Trypanosoma cruzi as an effective cancer antigen delivery vector

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One of the main challenges in cancer research is the development of vaccines that induce effective and long-lived protective immunity against tumors. Significant progress has been made in identifying members of the cancer testis antigen family as potential vaccine candidates. However, an ideal form for antigen delivery that induces robust and sustainable antigen-specific T-cell responses, and in particular of CD8^{+} T lymphocytes, remains to be developed. Here we report the use of a recombinant nonpathogenic clone of *Trypanosoma cruzi* as a vaccine vector to induce vigorous and long-term T cell-mediated immunity. The rationale for using the highly attenuated *T. cruzi* clone was (*i*) the ability of the parasite to persist in host tissues and therefore to induce a long-term antigen-specific immune response; (*ii*) the existence of intrinsic parasite agonists for Toll-like receptors and consequent induction of highly polarized T helper cell type 1 responses; and (*iii*) the parasite replication in the host cell cytoplasm, leading to direct antigen presentation through the endogenous pathway and consequent induction of antigen-specific CD8^{+} T cells. Importantly, we found that parasites expressing a cancer testis antigen (NY-ESO-1) were able to elicit human antigen-specific T-cell responses in vitro and solid protection against melanoma in a mouse model. Furthermore, in a therapeutic protocol, the parasites expressing NY-ESO-1 delayed the rate of tumor development in mice. We conclude that the *T. cruzi* vector is highly efficient in inducing T cell-mediated immunity and protection against cancer cells. More broadly, this strategy could be used to elicit a long-term T cell-mediated immunity and used for prophylaxis or therapy of chronic infectious diseases.

cytokine | prophylaxis | protozoa | innate immunity | *Trypanosoma cruzi* CL-14

The NY-ESO-1 antigen, a member of the cancer testis antigen (CTA) family, has been extensively characterized in terms of its distribution in tumor cells and immunological properties in humans (1). It induces integrated responses involving both the cellular and humoral arms of the immune system and is currently being tested in different clinical trials of antitumor vaccines (2). However, a major challenge for the development of an effective anticancer vaccine is the process and context of antigen delivery.

In addition to antigen retention and slow release, an inflammatory milieu is necessary for development of T cell-mediated immunity with the characteristics required to control tumor cells. The discovery of Toll-like receptors (TLRs), which recognize microbial molecular patterns, has led to significant progress in the field of immunological adjuvants, necessary to initiate adaptive immune responses and T cell-mediated immunity in vaccine protocols (3, 4). Although able to induce potent B- and T-cell responses, the frequency of antigen-specific T lymphocytes induced by TLR agonists rapidly decays. Furthermore, the conventional delivery of antigens associated to adjuvants favors antigen processing and presentation by MHC class II and results

in relatively weak responses of CD8^{+} T cells, which are critical for immunological control of different types of tumors (5).

The development of nonreplicative viral vectors partially resolved this question and brought advancement to the field (6). However, clinical trials with existing vectors have yielded poor results. Here, we propose that the intracellular protozoan *Trypanosoma cruzi* would be a highly effective vaccine vector to induce antitumor protective immunity mediated by T lymphocytes. The rationale for using *T. cruzi* as a tumor vaccine vector is based on earlier immunological studies demonstrating the capacity of this parasite to elicit a strong and persistent T cell-mediated immunity (7, 8). The critical immunostimulatory characteristics of this parasite are (*i*) the existence of intrinsic agonists for TLRs (9–13), which favor induction of highly polarized T helper cell type 1 (Th1) response; (*ii*) the ability to persist in host tissues and keep boosting the immune response; and (*iii*) the ability to replicate in the host cell cytoplasm, resulting in direct antigen presentation to CD8^{+} T-cell response (14).

To test our hypothesis, we have chosen to use the highly attenuated CL-14 clone of *T. cruzi* (15). Through the use of the CL-14 clone we generated three different *T. cruzi* lineages expressing NY-ESO-1. The transgenic parasites were shown to induce strong NY-ESO-1–specific immune responses, both in human cells in vitro and in the mouse model in vivo. The development of NY-ESO-1–specific immunity, including CD8^{+} T-cell responses, and antitumor activity were dependent on both IL-12 and MyD88 pathway. Furthermore, immunization with CL-14 stably expressing NY-ESO-1 resulted in prevention and delay of tumor development in prophylactic and therapeutic protocols, respectively. Hence, we demonstrated that *T. cruzi* is an effective antigen delivery vector for induction of T cell-mediated immunity and should be further explored as a potential vaccine vector for anticancer vaccines.

