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## T cell-derived lymphotoxin limits Th1 response during HSV-1 infection

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Though lymphotoxin (LT) is highly expressed by type I helper T (Th1) cells, its contribution to CD4<sup>+</sup> T cell differentiation during infections and diseases remains a mystery. In HSV-1 infection, we observed that LTβR signaling is required to limit the Th1 response. Using bone marrow chimeric mice, mixed-T-cell chimeric mice, and LTβR *in vivo* blockades, we unexpectedly observed that LT, especially T cell-derived LT, played an indispensable role in limiting the Th1 response. The LTβR-Ig blockade promoted the Th1 response by increasing infiltration of monocytes and monocyte-derived DCs and up-regulating IL-12 secretion in the lymphoid environment. Our findings identified a novel role for T cell-derived LT in manipulating Th1 differentiation.

During the adaptive immune response, activated CD4<sup>+</sup> T cells will expand and differentiate into several sub-populations, including Th1, Th2, Th17, Treg and T follicular helper (Tfh) cells, under regulation of distinct sets of cytokines and transcriptional factors<sup>1</sup>. Lymphotoxin (LT, in the form of membrane heterotrimer (LT<sub>α1β2</sub>) or secreted homotrimer (LT<sub>α3</sub>), is expressed on activated B and T cells, especially expressed constitutively on Th1 cells but not Th2 cells<sup>2</sup>. LT<sub>α3</sub> binds to TNFR1 and TNFR2<sup>3</sup>. The receptor for LT<sub>α1β2</sub>, Lymphotoxin-beta-Receptor (LTβR), is expressed on follicular dendritic cells (FDCs), DCs, macrophages and stromal cells<sup>4</sup>. Activation of the LTβR pathway stimulates the expression of pro-inflammatory mediators, adhesion molecules<sup>5</sup> and lymphocyte-recruiting chemokines, such as CCL19/CCL21 and CXCL13. Chemokine gradients help to define the B and T cell zones, thereby establishing the primary and secondary lymphoid structures<sup>6–8</sup>. Beyond that, the LT-LTβR signaling also helps to mediate the adaptive immune response. LT expression on activated helper T cells plays a critical role in mediating full DC maturation, indispensable for optimal CTL response<sup>9</sup>. The lymphotoxin-induced signaling deficiency leads to an increased susceptibility to some bacterial infections in association with the impaired Th1 response<sup>10,11</sup>. As a hallmark molecule of Th1 cells, LT is thought to facilitate Th1 differentiation by supporting the lymphoid tissue development<sup>12</sup>, but whether LT promotes Th1 differentiation in viral infections has not been proven experimentally.

HSV-1 (herpes simplex virus type 1) is a double-stranded DNA virus that can cause acute and latent infections in human beings, often used as an acute Th1-biased viral infection model in mice<sup>13,14</sup>. Our previous finding suggests that Tfh-like cells will acquire a Th1-like feature in the LT<sub>α1β2</sub>-LTβR signaling deficient environment post HSV-1 foot-pad infection<sup>15</sup>, based on which, we suppose that the LT-LTβR signaling may somehow limit Th1 over-differentiation. In this study, an LTβR *in vivo* blockade (LTβR-Ig) was employed to investigate how the LT<sub>α1β2</sub>-LTβR induced signaling is involved in controlling the Th1 differentiation within well-established lymphoid structures during HSV-1 infection. We revealed that T cell-derived LT<sub>α1β2</sub> could unexpectedly limit the Th1 response by constricting expression of IL-12 from monocyte-derived cells.

### Materials and Methods

**Mice.** The mice used in this work all were on a C57BL/6 background. Wild type C57BL/6 mice were purchased from Beijing Vital River Co., Ltd. *Ltb*<sup>-/-</sup>, *Lta*<sup>-/-</sup>, *Light*<sup>-/-</sup>, *Tcra*<sup>-/-</sup>, μMT, OT-II transgenic, *Lta*-deficient OT-II transgenic and *LysM*<sup>Δ*Ltr*</sup> mice were bred and housed under specific pathogen-free (SPF) conditions. *Tcra*<sup>-/-</sup> and OT-II transgenic mice were obtained from The Jackson Laboratory. Mice were used at 6–10 weeks of age. Animal care and experiments were performed in accordance with the guidelines of the Institute of Biophysics, Chinese

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Academy of Sciences, using protocols approved by the Institutional Laboratory Animal Care and Use Committee, including any relevant details.

**Foot-pad infection and vaccination model.** HSV-1 (strain 17) was kindly provided by Dr. Thomas Kristie, LVD/NIAID/NIH, and amplified with *vero* cells (ATCC) purified through sucrose-dextran gradient centrifuge<sup>16</sup>. A total of  $5 \times 10^7$  pfu of HSV-1 in 50  $\mu$ l of PBS was subcutaneously injected into the foot-pad of mice after anesthesia. In the Heat-iHSV (heat-inactivated HSV-1) infection model,  $5 \times 10^7$  pfu of HSV-1 was heat-inactivated at 60 °C for 30 min and injected into the mouse foot-pad. In an OVA-CpG foot-pad vaccination model, mixture of 100  $\mu$ g of OVA and 50  $\mu$ g of CpG-1826 was injected into the foot-pad.

**In vivo blockade of LT $\beta$ R signaling or cytokines.** LT $\beta$ R-Ig was i.p. administered, 100  $\mu$ g/mouse one day before HSV-1 infection<sup>17</sup>. Control mice were injected with the same volume of the carrier only (PBS) or human IgG (100  $\mu$ g/mouse). Anti-IFN $\gamma$  (XMG1.2, Bioxell, US), anti-IL-12p75 (R5-9A2, Bioxell, US) or control rat IgG (Biogen, US) was i.p. administered, 500  $\mu$ g/mouse every third day, three times in total, as described before<sup>18,19</sup>.

