Author

Though she hopes to attend medical school, Judy Wei wants to continue doing research. As she says, "Because research is the backbone to medical advancement, I don't want to miss the opportunity to be part of the future direction of science and medicine." Though she found her research topic extremely interesting, Wei says it is not the research alone that kept her going. "I believe that a great research experience comes also from the group of scientists that you interact with extensively," Wei said. "In my project, my input was incorporated and I was part of the research group." Wei has also presented her research in *The Journal of Undergraduate Research in Biological Sciences*.

Key Terms

- Chagas' Disease
- Epitopes
- IFN-γ
- Nitric Oxide
- Paraflagellar Rod Proteins
- *Trypanosoma cruzi*
- Vaccine

Mapping the T-Cell Epitope of the Paraflagellar Rod Protein 3 of Parasitic Hemoflagellate, *Trypanosoma Cruzi*

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Abstract

Trypanosoma cruzi is the causative agent of American trypanosomiasis, or Chagas Disease. Currently, 16-18 million people are affected worldwide and although it is an illness prevalent in S. America, evidence for human infection has also been found in the U.S. Previous studies have shown that the paraflagellar rod proteins (PFR) derived from the flagellum of the parasite are able to induce a protective T-helper type 1 response. That is, the paraflagellar rod is a unique structure of the parasite that has the capability of triggering the host immune system to attack *T. cruzi*. One of the four distinct proteins that compose this structure, PAR 3, is the focus of the present investigation. Via recombinant DNA techniques, six overlapping polypeptides of PAR 3, denoted as PAR 3 A-F, were prepared and screened for epitopes through *in vitro* analyses. Recognition by PFR-immune T cells and the degree of activation by the recombinant polypeptides were compared. This "low resolution" mapping identified two polypeptides as areas that potentially contain T-cell epitopes. With these findings and with reference to PAR 2 epitopes previously mapped, two synthetic 15mer peptides, starting at amino acid residues 377 and 392 were subjected to parallel cell culture experiments with PAR 3-immune T cells. Only peptide 377 of this "high resolution" search stimulated a high degree of T-cell and macrophage activation. Assays for IFN-γ further confirmed peptide 377 as the dominant PAR 3 epitope.

Faculty Mentor

The work described in Judy Wei's paper represents a substantial advancement in our efforts to develop a vaccine against American Trypanosomiasis (Chagas Disease). This was quite a difficult study, and Judy did an excellent job. She worked extremely hard on this project and made several insightful suggestions during the course of the work. Students like Judy are the reason that our undergraduate research program at UCI is so intellectually fulfilling both for the students and the faculty.

Jerry Manning
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Introduction

According to the World Health Organization, it is estimated that 16 to 18 million people are currently afflicted by American trypanosomiasis, or Chagas disease, caused by the parasitic hemoflagellate, *Trypanosoma cruzi* (WHO Tropical medicine research report, 1997). As the third largest disease in Latin America, it is a major health and economic burden; Chagas disease causes losses equivalent to 1.3% of the 1993 external debt of South America. Furthermore, more than 300,000 U.S. immigrants are victims of *T. cruzi* (Panosian, 1998), and serologic evidence for human infection has also been found in Georgia and northern California. Due to the limited effectiveness of chemotherapeutic agents and the fact that no successful vaccines have been produced, it is in our great interest to engineer a defined recombinant vaccine capable of generating a protective immune response against these invading parasites.

Previous studies in Dr. Jerry Manning's laboratory have shown that purified paraflagellar rod (PFR) proteins derived from the flagellum of *T. cruzi* can induce an immune response in mice capable of reducing the level of circulating parasites and protecting against an otherwise lethal challenge of *T. cruzi* trypomastigotes, with a 100% survival rate (Wrightsman et al., 1995; Wrightsman and Manning, 2000). Furthermore, it has been shown that the protective immunity in our PFR protein vaccine mouse model is associated with a T-helper type 1 (Th1) response (Miller et al., 1996). This finding allows the mapping of antigenic epitopes, or minimal peptide determinants, responsible for the Th1 response and the optimization of the immune response of the vaccine regimen.

The logic behind favoring epitope mapping and synthesizing a recombinant vaccine as opposed to immunizing with purified PFR from the parasite is twofold. First, PFR proteins are expensive to prepare, and second, PFR proteins may not provide the *optimal* immune response. Immunization with PFR could generate immunity that recognizes regions not conserved across diverse strains of *T. cruzi*. In addition, PFR could elicit immunity against epitopes that are not presented by infected macrophages, perhaps due to the processing differences between denatured recombinant antigen and native PFR from intact parasites. By focusing on peptides, we will be able to test each epitope and eliminate those that are not protective, and thereby create a defined vaccine that results in an optimal immune response.

There are four distinct protein components of the PFR, designated PAR 1, 2, 3, and 4 (Fouts et al., 1998). Similar techniques were used to map the Th1 epitopes of PAR 1 and PAR 2 and it is the focus of the present study to map T-cell epitopes of PAR 3. The first phase will begin with a broader search zone (i.e. low resolution). Once general regions containing epitopes are identified, the search shifts to "high resolution" to map more locally, testing overlapping synthetic peptides. This experimental design is necessary because PAR 3 consists of over 600 amino acid residues. Synthesizing peptides to span this entire length would be prohibitively expensive. Hence, it is crucial to start by narrowing the search to polypeptide segments of the whole protein.