Results

Stable Transgenic *T. cruzi-NY-ESO-1* Are Able to Express the Exogenous Protein. We engineered the CL-14 to express NY-ESO-1, a well-defined and highly immunogenic CTA, which is currently being tested as a vaccine candidate against cancer in different clinical trials (16–19). For homologous recombination, we used

the **pROCKNeo** plasmid (20) that promotes insertion of the transgene in one of the many copies of the *β-tubulin* gene in the *T. cruzi* genome (Fig. S1*A*). Parasites were stably transfected with three different constructs of *NY-ESO-1*. Two plasmids encoded the *NY-ESO-1* gene, with or without a 6x histidine (His) tag, were used to promote NY-ESO-1 expression in the parasite cytoplasm (Fig. S1 *B* and *C*). The third plasmid encoded the *NY-ESO-1* gene along with a signal peptide (SP) from a *T. cruzi* metalloprotease, named glycoprotein 63 (gp63) (21), and used to favor NY-ESO-1 secretion by the parasite (Fig. S1*D*). Fig. S1*E* shows an agarose gel confirming the plasmid constructs. As shown in Fig. 1*A*, each of the constructs containing *NY-ESO-1* gene were efficiently inserted into the genomic DNA from transgenic parasites. After digestion of parasite genomic DNA with BamHI and SacI, only one major band hybridized with the *NY-ESO-1* probe. Thus, we assume that *NY-ESO-1* gene was inserted in the *β-tubulin* locus. Fig. 1*A*, *Right*, shows control hybridization with the *T. cruzi* gene named *amastin*, which was present in the genome from all WT (22) and transgenic parasites. Recombinant NY-ESO-1 (rNY-ESO-1) was recovered in the pellet of parasites transfected with the coding region of the gene for NY-ESO-1 protein with 6x-His tag [CL-14-NY-ESO-1His(+)] or without the tag [CL-14-NY-ESO-1His(-)], being consistent with the localization in the cytoplasm. In contrast, the rNY-ESO-1 was found primarily in the supernatants of cultures with parasites transfected with *NY-ESO-1* gene, which included the gp63 SP (CL-14-NY-ESO-1gp63SP) (Fig. 1*B*). Immunofluorescence analyses indicate that rNY-ESO-1 is expressed in all different stages of the parasite (Fig. 1*C*), including the trypomastigote metacyclic form, which was used to vaccinate mice and infect human cells in all experiments described below.

The amastigote forms are the replicative intracellular stage of *T. cruzi* and persist in the host tissues for life. This is thought to be critical for eliciting long-lasting CD8$^+$ T lymphocytes during *T. cruzi* infection. We carefully examined the expression of rNY-ESO-1 by intracellular amastigotes in two human cell lines, SK-MEL-149 and SK-MEL-52, which were selected on the basis of their ability to constitutively express NY-ESO-1. SK-MEL-52 that expresses NY-ESO-1 was used as a positive control (Fig. 2*A*, *Upper*). As negative control, the SK-MEL-149 cell line, which does not express NY-ESO-1, was used uninfected or infected with WT parasite (Fig. 2*A*, *Lower*). The immunocytochemistry results clearly show the presence of NY-ESO-1 only in the SK-MEL-149 cell line infected with either CL-14-NY-ESO-1His(+) or CL-14-NY-ESO-1gp63SP. NY-ESO-1 was localized in the intracellular amastigotes expressing NY-ESO-1His(+) protein. Consistent with protein secretion, the recombinant CTA was homogeneously diffused in the cytoplasm of cells infected with CL-14-NY-ESO-1gp63SP (Fig. 2*A*, *Lower*).

Human T-Cell Immunity Induced by Transgenic Parasites. We also assessed the ability of CL-14-NY-ESO-1His(+) or CL-14-NY-ESO-1gp63SP to stimulate in vitro CD4$^+$ T and CD8$^+$ T cells purified from peripheral blood mononuclear cells (PBMCs) from unprimed healthy donors. Autologous antigen-presenting cells (APCs, CD4$^-$/CD8$^-$) were infected with WT (negative control) or transfected parasites and used for in vitro sensitization followed by evaluation of both CD4$^+$ T and CD8$^+$ T-cell responses specific for NY-ESO-1. To detect the T-cell response elicited by transgenic parasites, presensitized cells were washed and cocultured with target APCs (EBV-transformed B lymphocytes) from the same donor, infected with *T. cruzi*, fowlpox expressing or not NY-ESO-1, or APCs primed with a pool of immunostimulatory peptides derived from NY-ESO-1 amino acid sequence. Our results with two donors (NC235 and NC236) presented in Fig. 2 *B* and *C* demonstrate the ability of transgenic parasites to elicit NY-ESO-1–specific CD4$^+$ T as well as CD8$^+$ T lymphocytes. The latter results were confirmed by the increased frequency of CD8$^+$ T lymphocytes, which reacted with a tetramer construct containing HLA*0201 compatible to the donors used in the study, and the NY-ESO-1$_{157-165}$ peptide (Fig. 2*C*). Thus, we concluded that CL-14 clone expressing NY-ESO-1 was a potent inducer of both CD4$^+$ T as well as CD8$^+$ T-cell responses in humans.