**Measurement of IFN $\gamma$ -secreting CD4<sup>+</sup> T cells.** Cells isolated from the draining LNs (popliteal LN and inguinal LN) were re-suspended in R10 medium (RPMI1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin). CD8<sup>-</sup> cells were purified using an EasySep Biotin Selection Kit (STEMCELL, Canada), and a total of  $2 \times 10^5$  purified cells were stimulated with Heat-iHSV (heat-inactivated at 60 °C for 30 min, MOI = 10) for 24–48 hrs. The number of IFN $\gamma$ -secreting CD4<sup>+</sup> cells was determined by an IFN $\gamma$  ELISPOT Assay Kit (BD Biosciences, US). Spots were captured and calculated with the ImmunoSpot Analyzer (CTL, US).

**Detection of cytokines.** Lymph nodes were homogenized and centrifuged at 12,000  $\times$  g, 10 min, for supernatant collection. Protease Inhibitor Cocktail (100 $\times$ , CWBio, China) was added during supernatant collection. IFN- $\gamma$ , IL-12p70 and MCP-1 levels in the supernatant were detected by mouse Inflammation CBA assay (BD Biosciences, US).

**mRNA and DNA detection.** RNA was extracted using the RNeasy Plus Universal Micro kit (Qiagen, US). The quality and quantity of the total RNA were assessed with Nanodrop spectrophotometer (ND 2000C; Thermo Fisher Scientific, US). cDNA was reverse-transcribed using a First Strand cDNA Synthesis Kit (Thermo Scientific, US). Real-time RT-PCR was performed using an ABI7500 (Applied Biosystems, US). cDNA was amplified using SYBR Premix Ex Taq<sup>TM</sup> mix (Takara, Japan). HSV DNA was extracted using the TIANamp DNA kit (TIANGEN, China). HSV DNA level was normalized to GAPDH and murine gene expression was normalized to  $\beta$ -actin and calculated using 7500 software v2.0.6 (Applied Biosystems, US). Primers used are listed: HSV-gE: forward, 5'-GGGAGCACCACATAACCGACC-3'; reverse, 5'-GGCAAAGTCAACACAACAACGC-3'; GAPDH: forward, 5'-CGGACTGCAGCCCTCCC-3'; reverse, 5'-CCTTCCCAGTTTCCGACTGTCC-3'; *Tbx21*: forward, 5'-AGCAAGGACGGCGAATGTT-3'; reverse, 5'-GGGTGGACATATAAGCGGTTTC-3'; *Irfng*: forward, 5'-CTCTGAGACAATGAACGCTACA-3'; reverse, 5'-TCTTCCACATCTATGCCACTT-3'; *Gata3*: forward, 5'-CTCGGCCATTTCGTACATGGAA-3'; reverse, 5'-GGATACCTCTGCACCGTAGC-3'; *Il4*: forward, 5'-GGTCTCAACCCAGCTAGT-3'; reverse, 5'-GCCGATGATCTCTCTCAAGTGAT-3'; *Bcl6*: forward, 5'-CCGGCAGCTAGTGATGTT-3'; reverse, 5'-TGTCTTATGGGCTCTAAACTGCT-3'; *Il21*: forward, 5'-GGACCCTTGCTGTCTGGTAG-3'; reverse, 5'-TGTGGAGCTGATAGAAGTTCAGG-3';  $\beta$ -actin: forward, 5'-ACACCCGCCACCAGTTCGC-3'; reverse, 5'-ATGGGGTACTTCAGGGTCAGGGTCAGGATA-3'.

**Surface and intracellular staining.** Draining lymph nodes were digested with 1 mg/mL collagenase IV (Sigma-Aldrich, US) and 200  $\mu$ g/mL DNaseI (Sigma-Aldrich, US) at 37 °C for 30 minutes. Flow cytometry data were acquired on an LSRFortess (BD, US) with FACSDiva software (BD, US). Data were analyzed using FlowJo software (Tree Star, Ashland, USA). Dead cells were excluded by LIVE/DEAD<sup>®</sup> Fixable Yellow Dead Cell Stain Kit (Invitrogen, US). The antibodies used were: anti-CD11b (M1/70), anti-CD4 (RM4-5), anti-CD8 $\alpha$  (53-6.7) and anti-Tbet (eBio4B10) from eBioscience (US); anti-CD11c (N418), anti-CD8 $\alpha$  (53-6.7), anti-Ly6C (HK1.4) and anti-MHCII (M5/114.15.2) from BioLegend (US).

**Cell sorting.** Draining lymph nodes were digested with 1 mg/mL collagenase IV (Sigma-Aldrich, US) and 200  $\mu$ g/mL DNaseI (Sigma-Aldrich, US) at 37 °C for 30 minutes. CD4<sup>+</sup> T cells (CD3<sup>+</sup>CD4<sup>+</sup>), monocytes (CD11c<sup>-</sup>CD11b<sup>+</sup>Ly6C<sup>hi</sup>MHC-II<sup>+</sup>), moDCs (CD11c<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>hi</sup>MHC-II<sup>+</sup>) and non-monocyte-derived DCs (CD11c<sup>+</sup>Ly6C<sup>-</sup>MHC-II<sup>+</sup>, used as control) were sorted on an AriaII (BD, US). Purified cells were checked using LSRFortess (BD, US) with FACSDiva software (BD, US). Purity of the indicated cell population was more than 90%. The antibodies used were: anti-CD3 $\epsilon$  (145-2C11), anti-CD4 (RM4-5), anti-CD11b (M1/70) from eBioscience (US); anti-CD11c (N418), anti-CD8 $\alpha$  (53-6.7), anti-Ly6C (HK1.4) and anti-MHCII (M5/114.15.2) from BioLegend (US).

**Generation of bone marrow chimeras.** Bone marrow cells ( $2 \times 10^6$ ) from the indicated donors were transferred intravenously into each recipient mouse, which had been lethally irradiated with a single dose of 1000 rad. Donor-derived cells can be found in the peripheral blood or primary and secondary lymphoid organs of the recipient mice after reconstitution<sup>20</sup>. The bone-marrow chimeric mice were infected after an approximately 8-week reconstitution after check.

