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Supplemental Information

RNA Aptamers Recognizing Murine CCL17 Inhibit T Cell Chemotaxis and Reduce Contact Hypersensitivity *In Vivo*

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SUPPLEMENTAL FIGURES AND TABLES

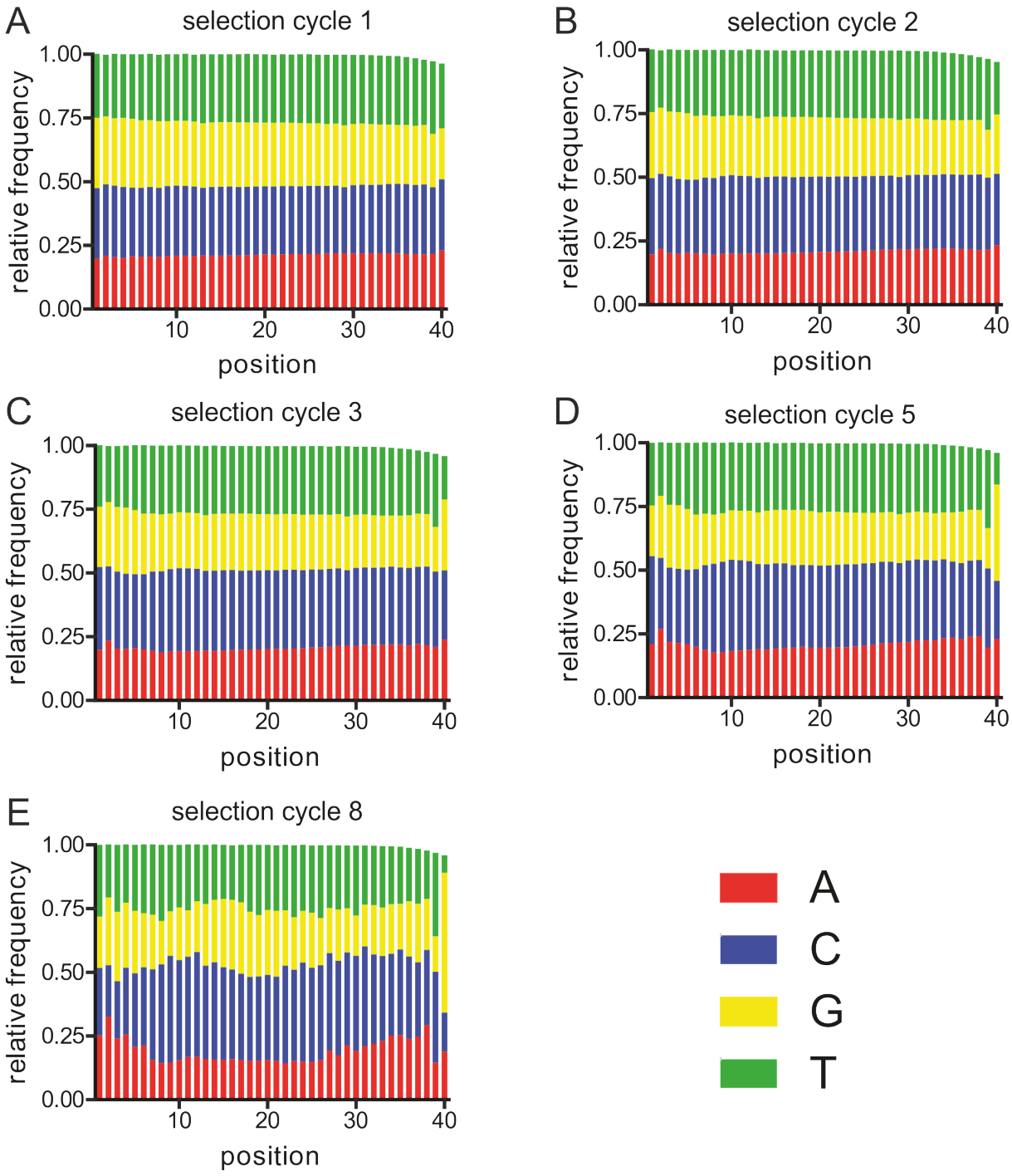

Figure S1: Nucleotide distribution at the different positions of the random region in selection cycles 1, 2, 3, 5, and 8

While the nucleotides are evenly distributed in the first selection cycle (**A**), preferences for certain nucleotides at certain positions evolve over the selection cycles 2 (**B**), 3 (**C**) and 5 (**D**) until selection cycle 8 (**E**). The nucleotide distributions for the starting library and selection cycle 10 are shown in **Figure 1B** and **C**.

