nature biomedical engineering

Article

https://doi.org/10.1038/s41551-026-01738-z

Chemically synthesized, non-capped and non-polyadenylated peptide-coding RNA efficiently induces antigen-specific CD8$^+$ T cells

Received: 16 February 2025

Accepted: 2 June 2026

Published online: 07 July 2026

Check for updates

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Recombinant in vitro-transcribed mRNA is broadly used for vaccination and is evaluated in numerous clinical studies for multiple indications. Typical features of mRNA are the 5′ cap, 5′ untranslated region, start and stop codons, 3′ untranslated region and 3′ poly(A) tail. Here, contrary to current dogma, we show that short, chemically synthesized RNA oligonucleotides lacking some or all of these features are efficiently translated when they encode epitopes recognized by CD8$^+$ T cells. In particular, one design that we termed ChemRNA with the structure 5′-OH-AUG-coding sequence-3′-OH strongly stimulates antigen-specific CD8$^+$ T cells both in vitro and in vivo. Our results challenge the current understanding of canonical mRNA structure and introduce the possibility that defective or supposedly non-coding RNA may encode human and murine major histocompatibility complex class I-associated peptides. Moreover, ChemRNA could help overcome challenges associated with the design and purification of individualized anti-cancer vaccines.

Several approved and emerging medicines are based on the utilization of in vitro-transcribed (ivt) mRNA as a molecule for vaccination (via the induction of an immune response against the encoded protein), protein replacement (for example, immunosilent expression of a therapeutic protein) and genome editing[1,2]. Such synthetic mRNAs are produced in vitro by enzymatic processes: typically, a linear DNA template is transcribed into RNA by an RNA polymerase, then the DNA is degraded by a DNase. The 5′ cap and 3′ poly(A) tail can be generated cotranscriptionally using cap analogues and an A stretch in the DNA matrix or post-transcriptionally, using capping enzymes and poly(A) polymerases[3,4]. The enzymatic production of mRNA is efficient, scalable and robust. However, one of its major shortcomings is that the production process is indirect, as it requires generation of a synthetic DNA plasmid or PCR product that is then transcribed into RNA and is therefore relatively laborious. Nevertheless, dedicated production plants, the first of which was certified as early as 2006[1], can deliver large

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amounts of pharmaceutical-grade ivt mRNA, which is currently being used to generate billions of doses of vaccines against coronavirus disease 2019 (COVID-19)[5,6]. However, for purposes such as individualized anti-cancer vaccines aimed at the stimulation of neoantigen-specific T cells, the generation of ivt mRNA is relatively cumbersome. Indeed, only small amounts of several mRNAs are needed for each patient. A solution to this challenge has been to generate ivt mRNA coding multiple neoantigen epitopes in which antigenic peptides are separated by linkers[7,8]. Each patient with cancer who is treated with this approach receives one or more of such polyepitope ivt mRNAs that are fully customized to the patient's mutanome, that is, to mutations identified in autologous cancer cells. Still, for each patient, DNA constructs must be designed, synthesized and transcribed before the mRNA is purified. Collectively, these steps pose a critical bottleneck to the rapid scale-up and broad implementation of individualized anti-cancer mRNA vaccines[9,10]. Thus, we evaluated whether fully chemically synthesized RNA oligonucleotides could be used to encode $CD8^+$ T cell-recognized epitopes.

Results

Oligonucleotides encoding the H-2K^b SIINFEKL epitope efficiently stimulate antigen-specific T cells in vitro

The 5′ untranslated regions (UTRs) in ivt mRNA frequently consist of over 40 residues corresponding to, for example, globin 5′ UTRs or to other selected sequences (that is, an eIF4G-binding aptamer[11]) and preceding the start codon in a Kozak sequence context. However, another translation initiation element distinct from Kozak has been identified, characterized by a very short 5′ UTR and termed translation initiator of short 5′ UTR (TISU)[12]. It can be as short as four nucleotides: for example, CAAG preceding the start codon AUG. We found that ivt mRNA with such a TISU 5′ UTR is well translated[13]. Thus, TISU can replace the longer 5′ UTRs usually used to produce recombinant ivt mRNA. Concerning the 3′ UTR, we have previously shown that the poly(A) tail can be added directly after the 'ochre' stop codon, producing an ivt mRNA devoid of a 3′ UTR but still highly functional[14]. This design of the TISU 5′ UTR and an absent 3′ UTR was adopted for the production of chemically synthesized mRNA.

A 38-residue RNA oligonucleotide (sequence in Supplementary Table 1) containing the TISU 5′ UTR and a sequence encoding MESIINFEKL (the SIINFEKL epitope preceded by a start codon encoding methionine (M) and a glutamate residue (E) that is present in the natural epitope produced from ovalbumin (OVA) by proteasomes and is recognized by SIINFEKL-specific $CD8^+$ T cells restricted by H2-K^b, commonly referred to as OT-I T cells[15]) was chemically synthesized and chemically capped[16] (SIINFEKL cap 5′ UTR in Fig. 1a). As a 3′ poly(A) tail is known to be required for stability and translation of eukaryotic mRNA[17], we extended this capped oligonucleotide enzymatically with a poly(A) sequence using a recombinant poly(A) polymerase (SIINFEKL cap 5′ UTR poly(A) in Fig. 1a). Surprisingly, however, in vitro lipofection of mouse splenocytes (an immunomonitoring method that we previously optimized[18]) with the non-polyadenylated and with the polyadenylated capped oligonucleotide encoding MESIINFEKL stimulated OT-1 T cells to a similar level (Fig. 1b). At all tested RNA concentrations (in μg ml^{-1}), chemically synthesized mRNA without a poly(A) tail equalled the performance of poly(A)-containing RNA and performed better than the ivt mRNA encoding the full-length OVA protein (OVA ivt mRNA sequence in Supplementary Table 2). Having established that the 3′ poly(A) tail is not needed in oligonucleotides encoding MESIINFEKL, we checked whether the 5′ cap was required. Again, most surprisingly, lipofected RNA oligonucleotides with a TISU 5′ UTR and a sequence encoding MESIINFEKL were equally capable of stimulating OT-1 cells whether they had a 5′ cap, a 5′-triphosphate, a 5′-monophosphate or even a 5′-OH (Fig. 1c).

We further tested uncapped RNA coding for MSIINFEKL (hereafter called SIINFEKL ChemRNA) without a 5′ TISU UTR and without a 3′ stop codon and found that this sequence design, which we termed ChemRNA and is only 27 nucleotides in length, still stimulates OT-1 cells efficiently (SIINFEKL ChemRNA in Fig. 1a,c). An oligonucleotide missing the AUG start codon in front of the sequence coding for SIINFEKL was still capable of OT-1 cell stimulation, albeit less efficiently than an oligonucleotide retaining the start codon (Fig. 1c). This and the fact that a deoxyribose oligonucleotide encoding MSIINFEKL (sequence in Supplementary Table 1) did not stimulate OT-1 T cells (Fig. 1d) indicate that the production of the peptide from such minimal chemically synthesized mRNA is performed by ribosomes and not by a so-far-undiscovered alternative translation machinery. For the SIINFEKL epitope, a codon-optimized ChemRNA sequence did not enhance functionality, probably due to the wild-type sequence's lack of very rare codons, reducing the potential for codon-optimization improvements (Fig. 1d). A sequence similar to SIINFEKL ChemRNA but produced by in vitro transcription (minSIINFEKL ivt mRNA no cap; sequence in Supplementary Table 1) performed as well as SIINFEKL ChemRNA (Fig. 1e). An optimized capped and polyadenylated ivt mRNA encoding SIINFEKL flanked with sequences that optimize antigen processing[19] (SIINFEKL ivt mRNA; sequence in Supplementary Table 2) gave T cell stimulation similar to the simple chemically produced 27-base ChemRNA (Fig. 1e). Comparing the efficacy of all these synthetic mRNAs encoding SIINFEKL with a T cell readout shows that they perform similarly. Lower stimulation by ivt mRNA encoding full-length OVA can be attributed to the larger size of this mRNA (1.5 kb), resulting in a lower total number of molecules in the experiment when compared with ivt or chemically synthesized mRNAs encoding only the SIINFEKL sequence. Indeed, using equimolar amounts of RNA, we saw similar or slightly lower stimulation of SIINFEKL-specific T cells with ivt mRNA coding for OVA and the different RNA oligonucleotides encoding SIINFEKL (Extended Data Fig. 1a). The SIINFEKL-K^b complexes were also directly detected at the surface of the dendritic cell (DC) line DC2.4 transfected with SIINFEKL ChemRNA using the 25D1.16 antibody, which is specific for the SIINFKEL-H2-K^b complex (Extended Data Fig. 1b,c), although less markedly than with ivt mRNA. Due to limited assay sensitivity, at least 1 μg ml^{-1} of ChemRNA was required to detect the staining. In equimolar amounts, this corresponds to 30 μg ml^{-1} of ivt mRNA, making a comparative equimolar experiment not technically feasible due to liposome toxicity. Translational frameshifting and AUG skipping were tested using both ivt and ChemRNA (sequences in Supplementary Tables 1 and 2). Both formats resulted in similar amounts of residual epitope production when the start codon was absent or when a frameshift occurred after the start codon (Extended Data Fig. 1d). This indicates that ChemRNA has a translational fidelity similar to that of ivt mRNA.

To deepen our understanding of ChemRNA translation, we explored various modifications. Elongating the ChemRNA on both sides of the coding sequence of the epitope (encoding extended epitopes QLESIINFEKLTEWT and LEQLESIINFEKLTEWTSS using oligonucleotides of 48 and 60 nucleotides in length, respectively; sequences in Supplementary Table 1) did not affect recognition by OT-1 cells (Fig. 1f). Replacing OT-1 splenocytes with the T cell hybridoma B3Z (expressing the T cell receptor (TCR) that recognizes SIINFEKL in H2-K^b and expressing β-galactosidase upon TCR engagement) as a readout allowed us to test translation of ChemRNA in selected cells instead of in total splenocytes from OT-1 mice. Measuring β-galactosidase activity in B3Z cells co-cultured with transfected splenocytes or DC2.4 cells recapitulated the results obtained with OT-1 cells (Extended Data Fig. 1e–g). However, when ChemRNA was transfected in tumour cells, interferon (IFN; α or γ) treatment was necessary for efficient presentation of SIINFEKL (Extended Data Fig. 1h). Thus, translation and epitope presentation from ChemRNA requires an immune environment (immune cells or non-immune cells stimulated with IFN). In the B3Z system, a wide range of base or sugar modifications were tested (Fig. 1g,h). They did not enhance recognition of transfected

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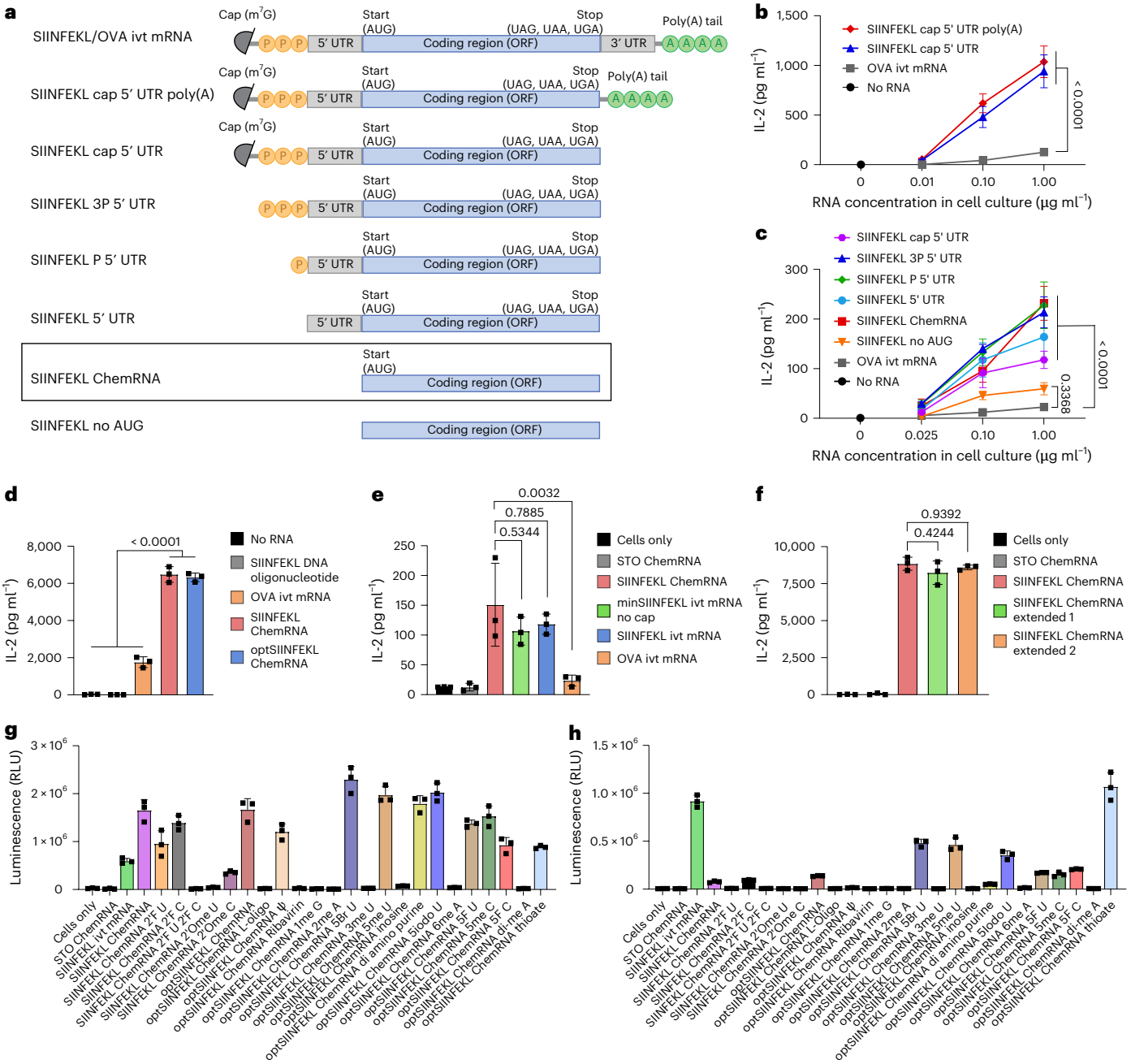


Fig. 1 | Design and testing of ChemRNA encoding the H2-K^b SIINFEKL epitope. **a**, Schematic depicting the design of chemically produced minimal RNAs. **b**–**f**, Stimulation of OT-1 T cells by lipofection of different chemically produced mRNA designs encoding SIINFEKL. Either 100,000 B16-F10 cells (**b**–**d**) or 1,000,000 C57BL/6 splenocytes (**e**,**f**) were seeded with 100,000 OT-1 splenocytes and cotransfected with different concentrations of RNA in culture medium (range, 0–1 μg ml^{-1}). At 24 (**b**,**c**,**f**) or 72 hours (**e**) after transfection, cell culture supernatants were subjected to IL-2 ELISA. Values below the LOD were substituted with LOD ÷ $\sqrt{2}$ before statistical analysis. **b**,**c**, Effect of 3′ poly(A) tail removal (**b**) or 5′ cap, 5′ UTR, 3′ stop codon and AUG start codon removal (**c**). **d**, Comparison of a DNA oligonucleotide, normal ChemRNA and codon-optimized ChemRNA encoding SIINFEKL (1 μg ml^{-1} RNA in culture medium). **e**, Comparison of uncapped ivt mRNA with a similar sequence to ChemRNA and ivt mRNA with an optimized sequence to ChemRNA. **f**, Testing of extended SIINFEKL ChemRNA sequences. **g**,**h**, Testing of different ChemRNA modifications in C57BL/6 splenocytes (**g**) or B16-F10 cells (**h**). Either 100,000 B16-F10 cells (**h**) or C57BL/6 splenocytes (**g**) were mixed with an equal number of B3Z cells and transfected with 1 μg ml^{-1} of ChemRNA encoding SIINFEKL. After 24 hours, as a readout for T cell activation, β-galactosidase was detected with the Invitrogen Galacto-Light Plus β-Galactosidase Reporter Gene Assay System (Thermo Fisher Scientific). In **b**–**f**, $n = 3$ samples for each group. Data are representative of three independent experiments and are presented as mean ± s.d. Two-way (**b**,**c**) or one-way (**d**–**f**) ANOVA with Tukey's multiple comparison test was used to determine the significance between the different groups (P values indicated). m^{7}G, 7-methylguanosine; optSIINFEKL, optimized SIINFEKL; P, phosphate; RLU, relative light unit.

cells by B3Z; furthermore, several of them diminished or abrogated (2′-deoxy, L-ribose enantiomers, ribavirin, 1-methylguanosine, 2-methyladenosine, 3-methyluridine, inosine, 6-methyladenosine and dimethyladenosine) the translation of ChemRNA in splenocytes (Fig. 1g). It was further shown with this method that in the tumour cell line B16-F10, canonical (capped and polyadenylated) ivt mRNA encoding full-length OVA or SIINFEKL is superior to ChemRNA (Fig. 1h and Extended Data Fig. 2a). The cap and poly(A) tail are

