identified larvae in the placenta and fetal blood vessels, histopathologically. They concluded that *T. canis* larvae were able to migrate through the placenta during pregnancy. However, because they did not examine the neonates after birth, they could not eliminate the possibility of trans-lactational transmission of the larvae from mother to neonates after delivery.

It is well documented that malaria infection induces placental injury, resulting in fetal loss in both humans and mice [19,20]. In murine toxocariasis, the litter sizes from infected mice are smaller than those from uninfected controls [21,22]. These data suggest that *T. canis* infection in mice can lead to mechanical injury of the placenta and a resultant decrease of litter size when the infection occurs during pregnancy.

Yet, in spite of these difficulties, newborns are still successfully delivered in most cases. In another previous study, larvae were found in offspring on day 5 after birth [15], suggesting suckling behavior might cause maternal–newborn transmission of *T. canis* larvae. In fact, our preliminary experiment revealed that larvae were first identified in offspring 11 days after birth (unpublished data). Thus, we hypothesized that larvae could migrate from mother to newborn mice through the mammary gland during suckling. The present findings support this hypothesis.

In general, *T. canis* larvae in mice settle in the brain and skeletal muscle after migration through the systemic circulation, and survive for a long period [4,8]. However, because we could not find any larvae in the mammary gland of non-pregnant infected mice, the larvae must be aroused by some sort of stimuli in order to migrate from those organs to the mammary gland. Prolactin, a lactogenic hormone, plays an essential role in the development of breast tissue. None of the non-pregnant mice not treated with prolactin showed the presence of larvae, in either the acute or chronic stage of infection, whereas prolactin-treated mice exhibited *T. canis* larvae infection in the mammary glands. One previous study discussed the relationship between *T. canis* infection and prolactin [23], reporting that the administration of prolactin led to a reduction in the number of larvae in infected mice. This may be related to the finding that prolactin acts as an immunomodulatory agent or proinflammatory cytokine in autoimmune diseases [24], and in several parasitic infections [25–28].

Eosinophil infiltration is a common feature in tissue-invading nematode infections, such as gnathostomiasis and trichinosis [29]. In toxocariasis, an eosinophilic granulomatous response is a typical pathological finding both in humans and in experimentally infected animals including mice [30,31]. Furthermore, eosinophil infiltration was demonstrated not only in the tissue adjacent to the larvae but also in that through which the larvae had passed [32]. These pathological changes are thought to be stimulated by the metabolic products from the larvae [29]. Therefore, we assumed that eosinophil infiltration around the capsule of the mammary gland in the prolactin-treated mice might be attributable to the migration of larvae into the mammary gland following stimulation of the tissue-arrested larvae.

The mechanism of this stimulation of tissue-arrested larvae during breast-feeding has yet to be elucidated. In hookworm infection, tissue-arrested larvae of *Ancylostoma caninum* were activated *in vitro* by TGF-β [33]. No such connection, however, has been demonstrated in *Toxocara* infection. The secretion of TGF-β is tightly regulated by the hormones estrogen and prolactin, and they are critical factors in the tissue-specific regulation of the local production of TGF-β in the mammary gland of the rat [34]. Therefore, we presumed that a similar cytokine reaction could be induced by prolactin, and may contribute to the reactivation of cryptic larvae in *Toxocara*-infected mice.

In the present study, we found clear evidence that prolactin is one of the factors in the lactational transmission of *T. canis* larvae from mother mice to offspring. Further investigation is needed to elucidate the precise mechanism of the stimulation of tissue-arrested larvae in mice.

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Trypanosoma cruzi infection from the view of CD8$^+$ T cell immunity — An infection model for developing T cell vaccine

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Abstract

Chagas' disease is caused by *Trypanosoma cruzi* (*T. cruzi*) which was once prevalent in Central and South America. Although the recent success in Triatoma vector control has made the disease being possibly "extinct" in the near future, the development of effective preventive and therapeutic vaccines is still necessary to prevent the resurgence of the neglected infection. In addition to the importance for containing the disease, *T. cruzi* infection presents unique features for elucidating hosts' immune responses against intracellular infectious agents. Due to its biological capacity for invading into principally any types of cells and for causing systemic infection which damages particularly muscle and neural cells, T cell immunity is critical for resolving its infection. Although T cell-mediated immune responses have been, so far, extensively investigated in viral and bacterial infections, parasitic infection such as malaria has presented epoch-making discovery in T cell immunity. Recent advances in the analyses of T cell-mediated immune responses against *T. cruzi* infection now make this infectious disease potentially more suitable for detecting subtle immunological changes in hosts' immune defense upon modifying immune system. The current review focuses on the usefulness of *T. cruzi* infection as a model for developing effective CD8$^+$ T cell-mediated vaccine against intracellular infectious agents.

Keywords: CD8$^+$ T cells; *Trypanosoma cruzi*; T cell vaccine

1. Introduction

Trypanosoma cruzi (*T. cruzi*) is the etiological agent of Chagas' disease in Central and South America [1]. We see drastic epidemiological changes in Chagas' disease since the initiation of large-scale vector control program which is called "Southern Cone Initiative" [2,3]. As the incidence of Triatoma vector-transmitted Chagas' disease has dramatically declined, the importance of the disease has become controversial from the clinical point of view. However, by considering the zoonotic feature of Chagas' disease which is even prevalent among wild and domestic animals, it is too optimistic to expect not only its eradication but even its control. One more important issue which we should consider in Chagas' disease is that there are thousands of chronically-infected people around the world [2,3], and that there is no concrete surveillance system to detect and control the disease. *T. cruzi* infection, therefore, still holds an enormous potential to resurge, once the economic back-up to the current effort for vector control program discontinues.

Since the successful vaccination and eradication campaign against smallpox [4], the induction of protective immune responses by vaccines has become a mainstream strategy to prevent the onset of and death from infectious diseases. Most of the diseases had been considered preventable by the induction of neutralizing antibodies [5,6]; however, it's now become obvious that some infectious agents such as the human immunodeficiency virus (HIV) could be hardly controlled well by the conventional vaccine strategies [6,7]. Among various immunological effector mechanisms including innate and acquired immunity, T cells are critical for resolving intracellular infectious diseases [8,9]. Since CD8$^+$ T cells possess effector function to suppress the replication of infectious pathogens in vivo [6], the research area aiming for the induction of CD8$^+$ T cell-mediated protective immunity has

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become a center of intense research efforts to find control measures against antibody-resistant pathogens [10,11].

Characterization of T cell immunity has been, so far, achieved mainly in viral and bacterial infections. Lymphocytic choriomeningitis virus infection is well known as a useful infection model in immunology especially represented by the discovery of MHC restriction [12]. Enormous efforts for developing T cell vaccine against HIV infection have led to the new discoveries in T cell immunity [13]. Analyses of hosts' T cell immunity upon *Listeria monocytogenes* infection contributed to the detailed characterization of $CD8^+$ T cell immune responses [14].

Regarding the parasitic infections, malaria is literally the sole parasitic disease which added substantial knowledge to the advancement of research on $CD8^+$ T cells [15]. Prime-boost vaccination regimen, which demonstrated, for the first time, that the augmentation or "boost" of $CD8^+$ T cell immune responses is feasible, was discovered during the research on malaria vaccine development [16,17]. In addition, the importance of interleukin (IL)-4 in $CD8^+$ T cell immune responses was first documented in malaria research [18].

Despite these intense efforts for developing preventive and therapeutic $CD8^+$ T cell-mediated vaccine against intracellular infectious agents, the goal is still far away to reach and there are still many unsolved questions to be answered in T cell immunity. Chagas' disease, besides its clinical importance, presents unique features in immunological interactions between host and infectious agent. Of particular interest, as it invades and replicates in essentially all types of cells of mammalian hosts, T cell-mediated immunity is critical for resolving the infection [19]. The depletion of $CD8^+$ or $CD4^+$ T cells results in unrelenting parasitemia and a fatal outcome in mice [20–22], supporting the importance of T cell immunity in Chagas' disease. Since *T. cruzi* possesses much more antigens than viruses or bacteria, the complexity of parasitic protozoa could be potentially disadvantageous for analyzing host–parasite relationships. However, taking advantage of *T. cruzi* infection could be, on the contrary, beneficial for searching for new phenomena in infections, which might be hardly visible, and therefore could not be elucidated in viral or bacterial infections. A newly-established experimental system to analyze T cell immunity in experimental Chagas' disease would be a useful infection model to develop effective T cell vaccine, a long-awaited strategy to combat intracellular infectious diseases.

