**FANNY TZELEPIS**

# RESPOSTA IMUNE MEDIADA POR LINFÓCITOS T CD8+ NA INFECÇÃO EXPERIMENTAL PELO *Trypanosoma cruzi*: ESPECIFICIDADE, CINÉTICA E MECANISMOS DE IMUNODOMINÂNCIA.

**Tese apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina, para obtenção do título de Doutor em Ciências.**

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**Orientador: Prof. Dr. Mauricio Martins Rodrigues**

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"A coisa mais importante no mundo não é aonde nós chegamos, mas em qual direção estamos nos movendo".

Oliver Wendall Holmes

## SUMÁRIO

## RESUMO

Embora esteja bem estabelecido, há mais de quinze anos, que linfócitos T CD8+ restritos por moléculas de MHC-Ia são importantes no controle da parasitemia e sobrevivência de camundongos infectados com *Trypanosoma cruzi*, pouco se sabia da especificidade desses linfócitos. A ausência de epítopos bem definidos impedia o estudo detalhado da cinética da resposta imune e dos mecanismos de controle da imunodominância. Assim, o objetivo inicial desta tese foi a identificação de epítopos reconhecidos pelos linfócitos T CD8+ ativados durante a infecção pelo *T. cruzi*. Uma vez definidos esses epítopos, estudamos a cinética de ativação dessas células e alguns dos parâmetros que a controlavam. Por fim, estudamos os possíveis mecanismos de imunodominância durante a resposta imune.

Durante a infecção experimental com a cepa Y de *T. cruzi,* nós observamos que os epítopos VNHRFTLV, IYNVGQVSI ou TEWETGQI foram reconhecidos por camundongos infectados C57BL/6, BALB/c ou B10.A, respectivamente. Esses epítopos, expressos por membros da família das *trans*-sialidases, geraram forte resposta imune medida pela citotoxicidade *in vivo* ou pela produção de IFN-γ ex vivo (Elispot). A cinética da ativação desses linfócitos T CD8+ se iniciou no pico da parasitemia e dependeu da carga parasitária. Ou seja, quanto mais tempo a parasitemia demorou para atingir o pico, mais lenta foi a aparição das células específicas. Uma vez que a resposta chegou ao máximo, essa se manteve alta por meses e só então começou a declinar lentamente. O fenótipo dos linfócitos T CD8+ específicos de memória é CD62Llow, característico de células de memória efetoras. Tanto a cinética, quanto o fenótipo dessas células diferiram dos achados observados para vírus, bactérias e outros protozoários intracelulares. Em animais previamente vacinados com DNA plasmidial ou proteínas recombinantes, uma significativa aceleração da resposta imune específica foi observada, a qual correlacionou com a imunidade protetora.

A fim de estudarmos os fatores que contribuíram para ativação dessas células, nós utilizamos camundongos geneticamente deficientes. Observamos que a deficiência para a produção de IL-12 e Interferon tipo I não causou nenhuma redução na geração das células citotóxicas específicas. O mesmo foi observado em animais deficientes para os receptores do tipo Toll 2, 4 ou 9. Por outro lado, animais

geneticamente deficientes que não expressavam as moléculas de MHC-II ou CD4 apresentaram significativa redução na resposta específica de linfócitos T citotóxicos.

O estudo dos mecanismos que controlam a imundominância da resposta imune mediada por linfócitos T CD8+ específicos foi feito, inicialmente, pela comparação da resposta imune em camundongos C57BL/6. Observamos que a resposta imune para o epítopo VNHRFTLV foi imunodominante sobre os demais epítopos restritos pelo MHC-Ia H-2K$^b$. A fim de determinar se essa imunodominância poderia ser exercida sobre epítopos restritos por MHC-Ia H-2K$^k$ (TEWETGQI) ou H-2K$^d$ (IYNVGQVSI), comparamos as respostas imunes em camundongos infectados homozigotos e heterozigotos. No caso do epítopo VNHRFTLV, nós observamos que a resposta imune se manteve alta em ambas as linhagens de camundongo. Já no caso dos dois outros epítopos restritos por MHC-Ia, H-2K$^k$ ou H-2K$^d$, observamos uma significativa redução na resposta imune dos animais heterozigotos em relação aos homozigotos. Essa competição não foi dependente do tempo ou da dose de parasitas inoculados. Também não foi observada quando os animais foram imunizados com adenovírus recombinantes contendo esses mesmos epítopos. O mais importante foi observar que a imunodominância pode ser evitada quando duas cepas de parasitas, contendo epítopos imundominantes distintos, foram utilizadas simultaneamente para infecção, sugerindo que há uma competição pelas células apresentadoras de antígenos durante o “priming”. Esse mecanismo de imundominância reduz a magnitude e o repertório da resposta imune e pode ser um mecanismo sofisticado de escape do parasita para evitar a eliminação completa pelos mecanismos efetores do hospedeiro.

## ABSTRACT

Although it is well established for more than 15 years that CD8+ lymphocytes restricted by MHC-Ia molecules are important for the control of the parasitemia and survival during rodent infection with *Trypanosoma cruzi*, little was known about the specificity of these lymphocytes. The lack of well defined epitopes hindered the detailed study of the kinetic of the immune response and the mechanisms of control of the immunodominance. Therefore, the initial objective of this thesis, was to identify epitopes recognized by CD8+ T lymphocytes activated during the *T. cruzi* infection. Once we defined these epitopes, we studied the kinetics of activation of these cells and some of the parameters that controlled it. Finally, it was possible to study the mechanisms of immunodominance during the immune response.

During the experimental infection with Y strain of *T. cruzi*, we observed that the epitopes VNHRFTLV, IYNVGQVSI or TEWETGQI were recognized by C57BL/6, BALB/c or B10.A infected mice, respectively. These epitopes, expressed by members of the *trans*-sialidase family of surface proteins, generated strong immune response as measured by the *in vivo* cytotoxicity or by the production of interferon-γ (Elispot). The kinetics of the activation of these CD8+ T cells initiated on the peak of parasitemia and depended on the parasite load. The longer delayed the parasitemia to reach its peak, slower was the appearance of these specific T cells. When the immune response reached the maximum, it was kept high for months and then, it declined slowly. The phenotype of memory CD8+ T lymphocytes was CD62LLow characteristic of effector memory cells. The kinetic one and phenotype of these cells had differed from the findings observed for other intracellular viruses, bacteria and protozoan parasites. In animals previously vaccinated with plasmidial DNA or recombinant proteins, a significant acceleration of the specific immune response was observed that correlated with the protective immunity.

To study the factors that contributed for the activation of these cells, we used genetically deficient mice. We observed that deficiency for production of IL-12 and Interferon type I did not cause any reduction in the specific cytotoxic response. The same was observed in animals deficient for the Toll-like receptors 2, 4 or 9. On the other hand, genetically deficient animals that did not express MHC-II or CD4

molecules had significant reduction in the cytotoxic response mediated by CD8+ T cells.

Finally, we described that following infection of mice with *T. cruzi*, an immunodominant $CD8^+$ T cell immune response was developed directed to the epitope VNHRFTLV. To determine whether this immunodominance was exerted over other non H-$2K^b$-restricted epitopes, we measured during infection of heterozygote mice, immune responses to three distinct epitopes, all expressed by members of the *trans*-sialidase family, recognized by H-$2K^b$, H-$2K^k$ (TEWETGQI), or H-$2K^d$ (IYNVGQVSI)- restricted $CD8^+$ T cells. Infected heterozygote or homozygote mice displayed comparably strong immune responses to the H-$2K^b$-restricted immunodominant epitope. In contrast, H-$2K^k$ or H-$2K^d$-restricted immune responses were significantly impaired in heterozygote infected mice when compared to homozygote ones. This interference was not dependent on the dose of parasite or the timing of infection. Also, it was not seen in heterozygote mice immunized with recombinant adenoviruses expressing *T. cruzi* antigens. Finally, we observed that the immunodominance was circumvented by concomitant infection with two *T. cruzi* strains containing distinct immunodominant epitopes, suggesting that the operating mechanism most likely involves competition of T cells for limiting APCs. This type of interference never described during infection with a human parasite may represent a sophisticated strategy to restrict priming of $CD8^+$ T cells of distinct specificities, avoiding complete pathogen elimination by host effector cells, and thus favoring host parasitism.

# *INTRODUÇÃO*

## 1. Reconhecimento antigênico por células T CD8+.

As células CD8+ que expressam as cadeias α e β dos receptores dos linfócitos são importantes mediadores da resposta imune contra infecções causadas por diversos patógenos, tais como vírus, bactérias e parasitas. Para iniciar uma resposta de células T CD8+ após infecção ou imunização, as células apresentadoras de antígeno (APC) precisam processar o antígeno derivado do patógeno e apresentá-lo no contexto do complexo de histocompatibilidade principal de classe Ia (MHC-Ia) para as células T CD8+ (JUNG *et al*., 2002 e LANZAVECCHIA & SALLUSTO, 2001).

### 1.1 Células apresentadoras de antígenos.

Todos os tipos celulares que apresentam moléculas de MHC classe Ia são capazes de apresentar antígenos para linfócitos T CD8+. Porém, a maioria deles somente é capaz de fazê-lo para linfócitos efetores e de memória. As únicas células capazes de apresentar antígeno para linfócitos T CD8+ naïve e induzir a ativação dos mesmos são as chamadas APC "profissionais". Este grupo de células inclui células dendríticas (DC), macrófagos (MØ) e linfócitos B.

Devido à sua distribuição nos tecidos e nos órgãos linfóides e às diversas características que refletem sua especialização para essa função, as DC são mais eficientes na apresentação de antígenos do que os MØ e linfócitos B (revisto por TROMBETTA & MELLMAN, 2005). Estudos recentes de depleção seletiva de DC confirmaram a importância dessas células para o desenvolvimento de respostas primárias e secundárias contra patógenos (JUNG *et al*., 2002).

O "amadurecimento" das DC é um dos eventos mais importantes no processo de apresentação de antígenos, compreendendo uma série de modificações funcionais e fenotípicas, que promovem a indução da resposta imune adaptativa convencional (revisto por BANCHEREU *et al*., 2000).

As DC "imaturas" se localizam nos tecidos e órgãos linfóides, apresentam alta capacidade endocítica e baixa expressão de moléculas de MHC e co-estimulatórias na superfície. Assim, elas são importantes para a captura de antígeno, mas são pobremente capazes de induzir uma resposta imune adaptativa clássica. Já as DC "maduras" se localizam nos órgãos linfóides secundários, apresentam baixa capacidade endocítica e altos níveis de expressão de moléculas de MHC e co-

estimulatórias em sua superfície. Desta forma, ao amadurecer, as DC tornam-se extremamente eficientes na indução de resposta adaptativa clássica (revisto por BANCHEREU *et al*., 2000).

Mais recentemente, se observou que as DC "imaturas" são capazes de processar e apresentar determinantes antigênicos para linfócitos T naïve. Entretanto, ao invés de estimular uma resposta imune clássica, essas células são capazes de induzir um potente estado de anergia/tolerância em nível periférico (HAWIGER *et al*., 2001). Este evento pode ser muito importante para o controle de linfócitos T auto-reativos e da auto-imunidade.

Diversos estímulos podem levar à maturação das DC. Ligantes dos receptores de PAMP ("pathogen-associated molecular patterns"), como por exemplo, receptores do tipo Toll (TLR) estão entre os mais potentes e comumente usados experimentalmente (revisto por GUERMONPREZ *et al*., 2002). Dentre os ativadores de TLR, o LPS bacteriano, que se liga ao TLR-4, é o mais usual. Mais recentemente, o CpG ODN, que se liga ao TLR-9, também tem recebido considerável atenção.

**Tabela I: Características das DC imaturas e maduras.**

| DC imaturas | DC maduras |
|---|---|
| Tecidos linfóides e periféricos | Tecidos linfóides secundários |
| Altamente endocítica | Baixa capacidade endocítica |
| Baixa expressão de MHC classe I e II e moléculas co-estimulatórias na superfície | Alta expressão de moléculas de MHC classe I, II e co-estimulatórias na superfície |
| Acumulação de antígeno | Estimulação de linfócitos T |

Vários tipos de DC já foram identificados *in vitro* e *in vivo*. As diferenças entre elas podem ser atribuídas às diferentes linhagens progenitoras, à distribuição nos tecidos ou aos marcadores de superfície. Os tipos de DC mais popularmente referidos são: 1) DC humanas $CD14^+$ diferenciadas a partir de monócitos humanos na presença de GM-CSF e IL-4; 2) DC mielóides de camundongos que são obtidas a partir da diferenciação de células progenitoras da medula óssea na presença de GM-CSF e IL-4; 3) células de Langerhans humanas derivadas de células

progenitoras $CD34^+$; 4) DC plasmacitóides (pDC) humana ou de camundongo. Some-se a estas 4 populações, as DC $CD8\alpha^+$ e $CD8\alpha^-$ (revisto por TROMBETTA & MELLMAN, 2005).

Devido a sua alta capacidade endocítica, os MØ são há muito considerados como protótipos de APC. Este tipo celular está envolvido na fagocitose de partículas, debris celulares, microorganismos, células apoptóticas, imunocomplexos, etc. Esse processo pode ocorrer de maneira inespecífica ou através de inúmeros receptores específicos presentes na sua superfície (receptores de Fc, complemento, lectinas, integrinas, etc.). Os MØ expressam em sua superfície diversas moléculas envolvidas no processo de apresentação de antígenos (moléculas de MHC classe I e II, co-estimulatórias e de adesão) e são capazes de aumentar a expressão destas, após estímulos inflamatórios. Entretanto, os níveis de expressão das moléculas de MHC classe I e II e co-estimulatórias não atingem os níveis observados nas DC (revisto por TROMBETTA & MELLMAN, 2005).

Linfócitos B não parecem ser as APC mais eficientes para iniciar uma resposta imune. Aparentemente, estas células retêm a função de APC para aumentar sua interação com linfócitos T específicos e, assim, sua eficiência na produção de anticorpos. Apresentam baixa capacidade endocítica, o que reduz sua eficiência como APC, principalmente no início da resposta imune. A principal forma de internalização de antígenos é através da Imunoglobulina de superfície (sIg). Uma vez que o antígeno é endocitado via sIg, este é rapidamente transportado para vesículas endocíticas, o que permite uma boa apresentação do antígeno, já que esse tipo de célula apresenta na sua superfície grande quantidade de moléculas de MHC classe II, co-estimulatórias e de adesão (revisto por TROMBETTA & MELLMAN, 2005).

Os linfócitos B também podem apresentar antígenos via MHC classe Ia para linfócitos T CD8+ através das vias endógena e exógena (ver abaixo), entretanto a importância destas células na iniciação de uma resposta imune mediada por linfócitos T CD8+ ainda é assunto de debate.

**1.2 Encontro dos linfócitos T CD8+ com as APC.**

Recentes avanços nas técnicas de vídeo microscopia *in vivo,* utilizando a tecnologia "multiphoton-based intravital microscopy", tornaram possível determinar onde os linfócitos T CD8+ específicos para um determinado antígeno encontram APC estimulatórias (HICKMAN *et al.*, 2008). Foi demonstrado que a maioria dos linfócitos T específicos para um determinado antígeno localizam-se centralmente nas zonas de linfócitos T dos nódulos linfáticos. Entretanto, após a entrada de um vírus, os linfócitos T específicos migram para as zonas periféricas dos nódulos linfáticos, perto do sinus sub-capsular (SCS), e formam contato com as células infectadas. A região peri-SCS que atrai os linfócitos T CD8+ é superficial à HEV (high endothelial venules), no paracórtex do nódulo linfático. Essa migração cessa após 6h de infecção e é dependente da presença de MHC-Ia, pois nos nódulos linfáticos dos camundongos geneticamente deficientes na expressão destas moléculas não ocorre redistribuição considerável. De forma semelhante, é dependente do antígeno específico, uma vez que a infecção com um vírus que não contém o epítopo reconhecido pelos linfócitos T específicos, não induz a migração substancial dessas células. Assim, o processo de migração dos linfócitos T CD8+ para as regiões onde APC infectadas estão presentes é específico e regulado pela presença do complexo peptídeo-MHC-Ia. Uma vez que os linfócitos T CD8+ chegam às células infectadas, estabelecem contato que perdura por várias horas. Embora as células infectadas por vírus sejam na sua maioria macrófagos, linfócitos T CD8+ específicos formam agregados somente em volta das DC. Muitas dessas DC chegam a interagir com mais de 10 linfócitos T específicos ao mesmo tempo, sendo a média de cerca de 5 linfócitos T CD8+ específicos por DC. Esse processo é completo após cerca de 12h do início da infecção.

Recentes estudos, também *in vivo,* utilizando anticorpos quiméricos dirigidos contra diferentes populações de células determinaram que as DC CD8$\alpha$+ DEC205+ são as principais APC para linfócitos T CD8+ (DUDZIAK *et al.*, 2007).

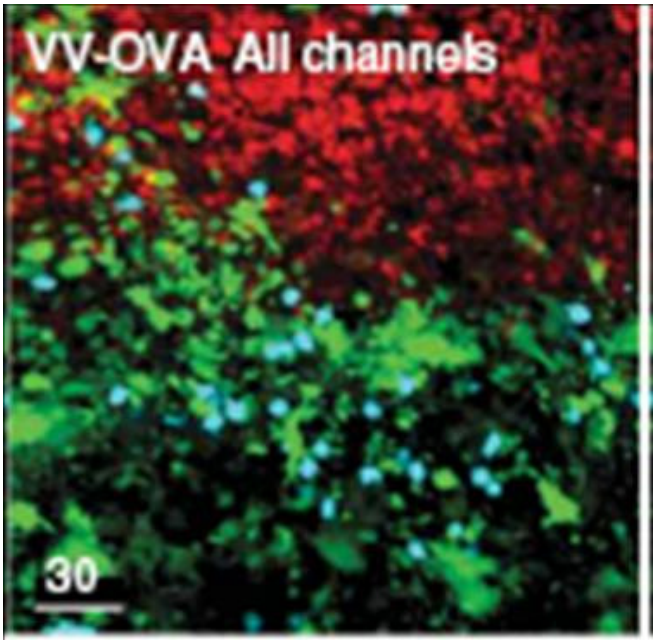


**Figura 1**

**MO, DC e Linfócitos T específicos** (HICKMAN *et al.*, 2007).

### 1.3 Processamento antigênico para a geração de complexo peptídeo-MHC-I (p-MHC-I).

Os peptídeos se ligam às moléculas de MHC-Ia nascentes no retículo endoplasmático (RE) de cada célula. Os antígenos doadores destes peptídeos são proteínas celulares e proteínas exógenas (virais, bacterianas, etc.). Quantitativamente, a maior fonte de peptídeos para a formação do complexo peptídeo-MHC-Ia são proteínas presentes no citoplasma das células. Essa via de processamento foi denominada de via endógena. Os polipeptídeos mais abundantes para o processamento são formas defeituosas de proteínas celulares denominadas DRiP ("defective ribossomal products", revisto por Kloetzel & Ossendorp, 2004). Além dos DRiPs, proteínas celulares e de microorganismos estáveis que se encontram no citoplasma também podem ser processadas e apresentadas por diferentes APC, pela via endógena.

O segundo grupo de proteínas que tem acesso ao citoplasma das APC são as proteínas produzidas nos ribossomos ligados ao RE. Neste caso, essas proteínas são rapidamente translocadas para dentro do RE e, portanto, necessitam ser retro-translocadas para o citoplasma das APC antes de seguirem o processamento

endógeno (revisto por TROMBETTA & MELLMAN, 2005). Dentro do RE, há um complexo protéico denominado ERAD ("Endoplasmic reticulum-associated protein degradation"), o qual elimina proteínas mal-formadas que são incapazes de adquirir uma estrutura secundária/terciária apropriada. Os alvos do ERAD são selecionados por um sistema de controle de qualidade no lúmen do RE. O transporte das proteínas defeituosas do RE para o citoplasma é mediado por um complexo hetero-trimérico formado pela proteína de membrana Sec61 (revisto por MEUSSER et al., 2005).

O terceiro grupo de proteínas que tem acesso ao citoplasma das APC são proteínas exógenas, ou seja, não produzidas no citoplasma destas células. Proteínas solúveis que escapam à proteólise são retro-translocadas, entram no lúmem do RE e de lá são deslocadas para o citoplasma das APC (revisto por TROMBETTA & MELLMAN, 2005). Antígenos particulados, por sua vez, seguem outra rota e ganham acesso a fagossomos que se fundiram ao RE periférico e são competentes para apresentação de antígenos. As proteínas presentes nesses novos fagossomos, agora denominados ergossomos, podem ser translocadas para o citoplasma, pela proteína Sec61 e processadas por proteossomos próximos ao complexo Sec61. As vias que utilizam proteínas exógenas são conhecidas como de apresentação cruzada ("cross-priming"). Na figura 2 pode-se ver um esquema detalhado das diferentes fontes de proteína que podem ser introduzidas no citoplasma das APC.

Antígenos que caem no citoplasma das APC podem ser alvos da ubiquitina, que se liga covalentemente às proteínas que deverão ser degradadas pelo complexo multi-catalítico denominado proteossomo. Os proteossomos são compostos por 14 distintas subunidades (sete $\alpha$ e sete $\beta$) dispostas em 4 anéis simétricos que formam uma espécie de barril com finais fechados. Esses finais podem ser abertos, criando um canal estreito por onde entram os substratos. A atividade proteolítica é exercida pelas sub-unidades $\beta$1,$\beta$2 e $\beta$5 (revisto por KLOETZEL, 2004). Este proteossomo, denominado 20S, é capaz de degradar proteínas linearizadas, *in vitro*, e há evidências de que é capaz de degradar proteínas não ligadas a ubiquitina. A degradação do complexo proteína-ubiquitina é feita por estruturas maiores conhecidas como proteossomos 26S. Essas estruturas compreendem os proteossomos 20S acrescidos de estruturas reguladoras chamadas de 19S. Os

reguladores de 19S são estruturas multi-catalíticas que exercem várias funções, como se ligar ao complexo proteína-ubiquitina, remover a ubiquitina, linearizar a proteína e ajudar na passagem do substrato pela abertura do complexo 20S. Por causa da dependência de ATP do regulador 19S, a degradação mediada pelo complexo 26S é dependente de energia (revisto por KESSLER *et al*., 2002).

Após o início da resposta imune, a presença de IFN-γ (Interferon-gama) leva à síntese dos imunoproteossomos. O processo de formação dos imunoproteossomos ocorre rapidamente através da síntese das cadeias β1i (chamada também de LMP2), β2i (MECL1) e β5i (LMP7). Estas cadeias substituem respectivamente as cadeias β1, β2 e β5 dos proteossomos convencionais (revisto por KESSLER *et al*., 2002). O padrão de regulação sugere uma função importante durante a resposta imune adaptativa. Em vários casos, a ausência de cadeias dos imunoproteossomos definem o padrão de resposta imune (PANG et al., 2006). Entretanto, estudos recentes não confirmaram uma função crítica das cadeias β1i e β5i durante a resposta imune de camundongos observada após a infecção com o vírus LCMV ou DNA plasmidial (NUSSBAUM *et al*., 2005).

Recentemente, os proteossomos foram implicados numa nova função. Foi observado que proteínas podem ser quebradas, re-ligadas e de novo quebradas por este complexo multi-catalítico. Assim, novos epítopos podem ser formados utilizando partes não contínuas de proteínas antigênicas. Esse fenômeno, denominado "splicing" de proteínas, pode servir para aumentar o número de epítopos apresentados para linfócitos T CD8+ (VIGNERON *et al*. 2004, WARREN *et a*l., 2006).

Cerca de $10^8$ peptídeos de 2 a 24 aminoácidos são gerados por minuto por célula através da degradação nos proteossomos. Dois terços destes peptídeos contém menos de 8 aminoácidos e, portanto não podem ser usados como epítopos para linfócitos T CD8+. Estes peptídeos curtos são degradados em segundos por um grupo heterogêneo de amino-peptidases citoplasmáticas. Os peptídeos com 6 a 17 aminoácidos são substratos da amino-peptidase denominada "thimet oligopeptidase", que acaba por agir reduzindo o grupo de peptídeos que poderia servir para a apresentação de antígenos (revisto por KLOETZEL & OSSENDORP,

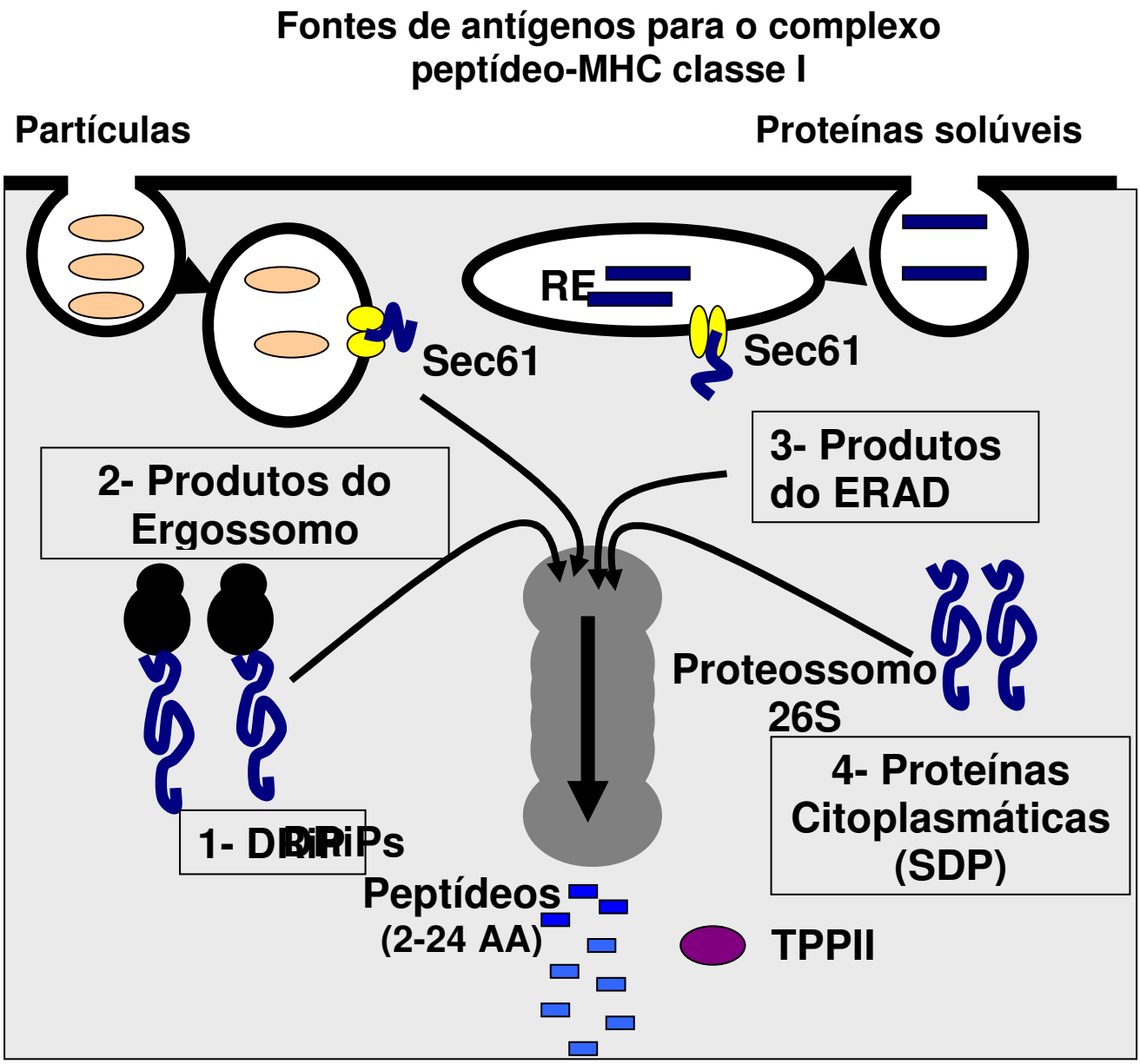


**Figura 2. Diferentes fontes de proteínas que tem acesso ao citoplasma das APC.**

1- Formas defeituosas de proteínas celulares e virais produzidas no citoplasma por ribossomos denominadas DRiP ("defective ribossomal products").
2- Partículas ou microrganismos endocitadas pelas APC são transportadas por fagossomos que se fundem com o RE formando os ergossomos. O transporte dos antígenos para o citoplasma é feito pela proteína Sec61.
3- Proteínas presentes no RE podem vir através da macropinocitose ou de proteínas que contém o peptídeo sinal. Proteínas reconhecidas pelo ERAD ("Endoplasmic reticulum-associated protein degradation") são retro-translocadas para o citoplasma através da proteína Sec61.
4- Proteínas celulares e virais produzidas no citoplasma por ribossomos e que apresentam uma degradação lenta. Estes produtos são chamados de SDP ("slowly degraded proteins") .
As proteínas presentes no citoplasma reconhecidas por ubiquitinas são carreadas para dentro do complexo multi-catalítico denominado proteossomo 26S. Os proteossomos liberam peptídeos de 2 a 24 aminoácidos que são processados pela enzima citoplasmática TPPII

2004). Essa degradação de peptídeos, potencialmente antigênicos, pode ser evitada pela ligação do antígeno na chaperonina TRiC que protege peptídeos antigênicos. Ao contrário das amino-peptidases que agem sobre peptídeos curtos, a endo- e exo-peptidase TPPII ("tripeptidyl peptidase II") clivam peptídeos com 15 ou mais aminoácidos (revisto por KLOETZEL, 2004). O processamento pela TPPII parece ser importante para a geração de peptídeos antigênicos menores, os quais serão translocados para o RE, onde serão acoplados ao MHC-I e servirão para apresentação antigênica.

