



# Antagonistic antibody prevents toll-like receptor 2–driven lethal shock-like syndromes

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**Hyperactivation of immune cells by bacterial products through toll-like receptors (TLRs) is thought of as a causative mechanism of septic shock pathology. Infections with Gram-negative or Gram-positive bacteria provide TLR2-specific agonists and are the major cause of severe sepsis. In order to intervene in TLR2-driven toxemia, we raised mAb's against the extracellular domain of TLR2. Surface plasmon resonance analysis showed direct and specific interaction of TLR2 and immunostimulatory lipopeptide, which was blocked by T2.5 in a dose-dependent manner. Application of mAb T2.5 inhibited cell activation in experimental murine models of infection. T2.5 also antagonized TLR2-specific activation of primary human macrophages. TLR2 surface expression by murine macrophages was surprisingly weak, while both intra- and extracellular expression increased upon systemic microbial challenge. Systemic application of T2.5 upon lipopeptide challenge inhibited release of inflammatory mediators such as TNF- $\alpha$  and prevented lethal shock-like syndrome in mice. Twenty milligrams per kilogram of T2.5 was sufficient to protect mice, and administration of 40 mg/kg of T2.5 was protective even 3 hours after the start of otherwise lethal challenge with *Bacillus subtilis*. These results indicate that epitope-specific binding of exogenous ligands precedes specific TLR signaling and suggest therapeutic application of a neutralizing anti-TLR2 antibody in acute infection.**

## Introduction

Host cells recognize specific microbial components through pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) that mediate immune responses (1, 2). LPS from the outer membrane of Gram-negative bacteria is a potent agonist for TLR4, whose effects on host organisms of different species have been studied extensively in experimental models of infection and septic shock (3–6). Hyperstimulation of host immune cells by microbial products causes the release of large amounts of inflammatory mediators such as the cytokine TNF- $\alpha$  (7). Its systemic presence at high concentrations is recognized as a major cause of septic shock, characterized by clinical parameters such as abnormal coagulation, profound hypotension, and organ failure (8–10). Also, further inflammatory cytokines such as macrophage inhibitory factor have been shown to directly bias host responsiveness to microbial challenge through modulation of TLR expression (11).

The concept of PRR-dependent induction of hyperinflammation by microbial products has been validated using both gene-targeted mice lacking the expression of respective receptors, and

receptor-specific inhibition of microbial product-induced host cell activation. For example, application of CD14-specific antibodies inhibited LPS-induced cell activation, protected rabbits against LPS-induced pathology, and is being evaluated in clinical trials (12, 13). Blockage of further LPS receptors or extracellular effector proteins such as high-mobility group 1 protein has been shown to be preventive as well (14). Another approach of therapeutic intervention in inflammation has been interference with the functions of proinflammatory cytokines such as TNF- $\alpha$  or IL-1 $\beta$ . For instance, competitive inhibition of the binding of a cytokine to its signaling receptors by application of recombinant extracellular domain (ECD) or naturalizing receptor antagonist proteins has been shown to be protective in LPS-induced shock-like syndrome (15). Alternatively, antagonistic antibodies targeting cytokines or ECDs of cytokine receptors have been applied for inhibition of inflammatory immune reactions (16). Therapeutic blockage of cytokines such as TNF- $\alpha$  and IL-6 is used already for treatment of chronic inflammations (17, 18).

Besides Gram-negative bacteria, Gram-positive bacteria lacking LPS play an equally important role in the clinical manifestation of shock (10). Cell wall components from these bacteria, such as peptidoglycan (PGN) and lipoteichoic acid (LTA), are considered major causative agents of Gram-positive shock (19, 20). PGN is a main component of Gram-positive and is also present in Gram-negative bacterial cell walls, and it consists of an alternating  $\beta(1,4)$ -linked *N*-acetylmuramyl and *N*-acetylglucosaminyl glycan cross-linked by small peptides (21). In contrast, the macroamphiphile LTA, a saccharide chain molecule consisting of repetitive oligosaccharides connected by alcohols such as ribitol and carrying acyl chains through which it is anchored to the bacterial cell

**Nonstandard abbreviations used:** electrophoretic mobility shift assay (EMSA); extracellular domain (ECD); flow cell (FC); heat inactivated (h.i.); human embryonic kidney 293 [cell line] (HEK293); intraperitoneal (i.p.); leucine-rich repeat (LRR); lipoteichoic acid (LTA); murine toll-like receptor 2 (mTLR2); murine TLR2ECD–human IgGFc fusion protein (T2EC); *N*-palmitoyl-S-(1,2-bis(hexadecyloxy-carbonyl)-ethyl-(R)-cysteinyl-seryl-(lysyl)3-lysine (PHCSK<sub>4</sub>); *N*-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2R,S)-propyl)-(R)-cysteinyl-seryl-(lysyl)3-lysine (P<sub>3</sub>CSK<sub>4</sub>); pattern recognition receptor (PRR); peptidoglycan (PGN); surface plasmon resonance (SPR); toll-like receptor (TLR).

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membrane, is specific for Gram-positive bacteria (22). For example, LTA has been described to carry the major stimulatory activity of *Bacillus subtilis* (23). Further, tripalmitoylated proteins, which have been identified in Gram-negative bacteria initially, are mimicked by the synthetic compound *N*-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2*R,S*)-propyl)-(R)-cysteinyl-seryl-(lysyl)3-lysine (P<sub>3</sub>CSK<sub>4</sub>) (24).

The bacteria and bacterial products named above are known to trigger the TLR2 signaling cascade (2). For example, the bacterial species *Listeria monocytogenes* and *Staphylococcus aureus*, recognized as clinically more important, are phylogenetically closely related to *B. subtilis*, and the Gram-positive bacteria of all three species produce TLR2 agonists (25–28). However, recent reports indicate that *L. monocytogenes* and *S. aureus* generate additional molecular patterns that elicit immune responses in a TLR2-independent manner *in vivo*. Susceptibilities of *TLR2*<sup>-/-</sup> as compared with wild-type mice to respective bacterial challenges differed to a limited degree or did not differ (29–31), implicating further PRRs in their cellular recognition. Of note, triacylated P<sub>3</sub>CSK<sub>4</sub> has been demonstrated to use TLR2 in combination with TLR1, while a diacylated mycoplasmal protein uses TLR6 in addition to TLR2 or cell activation (32–34). The TLR2ECD, whose N-terminal portion has been implicated in direct PGN recognition (35), contains an array of distinct leucine-rich repeat (LRR) motifs. The LRR-rich domain is followed by an LRR C-terminal, a transmembrane, and an intracellular C-terminal toll-IL-1 receptor typical signaling domain (TIR) (36).

Here, we show by application of surface plasmon resonance (SPR) biosensor technology that the TLR2-specific mAb T2.5 abrogated TLR2ECD binding to P<sub>3</sub>CSK<sub>4</sub>. Consequently, TLR2-mediated activation of murine and human cells was inhibited in the presence of T2.5, demonstrating ligand binding to a specific epitope within the TLR2ECD to cause signaling-receptor complex formation. Using two different TLR2-dependent shock models, we demonstrate the protective potential of neutralization of TLR2 function with this antibody *in vivo*. We propose that antagonism of extracellular TLR2ECD function might provide a therapeutic option for prevention of septic shock.

