COMPARATIVE ASPECTS OF T CELL ACTIVATION *IN VIVO* FOLLOWING STIMULATION WITH ANTI-CD3 MAB, ALLOGENEIC CELLS AND *TRYPANOSOMA CRUZI*

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INTRODUCTION

We have developed a quantitative *in vivo* model system for the assessment of T cell activation in the draining popliteal lymph node(PLN) population of the mouse, following footpad injection of the mitogen Concanavalin A(Con A;1), monoclonal anti-CD3 antibody(2), or allogeneic cells(3, 4). All three of these stimuli resulted in the induction of IL-2 production, IL-2 receptor expression, and in T cell proliferation when the freshly explanted lymph node was studied *in vitro*. The *in vivo* induced proliferative response observed after Con A or anti-CD3 stimulation was inhibited when the mice were treated with the immunosuppressive drug Cyclosporin A (CSA). In contrast, the proliferative response observed following the injection of allogeneic cells was resistant to the effects of CSA administration *in vivo* and appeared to be mediated by a CSA-resistant, IL-2 independent pathway of T cell activation(2, 4).

In the present report, we have used this model system to analyze the responses of the draining PLN population following footpad injection of irradiated culture-generated trypomastigotes (CMT) of a clone of *Trypanosoma cruzi*. We will demonstrate that injection of Trypanosomes results in the induction of IL-2 receptor expression, responsiveness to IL-2 and IL-4, as well as a proliferative response when the PLN cells are cultured *in vitro*. Although the kinetics of T cell activation *in vivo* observed after injection of Trypanosomes were similar to those observed following injection of allogeneic cells, the response to Trypanosomes was sensitive to the administration of CSA. This result strongly suggests that the early response of the mouse to this parasite is mediated by lymphokines such as IL-2 or IFN-γ which are sensitive to the immunosuppressive effects of this drug.

MATERIALS AND METHODS

Animals- BALB/c and C57BL/6 mice (female, 6 to 8wk of age) were supplied by the Division of Research Services, National Institutes of Health(Bethesda, MD-USA).

In vivo stimulation protocols- BALB/c mice were stimulated with allogeneic cells by injecting 1.5×10^7 normal C57BL/6 spleen cells, subcutaneously (SC) in the rear footpads(RFPs), as previously described(3). 145-2C11, a monoclonal antibody specific for CD3(a kind gift from Dr. J.A. Bluestone, U. of Chicago, Chicago IL-USA; 5) was injected SC, in the RFPs of BALB/c mice, at a dose of 25 µg/RFP, and the PLNs

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were harvested 24hr after challenge; previous studies have shown that this dose results in maximal stimulation(2). Stimulation with *T.cruzi* CMTs from the clone Sylvio-X10/4(6) was induced by injecting irradiated CMTs($1.5x10^5$ rad), SC in the RFPs of the mice(unless otherwise specified the dose was $1.5x10^7$ CMTs/RFP), and the PLNs were harvested three days after the challenge. The clone Sylvio-X10/4 was maintained *in vitro* in axenic conditions, and the CMTs were purified as previously described(7).

CSA treatment *in vitro* and *in vivo*- CSA(Sandimmune I.V.)was generously provided by Sandoz(Sandoz, Inc., East Hanover, NJ-USA).The drug was delivered continuously via an Alzet osmotic pump, model 2001(Alza Corp., Palo Alto CA-USA), implanted SC , at a daily dose of 35 mg/Kg.The osmotic pump was implanted two days before the injection of stimuli(2) . CSA for use *in vitro* was supplied as a powder, dissolved initially in absolute ethanol, and diluted in culture media at a final concentration of 250 ng/ml.