2. $CD8^+$ T cells as a main effector for developing T cell vaccine

The typical kinetics of $CD8^+$ T cell immune responses after infections or vaccinations is described in Fig. 1. Although $CD8^+$ T cells initiate their replication after just a brief encounter with specific antigen and expand swiftly (Expansion phase) [23,24], the responses of antigen-specific $CD8^+$ T cells dwindle quickly, leading to the steep decrease of its cell number (Contraction phase) [25]. The fade of $CD8^+$ T cell immune responses, however, does not imply that the primed antigen-specific $CD8^+$ T cells revert to the initial baseline, and in reality, a part of the cells remain alive as memory T cells (Memory phase) [26]. Naïve and memory T cells are different both in phenotypes and in biological features [27,28]. The maintenance of memory T cells requires IL-7 and IL-15 for their survival, but does not require the presence of any specific antigens [8]. The memory $CD8^+$ T cells are stably maintained and last even for life. One of the critical factors for determining the size of antigen-specific memory T cells is the initial burst size of $CD8^+$ T cell responses [26]. As shown in Fig. 1, the bigger the initial $CD8^+$ T cell burst size is, the more memory T cells remain for a long term. The genesis of memory T cells is recognized as one of the important factors for the development of effective T cell vaccine, since the remaining number of memory T cells and the induction of a balanced ratio of memory T cell subsets are correlated with the maintenance of long-term protective immunity against infections [29]. In another word, maintaining more memory T cells implies that the recall $CD8^+$ T cell responses after re-infection could be faster and more effective for suppressing the growth of re-invading pathogens. In that sense, to discover a regimen for the genesis of more memory T cells in number will be critically needed to develop an innovative future vaccination strategy.

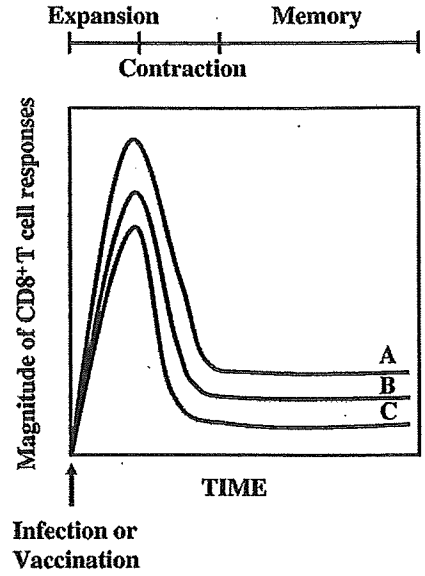
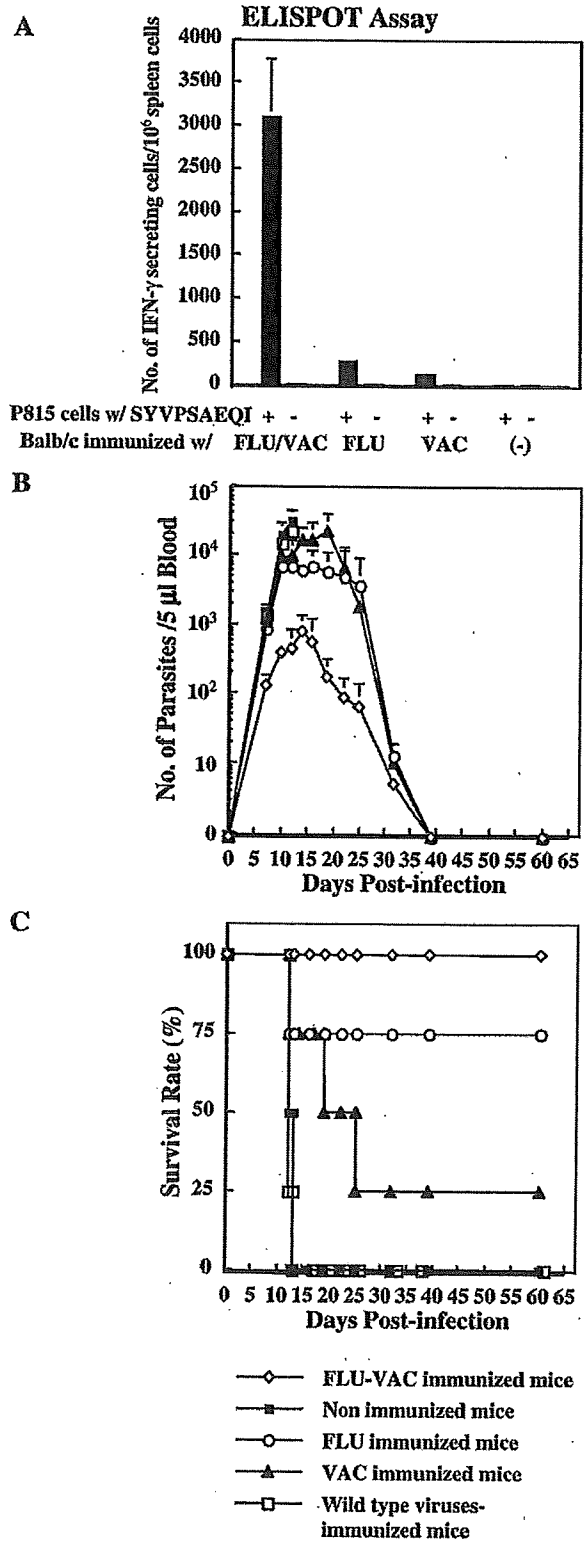


Fig. 1. The typical kinetics of $CD8^+$ T cell immune responses after infections or vaccinations. $CD8^+$ T cells initiate their replication after just a brief encounter with specific antigen and expand swiftly (expansion phase). The responses of antigen-specific $CD8^+$ T cells dwindle quickly, leading to the steep decrease of its cell number (contraction phase). The fade of $CD8^+$ T cell immune responses does not imply that the primed antigen-specific $CD8^+$ T cells revert to the initial baseline, and in reality, a part of the cells remain alive as memory T cells (memory phase). Naïve and memory T cells are different both in phenotypes and in biological features. The maintenance of memory T cells requires IL-7 and IL-15 for their survival, but does not require the presence of any specific antigens. The memory $CD8^+$ T cells are stably maintained and last even for life. One of the critical factors for determining the size of antigen-specific memory T cells is the initial burst size of $CD8^+$ T cell responses. The bigger the initial $CD8^+$ T cell burst size is, the more memory T cells remain for a long term (Line A>B>C). The genesis of memory T cells is recognized as one of the important factors for the development of effective T cell vaccine, since the remaining number of memory T cells is critical for the maintenance of long-term protective immunity against infections.



3. Prime-boost vaccination regimen

Enumeration of antigen-specific $CD8^+$ T cells became feasible by several methods [30–33]. Since $CD8^+$ T cells are well known for possessing cytolytic capability, ^{51}Chromium-release assay became a conventional way for detecting the presence of cells [30]. Technological advances for analyzing $CD8^+$ T cells include interferon-γ (IFN-γ) ELISPOT assay [31], intracellular staining for IFN-γ [32] and MHC-tetramer/pentamer technology [32,33]. Since IFN-γ is regarded as one of the most important cytokines for containing infectious agents, the enumeration of IFN-γ-secreting antigen-specific $CD8^+$ T cells by ELISPOT assay has become a valuable way for assessing vaccine efficacy [34]. In the majority of cases, the induction of more antigen-specific $CD8^+$ T cells in number enumerated by IFN-γ ELISPOT assay is well correlated with the improved protective immunity against infections [35].

Prime-boost vaccination regimen is an epoch-making vaccine strategy, since the protocol has demonstrated, for the first time, that the boost of $CD8^+$ T cell responses is feasible [16,17]. Although the protocol is now being applied to develop T cell vaccines against several infectious agents [34,35], there are still many points to be modified to improve its vaccine efficacy. In case of HIV vaccine development, the feasibility of $CD8^+$ T cell-mediated preventive vaccine is now questioned due to both the immunological features of $CD8^+$ T cell immune responses and the biological features of HIV itself [10,13]. The efficacy of prime-boost vaccination regimen against malaria in humans was also not as good as expected [36]. Therefore, despite the general agreement that the protocol is innovative, the more detailed characterization and the accumulation of knowledge on T cell immunity should be achieved to improve the protocol from the view of practical vaccine development.

Fig. 2. Protection of mice by immunization with the recombinant influenza and vaccinia viruses against the lethal infection of *P. yoelii* CSP-expressing *T. cruzi*. (A) Each of the six mice was first immunized with the 5×10^3 PFU SYVPSAEQI-expressing recombinant influenza virus, and then given a booster injection of the 2×10^7 PFU CSP-expressing recombinant vaccinia virus, i.p. Seventeen days after the last immunizing dose and 1 day before the *T. cruzi* challenge infection, two mice were sacrificed and their spleens were removed. The standard ELISPOT assay was performed by using freshly isolated spleen cells as effector cells. Spleen cells isolated from mice immunized with only recombinant influenza virus, from mice immunized with only recombinant vaccinia virus or from non-immunized mice were included for the assay. The results are described as mean values of IFN-γ-secreting cells per 10^6 freshly isolated spleen cells±SE. (B, C) Twenty mice in five groups, four mice in each group, were treated as follows. The first group received recombinant influenza virus with a booster injection of recombinant vaccinia virus (open diamonds). The second group received 5×10^3 PFU of recombinant influenza virus (open circles), while the third received 2×10^7 PFU of recombinant vaccinia virus for immunization (filled triangles). The remaining four mice were first immunized with 5×10^3 PFU wild-type influenza virus, s.c., and then given a booster injection of 2×10^7 PFU wild-type vaccinia virus, i.p., 2 weeks later (open squares). Non-immunized mice were included for the study (filled squares). After immunizations, all mice were challenged with 8×10^5 *P. yoelii* CSP-expressing *T. cruzi*, i.m., 16 days after the last immunizing dose. The time course of parasitemia (B) and the survival rate against the infection (C) of each group were monitored. The figure is reproduced from the Ref [42] with permission.

