Campylobacter and Toll-like receptors

Implications for vaccine development

Marcel de Zoete

Printed by Atalanta Drukwerkbemiddeling, Houten
ISBN 978-90-393-5353-0

Campylobacter and Toll-like receptors

Implications for vaccine development

Campylobacter en Toll-like receptoren

Implicaties voor vaccin ontwikkeling

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 26 mei 2010 des middags te 2.30 uur

door

Marcel Robert de Zoete

geboren op 3 november 1979 te Leiden

Promotoren: Prof. dr. J.P.M. van Putten
Prof. dr. J.A. Wagenaar

The research described in this thesis was financially supported by the Dutch Ministry of Agriculture, Nature and Food Quality

Printing of this thesis was financially supported by:

CamVac (Danish Research Council)
BioTRADING Benelux BV
Oxoid BV
Merial BV
AUV Dierenartsencoöperatie
Infection & Immunity Center Utrecht

Aan mijn ouders

Table of contents

1

General introduction

Marcel R. de Zoete

Parts of this chapter were published in:

Vaccine 25:5548-57. 2007
Immunology Letters 128:8-11. 2010

1. Introduction

The Gram-negative bacterium *Campylobacter* is responsible for an estimated 400 million human cases of enterocolitis each year, making it the leading cause of bacterial food-borne illness and a major causative agent of traveller's disease (2, 31, 33). In a limited number of cases the enteric manifestations are followed by sequelae such as reactive arthritis and the life-threatening neuropathy Guillain-Barré Syndrome (GBS) (2, 37, 46). Estimated incidences of human campylobacteriosis in industrialized countries vary from 21.9/100,000 (US) to 396/100,000 (New Zealand). In developing countries, approximately 40-60% of young children is estimated to become infected every year and high numbers of asymptomatic carriage are reported (7, 21, 103). Altogether, *Campylobacter* species affect the health of millions of people worldwide with an estimated annual economical burden of up to 8 billion dollars in the US alone (18).

At present, 17 *Campylobacter* species have been identified that can be separated into more than 60 Penner serotypes (heat-stable antigens) and more than 100 Lior serotypes (heat-labile antigens). Two thermophilic species, *C. jejuni* ssp. *jejuni* and *C. coli* (further referred to as *C. jejuni* and *C. coli*, or together as *Campylobacter*) are responsible for the vast majority of human campylobacteriosis (~90% and ~10%, respectively). Although *C. jejuni* and *C. coli* are frequently isolated from the digestive tract of a wide variety of warm-blooded animals, (broiler) chickens are considered a major source of human infection (35, 67): as much as 70% of raw poultry meat products sold in the US in 1999/2000 was found to be contaminated with *Campylobacter* (129).

2. *Campylobacter* in humans

Campylobacter infection in humans is initiated by ingesting as little as 500 bacteria that, aided by their corkscrew-shape and high motility, move easily through the intestinal tract and colonize preferably the distal ileum and colon (12, 56, 111, 125). Here, the bacterium resides specifically in the mucosal layer, disrupts the epithelial barrier, and initiates an inflammatory response. This may give rise to clinical symptoms that range from mild watery to voluminous and bloody diarrhea accompanied by headache, abdominal pain, fever, malaise and occasional vomiting (17). The molecular events that lead to acute intestinal inflammation largely remain to be defined. In recent years, a series of putative virulence determinants (Table I) that influence bacterial adhesion and/or invasion

of isolated eukaryotic cells have been identified (48, 111, 112). Furthermore, the presence of toxins in certain strains of *Campylobacter* has been suggested to enhance the inflammation (126). The contribution of each of these factors to the development of enterocolitis however, remains to be established.

Table I – Putative virulence factors of Campylobacter spp.

Virulence factor	Function	Reference
CapA	Host-cell binding	(5)
CadF	Fibronectin-binding, Rac1 and Cdc43 activation	(60, 62)
JlpA	Host-cell binding, NF-κB and MAPK activation	(49)
Peb1	Host-cell binding	(92)
FlaC	Host-cell binding, affects invasion	(112)
CiaB	Affects invasion (some strains)	(61)
FspA2	Induces apoptosis	(94)
Cytolethal distending toxin	Induces G2/M cell cycle arrest, NF-κB and IL-8 activation, and apoptosis	(30, 41, 42)
N-glycosylation	Host-cell binding, affects invasion	(51)
Flagellar motility	Host-cell binding, affects invasion	(79, 125)
Chemotaxis	Affects invasion	(121)
Capsule	Affects invasion	(6)

Campylobacter infections are generally self-limiting; diarrhea usually lasts three to five days, while other symptoms gradually resolve the following week (17). From the second week after infection, *Campylobacter*-specific antibodies against several antigens including flagellin (the major subunit of the bacterial motility apparatus, see Fig. 1), major outer membrane protein (MOMP), and lipopolysaccharides (LPS), can be detected in the serum and mucosal secretions (14, 76, 82). Several lines of evidence suggest that the generation of anti-*Campylobacter* antibodies during infection is beneficial for clearance of the bacterium, and that the presence of specific antibodies may provide (partial) protection against clinical disease upon re-infection. For instance, in developing countries, where people are frequently challenged with *Campylobacter* and are often colonized without clinical symptoms, the occurrence of asymptomatic carriage strongly correlates

with increased antibody titers (13). Furthermore, in human volunteers, a re-challenge with the homologous *Campylobacter* strain results in a less severe clinical outcome (12). Finally, breast milk with maternal IgA directed against several *Campylobacter* surface antigens protects young children against *Campylobacter*-induced diarrhea (97, 101, 118).

3. *Campylobacter* in chickens

Like humans, chickens are easily colonized with *Campylobacter*, although considerable variation between bacterial strains and the specific breed and age of the broiler has been reported (16, 99, 113, 114). Fecal shedding results in a rapid spread throughout the entire flock. Depending on the geographical region and season, the percentage of *Campylobacter*-positive flocks in Europe reaches up to 90% (84). The two dominant *Campylobacter* species that colonize chickens are also most prevalent in humans, although the proportion of *C. jejuni* and *C. coli* is different (~65% and ~35% in broilers, respectively) (Jacobs-Reitsma, W.F., personal communication). Thus far, molecular typing has not revealed intrinsic differences between chicken and human isolates. However, these studies are complex as most *Campylobacter* strains do not have a stable genotype due to high frequency DNA exchange between strains and frequent simultaneous colonization of chickens (but not humans) with multiple strains (25, 26).

In chickens *Campylobacter* does not elicit the potent inflammatory response that can be observed in humans but rather seems to behave as a commensal bacterium. The molecular basis for the apparent different lifestyle of *Campylobacter* in the chicken and human host remains to be defined. The chicken gut may contain up to 10^9 *Campylobacter* per gram of feces (11, 23) without apparent signs of pathology. They are found in the intestinal lumen and the mucus, and penetrate deep into the intestinal crypts in close proximity to the epithelium, seemingly without cellular adherence or invasion. In experimental infections, the rapid peak in intestinal bacterial numbers is often followed by a slow decrease from week 4 up to the point of slaughtering at 6 weeks of age, with some birds able to completely clear the bacteria from the cecum (1). Some studies report the presence of bacteria in the spleen, liver and blood in young chicks, suggesting that, shortly after hatching, *Campylobacter* may gain access to the deeper tissues (59, 104). Whether this is caused by insufficient maturation of the mucosal tissue, the absence of endogenous bacterial flora, and/or immune factors, is unknown.

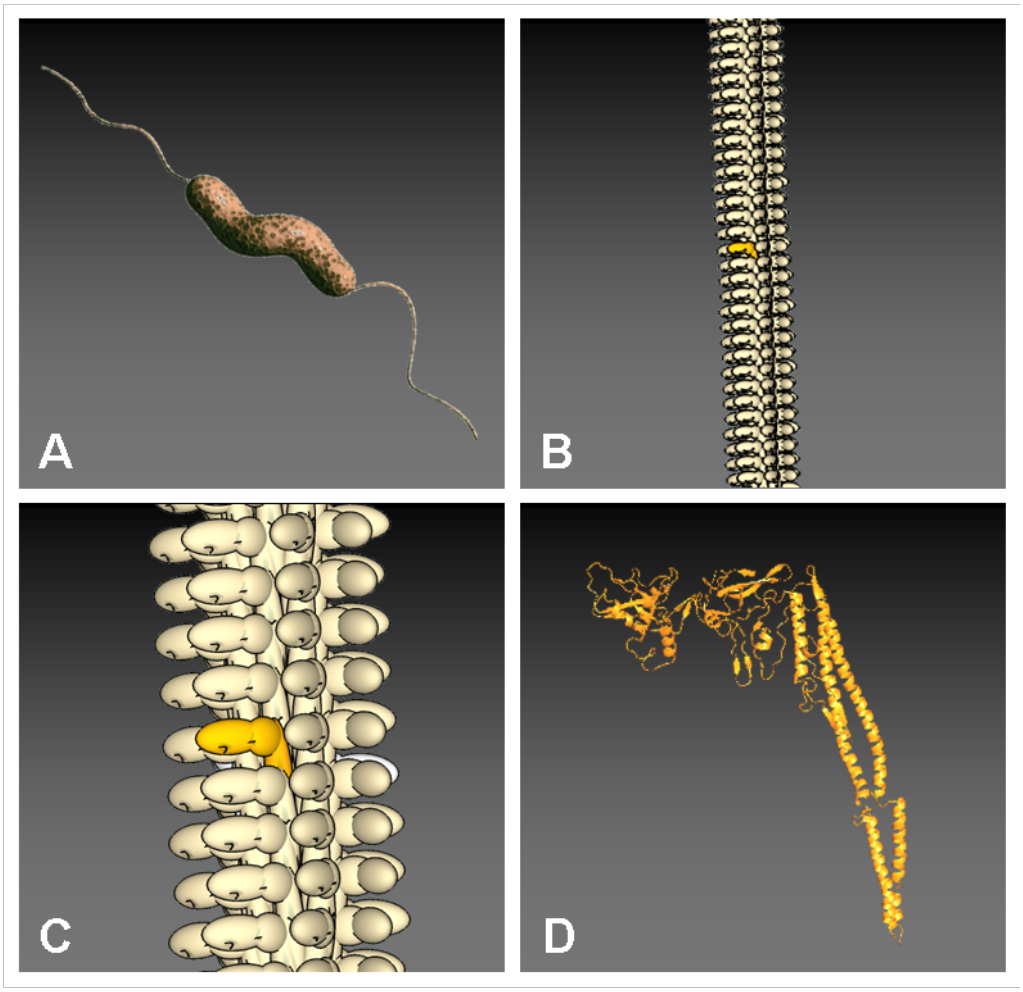

Figure 1. Schematic representation of Campylobacter flagellin. *(A) C. jejuni contains two polar flagella. (B, C) Model of the flagellum of C. jejuni, consisting of up to 10,000 copies of the polymerized flagellin protein (indicated in yellow). (D) Structural model of flagellin A from C. jejuni strain 81116.*

Several studies have shown a strong correlation between increasing levels of *C. jejuni* specific antibodies in chicken and the reduction of bacterial shedding observed with duration of the colonization, suggesting the development of an (partially) effective immune response (20, 81, 98). In general, *Campylobacter*-specific antibody levels rise gradually from week 2-3 after experimental inoculation. Flagellin is generally the first

antigen to be recognized by all antibody isotypes. During the following 8 weeks, antibodies directed against a number of other bacterial antigens, including the major outer membrane protein, are induced (20, 98). Overall, the main difference in host immune response towards *Campylobacter* between humans and chickens seems to be within the first week of infection, when the intestinal tracts of both species are heavily colonized. In humans but not in chicken, this is accompanied with an inflammatory response and tissue damage. The absence of a strong activation of the innate immune defence in the chicken may be an important reason for the relatively slow and weak antibody response against *Campylobacter* in this species.

4. Towards a *Campylobacter* vaccine for chickens

Several strategies have been applied to reduce *Campylobacter* counts on chicken meat, including attempts to eliminate *Campylobacter* from the farms by increasing biosecurity and the separation of contaminated flocks, and by improving hygiene during the process of slaughtering. In addition, several experimental approaches like the reduction of colonization by competitive exclusion, antibacterial agents, or phage therapy are being investigated for their efficacy (71, 123, 124). Although these measures undoubtedly may help to control shedding of *Campylobacter* by the animals and may reduce the number of positive flocks, vaccination of poultry against *Campylobacter* will probably be most effective and remains a major goal.

Two major problems hamper the development of a *Campylobacter* vaccine. First, conserved protection-inducing antigens have not yet been identified. Vaccination with killed whole bacteria (in several forms, with or without a specific mammalian mucosal adjuvants) does not result in the production of sufficient protective antibodies to prevent re-infection (34, 86, 98). While several highly immunogenic antigens have been described, only flagellin subunit vaccines have been tested several times in chicken vaccination trials. Despite the high immunogenicity of *Campylobacter* flagellin and its crucial role in efficient colonization (20, 40), results showed variable success (57, 83, 127, 128). Overall, flagellin-based vaccines seem to somewhat reduce *Campylobacter* colonization levels in the gut, but in order to be used in an effective vaccine, much higher levels of protection are desired.

The second major obstacle is the still rudimentary knowledge on the chicken immune system, in particular in the field of the innate immune system and the functionality

of adjuvants. In mammals, major breakthroughs in understanding of the cross-talk between the innate and adaptive immune system and the function of adjuvants have been achieved in the last decade (88). Clearly this knowledge cannot be readily extrapolated to the chicken; several adjuvants that are successfully utilized in mammals do not seem to effectively boost a strong intestinal antibody response in chickens (98, 127). As the induction of powerful immune responses seem to be indispensable for the development of an adequate immunity against *Campylobacter* antigens, thorough identification and evaluation of innate immunity, adjuvant activity, and early immune responses in chickens may greatly aid vaccine development.

5. Toll-like receptors

Effective vaccination requires targeted manipulation of both the innate and the adaptive immune system. In mammals, the significance of the innate immune response as a key regulatory element of the adaptive immune systems has become particularly evident with the discovery of the family of Toll-like receptors (TLRs) (88). TLRs, named after the *Drosophila melanogaster* Toll protein involved in embryogenesis and immune defense against invading fungi, presented the long-awaited missing link between pathogens and the adaptive immune system (73). Since the identification of TLR4 (initially named hTOLL) in 1997 (74), a total of 10 different TLR have been discovered in humans, and many more in other species.

Receptor architecture

TLRs are type I membrane receptors with a highly conserved architecture (Fig. 2) (19). The N-terminus consists of an extracellular ligand-binding domain, which is composed of 19-27 leucine-rich repeats (LRRs) that form a horse-shoe shape. Each TLR binds a specific (set of) molecule(s), resulting from variations in the LRR framework that create binding pockets or regions for their specific ligands. The cytoplasmic tail of the TLRs contains a highly conserved globular signaling region, which, because of its high homology with the Interleukin-1 receptor signaling domain, is termed the Toll/Inter-leukin Receptor (TIR) domain. The ligand-binding and TIR domains are spatially separated by a transmembrane helix that holds the protein within the membrane.

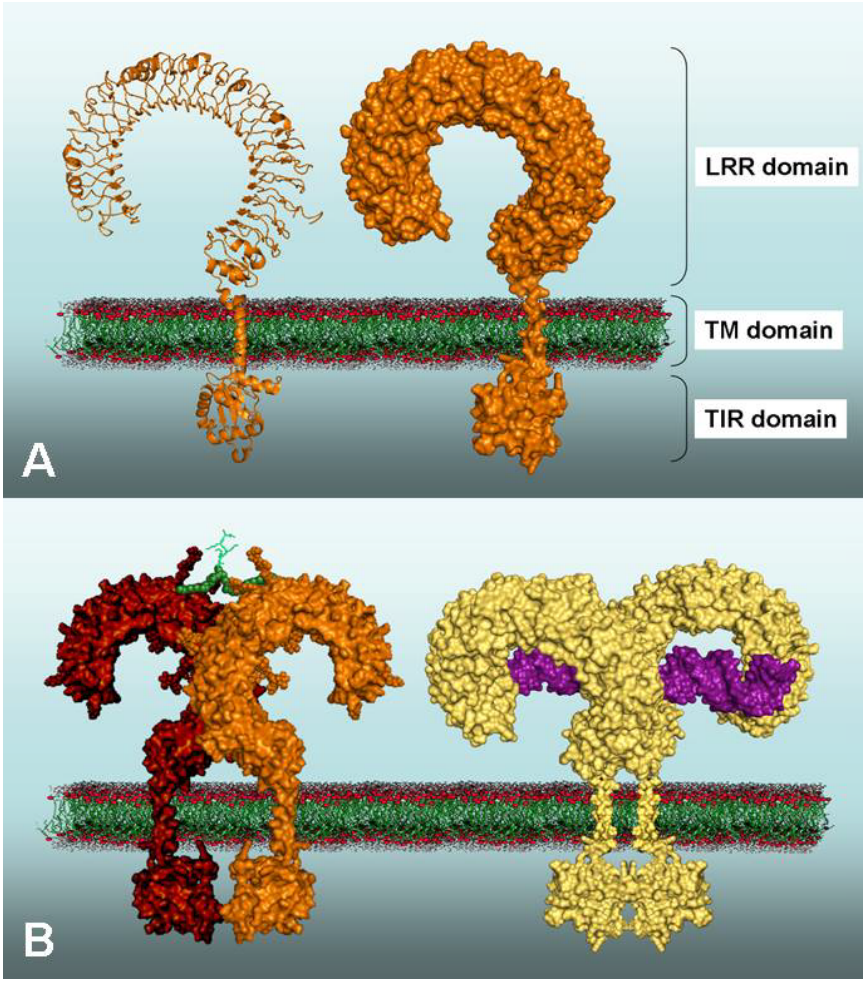

Figure 2. Schematic representation of Toll-like receptors. *(A) Toll-like receptors consist of a horse-shoe-shaped leucine-rich repeat (LRR) domain, a transmembrane (TM) domain, and a signaling TIR domain. (B) Model of TLR dimerization: (left) the TLR2 (orange) / TLR1 (red) heterodimer bound to the lipopeptide Pam_3CSK_4 (green), and (right) the TLR3 (yellow) homodimer bound to dsRNA (purple).*

TLR ligands

Classical TLR ligands are structurally highly conserved, not present in the host, and generally crucial for survival of the microbe. These features enable the host to safely detect a wide range of bacteria, viruses or fungi with only a handful of TLRs. TLR ligands can be

grossly divided into 3 groups: 1) glycolipids (LPS) and lipopeptides (di- and triacylated lipopeptides), which are recognized by TLR4/MD-2 (LPS), and the combination of either TLR2-TLR1 or TLR2-TLR6 (di- and triacylated lipopeptides, respectively); 2) conserved proteins, which are recognized by TLR5 (flagellin) and mouse TLR11 (profillin-like protein); and 3) DNA or RNA, recognized by TLR3 (dsRNA), TLR7 and TLR8 (ssRNA) and TLR9 (DNA) (63). The ligand for human TLR10 remains to be defined, as are the ligands for most of the TLRs absent in humans, including mouse TLR12 and TLR13, fish TLR23, and chicken TLR15 and TLR21.

In depth: TLR5 and flagellin

Bacterial flagellin is currently the only human TLR ligand of which the mechanistics of receptor binding are not elucidated or safely predicted by comparative modeling. Several amino acids in flagellin have been identified that are crucial for TLR5 activation, clustering mainly in two separate regions in the conserved N-terminal and C-terminal domains (80, 110). Interestingly, the vast majority of these residues are indispensable for flagella assembly and bacterial motility, providing a strong selective pressure against mutating the TLR5 binding sites. Still, several species of bacteria, including *Campylobacter*, have evolved a flagellin that evades TLR5 activation (4). Although the precise cause of this trait is not yet fully clear, alterations within the proposed TLR5 binding sites have been implicated to contribute to TLR5 evasion. The involvement of the negatively charged glycans on the flagellin surface and an alternative flagella assembly strategy in evasion remains to be studied. The role of TLR5 in guarding the body for infection has been demonstrated by studies in knockout mice, which show spontaneous colitis and increased susceptibility for urinary tract infection (3, 122), and in humans, where TLR5 has been shown to provide protection against *Legionella pneumophila* lung infections (38). Seemingly contradictory, TLR5-/- mice are less susceptible for systemic *Salmonella* infection, probably because the pathogen exploits TLR5-induced activation and migration of dendritic cells for dissemination through the host (120). The function of TLR5 evasion during infection or colonization by *Campylobacter* remains unclear, although, in contrast to *Salmonella*, TLR5-induced inflammatory responses in the intestine are likely detrimental for the bacterium.

From a microbiology point of view, the classification of the distinct types of microbial molecules as TLR ligands is a gross simplification and a denial of the tremendous variation in each of these types of molecules between bacterial species or even single

bacterial strains. The different microbial structures are not as conserved as often advertised. To better understand the role of TLR recognition in individual bacterial infections, the interaction between the receptors and the various putative TLR ligands of that specific microbe has to be investigated. The importance of this need is demonstrated by the finding that bacterial flagellins from *C. jejuni* and the gastric pathogen *Helicobacter pylori*, as well as of a whole category of commensal intestinal bacterial flora, fail to activate TLR5 (4). Similarly, variations in LPS structure can have drastic impact. For instance, *Yersina pestis*, the cause of the plague, activates TLR4 when grown at 25°C. However, at 37°C the bacterium changes its hexa-acylated LPS into a tetra-acylated form which acts as a TLR4 antagonist and prevents the development of immunity (77). Thus natural variation in TLR ligands between bacterial species are highly relevant and need to be considered to explain the immune response to diverse infectious agents.

In depth: TLR9 and DNA

Originally, TLR9 was reported to specifically recognize bacterial DNA containing non-methylated CpG motifs. These motifs were uncommon in mammalian DNA, and when present, often methylated (39). Later, the necessity for non-methylated CpG motifs was shown to be true only for synthetic DNA, which was designed with the more stable but non-natural phosphorothioate linking the deoxyribose sugars in DNA. In natural DNA, it is mainly the sugar backbone of DNA and not specific nucleotide motifs that activate TLR9 (36). TLR9 resides mainly in the ER, but (after DNA stimulation) is transported by the shuttle protein UNC93B to early endosomes and lysosomes, where it encounters and binds DNA (58, 66). The low pH of the lysosomes is believed to strongly increase the binding affinity for DNA. In lysosomes, TLR9 has to undergo a series of proteolytic cleavage steps in order to signal after DNA binding (27, 89, 106). Cleavage was shown to be dependent on the presence of lysosomal cysteine proteases, and may serve as an additional safety mechanisms to prevent recognition of self-DNA. The tight regulation, complex functionality, and the requirement of several helper proteins might reflect the danger of recognizing host nucleic acids. Indeed, several autoimmune disorders, like systemic lupus erythematosus and psoriasis are believed to result from aberrant TLR9 stimulation by self-DNA (32, 68).

TLR localization

Depending on the type of ligand, TLRs are functional either on the cytoplasmic membrane (TLR2/1/6, TLR4 and TLR5) or in endolysosomes (TLR3, TLR7, TLR8, TLR9). The

exact biological rationale for sensing DNA and RNA in the lysosomal compartment is currently unknown, although it can be imagined that microbial DNA and RNA is only efficiently released during lysosomal degradation. In addition, sensing of DNA/RNA in lysosomes may enable the host with a mechanism to distinguish between self and foreign DNA/RNA (8, 9). Transport of (the appropriate amounts of) TLRs to the correct location in the cell is a tightly regulated process. The determination of a TLR's final destination has been shown to be dependent on the presence of specific amino acid motifs in the transmembrane domain and/or region between the transmembrane domain and the TIR domain (50, 69, 85), but is highly influenced by ligand stimulation, receptor glycosylation, and a growing number of "helper" or "shuttle" proteins, like UNC93B (TLR9), MD2 and PRAT4A (TLR4), or the chaperone GP96 (TLR2/4/5/7/9) (28, 102).

TLR signaling

Signaling through TLRs is initiated by receptor dimerization that follows binding of the TLR ligand to its receptor. Although the exact mechanism is not yet fully clear, it is apparent that the ligand serves as a bridging molecule between the two TLR monomers. For instance, one molecule of dsRNA binds to the extracellular domains of two TLR3 molecules, one molecule of the synthetic lipopeptide Pam_3CSK_4 binds with two acyl chains to the ligand-binding domain of TLR2 and with one to that of TLR6, and one LPS molecule can crosslink two TLR4/MD-2 complexes (47). Although definitive evidence is currently lacking, the involvement of additional conformational changes following ligand binding are likely to strengthen the protein complex and/or initiate signal transduction.

The TLR dimerization and associated conformational changes are believed to bring the intracellular TIR domains in close proximity to form a scaffold that enables the recruitment of signaling adapter proteins (52). Not all TLRs make use of the same signaling pathway; the majority of TLRs recruit the adapter protein MyD88 to the TIR domains. For TLR2/1/6 and TLR4, this process requires an additional adapter protein named Mal. Through phosphorylation, (poly)ubiquitinylation, and binding of several signaling proteins like IRAK1, IRAK2, IRAK4, TRAF6 and NEMO (75), the transcription factor NF-κB is activated and translocated into the nucleus where it promotes the transcription of inflammatory cytokines. The second major TLR signaling route uses the adapter protein TRIF. This protein is recruited to the TIR domain of TLR3 and, via the adapter protein TRAM, to the TIR domain of TLR4. Recruitment of TRIF and TRAM results in the activation and translocation of transcription factor IRF3 to the nucleus to induce expression of IFN-β and IFN-inducible genes.

Role of TLRs during infection and vaccination

TLRs impact microbial infection on two levels. Firstly, TLR activation initiates rapid inflammatory responses that lead to the influx of phagocytes like macrophages, dendritic cells, and neutrophils. Through engulfment, production of reactive oxygen species, and secretion of proteases and antimicrobial peptides, these cells limit microbial spread and aid the clearance of infection (29). Secondly, activation of TLRs provides important signals to steer adaptive immunity, resulting in a streamlined production of neutralizing antibodies (91). Without TLRs, these processes are compromised. This is best demonstrated by specific knockout mice and natural polymorphisms in human populations. For instance, mice with a non-functional TLR2 are more susceptible for infection and subsequent septicemia or meningitis caused by several Gram-positive bacterial pathogens (24, 95, 116), TLR3 and TLR9-knockouts are highly susceptible to cytomegalovirus infection (115), humans with a defective TLR5 are more susceptible to Legionnaires' disease (38), and IRAK4-deficient humans, who cannot signal through the majority of TLRs, suffer from severe pyogenic infections during childhood (93). Although TLR stimulation unequivocally induces maturation of dendritic cells, the necessity for TLR signaling for humoral responses is still under debate. It is clear now that particularly IgG1 and IgG2c responses are TLR dependent (87). Interestingly, it was shown that B-cells themselves require stimulation through TLRs for the optimal generation of T-dependent antigen-specific antibody responses (90). Because of their immune stimulatory activity, all known TLR ligands can function as adjuvants and have been successfully used during experimental vaccination (64). In humans, currently only monophosphoryl lipid A (MPL), which activates TLR4, is used as an adjuvant in a cervical cancer vaccine, while the usage of CpG oligonucleotides is still in an experimental phase.

Toll-like receptors in chicken

TLRs are found throughout the entire eukaryotic kingdom, including plants, insects, fish birds and mammals (100). On the basis of their DNA sequence, the TLRs present in humans can be divided into 5 groups: TLR2/1/6/10, TLR3, TLR4, TLR5, and TLR7/8/9. Humans do not have a representative of an additional other class found in nature, comprising TLR11/12/13 and TLR21/22/23. Genome scanning revealed that chickens have TLRs from all groups (Table II) (117). Chicken TLR3, TLR4 and TLR5 are functional homologues of their human counterparts (54, 55, 105). This may also hold for the chicken members of the TLR2 group, although chicken possess two versions of TLR2, one TLR that comprises the function of both human TLR1 and TLR6 (TLR16), and an

additional truncated version of TLR16 (44, 53). In the DNA-sensing group of TLR7/8/9, chicken have only TLR7; TLR8 is disrupted by the insertion of a retroviral-like insertion elements, and TLR9 is absent entirely in chickens. In contrast to humans, chickens have TLR21 and TLR15. The latter TLR does not fit within any of the known TLR groups on the basis of its sequence. Both receptors are strongly upregulated at the mRNA level in the intestinal tissue following *Salmonella* infection (43, 107).

In depth: identification of TLR ligands and contamination
The identification of a biologically active TLR ligand of a bacterium is not an easy task. Apart from structural variation among bacterial populations, purification of the molecules free of contaminants is a major, often underestimated, challenge. This has led to misinterpretation of results. A classical example is the identification of TLR2 as LPS receptor. This erroneous conclusion was shown later to be caused by contamination of the 'pure' LPS with traces of (biologically active) lipopeptides (45). Other examples where contamination is suspected to, at least partially, contribute to TLR responses are the binding of peptidoglycan (with associated 'contaminating' lipopeptides) to TLR2 (119), and the recognition of heat-shock proteins (with tightly associated LPS) by TLR4 (10). TLRs also bind a rapidly growing repertoire of endogenous TLR ligands. Some of these molecules can also bind microbial products (bacterial DNA and LPS are found in serum), but whether this association contributes to TLR recognition, awaits co-crystallization of the various ligand-TLR complexes.

6. TLR recognition of *Campylobacter* in humans and chickens

At present, the knowledge of the interaction of *Campylobacter* with TLRs is still very limited. It has been demonstrated that *C. jejuni* DNA is able to stimulate human TLR9, but it appears less potent than DNA from other bacterial species such as *Salmonella* and *E. coli* (22). Studies with mouse macrophages have shown to potential of *Campylobacter* LPS to stimulate TNF-α in mouse macrophages, presumably through TLR4, although the LPS appears less biologically active than *Salmonella* LPS (78). In human dendritic cells, *Campylobacter* induced both MyD88- and TRIF-dependent cytokine responses through TLR4 (96). *Campylobacter*, like *Helicobacter pylori* and *Wolinella succinogenes*, belongs to the group of mucus-based ε-proteobacteria, that have evolved a flagellin that does not activate human TLR5 (4) (See *In depth: TLR5 and flagellin*).

Knowledge on TLR stimulation in chickens by *Campylobacter* is even more rudimentary. There are, however, several studies reporting the activation of cytokines after experimental *Campylobacter* infection of chickens or cultured chicken cells (70, 72, 108, 109). Overall, *Campylobacter* can induce early inflammatory responses in the chicken intestine and cultured cells. Some reports claim to observe less cytokine production in chicken cells than in human cells (15, 65). Indeed, species-specific differences in TLR-mediated innate responses may contribute to the differences in pathology between mammals and chicken. To make these claims, however, a more thorough and direct investigation is required.

Table II – Toll-like receptors and ligands in humans and chickens.

Ligand	TLRs in humans	TLRs in chickens
triacylated lipopetides	TLR2 + TLR1	TLR2t2 + TLR16, TLR2t1 + TLR1LB, TLR2t1 + TLR16
diacylated lipopetides	TLR2 + TLR6	TLR2t2 + TLR16, TLR2t1 + TLR1LB
dsRNA	TLR3	TLR3
LPS	TLR4	TLR4
Flagellin	TLR5	TLR5
ssRNA	TLR7	Possibly TLR7
ssRNA	TLR8	Not functional
DNA	TLR9	Not present
Unknown	TLR10	Not present
Unknown (Chapter 2)	Not present	TLR21
Unknown (Chapter 3)	Not present	TLR15

7. Outline of the thesis

With close to half a billion cases each year, *Campylobacter* is the most common causative agent for bacterial foodborne infections in humans. Decreasing the number of *Campylobacter* in the intestinal tract of chickens, a major source for human infections, provides an opportunity to effectively reduce the infections in humans. However, the development of an anti-*Campylobacter* vaccine for chickens seems to be hampered at least in part by the inability to induce strong immune responses against *Campylobacter* antigens. Toll-like receptors are specialized in boosting the immune responses during infection, and TLR-ligands are successfully used as adjuvants during vaccination. In chickens, the knowledge on TLRs, TLR-ligands, and TLR-stimulation by *Campylobacter* remains limited. Therefore, the aims of this PhD dissertation were (i) to elucidate the function of the chicken TLR with unknown function, (ii) to determine TLR interactions with *Campylobacter*, and (iii) to exploit gained knowledge for the construction of a candidate vaccine.

To achieve these objectives, we first investigated the function of the TLR21 and elucidated its role as an innate immune sensor **(Chapter 2)**. Using recombinational cloning, we expressed TLR21 in an *in vitro* cell system, examined in detail its cellular localization, and determined the TLR21 ligand to be (bacterial) DNA. Subsequently, in **Chapter 3**, we studied the function of TLR15, the last remaining TLR with unknown ligand in the chicken TLR repertoire. Using similar techniques, we discovered the microbial ligand for TLR15 and identified a mechanism of TLR activation previously undescribed for TLRs.

As *Campylobacter* induces strong innate and adaptive immune responses in humans but not in chickens, we analysed *Campylobacter*-induced TLR activation for the two species in **Chapter 4**. Through cloning and *in vitro* expression of hTLR2/1/6, hTLR4, hTLR5 and hTLR9, and chTLR2/16, chTLR4, chTLR5 and chTLR21, we revealed several basic and previously unknown differences in innate immune recognition between the two species.

In an attempt to restore the intrinsic adjuvant activity of *Campylobacter* flagellin, we examined the mechanism of TLR5-evasion in **Chapter 5**. By creating a series of recombinant chimeric proteins using *Salmonella* flagellin, we for the first time identified a defined region in flagellin required to activate human TLR5. This knowledge enabled the construction of *C. jejuni* flagellin with the ability to potently activate TLR5, and serves as a first generation tailormade candidate vaccine.

In **Chapter 6**, the major findings and conclusions of this thesis are summarized and discussed.

References

1. **Achen, M., T. Y. Morishita, and E. C. Ley.** 1998. Shedding and colonization of *Campylobacter jejuni* in broilers from day-of-hatch to slaughter age. Avian Dis **42:**732-737.
2. **Allos, B. M.** 2001. *Campylobacter jejuni* Infections: update on emerging issues and trends. Clin Infect Dis **32:**1201-1206.
3. **Andersen-Nissen, E., T. R. Hawn, K. D. Smith, A. Nachman, A. E. Lampano, S. Uematsu, S. Akira, and A. Aderem.** 2007. Cutting edge: Tlr5-/- mice are more susceptible to *Escherichia coli* urinary tract infection. J Immunol **178:**4717-4720.
4. **Andersen-Nissen, E., K. D. Smith, K. L. Strobe, S. L. Barrett, B. T. Cookson, S. M. Logan, and A. Aderem.** 2005. Evasion of Toll-like receptor 5 by flagellated bacteria. Proc Natl Acad Sci U S A **102:**9247-9252.
5. **Ashgar, S. S., N. J. Oldfield, K. G. Wooldridge, M. A. Jones, G. J. Irving, D. P. Turner, and D. A. Ala'Aldeen.** 2007. CapA, an autotransporter protein of *Campylobacter jejuni*, mediates association with human epithelial cells and colonization of the chicken gut. J Bacteriol **189:**1856-1865.
6. **Bacon, D. J., C. M. Szymanski, D. H. Burr, R. P. Silver, R. A. Alm, and P. Guerry.** 2001. A phase-variable capsule is involved in virulence of *Campylobacter jejuni* 81-176. Mol Microbiol **40:**769-777.
7. **Baker, M. G., E. Sneyd, and N. A. Wilson.** 2006. Is the major increase in notified campylobacteriosis in New Zealand real? Epidemiol Infect**:**1-8.
8. **Barton, G. M., and J. C. Kagan.** 2009. A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nature reviews **9:**535-542.
9. **Barton, G. M., J. C. Kagan, and R. Medzhitov.** 2006. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nat Immunol **7:**49-56.
10. **Bausinger, H., D. Lipsker, U. Ziylan, S. Manie, J. P. Briand, J. P. Cazenave, S. Muller, J. F. Haeuw, C. Ravanat, H. de la Salle, and D. Hanau.** 2002. Endotoxin-free heat-shock protein 70 fails to induce APC activation. European journal of immunology **32:**3708-3713.
11. **Beery, J. T., M. B. Hugdahl, and M. P. Doyle.** 1988. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. Appl Environ Microbiol **54:**2365-2370.

12. **Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser.** 1988. Experimental *Campylobacter jejuni* infection in humans. J Infect Dis **157:**472-479.
13. **Blaser, M. J., R. E. Black, D. J. Duncan, and J. Amer.** 1985. *Campylobacter jejuni*-specific serum antibodies are elevated in healthy Bangladeshi children. J Clin Microbiol **21:**164-167.
14. **Blaser, M. J., J. A. Hopkins, and M. L. Vasil.** 1984. *Campylobacter jejuni* outer membrane proteins are antigenic for humans. Infect Immun **43:**986-993.
15. **Borrmann, E., A. Berndt, I. Hanel, and H. Kohler.** 2007. *Campylobacter*-induced interleukin-8 responses in human intestinal epithelial cells and primary intestinal chick cells. Vet Microbiol **124:**115-124.
16. **Boyd, Y., E. G. Herbert, K. L. Marston, M. A. Jones, and P. A. Barrow.** 2005. Host genes affect intestinal colonisation of newly hatched chickens by *Campylobacter jejuni*. Immunogenetics **57:**248-253.
17. **Butzler, J. P.** 2004. *Campylobacter*, from obscurity to celebrity. Clin Microbiol Infect **10:**868-876.
18. **Buzby, J. C., B. M. Allos, and T. Roberts.** 1997. The economic burden of *Campylobacter*-associated Guillain-Barre syndrome. J Infect Dis **176 Suppl 2:**S192-197.
19. **Carpenter, S., and L. A. O'Neill.** 2009. Recent insights into the structure of Toll-like receptors and post-translational modifications of their associated signalling proteins. The Biochemical journal **422:**1-10.
20. **Cawthraw, S., R. Ayling, P. Nuijten, T. Wassenaar, and D. G. Newell.** 1994. Isotype, specificity, and kinetics of systemic and mucosal antibodies to *Campylobacter jejuni* antigens, including flagellin, during experimental oral infections of chickens. Avian Dis **38:**341-349.
21. **Coker, A. O., R. D. Isokpehi, B. N. Thomas, K. O. Amisu, and C. L. Obi.** 2002. Human campylobacteriosis in developing countries. Emerg Infect Dis **8:**237-244.
22. **Dalpke, A., J. Frank, M. Peter, and K. Heeg.** 2006. Activation of toll-like receptor 9 by DNA from different bacterial species. Infect Immun **74:**940-946.
23. **Dhillon, A. S., H. L. Shivaprasad, D. Schaberg, F. Wier, S. Weber, and D. Bandli.** 2006. *Campylobacter jejuni* infection in broiler chickens. Avian Dis **50:**55-58.
24. **Drennan, M. B., D. Nicolle, V. J. Quesniaux, M. Jacobs, N. Allie, J. Mpagi, C. Fremond, H. Wagner, C. Kirschning, and B. Ryffel.** 2004. Toll-like receptor 2-

deficient mice succumb to *Mycobacterium tuberculosis* infection. The American journal of pathology **164:**49-57.

25. **Duim, B., C. W. Ang, A. van Belkum, A. Rigter, N. W. van Leeuwen, H. P. Endtz, and J. A. Wagenaar.** 2000. Amplified fragment length polymorphism analysis of *Campylobacter jejuni* strains isolated from chickens and from patients with gastroenteritis or Guillain-Barre or Miller Fisher syndrome. Appl Environ Microbiol **66:**3917-3923.
26. **Duim, B., T. M. Wassenaar, A. Rigter, and J. Wagenaar.** 1999. High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with amplified fragment length polymorphism fingerprinting. Appl Environ Microbiol **65:**2369-2375.
27. **Ewald, S. E., B. L. Lee, L. Lau, K. E. Wickliffe, G. P. Shi, H. A. Chapman, and G. M. Barton.** 2008. The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor. Nature **456:**658-662.
28. **Ewaschuk, J. B., J. L. Backer, T. A. Churchill, F. Obermeier, D. O. Krause, and K. L. Madsen.** 2007. Surface expression of Toll-like receptor 9 is upregulated on intestinal epithelial cells in response to pathogenic bacterial DNA. Infect Immun **75:**2572-2579.
29. **Fang, F. C.** 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat Rev Microbiol **2:**820-832.
30. **Fox, J. G., A. B. Rogers, M. T. Whary, Z. Ge, N. S. Taylor, S. Xu, B. H. Horwitz, and S. E. Erdman.** 2004. Gastroenteritis in NF-kappaB-deficient mice is produced with wild-type *Campylobacter jejuni* but not with *C. jejuni* lacking cytolethal distending toxin despite persistent colonization with both strains. Infect Immun **72:**1116-1125.
31. **Gascon, J.** 2006. Epidemiology, etiology and pathophysiology of traveler's diarrhea. Digestion **73 Suppl 1:**102-108.
32. **Gilliet, M., W. Cao, and Y. J. Liu.** 2008. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nature reviews **8:**594-606.
33. **Girard, M. P., D. Steele, C. L. Chaignat, and M. P. Kieny.** 2006. A review of vaccine research and development: human enteric infections. Vaccine **24:**2732-2750.
34. **Glünder, G., N. Spiering, and K. H. Hinz.** 1998. Investigations on parental immunization of chickens with a *Campylobacter* mineral oil vaccine. Eds. Nagy, B. and Mulder. R. W. A. W., Proceedings COST Action 97**:**Pathogenic micro-

organisms in poultry and eggs; 5. Poultry and food safety, European Commision, Luxembourg: Office for Official Publications of the European Communities, 247-253.

35. **Grant, I. H., N. J. Richardson, and V. D. Bokkenheuser.** 1980. Broiler chickens as potential source of *Campylobacter* infections in humans. J Clin Microbiol **11:**508-510.
36. **Haas, T., J. Metzger, F. Schmitz, A. Heit, T. Muller, E. Latz, and H. Wagner.** 2008. The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation. Immunity **28:**315-323.
37. **Hannu, T., L. Mattila, H. Rautelin, P. Pelkonen, P. Lahdenne, A. Siitonen, and M. Leirisalo-Repo.** 2002. *Campylobacter*-triggered reactive arthritis: a population-based study. Rheumatology (Oxford) **41:**312-318.
38. **Hawn, T. R., A. Verbon, K. D. Lettinga, L. P. Zhao, S. S. Li, R. J. Laws, S. J. Skerrett, B. Beutler, L. Schroeder, A. Nachman, A. Ozinsky, K. D. Smith, and A. Aderem.** 2003. A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease. The Journal of experimental medicine **198:**1563-1572.
39. **Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira.** 2000. A Toll-like receptor recognizes bacterial DNA. Nature **408:**740-745.
40. **Hendrixson, D. R., and V. J. DiRita.** 2004. Identification of *Campylobacter jejuni* genes involved in commensal colonization of the chick gastrointestinal tract. Mol Microbiol **52:**471-484.
41. **Hickey, T. E., G. Majam, and P. Guerry.** 2005. Intracellular survival of *Campylobacter jejuni* in human monocytic cells and induction of apoptotic death by cytholethal distending toxin. Infect Immun **73:**5194-5197.
42. **Hickey, T. E., A. L. McVeigh, D. A. Scott, R. E. Michielutti, A. Bixby, S. A. Carroll, A. L. Bourgeois, and P. Guerry.** 2000. *Campylobacter jejuni* cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. Infect Immun **68:**6535-6541.
43. **Higgs, R., P. Cormican, S. Cahalane, B. Allan, A. T. Lloyd, K. Meade, T. James, D. J. Lynn, L. A. Babiuk, and C. O'Farrelly.** 2006. Induction of a novel chicken Toll-like receptor following *Salmonella enterica* serovar Typhimurium infection. Infect Immun **74:**1692-1698.
44. **Higuchi, M., A. Matsuo, M. Shingai, K. Shida, A. Ishii, K. Funami, Y. Suzuki, H. Oshiumi, M. Matsumoto, and T. Seya.** 2008. Combinational recognition of bac-

terial lipoproteins and peptidoglycan by chicken Toll-like receptor 2 subfamily. Developmental and comparative immunology **32:**147-155.

45. **Hirschfeld, M., Y. Ma, J. H. Weis, S. N. Vogel, and J. J. Weis.** 2000. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J Immunol **165:**618-622.
46. **Hughes, R. A., R. D. Hadden, N. A. Gregson, and K. J. Smith.** 1999. Pathogenesis of Guillain-Barre syndrome. J Neuroimmunol **100:**74-97.
47. **Jin, M. S., and J. O. Lee.** 2008. Structures of the toll-like receptor family and its ligand complexes. Immunity **29:**182-191.
48. **Jin, S., A. Joe, J. Lynett, E. K. Hani, P. Sherman, and V. L. Chan.** 2001. JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. Mol Microbiol **39:**1225-1236.
49. **Jin, S., Y. C. Song, A. Emili, P. M. Sherman, and V. L. Chan.** 2003. JlpA of *Campylobacter jejuni* interacts with surface-exposed heat shock protein 90alpha and triggers signalling pathways leading to the activation of NF-kappaB and p38 MAP kinase in epithelial cells. Cellular microbiology **5:**165-174.
50. **Kajita, E., T. Nishiya, and S. Miwa.** 2006. The transmembrane domain directs TLR9 to intracellular compartments that contain TLR3. Biochemical and biophysical research communications **343:**578-584.
51. **Karlyshev, A. V., P. Everest, D. Linton, S. Cawthraw, D. G. Newell, and B. W. Wren.** 2004. The *Campylobacter jejuni* general glycosylation system is important for attachment to human epithelial cells and in the colonization of chicks. Microbiology **150:**1957-1964.
52. **Kawai, T., and S. Akira.** 2007. TLR signaling. Seminars in immunology **19:**24-32.
53. **Keestra, A. M., M. R. de Zoete, R. A. van Aubel, and J. P. M. van Putten.** 2007. The central leucine-rich repeat region of chicken TLR16 dictates unique ligand specificity and species-specific interaction with TLR2. J Immunol **178:**7110-7119.
54. **Keestra, A. M., M. R. de Zoete, R. A. van Aubel, and J. P. M. van Putten.** 2008. Functional characterization of chicken TLR5 reveals species-specific recognition of flagellin. Molecular immunology **45:**1298-1307.
55. **Keestra, A. M., and J. P. M. van Putten.** 2008. Unique properties of the chicken TLR4/MD-2 complex: selective lipopolysaccharide activation of the MyD88-dependent pathway. J Immunol **181:**4354-4362.