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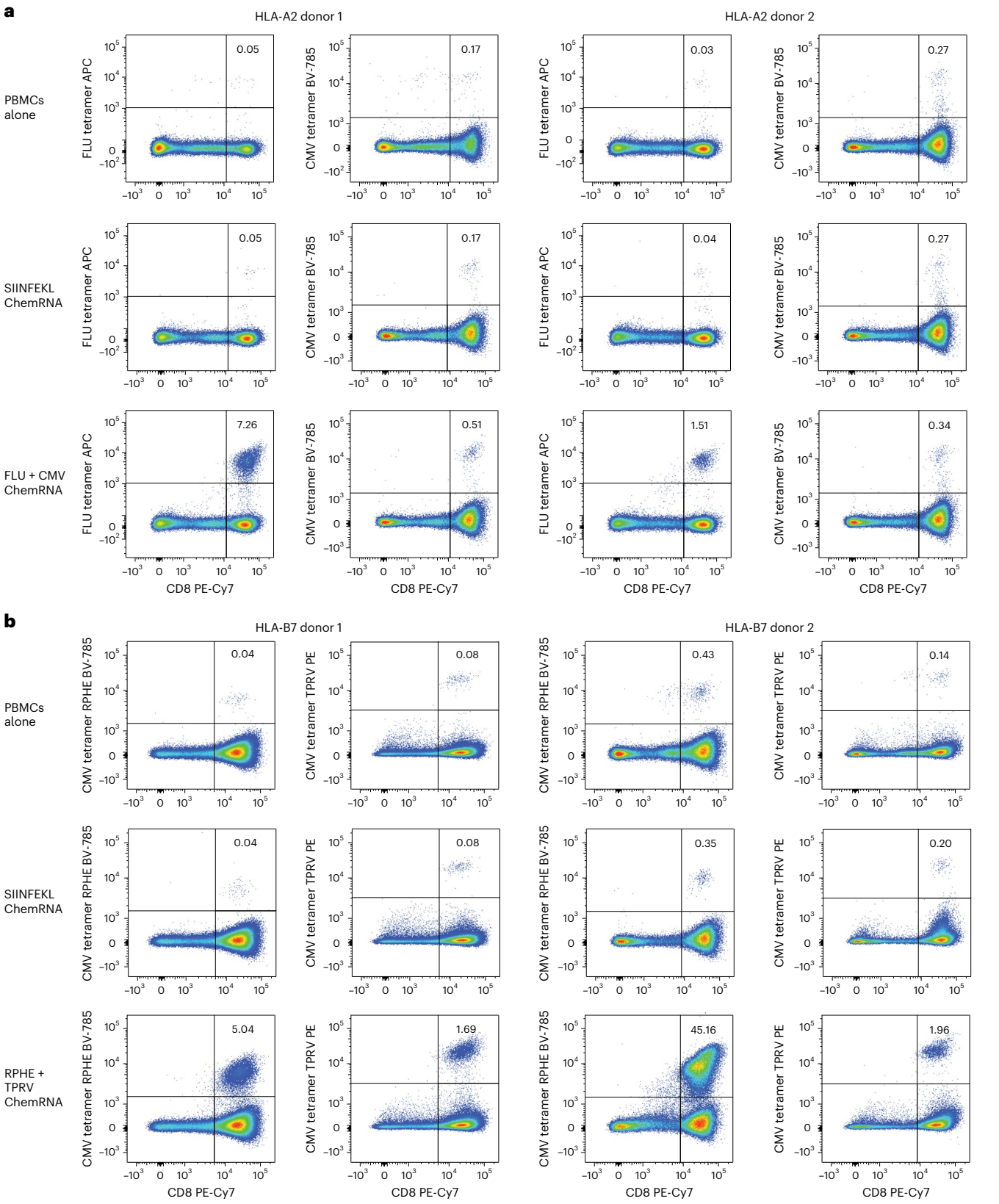


Fig. 2 | ChemRNA stimulates virus-specific T cells in vitro. a,**b**, Stimulation of antigen-specific T cells induced by lipofection of ChemRNA coding for HLA-A2-binding (**a**) or HLA-B7-binding (**b**) human viral epitopes. Human PBMCs from HLA-A2-positive (**a**) or HLA-B7-positive (**b**) donors were cultured for 10 days in the presence of 0.2 μg ml^{-1} ChemRNA coding for either influenza (FLU) matrix or CMV pp65 epitopes before flow cytometry staining for tetramer and subsequent analysis. Gating strategy: time (time versus FSC-A), singlets (FSC-A versus FSC-H), living cells (Zombie Aqua versus FSC-A), lymphocytes (FSC-A versus SSC-A), CD4$^-$ (CD4 versus CD8). The numbers in the top right quadrants show the percentages of tetramer$^+$ CD8$^+$ T cells. RPHE, RPHERNGFTVL; TPRV, TPRVTGGGAM.

required for ivt mRNA encoding full-length OVA to induce T cells in vitro (Extended Data Fig. 2a) and in vivo (Extended Data Fig. 2b,c) and antibody responses (Extended Data Fig. 2d). This is in agreement with current knowledge but different from what we observed for T cell stimulation with ChemRNA (uncapped and non-polyadenylated) in immune cells. Only stabilization of ChemRNA with a fully phosphorothioate backbone gave strong presentation of SIINFEKL in the context of B16-F10. Incorporation of phosphorothioate nucleotides by RNA polymerases is generally inefficient, yielding mRNAs that are poorly translated in both bacterial[20] and mammalian[21] systems. Of note, such ivt mRNAs contain the R_p enantiomer at each phosphorothioate linkage. Conversely, chemically synthesized RNA oligonucleotides contain a racemic mixture, featuring 50% R_p and 50% S_p enantiomers at each position. This difference in stereochemistry probably explains the better translational efficiency observed with the chemically synthesized phosphorothioate RNA oligonucleotides, with the S_p conformation potentially being more permissive to ribosomal activity. 5-Bromouridine, 5-methyluridine, 5-iodouridine and 5-fluorocytidine also allowed enhanced expression of SIINFEKL compared with unmodified ChemRNA in B16-F10 cells (Fig. 1h). Furthermore, we used the HiBiT system to determine whether ChemRNA could encode a functional free peptide. HiBiT is an 11-amino-acid peptide complementing Large BiT (LgBiT), the large subunit used in NanoLuc Binary Technology (NanoBiT; Promega)[22] (Extended Data Fig. 3a). In DC2.4 cells, both ChemRNA and capped, polyadenylated ivt mRNA resulted in similar amounts of HiBiT peptide expression as measured by luminescence (Extended Data Fig. 3b). In transfected B16-F10 cells, ChemRNA is inefficiently translated (Extended Data Fig. 3c) compared with capped polyadenylated ivt mRNA, as also reported in another study[23] that used chemically capped and synthesized mRNA transfected in HeLa cells. Again, a phosphorothioate backbone strongly increases expression of HiBiT from ChemRNA. We studied the intracellular stability of ChemRNA and it appears that using quantitative RT-PCR (RT-qPCR), ivt mRNA and ChemRNA could have similar intracellular stability (Extended Data Fig. 4a). However, using the indirect peptide presentation readout, it was evident that capped and polyadenylated ivt mRNA has a longer half-life than ChemRNA, unless ChemRNA is fully phosphorothioate-modified (Extended Data Fig. 4b). With quantitative RT-qPCR incomplete (partially degraded), ChemRNA may be detected, possibly accounting for the difference between the two readouts. Phosphorothioate ChemRNA is more abundant in the cytoplasm than ivt mRNA and unmodified ChemRNA in the first 3 days, possibly indicating resistance towards RNases involved in early degradation of exogenous RNA. Thus, higher stability and increased peptide expression from phosphorothioate ChemRNA probably accounts for the enhanced presentation of SIINFEKL in non-immune cells that have a weaker major histocompatibility complex (MHC) class I presentation machinery compared with immune cells or cells activated with IFNs. As with ivt mRNA, the presentation of MHC class I epitopes from ChemRNA is dependent on transporter associated with antigen processing (TAP) and the expression of HiBiT from ChemRNA is inhibited by ribosome inhibitors (Extended Data Fig. 5). Thus, from a mechanical point of view, the epitopes produced from ChemRNA and ivt mRNA meet the same requirements. On the basis of these results, it can be proposed that immune cells have a specialized ribosomal translation machinery (such as the hypothetical immunoribosome, an altered translation apparatus that could generate antigenic peptides from non-canonical open reading frames[24]) and the capacity to load peptides onto MHC class I molecules from non-classical mRNAs that may arise from infectious agents and may subsequently be modified or lack the features (5′ cap and 3′ poly(A) tail) required for normal translation in mammalian cells. With our investigations (using available inhibitors of translation initiation or elongation), however, we have so far been unable to detect whether specialized ribosomal subunits are involved in the translation of ChemRNA in immune cells.

ChemRNA encoding HLA-A2- and HLA-B7-binding human viral epitopes leads to the proliferation of antigen-specific CD8$^+$ T cells in vitro

On the basis of the model sequence of SIINFEKL ChemRNA (that is, 5′-OH, start codon followed by the coding sequence ending on the last codon, not on a stop codon), we tested ChemRNAs encoding human viral epitopes presented by HLA-A2 or HLA-B7. To this end, peripheral blood mononuclear cells (PBMCs) from pre-screened healthy HLA-A2- or HLA-B7-positive donors were cultured for 10 days in the presence of 0.2 μg ml^{-1} of RNA formulated in Lipofectamine MessengerMAX[18]. ChemRNAs encoding the HLA-A2 epitopes GILGFVFTL and NLVPMVATV from influenza matrix protein and cytomegalovirus (CMV) pp65, respectively, or the HLA-B7 epitopes TPRVTGGGAM and RPHERNGFTVL from CMV pp65, could stimulate proliferation of antigen-specific CD8$^+$ T cells (Fig. 2a,b). In the context of the HLA-A2-restricted cells, influenza-specific T cells proliferated more upon transfection with the corresponding ChemRNA than CMV-specific T cells (Fig. 2a). This might be due to the hyporesponsive nature of terminal late-stage T cells specific for the NLVPMVATV peptide[25]. In the context of HLA-B7, both CMV epitopes encoded by ChemRNAs stimulated a strong proliferation of antigen-specific CD8$^+$ T cells.

Lipofection of ChemRNA encoding tumour-derived epitopes leads to the stimulation of cancer-specific T cells in vitro

We aimed to further validate ChemRNA in the context of tumour-derived mutated epitopes. To test this, we synthesized ChemRNAs encoding the mutated epitopes identified in the murine MC-38 colorectal cancer cell line: mutated Adpgk[26] and mutated Rpl18 (ref. 27). Lipofection of ChemRNAs encoding these mutated sequences in mouse bone marrow-derived DCs (BMDCs) efficiently stimulated T cells expressing the TCR specific for these epitopes (Fig. 3a–c). The ChemRNAs were similarly active (for Adpgk) or showed even greater activity (for Rpl18) compared with conventional ivt mRNAs encoding the same minimal epitopes when transfected at identical amounts (1 μg RNA per transfection). As performed for the SIINFEKL ChemRNA, we tested extended versions of the Adpgk ChemRNA (encoding 20 or 27 amino acids with the mutation located in the centre; or extended by 3 flanking amino acids amino- and carboxy-terminally; or having a 5′ UTR; sequences in Supplementary Table 1) and found that none of these additions enhanced the functionality of the ChemRNA for T cell stimulation when transfecting equimolar (13 pmol) amounts of the different RNAs (Extended Data Fig. 6). In addition, ChemRNAs encoding the optimized version of the human tumour epitope MART-1 (HLA-A2-presented peptide ELAGIGILTV, an analogue of the natural EAAGIGILTV from Melan-A[28]) efficiently restimulated anti-cancer T cells from PBMCs of HLA-A2-positive cancer patients in vitro (Fig. 3d). Compared with minimal synthetic peptide epitopes, ChemRNAs induced a more pronounced proliferation of specific T cells. Thus, in these systems and using our routine experimental set-ups, compared with ivt mRNA or synthetic peptides, ChemRNAs were as good as or better at inducing stimulation of antigen-specific CD8$^+$ T cells.

Vaccination with ChemRNA induces expansion of antigen-specific T cells, restricts tumour growth and results in significantly prolonged survival

We next tested whether in vivo-administered ChemRNA could induce de novo immune responses and protect against tumours by using RNA lipoplexes for the delivery of RNA to lymphoid organs after intravenous injection[29]. In preclinical and clinical settings, RNA lipoplexes were shown to trigger production of anti-cancer mediators, such as type I IFN, and prime and recall antigen-specific T cell responses[29–31]. ChemRNA and an optimized ivt mRNA[19] encoding SIINFEKL or Reps1 were formulated as an RNA lipoplex and administered intravenously (Fig. 4a,b). The SIINFEKL ChemRNA induced both an innate immune response, measured by IFNα ELISA (Fig. 4c), and an

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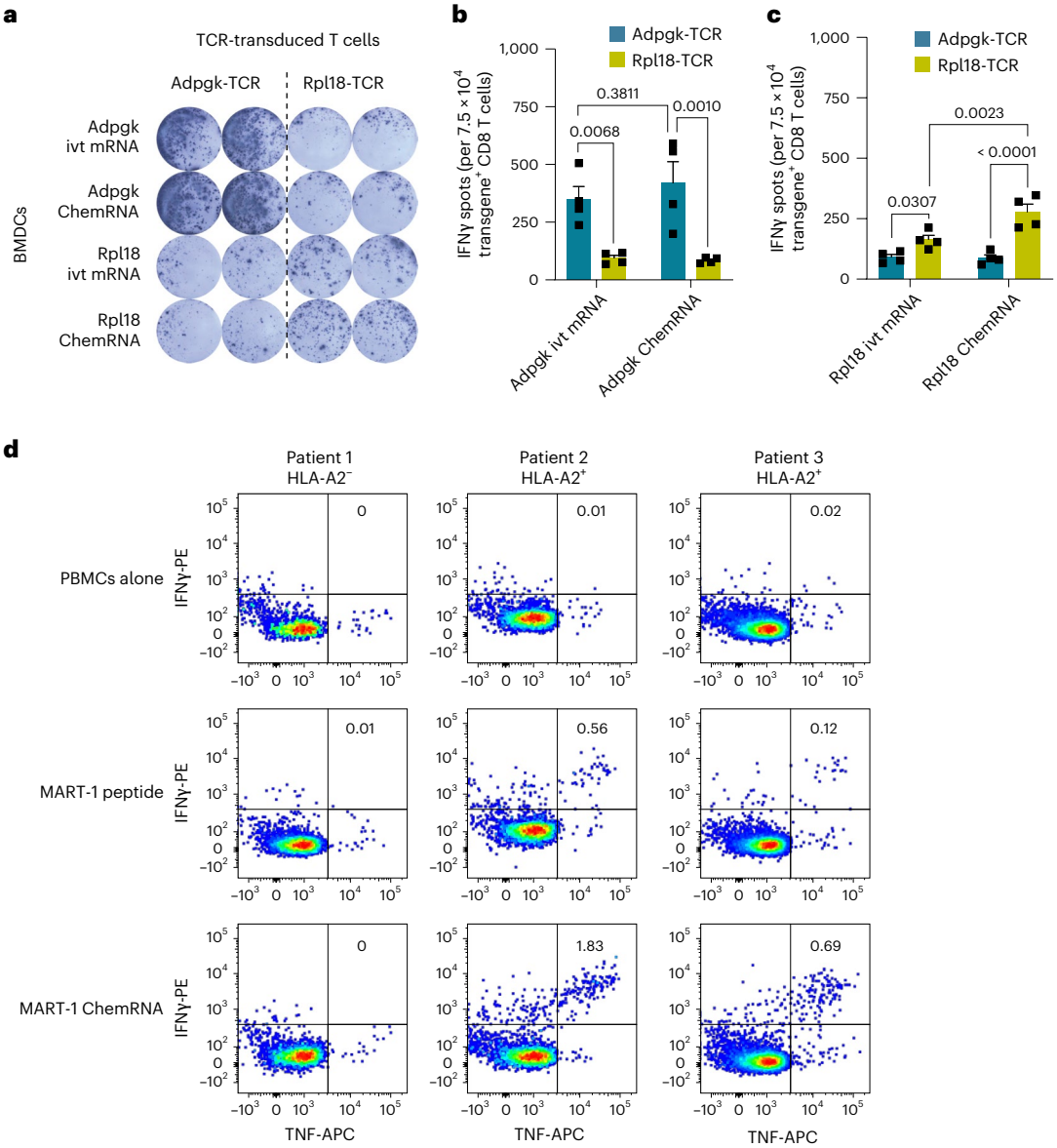


Fig. 3 | Stimulation of T cells in vitro with ChemRNAs encoding cancer cell epitopes. a–**c**, Transduced T cells expressing TCRs for the mutated MC-38-specific epitopes were co-cultured with BMDCs lipofected with ChemRNA or ivt mRNA coding for mutated MC-38-specific epitopes. At 16 hours after co-culture, IFNγ secretion was measured by ELISpot. **a**, Image of ELISpot wells. **b**,**c**, Quantification of IFNγ spots after lipofection with RNA coding for either Adpgk (**b**) or Rpl18 (**c**). **d**, Stimulation of PBMCs from HLA-A2-positive or -negative (as control) patients with melanoma, using either ChemRNA coding for the MART-1 epitope ELAGIGILTV or the MART-1 ELAGIGILTV synthetic peptide, before flow cytometry staining for IFNγ and TNF. Gating strategy: lymphocytes (SSC-A versus FSC-A), $CD3^+$ (SSC-A versus CD3), living cells (SSC-A versus Zombie Aqua), $CD4^-/CD8^+$ (CD4 versus CD8). The numbers in the top-right quadrants show percentages of TNF- and IFNγ-double-positive $CD8^+$ T cells. In **b**,**c**, $n = 4$ samples per group. Data are presented as mean ± s.e.m. of two independent experiments with two technical replicates each. Statistical significance was determined using two-way ANOVA with uncorrected Fisher's least significant difference test (*P* values indicated).

adaptive immune response, that is, antigen-specific $CD8^+$ T cells that could be detected ex vivo from blood 1 week after the boost (Fig. 4d and Extended Data Fig. 7). ChemRNA and ivt mRNA were equally capable of priming anti-SIINFEKL T cells in vivo (there was no significant difference in the frequency of SIINFEKL-specific T cells detected in blood). Accordingly, mice that received ChemRNA encoding SIINFEKL were able to efficiently control B16/OVA tumour cell growth, with a significant increase in survival compared with the negative controls and similar outcomes to mice vaccinated with capped and polyadenylated ivt mRNA encoding SIINFEKL (Fig. 4e,f). We found that other formulations, doses and administration sites can also be applied: for example, ChemRNA injected subcutaneously in Lipofectamine MessengerMAX induces T cell responses (Extended Data Fig. 8a,b). Titrating the amount of injected mRNA similarly lowers the amount of primed T cells (Extended Data Fig. 8c,d). As seen in vitro in immune cells, in vivo, after intravenous or subcutaneous injection, the induced immune response is similar whether the ChemRNA is unmodified or fully phosphorothioated (Extended Data Fig. 9).