To map for epitopes, polypeptides and peptides are screened individually, *in vitro*, by quantitating the degree of activation of immune-T lymphocytes by the tested antigen. Upon presentation of the antigen by antigen presenting cells (APC), cognate CD4^{+} T cells are activated and in turn secrete cytokines that induce other T cells and activate macrophages. Figure 1 depicts the interaction between the APCs, presented antigens, and the T cells. Macrophages are large mononuclear phagocytic cells that act as APCs and are important not only in innate immunity but in humoral and cell-mediated immu-
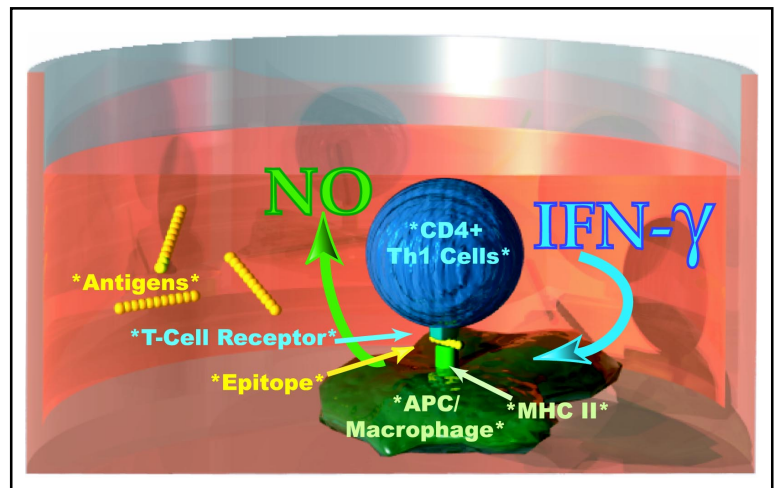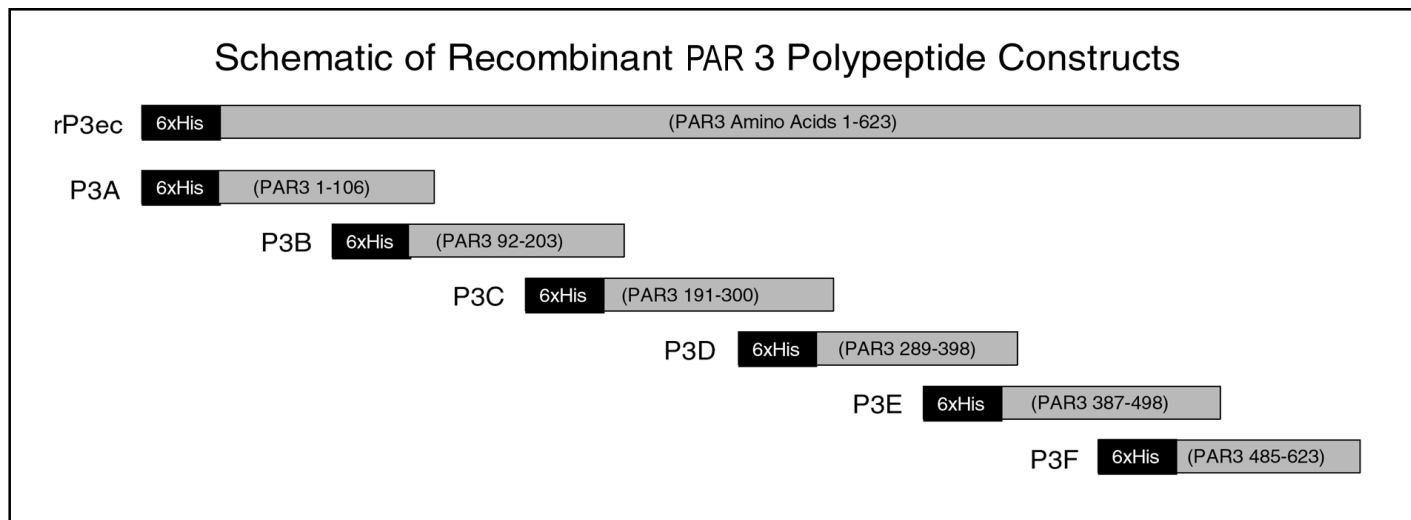

Figure 1

Illustration of the interaction between a T cell, presented antigen, and an APC. Following digestion and presentation of antigens by the major histocompatibility complex molecules (MHC) of APCs, T cells examine the presented antigenic fragments by binding with the T-cell receptor. In this case, a CD4+ T helper type 1 cell recognizes the epitope presented on the MHC class II molecule of a macrophage and becomes activated, triggering a number of responses, two of which are relevant to the present study. Interferon-gamma is released to activate the macrophage, and the activated macrophage, in turn, produces nitric oxide (NO). This diagram depicts an in vitro interaction, occurring in culture media.

Figure 2

Design of the PAR 3 polypeptides, PAR 3A-F. The six histidines are part of the amino terminal fusion protein. PAR 3 is abbreviated as P3 and rP3ec denotes recombinant PAR 3 from *E. coli* expression.

nity as well. T-helper type-1 cells, a subset of the CD4+ T cell population, are mainly involved in activating macrophages and inflammatory responses and produce interferon gamma (IFN-γ), among other cytokines, while T-helper type-2 cells stimulate B cells and produce interleukin 4 (IL-4).

Activated macrophages go through a number of changes that enhance their role as effector cells. One such change relevant to this study is the increased production of nitric oxide (NO) since it has been shown to be highly effective in killing *T. cruzi*. Measurement of the level of nitric oxide production allows us to indirectly detect the level of T-cell activation and, therefore, determine which antigens potentially encompass epitopes necessary for protection (Figure 1). Because different types of T-helper cells produce characteristic cytokines upon activation, a measure of the signature cytokine secreted would confirm which T-cell type is the key player in the immune response.

