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Neisseria meningitidis is a major cause of sepsis and/or meningitis. These bacteria normally cause disease only in humans, however, mice expressing human CD46 are susceptible to meningococcal disease. To explain the sensitivity of CD46 transgenic mice to meningococci, we evaluated early immune responses. Stimulation of TNF, IL-6, and IL-10 was stronger in CD46 transgenic mice compared with nontransgenic mice, and resembled human responses. In CD46 transgenic mice, bacterial clearance in blood started at later time points, and neutrophil numbers in blood were lower compared with nontransgenic mice. Further, elevated levels of activated microglia cells and cyclooxygenase-2 were observed in brain of infected CD46 transgenic mice. Intraperitoneal administration of meningococci led to increased levels of macrophages only in the i.p. cavity of CD46 transgenic mice. Most of the responses were impaired or absent using LPS-deficient meningococci, showing the importance of LPS in the early immune response to meningococcal infection. Taken together, these data demonstrate that responses in mice expressing human CD46 mimic human meningococcal disease in many aspects, and demonstrate novel important links between CD46 and the innate immune system. The Journal of Immunology, 2005, 175: 433–440.

Neisseria meningitidis (meningococcus) is a strict human pathogen and a major cause of sepsis, septic shock, and meningitis worldwide. These bacteria colonize the nasopharynx in 5–15% of healthy individuals for limited time periods during nonepidemic conditions (1). Nasopharyngeal carriage of these bacteria is normally entirely asymptomatic and only in a small percentage of colonized people N. meningitidis reaches the bloodstream, where it can cause meningococcemia and sepsis. From the blood the bacteria cross the blood-brain barrier (BBB), reach the cerebrospinal fluid (CSF), and cause meningitis.

Attachment of pathogenic Neisseria to epithelial cells is mediated by type IV pili and involves interaction with CD46 (2), a cell surface complement regulator (3, 4) and receptor for several other pathogens, e.g., adenovirus group B and D, human herpes virus 6, measles virus, and Streptococcus pyogenes (5–11). Adherence and colonization of host epithelial cells is followed by transcytosis and rapid meningococcal multiplication in the bloodstream, upon which three major cascade pathways are activated: the complement system, the coagulation and fibrinolysis pathway, and inflammatory responses (12).

Phagocytic leukocytes such as neutrophils and macrophages are essential for innate immune responses against invading bacteria. Interaction between bacteria and these host cells triggers potent antimicrobial activity. Although phagocytes aim to destroy bacteria, modulation of leukocyte apoptosis or cell death by bacteria has developed as a mechanism of pathogenesis (13). Neutrophils are short-lived cells produced in the bone marrow, circulating for 6–9 h, and then migrating into tissue with a life span of an additional 3 days. Neutrophils leave the blood by interaction with integrins and concentrate at the site of infection in response to chemotactic factors (14). Bacteria are phagocytosed by neutrophils and exposed to antibacterial substances. The mononuclear phagocyte system includes cells derived from monocytes, e.g., macrophages and microglia in the brain. These cells are readily mobilized to sites of infection for local activation, and macrophages are present at mucosal surfaces and occur systemically. The CSF lacks cells capable of initiating an effective immune response against invading pathogens. Meningeal macrophages act as phagocytic cells and facilitate the influx of leukocytes at the BBB and hence play a crucial role during clearance of bacteria entering the subarachnoidal space (15).

Neisserial LPS is responsible for the damage of human epithelial and endothelial cells, and is an important activator of immune responses upon infection (16). Morbidity and mortality of meningococcal bacteremia is directly correlated with circulating meningococcal LPS leading to activation of pro- and anti-inflammatory mediators resulting in septic shock and, occasionally, death (17–19). LPS binds to LPS-binding protein and eventually to CD14 expressed on monocytes, macrophages, and other host cells. Because CD14 lacks an intracellular signaling domain, interaction between CD14, TLR 4, and MD-2 is necessary to mediate an intracellular signal transduction. This signal results in production of cytokines such as IL-1β and TNF (20–21). Proinflammatory cytokines are believed to elicit the systemic cascade reactions seen in meningococcal disease such as multiorgan failure and death (22). These cytokines play a crucial role in enhancing the bactericidal capacity of phagocytes, recruiting additional innate cell populations to the site of infection, and directing immune responses to the...
invading microbe (23). LPS-deficient N. meningitidis may also induce proinflammatory cytokine production. This immune response is thought to involve bacterial components such as peptidoglycan, lipoprotein, and CpG DNA (24).

