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Immunization with a Circumsporozoite Epitope Fused to *Bordetella pertussis* Adenylate Cyclase in Conjunction with Cytotoxic T-Lymphocyte-Associated Antigen 4 Blockade Confers Protection against *Plasmodium berghei* Liver-Stage Malaria

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The adenylate cyclase toxoid (ACT) of *Bordetella pertussis* is capable of delivering its N-terminal catalytic domain into the cytosol of CD11b-expressing professional antigen-presenting cells such as myeloid dendritic cells. This allows delivery of $CD8^+$ T-cell epitopes to the major histocompatibility complex (MHC) class I presentation pathway. Recombinant detoxified ACT containing an epitope of the *Plasmodium berghei* circumsporozoite protein (CSP), indeed, induced a specific $CD8^+$ T-cell response in immunized mice after a single application, as detected by MHC multimer staining and gamma interferon (IFN-γ) ELISPOT assay. This CSP-specific response could be significantly enhanced by prime-boost immunization with recombinant ACT in combination with anti-CTLA-4 during the boost immunization. This increased response was accompanied by complete protection in a number of mice after a challenge with *P. berghei* sporozoites. Transient blockade of CTLA-4 may overcome negative regulation and hence provide a strategy to enhance the efficacy of a vaccine by amplifying the number of responding T cells.

The life cycle of malaria parasites in mammals can be separated into a liver stage and a blood stage. Infection starts with a liver stage during which sporozoites invade hepatocytes and replicate vigorously without provoking clinical illness. After the infected hepatocytes burst, merozoites are released into the bloodstream and infect red blood cells, where they undergo massive replication. This blood stage is associated with the typical symptoms of malaria. There is a large body of evidence that the clinical symptoms of severe malaria are caused by the production of proinflammatory cytokines by the host immune system that is activated by the large amount of parasitic antigens (6, 39). Therefore, strategies to enhance the cellular immune response during the blood stage might lead to exacerbation of disease (21, 22). Taking this into account, several vaccine strategies focused on the liver stage, where protection is achievable by gamma interferon (IFN-γ)-producing, major histocompatibility complex (MHC) class I-restricted $CD8^+$ T cells (13). A high level of protection was induced by irradiated sporozoites (18, 19, 36). Promising results were also obtained with genetically modified sporozoites that were not capable of establishing the infectious cycle (12, 35). With an experimental malaria model employing infection of BALB/c mice with *P. berghei* sporozoites, a peptide presented on $H\text{-}2K^d$ and derived from the sporozoite-specific circumsporozoite protein (CSP) was identified (41). Although several methods were employed to induce CSP-specific T cells and some of these strategies indeed induce promising numbers of these CSP-specific $CD8^+$ T cells, the degree of protection often varies (1, 46). Up to now, the most promising strategies have relied on heterologous prime-boost immunization (44).

Protective immunity against the liver stage of malaria might be hampered by the special status of the liver, which favors the persistence of certain pathogens by exerting suppressive effects on T cells (28). Therefore, the liver is considered a "graveyard of activated T cells" (4). Indeed, several recent studies indicate that activated T cells are entrapped and suppressed by liver-specific mechanisms that include an immunosuppressive cytokine environment mediated by transforming growth factor β and interleukin-10 (27). In addition, liver cells are capable of presenting antigens to T cells and rendering them tolerant (31). Taking this into account, special emphasis should be placed on the vaccine-induced immune response in the liver.

Another mechanism to control T cells involves signaling through CTLA-4 (CD152), which has a high degree of sequence homology with CD28 and binds to the same ligands, CD80 and CD86. CTLA-4 is a potent negative regulator of the immune response that is expressed on T cells upon activation and is also constitutively expressed on regulatory T (Treg) cells (7). Functional studies revealed that CTLA-4 is involved in the

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maintenance of peripheral tolerance and that mutations in the CTLA-4 molecule are linked to an increased incidence of autoimmune diseases. The role of CTLA-4 in dampening the immune response was further confirmed with antibodies (Abs) that block CTLA-4 ligation, thereby promoting an enhanced immune response against tumors and infectious diseases and exacerbation of autoimmune diseases (20, 38). Thus, manipulating CTLA-4 interactions became a valuable target for immunological therapies, including the use of anti-CTLA-4 Abs to enhance the effectiveness of vaccines (3, 25).

The adenylate cyclase toxoid (ACT) of *Bordetella pertussis* is capable of delivering its N-terminal catalytic domain into the cytosol of CD11b-expressing professional antigen-presenting cells such as myeloid dendritic cells (47). This allows the use of ACT for delivery of inserted cargo $CD8^+$ T-cell epitopes into the MHC class I presentation pathway (45). Indeed, recombinant detoxified ACTs containing different epitopes were repeatedly used to generate T cells against different model antigens, thus demonstrating the versatility of this tool as an antigen delivery system (37, 43).

In the present study, we used recombinant detoxified ACT containing an epitope of *Plasmodium berghei* CSP. This induced a high number of IFN-γ-secreting $CD8^+$ T cells, while no protective immunity was achieved. In contrast, when anti-CTLA-4 was administered during boost immunization, complete protection against challenge with *P. berghei* ANKA (ANtwerpKAtango) sporozoites was induced in a number of mice.

MATERIALS AND METHODS

Mice and parasites. BALB/c (*H-2K*^{d}), C57BL/6 (*H-2K*^{b}), and OT-1 (*H-2K*^{b}) mice were bred in the animal facility of the Bernhard Nocht Institute for Tropical Medicine. For all experiments, female mice (6 to 8 weeks old) were used. *P. berghei* ANKA was maintained by alternating cyclic passage of the parasite in *Anopheles stephensi* mosquitoes and BALB/c mice at the mosquito colony of the Bernhard Nocht Institute for Tropical Medicine. Sporozoites were collected by manual dissection of infected mosquito salivary glands in minimal essential medium 18 to 21 days after the mosquito had taken an infectious blood meal.

