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Supplemental Information

Adoptive Immunotherapy Based on Chain-Centric TCRs in Treatment of Infectious Diseases

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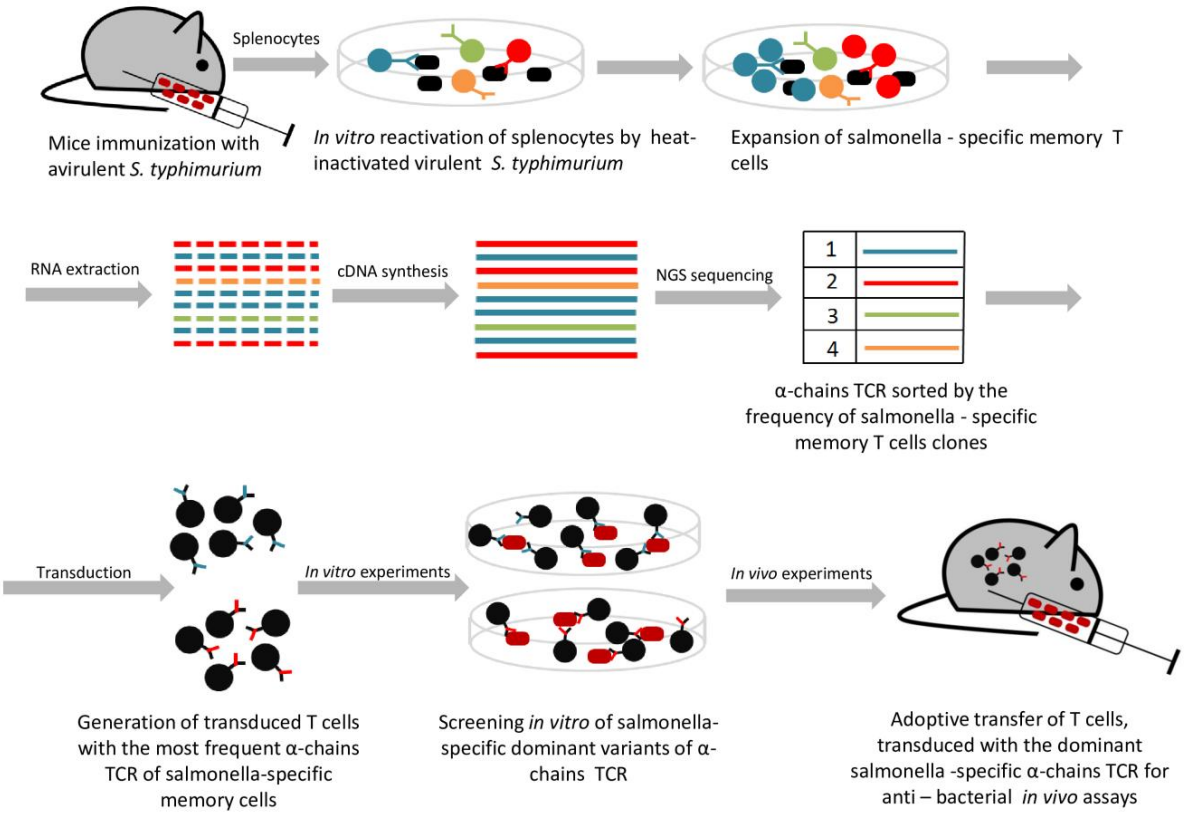

Figure S1. General scheme of experiments. Related to Figure 1, Figure 3, Figure 5, and Figure 6.

