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Supplemental Information

Fever Promotes T Lymphocyte Trafficking via a Thermal Sensory Pathway Involving Heat Shock Protein 90 and α4 Integrins

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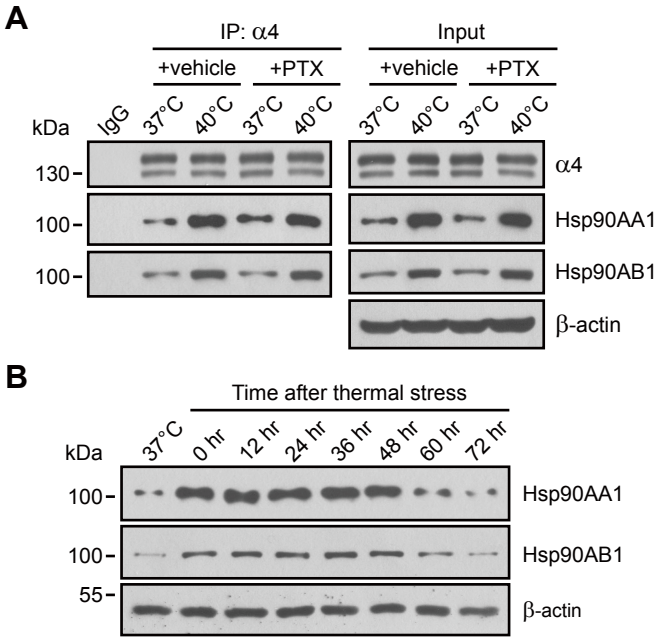


Figure S1 PTX does not affect fever-range thermal stress-induced Hsp90-α4 interaction and the enhanced expression of Hsp90 could last at least 48 hr under normal temperature after thermal stress, related to Figure 2.

(**A**) T cells were pre-treated at 37 °C or 40 °C in culture medium with or without 100 ng/ml PTX for 12 hr. Co-immunoprecipitation of Hsp90 with integrin α4 in the cell membrane fractions. (**B**) T cells were pre-treated at 37 °C or 40 °C in culture medium for 12 hr, respectively, followed by treatment at 37 °C for 0 hr, 12 hr, 24 hr, 36 hr, 48 hr, 60 hr or 72 hr. The expression of Hsp90AA1 and Hsp90AB1 were determined by immunoblot. One representative result of three independent experiments is shown.