We further show that ChemRNA can induce T cell responses against mutated tumour neoepitopes in vivo using a mutated epitope from the Reps1 gene, leading to significant protection against tumours expressing this neoepitope. Of note, in this case ChemRNA produces weaker anti-neoepitope T cell responses and tumour protection compared with ivt mRNA (Fig. 4g,h)[26,27,32].

Thus, short, non-capped and non-polyadenylated ChemRNA can be efficiently translated and can be used to deliver MHC class

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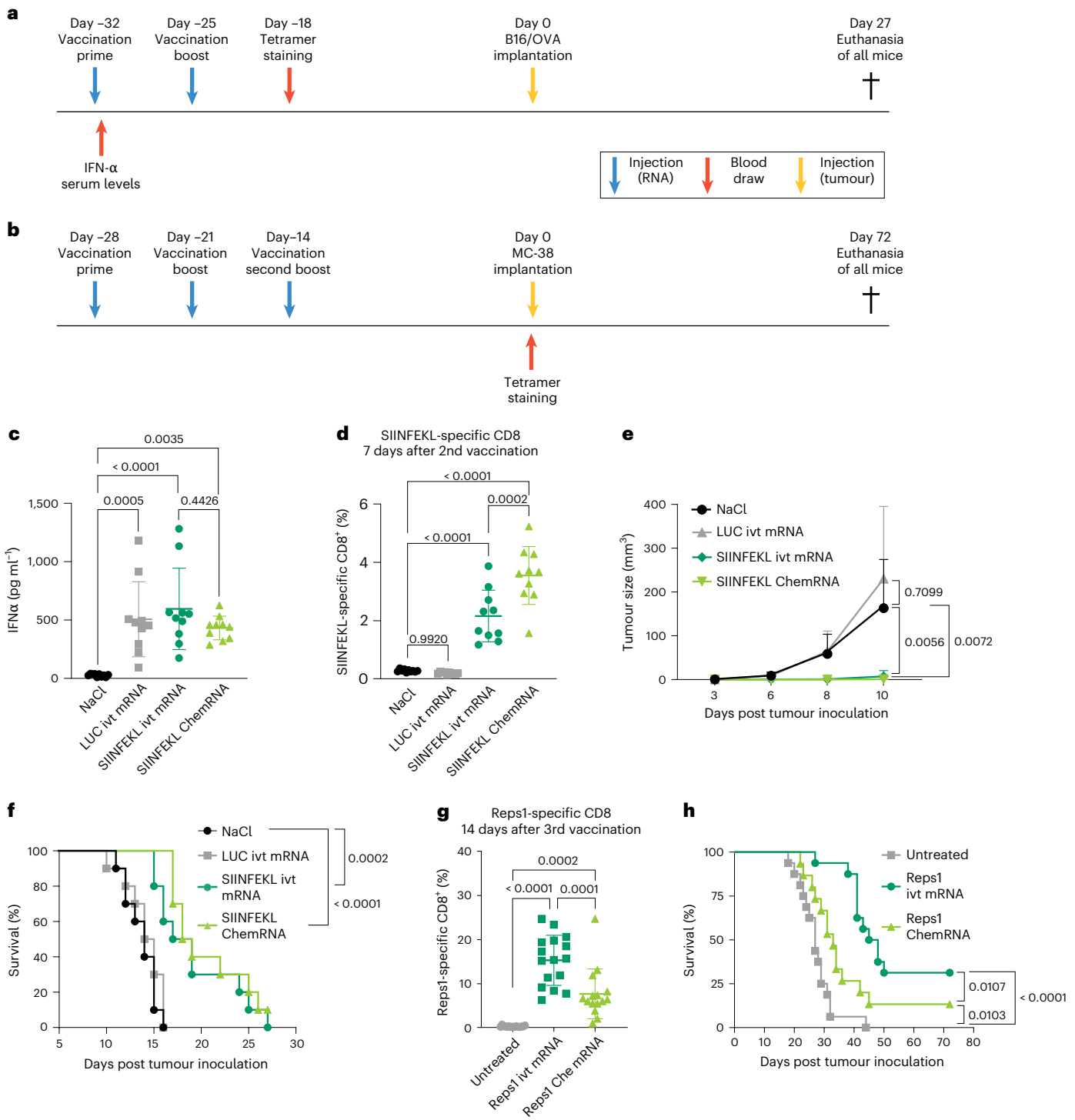


Fig. 4 | ChemRNA vaccination induces expansion of antigen-specific T cells, delays tumour growth and results in significantly prolonged survival. **a**–**h**, Six-week-old (**a**,**c**–**f**) or 12-week-old (**b**,**g**,**h**) female C57BL/6 mice were vaccinated either 2× (**a**,**c**–**f**) or 3× (**b**,**g**,**h**) by intravenous administration with 10 µg of ivt mRNA or ChemRNA coding for SIINFEKL (or ivt mRNA coding for firefly luciferase (LUC) as a negative control) (**a**,**c**–**f**) or 15 µg of ivt mRNA or ChemRNA coding for Reps1 (**b**,**g**,**h**). **a**,**b**, Experimental timelines for SIINFEKL (**a**) or Reps1 (**b**) vaccination and subsequent tumour challenge. **c**, At 3 hours after vaccination, IFNα serum levels were measured by ELISA. Values below the LOD were substituted with LOD ÷ $\sqrt{2}$ before statistical analysis. **d**, Quantification of SIINFEKL-specific CD8$^+$ T cells by flow cytometry, 7 days after the last vaccination. The representative dot plots are depicted in Extended Data Fig. 7. **e**, Tumour growth curve for B16/OVA-bearing mice until day 10. **f**, Survival curve of B16/OVA tumour-bearing mice. **g**, Percentage of Reps1-specific T cells among CD8$^+$ cells. **h**, Survival curve of MC-38 tumour-bearing mice. $n = 10$ animals per treatment group (**c**–**f**) or $n = 16$ animals for untreated and Reps1 ivt mRNA and $n = 15$ animals for Reps1 ChemRNA (**g**,**h**). Data from one independent experiment are presented as mean ± s.d. One-way (**c**,**d**,**g**) or two-way (**e**) ANOVA with Tukey's multiple comparison test was used to assess the significance between treatment groups (*P* values indicated). **f**,**h**, log-rank (Mantel–Cox) test was used for curve comparison (*P* values indicated).

I-associated peptides in vivo. Moreover, ChemRNA, like ivt mRNA[29], stimulates type I IFN production, which, in conjunction with epitope presentation, fosters priming and expansion of specific T cells capable of controlling tumours expressing the corresponding antigen.

Discussion

We found that chemically synthesized RNA oligonucleotides encoding $CD8^+$ T cell epitopes can be efficiently translated, particularly in immune cells, even though they lack structural mRNA features previously considered to be essential for translation. Of great interest is that the MHC class I processing and loading machinery appears to be very proficient in presenting peptides derived from ChemRNAs. When transfected into immune cells, non-capped, non-polyadenylated ChemRNA, even at low concentrations, effectively stimulates robust antigen-specific $CD8^+$ T cell responses both in vitro and in vivo and can be as potent as canonical capped and polyadenylated ivt mRNA. These findings bear significance in several respects.

First, our study shows that defective or supposedly non-coding RNAs could encode human and murine MHC class I-associated peptides. These findings suggest that MHC peptides can be generated from any short, non-capped RNA originating either from aberrant transcription, from endonucleolytic degradation of longer RNA or from pathogens. Our study does not provide an understanding of whether specialized ribosomes or cell compartments are involved in the translation of ChemRNA. Previous studies demonstrated defective ribosomal products as a source of MHC class I-associated peptides[33] and postulated the existence of 'immunoribosomes', a potential subgroup of ribosomes with specialized subunits, hypothesized to effectively provide MHC class I-associated peptides[24]. Recent discoveries also identified that long non-coding RNA or alternative open reading frames can encode peptides presented by MHC class I molecules[34]. Our results extend these phenomena, suggesting that the MHC class I antigen processing machinery uses non-coding or defective RNAs to generate epitopes. We also report here that many RNA modifications do not interfere with the production of MHC class I epitopes from the mRNA. This may be an adaptation, particularly of the immune system, to facilitate translation of mRNA from pathogens that are missing typical features of mammalian mRNA or that are heavily modified. This observation also prompts a reassessment of the biological activity of other RNA oligonucleotides, such as antisense molecules. Indeed, modified RNA oligonucleotides, typically designed to suppress the expression of a targeted gene, may unintentionally encode MHC class I-associated peptides. Although we found that the epitopes produced from ChemRNA and ivt mRNA meet the same basic requirements (ribosomes and TAP), additional research is required to unravel the mechanisms driving the initiation of translation of ChemRNAs, and potentially of endogenous non-capped, non-polyadenylated RNA (such as long non-coding RNA or microRNA).

Second, we believe that chemically synthesized mRNA in the form of ChemRNA offers a great alternative to ivt mRNA for developing individualized mRNA-based anti-cancer vaccines aimed at triggering cytotoxic T cells. The inherent nature of ChemRNA, being short, chemically synthesized oligonucleotides, offers distinct advantages in manufacturing scalability, particularly within the context of highly individualized medicines such as neoantigen-specific anti-cancer vaccines. Although ivt mRNA production is robust and highly scalable for bulk quantities (as demonstrated by the billions of anti-COVID-19 vaccine doses manufactured), its scalability is challenged by the requirement for sequential, multistep enzymatic processes (DNA template generation, in vitro transcription, capping, polyadenylation and complex purification). This multistep nature creates a critical bottleneck for the rapid, on-demand manufacturing needed when treating a single patient with a panel of customized neoantigens. In contrast, ChemRNA uses solid-phase oligonucleotide synthesis, a technology that is inherently optimized for a high-throughput, parallel production of diverse, short sequences. Commercially available oligonucleotide synthesizers are designed to create dozens, or even hundreds, of different short RNA molecules simultaneously and rapidly. This platform bypasses the most laborious upstream ivt steps, DNA template production and enzymatic transcription by directly synthesizing the final active molecule. This shift in platform manufacturing fundamentally changes the definition of scalability for personalized vaccines: it moves from optimizing for volume (kilograms of a single sequence) to optimizing for diversity and speed (rapid production of many different sequences in small, patient-specific batches). In addition, ChemRNA avoids peptide linkers used in multi-epitope ivt mRNA vaccines, thereby avoiding the generation and presentation of non-natural epitopes containing linker-derived sequences to T cells.

Despite the high efficiency and simplified workflow of this chemically synthesized, non-capped and non-polyadenylated RNA platform, maintaining very high oligonucleotide purity remains a critical factor for clinical translation. While the chemical synthesis of shorter sequences, such as the approximately 30-nucleotide-long strands used in this study, reliably yields high-purity products, scaling up to longer sequences poses distinct technical hurdles. For personalized anti-cancer vaccines that typically require the expression of 21-mer peptides, the corresponding RNA strands must span at least 66 nucleotides (comprising an initiating AUG start codon, followed by 63 coding nucleotides). At this length, the amount of $(n-1)$ shortmer impurities can increase, risking the introduction of frameshifts that code for unintended out-of-frame peptides. Consequently, the next critical milestone for this technology is optimizing synthesis and advanced purification techniques to consistently achieve ultra-high purity levels of 99% or greater for longer RNA oligonucleotides. Validating these high-fidelity manufacturing processes under Current Good Manufacturing Practice guidelines represents the essential next step towards safely advancing this platform into human clinical trials.

In summary, our discoveries extend the possibility of translation to short uncapped and non-polyadenylated RNA. This could expand the diversity of MHC class I-associated peptide epitopes that cells can produce, and could substantially influence the development and application of personalized anti-cancer mRNA vaccines.

Methods

Production of chemically synthesized mRNA

The sequences of all RNA oligonucleotides and modified versions are presented in Supplementary Table 1. 5′-N7-Methylguanosine-triphosphate capped RNA oligonucleotides were ordered from Bio-Synthesis. These capped oligonucleotides are used when triggering of cytosolic RNA sensors such as MDA5 or RIG-I should be prevented in the context of oligonucleotide-based therapies. The design of these capped RNA oligonucleotides consisted of either a TISU 5′ UTR or no 5′ UTR, followed by a sequence coding for MESIINFEKL (the SIINFEKL epitope preceded by a start codon and a glutamate residue that is present in the natural epitope produced from OVA by proteasomes[15]). The synthesis was performed by phosphoramidite solid-phase synthesis and the 5′-N7-methylguanosine-triphosphate cap was produced as reported by ref. 16. Uncapped 5′-triphosphate and 5′-monophosphate RNA oligonucleotides were also synthesized by Bio-Synthesis. The RNA oligonucleotides were purified by high-performance liquid chromatography. For polyadenylation, the oligonucleotides were incubated for 6 hours with recombinant poly(A) polymerase (New England Biolabs) in the conditions recommended by the manufacturer. Elongation of the RNA oligonucleotide was confirmed by comparing its size with the same RNA incubated in a polyadenylation reaction missing the enzyme on a 2% MOPS agarose gel. Non-modified RNA oligonucleotides without the 5′ cap, UTRs, stop codon or poly(A) tail (ChemRNA) and DNA oligonucleotides were purchased from Microsynth. Non-modified,

codon-optimized and modified RNA oligonucleotides without 5′ cap, UTRs, stop codon or poly(A) tail (ChemRNA) were purchased from Dharmacon.

Production of ivt mRNA

All ivt mRNAs used in this article were unmodified; sequences of in-house-produced ivt mRNAs are presented in Supplementary Table 2. The vectors for SIINFEKL ivt mRNA, Adpgk ivt mRNA, Rpl18 ivt mRNA and Reps1 ivt mRNA were pharmacologically optimized for RNA stability and protein translation and encoded a 5′ human α-globin UTR, a 3′ UTR of FI element and a poly(A) tail of 110 nucleotides. They were further equipped with a secretion signal for routing to the endoplasmic reticulum and the transmembrane domain derived from murine MHC class I (MITD) based on the human sequence described previously for improved presentation of MHC class I and II epitopes[7–9]. For in-house production, in vitro transcription was conducted with the HiScribe T7 mRNA kit (New England Biolabs). The nucleotide mixture consisted of 8 mM CleanCap (TriLink BioTechnologies), ATP, GTP, CTP and UTP, with a final volume of 100 µl. After incubation for 12 hours at 37 °C, 400 µl of a solution containing 50 µl of 10× poly(A) polymerase buffer, 5 µl of DNase and 5 µl of RNasin (all from New England Biolabs) were added to degrade residual DNA. The reaction mixture was incubated for 1 hour before the addition of 500 µl water and 500 µl 8 M LiCl. After incubation at −20 °C for 1 hour and centrifugation at 20,000*g* for 15 minutes, supernatants were removed, and 1 ml of 75% ethanol was added to wash the pellets. Next, the mRNAs were centrifuged for another 10 minutes at 20,000*g*, the supernatants were discarded and the mRNA pellets were dried in a vacuum centrifuge (Speed-Vac). The mRNAs were then resuspended in water, and the concentration was determined using a Nanodrop and adjusted to 1 µg µl^{-1}. Quality and integrity of ivt-produced mRNAs were checked by agarose gel electrophoresis and the mRNAs were subsequently stored at −20 °C.

RNA formulations

For the lipofection with Lipofectamine MessengerMAX, 2 µl MessengerMAX (Thermo Fisher Scientific) was added to 25 µl Opti-MEM (Gibco, Thermo Fisher Scientific) and incubated for 10 minutes at room temperature (RT) before mixing with a solution of 1 µg RNA in 25 µl of Opti-MEM and further incubation for 10 minutes.

For the lipofection with the spleen-targeting liposome from BioNTech[29–31], 10 µg RNA was diluted to 83 µl with water before addition of 10 µl of 1.5 M NaCl and then 7 µl of liposome was added. The mixture was then incubated for 10 minutes at RT.

Lipid nanoparticles (LNPs) were formulated in-house with a set-up including a herringbone mixer glass chip (Darwin Microfluidics), SGE gas tight glass syringes (Sigma-Aldrich) and syringe pumps. The ethanol phase consisted of 149.22 µl ethanol (Sigma-Aldrich), 28 µl of 50 mg ml^{-1} ionizable lipid ALC-0315 (MedChem Express), 9.81 µl of 15 mg ml^{-1} pegylated lipid ALC-0159 (Avanti Polar Lipids), 43.43 µl of 15 mg ml^{-1} cholesterol (Sigma-Aldrich) and 19.54 µl of 15 mg ml^{-1} DSPC (Avanti Polar Lipids). The aqueous phase was made up of 575 µl water, 75 µl of 100 mM citrate buffer pH 3 and 100 µl RNA (at 1 µg µl^{-1}). After loading of the two phases into the respective syringes, they were mixed by pumping them through the mixer chip at a flow rate of 900 µl min^{-1} for the aqueous phase and 300 µl min^{-1} for the ethanol phase. The collected LNPs were subsequently loaded into Pierce Slide-A-Lyzer Dialysis Cassettes (Thermo Fisher Scientific) and dialysed against 1× PBS overnight at 4 °C. Particle size and polydispersity index were measured with a Zetasizer (Malvern Panalytical). LNP encapsulation efficiency was determined using Quant-iT RiboGreen RNA reagent (Thermo Fisher Scientific). For this assay, 10 µl LNPs were diluted in 490 µl TE buffer, added to a black 96-well plate (50 µl per well) and mixed with either 50 µl per well TE buffer or 50 µl per well 4% Triton X-100. An RNA standard curve was prepared in both TE buffer and 4% Triton X-100. After incubating the plate for 15 minutes at 37 °C, 100 µl of RiboGreen reagent in TE buffer (1:100) was added to the wells and incubated again at 37 °C for 3 minutes before reading the fluorescence at 480 nm excitation and 520 nm emission. After calculating the RNA concentration for both total and free RNA from the standard curves, the encapsulation efficiency (E) was calculated with $E = (1 - \frac{\mathrm{RNA_{TE}}}{\mathrm{RNA_{Triton}}}) \times 100$.

Cells and in vitro experiments

Primary cells and cell lines. All primary murine cell lines were derived from either C57BL/6 or Rag2/OT-1 mice as listed in the article.