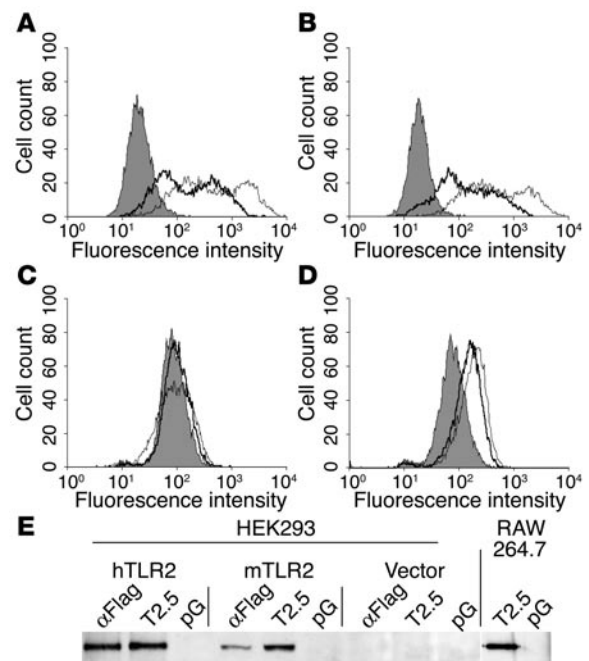
**Results**

*Application of murine mAb T2.5 for TLR2 expression analysis in vitro.* We have selected an IgG1κ anti-TLR2 mAb named T2.5, which recognized TLR2. Human embryonic kidney 293 (HEK293) cells stably expressing murine or human TLR2 were stained specifically on their surface by T2.5 (Figure 1, A and B). Furthermore, T2.5 did not bind to primary murine *TLR2*<sup>-/-</sup> but bound to wild-type macrophages cultured *in vitro* (Figure 1, C and D). T2.5 immunoprecipitated native murine and human TLR2 from lysates of HEK293 cells overexpressing one or the other of the two receptors (Figure 1E). Most importantly, T2.5 precipitated endogenous TLR2 from lysates of RAW264.7 macrophages (Figure 1E). We further analyzed T2.5 for its capacity to specifically detect TLR2 on the subcellular level. Detection of overexpressed murine and human TLR2 was specific (Figure 2A). Further, endogenous TLR2 was detectable on the surface of primary murine human macrophages, as well as within the cytoplasmic space (Figure 2B).

*Inhibitory effects of T2.5 on TLR2-specific cell activation in vitro and in vivo.* T2.5 inhibited murine and human TLR2-mediated cell activation by the TLR2-specific stimuli P<sub>3</sub>CSK<sub>4</sub> or *B. subtilis* applied to HEK293 cells overexpressing TLR2, as well as murine RAW264.7 and primary macrophages. NF-κB activation and IL-8 release, as

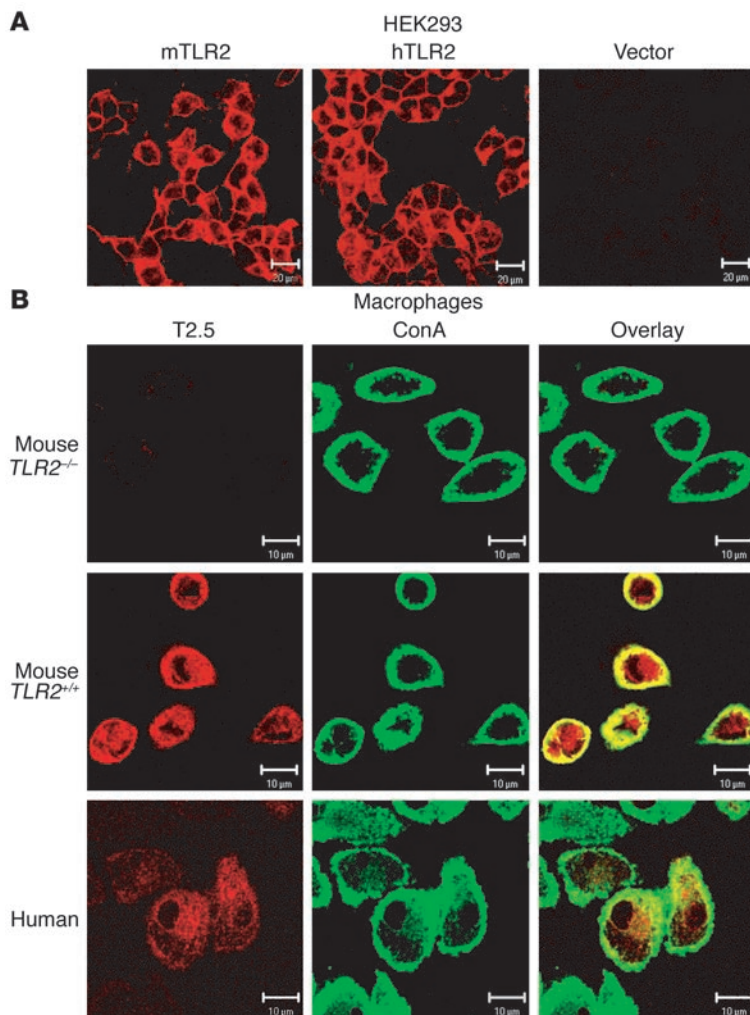
well as TNF-α and IL-6 release, respectively, were analyzed upon cellular challenge (Figure 3, A–D, and data not shown). A second newly generated IgG1κ anti-TLR2 mAb, conT2, was used as a control. This mAb bound to native murine TLR2 (mTLR2), as T2.5 did, but it did not bind to human TLR2 (data not shown) and failed to inhibit TLR2-dependent cell activation *in vitro* and *in vivo* (Figure 3). Also, no inhibition of IL-1 receptor or TLR4 signaling by T2.5 was evident, which indicates that TLR2-independent signaling pathways in T2.5-treated cells remain intact (Figure 3, A–D). Moreover, TLR2-mediated nuclear translocation of NF-κB was specifically inhibited by T2.5 in human macrophages (Figure 3E). NF-κB-specific electrophoretic mobility shift assay (EMSA), as well as anti-phospho-p38, anti-phospho-Erk1/2, and anti-phospho-Akt immunoblot analysis, revealed T2.5 but not conT2 dose-dependent inhibition of P<sub>3</sub>CSK<sub>4</sub>-induced NF-κB-DNA binding and cellular kinase phosphorylation (Figure 3, F and G).