Como pode ser visto na figura 3, os peptídeos são translocados para dentro do lúmen do RE por proteínas conhecidas como TAP 1 e 2 ("transporter associated proteins"). O transporte das principais fontes de peptídeos antigênicos que serão acoplados às moléculas de MHC-I é feito por essas proteínas. Entretanto, há outras formas de entrada no RE, já que células deficientes de TAP são capazes de apresentar alguns poucos tipos de peptídeos antigênicos (revisto por ACKERMAN & CRESSWELL, 2004).

Dentro do RE, a amino-peptidase denominada ERAP-1 ("endoplasmic reticulum amino-peptidase 1") é capaz de agir na região N-terminal de peptídeos maiores que 8 aminoácidos. Essa atividade enzimática parece crítica para a formação de epítopos de 8 a 10 (podendo chegar a 11) aminoácidos que se ligam ou estão ligados às moléculas de MHC-I. A montagem da molécula de MHC-I com a β2-microglobulina e o peptídeo antigênico é um processo complexo que envolve as chaperoninas calnexina, calreticulina, e a thiol-oxidoredutase Erp57, além de uma proteína acessória, a tapasina. A tapasina e a calreticulina são essenciais para a montagem eficiente da molécula de MHC-I, mas a natureza precisa das suas funções durante a montagem da molécula de MHC-I ainda é desconhecida. Uma vez montado, o complexo p-MHC-I é exportado para a superfície das APC, permitindo o reconhecimento pelo receptor dos linfócitos T CD8+ (revisto por ACKERMAN & CRESSWELL, 2004).

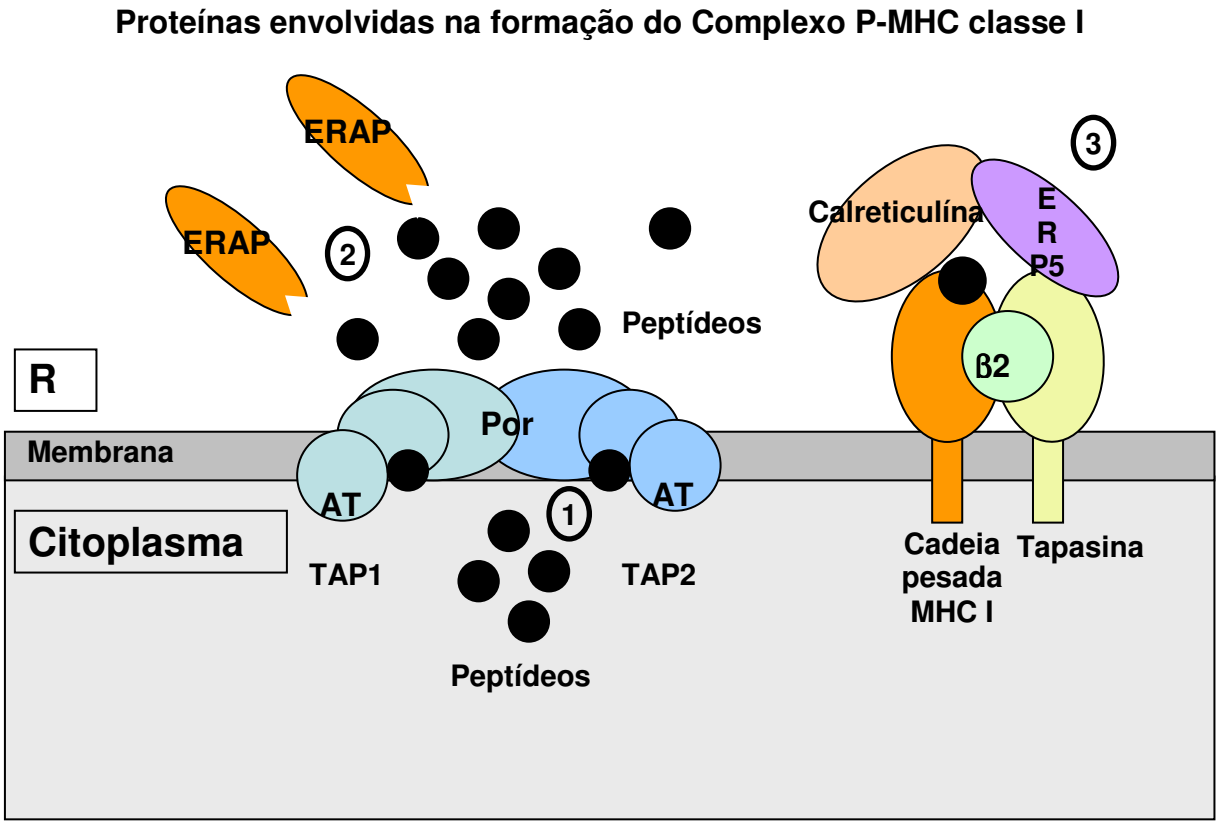


**Figura 3**
A figura ilustra as diferentes proteínas que participam da montagem do complexo P-MHC-I.
1- Peptídeos são transportados pelas proteínas TAP para dentro do RE.
2- Dentro do RE, os peptídeos sofrem um processamento proteolítico na região N-terminal feito pela enzima ERAP-1 ("endoplasmic reticulum amino-peptidase 1").
3- A montagem da molécula de MHC-I com a β2-microglobulina e o peptídeo antigênico é um processo que envolve a chaperonina, calreticulina, e a thiol-oxidoredutase Erp57, além de uma proteína acessória, a tapasina.

## 2. Cinética da resposta imune mediada por linfócitos T CD8+.

A resposta das células T CD8+ durante uma infecção, por exemplo, pode ser dividida em três fases: (a) fase de expansão (diferenciação/proliferação), (b) fase de contração e (c) geração de memória. A figura 4 ilustra as diferentes fases durante a resposta contra uma infecção.

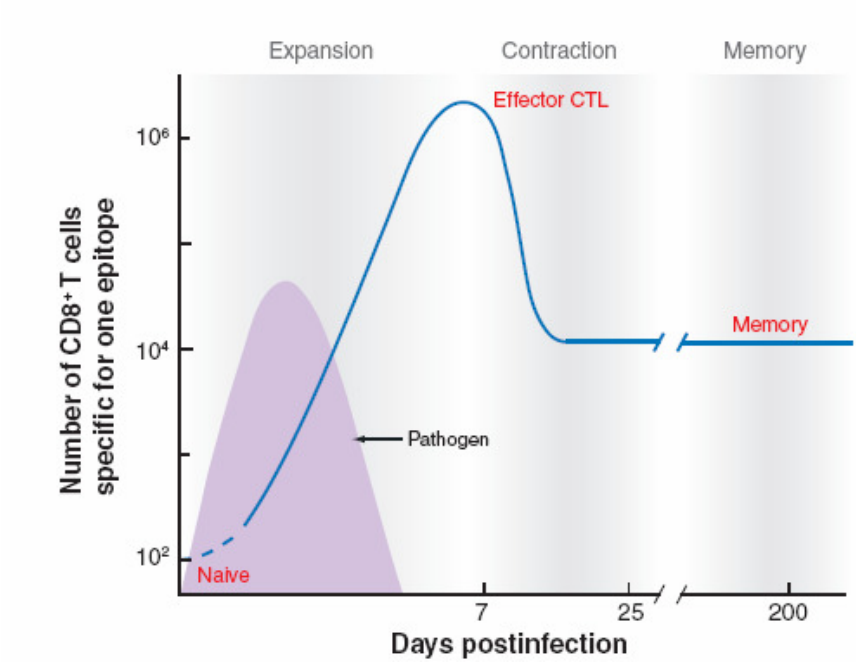


**Figura 4.** Cinética da massiva resposta proliferativa de células T CD8+ específicas após infecção de um camundongo com um patógeno virulento, tal como *Listeria monocytogenes*, LCMV, vírus da estomatite vesicular ou vírus vaccinia. Um camundongo contém 50-200 células T CD8+ naïve específicas para um epítopo qualquer. Depois de 24 horas, esses precursores sofrem de 7-15 divisões e geram milhares de linfócitos T CD8+ efetores nos dias 7 e 8 após infecção. Quando o patógeno é eliminado, a maioria das células efetoras morre, deixando um pool de células de memória (WILLIAMS & BEVAN, 2007).

Um dos mais bem estabelecidos modelos de estudo de linfócitos T CD8+ é o do LCMV (lymphocytic choriomeningitis vírus). Através da utilização de células TCD8+ transgênicas P14, as quais expressam um TCR específico para o peptídeo $GP_{33}$, foi demonstrado que após o contato com a APC, essas células sofrem ao menos sete divisões, podendo atingir até 15 a 20 divisões por precursor naïve, gerando assim, um significativo aumento no número de células T CD8+ específicas (BUTZ & BEVAN, 1998 e KAECH & AHMED, 2001). Reforçando essa idéia, um

estudo utilizando marcação com tetrâmero e BrdU (Bromodeoxiuridina) mostrou que a expansão das células T CD8+, após a infecção, ocorria devido à divisão das células específicas (MURALI-KRISHNA *et al*., 1998).

A cinética da expansão (proliferação) das células T CD8+ específicas pode variar de acordo com o tipo de infecção. Em camundongos infectados com LCMV, vírus influenza, *Listeria monocytogenes* (LM) ou *Plasmodium yoelli* a expansão dessas células ocorre entre os dias 4 e 8 após a infecção (BUTZ & BEVAN, 1998; STAMBAS *et al*., 2007; POPE *et a*l., 2001; SANO *et al*., 2001). Já em camundongos infectados com *Mycobacterium bovis* (bacillus Calmette Guérin – BCG)*, Toxoplasma gondii*, *Salmonella typhimurium* ou *Trypanosoma cruzi* a expansão das células específicas inicia-se mais tardiamente (IRWIN *et al*., 2005; KWOK *et al*., 2003; LUU *et al*., 2006; TZELEPIS *et al*., 2006; MARTIN *et al*., 2006). No caso do *T. gondii*, a resposta de células T CD8+ específicas para um epítopo transgênico só foi inicialmente detectada no 10º dia após a infecção e o pico dessa resposta ocorreu no 23º dia. Em camundongos infectados com *Salmonella typhimurium* ou com BCG recombinantes, o pico da resposta contra o epítopo transgênico ocorreu no 21º e 30º dia após a infecção, respectivamente. Após a infecção com a cepa Y de *Trypanosoma cruzi,* foi observado que a expansão das células T CD8+ específicas iniciou-se no 9º dia e atingiu o pico no 15º dia. Durante a infecção com outras cepas de *T. cruzi*, o pico da resposta de células T CD8+ específicas variou do 14º ao 24º dia após a infecção.

De forma semelhante, a modulação da cinética da expansão (proliferação) das células T CD8+ específicas também depende do tipo de infecção. Um recente trabalho demonstrou que camundongos que receberam transferência adotiva de células OT-I, as quais possuem TCR específico para OVA, e que foram infectados com $10^2$ ou $10^4$ LM-OVA, apresentaram semelhantes cinéticas de expansão de células T CD8+ específicas para OVA. Entretanto, camundongos que foram infectados com $10^4$ ou $10^6$ BCG-OVA apresentaram distintas cinéticas de expansão. O aumento na dose do desafio, nesse caso, provocou o adiantamento da ativação das células específicas devido ao aumento na quantidade de antígeno e não ao aumento dos sinais inflamatórios (RUSSEL *et al*., 2007). Da mesma forma, a alteração na quantidade de *T. cruzi* utilizado para o desafio de camundongos provocou uma modificação no tempo de ativação das células T CD8+ específicas.

Animais infectados com diferentes doses, as quais variaram de $10^2$ até $10^5$ parasitas, apresentaram diferentes tempos de ativação de células T CD8+ específicas (TZELEPIS *et al*., 2007). Nestes dois casos, os autores atribuíram o aumento da carga do patógeno como um fator crítico na determinação da cinética de ativação dos linfócitos T.

O estudo do tempo de exposição ao antígeno necessário para a ativação das células T CD8+ específicas sugeriu a ocorrência de uma "programação" para a expansão dessas células em um período muito curto de contato com a APC contendo o epítopo antigênico. No modelo da *Listeria monocytogenes*, Mercado *et al.* (2000) trataram camundongos infectados com ampicilina para controlar o tempo da infecção. Eles observaram que um período de 24 horas de exposição ao antígeno foi suficiente para aquisição de função efetora e para a geração de memória de células T CD8+ específicas. Entretanto, outro grupo, trabalhando com o mesmo modelo, demonstrou que esse curto período altera a capacidade máxima das células T CD8+ para se diferenciar em células de memória (WILLIAMS & BEVAN, 2004).

A resposta de células T CD8+ contra os diferentes patógenos difere na dependência das células T CD4+ para sua ativação. Durante a resposta primária contra a cepa Y de *T. cruzi*, por exemplo, foi demonstrado que a ativação de linfócitos T CD8+ é dependente dos linfócitos T CD4+ (TZELEPIS *et al*., 2007). Já a infecção de camundongos deficientes de MHC classe II com *L. monocytogenes* recombinante gerou uma intensa resposta primária. Porém, estes camundongos apresentaram uma resposta de células T CD8+ de memória qualitativamente pior. Nem mesmo a presença das células T CD4+ durante a resposta secundária de células T CD8+, primadas na ausência de linfócitos T CD4+, foi capaz de melhorar a qualidade da resposta. Isso demonstrou a importância das células T CD4+ durante o "priming" das células T CD8+ para a geração de memória (SUN & BEVAN, 2003). A infecção de camundongos deficientes de CD4 (CD4KO) com LCMV também demonstrou a importância dos linfócitos T CD4+ para a geração de células T de memória completamente funcionais (KHANOLKAR *et al*., 2004).

Durante a fase de expansão, além de proliferarem, as células T CD8+ também sofrem um processo de diferenciação. Este é representado pela alteração na expressão de certas moléculas de superfície celular, pela produção de citocinas e capacidade citolítica, o que as caracteriza como células efetoras.

A alteração da expressão de moléculas envolvidas na migração foi observada nas células T CD8+ ativadas. Estas apresentaram diminuição da expressão de CD62L e CCR7 (SALLUSTO *et al*., 1999), receptores que induzem a migração dos linfócitos para os órgãos linfóides secundários. Em contraste, foi observado o aumento da expressão de integrinas, as quais participam da migração para os tecidos, como VLA-4 (OSBORN, 1990) e VLA-1 (RAY *et al*., 2004).

A capacidade de produção de IFN-γ e/ou atividade citolítica são importantes mecanismos efetores das células T CD8+ ativadas. Diversos trabalhos, nos mais diferentes tipos de infecções e imunizações, utilizam estes dois parâmetros como abordagem para o estudo tanto da resposta primária como da secundária de células T CD8+ específicas. O TNF (fator de necrose tumoral), assim como o IFN-γ, produzido pelas células T CD8+, pode ser importante para a defesa contra infecções por microorganismos.

O IFN-γ pode agir na ativação de células hematopoiéticas, como macrófagos, ou não hematopoiéticas sendo importante nos dois casos para a imunidade a certos patógenos como, por exemplo, *T. cruzi* e *Toxoplasma gondii* (YAP & SHER, 1999). No caso de células hematopoiéticas, o IFN-γ induz a produção de óxido nítrico (NO), uma substância microbicida. De fato, foi demonstrado, *in vitro*, que a inibição de NO bloqueia a atividade anti-parasitária de macrófagos induzida por IFN-γ (GAZZINELLI *et al*., 1992) e, *in vivo,* que as células hematopoiéticas necessitam expressar a enzima NO sintase 2 (iNOS) para que os animais possam sobreviver à infecção experimental pelo *T. gondii* (YAP & SHER, 1999). Entretanto, este não parece ser o modo de ação do IFN-γ em células não hematopoiéticas, uma vez que animais iNOS KO, reconstituídos com células de medula óssea de animais selvagens são capazes de resistir à infecção experimental pelo *T. gondii* (YAP & SHER, 1999). Da mesma forma, camundongos iNOS KO infectados com a cepa Brazil de *T. cruzi* apresentaram resistência semelhante aos animais selvagens, sugerindo um mecanismo NO independente (CUMMINGS & TARLETON, 2004).

A atividade citotóxica mediada pelas células T CD8+ pode ocorrer através de duas vias: a mediada por Fas/FasL e a mediada por exocitose de grânulos (perforina/granzima). A estimulação do TCR pode induzir a expressão de FasL (ligante de Fas) nas células T ativadas, o qual interage com o Fas (membro da família do receptor TNF) presente nas células alvo. Esta interação provoca a

ativação de caspases nas células alvo, o que resultará na clivagem de substratos apoptóticos adicionais que levam à fragmentação do DNA e conseqüentemente a apoptose (revisto por RUSSEL & TIMOTHY, 2002).

A estimulação do TCR também pode levar à produção de grânulos citotóxicos (perforina/granzimas) pelas células T ativadas. Quando esta célula reconhece uma célula alvo e a ela se conjuga, a perforina (na presença de cálcio) polimeriza e forma uma estrutura do tipo anel que aparentemente contém um poro central. Supõe-se que este canal permite a passagem de granzimas, as quais ativarão as caspases e induzirão a apoptose como descrito acima (revisto por RUSSEL & TIMOTHY, 2002).

Entretanto, essa hipótese de que a perforina forma um canal através do qual passam outras enzimas indutoras de morte foi questionada. O tamanho do poro parece ser muito pequeno para a passagem de moléculas grandes como as granzimas. Assim sendo, uma segunda hipótese foi elaborada, a da endocitose reparativa: a perforina entra na célula alvo, cria um sinal para a mesma reparar o dano via endocitose da perforina e da membrana plasmática que circunda a região. As granzimas presentes na proximidade da lesão são também endocitadas e entregues para o citoplasma e núcleo, onde induzirão apoptose. Porém, ainda não se sabe se esse modelo é biologicamente relevante (revisto por RUSSEL & TIMOTHY, 2002).

A fase de expansão das células T CD8+ específicas para o antígeno é seguida por uma fase de contração, onde 90-95% dessas células são eliminadas por apoptose (MURALI-KRISHNA *et al.*, 1998). No modelo de infecção aguda é sugerido que a contração ocorre após a eliminação do patógeno. Entretanto, ainda não se sabe claramente o que influencia essa fase. Através da infecção de camundongos com *Listeria monocytogenes* virulenta ou atenuada, as quais provocam picos de infecção em diferentes dias e distintas cinéticas de contração, e através do tratamento desses camundongos com antibiótico, foi sugerido que a contração das células T CD8+ específicas é influenciada pelo pico da infecção e pela expressão de antígeno (PORTER & HARTY, 2006).

As células T CD8+ específicas para o antígeno, sobreviventes à fase de contração, constituem um estável pool de memória, o qual é capaz de ser mantido na ausência do antígeno. A IL-7 e a IL-15 parecem dar os sinais de sobrevivência e proliferação para as células T CD8+ de memória, respectivamente (revisto por

HARTY & BADOVINAC, 2008). Como descrito acima, o tempo inicial de exposição ao antígeno e o auxílio de células T CD4+ podem influenciar na qualidade das células T CD8+ de memória.

As características das células T CD8+ efetoras com potencial para compor o "pool" de memória ainda não estão estabelecidas. Um grupo sugeriu que a expressão de IL-7Rα (CD127) poderia ser um indicativo das células efetoras que sobrevivem à contração. Eles observaram que a transferência adotiva de células transgênicas P14 efetoras $CD127^{high}$ foi mais efetiva na geração de células de memória do que a transferência de células efetoras P14 $CD127^{low}$ (KAECH *et al*., 2003). Entretanto, outro estudo com células transgênicas efetoras demonstraram que mesmo mantendo alto os níveis de CD127, após a imunização com células dendríticas pulsadas com peptídeo, essas células não foram impedidas de sofrer contração (LACOMBE *et al*., 2005).

Como revisto por Badovinac e Harty (2006), as qualidades relevantes para uma boa célula T CD8+ de memória são: (a) persistir em número substancialmente maior que o repertório naïve; (b) ser capaz de rapidamente responder a re-infecção utilizando vários mecanismos efetores (citólise e produção de citocinas) e (c) gerar imunidade protetora após o re-encontro com o patógeno.

Entretanto, como descrito pela primeira vez com LCMV, as células T CD8+ presentes na fase crônica podem apresentar uma exaustão funcional. A inativação de distintas funções de células T ocorre de uma maneira hierárquica. A capacidade para produzir IL-2, de citólise e de proliferação robusta são as primeiras funções a serem perdidas. No estágio seguinte de exaustão, a célula perde a capacidade de produzir TNF-α e no estágio mais severo, a produção de IFN-γ fica comprometida. Altos níveis de antígeno e baixos níveis de auxílio de linfócitos T CD4+ podem correlacionar com maiores níveis de exaustão (revisto por SHIN & WHERRY, 2007).

Em 1999, dois subgrupos de células T de memória foram descritos baseados na localização anatômica, na expressão de marcadores de superfície celular e função efetora (SALLUSTO *et al*., 1999). As células T de memória que expressam as moléculas CD62L e CCR7 e, portanto, migram para os nódulos linfáticos, são chamadas de memória central ($T_{CM}$). Já as células T de memória que perdem a expressão dessas moléculas e estão localizadas nos tecidos periféricos são chamadas de células de memória efetoras ($T_{EM}$).

Desde então, os imunologistas adotaram esta nomenclatura e importantes questões vêm sendo abordadas a respeito destes dois subgrupos: (a) a relação entre eles e (b) a relação com a imunidade protetora.

Três modelos foram propostos para explicar a relação entre os dois grupos. O primeiro deles é o "modelo de diferenciação progressiva", no qual a diferenciação das células dependerá da força e da estimulação recebida pelas células T naïve ao longo da infecção. Enquanto algumas células T naïve tornam-se células T efetoras completamente diferenciadas durante a infecção, outras param num estado intermediário de diferenciação. No fim da resposta primária, as células do estado intermediário de diferenciação darão origem às células $T_{CM}$, as quais sofrerão a diferenciação terminal após o re-estímulo com o antígeno. Já as células T efetoras completamente diferenciadas permanecem vivas, dando origem às células $T_{EM}$, as quais serão capazes de providenciar proteção imediata em um próximo contato com o antígeno (LANZAVECCHIA & SALLUSTO, 2000).

O segundo é o "modelo stem cell" que propõe que as células T naïve são estimuladas por antígeno para diferenciarem-se em células $T_{CM}$, a qual foi proposto ter capacidade de auto-renovação semelhante às "stem cells". As $T_{EM}$ diferenciam-se a partir das $T_{CM}$, sofrem um limitado número de divisões e diferenciam-se totalmente em células efetoras sem capacidade de proliferação (FEARON *et al.*, 2001).

O terceiro modelo foi denominado "modelo de diferenciação linear". Este modelo sugere que após o encontro com o antígeno, as células T naïve diferenciam-se em células efetoras, estas por sua vez diferenciam-se em células $T_{EM}$. Após a eliminação do patógeno, as células $T_{EM}$ diferenciam-se em $T_{CM}$, as quais apresentam rápida resposta ao antígeno e capacidade de auto-renovação como as "stem-cells". Na presença de antígeno, as $T_{CM}$ convertem-se em $T_{EM}$. Assim sendo, eles não seriam dois subgrupos distintos e sim uma contínua diferenciação que termina com o desenvolvimento da $T_{CM}$, "a verdadeira" célula de memória (WHERRY *et al.*, 2003).

No estudo em que esta última hipótese foi formulada, eles também demonstraram, através de experimentos de transferência adotiva de células transgênicas, que as $T_{CM}$ foram substancialmente melhores no controle de infecções causadas por diferentes vias do que as $T_{EM}$. Essa imunidade protetora ocorreu por uma maior expansão das $T_{CM}$ após a infecção devido, em parte, a uma aumentada capacidade proliferativa destas células.

Entretanto, outro grupo demonstrou, também através de transferência adotiva de células transgênicas, que a proteção mediada por células T CD8+ de memória varia de acordo com a infecção. Eles observaram que, no modelo de infecção com LCMV, as células $T_{CM}$ apresentaram uma capacidade proliferativa maior, a qual correlacionou com a proteção. Porém, na infecção com vírus vaccinia recombinante, apesar de as $T_{CM}$ apresentarem maior capacidade proliferativa, as $T_{EM}$ foram as células capazes de conferir proteção (BACHMANN et al., 2005).

De acordo com a hipótese proposta neste último modelo, a conversão das $T_{EM}$ em $T_{CM}$ ocorre após a eliminação do antígeno. Talvez, essa possa ser a razão pela qual em infecções crônicas, como já demonstrado no caso *T. cruzi*, a maioria das células T CD8+ mantêm o fenótipo de $T_{EM}$ na fase tardia e não se diferenciam em $T_{CM}$ (MARTIN & TARLETON, 2005).

## 3. Importância dos linfócitos T CD8+ na imunidade contra patógenos intracelulares.

O uso de camundongos geneticamente modificados tornou-se uma importante ferramenta para o estudo da importância dos linfócitos T CD8+ nas diversas infecções. Além desta abordagem, a depleção dessas células com anticorpos específicos e a transferência adotiva de células purificadas ou clonadas continuam sendo importantes instrumentos de estudo.

### 3.1. Infecções virais.

A importância das células T CD8+ para o controle da infecção por LCMV foi demonstrada através da infecção de camundongos selvagens e deficientes de CD8 (CD8KO) com a cepa LCMV-WE. Enquanto os animais CD8KO apresentaram altos níveis de viremia, os selvagens controlaram a infecção, demonstrando assim, a importância dessas células na eliminação do patógeno (FUNG-LEUNG *et al*., 1991).

Foi demonstrado, *in vitro,* que as células T CD8+ provenientes de camundongos perforina KO infectados com a cepa LCMV-Armstrong eram ativadas, porém não tinham capacidade de lisar fibroblastos infectados com o vírus. Isso sugeriu que o mecanismo pelo qual as células T CD8+ específicas para o LCMV matam as células alvo é via perforina (WALSH *et al*., 1994). Mais recentemente, um

trabalho utilizando camundongos triplamente deficientes (Fas, granzima A e granzima B) infectados demonstrou que a perforina atua juntamente com o Fas (RODE *et al*., 2004). Já camundongos deficientes de granzima B foram capazes de eliminar o vírus, porém essa eliminação foi mais lenta do que a observada em camundongos selvagens. Isso sugeriu que outros mecanismos compensaram a ausência da granzima (ZAJAC *et al*., 2003).

Diversos trabalhos demonstraram que a transferência adotiva de células T citotóxicas específicas para o vírus influenza A conferiu proteção completa para camundongos infectados com doses letais do vírus, demonstrando dessa forma, que a citotoxicidade mediada por células T é um importante mecanismo antiviral na infecção experimental pelo vírus influenza (LUKACHER *et al*., 1984 e TAYLOR & ASKONAS, 1986).

No caso do vírus da hepatite de camundongos, a depleção de células T CD8+ de animais C57BL/6 infectados com a cepa JHM impediu a eliminação do vírus no sistema nervoso central, diferentemente do observado em camundongos selvagens (WILLIAMSON & STOHLMAN, 1990). Da mesma forma, a depleção de células T CD8+, juntamente com a de células T CD4+, impediu a eliminação do herpes vírus na infecção cutânea. O mecanismo pelo qual essas células eliminaram o vírus parece ser mediado por IFN-γ, uma vez que o tratamento com anti-IFN-γ também impediu a eliminação do mesmo (SMITH *et al*., 1994).

### 3.2. Infecções bacterianas.

Além de ter um papel essencial nas infecções virais experimentais, as células T CD8+ também têm um papel importante na proteção contra bactérias intracelulares.

Roberts *et al*. (1993) demonstraram que camundongos selvagens infectados com *Listeria monocytogenes* são capazes de controlar a infecção e eliminar a bactéria. Entretanto, animais β2-microglobulina KO são capazes de controlar a infecção, porém não são capazes de eliminar o patógeno, demonstrando assim a importância das células T CD8+ na eliminação da bactéria. Além disso, a transferência de linhagens de células T CD8+ específicas para o epítopo $LLO_{91-99}$, geradas *in vitro*, foi capaz de induzir proteção significativa em camundongos BALB/c infectados com *Listeria monocytogenes* (HARTY & BEVAN, 1992).