Primary human PBMCs were acquired from the blood of healthy donors or from patients with melanoma. For healthy volunteers, blood collection and study were approved by the Ethics Committee of the University of Tübingen (approvals 156/2012B01 and 713/2018BO2), and all participants gave written informed consent. For patients with cancer, PBMCs were obtained from a prospective observational cohort (ImmunoMonitoring of ImmunoTherapy; IMIT) of patients with metastatic melanoma receiving immune checkpoint inhibitor treatment (anti-PD-1, anti-PD-L1, anti-CTLA4 or combination treatment) at the Kantonsspital St. Gallen. The study was approved by the Ethics Committee of Eastern Switzerland (project number EKOS 2016-00998). All participants provided written informed consent before study inclusion and were not financially compensated.

Cells were maintained in RPMI medium (Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS) and 0.1 mg ml^{-1} of the antimicrobial reagent Normocin (InvivoGen) (complete medium). In addition, the complete medium of DC2.4 cells was supplemented with 10 ng ml^{-1} final concentration of murine granulocyte–macrophage colony-stimulating factor (GM-CSF) and the complete medium of B16/OVA and B3Z cells was supplemented with Geneticin (G418; InvivoGen) as a selection antibiotic at a final concentration of 0.2 mg ml^{-1}. The OVA(257–264) (SIINFEKL)-specific, H2-K^{b}-restricted CTL hybridoma, B3Z[35] was kindly provided by Prof. Guizhi Zhu, Department of Pathology and Immunology, Johns Hopkins University School of Medicine. B3Z cells carry a β-galactosidase (lacZ) construct driven by NF-AT elements from the interleukin-2 (IL-2) promoter. B16-F10 and B16/OVA were obtained from ATCC. DC2.4 were purchased from Creative Biolabs (catalogue number NCL2110P098). RMA/S were provided by Thomas Look, Department of Dermatology, University Hospital Zurich. All cell lines used in this article tested negative for mycoplasma. No commonly misidentified lines were used in this study.

Electroporation of RMA/S cells. The TAP2-deficient RMA/S cell line was electroporated with ivt mRNA coding for murine TAP2 (sequence in Supplementary Table 2) 24 hours before any in vitro experiments to restore functionality. A total of 1.5 × 10^{6} RMA/S cells were washed 2× in PBS and electroporated with 30 µg of mRNA encoding murine TAP2 by using the Neon Transfection System 100 µl Kit (Thermo Fisher Scientific). The cells were electroporated with three 10 ms pulses at 1,600 V, resuspended in RPMI containing 10% FBS (but no antibiotics) and incubated at 37 °C, 5% CO_2 for 24 hours.

Single-cell suspension of human PBMCs and mouse splenocytes. Mouse splenocytes were extracted from Rag2/OT-1 or C57BL/6 mouse spleens in complete medium with a 70 µm cell strainer (Becton Dickinson) and the plunger of a 2 ml syringe (Becton Dickinson). Subsequently, red blood cell lysis was performed by incubating the cells in 10 ml eBioscience 1× RBC Lysis Buffer (Invitrogen) for 5 minutes at RT. To stop the reaction, 30 ml PBS was added and the cells were washed 2× with PBS before resuspension in complete medium. Human PBMCs were obtained from whole blood of healthy volunteers or patients with cancer using centrifugation at 500*g* for 20 minutes either on Ficoll-Paque Plus (Cytiva) or on Lymphosep (Biowest). Subsequently, the PBMCs were washed 2× with PBS, resuspended in FBS containing 10% DMSO and stored frozen until further use.

Generation of mouse BMDCs. Bone marrow cells were flushed from femur and tibia bones of naive C57BL/6 mice, homogenized and filtered before removal of erythrocytes by hypotonic lysis. Cells were cultured in RPMI 1640 GlutaMAX containing 10% FBS, 50 U ml^{-1} penicillin, 50 µg ml^{-1} streptomycin, 1× non-essential amino acids (NEAA), 1× sodium pyruvate (all Gibco, Thermo Fisher Scientific) and 1,000 U ml^{-1} recombinant murine GM-CSF (PeproTech) at 37 °C, 5% CO_2. Fresh medium was added every 2–3 days. After 7 days, non-adherent cells containing BMDCs were harvested and stored in liquid nitrogen. The cells were more than 60% $CD11c^+$.

OT-1 experiments. To test different designs of chemically synthesized mRNA, 100,000 OT-1 splenocytes were mixed with an equal amount of either B16-F10 cells or C57BL/6 splenocytes before transfection (as described earlier) with either 0.01 µg ml^{-1}, 0.025 µg ml^{-1}, 0.1 µg ml^{-1} or 1 µg ml^{-1} of formulated RNA coding for OVA or SIINFEKL. For the OT-1 readout, after incubation for 24 hours or 72 hours at 37 °C, 5% CO_2, IL-2 was measured in the culture supernatants by ELISA (ELISA MAX Standard Set Mouse IL-2; BioLegend). Values below the limit of detection (LOD) were substituted with LOD ÷ $\sqrt{2}$ before statistical analysis.

Fluorescence activated cell sorting experiments. All antibodies used for fluorescence activated cell sorting (FACS) experiments are presented in Supplementary Table 3. For the flow cytometry staining to analyse H2-K^b SIINFEKL presentation, flow cytometry buffer was prepared by combining 488 ml PBS, 10 ml heat-inactivated FBS (2% final), 2 ml 0.5 M EDTA (2 mM final), and 0.5 ml 10% sodium azide. DC2.4 cells were transferred to 96-well U-bottom plates and pelleted by centrifugation at 460*g* for 4 minutes at RT. Supernatants were discarded. Cell pellets were resuspended by brief vortexing and washed with 200 µl PBS, followed by centrifugation and supernatant removal. For viability assessment and blocking of Fc receptors, 100 µl live/dead stain containing Fixable Viability Dye eFluor 780 and Mouse BD Fc Block was added to each well, mixed by pipetting and incubated for 15 minutes at 4 °C. After washing with 100 µl PBS and centrifugation, cells were stained with 50 µl extracellular antibody (PE anti-mouse H2-K^b bound to SIINFEKL) for 30 minutes at 4 °C. Following antibody staining, cells were washed with 200 µl flow cytometry buffer and fixed with 150 µl 2% paraformaldehyde (diluted from 4% ROTI Histofix; Carl Roth) for 15 minutes at 4 °C. Fixed cells were pelleted, resuspended in 200 µl flow cytometry buffer and analysed by flow cytometry using a BD FACSCelesta Cell Analyzer. Data were analysed using FlowJo v.10.10.0. The gating strategy was as follows: single cells (forward scatter height (FSC-H) versus forward scatter area (FSC-A)) → living cells (FSC-A versus viability dye) → FSC-H versus side scatter height (SSC-H) → K^b-SIINFEKL-positive cells (FSC-A versus H2-K^b SIINFEKL antibody).

For the experiment with PBMCs from healthy donors, 5 µl of the RNA-MessengerMax preparation were added to 1 ml of previously characterized (using tetramer staining) PBMC suspension (in Iscove's modified Dulbecco medium containing 10% heat-inactivated human serum, penicillin–streptomycin and 50 µM 2-mercaptoethanol) and incubated for 12 days (split on day 4) with addition of 2 ng ml^{-1} IL-2 (R&D Systems) every other day until staining and FACS analysis as described in ref. 36. A total of 1.5×10^6 cells were stained per test, first with 2.5 µg ml^{-1} of the tetramers (in-house production) for 30 minutes at RT, then with Zombie Aqua (1:200; BioLegend), CD4 APC-Cy7 (1:100; Becton Dickinson) and CD8 PE-Cy7 (1:400; Beckman Coulter) for 20 minutes at 4 °C. After a final wash, cells were resuspended in FACS buffer and acquired directly on a LSRFortessa SORP (Becton Dickinson) equipped with DIVA (v.6.1.3).

For the FACS experiment with PBMCs from patients with melanoma, the cryopreserved PBMCs were resuspended in RPMI containing 8% human serum (Biowest), 1% penicillin–streptomycin, 1% L-glutamine, 1% NEAA, 1% sodium pyruvate, 0.1 mg ml^{-1} kanamycin and 0.1% 2-β-mercaptoethanol, plated at a concentration of 0.5×10^6 cells ml^{-1} in low-adherence 24-well plates (Sarstedt) and maintained at 37 °C, 5% CO_2. The following day, either 4 µg single peptide MART-1 (ELAGIGILTV; JPT Peptide Technologies) or 1 µg of ChemRNA coding for MART-1 (20 µl of the RNA-MessengerMax preparation) were added to the cells. After 48 hours, 1 ml of medium was replaced with fresh RPMI (as mentioned earlier) containing IL-2 (final concentration 150 U ml^{-1}). Cells were kept in culture for 10 days and 1 ml medium was replaced with fresh IL-2-containing medium every other day. On day 10, cells were collected from the plates, washed and transferred to 96-well plates where they were restimulated with 0.4 µg of the single peptide or 0.1 µg ChemRNA in the absence of IL-2. Brefeldin A was added 1 hour after adding the peptide or the ChemRNA at a final concentration of 10 µg ml^{-1}. After an incubation for 6 hours at 37 °C and 5% CO_2, cells were washed, stained for viability (BV510; Becton Dickinson) and incubated with fluorescently labelled antibodies recognizing CD3 (APC-Cy7, 1:100; Lucerna Chem), CD4 (PE-Cy7, 1:100; Lucerna Chem), CD8 (PerCP, 1:100; Lucerna Chem) and CD45RA (BV711, 1:400; Lucerna Chem) in FACS buffer. Cells were then fixed and permeabilized using Cytofix/Cytoperm (Becton Dickinson) and incubated with fluorescently labelled antibodies recognizing IFNγ (PE, 1:100; Thermo Fisher Scientific), TNF (APC, 1:100; BioLegend), CD40L (FITC, 1:100; BioLegend) and IL-17A (BV421, 1:100; BioLegend) in Perm buffer (2% FCS, 0.5% saponin in PBS). Samples were acquired on a LSRFortessa (Becton Dickinson) and data were analysed using FlowJo software (v.10.6.2; Becton Dickinson). IFNγ and TNF production were analysed to identify antigen-specific T cells.

Lipofection of BMDCs. For the readout with TCRs specific of mutated mouse peptides, freshly thawed BMDCs were lipofected with MessengerMAX as described earlier and used for IFNγ enzyme-linked immunospot assay (ELISpot) 16–20 hours post-transfection.

Retroviral transduction of mouse T cells. Splenocytes of naive C57BL/6 mice were pre-activated with Dynabeads Mouse T-Activator CD3/CD28 (Gibco, Thermo Fisher Scientific) in mouse T cell media, RPMI 1640 GlutaMAX supplemented with 10% FBS, 1× NEAA, 1 mM sodium pyruvate, 10 mM HEPES, 50 µM β-mercaptoethanol, 50 IU ml^{-1} penicillin and 50 µg ml^{-1} streptomycin (all Gibco, Thermo Fisher Scientific) in the presence of 500 IU ml^{-1} recombinant human IL-7 (rhIL-7) and 50 IU ml^{-1} rhIL-15 (both from Miltenyi Biotec). At 24 hours following activation, cells were added on murine leukemia virus envelope-pseudotyped gamma-retroviral vector (Adpgk-TCR or Rpl18-TCR) pre-coated RetroNectin plates (Takara Bio) and spun down (1 hour, 37 °C, 300*g*). Freshly coated viral particle plates were used to repeat spin-down transduction the following day. Activation substance was removed 72 hours after initial activation and cells were expanded for 3 days in T cell media in the presence of rhIL-7 and rhIL-15. Following activation and expansion, the lymphocyte layer was isolated by Ficoll-Paque (Cytiva) density gradient centrifugation. Transgene expression on transduced murine T cells was measured by flow cytometry.

IFNγ ELISpot. IFNγ released by TCR-transduced murine T cells upon antigen encounter was detected by IFNγ ELISpot. A total of 75×10^3 TCR-transduced cells were cultured overnight with 1×10^4 BMDCs lipofected with antigen-encoding RNAs, that is, Adpgk/Rpl18 ivt mRNA or Adpgk/Rpl18 ChemRNA on anti-IFNγ (clone AN18; Mabtech) pre-coated MultiScreen filter plates (Merck Millipore). The spots were visualized with a biotin-conjugated anti-IFNγ antibody (Mabtech) followed by incubation with ExtrAvidin alkaline phosphatase (Sigma-Aldrich) and BCIP/NBT substrate (Sigma-Aldrich). Plates were scanned using CTL's ImmunoSpot Series S5 Versa ELISpot Analyzer (S5Versa-02-9038) and analysed by ImmunoCapture v.6.3 software. The IFNγ spot counts for wells with uncountable (saturated) signals were set to 1,000.

The samples were tested in duplicates for each biological replicate and spot counts were summarized as means of two biological and two technical duplicates using GraphPad Prism 9.

In vitro luminescence assays. For the luminescence assay using the Nano-Glo HiBiT Lytic Detection System (Promega), 1 μg ml^{-1} of formulated RNA was added to a white 96-well tissue culture plate. Next, 100,000 cells were added to the respective wells and incubated at 37 °C, 5% CO_2 for the indicated amount of time. For experiments using the ribosome inhibitors cycloheximide, lactimidomycin or harringtonine, cells were incubated with the inhibitors for 1 hour before transfection. For the readout, the supernatant was removed and replaced with 50 μl PBS. The amount of translated HiBiT protein in the cells was then determined by adding 50 μl Nano-Glo HiBiT Lytic Detection Reagent (Promega) containing the substrate furimazine and LgBiT, the large subunit used in NanoBiT (Promega). Luminescence was measured with a GloMax Discover Microplate Reader (Promega).

For the β-galactosidase readout using the Invitrogen Galacto-Light Plus β-Galactosidase Reporter Gene Assay System (Thermo Fisher Scientific), 100,000 B16-F10 cells, C57BL/6 splenocytes or DC2.4 cells were mixed with an equal amount of B3Z cells in complete medium and transfected with 1 μg ml^{-1} formulated RNA and incubated for 24 hours at 37 °C, 5% CO_2. For the IFNα or IFNγ stimulation of B16-F10 cells, 100,000 B16-F10 cells were treated with either 100 ng ml^{-1} IFNα or IFNγ and transfected with 1 μg ml^{-1} ChemRNA or ivt mRNA encoding SIINFEKL or OVA. After 24 hours, the culture medium was exchanged and 100,000 B3Z cells were added and incubated for another 24 hours at 37 °C, 5% CO_2. For the experiment looking at the stability of MHC class I presentation after transfection, 20,000 DC2.4 cells were transfected with 1 μg ml^{-1} of formulated RNA and incubated for 4 hours at 37 °C, 5% CO_2. Next, the cells were washed and supplemented with fresh RPMI complete medium to remove any formulated RNA outside of the cells. After incubating the transfected DC2.4 cells for 24 hours, 48 hours, 72 hours or 96 hours, 100,000 B3Z cells were added and incubated for another 24 hours at 37 °C, 5% CO_2. For the β-galactosidase readout, the cells were washed once with 150 μl PBS, before cell lysis with 10 μl lysis solution for 10 minutes. The cell lysate was then transferred to white 96-well plates and incubated with 70 μl reaction solution for 40–50 minutes, before addition of 100 μl accelerator and the luminescence readout with a GloMax Discover Microplate Reader (Promega).

RT-qPCR experiment. For the RT-qPCR experiment to determine the intracellular stability of ivt mRNA and ChemRNA, 10,000 DC2.4 cells were transfected with 50 pmol ml^{-1} of formulated RNA coding for SIINFEKL and incubated for 4 hours at 37 °C, 5% CO_2. Next, the cells were washed and supplemented with fresh RPMI complete medium to remove any formulated RNA outside of the cells. After incubating the transfected DC2.4 cells for 24 hours, 48 hours, 72 hours or 96 hours, the cells were washed with PBS and processed with the Cells-to-CT 1-Step Power SYBR Green Kit (Thermo Fisher Scientific). The RT-qPCR was performed in 96-well PCR plates with 1 μl cell lysate in 19 μl RT-PCR Master Mix with a Roche Light Cycler 480 (Roche).

Animals

Female (6–12 weeks old) C57BL/6J mice were kept under specific pathogen-free conditions with a 12-hour light/dark cycle and food and water provided ad libitum. All experiments were performed according to the governmental and institutional guidelines. Our studies 'Anti-cancer therapies based on RNA' (licence number ZH215/2017) and 'Testing mRNA formats and formulations for anti-cancer vaccinations' (licence number ZH158/2022) were approved by the Veterinary Office (Kanton Zurich, Health Direction, Veterinary Office). The Reps1 animal study was approved by the responsible authority Landesuntersuchungsamt Rheinland-Pfalz, Germany under the protocol number G22-12-061. Animals were purchased from Envigo. C57BL/6-Tg(TcraTcrb)1100Mjb mice (Rag2/OT-1 mice) were a generous gift from Prof. Pål Johansen (University Hospital Zurich) or purchased from Taconic Biosciences.

Tumour and vaccination experiments

For the tumour experiments comparing ChemRNA and ivt mRNA or the vaccination experiment comparing different ChemRNA modifications, female 6-week-old C57BL/6 mice were injected intravenously in the tail vein with 10 μg of ivt mRNA or ChemRNA coding for SIINFEKL or with 15 μg coding for Reps1, formulated as described earlier with a spleen-targeting liposome from BioNTech (lipoplex)[29–31]. Three hours after the mRNA injection, mice were anaesthetized with Attane isoflurane (veterinary-grade formulation; Minrad) and 50–70 μl blood was drawn from the sublingual vein. Serum was prepared from whole blood using BD Microtainer serum separation tubes (BD Biosciences) and subjected to IFNα quantification by ELISA (PBL Assay Science). Values below the LOD were substituted with LOD ÷ $\sqrt{2}$ before statistical analysis. One week after the first vaccination, mice received a boost.