***T. cruzi-NY-ESO-1* Parasites Induce Antigen-Specific Immune Response and Tumor Inhibition in Mice.** We next tested the ability of transgenic CL-14 (CL-14-NY-ESO-1) to elicit NY-ESO-1–specific humoral and cellular responses in mice. We first performed an experiment to define the protocol of immunization. One hour and 20 h after i.p. injection of 1×10^7 carboxyfluorescein succinimidyl ester-labeled CL-14-NY-ESO-1 metacyclics, a significant proportion of the dendritic cells in the lymph nodes (≈39%) and spleens (≈67%), respectively, contained intracellular parasites. Thus, we decided to use this dose and route of parasite inoculation. Furthermore, we tested a single vs. a homologous prime and boost protocol. As shown in Fig. S2 the prime-boost protocol, using CL-14-NY-ESO-1gp63, induced a stronger T-cell response to NY-ESO-1 peptides than a single-dose immunization. Thereafter, we used in all experiments described in this study two immunization doses 30 d apart. The results shown in Fig. 3 were obtained in vivo and demonstrated that all stably transfected parasites elicited high levels of antibodies specific for rNY-ESO-1 (Fig. 3*A*). In particular, we observed the induction of the isotype IgG2c, which is normally induced by IFN-γ produced by CD4$^+$ Th1 lymphocytes. Consistently, we demonstrated a strong IFN-γ response by splenocytes from vaccinated mice, stimulated in vitro with NY-ESO-1–derived peptides that encode epitopes recognized by either CD4$^+$ T or CD8$^+$ T lymphocytes (Fig. 3*B*). As control, we used the CD8$^+$ T cell-specific epitope

Fig. 1. Expression of recombinant NY-ESO-1 by stably transfected *T. cruzi* parasites. (*A*) Genomic DNA from WT and transgenic parasites was digested with BamHI (B) or SacI (S), submitted to Southern blot, and hybridized with *NY-ESO-1* or *amastin* probes. (*B*) Western blot analysis was used to detect NY-ESO-1 expression, in metacyclic lysates (lanes 1–3 and 5) or supernatants (lane 4) of parasite cultures, using anti-NY-ESO-1 or anti-His tag mAbs. (*C*) NY-ESO-1 expression by the four different stages of the CL-14-NY-ESO-1His(+) was analyzed by immunofluorescence. Parasites stained with anti-NY-ESO-1 mAb (green), or DAPI for DNA, were visualized by differential interference contrast (DIC).
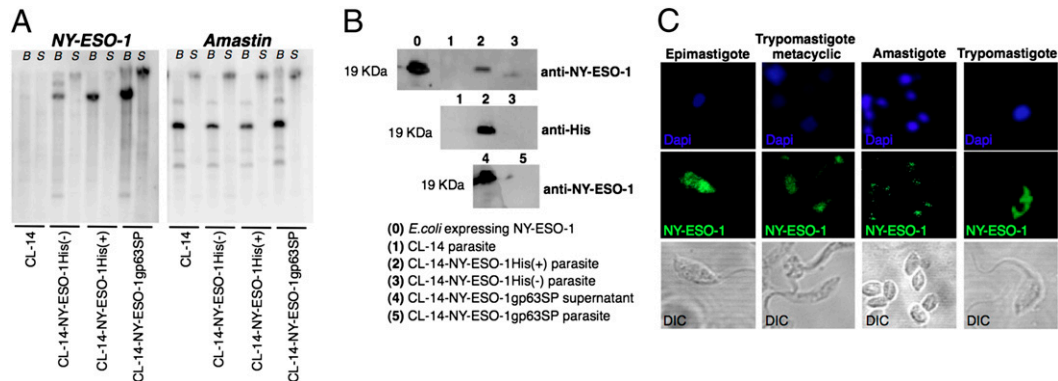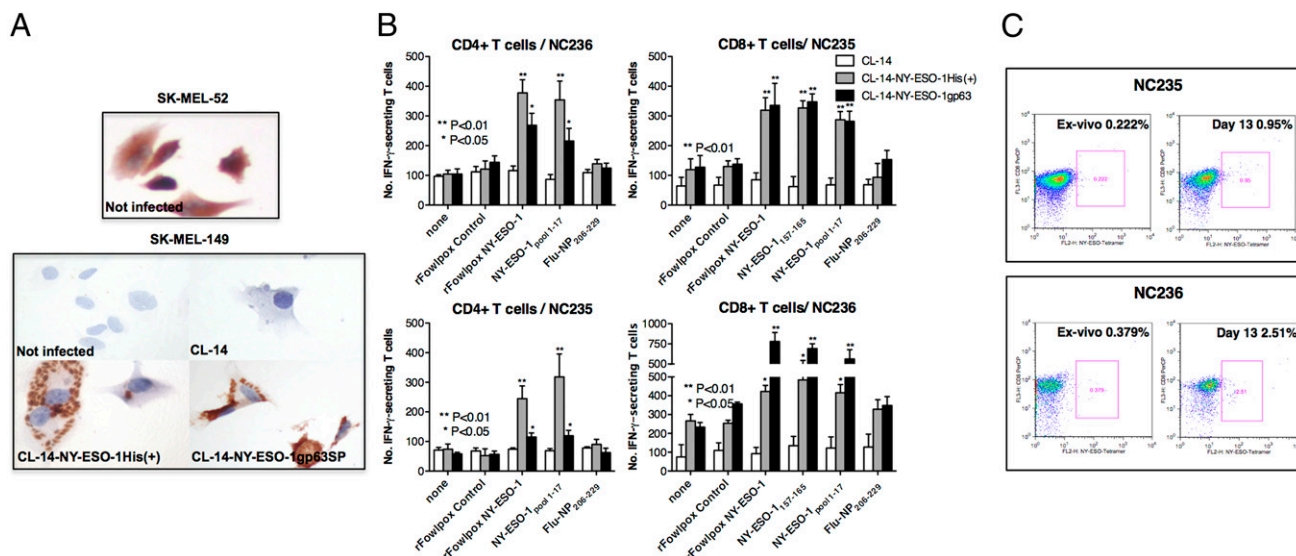
Fig. 2. Priming with transgenic parasites elicits IFN-γ–producing T cells specific for NY-ESO-1. (*A*) A melanoma cell line (SK-MEL-149) that does not express NY-ESO-1 was infected with either WT or transgenic parasites and expression of rNY-ESO-1 detected by immunohistochemistry. SK-MEL-52 cells were used as positive control. (*B*) CD8$^+$ T as well as CD4$^+$ T cells from healthy donors (NC235 and NC236) were cocultured with autologous APCs infected with WT or transgenic parasites expressing different forms of rNY-ESO-1. Induction of antigen-specific T cells was analyzed by quantifying the number of IFN-γ–producing cells by ELISPOT after restimulation with autologous EBV-B cells pulsed with peptides or infected with rFowlpox virus, as indicated. (*C*) The frequency of NY-ESO-1–specific CD8$^+$ T cells was determined by flow cytometry. Percentages indicate the frequency of CD8$^+$ T cells stained with NY-ESO-1 tetramer.