Na infecção com *Mycobacterium tuberculosis* virulenta, um estudo demonstrou que enquanto os camundongos selvagens sobreviveram à infecção, os β2-microglobulina KO morreram entre a 4ª e 10ª semana após infecção. Diferentemente dos selvagens, os camundongos β2-microglobulina KO apresentaram necrose pulmonar e os granulomas presentes no pulmão apresentaram maior número de bacilos que o dos selvagens. A imunização com BCG conferiu uma proteção inicial contra o desafio com a cepa virulenta nos camundongos β2-microglobulina KO, como refletido pelo aumento no tempo de sobrevivência de 3-4 semanas. Entretanto, isso não foi suficiente para impedir que os animais morressem (FLYNN *et al*., 1992).

O aumento da susceptibilidade dos camundongos β2-microglobulina KO também foi observado após infecção com uma cepa avirulenta de *Salmonella typhimurium* (LO *et al*., 1999). Entretanto, como revisto por Mittrucker and Kaufmann (2000), foi demonstrado que camundongos β2-microglobulina KO controlaram a infecção contra uma cepa atenuada de *S. typhimurium* de forma semelhante aos animais selvagens controle.

**3.3. Infecções parasitárias em geral.**

Em meados da década de oitenta, foi demonstrado que a imunidade induzida em camundongos vacinados com esporozoítas de *Plasmodium* irradiados era dependente da ativação de linfócitos T CD8+ (SCHOFIELD *et al*., 1987 e WEISS *et al*., 1988).

A identificação de epítopos da proteína CS de *P. berghei* e *P. yoelli* permitiu a geração de clones de células T CD8+. Esses clones gerados *in vitro*, caracterizados como células $Thy1^+$ $TCR\alpha\beta^+$ $CD4^-$ $CD8^+$, apresentaram forte atividade lítica contra células alvo cobertas por peptídeos da malária. Além disso, essas células produziram grande quantidade de IFN-γ, TNF-α e BLT-esterase (enzima N-carbozybenzoxy-L-lysine thiobenzil ester) quando estimuladas pelo antígeno *in vitro.* A transferência adotiva desses clones para camundongos selvagens protegeu completamente contra o desafio com esporozoítas (revisto por MORROT & ZAVALA, 2004).

Como revisto por Rodrigues *et al*., 2003, a importância dos linfócitos T CD8+ durante a infecção pelo *Toxoplasma gondii* foi demonstrada inicialmente pela

capacidade de células T CD8+, provenientes de animais imunes, serem capazes de proliferar, produzir citocinas, lisar células infectadas e bloquear a multiplicação do parasita *in vitro*. Em adição, a depleção dessa sub-população de células em animais vacinados com a cepa atenuada aboliu parcialmente a resistência à infecção com uma cepa altamente virulenta. Finalmente, a transferência adotiva de clones de células T específicos para um antígeno de *T. gondii* induziu imunidade protetora contra a infecção letal.

Ainda hoje, há controvérsias a respeito da via de ativação das células T CD8+ na leishmaniose, uma vez que este parasita reside dentro de vacúolos parasitóforos de macrófagos. Estudos recentes sugerem que antígenos de *Leishmania* são processados por uma via independente de TAP/proteossomos (BERTHOLET *et al*., 2006). Porém, diversos trabalhos já demonstraram que as células T CD8+ têm sido associadas com proteção e cura tanto em pacientes infectados como em camundongos (revisto por RUIZ & BECKER, 2007).

Em camundongos infectados com baixa dose de promastigotas de *L. major* foi demonstrado que as células T CD8+ são cruciais no controle da infecção primária por mudar a resposta do tipo Th2 para Th1. Em pacientes, um estudo mostrou que lesões de indivíduos com leishmaniose cutânea local, infectados com *L. braziliensis,* exibiram um grande número de linfócitos T CD8+ apoptóticos, enquanto que pacientes que sofreram cura espontânea apresentaram poucas células T CD8+ apoptóticas. Estudos *in vitro* apontam a secreção de IFN-γ como principal mecanismo de eliminação das formas amastigotas por linfócitos T CD8+ (BASU *et al*., 2007). Além disso, foi demonstrado que os linfócitos T CD8+ específicos para *Leishmania* foram capazes de lisar macrófagos infectados (revisto por RUIZ & BECKER, 2007). Já foi demonstrado que a citotoxicidade e o IFN-γ trabalham conjuntamente na eliminação de amastigotas, num modelo de infecção *in vitro* com *L. major,* por linfócitos T CD8+ (SMITH *et al*., 1991).

### 3.4. Doença de Chagas.

O agente etiológico da doença de Chagas é o *Trypanosoma cruzi* (CHAGAS, 1909). Este, protozoário intracelular obrigatório pertencente à ordem Kinetoplastida, família Trypanosomatidae, é um parasita digenético e apresenta um complexo ciclo de vida possuindo um hospedeiro vertebrado (diversos mamíferos) e um

invertebrado, o inseto vetor (triatomíneo hematófago, pertencente à família Reduviidae).

Ao picar o indivíduo, o triatomíneo defeca próximo ao sítio da picada. Nas fezes e urina do inseto encontram-se as formas tripomastigotas metacíclicas que penetram pela lesão causada no momento em que o indivíduo começa a coçar a região. Uma vez no hospedeiro vertebrado, os tripomastigotas metacíclicos invadem as células das camadas subjacentes à pele e, no citoplasma dessas células, diferenciam-se em amastigotas que se multiplicam por divisão binária. Os amastigotas diferenciam-se em tripomastigotas sanguíneos, os quais com a lise da célula hospedeira são liberados na corrente sanguínea de onde se espalham para os diferentes órgãos e tecidos. Se o hospedeiro vertebrado for novamente picado por um inseto triatomíneo, este ingere as formas tripomastigotas sanguíneas, reiniciando, desta forma, o ciclo.

A doença de Chagas atinge os países pertencentes às Américas do Sul, Central e Norte (México). Mais de 10 milhões de indivíduos encontram-se infectados na fase crônica da doença. Milhares de novos casos também são relatados anualmente. Graças a iniciativas regionais de combate ao vetor triatomíneo na América Latina, o número de casos novos por transmissão vetorial foi drasticamente reduzido nos últimos 20 anos, nos países do cone sul (Uruguai, Argentina, Chile e Brasil – revisto por SCHOEFIELD, 2006). Entretanto, uma epidemia foi recentemente relatada em cães, no sul dos Estados Unidos, demonstrando o perigo que a doença de Chagas ainda representa, mesmo em regiões onde não é considerada um problema grave de saúde pública (KJOS *et al*., 2008).

A doença de Chagas é caracterizada por apresentar um curso clínico variável, que incluiu uma fase inicial aguda, que pode ser assintomática ou sintomática, com febre, adenomegalia, conjuntivite unilateral (Sinal de Romaña), parasitemia no sangue periférico, miocardite e meningoencefalite. A fase inicial pode ser fatal em até 10% dos casos mais graves, com alta mortalidade em crianças abaixo de três anos de idade em conseqüência da meningoencefalite (COURA, 2003).

Após alguns meses, a parasitemia se torna sub-patente e há o estabelecimento da fase denominada crônica. Esses indivíduos, na sua maioria, continuam com anticorpos específicos, e em grande parte deles, os parasitas podem ser isolados por hemocultura ou por xeno-diagnóstico. Raramente, indivíduos

eliminam completamente os parasitas. Essa fase, denominada crônica, permanece por toda a vida do indivíduo e o DNA parasitário pode ser detectado, por PCR, a partir da necrópsia de pacientes, determinando que o *T. cruzi* muito possivelmente se manteve no indivíduo por até a sua morte (JONES *et al*., 1993).

Na fase crônica, após muitos anos de contato com o parasita, 27% dos indivíduos infectados desenvolvem sintomas cardíacos, levando à morte, 6% desenvolvem danos digestivos e 3% podem apresentar envolvimento do sistema nervoso periférico (WHO, 2006). Acredita-se hoje que a persistência do parasita é fator fundamental para o desenvolvimento da patologia chagásica.

O tratamento com as drogas nifurtimox (Lampit®), introduzida em 1967 pela Bayer e benzonidazole (Rochagan®, Radanil®) tem uma eficiência em parte das crianças infectadas. Nestes casos, os indivíduos se tornam sorologicamente negativos e não apresentam sintomas da doença. Entretanto, o quadro é bem mais complicado em adultos cronicamente infectados. Nestes, só uma parcela reduzida (~30%) responde ao tratamento, se tornando negativos sorologicamente (PEREIRA-CHIOCCOLA *et al*., 2003). Não se sabe, ao certo, quais os fatores que impedem o tratamento eficiente da maioria dos pacientes. Há a descrição de cepas que são resistentes às drogas, o que seria a explicação mais plausível para a falha no tratamento.

A importância dos linfócitos T CD8+ na infecção experimental pelo *T. cruzi* foi demonstrada através da infecção de camundongos geneticamente deficientes (CD8KO, β-2 microglobulina KO) ou tratados com anticorpos anti-CD8, os quais apresentaram parasitemia e susceptibilidade aumentada em relação aos controles (TARLETON *et al*., 1992; ROTTENBERG *et al*., 1993 e TARLETON, 1990). Os mecanismos antiparasitários mediados por essas células são múltiplos, incluindo secreção de citocinas e citotoxicidade direta contra células infectadas (MARTIN & TARLETON, 2004; MULLER *et al*., 2003, TZELEPIS *et al*., 2006).

Em 2007, Cardillo *et al*. sugeriram que os linfócitos B têm um importante papel na geração e manutenção de células T CD8+ efetoras/memória. Foi observado que a infecção, com tripomastigotas da cepa Tulahuén de *T. cruzi,* de camundongos deficientes de linfócitos B resultou em um número total de células T CD8+ menor que nos animais controle, menor produção de citocinas inflamatórias (IFN-γ e IL-12) e menor infiltrado inflamatório, o qual também apresentou menor

porcentagem de células T CD8+ ativadas/memória. Um maior parasitismo nos tecidos e maior parasitemia também foram observados.

Tanto as formas amastigotas como as tripomastigotas são alvos da resposta de linfócitos T CD8+. No fim da década de noventa, foi demonstrado que as proteínas ASP-1 (amastigote surface protein-1) e ASP –2 (amastigote surface protein 2), presentes na forma amastigota, e a TSA-1 (trypomastigote surface antigen 1) e TCTS (*trans*-sialidase de *T. cruzi*), presentes nas formas tripomastigotas, continham epítopos reconhecidos por células T CD8+ restritas por MHC-Ia (LOW *et al*., 1998; WIZEL *et al*., 1997; RODRIGUES *et al*., 1999).

Apesar de descrita na década passada, os epítopos alvos desta resposta imune mediada por linfócitos T CD8+, durante a infecção, eram desconhecidos até recentemente. Este fato também impedia que se estudasse a cinética do aparecimento dessas células durante a infecção. Trabalhos recentes, feitos pelo nosso grupo e por outro laboratório, finalmente descreveram epítopos reconhecidos por linfócitos T CD8+ durante a infecção natural com parasitas de diferentes cepas de *T. cruzi,* em camundongos de diferentes linhagens (TZELEPIS *et al*., 2006; TZELEPIS *et al*., 2007; MARTIN *et al*., 2006). Os epítopos descritos, reconhecidos por linfócitos T CD8+ de camundongos da linhagem C57BL/6 infectados com parasitas da cepa Y ou G de *T. cruzi* são o VNHRFTLV (da Proteína 2 da Superfície de amastigotas), TsKb-18 (ANYDFTLV, do antígeno TSA) e TsKb-20 (ANYKFTLV do antígeno TSA) (TZELEPIS *et al*., 2006; TZELEPIS *et al*., 2007; MARTIN *et al*., 2006, TZELEPIS *et al*., 2008). Por outro lado, no caso de camundongos C57BL/6 infectados com as cepas CL e Brazil foram observadas respostas aos epítopos TsKb-18 (ANYDFTLV) e TsKb-20 (ANYKFTLV), mas não ao epítopo VNHRFTLV (MARTIN *et al*., 2006, TZELEPIS *et al*., 2008). Os animais BALB/c, infectados com a cepa Y, reconhecem o epítopo da *trans*-sialidase (TS) IYNVGQVSI e os camundongos B10.A, infectados com essa mesma cepa, reconhecem o epítopo TEWETGQI (TZELEPIS *et al*., 2006, TZELEPIS *et al*., 2008).

A descrição desses epítopos permitiu o estudo da cinética do aparecimento das células T CD8+ específicas capazes de mediar atividade citotóxica *in vivo*, assim como a de células T CD8+ específicas capazes de produzir IFN-γ *ex vivo,* durante a infecção experimental (TZELEPIS *et al*., 2006).

A importância da resposta imune mediada por linfócitos T CD8+, na resistência à infecção pelo *T. cruzi,* fez com que vários grupos iniciassem uma busca pela indução dessas células específicas e a possibilidade de vacinação contra a infecção experimental. De fato, a imunização de camundongos, com diferentes protocolos capazes de induzir linfócitos T CD8+ específicos contra alguns dos epítopos descritos acima conferiu uma proteção contra a infecção letal. Esses protocolos incluem vacinação com DNA plasmidial, vetores virais, e proteínas recombinantes (COSTA *et al*., 1998, WIZEL *et al*., 1998, SEPULVEDA *et al*., 2000; VASCONCELOS *et al*., 2004; FUJIMURA *et al*., 2001, GARG & TARLETON, 2002, KATAE *et al*., 2002, FRALISH & TARLETON, 2003, BOSCARDIN *et al*., 2003, DUMONTEIL *et al*., 2004, ARAÚJO *et al*., 2005; MIYAHIRA *et a.l*, 2005, MACHADO *et al*., 2006; HOFT *et al*., 2007; de ALENCAR *et al*., 2007, SANCHEZ-BURGOS *et al*., 2007). Em conjunto, esses trabalhos reforçam a idéia de um papel essencial para as células T $CD8^+$ no controle da infecção experimental pelo *T. cruzi*.

**4. Imunodominância na resposta imune mediada por linfócitos T CD8+.**

Como já mencionado, linfócitos T CD8+ são um componente crítico na imunidade contra patógenos intracelulares. Embora os patógenos apresentem milhares de possíveis epítopos, a resposta de células T CD8+ é focada em apenas poucos. O foco da imunidade celular para estes epítopos também ocorre durante a resposta imune contra microorganismos complexos e é denominado imunodominância. Apesar de estabelecida experimentalmente, o significado evolutivo e implicações biológicas da imunodominância da resposta imune ainda estão longe de serem compreendidos.

Para melhorar a compreensão desse fenômeno básico da resposta imune, estudos sobre imunodominância estão sendo realizados após infecções com diferentes patógenos. Até pouco tempo, estudos mais detalhados só podiam ser feitos em vírus com genomas relativamente pequenos. Entretanto, mais recentemente, os estudos de imunodominância realizados em vírus com genomas maiores, como o citomegalovírus e vaccinia, têm sido executado com sucesso (TSCHARKE *et al*., 2005, MUNKS *et al*., 2006, MOUTAFTSI *et al*., 2006, revisto por YEWDELL 2006).

O estudo da imunodominância durante a resposta imune contra um patógeno complexo como o *T. cruzi* foi inicialmente considerado difícil devido ao grande número de proteínas potencialmente expressas por este. Embora o parasita apresente pelo menos doze mil genes (EL SAYED *et al*., 2005), trabalhos recentes sugerem que a resposta de células T CD8+ seja altamente focada em poucos epítopos expressos por diferentes membros da superfamília das *trans*-sialidases (TZELEPIS *et al*., 2006; MARTIN *et al*., 2006, TZELEPIS *et al*., 2007; TZELEPIS *et al*., 2008).

Devido a sua simplicidade estrutural, o patógeno mais bem estudado até o momento, do ponto de vista da imunodominância da resposta imune mediada por linfócitos T CD8+, é o vírus influenza, causador da gripe. Neste modelo, o padrão de imunodominância observado em camundongos BALB/c, infectados com a cepa PR8, é caracterizado pela resposta imune contra os epítopos ilustrados na figura 5, todos restritos pelo MHC-Ia H-2K$^d$. Este modelo, relativamente simples, permitiu a análise dos possíveis mecanismos responsáveis pelas discrepâncias nas freqüências de linfócitos T específicos, observados durante a resposta imune, e foi ilustrativo em demonstrar seu caráter multi-fatorial (CHEN *et al*., 2000).

Após a infecção, o epítopo ***NP147*** é imunodominante, pois apresenta o maior número de linfócitos T CD8+ específicos. A análise detalhada dos diferentes passos do processo de apresentação desse antígeno revelou que este epítopo não é o mais abundante no citoplasma da célula infectada, nem apresenta o mais lento "Off rate" após a ligação ao H-2K$^d$, e seu processamento e liberação para o RE é apenas melhor do que a média. Sua imunodominância, só pode assim ser explicada, pelo maior número de precursores específicos, ou pela melhor eficiência destes na multiplicação ou capacidade de exercer imuno-dominação sobre os demais clones.

O epítopo ***HA518***, o segundo mais reconhecido, apresenta o segundo mais lento "Off rate" após a ligação ao H-2K$^d$. Apesar de apresentar quantidade intermediária no citoplasma da célula infectada e aumento de disponibilidade, isso pouco influencia a resposta imunológica. Assim, sua limitação fundamental parece ser o restrito número de precursores de linfócitos T CD8+ específicos disponíveis.

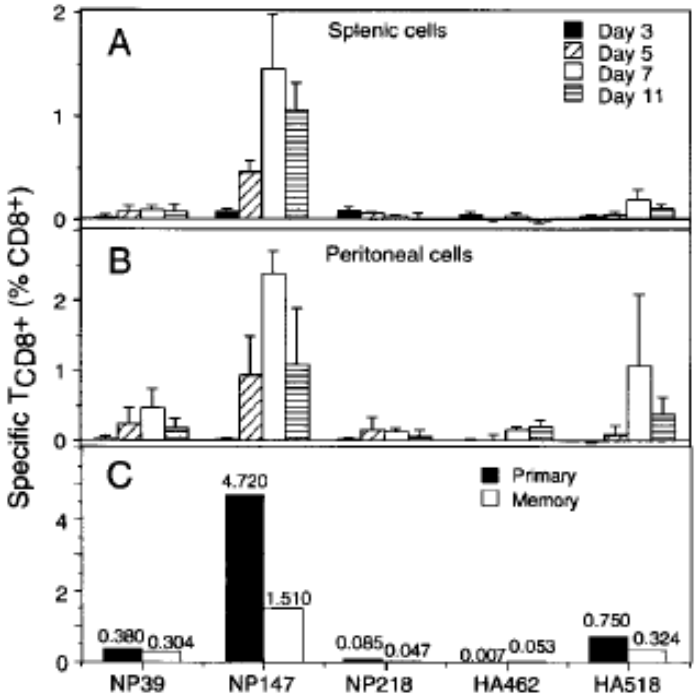


**Figura 5 – Hierarquia da resposta restrita ao H-2K$^d$ de camundongos BALB/c infectados com PR8**

O epítopo ***NP39*** é um exemplo interessante de falta adequada de processamento antigênico. Seu "Off rate", após a ligação ao H-2K$^d$, é o mais lento e na presença de epítopo já processado, sua resposta se eleva ao nível do epítopo imunodominante ***NP147***. Demonstrando assim, a presença de grande número de precursores de linfócitos T CD8+ específicos, que só não são ativados, pela limitação do complexo peptídeo-MHC-Ia na superfície da APC após a infecção por influenza.

O epítopo ***NP218*** apresenta a menor afinidade para a molécula de MHC-Ia, a qual correlaciona com sua baixa imunogenicidade. Por fim, o epítopo ***HA462*** é um exemplo de que mesmo sendo o epítopo mais abundante no citoplasma da célula e produzindo o maior número de complexos peptídeo-MHC-Ia na superfície da APC infectada, apresenta a resposta imune mais fraca. Isso significa, também, ausência de significante número de precursores de linfócitos T CD8+ específicos.

Pela simplicidade, esse modelo gerou as regras principais que influenciam a geração de imunodominância entre peptídeos restritos pelo mesmo MHC-Ia. A primeira e mais importante regra é uma afinidade mínima entre o peptídeo e a molécula de MHC-Ia. A importância deste fator levou a construção de modelos

teóricos de ligação que podem guiar o mapeamento de epítopos CD8. A segunda regra é a disponibilidade de antígeno e seu processamento no citoplasma da APC. A terceira regra é a presença de linfócitos T específicos no repertório de linfócitos T CD8+. Esta última pode ser influenciada pelo número, pela afinidade e pela capacidade proliferativa desses precursores. Por último, pode haver a competição de diferentes clones de linfócitos T CD8+ (imunodominação).

Diversos trabalhos, publicados recentemente, re-enfatizaram a importância desses mecanismos de geração de imunodominância específicos para um determinado alelo utilizando, por exemplo, vírus influenza recombinantes. No modelo de infecção com o vírus influenza A, a comparação da resposta de células T CD8+ específicas para $D^bNP_{366}$ e $D^bPA_{224}$ demonstrou que no desafio secundário, a resposta específica para $D^bNP_{366}$ é maior que a resposta específica para $D^bPA_{224}$. Vírus recombinantes contendo os peptídeos $NP_{366}$ ou $PA_{224}$ inseridos na neuraminidase viral (NA) foram construídos, tal que as diferenças na apresentação dos epítopos $D^bNP_{366}$ e $D^bPA_{224}$ foram eliminadas. Foi mostrado que no desafio primário e secundário a carga de antígeno, assim como o número de células T precursoras teve um papel importante em determinar a hierarquia de imunodominância (La GRUTA *et al*., 2006).

Apesar dos diferentes mecanismos descritos acima serem, possivelmente, os mais freqüentes responsáveis pela imunodominância da resposta imune, o mais potente mecanismo parece ser a competição entre células T específicas para diferentes complexos peptídeo-MHC-Ia. Durante este tipo de competição, a resposta imunodominante de uma célula T CD8+, específica para um determinado epítopo, "interfere" na expansão da resposta imune de outras células T CD8+, específicas para quaisquer outros epítopos ligados a quaisquer outras moléculas de MHC-Ia, gerando assim forte padrão de imunodominância.

Este mecanismo que age impedindo a expansão de linfócitos T CD8+ de diferentes especificidades foi demonstrado em diferentes modelos de imunizações experimentais (WILLIS *et al*., 2006; VAN DER MOST *et al*., 2003; GRUFMAN *et al*., 1999; KEDL *et al*., 2000; KEDL *et al*., 2002). Sua importância durante infecções é ainda motivo de controvérsia. Por um lado, há trabalhos que demonstram que epítopos subdominantes podem tornar-se imunodominantes através da eliminação do epítopo dominante (VAN DER MOST *et al*., 2003) ou que na ausência do epítopo

imunodominante, a resposta global de linfócitos T CD8+ se encontra somente um pouco reduzida (STOCK *et al*., 2006). Na mesma linha, há evidências de que no caso da infecção pelo vírus influenza, respostas imunes compensatórias se desenvolvem na ausência de epítopos imunodominantes (THOMAS e*t al*., 2007).

Por outro lado, um estudo com *Listeria monocytogenes* demonstrou que a eliminação dos epítopos imunodominantes não altera a resposta aos epítopos subdominantes (VIJH *et al*., 1999) e na infecção com o vírus LCMV, interferência não foi observada em distintos enfoques experimentais (BUTZ & BEVAN, 1998, PROBST *et al*., 2002).

Para tornar esta matéria ainda mais desafiante, um trabalho recente demonstrou que a competição entre células T de diferentes especificidades pode ocorrer durante a resposta imune secundária, após infecção com o vírus vaccinia, mesmo quando esta não ocorre durante a resposta imune primária (KASTENMULLER *et al*., 2007). Neste caso, os autores correlacionaram a competição cruzada com a expressão precoce do gene do antígeno imunodominante na resposta imune secundária. De qualquer forma, a competição de linfócitos T CD8+ durante a resposta imune a diferentes patógenos ainda é assunto em debate.

**5. Implicações do estudo de linfócitos T CD8+.**

Os conhecimentos básicos adquiridos sobre a resposta imune mediada pelos linfócitos T CD8+ podem representar passos importantes para a compreensão da imunopatologia das doenças agudas e crônicas e para o tratamento e prevenção de doenças (infecciosas e cânceres).

Do ponto de vista da prevenção de doenças infecciosas causadas por vírus, bactéria e parasitas intracelulares, até hoje não há nenhuma vacina comercial baseada na indução de linfócitos T CD8+. Entretanto, já foi visto que algumas das vacinas vivas atenuadas são capazes de induzir linfócitos T CD8+ específicos (varíola, BCG, adenovírus, gripe, etc.). Baseados na possibilidade de se induzir esse tipo de imunidade, inúmeros estudos vêm sendo realizados, principalmente em modelos experimentais, para o desenvolvimento dessas vacinas preventivas. Devido ao volume de trabalho realizado nesta área nos últimos 10 anos, seria impossível

citar todos os trabalhos feitos em modelos experimentais para os diversos patógenos. No caso específico do *Trypanosoma cruzi,* já foram citados anteriormente diversos trabalhos utilizando proteínas, DNA e vírus recombinantes com intuito de vacinação experimental.

Os resultados obtidos em modelos experimentais, também chamados de testes pré-clínicos, serviram de base para os "clinical trials" ou testes clínicos no homem. Vacinas recombinantes, visando à indução de imunidade mediada por linfócitos T CD8+, estão sendo testadas, por exemplo, em associação com adjuvantes sintéticos como o Iscomatrix (revisto por DRANE *et al.,* 2007), como vírus recombinantes (revisto por YANG *et al.,* 2007) ou como bactérias atenuadas (revisto por SZTEIN, 2007). Alternativamente, outros grupos estão usando a estratégia denominada "priming-boosting" heterólogo, no qual dois imunonógenos distintos, contendo o mesmo epítopo CD8, são utilizados seqüencialmente, visando uma maior expansão dos clones de linfócitos T CD4+ e CD8+ contra os transgenes, muito acima do que é obtido com doses seqüenciais dos mesmos antígenos (revisto por SANDER & MCSHANE, 2007). Este tipo de estratégia foi demonstrada primeiro em modelos experimentais e, posteriormente, no homem, como sendo capaz de induzir uma resposta imune celular consideravelmente mais alta (MCSHANE *et al.*, 2004).

Assim como as vacinas profiláticas, dezenas de vacinas terapêuticas estão sendo testadas contra as mais diversas formas de cânceres em modelos experimentais. Estas vacinas são constituídas também de proteínas, plasmídeos e vírus recombinantes, além de peptídeos sintéticos. Algumas dessas formulações já foram selecionadas para testes clínicos em pacientes com essa enfermidade. Os testes clínicos, ainda em fase I, têm demonstrado que é possível induzir resposta imune específica mediada por linfócitos T CD8+, em pacientes vacinados com peptídeos sintéticos ou proteína recombinante emulsificados em adjuvantes comerciais, na presença de um ativador de TLR-9 (SPEISER *et al.*, 2005, SPEISER *et al.*, 2008, VALMORI *et al.*, 2007).

Além da vacinação de pacientes com proteínas, plasmídeos, vírus recombinantes etc., uma linha que está sendo desenvolvida em muitos laboratórios é a de vacinação com DC pulsadas com antígenos específicos. Recentemente, SANTIN *et al.*, 2008 obtiveram sucesso na indução de imunidade contras o antígeno

E7 do vírus do papiloma humano usando esta estratégia. Esse tipo de vacinação, apesar de pouco prático e caro, pode vir a se tornar uma alternativa para o tratamento de determinados cânceres.

Além da vacinação, a transferência adotiva de linfócitos T CD8+ específicos já vem sendo usada em testes clínicos no homem. Em algumas circunstâncias, respostas objetivas de regressão de tumores foram descritas (MORGAN *et al*., 2006 e revisto por GATTINONI *et al*., 2006 e BLATTMAN & GREENBERG, 2004).