For the Reps1 tumour experiment, mice received a second boost 1 week after the first. At 1 week (SIINFEKL experiments) or 2 weeks (Reps1 experiment) after the final boost, mice were anaesthetized with Attane isoflurane (veterinary-grade formulation; Minrad) and either 50 μl (Reps1 experiment) or 200 μl (SIINFEKL experiments) blood was drawn from either the vena facialis (Reps1 experiment) or the sublingual vein (SIINFEKL experiments), respectively, in EDTA-treated BD Microtainer tubes (BD Biosciences) to perform flow cytometry analysis of tetramer$^+$ CD8$^+$ T cells. At 14 days (Reps1 experiment) or 25 days (SIINFEKL experiment) after the second mRNA injection, mice received a subcutaneous injection of either 5×10^5 MC38 tumour cells in 100 μl PBS (Reps1 experiment) or 1×10^6 B16/OVA (SIINFEKL experiment) tumour cells in 200 μl PBS. Health was monitored every day until the termination of the experiment and tumour size was recorded every other day until the first death by measuring the largest diameter (length) and the perpendicular diameter (width) with a calliper. Tumour volume (TV) was calculated according to the following formula, presuming that the tumours are idealized ellipsoids:

$$\mathrm{TV} = \frac{(\mathrm{width})^2 \times (\mathrm{length})}{2} = \mathrm{mm}^3 \ (\mathrm{width} < \mathrm{length})$$

Termination criteria applied according to the specifications within the respective animal licence. The maximal tumour size permitted by our licences was 1,000 mm^3 (Figs. 4a,e,f) and 1,500 mm^3 (Fig. 4b,h), respectively. Animals were killed once tumour size exceeded the maximum size, in accordance with the approved protocol. Tumour growth health condition was monitored daily. In rare instances, tumours grew so rapidly within a 24-hour period that the volume briefly exceeded the permitted maximum size before animals could be killed. Due to the close daily monitoring, this condition was of very short duration and has been accepted by the responsible authorities.

For the vaccination experiments, female 6-week-old C57BL/6 mice were injected either intravenously in the tail vein or subcutaneously in the neck with 2 μg of ChemRNA or ivt mRNA either formulated in lipoplex, Lipofectamine MessengerMAX or LNPs. Mice received a total of 3 injections, always 1 week apart. One week after the last boost, mice were anaesthetised with Attane isoflurane (veterinary-grade formulation; Minrad) and 200 μl blood was drawn from the sublingual vein in EDTA-treated BD Microtainer tubes (BD Biosciences) to perform flow cytometry analysis of SIINFEKL tetramer$^+$ CD8$^+$ T cells or ELISA quantification of serum antibody levels.

Single-cell suspension preparation and staining for FACS. Whole blood (200 μl) was treated with 2 ml eBioscience 1× RBC Lysis Buffer (Invitrogen) and incubated for 5 minutes at RT, while vortexing every minute. Next, the cells were centrifuged at 500*g* for 5 minutes, washed 2× with 2 ml of PBS and resuspended in 50 μl PBS. The murine

lymphocytes were stained with Zombie NIR Fixable Viability Kit (BioLegend) for 20 minutes at RT in the dark. After washing with 200 µl PBS, cells were resuspended in 50 µl PBS with 5 µl PE-conjugated SIINFEKL tetramer H2-K^b (The Tetramer Shop) and incubated for 15 minutes at RT in the dark. Without washing, 50 µl of FITC-conjugated anti-CD3, Pacific Blue-conjugated anti-CD8a and APC-conjugated anti-CD4 antibodies (BioLegend) at a final dilution of 1:100 in PBS were added and the cells were incubated for 1 hour at 4 °C in the dark. In a next step, cells were washed 2× with 200 µl PBS and fixed with 1% paraformaldehyde in PBS for 30 minutes at 4 °C in the dark. Subsequently, the cells were again washed 2× with 200 µl PBS, resuspended in 300 µl PBS and stored at 4 °C in the dark until sample acquisition with BD LSRFortessa (BD Biosciences) and BD FACSDiva Software (BD Biosciences). The obtained data were analysed using FlowJo (BD Biosciences). All antibodies used for FACS experiments are presented in Supplementary Table 3.

Tetramer generation for the Reps1 tumour experiment. For customized tetramer production, peptide-receptive easYmers (Immunaware) were diluted in PBS to reach a concentration of 500 nM. After adding the respective peptides to a final concentration of 3 µM, the monomers were incubated for 48 hours at 18 °C. Next, streptavidin–fluorochrome conjugates were added in 3 consecutive steps to reach a final concentration of 125 nM. In a first step, a third of the total streptavidin–fluorochrome volume was added to the peptide-loaded easYmers. The solution was mixed and incubated in the dark for 10 minutes at 4 °C. Another third of the total streptavidin–fluorochrome volume was added followed by 10 minutes incubation in the dark at 4 °C. After adding the last third of the total volume, the solution was incubated for another 30 minutes at 4 °C in the dark. The tetramers were stored at 4 °C until used for flow cytometry staining.

Flow cytometry for the Reps1 tumour experiment. For immunophenotyping of blood samples, 50 µl of whole blood was dispensed into a 96-well U-bottom plate and 100 µl PBS was added. After centrifugation (3 minutes, 460*g*, 2–8 °C), the supernatants were carefully aspirated and the cell pellet was resuspended in 200 µl ammonium–chloride–potassium lysis buffer (erythrocyte lysis). The cell suspension was incubated for 5 minutes at RT, centrifuged (3 minutes, 460*g*, 4 °C) and the supernatant was carefully aspirated. After repeating the erythrocyte lysis steps, the cells were washed once with 200 µl FACS buffer (PBS supplemented with 5 mM EDTA and 5% FBS) and were subsequently stained with antibodies (CD45, CD90.2, CD4 and CD8a) and tetramers in 40 µl Brilliant Stain Buffer for 30 minutes at 2–8 °C in the dark. After washing once with 200 µl PBS, cells were resuspended thoroughly in 200 µl Fix/Perm buffer (Foxp3/Transcription Factor Staining Buffer Set) and incubated for 30 minutes at 2–8 °C. After washing once with 200 µl Perm/Wash buffer (Foxp3/Transcription Factor Staining Buffer Set; 3 minutes, 460*g*, 2–8 °C), cells were stained with antibodies in 40 µl Perm/Wash buffer for 30 minutes at 4 °C. After washing 2× with 200 µl Perm/Wash buffer (3 minutes, 460*g*, 4 °C), cells were resuspended in 200 µl FACS buffer and 33 µl CountBright Absolute Counting Beads and stored at 2–8 °C until measurement.

Data were acquired on a BD FACSymphony flow cytometer and analysed with FlowJo software v.10.10.0. The gating strategy was as follows: single cells (FSC-H versus FSC-A) → FSC-H versus SSC-H → CD45$^+$ cells → CD90$^+$/CD19$^-$ cells → CD8$^+$ cells → Reps1-specific cells.

IgG anti-OVA ELISA. ELISA plates were coated with 1 µg ml^{-1} OVA (Sigma-Aldrich) in PBS and stored at 4 °C overnight. After washing with ELISA wash buffer (BioLegend), serum from mice taken 7 days after the last vaccination was diluted 1:100 in ELISA diluent (BioLegend), added to the plates and incubated for 1 hour at RT. After another wash step, plates were treated with an HRP-conjugated goat anti-mouse IgG antibody (Abcam), diluted 1:5,000 in ELISA diluent. After another wash step and TMB (BioLegend) addition, 2 M sulfuric acid (Sigma-Aldrich) was added to stop the reaction and absorbance was measured at 450 nm with a GloMax Discover Microplate Reader (Promega).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software). Parametric data were analysed using an unpaired, two-tailed Student's *t*-test when comparing two conditions or one-way analysis of variance (ANOVA) corrected with Tukey's comparison test factor when comparing more than two conditions. To compare data influenced by two factors, two-way ANOVA with Šidák's, Dunnett's or Tukey's multiple comparisons post-hoc test was used. For survival curves, a log-rank (Mantel–Cox) test was used for curve comparison. For all statistical tests, $P < 0.05$ was considered statistically significant; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Ethics statement

Our research complies with all relevant ethical regulations. Animal studies were approved by the Veterinary Office Kanton Zurich and the Landesuntersuchungsamt Rheinland-Pfalz. Human studies were approved by the Ethics Committee of the University of Tübingen and the Ethics Committee of Eastern Switzerland. All participants provided written informed consent before study inclusion and were not financially compensated.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data needed to evaluate the conclusions of the paper are present in the main text or the Supplementary Information. Additional data are available from the authors upon request. Correspondence and requests for materials should be addressed to the corresponding authors. Source data are provided with this paper.

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Acknowledgements

This work was supported by the University of Zurich; the Department of Dermatology at the University Hospital of Zurich; Swiss National Science Foundation NRP 78 program, grant number 4078PO_198321 (to S.P.); Innosuisse ('Development of a novel delivery system for mRNA COVID-19 vaccines', application number 52910.1 IP-LS; to S.P.); NCCR RNA&Disease (to S.P.); Swiss Cancer Research (KFS-5271-02-2021 'Enhancing therapy of cancer using immuno-chemotherapeutic RNA nanoparticles and immune check-point inhibition'; to S.P.); Kelm Stiftung; EU grants NEWmRNA (Horizon 2020 research and innovation programme number 965135) and REGeRNA (Horizon Europe number 101057318) (to S.P.); Swiss National Science Foundation, grant number 310030_215553 (to S.P.); Deutsche Forschungsgemeinschaft (DFG; German Research Foundation) under Germany's Excellence Strategy – EXC 2180 – 390900677 (to C.G.); and Collaborative Research Center (CRC) 1292, TP17 – 'Neo-epitope specific CD4$^+$ T cells—anti-tumoral mechanisms and their modulation' (to M.D. and I.G.Y.).

Author contributions

J.F., D.E., N.T.J., M.D., C.G., L.F., U.S. and S.P. conceptualized the study. Methodology was developed by J.F., D.E., N.T.J., I.G.Y., M.T.A., M.D., C.G. and S.P. Validation of experimental parameters was performed by J.F., D.E., N.T.J., I.G.Y., M.T.A., M.D., C.G., L.F. and S.P. Formal data analysis was conducted by J.F., D.E., N.T.J., L.F., M.D., C.G. and S.P. Resources for the study were provided by M.D., U.S., C.G., L.F., T.M.K. and S.P. The original draft of the paper was written by J.F., M.M. and S.P. Writing, review and editing of the final paper were performed by J.F., M.M., D.E., C.G., M.D., L.F., T.M.K., U.S. and S.P. Funding for this research was acquired by C.G., L.F., U.S., S.P. and M.M. All authors have read and agreed to the published version of the article.

Funding

Open access funding provided by University of Zurich.

Competing interests

S.P. is an inventor on patent WO2021038089A1 'Minimal Messenger RNAs and uses thereof', University of Zurich. M.D. and D.E. are employees and U.S. is cofounder and management board member at BioNTech SE (Mainz, Germany). M.D., D.E. and U.S. hold securities from BioNTech SE. The other authors declare no competing interests.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41551-026-01738-z.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41551-026-01738-z.

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Peer review information *Nature Biomedical Engineering* thanks Jeff Coller, Ru-Yi Zhu and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

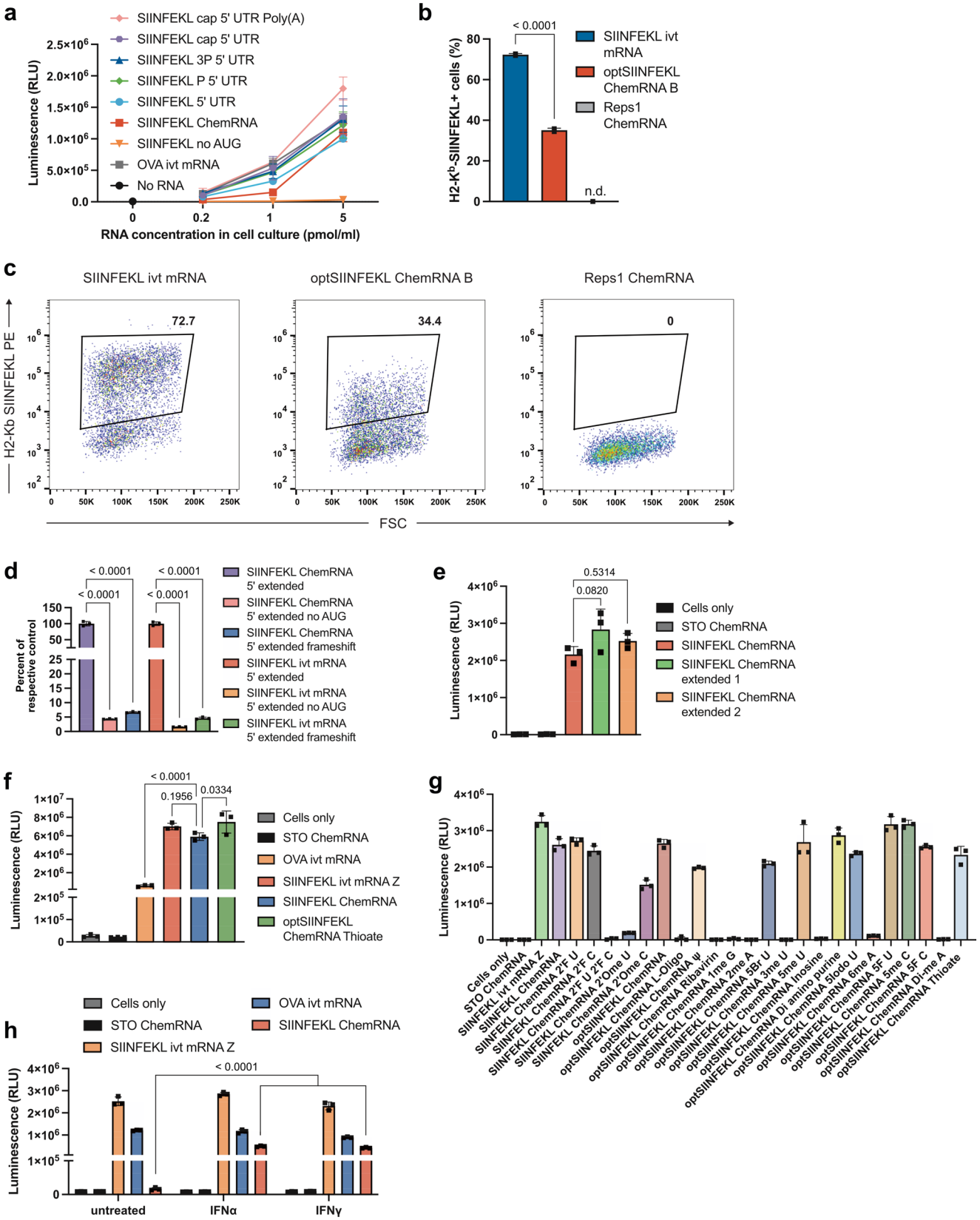
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https://doi.org/10.1038/s41551-026-01738-z

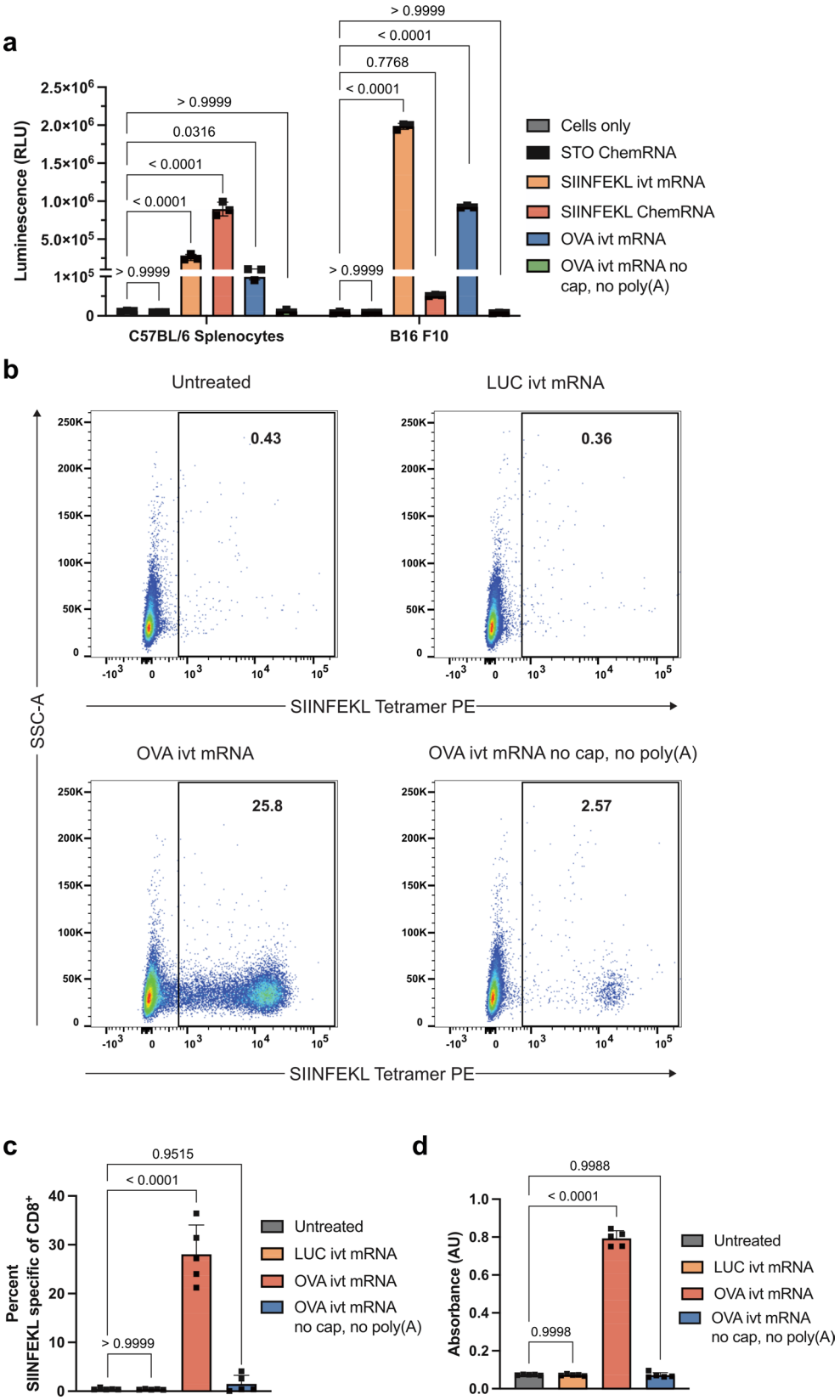


Extended Data Fig. 1 | See next page for caption.

