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

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During chronic viral infections, both CD8 and CD4 T cell responses are functionally compromised. Alongside exhaustion of CD8 T cells during chronic viral infections, it has also been documented that the CD4 T cells have an increased propensity to differentiate toward CXCR5+ T follicular helper cell (T\( _{FH} \)) lineage. Whether these T\( _{FH} \) cells contribute to the immune response to chronic viral infection has remained unclear. Using chronic lymphocytic choriomeningitis virus (LCMV) infection in conjunction with an in vivo system where T\( _{FH} \) cells can be conditionally ablated, we have established that these T\( _{FH} \) cells do in fact play an important protective function. Specifically, we demonstrate that these T\( _{FH} \) cells are essential for the late emergence of neutralizing LCMV-specific antibodies that keep viral titers in check and ultimately allow mice to clear the virus. By supporting the generation of neutralizing antibodies, we show that sustained activity of T\( _{FH} \) cells promotes control of the chronic infection in face of exhausted CD8 T cell responses.

INTRODUCTION

During chronic infections with viruses, such as HIV-1, hepatitis C, and hepatitis B virus in humans or lymphocytic choriomeningitis virus (LCMV) in mice, 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 diminution of virus-specific CD8 and T helper (T\( _{H} \)) T cells, collectively termed T cell exhaustion (1, 2). Instead, virus-specific CD4 T cells exhibit a differentiation bias toward follicular helper cells (T\( _{FH} \)) throughout chronic infection (3–5). In the context of acute infections or vaccinations, T\( _{FH} \) cells support induction and selection of T helper–dependent B cell responses (6). They express characteristic markers, including the transcriptional repressor Bcl6, the chemokine receptor CXCR5, the costimulatory molecules ICOS and CD40L, the coinhibitory molecule PD-1, and interleukin-21 (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). Here, T\( _{FH} \) cells (9, 10) instruct B cell differentiation and induce the germinal center (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 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).

Although CD8 T cell exhaustion has been intensively studied in persistent LCMV infection (1), a role of the pronounced differentiation of CD4 T cells toward T\( _{FH} \) cells in controlling established persistent infection remains unclear. Given the importance of T\( _{FH} \) cells in supporting B cell responses, it is of particular interest to establish whether and how they promote the generation of virus-binding and virus-neutralizing antibodies. The absence of T\( _{FH} \) cells at the onset of infection curtails the overall LCMV-specific humoral response and prevents viral clearance (3). LCMV-binding antibodies generated early and throughout the infection cannot prevent the infection (14), but can limit viral spread and exert indirect protective functions (15–18). However, LCMV-neutralizing antibodies directly inhibit infection and thereby limit viral replication and spread. However, neutralizing antibodies arise late [days (d) 60 to 80] during chronic LCMV infection (14, 19). Persistently infecting RNA viruses undergo mutational adaptations that escape pressure imposed by adaptive immunity, thereby promoting viral persistence (20, 21). B cells are equipped with the ability to counter adapt their antibodies to the currently prevailing antigen by the process of SHM and selection in the LZ. Whether these adaptations rely on sustained activity of T\( _{FH} \) cells is plausible, but it has remained elusive.

Here, we addressed whether the accentuated T\( _{FH} \) differentiation during chronic viral infections fulfills a relevant physiological function. 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 immunoglobulin G (IgG) responses, we determined 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 for the eventual termination of the chronic infection.

