining lymph node and activate T cells. A subset of DCs produces IL-12 upon activation and thus induces Th1 cells (Shortman and Heath, 2010).

Because IL-4 was originally thought to be produced by Th2 cells, the initial source of this cytokine was enigmatic. It was later shown that CD1d-restricted natural killer T (NKT) cells (Arase *et al.*, 1993; Yoshimoto and Paul, 1994), mast cells (Plaut *et al.*, 1989) and basophils (Piccinni *et al.*, 1991; Seder *et al.*, 1991) are able to produce IL-4 upon stimulation. Basophils produce IL-4 and accumulate in the liver and lung after *N. brasiliensis* infection (Min *et al.*, 2004). Many allergens are associated with cysteine protease activities and incubation of basophils with one of these cysteine proteases, papain, leads to the production of IL-4 (Sokol *et al.*, 2008). Basophils also produce TSLP. TSLP was originally identified from thymic stromal cells and thought to support growth and differentiation of T and B cells but is now considered to be a Th2-inducing cytokine (Liu, 2006). Intriguingly, basophils are transiently recruited to the draining lymph node where Th2 cells are predominantly induced after *T. muris* infection and present antigens to T cells (Perrigoue *et al.*, 2009; Sokol *et al.*, 2009; Yoshimoto *et al.*, 2009). However, other studies have concluded that the Th2 induction is independent of basophils (Hammad *et al.*, 2010; Ohnmacht *et al.*, 2010; Phythian-Adams *et al.*, 2010).

4. TH2-INDUCING CYTOKINES IN INNATE IMMUNE PHASES

Recent studies also revealed the importance of epithelial cells in the production of cytokines such as TSLP, IL-25, and IL-33 in response to allergens and helminths (Eisenbarth *et al.*, 2002; Zaph *et al.*, 2007). Innate stimuli inducing these cytokines include house dust mites that stimulate lung epithelial cells through TLR4 (Hammad *et al.*, 2009).

4.1. TSLP

TSLP acts to induce DCs capable of differentiating naïve $CD4^+$ T cells to Th2 cells producing IL-4, IL-5, and IL-13 (Soumelis *et al.*, 2002; Ying *et al.*, 2005). Interestingly, DCs activated by TSLP produce IL-8 (CXCL8) attracting neutrophils, eotaxin-2 (CCL24) attracting eosinophils, and TARC (thymus and activation-regulated chemokine: CCL17) and MDC (macrophage-derived chemokine: CCL22) attracting Th2 cells. These same DCs, however, do not produce TNF, IL-1β, IL-6, IL-10, or IL-12. Induction of Th2 cells by TSLP-stimulated DCs depends on OX40L specifically induced by TSLP (Ito *et al.*, 2005). Smooth muscle also produces IL-8 and eotaxin in response to TSLP (Shan *et al.*, 2010).

4.2. IL-25

IL-25 is a member of the IL-17 family (Fort *et al.*, 2001) but, unlike other family members, induces Th2 immune responses (Hurst *et al.*, 2002). Transgenic mice expressing either mouse or human IL-25 produce

increased levels of serum IL-5 and IL-13 and induce eosinophilia (Kim *et al.*, 2002; Pan *et al.*, 2001). IL-25 was originally considered as a Th2 cytokine produced by Th2 cells (Fort *et al.*, 2001) but further studies have shown that this cytokine is also produced by epithelial cells as well such as gut epithelial cells in mice infected with *N. brasiliensis* (Angkasekwinai *et al.*, 2007) and lung epithelial cells infected with *Aspergillus fumigatus* (Hurst *et al.*, 2002). Mast cells activated through FcεRI also produce IL-25 (Ikeda *et al.*, 2003).

The importance of IL-25 in the Th2 immune response was demonstrated using mice deficient for IL-25. Such mice were unable to elicit a Th2 response upon *T. muris* infection and thus unable to control infection (Owyang *et al.*, 2006). Similarly, the expulsion of *N. brasiliensis* was significantly delayed in IL-25 deficient mice (Fallon *et al.*, 2006). It should be noted that administration of recombinant IL-25 leads to the expulsion of *N. brasiliensis* even in Rag1 deficient mice that lack both T and B cells, suggesting a pivotal role for the innate immune response in this process and involvement of IL-25 and Th2 cytokines.

