The Journal of Immunology

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J Immunol 2015; 194:1806-1818; Prepublished online 16 January 2015;
doi: 10.4049/jimmunol.1303163
http://www.jimmunol.org/content/194/4/1806

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Deficiency of Antigen-Specific B Cells Results in Decreased *Trypanosoma cruzi* Systemic but Not Mucosal Immunity Due to CD8 T Cell Exhaustion

Nicole L. Sullivan,*[,1] Christopher S. Eickhoff,[†] John Sagartz,[‡] and Daniel F. Hoft*

Vaccines against mucosally invasive, intracellular pathogens must induce a myriad of immune responses to provide optimal mucosal and systemic protection, including CD4$^+$ T cells, CD8$^+$ T cells, and Ab-producing B cells. In general, CD4$^+$ T cells are known to provide important helper functions for both CD8$^+$ T cell and B cell responses. However, the relative importance of CD4$^+$ T cells, CD8$^+$ T cells, and B cells for mucosal protection is less clearly defined. We have studied these questions in detail using the murine model of *Trypanosoma cruzi* infection. Despite our initial hypothesis that mucosal Abs would be important, we show that B cells are critical for systemic, but not mucosal, *T. cruzi* protective immunity. B cell–deficient mice developed normal levels of CD8$^+$ effector T cell responses early after mucosal *T. cruzi* infection and *T. cruzi trans*-sialidase vaccination. However, after highly virulent systemic challenge, *T. cruzi* immune mice lacking *T. cruzi*–specific B cells failed to control parasitemia or prevent death. Mechanistically, *T. cruzi*–specific CD8$^+$ T cells generated in the absence of B cells expressed increased PD-1 and Lag-3 and became functionally exhausted after high-level *T. cruzi* systemic challenge. *T. cruzi* immune serum prevented CD8$^+$ T cell functional exhaustion and reduced mortality in mice lacking B cells. Overall, these results demonstrate that *T. cruzi*–specific B cells are necessary during systemic, but not mucosal, parasite challenge. *The Journal of Immunology*, **2015, 194: 1806–1818.**

Trypanosoma cruzi is a protozoan parasite and the etiological agent of Chagas' disease. Prevention and vector control practices throughout Latin America have reduced the current number of infected individuals to ~8–11 million people (1). However, movement of infected individuals to nonendemic areas poses an emerging public health problem. Up to 40% of infected individuals develop serious cardiac and/or gastrointestinal problems 1–30 y after infection, leading to significant morbidity and mortality. *T. cruzi* is transmitted to both humans and animals by reduviid insects of the subfamily Triatominae. Infectious parasites are present in the excreta of infected Triatominae insects and can transmit via breaks in the skin, mucosal tissues associated with the eye and gastrointestinal tract, congenital transmission from mother to child, as well as blood and tissue donation from infected individuals.

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Received for publication November 27, 2013. Accepted for publication December 13, 2014.

This work was supported by National Institutes of Health Grants R01 AI040196 and R56A104019 (to D.F.H.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: ASC, Ab-secreting cell; BFT, blood-form trypomastigote; CMT, culture-derived metacyclic trypomastigote; HEL, hen egg lysozyme; i.g., intragastric(ally); KO, knockout; LCMV, lymphocytic choriomeningitis virus; PD-1, programmed death 1; Tc immune, *Trypanosoma cruzi* infection–induced immune; TS, *trans*-sialidase; TS immune, TS vaccine–induced immune; TSKd1, *trans*-sialidase epitope IYNVGQVSI; WT, wild-type.

T cells and B cells have been shown to play critical roles in protection against *T. cruzi*. CD8$^+$ T cells are important in both primary (2) and secondary (3, 4) protective *T. cruzi* immunity. There are several highly immunodominant CD8$^+$ epitopes encoded in the *T. cruzi trans*-sialidase (TS) family of genes. In the BALB/c background, up to 30–40% of CD8$^+$ T cells during acute infection are responsive to the immunodominant H-2K^d–restricted TS epitope IYNVGQVSI (TSKd1) (5, 6). CD4 T cells have been shown to be necessary (7–9) but not sufficient (10) for generation of protective immunity against *T. cruzi* infection. B cells have also been shown to play an important role in systemic *T. cruzi* protection. Early work demonstrated that *T. cruzi*–specific Abs can provide systemic protection via complement activation and lysis (11–13), opsonization (14), and Ab-dependent cellular cytotoxicity (15). These early results demonstrated that B cells play an important role in systemic *T. cruzi* protection through the production of *T. cruzi*–specific Abs (16). Additionally, systemic infection of μMT B cell knockout (KO) mice with *T. cruzi* resulted in initial control of parasite replication but the mice eventually died due to increased parasitemia (16). Previous work by our laboratory demonstrated that *T. cruzi* mucosal infection induces protective immunity against subsequent challenge (17, 18). This mucosal protection was associated with increased levels of *T. cruzi*–specific IgG and IgA Ab-secreting cells in the gastric mucosa (17), the initial site of invasion after oral parasite exposures. However, a causal role for B cells in mucosal *T. cruzi* protection has not been mechanistically defined.

In the present study, we have further examined the importance of B cells for both mucosal and systemic *T. cruzi* immunity. First, we demonstrate that in contrast to what we initially hypothesized, B cells are not required for mucosal *T. cruzi* protection. We predicted that B cells producing secretory IgA would be very important in mucosal protection against an extracellular parasite life stage that invades through nasal and gastrointestinal epithelia, but this was found not to be the case. In contrast, we demonstrate that CD8$^+$ T cells are critical for mucosal *T. cruzi* protection. We confirm

that B cells are important for systemic *T. cruzi* protection in both KO and transient depletion models. After virulent systemic challenge, B cell–deficient/depleted mice are unable to control parasitemia and develop increased morbidity and mortality. We further demonstrate that *T. cruzi*–specific B cells are required for systemic protection, producing parasite-specific Abs that reduce parasite load, resulting in decreased $CD8^+$ T cell exhaustion and mortality in B cell KO mice. Finally, we demonstrate that B cells play a critical role in development and maintenance of multifunctional $CD8^+$ T cells, as well as in the prevention of $CD8^+$ T cell exhaustion. Overall, our new data suggest that memory $CD8^+$ T cells primed in mice lacking *T. cruzi*–specific B cells are sufficient to control low levels of tissue parasitism. However, these $CD8^+$ T cells eventually become exhausted after high-level systemic challenge due to prolonged exposure to high levels of Ag.

Materials and Methods

Mice, parasites, and infection/challenge protocols

BALB/c, SCID BALB/c, JhD B cell KO BALB/c, and hen egg lysozyme (HEL)–specific BCR transgenic and C57BL/6 mice were used throughout this study. In some instances, female BALB/c mice were B cell depleted or not using anti-CD20 (18B12) or IgG2a (2B8) mAb (provided by Biogen Idec). Mice were treated with 250 μg Ab in PBS i.p. every 2–3 wk. Mice were housed under pathogen-free conditions. All studies were conducted with the approval of the Institutional Animal Care and Use Committee/Animal Care Committee in an American Association for the Accreditation of Laboratory Animal Care–accredited facility at Saint Louis University. Tulahuén strain culture-derived metacyclic trypomastigotes (CMT) were used for mucosal infections/challenges and blood-form trypomastigotes (BFT) were used for systemic challenges as described (18, 19) and detailed in Fig. 1. Briefly, CMT can infect both mucosally (via oral, intranasal, conjunctival routes) and systemically whereas BFT can only infect systemically. These differences in infectivity are due to differential glycosylation resulting in increased inflammation induced by the BFT life stage, which is hypothesized to prevent infection at mucosal surfaces (20, 21). *T. cruzi* infection–induced immune mice (referred to as Tc immune mice throughout this study) mice were generated by repeated low-dose infection ($1–3 \times 10^6$ CMT intragastrically [i.g.]). For i.g. infection of mice, mice were first given 0.5 ml 1.5% sodium bicarbonate in HBSS i.g. using a ball-ended 1.5-inch, 22-gauge animal feeding needle and rested for 15 min to neutralize stomach pH. Parasites were then diluted in PBS plus 1% glucose, and 0.1 ml was delivered i.g. These mice are referred to as Tc immune mice throughout this study.

Vaccinations

To generate mucosal immunity, naive BALB/c mice (anti-CD20/IgG2a mAb treated) were vaccinated with 50 μg recombinant TS plus 10 μg CpG-containing oligodeoxynucleotide 1826 (InvivoGen, San Diego, CA) intranasally twice, 2 wk apart (TS/CpG). To generate systemic immunity, naive wild-type (WT), B cell KO, or anti-CD20/IgG2a mAb–treated BALB/c mice were vaccinated with our heterologous TS vaccination protocol (100 μg TS-DNA on days 0 and 14 i.m., and boosted on days 44 and 58 with 1×10^8 PFU adenovirus-expressing TS s.c. (base of tail) and intranasally (4). These mice are referred to as TS vaccine–induced immune (TS immune) mice throughout this study.

Assessment of protective immunity

To assess protective mucosal immunity, mice were challenged with $1–2 \times 10^7$ CMT i.g. Twelve days later, which is the peak time for *T. cruzi* replication in the gastric mucosa (17), mice were sacrificed and gastric DNA was used for quantitative PCR as described (18). Briefly, 100–200 ng gastric DNA purified using Qiagen DNeasy blood and tissue kits was added to each real-time PCR reaction containing 900 nM each primer (5′-AACCACCACGACAACCACAA-3′ and 5′-TGCAGGACATCTGCACAAAGTA-3′), 250 nM TaqMan probe (FAM/TAM 5′-TGCCCCAGGACCGTCCCCA-3′), and 1× TaqMan PCR master mix. Thermocycling conditions using an Applied Biosystems 7500 fast real-time PCR instrument were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A standard curve was generated using DNA purified from a known number of *T. cruzi* epimastigotes.

To assess protective systemic immunity, mice were challenged with 5000 *T. cruzi* (BFT) s.c. Hindlimb paralysis was assessed via paralysis scores similar to those used in experimental autoimmune encephalomyelitis studies: 0, no obvious changes in motor functions; 1, hindlimb weakness; 2, complete paralysis of hindlimbs; 3, complete hindlimb paralysis and partial front leg paralysis; 4, death. Morbidity and mortality were assessed weekly for 60–200 d after systemic BFT challenge.