RESULTS

Generation of a mouse model that allows specific depletion of CD4 T cells

To establish conditions in which CD4 T cells can be specifically depleted in chronically infected hosts, we generated a mouse model (“CD4-DTR”) expressing the primate diphtheria toxin receptor (DTR) specifically in CD4 T cells. A loxP-flanked stop cassette before the DTR gene was targeted into exon 2 of the CD4 locus, and gene-targeted mice were crossed to lck-Cre transgenic mice (fig. S1A) (22), leading to excision of the stop cassette during thymic T cell development and yielding CD4-DTR mice expressing the DTR, specifically in CD4 T cells. Heterozygous CD4-DTR mice were comparable to wild-type (WT) C57BL/6 mice regarding thymic T cell development and yielding CD4-DTR mice expressing the DTR, specifically in CD4 T cells. Heterozygous CD4-DTR mice were comparable to wild-type (WT) C57BL/6 mice regarding thymic T cell development and yielding CD4-DTR mice expressing the DTR, specifically in CD4 T cells.
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. CD45.1+ B6 BM and CD45.2+ CD4-DTR BM were cotransferred into lethally irradiated CD4−/− recipients (Fig. 1A). CD4 T cells derived from the CD4-DTR compartment (CD45.2+) were specifically and fully depleted from d25 pi onward (Fig. 1B) and were also efficiently depleted in the spleen, mLN, and lung at d40 pi (Fig. 1C). CD8 T cells derived from the CD4-DTR compartment were not affected by the DTx treatment (Fig. 1B). These were at slightly higher frequencies because the BM graft contained more CD4-DTR donor cells to achieve 50% contribution to the CD4 compartment, because CD4-DTR CD4 T cells seem to have a slight reconstitution disadvantage.

Furthermore, CXCR5+ PD-1+ T FH cells and CXCR5− non-T FH cells were 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). DTx treatment did not affect viral clearance or viral titers in the spleen at d40 pi (Fig. 1D). 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**

Next, we used CD4-DTR cells to generate mouse models permitting specific and conditional depletion of T FH or LCMV-specific CD4 T cell subsets during established chronic infection. We generated mixed BM chimeras harboring CD4-DTR T cells alongside CD4 T cells that cannot form CXCR5+/+ T FH cells (CXCR5−/− CD4 T cells) or do not contain LCMV-specific CD4 T cells [M25 T cell receptor (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 (23). CD4−/− recipients were irradiated and reconstituted with a mixture of CD4-DTR BM and (i) CD45.1+ WT BM (control chimeras), (ii) CD45.1+ CXCR5−/− BM (CXCR5−/− chimeras), or (iii) CD45.1+ M25 BM (M25 chimeras) (Fig. 2A). Chimeras were infected with LCMV clone 13; and longitudinal DTx treatment was initiated 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 (Fig. 2, B and C). Absolute numbers of CD4 T cells in the spleen were not notably reduced in control chimeras but reduced about twofold in DTx-treated CXCR5−/− and M25 chimeras (Fig. 2D). Although CXCR5+/+ T FH cells were ablated in DTx-treated CXCR5−/− and M25 chimeras, CXCR5+/+ T FH cells were maintained in untreated 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 ideal for analyzing the functional roles of CXCR5+/+ T FH and LCMV-specific CD4 T cells in 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**

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 initial establishment of the LCMV-specific IgG response. Analysis of the three mixed BM chimera groups at d70 pi (50 days after initiation of DTx treatment) revealed that the total cell count of splenic GC B cells (CD95+ CD38−/−) in the absence of CXCR5+/+ T FH or LCMV-specific CD4 T cells was fourfold, 2.5-fold reduced compared to untreated chimeras (Fig. 3A). Likewise, the total number of memory B cells (isotype-switched CD38+) in the spleen was reduced in the absence of CXCR5+/+ T FH or LCMV-specific CD4 T cells compared with untreated chimeras and DTx-treated control chimeras (Fig. 3A). Unexpectedly, numbers of LCMV-specific antibody-secreting cells (ASCs) were not reduced in the spleen (Fig. 3B) and BM (Fig. 3C) in the 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 BM in their prolonged absence (Fig. 3C).

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. 3, D and E). LCMV-specific antibody titers were significantly reduced at d100 pi in prolonged absence of LCMV-specific CD4 T cells (Fig. 3E, right), reflecting the reduced ASC counts in BM. Both LCMV nucleoprotein (NP)-specific and glycoprotein 1 (GP1)–specific IgG titers were reduced in serum of DTx-treated M25 chimeras (Fig. 3, F and G). Prolonged absence of CXCR5+/+ T FH cells did reduce overall LCMV-specific antibody titers at d100 pi (Fig. 3, E to G).

