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RESEARCH PAPER

Long noncoding RNA Malat1 is not essential for T cell development and response to LCMV infection

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ABSTRACT

Long noncoding RNAs (lncRNAs) are emerging as critical mediators of various biological processes in the immune system. The current data showed that the lncRNA Malat1 is highly expressed in T cell subsets, but the function of Malat1 in T cell remains unclear. In this study, we detected the T cell development and both $CD8^+$ and $CD4^+$ T cell response to LCMV infection using *Malat1*$^{-/-}$ mice model. To our surprise, there were no significant defects in thymocytes at different developmental stages and the peripheral T cell pool with ablation of Malat1. During LCMV infection, *Malat1*$^{-/-}$ mice exhibited normal effector and memory $CD8^+$ T cells as well as T_{FH} cells differentiation. Our results indicated that Malat1 is not essential for T cell development and T cell-mediated antiviral response though it expresses at very high level in different T cell populations.

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Introduction

Long noncoding RNAs (lncRNAs) are non-protein coding transcripts, which coordinate diverse aspects of cell and tissue development [1]. It is well documented that lncRNAs regulate the development of cardiomyocytes, stem cells, epithelial cells, erythrocytes, and adipocyte [2]. A large number of studies have indicated lncRNAs also regulate the development and differentiation of several immune cell lineages, such as myeloid cells and dendritic cell development [3,4]. Previous works from different labs have characterized that distinct T cell subsets express unique profiles of lncRNAs at different stages of development [5–8]. The lncRNA Malat1 (metastasis-associated lung adenocarcinoma transcript 1) is a nuclear localized RNA, which is a highly conserved transcript that is involved in alternative splicing [9,10]. The expression of Malat1 is correlated with tumorigenesis and metastasis in multiple myeloma and solid tumors, suggesting its universal role in cancer [11]. Ma et al. reported that Malat1 plays a critical role in regulating proliferation and maintaining undifferentiated status of early-stage hematopoietic cells [12]. To our knowledge, although Malat1 plays essential role in many kinds of cancer cells and emerging roles in immune system, its role in T cell development and T cell-mediated immune response remains largely unknown.

T cells, the essential regulators of cellular immunity, are produced in thymus and undergo a series of well-documented differentiation steps. Pluripotent precursors derived from hematopoietic stem cells in the bone marrow migrate to the thymus, which initiate and sustain T cell development at $CD4^-CD8^-$ double-negative (DN) stage [13,14]. The surface expression of CD44 and CD25 characterizes the four major DN cell subsets: $CD44^+CD25^-$ (DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$ (DN3), and $CD44^-CD25^-$ (DN4) cells. When cells proceed to differentiate from DN2 to DN4 stages, the pre-TCR is expressed, which is comprised of the non-rearranging pre-TCR α-chain and a rearranged TCR β-chain [15]. Successful pre-TCR expression leads to DN4 cells transition to $CD4^+CD8^+$ double-positive (DP) thymocytes and replacement of the pre-TCR α-chain with a newly rearranged TCR α-chain, which yields a complete αβ TCR [16]. The αβ-TCR^+ DP thymocytes undergo the processes of positive and negative selection based on their relative ability to interact with thymic selecting ligands [17]. The positive-selected DP cells first differentiate into $CD4^+CD8^{lo}$ intermediate (IM) thymocytes, which then give rise to mature major histocompatibility complex (MHC) class II–restricted $CD4^+$ or MHC class I–restricted $CD8^+$ single-positive (SP) T cells. After that, mature T cells emigrate and join the peripheral lymphocyte pool.

T cells play vital roles in controlling the adaptive immune response. As they not only control a multitude of immune responses directly, but also regulate B cell immune responses. CD4-bearing T cells are associated with helper functions and CD8-bearing T cells are associated with cytotoxicity. $CD4^+$ T cells produce functional T helper cells that are tailored to their respective roles in host defense. Follicular helper T (T_{FH}) cells, a unique subset of T helper cells, are the specialized providers of B cell help, and are essential for germinal center (GC) formation and the development of high-affinity antibodies-

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secreted plasma cells and memory B cells [18,19]. T_{FH} cell differentiation is a multistage, multifactorial process [20]. Priming by dendritic cells triggers upregulation of ICOS on $CD4^+$ T cells, which is required for the expression of Bcl-6, a master regulator of T_{FH} cells differentiation [21–23]. Bcl-6 in turn induces chemokine receptor CXCR5 expression [18], which allows the early T_{FH} cells migrate to the border of the B cell follicular and undergo further differentiation. At the T cell-B cell border, early T_{FH} cells interact with activated B cells presenting cognate antigen, results in the early T_{FH} cells providing help to B cells. Meanwhile, cognate help from B cells drives the full development of T_{FH} cells. Within GC, T_{FH} cells continue to provide help to the B cells, facilitating the establishment of GC reaction and promoting the GC B cells differentiate into long-lived plasma cells and memory B cells. Reciprocal signals provided by the B cells are also crucial for sustaining the T_{FH} cells [24].

Cytotoxic effector $CD8^+$ T cells are an essential component of the immune system against various intracellular pathogens including viruses and intracellular bacterial. Upon encountering antigen, antigen-presenting cells (APCs) initiate responses from rare, antigen-specific $CD8^+$ T cells. The activated pathogen-specific $CD8^+$ T cells embark on a proliferative expansion in numbers and differentiate into primary effector populations. These effector $CD8^+$ T cells acquire the ability to produce interferon-γ (IFN-γ), lesser extent tumor-necrosis factor (TNF), and the ability to perform cytolysis to manifest antimicrobial functions. Most of the effector cells succumb to apoptosis during contraction phase, and only a small portion of surviving $CD8^+$ T cells initiate the memory pool and differentiate to long-lived memory cells, capable of providing strengthened protection against same pathogens [25–27]. Memory $CD8^+$ T cells are heterogeneous, consisting of at least four distinct subsets: effector memory T cells (T_{EM}), central memory T cells (T_{CM}), tissue-resident memory T cells (T_{RM}), and stem memory T cells [28]. At effector phage, $CD8^+$ T cells downregulate the expression of CD127 (also known as IL-7Rα) and CD62L, but generally are $KLRG1^{hi}$. The differentiation of effector cells to memory $CD8^+$ T cells is accompanied with CD127 upregulation and KLRG1 downregulation. T_{EM} cells and T_{CM} cells have different expression pattern of homing molecules, chiefs among these are CCR7 and CD62L [29]. T_{EM} cells and T_{CM} cells are phenotypically and functionally vary. T_{CM} cells usually reside in secondary lymphoid organs and have greater proliferation potential, whereas T_{EM} cells constitutively exert effector functions such as cytotoxicity [30,31].