56. **Ketley, J. M.** 1997. Pathogenesis of enteric infection by *Campylobacter*. Microbiology **143 (Pt 1):**5-21.

57. **Khoury, C. A., and R. J. Meinersmann.** 1995. A genetic hybrid of the *Campylobacter jejuni flaA* gene with LT-B of *Escherichia coli* and assessment of the efficacy of the hybrid protein as an oral chicken vaccine. Avian Dis **39:**812-820.

58. **Kim, Y. M., M. M. Brinkmann, M. E. Paquet, and H. L. Ploegh.** 2008. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. Nature **452:**234-238.

59. **Knudsen, K. N., D. D. Bang, L. O. Andresen, and M. Madsen.** 2006. *Campylobacter jejuni* strains of human and chicken origin are invasive in chickens after oral challenge. Avian Dis **50:**10-14.

60. **Konkel, M. E., S. G. Garvis, S. L. Tipton, D. E. Anderson, Jr., and W. Cieplak, Jr.** 1997. Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. Mol Microbiol **24:**953-963.

61. **Konkel, M. E., B. J. Kim, V. Rivera-Amill, and S. G. Garvis.** 1999. Bacterial secreted proteins are required for the internaliztion of *Campylobacter jejuni* into cultured mammalian cells. Mol Microbiol **32:**691-701.

62. **Krause-Gruszczynska, M., M. Rohde, R. Hartig, H. Genth, G. Schmidt, T. Keo, W. Konig, W. G. Miller, M. E. Konkel, and S. Backert.** 2007. Role of the small Rho GTPases Rac1 and Cdc42 in host cell invasion of *Campylobacter jejuni*. Cellular microbiology **9:**2431-2444.

63. **Kumar, H., T. Kawai, and S. Akira.** 2009. Toll-like receptors and innate immunity. Biochemical and biophysical research communications **388:**621-625.

64. **Lahiri, A., P. Das, and D. Chakravortty.** 2008. Engagement of TLR signaling as adjuvant: towards smarter vaccine and beyond. Vaccine **26:**6777-6783.

65. **Larson, C. L., D. H. Shah, A. S. Dhillon, D. R. Call, S. Ahn, G. J. Haldorson, C. Davitt, and M. E. Konkel.** 2008. *Campylobacter jejuni* invade chicken LMH cells inefficiently and stimulate differential expression of the chicken CXCLi1 and CXCLi2 cytokines. Microbiology **154:**3835-3847.

66. **Latz, E., A. Schoenemeyer, A. Visintin, K. A. Fitzgerald, B. G. Monks, C. F. Knetter, E. Lien, N. J. Nilsen, T. Espevik, and D. T. Golenbock.** 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat Immunol **5:**190-198.

67. **Lee, M. D., and D. G. Newell.** 2006. *Campylobacter* in poultry: filling an ecological niche. Avian Dis **50:**1-9.

68. **Lee, P. Y., Y. Kumagai, Y. Li, O. Takeuchi, H. Yoshida, J. Weinstein, E. S. Kellner, D. Nacionales, T. Barker, K. Kelly-Scumpia, N. van Rooijen, H. Kumar, T. Kawai, M. Satoh, S. Akira, and W. H. Reeves.** 2008. TLR7-dependent and FcgammaR-independent production of type I interferon in experimental mouse lupus. The Journal of experimental medicine **205:**2995-3006.

69. **Leifer, C. A., J. C. Brooks, K. Hoelzer, J. Lopez, M. N. Kennedy, A. Mazzoni, and D. M. Segal.** 2006. Cytoplasmic targeting motifs control localization of toll-like receptor 9. J Biol Chem **281:**35585-35592.

70. **Li, Y. P., H. Ingmer, M. Madsen, and D. D. Bang.** 2008. Cytokine responses in primary chicken embryo intestinal cells infected with *Campylobacter jejuni* strains of human and chicken origin and the expression of bacterial virulence-associated genes. BMC microbiology **8:**107.

71. **Loc Carrillo, C., R. J. Atterbury, A. el-Shibiny, P. L. Connerton, E. Dillon, A. Scott, and I. F. Connerton.** 2005. Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens. Appl Environ Microbiol **71:**6554-6563.

72. **Meade, K. G., F. Narciandi, S. Cahalane, C. Reiman, B. Allan, and C. O'Farrelly.** 2009. Comparative in vivo infection models yield insights on early host immune response to *Campylobacter* in chickens. Immunogenetics **61:**101-110.

73. **Medzhitov, R.** 2009. Approaching the asymptote: 20 years later. Immunity **30:**766-775.

74. **Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr.** 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. Nature **388:**394-397.

75. **Meylan, E., and J. Tschopp.** 2008. IRAK2 takes its place in TLR signaling. Nat Immunol **9:**581-582.

76. **Mills, S. D., and W. C. Bradbury.** 1984. Human antibody response to outer membrane proteins of *Campylobacter jejuni* during infection. Infect Immun **43:**739-743.

77. **Montminy, S. W., N. Khan, S. McGrath, M. J. Walkowicz, F. Sharp, J. E. Conlon, K. Fukase, S. Kusumoto, C. Sweet, K. Miyake, S. Akira, R. J. Cotter, J. D. Goguen, and E. Lien.** 2006. Virulence factors of *Yersinia pestis* are overcome by a strong lipopolysaccharide response. Nat Immunol **7:**1066-1073.

78. **Moran, A. P.** 1995. Biological and serological characterization of *Campylobacter jejuni* lipopolysaccharides with deviating core and lipid A structures. FEMS Immunol Med Microbiol **11:**121-130.

79. **Morooka, T., A. Umeda, and K. Amako.** 1985. Motility as an intestinal colonization factor for *Campylobacter jejuni*. Journal of general microbiology **131:**1973-1980.
80. **Murthy, K. G., A. Deb, S. Goonesekera, C. Szabo, and A. L. Salzman.** 2004. Identification of conserved domains in *Salmonella muenchen* flagellin that are essential for its ability to activate TLR5 and to induce an inflammatory response in vitro. J Biol Chem **279:**5667-5675.
81. **Myszewski, M. A., and N. J. Stern.** 1990. Influence of *Campylobacter jejuni* cecal colonization on immunoglobulin response in chickens. Avian Dis **34:**588-594.
82. **Nachamkin, I., and X. H. Yang.** 1992. Local immune responses to the Campylobacter flagellin in acute *Campylobacter* gastrointestinal infection. J Clin Microbiol **30:**509-511.
83. **Newell, D. G., and S. A. Cawthraw.** 2006. Vaccine and nuleic acids. World Intellectual Property Organization**:**Patent nr.: WO 2006/046017.
84. **Newell, D. G., and C. Fearnley.** 2003. Sources of *Campylobacter* colonization in broiler chickens. Appl Environ Microbiol **69:**4343-4351.
85. **Nishiya, T., E. Kajita, S. Miwa, and A. L. Defranco.** 2005. TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. J Biol Chem **280:**37107-37117.
86. **Noor, S. M., A. J. Husband, and P. R. Widders.** 1995. In ovo oral vaccination with *Campylobacter jejuni* establishes early development of intestinal immunity in chickens. Br Poult Sci **36:**563-573.
87. **Palm, N. W., and R. Medzhitov.** 2009. Immunostimulatory activity of haptenated proteins. Proc Natl Acad Sci U S A **106:**4782-4787.
88. **Palm, N. W., and R. Medzhitov.** 2009. Pattern recognition receptors and control of adaptive immunity. Immunological reviews **227:**221-233.
89. **Park, B., M. M. Brinkmann, E. Spooner, C. C. Lee, Y. M. Kim, and H. L. Ploegh.** 2008. Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. Nat Immunol **9:**1407-1414.
90. **Pasare, C., and R. Medzhitov.** 2005. Control of B-cell responses by Toll-like receptors. Nature **438:**364-368.
91. **Pasare, C., and R. Medzhitov.** 2005. Toll-like receptors: linking innate and adaptive immunity. Adv Exp Med Biol **560:**11-18.
92. **Pei, Z., C. Burucoa, B. Grignon, S. Baqar, X. Z. Huang, D. J. Kopecko, A. L. Bourgeois, J. L. Fauchere, and M. J. Blaser.** 1998. Mutation in the *peb1A* locus

of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. Infect Immun **66:**938-943.

93. **Picard, C., A. Puel, M. Bonnet, C. L. Ku, J. Bustamante, K. Yang, C. Soudais, S. Dupuis, J. Feinberg, C. Fieschi, C. Elbim, R. Hitchcock, D. Lammas, G. Davies, A. Al-Ghonaium, H. Al-Rayes, S. Al-Jumaah, S. Al-Hajjar, I. Z. Al-Mohsen, H. H. Frayha, R. Rucker, T. R. Hawn, A. Aderem, H. Tufenkeji, S. Haraguchi, N. K. Day, R. A. Good, M. A. Gougerot-Pocidalo, A. Ozinsky, and J. L. Casanova.** 2003. Pyogenic bacterial infections in humans with IRAK-4 deficiency. Science (New York, N.Y **299:**2076-2079.

94. **Poly, F., C. Ewing, S. Goon, T. E. Hickey, D. Rockabrand, G. Majam, L. Lee, J. Phan, N. J. Savarino, and P. Guerry.** 2007. Heterogeneity of a *Campylobacter jejuni* protein that is secreted through the flagellar filament. Infect Immun **75:**3859-3867.

95. **Puliti, M., S. Uematsu, S. Akira, F. Bistoni, and L. Tissi.** 2009. Toll-like receptor 2 deficiency is associated with enhanced severity of group B streptococcal disease. Infect Immun **77:**1524-1531.

96. **Rathinam, V. A., D. M. Appledorn, K. A. Hoag, A. Amalfitano, and L. S. Mansfield.** 2009. *Campylobacter jejuni*-induced activation of dendritic cells involves cooperative signaling through Toll-like receptor 4 (TLR4)-MyD88 and TLR4-TRIF axes. Infect Immun **77:**2499-2507.

97. **Renom, G., M. Kirimat, A. J. Georges, J. C. Philippe, and P. M. Martin.** 1992. High levels of anti-*Campylobacter*-flagellin IgA antibodies in breast milk. Res Microbiol **143:**93-98.

98. **Rice, B. E., D. M. Rollins, E. T. Mallinson, L. Carr, and S. W. Joseph.** 1997. *Campylobacter jejuni* in broiler chickens: colonization and humoral immunity following oral vaccination and experimental infection. Vaccine **15:**1922-1932.

99. **Ringoir, D. D., and V. Korolik.** 2003. Colonisation phenotype and colonisation potential differences in *Campylobacter jejuni* strains in chickens before and after passage in vivo. Vet Microbiol **92:**225-235.

100. **Roach, J. C., G. Glusman, L. Rowen, A. Kaur, M. K. Purcell, K. D. Smith, L. E. Hood, and A. Aderem.** 2005. The evolution of vertebrate Toll-like receptors. Proc Natl Acad Sci U S A **102:**9577-9582.

101. **Ruiz-Palacios, G. M., J. J. Calva, L. K. Pickering, Y. Lopez-Vidal, P. Volkow, H. Pezzarossi, and M. S. West.** 1990. Protection of breast-fed infants against *Campylobacter* diarrhea by antibodies in human milk. J Pediatr **116:**707-713.

102. **Saitoh, S., and K. Miyake.** 2006. Mechanism regulating cell surface expression and activation of Toll-like receptor 4. Chemical record (New York, N.Y **6:**311-319.

103. **Samuel, M. C., D. J. Vugia, S. Shallow, R. Marcus, S. Segler, T. McGivern, H. Kassenborg, K. Reilly, M. Kennedy, F. Angulo, and R. V. Tauxe.** 2004. Epidemiology of sporadic *Campylobacter* infection in the United States and declining trend in incidence, FoodNet 1996-1999. Clin Infect Dis **38 Suppl 3:**S165-174.

104. **Sanyal, S. C., K. M. Islam, P. K. Neogy, M. Islam, P. Speelman, and M. I. Huq.** 1984. *Campylobacter jejuni* diarrhea model in infant chickens. Infect Immun **43:**931-936.

105. **Schwarz, H., K. Schneider, A. Ohnemus, M. Lavric, S. Kothlow, S. Bauer, B. Kaspers, and P. Staeheli.** 2007. Chicken toll-like receptor 3 recognizes its cognate ligand when ectopically expressed in human cells. J Interferon Cytokine Res **27:**97-101.

106. **Sepulveda, F. E., S. Maschalidi, R. Colisson, L. Heslop, C. Ghirelli, E. Sakka, A. M. Lennon-Dumenil, S. Amigorena, L. Cabanie, and B. Manoury.** 2009. Critical role for asparagine endopeptidase in endocytic Toll-like receptor signaling in dendritic cells. Immunity **31:**737-748.

107. **Shaughnessy, R. G., K. G. Meade, S. Cahalane, B. Allan, C. Reiman, J. J. Callanan, and C. O'Farrelly.** 2009. Innate immune gene expression differentiates the early avian intestinal response between *Salmonella* and *Campylobacter*. Vet Immunol Immunopathol **132:**191-198.

108. **Smith, C. K., M. Abuoun, S. A. Cawthraw, T. J. Humphrey, L. Rothwell, P. Kaiser, P. A. Barrow, and M. A. Jones.** 2008. *Campylobacter* colonization of the chicken induces a proinflammatory response in mucosal tissues. FEMS Immunol Med Microbiol **54:**114-121.

109. **Smith, C. K., P. Kaiser, L. Rothwell, T. Humphrey, P. A. Barrow, and M. A. Jones.** 2005. *Campylobacter jejuni*-induced cytokine responses in avian cells. Infect Immun **73:**2094-2100.

110. **Smith, K. D., E. Andersen-Nissen, F. Hayashi, K. Strobe, M. A. Bergman, S. L. Barrett, B. T. Cookson, and A. Aderem.** 2003. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol **4:**1247-1253.

111. **Snelling, W. J., M. Matsuda, J. E. Moore, and J. S. Dooley.** 2005. *Campylobacter jejuni*. Lett Appl Microbiol **41:**297-302.

112. **Song, Y. C., S. Jin, H. Louie, D. Ng, R. Lau, Y. Zhang, R. Weerasekera, S. Al Rashid, L. A. Ward, S. D. Der, and V. L. Chan.** 2004. FlaC, a protein of *Campylobacter jejuni* TGH9011 (ATCC43431) secreted through the flagellar apparatus, binds epithelial cells and influences cell invasion. Mol Microbiol **53:**541-553.

113. **Stas, T., F. T. Jordan, and Z. Woldehiwet.** 1999. Experimental infection of chickens with *Campylobacter jejuni*: strains differ in their capacity to colonize the intestine. Avian Pathol **28:**61-64.

114. **Stern, N. J., R. J. Meinersmann, N. A. Cox, J. S. Bailey, and L. C. Blankenship.** 1990. Influence of host lineage on cecal colonization by *Campylobacter jejuni* in chickens. Avian Dis **34:**602-606.

115. **Tabeta, K., P. Georgel, E. Janssen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, L. Alexopoulou, R. A. Flavell, and B. Beutler.** 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. Proc Natl Acad Sci U S A **101:**3516-3521.

116. **Takeuchi, O., K. Hoshino, and S. Akira.** 2000. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. J Immunol **165:**5392-5396.

117. **Temperley, N. D., S. Berlin, I. R. Paton, D. K. Griffin, and D. W. Burt.** 2008. Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. BMC genomics **9:**62.

118. **Torres, O., and J. R. Cruz.** 1993. Protection against *Campylobacter* diarrhea: role of milk IgA antibodies against bacterial surface antigens. Acta Paediatr **82:**835-838.

119. **Travassos, L. H., S. E. Girardin, D. J. Philpott, D. Blanot, M. A. Nahori, C. Werts, and I. G. Boneca.** 2004. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO reports **5:**1000-1006.

120. **Uematsu, S., and S. Akira.** 2009. Immune responses of TLR5(+) lamina propria dendritic cells in enterobacterial infection. Journal of gastroenterology **44:**803-811.

121. **van Alphen, L. B., N. M. Bleumink-Pluym, K. D. Rochat, B. W. van Balkom, M. M. S. M. Wösten, and J. P. M. van Putten.** 2008. Active migration into the subcellular space precedes *Campylobacter jejuni* invasion of epithelial cells. Cellular microbiology **10:**53-66.

122. **Vijay-Kumar, M., C. J. Sanders, R. T. Taylor, A. Kumar, J. D. Aitken, S. V. Sitaraman, A. S. Neish, S. Uematsu, S. Akira, I. R. Williams, and A. T. Gewirtz.**

2007. Deletion of TLR5 results in spontaneous colitis in mice. The Journal of clinical investigation **117:**3909-3921.

123. **Wagenaar, J. A., D. J. Mevius, and A. H. Havelaar.** 2006. *Campylobacter* in primary animal production and control strategies to reduce the burden of human campylobacteriosis. Rev sci tech Off int Epiz **25:**581-594.

124. **Wagenaar, J. A., M. A. Van Bergen, M. A. Mueller, T. M. Wassenaar, and R. M. Carlton.** 2005. Phage therapy reduces *Campylobacter jejuni* colonization in broilers. Vet Microbiol **109:**275-283.

125. **Walker, R. I., M. B. Caldwell, E. C. Lee, P. Guerry, T. J. Trust, and G. M. Ruiz-Palacios.** 1986. Pathophysiology of *Campylobacter* enteritis. Microbiol Rev **50:**81-94.

126. **Wassenaar, T. M.** 1997. Toxin production by *Campylobacter* spp. Clin Microbiol Rev **10:**466-476.

127. **Widders, P. R., R. Perry, W. I. Muir, A. J. Husband, and K. A. Long.** 1996. Immunisation of chickens to reduce intestinal colonisation with *Campylobacter jejuni*. Br Poult Sci **37:**765-778.

128. **Widders, P. R., L. M. Thomas, K. A. Long, M. A. Tokhi, M. Panaccio, and E. Apos.** 1998. The specificity of antibody in chickens immunised to reduce intestinal colonisation with *Campylobacter jejuni*. Vet Microbiol **64:**39-50.

129. **Zhao, C., B. Ge, J. De Villena, R. Sudler, E. Yeh, S. Zhao, D. G. White, D. Wagner, and J. Meng.** 2001. Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the Greater Washington, D.C., area. Appl Environ Microbiol **67:**5431-5436.

2

Chicken TLR21 is an innate CpG DNA receptor distinct from mammalian TLR9

Marcel R. de Zoete*
A. Marijke Keestra*
Lieneke I. Bouwman
Jos P. M. van Putten

*these authors contributed equally to this work

Journal of Immunology 2010, in press

Abstract

Toll-like receptors (TLRs) comprise a family of evolutionary conserved sensory receptors that respond to distinct classes of ligands. For one major evolutionary branch of TLRs, the ligands are still largely unknown. Here we report the cloning and function of one member of this group, chicken TLR21 (chTLR21). This TLR is absent in the human species but has homologues in fish and frog and displays similarity with mouse TLR13. Expression of chTLR21 in HEK293 cells resulted in activation of NF-κB in response to unmethylated CpG DNA, typically recognized by mammalian TLR9. Silencing of chTLR21 (but not chTLR4) in chicken macrophages inhibited the response to CpG-DNA (but not to LPS), indicating similar functionality of the endogenous receptor. ChTLR21 responded to human- and murine-specific TLR9 ligands, as well as to bacterial genomic DNA isolated from *Salmonella* Enteritidis. Confocal microscopy located chTLR21 in the same intracellular compartments as hTLR9. Inhibition of the chTLR21 response by the endosomal maturation inhibitor chloroquine suggested that the receptor is functional in endolysosomes, as known for TLR9. The analogous localization and function of the phylogenetically only distantly related chicken TLR21 and mammalian TLR9 suggest that during evolution different classes of TLRs have emerged that recognize the same type of ligands.

Introduction

The innate immune system is the first line of defense against invading pathogens. Central in the sensing of microbes are germline encoded pattern recognition receptors (PRRs) that recognize conserved microbial structures such as LPS, flagellin, lipoproteins and DNA/RNA (2, 12, 30). Activation of PRRs initiates one or more signaling pathways that ultimately activate transcription factors such as NF-κB that drive the innate immune response. One major PRR family comprises the Toll-like receptors (TLRs). These receptors function mainly at the cell surface (human TLR1, TLR2, TLR4, TLR5, TLR6) or in endolysosomes (TLR3, TLR7, TLR8, TLR9) (1, 8, 22, 29). The surface-located TLRs generally respond to conserved microbial cell wall constituents, whereas the endolysosomal TLRs typically recognize viral and bacterial nucleic acids. TLR9, which responds to unmethylated CpG DNA motifs (4, 17), is proteolytically cleaved in the endolysosomes, a process considered crucial for TLR9 activation (11, 26, 32).

TLRs are highly conserved throughout phylogeny and are present in insects, amphibians, birds and mammals (35, 38). In all cases, the receptors consist of an extracellular leucine-rich repeat (LRR) domain that is involved in ligand recognition, a single transmembrane domain, and a cytoplasmic Toll/interleukin-1 receptor (TIR) signaling domain. For most TLRs microbial ligands have been identified (reviewed in: (2, 30)). The type of microbial ligand recognized by TLR homologues in different species is generally conserved, although subtle species-specific differences in ligand recognition and signaling exist between TLR orthologues. One major subfamily of TLRs with a still largely unknown function consists of TLR21-23 mainly found in birds, amphibians and fish, and the related murine members TLR11-13 (35). For this group of TLRs, ligands have been identified only for TLR22 in fish (dsRNA) (27) and murine TLR11 (profilin-like protein) (45).

In the present study, we resolved the function of chicken TLR21 (chTLR21). Based on amino acid sequence similarity, this TLR appears unique compared to the human TLR repertoire and its ligand was thus far unknown. We provide evidence that chTLR21 is an nucleotide receptor that senses and responds to DNA, despite low sequence similarity with the functionally related mammalian TLR9.

Materials and Methods

Cells and reagents

HEK293 cells, HeLa 57A cells, and HD11 chicken macrophages were maintained as previously described (21). COS-7 cells were routinely cultured in IMDM (Gibco) supplemented with 10% FCS. The TLR ligands FSL-1, Pam_3CSK_4, Poly(I:C), CL097, ODN 1826 and ODN 2006 were purchased from InVivogen. LPS, flagellin and genomic DNA from *Salmonella enterica* serovar Enteritidis strain 706 were purified as described (10, 20, 21). Peptide-*N*-glycosidase F (PNGase F) and chloroquine were purchased from Roche and Sigma, respectively.

Construction of expression plasmids

Gene expression was established using a pTracer-CMV2 (Invitrogen) derivative that lacks the *gfp* gene and contains a 3xFLAG-tag at the C-terminal cloning site. The *gfp* gene was deleted from the vector by inverse PCR using the SphI-tailed primers that anneal just outside the *gfp* gene. The obtained PCR product was digested with SphI and religated, yielding pTracer-CMV2ΔGFP. The 3xFLAG sequence was amplified by PCR from the p3xFLAG-*Myc*-CMV-23 vector (Sigma) using a forward primer with a NotI site and a reverse primer with an XbaI site. The obtained PCR product and pTracer-CMV2ΔGFP were digested with NotI and XbaI and ligated, yielding pTracer-CMV2ΔGFP/3xFLAG. Full-length *tlr21* (including the intron) was amplified by PCR from chromosomal DNA of HD11 cells using *pfu* polymerase (Promega) and directly cloned into pTracer-CMV2, yielding pTracer-chTLR21. RNA was isolated from HEK293 cells transiently transfected with this plasmid, and cDNA encoding chTLR21 was obtained by reverse transcription using chTLR21 specific primers. Amplification of this intron-less chTLR21 with specific primers tailed with NotI sites enabled ligation into pTracer-CMV2ΔGFP/3xFLAG, yielding chTLR21-3xFLAG-C. Human TLR9 was amplified by PCR with *pfu* polymerase from the vector pUNO-hTLR9 (InVivogen), digested with the restriction enzyme NotI present in the primers, and cloned into pTracer-CMV2ΔGFP/3xFLAG, yielding hTLR9-3xFLAG-C. All primers used for cloning are listed in the Table I. Comparative sequence analysis was done using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Glycosylation sites were predicted using NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc). Protein modeling was performed using the CPHmodels 3.0 Server (http://www.cbs.dtu.dk/Services /CPHmodels).

Transfection of HEK293 and HD11 cells

For stimulation assays, HEK293 cells were grown in 48-well tissue culture plates in DMEM / 5% FCS for 24 h (~50% confluence) and transiently transfected with a total of 250 ng of plasmid DNA, consisting of 62.5 ng of the reporter plasmid pNF-κB-luc, 62.5 ng of normalization vector pTK-LacZ, and 125 ng of either chTLR21, hTLR9, or empty control vector. FuGENE 6 (Roche-Diagnostics) was used as transfection reagent at a lipid to DNA ratio of 3 to 1. After 48 h of incubation (37°C), the medium was replaced with fresh DMEM / 5% FCS and the cells stimulated with the appropriate ligands. HD11 cells, seeded at 50% confluency in 12-well plates, were transfected with ExGen 500 in vitro transfection reagent (Fermentas) according to the manufacturer's protocol. Briefly, 6.6 µl ExGen 500 was added to 2 µg of plasmid DNA in 200 µl of 150 mM NaCl and vortexed. After incubation (22ºC, 15 min), the mixture was added dropwise to the cells.

Table I. *Primers used in this study.*

Primers	Sequence (5'-3')*
cloning	
pTracerCMV2ΔGFP Fwd	ACATGCATGCGCCAAGTTGACCAGTGCCGTT
pTracerCMV2ΔGFP Rev	ACATGCATGCCATGGTTTAGTTCCTCACCTT
3xFLAG Fwd	ATTTGCGGCCGCGACTACAAAGACCATGACGGT
3xFLAG Rev	GCTCTAGATCACTTGTCATCGTCATCCTTGTA
chTLR21 Fwd	ATTTGCGGCCGCCACCATGATGGAGACACCGGAGAAGG
chTLR21 Rev	ATTTGCGGCCGCGCATCTGTTTGTCTCCTTCCCT
hTLR9 Fwd	ATTTGCGGCCGCCACCATGGGTTTCTGCCGCAGCGCC
hTLR9 Rev	ATTTGCGGCCGCGTTCGGCCGTGGGTCCCTGGCA
siRNA	
chTLR21-Fwd	GTACCTCGCAACTGCATTGAGGATGTCATCAAGAGTGAC
	ATCCTCAATGCAGTTGCTTTTTGGAAA
chTLR21-Rev	AGCTTTTCCAAAAAGCAACTGCATTGAGGATGTCACTCT
	TGATGACATCCTCAATGCAGTTGCGAG
chTLR4-Fwd	GTACCTCGTTTACAGGTCAACAGACTAATCAAGAGTTAG
	TCTGTTGACCTGTAAACTTTTTGGAAA
chTLR4-Rev	AGCTTTTCCAAAAAGTTTACAGGTCAACAGACTAACTCT
	TGATTAGTCTGTTGACCTGTAAACGAG

*Underlined sequences represent restriction sites used in cloning of the PCR products.

Luciferase assays

Transfected cells were stimulated with the indicated TLR ligands as described (19). When appropriate, chloroquine (150 μM) was added at 30 min prior to stimulation. TLR signaling was assessed using the NF-κB-luciferase reporter system (40). Results are from at least three independent experiments performed in duplicate. Data were analyzed by Student's paired *t* test. P values < 0.05 were considered significant.

RNA silencing

Knockdown of chTLR21 and chTLR4 were achieved using the psiRNA System (Invivogen). For each TLR, two complementary oligonucleotides (Table I) were designed using the RNA Wizard (http://www.sirnawizard.com), mixed at a final concentration of 1.7 μM each, and annealed in the presence of 100 mM NaCl at decreasing temperature starting at 80ºC. Annealed inserts were subsequently ligated into psiRNA-h7SKGFPzeo using the restriction enzymes Acc 65I/Hind III. The obtained plasmids were transfected to HD11 cells as described above. After 48 h, Zeocin (Invivogen) was added at 100 μg/ml to select for stable transfectants. Silenced HD11 cells were stimulated with ODN 2006 or *S*. Enteritidis LPS for 17 h, after which nitric oxide production was determined with the Griess assay (21).

Deglycosylation of chTLR21 and hTLR9

COS-7 cells grown overnight to ~50% confluence in 12-well plates were transfected with 2 μg of chTLR21-3xFLAG-C or hTLR9-3xFLAG-C per well using FuGENE 6 (Roche Diagnostics) at a lipid to DNA ratio of 2 to 1. After incubation (48 h, 37°C), the cells were rinsed with DPBS, resuspended in 250 μl of Reporter Lysis Buffer (Promega), and frozen at -80˚C to complete lysis. For deglycosylation of chTLR21 and hTLR9, 50 μl of cell lysate was mixed with 25 μl of 500 mM of K_2HPO_4, 250 mM of EDTA (pH 7.0), 60 μl of H_2O, 50 μl of 10% sodium dodecyl-sulphate (SDS) and 12.5 μl of 10% 2-mercaptoethanol and boiled (5 min). After addition of Nonidet P-40 (final concentration of 2%), the samples were cooled and incubated (16 h, 37°C) in the presence of 5 units of recombinant PNGase F. After inactivation of the enzyme (100°C, 3 min), the deglycosylated and mock-treated samples were subjected to SDS-PAGE and immunoblotting. ChTLR21 and hTLR9 were detected using M2 anti-FLAG antibodies (1:2,000, Sigma) and goat-anti-mouse-HRP (1:10,000) with Supersignal chemiluminescence substrate (Thermo Scientific).

Confocal laser microscopy

HEK293 cells grown on poly-L-Lysine coated glass coverslips were transfected with 250 ng of chTLR21-3xFLAG-C and/or pUNO-hTLR9 DNA as described above. After 48 h, the cells were rinsed with 2% paraformaldehyde (PFA)/100 mM phosphate buffer in DMEM, fixed in 4% PFA in 100 mM phosphate buffer (30 min, 20°C), quenched with 50 mM NH_4Cl (10 min), and permeabilized and blocked with DPBS/1.0% Triton X-100/1% BSA (30 min). For surface staining, cells were incubated with WGA-Alexa Fluor 633 (1:500, Invitrogen) (10 min, 37°C) and rinsed three times with DPBS before fixation. Organelles were stained (1 h, 20°C) using DPBS/2% BSA containing antibodies directed against CD63 (1:200, Immunotech), Calnexin (1:100, BD), hTLR9 (1:500, Imgenex) or chTLR21-3x-FLAG-C (anti-Flag M2, 1:500, Sigma), followed by incubation (1 h, 20°C) with goat Alexa488 anti-mouse or goat Alexa568 anti-rabbit (1:100) secondary antibodies. Stained cells were rinsed three times with DPBS, once with distilled water, and embedded in Fluorsave (Calbiochem). Cells were viewed in a Bio-Rad radiance 2100MP multiphoton confocal laser microscope and analyzed using ImageJ software.

Transfected HD11 cells were fixed in DPBS/4% paraformaldehyde (30 min, RT) and stained as described (20). WGA-biotin (10 min, 37°C, 1:2000 dilution in DPBS) in combination with streptavidin-conjugated Alex Fluor 488 (dilution 1:2000) was used as cell surface stain. After cell permeabilization using Triton X-100 (1%, 10 min, 20°C) and blocking in DPBS/2% BSA (30 min, 20°C), the cells TLRs were stained using anti-Flag M2 antibody (dilution 1:500) in combination with goat-anti-mouse-Alexa Fluor 568 (dilution 1:2,000). Organelle staining was not possible due to a lack of chicken cell compartment markers. HD11 cells viewed in a Leica TCS SP confocal laser microscope.

RNA isolation and quantitative RT-PCR

RNA extraction and measurement of RNA transcripts was essentially carried out as described (21). RNA was extracted from HD11 macrophages after stimulation (2 h) with the indicated ligands. RNA transcript levels were determined by quantitative reverse transcription PCR (RT-PCR) using an ABI PRISM 7000 sequence detection system. Primers and probes are described (21). Probes were labeled with the reporter dye FAM and the quencher TAMRA. RT-PCR was performed on 50 ng of DNase I (Fermentas) treated RNA with the One Step RT-PCR MasterMix kit for Probe Assays (Eurogentec). Real time cycler conditions were 30 min at 48°C, followed by 10 min at 95°C, 40 cycles of 15 s at

95°C, and 1 min at 60°C. Transcript levels were corrected to those for the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as described (21). Data shown are from three independent batches of isolated RNA.

	TM/TIR domain	LRR domain	Full protein
hTLR3	43	39	39
hTLR7	49	37	40
hTLR8	47	36	38
hTLR9	49	35	38
mTLR13	61	43	47
TrTLR9	46	34	35
TrTLR21	71	53	57
TrTLR22	61	42	46
TrTLR23	65	42	47
XtTLR9	51	35	38
XtTLR13	46	44	43
XtTLR21	74	58	61
XtTLR22	64	44	48

Table II. *Amino acid similarities (%) of the transmembrane (TM) / TIR domain, the LRR domain, and the full protein of chTLR21 compared to the indicated TLRs of human (h), murine (m), Takifugu rubripes (Tr), and Xenopus tropicalis (Xt) origin.*

Results

Cloning and characteristics of chTLR21.

Cloning and sequence analysis of the *tlr21* gene of chicken HD11 macrophages revealed one open reading frame of 2,919 nucleotides, encoding a protein of 972 amino acid residues. The obtained sequence was identical to GenBank accession number NP_001025729. The putative protein, designated chTLR21, is predicted to contain a signal peptide, an extracellular domain containing 27 LRRs with cysteine-rich capping structures at both ends, a single transmembrane region, and a cytoplasmic (TIR) domain,

conform the TLR consensus architecture. The TIR domain has a proline residue at position 844 in its BB-loop. This proline is conserved in all mammalian TLR (except TLR3) and is critical for activation of the MyD88-dependent signaling pathway (34, 39).

Comparative sequence analysis of chTLR21 revealed only low levels of similarity (< 40%) with other identified chicken TLRs (data not shown). Compared with TLRs of other species, chTLR21 is most similar to TLR21 of Xenopus tropicalis (61%) and Takifugu rubripes (57%) and, more distantly, to murine TLR13 (47%). Overall, the TIR domain appears more conserved than the extracellular LRR domain (Table II). Analysis of putative *N*-glycosylation sites using NetNGlyc software predicts ten putative *N*-glycosylation sites at the Asn residues at the positions 79, 146, 227, 267, 398, 443, 566, 667, 685 and 719 in the extracellular domain of chTLR21.

Modeling of the LRR domain using the CPHmodels 3.0 Server revealed that the extracellular domain of chTLR21 adopts a single horseshoe shape structure as predicted for most mammalian TLRs except TLR7, TLR8, and TLR9 (28) (data not shown). Structure predictions of the putative TIR domain of chTLR21 including the position of the conserved proline residue in the BB-loop, resembled those of the TIR domains of mammalian TLRs (44).

ChTLR21 responds to CpG DNA.

To assess the function of chTLR21, the encoding cDNA was cloned and transfected into HEK293 cells carrying an NF-κB-luciferase reporter gene. Stimulation of these cells with mammalian ligands for TLR2/1, TLR2/6, TLR3, TLR4 or TLR7/8 (Pam_3CSK_4, FSL-1, poly(I:C), LPS, and CL097, respectively) failed to activate NF-κB compared to control cells carrying empty expression vector (Fig. 1). TLR3 stimulation even slightly reduced NF-κB activation in chTLR21 transfected cells, perhaps because of common use of accessory proteins (7). As HEK293 cells can respond to flagellin (20), we measured the potential of bacterial flagellin to activate chTLR21 in HeLa 57A cells. No activation of NF-κB was detected in response to flagellin (data not shown). In contrast to these results, the synthetic CpG-oligodeoxynucleotide ODN 2006, which is a ligand for mammalian TLR9, significantly activated NF-κB in the chTLR21-transfected cells (Fig. 1). These results suggest that chTLR21 senses CpG DNA and thus appears to act as a functional homologue of mammalian TLR9.

To verify that chTLR21 acts as a DNA sensor in chicken cells, a plasmid encoding either chTLR21-specific or (as control) chTLR4-specific silencing mRNA was introduced into HD11 chicken macrophages. Knockdown of endogenous chTLR21 strongly reduced

the stimulation of nitric oxide production in response to ODN 2006, whereas LPS yielded a potent response (Fig. 2). Conversely, silencing of chTLR4 abolished the LPS response, while ODN 2006 induced high levels of nitric oxide (Fig. 2). These results indicate that also endogenous chTLR21 of HD11 cells is activated by DNA.

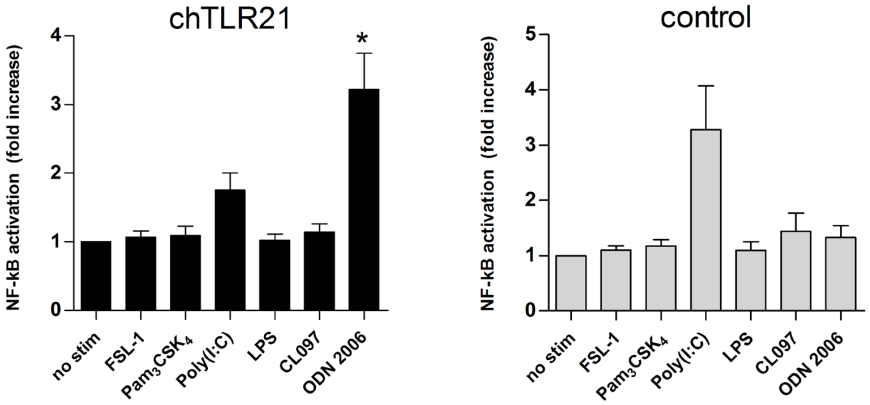

Figure 1. Response of chTLR21 to mammalian TLR ligands. *HEK293 cells transfected with an NF-κB luciferase reporter and either chTLR21 or empty control vector, were stimulated (5 h) with either of the following TLR ligands: FSL1 (100 ng/ml), Pam_3CSK_4 (100 ng/ml), Poly(I:C) (50 μg/ml), S. Enteritidis LPS (100 ng/ml), CL097 (5 μg/ml), and CpG ODN 2006 (0.5 μM). NF-κB luciferase activity was measured as relative light units (RLU) and expressed as fold increase compared to unstimulated transfected cells. Data represent the mean ± SEM of three independent experiments. * p<0.05.*

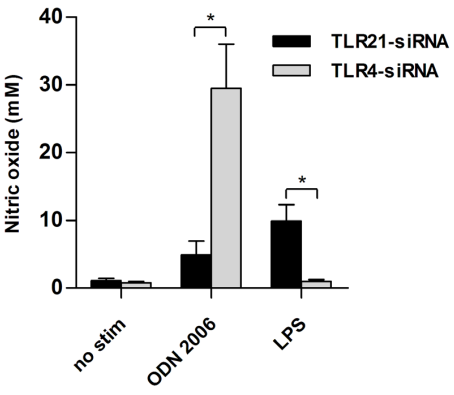

Figure 2. Nitric oxide production by HD11 cells silenced for TLR21 and TLR4. *HD11 cells were stimulated (17 h) with either ODN 2006 (50 nM) or S. Enteritidis LPS (1 ng/ml). Nitric oxide in the cell super-natants were measured using the Griess assay. Silencing of chTLR21 and chTLR4 selectively inhibited the ODN 2006 and LPS response, respectively. Values are the mean ± SEM of four independent experiments. Asterisks indicate statistically significant differences (p<0.05).*

Comparison of chicken TLR21 and human TLR9.

Chickens do not have a gene encoding TLR9. Closer inspection of chTLR21 and mammalian TLR9 revealed only 38% similarity in amino acids between the two receptors, while other chicken TLRs showed similarities of around 65% to their mammalian homologue. SDS-PAGE and immunoblotting of C-terminal FLAG-tagged chTLR21 and human TLR9 expressed in COS-7 cells showed a slightly different electrophoretic mobility (Fig. 3). This difference was maintained after treatment of the respective cell lysates with PNGase F, which removes *N*-linked sugars (Fig. 3). The deglycosylated chTLR21 and hTLR9 proteins migrated with molecular masses of ~109 kDa and ~116 kDa, respectively (Fig. 3). The lower apparent mass of chTLR21 is consistent with the reduced length of the extracellular domain compared to TLR9. The LRR modules of the central domains of the mammalian TLR7, TLR8 and TLR9 family have longer amino acid sequences than other TLRs (43). Although both chTLR21 and hTLR9 are predicted to have 27 LRRs, chTLR21 lacks the ~30 amino acid potential loop structure at the end of LRR15 of TLR9 (Fig. 4) (11, 28). The chicken receptor also lacks the 16-amino-acid segment containing two cysteine (CXXC) motifs present in LRR8 of human and murine TLR7-9 (Fig. 3) (6, 24). Both inserts may be essential for TLR7-9 function (5, 13, 16, 23, 33). Two amino acid residues important for the interaction with CpG DNA in TLR9 (Asp^{534} and Tyr^{536}) (36, 43) appear conserved in chTLR21 (Fig. 4).

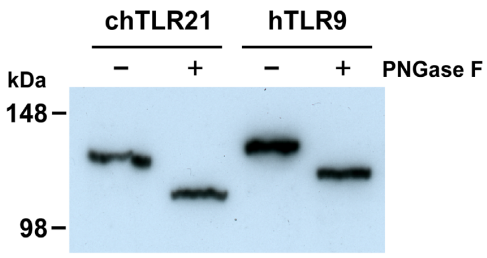

Figure 3. Electrophoretic mobility and N-glycosylation of chTLR21 and hTLR9. *Cell lysates of chTLR21-3xFLAG-C and hTLR9-3xFLAG-C transfected COS-7 cells treated with PNGase F (+) or control buffer (-) were subjected to SDS-PAGE and immuno-blotting. TLR proteins were detected with anti-FLAG M2 antibody in combination with HRP-conjugated goat-anti-mouse antibodies and chemi-luminescence substrate. Molecular masses are indicated in kilo-daltons (kDa).*

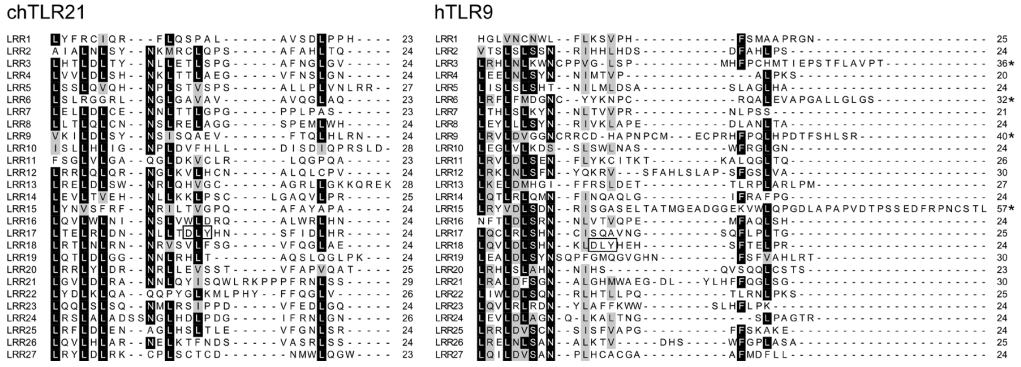

Figure 4. Sequence characteristics of chTLR21 and hTLR9. *The sequences and length of LRR1-27 of chTLR21 and hTLR9 are shown. Conserved amino acids typical of LRR of TLR are shaded in black, synonymous substitutions in gray. The asparagine and aspartate residues (Asp^{534} and D^{536}) implicated in hTLR9 DNA recognition are boxed. The length of the extended LRR in hTLR9 (absent in chTLR21) are indicated in bold numbers.*

Different ligand specificities of chTLR21 and hTLR9.

To better understand the (dis)similarities between chTLR21 and mammalian TLR9, we compared the ligand specificity of the receptors bearing in mind that human and murine TLR9 prefer the CpG-DNA hexameric motifs GTCGTT (present in ODN 2006) and GACGTT motif (present in ODN 1826), respectively (4). Stimulation of chTLR21 transfected cells with either of these compounds activated NF-κB in a chTLR21-dependent fashion (Fig. 5), while hTLR9 transfected cells showed the expected specific response to ODN 2006 (Fig. 5).

As unmethylated CpG motifs are mostly found in bacterial DNA, we also stimulated chTLR21 transfected cells with genomic bacterial DNA rather than with the synthetic DNA derivatives. Bacterial DNA isolated from *S*. Enteritidis activated NF-κB in chTLR21 transfected cells (Fig. 5), while no response was observed for hTLR9-transfected and control cells (Fig. 5). These results indicate that chTLR21 acts as a receptor both for synthetic ODNs as well as for DNA from bacterial origin.

Cellular localization of chTLR21.