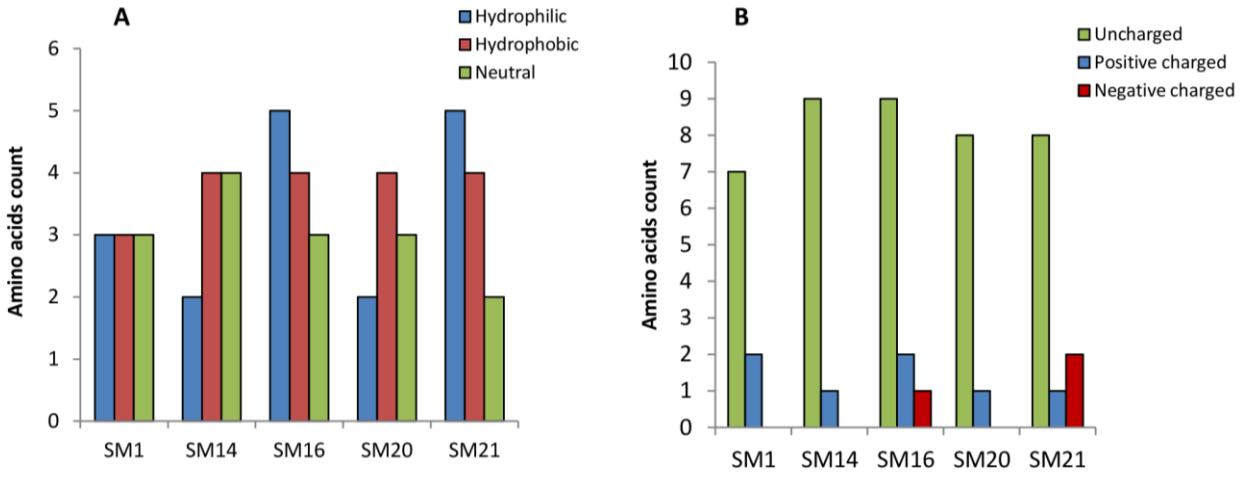

Figure S2. Amino acid (AA) distribution in the CDR3 region of dominant - active salmonella-specific TCRα SM1, SM14, SM16, SM20, and SM21. Related to main-text Results section "Physicochemical properties of the CDR3 region of dominant -active salmonella - specific TCRα**".** Related to Figure 3.
A) Utilization of hydrophobic, hydrophilic, and neutral AA in the CDR3 of each TCRα**. B)** Utilization of uncharged and charged AA in the CDR3 of each TCRα.

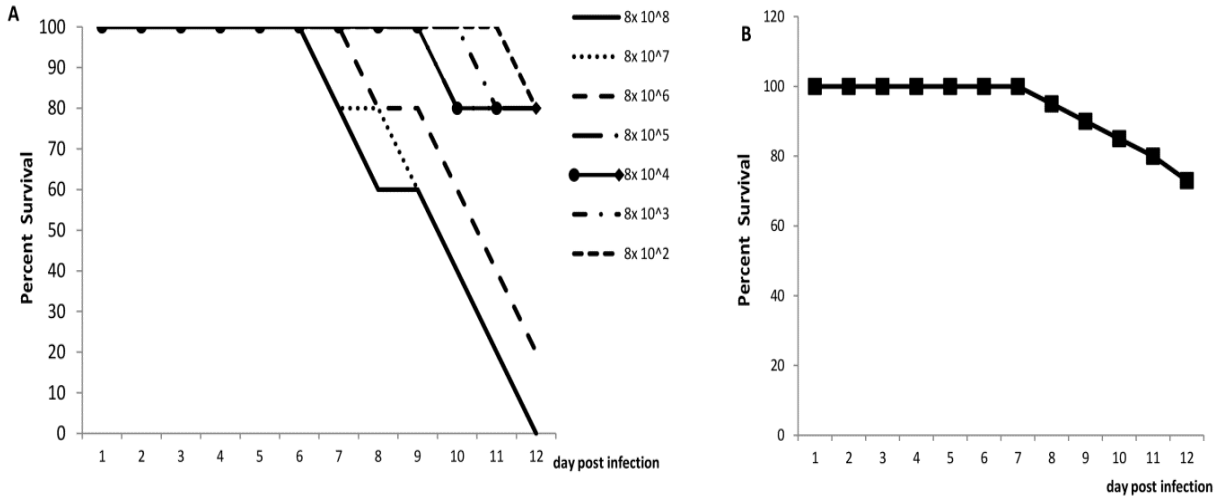

Figure S3. Death dynamics of B10/D2(R101) mice, infected with *S. typhimurium*. Related to Transparent methods. Related to Figure 5, Figure 6.

