表1 21年度出身国別トキソプラズマ感染状況

	Total	M	F
ARG	1/4	0/2	1/2
AUS	0/1	0/1	
BOL	20/24	9/11	10/12
BRA	20/48	6/17	14/31
CHL	0/1		0/1
COL	1/2		1/2
IRN	0/1	0/1	
JPN	1/6	0/3	1/3
NGA	2/3	2/2	
NZL	0/1	0/1	
PER	9/25	4/10	5/15
PHL	2/12	2/5	0/7
VNM	6/72	1/17	5/41
unknown	1/1		

表2 年齢別トキソプラズマ感染状況

20年度

	Total	M	F
Total	61/201	24/70	36/114
1-19	2/15	1/6	1/9
20-29	6/30	0/10	6/19
30-39	10/34	3/12	7/18
40-49	23/68	11/24	12/37
50-59	13/30	6/12	7/16
60-89	5/19	2/3	3/14

21年度

	Total	M	F
Total	38/139	15/65	23/74
1-19	1/54	0/29	1/25
20-29	2/11	0/3	2/8
30-39	16/35	6/14	10/21
40-49	13/28	5/12	8/16
50-59	3/8	2/5	1/3
60-	3/3	2/2	1/1

厚生労働科学研究費補助金(新興・再興感染症研究事業)
分担研究報告書

慢性寄生虫感染症の侵入監視及びその健康管理体制の確立
慢性期シャーガス病の調査研究

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研究要旨:在日ラテンアメリカ人のシャーガス病感染情況の把握・本疾患を媒介する昆虫が生息しない我が国では輸血感染、臓器移植感染、母子感染以外に感染経路は皆無である。輸血、細胞臓器移植治療による、感染防止対策上、医療現場で対応できる *T. cruzi* 抗体についてのスクリーニングを行うことの出来る迅速診断キットの検討を行う。在日外国人の本疾患感染情況の把握とその健康管理/教育体制整備のためのガイドライン作成に必要な情報を提供する。

A. 研究目的

① 在日ラテンアメリカ人の *Trypanosoma cruzi* (*T.cruzi*) 抗体保有者の検索。

② 献血現場で対応できる迅速抗体測定キットの開発検討。

B. 研究方法

1) 疫学調査:主任研究者と共に、調査研究参画への同意書が得られた方々を対象に抗体検査を行った。健診で得られた血清につい病原体 *T. cruzi* に対する IgG 抗体の有無をクロマト法、IHA,IFA、Dot-ELISA 法で調べた。

今回はラテンアメリカ人定住者の8割を占めるブラジル人に特化して群馬県、茨城県下のブラジル人学校関係者を対象に *T. cruzi* 抗体の有無について Chagas-Stat-Pack (CHEMBIO) および、開発途上キット Instant-ChagasCheck (EY-Labo) を用いて検討した。常総市の健診では母国での生活環境についてのアンケート調査を行った。

2) 簡易 *T.cruzi* 抗体スクリーニングキットの開発:既存の慢性シャーガス病患者血清(ブラジル人・ボリビア人)を用いて迅速スクリーニング用検査キットの開発を検討した。

開発キットの評価は中南米諸国で既に使用されている CHEMBIO 社の CHAGAS-STAT-PACK と結果の比較検討を行った。

C. 結果

結果は茨城県常総市および、群馬県大田のいずれの検診でも *T. cruzi* 抗体陽性者は居なかった。常総市で行った

健診アンケートから成人の平均年齢が37 歳であった。常総市の健診で判ったことは 63.8%がサンパウロ州出身、12.0%がパラナ州,ミナス州が 3.6%その他 7.3%であり。既に日本生まれが13.3%も居ることが判明した (表-1)。

表-1

受診者の出身地および男女比
平均日本滞在期間8. 9年(2～20年)

出身地	総数〔人〕 83	男性 30	女性 53
サンパウロ	53(63. 8%)	19	34
パラナ	10(12. 0%)	1	9
ミナス州	3 (3. 6%)	1	2
ロンドニア	3 (3. 6%)	2	1
リオ	1(1. 2%)	1	
ペルー	2(2. 4%)	1	1
日本	11(13. 3%)	5	6

表-2

シャーガス病認識度と生活環境(20091129)

年齢 (人数)	媒介昆虫	シャーガス病	木造家屋	土壁茅葺	レンガコンクリート	輸血	献血
11~20 (25)	7	5		1	1	1	
21~30 (13)	10	9			4		
31~40 (21)	16	17	3		6		
[illegible]	6	4	3	1	1	2	
[illegible]	2	1		1			
[illegible]					1		
年齢不詳計 25名	41/83	5/83	6	3	13	3	0

シャーガス病を知っていると回答の36家族中【5家族】13. 8%の家族にシャーガス病と診断されたものが居るた。
平均年齢27 73、成人平均年齢 36. 82

T. cruzi 抗体陽性者は居なかったが、その家族にブラジルでシャーガス病と診断を受けたものが居ると回答があったのは 36 家族中 5 家族 (13.8%) であった。健診対象者の若年層にサシガメを知っていると回答した者が多い。生活家屋の構造は若年層ではレンガコンクリート構造での生活、木造、土壁藁葺き構造に居住経験があるものは40歳以上に若干見られた。現地での輸血経験者も 3 名居たがいずれも抗体は陰性であった。日本での献血経験者は居なった (表-2)。

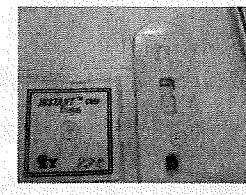
太田市で行ったものでも成人平均年齢は 38 歳と若く常総市と同様な結果と思われるが、Chagas 病に特化したアンケート調査は行われていない。

迅速キットの開発については在日ラテンアメリカ人のシャーガス病患者血清、血液、および保存血清(ブラジル現地採血)の材料を用いて、検討を行った。その結果既存の検査キットと結果は略一致し、なおかつ献血現場で充分判定が可能なキットの試作が出来た。

開発スクリーニングキット
Instant-Chagas-Check

抗原プロットに既があり判定が困難なものもあるが習熟すればその判定は可能であり、スクリーニング検査には使用できる

・ 全血を用いても充分に検出できる



D.考察

今回はシャーガス病慢性感染者を見出すために、南米からの定住者に特化して健診を行った。その結果ブラジル、ペルーからの定住者が健診を受けたが、いずれも対象集団の成人平均年齢が37 歳と若く、母国での感染リスクは低く

なってからの世代であることが示唆された。一方、アンケート結果 (表-2) から判るようにその家族には母国でシャーガス病と診断された者が居る、と回答を寄せたのは 5/36 家族 (13.8%) とかなり高率に日系家族で感染者があることが判明、協力者〔三浦〕の現地調査 (2007~2008) の結果、日系移住地でのシャーガス病感染状況は 10~15% と略一致している。また、同様に在日ラテンアメリカ人のシャーガス病検査依頼(日本各地の医療機関)のデータと比較すると、検査対象の平均年齢がブラジル人集団53歳、ボリビア人集団52歳と、明らかに今回の対象集団とは年齢的に隔たりが認められた。

1970 年代から南米ではシャーガス病感染予防対策として媒介昆虫の撲滅作戦が展開され、サシガメの生息が激減した。また、輸血による感染予防対策も 1985 年頃より輸血時にはシャーガス病感染に対する抗体スクリーニング検査が義務付けられていることから、今回健診対象になった世代はほとんどがリスクの少ない世代であると考えられる。一方現在世界的に最もシャーガス病感染リスクの高いボリビアからの在日定住者は僅かであるが、南米で現在最も感染リスクが高いボリビア人集団を組織的に健診が出来ないのは、彼らのコミュニティーを把握するリーダーが居ないことにあり、今後もこの集団に関する情報の把握は大変困難と思われる。