In conclusion, CXCR5+/+ T FH cells seem to be dispensable for the differentiation and maintenance of ASCs or the maintenance of the LCMV-specific IgG titers after initiation of the LCMV-specific IgG response. However, LCMV-specific CD4 T cells are essential for maintenance of LCMV-specific ASCs in the BM 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**

Because 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. However, neutralizing antibodies are believed to contribute to eventual viral clearance (24, 25). Hence, we measured the neutralizing capacity of sera isolated at d70 pi against the inoculum virus. Sera of untreated and DTx-treated control chimeras and sera of untreated M25 chimeras exhibited about 40% neutralization, whereas sera of untreated CXCR5−/− chimeras exhibited about 60% neutralization (Fig. 4A). In the absence of CXCR5+/+ T FH cells or all LCMV-specific CD4 T cells, neutralization was reduced to 1 to 10% (Fig. 4A). To evaluate the in vivo protective capacities of sera isolated from untreated or DTx-treated CXCR5−/− or control chimeras, we infused their immune serum or naïve serum, together with inoculum virus, into naïve C57BL/6 mice. Serum from untreated control, CXCR5−/−, or DTx-treated control mice, containing in vitro neutralizing antibodies,
conferred in vivo protection, whereas serum from naïve mice or DTx-treated CXCR5−/− chimeras were not protective (Fig. 4B). This shows that continued presence of CXCR5+/+ TFH cells is essential for the generation of in vitro neutralizing and in vivo protective IgG responses. Because LCMV-neutralizing antibodies may be key effectors for eventual clearance of persistent LCMV infection, we analyzed viral titers in blood at d20 and d70 pi, as well as in the spleen at d100 pi. At d20 pi, before DTx treatment was initiated, all chimeras had comparable virus titers in blood [about 10^5 focus-forming units (FFU/ml

Fig. 1. Specific depletion of CD4-DTR–derived CD4 T cells. (A) Lethally irradiated CD4−/− mice were reconstituted with CD4-DTR (CD45.2+) and CD45.1+ B6 BM and persistently infected with 2 × 10^6 FFU LCMV clone 13. From d20 pi onward, continuous DTx treatment every 3 to 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 the spleen, mLN, and lung at d40 pi. (D) Viral titers in the spleen on d40 pi. Statistical analysis was performed using Mann-Whitney U test; *P = 0.8413. (E) Ratios and numbers of CD4-DTR–derived (CD45.2+) and CD45.1+ B6–derived non-TFH (red gate; CXCR5−) and TFH (blue gate; CXCR5+ PD-1+) cells in the 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 bars represent means ± SD, ns, not significant.
of blood; Fig. 4C). Most of the mice from the untreated groups and DTx-treated control chimeras had either cleared or partially controlled the infection in blood at d70 pi (Fig. 4D) and in the spleen at d100 pi (Fig. 4D). DTx-treated CXCR5−/− and M25 chimeras showed still comparable viral titers to d20 pi (Fig. 4, C and D). Therefore, continuous absence of CXCR5+/+ TFH cells not only affected the generation of neutralizing antibodies but also abolished control of persistent LCMV infection.

We wanted to confirm our observations using a different in vivo experimental setup in which TFH cell generation was inhibited by the absence of Bcl6 [being essential for the differentiation of TFH cells (6)]. 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 Bcl6Δfl x CD4-Cre mice; Bcl6−/− chimeras) T cells into TCRα−/− hosts (fig. S2A). One week after transfer, mice were chronically infected with LCMV clone 13, and, after establishment of LCMV-specific IgG titers, half of the mice were treated with DTx starting from d20 pi onward (fig. S2A). CXCR5+/+ TFH cells could be effectively depleted in DTx-treated CXCR5−/− and Bcl6−/− splenocyte chimeras (fig. S2B). GC B cell numbers were reduced in DTx-treated CXCR5−/− and Bcl6−/− chimeras as compared with untreated chimeras and DTx-treated control chimeras (fig. S2C). Depletion of CXCR5+/+ TFH cells in CXCR5−/− and Bcl6−/− TFH 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 d20 pi (fig. S2, D and E). However, LCMV-neutralizing antibodies only emerged in the presence of CXCR5+/+ or Bcl6+/+ TFH cells (fig. S2F). DTx-treated CXCR5−/− and Bcl6−/− chimeras exhibited elevated viral titers in the spleen at d100 pi as compared with 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+/+ TFH cells during the entire course of infection was essential for the induction of LCMV-neutralizing antibody responses.

