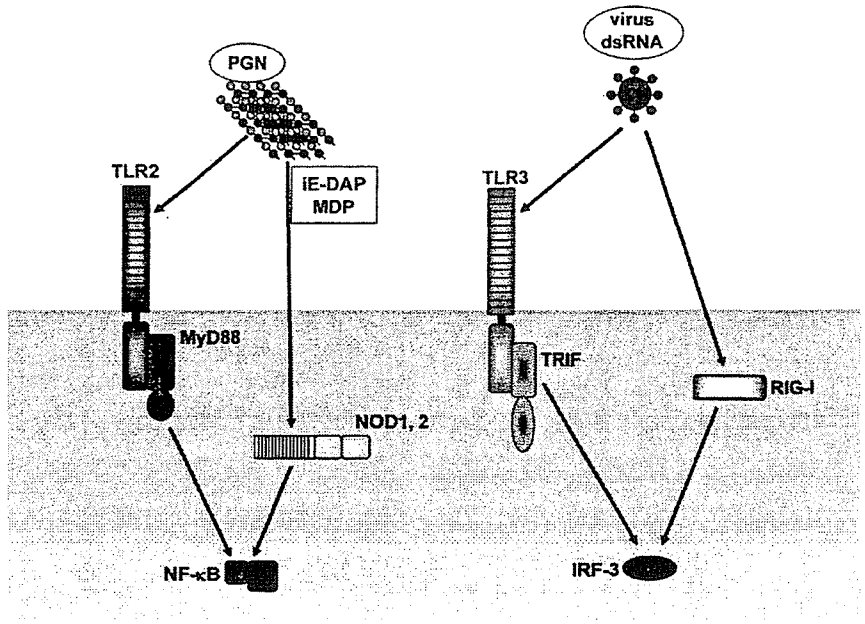

Fig. 2. TLR-dependent and -independent recognition of microbial components. TLR2 has previously been shown to mediate peptidoglycan (PGN) recognition. However, NOD1 and NOD2 have recently been shown to recognize motifs found in the layer of PGN. It is possible that TLR2 recognizes lipoprotein contamination in the PGN layer. Viral recognition is also mediated by TLR-dependent and -independent mechanisms. TLR3-mediated recognition of viruses or dsRNA results in TRIF-dependent activation of IRF-3 and NF-κB. However, viruses or dsRNA are recognized in a TLR3-independent manner, since the impairment of the responsiveness to viruses or dsRNA in TLR3-deficient mice is only partial. RIG-I is identified as a molecule that is responsible for viral recognition and that mediates activation of IRF-3.

patients are located in the NOD domain, leading to an increase in NF-κB activity. Thus, *NOD2* is associated with certain human diseases.

Recognition of PGN motifs by NOD1 and NOD2 results in their oligomerization, which induces the recruitment of Rip2/RICK, a serine/threonine kinase (64). Rip2/RICK has a CARD domain in its C-terminal portion and an N-terminal catalytic domain that shares sequence similarity with Rip, a factor essential for NF-κB activation through the TNF receptor. NODs and Rip2/RICK interact via their respective CARD domains, and induce recruitment of the IKK complex to the central region of Rip2/RICK. This in turn leads to activation of NF-κB. Rip2/RICK-deficient mice have been shown to be highly sensitive to infection with the intracellular pathogen *Listeria monocytogenes* (65). Introduction of NOD1 or NOD2 into Rip2/RICK-deficient embryonic fibroblast cells does not induce NF-κB activation (66). Thus, Rip2/RICK is essential for NOD1- and NOD2-mediated responses, although its involvement in the recognition of PGN motifs needs to be more precisely analyzed in Rip2/RICK-deficient mice.

Phagocytosis and TLRs

Phagocytosis is an important step for host defense against microbial pathogens, since it triggers both degradation of pathogens and subsequent presentation of pathogen-derived peptide antigen. TLR recognition of pathogens leads to expression of genes such as inflammatory cytokines and co-stimulatory molecules. Phagocytosis-mediated antigen presentation together with TLR-dependent gene expression of inflammatory cytokines and co-stimulatory molecules, instruct development of antigen-specific acquired immunity (Fig. 3). Therefore, it is of interest to characterize the relationship between phagocytosis and TLRs. In the absence of TLR2/TLR4 or MyD88, a common adaptor in TLR signaling, phagocytosis of bacteria including *Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus* has been shown to be impaired due to impaired phagosome maturation (67). Further studies indicate that TLR-mediated MyD88-dependent activation of p38 is required for phagosome maturation (67,68). Thus, TLRs are linked to phagocytosis of bacteria.

TLR signaling pathways

Stimulation of TLRs by microbial components triggers expression of several genes that are involved in immune responses. The molecular mechanisms by which TLRs induce gene expression are now rapidly being elucidated through analyses of TLR-mediated signaling pathways (69). Microbial recognition of TLRs facilitates dimerization of TLRs. TLR2 is shown to form a heterophilic dimer with TLR1 or TLR6, but in other cases TLRs are believed to form homodimers (70). Dimerization of TLRs triggers activation of signaling pathways, which originate from a cytoplasmic TIR domain. In the signaling pathways

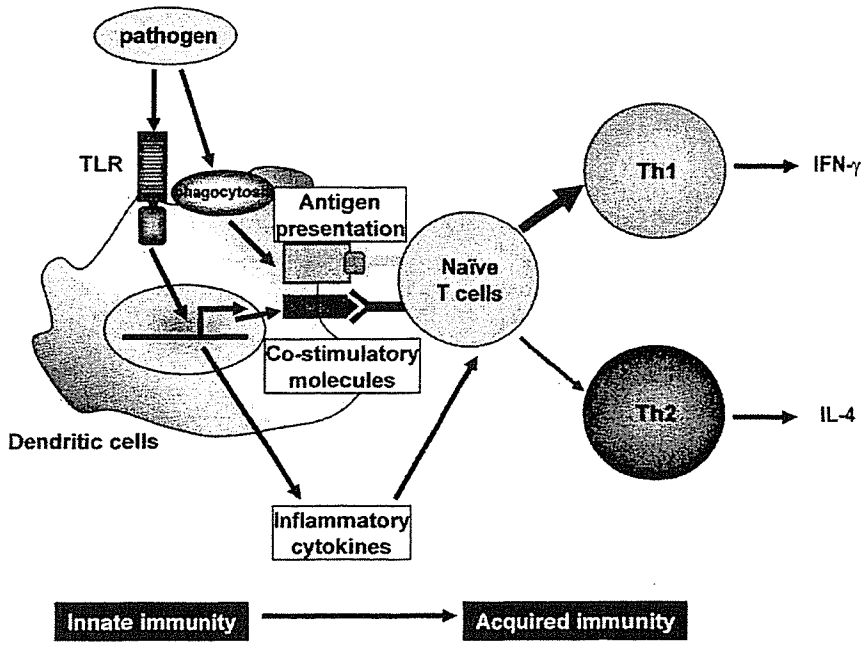

Fig. 3. Innate and adaptive immunity. Innate immune cells, such as dendritic cells and macrophages, engulf pathogens by phagocytosis, and present pathogen-derived peptide antigens to naïve T cells. In addition, TLRs recognize pathogen-derived components and induce expression of genes, such as co-stimulatory molecules and inflammatory cytokines. Phagocytosis-mediated antigen presentation, together with TLR-mediated expression of co-stimulatory molecules and inflammatory cytokines, instruct development of antigen-specific adaptive immunity, especially Th1 cells.

downstream of the TIR domain, a TIR domain-containing adaptor, MyD88, was first shown to be essential for induction of inflammatory cytokines such as TNF-α and IL-12 through all TLRs (21,26,71–74). However, activation of specific TLRs leads to slightly different patterns of gene expression profiles. For example, activation of TLR3 and TLR4 signaling pathways results in induction of type I interferons (IFNs), but activation of TLR2- and TLR5-mediated pathways does not (75–77). TLR7, TLR8 and TLR9 signaling pathways also lead to induction of type I IFNs through mechanisms distinct from TLR3/4-mediated induction (36,78). Thus, individual TLR signaling pathways are divergent, although MyD88 is common to all TLRs. It has also become clear that there are MyD88-dependent and MyD88-independent pathways (Fig. 4).

MyD88-dependent pathway

A MyD88-dependent pathway is analogous to signaling pathways through the IL-1 receptors. MyD88, harboring a C-terminal TIR domain and an N-terminal death domain, associates with the TIR domain of TLRs. Upon stimulation, MyD88 recruits IRAK-4 to TLRs through interaction of the death domains of both molecules, and facilitates IRAK-4-mediated phosphorylation of IRAK-1. Activated IRAK-1 then associates with TRAF6, leading to the activation of two distinct signaling pathways. One pathway leads to activation of AP-1 transcription factors through activation of MAP kinases. Another pathway activates the TAK1/TAB complex, which enhances activity of the IκB kinase (IKK) complex. Once activated, the IKK complex induces phosphorylation and subsequent degradation of IκB, which leads to nuclear translocation of transcription factor NF-κB.

As its name suggests, in the MyD88-dependent pathway, MyD88 plays a crucial role. MyD88-deficient mice do not show production of inflammatory cytokines such as TNF-α and IL-12p40 in response to all TLR ligands (21,26,71–74). Thus, MyD88 is essential for inflammatory cytokine production through all TLRs.

A database search for molecules that are structurally related to MyD88 led to identification of the second TIR domain-containing molecule TIRAP (TIR domain-containing adaptor protein)/Mal (MyD88-adaptor-like) (79,80). Similar to MyD88-deficient macrophages, TIRAP/Mal-deficient macrophages show impaired inflammatory cytokine production in response to TLR4 and TLR2 ligands (81,82). However, TIRAP/Mal-deficient mice are not impaired in their response to TLR3, TLR5, TLR7 and TLR9 ligands, Thus, TIRAP/Mal has been shown to be essential for the MyD88-dependent signaling pathway via TLR2 and TLR4.

MyD88-independent/TRIF-dependent pathway

In MyD88-deficient macrophages, TLR4 ligand-induced production of inflammatory cytokines is not observed; however, activation of NF-κB is observed with delayed kinetics (72). This indicates that although TLR4-mediated production of inflammatory cytokines completely depends on the MyD88-dependent pathway, a MyD88-independent component exists

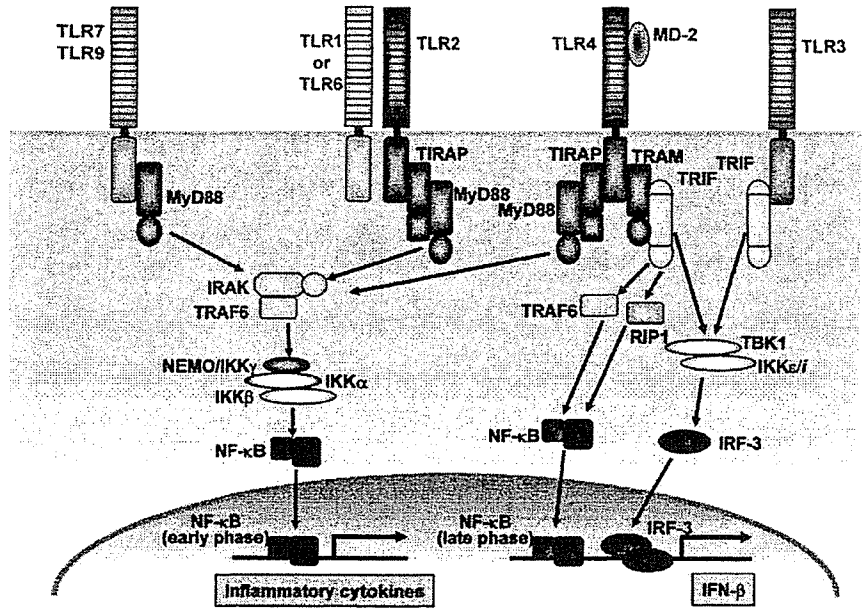

Fig. 4. TLR signaling pathway. TLR signaling pathways originate from the cytoplasmic TIR domain. A TIR domain-containing adaptor, MyD88, associates with the cytoplasmic TIR domain of TLRs, and recruits IRAK to the receptor upon ligand binding. IRAK then activates TRAF6, leading to the activation of the IκB kinase (IKK) complex consisting of IKKα, IKKβ and NEMO/IKKγ. The IKK complex phosphorylates IκB, resulting in nuclear translocation of NF-κB which induces expression of inflammatory cytokines. TIRAP, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signaling pathway via TLR2 and TLR4. In TLR3- and TLR4-mediated signaling pathways, activation of IRF-3 and induction of IFN-β are observed in a MyD88-independent manner. A third TIR domain-containing adaptor, TRIF, is essential for the MyD88-independent pathway. Non-typical IKKs, IKK*i*/IKKε and TBK1, mediate activation of IRF-3 downstream of TRIF. A fourth TIR domain-containing adaptor, TRAM, is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway.