Culture conditions-The culture medium used for all the in *vitro* assays was DMEM, supplemented with 2-mercaptoethanol($5x10^{-5}$M), L-glutamine(2mM), sodium pyruvate(1mM), nonessential amino acids(0.1mM), penicillin(100 U/ml), streptomycin(100 µg/ml), and 10% heat-inactivated fetal bovine serum(Biofluids Inc., Rockville, MD-USA). The *in vivo* induced proliferation was determined by culturing the PLNs($2x10^5$/well) in microtiter wells for 18 hr, in presence of 1µCi/well of tritiated thymidine(^{3}H-TdR, ICN Pharmaceuticals, Irvine, CA-USA). Responsiveness to lymphokines was evaluated by culturing the lymph node cells($2x10^5$/well) in presence of human recombinant IL-1-α(the gift of Hoffmann-La Roche Inc, Nutley,NJ-USA), human recombinant IL-2(the gift of CETUS Corp., Emeryville, CA-USA), and murine recombinant IL-4(the gift of Immunex,Seattle,WA-USA), for 42 hr. Cell proliferation was measured by the addition of 1µCi , ^{3}H-TdR, for the last 18hr of culture.

Flow microfluorimetry - Analysis of changes in volume, and expression of "activation antigens" was performed on the FACSCAN(Becton-Dickinson, Mountain View, CA-USA). The monoclonal antibodies(MAB) specific for the murine IL2 receptor(7D4), LY6-A/E(D7) and an activation-associated homodimer(H1-2F3) were previously described(8,10,9). The MABs were directly labelled with fluorescein(FITC) and the negative controls were stained with the FITC-labelled MABs in the presence of an excess amount of unlabelled antibody. The percentage of positive cells with each antibody was determined after subtraction of the values seen with antibody blockade.

RESULTS

Kinetics of the in vivo response to irradiated T. cruzi CMTs--We have previously demonstrated that the activation response in the draining PLN population peaked 1 day following injection of Con A or anti-CD3 MAB or 3 days following injection of allogeneic cells(1, 2, 3). Following the injection of either BALB/c or C57BL/6 mice with $1.5x10^7$ CMTs, a marked increased in the number of cells in the draining PLN was seen 3 days after footpad injection and the cell number in the PLN remained elevated until day 7 . The *in vivo* induced proliferative responses, and the levels of IL-2 receptor expression peaked on day 3 after injection and were reduced, but still above resting levels on day 7(Figs.1a, 1b). In addition to induction of the IL-2 receptor, the activation of T cells *in vitro* and *in vivo* by a number of stimuli is accom-

