T-cell STAT3 is required for the maintenance of humoral immunity to LCMV

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STAT3 is a critical transcription factor activated downstream of cytokine signaling and is integral for the function of multiple immune cell types. Human mutations in STAT3 cause primary immunodeficiency resulting in impaired control of a variety of infections, including reactivation of latent viruses. In this study, we investigate how T-cell functions of STAT3 contribute to responses to viral infection by inducing chronic lymphocytic choriomeningitis virus (LCMV) infection in mice lacking STAT3 specifically in T cells. Although mice with conditional disruption of STAT3 in T cells were able to mount early responses to viral infection similar to control animals, including expansion of effector T cells, we found generation of T-follicular helper (Tfh) cells to be impaired. As a result, STAT3 T cell deficient mice produced attenuated germinal center reactions, and did not accumulate bone marrow virus specific IgG-secreting cells, resulting in failure to maintain levels of virus-specific IgG or mount neutralizing responses to LCMV in the serum. These effects were associated with reduced control of viral replication and prolonged infection. Our results demonstrate the importance of STAT3 in T cells for the generation of functional long-term humoral immunity to viral infections.

Keywords: Chronic infection · Humoral immunity · LCMV · STAT3 · T follicular helper cells

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Introduction

JAK-STAT signaling is a major cytokine effector pathway critical for the development and function of different immune cell types [1, 2]. In humans, mutations in STAT3 cause autosomal dominant hyper IgE syndrome (AD-HIES), a disorder with immunological manifestations including pneumonia, skin infections, and susceptibility to *Staphylococcus aureus* and mucocutaneous candidiasis [3, 4]. In addition to misregulation of IgE, AD-HIES syndrome patients also have impaired long-term IgG production following immunization [5–7].

Recent reports indicate that STAT3 mutations are further associated with reactivation of Epstein-Barr virus and Varicella zoster virus, suggesting that long term control of viruses may require STAT3 [8, 9]. However, a precise mechanistic understanding of viral responses in the absence of STAT3 is lacking.

STAT3 deficiency in humans is associated with reduced quantities of CD8$^+$ memory T cells [8, 10]. Although memory CD8$^+$ T-cell defects have also been observed in mice with CD8$^+$ T-cell-deletion of STAT3 (GzB-cre$^+$; Stat3$^{fl/fl}$), effector function and ability to control primary acute lymphocytic choriomeningitis virus (LCMV) infection is preserved [11].

Antigen experienced CD4$^+$ T follicular helper (Tfh) cells seed germinal center reactions during infection. Germinal center reactions promote differentiation of follicular B cells into antibody producing long-lived plasma cells and memory B cells [12, 13]. STAT3 is an important mediator of signaling for cytokines involved in the generation of Tfh cells including IL-6 and IL-21 [12, 13]. CD4-Cre conditional STAT3 knockout mice were recently examined following acute LCMV infection, revealing Tfh cell defects resulting in limited germinal center reactions [14]. However, these effects have not yet been examined in a chronic model of LCMV infection. Importantly, AD-HIES patients are also known to have reduced quantities of circulating Tfh cells [3, 15, 16].

IL-21 signaling via STAT3 in germinal centers is important for generation of plasma cells. LCMV infection of IL-21 deficient mice revealed a failure to maintain long lived virus specific IgG plasma cells in the bone marrow, effects which appear to be both T-cell and B-cell-dependent [17]. These data support a model whereby STAT3 is involved in both differentiation of Tfh cells to produce IL-21, and subsequent IL-21 signaling via STAT3 in B cells promotes differentiation into plasmablasts [3, 14, 18]. The direct role of T-cell STAT3 in maintenance of virus specific IgG-producing plasma cells during chronic infection has not yet been reported.

In order to better understand STAT3 function in T cells during viral infection, we chose to examine mice lacking T-cell STAT3 infected with chronic LCMV. Whereas initial control of viral infection including generation of virus specific T cells and production of virus specific antibodies was largely normal in the absence of STAT3, reduced quantities of Tfh cells were present. At later time points, STAT3 deficiency resulted greatly diminished accumulation of virus-specific IgG-producing cells in the bone marrow, and an inability to produce LCMV neutralizing antibodies or maintain serum levels of virus-specific IgG. These defects were associated with impaired long-term control of LCMV infection and reduced survival.

Results

STAT3 is dispensable for generation of virus-specific T cells but necessary for Tfh cells

STAT3 is critically required for development as homozygous *Stat3* deficient mice arrest early during embryogenesis [19]. In an attempt to understand the role of STAT3 in T cells we investigated a model employing transgenic *Lck-Cre* to conditionally inactivate *Stat3* across all T-cell lineages [20, 21]. *Lck-Cre$^-$ Stat3$^{fl/fl}$* (referred to as WT in figures for simplicity) mice were indistinguishable from *Lck-Cre$^+$* Stat3$^{fl/fl}$ (also referred to as *Stat3$^{-/-}$*) littermate controls in terms of appearance and long-term survival (Supporting Information Fig. 1A). We investigated basal levels of B cells, T cells and NK cells in the spleen of these mice, finding no major defects in cell population numbers associated with presence or absence of STAT3 (Fig. 1A). Similarly, thymic T-cell populations were largely equivalent for *Lck-Cre$^+$* Stat3$^{fl/fl}$ and *Lck-Cre$^-$* Stat3$^{fl/fl}$ mice indicating grossly normal T-cell development (Supporting Information Fig. 1B, C; also see general flow cytometry gating strategy in Supporting Information Fig. 2).

