# Sustained follicular T helper cell response terminates chronic LCMV infection by promoting LCMV-neutralizing antibodies

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**One Sentence Summary**

We demonstrate that continued presence of CXCR5$^{+/+}$ $T_{FH}$ cells throughout chronic viral infection is dispensable for maintenance of overall virus-specific antibody titers but is vital for the generation of virus-neutralizing antibodies and eventual control of the infection.

**Abstract**

Virus-specific Th1 immunity is, like virus-specific CD8 T cell immunity, numerically and functionally compromised during chronic viral infections. Instead, the CD4 T cell response is increasingly and long-term skewed towards T follicular helper ($T_{FH}$) cells, yet their functional relevance during established chronic infection is unclear. Using chronic LCMV infection in combination with an *in vivo* system allowing conditional ablation of $T_{FH}$ cells or LCMV-specific CD4 T cells, we show that $CXCR5^{+/+}$ $T_{FH}$ cells - but not LCMV-specific CD4 T cells - are dispensable for the maintenance of LCMV-specific IgG antibody titers after their initial establishment. However, sustained activity of $CXCR5^{+/+}$ $T_{FH}$ cells is mandatory for the late emergence of LCMV-specific antibodies that are capable of neutralizing the viral inoculum and contemporary virus isolates. By supporting the generation of neutralizing antibodies, continued activity of $T_{FH}$ cells promotes eventual control of the chronic infection in face of exhausted CD8 T cell responses.

## Introduction

During chronic infections with viruses like HIV-1, Hepatitis C virus and Hepatitis B virus in humans or lymphocytic choriomeningitis virus (LCMV) in the mouse, adaptive immune cells are continuously confronted with cognate antigen. In this setting, multiple regulatory measures operate to avoid immunopathology, including the functional impairment and physical decimation of virus-specific CD8 T cells and Th1 T cells, collectively termed T cell exhaustion (*1, 2*). Instead, virus-specific CD4 T cells exhibit a differentiation bias towards follicular helper cells ($T_{FH}$) throughout the chronic infection (*3-5*). In the context of acute infections or vaccinations, $T_{FH}$ cells support induction and selection of T-help dependent B cell responses (*6*). They express characteristic markers, including the transcriptional repressor Bcl-6, the chemokine receptor CXCR5, the costimulatory molecules ICOS and CD40L, the co-inhibitory molecule PD-1 as well as IL-21 (*6*), which endows them to exert B cell helper functions. Localization of $T_{FH}$ cells to the CXCL13-rich B cell follicles is mediated by their expression of CXCR5 (*7, 8*). There $T_{FH}$ cells, and especially germinal center (GC) $T_{FH}$ cells (*9, 10*), instruct B cell differentiation and induce the GC response. The GC itself is partitioned into a dark zone (DZ), where B cells predominantly undergo sequential rounds of proliferation and somatic hypermutation (SHM), and a light zone (LZ), where B cells undergo selection based on their affinity for a given antigen (*11*). High-affinity B cell clones form the most intense interactions with GC $T_{FH}$ cells and receive most ICOSL-, CD40- and IL-21 signals, fostering their continued survival and differentiation into long-lived plasma cells or memory B cells (*11-13*).

In persistent LCMV infection, the pronounced differentiation of CD4 T cells towards $T_{FH}$ cells and their role in supporting antibody responses is of interest as LCMV-binding and neutralizing antibodies were shown to contribute to virus control. LCMV binding antibodies, generated early and throughout the infection, cannot prevent infection (*18*) but limit viral spread and exert indirect

protective functions (*14-17*). LCMV-neutralizing antibodies, however, directly inhibit infection of host cells and thereby limit viral replication and spread. Yet, neutralizing antibodies arise late, around day 60-80 during chronic LCMV infection (*18, 19*). Importantly, persistently infecting RNA viruses undergo mutational adaptations that escape pressure imposed by adaptive immune cells and antibodies, thereby promoting viral persistence (*20, 21*). In this scenario, B cells are equipped with the ability to counter-adapt their BCRs and antibodies to the currently prevailing antigen by the process of SHM and selection in the LZ. Whether such adaptations rely on sustained activity of $T_{FH}$ cells is plausible, but has never been formally shown.

The induction of LCMV-specific antibodies is dependent on CD4 T cells and in particular on $T_{FH}$ cells. Their absence at the onset of infection curtails the overall LCMV-specific humoral response and prevents viral clearance (*3*). However, the contribution of $T_{FH}$ cells for the generation of late appearing neutralizing antibodies is unclear.

In this paper we addressed whether the accentuated $T_{FH}$ differentiation during chronic viral infections fulfills a relevant biological and physiological function by continuously providing critical B cell helper functions even after the initial antibody response is induced. Using an experimental mouse model allowing conditional depletion of $CXCR5^{+/+}$ $T_{FH}$ or LCMV-specific CD4 T cells after the initial establishment of LCMV-specific IgG responses, we discovered that $T_{FH}$ cells, in contrast to LCMV-specific CD4 T cells, were dispensable for the maintenance of overall LCMV-specific IgG titers. However, $CXCR5^{+/+}$ $T_{FH}$ cells were essential for the generation of LCMV-neutralizing antibodies and thereby for the eventual termination of the chronic infection.

**Results**

*Specific depletion of CD4 T cells employing "CD4-DTR" cells*

To establish conditions in which CD4 T cells can be specifically depleted in chronically infected hosts, a mouse model ("CD4-DTR") was generated that expresses the primate diphtheria toxin receptor (DTR) specifically in CD4 T cells. To this end, a loxP-flanked stop cassette preceding the DTR gene was targeted into the exon 2 of the CD4 locus and gene-targeted mice were crossed to *lck*-Cre transgenic mice (*22*) (Fig. S1A). This leads to excision of the stop cassette during thymic T cell development, yielding "CD4-DTR" mice expressing the DTR specifically in CD4 T cells. Heterozygous CD4-DTR mice (in which one CD4 allele is targeted) were comparable to wildtype (wt) C57BL/6 mice with regards to thymic T development (Fig. S1B,C). Also, the percentage and total cell numbers of CD4 and CD8 T cells in spleen and mesenteric lymph node (mLN) were comparable between CD4-DTR and wt mice (Fig. S1D-G).

To test whether DTR-expressing CD4 T cells can be specifically depleted during chronic virus infection, we generated mixed bone marrow (BM) chimeras harboring wt and CD4-DTR T cells. To this end, CD45.1$^{+}$ B6 BM and CD45.2$^{+}$ CD4-DTR BM co-transferred into lethally irradiated CD4$^{-/-}$ recipients (Fig. 1A). After reconstitution, mixed BM chimeras were persistently infected with LCMV Clone13. On day (d) 20 post-infection (pi) continuous treatment with diphtheria toxin (DTx) was initiated and maintained over 20 days (Fig. 1A). Longitudinal depletion efficacy was analyzed in blood samples of DTx treated mice (Fig. 1B). CD4 T cells derived from the CD4-DTR compartment (CD45.2$^{+}$) were specifically and fully depleted from day 25 pi onwards (Fig. 1B). Importantly, CD8 T cells derived from the CD4-DTR compartment were not affected by the DTx treatment (Fig. 1B). CD4-DTR derived CD4 T cells were also efficiently and specifically depleted in spleen, mLN and lung at d40 pi (Fig. 1C).

Furthermore, $CXCR5^{+/+}$ PD-$1^{+}$ $T_{FH}$ cells were entirely and $CXCR5^{-}$ non-$T_{FH}$ cells almost entirely depleted when derived from the CD4-DTR BM compartment, however these subsets were not affected by DTx treatment when derived from the $CD45.1^{+}$ wt BM compartment (Fig. 1E). Moreover, DTx treatment did not affect viral clearance or viral titers in spleen at d40 pi (Fig. 1D), indicating that the remaining CD4 T cells after DTx treatment were sufficient to support eventual virus control. Thus, CD4-DTR cells can be specifically and efficiently depleted during chronic LCMV infection.

*Specific depletion of $CXCR5^{+/+}$ $T_{FH}$ or LCMV-specific CD4 T cells*

As our aim was to determine the role of LCMV-specific T helper cells and $CXCR5^{+/+}$ $T_{FH}$ cells during chronic LCMV infection, we employed CD4-DTR cells to generate mouse models that permitted specific and conditional depletion of these CD4 T cell subsets during established chronic LCMV infection. To this end, we generated mixed BM chimeras harboring CD4-DTR cells alongside a CD4 T cell compartment that cannot form $CXCR5^{+/+}$ $T_{FH}$ cells ($CXCR5^{-/-}$ CD4 T cells) or does not contain LCMV-specific CD4 T cells (M25 TCR transgenic cells). CXCR5 mediates the follicular localization of $T_{FH}$ cells (*7*, *8*). M25 TCR transgenic cells are specific for the M25 epitope of murine cytomegalovirus (MCMV) (*23*). We lethally irradiated $CD4^{-/-}$ recipients and reconstituted them with a mixture of CD4-DTR BM and (1) $CD45.1^{+}$ wt BM (control chimeras), (2) $CD45.1^{+}$ $CXCR5^{-/-}$ BM ($CXCR5^{-/-}$ chimeras) or (3) $CD45.1^{+}$ M25 BM (M25 chimeras) (Fig. 2A), infected them with LCMV Clone 13 and initiated longitudinal DTx treatment on d20 pi in half of the animals (Fig. 2A). Before DTx treatment all chimeras had generated LCMV-specific IgG titers (Fig. 3D).

Depletion efficiency of the CD4-DTR derived CD4 T cell populations was validated in longitudinal blood samples (Fig. 2B). From d30 pi onwards, CD4-DTR derived CD4 T cells were fully depleted in DTx treated control and $CXCR5^{-/-}$ chimeras and almost entirely in M25 BM chimeras (Fig. 2B). CD4 T cells derived from CD4-DTR compartment were also efficiently depleted in DTx treated control, $CXCR5^{-/-}$ and M25 chimeras in spleen and mLN at d70 pi (Fig. 2C). Absolute numbers of CD4 T cells in spleen were not significantly reduced in control chimeras, however the population was reduced about 2-fold in DTx treated $CXCR5^{-/-}$ and M25 chimeras (Fig. 2D). While $CXCR5^{+/+}$ $T_{FH}$ cells were ablated in DTx treated $CXCR5^{-/-}$ chimeras as well as DTx treated M25 chimeras, $CXCR5^{+/+}$ $T_{FH}$ cells were maintained in untreated chimeras and DTx treated control chimeras (Fig. 2E). Furthermore, overall numbers of LCMV gp61-80-specific CD4 T cells, identified by tetramer staining, were comparable in control and $CXCR5^{-/-}$ chimeras with or without DTx treatment (Fig. 2F).

Therefore, this experimental model was ideally suited to analyze the functional roles of $CXCR5^{+/+}$ $T_{FH}$ and LCMV-specific CD4 T cells in the setting of established chronic LCMV infection.

*LCMV-specific CD4 T cells, but not $CXCR5^{+/+}$ $T_{FH}$ cells are required for the maintenance of LCMV-specific antibody titers*

$T_{FH}$ cells play an important role in the regulation of the GC response, as well as in the selection of affinity matured B cell clones and their differentiation into memory B cells or plasma cells (*6, 11*). Whether this also holds true during chronic infection with abundant and prolonged presence of viral antigen remains unclear. Although absence of $CXCR5^{+/+}$ $T_{FH}$ cells or CD4 T cells from the onset of chronic LCMV infection compromises LCMV-specific IgG responses and leads to prolonged viral persistence (*3*), it remains unresolved whether $CXCR5^{+/+}$ $T_{FH}$ cells or LCMV-

specific CD4 T cells are still required after the induction and initial establishment of the LCMV-specific IgG response.

Analysis of the three mixed bone marrow chimera groups at day 70 pi (50 days after initiation of DTx treatment) revealed that the total cell count of splenic GC B cells ($CD95^{+}$ $CD38^{-/lo}$) in absence of $CXCR5^{+/+}$ $T_{FH}$ or LCMV-specific CD4 T cells was 4-fold, respectively 2.5-fold reduced compared to untreated chimeras (Fig. 3A). Likewise, the total number of memory B cells (isotype-switched $CD38^{+}$) in spleen was reduced in absence of $CXCR5^{+/+}$ $T_{FH}$ or LCMV-specific CD4 T cells compared to untreated chimeras and DTx treated control chimeras (Fig. 3A). Surprisingly, numbers of LCMV-specific antibody secreting cells (ASC) were not reduced in spleen (Fig. 3B) and bone marrow (Fig. 3C) in absence of $CXCR5^{+/+}$ $T_{FH}$ cells. The same holds true in the spleen in absence of LCMV-specific CD4 T cells, however ASC numbers were reduced in the bone marrow in their prolonged absence (Fig. 3C).

Subsequently, we determined LCMV-specific serum IgG titers. At d20 pi all chimeras exhibited similar titers of LCMV-specific antibodies, which were maintained in untreated chimeras and DTx treated control chimeras until d100 pi (Fig. 3D,E). LCMV-specific antibody titers were significantly reduced at d100 pi in prolonged absence of LCMV-specific CD4 T cells (Fig. 3E), reflecting the reduced ASC counts in BM. Both LCMV NP-specific and GP-specific IgG titers were reduced in serum of DTx treated M25 chimeras (Fig. 3F,G). Surprisingly, prolonged absence of $CXCR5^{+/+}$ $T_{FH}$ cells did not alter overall LCMV-specific antibody titers compared to untreated chimeras or DTx treated control chimeras at d100 pi (Fig. 3E), which was the case for both for GP and NP-specific IgG titers (Fig. 3F,G).

