ORIGINAL ARTICLE

Aminopeptidase N facilitates entry and intracellular survival of *Mycobacterium tuberculosis* in monocytes

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ABSTRACT

Background and objective: **Aminopeptidase N (CD13) is an ectoenzyme located in the outer membrane of a variety of cells. Proteomic profiling indicates an increased expression of CD13 in phagocytes during *Mycobacterium tuberculosis* infection. The purpose of this study was to investigate the role of CD13 on the internalization and intracellular survival of *M. tuberculosis* in monocytes.**
Methods: **Magnetic nanoparticles and confocal microscopy were used to observe interactions between CD13 and *M. tuberculosis*. Mycobacterial entry and intracellular survival in monocytes were assessed with and without anti-CD13 antibody (WM15 and WM47) using flow cytometry and colony formation assay.**
Results: **By using magnetic nanoparticles and confocal microscopy, *M. tuberculosis* was found to be capable of binding to either soluble CD13 or membranous CD13 on monocytes. Flow cytometry showed that pretreatment of monocytes with WM15 or WM47 reduced the number of intracellular *M. tuberculosis*. Collectively, the data suggest that CD13 is a binding and entry receptor for *M. tuberculosis* on monocytes. Treatment of infected monocytes showed a greater effect of WM47 than WM15 in reducing the intracellular colonization of *M. tuberculosis*, suggesting that specific epitopes of CD13 may play an important role modulating intracellular *M. tuberculosis* survival.**
Conclusions: **CD13 acts as a receptor for *M. tuberculosis* on human monocytes. The molecule facilitates internalization, and interaction of CD13 with an anti-CD13 antibody reduces intracellular *M. tuberculosis* survival.**

Key words: aminopeptidase N, CD13, *Mycobacterium tuberculosis*, receptor, tuberculosis.

Abbreviation: CD13, aminopeptidase N; MNP, magnetic nanoparticle.

SUMMARY AT A GLANCE

The particular receptor by which *M. tuberculosis* infects monocytes may have a distinct influence on its survival. CD13 appears to participate in the internalization of *M. tuberculosis* into human monocytes and modulate intracellular survival.

INTRODUCTION

Mycobacterium tuberculosis is one of the most successful pathogens estimated to have infected nearly one-third of the human population and killing approximately 1.7 million people each year.[1] Much of its success is due to essential virulence factors that allow it to survive within phagocytes rather than to be eliminated by these scavenger cells. Mycobacteria bind to macrophages in cholesterol and lipid-rich domains of the host cell plasma membrane called lipid rafts,[2] which are associated with various signalling mechanisms.[3] Once within the cell, the organism can degrade cholesterol as energy source to maintain a chronic infection in the host.[4] Additionally, mycobacterial interference with lipid-mediated signalling arrests phagosome maturation, thus protecting the bacterium against delivery to the lysosome.[5]

Aminopeptidase N (CD13) is a multifunctional protein expressed in many tissues and has been found to be partially localized in lipid rafts.[3] It influences plasma membrane protein organization[6] and cholesterol uptake.[7] It can exist both as a membrane-bound protein and as an active soluble protein secreted by certain cells or released by cleavage of the plasma membrane.[8] CD13 has been shown to be a cell-surface receptor for certain viruses, seemingly required for endocytosis enabling internalization of the virus into the cell.[9,10] By a modified isotope-coded affinity tag technology,[11] we previously found a significantly elevated expression of CD13 in human phagocytes infected with *M. tuberculosis* (Lu & Tsai, unpubl. data, 2007). We therefore speculated that CD13 might have a yet undefined role in mycobacterial infection. This study was designed to

assess the interaction between CD13 and *M. tuberculosis*, investigating both entry of the organism into monocytes and its subsequent intracellular survival.

METHODS

Ethics statement
This study was conducted according to the principles expressed in the International Conference on Harmonisation/World Health Organization Good Clinical Practice standards, and written informed consent was obtained for participation in the study, which was approved by the institutional review board of the Mackay Memorial Hospital.

M. tuberculosis preparation
M. tuberculosis strains were obtained from the culture collection of Mycobacteriology Laboratory, Mackay Memorial Hospital, Taipei, Taiwan. The organisms were cultured on Lowenstein-Jensen medium slant at 37°C in a 10% CO_2 humidified atmosphere. Detailed documentation of the experimental procedures was described in the Appendix S1 and S2 in the online supporting information.

