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The roles of CD4$^+$ T cell help, sex, and dose in the induction of protective CD8$^+$ T cells against a lethal poxvirus by mRNA-LNP vaccines

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The role of CD4$^+$ T cells in the induction of protective CD8$^+$ T cells by mRNA lipid nanoparticle (LNP) vaccines is unknown. We used B6 or *Tlr9*$^{-/-}$ mice depleted or not of CD4$^+$ T cells and LNP vaccines loaded with mRNAs encoding the ectromelia virus (ECTV) MHC class I H-2 K^b-restricted immunodominant CD8$^+$ T cell epitope TSYKFESV (TSYKFESV mRNA-LNPs) or the ECTV EVM158 protein, which contains TSYKFESV (EVM-158 mRNA-LNPs). Following prime and boost with 10 μg of either vaccine, K^b-TSYKFESV-specific CD8$^+$ T cells fully protected male and female mice from ECTV at 29 (both mRNA-LNPs) or 90 days (EVM158 mRNA-LNPs) post boost (dpb) independently of CD4$^+$ T cells. However, at 29 dpb with 1 μg mRNA-LNPs, males had lower frequencies of K^b-TSYKFESV-specific CD8$^+$ T cells and were much less well protected than females from ECTV, also independently of CD4$^+$ T cells. At 90 dpb with 1 μg EVM158 mRNA-LNPs, the frequencies of K^b-TSYKFESV-specific CD8$^+$ T cells in males and females were similar, and both were similarly partially protected from ECTV, independently of CD4$^+$ T cells. Therefore, at optimal or suboptimal doses of mRNA-LNP vaccines, CD4$^+$ T cell help is unnecessary to induce protective anti-poxvirus CD8$^+$ T cells specific to a dominant epitope. At suboptimal doses, protection of males requires more time to develop.

INTRODUCTION

Nucleoside-modified mRNA lipid nanoparticle (LNP) vaccines have recently attracted significant interest and revolutionized the vaccine field due to their many advantages over traditional vaccine platforms.[1–4] mRNA-LNP vaccines do not have the safety problems common to live attenuated vaccines.[1,5–7] Compared to other technologies, mRNA-LNP vaccines can be rapidly mass-produced with Good Manufacturing Practices, making them ideal to fight pandemics.[8] Certain nucleoside modifications have improved mRNA stability and translation, leading to strong immune responses and reduced inflammation.[9–12] The success of nucleoside-modified mRNA-LNP vaccines in fighting the SARS-CoV-2 pandemic has opened avenues for mRNA-LNP vaccines against other infectious diseases.[2,4,11–13] While mRNA vaccines are mostly evaluated for their capacity to induce antibody production,[14,15] they also induce potent T cell responses against infectious diseases and cancer.[16–20]

T cells, including CD8$^+$ and CD4$^+$ T cells, are arms of the adaptive immune system critical to controlling many viral infections. CD8$^+$ T cells recognize antigens as 8- to 10-amino-acid peptides presented by major histocompatibility complex I (MHC-I) molecules at the cell surface. These peptides derive from the proteasomal degradation of proteins in the cytosol. After cognate antigen recognition by the T cell receptor (TCR) and additional co-stimulatory signals, CD8$^+$ T cells exert their anti-viral function by releasing cytotoxic granules containing granzyme B and perforin to kill infected targets.[21–23] CD4$^+$ T cells recognize somewhat longer peptides presented by MHC-II molecules. While CD4$^+$ T cells can also have direct effector functions such as cytotoxicity,[24–27] they are better known as "helpers" of the B and CD8$^+$ T cell responses. In the case of B cells, CD4$^+$ T cell help is critical for proliferation, isotype switching, and antibody affinity maturation.[28,29] For CD8$^+$ T cells, the need for CD4$^+$ T cell help depends on the type of stimulus. For certain viral infection and tumor models, CD4$^+$ T cell help is necessary for the differentiation of CD8$^+$ T cells from naive (N) to effectors (E) via co-stimulation or cytokines.[22,30–35] For some infections, CD4$^+$ T cell help can also assist in the differentiation or maintenance of the CD8$^+$ T cell memory pool.[32,36] However, for other viral infections, including the poxviruses ectromelia virus (ECTV) and vaccinia virus (VACV), the differentiation and expansion of E CD8$^+$ T cells, or their differentiation and maintenance as memory cells, does not require CD4$^+$ T cell help.[37–39] Based on these observations, it is important to understand the role of CD4$^+$ T cell help in CD8$^+$ T cell responses to mRNA-LNP vaccines because it could directly impact the vaccine's effectiveness and design.

ECTV is a mouse-specific poxvirus that, in susceptible strains of mice, causes lethal mousepox, a disease characterized by high virus loads in

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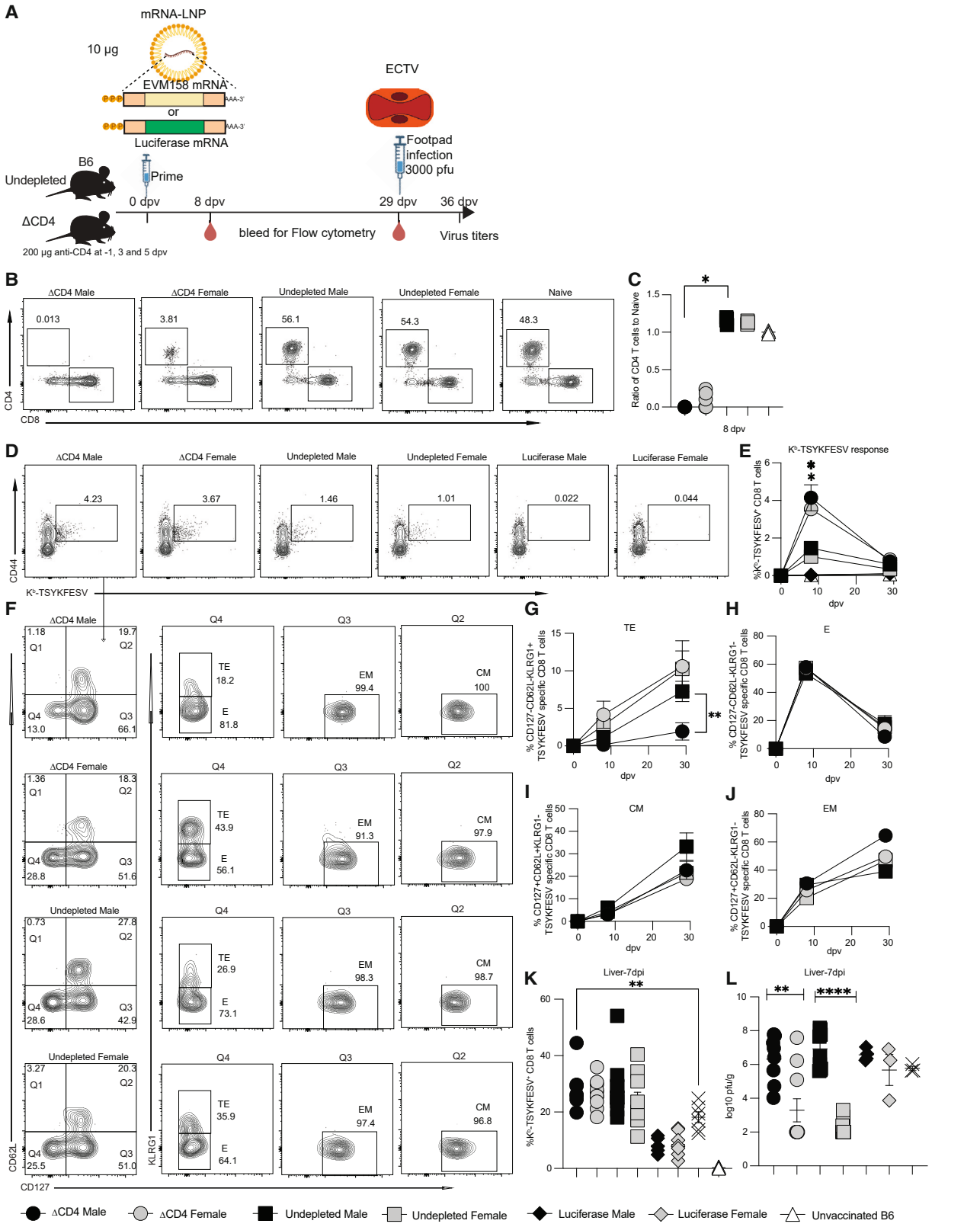

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the liver and fulminant hepatitis. C57BL/6 (B6) mice are highly resistant to mousepox. However, deficiencies in various immune-related genes render B6 mice highly susceptible to mousepox.[40] For example, mice deficient in TLR9, an innate DNA sensor, invariably succumb 7–8 days post infection (dpi) with ECTV in the footpad.[41–43] However, *Tlr9*$^{-/-}$ mice can be fully protected by CD8$^+$ T cell vaccination.[16]

The most immunodominant ECTV CD8$^+$ T cell epitope in B6 mice and derived strains is the H-2 K^b-restricted peptide TSYKFESV from the early/late EVM158 protein.[44,45] Whether EVM158 contains CD4$^+$ T cell epitopes is unknown. We previously used LNPs loaded with EVM158 mRNA (EVM158 mRNA-LNPs) and GFP fused to C-terminal TSYKFESV (GFP-TSYKFESV mRNA-LNPs) to show that mRNA-LNP vaccines can induce effector and memory CD8$^+$ T cells that protect mice against lethal mousepox.[16] Here, we use LNPs loaded with full-length EVM158 (EVM158 mRNA-LNP) in CD4$^+$ T cell-intact or -depleted mice or a "mini-mRNA" encoding for only TSYKFESV (TSYKFESV mRNA-LNP) to show that CD4$^+$ T cell help is not required during prime or prime/boost vaccination with mRNA-LNP to mount protective CD8$^+$ T cell responses against ECTV challenge. Additionally, we show that at suboptimal doses, CD8$^+$ T cell mRNA-LNP vaccines induce protective CD8$^+$ T cell responses more effectively in female than male mice at early but not late stages post vaccination.