Durante doenças virais crônicas, um estado de exaustão é observado nos linfócitos T CD8+ específicos, o qual impede a completa eliminação viral. Pela comparação dos genes expressos nestas células exauridas com linfócitos T CD8+ de memória, observou-se que as primeiras expressavam altos níveis de PD-1, um receptor inibitório da família CD28 e receptor para a molécula PD-L1. O bloqueio da interação PD-1/PD-L1 foi capaz de restaurar a função dessas células CD8+. Assim sendo, elas voltaram a apresentar capacidade de proliferação, secreção de citocinas e citotoxicidade, levando a uma diminuição significativa na carga viral (BARBER *et al*., 2006). Este trabalho apresentou uma nova estratégia alvo para o desenvolvimento de tratamentos. A importância do PD-1 foi confirmada pela presença deste receptor em linfócitos T CD8+ específicos para antígenos de HIV (DAY *et al*., 2006, PETROVAS *et al*., 2006) e hepatide C (URBANI *et al*., 2006, GOLDEN-MASON *et al*., 2008). Futuros tratamentos com moléculas capazes de bloquear a interação PD-1/PD-L1 poderão melhorar o desempenho funcional destes linfócitos T CD8+ em estado de exaustão, promovendo a melhora dos indivíduos. Bem recentemente, foi observado que no modelo experimental da infecção crônica por LCMV, a vacinação terapêutica com um vírus vaccinia recombinante pode ser melhorada pelo bloqueio combinado da ligação PD-1/PD-L1 (HA *et al*., 2008).

# *OBJETIVOS*

Apesar de ser bem estabelecido o papel dos linfócitos T CD8+ na imunidade protetora contra a infecção causada pelo *T. cruzi,* pouco se sabia da especificidade, cinética de expansão de células T CD8+ específicas e os possíveis mecanismos de geração de imunodominância. Assim sendo, os nossos objetivos foram:

1- Determinar a especificidade dos linfócitos T CD8+ na infecção por diferentes cepas de *T. cruzi*;

2- Analisar a cinética da resposta imune mediada por linfócitos T CD8+ após a infecção por *T. cruzi* em camundongos naïve ou vacinados;

3- Determinar alguns dos possíveis fatores que controlam a cinética da ativação das células T CD8+ específicas;

4- Entender os possíveis mecanismos de imunodominância durante a infecção pelo *T. cruzi*.

## ***ARTIGOS PUBLICADOS***

# *ARTIGO 1*

## Resumo 1

As células T CD8+ restritas para MHC classe I são críticas para a sobrevivência de camundongos selvagens e imunizados, infectados com o parasita *Trypanosoma cruzi*. Apesar da importância delas para a resistência do hospedeiro, havia somente limitada informação sobre a cinética das células T CD8+ efetoras após a infecção. O objetivo deste estudo foi a caracterização da cinética da resposta de células T CD8+ efetoras específicas para epítopos presentes na *trans*-sialidase ou na proteína 2 da superfície de amastigota (ASP-2). Essas respostas foram avaliadas em camundongos BALB/c ou C57BL/6 infectados, através da monitoração da atividade citotóxica *in vivo* e pela detecção de células produtoras de IFNγ *ex vivo* (ELISPOT). Essas atividades efetoras das células T tornaram-se detectáveis somente 9 dias após a infecção. Elas atingiram uma resposta máxima na segunda semana, permaneceram elevadas de 30 a 60 dias e declinaram lentamente após esse período. A atividade máxima das células T em camundongos selvagens ocorreu no mesmo tempo em que camundongos geneticamente deficientes para IFN-γ, CD8 ou perforina morreram. Essa cinética de resposta em camundongos selvagens diferiu daquelas observadas em outras infecções virais, bacterianas ou parasitárias. Em contraste, camundongos vacinados com o gene *asp-2* ou com uma proteína recombinante baseada no antígeno ASP-2 e desafiados com parasitas, exibiram uma resposta de células T CD8+ anamnéstica rápida e de grande magnitude, a qual correlacionou com a imunidade protetora. Nossos resultados sugeriram que a cinética lenta de expansão de células T CD8+ específicas permite o estabelecimento da infecção nos camundongos selvagens. Em contraste, em camundongos vacinados, a aceleração na resposta de células T CD8+ ajuda a sobrevivência do hospedeiro após o desafio.

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# Distinct Kinetics of Effector CD8$^+$ Cytotoxic T Cells after Infection with *Trypanosoma cruzi* in Naïve or Vaccinated Mice

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**The kinetics of effector CD8$^+$-T-cell responses to specific *Trypanosoma cruzi* epitopes was investigated after challenge. Our results suggest that the delayed kinetics differs from that observed in other microbial infections and facilitates the establishment of the disease in naïve mice. In contrast, in vaccinated mice, the swift CD8$^+$-T-cell response helps host survival after challenge.**

Major histocompatibility complex class Ia-restricted CD8$^+$ T cells are critical for the survival of naïve and vaccinated mice infected with the human protozoan parasite *Trypanosoma cruzi* (1, 5, 9, 11, 15, 16, 18). The antiparasitic mechanisms mediated by these cells are multiple, including cytokine secretion and possibly direct cytotoxicity against infected cells (6, 12, 13). In spite of their importance for host resistance, limited information is available regarding the kinetics of effector CD8$^+$ T cells following parasite challenge. Here, we describe studies aimed at characterizing the kinetics of effector CD8$^+$-T-cell responses specific to epitopes present in the *trans*-sialidase (TS) or the amastigote surface protein 2 (ASP-2), prime candidates for vaccine development against Chagas' disease (1, 4, 5, 18).

After infection with trypomastigotes of the Y strain, we observed that the parasitemia of wild-type (WT) C57BL/6, CD8α knockout (KO), gamma interferon (IFN-γ) KO, or perforin KO mice was not significantly different on day 8 after challenge (peak parasitemia of WT mice). From days 9 to 13, WT mice quickly reduced their parasitemia, and only 9% of them died after challenge (Fig. 1A and B). In contrast, IFN-γ KO mice were unable to control the parasitemia, dying faster than the other mouse groups. CD8α KO or perforin KO mice were unable to control their parasitemia at the same rate as WT animals, dying between days 15 and 21 after challenge (Fig. 1A and B). We concluded that IFN-γ secreted by non-CD8$^+$ cells is important until day 13. CD8$^+$ cells and perforin are critical for survival after day 14.

Based on the observations that CD8$^+$ cells, IFN-γ, and perforin are critical for mouse survival after challenge, we followed the kinetics of specific effector CD8$^+$ T cells by using the ex vivo enzyme-linked immunospot (ELISPOT) assay for IFN-γ (4) and the in vivo cytotoxicity assay, which measures the elimination of peptide-coated target cells mediated by perforin (3). Target cells were coated with synthetic peptides representing the CD8 T-cell epitope IYNVGQVSI (TS) or VNH RFTLV (ASP-2) (Table 1). The results of the in vivo cytotoxicity assay were obtained by measuring the carboxyfluorescein diacetate succinimidyl diester-labeled cells in the spleens of recipient mice (3). Identical results were seen when we analyzed labeled cells in lymph nodes (data not shown).

In BALB/c mice, the in vivo cytotoxicity and IFN-γ-secreting cells specific to the TS peptide IYNVGQVSI were first detected at the peak of parasitemia (day 9). Both T-cell activities rose quickly until the 15th day and were kept high until the 30th day after infection. During the subsequent period, they declined slowly but were still detectable by day 240 (Fig. 2A and B).

A similar picture emerged when we evaluated the kinetics of effector cells specific to the ASP-2 peptide VNHRFTLV. In C57BL/6 mice, both T-cell activities were not detectable at the peak of parasitemia (day 8). They rose quickly until the 16th day and were kept high until the 60th day after infection. During the subsequent period, both activities declined but were still detectable by day 240 (Fig. 2C and D).

Treatment of *T. cruzi*-infected BALB/c or C57BL/6 mice with anti-CD8 monoclonal antibody resulted in a selective depletion of splenic CD8$^+$ T cells in vivo (>98.6%) and a complete reversion of the in vivo cytotoxicity response to the peptide IYNVGQVSI or VNHRFTLV (Fig. 3A and B, respectively).

TABLE 1. Peptides used in the present study

| Peptide | Antigen | aa[a] | MHC[b] restriction | Form of the parasite | Reference |
|---|---|---|---|---|---|
| IYNVGQVSI | TS | 359–367 | *H-2K$^d$* | Trypomastigote | 14 |
| VNHRFTLV | ASP-2 | 553–560 | *H-2K$^b$* | Amastigote | 8 |
| TEWETGQI | ASP-2 | 320–327 | *H-2K$^k$* | Amastigote | 1 |

[a] aa, amino acids.
[b] MHC, major histocompatibility complex.

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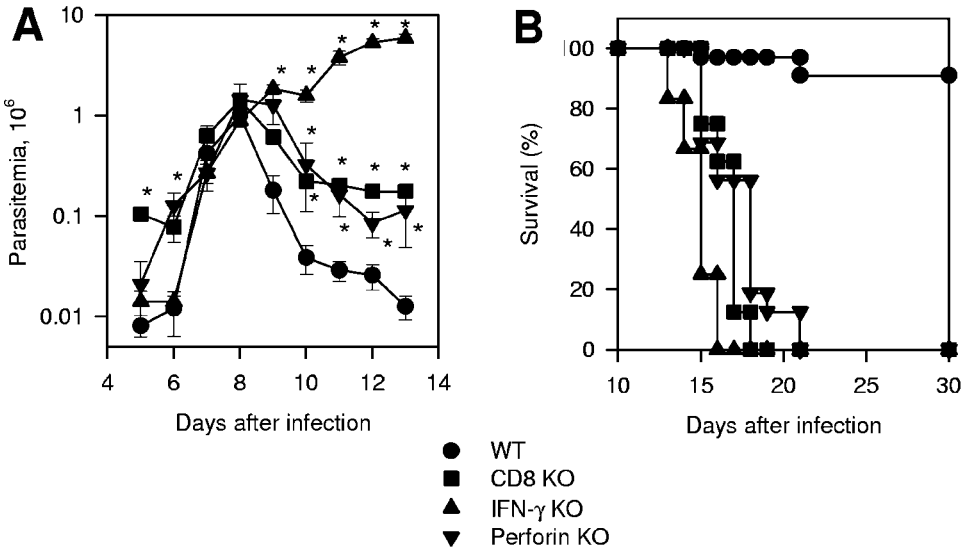



FIG. 1. Trypomastigote-induced parasitemia and mortality in WT, CD8α KO, IFN-γ KO, or perforin KO mice. Groups of mice were infected intraperitoneally with $10^4$ bloodstream trypomastigotes of the Y strain of *T. cruzi*. (A) Parasitemia was followed daily from days 5 to 13 after challenge. The results represent the mean of five or six mice ± standard deviation. The asterisks denote values statistically higher than the values in control WT mice ($P < 0.05$; one-way analysis of variance). The results are representative of two independent experiments. (B) Kaplan-Meier curves for survival of each mouse group: (i) WT, $n = 32$; (ii) CD8α KO, $n = 8$; (iii) IFN-γ KO, $n = 12$; (iv) perforin KO, $n = 16$. The results were pooled from two different experiments. Statistical analysis revealed significant differences in the survival of WT mice compared to the other mouse groups ($P < 0.0001$ in all cases; log rank test). CD8α KO and perforin KO mice survived longer than IFN-γ KO ($P < 0.01$ in both cases).

The phenotypes of splenic IFN-γ-secreting cells were determined by sorting, with the aid of a FACS Vantage (Becton Dickinson), $CD8^+$ cells costained with antibodies to CD62L (anti-CD8-Pe-CY5, clone 53-67, and anti-CD62L-FITC, clone Mel14; BD-Pharmingen) from C57BL/6 mice infected 80 days earlier. ELISPOT assays performed with these purified cells demonstrated that splenic IFN-γ-secreting cells were $CD8^+$ $CD62L^{Low}$ (Fig. 4). Similar results had been described earlier for mice infected with *T. cruzi* (Brazil strain) for more than 150 days (10).

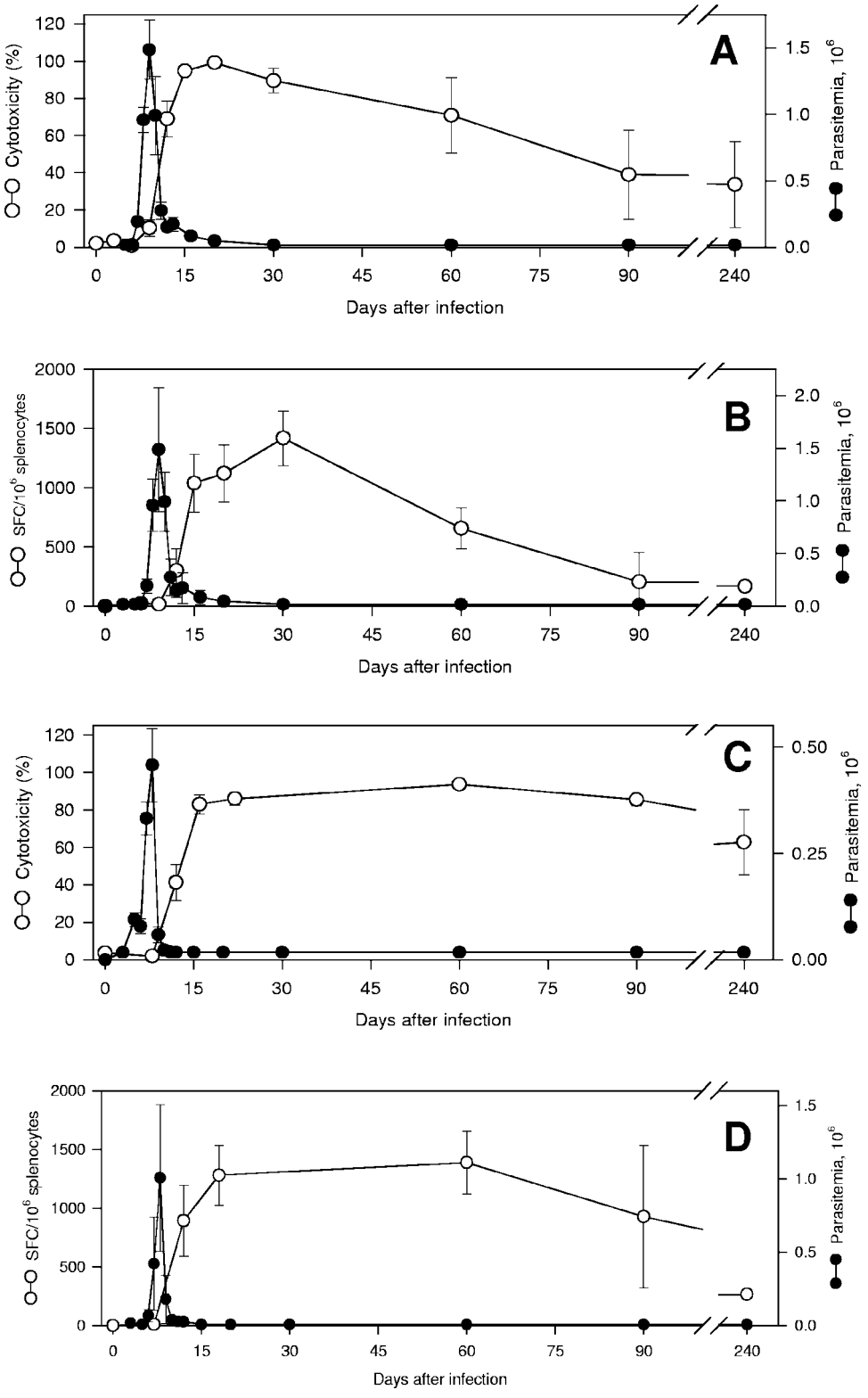
Finally, we compared the kinetics of effector $CD8^+$-T-cell expansion following infection of immune or naïve animals. C57BL/6 or A/Sn mice were immunized with the plasmid pIgSPclone 9 (*asp-2* gene) or with the recombinant protein gluthatione *S*-transferase (GST)-P4-P7, respectively (1, 4). After challenge, mice vaccinated with plasmid pIgSPclone9 (C57BL/6) or with the recombinant protein GST-P4-P7 (A/Sn) quickly developed in vivo specific cytotoxic cells against target cells coated with the peptide VNHRFTLV or TEWETGQI, respectively (Fig. 5A and C). Control mice injected with pcDNA3 or recombinant GST presented a delay in the generation of in vivo cytotoxicity.

The enumeration of specific $CD8^+$ T cells by ELISPOT showed a similar pattern of immune response. After challenge, mice vaccinated with the plasmid pIgSPclone9 or with the recombinant protein GST-P4-P7 displayed faster expansion and significantly higher numbers of IFN-γ-secreting cells (Fig. 5B and D).

An interesting observation we made in naïve mice was that the expansion of specific splenic $CD8^+$ T cells occurred in the days following the peak parasitemia, between days 9 and 15 after challenge of BALB/c or C57BL/6 mice. The delayed kinetics of specific $CD8^+$-T-cell expansion may be an important factor for the establishment of infection in naïve hosts. These results are in agreement with the data collected after the challenge of naïve KO mice. Mouse survival mediated by $CD8^+$ T cells, perforin, and IFN-γ occurred mainly between days 14 and 17 after challenge. This timing correlated closely with the moment that specific effector $CD8^+$ T cells of C57BL/6 mice reached their maximum activity (days 15 and 16).

Overall, the kinetics of *T. cruzi*-specific $CD8^+$ cytotoxic T cells differs sharply from the observations made with mice infected with lymphocytic choriomeningitis virus, *Listeria mono-*

FIG. 2. Kinetics of peptide-specific cell-mediated immune responses in BALB/c or C57BL/6 mice after challenge with trypomastigotes of *T. cruzi*. BALB/c mice or C57BL/6 mice were challenged with $2.5 \times 10^3$ or $2.5 \times 10^4$ bloodstream trypomastigotes of *T. cruzi*, respectively. On the indicated days, the parasitemia was monitored in these animals (closed symbols in all panels). The results represent the mean of six mice ± standard deviation (SD). (A) The in vivo cytotoxic activity of BALB/c mice against target cells coated with peptide IYNVGQVSI was determined (open symbols). The results represent the mean of three to nine mice ± SD per group. (B) IFN-γ-producing spleen cells of BALB/c mice specific to the peptide IYNVGQVSI were estimated by the ELISPOT assay (4). The results represent the mean number of spot-forming cells (SFC) per $10^6$ splenocytes ± SD ($n = 4$; open symbols). (C) The in vivo cytotoxic activity of C57BL/6 mice against target cells coated with peptide VNHRFTLV was determined. The results represent the mean of three or four mice ± SD per group (open symbols). (D) IFN-γ-producing spleen cells of C57BL/6 mice specific to the peptide VNHRFTLV were estimated by the ELISPOT assay. The results represent the mean number of SFC per $10^6$ splenocytes ± SD ($n = 4$; open symbols). The results are representative of two or more independent experiments.


A
Cytotoxicity (%)
Parasitemia, 10^6
Days after infection
B
SFC/10^6 splenocytes
Parasitemia, 10^6
Days after infection
C
Cytotoxicity (%)
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Days after infection
D
SFC/10^6 splenocytes
Parasitemia, 10^6
Days after infection

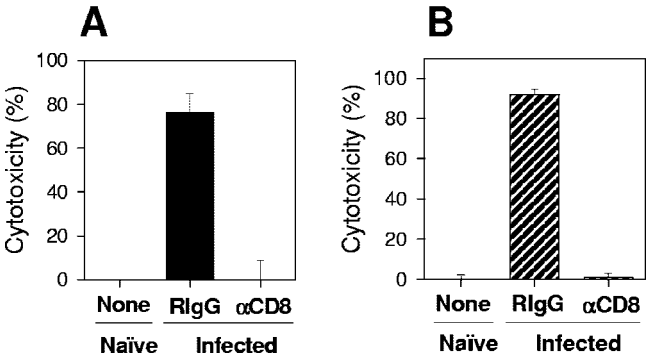


FIG. 3. Phenotypes of the cells mediating in vivo cytotoxic activity. BALB/c (A) or C57BL/6 (B) mice were challenged 30 days before the in vivo cytotoxic assay was performed with bloodstream trypomastigotes of *T. cruzi* as described in the legend to Fig. 1. Two days earlier, infected mice were treated with Rat immunoglobulin G (RIgG) or αCD8 monoclonal antibody (18). The in vivo cytotoxic activity against target cells coated with peptide IYNVGQVSI (A) or VNHRFTLV (B) was determined. The results represent the mean of three mice ± standard deviation and are representative of two independent experiments.

*cytogenes*, and *Plasmodium yoelli*. In these cases, maximum $CD8^+$-T-cell immune response was achieved between days 4 and 8 following challenge and rapidly declined after that period (2, 17, 19). Studies of mice infected with β-galactosidase-transgenic *Toxoplasma gondii* described much slower kinetics (7). The distinct kinetics can be explained by differences in the natures of these infections (acute versus chronic).

As opposed to naïve mice, vaccinated immune animals exhibited a significantly faster in vivo cytotoxic immune response. This swift immune response correlated with protective immunity, helping host survival after challenge.

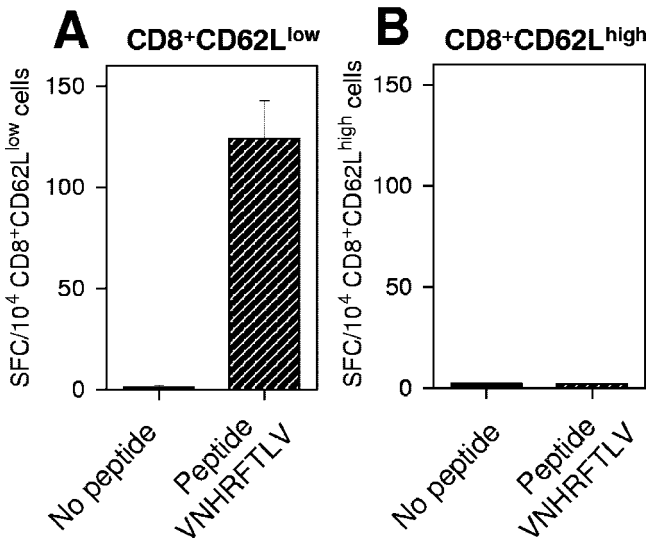


FIG. 4. Phenotypes of IFN-γ-producing cells. C57BL/6 mice were challenged 80 days before the assay was performed with bloodstream trypomastigotes of *T. cruzi* as described in the legend to Fig. 1. $CD8^+$ $CD62L^{Low}$ or $CD8^+$ $CD62L^{High}$ cells were 97.96% or 94.41% pure, respectively (data not shown). An ELISPOT assay was used to estimate the number of IFN-γ-producing cells specific to the peptide VNHRFTLV in purified $CD8^+$ $CD62L^{Low}$ (A) or $CD8^+$ $CD62L^{High}$ (B) cells. The results represent the mean number of spot-forming cells (SFC) per $10^4$ splenocytes ± standard deviation for triplicate cultures. The results are representative of two independent experiments.

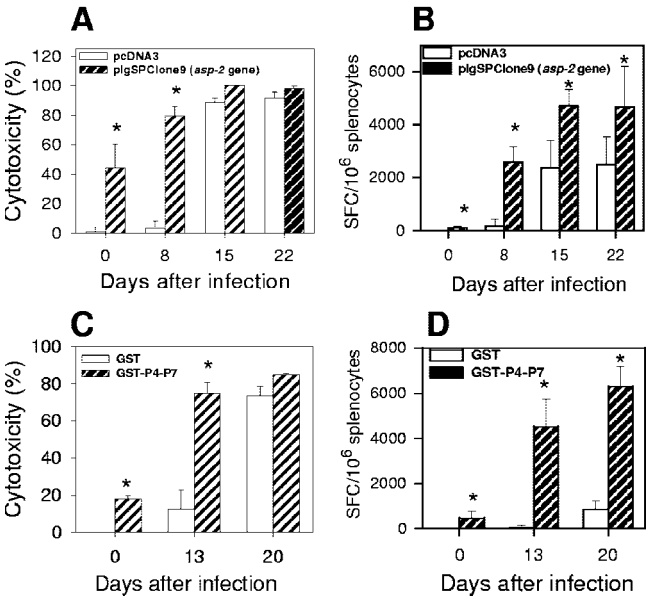


FIG. 5. Kinetics of the $CD8^+$-T-cell-mediated immune responses after challenge in mice vaccinated with the *asp-2* gene or a recombinant protein representing ASP-2 antigen. C57BL/6 mice were immunized intramuscularly with three doses of 100 μg plasmids pIgSPclone9 (*asp-2* gene; hatched bars) or pcDNA3 (control; white bars). Two weeks after the last dose, the mice were challenged or not intraperitoneally (i.p.) with $10^4$ bloodstream trypomastigotes. (A) On the indicated days, the in vivo cytotoxic activity against target cells coated with peptide VNHRFTLV was determined. The results represent the mean of three mice ± standard deviation (SD) per group. (B) On the indicated days, IFN-γ-producing spleen cells specific to the peptide VNH RFTLV were estimated by the ELISPOT assay. The results represent the mean number of spot-forming cells (SFC) specific to the peptide VNHRFTLV per $10^6$ splenocytes ± SD ($n = 4$). A/Sn mice were immunized with three doses of the recombinant protein GST-P4-P7 (hatched bars) or GST (white bars) as described previously (1). Two weeks after the last dose, the mice were challenged or not i.p. with 250 bloodstream trypomastigotes. (C) On the indicated days, the in vivo cytotoxic activity against target cells coated with peptide TEWETGQI was determined. The results represent the mean of three mice ± SD per group. (D) On the indicated days, numbers of IFN-γ-producing spleen cells specific to the peptide TEWETGQI were estimated by the ELISPOT assay. The results represent the mean number of SFC specific to the peptide TEWETGQI per $10^6$ splenocytes ± SD ($n = 4$). The asterisks denote values statistically higher than the values for control mice ($P < 0.05$; one-way analysis of variance). The results are representative of two independent experiments.

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# ***ARTIGO 2***

## Resumo 2

No trabalho anterior, nós descrevemos a cinética de ativação de células T CD8+ específicas para epítopos de *T. cruzi,* em camundongos selvagens e vacinados, após a infecção experimental. Neste trabalho, nós levantamos a hipótese de que a carga parasitária poderia ser um parâmetro crítico neste processo. Ao desafiarmos camundongos C57BL/6 com diferentes doses de parasitas, observamos uma aceleração no tempo da parasitemia conforme aumentávamos a dose. No entanto, não observamos aumento de mortalidade. Quando o mesmo experimento foi feito com camundongos CD8KO, resultado semelhante foi obtido com relação a parasitemia. Porém, observamos que quanto maior a dose, menor foi o tempo necessário para que os animais morressem. Esses resultados sugeriram que, em camundongo normal, as células T $CD8^+$ tornaram-se protetoras precocemente, seguindo o adiantamento da parasitemia. A avaliação da resposta citotóxica específica para diferentes epítopos (VNHRFTLV, TsKb18 e TsKb20), *in vivo,* confirmou que quanto maior a dose de parasitas injetados, mais rápido se observou a expansão das células específicas após a infecção. A diferenciação e expansão das células T $CD8^+$ específicas foram dependentes da replicação do parasita, uma vez que o desafio com parasitas irradiados não foi capaz de ativar essas células. Mostramos também, que a ativação das células T $CD8^+$ específicas foi dependente de células T $CD4^+$ restritas para MHC classe II. Esses resultados foram compatíveis com nossa hipótese inicial, na qual a carga parasitária é um parâmetro importante para controlar a cinética da expansão de células T $CD8^+$ específicas, dependentes de células T $CD4^+$.

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PLoS one

# Modulation of CD4$^+$ T Cell-Dependent Specific Cytotoxic CD8$^+$ T Cells Differentiation and Proliferation by the Timing of Increase in the Pathogen Load

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***Background.*** **Following infection with viruses, bacteria or protozoan parasites, naïve antigen-specific CD8$^+$ T cells undergo a process of differentiation and proliferation to generate effector cells. Recent evidences suggest that the timing of generation of specific effector CD8$^+$ T cells varies widely according to different pathogens. We hypothesized that the timing of increase in the pathogen load could be a critical parameter governing this process.** ***Methodology/Principal Findings.*** **Using increasing doses of the protozoan parasite *Trypanosoma cruzi* to infect C57BL/6 mice, we observed a significant acceleration in the timing of parasitemia without an increase in mouse susceptibility. In contrast, in CD8 deficient mice, we observed an inverse relationship between the parasite inoculum and the timing of death. These results suggest that in normal mice CD8$^+$ T cells became protective earlier, following the accelerated development of parasitemia. The evaluation of specific cytotoxic responses *in vivo* to three distinct epitopes revealed that increasing the parasite inoculum hastened the expansion of specific CD8$^+$ cytotoxic T cells following infection. The differentiation and expansion of *T. cruzi*-specific CD8$^+$ cytotoxic T cells is in fact dependent on parasite multiplication, as radiation-attenuated parasites were unable to activate these cells. We also observed that, in contrast to most pathogens, the activation process of *T. cruzi*-specific CD8$^+$ cytotoxic T cells was dependent on MHC class II restricted CD4$^+$ T cells.** ***Conclusions/Significance.*** **Our results are compatible with our initial hypothesis that the timing of increase in the pathogen load can be a critical parameter governing the kinetics of CD4$^+$ T cell-dependent expansion of pathogen-specific CD8$^+$ cytotoxic T cells.**

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## INTRODUCTION

MHC class Ia-restricted CD8$^+$ T cells are important mediators of the adaptive immune response against infections caused by intracellular microorganisms. Following infection with certain viruses, bacterias or parasites, naïve antigen-specific CD8$^+$ T cells go through a process of fast differentiation and proliferation, generating effector cytotoxic cells (expansion phase). These effector cells circulate between lymphoid and non-lymphoid tissues to restrain the multiplication of the infectious pathogen. Following pathogen elimination, the number of specific effector CD8$^+$ T cells is drastically reduced (contraction phase) and the establishment of a long-lived population of memory T cells responsible for perpetuating immunity against re-infection takes place. This kinetics of effector CD8$^+$ T cell expansion, contraction and establishment of a memory population has been fairly well reproduced and it is being thoroughly studied in a number of experimental models, including virus, bacterias and protozoan parasites (reviewed in ref. 1–3).