B10.D2(R101) mice were infected per os via gavage needle with *S. typhimurium* virulent strain IE 147 at doses **(A)** 8.0 x 10^8 - 8.0 x 10^2 CFU/mouse or **(B)** 4.0 - 6.0 x 10^6 CFU/mouse. Animal survival was monitored for 40 days, with animal mortality observed within 8 - 12 days post-infection

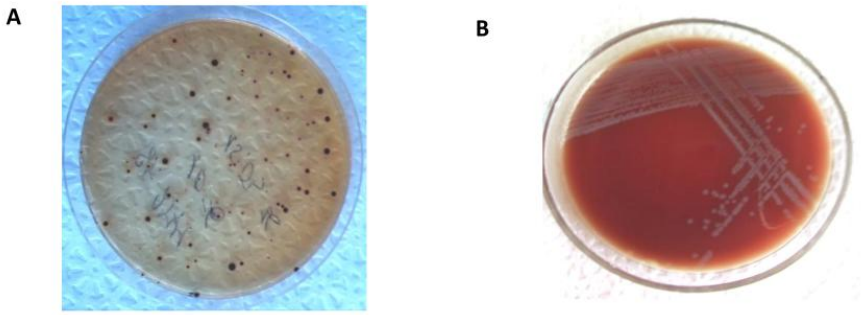

Figure S4. Phenotypic characteristics of *Salmonella typhimurium* virulent strain IE 147. Related to Transparent methods. Related to Figure 1, Figure 5, and Figure 6.
A) Formation of black colonies on SS-agar. **B)** Growth on blood agar without hemolysis.

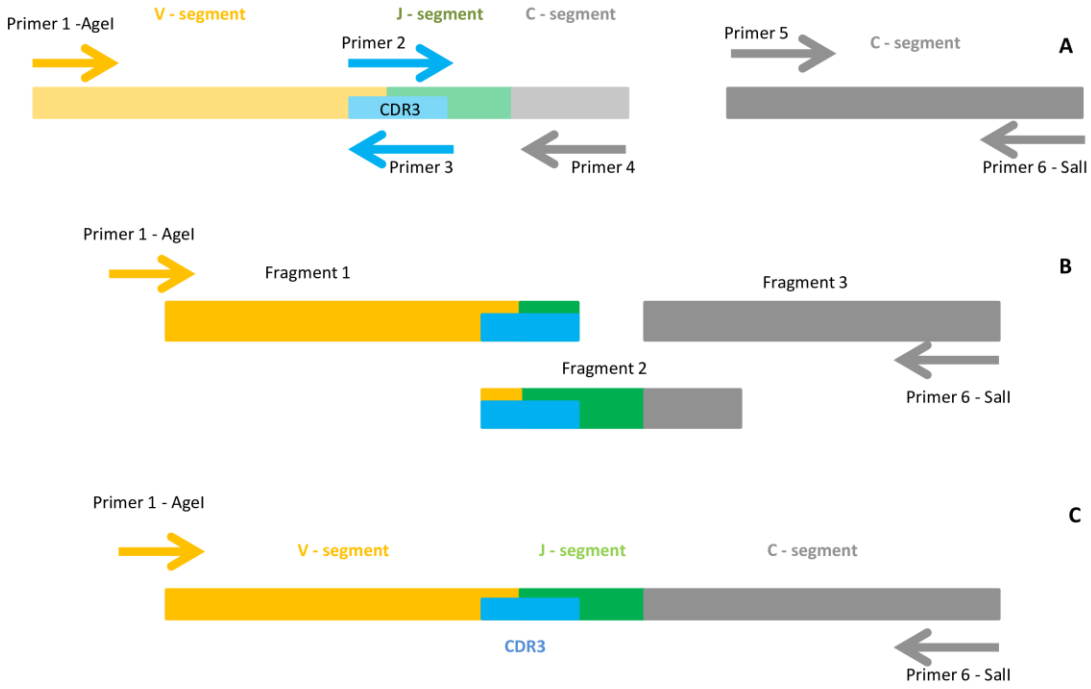

Figure S5. Scheme of selected α-chains TCR cloning. Related to Transparent methods. Related to Figure 3.
A) Schematic presentation of TCRα V-, J-, and C-segments and primers used in cloning. **B)** Three fragments were individually amplified using the combination of Primer 1 – AgeI + Primer 3 (Fragment 1), Primer 2 + Primer 4 (Fragment 2), and Primer 5 + Primer 6-SalI (Fragment 3). **C)** The full ORF of TCRα was amplified by the overlapping PCR using the combination of Primer 1 –AgeI + Primer 6- SalI.

