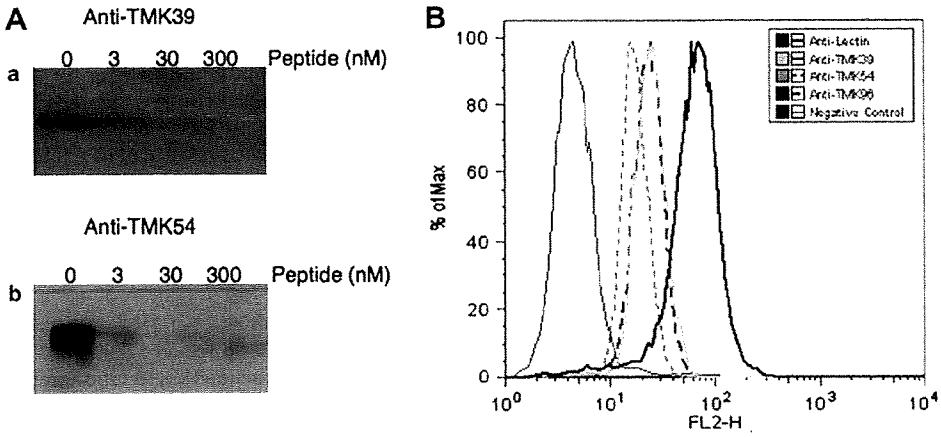

Fig. 1. Analysis of transmembrane kinase (TMK) protein expression in *Entamoeba histolytica* trophozoites. (A) Western blot with anti-peptide antibodies specific for TMK39 and TMK54. Lysate from 5.0×10^6 trophozoites was resolved in a large-well SDS–PAGE gel, transferred to polyvinylidene fluoride (PVDF) membrane and cut into strips. Each strip was incubated with 5 μg/mL of anti-TMK39 (a) or anti-TMK54 (b) that had been pre-incubated with the indicated amount of unconjugated peptide for 1 h at room temperature (RT). Anti-rabbit:AP (Sigma) was used for detection as recommended by the manufacturer. Antibodies recognised single bands of the expected size (127 kDa TMK39 and 100 kDa TMK54). (B) Populations of amoebae expressed more than one TMK. Permeabilized trophozoites were stained with antibodies against TMK39, TMK54, TMK-96 (PATMK), the Gal/GalNAc lectin or an irrelevant antibody (negative control) followed by an anti-rabbit:Cy3 conjugate. Flow cytometry was used to assess staining. Forward scatter (FSC) and side scatter (SSC) were used to gate on intact cells prior to data collection and 10,000 gated events were collected for each sample. The experiment was carried out three times and a representative histogram is shown.

analysis of the stained samples revealed homogenous expression of each TMK by more than 95% of cells within the population, compared with the anti-Ft negative control (Fig. 1B). The absence of distinct sub-populations strongly suggests that single amoebae express multiple TMKs at the protein level, further discounts the notion of antigenic variation, and points instead to the possibility of non-redundant function among TMK family members.

3.3. Localisation of TMK39 and TMK54

TMKs generally possess predicted signal-peptides of approximately 20 aa and single-pass transmembrane domains. Specific antibodies against PaTMK and cross-reactive antibodies against B_1 sub-family members have localised the corresponding proteins to punctate regions of the plasma membrane (Mehra et al., 2006; Boettner et al., 2008). As the cellular localisations of TMK39 and TMK54 are unknown, cellular fractionation and confocal microscopy were used to localise the two proteins in log-phase trophozoites. When an established method (Aley et al., 1980) was used to separate soluble, internal membrane and plasma membrane components, TMK39 and TMK54 were identified in both membrane fractions by Western blotting (Fig. 2A). As the proteins both contain canonical signal-peptides and membrane spanning regions, this was not surprising.

However, when TMK39 and TMK54 were localised using confocal microscopy, the pattern of plasma membrane staining in non-permeabilized cells was surprisingly different, with TMK39 in membrane microdomains (Fig. 2B). This difference was noticeable but less dramatic when the cells were permeabilized. In both instances, pre-incubation of the antibody (10 μg/ml) and corresponding peptide (300 nM) competitively inhibited the staining (Fig. 2B). The accumulation of TMK39 in discrete regions of the plasma membrane is reminiscent of membrane microdomains, such as aggregated lipid rafts and cavaolae that are thought to be centres of cell signalling in metazoan organisms (Parton and Hancock, 2004; Pike, 2006; Pani and Singh, 2009), as well as *E. histolytica* (Laughlin et al., 2004). In contrast, the even distribution of TMK54 throughout the plasma membrane mirrored the localisation of the *E. histolytica* Gal/GalNAc lectin (Petri et al., 1987). In permeabilized cells, both proteins were occasionally found associated with internal vesicles, however TMK54 was more consistently associated with some type of large intracellular compartment (Fig. 2B). While the biological significance of the proteins awaits further investigation, the distinct localisation patterns observed implied that TMK39 and TMK54 served non-redundant functions.

3.4. Functional analysis of TMK39 and TMK54

The kinase-containing intracellular regions of TMK39 and TMK54 were replaced with V5 and poly-histidine tags, and a tetracycline-inducible *E. histolytica* expression vector (Hamann et al., 1997) was used to over-express the truncated proteins, t-39 and t-54 respectively, in trophozoites (Fig. 3A). Parental vectors were transfected in parallel and served as a control for any unexpected effects of the induction of the tetO vector in the experiments. Each construct was used to generate at least two independently transfected clones. To confirm expression of the truncated proteins, cells were induced with 10 μg/ml of tetracycline for 24 h and cellular lysate was subjected to immunoprecipitation using anti-V5 agarose. The anti-peptide antibodies described above were used to probe Western blots of the immunoprecipitations, and both t-39 (Fig. 3B) and t-54 (Fig. 3C) cells expressed proteins of the expected size.

Importantly, wild type and truncated proteins appeared to interact, as endogenous TMK39 and TMK54 co-immunoprecipitated with the corresponding truncated protein (Fig. 3B and C). Receptor kinases are generally activated by ligand-induced dimerization followed by trans-autophosphorylation of kinase domains (Lemmon and Schlessinger, 1994; Heldin, 1995). Consequently, over-expression of dominant-negative receptors that lack cytoplasmic kinase domains has long been used to study the biological relevance of receptor kinases (Ueno et al., 1991). Such truncated receptors bind ligand, fail to propagate a downstream signal, and can inhibit wild type receptors through ligand-induced heterodimerization of the truncated and wild type proteins. These mutants therefore allowed us to address the biological functions of TMK39 and TMK54.

As shown in Fig. 4, cells induced to over-express t-54 had a severe growth defect during the first 24 h of induction but recovered to a near normal growth rate by 48 h (as indicated by the slope of

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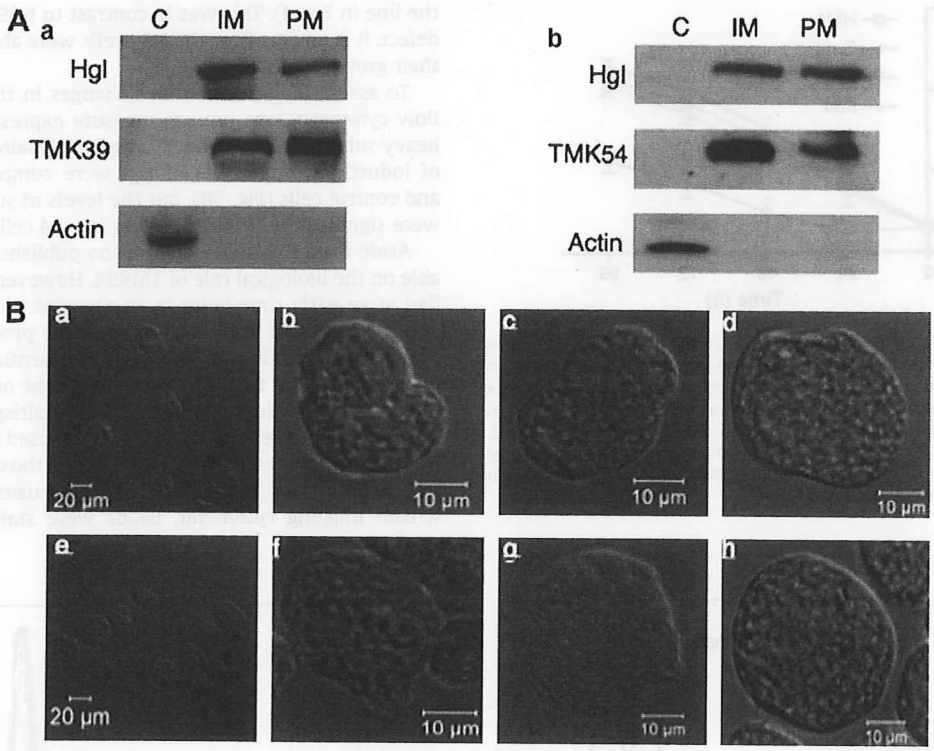

Fig. 2. Transmembrane kinases, TMK39 and TMK54 have discrete distributions at the cell surface. (A) *Entamoeba histolytica* trophozoites were lysed and fractionated. Trophozoite cytoplasmic (C), inner membrane (IM) and plasma membrane (PM) fractions were prepared for Western blot analysis. The panels were probed with anti-TMK39 (a) or anti-TMK54 (b), and in both instances, anti-Hgl and anti-actin antibodies were used as controls. Both TMK39 and TMK54 were expressed in membrane fractions. (B) Confocal images of non-permeabilized trophozoites are shown after staining with anti-peptide antibodies against TMK39 (a and b) and TMK54 (e and f). Permeabilized cells stained with anti-TMK39 (c) and anti-TMK54 (g) are also shown. As a control, antibodies were pre-incubated with the corresponding unconjugated peptides to competitively inhibit staining and staining with both anti-TMK39 (d) and anti-TMK54 (h) antibodies was abolished.

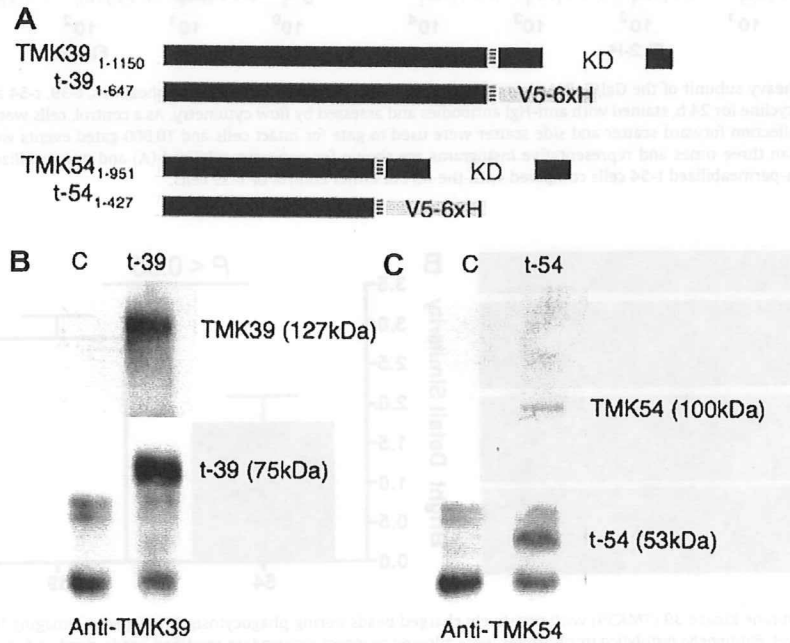

Fig. 3. Analysis of transmembrane kinase (TMK) function using a dominant-negative approach. (A) Depiction of the kinase domain (KD) deletions of TMK39 and TMK54 in t-39 and t-54, respectively. (B and C) Immunoprecipitation of t-39 and t-54. Amoebae were transfected with inducible expression vectors encoding t-39, t-54 or an empty vector control (C). Anti-V5 agarose was used to immunoprecipitate the truncated proteins from whole cell lysate. Western blots of immunoprecipitations are shown, probed with antibodies against TMK39 (B) or TMK54 (C). Heavy chain from the precipitating antibody is visible in every lane. As visualised, full-length wild type proteins co-immunoprecipitated with the truncated proteins in both instances. Separation between panels in B and C indicates different exposure times of blot to film.