In order to better understand the function of STAT3 in T cells during antiviral immunity, we chose to infect mice with the LCMV-Docile strain which can result in a chronic course of infection [22, 23]. Control of LCMV infection relies on expansion of virus specific T cell populations. Therefore we first examined the ability of isolated T cells from *Lck-Cre$^+$* Stat3$^{fl/fl}$ and *Lck-Cre$^-$* Stat3$^{fl/fl}$ mice to respond to non-specific proliferative signals. CD8$^+$ and CD4$^+$ T cells were isolated from spleens of uninfected Stat3$^{fl/fl}$ *Lck-Cre$^+$* Stat3$^{fl/fl}$ and *Lck-Cre$^-$* Stat3$^{fl/fl}$ mice and cultured for 72 h in the presence of anti-CD3 and anti-CD28 activating antibodies. CFSE labeling revealed that both control and STAT3 deficient CD8$^+$ T cells proliferated to the same extent (Fig. 1B). Similar results were found when examining proliferative capacity of CD4$^+$ T cells (Fig. 1C). Next, we examined T-cell responses between 12 and 65 days following infection of mice with 2×10^3 PFU of LCMV-Docile. Following infection, comparably large numbers of virus specific CD8$^+$ T cells recognizing the LCMV glycoprotein gp33 were evident in splenocytes isolated from both *Lck-Cre$^+$* Stat3$^{fl/fl}$ and *Lck-Cre$^-$* Stat3$^{fl/fl}$ animals (Fig. 1D). The ability of CD8$^+$ and CD4$^+$ T cells to be activated in response to LCMV peptides was tested by measuring interferon gamma (IFN-γ) production following re-stimulation with gp-33 and gp-61 peptides. Similar IFN-γ responses were detected following re-stimulation of both CD8$^+$ and CD4$^+$ T cells from infected *Lck-Cre$^+$* Stat3$^{fl/fl}$ and *Lck-Cre$^-$* Stat3$^{fl/fl}$ mice (Fig. 1E, F). As expected, we observed an overall decline in the quantity of virus specific cells and re-stimulated IFN-γ$^+$ CD8$^+$ and CD4$^+$ T cells between 12 and 65 days post-infection in both the presence or absence of STAT3 (Fig. 1D–F).
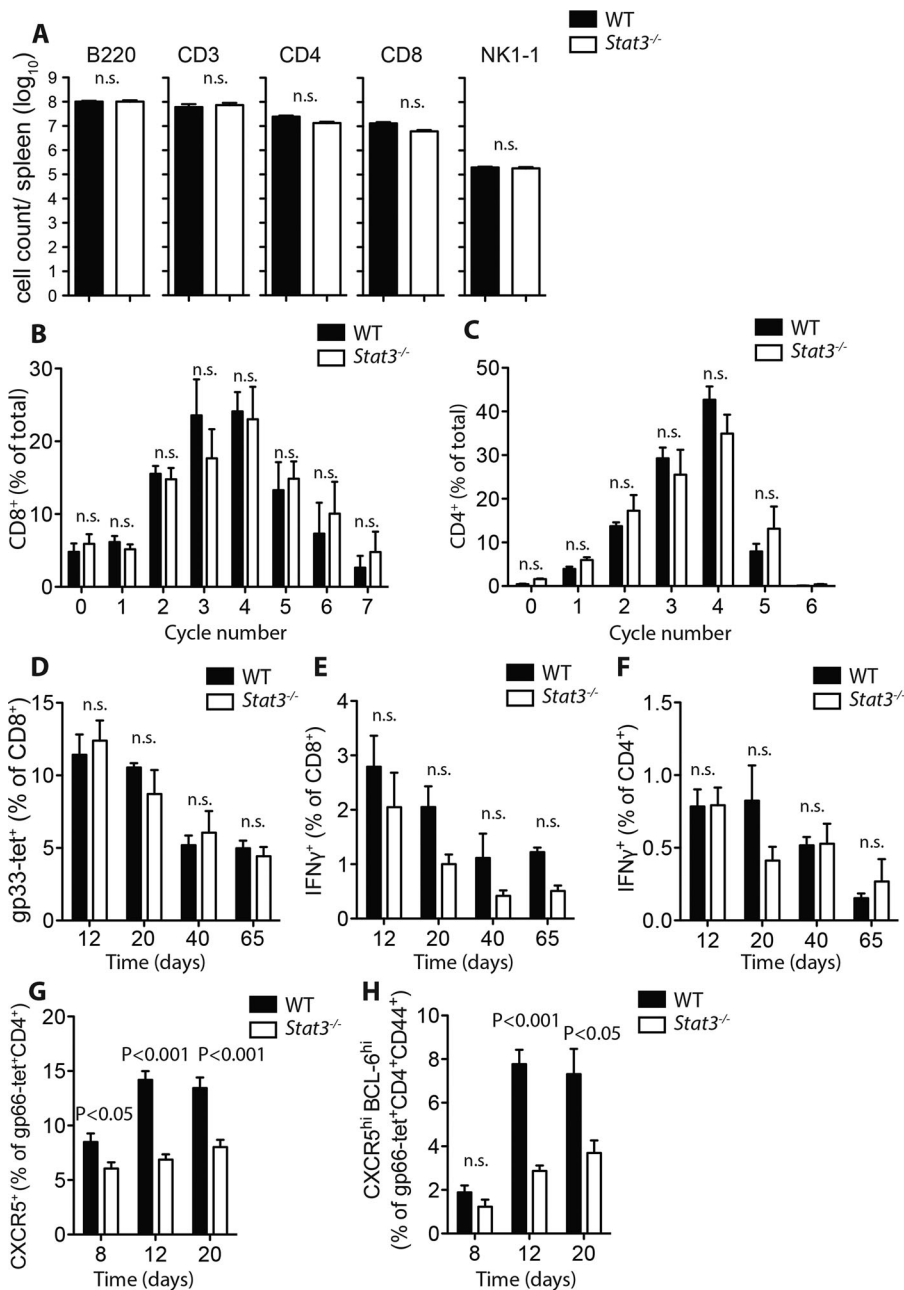
Figure 1. Generation of LCMV-specific Tfh cells requires STAT3. (A) Quantity of B220^{+}, CD3^{+}, CD4^{+}, CD8^{+}, and NK1-1^{+} cells were compared in naive spleens from *Lck-Cre⁻ Stat3^{fl/fl}* (WT) vs. *Lck-Cre⁺ Stat3^{fl/fl}* (*Stat3^{−/−}*) mice. (B–C) Quantification of CFSE dilution indicative of proliferation for *Lck-Cre⁻ Stat3^{fl/fl}* vs. *Lck-Cre⁺ Stat3^{fl/fl}* (B) CD8^{+} T cells or (C) CD4^{+} T cells (both pregated on CD3^{+}) in vitro. Percentage of cells in each division cycle for negatively selected, CFSE labeled *Lck-Cre⁻ Stat3^{fl/fl}* vs. *Lck-Cre⁺ Stat3^{fl/fl}* T cells stimulated with 5 μg/mL anti-CD3 and 2 μg/mL anti-CD28 for 72 h as measured by flow cytometry. (D–H) *Lck-Cre⁻ Stat3^{fl/fl}* vs. *Lck-Cre⁺ Stat3^{fl/fl}* mice were infected with 2×10^3 PFU LCMV-Docile. (D) Quantity of gp33 tetramer-specific CD8^{+} T cells in the spleen at days 12, 20, 40, and 65 after infection was measured (% of total CD8^{+} T cells). (E) Quantity of IFN-γ-producing CD8^{+} T cells present at days 12, 20, 40, and 65 after infection following in vitro restimulation with virus specific peptide gp33. (F) Quantity of IFN-γ-producing CD4^{+} T cells present at days 8, 12, 20, 40, and 65 after infection following in vitro restimulation with virus specific peptide gp61. (G) Quantity of virus-specific CXCR5^{+} cells in spleen at days 8, 12, and 20 postinfection was measured (% of gp66-tet^{+}CD4^{+} T cells). (H) Quantification of CXCR5^{hi}BCL-6^{hi} virus-specific cells in spleen at days 12 and 20 postinfection (% of gp66-tet^{+}CD44^{+}CD4^{+} T cells). (A–H) Data are shown as mean ± SEM from (A) $n = 5–6$ mice/group, (B and C) $n = 7$ mice/group, (D–F) $n = 4–7$ mice/group, (G) $n = 6$ mice/group, (H) $n = 3–4$ mice/group. Data are pooled from two independent experiments (A–C), or pooled from one or two independent experiments per time point (D–H). Statistical significance between groups was determined by Student's *t*-test.

