Streptavidin-HRP ELISPOT, and AEC Substrate Set, respectively; BD Biosciences, Franklin Lakes, NJ), according to the manufacturer's instructions. PBMCs (2×10^5) suspended in 200 μl of RPMI-1640 medium (Wako Pure Chemical Industries, Ltd.) containing 10% human AB serum (Sigma-Aldrich, St. Louis, MO) were stimulated in duplicate for 18 h with 1 μg of recombinant proteins (final concentration, 5 μg/ml) in MultiScreen 96-well plates (Millipore, Billerica, MA) precoated with anti-human IFN-γ capture antibody. Purified protein derivative (PPD; final concentration, 5 μg/ml) (Japan BCG Laboratory, Tokyo, Japan) and phytohemagglutinin (PHA; final concentration of 5 μg/ml) (Sigma-Aldrich) were also used as stimulators. The number of spot-forming cells (SFC) was manually counted under a dissecting microscope by two operators unaware of the clinical information. The results are expressed as the SFC count/10^6 cells after subtracting the mean SFC count in the medium control (<20 SFCs/10^6 PBMCs in all subjects). Assays were considered valid if there were at least 250 SFCs/10^6 PBMCs against PHA. The discordant results of SFCs were resolved by consensus between both operators.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as described[20] by using 96-well flat-bottomed half-well plates (Corning Inc., Corning, NY) precoated with DosR regulon-encoded proteins (100 ng/well). After blocking with Block One Blocking solution (Nacalai Tesque, Kyoto, Japan), 100 μl of diluted serum (1:100) with PBS containing 5% Block One Blocking solution and 0.05% Tween-20 (Sigma-Aldrich) was added to each well in duplicate. HRP-conjugated goat anti-human IgG (Invitrogen, Carlsbad, CA) was used as a secondary antibody, and color development was performed with TMB one component HRP microwell substrate (BioFX Laboratories, Owings Mills, MD). The optical density was measured by an ELISA reader (EZS-ABS; IWAKI, Tokyo, Japan), and the result of the negative control was subtracted from each experimental result.

Data analysis

Antigen-specific T-cell responses and serum antibody responses were compared between LTBI group and ATB group by using Mann-Whitney U test for non-parametric comparison. In all analyses, $P < 0.05$ was regarded as significant. All data were analyzed using commercially available software (JMP version 9.0.3; SAS Institute Inc, NC).

RESULTS

Establishment of ELISPOT assay system for antigen-specific T-cell response

We first established an ELISPOT assay and validated the specificity and sensitivity by using PBMCs from study subjects (Table 1). Stimulation with PHA induced >1,000 SFCs/10^6 PBMCs in all subjects (data not shown), indicating no obvious immunosuppression in study participants. As shown in Table S1 and Fig. S1, T-cell responses against ESAT-6 and CFP-10 in LTBI and ATB were significantly stronger than those in HC. Because SFCs against both antigens in HC were <30/10^6 PBMCs, the newly established ELISPOT assay system seemed comparable to the T-SPOT®.*TB* assay (Oxford Immunotec, Abingdon, UK) defining >32 SFCs/10^6 PBMCs to these antigens as positive. In addition, when this cut-off value was applied for the newly established ELISPOT assay system, the results were consistent with those of the QFT test conducted prior to this study.

T-cell responses against DosR regulon-encoded proteins

We next examined the T-cell responses against DosR regulon-encoded proteins by the ELISPOT assay comparing LTBI and ATB. Since we could not prepare some recombinant DosR-proteins in a bacterial expression system employed, we analyzed the immunogenicity of 33 DosR-proteins. As shown in Table S1 and Fig. 1, T-cell responses against some DosR regulon-encoded proteins in LTBI seemed stronger than those in ATB. When the results were statistically analyzed, T-cell responses against six DosR regulon-encoded proteins (Rv570, Rv1996c, Rv2004, Rv2028, Rv2029c, and Rv3133c) were significantly stronger in LTBI than in ATB (Fig. 2). Although some materials from LTBI and ATB showed strong T-cell responses against Rv2031c (Fig. 1 and Table S1), no statistical difference was observed between the groups.

Serum antibody responses against DosR regulon-encoded proteins

The antibody responses against DosR regulon-encoded antigens were also examined by ELISA employing additional 16 TB patients to the subjects for ELISPOT analysis (Table 2). However, the results were different from those of T-cell responses, showing no stronger antigen-specific antibody response in LTBI than in ATB (Table S2 and Fig. 3). In contrast, antibody responses to five antigens (Rv0080, Rv1738, Rv2007c, Rv2031c and Rv2032) were stronger in ATB than in LTBI (Fig. 4).

DISCUSSION

DosR regulon-encoded proteins are considered to be good targets of novel vaccines to prevent the reactivation of *Mtb* because of their unique expression profiles and functions in dormant *Mtb*,[8-10] and immune responses to the proteins have been studied in humans[11-16] and mice.[21, 22] In the present study, we found that human T-cell responses to at least six DosR regulon-encoded proteins were stronger in LTBI than in ATB in a Japanese population. Leyten *et al.* showed that cumulative T-cell responses to DosR regulon-encoded antigens were stronger in tuberculin skin test (TST)-positive healthy individuals than in ATB.[11] T-cell responses to PPD in LTBI were also reported to be stronger than in ATB.[23, 24] Although the populations of TST-positive and QFT-positive individuals are not necessarily the same, these observations together imply that immune responses to DosR regulon-encoded antigens, as well as whole TB antigens, might contribute to natural protection against dormant *Mtb* and that the lack or decrease of the responses might be associated with development of active TB. Therefore, the six DosR regulon-encoded proteins effectively inducing T-cell responses in LTBI are considered to be potential candidate antigens to prevent the reactivation of *Mtb*, although the vaccination effects should be confirmed by animal experiments.

Human T-cell responses to individual DosR regulon-encoded proteins in LTBI and/or ATB have also been reported by some groups.[11, 13, 16] Leyten *et al.* showed that Rv1733c, Rv2029c, Rv2627c, and Rv2628 induced strong IFN-γ responses in TST-positive individuals.[11] Black *et al.* showed that four DosR regulon-encoded proteins (Rv0080, Rv1733c, Rv1735c, and Rv1737c) were recognized most frequently by T-cells from TST-positive and/or ESAT-6/CFP10-positive individuals in three high-burden African countries.[13] Goletti *et al.* reported that IFN-γ response to Rv2628 might distinguish recent infection from remote infection.[16] However, the results including the present one are somewhat varied, which might stem from the differences in ethnic backgrounds, bacterial prevalence, BCG vaccination history, and detection methods, as well as genetic differences in local pathogens.