Adoptive transfers

To determine whether total lymphocytes from immune mice could transfer mucosal *T. cruzi* protection, one mouse equivalent ($4-8 \times 10^7$) of spleen cells plus draining lymph node cells from Tc immune and naive BALB/c mice were transferred i.v. into SCID mice. Both spleen and draining lymph nodes were used for transfer experiments, as Butcher and colleagues (22) have shown that there are more Ag-specific Ab-secreting cells (ASC) in the spleen after rotavirus infection. Furthermore, it was also shown that splenocytes from immune mice contain lymphocytes with mucosal homing capacity ($\alpha_4\beta_7$) that can transfer mucosal protection against rotavirus intestinal infection (22, 23). To determine whether B cells were necessary for the transfer of mucosal *T. cruzi* protection, B cells were depleted using anti-CD19 microbeads (Miltenyi Biotec, Auburn, CA) resulting in >93% $CD19^+$ depletion (Supplemental Fig. 2). $CD19^+$-depleted cells (2.4×10^7) from Tc immune or naive BALB/c mice were transferred i.v. into naive SCID mice. To determine whether $CD8^+$ T cells alone were sufficient to transfer mucosal *T. cruzi* protection, $CD8^+$ T cells were positively selected using anti-CD8 microbeads (Miltenyi Biotec) resulting in >98% $CD8^+$ purity. $CD8^+$ T cells ($5.2–6.95 \times 10^6$) from Tc immune and naive BALB/c mice were transferred i.v. into naive SCID mice. One day after transfer, recipient mice were challenged with 1.2×10^7 *T. cruzi* (CMT) i.g. Twelve days later, recipient mice were sacrificed and *T. cruzi* DNA was assessed in the gastric mucosa via real-time PCR. In some instances, $CD8^+Thy1.2^+$ T cells (>95% $CD8^+$) were purified from B cell KO, WT BALB/c Tc immune, and naive BALB/c mice and transferred into naive BALB/c $Thy1.1^+$ mice. To normalize the number of $CD8^+TSKd1^+$ T cells transferred, purified $CD8^+$ T cells were stained with a TSKd1 tetramer and analyzed with an LSR II flow cytometer (BD Biosciences) and FlowJo flow cytometry software (Tree Star, Ashland, OR). Equivalent numbers of $CD8^+TSKd1^+tetramer^+$ cells from B cell KO and WT Tc immune mice were transferred i.v. into naive BALB/c Thy1.1 mice. One day after transfer, recipient mice, along with naive control mice, were challenged systemically with 5000 *T. cruzi* (BFT) s.c. and followed for parasitemia and survival.

Passive serum transfers

Serum was collected from naive and WT Tc immune BALB/c mice (generated by repeated low-dose oral infection using CMT i.g.). B cell KO BALB/c Tc immune mice were given either 300 μl WT BALB/c Tc immune serum, WT BALB/c naive serum, or PBS i.p. every week starting either 2 wk prior to or at the time of *T. cruzi* (BFT) s.c. systemic challenge. *T. cruzi*–specific serum IgG endpoint titers were measured every 1–2 wk.

T. cruzi–*specific serum IgG ELISA*

Serum was collected at different time points and assessed for TS-specific serum IgG responses via ELISA as described (24). In some instances, 8 M urea was added to evaluate for the presence of high-affinity Abs. Following serum incubation, the ELISA plate was washed with PBS plus 0.05% Tween 20. Urea (8 M) was added for 5 min at room temperature, washed four times with PBS–Tween 20 with the last wash incubated for 5 min at room temperature before removal. Endpoint titers were calculated using Unitwin ELISA software (PhPlate, Stockholm, Sweden).

T. cruzi–*specific IFN-γ, IgG, and IgA ELISPOT*

Millititer HA 96-well microtiter plates with nitrocellulose bases (Millipore, Bedford, MA) were coated with either purified rat anti-mouse IFN-γ (clone R46A2; BD Pharmingen, San Diego, CA) or recombinant TS as described (25). For IFN-γ ELISPOT analyses, spleen cells (2.5×10^5 cells/well) plus APCs (1×10^5 A20J cells alone [control A20; American Type Culture Collection], A20J cells stably transfected with the TS gene [A20-TS], or A20J cells pulsed with 2.5 μg/ml immunogenic $CD8^+$ $H\text{-}2K^d$–restricted TS peptide IYNVGQVSI [A20-TSKd1]) were cultured overnight at 37°C. TS-specific Ab ELISPOT analyses were done as previously described (24). Briefly, $1–5 \times 10^5$ spleen cells were added to TS-coated ELISPOT plates. Following overnight incubation at 37°C, anti-mouse IgG or IgA biotin was added to detect *T. cruzi*–specific IgG and IgA Ab-secreting cells, respectively (SouthernBiotech). Results are represented as the number of spot-forming cells or ASC per million spleen cells or absolute number per spleen. Background responses to A20J cells were subtracted from experimental Ag responses in results shown from IFN-γ ELISPOT assays.

Flow cytometry and in vitro CD8$^+$TSKd1$^+$ expansion

All Abs were purchased from BD Biosciences (San Diego, CA) with the exception of anti–PD-1, anti–Lag-3, and anti-CD62L (eBioscience, San Diego, CA). The TS^{IYNVGQVSI}/H-2K^d–APC (TSKd1) tetramer was provided through the National Institutes of Health Tetramer Core Facility (Atlanta, GA). To evaluate in vitro TSKd1 expansion, spleen cells were labeled with CFSE (Vybrant CFDA SE cell tracer; Invitrogen) and cultured with 1×10^5 irradiated (12,500 rad) APCs (A20-negative control or A20-TS) for 6 d at 37°C. Cells were then counted using Guava ViaCount reagent and the EasyCyte flow cytometer (Millipore, Billerica, MA). Half of the cells were used for direct cell surface staining and the other half were stimulated with PMA (10 ng/ml), ionomycin (500 ng/ml), and cultured with monensin (GolgiPlug; 1 μl/ml) and brefeldin A (GolgiStop; 0.67 μl/ml) for 3 h at 37°C. Cells were stained as described (4) and analyzed with an LSR II flow cytometer (BD Biosciences) and FlowJo v7 software (Tree Star).

CD8$^+$TSKd1$^+$ stimulation indices were calculated by taking the absolute number of live CD19$^-$CD3$^+$CD4$^-$CD8$^+$tetramer$^+$CFSE^{lo} cells present after A20-TS stimulation divided by the number present after A20-negative control incubation. The percentages and absolute numbers of CD8$^+$TSKd1$^+$cytokine$^+$ T cells were calculated by taking the percentages or numbers of live CD19$^-$CD3$^+$CD4$^-$CD8$^+$tetramer$^+$CFSE^{lo}cytokine$^+$ cells present after stimulation with A20-TS subtracted by the number present after A20-negative control incubation.

Histopathology

Cardiac and skeletal muscle inflammation was assessed at days 30 or 87 after systemic *T. cruzi* (BFT) challenge. The heart and skeletal muscles from individual mice were fixed in 10% formalin, paraffin embedded, sectioned, and stained with H&E. Pathology slides were graded by a blinded pathologist as follows: 1, minimal; 2, mild; 3, moderate; 4, marked.

Statistical analysis

Statistical analyses were performed using Prism v4 software (GraphPad Software, La Jolla, CA). Mann–Whitney *U* tests or unpaired Student *t* tests were used to compare responses between groups. Log-rank tests were used to compare survival between groups.

Results

CD8$^+$ T cells, but not B cells, are required for protective mucosal T. cruzi *immunity*

Previous work has shown that repeated *T. cruzi* mucosal infections result in chronically infected mice that develop concomitant immunity protective against subsequent mucosal challenges (Tc immune) (17). These Tc immune mice developed high numbers of *T. cruzi*–specific ASC in the gastric mucosa, suggesting that B cells played an important role in mucosal *T. cruzi* protection (17). We first sought to directly address the potential causative role of B cells in protective mucosal *T. cruzi* immunity. Tc immune B cell KO (JhD) and WT BALB/c mice were generated as shown in Fig. 1A. One month after the last infection, Tc immune and naive B cell KO and WT BALB/c mice were challenged orally with a high dose of *T. cruzi*. Mucosal protection was assessed 12 d later via real-time PCR, as we have previously shown that the highest level of *T. cruzi* DNA can be measured in the gastric mucosa, the point of initial mucosal invasion after oral parasite inoculation, 10–14 d postchallenge (17) (Fig. 2A). B cell KO mice had significantly higher levels of *T. cruzi* DNA in the gastric mucosa compared with WT mice after primary mucosal parasite infection ($p = 0.0006$; Mann–Whitney *U* test), suggesting that B cells are important in the primary control of parasite replication. However, more relevant for vaccine development is the role of B cells in memory immune responses. Tc immune B cell KO mice did develop mucosal immunity, as they were significantly protected compared with the primary challenged B cell KO mice (Fig. 2A, $p < 0.0005$; Mann–Whitney *U* test). Additionally, Tc immune B cell KO mice developed similar levels of mucosal protection compared with WT Tc immune mice ($p = 0.1719$; Mann–Whitney *U* test). We also assessed the frequency of *T. cruzi*–specific T cells in B cell KO and WT Tc immune mice. There were similar frequencies of total T cells specific

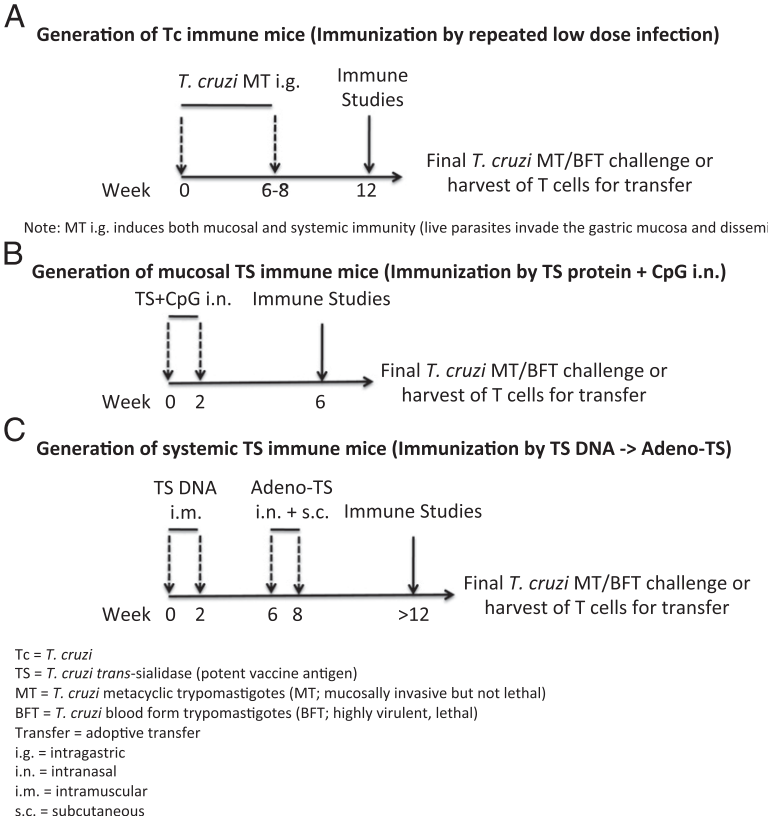

FIGURE 1. *T. cruzi* infection– and TS vaccine–induced memory models. Shown are the major models used in this study to demonstrate immunity induced by multiple low-dose *T. cruzi* infections (Tc immune model) and by immunization with various TS vaccines (TS immune models). (**A**) Generation of Tc immune mice. Mice are dosed at 0 and 6–8 wk with low doses of *T. cruzi* metacyclic trypomastigotes (MT) i.g. At least 4 wk later, these mice are ready to be used for immune studies, sources of immune cells for use in adoptive transfer models, final mucosal challenge with high doses of MT i.g., or systemic *T. cruzi* challenge with BFT. (**B**) Generation of mucosal TS immune mice. Mice are vaccinated at 0 and 2 wk with CpG-adjuvanted recombinant TS intranasally (i.n.), and 4 wk later mice are ready for use in various studies as described in (A). (**C**) Generation of systemic TS immune mice. Mice are vaccinated at 0 and 2 wk with DNA-TS (i.m.) and with adenovirus-expressing TS (s.c. and intranasal) on weeks 6 and 8. At least 4 wk later, mice are ready for use in various studies as described in (A). Importantly, note that Tc immune mice remain chronically infected with low levels of parasites and have so-called concomitant infection-induced natural immunity. In contract, TS immune mice are not infected until challenged later with *T. cruzi*.

