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Critical Contribution of CD28-CD80/CD86 Costimulatory Pathway to Protection from *Trypanosoma cruzi* Infection

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The CD28-CD80/CD86-mediated T-cell costimulatory pathway has been variably implicated in infectious immunity. In this study, we investigated the role of this costimulatory pathway in resistance to *Trypanosoma cruzi* infection by using CD28-deficient mice and blocking antibodies against CD80 and CD86. CD28-deficient mice exhibited markedly exacerbated *T. cruzi* infection, as evidenced by unrelenting parasitemia and 100% mortality after infection with doses that are nonlethal in wild-type mice. The blockade of both CD80 and CD86 by administering specific monoclonal antibodies also exacerbated *T. cruzi* infection in wild-type mice. Splenocytes from *T. cruzi*-infected, CD28-deficient mice exhibited greatly impaired gamma interferon production in response to *T. cruzi* antigen stimulation in vitro compared to those from infected wild-type mice. The induction of *T. cruzi* antigen-specific $CD8^+$ T cells was also impaired in *T. cruzi*-infected, CD28-deficient mice. In addition to these defects in natural protection against *T. cruzi* infection, CD28-deficient mice were also defective in the induction of $CD8^+$-T-cell-mediated protective immunity against *T. cruzi* infection by DNA vaccination. These results demonstrate, for the first time, a critical contribution of the CD28-CD80/CD86 costimulatory pathway not only to natural protection against primary *T. cruzi* infection but also to DNA vaccine-induced protective immunity to Chagas' disease.

Trypanosoma cruzi is the etiological agent of Chagas' disease in Central and South America (7, 17, 18, 35). As it invades and replicates in essentially all nucleated cells of mammalian hosts, T-cell-mediated immunity is critical for resolving infection (3, 9). In accordance with this notion, the depletion of $CD8^+$ or $CD4^+$ T cells results in unrelenting parasitemia and a fatal outcome in mice (37, 44, 45, 46). Considering the paucity of therapeutic drugs against *T. cruzi* (49), the development of a vaccine to induce effective T-cell-mediated immunity to control the infection has been eagerly expected. We recently identified a major epitope of trans-sialidase surface antigen (TSSA) recognized by $CD8^+$ T cells in *T. cruzi*-infected C57BL/6 (B6) mice. We demonstrated that vaccination with plasmid DNA encoding TSSA can induce $CD8^+$-T-cell-mediated protective immunity against lethal *T. cruzi* infection, although sterile immunity has never been achieved (15, 29).

Costimulatory molecules are essential for effective T-cell activation and differentiation (19, 33, 39, 52). CD28, together with its ligands CD80 and CD86, is one of the most pivotal costimulatory molecules for inducing functional T-cell responses (20, 38). Stimulation of T cells in the absence of CD28-CD80/CD86-mediated costimulation results in impaired proliferation, reduced cytokine production, and altered Th1/Th2 balance (22, 42, 47). CD28-deficient mice fail to develop germinal centers and exhibit impaired specific antibody production (11, 42). A pivotal role of the CD28 costimulatory pathway in protective immunity against microbial infections has been also demonstrated, since CD28-deficient mice exhibit increased susceptibility to *Salmonella enterica* serovar Typhimurium (23) and *Listeria monocytogenes* (24). However, CD28-deficient mice are normally resistant to various infections such as *Leishmania major* infection (4, 10) or primary *Toxoplasma gondii* infection (50). These findings indicate that the contribution of the CD28 costimulatory pathway to protective immunity varies depending on the type of infectious agent (4, 10, 23, 24, 43, 50, 52). Involvement of the CD28 costimulatory pathway in protective immunity against *T. cruzi* has not been determined.

In the present study we demonstrate a critical role of the CD28 costimulatory pathway in natural protective immunity against *T. cruzi* infection by using CD28-deficient mice and anti-CD80/CD86 blocking antibodies. We also found a critical contribution of the CD28 pathway to the induction of protective immunity against *T. cruzi* by DNA vaccination. These findings provide a new basis for improving vaccine strategies against Chagas' disease.

MATERIALS AND METHODS

Animals and parasite. Female, 5- to 8-week-old B6 ($H\text{-}2^b$) mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Genetically manipulated female CD28-deficient mice were bred and reared in the specific-pathogen-free animal facility at the Science University of Tokyo (Noda City, Chiba, Japan). Blood-form trypomastigotes of *T. cruzi* Tulahuen strain (25) were maintained in outbred CD1 or inbred BALB/c mice by intramuscular (i.m.) inoculation of 5,000 trypomastigotes into naive mice every 2 weeks. Tissue culture trypomastigotes of

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T. cruzi Sylvio X10/4 clone (25) were produced from Swiss 3T3 cell culture as previously described (28).