panied by induction of increased expression of the Ly-6A/E antigens and by the

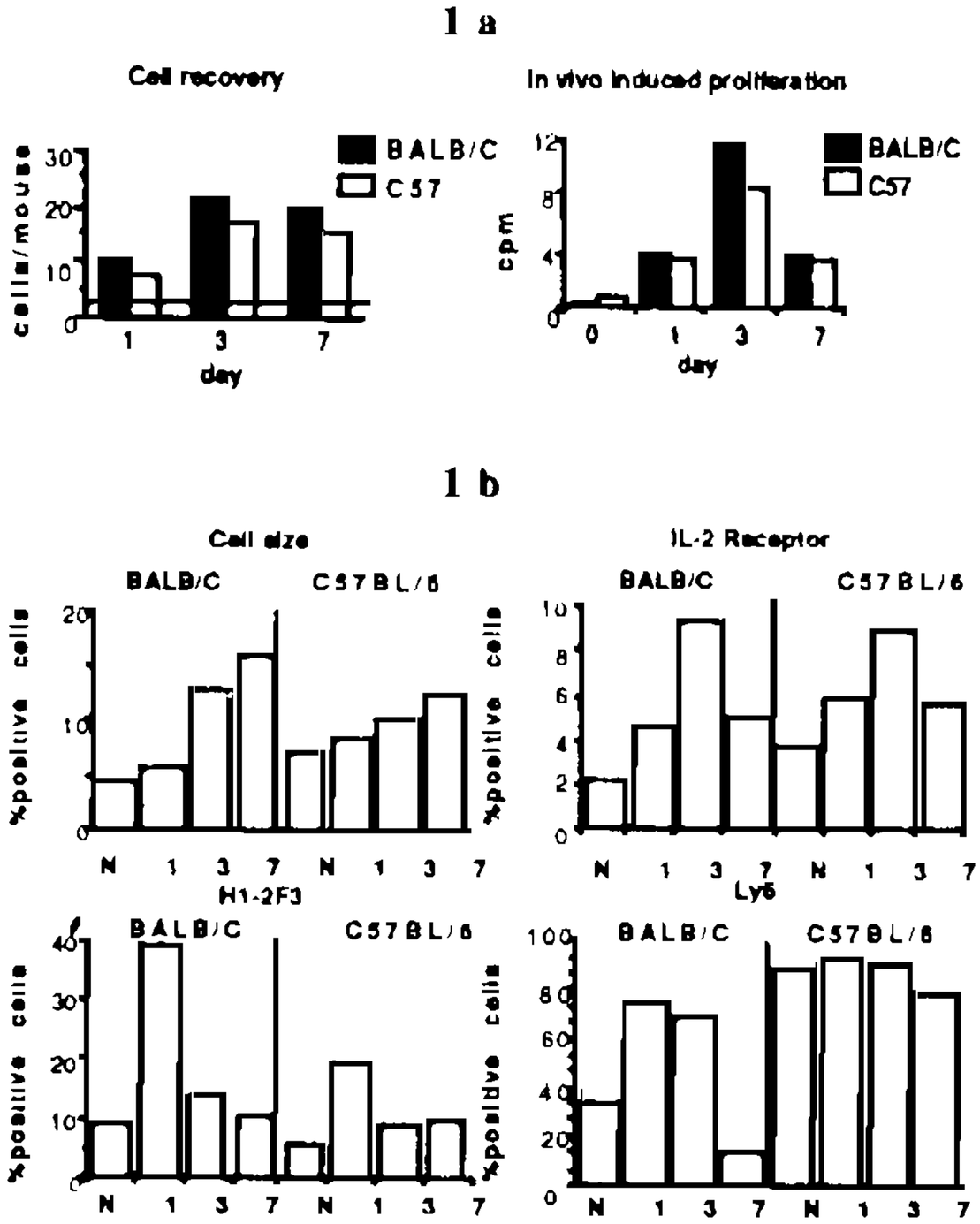

Fig.1a - Kinetics of PLN activation following stimulation *in vivo* with *T.cruzi* CMTs. BALB/c and C57BL/6 mice were injected with $1.5x10^7$ irradiated CMTs/RFP 1, 3, and 7 days before the experiment. The cell recovery is expressed in cells($x10^{-6}$)/mouse. The line parallel to the x axis indicates the number of cells recovered from normal mice. *In vivo* induced proliferation is determined by pulsing the *in vivo* stimulated PLN with ^{3}H-TdR for 18 hr *in vitro*.

Fig.1b - Time course of Cell size variation, and expression of lymphocyte activation antigens after stimulation *in vivo* with *T. cruzi* CMTs. Cell size distribution was evaluated by forward light-scatter(FSC) measurement, with channel 70 chosen for discrimination between large and small cells. Dead cells and debris were excluded by simultaneous use of FSC gating and exclusion of propidium iodide stained cells with a red fluorescence gate (In the x axis N=normal control; 1, 3 and 7 are days after immunization).

expression of a homodimer(murine CD28 homologue) recognized by the H1-2F3 MAB(9, 10). The injection of *T. cruzi* CMTs resulted in a rapid rise and fall in the expression of the dimer recognized by H1.2F3; similar kinetics of expression of this antigen have been observed after injection of Con A or anti-CD3 mAb(9). In contrast, following stimulation of BALB/c mice with the Trypanosomes a marked increase of the number of Ly-6 positive cells was observed one day following injection and this level remained elevated until day 3, but decreased to below the level observed in the resting PLN by day 7. No increase in the level of Ly-6 expression was seen in the C57BL/6 PLNs, but the level of Ly-6 expression in the resting lymph node population of this strain is much higher than that seen in the BALB/c(10).