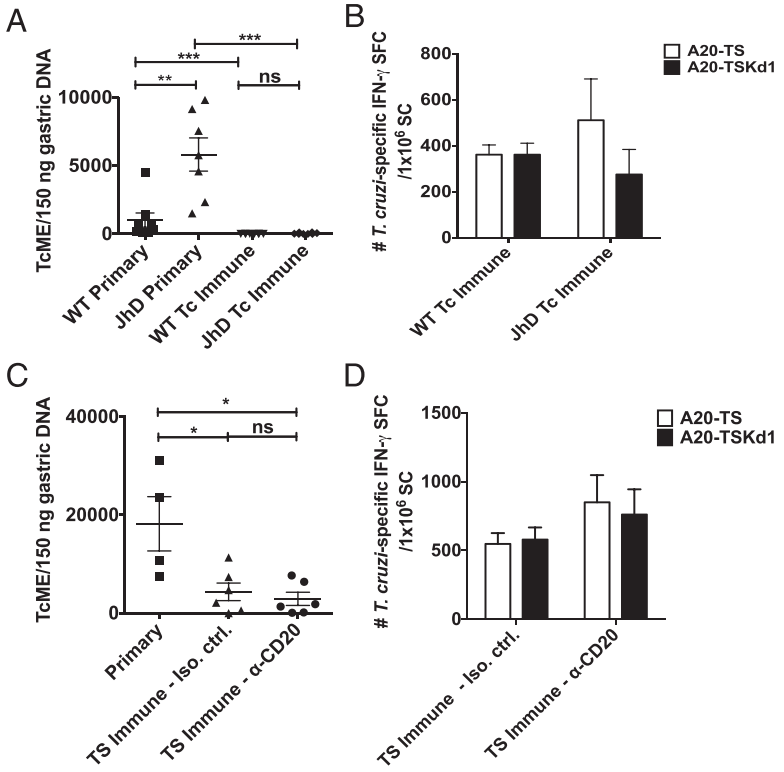

FIGURE 2. B cells are not required for protective mucosal *T. cruzi* immunity. (**A** and **B**) Tc immune JhD B cell KO and WT BALB/c mice were generated by repeated low-dose i.g. *T. cruzi* infections as described in Fig. 1. One month following the last i.g. infection, naive and Tc immune B cell KO and WT mice were challenged i.g. with a high dose of *T. cruzi*. Twelve days later (the point at which *T. cruzi* DNA is highest in the gastric mucosa) (17), mice were sacrificed and (A) gastric DNA was isolated to assess the level of *T. cruzi* DNA via quantitative PCR (n = 7–8 mice/group). Data are derived from two independent experiments pooled together. (B and **D**) Splenocytes (SC) were isolated and the frequency of IFN-γ–producing TS or TSKd1-specific T cells was measured via ELISPOT. Background responses were subtracted from the experimental values. (**C** and D) Mucosal TS immune mice were generated by intranasal vaccination with recombinant TS plus CpG 1826 twice, 2 wk apart. Mice were either B cell depleted (anti-CD20) or not (IgG1κ) starting 2 wk prior to vaccination. One month after vaccination, naive and TS immune mice were challenged i.g. with a high dose of *T. cruzi*. Twelve days later, mice were sacrificed and (C) gastric DNA was isolated to assess the level of *T. cruzi* DNA via quantitative PCR (n = 5 mice/group). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0005$ by Mann–Whitney U test. SFC, spot-forming cell; TcME, *T. cruzi* molecular equivalent. Error bars represent SEM.

for the *T. cruzi* protein TS (A20-TS response) and T cells reactive with an immunodominant CD8 T cell epitope (A20-TSKd1 response) in WT and B cell KO mice (Fig. 2B). We measured immune responses in the spleen, as it has been shown previously that immunity to mucosal pathogens can be efficiently measured in the spleen (22, 23). Ag-specific cells generated in mucosal lymph nodes traffic through the spleen before migrating back to mucosal tissues. Additionally, higher total numbers of rotavirus-specific sIgA ASC have been recovered from spleens than from mucosal tissues (22), and splenic $CD8^+$ T cells from rotavirus-immune mice can efficiently transfer mucosal protection (22, 23). Overall, our results demonstrate that B cells are not required for *T. cruzi* mucosal immunity.

Next, we wanted to determine whether B cells play an important role in TS vaccine–induced mucosal immunity (Fig. 1B). TS is an enzyme on the surface of *T. cruzi* that is important for parasite infectivity, as it has both neuraminidase and sialic acid transfer activity. We have previously shown that mice vaccinated with TS plus the TLR9 agonist CpG 1826 are protected against both mucosal and systemic *T. cruzi* challenges (25). B cells were first depleted in naive WT BALB/c mice using anti-CD20 mAb (Biogen Idec). TS immune mice (Fig. 1B) were intranasally vaccinated twice with recombinant TS plus CpG 1826 two week apart, rested for 1 mo, challenged with a high dose of *T. cruzi* orally (10^7), and assessed for mucosal protection via real-time PCR. Similar to the results seen in Tc immune mice, B cells were not required for TS vaccine–induced protective mucosal immunity (Fig. 2C). TS- and TSKd1-specific T cell responses were comparable between B cell–depleted and isotype control–treated TS-vaccinated mice (Fig. 2D), demonstrating that B cells are not required for the development, maintenance, and/or recall of TS- or TSKd1-specific T cells after mucosal *T. cruzi* challenge. Thus, B cells are not required for TS-specific vaccine-induced mucosal immunity.

To confirm that B cells are not critical for mucosal *T. cruzi* protection, we performed adoptive transfer studies. We first determined whether cells from Tc immune mice or TS immune mice generated as shown in Fig. 1C with a more potent vaccination regimen (heterologous TS DNA primed, adenovirus-TS boosted) could transfer mucosal protection, as this has not been shown previously. Owing to the low numbers of cells recovered from the gastric lymph nodes, mixed populations of splenocytes plus gastric lymph node cells were used to transfer maximal numbers of parasite-specific cells. Previous work by others has shown that splenocytes from rotavirus-immune mice contain lymphocytes with mucosal homing capacity that can transfer mucosal protection (22, 23). Both Tc immune (Fig. 3A) and TS immune (Fig. 3B) donor cells were able to transfer mucosal protection to naive SCID mice.

Next, we studied whether B cells are necessary and/or sufficient for the transfer of mucosal protection. We transferred highly purified $CD19^+$ B cells from either naive or Tc immune BALB/c mice into SCID mice. However, we could not confirm whether B cells were sufficient, as SCID mice receiving purified B cells (Supplemental Fig. 1A) alone did not develop *T. cruzi*–specific Ab responses (serum IgG by ELISA or ASC detected by ELISPOT; Supplemental Fig. 1C and 1D, respectively), even though B cells were clearly present (Supplemental Fig. 1B). Thus, we decided to perform B cell depletion studies to determine whether B cells were necessary for the transfer of mucosal protection. B cells were depleted from Tc immune and naive BALB/c splenocytes and gastric lymph node cells (Supplemental Fig. 2). These B cell–depleted fractions were transferred into naive SCID mice and recipient mice were orally challenged the following day with a high dose of *T. cruzi* i.g. Twelve days after challenge, recipient mice were assessed for the presence of B cells and *T. cruzi*–specific serum IgG and IgG ASC. Supplemental Fig. 2 shows that in SCID mice given CD19-depleted cells, B cells were undetectable. Fig. 3C demonstrates that B cells are not necessary for the transfer of mucosal protection, as SCID mice that received either total or B cell–depleted splenocytes plus gastric lymph node cells from Tc immune mice were similarly protected ($p < 0.05$, Mann–Whitney U test compared with transfer of cells from naive mice). There was no significant difference in protection transferred by naive total or naive B cell–depleted cell populations ($p = 0.22$, Mann–Whitney