4. Vaccine-induced CD8$^+$ T cells could protect mice from *T. cruzi* infection

There were circumstantial evidences that CD8$^+$ T cell-dependent protective immunity is induced in *T. cruzi* infection [19–22]. One example is that, by depleting CD8$^+$ T cells in vivo by administering anti-CD8 mAb, the course of *T. cruzi* infection was exacerbated [20]. Another example is that the *T. cruzi* antigen-encoding DNA vaccine-induced antigen-specific CD8$^+$ T cells [37–41]. However, in these initial DNA vaccine reports, there were no studies on the in vivo depletion of specific immune cells; therefore, indicating that the protective immunity might depend on not only the CD8$^+$ T cells but the other effector mechanisms such as antibodies. The first indication that the vaccine-induced CD8$^+$ T cells are effective for conferring protective immunity against *T. cruzi* infection came from an artificial system in which a murine malaria-derived antigen was used to transform *T. cruzi* [42]. The transformation of *T. cruzi* with a gene encoding *Plasmodium yoelii* circumsporozoite protein (CSP) was conducted due to the unavailability of identified CD8$^+$ T cell-inducing epitope on intrinsic *T. cruzi* antigens at that time. The generation of CSP-expressing *T. cruzi* made the analyses of CD8$^+$ T cell immunity during the infection feasible, since the CSP contains a well-characterized CD8$^+$ T cell-inducing, H-2K^d-restricted epitope, SYVPSAEQI [43]. As shown in Fig. 2, the prime-boost immunization of BALB/c mice with SYVPSAEQI-expressing influenza virus followed by the CSP-expressing vaccinia virus protected them from the lethal infection of CSP-expressing *T. cruzi* (Fig. 2B, C). The protective immunity depended on the induction of CD8$^+$ T cells (Fig. 2A), since their depletion by administering anti-CD8 mAb to immunized mice before infection completely abrogated the vaccine efficacy [42]. The results demonstrated, for the first time, that the vaccine-induced CD8$^+$ T cells could protect mice from *T. cruzi* infection.

5. *T. cruzi* antigens which confer CD8$^+$ T cell-dependent protective immunity

The identification of CD8$^+$ T cell-inducing epitope on intrinsic *T. cruzi* antigens had been awaited for years. It was first achieved in 1997, by identifying VDYNFTIV/K^b on a *T. cruzi trans*-sialidase antigen [44]. Identification of more CD8$^+$ T cell-inducing epitopes on *T. cruzi* antigens, VNHRFTLV/K^b [45], VNHDFTVV/K^b [45], IYNVGQVSI/K^d [46], ANYNFTLV/K^b [40], ELTMYKQLL/K^b [47], TEWETGQI/K^k [48] or ANYKFTLV/K^b [49], was followed (Table 1). Among these epitopes, there is an indication that the CD8$^+$ T cells responding against ANYNFTLV and ANYKFTLV bind to the same T cell receptor [49]. CD8$^+$ T cell responses against H-2K^b-restricted ANYNFTLV [40] or ANYKFTLV [49] consist of nearly 30% of all CD8$^+$ T cells at a peak after Brazil strain of *T. cruzi* infection [49] or after ANYNFTLV-expressing recombinant virus vector vaccination [50 and Miyahira et al., unpublished observation]. The epitopes, ANYNFTLV/ANYKFTLV, present one of the strongest documented responses against a single epitope in any infectious disease [49], and could be the best and suitable epitopes for elucidating hosts' CD8$^+$ T cell responses against *T. cruzi* infection.

6. DNA vaccine-induced protective immunity against *T. cruzi* infection

Since the DNA itself was demonstrated to be a potent immunomodulator for the induction of hosts' immune responses, DNA vaccine has been hoped to become a mainstream technology to perform immunizations which should be both immunogenic and safe [51]. Among several potential advantages, DNA is easier to be purified than protein; therefore, there is no need to perform laborious purification procedure by which protein-based immunogens must be processed to reach a clinical grade. Considering numerous reports on the efficacy of DNA vaccine against a variety of infectious diseases, it was not surprising to find that it was also effective for conferring protective immunity against *T. cruzi* infection [37,38,40]. The characterization of DNA vaccine-induced protective immunity against intracellular infectious agents revealed its dependence not only on the induction of neutralizing antibodies but on the induction of antigen-specific CD8$^+$ T cell responses [52]. Despite the optimism and expectations during the initial stage of technological advancement, however, recent studies demonstrated that DNA vaccine is not as immunogenic as expected in humans, and suggested that modifications for augmenting immune responses should be considered by incorporating adjuvants into DNA vaccine-based regimens [51]. Several approaches to the optimization of DNA vaccine are the appropriate choice of a vector including the promoter-enhancer complex, the optimization of codon usage, the use of N-terminal ubiquitination signals, the unmethylated CpG sequences [51]. Cytokines or chemokines as plasmids or proteins delivered

Table 1
Intrinsic *T. cruzi* antigen-derived, CD8$^+$ T cell-inducing epitopes recognized by murine cells

Sequence	H-2 haplotype	*T. cruzi* antigen	Reference
VDYNFTIV	K^b	TSA-1	Wizel et al., 1997 [44]
VNHDFTVV	K^b	ASP-1	Low et al., 1998 [45]
VNHRFTLV	K^b	ASP-2	
KNYPFSSI	K^b	ASP-1	
NTLVFPLV	K^b	ASP-1	
DNRQYSFV	K^b	ASP-1	
EKEANALYLWV	D^b/K^b	ASP-1	
IYNVGQVSI	K^d	TCTS	Rodrigues et al., 1999 [46]
ANYNFTLV	K^b	TSSA	Katae et al., 2002 [40]
ELTMYKQLL	K^b	LYT1	Fralish and Tarleton, 2003 [47]
TEWETGQI	K^k	ASP-2	Araujo et al., 2005 [48]
ANYKFTLV	K^b	*trans*-sialidase	Martin et al., 2006 [49]
ANYKFTLL	K^b	*trans*-sialidase	
ANYDFTLV	K^b	*trans*-sialidase	
ANYNFTLL	K^b	*trans*-sialidase	
VNYDFTLV	K^b	*trans*-sialidase	
VNYDFTIV	K^b	*trans*-sialidase	

simultaneously with DNA vaccines are other examples for achieving the optimization [51]. Table 2 summarizes genetic adjuvants which are effective for the enhanced/diminished induction of antigen-specific $CD8^+$ T cells. Some of them were also assessed with regard to the improved protective immunity against the infection-induced pathologies [40,41,51,53,54].

By combining *T. cruzi* antigen-encoding DNA with cytokine-encoding genetic adjuvant, both the enhancement of $CD8^+$ T cell responses and improved protective immunity against lethal infection could be achieved. Either an IL-12-encoding DNA alone [40] or an IL-12 gene with a gene encoding granulocyte-macrophage colony stimulating factor (GM-CSF) [41] was an effective genetic adjuvant for both the better induction of $CD8^+$ T cells and improved protection against *T. cruzi*. These results, which were demonstrated in *T. cruzi* infection, were in accordance with previous reports demonstrated against other infectious agents [51,53]. This consistency between *T. cruzi* infection and other infectious diseases indicates that the scientific achievements demonstrated in *T. cruzi* infection would be generalized to the better vaccination strategy against other infectious diseases. IL-12 is a crucial component for the induction of long-lasting protective immunity against intracellular infectious agents [55]. The better management for reducing its toxic effect would make the use of IL-12 as an adjuvant reach to a clinical application [56].

In addition to IL-12 and GM-CSF, the gene encoding the ligand to receptor activator for NFκB (RANKL) was also effective for enhancing antigen-specific $CD8^+$ T cell responses [54]. By co-administering a *T. cruzi* antigen-encoding plasmid DNA (pTSSA) with RANKL gene (pRANKL), the induced $CD8^+$ T cell responses exhibited approximately two- to three-fold increase in number (Fig. 3). The effect was not restricted only to the use of DNA vaccine, since the co-administration of RANKL gene with the recombinant influenza virus expressing *P. yoelii* CSP-derived $CD8^+$ T cell-inducing epitope significantly augmented the induction of $CD8^+$ T cell responses [54]. Considering the immunological effect of RANKL on hosts' immune responses [57], the adjuvant effect of RANKL gene could be probably attributed to the extended survival of dendritic cells which are potent antigen-presenting cells. Since the RANK-RANKL interaction is critical for bone metabolism [57], the over-expression of RANKL in vivo could cause adverse effect. This point will have to be taken into consideration to elucidate its immunological effect further and to achieve its future clinical application.