*Abrogation of TLR2ECD ligand binding by T2.5 and analysis of T2.5 epitope localization.* To investigate whether T2.5 blocked binding of TLR2 to its synthetic agonist P<sub>3</sub>CSK<sub>4</sub>, we established an SPR biosensor-based binding assay. P<sub>3</sub>CSK<sub>4</sub> was immobilized on a chip surface, and binding of murine TLR2ECD-human IgGFcγ



**Figure 1**

Application of mAb T2.5 for specific detection of TLR2. (A–D) Results of flow cytometry of HEK293 cells stably overexpressing Flag-tagged mTLR2 (A) or human TLR2 (B), as well as primary *TLR2*<sup>-/-</sup> (C) and wild-type murine macrophages (D), by staining with mAb T2.5 (bold line). Negative controls represent cells incubated with a mouse IgG-specific secondary antibody only (filled areas). For positive controls, Flag-specific (A and B) and mTLR2-specific (C and D) polyclonal antisera were used (thin line). (E) For immunoprecipitation with T2.5, lysates of HEK293 cells overexpressing murine or human TLR2, as well as of murine RAW264.7 macrophages, were applied as indicated. TLR2 precipitates were visualized by application of Flag-specific (HEK293) or mTLR2-specific (RAW264.7) polyclonal antisera. Flag-specific beads (αFlag) and protein G beads in the absence of antibodies (pG), as well as vector-transfected HEK293 cells, were used as controls. The size of TLR2 was 97 kDa.



**Figure 2** Subcellular localization of TLR2 in vitro. Monoclonal antibody T2.5 was used for cytochemical detection of overexpressed mTLR2 and human TLR2 (hTLR2) (A), as well as endogenous murine (*TLR2*<sup>+/+</sup>, wild-type) or human TLR2 in primary macrophages (B). Vector-transfected HEK293 cells as well as *TLR2*<sup>-/-</sup> primary macrophages were analyzed as controls. Concanavalin A (ConA) was used for staining of cellular membranes. The bar in the lower right corner of each field represents a distance of 20  $\mu\text{m}$  (A) or 10  $\mu\text{m}$  (B) on the slides analyzed.

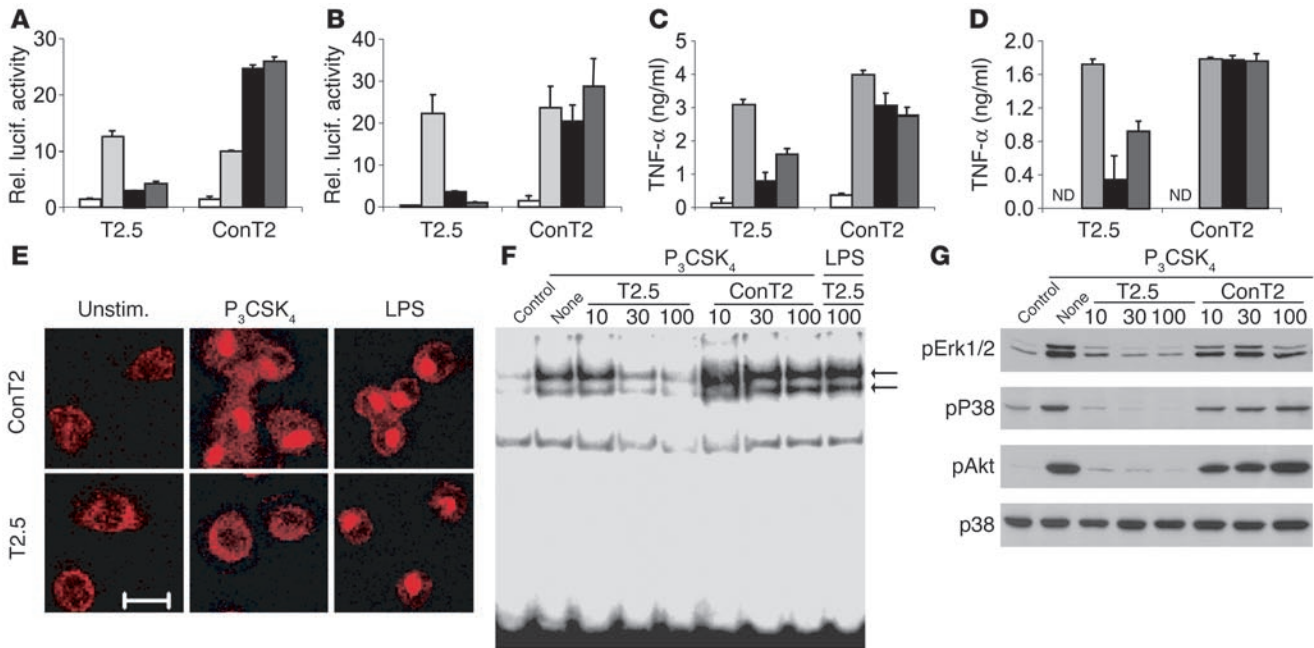
fusion protein (T2EC) was tested under various conditions. *N*-palmitoyl-S-(1,2-bis(hexadecyloxy-carbonyl)-ethyl)-(R)-cysteinyllseryl-(lysyl)3-lysine (PHCSK<sub>4</sub>), a nonactive analogue of P<sub>3</sub>CSK<sub>4</sub>, was used as a control, and sensorgrams are displayed as subtracted binding curves. Binding of T2EC to P<sub>3</sub>CSK<sub>4</sub> was specific (Figure 4A). When T2EC was preincubated with T2.5, the antibody dose-dependently inhibited T2EC-P<sub>3</sub>CSK<sub>4</sub> binding (Figure 4A). A molar ratio of 3.3 (T2.5/T2EC) was required to reduce binding to 50%. Preincubation of T2EC with T2.5 at tenfold molar excess abrogated T2EC-P<sub>3</sub>CSK<sub>4</sub> interaction (Figure 4A). In contrast, an isotype-matched control antibody did not block binding of T2EC to P<sub>3</sub>CSK<sub>4</sub> even when applied at tenfold molar excess (Figure 4B). When applied alone, both mAb's did not interact with the sensorchip surface (Figure 4, A and B). The N-terminal third of the LRR-rich domain of human TLR2 is not involved in lipopeptide recog-

nition (37), and T2.5 cross-reacts with human TLR2 (Figures 2B and 3E). Thus, we applied T2.5 to HEK293 cells overexpressing a mutant construct of human TLR2 that lacks the respective portion of the wild-type ECD (37). Specific abrogation of NF- $\kappa$ B-dependent reporter gene activation upon P<sub>3</sub>CSK<sub>4</sub> challenge after administration of T2.5 strongly suggests localization of the epitope recognized by T2.5 within the C-terminal portion of the TLR2ECD (Figure 4C).