derived from transialidase, a surface antigen from *T. cruzi*. Both WT and transgenic parasites primed CD8$^+$ T cells to produce high levels of IFN-γ in response to this epitope. We also observed that the induction of NY-ESO-1–specific IgG2c, the levels of IFN-γ, and the frequency of IFN-γ–producing CD4$^+$ T as well as CD8$^+$ T lymphocytes correlated with protection, which was significantly higher in mice vaccinated with transgenic parasites than with recombinant NY-ESO-1 protein associated to TLR agonists (Fig. S3 *A* and *B*).

Importantly, vaccination with transgenic parasites expressing NY-ESO-1gp63SP as well as NY-ESO-1His(+) induced complete protection and prevented mortality in mice challenged with a syngeneic transgenic melanoma, the B16F10 cell line expressing NY-ESO-1 (23) (Fig. 3 *C* and *D*). In contrast, the formulations using rNY-ESO-1 protein and TLRs agonists were only able to delay tumor development and mortality (Fig. S3 *C* and *D*). We also tested the ability of transgenic parasites in a therapeutic protocol to reverse/delay B16-NY-ESO-1 growth in C57BL/6 mice (Fig. S4 *A* and *B*) or in BALB/c mice challenged with fibrosarcoma (CMS5a) (24) or colon adenoma (CT26) (25) cell lines. The results shown in Fig. 4 also demonstrate that repeated injections with trypomastigote metacyclics expressing NY-ESO-1 were able to delay tumor growth and prolong survival for both tumor cell lines CMS5a-NY-ESO-1 (Fig. 4 *A* and *B*) and CT26-NY-ESO-1 (Fig. S4 *C* and *D*). It is noteworthy that no parasitemia/parasitism or any sign of *T. cruzi* infection was observed, even after 90 d of immunization with CL-14-NY-ESO-1 (Fig. 3*D* and Fig. S3*D*).

***T. cruzi*-NY-ESO-1 Leads to IL-12 and MyD88 Dependent Pathway.** To investigate the mechanism by which CL-14-NY-ESO-1gp63SP induces protective immunity against the B16-NY-ESO-1 cell line, we used the $MyD88^{-/-}$, $IL\text{-}12p40^{-/-}$, $iNOS^{-/-}$, and $\beta2\text{-}microglobulin^{-/-}$ (CD8 T cell-deficient) mice. None of these knockout mice that are otherwise highly susceptible to *T. cruzi* (11, 14, 26, 27) showed any sign of disease when vaccinated with the live transgenic parasites or the WT CL-14 (Fig. S5). Importantly, our results show that upon in vitro stimulation with either rNY-ESO-1, NY-ESO-1, or *T. cruzi*–derived peptide

(TSKB20), the IFN-γ production by CD4$^+$ T and CD8$^+$ T lymphocytes was severely impaired in both $MyD88^{-/-}$ and $IL\text{-}12p40^{-/-}$ mice vaccinated with CL-14-NY-ESO-1gp63SP (Fig. 5*A*). Similar results were obtained when we immunized knockout mice with either Alum/CpG/NY-ESO-1 or Alum/MPL/NY-ESO-1 (Fig. S6). On the other hand, we found no significant decrease in the frequency of CD8$^+$ T lymphocytes specific for NY-ESO-1 epitope in the various knockout (KO) mice vaccinated with the transgenic parasites (Fig. 5*B*).