In conclusion, $CXCR5^{+/+}$ $T_{FH}$ cells seem to be dispensable for the differentiation and maintenance of ASC or the maintenance of the LCMV-specific IgG titers after initiation of the LCMV-specific

IgG response. Yet, LCMV-specific CD4 T cells appear to be essential for maintenance of LCMV-specific ASCs in the bone marrow and for overall LCMV-specific IgG titers.

*CXCR5$^{+/+}$ $T_{FH}$ cells are essential for the generation of LCMV-neutralizing antibodies and control of chronic LCMV infection*

As CXCR5$^{+/+}$ $T_{FH}$ cells were dispensable for the overall maintenance of the LCMV-specific IgG titers, we next analyzed the quality of the IgG response. The LCMV-specific antibody response consists almost exclusively of LCMV-binding, non-neutralizing antibodies at early time points. Precursors of LCMV-neutralizing antibodies are infrequent and / or early ablated, contributing to late appearance (d40-d70 pi) of neutralizing antibodies during persistent LCMV infection (*14, 19, 24-26*). However, neutralizing antibodies are believed to contribute to eventual viral clearance (*27, 28*). Hence, we measured the neutralizing capacity of sera isolated at d70 pi of untreated or DTx treated chimeras against the inoculum virus. Sera of untreated and DTx treated control chimeras and sera of untreated M25 chimeras exhibited about 40% neutralization; sera of untreated CXCR5$^{-/-}$ chimeras an even higher neutralization of about 60% (Fig. 4A). Strikingly, in absence of CXCR5$^{+/+}$ $T_{FH}$ cells or all LCMV-specific CD4 T cells in DTx treated CXCR5$^{-/-}$ and M25 chimeras, neutralization was reduced to 1-10% (Fig. 4A). To evaluate the *in vivo* protective capacities of sera isolated from untreated or DTx treated CXCR5$^{-/-}$ or control chimeras, we infused immune serum from these chimeras or naïve serum together with inoculum virus into naïve C57BL/6 mice. At d4 pi we determined viral titers in spleen. Serum from untreated control, CXCR5$^{-/-}$ or DTx treated control mice, containing *in vitro* neutralizing antibodies, conferred *in vivo* protection while serum from naïve mice or DTx treated CXCR5$^{-/-}$ chimeras were not

protective (Fig. 4B). This clearly shows that continued presence of CXCR5$^{+/+}$ $T_{FH}$ cells is essential for the generation of *in vitro* neutralizing and *in vivo* protective IgG responses.

As LCMV-neutralizing antibodies may be key effectors for eventual clearance of persistent LCMV infection, we analyzed viral titers in blood at d20 pi and d70 pi (Fig. 4C) as well as in spleen (Fig. 4D) at d100 pi. At d20 pi, before DTx treatment was initiated, all chimeras had comparable virus titers in blood (about $10^5$ ffu per ml blood, Fig. 4C). Most of the mice from the untreated groups and of the DTx treated control chimeras had either cleared the infection or displayed reduced viral titers in blood at d70 pi (Fig. 4D), and in spleen at d100 pi (Fig. 4D). Importantly, DTx treated CXCR5$^{-/-}$ and M25 chimeras showed still comparable viral titers to d20 pi, and none of these mice had cleared the infection (Fig. 4C,D).

Therefore, continuous absence of CXCR5$^{+/+}$ $T_{FH}$ cells not only affected the generation of neutralizing antibodies, but also abolished control of persistent LCMV infection, indicating that CXCR5$^{+/+}$ $T_{FH}$-driven generation of neutralizing antibodies is directly responsible for control of the persistent infection.

We wanted to confirm our observations using a different *in vivo* experimental setup in which $T_{FH}$ cell generation was inhibited by absence of Bcl-6 (being essential for the differentiation of $T_{FH}$ cells (*6*)) instead of absence of CXCR5 (mediating the localization to the B cell follicle (*7, 8*)). To this end, we generated splenocyte chimeras, avoiding irradiation, by adoptive transfer of mixes of mature CD4-DTR and wt, CXCR5$^{-/-}$ or Bcl6$^{-/-}$ (derived from Bcl6$^{fl/fl}$ x CD4-Cre mice; Bcl6$^{-/-}$ chimeras) T cells into TCRβ$^{-/-}$ hosts (Fig. S2A). One week after transfer, mice were chronically infected with LCMV Clone13 and after establishment of LCMV-specific IgG titers, half of the mice were treated with DTx starting from d20 pi onwards (Fig. S2A). As before, CXCR5$^{+/+}$ $T_{FH}$ cells could be effectively depleted in DTx treated CXCR5$^{-/-}$ splenocyte chimeras, and also in DTx

treated Bcl-6$^{-/-}$ splenocyte chimeras (Fig. S2B). Furthermore, GC B cell numbers were reduced in DTx treated CXCR5$^{-/-}$ and Bcl6$^{-/-}$ chimeras as compared to untreated chimeras and DTx treated control chimeras (Fig. S2C). Moreover, depletion of CXCR5$^{+/+}$ $T_{FH}$ cells in CXCR5$^{-/-}$ and Bcl6$^{+/+}$ $T_{FH}$ cells in Bcl6$^{-/-}$ chimeras did not alter the overall LCMV-specific IgG titers in serum at d100 pi compared to untreated splenocyte chimeras or DTx treated control chimeras and compared to antibody titers at d20pi (Fig. S2D,E). Yet, again, LCMV-neutralizing antibodies only emerged in presence of CXCR5$^{+/+}$ or Bcl-6$^{+/+}$ $T_{FH}$ cells. Most sera of untreated or DTx treated control splenocyte chimeras as well as untreated CXCR5$^{-/-}$ and Bcl6$^{-/-}$ chimeras exhibited robust neutralization of the inoculating virus on d100 pi (around 40-60%), while sera of DTx treated CXCR5$^{-/-}$ and Bcl6$^{-/-}$ chimeras were unable to neutralize the inoculum (5-15% neutralization) (Fig. S2F). DTx treated CXCR5$^{-/-}$ and Bcl6$^{-/-}$ chimeras exhibited elevated viral titers in spleen at d100 pi as compared to untreated chimeras or DTx treated control chimeras (Fig. S2G). Thus, we could confirm with this second *in vivo* experimental model that prolonged presence of CXCR5$^{+/+}$ or Bcl6$^{+/+}$ $T_{FH}$ cells during the entire time course of infection was essential for the induction of LCMV-neutralizing antibody responses but dispensable for overall maintenance of the LCMV-specific IgG responses.

*CXCR5$^{-/-}$ CD4 T cells are found in the GC in absence of CXCR5$^{+/+}$ $T_{FH}$ cells*

As CXCR5$^{+/+}$ $T_{FH}$ cells were not essential for the maintenance of the overall LCMV-specific IgG titers once established, we wondered how CXCR5$^{-/-}$ LCMV-specific CD4 T cells might be able to take over B cell helper functions during chronic viral infection. It was previously shown that CXCR5$^{-/-}$ CD4 T cells are able to migrate to the T: B border (*29*) and might also be passively dragged into the B cell follicle by cognate B cells forming motile conjugates with antigen-specific

CD4 T cells (*30*). To analyze whether there is contact between CD4 T cells and B cells in CXCR5$^{+/+}$ $T_{FH}$ depleted chronically infected animals, we stained GC B cells (PNA$^{+}$), naïve mature B cells (IgD$^{+}$) and CD4 T cells in splenic thin sections. In all untreated or DTx treated chimeras GCs were localized regularly in the B cell follicle (Fig. 5A, Fig. S3A). However, in DTx treated M25 chimeras and therefore in absence of LCMV-specific CD4 T cells, GCs contained far less CD4 T cells compared to the untreated chimeras as the ratio of CD4 MFI to PNA MFI as well as the number of CD4 T cells per µm$^2$ of the PNA$^{+}$ area was significantly reduced (Fig. 5A-C, Fig. S3A). Surprisingly, GCs in DTx treated CXCR5$^{-/-}$ chimeras, lacking CXCR5$^{+/+}$ $T_{FH}$ cells, contained similar or even slightly more CD4 T cells compared to untreated CXCR5$^{-/-}$ chimeras (Fig. 5A-C; Fig. S3A).

As the emergence of LCMV neutralizing antibodies was dependent on continued presence of CXCR5$^{+/+}$ $T_{FH}$ cells, we wondered whether CXCR5$^{-/-}$ CD4 T cells might be differentially localized in the GC as compared to CXCR5$^{+/+}$ CD4 T cells. $T_{FH}$ cells are known to preferentially localize to the LZ of the GC where they exert their B helper functions and partake in the positive selection of affinity matured B cells (*31*). Therefore, we analyzed the micro-anatomical localization of CD4 T cells within the LZ and DZ of the GC in untreated or DTx treated control or CXCR5$^{-/-}$ splenocyte chimeras (Fig. S2) at d50 pi in more detail. We stained splenic thin sections for GC B cells (PNA$^{+}$, blue), CD4$^{+}$ cells (yellow) and mature B cells (IgD$^{+}$, red) (Fig. 5D, Fig. S3B). Localization of the LZ within the GC (PNA$^{+}$ area) was determined by staining of FDCs (preferentially localizing to the LZ) with a CD35/21 antibody. The PNA$^{+}$ area not containing FDCs was determined as DZ (Fig. 5D, Fig. S3B). Determining the number of CD4$^{+}$ cells contained per µm$^3$ of LZ and DZ, we found a preferential localization of CD4$^{+}$ cells to the LZ in untreated or DTx treated control chimeras and untreated CXCR5$^{-/-}$ chimeras (Fig. 5D,E, Fig. S3B). In contrast, CXCR5$^{-/-}$ CD4 T

cells in CXCR5$^{+/+}$ $T_{FH}$ depleted chimeras were distributed more evenly between LZ and DZ of the GC (Fig. 5D,E, Fig. S3B). A less polarized distribution of CXCR5$^{-/-}$ CD4 T cells could be one explanation why B cell help by CXCR5$^{-/-}$ CD4 T cells was not sufficient to confer the selection of B cell clones able to produce LCMV neutralizing antibodies, since B cells are only responsive to $T_{FH}$ cell mediated selection in the LZ of the GC (*11*, *31*).

*CXCR5$^{+/+}$ $T_{FH}$ cells drive adaptation of the neutralizing antibody response towards contemporary virus species*

So far, we had determined the serum IgG neutralization capacity against the inoculating virus. However, it is conceivable that mutational escape of LCMV from neutralizing antibodies or CD8 T cell recognition may occur during a chronic infection (*32, 33*). Therefore, we wondered if CXCR5$^{+/+}$ $T_{FH}$ cells played a role in the adaptation and shaping of the antibody response towards contemporary viral species. For this purpose, we produced virus isolates and antibodies from identical time points of single chimeras and tested the neutralization capacity of sera against the respective contemporary virus isolate of the same chimera.

At d20 pi, right before start of the DTx treatment, little neutralization (10-20%) of the inoculating virus could be detected, only a few chimeras depicted already a neutralization capacity of around 40% (Fig. 6A, upper left panel). Except for two chimeras, s (20%) and u (15%), no neutralization of the contemporary virus isolates from d20 pi could be detected (Fig. 6a, upper right panel).

At d70 pi sera of untreated chimeras and DTx treated control chimeras showed robust neutralization capacity of the inoculum virus (between 40% and 60%), with just one exception (chimera m) (Fig. 6A, lower left panel). Also, at this time point of infection, neutralization of the contemporary isolates was in general lower compared to the neutralization of the inoculum in the

untreated groups, ranging between 20% and 40%, except for chimeras j,n, and s which neutralized their contemporary viral isolates up to 80% (Fig. 6A, lower right panel). Sera of chimeras without $CXCR5^{+/+}$ $T_{FH}$ cells could still neutralize the inoculum to a minor extent (10-20%), comparable to neutralization on d20 pi (Fig. 6A, left panels), but not the contemporary isolates at d70 pi (Fig. 6A, lower right panel). In chimeras without any LCMV-specific CD4 T cells no neutralizing antibodies could be detected against either the inoculum or the contemporary isolate (Fig. 6A, lower panel). In general, the generation of neutralizing antibodies seemed to lag behind the evolution circulating viral species and $CXCR5^{+/+}$ $T_{FH}$ cells seemed to play an important role in driving the adaptation towards the contemporary virus species. Moreover, sera isolated on d100 pi of these chimeras were able to neutralize the virus isolates from d70 pi far better, with neutralizing capacities ranging from 40% to 60% (Fig. S4A). Finally, robust neutralization of the contemporary virus isolate - and less so of the inoculum - was generally linked to enhanced virus control (Fig 6B).

Furthermore, we wondered whether viral evolution in our infection setting is driven predominantly by neutralizing antibodies or by the CD8 T cell response. Therefore, we used the different virus isolates derived from d70 pi blood to infect thioglycollate-induced peritoneal macrophages and used these infected cells for re-stimulation of CD8 T cells derived from mice acutely infected with the inoculum virus. The hypothesis was, that d70 isolates evading control by CTLs would not be as potent in stimulating CD8 T cells as the inoculum. No significant differences in the total CD8 T cell number producing INF-ɣ (Fig. 6C) or TNF (Fig. 6D) after 6 hours of stimulation with either the inoculum or any of the d70 isolates could be detected. This also holds true for gp33-tetramer$^{+}$ CD8 T cells after 3h of stimulation (Fig. S4B,C,D). Therefore, we have no indication for CTLs being a main driving force for viral adaptation during chronic LCMV infection.