Table S1. Physicochemical properties of the amino acids (AA) in the CDR3 region of dominant -active salmonella - specific TCRα. Related to main-text Results section "Physicochemical properties of the CDR3 region of dominant - active salmonella - specific TCRα"**.** Related to Table 1 and Figure 3.

TCRα	CDR3α full sequence	Length, aa	**Central 5 AA (cCDR3α)**		
			Sequence*	GRAVY**	Normalized polarity
SM1	RVAGYNKLT	9	VAG**Y**N	0,16	0,38
SM14	SAWGNTGKLI	10	**W**GNTG	-1,18	0,28
SM16	REINQGGSAKLI	12	NQGGS	-1,72	0,37
SM20	ITASLGKLQ	9	TAS**L**G	0,74	0,31
SM21	REVMDSNYQLI	11	V**M**DSN	-0,34	0,47

* strongly interacting AA is indicated in red bold
**GRAVY - grand average of hydropathicity index

Table S2. Vα family of the selected dominant -specific α-chains TCR as determined by the amino acid sequence. Related to Table 1.

Variant of α-chain TCR	Vα family
SM1	Va2
SM14	Va3
SM16	Va16
SM20	Va5
SM21	Va16

Supplemental Experimental Procedures

Animals. Mice of inbred line B10.D2(R101) were obtained from the breeding facility of the Federal State Budgetary Institution «N.N. Blokhin National Medical Research Center of Oncology» of the Ministry of Health of the Russian Federation (N.N. Blokhin NMRCO, Moscow, Russia). Mice of inbred line I/StSnEgYCit (I/St), susceptible to *S. typhimurium (*Nesterenko *et al., 2006)*, were obtained from the breeding facility of "N.F. Gamaleya National Research Center of Epidemiology and Microbiology", the Ministry of Health of the Russian Federation ("N.F. Gamaleya NRCEM", Moscow, Russia). Mice were maintained in strict compliance with the NIH Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of N.N. Blokhin NMRCO and "N.F. Gamaleya NRCEM". Both female and male mice (18-22 g, 6-8-wk-of-age) were used in the experiments here.

Bacterial strains and growth conditions. *Salmonella typhimurium* virulent strain IE 147 was obtained from H. Tschape from the Robert Koch Institute branch at Wernigerode, Germany. Strain 274, resistant to rifampicin, was isolated, identified and patented in the "N.F. Gamaleya NRCEM". Rifampicin resistance of strain 274 is due to a mutation in the gene encoding RNA polymerase, which leads to a significant decrease in virulence in this strain. Both bacterial strains exhibited characteristic features of *S. typhimurium*, i.e. formed black colonies on SS-agar (Condalab, Madrid, Spain) and grew on blood agar (Condalab) without hemolysis (Figure S4). Bacterial strains were grown overnight in LB broth (AMRESCO, Inc. Solon, OH). To assess the bacteria growth, 10-fold serial dilutions of the cultures were seeded on SS agar. The numbers of colonies were assessed as described elsewhere. To confirm the avirulence of the strain 247, susceptible I/St mice were i.p. injected with 4.0 $x10^6$ CFU (a lethal dose for virulent strains). No animal mortality was detected during 1 month of observation. Freshly grown bacterial strains IE 147 and 247 were used for infection or immunization of B10.D2(R101) mice, respectively. Bacterial cells of virulent strain IE 147 were heat - inactivated (1h, 60ºC) and used in *in vitro* tests.