Bacterial strains, growth conditions, and plasmids. The *Escherichia coli* K-12 strain XL1-Blue (Stratagene) was used throughout this work for DNA manipulation and for expression of ACT-derived proteins. Bacteria transformed with appropriate plasmids derived from pT7CACT1 were grown at 37°C in Luria-Bertani medium supplemented with 150-μg/ml ampicillin. Plasmid pT7CACT1 is a construct for coexpression of *cyaC* and *cyaA* (37), and it allows production of recombinant CyaC-activated ACT in *E. coli* (r-*Ec*-ACT) under control of the *lacZp* promoter.

ACT-CSP toxoid construction. The ACT-CSP plasmid that was constructed for the present study was generated by in-frame insertion of corresponding pairs of annealed synthetic oligonucleotides with the sequences 5′-GTACGCGTACGCAAAACAACGACGACTCTTACATCCCGTCTGCTGAAAAAATCCTGGAATTCGTTAAACAG and 5′-GTACCTGTTTAACGAATTCCAGGATTTTTTCAGCAGACGGGATGTAAGAGTCGTCGTTGTTTTTGCGTACGC, which were introduced into a unique BsrGI site located between codons 335 and 336 of the *cyaA* open reading frame carried on pT7CACT1-BsrG (37). This yielded an insertion of the amino acid sequence VRVRKNNDDSYIPSAEKILEFVKQ. The inserted sequence comprises the MHC class I epitope SYIPSAEKI, corresponding to residues 245 to 253 from CSP and contained within the natural flanking sequences, to ensure appropriate processing by the proteasome. Additional positively charged amino acid residues were introduced toward the termini of the inserted sequence to compensate for the negatively charged amino acid residues present at the epitope-processing sites (24). To allow monitoring of the delivery of the AC domain with the inserted CSP epitope into the MHC class I pathway, the ACT construct was further tagged by insertion of the MHC class I epitope SIINFEKL, corresponding to residues 257 to 264 from OVA, as described previously (37). The generated ACT fusion protein was further genetically detoxified by ablating the catalytic adenylate cyclase activity by placing a GlyPhe dipeptide insert between residues 188 and 189, thereby disrupting the ATP binding site of ACT, as shown previously (10). The orientations and exact sequences of inserted oligonucleotides were verified by DNA sequencing.

Production and purification of ACT-derived proteins. The detoxified ACT carrying the inserted CSP and OVA epitopes and the control detoxified mock ACT (10) were produced in the presence of the activating protein CyaC with *E. coli* strain XL1-Blue (Stratagene) transformed with the appropriate plasmid construct derived from pT7CACT1. Exponential 500-ml cultures were induced with isopropyl-β-D-thiogalactopyranoside (1 mM), the cells were disrupted by ultrasound, and the insoluble cell debris was extracted with 8 M urea–50 mM Tris-HCl (pH 8.0)–0.2 mM $CaCl_2$. The proteins were further purified by ion-exchange chromatography on DEAE-Sepharose and hydrophobic chromatography on Phenyl-Sepharose as described previously (37). During hydrophobic chromatography, the resin with bound ACT was repeatedly washed with several bed volumes of 60% isopropanol to remove bacterial endotoxin (11). In the final step, the proteins were eluted with 8 M urea–2 mM EDTA–50 mM Tris-HCl (pH 8.0) and stored at −20°C. The integrity and purity of the purified proteins were monitored by visualizing the proteins by Coomassie staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The endotoxin content of the samples was determined by the *Limulus* amebocyte lysate assay (QCL-1000; Cambrex) according to the manufacturer's instructions and was below 100 IU/mg of purified protein.

Synthetic peptides. The synthetic peptides SYIPSAEKI and SIINFEKL, corresponding to $CD8^+$ T-cell epitopes $CSP_{245-253}$ and $OVA_{257-264}$, respectively, were purchased from MWG (Ebersberg, Germany).

Immunization and challenge. Mice were injected i.p. with 20 μg of recombinant ACT diluted in 200 μl of phosphate-buffered saline (PBS). In some experiments, a second immunization was performed after 21 days. Five hundred micrograms of anti-CTLA-4 monoclonal antibody 4F10 was administered intraperitoneally (i.p.) 1 day before priming or boosting. This dose was selected based on previous results (22). The endotoxin content of the purified Ab was below 10 IU/mg of protein. Seven days after the last immunization, mice were either sacrificed for IFN-γ ELISPOT assay and fluorescence-activated cell sorter analysis or challenged with 1,000 *P. berghei* sporozoites intravenously (i.v.). Parasitemia was determined by staining of blood smears with Wright's stain (Sigma, Taufkirchen, Germany).

Isolation of splenocytes, liver cells, and peripheral blood lymphocytes. Spleens were removed, and red blood cells were lysed by addition of ammonium chloride. The liver was flushed via the portal vein with 10% fetal calf serum–PBS and homogenized through a cell strainer. The suspension was centrifuged for 10 min at 380 × *g* and resuspended in 1 ml of complete RPMI 1640 medium. This suspension was mixed with a 30% Nycodenz solution (Nycomed Pharma AS, Oslo, Norway) and layered under 2 ml of RPMI 1640 medium. After centrifugation for 20 min at 4°C and 1,200 × *g*, the layer containing immune liver cells was recovered and washed once with 10% fetal calf serum–PBS. Blood was collected from the tail vein, and red blood cells were lysed by addition of ammonium chloride.