Materials and Methods

Recombinant polypeptide antigen preparation ("low resolution" mapping)
Six overlapping, by 36-45 base pairs, DNA constructs spanning the entire PAR 3 gene were designed. Expression of these constructs in *E. coli* results in polypeptides containing approximately 110 amino acids of PAR3 sequences, each overlapping adjacent fragments by 12-15 amino acids. These polypeptides are denoted as PAR 3 A-F (Figure 2). The individual DNA constructs were amplified using PCR techniques. The PCR products were then ligated into plasmid vector,

pTrcHis, and transformed into bacterial *E. coli* cells. This vector allows for induced expression in *E. coli* with isopropyl-1-thio-β-D-galactopyranoside (IPTG). Expressed proteins have an amino terminal fusion protein that contains six consecutive histidine residues. Taking advantage of the fact that polyhistidine has high affinity for Ni^{2+} (at appropriate pH), the six consecutive histidines serve as affinity tags that allow for purification using chelated Ni^{2+} columns on fast protein liquid chromatography (FPLC). Polypeptides were further purified on the basis of hydrophobicity using the Jupiter C_4 4.6 x 50 mm column on LKB HPLC (high performance liquid chromatography). Due to the volatile properties of the HPLC solvent (acetonitrile and trifluoroacetic acid), HPLC eluant were readily lyophilized and resuspended in dimethyl sulfoxide (DMSO) to desired concentrations. Between manipulations, the polypeptides were confirmed in size and concentration utilizing polyacrylamide gel electrophoresis (SDS-PAGE), mass spectrometry, and bicinchoninic acid (BCA) protein assay, while western blot analyses with monoclonal antibody against the fusion proteins were performed to further validate the identity of the recombinant polypeptides.

Immunization of mice with purified PFR proteins and recombinant PAR 3
Based on previous investigations that have shown PFR proteins purified from the parasite to be able to induce a protective immune response against *T. cruzi* trypomastigotes (non-dividing, circulating stage), mice were immunized with PFR to yield immune T cells. Immunization of mice with PFR and recombinant proteins have been described and approved

by the Institutional Animal Care and Use Committee (IACUC) protocol #981378, and an experienced research scientist performed the immunizations. Another set of mice was immunized with recombinant PAR 3 (rPAR 3) for the second phase of the study (to be described below). Each C57BL/6 mouse was initially injected sub-cutaneously with 40 μg of PFR (or rPAR 3) in complete Freund's adjuvant. This was followed by two booster immunizations of 20 μg of PFR (or rPAR 3) in incomplete Freund's adjuvant.

Macrophage activation cultures ("low resolution" mapping)

IC-21 cells are macrophage-like cells that serve as the APCs. These cells were plated on 96 well plates at 4×10^4 cells/well and allowed to adhere to the bottom of the wells (37 °C at 8% CO_2).

To prepare the T lymphocytes, spleens of both non-immune and PFR (purified from *T. cruzi*)-immune mice were removed, homogenized, and suspended in Dulbecco's Modification of Eagle's Medium (DMEM) (10% fetal calf serum (FCS)). The red blood cells were lysed and removed and the suspension was passed through prepared nylon wool columns. Unlike other splenocytes, such as B cells, T cells do not bind to the nylon wool which allowed them to be isolated and purified using this method. Previous studies had shown that the purity of the enriched T-cell population was >98% as determined by FACS analysis (Wrightsman and Manning, 2000). The T cells were counted using trypan blue and the appropriate volume was added to the 96 well plates at 5×10^5 cells/well. Half of the plate (48 wells) contained non-immune T cells while the other half contained PFR-immune T cells.

The purified recombinant polypeptide antigens (PAR 3A-F) at 5 μg/mL were added to co-cultures of either PFR-immune or non-immune T cells and IC-21 macrophages. Con A is a mitogen that non-specifically activates T cells. It functioned as a positive control to test the stimulatory capabilities of T cells and macrophages—both the PFR-immune and non-immune T cells were expected to induce high levels of NO production. PFR was used as the other positive control. This antigen allowed us to determine the success of the immunization. The wells containing no antigens functioned as the negative controls because these would confirm that T cells and IC-21 macrophages were not active to begin with and that the culture media were not contaminated with other substances that would activate the T cells or macrophages. Very little NO production was expected.

The cell cultures were incubated at 37 °C, 8% CO_2 for four to seven days and the supernatants were analyzed for NO production on the fourth day via the Greiss reaction.

Synthetic peptide antigen, N′-PAR 3 and C′-PAR 3 ("high resolution" mapping)

Following detection of polypeptides that contained epitopes, shorter synthetic peptides were subjected to parallel cell culture analyses. Two 15-mers, peptides 377 and 392, spanning amino acids 377-391 and 392-406, respectively, were purchased from Mimotopes Pty Ltd. Due in large part to the highly identical amino acid sequence between PAR 3 and PAR 2, another protein component of PFR, these two peptides were chosen rather than carrying out the original plan of testing all peptides spanning the "positive" polypeptide sequences from the "low resolution" phase. The reasoning will be further described later.

In addition, to confirm that the two peptides were the dominant epitopes, two constructs representing all regions of PAR 3 excluding the regions spanned by the peptides were prepared. The first, denoted as N′-PAR 3, is comprised of PAR 3 sequence beginning at the *amino*-terminus to the start of peptide 377 (covering amino acid residues 1-376), while the second, C′-PAR 3, spans from the end of peptide 392 to the *carboxy*-terminus of PAR 3 (covering amino acid residues 406-623). Please refer to Figure 2 for a visualization.

Macrophage activation cultures ("high resolution" mapping)

The same setup was used to test peptides 377 and 392 as was used during the initial "low resolution" search. Instead of testing polypeptides, peptides were the stimulation antigens, and rather than co-culturing with PFR-immune T cells, recombinant PAR 3 (rPAR 3)-immune T cells were added as the immune T cells. Additionally, N'-PAR 3 and C'-PAR 3 were tested in culture to confirm that the entire region spanning PAR 3 excluding the two peptide sequences was not immunogenic. Again, PFR was one of the positive controls since PAR 3 is one of the four protein components of PFR and, therefore, PFR is expected to be recognized by rPAR 3-immune T cells. As another positive control, rPAR 3 should also be able to stimulate rPAR 3-immune T cells to subsequently activate macrophages. In addition, Con A was used as the third positive control.