in TLR4 signaling. Subsequent studies have demonstrated that TLR4 stimulation leads to activation of the transcription factor IRF-3, as well as the late phase of NF-κB activation in a MyD88-independent manner (83). TLR4-induced activation of IRF-3 leads to production of IFN-β. IFN-β in turn activates Stat1 and induces several IFN-inducible genes (75–77). Viral infection or dsRNA was found to activate IRF-3 (84). Accordingly, the TLR3-mediated pathway also activates IRF-3 and thereby induces IFN-β in a MyD88-independent manner. Hence, TLR3 and TLR4 utilize the MyD88-independent component to induce IFN-β.

Characterization of MyD88 and TIRAP/Mal prompted us to hypothesize that TIR domain-containing molecules regulate the MyD88-independent pathway, and also facilitated the search for such molecules. A database search led to identification of a third TIR domain-containing adaptor, TIR domain-containing adaptor inducing IFN-β (TRIF) (85). This molecule was identified as a TLR3-associated molecule by two-hybrid screening and was named TIR domain-containing adaptor molecule (TICAM-1) (86). The physiological role of TRIF/TICAM-1 was then demonstrated by generation of TRIF-mutant mice. TRIF-deficient mice generated by gene targeting showed no activation of IRF-3 and had impaired expression of IFN-β- and IFN-inducible genes in response to TLR3 and TLR4 ligands (52). Another mouse strain mutated in the *Trif* gene generated by random germline mutagenesis also revealed that they were defective in TLR3- and TLR4-mediated induction of IFN-β- and IFN-inducible genes (87). Thus, TRIF has been demonstrated to be essential for TLR3- and TLR4-mediated MyD88-independent pathways.

Database searches further led to identification of a fourth TIR domain-containing adaptor, TRIF-related adaptor molecules (TRAM)/TICAM-2 (88–91). Studies with TRAM-deficient mice and RNAi-mediated knockdown of TRAM expression showed that TRAM is involved in TLR4-mediated, but not TLR3-mediated, activation of IRF-3 and induction of IFN-β- and IFN-inducible genes (88–90). Thus, TRAM is essential for the TLR4-mediated MyD88-independent/TRIF-dependent pathway.

In TRIF- and TRAM-deficient mice, inflammatory cytokine production induced by TLR2, TLR7 and TLR9 ligands was observed, as well as TLR4 ligand-induced phosphorylation of IRAK-1 (52,89). These findings indicate that the MyD88-dependent pathway is not impaired in these mice. However, TLR4 ligand-induced inflammatory cytokine production was not observed in TRIF- and TRAM-deficient mice. Therefore, activation of both the MyD88-dependent and MyD88-independent/TRIF-dependent components is required for the TLR4-induced inflammatory cytokine production, but the mechanisms are unknown.

Key molecules that mediate IRF-3 activation have been revealed to be non-canonical IKKs, TBK1 and IKK*i*/IKKε (92). Introduction of TBK1 or IKK*i*/IKKε, but not IKKβ, resulted in phosphorylation and nuclear translocation of IRF-3. RNAi-mediated inhibition of TBK1 or IKK*i*/IKKε expression led to impaired induction of IFN-β in response to viruses and dsRNA

(92,93). Embryonic fibroblast cells obtained from TBK1-deficient mice showed impaired activation of IRF-3 and expression of IFN-β and IFN-inducible genes in response to TLR3 and TLR4 ligands (94–96). In contrast, embryonic fibroblast cells from IKKi/IKKε-deficient mice were not defective in their response to TLR3 and TLR4 ligands (95). However, TLR3-mediated activation of IRF-3 and expression of IFN-β and IFN-inducible genes were almost completely abolished in embryonic fibroblast cells lacking both TBK1 and IKKi/IKKε. Thus, TBK1 and IKKi/IKKε are critical regulators of IRF-3 activation in the MyD88-independent pathway.

The mechanisms by which the TRIF-dependent pathway leads to activation of NF-κB and IRF-3 are now under investigation. The TIR domain of TRIF is located in the middle portion of this molecule, flanked by the N-terminal and C-terminal portions. Both N-terminal and C-terminal portions of TRIF mediate activation of the NF-κB-dependent promoter, whereas only the N-terminal portion is involved in IFN-β promoter activation (85). Accordingly, the N-terminal portion of TRIF was shown to associate with IKKi/IKKε and TBK1, which mediate IRF-3-dependent IFN-β induction (93,97). The N-terminal portion of TRIF was also shown to associate with TRAF6 (97,98). Since TRAF6 is critically involved in TLR-mediated NF-κB activation (99), TRAF6 may regulate NF-κB activation derived from the N-terminal portion of TRIF. The C-terminal portion of TRIF was shown to associate with RIP1 (100). Embryonic fibroblast cells from RIP1-deficient mice showed impaired NF-κB activation in response to the TLR3 ligand. Thus, RIP1 is shown to be responsible for NF-κB activation that originates from the C-terminal portion of TRIF.

Negative regulation of TLR signaling

Stimulation of TLRs by microbial components triggers the induction of inflammatory cytokines such as TNF-α, IL-6 and IL-12. When all these cytokines are produced in excess, they induce serious systemic disorders with a high mortality rate in the host. It is therefore not surprising that organisms have evolved mechanisms for modulating their TLR-mediated responses.

Exposure to microbial components such as LPS results in a severely reduced response to a subsequent challenge by LPS. This phenomenon was first described over 50 years ago and is now called endotoxin (or LPS) tolerance, but the precise mechanisms remain unclear (101). The mechanisms are now being analyzed in the context of TLR signaling, and several models are proposed. LPS stimulation of macrophages results in reduced surface expression of the LPS receptor complex composed of TLR4 and MD-2, a co-factor that facilitates LPS binding (102,103). TLR2, TLR7 and TLR4 ligands induce reduced expression of IRAK-1 (104–106). Several other mechanisms are also shown to be involved in LPS tolerance (107).

In addition, molecules that negatively regulate TLR signaling have been identified. IRAK-M, a member of the IRAK family of serine/threonine kinases, is induced by TLR stimulation in monocyte/macrophages, and lacks kinase activity (108). IRAK-M-deficient mice show increased production of inflammatory cytokines in response to TLR ligands and defective induction of LPS tolerance (109). Inhibitory activity of IRAK-M seems to be elicited by IRAK-M prevention of IRAK-1/IRAK-4 dissociation from MyD88, thereby preventing formation of the IRAK-1–TRAF6 complex.

An alternatively spliced variant of MyD88 that lacks the intermediary domain of MyD88 (MyD88s) is induced in monocytes upon LPS stimulation. Overexpression of MyD88s results in impaired LPS-induced NF-κB activation through inhibition of IRAK-4-mediated IRAK-1 phosphorylation (110).

SOCS1 is a member of the SOCS family of proteins that are induced by cytokines and that negatively regulate cytokine signaling pathways (111). In addition to cytokines, TLR ligands such as LPS and CpG DNA induced expression of SOCS1 in macrophages (112,113). SOCS1-deficient mice were hypersensitive to LPS-induced endotoxin shock and showed defective induction of LPS tolerance (114,115). Ectopic expression of SOCS1 resulted in impaired LPS-induced NF-κB activation in macrophages. These findings indicate that SOCS1 directly down-modulates TLR signaling pathways, although the precise mechanism by which SOCS1 inhibits TLR signaling remains unclear.

Membrane-bound proteins harboring the TIR domain, such as SIGIRR (single immunoglobulin IL-1 receptor-related molecule) and T1/ST2, have also been shown to be involved in negative regulation of TLR signaling. In both SIGIRR- and T1/ST2-deficient mice, the LPS-induced inflammatory response was enhanced (116,117).

Ubiquitination-mediated degradation of TLRs is also proposed as a mechanism to inhibit activation of the signaling pathway. A RING finger protein, Triad3A, is shown to act as an E3 ubiquitin ligase and enhance ubiquitination and proteolytic degradation of TLR4 and TLR9 (118). Thus, several molecules are postulated to modulate TLR signaling pathways (Fig. 5). Combination of these negative regulators may finely coordinate the TLR signaling pathway to limit exaggerated innate responses causing harmful disorders.

Involvement of TLRs and immune disorders

Several lines of evidence indicate that TLRs are implicated in inflammatory and immune disorders. For example, constitutive activation of innate immune cells caused by defective IL-10 signaling results in development of chronic enterocolitis (119). Introduction of TLR4 deficiency into these mutant mice results in improvement of intestinal inflammation, indicating that TLR-mediated microbial recognition in the intestine triggers development of chronic enterocolitis (120). The MyD88-dependent pathway is seemingly involved in allograft rejection (121). Development of atherosclerosis observed in apolipoprotein E-deficient mice is rescued by introduction of MyD88 deficiency, indicating that the TLR-mediated pathway is responsible for the development of atherosclerosis (122,123). Involvement of the TLR9–MyD88-dependent pathway in the induction of auto-antibodies in SLE and rheumatoid arthritis was also demonstrated, as described above. In addition to these immune-related disorders, TLR recognition of commensal bacteria has been shown to play a crucial role in the maintenance of intestinal epithelial homeostasis (124). Thus, TLR-mediated pathways are probably involved in many aspects of immune responses, even in the absence of infection.

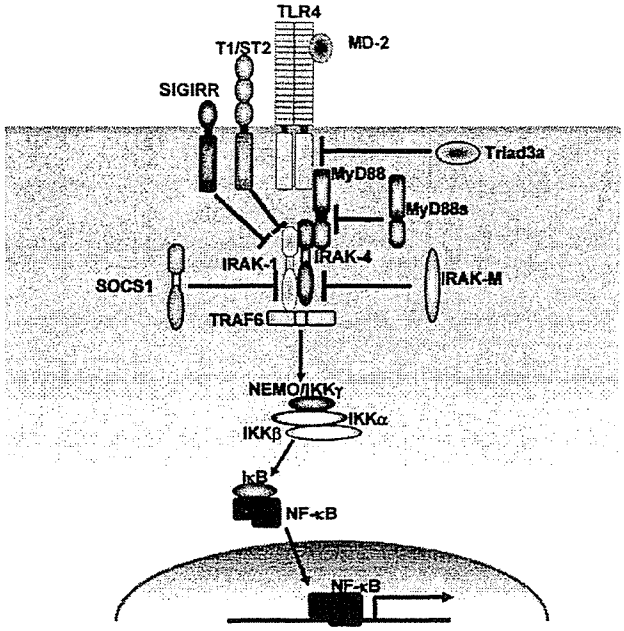

Fig. 5. Negative regulation of TLR signaling pathways. TLR signaling pathways are negatively regulated by several molecules. IRAK-M inhibits dissociation of IRAK-1/IRAK-4 complex from the receptor. MyD88s blocks association of IRAK-4 with MyD88. SOCS1 is likely to associate with IRAK-1 and inhibits its activity. TRIAD3A induces ubiquitination-mediated degradation of TLR4 and TLR9. TIR domain-containing receptors SIGIRR and T1/ST2 are also shown to negatively modulate TLR signaling.