A complete understanding of meningococcal disease requires an animal model that mimics the human host. Several experimental model systems using mice, rats, or rabbits have been evaluated over the last decades, but these require either infant animals or preinjection of inflammatory enhancers such as iron. Recently, transgenic mice expressing human CD46 were demonstrated to be susceptible to meningococcal disease (25). Crossing of the BBB occurred in CD46 transgenic mice, but not in nontransgenic mice. Additionally, CD46 transgenic mice showed 100% mortality 48 h postchallenge, whereas nontransgenic mice survived.

In this study, immune responses in CD46 transgenic mice challenged with N. meningitidis were investigated with the aim to explain the rapid course of disease and the high mortality rate observed in CD46 transgenic mice. In CD46 transgenic mice the production of pro- and anti-inflammatory cytokines such as TNF, IL-6, and IL-10, the ability of bacteria to cross the BBB, and the stimulation of inflammatory responses in the brain tissue seem to be major factors of the lethal outcome seen after meningococcal challenge. The CD46 transgenic mouse model mimics human meningococcal disease in many aspects and is therefore a very useful tool in future vaccine trials against meningococcal infection.

**Materials and Methods**

**Mouse strains**

The hCD46Ge transgenic mouse line was generated as previously described (26). CD46 in the mouse strain used (hCD46Ge) was detected in all tested tissues (25–27). Using immunohistochemistry, CD46 was found on epithelial cells, endothelial cells, glial cells, hepatocytes, in the glomerulus (in kidney) and adrenal gland, as well as on B cells, T cells, neutrophils, macrophages. The hCD46Ge transgenic mice breed normally but tend to become obese with time (27). C57BL/6 and hCD46Ge mice were bred at the animal facility. All mice were 5–8 wk old when challenged with bacteria. All mouse procedures were performed in accordance with institutional protocol guidelines under an approved protocol.

**Bacterial strains and growth conditions**

N. meningitidis FAM20 (PilC1−, PilC2−) belongs to serogroup C (28, 29). The LPS-deficient FAM20 (PilC1−, PilC2−, lpxA−) mutant has previously been described (30). The lpxA gene encodes an enzyme responsible for the first step of the lipid A biosynthesis pathway, adding the O-linked 3-OH fatty acid to UDP-acetylglucosamine. The meningococcal strains were grown on GC-agar containing Kellogg’s supplement (31) at 37°C in 5% CO2 atmosphere and were passaged every 18–20 h. The LPS from a commercial kit tyramide signal amplification (DuPont/NEN) was used. The sections were then incubated with a secondary Ab (1/40) from The Jackson Laboratory was used to visualize the signal and the sections were analyzed in a Nikon Fluorescence microscope. As the primary Ab for detecting COX-2 a rabbit anti-COX-2 (Cayman Chemical) was used at a dilution of 1/2000. For detection of ionized calcium-binding adaptor molecule 1 (Iba1) a rabbit Iba1 antiserum (1/2000) from Wako Chemicals was used. The sections were then incubated with a secondary swine anti-rabbit Ab conjugated with HRP and proceeded with reagents from a commercial kit tyramide signal amplification (DuPont/NEN) including FITC-conjugated streptavidin. The sections were analyzed in a Nikon fluorescence microscope.

**Statistics**

Statistical significance was determined using the two tailed t test; values of p < 0.05 were considered significant.