Human T-cell response to an antigen is restricted by HLA, and the distribution profiles of HLA alleles differ by ethnic groups. Therefore, differences in genetic backgrounds (European vs. African vs. Asian) seem to be a cause of the difference in immunogenicity. Bacterial prevalence may also affect the T-cell responses to DosR antigens. Rv2031c (also termed HspX or α-crystallin) was reported to be more strongly recognized by T-cells from LTBI and/or ATB than by those from HC.[11, 24-26] T-cell responses to Rv2031c in ATB are stronger than in LTBI in a low-burden country (the Netherlands), whereas those in LTBI are stronger in ATB in high-burden countries (African countries).[25] In the present study, although some strong responders to Rv2031c

were preferentially found in ATB, there was no statistical difference in the T-cell responses to Rv2031c as a whole between ATB and LTBI in Japan, an intermediate-burden country. Rv2031c was reported to be required for the maintenance of long-term viability during latent infection and replication during initial infection of *Mtb*.[27] Together with the reports demonstrating that vaccination with Rv2031c induced protective immunity in animal studies, Rv2031 seems to be one of the better target antigens for a novel vaccine(s),[28, 29] although racial and/or environmental factors may affect the immune responses. BCG vaccination also seems to affect the results, because DosR regulon genes are highly conserved between *Mtb* and BCG.[14] However, previous reports showed that BCG vaccination did not induce immune responses to DosR regulon-encoded antigens,[12, 23, 26] and we also have similar results (Fig. S2). In addition to the factors described above, differences in the detection methods might affect the results. Previous studies mainly employed IFN-γ ELISA with a long-term culture (approximately 1 week), which is more sensitive than a short-term cultured assay,[30] and the results reflect the entire amount of IFN-γ produced in the culture supernatant, but not the actual number of antigen-specific T-cells. In the present study, we employed a short-term ELISPOT assay, which reflects the number of antigen-specific T-cells and is more sensitive for low-level responses than other assays.[31, 32] A multi-color flow cytometric analysis may be the best way to investigate the status of antigen-specific T-cells such as memory/effector, cytokine profiles, and gene expression.[33] However, *ex vivo* multi-color flow cytometric analysis requires a large amount of blood, because the number of T-cells specific for DosR regulon-encoded antigen is low.[15]

In the present study, we also analyzed serum IgG responses against DosR regulon-encoded antigens. However, the results were not consistent with those of the ELISPOT assay. In contrast to T-cell-responses, antibody responses to five antigens were found to be stronger in ATB than in LTBI. We and others reported such discordance between T-cell and antibody immune responses to DosR-encoded antigens in DNA-vaccinated mice,[21, 22] but this may reflect the immunogenicity of individual antigens. In human, accumulating evidence suggests that many antibody responses reflect bacillary burden.[34, 35] Therefore, the five antigens invoking stronger antibody responses in ATB than in LTBI might also be produced in ATB, and the antibody responses to those might reflect the bacterial number in the body. If so, antibody responses to these antigens might be applicable for a biomarker to distinguish these groups, although a larger-scale study is necessary.

In conclusion, six DosR regulon-encoded proteins were found to induce stronger T-cell responses in LTBI than in ATB and are considered potential candidate antigens

for establishing novel vaccines to control *Mtb* reactivation in Japan.

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The authors state that they have no conflicts of interest to declare.

H.H., K.T., Y.Y., S.S., M.U., T.N., and Y.K. designed research; H.H., K.T., Y.Y., S.S., M.U., T.N., and Y.K. performed research; H.H., S.M., H.H, T.F., D.H., N.I., T.S., and K.C. contributed acquisition of data; H.H., K.T., S.M., H.H, T.F., D.H., N.I., T.S., and K.C. analyzed data; and H.H. and K.T. wrote the paper.

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Table 1. Characteristics of study participants for T-cell responses (ELISPOT).

	LTBI[a]	ATB[b]	Total
Number of participants	14	12	26
Age, median year (range)	71 (33-84)	78 (33-97)	75 (33-97)
Sex, Male (%)	10 (71.4)	2 (16.7)	12 (46)
Recent exposure[c], yes (%)	3 (21.4)	0 (0)	3 (11.5)
BCG vaccination[d], yes (%)	11 (78.6)	9 (75.0)	20 (76.9)

[a]Individuals with latent TB infection who were positive for QFT but had no clinical evidence of active TB.

[b]Patients with active TB confirmed by microbiological examination.

[c]History of contact with active TB patient within 6 months.

[d]Vaccination with *Mycobacterium bovis* bacillus Calmette-Guérin.

Table 2. Characteristics of study participants for antibody titration (ELISA).

	LTBI[a]	ATB[b]	Total
Number of participants	14	28	42
Age, median year (range)	71 (33-84)	81.5 (33-97)	73 (33-97)
Sex, Male (%)	10 (71.4)	14 (50.0)	24 (57.1)
Recent exposure[c], yes (%)	3 (21.4)	0 (0)	3 (7.1)
BCG vaccination[d], yes (%)	11 (78.6)	17 (60.7)	28 (66.7)

[a]Individuals with latent TB infection who were positive for QFT but had no clinical evidence of active TB.

[b]Patients with active TB confirmed by microbiological examination.

[c]History of contact with active TB patient within 6 months.

[d]Vaccination with *Mycobacterium bovis* bacillus Calmette-Guérin.