Using these experimental models, it was possible to establish that specific CD8$^+$ T cells differentiate and proliferate very quickly reaching a peak between days 4 and 8 after immunization with either lymphocytic choriomeningitis virus (LCMV), influenza virus, vaccinia virus, *Listeria monocytogenes* or *Plasmodium yoelli* [4–9]. Recent studies in mice infected with *Toxoplasma gondii*, *Mycobacterium bovis* bacille Calmette-Guerin (BCG), *Trypanosoma cruzi* and *Salmonella typhimurium* described significantly different kinetics of differentiation and proliferation of specific CD8$^+$ T cells. In the case of *T. gondii*, CD8$^+$ T cells specific for a transgenic epitope became detectable only 10 days after challenge, and the maximum number of epitope-specific T cells peaked at day 23 [10]. Similarly, in mice injected with recombinant *S. typhimurium* or BCG the peak response to the transgenic epitope was day 21$^{st}$ or 30$^{th}$ following challenge, respectively [11,12].

We recently described the kinetics of parasite-specific cytotoxic CD8$^+$ T cell responses following mouse infection with the human protozoan parasite *Trypanosoma cruzi* [13]. An interesting finding was that the initial inoculum of *T. cruzi* did not drive the differentiation and proliferation of effector CD8$^+$ T cells. The expansion phase of specific splenic CD8$^+$ T cells occurred after *in vivo* multiplication of parasites, between days 9 and 15 after i.p. challenge of C57BL/6 mice with $10^4$ parasites of the Y strain. More recently, Martin *et al.*, (2006) confirmed and extended our results in studies using different *T. cruzi* strains, which revealed that the peak of parasite epitope-specific CD8$^+$ T cells could vary from 14 to 24 days post-infection [14].

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The results obtained during *T. gondii*, BCG, *S. typhimurium* or *T. cruzi* infections sharply differed from the observations made in mice infected with LCMV, vaccinia, influenza, *L. monocytogenes* or *P. yoelli* and raised questions on the possible mechanisms controlling the kinetics of differentiation and proliferation following infection with different pathogens.

One mechanism that could influence the kinetics of specific $CD8^+$ T cells differentiation and proliferation is the amount of antigen and parasite-derived adjuvant both of which accumulate during the infection. Immediately after the initial infectious inoculum, the amount of antigen and parasite adjuvant available for $CD8^+$ T-cell priming may be limited, however both will increase after pathogen multiplication, and then may reach a certain threshold necessary to promote the maturation of antigen presenting cells (APC) - in the case of the adjuvant molecule - and trigger the activation of naïve $CD8^+$ T cells, in the case of the antigen. If this hypothesis is correct, the timing of increase of pathogen adjuvant/antigen would be a key parameter governing the process of $CD8^+$ T cell differentiation and expansion.

The aim of the present study was to determine whether modulation of the parasite load within a certain period of time can in fact alter the activation of effector/protective $CD8^+$ T cells. For this purpose, we used an experimental model where C57BL/6 mice were challenged with different doses of parasite of the Y strain of *Trypanosoma cruzi*. This strategy allowed us to modulate the timing of increase in the parasite load and determine the effect it may have on the *in vivo* differentiation and proliferation of effector/protective $CD8^+$ T cells. Using this experimental model, we were able to lend support to the hypothesis that the timing of accumulation of the parasite load can be a key factor influencing the differentiation and proliferation of $CD4^+$ T cell-dependent specific $CD8^+$ cytotoxic T cells following infection with a human pathogen.

## METHODS

### Mice and parasites

Female 8 to 10-week-old wild type (WT) C57BL/6, CD8α deficient C57BL/6 (CD8 KO), MHC-II deficient C57BL/6 (MHC-II KO), CD4 deficient C57BL/6 (CD4 KO), p40 deficient C57BL/6 (IL-12 KO), wild type 129 and 129 deficient for the IFN-I receptor (IFN-I receptor KO) were obtained from University of São Paulo. Perforin deficient C57BL/6 (Perforin KO) mice were bred on our own facility.

Parasites of the Y strain of *T. cruzi* were used in this study [13]. Bloodstream trypomastigotes were obtained from the plasma of A/Sn mice infected 7 days earlier. The concentration of parasites was adjusted and each mouse was inoculated intraperitoneally (i.p.) with 0.2 mL containing the indicated amount of trypomastigotes. Parasite development was monitored by counting the number of bloodstream trypomastigotes in 5 µl of fresh blood collected from the tail vein [13]. When the parasitemia was above $10^5$ trypomastigotes per mL, blood samples were diluted and the number of parasites estimated with the aid of a hemacytometer. Radiation-attenuated parasites were obtained by exposing them to gamma-irradiation (100 krads).

### Immunological assays

For the *in vivo* cytotoxicity assays, C57BL/6 splenocytes were divided into two populations and labeled with the fluorogenic dye carboxyfluorescein diacetate succinimidyl diester (CFSE Molecular Probes, Eugene, Oregon, USA) at a final concentration of 5 µM ($CFSE_{high}$) or 0.5 µM ($CFSE_{low}$). $CFSE_{high}$ cells were pulsed for 40 min at 37°C with 1 µM of the $H\text{-}2K^b$ ASP-2 peptide (VNHRFTLV), or TsKb-18 (ANYKFTLV) or TsKb-20 (ANYDFTLV). $CFSE_{low}$ cells remained unpulsed. Subsequently, $CFSE_{high}$ cells were washed and mixed with equal numbers of $CFSE_{low}$ cells before injecting intravenously (i.v.) 15 to $20\times10^6$ total cells per mouse. Recipient animals were mice that had been infected or not with *T. cruzi*. Spleen cells of recipient mice were collected 20 h after transfer, fixed with 3.7% paraformaldehyde and analyzed by fluorescence-activated cell sorting (FACS), using a Facscalibur Cytometer (BD, Mountain View, CA). The percentage of specific lysis was determined using the formula:

$$1-\frac{\%\ \mathrm{CFSE_{high}\ infected}/\%\ \mathrm{CFSE_{low}\ infected}}{\%\mathrm{CFSE_{high}\ naive}/\%\mathrm{CFSE_{low}\ naive}}\times 100\%. \quad (1)$$

The ELISPOT assay for enumeration of Interferon-gamma (IFN-γ) producing cells was performed essentially as described earlier [15].

### Statistical analysis

The values of were compared by One-Way Anova followed by Tukey HSD tests available at the site http://faculty.vassar.edu/lowry/VassarStats.html. The LogRank test was used to compare the mouse survival rate after challenge with *T. cruzi*. The differences were considered significant when the $P$ value was $<0.05$.

## RESULTS

In initial studies we investigated the development of *T. cruzi* parasitemia in wild type C57BL/6 mice challenged i.p. with different doses of trypomastigotes ($10^2$, $10^3$, $10^4$ or $10^5$ parasites per mouse). As shown in figure 1, infection with $10^2$ parasites generated a parasitemia that could be first detected at day 8 and peaked at day 11 post challenge. Doses of $10^3$, $10^4$ or $10^5$ parasites hastened the initial detection of parasitemia to days 5, 4 and 3, respectively. The peak parasitemia in mice receiving the highest parasite doses was also earlier, at days 9, 8 or 5 respectively. The magnitude of the peak parasitemias between the different mice groups was not significantly different when comparing groups of mice infected with $10^5$ or $10^4$. Similarly, no difference was found when comparing groups of mice infected with $10^3$ or $10^2$. Mice infected with $10^3$ parasites presented a peak parasitemia lower ($P<0.05$) than the mouse group infected with $10^4$ but not with $10^5$ parasites. In repeated experiments, mice infected with $10^2$ parasites presented a peak parasitemia lower than mouse groups infected with $10^4$ or $10^5$ ($P<0.01$ in both cases).

In view of these results, we concluded that there is an inverse relationship between parasite inoculum and the timing of the development of *T. cruzi* parasitemia, a feature that had not been described. In addition, we observed that the peak parasitemia did not differ significantly among groups of mice injected with $10^3$, $10^4$, or $10^5$ parasites. Mice infected with $10^2$ parasites consistently presented lower peak parasitemia than animals challenged with much higher parasite doses ($10^4$ or $10^5$ parasites per mouse). In spite of the fact that the parasitemia reached the peak earlier when the parasite inoculum was increased, these mice were capable of controlling the infection and survived the challenge. Because in this mouse model of infection $CD8^+$ T cells are critical for survival [13], the fact that animals injected with increasing parasite inoculum survived infection suggested that they were able to develop protective immunity in spite of increasing the infective dose. To determine whether protective immunity dependent on $CD8^+$ T cells could indeed be developed in these animals, we compared the parasitemia and mortality of wild type and CD8 KO mice following infection with different doses of parasites. As shown in figure 2A, when comparing infected wild type and CD8

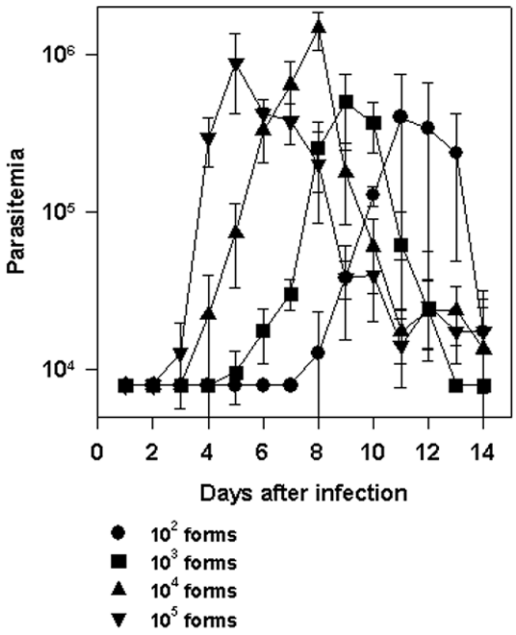


**Figure 1. Trypomastigote-induced parasitemia in C57BL/6 mice challenged with different doses of trypomastigotes of *T. cruzi*.** C57BL/6 mice were infected i.p. with $10^2$,$10^3$, $10^4$ or $10^5$ bloodstream trypomastigotes of the Y strain of *T. cruzi*. Parasitemia was followed daily from days 0 to 14 after challenge. The results represent the mean of 5–6 mice±SD. At the peak of infection, the parasitemia of mice infected with each different dose was compared by One-way Anova and Tukey HSD tests. The results of the comparisons were as follows: **i)** $10^2\times10^3$, Non-Significant (NS); **ii)** $10^2\times10^4$, $P<0.01$; **iii)** $10^2\times10^5$, $P<0.01$; **iv)** $10^3\times10^4$, $P<0.05$; **v)** $10^3\times10^5$, NS; **vi)** $10^4\times10^5$, NS. Results are representative of two independent experiments.
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KO mice, the ascendant part of the curve of parasitemia was not significantly different. These results indicated that $CD8^+$ T cells were not important for parasite control during that period regardless of the parasite dose used for challenge. After the day of the peak parasitemia, wild type mice rapidly controlled the number of parasites in the blood. In sharp contrast, CD8 KO mice were unable to control the parasitemia, became ill and eventually died.

When we compared the timing of death of each group of CD8 KO mice, we observed an inverse relationship between the size of parasite inoculum and the timing of death. Statistical analysis revealed a significant difference among the groups of infected CD8 KO mice ($P\leq0.01$ in all cases, Fig. 2B). As wild type mice survived, these results clearly confirmed the importance of $CD8^+$ T cells as a protective mechanism in mice infected with different doses of parasite. These results also suggest that the activation of protective $CD8^+$ T cells in wild type C57BL/6 mice takes place earlier as the parasite inoculum is increased.

To determine whether there was an inverse relationship between the parasite inoculum used for infection and the timing of expansion of specific $CD8^+$ cytotoxic T cells, we characterized the kinetics of effector $CD8^+$ T cell development. For this purpose we used a functional cytotoxic assay which measures the *in vivo* elimination of target cells coated with peptide VNHRFTLV [13]. The phenotype of effector cells mediating peptide-specific *in vivo* cytotoxicity was established earlier as being $CD8^+$ T cells [13]. As shown in Fig. 3A, at day 5 post-infection, none of the mouse groups presented peptide-specific cytotoxicity. At day 10 day post-infection, we observed a direct correlation between the size of parasite inoculum and intensity of the *in vivo* cytotoxicity (Fig. 3B). By day $15^{th}$, groups of mice infected with $10^3$, $10^4$ or $10^5$ parasites had reached their maximum cytotoxicity (close to 100% specific lysis). In contrast animals infected with $10^2$ parasites still displayed only ~50% cytotoxic activity (Fig. 3C). By day $30^{th}$ post-infection, the *in vivo* cytotoxicity reached frequencies close to 100% in all mouse groups (Fig. 3D). The *in vivo* cytotoxicity continued at a high level in all infected groups until tested 100 days after challenge (Fig. 3E).

IFN-$\gamma$ ELISPOT assays confirmed that at day 5 post-infection few peptide-specific T cells were detected in mice infected with increasing doses of *T. cruzi*. A direct correlation between the size of parasite inoculum and the number of peptide-specific cells was clearly evident by day 10 post-infection. By day $15^{th}$ or $30^{th}$, in all mice groups we detected a high frequency of IFN-$\gamma$ producing specific T cells (Fig. 3F).

To evaluate whether the results described above could also be extended to other parasite epitopes, we evaluated the kinetics of the *in vivo* cytotoxicity specific for two other sub-dominant epitopes (TsKb-18 and TsKb-20, ref. 14). We found that the kinetics of cytotoxicity for both sub-dominant epitopes was also dependent on the dose of parasites used for the challenge (Fig. 4).

The results presented above established an inverse relationship between the parasite inoculum and the timing of cytotoxic $CD8^+$ T cells differentiation and proliferation. However, it was not clear whether this event was in fact dependent on parasite multiplication or depended solely on dose of parasite used for challenge. To address this question, we challenged mice with irradiated or non-irradiated parasites. Irradiated parasites maintain their viability as assessed by their motility and capacity to infect host cells *in vitro*. However, they are unable to multiply (*in vitro* or *in vivo*) and establish an infection as determined by absence of parasitemia. Mice challenged with $10^3$ or $10^4$ irradiated parasites did not develop detectable *in vivo* cytotoxic activity or IFN-$\gamma$ producing cells (Fig. 5A and 5B, respectively). In contrast, animals challenged with $10^3$ or $10^4$ non-irradiated parasites developed strong cytotoxic responses and peptide-specific IFN-$\gamma$ producing cells (Fig. 5A and 5B, respectively). This result suggested that parasite replication was indeed an important factor to promote differentiation and proliferation of cytotoxic T cells.

Because certain genetically deficient mice are highly susceptible to *T. cruzi* infection, and die before specific $CD8^+$ T cells could be detected, it is difficult to study the importance that certain cells/molecules may have on proliferation and development of effector functions of $CD8^+$ cytotoxic T cells following *T. cruzi* infection. However, the fast development of cytotoxic T cells observed in mice infected with large doses of *T. cruzi* ($10^5$ parasites per mouse), allowed us to study some of the molecules of the immune system that could play an important role in the development of protective $CD8^+$ cytotoxic T cells. Using this strategy, we were able to study whether genetically deficient mice lacking MHC-II, CD4, IL-12, perforin or IFN-I receptor were capable of developing specific $CD8^+$ cytotoxic T cell responses. We found that MHC-II or CD4 KO mice developed negligible levels of specific cytotoxicity *in vivo* (Fig. 6A and B, respectively). In contrast, IL-12 KO or IFN-I receptor KO mice developed normal levels of specific cytotoxicity *in vivo* (Fig. 6A and 6C, respectively). Cytotoxicity mediated by $CD8^+$ T cell responses in Perforin KO mice were significantly reduced (~75%) compared to control wild type animals. The results of these studies clearly indicate that MHC-II and CD4 are key molecules for the induction of an effective cytotoxic $CD8^+$ T cell response following *T. cruzi* infection.

## DISCUSSION

Sufficient amounts of pathogen-derived adjuvant to mature APC and antigen to trigger T cells are possibly among the critical steps

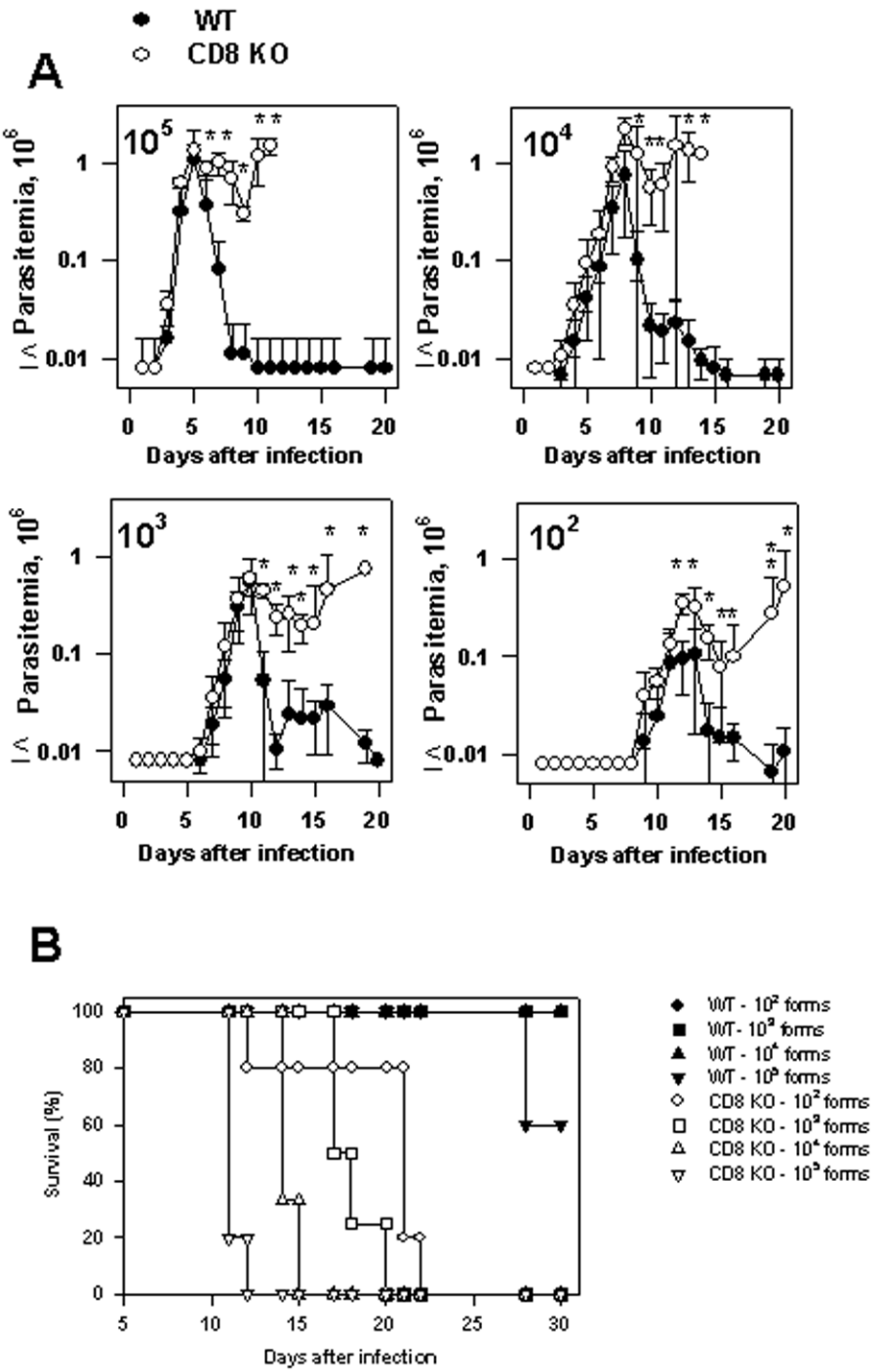


**Figure 2. Infection in WT C57BL/6 or CD8 KO mice challenged with different doses of *T. cruzi.*** Groups of WT C57BL/6 or CD8 KO were infected i.p. with $10^2$,$10^3$, $10^4$ or $10^5$ bloodstream trypomastigotes of the Y strain of *T. cruzi*. **(A)** Course of infection, estimated by the number of trypomastigotes per mL of blood. Results represent the mean values of 4–5 mice±SD. The parasitemias of WT C57BL/6 or CD8 KO mice were compared by One-way Anova. Asterisks denote statistically significant differences ($P<0.05$). **(B)** Kaplan-Meier curves for survival of WT C57BL/6 or CD8 KO infected mice with different doses of parasites. Statistical analyses were performed using LogRank test comparing the different mouse groups. Initially, we compared groups of WT C57BL/6 infected with different doses. The results of the comparison showed no statistically significant differences among them. Subsequently, we compared WT C57BL/6 or CD8 KO infected with each different dose of parasites. The results of the comparison showed statistically significant differences in between C56BL/6 or CD8 KO challenged with each parasite dose ($P<0.0001$, in all cases). Finally, statistical analyses were performed comparing the groups of CD8 KO infected with different doses. The results of the comparison were as follows: **i)** CD8 KO $10^2$×CD8 KO $10^3$ ($P=0.0025$); **ii)** CD8 KO $10^2$×CD8 KO $10^4$ ($P=0.0046$); **iii)** CD8 KO $10^2$×CD8 KO $10^5$ ($P=0.0016$); **iv)** CD8 KO $10^3$×CD8 KO $10^4$ ($P=0.01$); **v)** CD8 KO $10^3$×CD8 KO $10^5$ ($P=0.0035$); **vi)** CD8 KO $10^4$×CD8 KO $10^5$ ($P=0.0082$).
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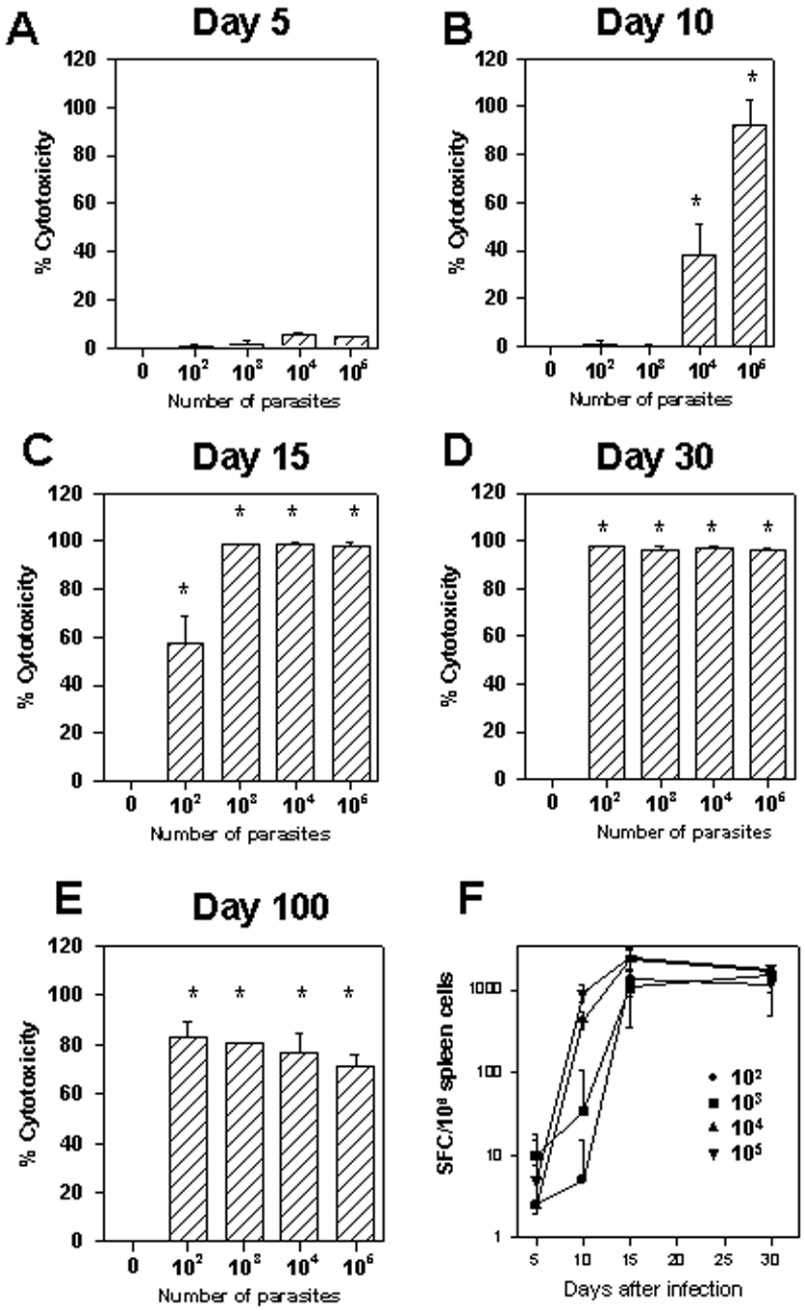


**Figure 3. Kinetics of specific CD8+ T-cell mediated immune responses following challenge with *T. cruzi.*** Groups of C57BL/6 mice were challenged or not i.p. with $10^2$,$10^3$, $10^4$ or $10^5$ bloodstream trypomastigotes of the Y strain of *T. cruzi*. **Panels A to E** - At the indicated days, the *in vivo* cytotoxic activity against target cells coated with peptide VNHRFTLV was determined as described in the Methods Section. The results represent the mean of 4 mice±SD per group. Asterisks denote statistically significant differences when we compared *T. cruzi* challenged with control mice ($P<0.05$). **Panel F**- At the indicated days, IFN-$\gamma$ producing spleen cells specific to the peptide VNHRFTLV were estimated by the ELISPOT assay. The results represent the mean number of peptide-specific spot forming cells (SFC) per $10^6$ splenocytes±SD (n = 4). Results are representative of two or more independent experiments.