These results indicate that in this setting, basal immune cell populations and the ability of the majority of T cells to be activated in response to chronic LCMV-Docile infection are not affected by the absence of STAT3. These results are also consistent with normal initial responses to acute infection with the LCMV-Armstrong strain (prior to the emergence of memory CD8^{+} defects) seen in CD8^{+} Stat3 conditional mice knockout mice (Granzyme B Cre⁺ Stat3^{fl/fl}) [11]. However, similar to recent observations involving deletion of STAT3 in T cells [14], we observed reduced C-X-C chemokine receptor type 5 (CXCR5^{+}) and BCL6^{+} virus specific CD4^{+} T cells in infected *Lck-Cre⁺* Stat3^{fl/fl} vs. *Lck-Cre⁻* Stat3^{fl/fl} mice, indicating diminished Tfh populations at days 12 and 20 postinfection (Fig. 1G and H).

STAT3 in T cells is necessary for sustained humoral immunity to LCMV

Since Tfh cells support germinal center reactions [13, 24, 25], we next examined the status of B-cell immunity to chronic LCMV in the presence or absence of STAT3. Total B-cell numbers in the spleen were similar across both types of animals (Fig. 2A), as were proliferative responses of B cells to LPS or CD40-L (Supporting Information Fig. 3A, B), indicating that T cell specific deletion of STAT3 did not result in gross B-cell defects. Germinal center reactions were strongly induced in both genotypes, with similar levels of germinal center B cells present at day 12 following infection (Fig. 2B). However, compared to controls, the quantity of
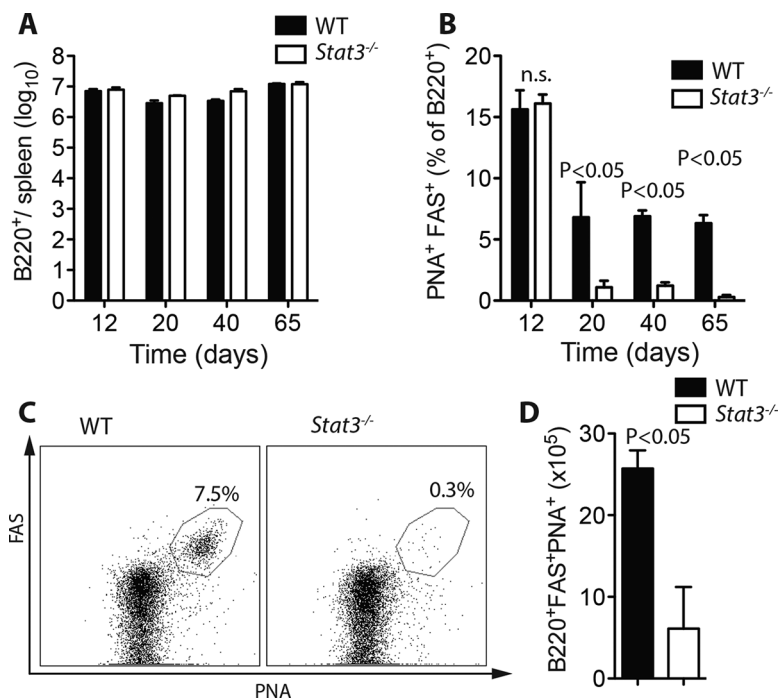
Figure 2. T-Cell STAT3 is required for the maintenance of germinal center B-cell reactions toward LCMV. (A–D) *Lck-Cre⁻ Stat3*$^{fl/fl}$ vs. *Lck-Cre⁺ Stat3*$^{fl/fl}$ mice were infected with 2×10^3 PFU LCMV-Docile. (A) Total quantity of (B220⁺) B cells in the spleen on days 12, 20, 40, and 65 postinfection was measured by flow cytometry (mean ± SEM; n = 4–7 mice/group). (B–D) GC B cells (B220⁺PNA⁺FAS⁺) were measured on days 12, 20, 40, and 65 postinfection (n = 4–7 mice/group). (B) Quantity of B220⁺PNA⁺FAS⁺ cells as a percent of total B cells (B220⁺) at each time point (mean ± SEM), (C) representative dot plot from day 65 (gated on B220⁺ cells), and (D) numerical quantification of B220⁺PNA⁺FAS⁺ cells in each group at day 65 (mean ± SEM). Data are pooled from —one or two independent experiments per time point. Statistical significance between groups was determined by Student's *t*-test.