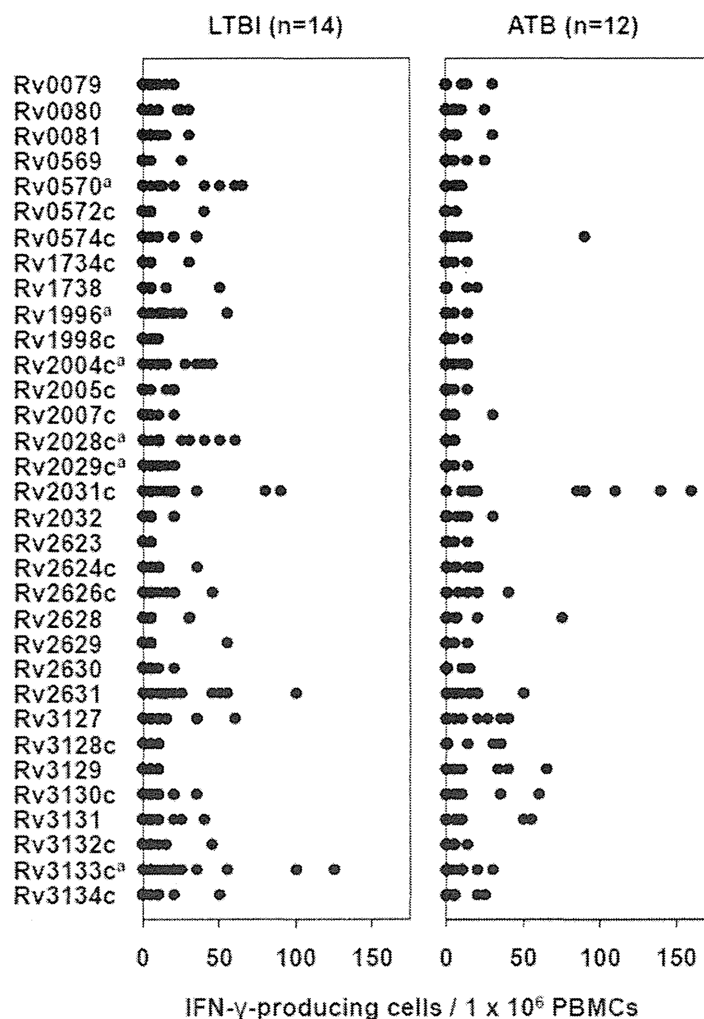

Fig. 1. T-cell responses to individual 33 DosR regulon-encoded antigens.

The number of IFN-γ-producing cells in response to 33 DosR regulon-encoded antigens was measured by ELISPOT assay. PBMCs (2×10^5) suspended in 200 μl of RPMI-1640 medium containing 10% human AB serum were stimulated in duplicate for 18 h with 1 μg of recombinant proteins (final concentration, 5 μg/ml) in MultiScreen 96-well plates precoated with anti-human IFN-γ capture antibody. The number of spot-forming cells (SFC) was manually counted under a dissecting microscope by two operators unaware of the clinical information. The results are expressed as the SFC count/10^6 cells after subtracting the mean SFC count in the medium control (<20 SFCs/10^6 PBMCs in all subjects). LTBI, individual with latent TB infection (for details, see Table 1); ATB, active pulmonary TB patients (for details, see Table 1).

[a] T-cell responses which were significantly stronger in LTBI than in ATB. Analyses were conducted by using Mann-Whitney U test for non-parametric comparison, and $P < 0.05$ was regarded as significant.

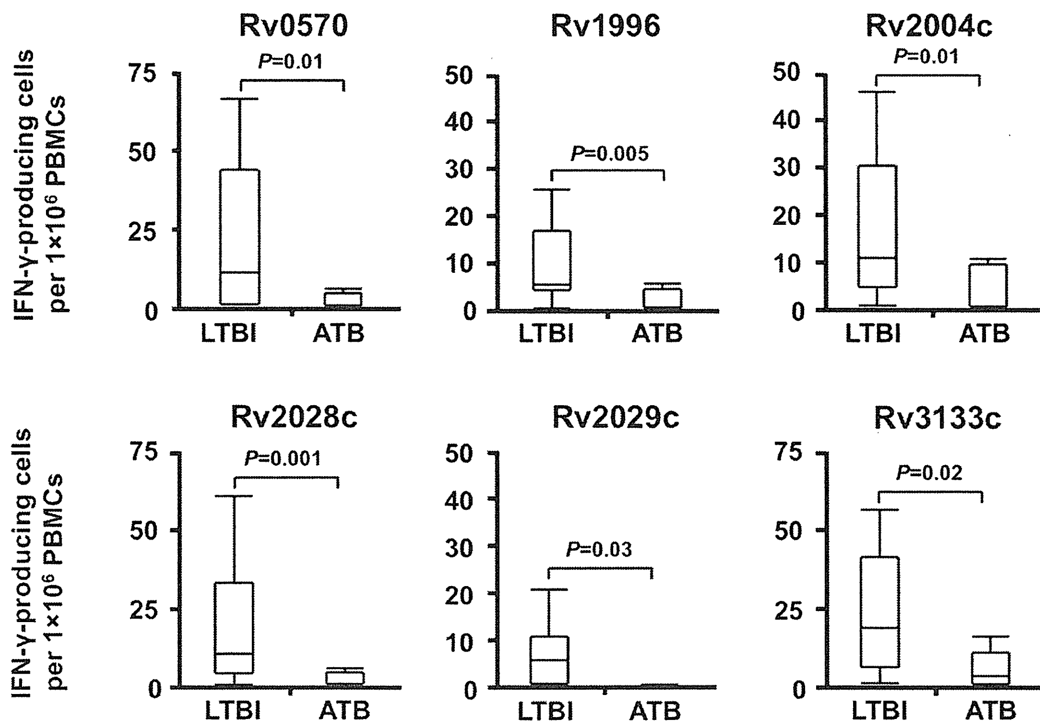

Fig. 2. T-cell responses to DosR regulon-encoded antigens with stronger immunogenicity in individuals with latent TB infection (LTBI) than in active pulmonary TB patients (ATB).

The number of IFN-γ-producing cells in response to 33 DosR regulon-encoded antigens was measured by ELISPOT assay. PBMCs (2 x 10^5) suspended in 200 μl of RPMI-1640 medium containing 10% human AB serum were stimulated in duplicate for 18 h with 1 μg of recombinant proteins (final concentration, 5 μg/ml) in MultiScreen 96-well plates precoated with anti-human IFN-γ capture antibody. The number of spot-forming cells (SFC) was manually counted under a dissecting microscope by two operators unaware of the clinical information. The results are expressed as the SFC count/10^6 cells after subtracting the mean SFC count in the medium control (<20 SFCs/10^6 PBMCs in all subjects). The results are shown by box-and-whisker plots. The line within the box shows the median value of spot-forming cells. The lower and upper boundaries of the box indicate the 25th and 75th percentile, respectively. The whiskers represent the lowest and highest data still within 1.5 inter-quartile ranges of the lower and upper quartiles, respectively. T-cell responses that were significantly higher in LTBI than in ATB are shown. Antigen-specific T-cell responses were compared between LTBI group and ATB group by using Mann-Whitney U test for non-parametric comparison. In all analyses, $P < 0.05$ was regarded as significant.