Cells and culture. The B6-derived thymoma cell line EL-4 was used as antigen-presenting cells for $CD8^+$-T-cell cultures and assays. These cells were cultured in high-glucose Dulbecco modified Eagle medium (DMEM; Life Technologies/BRL, Rockville, Md.) supplemented with 10% fetal calf serum, 2 g of sodium bicarbonate (Sigma, St. Louis, Mo.)/liter, 200 mg of L-arginine hydrochloride (Life Technologies/BRL)/liter, 36 mg of L-asparagine (Life Technologies/BRL)/liter, 2.6 g of HEPES (Sigma)/liter, 5×10^{-5} M 2-mercaptoethanol (Sigma), and antibiotics (complete DMEM). The medium used for enzyme-linked immunospot (ELISPOT) assays and the culture of lymphocytes was supplemented with phorbol myristate acetate-stimulated EL-4 cell culture supernatant as a source of 30 U of interleukin-2/ml (complete DMEM–IL-2). Insect-form (epimastigotes) of *T. cruzi* Sylvio X10/4 clone or Tulahuen strain were cultured in LIT medium (2) and incubated at 27°C. Tulahuen strain of *T. cruzi* epimastigotes were harvested from LIT culture, washed three times with sterile phosphate-buffered saline (PBS), suspended in PBS at 3×10^8 per ml, subjected to three rounds of freeze-thaw at −80°C, homogenized, and filter sterilized for use in the cytokine assay.

Plasmid DNA and peptide. A pCMV-tag epitope tagging mammalian expression vector (pCMV; Stratagene) was used to construct a *T. cruzi* TSSA gene-expressing plasmid DNA, and this was designated pTSSA (15, 29). A $CD8^+$-T-cell-inducing, *H-2K*^{b}-restricted peptide, ANYNFTLV, derived from TSSA (15, 29), was used for immunological assays.

Protection assay and evaluation of protective efficacy. For protection assays, naive mice were infected either i.m. with 10 blood-form trypomastigotes of *T. cruzi* Tulahuen strain or intraperitoneally (i.p.) with 10^6 tissue culture trypomastigotes of *T. cruzi* Sylvio X10/4 clone. Blood from all infected mice was obtained periodically from the tail vein, and the number of parasites in 5 μl of blood (parasitemia) was counted microscopically. The survival of host mice was monitored daily.

Administration of anti-CD80 and/or anti-CD86 MAbs. Anti-mouse CD80 (B7-1) monoclonal antibody (MAb; hybridoma 1G10, rat immunoglobulin G2a [IgG2a]) and anti-mouse CD86 (B7-2) MAb (GL1, rat IgG2a) were purchased from Pharmingen (San Diego, Calif.) and used to treat mice in vivo as described previously (41) with slight modifications. B6 mice were administered a 200 μg per dose i.p. of either anti-mouse CD80 or anti-mouse CD86 alone or both three times per week for 4 weeks. As a control, mice were administered the same dose of naive rat IgG (Sigma-Aldrich Chemical Co.). All mice were infected i.m. with 10 blood-form trypomastigotes of *T. cruzi* Tulahuen strain on the day of the first antibody administration.

In vitro stimulation of *T. cruzi*-infected-mouse-derived splenocytes and cytokine enzyme-linked immunosorbent assay. Whole splenocytes were isolated from *T. cruzi*-infected CD28-deficient or B6 mice 15 days after infection, cultured in complete DMEM at a cell density of 5×10^4 cells per well in 96-well round-bottom plates, and stimulated with 20 μg of soluble *T. cruzi* epimastigote antigen for 48 h/ml. Cell culture supernatants were analyzed for the cytokines gamma interferon (IFN-γ) and IL-4 as described previously (1) by using OptEIA kits (Pharmingen) according to the manufacturer's instructions.

Quantification of antigen-specific T cells by ELISPOT assay. The frequency of *T. cruzi* antigen-specific T cells was determined by ELISPOT assay for IFN-γ-secreting cells essentially as described previously (6, 26). Briefly, serial dilutions of splenocytes or T cells (1×10^4 to 100×10^4) were cocultured with irradiated EL-4 cells that had been pulsed with 1 μM peptide in anti-IFN-γ MAb-coated plates for 24 to 28 h. The spots formed by IFN-γ-secreting cells were detected with biotinylated anti-IFN-γ MAb, followed by the addition of peroxidase-labeled streptavidin and diaminobenzidine. The developed spots were counted under a microscope and are expressed as the number of spots per 10^6 cells.

