Induction of autophagy through CLEC4E in combination with TLR4: an innovative strategy to restrict the survival of *Mycobacterium tuberculosis*

Susanta Pahari[1,2], Shikha Negi[1], Mohammad Aqdas[1], Eusondia Arnett[2], Larry S. Schlesinger[2], Javed N Agrewala[1,3]*

[1]Immunology Division, CSIR-Institute of Microbial Technology, Chandigarh-160036, India.
[2]Host-Pathogen Interactions Program, Texas Biomedical Research Institute, San Antonio, TX-78227, USA.
[3]Biomedical Engineering Department, Indian Institute of Technology Ropar, Rupnagar-140001, Punjab, India.

*Corresponding author: Javed N Agrewala, Indian Institute of Technology Ropar, Rupnagar-140001, Punjab, India and Immunology Laboratory, CSIR-Institute of Microbial Technology, Chandigarh-160036, India. Email: jagrewala@iitrpr.ac.in

Supplemental material

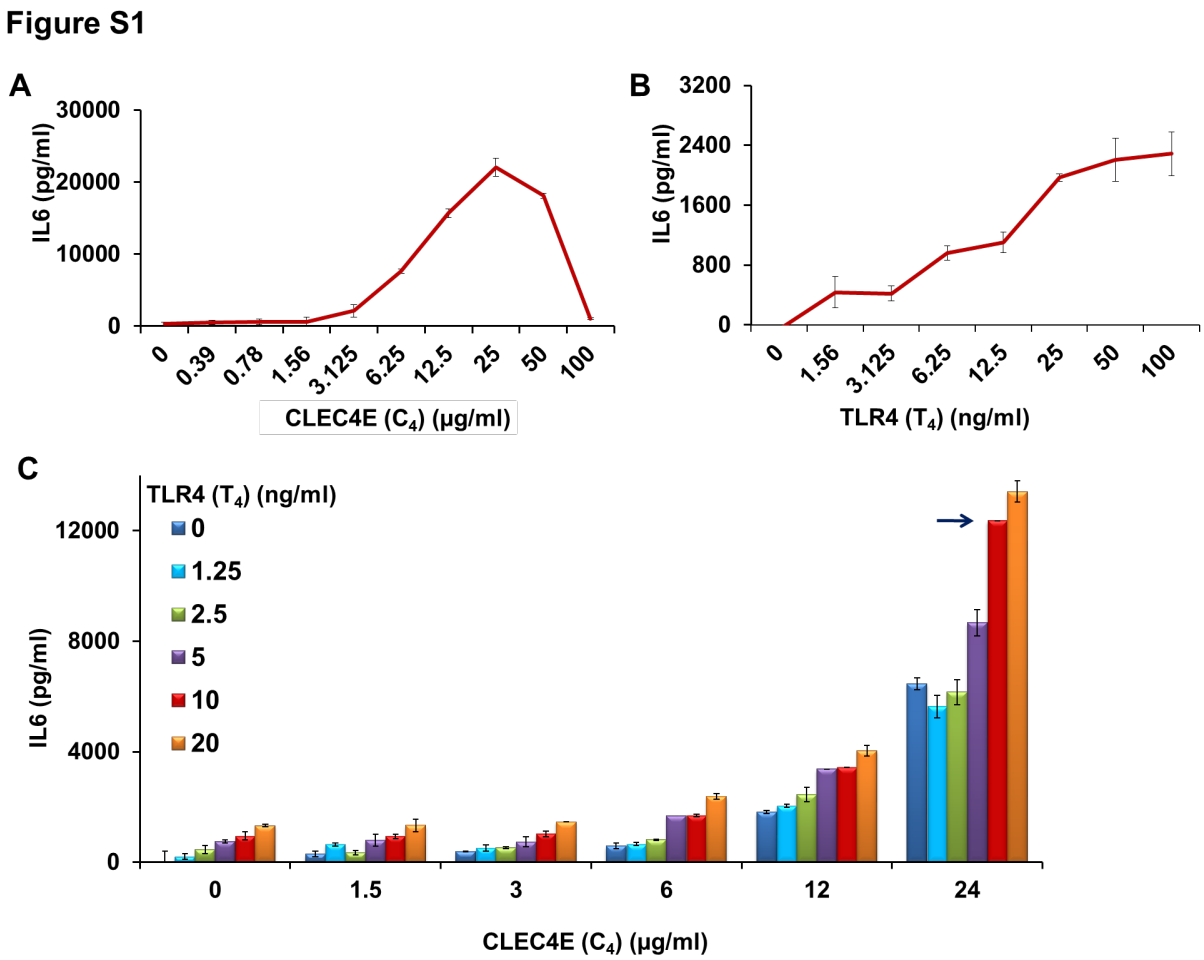

Figure S1. Macrophages stimulated by $C_4.T_4$ showed a dose-dependent increase in the secretion of IL6. BMDMs were stimulated with different concentrations of CLEC4E (C_4) and TLR4 (T_4). After 48 h, the SNs were collected and IL6 was measured following stimulation with (**A**) C_4; (**B**) T_4; or (**C**) $C_4.T_4$. Data are the mean ± SD of 4 wells and are representative of 3 independent experiments.