**Statistical Analysis.** Data were shown by GraphPad Prism software (version 7.0, US). For XY curves, statistical analyses were performed using the two-way ANOVA multiple comparisons, with data shown as the

mean  $\pm$  SEM. For histograms, statistical analyses were performed using a two-tailed unpaired Student's *t* test unless stated otherwise, with data shown as the mean  $\pm$  SEM. Differences with a *P*-value  $< 0.05$  were considered significant. n.s., not significant; \**P*  $< 0.05$ ; \*\**P*  $< 0.01$ ; \*\*\**P*  $< 0.001$ , \*\*\*\**P*  $< 0.0001$ .

## Results

**LT $\alpha_1\beta_2$ -LT $\beta$ 3R signaling is required to limit an excessive anti-HSV-1 Th1 response.** Differentiation of helper T cells is controlled by the LT $\beta$ 3R mediated signaling but may differ under specific infections or diseases. To describe the differentiation of helper T cells after HSV-1 infection, mRNA from CD4<sup>+</sup> T cells in the popliteal lymph nodes was analyzed on day 4 post HSV-1 foot-pad infection. In CD4<sup>+</sup> T cells purified from LT $\beta$ 3R-Ig-treated mice, lower expression of Th2 signature genes (*Il-4*) and Tfh signature genes (*Bcl6*, *Il-21*)<sup>21,22</sup> was observed, while the expression of Th1 signature genes (*Tbx21*, *Ifng*)<sup>23</sup> was overly up-regulated (Fig. 1a), showing an enhanced Th1-biased feature. Furthermore, during the process of helper T cell differentiation, the percentage of CD4<sup>+</sup>Tbet<sup>+</sup> cells remained high in LT $\beta$ 3R-Ig-treated mice but declined in wild-type (WT) mice (Fig. 1b, gating strategy shown in Supplementary Fig. 1a and number of CD4<sup>+</sup>Tbet<sup>+</sup> cells shown in Supplementary Fig. 1b). Further, IFN $\gamma$ -secreting CD4<sup>+</sup> T cells were detected on day 6, day 10 and day 14 post infection (p.i.) (Fig. 1c) using an enzyme linked immunospot assay (Elispot) under *ex vivo* stimulation with heat-inactivated HSV-1 (Heat-iHSV) for 24–48 hours. Consistent with the up-regulation of Th1 signature genes and the increasing of CD4<sup>+</sup>Tbet<sup>+</sup> cells, the anti-HSV-1 Th1 response was continuously enhanced in the LT $\beta$ 3R-Ig-treated mice over time. The LT $\beta$ 3R-Ig-induced prolonged increasing of Tbet<sup>+</sup>CD4<sup>+</sup> T cells from day 6 p.i. (Fig. 1b) finally resulted in an enhanced Th1 response on day 14 p.i. These together suggest that activation of the LT $\beta$ 3R pathway limits Th1 cell development in HSV-1 infection.

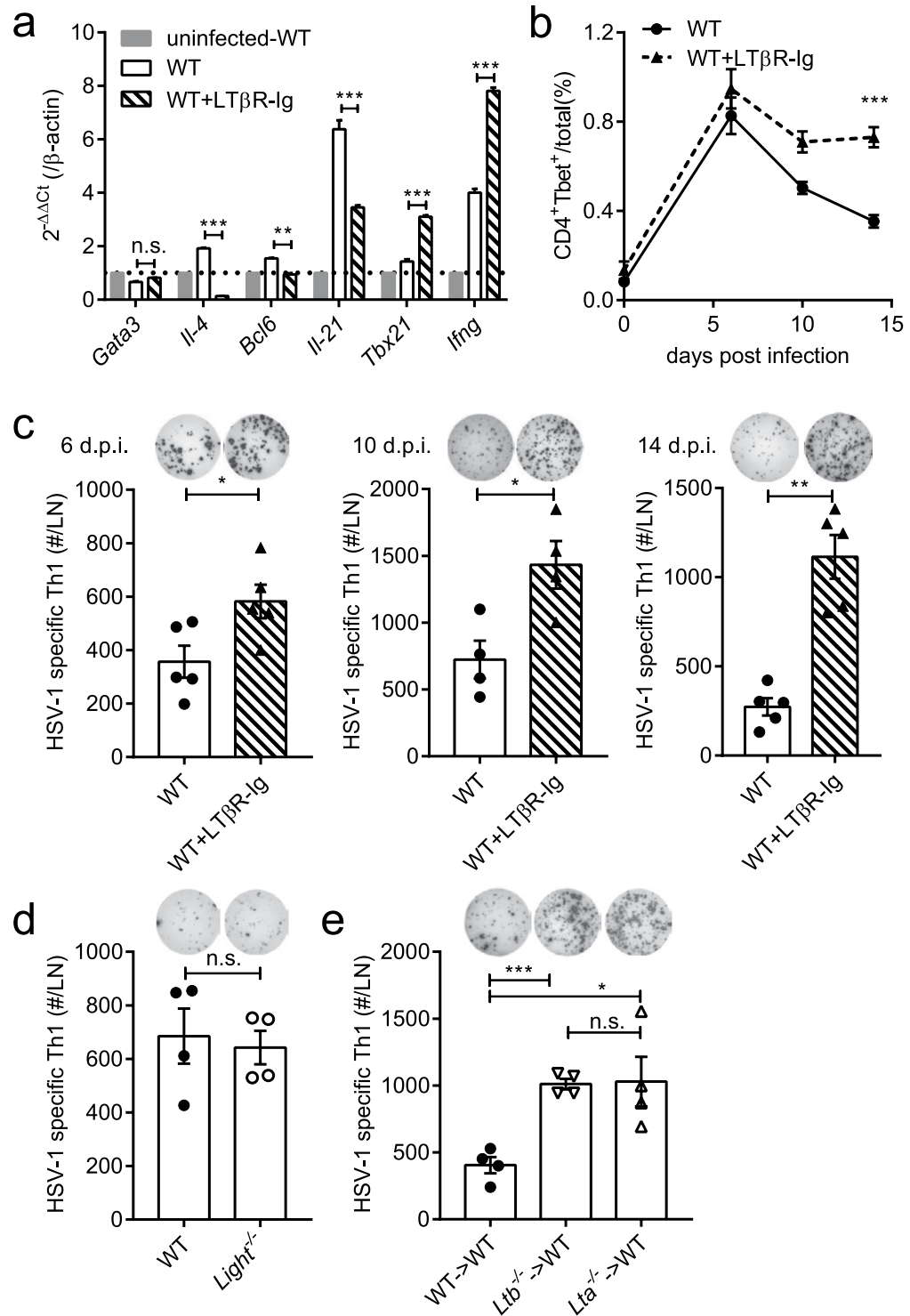
There are two ligands for activation of the LT $\beta$ 3R pathway, LT $\alpha_1\beta_2$  and LIGHT (homologous to LT $\alpha_1\beta_2$ ). We previously found that the innate LT $\alpha_1\beta_2$ /LIGHT signaling could promote an HSV-1-induced inflammation in immunocompromised mice<sup>24</sup>. To further identify the contributing ligand for LT $\beta$ 3R in limiting the Th1 response in a wild-type (WT) background, mice with homozygous deletion for LIGHT (*Light*<sup>-/-</sup>) or LT $\alpha_1\beta_2$  (*Ltb*<sup>-/-</sup> and *Lta*<sup>-/-</sup>) were used. Comparable Th1 responses were detected in *Light*<sup>-/-</sup> and WT mice (Fig. 1d). However, *Lta*<sup>-/-</sup> mice were reported to be more susceptible to HSV-1 infections<sup>25</sup>, where lack of lymph nodes (LNs) and CTL responses were thought to be the major cause. To rule out the interference from the disorganized lymphoid structures, bone marrow chimeric mice were prepared and infected. Bone marrow cells from *Ltb*<sup>-/-</sup>, *Lta*<sup>-/-</sup> vs. WT mice were adoptively transferred to the WT mice before infection, abbreviated as *Ltb*<sup>-/-</sup>  $\rightarrow$  WT, *Lta*<sup>-/-</sup>  $\rightarrow$  WT, and WT  $\rightarrow$  WT. *Ltb*<sup>-/-</sup>  $\rightarrow$  WT and *Lta*<sup>-/-</sup>  $\rightarrow$  WT mice both generated an enhanced anti-HSV-1 Th1 response, compared to the WT  $\rightarrow$  WT mice (Fig. 1e). Together, these indicate that LT $\alpha_1\beta_2$  induces a limitation of the Th1 response in HSV-1 infection.

