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Original Article

BTLA exhibits immune memory for αβ T cells in patients with active pulmonary tuberculosis

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Abstract: Despite past extensive studies, the role of B and T lymphocyte attenuator (BTLA) in αβ T cells in patients with active pulmonary tuberculosis (ATB) remains poorly understood. Here we demonstrate that BTLA expression on αβ T cells is decreased in patients with M. tuberculosis (Mtb) infection. Particularly, BTLA expression levels are likely critical for αβ T cells to manifest and maintain an active central memory phenotype with high capacity for secretion of IFN-γ and perforin, which are important for immune memory against TB infection. BTLA^{high} αβ T cells also exhibited higher capacity in response to Mtb peptide stimulation. In contrast to the role of BTLA played for negative regulation of immune responses, our data in the current studies suggest that BTLA expression on αβ T cells is likely associated with protective immune memory against Mtb infection in the setting of patients with active pulmonary tuberculosis. This previous unappreciated role for BTLA may have implications for prevention and treatment of patients with Mtb infection.

Keywords: Human active pulmonary tuberculosis, B and T lymphocyte attenuator, memory T cells, αβ T cells

Introduction

Tuberculosis (TB) is a global health problem, its prevalence in developing countries such China, is significantly higher than that in developed countries [1]. The spread of drug-resistant tuberculosis is becoming a formidable challenge to control its epidemic and mortality rate in China, which also poses a major threat to global tuberculosis control [2]. It has been well known that the host immune system plays a critical role both in the containment and cure of TB infection. Interestingly, the impact of αβ T cells on patients against TB infection has recently been highlighted [3-6], in which cytokine IFN-γ and cytotoxic molecule perforin produced by αβ T cells had been demonstrated implicated in the immune control of TB infection [3, 5, 6]. Furthermore, the M. tuberculosis (Mtb)-specific CD8$^+$ T cells are found to be capable of differentiating into a canonical effector memory state manifested by a CD127^{hi}CD62L^{lo}PD-1^{lo} phenotype [7]. Indeed, during the course of TB infection around 5 to 10% antigen-specific αβ T effector cells convert into long-lived memory T cells [8, 9]. Therefore, elucidation of the memory functions and regulatory pathways relevant to Mtb-specific αβ T cells would be important in the clinical setting for prevention and treatment of TB infection.

B and T lymphocyte attenuator (BTLA), a recently discovered inhibitory receptor belonging to the CD28 family, is expressed by most lymphocytes and shares structural and functional similarities with cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death 1 (PD1) [10]. BTLA interacts with the herpesvirus entry medi-

ator (HVEM), a TNFR super family member found on T, B, NK, DC and other cells [11]. More recent studies further demonstrated emerging evidence suggesting that BTLA could also initiate pro-survival signals for activated or effector T cells relevant to the generation of immune memory other than its negative regulatory role in diverse immune responses [5, 12-15]. However, the exact impact of BTLA expression or BTLA pathway on modulation of T-cell responses during the course of Mtb infection in humans is yet to be fully elucidated. To address this question, we thus examined the expression of BTLA in patients with active pulmonary tuberculosis (ATB) in the context of functional characteristics of Mtb antigen-specific αβ T cells. Surprisingly, in contrast to its previously identified inhibitory role in immune response, BTLA expression on αβ T cells is likely associated with protective immune memory against Mtb infection in the setting of patients with active pulmonary tuberculosis.

Materials and methods

Study subjects

A total of 68 patients with active pulmonary tuberculosis (ATB) were diagnosed based on clinical symptoms, chest X radiography, acid fast bacilli (AFB) staining of sputum smears, positive bacterial culture, bronchoalveolar lavage (BAL) or direct biopsy examination and culture, which were done in Dongguang Hospital for Prophylaxis and Treatment of Chronic Disease (Dongguan, China). These ATB patients were next subjected to individualized treatment with anti-tuberculosis drugs (ATDs) such as isoniazid, rifampicin, pyrazinamide and ethambutol. After the initial ATD treatment, the patients were evaluated again clinically and bacteriologically to determine the effectiveness of the therapy and the transition of disease. The patients with null initial ATD treatment were retreated with Pasiniazid, Rifapentin, Pyrazinamide, Ethambutol and Levofloxacin. All samples were undergone sputum smears and microbiological screenings (e.g., Ziehl-Neelsen acid fast staining) to record smear positivity along with cultures on Lowenstein-Jensen slants according to the standard method. Forty healthy volunteers (HV) manifested by the absence of bacteriological and clinical evidence of TB were served as controls. Subjects with HIV infection, diabetes, cancer, autoimmune diseases, immunosuppressive treatment and pulmonary tuberculosis history were excluded from the study. The study was approved by the Internal Review and the Ethics Board of Guangdong Medical College and Dongguang Hospital for Chronic Diseases, and informed consent was obtained from all study subjects.

