CD8⁺ T cell immunity is compromised by anti-CD20 treatment and rescued by IL-17A

Running title: IL-17A rescued deficient CD8⁺ T cell response

Facundo Fiocca Vernengo^{a,b*}, Cristian G. Beccaria^{a,b*}, Cintia L. Araujo Furlan^{a,b}, Jimena Tosello Boari^{a,b*}, Laura Almada^{a,b}, Melisa Gorosito Serrán^{a,b}, Yamila Gazzoni^{a,b}, Carolina L. Montes^{a,b}, Eva V. Acosta Rodríguez^{a,b} and Adriana Gruppi^{a,b#}

^aDepartamento de Bioquímica Clínica, Facultad de Ciencias Químicas (FCQ), Universidad Nacional de Córdoba (UNC), Córdoba, X5000HUA, Argentina

^bCentro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI - CONICET), Córdoba, X5000HUA, Argentina.

Correspondence:

Dr Adriana Gruppi. agruppi@fcq.unc.edu.ar

*Present address:

Facundo Fiocca Vernengo: Dept. of Internal Medicine - Infectious Diseases and Respiratory Medicine. Charité Universitätsmedizin Berlin, Berlin, Germany.

Cristian G. Beccaria: Division of Immunology, Transplantation and Infectious Diseases and Experimental Imaging Center, IRCCS, San Raffaele Scientific Institute, Milán, Italy.

Jimena Tosello Boari: SiRIC TransImm “Translational Immunotherapy Team”, Translational Research Department, Research Center, Institut Curie, Paris, France.

Abstract

Treatment with anti-CD20, used in many diseases in which B cells play a pathogenic role, has been associated with susceptibility to intracellular infections. Here, we studied the effect of anti-CD20 injection on CD8⁺ T cell immunity using an experimental model of *Trypanosoma cruzi* infection, in which CD8⁺ T cells play a pivotal role. C57BL/6 mice were treated with anti-CD20 for B cell depletion prior to *T. cruzi* infection. Infected anti-CD20-treated mice exhibited a CD8⁺ T cell response with a conserved expansion phase followed by an early contraction, resulting in a strong reduction in total and parasite-specific CD8⁺ T cells at 20 days postinfection. Anti-CD20 injection decreased the number of effector and memory CD8⁺ T cells and reduced the frequency of proliferating and cytokine producing CD8⁺ T cells. Accordingly, infected anti-CD20-treated mice presented a lower cytotoxicity of *T. cruzi* peptide-pulsed target cells *in vivo*. All of these alterations in CD8⁺ T cell immunity were associated with increased tissue parasitism. Anti-CD20 injection also dampened an established CD8⁺ T cell response, indicating that B cells were involved in the maintenance rather than the induction of CD8⁺ T cell immunity. Anti-CD20 injection also resulted in a marked reduction in the frequency of IL-6- and IL-17A-producing cells, and only rIL-17A injection partially restored the CD8⁺ T cell response in infected anti-CD20-treated mice. Thus, anti-CD20 reduced CD8⁺ T cell immunity, and IL-17A is a candidate for rescuing deficient responses either directly or indirectly.

Importance

Monoclonal antibody targeting the CD20 antigen on B cells is used to treat the majority of Non-Hodgkin lymphoma patients and some autoimmune disorders. This therapy generates adverse effects, notably opportunistic infections and activation of viruses from latency. Here, using the infection murine model with the intracellular parasite *Trypanosoma cruzi*, we report that anti-CD20 treatment not only affects B cell response but also CD8⁺ T cells, the most important immune effectors involved in control of intracellular pathogens. Anti-CD20 treatment, directly or indirectly, affects cytotoxic T cell number and function and this deficient response was rescued by the cytokine IL-17A. The identification of IL-17A as the cytokine capable of reversing the poor response of CD8⁺ T cells provide information about a potential therapeutic treatment aimed at enhancing defective immunity induced by B cell depletion.

Introduction

The anti-CD20 monoclonal antibody (mAb) has revolutionized the treatment of B-cell malignancies. This antibody, which depletes B cells, has been a success for the treatment of non-Hodgkin's lymphoma (1), chronic lymphocytic leukemia (2) and autoimmune disorders (3) and has also provided information about the antibody-independent role of B cells (4, 5).

B cells are known to produce antibodies (Abs), but they also take up, process and present soluble antigens (Ags) and secrete cytokines. B cells were shown to produce IL-10 and to have regulatory functions in autoimmune models of colitis, experimental autoimmune encephalitis (EAE) and arthritis (6-8). However, B cells can produce cytokines other than IL-10. *Salmonella* triggers IL-35 and IL-10 production by B cells (9, 10), and we demonstrated that *Trypanosoma cruzi* infection leads B cells to produce IL-17A (11). In addition, B cells can produce TGF- β 1, and through this cytokine, they downregulate the function of antigen presenting cells and encephalitogenic Th1/17 responses in a murine model of multiple sclerosis (12). Therefore, under particular microenvironmental conditions, namely, through different activation and differentiation signals, B cells are able to produce different cytokines (13).

The role of B cells in conditioning CD8⁺ T cell responses has been reported in autoimmunity (14), in bacterial (15) and viral (16, 17) infections and in cancer (18). B cells have been shown to shape the profile of CD8⁺ T cells, but the mediators involved in that process have not been completely elucidated.

In Chagas disease, which is caused by the protozoan parasite *T. cruzi*, CD8⁺ T cells capable of recognizing *T. cruzi*-infected cells are essential for control of the infection. Deleting or inhibiting CD8⁺ T cells results in uncontrollable parasite load early in infection and an exacerbation of infection in chronically infected hosts (19, 20). Strategies that generate a productive specific CD8⁺ T cell response will lead to increased host protection, a reduction in symptoms, and a decrease in disease transmission (21, 22). During *T. cruzi* infection B cells undergo polyclonal expansion (23), IL-17A production (11) and also regulate CD4⁺ T cell response (24). Considering these characteristics, and the key functions of CD8⁺ T cells in controlling parasite replication, we used this experimental model to investigate how B cell depletion by anti-CD20 injection conditions CD8⁺ T cell immunity.

Materials and Methods

Mice, parasites and experimental infection

All animal experiments were approved by and conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of FCQ-UNC (Res. No. 854/18 CICUAL-FCQ). Female and age-matched (2-3-month-old) mice were used. C57BL/6 wild-type (WT; JAX:000664) and μ MT mice (B6.129S2-Ighmtm1Cgn/J; JAX:002288) were obtained from The Jackson Laboratories (USA) and housed in our animal facility. Mice were inoculated intraperitoneally (ip) with 5×10^3 trypomastigotes of *T. cruzi* (Tulahuen strain)/0.2 ml PBS (25).

Anti-CD20 injection

Mice were injected ip with 50 μ g of anti-CD20 mAb (Genentech, clone 5D2) or with mouse IgG2a control isotype (BioXcell, clone C1.18.4.) 8 days before infection with *T. cruzi* and studied at different days postinfection (dpi). In an alternative setting, mice were injected with anti-CD20 at 12 dpi and studied at 20 dpi.

Quantification of parasite DNA

Genomic DNA was purified from 50 μ g of tissue using TRIzol Reagent (Life Technologies) following the manufacturer's instructions. Satellite DNA from *T. cruzi* (GenBank AY520036) was quantified by RT-PCR as reported (24).

Cell preparation

Blood, spleen and liver-infiltrating cells were obtained as described (25).

Antibodies and flow cytometry

Cell suspensions were washed in PBS and incubated with LIVE/DEAD Fixable Cell Dead Stain (Thermo Fisher Scientific) for 15 min at room temperature. Next, the cells were washed in ice-cold FACS buffer (PBS-2% FBS) and incubated with fluorochrome-labeled Abs for 20 min at 4°C. Different combinations of the following anti-mouse Abs (Thermo Fisher Scientific, Biolegend) were used: FITC-labeled anti-CD8 (53-6.7) and anti-CD44 (IM7); PE-labeled anti-CD127 (A7R34); PerCp-Cy5.5-labeled anti-CD19 (eBio1D3), anti-CD3 (145-2C11), and anti-CD62L (MEL-14); PECy7-labeled anti-B220 (RA3-6B2), anti-KLRG1 (2F1), Alexa Fluor 647-labeled anti-CD8 (53-6.7); and APC-eFluor 780-labeled anti-CD8 (53-6.7). *T. cruzi*-specific CD8⁺ T cells were evaluated using APC-labeled tetramer of H-2K(b) molecules loaded with *T. cruzi* trans-sialidase

immunodominant ANYKFTLV (Tskb20) peptide (NIH Tetramer Core Facility) (26). After staining, cells were washed and acquired in a FACSCanto II (BD Biosciences) and analyzed with FlowJo V10 software (TreesStar). Blood was directly incubated with the abovementioned antibodies, and erythrocytes were lysed with a 0.87% NH₄Cl buffer prior to acquisition. Transcription factors (TF) were detected after cell fixation and permeabilization with a Foxp3 Staining Buffer Set according to the manufacturer's protocol (Thermo Fisher Scientific) using the following antibodies: PECy7-labeled anti-Tbet (4B10) and PE-labeled anti-Ki67 (SolA15). For intracellular cytokine staining, cells were cultured for 5 h with 50 ng/ml PMA (phorbol 12-myristate 13-acetate) (Sigma), 1 µg/ml ionomycin (Sigma), brefeldin A (Thermo Fisher Scientific) and monensin (Thermo Fisher Scientific). Cells were fixed and permeabilized with BD Cytofix/Cytoperm and Perm/Wash (BD Biosciences) according to the manufacturer's instructions. Cells were incubated with surface-staining antibodies and PE-labeled anti-IL-17A (eBio17B7), anti-IL-6 (MP5-20F3), APC-labeled anti-IFN γ (XMG1.2), and anti-IL-10 (JES5-16E3).