**The LT $\alpha_1\beta_2$ -LT $\beta$ 3R induced Th1 limitation is not restricted to a specific viral infection.** To rule out the possibility that an enhanced Th1 response might result from a higher viral load caused by the LT $\beta$ 3R-Ig blockade, viral load in peripheral nervous tissues were detected on day 8 p.i. There was no significant difference in the peripheral viral load with or without LT $\beta$ 3R-Ig treatment (Fig. 2a). The levels of the HSV-1 genome DNA in the DLN were also comparable between these two groups (Fig. 2b). Moreover, the Heat-iHSV infection model was employed to exclude the interference of viral replication. Though Heat-iHSV induced a weaker Th1 response than HSV-1 infection, LT $\beta$ 3R signaling deficiency still resulted in an enhanced Th1 response in the Heat-iHSV infection (Fig. 2c).

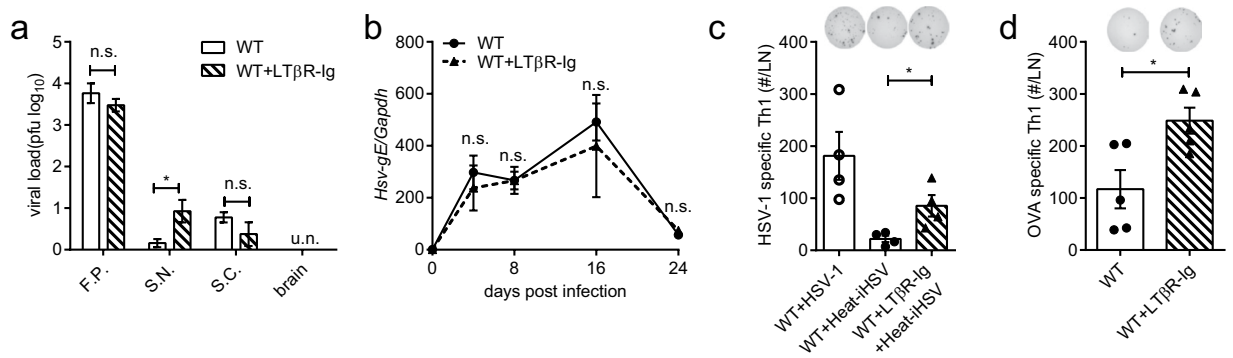
To further investigate whether the enhanced Th1 response by LT $\alpha_1\beta_2$ -LT $\beta$ 3R signaling deficiency is restricted to a specific viral infection, mice were immunized using OVA combined with CpG oligodeoxynucleotides (CpG-1826, a TLR9 agonist) as adjuvant (OVA-CpG). After OVA-CpG immunization, the LT $\beta$ 3R signaling blockade again resulted in an enhanced anti-OVA Th1 response (Fig. 2d).

