Science Advances
AAAS

# Supplementary Materials for

## Multiepitope supramolecular peptide nanofibers eliciting coordinated humoral and cellular antitumor immune responses

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*Sci. Adv.* **8**, eabm7833 (2022)
DOI: 10.1126/sciadv.abm7833

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Table S1
Figs. S1 to S12

**Table S1 Peptides used in this paper. All peptides were purified by HPLC, and the masses were confirmed by MALDI**

| Peptide | MW |
|---|---|
| **PEPvIII-Coil29**<br>$NH_2$-LEEKKGNYVVTDH SGSG QARILEADAEILRAYARILEAHAEILRAQ-$NH_2$* | MW= 5105 g/mol |
| **Trp2-Coil29**<br>$NH_2$- SVYDFFVWL AAYGG QARILEADAEILRAYARILEAHAEILRAQ-$NH_2$* | MW= 4880 g/mol |
| **Td-Coil29**<br>$NH_2$-FNNFTVSFWLRVPKVSASHLE SGSG QARILEADAEILRAYARILEAHAEILRAQ-$NH_2$* | MW= 6053 g/mol |
| **Coil29**<br>Ac-QARILEADAEILRAYARILEAHAEILRAQ-$NH_2$* | MW= 3345 g/mol |
| **PEPvIII**<br>$NH_2$-LEEKKGNYVVTDH-$NH_2$* | MW= 1531 g/mol |
| **Trp2**<br>$NH_2$-SVYDFFVWL-$NH_2$* | MW= 1174 g/mol |
| **Td**<br>$NH_2$-FNNFTVSFWLRVPKVSASHLE-$NH_2$* | MW= 2478 g/mol |
| **Biotin-PEPvIII**<br>Biotin-LEEKKGNYVVTDH-$NH_2$* | MW= 1757 g/mol |