Cell culture and mycobacterial infection
Peripheral blood mononuclear cells were isolated from the whole blood of healthy adult volunteers by Ficoll-Paque gradient centrifugation. Mononuclear cells were incubated with CD14 microbeads (Miltenyi Biotec, Auburn, CA, USA) and then the CD14-positive cells were separated by means of a magnetic force. These cells were seeded in U-bottom 96-well plates at a density of 2×10^5 cells in a volume of 200 µl of RPMI-1640 medium with 10 % fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Israel) and infected with *M. tuberculosis* (approximately 5×10^5). Monocytes were washed repeatedly to remove extracellular bacteria and incubated with monoclonal antibodies against CD13 (clone WM15, Biolegend or WM47, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or isotype (mouse IgG1κ, Biolegend, San Diego, CA, USA).

Flow cytometry
Binding of soluble CD13 to *M. tuberculosis* was examined by flow cytometry. A suspension of about 1×10^7 bacilli/mL was incubated with recombinant human CD13 (residues 69-967, R&D, Minneapolis, MN, USA) or CD4 (residues 26–226, Abcam, Cambridge, UK) for 30 min at 37°C, washed twice with phosphate buffer saline and centrifuged at 3500 *g* for 15 min at 4°C. The pellet was resuspended in phosphate buffer saline, after which phycoerythrin-conjugated mouse antibody against isotype (IgG1κ), or CD13 (clone L138) or CD4 (clone RPA-T4) (BD Pharmingen, San Jose, CA, USA) was added for 30 min at room temperature, followed by washing with phosphate buffer saline twice and centrifugation at 3400 *g* for 15 min at 4°C. The pellet resuspended in phosphate buffer saline was mixed with an equal volume of 4% formalin and incubated for 24 h prior to flow cytometric analysis. For the assay of bacteria entry, intracellular quantities of *M. tuberculosis* were measured by staining permeabilized cells with fluorescein isothiocyanate-conjugated anti-*M. tuberculosis* antibody. Monocytes were washed repeatedly to remove extracellular bacteria and incubated with 0.2% trypan blue for 2 min at 4°C to allow efficient quenching of surface fluorophore. The mean fluorescence intensity of stained cells was measured by fluorescence-activated cell sorting Calibur flow cytometry and analysed by CellQuest software (BD Bioscience, San Jose, CA, USA).

Magnetic nanoparticle preparation
Magnetic nanoparticles (MNP) composed of Fe_3O_4 were pre-labelled with nitrilotriacetic acid[12] and conjugated with recombinant histidine-tagged CD13 protein. MNP composed of Fe_3O_4 coated with nitrilotriacetic acid derivative was kindly donated by Professor Yu-Chie Chen, National Chiao Tung University, Hsinchu, Taiwan. CD13-MNP was prepared by pre-labelling the surface of MNP with Ni(II), which was then conjugated to recombinant histidine-tagged CD13 protein through the binding to Ni(II). *M. tuberculosis* (10^8 bacilli/mL, 200 µL) were mixed with either unbound nanoparticles or CD13-MNP (0.25 µg/mL, 100 µL) and then allowed to precipitate for 10 min. By applying an external magnetic field, nanoparticles and the attached mycobacteria were attracted to the tube wall. The amount of unbound *M. tuberculosis* was estimated by measuring the absorbance at 600 nm with a spectrophotometer. The nanoparticles were smeared onto slides and acid-fast stain was performed. The slides were observed under the microscope.

Confocal microscopy
Monocytes were cultured on 18-mm diameter cover glass placed in 12-well culture plate and infected with *M. tuberculosis* labelled with Auramine-Rhodamine T. After 30 min, unbound bacteria were washed away with phosphate buffer saline and monocytes were fixed in 4% formalin. CD13 were stained with Cy-Chrome 5-conjugated anti-CD13 antibody, and nuclei were stained with 4′-6-diamidino-2-phenylindole. Samples were analysed by Leica true confocal scanner SP5 confocal laser scanning microscopy (Leica Microsystems, Wetzlar, Germany).