Infection of mice. To determine LD50 of *S. typhimurium,* B10.D2(R101) mice were infected per os via a gavage needle with the virulent strain IE 147 at doses 8.0 x 10^2 - 8.0 x 10^8 CFU/mouse in 200 µl sterile saline (Figure S3A). The theoretical LD50 was calculated according to Kerber and amounted 1.0 x 10^7 CFU/mouse. For *in vivo* adoptive transfer experiments here, B10.D2(R101) mice were similarly infected with 4.0 - 6.0 x 10^6 CFU/mouse of the virulent strain IE 147 that corresponded to LD27 with animal mortality within 8 - 12 days post-infection (Figure S3B).

Immunization of mice. To generate salmonella-specific memory T cells, B10.D2(R101) mice were i.p. immunized with 1.0 $x10^6$ CFU/mouse of avirulent strain 247 in 500 µl sterile saline. Immunized mice were used in *in vitro* tests on Day 21 post-immunization. Intact (non-immunized) mice were used as the background control.

Isolation of spleen cells. Immunized and intact mice were sacrificed by cervical dislocation, spleens were aseptically isolated and homogenized in a Potter homogenizer in 3 ml of sterile PBS. Cell suspensions were centrifuged (200 g, 5 min, 4^0C) and resuspended in 3 ml of RPMI-1640 medium (PanEco, Moscow, Russia). Viable spleen cells were counted after trypan blue - eosin staining.

***In vitro* enrichment of salmonella - specific memory T cells.** 3.0 x 10^5 splenocytes of B10.D2(R101) mice, immunized with avirulent strain 247, were cultured with 2.0 x 10^3 - 2.0 x 10^7 CFU of heat-inactivated virulent strain IE 147 in 200 µl of RPMI-1640 medium (PanEco), supplemented with 10% fetal bovine serum (HyClone, GE Healthcare, Chicago, IL), 0.01 mg/ml ciprofloxacin (KRKA, Novo Mesto, Slovenia), 0.01 M HEPES (PanEco), and 10 mM 2-mercaptoethanol (Merck, Darmstadt, Germany) (complete medium, CM) at 37ºC, 5% CO_2 for 72 h. Cell proliferation was measured by incorporation of ^{3}H- thymidine (Saint-Petersburg 'Izotop', Saint-Petersburg, Russia), added for the last 16-18 h of culture. The level of the proliferative response of intact (non-immunized) B10.D2(R101) mice was used as the control. For two rounds of *in vitro* restimulation, spleen cells of immunized and intact mice were similarly cultured with 2.0 x 10^4 - 2.0 x 10^6 CFU of heat-inactivated virulent strain IE 147 for 72 h, then 2.0 x 10^5 CFU were added in 10 µl of CM and cells were cultured for another 72 h at 37ºC, 5% CO_2.

Flow cytometry analysis. Cell samples (5.0 x 10^5 cells) were incubated with Fc block (clone 2.4G2, BD Pharmingen) (10 min, 4ºC) and then stained with the following antibodies (40 min, 4ºC): Brilliant Violet 421 - conjugated anti-CD3 (clone 145-2C11, BioLegend, San Diego, CA); Pacific Blue-conjugated anti-CD8a (clone 53-6.7, eBioscience, San Diego, CA), PE-conjugated anti-CD4 (clone GK 1.5, BioLegend). The analysis was performed on FACS CantoII flow cytometer (BD, San Jose, CA) using the FACSDiva 6.0 program (BD). Leukocytes were gated based on the parameters of side (SSC-A) and forward (FSC-A) scatter followed by gating of singlets based on FSC-H vs. FSC-A parameters. Dead cells were excluded from the analysis by propidium iodide staining (PI, BD Bioscience). Further analyses of surface markers expression were performed in the population of live singlet leukocytes. The results were analyzed using Flow Jo 7.6. (TreeStar Inc., Ashland, OR).