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required for the differentiation and proliferation of CD8+ cytotoxic T cells. However, during infection with different pathogens, it is not clear the point at which the amount of adjuvant and antigen reach the necessary threshold to induce APC maturation and T cell activation. Considering that in most cases the initial pathogen inoculum is limited, pathogen multiplication may be a critical step in this process. In our experimental model, after infection with viable irradiated parasites of *T. cruzi*, we were unable to detect differentiation and proliferation of cytotoxic CD8+ T cells (Fig. 5). This result suggests that *T. cruzi* multiplication is critical to generate sufficient amounts of parasite adjuvant for the maturation of APC, and antigens for T cell activation. Our result contrasts with the data published using sporozoites of *P. yoelii*. In this case, relatively small doses ($10^4$–$10^5$ parasites per mouse) of non-replicating radiation-attenuated parasites efficiently prime effector/protective CD8+ T cells [16]. As for *Listeria*, non-replicating

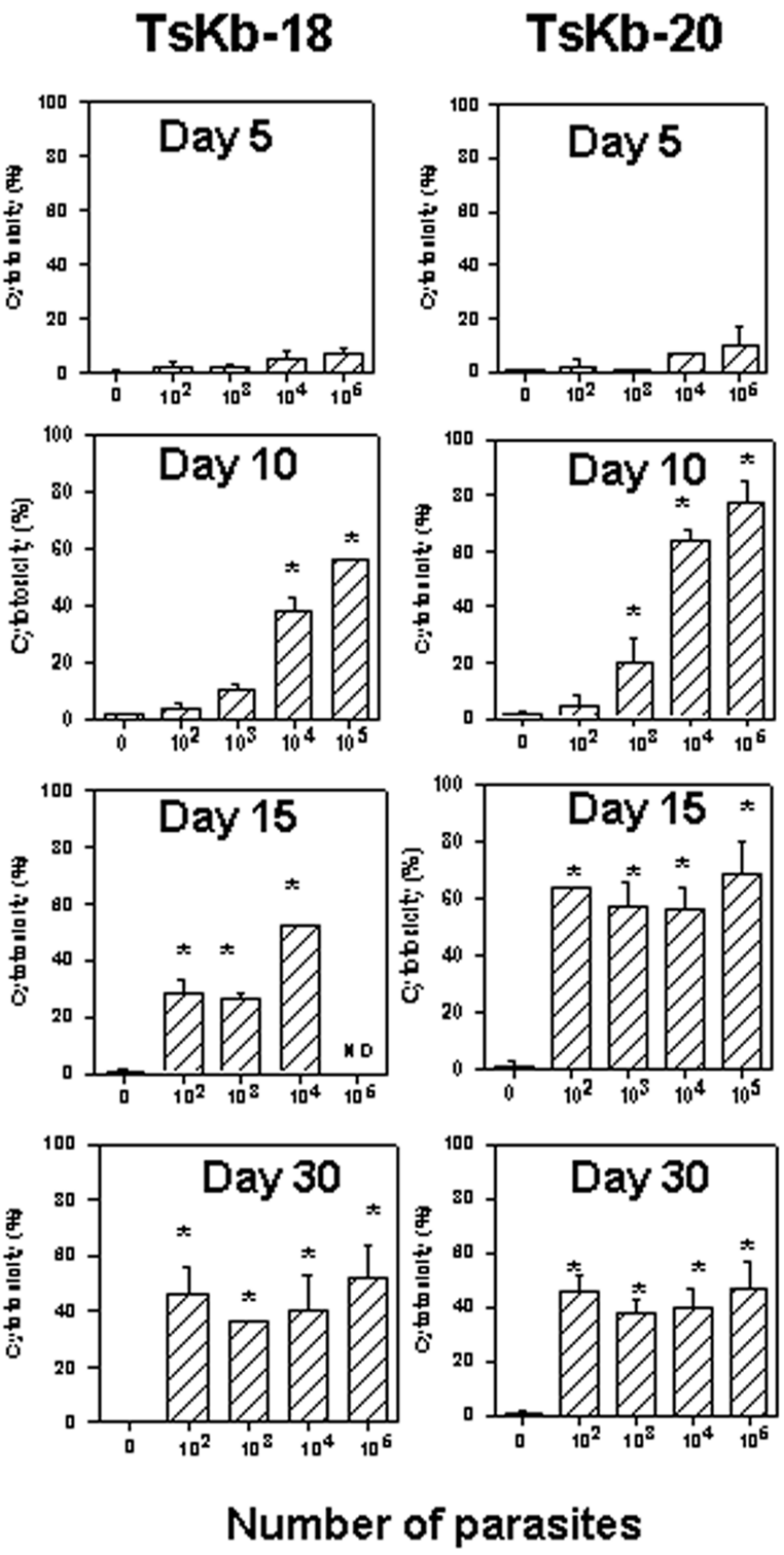


**Figure 4. Kinetics of CD8+ T-cell mediated immune responses specific for sub-dominant epitopes in C57BL/6 mice.** Groups of C57BL/6 mice were challenged or not i.p. with $10^2$,$10^3$, $10^4$ or $10^5$ bloodstream trypomastigotes of the Y strain of *T. cruzi*. At the indicated days, the *in vivo* cytotoxic activity against target cells coated with peptide TsKb-18 or TsKb-20 was determined as described in the Methods Section. The results represent the mean of 4 mice±SD per group. Asterisks denote statistically significant differences when we compared *T. cruzi* challenged with control mice ($P<0.05$). ND = Not done. Results are representative of two or more independent experiments.
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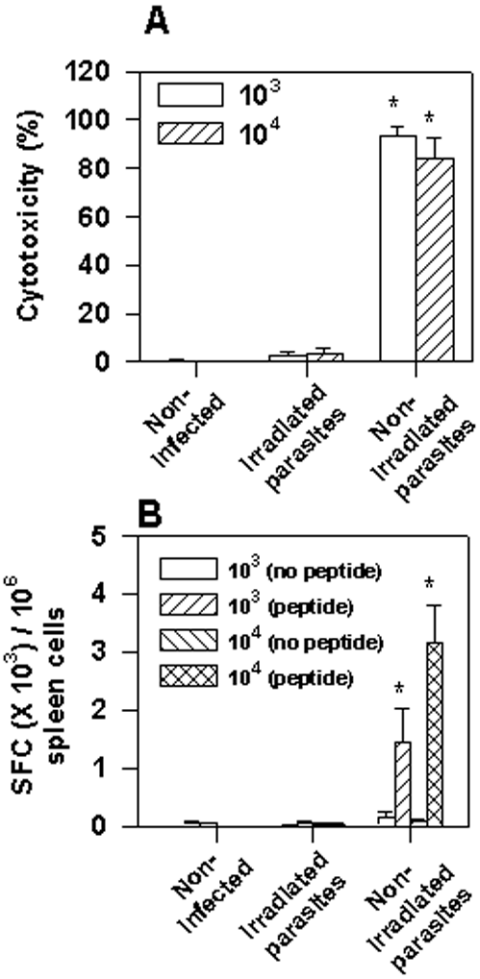


**Figure 5. Specific cytotoxicity in C57BL/6 mice challenged with irradiated or non-irradiated trypomastigotes of *T. cruzi*.** Groups of C57BL/6 mice were challenged or not i.p. with $10^3$ or $10^4$ irradiated or non-irradiated bloodstream trypomastigotes of the Y strain of *T. cruzi*. A) Fifteen days after challenge, the *in vivo* cytotoxic activity against target cells coated with peptide VNHRFTLV was determined. The results represent the mean of 4 mice±SD per group. B) Fifteen days after challenge, IFN-$\gamma$ producing spleen cells specific to the peptide VNHRFTLV were estimated by the ELISPOT assay. The results represent the mean number of SFC per $10^6$ splenocytes±SD (n = 4). Asterisks denote statistically significant differences ($P<0.05$) when we compared mice challenged with irradiated or non-irradiated trypomastigotes of *T. cruzi*. Results are representative of two independent experiments.
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irradiated bacterias were also used during priming of effector/protective $CD8^+$ T cells. However, large doses of bacteria ($10^9$ per mouse) were employed. This inoculum size of radiation-attenuated bacterias was considerably higher than the inoculum of $10^4$ non-irradiated bacterias per mouse used to prime protective $CD8^+$ T cells [17].

While the notion that the timing of accumulation of pathogen-derived antigen/adjuvant may influence the kinetics of expansion of effector $CD8^+$ T cells appears to be reasonable, few experimental models provide an opportunity to evaluate this important aspect of the $CD8^+$ T cell response. Our initial observation that increasing doses of *T. cruzi* caused an acceleration of *T. cruzi* parasitemia without increasing mouse susceptibility provided a suitable experimental model to study this issue. As observed with *T. cruzi* infection, an inverse relationship was described

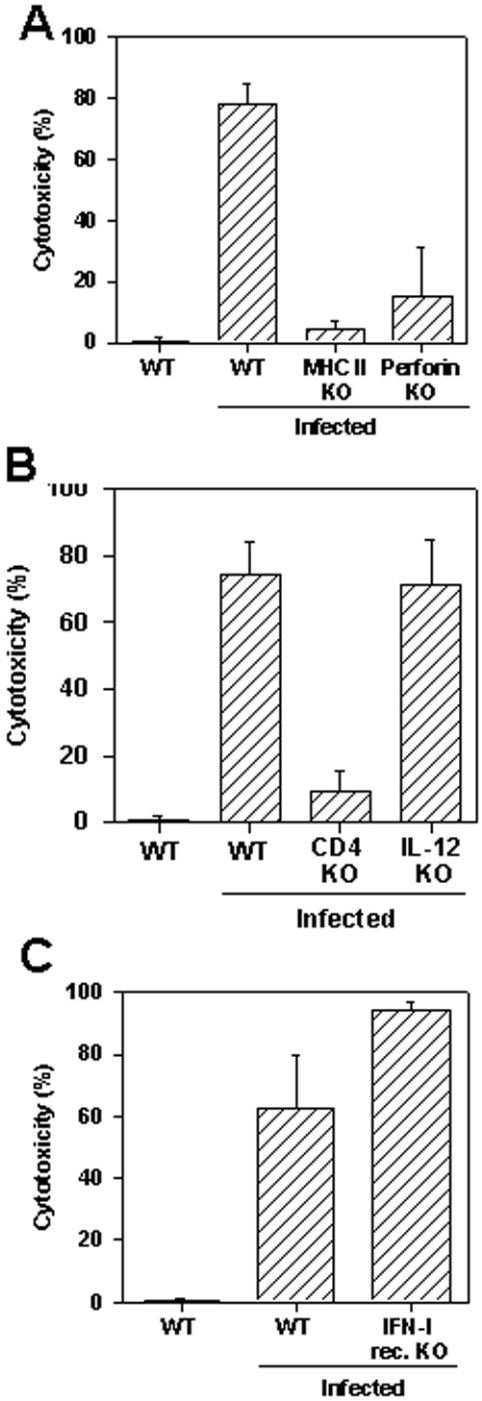


**Figure 6. Specific cytotoxicity in WT or genetically deficient mice challenged with *T. cruzi*.** Groups of WT C57BL/6 (n = 4), WT 129 mice (n = 4), MHC-II KO (n = 4), perforin KO (n = 8), CD4 KO (n = 4), IL-12 KO (n = 4), and IFN-I receptor KO (n = 4) were challenged or not i.p. with $10^5$ bloodstream trypomastigotes of the Y strain of *T. cruzi*. Ten days after challenge, the *in vivo* cytotoxic activity against target cells coated with peptide VNHRFTLV was determined. The results represent the mean of the above indicated number of mice±SD per group. The *in vivo* cytotoxicity was compared by One-way Anova and Tukey HSD tests. The results of the comparisons were as follows: **i)** WT C57BL/6×MHC-II KO ($P<0.01$); **ii)** WT C57BL/6×Perforin KO ($P<0.01$); **iii)** WT C57BL/6×CD4 KO ($P<0.01$); **iv)** WT C57BL/6×IL-12 KO (NS); **v)** WT 129×IFN-I receptor KO (NS). Results are representative of two or more independent experiments.
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between the inoculum size of influenza virus and the timing of accumulation of viral load in the lung. However, in this experimental model, the rapid increase in the viral load augmented the apoptosis of $CD8^+$ T cells mediated by Fas/FasL interaction, causing a reduction in the *in vivo* cytotoxicity mediated by $CD8^+$ T cells and and increased death rate in mice which received larger viral doses [18]. Differently, in our *T. cruzi* model we observed that increasing the size of parasite inoculum accelerated the parasitemia and the timing of differentiation and expansion of cytotoxic $CD8^+$ T cells (Fig. 3). However, the different doses of parasite did not modified the final magnitude of the specific $CD8^+$ T cell response as measured by the *in vivo* cytotoxicity or the ELISPOT assay (Fig. 3). Therefore, we concluded that differently to the influenza virus system, no inhibitory activity was generated in *T. cruzi* infection by the fast pace of parasite adjuvant/antigen accumulation in C57BL/6 mice.

The curves of parasitemia observed in WT C57BL/6 or CD8 KO mice challenged with different parasite doses, strongly suggest that protective $CD8^+$ T cells are important for mouse survival only after the parasitemia reached a peak (Fig. 1 and 2). Until that day, the amounts of parasites in the blood of both, WT C57BL/6 and CD8 KO mice, were similar (Fig. 2, ref. 13). After the peak parasitemia, a rapid reduction in the number of blood parasites was observed in WT mice while CD8 KO mice failed to control parasite growth, became severely ill, and eventually died. Confirmation of the importance of $CD8^+$ T cells in the period after the peak parasitemia was obtained in experiments in which we estimated the presence of cytotoxic T cells *in vivo*. For example, while the peak parasitemia of mice challenged with $10^5$ parasites was reached 5 days post-infection, no peptide-specific cytotoxicity was detected at that day (Fig. 3A). However, 5 days later, the *in vivo* cytotoxicity was already 100% indicating that specific $CD8^+$ T cells expanded vigorously during that period. Essentially the same sequence of events is observed in mice challenged with different doses of parasites. Based on these observations, we concluded that following challenge of naïve hosts with parasites of the Y strain of *T. cruzi*, the differentiation and expansion of splenic antigen-specific effector $CD8^+$ T cells occurs after the peak parasitemia. These results are in close agreement with the data published by us and others using 4 different *T. cruzi* epitopes in two different mouse strains [13,14]. We consider that our observations are consistent with the interpretation that the amount of *T. cruzi* antigen available before the peak of parasitemia is limited. When the parasitemia reaches its peak, the threshold for the level of adjuvant/antigen requirement may be achieved and only then, the triggering and fast activation of naïve $CD8^+$ T cells may occur. Studies performed in mice infected with LCMV or *L. monocytogenes* described comparable timing for expansion of specific $CD8^+$ T cells i.e., the peak of viral or bacterial numbers occurs approximately 2–3 days after infection. The peak of $CD8^+$ T cell response was approximately 5 days later at day 7–$8^{th}$ post-infection [1,2].

In the last part of our study, considering that a rapid induction of *T. cruzi*-specific $CD8^+$ T cells occurred after administration of a large inoculum of parasites, we evaluated the importance that certain molecules/cells may have on differentiation/proliferation and effector function of specific $CD8^+$ T cells following *T. cruzi* infection. For this purpose, we used genetically deficient mouse strains that are described as highly susceptible to infection with *T. cruzi* such as MHC-II KO, CD4 KO, IL-12 KO and perforin KO [13,19–22]. MHC II KO or CD4 KO mice failed to develop peptide-specific cytotoxicity. We therefore concluded that MHC II-restricted $CD4^+$ T cells are important for the maturation and/or expansion of *T. cruzi* specific cytotoxic $CD8^+$ T cells.

Our results indicating that $CD8^+$ T cells responses against *T.cruzi* are critically dependent on $CD4^+$ T cells differ from most pathogens. Following viral or bacterial infections, the maturation and expansion of specific $CD8^+$ T cells are not critically dependent on $CD4^+$ T cells [23–29]. Similarly, $CD4^+$ T cells are not required for the initial expansion of $CD8^+$ T cells specific for epitopes expressed by the protozoan parasites *P. yoelii* or *T. gondii* [11,30,31]. The precise role for MHC II-restricted $CD4^+$ T cells during the process of $CD8^+$ T cell activation in our model has yet to be investigated. An intriguing possibility is that $CD4^+$ T cells can license dendritic cells for the activation of highly cytotoxic $CD8^+$ T cells detected by an *in vivo* assay [32].

Using IL-12 or IFN-I receptor KO mice, we observed that neither IL-12 nor IFN type I are critically important for the efficient maturation and expansion of *T. cruzi*-specific cytotoxic $CD8^+$ T cells. Our results contrasts with previous observation that IL-12 can provide an important third signal that, in addition to the engagements of TCR-MHC and CD28-B7, it could provide an optimal environment for the efficient cytotoxic $CD8^+$ T cells differentiation and expansion [33–35].

Perforin KO mice were also severely impaired in their ability to eliminate peptide-coated targets *in vivo*. The low level killing detected in the absence of perforin may represent the contribution of perforin-independent killing mechanisms. Considering our previous results indicating that perforin KO mice are highly susceptible to infection with parasites of the Y strain of *T. cruzi*, we propose that the perforin-dependent granule exocytosis pathway represent an important mechanism of protection against *T. cruzi* infection. These results are in agreement with some viral models describing perforin as a key molecule for resistance against viral infection and mediating *in vivo* lysis of peptide-coated target cells [36,37]. However, they differ with the observations made for other protozoan parasites in which perforin KO mice have been shown to develop protective $CD8^+$ T cell mediated immunity [38–40].

In summary, our study provides new insights regarding the requirements for the differentiation and expansion of cytotoxic $CD8^+$ T cells during experimental infection with a human protozoan parasite. Using this experimental model, we determined the importance of parasite load and MHC-II restricted $CD4^+$ T cells for the maturation and expansion of highly cytotoxic $CD8^+$ T cells. Also, it established an important role for perforin as a mediator of the *in vivo* cytotoxicity against parasite-infected cells.

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### Author Contributions

Conceived and designed the experiments: MR FT PP. Performed the experiments: MR FT. Analyzed the data: MR FT PP. Contributed reagents/materials/analysis tools: MR FT PP. Wrote the paper: MR FT PP.

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# *ARTIGO 3*

## Resumo 3

Interferência ou competição entre linfócitos T CD8+ restritos por distintas moléculas de MHC-Ia pode ser uma forma potente para o estabelecimento de uma resposta imunodominante. Entretanto, sua importância durante infecções ainda é questionável. No presente estudo, nós descrevemos que após a infecção com o patógeno humano *Trypanosoma cruzi*, uma resposta imune de linfócitos T CD8+ imunodominante é desenvolvida e dirigida contra um epítopo restrito por H-2K$^b$, expresso por membros da família de proteínas de superfície *trans*-sialidase. A fim de determinar se esta imunodominância seria exercida não somente contra outros epítopos restritos pelo MHC H-2K$^b$, mas também sobre outros complexos peptídeo-MHC, nós estudamos a resposta imune contra epítopos restritos por distintos MHC durante a infecção de camundongos homozigotos e heterozigotos. Nós observamos que a resposta imune contra o complexo H-2K$^b$-VNHRFTLV era semelhante em camundongos homozigotos e heterozigotos. Em contraste, a resposta imune aos epítopos CD8 restritos pelo MHC H-2K$^d$ e H-2K$^k$ foram significativamente mais baixas em camundongos heterozigotos quando comparados com a resposta de camundongos homozigotos. Esta interferência não foi dependente da dose de parasitas ou do tempo da infecção. Também não ocorreu em camundongos heterozigotos imunizados com adenovírus recombinante que expressam estes mesmos antígenos de *T. cruzi,* demonstrando que a interferência é específica para a infecção por *T. cruzi*. Por fim, nós observamos que a infecção concomitante com duas cepas de *T. cruzi*, as quais apresentam epítopos imunodominantes diferentes, foi capaz de eliminar a competição/interferência entre células T de diferentes especificidades. Juntos, estes dados sugeriram fortemente que o mecanismo responsável por gerar a imunodominância durante a infecção pelo *Trypanosoma cruzi* foi o de competição de células T por APCs limitantes. Este tipo de interferência, nunca descrita durante infecção por um parasita humano, pode representar uma estratégia sofisticada para restringir o "priming" de linfócitos T CD8+ de distintas especificidades, evitando a eliminação completa do patógeno por células efetoras, e assim favorecendo o parasitismo.

# Infection with *Trypanosoma cruzi* Restricts the Repertoire of Parasite-Specific CD8$^+$ T Cells Leading to Immunodominance[1]

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**Interference or competition between CD8$^+$ T cells restricted by distinct MHC-I molecules can be a powerful means to establish an immunodominant response. However, its importance during infections is still questionable. In this study, we describe that following infection of mice with the human pathogen *Trypanosoma cruzi*, an immunodominant CD8$^+$ T cell immune response is developed directed to an H-2K$^b$-restricted epitope expressed by members of the *trans*-sialidase family of surface proteins. To determine whether this immunodominance was exerted over other non-H-2K$^b$-restricted epitopes, we measured during infection of heterozygote mice, immune responses to three distinct epitopes, all expressed by members of the *trans*-sialidase family, recognized by H-2K$^b$-, H-2K$^k$-, or H-2K$^d$-restricted CD8$^+$ T cells. Infected heterozygote or homozygote mice displayed comparably strong immune responses to the H-2K$^b$-restricted immunodominant epitope. In contrast, H-2K$^k$- or H-2K$^d$-restricted immune responses were significantly impaired in heterozygote infected mice when compared with homozygote ones. This interference was not dependent on the dose of parasite or the timing of infection. Also, it was not seen in heterozygote mice immunized with recombinant adenoviruses expressing *T. cruzi* Ags. Finally, we observed that the immunodominance was circumvented by concomitant infection with two *T. cruzi* strains containing distinct immunodominant epitopes, suggesting that the operating mechanism most likely involves competition of T cells for limiting APCs. This type of interference never described during infection with a human parasite may represent a sophisticated strategy to restrict priming of CD8$^+$ T cells of distinct specificities, avoiding complete pathogen elimination by host effector cells, and thus favoring host parasitism.** ***The Journal of Immunology,*** **2008, 180: 1737–1748.**

Major histocompatibility complex class Ia-restricted CD8$^+$ T cells are important mediators of the adaptive immune response against infections caused by intracellular microorganisms. Pathogens have a number of potential amino acid sequences that can bind to MHC-I molecules and provide targets for specific CD8$^+$ T cells. In contrast, hosts have a vast number of TCR clonally distributed on these CD8$^+$ T cells to recognize a variety of MHC class I (MHC-I)[3]-peptide complexes. Despite this large possible combination, pathogen-specific CD8$^+$ T cells seem to recognize preferentially a small number of epitopes. This preference, known as immunodominance, may occur due to different mechanisms including the formation of stable MHC-I peptide complexes on the surface of APC, higher amounts or higher affinity specific T cell precursors or competition of T cells for APC. The full biological implications of immunodominance during effector and memory immune responses are unknown and are being thoroughly studied during infection with viruses or bacteria (reviewed in Refs. 1–6).

Infection of humans or mice with the digenetic intracellular protozoan parasite *Trypanosoma cruzi* also induces MHC class Ia-restricted CD8$^+$ T cells specific for parasite epitopes (7, 8). This T cell subpopulation is critical for host survival following infection even when small challenge doses of parasites are used to initiate infection (Refs. 9–11 and reviewed in Ref. 12). Despite the fact that CD8$^+$ T cell mediated immune response is critical for host survival during acute infection, *T. cruzi* manages to survive within the host and establishes a lifelong chronic infection. Parasite persistence is considered a key element in the development of symptoms that occur many years or decades after initial infection in approximately one-third of these chronically infected individuals (13–15). Thus, understanding the specificity, magnitude, and longevity of CD8$^+$ T cell immune responses may greatly help us to explain the complex immunopathology caused by *T. cruzi* and to propose new means for intervention for Chagas' disease, which affects ~20 million people in Latin America.

Studies on the immunodominance of CD8$^+$ T cell immune responses during *T. cruzi* infection were thought not to be simple to accomplish due to the large parasite genome which contains >12,000 genes (16). Nevertheless, the description of epitopes targets of powerful CD8$^+$ T cell immune responses elicited during *T. cruzi* infection in distinct inbred mouse strains allowed us to

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[3] Abbreviations used in this paper: MHC-I, MHC class I; TS, *trans*-sialidase; SFC, spot forming cell; ASP-2, amastigote surface protein 2.

initiate studies on the immunodominance phenomenon (7, 8). Surprisingly, these studies suggest that CD8 response is highly confined to epitopes expressed by different members of a large family of *T. cruzi* surface Ags named *trans*-sialidases (TS). Thus, studies performed in our laboratory and elsewhere, show that C57BL/6, BALB/c, and more recently B10.A mice infected with *T. cruzi* develop a strong and long-lasting $CD8^+$ T cells specific for, respectively, H-$2K^b$-, H-$2K^d$-, and H-$2K^k$-restricted epitopes expressed by members of the TS family of surface Ags.

The purpose of the present study was to determine the mechanisms that established the immunodominance during naturally acquired immunity following experimental infection with *T. cruzi*. Toward that goal, we compared the magnitude of $CD8^+$ T cell immune responses to a number of different epitopes from members of the TS family, which are recognized by H-$2K^b$-restricted T cells. We found that the epitope VNHRFTLV was immunodominant following infection of C57BL/6 mice with parasites of two different strains. Subsequently, we investigated whether this immunodominance could be exerted on the immune response to CD8 T cell epitopes restricted by different MHC-I molecules. We accomplished that by comparing specific immune responses to three distinct H-$2K^b$-, H-$2K^d$-, or H-$2K^k$-restricted epitopes following *T. cruzi* infection of homozygote (C57BL/6, BALB/c, and B10.A) or heterozygote ($F_1$) mice. Our results provide strong evidence that homozygote (C57BL/6) and heterozygote mice develop similar immune responses to immunodominant H-$2K^b$-restricted epitope VNHRFTLV. However, immune responses to H-$2K^d$- or H-$2K^k$-restricted epitopes were severely diminished in heterozygote mice. This interference/competition was restricted to *T. cruzi* infection because following immunization with recombinant adenovirus, heterozygote mice responded to these same *T. cruzi* epitopes, as well as or better than homozygote strains. Finally, the possible mechanism mediating this strong immunodominance was evaluated by simultaneous infection with two *T. cruzi* strains containing distinct immunodominant epitopes. These mice responded equally well to the "dominant" and "subdominant" epitopes suggesting that the operating mechanism most likely involves competition of T cells for limiting APCs. We believe that this competition between T cells of different specificities is a sophisticated strategy that *T. cruzi* developed to restrict $CD8^+$ T cell responses and escape complete elimination by host effector cells, and thus favoring host parasitism.

## Materials and Methods

### *Mice and parasites*

Female 8- to 10-wk-old wild-type C57BL/6, BALB/c, B10.A, $F_1$ C57BL/6 × BALB/c, $F_1$ C57BL/6 × B10.A, $F_1$ B10.A × BALB/c mice were obtained from Federal University of São Paulo. Experimental procedures were approved by the Committee of Ethics of Federal University of São Paulo.

Parasites of Y, G, or CL-Brener strains of *T. cruzi* were used in this study (17, 18). Bloodstream trypomastigotes of Y strain were obtained from plasma of A/Sn mice infected 7 days earlier. Tissue culture trypomastigotes of the G or CL-Brener strain were used to infect mice. The concentration of parasites was adjusted and each mouse was inoculated i.p. with 0.2 ml containing the indicated amount of trypomastigotes. C57BL/6, B10.A, $F_1$ C57BL/6 × BALB/c, $F_1$ C57BL/6 × B10.A, $F_1$ B10.A × BALB/c were challenged, in most experiments, with $10^4$ bloodstream trypomastigotes i.p. BALB/c mice were challenged with only $2.5 \times 10^3$ parasites because they are more susceptible to infection and succumb to challenge doses higher than $5 \times 10^3$ parasites.

### *Peptide synthesis*

Peptides VNHRFTLV, TEWETGQI, TsKb-18 (ANYDFTLV), TsKb-20 (ANYKFTLV) were prepared by standard $N^{\alpha}$[9-fluorenylmethyloxycarbonyl] on a PSSM8 multispecific peptide synthesizer (Shimadzu) by solid-phase synthesis with a scale of 30 $\mu$M. Peptide was purified by HPLC in a Shimadzu system. Peptides were analyzed in a C18 Vydac column (10 × 250 mm, 5-$\mu$m particle diameter). Different peptide batches were obtained in a range of 80–90% purity. Their identities were confirmed by Q-TOF Micro equipped with an electrospray ionization source (Micromass). Peptide IYNVGQVSI was purchased from Neosystem. As estimated by HPLC analysis, peptide IYNVGQVSI was >90% pure.

Table I. *Peptides used in the study*

| Peptide/Epitope | Sequence | H-2K Restriction | Ref. |
|---|---|---|---|
| VNHRFTLV | VNHRFTLV | b | **7** |
| TsKb-18 | ANYDFTLV | b | **8** |
| TsKb-20 | ANYKFTLV | b | **8** |
| IYNVGQVSI | IYNVGQVSI | d | **7** |
| TEWETGQI | TEWETGQI | k | **7** |

### *Immunological assays*

For the in vivo cytotoxicity assays, splenocytes of the different mouse strains were divided into two populations and labeled with the fluorogenic dye CFSE (Molecular Probes) at a final concentration of 5 $\mu$M ($CFSE_{high}$) or 0.5 $\mu$M ($CFSE_{low}$). $CFSE_{high}$ cells were pulsed for 40 min at 37°C with 1–2.5 $\mu$M of H-$2K^b$ ASP-2 peptide (VNHRFTLV), or H-$2K^b$ TsKb-18 peptide (ANYDFTLV), H-$2K^b$ TsKb-20 peptide (ANYKFTLV), or H-$2K^d$ TS peptide (IYNVGQVSI) peptide or H-$2K^k$ ASP-2 peptide (TEWETGQI). $CFSE_{low}$ cells remained unpulsed. Subsequently, $CFSE_{high}$ cells were washed and mixed with equal numbers of $CFSE_{low}$ cells before injecting i.v. $15–20 \times 10^6$ total cells per mouse. Recipient animals were mice that had been infected or not with *T. cruzi*. Spleen cells of recipient mice were collected 20 h after transfer, fixed with 3.7% paraformaldehyde and analyzed by FACS, using a FACSCalibur Cytometer (BD Biosciences). Percentage of specific lysis was determined using the formula: $1 - ((\%\ CFSE_{high}\ \text{infected}/\%\ CFSE_{low}\ \text{infected})/(\%CFSE_{high}\ \text{naive}/\%\ CFSE_{low}\ \text{naive})) \times 100\%$. In experiments using adenovirus, we estimated the percentage of $CFSE_{high}$ or $CFSE_{low}$ cells in immunized, not infected mice. ELISPOT assay for enumeration of IFN-$\gamma$-producing cells was performed essentially as described earlier (19).