**T cell-derived LT $\alpha_1\beta_2$  is required to limit Th1 response by modulating the lymphoid microenvironment.** B cell-derived LT was proved essential in recruiting CXCR5<sup>+</sup> T cells into the germinal center in *Heligmosomoides polygyrus* infection<sup>26</sup>. To determine if B cell-derived LT $\alpha_1\beta_2$  could limit the Th1 response, splenic B cells purified from *Lta*<sup>-/-</sup> vs. WT mice were adoptively transferred to the B-cell-deficient ( $\mu$ MT) mice before HSV-1 infection, abbreviated as B-*Lta*<sup>-/-</sup> and B-WT mice. When analyzed on day 14 p.i., B-*Lta*<sup>-/-</sup> mice generated a weaker Th1 response compared to the B-WT mice (Supplementary Fig. 2a–c). Given that LT is highly expressed on Th1 cells and was traditionally thought as a hallmark molecule of Th1 cells, we wondered if T cell-derived LT $\alpha_1\beta_2$  would conversely limit an excessive Th1 response. To detect the role of LT $\alpha_1\beta_2$  from T cells, splenic T cells from *Lta*<sup>-/-</sup> vs. WT mice were adoptively transferred to the T-cell-deficient (*Tcra*<sup>-/-</sup>) mice before infection, abbreviated as T-*Lta*<sup>-/-</sup> and T-WT mice. When analyzed on day 14 p.i., an enhanced Th1 response was generated in the T-*Lta*<sup>-/-</sup> mice compared to the T-WT mice (Fig. 3a,b), suggesting that LT $\alpha_1\beta_2$  from T cells limits the Th1 response in HSV-1 infection.

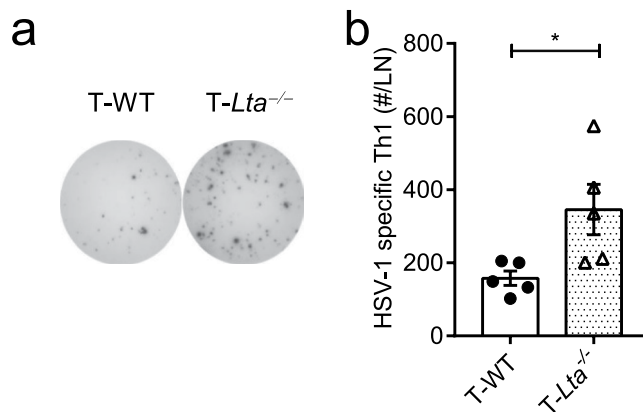
To further determine whether T cell-derived LT $\alpha_1\beta_2$  limits the Th1-biased differentiation in an intracellular manner or indirectly by modulating the lymphoid environment via LT $\beta$ 3R activation, mixed T cells from WT (CD45.1<sup>+</sup>) and *Lta*<sup>-/-</sup> (CD45.1<sup>-</sup>) mice (1:1) were adoptively transferred to *Tcra*<sup>-/-</sup> mice. After HSV-1 infection, there were no significant difference between the percentages of WT (CD45.1<sup>+</sup>) and *Lta*<sup>-/-</sup> (CD45.1<sup>-</sup>) CD4<sup>+</sup> cells in the LN of the recipient *Tcra*<sup>-/-</sup> mice. Percentages of CD4<sup>+</sup>Tbet<sup>+</sup> cells in WT (CD45.1<sup>+</sup>) vs. *Lta*<sup>-/-</sup> (CD45.1<sup>-</sup>) CD4<sup>+</sup> T cells were also comparable. *Lta*-deficient T cells differentiated in almost the same way as wild-type T cells when situated in the same environment (Supplementary Fig. 3a–c). We repeated this mixed-transferring experiment by transferring mixed WT-OT-II (Thy1.1<sup>-</sup>) T cells and *Lta*<sup>-/-</sup>-OT-II T (Thy1.1<sup>+</sup>) cells (1:1) to *Tcra*<sup>-/-</sup> mice, and the recipient mice were immunized with OVA-CpG. The OT-II T cell differentiation again showed no difference between *Lta*<sup>-/-</sup> and WT donor T cells in the recipient mice on day 10 post-immunization (Supplementary



**Figure 1.** LT-LTβR signaling deficiency enhances the anti-HSV-1 Th1 response. **(a)** Expression of the Th1/Th2/Tfh-related transcriptional factors and cytokines in purified CD4<sup>+</sup> T cells on day 4 p.i. in the popliteal LNs tested by real-time qPCR (n = 3/group). **(b)** Percentages of CD4<sup>+</sup>Tbet<sup>+</sup> cells in total cells in the draining LNs (popliteal LN and inguinal LN) from WT mice (solid line) and LTβR-Ig-treated mice (dotted line) (n = 5/group). **(c)** The Th1 response of LTβR-Ig-treated mice on 6 d.p.i. (n = 5/group), 10 d.p.i. (n = 4/group) and 14 d.p.i. (n = 5/group), including immunospots and absolute numbers of IFNγ-secreting CD4<sup>+</sup> cells per LN. **(d)** The Th1 response of *Light*<sup>-/-</sup> mice on day 14 p.i. (n = 4/group), including immunospots and absolute numbers of IFNγ-secreting CD4<sup>+</sup> cells per LN. **(e)** The Th1 response of *Lta*<sup>-/-</sup> → WT and *Ltb*<sup>-/-</sup> → WT bone marrow chimeric mice on day 14 p.i. (n = 4/group), including immunospots and absolute numbers of IFNγ-secreting CD4<sup>+</sup> cells per LN. Data are representative of three independent experiments, shown as mean ± SEM. n.s., not significant; \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001. (Two-way ANOVA multiple comparisons for b).