**CXCR5−/− CD4 T cells are found in the GC in the absence of CXCR5+/+ TFH cells**

Because CXCR5+/+ TFH cells were not essential for the maintenance of the overall LCMV-specific IgG, we wondered how CXCR5−/− LCMV-specific CD4 T cells might be able to take over B cell helper functions. It was previously shown that CXCR5−/− CD4 T cells are able to migrate to the T/B border (26) and might also be passively dragged into the B cell follicle by cognate B cells forming motile conjugates with antigen-specific CD4 T cells (27). To analyze whether there is contact between CD4 T and B cells in CXCR5+/+ TFH−depleted chimeras, we stained GC B cells (peanut agglutinin, 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 and fig. S3A). However, in DTx-treated M25 chimeras, GCs contained far fewer CD4 T cells compared with the untreated chimeras because the ratio of CD4 mean fluorescence intensity (MFI) to PNA MFI, as well as the number of CD4 T cells per square micrometers of the PNA+ area, was significantly reduced (Fig. 5, A to C, and fig. S3A). GCs in DTx-treated CXCR5+/− chimeras contained similar or even slightly more CD4 T cells compared to untreated CXCR5−/− chimeras (Fig. 5, A to C, and fig. S3A).

We wondered whether CXCR5−/− CD4 T cells might be differentially localized in the GC compared to CXCR5+/+ CD4 T cells. TFH cells are known for preferentially localizing to the LZ of the GC where they exert their B helper functions and partake in the positive selection of affinity-matured B cells (28). Therefore, we analyzed the localization of CD4 T cells within the LZ and DZ of the GC (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 and fig. S3B). Localization of the LZ within the GC (PNA+ area) was determined by staining of follicular dendritic cells (FDCs) with a CD35/21 antibody. The PNA− area not containing FDCs was determined as DZ (Fig. 5D and fig. S3B). By determining the number of CD4+ cells contained per cubic micrometer 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. 5, D and E, and 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, because B cells are only responsive to TFH cell–mediated selection in the LZ of the GC ([11, 28]).