As IL-21-producing CD4 T cells also play a role in maintaining the CD8 T cell response during persistent LCMV infection (*34, 35*), we also analyzed the LCMV-specific CD8 T cell response in the chimeras. The total number of activated ($CD44^+$) CD8 T cells, as well as the total number of exhausted ($PD\text{-}1^+$) CD8 T cells (Fig. S5A-C), was not affected by absence of $CXCR5^{+/+}$ $T_{FH}$ or LCMV-specific CD4 T cells as compared to untreated or DTx treated control chimeras. The production of effector cytokines IFN-ɣ (Fig. S5D) and TNF (Fig. S5E) after re-stimulation of CD8 T cells with gp33 peptide was also unaltered by absence of $CXCR5^{+/+}$ $T_{FH}$ or LCMV-specific CD4 T cells. However, due to the exhausted state of the CD8 T cells, cytokine production was overall very modest (Fig. S5D,E). Furthermore, as recent publications revealed the existence of a less exhausted $CXCR5^+$ CD8 T cell population localizing to the B cell follicle (*36*), we also analyzed the effect of DTx treatment in $CXCR5^{-/-}$ or M25 chimeras on this population. Yet, we could not detect any effect of the absence of $CXCR5^{+/+}$ $T_{FH}$ or LCMV-specific CD4 T cells on the frequency and number of $CXCR5^+$ CD8 T cells in the spleen (Fig. S5F,G).

In conclusion, clearance of persistent LCMV infection, as well as viral adaptation during persistent LCMV infection, is mainly driven by neutralizing antibodies whose robust generation is strictly dependent on continued activity of $CXCR5^{+/+}$ $T_{FH}$ cells. CD8 T cells might also contribute to eventual control but seem not to be decisive as CD8 T cell function and numbers were comparable in chimeras with and without $CXCR5^{+/+}$ $T_{FH}$ cells.

**Discussion**

Although chronicity of LCMV infection prevails over several weeks, being associated with severe dysfunction of CD8 and Th1 T cell immunity (*1, 37*), it is eventually controlled in most cases. This offers the unique opportunity to evaluate mechanistically how chronic viral infections can be naturally controlled in absence of overt immunopathology. However, it has not been satisfyingly

resolved which immune effector functions are naturally most relevant for resolution of a chronic infection. As recent studies have noticed that CD4 T cell responses are increasingly skewed towards $T_{FH}$ cells during chronic viral infections, including HIV-1 infection in humans and LCMV infection in mice (*3-5*), it is tempting to speculate that emphasis on $T_{FH}$ cells might have physiological relevance.

In chronic LCMV infection, constitutive absence of CXCR5 severely impairs LCMV specific IgG responses and virus control (*3*). Using our experimental *in vivo* system we were able to clearly demonstrate a pivotal role of continued activity of $CXCR5^{+/+}$ $T_{FH}$ cells in supporting the late generation of LCMV-neutralizing antibodies against the inoculating virus but also against contemporary circulating viruses, enabling control of LCMV infection. However, overall LCMV-specific IgG responses were maintained even in absence of $CXCR5^{+/+}$ $T_{FH}$ cells. Importantly, conditional ablation of $CXCR5^{+/+}$ $T_{FH}$ cells or LCMV-specific CD4 T cells did not affect LCMV-specific CD8 T cell immunity, as LCMV-specific CD8 T cell numbers (including $CXCR5^{+}$ CD8 T cells) and functions were comparable in mice with and without conditional depletion of $CXCR5^{+/+}$ $T_{FH}$ or LCMV-specific CD4 T cells. These results strongly support a key role for $CXCR5^{+/+}$ $T_{FH}$ cells and antibodies in natural resolution of chronic LCMV infection.

Concerning the maintenance of the LCMV-binding antibody response during chronic infection in absence of $CXCR5^{+/+}$ $T_{FH}$ cells, $CXCR5^{-/-}$ CD4 T cells were clearly capable of taking over some B helper functions, analogous to acute influenza infections of mice with constitutive $T_{FH}$ absence (*38*). This help might take place in the GC as even in absence of $CXCR5^{+/+}$ $T_{FH}$ cells or LCMV-specific CD4 T cells, the GC response was, in accordance with previous publications (*29, 39, 40*), reduced, but not absent. However, in absence of LCMV-specific CD4 T cells the GCs were devoid of CD4 T cells while LCMV-specific $CXCR5^{-/-}$ CD4 T cells could access the GC in absence of

CXCR5$^{+/+}$ $T_{FH}$ cells. We showed that these CXCR5$^{-/-}$ CD4 T cells are no longer preferentially localizing to the LZ, thereby likely being less able to assist selection of affinity matured or virus-adapted B cells. Moreover, in absence of Bcl6-expressing CD4 T cells after establishment of initial LCMV-specific IgG titers, GC B cells were drastically reduced without measurable decimation of overall LCMV-specific IgG titers, suggesting that extra-follicular help might be responsible for a substantial fraction of the overall LCMV-specific IgG response when $T_{FH}$ is missing. LCMV-specific CXCR5$^{-/-}$ CD4 T cells might encounter cognate B cells at the T:B border after downregulation of CCR7 upon T cell activation, as localization to the B cell follicle is dependent on both CXCR5 up- and CCR7 downregulation (*29, 41*). Through cognate interaction with B cells, CXCR5$^{-/-}$ CD4 T cells may be dragged into the GCs as B cells form motile, B cell led, conjugates with cognate CD4 T cells (*30*). Alternatively, recruitment of LCMV-specific CXCR5$^{-/-}$ CD4 T cells into the GC might be mediated by expression of CXCR4 (*42*). Interestingly, in the context of autoimmunity there have been reports about $T_{FH}$ - like cells which migrate to extrafollicular sites by expression of CXCR4. These cells were able to support antibody production, even class-switch reactions, by extrafollicular plasmablasts through secretion of IL-21 and expression of CD40L (*43*). It needs to be determined whether these cells might also play a role in maintaining the antibody response in absence of CXCR5$^{-/-}$ $T_{FH}$ cells during chronic viral infection. Furthermore, it might be of interest to resolve whether the LCMV-binding antibodies produced in absence of $T_{FH}$ cells actually arise from the GCs or are generated at extrafollicular sites.

On a functional level, our data suggest that CXCR5$^{-/-}$ CD4 T cells are able to support LCMV-specific ASC formation in absence of CXCR5$^{+/+}$ $T_{FH}$ cells, evidenced by comparable numbers of LCMV-specific ASCs in spleen and bone marrow in absence of CXCR5$^{+/+}$ CD4 T cells. However, they have an impaired ability in promoting generation / selection of ASCs producing LCMV-

neutralizing antibodies and therefore might not be qualitatively and functionally fully comparable to $CXCR5^{+/+}$ $T_{FH}$ cells. One possible explanation for their inability to induce/support production of LCMV neutralizing antibodies might be their less polarized localization within the GC as $CXCR5^{-/-}$ CD4 T cells were more evenly distributed throughout the LZ and DZ of the GC as compared to $CXCR5^{+/+}$ CD4 T cells which localized preferentially in the LZ, where B cells are mainly responsive to $T_{FH}$ cell-mediated selection.

Our results regarding the dependence of the emergence of LCMV neutralizing antibodies on the continued presence of $CXCR5^{+/+}$ $T_{FH}$ cells stands in contrast to the recent findings during acute influenza virus infection (*38*), where protective antibody responses emerged independent of $T_{FH}$ cells. Probably the maturation trajectories of protective neutralizing antibodies differ between LCMV and influenza virus infection in the sense that influenza neutralizing antibodies might be much faster and easier generated compared to LCMV-neutralizing antibodies and that the latter might therefore depend much more on continued activity of $CXCR5^{+/+}$ $T_{FH}$ cells. Furthermore, in absence of $T_{FH}$ cells during acute influenza virus infection the protective antibody response was of low-affinity, indicating that somatic hypermutation (SHM) and affinity selection might play an inferior role in creating a protective response. However, during chronic viral infections with LCMV, but also other non-cytopathic viruses, antibodies may have to undergo several rounds of SHM and affinity selection in the GC to achieve neutralizing and protective capacity (*19, 44*). Reasons might be that precursors of B cells producing neutralizing antibodies occur only in low frequencies and have a low affinity at first - or are rapidly depleted early during infection (*24-26*). In accordance with our results, it has been recently reported in the context of a HIV vaccination trial, that development of neutralizing antibodies against HIV correlated directly with the strength of the GC response and was dependent on the quality of the $T_{FH}$ cell response, while the emergence

of a binding antibody response was not affected by both these factors. Neutralizing antibodies only emerged in animals exhibiting extensive GC reactions (*45*). This indicates an essential role for $T_{FH}$ cells, and also their correct localization in the GC, in the selection process of B cells producing neutralizing antibodies during chronic viral infections, which is substantiated by the fact that we could not detect any significant LCMV-neutralizing antibodies in absence of $CXCR5^{+/+}$ $T_{FH}$ cells. Concomitant to the emergence of LCMV-neutralizing antibodies, the infecting virus may undergo rapid mutation to escape detection by these antibodies (*32, 33*). Occurrence of LCMV escape mutants evading detection by neutralizing antibodies has so far been mainly studied in systems that were either CTL depleted (*32*) or in mouse models with a reduced CD8 T cell response (*33*). Here, we were able to show that neutralization of contemporary virus isolates is generally delayed compared to neutralization of the inoculum, even in presence of a normal CD8 T cell response. Together with the finding that the contemporary isolates and the inoculum comparably activated LCMV-specific CD8 T cells, this indicates that also in a system in which CD8 T cells are present, neutralizing antibodies are the main drivers of viral escape. Further, as no neutralization of contemporary isolates can be detected in absence of $CXCR5^{+/+}$ $T_{FH}$ cells, these cells are essential in driving the adaptation of the humoral response towards the mutating virus. Importantly, the $T_{FH}$ driven appearance of LCMV-neutralizing antibodies leads to viral clearance of persistent LCMV infection, demonstrating that neutralizing antibodies decisive for viral clearance in established chronic LCMV infection.

A limitation of the present study is that the question of whether sustained activity of $T_{FH}$ cells is a general requirement for effective control of chronic viral infections remains open as well as the question how exactly $T_{FH}$ cells promote the emergence of neutralizing antibodies. Do $T_{FH}$ cells act as "gate-keepers", sending virus-specific B cells for multiple rounds of SHM - or do they act as

"selectors" that continuously identify B cells with the most virus-adapted antibodies? Clearly, in human HIV-1 infection elevated frequencies of $T_{FH}$ cells were reported (*5*), yet, virus clearance is almost never achieved. This raises an important question, namely how virus and host parameters must align to afford eventual control of a chronic infection; for instance, what is the relative kinetics of mutational escape compared to the kinetics of SHM and GC selection and how do these present in different chronic viral infections? Answering these questions will be important to advance our understanding of successful immune control of chronic viral infections.

## Materials and Methods

*Study design*

Aim of this study was to determine the long-term function of $CXCR5^{+/+}$ $T_{FH}$ cells and LCMV-specific CD4 T cells in the regulation of humoral immune responses towards persistent LCMV infection. For this purpose, we implemented an *in vivo* experimental mouse model, which allows conditional ablation of $CXCR5^{+/+}$ $T_{FH}$ cells and LCMV-specific CD4 T cells. We used this model to study the impact of depletion of $CXCR5^{+/+}$ $T_{FH}$ cells and LCMV-specific CD4 T cells after the initial establishment of the LCMV-specific IgG response on the GC response, formation of LCMV-specific ASC, overall LCMV-specific binding antibody responses, LCMV-neutralizing responses as well as eventual viral clearance and adaptation of the humoral immune response towards contemporaneous viral isolates. All experiments were performed at least twice. Every group consisted of at least 3 mice. No outliers were excluded from the data analysis and no randomization or blinding was used.

*Mice*

The CD4-DTR mouse line was engineered by targeting a loxP-flanked stop cassette preceding the primate diphtheria toxin receptor (DTR) into exon 2 of one CD4 allele in C57BL/6 ES cells (construct design, production, ES cell targeting, selection and generation of germline transmitters was done by Ozgene, Bentley, Australia). Gene-targeted mice were crossed to *lck*-Cre transgenic mice (*22*), leading to excision of the stop cassette during the CD4/ CD8 double positive (DP) stage of T cell development in the thymus. Heterozygous CD4-DTR mice, in which only one CD4 allele is targeted by the DTR, were consistently used in this study. CD4-DTR, $CXCR5^{-/-}$ (*46*), $CD4^{-/-}$ (*47*), M25 TCR transgenic (*23*), TCRβ-/- (*48*) and $CD45.1^{+}$ B6 mice were bred and maintained under specific pathogen-free conditions at the ETH Phenomics Center. All KO and transgenic mice strains were crossed to C57BL/6 background for more than 10 generations. $Bcl6^{fl/fl}$ x CD4-Cre mice were provided by Dr. Harker, obtained from Charles River Laboratories (London, Great Britain) and C57BL/6 mice from Janvier.