Phylogenetic divergence in the role of Toll

It is well established that mammalian TLRs recognize specific molecular patterns found in microbial components, possibly through a close physical interaction. The Toll receptor in *Drosophila melanogaster* plays an essential role in the host defense against infection by fungi and Gram-positive bacteria. In *Drosophila*, fungal and Gram-positive bacterial infection triggers activation of the Toll-mediated pathway. Toll signaling induces the activation of the Pelle serine/threonine kinase via the adaptor DmMyD88 and degradation of the ankyrin-repeat protein Cactus, and causes activation of the Rel-type transcription factors Dorsal and DIF. Thus, the *Drosophila* Toll signaling pathway is very similar to that of mammalian TLR, especially the participation of the adaptor MyD88, the serine/threonine kinase IRAK, the ankyrin-repeat protein IκB and the Rel-type transcription factor NF-κB (125,126). However, there are some functional differences between the mammalian TLR system and the *Drosophila* Toll system (Fig. 6). First, unlike the mammalian TLR-mediated pathway, the *Drosophila* Toll pathway does not seem to utilize homologs of the mammalian IKKβ and IKKγ/NEMO proteins. These molecules have been shown to be involved in the IMD pathway that senses Gram-negative bacterial infection (125,126). In addition, *Drosophila* Toll is activated by an endogenous ligand, Spätzle (127). Spätzle is initially produced as a pro-Spätzle, and is cleaved into the active signaling form by an as yet unidentified serine protease in response to invasion by fungi or Gram-positive bacteria. This indicates that Toll is not directly involved in the pattern recognition of micro-organisms. In the case of fungal infections, a serine protease that is encoded by the *Persephone* gene has been shown to activate Toll (128). The *Persephone* gene product possesses no obvious pattern recognition motif. Thus, the molecule that is responsible for the recognition of fungi remains unclear. In the case of Gram-positive bacterial infections, Gram-negative binding protein (GNBP) and PGRP-SA, a member of the peptidoglycan recognition protein (PGRP) family, play an essential role in the activation of Toll. This is demonstrated by the finding that mutant flies lacking GNBP or PGRP-SA are defective in the activation of the Toll-mediated pathway in response to Gram-positive bacterial infection (129,130). Subsequently, PGRP-SA has been shown to be responsible for the recognition of Gram-positive lysine-type peptidoglycan, demonstrating that PGRP-SA is a pattern recognition molecule in *Drosophila* (131). In addition, another member of the PGRP family, PGRP-LC, has been shown to play an essential role in the host defense against Gram-negative bacterial infection through the recognition of Gram-negative diaminopimelic acid-type peptidoglycan (132–134). In sharp contrast, mammalian PGRP-S has been shown to play only a minor role in the recognition of pathogens (135). Thus, although the signaling molecules associated with *Drosophila* Toll and mammalian TLR are shared, the actual pattern recognition of pathogens is mediated by quite different mechanisms. In particular, it is of note that only one Toll is actually involved in the *Drosophila* immune response (126). The complete lack of immune functions in other members (18-Wheeler/Toll-2 to Toll-9) of the *Drosophila* Toll family strongly suggests that the ancestral function of the Toll family is not to mediate immune response. In contrast, all members of the mammalian TLR family are specialized for functioning in immune responses, indicating that they have arisen from a common ancestor possessing immune recognition function. Thus, the systems by which pathogens are recognized in vertebrates and insects seem to have evolved separately.

Future prospects

We now know that innate immunity plays an important role in the initiation of an immune response that follows the activation of antigen-specific acquired immunity. Although signaling pathways via TLRs are now being unveiled, there still remain several unanswered questions. For example, activation of TLR7, TLR8 and TLR9 leads to induction of IFN-α/β in a MyD88-dependent manner in PDC. It is possible that there might be a unique pathway downstream of MyD88 that specifies the signaling cascade of these TLRs. The mechanism for regulation of TLR-mediated gene induction is also of interest. A TLR-inducible nuclear factor, IκBζ, has been shown to regulate a subset of TLR-inducible genes (136). In this model, IκBζ, which is immediately induced by TLR stimulation, mediates induction of a certain group of TLR-inducible genes such as IL-6, IL-12p40 and GM-CSF in macrophages. It is of interest to analyze whether this two-step TLR-mediated gene induction model can be applied to other subsets of genes that are induced by TLRs. A complete understanding of the mechanisms of innate immunity will be helpful for the future

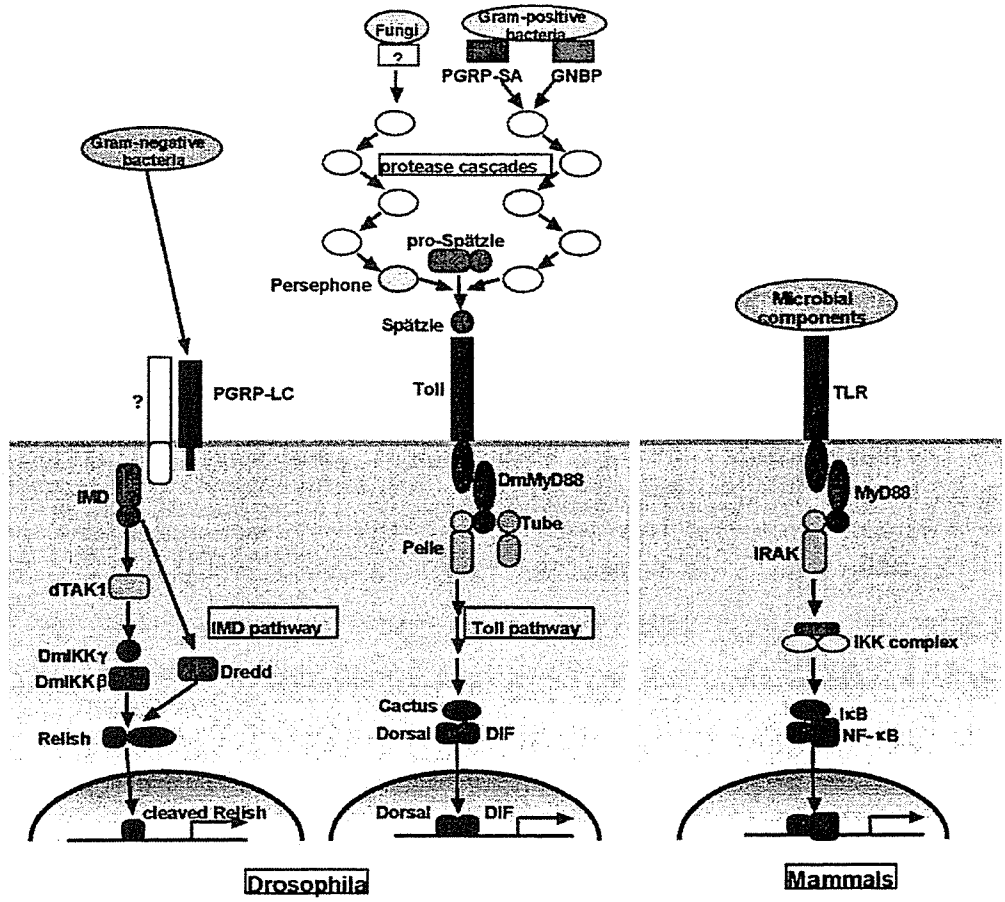

Fig. 6. Toll pathway in *Drosophila* and TLR pathway in mammals. In *Drosophila*, fungal and Gram-positive bacterial infections are sensed by pattern recognition proteins. GNBP1 and PGRP-SA are responsible for the recognition of Gram-positive bacteria. Recognition of micro-organisms is followed by activation of proteolytic cascades, leading to the cleavage of Spätzle. Spätzle activates Toll, which leads to degradation of Cactus and nuclear translocation of the Rel-type transcription factor DIF. In the case of Gram-negative bacterial infection, the IMD pathway is activated in *Drosophila*. In mammals, microbial infections are sensed by TLRs, which leads to activation of the Rel-type transcription factor NF-κB.

development of innovative therapies for manipulation of infectious diseases, cancer and allergies.

Acknowledgements

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Abbreviations

CARD	caspase-recruitment domain
dsRNA	double-stranded RNA
HSP	heat shock proteins
iE-DAP	γ-D-glutamyl-meso diaminopimelic acid
IKK	IκB kinase
LRRs	leucine-rich repeats
Mal	MyD88-adaptor-like
MCMV	mouse cytomegalovirus
MDP	MurNAc-L-Ala-D-isoGln
NOD	nucleotide-binding oligomerization domain
PDC	plasmacytoid dendritic cells
PGN	peptidoglycan
PGRP	peptidoglycan recognition protein
PKR	dsRNA-dependent protein kinase
RNAi	RNA interference
SIGIRR	single immunoglobulin IL-1 receptor-related molecule
ssRNA	single-stranded RNA
TICAM	TIR domain-containing adaptor molecule
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLRs	Toll-like receptors
TRIF	TIR domain-containing adaptor inducing IFN-β

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A Novel Neurotoxoid Vaccine Prevents Mucosal Botulism[1]

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The threat posed by botulism, classically a food- and waterborne disease with a high morbidity and mortality, has increased exponentially in an age of bioterrorism. Because botulinum neurotoxin (BoNT) could be easily disseminated by terrorists using an aerosol or could be used to contaminate the food or water supply, the Centers for Disease Control and Prevention and the National Institute of Allergy and Infectious Diseases has classified it as a category A agent. Although clearly the development of a safe and effective mucosal vaccine against this toxin should be a high priority, essentially no studies to date have assessed mucosal immune responses to this disease. To bridge this gap in our knowledge, we immunized mice weekly for 4 wk with nasal doses of BoNT type A toxoid and a mutant of cholera toxin termed E112K. We found elevated levels of BoNT-specific IgG Abs in plasma and of secretory IgA Abs in external secretions (nasal washes, saliva, and fecal extracts). When mice given nasal BoNT vaccine were challenged with 4×10^3 LD_{50} of BoNT type A (BoNT/A) via the i.p. route, complete protection was seen, while naive mice given the same dosage died within 2 h. To further confirm the efficacy of this nasal BoNT vaccine, an oral LD_{50} was determined. When mice were given an oral challenge of 5 μg ($2 \times$ oral LD_{50}) of progenitor BoNT/A, all immunized mice survived beyond 5 days, while nonimmunized mice did not. The fecal extract samples from nasally vaccinated mice were found to contain neutralizing secretory IgA Abs. Taken together, these results show that nasal BoNT/A vaccine effectively prevents mucosal BoNT intoxication. *The Journal of Immunology,* **2005, 174: 2190–2195.**

It is now clear that parenteral immunization usually fails to elicit protective mucosal immune responses (1). Various alternate routes of Ag-vaccine delivery have been suggested by researchers, but none has shown more promise for the induction of Ag-specific Ab responses in the upper mucosal compartments (e.g., the respiratory tract) than nasal administration or in the gastrointestinal (GI)[3] tract than oral administration (2–7). Our own previous studies have shown that gut-associated lymphoreticular tissue-directed oral immunization effectively induces Ag-specific secretory IgA (S-IgA) Ab responses in the small intestinal lamina propria (iLP) and the submandibular glands, but not in the lung or the lamina propria of the nasal or reproductive tracts (5–9). In most current nasal immunization studies, the vaccine is instilled into each nostril (usually ~5 μl/nostril), and normal inhalation results in the effective delivery of the vaccine, presumably into nasopharyngeal-associated lymphoreticular tissues (NALT). Such NALT-mediated nasal immunization has been shown to elicit significant immunity in the respiratory, GI, and reproductive tracts as well as in the nasal and oral cavities. These findings suggest that the nasal route of Ag delivery may possess potential advantages in the induction of mucosal immunity.