*N-terminal $NH_2$ indicates amidation

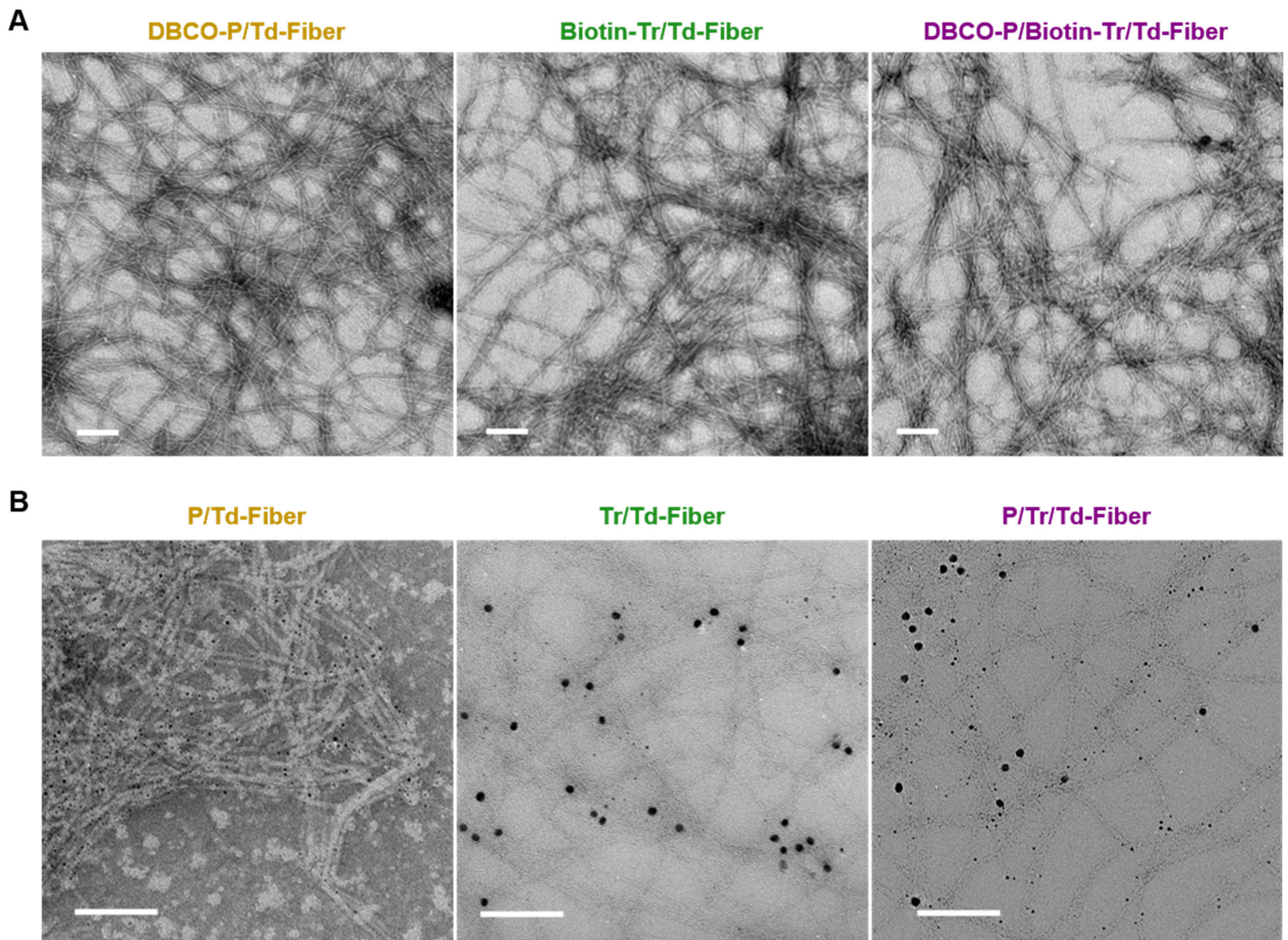
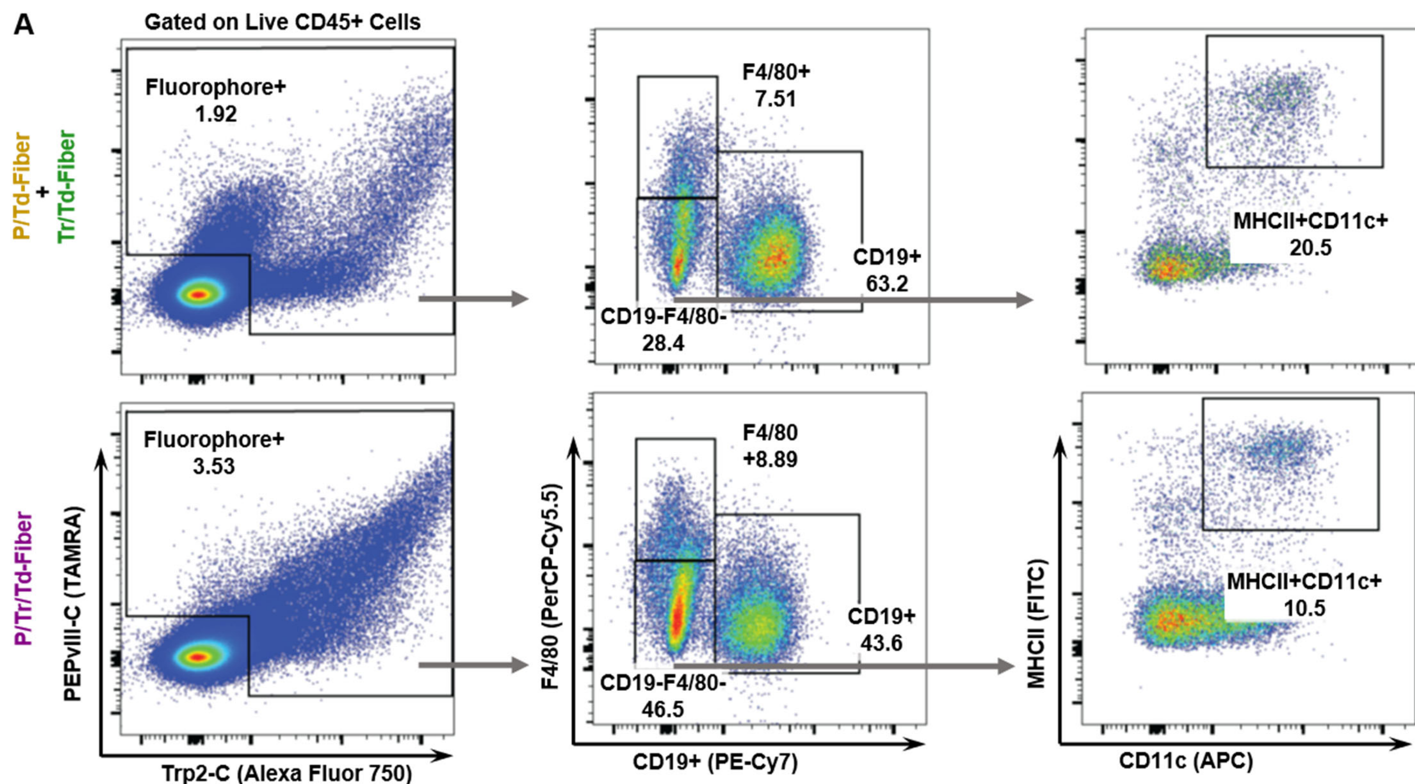