Reagents

Ag85B was synthesized and used as previously described [16]. Antibodies against human BTLA (MIH26) and mouse IgG2a (MG2a-53) were obtained from Biolegend (San Diego, CA), while antibodies against human CD28 (CD28.2), CD-49d (9F10), CD3 (SP34-2), CD8 (RPA-8), CD27 (M-T271), 62L (DREG-56), CD45RO (UCHL1), IFN-γ (4S.B3), Perforin (δG9), mouse IgG1 (MOPC-21), IgG2b (27-35), IgG2a (G155-178), IgG1 (κ) and IgG2b (κ) were purchased from BD Biosceinces (San Jose, CA, USA).

PBMC isolation and flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh peripheral blood by standard Ficoll (GE Health, Fairfield, USA) density gradient centrifugation. Cell viability was determined by trypan blue staining (>95% in all experiments). The isolated PBMCs were resuspended in 2% FBS-PBS, and then stained with indicated antibodies, and analyzed by flow cytometry (BD FACS Calibur II, San Jose, CA, USA).

For assessing BTLA expression and Perforin- or IFN-γ-producing αβ T cells, $3\sim10\times10^5$ lymphocytes were incubated in the presence or absence Ag85B (10 ug/ml) along with co-stimulatory CD28 (1 ug/ml) and CD49d (1 ug/ml) mAbs for 6h as described previously [16, 17]. The cells were then transferred into 5 ml polystyrene round bottom tubes for flow cytometry analysis of surface marker expression and intracellular cytokine expression. To ensure the specificity of immune staining, a matched isotype IgG was employed as negative controls. The BD FACS Calibur II (San Jose, CA, USA) platform was used to acquire data. All Data were analyzed by using the CellQuest v3.3 software (BD Bioscience, San Jose, CA, USA) as instructed.

Cell proliferation assay

Lymphocytes were cultured with 10% FBS-RPMI 1640 medium and labeled with CFDA

Table 1. The clinical data of studied subjects

Groups	PTB (n = 68)	HV (n = 40)
Age (years)	(17~65)	(20~65)
Mean ± SEM	39.0 ± 1.4	37.9 ± 1.9
% Female/Male	21/47	14/26
TST +/-	33/35	-/-
Sputum smear +/-	18/40	0/40
ATDs treatment (0~40 days)		
M0/M1	42/26	-/-

M0: ATDs treatment during 0~4 days. M1: ATDs treatment during 20~40 days. There were no signification differences among age and gender groups (P>0.05).

(Beyotime, China) for 15 min according to the manufacturer's instructions. The labeled cells were stimulated with Ag85B in the presence of co-stimulatory CD28 (1 ug/ml) and CD49d (1 ug/ml) mAbs for 10 days, followed by flow cytometry analysis to assess the proliferation ability of $BTLA^{high/low}$ αβ T cells by staining surface markers CD3, CD8 and BTLA, and the gating strategy is described detailed in Figure S1. The CFDA intensity of gated cells was measured through a 518 nm filter (FL1). The multiparametric data were analyzed using Flowjo.7.6.1 software (Treestar, Ashland, OR, USA).

Statistical analysis

Normality test was first performed to decide whether the data were in normal distribution. Student's *t-test* (two-sample two-tailed comparison) was employed to compare the differences of measured data, and Pearson correlation was used to measure the degree of dependency between variables by the GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, CA, USA). A P value of less than 0.05 (95% confidence interval) was considered with statistical significance.