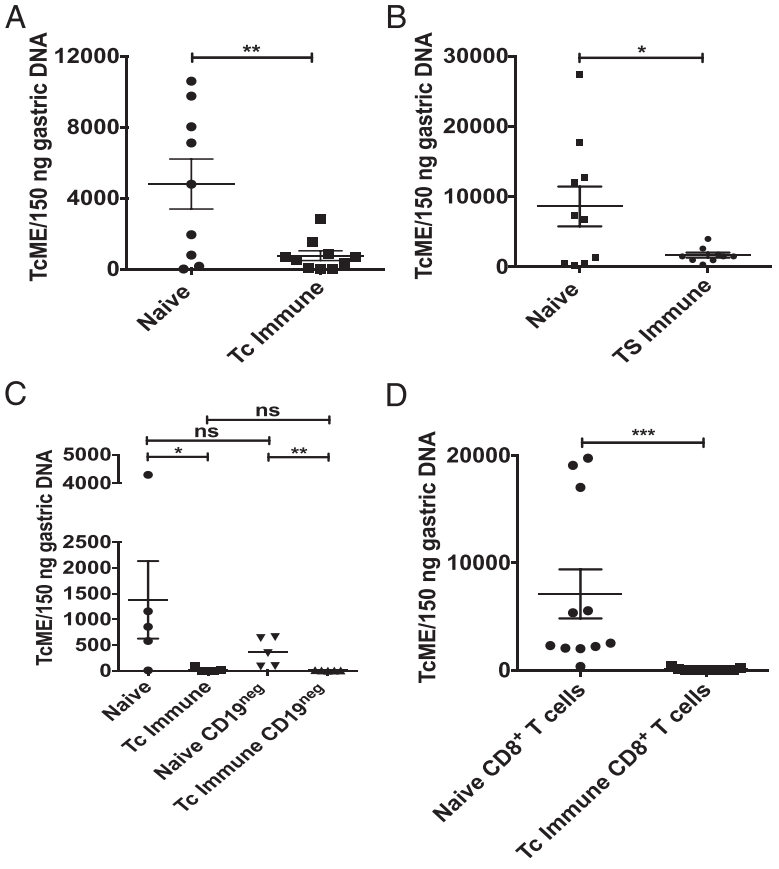

FIGURE 3. *T. cruzi*–specific CD8$^+$ T cells generated by infection or vaccination can transfer mucosal *T. cruzi* protection. (**A**–**D**) Subsets of immune cells from Tc immune or systemic TS immune mice were transferred into naive SCID mice. Recipient mice were challenged the following day with a high dose of *T. cruzi* i.g., and 12 d later mice were sacrificed and *T. cruzi* DNA was assessed in the gastric mucosa via quantitative PCR. (A) One mouse spleen cell plus draining lymph node cell equivalent from Tc immune or naive BALB/c mice was transferred i.v. into naive SCID mice (n = 9–10 mice/group). Data presented are derived from two independent experiments pooled together. $^{**}p < 0.01$ by Student t test. (B) One mouse equivalent of splenocytes from TS immune mice (TS DNA prime, adenovirus-TS boost) or naive BALB/c mice was transferred i.v. into naive SCID mice (n = 9–10 mice/group). $^{*}p < 0.05$ by Student t test. Data presented are derived from two independent experiments pooled together. (C) One mouse equivalent of total splenocytes or CD19-depleted splenocyte populations from Tc immune or naive BALB/c mice were transferred i.v. into naive SCID mice (n = 5 mice/group). $^{*}p < 0.05$, $^{**}p < 0.01$ by Mann–Whitney U test. Data are representative of two independent experiments. (D) One mouse spleen plus gastric lymph node equivalent of highly purified CD8$^+$ T cells (>98% CD8$^+$) from Tc immune or naive BALB/c mice were transferred i.v. into naive SCID mice (n = 11 mice/group). $^{***}p < 0.0001$ by Mann–Whitney U test. Data presented are derived from two independent experiments pooled together. Error bars represent SEM. TcME, *T. cruzi* molecular equivalent.

U test). These results confirm our earlier results detected in the B cell KO model that B cells are not required for mucosal *T. cruzi* protection.

Since B cells were not critical for mucosal *T. cruzi* protection, we examined whether CD8$^+$ T cells alone from Tc immune mice could transfer mucosal protection. Previous work has shown that CD8$^+$ T cells from Tc immune mice could transfer protection against normally lethal systemic parasite challenges (4, 26). However, in order for CD8$^+$ T cells to mediate mucosal protection, these cells must be able to migrate into the gastric tissue and provide effector functions. Highly purified CD8$^+$ T cells (Supplemental Fig. 3A) from Tc immune and naive BALB/c mice were transferred into naive SCID mice 1 d prior to oral challenge. SCID mice that received Tc immune CD8$^+$ T cells were significantly protected against oral *T. cruzi* challenge as compared with mice receiving naive control CD8$^+$ T cells (Fig. 3D), despite the absence of detectable *T. cruzi*–specific serum IgG (Supplemental Fig. 3B). Thus, CD8$^+$ T cells alone are able to confer mucosal *T. cruzi* protection.

B cells are important for systemic T. cruzi *immunity*

We next investigated the ability of B cells to contribute to systemic *T. cruzi* protection in our model systems. Previous work by Kumar and Tarleton (16) showed that B cell KO mice could initially control parasitemia after systemic infection with *T. cruzi* but eventually succumbed to the infection. First, we verified whether B cell KO mice could develop systemic protection. B cell KO and WT BALB/c mice were orally infected with a low dose of *T. cruzi* twice, 2 mo apart, to generate Tc immune mice (Fig. 1A). These mice did not succumb to infection or require antiparasitic drugs (data not shown). In contrast, we have previously shown that mice deficient in IL-12, IFN-γ, or β_2-microglobulin are unable to survive low-dose parasite infections even with antiparasite drug intervention (19). We then challenged B cell KO and WT Tc immune mice systemically with BFT, which are more virulent and induce higher levels of parasitemia in naive mice (25, 27). B cell KO Tc immune mice survived significantly longer than did their naive cohorts, but they eventually succumbed to infection (Fig. 4A), developing increased parasitemia (Fig. 4B), weight loss (Fig. 4C), and decreased TS- and TSKd1-specific CD8$^+$ T cell responses (Fig. 4D, 4E). Importantly, the frequencies and absolute numbers of IFN-γ–producing TSKd1-specific CD8$^+$ T cells were significantly reduced. These results indicate that B cells are important for the maintenance of *T. cruzi*–specific, CD8$^+$ T cell functional responses when systemic Ag levels are high. Previous work by our laboratory has shown that WT BALB/c mice require ~5000 TSKd1-specific IFN-γ–producing CD8$^+$ T cells to be protected from death (C.S. Eickhoff, N.L. Sullivan, and D.F. Hoft, manuscript in preparation). As shown in Fig. 4E, B cell KO Tc immune mice developed significantly lower numbers of TSKd1-specific IFN-γ–producing CD8$^+$ T cells per spleen (738 ± 198) compared with WT Tc immune mice (11,374 ± 1,764). Thus, B cell KO Tc immune mice probably were not protected because they either did not develop or sustain sufficient numbers of IFN-γ–producing, TSKd1-specific CD8$^+$ T cells. Consistent with the higher levels of parasitemia, Tc immune mice lacking B cells developed significantly higher levels of inflammation in cardiac and skeletal tissues, hallmarks of disease pathology induced by chronic *T. cruzi* infection (Fig. 4F, 4G). Overall, these results demonstrate that B cells play a critical role in protective *T. cruzi* systemic immunity.

Nonspecific B cells cannot reconstitute T. cruzi *immunity*

B cells provide many important functions such as Ab production, Ag presentation, costimulation, and cytokine production. Thus, B cell deficiency not only results in the absence of Abs, but it also could affect the development of T cell responses. To determine

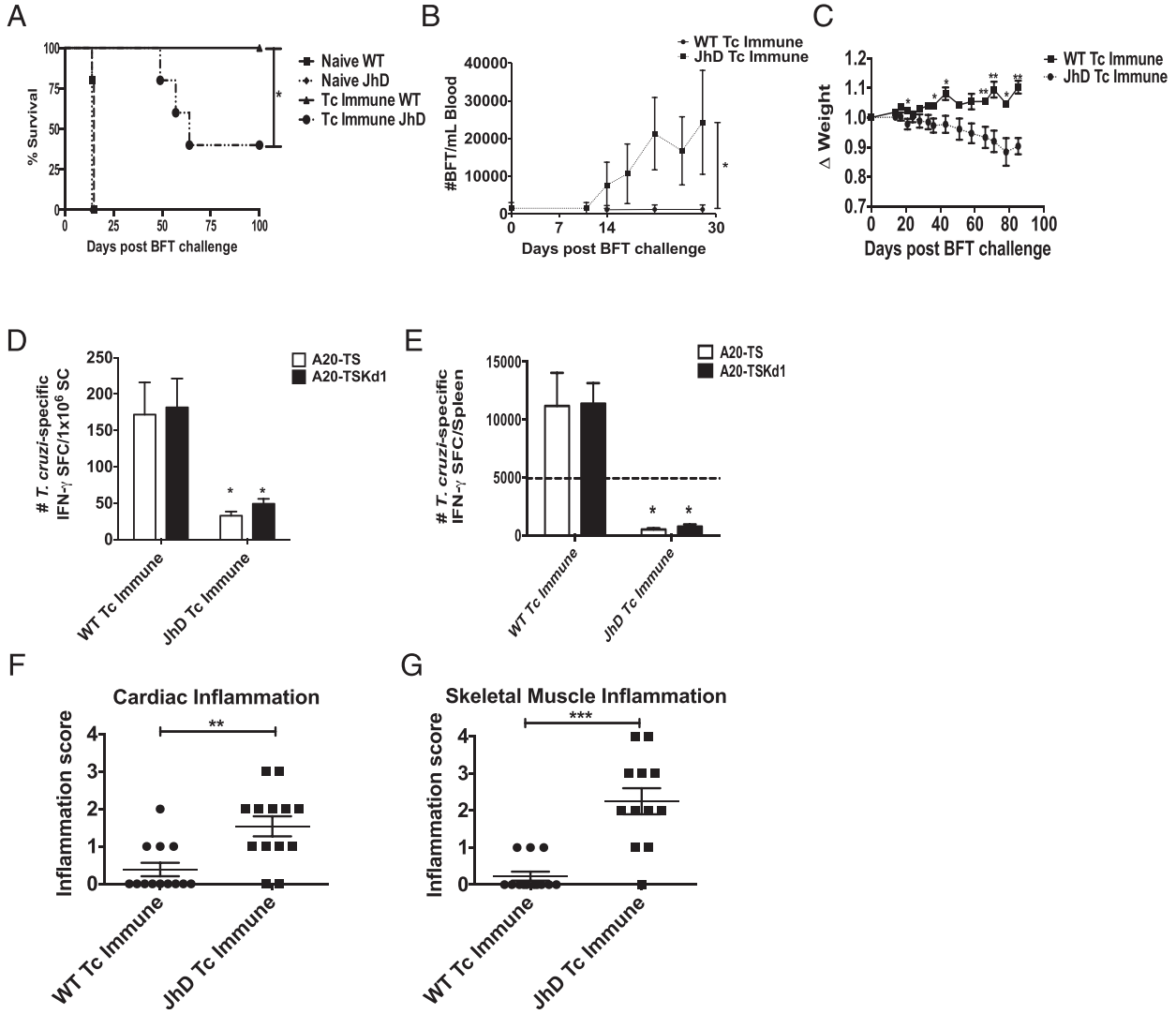

FIGURE 4. B cells are important for systemic *T. cruzi* immunity developing in response to parasite infection. JhD B cell KO and WT Tc immune mice were generated as described in Fig. 1. At least 1 mo following the last mucosal infection, Tc immune and naive B cell KO and WT BALB/c mice were challenged systemically with virulent *T. cruzi* BFT s.c. (**A**) Survival was assessed for up to 100 d. $*p < 0.05$ by log-rank test ($n = 5$/group). (**B**) Parasitemia was assessed prior to and up to 28 d after systemic *T. cruzi* (BFT) challenge. $**p < 0.01$, area under the curve was assessed for each individual mouse and used to compare groups in Mann–Whitney *U* test ($n = 5$/group). (**C**) Weight was measured prior to and through day 85 after systemic challenge. $*p < 0.05$, $**p < 0.01$ by Mann–Whitney *U* test. Data in (A)–(C) are representative of two independent experiments with similar results. Thirty days after systemic challenge, some mice were sacrificed to assess immune and inflammatory responses. (**D**) The frequency and (**E**) absolute number of IFN-γ–producing TS- and TSKd1-specific T cells were measured via ELISPOT using total splenocytes. Background responses were subtracted from the experimental values. The dashed line in (E) represents 5000 TSKd1-specific $CD8^+$ T cells, a number found in previous work to provide systemic protection (C.S. Eickhoff, N.L. Sullivan, and D.F. Hoft, manuscript in preparation). $*p < 0.05$ by Mann–Whitney *U* test ($n = 4$ mice/group). Data are representative of two independent experiments with similar results. (**F**) Inflammation of cardiac and (**G**) skeletal muscle was assessed at 30–87 d after systemic challenge: 1, minimal; 2, mild; 3, moderate; 4, marked. Naive B cell KO and WT mice showed no signs of inflammation (data not shown) ($n = 12$–13 mice/group). $**p < 0.005$, $***p < 0.0005$ by Mann–Whitney *U* test. Data presented are derived from two independent experiments with results pooled together. Error bars represent SEM.