**Figure S1. (A)** Labelling handle modified peptides showed self-assembled morphology consistent to unmodified Coil29 nanofibers. **(B)** Gold nanoparticle labeling confirmed co-assembly between PEPvIII-Coil29 and Trp2-Coil29 peptides (five-nanometer particles label PEPvIII epitopes and ten-nanometer particles label Trp2 epitopes; scale bar = 100 nm)



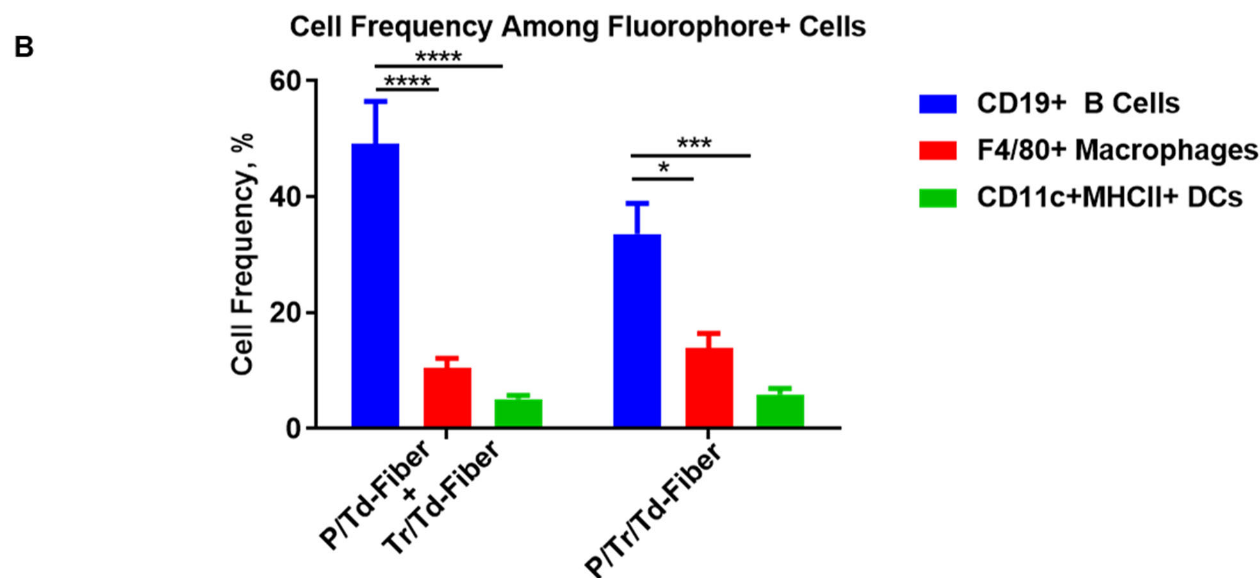




**Figure S2 The majority of fluorophore-labeled nanofibers were taken up by CD19+ B cells, with F4/80+ macrophages and CD11c+MHCII+ dendritic cells internalizing smaller proportions of the materials, 4 h after IP administration. (A)** Representative flow histogram **(B)** The frequencies of each cell types among fluorophore+ CD45+ cells (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ by multiple comparison calculated using two-way ANOVA with Tukey test. N=3).