RESULTS

CD8$^+$ T cells induced by one dose of 10 μg EVM158 mRNA-LNPs do not require CD4$^+$ T cell help during immunization for short-term protection in B6 mice

We have shown that unhelped polyclonal memory CD8$^+$ T cells induced by vaccinia immunization can protect susceptible mice from lethal ECTV challenge.[46] We have also shown that mice immunized with one dose of 10 μg EVM158 mRNA-LNPs generate moderate frequencies of TSYKFESV-specific CD8$^+$ T cells that partially protect mousepox-susceptible *Tlr9*$^{-/-}$ mice from lethal mousepox.[16] As an initial way to test whether CD4$^+$ T cell help is necessary to induce protective K^b-TSYKFESV-specific CD8$^+$ T cells, we transiently depleted CD4$^+$ T cells in groups of B6 mice (ΔCD4) by intraperitoneal inoculation of 200 μg anti-CD4 monoclonal antibody (mAb) GK1.5 at −1, 3, and 5 days post immunization with a single dose of 10 μg EVM158 mRNA-LNP (Figure 1A). As controls, we used undepleted mice, mice vaccinated with LNPs loaded with luciferase mRNA (mRNA-LNP), and unvaccinated, undepleted mice. GK1.5 mAb treatment efficiently depleted CD4$^+$ T cells as determined by the reduction in CD4$^+$ T cells in peripheral blood (Figures 1B and 1C). At 8 days post vaccination (dpv), ΔCD4 and undepleted male and female mice had higher frequencies than controls of CD8$^+$ T cells that stained with K^b-TSKYFESV tetramers (K^b-TSYKFESV$^+$ CD8$^+$ T cells) (Figures 1D and 1E). Interestingly, ΔCD4 male and female mice had significantly higher frequencies of K^b-TSYKFESV$^+$ CD8$^+$ T cells (∼4%) than their undepleted counterparts (∼1%) (Figures 1D and 1E). At 28 dpv, the frequencies of K^b-TSYKFESV$^+$ CD8$^+$ T cells in ΔCD4 and undepleted B6 mice decreased and stabilized at 0.5%–1% of total CD8$^+$ T cells, which were significantly higher than in control mice (Figures 1D and 1E). We also measured the frequencies and kinetics of differentiation of the K^b-TSYKFESV$^+$ CD8$^+$ T cells in EVM158 mRNA-LNP-vaccinated mice. At 8 dpv, the frequencies of terminally differentiated effector (TE) (CD127$^-$ CD62L$^-$ KLRG1$^+$), effector (E, CD127$^-$ CD62L$^-$ KLRG1$^-$), effector memory (EM, CD127$^+$ CD62L$^-$ KLRG1$^-$) and central memory (CM, CD127$^+$ CD62L$^+$ KLRG1$^-$) K^b-TSYKFESV$^+$ CD8$^+$ T cells was statistically similar in all groups. In comparison, at 29 dpv, ΔCD4 males had significantly fewer TE cells than the other groups (Figures 1F–1J).

In wild-type B6 mice, ECTV infection in the footpad is not lethal. However, the virus still spreads to and replicates in the liver, peaking 7 days post infection (dpi), albeit much lower than in mousepox-susceptible strains.[38,47,48] Thus, at 29 dpv, we infected the mice with ECTV in the footpad and determined CD8$^+$ T cell responses and viral titers in the liver at 7 dpi. The K^b-TSYKFESV$^+$ CD8$^+$ T cells in the livers of ΔCD4 and undepleted male and female mice, which were undergoing secondary exposure to K^b-TSYKFESV, expanded to similarly higher frequencies (∼30%–35%) compared to Luc mRNA-LNP-vaccinated and unvaccinated control mice, which were responding to K^b-TSYKFESV for the first time (Figure 1K). Surprisingly, regardless of CD4 depletion, EVM158 mRNA vaccinated female but not male mice had reduced virus loads in the liver compared to control mice (Figure 1L). This sex-biased response to vaccination suggests that male mice may need more antigen-specific CD8$^+$ T cells than females for improved virus control in the liver. The data also suggest that the

Figure 1. CD8$^+$ T cells induced by one dose of 10 μg EVM158 mRNA-LNPs do not require CD4$^+$ T cell help during immunization for short-term protection in B6 mice

B6 mice, CD4-depleted, or not were immunized intradermally (i.d.) once with 10 μg of mRNA-LNPs encoding EVM158. B6 males and females vaccinated once with 10 μg of luciferase mRNA-LNPs served as controls. (A) Schematic of the experimental timeline. (B) Concatenated flow cytometry plots of CD4 vs. CD8 staining in gated CD3$^+$ cells in the PBL at 8 dpv in the indicated mice. (C) Ratio of CD4$^+$ T cells to naive in the indicated mice at 8 dpv. (D) Representative flow cytometry plots for CD44 vs. K^b-TSYKFESV DimerX staining in gated CD3$^+$ CD8$^+$ cells in the PBL at 8 dpv. (E) Kinetics of the frequency of K^b-TSYKFESV$^+$ CD8$^+$ T cells within gated CD3$^+$ CD8$^+$ CD44$^+$ cells in PBL at the indicated dpv. (F) Representative flow cytometry plots for memory CD8$^+$ T cell subsets: terminally differentiated effector cells, TE: CD127$^-$ CD62L$^-$ KLRG1$^+$ (Q4, top); effector cells, E: CD127$^-$ CD62L$^-$ KLRG1$^-$ (Q4, bottom); effector memory, EM: CD127$^+$ CD62L$^-$ KLRG1$^-$ (Q3); and central memory, CM: CD127$^+$ CD62L$^+$ KLRG1$^-$ of TSYKFESV-specific CD8$^+$ T cells (Q2) in the PBL of vaccinated animals at 29 dpv. (G–J) Kinetics of the frequency of TE (G), E (H), EM (I), and CM (J) populations of TSYKFESV-specific CD8$^+$ T cells in PBL. In (K)–(L), mice were infected with 3,000 pfu WT ECTV in the footpad at 29 dpv. (K) Frequency of K^b-TSYKFESV$^+$-specific CD8$^+$ T cells within gated CD3$^+$ CD8$^+$ CD44$^+$ cells in the livers at 7 dpi. (L) Virus titers in the livers at 7 dpi as determined by plaque assay. (A–J) Data are representative of an independent experiment out of two or three experiments (n = 10). (K and L) Data were pooled from two independent experiments (n = 9–11). Experimental groups were compared to each other by t test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. E: effector CD8$^+$ T cell; TE: terminal effector CD8$^+$ T cells; EM: effector memory CD8$^+$ T cell; CM: central memory CD8$^+$ T cell; dpv: days post vaccination; dpi: days post infection; PBL: peripheral blood; pfu: plaque-forming units.