https://doi.org/10.1038/s41551-026-01738-z

Extended Data Fig. 1 | Functionality of ChemRNAs in immune and non-immune cells. a, Repetition of Fig. 1b and c with equimolar RNA amounts transfected in DC2.4 and co-cultured with B3Z. **b, c**, DC2.4 cells were seeded at 5×10^4 cells per well in 96-well plates and transfected with 500 ng RNA and incubated for 24 hours before staining with the Kb-SIINFEKL specific monoclonal antibody 25D1.16 and FACS analysis. (**b**) Percentage of H2-Kb SIINFEKL positive cells. Data represent mean ± SD of two technical replicates. (**c**) Representative Pseudocolour plots. Results are representative of two independent experiments. n.d. = not detected. Gating strategy: Single cells (FSC-H vs. FSC-A) -> Living cells (FSC-A vs. Viability Dye) -> FSC-H vs. SSC-H -> Kb-SIINFEKL positive cells (FSC-A vs. H2-Kb SIINFEKL antibody). **d**, Testing of translation fidelity of ivt mRNA or ChemRNA coding for SIINFEKL. ChemRNA designs were normalized to SIINFEKL ChemRNA 5′ extended (100%) and ivt mRNA designs were normalized to SIINFEKL ivt mRNA 5′ extended, background was set to 0%. **e**, Like Fig. 1f, testing of extended SIINFEKL ChemRNA sequences using the B3Z (tumour cell line) readout instead of the OT-1 (splenocytes) readout. (**e**) 100,000 C57BL/6 splenocytes or (**a, d, f, g**) 50,000–100,000 DC2.4 cells were mixed with 100,000 B3Z cells and transfected with 1 µg/ml of ChemRNA coding SIINFEKL. (**h**) 100,000 B16 F10 cells were treated with either 100 ng/ml IFN-α or IFN-γ and transfected with 1 µg/ml of ChemRNA or ivt mRNA coding SIINFEKL or Ovalbumin (OVA). After 24 hours, the culture medium was exchanged and 100,000 B3Z cells were added. (**a, d-h**) 24 hours later, as a readout for T cell activation, β-Galactosidase was detected with the Invitrogen™ Galacto-Light Plus™ β-Galactosidase Reporter Gene Assay System (Thermo Fisher Scientific). n = 3 replicates for each group. Data are representative of three independent experiments and are presented as mean ± SD. (**d-f**) One-way or (**h**) two-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used to determine the significance between the different groups (p values indicated).

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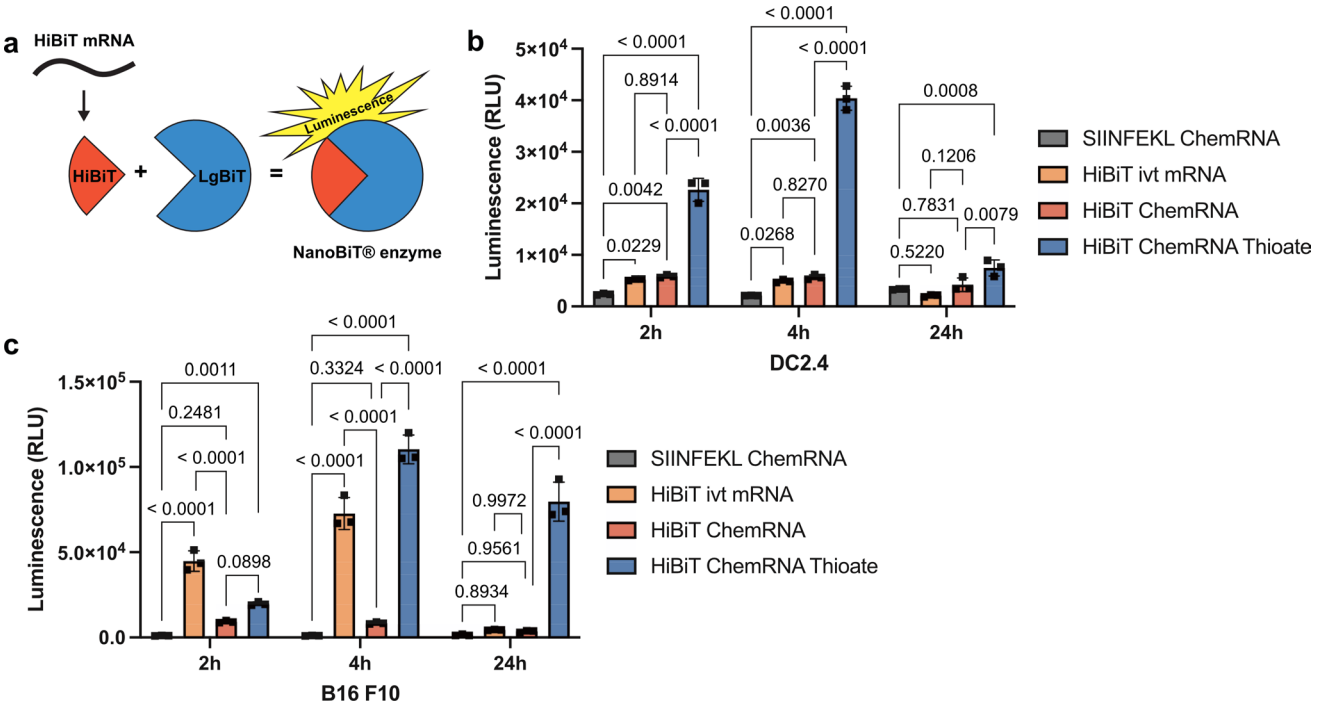


Extended Data Fig. 2 | See next page for caption.

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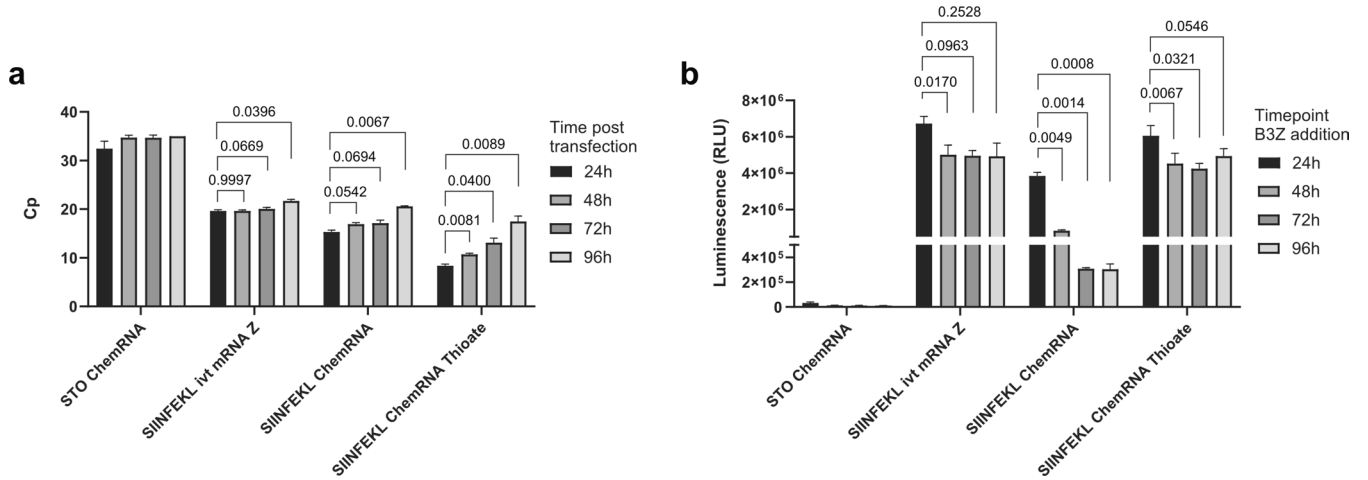
Extended Data Fig. 2 | Evaluation of non-capped and non-polyadenylated ivt mRNA coding for the full OVA protein in vitro and in vivo. a, 100,000 C57BL/6 splenocytes or B16 F10 cells were mixed with 100,000 B3Z cells and transfected with 1 µg/ml mRNA. N = 3 replicates for each group. Data are representative of three independent experiments and are presented as mean ± SD. **b-d**, 6-week-old female C57BL/6 mice were vaccinated 3 times by sub-cutaneous administration of 2 µg of mRNA formulated in LNPs. (**b**) Representative dot plots of SIINFEKL specific blood CD8+ T cells 7 days after the last vaccination. Gating strategy was: single cells (FSC-H/FSC-A), lymphocytes (SSC-A/FSC-A), living cells (SSC-A/Zombie NIR™), CD3+ (SSC-A/CD3), CD4-/CD8a+ (CD4/CD8a). Numbers on the right show percentages of SIINFEKL Tetramer+ CD8+ T cells. (**c**) Quantification of SIINFEKL specific CD8+ T cells. (**d**) ELISA quantification of IgG antibodies against OVA 7 days after the last vaccination. (**c, d**) n = 5 animals per treatment group. Data of one independent experiment are presented as mean ± SD. (**a**) Two-way or (**c, d**) one-way analysis of variance (ANOVA) with (**a**) Šídák's or (**c, d**) Tukey's multiple comparison test was used to assess the significance between treatment groups (p values indicated).

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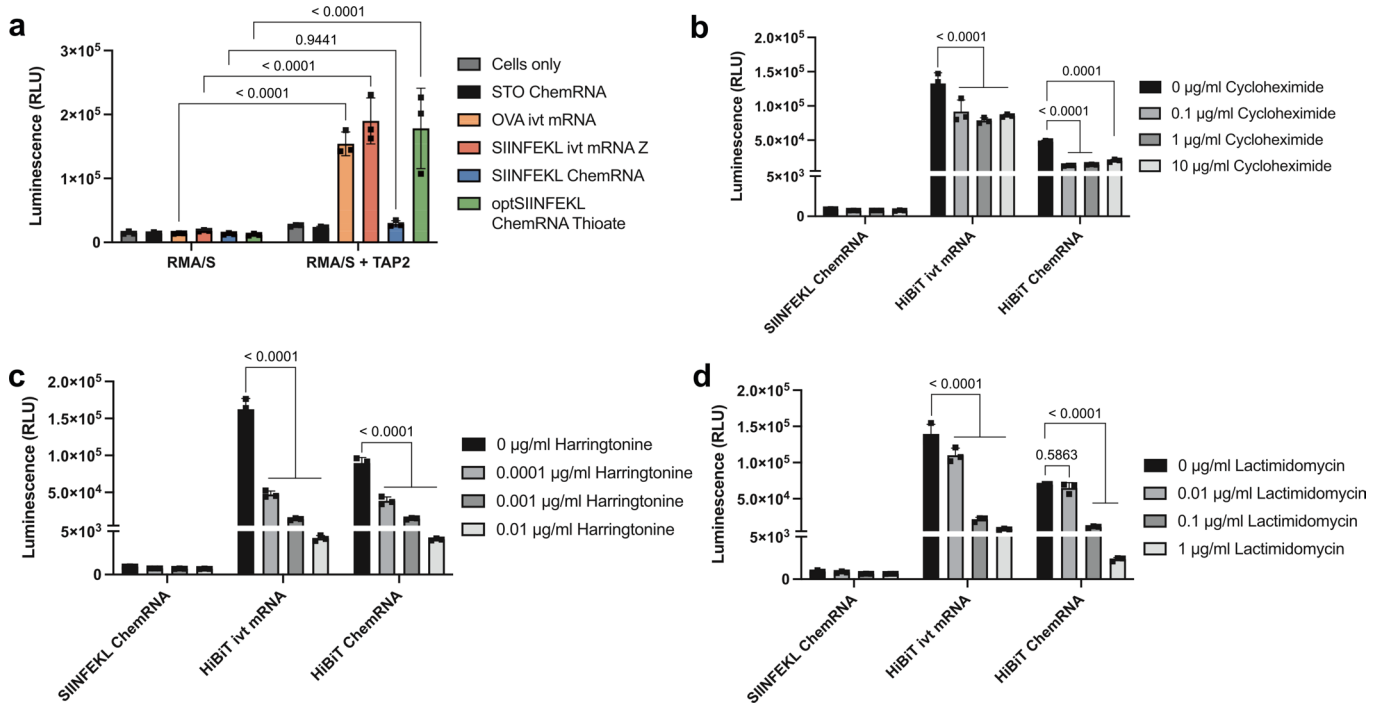
Extended Data Fig. 3 | Translation efficiency of ChemRNA coding for HiBiT is different between immune and non-immune cells. a, As a direct read-out of the translation efficiency from the different mRNAs, HiBiT, an 11-amino-acid peptide complementing Large BiT (LgBiT), the large subunit used in NanoLuc® Binary Technology (NanoBiT®) was used. (**b**) DC2.4 cells or (**c**) B16 F10 cells were seeded at 100,000 cells per well of a white 96-well plate and transfected with 1 µg/ml of ivt mRNA, ChemRNA or phosphorothioate modified ChemRNA coding for HiBiT. The amount of translated HiBiT protein in the cells was determined by measuring luminescence 2, 4 and 24 hours after transfection. n = 3 samples for each group. Data are representative of three independent experiments and are presented as mean ± SD. Two-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used to determine the significance between the different groups (p values indicated).

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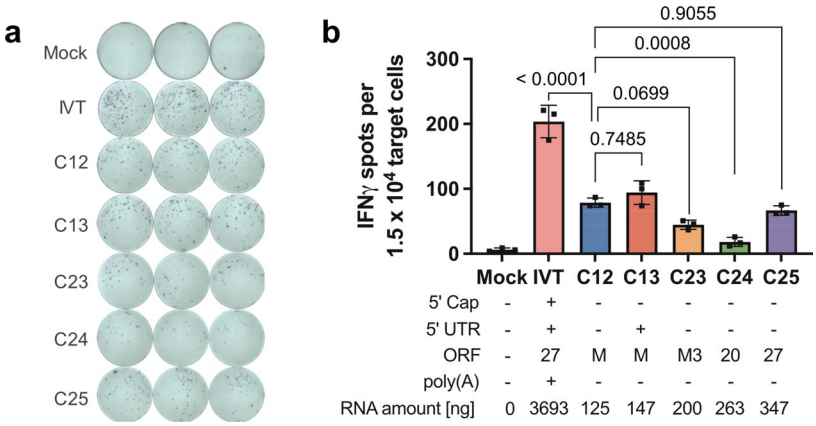
Extended Data Fig. 4 | Stability of ChemRNA and ivt mRNA coding for SIINFEKL. a, RT-qPCR of RNA extracted from DC2.4 cells. **b**, Stability of MHC class I presentation on DC2.4 cells. (**a**) 10,000 or (**b**) 20,000 DC2.4 cells were transfected with (**a**) 50 pmol/ml or (**b**) 1 µg/ml of ivt mRNA or ChemRNA coding for SIINFEKL. 4 hours later, cells were washed and after 24, 48, 72 or 96 hours (**a**) DC2.4 cells were processed for RNA extraction or (**b**) B3Z cells were added. (**a, b**) n = 3 samples for each group. Data are representative of three independent experiments and are presented as mean ± SD. Two-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used to determine the significance between the different groups (p values indicated).

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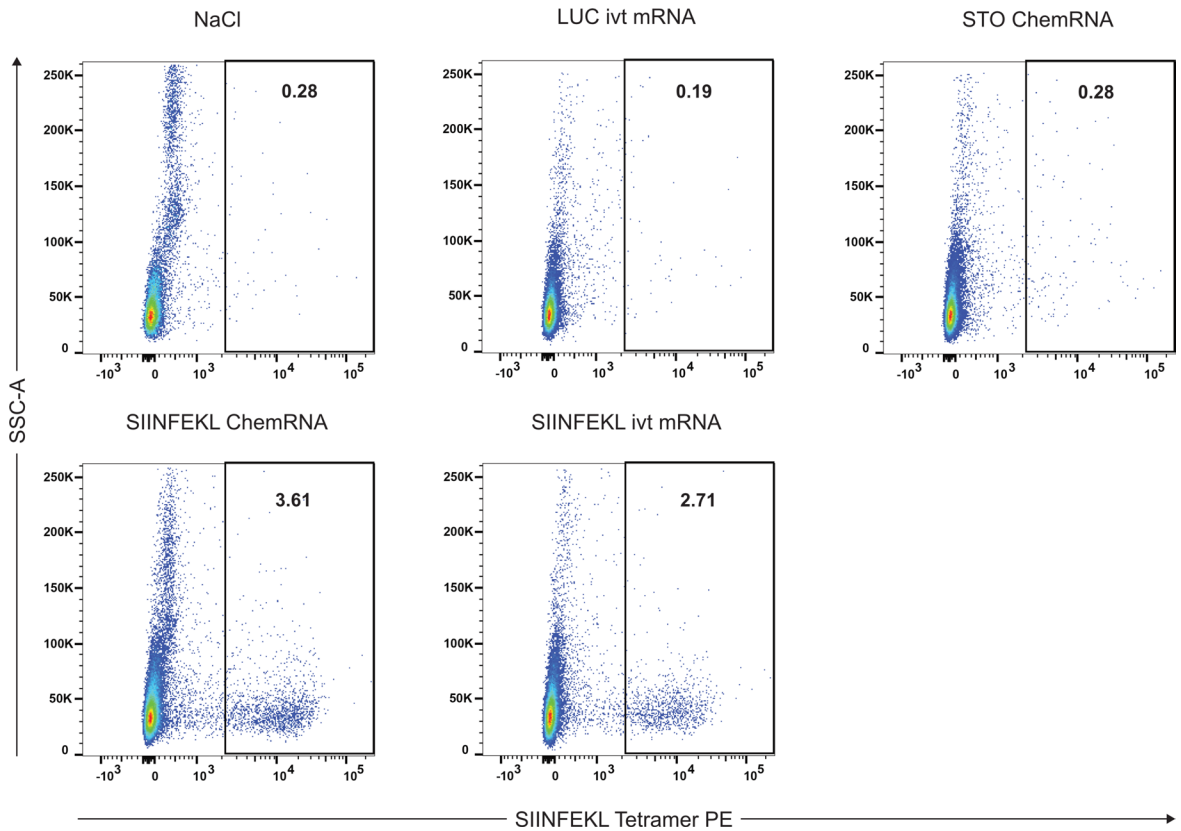
Extended Data Fig. 5 | ChemRNA and ivt mRNA translation is inhibited by ribosome inhibitors and MHC class I presentation of peptides generated from ChemRNA is TAP dependent. a, 100,000 RMA/S cells or RMA/S cells electroporated with TAP2 mRNA were lipofected one day later with ivt mRNA or ChemRNA coding for SIINFEKL. After 24 hours 100,000 B3Z cells were added and incubated another 24 hours before the readout. **b-d**, B16 F10 cells were treated with different concentrations of the ribosome inhibitors (**b**) cycloheximide, (**c**) harringtonine or (**d**) lactimidomycin before transfection with ivt mRNA or ChemRNA coding for HiBiT. The amount of translated HiBiT peptide was quantified 2 hours after transfection. (**a-d**) n = 3 samples for each group. Data are representative of three independent experiments and are presented as mean ± SD. Two-way analysis of variance (ANOVA) with (**a**) Šídák's or (**b-d**) Dunnett's multiple comparison test was used to determine the significance between the different groups (p values indicated).