MHC-I tetramer IYNVGQVSI/$K^d$ or pentamer VNHRFTLV/$K^b$ were synthesized at the Tetramer Core Facility (Emory University, Atlanta, GA) or were purchased from ProImmune, respectively. Mouse splenocytes were analyzed. Single-cell suspensions of splenocytes were washed in PBS, stained for 15 min at 37 °C with tetramers, then stained 30 min at 4°C with labeled anti-CD8 Abs as well as anti-CD4, -CD11b, and -B220. At least 1,000,000 cells were acquired on a FACSCalibur flow cytometer (BD Pharmingen) then analyzed with FlowJo (Tree Star) using a biexponential transform.

### *Recombinant adenoviruses and immunization*

pAdCMV-TS is an adenoviral transfer plasmid that contains an eukaryotic expression cassette formed by the CMV immediate-early promoter and the SV40 RNA polyadenylation sequences. Inside this cassette, we cloned the DNA sequences encoding *T. cruzi* TS protein signal peptide and catalytic domain obtained by restriction enzyme digestion of plasmid p154/13 (adeno-TS). Equivalently, pAdCMV-amastigote surface protein-2 (ASP2) encodes ASP2 sequences obtained by restriction digestion of plasmid pIgSP clone 9 (adeno-ASP-2). Viruses were generated and purified as described earlier (20).

Mice were inoculated i.m. in each tibialis anterioris muscle with 50 $\mu$l of viral suspension containing $5 \times 10^7$ PFU of each adenovirus. Immunological assays were performed 15 days after viral inoculation.

### *Statistical analysis*

Values were compared by one-way ANOVA followed by Tukey honestly significant difference tests available at http://faculty.vassar.edu/lowry/VassarStats.html. Differences were considered significant when the $p$ value was $<0.05$.

## Results

### *Immunodominance of the $CD8^+$ T cell immune response to the H-$2K^b$-restricted epitope VNHRFTLV in C57BL/6 mice infected with* T. cruzi *of Y or G strains*

Three epitopes recognized by H-$2K^b$-restricted $CD8^+$ T cells were described as targets of strong and long-lasting immune responses following infection of C57BL/6 mice with *T. cruzi* (Table I, Refs.

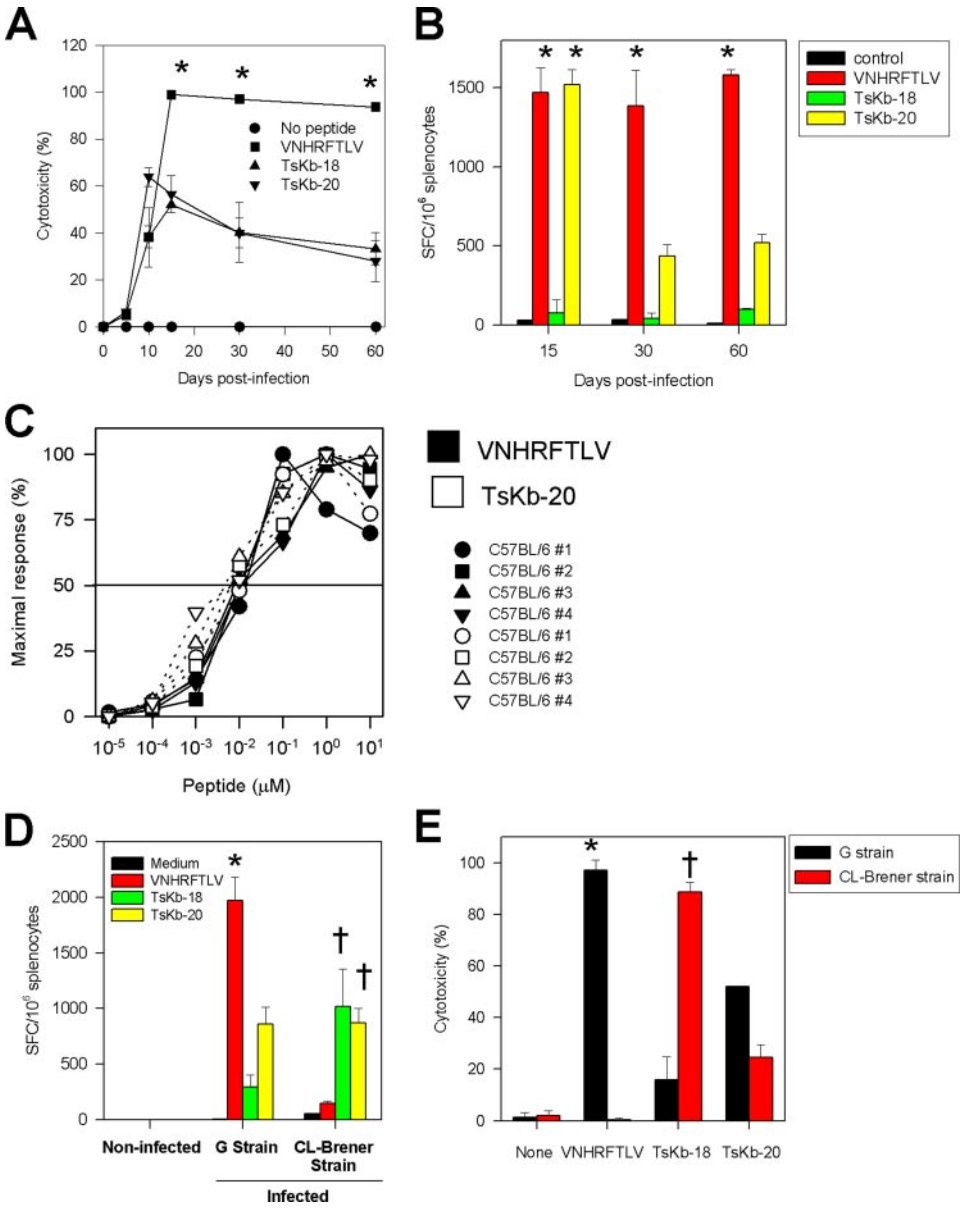


**FIGURE 1.** Specific CD8$^+$ T cell-mediated immune responses in C57BL/6 mice challenged with *T. cruzi*. C57BL/6 mice were challenged or not i.p. with $10^4$ bloodstream trypomastigotes of the Y strain (*A–C*), or $10^5$ trypomastigote culture forms of the G or CL-Brener strains (*D* and *E*) of *T. cruzi*. *A*, At the indicated days, the in vivo cytotoxic activity against target cells coated with peptides VNHRFTLV, TsKb-18, or TsKb-20 were determined as described in *Materials and Methods*. Results represent the mean of four mice ± SD per group. ∗, In vivo cytotoxicity against target cells coated with peptide VNHRFTLV was significantly higher than TsKb-18- or TsKb-20-coated cells ($p < 0.01$). *B*, At the indicated days, IFN-γ-producing spleen cells specific for peptides VNHRFTLV, TsKb-18, or TsKb-20 were estimated ex vivo by ELISPOT assay. Results represent the mean number of SFC per $10^6$ splenocytes ± SD ($n = 4$) following in vitro stimulation with the indicated peptide or medium only. ∗, At day 15, the numbers of SFC specific for peptide VNHRFTLV or TsKb-20 were significantly higher than the number of TsKb-18-specific SFC ($p < 0.01$). ∗, At days 30 and 60, the number of SFC specific for peptide VNHRFTLV was significantly higher than the number of TsKb-18- or TsKb-20-specific SFC ($p < 0.01$). *C*, Ex vivo ELISPOT assay was performed using in vitro the indicated concentrations of peptides VNHRFTLV or TsKb-20 to stimulate spleen cells from mice infected 15 days earlier. The data was normalized against the maximal values obtained with each peptide in each individual mouse. This value was considered 100%. Results of the titration curve of each individual mouse are shown. *D*, The numbers of IFN-γ-producing spleen cells specific for peptides VNHRFTLV, TsKb-18, or TsKb-20 were estimated ex vivo by ELISPOT assay in noninfected mice or animals infected with parasites of the G or CL-Brener strain 15 days earlier. Results represent the mean number of SFC per $10^6$ splenocytes ± SD ($n = 4$) following in vitro stimulation with the indicated peptide or medium only. ∗, The number of SFC specific for peptide VNHRFTLV was significantly higher than the number of TsKb-18- or TsKb-20-specific SFC ($p < 0.01$). †, The number of SFC specific for peptides TsKb-18 or TsKb-20 were significantly higher than the number of specific SFC for peptide VNHRFTLV ($p < 0.01$). *E*, The in vivo cytotoxic activity against target cells coated with peptides VNHRFTLV, TsKb-18, or TsKb-20 was determined in mice challenged with trypomastigotes of the G or CL-Brener strains. Results represent the mean of four mice ± SD per group. ∗, In vivo cytotoxicity against target cells coated with peptide VNHRFTLV was significantly higher than TsKb-18- or TsKb-20-coated cells ($p < 0.01$). †, In vivo cytotoxicity against target cells coated with peptide TsKb-18 was significantly higher than VNHRFTLV or TsKb-20-coated cells ($p < 0.01$). Results are representative of two or more independent experiments.

7 and 8). These epitopes are expressed by members of the TS family of surface proteins/Ags. The characterization of the immune response to the epitope VNHRFTLV was performed by our group following infection of C57BL/6 mice with parasites of the Y strain of *T. cruzi* (7). The immune responses to the other two epitopes (TsKb-18 and TsKb-20) were described in C57BL/6 mice

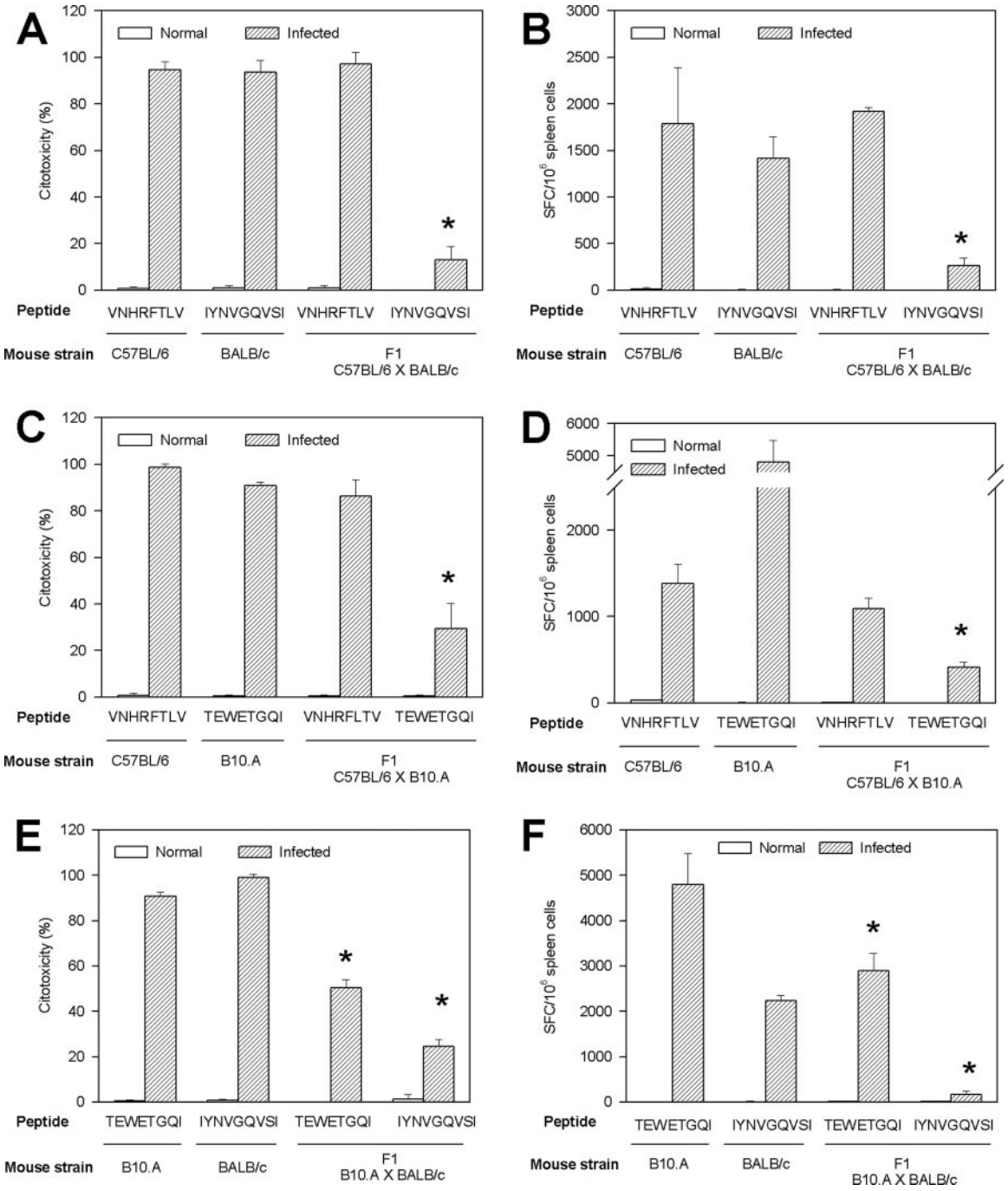


**FIGURE 2.** Comparison of specific $CD8^+$ T cell-mediated immune responses in homozygote or heterozygote mice challenged with *T. cruzi*. Mice of the indicated strains were challenged i.p. with $10^4$ bloodstream trypomastigotes of Y strain of *T. cruzi,* except for BALB/c mice which received 2500 trypomastigotes. Fifteen days later, specific immune responses were estimated in vivo by cytotoxicity (*A*, *C*, and *E*) or ex vivo by ELISPOT (*B*, *D*, and *F*). *A* and *B*, ∗, The in vivo cytotoxicity or the number of SFC from heterozygote $F_1$ C57BL/6 × BALB/c mice specific for peptide IYNVGQVSI were significantly lower than the values of homozygous BALB/c mice ($p < 0.01$ in both cases). *C* and *D*, ∗, The in vivo cytotoxicity or the number of SFC from heterozygote $F_1$ C57BL/6 × B10.A mice specific for peptide TEWETGQI were significantly lower than the values of homozygous B10.A mice ($p < 0.01$ in both cases). *E* and *F*, The in vivo cytotoxicity or the number of SFC from heterozygote $F_1$ B10.A × BALB/c mice specific for peptides TEWETGQI or IYNVGQVSI was significantly lower than the values of homozygous B10.A or BALB/c mice, respectively ($p < 0.01$ in both cases). Results are representative of two or more independent experiments.

infected with parasites of the Brazil, CL, and Y strain (8). TsKb-18 (ANYDFTLV) and TsKb-20 (ANYKFTLV) epitopes display a single amino acid substitution (D→K) but are recognized by $CD8^+$ T cells of distinct specificities (Table I and Ref. 8).

To evaluate the immunodominance pattern among these three epitopes after infection of C57BL/6 mice with *T. cruzi*, we estimated in vivo peptide-specific cytotoxicity and ex vivo the number of peptide-specific IFN-γ-producing cells following infection with parasites of the Y strain. In this mouse model, specific immune responses were first detected 9 days following infection. The maximum in vivo cytotoxicity occurred from days 15 to 90 following infection (7). Fig. 1*A* shows the kinetics of the in vivo cytotoxicity specific for target cells coated with peptide VNHRFTLV. More than 96% specific lyses were detected at day 15, 30, or 60 after

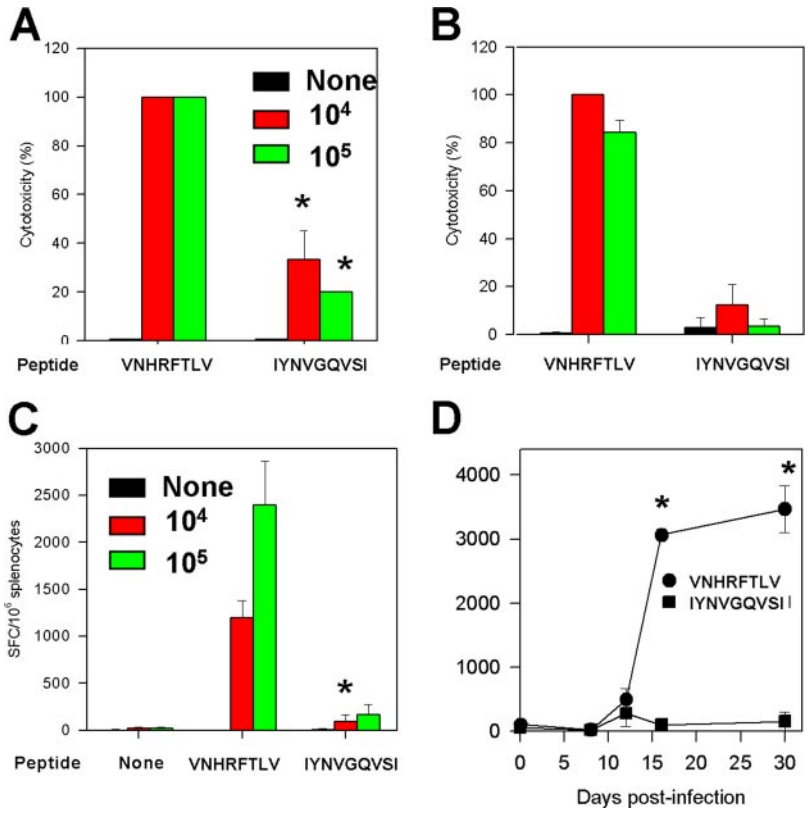


**FIGURE 3.** Specific $CD8^+$ T cell-mediated immune responses in heterozygote $F_1$ C57BL/6 × BALB/c mice challenged with different doses of *T. cruzi*. Groups of heterozygote $F_1$ C57BL/6 × BALB/c mice were challenged i.p. with $10^4$ or $10^5$ bloodstream trypomastigotes of the Y strain of *T. cruzi*. The in vivo cytotoxic activities against target cells coated with peptides VNHRFTLV or IYNVGQVSI were estimated 15 (*A*) or 30 days (*B*) following infection. Results represent the mean of four mice ± SD per group. ∗, The in vivo cytotoxicity against target cells coated with peptide IYNVGQVSI was significantly lower than against cells coated with peptide VNHRFTLV ($p < 0.01$). No statistically significant difference was detected when we compared the immune response in mice which received $10^4$ or $10^5$ parasites. *C*, IFN-γ-producing spleen cells specific for peptides VNHRFTLV or IYNVGQVSI were estimated ex vivo by ELISPOT 15 days following infection. Results represent the mean number of SFC per $10^6$ splenocytes ± SD ($n = 4$) following in vitro stimulation with the indicated peptide or medium only. ∗, The number of SFC specific for peptide IYNVGQVSI was significantly lower than VNHRFTLV-specific SFC ($p < 0.05$ in both doses). No statistically significant difference was detected in the number of SFC specific for peptide IYNVGQVSI of mice which received $10^4$ or $10^5$ parasites ($p > 0.05$). *D*, $F_1$ C57BL/6 × BALB/c mice were challenged or not (day 0) i.p. with $10^4$ bloodstream trypomastigotes of *T. cruzi*. At the indicated days, IFN-γ-producing spleen cells specific for peptides VNHRFTLV or IYNVGQVSI were estimated ex vivo by ELISPOT. Results represent the mean number of peptide-specific SFC per $10^6$ splenocytes ± SD ($n = 4$). ∗, The number of SFC specific for peptide VNHRFTLV was significantly higher than IYNVGQVSI-specific SFC ($p < 0.01$).

challenge. These values were significantly higher than cytotoxicity levels specific for target cells coated with peptides TsKb-18 or TsKb-20. In these cases, the maximum specific cytotoxicities were 52.0 ± 2.3 or 63.8 ± 4.0, respectively. The numbers of splenic IFN-γ-producing cells specific for peptides VNHRFTLV or TsKb-20 detected ex vivo by ELISPOT assay were quite similar at day 15 postinfection. However, at days 30 and 60 postinfection, the number of IFN-γ-producing cells specific for peptide VNHR FTLV was more than three times higher than cells specific for TsKb-20 (Fig. 1*B*). Despite the fact that TsKb-18 peptide has a single amino acid substitution when compared with TsKb-20, very few IFN-γ-producing cells specific for this epitope were detected ex vivo at day 15, 30, or 60 postinfection (Fig. 1*B*). Analysis of the immune T cell affinities for the peptides VNHRFTLV or TsKb-20 failed to provide any significant degree of difference (Fig. 1*C*).

We also estimated the number of IFN-γ-producing cells detected ex vivo following infection with a second strain of *T. cruzi* (G strain). We found that the number of IFN-γ-producing cells specific for peptide VNHRFTLV was significantly higher than the number of cells specific for peptides TsKb-18 or TsKb-20 (Fig. 1*D*). In mice infected with the G strain, the values of the in vivo cytotoxicity specific for target cells coated with peptide VNHR FTLV were also significantly higher than cytotoxicity levels specific for target cells coated with peptide TsKb-18 or TsKb-20 ($p < 0.001$, Fig. 1*E*).

Similar experiments performed with C57BL/6 mice infected with a third *T. cruzi* strain (CL-Brener) provided a distinct result. In these animals, we found that the number of IFN-γ-producing cells specific for peptide VNHRFTLV was significantly lower than the number of cells specific for peptides TsKb-18 or TsKb-20 (Fig. 1*D*). In mice infected with the CL-Brener strain, the values of the in vivo cytotoxicity specific for target cells coated with peptide VNHRFTLV were also significantly lower than cytotoxicity levels specific for target cells coated with peptide TsKb-18 or TsKb-20 (Fig. 1*E*).

In addition, a number of other peptides from *T. cruzi* previously described as capable of binding to $H\text{-}2K^b$ were also tested ex vivo by ELISPOT using splenic cells from C57BL/6 infected 15 or 30 days earlier with parasites of the Y strain (21–23). None of them stimulated IFN-γ-producing cells (data not shown). Based on these observations, it seems that the $CD8^+$ T cell immune response to VNHRFTLV is immunodominant during infection of C57BL/6 mice with parasites of the Y or G strain of *T. cruzi*. In contrast, during infection of C57BL/6 mice with parasites of the CL-Brener strain, $CD8^+$ T cell immune response to TsKb-18 and TsKb-20 are immunodominants.

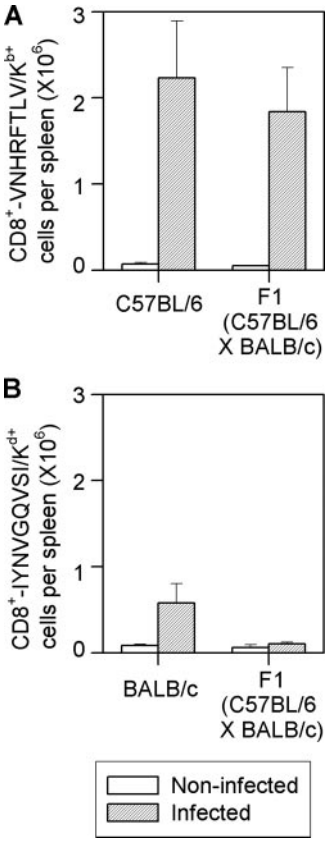


**FIGURE 4.** Staining of *T. cruzi*-specific $CD8^+$ T cells in infected homozygote and heterozygote mice. Spleen cells were collected from homozygote or heterozygote mice infected or not 35 days earlier with *T. cruzi*. The numbers of total splenic $CD8^+$ costained with either VNHR FTLV-$K^b$ pentamer (*A*) or IYNVGQVSI-$K^d$ tetramer (*B*) are shown. Data are representative of three mice ± SD. These experiments were performed twice with similar results.

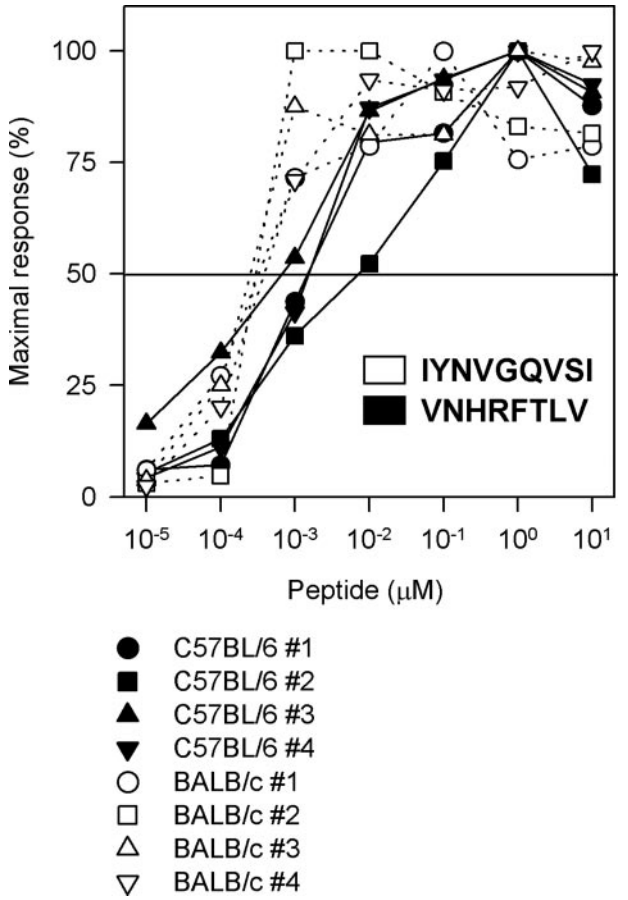


**FIGURE 5.** Recognition of epitopes VNHRFTLV and IYNVGQVSI by specific T cells as measured by the ELISPOT assays. C57BL/6 ($n = 4$) or BALB/c ($n = 4$) mice were challenged i.p. with $10^4$ or $2.5 \times 10^3$ bloodstream trypomastigotes of the Y strain of *T. cruzi*, respectively. Fifteen days after challenge, ex vivo ELISPOT assay was performed using in vitro the indicated peptide concentrations. Spleen cells from C57BL/6 or BALB/c mice were restimulated with peptides VNHRFTLV or IYN VGQVSI, respectively. The data were as normalized against the maximal values obtained with each peptide in each individual mouse. This value was considered 100%. Results of the titration curve of each individual mouse is shown.

### T. cruzi-*infected heterozygote mice displays significantly reduced $CD8^+$ T cell immune responses to certain parasites epitopes*

This immunodominance could be explained by several nonmutually exclusive reasons. The first possibility is that during *T. cruzi* infection the number of MHC-I-VNHRFTLV complexes formed on the surface of APC is higher than the other two epitopes. Alternatively, this complex could be exposed for a longer period. A third possibility is that C57BL/6 mice have a greater number of, or higher affinity, precursor T cells specific for MHC I-VNHRFTLV complexes. Also, it is possible that a competition occurs between specific T cells for APC. This last possibility could be caused, or increased, by the first three and may provide a much stronger mechanism of immunodominance. In this case, the immunodominant response would "interfere" with priming of immune responses specific for epitopes restricted by distinct MHC-I molecules (24).

To investigate some of these possibilities, we took advantage of the fact that we had previously identified two other epitopes recognized by H-$2K^k$- (TEWETGQI) or H-$2K^d$ (IYNVGQVSI)-restricted $CD8^+$ T cells from *T. cruzi* infected mice (Table I and Ref. 7). The presence of strong immune responses to epitopes restricted by distinct MHC-I alleles allowed us to investigate a possible interference of one $CD8^+$ T cell-mediated immune response on the others, or simultaneously on one another. To evaluate that possibility, we infected heterozygote and homozygote mice and compared the immune responses to three distinct epitopes. Fifteen days after challenge, homozygote mice (C57BL/6 or BALB/c) displayed >90% in vivo cytotoxicity specific for target cells coated with peptide VNHRFTLV (C57BL/6) or IYNVGQVSI (BALB/c). Heterozygote mice ($F_1$ C57BL/6 × BALB/c) also presented an in vivo cytotoxicity against target cells coated with peptide VNHR FTLV above 95%. In contrast, only 12.92 ± 5.91% of the target cells coated with peptide IYNVGQVSI were eliminated in vivo (Fig. 2*A*). Analysis of the number of splenic peptide-specific IFN-γ-producing cells revealed a similar picture. Although homozygote mice (C57BL/6 or BALB/c) had a significant number of T cells specific for the respective MHC-I-restricted epitope, heterozygote mice responded well only to H-$2K^b$-restricted epitope VNHR FTLV (Fig. 2*A*). In $F_1$ C57BL/6 × BALB/c mice, the number of T cells specific for H-$2K^d$-restricted epitope IYNVGQVSI was only 18.51% of the number detected in BALB/c mice.