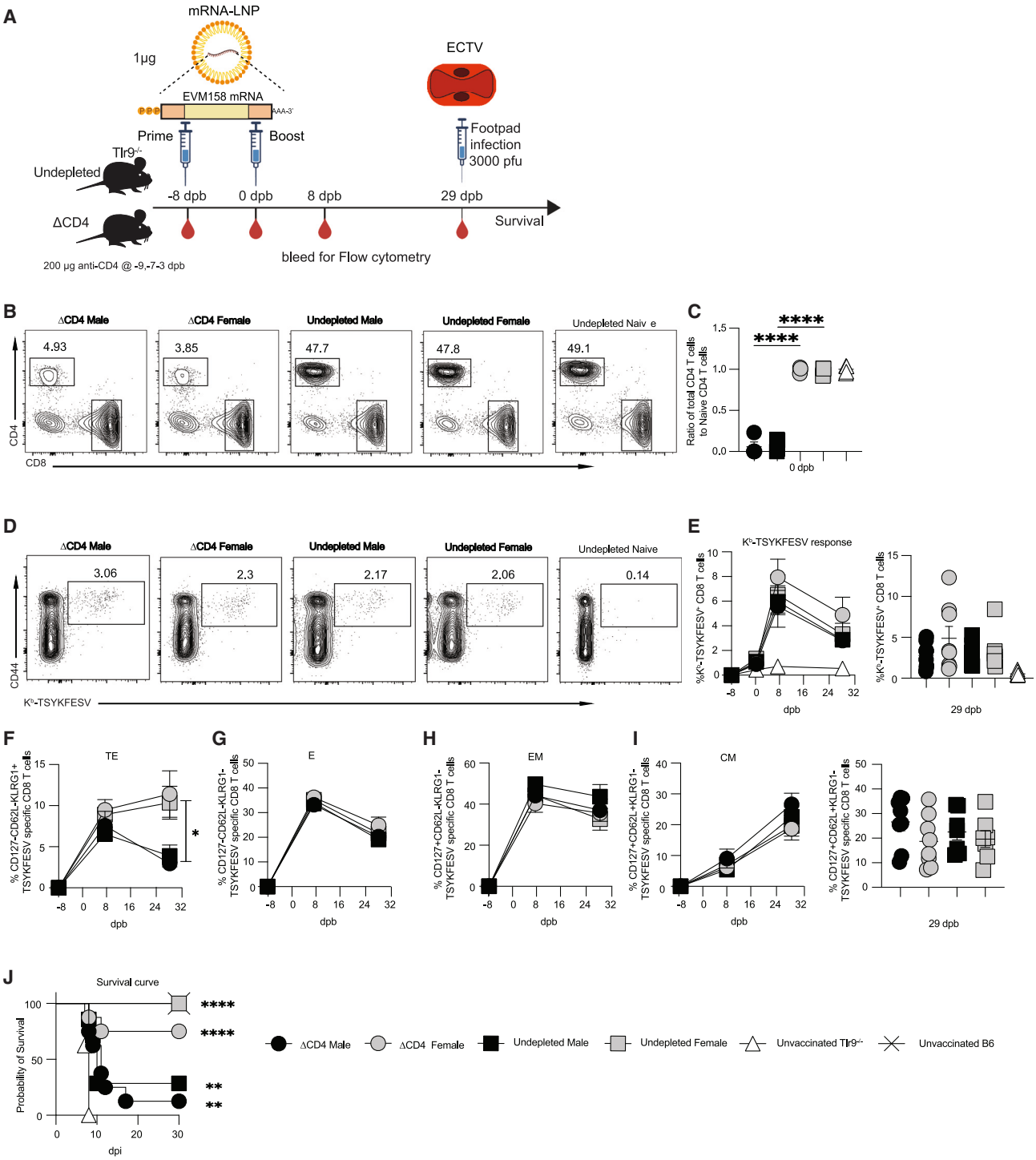

Figure 2. CD4⁺ T cell help does not alter the short-term protection of Tlr9$^{-/-}$ mice by CD8⁺ T cells induced with prime-boost immunization by 1 µg EVM158 mRNA-LNPs

Male and female *Tlr9*$^{-/-}$ mice were depleted or not of CD4⁺ T cells and primed and boosted i.d. 8 days apart with 1 µg of EVM158 mRNA-LNPs. CD8⁺ T cell responses and protection from ECTV infection were evaluated. (A) Schematic of the experimental timeline. (B) Representative flow cytometry plots of CD4 and CD8 staining in gated CD3⁺ cells in the PBL at 0 days post boost (dpb) in the indicated mice. (C) Ratios of CD4⁺T cells in vaccinated mice to naive mice at 0 dpb. (D) Representative flow cytometry plots for

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induction of protective CD8$^+$ T cells in females was independent of CD4$^+$ T cell help.

CD4$^+$ T cell help does not alter the short-term protection of *Tlr9*$^{-/-}$ mice by CD8$^+$ T cells induced by prime-boost immunization with 1 μg EVM158 mRNA-LNPs

Naive B6 mice deficient in the DNA sensor TLR9 (*Tlr9*$^{-/-}$) invariably succumb to ECTV infection at 7–8 dpi.[41,43] We have previously shown that *Tlr9*$^{-/-}$ mice are partially protected from mousepox when vaccinated once with EVM158 mRNA-LNPs but are fully protected when boosted 2 weeks after priming.[16] Using mRNA-LNPs loaded with SARS-CoV-2 spike mRNA, we have recently shown that prime-boost 8 days apart accelerates the expansion of the antigen-specific CD8$^+$ T cells without affecting the final frequency of memory CD8$^+$ T cells or anti-spike antibody in sera.[49] It has also been shown that 1 μg of the Moderna mRNA-1273 SARS-CoV-2 vaccine in the mouse was required to induce neutralizing Ab responses similar to those observed in humans vaccinated with 100 μg of the same vaccine.[50] Thus, to test whether CD4$^+$ T cell help is required for CD8$^+$ T cell protection from lethal mousepox after mRNA-LNP vaccination, male and female *Tlr9*$^{-/-}$ mice depleted or not of CD4$^+$ T cells were primed-boosted 8 days apart with 1 μg of EVM158 mRNA-LNPs (Figure 2A). Unvaccinated *Tlr9*$^{-/-}$ and B6 mice were susceptible and resistant controls, respectively. A comparison of the CD4$^+$ T cell frequencies in the peripheral blood (PBL) at 8 days post prime (dpp) demonstrated that the depletion was efficient (Figures 2B and 2C). Thus, the 8-day prime-boost regime was ideal because it allowed us to prime and boost without needing two series of CD4 T cell depletions. At 0, 8, 15, and 29 days post boost (dpb), we analyzed the PBL for the presence of H-2K^b-TSYKFESV-specific CD8$^+$ T cells and their effector/memory status (Figure 2A). ΔCD4 and undepleted *Tlr9*$^{-/-}$ male and female mice had similar frequencies of K^b-TSYKFESV$^+$ CD8$^+$ T cells (∼1%–3%) at 8 dpp, which hereafter we will refer to as 0 dpb, and the responses were similarly enhanced to ∼7%–9% at 8 dpb and decreased with similar kinetics to ∼3%–4% of the total CD8$^+$ T cells at 29 dpb (Figures 2D and 2E). Regardless of sex, the frequencies of E, EM, and CM cells within the K^b-TSYKFESV$^+$ CD8$^+$ T cells did not show significant differences between ΔCD4 and undepleted *Tlr9*$^{-/-}$ mice at either 8 or 29 dpb (Figures 2G–2I). Notably, regardless of CD4$^+$ T cell depletion, the TE cell frequencies were significantly higher in female than male mice at 29 dpb (Figure 2F). When challenged with wild-type (WT) ECTV in the footpad at 29 dpb, all control unvaccinated mousepox-resistant wild-type B6 mice survived, and all control unvaccinated mousepox-susceptible *Tlr9*$^{-/-}$ ones succumbed to the infection (Figure 2J). Notably, most vaccinated ΔCD4 and all undepleted females survived the infection, demonstrating strong protection. On the other hand, most but not all vaccinated ΔCD4 and undepleted males succumbed to the infection, indicating significant but poor protection (Figure 2J). Together, these results suggest that the protective K^b-TSYKFESV-specific CD8$^+$ T cell responses to prime-boost immunization 8 days apart with 1 μg EVM158 mRNA-LNPs are unaffected by CD4$^+$ T cell help. The results also suggest that the efficient short-term protection of males from lethal mousepox may require higher frequencies of total K^b-TSYKFESV-specific CD8$^+$ T cells than females or comparable frequencies of TE cells.

CD4$^+$ T cell help is dispensable for short-term protection of *Tlr9*$^{-/-}$ male and female mice by CD8$^+$ T cells induced with prime-boost immunization with 10 μg EVM158 mRNA-LNPs