Immunofluorescence of Spleen

Spleens were collected and frozen over liquid nitrogen. Frozen sections 7 µm in thickness were cut, fixed for 10 min in cold acetone, left to dry at 25 °C and stored at -80 °C until use. Slides were hydrated in TRIS buffer and blocked for 30 min at 25 °C with 10% normal mouse serum in TRIS buffer. After blocking, slides were incubated for 50 min at 25 °C with different combinations of the following anti-mouse Abs (Thermo Fisher Scientific, Biolegend, BD Biosciences): Alexa Fluor 488-labeled anti-CD3 (HM3420) and anti-CD8 (53-6.7), PE-labeled anti-B220 (RA3-6B2) and anti-IL-17A (eBio17B7), APC-labeled anti-CD138 (281-2). Slices were mounted with FluorSave (Merck Millipore). For immunofluorescent staining of intracellular IL-17A, tissue sections were prepared, fixed, permeabilized and blocked using the Image-iT Fixation/Permeabilization kit (Invitrogen, R37602) prior to IL-17A staining. Images were collected with an Olympus microscope (FV1000) and processed/analyzed using ImageJ64 1.52e (National Institutes of Health, USA). For B220+ area measurements and IL-17A expression analysis, we segmented cell objects by converting images into a binary mask using the default setting, after which, standard built-in functions were used to segment out cell objects.

Apoptosis

Mitochondrial depolarization was measured by FACS using 50 nM TMRE (Thermo Fisher Scientific) as described (27). The splenic cell suspensions were stained with anti-mouse CD8 and

Tskb20/Kb tetramer prior to staining with TMRE. Non-apoptotic cells were defined as TMRE^{hi} within live single cells.

CD8⁺ T cell effector function in vitro

CD8⁺ T cell effector function was determined *in vitro* by CD107a mobilization and cytokine production, as previously reported (28). Briefly, cell suspensions were cultured for 5 h with medium, 5 µg/ml TSKB20 (ANYKFTLV) peptide (Genscript Inc.) or 50 ng/mL PMA plus 500 ng/ml ionomycin (Sigma) in the presence of monensin (Thermo Fisher Scientific) and a PE-labeled anti-CD107a mAb (Thermo Fisher Scientific, eBio1D4B). After culture, the cells were stained with a PECy7-labeled anti-CD8 mAb, fixed and permeabilized with BD Cytfix/Cytoperm and Perm/Wash (BD Biosciences) according to the manufacturer's instructions. After permeabilization, the cells were incubated for 30 min at RT with the following anti-mouse Abs (Thermo Fisher Scientific): APC-labeled anti-IFNγ (XMG1.2) and PerCp-Cy5.5-labeled anti-TNF (MP6-XT22).

In vivo cytotoxicity assay

Spleen mononuclear cell suspensions from µMT mice were pulsed for 1 h with 1 µg/ml of the Tskb20, Tskb18 or PA8 (VNHRFTLV) peptides (target cells). In this setting, µMT mice, which lack mature B cells, were used to avoid circulating anti-CD20 killing of the transferred cells. Unpulsed splenocytes were used as a control. Target and control cells were washed and stained with 2 µM or 20 µM of CFSE and 5 µM of the Cell Proliferation Dye eFluorTM670 (Thermo Fisher Scientific). After staining, cells were mixed in equal proportions and injected iv into uninfected and infected control and anti-CD20-treated mice at 20 dpi. Mice were sacrificed 5 h later, and the frequency of injected cells in the spleen was evaluated by flow cytometry. The percentage of specific lysis was calculated between the unloaded and each of the different peptide-loaded cells independently as follows: $100 - \left(\frac{(\% \text{ peptide pulsed in infected} / \% \text{ unpulsed in infected})}{(\% \text{ peptide pulsed in uninfected} / \% \text{ unpulsed in uninfected})} \right) \times 100$.

Cytokine quantification

Serum IL-6 and IL-17A concentrations were assessed by ELISA according to the manufacturer's instructions (Thermo Fisher Scientific).

In vivo IL-6 and IL-17A treatment

Anti-CD20-treated mice were administered with recombinant IL-6 or IL-17 taking into account their production kinetics (Fig. 5A). Either IL-6 (200ng/dose) (29) or IL-17A (500ng/dose) (30) (Shenandoah – EEUU) were injected intraperitoneally at 12, 14, 16 and 18 dpi. Anti-CD20-treated mice injected with PBS were used as the non-cytokine-treated control group.

Statistics

The statistical significance of comparisons of mean values was assessed as indicated by a two-tailed Student's t test and two-way ANOVA followed by Bonferroni's posttest using GraphPad software.

Results

Anti-CD20 treatment decreased the number of CD8⁺ T cells and increased tissue parasitism

As reported by Tosello Boari et al (28), we determined by flow cytometry that the frequency and number of CD8⁺ T cells increased during *T. cruzi* infection. CD8⁺ T cell expansion peaked at 20 dpi and was followed by the contraction phase of the response (Fig. 1A, control mice). Anti-CD20 injection, 8 days prior to the infection, significantly decreased splenic B cell numbers (Fig. S1 A-C), which persisted at very low levels until 20 dpi (Fig. S1B) and influenced CD8⁺ T cells (Fig. 1A-C). Anti-CD20 injection resulted in a significantly reduced CD8⁺ T cell frequency and number at 20 dpi (Fig. 1A, αCD20). As expected from the depletion of B cells, normal uninfected anti-CD20-treated mice had a higher frequency of CD8⁺ T cells compared to uninfected isotype-treated control mice (Fig. 1A, day 0). The frequency and number of splenic Tskb20-specific CD8⁺ T cells (parasite specific CD8⁺T cells) detected in infected control mice were similar to those of infected anti-CD20-treated mice until 14 dpi (Fig. 1B). After that, the percentage dropped dramatically in anti-CD20-treated mice, which presented a significantly lower frequency and number of *T. cruzi*-specific CD8⁺ T cells at 20 dpi, remaining low up to 35 dpi (the last day of our analysis) (Fig. 1B). The frequency and number of Tskb20-specific CD8⁺ T cells was also reduced in liver, a *T. cruzi* infection target organ, of infected anti-CD20-treated mice at 20 dpi (Fig. 1C). In concordance with the low number of total and parasite-specific CD8⁺ T cells, anti-CD20-treated mice infected with *T. cruzi* had a higher parasite load than controls, as evidenced by the *T. cruzi* DNA fold increase in the liver, spleen and heart at 20 dpi (Fig. 1D).

Anti-CD20 treatment decreased the number of effector and memory CD8⁺ T cells and compromised their survival and proliferation

An assessment of CD8⁺ T cell subset distribution, based on CD62L versus CD44 expression, showed that the spleen of infected anti-CD20-treated mice at 20 dpi presented a higher percentage of naïve (CD62L^{hi}CD44^{neg}) and lower percentages of effector memory/effector (CD62L^{neg}CD44^{hi}) CD8⁺ T cells in comparison to their counterparts from infected control mice (Fig. 2A). When the values were expressed in absolute numbers, we determined that anti-CD20-treated mice exhibited a significant reduction in all CD8⁺ T cell subpopulations (Fig. 2A). The anti-CD20 injection decreased the frequency and number of splenic Tskb20/Kb⁺ CD8⁺ T cells with an effector phenotype but not the number of Tskb20/Kb⁺ CD8⁺ T cells with a naïve or central memory phenotype (Fig. 2B).

Considering that the general features of protective CD8⁺ T cell responses against intracellular pathogens consist of the generation and expansion of short-lived highly functional effector

populations, we also evaluated the phenotype of CD8⁺ T cells based on KLRG-1 and CD127 expression (31). The frequency and number of total and parasite-specific CD8⁺ T cells with a short-lived effector cell (SLEC) phenotype (CD44⁺KLRG1^{hi}CD127^{lo}) were significantly reduced in the group of anti-CD20-treated mice at 20 dpi (Fig. 2C-D). The frequencies of total (Fig. 2C) and Tskb20/Kb⁺ (Fig. 2D) CD8⁺ T cells with a memory precursor effector cell (MPEC) phenotype (CD44⁺KLRG1^{lo}CD127^{hi}) were similar in both experimental groups, but a strong reduction in the number of MPECs was observed in the infected anti-CD20-treated mice with respect to the infected control mice (Fig. 2C-D).

Splenic total and parasite-specific CD8⁺ T cells from infected anti-CD20-treated mice had a significantly lower frequency of proliferating Ki-67⁺ cells (Fig. 2E) and TMRE^{hi} cells, which is compatible with cellular viability (Fig. 2F). These results indicate that anti-CD20 injection (B cell depletion) partially arrested CD8⁺ T cell proliferation, leading to an early contraction of this protective response.