4.3. IL-33

IL-33 is a member of the IL-1 family and binds to a complex formed by T1/ST2 and IL-RAP (Sanada *et al.*, 2007). IL-33 is expressed in a variety of cells including fibroblasts, epithelial cells, adipocytes and endothelial cells (Moussion *et al.*, 2008; Sanada *et al.*, 2007; Wood *et al.*, 2009). Intriguingly, IL-33 is localized in the nucleus (Carrière *et al.*, 2007). Although IL-33 can be cleaved by caspase 1 *in vitro* (Schmitz *et al.*, 2005), it is thought that caspases 3 and 7 cleave IL-33 at the IL-1-like domain and inactivate it during apoptosis (Cayrol and Girard, 2009; Lüthi *et al.*, 2009). In contrast, full-length IL-33 is released from cells upon necrotic death and functions as an alarmin to stimulate a variety of cells (Cayrol and Girard, 2009). Similar alarmins include HMGB-1 (Scaffidi *et al.*, 2002), SAP130 (Yamasaki *et al.*, 2008) and IL-1α (Cohen *et al.*, 2010) that are also present in the nucleus and released upon cellular damage. IL-33R is expressed on various types of cells including Th2 cells (Xu *et al.*, 1998), mast cells (Ali *et al.*, 2007), basophils (Smithgall *et al.*, 2008; Suzukawa *et al.*, 2008), NK cells and NKT cells (Bourgeois *et al.*, 2009; Smithgall *et al.*, 2008). Interestingly, IL-33 induces Th1 cytokine production by NK and NKT cells (Bourgeois *et al.*, 2009; Smithgall *et al.*, 2008).

Administration of TSLP, IL-25, and IL-33 induces Th2 cytokine production and associated physiological changes in mice including IgE production, eosinophilia, and goblet cell hyperplasia, suggesting that these innate cytokines are involved in the induction of Th2 immune responses.

5. CELLS PRODUCING TH2 CYTOKINES IN INNATE IMMUNE RESPONSES

Although TSLP, IL-25, and IL-33 induce Th2 cytokines *in vivo*, the identity of the cell(s) responsible for the production of Th2 cytokines has been obscure. Mast cells and basophils have been reported to respond to those cytokines as described above. A fraction of NKT cells express IL-25R and respond to IL-25 to produce IL-13 (Terashima *et al.*, 2008). In addition, non-T/non-B (NTNB) cells of unknown origin have been reported to produce Th2 cytokines in response to IL-25, IL-33, *T. muris*, and *N. brasiliensis* (Fallon *et al.*, 2006; Fort *et al.*, 2001; Humphreys *et al.*, 2008; Hurst *et al.*, 2002; Voehringer *et al.*, 2006). We have recently identified a previously unrecognized lymphocyte population that we named natural helper (NH) cells. These cells are present in the adipose tissue of the peritoneal cavity and NH cells produce a large amount of Th2 cytokines (Moro *et al.*, 2010).

5.1. Natural Helper Cell

Infection of *N. brasiliensis* in Rag2 deficient mice resulted in the production of IL-5 and IL-13 in the sera of infected mice but such cytokine production was not observed in mice lacking the cytokine receptor common γ chain (γ_c) and Rag2. These observations suggest that γ_c is required for the development or function of NTNB cells producing Th2 cytokines upon helminth infection and prompted us to examine the γ_c-dependent mucosal immune tissues.

We noted a previously unrecognized γ_c-dependent lymphoid structure located along the blood vessels in the mouse mesentery, an adipose tissue in the peritoneal cavity (Fig. 2.3A). The size of each cluster is small in young mice but increases with age and clusters are easily identified in mice over 20 weeks of age. By H&E staining, the majority of cells appear to be lymphocytes and no fibrous capsule is present around the clusters such that, unlike in lymph nodes, lymphocytes are in direct contact with ambient adipocytes (Fig. 2.3A). These clusters were structurally similar to the "milky spot" in the omentum, which is considered a gateway of cells between the circulation and the peritoneal cavity (Cranshaw and Leak, 1990). However, unlike the milky spot (Rangel-Moreno *et al.*, 2009), T and B cell zones or germinal center structures are not observed in lymphoid clusters in the mesentery. Similar lymphocyte clusters distinct from lymph node are found in the human mesentery. We named these lymphoid clusters "fat associated lymphoid clusters" or FALC. FALC are present in $ROR\gamma^{GFP/GFP}$ and *aly*/*aly* mice, indicating that FALC are distinct from lymph nodes and the development of FALC is independent of lymphoid tissue inducer (LTi) cells (Nishikawa *et al.*, 2003).