T. cruzi 抗体スクリーニングキット開発については、評価対象として用いた Chagas-Stat-Pack (CHEMBIO-USA) は特異性が高く Recombinant antigen を用いて、中南米で広く用いられているスクリーニングキットである。 それに比べると Instant-Chagas-Check は粗抗原を用いているためか、False-positive の問題が多少残るが、ブラジルでの陽性患者血清を用いて Stat-Pack との相関を検討してみるとまったく問題はない。また全血を用いての反応性、スクリーニングの迅速性から考えても、充分に現場での使用に耐えうるものであり、コストパフォーマンスの面では前者に勝っている。今後在日ラテンアメリカ人を対象とした疫学調査を実施する場合は地域特性を把握し成人を対象に検査を実施することが望ましく、ラテンアメリカ人を中心に調査を継続すれば、更に潜在感染者が検出されるはずであり、早期発見につながれば彼らにとっても、自身の健康管理に検査結果がつながる。今回も百余名のラテンアメリカ人を健診できたのも NPO,NGO の協力が得られ結果の手渡し、詳細説明が行われたことは地域社会医療の面からも意義があったと思われ、今後の同地域での活動の推進につながる。

E. 結論

ラテンアメリカ人支援NPO,NGOの実施するネットワークを通じシャーガス病検診のみならず、ラテン諸国の知られざる感染症に対する啓蒙講演は彼らを受け入れる地域社会の医療機関関係者への呼びかけにもなりうる。献血現場で実施する問診票の改訂にも言及し、抗体スクリーニング検査実施方法などの提言をすべきである。

F;健康危険情報

一昨年健診を通じて検出された在日ボリビア人の抗体陽性者については本疾患の慢性感染を強く示唆する者であり、献血輸血の禁止など健康管理面での注意を喚起する必要がある。

協力者三浦の情報提供では昨年の成人健診、移動領事館健康相談においては 3/210(1.43%)に *T. cruzi* 抗体陽性者を検出している。シャーガス病慢性感染キャリアーの可能性を強く示唆する者は少なからず在日定住者の中に存在する。

ラテンアメリカ人定住者が多い地域医療関係者には注意を促す啓蒙口演など情報の発信が不可欠。

G:論分:なし
関連:小冊子
竹内勤、三浦左千夫
しのびよるシャーガス病・・・中南米の知られざる感染症、慶應義塾大学出版会
2009−3月
学会発表:
第 58 回日本感染症学会東日本地方会学術集会、2009 年 10 月(東京);輸入感染症:在日ラテンアメリカ人の慢性シャーガス病キャリアーと2次感染予防
慶應義塾大学医学部・熱帯医学寄生虫学○三浦　左千夫・竹内　勤

H:
特許取得:なし
実用新案登録:なし
その他:将来的に *Trypanosoma cruzi* 抗体スクリーニング用キットが評価されれば
特許出願の予定

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
赤尾信明	イヌ回虫症(トキソカラ症)	岸本寿男、山田章雄	ズーノーシスハンドブック	メディカルサイエンス社	東京	2009	83-85
竹内勤、三浦左千夫	しのびよるシャーガス病	竹内勤、三浦左千夫	しのびよるシャーガス病	慶応義塾大学出版会	東京	2009	i-iii, 1-110

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Hu T, Takamoto M, Hida S, Tagawa YI, Sugane K.	IFN-γdeficiency worsen Pneumocystis pneumonia with Th17 development in nude mice.	Immunol Lett.	127	55-59	2009
赤尾信明	ヒトの犬・猫回虫症	Clinic Note	52(November)	66-68	2009
赤尾信明	小児のイヌ・ネコ回虫症(トキソカラ症)	小児科臨床	62(4)	697-702	2009
菅沼真澄, 七戸和博, 友田弥里, 鈴木晟幹, 赤尾信明, 太田伸生	動物から感染するヒトの回虫症	臨床福祉ジャーナル	6(1)	39-43	2009
大友弘士, 赤尾信明	抗微生物薬の治療効果の判定　2.マラリア	検査と技術	37(10)	977-982	2009

寄生虫

イヌ回虫症(トキソカラ症)

Toxocariasis

病原体
Toxocara canis
Toxocara cati

媒介動物	保有動物	主な感染様式
ミミズ, コキブリ	イヌ, ネコ, 待機宿主(ニワトリ, ウズラ, ウシなど)	経口(砂場, 生肉)

この疾患について

図1

イヌ回虫症(Toxocariasis)は，イヌ回虫(*Toxocara canis*)あるいはネコ回虫(*Toxocaria cati*)の幼虫によって引き起こされる疾患である．幼虫はヒトの体内では発育することなく，体内を移行して炎症反応を引き起こす．従来，幼小児に好発する疾患とみなされてきたが，最近では成人例が増加している．

感染経路

図2

虫卵に汚染された砂場：糞便とともに排泄された虫卵は外界において2～4週間で，感染幼虫をもつ幼虫包蔵卵となる．幼虫包蔵卵の経口摂取によりヒトに感染する．卵殻の周りには「糊」の役割をもつタンパク膜があり，手指につくと脱落しづらい．

食物媒介感染：ニワトリやウシの肝臓の生食により感染する．これらの動物はイヌ回虫やネコ回虫の待機宿主となっている．幼虫はこれらの臓器の中で長期間生存しており，摂取された幼虫が直接感染すると考えられている．

潜伏期間

動物モデルでは，幼虫は1～2週間で全身(特に脳と筋肉)に分布し，さまざまな症状が出現するが，ヒトの場合，発症するまでに通常1ヵ月ほどが経過している場合が多い．眼型トキソカラ症では，前駆症状なしに発症する場合がほとんどで，感染時期を特定することは困難である．

症　状

幼虫は通常，腸管粘膜を穿通して血行性，あるいはリンパ行性に体内移行を開始する．肝臓から肺に移行する時期には肝炎・肺炎がみられる呼吸器症状を主訴として受診する例が多い(内臓型トキソカラ症)．幼虫が網膜内に移行すると，ぶどう膜炎や眼内炎を起こし，失明する場合もある(眼型トキソカラ症)．小児では，しばしば網膜芽細胞腫と誤診される．中程度の好酸球増加症を示す抗体陽性者で，症状のみられないものを潜在型トキソカラ症といい，アレルギー疾患や免疫疾患異常との関連が指摘されている．

検査・診断

内臓型では好酸球増多や血清中の幼虫排泄物抗原に対する特異的抗体上昇が必発するが，眼型ではみられないことが多い．眼内液中の抗体の有無と特徴的な眼底所見により診断される．

予防・治療

小児では外遊びやイヌ・ネコとの接触後の手洗い励行，成人では待機宿主となるニワトリやウシの肝臓・筋肉などの生食の禁止により予防が可能である．治療は抗炎症薬#と駆虫剤アルベンダゾール#（10～15 mg/kg/day）との併用による．

#：保険適応外

動物での状況

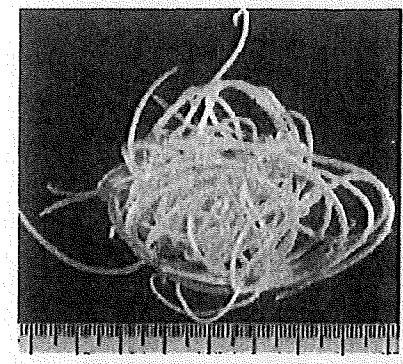
臨床症状　主として生後1年未満の仔イヌに感染がみられる．少数感染では無症状であるが，多数感染では軟便，下痢などの消化器症状がみられ，発育障害が生じる．糞便内や嘔吐物中に虫体の排泄をみることもある．仔イヌでのイヌ回虫の感染は，都会では年々低下しつつあるといわれているが，われわれが2007年に栃木県内の2ヵ月齢のイヌ26頭を検査したところ，19頭（73%）でイヌ回虫卵が陽性であった．成長すると，回虫は自然に排虫されてしまう．ネコではこのような自然排虫現象はみられず，成虫は成猫にも寄生する．

仔イヌへの感染は胎盤感染で起こり，成犬が虫卵を摂取しても腸管内で成長することはなく，筋肉内で幼虫のまま潜んでいる．雌イヌでは妊娠を契機として胎盤感染が起こり，仔イヌに移行する．ネコ回虫は乳汁感染によって仔ネコに感染し，成猫は虫卵の経口摂取によって感染が起こる．