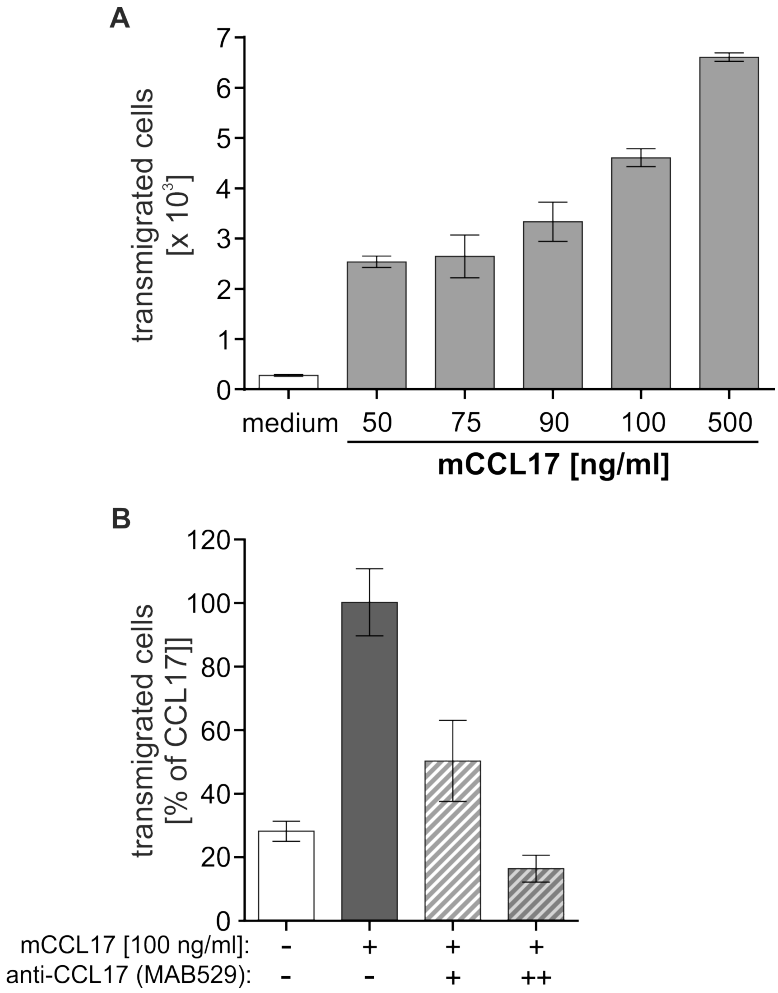

Figure S2: Establishment of the transwell migration assay

A: BW 5147.3 cells were subjected to a transwell migration assay with increasing concentrations of mCCL17 as indicated. After 2 hr, transmigrated cells were counted and plotted as technical duplicates. **B:** anti-CCL17 monoclonal antibody (MAB529) was used at 1.88 µg/ml (+) and 3.76 µg/ml (++) to inhibit migration of BW5147.3 cells towards 100 ng/ml mCCL17 (molar ratio of 1:1 and 2:1, respectively). Transmigrated cells were counted after 2 hr and are shown as percent of migration towards mCCL17 w/o addition of antibody. In both graphs, one representative experiment out of two is shown (mean ± SD).

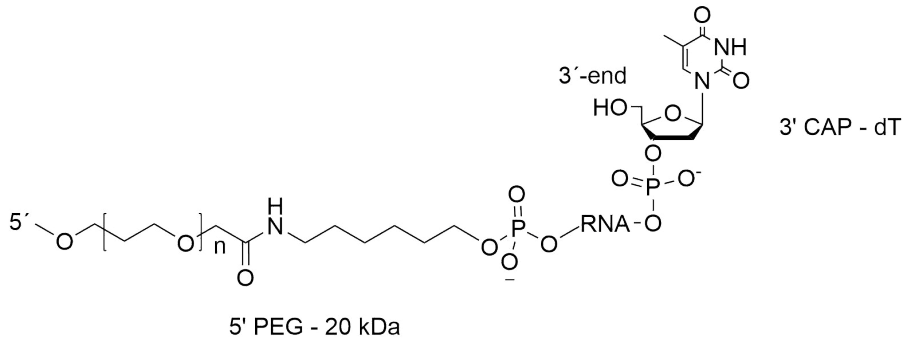

Figure S3: **5' and 3' modifications of the truncated aptamers**

Truncated 2'F-RNA aptamers were modified on the 5' position with a 20 kDa PEG in order to prevent rapid renal filtration and on the 3' position with an inverted dT in order to increase stability against nuclease degradation.

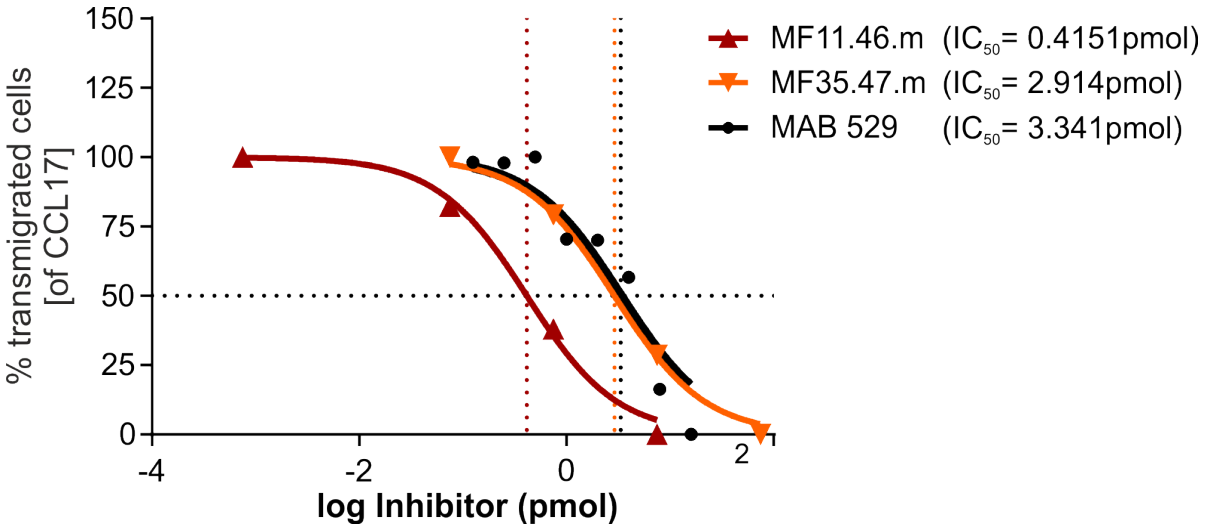

Figure S4: Comparison of the IC_{50} of anti-CCL17 antibody and aptamers

Effectiveness of MAB529, MF11.46.m and MF35.47.m was compared by determining the IC_{50} (half maximal inhibitory concentration). BW5147.3 cells were subjected to a transwell migration assay and increasing concentrations of MAB529, MF11.46.m and MF35.47.m were tested to inhibit migration towards 100 ng/ml mCCL17 (=7.5 pmol). Transmigrated cells were counted after 2 hr. Total cell counts were normalized to the medium control and shown as percent of migration towards mCCL17 w/o addition of antibody or aptamer. One representative experiment out of three is shown.