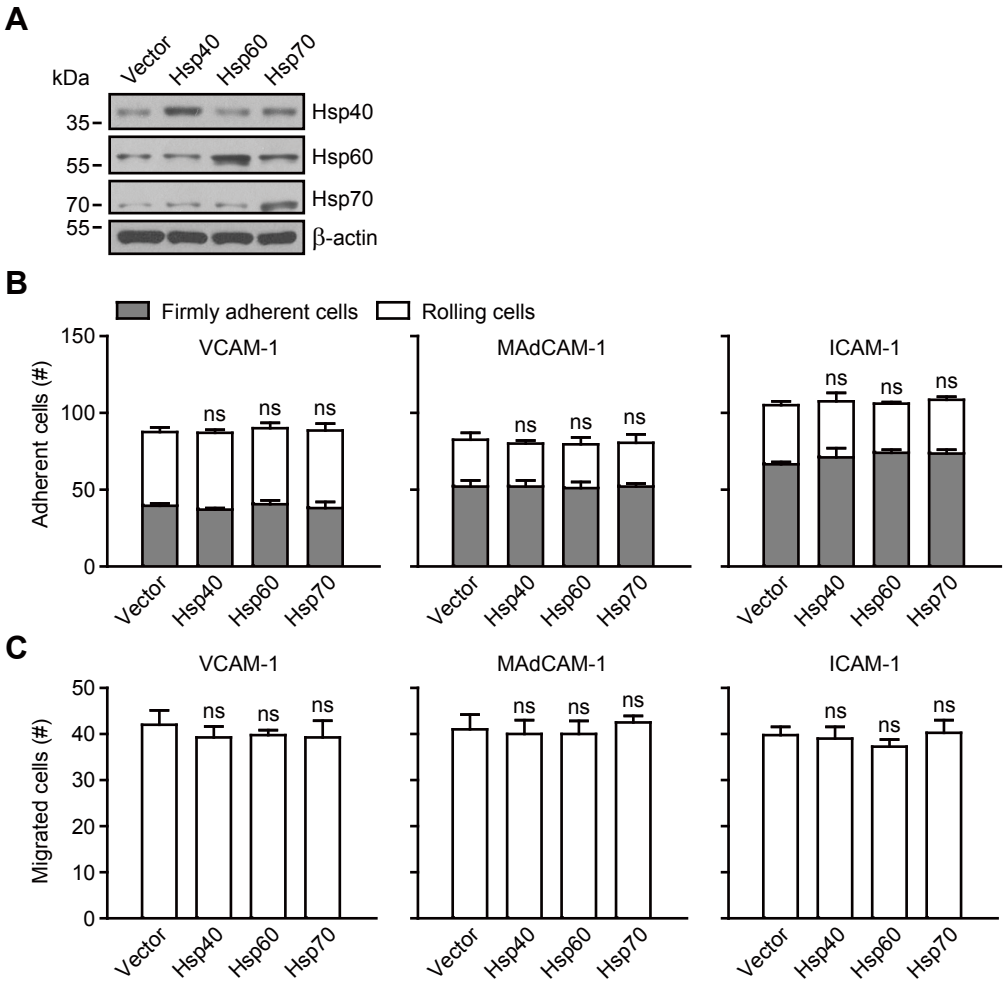
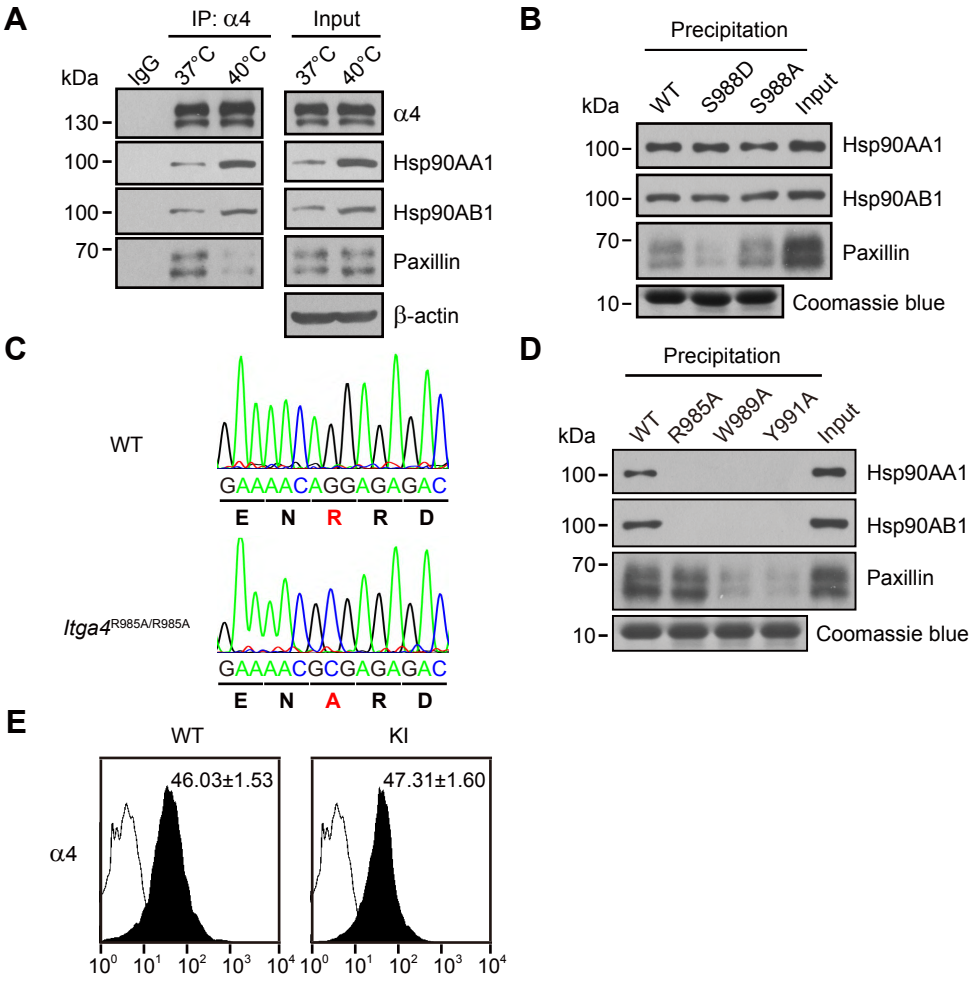


Figure S2 Overexpression of Hsp40, Hsp60 or Hsp70 does not affect α4- or β2-integrin-mediated T cell adhesion and transmigration, related to Figure 2.