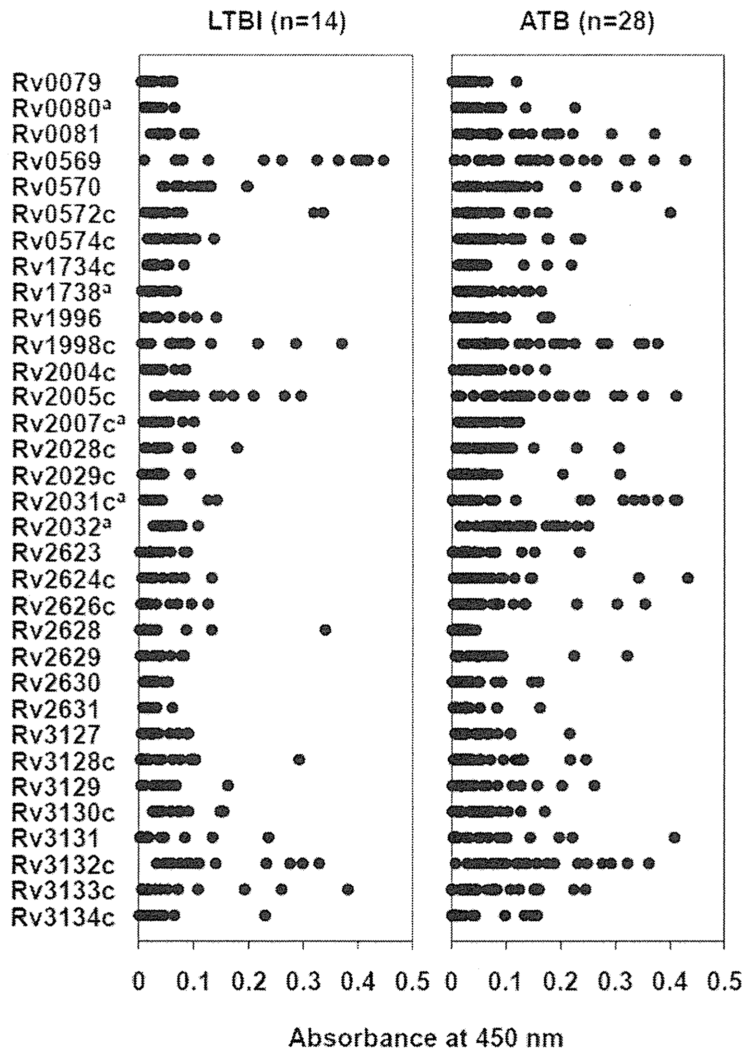

Fig. 3. Serum antibody responses against 33 DosR regulon-encoded antigens.

Serum IgG responses were measured by ELISA using 1:100 diluted sera and HRP-labeled polyclonal anti-human IgG antibodies. LTBI, individual with latent TB infection (for details, see Table 2); ATB, active pulmonary TB patients (for details, see Table 2). [a] Serum IgG responses which were significantly stronger in LTBI than in ATB. Analyses were conducted by using Mann-Whitney U test for non-parametric comparison, and $P < 0.05$ was regarded as significant.

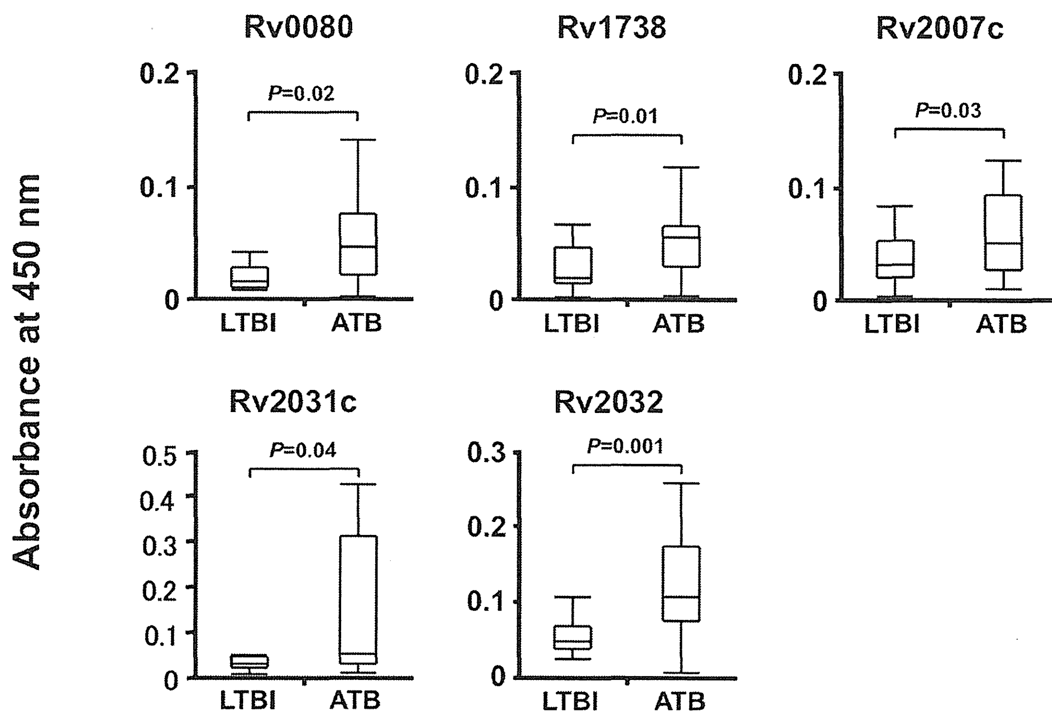

Fig. 4. Serum antibody responses against DosR regulon-encoded antigens with stronger immunogenicity in ATB than in LTBI.

Serum IgG responses were measured by ELISA using 1:100 diluted sera and HRP-labeled polyclonal anti-human IgG antibodies. IgG responses that were significantly higher in ATB than in LTBI are shown. Serum antibody responses were compared between LTBI group and ATB group by using Mann-Whitney U test for non-parametric comparison. In all analyses, $P < 0.05$ was regarded as significant.

Table S1. T-cell responses to DosR regulon-encoded antigens.