*Surface and intracellular TLR2 expression ex vivo as analyzed immediately after primary cell preparation.* Since LPS induces TLR2 expression in primary macrophages in vitro, we first compared T2.5-specific staining of CD11b<sup>+</sup> splenocytes from LPS-challenged wild-type and *TLR2*<sup>-/-</sup> mice by flow cytometry. Weak surface staining and more pronounced intracellular staining were evident (Figure 5A). In subsequent experiments, peritoneal cells and splenocytes from mice infected with Gram-positive *B. subtilis* bacteria were analyzed. While surface expression of TLR2 in primary murine macrophages was relatively strong upon in vitro culture (Figure 1D), surface expression was weak or not detectable in unchallenged CD11b<sup>+</sup>, CD11c<sup>+</sup>, CD19<sup>+</sup>, and peripheral neutrophil marker GR1<sup>+</sup> subpopulations of splenocytes and peritoneal washout cells (Figure 5, A and B, and data not shown). Upon microbial challenge, however, TLR2<sup>+</sup> cell numbers and TLR2 surface expression increased in CD11b<sup>+</sup> and GR1<sup>+</sup> cells (Figure 5B and data not shown). The increase in the numbers of cells expressing intracellular TLR2 because of prior challenge, however, was more pronounced than the propagation of extracellular TLR2<sup>+</sup> cells (Figure 5, A–C), and the signals detected were largely TLR2-specific (Figure 5, B and C).

*Antibody-mediated interference with TLR2-dependent cell activation in vivo leading to cytokine and chemokine release into the serum.* Next, we determined cytokine and chemokine serum concentrations in mice either pretreated or not pretreated with T2.5 and challenged with P<sub>3</sub>CSK<sub>4</sub>. While cytokine and chemokine concentrations were low in sera of untreated mice (see Methods), serum levels of TNF- $\alpha$ , GRO $\alpha$ /KC (a murine homolog of human IL-8), IL-6, and IL-12p40 were significantly lower in mice pre-injected with T2.5 than in controls upon challenge with P<sub>3</sub>CSK<sub>4</sub> (Figure 6, A–D).

*Antibody-mediated interference with systemic induction of shock-like syndromes through TLR2-specific challenge.* Both a high-dose (microbial product only) and a low-dose model (additional sensitization with D-galactosamine) have been established for bacterial product-induced shock-like syndromes in mice (38). In order to interfere in a specific model of septic shock, we applied the bacterial lipopeptide analogue P<sub>3</sub>CSK<sub>4</sub> (a TLR2 agonist) upon sensitization of mice with IFN- $\gamma$  and D-galactosamine (39, 40). Sensitization was used to mimic an underlying primary infection priming host defense. While mice that had received no mAb or conT2 30 minutes prior to injection succumbed to lethal toxemia within 24 hours, mice treated with T2.5 survived (Figure 7A). Intending to use a more complex challenge for a distinct shock model, we took advantage of the finding that shock-like syndrome induction by viable or heat-inactivated (h.i.) *B. subtilis* bacteria was TLR2-



**Figure 3** Inhibitory effect of mAb T2.5 on cell activation in vitro. (A–D) NF-κB-dependent luciferase activities in HEK293 cells overexpressing either murine (A) or human TLR2 (B), as well as TNF-α concentrations in supernatants of RAW264.7 (C) or primary murine macrophages (D) challenged with inflammatory agonists. Rel. lucif. activity, relative luciferase activity; ND, not detectable. Cells were incubated with T2.5 or conT2 only (white bars), or additionally challenged with IL-1β (A and B, light gray bars), ultrapure LPS (C and D, medium gray bars), P<sub>3</sub>CSK<sub>4</sub> (black bars), or h.i. *B. subtilis* (A–D, dark gray bars). (E) NF-κB/p65 nuclear translocation dependent on mAb, P<sub>3</sub>CSK<sub>4</sub> challenge, or LPS challenge in human macrophages was analyzed by cytochemical staining. Unstim., unstimulated. Scale bar: 20 μm; magnification was equal for all recordings. (F and G) NF-κB-dependent EMSA was analyzed by application of nuclear extracts from RAW264.7 macrophages, and phosphorylation of MAPKs Erk1/2 (pErk1/2), p38 (pP38), and Akt (pAkt) was analyzed by application of total extracts from RAW264.7 macrophages. Cells were preincubated with the indicated amounts of mAb T2.5 or conT2 (μg/ml) and challenged with P<sub>3</sub>CSK<sub>4</sub> or LPS subsequently for 90 minutes (F; arrows indicate specific NF-κB–DNA complexes) or 30 minutes (G; phosphorylation-independent p38-specific immunoblot analysis as positive control). Untreated cells were analyzed as controls (Control).

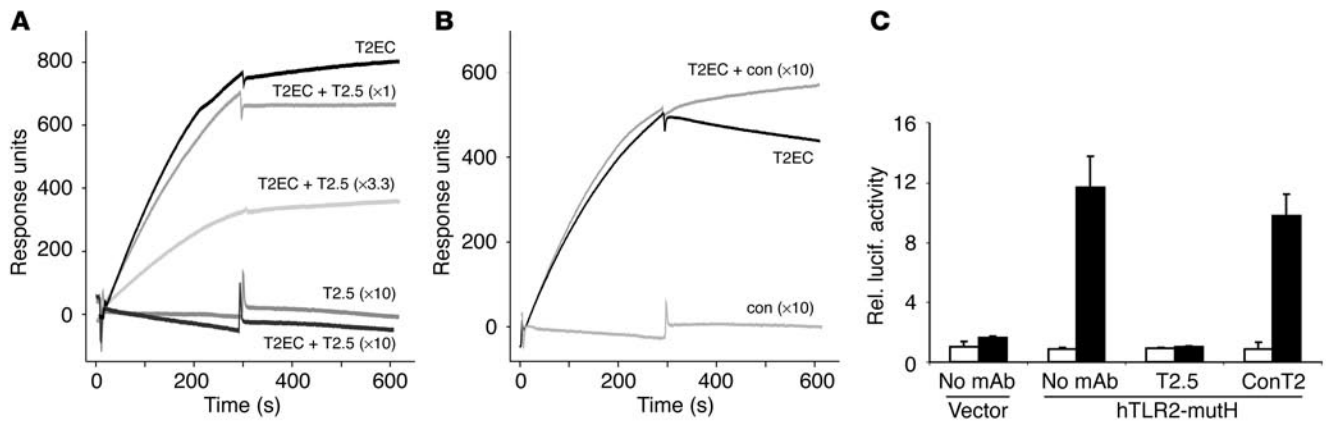
dependent. Clearance of *B. subtilis*, notably, was complete within 48 hours in *TLR2*<sup>-/-</sup> mice challenged intraperitoneally (i.p.) with viable *B. subtilis* at dosages lethal for wild-type mice. These findings encompassing TLR2 dependence in vivo indicated toxemia as the major pathophysiologic cause of wild-type mouse lethality in both experimental models (P<sub>3</sub>CSK<sub>4</sub>-induced and *B. subtilis*-induced experimental shock-like syndrome; A. Grabiec and C.J. Kirschning, unpublished observations). Thus, mice were pretreated with different doses of T2.5 prior to administration of h.i. *B. subtilis*. While pretreatment with 1 mg and 0.5 mg of T2.5 protected mice from lethal toxemia (protective protocol), lower amounts were ineffective (Figure 7B). Next, aside from T2.5, conT2 was also applied prior to administration of a principally lethal dose of h.i. *B. subtilis*. In a separate group of mice, we first administered h.i. *B. subtilis* and applied T2.5 up to 4 hours later (therapeutic protocol). In the absence of sufficient dosage of T2.5, the high-dose h.i. *B. subtilis* challenge was lethal for all mice tested (Figure 7, B and C). However, when given T2.5 either 1 hour before or up to 2 hours after microbial challenge, all mice challenged with h.i. *B. subtilis* survived. Most notably, treatment with T2.5 even 3 hours after otherwise lethal injection saved 75% of mice challenged (Figure 7C). When the order of experimental T2.5 and h.i. *B. subtilis* application was reversed, a completely protective effect of T2.5 administration was evident if the bacterial challenge was started 3 hours later (Figure 7D). While T2.5 treatment was effective for two out of three mice

even when applied for 4 hours, a protective effect was not detectable at the 5-hour and 6-hour time points in the respective experimental setting (Figure 7D).