T cells were transiently transfected with vector, Hsp40, Hsp60 and Hsp70, respectively. α4β7-VCAM-1 binding was disrupted by pre-treating the cells with 10 μg/ml α4β7-blocking antibody DATK32 when examining α4β1-mediated cell adhesion and migration on VCAM-1 substrate in (**B** and **C**). (**A**) Immunoblot analysis of Hsp40, Hsp60 and Hsp70. (**B**) Adhesion of T cells to immobilized VCAM-1-Fc (5 μg/ml), MAdCAM-1-Fc (5 μg/ml) or ICAM-1-Fc (5 μg/ml) substrate in 1 mM Ca^{2+} + Mg^{2+} at a wall shear stress of 1 dyn/cm^2. (**C**) Transmigration of T cells across VCAM-1-Fc (5 μg/ml), MAdCAM-1-Fc (5 μg/ml) or ICAM-1-Fc (5 μg/ml) coated membrane in the presence of CCL21 (500 ng/ml) in the lower chamber. One representative result of three independent experiments is shown in (**A**). Data represent the mean ± SEM (n ≥ 3) in (**B**) and (**C**). ns: not significant (one-way ANOVA with Dunnett post-tests); ns in (**B**) indicates the changes in total adherent cells.



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	CCR7 expression	
	WT	KI
37°C	6.94±0.70	7.64±0.81
40°C	7.09±0.52	7.97±0.76
p value	0.7707	0.6332

G

	CCR7 expression	
	WT	KI
NT	6.79±0.52	7.12±1.18
WBH	6.52±0.39	7.42±1.04
p value	0.5137	0.7522

Figure S3 Hsp90 competes with paxillin to bind to α4 integrins and generation of *Itga4*$^{R985A/R985A}$ (KI) C57BL/6J mice, related to Figure 3.

(**A**) Co-immunoprecipitation of Hsp90AA1, Hsp90AB1 and paxillin with integrin α4 in the cell membrane fractions of T cells pre-treated at 37 °C or 40 °C. (**B**) Precipitation of Hsp90AA1, Hsp90AB1 and paxillin from T cell lysate by Ni^{2+}-charged resins loaded with WT, S988D or S988A of α4 integrin tail model proteins. Coomassie blue staining of gels assessed the loading of each integrin tail model protein. (**C**) *Itga4*$^{R985A/R985A}$ (KI) C57BL/6J mice was generated by using the CRISPR/Cas9 system. Sequencing analysis of WT and KI mice. DNA sequencing confirmed an Arg985 to Ala substitution in mouse α4 integrin gene in KI mice. (**D**) Precipitation of Hsp90AA1, Hsp90AB1 and paxillin from T cell lysate by Ni^{2+}-charged resins loaded with WT or mutants of α4 tail model proteins. Coomassie blue staining of gels assessed the loading of each integrin tail model protein. (**E**) Integrin α4 expression on T cells was determined by flow cytometry. T cells were isolated form mouse spleen. Numbers within the panel showed the specific mean fluorescence intensities. Opened histogram: mock control. (**F**) CCR7 expression on T cells pre-treated at 37 °C or 40 °C was determined by flow cytometry. Numbers within the table showed the specific mean fluorescence intensities and p values. (**G**) WT and KI C57BL/6J mice were treated with normothermia (NT, core temperature 36.8 ± 0.2 °C) or fever-range whole-body hyperthermia (WBH, core temperature 39.5 ± 0.5 °C) for 6 hr (n = 3 mice per group), and then were sacrificed. Cell-surface expression of CCR7 on T cells was determined by flow cytometry. Numbers within the table showed the specific mean fluorescence intensities and p values. One representative result of three independent experiments is shown in (**A**)–(**E**). Data represent the mean ± SEM (n = 3) in (**E**)–(**G**) (Student's t test in **F** and **G**).