Figure S2

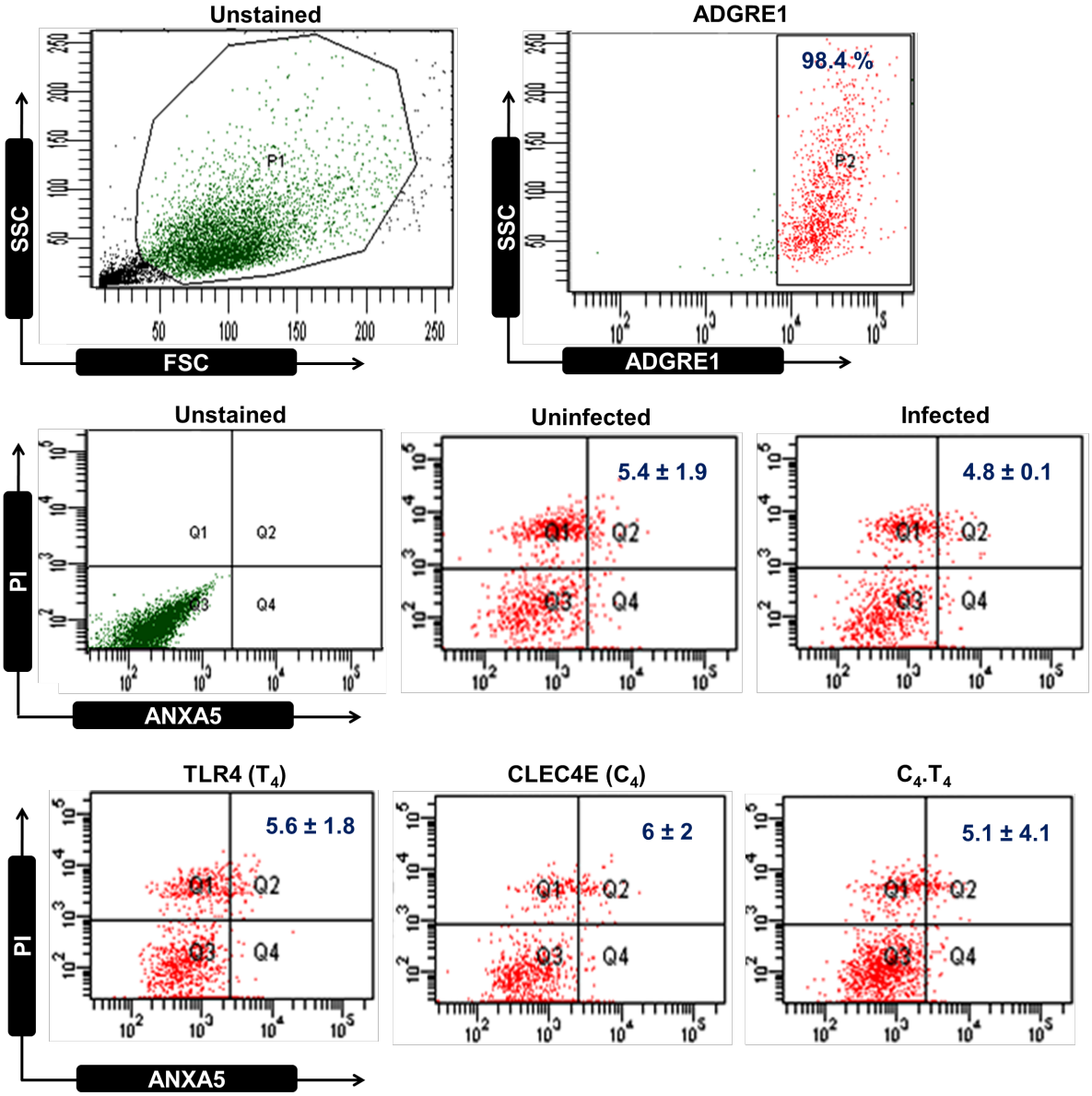

Figure S2. Stimulation with CLEC4E and TLR4 agonists did not affect the viability of *Mtb*-infected macrophages. BMDMs were infected with *Mtb* for 4 h and stimulated with $C_4.T_4$ for 48 h. Cells were then stained with ANXA5/annexin V-FITC followed by propidium iodide (PI) staining to check for the viability of cells. The number in the inset indicates the percentage of ANXA5- and PI-positive cells on ADGRE1/F4/80 gated cell populations. Data are the mean ± SD of 2 wells and are representative of 2 independent experiments.

Figure S3

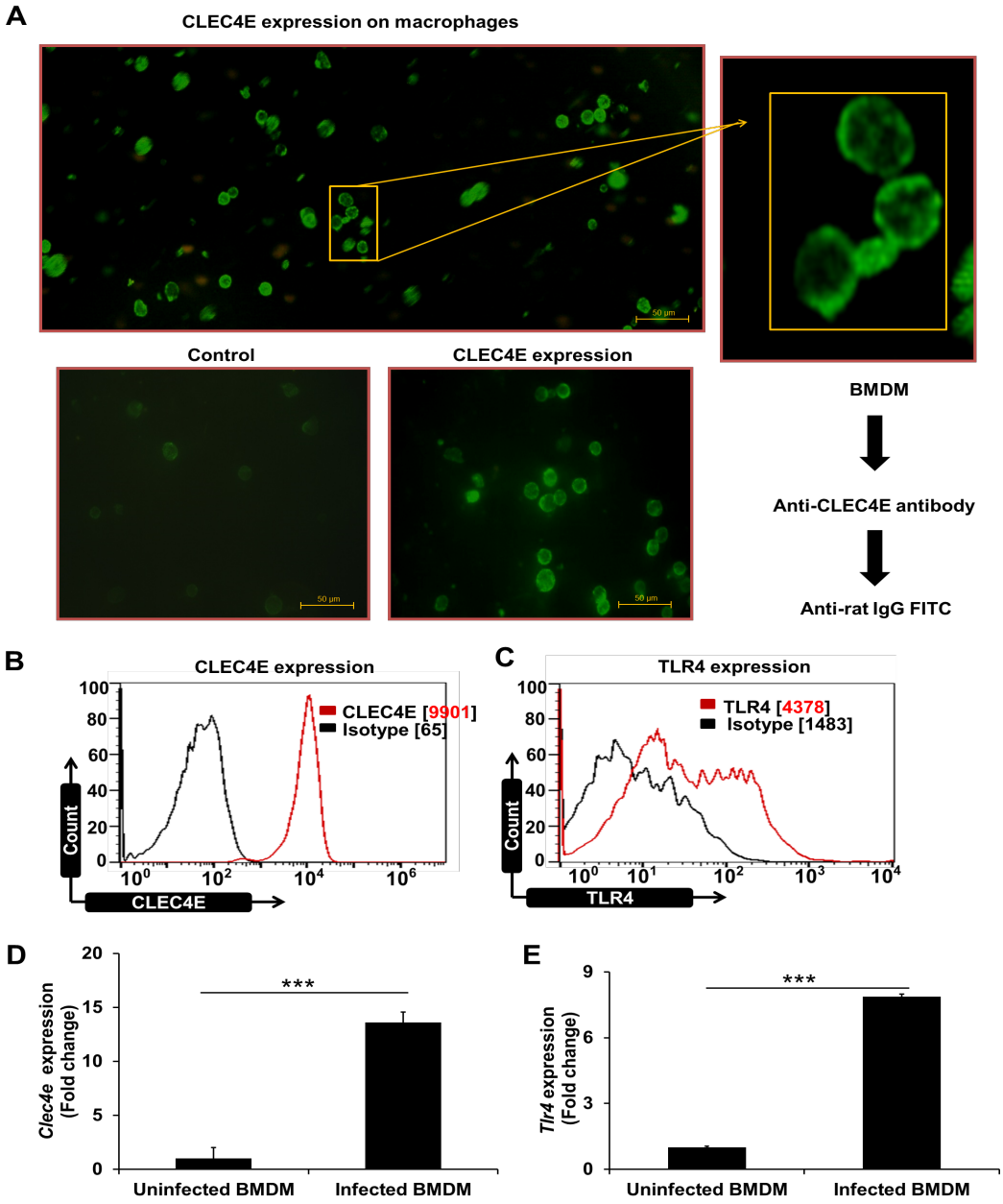

Figure S3. CLEC4E and TLR4 were highly expressed on macrophages. BMDMs were incubated with anti-CLEC4E and anti-TLR4 Abs. CLEC4E and TLR4 expression was confirmed by (**A**) fluorescent microscopy, (**B and C**) flow cytometry, (**D and E**) RT-qPCR. Scale bar: 50 µm and 20X magnification. Data are the mean ± SD of 4 wells and are representative of 3 independent experiments. Data were analyzed by an unpaired Student's 't' test ***p<0.0004.