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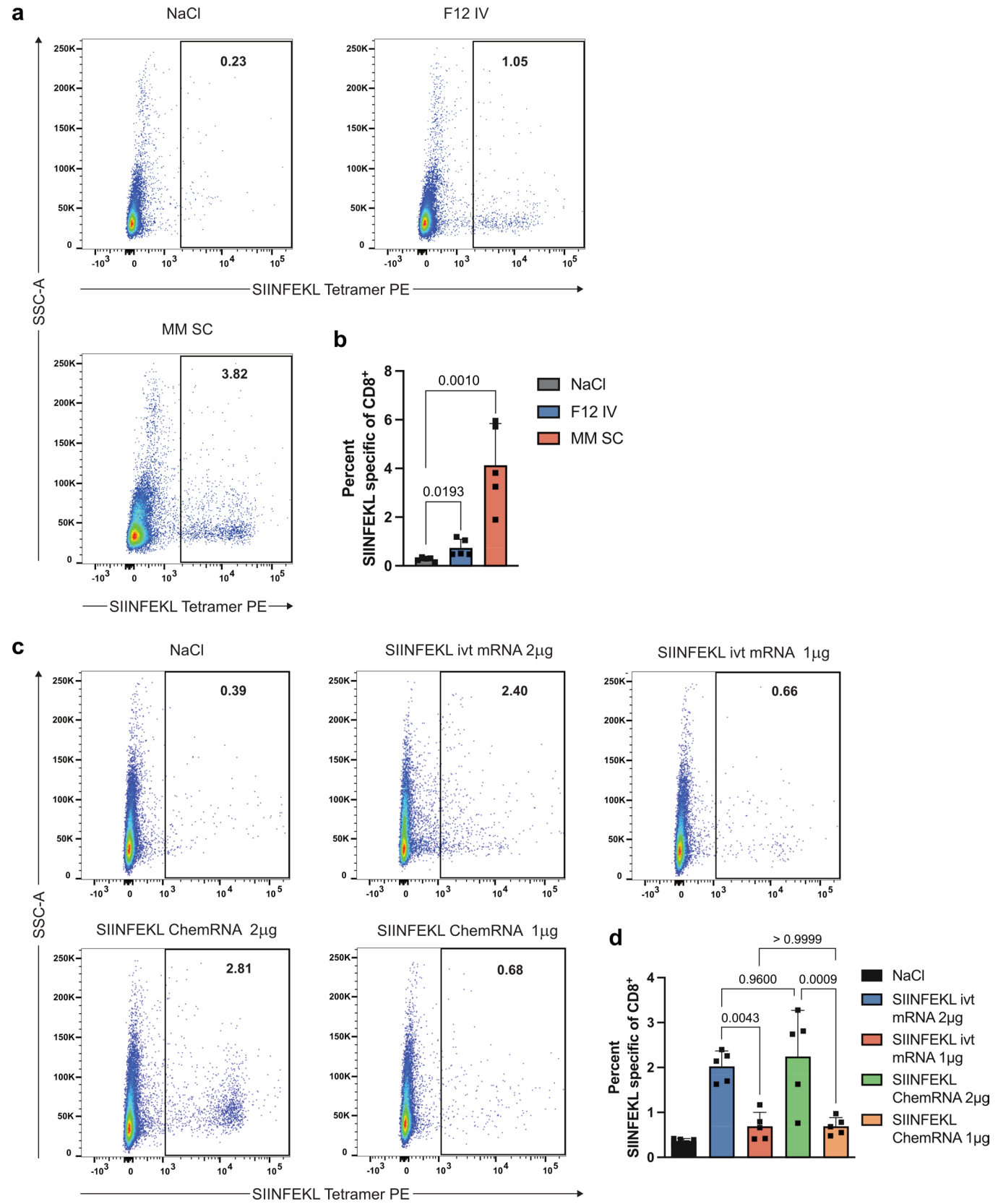
Extended Data Fig. 6 | Expanding the minimal epitope does not lead to increased T-cell activation. a,b, Splenocytes of C57BL/6 animals (100,000 cells per well) were isolated 6 days after the third ivt RNA-LPX vaccination and co-cultured with BMDCs lipofected with 13 pmol ChemRNA or ivt mRNA coding for Adpgk. IFN-γ secretion was assessed 16 hours post co-culture using an ELISpot assay. (**a**) Image of ELISpot wells. (**b**) Quantification of IFN-γ spots. n = 3 samples for each group. Data are shown as mean ± SD from a single experiment with three technical replicates. One-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used to determine the significance between the different groups (p values indicated). Mock: non-lipofected BMDCs; IVT: in vitro transcription; C: Construct; 5′ Cap: m7(3′OMeG)(5′)ppp(5′)(2′OMeA)pG; 5′ UTR: TISU sequence; poly(A): 18 adenosines; ORF 27: Minimal epitope extended to a 27mer with the mutation located in the center; ORF M: Minimal epitope; ORF M3: Minimal epitope extended by 3 flanking amino acids N- and C-terminally; ORF 20: Minimal epitope extended to a 20mer with the mutation located in the center.

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Extended Data Fig. 7 | Representative dot plots for SIINFEKL tetramer staining from Fig. 4d. Representative dot plots of SIINFEKL specific blood CD8+ T cells 7 days after the last vaccination. Gating strategy was: lymphocytes (SSC-A/FSC-A), CD3+ (SSC-A/CD3), living cells (SSC-A/Zombie NIRTM), CD4-/CD8a+ (CD4/CD8a). Numbers on the right show percentages of SIINFEKL Tetramer+ CD8+ T cells.

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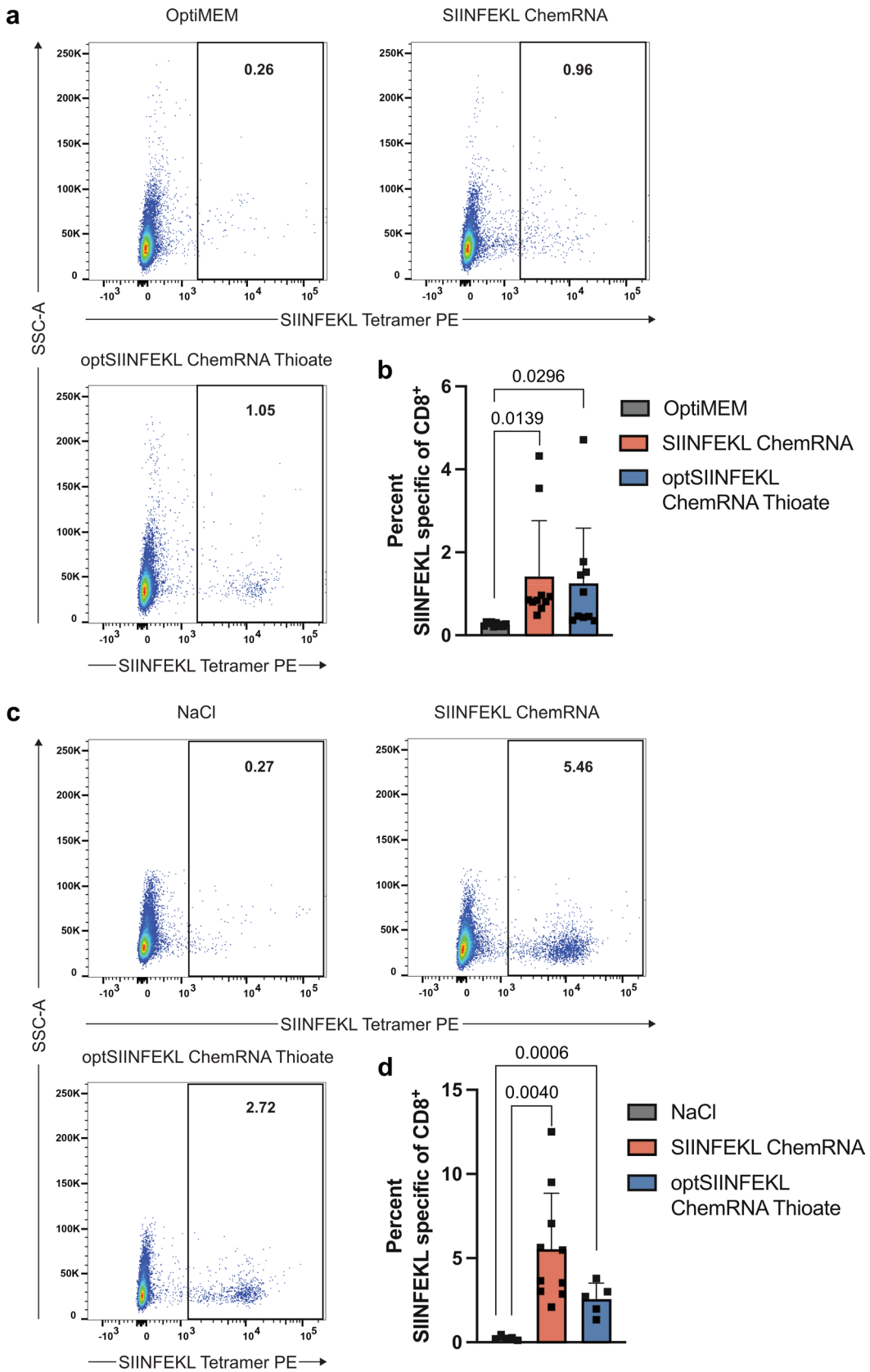


Extended Data Fig. 8 | See next page for caption.

https://doi.org/10.1038/s41551-026-01738-z

Extended Data Fig. 8 | Comparison of different liposome formulations and injections sites for vaccination. a-d, 6-week-old female C57BL/6 mice were vaccinated 3 times either by (**a, b**) intra-venous administration with 2 µg of ChemRNA coding for SIINFEKL formulated in lipoplex (F12 IV) or by (**a-d**) subcutaneous administration with (**a, b**) 2 µg of ChemRNA or (**c, d**) 1 or 2 µg of either ivt mRNA or ChemRNA formulated in Lipofectamine MessengerMAX (MM SC). (**a, c**) Representative dot plots of SIINFEKL specific blood CD8+ T cells 7 days after the last vaccination. Gating strategy was: single cells (FSC-H/FSC-A), lymphocytes (SSC-A/FSC-A), living cells (SSC-A/Zombie NIRTM), CD3+ (SSC-A/CD3), CD4-/CD8a+ (CD4/CD8a). Numbers on the right show percentages of SIINFEKL Tetramer+ CD8+ T cells. (**b, d**) Quantification of SIINFEKL specific CD8+ T cells. (**a-d**) n = 5 animals per treatment group. Data of one independent experiment are presented as mean ± SD. (**b**) Unpaired, two-tailed Student's t-test or (**d**) one-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used to assess the significance between each treatment group and the untreated negative control group (p values indicated).

https://doi.org/10.1038/s41551-026-01738-z



Extended Data Fig. 9 | See next page for caption.

https://doi.org/10.1038/s41551-026-01738-z

Extended Data Fig. 9 | Phosphorothioate modified ChemRNA induces antigen specific T cells in vivo. a-d, 6-week-old female C57BL/6 mice were vaccinated either (**c, d**) 2 or (**a, b**) 3 times by sub-cutaneous administration with either (**a, b**) 2 µg or (**c, d**) 10 µg of unmodified ChemRNA (SIINFEKL ChemRNA) or phosphorothioate modified ChemRNA (SIINFEKL ChemRNA Thioate) coding for SIINFEKL, both formulated either in (**a, b**) Lipofectamine MessengerMAX or (**c, d**) F12. (**a, c**) Representative dot plots of SIINFEKL specific blood CD8+ T cells 7 days after the last vaccination. Numbers on the right show percentages of SIINFEKL Tetramer+ CD8+ T cells. Gating strategy was: single cells (FSC-H/FSC-A), lymphocytes (SSC-A/FSC-A), living cells (SSC-A/Zombie NIRTM), CD3+ (SSC-A/CD3), CD4-/CD8a+ (CD4/CD8a). Numbers on the right show percentages of SIINFEKL Tetramer+ CD8+ T cells. (**b, d**) Quantification of SIINFEKL specific CD8+ T cells. (**a, b**) n = 10 animals per treatment group, (**c, d**) n = 5 animals for NaCl and SIINFEKL ChemRNA Thioate or n = 10 animals for SIINFEKL ChemRNA. Data of one independent experiment are presented as mean ± SD. Unpaired, two-tailed Student's t-test was used to assess the significance between each treatment group and the untreated negative control group (p values indicated).

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Last updated by author(s): Apr 7, 2026

Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
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☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
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☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	EXCEL, BD FACSDIVA Software
Data analysis	FlowJo v10.6.2; ImmunoCapture V6.3; GraphPad Prism 8

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

All data needed to evaluate the conclusions of the paper are present in the Main Text or the Extended Data. Additional data are available from the authors upon request. Correspondence and requests for materials should be addressed to Steve Pascolo, steve.pascolo@usz.ch.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	This information has not been collected
Reporting on race, ethnicity, or other socially relevant groupings	No reporting on race, ethnicity, or other socially relevant groupings
Population characteristics	See above
Recruitment	Patients with metastatic melanoma receiving immune checkpoint inhibitor treatment (anti–PD 1, anti–PD L1, anti-CTLA4, or combination treatment) at the Kantonsspital St. Gallen, Switzerland
Ethics oversight	Healthy volunteers: Blood collection and study were approved by the Ethics Committee of the University of Tübingen (approvals 156/2012B01 and 713/2018BO2), and all participants gave written informed consent" Cancer patients: PBMCs were obtained from a prospective observational cohort (IMIT: ImmunoMonitoring of ImmunoTherapy) of patients with metastatic melanoma receiving immune checkpoint inhibitor treatment (anti–PD 1, anti–PD L1, anti-CTLA4, or combination treatment) at the Kantonsspital St. Gallen, Switzerland. The study was approved by the Ethics Committee of Eastern Switzerland (project number EKOS 2016-00998). All participants provided written informed consent prior to study inclusion.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No sample size calculation was performed. Sample sizes were at least biological triplicate in all cases. Additional samples were conducted to provide a reasonable statistical conclusion balancing both time, ethics and budgetary constraints.
Data exclusions	No data exclusions
Replication	All attempts at replication were successful. Experiments were replicated at separate times by a variety of co-authors on the paper over the duration of the project.
Randomization	All mice were randomly organized into experimental groups.
Blinding	Blinding was not possible due to budgetary constraints.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

All antibodies are summarized in Extended Data Table 3

Validation

Target Fluorophore Dilution Supplier Catalog Nr. Validation
GILGFVFTL
(Influenza) Tetramer HLA-A2 APC 2.5 µg/ml In house - Chandran, P.A. et al. Validation of immunomonitoring methods for application in Clinical Studies: The hla-peptide multimer staining assay. Cytometry Part B: Clinical Cytometry 94(2), 342–353 (2016).
Hadrup, S.R. et al. Cryopreservation of mhc multimers: Recommendations for quality assurance in detection of antigen specific t cells. Cytometry Part A 87(1), 37–48 (2014).

NLVPMVATV
(CMV-pp65) Tetramer HLA-A2 BV-785 2.5 µg/ml In house - Chandran, P.A. et al. Validation of immunomonitoring methods for application in Clinical Studies: The hla-peptide multimer staining assay. Cytometry Part B: Clinical Cytometry 94(2), 342–353 (2016).
Hadrup, S.R. et al. Cryopreservation of mhc multimers: Recommendations for quality assurance in detection of antigen specific t cells. Cytometry Part A 87(1), 37–48 (2014).

RPHE (CMV-pp65) Tetramer HLA-B7 BV-785 2.5 µg/ml In house - Chandran, P.A. et al. Validation of immunomonitoring methods for application in Clinical Studies: The hla-peptide multimer staining assay. Cytometry Part B: Clinical Cytometry 94(2), 342–353 (2016).
Hadrup, S.R. et al. Cryopreservation of mhc multimers: Recommendations for quality assurance in detection of antigen specific t cells. Cytometry Part A 87(1), 37–48 (2014).

TRPV
(CMV-pp65) Tetramer HLA-B7 PE 2.5 µg/ml In house - Chandran, P.A. et al. Validation of immunomonitoring methods for application in Clinical Studies: The hla-peptide multimer staining assay. Cytometry Part B: Clinical Cytometry 94(2), 342–353 (2016).
Hadrup, S.R. et al. Cryopreservation of mhc multimers: Recommendations for quality assurance in detection of antigen specific t cells. Cytometry Part A 87(1), 37–48 (2014).