germinal center B cells detectable in STAT3 deficient animals was markedly lower on days 20, 40, and 65 postinfection, indicating that normal germinal center reactions are not sustained in the absence of T cell STAT3 (Fig. 2B–D).

We next examined the kinetics of LCMV-GP specific IgG production in the serum by ELISA. *Lck-Cre⁺* Stat3$^{fl/fl}$ and *Lck-Cre⁻* Stat3$^{fl/fl}$ mice had similar production of virus-specific IgG up until 20 days postinfection, however, while LCMV-GP-IgG titers continued to increase in control animals, titers progressively declined in the absence of STAT3, evident at time points up until 250 days postinfection (Fig. 3A). STAT3 associated impairment in LCMV-specific antibody production was not evident for virus specific serum IgM (Fig. 3B), suggesting that preclass switch B cell functions remained intact in T-cell STAT3 deficient animals. Serum antibodies with the capacity to neutralize LCMV appear with delayed kinetics relative to virus specific IgG in wild-type mice [26]. Consistently, we observed emergence of robust LCMV neutralization capacity in serum from infected control mice at day 65, which was maintained until at least day 250 (Fig. 3C). In sharp contrast, neutralizing capacity of serum from STAT3 deficient animals was nearly undetectable across all time points (Fig. 3C). Sustained IgG production is facilitated by long-lived IgG-producing plasma cells typically residing in the bone marrow [27]. Therefore, we measured the quantity of LCMV-specific IgG antibody-secreting cells (ASCs) present in both the spleen and bone marrow between zero and 65 days postinfection. Quantities of LCMV IgG ASCs appeared highest in the spleen at 12 days postinfection and were not significantly different between *Lck-Cre⁺* Stat3$^{fl/fl}$ and *Lck-Cre⁻* Stat3$^{fl/fl}$ mice at any of the time points tested (Fig. 3D). In contrast, STAT3 deficient mice possessed only minimal quantities of LCMV-specific IgG ASCs in the bone marrow compared to control mice (Fig. 3E), consistent with the inability to

sustain high serum titers of LCMV-specific IgG at later time points after infection. Taken together these data indicate that while early B-cell responses to chronic LCMV infection appear unaffected, absence of T-cell STAT3 is associated with reduced maintenance of germinal centers, failure to sustain serum virus-specific IgG levels or gain neutralization capacity and reduced accumulation of long lived virus specific IgG-producing cells in the bone marrow.