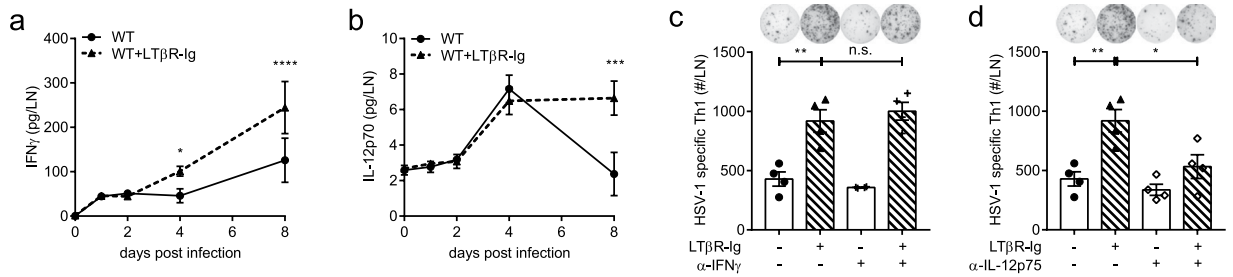
**Figure 2.** The LT $\beta$ R-Ig blockade results in an enhanced Th1 response in a non-viral-replication-dependent manner. **(a)** Viral load of peripheral nervous tissues tested on day 8 p.i. (F.P.: foot-pad; S.N.: sciatic nerves; S.C.: spinal cord.  $n = 4$ /group). **(b)** HSV-gE detected in the DLN ( $n = 4$ /group). **(c)** Th1 response in the Heat-iHSV infection model on day 14 p.i.  $5 \times 10^7$  pfu of HSV-1 was heat-inactivated at 60 °C for 30 min and injected into the foot-pad. ( $n = 4$ /group), including immunospots and absolute numbers of IFN $\gamma$ -secreting CD4 $^+$  cells per LN. **(d)** Th1 response in an OVA-CpG foot-pad vaccination model (100  $\mu$ g of OVA and 50  $\mu$ g of CpG-1826) on day 10 post immunization ( $n = 5$ /group), including immunospots and absolute numbers of IFN $\gamma$ -secreting CD4 $^+$  cells per LN. Data are representative of three independent experiments, shown as mean  $\pm$  SEM. n.s., not significant; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . (Two-way ANOVA multiple comparisons for **b**).



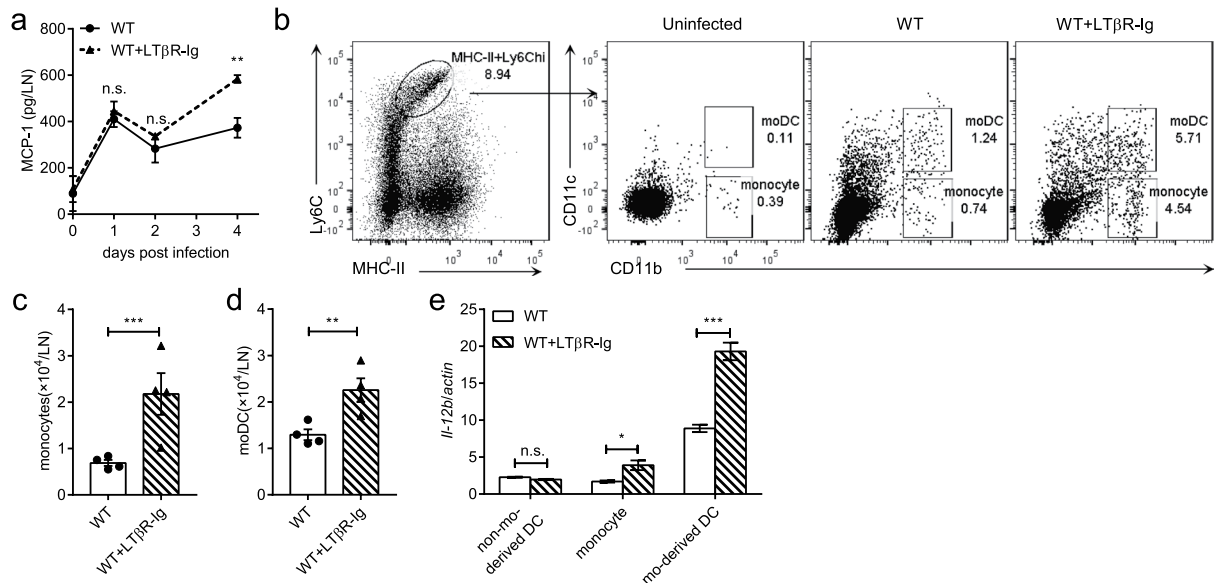
**Figure 3.** T cell-derived LT is required to limit Th1 response. T cells purified from WT and *Lta* $^{-/-}$  mice were transferred to *Tcra* $^{-/-}$  mice on the day before HSV-1 infection, abbreviated as T-WT and T-*Lta* $^{-/-}$  mice; Th1 response in the T-cell conditional LT $\alpha$ -deficient mice on day 14 p.i. ( $n = 5$ /group), including immunospots **(a)** and absolute number of IFN $\gamma$ -secreting CD4 $^+$  cells per LN **(b)**. Data are representative of three independent experiments, shown as mean  $\pm$  SEM. n.s., not significant; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

Fig. 3d–f). These indicate that the LT $\alpha_1\beta_2$  deficiency in T cells does not intrinsically enhance the activation of Th1 cells. The fact that LT $\alpha_1\beta_2$  from WT-T cells are sufficient to regulate a normal Th1 differentiation of *Lta* $^{-/-}$  donor T cells actually suggests that T cell-derived LT $\alpha_1\beta_2$  regulates the Th1 differentiation by modulating the lymphoid microenvironment.

**Blocking the LT $\alpha_1\beta_2$ -LT $\beta$ R signaling enhances the Th1 response through over-production of IL-12.** Based on the above, we tried to discover the potential Th1 promoting factors in the LT $\alpha_1\beta_2$ -LT $\beta$ R signaling deficient lymphoid microenvironment. Increased IFN $\gamma$  secretion was observed in the popliteal LN in LT $\beta$ R-Ig-treated mice from day 2 post HSV-1 infection (Fig. 4a). Release of IL-12 was also maintained at a high level after day 4 p.i. under the LT $\beta$ R-Ig blockade (Fig. 4b). As previously reported, IL-12 induces expression of both T-bet and Bcl6 via the transcription factor STAT4. During the transitional stage of both phenotypes, T-bet will finally promote a full Th1 polarization<sup>27,28</sup>. IFN $\gamma$  was also reported to support the Th1 polarization in an auto-crine manner<sup>29</sup>. To test if the LT $\beta$ R-Ig enhanced Th1 response would be repressed by blocking the potential IL-12 or IFN $\gamma$  pathways, neutralizing antibodies for the candidate Th1-promoting cytokines were employed. After systemic administration of the neutralizing antibodies every three days for three times in total, the enhanced Th1 response induced by the LT $\beta$ R-Ig blockade was kept enhanced under the neutralization of IFN $\gamma$  (Fig. 4c), but reversed by neutralizing IL-12 (Fig. 4d). These results indicate that the LT $\beta$ R-Ig induces an over-production of IL-12 which promotes an excessive Th1 response.



