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A taRNA vaccine candidate induces a specific immune response that protects mice against Chikungunya virus infections

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The arthritogenic alphavirus, chikungunya virus (CHIKV), is now present in almost 100 countries worldwide. Further spread is very likely, which raises public health concerns. CHIKV infections cause fever and arthralgia, which can be debilitating and last for years. Here, we describe a CHIKV vaccine candidate based on *trans*-amplifying RNA (taRNA). The vaccine candidate consists of two RNAs: a non-replicating mRNA encoding for the CHIKV nonstructural proteins, forming the replicase complex and a *trans*-replicon (TR) RNA encoding the CHIKV envelope proteins. The TR-RNA can be amplified by the replicase in *trans*, and small RNA amounts can induce a potent immune response. The TR-RNA was efficiently amplified by the CHIKV replicase *in vitro*, leading to high protein expression, comparable to that generated by a CHIKV infection. In addition, the taRNA system did not recombine to replication-competent CHIKV. Using a prime-boost schedule, the vaccine candidate induced potent CHIKV-specific humoral and cellular immune responses *in vivo* in a mouse model. Notably, mice were protected against a high-dose CHIKV challenge infection with two vaccine doses of only 1.5 μg RNA. Therefore, taRNAs are a promising safe and efficient vaccination strategy against CHIKV infections.

INTRODUCTION

Chikungunya virus (CHIKV) is a human pathogenic alphavirus belonging to the *Togaviridae* family. It has caused several epidemics and is of major public health concern. CHIKV is transmitted by mosquito vectors and has rapidly spread worldwide after vector extension from *Aedes aegypti* to *Aedes albopitcus*.[1] In contrast to *Aedes aegypti*, *Aedes albopictus* is not limited to the tropics and sub-tropics but is also present in temperate climate zones.[2] CHIKV has now been found in almost 100 countries worldwide, and due to climate change, further spread is very likely.[3,4] In humans, symptomatic CHIKV infections are characterized by an acute infection with high fever, rash, myalgia, and polyarthralgia. Although the mortality rate is low, patients can develop severe, debilitating arthralgia affecting multiple joints that can become chronic and last for years.[5] However, no licensed vaccine or specific antiviral treatment is currently available.

Several vaccine candidates against CHIKV infection have so far been explored, including live-attenuated, vector-based, virus-like particle, and nucleic-acid-based vaccines.[6,7] Although live-attenuated vaccines can efficiently induce humoral and cellular immune responses, they pose the risk of virus reversion.[8] In contrast, virus-like particle vaccines are safer but induce mainly humoral immune responses and are expensive to produce. The coronavirus disease 2019 (COVID-19) pandemic has demonstrated the advantages of RNA-based vaccinations, which allow a rapid response to emerging infections. Importantly, RNA vaccines can induce potent and protective humoral and cellular immune responses with an improved safety profile and might thus be suitable CHIKV vaccine candidates.[9]

Non-replicating RNAs (nrRNAs), self-amplifying RNAs (saRNAs), and *trans*-amplifying RNAs (taRNAs) have all been described as RNA vaccine candidates. nrRNAs are based upon naturally occurring mRNA with a synthetic cap analog and engineered UTRs.[10] In contrast, saRNAs and taRNAs are based on the genomes of positive-sense viruses like alphaviruses. For their construction, the viral structural proteins are deleted from the viral template RNA except for the virus replicase. The virus replicase can amplify the template RNA, allowing potent immune responses to be induced with lower RNA amounts compared with nrRNA.[11] In the case of saRNA, the antigen and replicase are encoded on the same RNA, whereas for taRNA, the replicase and the antigen are split onto two RNAs and the antigen RNA is amplified by the replicase in *trans*.[12,13]

The genome structure of alphaviruses allows straightforward adaptation to a taRNA vaccine candidate. Alphaviruses have a single-stranded,

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positive-sense RNA genome consisting of two open reading frames (ORFs). During the replication cycle, two RNAs are synthesized, the full-length genomic RNA (gRNA) and the subgenomic RNA (sgRNA). The first ORF on the gRNA encodes the four nonstructural proteins (nsP1–4), which assemble into the alphavirus replicase complex (replicase) responsible for RNA amplification and sgRNA synthesis. The second ORF encodes the structural proteins, which are the capsid and the envelope proteins E3–E2-6K-E1. Their expression from the sgRNA is under the control of the subgenomic promotor.[14]

The taRNA constructs contain the alphavirus replicase (nsP1–4 genes) encoded on a first RNA, which can be either an nrRNA or saRNA. The replicase can amplify a second so-called *trans*-replicon (TR)-RNA. TR-RNAs are generated by deletion of the replicase from the alphavirus genome and insertion of the respective antigen under the control of the subgenomic promotor. Amplification of this TR-RNA by the replicase is ensured by the viral 5′ and 3′ conserved sequence elements.[13,14] Recently, a taRNA vaccine candidate based on the Semliki Forest virus (SFV) replicase and a TR-RNA encoding the influenza hemagglutinin was used successfully to induce a potent influenza-specific immune response and protection against a challenge infection and was comparable to the saRNA vaccine candidate but at a much lower dose of antigen-encoding RNA.[15]

Here, we generated a taRNA vaccine candidate against the alphavirus CHIKV. The taRNA vaccine approach makes use of an nrRNA encoding the CHIKV replicase and a TR-RNA encoding the CHIKV envelope proteins. This system demonstrated high TR-RNA amplification and antigen expression by the replicase *in vitro* and did not generate replication-competent CHIKV. Moreover, the vaccine candidate was able to induce potent humoral and cellular immune responses *in vivo* in a mouse model, and animals were protected against a CHIKV challenge infection by a prime-boost vaccination each with only 1.5 μg total RNA.

RESULTS

CHIKV taRNAs are safe and do not reassemble a full-length infectious virus

The taRNA system consists of at least two RNAs. The first typically encodes the alphavirus replicase, and the TR-RNA encodes the protein of interest.[13] For a CHIKV taRNA vaccine candidate, the CHIKV replicase can either be encoded on an saRNA, which is derived from the CHIKV genome with deleted structural proteins, or on an nrRNA, which is a synthetic mRNA (Figure 1B).[15,16] In addition to these, a TR-RNA construct containing three different antigens was designed by deleting the replicase from a CHIKV genome and retaining only the first 231 nt of nsP1 and the last 1,818 nt of nsP4. The template included the alphavirus conserved sequence elements (5′CSE and 3′CSE), ensuring specific RNA amplification by the CHIKV replicase.[14] The CHIKV antigens encoded by the different TR-RNA constructs were the complete structural protein genes (capsid and envelope; TR-CS), only the envelope (TR-S), or the capsid (TR-C), which was used in combination with TR-S as two separate TR-RNAs (TR-S + TR-C) (Figure 1C). The antigens were placed under the control of the subgenomic promotor.

To assess whether the CHIKV taRNAs recombine to one infectious, full-length RNA, we first performed a luciferase assay detecting infectious CHIKV.[18] This assay makes use of HEK293T cells that were transfected before with a TR-luc reporter RNA, which is only amplified and translated in the presence of the CHIKV replicase. Consequently, luciferase signals rely on the presence of infectious virus. For the assay, HEK293T cells were transfected with the replicase-encoding RNAs together with the different TR-RNAs, and the supernatants were passaged on new HEK293T cells to enrich replication-competent virus in case of recombination. As a control, cells were transfected with the TR-CS-RNA only or infected with CHIKV. Supernatants after transfection (p1) and supernatants after further passaging (p2–p4) were then evaluated for the presence of recombined virus. The transfection of the saRNA-encoded replicase with the TR-CS-RNA generated infectious virus that could be detected as luciferase activity. Virus also amplified during the second passage, reaching virus titers of about 10^6 plaque forming units (PFUs)/mL, similar to a CHIKV infection, and was still detectable after further passaging (Figure 1D). In all tested combinations with the nrRNA-encoded replicase, no luminescence signals higher than background levels were measured, indicating that the nrRNAs and the TR-RNA did not reassemble to full-length infectious CHIKV RNA. Accordingly, the combination of the nr-replicase-RNA with any of the three different TR-RNA constructs is expected to be a safe vaccine candidate.