Antigen name	Gene	IFN-γ-producing cells (per 1×10^6 PBMCs)[a]		
		LTBI[b]	ATB[b]	P value[c]
Rv0079		5 (0-20)	0 (0-30)	0.08
Rv0080		5 (0-30)	2.5(0-25)	0.31
Rv0081		5 (0-30)	0 (0-30)	0.20
Rv0569		0 (0-25)	0 (0-25)	0.28
Rv0570	*nrdZ*	10 (0-65)	0 (0-5)	0.01[f]
Rv0572c		0 (0-40)	0 (0-5)	0.10
Rv0574c		7.5 (0-35)	0 (0-90)	0.18
Rv1734c		0 (0-30)	0 (0-10)	0.61
Rv1738		2.5 (0-50)	0 (0-20)	0.15
Rv1996		5 (0-55)	0 (0-10)	0.005[f]
Rv1998c		0 (0-10)	0 (0-10)	0.51
Rv2004c		3.75 (0-45)	0 (0-10)	0.01[f]
Rv2005c		0 (0-20)	0 (0-10)	0.38
Rv2007c	*fdxA*	0 (0-20)	0 (0-30)	0.59
Rv2028c		10 (0-60)	0 (0-5)	0.001[f]
Rv2029c	*pfkB*	5 (0-20)	0 (0-10)	0.03[f]
Rv2031c	*Acr*	16.3 (0-90)	51.3 (0-140)	0.15
Rv2032	*Acg*	2.5 (0-20)	0 (0-30)	0.60
Rv2623	*TB31.7*	0 (0-5)	0 (0-10)	0.45
Rv2624c		2.5 (0-35)	2.5 (0-20)	0.80
Rv2626c		5 (0-45)	0 (0-40)	0.55
Rv2628		0 (0-30)	0 (0-75)	0.72
Rv2629		0 (0-55)	0 (0-10)	0.59
Rv2630		0 (0-20)	0 (0-15)	0.18
Rv2631		15 (0-100)	7.5 (0-50)	0.11
Rv3127		10 (0-60)	2.5 (0-40)	0.30
Rv3128c		0 (0-10)	0 (0-35)	0.90
Rv3129		5 (0-10)	2.5 (0-65)	0.98
Rv3130c		5 (0-35)	0 (0-60)	0.19
Rv3131		5 (0-40)	0 (0-55)	0.34
Rv3132c	*devS*	2.5 (0-45)	0 (0-10)	0.31
Rv3133c	*dosR*	17.5 (0-125)	2.5 (0-30)	0.02[f]
Rv3134c		0 (0-50)	2.5 (0-25)	0.98
Rv3875 (ESAT-6[d])	*esxA*	100 (20-280)	90 (15-620)	0.66
Rv3874 (CFP-10[e])	*esxB*	42.5 (10-505)	45 (20-160)	0.92

[a]Median (range). [b]See Table 1.

[c]T-cell responses (IFN-g-producing cells) were compared between LTBI and ATB group using Mann-Whitney U test for non-parametric comparison.

[d]6 kDa early secretory antigenic target of *Mycobacterium tuberculosis*.

[e]10 kDa culture filtrate antigen.

[f]Statistically significant, P<0.05.

Table S2. Serum IgG titers for DosR regulon-encoded antigens.

		Titer of antigen specific antibody (ELISA)[a]		
Antigen name	**Gene**	**LTBI[b]**	**ATB[b]**	**P value[c]**
Rv0079		0.02 (0.002-0.06)	0.02 (0-0.12)	0.99
Rv0080		0.02 (0.01-0.06)	0.04 (0-0.23)	0.02[d]
Rv0081		0.05 (0.02-0.10)	0.08 (0.01-0.37)	0.07
Rv0569		0.24 (0-0.45)	0.14 (0-0.43)	0.19
Rv0570	*nrdZ*	0.10 (0.04-0.20)	0.10 (0.01-0.34)	0.49
Rv0572c		0.04 (0.01-0.34)	0.05 (0.01-0.40)	0.29
Rv0574c		0.06 (0.01-0.14)	0.07 (0.01-0.24)	0.37
Rv1734c		0.03 (0.01-0.08)	0.03 (0-0.22)	0.70
Rv1738		0.02 (0-0.07)	0.05 (0-0.16)	0.01[d]
Rv1996		0.03 (0.01-0.14)	0.05 (0-0.18)	0.77
Rv1998c		0.08 (0-0.37)	0.09 (0-0.38)	0.33
Rv2004c		0.04 (0.01-0.08)	0.04 (0-0.17)	0.35
Rv2005c		0.09 (0.03-0.29)	0.12 (0.01-0.41)	0.47
Rv2007c	*fdxA*	0.03 (0-0.10)	0.05 (0.01-0.12)	0.03[d]
Rv2028c		0.04 (0.01-0.18)	0.05 (0-0.31)	0.47
Rv2029c	*pfkB*	0.03 (0-0.09)	0.03 (0-0.31)	0.26
Rv2031c	*acr*	0.02 (0-0.14)	0.04 (0-0.41)	0.04[d]
Rv2032	*acg*	0.05 (0.02-0.11)	0.10 (0-0.25)	0.001[d]
Rv2623	*TB31.7*	0.02 (0-0.09)	0.03 (0-0.23)	0.46
Rv2624c		0.03 (0-0.13)	0.05 (0-0.43)	0.19
Rv2626c		0.01 (0-0.12)	0.04 (0-0.35)	0.05
Rv2628		0.02 (0-0.34)	0.02 (0-0.04)	0.58
Rv2629		0.03 (0-0.08)	0.05 (0-0.32)	0.16
Rv2630		0.02 (0.01-0.05)	0.02 (0-0.16)	0.80
Rv2631		0.02 (0-0.06)	0.02 (0-0.16)	0.58
Rv3127		0.02 (0-0.09)	0.03 (0-0.22)	0.96
Rv3128c		0.04 (0-0.29)	0.03 (0-0.25)	0.93
Rv3129		0.03 (0-0.16)	0.03 (0-0.26)	0.26
Rv3130c		0.06 (0.02-0.15)	0.05 (0-0.17)	0.46
Rv3131		0.02 (0-0.24)	0.02 (0-0.41)	0.98
Rv3132c	*devS*	0.10 (0.03-0.33)	0.12 (0.01-0.36)	0.93
Rv3133c	*dosR*	0.03 (0-0.38)	0.04 (0-0.25)	0.93
Rv3134c		0.01 (0-0.23)	0.01 (0-0.16)	0.75

[a]Median absorbance at 450 nm (range). Also see Meterials and Methods.

[b]See Table 2.

[c]Serum IgG titers for DosR regulon-encoded antigens were compared between LTBI and ATB group using Mann-Whitney U test for non-parametric comparison.

[d]Statistically significant, $P<0.05$.