Results

Clinical characteristics for the study subjects

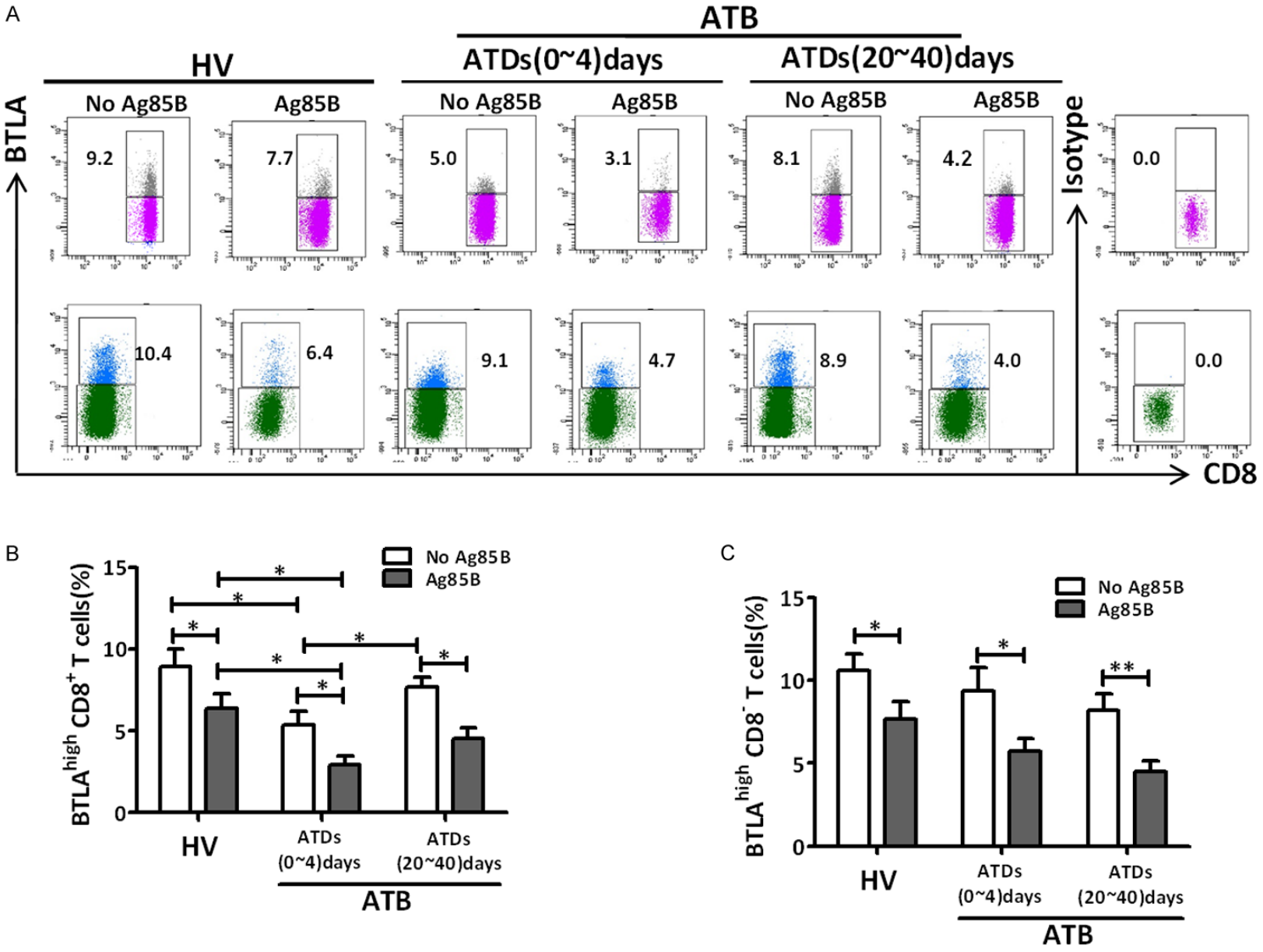
The demographic and clinical characteristics for all study subjects are shown in **Table 1** and Table S1. No significant difference in terms of age and gender was noted between patients with active pulmonary tuberculosis (ATB) and healthy volunteers (HV). Among ATB patients, 48.5% of them were positive for tuberculin skin test (TST), 26.5% of them were Mtb positive by sputum smear analysis. Twenty three (38.2%) ATB patients were treated by anti-tuberculosis drugs (ATDs) for more than 20 days but less than 40 days. Follow-up exams revealed that 5 ATB patients were successful from the initial ATD treatment (named M0 phase, treated for 0 to 4 days) to M1 phase (treatment for 20~40 days) as manifested by the absence of TB relapsed symptoms.