**Results**

CD46 transgenic mice are delayed in bacterial clearance from blood

Survival of bacteria in the blood and subsequent crossing of the BBB are considered critical for the development of meningococcal disease. Intraperitoneal challenge of CD46 transgenic mice with N. meningitidis is lethal, and bacterial crossing of the BBB occurs in CD46 mice, but not in nontransgenic mice (25). In representative experiments, 30% of the CD46 transgenic mice survived day 1 postchallenge, and none survived day 2, whereas all nontransgenic mice survived the challenge. To explore possible variations in bacterial blood counts after i.p. challenge with N. meningitidis FAM20, blood was collected at different time points during the first 24 h, diluted, and spread on GCB-agar plates for determination of CFUs. At 1, 3, and 6 h postchallenge there were no significant differences in CFU per milliliter between CD46 transgenic mice and nontransgenic mice (Fig. 1), supporting the idea that survival of nontransgenic mice most likely was not due to differences in bacterial numbers initially reaching the blood. However,
10-fold more bacteria were found in blood of CD46 transgenic mice compared with nontransgenic mice at 12 h postinoculation, suggesting that bacterial survival and avoidance of clearance was enhanced in CD46 transgenic mice.

LPS-deficient FAM20 (lpxA mutant) does not cause disease in CD46 transgenic mice at equivalent doses (25). As shown in Fig. 1, bacterial numbers in blood of CD46 transgenic and nontransgenic mice were 30-fold lower at 1 h postchallenge with LPS-deficient bacteria compared with the wild-type strain. Further, LPS-deficient meningococci were cleared 12 h postchallenge.

Increased TNF and IL-6 production in infected CD46 transgenic mice

Most death by meningococcal sepsis is not only due to the infection itself, but also from hypotension and organ failure characteristic of septic shock. This is a manifestation of the uncontrolled release of proinflammatory cytokines, such as TNF and IL-6. Host TNF and IL-6 responses were analyzed by measuring cytokine production in mouse serum by ELISA at different time points postinfection. As shown in Fig. 2A, TNF levels were raised after i.p. challenge with FAM20. Stimulation was significantly higher in CD46 mice compared with nontransgenic mice, and at 1 h postinfection TNF levels ranged between 271 and 425 pg/ml in CD46 transgenic mice and between 185 and 263 pg/ml in nontransgenic mice. In patients with septic shock caused by meningococci, TNF serum levels may reach ~500 pg/ml (35), which is comparable to TNF levels detected in CD46 transgenic mice challenged with N. meningitidis FAM20 (350 pg/ml). Infection of CD46 mice or nontransgenic mice with LPS-deficient FAM20 did not stimulate the production of TNF in
IL-10 levels of serum. Cytokines could not be detected in mice before infection or in mice injected with medium (data not shown).

IL-6 induction was strong at 3 and 6 h postchallenge in CD46 transgenic mice and then decreased rapidly (Fig. 2B). Nontransgenic mice showed significantly lower levels of IL-6. IL-6 was not detected in noninfected mice, mice inoculated with media, or mice inoculated with LPS-deficient meningococci (data not shown). In patients with meningococcal disease the pattern of TNF and IL-6 response resembled those seen in CD46 transgenic mice with an early peak and rapid decrease, and with the IL-6 peak occurring shortly after TNF (36).

**Increased IL-10 production in CD46 transgenic mice**

IL-10 is an anti-inflammatory cytokine with suppressive effects on the synthesis of proinflammatory cytokines and chemokines, such as TNF, IL-1β, IL-1α, IL-2, IL-6, and IL-8. IL-10 injection has been shown to increase survival rates in murine models of endotoxemia (37–39). High levels of IL-10 in sera have been reported to increase survival rates in murine models of endotoxemia and with the IL-6 peak occurring shortly after TNF (36).