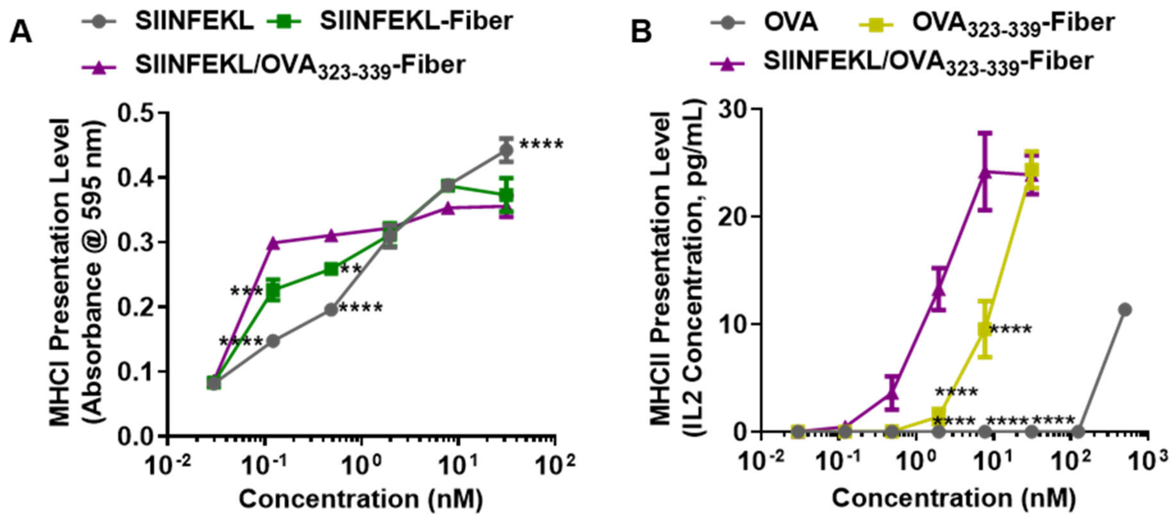



**Figure S3. Both CD4+ and CD8+ T cell epitopes were efficiently presented in vitro when delivered by co-assembled nanofibers. (A)** BMDCs were incubated with either SIINFEKL peptides or assembled nanofibers from indicated groups for 4 hours, followed by incubation with B3Z hybridoma reporter cells for 12 hours. Co-assembled nanofibers were found to efficiently present CD8+ T cell epitopes (**** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, when compared to SIINFEKL/$OVA_{323-339}$-fiber group by multiple comparison using two-way ANOVA with Dunnett test; N=3) **(B)** BMDC were incubated with either $OVA_{323-339}$ peptides or assembled nanofibers from indicated groups, followed by DOBW hybridoma reporter cells. IL-2 ELISA assays were carried out to determine the level of $OVA_{323-339}$/MHCII presentation for different treatment conditions. Co-assembled nanofibers were found to efficiently present CD4+ T cell epitopes (**** $p<0.0001$ when compared to co-assembled SIINFEKL/$OVA_{323-339}$-fiber group, as determined by multiple comparison using two-way ANOVA with Dunnett test; N=3).

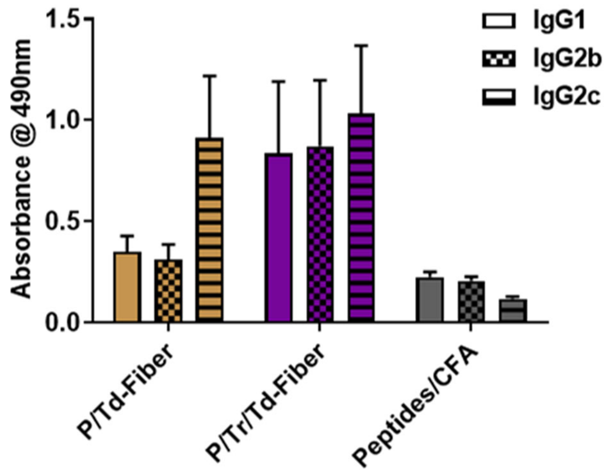



**Figure S4. CpG-adjuvanted nanofibers elicited Th1-biased IgG responses.** Sera were collected from mice in Fig 3A on week 5 and diluted 500-fold for isotype ELISA.

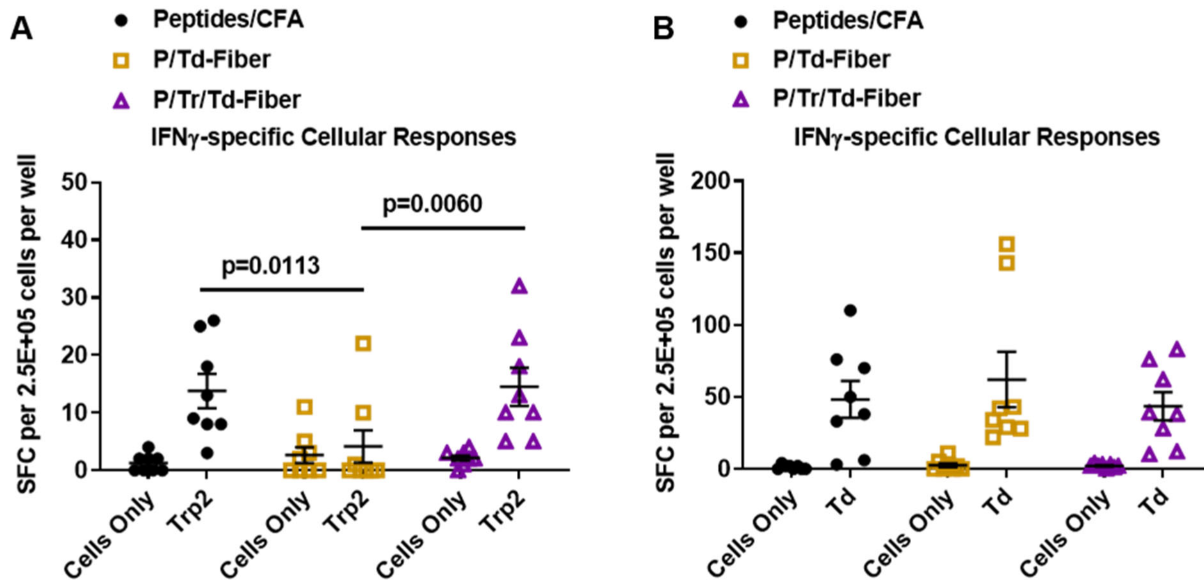



**Figure S5. Co-assembled P/Td-fibers elicited strong cellular responses against Td CD4+ T cell epitopes, but generated low background Trp2-specific responses.** Splenocytes from immunized mice in Fig 2A were harvested 7 days after final immunizations, and were examined for cellular responses against Trp2 **(A)** and Td **(B)**, using IFN-specific ELISPOT assay. (A) p-values calculated by multiple comparison using two-way ANOVA with Tukey test. N=8 (B) No statistical differences were found between groups for either stimulation condition by multiple comparison using two-way ANOVA with Tukey test. N=8.