IFN-γ ELISPOT assay. Single-cell suspensions (2×10^5/well) were cultivated in Millipore HTS HA plates coated with anti-mouse IFN-γ. Cells were stimulated with 1-μg/ml $CSP_{245-253}$ peptide. After 18 h, supernatants were removed and the number of IFN-γ-producing cells was determined by ELISPOT assay as described elsewhere (22). Abs and avidin-horseradish peroxidase were purchased from BD Pharmingen (Heidelberg, Germany).

Analysis of cytokine production and proliferation. Single-cell suspensions (10^5 cells/well) were cultivated in 96-well plates. Cells were stimulated with 0.01-μg/ml $OVA_{257-264}$, 50-μg/ml ovalbumin, and 0.01- to 1.0-μg/ml ACT-CSP. After 48 h, supernatants were removed and the IFN-γ concentration was determined by indirect sandwich enzyme-linked immunosorbent assay. Abs and cytokine standard were purchased from R&D Systems (Wiesbaden, Germany). Proliferation was analyzed after 24 h by incorporation of [^{3}H]thymidine. Therefore, cells were pulsed with 0.5 μCi of [^{3}H]thymidine for 6 h.

Abs and flow cytometry. Hybridoma cells that produce anti-CTLA-4 (UC10-4F10; hamster immunoglobulin G [IgG]) were kindly provided by J. Bluestone (University of Chicago). Abs were purified from supernatants with HiTrap protein G columns (Pharmacia, Uppsala, Sweden) by standard protocols. The functionality of the purified Ab was checked by staining CTLA-4 on concanavalin A-stimulated spleen cells. For flow cytometry, fluorescein isothiocyanate-labeled anti-CD8 (Caltag, Burlingame, CA) and phycoerythrin (PE)-labeled anti-CD62L (BD Pharmingen, Heidelberg, Germany) were used. The frequency of CSP-specific T cells was determined with an allophycocyanin-labeled H2-K^d pentamer loaded with the $CSP_{245-253}$ peptide (Proimmune, Oxford, United Kingdom). For analysis of Foxp3 expression, the PE–anti-mouse Foxp3 Staining Set (eBioscience, San Diego, CA) was used according to the manufacturer's instructions. The set contains PE-labeled anti-Foxp3 Ab (clone FJK-16S, rat IgG2a, κ).

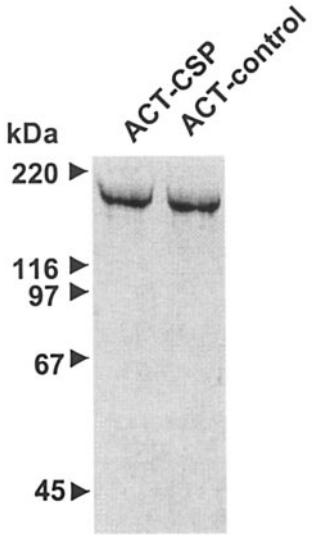

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified toxoids. The ACT proteins were expressed in the presence of the activating protein CyaC in a recombinant *E. coli* XL-1 strain transformed with pT7CACT1-derived plasmids and purified from urea extracts of cell debris by a combination of DEAE-Sepharose chromatography and Phenyl-Sepharose chromatography as described in Materials and Methods. Four-microgram samples of ACT-CSP and mock ACT were separated on a 7.5% acrylamide gel and visualized by Coomassie staining.

PE-labeled rat IgG2a, κ (clone R35-95), was used as an isotype control (BD Pharmingen, Heidelberg, Germany). Data were acquired on a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA) and analyzed with the CellQuest program (Becton Dickinson).

Statistical analysis. Student's *t* test of statistical significance was performed with the Prism software (GraphPad Software, San Diego, CA).

RESULTS

Delivery of T-cell epitopes into the MHC class I presentation pathway with ACT-CSP. An ACT was constructed that carries an insert of the *P. berghei* CSP-derived $CD8^+$ T-cell epitope SYIPSAEKI presented on H-$2K^d$ and, as a marker, the OVA-derived SIINFEKL epitope presented in the context of H-$2K^b$. The resulting ACT-CSP fusion protein was expressed in *E. coli* and purified close to homogeneity (Fig. 1). Particular attention was paid to endotoxin removal during the purification procedure, and the resulting protein preparations were essentially endotoxin free (<100 IU of lipopolysaccharide [LPS]/mg of ACT-CSP). To verify that the ACT-CSP fusion protein was capable of delivering antigens into the MHC class I compartment, the protein was incubated with spleen cells from T-cell receptor transgenic OT-1 mice capable of presenting the SIINFEKL peptide. Indeed, as shown in Fig. 2, spleen cells from OT-1 mice proliferated and produced IFN-γ in a dose-dependent manner upon incubation with ACT-CSP (0.01 to 1 μg/ml), whereas neither the free OVA protein (50 μg/ml) nor the mock ACT control protein (1 μg/ml) induced any OT-1 T-cell activation. These data show that the ACT-CSP construct was still capable of translocating the AC domain into the cytosol of antigen-presenting cells and allowed the inserted epitopes to access the MHC class I presentation pathway.