We also challenged the different KO mice with B16-NY-ESO-1 (Fig. 5*C*). We observed that protective immunity elicited by vaccination was not effective in either $MyD88^{-/-}$ or $IL\text{-}12p40^{-/-}$ mice, suggesting the critical role of TLRs in inducing IL-12 and consequently IFN-γ production and protection elicited by the CL-14-NY-ESO-1gp63SP. We also observed that vaccinated $\beta2\text{-}microglobulin^{-/-}$ mice (CD8$^{-/-}$), which lack CD8$^+$ T lymphocytes, were unable to control tumor growth, suggesting a critical role of this T-cell subset in host resistance to B16-NY-ESO-1. In contrast, mice deficient in inducible nitric oxide synthase controlled tumor growth when vaccinated with CL-14-NY-ESO-1 (Fig. 5*C*). Together, these results indicate that the adjuvant activity of CL-14 clone is highly dependent on MyD88 and IL-12, whereas CD4$^+$ T as well as CD8$^+$ T lymphocytes are the main cellular sources of IFN-γ.

Discussion

Both attenuated viruses and bacteria have been largely explored as vaccine vectors to elicit T cell mediated immunity and in particular CD8$^+$ T-cell responses (6, 24). Although effective, some of these vectors induce strong but not persistent immunity. A possible explanation for the decay of the antigen-specific T-cell response is related to the fact that these attenuated viruses or bacteria are often not replicative, and infection is rapidly eliminated by the immune system. One possible alternative is to vaccinate with vectors that persist in the host, ensuring long-term immunity. In this regard, like other parasites, *T. cruzi* coevolved with their hosts, to maintain a long-lived infection (8). As a consequence, the parasite induces a strong immunity that
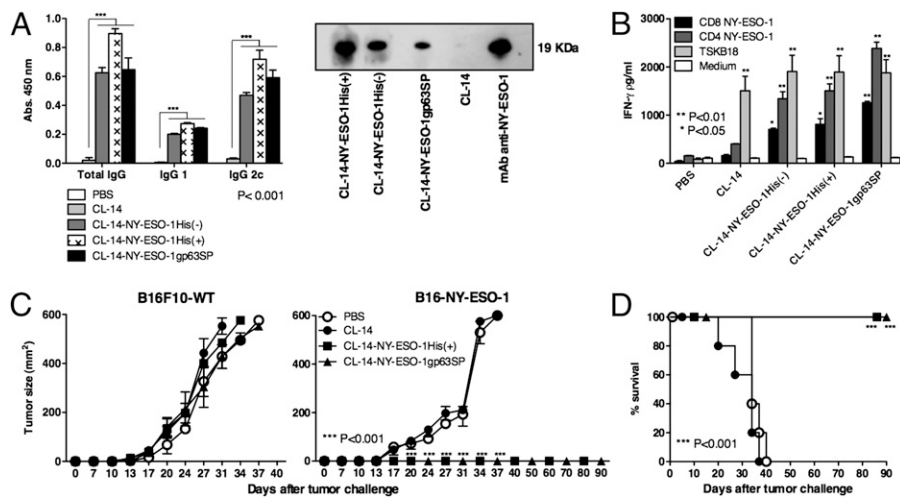
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Fig. 3. Antigen-specific humoral and cellular responses and complete protection induced by immunization with transgenic parasites expressing NY-ESO-1. (*A*) ELISA plates coated with rNY-ESO-1 were used to quantify the levels of NY-ESO-1–specific total IgG, IgG1, and IgG2c isotypes present in sera of control and immunized mice; Western blot membranes containing rNY-ESO-1 were individually incubated with sera of mice immunized with each of the three transgenic or WT parasites, or the anti-NY-ESO-1 mAb. (*B*) Splenocytes from control or immunized mice were cultured in the presence or absence of NY-ESO-1 peptides encoding epitopes specific for CD4⁺ T or CD8⁺ T cells. As positive control we used a *T. cruzi*–derived immunodominant epitope named TSKB18. IFN-γ production was measured at 72 h after stimulation by a sandwich ELISA. Control and immunized mice were challenged with 5×10^4 B16F10 melanoma cells expressing or not NY-ESO-1 (*n* = 4). Tumor growth (*C*) and survival (*D*) were measured for 90 d (*n* = 6).

persists throughout the infection, which prevents reemergence of intense parasitism and secondary infection with *T. cruzi*.