7. Roles of costimulatory molecules affecting the protective immunity against *T. cruzi*

Costimulatory molecules are vital for the appropriate activation and differentiation of T cells. There are a wide variety of costimulatory molecules which have been reported so far [58,59]. Each molecule presents unique features for immunological function. Targeting on the molecules would be one of the key elements for developing successful $CD8^+$ T cell vaccine. However, there are few reports elucidating the immunological function of those molecules during *T. cruzi* infection despite their enormous potential for contributing to the better understanding for the disease.

CD28–CD80/CD86 costimulatory pathway is one of the few which were analyzed relatively in detail in *T. cruzi* infection [60]. Deficiency of CD28 molecule or the simultaneous blockade of CD80/CD86 molecules resulted in the increased susceptibility against *T. cruzi* infection [60]. It was correlated with the decreased antigen-specific IFN-γ secretion and the decreased induction of antigen-specific $CD8^+$ T cell responses. CD28–CD80/CD86 costimulatory pathway is also critical to induce protective immunity when DNA vaccine is used as a vaccine immunogen [60]. Without CD28 molecule, both the induction of $CD8^+$ T cells and the DNA vaccine-induced protective immunity was abrogated.

Importantly, the requirement for this costimulatory pathway for the induction of immunological resistance has varied depending on infectious agents (Table 3) [60–76]. Although there are no definite explanations for the differential requirement of costimulations to achieve biodefense against various infections, it indicates that the elucidation of each costimulatory molecule against each infectious agent should be completed to explore for better immunological intervention strategies against either known or emerging infectious diseases.

8. Recombinant virus vector vaccination against *T. cruzi* infection

Vaccination using recombinant virus vectors has become a promising strategy to induce T cell immunity against

Table 2
Genetic adjuvants in vaccinations

Cytokines, chemokines, costimulatory molecules		Induction of $CD8^+$ T cell responses	Protective immunity against *T. cruzi*	Protective immunity against other infections	Refs.
IL-1α TNF-α TNF-β IFN-γ IL-2 IL-12+GM-CSF+ CD86 IL-12+CD80	IL-15 IL-18 CCR7 ligand SLC CCR7 ligand ELC CCL3 (MIP-1α) CCL5 (RANTES) CtB	↑	ND	ND	[51,53]
IL-12 IL-12+GM-CSF		↑	↑	↑	[40,41] [51,53]
RANKL		↑	↑	ND	[54]
CD80+ICAM-1+ LFA-3 Caspase-3 (apoptotic) BCL-xL (antiapoptotic) Fli-C (TLR-5 antagonist)		↑	ND	↑	[51,53]
IL-4 IL-5		↓	ND	→ ND	[51]

ND; Not Done.

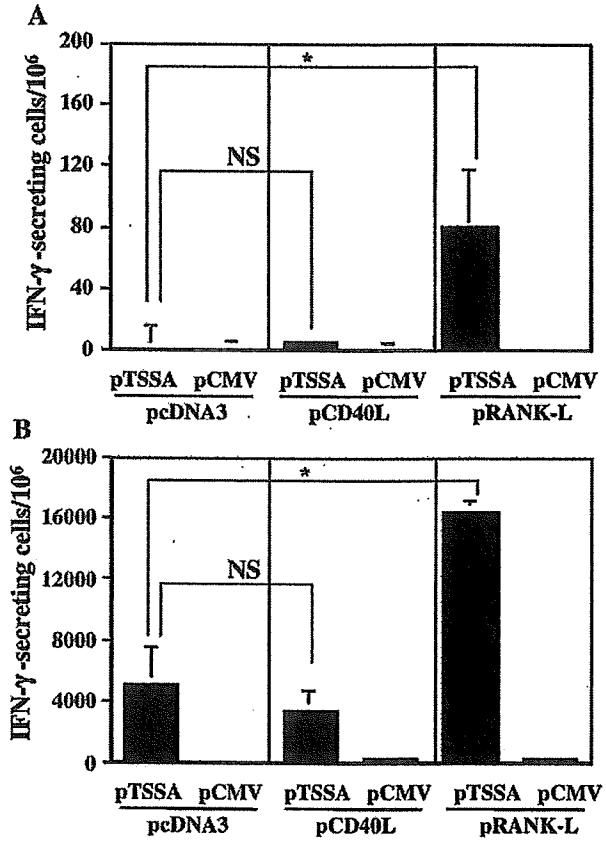


Fig. 3. Coadministration of pRANKL, but not pCD40L, with pTSSA enhances the induction of TSSA-specific $CD8^+$ T cells. (A, B) B6 mice were immunized with 100 μg of pTSSA or pCMV and 100 μg of pCD40L, pRANKL, or pcDNA3, which were all suspended in 50 μl of sterile PBS, into the right hind leg quadriceps twice at a 10-day interval. The mice were sacrificed 12 days after the second immunization, and their spleens were removed. Splenocytes from individual mice were cultured with irradiated EL-4 cells pulsed with ANYNFTLV peptide for 1 wk. The freshly isolated splenocytes (A) or the 1-wk cultured splenocytes (B) were subjected to the ELISPOT assay for IFN-γ-producing cells in response to ANYNFTLV peptide-pulsed EL-4 cells. The number of IFN-γ-secreting cells/1×10^6 cells was counted 24 h later. The number of IFN-γ-secreting cells that appeared against peptide-unpulsed EL-4 was subtracted from the number of IFN-γ-secreting cells that appeared against peptide-pulsed EL-4. Data represent the mean±SD of four mice in each group. *, $p<0.05$ by the Dunnett's two-tailed t-test. NS, not significantly different. The figure is reproduced from Ref. [54] with permission.

intracellular infectious agents [77]. Among virus vectors, adenovirus and vaccinia virus have been shown to be the most efficient vectors for inducing protective immune responses against HIV [78,79] and malaria [16,80,81].

The generation of replication-deficient virus mutants makes this strategy safer and more effective for containing the threat and spread of infections. As described above, the recombinant virus vector vaccination was also demonstrated effective for conferring protective immunity against *T. cruzi*, which was artificially engineered to express a well-characterized, immunogenic foreign antigen [42]. Since the intrinsic *T. cruzi* antigens which contained $CD8^+$ T cell-inducing epitope were identified, a question if the recombinant virus vectors expressing an intrinsic *T. cruzi* antigen are really effective for conferring immunological protection, should have been determined. In addition, it had also remained to be determined whether the vaccine-induced $CD8^+$ T cell responses against intrinsic antigens are sufficient for controlling the *T. cruzi* infection.

The $CD8^+$ T cell-inducing epitope, ANYNFTLV/K^b was used to generate recombinant attenuated adenovirus (replication-deficient) and recombinant attenuated vaccinia virus (modified vaccinia virus Ankara: MVA), which express only the small portion of *T. cruzi* antigen, the minimal 8-mer peptide (Fig. 4) [50]. The enumeration of ANYNFTLV-specific $CD8^+$ T cells revealed that the priming with ANYNFTLV-expressing adenovirus followed by the booster injection of ANYNFTLV-expressing MVA gave the highest number of induced epitope-specific $CD8^+$ T cells. In comparison, the combined immunization of DNA-priming with the recombinant MVA-boost was performed and was not as good as the $CD8^+$ T cell immune responses induced by the recombinant adenovirus-priming / the recombinant MVA-boost [50]. The infection of lethal or sub-lethal dose of *T. cruzi* resulted in the significant suppression of parasitemia and the improved survival of immunized mice, demonstrating that the immune responses against a single $CD8^+$ T cell-inducing epitope, ANYNFTLV, could control *T. cruzi* infection (Fig. 5) [50].