While most female *Tlr9*$^{-/-}$ mice were protected by prime-boost immunization with 1 μg of EVM158 mRNA-LNP, most males were not. We wondered whether this was because protecting males may require higher numbers of K^b-TSYKFESV-specific CD8$^+$ T cells. Given that we had previously observed far greater numbers of K^b-TSYKFESV$^+$ CD8$^+$ T cells in *Tlr9*$^{-/-}$ mice vaccinated with 10 μg of EVM158-or GFP-TSYKFESV mRNA-LNP[16] than those that we observed above with 1 μg, we primed/boosted 8 days apart ΔCD4 and undepleted *Tlr9*$^{-/-}$ male and female mice with 10 μg of EVM158 mRNA-LNP. Undepleted and ΔCD4 *Tlr9*$^{-/-}$ male and female mice had similarly high frequencies (∼10%–15%) of K^b-TSYKFESV$^+$ specific CD8$^+$ T cells at 0 dpb (Figure 3A, left). While the frequencies of K^b-TSYKFESV$^+$ CD8$^+$ T cells strongly increased at 8 dpb in all vaccinated groups, the responses were significantly higher in males than in females, irrespective of CD4$^+$ T cell depletion (Figure 3A). At 29 dpb, they decreased but were slightly but significantly higher in ΔCD4 males than females (Figure 3A, right). Compared to 1 μg, 10 μg EVM158 mRNA-LNPs induced ∼10 times higher frequencies of K^b-TSYKFESV$^+$ CD8$^+$ T cells at 29 dpb (Figure 3B). Interestingly, males had significantly higher frequencies of TE cells, and females had higher frequencies of CM K^b-TSYKFESV$^+$ CD8$^+$ T cells at 8 and 29 dpb (Figures 3C and 3F). The frequencies of E and EM cells were similar in ΔCD4 and undepleted *Tlr9*$^{-/-}$ mice of both sexes at every time point (Figures 3D and 3E). We also found that at 29 dpb, ΔCD4 mice did not have anti-EVM158 IgG in serum, while undepleted mice had high concentrations of it (Figure 3G). This demonstrated that the GK1.5 mAb inoculation regime effectively abolished CD4$^+$ T cell help. After challenge with WT ECTV in the footpad at 29 dpb, all vaccinated *Tlr9*$^{-/-}$ mice survived, regardless of CD4$^+$ T cell depletion status or sex, while all unvaccinated Tlr9$^{-/-}$ controls died (Figure 3H). These results show that the K^b-TSYKFESV$^+$ CD8$^+$ T cells induced by 10 μg EVM158-mRNA-LNP immunization do not require CD4$^+$ T cell help for primary or secondary expansion, contraction, TE, E, EM, or CM differentiation, or protection from lethal mousepox. Moreover, full protection requires a higher dose of mRNA-LNP vaccine and, possibly, higher frequencies of K^b-TSYKFESV-specific TE CD8$^+$ T cells in male than female mice.

CD44 and K^b-TSYKFESV DimerX staining in gated CD3$^+$ CD8$^+$ cells in the PBL at 29 dbp. (E) Frequency of K^b-TSYKFESV$^+$ CD8$^+$ T cells within gated CD3$^+$ CD8$^+$ CD44$^+$ cells in PBL at the indicated times. (F–I) The TE, E, EM, and CM frequency at the indicated dpb was determined by staining with the indicated markers within gated CD3$^+$ CD44$^+$ Kb-TSYKFESV$^+$ CD8$^+$ in the PBL, respectively. (J) Survival to infection with ECTV in the footpad. Unvaccinated *Tlr9*$^{-/-}$ mice and wild-type C57BL/6 (B6) mice were used as positive and negative controls, respectively. In (J), statistical differences marked by asterisks compared the survival of vaccinated vs. unvaccinated *Tlr9*$^{-/-}$ mice. (A–J) Data were pooled from two independent experiments (n = 9–11). Experimental groups were compared to each other by t test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

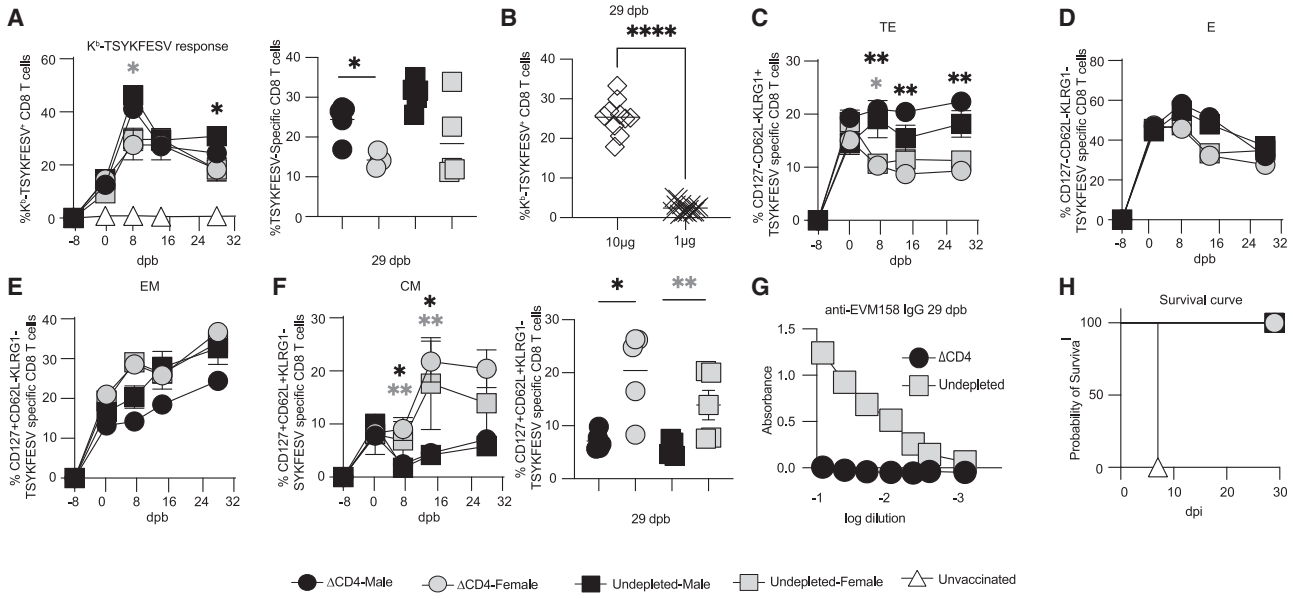

Figure 3. CD4⁺ T cell help is dispensable for short-term protection of Tlr9$^{-/-}$ male and female mice by CD8⁺ T cells induced with prime-boost immunization with 10 μg EVM158 mRNA-LNPs

Male and female *Tlr9*$^{-/-}$ mice were depleted or not of CD4⁺ T cells and primed and boosted i.d. 7 days apart with 10 μg of EVM158 mRNA-LNPs. The experimental setup was the same as in Figure 2A, but animals received a higher (10 μg) vaccine dose. (A) Frequency of K^b-TSYKFESV⁺ CD8⁺ T cells within gated CD3⁺ CD8⁺ CD44⁺ cells in PBL at the indicated times. Gray asterisks indicate statistical differences between undepleted males and females. Black asterisks indicate statistical differences between ΔCD4 males and females. (B) Comparison of frequencies of K^b-TSYKFESV⁺ CD8⁺ T cells within gated CD3⁺ CD8⁺ CD44⁺ cells in PBL at 29 dpb with 10- and 1-μg doses of EVM158 mRNA-LNPs. (C–F) The TE, E, EM, and CM frequency at the indicated dpb was determined by staining with the indicated markers within gated CD3⁺ CD44⁺ Kb-TSYKFESV⁺ CD8⁺ in the PBL, respectively. (G) EVM158-specific antibody titers in the serum of the indicated mice at 29 dpb as determined by ELISA. (H) Survival to infection with ECTV in the footpad. Unvaccinated *Tlr9*$^{-/-}$ mice were used as positive controls. (A–H) Data were pooled from two independent experiments (n = 9–11). Groups were compared by t test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Prime-boost immunization of *Tlr9*$^{-/-}$ mice with 1 μg TSYKFESV mRNA-LNPs provides stronger short-term CD8⁺ T cell protection to females than males

To confirm with another method that CD4⁺ T cell help is not required for protective CD8⁺ T cell responses against lethal ECTV, we designed a mini-mRNA encoding only for the minimal CD8⁺ T cell epitope TSYKFESV, which is not a CD4⁺ T cell epitope. We primed and boosted male and female *Tlr9*$^{-/-}$ mice 8 days apart with 1 μg of TSYKFESV mRNA-LNPs (Figure 4A). The frequencies of K^b-TSYKFESV⁺ CD8⁺ T cells in vaccinated female and male mice were comparable at 0 dpb (Figure 4B, left) but were significantly higher in females than in males at 8 and 29 dpb (Figure 4B, right). The E, EM, and CM populations were comparable between the two groups (Figures 4C–4G). However, like after immunization with 1 μg EVM158 mRNA-LNPs (Figure 2F), female *Tlr9*$^{-/-}$ mice had significantly higher frequencies of TE cells than males at 29 dpb (Figure 4D). Like after EVM158 mRNA-LNP vaccination, most TSYKFESV-mRNA-LNP-vaccinated males succumbed, while most females survived an ECTV challenge at 29 dpb. All control unvaccinated *Tlr9*$^{-/-}$ mice died (Figure 4H). These results indicate that 1 μg of mRNA-LNP vaccine containing a CD8⁺ but no CD4⁺ T cell epitopes can induce CD8⁺ T cell responses that are highly protective to females and poorly protective to male *Tlr9*$^{-/-}$ mice.