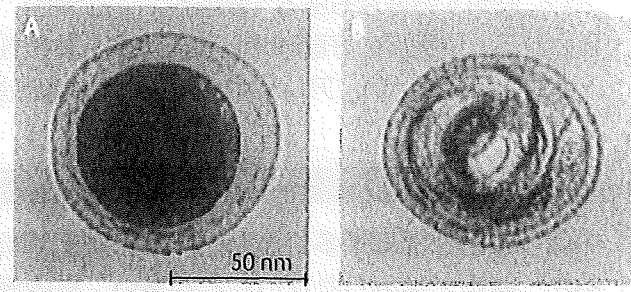
検査・診断　イヌ回虫，ネコ回虫とも多数の虫卵を産卵するので，糞便検査によって虫卵を確認することで診断する．

予防・治療　仔イヌは生後2ヵ月目までのできるだけ早い時期に獣医師の診断を受け，感染がみられたならば，駆虫剤の投与を行う．一度の検査で陰性の場合でも，3週ごとに検査を行い，感染の有無を確認する．雌成犬が感染すると，次の妊娠出産を契機に仔イヌへの感染が起こるので，散歩途中の拾い食いや感染したイヌとの接触を避ける．ネコでは生涯を通して感染の機会があるため，定期的な検診と駆虫が必要である．駆虫剤としてはミルベマイシン・オキシムやパモ酸ピランテルを用いる．

飼育ケージは常に清潔に保ち，排泄物は直ちに処理することが重要である．また，虫卵は被毛にも付着するので，定期的なシャンプーなどイヌやネコ自身を清潔に保つことも必要である．



【図1】イヌ回虫成虫



【図2】イヌ回虫卵
排卵直後（A），幼虫包蔵卵（B）．

ケーススタディ

事例1　ニワトリの肝臓を生食後，肺炎症状を呈した症例

患　者　45歳．男性

主　訴　全身倦怠感，微熱．

家族歴　71歳の父親も同様の症状を訴える．

既往歴　25歳よりアレルギー性鼻炎，29歳より扁桃腺炎．

経　過　2004年5月上旬より全身倦怠感，微熱，後頭部痛，右前腕のしびれが出現．近医にて末梢血好酸球増多と胸部X線異常を指摘され，好酸球性肺炎の診断にて抗菌薬とステロイド薬を投与された．異常陰影は改善傾向にあったが，好酸球数は低下しなかったため，当院紹介入院となる．

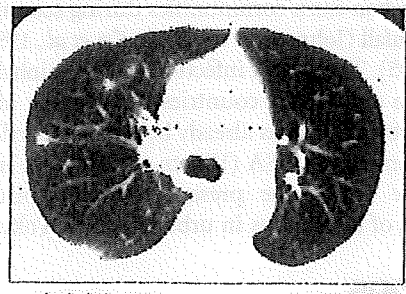
疫学的背景　入院後の詳細な問診によって4月下旬に父親とともに，名古屋コーチンの生肝を一口食べたことが判明した．

検査・診断　検査結果は次のとおり．血清および気管支洗浄液中のイヌ回虫幼虫排泄物抗原に対する抗体が著明に上昇していた．

血液検査

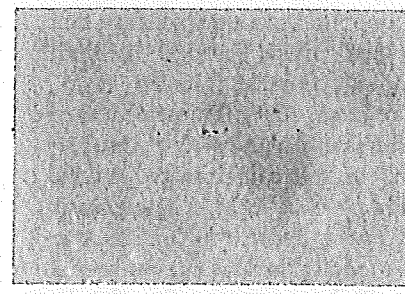
	WBC	Neutrophil 桿状核球 (%)	Neutrophil 分節核球 (%)	Eos (%)	Lym (%)	Mono (%)	Hb (mg/dL)	Plt	CRP (mg/dL)	IgE
症例	15,000	2	42	36	17	3	16.7	56,000	1.0	24,000
父親	25,900	4	14	63	16	3	14.5	114,000	1.0	3,220

CT検査

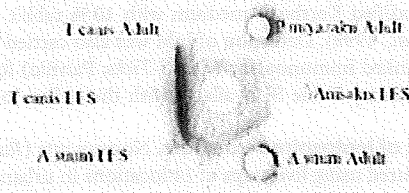


入院時胸部CT像
（写真提供：熊本病院呼吸器科 森松 嘉孝先生のご厚意による）

病理組織検査（肝生検）



好酸球浸潤を伴う壊死組織を認める．



免疫学的検査
寒天ゲル内沈降反応で患者血清中にイヌ回虫幼虫抗原に対する強い抗体が証明された．

「イヌ回虫症（トキソカラ症）」著：赤尾 信明

Journal of Medical Microbiology (2009), 58, 1227–1230 DOI 10.1099/jmm.0.011528-0

Human leptospirosis cases and the prevalence of rats harbouring *Leptospira interrogans* in urban areas of Tokyo, Japan

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Thirteen patients with leptospirosis were identified, as confirmed by laboratory analysis during the last 5 years in our laboratory, who came from urban areas of Tokyo, Japan. All of the patients came into contact with rats before the onset of illness. Seventeen per cent of Norway rats captured in the inner cities of Tokyo carried leptospires in their kidneys. Most of these rat isolates were *Leptospira interrogans* serovar Copenhageni/Icterohaemorrhagiae. Antibodies against these serovars and their DNA were detected in the patients. This suggests that rats are important reservoirs of leptospirosis, and that rat-borne leptospires occur in urban areas of Tokyo.

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INTRODUCTION

Leptospirosis is caused by infection with pathogenic *Leptospira*. It is a globally important zoonotic disease that affects humans in rural and urban settings, in both industrialized and developing countries (Bharti *et al.*, 2003; Levett, 2001; McBride *et al.*, 2005). Transmission of *Leptospira* pathogens to humans occurs mainly through indirect contact with water or soil contaminated by the urine of infected animals (Faine *et al.*, 1999). Leptospirosis has become an important public health problem in Asia and Latin America. In these tropical areas, large outbreaks of leptospirosis are most likely to occur after floods, hurricanes or other disasters. Leptospirosis has also become an urban problem in developing countries. Outbreaks occur in poor urban slum communities during seasonal periods of heavy rainfall (Johnson *et al.*, 2004; Ko *et al.*, 1999; LaRocque *et al.*, 2005). The risk of infection in urban inhabitants is not limited to developing countries because the importance of urban leptospirosis has already been recognized in inner-city populations of the USA (Vinetz *et al.*, 1996). In the present study, we report the presence of leptospirosis and rat reservoirs of leptospires in urban areas of Tokyo, Japan.

METHODS

Serodiagnosis of patients with clinically suspected leptospirosis. The microscopic agglutination test (MAT) for detection of anti-*Leptospira* antibodies in patient serum samples was performed (Faine *et al.*, 1999) using a battery of reference strains described previously (Koizumi *et al.*, 2008). These reference strains were cultivated in liquid modified Korthof's medium with 10 % rabbit serum at 30 °C (Faine *et al.*, 1999). Detection of IgM was also carried out by IgM dot enzyme-linked immunoassay (*Dip-S-Ticks*; PanBio) for cases 6 and 8 (Supplementary Table S1 available with the online journal).

Isolation of leptospires from rats. Norway rats (*Rattus norvegicus*) were captured using live traps at 14 locations in urban areas of Tokyo from 2002 to 2007. For the isolation of leptospires, rat kidneys were inoculated into medium and cultivated as described above.

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Abbreviation: MAT, microscopic agglutination test.

The GenBank/EMBL/DDBJ accession numbers for the *flaB* sequences of rat isolates and patient samples are AB454100–AB454125.

A table of detection test data and a figure of PFGE results are available as supplementary data with the online version of this paper.

PCR. DNA was extracted from *Leptospira* isolates, and the blood and urine samples of patients, using a DNeasy tissue kit (Qiagen). Extracted DNAs were subjected to PCR to detect the *Leptospira flaB* gene (*flaB*-PCR; Kawabata *et al.*, 2001; Koizumi *et al.*, 2003). Sequencing of amplicons was performed by the dideoxynucleotide chain-termination method using a BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems).