Colony forming units
Cells incubated with *M. tuberculosis* were lysed by adding 100 µL 0.1% sodium dodecyl sulfate, vortexing and incubated for 10 min. Additional 900 µL H_2O was added to the solution and centrifuged at 3400 *g* for 15 min at 4°C. The lysates were plated on 7H11 agar and incubated for 3 weeks. The resulting growth of *M. tuberculosis* was reported as mean colony forming units (CFU) per 10 000 cells.

Statistical analysis
Paired *t*-test was used for analysis. Data are reported as the mean ± standard error of the mean. Statistical

analysis was performed using Prism 3.0 software (GraphPad Software, Inc., San Diego, CA, USA). Two-sided tests were used, and a *P*-value of <0.05 was considered statistically significant.

RESULTS

Interactions between CD13 and *M. tuberculosis*

To assess whether *M. tuberculosis* interacts with CD13, bacilli were incubated with or without soluble recombinant human CD13 protein for 30 min. The binding of soluble CD13 to *M. tuberculosis* was detected by staining with phycoerythrin-conjugated anti-CD13 antibody and an isotype antibody as negative control. As demonstrated by the flow cytometry, a dose-dependent increase of CD13-positive *M. tuberculosis* organisms was observed with the binding of CD13 to *M. tuberculosis* reaching up to 6.53% (Fig. 1a,b). An irrelevant protein CD4 was concurrently utilized to test whether the binding of *M. tuber-*