CHIKV replicase efficiently amplifies CHIKV TR-RNAs, leading to high antigen expression

The three potential vaccine candidates were further evaluated *in vitro*. First, the amplification of the TR-RNAs by the CHIKV replicase was assessed by qRT-PCR. RNA levels of the envelope gene E2 and capsid were detected with specific primers and normalized to GAPDH. HEK293T cells were transfected with one of the three TR-RNA constructs together with the nr-replicase-RNA or with an irrelevant RNA (TR-luc). RNA was extracted 6 h and 16 h after transfection and compared with a CHIKV infection (multiplicity of infection [MOI] 3). After 6 h, the TR-RNA levels were already significantly increased in the presence of the viral replicase compared with the irrelevant RNA transfection, and they were further increased by 16 h (Figure 2). The E2 RNA levels at 16 h after transfection with TR-CS or TR-S were similar to that of virus-infected cells (Figure 2A). If the CHIKV capsid and envelope genes were separated onto two TR-RNAs, the replicase preferentially amplified the shorter capsid RNA (286.26-fold) (Figure 2B), whereas only a minor amplification of the E2 RNA was detected (14.68-fold) (Figure 2A).

In addition, cells were stained for the presence of double-stranded RNA (dsRNA), which indicates the formation of viral replication complexes.[19] Indeed, dsRNA could be visualized during amplification of the TR-RNAs by the CHIKV replicase (Figure S1). This was similarly observed during CHIKV infections, but dsRNA was absent in

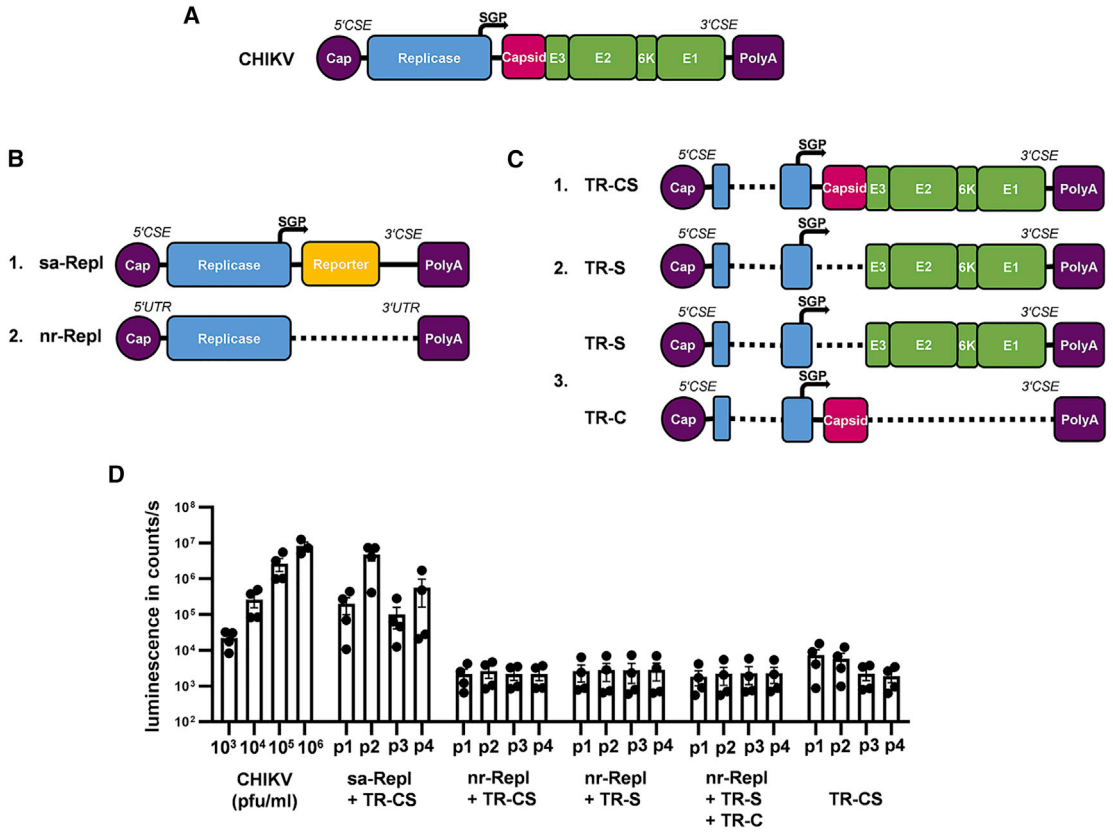

Figure 1. CHIKV *trans*-amplifying (ta)RNA vaccine constructs do not recombine to infectious CHIKV

(A) Schematic representation of CHIKV genome. (B) RNAs encoding replicase are shown. (1) Schematic illustration is shown of CHIKV replicase RNAs as self-amplifying (sa) RNA with viral conserved sequence elements (5′ CSE and 3′ CSE) and subgenomic promotor (SGP) (the sa-Repl RNA comprises nucleotides 1–7,566 and 11,314–11,840) or (2) as non-replicating (nr) RNA, where the CHIKV replicase ORF is flanked by alpha-globin 5′ UTR to ensure high translational activity and AES-mtRNR1 sequences as 3′ UTR to increase RNA stability as described previously.[17] (C) RNAs encoding CHIKV structural proteins are shown (capsid [C] and envelope [S] [E3–E1]). (1) Structural genes delivered on one TR-RNA, (2) the envelope delivered exclusively, or (3) envelope and capsid separated onto two TR-RNAs are shown. TR-RNAs contain the CHIKV conserved sequence elements (CSEs), the first 231 nt of nsP1, and the last 1,818 nt of nsP4. (D) To assess the recombination of the two RNAs into one full-length viral RNA, cells were transfected with the indicated RNA combinations and initial supernatants (p1), and supernatants obtained after further passaging (p2–p4) were evaluated for the presence of infectious CHIKV with a luciferase-based assay.[18] As control, 10^3–10^6 PFUs/mL CHIKV were analyzed. Data are mean ± SEM of four independent experiments.

cells transfected either only with the synthetic replicase RNA or TR-S-RNA.