The cell cultures were incubated at 37 °C, 8% CO_2 for four to seven days and the supernatants were quantitated for NO production on the fourth day and for cytokine IFN-γ on the fifth day.

Nitrite assay-Greiss reaction

The Greiss reaction is the basis for the quantification of nitrite—a stable metabolic by-product of NO synthesis—in the culture media. This was used as an indicator of the level of macrophage activation as compared to controls. On day four, 50 μl culture supernatants were combined on 96 well plates with a 1:1 mixture of 1% sulfanilamide in 2.5% H_3PO_4 and 0.1% naphthylethylenediamide in 2.5% H_3PO_4. The plates were incubated at room temperature for 10 min and absorbance data were determined at 550 nm on an automated microplate reader. The nitrite levels were calculated using a standard curve of sodium nitrite from 125 to 1 μM prepared in culture media.

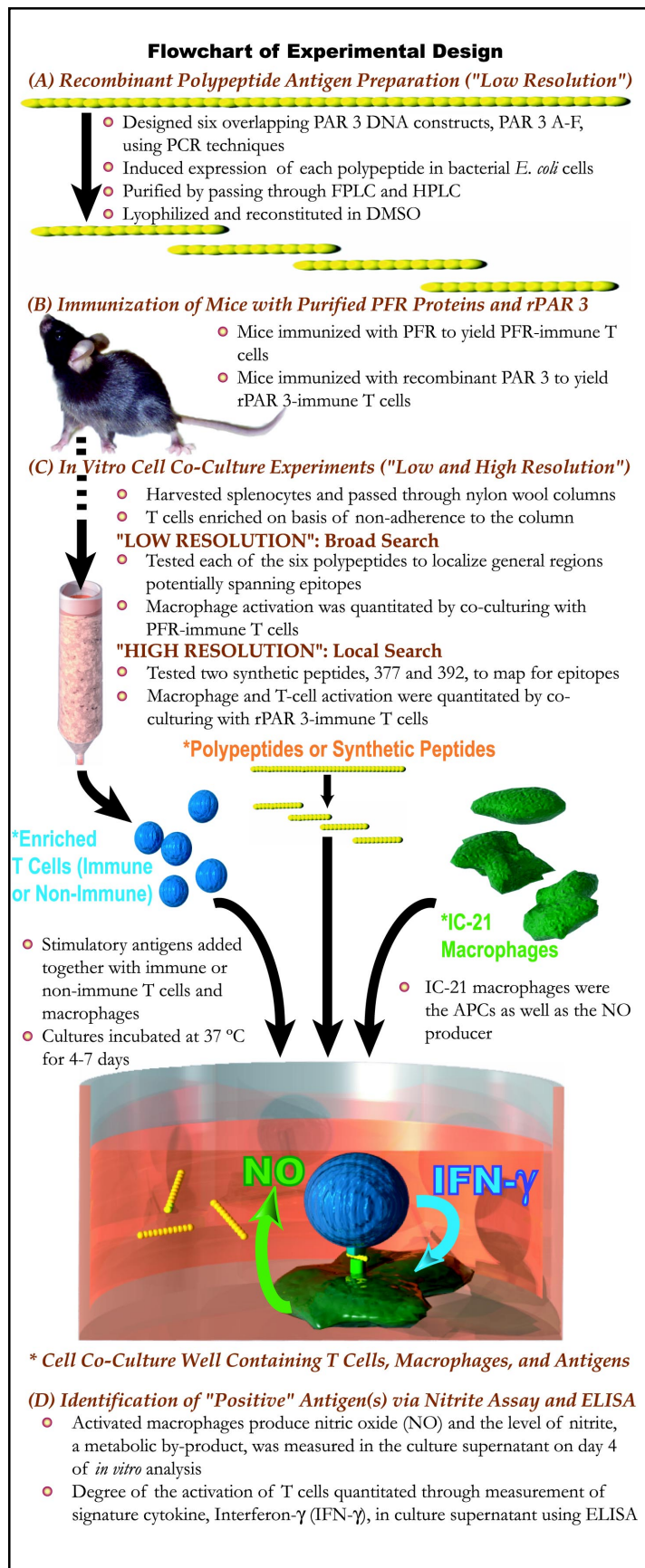## T-cell activation assay: measurement of IFN-γ

The level of the signature cytokine, IFN-γ, was measured by performing a capture Enzyme-Linked Immunosorbent Assay (ELISA) of the culture supernatants taken from the "high resolution" experiment on day five. 1-2 μg/ml of IFN-γ specific capture antibody was allowed to bind to 96 well plates in 0.1 M $NaHCO_3$, pH 8.2 at 4 °C overnight. Unbound antibody molecules were washed with Phosphate-Buffered Saline Tween 20 (PBST) buffer and the wells were blocked with 10% FCS in PBS for 2 hr at room temperature. Wells were subsequently washed with PBST and the appropriate concentration of biotinylated anti-IFN-γ-detecting antibody was added in a volume of 100 μl and incubated for 45 min at room temperature. Again, the wells were washed to remove unbound antibody molecules, and 100 μl of strepavidin-peroxidase solution was added and incubated at room temperature for 30 min. Following thorough washes with PBST, 100 μl of 2,2'-azino-di-3-ethylbenzthiazoline sulfonate substrate was added, and the plates were read at 405 nm on an automated ELISA plate reader. Cytokine concentrations were calculated from linear regions of a titration curve of IFN-γ standards, values for controls were subtracted, and final concentrations in ng/ml were determined (Wrightsman and Manning, 2000).