Prime-boost immunization of *Tlr9*$^{-/-}$ mice with 10 μg TSYKFESV mRNA-LNPs provides strong short-term CD8⁺ T cell protection to females and males

Because the 1-μg dose of TSYKFESV mRNA-LNPs poorly protected male mice from mousepox, we immunized male and female *Tlr9*$^{-/-}$ mice with 10 μg of TSYKFESV mRNA-LNPs (same timeline as in Figure 4A). The 10-μg dose eliminated the TSYKFESV-specific CD8⁺ T cell frequency difference between vaccinated male and female mice at all time points (Figure 5A). Notably, the frequency of K^b-TSYKFESV⁺ CD8⁺ T cells increased in males to the levels observed in females at the 1-μg dose but did not further increase in females (Figures 5A and 5B). The frequencies of E, TE, EM, and CM K^b-TSYKFESV⁺ CD8⁺ T cells in male and female mice were also similar (Figures 5C–5F). Following ECTV challenge at 29 dpb, most vaccinated *Tlr9*$^{-/-}$ female and male mice survived, while all the unvaccinated controls succumbed to mousepox (Figure 5G). Therefore, increasing the dose of TSYKFESV mRNA-LNPs to 10 μg increased the CD8⁺ T cell responses in males to levels like those in females and improved their survival.

CD8⁺ T cell protection is long lasting in females and males with 10 but not 1 μg EVM158 mRNA-LNPs

We next tested whether the levels of protection remained stable at 90 dpb. For this purpose, we used the EVM158 mRNA-LNP vaccine,

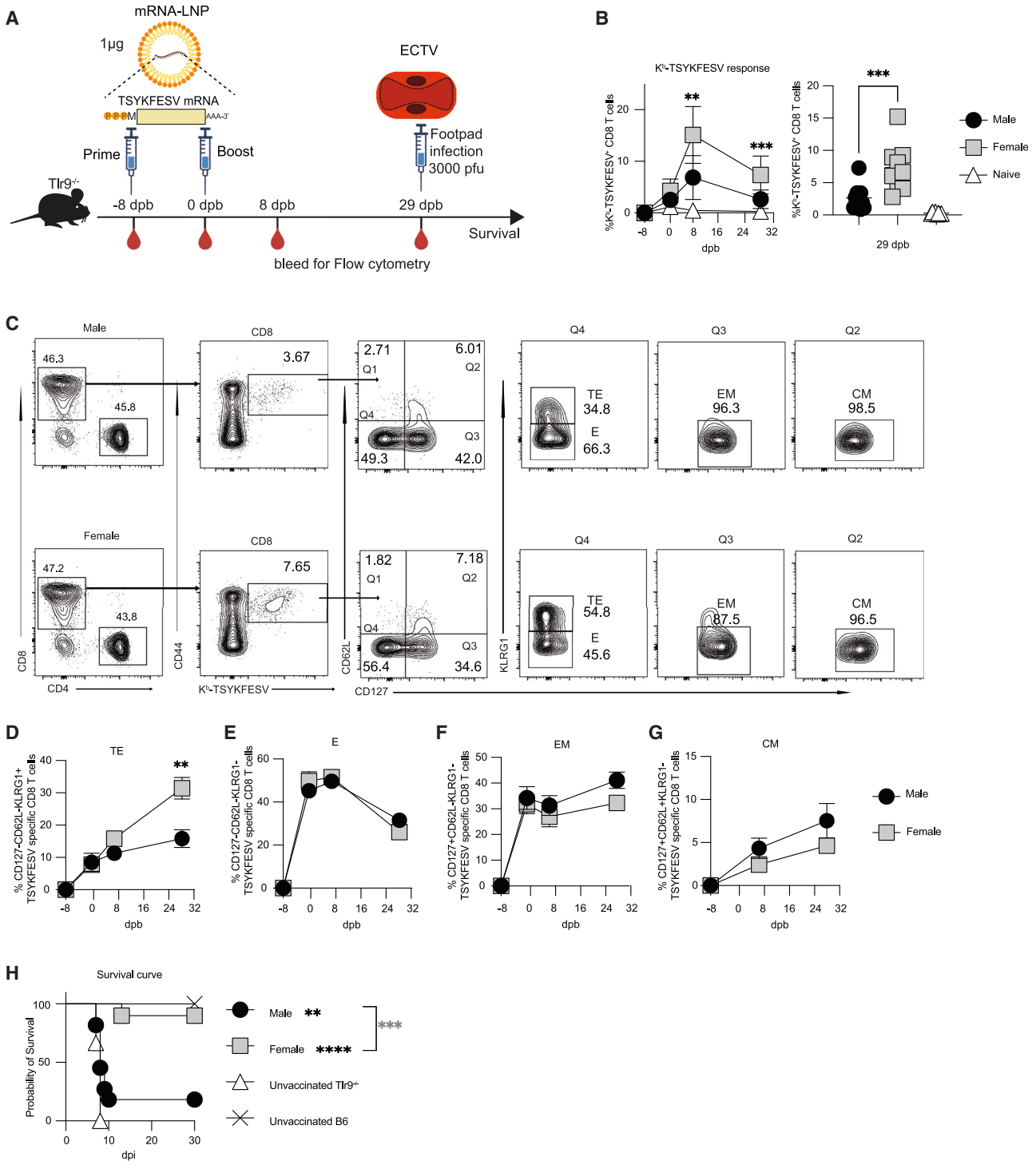

Figure 4. Prime-boost immunization of *Tlr9*$^{-/-}$ mice with 1 µg TSYKFESV mRNA-LNPs provides stronger short-term CD8$^+$ T cell protection to females than males

Male and female *Tlr9*$^{-/-}$ mice were primed and boosted 7 days apart with 1 µg of intradermally injected TSYKFESV mRNA-LNPs, and CD8$^+$ T cell responses and protection from ECTV infection were evaluated. (A) Schematics of the experimental timeline. (B) Frequency of K^b-TSYKFESV CD8$^+$ T cells in PBL over time (left) and at 29 dpb (right). (C) Representative flow cytometry plots of (left) TSYKFESV-specific CD4$^+$ and CD8$^+$ T cells using DimerX complexes at 29 dpb (top: male; bottom: female) and (right) memory CD8$^+$ T cell subsets: terminally differentiated effector, TE: CD127$^-$ CD62L$^-$ KLRG1$^+$ (Q4, top); effector, E: CD127$^-$ CD62L$^-$ KLRG1$^-$ (Q4, bottom), effector memory,

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which, with the 10-μg dose, was better than the TSYKFESV mRNA-LNP vaccine at inducing $CD8^+$ T cell responses.

At 90 dpb with 10 μg EVM158 mRNA-LNP, all ΔCD4 and undepleted B6 mice of both sexes had comparable, albeit widely variable, frequencies of circulating K^b-$TSYKFESV^+$-specific $CD8^+$ T cells (Figure 6A). No differences were observed in TE, E, EM, or CM cell frequencies (Figures 6B and 6C). When challenged with ECTV, all the mice survived the ECTV challenge without signs of disease (Figure 6D). Most mice immunized with 1 μg also had circulating K^b-$TSYKFESV^+$-specific $CD8^+$ T cells, albeit their frequencies were significantly lower ($p < 0.05$) than in mice immunized with 10 μg, except for the undepleted females (Figure 6E). The undepleted females had higher TE frequencies, but the E, EM, and CM cell frequencies were similar between the different groups (Figures 6F and 6G). After ECTV challenge, all groups were significantly protected from death when compared to unvaccinated *Tlr9*$^{-/-}$ mice ($p < 0.001$–0.0001), but only the undepleted females were fully protected (Figure 6H). Interestingly, both groups of males were better protected than when challenged at 29 dpb (compare to Figure 2J). Of note, most of the mice that succumbed to the infection had low overall frequencies of K^b-$TSYKFESV^+$-specific $CD8^+$ T cells and of TE cells (Figures 6E and 6F, red symbols). Taken together, these data indicate that TE, E, and EM cells persist for a long time after EVM158 mRNA-LNP vaccination, that $CD4^+$ T cell help at the time of vaccination is dispensable to generate long-lasting K^b-$TSYKFESV^+$ $CD8^+$ T cells that fully protect from mousepox with the 10-μg dose, and that with the suboptimal 1-μg dose, the protection of males increases and that of females decreases over time.

DISCUSSION

Our work demonstrates that contrary to Ab induction, the absence of $CD4^+$ T cell help during EVM158 or TSYKFESV mRNA-LNP vaccination does not significantly alter the potency, memory phenotype, or the ability of K^b-TSYKFESV-specific $CD8^+$ T cells to protect an otherwise susceptible mouse strain from lethal mousepox at doses of 1 or 10 μg. Interestingly, our data show that at 29 dpb with the 1-μg but not the 10-μg dose, female mice had higher frequencies of TSYKFESV-specific $CD8^+$ T cells and TE cells regardless of $CD4^+$ T cell depletion and were better protected than males, which were protected very poorly. At ∼90 dpb, these differences vanished for $CD4^+$ T cell-depleted but not for undepleted females.