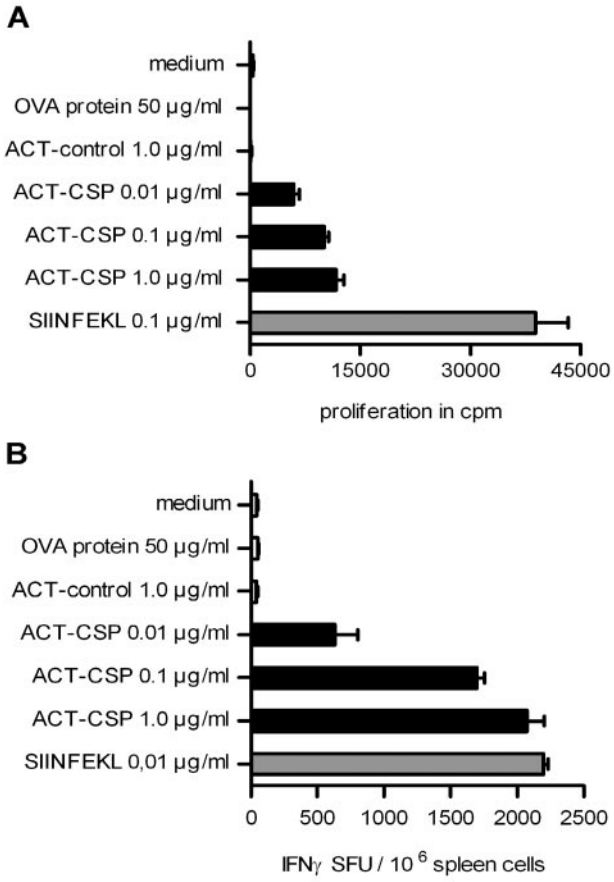

FIG. 2. In vitro delivery of OVA T-cell epitope into the MHC class I presentation pathway by ACT-CSP. (A) Spleen cells from T-cell receptor-transgenic OT-1 mice were incubated with medium, with OVA protein, with mock ACT control protein, with ACT-CSP containing both the SIINFEKL epitope from OVA and the CSP epitope, or with SIINFEKL peptide, respectively. After 24 h, cells were pulsed with [^{3}H]thymidine and proliferation was measured after an additional 6 h. (B) In parallel, cells were stimulated on Ab-coated ELISPOT assay plates and the numbers of IFN-γ-producing cells were determined. Data are expressed as the mean ± the standard deviation of values from three wells within a representative experiment of two performed.

Induction of OVA-specific $CD8^+$ T cells in vivo. To examine whether ACT-CSP could induce OVA-specific $CD8^+$ T cells in vivo, C57BL/6 mice were immunized i.p. with a single dose of 20 μg of ACT-CSP or with mock ACT as a control. As shown in Fig. 3, mice that received ACT-CSP produced high numbers of IFN-γ-secreting OVA-specific T cells, as revealed by ELISPOT assay with spleen cells restimulated in vitro with the SIINFEKL peptide. Moreover, immunization with ACT-CSP induced Th1 cytokine-producing T cells (data not shown) despite the very low levels of contaminating LPS in the ACT preparations (<1 IU of LPS per vaccine dose), which argues for an intrinsic capacity of the detoxified ACT vector to polarize the induced immune response, in line with previous reports (5).

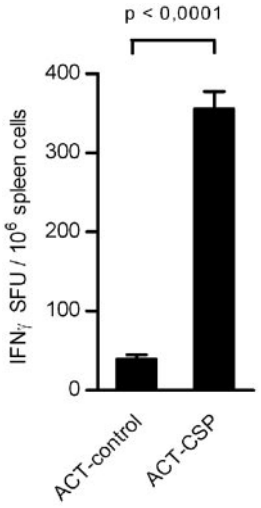

FIG. 3. Induction of OVA-specific CD8$^+$ T cells in vivo. Groups of C57BL/6 mice were immunized i.p. with 20 μg of mock ACT control or with 20 μg of ACT-CSP. Seven days later, spleen cells were isolated and restimulated in vitro with SIINFEKL peptide and the number of IFN-γ-producing cells was determined by ELISPOT assay. Data are expressed as the mean ± the standard deviation from two experiments ($n = 6$).

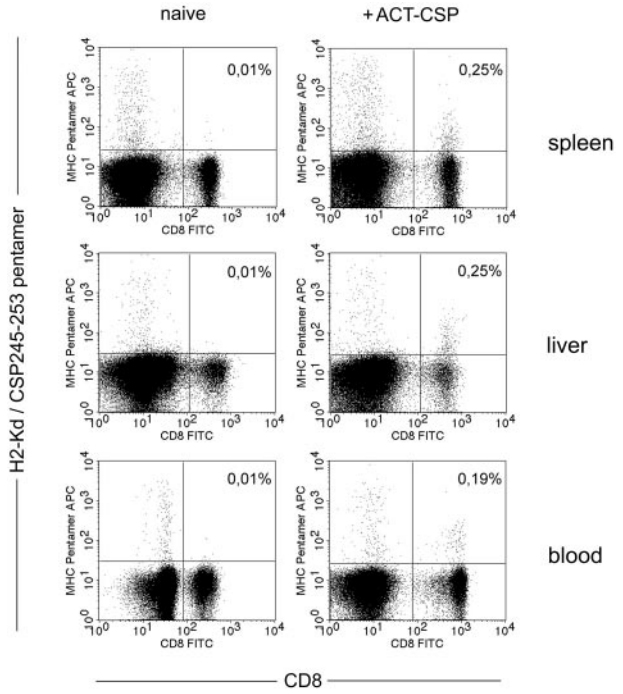

FIG. 4. Efficient induction of CSP-specific CD8$^+$ T cells by a single immunization with ACT-CSP. BALB/c mice were immunized i.p. with 20 μg of ACT-CSP. Seven days later, peripheral blood lymphocytes, liver cells, and spleen cells were isolated and stained with a CSP peptide-loaded H-2K^d pentamer. Results of a representative experiment out of five are shown.