ATB patients manifest attenuated BTLA expression on αβ T cells

We first sought to examine the differences of BTLA expression between ATB patients and HV controls, PBMCs isolated from ATB patients or HV controls were stimulated with Ag85B followed by flow cytometry analysis of BTLA expression. Surprisingly, a significant reduction of BTLA expression was noted on αβ T cells in ATB patients, especially in those patients undergone primary treatment. Interestingly, the reduction of BTLA expression on $CD8^+$ T cells was much more significant than that on $CD8^-$ T cells (**Figure 1**). More importantly, Mtb peptide stimulation further induced reduction of BTLA expression on αβ T cells. It is worthy of note, patients undergone ATD treatment for 20~40 days showed an increased BTLA expression on $CD8^+$ T cells (**Figure 1B**), but not on $CD8^-$ T cells (**Figure 1C**). Together, these results suggest that TB infection suppresses BTLA expression on αβ T cells, especially on $CD8^+$ T cells, while chemotherapy related bacterial clearance is manifested by the induction of BTLA expression on $CD8^+$ αβ T cells.

$BTLA^{high}$ αβ T cells originated from ATB patients display a memory or naïve phenotype

To address the functional relevance of BTLA expression on T cells, we analyzed surface markers on $BTLA^{high}$ and $BTLA^{low}$ αβ T cells by flow cytometry, in which markers CD27 and CD45RO were used to class T cell subsets. Four distinct T cell subsets were identified, and they were naïve T cells (TN, $CD27^+CD45RO^-$), central memory T cells (TCM, $CD27^+CD45RO^+$), effector memory T cells RA (TEMRA, also known as terminally differentiated; $CD27^-CD45RO^-$), and effector memory T cells (TEM, $CD27^-CD45RO^+$) [18-20]. Interestingly, the $BTLA^{high}$ $CD8^+$ T cells in ATB patients are predominantly characterized by a $CD27^+CD45RO^+$ TCM or a $CD27^+CD45RO^-$ TN phenotype as compared


A
ATB
HV
ATDs(0~4)days
ATDs(20~40)days
No Ag85B
Ag85B
No Ag85B
Ag85B
No Ag85B
Ag85B
BTLA
Isotype
CD8
9.2
7.7
5.0
3.1
8.1
4.2
0.0
10.4
6.4
9.1
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B
No Ag85B
Ag85B
BTLA^high CD8+ T cells(%)
15
10
5
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HV
ATDs (0~4)days
ATDs (20~40)days
ATB
C
No Ag85B
Ag85B
BTLA^high CD8- T cells(%)
15
10
5
0
HV
ATDs (0~4)days
ATDs (20~40)days
ATB

Figure 1. MTB infection exhibited down-regulation of BTLA expression on αβ T cells subsets, prominently in $CD8^+$ T cells. PBMC from 68 active pulmonary tuberculosis (ATB) patients and 40 healthy individuals (HV) were stained directly or stimulated ex vivo with Ag85B plus CD49d/CD28 antibodies, and then analyzed by polychromatic flow cytometry. (A) Representative flow cytometric dot plots showing BTLA expression on αβ T cells subsets ($CD3^+CD8^+$ T cells and $CD3^+CD8^-$ T cells defines as $CD3^+CD4^+$ T cells) in ATB patients and in healthy individuals. Gating strategy show in Figure S1. (B and C) Are bar graph data showing that the percentages (%) of BTLA expression on $CD8^+$ and $CD8^-$ T cells from HV and ATB patients with ATDs treatment during 0~4 days, or ATDs treatment during 20~40 days. Horizontal bars depict the mean percentage of Ag85B-specific BTLA expression on $CD8^+$ and $CD8^-$ T cells. Groups were compared by t test with GraphPad Prism Version 5.0. Significant differences are indicated by *P<0.05, **P<0.01, ***P<0.001.