**Discussion**

Our results suggest a therapeutically useful function of an antagonistic TLR2 mAb in TLR2-driven toxemia. We found that application of TLR2 agonists was lethal in two experimental models of septic shock and aimed to identify antibodies that recognize TLR2. One mAb, named T2.5, blocked mTLR2-dependent cell activation. T2.5 also blocked human TLR2 function, since subcellular NF-κB translocation upon TLR2-specific challenge of primary human macrophages was inhibited upon its application. The neutralizing effect of T2.5 application is based on abrogation of TLR2ECD-agonist binding as revealed by SPR analysis upon immobilization of P<sub>3</sub>CSK<sub>4</sub>. Here we show that T2.5 prevents lethal shock-like syndromes induced by P<sub>3</sub>CSK<sub>4</sub> or Gram-positive bacteria (*B. subtilis*) in mice.

The lack of TLR functions negatively affects humans, at least upon acute infection (41, 42). However, in a systemic model of polymicrobial sepsis encompassing standardized influx of the gut flora into the peritoneal cavity, mice benefit from the lack of TLR functions (43), which indicates TLR-dependent mediation of harmful effects in acute infection. Accordingly, blockage of LPS-binding protein (LBP) (44), as well as application of LBP, of peptides rep-

**Figure 4**

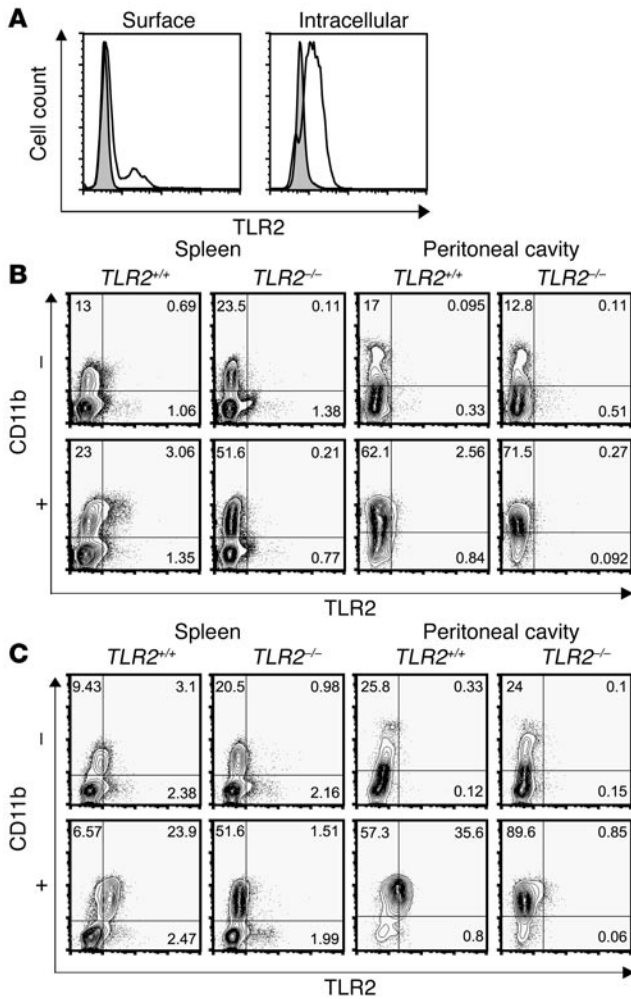
Molecular analysis of the effects of mAb T2.5 on TLR2ECD- $P_3$ CSK<sub>4</sub> interaction. (A and B) Binding of recombinant TLR2ECD-Fc fusion protein (T2EC, positive controls) to immobilized  $P_3$ CSK<sub>4</sub> upon preincubation with T2.5 (T2EC + T2.5) at different molar excesses (A,  $\times 1$ ,  $\times 3.3$ ,  $\times 10$ ) or with an isotype-matched control mAb (T2EC + con) at tenfold molar excess only (B,  $\times 10$ ). Binding was continuously monitored in an SPR biosensor device, and amounts of antibodies used to gain high molecular excess over T2EC (coincubation) were applied alone as negative controls (A, T2.5; B, Con). Response units at 300 seconds are a measure for  $P_3$ CSK<sub>4</sub>-binding capacities of T2EC and T2EC plus mAb. (C) For analysis of approximate localization of T2.5 epitope within the TLR2ECD, a mutant human TLR2 construct lacking the N-terminal third of the LRR-rich ECD (hTLR2-mutH) was used for NF- $\kappa$ B-dependent luciferase assay upon transient transfection, preincubation with mAb (T2.5, conT2), and  $P_3$ CSK<sub>4</sub> challenge (black bars). Absence of mAb treatment (No mAb) and/or of  $P_3$ CSK<sub>4</sub> challenge (white bars), and empty vector (Vector), represent respective controls.

representing its subdomains, or of bactericidal/permeability-increasing protein (BPI), has been effective in inhibiting LPS-induced pathology (45–49). Attempting to inhibit a TLR-specific immune activation as has been exemplified by systemic tolerance induction through TLR2-specific challenge prior to principally fatal microbial challenge (40), we applied an antagonistic mAb T2.5 raised against the murine TLR2ECD. Its application enabled analysis of murine and human TLR2 localization on the surface and inside of immune cells (Figures 1 and 2). Direct interaction between TLR2 and  $P_3$ CSK<sub>4</sub> was demonstrated and allowed comparison of the affinities of TLR2 and of the TLR2-T2.5 complex to this ligand. SPR analysis showed the direct and specific interaction between TLR2ECD and  $P_3$ CSK<sub>4</sub>, as well as a specific and dose-dependent inhibition of this interaction by T2.5 (Figure 4, A and B), indicating that binding of T2.5 masked the ligand-binding domain in TLR2. Accordingly, T2.5 antagonized not specifically  $P_3$ CSK<sub>4</sub>, but also h.i. *B. subtilis*, PGN, LTA, and TLR2-dependent cell activation induced by mycoplasmal macrophage-activating protein (Figure 3 and data not shown). Blockage was specific and dose-dependent (Figure 3). Taken together, these findings show that specific binding of ligands to a discrete site within the TLR2ECD is a prerequisite for TLR2-mediated signaling.