STAT3 T-cell deficiency results in impaired control of chronic LCMV

Cytotoxic T-cell responses toward LCMV are well described, yet viral persistence occurs in mice with impaired Tfh responses and impaired virus-specific antibody formation, indicating that humoral immunity is also important to control LCMV infection [14, 26, 28]. We therefore examined whether defects observed in Tfh cells and virus specific serum IgG production in the absence of STAT3 would be associated with reduced control of LCMV replication. Viral titers were measured in spleen, liver, lung, kidney, and brain of *Lck-Cre⁺* Stat3$^{fl/fl}$ and *Lck-Cre⁻* Stat3$^{fl/fl}$ mice between 12 and 65 days postinfection. Virus was detected in all organs and was present in similar quantities in samples from control and STAT3 deficient mice at days 12 and 20 following infection (Fig. 4A–E). However, by day 65 while many control mice had successfully cleared the infection, viral titers remained significantly elevated in organs from STAT3 deficient animals (Fig. 4A–E). Particularly large differences between *Lck-Cre⁺ Stat3*$^{fl/fl}$ and *Lck-Cre⁻ Stat3*$^{fl/fl}$ mice were evident in the liver (Fig. 4B). Liver sections from these mice harvested at 65 days postinfection showed evidence of lymphocyte infiltration by hematoxylin and eosin staining in both settings, but strong LCMV-NP staining only in STAT3 deficient livers (Fig. 4F). Taken together, viral replication appears to occur at
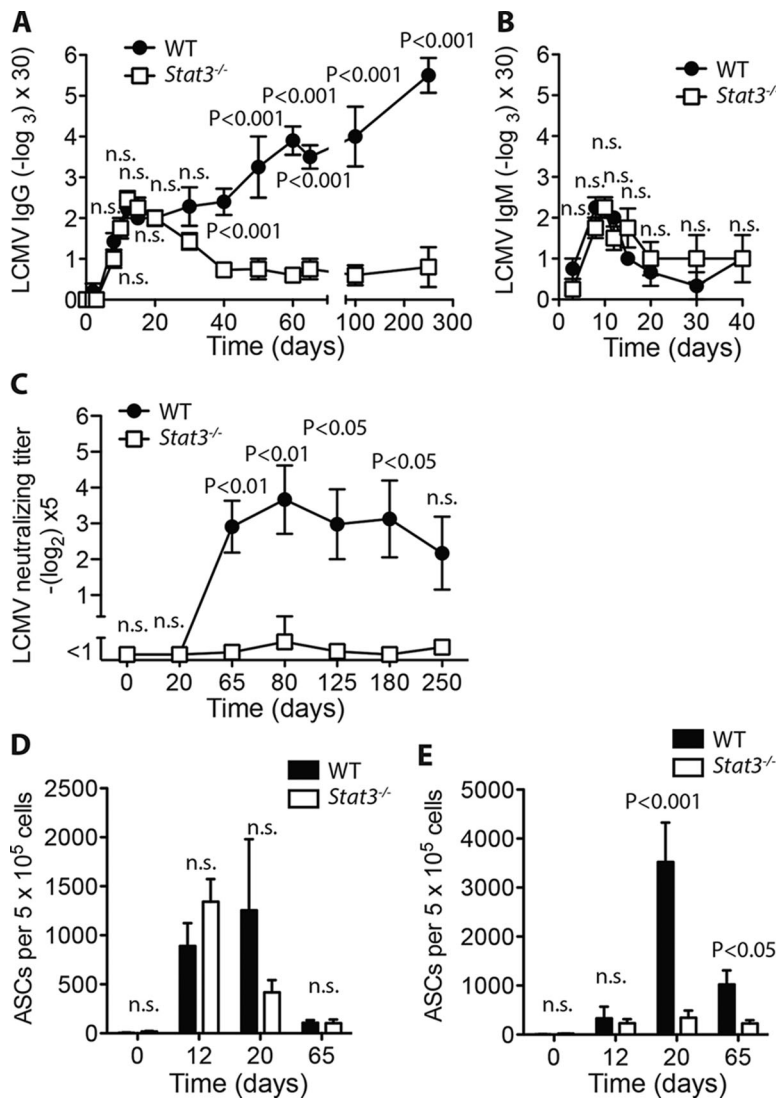
Figure 3. T-cell STAT3 is necessary for humoral immunity to chronic LCMV. (A–E) *Lck-Cre⁻ Stat3*^fl/fl vs. *Lck-Cre⁺ Stat3*^fl/fl mice were infected with 2×10^3 pfu LCMV-Docile. (A) LCMV-specific IgG antibodies in serum were measured by serial sampling and ELISA on days 0, 2, 3, 8, 10, 12, 15, 20, 30, 40, 50, 60, 65, 100, and 250 postinfection (mean ± SEM; n = 4–15 mice/group (B) LCMV-specific IgM antibodies in serum were measured by serial sampling and ELISA at days 3, 8, 10, 12, 15, 20, 30, and 40 postinfection (mean ± SEM, n = 3–4 mice/group). (C) LCMV- neutralizing antibodies were measured in the serum after serial sampling on days 0, 20, 65, 80, 125, 180, 250 postinfection (mean ±SEM; n = 4–10 mice/group). (D–E) LCMV-specific ASC in (D) spleen or (E) bone marrow were counted on day 0, 12, 20, and 65 postinfection by ELISPOT (mean ± SEM; n = 4–7 mice/group). Data shown are pooled from (A) three independent experiments, (B and C) two independent experiments, or (D–E) —one or two experiments per time-point. Statistical significance between groups was determined by Student's *t*-test, significance between multiple time points, was determined by two-way ANOVA with Bonferroni posttest.

normal levels in the initial phases following LCMV infection where CTL and early B-cell responses are also not significantly different between control and STAT3 deficient mice, however virus persists at later time points in STAT3 deficient animals coinciding with declining virus specific IgG levels.

Prolonged infections of noncytolytic viruses can lead to toxic immunopathology [29–31]. When we tested long-term survival following LCMV infection we found that STAT3 deficiency was associated with significantly earlier demise (Fig. 5). Thus impaired Tfh function, reduced humoral immunity, and diminished control of viral replication appears to reduce long-term survival of *Lck-Cre⁺ Stat3*^fl/fl vs. *Lck-Cre⁻ Stat3*^fl/fl mice following chronic LCMV infection.

Discussion

In the setting examined here, chronic infection with LCMV revealed STAT3 to be dispensable for expansion and IFN–γ production by virus specific CD4⁺ and CD8⁺ T cells (Fig. 1D–F). Mice

lacking IL-21, a critical activator of STAT3, also generate normal levels of virus specific cytokine producing CD4⁺ T cells [17]. Furthermore, studies examining early time points after acute LCMV infection using mice with individual conditional deletion of STAT3 in either CD4⁺ [14] or CD8⁺ cells (Granzyme B-Cre) [11] corroborate that, although memory CD8⁺ T-cell responses are perturbed, virus specific CD4⁺ and CD8⁺ effector cells are generated in the absence of STAT3 signaling.