Figure S4

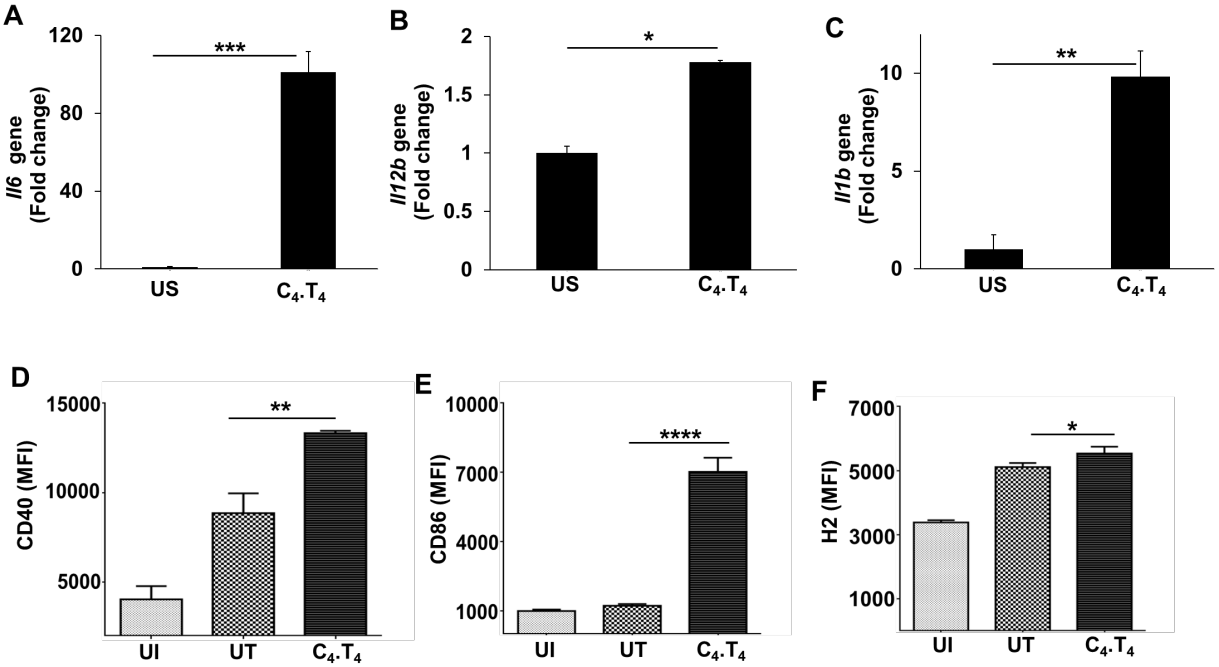

Figure S4. Signaling of macrophages through CLEC4E and TLR4 induced activation and maturation. BMDMs were stimulated with CLEC4E (C_4 [24 µg/ml]) and TLR4 (T_4 [10 ng/ml]) individually or in combination ($C_4.T_4$) for 6 h. RNA was isolated and (**A**) *Il6*; (**B**) *Il12b* and (**C**) *Il1b* gene expression was confirmed by RT-qPCR. Further, BMDMs were infected with *Mtb* for 4 h and treated with $C_4.T_4$ were assessed for the expression of (**D**) CD40; (**E**) CD86 or (**F**) H2/MHC-II on ADGRE1/F4/80 gated cell populations by flow cytometry. Data presents the mean ± SEM of 3 wells and are from 2-3 independent experiments. US, unstimulated; C_4, CLEC4E agonist (TDB); T_4, TLR4 agonist (ultra-pure LPS); UI, uninfected; UT, untreated. Data were analyzed by an unpaired Student's 't' test *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Figure S5

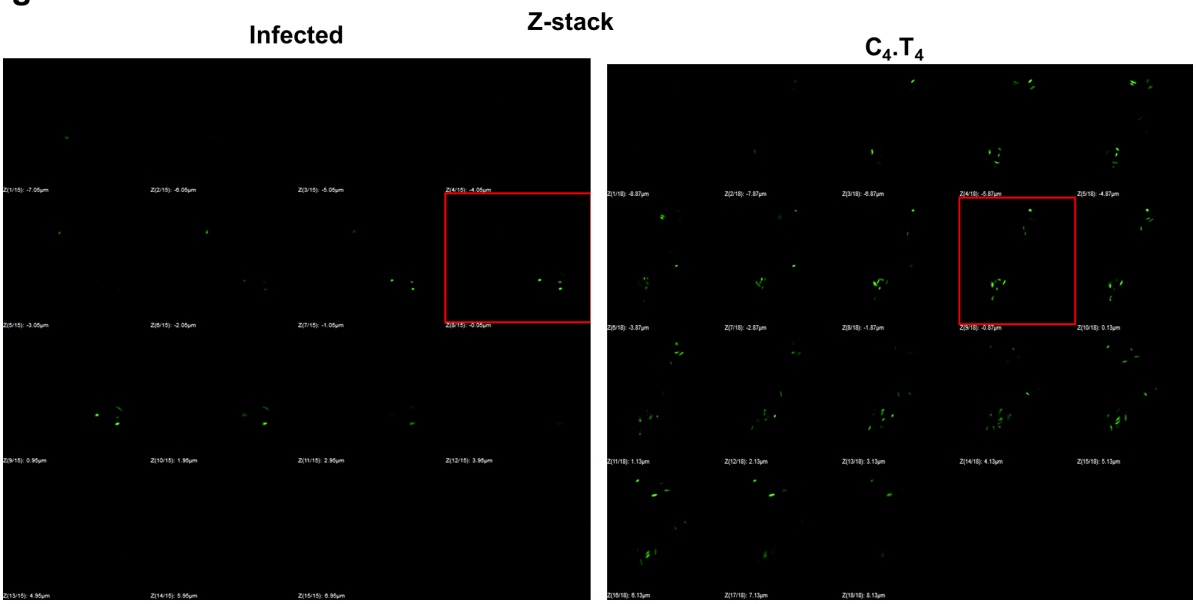

Figure S5. Signaling of BMDMs through $C_4.T_4$ enhanced the phagocytosis of *Mtb*. BMDMs were stimulated with C_4 (24 µg/ml) and T_4 (10 ng/ml) for 48 h. The cells were infected with GFP-H37Ra and uptake was monitored by confocal microscopy. The z-stack images showed the intracellular location of *Mtb* in macrophages. The number on the confocal stacks image indicates total 15 optical sections in untreated macrophage and 18 optical sections of $C_4.T_4$ treated macrophage with an optical section separation (z-interval) of 1 µm. Untreated macrophage z-stack section images are shown side by side (sequences shown in ascending section number from top to bottom) layer 1-15: -7.05 µm; -6.05 µm; -5.05 µm; -4.05 µm; -3.05 µm; -2.05 µm; -1.05 µm; -0.05 µm; 0.95 µm; 1.95 µm; 2.95 µm; 3.95 µm; 4.95 µm; 5.95 µm; 6.95 µm. $C_4.T_4$ treated macrophage z-stack section images are shown side by side (sequences shown in ascending section number from top to bottom) layer 1-18: -8.87 µm; -7.87 µm; -6.87 µm; -5.87 µm; -4.87 µm; -3.87 µm; -2.87 µm; 1.87 µm; -0.87 µm; 0.13 µm; 1.13 µm; 2.13 µm; 3.13 µm; 4.13 µm; 5.13 µm; 6.13 µm; 7.13 µm; 8.13 µm. Data are representative from 2 independent experiments.