In most cell types, the mammalian nucleotide-sensing TLRs (TLR3, TLR7, TLR8 and TLR9) reside in the endoplasmic reticulum (ER), but are functional in the endolysosomal compartment (reviewed in (30)). Confocal laser microscopy on transfected HEK293 cells

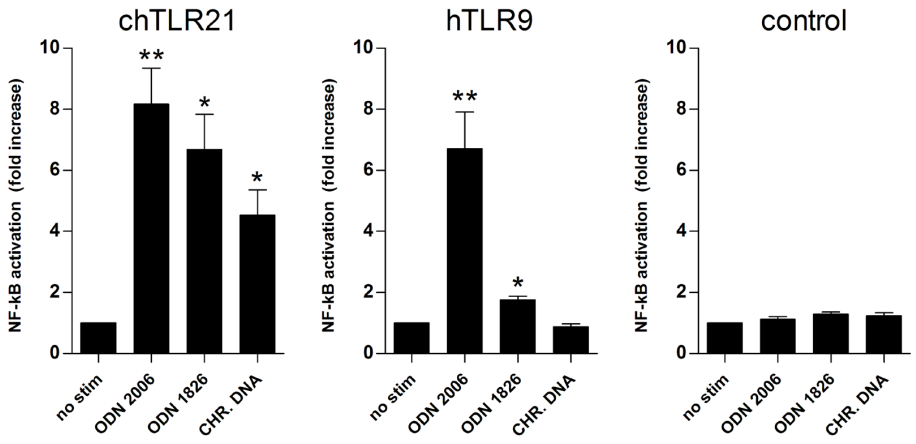

Figure 5. Ligand specificity of chTLR21. *HEK293 cells transfected with an NF-κB luciferase reporter together with either chTLR21, hTLR9, or control vector were stimulated (5 h) with the human-specific ODN 2006 (0.5 μM), the mouse-specific ODN 1826 (0.5 μM) or S. Enteritidis chromosomal (chr.) DNA (30 μg/ml). NF-κB luciferase activity was measured as relative light units (RLU) and expressed as fold increase compared to unstimulated transfected cells. Data represent the mean ± SEM of three experiments. ** p<0.01, * p<0.05.*

that co-expressed chTLR21-FLAG and hTLR9 revealed similar localization of both types of receptors inside the cells (Fig. 6A). Staining of cellular organelles using the lysosome and ER markers CD63 and calnexin respectively, localized chTLR21 mainly in the ER with possibly small amounts present in the CD63 positive compartment (Fig. 6B and C). This localization resembles that of mammalian TLR9 (30).

As TLR9 is functional in endolysosomes we pretreated chTLR21, hTLR9, and (as control) hTLR2/1-transfected HEK293 cells with chloroquine prior to stimulation with ODN 1826 and ODN 2006. Chloroquine inhibits endosomal maturation but not cellular uptake of CpG DNA (14). The compound effectively inhibited both the chTLR21 and hTLR9-mediated NF-κB luciferase and cytokine responses, while the response of the surface-located TLR2/1 complex to the TLR2 ligand Pam_3CSK_4 was unaltered (Fig. 7).

In comparable experiments with HD11 chicken macrophages from which the cloned chTLR21 was derived, chTLR21 was also detected inside the cells (Fig. 6D), although the exact location could not be determined due to a lack of markers. Functionally, chloro-

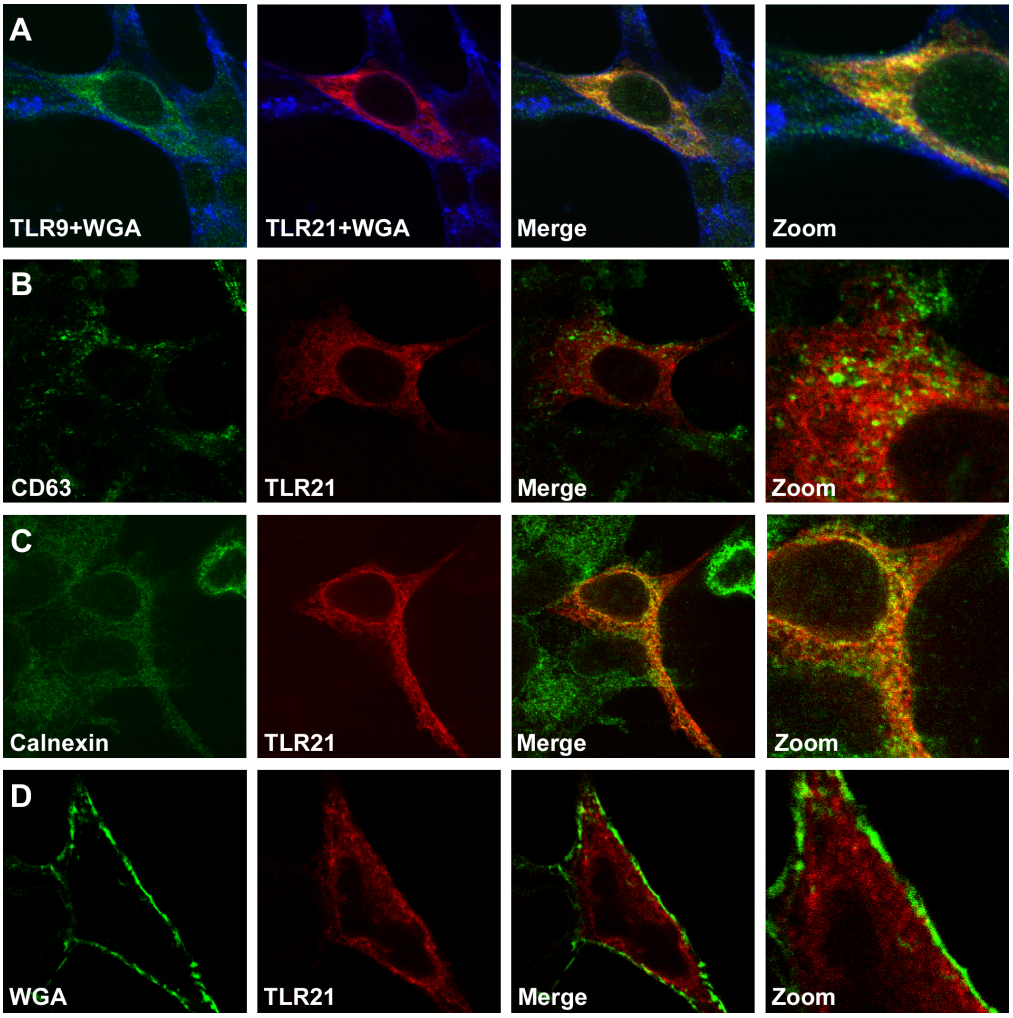

Figure 6. Cellular localization of chTLR21 in HEK293 and HD11 cells. *(A-C) HEK293 cells transfected with either FLAG-tagged-chTLR21 or (non-tagged) hTLR9 were fixed, permeabilized, and stained with late endosome (CD63), ER (Calnexin), or surface membrane (WGA) markers and the appropriate Alex Fluor conjugates. ChTLR21 and hTLR9 were detected using rabbit anti-FLAG M2 and anti-TLR9 antibody in combination with the appropriate conjugate. Cells were viewed using a Bio-Rad radiance 2100MP multi-photon confocal laser microscope and analyzed using ImageJ software. Yellow staining in the merged figures (Merge and Zoom) indicates co-localization of the probed proteins. (D) HD11 cells transfected with chTLR21 stained with the surface membrane tracker WGA-biotin and streptavidin Alexa Fluor 488 (green) and, after cell permeabilization, with anti-FLAG M2 antibody in combination with goat-anti-mouse-Alexa Fluor 568 (red). Cells were viewed in a Leica confocal microscope.*

quine completely inhibited ODN 1826- and ODN 2006-induced IL-1β and IL-8 responses in the chicken cells, consistent with localization of chTLR21 in the endolysosomal compartment (Fig. 8). Chloroquine also inhibited the enhanced transcription of IFNβ by the TLR3 ligand poly(I:C) but not the cytokine response to bacterial flagellin (Fig. 8), in agreement with endolysosomal and surface-localization of TLR3 and chTLR5, respectively (20). Together, the results classify chTLR21 as a unique intracellular chicken nucleotide receptor with a broad DNA ligand specificity.

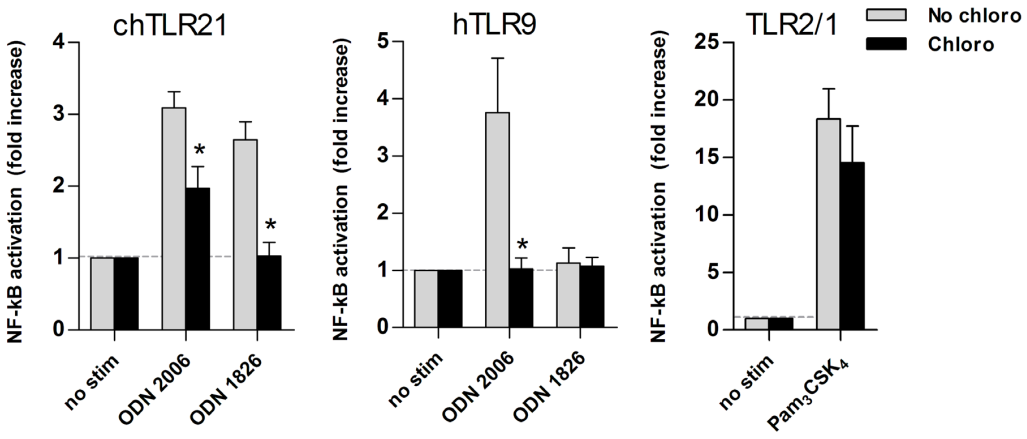

Figure 7. Effect of chloroquine on the chTLR21, hTLR9 and hTLR2/TLR1 response in HEK293 cells. *Cells were treated with 150 μM of the endosomal maturation inhibitor chloroquine (chloro) or control buffer (no chloro) for 30 min prior to stimulation (5 h) with the indicated TLR ligands. NF-κB luciferase activity was measured as relative light units (RLU) and expressed as fold increase compared to unstimulated transfected cells. Data represent the mean ± SEM of three experiments. Statistically lower NF-kB activatation in the presence of chloroquine is indicated with an asterisk. * p<0.05.*

Discussion

In the present study, we provide evidence that chicken TLR21 is an intracellular nucleotide receptor that senses synthetic CpG DNA and bacterial genomic DNA. The receptor shares many functional characteristics but displays minimal sequence similarity with mammalian TLR9. As the chicken genome lacks a TLR9 homologue, the results suggest that during evolution separate TLRs with seemingly similar functionality have emerged in birds and mammals.

ChTLR21 has hitherto been indicated as TLR protein based on shared sequence characteristics with members of the TLR receptor family (35, 38). The protein is annotated in the databases as hypothetical protein similar to TLR21. The successful cloning and expression in this study demonstrates that the gene encodes a ~109 kDa mature protein that is extensively modified with *N*-linked glycans. The protein is predicted to consist of an extracellular domain containing 27 LRRs, a single transmembrane domain, and an TIR domain which contains the conserved proline residue in the BB-loop implicated in MyD88-dependent signaling of human TLR4 (34), human TLR2 (39), and also chicken TLR5 (20). These traits together with the identification of CpG DNA as ligand and activator of NF-κB and pro-inflammatory gene transcription, strongly support the classification of chTLR21 as a member of the TLR family.

The identification of chTLR21 as endolysosomal DNA sensor is based on (i) the acquisition of cellular responsiveness to DNA after transfer and expression of chTLR21 in a heterologous (human) system, (ii) the loss of response after silencing of endogenous TLR21 but not TLR4 in chicken macrophages, and (iii) the susceptibility of the TLR21 response to the endosomal maturation inhibitor chloroquine. All these traits are shared with the known mammalian nucleotide receptors. One unique characteristic of chTLR21 is its broader CpG DNA ligand specificity compared to mammalian TLR9 as apparent from the response to both human- and murine-specific TLR9 ligand as well bacterial genomic DNA. The molecular basis of the species-specific DNA response of TLR9 still is still poorly understood. Human TLR9 binds DNA with little sequence specificity (22) and artificial targeting of a TLR9-TLR4 chimeric receptor to the cell surface relieves the species-specific TLR9 response (3, 16). Other reports indicate that the species-specific response co-transfers with TLR9 itself (4) and that binding of stimulatory but not inhibitory CpG DNA results in a conformational change in TLR9 and receptor activation (23).

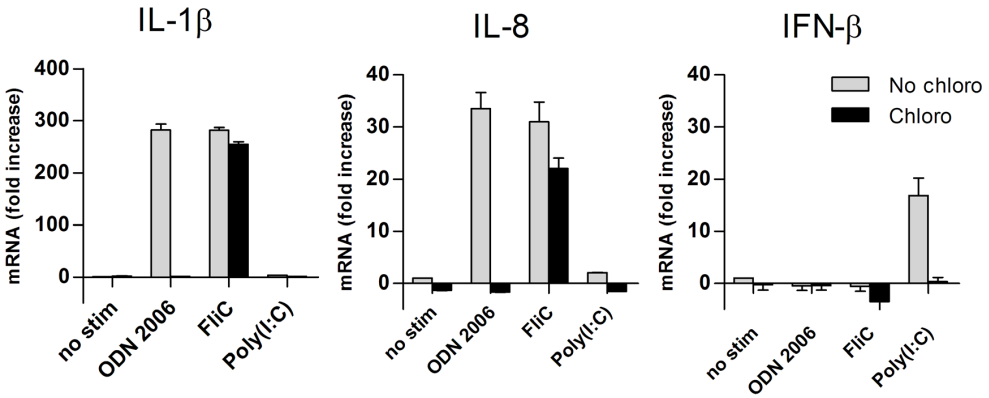

***Figure 8**. Effect of chloroquine on the DNA response of chicken macrophages. Cells were treated with 150 μM of the endosomal maturation inhibitor chloroquine (chloro) or control buffer (no chloro) for 30 min prior to stimulation (2 h) with the indicated TLR ligands. Cells were analyzed for IL-1β, IL-8, and IFNβ transcripts by real time RT-PCR. Data represent triplicate values from one of three representative assays with different batches of RNA, and expressed as fold increase in mRNA levels after treatment compared to control-treated cells.*

These data suggest that DNA binding and responsiveness are distinct events and that intrinsic structural differences between mammalian TLR9 contribute to the species-specific response to ODNs. Recent evidence shows that full length TLR9 (i.e. not the TLR chimeric protein) targeted to the cell surface using a yeast sorting sequence is unresponsive, and that the receptor requires a low pH (13) and protease activity (11) in the endolysosomes to gain functionality. As the uptake of CpG DNA via endocytosis is not DNA sequence specific (1), these results suggest that the physical properties of the (polyanionic) ligand at low pH (36), possible regulatory molecules (37), and/or intracellular processing of TLR9 (11, 32) are important for the species-specific response to CpG DNA (8). Our confocal microscopy results and the sensitivity of the chTLR21 response to the endosomal maturation inhibitor chloroquine, indicate that the receptor is mainly located in the ER and functional in endolysosomes, as reported for mammalian TLR9. This seems to exclude differences in pH-related differences in ligand affinity or receptor localization as a cause of the different ligand specificity of chTLR21 and hTLR9.

It has been proposed that proteolytic cleavage of TLR9 is important for receptor activation (11, 32). Although the exact cleavage site in TLR9 is unknown, it is believed to be

located in the extended loop between LRR15 and 16 (32). This insertion sequence appears missing in chTLR21 (Fig. 4). This may indicate that chTLR21 and TLR9 signaling are initiated by different mechanisms.

Comparative analysis of the protein sequence of chTLR21 and mammalian TLR9 indicates considerable additional differences in protein architecture. Apart from the absence of the ~30 amino acid linker between LRR15 and LRR16 typical for mammalian TLR7-9 and implicated in TLR9 function (11, 23, 32, 33), chTLR21 lacks the double CXXC motif that is present in LRR8 of murine and human TLR7-9 (Fig. 4) (5) and several unrelated DNA binding proteins (24). ChTLR21 also does not contain the extended irregular LRR2 and LRR5 of mammalian TLR9 as well as the K^{51} residue that all seem critical for TLR9 responsiveness (33). The assumed involvement of N-terminal LLR2, 5 and 8 in TLR9 receptor binding and activation (33) seems at variance with the scenario that the C-terminal fragment starting from LLR15 of TLR9 mediates ligand recognition (11, 32). The data may indicate that full length and cleaved mammalian TLR9 display different ligand binding sites. The fundamental structural differences in both N-terminal LRRs and the putative proteolytic cleavage region of mammalian TLR9 and chTLR21 suggest that the chTLR21 may have evolved yet another mechanism of DNA binding and receptor activation.

The identification of chTLR21 as a DNA receptor was unexpected from an evolutionary point of view. At the sequence level, chTLR21 is most similar to TLR21 of frog (*Xenopus tropicalis*) and fish (*Takifugu rubripes*) (Table II). These species however, also contain a TLR9 homologue in contrast to the chicken (18, 31). Whether this indicates the existence of redundant DNA receptors (TLR21 and TLR9) in these species awaits elucidation of the function of these receptors. Recently, TLR22 of fugu (*Takifugu rubripes*) was shown to respond to long-sized dsRNA, whereas fugu TLR3 recognized short-sized dsRNA (27). This indicates that fish have two RNA receptors that distinguish different RNA structures. The presence in different species of either TLR9 (mammals) or TLR21 (chicken), or both (fish), may indicate that during evolution chicken may have lost their TLR9, perhaps because of lack of a need to distinguish different DNA patterns. We previously demonstrated that chicken express a unique TLR16 that combines the ligand specificities of mammalian TLR1 and TLR6 in a single molecule (19) and that chTLR4 fails to activate the TRAM/TRIF signaling pathway (21). These data indicate different selection forces on the innate immune system of chicken and mammals during evolution.

CpG DNA activated NF-κB in chTLR21 transfected human cells and enhanced the transcription of a number of cytokines in HD11 chicken macrophages. CpG DNA has been successfully applied to stimulate the immune response in chicken and to provide protec-

tion against *Salmonella* infection (15, 25, 41). CpG DNA is also considered as an adjuvant in vaccines against e.g. avian influenza virus (9, 42). The present identification chTLR21 as innate DNA sensing receptor may provide for the first time a molecular basis for the adjuvant activity. The identification and expression of chTLR21 may be an important step in further rational optimization of the use of CpG DNA as an adjuvant in chicken.

Finally, we believe that the identification of chTLR21 as a DNA receptor with different structural and functional characteristics compared to mammalian TLR9 is of particular interest to better define the exact function of the different TLR receptor domains in the recognition of foreign (microbial) DNA as well as self-DNA. Comparison of the crystal structures of TLR9 and chTLR21 may shed light on how evolution succeeded in the development of distinct TLR receptors that display similar ligand recognition and function.

Footnotes

Abbreviations used in this paper: chTLR21, chicken TLR21; LRR, leucine rich repeat; TIR, Toll/IL-1R; *S*. Enteritidis, *Salmonella enterica* serovar Enteritidis; PNGase F, peptide-*N*-glycosidase F.

Acknowledgements

This work was supported in part by the Netherlands Organization for Health Research and Development (grant number Zon-MW 9120.6150).

References

1. **Ahmad-Nejad, P., H. Hacker, M. Rutz, S. Bauer, R. M. Vabulas, and H. Wagner.** 2002. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. European journal of immunology **32:**1958-1968.
2. **Akira, S., S. Uematsu, and O. Takeuchi.** 2006. Pathogen recognition and innate immunity. Cell **124:**783-801.
3. **Barton, G. M., J. C. Kagan, and R. Medzhitov.** 2006. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nature immunology **7:**49-56.
4. **Bauer, S., C. J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford.** 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proceedings of the National Academy of Sciences of the United States of America **98:**9237-9242.
5. **Bauer, S., and H. Wagner.** 2002. Bacterial CpG-DNA licenses TLR9. Current topics in microbiology and immunology **270:**145-154.
6. **Bell, J. K., G. E. Mullen, C. A. Leifer, A. Mazzoni, D. R. Davies, and D. M. Segal.** 2003. Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends in immunology **24:**528-533.
7. **Brinkmann, M. M., E. Spooner, K. Hoebe, B. Beutler, H. L. Ploegh, and Y. M. Kim.** 2007. The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling. The Journal of cell biology **177:**265-275.
8. **Chaturvedi, A., and S. K. Pierce.** 2009. How location governs toll-like receptor signaling. Traffic (Copenhagen, Denmark) **10:**621-628.
9. **Cooper, C. L., H. L. Davis, M. L. Morris, S. M. Efler, A. M. Krieg, Y. Li, C. Laframboise, M. J. Al Adhami, Y. Khaliq, I. Seguin, and D. W. Cameron.** 2004. Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine. Vaccine **22:**3136-3143.
10. **Dalpke, A., J. Frank, M. Peter, and K. Heeg.** 2006. Activation of toll-like receptor 9 by DNA from different bacterial species. Infection and immunity **74:**940-946.
11. **Ewald, S. E., B. L. Lee, L. Lau, K. E. Wickliffe, G. P. Shi, H. A. Chapman, and G. M. Barton.** 2008. The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor. Nature **456:**658-662.

12. **Gay, N. J., and M. Gangloff.** 2007. Structure and function of Toll receptors and their ligands. Annual review of biochemistry **76:**141-165.
13. **Gibbard, R. J., P. J. Morley, and N. J. Gay.** 2006. Conserved features in the extracellular domain of human toll-like receptor 8 are essential for pH-dependent signaling. The Journal of biological chemistry **281:**27503-27511.
14. **Häcker, H., H. Mischak, T. Miethke, S. Liptay, R. Schmid, T. Sparwasser, K. Heeg, G. B. Lipford, and H. Wagner.** 1998. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. The EMBO journal **17:**6230-6240.
15. **He, H., K. J. Genovese, C. L. Swaggerty, D. J. Nisbet, and M. H. Kogut.** 2007. *In vivo* priming heterophil innate immune functions and increasing resistance to Salmonella enteritidis infection in neonatal chickens by immune stimulatory CpG oligodeoxynucleotides. Veterinary immunology and immunopathology **117:**275-283.
16. **Heeg, K., A. Dalpke, M. Peter, and S. Zimmermann.** 2008. Structural requirements for uptake and recognition of CpG oligonucleotides. Int J Med Microbiol **298:**33-38.
17. **Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira.** 2000. A Toll-like receptor recognizes bacterial DNA. Nature **408:**740-745.
18. **Ishii, A., M. Kawasaki, M. Matsumoto, S. Tochinai, and T. Seya.** 2007. Phylogenetic and expression analysis of amphibian Xenopus Toll-like receptors. Immunogenetics **59:**281-293.
19. **Keestra, A. M., M. R. de Zoete, R. A. van Aubel, and J. P. van Putten.** 2007. The central leucine-rich repeat region of chicken TLR16 dictates unique ligand specificity and species-specific interaction with TLR2. J Immunol **178:**7110-7119.
20. **Keestra, A. M., M. R. de Zoete, R. A. van Aubel, and J. P. van Putten.** 2008. Functional characterization of chicken TLR5 reveals species-specific recognition of flagellin. Molecular immunology **45:**1298-1307.
21. **Keestra, A. M., and J. P. van Putten.** 2008. Unique properties of the chicken TLR4/MD-2 complex: selective lipopolysaccharide activation of the MyD88-dependent pathway. J Immunol **181:**4354-4362.
22. **Latz, E., A. Schoenemeyer, A. Visintin, K. A. Fitzgerald, B. G. Monks, C. F. Knetter, E. Lien, N. J. Nilsen, T. Espevik, and D. T. Golenbock.** 2004. TLR9 sig-

nals after translocating from the ER to CpG DNA in the lysosome. Nature immunology **5:**190-198.

23. **Latz, E., A. Verma, A. Visintin, M. Gong, C. M. Sirois, D. C. Klein, B. G. Monks, C. J. McKnight, M. S. Lamphier, W. P. Duprex, T. Espevik, and D. T. Golenbock.** 2007. Ligand-induced conformational changes allosterically activate Toll-like receptor 9. Nature immunology **8:**772-779.
24. **Lee, J. H., K. S. Voo, and D. G. Skalnik.** 2001. Identification and characterization of the DNA binding domain of CpG-binding protein. The Journal of biological chemistry **276:**44669-44676.
25. **Mackinnon, K. M., H. He, C. L. Swaggerty, J. L. McReynolds, K. J. Genovese, S. E. Duke, J. R. Nerren, and M. H. Kogut.** 2009. *In ovo* treatment with CpG oligodeoxynucleotides decreases colonization of *Salmonella enteriditis* in broiler chickens. Veterinary immunology and immunopathology **127:**371-375.
26. **Matsumoto, F., S. Saitoh, R. Fukui, T. Kobayashi, N. Tanimura, K. Konno, Y. Kusumoto, S. Akashi-Takamura, and K. Miyake.** 2008. Cathepsins are required for Toll-like receptor 9 responses. Biochemical and biophysical research communications **367:**693-699.
27. **Matsuo, A., H. Oshiumi, T. Tsujita, H. Mitani, H. Kasai, M. Yoshimizu, M. Matsumoto, and T. Seya.** 2008. Teleost TLR22 recognizes RNA duplex to induce IFN and protect cells from birnaviruses. J Immunol **181:**3474-3485.
28. **Matsushima, N., T. Tanaka, P. Enkhbayar, T. Mikami, M. Taga, K. Yamada, and Y. Kuroki.** 2007. Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors. BMC genomics **8:**124.
29. **Nishiya, T., and A. L. DeFranco.** 2004. Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors. The Journal of biological chemistry **279:**19008-19017.
30. **O'Neill, L. A.** 2008. The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. Immunological reviews **226:**10-18.
31. **Oshiumi, H., T. Tsujita, K. Shida, M. Matsumoto, K. Ikeo, and T. Seya.** 2003. Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome. Immunogenetics **54:**791-800.
32. **Park, B., M. M. Brinkmann, E. Spooner, C. C. Lee, Y. M. Kim, and H. L. Ploegh.** 2008. Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. Nature immunology **9:**1407-1414.

33. **Peter, M. E., A. V. Kubarenko, A. N. Weber, and A. H. Dalpke.** 2009. Identification of an N-terminal recognition site in TLR9 that contributes to CpG-DNA-mediated receptor activation. J Immunol **182:**7690-7697.

34. **Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler.** 1998. Defective LPS signaling in C3H/HeJ and C57BL /10ScCr mice: mutations in Tlr4 gene. Science (New York, N.Y **282:**2085-2088.

35. **Roach, J. C., G. Glusman, L. Rowen, A. Kaur, M. K. Purcell, K. D. Smith, L. E. Hood, and A. Aderem.** 2005. The evolution of vertebrate Toll-like receptors. Proceedings of the National Academy of Sciences of the United States of America **102:**9577-9582.

36. **Rutz, M., J. Metzger, T. Gellert, P. Luppa, G. B. Lipford, H. Wagner, and S. Bauer.** 2004. Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. European journal of immunology **34:**2541-2550.

37. **Saitoh, S., and K. Miyake.** 2009. Regulatory molecules required for nucleotide-sensing Toll-like receptors. Immunological reviews **227:**32-43.

38. **Temperley, N. D., S. Berlin, I. R. Paton, D. K. Griffin, and D. W. Burt.** 2008. Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. BMC genomics **9:**62.

39. **Underhill, D. M., A. Ozinsky, A. M. Hajjar, A. Stevens, C. B. Wilson, M. Bassetti, and A. Aderem.** 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature **401:**811-815.

40. **van Aubel, R. A., A. M. Keestra, D. J. Krooshoop, W. van Eden, and J. P. van Putten.** 2007. Ligand-induced differential cross-regulation of Toll-like receptors 2, 4 and 5 in intestinal epithelial cells. Molecular immunology **44:**3702-3714.

41. **Vleugels, B., C. Ververken, and B. M. Goddeeris.** 2002. Stimulatory effect of CpG sequences on humoral response in chickens. Poultry science **81:**1317-1321.

42. **Wang, Y., C. Shan, S. Ming, Y. Liu, Y. Du, and G. Jiang.** 2009. Immunoadjuvant effects of bacterial genomic DNA and CpG oligodeoxynucleotides on avian influenza virus subtype H5N1 inactivated oil emulsion vaccine in chicken. Research in veterinary science **86:**399-405.

43. **Wei, T., J. Gong, F. Jamitzky, W. M. Heckl, R. W. Stark, and S. C. Rossle.** 2009. Homology modeling of human Toll-like receptors TLR7, 8, and 9 ligand-binding domains. Protein Sci **18:**1684-1691.

44. **Xu, Y., X. Tao, B. Shen, T. Horng, R. Medzhitov, J. L. Manley, and L. Tong.** 2000. Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. Nature **408:**111-115.

45. **Yarovinsky, F., D. Zhang, J. F. Andersen, G. L. Bannenberg, C. N. Serhan, M. S. Hayden, S. Hieny, F. S. Sutterwala, R. A. Flavell, S. Ghosh, and A. Sher.** 2005. TLR11 activation of dendritic cells by a protozoan profilin-like protein. Science **308:**1626-1629.

3

Cleavage and activation of a Toll-like receptor by fungal proteases

Marcel R. de Zoete
Lieneke I. Bouwman
Jos P. M. van Putten

Manuscript in preparation.

Abstract

Toll-like receptors (TLRs) are eukaryotic immune receptors that detect the presence of infection by sensing structurally conserved pathogen-associated molecular patterns (PAMPs). While the microbial ligands for the majority of the mammalian TLRs have been identified, several non-mammalian TLRs are still orphan receptors. In birds, TLR15 is the only remaining TLR with unknown ligand specificity. Here, we identify secreted fungal proteases as the activating ligand for the TLR15. In a mechanism different from other TLR activation, TLR15 is proteolytically cleaved at the cell surface, resulting in receptor activation and innate immune responses. Activation and cleavage by fungal proteases is specific for TLR15. The presence of an immune sensor for extracellular proteolytic activity provides birds with a unique mechanism to selectively respond to organisms that exploit secreted proteases as virulence factors.

Introduction

The ability to sense the presence of infectious microbes is crucial for survival in all animals. For this reason, both mammalian and non-mammalian species have developed several highly conserved immunological detection systems that recognize common microbial molecules and initiate rapid defensive immune responses (25, 31). The pattern recognition receptor proteins that form the basis of these innate systems have been the subject of extensive research in the last decade. In mammals, several major families of innate immune receptors are well described: the Toll-like receptors (TLRs), the Nod-like receptors (NLRs), the RIG-like helicase (RLH) receptors, and the Lectin receptors (LRs) (8, 12). Combined, these receptors are able to sense the vast majority of both intra- and extracellular bacteria, viruses, parasites and fungi.

TLRs are the most well-studied group of innate immune receptors. TLRs are membrane proteins that operate either on the cell surface (e.g. TLR2/1/6, TLR4 and TLR5, which sense lipoproteins, lipopolysaccharide and bacterial flagellin, respectively) or within endolysosomes (TLR3, TLR7, TLR8 and TLR9, sensing varies types of DNA and RNA) (2, 22, 41). The evolutionary conservation of TLRs is very high amongst different species; for instance, although birds have diverted from mammals around 310 million years ago (1), most of the 10 human TLRs have functional homologues in chickens (19-21, 34, 40). At the same time, several TLRs are found only in specific groups of animals and have yet unknown ligand specificity. Identification of these ligands can provide important information about immunological evolution and function of the innate immune system, and may lead to the development of novel therapeutic agents.

TLRs consist of a leucine-rich repeat domain that serves as the ligand sensing domain, a single transmembrane domain, and a signaling TIR domain. Numerous functional and crystallization studies have clarified the overall basics of the receptor-activation mechanism (13, 14, 17, 26, 33). While structurally diverse, all TLR ligands appear to form a bridge between two TLR ligand sensing domains. For instance, the acyl chains of one molecule of the lipopeptide Pam_3CSK_4 binds TLR2 and TLR1 at the same time (13), two TLR3 receptors bind the same stretch of dsRNA (26), and LPS connects two TLR4/MD2 protein complexes (33). Ligand-binding induces receptor dimerization (generally as homodimers or, in the case of TLR2/1/6 as heterodimers), which enables the intracellular TIR domains to recruit the adaptor proteins MyD88, Mal, TRIF and/or TRAM. Subsequent downstream signaling leads to the activation and translocation of the transcription factors such as NF-kB and IRF3 to the nucleus, where they initiate the pro-

duction of cytokines and other immune mediators (18). Recent studies have added an additional layer of complexity to the TLR-activation mechanism, as TLR9 and TLR7 were shown to require proteolytic cleavage in lysosomes in a multistep process of activation (7, 32, 36). The exact mechanism by which TLR9/7 cleavage influences DNA binding and/or initiation of the signaling cascade remains to be elucidated.

Fungal infections pose serious health threats throughout the entire animal kingdom. The importance of efficient innate immune recognition in the defense against fungi is clear; in *Drosophila melanogaster*, Toll knockouts succumb rapidly by infectious fungi (24), while dectin-1 knockout mice, which are unable to induce immune responses after fungal β-glucan recognition, are more susceptible to fungal infections (35, 39). In the present study, we identify secreted fungal proteases as the ligand for the surface localized chicken TLR15. Through cleavage of the TLR15 extracellular domain, secreted fungal proteases activate TLR15 and induce an inflammatory response. Identification of this unique mechanism of TLR activation adds a new dimension to the existing knowledge on TLR recognition and function.

Materials and Methods

Cell lines and chemicals

HeLa 57A and HEK293 were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS); COS-7 cells were maintained in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 10% FCS; chicken DF-1 and HD11 cells were maintained in DMEM supplemented with MEM non-essential amino acids and 10% FCS. PMSF, chloroquine, and proteases (pronase, pancreatic porcine elastase, collagenase, trypsin, chymotrypsin, thrombin, and papain) were purchased from Sigma-Aldrich; TLR ligands were purchased from Invivogen; proteinase K was purchased from Roche; PNGase F was purchased from New England Biolabs.

Plasmid constructs

For the construction of N-terminal flag-tagged TLR15 (TLR15-flag-N), the TLR15 open reading frame was amplified by PCR using *pfu* polymerase (Promega) from HD11 chromosomal DNA using primers 5'-CCGAATTCATTCCTAACTCAGAGAACATCTCC-3' (forward) and 5'- GGTCTAGATTCCATCTCAATTACATCCTC-3' (reverse), and cloned into p3Xflag-CMV23-c-Myc (Sigma-Aldrich) with the restriction enzymes EcoRI and XbaI. For

the construction of C-terminal flag-tagged TLR15 (TLR15-flag-C), TLR15 was amplified from HD11 chromosomal DNA using primers 5'-CCGAATTCGCCACCATGGGGATCCTTA TTGGGAGTC-3' (forward) and 5'-GGGCGGCCGCCTTCCATCTCAATTACATCCTC-3' (reverse), and cloned into p3XTracer (de Zoete er al, Chapter 2) with restriction enzymes EcoRI and NotI. C-terminal flag-tagged chTLR5 (chTLR5-flag-C) was amplified from pTracer-chTLR5 (20) with primers 5'-CCGGATCCGCCACCATGGTACATCAACGGCTAAT AATTG-3' (forward) and 5'-GGGCGGCCGCCTTCCATCTCAATTACATCCTC-3' (reverse), and cloned into p3XTracer with restriction enzymes BamHI and NotI. chTLR21, hTLR9, hTLR1, hTLR6, hTLR2, chTLR2t2, chTLR2t2, chTLR16, chTLR5 and hTLR5 were constructed previously (19-21) (de Zoete et al, Chapter 2).

Deglycosylation of TLR15

N-terminal Flag-tagged TLR15 plasmid DNA (500 ng) was transfected into COS-7 cells in 12-well plates using FuGENE 6 at a DNA:lipid ratio of 1:2. After 48 h, cells were washed 3 times with Dulbecco's Phosphate Buffered Saline (DPBS) and lysed using Reporter Lysis Buffer (RLB, Promega) at -80°C. Protein lysates were deglycosylated using PNGase F (New England Biolabs) according to the manufacturer's protocol and analysed by immunoblotting using M2-α-Flag (Sigma-Aldrich).

Stimulation assays

For stimulation assays, HeLa 57A and HEK293 cells were transfected in 48-well plates with 125 ng receptor plasmid DNA and 125 ng LacZ plasmid DNA (HeLa 57A), or 125 ng receptor plasmid DNA, 62.5 ng LacZ plasmid DNA, and 62.5 ng NF-κB plasmid DNA (HEK293) using FuGENE 6 (Roche), at a DNA:lipid ratio of 1:3. After 48 h, cells were washed 3 times with DMEM and stimulated with the appropriate amount of ligand. After 5 of stimulation, cells were washed twice with DPBS and lysed using RLB at -80°C. Luciferase activity in the lysates was measured in a Luminometer (TD-20-20, Turner Designs) using Luciferase Reagent (Promega). Experiments were performed at least three times independently. Cecal content was collected from free ranging chickens, diluted 1:10 in DPBS and filtered; fungal supernatants were prepared by overnight incubation of maltose-cultivated fungi in DMEM at 37°C, and subsequently filter sterilized.

Detection of TLR15 cleavage

For detection of TLR15 cleavage, COS-7 and DF-1 cells were transfected in 12-well plates with 2 μg of receptor plasmid DNA using FuGENE 6 at a DNA:lipid ratio of 1:2 (COS-7)

and 1:3 (DF-1). After 42 h, cells were washed 3 times with IMDM (COS-7) or DMEM (DF-1) and incubated for 2 h with proteases. Cells were subsequently lysed in RLB and subjected to immunoblot analysis. TLRs were detected using M2-α-Flag antibody.

Confocal laser microscopy

Cells were grown in 24-well plates on glass coverslips and transfected with the appropriate C-terminal flag-tagged TLR. After 48 h, cells were prepared for confocal laser microscopy as described previously (19). Briefly, cells were washed with DPBS and incubated with WGA-biotin for 10 min at 37ºC. After washing with DPBS, cells were fixed with DPBS/2% paraformaldehyde for 30 min and permeabilized with 0.2% Triton-X-100 for 15 min. Following blocking with DPBS/2% BSA (60 min), cells were incubated with M2-α-Flag antibody (60 min), washed with DPBS, and incubated with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) and Alexa Fluor 568 Streptavidin (Invitrogen) for 60 min. Cells were subsequently embedded in FluorSave (Calbiochem) and viewed in a Leica TCA SP confocal laser-scanning microscope.

Homology modeling

The amino acid sequence of the TLR15 ectodomain and TIR domain were modeled using the automative ESyPred3D Web Server 1.0 on the ectodomain of human TLR3 (PDB: 2A0Z), and the TIR domain of human TLR1 (PDB: 1FYV), respectively.

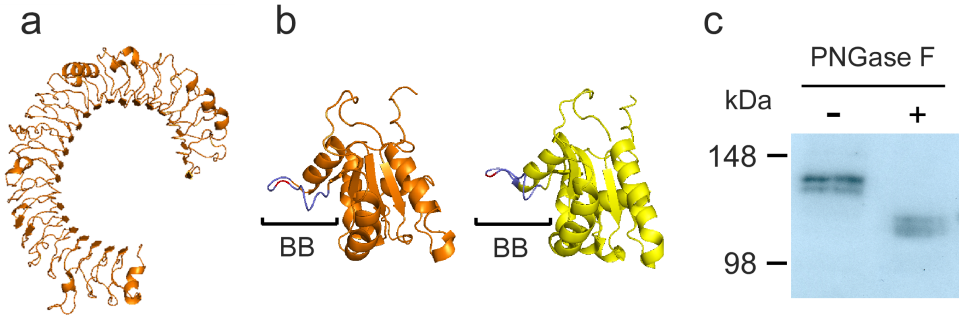

Figure 1. TLR15 is a glycoprotein. *(A) Computational modeling of the extracellular domain of TLR15 predicts a horseshoe-shape structure containing 23 leucine-rich repeats (LRR). (B) Predicted structure of the TLR15 TIR domain (orange), modeled on the human TLR1 TIR domain (yellow). The conserved signaling BB loop (BB) and proline residue are indicated in blue and red, respectively. (C) TLR15 is expressed as two high molecular mass glycoproteins. Flag-tagged TLR15 was expressed in COS-7 cells, deglycosylated using PNGase F, and detected by immunoblotting with M2-α-FLAG.*

Results

TLR15 is a glycoprotein with a typical TLR architecture.

Sequence analysis of TLR15 revealed the typical TLR make-up, comprising a signal sequence, an N-terminal leucine-rich repeat domain, a transmembrane domain, and a TIR domain. Comparative modeling of the LRR domain of TLR15 revealed a horseshoe-shaped form (consisting of 23 leucine-rich repeats) typical for TLR (Fig. 1A). Modeling of the TLR15 TIR domain revealed the presence of conserved surface patch named the BB-loop required for signaling in other TLRs, and the presence of a conserved proline residue, shown to be required for MyD88-dependent signaling in human TLRs (Fig. 1B). In order to investigate its biological function, flag-tagged TLR15 was cloned and expressed in human COS-7 cells. This resulted in the expression of TLR15 that migrated in SDS-PAGE as a protein doublet with apparent molecular masses of ~120 kDa and ~130 kDa, respectively (Fig. 1C). Deglycosylation of the cell lysates with PNGase F decreased the electrophoretic mobility of both TLR15 forms (Fig. 1C), indicating that TLR15 is decorated with N-glycans.

TLR15 is activated by secreted fungal proteases.

Although no sequence homology between TLR15 and other TLRs was apparent, all known TLR ligand (LPS, di- and tri-acylated lipopeptides, zymosan, flagellin, TLR7/8 ligand, CpG DNA and profillin-like protein) were tested for their ability to activate TLR15 expressed in HeLa 57A cells. In addition, as several TLR form heterodimers with other TLRs, cells expressing combinations of TLR15 with chicken TLR2t1 and TLR2t2, TLR16, TLR4 and TLR5 were stimulated with all known TLR ligands. Both sets of experiments did not result in TLR15-mediated NF-κB activation, suggesting that the ligand for TLR15 differs from the known TLR activators (data not shown). Previous reports indicated that TLR15 is expressed in the chicken cecum(10, 37). In search for the ligand of TLR15, we therefore tested freshly isolated diluted chicken cecal content for TLR15-stimulating activity in transfected HeLa 57A cells. This caused potent TLR15-dependent NF-kB activation (Fig. 2A). Heat-treatment (100°C) of the active material abolished TLR15 activation. These results identify a heat-sensitive ligand present in cecal content as ligand for TLR15.

One of the organisms recovered and isolated from the cecum was typed as the yeast *Candida guilliermondii*. Sterile supernatant of the isolated pure yeast culture, grown overnight in DMEM medium at 37°C, activated TLR15 in a similar manner as the cecal

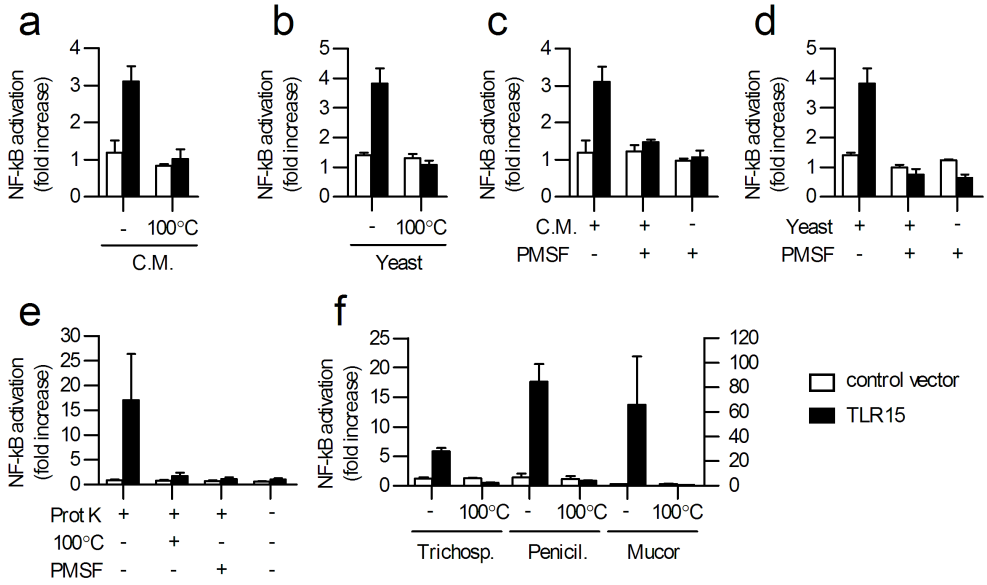

Figure 2. TLR15 is activated by fungal proteases. *NF-κB activation of TLR15- or control-transfected HeLa 57A stimulated for 5 h with (A) cecal matter (CM) or (B) yeast supernatant. Heat-inactivation (100ºC, 10 min) of CM and yeast supernatant abolished TLR15-mediated NF-κB activation. (C) FM- or (D) yeast supernatant-induced TLR15-mediated NF-κB activation in HeLa 57A cells is inhibited by PMSF (1 mM) treatment. (E) Proteinase K (10 ng ml^{-1}, 5 h) specifically activated TLR15- but not control-transfected HeLa 57A cells in a heat- and PMSF treatment dependent manner. (F) TLR15-mediated NF-κB activation in HeLa 57A cells induced by culture supernatant of Trichosporon spp., Aspergillus spp. (left Y-axis), and Mucor spp. (right Y-axis), which is abolished by heat-treatment (100ºC, 10 min). Data are presented as mean ± SEM of stimulated compared to unstimulated cells from three independent experiments.*

content. Again, this activity was destroyed by heat-treatment (Fig. 2B). Fungi are notorious for secreting high levels of (heat-labile) proteases as virulence factors into the environment. To test the involvement of secreted fungal proteases in the activation of TLR15, the protease inhibitor PMSF was added to the cecal content and the fungal supernatant (Fig. 2C and D). In both cases, TLR15 activation was reduced to background levels, indicating a crucial role for proteases in the activation of the receptor. Control experiments confirmed that NF-κB activation by other human and chicken TLRs is not affected by the addition of PMSF. To corroborate our findings, we stimulated TLR15-expressing cells with the recombinant purified fungal protease proteinase K. The enzyme activated NF-κB in a TLR15-dependent fashion. The activation was abolished by heat and PMSF

treatment of the proteinase K (Fig. 2E). Sterile culture supernatants of three other fungi (*Trichosporon* spp, *Penicillium* spp., and *Mucor* spp.) were similarly able to specifically activate TLR15 in a heat-dependent manner (Fig. 2F). Together, these data indicate that TLR15 is activated by fungal proteases.