Identification of serogroups of rat isolates. The serogroups of the isolates were identified by MAT using a panel of anti-*Leptospira* rabbit sera for serovars Australis, Autumnalis, Canicola, Copenhageni, Hebdomadis and Icterohaemorrhagiae, which are present in the main island of Japan.

PFGE. PFGE of rat isolates was carried out as described previously (Koizumi *et al.*, 2009).

RESULTS AND DISCUSSION

Human leptospirosis cases in urban areas of Tokyo

In the last 5 years (from the first case on 4 September 2003 to the last on 18 September 2008), we carried out laboratory examinations for leptospirosis for 55 cases. According to their physicians in Tokyo, the symptoms in these patients matched those of leptospirosis. A total of 16 cases were revealed to be positive for leptospirosis during the period of the study; 13 were from Tokyo (Table 1, Supplementary Table S1 available with the online journal) and the other 3 cases were from Bali (Indonesia), Borneo (Malaysia) and Fiji (data not shown). Among the 13 cases in Tokyo, 12 patients were definitively diagnosed by MAT (fourfold increase in antibody titre between acute and convalescent serum samples or reciprocal MAT titre of at least 400 in a single serum sample), including 4 patients who were also positive for the leptospiral *flaB* gene by PCR using urine or blood specimens. One probable case was demonstrated by anti-leptospiral IgM and a MAT titre of 1 : 160. All patients were hospitalized with severe manifestations, such as acute renal failure and jaundice, indicating that they had contracted Weil's disease (a severe type of leptospirosis). All patients declared that they had come into contact with rats (Table 1). Two patients (nos 1 and 2) worked at a place where they came in contact with rats, and rats were frequently found in the houses or stores of other patients (nos 3–13). The patients neither performed agricultural work nor undertook recreational activity in an endemic area, nor were they exposed to possible reservoir animals other than rats, which are generally considered as high risk behaviours. Among the 39 leptospirosis-negative cases, only 4 patients came in contact with rats (1 patient was a construction worker, and rats were seen at the houses of the other 3 patients). This indicated that the ratio of contact with rats among leptospirosis-positive cases was significantly higher than that among leptospirosis-negative cases in urban areas of Tokyo (Fisher's exact test, $P<0.01$). Since leptospirosis became a reportable disease in Japan (from November 2003), another laboratory-confirmed case other than the 13 cases mentioned above was reported from a regional medical centre in Tokyo in September 2006. The patient saw rats at his restaurant (T. Iida, personal communication).

Isolation and characterization of *Leptospira interrogans* from Norway rats captured in urban areas of Tokyo

We captured 127 Norway rats (*R. norvegicus*) at 14 locations in urban areas of Tokyo from 2002 to 2007. Leptospires were isolated from 22 of the 127 rats from 6 of

Table 1. Human leptospirosis in urban areas of Tokyo diagnosed in our laboratory from 2003 to 2008

Patient no.	Year	Sex	Age (years)	Occupation	Association with rats
1	2003	M	66	Construction worker	Probable environmental contamination with rat urine
2	2004	M	35	Sewer worker	Probable environmental contamination with rat urine
3	2005	F	53	Housewife	Rats appeared frequently in patient's house
4	2005	M	65	Butcher	Patient cleaned the urine and faeces of rats in his store without wearing gloves
5	2006	M	51	Fish dealer	Rats appeared frequently in patient's store
6	2006	M	62	Unknown	Rats appeared frequently in patient's house
7	2006	M	54	Fish dealer	Patient cleaned the urine and faeces of rats in his store without wearing gloves
8	2007	M	51	Day worker	Rats appeared frequently in patient's house (he had been bitten by a rat at his house)
9	2007	F	57	Restaurant worker	Rats appeared frequently in patient's restaurant
10	2008	M	57	Supermarket salesman	Patient was involved in killing rats captured at his store
11	2008	M	56	Fish market worker	Rats appeared frequently in the fish market
12	2008	M	58	Unknown	Patient had contact with rat urine in his house
13	2008	M	68	Restaurant chef	Patient had been bitten by a rat in his restaurant

F, Female; M, male.

the 14 places (Table 2). Nucleotide sequences of the partial *flaB* gene from 18 rats captured at locations F, G, H, K and M were identical (GenBank accession numbers AB454100–AB454117) and those from 4 rats at location A (the *flaB* sequences were identical among the four; GenBank accession numbers AB454118–AB454121) were different in six bases from those described above. The sequences from the 18 rats were identical to those of the reference strains of *L. interrogans* serovar Copenhageni and Icterohaemorrhagiae, suggesting that all the isolates were *L. interrogans*. These isolates reacted equally with anti-Copenhageni and anti-Icterohaemorrhagiae sera, but not with the other sera (data not shown). The *Not*I restriction patterns of the genomes of the isolates from the 18 rats were identical on PFGE not only to each other, but also to the reference strains of serovars Copenhageni and Icterohaemorrhagiae (Supplementary Fig. S1 available with the online journal). It has been determined by PFGE that the genomes of leptospiral serovars have been remarkably conserved over time and across a wide geographical distribution (Herrmann *et al.*, 1991, 1992). Most (but not all) serovars give unique patterns on PFGE carried out using the restriction enzyme *Not*I, although the *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae are indistinguishable. These two serovars are also very difficult to distinguish by serological methods (Kobayashi *et al.*, 1984). These results indicate that isolates from the 18 rats belonged to *L. interrogans* serovar Copenhageni or Icterohaemorrhagiae, which are known to frequently cause Weil's disease in Japan and other countries. We could not carry out MAT for serogroup identification and PFGE on the four isolates at location A due to poor growth.

Table 2. Isolation of leptospires from rats captured in urban areas of Tokyo

Location*	No. of rats captured	No. of rats from which *Leptospira* was isolated (%)
A – park	15	4 (27)
B – park	2	0
C – street	4	0
D – street	8	0
E – building	12	0
F – street	29	12 (41)
G – street	1	1 (100)
H – garden (house)	4	3 (75)
I – park	1	0
J – street	12	0
K – street	13	1 (8)
L – street	5	0
M – store	2	1 (50)
N – street	19	0
Total	127	22 (17)

*Location M was a store in which patient 7 used to work; other locations are not related to the putative exposure sites of the patients.

Conclusion

In 5 of the 13 human leptospirosis cases (cases 1–3, 6 and 7), the patients were infected with serovar Copenhageni or Icterohaemorrhagiae as shown by serological and PCR-based evidence (Supplementary Table S1 available with the online journal). Cross-agglutination and even paradoxical reactions are observed in MAT, but the existence of antibodies against serovars Copenhageni and Icterohaemorrhagiae in all other serum samples suggests the possibility of infection with these serovars (Supplementary Table S1 available with the online journal). Nucleotide sequences of the partial *flaB* gene from urine and blood (patients 1, 2, 3 and 7; GenBank accession numbers AB454122–AB454125) were identical to those from the rat isolates. In particular, *Leptospira* was isolated from a rat captured at the store where patient 7 previously worked. Although there is a possibility of recall bias, all the patients said they had rat contact (Table 1). Dogs and cats may also serve as reservoir hosts in urban settings. We attempted to isolate leptospires from the kidney tissues of stray or abandoned dogs and cats in Tokyo (304 dogs and 77 cats), but *Leptospira* was not obtained. These results strongly suggest that the patients contracted leptospirosis (Weil's disease) from rats in urban areas of Tokyo, though the possibility of involvement of other reservoir animals cannot be excluded.