Comparison of the popliteal LN responses to activation by anti-CD3, allogeneic cells, and *T. cruzi*-- No increases in the number of recovered cells were seen in the PLN at the peak of the response 24 hours following injection of anti-CD3. The number of cells recovered 3 days after the injection of allogeneic cells was very similar to that seen after the injection of the maximal concentration of *T. cruzi*. All three stimuli induced a proliferative response when the LN cells were cultured *in vitro*. However, the response to anti-CD3 stimulation was an order of magnitude greater than that seen

following stimulation with allogeneic cells or *T. cruzi*(Figs. 2a, 2b). The most likely explanation for this result is that the response to anti-CD3 is polyclonal, while the responses to allogeneic cells or *T. cruzi* are of a more restricted nature. All three stimuli were able to induce responsiveness to IL-2(Fig. 2c). In all cases the responses

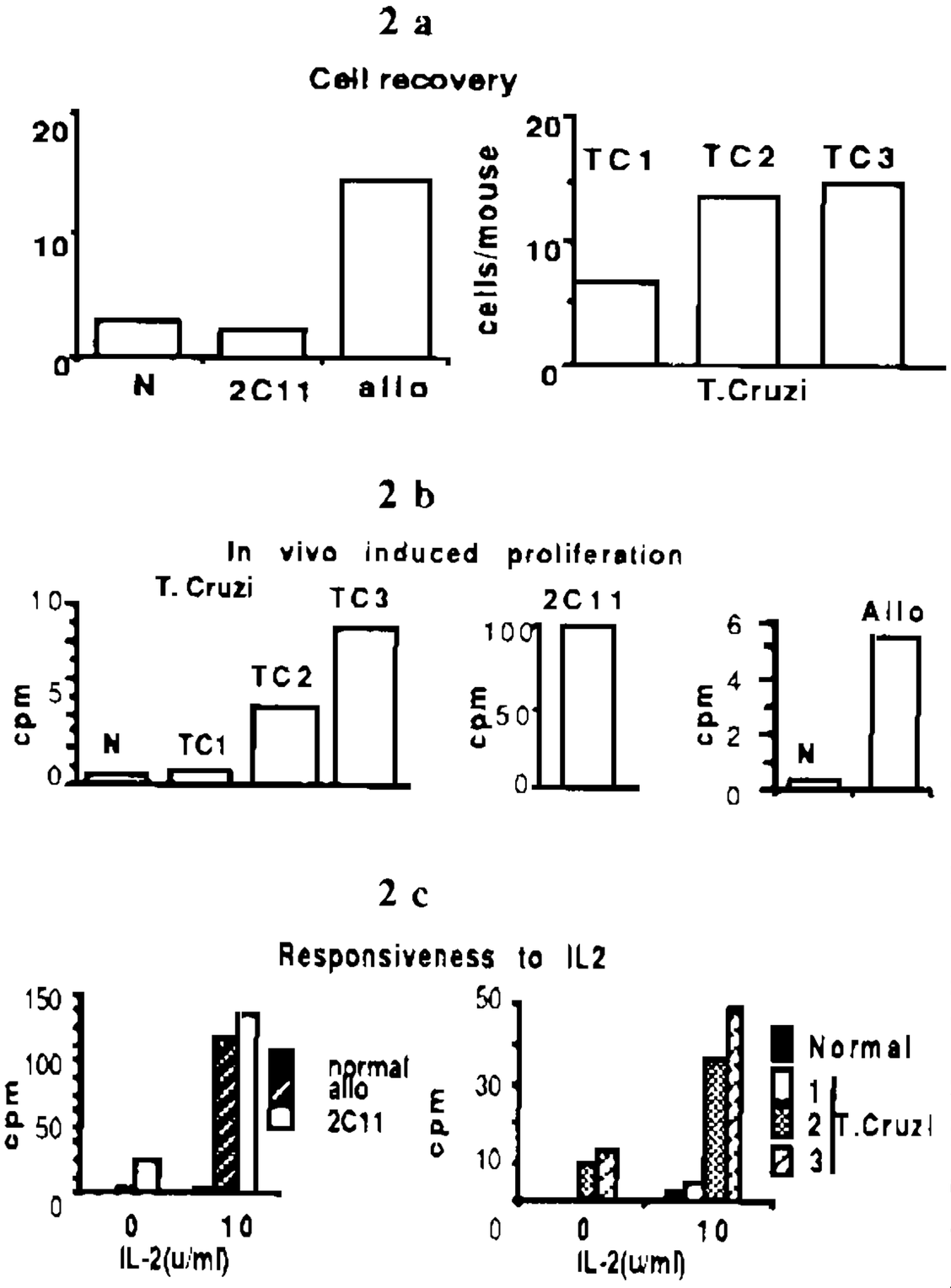

Fig.2a - Comparative analysis of lymphocyte activation parameters after stimulation *in vivo* with anti-CD3 MAB, allogeneic cells, and *T.cruzi* CMTs. N is the normal group, 2C11 corresponds to stimulation with the anti CD3 MAB, and allo is the group stimulated with normal allogeneic spleen cells. BALB/c mice were primed with three different doses of irradiated *T. cruzi* CMTs(TC1=$1.5x10^5$,TC2=$1.5x10^6$ and TC3=$1.5x10^7$ CMTs/RFP). Cell recovery is expressed in PLN cells/mouse($x10^{-6}$) .

Fig 2b - *In vivo* driven proliferation after stimulation with anti-CD3 MAB, allogeneic cells, or *T. cruzi* CMTs. PLN cells from normal controls, mice stimulated SC with 2C11, allogeneic cells or T. cruzi were incubated for 18 hr in the presence of ^{3}H-TdR($cpmx10^{-3}$).