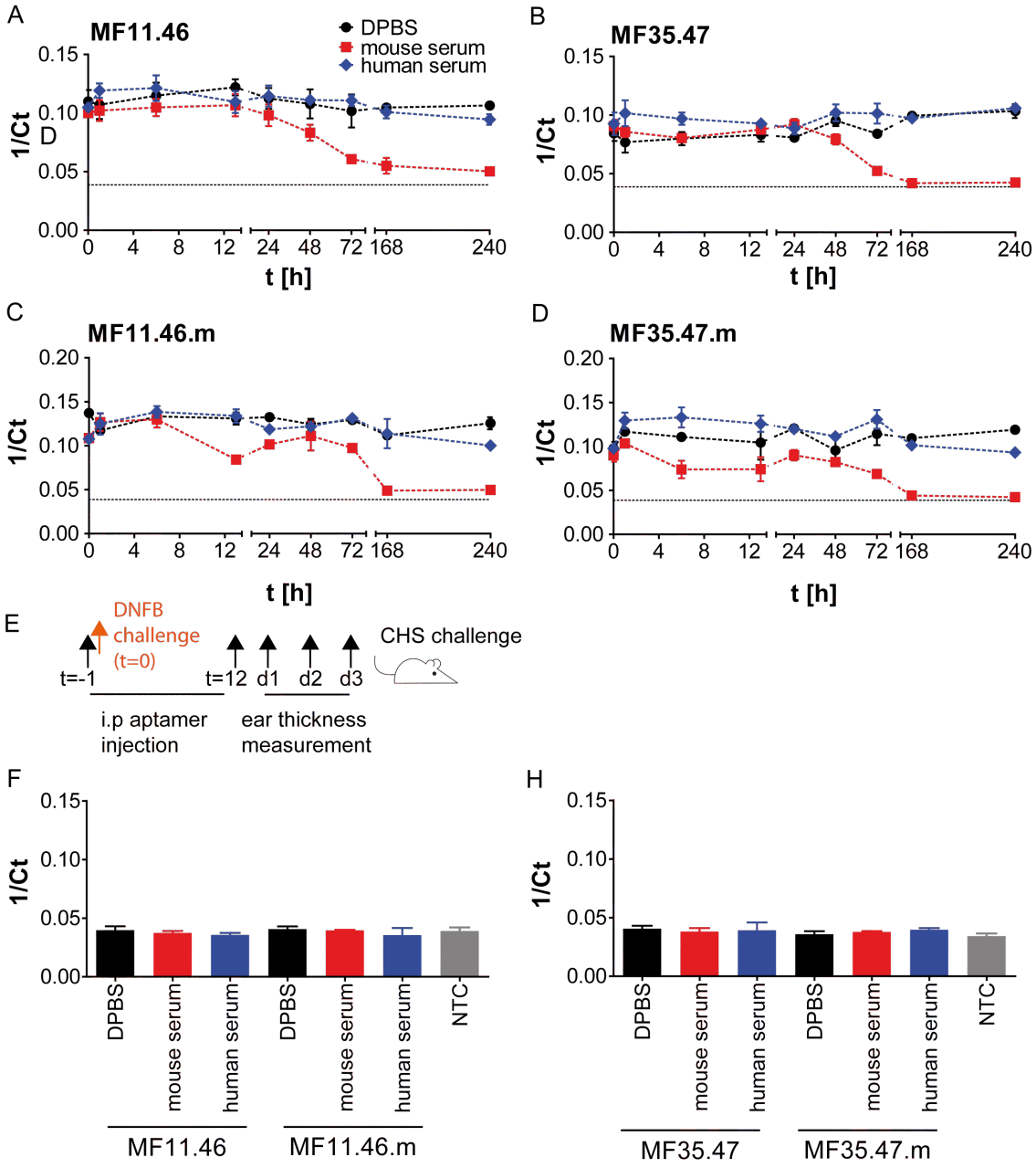

Figure S5 – Serum stability of unmodified and modified aptamers in DPBS, mouse and human serum

Serum stability of MF11.46 (**A**), MF35.47 (**B**), MF11.46.m (**C**), and MF35.47.m (**D**) in 90% Dulbecco's PBS (DPBS) (black dots), mouse (red rectangles), and human serum (blue diamonds). After reverse transcription, the samples were evaluated using qPCR. Depicted is 1/Ct for each investigated time point (t = 0, 1, 6, 13, 24, 48, 72, 168, and 240 hr) to show the decrease of RNA over time. The dashed line indicates the 1/Ct of the respective no template control (NTC). **E**: The timeline of the *in vivo* contact hypersensitivity assay correlates to the time points of the serum stability assay minus 1 hr. **F and G**: Additional serum stability samples were taken at t = 0 hr, digested by RNAse T1, reverse transcribed and evaluated by qPCR. 1/Ct is depicted side by side with the value for the respective no template control (NTC) for MF11 (**F**) and MF35 (**G**) (n=2; mean ± SD).