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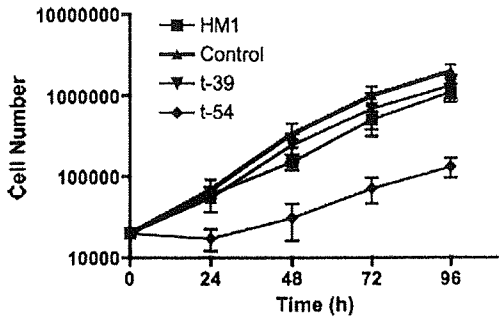

Fig. 4. Induction of t-39 and t-54 expression in *Entamoeba histolytica* trophozoites resulted in different phenotypes with respect to growth. Trophozoites were harvested during log-phase growth and 10,000 cells were seeded into media containing 10 μg/mL tetracycline. Cell numbers were assessed every 24 h for 4 days. For each construct at least two independently transfected clones were analysed. Each sample was assayed in duplicate and the graph represents the mean of three independent experiments +/− SD. For t-54 cells $P < 0.05$ at every time point after 0 h compared with any of the other cell types.

the line in Fig. 4). This was in contrast to t-39 cells, which had no defect. It is unclear how the t-54 cells were able to compensate for their growth defect.

To assess major cell surface changes in the mutant cell lines, flow cytometry was used to measure expression of the 170-kDa heavy subunit (Hgl) of the parasite's Gal/GalNAc lectin. After 24 h of induction, total levels of Hgl were comparable in t-39, t-54 and control cells (Fig. 5B), but the levels of surface expressed Hgl were significantly ($P < 0.05$) lower in t-54 cells (Fig. 5A).

Aside from this study, there is no published information available on the biological role of TMK54. However, TMK39 was identified at an early time point in an amoebic phagosomal proteome, and has therefore been implicated in the process of phagocytosis (Okada et al., 2006). To address the potential role of TMK39 in phagocytosis, we first assessed the extent of co-localisation between TMKs and ingested beads using multispectral imaging flow cytometry. Anti-peptide antibodies were used to stain trophozoites that had been allowed to ingest 2 μm carboxylate-modified fluorescent beads and samples were imaged using the Amnis Imagestream imaging cytometer. Beads were stained in an identical

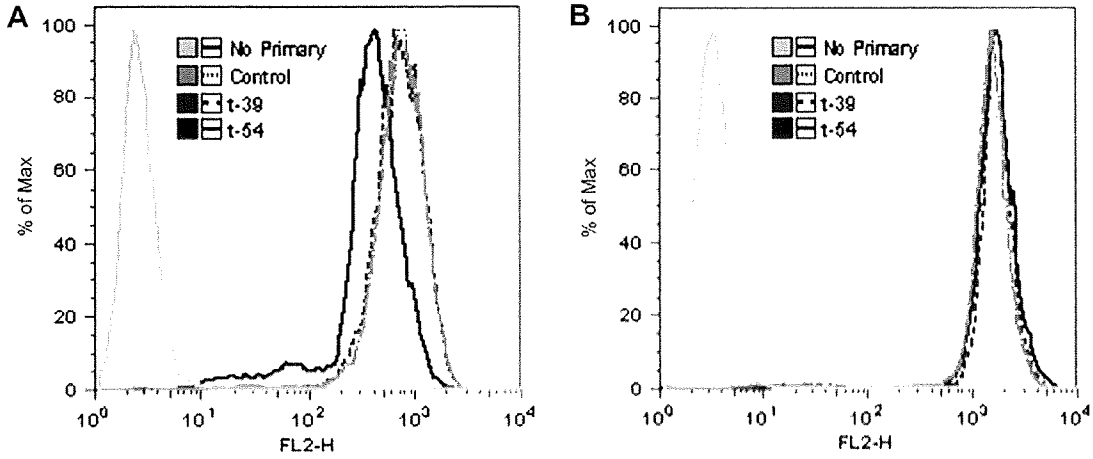

Fig. 5. Expression analysis of the heavy subunit of the Gal/GalNAc lectin (Hgl) in mutant *Entamoeba histolytica* trophozoites. t-39, t-54 and empty vector transfected cells were induced with 10 μg/mL tetracycline for 24 h, stained with anti-Hgl antibodies and assessed by flow cytometry. As a control, cells were stained with secondary antibody only (no primary). Prior to data collection forward scatter and side scatter were used to gate for intact cells and 10,000 gated events were collected for each sample. The experiment was repeated more than three times and representative histograms are shown for non-permeabilized (A) and permeabilized (B) cells. $P < 0.05$ for the mean fluorescence intensity (MFI) of non-permeabilized t-54 cells compared with the MFI of either control or t-39 cells.

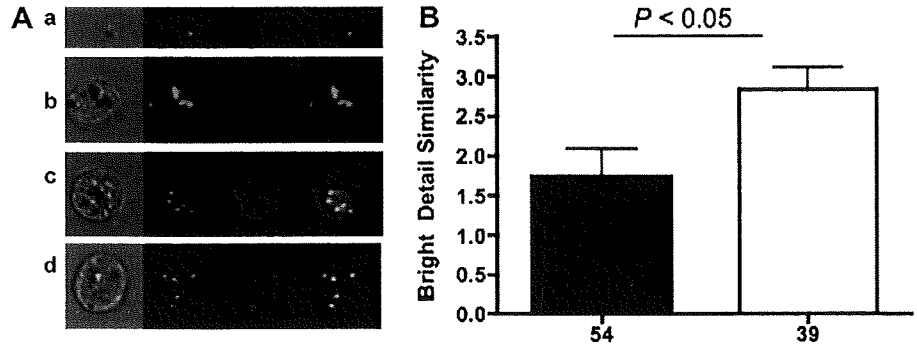

Fig. 6. Co-localisation of transmembrane kinase 39 (TMK39) with negatively charged beads during phagocytosis. Multispectral imaging flow cytometry was used to assess co-localisation at the population level. *Entamoeba histolytica* trophozoites were allowed to ingest carboxylate-modified beads, fixed and stained with anti-peptide antibodies. Beads were stained alongside bead-containing cells. Samples were imaged using the Amnis Imagestream imaging cytometer. (A) Representative images are shown for beads stained (as a control) with anti-TMK39 (a), permeabilized, bead-positive cells stained with secondary antibody alone (b), anti-TMK54 (c) or anti-TMK39 (d). (B) ImageStream Data Exploration and Analysis Software (IDEAS) was used to calculate the Bright Detail Similarity score (a measure of co-localisation between fluorescence in different channels with higher numbers indicating more co-localisation and a score ⩾ 3 indicating co-localisation) for permeabilized cells stained with anti-TMK39 and anti-TMK54, and results were plotted as mean Bright Detail Similarity (BDS) +/− SD for three independent experiments (*P*-value = 0.039).

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manner to bead-containing cells to ensure that the anti-peptide antibodies and beads did not directly interact. While there was no evidence of non-specific binding of antibodies to beads, or of (as a control) co-localisation between TMK54 and ingested particles (Fig. 6A), TMK39 did appear to co-localise with ingested beads (Fig. 6A). Amnis IDEAS software was used to calculate the BDS score of single, in-focus, bead-positive cell images and the results are plotted in Fig. 6B as mean BDS +/− SD BDS scores are indicative of co-localisation between fluorescence in two channels: in this instance between ingested beads and TMKs. The mean BDS scores for permeabilized cells stained with TMK39 approached three (the accepted value for co-localised images) and TMK39 had higher BDS scores ($P < 0.05$), indicating a greater extent of co-localisation.

As TMK54 did not appear in any phagosomal proteome (Marion et al., 2005; Okada et al., 2006; Boettner et al., 2008) or co-localise with ingested beads, and as t-54 cells had striking defects in growth and surface expressed Hgl levels, the ability of t-54 cells to phagocytose was not assessed. However, flow cytometry was used to measure the ability of t-39 cells to ingest carboxylate-modified fluorescent beads, CFSE-labelled apoptotic Jurkat cells and Ca^{2+} treated erythrocytes. Uptake of FITC-labelled dextran was also measured as a marker of fluid phase pinocytosis. As shown in Fig. 7A and B, t-39 cells were significantly impaired in their ability to ingest carboxylate-modified beads and apoptotic Jurkat cells. The defect was specific, as t-39 cells were unimpaired in their ability to uptake FITC-dextran and Ca^{2+} treated erythrocytes.

4. Discussion

The most important conclusion from these studies is that single *E. histolytica* trophozoites express multiple members of a large TMK family and utilise the TMKs for non-redundant functions. While large families of TMKs have been considered hallmarks of multi-cellularity, the life cycle of *E. histolytica* provides clues as to why the protist would require an extensive network of cell surface signalling molecules. In the complex intestinal microenvironment, the organism must compete with bacteria for nutrients and space, sense stressors to regulate developmental changes between cyst and trophozoite, subvert host defences, ingest bacteria and control its invasive behaviour. Upon invasion, trophozoites continue to face a battery of challenges that require the ability to chemotax, adhere, kill and ingest human cells, and obtain sufficient nutrients. Survival of *E. histolytica* within its human host must require a profound ability to sense and respond to environmental challenges and utilisation of the extensive TMK network may therefore be critical.

Single cell microarray analysis demonstrated expression of multiple members of TMK sub-families A, B_1, D_1 and E. Expression of multiple sub-family members by a single cell raises the possibility that the TMK sub-families function in a complex manner similar to ErbB receptors in mammalian cells. ErbB family members form homo- and hetero-dimers, bind to different ligands and can be transactivated by other proteins (Linggi and Carpenter, 2006). It will be important to keep in mind the potential for such complexity while initiating preliminary studies on TMKs.

A limitation of this analysis was the minute quantity of RNA obtained from a single cell. Previous reports indicate that trophozoite populations in culture or isolated from mice cumulatively express 65–80% of *E. histolytica* genes (Gilchrist et al., 2006). However, only 15–20% of genes were detected as expressed within a single cell. While the averaging of population data in previous studies may have partially contributed to such a discrepancy, it is more likely that false-negative calls were generated in this study, as single cell microarray analysis is an inherently insensitive technique (Esumi et al., 2008), and members of the TMK gene family have been generally shown to be expressed at low to medium levels in other

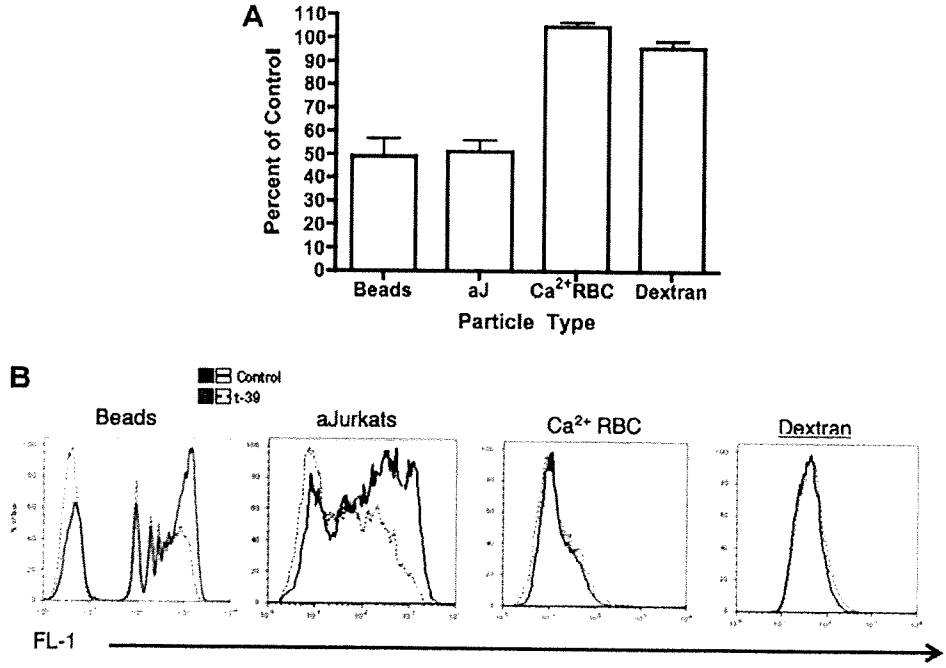

Fig. 7. Phagocytosis and pinocytosis in *Entamoeba histolytica* trophozoites induced to express t-39. (A) t-39 and empty vector transfected control cells were induced for 24 h with 10 μg/mL tetracycline and allowed to ingest carboxylate-modified beads, carboxyfluorescein succinimidyl ester (CFSE) labelled apoptotic Jurkat (aJ) cells, CFSE-labelled Ca^{2+} treated erythrocytes or FITC-labelled dextran. For each vector, at least two independently transfected clones were used in each experiment and the experiments were carried out more than three times. Samples were analysed by flow cytometry and the graph represents the mean fluorescent intensity (MFI) of t-39 cells plotted as a percentage of control cells. Error bars represent SD and $P < 0.005$ for beads and apoptotic Jurkats compared with either Ca^{2+} treated erythrocytes or dextran. (B) Representative histograms are shown.