Next, the protein expression of the vaccine candidates was investigated. The envelope E2 proteins on the cell surface of transfected cells were stained and analyzed by flow cytometry. The mean fluorescent intensity (MFI) indicates the amount of protein on the cell surface. In line with the RNA amplification by the CHIKV replicase, E2 protein expression also required the replicase activity (Figure 3A). Cells transfected with an irrelevant RNA did not show E2 expression (Figure 3A). The E2 protein levels of cells transfected with TR-CS- or TR-S-RNA were comparable to a CHIKV infection (MOI 3). Moreover, western blot analysis showed that the replicase proteins nsP1–4, which are encoded on the nrRNA, were already expressed 6 h after transfection and protein levels decreased slightly by 24 h (Figure 3B). The two structural proteins capsid and E2, encoded on the TR-RNA, could also be detected at 6 h post-transfection in low amounts, but protein levels increased over time due to RNA amplification. In CHIKV-infected cells, protein expression was only detected 24 h post-infection, with a higher level of nsP1–4 expression compared with RNA-transfected cells, although structural proteins were expressed at similar levels (Figure 3B). The levels of E2 protein in lysates of cells transfected with TR-CS- or TR-S-RNA were comparable (Figure 3C). In contrast, co-transfection of two RNAs, TR-S- and TR-C-RNA, resulted in almost non-detectable E2 protein expression in cell lysates and only the capsid protein was highly expressed (Figure 3C). Intracellularly, the E2 protein is transported via the endoplasmic reticulum (ER)-Golgi pathway to the plasma membrane, and this localization was detected by immunofluorescence analysis after RNA transfection or during a CHIKV infection (Figure S2). Furthermore, the E2 and capsid protein were also secreted into the cell supernatant, with the highest release from TR-CS-transfected cells (Figure 3C). In summary, the taRNA system was safe and induced similar

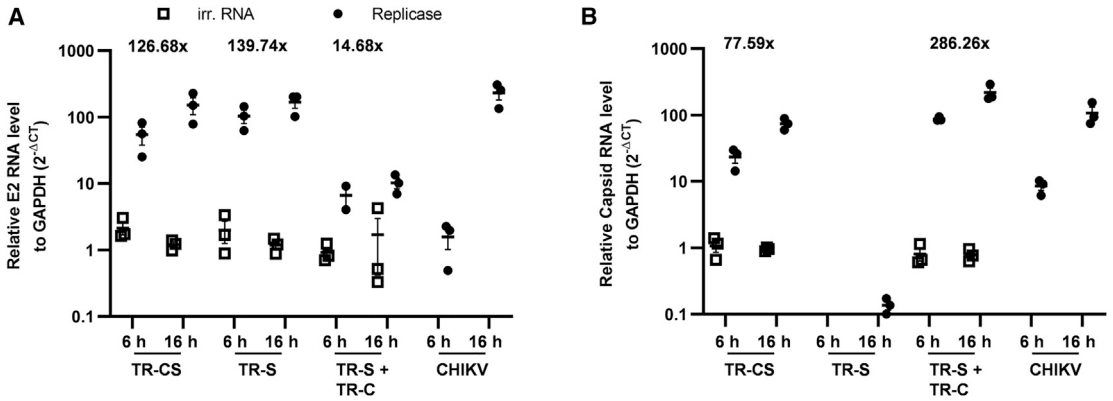

Figure 2. TR-RNAs are efficiently amplified by CHIKV replicase

For the *in vitro* characterization of the taRNA vaccine combinations, HEK 293T cells were transfected with the replicase (nrRNA) or irrelevant RNA (TR-luc) together with the different TR-RNAs or infected with CHIKV (MOI 3). RNA levels were measured by qRT-PCR after 6 h and 16 h with specific primers directed against the E2 gene (A) or the capsid gene (B) and normalized to GAPDH. Numbers indicate the fold change in TR-RNA amount after 16 h mediated by the replicase expression. Black circles indicate nr-replicase-RNA-transfected cells and gray rectangles irrelevant RNA. Data are mean ± SEM of three independent experiments.

protein expression patterns as a CHIKV infection. The high antigen expression with low RNA amounts qualifies it as a potent vaccine candidate.

The CHIKV taRNA vaccine candidate induces potent CHIKV-specific B and T cell responses

In humans, most CHIKV-neutralizing antibodies are directed against the envelope protein E2,[20,21] because interference with E2 inhibits virus attachment to cells and receptor binding. Hence, the third vaccine candidate (TR-S and TR-C) with the lowest E2 protein expression would potentially provide less efficient protection against a CHIKV infection. Although the release of the E2 protein into the cellular supernatant was higher in cells transfected with TR-CS-RNA, the E2 RNA amplification and total E2 protein expression were almost identical for TR-CS and TR-S *in vitro* (Figure 3C). Although infectious virus was not detected when the nr-replicase-RNA and the TR-CS-RNA were used together in *in vitro* experiments (Figure 1C), this cannot be entirely excluded *in vivo*. A previously described capsid-deleted CHIKV vaccine was severely attenuated *in vivo*.[22] Consequently, as an additional safety measure, the capsid-deleted TR-S-RNA in combination with the nr-replicase-RNA were tested as a potential CHIKV vaccine candidate.

In a dose-finding study, the vaccine candidate was injected intradermally into BALB/c mice as a homologous prime-boost vaccination on day 0 and day 28 (Figure 4A). Mice were immunized with either 5 μg or 1.25 μg nr-replicase-RNA and 1.25 μg, 0.25 μg, or 0.05 μg TR-S-RNA, both diluted in RNase-free PBS. As a negative control, mice received only PBS, and as a positive control, mice were infected intranasally with 10^6 PFUs CHIKV. Blood was collected for the analysis of humoral immune responses prior to the boost on day 28 and finally on day 56.

First, CHIKV-binding antibodies in mouse sera were measured by ELISA. CHIKV-specific immunoglobulin G (IgG) antibodies could be detected in all vaccinated groups, and antibody responses were boosted by the second immunization (Figure 4B; dilution curves can be found in Figure S3). Highest endpoint antibody titers were achieved with the highest RNA dose (5 μg nr-replicase-RNA and 1.25 μg TR-S-RNA), and these almost reached the antibody titers of CHIKV-infected mice (Figure 4B). In some animals, CHIKV-specific antibodies could be induced with as little as 0.05 μg TR-S-RNA; however, more robust antibody responses were seen with 1.25 μg and 0.25 μg TR-S-RNA. Using pseudotyped lentiviral vectors, CHIKV-neutralizing antibodies could also be measured in some vaccinated mice (Figure 4C; neutralization curves can be found in Figure S4).[23] However, in most mice, no significant CHIKV-neutralization capacity was observed. For further characterization of the induced CHIKV-specific antibodies, the IgG subtypes were determined on day 56 (Figure 4D). IgG2a and IgG2b, which can mediate protective Fc receptor functions, were predominantly found in mice vaccinated with 5 μg nr-replicase-RNA/1.25 μg TR-S-RNA. With lower TR-S-RNA doses, IgG1 responses were more dominant, although IgG2a and IgG2b antibodies could also be detected (Figure 4D). IgG3 antibodies were not observed in either vaccinated or infected mice (Figure 4D).

In addition to antibodies, CHIKV-specific T cell responses were analyzed in the vaccinated mice. Splenocytes were harvested on day 56, and interferon (IFN)-γ-secreting T cells reactive to CHIKV were re-stimulated and quantified by enzyme-linked immunosorbent spot (ELISpot). Therefore, splenocytes were first stimulated with CHIKV particles to analyze the total CHIKV-specific T cell responses. All vaccinated mice had profound CHIKV-reactive T cell titers, and these were significantly higher in mice vaccinated with 1.25 μg or 0.25 μg TR-S-RNA. The amount of nr-replicase-RNA did not significantly influence the T cell response (Figure 5). T cell titers of vaccinated mice were comparable to those of CHIKV-infected mice, and some vaccinated mice had even higher levels of CHIKV-reactive