whether *T. cruzi*–specific B cells are critical for systemic protection, we studied HEL-specific BCR transgenic mice. Advantages of using these mice are that in contrast to complete B cell KO mice, lymphoid tissue development and cytokine production are relatively normal. However, these mice cannot generate B cells that produce high-affinity *T. cruzi*–specific Abs, which could be important for both direct protective effects as well as enhanced Ag presentation. HEL and WT Tc immune mice were generated by repeated low-dose *T. cruzi* oral infections (Fig. 1A). Prior to and following systemic *T. cruzi* (BFT) challenge (at least 1 mo after the second oral infection), mice were assessed for *T. cruzi*–specific IgG Abs, parasitemia, weight loss, and death (Fig. 5).

HEL Tc immune sera did have some background reactivity in our recombinant TS-specific ELISA, but very little of this reactivity persisted in the presence of 8 M urea, demonstrating that minimal if any *T. cruzi*–specific, high-affinity Abs were present (Fig. 5A). In contrast, WT Tc immune sera had markedly higher reactivity with recombinant TS and much of this persisted despite 8 M urea treatment.

Even before the high-level *T. cruzi* BFT systemic challenge, parasitemia levels were increased in the HEL Tc immune mice (Fig. 5B). These results could be interpreted to suggest that the absence of high-affinity, *T. cruzi*–specific B cell responses resulted in a deficiency in protective mucosal memory immunity, despite

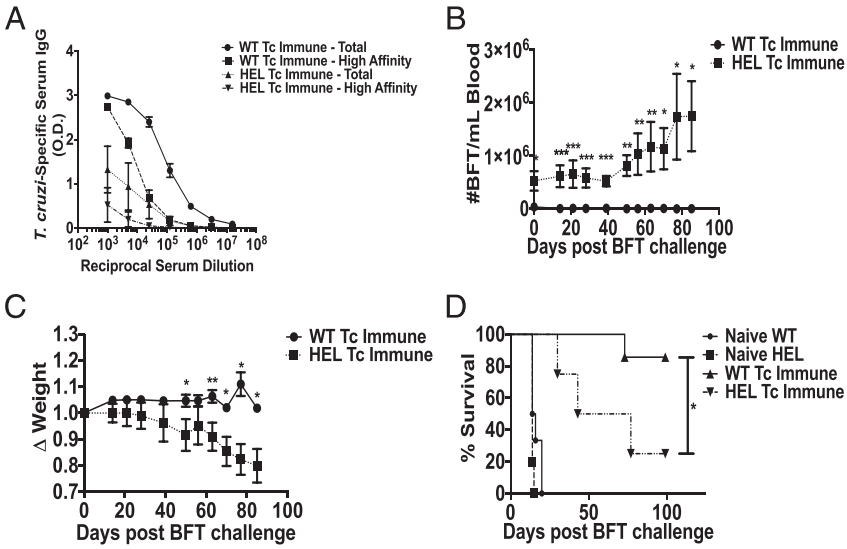

FIGURE 5. Nonspecific B cells cannot reconstitute *T. cruzi*–induced immunity. Tc immune HEL-specific BCR transgenic or control C57BL/6 mice were generated by repeated low-dose intragastric infections with *T. cruzi*. At least 1 mo after the last infection, Tc immune and naive HEL and WT mice were challenged systemically with virulent *T. cruzi* BFT s.c. (**A**) Total and high-affinity recombinant TS–specific serum IgG responses were measured 50 d after systemic challenge via ELISA. (**B**) Parasitemias were assessed at different time points before and after final systemic challenge (n = 7–10/group). $^{**}p <$ 0.01, $^{***}p < 0.005$ by Mann–Whitney U test. (**C**) Weight loss assessed at different time points after systemic challenge (n = 7–10/group). $^{**}p < 0.01$ Mann–Whitney U test. (**D**) Mice were followed for survival for 100 d after systemic challenge (n = 4-7 mice/group). $^{*}p < 0.05$ by log-rank test. Data are representative of two independent experiments with similar results. Error bars represent SEM.

our earlier results shown in Figs. 2 and 3 demonstrating that B cells were not important for either infection- or vaccine-induced mucosal *T. cruzi*–specific protective immunity. However, parasitemia is a systemic endpoint largely controlled by systemic immunity. After oral *T. cruzi* challenges, the parasite drains to the gastric lymph node and disseminates throughout the body, causing systemic infection. All Tc immune mice develop chronic systemic infection requiring systemic immunity to control parasitemia. The fact that HEL Tc immune mice had higher parasitemias prior to the high-level BFT systemic challenge indicates that these mice have profound defects in systemic immunity, resulting in the inability to control systemic parasite infection even after low-dose *T. cruzi* mucosal challenge.

Consistent with the results presented in Fig. 4, parasitemia levels increased further in HEL Tc immune mice after systemic challenge (Fig. 5B), resulting in increased weight loss (Fig. 5C) and death (Fig. 5D). These results further demonstrate that *T. cruzi*–specific B cells are critical for systemic *T. cruzi* protection.

Similar levels of memory CD8$^+$ T cells develop in the absence of infection but fail to protect after systemic challenge

In our Tc immune model, mice are chronically infected, making it difficult to study the development of memory CD8$^+$ T cell responses in the absence of persistently high levels of *T. cruzi* Ag. Thus, we examined the development of CD8$^+$TSKd1$^+$ T cells in our TS immune memory model (heterologous TS DNA primed, adenovirus-TS boosted; Fig. 1C). As described earlier, B cells were depleted in vivo with anti-CD20 Ab (or isotype control) throughout the vaccination/treatment regimen. TS immune B cell–depleted and control mice developed similar levels of immunodominant CD8$^+$TSKd1$^+$ T cells detectable in blood (Fig. 6A–C). Thus, Ag-specific CD8$^+$ T cells can be primed normally in B cell–depleted mice. These mice were then challenged systemically with *T. cruzi* and assessed for immune responses and protection. The percentages of the total CD8 populations that were CD8$^+$TSKd1$^+$ T cells expanded from ~5.3 ± 1.0% at 4 mo after boost (Fig. 6C) to 38.5 ± 4.4% 15 d after the systemic *T. cruzi* challenge in isotype control–treated TS immune mice (Fig. 6D). Anti-CD20–treated TS immune mice had frequencies of CD8$^+$TSKd1$^+$ T cells similar to control mice prior to systemic challenge (4.62 ± 1.5%; Fig. 6C). However, these cells did not expand optimally after systemic *T. cruzi* challenge (Fig. 6D), resulting in significantly fewer CD8$^+$TSKd1$^+$ T cells after challenge than in control TS immune mice. These reduced numbers of CD8$^+$TSKd1$^+$ T cells in anti-CD20–treated TS immune mice were associated with increased parasitemia (Fig. 6E) and death (Fig. 6F). Although similar levels of immunodominant CD8$^+$ T cells developed in response to TS vaccination in the absence of B cells, these CD8$^+$ T cells were functionally deficient and failed to protect against *T. cruzi* systemic challenge. Thus, memory CD8$^+$ T cells that develop in the absence of B cells were impaired in their ability to respond to and protect against virulent systemic *T. cruzi* challenges.

B cells are important for multifunctional CD8$^+$ T cell responses and prevention of CD8$^+$ T cell exhaustion

As we have shown in Figs. 4–6, in the absence of B cells, mice are unable to develop immunity that prevents death after virulent *T. cruzi* systemic challenge. Interestingly, these mice are able to develop CD8$^+$ T cell responses and survive longer than do their naive cohorts (Fig. 4A). However, parasitemia increases over time in these B cell–deficient immune mice after virulent systemic challenge, ultimately resulting in death. These data suggested that although similar numbers of *T. cruzi*–specific CD8$^+$ T cells could be induced in B cell–deficient mice, these CD8$^+$ T cells were functionally deficient, especially when confronted with high Ag loads. To test this hypothesis, we first examined the ability of TSKd1-specific CD8$^+$ T cells to expand and produce cytokines after ex vivo restimulation. B cell KO and WT Tc immune mice were challenged systemically with high doses of *T. cruzi* and immune responses were evaluated 30 d later. Splenocytes were labeled with CFSE and cocultured with either negative control APCs or APCs that were stably transduced with TS (27). Significant reductions in Ag-specific CD8$^+$TSKd1$^+$ T cell proliferative capacity (Fig. 7A) and cytokine production (IFN-γ and TNF-α;