**Mice** were challenged i.p. and blood samples were collected at different time points postchallenge. Neutrophils vs total number of immune cells were determined by microscopical examination of blood smears. As shown in Fig. 3A, the neutrophil levels were stable over time in CD46 transgenic mice, but increased in nontransgenic mice at 6, 12, and 24 h postchallenge. These levels were significantly higher than neutrophil levels at earlier time points and levels in nontransgenic mice injected with medium (control). Transgenic and nontransgenic mice challenged with LPS-deficient meningococci did not show increased levels of neutrophils compared with control mice (Fig. 3B). Following the same procedure as described above blood smears were also analyzed for presence of monocytes. No significant differences were detected between transgenic and nontransgenic mice challenged with wild-type FAM20 or the lpxA mutant (data not shown). Taken together, these data show that after meningococcal challenge, neutrophil counts in CD46 transgenic mice are not increased as compared with nontransgenic mice. Possible explanations for the impaired neutrophil recruitment could be induction of neutrophil apoptosis/cell death, or increased adherence of neutrophils to vascular endothelial surfaces.

**Serum C5a levels differ in CD46 transgenic and nontransgenic mice**

C5a is generated upon activation of the complement cascade. It has both anaphylatoxin and chemotactic activity, and can trigger degranulation of granulocytes. Excessive production of C5a in humans correlates with increased cytokine release and severe sepsis (40). Complement activation after meningococcal challenge was determined by measuring C5a levels in mouse serum at different times postinfection. CD46 transgenic mice contained higher basal levels of C5a in serum compared with nontransgenic mice after injection with medium or FAM20 (data not shown). The control level of C5a was 9 U in CD46 transgenic mice, and 3 U in nontransgenic mice, indicating that the presence of human CD46 increased basal C5a levels in the mice. The high levels of C5a in CD46 transgenic mice occurred at the same time as high bacterial blood counts, indicating that in this system meningococci show resistance to complement-mediated killing. As shown in Fig. 4, serum C5a was significantly raised in both CD46 transgenic and nontransgenic mice after i.p. challenge with FAM20, however, with delayed kinetics in the nontransgenic mice. Production of C5a in CD46 transgenic mice peaked at 3 h postchallenge and then decreased over time. At 24 h postchallenge, no difference in serum C5a was detected compared with control mice. In nontransgenic mice, C5a was maximal at 12 h postchallenge. It is unlikely that the correlation between high C5a levels resulted in the increased neutrophil numbers in nontransgenic mice, because there was no relationship between high C5a and neutrophil recruitment in CD46 transgenic mice. We cannot discount that the neutrophil recruitment could be mediated by another chemotactic factor.
Activation of microglia cells in CD46 transgenic mice

Bacteria cross the BBB in CD46 transgenic mice challenged i.p. with N. meningitidis (25). It is likely that the presence of bacteria in the meninges activate inflammatory responses in the brain. Microglia share many phenotypic and functional characteristics with macrophages and provide an initial line of defense in the brain against invading pathogens into the CNS (41). Iba1 is a calcium-binding protein whose expression is restricted to macrophages/microglia (42), which is further enhanced in activated microglia (43). CD46 transgenic mice challenged i.p. with wild-type N. meningitidis were perfused with fixative and the expression of Iba1 was investigated immunohistochemically at different time points (3, 6, 12, and 24 h). Activated microglia were detected throughout the brain tissue of challenged CD46 transgenic mice at all investigated time points (Fig. 5A; piriform cortex, C; striatum). Few activated microglia cells were detected in naive transgenic mice (Fig. 5B; piriform cortex, D; striatum).

Induction of cyclooxygenase-2 (COX-2) expression in brain of CD46 mice

COX-2 is an enzyme responsible for the production of PG H₂, the first step in the prostanoïd biosynthesis (44). TNF and IL-1β induce COX-2 production (45). CD46 transgenic mice were challenged i.p. with wild-type N. meningitidis and perfused with fixative at 3, 6, 12 and, 24 h postchallenge.Brains were removed, frozen, and expression of COX-2 was investigated immunohistochemically. Three hours postinfection COX-2 expression was detected in piriform cortex and expression peaked at 6 h (Fig. 5E). COX-2 expression could not be detected at 12 and 24 h postinfection (data not shown). Noninfected CD46 transgenic mice did not express detectable levels of COX-2 (Fig. 5F). Further, an activation of astrocytes was detected by GFAP-staining in the infected brains, but there was no difference between transgenic and nontransgenic animals in this respect (data not shown). Because bacteria did not reach the brain in nontransgenic mice, it is possible...
that the activation of astrocytes arises from signaling mediated by cytokines, immune cells, or LPS.