culosis was specific for CD13. No apparent binding was observed between CD4 (0.5 μg) and *M. tuberculosis* (Fig. 1a, bottom row). CD13-MNP was also prepared to evaluate the binding affinity between CD13 and *M. tuberculosis*. While applying an external magnetic field to accelerate the process of aggregation, we noticed that CD13-MNP aggregates were lightly coloured in comparison with those of MNP (Fig. 1c). We speculated that abundant *M. tuberculosis* organisms bound to CD13-MNP shuttered the natural brown colour of MNP. To estimate the amount of unbound *M. tuberculosis* in the solution, we removed the aggregate pellets by applying an external magnetic field and estimated the density of mycobacteria in the solution by measuring absorbance at 600 nm (Fig. 1d). Prior to the addition of MNP, mycobacterial concentration was $3.7 \pm 0.2 \times 10^7$/mL. At 10 min after mixing, much less unbound *M. tuberculosis* ($0.4 \pm 0.1 \times 10^7$/mL) was detected in the CD13-MNP solution than in the MNP solution ($2.6 \pm 0.2 \times 10^7$/mL). Following acid-fast staining, the majority of
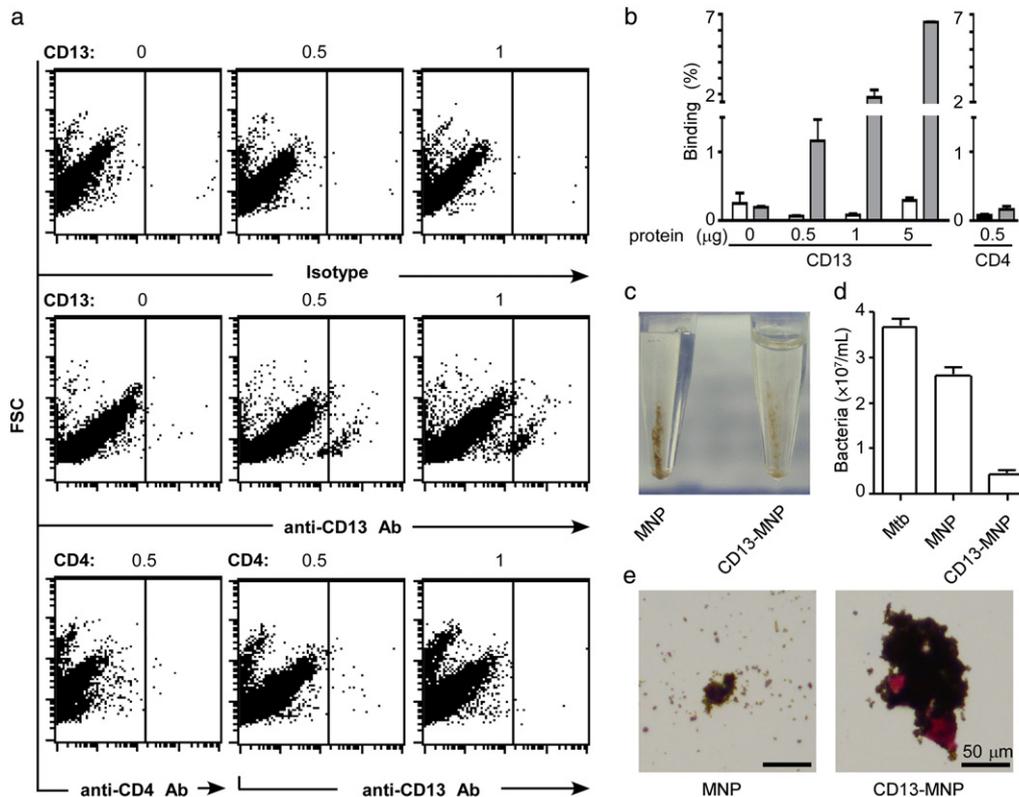
Figure 1 Interactions between soluble aminopeptidase N (CD13) and *Mycobacterium tuberculosis* (Mtb). (a) Mtb were incubated with CD13 or CD4 protein and stained with phycoerythrin-conjugated isotype or anti-CD13 or anti-CD4 antibody (Ab). Bacterial binding was assessed by flow cytometry. The cut-off lines were determined using isotype controls. (b) A dose-dependent increase of CD13-positive Mtb organisms was observed with the binding of CD13 to Mtb reaching up to 6.53%. (□) isotype; (▨) anti-CD13; (■) anti-CD4. (c) Magnetic nanoparticles (MNP) or CD13-MNP were incubated with Mtb for 10 min followed by imposition of an external magnetic force. Aggregates of plain MNP were dark, whereas those of CD13-MNP were lighter in colour, implying that Mtb in the latter aggregates partially covered the original nanoparticle color. (d) The bacterial density of Mtb in suspension was estimated by measuring the absorbance at 600 nm with a spectrophotometer. A significant majority of the Mtb was bound and precipitated by CD13-MNP leaving only $0.4 \pm 0.1 \times 10^7$/mL of unbound bacteria being detected in the solution. Bars represented means ± standard error of the mean (*n* = 3). (e) The aggregates of nanoparticles were examined by acid-fast stain. Scale bar, 50 μm. The majority of plain nanoparticle aggregates were found to be small, with only occasional acid-fast organisms seen. By contrast, CD13-MNP aggregates occurred in large clusters surrounded by and intermingled with abundant acid-fast organisms.
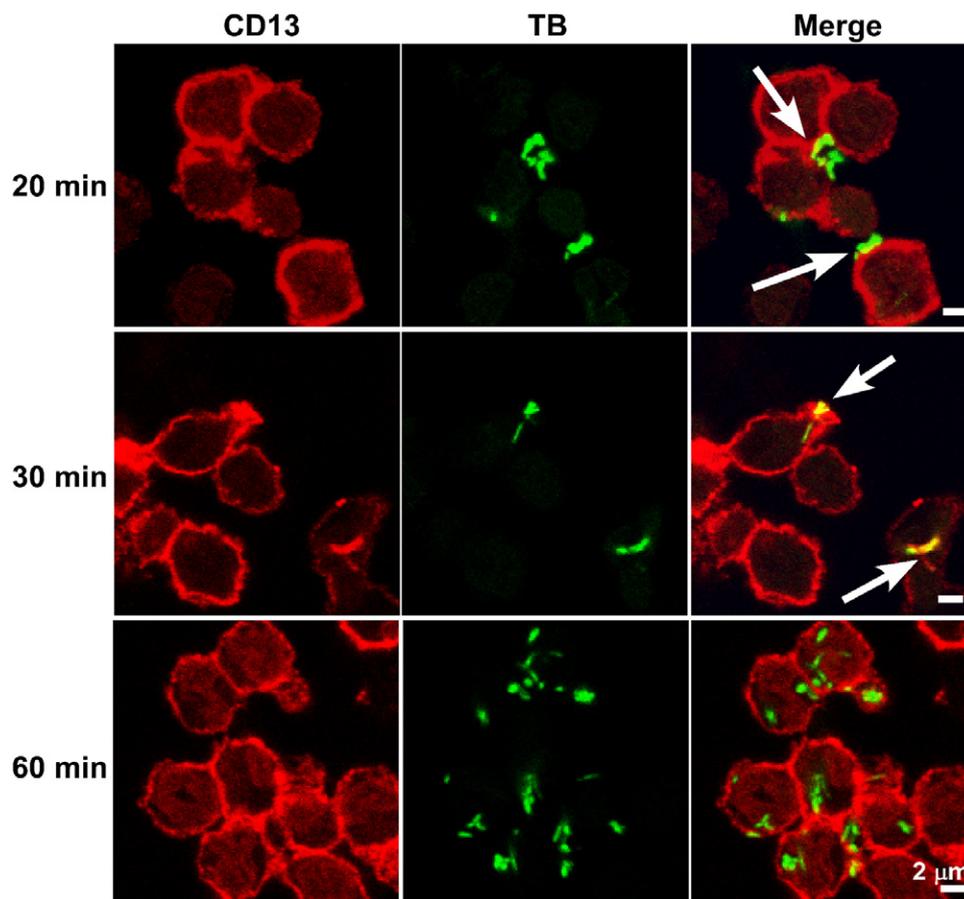
Figure 2 The binding of *Mycobacterium tuberculosis* to aminopeptidase N (CD13) on the surface of monocytes. Confocal microscopy showing CD13 on monocyte surface stained with anti-CD13 antibody (red); *M. tuberculosis* stained with auramine-rhodamine T (green); colocalization of *M. tuberculosis* with membrane-bound CD13 on monocytes (yellow). Each panel showed a representative cell. Arrows point out to the colocalization of CD13 with *M. tuberculosis* (yellow). Scale bar, 2 μm. At 20 min of incubation with monocytes, *M. tuberculosis* was mostly found in the extracellular location with the bacteria partly colocalized with CD13 (top panel). Complete colocalization of *M. tuberculosis* with the surface CD13 could be observed at 30 min of incubation (middle panel). At 60 min of incubation, most *M. tuberculosis* was found inside the monocytes (bottom panel). TB, tuberculosis.