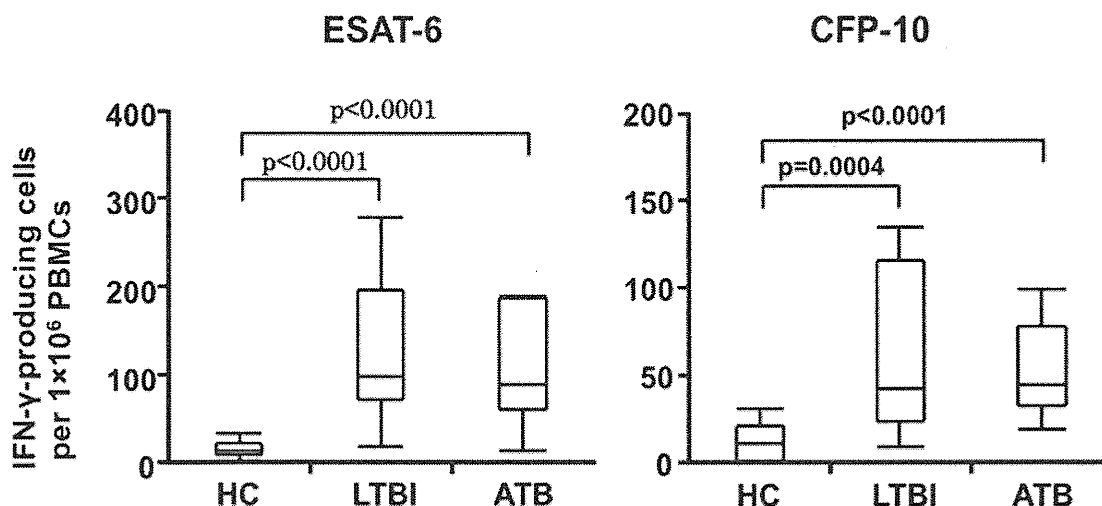

Fig. S1. T-cell responses to ESAT-6 and CFP-10.

The number of IFN-γ-producing cells in response to the antigens was measured by ELISPOT assay, and the results are shown by box-and-whisker plots. The line within the box shows the median value of spot-forming cells. The lower and upper boundaries of the box indicate the 25th and 75th percentile, respectively. The whiskers represent the lowest and highest data still within 1.5 inter-quartile ranges of the lower and upper quartiles, respectively. In addition to individuals with latent TB infection (LTBI) and active pulmonary TB patients (ATB) described in Materials and methods, 11 healthy controls (HC) were recruited. All HC were negative for QFT and had no history of close TB exposure, anti-TB treatment, and prior active TB. Their chest radiography did not show any abnormal findings, and they did not have any respiratory or systemic symptoms. Antigen-specific T-cell responses were compared between three groups (HC, LTBI, and ATB) by using Steel-Dwass test for non-parametric multiple comparison. In all analyses, $P < 0.05$ was regarded as significant. ESAT-6, 6-kDa early secretory antigenic target of *Mycobacterium tuberculosis*; CFP-10, 10-kDa culture filtrate antigen.

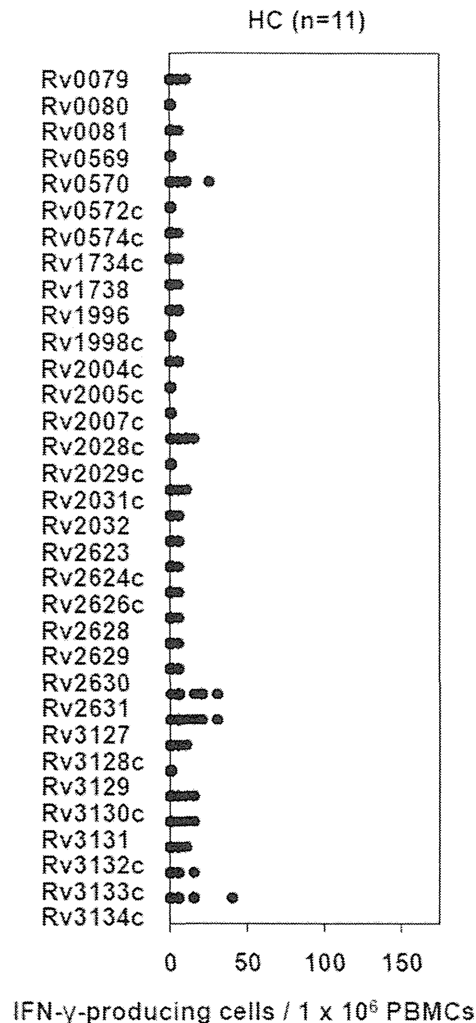

Fig. S2. T-cell responses to individual 33 DosR regulon-encoded antigens in HC.

The number of IFN-γ-producing cells in response to 33 DosR regulon-encoded antigens was measured by ELISPOT assay. PBMCs (2 x 10^5) suspended in 200 μl of RPMI-1640 medium containing 10% human AB serum were stimulated in duplicate for 18 h with 1 μg of recombinant proteins (final concentration, 5 μg/ml) in MultiScreen 96-well plates precoated with anti-human IFN-γ capture antibody. The number of spot-forming cells (SFC) was manually counted under a dissecting microscope by two operators unaware of the clinical information. The results are expressed as the SFC count/10^6 cells after subtracting the mean SFC count in the medium control (<20 SFCs/10^6 PBMCs in all subjects). All HC were negative for QFT and had no history of close TB exposure, anti-TB treatment, and prior active TB. Their chest radiography did not show any abnormal findings, and they did not have any respiratory or systemic symptoms. In the present study, all HC had ever received BCG-vaccination.

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Coronin-1a inhibits autophagosome formation around *Mycobacterium tuberculosis*-containing phagosomes and assists mycobacterial survival in macrophages

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Summary

Mycobacterium tuberculosis **is an intracellular bacterium that can survive within macrophages. Such survival is potentially associated with Coronin-1a (Coro1a). We investigated the mechanism by which Coro1a promotes the survival of *M. tuberculosis* in macrophages and found that autophagy was involved in the inhibition of mycobacterial survival in Coro1a knock-down (KD) macrophages. Fluorescence microscopy and immunoblot analyses revealed that LC3, a representative autophagic protein, was recruited to *M. tuberculosis*-containing phagosomes in Coro1a KD macrophages. Thin-section electron microscopy demonstrated that bacilli were surrounded by the multiple membrane structures in Coro1a KD macrophages. The proportion of LC3-positive mycobacterial phagosomes colocalized with p62/SQSTM1, ubiquitin or LAMP1 increased in Coro1a KD macrophages during infection. These results demonstrate the formation of autophagosomes around *M. tuberculosis* in Coro1a KD macrophages. Phosphorylation of p38 mitogen-activated protein kinase (MAPK) was induced in response to *M. tuberculosis* infection in Coro1a KD macrophages, suggesting that Coro1a blocks the activation of the p38 MAPK pathway involved in autophagosome formation. LC3 recruitment to *M. tuberculosis*-containing phagosomes was also observed in Coro1a KD alveolar or bone marrow-derived macrophages. These results suggest that Coro1a inhibits autophagosome formation in alveolar macrophages, thereby facilitating *M. tuberculosis* survival within the lung.**