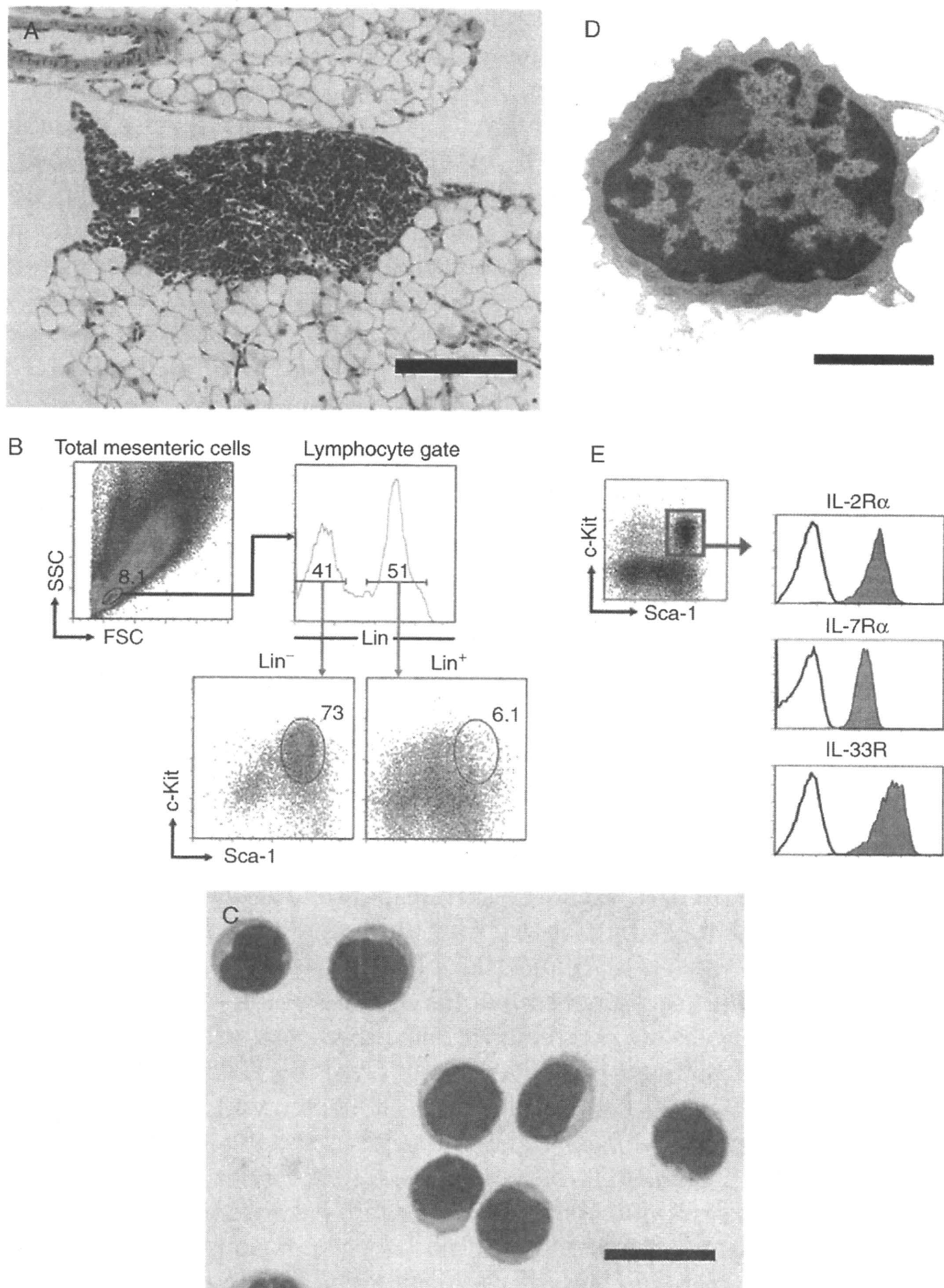

FIGURE 2.3 Fat-associated lymphoid cluster (FALC) and FALC-derived NH cells. (A) H&E stained specimen of a lymphoid cluster in the mesentery. Bar = 200 μm. (B) Flow cytometry of FALC-derived cells. FALC-derived cells were stained with monoclonal antibodies against c-Kit, Sca-1, and Lin markers (CD3, CD4, CD8α, TCRβ, TCRδ, CD5, CD19, B220, NK1.1, TER119, Gr-1, Mac-1, CD11c, FcεRIα). Staining patterns of cells in the lymphoid gate are shown. (C, D) Giemsa staining (C) and electron micrograph (D) of sorted FALC-derived NH cells. Bars = 20 μm (C) and 2 μm (D). These figures are reproduced from Moro *et al.* (2010) by the courtesy of *Nature*.

Flow cytometric analysis of FALC-derived cells shows that nearly 50% of cells in a lymphocyte gate express c-Kit, IL-2R, IL-7R, and IL-33R but lack lineage (Lin) markers (CD3, CD4, CD8α, TCRβ, TCRδ, CD5, CD19, B220, NK1.1, TER119, Gr-1, Mac-1, CD11c, FcεRIα) (Fig. 2.3B). Giemsa staining and electron microscopic analysis of sorted cells demonstrate that these cells are lymphocytes (Fig. 2.3C and D). The fact that these cells express IL-7R and are absent in $\gamma_c^{-/-}$ and IL-$7^{-/-}$ mice also supports the notion that these cells are of lymphoid origin. Among the cytokines tested including Flt3L, SCF, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-15, IL-25, IL-33, M-CSF, GM-CSF, TNFα, and TGF-β1, only SCF and IL-7 supported the survival of FALC-derived Lin$^-$c-Kit$^+$IL-2R$^+$IL-7R$^+$IL-33R$^+$ cells and IL-2 induced proliferation of these cells without changing their surface phenotype. Based on the observations that these lymphocytes proliferate in response to IL-2 and exhibit innate-type effector functions by producing Th2 cytokines (see below), we named these cells as NH cells. NH cells are also present in adipose tissues around the kidney and genitalia but very few are found in the subcutaneous fat tissue or the omentum.