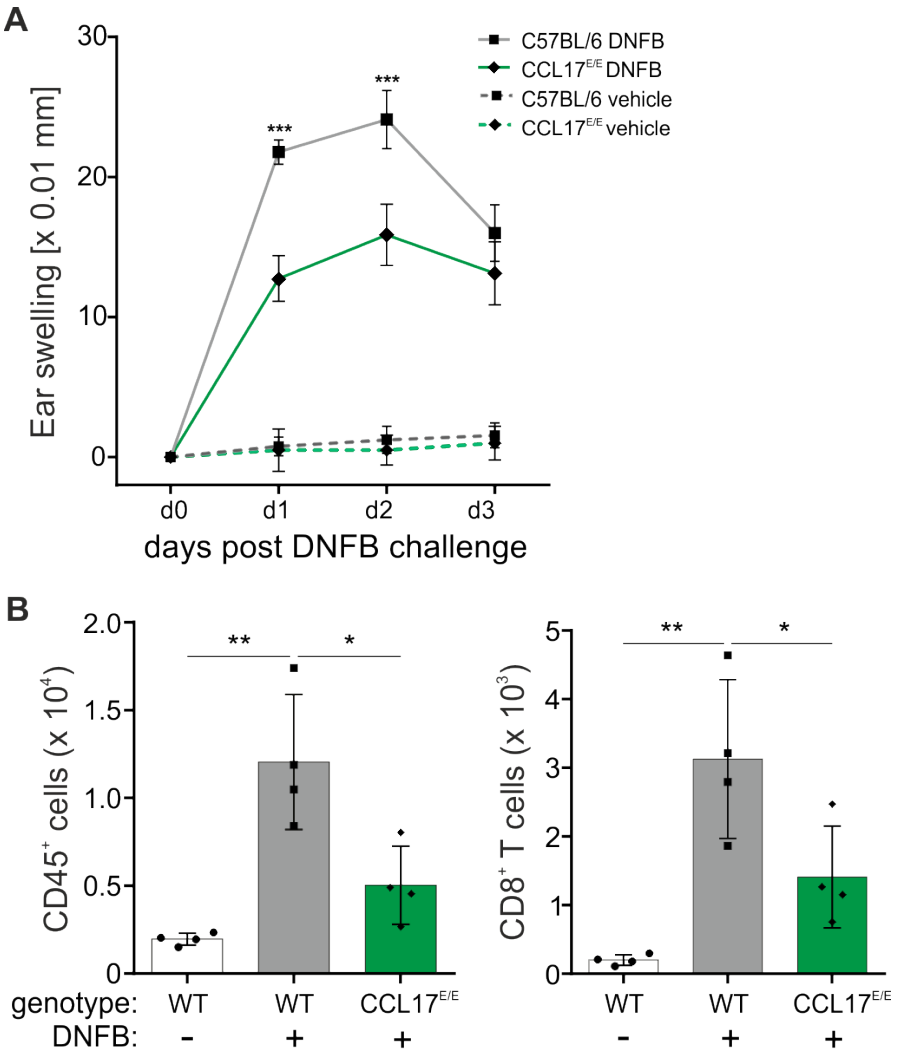

Figure S6: CCL17-deficient mice are protected from contact hypersensitivity

A: CHS response of C57BL/6 (grey lines) and CCL17$^{E/E}$ mice (green lines) after sensitization with DNFB on day -5 and day -4. At day 0, mice were challenged with DNFB at the right ear (solid lines), whereas vehicle only was applied to the left ear as a control (dashed lines). Thickness of the ears was determined the three following days. Data is shown as ear swelling response (day 1, 2 or 3 minus day 0); n=8-9 animals per group, mean ± SEM. One representative of three experiments is shown. Data was tested for statistical significance by 2-way ANOVA with Bonferroni post-hoc test for multiple comparisons (***p<0.001). **B**: Flow cytometric analysis of the immune cell infiltrate. At day 4 ears were digested and the isolated cells subjected to staining for flow cytometry. Absolute numbers of CD45$^+$ and CD3$^+$CD8$^+$ cells were determined by flow cytometry (n=4 per group, mean ± SD). The gating strategy is depicted in Fig. S7. Data was tested for statistical significance by student's t-test compared to the DNFB treated WT group (**p<0.01; *p<0.05).

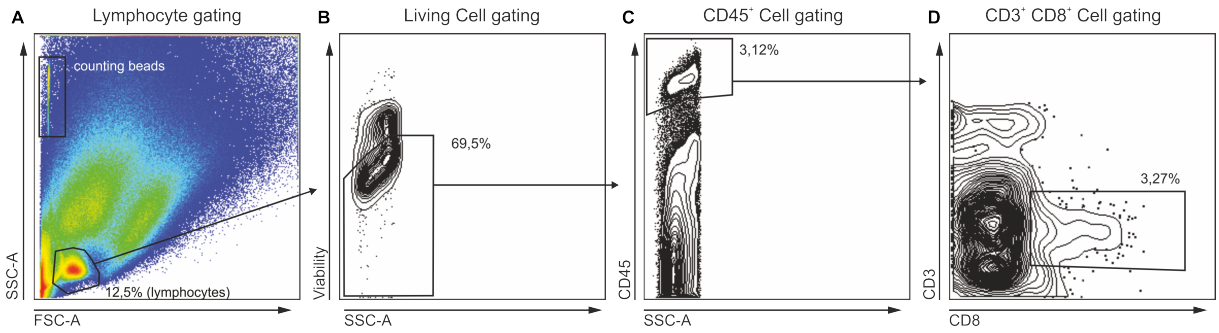

Figure S7: Gating strategy to identify skin lymphocytes and T cells

Lymphocytes were identified based on their characteristic FSC-A and SSC-A profiles (**A**). Using a viability staining, dead cells were excluded from further analysis (**B**). $CD45^+$ cells were identified among all living cells (**C**) and then further analyzed for CD3 and CD8 expression (**D**). Total cell numbers of $CD45^+$ and $CD45^+CD3^+CD8^+$ cells are depicted in **Figure S6B and Figure 6B**

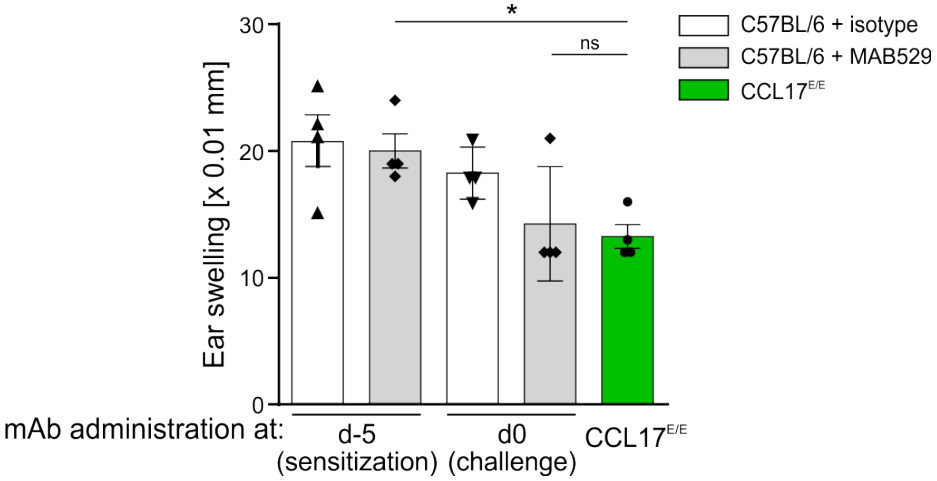

Figure S8: Determination of the optimal time point for intervention in the CHS model