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Introduction

Mycobacterium tuberculosis, a causative bacterium of tuberculosis, infects one-third of the world population and causes approximately 1.7 million deaths per year (World Health Organization, 2010). The ability to survive and persist in host macrophages is a major determinant of *M. tuberculosis* pathogenicity (Hingley-Wilson *et al.*, 2003). Studies suggest that this bacterium gains the ability to proliferate within infected macrophages by blocking phagolysosome biogenesis, because mycobacterial phagosomes do not fuse with lysosomal vesicles (Armstrong and Hart, 1971; Clemens and Horwitz, 1995). *M. tuberculosis* can interfere with intracellular membrane trafficking and subsequently cause phagosome maturation arrest in infected macrophages (Vergne *et al.*, 2003; 2004; Deretic *et al.*, 2004; 2006). We recently demonstrated that *M. tuberculosis* modulated the trafficking of Rab GTPases regulating phagosome maturation and that this modulation is relevant to the maturation arrest of mycobacterial phagosomes in macrophages (Seto *et al.*, 2009; 2010; 2011; Sugaya *et al.*, 2011). However, there is currently a lack of direct evidence that phagosome maturation arrest facilitates *M. tuberculosis* survival in macrophages. For example, some mutants generated by transposon mutagenesis survived and persisted within infected macrophages despite the progression of their phagosome maturation (Pethe *et al.*, 2004).

Autophagy is a unique lysosomal degradation pathway for cytoplasmic materials. This process is involved in the innate and adaptive immune systems (Deretic and Levine, 2009). The same process is also involved in the selection and exclusion of intracellular parasites especially in innate immunity and is termed xenophagy (Levine, 2005). *M. tuberculosis* is eliminated from infected macrophages by the induction of autophagy as a consequence of nutrient starvation, drug inducer or interferon-γ (Gutierrez *et al.*, 2004; Singh *et al.*, 2006). Autophagy also potentially controls the intracellular burdens of *M. tuberculosis* in macrophages (Kumar *et al.*, 2010).

These studies suggest that new therapeutic or vaccine regimens against tuberculosis might be successfully developed by targeting strategies that induce autophagy (Jagannath *et al.*, 2009; Yuk *et al.*, 2009; Jo, 2010).

Coronin-1a (Coro1a) is a member of the coronin family associated with F-actin (de Hostos, 1999) and localizes to mycobacterial phagosomes (Ferrari *et al.*, 1999). Gene silencing of Coro1a was found to prevent *M. tuberculosis* survival in macrophages (Jayachandran *et al.*, 2007; 2008; Kumar *et al.*, 2010). These results suggest that *M. tuberculosis* perverts Coro1a function to allow the survival of this bacterium in macrophages (Pieters, 2008). Currently, the survival mechanisms involved in this process are not fully understood. In this study, we found that the depletion of Coro1a promoted autophagosome formation around *M. tuberculosis*-containing phagosomes in macrophages. The molecular mechanisms of Coro1a-mediated *M. tuberculosis* survival are also discussed.

Results

Depletion of Coro1a in macrophages by siRNA prevents M. tuberculosis *survival*

To deplete Coro1a expression in Raw264.7 macrophages, we employed Coro1a-specific small interfering ribonucleic acids (siRNA) and verified the knock-down (KD) efficiency of Coro1a by immunoblot analysis. We designed two sets of the Coro1a-specific siRNA sequences, one of which is based on the report by Jayachandran *et al.* (2007). More than 90% KD efficiency for the Coro1a protein was obtained by transfection with siRNA duplexes (Fig. 1A). We then examined the survival of *M. tuberculosis* in Raw264.7 macrophages transfected with Coro1a-specific siRNA and confirmed the inhibition of *M. tuberculosis* survival in Coro1a KD macrophages (Fig. 1B) (Jayachandran *et al.*, 2007; 2008; Kumar *et al.*, 2010).

Previous studies demonstrated that lysosomes fused with mycobacterial phagosomes in Coro1a-depleted macrophages (Jayachandran *et al.*, 2007; 2008), suggesting that the inhibition of phagolysosomal biogenesis is associated with the Coro1a-dependent survival of *M. tuberculosis*.

In this study, we examined the acidification of mycobacterial phagosomes and their fusion with lysosomes in Coro1a KD macrophages (Fig. 2). Coro1a KD macrophages were infected with DsRed-expressing *M. tuberculosis* for 24 h and examined the acidification of mycobacterial phagosomes and their fusion with lysosomes by staining with LysoTracker and anti-LAMP1 antibody respectively. The proportion of LysoTracker-positive (Fig. 2A and B) and LAMP1-positive (Fig. 2C and D) mycobacterial phagosomes increased in Coro1a KD macrophages. These results suggest that the maturation of mycobacterial phagosomes is promoted in Coro1a KD macrophages.

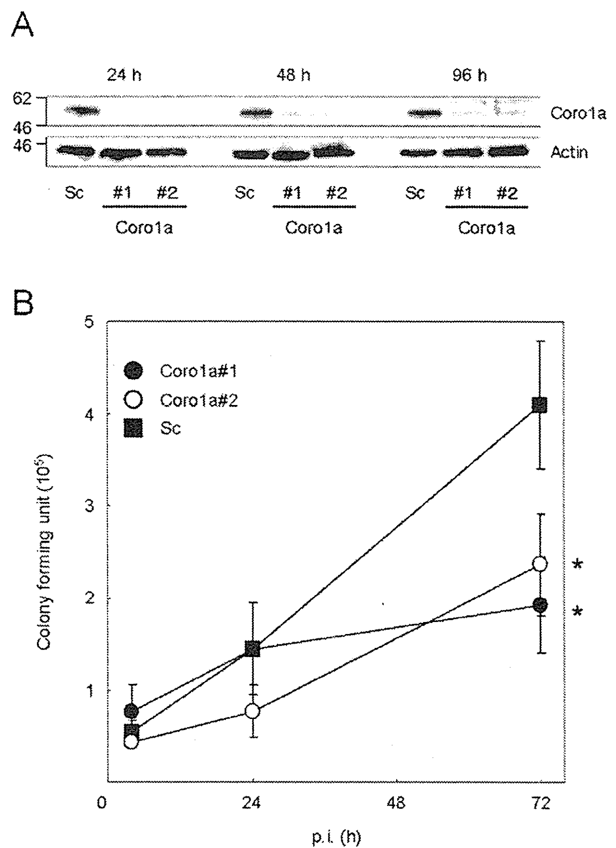

Fig. 1. Survival of *M. tuberculosis* in Coro1a KD macrophages.
A. Immunoblot analysis of Coro1a KD macrophages. Raw264.7 macrophages were transfected with Coro1a-specific or scrambled (Sc) siRNA for 24, 48 and 96 h. Whole-cell lysates from transfected macrophages were subjected to SDS-PAGE, followed by immunoblot analysis using the indicated antibodies.
B. Proliferation of *M. tuberculosis* in Coro1a KD macrophages. Macrophages were transfected with Coro1a or scrambled siRNA for 24 h and then infected with *M. tuberculosis*. The number of viable mycobacteria was determined using a colony-forming unit (cfu) assay at 4, 24 and 72 h post infection (p.i.). The data represent the means and the standard error of the means (SEM) of three independent experiments. The numbers of cfu at 72 h p.i. in macrophages transfected with Coro1a and scrambled siRNA were compared. $^{*}P < 0.05$ (unpaired Student's *t*-test).