Figure S6

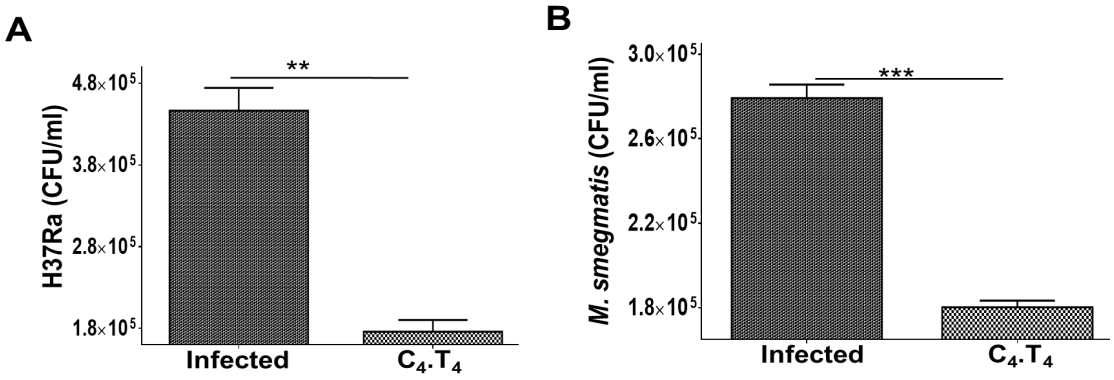

Figure S6. Activation of macrophages through $C_4.T_4$ effectively restricted the survival of *Mtb*. BMDMs were infected with (**A**) *Mtb*-H37Ra for 4 h or (**B**) *Mycobacterium smegmatis* for 3 h. Cells were then stimulated with $C_4.T_4$ for 48 h (H37Ra) and 18 h (*M. smegmatis*). The infected macrophages were lysed and CFUs were enumerated after 21 d (H37Ra) or 3 d (*M. smegmatis*). Data are presented as mean ± SEM of 4 wells and are representative from 3 independent experiments. Data were analyzed by an unpaired Student's 't' test **$p \leq 0.01$, ***$p \leq 0.001$.

Figure S7

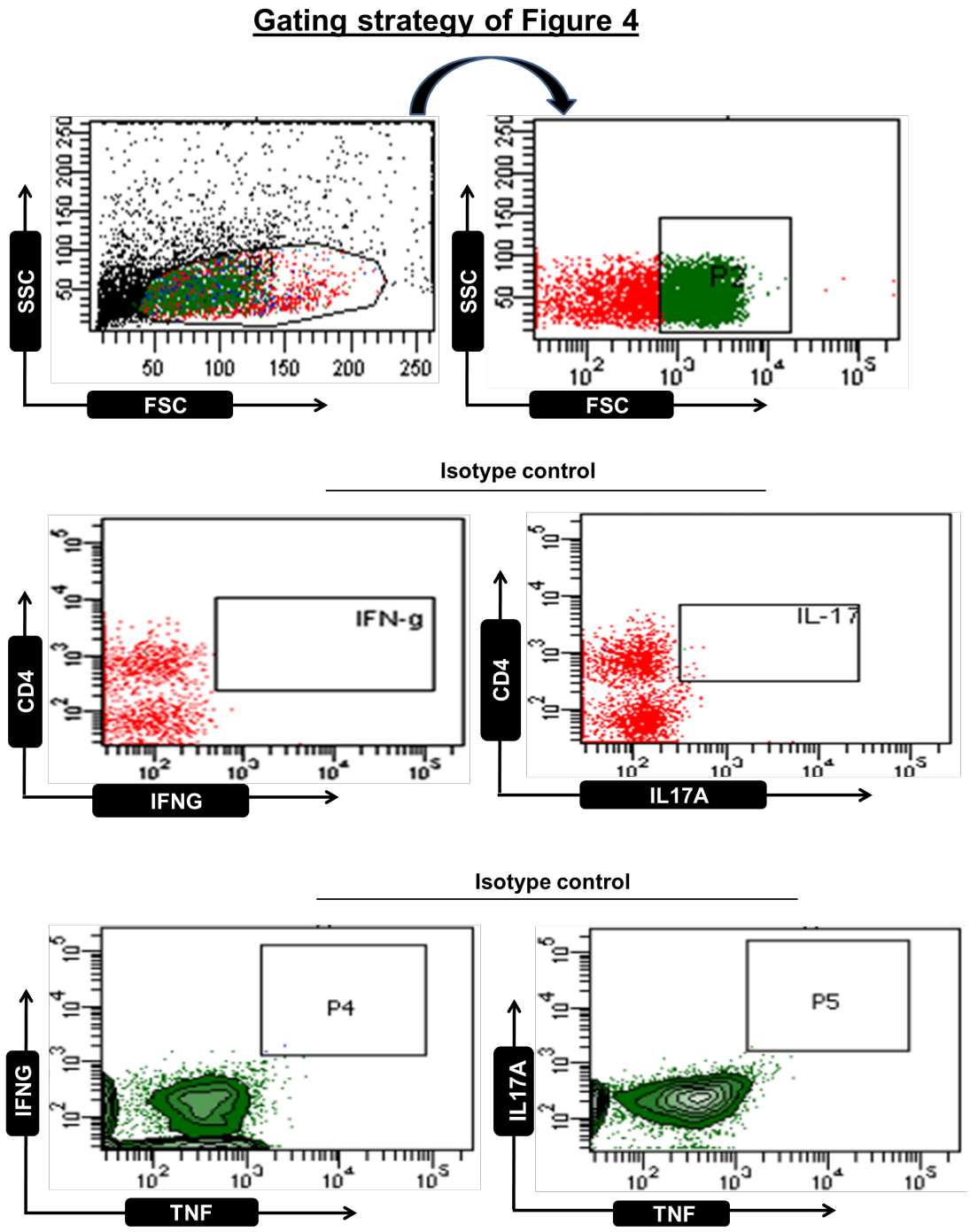

Figure S7. Treatment with $C_4.T_4$ evoked Th1 and Th17 immune responses in *Mtb*-infected mice. The diagram shows the gating strategy for **Figure 4**.