Generation of cDNA libraries. Cell suspensions were prepared as described above from spleens harvested individually from two B10.D2(R101) mice, immunized with avirulent strain 247 as described above. The splenocytes were cultured *in vitro* with two- rounds of re-stimulation by salmonella using 2.0 x 10^6 and 2.0 x 10^5 CFU of heat-inactivated *S. typhimurium* virulent strain IE 147 for the first (72 h) and the second (72 h) re-stimulation, respectively. After two-rounds of *in vitro* re-stimulation by salmonella (6 days *in vitro* cultivation in total) 5.0 x 10^5 - 1.0 x 10^6 spleen cells of

each mouse were used for RNA isolation using TRI reagent (MRC, Inc., Cincinnati, OH). In parallel, splenocytes of these two immunized B10.D2(R101) mice, similarly cultivated for 6 days without the antigen stimulation were harvested for RNA extraction. Using all amount of isolated RNA, cDNA libraries of TCRα of each mouse were prepared as described earlier (Egorov *et al., 2015)*. After two-rounds of PCR amplification as described in ref (Egorov *et al., 2015),* the samples were purified and true-seq adapters were ligated according to the manufacturer`s recommendations (Illumina, San Diego, CA). Next-generation sequencing was performed on MiSeq platform (Illumina) using the Myseq reagent kit (300 cycles). Raw data was processed using MiGEC (Shugay et al., 2014). Briefly, a total of 2,194606 raw reads were obtained which contained 438,151 unique molecular identifiers (UMIs). The data was processed with the threshold 6 reads per UMI for all samples. CDR3-containing reads labeled with identical UMIs were assembled into a single molecular identifier group. Further clonotype extraction from the MiGEC-assembled data was performed using MiXCR software (Bolotin et al., 2015). As a result, two cDNA libraries were generated for each immunized B10.D2(R101) mouse that contained TCRα clonotypes with or without *in vitro* re-stimulation by salmonella. Next, two cDNA libraries (with and without re-stimulation) of each mouse were compared, and clones with at least 1.5-fold increased frequency after re-stimulation were selected for subsequent TCRα cloning.

Cloning of TCRα. Using cDNA generated for NGS-sequencing as the template, V-, J-, and C-segments of 23 selected variants of TCRα were amplified. For this, several oligonucleotides were used: three primers within C-segment, common for all TCRα; one primer to the 5'-end of the ORF, generally unique for different α-chains but common for several α-chains; and two primers (forward and reverse) strictly unique for the CDR3 of each TCRα (Figure S5; Table S3). The resulted amplified V-, J-, and C-segments had the pair wise overlap (Figure S5B), and to obtain the full ORF of each TCRα PCR was performed with 5'-V (Primer 1 -AgeI) and 3'-C (Primer 6 - SalI) primers using the mixture of respective three segments as the template (Figure S5C; Table S3). AgeI and SalI restriction sites were additionally introduced into the 5'-V and 3'-C primers, respectively, to clone the full ORF of each TCRα into the MigRI expression vector. All primers used in TCRα cloning are listed in the Table S3. All cloned 23 variants of TCRα were sequenced and compared with the predicted sequences. No mismatches were found.

Transfection. The full-length cDNA of α-chain TCR or GFP were cloned into the MigRI retroviral vector under the PGK-promoter. pCL-Eco plasmid, kindly provided by Beliavskiĭ A.V. (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia) was used as a packaging plasmid for retroviruses. The 293T cell line was transfected with the plasmids by the calcium-phosphate method.

Transduction. Splenocytes of B10.D2(R101) mice were isolated as described above and activated with 3.0 μg/ml concanavalin A, a T cell mitogen and activator (Ando et al., 2014) (ConA, Sigma Aldrich, St. Louis, MO) and 10.0 U/ml murine interleukin-2 (IL-2, BioLegend) for 24h (Kazansky et al., 2018). Retroviral transduction of activated lymphocytes was performed by two rounds of spinoculation (2000 g, 2 h, 22ºC) with viruses collected 48h and 72h post-transfection for the first and the second spinoculation, respectively (Kazansky et al., 2018). 2 - 4 h post-second spinoculation cells were washed in CM (200 g, 5 min, 4ºC) to remove redundant viruses and then cultured in CM supplemented with 10.0 U/ml IL-2 for 72 h (Kazansky et al., 2018). The efficiency of transduction was determined on Day 3 post-transduction using flow cytometry by staining with APC-conjugated anti-Vα2 antibodies (clone B20.1, BioLegend) to evaluate the expression of corresponding transduced TCRα (e.g., SM1) or by measuring GFP fluorescence in parallel control probes transduced with GFP to evaluate the expression of transduced TCRα that belong to Vα families, for which no commercial antibodies were available. The transduction efficiency amounted 40 - 70%.