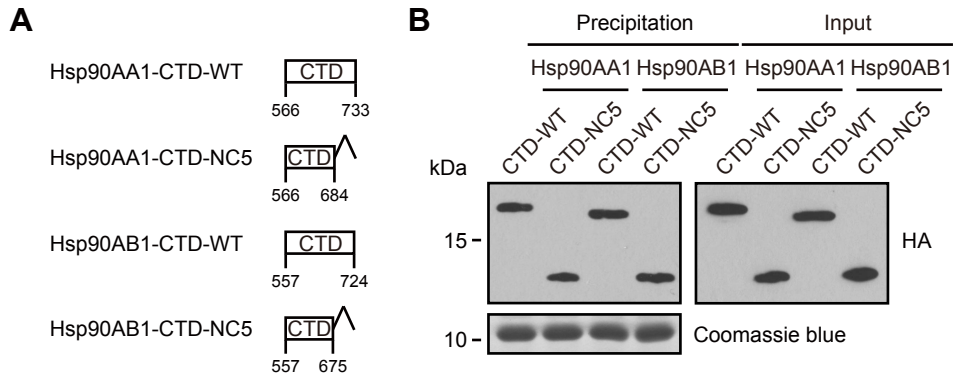

Figure S4 Deletion of the C-terminal 49 amino acids in Hsp90 does not affect Hsp90 C-terminal domain (CTD-NC5)-α4 binding, related to Figure 5.

(**A**) Schematic diagram of CTD structures of Hsp90 WT and NC5 mutant with deletion of the C-terminal 49 amino acids. (**B**) Precipitation of HA-tagged WT CTD or CTD-NC5 mutant of Hsp90 from T cell lysates by Ni^{2+}-charged resins loaded with α4 tail model protein. Coomassie blue staining of gels assessed the loading of each integrin tail model protein. One representative result of three independent experiments is shown in (**B**).