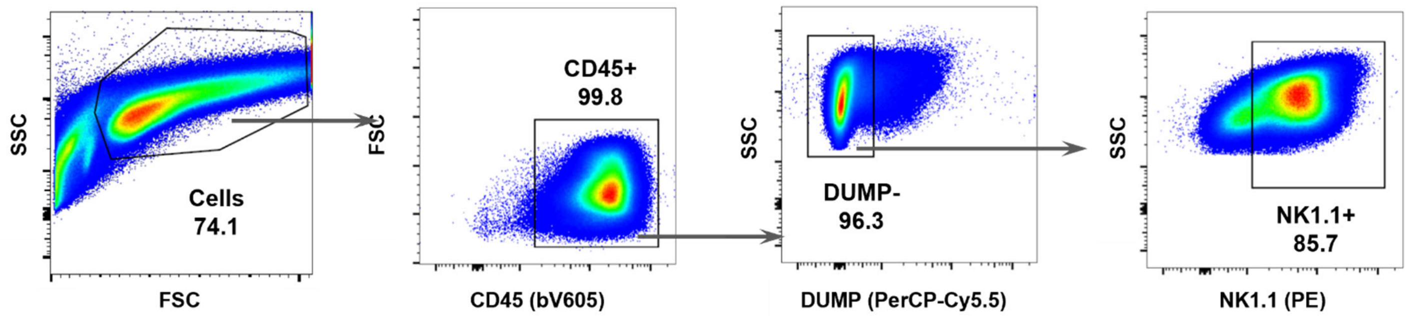



**Figure S6. NK1.1+ cells were expanded and harvested for ADCC assays after 12-day culture of splenocytes in the presence of IL-2 at 1000 U/mL.**

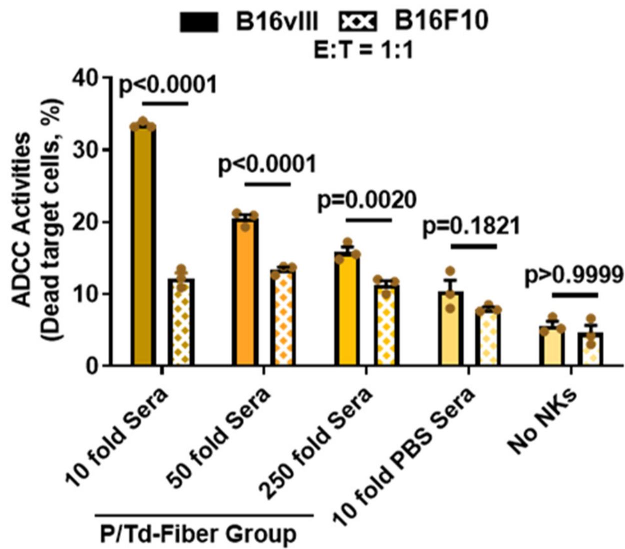



**Figure S7. Sera from mice immunized with P/Td-fibers mediated ADCC activity specifically against B16 cells with EGFRvIII receptors.** CFSE-labelled B16vIII or B16F10 cells were incubated with mouse NK cells with an E:T ratio of 1:1 in the presence of mouse sera after serial dilutions. The viability of B16vIII were assessed after 7 hour incubation, and the percentage of dead cells is defined as ADCC activity. The p values were calculated between different cell types within each treatment group by multiple comparison using two-way ANOVA with Bonferroni test. N=3