**CXCR5+/+ TFH cells drive adaptation of the neutralizing antibody response toward contemporary virus species**

So far, we had determined the serum IgG neutralization capacity against the inoculating virus. We wondered whether CXCR5+/+ TFH cells played a role in the adaptation and shaping of the antibody response toward contemporary adapting viral species. 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 to 20%) of the inoculating virus could be detected, with only a few chimeras depicting already a neutralization capacity of around 40% (Fig. 6A, top left). Except for two chimeras, s (20%) and u (15%), no neutralization of the contemporary virus isolates from d20 pi could be detected (Fig. 6A, top right). At d70 pi, sera of untreated chimeras and DTx-treated control chimeras showed robust neutralization capacity against the inoculum virus (between 40 and 60%), with just one exception (chimera m) (Fig. 6A, bottom left). In addition, at this time point of infection, neutralization of the contemporary isolates was in general lower compared with 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, bottom right). Sera of chimeras without CXCR5+/+ TFH−depleted chimeras could still neutralize the inoculum to a minor extent (10 to 20%), comparable to neutralization on d20 pi (Fig. 6A, left), but not the contemporary isolates at d70 pi (Fig. 6A, bottom right). 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, bottom). In general, the generation of neutralizing antibodies seemed to lag behind the evolution of circulating viral species, because sera isolated on d100 pi were able to neutralize the virus isolates from d70 pi far better, with neutralizing capacities ranging from 40 to 60% (fig. S4A). Last, robust neutralization of
Fig. 2. Specific depletion of TFH and LCMV-specific CD4 T cells. (A) Experimental approach. Lethally irradiated CD4−/− mice were reconstituted with CD4-DTR (CD45.2+) BM and CD45.1+ B6 (control chimeras), CXCR5−/− (CXCR5−/− chimeras), or M25 (M25 chimeras) BM and persistently infected with 2 × 10⁸ FFU LCMV clone 13. From d20 pi onward, continuous DTx treatment was initiated. (B) Longitudinal analysis of ratios of CD4-DTR–derived (CD45.2+) and CD45.1+ B6 (left), CXCR5−/− (CD45.1+; middle), or M25 (CD45.1+; right)-derived CD4 T cells in blood in untreated (upper) or DTx-treated (lower) 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 the spleen (left) and mLN (right) on d70 pi in DTx-treated chimeras. (D) Total CD4 T cells in the spleen d70 pi of untreated or DTx-treated control (black), CXCR5−/− (red), or M25 (blue) chimeras. (E) Flow cytometric analysis of TFH cells (CXCR5+ PD-1+) in the spleen on d70 pi in untreated or DTx-treated control (black), CXCR5−/− (red), or M25 (blue) chimeras. (F) Number of gp61-tetramer+ CD4 T cells in untreated or DTx-treated control (dark gray) or CXCR5−/− (red) chimeras at d100 pi in the spleen. (B, C, and F) One representative experiment of three is shown, n = 3 to 4 mice per group. Error bars represent means ± SD. (D and E) Pooled data of two independent experiments, n = 3 to 4 mice per group. Error bars represent means ± SEM. Statistical analysis was performed using Mann-Whitney U test, P > 0.05.
Fig. 3. CXCR5+/+ T FH cells are dispensable for maintenance of the LCMV-specific antibody response. (A) GC B cell (CD95+ CD38^high[/sup]IgM^low[/sup]) and memory B cells (CD38^high[/sup]IgM^low[/sup]) in the spleen as identified by gating on CD19^+ and switched IgD^−IgM^− B cells. Representative FACS blots of untreated (upper) and DTx-treated (lower) control (left), CXCR5^−/−[/sup] (middle), and M25 (right) chimeras are shown. Below quantifications of total cell counts are depicted. Data of three independently conducted experiments were pooled, n = 3 to 5 mice per group. (B) Number of LCMV-specific ASC as determined by ELISPOT in the spleen. (C) Number of LCMV-specific ASC as determined by ELISPOT in BM. One representative of two experiments is shown, n = 3 to 5 mice per group. (A to C) Error bars represent means ± SD. Mann-Whitney U test was used for statistical analysis. *P < 0.05. (D) Titers of LCMV-specific IgG in the sera of control, CXCR5^−/−[/sup], and M25 chimeras on d20 pi and (E) d100 pi in untreated (filled squares) and DTx-treated (empty squares) mice as determined by ELISA. Titers of LCMV-specific IgG were determined by ELISA analysis and further diluted in a threefold dilution series. Serum from naive C57BL/6 mice was used as negative control. (D to G) One representative experiment of two is shown, n = 3 to 5 mice per group. Error bars represent means ± SD.


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 was driven predominantly by neutralizing antibodies or by the CD8 T cell response. We used the different virus isolates derived from d70 pi blood to infect thioglycollate-induced peritoneal macrophages and used these infected cells for restimulation of CD8 T cells derived from mice acutely infected with the inoculum virus. No significant differences in the total CD8 T cell number producing interferon-γ (IFN-γ) (Fig. 6C) or tumor necrosis factor (TNF) (Fig. 6D) after 6 hours of stimulation with either the inoculum or any of the d70 isolates could be detected. This also held true for gp33-tetramer^+ CD8 T cells after 3 hours of stimulation (fig. S4, B to D). Therefore, we have no indication for cytotoxic T lymphocytes (CTLs) being a main driving force for viral adaptation during chronic LCMV infection.