Anti-CD20 treatment resulted in lower CD8⁺ T cell functional activity

In comparison to counterparts from infected control mice, splenic CD8⁺ T cells from infected anti-CD20-treated mice had a reduced frequency of polyfunctional CD8⁺ T cells (Fig. 3A), which are characterized by the simultaneous secretion of multiple cytokines and degranulation (see gating strategies in Fig. S2). Infected anti-CD20-injected mice exhibited a reduced frequency of IFNγ⁺ TNF⁺ CD107a⁺ (triple-positive), CD8⁺ T cells and IFNγ⁺ TNF⁺ or IFNγ⁺ CD107a⁺ (double-positive) CD8⁺ T cells after *in vitro* stimulation with PMA and ionomycin in comparison to infected control mice (Fig. 3A). Not only polyfunctional CD8⁺ T cells were affected but the frequency of total IFNγ- or TNF-producing CD8⁺ T cells was also reduced in infected anti-CD20-treated mice (Fig. S3A, see polyclonal stimulation). In addition, splenocytes from infected anti-CD20-treated mice cultured with the parasite peptide Tskb20 had a reduced frequency of total IFNγ⁺, TNF⁺ and CD107a⁺ and triple- and double-positive CD8⁺ T cells when compared with splenocytes from controls (Fig. S3 A-B, see Ag-specific stimulation). As highlighted by the reduced MFI values in flow cytometry evaluation, we observed that Tskb20-specific CD8⁺ T cells had lower IFNγ expression, indicating less IFNγ production by parasite-specific CD8⁺ T cells from infected anti-CD20-treated mice (Fig. S3C). In agreement with the reduction in the frequency of IFNγ-producing CD8⁺ T cells, total and parasite-specific CD8⁺ T cells from infected anti-CD20-treated mice exhibited lower Tbet expression than CD8⁺ T cells from control mice (Fig. 3B). Interestingly, CD8⁺ T cells from infected anti-CD20-treated mice had similar levels of Tbet compared to CD8⁺ T cells from uninfected mice (Fig. 3B, see histograms).

Next, we evaluated *in vivo* the cytotoxic capacity elicited in infected anti-CD20-treated mice in comparison to that in infected control and uninfected mice. For the evaluation, differentially stained antigen-parasite-loaded cells were transferred to the different groups of mice. Fig. 3C shows no specific lysis of PA8-loaded cells since this peptide is mainly present in other strains of the parasite, which are different from the strain used in this study (32). In addition, in infected anti-CD20-treated mice, the frequency of CFSE^{hi} eFluor670^{neg} Tskb20-pulsed cells and CFSE^{neg} eFluor670⁺ Tskb18-pulsed cells was higher than that in infected controls. The results indicate that infected anti-CD20-treated mice displayed a reduced capacity to specifically kill target cells pulsed with the parasite antigens Tskb20 and Tskb18.

Anti-CD20 treatment affected an established CD8⁺ T cell response

To analyze whether anti-CD20 injection affected an already established CD8⁺ T cell response, *T. cruzi*-infected mice were injected with anti-CD20 mAb at 12 dpi when the parasite-specific CD8⁺ T cell frequency had nearly peaked (see Fig. 1B). We determined that the injection of anti-CD20 at day 12 dpi induced a reduction in the frequency and number of splenic parasite-specific CD8⁺ T cells after 20 dpi, which was comparable with the effect of the treatment initiated before infection (Fig. 4A). When compared to infected control mice, mice treated with anti-CD20 after 12 dpi exhibited a significantly reduced number of CD8⁺ T cells with SLEC and MPEC phenotypes (Fig. 4B) and a diminished frequency of IFN γ ⁺ CD8⁺ T cells in splenocytes evaluated after polyclonal or antigen-specific stimulation (Fig. 4C). In line with the reduced frequency of IFN γ ⁺-producing cells, total and parasite-specific CD8⁺ T cells from infected mice treated with anti-CD20 mAb at 12 dpi expressed lower levels of Tbet than CD8⁺ T cells from infected control mice (Fig. 4D). In our experimental model, the results indicated that anti-CD20 injection did not affect the induction of the CD8⁺ T cell response (see Fig. 1A) but probably affected their survival/maintenance.

B cells from T. cruzi-infected mice produce cytokines involved in CD8⁺ T cell survival

Cytokines contribute to the regulation of the contraction of the response, as well as the long-term maintenance of memory CD8⁺ T cells (28, 33-35). Based on this and considering that anti-CD20 injection depletes B cells from mice, we hypothesize that B cells could be the source of cytokines involved in the maintenance of the CD8⁺ T cell response. By flow cytometry, we observed that *T. cruzi*-infected mice had IL-6 and IL-17A-producing B cells, and the numbers of these cells peak at 15 dpi and remained high at 20-30 dpi (Fig. 5A). In comparison to lymphoid non-B cells, B cells

were the main source of IL-6 and IL-17A (Fig. 5A), while the main source of IL-10, IFN γ and TNF within the lymphoid population were non-B cells (Fig. S4).

When mice were treated with anti-CD20 mAb prior to infection, a significant decrease in IL-6- and IL-17A-producing lymphoid cells was observed (Fig. 5B). Most of the cytokine-producing B cells were CD19^{low}, which is compatible with the plasmablast phenotype (11). Interestingly, anti-CD20 injection before the infection did not affect serum IL-6 concentration since IL-6 values were similar either at 15 and 20 dpi (Fig. 5C). The IL-17A concentration was undetectable in the sera from both groups of infected mice (data not shown).

Next, by immunofluorescence, we evaluated the spatial distribution of splenic B-, CD8⁺ T- and IL-17A-producing cells. Fig. 5D shows B cell follicles (B220⁺) and CD3⁺ T cells in uninfected and infected control mice. As expected, the extrafollicular plasmablasts (CD138⁺) were located in the T cell zone only in the spleen of infected control mice (23). Interestingly, IL-17A-producing plasmablasts and other IL-17A-producing cells were close to CD8⁺ T cells (Fig. 5E), suggesting a potential interaction/cross talk between IL-17A-producing cells and CD8⁺ T cells.

Interestingly we observed that anti-CD20 injection previous to infection did not affect neither the frequency and number of total CD4⁺ T cells (Fig. S5A) nor Tbet expression in CD4⁺ T cells (Fig. S5B). However, anti-CD20 treatment in infected mice also decreased the frequency and number of IL-17A⁺CD4⁺T cells (Fig. S5C)

Recombinant IL-17A, but not IL-6, partially restored the number and function of CD8⁺ T cells in infected anti-CD20-treated mice

To evaluate the hypothesis that the absence/diminution of IL-17A could affect the maintenance of the CD8⁺ T cell response in infected anti-CD20-treated mice, groups of these mice were injected with rIL-17A or PBS. Fig. 6 shows that injections of rIL-17A partially increased the frequency and number of total and Tskb20-specific CD8⁺ T cells (Fig. 6A). In particular, the frequency and number of total and Tskb20-specific SLEC CD8⁺ T cells, but not the frequency and number of the MPEC CD8⁺ T cell population, were increased by rIL-17A supplementation (Fig. 6B).

In addition, rIL-17A partially increased the frequency and number of IFN γ -producing CD8⁺ T cells and restored the frequency of TNF-producing CD8⁺ T cells (Fig. 7A). The increase in the frequency of IFN γ -producing CD8⁺ T cells in infected anti-CD20-treated mice injected with rIL-17A was accompanied by an increase in Tbet expression in total and Tskb20-specific CD8⁺ T cells (Fig. 7B).

322 As expected, the increase in the number and functionality of CD8⁺ T cells in infected anti-CD20-
 323 treated mice injected with rIL-17A was associated with a strong reduction in the parasite load in the
 324 liver, spleen and heart (Fig. 7C).
 325 Recombinant IL-6 injection in infected anti-CD20 treated mice did not increase the frequency and
 326 number of total and Tskb20-specific CD8⁺ T cells (Fig. 8A) and did not modify the frequency and
 327 number of SLECs and MPECs CD8⁺ T cell subsets (Fig. 8B). In addition, IL-6 injection to infected
 328 anti-CD20 treated mice was not able to modify total and Tskb20-specific INFγ-producing CD8⁺ T
 329 cells (Fig. 8C), nor Tbet expression in CD8⁺ T cells (Fig. 8D).

Discussion

An understanding of the effects of anti-CD20 treatment, which leads to the elimination of B cells, on other cell types allows the identification of different side effects of this therapy and highlights the Ab-independent functions of B cells. In this study, we show that anti-CD20 injection altered antiparasitic CD8⁺ T cell immunity when administered prior to the infection or after when the specific CD8⁺ T cell response was already established. In our work, we determined that the CD8⁺ T cell decrease in infected anti-CD20-treated mice affected both effector and memory cell numbers. Our kinetics studies indicated that treatment with anti-CD20 prior to infection did not affect the induction phase of the CD8⁺ T cell response, indicating that B cells are not involved in the initial events that drive CD8⁺ T cell immunity during *T. cruzi* infection. Similar results were reported for *Listeria monocytogenes* infection (15), in which B cells did not play any role in the initial activation or microorganism-driven expansion of CD8⁺ T cells. Instead, we determined that, similar to the *L. monocytogenes* infection model, depletion of B cells by anti-CD20 injection significantly accelerated the contraction phase of the CD8⁺ T cell response in *T. cruzi*-infected mice. Given the lower frequency of viable (TMRE^{hi}) and proliferating CD8⁺ T cells determined in infected anti-CD20-treated mice, it is likely that a reduced expansion rate could be responsible for the early contraction of the CD8⁺ T cell response. In a similar way, a significant, long-lasting and reversible depletion effect on CD8⁺ T cell counts was reported in patients with rheumatoid arthritis after 12 and 24 weeks of treatment with rituximab (36).