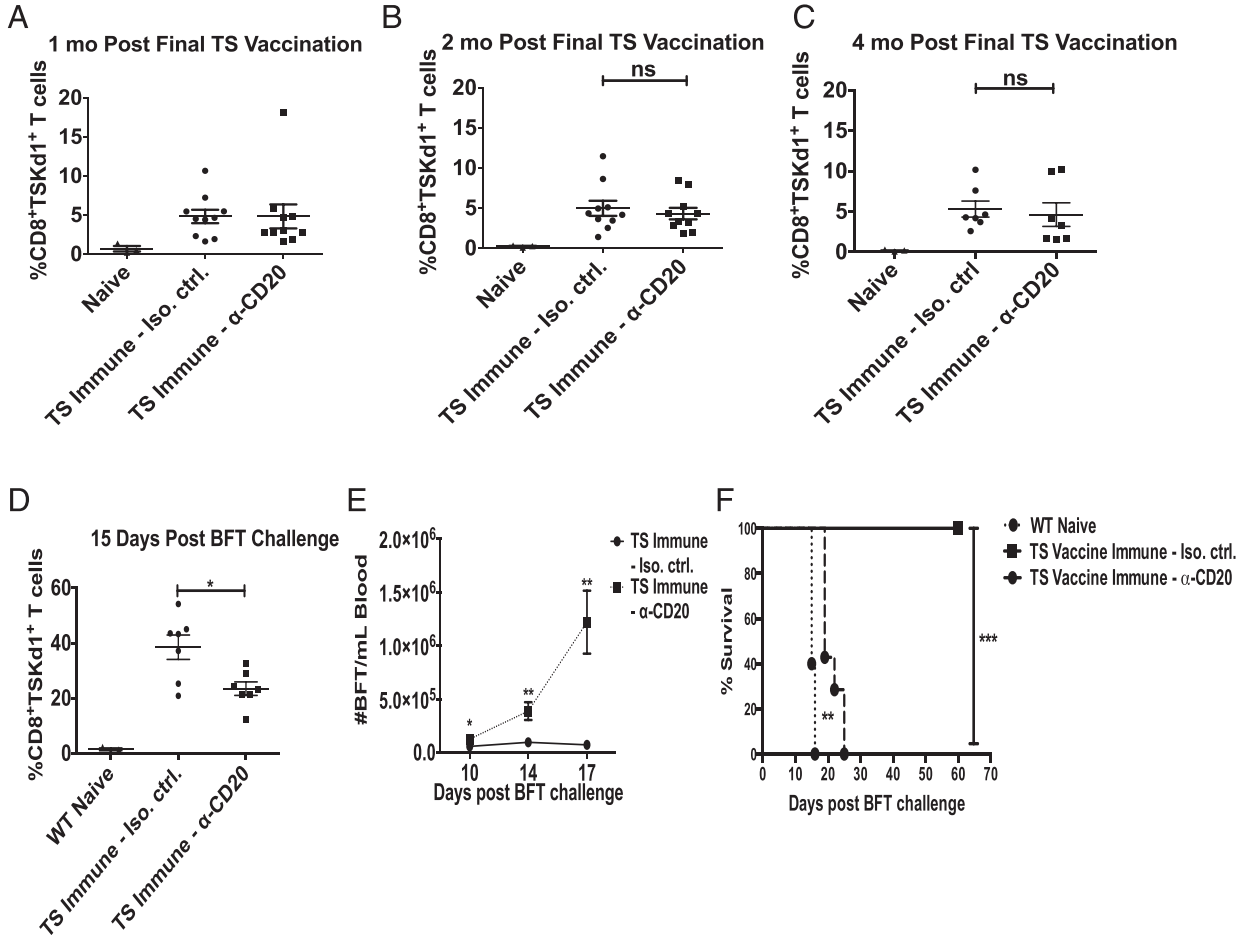

FIGURE 6. B cells are important for TS vaccine–induced systemic immunity. B cell–depleted (anti-CD20) and isotype control–treated BALB/c mice were vaccinated with our heterologous TS DNA prime, adeno-TS boost vaccination regimen (systemic TS immune mice). Mice were treated with anti-CD20 or isotype control Ab every 2–3 wk for the duration of the study and assessed for B cell depletion via flow cytometry and *T. cruzi*–specific serum IgG (data not shown). The percentages of $CD8^+TSKd1^+$ T cells were measured in blood via flow cytometry at (**A**) 1, (**B**) 2, and (**C**) 4 mo after the final TS vaccination. TS immune and naive BALB/c mice were challenged systemically with virulent *T. cruzi* BFT s.c. ~4.5 mo after the last TS vaccination (n = 7–10/group). (**D**) Fifteen days after systemic challenge, the percentages of $CD8^+TSKd1^+$ T cells were measured in blood via flow cytometry (n = 7/group). $^*p < 0.05$ by Mann–Whitney U test. (**E**) Parasitemia was measured through day 17 after systemic challenge (n = 7/group). $^*p < 0.05$, $^{**}p < 0.01$ by Mann–Whitney U test. (**F**) Survival was assessed through day 60 after systemic challenge (n = 5–7/group). $^{**}p < 0.01$, $^{***}p < 0.005$ by log-rank test. Error bars represent SEM.

Fig. 7B, 7C) were detected in mice lacking B cells compared with WT mice. To evaluate $CD8^+TSKd1^+$ in vivo expansion, equivalent numbers of $CD8^+Thy1.2^+TSKd1^+$-specific T cells from B cell KO and WT Tc immune mice (based on tetramer staining) were transferred into naive $Thy1.1^+$ BALB/c mice with normal B cell numbers and function (Fig. 7D–H). Recipient mice were then challenged systemically 1 d later. $CD8^+Thy1.2^+$ T cells from B cell KO Tc immune mice failed to expand normally in vivo compared with WT infection-immune $CD8^+$ T cells (Fig. 7E), resulting in a reduced ability to control parasitemia (Fig. 7F). These results identify intrinsic functional defects in *T. cruzi*–specific $CD8^+$ T cells generated in the absence of B cells.

To further examine the mechanism responsible for $CD8^+$ T cell dysfunction in B cell–deficient mice, we evaluated expression of the inhibitory receptors PD-1 and Lag-3 on $tetramer^+CD8^+$ T cells. There were significant increases in the percentages (Fig. 7G) and absolute numbers (Fig. 7H) of $CD8^+TSKd1^+PD\text{-}1^+Lag\text{-}3^+$ T cells in the spleens of B cell KO Tc immune mice compared with WT Tc immune mice. We also found significant increases in $CD8^+TSKd1^+Lag\text{-}3^+$ T cells in the blood of TS-vaccinated and B cell–depleted mice 15 d after systemic *T. cruzi* challenge (Supplemental Fig. 4). Overall, these results demonstrate that B cells play a critical role for both the induction of optimally functional $CD8^+$ T cell responses and the prevention of $CD8^+$ T cell exhaustion.

Passive immune serum transfer prevents $CD8^+$ T cell exhaustion and increases survival after challenge

Previous work by others has shown that passive serum transfers from Tc immune into naive mice can transfer systemic protection (11–15). We hypothesized that the lack of *T. cruzi*–specific Abs in B cell KO Tc immune mice resulted in increased tissue parasitism and death due to the inability of functionally exhausted $CD8^+$ T cells to properly control *T. cruzi* infection. To test this hypothesis, we performed passive serum transfers. Prior to and after systemic *T. cruzi* challenge, B cell KO Tc immune mice received WT Tc immune serum, naive serum, or PBS. As shown in Fig. 8A, *T. cruzi*–specific Ab responses were present in B cell KO mice after transfer of WT Tc immune serum. The immune serum protected B cell KO mice from increased morbidity and mortality (Fig. 8B, 8C). This protection was associated with increased TSKd1-specific $CD8^+$ T cell numbers (Fig. 8D) and total TS-specific and TSKd1-specific T cell function (Fig. 8E). Finally, Tc immune serum transfer resulted in reduced expression of the