Neutrophil and macrophage levels in i.p. cavity

To investigate the primary neutrophil and monocyte recruitment at the site of inoculation, CD46 transgenic mice and nontransgenic mice were challenged i.p. with wild-type \(N.\) meningitidis FAM20. The i.p. fluid was collected by peritoneal wash at 1 and 3 h postchallenge, stained for live neutrophils and macrophages, and analyzed by flow cytometry. Both CD46 transgenic mice and nontransgenic mice demonstrated a decrease of neutrophil numbers after 3 h postinoculation with wild-type \(N.\) meningitidis compared with mice injected with medium alone (control) (Fig. 6A). On the contrary, inoculation with LPS-deficient meningococci in CD46 transgenic mice resulted in a 4-fold increase of neutrophils i.p. at 3 h postchallenge (Fig. 6B). The corresponding low numbers of LPS-deficient meningococci in the blood at this time suggest that the bacteria remain within the peritoneal cavity, and recruit neutrophils to the site of increased infection. This increase was not seen in nontransgenic mice. Taken together these data indicate that wild-type meningococci trigger a decrease of viable neutrophils in both transgenic and nontransgenic mice, and that LPS deficiency leads to elevated neutrophil numbers in CD46 mice but not in nontransgenic mice.

A significant influx of macrophages i.p. occurred at 1 and 3 h postchallenge of \(N.\) meningitidis wild-type strain FAM20 in CD46 transgenic mice but not in nontransgenic or in control mice (Fig. 6C), indicating that CD46 plays an important role in inducing macrophage responses. Challenge with the LPS-deficient meningococci did not induce an influx of macrophages i.p. in transgenic or nontransgenic mice (Fig. 6D). These data argue that LPS is also required to stimulate increased local macrophage levels.

Discussion

In the present study we demonstrate important differences in early immune responses against meningococcal infection in transgenic mice expressing human CD46 compared with nontransgenic mice. Responses in CD46 transgenic mice mimic human meningococcal disease in many aspects. Further, the data indicate novel important functions of human CD46, and show additional links between CD46 and innate immunity.

Mice expressing the human cell surface protein CD46 are susceptible to meningococcal disease, in contrast to nontransgenic mice. Following i.p. challenge of transgenic CD46 mice and nontransgenic mice with \(N.\) meningitidis, similar bacterial counts in blood during the first hours postinjection were observed. To elucidate the mechanisms for the lethal outcome after bacterial challenge of CD46 transgenic mice, we investigated cytokine responses during the first 24 h after infection. The cytokines of major interest are the proinflammatory cytokines TNF and IL-6, and the anti-inflammatory cytokine IL-10, all shown to be elevated during meningococcal disease (36). Further, high levels of inflammatory cytokines can be correlated with meningococcal disease severity. TNF and IL-6 were significantly higher in CD46 transgenic mice compared with nontransgenic mice with peak values occurring during the first hours after infection. Interestingly, levels of proinflammatory cytokines at early time points in CD46 transgenic mice show comparable levels to human patients with meningococcal disease. Further, a similar pattern is observed in patients with meningococcal disease with an early peak and rapid decrease of TNF and IL-6, and with the IL-6 peak following the TNF peak (36). The anti-inflammatory cytokine IL-10 concentration was higher in CD46 mice at 3–24 h postchallenge. Previous studies have shown that patients with lethal meningococcal disease have IL-10 levels of \(\sim 1000\) pg/ml (22). Similar values were detected in CD46 transgenic mice at 24 h after infection. The uncontrolled release of cytokines in transgenic CD46 mice might explain the rapid course of disease leading to death within 48 h. In patients with sepsis, death is correlated with increased levels of cytokines that eventually lead to total organ failure rather than to the actual bacterial load circulating in the bloodstream (46–48).