To determine the optimal time point of pharmaceutical intervention C57BL/6J (WT) mice were injected with an anti-CCL17 antibody (MAB529, 200 µg/mouse) immediately before sensitization (day -5), or before challenge (day 0) as indicated. Ear swelling was compared to WT mice injected with an isotype control antibody (200 µg/mouse) and to non-injected $CCL17^{E/E}$ mice (n=4, mean ± SD). Data was tested for statistical significance by 1-way ANOVA with Bonferroni post-hoc test for multiple comparisons (*$p<0.05$, ns=not significant).

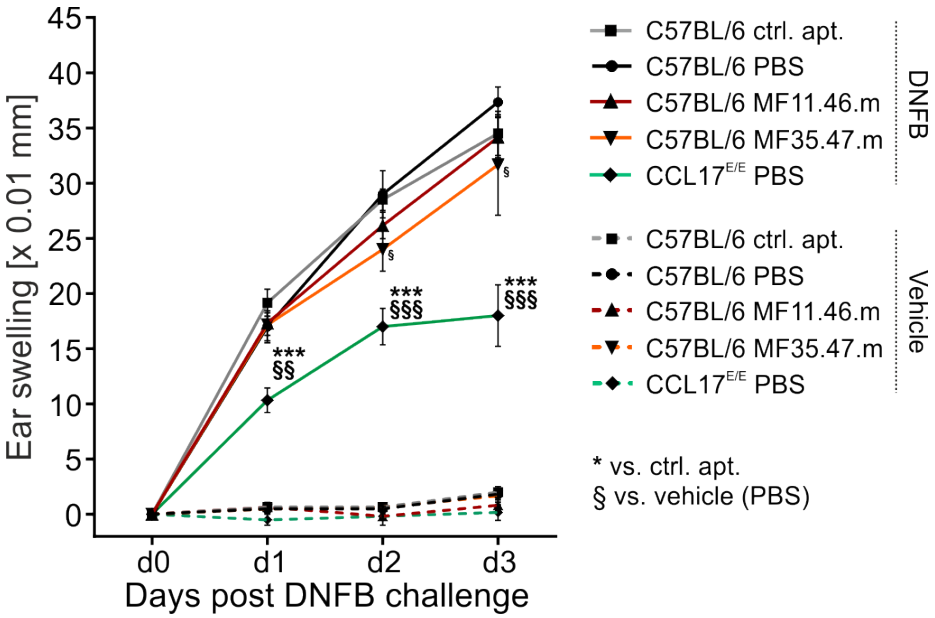

Figure S9: CHS response after low-dose injections of aptamers

C57BL/6 WT and CCL17$^{E/E}$ mice were sensitized with DNFB on day -8 and day -7. On day 0, WT mice were injected i.p. with 1 nmol of MF11.46.m and MF35.47.m, the control aptamer or PBS 1 hr before and 12 hr after DNFB challenge. CCL17$^{E/E}$ mice received PBS only. Depicted is the ear swelling of WT and CCL17$^{E/E}$ mice 24 hr (d1), 48 hr (d2) and 72 hr (d3) after application of DNFB (solid lines) or vehicle (dashed lines). (n=6 per group, mean ± SEM). Data was tested for statistical significance by 2-way ANOVA with Bonferroni post-hoc test for multiple comparisons (***p<0.001; §§§p<0.001; §§p<0.005).

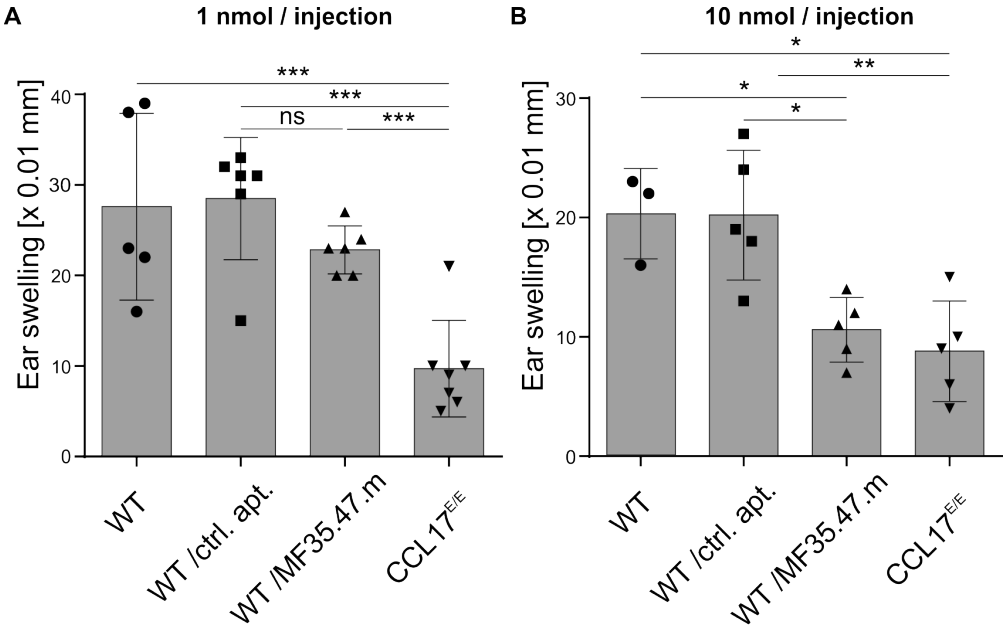

Figure S10: Testing of different aptamer doses in the CHS model

C57BL/6J (WT) and CCL17$^{E/E}$ mice were sensitized with DNFB on day -5 and day -4. 1 hr before and 12 hr after DNFB challenge on day 0 WT mice were injected i.p. with the CCL17 specific aptamer MF35.47.m or the control aptamer. **A**: mice received 1 nmol aptamer per injection and **B**: mice received 10 nmol aptamer per injection. Ear swelling was assessed 24 hr after challenge (n=3-7, mean ± SD). Data was tested for statistical significance by 1-way ANOVA with Bonferroni post-hoc test for multiple comparisons (***p<0.001, **p<0.01, *p<0.05, ns=not significant).