The overview of the experimental procedure, from the preparation of the DNA constructs to the cell co-culture set-up is illustrated in figure 3.

Figure 3

Schematic flowchart of the experimental design, beginning with the design and preparation of the polypeptide constructs of the "low resolution" search to the setup of the in vitro cell co-culture experiments.
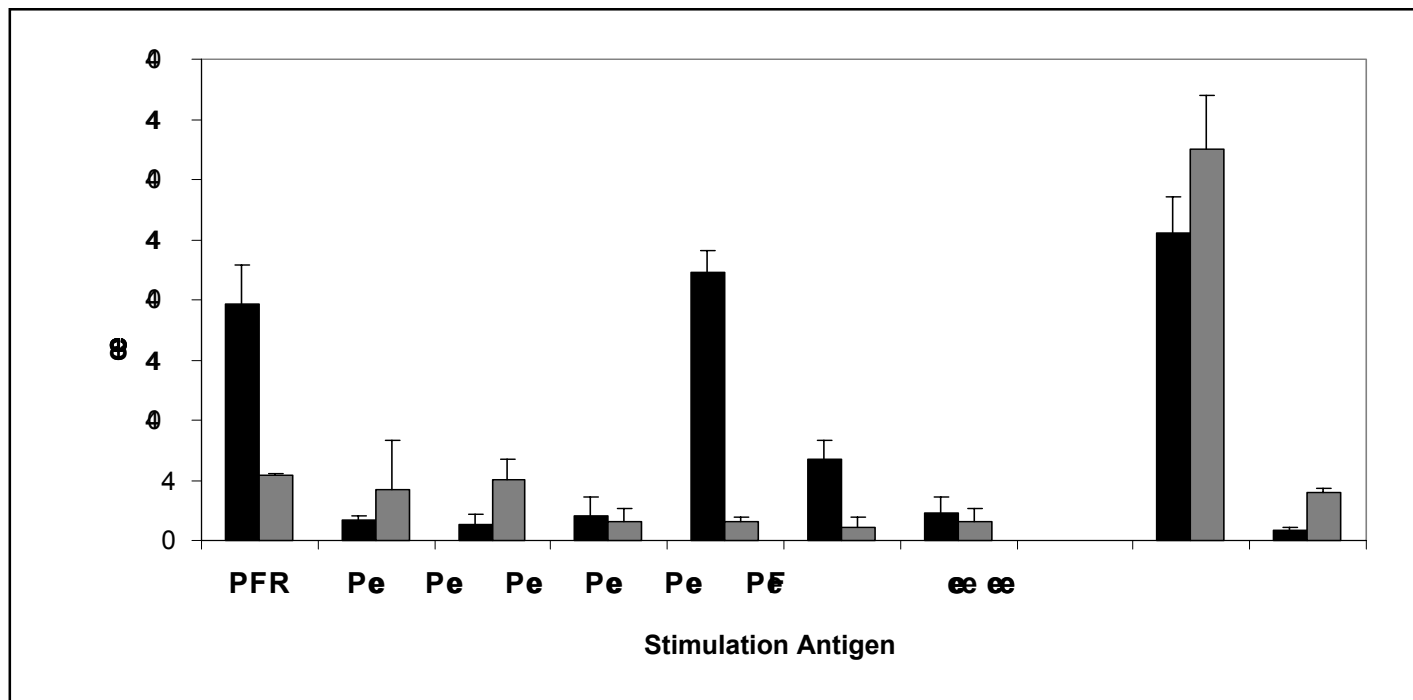

Flowchart of Experimental Design

(A) Recombinant Polypeptide Antigen Preparation ("Low Resolution")
- Designed six overlapping PAR 3 DNA constructs, PAR 3 A-F, using PCR techniques
- Induced expression of each polypeptide in bacterial *E. coli* cells
- Purified by passing through FPLC and HPLC
- Lyophilized and reconstituted in DMSO

(B) Immunization of Mice with Purified PFR Proteins and rPAR 3
- Mice immunized with PFR to yield PFR-immune T cells
- Mice immunized with recombinant PAR 3 to yield rPAR 3-immune T cells

(C) In Vitro Cell Co-Culture Experiments ("Low and High Resolution")
- Harvested splenocytes and passed through nylon wool columns
- T cells enriched on basis of non-adherence to the column

"LOW RESOLUTION": Broad Search
- Tested each of the six polypeptides to localize general regions potentially spanning epitopes
- Macrophage activation was quantitated by co-culturing with PFR-immune T cells

"HIGH RESOLUTION": Local Search
- Tested two synthetic peptides, 377 and 392, to map for epitopes
- Macrophage and T-cell activation were quantitated by co-culturing with rPAR 3-immune T cells

*Polypeptides or Synthetic Peptides

*Enriched T Cells (Immune or Non-Immune)

*IC-21 Macrophages

- Stimulatory antigens added together with immune or non-immune T cells and macrophages
- Cultures incubated at 37 ºC for 4-7 days

- IC-21 macrophages were the APCs as well as the NO producer

NO   IFN-γ

* Cell Co-Culture Well Containing T Cells, Macrophages, and Antigens

(D) Identification of "Positive" Antigen(s) via Nitrite Assay and ELISA
- Activated macrophages produce nitric oxide (NO) and the level of nitrite, a metabolic by-product, was measured in the culture supernatant on day 4 of *in vitro* analysis
- Degree of the activation of T cells quantitated through measurement of signature cytokine, Interferon-γ (IFN-γ), in culture supernatant using ELISA

Figure 4

This graphs the results of the broader, "low resolution," search for epitopes by screening PAR 3 polypeptides with PFR-immune T lymphocytes. (PAR 3 is abbreviated as P3). PAR 3D stimulated high degrees of macrophage and cognate T-cell activation, and PAR 3E resulted in relatively higher stimulation, as well. The dotted bars are from co-cultures with non-immune T cells, while the solid bars are with PFR-immune T cells.