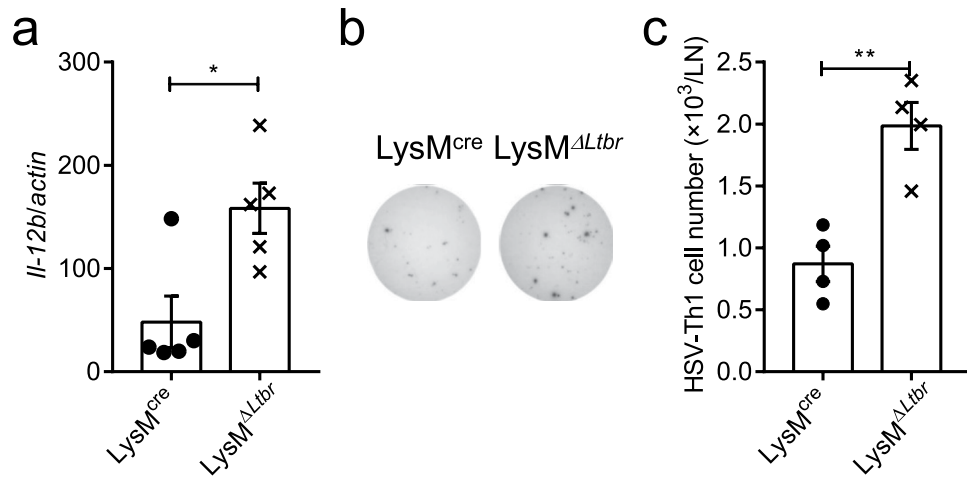
**Figure 4.** Blocking LT-LT $\beta$ R signaling enhances Th1 response by over-production of IL-12. **(a)** IFN $\gamma$  secretion in the popliteal LN from WT mice (solid line) and LT $\beta$ R-Ig-treated mice (dotted line) detected by CBA test ( $n = 5$ /group). **(b)** IL-12 secretion in the popliteal LN from WT mice (solid line) and LT $\beta$ R-Ig-treated mice (dotted line) detected by CBA test ( $n = 5$ /group). **(c)** Anti-IFN $\gamma$  (clone XMG1.2, 500  $\mu$ g/mouse) was i.p. administered every third day from the day before HSV-1 infection. Th1 response was detected on day 14 p.i. by Elispot ( $n = 4$ /group). Immunospots and absolute numbers of IFN $\gamma$ -secreting CD4 $^{+}$  cells per LN are shown. **(d)** Anti-IL-12p75 (clone R5-9A2, 500  $\mu$ g/mouse) was i.p. injected every third day from the day before HSV-1 infection. Th1 response was detected on day 14 p.i. by Elispot ( $n = 4$ /group). Immunospots and absolute numbers of IFN $\gamma$ -secreting CD4 $^{+}$  cells per LN are shown. Data are representative of three independent experiments, shown as mean  $\pm$  SEM. n.s., not significant; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . (Two-way ANOVA multiple comparisons for **a,b**).



**Figure 5.** Blocking the LT-LT $\beta$ R signaling promotes infiltration of monocytes and monocyte-derived DCs in the draining lymph node. **(a)** MCP-1 secretion in the popliteal LN from WT mice (solid line) and LT $\beta$ R-Ig-treated mice (dotted line) detected by CBA test ( $n = 5$ /group). **(b)** Gating strategy of monocytes and moDCs, shown by representative dot plots from day 4 p.i. **(c)** Absolute number of monocytes in the popliteal LNs ( $n = 4$ /group). **(d)** Absolute number of moDCs in the popliteal LNs ( $n = 4$ /group). **(e)** Monocytes, moDCs and non-monocyte-derived DCs (CD11c $^{+}$ CD11b $^{-}$ Ly6C $^{-}$ MHC-II $^{+}$ ) were purified on day 4 p.i. Expression of IL-12 was analyzed by RT-PCR ( $n = 3$ /group). Data are representative of three independent experiments, shown as mean  $\pm$  SEM. n.s., not significant; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . (Two-way ANOVA multiple comparisons for **(a)**; two-tailed paired Student's  $t$  test for **c–e**).

### LT $\alpha_1\beta_2$ -LT $\beta$ R signaling limits the Th1 response by controlling infiltration of monocytes and monocyte-derived DCs.

Who is the provider of the increased IL-12 during the LT $\alpha_1\beta_2$ -LT $\beta$ R signaling deficiency? Release of MCP-1 was raised after the LT $\beta$ R-Ig blockade in the popliteal LN early post HSV-1 infection (Fig. 5a). MCP-1, also known as CCL2, is a monocyte-recruiting chemokine<sup>30</sup>. Monocytes are known for secreting IL-12 during inflammation<sup>31</sup>, but monocyte-derived DCs (moDCs, CD11c $^{+}$ CD11b $^{+}$ Ly6C $^{hi}$ MHC-II $^{+}$ ) and CD8 $\alpha^{+}$  DCs are the major cellular source of IL-12 in *Toxoplasma gondii* cysts infection<sup>32,33</sup>. However, in the murine model of acute HSV-1 infection, CD8 $\alpha^{+}$  DCs were considered critical for the CTL response<sup>34–38</sup> while CD4 $^{+}$  T cells were proved to be primed by migratory APCs (no clarification of which subset of APCs)<sup>38,39</sup>. IL-12 production increased soon after HSV-1 infection and decreased after day 4 p.i. in wild-type mice. However, the LT $\beta$ R-Ig