with that of $BTLA^{low}$ $CD8^+$ T cells (**Figure 2B** to **2E**). Similarly, as compared with $BTLA^{low}$ $CD8^-$ T cells, most $BTLA^{high}$ $CD8^-$ T cells in ATB patients were manifested by a TCM phenotype. Collectively, those data indicate that ATB patient-derived $BTLA^{high}$ αβ T cells manifest either a central memory or a naïve phenotype. To further confirm that BTLA expression is associated with a central memory phenotype, we examined CD62L expression, which usually highly expressed on central memory phenotype T cells [21, 22]. Indeed, $BTLA^{high}$ αβ T cells displayed much higher levels of CD62L expression than that in $BTLA^{low}$ αβ T cells (**Figure 2**).

To demonstrate whether the central memory phenotype for αβ T cells relevant to BTLA expression was affected by Mtb infection, we followed-up five sputum smear positive patients for one month after ATD treatment. Surprisingly, the number of TCM cells was increased, while the amount of TEMRA cells was decreased in $BTLA^{high}$ αβ T cells after ATD treatment, which was accompanied by the clearance of bacterium (**Figure 3**). Taken all data together, $BTLA^{high}$ αβ T cells in ATB patients are characterized by a central memory phenotype during the course of bacterial clearance, and ATD treatment further increases the number of $BTLA^{high}$ $CD27^+CD45RO^+$ memory αβ T cells.

$BTLA^{high}$ αβ T cells possess high potency for proliferation

Given that proliferation in response to chronic infection is a typical functional property for central memory T cells, and by which they differentiate into effector memory T cells for eradication of invaded bacteria, we thus next compared the difference of proliferation capability between $BTLA^{high}$ and $BTLA^{low}$ αβ T cells. The results revealed that $BTLA^{high}$ αβ T cells exhibited greater potency for proliferation than the $BTLA^{low}$ counterparts in ATB patients, especially in the presence of antigen Ag85B stimulation (**Figure 4**). These results suggest that BTLA expression could be important for αβ T cell proliferation in ATB patients.

$BTLA^{high}$ αβ T cells exhibit higher capacity for secretion of IFN-γ and perforin

In general, cytokines and cytotoxic molecules produced by central memory T cells are very important in protective memory against TB infection [7, 23, 24]. We therefore next examined the impact of BTLA expression on the capacity of αβ T cells for secretion of cytokine IFN-γ and cytotoxic molecule perforin. $BTLA^{high}$ αβ T cells (both $CD8^+$ and $CD8^-$ T cells) demonstrated significantly higher capacity for secretion of IFN-γ (**Figure 5**) and perforin (**Figure 6**) than the $BTLA^{low}$ counterparts both in ATB patients and HV controls. Similar as above, Ag85B stimulation significantly enhanced the capacity of $BTLA^{high}$ αβ T cells for secretion of IFN-γ and perforin both in ATB patients and HV controls (**Figures 5** and **6**). In sharp contrast, Ag85B stimulation failed to induce a significant change for $BTLA^{low}$ αβ T cells in terms of their capacity for secretion of IFN-γ and perforin (**Figures 5** and **6**), suggesting that $BTLA^{high}$ αβ T memory cells are likely Ag85B-specific.

Discussion

In the present study, we demonstrated evidence indicating that BTLA expression on αβ T cells is decreased in patients with Mtb infection. Particularly, BTLA expression levels are likely critical for αβ T cells to manifest and maintain an active central memory phenotype with high capacity for secretion of IFN-γ and perforin, which are important for immune memory against TB infection. These findings uncovered a previously unknown mechanism for the regulation of T-cell immune responses during the course of TB infection, which may have significant implications in terms of treatment and prognosis of ATB patients in clinical settings.