Results

Macrophage activation cultures ("low resolution" mapping)

On day four of co-culture, the levels of nitrite in the culture supernatants were quantitated and plotted (Figure 4). The x-axis lists the individual stimulatory antigen added to co-cultures of T cells and macrophages, and the vertical axis shows the nitrite concentration in μM. PFR and Con A were the positive controls while "no antigen" was the negative control. When PFR was added, nitrite concentration was higher in wells cultured with PFR-immune T cells than those with non-immune T cells. Con A, however, activated both the PFR-immune T-cell population and the non-immune T-cell population. Furthermore, when no antigens were added, there was little (<<5 mM) nitrite production in both immune and non-immune T cultures. One recombinant polypeptide, PAR 3D, specifically stimulated PFR-immune but not non-immune T cells, while another, PAR 3E, also appeared to induce greater nitrite production within the PFR-immune T-cell cultures, but to a lesser degree. The two recombinant polypeptides span amino acid sequence 289-398 (PAR 3D) and 387-498 (PAR 3E) of the PAR 3 protein (Figure 2).

Macrophage activation cultures ("high resolution" mapping)

Again, fourth day nitrite concentrations of culture supernatants were plotted in Figure 5 for the individually-added antigens. The mice in this case were immunized with rPAR 3 rather than the entire PFR. PFR, rPAR 3 and Con A were the positive stimulatory antigenic controls and "no antigen" was the negative control. When T cells were cultured with IC-21s in the absence of antigens, both the rPAR 3-immune and non-immune T-cell cultures resulted in little nitrite production (nitrite levels in non-immune T-cell cultures are not shown). When PFR or rPAR 3 were added, however, rPAR 3-immune T-cell populations were activated, as seen in the higher nitrite concentration. Con A, moreover, was able to stimulate at a much greater level than either PFR or rPAR 3 in both immune and non-immune cultures. Peptide 377 stimulated higher levels of nitrite production and peptide 392 did not result in an appreciable amount of nitrite. Peptide 377 lies within PAR 3D, while 392 is located within the overlapping portion between PAR 3D and 3E. Additionally, both N'-PAR 3 and C'-PAR 3 were not recognized by the rPAR 3-immune T cells—nitrite concentrations for both antigens were similar to those seen with the negative control. Only the region of PAR 3 spanned by peptide 377, compared with the rest of the amino acid sequences, induced higher levels of
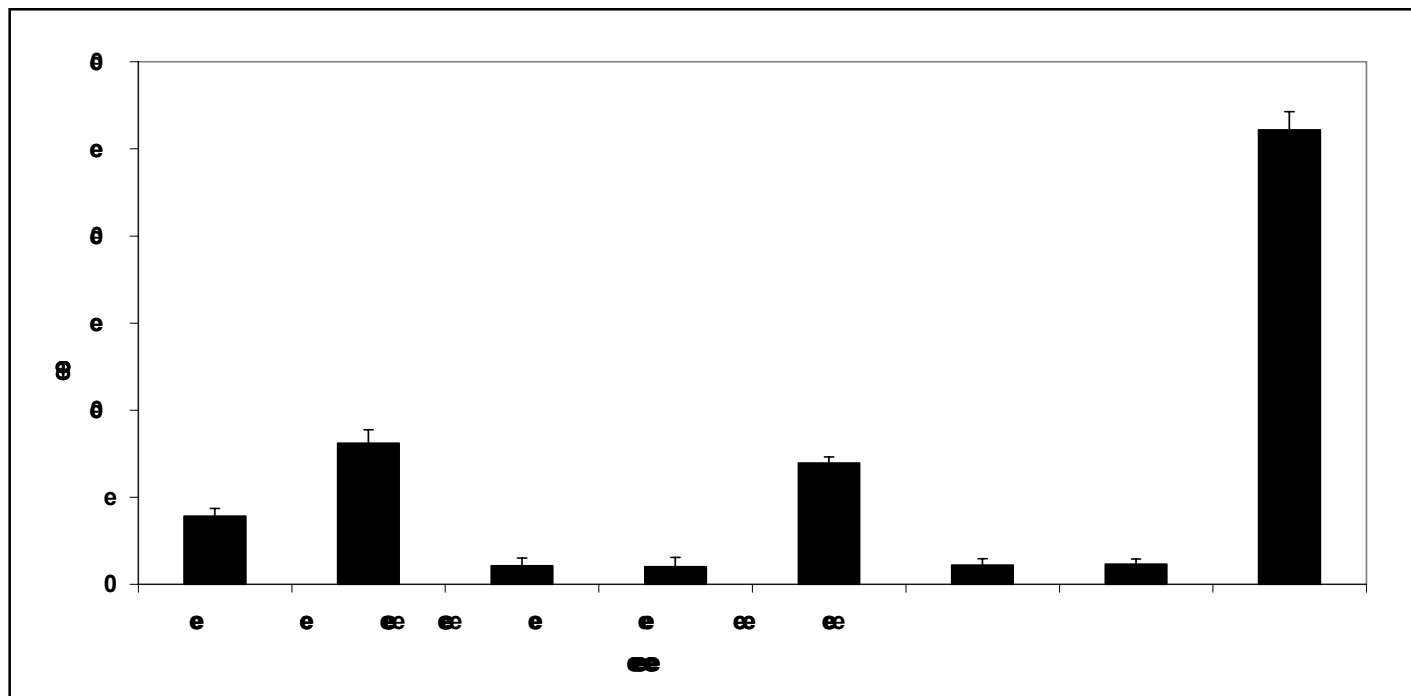
Figure 5

This graphs the results of the fine-tuned, "high resolution," search for epitopes via screening synthetic peptides, 377 and 392, with rPAR 3-immune T cells. N'-PAR 3 and C'-PAR 3 span all regions of PAR 3 excluding sequences covered by the peptides and did not appear to be recognized by rPAR 3-immune T cells. Peptide 377, on the other hand, elicited high levels of nitrite production, which suggests that this is the dominant PAR 3 epitope. The non-immune T-cell cultures are not shown. (Recombinant PAR 3 is abbreviated as rP3).

nitrite production in rPAR 3-immune T-cell cultures specifically.