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studies (expression levels in prior studies are accessible through NCBI's Gene Expression Omnibus GEO Series accession numbers GSE8484, GSE13023, GSE6648, GSE6650). Moreover, TMK54 was detected as present by Affymetrix® GCOS (in both cells), but TMK39 and PaTMK were both GCOS absent in this study. In contrast, more than 95% of trophozoites within a population expressed each TMK at the protein level. Consequently, we believe that the single cell TMK transcriptome described in this study should be considered a minimal estimate.

Additional similarities between metazoan and *Entamoeba* TMKs are likely to exist. For example, the widely studied metazoan TMKs are activated by ligand-induced dimerization (Lemmon and Schlessinger, 1994; Heldin, 1995) and typically contain extracellular furin-like and/or epidermal growth factor – (EGF) like moieties. Furin-like domains are thought to be involved with the aggregation of metazoan receptor tyrosine kinases and EGF-like moieties contribute to protein–protein interactions. Although the pairing of TMK and EGF or furin-like domains is a rare occurrence in protozoa, many TMKs (including both TMK39 and PaTMK) possess cysteine-rich extracellular domains containing furin-like and/or EGF-like moieties. We observed hetero-dimerization between wild type and truncated receptors in this study, which may indicate that TMKs are subject to the same ligand-induced dimerization events as their metazoan counterparts.

Without identification of receptor ligand or kinase substrate, it is not possible to definitively ascribe functions to any of the TMKs that have been studied thus far. However, we have discovered a variety of clues that can help us begin to understand the function of these proteins. The striking difference in the surface localisation patterns of TMK39 and TMK54 was the first indication that these proteins served non-redundant functions. The uniform cell surface staining of TMK54 was similar to that of the heterotrimeric Gal/GalNAc lectin that mediates adhesion, cytotoxicity, phagocytosis and complement resistance (Petri et al., 2002). It is currently unknown how the Gal/GalNAc lectin orchestrates such a wide variety of events but sequence similarity between the short cytoplasmic tail of Hgl and the cytoplasmic tails of β2 and β7-integrins is considered to play a key role (Vines et al., 1998). Tyrosine phosphorylation of β-integrins stimulates their translocation to the cell surface (Naccache et al., 1994). Interestingly, t-54 cells expressed less Hgl on their surface compared with both t-39 and control cells, indicating that TMK54 may regulate Hgl surface expression. Additionally, t-54 cells had a striking growth defect during the first 24 h of t-54 protein induction, indicating that TMK54 may represent a major growth factor receptor. Cross-talk between growth factor receptors and integrins is also known to affect surface integrin levels (Somanath et al., 2009). Members of the B_1 family of TMKs have also been found to impact cellular proliferation but any impact on surface Hgl expression has not been described (Mehra et al., 2006). Future studies in our laboratory will address the biological role of TMK54 directly and examine the relationship between TMK54 and the Gal/GalNAc lectin.

In contrast to TMK54, TMK39 was localised to punctate regions of the plasma membrane, in a pattern reminiscent of membrane microdomains such as lipid rafts. As expected from its prior identification as a member of the phagosomal proteome (Okada et al., 2006), TMK39 was found to co-localise with ingested beads at the surface and to a greater extent within cells. Additionally, t-39 cells had a specific defect in their ability to ingest carboxylate-modified beads and apoptotic lymphocytes, but not Ca^{2+} treated erythrocytes. PaTMK has been previously shown to play a role in uptake of Ca^{2+} treated red blood cells (Boettner et al., 2008), providing additional evidence that TMKs serve non-redundant cellular functions. Considering previous studies that identified TMK39 as a component of the phagosomal proteome at early time points and the phenotype of t-39 cells, it is tempting to speculate that TMK39 may function as a scavenger receptor and mediate the internalisation of polyanionic macromolecules. Although phosphatidyl serine (PS) is a critical "eat-me" signal recognised by phagocytes (Grimsley and Ravichandran, 2003), it is unlikely that TMK39 recognises the molecule because recognition of exposed PS on the surface of aged or Ca^{2+} treated erythrocytes is known to impact uptake of red blood cells by *E. histolytica* (Boettner et al., 2005). Alternative "eat-me" signals, such as modified low density lipoprotein (LDL), are recognised by scavenger receptors that mediate the uptake of bacteria and apoptotic corpses in other systems (Grimsley and Ravichandran, 2003). TMK39 shares 30% identity with the *Drosophila* scavenger receptor eater across the first 200 aa of the proteins. Eater binds modified LDL and mediates uptake of bacteria, and the first 200 aa of eater are known to facilitate binding to polyanionic ligands (Kocks et al., 2005). Consequently, modified LDL is a candidate recognition signal for TMK39 and for *E. histolytica* phagocytosis of apoptotic lymphocytes.

In plants and metazoa, TMKs are known to regulate a myriad of cellular processes including cellular proliferation, survival, differentiation, migration, metabolism and host defence. This study suggests that TMKs are likely to mediate a similarly diverse and essential set of processes for this *E. histolytica*.

Acknowledgements

This work was supported by NIH grant 5RO1 AI026649 (to W.A. Petri) and Grant-in-Aid for Scientific Research (to S. Hamano).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2009.12.007.

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Please cite this article in press as: Buss, S.N., et al. Members of the *Entamoeba histolytica* transmembrane kinase family play non-redundant roles in growth and phagocytosis. Int. J. Parasitol. (2010), doi:10.1016/j.ijpara.2009.12.007

STRONGYLOIDES RATTI: IMPLICATION OF MAST CELL-MEDIATED EXPULSION THROUGH FcεRI-INDEPENDENT MECHANISMS

WATANABE K.*,**, KISHIHARA K.*,***, HAMANO S.*, KOGA M.*, NOMOTO K.* & TADA I.*

Summary:

In order to examine whether FcεRI-dependent degranulation of intestinal mast cells is required for expulsion of intestinal nematode *Strongyloides ratti*, CD45 exon6-deficient (CD45-/-) mice were inoculated with *S. ratti*. In CD45-/- mice, egg excretion in feces persisted for more than 30 days following *S. ratti* larvae inoculation, whereas in wild-type (CD45+/+) mice, the eggs completely disappeared by day 20 post-infection. The number of intestinal mucosal mast cells, which are known effector cells for the expulsion of *S. ratti*, was 75 % lower in CD45-/- mice compared with that in CD45+/+ mice. Adoptive transfer of wild-type T cells from CD45+/+ mice into CD45-/- mice reduced the duration of *S. ratti* infection to comparable levels observed in CD45+/+ mice, with concomitant increases in intestinal mucosal mast cells. These results showed that CD45 is not involved in the effector function of intestinal mucosal mast cells against *S. ratti* infection. Since FcεRI-dependent degranulation of mast cells is completely impaired in these CD45 knockout mice, we conclude that FcεRI-dependent degranulation is not required in the protective function of intestinal mucosal mast cells against primary infection of *S. ratti*.

KEY WORDS : *Strongyloides ratti*, expulsion, mast cell, degranulation, FcεRI.

Résumé : L'EXPULSION DE *STRONGYLOIDES RATTI* EST INDÉPENDANTE DE LA DÉGRANULATION DES MASTOCYTES *VIA* LA VOIE DE TRANSDUCTION PAR LE RÉCEPTEUR FcεRI

Afin de déterminer si la dégranulation des mastocytes, après agrégation du récepteur de haute affinité des IgE (FcεRI), est responsable de l'expulsion des nématodes intestinaux Strongyloides ratti, *des souris CD45-/- (déficiente pour l'exon 6 de CD45) sont infectées par le nématode. Chez les souris CD45-/-, l'excrétion des œufs persiste plus de 30 jours après l'inoculation de larves de* S. ratti *alors que chez les souris de type sauvage (CD45+/+), il n'y a plus d'excrétion 20 jours post-infection. Il y a 75 % en moins de mastocytes au niveau de la muqueuse intestinale chez les souris CD45-/- par rapport aux souris CD45+/+. Le transfert de lymphocytes T de souris sauvage (CD45+/+) aux souris CD45-/- réduit la durée de sécrétion à des niveaux comparables à ceux observés dans le type sauvage avec une augmentation concomitante de mastocytes au niveau de la muqueuse intestinale. Ces résultats montrent que CD45 n'est pas impliquée dans la fonction effectrice des mastocytes de la muqueuse intestinale lors d'une infection par* S. ratti. *Étant donné que la dégranulation des mastocytes après agrégation du récepteur FcεRI est impossible chez les souris CD45-/-, nous en concluons que la dégranulation des mastocytes, dépendante du récepteur FcεRI, n'est pas nécessaire pour l'expulsion de* S. ratti.

MOTS CLÉS : Strongyloides ratti, *expulsion, mastocyte, dégranulation, FcεRI.*

INTRODUCTION

Intestinal parasites that reside in the gut face several host immune factors, including mast cells (Nawa *et al.*, 1994). Parasites deploy several strategies to escape these host immune factors; for example, parasites induce host regulatory T cells to dampen the immune mechanism or induce host polyclonal IgE production which competes with specific IgE (Maizels *et al.*, 2004)

Mast cells express FcεRI (high affinity IgE receptor) on their surface and crosslinking of FcεRI with IgE/antigen complexes results in degranulation, which leads to allergic or anaphylactic reaction (Metcalfe *et al.*, 1997). There are also reports which suggest that mast cells have various functions including cytokine secretion, such as IL-4, which is essential for protective Th2 response against bacteria and parasites (Nawa *et al.*, 1994; Frandji *et al.*, 1998; Heib *et al.*, 2008). In wild-type mice infected with *Strongyloides*, intestinal mast cell levels were increased in accordance with expulsion of the worms (Nawa *et al.*, 1994). In studies performed in mast cell-deficient W/W^v mice, the duration of the infection was markedly prolonged (Nawa *et al.*, 1985; Abe *et al.*, 1987). As such, mast cells are critical for expulsion of the intestinal nematodes *S. ratti* and *S. venezuelensis* (Nawa *et al.*, 1994). Sulfated proteoglycans from mast cells were shown to be involved in the expulsion of the worm from the intestine (Maruyama *et al.*, 2000).