T. cruzi is well known for its heterogeneity among isolates which could be a serious obstacle for developing $CD8^+$ T cell vaccine [82]. However, the peptide ANYNFTLV on *T. cruzi*

Table 3
Immunological effect of deficiency for CD28–CD80/CD86 costimulation on the induction of $CD8^+$ T cells and protective immunity against infections

Pathogen	$D8^+$ T cell induction	Protective immunity	References
Trypanosoma cruzi	↓	↓	[60]
Leishmania major	ND	↑→↓	[61–63]
Plasmodium chabaudi	ND	↓	[64,65]
Toxoplasma gondii	ND	→	[66,67]
Coxiella burnetti	ND	↑	[68]
Listeria monocytogenes	↓	↓	[69]
Mycobacterium bovis	ND	→	[70]
Salmonella typhimurium	ND	↓	[71]
Ectromelia virus	→	↓	[72]
Gammaherpesvirus 68	→	→	[73]
Influenza virus	↓	↓	[74]
LCMV	↓→	→	[75,76]
Vesicular stomatitis virus	↓	↓	[76]

ND; Not Done.

trans-sialidase antigen is, interestingly, well conserved among isolates [49] despite the $CD8^+$ T cell-mediated immune responses against the sequence could protect mice. It is suggested that $CD8^+$ T cell is one of the most powerful driving forces to generate antigenic polymorphism of infectious agents [13], which will confer resistance against $CD8^+$ T cells. In this regard, the conservation of the sequence among various *T. cruzi* isolates is puzzling, since the immune response against ANYNFTLV could be regarded as one of the strongest ones documented in any infectious disease [49]. If the epitope ANYNFTLV had given an influential impact on the survival of *T. cruzi*, it should have been, theoretically, more polymorphic. This might indicate that $CD8^+$ T cell responses are only partially effective for the suppression of expanding infection and might not be very efficient for completely eliminating *T. cruzi* [50]. If *T. cruzi* has acquired evasion mechanisms against hosts' $CD8^+$ T cell immune responses, it will be an important subject to be analyzed and will have to be clarified to develop better $CD8^+$ T cell vaccine strategy. These unknown mechanisms might be the reasons why *T. cruzi* infection frequently becomes chronic [83].

9. *T. cruzi* infection as a unique model for developing T cell vaccine

T. cruzi infection as a model for elucidating hosts' $CD8^+$ T cell responses presents both advantageous and disadvantageous aspects. *T. cruzi* is a eukaryotic cell which contains much more antigens than bacteria or viruses. Numerous antigens mean that the antigens which confer protective immunity will not be easy to be identified. Analyses of $CD8^+$ T cell responses are particularly troublesome, since they are regulated by an immunological phenomenon called immunodominance [9,84]. There is an estimate calculating approximately one $CD8^+$ T cell-inducing epitope per 500 to 1000 amino acid sequences [9]. There are, therefore, numerous potential epitopes in numerous *T. cruzi* antigens. However, $CD8^+$ T cell responses in vivo direct to only a few, or quite frequently, to only one [9,84]. The immunodominance clearly suggests that the identification of epitope is crucial for the development of effective T cell vaccine. The more a pathogen contains antigens, the more difficult the identification of immunodominant and protective epitope is. In this regard, the $CD8^+$ T cell-inducing epitopes, ANYNFTLV/ANYKFTLV present on *T. cruzi* antigens, would be the optimal and appropriate ones to conduct $CD8^+$ T cell research when taking advantages of experimental Chagas' disease as a disease model.

T. cruzi replicates and grows relatively slowly when compared to bacterial or viral infectious agents. The doubling time for replication of *T. cruzi* varies depending on isolates but is approximately between 24 to 72 h [82]. Since infection can be regarded as numbers game between infectious agents and hosts' immune cells [29], slow replication of *T. cruzi* indicates that there could be better chance for immune cells to outnumber *T. cruzi*. Although *T. cruzi* replicates inside cells, it is well known

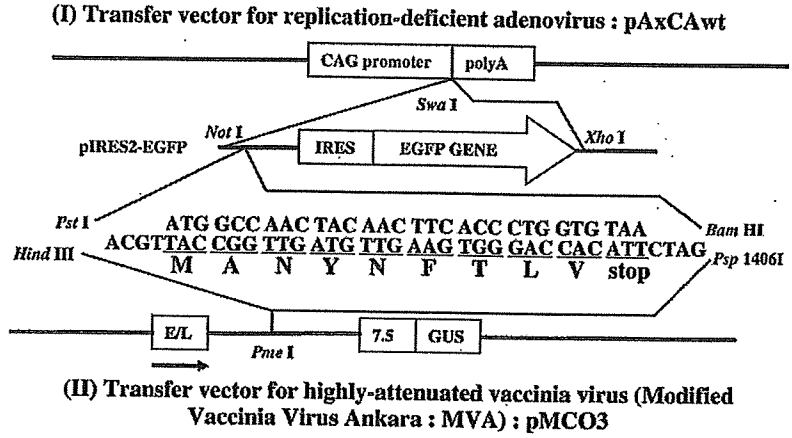


Fig. 4. Generation of recombinant viral vectors. An H-$2K^b$-restricted $CD8^+$ T cell epitope, ANYNFTLV was identified on *T. cruzi trans*-sialidase surface antigen (TSSA). A mini-gene encoding the MANYNFTLV peptide was inserted either into pAxCAwt, a transfer vector for replication-deficient adenovirus, or into pMCO3, a transfer vector for highly-attenuated vaccinia virus, modified vaccinia virus Ankara (MVA). CAG, modified chicken β-actin promoter with CMV-IE enhancer; poly A, poly A addition signal; IRES, internal ribosome entry site; EGFP, enhanced green fluorescent protein; E/L, synthetic early/late MVA promoter; 7.5, MVA P7.5 promoter; GUS, gene encoding *E. coli* β-glucuronidase. The figure is reproduced from the Ref. [50] with permission.

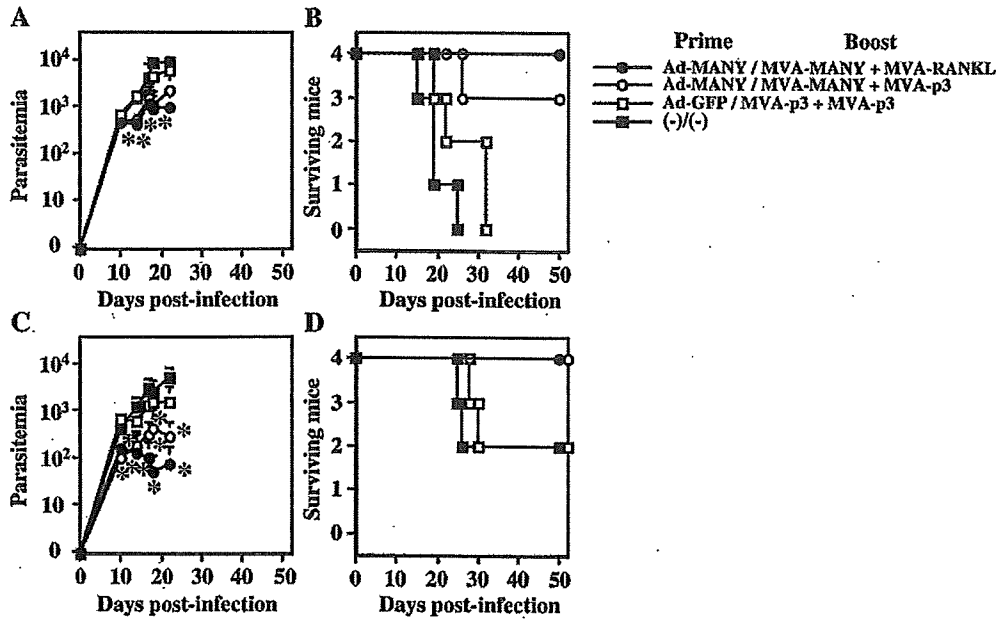


Fig. 5. Prime-boost immunization with Ad-MANY/MVA-MANY+MVA-RANKL can control lethal *T. cruzi* infection. B6 mice (n=4) were first primed with 5×10^8 PFU of Ad-MANY or Ad-GFP, and then boosted with 5×10^8 PFU of MVA-MANY or MVA-p3 and 5×10^7 PFU of MVA-RANKL or MVA-p3 11 days later. The mice were infected i.p. with 10,000 (A, B) or 2,000 (C, D) Tulahuen strain of *T. cruzi* blood-form trypomastigotes 14 days after the boost immunization. The number of parasites in 5 μl of peripheral blood (parasitemia) was counted periodically, and the data represent the mean±SD of four mice in each group (A, C). Survival was monitored daily (B, D). *, $p<0.05$ compared to the un-immunized mice by the Dunnett's two-tailed t-test (A, C). The longer survival of Ad-MANY/MVA-MANY+MVA-RANKL group was significantly different ($p<0.05$ by the unpaired Mann–Whitney U-test) from that of Ad-GFP/MVA-p3+MVA-p3 group of mice (B). The survival of other groups was not significantly different from that of Ad-GFP/MVA-p3+MVA-p3 group of mice (B, D). Ad-MANY: recombinant adenovirus expressing ANYNFTLV epitope, Ad-GFP: recombinant adenovirus expressing GFP, MVA-MANY: recombinant MVA expressing ANYNFTLV epitope, MVA-p3: recombinant MVA transformed with a transfer vector, MVA-RANKL: recombinant MVA expressing murine RANKL. The figure is reproduced from the Ref. [50] with permission.

that the increment of non-replicative *T. cruzi* trypomastigote form in the peripheral blood is well correlated with the progression of pathologies. In addition to its easy performance to determine the cell number without sacrificing animals, the slow progression of disease caused by *T. cruzi* could make the assessment of various $CD8^+$ T cell vaccination protocols more efficient to detect subtle immunological changes when modifying immune system.