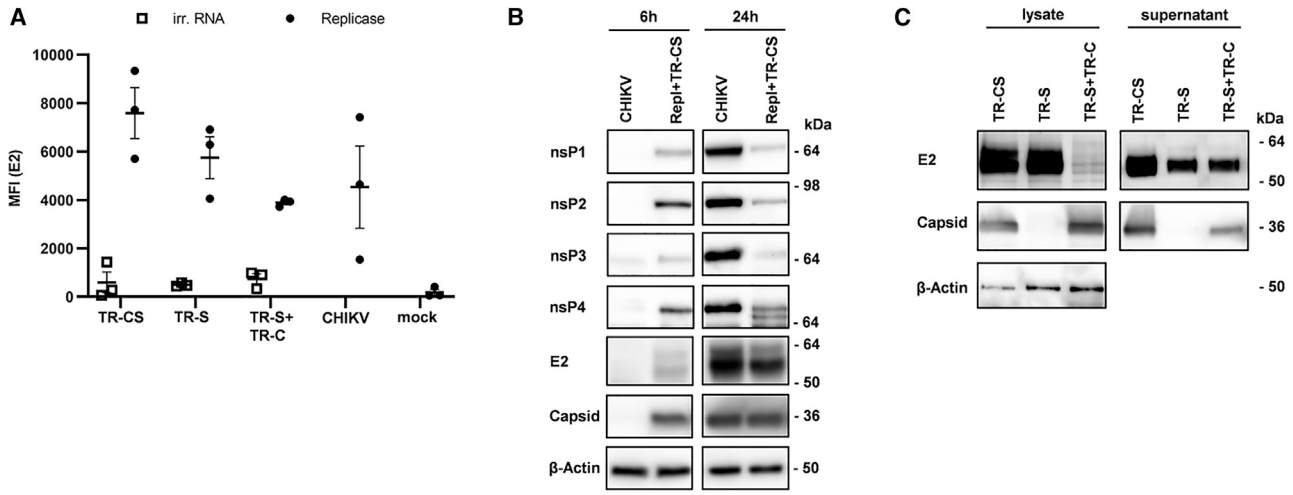

Figure 3. *In vitro* antigen expression of the taRNA constructs

(A) E2 protein expression on the cell surface was determined 16 h after RNA transfection or CHIKV infection (MOI 3) by flow cytometric analysis. Data are mean ± SEM of three independent experiments. As a control, irrelevant RNA (TR-luc) instead of the replicase RNA (nrRNA) was transfected. The mean fluorescent intensity (MFI) indicates the amount of protein on the cell surface. (B) Expression of the indicated proteins was determined in cellular lysates of cells transfected with nr-replicase-RNA and TR-CS-RNA or CHIKV-infected cells (MOI 3) after 6 h and 24 h. (C) E2 and capsid protein expression after transfection of the nr-replicase-RNA with the TR-RNA combinations of either TR-CS, TR-S alone, or with both TR-S and TR-C was determined in cellular lysates or concentrated supernatants 48 h after transfection. The depicted western blots are representative of three independent experiments.

T cells than infected mice (Figure 5). To further evaluate the induced T cell responses, the splenocytes were additionally stimulated with major histocompatibility complex class I (MHC class I)- or MHC-class-II-specific peptide pools containing only peptides from the nonstructural or only peptides from the structural proteins. As expected, CHIKV-specific T cells of vaccinated or infected mice preferentially reacted with MHC-I-specific replicase peptide pools and MHC II envelope peptide pools. Although the T cells of not all mice reacted to these peptide pools, this indicates the potential induction of cytotoxic T cells directed against the replicase and a humoral immune response against the envelope proteins (Figure S5).

The CHIKV taRNA vaccine candidate protects mice from a CHIKV infection

To evaluate whether the CHIKV-specific immune response elicited by the CHIKV vaccine candidate is also protective against a CHIKV infection, mice were immunized again by a prime-boost vaccination and were challenged intranasally with 10^6 PFUs CHIKV on day 56 after the first vaccination. Viral loads in serum were measured 2 and 4 days post-infection by qRT-PCR. Whereas viral loads of 10^4 viral genome copies per microliter were detected on day 2 in mock-vaccinated mice, almost no CHIKV RNA could be detected in vaccinated animals 2 days post-infection (Figure 6B). The reduction in viral titers was highly significant for all three tested RNA doses. On day 4 post-infection, CHIKV RNA was still detectable in mock-vaccinated mice (Figure 6C). Moreover, a low number of CHIKV genome copies was detected in two mice vaccinated with 5 μg nr-replicase-RNA and 0.25 μg TR-S-RNA on day 4 post-infection. In contrast, CHIKV RNA was completely absent in mice vaccinated with 1.25 μg nr-replicase-RNA and 0.25 μg TR-S-RNA, showing baseline values. Viral loads returned to baseline levels in all mice 14 days post-infection (Figure 6D). Importantly, the protective activity against CHIKV infection was in line with the induced CHIKV-specific antibody responses (Figures 6E, 6F, S6, and S7). For the induction of potent humoral immune responses, a 4:1 ratio of nr-replicase-RNA to TR-S-RNA was advantageous, producing higher total IgG titers and CHIKV-neutralization capacity. Moreover, a balanced IgG1 to IgG2a response was observed in protected mice (Figure 6G). Accordingly, the CHIKV taRNA vaccine candidate was able to induce a potent immune response, which was protective against viral infection with a prime-boost vaccination at a single dose of 1.5 μg total RNA.

DISCUSSION

CHIKV is of increasing public health concern after its rapid global spread following vector extension to *Aedes albopictus*.[1] It has caused massive outbreaks with debilitating arthralgia in infected patients, which can last for several years.[5] Thus, safe and potent vaccines are urgently needed. Here, we present the proof of principle for a CHIKV vaccine candidate based on taRNA. A taRNA vaccine candidate was generated that uses an nr-replicase-RNA and a TR-RNA encoding for the CHIKV envelope protein.

CHIKV taRNA systems have been described before mainly for basic virology studies, for example, to study alphavirus RNA replication mechanisms.[16,24] Here, a vaccine candidate was constructed with replicons that rely solely on the genome of CHIKV. Both the replicase and the amplified TR-RNAs contain homologous sequences; therefore, initial safety concerns had to be addressed. If the replicase and