Figure S8

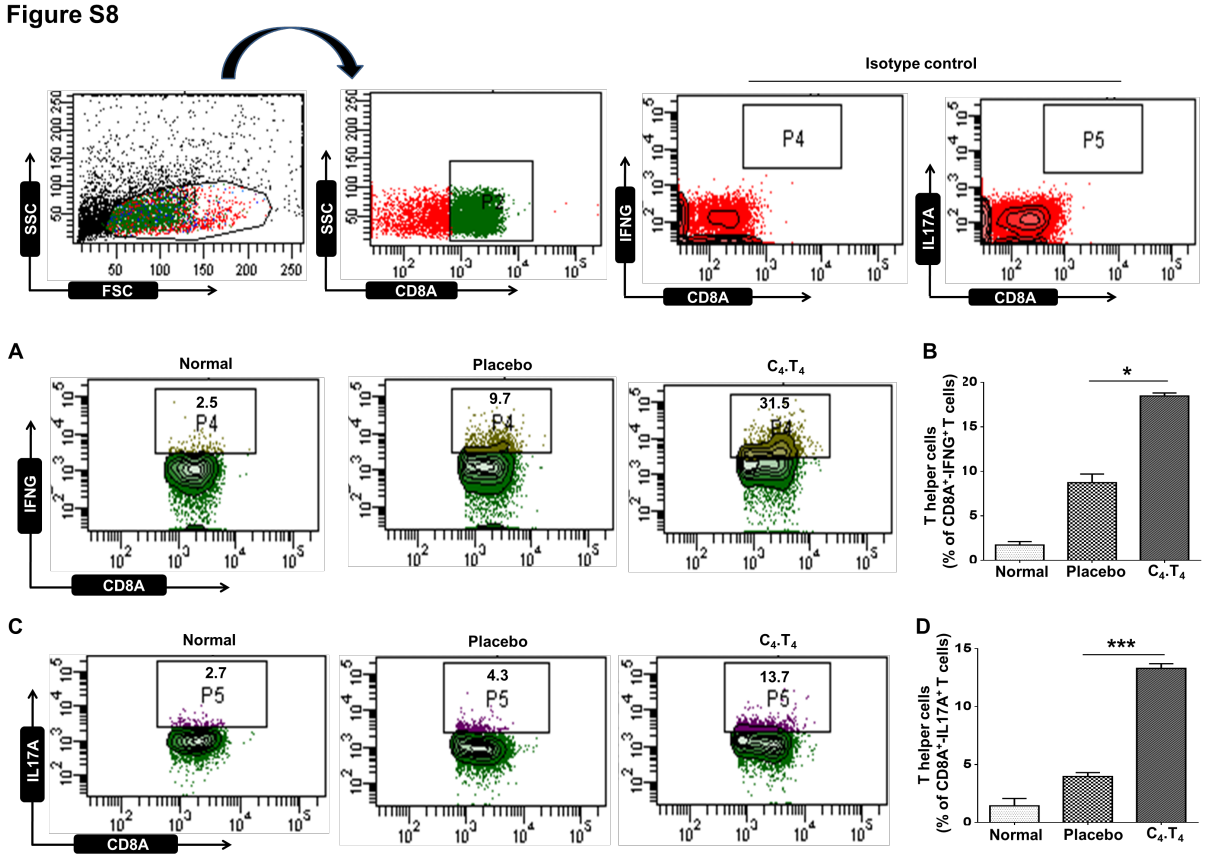

Figure S8. Treatment with $C_4.T_4$ evoked a Th1 and Th17 immune response in *Mtb*-infected mice. The top panel shows the gating strategy of CD8A-positive T cells. *Mtb*-challenged animals were treated with the $C_4.T_4$. After 45 d, cells were isolated from lungs, *in vitro* cultured with PPD for 72 h and intracellular staining of (**A and B**) $IFNG^+$ or (**C and D**) $IL17A^+$ was measured in CD8A-positive T cells by flow cytometry. The number in the inset of the flow cytometry dot plots depict percentage of cells (**A and C**) and bar diagrams (**B and D**) illustrate the percentage of activated T cells. Data shown as the mean ± SEM are represented from 3 independent experiments (n=4 mice/group). Data were analyzed by an unpaired Student's 't' test *$p \leq 0.05$, ***$p \leq 0.001$.

Figure S9

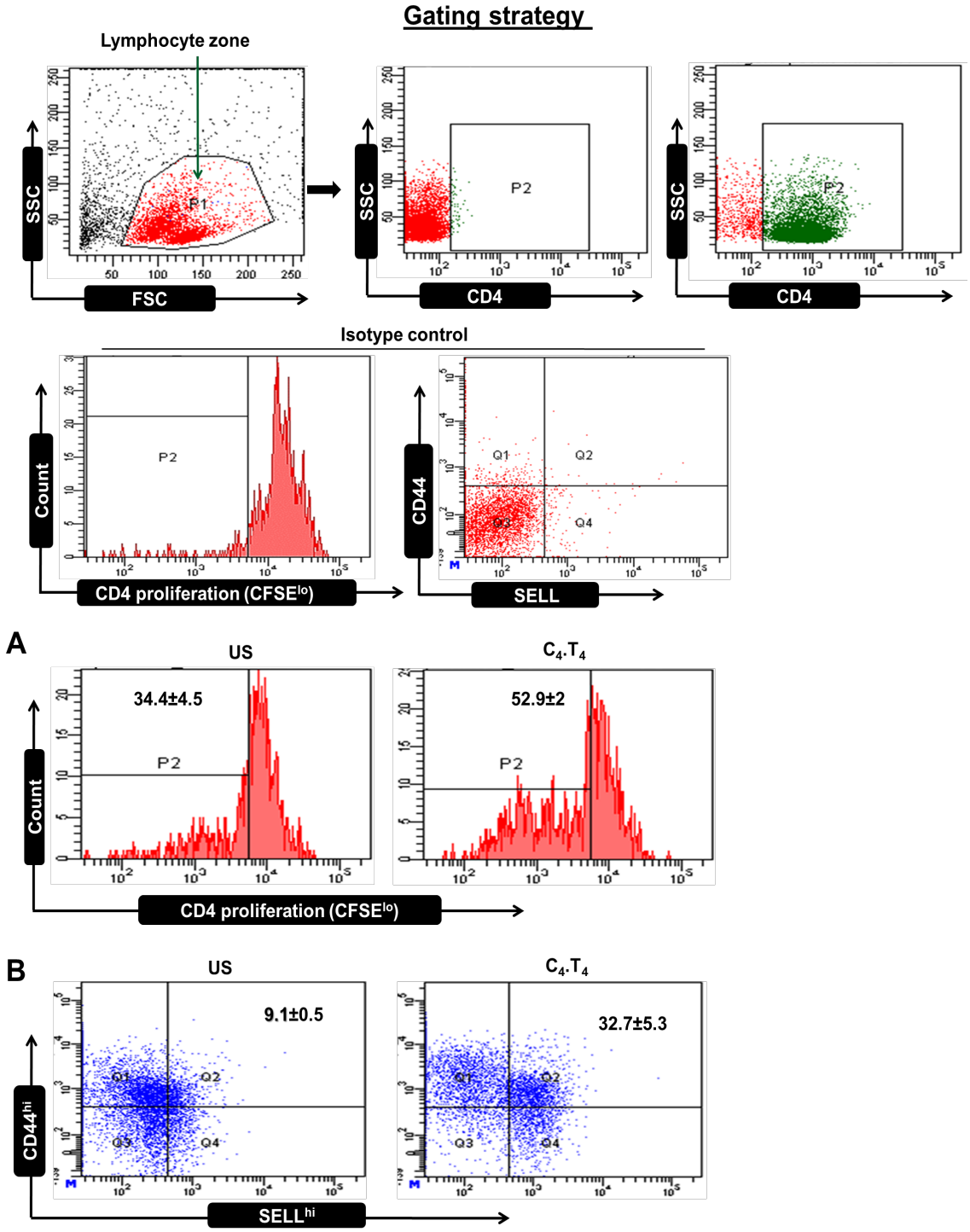

Figure S9. Stimulating macrophages with $C_4.T_4$ enhanced their ability to activate *Mtb*-specific CD4-positive T cells. CD4-positive T cells isolated from the lungs of *Mtb*-challenged animals were labeled with CFSE-dye. Labeled CD4-positive T cells were co-cultured with $C_4.T_4$ pre-

stimulated macrophages for 72 h. PPD was added to the co-culture to activate *Mtb*-specific CD4 T cells. The upper two panels show the gating strategy and isotype control. (**A**) CD4-positive T cell proliferation was examined by flow cytometry; (**B**) expression of CD44^{hi}-SELL/CD62L^{hi} (central memory phenotype) was assessed on CD4 gated T cells by flow cytometry. Data in the inset illustrate the percentage of cells. Data shown as mean ± SD are represented from 2 independent experiments.

Figure S10

Gating strategy of Figure 5.