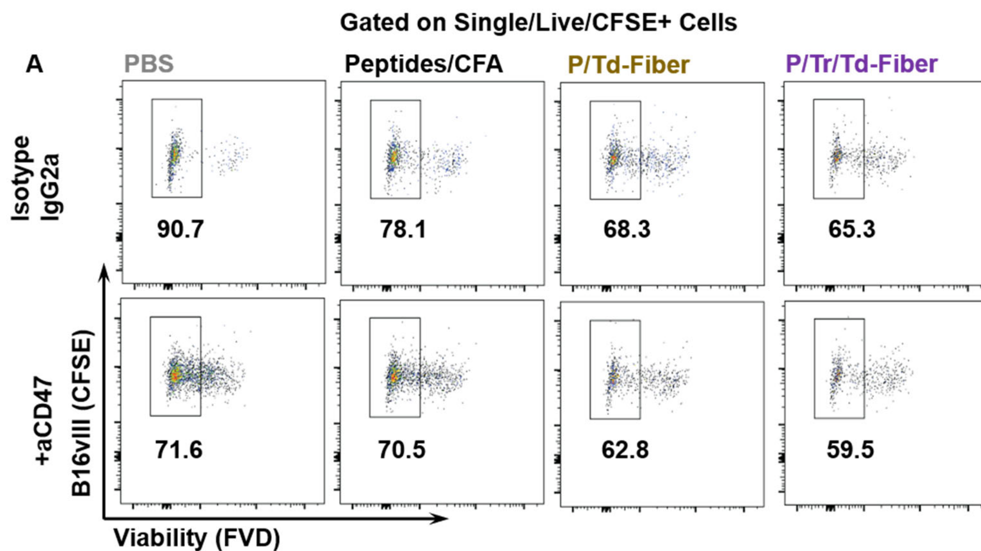



**Figure S8. Representative flow cytometry of CFSE-labeled B16vIII cells 7 h after coculturing with NK cells in the presences of sera from four immunization groups (PBS, Peptides/CFA, P/Td-fiber and P/Tr/Td-fiber)**

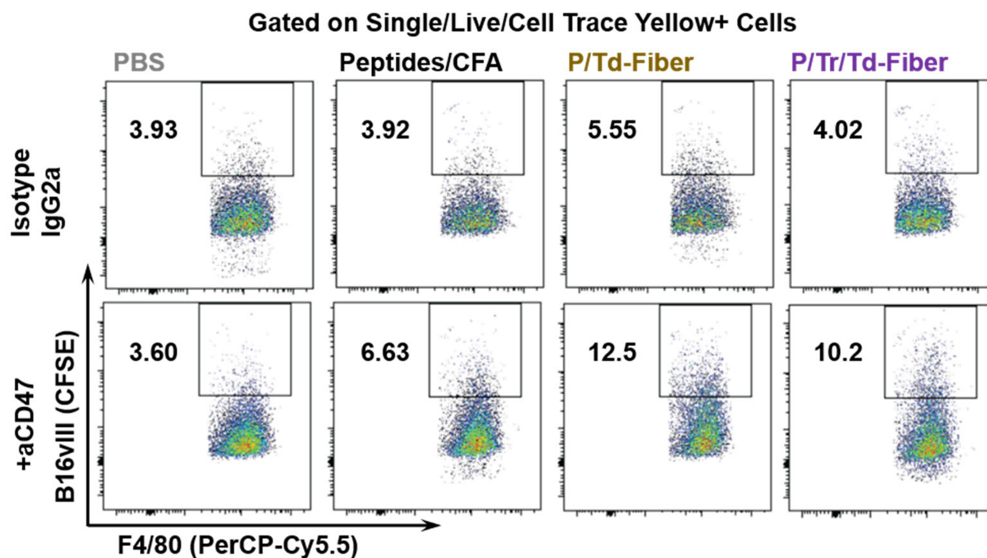



**Figure S9. Representative flow cytometry of phagocytosis activity 5 h after coculturing B16vIII and BMDMs in the presences of sera from four immunization groups (PBS, Peptides/CFA, P/Td-fiber and P/Tr/Td-fiber)**