Native cholera toxin (CT) and mutants of CT (mCTs) are effective mucosal adjuvants and have been widely used for nasal immunization with protein Ags, bacterial components, viruses, or virus-related peptides for the induction of protective immunity associated with S-IgA and plasma IgG Ab responses (4, 10–12). Nasal immunization with the weakly immunogenic OVA along with mCT as adjuvant resulted in S-IgA anti-OVA Ab responses in various mucosal external secretions (4). Furthermore, mice nasally immunized with pneumococcal surface protein A Ag plus mCT revealed pneumococcal surface protein A-specific S-IgA Ab responses associated with effective protection against capsular serotype 3 *Streptococcus pneumoniae* A66 (10). These Ag-specific S-IgA Ab responses were associated with polarized Th2-type responses in cervical lymph nodes (4, 10). Nasal immunization with diphtheria toxoid plus mCT E112K has also been shown to induce protective immunity to the diphtheria exotoxin (11). Furthermore, our recent studies showed that young adult as well as aged mice given a nasal vaccine of tetanus toxoid along with a chimera of mCT-A E112K and heat labile toxin (LT) B subunit were protected when mice were challenged via the systemic route with tetanus toxin (12, 13).

Clostridium botulinum is an anaerobic bacterium that produces a powerful exotoxin termed botulinum neurotoxin (BoNT), which

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[3] Abbreviations used in this paper: GI, gastrointestinal; AFC, Ab-forming cell; BoNT, botulinum neurotoxin; BoNToxoid, botulinum neurotoxoid; CT, cholera toxin; iLP, intestinal lamina propria; LT, labile toxin; mCT, mutant of CT; NALT, nasopharyngeal-associated lymphoreticular tissue; NP, nasal passage; S-IgA, secretory IgA.

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induces flaccid paralysis (14–17). The BoNTs, serologically divided into seven immunologically different types (A through G), are released from the bacterium as a single polypeptide chain (14–17). Among these seven serotypes, types A, B, and E are typically associated with botulism in humans, and type C has been the most common cause of disease in domestic animals. However, both C and D induce botulism in cows (15–18). As with foodborne botulism, type A is responsible for 60%, type B for 30%, and type E for 10% (18) of cases in humans. It should be emphasized that bioterrorists could potentially use any or all seven serotypes. The molecular mass of each exotoxin type is ~150 kDa (14–17). Each consists of two polypeptide chains, an L chain (50-kDa) and an H (100-kDa) chain. The main targets of BoNT are the peripheral cholinergic nerve endings and particularly the cholinergic neuromuscular junction. BoNT inhibits exocytosis, inducing the flaccid paralysis characteristic of botulism and eventually leading to death (19–25).

The bacterium *C. botulinum* and its derived BoNTs enter the body most commonly through the GI tract via tainted food. In the case of a bioterrorist event, the BoNT would most likely be disseminated by airborne or possibly waterborne routes. BoNTs are classified as one of the six highest risk agents for bioterrorism (category A agents) by the Centers for Disease Control and Prevention and the National Institute of Allergy and Infectious Diseases. Any chance of surviving this extremely potent and lethal toxin rests upon prolonged intensive care. The current pentavalent botulinum vaccine (serotypes A-E) is used for at-risk populations such as scientists researching BoNTs, health care providers dealing with their clinical manifestations, and the military (26). Furthermore, the current pentavalent vaccine is administered via the parenteral route and induces prolonged pain and swelling at the site of injection (26) (H. F. Staats, unpublished results) To minimize these side effects, several research groups have attempted to develop BoNT vaccines using recombinant C-terminal half of the H chain of BoNT peptide or synthetic epitopes (27–32). Furthermore, a Venezuelan equine encephalomyelitis virus replication vector system provided effective protection against BoNT intoxication (33). However, given the nature of botulinum intoxication, it is essential to devise more effective mucosal vaccines to prevent the intoxication at both mucosal and systemic tissue compartments.

In this study, we have examined whether a nasal vaccine consisting of botulinum neurotoxoid type A (BoNToxoid/A) and mCT E112K as adjuvant would effectively induce protective immunity to BoNT/A challenge. Our results indicate that this nasal vaccine induced BoNT-specific Ab responses in both mucosal and systemic lymphoid tissues. Furthermore, the nasal vaccine prevented botulinum intoxication following either mucosal or parenteral challenge.

Materials and Methods

Mice

Young adult (6–8 wk old) C57BL/6 mice were purchased from the Frederick Cancer Research Facility (National Cancer Institute). Upon arrival, all mice were immediately transferred to microisolators and maintained in horizontal laminar flow cabinets and provided sterile food and water ad libitum. Experiments were performed using young adult C57BL/6 mice between 6 and 8 wk of age. The health of the mice was tested semiannually, and mice of all ages used in these experiments were free of bacterial and viral pathogens.

Preparation of BoNToxoid/A

The BoNT/A from *C. botulinum* type A 62 was used for toxoid preparation. The BoNT/A was purified using previously described methods (15). Briefly, purified BoNT/A was detoxified at 30°C by dialyzing against 0.2% Formalin in 0.1 M phosphate buffer (0.2 mg/ml, type A toxin containing 3.5, 10^6 LD_{50}/ml). The detoxification proceeded at 30°C. The formalized toxin was sampled at intervals for mouse inoculation until it became completely nontoxic. At each interval, two mice were i.p. administered with 0.5 ml of the sample and then observed for 4 days. The formalized toxoid was shown to be nontoxic because mice neither died from intoxication nor showed any specific symptoms of intoxication such as muscle spasms, stiffening, or any other abnormal signs during the observation period. The toxoid was kept at 4°C until used (15, 34).

Nasal immunization and sample collection

Mice were nasally immunized at weekly intervals for 4 wk with 20 μg of BoNToxoid/A and 5 μg of mCT E112K in PBS (4, 10–13). Plasma and mucosal external secretions (nasal washes, saliva, and fecal extracts) were collected on day 28. Saliva was obtained from mice following i.p. injection with 100 μg of sterile pilocarpine (7). Fecal pellets (100 mg) were suspended into 1 ml of PBS containing 0.1% sodium azide and were then extracted by vortexing for 5 min. The samples were spun at 10,000 × *g* for 5 min, and the supernatants were collected as fecal extracts (5–7). The mice were sacrificed 7 days after the last immunization. The nasal washes were obtained by injecting 1 ml of PBS on three occasions into the posterior opening of the nasopharynx with a hypodermic needle (13).

Ab assays

Ab titers in plasma and mucosal secretions were determined by an ELISA (4–7, 11–13). Briefly, Falcon microtest assay plates (BD Biosciences) were coated with an optimal concentration of purified BoNT/A (100 μl of 2 μg/ml) in PBS overnight at 4°C (35). Two-fold serial dilutions of samples were added after blocking with 1% BSA. To detect BoNT/A-specific Ab levels, HRP-conjugated, goat anti-mouse γ or α, H chain-specific Abs were used (Southern Biotechnology Associates). For IgG Ab subclass determinations, biotinylated mAbs specific for IgG1 IgG2a, IgG2b, and IgG3 (BD Pharmingen) and peroxidase-conjugated goat anti-biotin Ab were used, as described elsewhere (11–13). Endpoint titers were expressed as the last dilution yielding an OD at 414 nm (OD_{414}) of >0.1 U above negative control values after a 15-min incubation.

Enumeration of Ab-forming cells (AFCs)

The spleens were removed aseptically and single cell suspensions were prepared, as described elsewhere (4, 10, 13). For isolation of mononuclear cells from nasal passages (NPs) and iLP, a modified dissociation method was used. This method was based upon a previously described protocol using collagenase type IV (0.5 mg/ml; Sigma-Aldrich) enzymatic dissociation to obtain single cell preparations (4, 10, 13). Mononuclear cells were purified using a discontinuous Percoll gradient (Pharmacia Fine Chemicals). Mononuclear cells at the interface between the 40 and 75% layers were removed, washed, and resuspended in RPMI 1640 (Mediatech) supplemented with HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FCS (complete medium). An ELISPOT assay was used to detect cells producing IgG and IgA Abs (5–7, 12, 13). Ninety-six-well nitrocellulose plates (Millititer HA; Millipore) were coated with 10 μg/ml BoNT/A for analysis of anti-BoNT/A-specific AFCs.

BoNT/A challenge

A 100-μl aliquot (2 μg/ml) of progenitor BoNT/A (2×10^7 i.p. LD_{50}/mg; WAKO) diluted in 0.2% gelatin/PBS was given to each mouse via the i.p. route. For oral challenge, mice were starved for 12 h before oral delivery of BoNT/A and were then gastrically intubated with a 7.5% sodium bicarbonate isotonic solution 30 min before actual intubation of BoNT/A (5–7). The various doses (25 ng, 250 ng, and 2.5 μg/mouse) of BoNT/A were gastrically intubated into the duodenum of each mice (six mice/group) using a 22-gauge ball-tip intubation needle to establish the oral LD_{50} for BoNT/A (5–7). Individual mice were monitored daily for paralysis and death.

S-IgA Ab neutralization assay

Undiluted fecal extract samples (200 μl) from mice given either BoNT/A vaccine or a nonrelevant Ag (OVA) were incubated with 50 pg of progenitor BoNT/A (WAKO) in 0.2% gelatin/PBS for 30 min at room temperature (36). The solution mixture was then injected into mice via the i.p. route, and individual mice were monitored daily for paralysis and death.

Statistics

The data are expressed as the mean ± SEM, and mouse groups were compared with control mice using a Mann-Whitney *U* test with Statview II software (Abacus Concepts) designed for Macintosh computers. A p value of <0.05 or less was considered significant.

Results

Induction of BoNT-specific S-IgA Ab responses

To date, no studies have assessed BoNT-specific mucosal IgA Ab responses. To bridge this gap in our knowledge, we first examined BoNT/A-specific mucosal immune responses in mice given nasal BoNToxoid/A plus mCT E112K as mucosal adjuvant. Nasal washes, saliva, and fecal extracts were collected 1 wk after the last nasal immunization with BoNToxoid/A plus mCT E112K and were then subjected to BoNT/A-specific ELISA. Significant S-IgA Ab responses were induced in external secretions of mice given nasal BoNToxoid/A plus mCT E112K; however, mice given nasal BoNToxoid alone did not exhibit BoNT-specific S-IgA Ab responses (Fig. 1). Interestingly, higher levels of BoNT/A-specific S-IgA Abs were detected in fecal extracts than in other mucosal secretions such as saliva and nasal washes. In contrast, nasal washes contained significant levels of anti-BoNT/A-specific IgG Abs (reciprocal $\log_2$ titer ~5). As one might expect, mice systemically immunized with BoNToxoid/A plus mCT E112K showed high titers of BoNT/A-specific plasma IgG (reciprocal $\log_2$ titer ~22), but not S-IgA Abs (data not shown). These results are the first evidence that BoNT/A-specific S-IgA Ab responses can be induced by nasal immunization with BoNToxoid plus an appropriate mucosal adjuvant (e.g., mCT E112K).