Specificity of protease-dependent activation of TLR15.

To investigate whether protease-dependent activation is common for TLRs or specific for TLR15, we expressed human TLR2/TLR1, TLR2/TLR6, TLR4, TLR5 and TLR10, and chicken TLR2t2/TLR16, TLR4, TLR5 and TLR21 in HeLa 57A cells and stimulated the cells with either proteinase K or the appropriate TLR ligand. Whereas all TLRs activated NF-κB after stimulation with their own ligand (with the exception of TLR10, of which the ligand is still unknown), only TLR15 was activated (~40-fold) by the addition of protease (Fig. 3A). As human TLR9 is not functional in the HeLa 57A cell line, HEK293 cells were transfected with TLR15 and TLR9. As observed for the other TLRs, TLR9 was successfully activated by the CpG DNA ligand ODN 2006 but not by proteinase K (Fig. 3B). The results show that the protease-dependent activation is specific for TLR15.

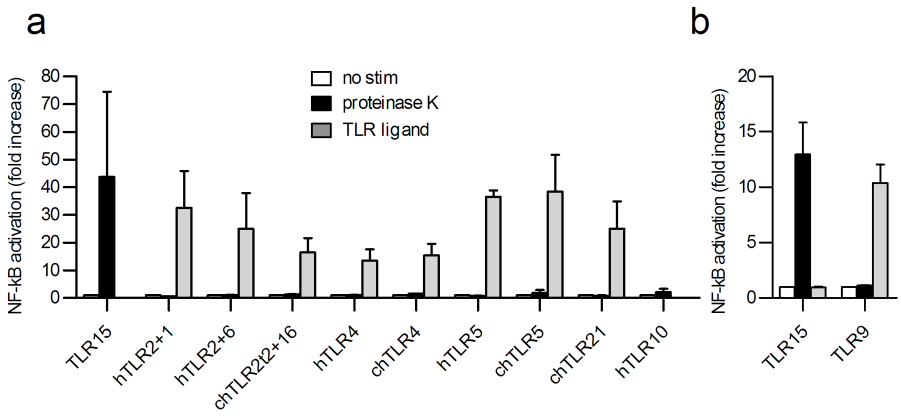

Figure 3. Protease-induced activation is specific for TLR15. *NF-κB activation in (A) HeLa 57A or (B) HEK293 cells transfected with (combinations of) human or chicken TLRs, and stimulated with 10 ng ml^{-1} proteinase K, or with 100 ng ml^{-1} Pam$_3$CSK$_4$ (hTLR1+2, chTLR2t2+16), 100 ng ml^{-1} FSL-1 (hTLR2+6), 100 ng ml^{-1} LPS (hTLR4, chTLR4), 1 μg ml^{-1} flagellin (hTLR5, chTLR5), or 0.5 μM ODN2006 (chTLR21, hTLR9) for 5 h. hTLR10 was stimulated with 10 ng ml^{-1} proteinase K only. Data are presented as mean ± SEM of stimulated compared to unstimulated cells from three independent experiments.*

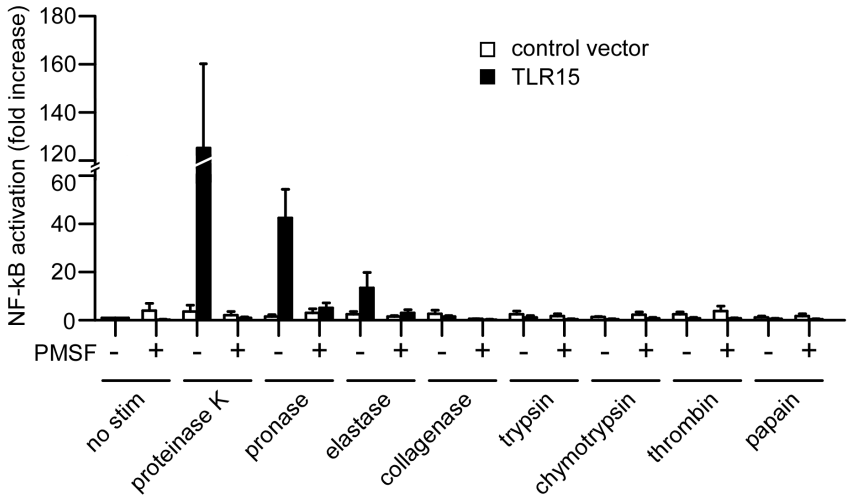

Figure 4. *TLR15 is activated exclusively by non-specific proteases. NF-κB activation of TLR15- or control-transfected HeLa 57A cells stimulated (3 h) with 25 ng ml^{-1} proteinase K, 25 ng ml^{-1} pronase, 25 ng ml^{-1} bovine elastase, 25 ng ml^{-1} collagenase, 25 ng ml^{-1} trypsin, 25 ng ml^{-1} chymotrypsin, 25 ng ml^{-1} thrombin, or 25 ng ml^{-1} papain. Proteases were inhibited by PMSF (1 mM) treatment. Data are presented as mean ± SEM of stimulated compared to unstimulated cells from three independent experiments.*

To determine which type/class of proteases is able to activate TLR15, a panel of 7 proteases with variable substrate specificity was selected (Fig. 4). When HeLa 57A cells transfected with TLR15 were stimulated with 25 ng ml^{-1} proteinase K or pronase, a strong increase in NF-kB activation was observed compared to unstimulated cells, which was absent in HeLa 57A cells transfected with the control vector (Fig. 4). When TLR15 was stimulated with 25 ng ml^{-1} porcine elastase, moderate but significant activation was observed (~15-fold increase). Serine proteases with more strict substrate specificity (collagenase, trypsin, chymotrypsin, and thrombin) and the cysteine protease papain did not activate TLR15. These results indicate that TLR15 is activated exclusively by proteases with low substrate specificity.

TLR15 is localized at the cell surface.

Mammalian TLR9 undergoes a series of proteolytic cleavage steps by cysteine endopeptidases in order to form a functional receptor (7, 32, 36). Proteolytic cleavage occurs in

the endolysosomes, where TLR9 binds and responds to DNA. To investigate a possible similarity between the mechanism of activation of TLR15 and TLR9, we first compared the localization of the receptors. HeLa 57A transfected with flag-tagged TLR15 showed clear co-localization of the receptor with the cell surface marker wheat germ agglutinin (WGA) (Fig. 5A). Similar TLR15 surface localization was found in human HEK293 cells (data not shown) and chicken DF-1 cells (Fig. 5C). Control experiments using TLR9-transfected HeLa 57A cells (Fig. 5B), HEK293 cells (data not shown), and chicken DF-1 cells (Fig. 5D), showed strong staining but failed to detect this receptor at the cell surface, consistent with its well-documented intracellular localization(23).

Chloroquine can block TLR9 activation by inhibiting lysosomal acidification. To determine the sensitivity of TLR15 for chloroquine, HEK293 cells transfected with either TLR15, TLR9, or control vector were stimulated in the presence or absent of the drug. Pretreatment of the cells with chloroquine completely abolished TLR9 activation by ODN 2006 (Fig. 5E). In contrast, chloroquine did not inhibit TLR15 activation by proteases. Together, these results show that, while proteases are required for the activation of both receptors, the localization and mechanism of activation TLR15 and TLR9 are different.

TLR15 is proteolytically cleaved by activating proteases.

Protease may activate TLR15 by the release of cellular factors and/or cleavage of the receptor. To investigate whether released cellular factors were involved in the activation of TLR15, we performed transfer experiments in which the cell supernatant of protease-treated cells (transfected with either TLR15 or control vector), after inhibition of the protease with PMSF, was added to non-stimulated TLR15 transfected cells. Transfer of protease activity-free supernatant never resulted in TLR15-dependent NF-κB activation (data not shown).

To test whether proteases directly targeted TLR15, COS-7 cells were transfected with TLR15 with a C-terminal flag-tag and incubated with proteinase K in the absence and presence of PMSF. Immunoblotting of the cell lysates revealed only full-length TLR15 for the untreated or proteinase K/PMSF treated cells. Incubation with active protease however, resulted in the appearance of a ~70 kD C-terminal TLR15 cleavage product, which increased in intensity at prolonged exposure to the protease (Fig. 6A). The appearance of the novel band coincided with disappearance of the upper band of the TLR15 doublet, suggesting that only this band was protease sensitive and at the cell surface.

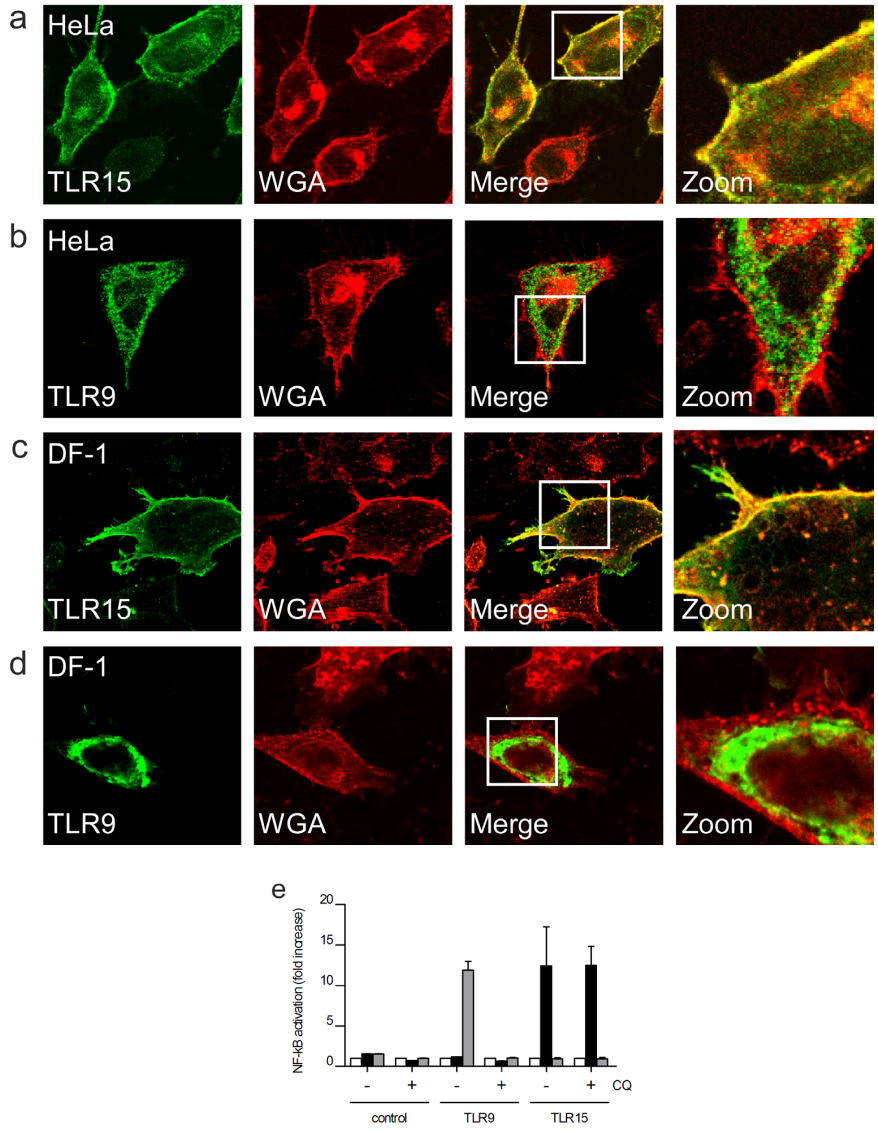

Figure 5. *TLR15 is localized at the cell surface and is insensitive to chloroquine. Confocal microscopy of HeLa 57A and chicken DF-1 cells transfected with flag-tagged TLR15 or TLR9. Both in (A) HeLa 57A and (C) DF-1 cells, TLR15 (green) co-localizes with the cell surface marker wheat germ agglutinin (WGA, red). (B, D) No co-localization of TLR9 (green) with WGA is observed. (E) NF-κB activation of TLR15-, TLR9-, or control-transfected HEK293 cells left untreated (white bars), stimulated with 20 ng ml^{-1} proteinase K (black bars), or with 0.5 μM ODN 2006 (grey bars) for 5 h. (+) Cells were preincubated with chloroquine (CQ) or buffer for 30 min prior to stimulation. Data are presented as mean ± SEM of stimulated compared to unstimulated cells from three independent experiments.*

Treatment of chicken DF-1 cells expressing TLR15 yielded similar results (Fig. 6B). All other (control) TLRs treated with identical amount of proteinase K yielded no cleavage products (Fig. 6C). Finally, only proteases that were shown to activate TLR15 (Fig. 4) gave rise to the specific ~70 kD protein product (Fig. 6D). These results show that activation of TLR15 by proteases is accompanied by the formation of a ~70 kD cleavage product of the receptor.

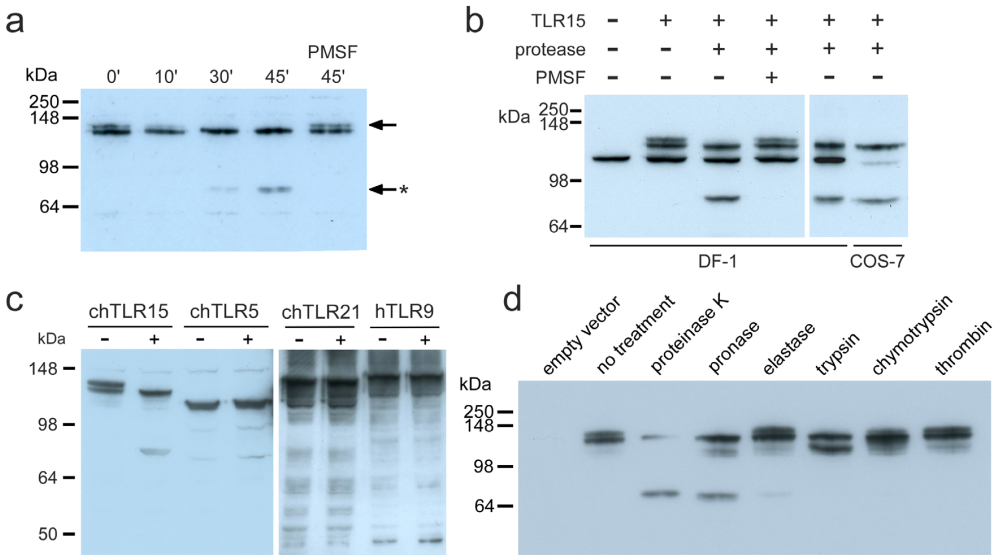

Figure 6. Proteolytic cleavage of TLR15. *(A) Immunoblot analysis of COS-7 cells expressing C-terminal flag-tagged TLR15 treated with 100 ng ml^{-1} proteinase K for the indicated times. The upper band of the TLR15 doublet (arrow) is proteolytically cleaved to a C-terminal ~70 kDa truncated receptor (arrow with asterisk). Cleavage does not occur after inactivation of the protease by PMSF (1 mM). TLR15 was detected using M2-α-Flag. (B) Proteinase K treatment of flag-tagged TLR15 expressed in chicken DF-1 resulted in an identical C-terminal receptor cleavage fragment. (C) COS-7 cells expressing chTLR15, chTLR5, chTLR21, or hTLR9 were stimulated with 100 ng ml^{-1} proteinase K for 45 min and immunoblotted. C-terminal flag-tags were detected using M2-α-Flag. (D) Immunoblot analysis of TLR15 cleavage in COS-7 stimulated with a panel of proteases (100 ng ml^{-1}) for 45 min. C-terminal flag-tagged TLR15 was detected using M2-α-Flag.*

Proteinase K induces an inflammatory response in chicken macrophages.
As the previous results were obtained with recombinant TLR15 expressed in human cells, we investigated the function of endogenous TLR15 in the anti-fungal immune response in chicken cells. Hereto, the mRNA transcripts for inducible nitric oxide synthase (iNOS) were determined in non-transfected HD11 chicken macrophages that endogenously express TLR15. Following treatment with low concentrations of proteinase K, high levels of iNOS mRNA were induced (Fig. 7). Chicken macrophages stimulated with PMSF-inactivated protease or PMSF alone did not show this increase in mRNA levels. These result indicate that, as observed with TLR15-transfected mammalian cells, HD11 macrophages endogenously expressing TLR15 respond to protease with an inflammatory response.

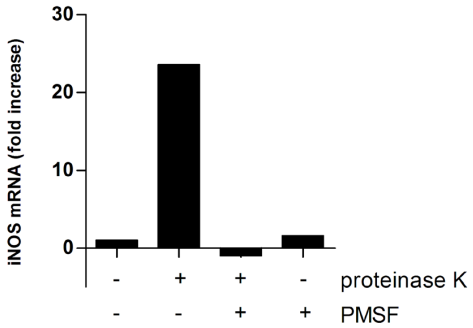

Figure 7. *Proteinase K initiates an inflammatory response in chicken macrophages. Chicken HD11 cells were stimulated for 2 h with control buffer, proteinase K (20 ng ml^{-1}), PMSF (1 mM)-inactivated proteinase K (20 ng ml^{-1}), or PMSF (1 mM). Cells were analyzed for inducible nitric oxide synthase (iNOS) transcripts by real time RT-PCR. Data represent fold increase in mRNA levels after treatment compared to control-treated cells.*

Discussion

The discovery that chicken TLR15 is activated through cleavage of secreted fungal proteases presents a novel innate immune defense strategy and a unique mechanism of TLR activation. As secreted proteases are widely present as virulence factors of numerous fungal, parasitic and bacterial pathogens, the detection of proteolytic activity may enable birds to selectively respond to organisms that induce cellular proteolytic damage. Our results demonstrate that chicken TLR15 is selectively activated through the enzymatic activity of fungi-derived proteases. We excluded contaminating agents like LPS, zymosan, and DNA as potential TLR15 ligands through several methods: i) proteases from several different microbial and non-microbial sources were able to activate the receptor,

ii) TLR15 activation was specifically inhibited after inactivation of the proteases, iii) a series of highly sensitive TLRs, including TLR2, TLR4 and TLR9, could not be activated by the purified TLR15-activating protease, and iv), a high dose of purified microbial TLR ligands did not activate TLR15. Recent advances in innate immunity have revealed an immune-stimulating function for damage-associated molecular patterns (DAMPs) like HMGB1, heat shock proteins, and uric acid, which are released by stressed or damaged host cells (4, 15). A**s** the culture supernatant of protease-stimulated cells did not gain the ability, after protease inhibition with PMSF, to activate TLR15, DAMPs were eliminated as potential TLR15 ligands.

Immune activation through TLR15 is accompanied by cleavage of the ligand sensing domain. Both activation and cleavage can be completely blocked by inactivation of the stimulating protease. Immunoblotting of cleaved TLR15 indicates that only the upper (~130 kDa) band of the TLR15 protein doublet is cleaved, presumably representing the surface localized, protease-accessible TLR15 fraction. Cleavage leads to the generation of a C-terminal TLR15 fragment of ~70 kDa. Further deglycosylation of the truncated receptor predicts a final molecular mass of ~63 kDa (data not shown), pin-pointing the cleavage site to insertion region of leucine-rich repeat 12 (LRR12). Structural modeling

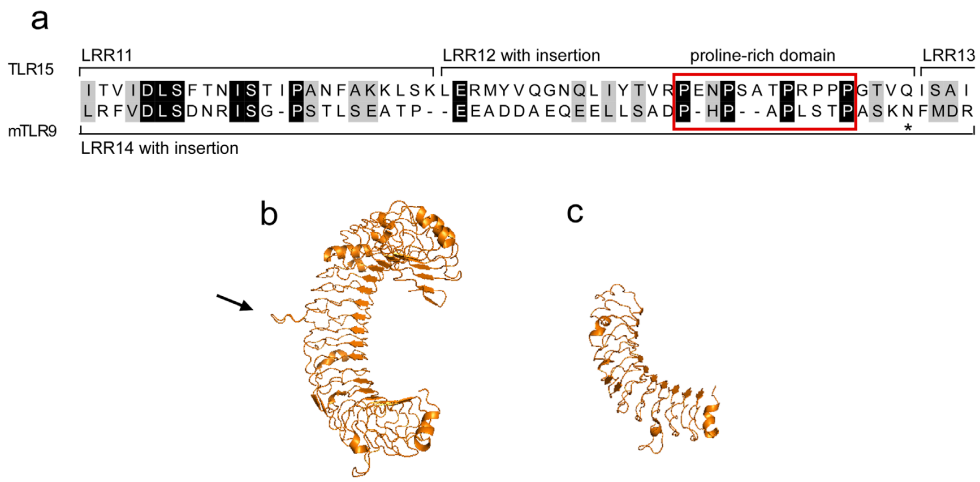

Figure 8. Model of TLR15 cleavage. *(A) ClustalW alignment of the cleavage regions of TLR15 and mTLR9. Boxed in red is the proline-rich domain within the insertion of LRR12 of TLR15 and LRR14 of mTLR9. The asterisk indicates the proposed mTLR9 cleavage site. (B) Model of TLR15 showing the extrusions from LRR12 (TLR15). (C) Model of truncated TLR15.*

shows an insertion present within LRR12 that forms a disordered loop extending outwards from the ligand sensing domain (Fig. 8B), similar to the cleavage region in LRR14 of mTLR9 (7, 32, 36). Alignment of these regions shows the conservation of a proline-rich domain, just prior to the asparagine predicted to be cleaved in mTLR9 (Fig. 8A). As proline-rich domains are strongly associated with disordered regions (27), the presence of this domain might be a prerequisite for protease-accessibility in both TLR9 and TLR15.

TLRs are believed to dimerize upon ligand-binding, resulting in the initiation of a signaling cascade through the intracellular TIR domain (14). Activation of TLR15 through receptor cleavage suggests an entirely different mechanism of dimerization. Although speculative, several mechanisms can be proposed for TLR15 activation. For instance, proteolysis may release an inhibitory element, either in the form of a TLR15 protein region or a (membrane) bound adapter/helper protein. Alternatively, TLR15 cleavage may induce conformational changes that initiate signaling, or the proteolytic cleavage may liberate a self-ligand that subsequently binds and dimerizes the receptors. Transfer of the supernatant of protease-treated cells, however, was not sufficient to activate full length TLR15 (data not shown). As TLR15-activation may be evolutionary related to the activation of mammalian TLR9, further elucidation of the mechanism of could provide important insights in both systems.

Mammals appear to lack a TLR that is able to directly sense proteolytic activity. However, several alternative systems may have evolved that initiate immune responses through pathogenic proteases in the mammalian species. The most-studied are the cell surface G-protein-coupled protease-activated receptors (PARs 1-4) (30). Particularly PAR2 has been shown to be activated by proteases from exogenous sources, like the bacterial pathogens *Porphyromonas gingivalis* (11) and *Helicobacter pylori* (16), and dust mite proteases (6). Also, TLR4 can be activated indirectly by elastase-cleaved extracellular matrix components (5), and dust mite proteases are proposed to activate an unidentified receptor present on human basophils (38). If these mechanisms also contribute to the defense against fungi remains to be investigated. Generally, detection of fungi in mammals occurs via a number of pattern recognition receptors, including dectin-1 (β-glucan), mannose receptor, DC-SIGN, TLR2 and TLR4 (29, 42). Although homologues of several PARs and TLR4 are present in the chicken genome, it remains unclear if these pathways are functional in birds. Genome analysis revealed no clear homologues of Dectin-1 and DC-SIGN in chicken. However, as chicken cells can be activated by the Dectin-1 agonist curdlan (28), functional homologues of this receptor may exist. Its tempting to speculate that alternative/different microbial pressure in birds and mammals has

resulted in a different approach in dealing with fungi. If the presence of TLR15 leads to a different susceptibility to pathogens with protease virulence factors awaits methods to inactivate the receptor in chickens.

Previous studies showed the expression of TLR15 in the intestinal tract of chickens, similar to where PAR2 is activated by pathogen-induced host protease in mice (9). Although the gut lumen is a rich environment loaded with multiple types of potentially activating proteases, PAR2 and presumably TLR15 are not constantly activated by the normal flora. Under normal conditions, the mucus layer functions as a barrier between the content of the intestinal lumen and the epithelial cell layer, which is further protected from aberrant protease activity by secretory leukocyte peptidase inhibitor (SLPI) (3). In addition, previous studies have shown dramatic upregulation of TLR15 in intestinal tissue after *Salmonella* infection or TLR stimulation (10, 37), suggesting that under healthy homeostatic conditions, the contact between TLR15 and normal intestinal proteases is limited. This form of regulation may extra safeguard the chicken for activation of the innate immune system by accidental exposure to proteases.

In conclusion, we show that chicken TLR15 is activated by secreted fungal proteases after cleavage of the extracellular receptor domain. The activation of TLR15 through proteolytic cleavage likely evolved through alternative evolutionary pressure of pathogens in birds compared to mammalian species, and suggests that mammals and birds exploit different classes of receptors (namely TLRS vs. PARs) to detect proteases in the environment.

References

1. **International Chicken Genome Sequencing Consortium.** 2004. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature **432:**695-716.
2. **Barton, G. M., and J. C. Kagan.** 2009. A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nature reviews **9:**535-542.
3. **Bergenfeldt, M., M. Nystrom, M. Bohe, C. Lindstrom, A. Polling, and K. Ohlsson.** 1996. Localization of immunoreactive secretory leukocyte protease inhibitor (SLPI) in intestinal mucosa. Journal of gastroenterology **31:**18-23.
4. **Bianchi, M. E.** 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. Journal of leukocyte biology **81:**1-5.
5. **Brunn, G. J., M. K. Bungum, G. B. Johnson, and J. L. Platt.** 2005. Conditional signaling by Toll-like receptor 4. Faseb J **19:**872-874.
6. **Chignard, M., and D. Pidard.** 2006. Neutrophil and pathogen proteinases versus proteinase-activated receptor-2 lung epithelial cells: more terminators than activators. American journal of respiratory cell and molecular biology **34:**394-398.
7. **Ewald, S. E., B. L. Lee, L. Lau, K. E. Wickliffe, G. P. Shi, H. A. Chapman, and G. M. Barton.** 2008. The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor. Nature **456:**658-662.
8. **Geijtenbeek, T. B., and S. I. Gringhuis.** 2009. Signalling through C-type lectin receptors: shaping immune responses. Nature reviews **9:**465-479.
9. **Hansen, K. K., P. M. Sherman, L. Cellars, P. Andrade-Gordon, Z. Pan, A. Baruch, J. L. Wallace, M. D. Hollenberg, and N. Vergnolle.** 2005. A major role for proteolytic activity and proteinase-activated receptor-2 in the pathogenesis of infectious colitis. Proceedings of the National Academy of Sciences of the United States of America **102:**8363-8368.
10. **Higgs, R., P. Cormican, S. Cahalane, B. Allan, A. T. Lloyd, K. Meade, T. James, D. J. Lynn, L. A. Babiuk, and C. O'Farrelly.** 2006. Induction of a novel chicken Toll-like receptor following *Salmonella enterica* serovar Typhimurium infection. Infection and immunity **74:**1692-1698.
11. **Holzhausen, M., L. C. Spolidorio, R. P. Ellen, M. C. Jobin, M. Steinhoff, P. Andrade-Gordon, and N. Vergnolle.** 2006. Protease-activated receptor-2 activation: a major role in the pathogenesis of *Porphyromonas gingivalis* infection. The American journal of pathology **168:**1189-1199.

12. **Ishii, K. J., S. Koyama, A. Nakagawa, C. Coban, and S. Akira.** 2008. Host innate immune receptors and beyond: making sense of microbial infections. Cell host & microbe **3:**352-363.
13. **Jin, M. S., S. E. Kim, J. Y. Heo, M. E. Lee, H. M. Kim, S. G. Paik, H. Lee, and J. O. Lee.** 2007. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell **130:**1071-1082.
14. **Jin, M. S., and J. O. Lee.** 2008. Structures of the toll-like receptor family and its ligand complexes. Immunity **29:**182-191.
15. **Kaczorowski, D. J., K. P. Mollen, R. Edmonds, and T. R. Billiar.** 2008. Early events in the recognition of danger signals after tissue injury. Journal of leukocyte biology **83:**546-552.
16. **Kajikawa, H., N. Yoshida, K. Katada, F. Hirayama, O. Handa, S. Kokura, Y. Naito, and T. Yoshikawa.** 2007. *Helicobacter pylori* activates gastric epithelial cells to produce interleukin-8 via protease-activated receptor 2. Digestion **76:**248-255.
17. **Kang, J. Y., X. Nan, M. S. Jin, S. J. Youn, Y. H. Ryu, S. Mah, S. H. Han, H. Lee, S. G. Paik, and J. O. Lee.** 2009. Recognition of Lipopeptide Patterns by Toll-like Receptor 2-Toll-like Receptor 6 Heterodimer. Immunity.
18. **Kawai, T., and S. Akira.** 2008. Toll-like receptor and RIG-I-like receptor signaling. Annals of the New York Academy of Sciences **1143:**1-20.
19. **Keestra, A. M., M. R. de Zoete, R. A. van Aubel, and J. P. M. van Putten.** 2007. The central leucine-rich repeat region of chicken TLR16 dictates unique ligand specificity and species-specific interaction with TLR2. J Immunol **178:**7110-7119.
20. **Keestra, A. M., M. R. de Zoete, R. A. van Aubel, and J. P. M. van Putten.** 2008. Functional characterization of chicken TLR5 reveals species-specific recognition of flagellin. Molecular immunology **45:**1298-1307.
21. **Keestra, A. M., and J. P. M. van Putten.** 2008. Unique properties of the chicken TLR4/MD-2 complex: selective lipopolysaccharide activation of the MyD88-dependent pathway. J Immunol **181:**4354-4362.
22. **Kumar, H., T. Kawai, and S. Akira.** 2009. Toll-like receptors and innate immunity. Biochemical and biophysical research communications **388:**621-625.
23. **Latz, E., A. Schoenemeyer, A. Visintin, K. A. Fitzgerald, B. G. Monks, C. F. Knetter, E. Lien, N. J. Nilsen, T. Espevik, and D. T. Golenbock.** 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nature immunology **5:**190-198.

24. **Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann.** 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. Cell **86:**973-983.
25. **Litman, G. W., J. P. Cannon, and L. J. Dishaw.** 2005. Reconstructing immune phylogeny: new perspectives. Nature reviews **5:**866-879.
26. **Liu, L., I. Botos, Y. Wang, J. N. Leonard, J. Shiloach, D. M. Segal, and D. R. Davies.** 2008. Structural basis of toll-like receptor 3 signaling with double-stranded RNA. Science (New York, N.Y **320:**379-381.
27. **Neduva, V., and R. B. Russell.** 2007. Proline-rich regions in transcriptional complexes: heading in many directions. Sci STKE **2007:**pe1.
28. **Nerren, J. R., and M. H. Kogut.** 2009. The selective Dectin-1 agonist, curdlan, induces an oxidative burst response in chicken heterophils and peripheral blood mononuclear cells. Veterinary immunology and immunopathology **127:**162-166.
29. **Netea, M. G., G. D. Brown, B. J. Kullberg, and N. A. Gow.** 2008. An integrated model of the recognition of *Candida albicans* by the innate immune system. Nat Rev Microbiol **6:**67-78.
30. **Ossovskaya, V. S., and N. W. Bunnett.** 2004. Protease-activated receptors: contribution to physiology and disease. Physiological reviews **84:**579-621.
31. **Palm, N. W., and R. Medzhitov.** 2009. Pattern recognition receptors and control of adaptive immunity. Immunological reviews **227:**221-233.
32. **Park, B., M. M. Brinkmann, E. Spooner, C. C. Lee, Y. M. Kim, and H. L. Ploegh.** 2008. Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. Nature immunology **9:**1407-1414.
33. **Park, B. S., D. H. Song, H. M. Kim, B. S. Choi, H. Lee, and J. O. Lee.** 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature **458:**1191-1195.
34. **Roach, J. C., G. Glusman, L. Rowen, A. Kaur, M. K. Purcell, K. D. Smith, L. E. Hood, and A. Aderem.** 2005. The evolution of vertebrate Toll-like receptors. Proceedings of the National Academy of Sciences of the United States of America **102:**9577-9582.
35. **Saijo, S., N. Fujikado, T. Furuta, S. H. Chung, H. Kotaki, K. Seki, K. Sudo, S. Akira, Y. Adachi, N. Ohno, T. Kinjo, K. Nakamura, K. Kawakami, and Y. Iwakura.** 2007. Dectin-1 is required for host defense against *Pneumocystis carinii* but not against *Candida albicans*. Nature immunology **8:**39-46.

36. **Sepulveda, F. E., S. Maschalidi, R. Colisson, L. Heslop, C. Ghirelli, E. Sakka, A. M. Lennon-Dumenil, S. Amigorena, L. Cabanie, and B. Manoury.** 2009. Critical role for asparagine endopeptidase in endocytic Toll-like receptor signaling in dendritic cells. Immunity **31:**737-748.

37. **Shaughnessy, R. G., K. G. Meade, S. Cahalane, B. Allan, C. Reiman, J. J. Callanan, and C. O'Farrelly.** 2009. Innate immune gene expression differentiates the early avian intestinal response between *Salmonella* and *Campylobacter*. Veterinary immunology and immunopathology **132:**191-198.

38. **Sokol, C. L., G. M. Barton, A. G. Farr, and R. Medzhitov.** 2008. A mechanism for the initiation of allergen-induced T helper type 2 responses. Nature immunology **9:**310-318.

39. **Taylor, P. R., S. V. Tsoni, J. A. Willment, K. M. Dennehy, M. Rosas, H. Findon, K. Haynes, C. Steele, M. Botto, S. Gordon, and G. D. Brown.** 2007. Dectin-1 is required for beta-glucan recognition and control of fungal infection. Nature immunology **8:**31-38.

40. **Temperley, N. D., S. Berlin, I. R. Paton, D. K. Griffin, and D. W. Burt.** 2008. Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. BMC genomics **9:**62.

41. **van Putten, J. P. M., L. I. Bouwman, and M. R. de Zoete.** 2009. Unraveling bacterial interactions with Toll-like receptors. Immunology letters.

42. **Willment, J. A., and G. D. Brown.** 2008. C-type lectin receptors in antifungal immunity. Trends in microbiology **16:**27-32.

4

Activation of human and chicken Toll-like receptors by *Campylobacter* spp.

Marcel R. de Zoete
A. Marijke Keestra
Paula Roszczenko
Jos P. M. van Putten

Infection and Immunity 78:1229-1238. 2010.

Abstract

Campylobacter infection in humans is accompanied by severe inflammation of the intestinal mucosa, in contrast to colonization of chicken. The basis for the differential host response is unknown. Toll-like receptors (TLRs) sense and respond to microbes in the body and participate in the induction of an inflammatory response. Thus far, the interaction of *Campylobacter* with chicken TLRs has not been studied. Here, we investigated the potential of four *Campylobacter* strains to activate human TLR1/2/6, TLR4, TLR5, and TLR9 and chicken TLR2t2/16, TLR4, TLR5, and TLR21. Live bacteria showed no or very limited potential to activate TLR2, TLR4, and TLR5 of both the human and chicken species, with minor but significant differences between *Campylobacter* strains. In contrast, lysed bacteria induced strong NF-κB activation through human TLR1/2/6 and TLR4 and chicken TLR2t2/16 and TLR4 but not via TLR5 of either species. Interestingly, *C. jejuni* induced TLR4-mediated beta interferon in human but not chicken cells. Furthermore, isolated chromosomal *Campylobacter* DNA was unable to activate human TLR9 in our system, whereas chicken TLR21 was activated by DNA from all of the campylobacters tested. Our data are the first comparison of TLR-induced immune responses in humans and chickens. The results suggest that differences in bacterial cell wall integrity and in TLR responses to *Campylobacter* LOS and/or DNA may contribute to the distinct clinical manifestation between the species.

Introduction

Campylobacter jejuni, a Gram-negative highly motile bacterium, is a major cause of intestinal enteritis in humans (2). The infection of the gut is accompanied by high numbers of infiltrating neutrophils, loss of epithelial barrier integrity, and a watery or bloody diarrhea (7, 57). The inflammatory pathology suggests a strong induction of innate immune responses. Indeed, experimental infection of several types of human tissue culture cells has demonstrated the potential of *Campylobacter* to initiate a range of immune responses. For instance, intestinal INT407 and T84 cells respond to *C. jejuni* by producing cytokines interleukin-8 (IL-8) and/or tumor necrosis factor alpha (TNF-α) (4, 8, 20, 25, 58, 60), whereas infected THP-1 monocytes and dendritic cells secrete an even broader range of cytokines (23, 26). Although some reports clearly show the requirement of the cytolethal distending toxin and/or bacterial invasion for cytokine responses (21, 25), most of the observed responses seem independent of these traits.

Chickens are considered to be the main source of human *Campylobacter* infection. *Campylobacter* colonization of chickens is rapid and widespread, and results in long-term persistence and shedding (5, 33). Although large numbers of bacteria are in close contact with the epithelial cell layer of particularly the ceca, no intestinal inflammation is seen in these birds (13), in clear contrast to humans. Despite the lack of pathology, several reports have shown induction of immune-associated gene and protein expression after *Campylobacter* colonization of chicken. Analysis of isolated chicken tissue displayed an increase in cytokine expression (48) and circulating monocytes/macrophages (38), and several different types of chicken cells produce or upregulate cytokines during in vitro infection (32, 34, 49).

Innate immune responses against infectious microbes are for a large part mediated by the activation of Toll-like receptors (TLRs) (31). TLRs are a group of membrane receptors that safeguard the host-microbe boundaries by sensing conserved microbial patterns (41). Bacteria are mainly sensed by TLR2/1/6, TLR4/MD-2, TLR5, and TLR9 that detect lipoproteins, lipopolysaccharide (LPS), flagellin, and DNA, respectively. After TLR activation, a signaling cascade involving the adapter proteins MyD88 (all TLRs but TLR3) or TRIF (TLR3 and TLR4 only) results in the activation and the translocation of nuclear transcription factors such as NF-κB and IRF3, which in turn induce the transcription of cytokines and other immune genes (1).

C. jejuni initiates both MyD88 and TRIF-dependent immune responses through the activation of human TLR2 and TLR4 (17, 43), whereas stimulation of human TLR9 only

resulted in low levels of IL-8 secretion (12). Furthermore, *C. jejuni* is unable to activate human TLR5 (3, 58). The potential of *Campylobacter* to activate TLRs of the chicken has thus far not been explored. Identification and characterization of the chicken TLRs revealed major species-specific characteristics. For instance, the chicken TLR2 complex does not seem to distinguish between different lipoproteins in contrast to its human counterpart (22, 28), whereas chickens lack TLR9 but express the unique DNA-sensing TLR21 (9; A. M. Keestra et al., unpublished data). Reports on differences in *Campylobacter*-induced (innate) immune responses between humans and chickens are scarce and somewhat contradictory. Larson et al. showed differences in the amount of IL-8 induction and secretion between *C. jejuni*-infected human INT407 cells and chicken LMH cells (32), whereas Borrmann et al. reported similar levels of IL-8 upregulation when comparing INT407 and chicken PIC cells (8). In the present study, we for the first time directly compared the potential of *Campylobacter* to activate individual human and chicken TLRs. Although the majority of TLRs responded similarly to four different *Campylobacter* strains, chicken TLR21, but not human TLR9, was activated by *Campylobacter* chromosomal DNA. In addition, *Campylobacter* was unable to activate MyD88-independent IFN-β transcription via TLR4 signaling in the chicken. Interestingly, live *Campylobacter* showed no or weak activation of TLRs of both species, suggesting that in vivo differences in bacterial integrity may also influence the innate immune response.

Materials and Methods

Bacteria, cell lines, and reagents

C. jejuni strains 81116 (42), GB18 (14), and RM1221 (40) and the *Campylobacter coli* strain H1 (a fresh chicken isolate) were routinely cultured on plates with 5% saponin-lysed horse blood (Biotrading) at 37°C under a microaerophilic atmosphere of 10% CO_2, 5% O_2, and 85% N_2. Liquid cultures were grown under the same conditions in heart infusion (HI) broth (Biotrading). Human Mono Mac 6 (MM6) monocytic cells (61) and chicken HD11 macrophages (6) were propagated in RPMI (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS) under 5% CO_2 at 37°C. HeLa 57A cells containing a stably transfected NF-κB luciferase reporter plasmid (45), and HEK293 cells were cultured in Dulbecco modified Eagle medium (DMEM; Invitrogen) supplemented with 5% heat-inactivated FCS under 10% CO2 at 37°C. The TLR ligands FSL-1, Pam_3CSK_4, poly(I:C), and ODN 2006 were purchased from Invivogen. Highly purified LPS from *Sal-*

monella enterica serovar Enteritidis 90-13-706 and highly purified lipo-oligosaccharide (LOS) from *C. jejuni* 81116 were isolated as described previously (30). Flagellin from *S.* Enteritidis was purified as described previously (24).

Preparation of live and lysed *Campylobacter*

Campylobacter starter cultures were inoculated in 5 ml of HI broth from saponin plates grown for 48 h at 37°C. After 6 to 8 h of incubation (37°C, shaking at 160 rpm), starter cultures were used to inoculate fresh cultures at a starting optical density at 550 nm (OD_{550}) of 0.00125 in 10 ml of HI broth to virtually exclude the presence of dead bacteria from the plates. After 17 h of growth, bacteria reached an OD_{550} of ~1.0, corresponding to 2 x 10^9 CFU ml^{-1}. Then, 1 ml of this suspension was pelleted by centrifugation (5,000 x g, 10 min, 22°C), washed twice with Dulbecco phosphate-buffered saline (DPBS), and resuspended in DPBS to a final OD_{550} of 1.0. Live bacteria were analyzed by using the Live/Dead BacLight kit (Invitrogen) and were only used if the percentage dead bacteria was <0.5%. Then, 1 ml of the suspended bacteria was heat killed at 65°C for 30 min and subsequently sonicated on ice (a 15-s pulse followed by a 30-s pause, repeated six times) to further release TLR ligands.

RT-PCR

MM6 and HD11 cells were cultured in 12-well plates overnight to a confluence of ~80%. Live or dead bacteria were added at a multiplicity of infection (MOI) of 100. After 2 h of incubation (37°C, 5% CO_2), the cells were washed twice with DPBS to remove the cell culture medium and bacteria. RNA from MM6 cells was isolated by using an RNeasy mini-kit (Qiagen), and RNA from HD11 cells was isolated by using RNA-Bee (Bio-Connect), both according to the manufacturer's protocol. Prior to reverse transcription-PCR (RT-PCR), the RNA was treated with 1 μg of DNase (Fermentas) per μg of RNA for 30 min at 37°C, after which the DNase was inactivated by heating at 65°C for 10 min in the presence of EDTA (2.5 mM, final concentration). Primers (Invitrogen) and probes (Table 1) were designed by using Primer Express software (Applied Biosystems). Probes, labeled with the reporter dye carboxyfluorescein (FAM) and the quencher tetramethyl-6-carboxyrhodamine (TAMRA), were either purchased from Isogen Life Science (chicken GAPDH [glyceraldehyde-3-phosphate dehydrogenase], IL-1β, IL-8 [also known as CXCLi2], iNOS, and beta interferon [IFN-β]) or Eurogentec (human β-actin, IL-1β, IL-8 [CXCL8], and IFN-β). RNA transcript levels were determined by quantitative RT-PCR with an ABI Prism 7000 sequence detection system (Applied Biosystems) using a One

Step RT-PCR MasterMix kit for probe assays (Eurogentec). Per reaction, 50 ng of DNase-treated RNA was used. Real-time cycler conditions and normalization against housekeeping genes β-actin (HD11) and GAPDH (MM6) were as described previously (30).

Chromosomal DNA isolation

Chromosomal DNA was isolated from *Campylobacter* grown in 100 ml of HI broth at 37°C overnight using CTAB (cetyltrimethylammonium bromide). Briefly, bacteria were pelleted by centrifugation and resuspended in 9.5 ml of TE buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA [pH 8.0]). Bacteria were lysed by adding 1 µg of proteinase K and 0.5 ml of 10% sodium dodecyl sulfate, followed by incubation at 37°C for 1.5 h. Subsequently, 1.8 ml of NaCl (5 M) and 1.5 ml of 10% CTAB in 0.7 M NaCl were added, and the mixture was incubated at 65°C for 20 min. DNA was extracted into the aqueous phase after the addition of an equal volume of chloroform-isoamyl alcohol (24:1). After centrifugation (6,000 x g, 10 min, 22°C), the aqueous phase was mixed with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and again centrifuged. DNA was precipitated from the aqueous phase with 0.6 volumes of isopropanol and collected by centrifugation (5,000 x g, 5 min, 22°C). The DNA pellet was washed with 5 ml of 70% ethanol, air dried, and resuspended in H_2O. DNA concentration was determined by measuring the absorption at 260 nm.