DNA vaccination schedule, dosages, and challenge infection. For the induction of immune T cells by DNA immunization, mice were injected i.m. with 100 μg of pTSSA or control pCMV vector suspended in 50 μl of sterile PBS into the right hind leg quadriceps twice at an 11-day interval. The mice were sacrificed 12 days after the second immunization, and their spleens were removed. The number of ANYNFTLV-specific $CD8^+$ T cells was quantified by ELISPOT assay. The immunized mice were challenged i.p. with 5,000 Tulahuen strain of *T. cruzi* blood-form trypomastigotes 9 days after the last immunization. Blood from all infected mice was obtained periodically from the tail vein, and the number of parasites in 5 μl of blood (parasitemia) was counted microscopically. Survival of host mice was monitored daily.

Statistical analyses. Statistical analyses were performed by using the unpaired Student *t* test or Dunnett two-tailed *t* test for the ELISPOT assays and for the counts of parasitemia. The unpaired Mann-Whitney U test determined significant differences in survival data. *P* values of <0.05 were considered significant.

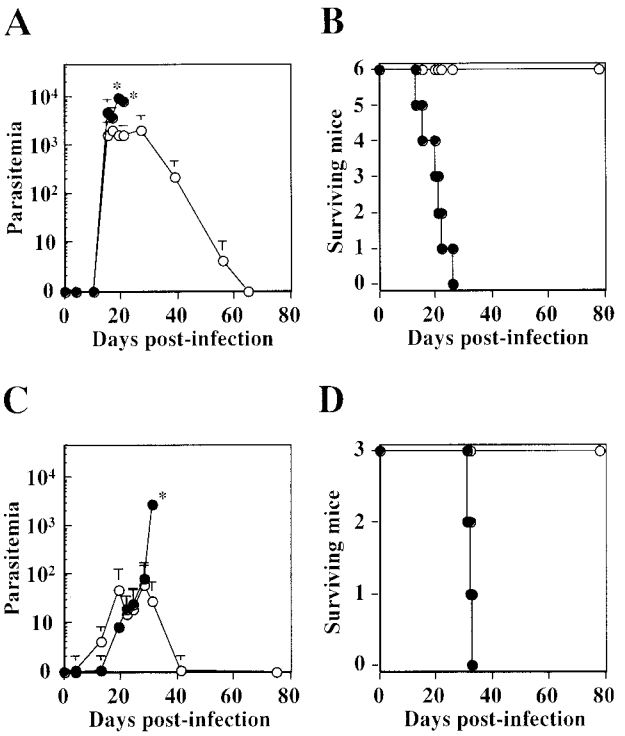

FIG. 1. CD28-deficient mice succumb to sublethal *T. cruzi* infection. CD28-deficient (●) or wild-type (○) B6 mice were infected i.m. with 10 blood-form trypomastigotes of *T. cruzi* Tulahuen strain ($n = 6$) (A and B) or i.p. with 10^6 tissue-culture trypomastigotes of *T. cruzi* Sylvio X10/4 clone ($n = 3$) (C and D). The number of parasites in 5 μl of peripheral blood (parasitemia) was counted periodically (panels A and C), and survival was monitored daily (panels B and D). ✻, $P < 0.01$ compared to wild-type mice (as determined by unpaired Student *t* test) (A and C). The survival of B6 mice was significantly different ($P < 0.05$ [unpaired Mann-Whitney U test]) from that of CD28-deficient mice (B and D).

RESULTS

CD28 deficiency increases the susceptibility of mice to *T. cruzi* infection. In order to clarify the role of CD28 costimulatory molecule in resistance to *T. cruzi* infection, we first monitored the disease course of *T. cruzi* infection in naive CD28-deficient mice. *T. cruzi* Tulahuen is one of the most virulent strains exhibiting patent parasitemia and death 2 to 4 weeks postinfection. However, the infection usually becomes sublethal in naive B6 mice at infection doses of 10 or fewer trypomastigotes per mouse. When both CD28-deficient and B6 mice were infected i.m. with blood-form *T. cruzi* Tulahuen strain trypomastigotes at a dose of 10 per mouse, CD28 deficiency resulted in unrelenting parasitemia (Fig. 1A) and 100% mortality (Fig. 1B), whereas the wild-type mice eventually suppressed the rising parasitemia, and all survived the infection (Fig. 1A and B). The increased susceptibility of CD28-deficient mice to primary *T. cruzi* infection was confirmed by infecting mice i.p. with 10^6 tissue culture trypomastigotes of the low-virulence *T. cruzi* Sylvio X10/4 clone. Infection with this clone rarely results in patent parasitemia and is typically nonlethal in acute infection even upon infection of naive B6 mice with $>10^6$ trypomastigotes (34). However, CD28 deficiency results in un-