Bone marrow (BM) chimeras were generated as previously described (*49*) and $TCR\beta^{-/-}$ mixed splenocyte chimeras were generated by adoptive transfer of splenocytes mixtures consisting of each ¼ of the splenocytes isolated from one donor spleen per recipient. BM chimeras were infected 8 weeks and splenocyte chimeras 1 week after reconstitution. Persistent infection was induced by i.v. infection with $2x\ 10^6$ ffu LCMV Clone13. Acute infection was induced with 200 ffu LCMV Clone13 i.v. BM or splenocyte chimeras containing a WT C57BL/6 CD4 T cell compartment next to the CD4-DTR CD4 T cell compartment were chosen as controls.

All animal experiments were performed according to institutional guidelines and Swiss federal regulations, and were approved by the veterinary office of the canton of Zürich (animal experimentation permission 147/2014).

*Virus and viral peptides*

The viral peptide gp33-41 (gp33; KAVYNFATM) was purchased from NeoMPS. 1µg / ml of viral peptide was used to restimulate gp33-specific CD8 T cells.

LCMV Clone13 was propagated on BHK-21 cells and viral titers were determined as described before (*50*). Ex vivo isolates of LCMV were obtained from blood of persistently infected mixed BM chimeras and propagated in two consecutive rounds on MC57G cells. Viral titers of the virus isolates were determined as described (*50*).

*Depletion of CD4-DTR derived cells*

CD4 T cells derived from CD4-DTR BM compartments were depleted by intraperitoneal (i.p.) administration of 250 ng / mouse diphtheria toxin (DTx, Sigma-Aldrich Chemie GmbH) diluted in PBS. For longitudinal depletion, DTx was injected every three to four days.

*Flow cytometry and lymphocyte stimulation*

All antibodies used for flow cytometry staining were purchased by BioLegend, eBioscience and BD Biosciences. Surface stainings were performed at 4°C for 20 minutes (min), except for CXCR5 (staining RT for at least 30 min) after Fc block using 2.4G2 (rat anti-mouse CD16/CD32). Afterwards, all samples were treated with 1ml ACK lysis buffer for 10 min at RT. Cells were then fixed in PBS containing 1% paraformaldehyde (PFA). Cytokine expression was analyzed by intracellular staining in stimulated lymphocytes. CD8 T cells were either stimulated for 3 or 6 hours (hrs) at 37°C in the presence of Monensin A on thio-macrophages infected with 0.1 MOI of the inoculating virus or different virus isolates. Or, CD8 T cells were stimulated with 1µg/ml of

the gp33 peptide in presence of Monensin-A for 6 hrs at 37°C. Degranulation and cytokine production was measured as described before (*49*). Multiparameter flow analysis was performed on a FACS LSR II flow cytometer (BD) with FACSDiva software. Data analysis was performed with FlowJo software (FlowJo Enterprise, Version 10).

*ELISA*

ELISA were carried out in 96-well Nunc Maxisorp Immunoplates (VWR International AG). For further information, see the Supplemental Materials.

*ELISPOT*

ELISPOT assays for detection of LCMV-specific antibody-secreting cells (ASC) in spleen or bone marrow were carried out in 96-well Multiscreen-IP PVDF Filter Plates (Millipore). For more information, see the Supplemental Materials.

*LCMV-neutralization assay*

Neutralizing capacities of sera isolated from infected mice were tested in a foci-reduction assay (*50*). Sera were heat-inactivated for 10 min at 56°C. Neutralization capacities were determined as the relative reduction of foci in presence or absence of immune serum. Thresholds for neutralization were determined as 1.5 higher than the SD of the control condition.

*Immunohistochemistry and fluorescence microscopy*

7 µm thin sections were prepared from frozen spleens embedded in Optimal Cutting Temperature (O.C.T.) compound (Sakura). Further information about the staining is provided in Supplementary Materials.

*Statistical analysis*

For statistical analysis non-parametric Mann-Whitney U tests were performed using GraphPad Prism Software. For statistical analysis of ELISA data multiple unpaired t tests were performed using GraphPad Prism Software.

**Supplementary Materials**

Materials and Methods (Production of LCMV Clone13 GP1-Fc and NP, ELISA, ELISPOT, Immunohistochemistry and fluorescence microscopy)

Fig. S1. Basic characterization of the CD4-DTR mouse line

Fig. S2. Sustained activity of CXCR5 or Bcl6 expressing $T_{FH}$ cells is required for the development of LCMV-neutralizing antibodies and control of protracted infection

Fig. S3: Immunofluorescence stainings in splenic thin sections

Fig. S4 Viral escape is not driven by pressure of CTLs

Fig S5 CD8 T cell responses are unaffected by absence of $T_{FH}$ or LCMV-specific CD4 T cells

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**Figure legends:**

**Fig. 1: Specific depletion of CD4-DTR derived CD4 T cells.** A) Lethally irradiated $CD4^{-/-}$ mice were reconstituted with a 60:40 mixture of CD4-DTR ($CD45.2^+$) and $CD45.1^+$ B6 BM. After 8 weeks of reconstitution, mixed BM chimeras were persistently infected with 2x $10^6$ ffu LCMV Clone13. From d20 pi onwards, continuous DTx treatment every 3-4 days was initiated. B) Longitudinal analysis of CD4 and CD8 T cell ratios in blood. CD4-DTR derived CD4/CD8 T cells ($CD45.2^+$) and $CD45.1^+$ B6 derived CD4/CD8 T cells are separately plotted. C) Ratios and total cell numbers of CD4-DTR derived CD4 T cells to $CD45.1^+$ B6 derived CD4 T cells in spleen, mLN and lung at d40 pi. D) Viral titers in spleen on d40 pi. Statistical analysis was performed using Mann Whitney U test; p=0.8413. E) Ratios of CD4-DTR derived ($CD45.2^+$) and $CD45.1^+$ B6 derived non-$T_{FH}$ (red gate; $CXCR5^-$) and $T_{FH}$ (blue gate; $CXCR5^+$ $PD\text{-}1^+$) cells in spleen at d40 pi in untreated or DTx treated animals. Pregated on $CD4^+$ single lymphocytes. One representative experiment of two is shown, n=4. Error bares represent means ±SD.

**Fig. 2: Specific depletion of $T_{FH}$ and LCMV-specific CD4 T cells employing CD4-DTR BM chimeras.** A) Experimental approach. Lethally irradiated $CD4^{-/-}$ mice were reconstituted with a 60:40 mixture of CD4-DTR ($CD45.2^+$) bm and $CD45.1^+$ B6 (control chimeras), $CXCR5^{-/-}$ ($CXCR5^{-/-}$ chimeras) or M25 (M25 chimeras) bm. After 8 weeks of reconstitution, mixed bm chimeras were persistently infected with 2x $10^6$ ffu LCMV Clone13. From d20 pi onwards, continuous DTx treatment every 3-4 days was initiated. B) Longitudinal analysis of ratios of CD4-DTR derived ($CD45.2^+$) and $CD45.1^+$ B6 (left panel), $CXCR5^{-/-}$ ($CD45.1^+$; middle panel) or M25 ($CD45.1^+$; right panel) derived CD4 T cells in blood in untreated (upper panels) or DTx treated (lower panels) mixed BM chimeras. C) Ratios of CD4-DTR derived ($CD45.2^+$, white) and

CD45.1$^+$ B6 (black), CXCR5$^{-/-}$(CD45.1$^+$; red) or M25 (CD45.1$^+$; blue) derived CD4 T cells in spleen (left panel) and mLN (right panel) on d70 pi in DTx treated chimeras. D) Total CD4 T cells in spleen d70 pi of untreated or DTx treated control (black), CXCR5$^{-/-}$ (red) or M25 (blue) chimeras. E) Flow cytometric analysis of $T_{FH}$ cells (CXCR5$^+$ PD-1$^+$) in spleen on d70 pi in untreated or DTx treated control (black), CXCR5$^{-/-}$ (red) and M25 (blue) chimeras. F) Number of gp61-tetramer$^+$ CD4 T cells in untreated or DTx treated control (gark grey) or CXCR5$^{-/-}$ (red) chimeras at d100 pi in spleen. B) – C), F) One representative experiment of three is shown, n= 3-4 mice per group. Error bars represent mean ±SD. D)-E) Pooled data of two independent experiments, n= 3-4 mice per group. Statistical analysis was performed using Mann Whitney U test: ns p>0.05.

**Fig. 3: CXCR5 $^{+/+}$ $T_{FH}$ cells are dispensable for maintenance of the general LCMV-specific antibody response.** A) GC B cell (CD95$^+$ CD38$^{-/lo}$) and memory B cells (CD38$^+$) in spleen as identified by gating on CD19$^+$ and switched IgD$^-$ IgM$^-$ B cells. Shown are representative FACS blots of untreated (upper panel) and DTx treated (lower panel) control (left), CXCR5$^{-/-}$ (middle) and M25 (right) chimeras. Below quantifications of total cell counts are depicted. Data of three independently conducted experiments were pooled, n=3-5 mice per group. B) Number of LCMV-specific ASC as determined by ELISPOT in spleen. C) Number of LCMV-specific ASC as determined by ELISPOT in bone marrow. One representative of two experiments is shown, n=3-5 mice per group. A-C) Error bars represent means ±SD. Mann-Whitney test used for statistical analysis: ns p>0.05. D) Titers of LCMV-specific IgG in sera of control, CXCR5$^{-/-}$ and M25 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: * p=0.0086.

D20 pi corresponds to the first day of DTx treatment. F) Titers of LCMV-Clone13-GP1-Fc-specific or G) LCMV-Clone13-NP-specific antibodies in untreated (left) and DTx treated (right) control, CXCR5$^{-/-}$ and M25 chimeras. Multiple unpaired t test used for statistical analysis: *p= 0.007. D)-G) Serum was pre-diluted 1:20 for ELISA analysis and further diluted in a 3-fold dilution series. Serum obtained from naïve C57BL/6 mice was used as negative control. D)-G) One representative experiment of two is shown, n=3-5 mice per group. Error bars represent means ±SD.

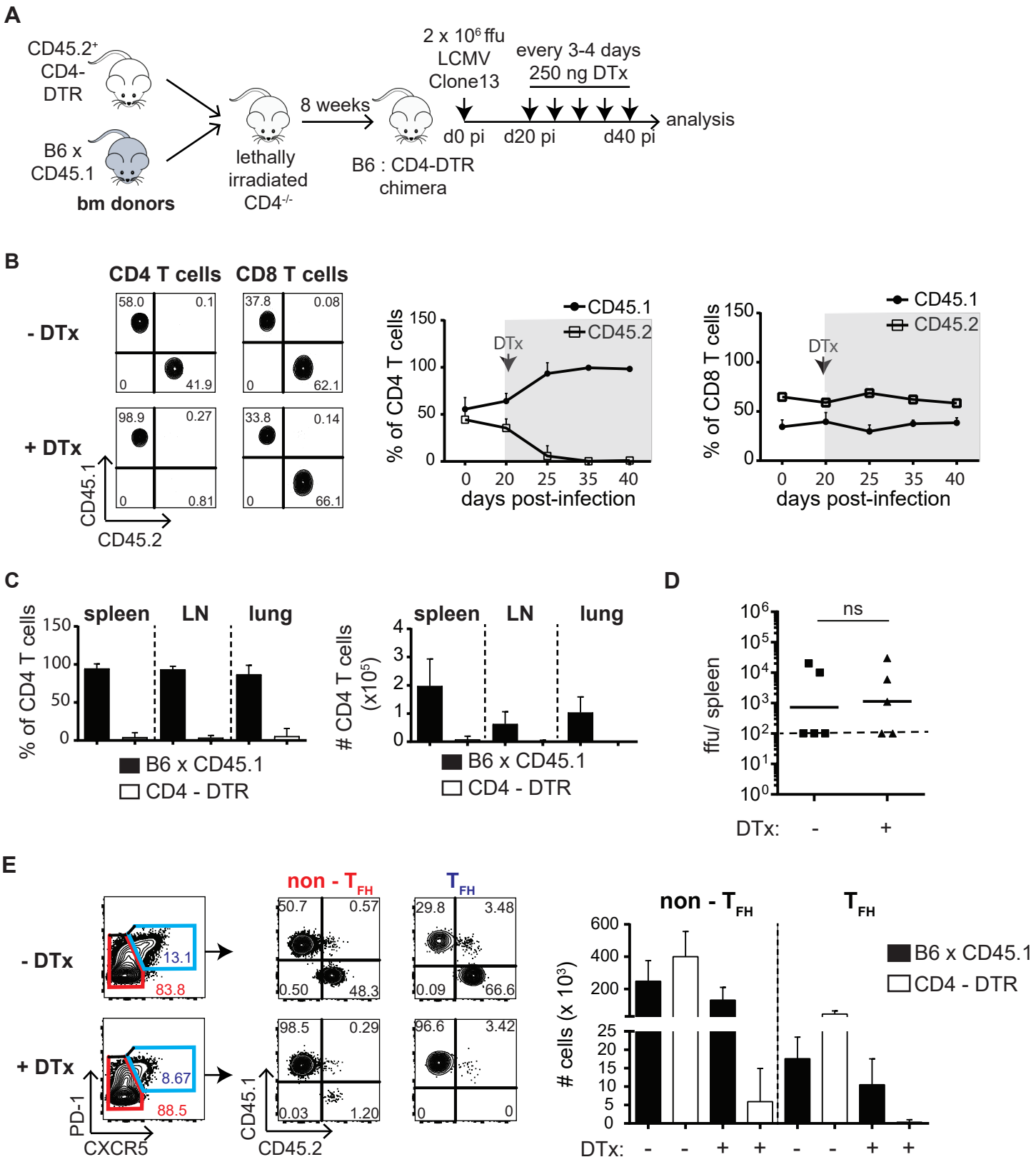
**Fig. 4: CXCR5$^{+/+}$ T$_{FH}$ cells are essential for the generation of LCMV-neutralizing antibodies and eventual clearance of a persistent LCMV infection.** A) Neutralization of LCMV by serum of untreated or DTx treated control, CXCR5$^{-/-}$ and M25 chimeras. Pooled data of three independent experiments, n=3-5 mice per group. Error bars represent means ±SD. B) Viral titers in spleen of naïve C57BL/6 mice infected with Clone13 together with either naïve (grey, -DTx) or serum derived from untreated (-DTx) or DTx treated (+DTx) control or CXCR5$^{-/-}$ chimeras at d4pi. Representative data of 2 independent experiments, n=3. C) Viral titers in blood at d20 and d70 pi in untreated and DTx treated control, CXCR5$^{-/-}$ and M25 chimeras. D) Viral titers in spleen at d100 pi in untreated and DTx treated control, CXCR5$^{-/-}$ and M25 chimeras.. C)-D) Pooled data of three independent experiments, n=3-5 mice per group. Mann-Whitney U test used for statistical analysis: ns p>0.05.