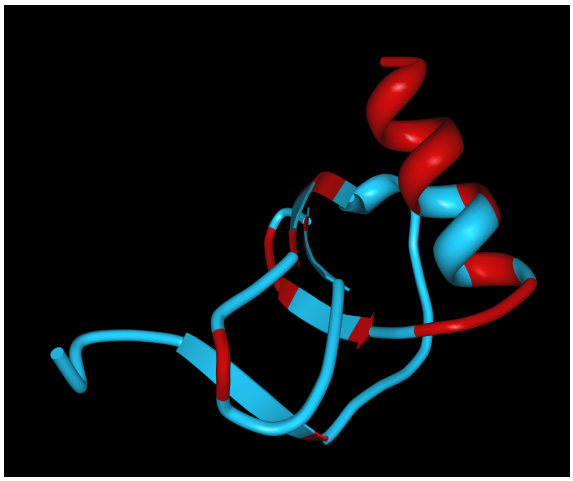

Figure S11: Structural comparison of human and mouse CCL17

Shown is the crystal structure of the human CCL17 (*PDB: 1NR4*).[1] Amino acids that differ between mouse and human CCL17 have been depicted in red. Identical amino acids are depicted in cyan.

Table S1: Selection conditions

Depicted are the amount of beads, washing steps and volume applied during the different selection cycles as well as the number of RT-PCR cycles used to amplify the eluted RNA.

Selection cycle	Empty beads	CCL17 beads	Wash	Total wash volume	RT-PCR cycles
1		80 µL	1 x 30 s 1 x 3 min	200 µL	5
2	80 µL	80 µL	1 x 30 s 1 x 3 min	200 µL	11
3	80 µL	80 µL	2 x 30 s 2 x 3 min	400 µL	13
4	80 µL	80 µL	2 x 30 s 2 x 3 min	400 µL	13
5	80 µL	80 µL	3 x 30 s 3 x 3 min	600 µL	12
6	80 µL	80 µL	3 x 30 s 3 x 3 min	600 µL	11
7	80 µL	80 µL	4 x 30 s 4 x 3 min	800 µL	8
8	80 µL	80 µL	4 x 30 s 4 x 3 min	800 µL	8
9	80 µL	80 µL	4 x 30 s 4 x 3 min	800 µL	7
10	80 µL	80 µL	4 x 30 s 4 x 3 min	800 µL	6

Table S2: Frequency of tested clones in Sanger and next-generation sequencing

Depicted is the sequence of the tested clones as determined by Sanger sequencing and NGS of the 10th selection cycle. Sequences with a single point mutation found by Sanger sequencing were considered identical. For NGS, sequences with up to 5 mutations (point mutation, insertion, deletion) were considered identical. Analyzed were 27 clones from Sanger sequencing and 4.23 million sequences from NGS.
*indicates that the sequence could not be detected in the next-generation sequencing data of the 10th selection cycle. In this case, we included the frequency of the respective sequence in the selection cycle 8.

Sequence Name	Random Region	Sanger Seq. [%]	NGS [%]
MF1*	ATACATGATCGTCTTCTCGACTGCATCGCACTTCCCTGAGCA	unique	0.0119
MF2	GTGAAGCCGCACACTCGCACTTCCCCTAAAACAAACCAGGCA	unique	0.923
MF4	TAGTCGTCGCGGGTTGCTCCATGAATGGGGCGGTACA	unique	0.165
MF7	CCGTGTCCTCTACCGCGGTAACGGACCTCCCGTTTGCTGGCA	7.69 (2x)	0.52
MF8*	AGTCTTTGCATCGCTTCCCTTTACATCAGTCCACTCATTGCA	7.69 (2x)	0.0358
MF11	AATAGAGTCGTCGCGGGTTGGCTCGTAGATCGGGCCGGTACA	19.3 (5x)	24.5
MF15	TGGACACTAACCTGCCGCAGTTGTTCTTCCCCTACCGCCGCA	unique	0.005
MF19	CGGACCTCCACGTCGCGTCGCTTTCCCATATACACTACTGCA	unique	1.73
MF35	GAGCAGCATTTGTGGTTTCCCGATCGCTTCCCCTAAACAGCA	unique	0.452

SUPPLEMENTAL MATERIALS AND METHODS

Coupling of CCL17 to magnetic beads. 25 µg recombinant mCCL17 (BioLegend, Koblenz, Germany) was incubated with EZ-Link Sulfo-NHS-LC-Biotin in accordance with the manufacturer's guidelines (Thermo Fisher Scientific, Darmstadt, Germany). 17 µg biotinylated CCL17 was immobilized on 5.5 mg Dynabeads M-280 Streptavidin (Thermo Fisher Scientific), washed and resuspended in 1500 µl PBS, pH 7.4, containing 0.01 mg/ml BSA (Merck Millipore, Darmstadt, Germany).