We have previously shown that vaccination with LNPs loaded with 10 μg mRNA encoding TSYKFESV fused to the C terminus of GFP (GFP-TSYFESV mRNA-LNP) induced potent TSYKFESV-specific $CD8^+$ T cell responses that protected *Tlr9*$^{-/-}$ mice from lethal mousepox.[16] At the time, we used GFP as a putative source of $CD4^+$ T cell help. Recently, we showed that mini-mRNAs encoding only the minimal VNFNFNGL epitope from the spike-2 (S-2P) mRNA of SARS-CoV2 induced similar frequencies of antigen-specific CD8+ T cells to full-length S-2P mRNA and protected B6 mice from SARS-CoV-2 lethality, providing the first circumstantial evidence that mRNA-LNP vaccines may not require $CD4^+$ T cell help to induce protective anti-viral $CD8^+$ T cell responses.[49] Here, we show that LNPs loaded with a mini-mRNA encoding only the immunodominant ECTV epitope TSYKFESV from EVM158 can induce $CD8^+$ T cells, which, similar to the full-length EVM158 mRNA-LNP, are fully protective for males and females at a dose of 10 μg, but are highly protective for females and poorly protective for males at a dose of 1 μg, when challenged at 29 dpb. Because TSYKFESV is not a $CD4^+$ T cell epitope, these data emphasize that $CD4^+$ T cell help is unnecessary to induce protective K^b-TSYKFESV-specific $CD8^+$ T cells.

The $CD8^+$ T cell responses to 10 μg EVM158 mRNA-LNPs were much higher than to 10 μg TSYKFESV mRNA-LNPs. This was surprising because minigenes encoding minimal epitopes induce more potent $CD8^+$ T cell responses than the full-length protein in the context of recombinant VACV.[51] Our recent work with SARS-CoV2 mRNA vaccines also suggested a better response to the full-length spike mRNA-LNP vaccine in B6 mice.[49] The exact reason for this difference must be determined. Still, it is independent of $CD4^+$ T cell help, as evidenced by the ΔCD4 and depleted mice vaccinated with 10 μg EVM158 mRNA-LNPs that had similarly high responses. We speculate it may be related to the stability of the mRNA or the translated product *in vivo*.

At 29 dpb, female *Tlr9*$^{-/-}$ mice were much better protected than male mice following vaccination with 1 μg TSYKFESV or EVM158 mRNA-LNPs, but the $CD4^+$ T cell depletion did not affect these outcomes. Sex affects the immune response to some vaccines in humans, such as influenza, yellow fever, hepatitis A and B, and dengue.[52–64] Generally, females develop a higher antibody response, cellular immunity, and more adverse vaccine reactions.[64,65] We do not know the exact reason behind the sex differences that we observed, but we can certainly speculate. Several immune-related genes, such as the pattern recognition receptors TLR7 and TLR8, and the transcription factors NF-kB and FoxP3 reside in the X chromosome, and these could play a role in the sex differences.

Additionally, many immune cells, such as B cells, T cells, NK cells, dendritic cells, and macrophages, express the estrogen receptor, suggesting that sex hormones are actively involved in the immune response.[64,66] Indeed, estrogen is an immune activator, and testosterone is an immune suppressor.[64,66] In the *Tlr9*$^{-/-}$ mouse model, the *Tlr9* gene resides in chromosome 9 and thus is not X-linked.

EM: $CD127^+$ $CD62L^-$ $KLRG1^-$ (Q3); and central memory, CM: $CD127^+$ $CD62L^+$ $KLRG1^-$ of TSYKFESV-specific $CD8^+$ T cells (Q2) in the PBL of vaccinated animals. (D–G) Kinetics of the frequency of TE (D), E (E), EM (F), and CM (G) cells at 29 dpb in the PBL. (H) At 29 dpb, the mice were infected with 3,000 pfu ECTV in the footpad, and their survival was determined. Unvaccinated *Tlr9*$^{-/-}$ mice were used as positive controls. *Highlighted statistical differences compared survivals of vaccinated groups to unvaccinated *Tlr9*$^{-/-}$ mice unless otherwise indicated. (A–H) Data were pooled from two independent experiments (n = 9–11). Experimental groups were compared to each other by t test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

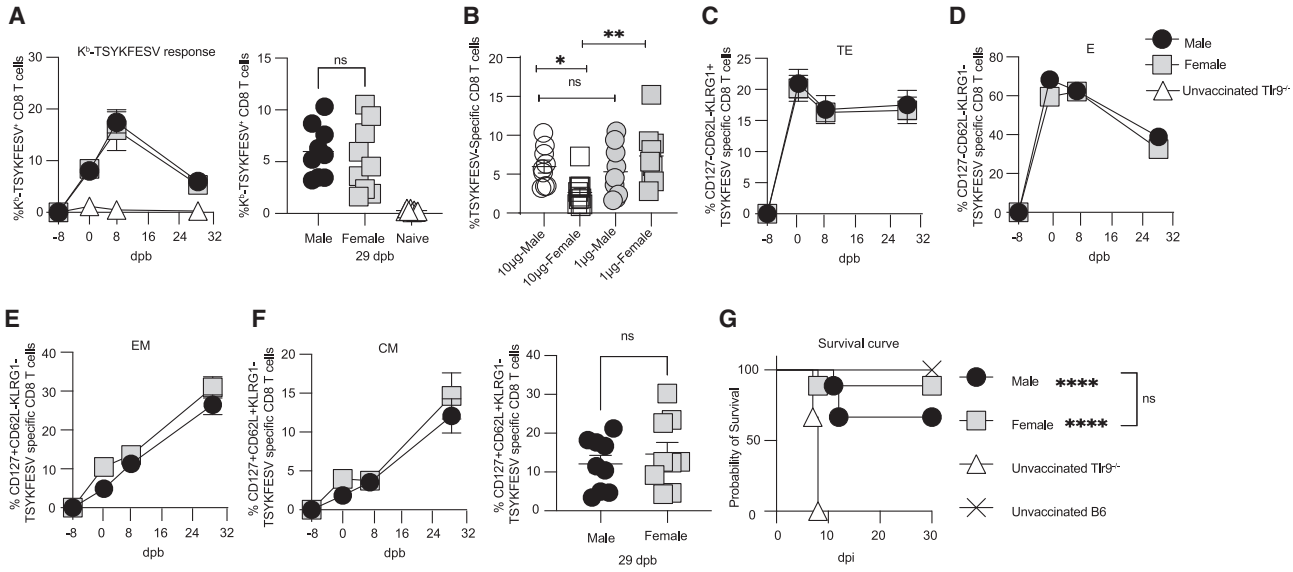

Figure 5. Prime-boost immunization of *Tlr9*$^{-/-}$ mice with 10 μg TSYKFESV mRNA-LNPs provides strong short-term CD8$^+$ T cell protection to females and males

Male and female *Tlr9*$^{-/-}$ mice were primed and boosted 7 days apart with 10 μg of i.d. injected TSYKFESV mRNA-LNP. The experimental setup was the same as in Figure 1A, but animals received a higher (10 μg) vaccine dose. (A) Frequency of K^b-TSYKFESV$^+$ CD8$^+$ T cells within gated CD3$^+$ CD8$^+$ CD44$^+$ cells in PBL at the indicated times. (B) Comparison of the frequencies of K^b-TSYKFESV$^+$ CD8$^+$ T cells within gated CD3$^+$ CD8$^+$ CD44$^+$ cells in PBL of the 10 and 1 μg TSYKFESV mRNA-LNP-vaccinated males and females at 29 dpb. (C–F) The TE, E, EM, and CM frequency at the indicated dpb was determined by staining with the indicated markers within gated CD3$^+$ CD44$^+$ Kb-TSYFESV$^+$ CD8$^+$ in the PBL, respectively. (G) Survival to infection with ECTV in the footpad. Unvaccinated *Tlr9*$^{-/-}$ mice and wild-type C57BL/6 mice were used as positive and negative controls, respectively. *Highlighted statistical differences compared survivals of vaccinated groups to unvaccinated *Tlr9*$^{-/-}$ mice unless otherwise indicated. Data were pooled from two independent experiments (n = 9–11). Experimental groups were compared to each other by t test; **$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

The differences observed between the sexes were also dose dependent. Therefore, it is possible that males inherently require a higher frequency of antigen-specific CD8$^+$ T cells for rapid CD8$^+$ T cell protection. This is consistent with the observation that women who were given a half-dose of the trivalent influenza vaccine mounted similar or higher antibody responses than males who received the full dose.[52] It is also possible that the dynamics of antigen processing for presentation on MHC-I are different between males and females, contributing to variable cellular responses. This needs to be further studied.

Compared to males, female mice immunized with 1 μg of either vaccine had significantly higher frequencies of K^b-TSYKFESV-specific TE cells at 29 dpb. This suggests that TE cells might be responsible for the increased protection of females. In agreement with this hypothesis, increasing the dose of either vaccine to 10 μg greatly increased the frequency of TE cells in the males and their survival to ECTV challenge. Our data at 90 dpb suggest that EVM158 mRNA-LNPs generate robust, long-lived TE CD8$^+$ T cells that provide complete protection in undepleted females for up to 3 months post vaccination, even at a low dose. We do not know why females have a higher frequency of TE cells than males. One possibility is that female CD8$^+$ T cells have an enhanced capacity to respond to interleukin-12, which drives TE differentiation, as was observed with VACV.[67] While we did not see differences in the CM populations with 1 μg, following vaccination with 10 μg EVM158 mRNA-LNP, females had higher frequencies of CM and stable TE cells, while males had an increase in TE CD8$^+$ T cells. This agrees with our hypothesis that protection from ECTV lethality requires higher frequencies of overall antigen-specific CD8+ T cells and, in particular, those with a TE phenotype in males than in females. Future studies are needed to determine the exact mechanisms for the sex differences we observed.