As observed in other helminth infections, *Strongyloides* infection also induces IgE production. However, it has been suggested that IgE might not be involved in expulsion of *Strongyloides* from the intestine because IgE levels specific to intestinal worm antigens increased

* Department of Microbiology (Parasitology), Graduate School of Medical Science and Department of Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan.
** Department of Parasitology, Institute of Tropical Medicine, Nagasaki University (NEKKEN), Nagasaki, Japan.
*** Present Address: School of Pharmaceutical Sciences, Nagasaki International University, Sasebo, Japan.
Correspondence: Kanji Watanabe, Department of Parasitology, Institute of Tropical Medicine (NEKKEN), Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523 Japan.
Tel.: +81 95 819 7825 - Fax: +81 95 819 7805
E-mail: watakanj@nagasaki-u.ac.jp

after, rather than before, expulsion of adult worm from intestine (Korenaga *et al.*, 1986). In addition, neutralization of IgE does not affect the duration of infection (Korenaga *et al.*, 1991). Furthermore, IL-4 knockout mice, in which IgE was not measurable (Kurup *et al.*, 1999), expelled *Strongyloides* from the intestine on almost the same day post-inoculation as wild-type mice (Watanabe *et al.*, 2001). On the other hand, there are reports that FcR-γ deficient mice which lack IgE/FcεR signaling in mast cells could not expel *Strongyloides venezuelensis* (Onah *et al.*, 2001, Onah & Nawa, 2004).

CD45 is a transmembrane tyrosine phosphatase expressed on all nucleated hematopoietic cells (Trowbridge *et al.*, 1994). CD45 activates *src* family protein tyrosine kinases, essential for antigen receptor-mediated signaling in lymphocytes (Penninger *et al.*, 1993). In CD45 exon6-deficient (CD45-/-) mice, a marked decrease in mature thymocyte levels was observed, suggesting that CD45 is important for thymocyte development and maturation (Kishihara *et al.*, 1993). Moreover, antigen-specific lymphocyte responses were defective in CD45-/- mice (Kishihara *et al.*, 1993; Kong *et al.*, 1995). However, analysis of macrophages showed normal antigen presentation and phagocytosis in CD45-/- mice (Berger *et al.*, 1994; Fujise *et al.*, 1997). Interestingly, in CD45-/- mice, the FcεRI-mediated degranulation of bone marrow derived mast cells (BMMC) was completely impaired and systemic anaphylaxis could not be induced (Berger *et al.*, 1994).

In this study, to address the involvement of FcεRI signaling in intestinal mast cell-mediated immunity against *S. ratti* infections, *S. ratti* infection was investigated using CD45 exon-6 knockout mice, which also has a defect in IgE/FcεRI-dependent mast cells degranulation (Berger *et al.*, 1994).

MATERIAL AND METHODS

Mice

Male C57BL/6 mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and maintained in the Laboratory for the Animal Experiments of Kyushu University under the SPF conditions. CD45 exon6 knockout (CD45-/-) mice were kindly supplied by Dr Tak W. Mak (AMGEN Institute/Ontario Cancer Institute, Toronto, Canada) and maintained in our laboratory. Mice aged 8-12 weeks were used throughout the study. CD45-/- mice have been backcrossed to C57BL/6 mice over seven times. All the experiments were conducted in accordance with the principles and procedures outlined in Guidelines for the Care and Use of Laboratory Animals in Kyushu University.

Preparation and inoculation of parasite

Strongyloides ratti, TMDU strain, has been maintained in our laboratory by serial passage in retired Wistar Rats. Infective larvae (L3) were obtained by filter paper culture of the feces of infected Wistar Rats. Two thousand L3 larvae were suspended in 0.2 ml saline and injected subcutaneously into the lower abdomen of the mouse.

Analysis of egg, output in feces and larval/adult worms in the tissues

Daily egg output in feces was monitored as follows. Fresh feces (50-80 mg) were collected in a 2 ml plastic microtube, weighed and suspended in 1 ml distilled water. The number of eggs in 50 µl of fecal suspension was counted under a microscope. The egg output in feces was presented as eggs per gram of feces (EPG). At 0, 24, 48, 72, 120 hours after infection, mice were sacrificed, organs (head, lung and intestine) were removed and cut into pieces. Minced tissues were incubated in Petri dishes containing saline at 37 °C for three hours. Minced tissues were then removed and the worms remaining in the Petri dishes were counted under dissecting microscope. In the head and lungs, third stage larvae were recovered and counted. In the intestine at 120 hours after infection, fourth stage larvae and mainly young adult worms were recovered and counted.

Histopathological analysis of intestinal mast cells

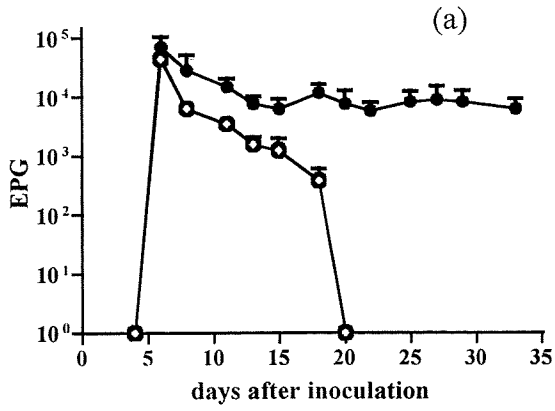
Intestinal sections (1 cm length) located 10 cm distal to the pylorus were removed and then immersed in Carnoy's fixative solution for two hours. The paraffin-embedded samples were cut into sections of 5 µm thickness and then stained with Alcian blue (pH 0.3) and Safranin-O according to Abe *et al.* (1987). The number of intestinal mast cells was counted in 40-60 villous-crypt units (VCU) in each two sections of each mouse. The densities were estimated as intestinal mast cell number per 10 VCU.

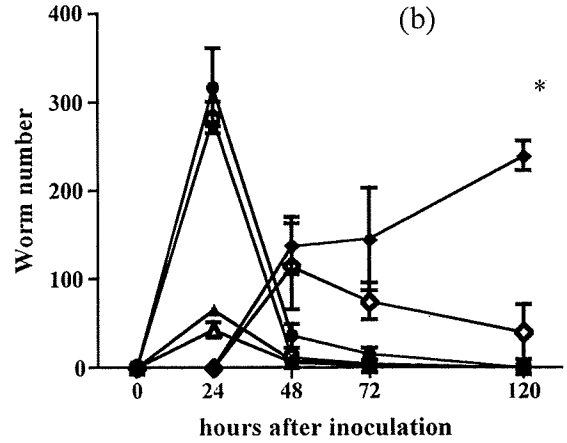
Adoptive transfer of wild-type T cells

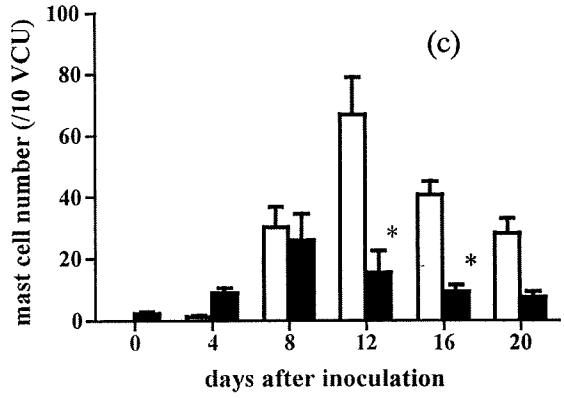
Spleen and mesenteric, popliteal and axial lymph nodes were aseptically removed from naïve C57BL/6 mice. The pooled single cell suspension in RPMI 1640 medium supplemented with 10 % heat-inactivated FCS, 2 mM HEPES, 0.2 % sodium bicarbonate and 50 µM 2-mercaptoethanol was passed through a nylon wool column to enrich T cells. The recovered cells (1×10^7/mouse; T cell purity: > 90 %) were intraperitoneally injected into CD45-/- mice one day before infection.

STATISTICAL ANALYSIS

In this study, the Welch test was used to determine statistical significance between two groups. Kruskal-Wallis and *post-hoc* tests (Dunn test) were used to make comparisons between three groups. Calculations were performed by GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, California USA, www.graphpad.com). Numerical data obtained from each experiment were displayed as a formula of mean ± SEM.








(d)

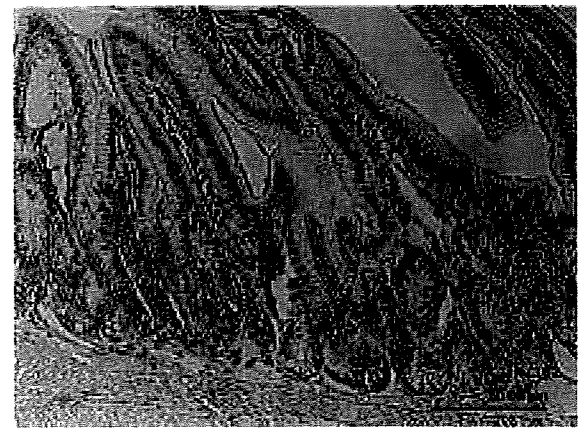

(e)

Fig. 1. – *S. ratti* infection persisted in CD45 knockout mice.
a. *S. ratti* infection monitored by egg excretion into feces.
CD45 exon-6 knockout mice (CD45-/-) and wild type mice (CD45+/+) were inoculated with 2000 *S. ratti* infective larvae. The number of eggs excreted in feces was monitored and the results are presented as egg number per gram feces. Data represent one of three independent experiments and are shown as mean ± SEM of four mice in each group. Open symbols, CD45+/+, Closed symbols, CD45-/-.
b. Worm number in head, lung and intestine in each group of mice. Mice were sacrificed at indicated hours and the number of larvae in each organ was counted as described in material and methods. Data are shown as mean ± SEM of three mice and represent one of two independent experiments. Open symbols: CD45+/+; Closed symbols: CD45-/-. Circles: the number of larvae in the head; Triangles: the number of larvae in the lung; Diamond: the number of larvae in the intestine. * $p < 0.05$ compared with the number in the intestine in CD45+/+.
c. Kinetics of intestinal mast cell numbers during the course of *S. ratti* infection.
Mice were inoculated with *S. ratti* as for figure 2a and sacrificed on indicated days. Mast cell numbers in the intestine at 10 cm distal to the pyloric ring were counted. Data are shown as mean ± SEM of six mice in each group at indicated days. Open bar: CD45+/+; Closed bar: CD45-/-. * $p < 0.05$ compared with CD45+/+.
d. Section of intestine of CD45+/+ mice on day 12, stained with Alcian Blue and Safranin-O. Blue stained spots showed mast cells.
e. Section of intestine of CD45-/- mice on day 12 treated same as d. Few blue stained cells were observed.

RESULTS

Decreased induction of intestinal mast cells and persistent infection in CD45-/- mice during *S. ratti* infection

In order to estimate the contribution of IgE/FcεRI-dependent mast cell degranulation to host defense against *S. ratti*, CD45-/- mice were inoculated with subcutaneous injection of 2,000 *S. ratti* L3.

The infection was monitored by the number of eggs excreted into feces (Fig. 1a). The presence of eggs in the feces of CD45-/- mice was detected up to 30 days after inoculation, whereas eggs were not detected from approximately 18-20 days after inoculation in wild type (CD45+/+) mice, indicating that the expulsion of this parasite was impaired in CD45-/- mice. Since *S. ratti* L3 migrate from the inoculated site to the intestine via the head and lung, the number of migrating larvae in the head, lung and intestine were counted at specified hours after inoculation in order to examine whether innate host defense against *S. ratti* migrating larvae was affected in these knockout mice (Fig. 1b). There was no difference in the number of migrating larvae in either the head or lung between the two groups of mice, and a comparable number of worms first appeared in the small intestine 48 hours after infection in both knockout and wild-type mice. The increase of worm numbers was observed in the intestine of CD45-/- mice from 72 hours after inoculation.

The number of intestinal mast cells which was shown to be essential for expulsion of *Strongyloides*, was counted (Fig. 1c, d, e). Before infection, the number of intestinal mast cells observed in CD45-/- mice was slightly higher than that observed in CD45+/+ mice (Day 0, CD45+/+: 0.8 ± 0.3/10 VCU; CD45-/-: 2.1 ± 0.6/10 VCU). The number of intestinal mast cells increased and peaked on day 12 after inoculation in CD45+/+ mice, as previously reported, in correlation with *S. ratti* expulsion. The number of mast cells in CD45-/- mice also increased and peaked on days 8-12 after inoculation, with a timing similar to that of CD45+/+ mice; however, the numbers were 75 % lower than those observed in CD45+/+ mice (day 12, CD45+/+: 66.7 ± 12.4/10 VCU; CD45-/-: 15.5 ± 6.8/10 VCU).