It has been reported that B cells can tolerize CD8⁺ T cells (37), but we found that during *T. cruzi* infection, CD8⁺ T cells became less functional in the absence of B cells. Anti-CD20 treatment reduced the functionality of CD8⁺ T cells, as evidenced by a strong reduction in the frequency of cytokine-producing (IFN γ or TNF) and polyfunctional (cytokine-producing and degranulating) CD8⁺ T cells. *T. cruzi* peptide-pulsed cell transfer demonstrated that infected anti-CD20-treated mice had a significantly reduced capability to lyse not only Tskb20-loaded target cells but also Tskb18-loaded cells *in vivo*, suggesting that this treatment affected not only the immunodominant CD8⁺ T cell response but also other *T. cruzi*-specific CD8⁺ T cell responses. Administration of the anti-CD20 treatment before or after infection led to an early contraction of the CD8⁺ T cell response, suggesting that B cells, either directly or indirectly, participate in the maintenance of CD8⁺ T cells.

Defective CD8⁺ T cell functional activity has been associated with increased pathogen load during chronic infections (38). The role of polyfunctional CD8⁺ T cells in the control of intracellular microorganisms was highlighted in patients infected with HIV, in which the frequency and proportion of the HIV-specific T cell response with the highest functionality inversely correlated

with the viral load in progressors (39). Accordingly, the deficient polyfunctionality of CD8⁺ T cells from infected anti-CD20-treated mice was associated with a higher parasite load at 20 dpi. Notably, it has been reported that patients treated with anti-CD20 can develop different viral infections, which are the most common non-hematological adverse effects of this therapy. Viral infections in patients receiving anti-CD20 include severe respiratory tract infections, hepatitis B virus reactivation and varicella-zoster virus infection (40-42). Based on our results, we hypothesize that treatment of patients with anti-CD20 not only affects Ab-secreting cells, which can neutralize viruses, but could also affect the development or establishment of CD8⁺ T cell responses, which are necessary for the control of intracellular infections. Additionally, we also observed that anti-CD20 did not modify CD4⁺ T cell frequency and number but significantly affected IL-17 producing CD4⁺ T cells. The results indicate that B cell depletion, directly or indirectly through the reduction of IL-17 producing cells reduce CD8⁺ T cell immunity. Indeed, we observed that rIL-17 was able to partially reverse deficient CD8⁺ T cell response.

In *T. cruzi* infection, two cytokines, IL-6 and IL-17A, have been reported to be involved in the improvement and maintenance of the CD8⁺ T cell response (28, 33). IL-6 improves the cytotoxic CD8⁺ T cell dysfunction triggered by nitric oxide in patients with Chagas Disease (33); additionally, we recently demonstrated that IL-17RA and IL-17A are critical factors for sustaining CD8⁺ T cell immunity to *T. cruzi* (28). A possible role of IL-6 in the maintenance of the CD8⁺ T cell response in our experimental model was ruled out by the fact that anti-CD20 treatment did not modify the concentration of serum IL-6 in infected mice and that rIL-6 injection did not improve or revert the deficient CD8⁺ T cell response. In contrast, we observed that rIL-17A was able to reverse the quantitative and functional reduction in CD8⁺ T immunity observed in *T. cruzi*-infected anti-CD20-treated mice. The results reported here reinforce the recent data obtained in our lab about the IL-17A/IL-17RA pathway-mediated roles of CD8⁺ T cells (28) and position IL-17A as a key cytokine in the maintenance of the cytotoxic T cell response. Our results are also supported by the findings reported by Acharya et al (43), which showed that IL-17A directly potentiates CD8⁺ T cell cytotoxicity against West Nile Virus infection.

In line with our findings, it was reported that μ MT mice infected with *T. cruzi* Tulahuén strain exhibited a marked reduction in the CD8⁺ T-cell subpopulation (44). Also, Sullivan and colleagues (45) observed that mice deficient in B cells that are infected with *T. cruzi* had a defective CD8⁺ T cell response. They postulated that specific antibodies are capable of restoring the deficient CD8⁺ T cell response, as the passive transfer of serum from infected but not normal mice reversed the magnitude and functionality as well as the exhausted phenotype observed in CD8⁺ T cells (45). Considering that the transfer of purified antibodies was not performed in that study, it is possible that

the observed effect may be associated with the presence of IL-17A in addition to the specific antibodies.

High microorganism load, together with persistent antigenic stimulation, are considered the most important factors influencing CD8⁺ T cell exhaustion and consequent loss of functionality. In *T. cruzi* infection, the relationship between parasite load and CD8⁺ T cell dysfunction is controversial. We (28) and others (46) have reported that infection of C57BL/6 mice with increasing parasite loads does not result in reduced CD8⁺ T cell cytotoxic effector function or the deletion of parasite-specific CD8⁺ T cells. It is difficult to determine whether antigen persistence or parasite load contribute to the dysfunctional CD8⁺ T cell response that we observed in infected anti-CD20-treated mice since rIL-17A injection substantially reduced the parasite load while simultaneously improve CD8⁺ T cell response.

In this work, we have not established whether IL-17A acts, directly or indirectly, on CD8⁺ T cells. In line with a direct effect, we reported that CD8⁺ T cells with a memory phenotype express the highest levels of the IL-17A receptor in comparison to naïve and effector CD8⁺ T cells (28). However, in our experimental model, IL-17A injection increased the number of CD8⁺ T cells with the SLEC but not with the MPEC phenotype. Since MPEC are not terminally differentiated cells, it is possible that IL-17A may favor MPEC survival and simultaneously promote MPEC differentiation into SLECs. Nevertheless, we cannot discount an indirect function of IL-17A since this cytokine has been reported to favor cytotoxic T cell responses against *L. monocytogenes* infection by enhancing dendritic cell cross presentation (47).

In conclusion, our work provides evidence that anti-CD20 treatment affects not only B cell numbers but also IL-17 producing cells and CD8⁺ T cell responses. This knowledge may be relevant for the clinical management of patients with autoimmune diseases or lymphomas who are receiving anti-CD20 treatment. Furthermore, given that IL-17A was able to revert CD8⁺ T cell dysfunction in treated hosts, targeting the IL-17R pathway can help to control not only viral infections but also fight against other microbes and eventually tumors.

Acknowledgments

This work was supported by grants from the Agencia Nacional de Promoción Científica y Técnica (Foncyt, PICT 2011-2647 and PICT 2015-0645), Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET, (PIP 112-20110100378), the Secretaría de Ciencia y Técnica-Universidad Nacional de Córdoba and the National Institute of Allergy And Infectious Diseases of the National Institutes of Health under Award Numbers R01AI116432 and R01AI110340.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication

The authors would like to thank M.P. Abadie, M.P. Crespo, V. Blanco, F. Navarro, D. Lutti, A. Romero, L. Gatica, G. Furlán, C. Sampredo and C.R. Mas for their excellent technical assistance.

C.L.M., E.V.A.R. and A.G. are researchers from CONICET. F.F.V., C.G.B., C.L.A.F., J.T.B., L.A., Y.G. and M.G.S. thanks CONICET for the fellowship awarded.

We also acknowledge Genentech (S. San Francisco, CA) for provision of the anti-mouse-CD20 mAb and the NIH Tetramer Core Facility for provision of the APC-labeled Tskb20/Kb tetramer.

Author contributions

Conceptualization: F.F.V. and A.G.; Methodology: F.F.V. and A.G.; Formal Analysis: F.F.V. and C.G.B.; Investigation: F.F.V., C.G.B., C.L.A.F., J.T.B., L.A. Y.G. and M.G.S; Resources: A.G.; Writing – Original Draft: F.F.V. and A.G.; Writing – Review & Editing: C.L.M. and E.V.A.R.; Visualization: F.F.V.; Supervision: A.G.; E.V.A.R. and C.L.M.; Project Administration: A.G.; Funding Acquisition: A.G.; E.V.A.R. and C.L.M.

Conflicts of interest

The authors declare no competing interests.