Surface expression of TLR2 in vivo was a precondition of systemic effects of T2.5 application. Relatively weak surface expression of TLR2 even upon LPS or bacterial challenge ex vivo (Figure 5, A and B), however, was in contrast with relatively high surface expression on unchallenged primary murine (Figures 1D and 2B) as well as human myeloid cells upon in vitro culture (50). However, comparative TLR2 staining of nonpermeabilized and permeabilized cells indicated localization of a major portion of TLR2 in the intracellular compartment of murine CD11b<sup>+</sup> and GR1<sup>+</sup> cells, as well as human macrophages (Figure 5, Figure 2B, and data not shown). In fact, we noted increased surface and, to a larger extent, intracellular TLR2 expression in specific cell

populations 24 hours after bacterial infection, which was similar upon LPS challenge (Figure 5 and data not shown). Weak unspecificity of intracellular staining with T2.5, detected mostly in permeabilized spleen cells, had to be taken into account (Figure 5C). The time course of TLR2 regulation in distinct immune cells upon microbial contact needs to be investigated in more detail, because it might determine the time frame within which intervention based on TLR2 blockage can be effective.

Perhaps it is the surprisingly low constitutive surface expression of TLR2 in host cells such as CD11b<sup>+</sup> (macrophage) cells, GR1<sup>+</sup> (granulocyte) cells, CD19<sup>+</sup> (B) cells, and CD11c<sup>+</sup> (dendritic) cells in vivo (Figure 5 and data not shown) that explains the high efficacy of T2.5-mediated prevention of TLR2-driven hyperinflammation (Figures 6 and 7). Application of T2.5 30 minutes prior to application of a principally lethal dose of  $P_3$ CSK<sub>4</sub> or 1 hour prior to administration of a principally lethal dose of h.i. *B. subtilis* protected mice against the otherwise lethal effects of both stimulants (Figure 7, A–C), but not against the lethal effects of LPS (data not shown). In fact, *B. subtilis*-induced toxemia was prevented upon application of T2.5 2 hours or even 3 hours after shock-like syndrome induction (100% or 75% of survival, respectively). In contrast, application of T2.5 was not effective after 4 hours (Figure 7C). However, the onset of septic shock upon acute infection in the clinical situation may be delayed as compared with sudden induction of toxemia by experimental injection of large amounts of stimulant and may allow interference within a larger time window. Our results indicate that complement-mediated depletion of TLR2<sup>+</sup> cells is unlikely to be a mechanism of prevention of T2.5-dependent prevention of TLR2-driven shock-like syndrome, since application of the mTLR2-specific isotype-matched mAb conT2 in vivo did not result in protection (Figure 7A). This is in line with reversibility of mAb-mediated TLR2 blockage within 5 hours (Figure 7D), which may be important for timely recovery of TLR2-dependent cellular responsiveness



**Figure 5**

TLR2 expression ex vivo immediately after primary cell isolation. Flow cytometry of splenocytes and peritoneal washout cells from wild-type (*TLR2*<sup>+/+</sup>) and *TLR2*<sup>-/-</sup> mice ex vivo immediately after isolation (*n* = 5, cells pooled for each sample). (A) CD11b<sup>+</sup> splenocytes from mice challenged with LPS for 24 hours were analyzed for surface and intracellular TLR2 expression by staining with T2.5 (bold line, *TLR2*<sup>+/+</sup>; filled area, *TLR2*<sup>-/-</sup>). (B and C) For analysis of TLR2 regulation upon infection, mice were either left uninfected (-) or infected with *B. subtilis* and sacrificed after 24 hours (+). Upon staining of CD11b, cells were stained with T2.5 (TLR2) either without permeabilization (B) or after permeabilization (C). Numbers in quadrants represent the percentage of single- or double-stained cells with respect to the total number of viable cells analyzed.

candidate, in order to facilitate inhibition of cell activation. Conversely, failure of therapy to compensate for a decrease in biocidal immune cell activity upon TLR blockage by antibiotic treatment might compromise a beneficial outcome (13).

We have identified exclusively antagonistic or neutral TLR2-specific mAb's, and antagonistic properties have recently been demonstrated in vitro also for two different human TLR2-specific mAb's (28, 51). Active complex formation of TLRs as compared with receptors for which agonistic antibodies have been identified might differ. However, T2.5 antagonized TLR2 function through inhibition of ligand-TLR2-complex formation (Figure 4A), which is a prerequisite of TLR2-driven cell activation. T2.5 may therefore recognize the possibly single ligand-binding site within the C-terminal portion of the TLR2ECD. We expect that identification of the epitope will show its conservation between mice and humans. In conclusion, our results implicate antibody-mediated TLR blockage on immune cells as a promising strategy for attenuation of potentially fatal host-response amplification in the course of acute infection.

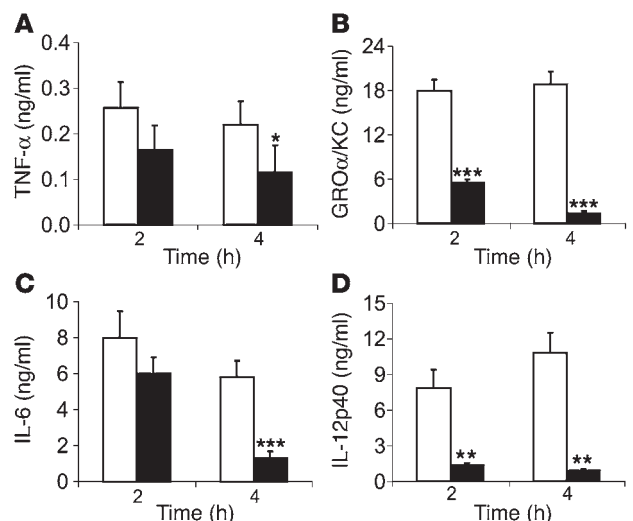
in later phases of sepsis at which diminished immune function is fatal (9). Systemic presence of T2.5 1 hour prior to challenge did not interfere with resistance of a *TLR2*<sup>-/-</sup> mouse challenged with h.i. *B. subtilis* at a dose that was lethal for wild-type mice in the absence of T2.5 application (data not shown). The demonstration of beneficial and specific effects of T2.5 in both a sensitization-dependent and a high-dose TLR2-specific experimental model may support transferability of our results to elimination of the TLR2-dependent share in septic shock induction (9). Specifically, TLR2 blockage upon antibiotic therapy may substantially contribute to prevention of an excessive host immune reaction upon sudden release of large amounts of microbial products from disintegrating microbial cells. It may have to be complemented by blockage of further surface receptors, for which TLR4 is a prime