Outbreaks of leptospirosis are recognized through occupational exposure, such as rice farming and other agricultural activities in rural areas of the tropics (Tangkanakul *et al.*, 2000). Leptospirosis has also become a health problem in urban slums in developing countries (Johnson *et al.*, 2004; Ko *et al.*, 1999; LaRocque *et al.*, 2005). In 'developed countries', recreational activities have recently been identified as a significant risk factor for leptospirosis (Bharti *et al.*, 2003; Levett, 2001; McBride *et al.*, 2005). The present study suggests that humans could contract leptospirosis through occupational exposure or exposure during activities of daily life in environments contaminated with rat urine containing leptospires in urban areas in Tokyo. Leptospirosis constitutes one of the neglected diseases in Japan except for Okinawa Prefecture (Nakamura *et al.*, 2006; Narita *et al.*, 2005). This is one of the reasons why few cases have been identified over the 5 year period despite the high carriage of leptospires in rats in Tokyo. A high prevalence of *Leptospira* spp. in Norway rats from urban settings has also been reported from temperate and tropical endemic countries, but human leptospirosis in urban areas is underreported even in an endemic country (Ariyapruchya *et al.*, 2003; Demers *et al.*, 1985; Doungchawee *et al.*, 2005; Easterbrook *et al.*, 2007; Krøjgaard *et al.*, 2009). Physicians and public health authorities should, therefore, be aware of the severe risk of contracting leptospirosis associated with rats in urban areas.

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IMMUNOLOGY ORIGINAL ARTICLE

STAT6 signalling is important in CD8$^+$ T-cell activation and defence against *Toxoplasma gondii* infection in the brain

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Summary

Signal transducer and activator of transcription (STAT) 6 is a molecule involved in interleukin (IL)-4 and -13 signalling. We investigated the role of STAT6 signalling in *Toxoplasma gondii*-infected mice using STAT6-deficient (STAT6$^{-/-}$) and wild-type (WT) mice. A significantly larger number of cysts were recovered from the brain in STAT6$^{-/-}$ than in WT mice on days 28 and 56 post-infection. CD8$^+$ T cells in cerebrospinal fluid and spleen stimulated with *T. gondii* antigen produced higher levels of interferon (IFN)-γ in WT than in STAT6$^{-/-}$ mice. CD8$^+$ T-cell function, estimated by expression of CD25 and cytotoxic activity, was lower in STAT6$^{-/-}$ than in WT mice. Transfer of CD8$^+$ but not CD4$^+$ T cells, purified from infected WT mice, into STAT6$^{-/-}$ mice successfully prevented formation of cysts in the brain. However, transfer of naïve CD8$^+$ T cells from WT into STAT6$^{-/-}$ mice did not show either activation of CD8$^+$ T cells or a decrease in the number of cysts in the brain. Transfer of splenic adherent cells from WT into STAT6$^{-/-}$ mice induced activation of CD8$^+$ T cells and decreased the number of cysts in the brain. Expression of CD86 on splenic dendritic cells and IL-12 p40 production were weaker in STAT6$^{-/-}$ than in WT mice after *T. gondii* infection. These results indicate that STAT6 signalling is important in CD8$^+$ T-cell activation, possibly through regulation of antigen-presenting cells, which could suppress *T. gondii* infection in the brain.

Keywords: CD8$^+$ T cell; signal transducer and activator of transcription 6; *Toxoplasma gondii*

Introduction

Toxoplasma gondii is an intracellular protozoan parasite that infects humans and other mammals. After peroral infection, *T. gondii* affects multiple organs including the spleen, liver, heart, lung and brain. The ensuing immune responses eliminate the pathogen from most organs, but not from the brain, where the parasite persists with development of chronic toxoplasmic encephalitis (TE).[1] This intracerebral parasite is regulated by interferon-γ (IFN-γ)-producing CD4$^+$ and CD8$^+$ T cells, which are recruited into the brain.[2] CD8$^+$ T cells are the major lymphocyte subpopulation involved in the protective immune response to TE.[3,4] CD8$^+$ T cell- or IFN-γ-deprived mice are unable to control both acute and chronic toxoplasmosis.[5,6]

In contrast to the important role of IFN-γ, the role of interleukin-4 (IL-4) in *T. gondii* infection is still unclear. Significantly greater acute focal inflammation with tachyzoites and a larger number of cysts in the brain were observed in IL-4-deficient (IL-4$^{-/-}$) than in wild-type (WT) mice on days 28 and 56 post-infection (pi). Mortality was also higher in IL-4$^{-/-}$ mice compared with WT mice during the late stage of infection. These results indicate that IL-4 is protective against TE by preventing formation of cysts and proliferation of tachyzoites in the brain.[7] In contrast, another group reported that IL-4$^{-/-}$ mice were resistant to *T. gondii* infection, showing a

higher survival rate than WT mice during the early acute phases of infection. Pathology in the small intestine was less severe in IL-$4^{-/-}$ mice although conversely liver pathology was greater than in WT mice.[8]

Intracellular signalling mechanisms provide the link between binding of a cytokine with its receptor and the effect of the cytokine on cellular function. The janus kinase (JAK) and signal transducer and activator of transcription (STAT) family plays a critical role in the signalling of many cytokine receptors. The IL-4 receptor (IL-4R) is associated with JAK1-3 and STAT6. STAT6-deficient (STAT$6^{-/-}$) mice are unable to process IL-4R-induced signals.[9,10] Furthermore, IL-13, which is closely related to IL-4 in biological function, shares receptor components and signalling through the STAT6 pathway with IL-4.[11]

In this study, the role of STAT6 signalling in cyst formation, TE in the brain and the immune response following *T. gondii* infection was investigated.

Materials and methods

Animals

STAT$6^{-/-}$ mice were donated by Dr S. Akira (Osaka University, Suita City, Japan)[10] and backcrossed to C57BL/6 (B6) mice at least 10 times. Six-week-old WT B6 mice were purchased from Clea Japan (Tokyo, Japan). Animals were housed in polycarbonate cages and fed with a commercial diet (Funabashi Farm, Chiba, Japan) in the Shinshu University Animal House. All mice were maintained under a 12 : 12 hr light/dark cycle (lights on at 9:00 AM) at 24 ± 2° and 55 ± 10% relative humidity. The Animal Ethics Committee of Shinshu University approved all protocols used in this study.

Monoclonal and polyclonal antibodies

Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) against murine CD4 (RM4-5), CD8 (53-6·7), B220 (RA3-6B2), CD11b (M1/70), CD11c (HL3), CD25 (7D4), CD44 (IM7), CD62L (MEL-14), H-$2D^b$ (KH95) and IFN-γ (XMG1·2) were purchased from BD Biosciences (San Jose, CA). Phycoerythrin (PE)-conjugated mAbs against CD4 (RM4-5), CD8 (53-6·7), CD11c (HL3), I-A^b (25-9-7), CD40 (3/23), CD80 (16-10A1) and CD86 (GL1) and PE-Cy5-conjugated anti-CD4 (RM4-5) mAb were also purchased from BD Biosciences. Rat immunoglobulin G (IgG) was purchased from Sigma-Aldrich (St Louis, MO).

Monoclonal Abs against murine CD4 (GK1·5) and CD8 (53-6·7) were purified as described previously.[12] Mice were injected intraperitoneally with anti-CD4 and anti-CD8 mAbs (0·5 mg/week) to deplete $CD4^+$ and $CD8^+$ T cells, respectively. Control mice received rat IgG. Depletion was confirmed by flow cytometric analysis.

Infection, cyst count and antigen preparation of T. gondii

An avirulent Fukaya strain and a virulent RH strain of *T. gondii* were donated by Dr N. Watanabe (Jikei Medical School, Tokyo, Japan). The Fukaya strain was maintained in B6 mice. Brains of infected mice were gently homogenized with a tissue homogenizer (UltraTurax; IKA-WERK, Staufen, Germany). Cysts were enriched by centrifugation of the homogenate. They were counted under a microscope in portions of precipitates that were smeared on cover-slipped glass slides. Mice were orally inoculated with brain homogenate containing 10 cysts using a stomach tube with a 1-ml syringe.[13]

Tachyzoites of the RH strain were collected from the peritoneal cavity of B6 mice injected 4 days previously. *Toxoplasma gondii* crude antigen was prepared by sonication of the tachyzoites followed by centrifugation.[14] The supernatant was stored as *T. gondii* antigen at −30° until use.