In summary, our data demonstrate that in mice of the B6 background, the induction of protective CD8$^+$ T cells by mRNA-LNPs but not of Abs is independent of CD4$^+$ T cell help, that mini-mRNA-LNPs can effectively induce CD8$^+$ T cell-focused immune responses, and that protective immunity by CD8$^+$ T cells induced by mRNA-LNP vaccines at suboptimal doses is more effective in females than in males short but not long term. Recent studies with mRNA-LNP vaccines against SARS-CoV2 have revealed important roles of CD4$^+$ T follicular helper cells in the formation of germinal centers and the induction of long-lived interferon-gamma-producing CD8$^+$ T cells.[5] In contrast, our results suggest that unhelped CD8$^+$ T cells can expand and provide protective immunity for at least 3 months. It remains unknown whether the absence of CD4$^+$ T cells at the time of mRNA-LNP vaccination is dispensable to induce protective CD8$^+$ T cells

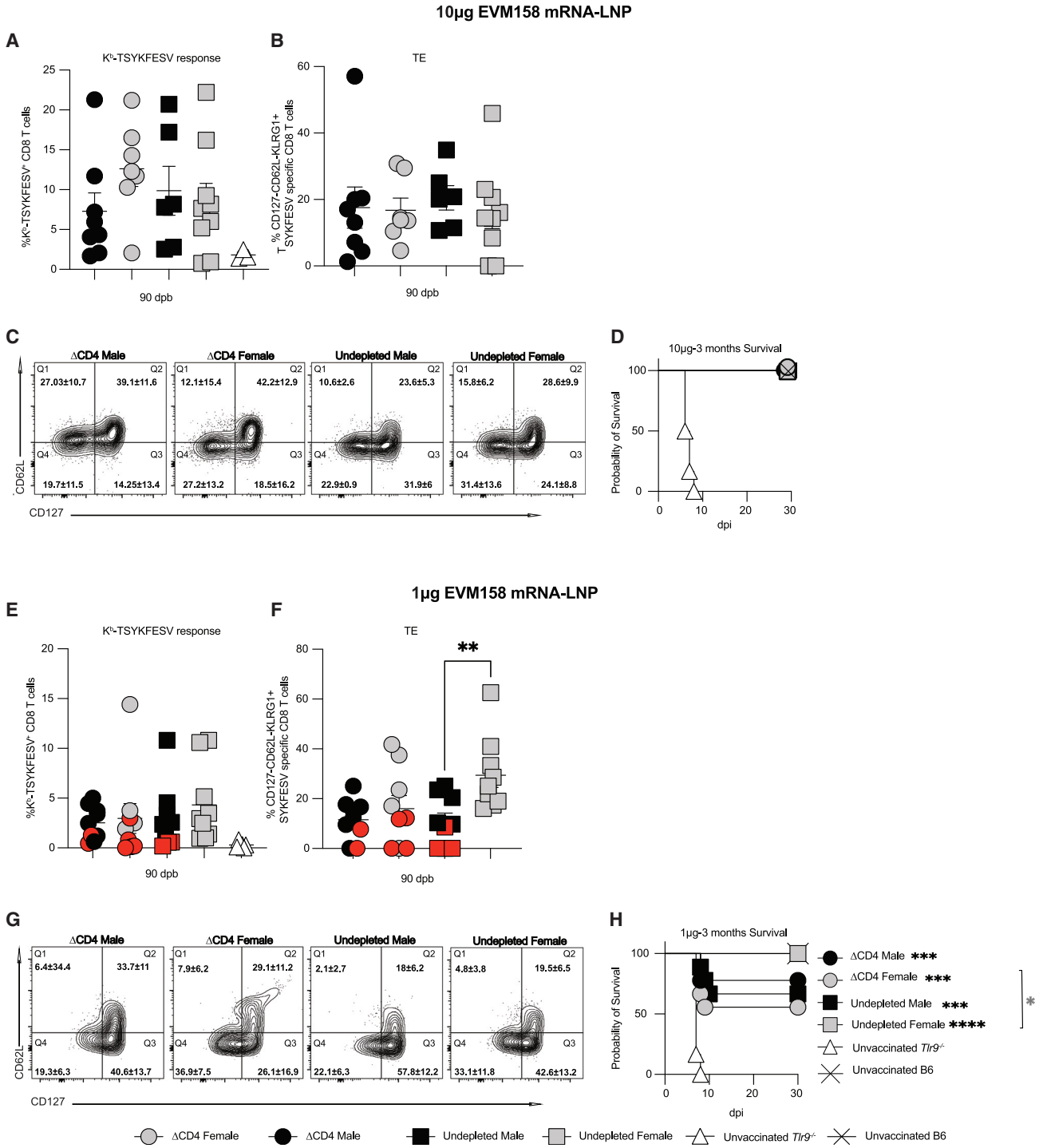

Figure 6. A higher dose of EVM158 mRNA-LNPs provides long-term protection in both sexes, while a lower dose is suboptimal for males

(A–F) Male and female *Tlr9*$^{-/-}$ mice were depleted or not of CD4$^+$ T cells and primed and boosted i.d. 8 days apart with either 1 µg (A–C) or 10 µg (D–F) of EVM158 mRNA-LNPs and followed up to 3 months post vaccination. (A–C) 1 µg EVM158 mRNA-LNPs. (A) Frequency of K^b-TSYKFESV$^+$ CD8$^+$ T cells within gated CD3$^+$ CD8$^+$ CD44$^+$ cells in PBL at 90 days post vaccination (dpv). Red circles indicate mice that subsequently succumbed to lethal challenge. (B) Frequency of TE cells at the indicated dpb as determined by staining with the indicated markers within gated CD3$^+$ CD44$^+$ K^b-TSYFESV$^+$ CD8$^+$ cells in the PBL. (C) Concatenated flow cytometry plots for memory CD8$^+$ T cell subsets of TSYKFESV-specific CD8$^+$ T cells in the PBL of vaccinated animals at 90 dpv. (D–F) 10 µg EVM158 mRNA-LNPs. (D) Frequency of K^b-TSYKFESV$^+$ CD8$^+$ T cells within gated CD3$^+$ CD8$^+$ CD44$^+$ cells in PBL at 90 days post vaccination (dpv). (E) Frequency of TE at the indicated dpb as determined by staining with the indicated

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against other viruses or species. However, our recent work showing protection from COVID-19-like disease in B6 mice following vaccination with LNPs loaded with mRNA encoding the minimal SARS-CoV-2 epitope VNFNFNGL suggests that help is also unnecessary for SARS-CoV-2, as well as in the B6 mouse model. The efficacy of mRNA vaccines in the context of sex and dosage has also not been properly studied. Our findings add to the growing knowledge of how mRNA vaccines can be exploited to study T cell function and urge the need to consider sex as a determining factor when optimizing the dosage during mRNA-LNP vaccine development.

Our studies have limitations. All the experiments (except Figure 1) were performed with a 1-week prime-boost regime. Whether a different prime-boost regime could improve responses and protection of males is unlikely but possible. It is also possible that additional boosts with the 1-μg dose can further increase short-term protection in males and long-term protection in both sexes. Additionally, our experiments targeted only the immunodominant $CD8^+$ T cell epitope TSYKFESV from ECTV EVM158. Further studies should test whether mRNA-LNP vaccines containing other conserved subdominant $CD8^+$ T cell epitopes from ECTV or dominant and subdominant epitopes from other viral proteins are also $CD4^+$ T cell independent.

MATERIALS AND METHODS

Mice

All experiments were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. WT C57BL/6N mice (B6) were purchased from Charles River. B6.129-$Tlr9^{tm1Aki/Obs}$ ($Tlr9^{-/-}$) mice were produced by Dr. S. Akira (Osaka University, Japan) and generously provided by Dr. Robert Finberg (University of Massachusetts, Worcester, MA).[68] All mice were bred and maintained in-house. Mice used for all experiments were gender and age matched and were between 6 and 12 weeks of age.

mRNA in LNP production and vaccination

Codon-optimized sequences for ECTV EVM158, TSYKFESV (preceded by an M for translation initiation), and chicken OVA were codon-optimized, synthesized (GenScript), and cloned into an mRNA production plasmid as previously described.[69] Briefly, plasmids were linearized, and mRNAs were generated using MEGAscript T7 RNA polymerase (Ambion). mRNAs were transcribed to contain poly(A) tails of 101 nucleotides in length. Uridine 5′-triphosphates were substituted for N(1)-methylpseudouridine 5′-triphosphates (TriLink), and cap1 structure was generated using CleanCap (TriLink). mRNA was purified by cellulose purification as previously described[69] and analyzed by agarose gel electrophoresis. Purified mRNAs were encapsulated in LNPs using a self-assembly process by rapidly mixing an aqueous solution of mRNA at pH = 4.0 that is rapidly mixed with a solution of lipids dissolved in ethanol; LNPs were similar in composition to those described previously, which contain a cationic lipid proprietary to Acuitas Therapeutics/phosphatidylcholine/cholesterol/PEG-lipid29. The proprietary lipid and LNP composition are described in US patent US10221127. LNPs had a diameter of ∼80 nm as measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) instrument. For vaccinations, the hind backs of mice were shaved, and mRNA was injected intradermally at four distinct locations approximately 1 cm apart with 20 μL of mRNA (80 μL total) using insulin syringes (29G). A graphical abstract depicting mRNA-LNP vaccination was generated using BioRender.