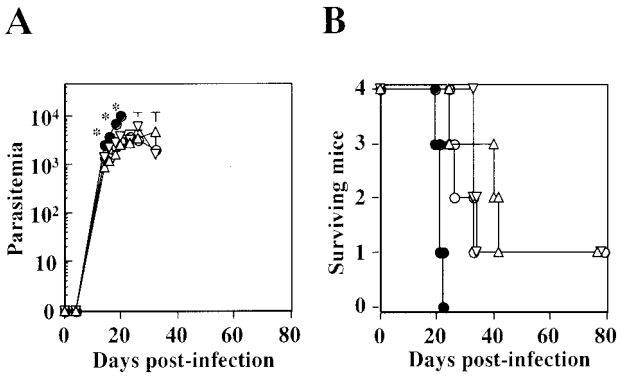

FIG. 2. Simultaneous administration of anti-CD80 and anti-CD86 MAbs exacerbates *T. cruzi* infection. Wild-type B6 mice ($n = 4$ in each group) were administered 200 μg of anti-CD80 MAb (△), anti-CD86 MAb (▽), both anti-CD80 and anti-CD86 MAbs (●), or control rat IgG (○) i.p. three times per week for 4 weeks. Mice were infected i.m. with 10 blood-form trypomastigotes of *T. cruzi* Tulahuen strain on the day of the first antibody inoculation. The number of parasites in 5 μl of peripheral blood (parasitemia) was counted periodically (A), and survival was monitored daily (B). ✱, $P < 0.05$ compared to control IgG-treated mice (as determined by Dunnett two-tailed *t* test) (A). The survival of mice administered both anti-CD80 and anti-CD86 MAbs was significantly different ($P < 0.05$ [unpaired Mann-Whitney U test]) from that of control IgG-treated mice (B).

relenting parasitemia (Fig. 1C) and 100% mortality (Fig. 1D) even in *T. cruzi* X10/4 infection.

Blockade of both the CD80 and CD86 molecules results in exacerbated *T. cruzi* infection. Since it is well established that the CD28 molecule interacts with two distinct ligands, CD80 (B7-1) and CD86 (B7-2), expressed on antigen-presenting cells (APCs) (20), we next tested whether one molecule or the other is more important and responsible for the increased susceptibility of mice to *T. cruzi* infection. When we administered specific MAbs into mice during *T. cruzi* infection, we found that the simultaneous blockade of CD80 and CD86 molecules significantly exacerbated infection, as assessed by analyzing parasitemia (Fig. 2A) and mortality (Fig. 2B). In contrast, the blockade of either molecule alone did not alter the progression of *T. cruzi* infection compared to rat IgG-treated mice as reflected by parasitemia and mortality (Fig. 2).

CD28 deficiency results in impaired *T. cruzi* antigen-specific IFN-γ secretion from infected mouse splenocytes. It has been shown that increased susceptibility to *Salmonella* infection is attributable in part to reduced antigen-specific IFN-γ production from splenocytes derived from CD28-deficient mice (23). We therefore tested the possibility that a similar immunological mechanism is responsible for the increased susceptibility of CD28-deficient mice to *T. cruzi* infection. We isolated splenocytes from *T. cruzi*-infected CD28-deficient or wild-type mice at either 11 or 15 days postinfection. The mean values of parasitemia (per 5 μl of blood) were 37 ± 21 in CD28-deficient mice and 47 ± 38 in wild-type mice at 11 days postinfection and 2,550 ± 1,348 in CD28-deficient mice and 1,130 ± 641 in wild-type mice at 15 days postinfection. The antigen-specific secretion of IFN-γ in the culture supernatant from *T. cruzi*-infected wild-type mouse-derived splenocytes 15 days postinfection increased to 7,732 ± 923 pg/ml, whereas that from *T. cruzi*-infected, CD28-deficient mouse-derived splenocytes was significantly lower at 756 ± 91 pg/ml ($P < 0.05$) (Fig. 3). Similar results were obtained at 11 days postinfection, although the secretion of IFN-γ into the culture supernatant was much lower (data not shown). The antigen-specific secretion of IL-4 in the same samples exhibited no significant difference between the groups (data not shown), suggesting that the capacity for antigen-specific cytokine secretion is not always impaired in CD28-deficient mice.