Here we set out to investigate the function of Malat1 in T cell development and T cell-mediated immune response. To our surprise, despite Malat1 is highly expressed in T cell subsets, the role of Malat1 in T cell development and T cell-mediated antiviral response is nonessential, which suggests Malat1 may not be a master regulator in T cells.

Results

To identify new regulators of T cell development, we analyzed published RNA-seq data from T cell at the different developmental stages (GSE109125). Comparing with several well-known T cell specific genes (*Bcl11b, Id2, Lef1, Notch1, Rag1, Tcf3, Tcf7 and Tox*), we found the relative expression of the lncRNA Malat1 was especially higher in T cell subsets (Figure 1(a)). In addition, Malat1 was identified to play key roles in tumorigenesis, so that we chose this gene for further investigation in order to characterize its function in T cell development and response to infection. We isolated different T cell subsets and subjected them to quantitative RT-PCR by three pairs of primers located at 5′-region, middle of gene body and 3′-region, respectively. *Malat1* was detected in all populations of T cells at very high level, with peaking in DP thymocytes (Figure 1(b)).

To address the functional importance of Malat1 in the T cell lineage, we investigated the T cell development in *Malat1*$^{-/-}$ mice. Quantitative RT-PCR results indicated that the expression of *Malat1* was significantly decreased in the thymus, spleen and bone marrow from *Malat1*$^{-/-}$ mice (Figure 1(c)) as well as all subsets of thymocytes and peripheral T cells (**Data not shown**). These results confirmed that *Malat1* gene was completely inactivated in *Malat1*$^{-/-}$ mice. Mice with a null Malat1 allele were viable and fertile and showed no obvious signs of immune deficiency syndrome. The size and cellularity of thymi and spleens were similar in *Malat1*$^{-/-}$ mice and their littermate control mice (Figure 1(d, e)). The frequency and numbers of DN, CD4 SP, CD8 SP, and DP thymocytes were similar in *Malat1*$^{-/-}$ and their littermate control mice. The percentages and numbers of DN1-DN4 subsets based on expression of CD44 and CD25 were showed no difference between *Malat1*$^{-/-}$ mice and their littermate control mice (Figure 1(f-h)). TCRβ gene rearrangements occurs in DN3 transition to DN4 and DP stage, namely V(D)J recombination, and β-selection [16]. Intracellular staining of TCRβ in DN3, DN4 and DP thymocytes indicated *Malat1*$^{-/-}$ mice have no defects in TCRβ expression (Figure 1(i)). These result suggested Malat1 is not essential for maturation of T cell from the DN to SP stage.

Next we explored whether Malat1 is essential for negative and positive selection and/or lineage commitment of αβ thymocytes. The frequency of surface $TCRβ^{hi}$ subsets was similar in mice of both genotypes (Figure 2(a, b)). Downregulation of the expression of CD24 and CD69 states the intrathymic positively selected $TCRβ^{hi}$ thymocytes maturation [32]. We found that the maturation of $CD24^+CD69^+$ subsets to $CD24^-CD69^-$ cells was not blockaded in *Malat1*$^{-/-}$ mice (Figure 2(c, d)). $CD24^+CD69^+TCRβ^{hi}$ cells contains post-selection DP thymocytes and $CD4^+CD8^{lo}$ intermediate thymocytes, which are the precursors of immature $CD4^+$ or $CD8^+$ SP thymocytes [17]. We observed the numbers of DP, $CD4^+$, $CD8^+$ SP and $CD4^+CD8^{lo}$ thymocytes were not altered in Malat1-deficiency mice (Figure 2(e, f)). $CD24^-CD69^-TCRβ^{hi}$ cells only contains mature thymocytes [17], and we noted ablation of Malat1 did not affect the frequency and numbers of mature thymocytes (Figure 2(g, h)). Collectively, these results indicated that Malat1 is not required for late T cell development and lineage commitment. Given Malat1 is not essential for T cell development, we questioned whether deficiency in Malat1 changed the populations of peripheral T cells. We noted peripheral $CD4^+$ and $CD8^+$ T cells were similar in *Malat1*$^{-/-}$ mice and their littermate control mice (Figure 2(i, j)).