Similar experiments were performed by comparing $CD8^+$ T cell immune responses of homozygote mice (C57BL/6 or B10.A) with heterozygote $F_1$ C57BL/6 × B10.A. Homozygote mice presented an immune response capable of eliminating in vivo >90% of peptide-coated target cells. Heterozygote animals also displayed an in

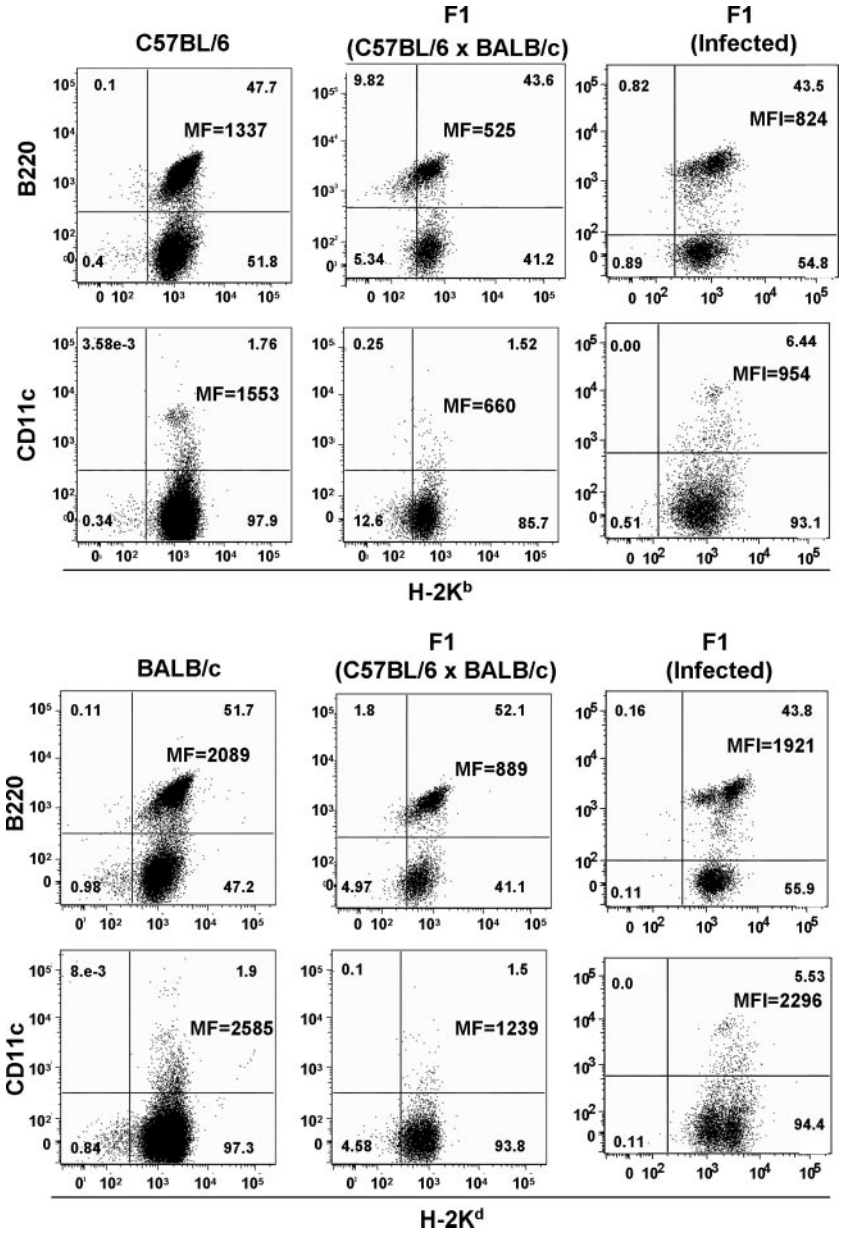


**FIGURE 6.** MHC class I expression in splenic APC cells from homozygote or heterozygote mice. Splenic cells were stained with anti-H-2K$^d$-FITC or anti-H-2K$^b$-FITC and anti-B220-PE or anti-CD11c-PE. The frequency of each cell population is indicated in the corners. The mean fluorescence (MF) of the MHC I molecules (H-2K$^b$ or H-2K$^d$) of the double-positive cells are also shown. Infected $F_1$ C57BL/6 × BALB/c mice were infected 30 days before with $10^4$ blood stream trypomastigotes. Results of each mouse group (infected and noninfected) were reproduced at least twice with identical results.

vivo cytotoxicity against target cells coated with peptide VNHR FTLV of 86.39 ± 6.90%. In contrast, in these animals only 29.5 ± 10.6% of target cells coated with peptide TEWETGQI were eliminated in vivo (Fig. 2*C*).

Analysis of the number of splenic peptide-specific IFN-γ-producing cells revealed that homozygote mice had a significant number of T cells specific for the respective MHC-I-restricted epitope. Heterozygote mice responded to the H-2K$^b$-restricted epitope VNHRFTLV similarly to homozygote animals (Fig. 2*D*). In contrast, in $F_1$ C57BL/6 × B10.A mice, the number of TEWETGQI-specific cells was 8.59% of the number detected in homozygote B10.A mice.

The last group of heterozygote mice we studied ($F_1$ B10.A × BALB/c) presented an interesting picture. When measured in vivo by cytotoxicity, the elimination of target cells coated with peptide TEWETGQI or IYNVGQVSI was lower in heterozygote mice than in homozygote ones, suggesting that immune responses of both specificities interfered with one another (Fig. 2*F*). Analysis of the number of splenic peptide-specific IFN-γ-producing cells revealed that homozygote mice had a significantly higher number of T cells specific for the respective MHC-I-restricted epitope. Heterozygote mice had <40% inhibition of the immune response to the H-2K$^k$-restricted epitope TEWETGQI when compared with homozygote animals. However, a drastic reduction of 92.75% was seen in the immune response to H-2K$^d$-restricted epitope IYN VGQVSI (Fig. 2*F*).

The impaired immune responses observed against these parasite epitopes in heterozygote mice could be dependent on the dose of *T. cruzi* used for challenge. To test this hypothesis, we challenged each mouse with $10^5$ parasites, a dose 10 times higher than used in the previous experiments. An increase in the dose of parasite challenge did not improve the limited immune response observed in $F_1$ C57BL/6 × BALB/c mice to the epitope IYNVGQVSI. Both, the in vivo cytotoxicity or the number of IFN-γ-producing cells were similar in mice challenged with $10^4$ or $10^5$ parasites (Fig. 3, *A–C*). In contrast, the number of IFN-γ-producing cells specific for the immunodominant epitope VNHRFTLV increased in mice challenged with $10^5$ parasites (Fig. 3*C*). Also, the reduced immune responses observed against the epitope IYNVGQVSI in $F_1$ C57BL/6 × BALB/c mice was not due to a delay in the priming

and/or expansion of specific $CD8^+$ T cells. The kinetics of IFN-$\gamma$-producing T cells specific for VNHRFTLV showed a vigorous expansion during the first 30 days following infection while cells specific for IYNVGQVSI expanded very little in that period (Fig. 3*C*). Specific immune response measured by in vivo cytotoxicity to the epitope IYNVGQVSI or VNHRFTLV was performed in mice infected 60 days earlier. Although the immune response to VNHR FTLV was still high (91.1 $\pm$ 0.5%, $n = 3$) at that point in time, the immune response to the epitope IYNVGQVSI was still negative (0.0 $\pm$ 0.0, $n = 3$, data not shown).

Finally, we used the staining of specific T cells to visualize whether in fact there was a significantly lower number of $CD8^+$ T cells specific for the epitope IYNVGQVSI in $F_1$ C57BL/6 $\times$ BALB/c mice. As shown in Fig. 4*A*, the total number of $CD8^+$ spleen cells from infected $F_1$ C57BL/6 $\times$ BALB/c mice stained with the pentamer VNHRFTLV/$K^b$ was only 18.35% lower than the number of cells in C57BL/6. When similar analysis was performed to estimate the number $CD8^+$ spleen cells stained with the tetramer IYNVGQVSI/$K^d$, we found that infected $F_1$ C57BL/6 $\times$ BALB/c mice had 91.5% less cells than BALB/c mice (Fig. 4*B*).

Analysis of the immune T cell affinities for the peptides VNHR FTLV or IYNVGQVSI were performed using immune T cells from C57BL/6 or BALB/c mice, respectively. This analysis provided evidence that the affinity of T cells specific for the H-$2K^d$-restricted epitope IYNVGQVSI was slightly higher than the affinity of the T cells specific for the H-$2K^b$-restricted epitope VNHRFTLV, a fact that cannot explain the immunodominance of the later (Fig. 5).

Analysis of the expression of MHC-I molecules H-$2K^b$ and H-$2K^d$ were performed using spleen cells of C57BL/6, BALB/c, and $F_1$ C57BL/6 $\times$ BALB/c mice (Fig. 6). The expression of either H-$2K^d$ or H-$2K^b$ molecules on the surface of $F_1$ C57BL/6 $\times$ BALB/c mice was lower than the homozygote animals. Nevertheless, in normal $F_1$ C57BL/6 $\times$ BALB/c mice, the expression of H-$2K^d$ molecules (nonresponder allele) in $B220^+$ or $CD11c^+$ or other cell types were higher than the expression of H-$2K^b$ molecules (responder allele). The same picture was observed when we analyzed spleen cells from infected $F_1$ C57BL/6 $\times$ BALB/c. Therefore the absence of immune response in the $F_1$ C57BL/6 $\times$ BALB/c to the H-$2K^d$-restricted epitope IYNVGQVSI cannot be attributed to a significantly lower expression of H-$2K^d$ molecules on APCs. Other experiments provided below also contribute to this interpretation.

### *Immune responses of heterozygote mice to* T. cruzi *epitopes following immunization with recombinant adenovirus are not impaired*

A possible explanation for the impaired immune response to the H-$2K^d$-restricted epitope IYNVGQVSI in heterozygote mice could be the development of a severely skewed T cell repertoire in the presence of MHC-I H-$2K^b$ or H-$2K^k$ molecules. Also, the number of H-$2K^d$ molecules on the surface of cells from heterozygote mice could be responsible for the reduced response to the epitope IYNVGQVSI. To test these hypotheses in vivo, we immunized homozygote (BALB/c) and heterozygote ($F_1$ C57BL/6 $\times$ BALB/c or $F_1$ B10.A $\times$ BALB/c) mice with a recombinant adenovirus expressing the TS protein, the Ag which contains the epitope IYNVGQVSI (20). Following immunization with recombinant adenovirus, the peak $CD8^+$ T cell immune response is at 15 days (25). The immune responses of heterozygote mice measured by the in vivo cytotoxicity against target cells coated with the peptide IYNVGQVSI was higher than homozygote BALB/c (Fig. 7*A*). Therefore, after immunization with a recombinant virus, the heterozygote mice immune response to the peptide IYNVGQVSI was not impaired.

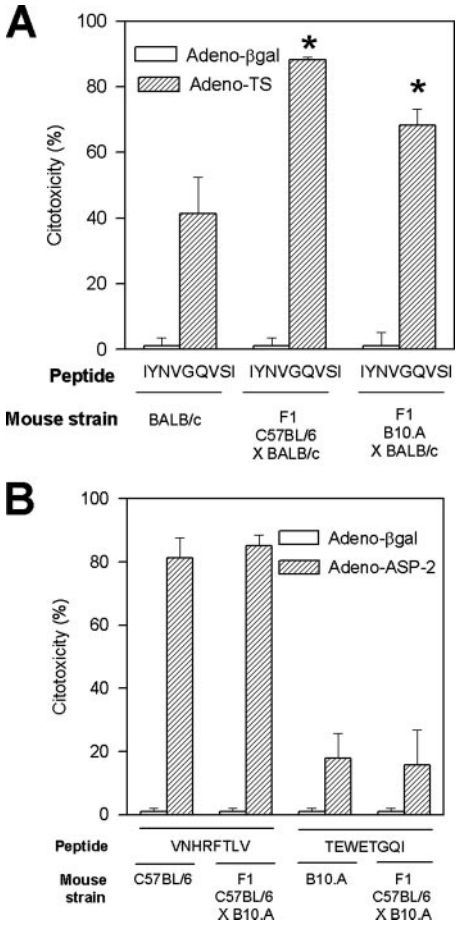


**FIGURE 7.** Immune responses of homozygote and heterozygote mice to *T. cruzi* epitopes following immunization with recombinant adenovirus. The indicated mouse strains were immunized i.m. 15 days earlier with recombinant adenovirus expressing a control protein (Adeno-$\beta$gal), *T. cruzi trans*-sialidase (adeno-TS) (*A*), or amastigote Surface Protein-2 (Adeno-ASP-2) (*B*). Specific immune responses to the indicated peptides were estimated by the in vivo cytotoxicity assay. Results represent the mean of four mice $\pm$ SD per group. *, The in vivo cytotoxicity of heterozygote $F_1$ C57BL/6 $\times$ BALB/c or $F_1$ B10.A $\times$ BALB/c mice against target cells coated with peptide IYNVGQVSI were significantly higher than homozygote BALB/c mice ($p < 0.01$ in both cases). Results are representative of three independent experiments.

The limited immune response to epitope TEWETGQI in heterozygote ($F_1$ C57BL/6 $\times$ B10.A) could be explained by a reduced Ag presentation in vivo to H-$2K^k$-restricted T cells specific for the epitope TEWETGQI caused by some intrinsic property of the H-$2K^b$-restricted epitope VNHRFTLV. Alternatively, T cells specific for VNHRFTLV could inhibit the activation of T cells specific for TEWETGQI by a direct interaction between $CD8^+$ T cells. To approach these questions, we used a second recombinant adenovirus expressing the ASP-2 of *T. cruzi.* This Ag contains both of these epitopes (20). Homozygote (C57BL/6 or B10.A) or heterozygote ($F_1$ C57BL/6 $\times$ B10.A) mice immunized with the recombinant adenovirus adeno-ASP-2 developed immune response specific for H-$2K^b$-restricted epitope VNHRFTLV significantly higher than the response to H-$2K^k$-restricted epitope TEWETGQI. Nevertheless, the immune response to the epitope TEWETGQI was identical in homozygote or heterozygote mice (Fig. 7*B*). These results indicated that although the immune response to the epitope VNHR FTLV seems to be stronger following immunization with adeno-ASP-2 virus, it did not interfere with the smaller response to the epitope TEWETGQI.

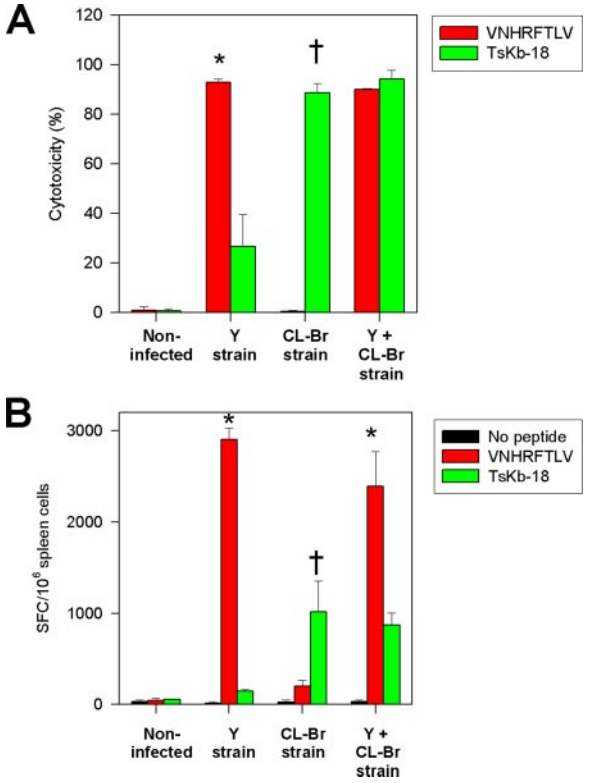


**FIGURE 8.** Specific $CD8^+$ T cell-mediated immune responses in C57BL/6 mice challenged with one or two strains *T. cruzi*. C57BL/6 mice were challenged or not i.p. with $10^4$ bloodstream trypomastigotes of the Y strain and/or $10^5$ trypomastigote culture forms of the CL-Brener strain of *T. cruzi*. *A*, Fifteen days after infection, the in vivo cytotoxic activity against target cells coated with peptides VNHRFTLV or TsKb-18 were determined. Results represent the mean of four mice ± SD per group. *B*, Fifteen days after infection, IFN-γ-producing spleen cells specific for peptides VNHRFTLV or TsKb-18 were estimated ex vivo by ELISPOT assay. Results represent the mean number of SFC per $10^6$ splenocytes ± SD ($n = 4$) following in vitro stimulation with the indicated peptide or medium only. *, Immune response specific for the peptide VNHRFTLV was significantly higher than to peptide TsKb-18 ($p < 0.01$). †, The immune response specific for the peptide TsKb-18 was significantly higher than to peptide VNHRFTLV ($p < 0.01$). Results are representative of two independent experiments.

Comparison of the CD4 T cell dependence on the immune response following infection with *T. cruzi* or immunization with recombinant adeno-ASP-2 was performed using CD4 knockout mice. We observed that these mice had a severe reduction on the in vivo cytotoxic immune response to the epitope VNHRFTLV following infection or immunization proving that both cytotoxic immune responses were dependent on $CD4^+$ T cell activation (data not shown).

### *Immune responses of C57BL/6 mice to* T. cruzi *epitopes following infection with two parasite strains containing distinct immunodominant epitopes*

Precisely how this immunodominant response to VNHRFTLV epitope interferes with T cells of other specificities during *T. cruzi* infection was not clear. It was possible that T cells specific for the epitope VNHRFTLV prevented the presentation of the other epitopes when both were present in the same APCs. In that case, we predicted that strong responses to both epitopes (dominant and subdominant) would be induced when the epitopes were expressed in different APCs. To test this hypothesis, we infected C57BL/6 mice with two different parasite strains expressing distinct immunodominant epitopes. As described above, infection with the Y strain of *T. cruzi* induced a strong response to the epitope VNHR FTLV, but not to TsKb-18, as measured by in vivo cytotoxicity or ELISPOT assay (Fig. 8). In contrast, infection with parasites of the CL-Brener strain elicited strong immune responses to the epitope TsKb-18, but not to VNHRFTLV (Fig. 8). As we predicted, mice infected with both parasite strains at the same time displayed strong immune response to both epitopes (Fig. 8). This result suggests that separation of the "dominant" and "subdominant" epitopes can circumvent the immunodominance.

## Discussion

Interference or competition between $CD8^+$ T cells restricted by distinct MHC-I molecules have been described following immunization with different Ags (24, 26–28). It is a powerful means used by $CD8^+$ T cells specific for a particular MHC-I-peptide complex to restrict expansion of T cells of all other specificities establishing an immunodominant response. Whether this type of interference/competition between $CD8^+$ T cells restricted for different MHC-I molecules could in fact be critical during infections is still a matter of debate (29–31). Here, we demonstrate that $CD8^+$ T cell immune response specific for H-$2K^b$-restricted epitope VNHRFTLV is immunodominant during infection of C57BL/6 mice with *T. cruzi* of Y and G strains. In addition, we have demonstrated that $CD8^+$ T cells specific for the immunodominant epitope VNHRFTLV greatly interferes with the generation of T cells restricted for other MHC-I molecules. Thus, following infection of heterozygote mice with *T. cruzi*, a significant reduction in the priming of $CD8^+$ T cells restricted for H-$2K^d$ or H-$2K^k$ molecules was observed providing evidence that interference/competition is an important factor down-modulating immune response during experimental infection with this human pathogen. In the case of infection of $F_1$ B10.A × BALB/c mice, a mutual interference takes place reducing both specific responses to the H-$2K^d$- or H-$2K^k$-restricted T cells.

Precisely how this immunodominant response to VNHRFTLV epitope interferes with T cells of other specificities is not clear. The interference observed following infection is not related to the epitopes themselves or caused by a skewed T cell repertoire in heterozygote mice as $F_1$ C57BL/6 × BALB/c mice immunized with recombinant adenovirus developed immune responses higher than the homozygote BALB/c to the H-$2K^d$-restricted epitope IYNVGQVSI.

A few nonmutually excluding hypotheses can be proposed. Interference could be due to the presence of $CD4^+CD25^+$ regulatory T cells. Thus, Haeryfar et al. (32) found that $CD4^+$ $CD25^+$ regulatory T cells selectively suppressed responses to the most immunodominant $CD8^+$ T cell epitopes in three distinct systems. However, in a recent study, Kotner and Tarleton (33) described that depletion of these cells had little impact on the immune response of $CD8^+$ T cells specific for *T. cruzi* immunodominant or subdominant epitopes. Alternatively, it could be possible that $CD8^+$ T cells specific for VNHRFTLV inhibit expansion/proliferation of T cells of other specificities by acting directly on responder T cells. This possibility also seems unlikely because after immunization with adeno-ASP-2, heterozygote and homozygote mice presented a strong response to VNHRFTLV and also generated immune responses of a similar degree to TEWETGQI epitope. Also, during concomitant infection with two parasites strains, the immune response to VNHRFTLV did not interfere with the expansion of TsKb-18 specific cells.

The explanation that we favor is that $CD8^+$ T cells specific for VNHRFTLV compete with T cells of other specificities in limiting APC resources during priming with *T. cruzi* (23, 26, 27). This

hypothesis is supported by the experiment where we infected C57BL/6 mice with two parasite strains containing distinct immunodominant epitopes. In this scenario, distinct parasite strains are most likely processed by different APCs, allowing strong immune response to be developed against both epitopes.

To generate an immunodominant response, H-2K$^b$-VNHR FTLV complexes should be formed in a larger number, or first, leading to a rapid expansion of specific T cells. These T cells would use the available APC resources precluding or reducing activation of T cells of other specificities. The possibility that more H-2K$^b$-VNHRFTLV complexes are formed has no experimental support. In fact, the observation that homozygote mice develop powerful immune responses to H-2K$^d$- IYNVGQVSI or H-2K$^k$-TEWETGQI complexes suggests that the number of complexes formed on the surface of APCs from heterozygote mice should be sufficient to promote a strong T cell response. Alternatively, the number of T cell precursors and/or their affinities could be factors that accelerate the immune response causing an immunodominance to the epitope VNHRFTLV. In experiments designed to evaluate whether CD8$^+$ T cells from infected BALB/c had a lower affinity for H-2K$^d$-IYNVGQVSI complexes than cells from infected C57BL/6 mice for H-2K$^b$-VNHRFLTV, we have found that this is not the case (Fig. 5). Therefore, it seems that the TCR affinity cannot explain the discrepant activation in heterozygote mice.

After contact with APCs, the expansion of T cells with higher affinity or more precursors for the MHC-I-peptide complex would overcome T cells specific for other parasite peptides. However, following immunization with recombinant adenovirus, we were not able to detect any interference in heterozygote mice when compared with homozygote ones, indicating that the affinity/more precursor hypotheses are not a likely explanation for the studied competition phenomenon.

Hence, in our opinion, the most plausible explanation is that this immunodominance is regulated by the timing and duration of H-2K$^b$-VNHRFTLV display on the surface of APCs. Earlier studies have shown that prolonged Ag exposure influences the generation of immune responses improving priming of T cells specific for immunodominant epitopes (34–36). Indeed, VNHRFTLV epitope is present in proteins expressed by trypomastigotes (infective forms) and amastigotes (intracellular stages) of the Y strain of *T. cruzi* (37, 38). This fact may accelerate and improve immune response by providing an advantage for T cells specific for H-2K$^b$-VNHRFTLV complexes on the surface of APC. In contrast, the epitopes IYNVGQVSI or TEWETGQI are expressed only by trypomastigotes (infective forms) or amastigotes (intracellular stages), respectively (38, 39). This last hypothesis would explain the differences observed following infection or immunization of heterozygote mice with adenovirus-expressing parasite Ag. Importantly, evidence exists that immunodominance may be dictated by the infective strain of *T. cruzi*. Thus, while expressing the VNHR FTLV epitope (21), the Brazil strain of *T. cruzi* induced an immunodominant response specific for TsKb-20 and TsKb-18 epitopes (8). This strain-dependent switch has no immediate explanation, but it indicates the importance of parasites in controlling the immunodominant CD8$^+$ T cell response. In the case of the CL-Brener strain, we found during sequence analysis of the cDNA of ASP-2 that the deduced AA sequence was VNYDFTIV (amino acid substitutions are underlined). A synthetic peptide representing this epitope was not recognized by CD8$^+$ T cells specific for the epitope VNHRFTLV (data not shown).

Although previous studies provided compelling evidence of competition between CD8$^+$ T cells for APCs expressing simultaneously two distinct MHC-I molecules, the substrate they compete for is not yet known. This competition occurs early in the activation process of T cells (24), and involves membrane proteins required for full T cell activation and/or soluble factor(s) such as cytokines (1, 24, 40). Despite its potential as a mechanism of immunodominance, during mouse infection with lymphocytic choriomeningitis virus, this interference/competition was not apparent (29, 30). Also, ablation of immunodominant epitopes from *Listeria monocytogenes* did not cause an increase in the immune responses to subdominant epitopes during infection, suggesting that there was no apparent interference/competition among CD8$^+$ T cells (31). Very recent reports suggest the opposite, that there is interference between CD8$^+$ T cells of different MHC-I specificities during viral infection with HIV, SIV. Severe acute respiratory syndrome and vaccinia (41–44). Thus, we believe that our observation reinforces the idea that a competitive mechanism for CD8$^+$ T cell immunodominance occurs during infectious diseases with different pathogens.

Although we do not yet know precisely the mechanism used to generate this strong non-MHC specific immunodominance, we would like to suggest that it has an important biological implication for a chronic parasitic disease. In the case of self-resolving common acute viral or bacterial infections, immunodominance focuses the response on few determinants. It was suggested that this strategy successfully maximizes the power of effector immune responses and, at the same time, minimizes the risk of autoimmunity (6). In the case of *T. cruzi* infection (Chagas' disease), immunodominance may have a similar function and may guarantee host survival for a long period of time. However, on the negative side, the immunodominance described here seems to hamper *T. cruzi*-infected mice (homozygotes or heterozygotes) in their development of stronger, and/or broader, specific immune responses. In addition, an immune response relying mainly on few immunodominant CD8$^+$ T cell epitopes may favor the escape of immune response associated with parasite mutation or any particular antigenic variation process. An example supporting this hypothesis was described in a chronic viral infection with hepatitis C virus. A strong immunodominant human CD8$^+$ T cells response was developed causing the selection of an escape mutant (45). Regardless, we favor the hypothesis that either restriction or avoidance of maximal host effector functions is a critical step to prevent parasite elimination, allowing parasite persistence and leading to a chronic lifelong infection.

One important immunological aspect to be considered in the rational design of a vaccine for Chagas' disease is the pathogenesis and the intense myocarditis composed mainly of CD8$^+$ T cells elicited during *T. cruzi* infection. Thus, following vaccination with plasmid DNA or recombinant proteins, we generated memory CD8$^+$ T cells that, after a challenge with *T. cruzi,* displayed a very fast anamnestic immune response (7). These cells were specific for immunodominant epitopes VNHRFTLV or TEWETGQI (7). Such a strong immune response did not cause any discernible immunopathology. It in fact led to a reduced parasite development and prevented the establishment of chronic phase tissue pathologies (46, 47). Also, some of these vaccinated mice completely cleared the parasite and established sterile immunity (46, 47). These findings are also supported by our vaccination studies when we obtained reduced parasitism and limited disease development by increasing the immune responses to these same immunodominant epitopes (20, 46, 47). Thus, the development of a restricted T cell response confined to a limited number of epitopes may be important to avoid elicitation of potentially self-reactive or pathogenic T cell clones.

In summary, our results provide evidence of a competitive expansion of CD8$^+$ T cells restricted by different MHC-I molecules.

*T. cruzi* infection may be an interesting model to evaluate the molecular basis for $CD8^+$ T cells competition and the biological role of immunodominance during infection with intracellular protozoan parasites, which are major causes of life-threatening chronic infection in humans. Among the protozoan parasites, $CD8^+$ T cells have also been shown to greatly influence immunity against *Plasmodium*, *Toxoplasma gondii*, and *Leishmania sp.* (48–53). Whether this competitive priming and expansion of T cells restricted by different MHC-I molecules also occurs during infection with these parasites also needs to be addressed.

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## Disclosures

The authors have no financial conflict of interest.

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# ***CONCLUSÕES***

1- Linfócitos T CD8+ na infecção pelo *T. cruzi* são restritos a epítopos provenientes de antígenos pertencentes à super família das *trans*-sialidases.

2- Há um atraso na ativação das células T CD8+ específicas durante a infecção pelo *T. cruzi*.

3- A vacinação de camundongos acelera a ativação das células T CD8+ após a infecção pelo *T. cruzi*.

4- A expansão das células T CD8+ é dependente da carga parasitária e da replicação do parasita. A ativação delas é dependente de LT CD4+ restritos por MHC-II.

5- Os mecanismos efetores dos linfócitos T CD8+ são produção de IFN-γ e citotoxicidade mediada por perforina.

6- Durante a infecção pelo *T. cruzi* ocorre o fenômeno de imunodominância, o qual é causado por uma competição entre células T de diferentes especificidades.

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