Adoptive transfer of CD45+ wild-type T cells restored expulsion of *S. ratti* from CD45 knockout mice

As the antigen-specific T cell response was completely abrogated in CD45-/- mice (Kishihara *et al.*, 1993; Kong *et al.*, 1995) and the expulsion of nematode from intestine was known to be T cell-dependent, we transferred 1 × 10^7 CD45+/+ T cells to CD45-/- mice in order to compensate for defective T cell function. One day after T cell transfer, mice were inoculated with *S. ratti*. As shown in Figure 2a, the T cell-transferred CD45-/- mice expelled *S. ratti* as effectively as the CD45 +/+ mice. The number of mast cells in the intestine was evaluated in the T cell-transferred CD45-/- mice (Fig. 2b).

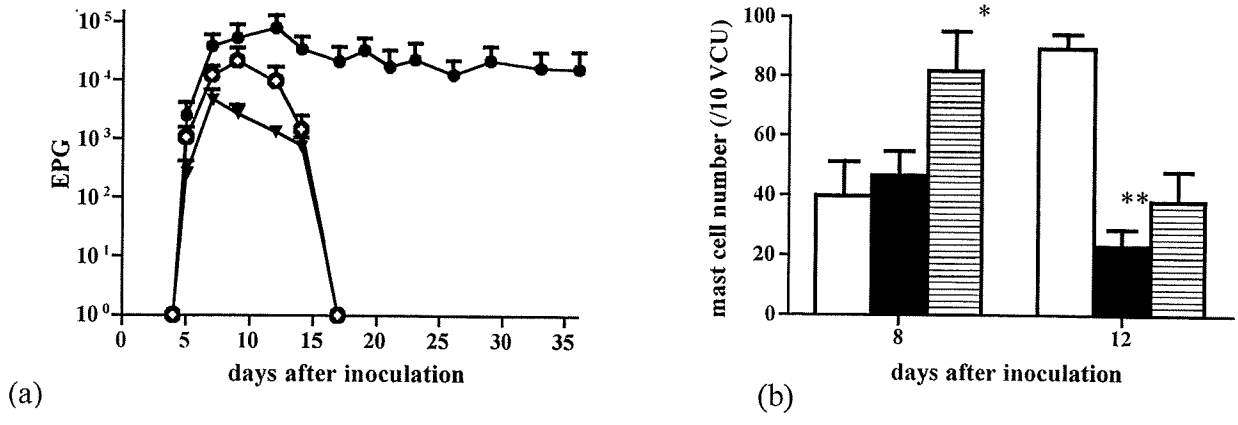


Fig. 2.

a. Adoptive transfer of T cell into CD45 knockout mice curtailed the *S. ratti* infection.

Purified T cells from wild-type mice were intraperitoneally transferred into CD45 knockout mice. Mice were then inoculated with 2,000 *S. ratti* infective larvae. The number of eggs excreted into feces was monitored and the results are presented as egg number per gram feces. Data are shown for one of two independent experiments. Data are shown as mean ± SEM in five mice in each group. Open circles: CD45+/+; Closed circles: CD45-/-; Closed inverted triangle: CD45-/- mice transferred CD45+/+ T cells.

b. Intestinal mast cells number in T cell-transferred CD45 knockout mice on day 8 and day 12 after inoculation.

Mast cells in the intestine were counted on day 8 and day 12 after larval inoculation. Data are shown as mean ± SEM of four to seven mice. Data are shown for one of two independent experiments. Open bar: CD45+/+; Closed bar: CD45-/-; Shaded bar: T cell-transferred CD45-/-. $p < 0.05$ within group by Kruskal Wallis test. * $p < 0.05$ T cell transferred CD45-/- *vs* CD45+/+ ** $p < 0.05$ CD45-/- *vs* CD45+/+ by Dunn test.

Interestingly, on day 8, the increase in mast cell numbers was higher in T cell-transferred CD45-/- mice compared with CD45+/+ and CD45-/- mice (CD45+/+: 40.4 ± 9.7; CD45-/-: 46.3 ± 8.6; T cell transferred CD45-/-: 81.8 ± 13.5/10 VCU). On day 12, when mast cell numbers peaked in CD45+/+ mice, those in T cell transferred CD45-/- mice continued to increase, although to a lesser extent than that observed on day 8 (CD45+/+: 89.6 ± 4.4; CD45-/-: 23.0 ± 5.9; T cell transferred CD45-/-: 39.2 ± 12.4/ 10VCU).

DISCUSSION

Nematode infection induces stereotypic Th2 responses, such as eosinophilia, mast cell and goblet cell hyperplasia, and elevated serum IgE titer (Maizels *et al.*, 2004), suggesting that these responses may be protective against intestinal nematode infection.

Among the various Th2 responses, it has been shown that mast and goblet cells function as effector cells in the intestine against nematode infection (Nawa *et al.*, 1985; Abe *et al.*, 1987; McKenzie *et al.*, 1998). The significance of each cell varies, depending on the species of nematode. In *Strongyloides* infection, intestinal mast cells are considered to be more important in enabling the host to expel the parasite, whilst in *Nippostrongylus* infection, goblet cells are thought to play a more significant role (Nawa *et al.*, 1994). Furthermore, non-bone marrow-derived cells, which included intestinal epithelium, were reported to contribute to the expulsion of *Nippostrongylus* through increased contractility of intestine and enhanced permeability of epithelium (Urban *et al.*, 2001).

Mast cells express high affinity receptors for IgE (FcεRI) and cross-linking of IgE/FcεRI induces degranulation which contains many physiologically active molecules (Metcalfe *et al.*, 1997). In this study, we examined the involvement of IgE/FcεRI signaling in the mast cell-dependent expulsion of *S. ratti* using CD45 exon6 knockout mice.

We showed that knockout mice supplemented with wild type T cells could expel *S. ratti* from the intestine. It has been reported that CD45-deficient mast cells cannot induce IgE-dependent degranulation either *in vitro* or *in vivo* (Berger *et al.*, 1994). Taken together, our study suggests that IgE-dependent degranulation is not essential for the protective function of intestinal mast cells against *S. ratti.* Degranulation itself is considered necessary for protection as sulfated proteoglycan, which is contained in mast cell granules, is essential for expulsion of *S. venezuelensis* (Metcalfe *et al.*, 1997; Maruyama *et al.*, 2000). Two reports showed results which conflicted with our study that *S. venezuelensis* expulsion was delayed in accordance with decreased release of proteoglycan into intestinal lumen using FcR-γ chain knockout mice which also had defect in IgE mediated degranulation of mast cells (Onah *et al.*, 2000, Onah & Nawa, 2004). So further study is expected to reveal which machinery of mast cell are essential for expulsion of parasite.

Since the antigen-specific T cell response is completely abrogated in CD45-/- mice (Kishihara *et al.*, 1993; Kong *et al.*, 1995) and protective immunity against the nematode is dependent on CD4 T cells (Urban *et al.*, 1995; Fowell *et al.*, 1997), it was anticipated that the transfer of wild-type T cells would be essential in enabling CD45-/- mice to expel the parasite. In our study, wild-type T cell transfer led to successful expulsion of the parasite from the host in CD45-/- mice with a concomitant increase in intestinal mast cells.

With regard to induction of intestinal mast cells, CD4 T cells, particularly Th2 cells, are thought to play an important role because they produce cytokines, IL-4, IL-9, and IL-10, all of which are necessary for effective proliferation, maturation and survival of mast cells (Okayama *et al.*, 2006). It can therefore be assumed that, in our study, the transferred T cells supported the effective proliferation and maturation of intestinal mast cells through cytokine production.

Surprisingly, mast cell numbers in T cell transferred CD45-/- mice had increased as early as day 8. A study reported that CD45 suppresses JAK kinase and negatively regulates cytokine receptor signaling (Irie-Sasaki *et al.*, 2001). In that report, BMMC from CD45-/- mice exhibited higher levels of proliferation in response to IL-3 compared with BMMC from CD45+/+ owing to increased JAK-STAT signaling in the absence of CD45. In our experiment, mast cells in T cell-transferred CD45-/- mice effectively proliferated, presumably in response to the cytokine produced by the transferred T cells, and increased as early as day 8.

In summary, we showed that CD45-deficient mice supplemented with T cell from wild type mice could expel *S. ratti* from the intestine. Because mast cells of CD45 knockout mice fail to induce degranulation in a IgE/FcεRI-dependent manner (Berger *et al.*, 1994), our study implicated that IgE/FcεRI signaling is not required for mast cell-dependent worm expulsion in *Strongyloides ratti* primary infection.

ACKNOWLEDGEMENTS

This work is supported, in part, by the Grant in aid for scientific research from the Japanese Ministry of Education, Science, Sports and Culture, Tokyo, Japan (No 08457081).

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Reçu le 20 janvier 2009
Accepté le 27 avril 2009

 DOI 10.1002/eji.200939433 Eur. J. Immunol. 2009. 39: 2822–2830

Concurrent infection with *Heligmosomoides polygyrus* suppresses anti-*Plasmodium yoelii* protection partially by induction of $CD4^+CD25^+Foxp3^+$ Treg in mice

Kohhei Tetsutani[1], Kenji Ishiwata[2], Hidekazu Ishida[1], Liping Tu[1], Motomi Torii[3], Shinjiro Hamano[1,4], Kunisuke Himeno[1] and Hajime Hisaeda[1]

[1] Department of Parasitology, Kyushu University Graduate School of Medical Science, Fukuoka, Japan
[2] Department of Tropical Medicine, The Jikei University School of Medicine, Tokyo, Japan
[3] Department of Molecular Parasitology, Ehime University School of Medicine, Ehime, Japan
[4] Department of Parasitology, Institute of Tropical Medicine (NEKKEN) and the Global COE program, Nagasaki University, Nagasaki, Japan

Malaria and intestinal nematode infection are widespread and co-infection frequently occurs. We investigated whether co-infected intestinal nematodes modulate immunity against co-existing malaria parasites. Infection of C57BL/6 mice with *Plasmodium yoelii* 17XNL (Py) was transient and self-limiting, but preceding infection with *Heligmosomoides polygyrus* (Hp), a mouse intestinal nematode, exacerbated malaria resulting in higher parasite burdens and poor survival of the mice. Co-infection with Hp led to reduced Py-responsive proliferation and IFN-γ production of spleen cells, and higher activation of $CD4^+CD25^+Foxp3^+$ Treg. *In vivo* depletion of Treg recovered anti-Py immunity and rescued co-infected mice from exacerbated malaria. However, we did not observe any obvious *ex vivo* activation of Treg by either Hp products or living worms. Our results suggest that intestinal nematodes moderate host immune responses during acute malaria infection by aggressive activation of Treg. Elucidation of the mechanisms of Treg activation *in situ* is a target for future analyses.

Key words: Adaptive immunity · Malaria · Intestinal nematode · Regulatory T cell

Introduction

Malaria is the most widespread and deadliest parasitic disease, and it causes hundreds of millions of clinical cases and millions of deaths annually worldwide. The severity of the disease is strongly related to the malaria parasite species, parasite density and immune responses of the host. Protective immunity against malaria develops very slowly, and individuals living in endemic areas suffer from repeated infections. The major reason why immunity to malaria is difficult to develop is that malaria parasites effectively evade host immunity in several ways. Antigenic diversity/variation allows parasites to escape immune recognition [1, 2]. They also actively suppress immunity *via* induction of Treg [3, 4], effector T-cell apoptosis [5] or dysfunctions of APCs [6]. These immune evasion mechanisms also make it difficult to develop effective vaccines against malaria.