BoNT/A-specific plasma Abs in mice given the nasal vaccine

Since it has been shown that nasal immunization induces Ag-specific immune responses in systemic as well as mucosal sites, we sought to assess BoNT/A-specific plasma Ab levels after nasal vaccine delivery and found that significant responses were induced (Fig. 2*A*). The level of BoNT/A-specific IgG Abs in mice given nasal vaccine (reciprocal $\log_2$ titer ~23) was comparable to that of systemically immunized mice (reciproal $\log_2$ titer ~22). Furthermore, mice given the nasal vaccine with mCT E112K as mucosal adjuvant also showed elevated BoNT/A-specific IgG Ab levels in plasma (Fig. 2*A*), while mice given nasal BoNToxoid alone exhibited only minimally detectable levels of BoNT/A-specific plasma IgG or IgA Ab responses (Fig. 2*A*). Furthermore, mice given nasal BoNToxoid/A plus mCT E112K showed significant levels of plasma IgG1, IgG2a, and IgG2b, but not IgG3 Ab responses (Fig. 2*B*). However, anti-BoNT/A IgG2a Ab levels were lower than IgG1 and IgG2b subclass Ab responses (Fig. 2*B*). These results clearly show that our mucosal BoNT vaccine evokes two layers of BoNT/A-specific immune responses: S-IgA Abs in mucosal external secretions, and IgG and IgA Abs in plasma.

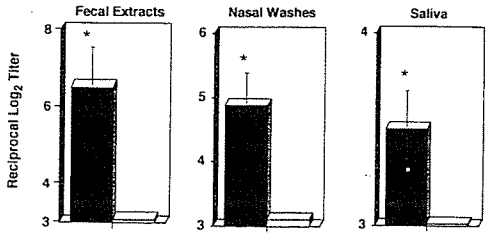

FIGURE 1. Comparison of BoNT/A-specific IgA Ab responses in nasal washes, fecal extracts, and saliva of mice given nasal BoNT vaccine with (■) or without (□) mCT E112K. Each mouse group was nasally immunized once/wk for 4 consecutive weeks with 20 μg of BoNToxoid alone or in together with 5 μg of mCT E112K as mucosal adjuvant. Seven days after the last nasal immunization, S-IgA Ab levels in nasal washes, fecal extracts, and saliva were determined by a BoNT/A-specific ELISA. The values shown are the mean ± SEM for 12 mice in each experimental group. The asterisks indicate a significant difference between the mean of groups given BoNT/A vaccine in the presence or absence of adjuvant; *, $p < 0.05$.

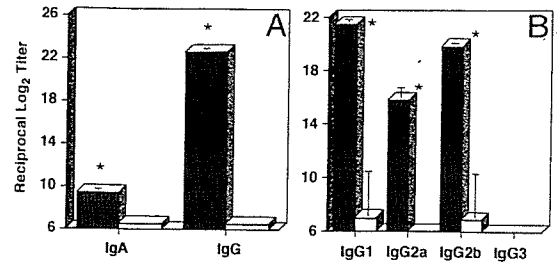

FIGURE 2. Comparison of BoNT/A-specific IgG, IgA, and IgG subclass Ab responses in plasma of mice given nasal BoNT vaccine alone (□) or together with mCT E112K as adjuvant (■). Each mouse group was nasally immunized once/wk for 4 consecutive weeks with 20 μg of BoNToxoid alone or together with 5 μg of mCT E112K as mucosal adjuvant. Seven days after the last nasal immunization, IgG or IgA (*A*) and IgG subclass (*B*) Ab levels in plasma were determined by a BoNT/A-specific ELISA. The values shown are the mean ± SEM for 12 mice in each experimental group. The asterisks indicate a significant difference between the mean of groups given BoNT/A vaccine in the presence or absence of adjuvant; *, $p < 0.01$.

BoNT/A-specific AFCs in mice given the nasal vaccine

To confirm that BoNT/A-specific Ab responses were induced in both mucosal and systemic lymphoid tissues, mononuclear cells from NP, iLP, and spleen were taken from mice nasally immunized with BoNToxoid/A plus mCT E112K as mucosal adjuvant. These lymphoid cells were then subjected to a BoNT/A-specific ELISPOT assay to determine the numbers and isotypes of AFCs present. Mice given nasal vaccine exhibited high numbers of BoNT/A-specific AFCs in IgA effector sites such as NPs and iLP (Fig. 3). Increased numbers of BoNT/A-specific IgG AFCs were noted in NP and iLP of mice given mCT E112K as nasal adjuvant when compared with the group of mice given BoNToxoid/A alone. Furthermore, mice given BoNToxoid/A and mCT E112K as nasal adjuvant showed significant numbers of anti-BoNT IgA and IgG AFCs in spleen, while mice given BoNToxoid alone showed only minimal numbers of BoNT/A-specific IgA or IgG AFCs (Fig. 3). These results clearly show that BoNT/A-specific immunity mediated through the NALT immune system is robustly induced in both mucosal and systemic lymphoid compartments.

Induction of protective systemic immunity by the nasal BoNT vaccine

Because nasal immunization with BoNToxoid/A plus mCT E112K as mucosal adjuvant induced both mucosal and systemic Ab responses, it was important to determine whether these Ag-specific

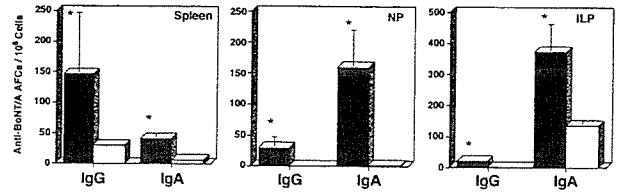

FIGURE 3. Analysis of BoNT/A-specific AFCs in mice given nasal BoNT vaccine with (■) or without (□) mCT E112K as adjuvant. Seven days after the last nasal immunization, mononuclear cells isolated from the NP, iLP, and spleen were examined using a BoNT/A-specific ELISPOT assay to determine the numbers of IgG and IgA AFCs. The results represent the mean values ± SEM for 15 mice in each experimental group. The asterisks indicate a significant difference between the mean of groups given BoNT/A vaccine in the presence or absence of adjuvant; *, $p < 0.01$.

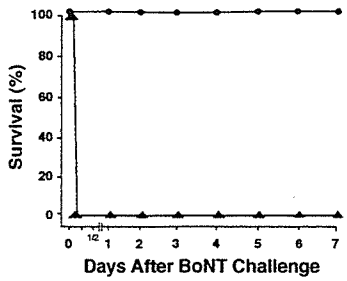

FIGURE 4. Nasal immunization with BoNToxoid/A together with mCT E112K as mucosal adjuvant elicits protection against systemic challenge with BoNT/A. Mice were immunized nasally with either 15 μg of BoNToxoid/A and 5 μg of mCT E112K (●) or 20 μg of BoNToxoid without adjuvant (▲) three times at weekly intervals. Mice were challenged with a lethal dose (4000 × i.p. LD_{50}) of BoNT/A in 0.1 ml of PBS including 0.2% gelatin via the i.p. route. Each group consisted of six mice, and the data are representative of three separate experiments.

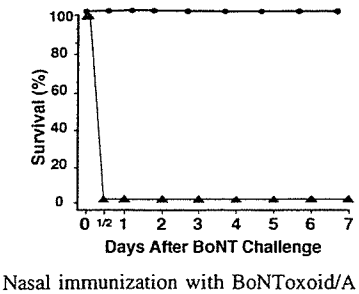

FIGURE 5. Nasal immunization with BoNToxoid/A and mCT E112K as mucosal adjuvant prevents enteric intoxication. Mice were immunized nasally with 20 μg of BoNToxoid/A and 5 μg of mCT E112K (●) or 15 μg of BoNToxoid without adjuvant (▲) three times at weekly intervals. Mice were challenged with a lethal dose (2 × oral LD_{50}) of BoNT/A in 0.25 ml of PBS including 0.2% gelatin via the oral route. Each group consisted of six mice, and the data are representative of three separate experiments.

Ab responses could protect mice from BoNT/A intoxication. To make this determination, groups of mice were immunized nasally with BoNToxoid/A plus mCT E112K or BoNToxoid without adjuvant at weekly intervals for 4 consecutive weeks. One week after the last immunization, the mice were then challenged i.p. with a lethal dose (0.2 μg/mouse: 4000 × i.p. LD_{50}) of BoNT/A, which induces paralysis and death within 30 min. Mice given nasal BoNToxoid/A plus mCT E112K as mucosal adjuvant were completely protected for the first 24 h (Fig. 4), while mice given BoNToxoid alone were not protected from paralysis and died within 24 h of challenge (Fig. 4). These findings clearly suggest that BoNT/A-specific, plasma IgG Abs in mice induced by the BoNT/A nasal vaccine were protective.

Mucosal BoNT/A-specific S-IgA Abs prevent enteric botulism

Because our nasal BoNT/A vaccine induced BoNT/A-specific S-IgA Ab responses in mucosal sites, it was important to determine whether these mucosal S-IgA Abs also provided protection against mucosal BoNT/A intoxication. To do so, we initially established an oral challenge system using gastric intubation. Because nasal washes, but not fecal extracts, contained significant levels of BoNT/A-specific IgG Abs, it was logical to devise an oral challenge system to elucidate the role of BoNT-specific S-IgA Abs for mucosal intoxication. Mice were starved and pretreated orally with 7.5% sodium bicarbonate isotonic solution before oral challenge. When various doses (25 ng, 250 ng, and 2.5 μg/mouse) of BoNT/A were administered into the duodenum of each group of mice (six mice/group), three of six mice given the highest dose (2.5 μg/mouse) died within 24 h. All mice in the other groups survived until the end of the experiment (7 days) (Table I). Thus, we established that 2.5 μg was a LD_{50} for oral challenge. Based upon these results, mice given nasal vaccine consisting of BoNToxoid/A and mCT E112K were orally challenged with a dose of 2 × LD_{50} of BoNT/A 1 wk after the last immunization. All mice in this mucosally vaccinated group survived for 7 days after oral challenge with BoNT/A (Fig. 5). Conversely, naive mice and mice given BoNToxoid alone died within 24 h after challenge (Fig. 5). These results indicate that mucosal BoNT/A-specific S-IgA Ab responses induced by our nasal vaccine containing BoNToxoid/A and mCT E112K protect mice from mucosal BoNT/A intoxication.

Mucosal BoNT/A-specific S-IgA Abs possess neutralization activity

To further establish a role for BoNT/A-specific S-IgA Abs, mice were challenged with BoNT/A pretreated with fecal extract samples. When mice were challenged with 1 × LD_{50} i.p. dose of BoNT/A that had been preincubated with a fecal extract sample, one-half survived for 3 days and one-third survived until the end of the experiment. In contrast, when mice were injected with BoNT/A and fecal extracts from mice nasally immunized with OVA plus mCT, three-quarters died of BoNT/A intoxication within 1 day (Fig. 6). These results indicate that BoNT/A-specific S-IgA Abs play a key role in the protection against BoNT/A intoxication.