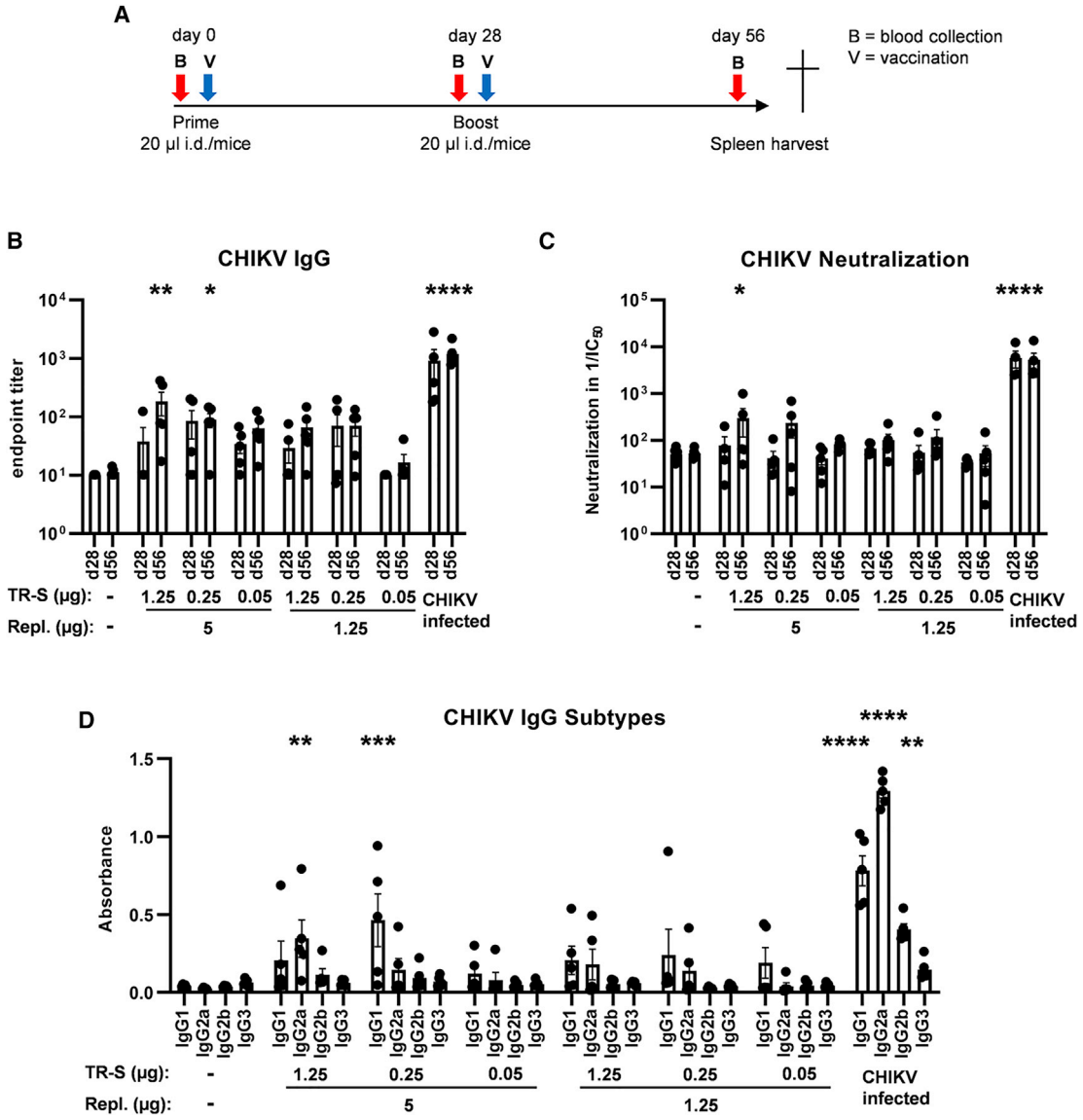

Figure 4. The CHIKV taRNA vaccine candidate induces potent CHIKV-specific antibody responses

(A) Graphic illustration of the vaccination scheme. Mice were immunized intradermally (i.d.) on day 0 and day 28. As negative control, mice received only PBS, and as positive control, mice were infected with 10^6 PFUs CHIKV. Blood was collected prior to the boost on day 28 and finally on day 56. (B) CHIKV-binding IgG antibodies in sera were determined by ELISA. Endpoint titers were calculated and are indicated. Samples, which were below the cutoff, were set to 10 for plotting and statistical analysis. The RNA amounts used for vaccination are indicated. (C) Neutralization of CHIKV-pseudotyped lentiviral vector particles by sera of immunized mice. Neutralization is represented by the reciprocal IC_{50} values. (D) CHIKV-specific IgG subtypes were determined by CHIKV-binding ELISA. For all assays, the means ± SEM of each group (n = 5) are depicted. For statistical analysis, a two-way ANOVA with Dunnett's multiple comparison post-test was performed (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

the structural proteins are both encoded on amplified RNAs, the continuous RNA amplification by the CHIKV replicase could lead to the recombination of the two RNAs into one full-length infectious viral RNA genome. Importantly, we could demonstrate that no infectious virus was generated when the replicase was encoded on an nrRNA, and the combination with TR-RNAs could be regarded as safe. As a second safety mechanism, we decided to use a capsid-deleted TR-RNA, which only encodes E3–E1. A CHIKV with a capsid deletion has been previously shown to be severely attenuated and was proposed as a potential live-attenuated vaccine candidate.[22]

The CHIKV taRNA vaccine candidate demonstrated efficient amplification of the TR-RNAs by the replicase, with a high increase in TR-RNA levels as early as 6 h after RNA transfection into HEK293T cells. As expected, the replicase was required for antigen expression. For the expression of proteins, which are under control of the subgenomic

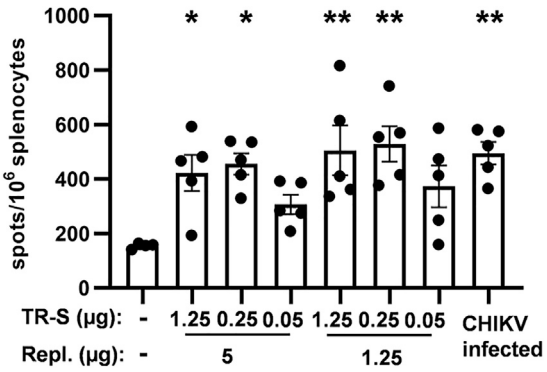

Figure 5. The CHIKV taRNA vaccine candidate induces a high frequency of CHIKV-reactive T cells

Mouse splenocytes isolated on day 56 after vaccination were re-stimulated with CHIKV, and IFN-γ-secreting T cells were quantified by ELISpot. The means ± SEM of each group (n = 5) are depicted. For statistical analysis, a one-way ANOVA with Dunnett's multiple comparison post-test was performed (*$p < 0.05$; **$p < 0.01$).

promotor, the respective virus replicase first has to synthesize sgRNA.[25] Importantly, the expression is transient, since the replicase RNA and replicase proteins are degraded. This gives another degree of safety to this vaccine approach, as no genetic material is integrated into target cells. Here, the levels of the replicase proteins nsP1–4 were already decreasing at 24 h after transfection. Interestingly, compared with a wild-type CHIKV infection, the taRNA system showed similar characteristics with a high RNA load and protein expression. In contrast, a slightly lower expression level compared with infection was described for a DNA-launched SFV-based taRNA system, further confirming the high antigen expression of the CHIKV taRNA system.[13] We also observed the release of the CHIKV E2 protein into the cellular supernatant. Previous studies have shown that the expression of alphavirus structural proteins leads to the assembly of virus-like particles.[26,27] Moreover, capsid-deleted alphaviruses can also build infectious microparticles, and these have a similar antigenicity to wild-type virus.[22,28]

The high antigen expression and particle release induced by the taRNA vaccine candidate could result in an efficient induction of a CHIKV-specific immune response. Indeed, intradermal RNA injection induced potent humoral and cellular immune responses in an *in vivo* mouse model, which were sufficient to protect against a CHIKV challenge infection. Since the taRNA vaccine candidate relies solely on components from the CHIKV genome, cellular immune responses could also be directed against the CHIKV replicase. This concept is in contrast to a typical mRNA vaccine, where only one antigen is used. Even if the overall T cell response was not influenced by the amount of replicase RNA, MHC-class-I-dependent replicase peptide pools were able to re-stimulate splenocytes from vaccinated mice. Although the role of T cells in CHIKV infection is still not fully understood, they most likely contribute to the protection against infection, since CHIKV is an intracellular pathogen.[29] Interestingly, some mice had only low IgG antibody titers but high T cell responses, and no correlation between antibody titers and T cell responses was found. Despite the induction of CHIKV-specific IgG antibodies, these were mainly non-neutralizing *in vitro*. However, protection against alphavirus infection can also be mediated by non-neutralizing antibodies of the IgG2a/c subtype.[30] Here, taRNA injection induced a balanced IgG1 and IgG2a response and the mice were protected against a CHIKV challenge infection. These data further highlight the problem of defining a clear correlate of protection for CHIKV vaccines.[31] Although neutralizing antibodies are the most straightforward measure, non-neutralizing antibodies and T cell responses can also mediate protection against CHIKV infection.