plain nanoparticle aggregates were found to be small, with only occasional acid-fast organisms seen (Fig. 1e). By contrast, CD13-MNP aggregates occurred in large clusters surrounded by and intermingled with abundant acid-fast organisms. These results indicate that soluble CD13 binds *M. tuberculosis*.

To find out whether *M. tuberculosis* interacts with CD13 on the surface of monocytes, *M. tuberculosis* were incubated with monocytes at different time points and examined by confocal microscopy. At 20 min of incubation with monocytes, *M. tuberculosis* was mostly found in the extracellular location with the bacteria partly colocalized with CD13 (Fig. 2, top panel). Complete colocalization of *M. tuberculosis* with the surface CD13 could be found at 30 min of incubation (Fig. 2, middle panel), and the rate of colocalization was $13.1 \pm 4.7\%$ ($n = 3$). At 60 min of incubation, most of *M. tuberculosis* were found inside the monocytes (Fig. 2, bottom panel). Furthermore, we also tested whether CD13 mediates the binding of mycobateria with human macrophages (Appendix S3 in the online supporting information). Hence, CD13,

in both soluble and membrane-bound forms, is capable of binding *M. tuberculosis*. In other words, CD13 may serve as a receptor to bind *M. tuberculosis* onto the monocyte and macrophage surface.