Autophagy is involved in the inhibition of M. tuberculosis *survival in Coro1a KD macrophages*

We next focused on the effect that Coro1a depletion had on the induction of autophagy in macrophages infected with *M. tuberculosis*, because autophagy is known to be associated with *M. tuberculosis* eradication (Deretic *et al.*, 2006; 2009; Jo, 2010; Lerena *et al.*, 2010). To investigate the involvement of autophagy, Coro1a KD macrophages were treated with 3-methyladenine (3-MA), an autophagy

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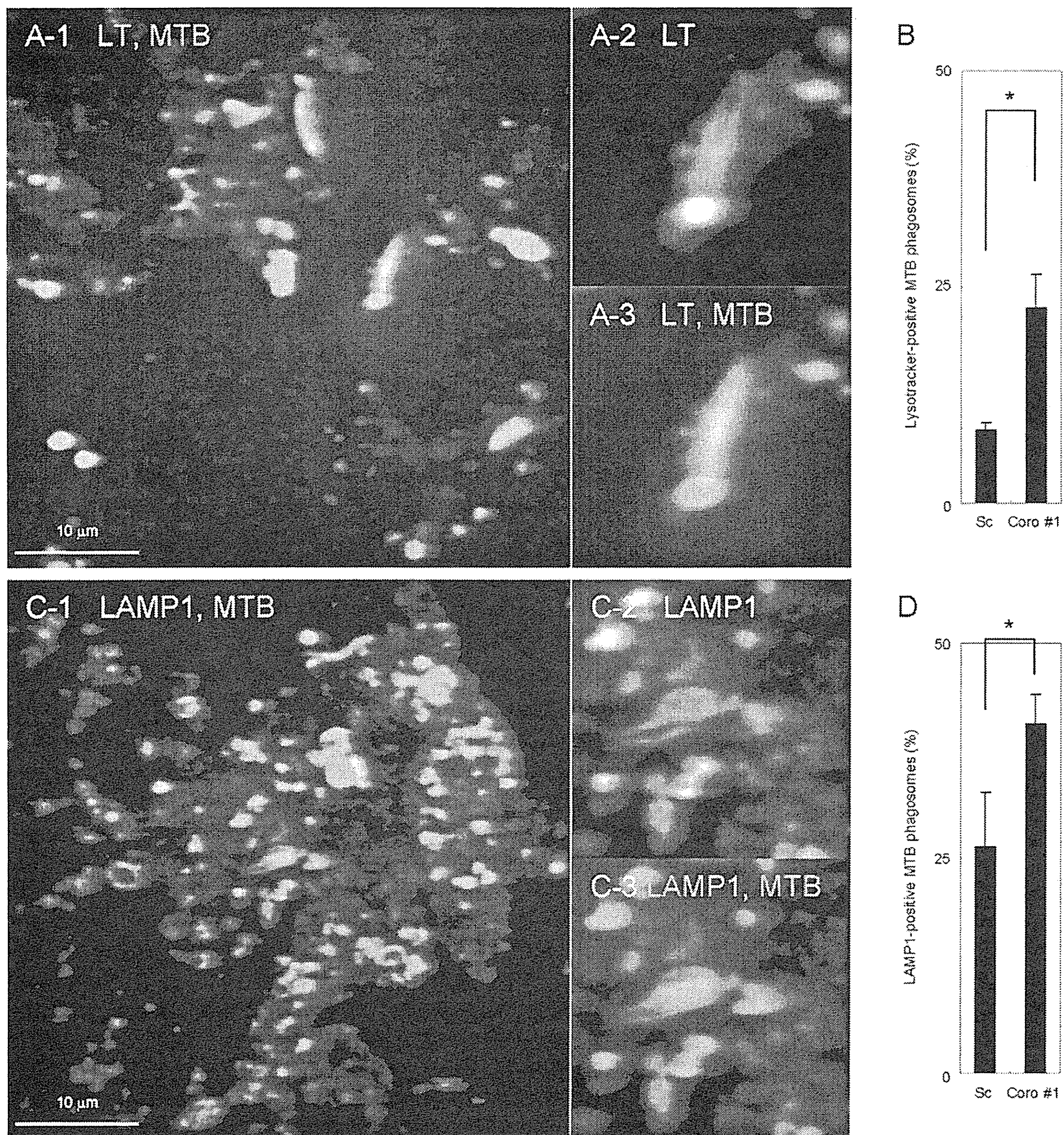

Fig. 2. Maturation of *M. tuberculosis*-containing phagosomes in Coro1a KD macrophages.
A. Acidification of mycobacterial phagosomes in Coro1a KD macrophages. Macrophages transfected with Coro1a-specific siRNA were infected with DsRed-expressing *M. tuberculosis* for 24 h and stained with LysoTracker. Infected macrophages were fixed and observed by laser scanning confocal microscopy (LSCM). Enlarged images of A-1 are represented in A-2 and A-3.
B. The proportion of *M. tuberculosis*-containing phagosomes labelled with LysoTracker in Coro1a KD macrophages. The numbers of LysoTracker-positive *M. tuberculosis*-containing phagosomes in macrophages transfected with Coro1a-specific or scrambled siRNA were counted.
C. LAMP1 localization to mycobacterial phagosomes in Coro1a KD macrophages. Macrophages transfected with Coro1a-specific siRNA were infected with DsRed-expressing *M. tuberculosis* for 24 h. Infected macrophages were stained with anti-LAMP1 antibody and observed by LSCM. Enlarged images of C-1 are represented in C-2 and C-2.
D. The proportion of *M. tuberculosis*-containing phagosomes labelled with anti-LAMP1 antibody in Coro1a KD macrophages. The numbers of LAMP1-positive *M. tuberculosis*-containing phagosomes in macrophages transfected with Coro1a-specific or scrambled siRNA were counted. Data represent the means and the standard deviations (SD) of three independent experiments in which more than 200 phagosomes were counted for each condition. $^{*}P < 0.05$ (unpaired Student's *t*-test); Sc, scrambled; Coro, Coro1a; MTB, *M. tuberculosis*; LT, LysoTracker.