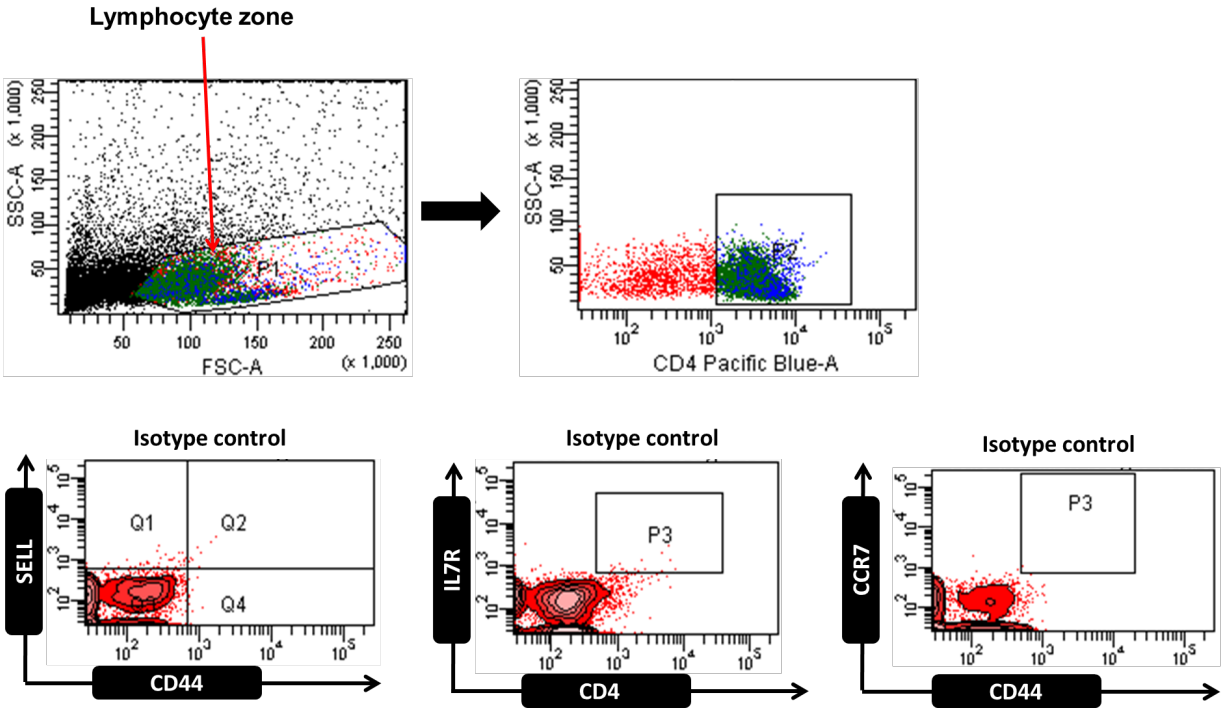

Figure S10. Signaling of macrophages through $C_4.T_4$ generated enduring memory CD4 T cells. The diagram shows the gating strategy for **Figure 5**. The data shows the central memory (CD4^{+}-CD44^{hi}-SELL/CD62L^{hi}) and effector memory (CD4^{+}-CD44^{hi}-SELL/CD62L^{low}); CD4^{+}-IL7R/CD127^{hi}; CD4^{+}-CD44^{+}-CCR7^{hi}.

Figure S11

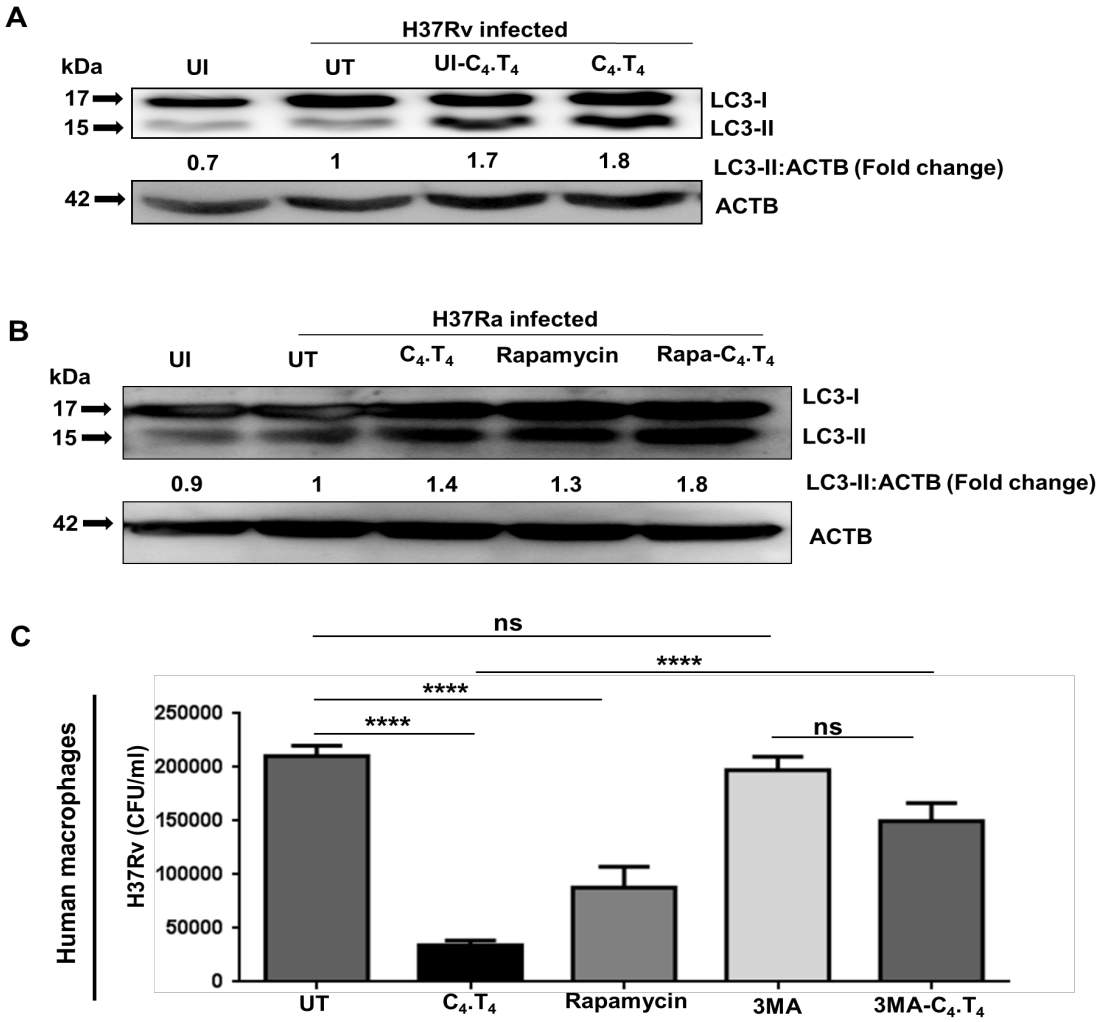

Figure S11. $C_4.T_4$ induced autophagy and restricted *Mtb* growth in murine and human macrophages. BMDMs were infected with (**A**) H37Rv or (**B**) H37Ra for 4 h, then stimulated with $C_4.T_4$ for 4 h. The conversion of LC3-I to LC3-II was checked by western blot. The over expression of LC3-II was confirmed after $C_4.T_4$-rapamycin (1 μM) treatment. Data depicted are representative of 3 independent experiments. (**C**) THP-1 macrophages were infected with H37Rv for 4 h, treated with 3MA (10 mM) for 1 h, and then stimulated with $C_4.T_4$ agonists for 48 h. Infected cells were lysed and CFUs were enumerated after 21 d. Data represent the mean ± SEM of 4 wells and are from 2 independent experiments. UI, uninfected; UT, untreated; 3MA, 3 methyladenine; ns, non-significant. Data were analyzed by one-way ANOVA repeated measure ****$p \leq 0.0001$.