Different studies associate the presence of local tissue parasitism with the development of chronic cardiac and digestive pathology, both in humans and mice (28–30). Hence, we used CL-14, a highly attenuated clone of *T. cruzi* that was derived from the CL strain (15, 31) in the early 1980s and kept attenuated, even after continuous passages in liquid cultures. The CL-14 clone is consistently avirulent, and both parasitemia and tissue parasitism are absent or very scarce, even when infecting newborn mice that are highly susceptible to *T. cruzi* infection (31). In addition, our results show that various KO mice, which are otherwise very susceptible to *T. cruzi* infection (11, 14, 26, 27), did not show signs of parasitemia or lethality upon vaccination with the live CL-14 clone or the CL-14 clone expressing NY-ESO-1. As a consequence, the systemic aberrant activation of the immune system and pathogenesis are not observed even in highly susceptible strains of mice, as one would predict from studies with virulent strains of *T. cruzi* (32). Importantly, vaccination with live CL-14 was repeatedly shown to induce a potent and long-lasting parasite-specific antibody and T cell-mediated immunity, characterized by high levels of IgG2, IFN-γ, and CD8⁺ T-cell responses, as well as solid protection against highly virulent strains of *T. cruzi* (33). Thus, we assume that the immunological adjuvant properties of CL-14 are still effective. *T. cruzi* parasites have intrinsic TLR agonists, such as glycosylphosphatidylinositol anchors (9, 10), unmethylated CpG motifs found on its nuclear DNA (11, 12) and RNA (13) that ensure the continuous stimulation of polarized antigen-specific Th1 lymphocytes. In addition, long-lived intracellular parasitism is likely to be another important characteristic of CL-14-NY-ESO-1 for the prolonged stimulation

of CD8⁺ T cells by MHC class I from parasitized host cells (34) and persistent immunity.

Inspired by the "malariotherapy," which used infection with *Plasmodium* to treat neurosyphilis (35), Roskin and colleagues thought that parasitic infections that cause serious diseases produced a "toxin" that could act on and kill tumor cells (36–38). Almost 10 y after he started his studies, Roskin published an article demonstrating tumor regression after infection with *T. cruzi* in mice (37). Later, he published an article demonstrating tumor regression in human patients infected with *T. cruzi* (38). A growing interest in using *T. cruzi* to treat cancer was pursued by different research groups (39, 40), and *T. cruzi* preparations for cancer therapy were launched by French and Russian pharmaceutical companies (36). Although similar results were found, the ambiguity of the findings obtained by various investigators regarding efficacy against experimental tumors (41) led to the discontinuation of this line of investigation and commercial distribution of this anticancer compound. We assume that, at least in part, the antitumor activity of *T. cruzi* infection was due to the strong activation of the innate immune system. The immunostimulatory effect of *T. cruzi* infection results in the production of high levels of proinflammatory cytokines, such as TNF-α (42), which leads to activation of effector mechanisms that efficiently destroy tumor cells (43).

Importantly, either CL-14-NY-ESO-1-His(+) or CL-14-NY-ESO-1gp63 induced in vitro, both human CD4⁺ T as well as CD8⁺ T lymphocytes to produce IFN-γ after restimulation with either fowlpox expressing NY-ESO-1 or NY-ESO-1 peptides. Our results also show that in a prophylactic protocol, CL-14-NY-ESO-1 was highly effective in inducing immunity to tumor development, compared with vaccination with recombinant NY-
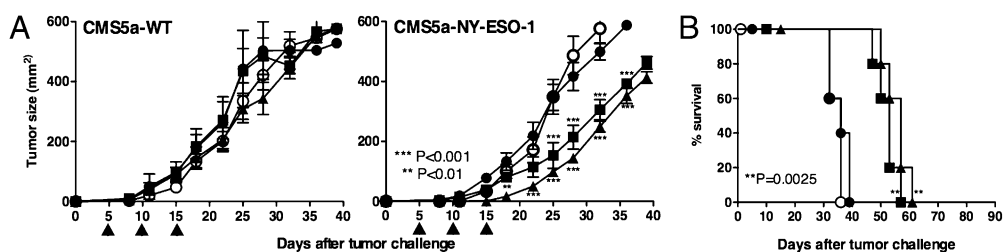