Efficient induction of CSP-specific T cells. Next, the ability of ACT-CSP to induce CSP-specific CD8$^+$ T cells was examined. As shown in Fig. 4, a single i.p. immunization of BALB/c mice with 20 μg of ACT-CSP was sufficient to induce high numbers of CSP-specific CD8$^+$ T cells. These could be detected in the spleens, blood, and livers of immunized mice with a fluorescence-labeled H-2K^d pentamer loaded with CSP peptide, indicating that CSP-specific CD8$^+$ T cells were capable of reaching the liver, the target organ (Fig. 4). In addition, a high number of cells producing IFN-γ were detected in splenocyte suspensions upon ex vivo restimulation with CSP peptide, as shown in Fig. 5. Moreover, compared to the yield of a single immunization, a prime-boost immunization regimen did not result in any further enhancement of the frequency of CSP-specific T cells secreting IFN-γ in the spleens of immunized animals (Fig. 5 A), suggesting that expansion of the response following booster immunization with ACT-CSP was suppressed.

CTLA-4 blockade results in an increased frequency of CSP-specific cells upon booster immunization with ACT-CSP. CTLA-4 expression on either Treg cells or activated T cells was previously reported to dampen the immune response, suggesting that blockade of CTLA-4 during immunization with ACT-CSP might provide a tool for further enhancement of the frequency of induced CSP-specific T cells. To test this strategy, anti-CTLA-4 Ab (500 μg) was administered i.p. 1 day before the primary or booster immunization with ACT-CSP, respectively. As shown in Fig. 5B, when anti-CTLA-4 was administered prior to the primary immunization, no statistically significant increase in the number of IFN-γ-producing T cells was observed, compared to administration of ACT-CSP alone. In contrast, when anti-CTLA-4 was given prior to the booster immunization with ACT-CSP, a slightly but significantly increased number of CSP-specific IFN-γ-producing cells was detected (Fig. 5C). Furthermore, compared to mice that received only ACT-CSP, significantly higher frequencies of CSP-MHC multimer-positive and CD62L^{low} cells were detected in the spleens, livers, and blood of mice that received anti-CTLA-4 prior to booster immunization, as shown in Fig. 6. These data show that not only the proportion of IFN-γ-producing cells was enhanced but also higher overall numbers of CSP-specific CD8$^+$ T cells were induced when CTLA-4 was blocked during booster immunization with ACT-CSP.

CTLA-4 blockade during booster immunization leads to enhanced protection against a sporozoite challenge. Given that IFN-γ-producing CD8$^+$ T cells recognizing antigens from sporozoites are capable of mediating protective immunity, we tested whether the immune response induced by ACT-CSP could protect mice against a challenge with *P. berghei*, as shown in Fig. 7 and summarized in Table 1. Despite inducing fairly high numbers of CSP-specific CD8$^+$ T cells in the spleens and blood, as well as the livers, of immunized animals (Fig. 6), neither a single nor a prime-boost immunization with ACT-CSP alone conferred any protection against parasitemia on mice challenged i.v. with 10^3 *P. berghei* sporozoites. In contrast, when mice were treated with anti-CTLA-4 (500 μg) a day before booster immunization with ACT-CSP to allow induction of further increased numbers of CSP-specific T cells (Fig. 6), a statistically significant degree of protection was achieved (Fig. 7 and Table 1), with 60% of the animals being fully protected and not developing any parasitemia over the course of the experiment, up to 14 days postinfection (p.i.). Moreover,

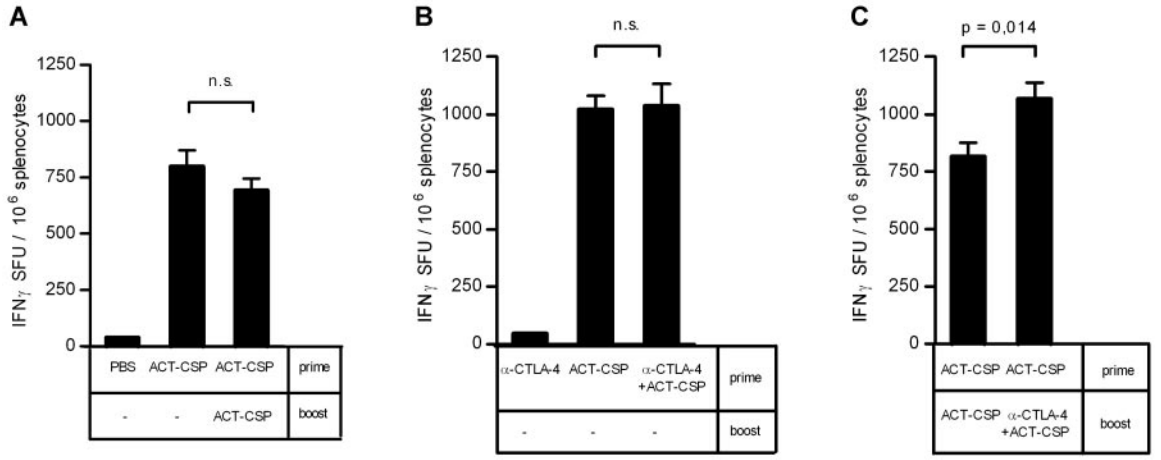

FIG. 5. CTLA-4 blockade allows induction of higher numbers of CSP-specific T cells that produce IFN-γ upon booster immunization. Spleen cells from BALB/c mice that received a single immunization with ACT-CSP ($n = 5$) on day 0 or from mice that received a booster immunization ($n = 6$) on day 21 were stimulated with CSP-specific peptide on Ab-coated ELISPOT assay plates, and the number of IFN-γ-producing cells was determined (A). A prime-boost immunization regimen does not lead to a statistically significant increase in the number of IFN-γ-producing cells. (B) Mice received i.p. 500 μg of anti-CTLA-4 ($n = 6$) or 20 μg of ACT-CSP ($n = 6$) or a combination of anti-CTLA-4 and ACT-CSP ($n = 6$). Seven days after the last immunization, spleen cells were isolated and stimulated with the CSP peptide on Ab-coated ELISPOT assay plates and the number of IFN-γ-producing cells was determined. (C) In a third set of experiments, mice were immunized at day 0 and day 21 with ACT-CSP ($n = 9$). One group received, in addition, anti-CTLA-4 during the booster immunization ($n = 9$). Seven days after the last immunization, spleen cells were isolated and restimulated with CSP peptide and the number of IFN-γ-producing cells was determined by ELISPOT assay. Data are expressed as the mean ± the standard deviation. n.s., no statistically significant difference.