Viruses and infection

ECTV strain Moscow was obtained from ATCC (VR-1374) and propagated as previously described. For all challenge experiments, mice were infected with 3,000 plaque-forming units (pfu) of the virus in 30 μL PBS subcutaneously through a footpad. For survival experiments, infected mice were observed daily for signs of morbidity and weighed in frequent intervals for up to 4 weeks post infection.

$CD4^+$ T cell depletion

Mice were inoculated intraperitoneally with 200 μg of the anti-CD4 monoclonal antibody (mAb) GK1.5 (Bio-X-cell) at −1, 3, and 5 dpp.

Plaque assay

Titers of ECTV were determined by plaque assay as previously outlined with slight modifications (ref). Briefly, BS-C-1 cells (ATCC CCL-26) were grown in 12-well tissue culture plates to 80%–90% confluency in DMEM tissue culture medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 4.5 g/L glucose, 4.5 g/L L-glutamine, 4.5 g/L sodium pyruvate, 1× non-essential amino acids, and 100 IU/mL penicillin and streptomycin (complete DMEM). BS-C-1 monolayers were infected with 10-fold dilutions of organ homogenate for 1.5 h at 37°C with 5% CO_2 in complete DMEM. Organ homogenates (spleen) were made while processing samples for flow cytometry or by whole organ mechanical disruption using a TissueLyser (QIAGEN) with 30 iterations/s frequency for 2 min. Following incubation, the virus was removed, and the monolayers were overlaid with a 1:1 mixture of 2% carboxymethyl cellulose in complete DMEM containing 5% FBS. After incubating for 5 days at 37°C with 5% CO_2, the monolayers were fixed for 20 min at room temperature in 1% crystal violet in 20% ethanol solution and 4% paraformaldehyde. Excess crystal violet was washed off in a pool of water, and the plaques were quantified. Virus titers were assessed at 7 dpi in B6 mice during the peak of infection.

markers within gated $CD3^+$ $CD44^+$ K^b-$TSYFESV^+$ $CD8^+$ cells in the PBL. (F) Concatenated flow cytometry plots for memory $CD8^+$ T cell subsets of TSYKFESV-specific $CD8^+$ T cells in the PBL of vaccinated animals at 90 dpv. (G and H) Survival to infection with ECTV in the footpad with 1 μg (G) or 10 μg (H) vaccinated animals after 90 dpv. Unvaccinated $Tlr9^{-/-}$ mice were used as positive controls. *Highlighted statistical differences compared survivals of vaccinated groups to unvaccinated $Tlr9^{-/-}$ mice unless otherwise indicated. (A–H) Flow data compiled from two independent experiments. Survival data were pooled from two independent experiments (n = 9–11). Groups were compared by t test; $^{**}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Flow cytometry

Mice were retro-orbitally bled every week post vaccination, and ~75 μL of blood was collected in hematocrit capillary tubes containing heparin (Fisher Scientific). Spleens were processed into single-cell suspensions by gentle tissue dissociation using frosted microscope slides (Fisher Scientific). The splenocytes or blood was treated with 1× ammonium chloride potassium (ACK) buffer (155 mM NH4Cl, 1 mM KHCO3, 0.1 mM EDTA) for 5–15 min to lyse red blood cells and washed with RPMI-1640 medium. To prevent non-specific Fc receptor binding to Abs, cells were stained with anti-CD16/32 (Fc-Block; 2.4G2 ATCC). To detect TSYKFESV-specific $CD8^+$ T cells, BD DimerX Kb (BD Biosciences) molecules were incubated with TSYKFESV peptide and PBS (0.2:0.075:0.725 volume ratio) overnight at 37°C. DimerX Kb-TSYKFESV complexes were conjugated with anti-mouse IgG1 at a 1:4 volume ratio (clone RMG1-1; PE) for 1 h at room temperature. Cells were incubated with 1 μL DimerX complexes for 30 min at 4°C before surface staining. For extracellular staining of surface molecules, single-cell suspensions were incubated with Abs in an Fc-block buffer for 30 min at 4°C. For intracellular staining, samples were stained as above and then fixed for 10–15 min in 1% paraformaldehyde in PBS. Cells were then incubated in 1× Perm/Wash buffer (BD Biosciences) for 5 min at 4°C and stained for 30 min with Abs in 1× Perm/Wash buffer. Data were acquired using the BD LSRFortessa cytometer (BD Biosciences) and analyzed with FlowJo cytometry software (BD Biosciences). For infected samples, all procedures were performed at a BSL-2 hood.

The following antibodies were used: CD4 (clone M4-5; BV785), CD8a (clone 53–6.7; BV711), CD44 (clone IM7; BV421 BioLegend, BUV395 BD Biosciences), CD45 (clone 30-F11; PerCP/Cy5.5), CD62L (clone MEL-14; FITC), CD90.2 (clone 53–2.1; BV605), CD127 (clone SB/199; APC), KLRG-1 (clone 2F1/KLRG1; PE/Cy7), TCRβ (clone H57-597; BV605), and granzyme B (clone GB11; Pacific Blue). All Abs were purchased from BioLegend unless otherwise stated.

ELISA

Serum samples collected from PBL were assessed for antibody response against the EVM158 by ELISA. Briefly, polystyrene 96-well flat-bottom plates (Costar 9018) were coated with 50 μL/well of mouse interferon-gamma (5 μg/mL) diluted in carbonate-bicarbonate coating buffer (3.7g sodium bicarbonate and 0.64g sodium carbonate in 1 L distilled water) and incubated overnight at 4°C. Plates were washed 3× with 200 μL of PBST (0.05% PBS-Tween 20), blocked by adding 200 μL of 5% non-fat dry milk in PBS and incubated at 37°C for 2 h. Next, 50 μL of cell culture supernatant from BSC-1 cell-infected WT ECTV (containing EVM158 protein) or, as control, ECTV-Δ158 (no EVM158) was added to each well and incubated for 2 h before washing 3× with PBST and adding serum dilutions. 100 μL of 1:500 detection antibody in dilution buffer (Goat α-mouse IgG1, Invitrogen A10551) was then added and incubated for 1 h at 37°C. Plates were washed again, and 200 μL of OPD substrate (Sigma-Aldrich P9187) was added and incubated for 30 min or until color change was visible. The reaction was stopped by adding 50 μL of 3 M HCL. Plates were read at 492 nm using the KCJunior Program.

Statistical analysis

Data were analyzed using Prism (GraphPad) software. Groups were assessed for normal distribution using both Anderson-Darling and D'Agostino and Pearson tests. If all groups passed normality tests ($p > 0.05$), two groups were analyzed using unpaired t test or one-way ANOVA with Tukey's multiple comparisons test for more than two groups. If any groups failed to pass one of the normality tests, we compared two groups using the non-parametric Mann-Whitney test and more than two groups using Kruskal-Wallis with Dunn's multiple comparisons tests. Survival curves were analyzed using the log rank Mantel-Cox test. For tracking TSYKFESV-specific $CD8^+$ T cells in the PBL, comparisons were always made between groups for each day using the appropriate t test or Mann-Whitney test. For most experiments with more than two groups, highlighted statistical differences were compared with the males and females or CD4-depleted and undepleted groups unless indicated otherwise. All experiments were done a minimum of 2 times, and where possible, experiments have been compiled and displayed as mean ± SEM. For all figures, p values are represented by the following symbols: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

DATA AND CODE AVAILABILITY

Not applicable.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

S.K. designed and performed most of the experiments and wrote the paper. B.M. collaborated in experiments. L.T. collaborated in experiments. Y.K.T. supervised the production of the LNPs. H.M. produced all mRNAs. N.P. designed mRNAs and contributed to the funding. L.J.S. was responsible for the project's overall design, supervised most of the work, edited the paper, and contributed to the funding.

DECLARATION OF INTERESTS

L.J.S is a member of the Scientific Board of RNA Advanced Technologies. N.P. is named on patents describing the use of nucleoside-modified mRNA in lipid nanoparticles as a vaccine platform. He has disclosed those interests fully to the University of Pennsylvania,

and he has an approved plan for managing any potential conflicts arising from licensing those patents. N.P. served on the mRNA strategic advisory board of Sanofi Pasteur in 2022. N.P. is a member of the Scientific Advisory Board of AldexChem. Y.K.T. is an employee of Acuitas Therapeutics.

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