449 References

- 450 1. Coiffier B, Thieblemont C, Van Den Neste E, Lepeu G, Plantier I, Castaigne S, Lefort S,
451 Marit G, Macro M, Sebban C, Belhadj K, Bordessoule D, Ferme C, Tilly H. 2010. Long-term
452 outcome of patients in the LNH-98.5 trial, the first randomized study comparing rituximab-
453 CHOP to standard CHOP chemotherapy in DLBCL patients: a study by the Groupe d'Etudes
454 des Lymphomes de l'Adulte. *Blood* 116:2040-5.
- 455 2. Hallek M, Fischer K, Fingerle-Rowson G, Fink AM, Busch R, Mayer J, Hensel M, Hopfinger
456 G, Hess G, von Grunhagen U, Bergmann M, Catalano J, Zinzani PL, Caligaris-Cappio F,
457 Seymour JF, Berrebi A, Jager U, Cazin B, Trneny M, Westermann A, Wendtner CM,
458 Eichhorst BF, Staib P, Buhler A, Winkler D, Zenz T, Bottcher S, Ritgen M, Mendila M,
459 Kneba M, Dohner H, Stilgenbauer S, International Group of I, German Chronic Lymphocytic
460 Leukaemia Study G. 2010. Addition of rituximab to fludarabine and cyclophosphamide in
461 patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. *Lancet*
462 376:1164-74.
- 463 3. Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowska A, Emery P, Close DR,
464 Stevens RM, Shaw T. 2004. Efficacy of B-cell-targeted therapy with rituximab in patients
465 with rheumatoid arthritis. *N Engl J Med* 350:2572-81.
- 466 4. Kelly-Scumpia KM, Scumpia PO, Weinstein JS, Delano MJ, Cuenca AG, Nacionales DC,
467 Wynn JL, Lee PY, Kumagai Y, Efron PA, Akira S, Wasserfall C, Atkinson MA, Moldawer
468 LL. 2011. B cells enhance early innate immune responses during bacterial sepsis. *J Exp Med*
469 208:1673-82.
- 470 5. Elsegeiny W, Eddens T, Chen K, Kolls JK. 2015. Anti-CD20 antibody therapy and
471 susceptibility to *Pneumocystis pneumonia*. *Infect Immun* 83:2043-52.
- 472 6. Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK. 2002. Chronic intestinal
473 inflammatory condition generates IL-10-producing regulatory B cell subset characterized by
474 CD1d upregulation. *Immunity* 16:219-30.
- 475 7. Fillatreau S, Sweeney CH, McGeachy MJ, Gray D, Anderton SM. 2002. B cells regulate
476 autoimmunity by provision of IL-10. *Nat Immunol* 3:944-50.
- 477 8. Mauri C, Gray D, Mushtaq N, Londei M. 2003. Prevention of arthritis by interleukin 10-
478 producing B cells. *J Exp Med* 197:489-501.
- 479 9. Neves P, Lampropoulou V, Calderon-Gomez E, Roch T, Stervbo U, Shen P, Kuhl AA,
480 Loddenkemper C, Haury M, Nedospasov SA, Kaufmann SH, Steinhoff U, Calado DP,
481 Fillatreau S. 2010. Signaling via the MyD88 adaptor protein in B cells suppresses protective
482 immunity during *Salmonella typhimurium* infection. *Immunity* 33:777-90.
- 483 10. Shen P, Roch T, Lampropoulou V, O'Connor RA, Stervbo U, Hilgenberg E, Ries S, Dang
484 VD, Jaimes Y, Daridon C, Li R, Jouneau L, Boudinot P, Wilantri S, Sakwa I, Miyazaki Y,
485 Leech MD, McPherson RC, Wirtz S, Neurath M, Hoehlig K, Meinl E, Grutzkau A, Grun JR,
486 Horn K, Kuhl AA, Dorner T, Bar-Or A, Kaufmann SHE, Anderton SM, Fillatreau S. 2014.
487 IL-35-producing B cells are critical regulators of immunity during autoimmune and
488 infectious diseases. *Nature* 507:366-370.
- 489 11. Bermejo DA, Jackson SW, Gorosito-Serran M, Acosta-Rodriguez EV, Amezcua-Vesely MC,
490 Sather BD, Singh AK, Khim S, Mucci J, Liggitt D, Campetella O, Oukka M, Gruppi A,
491 Rawlings DJ. 2013. *Trypanosoma cruzi* trans-sialidase initiates a program independent of the
492 transcription factors ROR γ and Ahr that leads to IL-17 production by activated B cells.
493 *Nat Immunol* 14:514-22.
- 494 12. Bjarnadottir K, Benkhoucha M, Merkler D, Weber MS, Payne NL, Bernard CC, Molnarfi N,
495 Lalive PH. 2016. B cell-derived transforming growth factor- β expression limits the
496 induction phase of autoimmune neuroinflammation. *Sci Rep* 6:34594.

- 497 13. Vazquez MI, Catalan-Dibene J, Zlotnik A. 2015. B cells responses and cytokine production
498 are regulated by their immune microenvironment. *Cytokine* 74:318-26.
- 499 14. Guo L, Kapur R, Aslam R, Speck ER, Zufferey A, Zhao Y, Kim M, Lazarus AH, Ni H,
500 Semple JW. 2016. CD20+ B-cell depletion therapy suppresses murine CD8+ T-cell-mediated
501 immune thrombocytopenia. *Blood* 127:735-8.
- 502 15. Shen H, Whitmire JK, Fan X, Shedlock DJ, Kaech SM, Ahmed R. 2003. A specific role for B
503 cells in the generation of CD8 T cell memory by recombinant *Listeria monocytogenes*. *J*
504 *Immunol* 170:1443-51.
- 505 16. Lykken JM, DiLillo DJ, Weimer ET, Roser-Page S, Heise MT, Grayson JM, Weitzmann
506 MN, Tedder TF. 2014. Acute and chronic B cell depletion disrupts CD4+ and CD8+ T cell
507 homeostasis and expansion during acute viral infection in mice. *J Immunol* 193:746-56.
- 508 17. Asano MS, Ahmed R. 1996. CD8 T cell memory in B cell-deficient mice. *J Exp Med*
509 183:2165-74.
- 510 18. DiLillo DJ, Yanaba K, Tedder TF. 2010. B cells are required for optimal CD4+ and CD8+ T
511 cell tumor immunity: therapeutic B cell depletion enhances B16 melanoma growth in mice. *J*
512 *Immunol* 184:4006-16.
- 513 19. Tarleton RL. 2007. Immune system recognition of *Trypanosoma cruzi*. *Curr Opin Immunol*
514 19:430-4.
- 515 20. Fernandes MC, Andrews NW. 2012. Host cell invasion by *Trypanosoma cruzi*: a unique
516 strategy that promotes persistence. *FEMS Microbiol Rev* 36:734-47.
- 517 21. Jordan KA, Hunter CA. 2010. Regulation of CD8+ T cell responses to infection with
518 parasitic protozoa. *Exp Parasitol* 126:318-25.
- 519 22. Padilla AM, Simpson LJ, Tarleton RL. 2009. Insufficient TLR activation contributes to the
520 slow development of CD8+ T cell responses in *Trypanosoma cruzi* infection. *J Immunol*
521 183:1245-52.
- 522 23. Bermejo DA, Amezcua Vesely MC, Khan M, Acosta Rodriguez EV, Montes CL, Merino
523 MC, Toellner KM, Mohr E, Taylor D, Cunningham AF, Gruppi A. 2011. *Trypanosoma cruzi*
524 infection induces a massive extrafollicular and follicular splenic B-cell response which is a
525 high source of non-parasite-specific antibodies. *Immunology* 132:123-33.
- 526 24. Gorosito Serran M, Tosello Boari J, Fiocca Vernengo F, Beccaria CG, Ramello MC, Bermejo
527 DA, Cook AG, Vinuesa CG, Montes CL, Acosta Rodriguez EV, Gruppi A. 2017.
528 Unconventional Pro-inflammatory CD4(+) T Cell Response in B Cell-Deficient Mice
529 Infected with *Trypanosoma cruzi*. *Front Immunol* 8:1548.
- 530 25. Tosello Boari J, Amezcua Vesely MC, Bermejo DA, Ramello MC, Montes CL, Cejas H,
531 Gruppi A, Acosta Rodriguez EV. 2012. IL-17RA signaling reduces inflammation and
532 mortality during *Trypanosoma cruzi* infection by recruiting suppressive IL-10-producing
533 neutrophils. *PLoS Pathog* 8:e1002658.
- 534 26. Martin DL, Weatherly DB, Laucella SA, Cabinian MA, Crim MT, Sullivan S, Heiges M,
535 Craven SH, Rosenberg CS, Collins MH, Sette A, Postan M, Tarleton RL. 2006. CD8+ T-Cell
536 responses to *Trypanosoma cruzi* are highly focused on strain-variant trans-sialidase epitopes.
537 *PLoS Pathog* 2:e77.
- 538 27. Amezcua Vesely MC, Schwartz M, Bermejo DA, Montes CL, Cautivo KM, Kalergis AM,
539 Rawlings DJ, Acosta-Rodriguez EV, Gruppi A. 2012. FcγRIIb and BAFF differentially
540 regulate peritoneal B1 cell survival. *J Immunol* 188:4792-800.
- 541 28. Tosello Boari J, Araujo Furlan CL, Fiocca Vernengo F, Rodriguez C, Ramello MC, Amezcua
542 Vesely MC, Gorosito Serran M, Nunez NG, Richer W, Piaggio E, Montes CL, Gruppi A,
543 Acosta Rodriguez EV. 2018. IL-17RA-Signaling Modulates CD8+ T Cell Survival and
544 Exhaustion During *Trypanosoma cruzi* Infection. *Front Immunol* 9:2347.
- 545 29. Quinton LJ, Blahna MT, Jones MR, Allen E, Ferrari JD, Hilliard KL, Zhang X, Sabharwal V,
546 Algul H, Akira S, Schmid RM, Pelton SI, Spira A, Mizgerd JP. 2012. Hepatocyte-specific

mutation of both NF-kappaB RelA and STAT3 abrogates the acute phase response in mice. *J Clin Invest* 122:1758-63.

30. Yang XO, Chang SH, Park H, Nurieva R, Shah B, Acero L, Wang YH, Schluns KS, Broaddus RR, Zhu Z, Dong C. 2008. Regulation of inflammatory responses by IL-17F. *J Exp Med* 205:1063-75.

31. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, Gapin L, Kaech SM. 2007. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27:281-95.

32. Low HP, Santos MA, Wizel B, Tarleton RL. 1998. Amastigote surface proteins of *Trypanosoma cruzi* are targets for CD8+ CTL. *J Immunol* 160:1817-23.

33. Sanmarco LM, Visconti LM, Eberhardt N, Ramello MC, Ponce NE, Spitale NB, Vozza ML, Bernardi GA, Gea S, Minguez AR, Aoki MP. 2016. IL-6 Improves the Nitric Oxide-Induced Cytotoxic CD8+ T Cell Dysfunction in Human Chagas Disease. *Front Immunol* 7:626.