Despite similar overall quantities of T cells responding to virus in both genotypes, in the absence of STAT3 we observed a specific decrease in CXCR5⁺ Tfh cells during chronic LCMV infection, which are necessary to prime germinal center B cells (Fig. 1G and H). These results are similar in magnitude to decreased Tfh cell populations recently observed during acute LCMV infection of *CD4-Cre⁺ Stat3*^fl/fl mice [14]. Results are further consistent with previous work showing the importance of STAT3 in Tfh-cell function during acute LCMV infection in T cell transfer models [32], or following KLH antigen immunization [33]. Importantly, humans with STAT3 mutations also have reduced CD4⁺ CXCR5⁺ Tfh cells
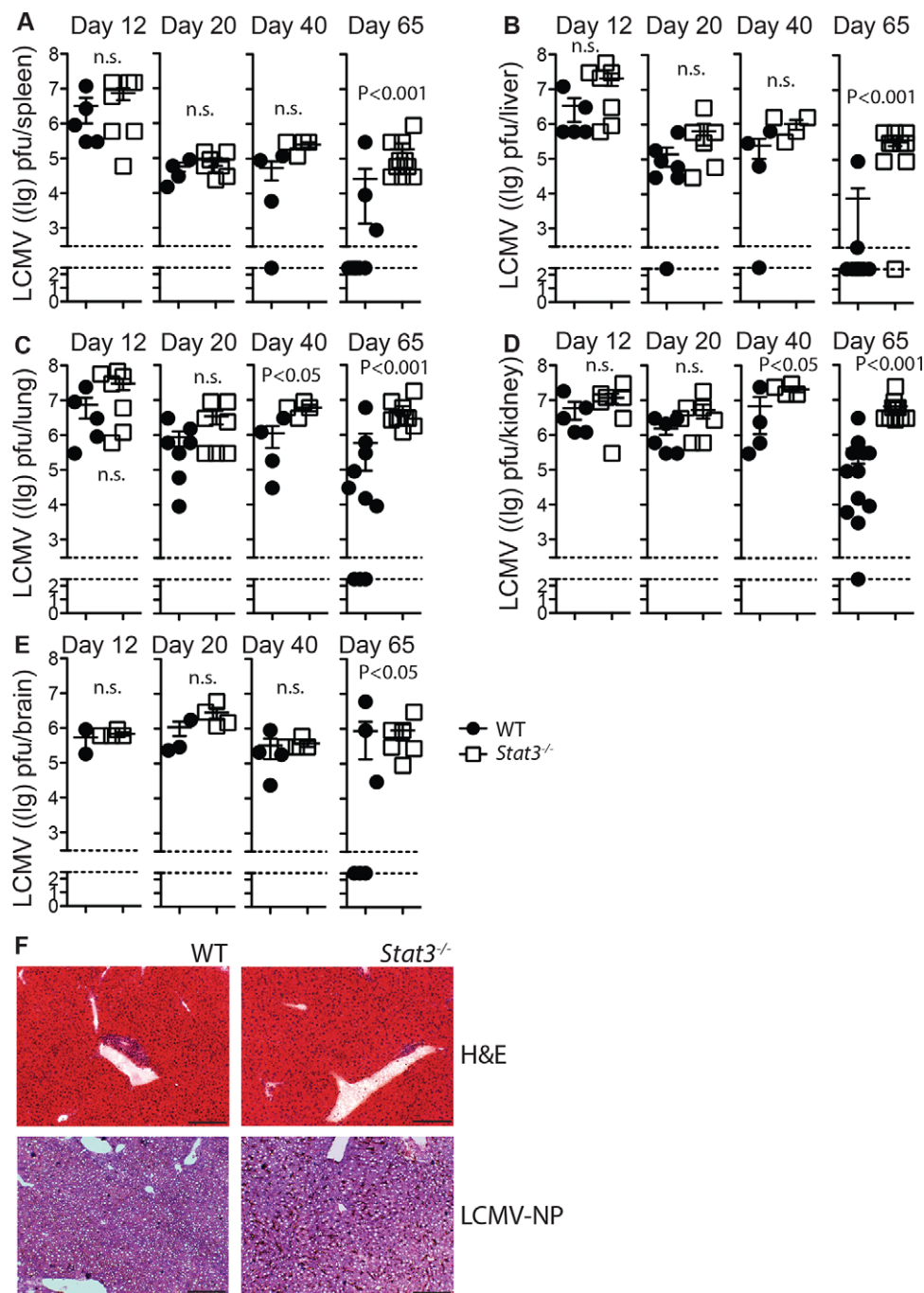
Figure 4. Long-term control of LCMV replication requires STAT3 in T cells. (A–F) *Lck-Cre⁻ Stat3*$^{fl/fl}$ (closed circles) vs. *Lck-Cre⁺ Stat3*$^{fl/fl}$ (open squares) mice were infected with 2×10^3 pfu LCMV-Docile. On day 12, 20, 40, and 65 postinfection virus titer were determined in (A) spleen, (B) liver, (C) lung, (D) kidney, and (E) brain tissue (symbols represent individual mice, bars show mean ± SEM, $n = 4$–12 mice/group, data shown are pooled from one or two independent experiments per time point). (F) Snap-frozen liver sections from *Lck-Cre⁻ Stat3*$^{fl/fl}$ and *Lck-Cre⁺ Stat3*$^{fl/fl}$ animals were stained with hematoxylin and eosin (upper panels), or immunostained for LCMV-NP with hematoxylin counterstaining (lower panels). One representative image from $n = 4$ mice/group from a single experiment is shown (10×, scale bar, 200 µm). Statistical significance between groups was determined by Student's *t*-test.

in vivo and defects in the ability of CD4⁺ T cells to help B cells in vitro [3, 15, 16].