Figure S12

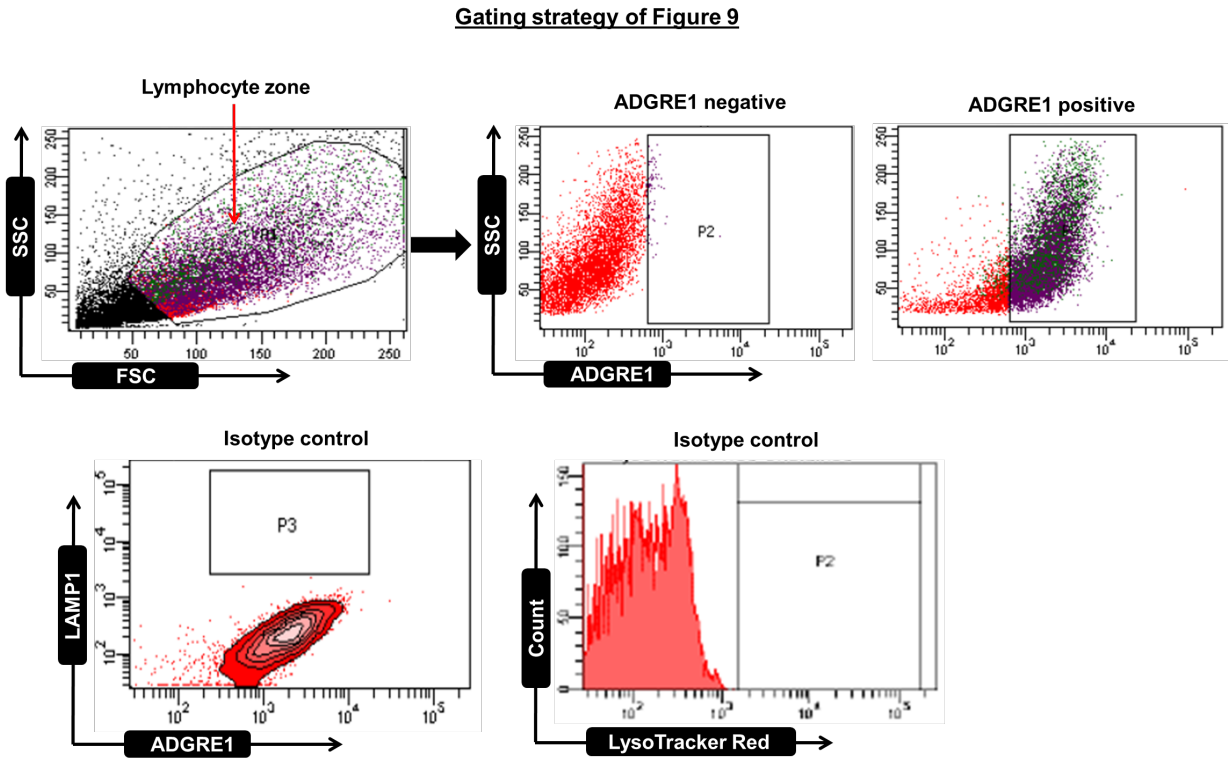

Figure S12. $C_4.T_4$ stimulation induced autophagosome and lysosome formation in macrophages. The diagram shows the gating strategy for **Figure 9**.

Figure S13

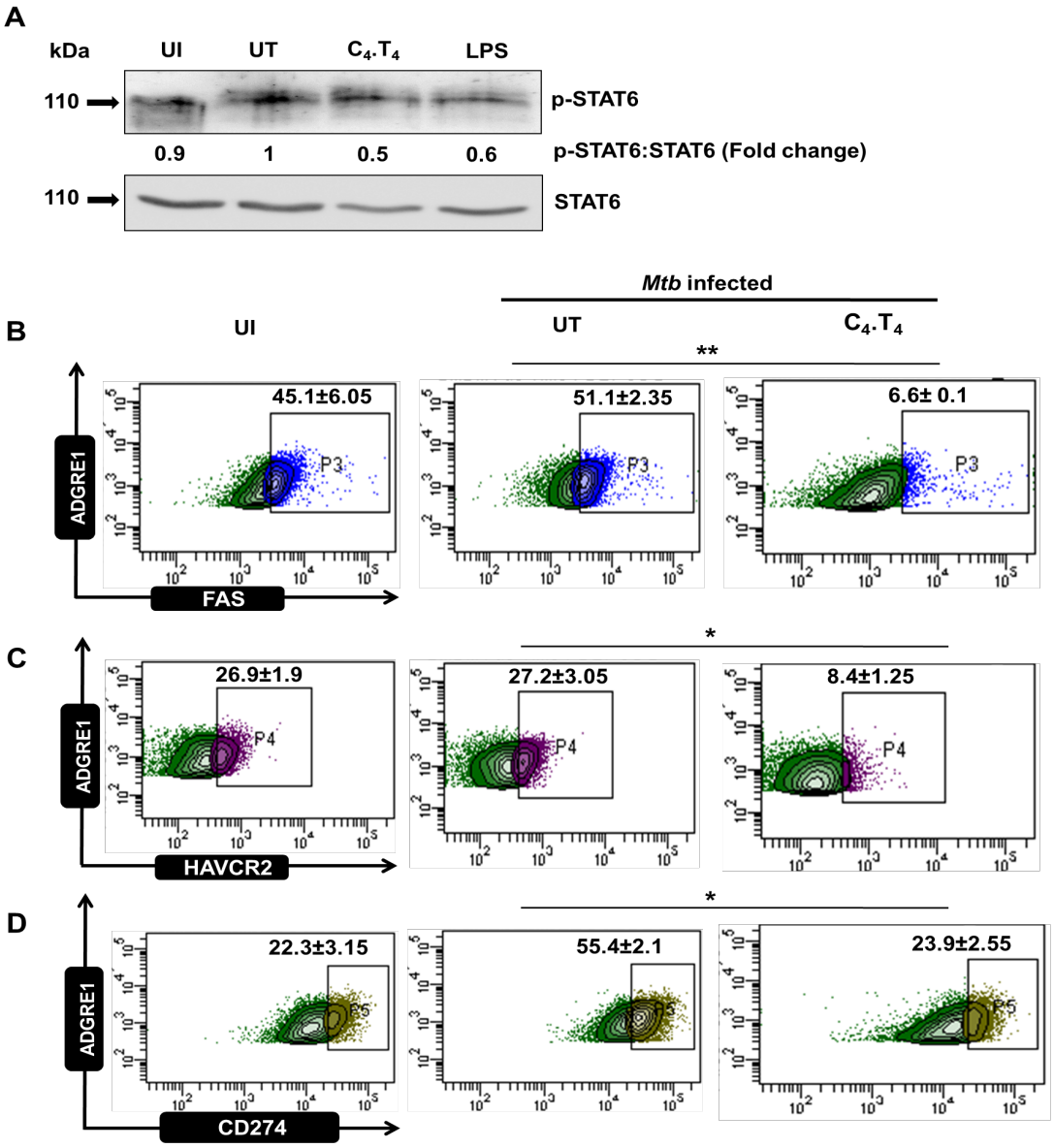

Figure S13. CLEC4E and TLR4 signaling induced macrophage survival. **(A)** Bone BMDMs were infected with H37Rv for 4 h and cultured with $C_4.T_4$ for 20 min. Cell lysates were prepared and western blot was completed to monitor the expression of p-STAT6. The densitometry data represent fold change. The ratio for untreated cells was considered to be 1. LPS was used as a positive control. (**B-D**) BMDMs were infected with H37Rv for 4 h, then stimulated with $C_4.T_4$ for 48 h. Cells were stained for expression of (**B**) FAS; (**C**) HAVCR2/TIM-3; and (**D**) CD274/PD-L1 on ADGRE1/F4/80 gated cells. UI, uninfected; UT, untreated and *Mtb* infected. Data depicted are representative of 2 independent experiments. *$p \leq 0.05$, **$p \leq 0.01$.