RNA sequences and preparation

MF11: 5'-GGG AGG ACG AUG CGG AAU AGA GUC GUC GCG GGU UGG CUC GUA GAU CGG GCC GGU ACA GAC GAC UCG CUG AGG AUC CGA GA-3'

MF35: 5'-GGG AGG ACG AUG CGG GAG CAG CAU UUG UGG UUU CCC GAU CGC UUC CCC UAA ACA GCA GAC GAC UCG CUG AGG AUC CGA GA-3'

MF11.46: 5'-GG GUC GUC GCG GGU UGG CUC GUA GAU CGG GCC GGU ACA GAC GAC CC-3'

MF35.47: 5'-GGG AGG ACG AUG CGG GAG CAG CAU UUG UGG UUU CCC GAU CGC UUC CC-3'

control aptamer: 5'-GGG GGC GUG UAU GCC AGA CCU GCC GAU GUC CUU AAG GUC GAC GCU GU-3'

The full-length aptamers and the non-modified short versions for the transwell assays were prepared by *in vitro* transcription as described under "Selection of CCL17-binding aptamers".

Synthesis of the truncated 2'F-RNA. Biotinylated MF11.46, MF35.47 and control aptamer for the surface plasmon resonance measurement as well as the modified

versions of the truncated aptamers for the *in vivo* and transwell assays were synthesized as follows:

Synthesis was performed on an ABI394 according to the manufacturer's recommendations: deblock: 3% (w/v) in methylene chloride; CAP A: *80% tetrahydrofuran, 10% acetic anhydride, 10% 2,6-lutidine*; CAP B: (tetrahydrofurane/N-methylimidazole 84/16 (v/v)); oxidizer (tetrahyrofurane/water/pyridine/iodine 66/12/22/0.6 (v/v/v/w)); activator: 0,25 M ETT (5-ethylthio-1H-tetrazole) in acetonitrile (Sigma-Aldrich, Munich, Germany).

The commercially available 2´-TBDMS protected amidites (Bz-A-CEPhosphoramidite, ibu-G-CEP) as well as the 2´-F amidites (2´F-dC-Ac-CEP and 2´F-dU-CEP) (Sigma-Aldrich) were used as 0.1 M and coupled under standard conditions as recommended by the synthesizer manufacturer (6 min). For further PEG functionalization, the oligos were labeled with a 5´-MMT-amino modification. All other amidites were commercially available and coupled under standard conditions, as recommended by the synthesizer manufacturer.

Oligonucleotides were deprotected using ammonium hydroxide for 17 hr at 55°C. Afterwards, the TBDMS protecting group was removed using tetrabutylammonium fluoride (TBAF, 1.0 M in THF). The oligos were then precipitated and desalted using Glen-Pak RNA cartridge ® and purified using reversed phase HPLC. Afterwards MMT/DMT-groups were removed.

In case of the PEGylated oligonucleotides, the PEG-NHS Ester (Creative PEGWorks, Chapel Hill, USA) was attached to the amino-labeled oligonucleotide in conjugation buffer (sodium borate buffer 0.1 M pH 8.5), and reacted o/n using 5 equiv. of the PEG-NHS ester, followed by a second RP-HPLC purification to remove unreacted PEG and unmodified oligonucleotides.

Preparation of 2'F-RNA for filter retention assays. The 2'F-RNA was transcribed *in vitro* as described under "Selection of CCL17-binding aptamers", but in the presence of 0.5 µl α-^{32}P-GTP (PerkinElmer LAS, Solingen, Germany). The radiolabeled 2'F-RNA was purified with the Nucleospin Gel and PCR cleanup kit according to the manufacturer's instructions and eluted in 50 µl ddH_2O (Macherey & Nagel, Düren, Germany).

Filter retention assay. 1 µl radioactive *in vitro* transcription product was mixed with the protein (none, 1 µM mCCL17 or streptavidin) in binding buffer (PBS pH 7.4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.000266% w/v BSA) and 0.01 mg/ml heparin sodium salt from porcine intestinal mucosa (Sigma-Aldrich). The samples were incubated for 30 min at 37°C. PROTAN nitrocellulose membrane (Sigma.Aldrich) was soaked for 15 min in 27 mM Tris, 40 mM 6-aminocaproic acid (Alfa Aesar, Karlsruhe, Germany), pH 9.4 and for 5 min in PBS. The samples were passed through the membrane, which was subsequently washed three times with 200 µl washing buffer (PBS, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) and dried. An additional piece of membrane was spotted with the same amount of 2'F-RNA and used for normalization of the intensities. Radiolabeled 2'F-RNA on the membranes was quantified using a Fujifilm FLA-3000.

Aptamer stability assay. 200 pmol of MF11.46, MF11.46.m, MF35.47, or MF35.47.m were incubated in Dulbecco's PBS (DPBS) (Gibco) or 90% mouse (Sigma Aldrich) or human serum (kindly provided by PD Dr. Jens Müller, University Hospital Bonn) at 37 °C for 0, 1, 6, 13, 24, 48, 72, 168, or 240 hr in a final volume of 20 µl. At each time point, 1 µl of each sample was reverse transcribed using the reverse primer MF11.46_rv 5'-GGGTCGTCTGTACCGGCC-3' or MF35.47_rv 5'-GGGAAGCGATCGGGAAACCA-3'. In addition, 1 µl aptamer (t = 0 hr) was digested

using RNase T1 (Thermo Fisher Scientific) for 30 min at 37 °C before the reverse transcription. The cDNA was diluted 1:50 and amplified by qPCR using SYBR Green I (Sigma Aldrich), the above mentioned reverse primer and the forward primer MF11.46_fw 5'-GGGTCGTCGCGGGTTGG-3' or MF35.47_fw 5'-GGGAGGACGATGCGGGAG-3'. 40 PCR cycles were performed in an iCycler Thermal Cycler upgraded with the iQ5 real-time PCR detection system (Bio-Rad). Serial dilutions of the t = 0 hr samples were used as standards. For analysis, the threshold was set to ~100 RFU and the resulting Ct-values are depicted as 1/Ct. All standard curves had an $r^2 \geq 0.98$.