The total number of activated (CD44^+[/sup]) CD8 T cells, as well as the total number of exhausted (PD-1^+[/sup]) CD8 T cells (fig. S5, A to C), was not affected by the absence of CXCR5^+[/sup] T FH or LCMV-specific CD4 T cells as compared to untreated or DTx-treated control chimeras. The production of effector cytokines IFN-γ (fig. S5D) and TNF (fig. S5E) after restimulation of CD8 T cells with gp33 peptide was also unaltered by the absence of CXCR5^+[/sup] T FH or LCMV-specific CD4 T cells. However, because of the exhausted state of the CD8 T cells, cytokine production was overall very modest (fig. S5, D and E). Because recent publications revealed the existence of a less exhausted CXCR5^+[/sup] CD8 T cell population localizing to the B cell follicle (29), we also analyzed the effect of DTx treatment in CXCR5^−/− or M25 chimeras on this population. However, we could not detect any effect of the absence of CXCR5^+[/sup] T FH or LCMV-specific CD4 T cells on the frequency and number of CXCR5^+[/sup] CD8 T cells in the spleen (fig. S5, F and G). In conclusion, clearance of persistent LCMV infection, as well as viral adaptation during persistent LCMV infection, is decisively driven by neutralizing antibodies whose robust generation is strictly dependent on continued activity of CXCR5^+[/sup] T FH cells.
CXCR5+/+ T FH cells are essential for the generation of LCMV-neutralizing antibodies and 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 to 5 mice per group. Error bars represent means ± SD. (B) Viral titers in the spleen of naïve C57BL/6 mice infected with clone 13 together with either naïve (gray, −DTx) or serum derived from untreated (−DTx), DTx-treated (+DTx) control, or CXCR5−/− chimeras at d4 pi. Representative data of two 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 the spleen at d100 pi in untreated and DTx-treated control, CXCR5−/−, and M25 chimeras. (C and D) Pooled data of three independent experiments, n = 3 to 5 mice per group. Mann-Whitney U test was used for statistical analysis, P > 0.05.

**DISCUSSION**

Although chronic LCMV infection prevails over several weeks and is associated with severe dysfunction of CD8 and T H 1 T cell immunity (1, 30), it is eventually controlled in most cases. This offers the opportunity to evaluate mechanistically how chronic viral infections can be naturally controlled in the absence of overt immunopathology. Because CD4 T cell responses are increasingly skewed toward T H 1 cells during chronic viral infections, it is tempting to speculate that emphasis on T FH cells might have physiological relevance.

We were able to demonstrate a pivotal role of continued activity of CXCR5+/+ T FH cells in supporting the late generation of LCMV-neutralizing antibodies not only against the inoculating virus but also against contemporary circulating viruses, enabling control of LCMV infection. Conditional ablation of CXCR5+/+ T FH cells or LCMV-specific CD4 T cells did not affect LCMV-specific CD8 T cell immunity, because 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.

CXCR5−/− CD4 T cells were previously shown to be able to take over some B helper functions, for instance, in acute influenza virus infection with constitutive T FH absence (31). This help might take place in the GC because even in the absence of CXCR5+/+ T FH cells, the GC response was, in accordance with previous publications (26, 32, 33), reduced but not absent. However, in the absence of LCMV-specific CD4 T cells, the GCs were devoid of CD4 T cells. We showed that 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 the absence of Bcl6-expressing CD4 T cells after establishment of initial LCMV-specific IgG titers, GC B cells were drastically reduced without measurable reduction 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.

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 the spleen and BM in absence of CXCR5+/+ CD4 T cells. However, they have an impaired ability in promoting
Fig. 5. GCs contain CXCR5−/− CD4 T cells in the absence of CXCR5+/+ T FH cells. (A) Immunofluorescence staining of splenic thin sections of untreated (upper) or DTx-treated (lower) control (left), CXCR5−/− (middle), and M25 (left) chimeras for GC B cells (PNA+, red), B cell follicles (IgD+, red), and CD4 T cells (yellow). Scale bars, 100 μm. (B) Ratio of CD4/PNA MFI and (C) quantification of CD4 T cells per square micrometer in the area of the GC. Twenty to 30 GCs in two independent experiments were analyzed in total, n = 3 to 4 mice per group. Error bars represent means ± SD. Mann-Whitney U test was used, P > 0.05.

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 recent findings during acute influenza virus infection (31), 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 more readily generated compared with LCMV-neutralizing antibodies and that the latter might therefore depend much more on continued activity of CXCR5+/+ T FH cells. In the absence of T FH cells during acute influenza virus infection, the protective antibody response was of low affinity, indicating that SHM and affinity selection might play an inferior role in creating a protective response. However, during chronic viral infections not only with LCMV but also with other noncytopathic viruses, antibodies may have to undergo several rounds of SHM and affinity selection to achieve neutralizing and protective capacity (19, 34). 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 (35–37).