Discussion

The current botulinum vaccines, both pentavalent (serotpyes A-E) and monovalent (serotype F), are used for at-risk populations such as scientists, health care providers, and the military (26, 37). Professional health care workers are well trained in avoiding mucosal BoNT intoxication while working with BoNTs. Thus, the current vaccines that induce only systemic immunity to BoNTs may be sufficient to protect these professional or military populations. However, natural botulism is disseminated by food, water, or air, and so a bioterrorist attack would most likely target mucosal surfaces. It is feared that the currently available botulinum vaccine will most likely not prevent such mucosal BoNT intoxication. Although passive immunization has been reported to prevent inhalational BoNT intoxication (38), it is not at all certain that such a systemic BoNT-specific Ab treatment would provide effective protection against food- or waterborne botulism. Furthermore, the mucosal tissue damage induced by BoNTs needs to be carefully evaluated. Our studies are aimed at developing a mucosal vaccine that

Table I. *Determination of the oral challenge dose for BoNT/A*

Oral Dose of BoNT/A (ng/mouse)	Numbers of Mice Surviving Challenge						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
25	6/6	6/6	6/6	6/6	6/6	6/6	6/6
250	6/6	6/6	6/6	6/6	6/6	6/6	6/6
2500	3/6	3/6	3/6	3/6	3/6	3/6	3/6

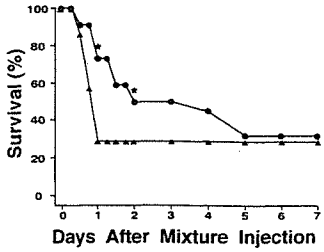

FIGURE 6. BoNT/A-specific S-IgA Abs in fecal extracts of mice given nasal vaccine neutralize BoNT/A toxicity. Fecal extracts (200 μl) from mice given nasal BoNToxoid/A (●) or OVA (▲) plus mCT E112K were incubated with 1 × i.p. LD_{50} of BoNT/A in 0.2 ml of PBS including 0.2% gelatin for 30 min at room temperature. Each mixture was then injected into mice via the i.p. route. Each group consisted of nine mice, and the data are representative of two separate experiments. The asterisks indicate a significant difference between the groups; *, $p < 0.01$.

can prevent mucosal BoNT intoxication as well as provide systemic immune protection comparable to that offered by the two current vaccines.

Our study provides the first evidence that nasal administration of BoNToxoid/A plus mCT E112K effectively induces BoNT/A-specific S-IgA Ab responses in addition to plasma IgG Abs. Thus, Ag-specific Ab responses were seen in plasma and mucosal secretions, while Ag-specific AFCs were detected in the NPs, the iLP, and the spleen, clearly showing that both mucosal and systemic immunity were induced in mice given the nasal BoNT vaccine. In this study, we have developed a unique oral BoNT/A challenge model that allows for investigation of protective mucosal S-IgA Ab responses. With this novel method, the levels of BoNT required for intoxication can be quantitated.

In showing that mice given a nasal BoNToxoid with mCT as mucosal adjuvant were protected from an oral challenge dose of 2 × LD_{50} BoNT/A, this study provided the first evidence that BoNT/A-specific S-IgA Abs play an important role in protecting against mucosally delivered BoNT/A. Furthermore, our study has also shown that BoNT/A-specific IgG Ab responses in plasma of mice given nasal vaccine also provide effective protection against i.p. BoNT/A intoxication. These results clearly demonstrate that this nasal BoNT/A vaccine establishes two layers of immune protection, one at the mucosal surfaces themselves and the second in the blood circulation via protective IgG Abs.

To assess the potential of mucosal vaccines, studies have relied on the oral delivery of mutant BoNT/B and on the nasal administration of the whole H chain component of BoNT/A. Although these reports showed successful induction of BoNT-specific Ab responses, they limited themselves to an examination of the IgG Ab isotype and did not investigate a role for S-IgA Ab responses in BoNT intoxication (39, 40). In contrast, our study has shown that BoNT/A-specific S-IgA Ab responses were induced in various mucosal secretions when mice were given a nasal BoNT/A vaccine. Indeed, like Ag-specific neutralizing plasma IgG Abs, BoNT/A-specific S-IgA Abs in fecal extract samples neutralized biologic activity and prevented BoNT/A intoxication. Furthermore, mice possessing high levels of BoNT/A-specific S-IgA Abs were completely protected against oral challenge with BoNT/A. These results indicate that intestinal mucosal BoNT/A-specific Abs, which are predominantly of the S-IgA isotype, play an important role in the prevention of GI tract botulism. To directly confirm the role of BoNT-specific S-IgA Abs in BoNT/A intoxication, we are currently testing the efficacy of nasal BoNT/A vaccine in both IgA-deficient and polymeric Ig receptor-deficient mice.

It is not enough, however, to consider the effects of BoNT on the GI tract. Because bioterrorists are more likely to disseminate BoNT by air than by food or water, it is of paramount importance to protect again nasal BoNT intoxication. For an example of terrorists' predilection for an airborne method of delivery, one need go no farther than the failed attempt by the Japanese cult Aum Shinrikyo to disseminate BoNT in downtown Tokyo (37).

The immunopathologic threat posed by nasal BoNT intoxication may be of a different order than those posed by orally ingested BoNT, because the nasal cavity is directly connected with the CNS through the olfactory nerves and epithelium as well as the olfactory bulbs. These neuronal tissues express an abundance of different types of gangliosides, including GD1a and GT1b, which are potent binding receptors for the BoNT/B serotype (41). The other type of ganglioside-binding toxins, such as CT and heat LT of *Escherichia coli*, has been shown to cause significant neuronal tissue damage when administered via the nasal route (42). Such a prospect of neuronal tissue damage underlines once again how imperative it is to devise a novel mucosal vaccine for the prevention of nasal BoNT intoxication. Our current study has shown that nasal BoNToxoid/A plus mCT E112K induces significant levels of BoNT/A-specific S-IgA and IgG Ab responses in nasal washes, indicating that this nasal vaccine protocol provides potent protection against nasal BoNT intoxication as well as neuronal tissue damage. We are currently developing a nasal BoNT challenge system to examine the efficacy of this mucosal BoNT vaccine and to assess any potential BoNT toxicity for the olfactory tissues and the CNS.

Although the nasal vaccine regimen helped induce two layers of Ag-specific responses in mucosal and systemic lymphoid tissues, it succeeded in doing so only with the aid of a mucosal adjuvant. Concern has been expressed that the potent toxicity of toxin-based mucosal adjuvants such as CT and LT may induce CNS damage (42). To circumvent this problem, both current and our previous studies have examined whether nontoxic mCTs as well as cytokines and chemokines provide mucosal adjuvanticity. These studies have clearly shown that nasal application of cytokines or chemokines as mucosal adjuvants together with selected Ags induces Ag-specific Ab responses in both systemic and mucosal compartments (5, 10–12, 43–47). Although the toxicity of these cytokines and chemokines needs to be elucidated, our most recent studies show that nasal mCT E112K does not elicit nerve growth factor-β1 induction indicative of neuronal tissue damage in the CNS of nonhuman primates (48). Thus, we feel confident that our current approach may yield a nasal vaccine able to generate effective immunity against botulism.

Ag-specific plasma IgG subclass Ab responses are excellent indicators of Th1- or Th2-type cytokine responses by $CD4^+$ T cells. Our results showed significantly increased levels of IgG1, IgG2a, and IgG2b Ab responses in plasma of mice given nasal BoNToxoid/A plus mCT E112K when compared with those mice given nasal toxoid only. Among these elevated IgG subclass Abs, levels of anti-BoNT/A IgG1 and IgG2b Abs were higher than the IgG2a subclass Ab response. A similar pattern of Ag-specific IgG subclass responses has been seen in mice given native CT or mCT as nasal adjuvants, which are known to induce Th2-type cytokine responses (3, 4). Thus, we anticipate that BoNT/A-specific Ab responses induced by nasal BoNToxoid plus mCT E112K are mediated through Th2-type cytokine responses. To our knowledge, no studies have reported potential roles of Th1- and Th2-type responses by BoNT-specific $CD4^+$ T cells. To this end, our studies are the first to suggest that BoNT/A-specific and dominant Th2-type responses are associated with protective immunity against botulism. More precise and direct studies of BoNT vaccine-induced Th1 and Th2 cytokine responses are currently underway in our laboratory.

In summary, our studies have now provided the first evidence that nasal immunization with BoNToxoid/A plus mCT E112K induces BoNT/A-specific S-IgA Ab responses in mucosal secretions as well as plasma IgG Ab responses. Furthermore, mice given nasal BoNT/A vaccine were protected from both parenteral and oral challenge with BoNT/A. These results directly demonstrate that mucosal immunization is able to provide two layers of immunity, a first line of defense at mucosal surfaces and a second line via systemic blood circulation. The precise roles of BoNT-specific S-IgA Abs will require further investigation; however, our findings in this study show that this BoNT-based mucosal vaccine is an effective and perhaps essential strategy for the protection of the population against botulism used as a weapon of bioterrorists.

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Prenatal Blockage of Lymphotoxin β Receptor and TNF Receptor p55 Signaling Cascade Resulted in the Acceleration of Tissue Genesis for Isolated Lymphoid Follicles in the Large Intestine[1]

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Signaling by lymphotoxin (LT) and TNF is essential for the organogenesis of secondary lymphoid tissues in systemic and mucosal compartments. In this study, we demonstrated that the progeny of mice treated with fusion protein of LTβR and IgGFc (LTβR-Ig) or LTβR-Ig plus TNFR55-Ig (double Ig) showed significantly increased numbers of isolated lymphoid follicles (ILF) in the large intestine. Interestingly, double Ig treatment accelerated the maturation of large intestinal ILF. Three-week-old progeny of double Ig-treated mice showed increased numbers of ILF in the large intestine, but not in the small intestine. Furthermore, alteration of intestinal microflora by feeding of antibiotic water did not affect the increased numbers of ILF in the large intestine of double Ig-treated mice. Most interestingly, mice that developed numerous ILF also had increased levels of activation-induced cytidine deaminase expression and numbers of IgA-expressing cells in the lamina propria of the large intestine. Taken together, these results suggest that ILF formation in the large intestine is accelerated by blockage of LTβR and TNFR55 signals in utero, and ILF, like colonic patches, might play a role in the induction of IgA response in the large intestine. *The Journal of Immunology,* **2005, 174: 4365–4372.**

The gut-associated lymphoid tissues are characterized as the initiation sites for the induction of IgA-mediated immunity and mucosally induced tolerance (1, 2). The mucosal immune system possesses a network of lymphoid organs that are composed of inductive sites (e.g., Peyer's patches (PP))[3] and effector sites (the intraepithelial and the lamina propria (LP) region) (1, 2). It had been believed that PP is the major inductive site for the initiation of Ag-specific IgA responses to a variety of exogenous Ag (3, 4); however, we and others have demonstrated that PP contribute to, but are not essential for, the induction of Ag-specific mucosal IgA responses (5–7). A recent study revealed the existence of isolated lymphoid follicles (ILF) in the small intestine that resemble PP in terms of architecture and cellular composition (8). The fact that ILF possess germinal centers and an overlying follicle-associated epithelium (FAE) containing M cells suggests their possible role as mucosal inductive sites (8).

Lymphotoxin (LT), a TNF family member, can be found in two forms: a membrane-bound heterotrimer and a soluble homotrimer (9, 10). The membrane-bound heterotrimer is comprised of two β-chains and one α-chain (LTα1β2) and is a ligand for LTβR, while the soluble homotrimer (LTα3) is ligand for both TNFR55 and TNFR75 (11, 12). Unlike the LTα trimer and TNF, which are secreted proteins, LT$\alpha\beta$ remains membrane bound and is expressed on the restricted hemopoietic lineage, particularly by T cells, B cells, and NK cells (13). The interaction of LT$\alpha\beta$ with LTβR is the critical molecular event triggering secondary lymphoid organ genesis and controlling spleen organization. For example, congenital lack of LTα, LTβ, or LTβR genes disrupted PP and lymph node (LN) organogenesis, and altered splenic architecture as characterized by the absence of distinct T and B cell areas and disruption of the marginal zone (14–17). Furthermore, administration of LTβR-Ig fusion protein to mice during the selected time window of embryogenesis disrupted LN and PP formation in the progeny (18, 19), suggesting that the molecular interaction of membrane-bound LT with LTβR during the gestational period is essential for the initiation of LN and PP development. In contrast to PP, a recent study demonstrated that ILF formation was not influenced by the blockage of LTβR signaling with LTβR-Ig fusion protein during gestation (8). However, no ILF was found in LT$\alpha^{-/-}$ or *aly/aly* mice, implying that ILF do require signals dependent on LT and NF-κB-inducing kinase, a critical downstream signaling molecule associated with LTβR, postgestation (8). It has recently been confirmed that, unlike PP formation, ILF formation requires LT-LTβR interaction in adulthood, as well as TNFR55-mediated signaling for their maturation (20).