NH cells constitutively produce IL-5 and IL-6. IL-5 is a critical growth factor for B1 cells, which are abundant in the peritoneal cavity and play an important role in innate-type immune responses by producing natural antibodies (Erickson *et al.*, 2001; Martin and Kearney, 2000). IL-5 and IL-6 regulate B cell antibody production (Beagley *et al.*, 1989; Sonoda *et al.*, 1989). Indeed, NH cells support the production of IgA from B cells and self-renewal of natural antibody-producing B1 cells (Moro *et al.*, 2010), which are apparently the steady-state functions of NH cells. NH cells produce large amounts of IL-5, IL-6, and IL-13 in response to IL-33 and a combination of IL-2 and IL-25 (Fig. 2.4A). Five thousand NH cells are able to produce μg amounts of IL-5 and IL-13 in response to a combination of IL-2 and IL-33 during a 5-day culture period. The amounts of IL-5 and IL-13 produced by NH cells are much higher than those from mast cells (Ali *et al.*, 2007), basophils (Smithgall *et al.*, 2008; Suzukawa *et al.*, 2008), and polarized Th2 cells (Xu *et al.*, 1998; Fig. 2.4B). It should be noted that NH cells do not respond to IL-25 without IL-2. Whereas basophils are able to produce IL-4, NH cells do not produce IL-4 in response to IL-33 or a combination of IL-2 and IL-25 although stimulation of NH cells with a combination of phorbol myristate acetate and ionomycin induced IL-4 production.

As observed in the *N. brasiliensis* infection, administration of IL-33 to Rag$2^{-/-}$ but not $\gamma_c^{-/-}$Rag$2^{-/-}$ mice induced the production of IL-5 and IL-13 and goblet cell hyperplasia in the intestine. Adoptive transfer of isolated NH cells into $\gamma_c^{-/-}$Rag$2^{-/-}$ mice restored the production of IL-5 and IL-13 and goblet cell hyperplasia in response to IL-33 administration and *N. brasiliensis* infection (Moro *et al.*, 2010). We conclude from these results that NH cells play a major role in both the innate production of IL-5 and IL-13 and in goblet cell hyperplasia upon *N. brasiliensis* infection independent of adaptive immunity (Fig. 2.5).