Impaired induction of antigen-specific $CD8^+$ T cells in CD28-deficient mice upon *T. cruzi* infection. CD28 deficiency resulted in a reduced induction of epitope-specific $CD8^+$ T cells upon *Listeria* (24) or lymphocytic choriomeningitis virus (LCMV) (43) infection. We therefore tested whether the increased susceptibility of CD28-deficient mice to *T. cruzi* infection could also be explained in part by the impaired induction of epitope specific $CD8^+$ T cells. We isolated splenocytes from either *T. cruzi*-infected CD28-deficient or wild-type mice 13 days or 16 days postinfection and performed ELISPOT assays to enumerate ANYNFTLV-specific $CD8^+$ T cells (6, 26). The mean values of parasitemia were 200 ± 115 in CD28-deficient mice and 400 ± 271 in wild-type mice at 13 days postinfection and 1,550 ± 778 in CD28-deficient mice and 875 ± 263 in wild-type mice at 16 days postinfection. We detected few ANYNF TLV-specific $CD8^+$ T cells in splenocytes that were stimulated in vitro for 1 week with antigen derived from CD28-deficient or wild-type mice at 13 days postinfection (data not shown). In the same assay with splenocytes derived from mice 16 days postinfection, we observed extensive deaths of in vitro antigen-stimulated splenocytes from both *T. cruzi*-infected CD28-deficient and wild-type mice compared to the ones derived from noninfected wild-type mice. This was assessed by the trypan blue dye exclusion test, which could constantly determine about one-fifth of 1-week-antigen-stimulated splenocytes derived from noninfected mice as viable cells. In contrast,

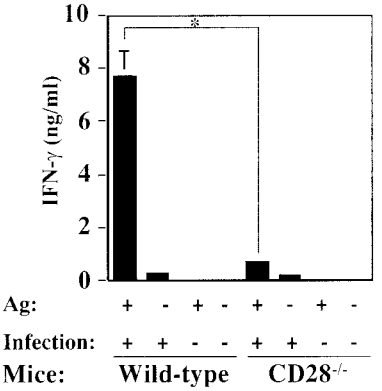

FIG. 3. *T. cruzi* antigen-specific IFN-γ production by splenocytes is impaired in CD28-deficient mice. Wild-type or CD28-deficient B6 mice were infected i.m. with 10 Tulahuen strain *T. cruzi* blood-form trypomastigotes and sacrificed 15 days later. Uninfected mice were also included as controls. Splenocytes from infected CD28-deficient mice (parasitemia = 2,550 ± 1,348), wild-type mice (parasitemia = 1,130 ± 641), or uninfected mice were cultured with or without 20 μg of *T. cruzi* epimastigote-soluble antigen/ml for 48 h. The concentration of IFN-γ in the culture supernatants was measured by enzyme-linked immunosorbent assay. The data represent the means ± the standard deviations (SDs) of three mice in each group. ✱, $P < 0.05$ (as determined by unpaired Student *t* test).

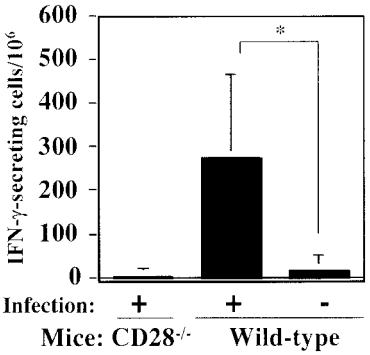

FIG. 4. Expansion of *T. cruzi* antigen-specific $CD8^+$ T cells is impaired in CD28-deficient mice. Wild-type or CD28-deficient B6 mice were infected i.m. with 10 Tulahuen strain *T. cruzi* blood-form trypomastigotes. Uninfected wild-type mice were also included as a control. The mice were sacrificed 16 days postinfection, and their spleens were removed. Splenocytes from individual mice were cultured with irradiated EL-4 cells pulsed with ANYNFTLV peptide for 1 week and then subjected to ELISPOT assay for IFN-γ-producing cells in response to ANYNFTLV peptide-pulsed EL-4 cells. The number of IFN-γ-secreting cells/10^6 cells was counted 24 h later. The number of IFN-γ-secreting cells that appeared in response to peptide-unpulsed EL-4 was subtracted from the number of IFN-γ-secreting cells that appeared in response to peptide-pulsed EL-4. Data represent the means ± the SDs of four mice in each group. ✱, $P < 0.05$ compared to uninfected wild-type mice (as determined by Dunnett two-tailed *t* test).