T-cell activation assay: cytokine capture ELISA for IFN-γ

On day five of co-culture, culture supernatants were assayed for the concentration of cytokine IFN-γ (Figure 6). The horizontal axis plots the individual antigens added and the vertical axis denotes the concentration of IFN-γ in ng/ml. In this experiment, rPAR 3-immune and non-immune T-cell populations were compared. rPAR 3 and Con A were the positive controls; rPAR 3 stimulated only the rPAR 3-immune T-cell cultures but not the non-immune T-cell cultures while Con A stimulated both cell populations. In the absence of antigens, T cells were not actively secreting large quantities of IFN-γ in either cell cultures.

Peptides 174 and 179 are T-cell epitopes previously mapped within PAR 2. These two antigens resulted in little IFN-γ production (concentrations similar to negative control) in rPAR 3-immune and non-immune T-cell cultures (Figure 6). Additionally, N'-PAR 3, C'-PAR 3 and peptide 392 did not induce a high degree of T-cell activation (also similar to negative control) in either cell cultures. In contrast, peptide 377 acti-

vated a much higher level of IFN-γ secretion specifically in cultures with rPAR 3-immune T cells but not with non-immune T cells, compared to controls.

Discussion

The present investigation is an attempt to map epitopes of one of the PFR component proteins, PAR 3, by way of *in vitro* analyses. By preparing overlapping fragments stretching the entire PAR 3 protein, the search is narrowed down to polypeptides in the range of 100-130 amino acid residues (compared to 600 amino acids of the entire PAR 3). Although it is possible to use exclusively synthetic peptides for the co-culture experiments, the cost would be exceedingly high. Therefore, the balanced decision between cost and time efficiency is to initially minimize the search range to polypeptides.

Once the co-cultured recombinant polypeptides were tested, overlapping synthetic peptides were made covering the regions that are capable of inducing T-cell/macrophage activation. T-cell epitopes average around 12 amino acid residues in length, with 9 amino acids bound to the major histocompatibility complex (MHC) II molecules. This is the rea-
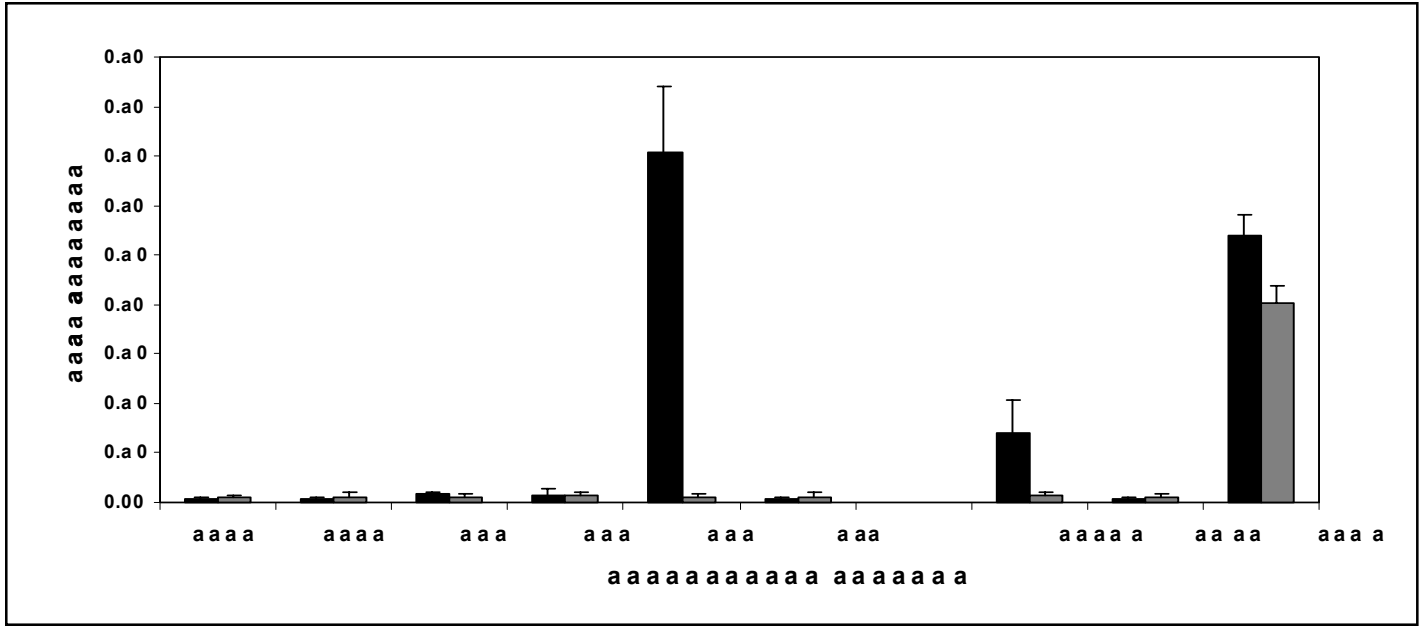
Figure 6

This plots the cytokine ELISA assay for IFN-γ within the culture media of co-cultures of peptides 377 or 392 of PAR 3 with rPAR 3-immune and non-immune T cells and IC-21s. PAR 2 peptides 174 and 179 were also tested in culture with rP3-immune T cells to confirm no cross reactivity. Peptide 377 resulted in high degrees of rP3-immune T-cell activation, indicated by the intense amount of IFN-γ, as compared to the rest of PAR 3 (N'-PAR 3, C'-PAR 3 and 392). Furthermore, from the way PAR 2 peptides responded, there is little doubt concerning cross-reactivity. The solid bars denote the rPAR 3-immune T cells and dotted bars denote the non-immune T cells. (N'-PAR 3 and C'-PAR 3 are abbreviated as N-P3 and C-P3, respectively).

son behind basing the size of the synthetic peptides (~15 amino acid) to range around that of physiological epitopes. Although CD4+ Th type 1 cells are mainly responsible for the activation of macrophages (with subsequent NO synthesis) and prior studies linked Th1 cells with the immune response, cytokine ELISA for IFN-γ was performed to confirm the type of T cells responsible for the generated immune response.