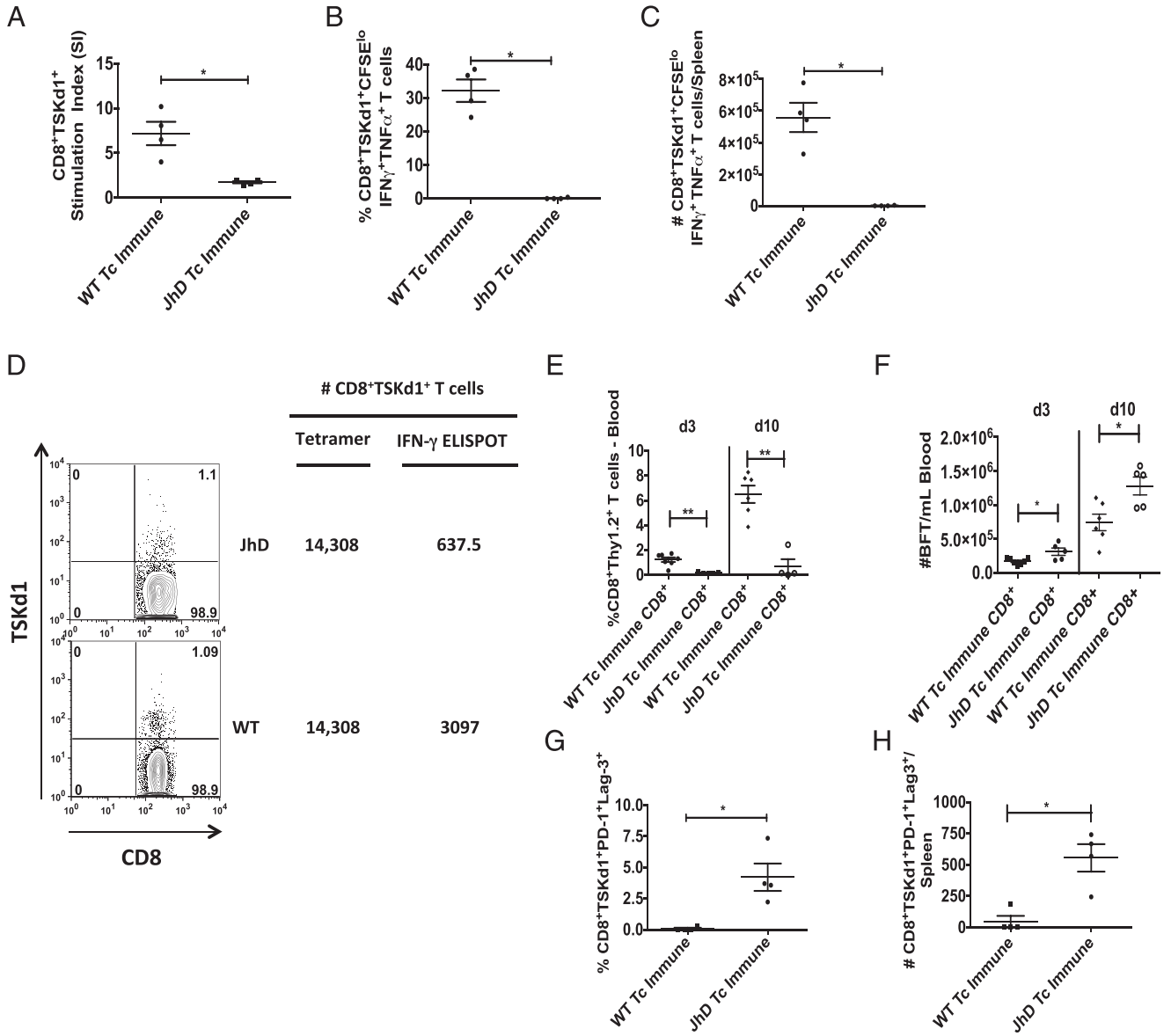

FIGURE 7. B cells prevent *T. cruzi*–specific CD8$^+$ T cell exhaustion. At least 1 mo following the last sublethal mucosal *T. cruzi* infection, Tc immune and naive B cell KO and WT BALB/c mice were systemically challenged with virulent *T. cruzi* BFT. Thirty days later, mice were sacrificed and *T. cruzi*–specific CD8$^+$ T cell responses were measured. (**A**–**C**) Splenocytes were stained with CFSE and cocultured with either control A20 or A20-TS APCs for 6 d. Some cells were used to assess CD8$^+$TSKd1$^+$ proliferation via flow cytometry (A). CD8$^+$TSKd1$^+$ stimulation indices were calculated by taking the number of live CD19$^-$CD3$^+$CD4$^-$CD8$^+$TSKd1$^+$CFSE^{lo} cells present after A20-TS stimulation divided by the number after control A20 incubation. (B and C) PMA/onomycin, GolgiStop, and GolgiPlug were added to the remaining cells and placed back in culture for 3 h prior to surface and intracellular cytokine staining. The percentages (B) and absolute numbers (C) of CD8$^+$TSKd1$^+$cytokine$^+$ T cells were calculated by taking the number of live CD19$^-$CD3$^+$CD4$^-$CD8$^+$TSKd1$^+$CFSE^{lo}cytokine$^+$ cells after stimulation with A20-TS divided by the number present after control A20 incubation. In (A)–(C), $*p < 0.05$ by Mann–Whitney *U* test. Data are representative of two independent experiments with similar results. (**D**) CD8$^+$ T cells were purified from the spleens of Tc immune B cell KO and WT BALB/c mice 87 d after systemic *T. cruzi* (BFT) challenge, and then equivalent numbers of TSKd1$^+$ T cells were transferred i.v. into naive Thy1.1$^+$ BALB/c mice. One day later, these mice were challenged systemically with virulent *T. cruzi* BFT s.c. (**E**) Three and 10 d after systemic challenge, recipient mice were bled to assess for the percentage of CD8$^+$Thy1.2$^+$ T cells in the blood via flow cytometry ($n = 4$–7 mice/group). $**p < 0.01$ by Mann–Whitney *U* test. (**F**) Ten and 14 d after systemic challenge, parasitemia was assessed in recipient mice ($n = 5$–7 mice/group). $*p < 0.05$ by Student *t* test. (**G** and **H**) Splenocytes were directly stained on day 30 after BFT challenge and assessed for the percentages (G) and absolute numbers (H) of CD8$^+$TSKd1$^+$PD-1$^+$Lag-3$^+$ T cells via flow cytometry ($n = 4$ mice/group). Data are representative of two independent experiments with similar results. Error bars represent SEM.

T cell exhaustion markers PD-1 and Lag-3 on TSKd1-specific CD8$^+$ T cells (Fig. 8F). Overall, these results demonstrate that *T. cruzi*–specific Abs could prevent death and CD8$^+$ T cell exhaustion.

Discussion

In this study, we evaluated the importance of B cells in mucosal and systemic *T. cruzi* protection. We first examined whether B cells were important for mucosal *T. cruzi* protection. Previous results suggested that B cells would play a critical role in mucosal *T. cruzi* protection (17, 25). In fact, B cells have been shown to play important roles in mucosal protection against several mucosal pathogens such as helminthes (28, 29) and rotavirus (30, 31). Although we failed to identify a critical role for B cells in mucosal *T. cruzi* protection, this does not rule out the possibility that B cell

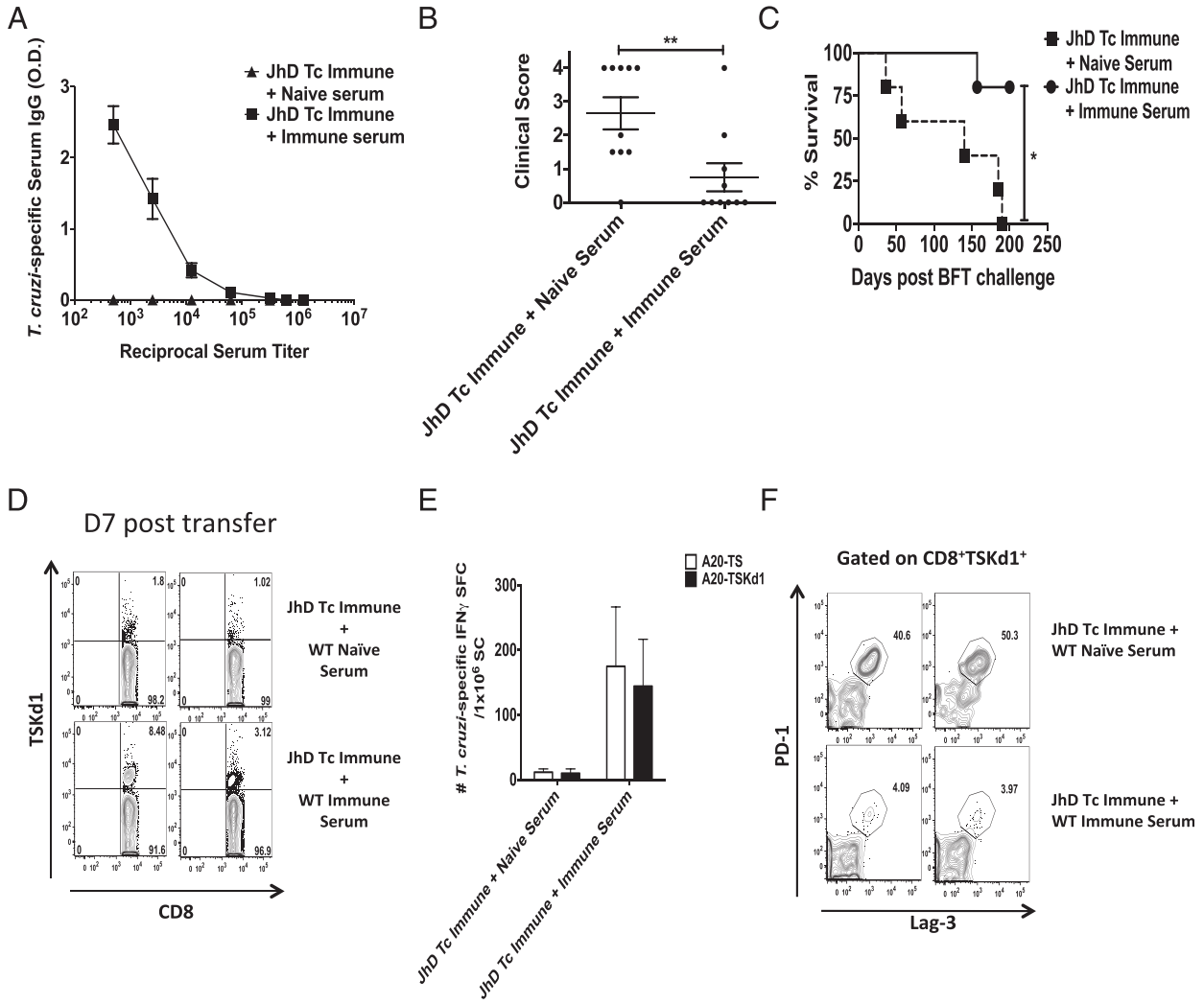

FIGURE 8. Tc immune serum can rescue CD8-mediated systemic protection in B cell KO mice. JhD B cell KO Tc immune mice were generated as described in Fig. 1 and *Materials and Methods*. Beginning at least 1 mo following the last mucosal infection, mice were injected i.p. weekly with 300 μl of either WT naive or WT Tc immune serum. (**A**) Passive serum transfers reconstitute *T. cruzi* TS-specific IgG levels. Serum-treated Tc immune B cell KO mice were then challenged systemically with virulent *T. cruzi* BFT and monitored for morbidity (**B**) and mortality (**C**). (B) Hindlimb paralysis was measured on days 30 and 87 after systemic *T. cruzi* challenge; see *Materials and Methods* for scoring system ($n = 10$ mice/group). $^{**}p = 0.0115$ by Mann–Whitney *U* test; data are representative of two independent experiments with similar results. In (C), $n = 5$ mice/group; data are representative of two independent experiments with similar results. $^{*}p < 0.05$ by log-rank test. (**D**) Five weeks after systemic challenge, spleen cells from serum-rescued B cell KO mice were assessed by flow cytometry and ELISPOT. (D) Percentage of $CD8^{+}TSKd1^{+}$ T cells measured via flow cytometry. (**E**) Frequency of IFN-γ–producing TS- and TSKd1-specific T cells measured by ELISPOT. Background responses were subtracted from the experimental values. (**F**) Frequencies of $PD\text{-}1^{+}LAG\text{-}3^{+}$ TSKd1-specific $CD8^{+}$ T cells. Error bars represent SEM.

responses could provide some level of mucosal protection. However, using both B cell–deficient mice and adoptive transfer studies, we showed that $CD8^{+}$ T cells, but not B cells, are critical for mucosal *T. cruzi* immunity.

Next, we examined the importance of B cells in systemic protection induced by multiple low-dose parasite infections or highly potent vaccine regimens (TS DNA prime/adenovirus-TS boost). B cell–deficient mice infected orally with *T. cruzi* developed higher levels of parasitemia compared with WT mice, but this did not result in increased morbidity and mortality. These results indicate that B cells are not required when systemic Ag load is low after an oral infection. In contrast, we did show that B cell responses are absolutely required for protection after systemic challenge with the more virulent BFT that leads to higher systemic Ag loads. Tc immune B cell–deficient mice survived longer than did their naive cohorts after virulent systemic parasite challenge. However, Tc immune B cell–deficient mice developed increased parasitemia over time compared with WT Tc immune mice, resulting in significant morbidity and eventual death. We also determined that *T. cruzi*–specific B cells, not polyclonal nonspecific B cells, are critically important for *T. cruzi* systemic protection using HEL BCR transgenic mice. Decreased survival in B cell KO $\mu MT^{-/-}$ mice after *T. cruzi* systemic challenge has been shown previously (16, 25). Additionally, our results are similar to recently published data demonstrating that after disseminating virus infection (lymphocytic choriomeningitis virus [LCMV]-t1b), μMT KO and anti-CD20–treated mice could not control viral replication, resulting in weight loss and decreased LCMV-specific $CD8^{+}$ T cell functionality (32). Overall, these previous results combined with our new data suggest that memory $CD8^{+}$ T cells primed in mice lacking *T. cruzi*–specific B cells are sufficient to control low levels of tissue parasitism, but they eventually become exhausted after systemic challenge due to prolonged exposure to high levels of Ag. These findings are consistent with previous data

showing that high Ag load results in $CD8^+$ T cell exhaustion (33–35). The role of B cells in preventing $CD8^+$ T cell exhaustion is nicely shown when examining the $CD8^+$ T cell response after TS vaccination. B cell–deficient mice developed similar frequencies of TSKd1-specific $CD8^+$ T cells compared with WT mice after our heterologous TS vaccine regimen. These results confirm that B cells are not required for the initial priming of $CD8^+$ T cell memory. However, after systemic challenge (resulting in high Ag load), $CD8^+TSKd1^+$ T cells from B cell–deficient TS immune mice did not expand as well as did $CD8^+$ T cells primed in WT mice. This suboptimal $CD8^+TSKd1^+$ T cell secondary expansion capacity in B cell–deficient, TS-vaccinated mice was associated with increased parasitemia and death. Thus, although *T. cruzi*–specific $CD8^+$ T cells can be primed in the absence of B cells, they become functionally deficient, severely impairing protective immunity when Ag level is high (Fig. 9).

