Supplementary Materials for

Sustained T follicular helper cell response is essential for control of chronic viral infection

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DOI: 10.1126/sciimmunol.aam8686

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Supplementary Materials and Methods

Production of recombinant LCMV-clone 13-GP1-Fc and LCMV-clone 13-NP

The sequence of the GP1 (amino acids 1 to 265) of the LCMV clone 13 strain was amplified and cloned into a pHPI vector containing the sequence of the LCMV-WE GP1 and the human IgG1 Fc part (Eschli et al., 2007). Cloning was performed so that the LCMV-WE GP1 sequence was replaced by the sequence of the clone 13 GP1. Expression of clone 13-GP1-Fc was performed by transient transfection of CHO cells with jetPEI according to the manufacturers’ protocol. Transfected cells were cultured for 2 to 3 days, afterward the supernatant containing the GP1-Fc was harvested and used for ELISA analysis.

The gene sequence of the LCMV-clone 13 nucleoprotein (NP) was cloned into the IPTG-inducible bacterial expression vector pET28b (Novagene) under control of the T7 promoter and linked to a N-terminal His-Tag (6x His). Production of clone 13-NP-His was performed in BL21 bacteria cultured in TB medium. Expression was induced with 1 mM IPTG (Sigma-Aldrich) at an OD of 0.8 to 1. Afterward, bacteria were cultured at 16°C o/n. Then bacteria were harvested, lysed with lysozyme (Sigma-Aldrich) for 1h at 4°C and further sonicated (140 s, 10s per run and a 20% amplitude). The supernatant was bound to Protino® Ni-NTA Agarose beads (Macherey-Nagel) (100 µl per 2 ml sample) for 1h at 4°C. Thereafter the sample was loaded to a column and the residue was washed three times with wash buffer containing increasing amounts of imidazole (0 mM, 40 mM and 150 mM; Sigma-Aldrich). This was followed by three elution steps with elution buffer containing 250 mM imidazole. Every washing and elution step was kept for analysis and stored at 4°C. Presence of clone 13-NP-His was confirmed by SDS-Page and ELISA.
**ELISA**

Plates were coated overnight at 4°C with the respective specific antigen diluted in 0.1M sodium carbonate buffer (pH=9.6). For detection of LCMV-specific antibodies, plates were coated with 20 µg/ml lysate of LCMV-clone 13 infected MC57G cells or as negative control with 20 µg/ml lysate of uninfected MC57G cells. For detection of GP1-specific antibodies, plates were coated with 1:50 diluted GP1-humanFc and NP-specific antibodies were detected on plates coated with 10 µg/ml of recombinant clone 13-NP. Development of the ELISA was performed as described in Eschli et al, 2007 (19).

**ELISPOT**

Plates were coated for 90 min at 37°C with the respective antigen diluted in 50µl PBS. For coating, 20 µg/ml lysate of LCMV-clone 13 infected MC57G cells or 20 µg/ml lysate of uninfected MC57G cells (as negative control) were used, or wells were treated with PBS only (as further negative control). Plates were blocked with Roswell Park Memorial Institute (RPMI) medium containing 10% FCS. To obtain single splenocyte suspensions, spleens were smashed through a metal grid with a syringe plunger. Single cell suspensions from bone marrow (bm) were obtained by flushing the bone of a hind leg with medium and resuspending the bone marrow several times with a 10 ml pipet. Single cell suspensions were treated with ACK lysis buffer for 5 min at room temperature to lyse red blood cells and after a washing step cell numbers were determined using a Neubauer counting chamber. 1x10^5 cells per well were transferred in 100 µl RPMI medium (10% FCS) onto the coated ELISPOT plates. Afterwards, plates were incubated overnight at 37°C without disturbing or moving them. Then, plates were incubated with 50 µl of a Biotin-coupled anti-mouse IgG antibody (Jackson Immunoresearch Laboratories Inc.) diluted 1:1000 in PBS. Thereafter, the biotinylated antibody was detected with AP-coupled Streptavidin (Vector Laboratories) diluted 1:1000 in
50µl PBS. Color reaction was performed using the AP Conjugate Substrate Kit (Biorad). Between every step, plates were washed six times with PBS-Tween 20 (0.05%). The spots were counted using the AID ELISpot reader (CTL, Germany) with the AID ELISpot software (Version 7.0).