Fig. 4. Therapeutic protocols using transgenic parasites delay the growth of tumor cells expressing NY-ESO-1. 1×10^6 cells of the fibrosarcoma CMS5a (*A* and *B*) expressing or not rNY-ESO-1 were used to challenge BALB/c mice (*n* = 6). Mice were treated with three doses of 10^7 metacyclic forms of WT (CL-14) or transgenic parasites given 5 d apart (arrows), starting at day 5 after challenge. Tumor growth (*A*) and survival (*B*) were monitored for 40 and 90 d, respectively.
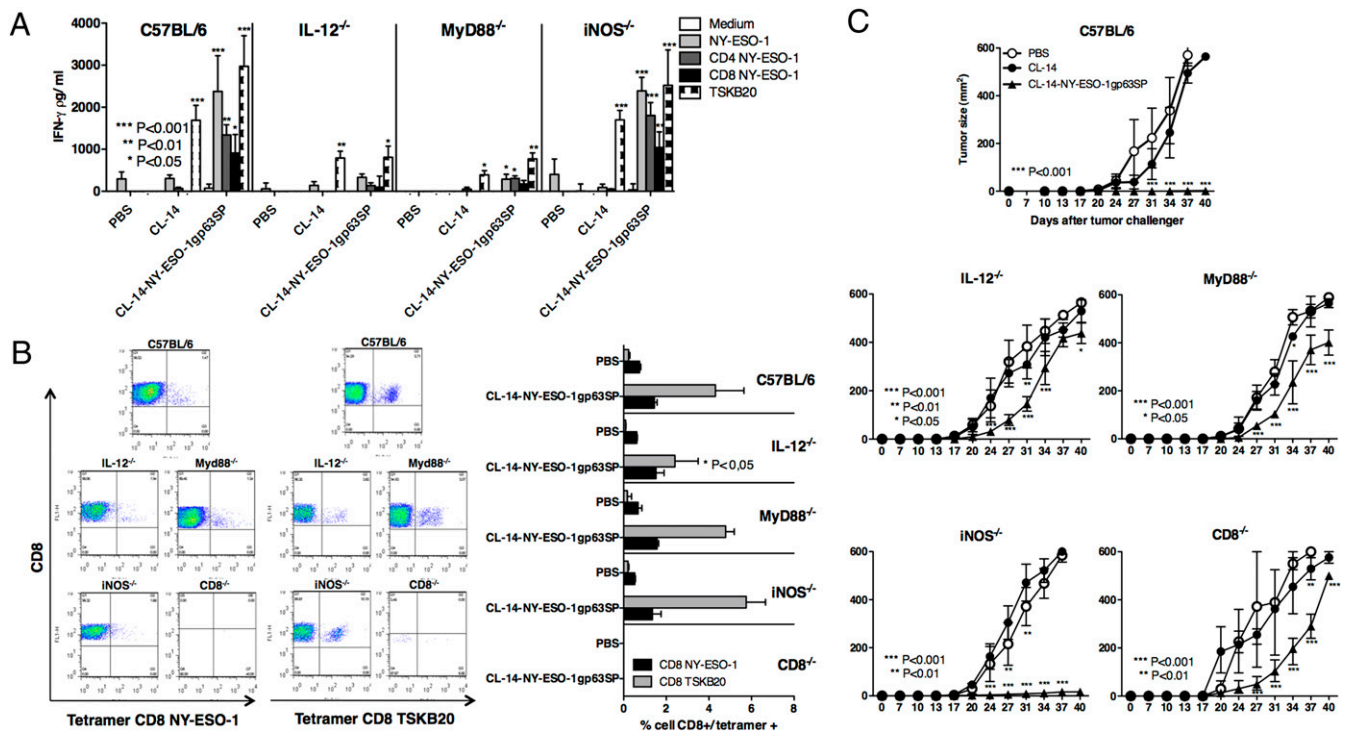
Fig. 5. MyD88- and IL-12–dependent induction of IFN-γ–producing T cells and protective immunity elicited by immunization with CL-14 expressing NY-ESO-1. (A) Splenocytes from vaccinated WT (C57BL/6) and KO mice (MyD88$^{-/-}$, IL-12$^{-/-}$, iNOs$^{-/-}$, and CD8$^{-/-}$) were restimulated with T CD4$^+$- or T CD8$^+$-specific peptides or with rNY-ESO-1 protein (n = 4). IFN-γ production was measured by ELISA at 72 h after stimulation. (B) Before restimulation, the splenocytes were stained with anti-CD3, anti-CD8, and NY-ESO-1 or TSKB20 tetramers and analyzed by flow cytometry. Representative dot blots and a graph summarizing the percentage of double-positive cells are shown at *Left* and *Right*, respectively. (C) Immunized C57BL/6 and KO mice were challenged with 5 × 10^4 of the B16F10 melanoma cells expressing the NY-ESO-1 and monitored 40 d for tumor growth (n = 6).

ESO-1 associated with classic TLR agonists. Furthermore, we show that the levels of IFN-γ responses and frequency of IFN-γ–producing T cells nicely correlate with protective immunity and were dependent of MyD88, IL-12, and CD8$^+$ T lymphocytes. In contrast, the effect of therapeutic vaccination with CL-14-NY-ESO-1 was only partial. We believe that this might be explained in part by the ability of tumors to elicit immunoregulatory mechanisms, such as regulatory T cells, expression of CTLA-4, IL-10, and TGF-β (44–47), that prevent establishment of an optimal immune response and complete reversion of tumor growth. This is an important question, which is currently being investigated in our laboratory.