Concomitant to the emergence of LCMV-neutralizing antibodies, the infecting virus may undergo rapid mutation to escape detection by these antibodies (38, 39). Occurrence of LCMV escape mutants evading detection by neutralizing antibodies has so far been mainly studied in systems with complete or partial CD8 T cell deficiency (38, 39). We show that neutralization of contemporary virus isolates is generally delayed compared with neutralization of the inoculum, even in presence of a normal CD8 T cell response, indicating that neutralizing antibodies are the main drivers of viral escape. Furthermore, because no
Fig. 6. TFH cells drive the adaptation of the neutralizing antibody response toward contemporary virus species. (A) Neutralization of sera isolated from single chimeras (indicated by letters) isolated at d20 (upper) or d70 pi (lower) against the inoculum (left) or the contemporary virus isolates (right). Untreated chimeras are marked in green, and DTx-treated chimeras are marked in orange. Lines indicate effective neutralization (>1.5 SD of control). One representative experiment of two is shown. (B) Correlation between neutralization against the inoculum (upper) or the contemporary isolate (lower) 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 from mice acutely infected with the inoculum and restimulated for 6 hours with macrophages infected with the inoculum (red) or contemporary virus isolates from d70 pi 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 cytokine expression by CD8 T cells stimulated by thio-macrophages infected with the inoculating virus. One representative experiment of two is shown. PMA, phorbol 12-myristate 13-acetate.
neutralization of contemporary isolates can be detected in the absence of CXCR5<sup>−/−</sup> T<sub>FH</sub> cells, these cells are essential in driving the adaptation of the humoral response toward the mutating virus. The T<sub>FH</sub>-driven appearance of LCMV-neutralizing antibodies leads to viral clearance of persistent LCMV infection, demonstrating that neutralizing antibodies are 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<sub>FH</sub> cells is a general requirement for effective control of chronic viral infections remains open, and the question how exactly T<sub>FH</sub> cells promote the emergence of neutralizing antibodies. Do T<sub>FH</sub> 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? In human HIV-1 infection, elevated frequencies of T<sub>FH</sub> cells were continuously identify B cells with the most virus-adapted antibodies? 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**

The aim of this study was to determine the long-term function of CXCR5<sup>−/−</sup> T<sub>FH</sub> cells and LCMV-specific CD4 T cells in the regulation of humoral immune responses toward persistent LCMV infection. We implemented an in vivo experimental mouse model, allowing conditional ablation of CXCR5<sup>−/−</sup> T<sub>FH</sub> cells and LCMV-specific CD4 T cells to study their impact on the GC response, formation of LCMV-specific ASC, overall LCMV-specific binding antibody responses, LCMV-neutralizing responses, and viral clearance and adaptation of the humoral immune response toward contemporaneous viral isolates. All experiments were performed at least twice. Every group consisted of at least three 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 before the primate DTR into exon 2 of one CD4 allele in C57BL/6 embryonic stem (ES) cells (construct design, production, ES cell targeting, selection, and generation of germline transmitters done by Ozgene). Gene-targeted mice were crossed to lck-Cre transgenic mice (22), leading to excision of the stop cassette during the CD4/CD8 double-positive stage of T cell development. Heterozygous CD4-DTR mice, in which only one CD4 allele is targeted by DTR, were consistently used in this study. CD4-DTR, CXCR5<sup>−/−</sup> (40), CD4<sup>−/−</sup> (41), M25 TCR transgenic (23), TCRβ<sup>−/−</sup> (42), and CD45.1<sup>+</sup> B6 mice were bred and maintained under specific pathogen-free conditions at the ETH Phenomics Center. All knockout and transgenic mouse strains were crossed to C57BL/6 background for >10 generations. B6/C57BL/6 × CD4-Cre mice were provided by J. Harker (London, Great Britain) and C57BL/6 mice by Janvier Labs.