In the field of infectious diseases, the control of chronic infections such as HIV infection or chronic hepatitis as well as the control of emergent, acute and fatal infectious diseases is two of the most important subjects to be solved. The precise mechanisms why hosts cannot completely eliminate invading pathogens should be elucidated in infectious diseases which progress toward a chronic state. Chagas' disease presents not only the acute phase pathologies but the chronic ones in approximately 20–35% of infected individuals [3]. There is an animal model representing chronic Chagas' disease in humans by combining certain *T. cruzi* isolates with certain mouse strains [85]. The etiology of chronic Chagas' disease is still controversial [86,87]. There are two major hypotheses to explain the pathologies of chronic Chagas' disease; one is "autoimmune hypothesis" [86], and another is "persistent infection hypothesis" [87]. Whether the etiological cause of chronic Chagas' disease is due to either mechanism, the elucidation of the roles of antigen-specific $CD8^+$ T cells in chronic infectious pathologies could be performed in the current *T. cruzi* infection model.

The last merit for taking advantage of *T. cruzi* infection to conduct $CD8^+$ T cell research comes from the nature of infectious agent itself. Malaria is well known for its critical contribution to $CD8^+$ T cell immunity, as represented by the discovery of prime-boost vaccination regimen [17,34,35]. However, $CD8^+$ T cells exert their immunological function against only the part of the complicated life cycle of malaria parasite. After the inoculation of sporozoites during the anopheline mosquito feeding, they invade almost exclusively into hepatocytes, where $CD8^+$ T cells could suppress the development of malaria liver stage. The assessment of immunological function of $CD8^+$ T cells is, therefore, based on their inhibitory effect in the restricted organ, only in liver. In addition, malaria liver stage causes no clinical symptoms. In contrast and different from malaria, *T. cruzi* invades principally into any types of cells except red blood cells. Despite it exhibits relatively stronger affinity to muscle or neural cells, every organ could be damaged by the infection. Based on the unique features of *T. cruzi* infection, the vaccine efficacy, especially the $CD8^+$ T cell immunity, could be assessed with regard to the improvement of systemic condition. This point may be another advantage for choosing *T. cruzi* infection to search for better $CD8^+$ T cell-mediated intervention strategies.

10. Concluding remarks

Chagas' disease has been afflicting people living in the Central and South America for thousands years [88]. However,

due to its relatively restricted endemic area and the dramatic success of Southern Cone Initiative [2,3] for eliminating Triatoma vectors by insecticides, it is now called as a neglected disease. Besides there are still thousands people who are chronically-infected with *T. cruzi*, the unique features of its infection should not be neglected only based on its clinical significance. Different from bacterial or viral infections, *T. cruzi* infection could present new scientific insight on phenomena of infection and could be a useful model for adding new knowledge to the $CD8^+$ T cell immunity. In this regard, *T. cruzi* infection holds an enormous potential for contributing to the discovery of universally effective T cell vaccine strategy against intracellular infectious agents.

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Review

Toxocariasis in Japan

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Abstract

Toxocariasis has long been considered a parasitic disease affecting pet owners and children who often play in sandboxes at public parks. Recent cases of this animal-borne infection, however, indicate that its clinical manifestations and etiologies are changing. In this article, we will describe the critical characteristic features of toxocariasis alongside the contributions of Japanese researchers to a better understanding of the disease.

Keywords: *Toxocara canis*; *Toxocara cati*; Toxocariasis; Visceral larva migrans

Contents

1. Introduction

Among animal-borne diseases, toxocariasis is one of the most popular parasitic infections in the world, caused by the larval stage of *Toxocara* spp. Humans are infected mainly by the tiny developmental stage of the parasite, which belong to the family Ascaridoidea, through their pet dogs and cats. Other natural hosts include wild Canidae for *Toxocara canis* and wild felines for *Toxocara cati*. Symptoms depend on organs affected and the magnitude of infection. It is usually a non-fatal disease, but the larvae migrate through the eyes and can cause severe vision disability or even blindness.

In 1950, Dr. Wilder, an American ophthalmologist, histopathologically identified a nematode of unknown etiology in the retinas of 26 out of 46 enucleated eyes with retinoblastoma [1].

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Two years later, Beaver et al. [2] recognized the same parasite in the liver of three young children. Shortly afterwards, the parasite was correctly identified as an infectious stage larva of *T. canis* [3–5]. Since then, many clinicians and biologists have been accumulating knowledge of *Toxocara* and toxocariasis.

In this review article, we describe the lesser-known contributions of Japanese researchers to the understanding of *Toxocara* and toxocariasis. This articles builds on the work of Kondo [6], focusing on the topics that he did not cover in his review and on new findings since his publication.

2. Toxocariasis in humans

2.1. Clinical cases

Toxocariasis is clinically classified into four types: visceral, ocular, neurologic, and covert [7,8]. In 1963, the first report on toxocariasis in Japan was presented orally at the 32nd Annual Meeting of the Japanese Society of Parasitology by Fushimi et al. [9]. A 14 year-old boy was admitted to a university hospital because of fever, hepatomegaly and persistent eosinophilia. The patient died from severe anemia six months later. Though no autopsy or serological examinations were performed, the patient was strongly suspected to have suffered from visceral toxocariasis. In the early 1960s, immunological tests for parasitic infections, especially for helminthiasis, had only just begun, and antigen for the diagnosis of toxocariasis was not yet known.

Just as in other parasitic infections, direct demonstration is the only way to make definite diagnosis of toxocariasis. However, it is difficult to find the larva in either tissue biopsies or autopsies due to its very small size. So far in Japan, one morphologically and two pathologically confirmed cases have been reported [10–12]. Two additional reports, both of ocular toxocariasis, were doubtful because of the lack of characteristic features of the parasite; the authors nevertheless reproduced the microscopic findings of the purported larva in their papers [13,14]. One of these two cases showed increased antibody production in vitreous fluid against *Toxocara* antigen prepared from larval excretory–secretory product (LES), suggesting that the case might be attributable to ocular toxocariasis.

Serology is an alternative method for the diagnosis of toxocariasis. A method has been established for *in vitro* cultivation of the larvae, with LES prepared from the culture medium serving as an antigen. Detection of specific antibodies against LES provides evidence of *Toxocara* infection in individual patients and useful tool for understanding the epidemiological characteristics of this disease. The first serological survey in Japan was reported by Matsumura and Endo [15] using sera of 83 clinically healthy children. In their sample, 3.6% tested were positive for LES. In another study, Matsumura and Endo [16] demonstrated that 20 of 530 adults possessed the IgG antibody to LES. The positive individuals were thought to have a latent or past infection. In a large-scale seroepidemiolgical survey, Kondo et al. [17] collected 3277 sera from 14 prefectures in Japan and tested for LES antibodies. Antibodies were confirmed in 52 individuals (1.6%), but geographical patterns were notable: the highest prevalence rate was observed in Miyagi Prefecture (6.1%), and the lowest was in Ibaragi Prefecture (0.5%). The researchers concluded that the overall seroprevalence rate was in good agreement with those reported from other countries [17–19].

Based on improvements in the field of serology, diagnosis of toxocariasis is usually made by detection of the specific antibody to LES, along with clinical manifestations such as eosinophilia, eosinophilic pneumonia, or ophthalmoscopic findings.

2.2. Characteristic features of toxocariasis

2.2.1. Toxocariasis as a food-borne infectious disease

Using serological methods, there were nearly 200 reports of toxocariasis in the database of Japana Centra Revuo Medicina, and almost 300 cases have been diagnosed in Japan in the past two decades. Among these cases, some significant reports have provided a new perspective on the pathogenic mechanisms of toxocariasis.

Since Beaver et al. [2] introduced the concept of visceral larva migrans, characterized by chronic eosinophilia with granulomatous lesions in the liver, toxocariasis was regarded as a disease in children who were infected by soil contaminated with embryonated eggs [20]. In 1983, Sakai et al. [21] reported a case toxocariasis after ingestion of raw chicken liver. The 57-year-old man was admitted to a hospital due to cough, fever and weight loss. Complete blood count revealed a marked increase in eosinophils in peripheral blood with leukocytosis, and serum antibody against *T. canis* was strongly positive. Before onset, he and his friends had eaten raw chicken livers derived from his poultry and boar farm. Soon after the meal, they experienced abdominal pain, vomiting and diarrhea, but the symptoms improved within two days after ingestion. One month later, his chief complaints emerged. Two similar cases were subsequently reported by the same group [22].