those mice that received the protective immunization regimen and nevertheless developed a blood-stage infection exhibited decreased parasitemia on day 9 p.i., whereas on day 13 p.i. they had a parasite load as great as that of the control mice. These results indicate that the protection during the liver stage of infection has to be complete, and if even one infected hepatocyte escapes immune destruction and releases its contents into the circulation, this can be sufficient to establish blood-phase parasitemia later in the infection (Fig. 7).

Booster immunization does not induce an increased number of Foxp3$^+$ Treg cells. To test whether the primary immunization with ACT-CSP-induced Treg cells, which might have limited the efficacy of the booster immunization, we analyzed the number of Treg cells in untreated control mice and in mice subjected to a single priming immunization or to a prime-boost immunization with ACT-CSP, respectively. Since intracellular expression of Foxp3 is a reliable marker for Treg cells, intracellular staining for this molecule was performed and cell samples were analyzed by flow cytometry. However, compared to control mice, neither in the livers nor in the spleens of mice immunized with AST-CSP could any significant change in Treg cell frequency be observed, as shown in Fig. 8. These results, however, do not exclude the possibility that CTLA-4-expressing Treg cells might control the expansion of activated CSP-

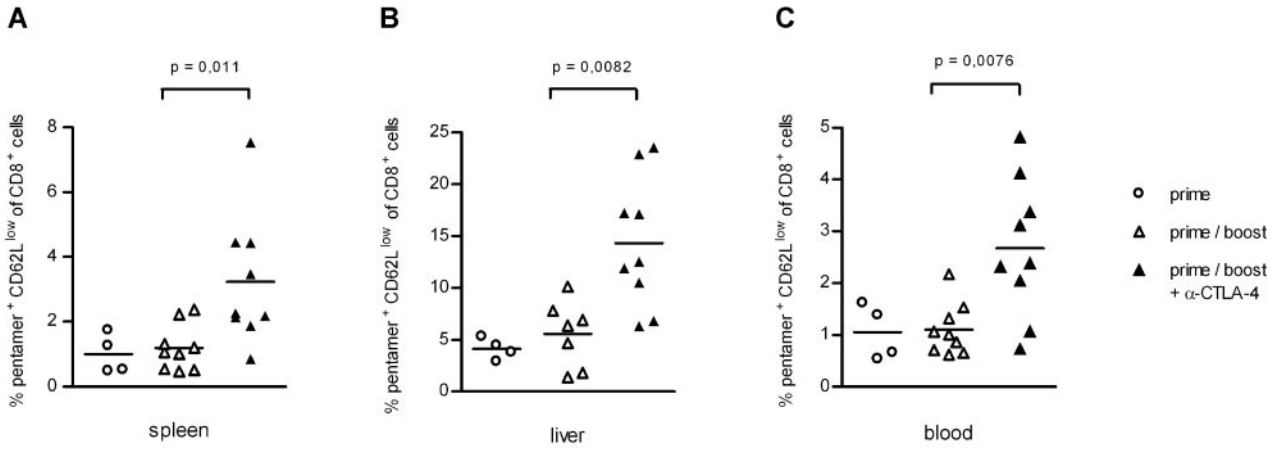

FIG. 6. Enhancement of the number of CSP-specific T cells by anti-CTLA-4 during booster immunization. The indicated numbers of BALB/c mice were immunized i.p. at day 0 and day 21 with 20 μg of ACT-CSP. One group received, in addition, anti-CTLA-4 i.p. (500 μg) on day 20. Seven days after administration of the second vaccine dose, peripheral blood lymphocytes, liver cells, and spleen cells were stained with a CSP peptide-loaded H-2K^d pentamer and analyzed by flow cytometry.

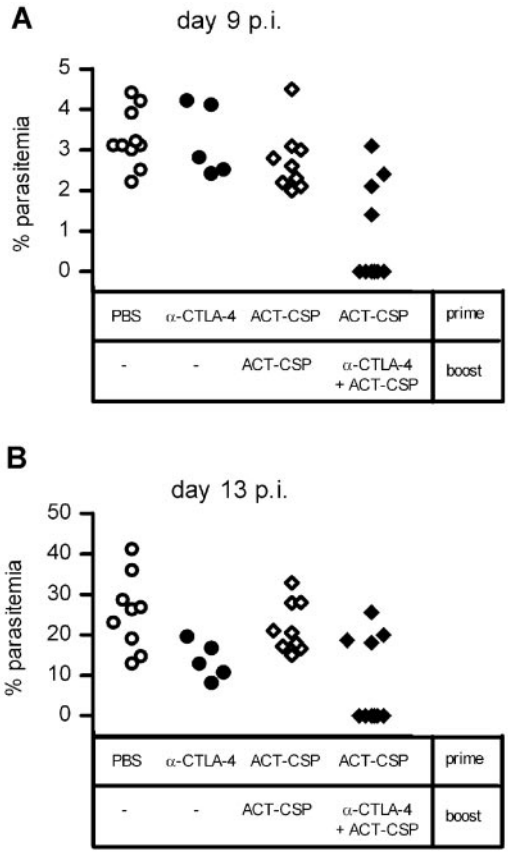

FIG. 7. Parasitemia of mice after challenge with sporozoites. Mice were treated with PBS, anti-CTLA-4, or a prime-boost regimen with ACT-CSP with or without anti-CTLA-4 during the boost. Seven days later, mice were challenged i.v. with 1,000 sporozoites and parasitemia was analyzed on day 9 and day 13.

specific $CD8^+$ T cells during a secondary encounter without an increasing frequency.