CD13 mediates the internalization of *M. tuberculosis*

To understand whether the expression of CD13 on monocytes is involved in the process of *M. tuberculosis* internalization, cells were pretreated with two clones of monoclonal antibodies against CD13, WM15 and WM47. Both decrease CD13 expression, but WM15 also inhibits the aminopeptidase activity.[13,14] To explore whether mycobacterial internalization was mediated through CD13, monocytes pretreated with WM15 and WM47 were then incubated with *M. tuberculosis* for 24 h. Our results show that the ratio of *M. tuberculosis*-positive monocytes was significantly reduced by the treatment of 10 μg/mL WM15 ($72.1 \pm 4.9\%$, $P = 0.0046$) or 10 μg/mL WM47 ($66.2 \pm 5.3\%$, $P = 0.003$) as compared
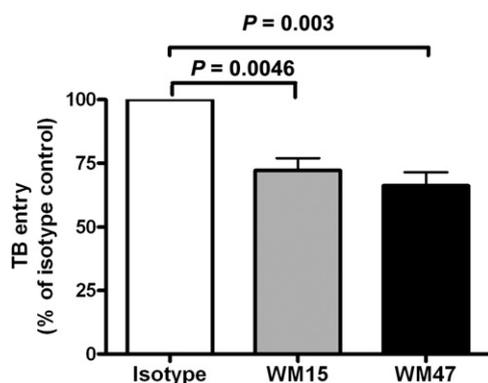
Figure 3 CD13 mediates the internalization of *Mycobacterium tuberculosis*. Monocytes pretreated with WM15 and WM47 were then incubated with *M. tuberculosis* for 24 h, intracellular quantities of *M. tuberculosis* were measured by staining permeabilized cells with fluorescein isothiocyanate-conjugated anti-*M. tuberculosis* antibody. The percentage of intracellular *M. tuberculosis* was measured relative to the isotype control (100%) by flow cytometry. Data represented means ± standard error of the mean (*n* = 5). The ratio of *M. tuberculosis*-positive monocytes was significantly reduced by the treatment of 10 μg/mL WM15 (72.1 ± 4.9%, *P* = 0.0046) or 10 μg/mL WM47 (66.2 ± 5.3%, *P* = 0.003) as compared with isotype control. TB, tuberculosis.

with that of isotype control (Fig. 3). As with monocytes, anti-CD13 antibody also reduces the entry of mycobateria into macrophages (Appendix S3 in the online supporting information). Such data support that CD13 is one of the entry receptors on monocytes and macrophages for *M. tuberculosis*.

The role of CD13 in intracellular survival of *M. tuberculosis*

To study the effects of CD13 on intracellular mycobacterial growth, monocytes were infected with *M. tuberculosis* for 1 h followed by 24-h anti-CD13 antibody treatment. After 3-week culture from the monocyte lysates, the CFU counts of *M. tuberculosis* with WM47 (2635 ± 430, *P* = 0.0002) as well as WM15 (3248 ± 322, *P* = 0.049) treatment were significantly less than that with isotype control (3827 ± 483) (Fig. 4). Our data showed that WM47 was superior in suppressing intracellular bacterial growth compared with WM15 (*P* = 0.0324). Collectively, treatment with anti-CD13 antibodies could reduce not only the entry of *M. tuberculosis* into monocytes but also the survival numbers of intracellular bacteria. These findings suggest that CD13 may facilitate the internalization of *M. tuberculosis* into monocytes as well as modulate intracellular survival of the organisms.

DISCUSSION

The current study employing recombinant soluble CD13, nanoparticle-bound CD13 and membrane-bound CD13 provides evidences that CD13 serves as a receptor on monocytes to bind *M. tuberculosis*.
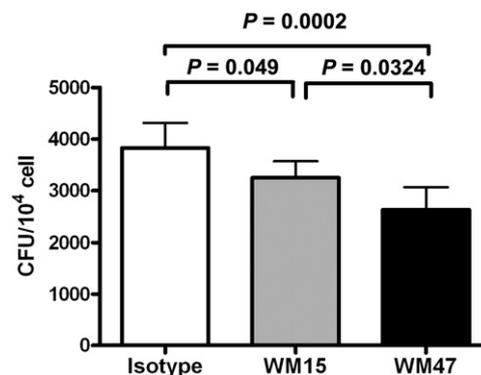
Figure 4 Intracellular survival of *Mycobacterium tuberculosis*. Monocytes were infected with *M. tuberculosis* for 1 h and then post-treated with isotype, or WM15 or WM47 for 24 h. The lysates were cultured for *M. tuberculosis* on 7H11 agar for 3 weeks, after which colony forming units (CFU) were counted. Bars represented the CFU numbers as means ± standard error of the mean (*n* = 13). The mean CFU of *M. tuberculosis* with WM47 (2635 ± 430, *P* = 0.0002) as well as WM15 (3248 ± 322, *P* = 0.049) treatment were significantly less than that with isotype control (3827 ± 483).