During the macrophage activation experiment testing PAR 3 polypeptides, patterns of nitrite levels of the positive controls, PFR and Con A, were as expected: PFR specifically activated PFR-immune T cells that subsequently activated macrophages to produce NO, whereas Con A non-specifically activated both immune and non-immune cells (Figure 4). The negative control also confirmed that the cells were not active to begin with and that the culture media was not contaminated with non-specific activators. Our findings of the "low resolution" search point to two polypeptides, PAR 3D and PAR 3E, as potential regions that contain T-cell epitopes. PAR 3D induced a greater level of nitrite production (an indication of the higher degree of macrophage activation) specifically in the immune-T-cell cultures, which suggests that sequences within this region are recognized by PFR-

immune T cells. Although PAR 3E resulted in a lower concentration of nitrite production than PAR 3D, the level was relatively higher than the negative control and the other PAR 3 polypeptides.

Initially, there were reasons to believe that the two synthetic peptides, 377 and 392, were potential candidates as T-cell epitopes due to the high sequence identity between PAR 3 and PAR 2. In fact, PAR 2 and 3 share 64.8% amino acid sequence identity. Moreover, when mapping PAR 2 epitopes, peptides homologous to 377 and 392 also induced a strong immune response. These factors, together with the prior finding that PAR 2 is protective against *T. cruzi* infections, constitute the reasoning behind choosing to test only the two PAR 3 peptides rather than testing all overlapping peptides spanning PAR 3D and PAR 3E. Not only was cost cut down substantially, but time consumption was also minimized.

The second macrophage activation experiment was performed to "fine-tune" the epitope mapping by testing the two peptides, 377 and 392. In addition to the peptides, N'-PAR 3 and C'-PAR 3 were also examined to see whether sequences other than the two peptides are recognized by cognate T cells. PFR and rPAR 3 resulted in higher levels of

nitrite in rPAR 3-immune T cell than non-immune T-cell cultures, as expected (Figure 5). Since PAR 3 is one of the components of PFR, PFR is expected to be recognized by rPAR 3-immune T cells. Con A stimulated a higher level of activation than either PFR or rPAR 3 and is most probably due to the fact that Con A non-specifically activates T cells while PFR and rPAR 3 specifically activate immune T cells. With Con A, the entire T-cell population is activated while the cognate T cells activated by specific antigens constitute a smaller percentage of the entire population.

Peptide 377 was able to activate specifically rPAR 3-immune T cells (Figure 5) as indicated by the higher nitrite concentration. Neither N'-PAR 3 and C'-PAR 3 or peptide 392 were capable of activating macrophages, which suggests that cognate T cells do not recognize sequences spanned by these constructs. Since N'-PAR 3 and C'-PAR 3 and peptide 392 encompass all sequences of PAR 3 except amino acids 377-391, and from the way these antigens reacted *in vitro* with cognate T cells, peptide 377 is shown to be the dominant epitope of PAR 3.

In the macrophage activation assay, only peptide 377 induced a greater level of nitrite synthesis. In the T-cell activation experiment, this peptide again resulted in IFN-γ concentrations much greater than the controls (Figure 6). By detecting the IFN-γ level within the media, the degree of rPAR 3-immune T-cell activation may be quantified. Compared to the rest of PAR 3 (covered by N'-PAR 3, C'-PAR 3 and peptide 392), peptide 377 is responsible for activating rPAR 3-immune T cells but not non-immune T cells. Furthermore, both PAR 2 epitopes, 174 and 179, were not recognized by rPAR 3-immune T cells. Since the core MHC binding region of both PAR 3 and PAR 2 "positive" peptides differs by only a single amino acid residue, this finding suggests that the rPAR 3-immune T cells are so specific that epitopes differing by one amino acid residue can be discerned. There is, therefore, no cross reactivity between rPAR 3-immune T cells and PAR 2 epitopes. Furthermore, since IFN-γ was secreted upon T-cell activation, the generated immune response was, indeed, of T-helper type 1.

Due to the complexity of the bodily functions, and especially of the immune system, the next step in analyzing the PAR 3 epitope is to conduct *in vivo* experiments by immunizing mice with peptide 377. The mapped PAR 3 epitope may serve in the ultimate goal as a candidate (alone or in combination with other recombinant proteins, engineered viruses, or synthetic peptides) for the defined vaccine against American trypanosomiasis.

Conclusion

With the goal of mapping the regions of PAR 3 responsible for generating a protective immune response against the parasite *T. cruzi*, several experiments were performed to locate the T-cell epitopes. An initial, broader search was done by testing PAR 3 polypeptides via cell co-culture experiments. The level of macrophage activation was quantitated to determine the regions that are recognized by immune T cells. From there, a more "fine-tuned" search aided in mapping epitopes down to sequences of 15 amino acid residues. Both the levels of macrophage and T-cell activation were analyzed, and peptide 377 appeared to be the dominant epitope of PAR 3 responsible for inducing an immune response that might be capable of protection against *T. cruzi* infection.

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