In addition to the total RNA dose, the ratio of replicase-RNA to antigen TR-RNA is also important for a robust taRNA vaccine. For the SFV-based taRNA vaccine candidate against influenza described recently, a combination of 20 µg replicase RNA plus 50 ng TR-RNA was superior compared with higher amounts of TR-RNA.[15] In contrast, the CHIKV taRNA vaccine candidate described here required only 1.25 µg replicase RNA and 250 ng TR-RNA to induce a protective immune response. Accordingly, the optimal dose and ratio for taRNA vaccines cannot be predicted and need to be determined individually for each vaccine candidate. Compared with the currently used COVID-19 mRNA vaccines, which use either 30 or 100 µg per dose in humans, the amount of replicon RNA is rather small.

Further optimization of the vaccine candidate is also possible. The usage of two separate RNAs for the vaccine antigen and the replicase allows the independent optimization of each RNA. Although we used unmodified RNA, nucleoside modification of nrRNA is, in principle, possible, but not of saRNA.[32,33] Importantly, such nucleoside modification of the nrRNA reduces innate immune responses, leading to a higher antigen expression, superior immune responses, and fewer unwanted side effects.[34] Moreover, formulation with, e.g., lipid nanoparticles might improve RNA stability and transmission, allowing standard intramuscular vaccine application.

This taRNA vaccine approach might also be suited for the induction of immune responses against multiple antigens. However, we observed a preferential amplification and expression of the capsid TR-RNA compared with the envelope TR-RNA when both RNAs were transfected into cells. This might be caused by the different lengths of the TR-RNAs. Such length dependency has been described before.[35] Longer TR-RNA templates amplified more slowly and also reached a lower total RNA level.[13] The capsid gene is only 0.78 kb in length, while the envelope gene is 2.8 kb. However, further study of the mechanisms of TR-RNA amplification by alphavirus replicases is required to enable the design of TR-RNAs that are equally amplified and expressed in a combined vaccine. In addition, taRNA vaccines can also be easily adapted if mutations appear in the pathogen.

Our study might be limited by the sole use of female mice, although sex differences in CHIKV infections have not been described. Immunocompetent mice do not develop clinical signs of CHIKV disease, and accordingly, we only measured protection by serum viral load.

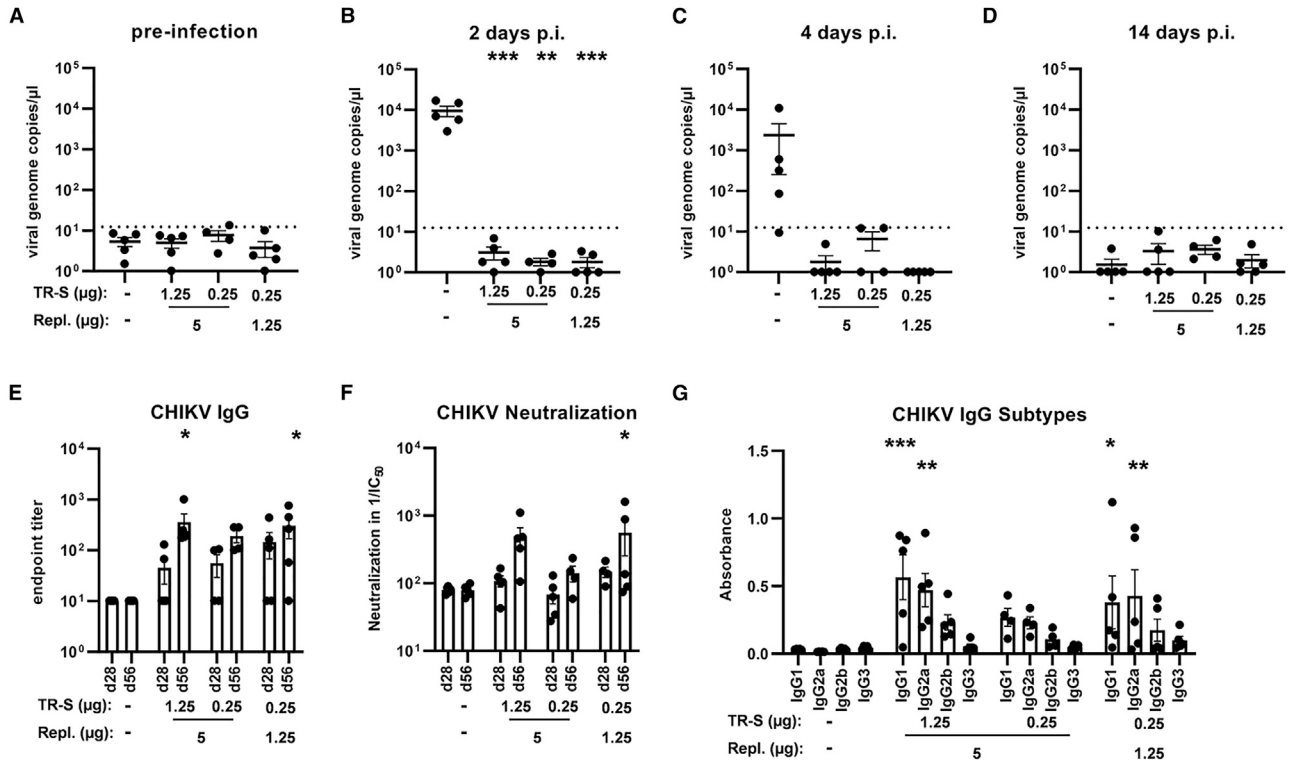

Figure 6. The CHIKV taRNA vaccine candidate protects against challenge infection *in vivo*

Mice were vaccinated on day 0 and day 28 and challenged on day 56 with 10^6 PFUs CHIKV. (A) CHIKV viral genome copies per microliter serum on day 56 pre-infection and (B) 2 days, (C) 4 days, and (D) 14 days post-infection (p.i.) measured by qRT-PCR are shown. The dotted line indicates the background level of the assay (mean pre-infection + two standard deviations). (E) CHIKV-binding IgG antibody endpoint titers of sera as determined by ELISA are shown. (F) CHIKV-neutralization capacity of sera represented as reciprocal IC_{50} values is shown. (G) CHIKV-specific IgG subtypes of antibodies are shown. For all assays, the means ± SEM of each group (n = 5) are depicted. For statistical analysis, a two-way or one-way ANOVA with Dunnett's multiple comparison post-test was performed (*p < 0.05; **p < 0.01; ***p < 0.001).

Furthermore, studies in non-human primates are needed to evaluate the immune responses in larger animals and to describe the mechanisms of protection against infection provided by this vaccine candidate.

In summary, a new CHIKV vaccine candidate based on taRNA was developed, using an nr-replicase-RNA together with a TR-RNA encoding the CHIKV envelope proteins. This vaccine approach demonstrated a high antigen expression with low amounts of RNA and induced potent humoral and cellular immune responses *in vivo* that protected mice against CHIKV infection. Accordingly, taRNA is a promising new vaccine concept to combat diseases of humans and animals caused by alphaviruses and maybe other RNA viruses.

MATERIALS AND METHODS

Cell culture

Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C. HEK293T (CRL-1573), HeLa (ATCC CCL-2), and Vero E6 cells (ATCC CRL-1586) were grown in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Verviers, Belgium) and BHK 21 (CCL-10) cells and isolated mouse splenocytes in Roswell Park Memorial Institute (RPMI) medium (Biowest, Nuaille, France). Media were supplemented with 10% fetal bovine serum (PAA, Pasching, Austria), 5% L-glutamine (200 mM; Lonza, Verviers, Belgium), and 1% penicillin/streptavidin (Fisher Scientific, Schwerte, Germany).