DISCUSSION

It was reported previously that vaccination against the liver stage of malaria was feasible with irradiated sporozoites (19, 35) or attenuated sporozoite mutants (34). The major advantage of an efficient vaccine targeting of the liver stage of malaria would be that this infection stage is not accompanied by the characteristic symptoms of malaria. These were solely found to be associated with the blood stage of infection. Hence, complete protective immunity against infection at the liver stage can reasonably be expected to prevent any symptoms of illness. However, mass treatment with attenuated sporozoites is an elaborate process. Thus, current malaria vaccine strategies are focused on subunit vaccines with antigens that are expressed during the liver stage. However, these strategies are hampered by (i) the limited numbers of immunologically characterized antigens, (ii) limitations in the ability to introduce antigens into the MHC class I pathway, and (iii) the shortage of appropriate adjuvants that would steer the immune system in the desired direction.

Recent studies have shown that the cell-invasive *B. pertussis* ACT is capable of delivering antigens into the MHC class I and II presentation pathways in parallel (32, 43). Whereas MHC class II targeting is achieved by conventional endocytosis, ACT is capable of delivering its N-terminal catalytic (AC) domain into the cytosol of CD11b-expressing professional antigen-presenting cells, and hence antigens inserted into the permissive sites within the AC domain of ACT can be targeted into the MHC class I-restricted presentation pathway. The usefulness of detoxified ACT as a vaccine carrier is further underscored by its high affinity for the $\alpha_M\beta_2$ integrin (CD11b/CD18), which is particularly prominently expressed on antigen-presenting cells such as myeloid dendritic cells. Hence efficient delivery of antigens to antigen-presenting cells is provided (8, 24, 43). In the present study, we employed as an experimental vaccine an ACT-CSP construct bearing $CD8^+$ T-cell epitopes of *P. berghei* CSP and OVA in parallel. A single immunization with ACT-CSP was capable of inducing high numbers of T cells specific either for OVA or for the CSP, showing that ACT-CSP was capable of targeted delivery of multiple epitopes into the MHC class I pathway simultaneously. Interestingly, ACT-CSP administration elicited IFN-γ-producing T cells, which indicates induction of a Th1 type of immune response in the absence of further adjuvants. Due to the very low level of endotoxin in the ACT preparations used that would finally result in the application of less than 1 IU of LPS per mouse, it is quite unlikely that endotoxin contamination accounts for the observed bias toward a Th1 type of response. An alternative explanation is that binding of ACT to CD11b/CD18, subsequent translocation of ACT across the membrane, or both might provide signals that foster a Th1 response by an as-yet-unknown mechanism (5). However, despite the induction of a high number of CSP-specific $CD8^+$ T cells by a single administration of ACT-CSP, no protection was achieved, although CSP-specific cells were readily detectable in the liver. This failure might be explained by the special status of the liver, which is known to dampen cytokine production by T cells or to induce their anergy (26, 31). Moreover, the CSP does not appear to be an ideal antigen candidate, since it is highly expressed on sporozoites in the mosquito while it rapidly vanishes after sporozoite infection of hepatocytes (2). Thus, it can be expected that small amounts of CSP-derived peptides will be presented on MHC

TABLE 1. A prime-boost vaccination regimen using ACT-CSP in combination with anti-CTLA-4 induces protection against infection with *P. berghei* sporozoites

Treatment[a]		No. infected/ total[b]	% Protection
Prime	Boost		
PBS	PBS	10/10	0
PBS	Anti-CTLA-4	5/5	0
ACT-CSP	ACT-CSP	9/9	0
ACT-CSP	Anti-CTLA-4^+–ACT-CSP	4/10	60

[a] In this regimen, mice were treated at day 0 with the indicated antigen (20-μg ACT-CSP prime) and on day 20 with anti-CTLA-4 (500 μg), followed by another 20-μg ACT-CSP dose on day 21 (boost).

[b] Mice were challenged i.v. with 10^3 sporozoites and examined at day 14 p.i. for parasitemia. Animals were scored as protected when no infected erythrocytes were detectable in blood smears. The protected mice did not develop a blood-phase disease even at time points beyond day 14 p.i. Variations in the course of parasitemia were not considered.