Zombie Aqua BV 510 1:200 BioLegend 423101 https://www.biolegend.com/de-de/products/zombie-aqua-fixable-viability-kit-8444
CD4 APC-Cy7 1:100 BD Biosciences 557871 https://www.bdbiosciences.com/en-ch/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/apc-cy-7-mouse-anti-human-cd4.557871
CD8 PE-Cy7 1:400 Beckman Coulter 301040
Fixable Viability Stain 510 BV510 1:1000 Becton Dickinson 564406
https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/fixable-viability-stain-510.564406
CD3 APC-Cy7 1:100 BioLegend 317342
CD4 PE-Cy7 1:100 BioLegend 317414
CD8 PerCP, 1:100 BioLegend 344708
CD45RA BV711 1:400 BioLegend 304138
IFNγ PE 1:100 Thermo Fisher Scientific MHCIFG04 https://www.thermofisher.com/antibody/product/IFN-gamma-Antibody-clone-B27-Monoclonal/MHCIFG04
TNF APC 1:100 BioLegend 502912
CD40L FITC 1:100 BioLegend 310804
IL-17A BV421 1:100 BD Biosciences 562933
Zombie NIR APC-Cy7 1:100 BioLegend 423105 https://www.biolegend.com/de-de/products/zombie-nir-fixable-viability-kit-8657
SIINFEKL Tetramer H-2Kb PE 1:20 The Tetramer Shop MKb-001
CD3 FITC 1:100 BioLegend 100203 https://www.biolegend.com/nl-be/products/fitc-anti-mouse-cd3-antibody-45
CD4 APC 1:100 BioLegend 100515 https://www.biolegend.com/nl-nl/products/apc-anti-mouse-cd4-antibody-477
CD8a Pacific Blue 1:100 BioLegend 100728 https://www.biolegend.com/de-de/products/pacific-blue-anti-mouse-cd8a-antibody-2856
Fixable Viability Dye eFluor™ 780 APC-eFluor™ 780 1:1600 eBioscience 65-0865-14 https://www.thermofisher.com/order/catalog/product/65-0865-14
Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™) N/A 1:100 BD Bioscience 553142 https://www.bdbiosciences.com/en-nl/products/reagents/western-blotting-and-molecular-reagents/purified-rat-anti-mouse-cd16-cd32-mouse-bd-fc-block.553142?tab=product_details
anti-mouse H-2Kb bound to SIINFEKL PE 1:100 BioLegend 141603 https://www.biolegend.com/fr-ch/products/pe-anti-mouse-h-2kb-bound-to-siinfekl-antibody-7247
Rat Anti-Mouse CD45 BUV395 1:100 BD Bioscience 564279 https://www.bdbiosciences.com/en-eu/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/buv395-rat-anti-mouse-cd45.564279?tab=product_details
Rat Anti-Mouse CD90.2 BUV496 1:800 BD Bioscience 741046 https://www.bdbiosciences.com/en-dk/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/buv496-rat-anti-mouse-cd90-2.741046?tab=product_details

Rat Anti-Mouse CD4 BUV615 1:800 BD Bioscience 613006 https://www.bdbiosciences.com/en-de/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/buv615-rat-anti-mouse-cd4.613006?tab=product_details
Rat Anti-Mouse CD8a BUV737 1:400 BD Bioscience 612759 https://www.bdbiosciences.com/en-pt/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/buv737-rat-anti-mouse-cd8a.612759?tab=product_details
Rat Anti-Mouse CD19 BUV661 1:400 BD Bioscience 612971 https://www.bdbiosciences.com/en-de/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/buv661-rat-anti-mouse-cd19.612971?tab=product_details
Rat Anti-Mouse FoxP3 PE-eFluor610 1:100 eBioscience 61-5773-82 https://www.thermofisher.com/antibody/product/FOXP3-Antibody-clone-FJK-16s-Monoclonal/61-5773-82
EasYmers (H2-Db) N/A 1:9 Immunaware 5001-01 https://immunaware.com/easYmer/h2-db-easymers/
Streptavidin AF647 AF647 Thermo Fisher Scientific S32357 https://www.thermofisher.com/order/catalog/product/S32357

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	All primary murine cell lines were derived from either C57BL/6 or Rag2/OT-1 mice as listed in the manuscript. All mouse experiments were performed according to the governmental and institutional guidelines. Our studies "Anti-cancer therapies based on RNA" (license number ZH215/2017) and "Testing mRNA formats and formulations for anti-cancer vaccinations" (license number ZH158/2022) were approved by the Veterinary Office (Kanton Zürich, Health Direction, Veterinary Office, Zollstrasse 20; 8090 Zurich). Primary human PBMCs were acquired from the blood of healthy donors or melanoma patients. Healthy volunteers: Blood collection and study were approved by the Ethics Committee of the University of Tübingen (approvals 156/2012B01 and 713/2018BO2), and all participants gave written informed consent" Cancer patients: PBMCs were obtained from a prospective observational cohort (IMIT: ImmunoMonitoring of ImmunoTherapy) of patients with metastatic mel anoma receiving immune checkpoint inhibitor treatment (anti–PD 1, anti–PD L1, anti-CTLA4, or combination treatment) at the Kantonsspital St. Gallen, Switzerland. The study was approved by the Ethics Committee of Eastern Switzerland (project number EKOS 2016-00998). All participants provided written informed consent prior to study inclusion. B16 F10 and B16/OVA were obtained from ATCC. DC2.4 were purchased from Creative Biolabs CAT#: NCL2110P098. RMA/S were provided by Thomas Look, Department of Dermatology, University Hospital Zürich, Switzerland. The OVA(257–264) (SIINFEKL) specific, H-2Kb-restricted CTL hybridoma, B3Z was provided by Dr. N. Shastri, University of California, Berkeley, USA.
Authentication	B3Z, B16, RMA/S and B16/OVA were not authenticated prior to being used.
Mycoplasma contamination	All cell lines used in the manuscript tested negative for mycoplasma.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified lines were used in this study.

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals	C57BL/6J mice were were purchased from Envigo (Netherlands). C57BL/6-Tg(TcraTcrb)1100Mjb mice (Rag2/OT-1 mice), were a generous gift from Prof. Pål Johansen (University Hospital Zurich) or purchased from Taconic Biosciences.
Wild animals	No wild animals were used in this study
Reporting on sex	Sex was not considered in study design
Field-collected samples	No field-collected samples were used in this study.
Ethics oversight	Our studies "Anti-cancer therapies based on RNA" (license number ZH215/2017) and "Testing mRNA formats and formulations for anti-cancer vaccinations" (license number ZH158/2022) were approved by the Veterinary Office (Kanton Zürich, Health Direction, Veterinary Office, Zollstrasse 20; 8090 Zurich). The Reps1 animal study was approved by the responsible authority Landesuntersuchungsamt Rheinland-Pfalz, Germany under the protocol number G22-12-061. Animals were purchased from Envigo (Netherlands). C57BL/6-Tg(TcraTcrb)1100Mjb mice (Rag2/OT-1 mice), were a generous gift from Prof. Pål Johansen (University Hospital Zurich) or purchased from Taconic Biosciences.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks

Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.

Novel plant genotypes

Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.

Authentication

Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosiacism, off-target gene editing) were examined.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

All antibodies used for FACS experiments are presented in Extended data Table 3. For the flow cytometry staining to analyse H2-Kb SIINFEKL presentation, flow cytometry buffer was prepared by combining 488 ml PBS, 10 ml heat-inactivated FBS (2% final), 2 ml 0.5 M EDTA (2 mM final), and 0.5 ml 10% sodium azide. DC2.4 cells were transferred to 96-well U-bottom plates and pelleted by centrifugation at 460 × g for 4 minutes at room temperature. Supernatants were discarded. Cell pellets were resuspended by brief vortexing and washed with 200 µl PBS, followed by centrifugation and supernatant removal. For viability assessment and blocking of Fc-receptors, 100 µl live/dead stain containing Fixable Viability Dye eFluor™ 780 and Mouse BD Fc Block™ was added to each well, mixed by pipetting, and incubated for 15 minutes at 4°C. After washing with 100 µL PBS and centrifugation, cells were stained with 50 µl extracellular antibody (PE anti-mouse H-2Kb bound to SIINFEKL) for 30 minutes at 4°C. Following antibody staining, cells were washed with 200 µL flow cytometry buffer and fixed with 150 µL 2% paraformaldehyde (diluted from 4% ROTI®Histofix, Carl Roth) for 15 minutes at 4°C. Fixed cells were pelleted, resuspended in 200 µL flow cytometry buffer, and analyzed by flow cytometry using a BD FACSCelesta Cell Analyzer. Data was analyzed using FlowJo Version 10.10.0. Gating strategy: Single cells (FSC-H vs. FSC-A) -> Living cells (FSC-A vs. Viability Dye) -> FSC-H vs. SSC-H -> Kb-SIINFEKL positive cells (FSC-A vs. H2-Kb SIINFEKL antibody).

For the experiment with PBMCs from healthy donors, 5 µl of the RNA-MessengerMax preparation were added to 1 ml of previously characterized (using tetramer staining) PBMC suspension (in IMDM containing 10% heat inactivated human serum, Pen/Strep and 50 µM 2-mercaptoethanol) and incubated for 12 days (split on day 4) with addition of IL-2 2ng/ml (R&D Systems, Minneapolis, MN, USA) every other day until staining and FACS analysis as described in Eckert et al.36. 1.5 x 106 cells were stained per test with first 2.5 µg/ml of the tetramers (in-house production) for 30 min at RT, then with Zombie Aqua (1:200, BioLegend), CD4 APC-Cy7 (1:100, Becton Dickinson) and CD8 PE-Cy7 (1:400, Beckman Coulter, Brea, CA, USA) for 20 min at 4°C. After a final wash, cells were resuspended in FACS buffer and acquired directly on a LSRFortessaTM SORP (Becton Dickinson) equipped with DIVA (Version 6.1.3).

For the FACS experiment with PBMCs from melanoma patients, the cryopreserved PBMCs were resuspended in RPMI containing 8% human serum (Biowest), 1% penicillin-streptomycin, 1% L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 0.1 mg/ml kanamycin and 0.1% 2-β-mercaptoethanol and plated at a concentration of 0.5x106 cells/ml in low-adherence 24-well plates (Sarstedt, Nümbrecht, Germany) and maintained at 37°C, 5% CO2. The following day, either 4 µg single peptide MART-1 (ELAGIGILTV, JPT Peptide Technologies GmbH, Berlin, Germany) or 1 µg of ChemRNA coding for MART-1 (20 µl of the RNA-MessengerMax preparation) were added to the cells. After 48 hours, 1 ml of medium was replaced with fresh RPMI (as above) containing IL-2 (final concentration 150 U/ml). Cells were kept in culture for 10 days and 1 ml medium was replaced with fresh IL-2-containing medium every other day. On day 10, cells were collected from the plates, washed and transferred to 96-well plates where they were re-stimulated with 0.4 µg of the single peptide or 0.1 µg of ChemRNA in the absence of IL-2. Brefeldin A was added 1 hour after adding the peptide or the ChemRNA, at a final concentration of 10 µg/ml. After an incubation for 6 hours at 37°C, 5% CO2, cells were washed, stained for viability (BV510, Becton Dickinson) and incubated with fluorescently-labelled antibodies recognizing CD3 (APC-Cy7, 1:100, Lucerna Chem GmbH, Luzern, Switzerland), CD4 (PE-Cy7, 1:100, Lucerna Chem GmbH), CD8 (PerCP, 1:100, Lucerna Chem GmbH) and CD45RA (BV711, 1:400, Lucerna Chem GmbH) in FACS buffer. Cells were then fixed and permeabilized using Cytofix/ Cytoperm (Becton Dickinson) and incubated with fluorescently-labelled antibodies recognizing IFN-γ (PE, 1:100, Thermo Fisher Scientific), TNF-α (APC, 1:100, BioLegend, San Diego CA, USA), CD40L (FITC, 1:100, BioLegend) and IL-17A (BV421, 1:100, BioLegend) in Perm buffer (2 % FCS, 0.5 % saponin in PBS). Samples were acquired on a LSRFortessa (Becton Dickinson) and data were analyzed using FlowJo™ software (Version 10.6.2, Becton Dickinson). IFN-γ and TNF-α production were analyzed to identify antigen-specific T cells.

Single cell suspension preparation and staining for FACS
200 µl of whole blood was treated with 2 ml of eBioscience™1x RBC Lysis Buffer (Invitrogen, USA) and incubated for 5 min at RT, while vortexing every minute. Next, the cells were centrifuged at 500 g for 5 min, washed twice with 2 ml of PBS and resuspended in 50 µl PBS. The murine lymphocytes were stained with Zombie NIR™ Fixable Viability Kit (BioLegend) for 20 min at RT in the dark. After washing with 200 µl PBS, cells were resuspended in 50 µl PBS with 5 µl PE-conjugated SIINFEKL Tetramer H-2Kb (The Tetramer Shop, Kongens Lyngby, Denmark) and incubated for 15 min at RT in the dark. Without washing, 50 µl of FITC-conjugated anti-CD3, Pacific Blue™-conjugated anti-CD8a and APC-conjugated anti-CD4 antibodies (BioLegend, San Diego CA, USA) at a final dilution of 1:100 in PBS were added and the cells were incubated for 1 hour at 4°C in the dark. In a next step, cells were washed twice with 200 µl PBS and fixed with 1% paraformaldehyde (PFA) in PBS for 30 min at 4°C in the dark. Subsequently, the cells were washed again twice with 200 µl PBS, resuspended in 300 µl PBS and stored at 4°C in the dark until sample acquisition with BD LSRFortessaTM (BD Biosciences, New Jersey, NJ, USA) and BD FACSDiva™ Software (BD Biosciences). The obtained data were analysed using FlowJo™ (BD Biosciences). All antibodies used for FACS experiments are presented in Extended data Table 3.

Tetramer generation for the Reps1 tumour experiment
For customized tetramer production peptide receptive easYmers (Immunaware) were diluted in PBS to reach a concentration of 500 nM. After adding the respective peptides to a final concentration of 3 µM, the monomers were incubated for 48 h at 18ºC. Next, streptavidin-fluorochrome conjugates were added in three consecutive steps to reach a final concentration of 125 nM. In a first step, one third of the total streptavidin-fluorochrome volume was added to the peptide loaded easYmers. The solution was mixed and incubated in the dark for 10 min at 4ºC. Another third of the total streptavidin-fluorochrome volume was added followed by 10 min incubation in the dark at 4ºC. After adding the last third of the total volume, the solution was incubated for another 30 min at 4ºC in the dark. The tetramers were stored at 4ºC until used for flow cytometry staining.

Flow cytometry for the Reps1 tumour experiment
For immunophenotyping of blood samples, 50 µL whole blood were dispensed to a 96-well U bottom plate and 100 µL PBS was added. After centrifugation (3 min, 460 x g, 2–8°C), the supernatants were carefully aspirated, and the cell pellet resuspended in 200 µl Ammonium-Chloride-Potassium (ACK) lysis buffer (erythrocyte lysis). The cell suspension was incubated for 5 min at room temperature, centrifuged (3 min, 460 x g, 4°C) and the supernatant was carefully aspirated. After repeating the erythrocyte lysis steps, the cells were washed once with 200 µL Fluorescence Activated Cell Sorting (FACS) buffer (PBS supplemented with 5 mM EDTA and 5% fetal bovine serum (FBS) and were subsequently stained with antibodies (CD45, CD90.2, CD4, CD8a) and tetramers in 40 µL Brilliant stain buffer for 30 minutes at 2-8°C in the dark. After washing once with 200 µL PBS, cells were resuspended thoroughly in 200 µL Fix/Perm buffer (Foxp3/Transcription Factor Staining Buffer Set) and incubated for 30 minutes at 2–8°C. After washing once with 200 µL Perm/Wash buffer (Foxp3/Transcription Factor Staining Buffer Set; 3 min, 460 x g, 2–8°C), cells were stained with antibodies in 40 µL Perm/Wash buffer for 30 min at 4°C. After washing twice with 200 µL Perm/Wash buffer (3 min, 460 x g, 4°C), cells were resuspended in 200 µL FACS buffer + 33 µL CountBright Absolute Counting Beads and stored at 2–8°C until measurement.
Data were acquired on a BD FACSymphony flow cytometer and analyzed with FlowJo software version 10.10.0. Gating strategy: Single cells (FSC-H vs. FSC-A) → FSC-H vs. SSC-H → CD45+ cells → CD90+/CD19- cells → CD8+ cells → Reps1 specific cells.

Instrument: BD LSR FortessaTM Cell Analyzer

Software: FlowJo™ Version 10

Cell population abundance: No sorting involved in our studies

Gating strategy:
Figure 2: time (time/FSC-A), singlets (FSC-A/FSC-H), living cells (Zombie Aqua/FSC-A), lymphocytes (FSC-A/SSC-A), CD4- (CD4/CD8). Numbers in upper right quadrants show percentages of Tetramer+ CD8+ T cells.

Figure 3c: lymphocytes (SSC-A/FSC-A), CD3+ (SSC-A/CD3), living cells (SSC-A/Zombie Aqua), CD4-/CD8+ (CD4/CD8). Numbers in upper right quadrants show percentages of TNF→/IFNγ double positive CD8+ T cells.

Figure 4d and extended data figure 7: lymphocytes (SSC-A/FSC-A), CD3+ (SSC-A/CD3), living cells (SSC-A/Zombie NIR), CD4-/CD8a+ (CD4/CD8a). Numbers on the right show percentages of SIINFEKL Tetramer+ CD8+ T cells.

Figure 4g: Single cells (FSC-H/FSC-A), FSC-H/SSC-H, CD45+ cells, CD90+/CD19- cells, CD8+ cells, Reps1 specific cells.

Extended data figure 1bc: Single cells (FSC-H vs. FSC-A) -> Living cells (FSC-A vs. Viability Dye) -> FSC-H vs. SSC-H -> Kb-SIINFEKL positive cells (FSC-A vs. H2-Kb SIINFEKL antibody).

Extended data figures 2bc, 8 and 9: single cells (FSC-H/FSC-A), lymphocytes (SSC-A/FSC-A), living cells (SSC-A/Zombie NIR), CD3+ (SSC-A/CD3), CD4-/CD8a+ (CD4/CD8a). Numbers on the right show percentages of SIINFEKL Tetramer+ CD8+ T cells.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.