34. Badovinac VP, Porter BB, Harty JT. 2004. CD8+ T cell contraction is controlled by early inflammation. *Nat Immunol* 5:809-17.

35. Cousens LP, Peterson R, Hsu S, Dorner A, Altman JD, Ahmed R, Biron CA. 1999. Two roads diverged: interferon alpha/beta- and interleukin 12-mediated pathways in promoting T cell interferon gamma responses during viral infection. *J Exp Med* 189:1315-28.

36. Melet J, Mulleman D, Goupille P, Ribourtout B, Watier H, Thibault G. 2013. Rituximab-induced T cell depletion in patients with rheumatoid arthritis: association with clinical response. *Arthritis Rheum* 65:2783-90.

37. Bennett SR, Carbone FR, Toy T, Miller JF, Heath WR. 1998. B cells directly tolerize CD8(+) T cells. *J Exp Med* 188:1977-83.

38. Mueller SN, Ahmed R. 2009. High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A* 106:8623-8.

39. Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, Lederman MM, Benito JM, Goepfert PA, Connors M, Roederer M, Koup RA. 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 107:4781-9.

40. Aksoy S, Harputluoglu H, Kilickap S, Dede DS, Dizdar O, Altundag K, Barista I. 2007. Rituximab-related viral infections in lymphoma patients. *Leuk Lymphoma* 48:1307-12.

41. Cohen SB, Emery P, Greenwald MW, Dougados M, Furie RA, Genovese MC, Keystone EC, Loveless JE, Burmester GR, Cravets MW, Hessey EW, Shaw T, Totoritis MC, Group RT. 2006. Rituximab for rheumatoid arthritis refractory to anti-tumor necrosis factor therapy: Results of a multicenter, randomized, double-blind, placebo-controlled, phase III trial evaluating primary efficacy and safety at twenty-four weeks. *Arthritis Rheum* 54:2793-806.

42. Hua Q, Zhu Y, Liu H. 2015. Severe and fatal adverse events risk associated with rituximab addition to B-cell non-Hodgkin's lymphoma (B-NHL) chemotherapy: a meta-analysis. *J Chemother* 27:365-70.

43. Acharya D, Wang P, Paul AM, Dai J, Gate D, Lowery JE, Stokic DS, Leis AA, Flavell RA, Town T, Fikrig E, Bai F. 2017. Interleukin-17A Promotes CD8+ T Cell Cytotoxicity To Facilitate West Nile Virus Clearance. *J Virol* 91.

44. Cardillo F, Postol E, Nihei J, Aroeira LS, Nomizo A, Mengel J. 2007. B cells modulate T cells so as to favour T helper type 1 and CD8+ T-cell responses in the acute phase of *Trypanosoma cruzi* infection. *Immunology* 122:584-95.

45. Sullivan NL, Eickhoff CS, Sagartz J, Hoft DF. 2015. Deficiency of antigen-specific B cells results in decreased *Trypanosoma cruzi* systemic but not mucosal immunity due to CD8 T cell exhaustion. *J Immunol* 194:1806-18.

46. Tzelepis F, Persechini PM, Rodrigues MM. 2007. Modulation of CD4(+) T cell-dependent specific cytotoxic CD8(+) T cells differentiation and proliferation by the timing of increase in the pathogen load. *PLoS One* 2:e393.

- 597 47. Xu S, Han Y, Xu X, Bao Y, Zhang M, Cao X. 2010. IL-17A-producing gammadeltaT cells
598 promote CTL responses against *Listeria monocytogenes* infection by enhancing dendritic cell
599 cross-presentation. *J Immunol* 185:5879-87.
600
601

Legends for Figures

FIGURE 1. Anti-CD20 treatment reduced CD8⁺ T cells and increased tissue parasitism.

Mice injected with isotype control (control; in white circles) or anti-CD20 (α CD20; in black circles) mAb were infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain. Representative dot plot and statistical analysis of the mean $\pm$ SD of the percentage and number of: (A) CD8⁺CD3⁺ T cells and (B) Tskb20/Kb⁺CD8⁺ T cells, within the lymphocyte gate, in the spleen from uninfected (day 0) or infected mice at different dpi. (C) Representative plots and statistical analysis of the mean $\pm$ SD of the percentage and number of Tskb20/Kb⁺CD8⁺ T cells in liver at 20 dpi in control (white bars) or anti-CD20 treated mice (black bars). Numbers within the plots indicate the frequency of cells in each region. N=4-5 mice per group. (D) Relative amount of *T. cruzi* satellite DNA in liver, spleen and heart from infected control and anti-CD20-treated mice determined at 20 dpi. Murine GAPDH was used for normalization. Data are presented as mean $\pm$ SD, N=4 mice per group. P values calculated with two tailed T test. Data are representative of four (A-C), and two (D) independent experiments.

FIGURE 2. Anti-CD20 treatment decreased effector and memory CD8⁺ T cell number and compromised their survival and proliferation.

Mice injected with isotype control (control; in white bars) or anti-CD20 (α CD20; in black bars) mAb were infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain. Splenic cells were obtained at 20 dpi and analyzed by flow cytometry. (A) Representative plots of CD62L vs CD44 expression on CD8⁺ T cells. Statistical analysis of the frequency and number of naïve (CD62L^{hi}CD44^{lo}), effector memory/effector (CD62L^{lo}CD44^{hi}) and central memory (CD62L^{hi}CD44^{hi}) cells of: (A) total CD8⁺ and (B) Tskb20/Kb⁺CD8⁺ T cells. (C) Representative plots of KLRG-1 vs CD127 expression on CD44⁺CD8⁺ T cells. Statistical analysis of the frequency and number of short-lived effector cells (SLEC: KLRG^{hi}CD127^{lo}) and memory precursor effector cells (MPEC: KLRG^{lo}CD127^{hi}) of (C) total CD44⁺CD8⁺ and (D) CD44⁺Tskb20/Kb⁺CD8⁺ T cells. (E) Plots and bar graphs representing the frequency of Ki67⁺ cells on gated CD8⁺ or Tskb20/Kb⁺CD8⁺ T cells. (F) Plots and bar graphs representing the frequency of viable non-apoptotic TMRE^{hi} cells on gated CD8⁺ or Tskb20/Kb⁺CD8⁺ T cells. Numbers within the plots indicate the frequency of cells in each region. Bar graphs represent data as mean $\pm$ SD, N=4-5 mice. All P values calculated with two-tailed T test. Data are representative of three (A-D) and two (E-F) independent experiments.

FIGURE 3: Anti-CD20 treatment resulted in a lower CD8⁺ T cell functional activity

Mice injected with isotype control (control) or anti-CD20 (α CD20) mAb were infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain. Uninfected mice were processed in parallel. Splenic cells were obtained at 20 dpi and analyzed by flow cytometry. (A) Chart pie with the frequency $\pm$ SD of polyfunctional CD8⁺ T cells upon PMA+Ionomycin stimulation. References of the different populations (IFN γ ⁺TNF⁺CD107a⁺, triple positive; IFN γ ⁺TNF⁺ or IFN γ ⁺CD107a⁺, double positive; IFN γ ⁺ single positive CD8⁺ T cells) are indicated in the table at the right. (B) Representative histograms and statistical analysis of Tbet expression in total and Tskb20/Kb⁺CD8⁺ T cells from infected control (empty solid line) or anti-CD20-treated (empty dashed line) mice or uninfected mice (gray fill solid line). (C) Representative dot plots of the frequency of transferred antigen-pulsed and unpulsed cells in the spleen of uninfected and infected control or anti-CD20-treated mice at 20 dpi (left panel), and statistical analysis of percentage of specific lysis (right panel) in infected control (white bars) or anti-CD20-treated (black bars) mice. N= 5-6 (A-B) and 4-5 (C) mice per group. P values calculated with two tailed T test. Data are representative of three (A-B) and two (C) independent experiments.

FIGURE 4. *T. cruzi* infected mice treated with anti-CD20 mAb at 12 dpi also had a reduced CD8⁺ T cell response.

Mice infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain were injected with isotype control (control; white bars) or anti-CD20 mAb at 12 dpi (12dpi α CD20; gray bars). Mice injected with anti-CD20 mAb 8 days before the infection with 5000 trypomastigotes of *T. cruzi* Tulahuén strain were processed in parallel (α CD20; black bars). The spleens of the different groups of mice were obtained at 20 dpi. (A) Representative dot plot showing the percentage of Tskb20/Kb⁺CD8⁺ T cells, within the lymphocyte gate; and statistical analysis of the mean $\pm$ SD of the percentages and number of indicated cells. (B) Statistical analysis of the number of SLECs and MPECs in total CD8⁺ and Tskb20/Kb⁺CD8⁺ T cells. (C) Statistical analysis of total IFN γ ⁺CD8⁺ T cell frequency of splenic cells stimulated with PMA+Ionomycin (Polyclonal stimulation) or with Tskb20 (Ag-specific stimulation) after 5h of culture. (D) Statistical analysis of Tbet expression on total and TSKB20/Kb⁺CD8⁺ T cells. N= 5-6 (A) and 4-5 (B-D) mice per group. P values were calculated with one-way ANOVA followed by Bonferroni's posttest. Data are representative of three (A) and two (B-D) independent experiments.