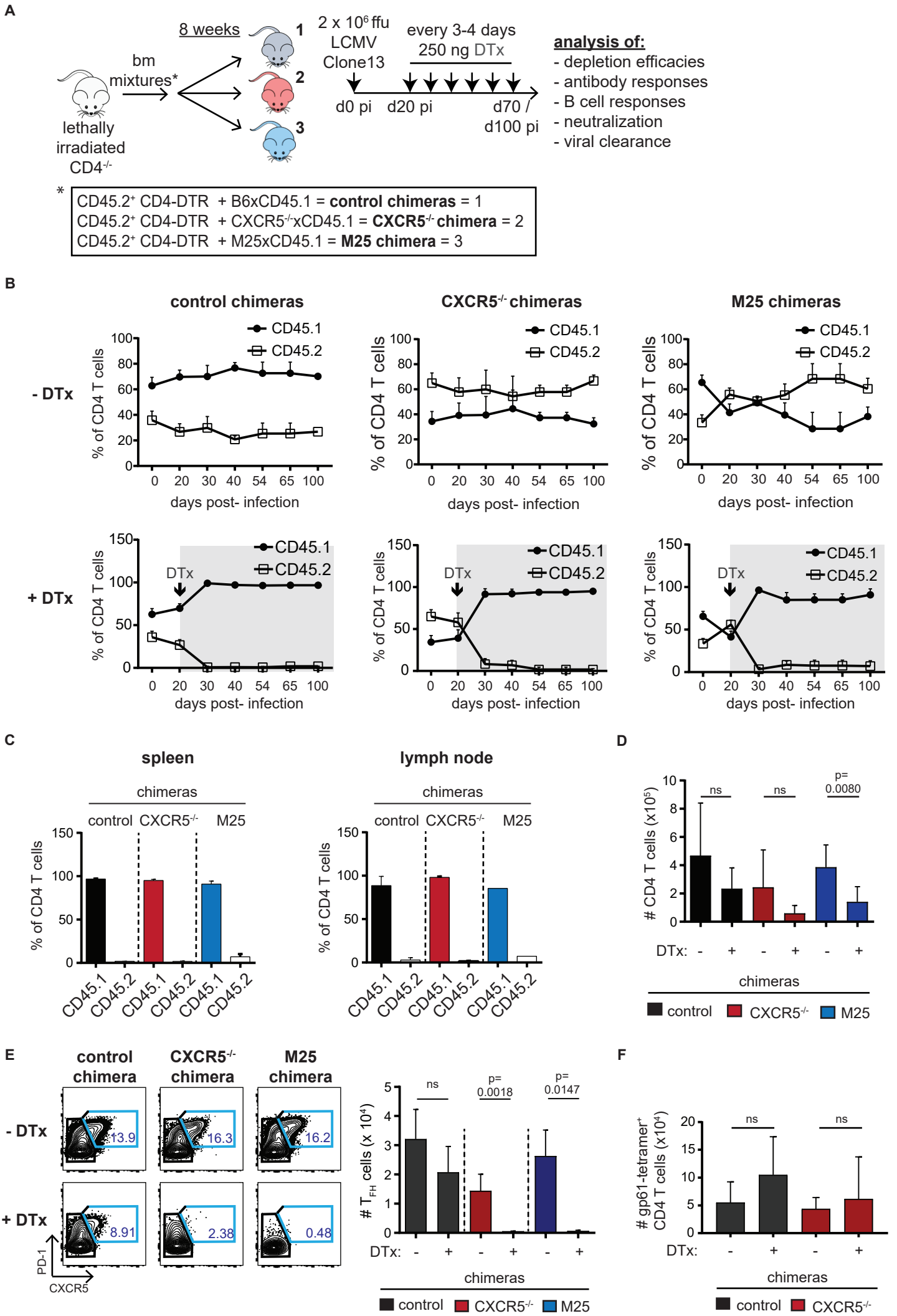
**Fig. 5: GCs contain CXCR5$^{-/-}$ CD4 T cells in absence of CXCR5$^{+/+}$ T$_{FH}$ cells.** A) Immunofluorescence staining of splenic thin sections of untreated (upper panel) or DTx treated (lower panel) control (left), CXCR5$^{-/-}$ (middle) and M25 (left) chimeras for GC B cells (PNA$^{+}$;

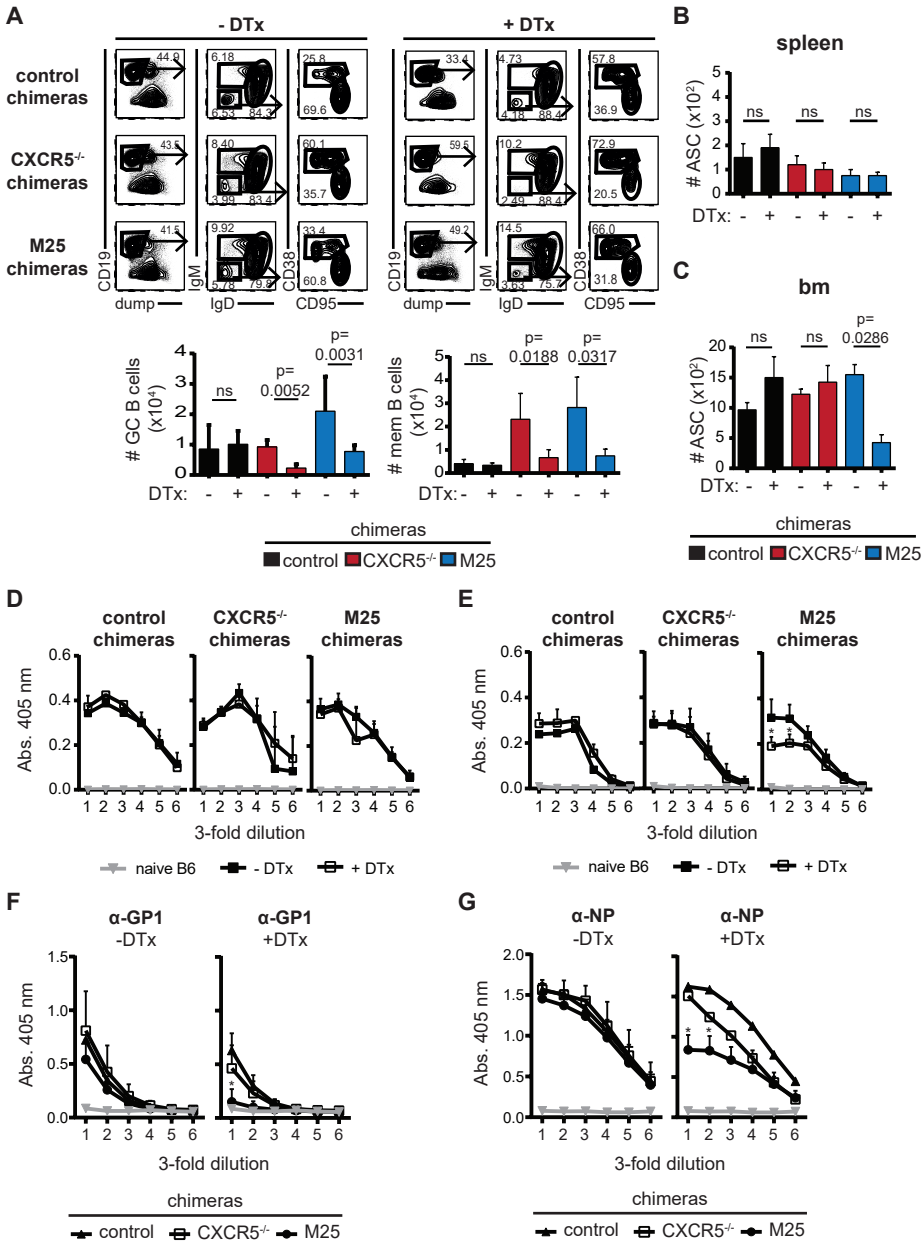
blue), B cell follicles ($IgD^+$; red) and CD4 T cells (yellow). Scale bar: 100 µm. B) Ratio of the CD4 and PNA MFI and C) Quantification of CD4 T cells per $µm^2$ in the area of the GC. 20-30 GCs in two independent experiments were analyzed in total, n=3-4 mice per group. Error bars represent means ±SD. D) Immunofluorescence staining of splenic thin sections of untreated (upper panel) or DTx treated (lower panel) control (left), $CXCR5^{-/-}$ (red) splenocyte chimeras at d50 pi. Staining for GC B cells (PNA, blue), CD4 (yellow), mature B cells (IgD, red) and FDCs ($CD35/21^+$, white). LZ (white circle) was identified as FDC containing $PNA^+$ area, DZ (green marked area) as non-FDC containing $PNA^+$ area. Scale bar: 100 µm. E) Quantification of ratio of CD4 T cells per $µm^3$ contained in LZ to CD4 T cells per $µm^3$ contained in the DZ. 20 to 50 GC were analyzed in total, n=3 per group. Error bars represent means ±SD. Mann-Whitney U test used: ns $p>0.05$.

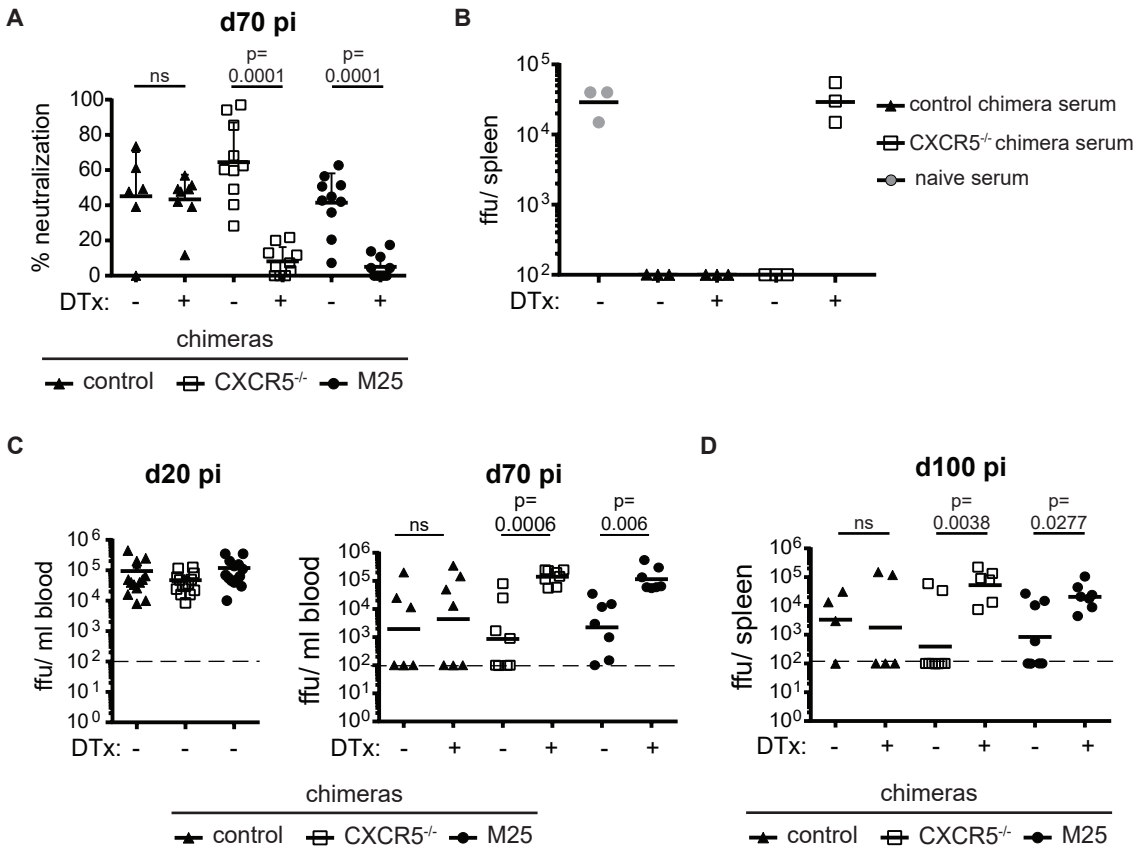
**Fig. 6: $T_{FH}$ cells drive the adaptation of the neutralizing antibody response towards the contemporary virus species.** A) Neutralization of sera isolated from single chimeras (indicated by letters) isolated at d20 (upper panel) or d70 (lower panel) pi against the inoculum (left panels) or the contemporary virus isolates (right panels). 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 condition. One representative experiment of two is shown. B) Correlation between neutralization against the inoculum (upper panel) or the contemporary isolate (lower panel) and viral clearance in untreated (green) and DTx treated (orange) control, $CXCR5^{-/-}$ and M25 chimeras at d70 pi. Pooled data of three (inoculating virus) or two (contemporary isolates) independent experiments are shown. C) Total number of IFN-γ or D) TNF producing CD8 T cells isolated from mice acutely infected with the inoculum and restimulated for 6h with macrophages infected with