These cases clearly indicates that the disease should be considered a food-borne parasitic infection. Four additional papers describing six patients were published in Japan in the 1980s [22–25]. These patients, all male and between 22 and 51 years of age, had a history of eating raw meat or liver of fowl and/or cattle before onset of symptoms. The possibility that raw liver of domestic animals can transmit the pathogens of human visceral larva migrans was substantiated by Lee et al. [26] of Yonsei University College of Medicine in Korea. They found that a dietary habit of raw liver was much more frequently seen in males than in females, especially in the 31–40 age group. Experimental studies revealed that chicken, cattle and swine were able to act as paratenic hosts for *T. canis* [27–29]. Most of the adult cases reported in recent years in Japan are categorized as this type of infection [30].

2.2.2. Respiratory illness and toxocariasis

In animal models in rodents, hatched larvae migrate into the lungs through the liver after ingestion, resulting in liver dysfunction and pneumonia [31–33]. In humans, similar manifestations are well documented in the literature [30,34–36]. Pulmonary lesions appear on computed tomography as multifocal subpleural nodules with halos or ground-glass

opacities and ill-defined margins. Additionally, transient pulmonary infiltrates are a characteristic finding. Morimatsu et al. [30] recently reported a familial case of visceral toxocariasis after consumption of raw chicken livers. In this case, the patients, a father (71 years old) and his son (45 years old), ate raw chicken livers three weeks before onset and then developed mild fever, general fatigue, headache and respiratory disorder. The specific antibody to LES was identified both in their serum samples and in bronchoalveolar lavage fluid (BALF). *T. canis* larvae were recovered from chicken liver from the same source as that ingested by the patients. These cases showed that BALF is a reliable specimen to demonstrate LES antibodies when the patient shows respiratory illness.

2.2.3. Urticaria-like skin lesions and toxocariasis

Parasitic infection is often said to be associated with chronic urticaria [37]. This is still a controversial issue, but acute urticaria is certainly associated with infection with larva from the marine fish parasite, *Anisakis simplex* [38]. Japanese have long tradition of eating raw fish, sashimi and sushi, and anisakidosis is a common parasitic infection in Japan. It is well documented that urticaria is closely related to the infestation of *Anisakis* larva [38,39]. As with anisakidosis, an allergic reaction could be elicited by the invasion of *Toxocara* larvae and result in skin rash that looks like hives. These skin manifestations might occur as a result of immunological response to larval metabolites [40,41].

In 1999, the first confirmed case of toxocariasis with larva in subcutaneous tissue was reported [11]. A 26-year-old female with fever, headache, and dry cough was admitted to a university hospital. Her peripheral blood smear showed an eosinophilia (61%) and her chest radiograph revealed multiple nodules. A diagnosis of visceral toxocariasis was made after detection of LES antibodies. During her hospitalization, several brown itchy nodules, which were thought to be prurigo, developed on her legs. Histological examination showed *Toxocara* larva in the center of an eosinophilic and lymphocytic abscess. The patient admitted frequently eating raw beef liver almost one year before her hospitalization for its purported health benefits. We can learn from this case that larvae migrating into subcutaneous tissue directly elicit pruriginous skin lesions.

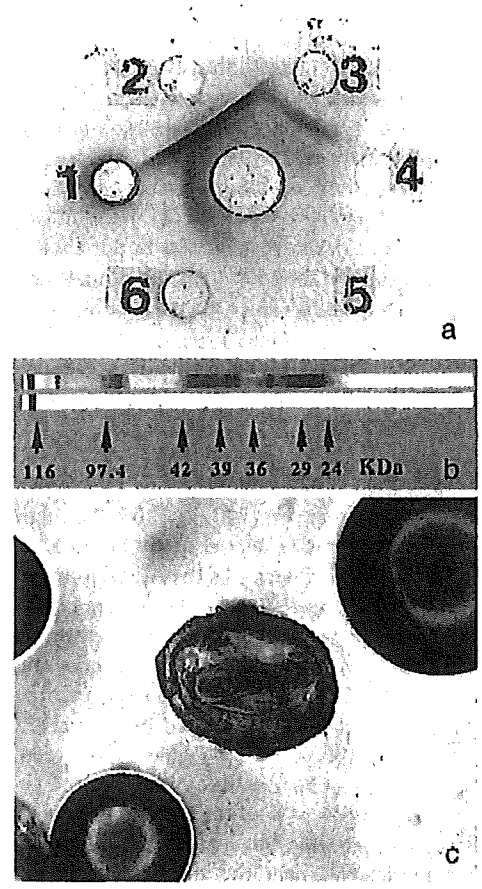

Fig. 1. The results of double gel diffusion (a) and western blot (b) tests of a patient of visceral toxocariasis. Strong precipitin bands were obviously observed between larval excretory–secretory products (LES) of *Toxocara canis* and patient's serum by means of double gel diffusion test. Antigens used in this test were adult worm extract (AEX) of *T. canis* (1), LES of *T. canis* (2), AEX of *Dirofilaria immitis* (3), AEX of *Ascaris suum* (4), LES of *Anisakis simplex* (5) and AEX of *Ascaris lumbricoides* (6). Western blot test shows a whole range of LES molecules were reacted with the patient's serum (upper strip) but not with a normal control serum (bottom strip). An embryonated egg recovered from the soil in the park where the patient often played (c). A fully developed and live *Toxocara* larva was found in the egg.

2.2.4. Toxocariasis is a disease that affects adults rather than children

Many reviews from western countries indicated that children under 12 years old, who often play outside, are the most affected age group for toxocariasis [42,43]. They are accidentally infected with *T. canis*/*T. cati* eggs, which expelled in feces puppies and fully develop in the surrounding environment within two to four weeks. Therefore, contaminated soil is the most important etiological source for toxocariasis [44,45]. Hori et al. [46] reported a case of visceral toxocariasis in a 1.5-year-old girl with fever, hepatomegaly, and eosinophilia (73%). The patient had a history of pica, particularly eating soil from a nearby park where she frequently played with her brother. Serological examination strongly suggested that she was suffering from *Toxocara* infection (Fig. 1a, b). They also found many embryonated eggs from the soil in the park that contained a live larva closely resembling *T. canis* eggs (Fig. 1c). Fortunately, her brother showed a negative result in serological tests.

In a review article of Barriga [47], the average age of visceral toxocariasis was 9.5 years, and only 18% of patients were adults. However, in recent investigations, adults rather than young children were more frequently affected by this parasite. This tendency is particularly true for ocular toxocariasis. Yoshida et al. [48] described that, among 38 Japanese cases of ocular toxocariasis, 34 (89%) were older than 20 years of age, and suggested that clinical features observed in these patients were somewhat different from those of previously reported cases [49]. Therefore, ocular toxocariasis is no longer merely a disease of young children, but affects any age group having a risk factor such as consumption of raw meat or close contact with contaminated soil.

As of the end of 2006, 584 clinically suspected cases of toxocariasis (112 of visceral type and 472 of ocular type) have been referred to our laboratory for detection of the anti-*Toxocara* antibody. We omitted 109 cases from this study due to a lack of description of the patient's age and sex. In visceral toxocariasis, the male-to-female ratio in the remaining sample was 2.04 (male: 53, female 26). The average age was 39.2± 21.7 (range, 0–83 years old) in male and 31.3±23.9 8range, 0.5–82 years old) in female. On the other hand, the male-to-female ratio in ocular toxocariasis group was 1.16 (male: 213, female: 183). The average age was 39.3±18.5 among males (range, 2–83 years old) and 37.6±18.2 among females (range, 2–74 years old). There were no significant differences in age distribution between males and females (Fig. 2). A similar result was obtained by Fujino et al. in 1998 [50].

2.2.5. Myelitis and toxocariasis

According to the case-control study by Magnaval et al. [51], migration of *T. canis* larvae in the human brain does not frequently induce recognizable neurological signs, but is possibly responsible for repeated low-dose infections. These light parasitic burdens usually do not appear to elicit a special clinical symptom, but in some cases, sever neurological disorders such as encephalitis, myelitis and meningitis are manifested [52]. In Japan, Ota et al. [53] reported a case of eosinophilic meningo-encephalo-myelitis due to *Toxocara* infection. The patient, a 21-year-old woman, showed frontal headache, low-grade fever and convulsion. She had a long history of close contact with her pet dog. Immunological tests were strongly positive for LES antigen in both her serum and cerebrospinal fluid. Based on clinical evidence and characteristic features in similar patients, Kira and his colleagues proposed a new disease entity: "atopic myelitis" or "parasitic myelitis." They assumed that allergic reaction to LES might be involved in this neurologic disorder [54]. Interestingly, most of the patients lived in Kyushu District, in the south of Japan, suggesting that myelitis due to *Toxocara* infection might be a regional clustering disease.