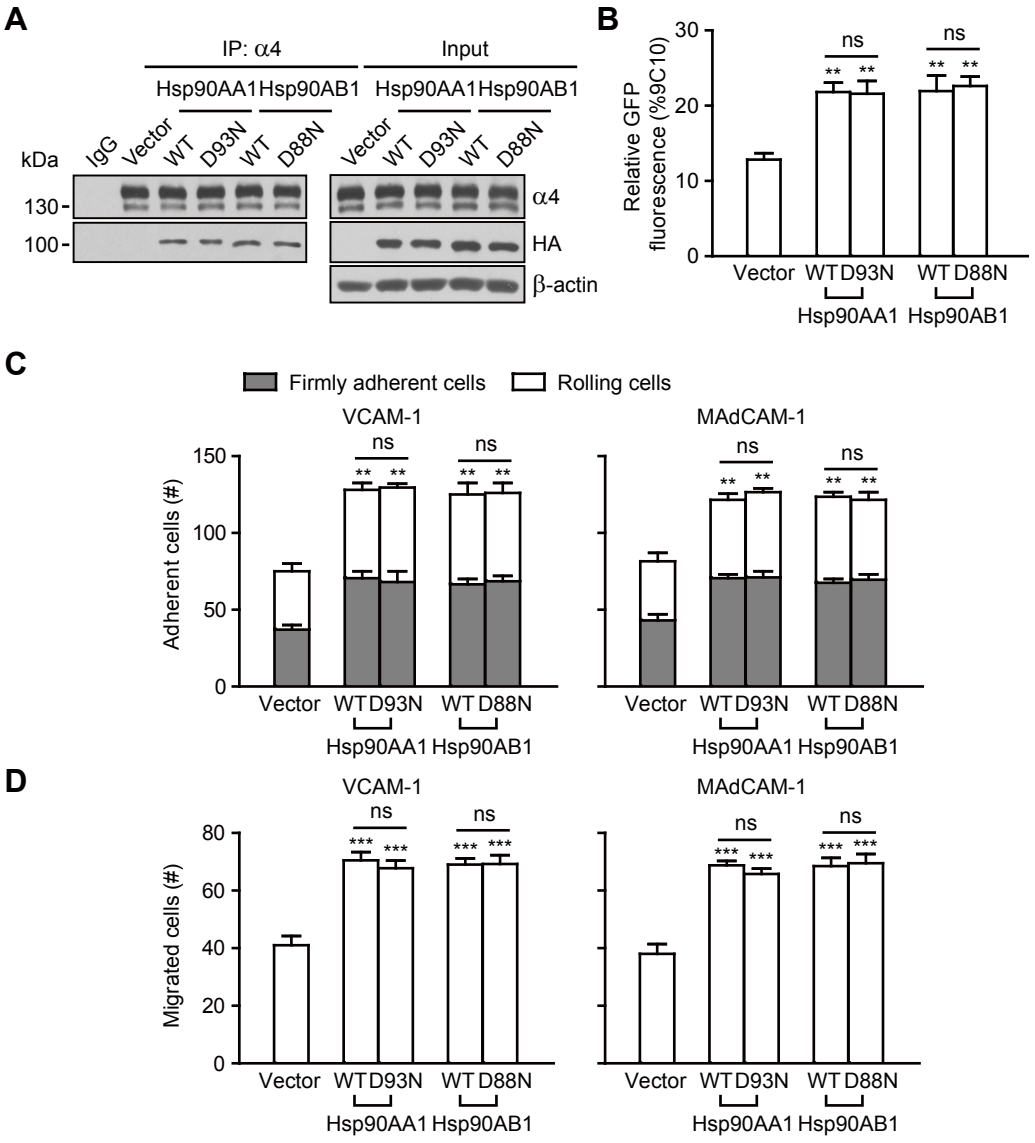

Figure S5 Hsp90 ATPase activity is not required for the regulation of α4 integrin functions by Hsp90, related to Figure 5.

T cells (**A**, **C** and **D**) or T cells expressing α4 integrin-split GFP (**B**) were transiently transfected with vector, Hsp90 WT or ATPase dominant-negative mutant (Hsp90AA1-D93N or Hsp90AB1-D88N), respectively. α4β7-VCAM-1 binding was disrupted by pre-treating the cells with 10 μg/ml α4β7-blocking antibody DATK32 when examining α4β1-mediated cell adhesion and migration on VCAM-1 substrate in (**C** and **D**). (**A**) Co-immunoprecipitation of HA-tagged Hsp90 WT and ATPase dominant-negative mutants with integrin α4 in T cells. (**B**) Relative GFP fluorescence of T cells expressing α4 integrin-split GFP was calculated by the mean fluorescence intensity of GFP and quantified as a percentage of α4 integrin expression. (**C**) Adhesion of T cells to immobilized VCAM-1-Fc (5 μg/ml) or MAdCAM-1-Fc (5 μg/ml) substrate in 1 mM Ca^{2+} + Mg^{2+} at a wall shear stress of 1 dyn/cm^2. (**D**) Transmigration of T cells across VCAM-1-Fc (5 μg/ml) or MAdCAM-1-Fc (5 μg/ml) coated membrane in the presence of CCL21 (500 ng/ml) in the lower chamber. One representative result of three independent experiments is shown in (**A**). Data represent the mean ± SEM (n ≥ 3) in (**B**)–(**D**). ** $p < 0.01$, *** $p < 0.001$, ns: not significant (one-way ANOVA with Dunnett post-tests and Student's t test); asterisk in (**C**) indicates the changes in total adherent cells.