the same test with *T. cruzi*-infected-mouse-derived splenocytes, which were isolated when the *T. cruzi* parasitemia level reached generally more than 1,000 trypomastigotes per 5 μl of blood, revealed that only 1/40 or 1/50 of the cells were determined to be viable. This phenomenon is probably due to the presence of active *T. cruzi* infection and increased activation-induced cell death as reported previously (21, 31). Despite the perturbed cell condition due to the progression of active disease, the ELISPOT assay detected 273 ± 190 ANYNFTLV-specific $CD8^+$ T cells per 10^6 1-week antigen-stimulated wild-type mouse-derived splenocytes ($P < 0.05$ compared to those derived from noninfected wild-type mice), whereas few ANYNFTLV-specific $CD8^+$ T cells were detected in CD28-deficient mouse-derived splenocytes (Fig. 4).

pTSSA vaccine-induced protective immune responses are completely abrogated in CD28-deficient mice. Finally, we examined the immunological role of the CD28 molecule in DNA vaccine-induced adaptive immunity. CD28-deficient and wild-type mice were immunized twice with pTSSA or control pCMV at an 11-day interval. At 9 days after the second immunization, the mice were infected i.p. with a lethal dose of *T. cruzi*, and the immunological efficacy of DNA vaccination depending on CD28 costimulation was evaluated. When epitope-specific $CD8^+$ T cells were enumerated by ELISPOT assay 12 days after the second immunization, no induction of ANYNFTLV-specific $CD8^+$ T cells was detected in CD28-deficient mice under conditions in which the cells were significantly induced in wild-type mice ($P < 0.05$) (Fig. 5A). In accordance with the impaired $CD8^+$-T-cell responses in CD28-deficient mice, pTSSA vaccination was totally ineffective in conferring protective immunity in CD28-deficient mice, as evidenced by the unrelenting parasitemia (Fig. 5B) and 100% mortality (Fig. 5C).