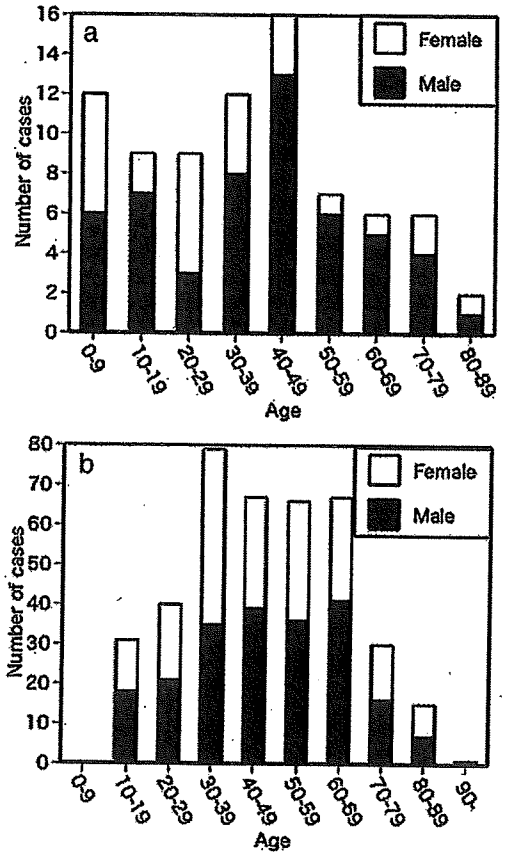

Fig. 2. Age distribution of suspected cases of visceral (n=79)(a) and ocular toxocariasis (n=396)(b) referred to our laboratory from August 1994 to December 2006.

2.3. T. cati

Because morphological differences between *T. canis* and *T. cati* in the adult stage are apparent [55], *T. cati* is easy to identify when patients expel adult worms. It has been suggested that *T. cati* could develop in children through the ingestion of the immature worm of *T. cati* [56]. More than 26 cases were reported so far [56,57], but there was only one case was reported from Japan. A 5-year-old male boy was admitted to a hospital due to a complaint of vomiting 3 worm-like foreign bodies. These worms were morphologically identified as two female and one male immature worms [58].

On the contrary, there are few reports of human intestinal infection with adult worms of *T. canis* [59], and many of these are believed to be erroneous observations [60]. Serological discrimination between toxocariasis canis and toxocariasis cati, however, is not so apparent, because of complete cross-reactivity between the two LESs, although *T. cati*-specific LES has been identified [61]. Therefore, distinguishing between *T. canis* and *T. cati* is even more difficult if somatic antigens are used in the serological diagnosis [62–64]. For the precise serodiagnosis of toxocariasis, a great deal of additional research effort is needed to obtain *T. cati*-specific LES antigens.

3. Advances in serological diagnosis

3.1. Antigens

As mentioned above, the most reliable and suitable antigen for the diagnosis of toxocariasis is LES from *T. canis*. Once the larvae are cultivated *in vitro*, they are viable for up to two years. During this period, no morphological changes have been observed, but chemosusceptibility to some compounds were found to have changed [65], suggesting that the physiological natures of the larva do change over this time period. The nature of LES was extensively studied by Maizels and colleagues [61,66–68]. Around the same time, Sugane and Oshima demonstrated that LES had an ability to induce not only IgG and IgM antibodies, but also IgE antibody in mice. Allergenic activity was lost when LES was treated with guanidine hydrochloride and 2-mercaptoethanol. LES also showed a cross-reaction with serum from *Ascaris suum*-infected mice

[69]. In addition, studies have identified numerous lectin-specific glycoconjugates on the surface of the larvae [61,66–68,70–73], and these have been found to dynamically change during the course of infection in murine [74] and rabbit models [75].

Although the antigenicity and specificity of LES is fairly high, cross-reaction to other parasites, especially nematode parasites, have been observed [76]. To overcome this problem, Yamasaki et al. [77] produced a recombinant antigen that reacted with serum from patients with toxocariasis but not from those with roundworm or hookworm infections.

3.2. Rapid diagnostic test for toxocariasis

For many years, numerous diagnostic measures, such as the double gel diffusion test, immunoelectrophoresis, indirect hemagglutination test, latex agglutination test, plate-based ELISA, membrane-based dot-ELISA, etc., have been employed to detect specific antibodies against LES. However, these tests require 1.5 hours or more to obtain an accurate result. In 1997, a new rapid diagnostic test kit for the detection of anti-LES antibody was introduced by us [78]. The test is based on the antigen-sensitized nitrocellulose membrane-based assay. It is easy to perform, does not require any sophisticated apparatus or expertise and the results can be obtained within 3 min. This test kit can even detect the antibody in intraocular fluid.

4. Conclusion

In this review article, we present an overview of human toxocariasis in Japan. Due to space limitations, we do not describe in detail the aspects of experimental investigations concerning biology, immunology and molecular biology using animal models. However, we briefly pay special attention to Japanese investigators who contributed to advance the understanding of toxocariasis. In early studies, Oshima established a standard method for the oral inoculation of eggs, in which the albuminoid coat of the egg is first removed in order to prevent the adhesion of eggs onto glassware [79]. Sugane is a longtime co-worker of Oshima, and his colleagues are actively engaged in the field of immunology [80–88]. They demonstrated many examples of cellular immunity to *Toxocara* infection in mice. The late Dr. Tsuji made pioneering efforts to develop immunodiagnostic techniques for toxocariasis [50,89,90]. Recently, Mongolian gerbils, *Meriones unguiculatus* have been established as a suitable animal model for experimental ocular and neurologic toxocariasis [91–94].

Human toxocariasis is a public health hazard not only in children but also in adults, both in developing and developed countries. There are still questions to which we have no answers: How does ocular toxocariasis develop? Why do nearly half of ocular toxocariasis patients not produce detectable antibody to LES? What is the pathogenesis of neurologic toxocariasis? What mechanisms are involved in the reemergence of *Toxocara* larvae during pregnancy both in definitive and undefinitive hosts? In addition, we have not yet established an effective anthelmintic against *Toxocara* parasites in the tissue stage, especially for the ocular toxocariasis. Continuous efforts should be made to address these issues. Finally, toxocariasis is a disease that afflicts two of the very best and oldest friends of humans: dogs and cats. Therefore, we must continue to study this puzzling disease both for the sake of humans, and for that of our animal friends.

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Migration behaviour and pathogenesis of five ascarid nematode species in the Mongolian gerbil *Meriones unguiculatus*

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Abstract

To understand the characteristic features of the Mongolian gerbil, *Meriones unguiculatus*, as an animal model of ascarid infections, the migration behaviour and pathogenesis of larvae were investigated in experimentally infected gerbils. Embryonated eggs from each of *Toxocara canis*, *Baylisascaris procyonis*, *B. transfuga*, *Ascaris suum*, and *A. lumbricoides* were orally inoculated into gerbils and larvae were recovered from various organs at designated periods. In *T. canis*-infected gerbils, larvae were present in the liver 3 days after infection and in the skeletal muscle and brain via the heart and lungs at a similar rate. In *B. procyonis*- and *B. transfuga*-infected gerbils, larvae were present in the lungs within 24 h after infection, with some having reached the brain by that time. After 24 h, larvae of *B. procyonis* tended to accumulate in the brain, while those of *B. transfuga* accumulated in skeletal muscles. In *A. suum*- and *A. lumbricoides*-infected gerbils, larvae remained in the liver on day 5 post-infection and elicited pulmonary haemorrhagic lesions, which disappeared 7 days after initial infection. Thereafter, no larvae of any type were recovered. Ocular manifestations were frequently observed in *T. canis*- and *B. procyonis* infected gerbils, but were rare in *B. transfuga*-infected gerbils. In the cases of *A. suum* and *A. lumbricoides*, migration to the central nervous system and eyes was extremely rare, and larvae had disappeared by 2 weeks post-infection. Fatal neurological disturbances were observed in *B. procyonis*-infected gerbils, whereas irreversible non-fatal neurological symptoms were observed in the case of *B. transfuga*.

Introduction

Larval stages of ascarid nematodes elicit severe tissue damage when they invade hosts which are not normally the definitive host. The racoon roundworm, *Baylisascaris procyonis*, is particularly prone to cause a fatal neurological disturbance (Huff *et al.*, 1984; Kuchle *et al.*, 1993; Moertel *et al.*, 2001; Wise *et al.*, 2005). The dog roundworm, *Toxocara canis*, and the cat roundworm, *T. cati*, are also responsible for the visceral larva migrans syndrome (VLM) in humans (Glickman & Magnaval, 1993; Fisher, 2003). The VLM caused by these two roundworms is commonly known as toxocariasis, which is considered a disease of infants and children, although adults are also infected (Glickman *et al.*, 1987; Aragane *et al.*, 1999; Yoshida *et al.*, 1999). Moreover, outbreaks of VLM due to the pig roundworm, *Ascaris suum*, have been reported from the southern part of Japan (Maruyama *et al.*, 1996; Sakakibara *et al.*, 2002). Meningitis can occur when *Ascaris* larvae migrate into the central nervous system

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