To determine whether the defect in memory $CD8^+$ T cell secondary expansion present in Tc immune B cell KO mice was due to poor priming or deficient support for re-expansion, we studied TSKd1-specific memory $CD8^+$ T cells generated in B cell KO and WT Tc immune mice after subsequent transfer into immunocompetent BALB/c mice (Fig. 7D–H). Memory $CD8^+$ T cells from B cell KO mice failed to expand in response to systemic parasite challenge after transfer into normal hosts containing polyclonal B cells, demonstrating an intrinsic defect in $CD8^+$ T cell responses generated in mice lacking B cells. This failure of expansion resulted in significantly reduced protection. We then examined expression of the inhibitory receptors PD-1 and Lag-3 by $CD8^+TSKd1^+$ T cells after TS vaccination and *T. cruzi* infection. After systemic challenge of Tc immune and TS immune B cell–deficient mice, significant increases in $CD8^+TSKd1^+$ T cells expressing PD-1^+ and Lag-3^+ were observed. Overall, the lack of both direct ex vivo and in vivo expansion capacity, decreased cytokine production, increased inhibitory receptor expression, and reduced protection associated with memory T cells primed in the absence of B cells all indicate an important role for B cells in the prevention of $CD8^+$ T cell exhaustion. To our knowledge, these results are the first to mechanistically demonstrate the importance of B cells for prevention of *T. cruzi* $CD8^+$ T cell exhaustion.

Few studies have evaluated the role of B cells in the development of $CD8^+$ T cell memory. During primary responses to an acute LCMV Armstrong infection, $CD8^+$ T cell responses in mice lacking B cells were normal at early time points (36), but they were reduced at 154 d after infection (36, 37). In a vaccination model using *Listeria monocytogenes*, B cells were not required for the initial activation and expansion of Ag-specific $CD8^+$ T cell responses (38). However, in the absence of B cells, a more profound contraction phase occurred leading to lower numbers of persisting Ag-specific memory $CD8^+$ T cells (38). Another group showed that lack of B cells affected the long-term maintenance of $CD8^+$ T cell memory (39). Previous *T. cruzi* studies have also indicated that B cells might be important in *T. cruzi*–specific memory T cell development, as lack of mature B cells ($\mu MT^{-/-}$ mice) resulted in increased susceptibility to primary parasite infection and failure to develop robust vaccine-induced protection (16, 25). However, none of these previous studies investigated the specific role of CD8 T cell exhaustion in the absence of B cells. Our present results confirm these previous data and further demonstrate that B cells are critically important for both the development of fully functional $CD8^+$ T cell responses as well as the prevention of $CD8^+$ T cell exhaustion.

B cells may provide help for optimal $CD8^+$ T cell memory responses by several mechanisms. First, B cells can present Ag and produce cytokines. Prior to high-level systemic challenge, the frequencies of $CD8^+TSKd1^+$ T cells were similar in Tc immune B cell KO and WT mice, suggesting that general T cell priming is not affected by the absence of B cells. However, $CD8^+TSKd1^+$ T cells induced in the absence of B cells and restimulated in the presence of high systemic Ag load in vivo were functionally deficient, producing reduced multifunctional cytokine responses (IFN-γ^+TNF-α^+). It has been reported previously that B cells

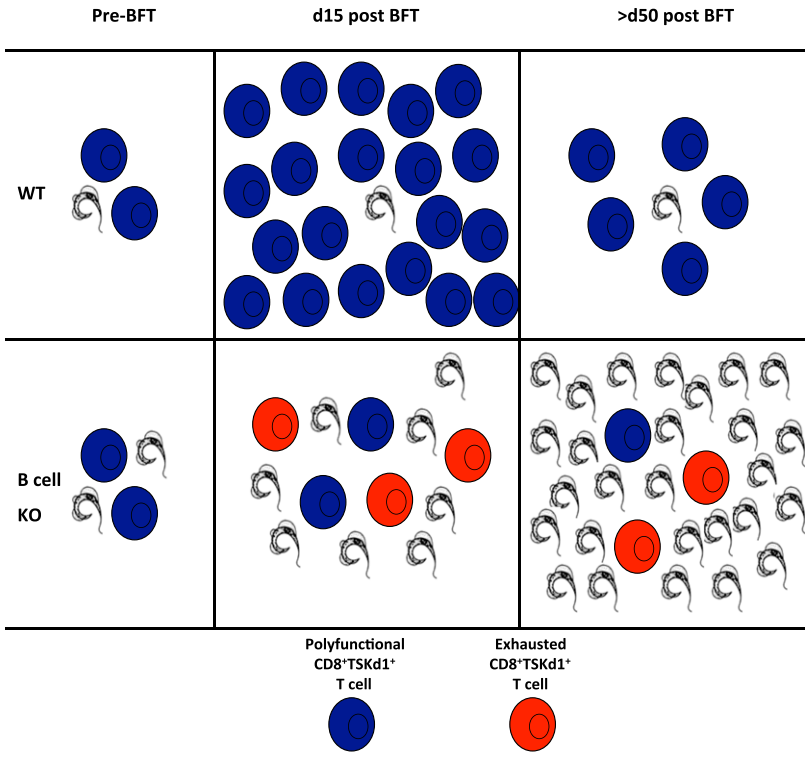

FIGURE 9. Proposed model of *T. cruzi*–specific $CD8^+$ T cell dysfunction in the absence of B cells. In both WT and JhD B cell KO mice, *T. cruzi* TSKd1-specific $CD8^+$ T cells were generated after low-dose mucosal *T. cruzi* challenge. Prior to systemic *T. cruzi* BFT challenge, there is higher parasitemia in the blood of B cell KO Tc immune mice, suggesting the early stages of $CD8^+$ T cell dysfunction. After systemic *T. cruzi* BFT challenge, polyfunctional TSKd1-specific $CD8^+$ T cells expand to high numbers in WT Tc immune mice by 15 d postchallenge. These T cells contract and are maintained at a higher number through day 50 after systemic challenge. This typical memory T cell response in WT infection-immune mice results in higher numbers and better quality T cells associated with limited parasite persistence. In B cell KO Tc immune mice, however, $CD8^+$ T cell expansion is significantly impaired. By day 15 after systemic challenge, TSKd1-specific $CD8^+$ T cells are markedly reduced in B cell–deficient mice, associated with increased parasitemia levels. By day 50, most TSKd1-specific $CD8^+$ T cells appear to be functionally exhausted due to limited proliferative and cytokine-producing capacity, and they express increased levels of the inhibitory receptors PD-1 and Lag-3. This functional exhaustion of $CD8^+TSKd1^+$ T cells in mice lacking B cells results in increased parasitemia and death.

amplify IFN-γ production by T cells in a contact-dependent manner (40). Our data also indicate that the production of Ag-specific Abs is another function of B cells critical for protective *T. cruzi* systemic immunity. Thus, in the absence of *T. cruzi*–specific IgG, increases in parasitemia and subsequent increases in overall Ag load occurred. Furthermore, passive transfer of *T. cruzi* immune sera known to reduce systemic parasite burden also reduced $CD8^+$ T cell exhaustion and mortality in B cell KO Tc immune mice after virulent systemic challenge.

Although we did not study *T. cruzi*–specific $CD4^+$ T cells, we do not think that $CD4^+$ T cells are irrelevant for inducing protective $CD8^+$ T cells. We and others have shown that $CD4^+$ T cell help is required for effective $CD8^+$ T cell memory (10, 41) and can rescue exhausted $CD8^+$ T cells (42). Work recently published showed that $CD4^+$ T cells are dysfunctional in the absence of B cells in the LCMV model (32). These data support the importance of our work, which, to our knowledge for the first time, demonstrates the principle that B cell responses are important for preventing the exhaustion of T cells directed against a major human pathogen.

Our work clearly demonstrates that previously activated memory $CD8^+$ T cells alone can protect against mucosal *T. cruzi* challenge. However, after highly virulent *T. cruzi* systemic challenges, $CD8^+$ T cells failed to provide optimal protection in the absence of B cells because $CD8^+$ T cells became functionally exhausted. We further showed that HEL-specific B cells cannot prevent $CD8^+$ T cell exhaustion, indicating that *T. cruzi*–specific B cells/Ab are needed for protection. Using both infection- and vaccine-induced memory immune models, we demonstrate that the absence of B cells leads to $CD8^+$ T cell exhaustion in mice with high Ag load. Overall, these data show that B cells are important for two interrelated aspects of Ag-specific $CD8^+$ T cell responses. First, Ag-specific B cells are critically important for the optimal development of multifunctional $CD8^+$ T cell memory. Second, *T. cruzi*–specific Abs can directly reduce overall parasite load, preventing $CD8^+$ T cell exhaustion. In conclusion, our results demonstrate that although B cells are not directly important for mucosal immunity, they do play critical roles for the induction and functional responses of $CD8^+$ memory T cells, which are absolutely essential for both mucosal and systemic protective *T. cruzi* immunity. These studies identify an important role for B cells in the prevention of $CD8^+$ T cell exhaustion, providing critical new information for vaccine design.

Acknowledgments

We thank Robert Dunn and Biogen Idec for providing the anti-CD20/isotype control Abs, Paul Garside at the University of Glasgow for providing the HEL BCR transgenic mice, Joy Eslick, Sherri Koehm, Erin Touchette, Kelly O'Shea, and Stella Hoft for technical assistance, Andreas Wieland at Emory University for critical reading of this manuscript, and the National Institutes of Health Tetramer Core Facility at Emory University for providing the $TS^{IYNVGQVSI}$/H-$2K^d$–APC tetramer.

Disclosures

The authors have no financial conflicts of interest.

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