Preparation of antigen - presenting cells (APC). We used a mix of the syngenic macrophages feeder layer and syngenic splenocytes as antigen-presenting cells. To generate the macrophages feeder layer, B10.D2(R101) mice were i.p. injected with 3 ml of 3.0% sterile peptone (Sigma Aldrich), and 3 days later macrophages were harvested from the peritoneal cavity in 3 ml of ice-cold PBS. 1.0×10^4 macrophages were cultured with 1.0×10^5 CFU of heat-inactivated virulent strain IE 147 in 200 μl of CM per well of flat-bottom 96-well plates (Corning Costar, Sigma Aldrich, St. Louis, MO) for 72 h at 37°C, 5% CO_2. Then macrophages were washed 2 times with pre-warmed CM (37°C) to remove bacterial cells. In parallel, control macrophages were similarly cultured without bacterial cells to generate the unloaded feeder layer. 5.0×10^5 syngenic intact splenocytes were treated with mitomycin C (25 μg/ml, 30 min, 37°C, Kyowa Hakko Kogyo Co., Ltd., Japan) to prevent cells proliferation, washed twice in CM (200 g, 5 min, 4ºC) and then added to the resulted unloaded macrophages feeder layer (unloaded APC) or the macrophages feeder layer, loaded with salmonella (loaded APC) in 100 μl of CM.

TCRα *in vitro* screening. T cells, transduced with TCRα or GFP, were added in the amount of 3.0×10^5 cells to loaded APC in 100 μl of CM and cultured for 72 h. To estimate the background proliferation, transduced T cells were similarly cultured with unloaded APC. Transduced T cells were seeded in three technical repeats. Cell proliferation was measured by ^{3}H-thymidine incorporation as described above. To determine the level of the antigen-induced response of transduced T cells, the level of the background cell proliferation was subtracted from the level of cell proliferation in the presence of loaded APC. The level of antigen-induced proliferation of non-transduced cells (NTC) was used as the reference. The antigen - induced proliferation of TCRα - transduced T lymphocytes at least 2.0-fold exceeding the reference level was considered as an indication of the dominant active α-chain that formed chain-centric TCRs after pairing with random endogenous α-chains of T lymphocytes.

Analyses of physicochemical properties of CDR3 regions of dominant-active TCRα. Evaluation of physicochemical properties of amino acids (AA) in the CDR3 region of 5 functional dominant - active salmonella - specific TCRα chains (SM1, SM14, SM16, SM20, and SM21) were performed using VDJ tools (Shugay et al., 2015). All physicochemical properties of each AA were obtained from the IMGT database (Lefranc et al., 1999). The first 3 and the last AA were removed from the analyses of the CDR3α sequence (obtained from the generated cDNA libraries, Table 1) as described previously (Wang et al., 2012; Yu et al. 2019), and AAs distribution was analyzed in the resulted CDR3α sequence (Table S1, Fig. S2). Averaged values of strength, hydropathicity, and polarity were calculated for the central 5 AAs of the CDR3α of each TCRα.

Adoptive transfer. T cells, transduced with the salmonella-specific TCRα were i.v. injected to B10.D2(R101) mice in a dose 3.0 x 10^5 cells/mouse in 200 μl of PBS 3 days before or 3 days after infection with virulent strain IE 147 for analysis of their prophylactic or therapeutic activity, respectively. NTC were similarly transferred to animals as the negative control.

Analysis of bacterial loads. On Day 7 post - infection spleens of mice were aseptically isolated and homogenized in a SilentCrasher M homogenizer (Heidolph, *Schwabach, Germany)* in 1 ml of sterile saline. To estimate salmonella loads, serial dilutions of spleen homogenates were plated onto SS-agar. Colonies were counted following 12 h incubation at 37 º C.

Statistical analysis. Data are presented as mean ± SD. All statistical analyses were performed using the unpaired Student`s t-test and one-way analysis of variance (ANOVA). A p-value < 0.05 was considered significant. All statistical analyses were performed using Prism software (v. 8.1.2, GraphPad, San Diego, CA).

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