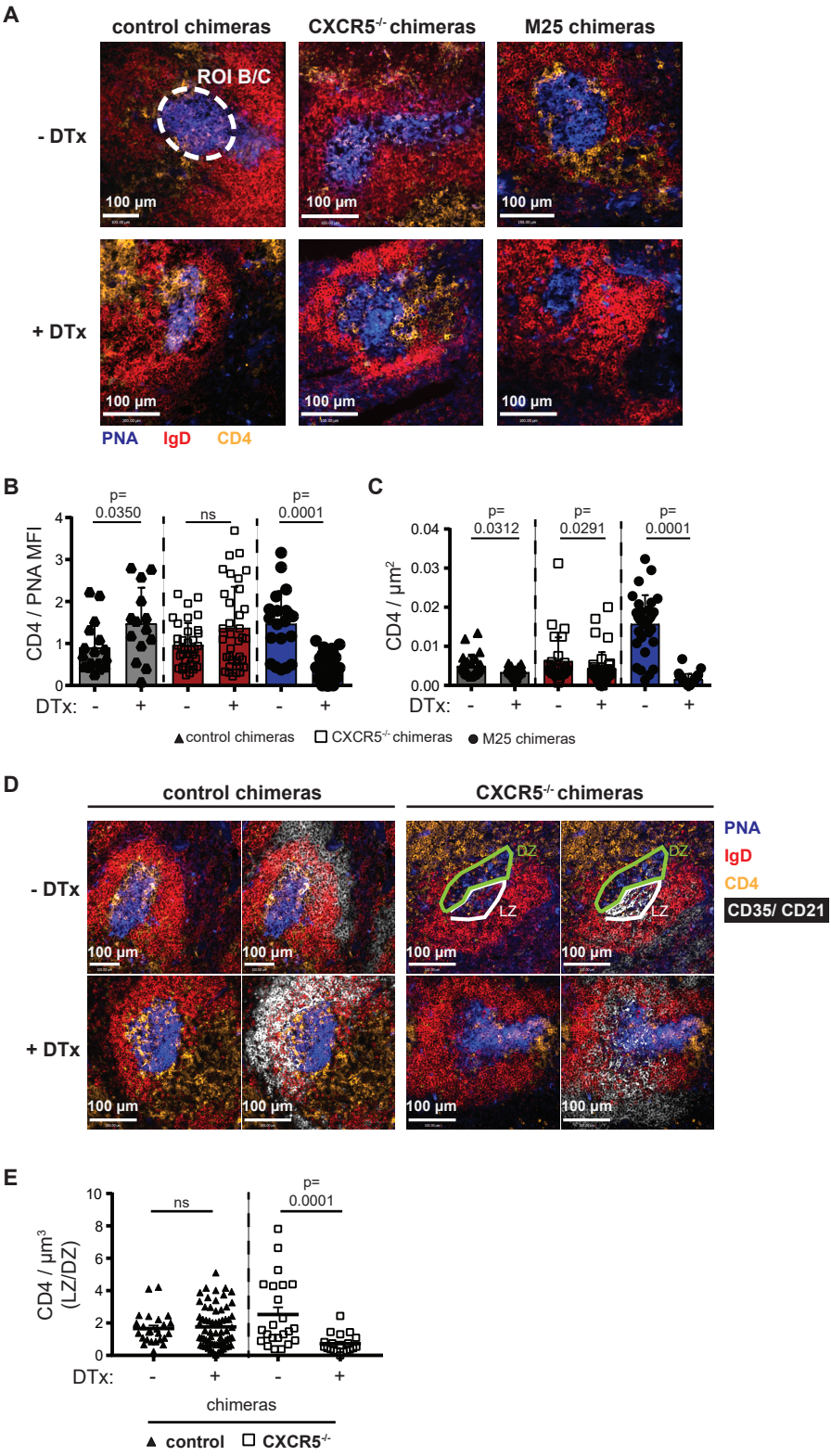
the inoculum (red) or contemporary virus isolates isolated on d70 pi of single chimeras (indicated with letters) of untreated (green) or DTx treated (orange) control, $CXCR5^{-/-}$ and M25 chimeras. Uninfected macrophages (white) were used as negative control and PMA/Ionomycin restimulation as positive control (black). Lines indicate the cytokine expression by CD8 T cells stimulated by thio-macrophages that were infected with the inoculating virus. One representative experiment of two shown.

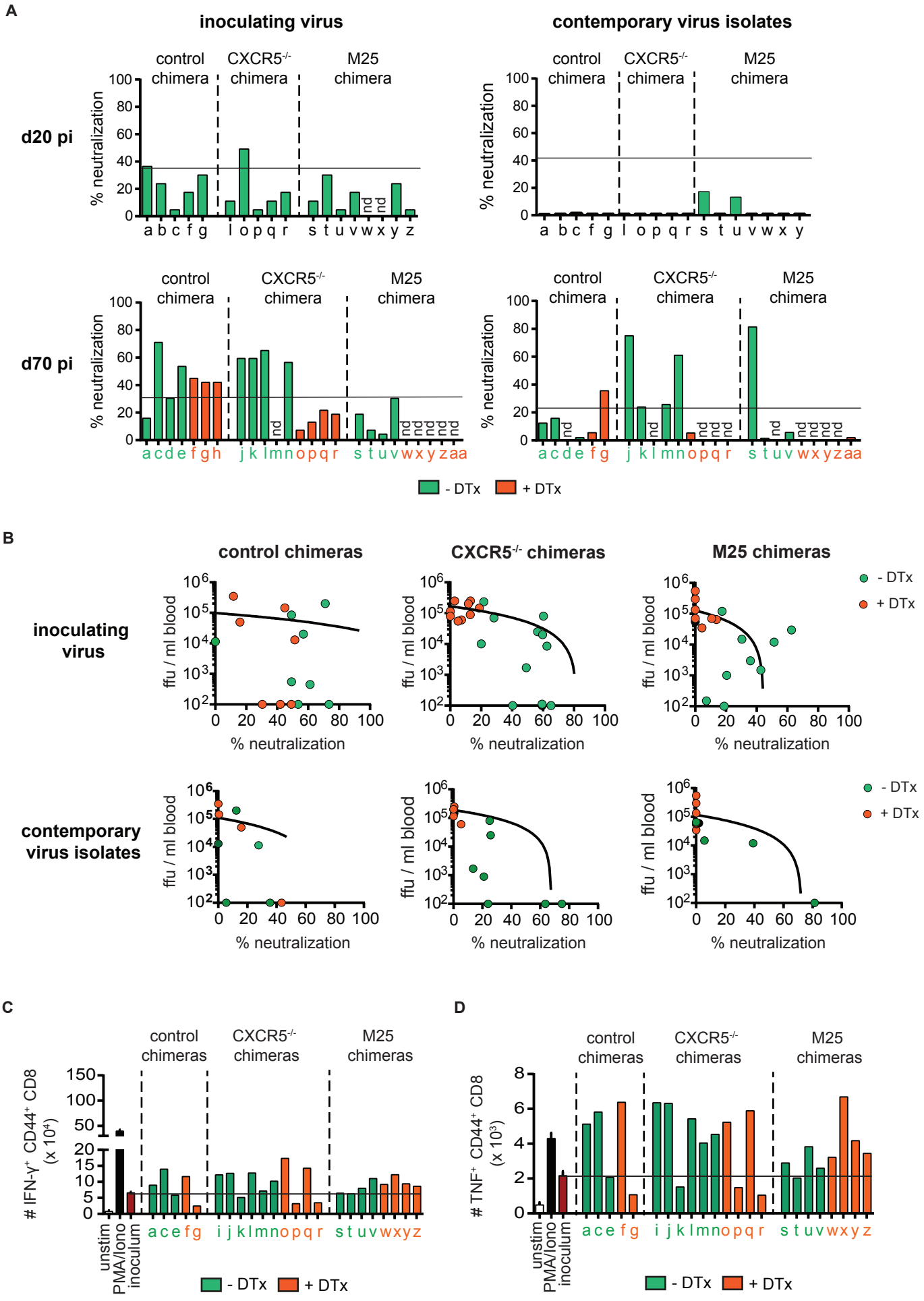

A
CD45.2+ CD4- DTR
B6 x CD45.1
bm donors
lethally irradiated CD4-/-
8 weeks
B6 : CD4-DTR chimera
2 x 10^6 ffu LCMV Clone13
every 3-4 days 250 ng DTx
d0 pi
d20 pi
d40 pi
analysis
B
CD4 T cells
CD8 T cells
- DTx
+ DTx
58.0
0.1
0
41.9
37.8
0.08
0
62.1
98.9
0.27
0
0.81
33.8
0.14
0
66.1
CD45.1
CD45.2
% of CD4 T cells
% of CD8 T cells
DTx
days post-infection
C
spleen
LN
lung
% of CD4 T cells
# CD4 T cells (x10^5)
B6 x CD45.1
CD4 - DTR
D
ffu/ spleen
ns
DTx: - +
E
non - T_FH
T_FH
- DTx
+ DTx
13.1
83.8
8.67
88.5
50.7
0.57
0.50
48.3
29.8
3.48
0.09
66.6
98.5
0.29
0.03
1.20
96.6
3.42
0
0
PD-1
CXCR5
CD45.1
CD45.2
# cells (x 10^3)
DTx: - - + + - - + +


A
8 weeks
bm mixtures*
lethally irradiated CD4-/-
1
2
3
2 x 10^6 ffu LCMV Clone13
every 3-4 days 250 ng DTx
d0 pi
d20 pi
d70 / d100 pi
analysis of:
- depletion efficacies
- antibody responses
- B cell responses
- neutralization
- viral clearance
* CD45.2+ CD4-DTR + B6xCD45.1 = control chimeras = 1
CD45.2+ CD4-DTR + CXCR5-/-xCD45.1 = CXCR5-/- chimera = 2
CD45.2+ CD4-DTR + M25xCD45.1 = M25 chimera = 3
B
control chimeras
CXCR5-/- chimeras
M25 chimeras
- DTx
+ DTx
CD45.1
CD45.2
% of CD4 T cells
days post- infection
DTx
C
spleen
lymph node
chimeras
control
CXCR5-/-
M25
% of CD4 T cells
CD45.1
CD45.2
D
# CD4 T cells (x10^5)
ns
p= 0.0080
DTx: - + - + - +
chimeras
control
CXCR5-/-
M25
E
control chimera
CXCR5-/- chimera
M25 chimera
- DTx
+ DTx
13.9
16.3
16.2
8.91
2.38
0.48
PD-1
CXCR5
# TFH cells (x 10^4)
p= 0.0018
p= 0.0147
F
# gp61-tetramer+ CD4 T cells (x10^4)


A
- DTx
+ DTx
control chimeras
CXCR5-/- chimeras
M25 chimeras
CD19
IgM
dump
IgD
CD38
CD95
# GC B cells (x10⁴)
# mem B cells (x10⁴)
DTx:
ns
p= 0.0052
p= 0.0031
p= 0.0188
p= 0.0317
chimeras
control
CXCR5-/-
M25
B
spleen
# ASC (x10²)
C
bm
p= 0.0286
D
E
Abs. 405 nm
3-fold dilution
naive B6
- DTx
+ DTx
F
α-GP1
-DTx
+DTx
G
α-NP


A
d70 pi
ns
p= 0.0001
p= 0.0001
% neutralization
DTx: - + - + - +
chimeras
control
CXCR5-/-
M25
B
ffu/ spleen
DTx: - - + - +
control chimera serum
CXCR5-/- chimera serum
naive serum
C
d20 pi
d70 pi
ffu/ ml blood
ns
p= 0.0006
p= 0.006
DTx: - - - - + - + - + - +
chimeras
control
CXCR5-/-
M25
D
d100 pi
ffu/ spleen
ns
p= 0.0038
p= 0.0277
DTx: - + - + - +
chimeras
control
CXCR5-/-
M25


A
control chimeras
CXCR5-/- chimeras
M25 chimeras
- DTx
+ DTx
ROI B/C
100 µm
PNA IgD CD4
B
CD4 / PNA MFI
p= 0.0350
ns
p= 0.0001
DTx: - + - + - +
C
CD4 / µm2
p= 0.0312
p= 0.0291
p= 0.0001
DTx: - + - + - +
control chimeras
CXCR5-/- chimeras
M25 chimeras
D
control chimeras
CXCR5-/- chimeras
- DTx
+ DTx
DZ
LZ
PNA
IgD
CD4
CD35/ CD21
E
CD4 / µm3 (LZ/DZ)
ns
p= 0.0001
DTx: - + - +
chimeras
control
CXCR5-/-


A
inoculating virus
contemporary virus isolates
control chimera
CXCR5-/- chimera
M25 chimera
d20 pi
d70 pi
% neutralization
- DTx
+ DTx
B
control chimeras
CXCR5-/- chimeras
M25 chimeras
inoculating virus
contemporary virus isolates
ffu / ml blood
% neutralization
C
# IFN-γ+ CD44+ CD8 (x 10⁴)
unstim
PMA/Iono
inoculum
D
# TNF+ CD44+ CD8 (x 10³)

# Supplementary Materials

## Supplementary Materials and Methods

*Production of recombinant LCMV-Clone13-GP1-Fc and LCMV-Clone13-NP*

The sequence of the GP1 (amino acids 1 to 265) of the LCMV Clone13 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 Clone13-GP1-Fc was performed by transient transfection of CHO cells with jetPEI according to the manufactures protocol. Transfected cells were cultured for 2 to 3 days, afterwards the supernatant containing the GP1-Fc was harvested and used for ELISA analysis.