Germinal center B cells were present at similar levels to control early after infection, however these declined more quickly in STAT3 deficiency (Fig. 2B–D). Thus Tfh cells generated in

response to LCMV in STAT3 deficient animals are able to seed, but not maintain germinal center reactions in the spleen. STAT3 deficiency was associated with reduced quantities of Tfh cells relative to controls, with differences more apparent at later stages after infection (Fig. 1G). Interestingly, although the magnitude
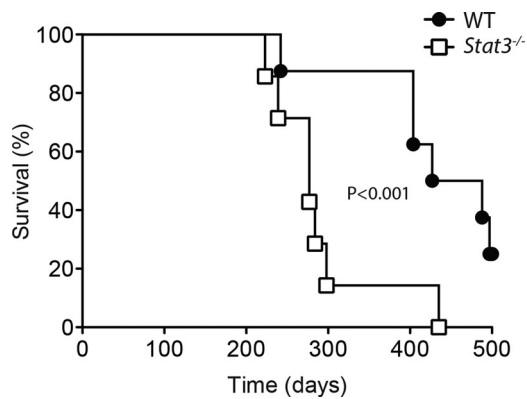
Figure 5. Reduced longevity of chronically infected STAT3 T-cell-deficient mice. *Lck-Cre⁻ Stat3*^fl/fl vs. *Lck-Cre⁺ Stat3*^fl/fl mice were infected with 2×10^3 PFU LCMV-Docile. Survival of infected mice was monitored over time ($n = 7$–8 mice/group). Data shown are pooled from two independent experiments. Statistical significance was determined by Kaplan–Meier log rank test.

of the observed Tfh defect appeared similar between 12 and 20 days postinfection, the quantity of germinal center B cells dropped dramatically in STAT3 deficient mice during this same time frame (Figs. 1G–H and 2B–D). While Tfh cells in STAT3 deficient mice may not have reached sufficient numbers to trigger maintenance of germinal center B-cell reactions, this data raises an interesting alternative possibility for future investigation— that following infection, the functional capacity of Tfh cells may be impaired and progressively decline in the absence of STAT3. The kinetics of virus specific IgG production and germinal center involution in *Lck-Cre⁺ Stat3*^fl/fl mice are also strikingly similar to those reported for mice lacking IL-21, suggesting that T cell STAT3 is a critical mediator of IL-21 in supporting germinal center reactions and long lived antiviral IgG production [17].

Mice with deletion of STAT3 in CD8⁺ T cells are able to control viral replication during primary LCMV infection [11]. Interestingly we find that mice lacking STAT3 throughout the T-cell compartment also have similar viral load to control animals at early time points postinfection, but fail to control viral replication at later stages (Fig. 4A–E). Reduced ability to control chronic LCMV at later time points coincided with a decline in virus specific serum IgG from control levels at day 20, to dramatically lower levels by day 65 postinfection in STAT3 deficient animals (Figs. 3A, and 4A–E). These kinetics may be further explained by the emergence of serum antibodies with strong capacity to neutralize LCMV by day 65 postinfection in control mice, whereas neutralizing antibodies were nearly undetectable in STAT3 deficient mice for the duration of the observed period (Fig. 3C).

Ray, et al. also recently observed decreased serum virus-specific IgG in the absence of STAT3 in T cells [14]. In addition to these findings, here we identify T-cell-specific expression of STAT3 to be important for the elimination of LCMV. Virus specific antibodies are known to be necessary to control persistent LCMV infection [26] and chronic infection can result in the demise of the host [30, 34]. Consistently, we observed reduced long term survival

of infected *Lck-Cre⁺ Stat3*^fl/fl mice compared to *Lck-Cre⁻ Stat3*^fl/fl controls (Fig. 5).

Maintenance of long-term IgG responses to LCMV depend on accumulation of plasma cells in the bone marrow, independent of memory B cells [27]. We found significantly lower quantities of LCMV specific ASCs appearing in the bone marrow during STAT3 deficiency at later time points postinfection, coincident with declining serum anti-LCMV IgG (Fig. 3E). Similar effects and kinetics have been observed in the absence of IL-21 [17]. Thus long-lived plasma cell maintenance and/or migration to the bone marrow appears to depend on both STAT3 production in T cells and IL-21 during LCMV infection.

Our observations support a model whereby limited IgG production in immunized AD-HIES patients reflects a failure to maintain long-lived plasma cells in the bone marrow [5–7]. In patients with inherited systemic mutations in STAT3, this could be a consequence of Tfh cell intrinsic defects, B-cell-intrinsic defects, or a combination of both. Nevertheless, our results demonstrate that absence of STAT3 in T cells is sufficient to prevent normal Tfh-cell development, block accumulation of long lived virus-specific antibody secreting cells in the bone marrow, and prevent maintenance of antiviral serum IgG.

Interestingly, in a report by Sheerin et al., primary antibody response to immunization in some AD-HIES patients was normal at early time points, but later declined below physiological levels [5], mimicking results seen in our mice. Similar defects to those observed in our mouse model may contribute to re-emergence of latent viral infections in humans with STAT3 mutations.

Materials and methods

Mice, viruses, virus titer

Mice used in this study were maintained on the C57BL/6-J genetic background. *Stat3*^fl/fl conditional knockout mice [20] and *Lck-Cre* transgenic mice [21] have been previously described. LCMV DOCILE (provided by R. M. Zinkernagel, University of Zurich) was propagated in L929 cells as previously described [35]. Virus titers were measured using a plaque forming assay also as previously described [36]. Mice were infected intravenously with 2×10^3 plaque forming units (PFU) of LCMV DOCILE. All experiments were performed in single ventilated cages. Animal experiments were carried out in accordance with the guidelines of the Ontario Cancer Institute and the German law for animal protection.