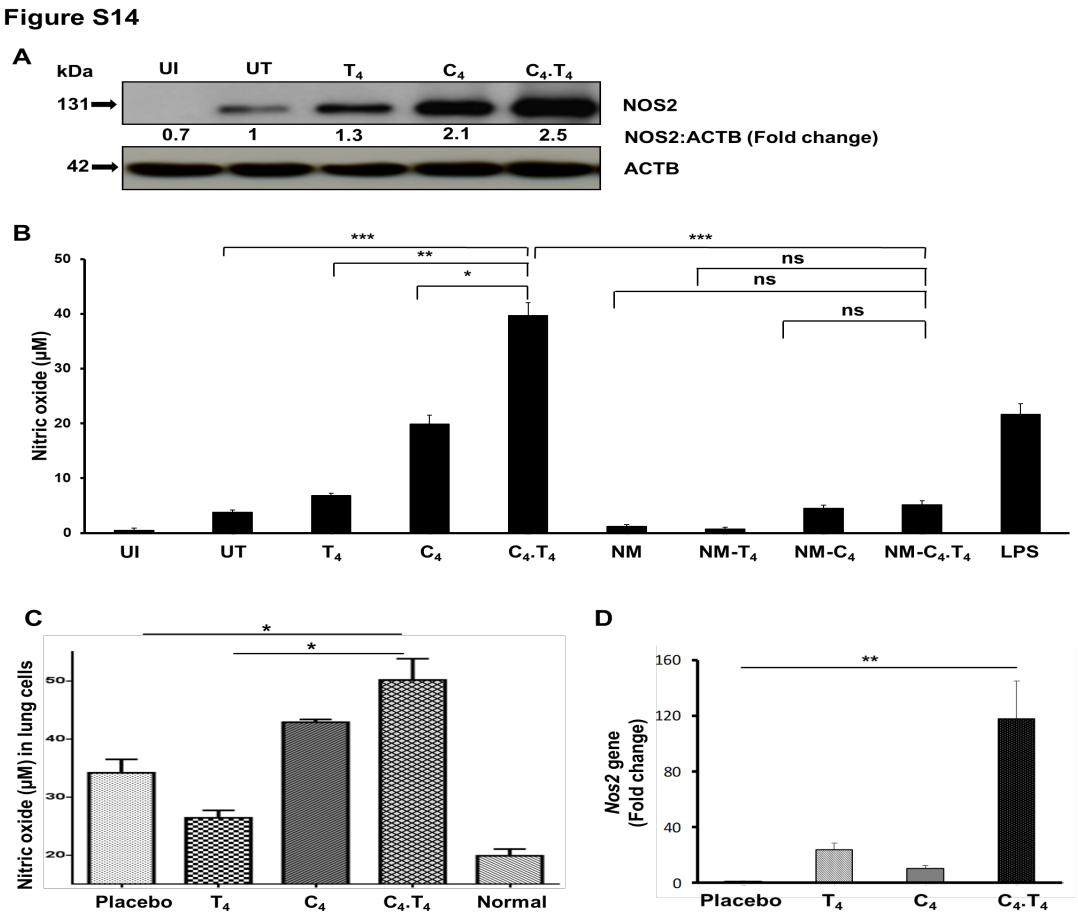

Figure S14. Signaling through $C_4.T_4$ in *Mtb*-infected macrophages increased the expression of NOS2. BMDMs were infected with H37Rv and stimulated with $C_4.T_4$ for 16 h. The cells were lysed by cytosolic extraction buffer and the expression of (**A**) NOS2 was demonstrated by western blot. (**B**) Infected BMDMs were treated with an NOS2 inhibitor (N-monomethyl-L-arginine) for 1 h and the secretion of NO was monitored by Griess method. (**C**) Secretion of NO was measured in the supernatant where the *Mtb*-challenged, $C_4.T_4$ treated lung cells were cultured *in vitro* in the presence of PPD; (**D**) quantified *Nos2* gene in the lungs of *Mtb* challenged and $C_4.T_4$ treated mice by RT-qPCR. Data depicted are representative of 3 independent experiments. UI, uninfected; UT, untreated; C_4, CLEC4E agonist (TDB); T_4, TLR4 agonist (ultra-pure LPS); Normal, animals not exposed to *Mtb*; ns, non-significant. Data were analyzed by one-way ANOVA repeated measure *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Figure S15

Genes	Primers
Actb	Fwd 5′-AGAGGGAAATCGTGCGTGAC-3′ Rev 5′-CAATAGTGATGACCTGGCCGT-3′
Il1b	Fwd 5′- CAACCAACAAGTGATATTCTCCATG-3′ Rev 5′- GATCCACACTCTCCAGCTGCA-3′
Il4	Fwd 5′- ACAGGAGAAGGGACGCCAT-3′ Rev 5′- GAAGCCCTACAGACGAGCTC-3′
Il6	Fwd 5′-GAGGATACCACTCCCAACAGACC-3′ Rev 5′-AAGTGCATCATCATCGTTGTTCATACA-3′
Il12b	Fwd 5′-GGAAGCACGGCAGCAGCAGAATA-3′ Rev 5′-AACTTGAGGGAGAAGTAGGAATGG-3′
Il10	Fwd 5′-GGTTGCCAAGCCTTATCGGA-3′ Rev 5′-ACCTGCTCCACTGCCTTTGCT-3′
Nos2	Fwd 5′-AACGGAGAACGTTGGATTTG-3′ Rev 5′-CAGCACAAGGGGTTTTCTT-3′
Becn1	Fwd 5′-TGCTCTGGCCAATAAGATGGGTCT-3′ Rev 5′-GGAAAGCCACCATTGCATGGTCAA-3′
Atg5	Fwd 5′- GAGGGTGACTGGACCTACGG-3′ Rev 5′-CCTTCAACCAAAGCCAAACCG-3′
Atg7	Fwd 5′-TCCCATGCCTCCTTTCTGGTTCTT-3′ Rev 5′-AGCCCACAGATGGAGTAGCAGTTT-3′
Lc3	Fwd 5′-CCGCAGCCCTTGAGCTCGAG-3′ Rev 5′-GGGTGCTGGTCGCGGATCTG-3′
Atg12	Fwd 5′-GGACCCATCTACAGAGGCTG-3′ Rev 5′-ATCACAATGGTGGAGGGTGC-3′
Lamp1	Fwd 5′-GCAGCAGGCCTTGCACAT-3′ Rev 5′-AATTGTGAGGCTGGGGTCAG-3′
Eea1	Fwd 5′-TGGAGGCTACAATAAACCAGC-3′ Rev 5′-AGGGATGCCTGGAGAGTCT-3′
Clec4e/Mincle	Fwd 5′-TGCTACAGTGAGGCATCAGG-3′ Rev 5′-GGTTTTGTGCGAAAAAGGAA-3′
Tlr4	Fwd 5′-ACCTGGCTGGTTTACACGTC-3′ Rev 5′-CTGCCAGAGACATTGCAGAA-3′

Figure S15. The primer sequences of autophagy related genes.