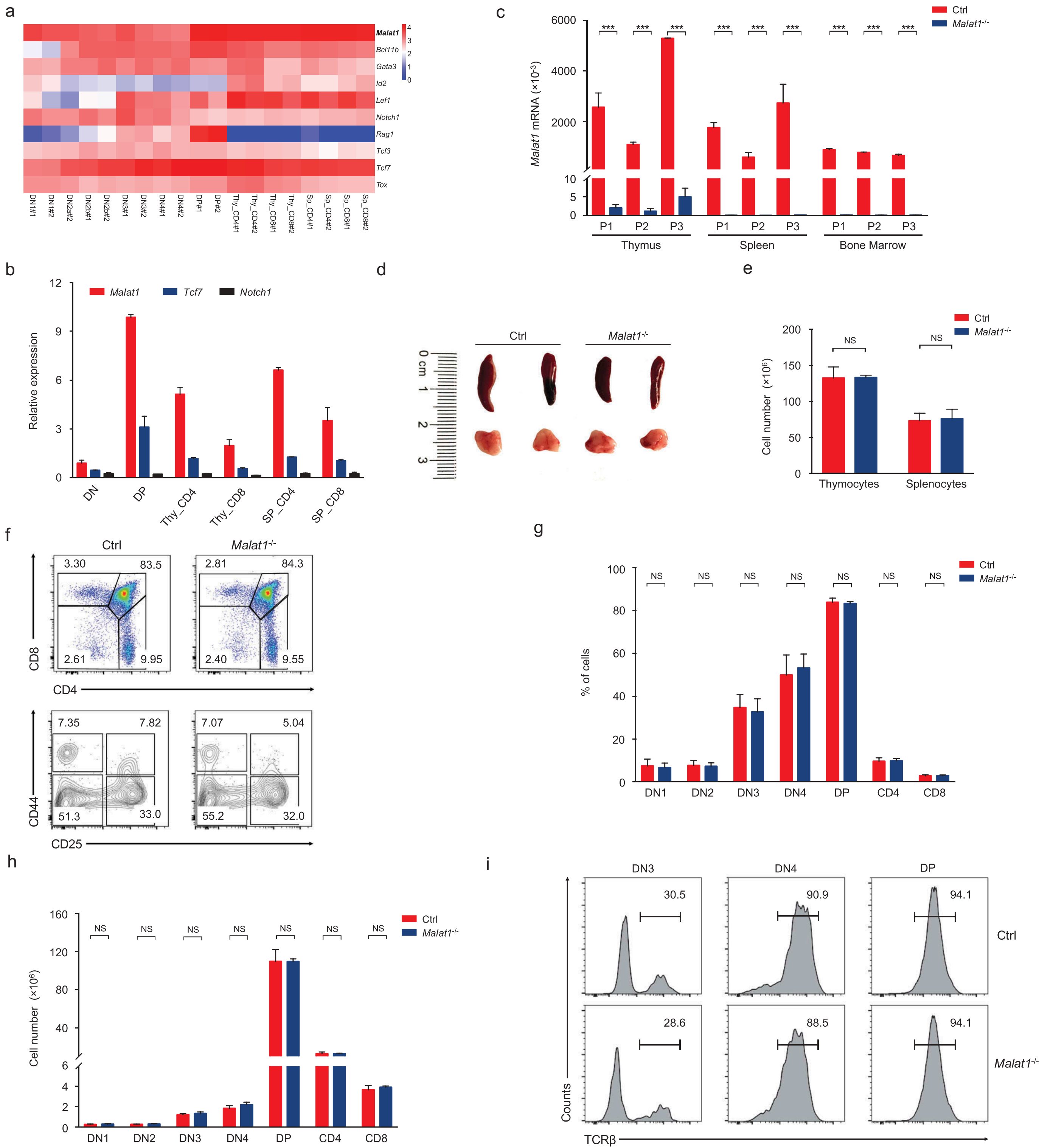

Figure 1. Malat1 is not required for early T cell development. (a) Expression of *Malat1* and key transcription factors of T cell development in different T cell subsets from published RNA-seq. (b) Analysis of *Malat1* expression in thymocytes and peripheral T cells by quantitative RT-PCR. Relative gene expression levels in each sample were normalized to *Hprt1*. (c) Analysis of *Malat1* expression in thymus, spleen and bone marrow from wild-type (Ctrl) and *Malat1*$^{-/-}$ mice. Relative gene expression levels in each sample were normalized to *Hprt1*. (d-e) The size (d) and cellularity (e) of thymus and spleen from wild-type (Ctrl) and *Malat1*$^{-/-}$ mice. (f) Surface staining of CD4 and CD8 on thymocytes and CD25 and CD44 on DN thymocytes. (g-h) The percentages (g) and numbers (h) of thymocytes in f. (i) Expression of TCRβ in DN3, DN4 and DP thymocytes. Data represent mean ± s.d.

Due to $CD4^+$ and $CD8^+$ T cells are essential mediators of cellular immune response. We next investigated whether Malat1 plays an important role in T cell-mediated antiviral immune. The mice were infected with lymphocytic choriomeningitis virus (LCMV), and the T_{FH} cells differentiation as well as effector and memory $CD8^+$ T cells were analyzed. We found that ablation of Malat1 did not alter $CD4^+$ T cells pool (Figure 3(a, b)) at 8 days after infection.