**Immunohistochemistry and fluorescence microscopy**

7 µm thin sections were air-dried and fixed in ice-cold acetone for 10 min at RT. Then, thin sections were rehydrated in PBS and blocked with PBS containing 10% goat serum for 1 hour at room temperature. After blocking, Biotin and Avidin binding sites in tissues were blocked with an Avidin/Biotin blocking kit (Vector Laboratories) following the manufacturer’s instructions. Primary staining was performed with the following reagents: PNA-Biotin (Sigma-Aldrich), anti-IgD-APC (Biolegend), anti-CD4-PE (Biolegend) and anti-CD35/21-FITC (Biolegend). The reagents were diluted in blocking buffer and staining was performed for 1 hour at room temperature in the dark. After three washing steps with PBS a secondary staining was performed for PNA-biotin. Streptavidin-PB was diluted in blocking buffer and slides were incubated for 45 minutes at room temperature in the dark. Afterwards, slides were again washed three times with PBS. Slides were mounted with Mowiol (Sigma-Aldrich Chemie GmbH) and air-dried until the Mowiol had hardened. Images were acquired on the Visitron Confocal System (inverse confocal microscope, Visitron Systems GmbH) and analysed using Volocity software (version 6.3, PerkinElmer).
**Supplementary Figures**

**Fig. S1: Basic characterization of the CD4-DTR mouse line.** A) Schematic illustration of the generation of the CD4-DTR mouse line. B) Total cell counts C) and percentage of cells in different T cell developmental stages in the thymus of CD4-DTR (white) and wt C57BL/6 (black) mice. DN = double negative, DN1-DN4: defined by CD25 and CD44 expression. DP = double positive. SP = single positive. D) Total cell counts and E) percentage of CD4, CD8 and B cells in spleen of CD4-DTR (white) and wt C57BL/6 (black) mice. F) Total cell counts and G) percentage of CD4, CD8 and B cells in mesenteric lymph nodes (mLN) of CD4-DTR (white) and wt C57BL/6 (black) mice. One representative experiment is shown, n=6. Error bars represent means ±SD. Statistical analysis performed using multiple unpaired t tests with Holm-Sidak Correction. ns p≥0.05.
A

1 weeks

1
2
spleocyte mixtures

TCRβ

2 x 10^6 flu

LCMV

Clone13
d0 pi
d20 pi
d100 pi

every 3-4 days

250 ng DTx

analysis of:

- T<sub>reg</sub> depletion efficacy
- antibody responses
- neutralization
- viral clearance

* CD45.2<sup>+</sup> CD4-DTR<sup>+</sup> B6xC57.l = control chimeras = 1
CD45.2<sup>+</sup> CD4-DTR<sup>+</sup> CXCR5<sup>+</sup> CXCR5<sup>+</sup> chimeras = 2
CD45.2<sup>+</sup> CD4-DTR<sup>+</sup> B6<sup>+</sup>x C57.Crea.1 = B6<sup>+</sup> chimeras = 3