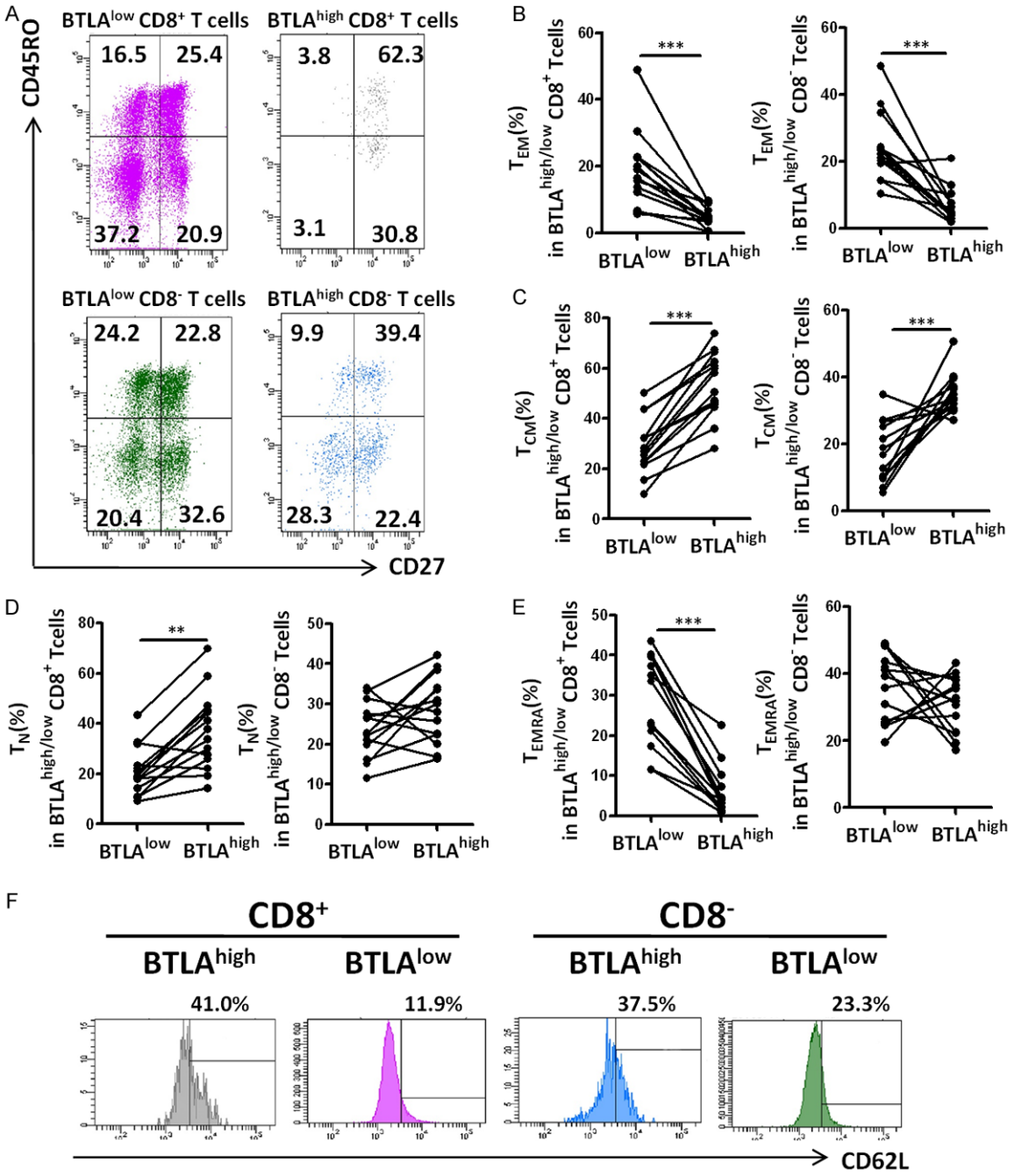


Figure 2. BTLA^{high} αβ T cells in ATB patients displayd more actively central memory/naïve and less effector memory phenotypes, than BTLA^{low} αβ T cells. Expression of CD27 and CD45RO were used to detect phenotypic profiles of BTLA$^{low/high}$ αβ T cells by polychromatic flow cytometry. 4 distinct T cell populations were classified as naïve T cells (TN, CD27^{+}CD45RO^{-}), central memory T cells (TCM, CD27^{+}CD45RO^{+}), effector memory T cells RA (TEMRA, also known as terminally differentiated; CD27^{-}CD45RO^{-}) and effector memory T cells (TEM, CD27^{-}CD45RO^{+}). (A) The representative flow cytometric dot plots showing the phenotypic profiles of BTLA$^{low/high}$ αβ T cells. (B) Indicate the percentages of TEM in BTLA$^{low/high}$ CD8$^{+/-}$ T cells. (C) Indicate the percentages of TCM in BTLA$^{low/high}$ CD8$^{+/-}$ T cells. (D) Indicate the percentages of TN in BTLA$^{low/high}$ CD8$^{+/-}$ T cells. (E) Indicate the percentages of TEMRA in BTLA$^{low/high}$ CD8$^{+/-}$ T cells. (F) Expression of CD62L was used to detect phenotypic profiles of BTLA$^{low/high}$ αβ T cells by polychromatic flow cytometry. Groups were compared by t test with GraphPad Prism Version 5.0. Significant differences are indicated by *P<0.05, **P<0.01, ***P<0.001.