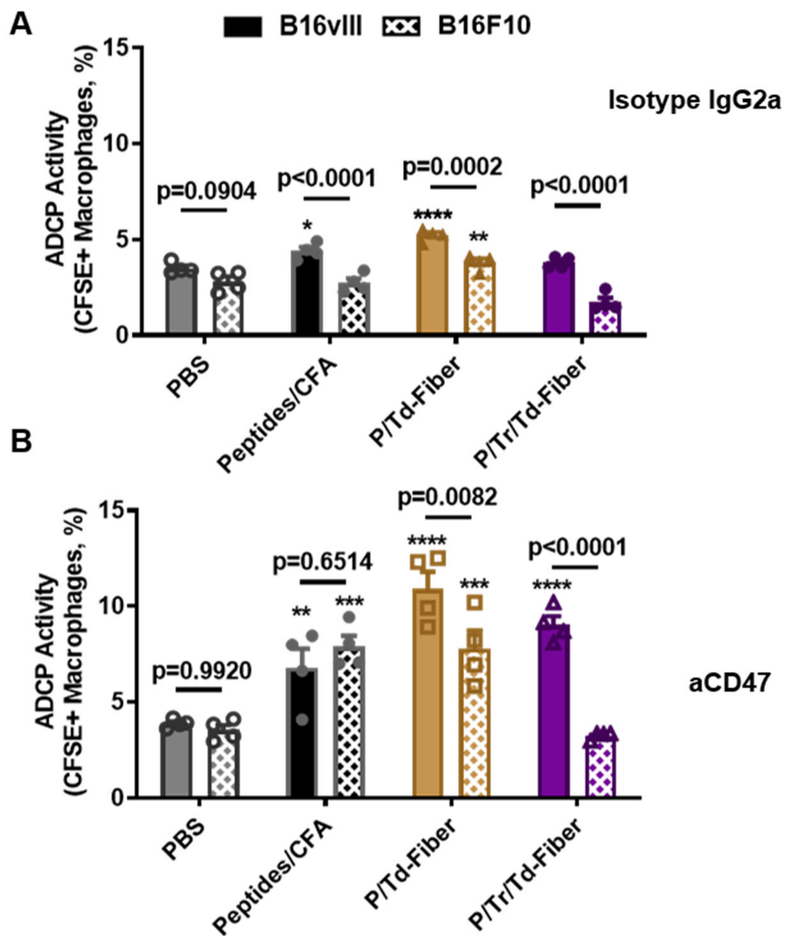



**Figure S10. Sera from immunized mice mediated ADCP phagocytosis specifically against cells with EGFRvIII receptors; and aCD47 antibodies enhanced ADCP against target cells.** CFSE-labelled B16vIII cells were incubated with CellTrace yellow-labelled BMDM with an E:T ratio of 2:1 in the presence of mouse sera from indicated groups, in addition to isotype control antibodies **(A)** or aCD47 **(B)**. The phagocytosis activity against tumor cells (the percentage of CFSE+ macrophages) were assessed after a 5 hour incubation. The p-values were calculated between target cell types within each sera group, by multiple comparison using two-way ANOVA with Bonferroni test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared with PBS within B16vIII or B16F10 groups, by multiple comparison using two-way ANOVA with Dunnett test (N=4).

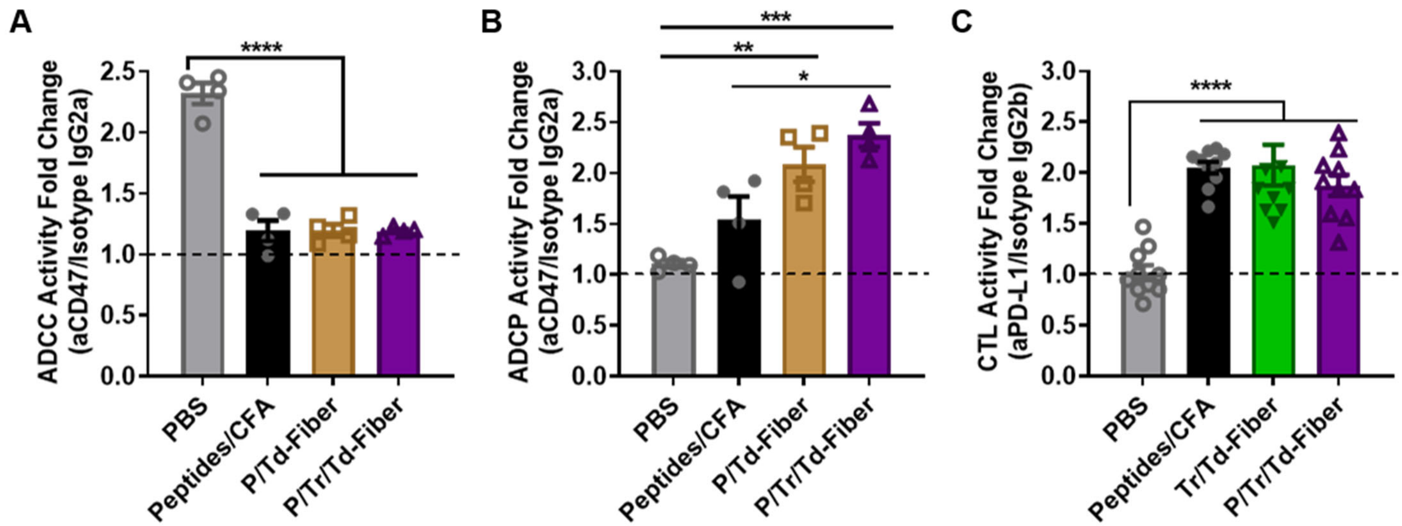



**Figure S11. Immune checkpoint and phagocytosis blockade antibodies enhanced cytotoxic activity against B16vIII cells *in vitro*.** Activity fold changes of the blockade antibodies relative to isotype control (experimental data from Figure 6A, 6B, and 6C): **(A)** ADCC (N=4) **(B)** ADCP (N=4) **(C)** CTL (N=10). * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 by multiple comparison using one-way ANOVA with Tukey test).