BM chimeras were generated as previously described (43), and TCRβ<sup>−/−</sup>-mixed splenocyte chimeras were generated by adoptive transfer of splenocyte mixtures derived from each one-fourth donor spleen per recipient. BM chimeras were infected 8 weeks, and splenocyte chimeras were infected 1 week after reconstitution with 2 × 10<sup>6</sup> FFU LCMV clone 13 intravenously. Acute infection was induced with 200 FFU intravenous LCMV clone 13 infection. BM or splenocyte chimeras containing WT CD4 T cell compartment next to the CD4-DTR CD4 T cell compartment were used 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 experiment permission 147/2014).

**Virus and viral peptides**

The viral peptide gp33-41 (gp33; KAVYNFATM) was purchased from NeoMPS. Viral peptide (1 μg/ml) was used for restimulating gp33-specific CD8 T cells. LCMV clone 13 was propagated on baby hamster kidney 21 cells, and viral titers were determined as described previously (44). 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 (44).

**Depletion of CD4-DTR-derived cells**

CD4 T cells derived from CD4-DTR BM compartments were depleted by intraperitoneal administration of 250 ng per mouse DTx (Sigma-Aldrich) diluted in phosphate-buffered saline (PBS). For longitudinal depletion, DTx was injected every 3 to 4 days.

**Flow cytometry and lymphocyte stimulation**

All antibodies used for flow cytometry staining were purchased from BioLegend, eBioscience, and BD Biosciences. Surface stainings were performed at 4°C for 20 min, except for CD4 and CD83 (staining at room temperature for at least 30 min) after Fc block using 2.4G2 (rat anti-mouse CD16/CD32). Antigen peptide gp33 peptide (1 μg/ml) was used for restimulating splenocytes. CD4 T cells derived from CD4-DTR BM compartments were used as controls. All knockout and transgenic mouse strains were crossed to C57BL/6 background for >10 generations. B6/C57BL/6 × CD4-Cre mice were provided by J. Harker (London, Great Britain) and C57BL/6 mice by Janvier Labs.

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**Enzyme-linked immunosorbent assay**

Enzyme-linked immunosorbent assay (ELISA) was carried out in 96-well Nunc MaxiSorp Immunoplates (VWR International AG). For further information, see the Supplementary Materials.

**Enzyme-linked ImmunoSpot**

Enzyme-linked ImmunoSpot (ELISpot) assays were carried out in a 96-well MultiScreen-IP polyvinylidene difluoride Filter Plates (Millipore). For more information, see the Supplementary Materials.

**LCMV-neutralization assay**

Neutralizing capacities of sera isolated from infected mice were tested in a foci-reduction assay (44). Sera were heat-inactivated for 10 min and
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 times higher than the SD of the control condition.

**Immunohistochemistry and fluorescence microscopy**

Seven-micrometer thin sections were prepared from frozen spleens embedded in O.C.T. compound (Sakura). Further information about the staining is provided in the Supplementary Materials.

**Statistical analysis**

For statistical analysis, nonparametric 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**

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Materials and Methods

Fig. 51. Basic characterization of the CD4-CCR5 mouse line.

Fig. 52. Sustained activity of CXCR5 or Bcl6-expressing TFH cells is required for the development of LCMV-neutralizing antibodies and control of protracted infection.

Fig. 53. Immunofluorescence stainings in splenic thin sections.

Fig. 54. Viral escape is not driven by pressure of CTLs.

Fig. 55. CD8 T cell responses are unaffected by the absence of Fkh or LCMV-specific CD4 T cells.

Source data

**REFERENCES AND NOTES**


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Sustained T follicular helper cell response is essential for control of chronic viral infection
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Clearing chronic viral infections
Although chronic lymphocytic choriomeningitis virus (LCMV) infection in mice persists for several months and is accompanied by exhaustion of CD8+ T cells, eventually the infection is controlled in most cases. Here, Greczmiel et al. have examined how these chronically infected mice clear LCMV. By developing mouse models to selectively deplete CD4+ T follicular helper cells (TFH), the authors found these TFH cells to be vital for the generation of neutralizing antiviral antibodies that promote viral clearance. The study underscores the importance of TFH cells in driving B cell responses in the context of CD8+ T cell exhaustion.