Environmental factors, such as exposure to infective vectors [7], nutritional status [8], medical/public health interventions [9] and concurrent infections with other pathogens [10], also affect the outcome of the disease. Among infections, chronic but mostly asymptomatic infection with intestinal helminths is the most

Correspondence: Dr. Kohhei Tetsutani
e-mail: tetztani@gmail.com

prevalent in malarial areas [11, 12] (http://gamapserver.who.int/mapLibrary/Files/Maps/global_cases.jpg, http://www.who.int/intestinal_worms/epidemiology/map/en/index.html) and the population in a given area tends to suffer from both infections. Generally, as well as schistosomes [13] or filarias [14], intestinal helminths are known to modulate, and mainly suppress, host immune responses [15]. Indeed, studies in Thailand showed that infection with intestinal helminths increased the frequency of malaria episodes, but decreased malaria-associated serious inflammations, such as cerebral malaria, acute renal failure or pulmonary edema [16, 17]. These observations suggest that intestinal helminths suppress host responses of both protective immunity and inflammation during malaria infection. Therefore, it is important to understand the relationship between co-infection and host immune responses for effective control of malaria.

Several researchers have studied the effects of co-infection with intestinal helminths on the course of malaria using mouse models and reported that co-infection causes rapid growth of malaria parasites *in vivo* [18–20]. A good experimental model is the mouse intestinal nematode *Heligmosomoides polygyrus* (Hp), which resides in the upper small intestine for a long time and is known to modulate host responses through various mechanisms [21, 22]. Su *et al.* [18] described that proliferation of immune cells in response to the malaria Ag or production of anti-malaria Ab was suppressed with the induction of regulatory cytokines during co-infection with Hp and *Plasmodium chabaudi*. Unlike the results from human studies in Thailand, Hp did not attenuate inflammation-associated experimental cerebral malaria, but it did suppress anti-parasite immunity during infection with *P. berghei* ANKA [20]. These observations are compatible with our findings from studies on mice infected with Hp [21]. However, the interactions between nematodes inside the intestine and immune systems are still not well understood.

Here, we examined the effects of co-infection with Hp on the course of infection with the rodent malaria parasite *P. yoelii* 17XNL (Py) by focusing on $CD4^+CD25^+Foxp3^+$ Treg as key factors linking nematodes and suppressed host protection. We found that Hp and Py co-infection induced strongly activated Treg, which suppressed anti-Py effector mechanisms, increased malaria parasite growth *in vivo* and deteriorated survival of mice.

Results

Preceding infection with Hp deteriorates Py infection

First, we infected Hp-harboring mice with the non-lethal malaria strain Py. As previously reported [4], infection with Py alone exhibited transient elevation of parasitemia and a spontaneous cure within 3–4 wk (Fig. 1A). Co-infection with Hp caused rapid growth of Py at the early and late phases of infection (Fig. 1A), and all the mice died (Fig. 1B). Unlike the rodent parasite *P. berghei*, Py is widely considered not to induce fatal host-damaging inflammations, such as liver injury [23]. Therefore, the cause of death of the co-infected mice was supposed to be failure to eradicate the parasites, suggesting that the presence of Hp suppressed host immune responses against Py.

Co-infection with Hp suppresses adaptive immune responses against Py

Next, we analyzed the immune responses to Py in Hp-harboring mice after infection with Py. Splenocytes isolated from co-infected mice were stimulated with Py-parasitized RBC (pRBC) and analyzed for their proliferative responses (Fig. 2A). Whole spleen cells from mice infected with Py alone showed remarkable proliferation in response to pRBC. However, preceding infection with Hp significantly suppressed this proliferation. Similar results were obtained when $CD4^+CD25^-$ spleen cells were examined. The suppression was not specific for Py Ag because responses to ConA were also suppressed. We also analyzed production of IFN-γ, one of the indispensable effector molecules against malaria parasites [24]. Consistent with the proliferative responses, production of IFN-γ by spleen cells of mice infected

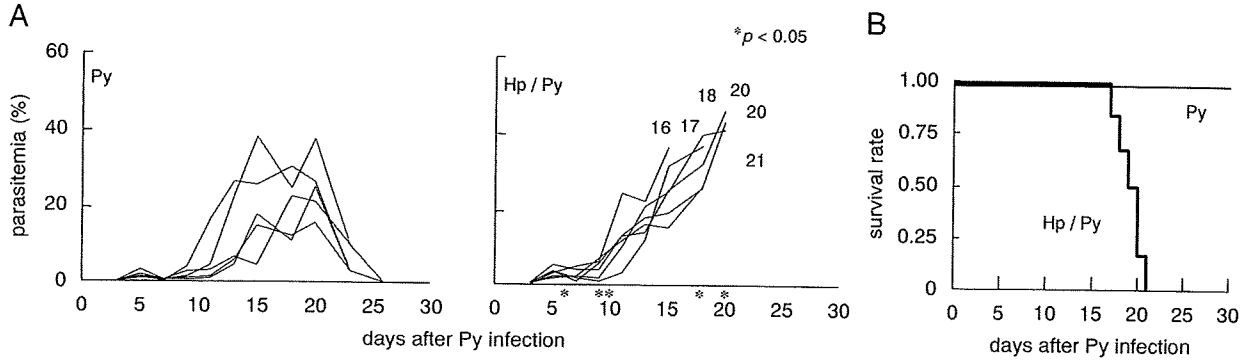

Figure 1. *H. polygyrus* and *P. yoelii* NL co-infection in C57BL/6N male mice. Mice ($n = 5–7$) were infected with Hp larvae orally and then i.p. infected with Py pRBC (2.5×10^4 cells/mouse) 2 wk later. Parasitemia (A) and survival (B) of the mice were monitored daily. Each line shows the parasitemia curve of an individual mouse, and the numbers show the death day of each individual (A). Parasitemia was analyzed statistically by Student's *t*-test. Asterisks show significant differences at the indicated *p* value. The experiment was repeated three times with similar results.

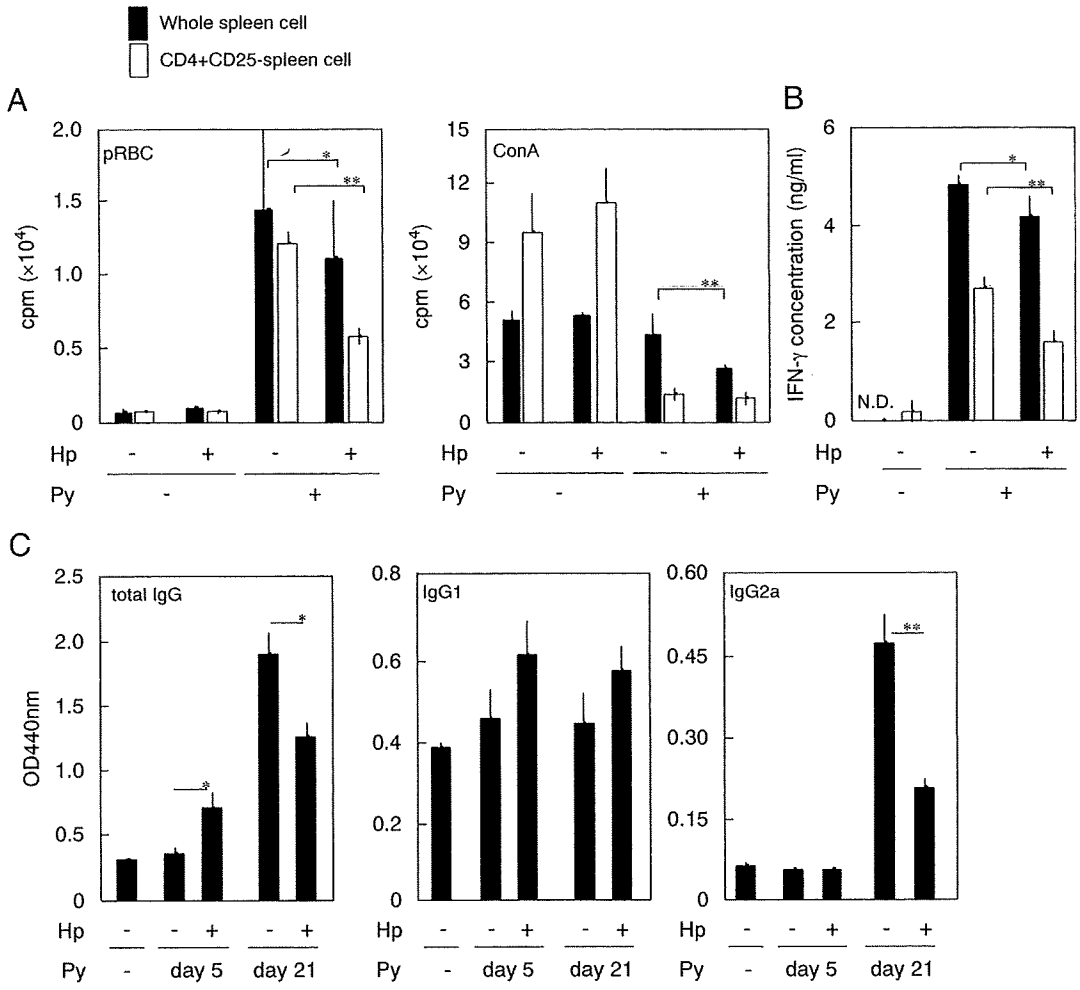

Figure 2. Effects of co-infection with Hp on immune responses against Py. Spleen cells or $CD4^+CD25^-$ spleen cells were isolated from mice co-infected with Hp and Py at 5 days after Py infection. Whole spleen cells (2×10^5; closed bars) or $CD4^+CD25^-$ cells (1×10^5) and $CD11c^+$ cells (1×10^4) from uninfected mice (open bars) were cultured with pRBC (2×10^5) or ConA (2.5 μg/mL). (A) Proliferation was analyzed by ^{3}H-thymidine uptake. (B) IFN-γ concentrations in the culture supernatants with pRBC were measured by ELISA. (C) Anti-Py IgG, IgG1 or IgG2a Ab in sera taken at the indicated days after Py infection were measured by ELISA using Py Ag. (D) $CD11c^+$ cells (1×10^4) were isolated from mice co-infected with Hp and Py at 5 days after Py infection and cultured with $CD4^+CD25^-$ cells (1×10^5) from Py-infected mice and pRBC (2×10^5). Proliferation was analyzed by ^{3}H-thymidine uptake. Data represent the means ±SE of triplicate samples in a representative of 3–6 repeated experiments. $^*p<0.05$, $^{**}p<0.01$; between the samples linked with horizontal lines, Student's t-test.

with Py was significantly suppressed by preceding infection with Hp (Fig. 2B). These results clearly indicate that co-infection with Hp suppresses cellular immune responses. Humoral immunity, represented by IgG specific for malaria parasites, was subsequently analyzed (Fig. 2C). Infection with Py alone led to the development of IgG2a-dominant Ab responses at 21 days after infection, but had no effect at 5 days after infection. Despite the higher parasitemia at the early phase, co-infection with Hp rather enhanced the production of IgG1. Conversely, it remarkably reduced the production of IgG2a in curable mice singly infected with Py, suggesting that the Ab responsible for protection at the late phase may be IgG2a. These results demonstrate that the increased Py parasitemia at the early stage of infection is due to suppression of cellular responses, rather than Ab responses. Therefore, we subsequently focused on the suppression of cellular responses at the early phase.