Histopathological evaluation

Mice were killed on days 14, 28 and 56 pi and their heads were fixed in 10% neutral buffered formalin solution. The cranium was then decalcified by immersion in K-CX (Fujisawa Co., Osaka, Japan) for 16 hr, washed with tap water for 12 hr and embedded in paraffin. Sections of 5 μm thickness were cut. Serial sections at 500-μm intervals were stained with haematoxylin and eosin. The histopathological changes in the brain were evaluated under a microscope.[13]

Collection of cerebrospinal fluid (CSF)

Mice were anaesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg; Dainippon Sumitomo Pharma, Osaka, Japan) and perfused through the heart with phosphate-buffered saline (PBS) to remove contaminating intravascular leucocytes. CSF was harvested by suboccipital puncture as follows.[13] The dura mater above the cisterna magna was exposed and cut. Then, 10–15 μl of CSF per mouse was aspirated with a micropipette. Total CSF cells were stained with Turk solution and counted under a microscope. The percentages of $CD4^+$, $CD8^+$, $B220^+$ and $CD11b^+$ cells were determined by flow cytometric analysis on cells stained with FITC-conjugated respective mAbs.

Culture of CSF cells and splenocytes

CSF cells (2×10^4 cells/well) in 200 μl or a single suspension of splenocytes (4×10^6 cells/well) in 1 ml of RPMI-1640 medium containing 10% fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 μg/ml),

and amphotericin B (0·25 mg/ml) were incubated with *T. gondii* antigen (12 μg/ml) at 37° in a humidified atmosphere of 5% CO_2 and 95% air for 48 hr. In order to block activation of CD4$^+$ and/or CD8$^+$ T cells in culture conditions, mAbs against murine CD4 (GK1·5) and/or CD8 (53–6·7) were added to the culture medium at 5 μg/ml before *T. gondii* antigen stimulation.[12] Supernatants were then collected and stored at −30° until use.

Flow cytometric analysis

Single nucleated cell suspensions of spleen, CSF and peripheral blood were stained with fluorescence-labelled mAbs for 30 min on ice in the dark after lysing erythrocytes with ammonium chloride solution. Cell populations were analysed by FACSCalibur (BD Biosciences).

Determination of cytokines using cytometric bead array (CBA)

The cytokine concentrations in CSF and culture supernatants of splenocytes and CSF cells were determined using a CBA mouse inflammation kit (BD Biosciences) according to the manufacturer's instructions.[15] The concentration of IFN-γ was also determined using the CBA Flex set (BD Biosciences). IL-12 p40 was determined using an OptEIA enzyme-linked immunosorbent assay (ELISA) set (BD Biosciences).

Intracellular IFN-γ staining

Splenocytes and CSF cells were incubated with *T. gondii* antigen for 24 hr as described above. Brefeldin A (10 μg/ml) was added during the last 4 hr of incubation. Cells were harvested, and stained with PE-Cy5-labelled anti-CD4 mAb and PE-labelled anti-CD8 mAb, followed by fixation with paraformaldehyde. After washing, cells were permeabilized with saponin and stained with FITC-labelled anti-IFN-γ mAb. Double-stained cells were analysed by flow cytometric analysis.

Separation and transfer of CD8$^+$ T cells, splenic adherent cells (SACs) and dendritic cells (DCs)

CD8$^+$ T cells were purified from splenocytes using a nylon wool column and magnetic beads. Briefly, 1×10^8 splenocytes were added to an autoclaved nylon wool column and incubated at 37° for 1 hr. The non-adherent T cells were collected by washing the column. Then, CD8$^+$ T cells were separated by indirect negative selection using Dynabeads M-450 (Dynal, Oslo, Norway), which bind to sheep anti-rat IgG after treatment of the cells with anti-CD4 mAb (GK1·5). The purity of CD8$^+$ T cells was > 95% as determined by flow cytometric analysis. Then, 5×10^6 CD8$^+$ T cells were injected into the tail vein of STAT6$^{-/-}$ mice at the indicated time.

SACs were separated from splenocytes by attachment to a polystyrene dish. Bone marrow (BM)-derived DCs were generated as described previously.[16] Briefly, BM cells were harvested from the femur of mice, and cultured at 1×10^5 cells/ml in the presence of 10 ng/ml recombinant murine granulocyte–macrophage colony-stimulating factor (GM-CSF; Endogen, Woburn, MA). Every 2 days, non-adherent cells were discarded and the remaining cells were fed with fresh medium containing 10 ng/ml GM-CSF. On day 6, loosely adherent cells were harvested by gentle pipetting. The purity of the cell population was determined to be more than 80% CD11c$^+$ by flow cytometry. SACs (1×10^6 cells/mouse) and DCs (1×10^6 cells/mouse) were injected into the tail vein of STAT6$^{-/-}$ mice before *T. gondii* infection.

Cytotoxic analysis

Cytotoxic activity of CD8$^+$ T cells was analysed as described previously with some modifications.[17] Briefly, peritoneal macrophages were harvested by lavage from untreated B6 mice. They were washed three times with PBS and labelled with PKH67 (Sigma-Aldrich). The cells were incubated with an optimal concentration of *T. gondii* antigen in 5% CO_2 and 95% air at 37° for 1 hr. They were then washed three times and incubated with purified CD8$^+$ T cells at various effector to target cell (E:T) ratios at 37° for 3·5 hr. Dead cells were determined by flow cytometry following labelling with 7-amino-actinomycin D (7-AAD) (BD Biosciences).

Statistical analysis

Statistical analysis of the data was performed using Student's *t*-test. A value of $P < 0·05$ was accepted as indicating significance.

Results

Histopathological evaluation and cyst burden in the brain

STAT6$^{-/-}$ and WT mice were orally infected with 10 cysts of an avirulent Fukaya strain of *T. gondii*. Less than 10% of infected mice died between days 10 and 14 pi. The number of cysts in the brain was greater in STAT6$^{-/-}$ than in WT mice on days 28 and 56 pi (Fig. 1a). Before day 14 pi, little evident inflammatory change was observed in the brains of STAT6$^{-/-}$ and WT mice (data not shown). However, on day 28 pi, cellular infiltrate was observed in the subarachnoid space and cerebral cortex in both STAT6$^{-/-}$ and WT mice (Fig. 1b). It was relatively mild in STAT6$^{-/-}$ compared with WT mice, in contrast

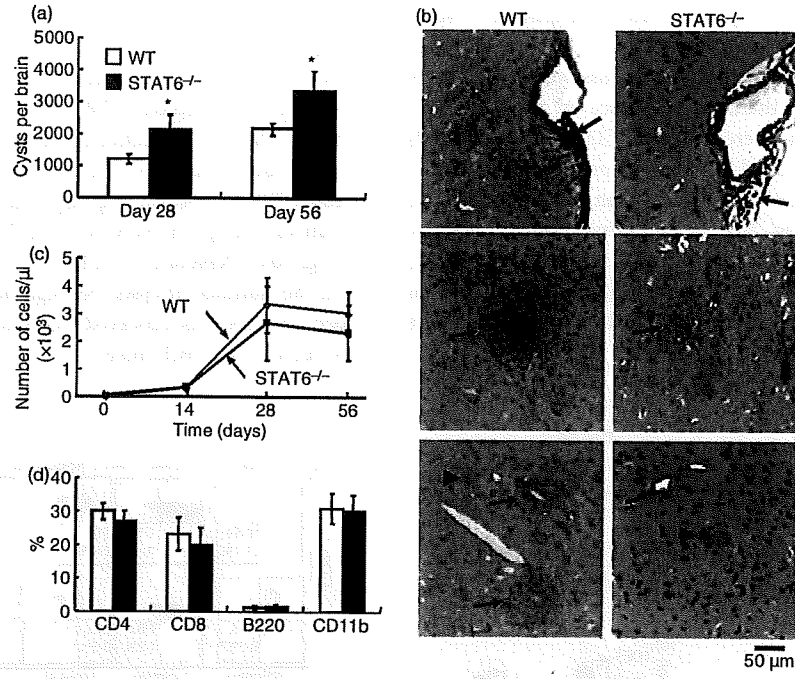

Figure 1. Cyst burden, histopathological findings and immune responses in the brain. (a) Brains were removed on days 28 and 56 pi, and the number of cysts was counted under a microscope. Data are expressed as mean ± standard deviation (SD) (n = 5). Similar results were obtained from five replicate experiments. (b) Representative photographs of the brain on day 28 post-infection (pi) are shown. Sections were fixed and stained with haematoxylin and eosin. Arrows indicate cellular infiltration in the subarachnoid space and cerebral cortex. Arrow heads indicate *Toxoplasma gondii* cysts. Note that the area of inflammation is wider in wild-type (WT) than in STAT6$^{-/-}$ mice. (c) Cerebrospinal fluid (CSF) was obtained from uninfected and infected mice on days 14, 28 and 56 pi. Nucleated cells were stained with Turk solution, and the total number of cells was counted. (d) Classification of cells in CSF was determined by flow cytometric analysis. Data are expressed as mean ± SD (n = 5). Similar results were obtained from three replicate experiments. *Significantly different from WT mice ($P < 0{\cdot}05$).