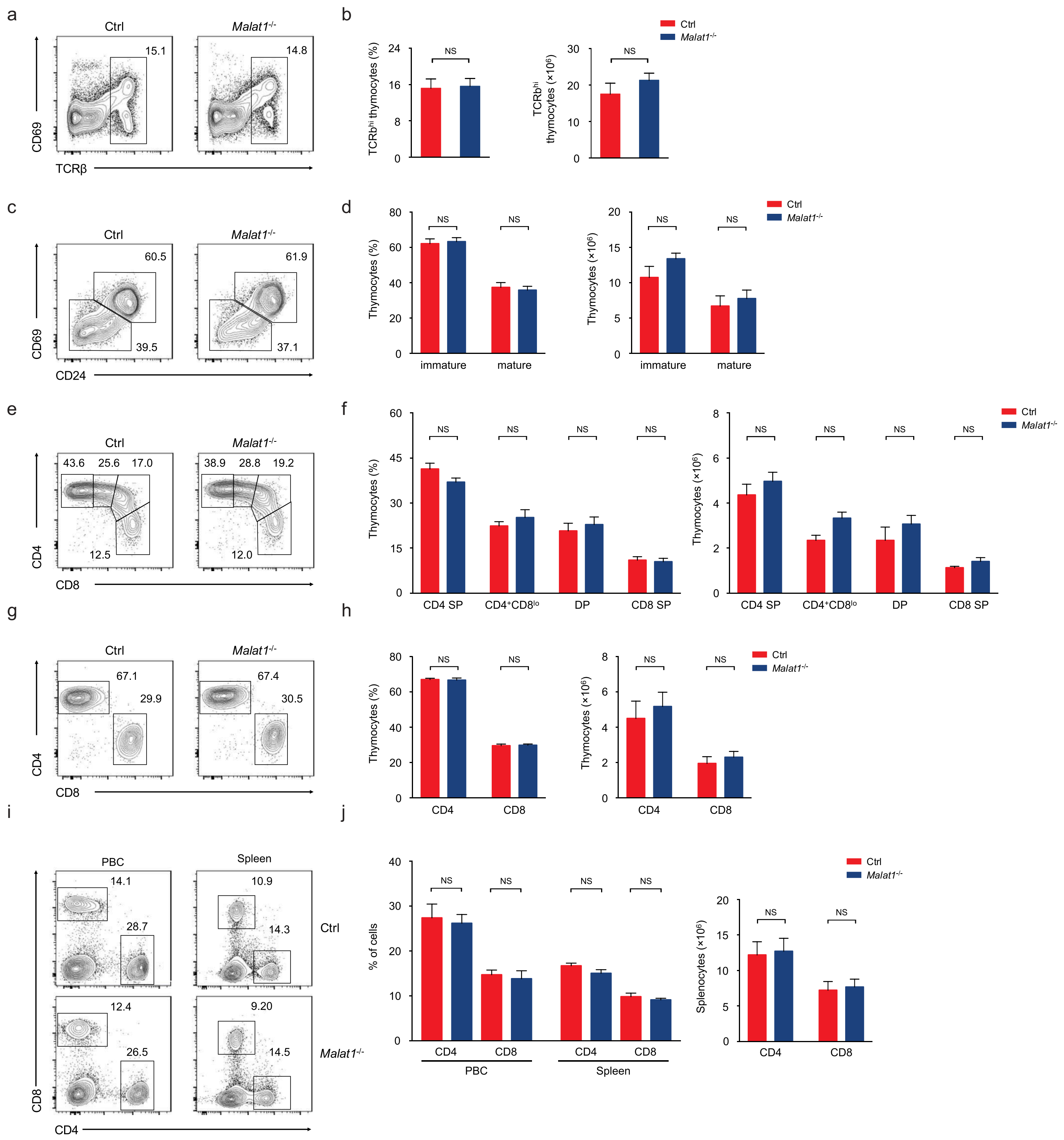

Figure 2. Ablation of Malat1 does not alter late T cell development and migration. (a-b) Flow cytometry analysis of post-selection TCRβ^{hi} thymocytes. The percentages (left) and numbers (right) of post-selection TCRβ^{hi} thymocytes are shown in b. (c-d) Flow cytometry analysis of mature (CD24^{-}CD69^{-}) thymocytes (bottom left) and immature (CD24^{+}CD69^{+}) thymocytes (top right) gated on post-selection TCRβ^{hi} thymocytes. The frequency (left) and numbers (right) of immature and mature subsets are shown in d. (e-f) Flow cytometry analysis of CD4^{+} SP, CD4^{+}CD8^{lo}, DP and CD8^{+} SP subsets gated on immature CD24^{+}CD69^{+} TCRβ^{hi} thymocytes. The percentages (left) and numbers (right) of indicated subsets are shown in f. (g-h) Flow cytometry analysis of CD4^{+} and CD8^{+} subsets gated on mature CD24^{-}CD69^{-} TCRβ^{hi} thymocytes. The frequency (left) and numbers (right) of CD4^{+} and CD8^{+} subsets are shown in h. (i-j) Flow cytometry analysis of CD4^{+} and CD8^{+} T cells in peripheral blood cells (left column) and spleen (right column). The percentages (left) and numbers (right) of peripheral T cells are shown in j. Data represent mean ± s.d.

Analysis of CD44^{hi}CD62L^{-} activated CD4^{+} T cells revealed that the percentages and numbers of SLAM^{lo}CXCR5^{+} T_{FH} cells, PD-1^{hi}CXCR5^{+} GC T_{FH} cells and Bcl-6^{hi}CXCR5^{+} GC T_{FH} were similar in *Malat1*$^{-/-}$ mice compared with that of littermate control mice (Figure 3(c-e)). Moreover, *Malat1*$^{-/-}$ mice exhibited similar frequency and numbers of GL7^{+}Fas^{+} GC B cells and B220^{-}CD138^{+} plasma cells and equivalent antigen-specific Ig secretion with that of littermate control