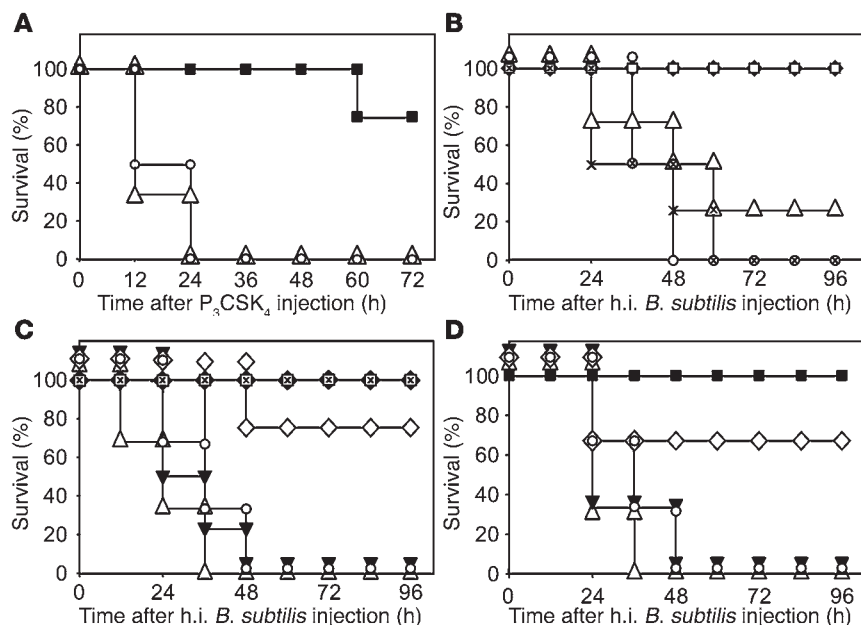
**Figure 6**

Inhibitory effect of mAb T2.5 on host activation by microbial challenge in vivo. Mice were pretreated i.p. with 1 mg mAb T2.5 (black bars) or left untreated (white bars). Mice were challenged i.p. with P<sub>3</sub>CSK<sub>4</sub> and D-galactosamine after 1 hour and sacrificed 2 or 4 hours later (*n* = 4 for each group at each time point). Serum concentrations of TNF-α (A), GROα/KC (human IL-8 homolog) (B), IL-6 (C), and IL-12p40 (D) were analyzed by ELISA. \**P* < 0.05, \*\**P* < 0.005, \*\*\**P* < 0.001, Student's *t* test for unconnected samples.

**Methods**

**Material.** Overnight *B. subtilis* (DSMZ.1087) cultures in brain-heart medium containing approximately 1 × 10<sup>9</sup> CFUs/ml were used immediately or heat-inactivated at 56°C for 50 minutes. Synthetic P<sub>3</sub>CSK<sub>4</sub> and, as a negative control, PHCSK<sub>4</sub>, a nonstimulatory derivative thereof (52), were purchased from EMC microcol-



**Figure 7**

Effects of mAb T2.5 administration on viability after TLR2-specific systemic challenge. **(A)** IFN- $\gamma$ - and D-galactosamine-sensitized mice received no mAb, 1 mg of mAb T2.5, or 1 mg of conT2 i.p. 30 minutes prior to microbial challenge with bacterial lipopeptide analogue  $P_3CSK_4$  (open circles, no mAb,  $n = 4$ ; open triangles, mAb conT2,  $n = 3$ ; filled squares, mAb T2.5,  $n = 4$ ). **(B–D)** Mice challenged with a high dose of h.i. *B. subtilis* were left untreated, treated 1 hour later with the indicated dosages of mAb T2.5 **(B)**: filled diamonds, 1 mg,  $n = 3$ ; open squares, 0.5 mg,  $n = 3$ ; open triangles, 0.25 mg,  $n = 4$ ; x's, 0.13 mg,  $n = 4$ ; open circles, no mAb T2.5,  $n = 4$ , or treated with 1 mg of mAb's at the different time points indicated below **(C and D)**. **(C)** TLR2-specific mAb was administered before (–) or after (+) bacterial challenge (filled inverted triangles, no mAb,  $n = 8$ ; open circles, mAb conT2, –1 hour,  $n = 3$ ; filled diamonds, mAb T2.5, –1 hour,  $n = 4$ ; open squares, mAb T2.5, +1 hour,  $n = 3$ ; x's, mAb T2.5, +2 hours,  $n = 3$ ; open diamonds, mAb T2.5, +3 hours,  $n = 4$ ; open triangles, mAb T2.5, +4 hours,  $n = 3$ ). **(D)** TLR2-specific mAb T2.5 was administered before (–) bacterial challenge (open triangles, no mAb; filled squares, mAb T2.5, –3 hours; open diamonds, mAb T2.5, –4 hours; open circles, mAb T2.5, –5 hours; filled inverted triangles, mAb T2.5, –6 hours;  $n = 3$  for all groups).

lections GmbH (Tuebingen, Germany); both carried biotin tags. Ultrapure LPS from *Salmonella minnesota Re595* was from List Laboratory (Campbell, California, USA), recombinant murine IFN- $\gamma$  and IL-1 $\beta$  were from PeproTech EC Ltd. (London, United Kingdom), and D-galactosamine was from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany).

**Mice.** Matched groups of wild-type ( $TLR2^{+/+}$ ) C57BL/6 and  $TLR2^{-/-}$  (39) mice generated by Deltagen Inc. (Redmond City, California, USA) were kindly provided by Tularik Inc. (South San Francisco, California, USA) and crossed ninefold from a mixed Sv129 $\times$ C57BL/6 toward a C57BL/6 genetic background. Experiments were approved by the government of Upper Bavaria, Germany.

**Generation of TLR2ECD-specific antibodies and ELISA.** A cDNA fragment encoding the N-terminal 587 amino acids of mTLR2 (53) was amplified from a RAW264.7 cell cDNA library (Advantage kit; BD Biosciences Clontech, Heidelberg, Germany). The murine TLR2ECD was fused to a C-terminal thrombin cleavage site followed by a human IgGFc $\gamma$  moiety (T2EC). The murine TLR2ECD protein was purified upon overexpression in HEK293 cells and thrombin digestion. A  $TLR2^{-/-}$  mouse was immunized three times within 8 weeks by i.p. injection of 50  $\mu$ g of TLR2ECD and 10 nmol of a thioated DNA oligonucleotide (5'-TCCATGACGTTCCCTGA-

3'; TIB MOLBIOL, Berlin, Germany). Its splenocytes were fused with murine P3X cells, and hybridomas were selected (54). Monoclonal antibody specificities for TLR2ECD, as well as cytokine and chemokine concentrations, in cell supernatants or murine sera were analyzed by ELISA (R&D Systems Inc., Minneapolis, Minnesota, USA). Significance of serum-concentration differences was determined by application of the Student's *t* test for unconnected samples.

**Flow cytometry.** Stably transfected HEK293 cell clones, as well as uninduced peritoneal washout macrophages, were cultured overnight as described previously (37). Flow cytometry was performed upon staining with T2.5 and a secondary mouse IgG-specific mAb, as well as affinity-purified polyclonal antisera specific for the murine TLR2ECD (55) or the Flag-tag (Sigma-Aldrich Chemie GmbH) and a rabbit IgG-specific secondary mAb. Secondary mAb's were phycoerythrin-labeled (BD Biosciences Pharmingen, Heidelberg, Germany). For establishment of mTLR2 expression analysis in primary cells, surface and intracellular T2.5-dependent staining of CD11b $^+$  splenocytes (54) from wild-type versus  $TLR2^{-/-}$  mice challenged with LPS (0.5 mg, i.p., 24 hours) was compared by flow cytometry (CyAn; DakoCytomation, Fort Collins, Colorado, USA). Cells were stained with photoactivated ethidium monoazide (Molecular Probes Europe BV, Amsterdam, The Netherlands) immediately upon isolation, followed by TLR2-specific surface staining, or intracellular staining (Cytotfix/Cytoperm; BD Pharmingen). In order to analyze TLR2 expression in uninfected or *B. subtilis*-infected mice ( $5 \times 10^8$  CFUs, i.p., 24 hours), peritoneal washout cells and splenocytes (54) from five uninfected or infected wild-type or  $TLR2^{-/-}$  mice were pooled. Fluorescence-labeled cell surface marker antibodies (BD Pharmingen) and T2.5 counterstained with secondary anti-mIgG1 were used as indicated.