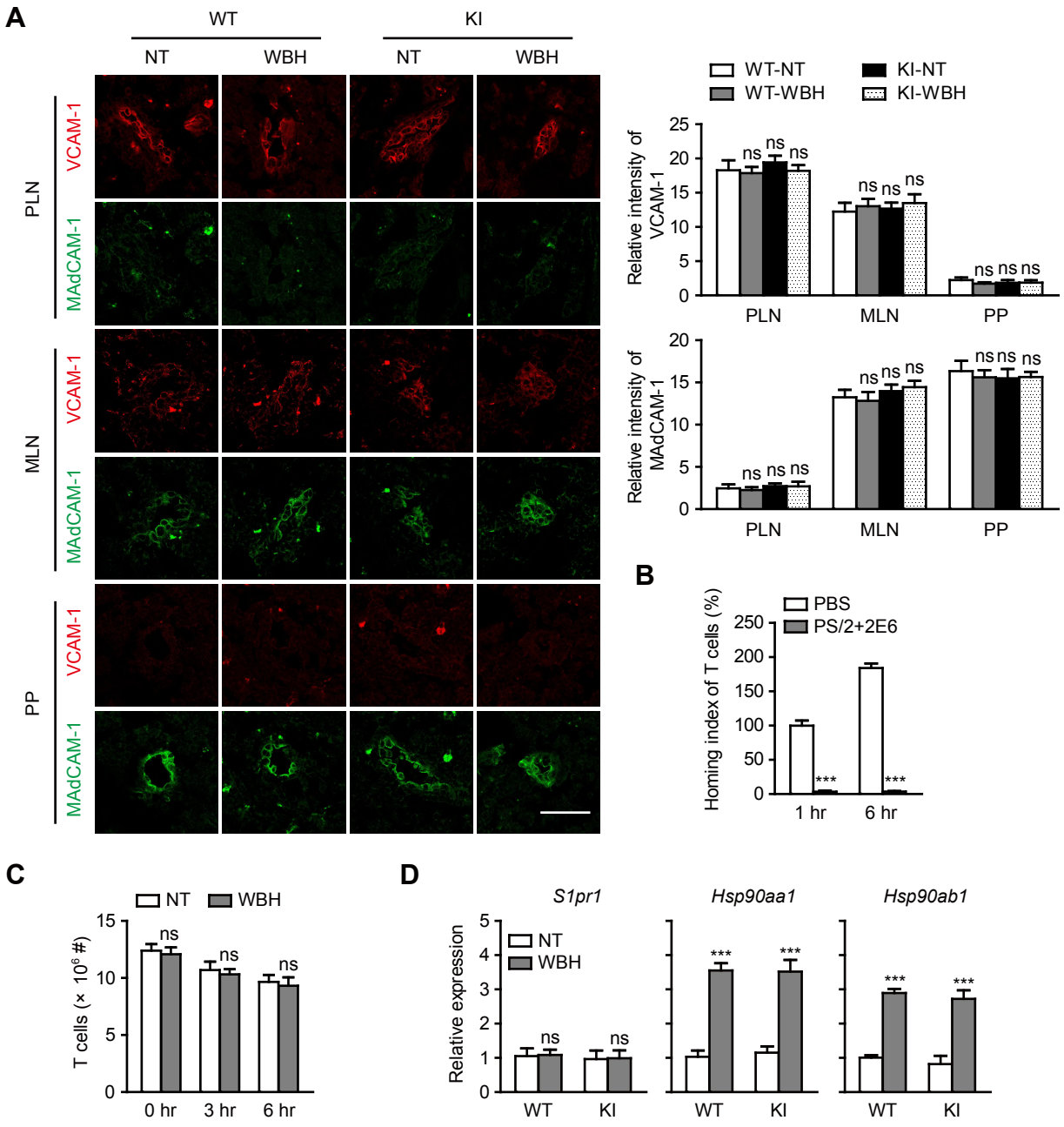

Figure S6 Effect of thermal stress on T cell homing to and egress from draining lymph nodes in mice, related to Figure 7.

(**A**) WT and *Itga4*$^{R985A/R985A}$ (KI) C57BL/6J mice were treated with normothermia (NT, core temperature 36.8 ± 0.2 °C) or fever-range whole-body hyperthermia (WBH, core temperature 39.5 ± 0.5 °C) for 6 hr (n = 3 mice per group). Representative images from confocal microscopy of PLN, MLN and PP frozen sections dually stained for VCAM-1 (red) and MAdCAM-1 (green). Scale bar, 50 μm. 6 sections for each condition were analyzed and the quantification of the relative fluorescence intensity was shown on the right. (**B**) Blocking in vivo homing of calcein-labelled WT T cells to inguinal lymph nodes. WT mice were pre-injected with 300 μg PS/2 and 2E6 (PS/2+2E6) antibodies or PBS as a control. After 20 min, 5 × 10^6 calcein-labelled T cells of WT mice were injected intravenously into WT C57BL/6J mice. Inguinal lymph nodes were collected 1 hr or 6 hr after cell transfer. The homing index was calculated as the percentage of the homed T cells in inguinal lymph nodes relative to the value in control mice 1 hr after cell transfer. (**C**) T cell egress during normothermia or WBH treatment. WT C57BL/6J mice were pre-injected with 300 μg PS/2 and 2E6 antibodies to block the recruitment of lymphocytes into lymph nodes. After 20 min, the mice were treated with normothermia or WBH for 0 hr, 3 hr and 6 hr (n = 3 mice per group). The total numbers of T cells in PLNs were quantified at each time point. (**D**) Expression of *S1pr1*, *Hsp90aa1* and *Hsp90ab1* mRNAs in T cells in PLNs in WT and KI mice treated with normothermia or WBH was measured. One representative result of three independent experiments is shown in (**A**). Data represent the mean ± SEM (n ≥ 3). *** p < 0.001, ns: not significant (one-way ANOVA with Dunnett post-tests in **A**; Student's t test in **B–D**).