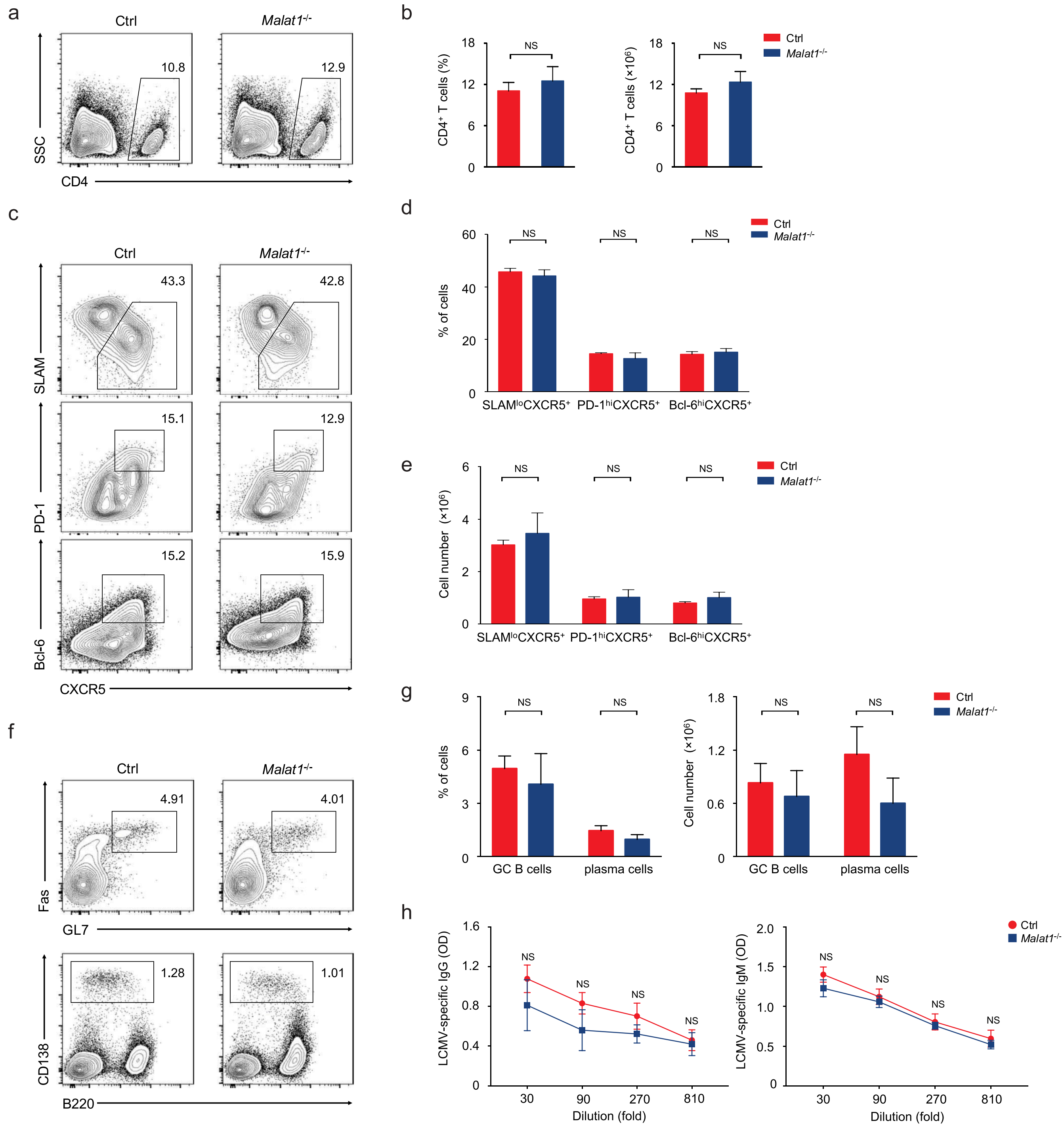

Figure 3. Malat1 is dispensable for T_{FH} differentiation. (a-b) Flow cytometry analysis of $CD4^{+}$ T cell pool at 8 days after infection. The percentage (left) and number (right) of effector $CD4^{+}$ T cells are shown in b. (c) Flow cytometry analysis of $SLAM^{-}$ $CXCR5^{+}$ T_{FH} cells (top), PD-$1^{hi}CXCR5^{+}$ GC T_{FH} cells (middle), and Bcl-$6^{hi}CXCR5^{+}$ GC T_{FH} cells (bottom) at day 8 after LCMV infection. (d-e) The percentages (d) and numbers (e) of T_{FH} cells in c. (f) Analysis of GC B cells (top) and plasma cells (bottom) in spleen by flow cytometry. (g) The percentages (left) and numbers (right) of GC B and plasma cells in f. (h) Analysis of LCMV-specific IgG (left) and IgM (right) in the sera after LCMV infection. Data represent mean ± s.d.

mice (Figure 3(f-h)). In summary, these data indicated that Malat1 is dispensable for T_{FH} cells differentiation and B cell helping functions.

We next examined effector differentiation of $CD8^{+}$ T cells on day 8 after infection. We found normal $CD8^{+}$ T cells expansion in *Malat1*$^{-/-}$ mice compared with that in wild-type mice (Figure 4(a, b)). *Malat1*$^{-/-}$ effector cells exhibited similar downregulation of CD62L and CD127, and similar upregulation of CD44 and KLRG1 (Figure 4(c-e)). Furthermore, *Malat1*$^{-/-}$ effector cells showed equivalent secretion of interferon-γ (IFN-γ) and similar tumor necrosis factor-α (TNF-α) and Granzyme B production compared with that of littermate control mice (Figure 4(f-h)). We also noted that the expression of Eomes and T-bet were similar between *Malat1*$^{-/-}$ and their littermate control mice