TNF-α homogeneous time-resolved fluorescence assay. The TNF-α homogeneous time-resolved fluorescence (HTRF) assay was performed in accordance with the manufacturer's guidelines (Cisbio, Berlin, Germany). Briefly, immortalized murine embryonic stem cell-derived macrophages were treated with rising concentrations of oligonucleotides for 24 hr. The cell supernatants were collected and stained with anti-TNF-α antibodies conjugated to FRET molecules. Changes in the fluorescence emission spectrum were proportional to the TNF-α concentration.

Surface Plasmon Resonance (SPR). SPR was performed using a BIAcore 3000 (GE Healthcare Europe GmbH, Munich, Germany). Running buffer (1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.000266% w/v BSA in 1x PBS) and regeneration buffer (50 mM EDTA) were filtered (0.22 µm) and degassed prior to use. 50 nM biotinylated aptamers (in 0.5 M NaCl) were immobilized on Xantec SAD chips at 25°C and a flow rate of 10 µl/min until a response of ~500 response units was reached. The control aptamer was immobilized as a non-binding control on the flow cell (Fc) 1, MF35.47

and MF11.46 were immobilized on Fc2 and Fc3, respectively. Proteins were injected at a flow rate of 80 µl/min for 120 s with a dissociation phase of 500 s. Afterwards, the flow cells were regenerated for 30 s with 50 mM EDTA. Immobilization was done at 25°C whereas binding analysis was performed at 37°C.

K_D determination was performed by equilibrium analysis. For this, the response of the steady state is plotted against the concentration of the protein. The K_D was determined with Graph Pad Prism 5.01 using one site nonlinear regression.

Next generation sequencing. Samples of the selection cycles 1, 2, 3, 5, 8 and 10 as well as the starting library were prepared for next generation sequencing according to Tolle et al.[2] Briefly, the samples were PCR-amplified with primers containing different bar codes for each cycle. The samples were then mixed and phosphorylated with 1 µl 10 U/µl T4 PNK (NEB, Frankfurt, Germany), 5 µl 10x PNK buffer and 0.5 µl 100 mM ATP in a final volume of 50 µl at 37°C for 60 min. After cleanup with a Gel and PCR cleanup kit (Macherey & Nagel) according to the manufacturer's instructions, they were ligated with an adaptor that allows hybridization to the sequencing flow cell. After agarose gel purification, the libraries were quantified using the KAPA library quantification kit for Illumina libraries according to manufacturer's instructions on a Roche LightCycler 480. Libraries were clustered at 7 pM supplemented with PhiX on a TruSeq SR v3 flow cell and sequenced over 76 base pairs and 7 index bases on a HiSeq1500 (Illumina, San Diego, USA). Sequencing data were demultiplexed using CASAVA v1.8.2.

Analysis of NGS-data was accomplished with the software tool COMPAS.[3,4] Sequences were directly parsed from FASTQ files. For this purpose, selection cycle specific bar codes where used to assign sequences to the respective datasets. In the next step, the random region of each sequence was defined by teaching the

COMPAS software the flanking, constant primer regions. The relative distribution of the A, T, G, T nucleotide building blocks over the random region was calculated for the datasets of the starting library as well as selection cycles 1, 2, 3, 5, 8 and 10. Potential CCL17-binding aptamer candidates were identified *in silico* in datasets of selection cycles 5, 8 and 10. For each cycle, in the first step, similar sequences were clustered by using relative information entropy as a measure to group sequences to patterns of related sequences. In the second step, sequences of each cluster were counted to calculate the relative frequency of the entire cluster as well as for each monoclonal sequence of each cluster. To trace the enrichment behavior of defined aptamers, COMPAS was used to calculate the relative frequency of aptamers MF11, MF35, MF1, MF2, MF4, MF7, MF8 and MF19 in datasets of selection cycles cycles 1, 2, 3, 5, 8 and 10 and the starting library. The quotient of the relative frequency values of successive selection cycles yielded the fold amplification values, as a measure for aptamer enrichment.

Flow cytometric analysis of leukocyte infiltrates. Four days after challenge (day 4), treated ears were removed, dorsal and ventral sides separated and placed with the dermal side down on 0.25% Trypsin in PBS-EDTA for 45 minutes at 37°C. Afterwards the tissue was minced and digested in 500 µl PBS with 0.154mg/ml Liberase™ (Sigma-Aldrich) and 0.1 mg/ml DNase I (Biomatik, Cambridge/Ontario, Canada) in a 12-well cell culture plate. Digestion was performed at 37°C for 90 minutes in a shaking incubator at 100 rpm. The lysate was subsequently resuspended using a blunted 1000 µl pipette tip and pushed through a 100 µm filter. Subsequently, the cell suspension was filtered through a 70 µm filter and centrifuged at 4000 rpm for 10 minutes. Cells were stained with antibodies against CD3 (145-2C11), CD8 (53-6.7), F4/80 (CI:A3-1, Bio-Rad Abd Serotec, Puchheim, Germany),

CD11b (M1/70), MHCII (M5/114.15.2), CD45 (30-F11, Biolegend). If not indicated otherwise, antibodies were purchased from Thermo Fisher Scientific. For identification of living cells Fixable Viability Dye eFluor® 450 from Thermo Fisher Scientific was used. Cells were analyzed on a BD LSR II Cytometer.

Amino acid sequence comparison of mouse and human CCL17

The structure of human CCL17 is available in the protein data bank under the ID 1NR4.[1] Using the freeware program RCSB PDB Protein Workshop 4.2.0,[5] the amino acids of mouse CCL17 that differ from those of the human protein were colored in red. All other amino acids have been depicted in cyan. As the crystal structure does not include the residues 1 to 3, the deviation from glycine (human) to alanine (mouse) at position three could not be depicted. The protein sequences to determine amino acid deviations were from http://www.prospecbio.com/CCL17_Human_4_213 and http:/www.prospecbio.com/TARC_Mouse_4 (both extracted 19.05.2016). The online program clustal O (1.2.1.)[6,7] was used to align the two sequences and determine differences.

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