FIGURE 5. Splenic B cells from *T. cruzi* infected mice were the main IL-6 and IL-17A-producing lymphoid cells

(A) C57BL/6 mice were infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain and analyzed at different dpi. Zero dpi indicates uninfected mice. Statistical analysis of the percentage of IL-6 or IL-17A-producing CD19⁺ (B) or CD19^{neg} (Non-B) cells within the lymphocyte gate, in the spleen from uninfected (0dpi) or infected mice at different dpi. (B) Representative plots and statistical analysis of IL-6 or IL-17A-producing lymphoid cells at 15 dpi, obtained from infected control (white bars) or anti-CD20-treated (black bars) mice. (C) Serum IL-6 concentration in infected control or α CD20-treated mice determined at 15 and 20 dpi. (D) Immunofluorescence of spleen sections from uninfected and *T. cruzi* infected mice obtained at 15 dpi, stained with anti-CD3 (green), anti-B220 (red) and anti-CD138 (blue). (E) Immunofluorescence of spleen sections from *T. cruzi* infected mice obtained at 15 dpi, stained with anti-CD8 (green), anti-IL-17A (red) and anti-CD138 (blue). The image on the right represents the binary expression of the positive fluorescence observed in the image on the left. N= 4-5 (A-C) and 3-4 (D-E) mice per group. P values were calculated with two tailed T test. Data are representative of three (A-B) and two (C-E) independent experiments.

FIGURE 6. IL-17A rescued the magnitude and effector phenotype of the CD8⁺ T cell response observed in infected anti-CD20-treated mice

Mice infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain were injected with isotype control (control; white bars) or anti-CD20 mAb 8 days before infection. Infected mice injected with anti-CD20 mAb were also injected with PBS (α CD20, black bars) or rIL-17A (α CD20 + rIL-17A, gray bars) at 12, 14, 16 and 18 dpi. The spleens of the different groups of mice were obtained at 20 dpi. (A) Representative dot plot showing the percentage of total and TSKB20/Kb⁺CD8⁺ T cells, gated on lymphoid cells; and statistical analysis of the mean $\pm$ SD of the percentages and number of indicated cells. (B) Statistical analysis of the number of SLECs and MPECs in total and Tskb20/Kb⁺-specific CD8⁺ T cells. N= 4-5 (A-B) mice per group. P values were calculated with one-way ANOVA followed by Bonferroni's posttest. Data are representative of three independent experiments.

FIGURE 7. IL-17A increased the functionality of CD8⁺ T cells and favored parasite control in infected anti-CD20-treated mice

Mice infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain were injected with isotype control (control; white bars) or anti-CD20 mAb 8 days before infection. Infected mice injected with

anti-CD20 mAb were injected with PBS (α CD20, black bars) or rIL-17A (α CD20 + rIL-17A, gray bars) at 12, 14, 16 and 18 dpi. The spleens of the different groups of mice were obtained at 20 dpi. (A) Representative dot plot and statistical analysis of the percentage and number of IFN γ ⁺ or the percentage TNF⁺ cells, gated on CD8⁺ T cells, obtained after polyclonal or Ag-specific stimulation. (B) Representative histograms and statistical analysis of Tbet expression in total and Tskb20/Kb⁺CD8⁺ T cells from infected control (black solid line) or anti-CD20-treated mice injected with PBS (black dashed line) or with rIL-17A (gray solid line). (C) Relative amount of *T. cruzi* satellite DNA in liver, spleen and heart determined at 20 dpi. Murine GAPDH was used for normalization. N= 5-6 (A-B) and 3-4 (C) mice per group. P values were calculated with one-way ANOVA followed by Bonferroni's posttest. Data are representative of three (A-B) and two (C) independent experiments.

FIGURE 8. IL-6 did not modify the magnitude and effector phenotype of the CD8⁺ T cell response observed in infected anti-CD20-treated mice

Mice infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain were injected with isotype control (control; white bars) or anti-CD20 mAb 8 days before infection. Infected mice injected with anti-CD20 mAb were also injected with PBS (α CD20, black bars) or rIL-6 (α CD20 + rIL-6, gray bars) at 12, 14, 16 and 18 dpi. The spleens of the different groups of mice were obtained at 20 dpi. (A) Representative dot plot showing the percentage of total and TSKB20/Kb⁺CD8⁺ T cells, gated on lymphoid cells; and statistical analysis of the mean $\pm$ SD of the percentages and number of indicated cells. (B) Statistical analysis of the number of SLECs and MPECs in total and Tskb20/Kb⁺-specific CD8⁺ T cells. (C) Statistical analysis of total IFN γ ⁺CD8⁺ T cell frequency of splenic cells stimulated with PMA+Ionomycin (Polyclonal stimulation) or with Tskb20 (Ag-specific stimulation) after 5h of culture. (D) Statistical analysis of Tbet expression on total and TSKB20/Kb⁺CD8⁺ T cells. N= 4-6 (A-D) mice per group. P values were calculated with one-way ANOVA followed by Bonferroni's posttest. Data are representative of two independent experiments.

Legends for Supplemental Figures

Fig. S1: B cell depletion by anti-CD20 injection. (A) C57BL/6 mice were injected with isotype control (control; in white bars) or anti-CD20 (in black bars) mAb, and B cell (CD19+B220+) frequency was determined in the spleen and blood at 8 days post-injection. (B-C) Mice injected with isotype control (control; in white circles) or anti-CD20 (in black circles) mAb were infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain at 8 days post anti-CD20 injection. C57BL/6 untreated uninfected mice were processed in parallel (in gray). (B) Number of B cells determined by flow cytometry. Statistical differences were evaluated between infected control and anti-CD20-treated mice at different dpi. (C) Immunofluorescence of spleen sections (7 µm) from control and anti-CD20-treated mice at 14dpi, stained with PE-labeled anti-B220 (white). Magnification: ×200. Right, statistical analysis of the percentage of area occupied by B220⁺ cells (n=4 for infected control (white bar) or anti-CD20-treated (black bar) mice). P values calculated with two tailed T test. Data are representative of two independent experiments.

Fig. S2: Flow cytometric gating strategy used to identify polyfunctional CD8⁺ T cells. Representative dot plots showing the frequency of IFNγ⁺, CD107a⁺ and TNF⁺ (single positive) cells, gated on splenic CD8⁺T cells, from infected control or anti-CD20-treated mice incubated with Medium or with PMA+Ionomycin (Polyclonal stimulation) or Tskb20 (Ag-specific stimulation) after 5h of culture.

Fig. S3: CD8⁺ T cell functionality after polyclonal and parasite-specific stimulation. (A) Statistical analysis of the frequency of total IFNγ⁺, TNF⁺ or CD107a⁺ CD8⁺ T cells in the spleen of infected control (white bars) or anti-CD20-treated (black bars) mice obtained at 20 dpi and stimulated with PMA+Ionomycin (Polyclonal stimulation) or with Tskb20 (Ag-specific stimulation) after 5h of culture. (B) Frequency of the polyfunctional CD8⁺ T cells in the spleen of infected control (white bars) or anti-CD20-treated (black bars) mice after *in vitro* Tskb20 stimulation. Data are presented as mean of 5-6 mice per group ± SD. P values calculated with two tailed T test. Data are representative of three independent experiments.

Fig. S4: Th17 response in infected anti-CD20-treated mice. (A) Statistical analysis of the frequency and number of CD4⁺ T cells in the spleen of control (white bars) or anti-CD20-treated (black bars) mice analyzed after 20 dpi with *T. cruzi*. (B) Statistical analysis of Tbet expression in CD4⁺ T cells in the spleen of control or anti-CD20-treated mice evaluated after 20 dpi with *T. cruzi*. (C) Representative plots and statistical analysis of the frequency and number of spleen Th17 cells determined at 15 dpi in control and anti-CD20-treated mice, after PMA+Ionomycin stimulation. Data are presented as mean of 5-6 mice per group ± SD. P values calculated with two tailed T test. Data are representative of two independent experiments.

Fig. S5: Source of IL-10, IFNγ and TNF in lymphoid splenic cells from *T. cruzi* infected mice. C57BL/6 mice were infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain and evaluated at different dpi. Zero dpi indicate uninfected mice. Statistical analysis of the percentage IL-10, IFNγ and TNF-producing CD19⁺ (B) or CD19^{neg} (Non-B) cells within lymphocyte gate, in the spleen from uninfected or infected mice at different dpi.

Figure 1

A

B

C

D

FIGURE 1. Anti-CD20 treatment reduced CD8⁺ T cells and increased tissue parasitism. Mice injected with isotype control (control; in white circles) or anti-CD20 (αCD20; in black circles) mAb were infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain. Representative dot plot and statistical analysis of the mean ± SD of the percentage and number of: (A) CD8⁺CD3⁺ T cells and (B) Tskb20/Kb⁺CD8⁺ T cells, within the lymphocyte gate, in the spleen from uninfected (day 0) or infected mice at different dpi. (C) Representative plots and statistical analysis of the mean ± SD of the percentage and number of Tskb20/Kb⁺CD8⁺ T cells in liver at 20 dpi in control (white bars) or anti-CD20 treated mice (black bars). Numbers within the plots indicate the frequency of cells in each region. N=4-5 mice per group. (D) Relative amount of *T. cruzi* satellite DNA in liver, spleen and heart from infected control and anti-CD20-treated mice determined at 20 dpi. Murine GAPDH was used for normalization. Data are presented as mean ± SD, N=4 mice per group. P values calculated with two tailed T test. Data are representative of four (A-C), and two (D) independent experiments.

Figure 2

FIGURE 2. Anti-CD20 treatment decreased effector and memory CD8⁺ T cell number and compromised their survival and proliferation.