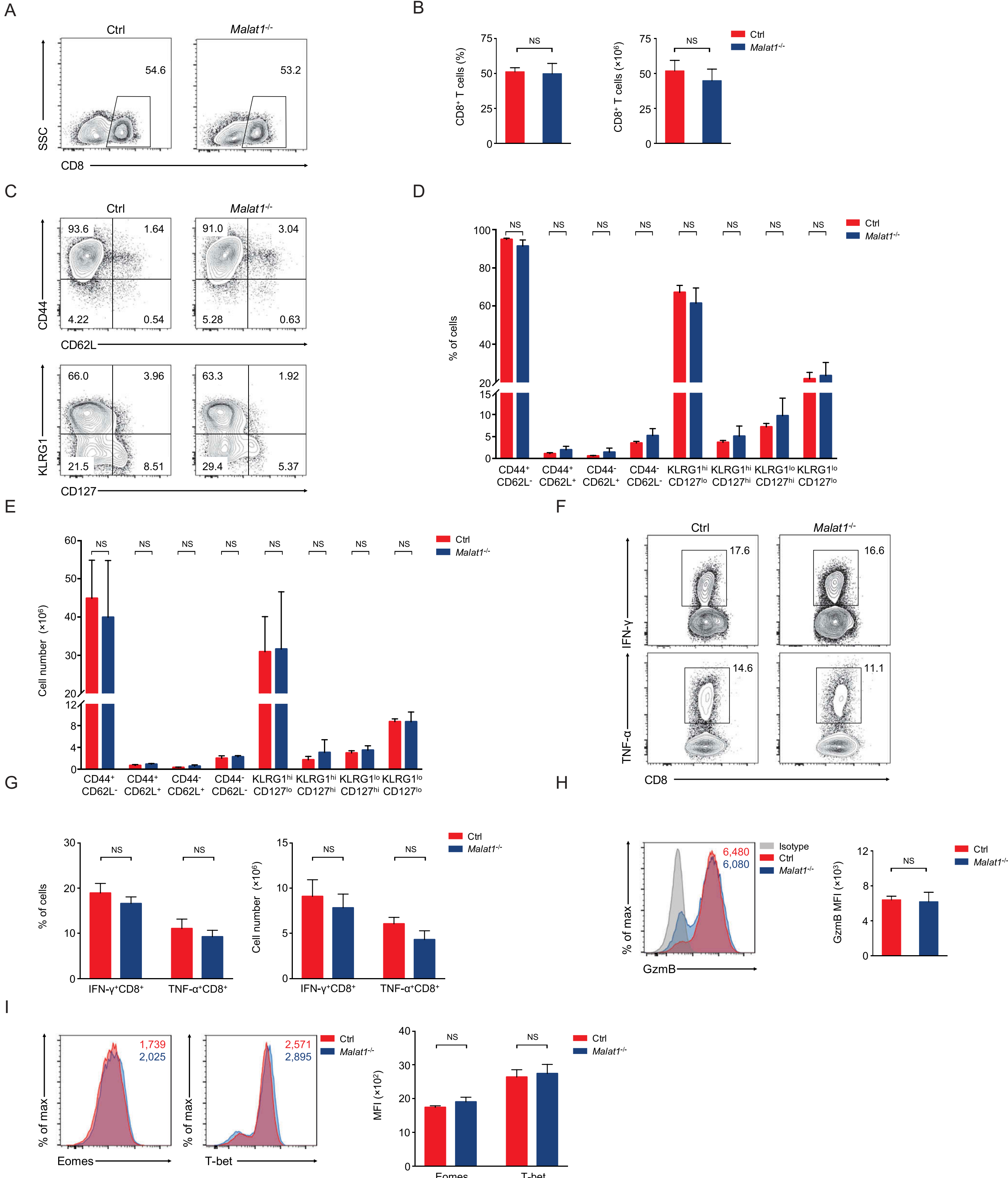

Figure 4. Malat1 is not essential for effector differentiation of CD8+ T cell. (a) Analysis of CD8+ T cells expansion at 8 days after infection. (b) The percentage (left) and number (right) of effector CD8+ T cells in a. (c) Analysis of surface expression of CD44, CD62L, KLRG1 and CD127 from day 8 effector T cells. (d-e) The percentages (d) and numbers (e) of effector T cells in c. (f) Production of effector molecules IFN-γ and TNF-α by effector T cells. (g) The percentages (left) and numbers (right) of IFN-γ+CD8+ and TNF-α+CD8+ T cells in f. (h) Expression of Granzyme B (GzmB) from day 8 effector T cells. (i) Intracellular staining of Eomes and T-bet expression in CD8+ T cells 8 days post infection. Data represent mean ± s.d.

(Figure 4(i)). Collectively, our results demonstrated that Malat1 is not essential for effector CD8^{+} T cells formation and expansion to response to LCMV infection.

Giving its dispensable role in effector differentiation of CD8^{+} T cells, we questioned whether deficiency of Malat1 altered the memory T cells formation. Analysis of memory CD8^{+} T cells at 72 days after infection indicated that the Malat1 deficiency did not affect memory CD8^{+} T cells pool formation (Figure 5(a, b)). We also noted that the percentages and numbers of CD44^{+}CD62L^{+}T$_{CM}$ and CD44^{+}CD62L^{-}T$_{EM}$ were similar in *Malat1$^{-/-}$* mice compared with that of littermate control mice (Figure 5(c, d)). Moreover, the expression of CCR7, CD127 and KLRG1 was considerable in *Malat1$^{-/-}$* and wild-type memory T cells (Figure 5(e)). Upon peptide stimulation *in vitro, Malat1$^{-/-}$* and wild-type memory CD8^{+} T cells were both capable of secreting IFN-γ, TNF-α and producing Granzyme B (Figure 5(f-h)). Furthermore, we noted that the protein expression of key transcription factors T-bet and Eomes were not altered in *Malat1$^{-/-}$* memory CD8^{+} T cells compared with that in wild-type memory CD8^{+} T cells (Figure 5(i)). Taken together, these results revealed that Malat1 is unnecessary for memory CD8^{+} T cells formation.

Discussion

Recent studies have reported that lncRNAs are essential for the differentiation and activation of immune cells, and they may be critical determinants of various biological processes. The differentiation of CD4^{+} T cells into T helper cell subsets is vital for the initiation of adaptive immune responses, and several studies have indicated that distinct T cell subsets express unique profiles of lncRNAs. lncRNA linc-MAF-4 controls the differentiation of T$_{H}$1 cells by suppress expression of the *MAF*, a T$_{H}$2 cells-associated transcription factor. The genomic regions of linc-MAF-4 and MAF form a long-distance interaction, and linc-MAF-4 recruits chromatin modifiers LSD1 and EZH2 to deposit H3K27me3 marks at the promoter of MAF to silence its expression in T$_{H}$1 cells [8]. In T$_{H}$2 cells, lncRNA LincR-Ccr2-5′AS, together with Gata3, is a 'master' component of a regulatory circuit in gene expression specific to the T$_{H}$2 cells and is essential for the migration of T$_{H}$2 cells [6]. Besides CD4^{+} T cells, lncRNAs also involve in CD8^{+} T cell immune response [33]. CD244 signaling induces the expression of lncRNA-CD244 and mediates the repression of IFN-γ/TNF-α expression in CD8^{+} T cells. Similar to linc-MAF-4, lncRNA-CD244 recruits EZH2 to the promoters of *IFNG* and *TNF*, and mediates the deposition of repressive chromatin marks.