Purification of T cells and B cells

Total T-cell populations were purified from single cell suspended splenocytes by negative selection, and B220⁺-cell populations were purified from single cell suspended splenocytes by positive selection, both following the manufacturer's instructions (mouse

In vitro B-cell proliferation

Isolated B cells were labeled with 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen, Carlsbad, CA, USA) for 10 min at 37°C. Labeled B cells were washed two times with RPMI 1640 culture medium (Biochrom, Berlin, Germany) with supplements (10% FCS, Biochrom, Berlin, Germany). B cells were cultured in 96-well flat-bottom tissue culture plates with either 100 ng/mL LPS or 10 ng/mL Il-4 (R&D Systems, Minneapolis, MN, USA) and 2 μg/mL CD40-L (2×10^5 cells/well). Cells were cultured at 37°C in humidified 5% CO_2 incubator for 72 h prior to analysis by flow cytometry.

In vitro T-cell proliferation

Isolated CFSE-labeled T cells were cultured in 96 well flat-bottom tissue culture plates precoated with 5 μg/mL anti-CD3 (eBioscience, San Diego, CA, USA) and 2 μg/mL soluble anti-CD28 (BD Pharmingen, Franklin Lakes, New Jersey, USA) (2×10^5 cells/well). Cells were cultured at 37°C in humidified 5% CO_2 incubator for 72 h prior to analysis by flow cytometry.

Flow cytometry analysis

gp33-H-2D[b] and np396-H-2D[b] tetramer production, surface, and intracellular staining, and analysis were performed as previously described [35]. Intracellular staining for IFN-γ was performed on single cell suspended splenocytes incubated with LCMV specific peptides (gp33 or gp61) for 1 h at 37°C followed by addition of Brefeldin A (eBioscience, San Diego, CA, USA) and subsequent incubation for 5 h at 37°C. After surface staining, cells were fixed with 2% formalin and permeabilized with 0.1% Saponin (Sigma-Aldrich, St. Louis, MO, USA) prior to staining with intracellular antibodies. Gp66-I-A(b) tetramer staining was performed for 30 min at 37°C prior to surface antibody staining at 4°C. Anti-CD3, anti-CD8, anti-IFN-γ, anti-CD4, anti-B220, anti-NK1.1, anti-CD25, anti-CD44, and anti-CD95 were obtained from eBioscience, San Diego, CA, USA. Anti-CXCR5 was obtained from BD Bioscience. PNA was procured from Vector Laboratories, Burlingame, CA, USA.

LCMV antibody Elisa

Analysis of LCMV glycoprotein specific antibodies was performed as previously described [37]. Briefly, plates were coated overnight with anti-human Fc (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), blocked for 120 min with PBS + 2%BSA at RT and incubated with 100 μL/well Gp1-Fc supernatant overnight at

4°C. Sera were serial diluted on the plate in PBS + 2% BSA and incubated for 90 min at RT. Plates were washed with PBS + 0.1% Tween-20 (PBS-T) and diluted antimouse IgG (γ-chain specific)-HRP or anti-mouse IgM-HRP (Sigma-Aldrich, St. Louis, MO, USA) was added for 1 h at RT. Plates were washed and stained with 0.1M NaH_2PO_4 (pH = 4) + ABST (10 102 946 001, Roche Diagnostics, Basel, Switzerland). Plates were read at 405 nm.

Histology

Immunohistochemistry was performed on snap-frozen samples. Tissue sections were stained with a monoclonal rat antibody recognizing LCMV-NP (clone: VL-4) and a biotinylated anti-rat secondary antibody(eBioscience, San Diego, CA, USA). Detection was performed by incubation with Streptavadin-peroxidase (Thermo Scientific, Waltham, MA, USA) and ImmPACT Nova Red substrate (Vector Laboratories, Burlingame, CA, USA). Immunohistochemistry sections were counterstained with hematoxylin (Vector Laboratories, Burlingame, CA, USA). Hematoxylin and Eosin (Sigma-Aldrich, St. Louis, MO, USA) staining was performed by standard methods on similarly processed tissue sections.

ASC (ELISPOT) LCMV assay

ELISPOT assay was performed as previously described [17]. Briefly, filter plates were coated overnight at RT with LCMV-infected BHK-cell lysates. Plates were washed with PBS-T and PBS and blocked with RPMI with supplements for 2 h at RT (10% FCS). Splenocytes and bone marrow cells were serial diluted onto filter plates and incubated for 8 h 37°C in humidified 5% CO_2 incubator. After washing with PBS and PBS-T the plates were incubated with biotinylated anti-IgG (Vector Laboratories, Burlingame, CA, USA) overnight at 4°C. The plates were then incubated with HRP-conjugated avidin-D (Vector Laboratories, Burlingame, CA, USA) for 60 min at RT. Following washing with PBS-T and PBS plates were stained with AEC working solution and rinsed with dH_2O.

LCMV neutralizing antibodies

Identification of serum antibodies capable of neutralizing LCMV was performed using an immunofocus reduction assay, as previously described [26]. Briefly, serum samples were diluted 1:5, then and serial diluted in α-MEM + 2% FCS (Biochrom, Berlin, Germany). Diluted serum samples were incubated with LCMV DOCILE for 90 min at 37°C followed by addition of MC57 cells (ATCC, Manassas, VA, USA) and 4 h incubation at 37°C to allow the virus to infect cells. Overlay-Media containing methylcellulose (Sigma-Aldrich, St. Louis, MO, USA) was added following the 4 h incubation and cells were fixed and stained after 48 h in a similar manner to the plaque forming assay indicated above.

Pan T cell Isolation Kit II, CD45R (B220) MicroBeads, mouse, Miltenyi, Bergisch Gladbach, Germany).

Statistical analysis

Data are expressed as mean ± SEM. Statistical significance between two groups was analyzed using Student's *t*-test. For experiments involving analysis of multiple time points, two-way ANOVA with an additional Bonferroni post-test was used. Mantel-Cox test was used for analysis of survival curves. *p*-values < 0.05 were considered as statistically significant. Analysis performed using GraphPad Prism.

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