C5a is an important mediator which is involved in stimulating mononuclear cells and the release of proinflammatory cytokines. To investigate the possible contribution of complement activation
to sepsis in CD46 transgenic mice we measured the complement activation marker C5a in serum from infected CD46 transgenic and nontransgenic mice at different time points. A higher level of complement activation was observed in CD46 transgenic mice compared with nontransgenic mice, moreover, the maximum level of activation was also observed at earlier time points postchallenge in CD46 transgenic mice. These results are in accordance with the TNF and IL-6 release in CD46 transgenic mice challenged with wild-type meningococci. It is possible that C5a leads to early activation of cells and mediates cytokine release, resulting in sepsis in CD46 transgenic mice. There is accumulating evidence that neutrophils are a significant source of serum IL-6 during sepsis (40). In the current study, a similar level of monocytes and lower levels of neutrophils were observed in CD46 transgenic mice compared with nontransgenic mice after challenge with wild-type FAM20. Although a time correlation exists between serum C5a levels and sepsis, there is no link between neutrophil levels and serum C5a was observed in CD46 transgenic mice.

In human meningococcal disease bacteria cross the BBB, reach the CSF, and cause meningitis. In cells of the BBB, proinflammatory cytokines have the ability to trigger transcription of different genes, including COX-2 (49). We found that meningococcal infection induced expression of COX-2 immunoreactivity in piriform cortex in CD46 transgenic mice. This response became evident by 3 h, and was most prominent at 6 h postchallenge. COX-2 expression could not be detected in challenged mice at 12 and 24 h postinfection or in noninfected mice. In vivo, local increases in COX-2 expression have been associated with inflammation, seizures, and ischemia (50–52).

In CD46 transgenic mice, activation of microglia occurred at all tested time points postchallenge. Microglia rapidly respond to CNS injury, yet the mechanisms leading to their activation and inactivation remain poorly defined. It has been shown in mice that microglia, which normally have small bodies with finely branched appendages, are activated to proliferate and migrate to the site of damage, and then drastically transform into expanded amoeboid shapes with the ability to clear invading pathogens (53, 54). Microglia arise from macrophages outside the nervous system and are unrelated to other cells of the nervous system. Activation of microglia has been regarded as a brain tissue reaction to cell death and infection, the main purpose of which is to remove cellular debris. Mounting evidence indicates that activated microglia, beside removing cellular debris, may be actively involved in neurodegenerative processes. In contrast, it is also suggested that microglia may exert neuroprotective effects, depending on the situation.

After i.p. challenge with wild-type meningococci, both CD46 transgenic and nontransgenic mice demonstrated decreased neutrophil numbers in the i.p. cavity. Because the neutrophil numbers did not differ between transgenic and nontransgenic mice after challenge with wild-type bacteria, the reason for this specific neutrophil change is independent of CD46. Interestingly, LPS prepared from Pseudomonas aeruginosa has been shown to decrease neutrophils i.p. by TNF-induced apoptosis (55). In this study, challenge with LPS-deficient meningococci resulted in several-fold elevated neutrophil number in CD46 transgenic, but not in nontransgenic, mice indicating that CD46 plays an important role in the initial recognition and response to LPS in N. meningitidis infection. As described previously (24, 56), LPS-deficient N. meningitidis induces lower levels of serum cytokines than the wild-type strain. Absence of high levels of TNF might lead to neutrophil accumulation in the i.p. cavity of mice challenged i.p. with LPS-deficient bacteria, rather than neutrophil apoptosis.

Macrophages, in contrast, were present in significantly higher numbers in CD46 mice infected with meningococci compared with nontransgenic mice and mice injected with medium alone. These data argue that in CD46 transgenic mice, macrophages are stimulated to migrate to the site of infection. Challenge with LPS-deficient bacteria did not induce a comparable influx of macrophages into the i.p. cavity.

By investigating immune responses to meningococcal infection in transgenic CD46-expressing mice, we found similar patterns of immune responses as compared with human meningococcal disease. The knowledge about these responses is important from a therapeutic point of view, where new drugs targeted against these factors could limit and impede the fulminating development of disease. The importance of an animal model mimicking human meningococcal disease is also crucial to trial new vaccine candidates.

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Disclosures
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