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[3] Abbreviations used in this paper: PP, Peyer's patch; AID, activation-induced cytidine deaminase; CP, colonic patch; FAE, follicle-associated epithelium; ILF, isolated lymphoid follicle; LI-ILF, large intestinal ILF; LN, lymph node; LP, lamina propria; LT, lymphotoxin; PNA, peanut agglutinin.

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An additional component of the gut immune system is the colonic patch (CP). The cytoarchitectural components and immune functions of CP and PP were remarkably similar, despite differences in the surrounding environment of mucosa and luminal microbial exposure (21). The presence of organized lymphoid tissue with M cells and germinal centers in CP suggests that Ag uptake and recognition can take place in the rectum (22, 23). Similar to the PP, in utero treatment with LTβR-Ig fusion protein depleted CP formation in progeny (23). These results suggested that PP and CP were developmentally and functionally related components of the small intestine and large intestinal (colonic) immune systems, respectively. In addition to CP, it was shown that ~50 ILF were dispersed throughout the large intestine of BALB/c mice (8). Recently, it was reported that in utero treatment of mice with LTβR-Ig and TNFR55-Ig fusion proteins caused an increase in the number of submucosal lymphoid patches in the large intestine (24). This suggests that ILF in the small and large intestine are developmentally similar, although little else is known about these immunological structures and functions. In particular, the function of ILF in the large intestine and the precise contribution of LTβR and TNFR55 for their genesis, maturation, and the subsequent induction of IgA responses remain to be elucidated.

In this study, we provide several new findings regarding the unique contribution of the inflammatory cytokines LT and TNF in the genesis and function of ILF in the large intestine. In particular, the tissue genesis signals provided by the cytokine receptors of LTβR and TNFR55 are essential for the postnatal development of large intestinal ILF (LI-ILF). Our present findings suggest that the receptors behave as negative regulators for the genesis of LI-ILF because the blockage of prenatal LT/LTβR and TNF/TNFR55 signaling cascades accelerated the formation and maturation of ILF in the large intestine. Secondly, environmental factors, such as microflora-associated Ags, did not affect the formation and maturation of ILF in the large intestine. Finally, ILF in the large intestine play an important role for IgA^+ B cell development.

Materials and Methods

Mice

Timed pregnant BALB/c mice were purchased from Japan CLEA. These mice were maintained in the experimental facility under pathogen-free conditions in the Research Institute for Microbial Diseases at Osaka University and International Vaccine Institute and received sterilized food (certified diet MF; Oriental Yeast) and tap water ad libitum. TNF and LTα double-knockout (TNF/$LT\alpha^{-/-}$ mice; 129 × C57BL/6) mice were kindly provided by H. Bluethmann (Roche Center for Medical Genomics, Basel, Switzerland) (25). Germfree mice (BALB/c Yit) were kindly provided by H. Funabashi (Yakult Central Institute for Microbiological Research, Japan).

Fusion proteins and treatment protocol

Proteins comprised of the extracellular domain of either murine TNFR55 or LTβR fused to the hinge, C_H2, and C_H3 domains of human IgG1 (LTβR-Ig, TNFR55-Ig, and LFA-3-Ig, respectively) were used in our studies, as described elsewhere (19, 26, 27). Timed pregnant mice were injected i.v. with 200 μg of LTβR-Ig and/or 200 μg of TNFR55-Ig on gestational days 14 and 17, as described previously (5, 19). In some experiments, progeny of mice treated i.v. with LTβR-Ig and TNFR55-Ig on gestational period were further injected i.p. with 20 μg of LTβR-Ig, TNFR55-Ig, or human IgG1 (control) at weekly intervals from 7 days after birth and 50 μg of each Ig fusion protein from 4 wk old until age of 6 wk.

Cell purification

The mononuclear cells from CP and ILF of the large intestine were obtained with modified method, as described previously (8). In brief, the large intestine was opened longitudinally along the mesenteric wall, and mucus and feces were vigorously washed in the RPMI 1640 medium and wiped with filter paper. Subsequently, a section of intestine ~30 mm long was pasted on a plastic culture dish. The structures of CP and ILF are both circular in appearance, but the CP are larger than triple diameter of the ILF. Morphologically, the center of CP forms protruding configuration, and thus CP appears as dome-shaped tissue under a transillumination seromicroscope (Olympus TH3). In contrast, the ILF are recognized as flat shape. Blind test was conducted to count the number of ILF by three independent investigators by a transillumination seromicroscope. Then CP were taken with two sharp forceps and isolated, and a tiny fragment of ILF was isolated using a sharp needle (23 gauge; inner diameter, μm). CP and ILF were separately digested with collagenase (type IV, 0.5 mg/ml in RPMI 1640 including 2% FBS; Sigma-Aldrich) for 20 min in a 37°C incubator. This step was repeated until the architecture of tissue was totally disrupted. The single cell suspensions were pooled, washed, and placed on a discontinuous 40 and 70% Percoll gradient (Pharmacia). After centrifugation for 20 min at 600 × *g*, the cells were collected from the interface (8). To isolate the LP lymphocytes from the large intestine, mononuclear cells were dissociated using the collagenase digestion method after removal of CP and ILF, as described previously (28).

Flow cytometric analysis

A single lymphoid cell suspension was incubated with anti-FcγRII/III mAb (BD Pharmingen) and stained with FITC- or PE-conjugated anti-B220, CD3, IgD, IgM, or IgA mAbs (BD Pharmingen). The other aliquots of cells were incubated with each isotype control mAb, including rat IgG2b or rat IgG2a (BD Pharmingen). The profiles were analyzed using FACScan with CellQuest software (BD Biosciences). Reactivity with peanut agglutinin (PNA) was demonstrated using biotinylated PNA (Vector Laboratories), followed by streptavidin-PE.

Histochemical analysis

For the H&E staining, the large intestine was fixed in 4% paraformaldehyde and embedded in paraffin. The tissues were cut into 5-μm sections and stained with H&E (28). The sections were mounted and viewed under ×20 optics using a digital light microscope. Each of the images was analyzed with Photoshop (Adobe Systems). For the immunohistochemical study, freshly obtained large intestine was rapidly frozen in OCT embedding medium (Tissue-Tek) and stored at −80°C until processing (28). Cryostat sections (5 μm) were fixed in ice-cold acetone for 10 min, dried, and preblocked with anti-FcγRII/III mAb (BD Pharmingen) in PBS. Cells were stained with FITC-conjugated anti-CD11c mAb (BD Pharmingen) and PE-conjugated anti-B220 or CD3 mAbs (BD Pharmingen). The other aliquots of cells were incubated with each isotype control mAb, including hamster IgG, rat IgG2b, or rat IgG2a (BD Pharmingen). IgA-containing cells were visualized by FITC-conjugated anti-IgA mAb (BD Pharmingen). The sections were mounted and viewed under a dual red/green filter by confocal microscopy (Bio-Rad). Each of the images was analyzed with Photoshop (Adobe Systems) in a consistent manner, followed by overlying of the green and red images in the screen mode.

Scanning electron microscope analysis

For scanning electron microscopy analysis, large intestinal fragments of the double Ig-treated mice were cleaned of mucus and fixed in 2% glutaraldehyde and 2% paraformaldehyde in PBS containing 100 mM HEPES for 1 h at room temperature. After being washed with PBS, specimens were treated with 1% osmium tetroxide for 1 h at room temperature and then dehydrated in graded ethanol solution. Dehydrated tissues were critical point dried with CO_2, and sputter coated and observed with a scanning electron microscope (Hitachi).

Treatment with antibiotic water

For the antibiotic treatment, each group of 6-wk-old progenies was given antibiotics in drinking water for a period of 4 wk. The antibiotic water contained 500 mg/L ampicillin, 1 g/L neomycin sulfate, and 2 g/L of streptomycin (29).

RT-PCR

A standard quantitative RT-PCR protocol was used for the study for GAPDH-based quantitative RT-PCR (30). Total RNA was extracted from mononuclear cells isolated from LP of the large intestine or CP of naive mice or ILF of double Ig-treated mice by using the RNeasy mini kit (Qiagen), according to the manufacturer's protocol. A total of 1 μg of total RNA was reverse transcribed into cDNA using *Taq* Man reverse transcription kit (Applied Biosystems). Activation-induced cytidine deaminase (AID) mRNA levels were measured by real-time quantitative PCR method performed on the ABI PRISM 7500 (Applied Biosystems). For each treatment, two distinct amplifications were conducted in parallel to amplify

AID cDNA and GAPDH cDNA. The amplification reactions were performed in 25 μl vol containing 100 ng of cDNA per treatment, 12.5 μl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), and 1.25 μl of 20× Assays-on-Demand Gene Expression probe for AID (Applied Biosystems) or TaqMan GAPDH probe (Applied Biosystems). AID mRNA levels from each treatment were normalized to the corresponding amount of GAPDH mRNA levels. Water controls and samples without PCR mixtures were set up to eliminate the possibility of significant DNA contamination.

ELISPOT assay for total IgA Ab-forming cells

An ELISPOT assay was adopted to detect total numbers of IgA Ab-forming cells in the large intestine, as described previously (31).

Statistics

The data are expressed as the mean ± SE and compared using *t* test in Microsoft Excel Program.

Results

In utero blockade of LTβR- and TNFR-mediated signals induces the accelerated formation of ILF in the large intestine of mice

Using LTβR- and TNFR55-Ig fusion proteins as soluble antagonists, we tested whether LTαβ- and TNF-mediated signals influenced the formation of organized lymphoid tissues in the large intestine. For this purpose, mice were treated with TNFR55-Ig or LTβR-Ig or LTβR-Ig plus TNFR55-Ig (double Ig) fusion protein at gestational days 14 and 17. The exposure to LTβR-Ig or double Ig during the gestation period disrupted CP formation in the progeny, which confirmed our previous finding (23). Unexpectedly, however, careful light microscopy and H&E staining analysis revealed that numerous ILF existed throughout the mucosa of the large intestine of 6-wk-old mice treated in utero with LTβR-Ig or with double Ig fusion proteins (Fig. 1*A*). These changes in ILF development were not seen in the small intestine of these treated mice (6 wk old), but were unique to the large intestine. Interestingly, the progeny of mice treated with double Ig fusion protein in utero possessed more numerous ILF of larger size in the large intestine than those treated with LTβR-Ig alone. When the total number of ILF in the whole large intestine was counted under light microscopy, ~250 ILF were found in the progeny of mice treated with double Ig during gestation (Fig. 1*B*). In contrast, the numbers of ILF in control Ig-treated mice were 50 per large intestine (Fig. 1*B*). When mice were treated with TNFR55-Ig alone, no significant changes were seen in the total number of ILF in the large intestine. In all mice, ILF were preferentially located in the distal region of the large intestine (data not shown). An average of total recovered cell numbers of ILF isolated from the large intestine of the progeny of mice treated with double Ig fusion protein was 10-fold higher than control Ig-treated mice (3.0×10^6 vs 0.3×10^6) and 3-fold higher than LTβR-Ig-treated mice (3.0×10^6 vs 1.0×10^6). These observations suggest that ILF formation in the large intestine was accelerated by blockage of prenatal LTβR-mediated signals, and their maturation was further enhanced by the coblockage of TNFR-mediated signals during the selected gestational period.