However, internalization of *M. tuberculosis* is evidently not dependent on the enzymatic activity CD13, even though this activity is considered an essential biological function of the receptor.[15] Similar results have been reported for *in vitro* experiments with a human coronavirus and cytomegalovirus infection,[16,17] showing that CD13-mediated uptake of virus was not dependent on its enzymatic activity.

We also observed that soluble CD13 nanoparticles bound about 6% of the mycobacteria in solution, but treatment of monocytes with anti-CD13 antibodies led to a 30% reduction in the internalization of *M. tuberculosis*. *M. tuberculosis* possesses numerous dissimilar ligands on its surface and is therefore likely to engage multiple receptors on phagocytes,[18] including complement receptor 3,[19] mannose receptor,[20] surfactant protein A,[21] class A scavenger receptors on monocytes/macrophages[22] and dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin.[23] Most of these phagocyte receptors are raft-associated and play major or auxiliary roles in the process of binding, phagocyting and transporting *M. tuberculosis* into the cell.

It has been suggested that the receptors involved in phagocytic entry of the bacilli have a major influence on the pathogens' intracellular survival.[24] For example, complement receptor 3-mediated phagocytosis does not result in the same degree of inflammatory response associated with the invasion of macrophages by *M. leprae*.[25] Internalization of *M. tuberculosis* via the mannose receptor generates a negative signal delivered through the mannose receptor[26] and inhibits the phagosome-lysosome fusion.[27] The use of these entry points therefore apparently confers a survival benefit. Nevertheless, Zimmerli *et al*. found that blocking these two particular receptors with specific antibody did not alter the survival and growth of *M. tuberculosis* in human macrophages.[22] Our

data showed a somewhat different outcome because treatment with two dissimilar anti-CD13 antibodies affected not only the internalization but also intracellular survival of *M. tuberculosis* in monocytes, particularly the WM47 antibody. This implies that WM47-specific epitope on CD13 may be responsible for reducing intracellular *M. tuberculosis* survival. It has been shown that cross-linking CD13 with a defined clone of anti-CD13 antibodies induces cell activation, including mitogen-activated protein kinase phosphorylation, calcium-fluxing and homotypic aggregation of monocytes in an epitope-dependent way.[28–30] It seems likely that a series of interactions provoked by the treatment with anti-CD13 may link to intracellular growth inhibition of *M. tuberculosis* in monocytes.

To examine mechanisms underlying CD13-associated modulation of mycobacterial growth in monocytes, representative bactericidal activities (reactive oxygen species and cytokine production) were examined, but no significant differences were found (Appendix S4 in the online supporting information). One of the strategies used by *M. tuberculosis* to escape from phagosomes and grow within the cytosol of phagocytes is through modulating the phagosome acidification.[31–33] We found that significantly lower pH value and higher number of active lysosomes (pH < 4.8) were observed in cells treated with WM47 but not with isotype control or WM15 (Appendix S5 in the online supporting information). CD13 has been proposed as a possible receptor of the cholesterol absorption inhibitor ezetimibe,[7] raising the possibility that CD13 participates in the uptake of cholesterol, the important nutrient for intracellular *M. tuberculosis* persistence. Further studies are needed to clarify these aspects.

In conclusion, this study highlights a putative role for CD13 as a novel monocyte receptor for *M. tuberculosis*. CD13 is capable of facilitating internalization and intracellular survival of *M. tuberculosis* in human monocytes. This information adds to our growing understanding about how *M. tuberculosis* can infect cells and continue to survive within them over a long period of time—and to develop novel anti-tuberculosis therapies.

Acknowledgement

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 The interaction of *M. tuberculosis* with surface CD13 of macrophages.

Figure S2 The effect of CD13 on microbicidal capacity of monocytes.

Figure S3 The effect of CD13 on phagosomal acidification of monocytes.

Appendix S1 Detailed experimental procedures.

Appendix S2 Method of *M. tuberculosis* preparation and infection.

Appendix S3 The interaction of *M. tuberculosis* with surface CD13 of macrophages.

Appendix S4 The effect of CD13 on microbicidal capacity of monocytes.

Appendix S5 The effect of CD13 on phagosomal acidification of monocytes.