to cyst burden. Cysts were not surrounded by inflammatory cells. The number of CSF cells increased after infection and peaked on day 28 pi, and then decreased in both STAT6$^{-/-}$ and WT mice (Fig. 1c). The total number of CSF cells in STAT6$^{-/-}$ mice was comparable to that in WT mice. Monocytes and CD4$^+$ and CD8$^+$ T cells were present in CSF, and there was no difference in the the percentage of each population between STAT6$^{-/-}$ and WT mice (Fig. 1d).

IFN-γ concentration in CSF and *in vitro* production by CD8$^+$ T cells were reduced in *T. gondii*-infected STAT6$^{-/-}$ mice

Inflammatory cytokine levels in CSF were determined on days 14, 28 and 56 pi. On day 14 pi, no significant difference was observed in levels of IL-6, IL-10, monocyte chemotactic protein (MCP)-1, IFN-γ, and tumour necrosis factor (TNF), which were very low in STAT6$^{-/-}$ and WT mice. However, on days 28 and 56 pi, their concentrations in CSF were markedly increased, and the IFN-γ level was significantly lower in STAT6$^{-/-}$ than in WT mice (Fig. 2a). After *in vitro* cultivation of CSF cells with *T. gondii* antigen, IFN-γ production by CD8$^+$ but not CD4$^+$ T cells and the percentage of IFN-γ-producing cells in CD8$^+$ T cells were greater in WT than in STAT6$^{-/-}$ mice (Fig. 2b and 2d). Splenocytes recovered on day 28 pi were cultured with *T. gondii* antigen *in vitro*. IFN-γ production by CD4$^+$ T cells in STAT6$^{-/-}$ mice was comparable to that in WT mice. The percentage of IFN-γ-producing CD4$^+$ T cells in STAT6$^{-/-}$ mice was not different from that in WT mice, whereas IFN-γ production by CD8$^+$ T cells was significantly lower in STAT6$^{-/-}$ mice than in WT mice. The percentage of IFN-γ-producing CD8$^+$ T cells was also significantly lower in STAT6$^{-/-}$ mice than in WT mice (Fig. 2c and 2e). These results indicate that IFN-γ production by CD8$^+$ T cells, but not by CD4$^+$ T cells, was systemically reduced in *T. gondii*-infected STAT6$^{-/-}$ mice.

Activated CD8$^+$ T cells were decreased in STAT6$^{-/-}$ mice

We further investigated CD8$^+$ T cell activation and function during *T. gondii* infection in STAT6$^{-/-}$ and WT mice. CD25$^+$, CD62L^{low} and CD44$^+$ CD8$^+$ T cells

increased in CSF, spleen and peripheral blood after infection. The percentage of CD25$^+$ CD8$^+$ and CD62L^{low} CD8$^+$ T cells in CD8$^+$ T cells was significantly lower in STAT6$^{-/-}$ than in WT mice in the CSF and spleen (Fig. 3a). The percentage of CD25$^+$ CD8$^+$ T cells in CD8$^+$ T cells peaked on day 10 pi, and was significantly lower in STAT6$^{-/-}$ mice than in WT mice in peripheral blood (Fig. 3b). In addition, cytotoxic activity of CD8$^+$ T cells in infected mice was significantly lower in STAT6$^{-/-}$ than in WT mice (Fig. 3c). The role of CD8$^+$ T cells in protection against *T. gondii* cyst formation in the brain was then studied. As shown in Fig. 1c, infiltration of T cells into the brain started on day 14 pi. Therefore, we injected anti-CD8 mAb on days 14 and 21 pi to deplete CD8$^+$ T cells. Injection of the mAb before day 14 pi induced the death of mice. A significantly larger number of cysts formed in the brains of the anti-CD8 mAb-treated group than in the rat IgG-treated control group in STAT6$^{-/-}$ and WT mice, respectively (Fig. 3d). Depletion of CD4$^+$ T cells had little influence on the number of cysts. No significant difference in number of cysts was observed between CD8$^+$ T cell-depleted STAT6$^{-/-}$ and WT mice.

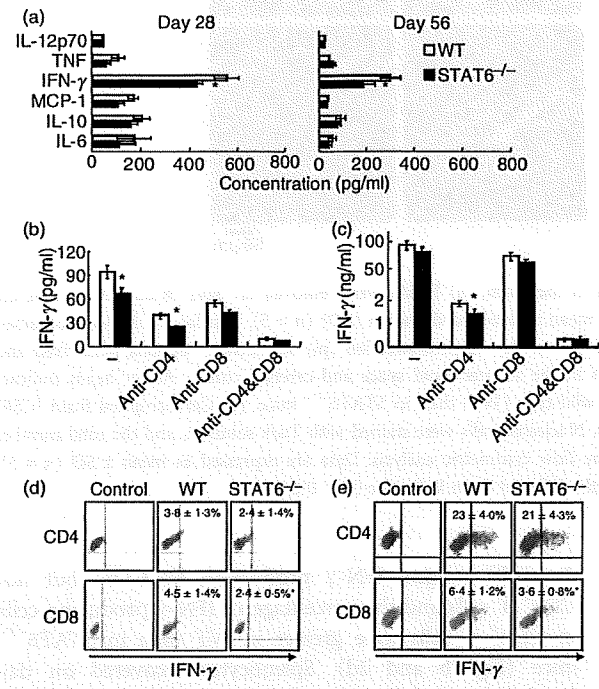

Figure 2. Production of interferon (IFN)-γ by T cells in the cerebrospinal fluid (CSF) and spleen. (a) CSF was taken from signal transducer and activator of transcription (STAT) 6$^{-/-}$ and wild-type (WT) mice on days 28 and 56 post-infection (pi). Concentrations of cytokines in CSF were determined using a cytometric bead array (CBA) kit. IFN-γ level was significantly lower in STAT6$^{-/-}$ as compared with WT mice. Data are mean ± standard deviation (SD) (n = 5). CSF cells (b) and splenocytes (c) were taken from STAT6$^{-/-}$ and WT mice on day 28 pi, and were incubated with *Toxoplasma gondii* antigen. Anti-CD4 and/or anti-CD8 monoclonal antibodies (mAbs) (5 μg/ml) were added to the culture to block activation of CD4$^+$ and/or CD8$^+$ T cells. The IFN-γ concentration in culture supernatants was determined using a CBA kit. Data are mean ± SD (n = 5). CSF cells (d) and splenocytes (e) were incubated with *T. gondii* antigen, and stained for T-cell markers and IFN-γ. Cells without antigen stimulation were used as a control. Numbers indicate the percentage of IFN-γ-producing cells in CD4$^+$ and CD8$^+$ T cells. Data are mean ± SD (n = 5). Experiments were carried out three times with similar results. *Significantly different from WT mice (P < 0·05).