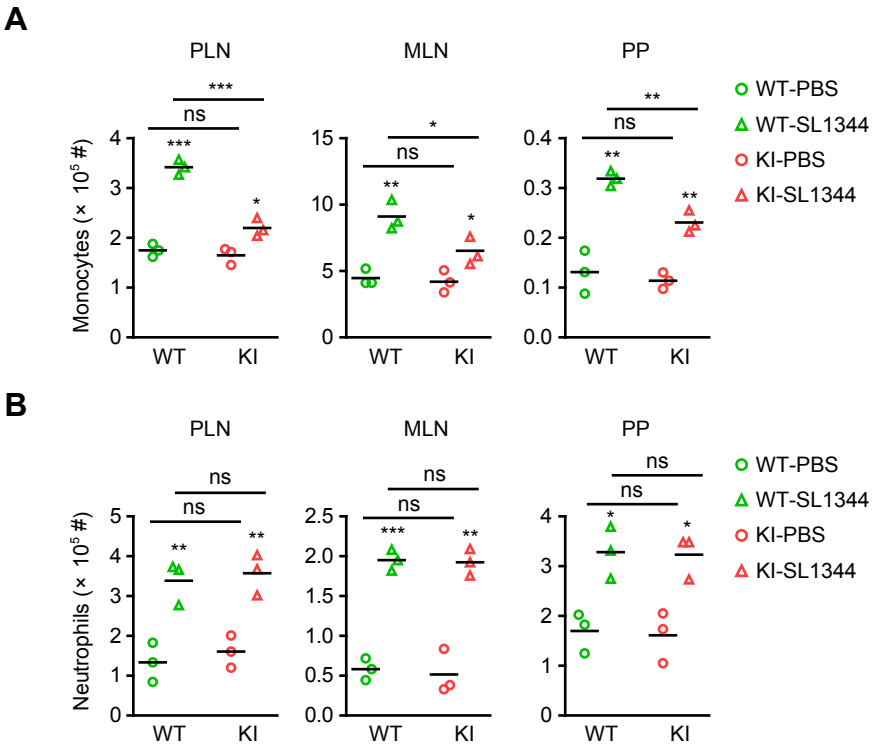

Figure S7 Monocyte and neutrophil recruitment to draining lymph nodes in mice during *Salmonella typhimurium* infection, related to Figure 7.

WT and *Itga4*$^{R985A/R985A}$ (KI) mice were orally injected with PBS or *S. typhimurium* strain SL1344 (10^8 CFU per mouse, n = 3 mice per group). The total numbers of CD11b$^+$Ly6C^{high}Ly6G^{low} monocytes (**A**) and CD11b$^+$Ly6G^{high}Ly6C^{low} neutrophils (**B**) in PLNs, MLNs and PPs were quantified at day 3 post infection. Data represent the mean ± SEM (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant (Student's t test).

integrin	α4	β1	β7	β2
Vector	47.23±1.75	26.28±0.42	351.5±6.72	937.06±6.04
Hsp90AA1	48.1±1.58	25.83±0.45	346.5±3.89	929.08±8.14
Hsp90AB1	47.33±1.87	25.63±0.30	347±9.90	934.52±10.89
p value	0.9756	0.5291	0.8690	0.8047

Table S1 Hsp90 overexpression does not affect T cell surface expression of either α4 or β2 integrins, related to Figure 2.

T cells were transiently transfected with vector, Hsp90AA1 or Hsp90AB1, respectively. Cell surface expression of α4, β1, β7 and β2 was determined by flow cytometry. Numbers within the table showed the specific mean fluorescence intensities and p values. Data represent the mean ± SEM (n = 3) (one-way ANOVA).