Fig 2c - Responsiveness to IL2 after stimulation *in vivo* with anti-CD3 MAB,allogeneic cells, or *T. cruzi* CMTs. Cells from the same groups shown in fig.2a and 2b were incubated for 42hr in presence or in absence(0) of human recombinant IL-2(10U/ml).The cells were pulsed with ^{3}H-TdR for the last 18hr of culture($cpmx10^{-3}$).

induced by *T. cruzi* were proportional to the number of irradiated parasites injected.

Comparison of the effects of CSA on the responses to *T. cruzi* and allogeneic cells-- If the responder mice were treated with CSA prior to the injection of allogeneic cells, a marked enhancement of the *in vivo* induced proliferative response was seen when the PLN cells from the CSA treated animals were cultured *in vitro*. If CSA was added to these cultures *in vitro*, the enhancement of the proliferative response was eliminated, but a significant proliferative response remained which was resistant to inhibition by CSA. In contrast, treatment of *T. cruzi* injected mice with CSA resulted in a marked inhibition of the proliferative response of the popliteal LN population when these cells were cultured *in vitro*(Fig. 3a).

Although cells from CSA treated mice that had been stimulated with anti-CD3 or allogeneic cells respond normally to IL-2 *in vitro*(2, 4), CSA treatment of mice, that had been injected with *T. cruzi* CMTs, resulted in a marked inhibition of IL-2 responsiveness (Fig. 3b). In addition to demonstrating a vigorous proliferative response to

IL-2. PLN cells from mice that had been injected with *T. cruzi* in the absence of CSA proliferated when stimulated *in vitro* with IL-1 and IL-4; however, marked inhibition of proliferation was seen in the presence of CSA when IL-1 or IL-4, but not IL-2, were used as the stimulatory lymphokines (Fig. 3c). This result raises the possibility that the proliferative response induced in *T. cruzi* primed cells by IL-1 and IL-4 is mediated by a CSA sensitive lymphokine such as IL-2.