**Figure 6.** Blocking LT-LT $\beta$ R signaling enhances Th1 response by up-regulating IL-12 expression from monocyte-derived cells. **(a)** Expression of IL-12 in the popliteal LN of LysM $\Delta$ Ltbr mice on day 4 p.i. (n = 5/group). **(b,c)** Th1 response of LysM $\Delta$ Ltbr mice on day 14 p.i. (n = 4/group). Immunospots **(b)**, and absolute number of IFN $\gamma$ -secreting CD4<sup>+</sup> cells per LN **(c)**. Data are representative of three independent experiments, shown as mean  $\pm$  SEM. n.s., not significant; \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001. (Two-tailed paired Student's *t* test for **a,c**).

blockade caused a prolonged IL-12 production after day 4 p.i., suggesting that the turning point of IL-12 provider cells happens around day 4 p.i. due to the LT $\beta$ R-Ig treatment. With the LT $\beta$ R-Ig administered, monocytes (CD11c<sup>-</sup>CD11b<sup>+</sup>Ly6C<sup>hi</sup>MHC-II<sup>+</sup>) and moDCs (CD11c<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>hi</sup>MHC-II<sup>+</sup>) were both found increased post HSV-1 infection in the LN (Fig. 5b–d). To further identify the main affected provider cells of IL-12, monocytes, moDCs and non-monocyte-derived DCs (CD11c<sup>+</sup>Ly6C<sup>-</sup>MHC-II<sup>+</sup>, used as control) were purified from the popliteal LN on day 4 p.i. and expression of IL-12 was analyzed by RT-PCR. Higher IL-12 expression was detected in moDCs and monocytes in the LT $\beta$ R-Ig treated mice than control mice (Fig. 5e). Finally, LysM $\Delta$ Ltbr mice (hybridized from *Ltbr*<sup>flx/flx</sup> × LysM<sup>cre</sup>) were utilized to confirm the function of LT $\beta$ R signaling in monocytes and monocyte-derived cells *in vivo* (Supplementary Fig. 4a,b). Though no more infiltration of monocytes or moDCs were found in LysM $\Delta$ Ltbr mice post infection (Supplementary Fig. 4c,d), a significant up-regulation of IL-12 in the DLNs of the LysM $\Delta$ Ltbr mice was observed on day 4 p.i. (Fig. 6a). Further, LysM $\Delta$ Ltbr mice also generated an enhanced Th1 response in HSV-1 infection (Fig. 6b,c). LT $\beta$ R activation of monocytes could inhibit the virus-induced up-regulation of IL-12 and limit the over-active Th1 response. Therefore, the accumulation of monocytes and moDCs with up-regulation of IL-12 promoted an enhanced Th1 response in the LT $\beta$ R-Ig-blocked mice.

## Discussion

LT is expressed on naive T cells and constitutively expressed on Th1 but not Th2 cells. Similar to IFN $\gamma$ <sup>29</sup>, LT is a key cytokine released from Th1 cells, which raises the possibility that LT may facilitate Th1 differentiation. In contrast, our data reveal that T cell-derived LT $\alpha_1\beta_2$  suppresses the Th1 response during HSV-1 infection. LT $\beta$ R-Ig treatment could also enhance the Th1 response to Heat-iHSV infection and OVA-CpG immunization. Thus, LT $\alpha_1\beta_2$  helps to limit the Th1 response in a negative feedback manner, which is opposite to the positive feedback regulation of IFN $\gamma$ . High expression of LT $\alpha_1\beta_2$  on Th1 cells may counter-regulate the excessive Th1 differentiation and induce a balanced helper T cell response.

LT $\alpha_1\beta_2$ -LT $\beta$ R signaling has been reported to regulate the helper T cell response. The LT $\beta$ R-deficiency-caused defective Th1 response was thought to increase the susceptibility to *Citrobacter rodentium*<sup>10</sup>. However, several studies reveal that LT from type III innate lymphoid cells (ILC3) is essential for control of *Citrobacter rodentium*<sup>40,41</sup>. In *Leishmania major* infection, *Ltbr*<sup>-/-</sup> and *Lta*<sup>-/-</sup> mice both favored Th2 cells rather than Th1 cells, but the LN structure was then considered to be important<sup>11</sup>. It is possible that parasites induce strong Th2 dominant responses in the infected microenvironment. These suggest that Th1/Th2 biased responses regulated by the LT $\alpha_1\beta_2$ -LT $\beta$ R signaling may differ depending on the pathogen-specific microenvironment to limit pathology and/or promote clearance of pathogens.

Recently, IFN $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells were reported to be increased in *Ltbr*<sup>-/-</sup> → WT mice in the small intestinal lamina after oral infection of rotavirus<sup>42</sup>. In our study, no increase of IFN $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cells were found after the LT $\beta$ R-Ig blockade. Given that CD8 $\alpha$ <sup>+</sup> DCs have been considered critical for an optimal CTL response in HSV-1 infection<sup>34–38</sup> and the increase of CD8 $\alpha$ <sup>+</sup> DCs in the draining LNs were found inhibited by the LT $\beta$ R-Ig, anti-HSV-1 CTL responses were not enhanced. Together, the LT $\alpha_1\beta_2$ -LT $\beta$ R signaling may play different roles in the Th1 and CTL responses in HSV-1 infection.

Altogether, this work uncovered a novel function of T cell-derived LT $\alpha_1\beta_2$  in controlling the defined Th1 differentiation in the adaptive immune response, which enhances our understanding of LT $\beta$ R-dependent Th1-induced autoimmune or inflammatory diseases. Targeting the LT $\beta$ R-induced pathway may provide insights for Th1-oriented vaccine designs and treatments of inflammatory diseases.

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### Author Contributions

K.Y., H.P. and Y.X.F. designed the experiments and analyzed the data; K.Y., Y.L. and Z.S. conducted the experiments; L.L., J.L. and H.X. contributed to reagents/materials; M.Z. provided important suggestions and comments. H.P. and Y.X.F. supervised the experiments; K.Y., H.P., and Y.X.F. wrote the manuscript.

### Additional Information

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