The lncRNA Malat1 has been reported that it can regulates alternative splicing of pre-mRNAs by modulating SR splicing factor phosphorylation [10]. More importantly, the emerging evidences indicated Malat1 plays an important role in cancers and metastasis [34,35]. Malat1-deficiency cancer cells showed impaired cell migration and defective in metastatic tumor nodules formation, which indicated Malat1 is a critical regulator of the metastasis of cancer cells [36]. Numerous studies have also reported Malat1 functions in lung cancer, liver cancer, breast cancer, bladder cancer, and osteosarcoma [34,37–39]. The existing data reveal that Malat1 is higher expressed in various immune cell subsets, but its roles in immune cells are rarely reported. It should be a significant work to identify the function and mechanism of Malat1 in T cells.

In this study, we identified the different developmental stages in T cell using *Malat1$^{-/-}$* mice, but no significant phenotypes were observed. Subsequently, we detected the effector and memory CD8^{+} T cells and T$_{FH}$ cell response to LCMV infection, but no defects were detected after *Malat1$^{-/-}$* ablation. Taken together, the lncRNA Malat1-deficiency mice have normal T cell development and peripheral T cells, and Malat1 is not required for effector and memory CD8^{+} T cell formation and function as well as T$_{FH}$ cell differentiation. Our study demonstrated Malat1 is not a key regulator in T cell no matter how high expression level it is. Undoubtedly, these data will contribute to a better understanding of lncRNA Malat1 as well as lncRNA in regulating T cell development and antiviral response.

Materials and methods

Mice

Malat1$^{-/-}$ mice were from Texas A&M Institute for Genomic Medicine (TIGM). All animals were on a fully C57BL/6J background. 6- to 10-week-old mice were used in this study, and both male and female mice were included without randomization or 'blinding'. Mice were bred and housed in specific pathogen-free conditions in accordance with the Institutional Animal Care and Use Committee of China Agricultural University.

LCMV infection

LCMV-Armstrong strain was grown on BHK-21 cells (ATCC, Manassas, VA, USA) and titers were determined as described before [40]. Age and sex matched wild-type mice and *Malat1$^{-/-}$* mice were infected by 2×10^{5} plaque-forming units LCMV-Armstrong strain intraperitoneally.

Flow cytometry and cell sorting

Single cell suspensions were prepared from bone marrow, spleen, or/and thymus and used for flow cytometry analysis or cell sorting. Surface staining was performed in FACS Buffer (PBS supplemented with 2% fetal bovine serum). The following fluorescence-labeled monoclonal antibodies were used: anti-CD19 (1D3), anti-CD25 (PC61.5), anti-CD279 (J43), anti-CD4 (RM4-5), anti-CD44 (IM7), anti-CD45R (RA3-6B2), anti- CD62L (MEL-14), anti-CD8a (53–6.7), anti-GL7 (GL7), anti-Granzyme B (GB11), anti-KLRG1 (2F1), anti-TCRβ (H57-597), anti-TNF-α (MP6-XT22) (from ebiosciences, CA, USA); anti-CD138 (281–2), anti-CD95 (Jo2), anti-IFN-γ (XMG1.2) (from BD Biosciences, San Jose, CA, USA). CXCR5 staining was done using purified anti-CXCR5 (2G8, BD Biosciences, San Jose, CA, USA) for 1 h, followed by biotin-conjugated goat anti-rat IgG (Cat # 112–066-143, Jackson Immunoresearch, West Grove, PA, USA) for 30 min, and then by fluorescence-conjugated streptavidin at 4 °C for 30 min in PBS supplemented with 2% normal mouse serum, 2% FCS, and 0.5% BSA. Staining for Bcl-6 (K112-91,