The gene sequence of the LCMV-Clone13 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 Clone13-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 -1. Afterwards 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$^{R}$ Ni-NTA Agarose beads (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 Clone13-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-Clone13 infected MC57G or as negative control with 20 µg/ml lysate of uninfected MC57G. 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 Clone13-NP (for production see Supplementary Materials). Development of the ELISA was then 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-Clone13 infected MC57G or 20 µg/ml lysate of uninfected MC57G (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) was 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 5min at RT 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. Eventually detection was

performed using the AP Conjugate Substrate Kit (Biorad). Between every step plates were washed six times with PBS-T. 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

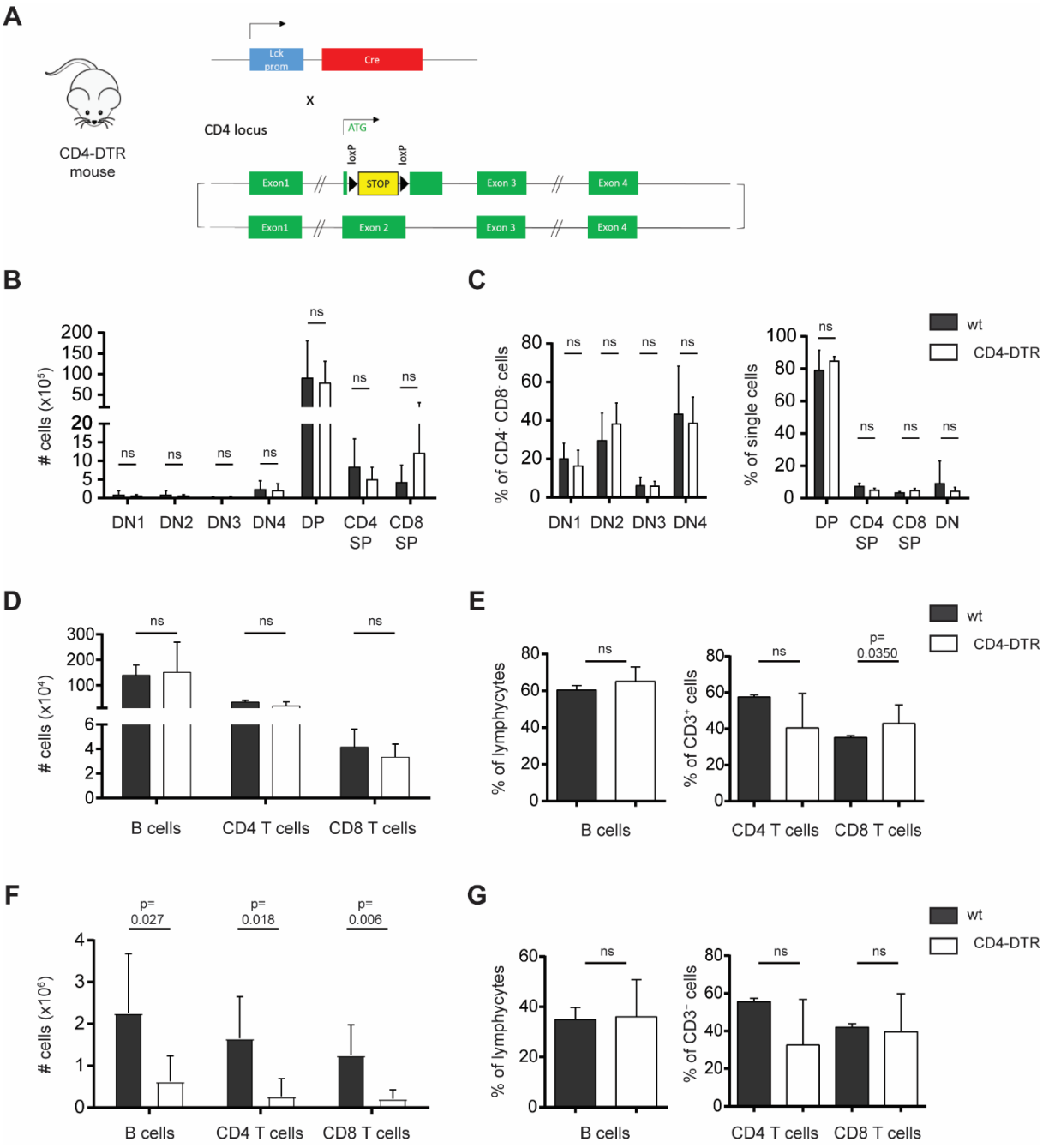



**Fig. S1: Basic characterization of the CD4-DTR mouse line.** A) Schematic illustration of the generation of the CD4-DTR mouse line. B) Total cell counts C) and percentage of cells in different T cell developmental stages in the thymus of CD4-DTR (white) and wt C57BL/6 (black) mice. DN = double negative, 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 $p \geq 0.05$.

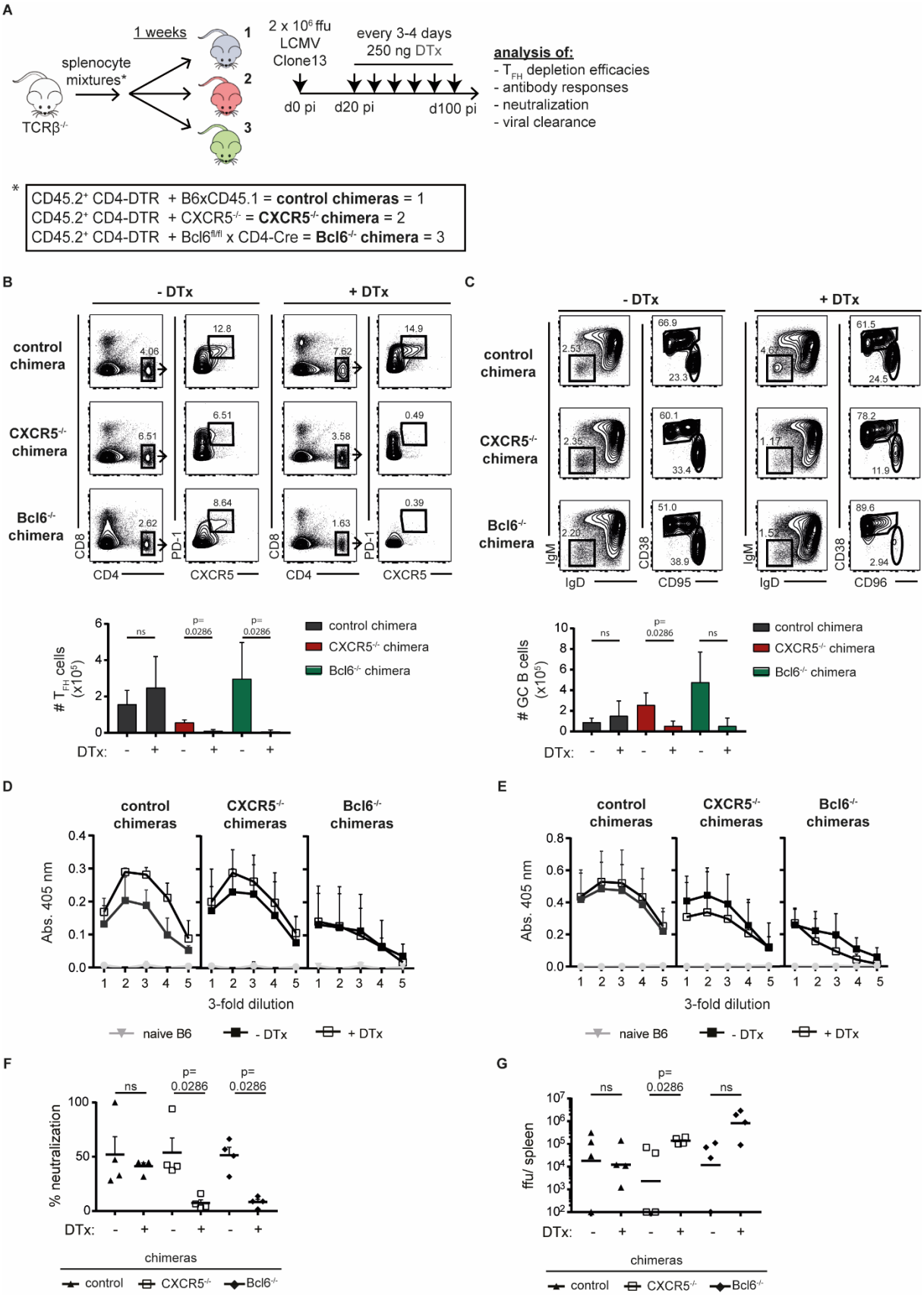
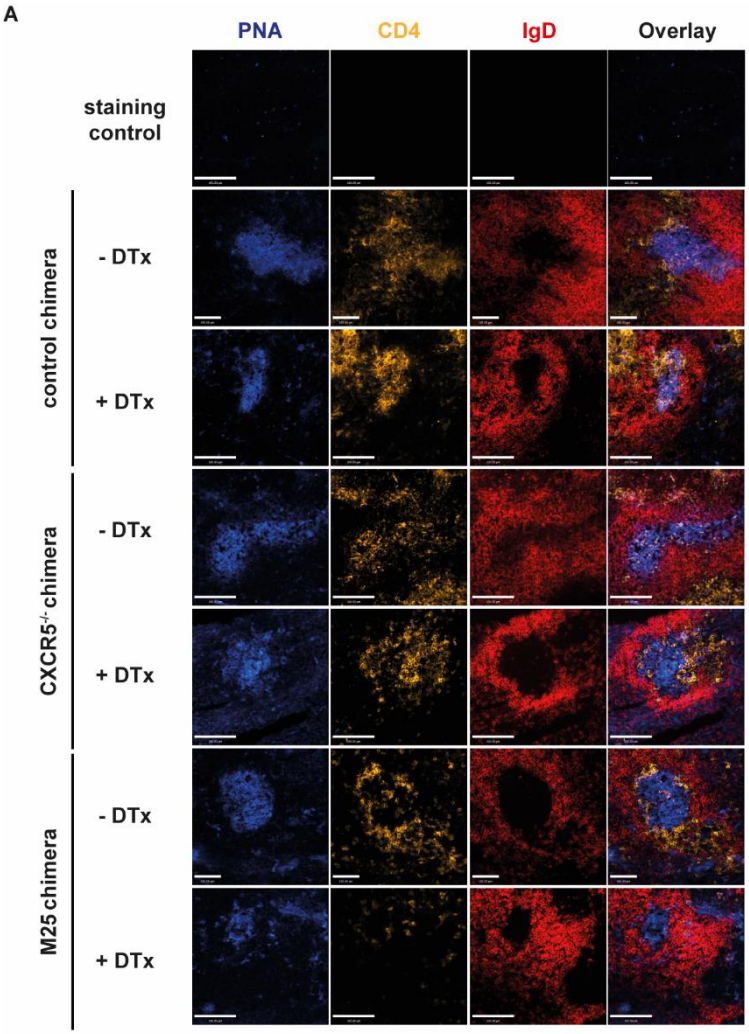

A
1 weeks
splenocyte mixtures*
TCRβ-/-
1
2
3
2 x 10^6 ffu LCMV Clone13
every 3-4 days 250 ng DTx
d0 pi
d20 pi
d100 pi
analysis of:
- TFH depletion efficacies
- antibody responses
- neutralization
- viral clearance
* CD45.2+ CD4-DTR + B6xCD45.1 = control chimeras = 1
CD45.2+ CD4-DTR + CXCR5-/- = CXCR5-/- chimera = 2
CD45.2+ CD4-DTR + Bcl6fl/fl x CD4-Cre = Bcl6-/- chimera = 3
B
- DTx
+ DTx
control chimera
CXCR5-/- chimera
Bcl6-/- chimera
CD8
CD4
PD-1
CXCR5
4.06
12.8
7.62
14.9
6.51
6.51
3.58
0.49
2.62
8.64
1.63
0.39
C
- DTx
+ DTx
IgM
IgD
CD38
CD95
CD96
2.53
66.9
23.3
61.5
24.5
2.35
60.1
33.4
78.2
11.9
2.20
51.0
38.9
89.6
2.94
# TFH cells (x10^6)
# GC B cells (x10^6)
ns
p= 0.0286
DTx: - + - + - +
control chimera
CXCR5-/- chimera
Bcl6-/- chimera
D
E
control chimeras
CXCR5-/- chimeras
Bcl6-/- chimeras
Abs. 405 nm
3-fold dilution
naive B6
- DTx
+ DTx
F
G
% neutralization
ffu/ spleen
chimeras
control
CXCR5-/-
Bcl6-/-