B

control chimeras
CXCR5<sup>+</sup> chimeras
B6<sup>+</sup> chimeras

CD4
CXCR5

- DTx

+ DTx

- DTx

+ DTx

CD4
CXCR5

D

control chimeras
CXCR5<sup>+</sup> chimeras
B6<sup>+</sup> chimeras

Abs. 405 nm

3-fold dilution

naive B6
- DTx
+ DTx

E

control chimeras
CXCR5<sup>+</sup> chimeras
B6<sup>+</sup> chimeras

Abs. 405 nm

3-fold dilution

naive B6
- DTx
+ DTx

F

% neutralization

DTx

control
CXCR5<sup>+</sup>
B6<sup>+</sup>

G

flu solution

control
CXCR5<sup>+</sup>B6<sup>+</sup>
Fig. S2: Sustained activity of CXCR5 or Bcl6 expressing T<sub>FH</sub> cells is required for the development of LCMV-neutralizing antibodies and control of protracted infection. A) Experimental approach. TCRβ<sup>−/−</sup> mice received a 50:50 mixture of mature CD4-DTR (CD45.2<sup>+</sup>) splenocytes and CD45.1<sup>+</sup> B6 (control chimeras), CXCR5<sup>−/−</sup> (CXCR5<sup>−/−</sup> chimeras) or CD4-Cre x Bcl6<sup>fl/fl</sup> CD4-CrexBcl6<sup>fl/fl</sup> (Bcl6<sup>−/−</sup> chimeras) splenocytes per adoptive transfer. One week after transfer, chimeras were persistently infected with 2x10<sup>6</sup> FFU LCMV clone 13. From d20 pi onward, continuous DTx treatment every 3 to 4 days was initiated. B) Representative FACS plots and quantification of T<sub>FH</sub> cells (CXCR5<sup>+</sup> PD-1<sup>+</sup> CD4 T cells) in untreated (-DTx) or DTx treated (+DTx) control (dark grey), CXCR5<sup>−/−</sup> (red) or Bcl6<sup>−/−</sup> chimeras on d100 pi. FACS blots pre-gated on lymphocytes and single cells. C) Representative FACS blots and quantification of isotype-switched GC B cells (CD19<sup>+</sup> IgD<sup>−</sup> IgM<sup>−</sup> CD95<sup>+</sup> CD38<sup>−/lo</sup>) in untreated (-DTx) or DTx treated (+DTx) control (dark grey), CXCR5<sup>−/−</sup> (red) or Bcl6<sup>−/−</sup> (green) chimeras on d100 pi. FACS blots pre-gated on lymphocytes and single cells. B+C) Mann-Whitney U test used for statistical analysis: ns P>0.05. n=4 D) Titers of LCMV-specific IgG in sera of control, CXCR5<sup>−/−</sup> and Bcl6<sup>−/−</sup> chimeras on d20 pi and E) d100 pi in untreated (filled squares) and DTx treated (empty squares) mice as determined by ELISA. Multiple unpaired t test used for statistical analysis. D20 pi corresponds to the first day of DTx treatment. Serum was pre-diluted 1:20 for ELISA analysis and further diluted in a threefold dilution series. Serum obtained from naïve C57BL/6 mice was used as negative control. F) Neutralization of LCMV by serum of untreated or DTx treated control, CXCR5<sup>−/−</sup> and Bcl6<sup>−/−</sup> chimeras. G) Viral titers in spleen at d100 pi in untreated and DTx treated control, CXCR5<sup>−/−</sup> and Bcl6<sup>−/−</sup> chimeras. Mann-Whitney U test used for statistical analysis: ns P>0.05. n=4
**Fig. S3:** Immunofluorescence stainings of splenic thin sections. A) Staining of GC B cells (PNA, blue), CD4 (yellow) and IgD (red) in splenic thin sections of untreated or DTx treated control, CXCR5−/− or M25 chimeras on d70-d100 pi. Upper panels: staining controls for the different channels. B) Staining of GC B cells (PNA, blue), FDCs (CD35/21, white), CD4 (yellow) and IgD (red) in splenic thin sections of untreated or DTx treated control or CXCR5−/− splenocyte chimeras on d50 pi. Upper panels: staining controls for the different channels. Scale bar: 100 µm.
Fig. S4: Viral escape is not driven by pressure of CTLs. A) Neutralization of sera isolated from single chimeras on d100 pi against the virus isolates extracted on d70 pi of the respective chimera. Untreated chimeras are marked in green and DTx treated chimeras are marked in orange. Lines indicate effective neutralization of >1.5 SD of the control. One representative experiment of two conducted experiments shown. B) FACS staining for IFN-γ and TNF expression by gp33-tetramer positive CD8 T cells after 3h of stimulation on thio-macrophages infected with either the inoculum or contemporary isolates isolated on d70 pi. C) Total number of IFN-γ or D) TNF producing gp33-tetramer+ CD8 T cells isolated from mice acutely infected with the inoculum and restimulated for 3h on thio-macrophages infected with the inoculum (red) or contemporary virus isolates isolated on d70 pi of single chimeras (indicated with letters) of untreated (green) or DTx treated (orange) control, CXCR5−/− and M25 chimeras. Uninfected thio-macrophages (white) were used as negative control and PMA/ionomycin restimulation as positive control (black). Lines indicate cytokine expression by CD8 T cells stimulated by thio-macrophages infected with the inoculating virus. One representative experiment of two conducted experiments shown.
Fig. S5: CD8 T cell responses are unaffected by absence of CXCR5+/+ T<sub>FH</sub> or LCMV-
specific CD4 T cells. A) FACS staining and B) + C) quantification of total cell numbers of
CD44<sup>hi</sup> (B) or PD-1<sup>+</sup> (C) CD8 T cells in untreated or DTx treated control (black), CXCR5<sup>−/−</sup>
(red) and M25 (blue) chimeras. D) + E) FACS staining and quantification of ΔIFN-γ (D) and
ΔTNF (E) production, substracting values of unstimulated control condition from a gp33-
peptide stimulated condition, in untreated or DTx treated control (black), CXCR5<sup>−/−</sup> (red) and
M25 (blue) chimeras. Mann-Whitney U test used. D: ns P=0.1111 (control), ns P=0.0571 (CXCR5−/−), ns P=0.1143 (M25). E: ns P=0.5556 (control), ns P=0.6857 (CXCR5−/−), ns P=0.2857 (M25). F) +G) FACS staining and quantification of CXCR5+/− CD8 T cells in spleen of untreated or DTx treated control (black), CXCR5−/− (red) or M25 (blue) chimeras. FACS blots are pre-gated on lymphocytes, single cells and CD8+ T cells. One representative experiment of two conducted experiments shown, n=3-5 mice per group. Error bars represent mean ±SD. Mann-Whitney U test used: ns P≥0.05.