Transfection

HeLa 57A cells and HEK293 cells were grown overnight to a confluence of ~60%. Individual wells were transfected with a total amount of 250 ng of plasmid DNA mixture using FuGENE-6 (Roche) at a lipid/DNA ratio of 3:1. Plasmids encoding the human and chicken TLRs and adapter proteins were obtained as described previously (28-30). Human TLR1, TLR2, TLR4, and TLR9 and chicken TLR4 and TLR21 were expressed with their natural leader peptide, and chicken TLR2t2, TLR5, and TLR16 and human TLR5 were expressed with the pFLAG-CMV1 preprotrypsin leader peptide for secreted proteins. Combinations of the following plasmids were transfected: human TLR2, human TLR1, human CD14, and LacZ (62.5 ng per plasmid per well); chicken TLR2t2, chicken TLR16, human CD14, and LacZ (62.5 ng per plasmid per well); human TLR4, human MD-2, human CD14, and LacZ (62.5 ng per plasmid per well); chicken TLR4, chicken MD-2, human CD14, and LacZ (62.5 ng per plasmid per well); human TLR5 and LacZ (125 ng

Table I. RT-PCR primers and probes used in this study

Gene (species)	Probe / primer	Sequence[a]
β-actin (human)	Forward	5'-ACCGAGCGCGGCTACAG-3'
	Reverse	5'-CTTAATGTCACGCACGATTTCC-3'
	Probe	5'-(FAM)-TTCACCACCACGGCCGAGC-(TAMRA)-3'
IL-1β (human)	Forward	5'-CGAATCTCCGACCACCACTAC-3'
	Reverse	5'-TCCATGGCCACAACAACTGA-3'
	Probe	5'(FAM)-AGGGCTTCAGGCAGGCCGC-(TAMRA)-3'
IL-8 (human)	Forward	5'-CTGGCCGTGGCTCTCTTG-3'
	Reverse	5'-CCTTGGCAAAACTGCACCTT-3'
	Probe	5'-(FAM)-CAGCCTTCCTGATTTCTGCAGCTCTGTGT-(TAMRA)-3'
IFN-β (human)	Forward	5'-TTCTCCACGACAGCTCTTTCC-3'
	Reverse	5'-CACTGACAATTGCTGCTTCTTTG-3'
	Probe	5'-(FAM)-TGAGCTACAACTTGCTTGGATTCC-(TAMRA)-3'
GAPDH (chicken)	Forward	5'-GCCGTCCTCTCTGGCAAAG-3'
	Reverse	5'-TGTAAACCATGTAGTTCAGATCGATGA-3'
	Probe	5'-(FAM)-AGTGGTGGCCATCAATGATCCC-(TAMRA)-3'
IL-1β (chicken)	Forward	5'-GCTCTACATGTCGTGTGTGATGAG-3'
	Reverse	5'-TGTCGATGTCCCGCATGA-3'
	Probe	5'-(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)-3'
IL-8 (chicken)	Forward	5'-GCCCTCCTCCTGGTTTCAG-3'
	Reverse	5'-CGCAGCTCATTCCCCATCT-3'
	Probe	5'-(FAM)-TGCTCTGTCGCAAGGTAGGACGCTG-(TAMRA)-3'
IFN-β (chicken)	Forward	5'-ACAACTTCCTACAGCACAACAACTA-3'
	Reverse	5'-GCCTGGAGGCGGACATG-3'
	Probe	5'-(FAM)-TCCCAGGTACAAGCACTG-(TAMRA)-3'

[a] FAM, carboxyfluorescein; TAMRA, tetramethyl-6-carboxyrhodamine

per plasmid per well); chicken TLR5 and LacZ (125 ng per plasmid per well); human TLR9 and LacZ (125 ng per plasmid per well); and chicken TLR21 and LacZ (125 ng per plasmid per well). Cells were incubated with a DNA-FuGENE-6 mixture for 48 h. Prior to stimulation, medium was replaced with fresh DMEM-5% FCS.

Stimulation and luciferase measurement

Individual wells were stimulated with 2.5 x 10^7 live or lysed bacteria, 30 μg of chromosomal DNA ml^{-1}, 20 ng of LPS ml^{-1}, 100 ng of Pam_3CSK_4 ml^{-1}, 100 ng of FSL-1 ml^{-1}, 1 μg of flagellin ml^{-1}, or 0.5 μM ODN 2006. After 5 h of stimulation, the cells were washed twice with 0.5 ml of DPBS and lysed in 100 μl of RLB buffer (Promega) at –80°C. The luciferase activity in thawed lysates was measured in a luminometer (TD-20/20; Turner Designs) using luciferase reagent (Promega). To normalize transfection efficiency, the luciferase results were corrected with LacZ values determined with a β-galactosidase assay (Promega). Statistical analysis was performed by using a paired *t* test with GraphPad Prism 5 software, where a two-tailed *P* of <0.05 was considered significant. All experiments were performed three times independently. Chromosomal DNA from all four *Campylobacter* strains was prepared twice.

Results

Human cytokine responses toward live and disrupted *C. jejuni*.

Prior to testing the activation of TLRs by *Campylobacter*, we assessed the effect of *C. jejuni* strain 81116 on human MM6 monocytic cells. As host cells likely encounter both live and disrupted *C. jejuni* during the course of infection, we determined the ability of both viable and lysed *C. jejuni* to induce innate immune responses. Highly sensitive RT-PCR on isolated RNA was performed to be able to measure early changes (2 h) in IL-1β and IL-8 gene transcription. MM6 cells were infected with live or lysed *C. jejuni* strain 81116 at an MOI of 100 (5 x 107 CFU ml–1). No significant reduction in viable bacteria was noticed during the period of infection (data not shown). As a positive control, cells were activated by the addition of 1 μg of purified *S*. Enteritidis LPS ml–1. This approach demonstrated that live *C. jejuni* induced minimal upregulation of human IL-1β transcript (~4-fold increase). In contrast, lysed *C. jejuni* increased the level of IL-1β transcript by factor ~175 (Fig. 1A). Similarly, live *C. jejuni* increased human IL-8 transcript by ~16-fold, whereas disrupted bacteria induced an ~125-fold increase in IL-8 mRNA (Fig. 1A). These results indicate that *C. jejuni* strain 81116 is capable of inducing a powerful innate immune response in human monocytes, particularly once the bacteria disintegrate.

Chicken cytokine responses toward live and lysed *C. jejuni*.

Since chickens can be colonized heavily by *Campylobacter* without obvious signs of pathology, we next investigated chicken HD11 macrophages for cytokine expression after infection with *C. jejuni*. We used similar conditions as described for the MM6 cells, except that primer pairs were optimally adapted to the chicken cytokine gene sequences. In these assays both viable and lysed *C. jejuni* increased IL-1β and IL-8 mRNA levels (Fig. 1B). Again, the cellular response to disrupted *C. jejuni* was significantly stronger than observed for live microorganisms (Fig. 1B), although the difference was markedly smaller than observed for the MM6 cells. Overall, the results indicate that *C. jejuni* strain 81116 activates both human and chicken immune innate genes and that bacterial cell wall integrity is an important determinant of the magnitude of the response in both species.

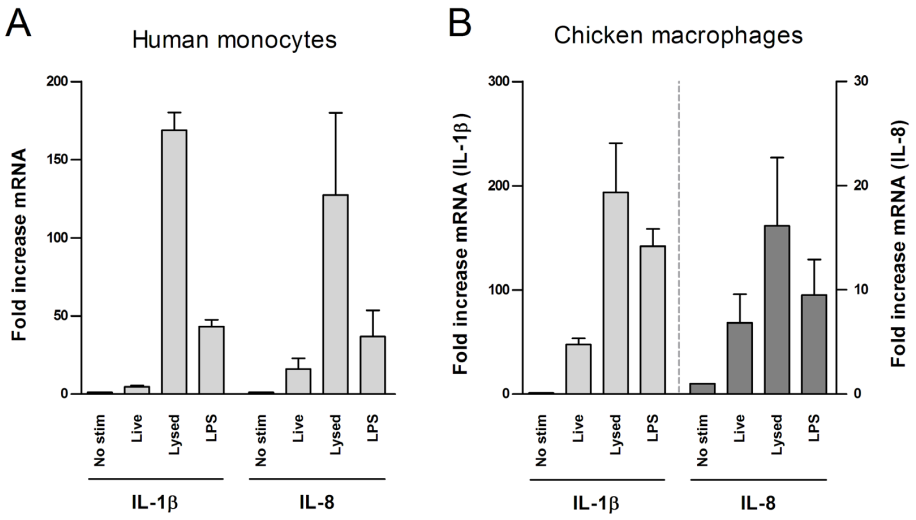

Figure 1. Induction of IL-1β and IL-8 by live and disrupted C. jejuni. *MM6 cells (A) and chicken HD11 cells (B) were stimulated for 2 h with 5 x 10^7 CFU of live or lysed C. jejuni strain 81116 ml^{-1}. As a positive control, 1 µg of LPS from S. Enteritidis was used. IL-1β and IL-8 transcripts were analyzed by RT-PCR and are presented as fold increase mRNA levels after stimulation compared to non-stimulated cells. Live Campylobacter induced statistically significant levels of IL-1β and IL-8 mRNA in MM6 cells ($P < 0.05$), whereas in HD11 cells only the induction of IL-1β was statistically significant ($P < 0.05$). Stimulation of MM6 and HD11 cells with lysed Campylobacter resulted in significantly higher levels of IL-1β and IL-8 mRNA compared to mock stimulation or stimulation with live bacteria ($P < 0.05$). Values are means ± the standard errors of the mean (SEM) of three independent experiments.*

Disrupted but not live *Campylobacter* activate both human and chicken TLR2.

To investigate the contribution of individual TLRs to the *Campylobacter* innate immune response in humans and chickens, we first tested the ability of live and lysed bacteria to activate the TLR2 complex of both species. Humans recognize bacterial di- and triacylated lipoproteins by the combinations TLR2-TLR6 and TLR2-TLR1, respectively. HeLa 57A cells, which endogenously express TLR6, were transfected with human TLR2 together with TLR1 and stimulated with 5 x 10^7 CFU ml^{-1} (MOI of 100) live or lysed *Campylobacter*. We tested four different *Campylobacter* strains—*C. jejuni* 81116, *C. jejuni* GB18, *C. jejuni* RM1221, and *C. coli* H1—to reduce possible strain-specific effects. Cellular activation was measured using the NF-κB luciferase reporter that is stably expressed in HeLa 57A cells. Viable bacteria of none of the strains significantly activated NF-κB compared to control transfected cells after 5 h of infection (Fig. 2A), although strain GB18 tended to show slightly elevated NF-κB activation. Stimulation of live *C. jejuni* together with the TLR2 ligand Pam_3CSK_4 yielded a response similar to that obtained with the TLR2 ligand alone, excluding potential inhibition of the TLR/NF-κB response by *C. jejuni* (data not shown). In contrast to live *Campylobacter*, disrupted bacteria induced strong levels of NF-κB translocation irrespective of the *Campylobacter* strain or species tested (Fig. 2B).

To assess the chicken TLR2 response against *Campylobacter*, HeLa 57A cells were transfected with chTLR2t2 and chTLR16. This receptor complex recognizes both di- and triacylated lipopeptides (28). The addition of live *Campylobacter* to these cells did not result in significant translocation of NF-κB (Fig. 2C). Again, GB18 showed an increased but not statistically significant NF-κB activation, as was observed for human TLR2/1/6. With lysed bacteria, all four strains were able to potently activate chicken TLR2t2/16-transfected cells (Fig. 2D). These results show that disrupted *Campylobacter* strains, unlike viable strains, are able to effectively activate both human TLR2 and chicken TLR2.

***C. jejuni* induces a TLR4-mediated IFN-β response in human but not chicken cells.**

TLR4 is one of the central TLRs in innate immunity. Both lipid A structures with different biological activity toward TLR4 and differences in TLR4 specificity among host species have been described (15, 30, 50). To assess the effect of *Campylobacter* on the human and chicken TLR4 complex, we expressed TLR4 together with the homologous MD-2 in HeLa 57A cells. Incubation of the cells expressing human TLR4/MD-2 with lysed *Campylobacter* resulted in high levels of NF-κB translocation for all four strains tested

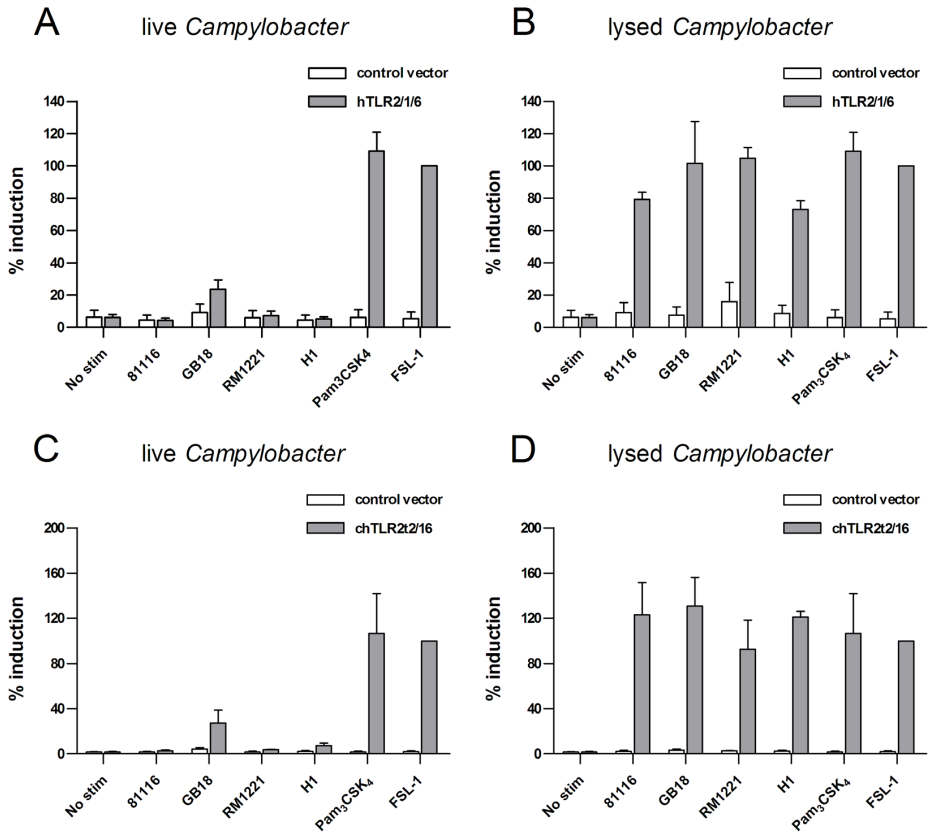

Figure 2. Activation of human and chicken TLR2 by Campylobacter. *HeLa 57A cells expressing human TLR2, TLR1, TLR6, and CD14 (A and B) or chicken TLR2t2, TLR16, and human CD14 (C and D) were stimulated with live or disrupted C. jejuni strains 81116, GB18, RM1221, or C. coli strain H1 for 5 h. Cells transfected with control vector were stimulated simultaneously to ensure TLR-specific NF-κB activation. Pam_3CSK_4 (100 ng ml^{-1}) and FSL-1 (100 ng ml^{-1}) were used as positive controls. Values are the percent induction of NF-κB activation after stimulation with the positive control and are means ± the SEM of three independent experiments. The P values for human and chicken TLR2 responses were as follows: live Campylobacter versus control, not significant; lysed Campylobacter versus control, $P < 0.05$; lysed versus live Campylobacter: $P < 0.05$.*

(Fig. 3B). With viable bacteria, moderate human TLR4 activation was detected for the *C. coli*, while *C. jejuni* strains yielded no response (Fig. 3A). For cells transfected with chicken TLR4/MD-2, similar results were obtained (Fig. 3C and D), suggesting no major differences in the recognition of *Campylobacter* by the human and chicken TLR4 complex.

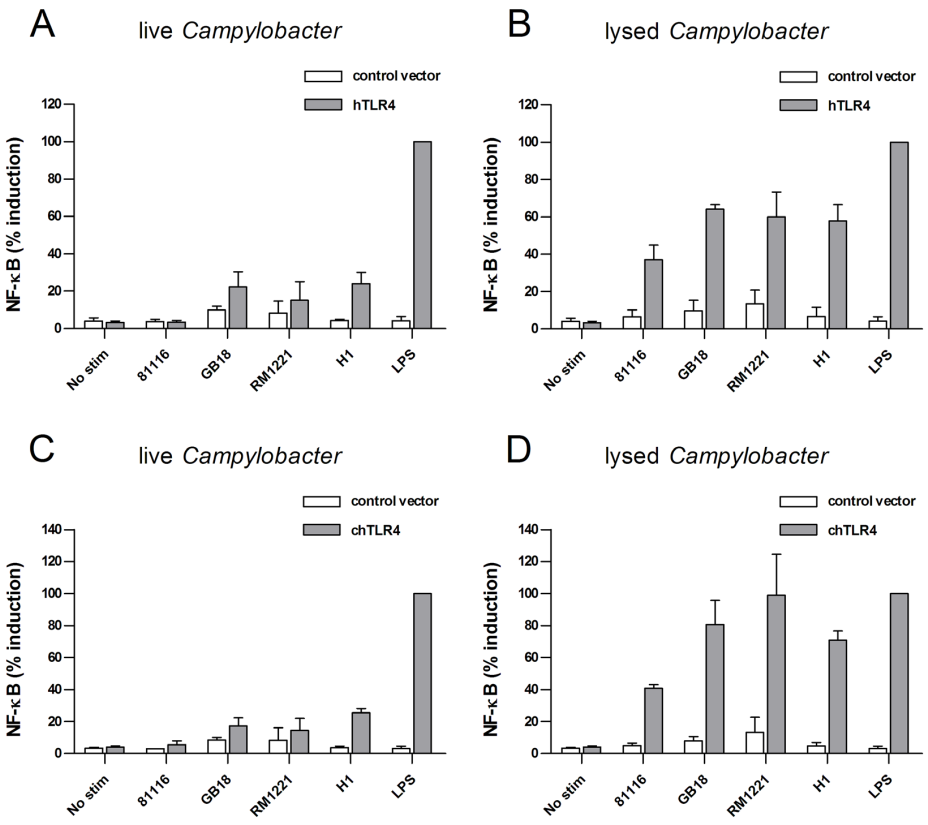

Figure 3. Activation of human and chicken TLR4 by Campylobacter. *HeLa 57A cells expressing human TLR4, MD-2, and CD14 (A and B) or chicken TLR4, MD-2, and human CD14 (C and D) were stimulated with live or lysed C. jejuni strains 81116, GB18, RM1221, or C. coli strain H1 for 5 h. Cells transfected with control vector were stimulated simultaneously to ensure TLR-specific NF-κB activation. LPS from S. Enteritidis (20 ng ml^{-1}) was used as positive control. Values are the percent induction of NF-κB activation after stimulation with the positive control and are means ± the SEM of three independent experiments. The P values for human and chicken TLR4 response were as follows: live Campylobacter versus control, not significant, except C. coli H1 ($P < 0.05$); lysed Campylobacter versus control: $P < 0.05$; lysed versus live Campylobacter: $P < 0.05$ for chicken TLR4 and $P < 0.001$ for human TLR4.*

As the mammalian TLR4/MD-2 complex not only signals via the MyD88-dependent pathway but also through the MyD88-independent pathway, we next compared the upregulation of IFN-β mRNA mediated via the MyD88-independent pathway in MM6 and

HD11 cells. Cells were stimulated with viable and lysed *Campylobacter* strains, as well as with purified *C. jejuni* 81116 LOS. Both viable and killed *C. jejuni*, as well as *C. jejuni* LOS, induced elevated levels of IFN-β transcript in the human cells (Fig. 4A). In contrast, stimulation of chicken cells with neither live nor dead *C. jejuni*, nor stimulation with purified LOS, induced IFN-β transcription, although a strong increase was observed after stimulation of the cells with the TLR3 agonist with poly(I:C) (Fig. 4B). These results indicate a major difference in response to *Campylobacter* between human and chicken cells.

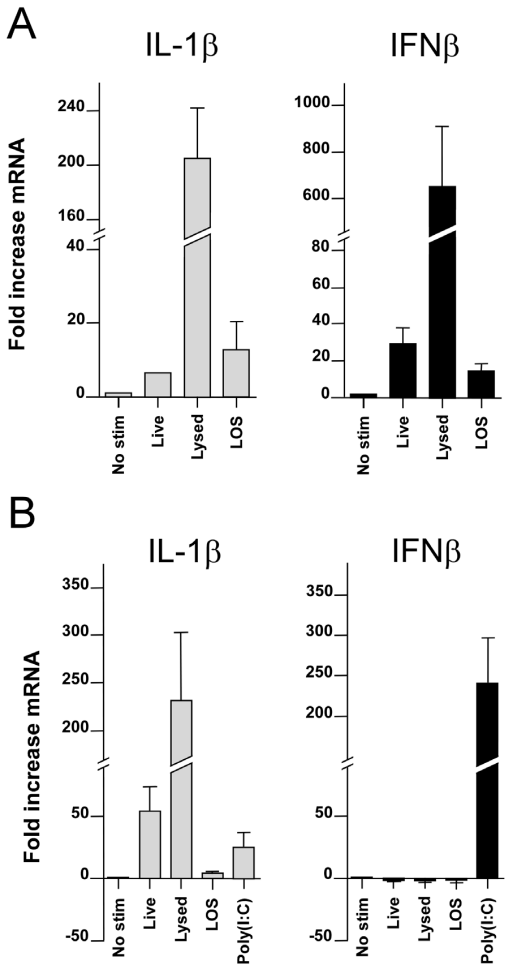

Figure 4. Induction of IFN-β by live, lysed, and purified LOS of C. jejuni. *MM6 cells (A) and chicken HD11 cells (B) were stimulated for 2 h with 5 x 10^7 CFU of live or lysed C. jejuni strain 81116 ml^{-1} or 1 µg of LOS ml^{-1}. As a positive control, 500 ng of poly(I:C) ml^{-1} with FuGENE 6 was used. IL-1β and IFN-β transcripts were analyzed by RT-PCR and are presented as fold increase mRNA levels after stimulation compared to non-stimulated cells. Stimulation of MM6 cells with live Campylobacter, lysed Campylobacter, or LOS induced statis-tically significant levels of IL-1β and IFN-β mRNA ($P < 0.05$). In HD11 cells, stimu-lation with live Campylobacter, lysed Campylobacter, or LOS but not poly(I:C) induced statistically significant levels of IL-1β, whereas IFN-β was only induced ($P < 0.05$) after stimulation with poly(I:C). Values are means ± the SEM of three independent experiments.*

***Campylobacter* is unable to activate chicken TLR5.**

TLR5 is considered to be important for maintenance of intestinal barrier (55). Stimulation of human TLR5 transfected HeLa 57A cells with either live or lysed *Campylobacter* showed a general lack of activation for all strains tested (Fig. 5A and B), confirming previous reports (3, 58). The identification and cloning of chicken TLR5 in our lab presented the opportunity to investigate its sensitivity toward *Campylobacter* flagellin. Whereas purified *S*. Enteritidis flagellin induced a robust NF-κB response in transfected HeLa 57A cells, stimulation with both live and disrupted *Campylobacter* strains did not result in activation of chTLR5 (Fig. 5C and D). Also, purified native flagellin of *C*. *jejuni* strain 81116 did not activate chTLR5, even at concentrations of >10 μg ml^{-1} (data not shown). These results indicate that the difference in *Campylobacter* intestinal pathology between humans and chicken is not due to differential recognition of flagellin by the respective TLR5 receptors.

***Campylobacter* chromosomal DNA is sensed by chicken TLR21 but not human TLR9.**

In the mammalian species, bacterial DNA is sensed by TLR9. This receptor is absent in chicken. Instead, chicken express TLR21 which responds to DNA and acts as a functional homologue of mammalian TLR9 (9; Keestra et al., unpublished). To test these receptors for the ability to respond to *Campylobacter* DNA, we expressed human TLR9 and chicken TLR21 in HEK293 cells. HEK293 cells were used in these experiments since HeLa 57A lack the ability to sense DNA and activate NF-κB when transfected with human TLR9. After 5 h of stimulation of HEK293 cells with chromosomal DNA (30 μg ml^{-1}) from any of the four *Campylobacter* strains, no significant NF-κB activation was detected for the cells expressing human TLR9. Increasing the internalization of *Campylobacter* DNA using the transfection reagent FuGENE-6 did not result in NF-κB activation either (data not shown). Stimulation with the synthetic TLR9 ligand ODN 2006 caused a 10-fold increase in TLR9-mediated NF-κB activation, indicating the functionality of the TLR9 receptor under the conditions used (Fig. 6A). The stimulatory effect of ODN 2006 was maintained in the presence of 30 μg of chromosomal *Campylobacter* DNA ml^{-1} (data not shown), excluding potential inhibitory factors in the DNA preparation as a cause for the lack of TLR9 activation. Similar sets of experiments with cells expressing chicken TLR21 demonstrated strong activation of NF-κB by ODN 2006, as well the chromosomal DNA of all four *Campylobacter* strains (Fig. 6B). These results suggest that, in addition to the response to *Campylobacter* LOS, humans and chickens may also differ in their ability to detect and respond to *Campylobacter* DNA.

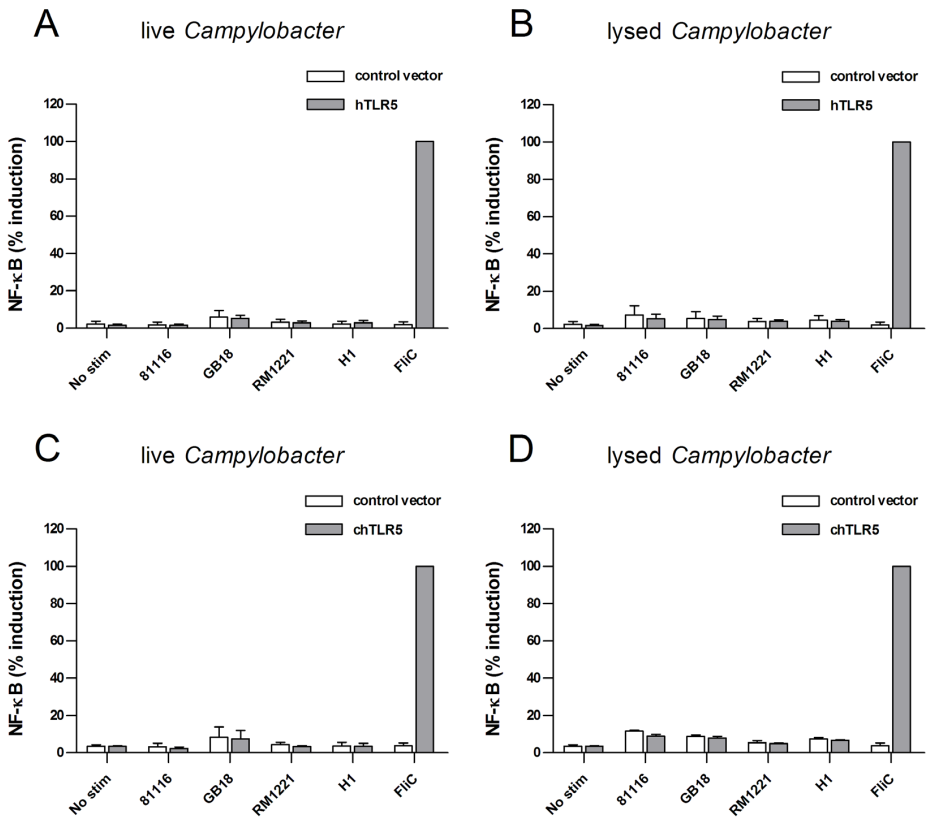

Figure 5. Activation of human and chicken TLR5 by Campylobacter. *HeLa 57A cells expressing human TLR5 (A and B) or chicken TLR5 (C and D) were stimulated with live or disrupted C. jejuni strains 81116, GB18, RM1221, or C. coli strain H1 for 5 h. Cells transfected with control vector were stimulated simultaneously to ensure TLR-specific NF-κB activation. Flagellin from S. Enteritidis (1 μg ml^{-1}) was used as positive control. None of the Campylobacter strains, either live or lysed, induced statistically significant activation of NF-κB in HeLa 57A cells transfected with human or chicken TLR5. Values are the percent induction of NF-κB activation after stimulation with the positive control and are means ± the SEM of three independent experiments.*

Discussion

Although *Campylobacter* is the most common cause of human bacterial enteritis, the mechanism that induces inflammation remains obscure. Similarly, the characteristics that determine its lifestyle as a harmless commensal in many animals, including chickens, are

still to be elucidated (54). In many bacterial infections, the level of recognition by the innate immune system of the host plays a critical role in determining whether or not pathology is induced. For instance, *Yersinia pestis* normally expresses an LPS that is not recognized by mammalian TLR4. In strains that artificially express TLR4-stimulating LPS, virulence is completely overcome by an activated host immune system (39).

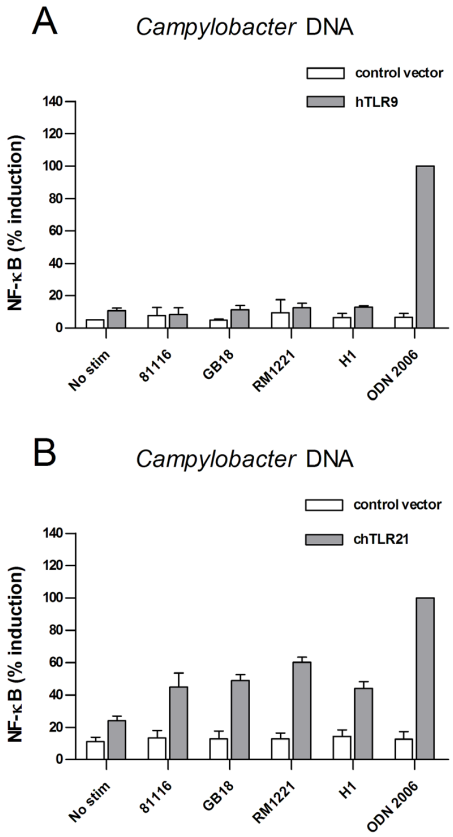

Figure 6. Activation of human TLR9 and chicken TLR21 by Campylobacter. *HEK-293 cells expressing human TLR9 (A) or HeLa 57A cells expressing chicken TLR21 (B) were stimulated with 30 μg of purified chromosomal DNA ml^{-1} from C. jejuni strains 81116, GB18, RM1221, or C. coli strain H1 for 5 h. Cells transfected with control vector were stimulated simultaneously to ensure TLR-specific NF-κB activation. ODN 2006 (0.5 μM) was used as positive control. None of the chromosomal Campylobacter DNA signifi-cantly activated TLR9. In contrast, DNA from all Campylobacter strains induced significant NF- κB activation in TLR21-transfected HEK293 cells ($P < 0.05$). Values are the percent induction of NF-κB activation after stimulation with the positive control and are means ± the SEM of three independent experiments.*

Likewise, studies with knockout mice clearly show the need for TLR activation to control bacterial virulence: TLR5 knockout mice exhibit increased bacterial load and inflammation in the intestine (56), TLR9 deficiency leads to uncontrolled Neisseria meningitidis infection (47), and MyD88-deficient mice are more sensitive to a wide range of bacterial infections (16, 36, 51). The extent to which TLR activation influences *Campylobacter* infection in humans and animal species is currently unknown. We present here the first

systematic comparison of the human and chicken TLR response to *Campylobacter*. We provide evidence of *Campylobacter* activation of human and chicken TLR2 and TLR4 and the lack of activation of human and chicken TLR5. Major differences in the human and chicken TLR response to *Campylobacter* are the failure of chicken cells to produce IFN-β after LOS stimulation in contrast to human cells and the activation of chicken TLR21 but not human TLR9 by *Campylobacter* chromosomal DNA. Another striking result is the poor activation of TLRs by viable *Campylobacter* compared to lysed bacteria. This may indicate bacterial integrity in the intestine as a potential determinant of inflammatory pathology.

The activation of the human and chicken TLR2 complexes by *Campylobacter* indicates that *Campylobacter* lipoproteins can act as TLR2 ligand and are recognized by both species. Based on genome sequence analysis *C. jejuni* is predicted to express up to 40 different lipoproteins and to produce both di- and triacylated lipoproteins (M. R. de Zoete, unpublished results). Humans can differentiate between di- and triacylated lipoproteins by utilizing either TLR2-TLR1 or TLR2-TLR6 combinations. Chickens have two versions of TLR2 (TLR2t1 and TLR2t2) that differ mainly in a 192-amino-acid region in the central region of the ligand-binding domain), one TLR16 protein (also known as TLR1t1 and TLR1LA), and a truncated TLR1t2 protein (also known as TLR1LB) (11, 52). The combinations of chicken TLR2t2/TLR16 and chicken TLR2t1/TLR1LB sense both di- and triacylated lipopeptides, whereas the TLR2t1/TLR16 complex only seems to respond to high concentrations of triacylated peptide (22, 28). The fact that both di- and/or triacylated peptides appear to be recognized by the different TLR2 complexes likely explains the TLR2 response to *Campylobacter* in humans and chicken.

For TLR4, differences in LPS specificity between humans and chickens are reported (30). We found that *Campylobacter* induced potent NF-κB activation via human TLR4/MD-2 and chicken TLR4/MD-2 (Fig. 3). One striking difference between humans and chickens, however, was the absence of *C. jejuni*-induced IFN-β upregulation through TLR4/MD-2 in chicken cells. Stimulation of these cells with the TLR3 ligand poly(I:C) yielded a potent IFN-β response, indicating that the cells are capable of producing this cytokine after TLR activation. In mammals, IFN-β is induced through TLR4 via the MyD88-independent TRIF pathway (53). At present, the adapter protein TRAM, which links the TLR4 signaling domain to the TRIF protein, has not been identified in the chicken genome (30), which may explain the inability to induce a TLR4/MD-2-dependent IFN-β response in this species. IFN-β is a key inducer of systemic inflammation, illustrated by the complete resistance to endotoxic shock in IFN-β knockout mice (27). Also, TLR4-induced IFN-β signaling is critical to control the growth of the intracellular bacterium

Chlamydia pneumoniae by enhancing the production of IFN-γ, the major macrophage-activating cytokine (46). The impact of the absence of this pathway during *Campylobacter* colonization of chickens is unclear. Although additional research is required to determine the precise role of the type I and II IFNs in bacterial infection in chickens, it can be speculated that a relative insensitivity for LOS-induced systemic inflammation and macrophage activation may help to retain the commensal nature of *Campylobacter* in the chicken gut.

TLR5 signaling plays an important role in preventing intestinal pathology, as demonstrated by TLR5-deficient mice, which develop spontaneous intestinal colitis (56). The successful evasion of human TLR5 by *Campylobacter* (Fig. 5) (3, 58) may contribute to intestinal inflammation. Likewise, the lack of intestinal pathology in chickens could be the result of recognition of *Campylobacter* flagellin by the chicken version of TLR5. However, our data indicate equal unresponsiveness of TLR5 from both species to *Campylobacter* flagellin. Therefore, TLR5 evasion seems unlikely to be a major cause of the difference in inflammation between infected humans and chickens.

While the ability of CpG DNA to activate chicken cells has been reported for years (18, 59), the receptor mediating this activation remained elusive due to the absence of a TLR9 homologue. The recent identification of chicken TLR21 as the receptor that senses DNA presented for the first time the opportunity to test the stimulatory potential of *Campylobacter* chromosomal DNA. TLR21 was activated by chromosomal DNA of all tested *Campylobacter* strains (Fig. 6). In contrast, HEK293 cells transfected with human TLR9 did not show an increase in NF-κB activation, although the cells did respond to the TLR9 ligand ODN 2006. These results substantiate that *C. jejuni* DNA is a very weak TLR9 ligand (12). The recognition of both synthetic ODN 2006 and bacterial chromosomal DNA suggest broad ligand specificity for chTLR21. In contrast, mammalian TLR9 (human and murine TLR9) display species-specific ligand recognition and show distinctive responses to ODN 2006. Although highly speculative, these results may indicate that during evolution the presence of a stronger selective advantage for broad recognition of DNA ligands resulted in a divergence between mammals and birds, which may have contributed to the loss of the ligand-specific TLR9 and the gain of the less-specific TLR21 receptor in chickens (44, 52). The role of TLR21 activation by *Campylobacter* during the colonization of chickens remains speculative. It can be imagined that in the nutrient-rich environment of the chicken cecum, bacterial lysis and thus the availability of TLR21 ligand is limited. Alternatively, it can be argued that activation of this receptor may induce an effective but mild local immune response that helps to retain the host-microbe bal-

ance. Lacking a response to *Campylobacter* DNA may result in spread of the bacteria to the deeper tissue, resulting in an uncontrolled infection and enteritis. In addition, as DNA signaling is known to provide strong adjuvant activity in both humans and chickens (10, 19, 35, 37), it can be speculated that the differential TLR activation by *Campylobacter* DNA may influence the host antibody response during infection. However, much more knowledge of the in vivo expression and function of TLR9 and TLR21 in the guts of humans and chickens is needed to determine the exact role of the bacterial DNA as a determinant of infection.

Another major conclusion of our work is that live bacteria induce relatively weak TLR responses compared to disrupted bacteria. This observation was noted for both defined TLR2 and TLR4 responses and for MM6 cells and, to a lesser extent, HD11 cells that express multiple TLR receptors. The weak immune response of intact bacteria could not be contributed to a lack of physical interaction between the bacteria and the TLRs on the cell surface, since centrifugation of *Campylobacter* onto TLR-expressing HeLa 57A cells did not result in increased levels of TLR activation (data not shown). When only heat-killed bacteria were used, the overall responses were significantly lower (M. R. de Zoete, unpublished) than with disrupted bacteria. This suggests that bacterial lysis may be needed to expose their full TLR-stimulative potential. In a hostile environment, *Campylobacter* will likely be disrupted, e.g., after exposure to antimicrobial peptides or after phagocytosis by macrophages and dendritic cells. It is currently unknown whether *Campylobacter* viability and lysis is different in the human gut compared to the nutrient-rich environment of the chicken cecum and thus may contribute to the difference in intestinal inflammatory response.

Taken together, our results indicate that *Campylobacter* is sensed by chicken TLRs in a fashion largely comparable to that of human TLRs. Major differences in the TLR responses are the lack of MyD88-independent IFN-β production in chickens after TLR4 activation and the potent activation of chTLR21 by chromosomal *Campylobacter* DNA. Together with the apparent requirement for bacterial cell lysis for strong innate immune activation, these data may provide a valuable basis for further elucidation of the basis for the different outcomes of *Campylobacter* infection of human and chicken intestines.

References

1. **Akira, S.** 2006. TLR signaling. Curr Top Microbiol Immunol **311:**1-16.
2. **Allos, B. M.** 2001. *Campylobacter jejuni* Infections: update on emerging issues and trends. Clin Infect Dis **32:**1201-1206.
3. **Andersen-Nissen, E., K. D. Smith, K. L. Strobe, S. L. Barrett, B. T. Cookson, S. M. Logan, and A. Aderem.** 2005. Evasion of Toll-like receptor 5 by flagellated bacteria. Proc Natl Acad Sci U S A **102:**9247-9252.
4. **Bakhiet, M., F. S. Al-Salloom, A. Qareiballa, K. Bindayna, I. Farid, and G. A. Botta.** 2004. Induction of alpha and beta chemokines by intestinal epithelial cells stimulated with *Campylobacter jejuni*. J Infect **48:**236-244.
5. **Beery, J. T., M. B. Hugdahl, and M. P. Doyle.** 1988. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. Appl Environ Microbiol **54:**2365-2370.
6. **Beug, H., A. von Kirchbach, G. Doderlein, J. F. Conscience, and T. Graf.** 1979. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell **18:**375-390.
7. **Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser.** 1988. Experimental *Campylobacter jejuni* infection in humans. J Infect Dis **157:**472-479.
8. **Borrmann, E., A. Berndt, I. Hanel, and H. Kohler.** 2007. *Campylobacter*-induced interleukin-8 responses in human intestinal epithelial cells and primary intestinal chick cells. Vet Microbiol **124:**115-124.
9. **Brownlie, R., J. Zhu, B. Allan, G. K. Mutwiri, L. A. Babiuk, A. Potter, and P. Griebel.** 2009. Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. Mol Immunol.
10. **Chu, R. S., O. S. Targoni, A. M. Krieg, P. V. Lehmann, and C. V. Harding.** 1997. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J Exp Med **186:**1623-1631.
11. **Cormican, P., A. T. Lloyd, T. Downing, S. J. Connell, D. Bradley, and C. O'Farrelly.** 2009. The avian Toll-Like receptor pathway--subtle differences amidst general conformity. Dev Comp Immunol **33:**967-973.
12. **Dalpke, A., J. Frank, M. Peter, and K. Heeg.** 2006. Activation of toll-like receptor 9 by DNA from different bacterial species. Infect Immun **74:**940-946.
13. **Dhillon, A. S., H. L. Shivaprasad, D. Schaberg, F. Wier, S. Weber, and D. Bandli.** 2006. *Campylobacter jejuni* infection in broiler chickens. Avian Dis **50:**55-58.

14. **Endtz, H. P., C. W. Ang, N. van Den Braak, B. Duim, A. Rigter, L. J. Price, D. L. Woodward, F. G. Rodgers, W. M. Johnson, J. A. Wagenaar, B. C. Jacobs, H. A. Verbrugh, and A. van Belkum.** 2000. Molecular characterization of *Campylobacter jejuni* from patients with Guillain-Barré and Miller Fisher syndromes. J Clin Microbiol **38:**2297-2301.

15. **Erridge, C., E. Bennett-Guerrero, and I. R. Poxton.** 2002. Structure and function of lipopolysaccharides. Microbes Infect **4:**837-851.

16. **Feng, C. G., C. A. Scanga, C. M. Collazo-Custodio, A. W. Cheever, S. Hieny, P. Caspar, and A. Sher.** 2003. Mice lacking myeloid differentiation factor 88 display profound defects in host resistance and immune responses to *Mycobacterium avium* infection not exhibited by Toll-like receptor 2 (TLR2)- and TLR4-deficient animals. J Immunol **171:**4758-4764.

17. **Friis, L. M., M. Keelan, and D. E. Taylor.** 2009. *Campylobacter jejuni* drives MyD88-independent interleukin-6 secretion via Toll-like receptor 2. Infect Immun **77:**1553-1560.

18. **He, H., T. L. Crippen, M. B. Farnell, and M. H. Kogut.** 2003. Identification of CpG oligodeoxynucleotide motifs that stimulate nitric oxide and cytokine production in avian macrophage and peripheral blood mononuclear cells. Dev Comp Immunol **27:**621-627.

19. **He, H., V. K. Lowry, C. L. Swaggerty, P. J. Ferro, and M. H. Kogut.** 2005. In vitro activation of chicken leukocytes and in vivo protection against *Salmonella enteritidis* organ invasion and peritoneal *S. enteritidis* infection-induced mortality in neonatal chickens by immunostimulatory CpG oligodeoxynucleotide. FEMS Immunol Med Microbiol **43:**81-89.

20. **Hickey, T. E., S. Baqar, A. L. Bourgeois, C. P. Ewing, and P. Guerry.** 1999. *Campylobacter jejuni*-stimulated secretion of interleukin-8 by INT407 cells. Infect Immun **67:**88-93.

21. **Hickey, T. E., A. L. McVeigh, D. A. Scott, R. E. Michielutti, A. Bixby, S. A. Carroll, A. L. Bourgeois, and P. Guerry.** 2000. *Campylobacter jejuni* cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. Infect Immun **68:**6535-6541.

22. **Higuchi, M., A. Matsuo, M. Shingai, K. Shida, A. Ishii, K. Funami, Y. Suzuki, H. Oshiumi, M. Matsumoto, and T. Seya.** 2008. Combinational recognition of bacterial lipoproteins and peptidoglycan by chicken Toll-like receptor 2 subfamily. Dev Comp Immunol **32:**147-155.

23. **Hu, L., M. D. Bray, M. Osorio, and D. J. Kopecko.** 2006. *Campylobacter jejuni* induces maturation and cytokine production in human dendritic cells. Infect Immun **74:**2697-2705.
24. **Ibrahim, G. F., G. H. Fleet, M. J. Lyons, and R. A. Walker.** 1985. Method for the isolation of highly purified *Salmonella* flagellins. J Clin Microbiol **22:**1040-1044.
25. **Johanesen, P. A., and M. B. Dwinell.** 2006. Flagellin-independent regulation of chemokine host defense in *Campylobacter jejuni*-infected intestinal epithelium. Infect Immun **74:**3437-3447.
26. **Jones, M. A., S. Totemeyer, D. J. Maskell, C. E. Bryant, and P. A. Barrow.** 2003. Induction of proinflammatory responses in the human monocytic cell line THP-1 by *Campylobacter jejuni*. Infect Immun **71:**2626-2633.
27. **Karaghiosoff, M., R. Steinborn, P. Kovarik, G. Kriegshauser, M. Baccarini, B. Donabauer, U. Reichart, T. Kolbe, C. Bogdan, T. Leanderson, D. Levy, T. Decker, and M. Muller.** 2003. Central role for type I interferons and Tyk2 in lipopolysaccharide-induced endotoxin shock. Nat Immunol **4:**471-477.
28. **Keestra, A. M., M. R. de Zoete, R. A. van Aubel, and J. P. M. van Putten.** 2007. The central leucine-rich repeat region of chicken TLR16 dictates unique ligand specificity and species-specific interaction with TLR2. J Immunol **178:**7110-7119.
29. **Keestra, A. M., M. R. de Zoete, R. A. van Aubel, and J. P. M. van Putten.** 2008. Functional characterization of chicken TLR5 reveals species-specific recognition of flagellin. Mol Immunol **45:**1298-1307.
30. **Keestra, A. M., and J. P. M. van Putten.** 2008. Unique properties of the chicken TLR4/MD-2 complex: selective lipopolysaccharide activation of the MyD88-dependent pathway. J Immunol **181:**4354-4362.
31. **Kopp, E., and R. Medzhitov.** 2003. Recognition of microbial infection by Toll-like receptors. Curr Opin Immunol **15:**396-401.
32. **Larson, C. L., D. H. Shah, A. S. Dhillon, D. R. Call, S. Ahn, G. J. Haldorson, C. Davitt, and M. E. Konkel.** 2008. *Campylobacter jejuni* invade chicken LMH cells inefficiently and stimulate differential expression of the chicken CXCLi1 and CXCLi2 cytokines. Microbiology **154:**3835-3847.
33. **Lee, M. D., and D. G. Newell.** 2006. *Campylobacter* in poultry: filling an ecological niche. Avian Dis **50:**1-9.
34. **Li, Y. P., H. Ingmer, M. Madsen, and D. D. Bang.** 2008. Cytokine responses in primary chicken embryo intestinal cells infected with *Campylobacter jejuni*

strains of human and chicken origin and the expression of bacterial virulence-associated genes. BMC Microbiol **8:**107.