**Immunoprecipitation and immunoblot analysis.** Lysates of Flag-TLR2-transfected HEK293 cells or macrophages were mixed with 1  $\mu$ g of antibody and protein G beads (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) for overnight precipitation. Immune complexes or cell lysates were analyzed by immunoblot analysis as described previously (37). Precipitations were controlled by application of Flag-specific (mAb M2; Sigma-Aldrich Chemie GmbH) or protein G beads only. Flag- or mTLR2-specific antisera were applied for immunoblot analyses of HEK293 or RAW264.7 cell lysates, respectively. In contrast, total lysates of macrophages were analyzed for phosphorylation of kinases as indicated.

**Cytochemical staining of TLR2 or NF- $\kappa$ B.** Transfected HEK293 cell clones, as well as primary murine or human macrophages, the latter isolated as CD14 $^+$  peripheral blood leukocytes and cultured in 20% of autologous serum (56), were grown on slides. Cells were washed with PBS, permeabilized, and incubated with 5  $\mu$ g/ml TLR2-specific mAb or anti-NF- $\kappa$ B/p65 (polyclonal



rabbit; Santa Cruz Biotechnology Inc.) (37). Specific secondary anti-IgG antibodies labeled with Alexa Fluor 546 (anti-TLR2) or Cy5 (anti-NF- $\kappa$ B; both from BD Biosciences Pharmingen) were applied. Cell membranes were stained with labeled concanavalin A (Molecular Probes Europe BV).

**Inhibition of TLR2-dependent cell activation in vitro and in vivo.** Transiently transfected HEK293 cells as well as murine RAW264.7 and primary macrophages were used. Fifty micrograms per milliliter of antibodies were applied 30 minutes prior to challenge with 100 ng/ml of LPS, IL-1 $\beta$ , or P<sub>3</sub>CSK<sub>4</sub> or 1  $\times$  10<sup>6</sup> CFUs/ml of h.i. *B. subtilis*. HEK293 cells were cotransfected with reporter (57), human wild-type TLR2, human mutant TLR2 (lacking the N-terminal third of the LRR-rich domain; ref. 37), or mTLR2, as well as MD2 and CD14 (provided by Tularik Inc., South San Francisco, California, USA; D. Golenbock, University of Massachusetts Medical School, Worcester, Massachusetts, USA; H. Heine, Research Center Borstel, Borstel, Germany; and K. Miyake, University of Tokyo, Tokyo, Japan) expression plasmids, and NF- $\kappa$ B-dependent reporter gene activity was assayed after 6 hours of stimulation. TNF- $\alpha$  concentrations in supernatants of RAW264.7 and primary murine macrophages were analyzed 24 hours after challenge, and NF- $\kappa$ B translocation in human macrophages (56) was analyzed 90 minutes after challenge. RAW264.7 macrophages were used for analysis of challenge and of antibody-dose-dependent activation of NF- $\kappa$ B and MAPK. NF- $\kappa$ B-specific EMSA and p38, Erk1/2, and Akt phosphorylation-specific immunoblot analysis (Cell Signaling Technology, Frankfurt, Germany) were carried out. Prior to TLR-specific challenge of 1  $\times$  10<sup>6</sup> cells, as described above, for 90 minutes (EMSA) or 30 minutes (kinase-phosphorylation analysis) (37), antibody was administered at various concentrations. For analysis of TLR2 inhibition in vivo, mice were injected i.p. with 1 mg of T2.5 or left untreated. One hour later, 100  $\mu$ g of P<sub>3</sub>CSK<sub>4</sub> and 20 mg of D-galactosamine were injected i.p. Serum concentrations of TNF- $\alpha$ , GRO $\alpha$ /KC (murine homolog of human IL-8), IL-6, and IL-12p40 in five unchallenged control mice were 0.05 ng/ml, 0.43 ng/ml, not detectable, and 0.44 ng/ml, respectively. Significance of results was determined by the Student's *t* test for unconnected samples.

**SPR biosensor measurements.** Real-time binding analysis was performed using SPR detection on a Biacore X device (Biacore AB, Uppsala, Sweden). The two flow cells (FCs) of a streptavidin-precoated chip were loaded with biotinylated PHCSK<sub>4</sub> (FC1) and P<sub>3</sub>CSK<sub>4</sub> (FC2), respectively. Specific binding of a recombinant T2EC protein was controlled by application of a human mAb carrying the same Fc $\gamma$  domain. This antibody did not bind in either FC1 or FC2 (data not shown). After prior incubation in 45  $\mu$ l of running buffer (50 mM morpholino ethane sulfonic acid, 150 mM MgCl<sub>2</sub>, pH 6.5) at 25°C for 15 minutes, 200 nmol of purified T2EC alone (maximum control) or in combination with mAb's

(T2.5 or an isotype-matched irrelevant mAb at the molar excesses indicated) was injected over FC1 and FC2 at a flow rate of 10  $\mu$ l/min. As negative control, mAb's alone were administered at the highest amounts also used for blocking analysis of TLR2 ligand binding. The values obtained upon continuous resonance monitoring at 25°C over 570 seconds (delay time 300 seconds) from the control FC1 were subtracted from the respective values resulting from simultaneously performed analysis of FC2. Generally, biomolecular interaction between receptor and its respective ligands immobilized on the sensor chip is optically monitored as a function of time and expressed in response units. Regeneration of the chip was achieved by washes with 50 mM NaOH and 1 M NaCl and extensive re-equilibration with running buffer.

**Systemic induction of shock-like syndrome.** In an experimental sensitization-dependent model (39), mice were injected i.v. with 1.25  $\mu$ g of murine IFN- $\gamma$ . Twenty minutes later, mice were injected i.p. with doses of mAb as indicated. Fifty minutes after IFN- $\gamma$  injection, 100  $\mu$ g of synthetic P<sub>3</sub>CSK<sub>4</sub> and 20 mg of D-galactosamine were injected i.p. as well. The experimental high-dose shock model encompassed a single i.p. injection of 1  $\times$  10<sup>10</sup> CFUs of h.i. *B. subtilis*, with i.p. injection of 1 mg of mAb 1 hour to 6 hours earlier or 1 hour to 4 hours later as indicated. Survival was monitored and did not change within 7 days after injection after the latest time points indicated in Figure 7.

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