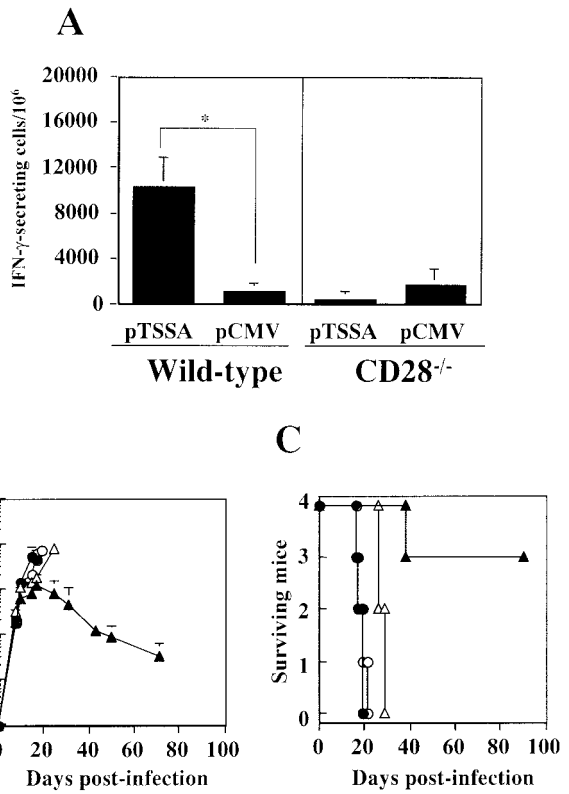

FIG. 5. CD28 deficiency abrogates the induction of ANYNFTLV-specific $CD8^+$ T cells and protective immunity against *T. cruzi* infection by DNA vaccination. (A) Wild-type or CD28-deficient B6 mice were immunized i.m. with 100 μg of pTSSA or pCMV twice at an 11-day interval into the right hind leg quadriceps. The mice were sacrificed 12 days after the second immunization, and their spleens were removed. Splenocytes from individual mice were cultured with irradiated EL-4 cells pulsed with ANYNFTLV peptide for 1 week. The antigenic stimulation was repeated for one more week, and the stimulated splenocytes were then subjected to the ELISPOT assay as described in the legend to Fig. 4. Data represent the means ± the SDs of three mice in each group. ✱, $P < 0.05$ (as determined by unpaired Student *t* test). (B and C) Wild-type or CD28-deficient B6 mice were immunized as described above and infected i.p. with 5,000 Tulahuen strain *T. cruzi* blood-form trypomastigotes 9 days after the second DNA immunization. The number of parasites in 5 μl of peripheral blood (parasitemia) was counted; the data represent the means ± the SDs of four mice in each group (B), and survival was monitored daily (C). The symbols represent parasitemia and survival of wild-type mice immunized with pTSSA (▲), wild-type mice immunized with pCMV (△), CD28-deficient mice immunized with pTSSA (●), and CD28-deficient mice immunized with pCMV (○). ✱, $P < 0.05$ compared to the pCMV-immunized wild-type mice (as determined by the Dunnett two-tailed *t* test) (B). The longer survival of pTSSA-immunized wild-type mice and the shorter survival of CD28-deficient mice immunized with either pTSSA or pCMV were significantly different ($P < 0.05$ as determined by the unpaired Mann-Whitney U test) from that of pCMV-immunized wild-type mice (C).

DISCUSSION

We evaluated here the immunological role of the CD28-CD80/CD86 costimulatory pathway in *T. cruzi* infection. As expected rationally in consideration of its pivotal role in T-cell activation and differentiation (14, 19, 33, 39, 51, 52), CD28 deficiency exacerbated *T. cruzi* infection (Fig. 1). In addition, the blockade of the CD80 and CD86 molecules by specific

MAbs also resulted in the exacerbation of *T. cruzi* infection (Fig. 2). The results, at a glance, do not seem surprising and look quite rational; however, the phenomenon is unique compared to other protozoan diseases such as *Leishmania major* (4, 10) or primary *Toxoplasma gondii* (50) infection, in which CD28 deficiency does not exacerbate infection. Earlier work has suggested that CD28 is also uninvolved in the case of other viral infectious agents such as LCMV (42), toward which CD28-deficient mice exhibit no significant difference in susceptibility in vivo compared to wild-type littermates. In contrast, Mittrucker et al. demonstrated that CD28 deficiency results in the exacerbation of *Salmonella* (23) and *Listeria* (24) infections. In this regard, *T. cruzi* is grouped among infectious diseases in which CD28 plays a critical role in resolving the infection. Furthermore, although CD80/CD86 can interact with the CTLA-4 molecule to promote the appropriate T-cell activation and differentiation (20), the results shown in Fig. 1 and 2 suggest that signal transduction via the CD28-CD80/CD86 pathways is critical for conferring resistance to *T. cruzi* infection.

As for the immunological mechanisms that cause the increased susceptibility of CD28-deficient mice to *T. cruzi* infection, we demonstrated reduced antigen-specific IFN-γ secretion and impaired antigen-specific $CD8^+$-T-cell responses as possible determining factors in host susceptibility (Fig. 3 and 4). Since IFN-γ is regarded as one of the most crucial cytokines for resolving intracellular infections, including infection by *T. cruzi* (5, 16), the reduced secretion of IFN-γ during *T. cruzi* infection might play a role in the increased susceptibility and disease outcome of CD28-deficient mice to *T. cruzi* infection. Similar results have been reported in the cases of *Salmonella* (23) and *Listeria* (24) infections, in which the increased susceptibility of CD28-deficient mice was explained in part by the impaired production of IFN-γ. However, since IFN-γ is also regarded critical for resolving *L. major* or *T. gondii* infection, only the impaired IFN-γ production in CD28-deficient mice could not fully explain the drastic difference of disease susceptibility against these three protozoan infections. Even if CD28-CD80/CD86 interactions are not essential for eliciting primary $CD8^+$-T-cell responses after LCMV infection, the induction of antigen-specific $CD8^+$ T cells in CD28-deficient mice is consistently lower than in wild-type mice (43). Reduced antigen-specific $CD8^+$-T-cell responses have also been reported in the case of *Listeria* infection (24), suggesting the indispensable role of the CD28-CD80/CD86 costimulatory pathway for the optimal induction of $CD8^+$ T cells (52). The variation in ANYNF TLV-specific $CD8^+$-T-cell enumeration was quite large, as shown in Fig. 4; this is probably due to the presence of active *T. cruzi* infection. However, we believe that the reduced induction of epitope-specific $CD8^+$ T cells can explain, at least in part, the increased susceptibility of CD28-deficient mice to *T. cruzi* infection and probably is more important than reduced IFN-γ secretion for the determination of disease susceptibility.