Co-infection with Hp and Py induces aggressively suppressive Treg

In a previous report, we demonstrated that activation of Treg occurs during infection with a lethal strain of *P. yoelii* [4]. In addition, it has been reported that the functions of Treg are altered in mice infected with Hp [25]. These observations led us to analyze the Treg behaviors in co-infected mice. As early as 5 days after infection with Py, the proportion of $CD4^+CD25^+$ $Foxp3^+$ cells was increased in the spleen, as well as MFI of either

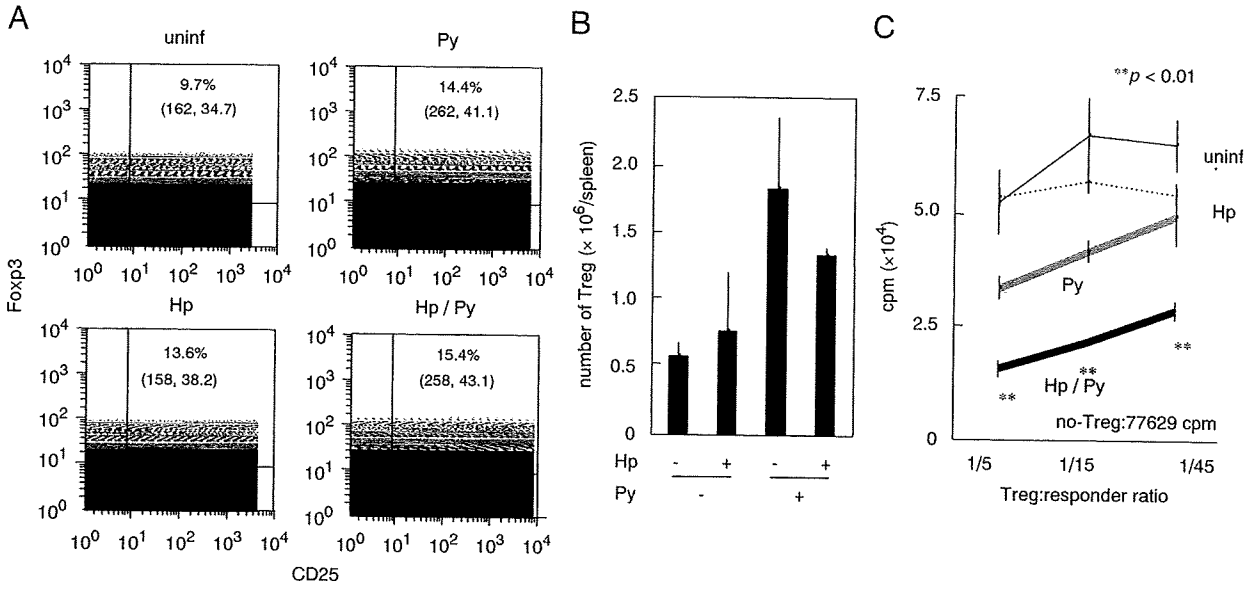

Figure 3. $CD4^+CD25^+Foxp3^+$ cells in Hp+Py co-infection. Spleen cells stained with fluorochrome-conjugated anti-CD4, anti-CD25 and anti-Foxp3 Ab were analyzed by flow cytometry at 5 days after infection with Py. (A) The dot plots represent the expression levels of CD25 and Foxp3 in CD4-gated cells from the indicated mice. Percentage inside of each plot shows the frequency of $CD4^+CD25^+Foxp3^+$ cells in total $CD4^+$ cells. The number inside of brackets shows the mean fluorescent intensity of CD25 (left) and Foxp3 (right). (B) The $CD4^+CD25^+Foxp3^+$ cell populations in individual spleens were calculated. (C) The suppressive functions of Treg from co-infected mice were analyzed at 5 days after infection with Py. Splenic Treg from the indicated mice were cultured with $CD4^+CD25^-$ cells (1×10^5) as responders and $CD11c^+$ cells (1×10^4) obtained from the spleens of uninfected mice in the presence of ConA (2.5 μg/mL) at the indicated frequencies. The proliferation of responders was analyzed by ^3H-thymidine uptake. Data are presented as the means ±SE of three samples in a representative of three repeated experiments. Asterisks show significant differences by Student's *t*-test between Treg from Py-infected and Hp+Py-infected mice at the indicated *p* value.

CD25 or Foxp3 (Fig. 3A). The absolute number of $CD4^+CD25^+$ $Foxp3^+$ cells in individual spleens, calculated from the cell frequency by flow cytometry, was also increased (Fig. 3B). The amount of Foxp3 expressed in Treg may influence their functions [26], suggesting that Treg with the higher Foxp3 MFI observed in co-infection may have enhanced suppressive function. Thus, the suppressive function of Treg was determined by analyzing the degree of suppression of TCR-triggered T-cell proliferation at this time point. Co-infection with Hp enhanced the suppressive activity of Treg, but did not affect the number of Treg (Fig. 3B, C). These results suggest that co-infection with Hp and Py induces Treg more aggressively than single infection with Py, resulting in deteriorated malaria infection associated with suppressed cellular responses against Py.

In vivo depletion of Treg partially abolishes the suppressed protection against Py

To confirm that induction of Treg in mice co-infected with Hp and Py is responsible for the deteriorated infection with Py, we depleted Treg by i.p. application of an anti-CD25 Ab. This treatment depleted 85% of the $CD4^+CD25^+Foxp3^+$ Treg in the spleen, as evaluated by Foxp3 intracellular flow cytometry, at 5 days after infection (Fig. 4A). However, the depletion effect was transient and Treg were detected in mice treated with the anti-CD25 Ab at similarly high levels to those in control mice at 2 wk after the treatment (data not shown). As previously shown, infection with Py was self-limiting, and again mice co-infected with Hp suffered from higher parasitemia and finally succumbed to infection with Py (Fig. 4B, C). Depletion of Treg significantly decreased the parasitemia at the early phase of infection in co-infected mice. Furthermore, 40% of the co-infected mice depleted of Treg were able to limit the parasitemia and they survived (Fig. 4B, C).

The anti-malarial effector mechanisms were altered by depletion of Treg. The suppression of IFN-γ production by splenic $CD4^+$ T cells in response to pRBC observed in co-infected mice was clearly reversed after removal of Treg (Fig. 4D). Alterations in Ab responses due to co-infection with Hp, namely suppression of IgG and IgG2a at the later phase and enhancement of IgG at the early phase, were all offset by the removal of Treg (Fig. 4E). These results suggest that the deteriorated malaria infection in mice co-infected with Hp and Py was partially due to aggressive activation of Treg.

Hp worm products and living worms do not directly activate Treg *in vitro*

The preceding existence of Hp activates Treg in terms of enhanced suppressive functions during infection with Py. To investigate how co-infection with Hp induces stronger Treg, we performed *in vitro* Treg activation assays using various Hp products. Splenic Treg from Py-infected mice were stimulated

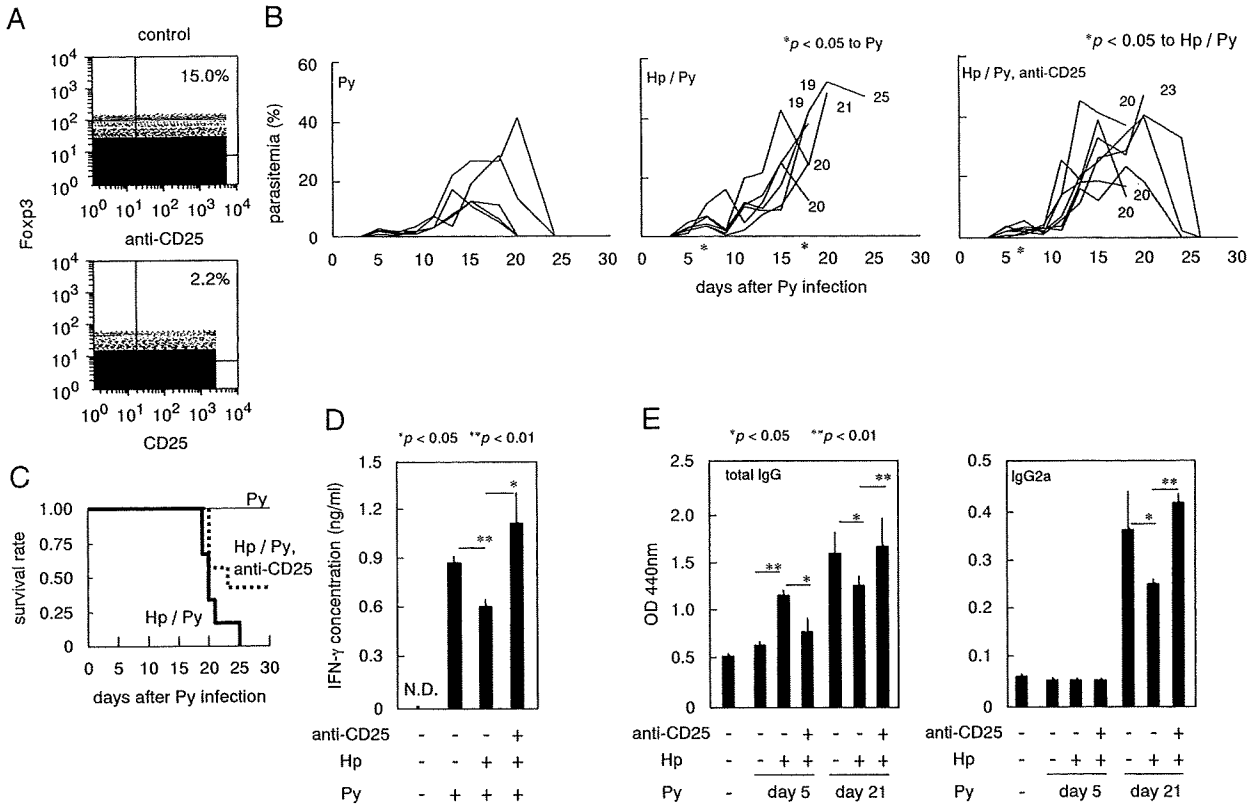

Figure 4. Effects of depletion of Treg in mice co-infected with Hp and Py. Spleen cells from mice depleted of Treg were analyzed as described in the legend for Fig. 3A. (A) The treatment depleted 85% of Treg in the spleen, as evaluated by flow cytometry on day 5 after Py infection. (B,C) Py infection in mice with or without depletion of Treg was monitored by the parasitemia (B) and survival (C) as described in the legend for Fig. 1. The infection experiments were repeated three times with similar results. (D, E) Immune responses and IFN-γ production in $CD4^+$ cells in response to pRBC (D) and serum levels of anti-Py IgG (E) were analyzed as described in the legend for Fig. 2. Data are presented as the means ±SE of three samples in a representative of 3–6 repeated experiments. Asterisks show significant differences at the indicated *p* values by Student's t-test.

with excretory/secretory (ES) Ag of Hp, an extract of adult Hp worms or living Hp worms in the presence of $CD11c^+$ DCs (Fig. 5). The cultured Treg were then analyzed for their suppressive activities. Treg cultured with medium alone still exhibited suppressive functions, although their effects were lower than those of freshly isolated Treg. However, none of the stimulatory conditions changed the suppressive abilities of the cultured Treg. The same tendency was observed when Treg were cultured without DC (data not shown) and when Treg from uninfected mice were stimulated with Hp preparations in combination with DC and pRBC, as well as without DC (data not shown). These results indicate that Hp worms residing in the intestine do not directly activate Treg.

Discussion

In the present study, we have demonstrated that a mouse intestinal nematode, Hp, was able to suppress anti-malaria protection *via* induction of Treg. Infection with malaria parasites is already known to activate Treg in humans and experimental models [3, 4], which allows rapid growth of malaria parasites. As previously reported [4], the non-lethal malaria parasite Py used in the present study does not strongly activate Treg and its low pathogenicity/virulence is closely linked to its failure to activate Treg. Therefore, it seems likely that activation of Treg in the presence of Hp converted the pathogenic behaviors of Py, as observed for a highly virulent *P. yoelii* strain [4]. Furthermore, the involvement of Treg activation for conversion to high virulence was clearly confirmed by the partial reversal of high parasitemia and mortality in mice depleted of Treg.