35. **Mahmood, M. S., M. Siddique, I. Hussain, A. Khan, and M. K. Mansoor.** 2006. Protection capability of recombinant plasmid DNA vaccine containing VP2 gene of very virulent infectious bursal disease virus in chickens adjuvanted with CpG oligodeoxynucleotide. Vaccine **24:**4838-4846.
36. **Mancuso, G., A. Midiri, C. Beninati, C. Biondo, R. Galbo, S. Akira, P. Henneke, D. Golenbock, and G. Teti.** 2004. Dual role of TLR2 and myeloid differentiation factor 88 in a mouse model of invasive group B streptococcal disease. J Immunol **172:**6324-6329.
37. **McCluskie, M. J., and A. M. Krieg.** 2006. Enhancement of infectious disease vaccines through TLR9-dependent recognition of CpG DNA. Curr Top Microbiol Immunol **311:**155-178.
38. **Meade, K. G., F. Narciandi, S. Cahalane, C. Reiman, B. Allan, and C. O'Farrelly.** 2009. Comparative in vivo infection models yield insights on early host immune response to *Campylobacter* in chickens. Immunogenetics **61:**101-110.
39. **Montminy, S. W., N. Khan, S. McGrath, M. J. Walkowicz, F. Sharp, J. E. Conlon, K. Fukase, S. Kusumoto, C. Sweet, K. Miyake, S. Akira, R. J. Cotter, J. D. Goguen, and E. Lien.** 2006. Virulence factors of *Yersinia pestis* are overcome by a strong lipopolysaccharide response. Nat Immunol **7:**1066-1073.
40. **Parker, C. T., B. Quinones, W. G. Miller, S. T. Horn, and R. E. Mandrell.** 2006. Comparative genomic analysis of *Campylobacter jejuni* strains reveals diversity due to genomic elements similar to those present in *C. jejuni* strain RM1221. J Clin Microbiol **44:**4125-4135.
41. **Pasare, C., and R. Medzhitov.** 2003. Toll-like receptors: balancing host resistance with immune tolerance. Curr Opin Immunol **15:**677-682.
42. **Pearson, B. M., D. J. Gaskin, R. P. Segers, J. M. Wells, P. J. Nuijten, and A. H. van Vliet.** 2007. The complete genome sequence of *Campylobacter jejuni* strain 81116 (NCTC11828). J Bacteriol **189:**8402-8403.
43. **Rathinam, V. A., D. M. Appledorn, K. A. Hoag, A. Amalfitano, and L. S. Mansfield.** 2009. *Campylobacter jejuni*-induced activation of dendritic cells involves cooperative signaling through Toll-like receptor 4 (TLR4)-MyD88 and TLR4-TRIF axes. Infect Immun **77:**2499-2507.

44. **Roach, J. C., G. Glusman, L. Rowen, A. Kaur, M. K. Purcell, K. D. Smith, L. E. Hood, and A. Aderem.** 2005. The evolution of vertebrate Toll-like receptors. Proc Natl Acad Sci U S A **102:**9577-9582.
45. **Rodriguez, M. S., J. Thompson, R. T. Hay, and C. Dargemont.** 1999. Nuclear retention of IkappaBalpha protects it from signal-induced degradation and inhibits nuclear factor kappaB transcriptional activation. J Biol Chem **274:**9108-9115.
46. **Rothfuchs, A. G., D. Gigliotti, K. Palmblad, U. Andersson, H. Wigzell, and M. E. Rottenberg.** 2001. IFN-alpha beta-dependent, IFN-gamma secretion by bone marrow-derived macrophages controls an intracellular bacterial infection. J Immunol **167:**6453-6461.
47. **Sjolinder, H., T. H. Mogensen, M. Kilian, A. B. Jonsson, and S. R. Paludan.** 2008. Important role for Toll-like receptor 9 in host defense against meningococcal sepsis. Infect Immun **76:**5421-5428.
48. **Smith, C. K., M. Abuoun, S. A. Cawthraw, T. J. Humphrey, L. Rothwell, P. Kaiser, P. A. Barrow, and M. A. Jones.** 2008. *Campylobacter* colonization of the chicken induces a proinflammatory response in mucosal tissues. FEMS Immunol Med Microbiol **54:**114-121.
49. **Smith, C. K., P. Kaiser, L. Rothwell, T. Humphrey, P. A. Barrow, and M. A. Jones.** 2005. *Campylobacter jejuni*-induced cytokine responses in avian cells. Infect Immun **73:**2094-2100.
50. **Steeghs, L., A. M. Keestra, A. van Mourik, H. Uronen-Hansson, P. van der Ley, R. Callard, N. Klein, and J. P. M. van Putten.** 2008. Differential activation of human and mouse Toll-like receptor 4 by the adjuvant candidate LpxL1 of *Neisseria meningitidis*. Infect Immun **76:**3801-3807.
51. **Takeuchi, O., K. Hoshino, and S. Akira.** 2000. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. J Immunol **165:**5392-5396.
52. **Temperley, N. D., S. Berlin, I. R. Paton, D. K. Griffin, and D. W. Burt.** 2008. Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. BMC Genomics **9:**62.
53. **Uematsu, S., and S. Akira.** 2007. Toll-like receptors and Type I interferons. J Biol Chem **282:**15319-15323.
54. **van Putten, J. P. M., L. B. van Alphen, M. M. S. M. Wösten, and M. R. de Zoete.** 2009. Molecular Mechanisms of *Campylobacter* Infection. Curr Top Microbiol Immunol **337:**In press.

55. **Vijay-Kumar, M., J. D. Aitken, and A. T. Gewirtz.** 2008. Toll like receptor-5: protecting the gut from enteric microbes. Semin Immunopathol **30:**11-21.

56. **Vijay-Kumar, M., C. J. Sanders, R. T. Taylor, A. Kumar, J. D. Aitken, S. V. Sitaraman, A. S. Neish, S. Uematsu, S. Akira, I. R. Williams, and A. T. Gewirtz.** 2007. Deletion of TLR5 results in spontaneous colitis in mice. J Clin Invest **117:**3909-3921.

57. **Walker, R. I., M. B. Caldwell, E. C. Lee, P. Guerry, T. J. Trust, and G. M. Ruiz-Palacios.** 1986. Pathophysiology of *Campylobacter* enteritis. Microbiol Rev **50:**81-94.

58. **Watson, R. O., and J. E. Galan.** 2005. Signal transduction in *Campylobacter jejuni*-induced cytokine production. Cell Microbiol **7:**655-665.

59. **Xie, H., R. B. Raybourne, U. S. Babu, H. S. Lillehoj, and R. A. Heckert.** 2003. CpG-induced immunomodulation and intracellular bacterial killing in a chicken macrophage cell line. Dev Comp Immunol **27:**823-834.

60. **Zheng, J., J. Meng, S. Zhao, R. Singh, and W. Song.** 2008. *Campylobacter*-induced interleukin-8 secretion in polarized human intestinal epithelial cells requires *Campylobacter*-secreted cytolethal distending toxin- and Toll-like receptor-mediated activation of NF-kappaB. Infect Immun **76:**4498-4508.

61. **Ziegler-Heitbrock, H. W., E. Thiel, A. Futterer, V. Herzog, A. Wirtz, and G. Riethmuller.** 1988. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. Int J Cancer **41:**456-461.

5

Reconstitution of a functional Toll-like receptor 5 binding site in *Campylobacter jejuni* flagellin

Marcel R. de Zoete
A. Marijke Keestra
Jaap A. Wagenaar
Jos P. M. van Putten

Journal of Biological Chemistry 285:12149-12158. 2010.

Abstract

Bacterial flagellin is important for intestinal immune homeostasis. Flagellins from most species activate Toll-like receptor 5 (TLR5). The principal bacterial food-borne pathogen *Campylobacter jejuni* (*C. jejuni*) escapes TLR5 recognition, probably due to an alternate flagellin subunit structure. We investigated the molecular basis of TLR5 evasion by aiming to reconstitute TLR5 stimulating activity in live *C. jejuni*. Both native glycosylated *C. jejuni* flagellins (FlaA and FlaB) and recombinant proteins purified from *E. coli* failed to activate NF-κB in HEK293 cells expressing TLR5. Introduction of multiple defined regions from *Salmonella* flagellin into *C. jejuni* FlaA via a recombinatorial approach revealed three regions critical for the activation of human and mouse TLR5, including a β-hairpin structure not previously implicated in TLR5 recognition. Surprisingly, this domain was not required for the activation of chicken TLR5, indicating a selective requirement for the β-hairpin in the recognition of mammalian TLR5. Expression of the active chimeric protein in *C. jejuni* resulted in secreted glycosylated flagellin that induced a potent TLR5 response. Overall, our results reveal a novel structural requirement for TLR5 recognition of bacterial flagellin and exclude flagellin glycosylation as an additional mechanism of bacterial evasion of the TLR5 response.

Introduction

Flagellin, the monomeric subunit of the bacterial motility apparatus, is the natural ligand of the innate immune sensor Toll-like receptor 5 (TLR5) (14). Activation of TLR5 by flagellin initiates a powerful host response that provides crucial signals for maintaining intestinal immune homeostasis (45, 50). The immunostimulatory properties make flagellin an attractive vaccine carrier protein and potent vaccine adjuvant. Its intrinsic adjuvant activity is currently being employed in experimental recombinant vaccines against human influenza, West Nile fever, malaria, tuberculosis and plague (4, 15, 21, 27, 36, 38). In addition, flagellin-induced immune activation protects the intestine and other tissues against lethal irradiation due to potent TLR5-mediated anti-apoptotic effects (5, 31).

The immunological impact of flagellin stimulation has driven several bacterial pathogens to evolve mechanisms to escape the effective TLR5-mediated host defense. In *Salmonella enterica* serotype Typhi, this is achieved by repression of flagellin expression and secretion (54), while *Listeria* shuts off flagellin expression at the host temperature of 37°C (53). The flagellins of the α- and ε-proteobacteria, which include the major foodborne pathogen *Campylobacter jejuni* and the gastric pathogen *Helicobacter pylori*, fail to activate TLR5 altogether (2, 11, 18, 22, 52). For these organisms the consequences of TLR5 evasion for infection are currently unknown. It has been demonstrated that purified *H. pylori* flagellin induces severely impaired adaptive immune responses in comparison to TLR5-activating flagellins (35).

The flagellin protein of *C. jejuni* clearly differs from bacterial flagellins that do activate TLR5. Electron microscopy shows that the *C. jejuni* flagellar filament comprises seven longitudinal helical arrays of stacked flagellin subunits (protofilaments) instead of the eleven present in e.g. *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium), suggesting differences in flagellin polymerization between these species (10). Consistent with this hypothesis, the amino acid regions of flagellin involved in filament assembly in *Salmonella* have diverged in *Campylobacter*. These changes may contribute to the TLR5 evasion in *C. jejuni* (2, 37). An additional difference between *C. jejuni* flagellin and most TLR5-activating flagellins is the presence of pseudaminic acid derivatives that cover the putative surface exposed region of *C. jejuni* flagellin and that may comprise up to 10% of their total weight (40). The contribution of the post-translational modification of *Campylobacter* flagellin to evasion of the TLR5 response is currently unknown.

Considering the important role of TLR5 in intestinal biology and the potential of bacterial flagellin as a vaccine adjuvant, we sought to better define the molecular basis of

the TLR5 evasion by *C. jejuni* flagellin by attempting to restore TLR5-stimulating activity. Mutagenesis or replacement of larger amino acid regions between flagellins has previously been instrumental in defining residues critical for TLR5 recognition (2, 7, 8, 17, 30, 37). Using a series of recombinant chimeric flagellins, we succeeded in reconstituting a *Campylobacter* flagellin with TLR5 activating ability and in the engineering of *C. jejuni* that processes, glycosylates and secretes flagellins that yield a potent TLR5 response. Activation of human TLR5 by *Campylobacter* was not influenced by flagellin glycosylation but required introduction of three defined domains of *Salmonella* flagellin. Differential activation of mammalian and chicken TLR5 by recombinant flagellins led to the discovery of a β-hairpin structure not previously implicated in mammalian TLR5 recognition.

Materials and Methods

Cell lines and bacterial strains

HeLa 57A cells stably transfected with a NF-κB luciferase reporter construct (34), HEK293 cells, and HT-29 intestinal epithelial cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 5% fetal calf serum (FCS) at 37°C under 5% CO_2. NHEK human primary keratinocytes were propagated under the same conditions in KGM-2 Keratinocyte Growth Medium-2 (Lonza). *C. jejuni* strains 81116 (NCTC11828) (33), NCTC 11168H1 (19) and their derivatives were grown at 37°C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) on saponin agar medium containing 4% lysed horse blood with the appropriate antibiotics. *Salmonella enterica* ssp. Enteritidis 90-13-706 and *S*. Enteritidis 90-13-706Δ*fliC* (44), and *Escherichia coli* BL21(DE3) Star (Invitrogen), used to express recombinant flagellins, were grown in Luria Bertani broth at 37°C.

Purification of native flagellin

Native flagellin of *S*. Enteritidis was purified overnight cultures as described (16), with minor modifications. Briefly, bacteria were resuspended in 10 mM Tris·HCl / 145 mM NaCl (pH 7.4), homogenized (2 min) and centrifuged twice (10,000 x *g*, 20 min, 4°C), discarding the pellet after each centrifugation. Flagella were collected from the supernatant by centrifugation (100,000 x *g*, 60 min, 4°C) and depolymerized in 0.2 M of glycine (pH 2) (30 min, 20°C, with stirring). After centrifugation (100,000 x *g*, 60 min, 4°C), the supernatant containing monomeric flagellin was adjusted to pH 7.2 with 1 M of NaOH and am-

monium sulphate was added to a final concentration of 2.67 M. Following overnight incubation (20°C), repolymerized flagellins were collected by centrifugation (14,000 x *g*, 15 min, 4°C), dissolved in H_2O, and dialyzed against H_2O (24 h, 4°C). Native flagellin of *C. jejuni* strain 81116 was purified as described (26) and stored at -20°C.

Construction and purification of chimeric flagellins

The construction of recombinant *Salmonella* FliC has been described (20). Recombinant FlaA and FlaB of *C. jejuni* strain 81116 were obtained after amplification of the corresponding genes with *pfu* polymerase (Promega) using primer pairs 6xhis-FlaA-F and 6xhis-FlaA-R, and 6xhis-FlaB-F and 6xhis-FlaB-R, respectively (Table I). Products were ligated into expression vector pT7-7 (41) using restriction enzymes *Bam*HI and *Cla*I. Chimeric flagellins were constructed by overlap extension PCR using primers and template as depicted in Table I, unless stated otherwise. For construction of chimera $FlaA^N$ the template was a mixture of FlaA(1-52), FliC(N) and FlaA(123-576). For chimera $FlaA^C$: FlaA(1-491), FliC(C) and FlaA(527-576); for $FlaA^{NC}$: FlaA(1-491) from template $flaA^N$, FliC(C) and FlaA(527-576); for $FlaA^{NVC}$: FlaA(1-52), FliC(NVC) and FlaA(527-576); for $FlaA^H$: FlaA(1-122), FliC(H) and FlaA(177-576); for $FlaA^{NH}$: FlaA(1-122) from template $flaA^N$, FliC(H) and FlaA(177-576); for $FlaA^{HC}$: FlaA(1-122), FliC(H) and FlaA(177-576) from template $flaA^C$; for $FlaA^{NHC}$: FlaA(1-122) from template $flaA^N$, FliC(H) and FlaA(177-576) from template $flaA^C$. The obtained chimeric flagellin genes were amplified using primers FlaA-topoF and FlaA-topoR and ligated in pET101/D-TOPO (Invitrogen). All plasmid constructs were propagated in *E. coli* DH5α and transformed into *E. coli* BL21(DE3) Star for protein expression.

Recombinant 6xHis-tagged proteins were obtained by incubating pellets of IPTG-induced (1 mM, 5 h, 37°C) bacterial cultures in 8 M urea for 17 h (20°C). After sonication and centrifugation (5000 x *g*, 15 min, 20°C) to remove debris, 6 xHis-tagged flagellin was purified with Ni-NTA agarose (Qiagen). After washing with 8 M urea, pH 6.4, flagellin was eluted in steps with 8 M urea, pH 5.3 and 8 M urea, pH 4.5, respectively. For recombinant FliC, FlaA and FlaB, fractions containing flagellin were pooled, dialyzed (24 h, 4°C) against 10 mM of Tris·HCl (pH 9.0), and centrifuged (100,000 x g, 60 min) to remove protein aggregates. Chimeric flagellins and control recombinant FlaA and FliC were kept at -20°C in 8 M urea, pH 4.5 at a concentration of 500 μg ml^{-1}. Proteins were analyzed on SDS-PAGE and concentrations were determined by using the BCA Protein Assay kit (Thermo Scientific Pierce).

Construction of *C. jejuni* mutants

The complete *flaA-flaB* region of *C. jejuni* 81116 was amplified by PCR with primers FlaA3-topoF and FlaAB-mutant-R (Table 1) and ligated into pGEM-T easy (Promega). *Eco*RV was used to remove the last 714 nucleotides of *flaA* and the N-terminal 1017 nucleotides of *flaB*. This fragment was replaced with a chloramphenicol resistance cassette obtained by digestion of pAV35 (47) with *Pvu*II (resulting in plasmid pMR108). *C. jejuni* mutant strain 11168H1Δ*flaAB* was constructed by homologues recombination through electroporation using the pMR108 deletion plasmid and strain *C. jejuni* 11168H1, as described (51). The σ28 *flaA* promoter region was amplified from *C. jejuni* 81116 with primers FlaAFSphI and FlaARNsiI, and cloned into pMA1 (46) using *Sph*I and *Nsi*I, resulting in pMA3. For the expression of flagellin proteins in *C. jejuni*, *flaA*, *flaA*^{NC} and *flaA*^{NHC} were PCR amplified with primers Flagellin-pMA3-F and Flagellin-pMA3-R using the his-tagged expression constructs as template, digested with *Sac*I and *Sac*II, and cloned into the multiple cloning site of pMA3. Conjugation to *C. jejuni* was performed as described (46).

Transient transfection

HEK293 and HeLa 57A cells (~70% confluent) kept in 48-well plates were transiently transfected with 50 µl of a mixture of plasmid DNA and FuGENE 6 (Roche Diagnostics) in DMEM at a lipid to DNA ratio of 3 to 1. HEK293 cells were transfected with 50 ng of NF-κB-luc plasmid and 70 ng of pTK-LacZ, which was used for normalization of transfection efficiency. HeLa 57A cells were transfected with 125 ng of pFlag-human-TLR5, pFlag-mouse-TLR5, pFlag-chicken-TLR5 (20), or pFlag-CMV1 empty vector (Sigma-Aldrich) together with 125 ng of pTK-LacZ. Cells were used in TLR5 stimulation assays at 48 h after transfection.

Toll-like receptor 5 stimulation assays

Transfected cells were placed in 0.5 ml of fresh DMEM with 5% FCS prior to stimulation with bacteria or purified flagellin. For cell stimulation, *S*. Enteritidis and *C. jejuni* were grown (17 h) in Luria Bertani broth and Heart Infusion broth respectively, collected by centrifugation (5,000 x *g*, 10 min, 22°C) and resuspended in Dulbecco's phosphate buffered saline (DPBS). Bacteria were added to the transfected cells at an m.o.i. of 1:100. After 3.5 h of stimulation, cells were rinsed three times with DMEM-5% FCS to prevent bacterial overgrowth, and further incubated in fresh DMEM-5%FCS. Bacterial culture supernatants were collected (5,000 x *g*, 10 min, 22°C) after 16 hrs of growth, filtered (0.22 µm, Millipore), and added to transfected cells (10 µl per well). Native flagellin was depolyme-

rized at 70°C for 20 min prior to stimulation and added to the cells at the indicated concentrations (ng ml^{-1}). Recombinant FlaA, FliC and chimeric flagellins, stored in 8 M urea pH 4.5 at a concentration of 500 µg ml^{-1} (see above) were instantly diluted 500-fold by adding 1 µl of protein solution per well. All flagellin stimulations were stopped after 5 h by rinsing the cells twice with DPBS, lysis in 0.1 mL of Reporter Lysis Buffer (Promega), and freezing at -80°C. Luciferase activity was measured in a luminometer (TD-20/20, Turner Designs) after mixing 20 µL of thawed cell lysate with 0.1 mL of Luciferase Reagent (Promega). For normalization of transfection efficiency, luciferase values were adjusted to β-galactosidase values determined with the β-galactosidase assay (Promega). Results were expressed in relative light units and represent the means of duplicate values of three independent experiments.

RT-PCR

RNA from HT-29 and NHEK cells stimulated for 2 h with 1 µg ml^{-1} of recombinant flagellins, was isolated using RNA-Bee (Bio-Connect). Subsequent DNase I treatment and RT-PCR analysis for actin and IL-8 mRNA was performed as described previously (6).

Detection of flagellins produced by *C. jejuni*

Whole bacterial lysates and culture supernatant were prepared from 17 h of *C. jejuni* cultures. After centrifugation (5,000 x *g*, 10 min, 22°C), the supernatant was collected and the pellet was resuspended in an equal amount of DPBS for SDS-PAGE analysis. Secreted and intracellular flagellins were detected by Western blot analysis using anti-FlaA antibody CF1 (1:500 dilution) (32) and HRP-conjugated Goat-anti mouse IgG (Sigma-Aldrich). Reactive bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermal Scientific Pierce). CF1 recognizes an epitope in a 193 amino acid stretch in the variable domain of *C. jejuni* 81116 FlaA. In chimera $FlaA^{NHC}$ the first 30 amino acids of this stretch has been replaced by corresponding *S.* Enteritidis sequence, which did not influence CF1 recognition. For 2D electrophoresis, culture supernatant was concentrated 20 times using Centricon YM-30 filters (Millipore) and mixed with rehydration solution (7 M urea, 2 M thiourea, 4% (w/v) chaps, 0.5% IPG buffer, pH 4-7 (Amersham Biosciences) and 0.3% (w/v) dithiothreitol). First-dimension isoelectric focusing was performed on an IPGphor (Amersham Biosciences) with immobilized non-linear pH (3-10) gradient strips (Amersham Biosciences) using the following IEF parameters: 12 hrs at 30 V, 30 min 500 V, 30 min 100 V, 1 hrs 40 min 6000 V and 2 hrs 500 V. IEF strips were equilibrated for 15 min in 50 mM Tris-HCl pH 8.8, 6 M urea, 2% SDS,

30% glycerol and 10 mg ml^{-1} DTT, followed by 15 min in 50 mM Tris-HCl pH 8.8, 6 M urea, 2% SDS, 30% glycerol and 25 mg ml^{-1} iodoacetamide. Second-dimension SDS-PAGE was performed using 10% polyacrylamide gels. Flagellins were detected as described above.

Results

TLR5-activating properties of *Campylobacter* flagellin.

The inability of *Campylobacter jejuni* flagellin to activate TLR5 has been demonstrated for native and recombinant flagellin from strain 81-176 (2, 18, 52). As *C. jejuni* flagellins show considerable sequence variation between different strains, are present in two differentially regulated isoforms (FlaA and FlaB) (55), and show variable glycosylation (24, 42, 43), we first examined the efficacy of *C. jejuni* strain 81116 to signal via TLR5. Flagellin activity was measured in HEK293 cells expressing TLR5 with an NF-κB-luciferase reporter as a read-out system. In this system, both *Salmonella* Enteritidis and its culture supernatant (containing secreted flagellin) but not the flagellin deficient (Δ*fliC*)strain induced a robust TLR5 response (Fig. 1A and 1B). In contrast, neither C. jejuni strain 81116 nor its culture supernatant activated NF-κB in the TLR5-expressing cells (Fig. 1A and 1B). Analysis of 10 additional clinical *C. jejuni* isolates confirmed the evolutionary conservation of this trait (data not shown). To exclude limited monomeric flagellin release as a cause of the inability to activate TLR5, native *C. jejuni* flagellin was purified. Isolated *C. jejuni* 81116 flagellin was also unable to activate TLR5, even at concentrations 10,000 fold higher than native flagellin of *S.* Enteritidis (Fig. 1C). Experiments with HeLa 57A cells transfected with TLR5 instead of HEK293 cells yielded similar results (Fig. 1D). Competition assays showed that an excess of native *C. jejuni* 81116 flagellin did not antagonize HEK293 activation by flagellin of *S.* Enteritidis (data not shown).

TLR5-stimulating activity of recombinant *C. jejuni* FlaA and FlaB.

TLR5 activation by *Salmonella* flagellin requires the amino acids 89-96 at the bridge of the α-helices ND1a and ND1b in the N-terminal conserved domain (Fig. 2, black box) (2, 37). An additional region, located in the center of the conserved C-terminal CD1 α-helix (Fig. 2, grey box) appears critical for stability of the N-terminal TLR5-binding domain (2, 30, 37). Sequence analysis of *C. jejuni* FlaA shows considerable deviation of both the rele-

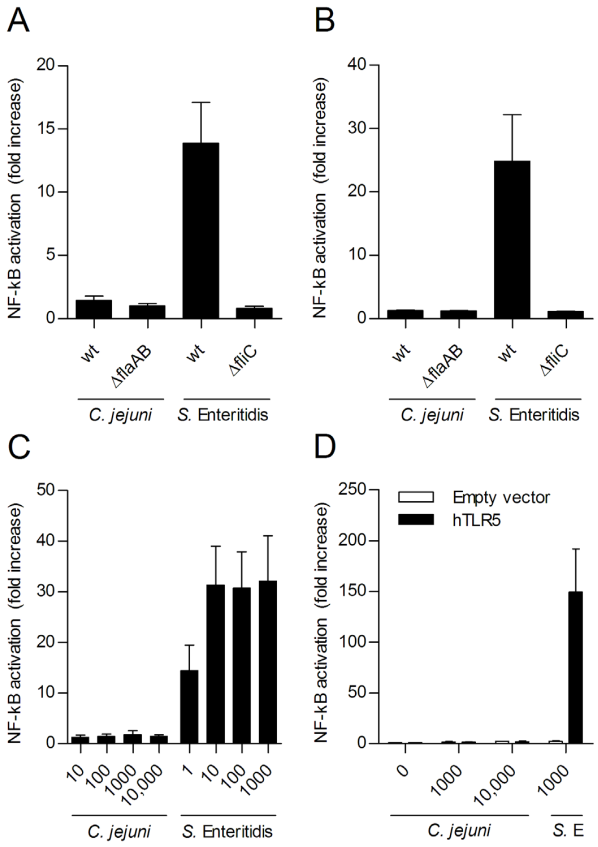

Figure. 1. C. jejuni fails to activate TLR5. *A and B, NF-κB activation was measured for TLR5-expressing HEK-293 cells stimulated with (A) live bacteria or (B) culture supernatant of wildtype (wt) C. jejuni, C. jejuni ΔflaAB, wt S. Enteritidis or S. Enteritidis ΔfliC. C, NF-κB activation was measured for TLR5-expressing HEK-293 cells stimulated for 5 h with purified native C. jejuni flagellin or S. Enteritidis flagellin at the indicated concentrations (ng ml^{-1}). D, NF-κB activation was measured for human TLR5-transfected or control HeLa 57A cells stimulated for 5 h with purified native C. jejuni and S. Enteritidis (S. E) flagellin at the indicated concentrations (ng ml^{-1}). Values represent the increase of NF-κB-induced luciferase activity in stimulated cells compared to non-stimulated cells and are the mean ± SEM of three independent experiments.*

vant N- and C-terminal regions from the corresponding regions of *Salmonella* FliC ((2) and Fig. 2). The independently expressed *C. jejuni* FlaB subunit is identical to FlaA in its N-terminal TLR5 binding site but differs at several amino acids in the center of the CD1 α-helix (Fig. 2, indicated by asterisks). To examine the potential relevance of these changes in amino acid composition for TLR5 activation, we expressed both FlaA and FlaB of *C. jejuni* strain 81116 as polyhistidine-tagged proteins in *Escherichia coli* and purified them by Ni^{2+}-affinity chromatography. SDS-PAGE analysis of the native and recombinant *C. jejuni* flagellins demonstrated a markedly lower apparent molecular mass for the recombinant proteins compared to native *C. jejuni* flagellin consistent with the absence of attached glycan moieties (Fig. 3A). The difference in electrophoretic mobility was not observed for recombinant and native *S*. Enteritidis flagellin (FliC) in agreement with the lack of flagellin glycosylation in this species. Functional assays using TLR5 expressing

HEK293 cells showed that both recombinant FlaA and FlaB failed to activate NF-κB (Fig. 3B), while purified recombinant *Salmonella* FliC induced a potent response. These results demonstrate that the non-glycosylated forms of both *C. jejuni* FlaA and FlaB lack TLR5-stimulating activity. As glycosylation of FlaA and FlaB is needed for flagella assembly (12) and thus possibly for appropriate folding of the protein, we also tested native flagellins isolated from *Campylobacter* 81116 FlaA and FlaB mutant strains. These proteins also failed to activate TLR5 (data not shown). Together, the data indicate that neither of the *C. jejuni* flagellins is able to activate TLR5, irrespective of their state of glycosylation.

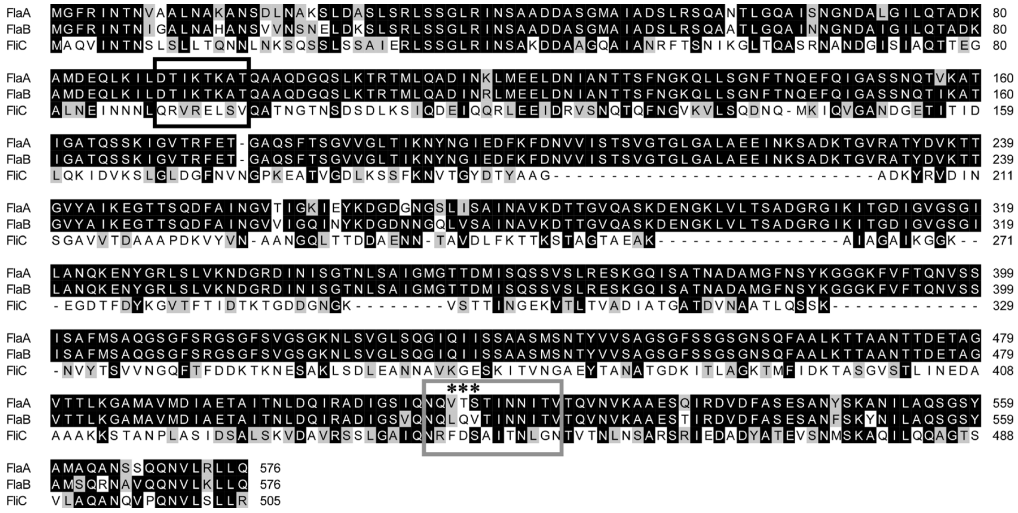

Figure. 2. ClustalW alignment of C. jejuni 81116 FlaA, FlaB and S. Enteritidis 706 FliC. *The stretch of amino acids proposed to bind TLR5 in the N-terminal conserved domain are boxed in black, the crucial residues for TLR5 activation in the C-terminal conserved domain are boxed in grey. Asterisks indicate differences in amino acid sequence between FlaA and FlaB in the C-terminal domain.*

Construction and function of chimeric flagellins.

In an attempt to restore the ability of *C. jejuni* flagellin to bind and activate TLR5, we replaced a part of its ND1 α-helix region with the corresponding region of *S.* Enteritidis FliC that contains the putative TLR5 binding site (chimera FlaA^{N}, Fig. 4). Comparative modeling of FlaA on the structure of *S*. Enteritidis flagellin was used to select amino acids re-

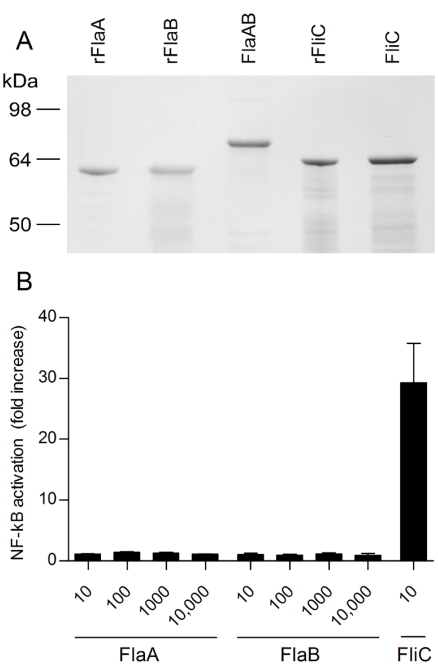

Figure. 3. Recombinant, non-glycosylated C. jejuni FlaA and FlaB fail to activate TLR5. *A, SDS-PAGE was performed to examine differences in electrophoretic mobility between recombinant non-glycosylated C. jejuni flagellins (rFlaA and rFlaB) and native glycosylated C. jejuni flagellin (FlaAB). As a control, recombinant and native S. Enteritidis flagellin (rFliC and FliC, respectively) were analyzed. B, NF-κB translocation was measured in TLR5-expressing HEK293 cells after stimulation (5 h) with recombinant non-glycosylated C. jejuni FlaA and FlaB at the indicated concentrations (ng ml^{-1}). S. Enteritidis FliC (10 ng ml^{-1}) was used as a positive control. Values represent the increase of NF-κB-induced luciferase activity in stimulated cells compared to non-stimulated cells and are the mean ± SEM of three independent experiments.*

gions that were predicted to yield minimal changes in the overall protein configuration. A second chimeric flagellin was constructed in which the CD1 α-helix region was replaced (chimera FlaA^{C}) and a third by exchanging both the α-helices ND1 and CD1 (chimera FlaA^{NC}). Finally, a control chimera was constructed that contained both *S*. Enteritidis α-helices ND1 and CD1 together with the entire central variable region (chimera FlaA^{NVC}, Fig. 4). All recombinant proteins were expressed in *E. coli*, purified, and tested for their ability to activated TLR5 in HEK293 cells. The control FlaA^{NVC} chimera was fully able to induce NF-κB translocation (Fig. 5A), confirming data that the structurally disordered extreme N- and C-terminal regions of flagellin are not involved in TLR5 engagement (8), and excluding the possibility these regions inhibit receptor activation. Functional analysis of the other three chimeric proteins unexpectedly showed that none of the chimeric flagellins had regained the ability to activate NF-κB (Fig. 5A), regardless the presence of both conserved regions critical for *Salmonella* flagellin to activate TLR5.

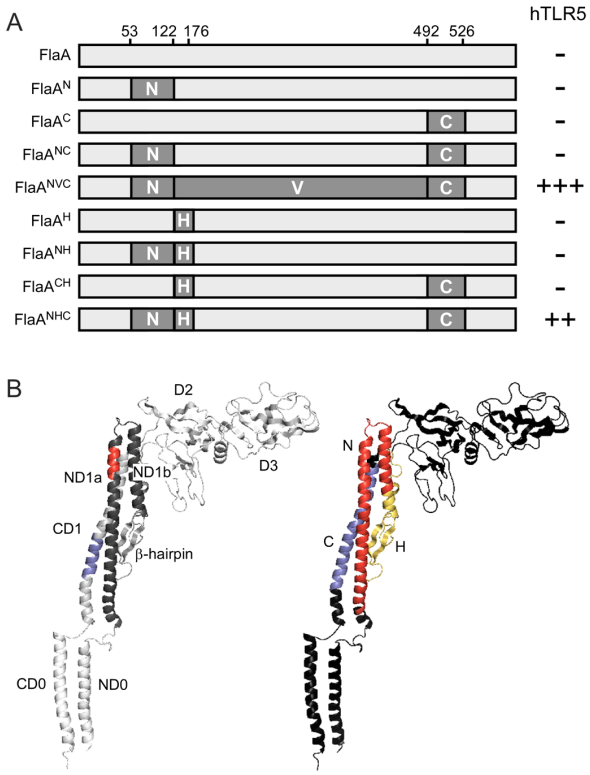

Figure. 4. Characteristics of chimeric flagellin proteins. *A, Schematic overview of the constructed chimeric flagellins. Numbers refer to the start and end amino acid positions of the exchanged FlaA domains N, H, V, and C. B, The structure of S. Typhimurium flagellin (PDB: 1UCU) shows (left) the different flagellin domains as well as the N- and C-terminal regions proposed to be involved in TLR5 activation (in red and blue, respectively), and (right) the location of the exchanged N (red), H (yellow) and C (blue) domains. The potential of the recombinant flagellins to activate human TLR5 is indicated on the right as -, +, ++, or +++.*

Reconstitution of human TLR5 recognition in *C. jejuni* FlaA requires a variable β-hairpin region of *S. Enteritidis* FliC.

In search for additional regions required for restoration of TLR5 activation in a *Campylobacter* flagellin backbone, we focused on the β-hairpin region following the ND1b helix in *Salmonella* flagellin. This hairpin structure is involved in multimerization of flagellin subunits and may further stabilize the intramolecular structure formed by the highly conserved α-helices (56). Due to low sequence homology between bacterial species, the β-hairpin region has thus far been ignored as part of a direct TLR5 binding site. To assess the role of the 56 amino acid β-hairpin, we constructed a second series of chimeric flagellins (Fig. 4). Replacement of the β-hairpin region of *C. jejuni* FlaA with the β-hairpin from *S.* Enteritidis FliC ($FlaA^{H}$) was not sufficient to induce TLR5 activation (Fig. 5B). Similarly, chimeras consisting of *C. jejuni* flagellin with two out of three *Salmonella* regions (chimera $FlaA^{NH}$ and chimera $FlaA^{CH}$) were inactive (Fig. 5B). However, a chimeric flagellin

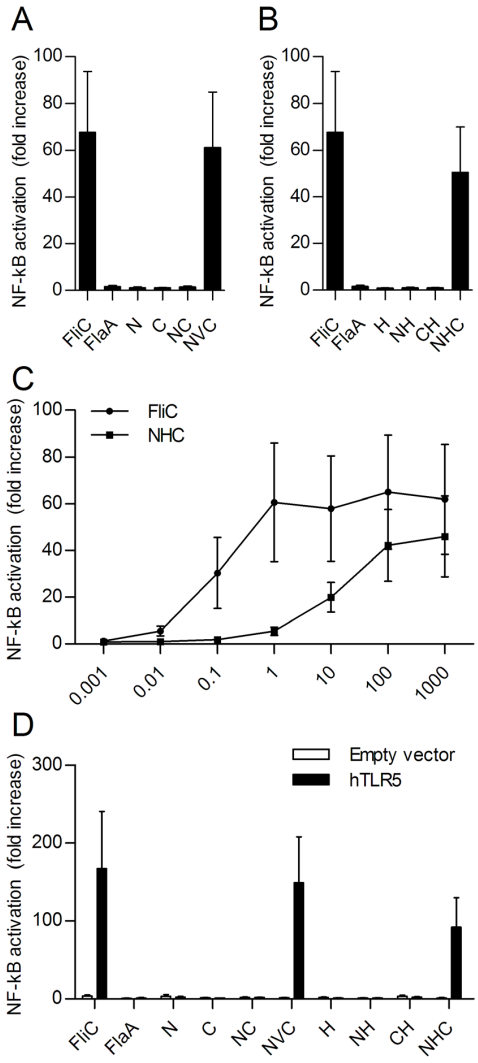

Figure 5. The TLR5 stimulatory activity of the recombinant chimeric flagellins. *A and B, NF-κB activation was measured for TLR5-expressing HEK293 cells after 5 h of stimulation with the indicated flagellins (1 μg ml^{-1}). C, TLR5-expressing HEK293 cells stimulated with increasing concentrations (ng ml^{-1}) of recombinant FlaA^{NHC} or FliC shows the dose-response relationship. D, NF-κB activation in HeLa 57A cells transfected with either human TLR5 or empty vector after stimulation (5 h) with 1 μg ml^{-1} of the indicated recombinant flagellins. Values represent the increase of NF-κB-induced luciferase activity in stimulated cells compared to non-stimulated cells and are the mean ± SEM of three independent experiments.*

containing the conserved ND1 and CD1 regions together with the variable β-hairpin region (chimera FlaA^{NHC}) strongly activated TLR5 in both HEK293 cells (Fig. 5B and 5C) and HeLa 57A carrying human TLR5 but not empty vector (Fig. 5D). To further verify that the β-hairpin plays a role in TLR5 stimulation, we tested the activity of the chimeric flagellins FlaA^{NC} and FlaA^{NHC} in the non-transfected human intestinal epithelial cell-line HT-29 and in non-transformed primary human epithelial cells, which both endogenously express TLR5. FlaA^{NHC}, but not FlaA or FlaA^{NC} enhanced IL-8 transcript levels in both cell

types (Fig. 6). Together, these results indicate that at least three distinct sections of *Salmonella* flagellin are required to reconstitute human TLR5-stimulating activity in *Campylobacter* flagellin.

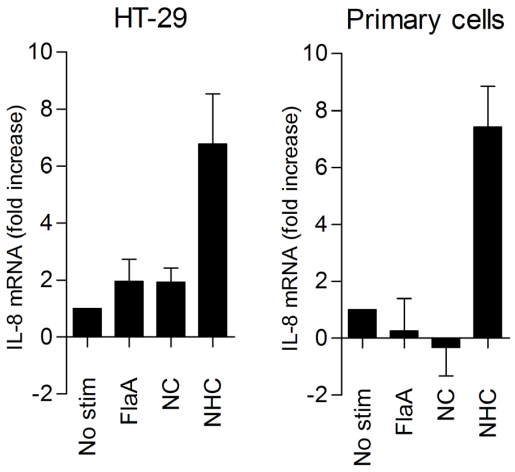

Figure 6. IL-8 mRNA induction by FlaA^{NHC} in human non-transfected intestinal and primary epithelial cells. *HT-29 cells (A) and non-transformed primary human cells (B) were stimulated (2 h) with 1 µg ml^{-1} of recombinant FlaA, FlaA^{NC}, or FlaA^{NHC}. IL-8 transcripts were analyzed by RT-PCR and are presented as fold increase in mRNA levels in stimulated versus non-stimulated cells. Values are the mean ± SEM of three independent experiments.*

The β-hairpin region of flagellin determines TLR5 species specificity.

To further explore the importance of the β-hairpin region in TLR5 recognition, we tested the abilities of constructed chimeric flagellins to activate TLR5 from different species. This approach has previously been instrumental in dissecting ligand properties required for TLR activations (1, 20, 39). All constructed chimeras that failed to activate human TLR5 were unable to activate mouse TLR5, except for FlaA^{NHC} (Fig. 7A and 7B). FlaA^{NHC} induced lower levels of NF-κB activation in mouse TLR5 than in human TLR5 transfected cells. This effect was also observed for *Salmonella* FliC (data not shown) and is likely caused by intrinsic differences in TLR5, different expression levels, and/or the expression of mTLR5 in a heterologous (human) background. Chimeric flagellin FlaA^{NHC} was also able to activate chicken TLR5 (Fig. 7C). Unexpectedly however, chicken TLR5 responded also to chimeric flagellin FlaA^{NC}, in clear contrast to human and mouse TLR5. As the only difference between flagellin FlaA^{NC} and FlaA^{NHC} is the presence of the *S*. Enteritidis β-hairpin, these results indicate that this hairpin structure is not merely needed for proper folding of the flagellin but rather is essential for activation of mammalian TLR5 but not chicken TLR5.

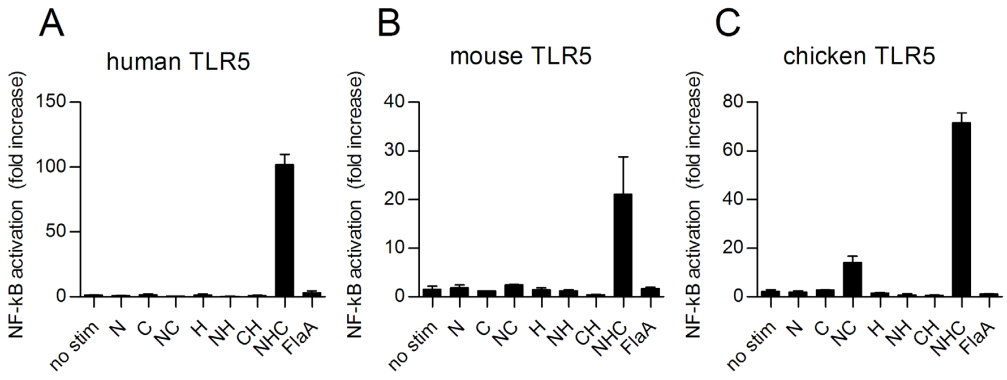

Figure 7. Species-specific activation of TLR5 by FlaA^{NHC}. *A, B and C, HeLa 57A cells were transfected with either (A) human, (B) mouse or (C) chicken TLR5. NF-κB translocation was measured after stimulation with 1 μg ml^{-1} of the indicated recombinant flagellins. Values represent the increase of NF-κB-induced luciferase activity in stimulated cells compared to non-stimulated cells and are the mean ± SEM of three independent experiments.*

Glycosylation and secretion of biologically active FlaA^{NHC} by *C. jejuni*.