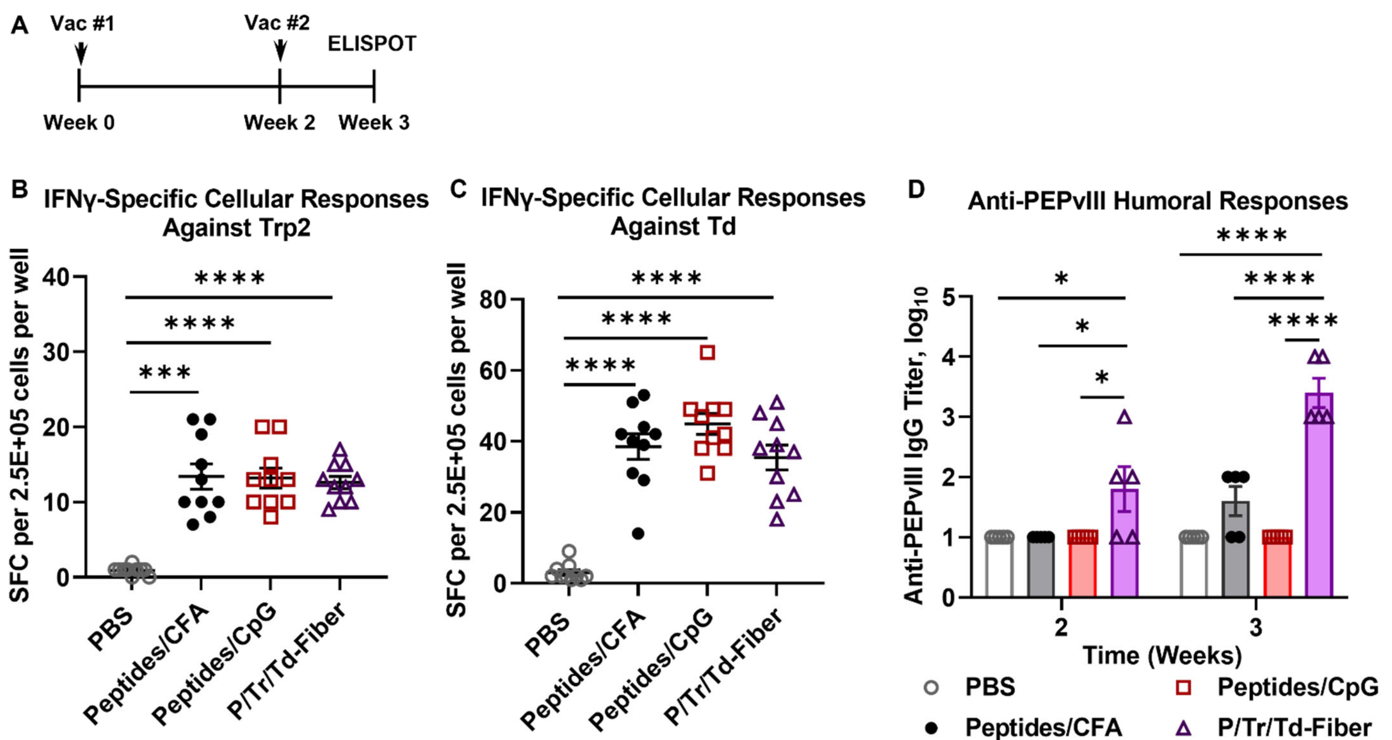



**Figure S12. P/Tr/Td-Fiber vaccine, CFA and CpG showed similar effect in eliciting T cell responses, while multi-epitope nanofibers exhibited superior ability in eliciting antibody responses against PEPvIII antigens. (A)** C57BL/6 mice received two subcutaneous immunizations (black arrows) and splenocytes were harvested on week 3 for ELISPOT assays. PEPvIII-specific IgG titers in sera were monitored on week 2 before booster immunizations and week 3. **(B)** P/Tr/Td-fiber, peptides/CFA emulsion group, and peptides/CpG group all stimulated significant level of Trp2-specific T cell responses on week 3 (*** p<0.001, **** p<0.0001 by multiple comparison using one-way ANOVA with Tukey test, N=10). **(C)** P/Tr/Td-fiber, peptides/CFA emulsion group, and peptides/CpG group all stimulated significant level of Td-specific T cell responses on week 3 (**** p<0.0001 by multiple comparison using one-way ANOVA with Tukey test, N=10). **(D)** P/Tr/Td-fiber elicited stronger PEPvIII-specific IgG responses after primary and booster immunizations, while neither CFA nor CpG was effective in inducing antibody responses when delivered with peptide epitopes. (* p<0.05, **** p<0.0001 by multiple comparison via one-way ANOVA with Tukey test, N=5).