It is noteworthy that the technology we developed for stable transfection targets the β-tubulin gene, which has multiple copies (≈40 copies) in the *T. cruzi* genome (48). Although CL-14 is highly susceptible to the existing drug (benznidazole) used to treat Chagas disease (49), we also have engineered *T. cruzi* to simultaneously express both NY-ESO-1 and the herpes virus thymidine kinase. Thus, an additional safety mechanism for eliminating persistent parasites could be the use of acyclovir therapy. Another attractive idea that has already been tested in the laboratory is the generation of CL-14 expressing different tumor antigens, which could be used as polyvalent antitumor vaccine. One important question that is currently being addressed in our laboratory concerns the molecular basis of the attenuated phenotype of the CL-14 clone. CL-14 has been kept stable for 3 decades, and this strategy could lead us to develop genetically engineered parasites with lower risk of reversion of the attenuated phenotype.

In conclusion, on the basis of the ability of *T. cruzi* parasites to strongly activate both innate and acquired immunity, we explored genetically engineered attenuated parasites to express tumor antigens as a strategy to develop an anticancer vaccine. Our initial hypothesis was that by triggering innate immunity and production of proinflammatory cytokines, in particular IL-12, *T. cruzi* parasites stimulate strong and long-lasting antigen-specific CD4$^+$ Th1 as well as CD8$^+$ T-cell responses. As a consequence, vaccination with *T. cruzi* infection would lead to activation of immunological effector mechanisms, including the production of TNF-α, that are highly effective in killing or controlling the growth of tumor cells. We envisage ethical barriers of using *T. cruzi* parasites to prevent or treat cancer. Nevertheless, this study highlights a new strategy to induce a highly and long-lasting T cell-mediated immune response, which is often poorly achieved in vaccine protocols against different types of tumors. Hence, the experiments presented here indicate that use of *T. cruzi* parasites as a live vector for an anticancer vaccine should be further explored.

Materials and Methods

Parasite Construction and Characterization. Transgenic parasites were obtained by cloning the *NY-ESO-1* Open reading frame (ORF), fused to His tag or to the gp63 SP sequences into the pROCKNeo plasmid. *T. cruzi* epimastigotes were transfected and maintained as previously described (20). The integration of the exogenous gene into the parasite genome was confirmed by Southern blot analysis of genomic DNA digested with BamHI and SacI and hybridized with *NY-ESO-1* and *amastin* probes labeled with ^{32}P. Expression of rNY-ESO-1 was detected by Western blot and fluorescence microscopy using the anti-NY-ESO-1 or anti-His tag mAbs.

Immunohistochemistry and Human T-Cell Immune Response. For immunohistochemistry, the SK-MEL-149 melanoma cell line infected or not with WT or transgenic parasites and the SK-MEL-52 cell line that constitutively expresses NY-ESO-1 (positive control) were stained with anti-NY-ESO-1 mAb (PowerVison Kit; Leica Microsystems). For human T-cell responses we used PBMC-derived irradiated APCs (CD4$^-$CD8$^-$) infected with 30 parasites per cell and cocultured with purified autologous CD8$^+$ T or CD4$^+$ T cells (50). The cellular immune

response was evaluated by an IFN-γ enzyme-linked immunosorbent spot assay (ELISPOT), 72 h after restimulation with autologous EBV-B cells pulsed with peptides or infected with recombinant Fowlpox (rFowlpox) virus. For flow cytometry analysis, CD8^{+} T cells were stained with anti-CD8 mAb and NY-ESO-1 tetramer using the HLA-A*0201 and NY-ESO$_{157-165}$ peptide.

Mouse Immune Response and Tumor Challenge. WT and KO mice received two i.p. doses, 30 d apart, of 10^7 metacyclic forms of *T. cruzi*. At 21 d after the boost, sera was collected to measure the levels of anti-NY-ESO-1 IgG isotypes by ELISA or Western blot; and spleen cells were cultured with or without 10 μM of either CD4-NY-ESO-1, CD8-NY-ESO-1, or TSKB18 peptides. IFN-γ was quantified in tissue culture supernatants by ELISA (R&D Systems). For flow cytometry analysis, splenocytes were stained with anti-CD3, anti-CD8 (BD Pharmingen), and NY-ESO-1 or TSKB18 tetramers. Alternatively, mice were challenged s.c. with 5×10^4 B16 melanoma cell lines expressing or not NY-ESO-1. For immunotherapy, mice were challenged s.c. with either 5×10^4 B16 melanoma, 1×10^6 CT26 colon adenoma, or CMS5a fibrosarcoma cell lines expressing or not NY-ESO-1 and treated with three doses of live parasites, 5 d apart, starting at day 5 after challenge.

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