Mice injected with isotype control (control; in white bars) or anti-CD20 (αCD20; in black bars) mAb were infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain. Splenic cells were obtained at 20 dpi and analyzed by flow cytometry. (A) Representative plots of CD62L vs CD44 expression on CD8⁺ T cells. Statistical analysis of the frequency and number of naïve (CD62LhiCD44lo), effector memory/effector (CD62LloCD44hi) and central memory (CD62LhiCD44hi) cells of: (A) total CD8⁺ and (B) Tskb20/Kb⁺CD8⁺ T cells. (C) Representative plots of KLRG-1 vs CD127 expression on CD44⁺CD8⁺ T cells. Statistical analysis of the frequency and number of short-lived effector cells (SLEC: KLRGhiCD127lo) and memory precursor effector cells (MPEC: KLRGloCD127hi) of (C) total CD44⁺CD8⁺ and (D) CD44⁺ Tskb20/Kb⁺ CD8⁺ T cells. (E) Plots and bar graphs representing the frequency of Ki67⁺ cells on gated CD8⁺ or Tskb20/Kb⁺CD8⁺ T cells. (F) Plots and bar graphs representing the frequency of viable non-apoptotic TMREhi cells on gated CD8⁺ or Tskb20/Kb⁺CD8⁺ T cells. Numbers within the plots indicate the frequency of cells in each region. Bar graphs represent data as mean ± SD, N=4-5 mice. All P values calculated with two-tailed T test. Data are representative of three (A-D) and two (E-F) independent experiments.

Figure 3

FIGURE 3: Anti-CD20 treatment resulted in a lower CD8⁺ T cell functional activity
 Mice injected with isotype control (control) or anti-CD20 (α CD20) mAb were infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain. Uninfected mice were processed in parallel. Splenic cells were obtained at 20 dpi and analyzed by flow cytometry. (A) Chart pie with the frequency $\pm$ SD of polyfunctional CD8⁺ T cells upon PMA+Ionomycin stimulation. References of the different populations (IFN γ +TNF+CD107a+, triple positive; IFN γ +TNF+ or IFN γ +CD107a+, double positive; IFN γ + single positive CD8⁺ T cells) are indicated in the table at the right. (B) Representative histograms and statistical analysis of Tbet expression in total and Tskb20/Kb⁺CD8⁺ T cells from infected control (empty solid line) or anti-CD20-treated (empty dashed line) mice or uninfected mice (gray fill solid line). (C) Representative dot plots of the frequency of transferred antigen-pulsed and unpulsed cells in the spleen of uninfected and infected control or anti-CD20-treated mice at 20 dpi (left panel), and statistical analysis of percentage of specific lysis (right panel) in infected control (white bars) or anti-CD20-treated (black bars) mice. N= 5-6 (A-B) and 4-5 (C) mice per group. P values calculated with two tailed T test. Data are representative of three (A-B) and two (C) independent experiments.

Figure 4

A

B

C

D

FIGURE 4. *T. cruzi* infected mice treated with anti-CD20 mAb at 12 dpi also had a reduced CD8⁺ T cell response.

Mice infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain were injected with isotype control (control; white bars) or anti-CD20 mAb at 12 dpi (12dpi αCD20; gray bars). Mice injected with anti-CD20 mAb 8 days before the infection with 5000 trypomastigotes of *T. cruzi* Tulahuén strain were processed in parallel (αCD20; black bars). The spleens of the different groups of mice were obtained at 20 dpi. (A) Representative dot plot showing the percentage of Tskb20/Kb⁺CD8⁺ T cells, within the lymphocyte gate; and statistical analysis of the mean $\pm$ SD of the percentages and number of indicated cells. (B) Statistical analysis of the number of SLECs and MPECs in total CD8⁺ and Tskb20/Kb⁺CD8⁺ T cells. (C) Statistical analysis of total IFN γ +CD8⁺ T cell frequency of splenic cells stimulated with PMA+Ionomycin (Polyclonal stimulation) or with Tskb20 (Ag-specific stimulation) after 5h of culture. (D) Statistical analysis of Tbet expression on total and TSKB20/Kb⁺CD8⁺ T cells. N= 5-6 (A) and 4-5 (B-D) mice per group. P values were calculated with one-way ANOVA followed by Bonferroni's posttest. Data are representative of three (A) and two (B-D) independent experiments.

Figure 5

A

B

C

D

E

FIGURE 5. Splenic B cells from *T. cruzi* infected mice were the main IL-6 and IL-17A-producing lymphoid cells

(A) C57BL/6 mice were infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain and analyzed at different dpi. Zero dpi indicates uninfected mice. Statistical analysis of the percentage of IL-6 or IL-17A-producing CD19+ (B) or CD19neg (Non-B) cells within the lymphocyte gate, in the spleen from uninfected (0dpi) or infected mice at different dpi. (B) Representative plots and statistical analysis of IL-6 or IL-17A-producing lymphoid cells at 15 dpi, obtained from infected control (white bars) or anti-CD20-treated (black bars) mice. (C) Serum IL-6 concentration in infected control or αCD20-treated mice determined at 15 and 20 dpi. (D) Immunofluorescence of spleen sections from uninfected and *T. cruzi* infected mice obtained at 15 dpi, stained with anti-CD3 (green), anti-B220 (red) and anti-CD138 (blue). (E) Immunofluorescence of spleen sections from *T. cruzi* infected mice obtained at 15 dpi, stained with anti-CD8 (green), anti-IL-17A (red) and anti-CD138 (blue). The image on the right represents the binary expression of the positive fluorescence observed in the image on the left. N= 4-5 (A-C) and 3-4 (D-E) mice per group. P values were calculated with two tailed T test. Data are representative of three (A-B) and two (C-E) independent experiments.

Figure 6

A

B

FIGURE 6. IL-17A rescued the magnitude and effector phenotype of the CD8⁺ T cell response observed in infected anti-CD20-treated mice

Mice infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain were injected with isotype control (control; white bars) or anti-CD20 mAb 8 days before infection. Infected mice injected with anti-CD20 mAb were also injected with PBS (α CD20, black bars) or rIL-17A (α CD20 + rIL-17A, gray bars) at 12, 14, 16 and 18 dpi. The spleens of the different groups of mice were obtained at 20 dpi. (A) Representative dot plot showing the percentage of total and TSKB20/Kb⁺CD8⁺ T cells, gated on lymphoid cells; and statistical analysis of the mean $\pm$ SD of the percentages and number of indicated cells. (B) Statistical analysis of the number of SLECs and MPECs in total and Tskb20/Kb⁺-specific CD8⁺ T cells. N = 4-5 (A-B) mice per group. P values were calculated with one-way ANOVA followed by Bonferroni's posttest. Data are representative of three independent experiments.

Figure 7

FIGURE 7. IL-17A increased the functionality of CD8 $^{+}$ T cells and favored parasite control in infected anti-CD20-treated mice

Mice infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain were injected with isotype control (control; white bars) or anti-CD20 mAb 8 days before infection. Infected mice injected with anti-CD20 mAb were injected with PBS (α CD20, black bars) or rIL-17A (α CD20 + rIL-17A, gray bars) at 12, 14, 16 and 18 dpi. The splees of the different groups of mice were obtained at 20 dpi. (A) Representative dot plot and statistical analysis of the percentage and number of IFN γ^{+} or the percentage TNF $^{+}$ cells, gated on CD8 $^{+}$ T cells, obtained after polyclonal or Ag-specific stimulation. (B) Representative histograms and statistical analysis of Tbet expression in total and Tskb20/Kb $^{+}$ CD8 $^{+}$ T cells from infected control (black solid line) or anti-CD20-treated mice injected with PBS (black dashed line) or with rIL-17A (gray solid line). (C) Relative amount of *T. cruzi* satellite DNA in liver, spleen and heart determined at 20 dpi. Murine GAPDH was used for normalization. N= 5-6 (A-B) and 3-4 (C) mice per group. P values were calculated with one-way ANOVA followed by Bonferroni's posttest. Data are representative of three (A-B) and two (C) independent experiments.

Figure 8

FIGURE 8. IL-6 did not modify the magnitude and effector phenotype of the CD8⁺ T cell response observed in infected anti-CD20-treated mice

Mice infected with 5000 trypomastigotes of *T. cruzi* Tulahuén strain were injected with isotype control (control; white bars) or anti-CD20 mAb 8 days before infection. Infected mice injected with anti-CD20 mAb were also injected with PBS (α CD20, black bars) or rIL-6 (α CD20 + rIL-6, gray bars) at 12, 14, 16 and 18 dpi. The splees of the different groups of mice were obtained at 20 dpi. (A) Representative dot plot showing the percentage of total and TSKB20/Kb+CD8⁺ T cells, gated on lymphoid cells; and statistical analysis of the mean $\pm$ SD of the percentages and number of indicated cells. (B) Statistical analysis of the number of SLECs and MPECs in total and Tskb20/Kb+-specific CD8⁺ T cells. (C) Statistical analysis of total IFN γ +CD8⁺ T cell frequency of splenic cells stimulated with PMA+Ionomycin (Polyclonal stimulation) or with Tskb20 (Ag-specific stimulation) after 5h of culture. (D) Statistical analysis of Tbet expression on total and TSKB20/Kb+CD8⁺ T cells. N= 4-6 (A-D) mice per group. P values were calculated with one-way ANOVA followed by Bonferroni's posttest. Data are representative of two independent experiments