Mouse experiments

The 6- to 10-week-old mice (female, BALB/c_Rj) were purchased from Janvier (Saint-Berthevin Cedex, France). Mice were immunized on day 0 and day 28 by intradermal injection of 20 µL of the respective RNAs diluted in RNase-free PBS. On day 0 and day 28 before the immunizations, 50 µL blood was collected by retro-orbital bleeding. On day 56, mice were euthanized, final blood was collected, and splenocytes were harvested.

For challenge experiments, mice were immunized as described above and challenged on day 56 by intranasal infection with 10^6 PFUs CHIKV. Blood was collected prior to challenge and on days 2 and 4 post-infection by tail-vein punctuation. Mice were sacrificed 2 weeks post-infection.

Ethics statement

All animal experiments were performed in accordance with legal requirements (German protection of animals act and experimental

animal regulation) after approval of the Darmstadt regional council, Germany (animal license nos. F107/1062 and F107/2008; Regierungspräsidium Darmstadt, Germany).

RNA vectors and *in vitro* transcription

CHIKV sequences used in this work are based on the isolate LR2006 OPY1 (isolated from a French patient returning from Reunion Island during the 2006 outbreak)[36] and following numberings refer to the gene bank entry GenBank: DQ443544.2. A codon-optimized sequence was used only for the replicase gene encoded on nr-Repl, whereas all other sequences match the wild-type sequence.

Generation of plasmids TR-luc and TR-CS was previously described.[18] To generate TR-C, a TAA stop codon was inserted and the envelope ORF was deleted from TR-CS. Therefore, parts of TR-CS were amplified by two PCRs: first with primer pair 5′-GGCAGA CCCGCTAAAAAGGCTT-3′ and 5′-GCGGCCGCCTCGAGCTAT TACCACTCTTCGGCCCCCTCG-3′ and the second with 5′-TAAT AGCTCGAGGCGGCCGCTTGACAATTAAGTATGAAGG-3′ and 5′-TACTTCTCAAAGTGAGTTC-3′. TR-CS was opened via *Hind*III and *Nhe*I sites, and both PCR products were inserted by using Cold Fusion system (System Biosciences, Palo Alto, USA). The TR-C RNA comprises nucleotides 1–309, 5,682–8,349, and 11,314–11,840.

To generate the TR-S plasmid, the capsid ORF from TR-CS was deleted and start codon including Kozak-sequence (5′-GCCACCA TG-3′) was inserted upstream of the envelope ORF by PCR, using primer pairs 5′-AGCAAGTATCTAAACACTAATCAGCTACACT AGTGCCACCATGAGTCTTGCCATCCCAGTTA-3′ and 5′-TACT TCTCAAAGTGAGTTC-3′. The PCR product was inserted into TR-luc via *Spe*I and *Hind*III sites by using Cold Fusion system. The TR-S RNA comprises nucleotides 1–309, 5,682–7,566, and 8,350–11,840.

The sa-Repl RNA contains all viral CSE and UTR elements, and only the ORF of the structural genes was deleted from the CHIKV genome. The sa-Repl RNA comprises nucleotides 1–7,566 and 11,314–11,840 of CHIKV. To generate a plasmid bearing the sequence of CHIKV sa-Repl RNA encoding reporter genes EGFP and secNLuc separated by P2A site, first luciferase ORF from TR-luc was exchanged by GFP-GSG-P2A-secNLuc cassette via *Spe*I and *Xho*I sites to obtain TR-GFP-SNL. To restore the complete replicase ORF, a fragment between *BamH*I and *Age*I site from wild-type (WT) replicase plasmid was inserted into TR-GFP-SNL by ligation.

In contrast to sa-Repl RNA, the nr-Repl RNA contains only the ORF of the nonstructural proteins (the replicase) with codon-optimized sequences and no other CHIKV sequence. The replicase ORF was amplified from the plasmid pUC-57-Kan-T7-CHIKV-Rep encoding CHIKV replicase sequence optimized according to human codon usage, which was kindly provided by Andres Merits.[16] The SFV replicase ORF in the previously described plasmid nrRNA-REPL[15] was exchanged by codon-optimized CHIKV replicase ORF by using Cold Fusion system. Thereby, the CHIKV replicase ORF was flanked by alpha-globin 5′ UTR to ensure high translational activity and AES-mtRNR1 sequences as 3′ UTR to increase RNA stability as described previously.[17]

Prior to *in vitro* transcription, template plasmids were linearized using the type IIS restriction enzyme SapI, which generates an unmasked poly (A) tail. Synthesis and purification of RNA were previously described.[18]

Virus

The CHIKV of the La Réunion strain (GenBank: DQ443544.2) was a kind gift from Matthias Niedrig (Robert-Koch-Institut, Berlin, Germany).[37] Viral titers (in PFUs/mL) were determined by plaque titration on Vero E6 cells.

RNA transfection

At 24 h after seeding, cells were transfected with the respective RNAs using Lipofectamine MessengerMAX in accordance with the manufacturer's protocol (Thermo Fisher Scientific, Darmstadt, Germany). Replicase and TR-RNAs were transfected at a ratio of 4:1.

Luciferase assay for detection of infectious CHIKV

HEK293T cells were transfected in 6-well plates with 2.5 μg of the respective RNA combinations. After 48 h incubation at 37°C, 100 μL of cell supernatants were passaged onto new HEK293T cells, and supernatants thereof were collected again after a further 48 h. Passaging was repeated twice.

Supernatants were evaluated for the presence of infectious CHIKV with a luciferase assay, as previously described.[18] Briefly, 100 μL of supernatant was added to TR-RNA-luc-transfected cells in 96-well plates. In TR-RNA-luc, luciferase expression is controlled by the subgenomic promoter and requires the presence of nsPs (replication-competent CHIKV) in the cells. After 16 h incubation, 50 μL BriteLite substrate (PerkinElmer, Rodgau, Germany) was added to each well, and the luciferase signal was detected as counts/s with a Tecan Spark reader (Tecan, Männedorf, Switzerland).

qRT-PCR

To evaluate TR-RNA amplification, RNA levels were quantified by qRT-PCR. HEK293T cells were transfected with the indicated RNA combinations or infected with CHIKV (MOI 3). After 6 h and 16 h, cells were harvested and RNA was extracted using the Direct-zol RNA Miniprep Kit (Biozol, Eching, Germany) according to the manufacturer's instructions. Specific primers and fam-labeled probes for CHIKV E2 RNA (CHIKV E2 fw 5′-CAT GCT ACT GTA TCC TGA CCA C-3′; CHIKV E2 rev 5′-ATG GGC TGT ACC GTT TG TAG-3′; CHIKV E2 probe 5′-TGC TAA CCG TGC CGA CTG AAG G-3′) and CHIKV capsid RNA (CHIKV capsid fw 5′-GGG ACA AAG TAA TGA AAC CAG C-3′; CHIKV capsid rev 5′-TTA GAT GAC CGC TTA AAG GCC-3′; CHIKV capsid probe 5′-CAG GTC CGC GTT ATC GAT GGT CC-3′) were used. RNA levels were normalized to GAPDH levels (PrimePCR Probe Assay: GAPDH, Human; Bio-Rad, Munich, Germany). The qRT-PCR

analysis was performed using a CFX96 Real-Time PCR detection system (Bio-Rad, Munich, Germany).