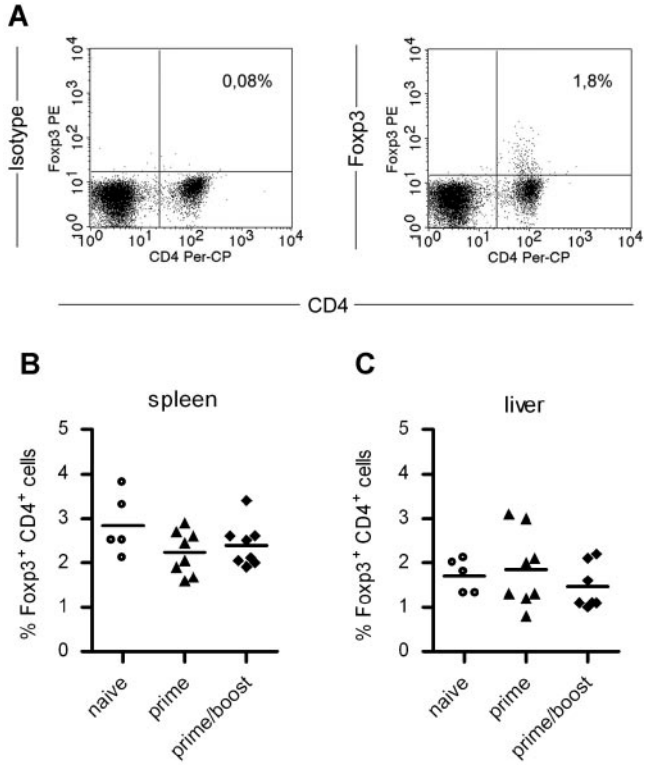

FIG. 8. A prime-boost vaccination regimen does not induce increased numbers of Foxp3$^+$ Treg cells. The number of Treg cells was evaluated by surface staining with anti-CD4, followed by intracellular staining with anti-Foxp3 (A, right side) or an isotype control Ab (left side). The individual number of CD4$^+$ Foxp3$^+$ T cells of control mice, prime-immunized mice, or mice that received the prime-boost immunization was analyzed in the spleen (B) and liver (C).

class I molecules for a limited time early upon infection of cells. New experimental strategies led to the identification of proteins that are expressed during the liver stage, and the delivery method described herein, based on the use of ACT as an antigen carrier, can be used to introduce these proteins into the MHC class I presentation pathway, which would allow their rapid immunological characterization (23, 34).

Most successful vaccines rely on a heterologous prime-boost regimen in order to yield an adequate immune response (44). In the present study, a single application of ACT-CSP was accompanied by the induction of high numbers of antigen-specific T cells, while a booster immunization, surprisingly, did not elicit any statistically significant increase in the frequency of CSP-specific T cells. In order to analyze if counterregulation of the immune response might have restricted the desired T-cell response, we combined our ACT-CSP-based vaccination protocol with a blockade of CTLA-4, which was previously shown to play a key role in restraining T-cell responses. CTLA-4 is a negative regulator of the T-cell response and is expressed on activated T cells and on Treg cells (3). Interestingly, anti-CTLA-4 treatment prior to the primary immunization neither induced an increased T-cell frequency nor allowed induction of protective immunity against a challenge with *P. berghei* sporozoites. In contrast, anti-CTLA-4 treatment prior to the booster immunization resulted in further increased numbers of CSP-specific T cells and induced protection against the liver phase of the infection and subsequent blood-stage disease development. This is in concordance with several animal studies, as well as clinical studies that employed anti-CTLA-4 blockade to enhance the immune response (9, 16, 17). It is interesting that in the present study, as well as in another report (14), a blockade of CTLA-4 had a major impact during the booster immunization and not on the efficacy of the primary immunization.

A major drawback that dampens the enthusiasm for using an anti-CTLA-4 treatment is the risk of inducing severe inflammatory side effects, such as those observed in clinical trials of immunotherapy of cancer, where autoimmune processes could be triggered by repeated administration of anti-CTLA-4 (38, 40, 42). We recently found that anti-CTLA-4 treatment during the blood phase of malaria—a phase where massive activation of T cells occurs—was a double-edged sword. An improved T-cell response induced an exacerbation of disease with *P. berghei* blood-stage infection (21, 22). These findings could be due to the fact that, during the blood phase, T cells contribute to protection via activation of macrophages by cytokines, since direct interaction with infected erythrocytes is not possible because of the lack of MHC molecules on erythrocytes. In contrast, T cells that produce proinflammatory cytokines were also shown to be involved in the pathogenicity of severe malaria. Thus, the use of anti-CTLA-4 in therapeutic vaccines has to be considered with caution. Nevertheless, this study demonstrates that counterregulation of the immune system dampens the elicited response induced by the vaccine. Recent findings demonstrated that CD4$^+$ CD25$^+$ Treg cells can control CD8$^+$ T cells and that depletion of CD4$^+$ T cells leads to an increased frequency of CD8$^+$ T cells in an infection model with *Listeria monocytogenes* (29, 30). It was also shown that during *Plasmodium yoelii* infection of mice, activation of Treg cells helps the parasites to evade the immune system of the host (15). Similar findings were reported when volunteers were infected with sporozoites. There, the blood stage was accompanied by rapid induction of CD4$^+$ CD25$^+$ Foxp3$^+$ T cells and was associated with a decreased antigen-specific immune response (48). With ACT-CSP as a vaccine carrier, we observed induction of Foxp3$^+$ Treg cells neither during prime immunization nor during prime-boost immunization. However, these data do not exclude the possibility that the pool of naturally occurring Treg cells limited the expansion of vaccine-induced T cells. An alternative explanation for the observed effects of CTLA-4 blockade is that CTLA-4 expression on T effector or memory cells was induced during the primary immunization and prevented the clonal expansion during secondary antigen encounter following the booster immunization. In both cases, temporary blockade of the regulatory pathway during a booster immunization in vaccination regimens would lead to an increase in the activation of T cells and hence would represent a promising means to potentiate vaccine-induced T-cell responses. Present limitations might be overcome by the development of new reagents capable of more-selective modulation of the immune response. It has, indeed, been shown with CTLA-4 knock-in mice that a number of Abs against human CTLA-4 differ in the ability to induce tumor rejection or autoimmunity. Most interestingly, the Ab that conferred the greatest protection induced the fewest undesired side effects

(33). These findings may provide the basis for the development of novel tools modulating the immune response without causing side effects. In addition, side effects associated with the use of anti-CTLA-4 treatments in the course of vaccine administration might be controllable, since CTLA-4 blockade has to be achieved only for a very short time during booster immunization, compared to the repeated administration of the Ab used in current clinical trials of tumor immunotherapy.

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