**Fig. S2: Sustained activity of CXCR5 or Bcl6 expressing $T_{FH}$ cells is required for the development of LCMV-neutralizing antibodies and control of protracted infection.** A) Experimental approach. TCRβ$^{-/-}$ mice received a 50:50 mixture of mature CD4-DTR (CD45.2$^{+}$) splenocytes and CD45.1$^{+}$ B6 (control chimeras), CXCR5$^{-/-}$ (CXCR5$^{-/-}$ chimeras) or CD4-Cre x Bcl6$^{fl/fl}$ (Bcl6$^{-/-}$ chimeras) splenocytes per adoptive transfer. One week after transfer, chimeras were persistently infected with 2x10$^{6}$ ffu LCMV Clone13. From d20 pi onwards, continuous DTx treatment every 3-4 days was initiated. B) Representative FACS plots and quantification of $T_{FH}$ cells (CXCR5$^{+}$ PD-1$^{+}$ CD4 T cells) in untreated (-DTx) or DTx treated (+DTx) control (dark grey), CXCR5$^{-/-}$ (red) or Bcl6$^{-/-}$ chimeras on d100 pi. FACS blots pre-gated on lymphocytes and single cells. C) Representative FACS plots and quantification of isotype-switched GC B cells (CD19$^{+}$ IgD$^{-}$ IgM$^{-}$ CD95$^{+}$ CD38$^{-/lo}$) in untreated (-DTx) or DTx treated (+DTx) control (dark grey), CXCR5$^{-/-}$ (red) or Bcl6$^{-/-}$ 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$^{-/-}$ and Bcl6$^{-/-}$ 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 3-fold 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$^{-/-}$ and Bcl6$^{-/-}$ chimeras. G) Viral titers in spleen at d100 pi in untreated and DTx treated control, CXCR5$^{-/-}$ and Bcl6$^{-/-}$ chimeras. Mann-Whitney U test used for statistical analysis: ns p>0.05. n=4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PNA
CD4
IgD
Overlay
staining control
control chimera
- DTx
+ DTx
CXCR5-/- chimera
- DTx
+ DTx
M25 chimera
- DTx
+ DTx

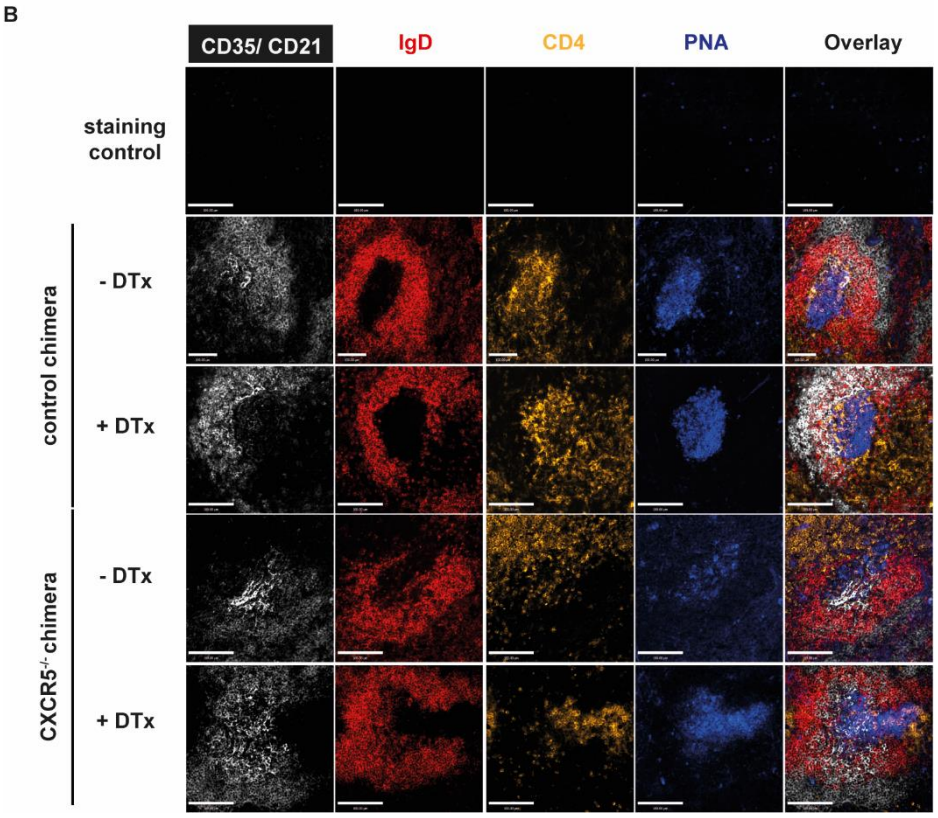
B
CD35/ CD21
IgD
CD4
PNA
Overlay
staining control
control chimera
- DTx
+ DTx
CXCR5-/- chimera
- DTx
+ DTx

**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.

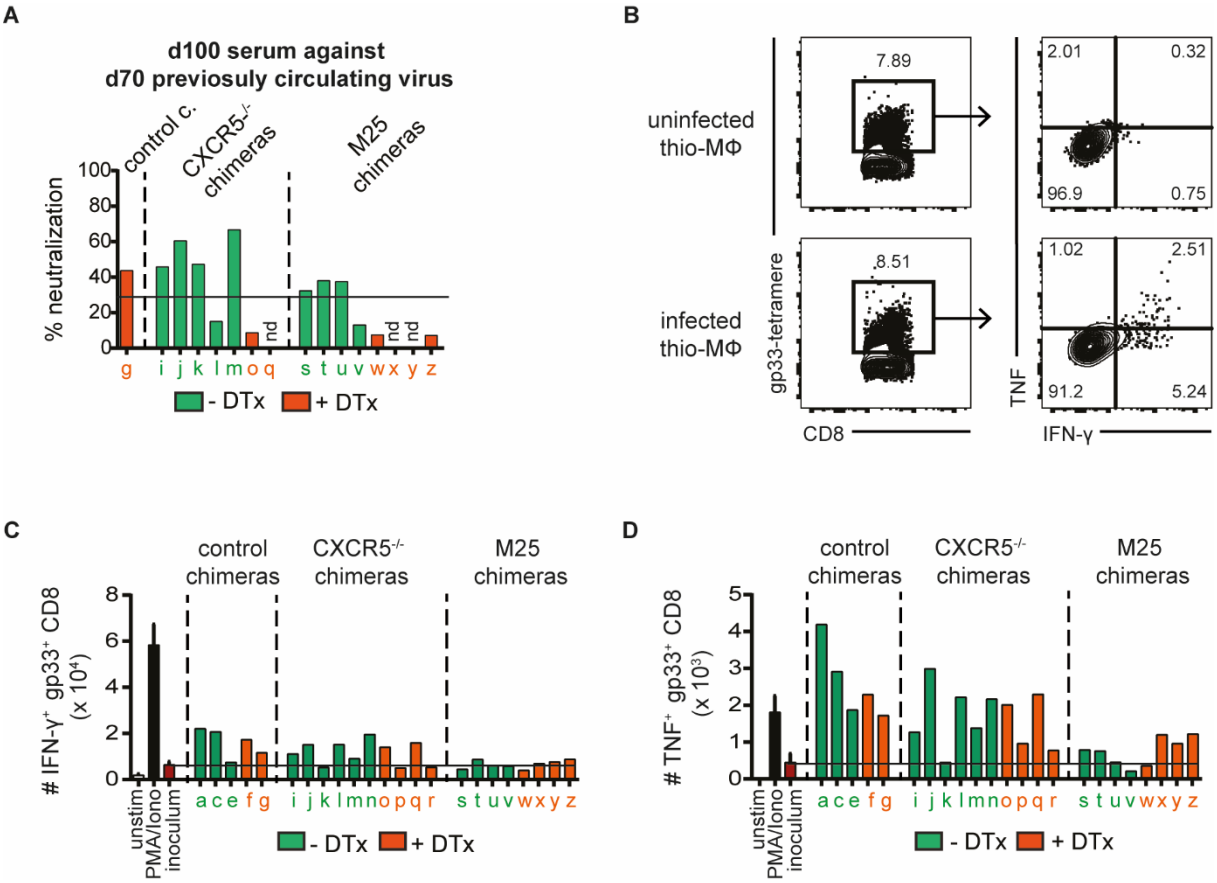


**Fig. S4: Viral escape is not driven by pressure of CTLs.** A) Neutralization of sera isolated from single chimeras on d100 pi against the virus isolates extracted on d70 pi of the respective chimera. Untreated chimeras are marked in green and DTx treated chimeras are marked in orange. Lines indicate 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.

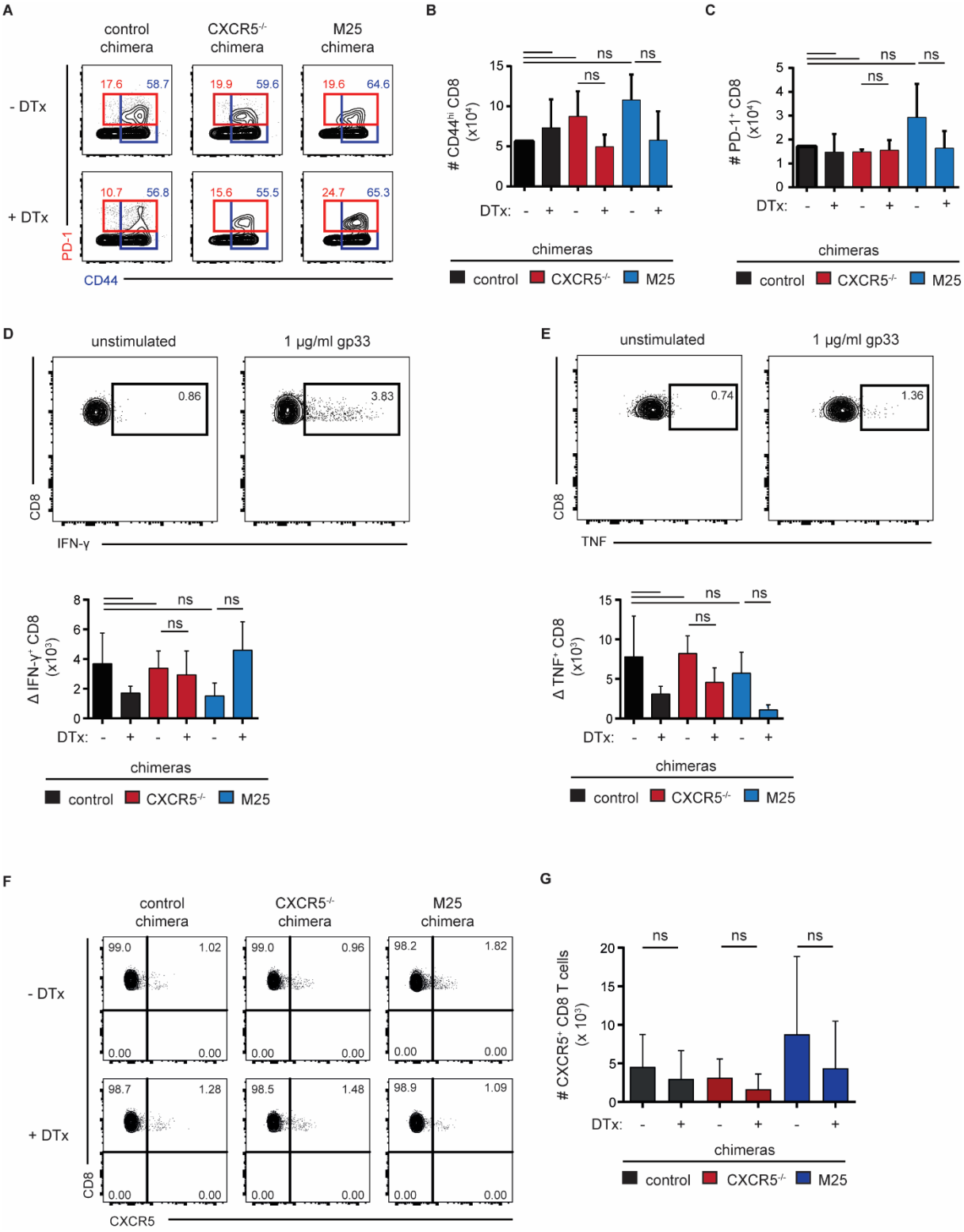


**Fig. S5: CD8 T cell responses are unaffected by absence of CXCR5$^{+/+}$ $T_{FH}$ or LCMV-specific CD4 T cells.** A) FACS staining and B) + C) quantification of total cell numbers of CD44$^{hi}$ (B) or PD-1$^{+}$ (C) CD8 T cells in untreated or DTx treated control (black), CXCR5$^{-/-}$(red)

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), $CXCR5^{-/-}$ (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 $p \geq 0.05$.