Flow cytometric analysis

To assess E2 protein expression by flow cytometry, HEK293T cells were seeded into 6-well plates, transfected with 2 μg replicase RNA plus 0.5 μg TR-RNA, or infected with CHIKV (MOI 3). After 16 h incubation at 37°C, E2 proteins on the cellular surface were stained with the monoclonal antibody CHK-265 (1 μg/mL; kind gift of Michael Diamond, Washington University, USA)[38] and anti-mouse IgG secondary AF555-coupled antibodies (1:200; Cell Signaling Technology, Frankfurt am Main, Germany; no. 4409S). Paraformaldehyde-fixed cells were then evaluated with the BD LSRFortessa Cell Analyzer (BD Biosciences, Heidelberg, Germany) and BD FACSDiva software.

Western blot analysis

Protein expression was evaluated by western blot analysis at indicated time points after RNA transfection. Cell lysates were prepared or supernatants were collected and concentrated by ultracentrifugation at 25,000 rpm for 2 h. Protein concentrations were determined with the Pierce BCA Protein-Assay Kit (Thermo Fisher Scientific, Schwerte, Germany). For western blot analyses, 20 μg protein was loaded per slot onto a gel and separated by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes with a Bio-Rad semidry blotter. Membranes were blocked with Roti-Block (Carl Roth, Karlsruhe, Germany), and proteins were then detected with primary antibodies directed against nsP1–4 (kind gift of Andre Merits, University of Tartu, Estonia),[16] CHIKV E2 (Eurogentec, Cologne, Germany; custom made), capsid (Fitzgerald, Acton, MA, USA; no. 10–1438), β-actin (Sigma, Munich, Germany; no. A5441), and appropriate secondary horseradish peroxidase (HRP)-coupled antibodies. Detection was performed with the ECL detection system (Amersham, Freiburg, Germany) and Fusion FX7 (Vilber, Eberhardzell, Germany).

Immunostaining

HeLa cells were transfected with RNA or infected with CHIKV (MOI 3). After 24 h incubation at 37°C, cells were fixed, permeabilized with 0.5% Triton X-100 in PBS, and stained with primary antibodies directed against CHIKV E2 (Eurogentec, Cologne, Germany; custom made) or dsRNA (Scicons, Budapest, Hungary; no. 10010200). Cell nuclei were stained with DAPI (1:5,000; Sigma-Aldrich Chemie, Taufkirchen, Germany). Cells were analyzed with an ApoTome microscope (Zeiss, Jena, Germany), and representative images were acquired with 64× magnification objectives.

CHIKV ELISA

ELISA Nunc-Immuno 96-well MaxiSorp ELISA plates (Thermo Fisher Scientific, Darmstadt, Germany) were coated with 10^6 PFUs CHIKV per well in 100 μL PBS at 4°C overnight. After blocking for 1 h with 1% BSA in PBS, serum samples were serially diluted (1:50, 1:250, 1:1,000, and 1:5,000) for IgG endpoint titer determination, or for isotype determination, serum samples were diluted 1:100 in PBS containing 0.05% Tween 20 and 1% BSA, and incubated for 1 h at 37°C. The following anti-mouse secondary HRP-coupled antibodies were then added for 1 h at room temperature: rabbit anti-mouse total IgG (1:2,000; dianova, Hamburg, Germany; no. 115-035-003), goat-anti-mouse IgG1 (1:5,000; Abcam, Cambridge, UK; no. ab97240), goat-anti-mouse IgG2a (1:5,000; Abcam; no. ab97245), goat-anti-mouse IgG2b (1:5,000; Abcam; no. ab97250), or goat-anti-mouse IgG3 (1:5,000; Abcam; no. ab97260). For detection, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Merck Millipore, Darmstadt, Germany) was added, and the reaction was stopped after 15 min with 1 M H_2SO_4. Absorbance was measured at 450 nm (reference wavelength 620 nm) with a Tecan spark reader (Tecan, Männedorf, Switzerland).

CHIKV neutralization assay with pseudotyped lentiviral vector particles

Neutralization assays were performed as described previously.[39] Briefly, 6,000 HEK293T cells were seeded in a volume of 20 μL DMEM in white CELLSTAR 384-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany). After 24 h incubation at 37°C, CHIKV vector particles in 10 μL DMEM plus 10 μL heat-inactivated mouse serum (dilution 1:60 to 1:14,580) were added to the cells. After 20 h of incubation, luciferase activity was detected.

ELISpot

For the evaluation of CHIKV-reactive T cells, murine IFN-γ ELISpot assays were performed. The mouse IFN-γ ELISpot Kit (BD Biosciences, Franklin Lakes, NJ, USA) and HRP streptavidin (BD Biosciences) were used in accordance with the manufacturer's protocol. Samples of 10^6 splenocytes were cultured with different stimuli on multiscreen immunoprecipitation (IP) ELISpot PVDF 96-well plates (Merck Millipore, Darmstadt, Germany) for 24 h. As stimuli, virus (CHIKV or the vaccinia virus modified Vaccinia virus Ankara [MVA] as control) at an MOI of 5 was used. In addition, CHIKV MHC-class-I- or MHC-class-II-specific peptide pools (10 μg/mL) containing either replicase- or envelope-specific peptides were used. For spot development, 100 μL TMB substrate for ELISpot (Mabtech, Stockholm, Sweden) was added to each well. Spots were counted using an Eli.Scan ELISpot scanner (AE.L.VIS, Hamburg, Germany) and the ELISpot analysis software Eli.Analyse v.5.0 (AE.L.VIS).

Detection of viremia in CHIKV-infected mice

For detection of viremia in serum samples, RNA was extracted from mouse serum using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Virus was detected with the Realstar Chikungunya RT-PCR Kit 2.0 (Altona Diagnostics, Hamburg, Germany) as viral genome copies/μL. The background was defined by analysis of the serum samples pre-infection (day 56) and set to the mean value + two standard deviations. The readout was performed using a CFX96 Real-Time PCR detection system (Bio-Rad, Feldkirchen, Germany).

Statistical analysis

Mean values and standard deviations were calculated with Excel. ELISA IgG data of serum dilutions were analyzed by setting a cutoff

value based on the optical density (OD) values from naive control sera (average at lowest dilution + two standard deviations). By using the GraphPad Prism 7.04 software (La Jolla, CA, USA) a four-parameter sigmoidal regression curve was employed to interpolate the endpoint titers. Samples, which were below the cutoff, were set to 10 for plotting and statistical analysis. Half-maximal inhibitory concentration (IC_{50}) values, representing the dilution factor of the serum sample to obtain 50% inhibition of pseudotyped vector transduction, were determined using the GraphPad Prism 7.04 software as nonlinear regression (log(inhibitor) versus response [three parameters, constrain equal to 0]). Data were analyzed using one-way or two-way analysis of variance (ANOVA) with appropriate post-tests, as indicated in the figure legends, using GraphPad Prism 7.04 software.

Artwork

The graphical abstract was performed with the Servier Medical Art software (https://smart.servier.com/).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.04.036.

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AUTHOR CONTRIBUTIONS

Conceptualization, C.S., T.B., M.P., U.S., and B.S.S.; methodology, C.S., E.H., J.G., and M.P.; validation, C.S., E.H., M.P., and B.S.S.; formal analysis, C.S. and E.H.; investigation, C.S., T.B., U.S., and M.P.; data curation, C.S., E.H., and J.G.; writing – original draft preparation, C.S. and B.S.S.; writing – review & editing, C.S., E.H., T.B., M.P., and B.S.S.; supervision, M.P. and B.S.S.; project administration, B.S.S.; funding acquisition, B.S.S.

DECLARATION OF INTERESTS

U.S., T.B., and M.P. are inventors on patents and patent applications that cover parts of this article. U.S. is an employee at BioNTech Corporation (Mainz, Germany), a privately owned company developing therapeutic RNA. All others declare no competing interests.

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