Postnatal blockade of LTβR-mediated signals inhibits the accelerated formation of ILF in the large intestine of mice

To establish an exact role of TNFR and LTβR signaling after birth on the accelerated formation of ILF in the large intestine, the

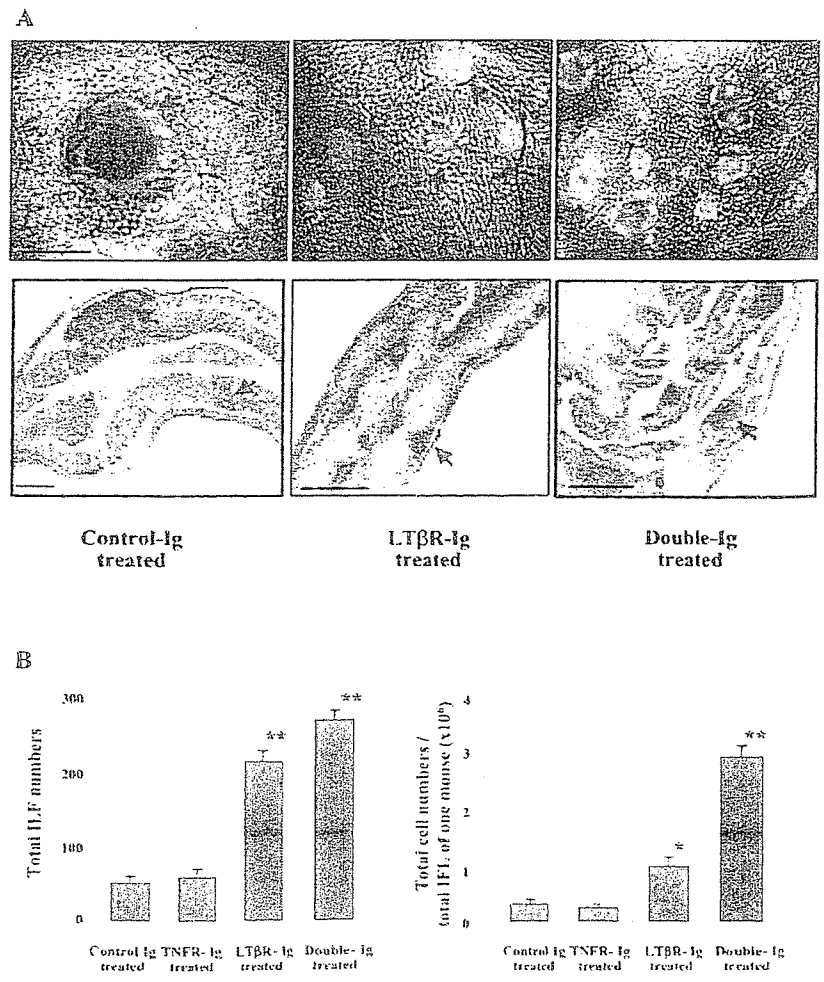


FIGURE 1. Effects of prenatal blockage of LT/LTβR and TNF/TNFR55 signal on the formation of ILF in the large intestine. Timed pregnant BALB/c mice were injected i.v. with TNFR55-Ig or LTβR-Ig and/or TNFR55-Ig (double IgG1) on gestational days 14 and 17. Purified human Ig was used as control Ig. *A*, Morphology of colonic patch (red arrow) and ILF (blue arrow) in the large intestine of each 6-wk-old progeny. The large intestine was opened longitudinally along the mesenteric wall, and a ~30-mm-long length of intestine was pasted onto the plastic culture dish. The picture was taken under the stereomicroscopy (*upper*). For the H&E staining, the large intestine was fixed in 4% paraformaldehyde and embedded in paraffin. The sections were viewed under ×20 optics with a digital light microscope (*below*). The bar indicates 50 μm. *B*, Total number of ILF in a whole large intestine was counted under a light microscope (*left*). Each ILF was then taken off with a sharp needle, and mononuclear cells were isolated and counted (*right*). The results are expressed as the mean ± SE from three mice per group and from a total of three experiments. *, $p < 0.05$, and **, $p < 0.01$ when compared with the number of ILF in the large intestine of control Ig-treated mice.

progenies of double Ig-treated mice on gestational period were further injected i.p. with LTβR-Ig or TNFR55-Ig or control Ig at weekly intervals from 1 wk after birth to age of 6 wk. Although the numbers of ILF were slightly lower, no significant difference was detected on the numbers of ILF in the large intestine of TNFR55-Ig-treated progenies compared with the control Ig-treated one. Interestingly, however, the large intestine of progenies postnatal treated with LTβR-Ig did not possess any ILF (Fig. 2). Furthermore, we have assessed the presence of ILF in the large intestine of TNF/LTα double-knockout mice that lack signaling pathways through both TNFR55 and LTβR. Interestingly, there are no ILF in the large intestine of those mice (Fig. 2). These findings indicate that the tissue genesis signaling pathway through LTβR, but not TNFR55, is required for the formation of ILF in the large intestine.

ILF in the large intestine contain B220$^+$, CD11c$^+$, and CD3$^+$ cells

To clarify the cell population in ILF of the large intestine, flow cytometric and immunohistochemical analyses were conducted using 6-wk-old progeny of mice treated in utero with control Ig or double Ig. Similar to the ILF in the small intestine (8, 20), immunohistochemical study revealed that LI-ILF were enriched with B cells (B220$^+$), with a limited frequency of dendritic stromal cells (CD11c$^+$) and T cells (CD3$^+$) (Fig. 3). Their cell population is phenotypically similar to the CP, but LI-ILF possesses more B220$^+$ cells and less CD3$^+$ cells than CP (Fig. 3). A major population of B220$^+$ cells in LI-ILF belongs to the IgD$^+$ and IgM$^+$ cells with some B220$^+$IgA$^-$ cells (Fig. 3). Similar to the phenotype of CP, LI-ILF possess detectable levels of B220$^+$IgA$^+$ cells, implying a role of LI-ILF as an inductive site for mucosal IgA responses. Interestingly, LI-ILF of mice treated with double Ig fusion protein in utero showed a higher density of CD3$^+$ cells when compared with those of control Ig-treated progeny (Fig. 3). Although the frequency of CD3$^+$ cells is increased in the treated LI-ILF, the follicle is still considered a B cell-enriched tissue because the frequency of B220$^+$ cells outnumbers CD3$^+$ cells (Fig. 3). In addition, flow cytometry analysis showed that LI-ILF of control Ig-treated progeny contained low numbers of germinal center-forming PNA$^+$ B cells; however, blockage of LTβR and TNFR55 signals during gestation enhanced the formation of germinal center-forming PNA$^+$ B cells (Fig. 3). These findings suggest that LI-ILF, similar to ILF in the small intestine, is one of key

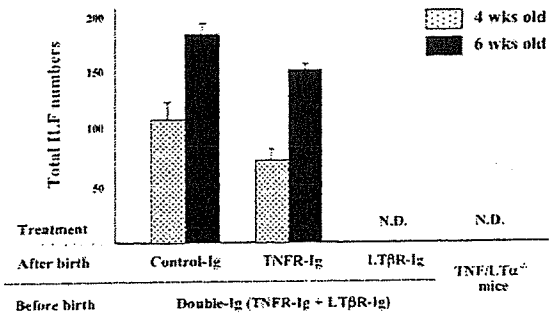
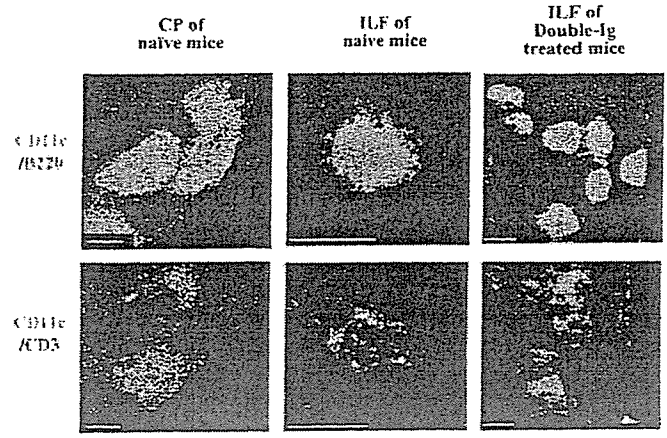


FIGURE 2. Effects of postnatal blockage of LT/LTβR or TNF/TNFR55 signal on the formation of ILF in the large intestine of the progenies treated in utero with the LTβR-Ig and TNFR55-Ig (double Ig). Progeny of mice treated i.v. with double Ig on gestational days 14 and 17 were further injected i.p. with 20 μg of LTβR-Ig or TNFR55-Ig or control Ig at weekly intervals from 7 days after birth and 50 μg of each Ig fusion protein from 4 wk old. Total numbers of ILF in a whole large intestine were counted under the stereomicroscope. The results are expressed as the mean ± SE from three mice per group and from a total of two experiments. N.D., not detectable.



Mononuclear Cells	CP	ILF	
	Control-Ig	Control-Ig	Double-Ig
B220$^+$	77.4± 2.5	86.9±3.4	86.2±3.9
CD3$^+$	13.3±1.2	5.7± 0.5	10.0± 2.2
IgD$^+$/IgM$^+$	57.5±3.8	32.0± 1.8	44.8± 2.2
PNA$^+$/B220$^+$	5.4± 1.1	1.0± 0.2	6.4± 1.0
B220$^+$/IgA$^+$	10.5± 1.2	5.6± 1.0	6.3± 0.2

FIGURE 3. Characterization of mononuclear cells in the LI-ILF and CP of mice in utero treated with LTβR-Ig and TNFR55-Ig fusion proteins, as described in Fig. 1 legend. Immunofluorescence staining (*upper picture*) and FACS analysis of mononuclear cells isolated from CP and ILF of the large intestine (table). For the immunohistochemical study, the large intestine of 6-wk-old progenies was rapidly frozen, and cryostat sections were stained with FITC-conjugated anti-CD11c mAb and PE-conjugated anti-B220 mAb or PE-conjugated anti-CD3 mAb. The FACS results are expressed as the mean ± SE from three mice per group and from a total of two experiments. The bar indicates 50 μm.

mucosal inductive tissues for initiation of IgA responses. Furthermore, LTβR- and TNFR55-mediated signal play a critical role in the control of LI-ILF development.

Chronological analysis of escalated LI-ILF formation in double Ig-treated mice

Because we found large numbers of ILF in the large intestine of 6-wk-old progeny following the gestational blockage of LTβR- and TNFR55-mediated signals, we further examined the effect of double Ig treatment on kinetics of their development. For this purpose, we compared ILF number in the small and large intestine of progeny of mice treated with control Ig or double Ig at age of 3, 10, and 24 wk. When the control Ig-treated mice were examined, the numbers of ILF in both small and large intestine were gradually increased (Fig. 4). Interestingly, maximum numbers of LI-ILF were already reached as early as 3 wk old among progeny of mice treated with double Ig (Fig. 4). The total numbers of ILF were then maintained up to 24 wk old. In contrast, ILF in the small intestine of progeny treated with control Ig or double Ig fusion protein gradually developed and reached the maximum numbers at the age of 24 wk old. It was also noted that total numbers of small intestinal ILF were higher in mice treated with the double Ig in utero when compared with the control Ig-treated mice. The fact that the accelerated ILF formation in the large intestine by gestational blockage of LTβR and TNFR55 signals occurred very early after birth implied that their formation and development might not be influenced by exogenous environmental factors, such as gut microflora.