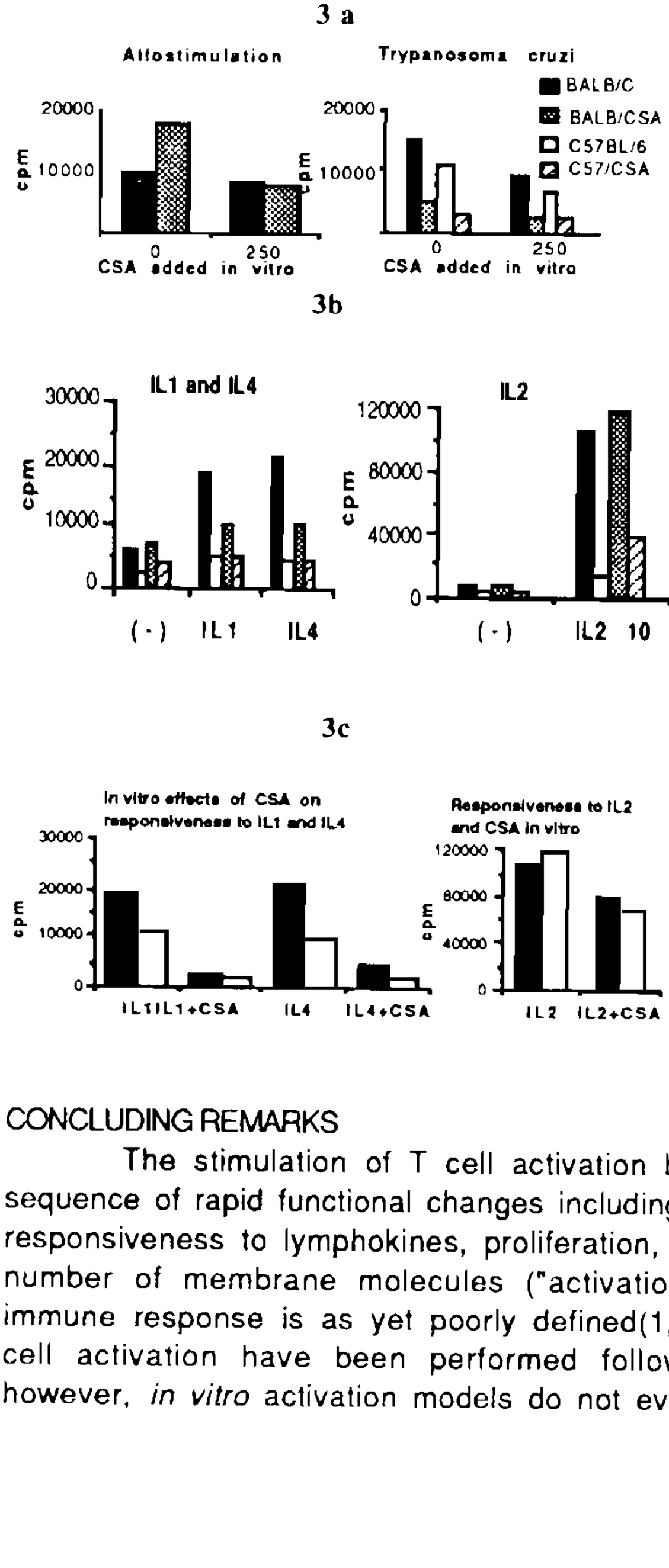

Fig.3a - Differential effects of CSA on the *in vivo* induced proliferation after stimulation by allogeneic cells or *T. cruzi* CMTs. BALB/c mice were primed SC with normal spleen cells from C57BL/6 three days before the experiment ($1.5x10^7$cells/RFP). One group was just immunized(Allostimulation graphic .BALB/c),and the second group begun receiving CSA five days before the experiment,at a daily dose of 35 mg/Kg (Allostimulation graphic, BALB/CSA). BALB/c and C57BL/6 mice were challenged with SC injection of $1.5x10^7$ irradiated T. cruzi CMTs/RFP. For each mouse strain one group was immunized(BALB/c, C57BL/6) and a second group received CSA following the same protocol used in the allostimulation groups. The *in vivo* induced proliferation was determined after an 18 hr pulse with ^{3}H-TdR in the presence or absence of CSA.

Fig.3b - Effects of CSA on responsiveness to lymphokines *in vitro* by PLN cells from mice immunized with *T.cruzi* irradiated CMTs. The same cell populations shown in Fig.3a (*Trypanosoma cruzi*) were incubated for 42 hr in the presence of IL1-a(10U/ml), IL2(10U/ml) and IL4(1000U/ml), and ^{3}H-TdR incorporation determined as in fig. 3a.