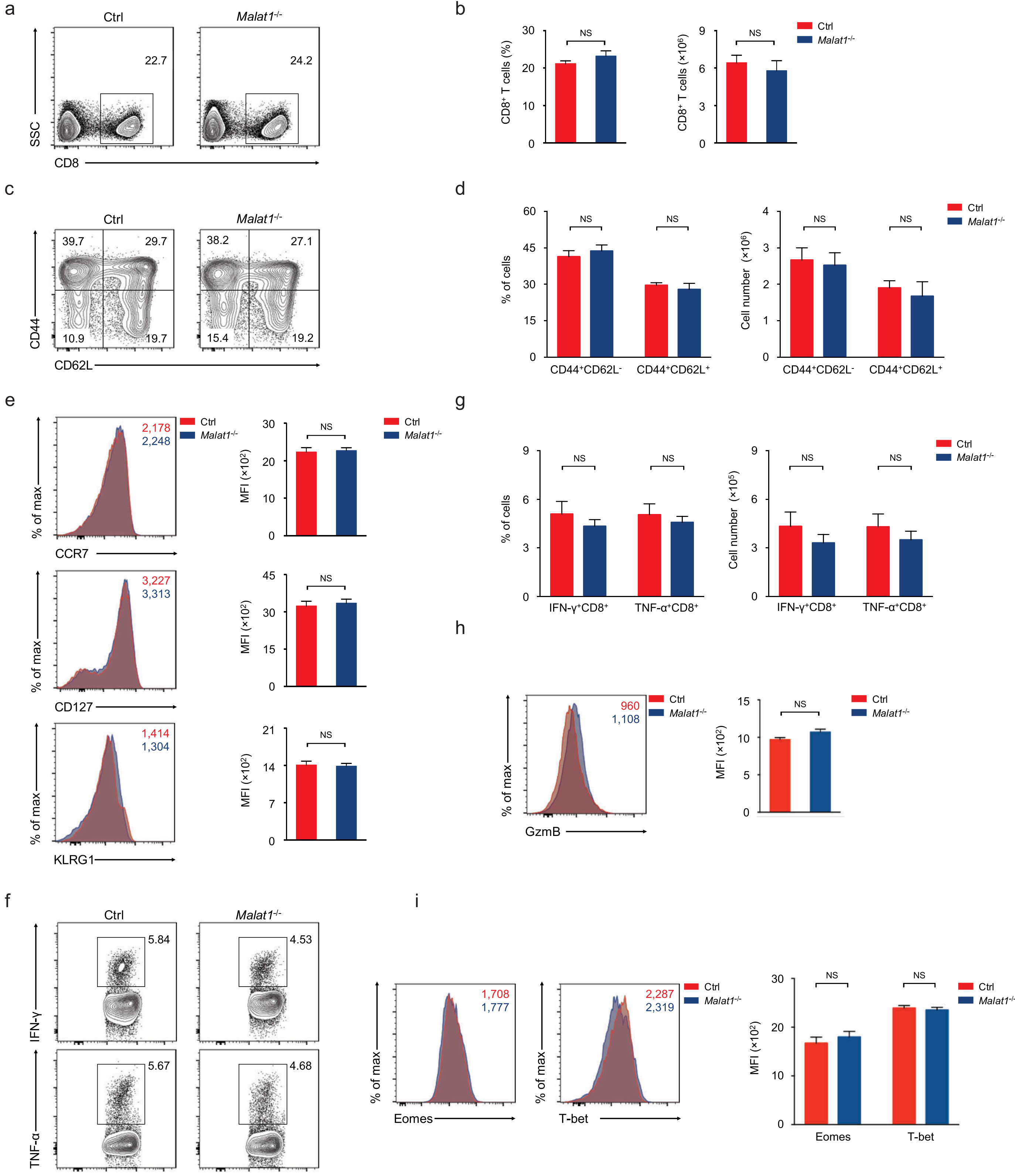

Figure 5. Ablation of Malat1 exhibited normal memory CD8$^+$ T cell pool formation. (a) Analysis of memory CD8$^+$ T cells pool at 72 days after infection. (b) The percentage (left) and number (right) of effector CD8$^+$ T cells in a. (c) Analysis of T_{EM} (CD44$^+$CD62L$^-$) and T_{CM} (CD44$^+$CD62L$^+$) gated on CD8$^+$ T cells. (d) The percentages (left) and numbers (right) of T_{EM} (CD44$^+$CD62L$^-$) and T_{CM} (CD44$^+$CD62L$^+$) in c. (e) Analysis of the expression of CCR7 (top), CD127 (middle), and KLRG1 (bottom) by memory CD8$^+$ T cells. (f) Analysis of the secretion of IFN-γ (top) and TNF-α (bottom) by memory CD8$^+$ T cells. (g) The percentages (left) and numbers (right) of IFN-γ$^+$CD8$^+$ and TNF-α$^+$CD8$^+$ T cells in f. (h) Analysis of the production of Granzyme B by memory CD8$^+$ T cells. (i) Analysis of the expression of Eomes (left) and T-bet (right) by memory CD8$^+$ T cells. Data represent mean ± s.d.

BD Biosciences, San Jose, CA, USA) was performed with Foxp3/Transcription Factor Staining Buffer Set following the manufacturer's instructions. Data were acquired on a FACSVerse (BD Biosciences, San Jose, CA, USA) and were analyzed with FlowJo software (Treestar, Ashland, Oregon, USA). All cell sorting experiments were carried out on a FACSAria II sorter (BD Biosciences, San Jose, CA, USA).

Functional characterization of antigen-specific CD8$^+$ T cells

For functional characterization of antigen-specific CD8$^+$ T cells, splenocytes were stimulated with 1 μM of GP_{33-41} (KAVYNFATC), $NP_{396-404}$ (FQPQNGQLI) for 5 hours in the presence of GolgiStop and GlogiPlug (BD Biosciences, San Jose, CA, USA). The stimulated cells were then surfaced stained, fixed and permeabilized using BD Cytofix/Cytoperm and BD Perm/Wash solutions (BD Biosciences, San Jose, CA, USA), and intracellularly stained for IFN-γ, IL-2, TNF-α following the manufacturer's instructions.

Quantitative RT-PCR

Cells were sorted and subsequently lysed in TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Total RNA was extracted followed by cDNA synthesis with FastQuant RT Kit (Tiangen, Beijing, China). cDNA was analyzed for expression of various genes with the SYBR Green SuperReal PreMix (Tiangen, Beijing, China) on a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). The following primers were used: for *Gapdh*, 5′-ACTCCACTCACGGCAAATTCA-3′ and 5′-GGCCTCACCCC ATTTGATG-3′; for *Hprt1*, 5′- GCGTCGTGATTAGCGATGA TG-3′ and 5′-CTCGAGCAAGTCTTTCAGTCC-3′; for *Malat1* (P1), 5′- AGAGTGAGTTCCAGGACAGC-3′ and 5′-GCTTCC TACTACCTGTGCCT-3′; for *Malat1* (P2), 5′- CATCTCGGA GCAGGAAACAG-3′ and 5′-CCATCATGAAAGCCCATCGG-3′ for *Malat1* (P3), 5′-TGAGGACAACAGGTGAACGA-3′ and 5′-GGCTCCGCTGTCCTACATTA-3′; for *Tcf7*, 5′-CCCTTCCTGCGGATATAGAC-3′ and 5′-GGTACACCAGA TCCCAGCAT-3′; and for *Notch1*, 5′- CCCTTGCTCTGCCTAA CGC-3′ and 5′-GGAGTCCTGGCATCGTTGG-3′.

Statistical analysis

Statistical analysis was conducted with Prism 7.0 (GraphPad, La Jolla, CA, USA). An unpaired Student's *t*-test with a 95% confidence interval was used for calculation of *P* value. NS *not significant*, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Author Contributions

Y.Y., W.G., J.C., P.G., G.Y., Juanjuan L., Jingjing L. and M.Y. performed the experiments; F.W. analyzed the high-throughput data and assisted the Figures processing; T.Z., Y.K. and X.M. provided scientific insights and helped the overall study; X.M. revised the manuscript; Y.Y. and S.Y. analyzed the data and wrote the paper; and S.Y. conceived of the project and supervised the whole study.

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