However, anti-CD25 treatment did not rescue all of the co-infected mice, partially because the depletion of Treg did not last longer than 2 wk. This finding may be supported by a previous observation that anti-CD25 treatment is insufficient because of a rapid expansion of Treg after infection with *P. yoelii* [27]. Interestingly, depletion of Treg recovered the suppression of presumably protective IgG responses at the late phase when Treg themselves reappeared comparably with the control Treg-sufficient mice. Therefore, besides activation of Treg, we cannot exclude various mechanisms supposed to cause the global immune suppression by intestinal helminths, regardless of the importance of Treg. For instance, the elevation of IgG1 observed at the early phase of infection suggests that Th2

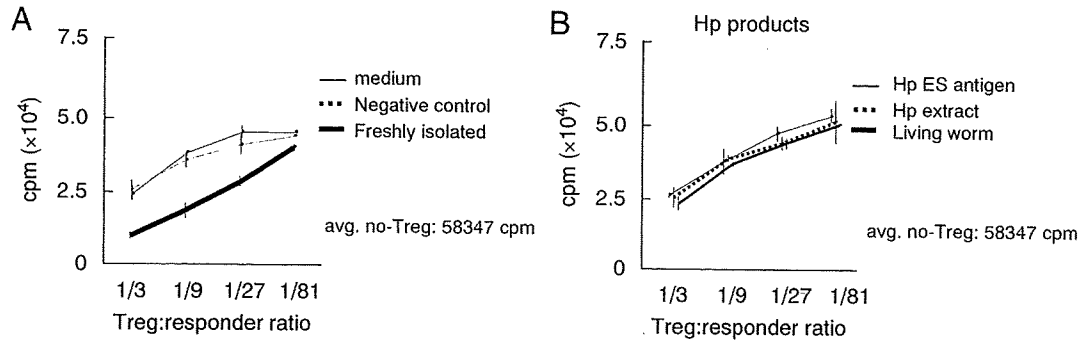

Figure 5. Stimulation of Treg with Hp products or living Hp worms *in vitro*. Splenic Treg isolated from mice at 5 days after Py infection were cultured with Hp products (100 μg/mL) or living Hp worms using a transwell system in the presence of DCs. Cultured Treg were used for suppressive analyses as described in the legend for Fig. 3C. Data are presented as the means ±SE of triplicate samples in a representative of 3–5 repeated experiments. There are no significant differences among the Treg conditioning treatments by Student's *t*-test.

responses in mice co-infected with Hp may prohibit the development of protective Th1 responses such as the production of IFN-γ.

Infections with helminths have also been reported to activate Treg [25, 28], and Hp suppresses allergic responses to innoxious Ag or protective immunity against pathogens in a Treg-dependent manner [29]. Su *et al.* [18] described the induction of regulatory cytokines, such as TGF-β and IL-10, during co-infection with Hp and *P. chabaudi*. We observed as well that transcription of TGF-β and IL-10 of Treg isolated from co-infected mice was slightly increased compared with those from non-infected animals or infected with either Py or Hp (data not shown). It would be interesting to evaluate the importance of these regulatory cytokines in *in vitro* suppressive function analysis, as done in Fig. 3C for instance.

The ES Ag of Hp were reported to be immunosuppressive toward T cells [30, 31]. However, we observed that infection with Hp alone did not activate Treg *in vivo* and that neither Hp products nor living worms directly activated Treg *in vitro*. Previous report suggested that Hp activates Treg *via* DC [32], but our *in vitro* results showed that Hp did not induce significant activation of Treg even in the co-culture with DC, suggesting less probability of the pathway between Hp *ex vivo* and strongly activated Treg, either dependently or independently of DC. One possibility may be that Hp infection takes longer than 2 wk to activate Treg by itself. Su *et al.* [18] described that mortality of co-infected mice increased if *P. chabaudi* infection was performed later than 2 wk after Hp infection. However, when we compared the effect of interval from preceding Hp to following Py infection on the induction of Treg between 2 and 6 wk (data not shown), Treg from both sets of co-infected mice showed similar and significant suppression compared with those from Py-infected mice, while Treg from Hp-infected mice did not show significant suppression. Taken together, our results suggest that Hp infection does not directly induce Treg activation, but probably conditions the host to stand by for Treg activation on exposure to incoming Ag /pathogens and/or enhances Treg activation by other stimulations such as Py.

Finally, our results demonstrate that co-infection with intestinal nematodes deteriorates the course of malaria, which is supposed to reflect the infectious state in malaria-endemic areas. Although we did not address whether treatment with anti-helminthic drugs improves malaria, chemotherapy against helminths would be included in global malaria control.

Materials and methods

Mice and parasites

Male 8- to 10-wk-old C57BL/6 mice were purchased from Kyudo (Tosu, Japan). All experiments using mice were conducted according to the guidelines for animal experimentation of Kyushu University.

Hp was kindly provided by Dr. J. F. Urban, Jr. (Beltsville Human Nutrition Research Center, US Department of Agriculture, Beltsville, MD, USA) and maintained by *in vivo* passages using male ICR mice. For infection, feces containing eggs were incubated on wet filter paper to allow the eggs to develop into infective larvae. Mice were infected orally with 200 infective larvae by gastric intubation. Infection was confirmed by Hp egg detection in feces before and after Py infection.

pRBC (2.5×10^4 cells/mouse) were commonly injected i.p., and the percent parasitemia (ratio of pRBC to total RBCs) was monitored by microscopic evaluation of thin blood films stained with Giemsa solution. For depletion of $CD25^+$ T cells *in vivo*, mice were injected i.p. with 100 μg of anti-CD25 Ab 1 day before and 1 day after Py infection. Each infection experiment was repeated three times with 5–7 mice *per* group.

Reagents

PE-anti-CD25 (PC61.5), allophycocyanin-anti-CD4 (GK1.5) and FITC-anti-Foxp3 (FJK-16s) staining kits, as well as FITC-anti-CD11c

(N418) Ab were obtained from eBioscience (San Diego, CA, USA). Anti-PE, anti-allophycocyanin and anti-FITC microbeads (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany) were used for cell purification. Monoclonal rat anti-mouse CD25 (7D4) IgM purified from the ascites of hybridoma-injected athymic nude mice was used for *in vivo* treatments.

Preparation of parasites and their products

Worm products were prepared as described previously [30, 31] with modifications. Adult Hp worms were collected from the upper gastrointestinal tract of infected mice at 3–4 wk after larvae infection. The worms were washed three times with PBS containing ampicillin (150 μg/mL; Sigma, St. Louis, MO, USA) and streptomycin (50 μg/mL; Sigma), and approximately 500–1000 worms were incubated in 0.5 mL of ampicillin/streptomycin/PBS for 24–48 h at 37°C. The culture supernatant was collected by centrifugation at 10 000 × *g* for 10 min at 4°C and used as the Hp ES Ag. Worms were mechanically homogenized after or before incubation, and frozen and thawed 3–5 times. The supernatant was collected by centrifugation at 10 000 × *g* for 10 min at 4°C and used as the Hp extract. As a negative control, non-Hp-infected mouse intestinal contents were prepared in the same manner as for Hp ES Ag. All products were filtered through 0.45-μm pore filters, quantified by the BCA™ Protein Assay (Pierce, Rockford, IL, USA) and stored at –80°C until use. The products were pretreated with polymyxin B (50 μg/mL; Sigma) for 30 min before dilution with cell culture medium to a final concentration of 100 μg/mL, including 12.5 μg/mL polymyxin B. When required, living worms were freshly prepared by washing three times with PBS containing ampicillin and streptomycin and pretreated with polymyxin B for 30 min at room temperature. Hp products were prepared for each cellular experiment.

pRBC were isolated using a Percoll enrichment technique [33]. Briefly, blood from Py-infected mice was collected into heparinized PBS and passed through a cellulose column to remove leukocytes and platelets. After addition of the RBC solution to 63% v/v Percoll/PBS and centrifugation, pRBC were collected from the interphase. pRBC were freshly prepared for each cellular experiment. When required, isolated pRBC were frozen and thawed three times, and the supernatant was collected by centrifugation at 10 000 × *g* for 10 min at 4°C for use as a coating Ag in ELISA.

Cell purification

Cell purification was performed using a magnetic cell sorting system (Miltenyi Biotech GmBH) according to the manufacturer's instructions. Spleens of mice were reduced to single cell suspensions by hemolysis with 0.86% NH_4Cl. To purify $CD4^+CD25^-$ cells, the suspensions were incubated with FITC-anti-CD4 and PE-anti-CD25 Ab, followed by the addition of anti-PE microbeads and removal of $CD25^+$ cells. Anti-FITC microbeads were added to the flowthrough and $CD4^+CD25^-$ cells were obtained. DCs were purified using an FITC-anti-CD11c Ab. Treg were purified using a PE-anti-CD25 Ab, and 90% of them were confirmed to express CD4, CD25 and Foxp3 by flow cytometry. The purity of each cell subset usually exceeded 90%.

Cell culture

Purified T cells were cultured with pRBC in the presence of $CD11c^+$ cells in 200 μL of RPMI1640 supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 20 mM HEPES, 50 mM $NaHCO_3$, 2 mM L-glutamine, 100 μM 2-mercaptoethanol and 10% inactivated fetal bovine serum on round-bottomed 96-well plates. ConA was added as an assay control at a final concentration of 2.5 μg/mL. $CD11c^+$ cells were irradiated with 30 Gy before coculture with other cells. Typically, 1×10^5 T cells, $1–2 \times 10^5$ pRBC and 1×10^4 $CD11c^+$ cells were cocultured *per* well. Cultures were performed for 68–76 h at 37°C in air supplemented with 5% CO_2, including 10–16 h of coculture with ^{3}H-thymidine (1 μCi/well). Cells were harvested onto glass–fiber filter mats, dried and measured for their ^{3}H-thymidine uptake using a liquid scintillation counter. When required, the supernatant was collected before the addition of ^{3}H-thymidine and kept at –80°C until use. Each triplicate experiment was repeated 3–6 times.

Treg suppression assay

To analyze Treg functions, purified $CD4^+CD25^-$ cells (1×10^5 cells/well) from uninfected or infected mouse spleens stimulated with soluble ConA (2.5 μg/mL) and $CD11c^+$ cells (1×10^4 cells/well) from uninfected mouse spleens were cultured with a variety of freshly isolated or preconditioned Treg at various populations for 72 h and incubated with ^{3}H-thymidine (1 μCi/well) for the last 8–12 h. Radioactivity was measured using a liquid scintillation counter.

When required, purified Treg isolated from uninfected or Py-infected mouse spleens were preconditioned with $CD11c^+$ cells from uninfected mouse spleens under various conditions for 72 h. Conditioning with living Hp worms was performed using a transwell system (0.2-μm Anopore membrane; Nalge Nunc International, Rochester, NY, USA), in which the cells were in the lower chamber and 10 worms were in the upper chamber. Conditioned Treg were washed once, counted for living cells by Trypan blue staining and used for suppression assays as described above. Each triplicate experiment was repeated 3–5 times.

ELISA

The IFN-γ concentrations in the above-described cell culture supernatants were measured using a Mouse IFN-γ ELISA

Development Kit, DuoSet (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Each duplicate experiment was repeated three times. The anti-malaria Ab titers in the sera of mice were measured by ELISA by the OD at 440 nm using HRP-anti-mouse IgG(H+L), HRP-anti-mouse IgG1 and HRP-anti-mouse IgG2a Ab as previously described [34]. Sera were collected and kept at –80°C until analysis. Malaria Ag was prepared as described above and used as a coating Ag. Each triplicate experiment was repeated three times.

Flow cytometry

Splenocytes were prepared as single cell suspensions by hemolysis with 0.86% NH_4Cl. After staining of cell surface molecules or intracellular staining of Foxp3, the cells were evaluated using a FACSCalibur (BD, Franklin Lakes, NJ, USA) according to the manufacturer's instructions and the data were analyzed with the CellQuest Pro software (BD). Each infection experiment using 2–3 mice was repeated three times.

Statistical analysis

Student's t-test was used for statistical analyses. Values of $p<0.05$ were considered significant.

Acknowledgements: This study was supported by the Ministry of Education, Science, Sport and Culture of Japan (Grant 1890400, 19041056). The authors appreciate the special kindness of Dr. J. F. Urban Jr., Beltsville Human Nutrition Research Center, US Department of Agriculture, Beltsville, MD, USA, who provided the larvae of *Heligmosomoides polygyrus* used in this study.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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