In addition to the defective protective immune responses against primary *T. cruzi* infection in CD28-deficient mice, we found that CD28 deficiency also impairs DNA vaccine-induced protective immune responses against lethal *T. cruzi* infection (Fig. 5). The induction of ANYNFTLV-specific $CD8^+$-T-cell responses was totally abrogated in CD28-deficient mice (Fig. 5A), indicating the absolute requirement of the CD28 molecule for DNA vaccine-induced protective immunity. The induction of antigen-specific $CD8^+$ T cells in CD28-deficient mice depends on the immunogens used for vaccination, among which naked DNA-induced immune responses require CD28 costimulation (13). Our results (shown in Fig. 5) assessed by the ELISPOT and *T. cruzi* protection assays in CD28-deficient mice are therefore in agreement with the previous report (13) that demonstrated the critical role of the CD28 molecule for an effective DNA vaccination strategy. Although Horspool et al. thoroughly investigated the effect of CD28 deficiency on DNA vaccine-induced humoral and cell-mediated immunity, they did not perform protection assays against infectious agents (13). Our results strongly suggest that pTSSA-vaccinated CD28-deficient mice lack vaccine-induced immunity, i.e., these mice show no detectable ANYNFTLV-specific $CD8^+$-T-cell responses (Fig. 5A) and exhibit unrelenting parasitemia and earlier death compared to pCMV-vaccinated wild-type mice (Fig. 5B and C).

The inability to detect antigen-specific T-cell responses in either *T. cruzi*-infected or pTSSA-vaccinated CD28-deficient mice is intriguing. There might be different requirements for costimulatory molecules in order to mount appropriate T-cell responses depending on the infectious agent or individual immunogens (52). Although *T. cruzi* can infect essentially any nucleated cells, it preferentially infects and proliferates in skeletal muscle or cardiac muscle cells. In contrast, *L. major* infects mostly macrophages and dendritic cells. *T. gondii* also infects any cell type; however, it vigorously infects macrophages at the early stage of infection and later infects cells in the central nervous system in the chronic stage. The different tissue tropism of each microorganism might be linked to the different requirements for costimulatory molecules for the optimal induction of the host immune response. Considering a report that suggests that the DNA vaccine works through local myocytes functioning as APCs (12) and that myocytes express CD80 and CD86 molecules (30, 40), the CD28-CD80/CD86 costimulatory pathway might be important for the induction of effective T-cell-mediated immunity when myocytes work as APCs.

It is also intriguing that the CD28-CD80/CD86 costimulatory pathway is required for memory responses to *T. gondii* infection, since the CD28-deficient mice that were chronically infected with a low-virulence strain of *T. gondii* were susceptible to rechallenge with a virulent strain (50). This deficiency in the protective memory response by CD28-deficient mice correlated with a lack of IL-2 and IFN-γ in recall responses and reduced numbers of $CD4^+$ T cells expressing a memory phenotype (50). The results have suggested that the requirement of this costimulatory pathway also depends on the stages of infection. We could not perform similar experiments in *T. cruzi* infection, since the CD28-deficient mice could not survive its primary infection. However, it might be important to analyze how this costimulatory pathway functions during the chronic stage of *T. cruzi* infection.

The present study demonstrates, for the first time, the immunological role of the CD28 costimulatory molecule against *T. cruzi* infection. Chagas' disease exhibits two distinct clinical phases: the acute and chronic stages (3). T-cell-mediated immunity is crucial for both protective and pathogenic immune responses in both stages (3, 9). In this respect, analyses of

T-cell costimulation are important in order to understand the underlying immunological mechanisms that cause specific pathologies in the chronic stage. In addition, modifying costimulation could be potentially useful for developing new vaccination strategies (14, 51) in order to achieve sterile immune responses by combining vaccination with other attractive strategies, such as the prime-boost strategy (27, 32, 53). Since sterile immunity would be a desirable outcome from an immunization to protect from late complications of Chagas' disease, the recent repertoire expansion of costimulatory molecules (8, 19) provides a continuously attractive target for effective vaccine development, particularly in the search for an IL-12 substitute for future clinical application (36, 48). In this regard, research on Chagas' disease should include further efforts to analyze other costimulatory molecules in addition to the CD28 molecule.

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