The TLR5-activating *Campylobacter* flagellins used above were overexpressed in *E. coli*, purified under denaturing conditions, and refolded *in vitro*. In *Campylobacter*, flagellins are only successfully secreted after post-translational modification. In order to engineer *C. jejuni* that express TLR5-activating flagellins, we expressed the genes encoding wild-type FlaA, the chimeric flagellin FlaA^{NC} and TLR5-activating chimeric flagellin FlaA^{NHC} in *C. jejuni* strain 81116. The genes were cloned into plasmid pMA3, a shuttle vector suitable for protein expression in *C. jejuni* under the control of the endogenous *flaA* σ^{28} promoter, and transformed into a flagella-deficient and non-motile *C. jejuni* 11168H1Δ*flaAB* mutant. Introduction of the plasmid encoding FlaA but not FlaA^{NC} or FlaA^{NHC} flagellin restored flagella formation and bacterial motility in a Δ*flaAB* background (data not shown). Western blot analysis of whole cell lysates using anti-FlaA antibody CF1 as a probe yielded reactive proteins for both FlaA^{NC} and FlaA^{NHC} (Fig. 8A). Analysis of the bacterial culture supernatants also yielded reactive flagellin bands for both strains, which were larger in size than the non-secreted intracellular proteins consistent with the attachment of glycan moieties during protein export (Fig. 8A). Two-dimensional gel electrophoresis followed by immunoblotting with anti-FlaA antibodies demonstrated that both wildtype flagellin produced by 11168H1Δ*flaAB*+FlaA and secreted FlaA^{NHC} appeared as an array of

similarly sized proteins of different isoelectric points, a pattern shown by mass spectrometry to be typical for variable glycosylation of the protein (25, 43) (Fig. 8B and 8C). Overall, these results indicate that the chimeric flagellins were expressed, processed, and secreted in the *C. jejuni* native background.

Infection of TLR5-expressing HEK293 cells with live *C. jejuni* that secrete chimeric glycosylated FlaA^{NHC} flagellin yielded a potent NF-kB response, while no activation was observed for *C. jejuni* producing the chimera FlaA^{NC} and wildtype FlaA (Fig. 9A and 9B). Similar results were obtained with sterile culture supernatants of *C. jejuni* secreting FlaA^{NHC}. These results indicate that viable *C. jejuni* strains can be engineered that activate TLR5 and that glycosylation of flagellin does not interfere with TLR5 receptor recognition.

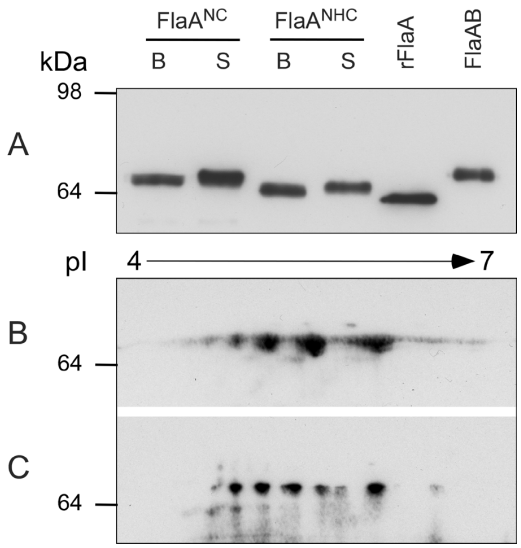

Figure 8. C. jejuni expresses, glycosylates and secretes chimeric flagellins FlaA^{NC} and FlaA^{NHC}. *A, Western blotting was performed to examine the electrophoretic mobility of bacteria-associated (B) and secreted (S) chimeric flagellins FlaA^{NC} and FlaA^{NHC} produced by C. jejuni. Blots were probed with the flagellin-specific antibody CF5. As controls, recombinant FlaA (rFlaA) and native C. jejuni flagellin (FlaAB) were used. B and C, 2D electrophoresis followed by Western blotting using antibody CF5 was performed to visualize the flagellin glycosylation pattern on C. jejuni produced and secreted (B) FlaA and (C) FlaA^{NHC}.*

Discussion

Knowledge of the molecular basis of TLR recognition is important to understand bacteria-host interactions and to exploit bacterial components for targeted modulation of the immune system. In the present study, we took advantage of the inability of *C. jejuni* flagellin to activate TLR5 to better define the molecular requirements for TLR5 recognition.

Using a reverse-engineering approach, we reconstituted TLR5-stimulating activity in *C. jejuni* and discovered that besides the conserved N-terminal ND1a and ND1b α-helices and the C-terminal CD1 α-helix in flagellin, an adjacent β-hairpin structure is required for activation of mammalian TLR5. This β-hairpin was not required for activation of chicken TLR5 (Fig. 7c), indicating species-specific interaction of the flagellin with TLR5. *C. jejuni O*-linked glycosylation of flagellin did not interfere with TLR5 activation, which may holds promise for modification of flagellin to alter its physical properties when used e.g. as a vaccine adjuvant.

Previous studies on the ability of *Campylobacter* to avoid TLR5 recognition were performed with *C. jejuni* strain 81-176 (2, 18, 52). However, *C. jejuni* flagellins are known to show high sequence variability between strains and are present in two differentially regulated isoforms, FlaA and FlaB, which consistently differ at twelve amino acid positions in their conserved N- and C-terminal regions (28). Our results demonstrate that the evasion of the TLR5 response is a conserved trait among the *C. jejuni* species and holds for both FlaA and FlaB (Fig. 3b and data not shown), irrespective of the composition of the flagella (FlaA/FlaB subunit ratio) or the variable glycan modification. These results lend support to the notion that this species and other *α*- and *ε-Proteobacteria* have evolved an alternate class of flagellins that may provide a selective advantage in the host by evading the TLR5 innate immune response (Andersen-Nissen, 2005). The different flagellin structure may explain why the *Campylobacter* flagella fiber is formed by seven in stead of eleven subunit helices (Gaskin, 2008). Although its contribution to pathogenesis is unknown, the widespread evolutionary conservation of TLR5 evasion suggests that this trait adds a valuable selective advantage during colonization or infection.

The evasion of the mammalian TLR5 sensing machinery by *C. jejuni* has thus far been mostly attributed to deviations in the proposed TLR5 binding region, a stretch of 8 amino acids located in the N-terminal conserved domain flagellin and crucial for flagella formation in *Salmonella* (2). The successful engineering of a recombinant *Campylobacter* flagellin that activates human TLR5 required, besides the known N- and C-terminal regions, the presence of the β-hairpin domain from *Salmonella* FliC (Fig. 5). This domain has previously been discarded as a potential binding region for TLR5 due to low sequence homology among bacteria, although disruption of the β-hairpin domain by transposon insertion of a 31-amino-acid polypeptide resulted in a significant decrease in TLR5 activation (37). As the construction of chimeric proteins brings the possibility of incorrect protein folding, it could be argued that proper flagellin folding and subsequent

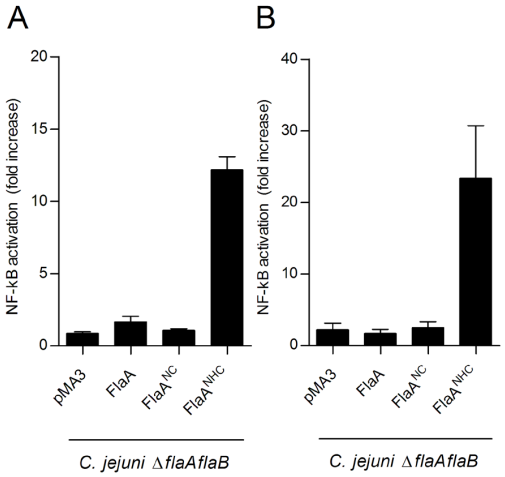

Figure 9. C. jejuni expressing FlaA^{NHC} induces NF-κB activation in HEK293 cells. *NF-κB activation in TLR5-expressing HEK293 cells was measured after stimulation (5 h) with (A) live bacteria or (B) culture supernatant of C. jejuni ΔflaAflaB expressing FlaA, FlaA^{NC} or FlaA^{NHC}. As a control, C. jejuni ΔflaAflaB carrying the empty expression vector pMA3 was used. Values represent the increase of NF-κB-induced luciferase activity in stimulated cells compared to non-stimulated cells and are the mean ± SEM of three independent experiments.*

TLR5 activation in humans is only achieved with the presence of three distinct flagellin domains from the same origin. Indeed, in *Salmonella* flagellin, the ND1 and CD1 helices form multiple intramolecular domain-domain interactions that provide structural strength in the flagellin protein. Absence of the interactions in FlaA^{N} and FlaA^{C}, which contain one helix of *Salmonella* and one of *Campylobacter*, may explain the biological inactivity of these chimeras. Evidence that the β-hairpin structure likely confers more than protein folding and stability is that the *Salmonella* β-hairpin proved necessary for activation of human and mouse TLR5, but not chicken TLR5. This receptor was activated by both the chimeras FlaA^{NC} and FlaA^{NHC} (Fig. 7c). The activation of chicken TLR5 by Fla^{NC} (but not Fla^{N} or Fla^{C}) indicates that the protein is folded into a TLR5 activating state.

Amino acid sequence analysis suggests that *C. jejuni* flagellin contains a β-hairpin structure at grossly the same position as in *Salmonella* flagellin. Although several amino acids are conserved between the β-hairpin of *Campylobacter* and *Salmonella*, the inactivity of chimera FlaA^{NC} towards human and mouse TLR5 shows that the *C. jejuni* β-hairpin

domain cannot substitute for the *Salmonella* β-hairpin structure in the receptor interaction. Chicken TLR5 is activated by flagellins that contain either the *Salmonella* or *Campylobacter* β-hairpin. This may indicate that chicken TLR5 has a more relaxed ligand specificity than mammalian TLR5 with respect to the β-hairpin. Indeed, we previously demonstrated that chicken TLR5 has different flagellin sensing qualities compared to human TLR5 (20). In addition, Smith *et al* showed that the disruption of the β-hairpin in flagellin in *Salmonella* significantly decreased biological activity of flagellin for human TLR5 but not mouse TLR5 (37). Together, these data suggest the hairpin stretch contributes to the species specificity of flagellin recognition by TLR5.

Campylobacter flagellins are heavily decorated with an array of variably modified pseudaminic acid residues, which presence is needed for flagella formation (12). In *Pseudomonas aeruginosa* (*P. aeruginosa*), flagellin glycosylation promotes TLR5 stimulation (49). The glycosylation moieties present on the flagellin of *C. jejuni* are located on the predicted surface exposed variable domain, mostly in a 200 amino acid hydrophobic patch. Structural modeling of *C. jejuni* FlaA on the crystal structure of *Salmonella* flagellin reveals that the sugar moieties are not in close proximity to the predicted TLR5 binding site. Expression of chimeric $FlaA^{NHC}$ by live *Campylobacter*, which resulted in glycosylated and secreted proteins, presented us with the opportunity to, for the first time, directly assess the role of the flagellin-glycosylation on TLR5 activation. Culture supernatants containing glycosylated $FlaA^{NHC}$ as well as live *C. jejuni* secreting glycosylated flagellins showed able to strongly activate TLR5, suggesting that the modification of *C. jejuni* flagellin does not serve as an additional mechanism to prevent or promote activation of TLR5.

The successful engineering of *Campylobacter* strains which secrete flagellins that variably activate TLR5 indicates that the modified regions are not critical for transport through the *C. jejuni* flagellar secretion apparatus. Successful secretion of flagellin through the flagellar basal body requires the ND0 domain, which contains a secretion signal (48), and the CD0 region, which binds chaperone FliS to inhibit cytosolic flagellin polymerization (3, 9). None of the chimeric flagellins constructed in this study have alterations in either the putative secretion signal in domain ND0 or in domain CD0, and the chimeras are thus predicted to bind *C. jejuni* FliS in a similar fashion as the wildtype flagellin. Furthermore, as in *Campylobacter* flagellin glycosylation is essential for secretion (12), all putative glycosylation sites in the constructed chimeric flagellins were left intact. The conservation of the basic *Campylobacter* flagellin architecture may explain the successful secretion of flagellins with the incorporated foreign domains needed for TLR5 activation. However, despite secretion, none of the chimeric flagellins in *C. jejuni* assembled

into a filament. This may indicate a dysregulation of the flagellar components needed for fiber assembly and/or incompatibility of the chimeric structure with e.g. the *Campylobacte*r filament capping protein FliD, a defective multimerization, or altered axial interactions between the flagellin subunits. Elucidation of the crystal structure of *C. jejuni* flagellin may resolve this issue.

Recent studies have identified two additional cellular receptors for flagellin, the intracellular Nod-like Receptor (NLR) Ipaf and NLR apoptosis inhibitory protein 5 (Naip5) (13, 29). Localized intracellularly, these receptors are involved in sensing flagellin that is injected into the host cell, for instance through the type III secretion system (T3SS) of *S.* Typhimurium, or the type IV secretion system (T4SS) of *Legionella pneumophila*. Activation of Ipaf and/or Naip5 results in caspase-1 dependent IL-1β and IL-18 secretion. So far, a functional injection machinery like T3SS or T4SS has not been found in *Campylobacter*. In the case that *Campylobacter* flagellin gains access to the cytosol, it may activate Ipaf and Naip5, as the C-terminal 35 amino acid flagellin domain that is sensed by these receptors, is highly conserved when compared to *Legionella* FlaA (23). As this region is not altered in FlaA^{NHC}, this chimeric flagellin is predicted to activate both TLR5 and Ipaf/Naip5.

In conclusion, we constructed live *C. jejuni* secreting glycosylated flagellins with reconstituted TLR5 activity by the introduction of multiple domains from *Salmonella* flagellin. Through the construction of a series of chimeric flagellins, we identified a previously unknown role for the flagellin β-hairpin domain in the activation of TLR5 and showed that this structure determines TLR5 species specificity in flagellin response. These results provide more insight in the flagellin-TLR5 interaction, and contribute to the current knowledge on the application of flagellin for vaccination purposes.

Acknowledgements

The work presented in this study was supported by the Dutch Ministry of Agriculture, Nature and Food Quality.

Footnote

The abbreviations used in this report are: TLR5, Toll-like Receptor 5; FlaA, *C*. *jejuni* flagellin A; FlaB, *C*. *jejuni* flagellin B; FliC, *S*. Enteritidis or *S*. Typhimurium Phase I flagellin; IPTG, isopropyl-1-thio-β-d-galactopyranoside; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; DPBS, Dulbecco's phosphate buffered saline; HRP, horseradish peroxidase; SE, *S*. Enteritidis; MOI, multiplicity of infection.

TABLE I. *Primers used in this study.*

Product/primer	Primer sequence*	Template DNA
6xhis-FlaA-F	Forward: 5'- GGATCCCACCACCACCACCACCACATGGGATTTCGT -3'	*C. jejuni* 81116
6xhis-FlaA-R	Reverse: 5'-ATCGATCTATTGTAATAATCTTAAAACATTTTGCTG -3'	
6xhis-FlaB-F	Forward: 5'-GGATCCCACCACCACCACCACCACATGGGTTTTAGG-3'	*C. jejuni* 81116
6xhis-FlaB-R	Reverse: 5'-ATCGATTTATTGTAATAGTTTTAAAACATTTTGCTG-3'	
FlaA(1-52)	Forward: 5'-CACCATGGGATTTCGTATTAACAC-3'	*C. jejuni* 81116
	Reverse: 5'-GATATTAGAAGTGAAGCGATCTGCTATCGCCATCCC-3'	
FliC(N)	Forward: 5'-GGGATGGCGATAGCAGATCGCTTCACTTCTAATATC-3'	*S.* Enteritidis 706
	Reverse: 5'-GTATTAGCGATATTATCAAGTTCTTCCAGACGTTGCTGAA-3'	
FlaA(123-576)	Forward: 5'-TTCAGCAACGTCTGGAAGAACTTGATAATATCGCTAATAC-3'	*C. jejuni* 81116
	Reverse: 5'-TTGTAATAATCTTAAAACATTTTGC-3'	
FlaA(1-491)	Forward: 5'-CACCATGGGATTTCGTATTAACAC-3'	*C. jejuni* 81116
	Reverse: 3'-ACAATGCAGAATCAATTGAATCCATAACCGCCATTGC-3'	
FliC(C)	Forward: 5'-GCAATGGCGGTTATGGATTCAATTGATTCTGCATTGT-3'	*S.* Enteritidis 706
	Reverse: 5'-ATTCTGCTGCTTTAACATTGGTTACCGTATTGCCAAG-3'	
FlaA(527-576)	Forward: 5'-CTTGGCAATACGGTAACCAATGTTAAAGCAGCAGAAT-3'	*C. jejuni* 81116
	Reverse: 5'-TTGTAATAATCTTAAAACATTTTGC-3'	
FlaA(1-122)	Forward: 5'-CACCATGGGATTTCGTATTAACAC-3'	*C. jejuni* 81116
	Reverse: 5'-TTAGAAACGCGATCGATATCTGCTATCGCCATCC-3'	
FliC(H)	Forward: 5'-GGATGGCGATAGCAGATATCGATCGCGTTTCTAA-3'	*S.* Enteritidis 706
	Reverse: 5'-GTAAAACTTTGAGCACCAACATTGAACCCATCAA-3'	
FlaA(177-576)	Forward: 5'-TTGATGGGTTCAATGTTGGTGCTCAAAGTTTTAC-3'	*C. jejuni* 81116
	Reverse: 5'-TTGTAATAATCTTAAAACATTTTGC-3'	
FliC(NVC)	Forward: 5'-GGGATGGCGATAGCAGATCGCTTCACTTCTAATATC-3'	*S.* Enteritidis 706
	Reverse: 5'-ATTCTGCTGCTTTAACATTGGTTACCGTATTGCCAAG-3'	
FlaA3-topoF	5'-CACCATGGGATTTCGTATTAACAC-3'	
FlaA6-topoR	5'-TTGTAATAATCTTAAAACATTTTGC-3'	
FlaAB-mutant-R	5'-AAAGCTATTATTCCCTTACAGGATGAG-3'	*C. jejuni* 81116
FlaAFSphI	5'-GCATGCTAGTAAAATTGAAGATGAAAGAGAG-3'	*C. jejuni* 81116
FlaARNsiI	5'-ATGCATTTTAAATCCTTTAAATAATTTC-3'	
Flagellin-pMA3-F	5'-CCGAGCTCAAAAGGATTTAAAATGGGATTTCGTATTAACACAAATGT-3'	
Flagellin-pMA3-R	5'-CCCCGCGGCTATTGTAATAATCTTAAAACATTTTGCTG-3'	

*underlined are restriction sites used for cloning

References

1. **Andersen-Nissen, E., K. D. Smith, R. Bonneau, R. K. Strong, and A. Aderem.** 2007. A conserved surface on Toll-like receptor 5 recognizes bacterial flagellin. J Exp Med **204:**393-403.
2. **Andersen-Nissen, E., K. D. Smith, K. L. Strobe, S. L. Barrett, B. T. Cookson, S. M. Logan, and A. Aderem.** 2005. Evasion of Toll-like receptor 5 by flagellated bacteria. Proc Natl Acad Sci U S A **102:**9247-9252.
3. **Auvray, F., J. Thomas, G. M. Fraser, and C. Hughes.** 2001. Flagellin polymerisation control by a cytosolic export chaperone. J Mol Biol **308:**221-229.
4. **Bargieri, D. Y., D. S. Rosa, C. J. Braga, B. O. Carvalho, F. T. Costa, N. M. Espindola, A. J. Vaz, I. S. Soares, L. C. Ferreira, and M. M. Rodrigues.** 2008. New malaria vaccine candidates based on the *Plasmodium vivax* Merozoite Surface Protein-1 and the TLR-5 agonist *Salmonella* Typhimurium FliC flagellin. Vaccine **26:**6132-6142.
5. **Burdelya, L. G., V. I. Krivokrysenko, T. C. Tallant, E. Strom, A. S. Gleiberman, D. Gupta, O. V. Kurnasov, F. L. Fort, A. L. Osterman, J. A. Didonato, E. Feinstein, and A. V. Gudkov.** 2008. An agonist of toll-like receptor 5 has radioprotective activity in mouse and primate models. Science **320:**226-230.
6. **de Zoete, M. R., A. M. Keestra, P. Roszczenko, and J. P. M. van Putten.** 2009. Activation of Human and Chicken Toll-like Receptors by *Campylobacter*. Infect Immun.
7. **Donnelly, M. A., and T. S. Steiner.** 2002. Two nonadjacent regions in enteroaggregative *Escherichia coli* flagellin are required for activation of toll-like receptor 5. J Biol Chem **277:**40456-40461.
8. **Eaves-Pyles, T. D., H. R. Wong, K. Odoms, and R. B. Pyles.** 2001. *Salmonella* flagellin-dependent proinflammatory responses are localized to the conserved amino and carboxyl regions of the protein. J Immunol **167:**7009-7016.
9. **Evdokimov, A. G., J. Phan, J. E. Tropea, K. M. Routzahn, H. K. Peters, M. Pokross, and D. S. Waugh.** 2003. Similar modes of polypeptide recognition by export chaperones in flagellar biosynthesis and type III secretion. Nat Struct Biol **10:**789-793.
10. **Galkin, V. E., X. Yu, J. Bielnicki, J. Heuser, C. P. Ewing, P. Guerry, and E. H. Egelman.** 2008. Divergence of quaternary structures among bacterial flagellar filaments. Science **320:**382-385.

11. **Gewirtz, A. T., Y. Yu, U. S. Krishna, D. A. Israel, S. L. Lyons, and R. M. Peek, Jr.** 2004. *Helicobacter pylori* flagellin evades toll-like receptor 5-mediated innate immunity. J Infect Dis **189:**1914-1920.
12. **Goon, S., J. F. Kelly, S. M. Logan, C. P. Ewing, and P. Guerry.** 2003. Pseudaminic acid, the major modification on *Campylobacter* flagellin, is synthesized via the Cj1293 gene. Mol Microbiol **50:**659-671.
13. **Grassl, G. A., and B. B. Finlay.** 2008. Pathogenesis of enteric *Salmonella* infections. Current opinion in gastroenterology **24:**22-26.
14. **Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem.** 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature **410:**1099-1103.
15. **Honko, A. N., N. Sriranganathan, C. J. Lees, and S. B. Mizel.** 2006. Flagellin is an effective adjuvant for immunization against lethal respiratory challenge with *Yersinia pestis*. Infect Immun **74:**1113-1120.
16. **Ibrahim, G. F., G. H. Fleet, M. J. Lyons, and R. A. Walker.** 1985. Method for the isolation of highly purified *Salmonella* flagellins. J Clin Microbiol **22:**1040-1044.
17. **Jacchieri, S. G., R. Torquato, and R. R. Brentani.** 2003. Structural study of binding of flagellin by Toll-like receptor 5. J Bacteriol **185:**4243-4247.
18. **Johanesen, P. A., and M. B. Dwinell.** 2006. Flagellin-independent regulation of chemokine host defense in *Campylobacter jejuni*-infected intestinal epithelium. Infect Immun **74:**3437-3447.
19. **Karlyshev, A. V., D. Linton, N. A. Gregson, and B. W. Wren.** 2002. A novel paralogous gene family involved in phase-variable flagella-mediated motility in *Campylobacter jejuni*. Microbiology **148:**473-480.
20. **Keestra, A. M., M. R. de Zoete, R. A. van Aubel, and J. P. M. van Putten.** 2008. Functional characterization of chicken TLR5 reveals species-specific recognition of flagellin. Mol Immunol **45:**1298-1307.
21. **Le Moigne, V., G. Robreau, and W. Mahana.** 2008. Flagellin as a good carrier and potent adjuvant for Th1 response: study of mice immune response to the p27 (Rv2108) *Mycobacterium tuberculosis* antigen. Mol Immunol **45:**2499-2507.
22. **Lee, S. K., A. Stack, E. Katzowitsch, S. I. Aizawa, S. Suerbaum, and C. Josenhans.** 2003. *Helicobacter pylori* flagellins have very low intrinsic activity to stimulate human gastric epithelial cells via TLR5. Microbes Infect **5:**1345-1356.

23. **Lightfield, K. L., J. Persson, S. W. Brubaker, C. E. Witte, J. von Moltke, E. A. Dunipace, T. Henry, Y. H. Sun, D. Cado, W. F. Dietrich, D. M. Monack, R. M. Tsolis, and R. E. Vance.** 2008. Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin. Nat Immunol **9:**1171-1178.

24. **Logan, S. M., J. P. Hui, E. Vinogradov, A. J. Aubry, J. E. Melanson, J. F. Kelly, H. Nothaft, and E. C. Soo.** 2009. Identification of novel carbohydrate modifications on *Campylobacter jejuni* 11168 flagellin using metabolomics-based approaches. Febs J.

25. **Logan, S. M., J. F. Kelly, P. Thibault, C. P. Ewing, and P. Guerry.** 2002. Structural heterogeneity of carbohydrate modifications affects serospecificity of *Campylobacter* flagellins. Mol Microbiol **46:**587-597.

26. **Logan, S. M., and T. J. Trust.** 1983. Molecular identification of surface protein antigens of *Campylobacter jejuni*. Infect Immun **42:**675-682.

27. **McDonald, W. F., J. W. Huleatt, H. G. Foellmer, D. Hewitt, J. Tang, P. Desai, A. Price, A. Jacobs, V. N. Takahashi, Y. Huang, V. Nakaar, L. Alexopoulou, E. Fikrig, and T. J. Powell.** 2007. A West Nile virus recombinant protein vaccine that coactivates innate and adaptive immunity. J Infect Dis **195:**1607-1617.

28. **Meinersmann, R. J., and K. L. Hiett.** 2000. Concerted evolution of duplicate fla genes in *Campylobacter*. Microbiology **146 (Pt 9):**2283-2290.

29. **Miao, E. A., E. Andersen-Nissen, S. E. Warren, and A. Aderem.** 2007. TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system. Semin Immunopathol **29:**275-288.

30. **Murthy, K. G., A. Deb, S. Goonesekera, C. Szabo, and A. L. Salzman.** 2004. Identification of conserved domains in *Salmonella muenchen* flagellin that are essential for its ability to activate TLR5 and to induce an inflammatory response in vitro. J Biol Chem **279:**5667-5675.

31. **Neish, A. S.** 2007. TLRS in the gut. II. Flagellin-induced inflammation and antiapoptosis. Am J Physiol Gastrointest Liver Physiol **292:**G462-466.

32. **Nuijten, P. J., B. A. van der Zeijst, and D. G. Newell.** 1991. Localization of immunogenic regions on the flagellin proteins of *Campylobacter jejuni* 81116. Infect Immun **59:**1100-1105.

33. **Pearson, B. M., D. J. Gaskin, R. P. Segers, J. M. Wells, P. J. Nuijten, and A. H. van Vliet.** 2007. The Complete Genome Sequence of *Campylobacter jejuni* Strain 81116 (NCTC11828). J Bacteriol **189:**8402-8403.

34. **Rodriguez, M. S., J. Thompson, R. T. Hay, and C. Dargemont.** 1999. Nuclear retention of IkappaBalpha protects it from signal-induced degradation and inhibits nuclear factor kappaB transcriptional activation. J Biol Chem **274:**9108-9115.
35. **Sanders, C. J., Y. Yu, D. A. Moore, 3rd, I. R. Williams, and A. T. Gewirtz.** 2006. Humoral immune response to flagellin requires T cells and activation of innate immunity. J Immunol **177:**2810-2818.
36. **Skountzou, I., M. D. Martin, B. Wang, L. Ye, D. Koutsonanos, W. Weldon, J. Jacob, and R. W. Compans.** 2009. *Salmonella* flagellins are potent adjuvants for intranasally administered whole inactivated influenza vaccine. Vaccine.
37. **Smith, K. D., E. Andersen-Nissen, F. Hayashi, K. Strobe, M. A. Bergman, S. L. Barrett, B. T. Cookson, and A. Aderem.** 2003. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol **4:**1247-1253.
38. **Song, L., V. Nakaar, U. Kavita, A. Price, J. Huleatt, J. Tang, A. Jacobs, G. Liu, Y. Huang, P. Desai, G. Maksymiuk, V. Takahashi, S. Umlauf, L. Reiserova, R. Bell, H. Li, Y. Zhang, W. F. McDonald, T. J. Powell, and L. Tussey.** 2008. Efficacious recombinant influenza vaccines produced by high yield bacterial expression: a solution to global pandemic and seasonal needs. PLoS ONE **3:**e2257.
39. **Steeghs, L., A. M. Keestra, A. van Mourik, H. Uronen-Hansson, P. van der Ley, R. Callard, N. Klein, and J. P. M. van Putten.** 2008. Differential activation of human and mouse Toll-like receptor 4 by the adjuvant candidate LpxL1 of Neisseria meningitidis. Infect Immun **76:**3801-3807.
40. **Szymanski, C. M., S. M. Logan, D. Linton, and B. W. Wren.** 2003. *Campylobacter*--a tale of two protein glycosylation systems. Trends Microbiol **11:**233-238.
41. **Tabor, S., and C. C. Richardson.** 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc Natl Acad Sci U S A **84:**4767-4771.
42. **Thibault, P., S. M. Logan, J. F. Kelly, J. R. Brisson, C. P. Ewing, T. J. Trust, and P. Guerry.** 2001. Identification of the carbohydrate moieties and glycosylation motifs in *Campylobacter jejuni* flagellin. J Biol Chem **276:**34862-34870.
43. **van Alphen, L. B., M. Wuhrer, N. M. Bleumink-Pluym, P. J. Hensbergen, A. M. Deelder, and J. P. M. van Putten.** 2008. A functional *Campylobacter jejuni* maf4 gene results in novel glycoforms on flagellin and altered autoagglutination behaviour. Microbiology **154:**3385-3397.
44. **Van Asten, F. J., H. G. Hendriks, J. F. Koninkx, B. A. Van der Zeijst, and W. Gaastra.** 2000. Inactivation of the flagellin gene of *Salmonella enterica* serotype

Enteritidis strongly reduces invasion into differentiated Caco-2 cells. FEMS Microbiol Lett **185:**175-179.

45. **van Aubel, R. A., A. M. Keestra, D. J. Krooshoop, W. van Eden, and J. P. M. van Putten.** 2007. Ligand-induced differential cross-regulation of Toll-like receptors 2, 4 and 5 in intestinal epithelial cells. Mol Immunol **44:**3702-3714.
46. **van Mourik, A., N. M. Bleumink-Pluym, L. van Dijk, J. P. M. van Putten, and M. M. S. M. Wösten.** 2008. Functional analysis of a *Campylobacter jejuni* alkaline phosphatase secreted via the Tat export machinery. Microbiology **154:**584-592.
47. **van Vliet, A. H., K. G. Wooldridge, and J. M. Ketley.** 1998. Iron-responsive gene regulation in a *Campylobacter jejuni* fur mutant. J Bacteriol **180:**5291-5298.
48. **Vegh, B. M., P. Gal, J. Dobo, P. Zavodszky, and F. Vonderviszt.** 2006. Localization of the flagellum-specific secretion signal in *Salmonella* flagellin. Biochem Biophys Res Commun **345:**93-98.
49. **Verma, A., S. K. Arora, S. K. Kuravi, and R. Ramphal.** 2005. Roles of specific amino acids in the N terminus of *Pseudomonas aeruginosa* flagellin and of flagellin glycosylation in the innate immune response. Infect Immun **73:**8237-8246.
50. **Vijay-Kumar, M., J. D. Aitken, and A. T. Gewirtz.** 2008. Toll like receptor-5: protecting the gut from enteric microbes. Semin Immunopathol **30:**11-21.
51. **Wassenaar, T. M., B. N. Fry, and B. A. van der Zeijst.** 1993. Genetic manipulation of *Campylobacter*: evaluation of natural transformation and electrotransformation. Gene **132:**131-135.
52. **Watson, R. O., and J. E. Galan.** 2005. Signal transduction in *Campylobacter jejuni*-induced cytokine production. Cell Microbiol **7:**655-665.
53. **Way, S. S., L. J. Thompson, J. E. Lopes, A. M. Hajjar, T. R. Kollmann, N. E. Freitag, and C. B. Wilson.** 2004. Characterization of flagellin expression and its role in *Listeria monocytogenes* infection and immunity. Cell Microbiol **6:**235-242.
54. **Winter, S. E., M. Raffatellu, R. P. Wilson, H. Russmann, and A. J. Baumler.** 2008. The *Salmonella enterica* serotype Typhi regulator TviA reduces interleukin-8 production in intestinal epithelial cells by repressing flagellin secretion. Cell Microbiol **10:**247-261.
55. **Wösten, M. M. S. M., J. A. Wagenaar, and J. P. M. van Putten.** 2004. The FlgS/FlgR two-component signal transduction system regulates the fla regulon in *Campylobacter jejuni*. J Biol Chem **279:**16214-16222.

56. **Yonekura, K., S. Maki-Yonekura, and K. Namba.** 2003. Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy. Nature **424:**643-650.

The research described in this chapter was originally published in Journal of Biological Chemistry.

6

Summarizing discussion

Marcel R. de Zoete

1. Aim of the thesis

Campylobacter induces acute intestinal inflammation and elicits adaptive immune responses in humans (12). In chickens, colonization of the intestinal tract with *Campylobacter* can be massive but does not lead to inflammation of the mucosa (18). While the differences in clinical manifestation in these hosts are clear, the underlying cause is not. *C. jejuni* lacks virulence factors commonly present in many other human intestinal pathogens (22), and frequently exhibits commensal colonization behavior (11). This may hint at the bacterium's preferred lifestyle in chickens; stealthily colonizing the intestine without having to fight an activated host immune system.

The behavior of *Campylobacter* poses a complicated problem for the development of a *Campylobacter* vaccine: how to elicit a protective immune response to an organism that has adopted a commensal lifestyle? *C. jejuni* actively minimizes the induction of inflammation, for instance through effective shielding of immunogenic epitopes, the development of a great arsenal of phase variable (antibody avoiding) surface structures, the presence of at least one polysaccharide capsule, and a relatively poor recognition by innate immune receptors (32). Adding to the problem, *Campylobacter* colonizes chickens generally within two weeks after hatching, resulting in $>10^9$ bacteria per gram feces. This leaves only a small window for vaccination during a period when the immune system is still in development (5). Tackling these problems requires a multidisciplinary research approach.

At the start of the work presented in this thesis, it was apparent that classical vaccination strategies using killed whole bacteria in combination with (mammalian) adjuvants, did not sort the effect needed to reduce the number of *Campylobacter* in the chicken gut (5). Growing awareness of the crucial role of Toll-like receptors (TLR) in orchestrating the innate and adaptive immune responses and in controlling bacterial infections, led to the hypothesis that TLR ligands may be exploited as natural vaccine adjuvants. Fundamental knowledge on the function of the TLR family of innate receptors in chickens however, was still incomplete. The first aim of the work described in this thesis was to elucidate the function of the orphan chicken TLR21 and TLR15. As a next step, we, for the first time, determined the interaction of *Campylobacter* with the full repertoire of chicken and human TLRs. Finally, we constructed a *Campylobacter* flagellin that activates TLR5, and engineered a *Campylobacter* vaccine strain that produces this recombinant flagellin as a first step towards the development of a subunit vaccine.

2. Ligand identification of orphan chicken Toll-like receptors

Both the human and chicken genome encode 10 intact TLRs (**Chapter 1**). Yet, only 8 of the 10 chicken TLRs have similar ligand specificity as human TLRs. Elucidation of the function of the two chicken-specific TLRs, i.e. TLR21 and TLR15, was considered important as this may provide important novel insights in the organization of the innate chicken defense against microbial infections. In addition, the identification of ligands for these receptors could aid vaccine development by providing novel adjuvants.

TLR21

Previous work has shown that chicken monocytes respond to stimulation with CpG DNA by the upregulation and secretion of nitric oxide and several cytokines (8, 31). In mammals, CpG DNA initiates pro-inflammatory signals via TLR9. Inspection of the chicken genome (in 2004) however, revealed the absence of a homologue of TLR9. In **Chapter 2**, we clarify the CpG DNA responsiveness of chicken cells by identifying TLR21 as the chicken sensor for DNA. This raises the question: why do chickens and humans utilize different receptors for the sensing of DNA?

To address this point, a detailed comparison of the functions of TLR21 and TLR9 is required. In **Chapter 2**, we describe several similarities and differences. Like TLR9, TLR21 is an intracellular receptor. Comparison of the subcellular localization of TLR21 and TLR9 revealed that both proteins are abundantly present in the endoplasmatic reticulum (ER). A small fraction of the TLR9 pool shuttles from the ER to the endolysosomes, which is requires for DNA binding, proteolytic processing and signaling (6, 16, 21). TLR9 activity is inhibited by the endosomal maturation blocker chloroquine. For TLR21, we could not definitively show endolysosomal localization. However, the response of the receptor to DNA was highly sensitive to chloroquine. Whether TLR21 requires proteolytic cleavage for activation, as has been reported for TLR9, still remains to be determined.

Transport of the mammalian nucleotide TLR receptors from the ER to the endolysosomes requires and appears to compete for the shuttle protein UNC93B (15). Interestingly, HEK293 cells transfected with TLR21 showed a reduced cellular response to the TLR3 ligand poly(I:C) than control cells (**Chapter 2**, Fig. 1). This may point to the use of UNC93B by TLR21. The chicken genome contains a putative, albeit considerably truncated homologue of UNC93B, which may have a similar function. On the other hand, it has been shown that human and mouse UNC93B are not interchangeable between the

species. This suggests that other (unknown) proteins and/or pathways are shared by the intracellular TLR receptors. Clearly, the regulation of the trafficking of TLR receptors between cellular compartments deserves more study both in chicken and mammalian cells.

The most notable difference between TLR21 and TLR9 became apparent when the ligand specificity of the receptors was analyzed (**Chapter 2**). Whereas TLR9 displayed a rather narrow ligand specificity for synthesized CpG ODNs, TLR21 sensed all CpG ODNs examined, and, moreover, responded to chromosomal bacterial DNA. The cause for this variable response is currently unknown, but indicates differences in DNA-binding characteristics of the ligand binding domain and/or of potential helper proteins. Chicken macrophages displayed similar broad ligand specificity as the recombinant receptor expressed in human cells, and this response was abolished by specific siRNA silencing of TLR21. This discards species incompatibility (chicken receptor in a human background) as a cause of the aspecificity of TLR21 for DNA.

Why have birds and mammals evolved different TLR receptors to sense DNA? Strikingly, fish and amphibians still contain orthologs of both TLR21 and TLR9. It has been suggested that perhaps also mammals and chickens may have carried both types of receptors but that one of the receptor is now absent due to secondary gene loss (27). This may indicate that during evolution, the presence of either TLR21 or TLR9 may have provided a selective advantage to birds and mammals, respectively. The selective forces that have driven this evolution remain to be defined. Perhaps for chickens, the ability to sense a broad range of different large and small DNAs through TLR21 has made TLR9 obsolete, while mammals required a mechanism to restricting the DNA sensing capacity to limit the development of autoimmunity against self-DNA. Whether the presence of TLR21 predisposes birds (and perhaps also fish and amphibians) to autoimmune diseases is currently unknown. Irrespective of the underlying mechanism, the broad ligand specificity of TLR21 provides a solid basis for further rational use and development of DNA adjuvants for use in chicken vaccines.

TLR15

Genome analysis indicates that TLR15 is unique for birds. By the use of recombinatorial cloning, expression, and extensive ligand screening, we elucidated the function of this last remaining orphan chicken TLR, as described in **Chapter 3**. Through TLR15, chicken are able to sense proteolytic activity at the cell surface. Particularly fungi, but also bacteria, exploit proteases as virulence factors, and may be the prime target for TLR15. We demonstrated that TLR15 is activated by fungal proteases. Current knowledge on the

chicken defense against fungi is very limited; several mammalian innate immune receptors involved in the detection of fungi are predicted to be present in the genome, but functional studies are mostly lacking. Our results are the first that indicate that TLR15 may be of importance. Future studies using TLR15-deficient chickens are needed to elucidate the exact role of TLR15 in fungal infection.

Despite its presence in the avian species only, TLR15 was functional when transiently expressed at the surface of human cells. This suggests that no other chicken species-specific molecules are involved in the activation of the receptor. We could demonstrate that TLR activation was accompanied by cleavage of the receptor. Proteolytic cleavage and activation of a TLR without the addition of a specific TLR ligand has never been reported and appears unique for TLR15. This mechanism of activation seems to deviate from the general consensus that TLR activation is induced by 'dimerizing ligands' (**Chapter 1**, Fig. 2). To some extent, the activation of TLR15 may be evolutionary related to the activation of mammalian TLR9, which also needs to be proteolytically cleaved before signaling can occur. In this case, however, a dimerizing ligands (DNA) is still required. Theoretically, it is possible that protease treatment releases endogenous ligands that activate the cleaved TLR15. We consider this unlikely as transfer of supernatant from protease-treated cells to TLR15 transfected cells did not result in activation. However, we cannot exclude that released ligands only activated cleaved TLR15. It should be noted that plants have evolved a similar immunoreceptor activation mechanism that seem to share some properties with TLR15. In *Arabidopsis*, the leucine-rich repeat (LRR) domain of the intracellular disease-resistance protein RPS5 forms a complex with the adapter protein PBS1, together operating as a negative regulator for an adjacent signaling domain. Cleavage of PBS1 by bacterial effector proteases is believed to release the LRR domain from the signaling domain, enabling protective immune responses (23). Albeit not through direct cleavage of a LRR domain and occurring inside the cell, plants seem to have developed the ability to sense the presence of pathogenic proteases as found for TLR15. Further studies are needed to identify similar systems in other species.

The ability of TLR15 to respond to proteases may impose a risk at tissues sites with high extracellular proteolytic activity. TLR15 is expressed in intestinal tissue. However, continuous TLR15-mediated inflammation of the intestine may be prevented via the protective properties of the mucus layer and the presence of secreted protease inhibitors (2). In addition, TLR15 expression is strongly upregulated during intestinal infection (10, 24), thereby further limiting the activation by the natural flora or endogenous enzymes.

The signals that stimulate the upregulation of TLR15 are still unknown. For use as a target of adjuvants, TLR15 may be less suitable as this would require a vaccine which contains proteolytic activity as well as signals to upregulate the receptor.

3. The role of Toll-like receptors during *Campylobacter* infection

The strong intestinal inflammatory symptoms during *Campylobacter* infection in humans likely reflect a strong activation of the innate immune defense. In **Chapter 4**, we analyzed the contribution of the various TLRs to the response to *Campylobacter* and investigated differences in the human and chicken TLR-response as possible cause of the different pathology in these species.

The role of TLR21 and TLR15 during Campylobacter infection

In contrast to human TLR9, chicken TLR21 is activated by purified chromosomal DNA from *Campylobacter* spp., as presented in **Chapter 4**. Although these experiments are done in a heterologous (human cell) background, the results suggest a more potent response to the bacterium in chickens than in humans. This seems contradictory, as colonization of humans but not chickens is frequently accompanied by strong inflammatory responses. One possible explanation for this apparent discrepancy is that the commensal nature of *Campylobacter* in chickens prevents TLR21 activation during colonization. An alternative scenario involves a more active role for TLR21; by an earlier and more sensitive detection of *Campylobacter*, locally induced (mild) immune responses may limit the intrusion of the bacterium into the deeper tissues of the intestine, and the subsequent widespread enteritis. In this case, *Campylobacter* either has to be endocytosed and lysed by the chicken cell, or lysed extracellularly to release its DNA.

Campylobacter contains several proteases like HtrA, ClpP and ClpX (3, 4). These enzymes are mainly involved in the degradation of misfolded proteins during stress responses, but it can be imagined that, once released into the environment, they may be sensed by TLR15 and activate the receptor. In addition, the genome of *C. jejuni* strains 81116 predicts the presence of at least two putative secreted proteases of unknown function. In our hands *C. jejuni* supernatants or cell lysates were unable to activate TLR15. Experimental infection of chickens with *C. jejuni* similarly failed to induce cecal TLR15 mRNA levels, in contrast to infection with *Salmonella* Typhimurium (24). Combined, these results indicate that TLR15 has no role during *C. jejuni* colonization of

chickens. Whether other *Campylobacter* species, potentially under alternative environmental conditions, have the ability to stimulate TLR15 remains to be studied.

Interaction of Campylobacter with TLR2, TLR4, and TLR5

Stimulation of human TLR2/1/6 and TLR4, and chicken TLR2t2/16 and TLR4 by lysed *Campylobacter* showed comparable activation for each class of receptor (**Chapter 4**), except for the LPS-dependent interferon production. The latter finding supports previous work suggesting the absence of a functional TLR4-TRAM-TRIF pathway in chickens (14). Another important finding was that both human and chicken TLR were unable to respond to *Campylobacter* flagellin (**Chapter 4**). This for the first time excluded the possibility that differential response to flagellin accounted for variable different clinical manifestation in both species.

A surprising finding was the general lack of activation of TLRs of either species by live *Campylobacter* (**Chapter 4**). This shows that, while the bacterium has the potential to strongly stimulate different TLRs, it only does so when the bacterial integrity becomes compromised. During infection this may happen due to shortage of nutrients, lysis by the intestinal flora or bacteriophages, or after attack by the host immune system. The chicken cecum is considered to be a nutrient-rich environment and the optimal niche for *Campylobacter*, which may results in relative low release of TLR ligands. On the other hand, potential sub-optimal conditions in the humans gut could result in bacterial damage which would induce an inflammatory response and cause snowball effect due to increased bacterial lysis and influx of inflammatory cells. Based on our results it is tempting to speculate that the difference in clinical outcome of *C. jejuni* infection in humans and chicken is related to the different environmental conditions that results in a difference in release of TLR ligands. A closer investigation of the nutritional requirements of *Campylobacter* and their availability in the host's intestine may provide new insights in the pathogenesis of the infection.