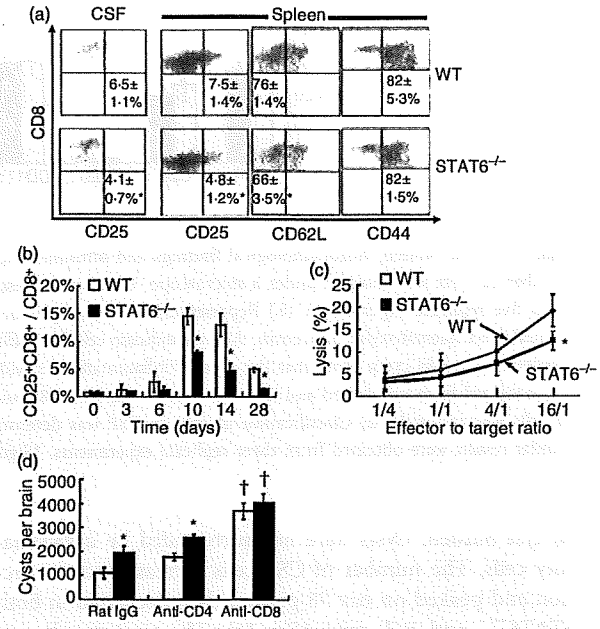

Figure 3. Activation of CD8$^+$ T cells after *Toxoplasma gondii* infection. (a) Cerebrospinal fluid (CSF) cells and splenocytes were taken from mice on day 28 post-infection (pi), and stained with fluorescein isothiocyanate (FITC)-anti-CD25, FITC-anti-CD62L, FITC-anti-CD44 and phycoerythrin (PE)-anti-CD8 monoclonal antibodies (mAbs) and then analysed by flow cytometry. Numbers indicate the percentage of CD25$^+$ CD8$^+$, CD62L^{low} CD8$^+$ and CD44$^+$ CD8$^+$ T cells in CD8$^+$ T cells in the CSF and spleen. Data are mean ± standard deviation (SD) (n = 4). (b) The percentage of CD25$^+$ CD8$^+$ T cells in CD8$^+$ T cells in peripheral blood is shown. Data are mean ± SD (n = 5). (c) Purified CD8$^+$ T cells and peritoneal macrophages labelled with PKH67 were co-cultured at various effector to target cell (E:T) ratios. Dead cells were determined by flow cytometry after staining with 7-amino-actinomycin D (7-AAD). Data are mean ± SD (n = 4). (d) *Toxoplasma gondii*-infected mice were intraperitoneally injected with 0·5 mg of anti-CD4 mAb, anti-CD8 mAb or rat immunoglobulin G (IgG) on days 14 and 21 pi. Cysts were counted on day 28 pi. Data are mean ± SD. (n = 5). Experiments were carried out three times with similar results. *Significantly different from WT mice (P < 0·05). †Significantly different from rat IgG-treated mice (P < 0·05).

Transfer of immune CD8$^+$ T cells was effective in reducing cyst number in the brain of STAT6$^{-/-}$ mice

Activation of CD8$^+$ T cells was considered to be important for preventing cyst formation in the brain. Therefore, we transferred CD8$^+$ T cells from either infected or uninfected WT mice into STAT6$^{-/-}$ mice to determine the effects of cyst burden in the brain. Transfer of CD8$^+$ T cells but not of CD4$^+$ T cells, recovered from infected WT mice, into STAT6$^{-/-}$ mice on day 14 pi successfully decreased the formation of cysts in the brain. The number of cysts in the brain of WT CD8$^+$ T cell-transferred mice was comparable to that of WT mice without cell transfer. Transfer of CD8$^+$ T cells from infected STAT6$^{-/-}$ mice showed only a slight reduction in cyst number (Fig. 4a). The percentage of CD25$^+$ CD8$^+$ T cells in CSF CD8$^+$ T cells was higher in WT CD8$^+$ T cell-transferred mice than in STAT6$^{-/-}$ CD8$^+$ T cell-transferred mice (Fig. 4b). Interestingly, transfer of native WT CD8$^+$ T cells (5×10^6 cells/mouse) into STAT6$^{-/-}$ mice that were infected with *T. gondii* simultaneously resulted in neither an increase in the percentage of CD25$^+$ CD8$^+$ T cells in peripheral blood nor a decrease in the number of cysts in the brain (Fig. 4c and 4d). These results indicate that STAT6 signalling in CD8$^+$ T cells is not important in their activation and that activated CD8$^+$ T cells predominantly suppress cyst formation in the brain.

Activation of CD8$^+$ T cells by antigen-presenting cells (APCs) may be impaired in STAT6$^{-/-}$ mice

Transfer of SACs from uninfected WT mice into STAT6$^{-/-}$ mice 10 days before infection successfully increased the percentage of CD25$^+$ CD8$^+$ T cells in peripheral blood, and decreased the number of cysts in the brain (Fig. 5a and 5b). Transfer of SACs from uninfected STAT6$^{-/-}$ mice into STAT6$^{-/-}$ mice did not change the percentage of CD25$^+$ CD8$^+$ T cells in peripheral blood or the number of cysts in the brain. Transfer of BM-derived DCs from uninfected WT mice also resulted in a decreased cyst burden in STAT6$^{-/-}$ mice (data not shown). The expression level of CD86 on splenic DCs after *T. gondii* infection was lower in STAT6$^{-/-}$ than in WT mice ($17{\cdot}5 \pm 1{\cdot}3$ versus $21{\cdot}0 \pm 1{\cdot}1$, $n = 4$, $P < 0{\cdot}05$) (Fig. 5c). The concentration of IL-12 p40 in serum was significantly lower in STAT6$^{-/-}$ mice on day 7 pi (Fig. 5d). In addition, SACs from infected STAT6$^{-/-}$ mice produced significantly lower IL-12 p40 than those from WT mice after incubation with *T. gondii* antigen (Fig. 5e).

Discussion

Our current study has demonstrated that STAT6 signalling is important in activation of CD8$^+$ T cells, resulting in a decrease in cyst number in the brain of *T. gondii*-

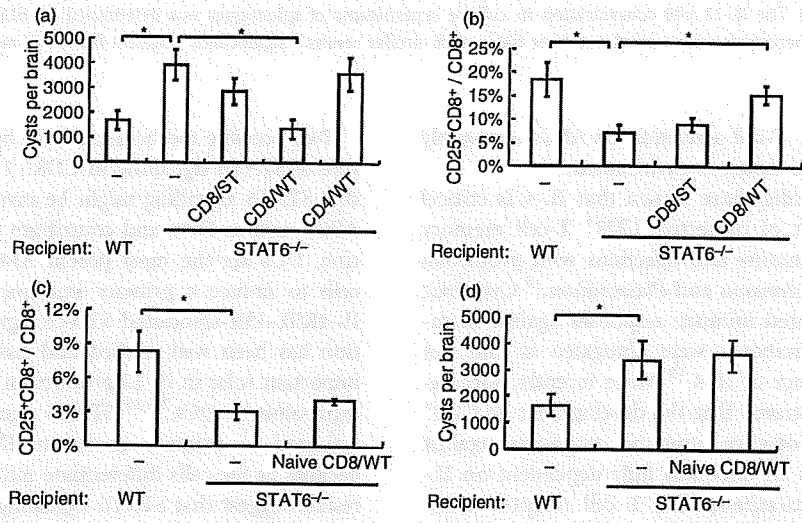

Figure 4. Effects of transfer of CD8$^+$ T cells on cyst burden in the brain. (a) Wild-type (WT) CD8$^+$ T cells (CD8/WT), WT CD4$^+$ T cells (CD4/WT) and signal transducer and activator of transcription (STAT) 6$^{-/-}$ CD8$^+$ T cells (CD8/ST) were purified from infected mice on day 14 post-infection (pi). Then 5×10^6 of these cells were injected intravenously into STAT6$^{-/-}$ mice that had been infected 14 days previously. The cyst burden in the brain was then determined on day 28 pi. (b) The percentage of CD25$^+$ CD8$^+$ T cells in CD8$^+$ T cells in cerebrospinal fluid (CSF) was determined on day 28 pi. Data are mean ± standard deviation (SD) ($n = 5$). (c) Five million CD8$^+$ T cells from uninfected WT mice (naïve CD8/WT) were transferred into STAT6$^{-/-}$ mice which were simultaneously infected with *Toxoplasma gondii*. The percentage of CD25$^+$ CD8$^+$ T cells in CD8$^+$ T cells in peripheral blood was determined on day 14 pi. (d) Cysts in the brains of these mice were determined on day 28 pi. Data are mean ± SD ($n = 5$). Experiments were carried out three times, with similar results. *Significantly different ($P < 0{\cdot}05$).