Fig.3c - CSA added during culture *in vitro* and responsiveness to IL1,IL4 and IL2 by cells from mice stimulated with *T. cruzi* CMT's.

CONCLUDING REMARKS

The stimulation of T cell activation by a large number of stimuli results in a sequence of rapid functional changes including increases in cell size, production of and responsiveness to lymphokines, proliferation, as well as the enhanced expression of a number of membrane molecules ("activation antigens") whose specific role in the immune response is as yet poorly defined(1, 3, 9, 10). The majority of studies of T cell activation have been performed following the activation of T cells *in vitro*; however, *in vitro* activation models do not evaluate a number of important factors such

as cell traffic to and from the site of the ongoing immune response, bioavailability of drugs and cytokines, and the influence of non-lymphoid cells on the immune response. The quantitative *in vivo* model of lymphocyte activation described by us allows a direct *in vitro* measurement of a number of parameters of lymphocyte activation which accurately reflects the result of all parameters of T cell activation as they occur *in vivo*.

In the present report, we have extended our studies to the analysis of the immune response to an infectious agent, *T. cruzi*. Although we used an *in vitro* adapted clone of *T. cruzi* which was irradiated prior to injection, the studies resembled the natural course of infection by this agent in that the parasite was injected SC in the footpad and the immune response was analyzed at the level of the draining lymph node. It is thus quite likely that the model we have developed can be expanded to analyze the production of mediators during the course of infection by *T. cruzi* and other infectious agents.

Two major points should be emphasized about our findings. First, the injection of *T. cruzi* resulted in T cell activation which appeared to be primarily mediated by an IL-2 dependent pathway. This finding must be contrasted to a number of studies of experimental Chagas' disease which have suggested that infection with T. cruzi results in a marked inhibition of IL-2 dependent T cell activation(10, 11, 12, 13). Secondly, the IL-2 dependent T cell activation seen in response to *T. cruzi* was totally inhibited by the immunosuppressive drug, CSA. This result must be contrasted to our findings with T cell activation induced by injection of allogeneic cells which is apparently mediated, by a CSA-resistant, IL-2 independent T cell activation pathway(2). Further analysis of the differences in these two activation systems as well as a comparison of the immune responses of different mouse strains to *T. Cruzi* should also yield important information about the basic immunologic mechanisms which govern the immune response to infectious agents at the local site of infection.

ABSTRACT

The *in vivo* administration of the immunosuppressive drug, Cyclosporin A (CSA), has allowed us to define IL-2 dependent and IL-2 independent pathways of T cell activation *in vivo*. Thus, CSA inhibited T cell activation and the production of IL-2 mRNA in the draining lymph node (LN) population following footpad injection of anti-CD3 mAb. In contrast, even though CSA completely inhibited the induction of IL-2 mRNA in the draining LN following the injection of allogeneic cells, T cell activation proceeded normally. In the present study, we have analyzed the effects of CSA on the T cell activation induced *in vivo* by *T. cruzi*. BALB/c and C57BL/6 mice were injected subcutaneously in the footpad with irradiated, cultured *T. cruzi* trypomastigotes (CMTs, clone sylvio-X10/4). CSA was delivered to the mice via an osmotic pump, Alzet 2001 at a concentration of 35mg/Kg/day. The injection of CMTs resulted in a dose dependent activation of the draining LN population including an increase in the number of cells, an increase in cell size, induction of expression of the IL-2 receptor and other T cell activation antigens (Ly-6, CD28), induction of responsiveness to IL-2, and a vigorous proliferative response when the freshly explanted node was cultured for 18 h in vitro in the presence of ^{3}H-TdR. CSA markedly inhibited all of these parameters of T cell activation. Thus, the early T cell activation response observed

after injection of irradiated T. cruzi CMTs appears to be mediated by an IL-2 dependent, CSA sensitive T cell activation pathway.

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