4. Targeting TLRs for vaccination against *Campylobacter*

Exploitation of TLRs for chicken vaccination

TLR ligands are successfully being employed as adjuvant in (experimental) mammalian vaccines (28). In chickens, however, much less is known about the immunomodulatory effects of TLR ligands. The work described in this thesis indicates that several chicken

TLRs may be exploited as adjuvant targets. One suitable TLR is presented by the CpG DNA-sensing TLR21. CpG DNA has previously been shown to boost adaptive immune responses in experimental chicken immunization against several infectious agents, like fowl cholera (9), avian influenza (29, 30), and pathogenic *E. coli* (7). Our work explains the mechanism behind the immunostimulatory effect of CpG DNA. In addition, the identification, cloning, and successful expression of TLR21 provide the tools to develop stronger or modulating CpG DNA-based immunostimulatory adjuvants.

A second potential adjuvant in chickens may be LPS, targeting the TLR4/MD-complex. LPS induces a potent NF-kB response and nitric oxide production (14). However, as indicated above, activation of TLR4 by LPS does not induce TRIF signaling in chickens, in contrast to activation of human TLR4. This is highly relevant, as this arm of the signaling pathway adds significantly to the adjuvant activity (20, 25), and is targeted specifically by monophosphoryl lipid A (MPL), which is the functional LPS-based adjuvant used in human cervix cancer vaccines (19). Because of the lack of LPS-mediated TRIF-signaling, the use of LPS or MPL as adjuvants in chickens may proof to be a rather ineffective in comparison to their use in humans, and does not seems to be a valuable component of a *C. jejuni* vaccine.

In theory, activation of TLR15 would provide the immunostimulatory signals needed to boost humoral responses to antigens during immunization. In practise, however, stimulation of TLR15 by proteases presents several potential problems. For instance, the dose and location of administration should be carefully controlled to limit collateral tissue damage. Obviously, the co-administered antigens should be (relatively) protease sensitive. Novel methods of vaccine administration, e.g. by using specialized delivery vehicles, may help solve these issues, but currently, TLR15 does not seem to be the preferred target of adjuvants.

A tailormade candidate vaccine based on TLR5 activation: FlaA^{NHC}

Another promising immune receptor for targeting during immunization is TLR5. Its ligand flagellin has been successfully used as an adjuvant in experimental vaccination against malaria, tuberculosis, and influenza in mammals (1, 17, 26). As chickens are highly responsive to *Salmonella* flagellin (13), we explored flagellin from *Campylobacter* for its use as an immunostimulatory vaccine adjuvant.

In humans, flagellin is the immunodominant antigen during *Campylobacter* infection. The generation of neutralizing antibodies against the major component of the essential motility apparatus of *Campylobacter* may well contribute to the clearance of the

bacterium in humans. In chickens, the immunodominance of flagellin is also evident. However, immune responses against the protein are induced considerably slower and less potent. In addition, anti-flagellin antibodies in chickens were shown to be directed mostly against non-surfaced exposed region of the protein, and thus mostly useless to protect against *Campylobacter* (5). A more potent response to *Campylobacter* flagellin may therefore be required to actively influence the number of *Campylobacter* present in the intestine of chickens.

To achieve this goal, we engineered a *Campylobacter* flagellin that is able to activate TLR5 (**Chapter 5)**. Hereto, we first investigated the molecular basis of the TLR5 evasion. Several conserved flagellin domains from *Salmonella* flagellin were exchanged with the corresponding region of *Campylobacter* flagellin, which resulted in the construction of a series of chimeric flagellins. When tested for their TLR5-stimulatory activity, two chimeras showed immunostimulatory potential: FlaA^{NC} for chicken TLR5, and FlaA^{NHC} for both chicken and human TLR5. The next step is to test these recombinant flagellins for their adjuvant activity *in vivo*.

In a further step to development of a flagellin-based *C. jejuni* vaccine, we engineered *C. jejuni* that expressed the recombinant TLR5-activating flagellin. This approach enables to evaluate the potentially immune-protection inducing activity of the glycans that are attached to the *C. jejuni* flagellin. Although the recombinant flagellin expressed in *C. jejuni* did not assemble into a filament, the bacteria did secrete glycosylated recombinant flagellin that activated TLR5. Vaccination trials are being planned to examine the antibody responses against FlaA^{NHC} in chickens.

5. Concluding remarks and future perspectives

A successful broiler chicken vaccine should meet several standards. First and foremost, a potent protective response has to be induced quickly as young chicks come into contact with *Campylobacter* very early on in life. Also, immunity should be cross-protective for the vast majority of different *C. jejuni* and *C. coli* serotypes. Finally, the vaccine should be safe, cost-effective, and easy to deliver as massive numbers of chicken have to be immunized. Overall, vaccination should prevent colonization or at least cause a strong (more than 2-3 log) reduction of bacterial numbers in colonized animals.

In this thesis, we added a new chapter to our knowledge of the chickens' innate immune system by identifying the ligand and function of TLR21 and TLR15. Furthermore,

we characterized the previously unknown interactions of *Campylobacter* with TLRs from human and chicken origin. Our findings provide valuable insights into the unique recognition of microorganisms by chickens and offer new strategies to design or improve vaccine for chickens. In addition, our results contribute to the understanding of the distinct clinical manifestation of *Campylobacter* infection in the different hosts. The construction of a rationally designed first generation candidate vaccine based on *Campylobacter* flagellin is a first step in translating gained knowledge into product development. Ongoing research will evaluate the immune response, dose, safety and cross-protection of the FlaA^{NHC} candidate vaccine, and assess its ability to reduce *Campylobacter* colonization of the chicken gut.

References

1. **Bargieri, D. Y., D. S. Rosa, C. J. Braga, B. O. Carvalho, F. T. Costa, N. M. Espindola, A. J. Vaz, I. S. Soares, L. C. Ferreira, and M. M. Rodrigues.** 2008. New malaria vaccine candidates based on the *Plasmodium vivax* Merozoite Surface Protein-1 and the TLR-5 agonist *Salmonella* Typhimurium FliC flagellin. Vaccine **26:**6132-6142.
2. **Bergenfeldt, M., M. Nystrom, M. Bohe, C. Lindstrom, A. Polling, and K. Ohlsson.** 1996. Localization of immunoreactive secretory leukocyte protease inhibitor (SLPI) in intestinal mucosa. Journal of gastroenterology **31:**18-23.
3. **Brondsted, L., M. T. Andersen, M. Parker, K. Jorgensen, and H. Ingmer.** 2005. The HtrA protease of *Campylobacter jejuni* is required for heat and oxygen tolerance and for optimal interaction with human epithelial cells. Applied and environmental microbiology **71:**3205-3212.
4. **Cohn, M. T., H. Ingmer, F. Mulholland, K. Jorgensen, J. M. Wells, and L. Brondsted.** 2007. Contribution of conserved ATP-dependent proteases of *Campylobacter jejuni* to stress tolerance and virulence. Applied and environmental microbiology **73:**7803-7813.
5. **de Zoete, M. R., J. P. van Putten, and J. A. Wagenaar.** 2007. Vaccination of chickens against *Campylobacter*. Vaccine **25:**5548-5557.
6. **Ewald, S. E., B. L. Lee, L. Lau, K. E. Wickliffe, G. P. Shi, H. A. Chapman, and G. M. Barton.** 2008. The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor. Nature **456:**658-662.
7. **Gomis, S., L. Babiuk, D. L. Godson, B. Allan, T. Thrush, H. Townsend, P. Willson, E. Waters, R. Hecker, and A. Potter.** 2003. Protection of chickens against *Escherichia coli* infections by DNA containing CpG motifs. Infection and immunity **71:**857-863.
8. **He, H., T. L. Crippen, M. B. Farnell, and M. H. Kogut.** 2003. Identification of CpG oligodeoxynucleotide motifs that stimulate nitric oxide and cytokine production in avian macrophage and peripheral blood mononuclear cells. Developmental and comparative immunology **27:**621-627.
9. **Herath, C., P. Kumar, M. Singh, D. Kumar, S. Ramakrishnan, T. K. Goswami, A. Singh, and G. C. Ram.** 2010. Experimental iron-inactivated *Pasteurella multocida* A: 1 vaccine adjuvanted with bacterial DNA is safe and protects chickens from fowl cholera. Vaccine. 28:2284-2289

10. **Higgs, R., P. Cormican, S. Cahalane, B. Allan, A. T. Lloyd, K. Meade, T. James, D. J. Lynn, L. A. Babiuk, and C. O'Farrelly.** 2006. Induction of a novel chicken Toll-like receptor following *Salmonella enterica* serovar Typhimurium infection. Infection and immunity **74:**1692-1698.
11. **Horrocks, S. M., R. C. Anderson, D. J. Nisbet, and S. C. Ricke.** 2009. Incidence and ecology of *Campylobacter jejuni* and *coli* in animals. Anaerobe **15:**18-25.
12. **Janssen, R., K. A. Krogfelt, S. A. Cawthraw, W. van Pelt, J. A. Wagenaar, and R. J. Owen.** 2008. Host-pathogen interactions in *Campylobacter* infections: the host perspective. Clinical microbiology reviews **21:**505-518.
13. **Keestra, A. M., M. R. de Zoete, R. A. van Aubel, and J. P. van Putten.** 2008. Functional characterization of chicken TLR5 reveals species-specific recognition of flagellin. Molecular immunology **45:**1298-1307.
14. **Keestra, A. M., and J. P. van Putten.** 2008. Unique properties of the chicken TLR4/MD-2 complex: selective lipopolysaccharide activation of the MyD88-dependent pathway. J Immunol **181:**4354-4362.
15. **Kim, Y. M., M. M. Brinkmann, M. E. Paquet, and H. L. Ploegh.** 2008. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. Nature **452:**234-238.
16. **Latz, E., A. Schoenemeyer, A. Visintin, K. A. Fitzgerald, B. G. Monks, C. F. Knetter, E. Lien, N. J. Nilsen, T. Espevik, and D. T. Golenbock.** 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nature immunology **5:**190-198.
17. **Le Moigne, V., G. Robreau, and W. Mahana.** 2008. Flagellin as a good carrier and potent adjuvant for Th1 response: study of mice immune response to the p27 (Rv2108) *Mycobacterium tuberculosis* antigen. Molecular immunology **45:**2499-2507.
18. **Lee, M. D., and D. G. Newell.** 2006. *Campylobacter* in poultry: filling an ecological niche. Avian diseases **50:**1-9.
19. **Mata-Haro, V., C. Cekic, M. Martin, P. M. Chilton, C. R. Casella, and T. C. Mitchell.** 2007. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science (New York, N.Y **316:**1628-1632.
20. **McAleer, J. P., and A. T. Vella.** 2008. Understanding how lipopolysaccharide impacts CD4 T-cell immunity. Critical reviews in immunology **28:**281-299.

21. **Park, B., M. M. Brinkmann, E. Spooner, C. C. Lee, Y. M. Kim, and H. L. Ploegh.** 2008. Proteolytic cleavage in an endolysosomal compartment is required for activation of Toll-like receptor 9. Nature immunology **9:**1407-1414.

22. **Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. van Vliet, S. Whitehead, and B. G. Barrell.** 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature **403:**665-668.

23. **Shao, F., C. Golstein, J. Ade, M. Stoutemyer, J. E. Dixon, and R. W. Innes.** 2003. Cleavage of *Arabidopsis* PBS1 by a bacterial type III effector. Science (New York, N.Y **301:**1230-1233.

24. **Shaughnessy, R. G., K. G. Meade, S. Cahalane, B. Allan, C. Reiman, J. J. Callanan, and C. O'Farrelly.** 2009. Innate immune gene expression differentiates the early avian intestinal response between *Salmonella* and *Campylobacter*. Veterinary immunology and immunopathology **132:**191-198.

25. **Shen, H., B. M. Tesar, W. E. Walker, and D. R. Goldstein.** 2008. Dual signaling of MyD88 and TRIF is critical for maximal TLR4-induced dendritic cell maturation. J Immunol **181:**1849-1858.

26. **Skountzou, I., M. D. Martin, B. Wang, L. Ye, D. Koutsonanos, W. Weldon, J. Jacob, and R. W. Compans.** 2009. *Salmonella* flagellins are potent adjuvants for intranasally administered whole inactivated influenza vaccine. Vaccine.

27. **Temperley, N. D., S. Berlin, I. R. Paton, D. K. Griffin, and D. W. Burt.** 2008. Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. BMC genomics **9:**62.

28. **Warshakoon, H. J., J. D. Hood, M. R. Kimbrell, S. Malladi, W. Y. Wu, N. M. Shukla, G. Agnihotri, D. Sil, and S. A. David.** 2009. Potential adjuvantic properties of innate immune stimuli. Human vaccines **5:**381-394.

29. **Wong, J. P., M. E. Christopher, S. Viswanathan, N. Karpoff, X. Dai, D. Das, L. Q. Sun, M. Wang, and A. M. Salazar.** 2009. Activation of toll-like receptor signaling pathway for protection against influenza virus infection. Vaccine **27:**3481-3483.

30. **Xiaowen, Z., Y. Qinghua, Z. Xiaofei, and Y. Qian.** 2009. Co-administration of inactivated avian influenza virus with CpG or rIL-2 strongly enhances the local immune response after intranasal immunization in chicken. Vaccine **27:**5628-5632.

31. **Xie, H., R. B. Raybourne, U. S. Babu, H. S. Lillehoj, and R. A. Heckert.** 2003. CpG-induced immunomodulation and intracellular bacterial killing in a chicken macrophage cell line. Developmental and comparative immunology **27:**823-834.
32. **Young, K. T., L. M. Davis, and V. J. Dirita.** 2007. *Campylobacter jejuni*: molecular biology and pathogenesis. Nature reviews **5:**665-679.

Nederlandse samenvatting

Inleiding

Campylobacter jejuni is een Gram-negatieve, spiraalvormige bacterie die voorkomt in het darmkanaal van een groot aantal verschillende dieren, waaronder pluimvee, runderen en de meeste huisdieren. Door zijn vorm en twee propellerachtige zweepstaarten, de zogeheten flagellen, is *Campylobacter* in staat zich zeer efficiënt door het slijmvlies van de darm heen te bewegen. In de meeste dieren vormt *Campylobacter* geen probleem; in de slijmlaag vermenigvuldigt de bacterie zich tot grote aantallen zonder dat de gastheer er hinder van ondervindt. In de mens is dit echter anders; na het binnenkomen in het darmkanaal via besmet voedsel of drinkwater, ontstaat een darmontsteking die gepaard gaat met acute (soms bloederige) diarree, hevige buikkrampen en koorts, en die ruim een week kan duren. In gezonde mensen wordt de infectie zonder de hulp van medisch ingrijpen door het immuunsysteem van het lichaam vanzelf opgeruimd. In mensen met een verzwakt immuunsysteem kan de bacterie echter tot langdurige darmontstekingen en sepsis leiden. In Nederland wordt het aantal gevallen van voedselvergiftiging door *Campylobacter* geschat op ~80.000 per jaar, wereldwijd ligt dit aantal waarschijnlijk rond de ~400 miljoen. *Campylobacter* infecties zijn sterk gecorreleerd met de autoimmuunziekte Guillain-Barré Syndroom. Zo wordt ongeveer 1 op de 5000 gevallen van *Campylobacter* bij gezonde mensen gevolgt door deze polyneuropathy, waarbij patiënten verlammingsverschijnselen krijgen en (landurige) ziekenhuisopname vaak noodzakelijk is.

Kip, een belangrijke bron van besmetting

Hoewel mensen een *Campylobacter* infectie kunnen oplopen door het binnenkrijgen van besmet oppervlaktewater of het drinken van niet-gepasteuriseerde koemelk, wordt algemeen geaccepteerd dat in Nederland de consumptie van besmet kippenvlees de belangrijkste bron van infectie is. Het merendeel van de (vlees)kippen in Nederland is besmet met grote hoeveelheden *Campylobacter* (rond de 10 miljard per kip), die tijdens het slachtproces vanuit het darmkanaal het kippenvlees besmetten. Dit leidt tot een groot aantal levende *Campylobacter* bacteriën op het kippenvlees in de supermarkt, terwijl slechts 500 bacteriën gemiddeld nodig zijn om iemand ziek te maken.

Eén van de manieren om de hoeveelheid *Campylobacter* op het kippenvlees te verminderen (en daardoor dus ook het aantal besmettingen in de mens) is het voorkomen of verlagen van de hoeveelheid *Campylobacter* in de kippendarm door middel van vaccinatie. Een dergelijk vaccin, wat doorgaans bestaat uit intacte bacteriën of onderdelen daarvan, moet een sterke immuunreactie opwekken waardoor ook gewone *Campylobac-*

ter infecties kunnen worden voorkomen. De ontwikkeling van een *Campylobacter* vaccin is in de praktijk echter moeilijk gebleken; de bacterie is gespecialiseerd om te leven als een (kippen)commensaal en is dus zeer goed in staat te overleven in de kippendarm zonder tot last te zijn voor de kip of immuunreacties op te wekken. Om dit te bereiken heeft *Campylobacter* een aantal eigenschappen ontwikkeld om onzichtbaar te blijven voor de gastheer. Zo dringt de bacterie niet de darmwand van de kip binnen, is hij omgeven door een zogeheten kapsel van suikers tegen aanvallen van het immuunsysteem, zijn de meeste oppervlakte eiwitten afgeschermd en induceert hij niet actief een immuunreactie. Al deze eigenschappen maken vaccinatie complex. Om toch een efficiënte immuunreactie tegen *Campylobacter* op te wekken tijdens vaccinatie, moet daarom het immuunsysteem actief gestimuleerd worden. Hiervoor is een gedegen kennis van de kippen immunologie vereist.

Toll-like receptoren

Het immuunsysteem is ruwweg in te delen in een "aangeboren" (innate) immuunsysteem, en een "verworven" (adaptive) immuunsysteem. De belangrijkste functie van de laatste is de productie van antilichamen die specifiek bacteriën, virussen en andere micro-organismen binden en neutraliseren. De aanmaak van deze antilichamen, wat ongeveer een week in beslag neemt, is een complex proces waarbij verschillende immuuncellen betrokken zijn. Maar hoe weten deze cellen wanneer en waartegen ze antilichamen moeten gaan maken? Hier ligt een belangrijke functie van het innate immuunsysteem. Dit systeem herkent (binnen seconden) bepaalde componenten die voorkomen in micro-organismen maar niet in mensen, en detecteert hierdoor de aanwezigheid van een potentieel gevaarlijke infectie. Na herkenning wordt een proces in werking gesteld dat leidt tot ontsteking en de aanmaak van de juiste antilichamen door het adaptive immuun systeem. Sleuteleiwitten van het innate immuunsysteem zijn de Toll-like receptoren (TLRs). Dit zijn de sensoren die de verschillende componenten van de micro-organismen herkennen. De mens bezit tien verschillen TLRs, TLR1-10. Afhankelijk van de TLR zitten deze receptoreiwitten aan het oppervlakte van de cel of in de intracellulaire lysosomen. De oppervlakte TLRs herkennen voornamelijk bacteriële componenten; TLR2 in combinatie met TLR1 of TLR6 bindt verschillende bacteriële lipoproteïne, TLR4 bindt lipopolysaccharide (een belangrijk onderdeel van de Gram-negatieve bacteriële celwand), en TLR5 bindt flagelline (een bestanddeel van de bacteriële zwemstaart). De intracellulaire TLRs (TLR3, TLR7, TLR8 en TLR9) binden voornamelijk opgenomen DNA of RNA, van zowel bacteriën als virussen. Na het binden van de microbiële component, of "ligand",

gaan twee TLRs een interactie aan die leidt tot TLR-activatie en de start van een signaal transductie route. Via de regulatoire eiwitten NF-κB (alle TLRs behalve TLR3) of IRF3 (TLR3 en TLR4) resulteert dit proces uiteindelijk in de productie van immunomodulatoire eiwitten, de zogeheten cytokines. Deze cytokines zijn de drijvende kracht voor het sturen van de juiste adaptive immuunreactie, de aanmaak van antilichamen, en de bestrijding van de infectie.

Het belang van een goed werkend innate immuunsysteem wordt vooral duidelijk als het fout gaat. Zo zijn bijvoorbeeld mensen met een niet-functioneel TLR5 eiwit (ongeveer 10% van de bevolking) veel gevoeliger voor een infectie met *Legionella*, en hebben kinderen met een mutatie in TLR3 vaker problemen met het *Herpes simplex* virus.

Doel van het onderzoek

Voor vaccinatiedoeleinden zijn TLRs zeer interessant. TLR liganden zijn namelijk natuurlijke "adjuvantia", stoffen met een krachtige immuunstimulerende werking die voor een goede immuunreactie tegen een vaccin vaak essentieel zijn. In kippen is de kennis over de functie van TLRs nog gering, zeker in combinatie met *Campylobacter*. In het DNA van de kip (het genoom) zijn net als bij mensen 10 TLRs aanwezig, maar er zijn een aantal duidelijke verschillen in het soort TLRs. Zo kunnen kippen met één combinatie van TLR2(type 2) en TLR16 dezelfde liganden herkennen als de mens met TLR2+TLR1 en TLR2+TLR6, hebben ze geen functioneel TLR8, én ontbreekt TLR9, die belangrijk is voor de herkenning van bacterieel DNA. In plaats daarvan zijn er twee sensoren die niet in zoogdieren voorkomen, namelijk TLR21 en TLR15. Aangezien TLRs een cruciale rol kunnen spelen bij vaccinatie, hebben wij onderzocht wat de preciese functie is van deze twee onbekende TLRs. Vervolgens hebben we gekeken naar de interactie tussen *Campylobacter* en TLRs van mens en kip, om zo mogelijke verschillen in de herkenning van de bacterie door de twee gastheren in kaart te brengen. Uiteindelijk hebben we geprobeerd deze kennis om te zetten in de ontwikkeling van een TLR-gebaseerde vaccin-kandidaat.

Opheldering van onbekende kippen TLRs

TLR21

De herkenning van bacterieel DNA is een belangrijke marker voor infectie en een krachtige immuunstimulans tijdens de ontwikkeling van antilichamen. Mensen hebben hiervoor het sensor eiwit TLR9. Echter, het genomisch DNA van de kip bevat geen versie van

"kippen TLR9". In **hoofdstuk 2** hebben wij het gen van kippen TLR21 geïdentifieerd en gecloneerd, waarna we het in een celkweeksysteem tot expressie hebben gebracht. Op deze manier werd het TLR21 eiwit door menselijke cellen geproduceerd en kon de functie onderzocht worden. Door het screenen van een serie verschillende microbiële liganden bleek dat de cellen met TLR21 (maar *niet* cellen zonder) het immunoregulatoire eiwit NF-kB activeerden na toevoeging van synthetisch DNA. Een zorgvuldige lokalisatie studie liet zien dat TLR21 intracellulair gelokaliseerd was, wat in lijn is met de huidige kennis over de immuunherkenning van DNA. Door middel van binding studies hebben we laten zien dan DNA inderdaad een direct contact aanging met de TLR21 receptor, net als TLR9 van de mens, en specifieke inhibitie van de productie van TLR21 in kippencellen resulteerde in een duidelijk verminderde reactie op het synthetische DNA, suggererend dat ook in een meer natuurlijke omgeving TLR21 in staat is DNA te herkennen. Vergeleken met TLR9 werd TLR21 geactiveerd door een breder scala aan verschillende DNA's; zo herkende TLR21 geïsoleerd chromosomaal DNA van bacteriën veel efficiënter dan TLR9, iets wat van cruciaal belang kan zijn tijdens infecties.

TLR15

De enige overgebleven TLR waarvan de functie nog opgehelderd moest worden in de kip was TLR15. TLR15 heeft geen enkele gelijkenis met TLRs met een bekende functie; sterker nog, het meest dichtbije "familielid" is aanwezig in insecten. In **hoofdstuk 3** is, net als bij TLR21, het gen van TLR15 geïdentificeerd, geanalyseerd, en gecloneerd voor expressie in een celkweeksysteem. TLR15 bleek een glycoproteïne dat in overvloed voorkomt op het celoppervlak van zowel kippencellen als de menselijke cellen van het celkweeksysteem. Een uitgebreide screening om het activerende ligand te vinden (door toevoeging van zowel alle bekende TLR liganden als een uitgebreide verzameling van micro-organismen) leverde echter niks op. Pas na toevoeging van gefiltreerd intestinaal materiaal van kippen induceerde TLR15 een sterke NF-κB activatie, wat niet gebeurde in cellen zonder TLR15. Uit dit materiaal was een schimmel te isoleren die, als zuivere cultuur, ook TLR15 kon activeren. Na verdere analyse van het schimmel-afkomstige TLR15 ligand bleek dat de immuunactivatie afhankelijk was van een zogeheten protease, een eiwit dat andere eiwitten kan afbreken. Deze proteases worden vaak uitgescheiden door schimmels. Een opgezuiverde protease kon TLR15 sterk activeren, en specifieke inhibitie van de enzymatische activiteit van het protease verhinderde NF-κB activatie. Deze resultaten geven aan dat de kip een unieke manier heeft gevonden om micro-organismen die proteases uitscheiden te herkennen door het immuunsysteem.

Aangezien het ligand van TLR15, het protease eiwit, compleet anders is dan de liganden die tot nu toe bekend zijn voor andere TLRs, hebben we verder onderzocht hoe het mechanisme van activatie precies werkt. Door middel van eiwit analyse op TLR15 voor en na "stimulatie" met de protease bleek dat de receptor na activatie ongeveer de helft kleiner was, en dus gedeeltelijk was afgebroken door de protease. Alhoewel TLR9 van de mens ook wordt "afgeknipt" voordat het op DNA kan reageren, is een directe activatie van een TLR door een protease niet eerder beschreven en uniek voor TLR15.

De interactie van *Campylobacter* met de TLRs van mens en kip

Campylobacter veroorzaakt acute darmontsteking in de mens, maar in de kip lijkt niks te gebeuren. Het innate immuunsysteem, en specifiek het TLR repertoire, ligt vaak ten grondslag ligt aan ontstekingen. Hierdoor kan één van de oorzaken van het kip / mens verschil liggen in een andere herkenning van *Campylobacter* door het immuunsysteem van beide gastheren. Kennis hierover kan vervolgens ook leiden tot een verbetering van kippen vaccinatie doordat er specifiek innate immuun stimulatie kan worden gegeven door middel van het toevoegen van TLR liganden (die misschien ontbreken in *Campylobacter*) als adjuvantia. In **hoofdstuk 4** hebben we daarom gekeken in hoeverre *Campylobacter* in staat is om TLRs van de mens en de kip te activeren.

In **hoofdstuk 4** laten we zien dat *Campylobacter* is in staat de productie cytokines te initiëren in immuuncellen van zowel mens als kip. Om te onderzoeken welke TLRs bij dit proces betrokken zijn, en of er gastheer specifieke verschillen zijn in het TLR repertoire dat door *Campylobacter* wordt geactiveerd, zijn alle menselijke en kippen TLRs die betrokken zijn bij de mogelijke herkenning van de bacterie afzonderlijk tot expressie gebracht in een celkweeksysteem en getest op activatie door zowel levende als gedode *Campylobacter*.

Een interessante bevinding was dat levende bacteriën niet of nauwelijks TLRs kunnen activeren, en er dus duidelijk bacteriële celschade of actieve uitscheiding van bacteriële liganden moet zijn voor het ontstaan van een immuunreactie, in zowel de mens als de kip. Dit zou een strategie van *Campylobacter* kunnen zijn om zo onzichtbaar mogelijk te blijven voor de gastheer. De lipoproteïne aanwezig in dode *Campylobacter* waren goed in staat een NF-κB reactie te starten via de combinatie TLR2/1/6 van de mens, en TLR2t2/TLR16 van de kip. Hetzelfde gold voor TLR4 van de kip en de mens, die beide het lipo-oligosaccaride aanwezig in gedode *Campylobacter* goed herkenden. Een belangrijk verschil in de activatie van TLR4 was dat één van de twee signaal transductie routes na TLR4 activatie, de IRF3-afhankelijk route die leidt to de productie van de cytokine interfe-

ron-β, in de mens wél wordt geactiveerd maar in de kip niet. Aangezien interferon-β productie in muizen leidt tot systemische ontsteking, zou het gebrek aan interferon-β tijdens infectie van *Campylobacter* in de kip grote gevolgen kunnen hebben op het verloop van de infectie.

TLR5 van zowel mens als kip bleek niet te kunnen reageren op de verschillende *Campylobacter* stammen die getest zijn. Hoewel dit een interessante bevinding is, is de functie hiervan tijdens infectie nog onduidelijk en lijkt het niet het verschil uit te maken tussen het verschil in ziektebeeld in mens en kip. Als laatste is de activatie van TLR9 van de mens en TLR21 van de kip door geïsoleerd chromosomal DNA van *Campylobacter* met elkaar vergeleken. Hoewel TLR9 wel goed reageerde op het synthetische DNA, wekte *Campylobacter* DNA geen NF-κB reactie op via deze receptor. Daarentegen kon TLR21 wel goed worden geactiveerd door *Campylobacter* DNA. Het verschil in innate immuun herkenning door de DNA receptors, wellicht in samenwerking met het gebrek aan interferon-β activatie door TLR4, zou kunnen bijdragen aan het verschillende verloop van een *Campylobacter* infectie in de mens en de kip.

Modulatie van TLR5 activatie door *Campylobacter* flagelline

Veel bacteriesoorten kunnen zich efficiënt voortbewegen (zwemmen) door middel van één of meerdere flagellen, propellers die razendsnel ronddraaien. Deze flagellen zijn opgebouwd uit tienduizenden kopieën van het eiwit flagelline. Aangezien dit eiwit voorkomt bij veel bacteriën, zeer geconserveerd is, en niet voorkomt bij de mens, is het een ideaal target voor het immuunsysteem. flagelline wordt daarom ook herkend door TLR5, waarna een sterke afweerreactie volgt. Zoals hierboven al is beschreven, kan flagelline van *Campylobacter* TLR5 niet activeren. Om te onderzoeken wat de basis hiervan is, en vervolgens de TLR5 activiteit te versterken, hebben we in **hoofdstuk 5** gekeken naar de interactie tussen *Campylobacter* flagelline en TLR5.

Campylobacter heeft twee flagellines, FlaA en FlaB, die bedekt zijn met ~20 suikermoleculen. Na onderzoek bleek dat beide flagellines niet in staat zijn TLR5 te activeren, en dat deze eigenschap na verwijdering van de suikers niet veranderde. Deze resultaten suggereerde dat er in het DNA dat codeert voor de flagellines veranderingen zijn ontstaan die er voor zorgen dat de binding tussen flagelline en TLR5 niet meer kan plaatsvinden. Een vergelijkende DNA analyse van de flagelline genen van *Campylobacter* en *Salmonella* (die wél TLR5 activeert), liet inderdaad zien dat er DNA veranderingen zijn die coderen voor plekken in het eiwit waarvan wordt vermoedt dat ze belangrijk zijn voor TLR5-binding. Via DNA recombinatie technieken hebben we vervolgens regio's van

Salmonella flagelline uitgewisseld met die van *Campylobacter*, om zo het TLR5-activerende effect in *Campylobacter* flagelline te herstellen. Uitwisseling van twee van deze regio's was voldoende om TLR5 van de kip te activeren, maar voor activatie van menselijk TLR5 was een derde, tot dan toe onbekende regio voor TLR5 activatie nodig. De gecreëerde "chimeer" flagelline eiwitten zijn vervolgens bij het genomisch DNA van *Campylobacter* geplaatst, waardoor de bacterie het eiwit kon produceren, er suikers op kon zetten, en kon uitscheiden.

Het *Campylobacter* flagelline is in het verleden vaak getest als vaccin kandidaat, omdat van antilichamen tegen flagelline wordt verwacht dat ze de bacterie onbeweeglijk, en dus zo goed als ongevaarlijk, maken. Echter, één van de problemen van deze vaccinatie experimenten is een relatief lage effectieve immuunreactie tegen het flagelline. Omdat het nieuwe chimeer flagelline, in tegenstellin tot het natuurlijke *Campylobacter* flagelline, is staat is zélf de immuunreactie via TLR5 te verhogen, heeft dit eiwit goede potentie als verbeterd flagelline vaccin kandidaat.

Conclusie en vooruitzichten voor de toekomst

Campylobacter is een groot probleem voor de mens, maar niet voor de kip. Hierin ligt de moeilijkheid voor de ontwikkeling van een vaccin tegen *Campylobacter* voor kippen. De grote efficiëntie waarmee de bacterie de kip al op jonge leeftijd koloniseert maakt het zeer moeilijk een sterke afweer op te roepen, zeker omdat *Campylobacter* in de kip van nature gespecialiseerd is om zich juist in de luwte te vermenigvuldigen. Om gericht een vaccin tegen deze commensale bacterie te ontwikkelen is een grondige kennis nodig van het immuunsysteem. In dit onderzoek is daarom de functie van twee innate immuun receptoren opgeheldert. Aangezien hun liganden kunnen dienen als adjuvantia, hebben beide receptoren de potentie om bij te dragen aan een versterkte afweer reactie tijdens vaccinatie. Een grondige inspectie van de TLRs die een rol spelen bij infectie in mens en kip heeft geleidt tot de ontwikkeling van het eerste "tailor-made" flagelline vaccin dat in staat is actief het immuunsysteem van de kip te moduleren, en een sterke afweerreactie.

Toekomstige vaccinatie experimenten zullen in de praktijk de werking van het flagelline vaccin moeten uitwijzen. Tot die tijd is het beste advies nog altijd: was je handen met zeep na het bereiden van kippenvlees, gebruik niet dezelfde snijplank voor de groente, en zorg dat de kip goed doorbakken is!

Dankwoord

En dan eindelijk het Dankwoord! Ook voor mij geldt helaas het cliché: op het allerlaatste moment met grote (deadline)druk geschreven, terwijl dit gedeelte juist door iedereen als eerste (en vaak enige) wordt gelezen. Ik ga mijn best doen om niemand te vergeten!

Natuurlijk moet ik als eerste mijn promotor Jos van Putten bedanken. Jos, ik schijn tijdens mijn sollicitatiegesprek in de richting van je bureaustoel te hebben gekeken, wat meteen werd geïnterpreteerd als zeer ambitieus; daar wilde ik blijkbaar zo snel mogelijk komen te zitten. Zelf kan ik het me niet meer herinneren, maar het verhaal is in de loop van de jaren, mede dankzij mijn tweede promotor, steeds sterker geworden. In ieder geval klikte het vanaf het eerste begin erg goed en dat is zo gebleven. Het meest zal me bijblijven de vrijdagmiddagen vanaf 16:00 uur, de vaste afsluiter van de week. Zowel grote als kleine mysteries werden behandeld, en regelmatig ook nog eens opgelost ("het wekelijkse Nature paper"). Vooral het vertrouwen in de dingen die ik bedacht, het subtiele bijsturen als het weer eens alle kanten op ging, het "groots denken", en de kiwi's zijn vreselijk belangrijk voor me geweest de afgelopen jaren. Wat nog blijft is de discussie hitteblok versus waterbad; daar komen we nog wel eens uit. Jos, ontzettend bedankt!

Dan Jaap Wagenaar, begonnen als copromotor maar halverwege zelf gepromoveerd tot promotor. Jaap, het eerste jaar schoof je nog netjes aan op vrijdagmiddag, maar plande speciaal een sabbatical om daar onderuit te komen. Na je terugkomst was je gelukkig slechts een gang verwijderd. Ik ben blij dat je gedurende mijn hele AIO-tijd bij mijn onderzoek betrokken bent gebleven, ik heb zowel de serieuze als totale onzin gesprekken erg gewaardeerd. Voor de rest was er natuurlijk de onvergetelijke Meldkamer, de *in ovo* vaccinatie, en *al* het geld. Jaap, heel erg bedankt, het was leuk! (Zie je wel? Niks gezegd over alle "vrijheid!")

Verder wil ik alle collega's van Infectiebiologie zeer bedanken voor de leuke tijd die ik er heb gehad. Eerst de harde kern: Marc, je kennis over bacteriën (en tegenwoordig ook schimmelinfecties bij gezelschapsdieren) was van grote waarde voor mijn onderzoek. Hetzelfde geldt voor Nancy; *Campylobacter* heeft zo langzamerhand weinig geheimen meer voor jullie! Wim, bedankt voor de inbreng en de papers die ik mocht reviewen.

Dan de mensen uit de eerste periode: Lieke, heel lang mijn kamergenoot, bedankt voor de gezelligheid, de wetenschappelijke discussies en het delen van de *Campylobacter*-frustraties. De grote vismoord van 2005 blijft één van mijn succesverhalen. Nog even en jullie zijn met z'n drieën, veel geluk! An, my second roommate, thank you for choosing me as your paranimf: it was an honor. Liana, Rémon, en Nina, jullie kennis en enthousiasme heb ik altijd zeer gewaardeerd. We komen elkaar in de toekomst ongetwijfeld nog tegen!

Vervolgens de huidige generatie, met om te beginnen Andries, mijn laatste kamergenoot. We hadden altijd een hoop te bespreken (er zijn dan ook *zoveel* mafketels in de wereld!). Succes met het afronden van je proefschrift, en je volgende stap. Andreas, onderzoeker in hard en nieren. Volgens mij zijn er weinig mensen die zo graag over wetenschap praten als jij. Daarnaast was het ook nog eens altijd lachen! Veel succes de komende periode, en we blijven pokeren! Lieneke, begonnen als mijn eerste fulltime student en dan tòch nog doorstromen als AIO: dat is pas doorzettingsvermogen! Je hebt een wezenlijke bijdrage geleverd aan dit boekje, heel erg bedankt! May Young, het was leuk om met jou in het lab te staan, zorg goed voor de plasmiden! Medi, a couple more years and then finally back to beautiful Iran!

Verder wil ik graag bedanken Stephanie (merci), Jésus, Irati (gracias), de "nieuwe" mensen van Moleculaire Afweer (in het bijzonder Edwin, Albert en Martin), Twan (gracias), uit Lelystad Esther, Fimme Jan, Marcel, Miriam en Frans, mijn andere studenten Paula (dzięki) en Berit (op de valreep), Anton, bedankt voor de hulp met de lay-out van mijn proefschrift, alle verdere studenten van de afgelopen jaren met in het bijzonder Matt (thanks), Martijn, Frederique, Marloes, en Anne Marie!

Ook bedank ik de mensen van de KLIF, met in het bijzonder Birgitta, Barend, Lea (ik zal je missen! Als je ooit in de buurt bent, kom langs!), en Rolf (wanneer is de volgende borrel??). Frans, je bent een goeie gozer. We gaan snel weer eens pokeren of schaken (hoewel er niemand in Utrecht en omstreken *zo* slecht schaakt als ik).

Linda, Linda, Linda… Ik kan met jou altijd praten, zowel over serieuzere zaken als over totale onzin. We hebben dezelfde humor, en ik zal het zeker gaan missen (ik weet namelijk zeker dat *niet* ieder lab een Linda heeft). En je weet het: iedereen is gek behalve wij!

Zorica, moet ik dit nu in het Nederlands, Engels of Servisch doen? Is het vrijdag? Thanks so much for the dinners, squash, and all those couples of minutes to spare… you're a good friend! Хвала!

Natuurlijk wil ik ook mijn vrienden bedanken, voor alle uren in de kroeg, etentjes in eetcafés, verre vakanties, of koffie in de oefenruimte. In het bijzonder Roel (succes met je eigen onderzoek!), Yo, Ramon (veel geluk met je gezinnetje!), en Chris. Dave, die wedstrijd in Spanje of Engeland, die doen we echt nog een keer!

Dan mijn paranimfen en vrienden Rob en Marijke. Rob, de wereld zal helaas nooit meer te weten komen wat voor baanbrekende wetenschappelijke ontdekkingen je had kunnen doen (hoewel, je weet het: "het is nog niet klaar!"). Gelukkig is de medische we-

reld er wel een stuk beter op geworden. Minimaal één keer per jaar in de californische zon, dat is een deal!

Marijke, zonder jou had mijn AIO periode er volledig anders uitgezien. Ik kan me niet voorstellen dat het net zo leuk en productief zou zijn geweest! Zonder jou naast me in het lab was het nog steeds heel leuk, maar toch niet helemaal hetzelfde. De huidige generatie kan gewoon niet zo goed zingen en dansen als wij! Ik weet zeker dat we elkaar blijven tegenkomen, zo niet in Cali, dan wel weer in Utrecht en omstreken. Bedankt! Wat ga ik trouwens eten vanavond?

Astrid en Sabine (en natuurlijk Dwight), geen idee wat jullie broer(tje) ergens in Utrecht in één of ander lab de hele dag uit aan het uitspoken was ("*Maar wat dóe je dan zo'n hele dag?*"). Bedankt dat jullie er altijd waren om weer over normale dingen te praten. Stella en Femke, mochten jullie over een jaar of 15 toevallig dat rare kleine boekje van jullie oom doorbladeren: jullie staan er ook in!

Als laatste pap en mam: bedankt voor alles.

Curriculum Vitae

List of Publications

Curriculum Vitae

Marcel Robert de Zoete werd geboren op 3 november 1979 te Leiden. In 1998 behaalde hij het Gymnasium diploma aan het Visser 't Hooft Lyceum in Leiden, waarna werd begonnen met de studie Medische Biologie aan de Vrije Universiteit in Amsterdam. Tijdens deze studie werd bij de afdeling Maag-, Darm-, en Leverziekten van het Erasmus Medisch Centrum in Rotterdam onder begeleiding van prof. dr. Hans Kusters en dr. Monique Gerrits een stage gevolgd waarin werd gewerkt aan antibiotica resistentie in *Helicobacter pylori*. Een tweede stage werd gevolgd bij de afdeling Medical Microbiology and Immunology aan de Texas A&M University in de Verenigde Staten. Hier werd onder begeleiding van prof. dr. Andreas Bäumler en dr. Rob Kingsley onderzoek gedaan naar adhesie eiwitten van *Salmonella*. In een afrondende literatuurstudie onder leiding van prof. dr. Andreas Bäumler en prof. dr. Hans Kusters werd gekeken naar virulentiefactoren van *Salmonella*. Na het behalen van zijn doctoraalexamen begon hij in september 2003 als assistent in opleiding bij de afdeling Infectiebiologie van de faculteit Diergeneeskunde, Universiteit Utrecht, waarbij onder begeleiding van prof. dr. Jos van Putten en prof. dr. Jaap Wagenaar onderzoek werd gedaan naar *Campylobacter jejuni*. Tot 1 september 2010 zal hij bij dezelfde afdeling als post-doc werkzaam blijven.

List of publications

1. **Keestra A. M.*, de Zoete M. R.*, Bouwman L. I., van Putten J. P. M.**
   Chicken TLR21 is an innate CpG DNA receptor distict from mammalian TLR9
   Journal of Immunology. **2010**. *In press*.
   *****Authors contributed equally

2. **de Zoete M. R., Keestra A. M., Wagenaar J. A., van Putten J. P. M.**
   Reconstitution of a functional Toll-Like receptor 5 binding site for Campylobacter jejuni Flagellin.
   Journal of Biological Chemistry. 285:12149-58. **2010**.

3. **de Zoete M. R., Keestra A. M., Roszczenko P., van Putten J. P. M.**
   Activation of human and chicken Toll-like receptors by Campylobacter spp.
   Infection and Immunity 78:1229-1238. **2010**.

4. **van Putten J. P. M., Bouwman L. I., de Zoete M. R.**
   Unraveling bacterial interactions with Toll-like receptors.
   Immunology Letters 128:8-11. **2010**.

5. **Wösten M. M. S. M., van Dijk L., Veenendaal A. K. J., de Zoete M. R., Bleumink-Pluijm N. M. C., van Putten J. P. M.**
   Temperature-dependent FlgM/FliA complex formation 1 regulates Campylobacter jejuni flagella length.
   Molecular Microbiology. **2010**. *In press*

6. **van Putten J. P. M., van Alphen L. B., Wösten M. M. S. M., de Zoete M. R.**
   Molecular mechanisms of Campylobacter infection.
   Current Topics in Microbiology and Immunology 337:197-229. **2009**.

7. **Keestra A. M., de Zoete M. R., van Aubel R. A., van Putten J. P. M.**
   Functional characterization of chicken TLR5 reveals species-specific recognition of flagellin.
   Molecular Immunology 45:1298-1307. **2008**.

8. **Keestra A. M., de Zoete M. R., van Aubel R. A., van Putten J. P. M.**
   The central leucine-rich repeat region of chicken TLR16 dictates unique ligand specificity and species-specific interaction with TLR2.
   Journal of Immunology 178:7110-7119. **2007**.

9. **de Zoete M. R., van Putten J. P. M., Wagenaar J. A.**
   Vaccination of chickens against Campylobacter.
   Vaccine 25:5548-5557. **2007**.

10. **Kingsley R. A., Keestra A. M., de Zoete M. R., Bäumler A. J.**
    The ShdA adhesin binds to the cationic cradle of the fibronectin 13FnIII repeat module: evidence for molecular mimicry of heparin binding.
    Molecular Microbiology 52:345-355. **2004**.

11. **Kingsley R. A., Humphries A. D., Weening E. H., de Zoete M. R., Winter S., Papaconstantinopoulou A., Dougan G., Bäumler A. J.**
    Molecular and phenotypic analysis of the CS54 island of Salmonella enterica serotype Typhimurium: identification of intestinal colonization and persistence determinants.
    Infection and Immunity 71:629-640. **2003**.

12. **Gerrits M. M., de Zoete M. R., Arents N. L., Kuipers E. J., Kusters J. G.**
    16S rRNA mutation-mediated tetracycline resistance in Helicobacter pylori.
    Antimicrobial Agents and Chemotherapy 46:2996-3000. **2002**.