BTLA was first identified as an inhibitory receptor on T cells based on that *Btla*-knockout mice manifest enhanced T cell responses [10]. In general, BTLA is expressed by lymphoid and

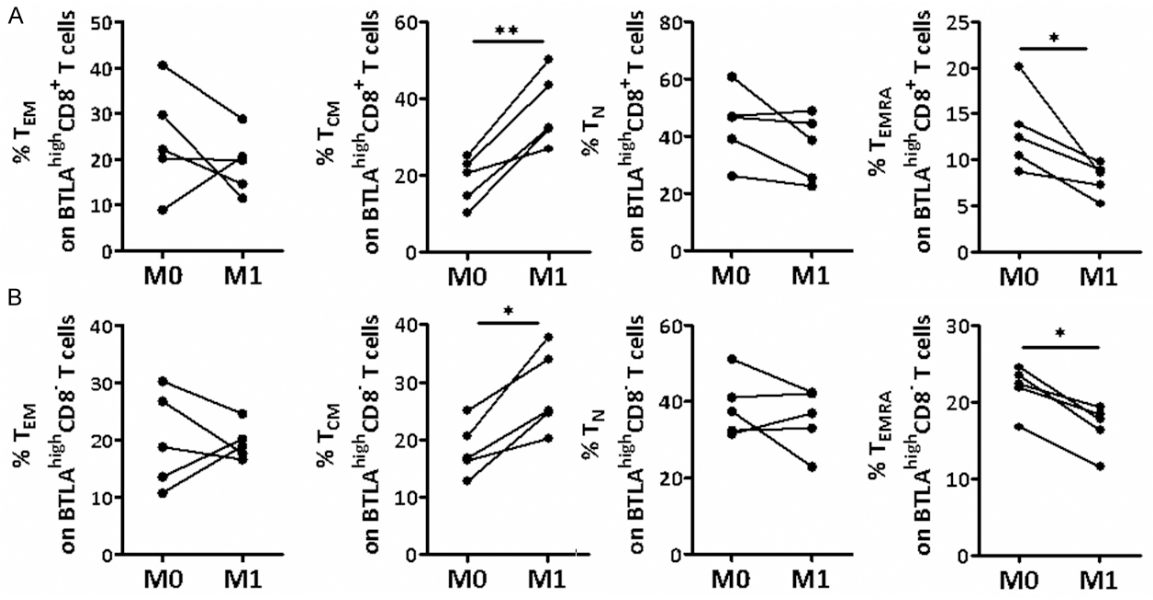


Figure 3. TCM increased and TEMRA decreased in $BTLA^{high}$ αβ T cells after ATDs treatments. 5 cases of sputum smear positive patients were follow-up to determain the changes of each T cell subset in $BTLA^{high}$ $CD8^+$ or $CD8^-$ T cells, respetively, after ATDs analyzed further by flow cytometery. (A and B) Show the percentage of TN, TCM, TEMRA and TEM in $BTLA^{high}$ $CD8^+$ and $BTLA^{high}$ $CD8^-$ T cells. Groups were compared by *t* test with GraphPad Prism Version 5.0. Significant differences are indicated by $*P<0.05$, $**P<0.01$, $***P<0.001$.

myeloid cells, and particularly, peripheral B cells and plasmacytoid dendritic cells express higher levels of BTLA, while lower levels of BTLA are found in $CD11c^+$ DCs and naive T cells [25]. Although altered BTLA expression has been characterized in different pathological conditions [26], its expression and functionality on T cells in patients with TB infection, however, are yet to be elucidated. We now demonstrated evidence that ATB patients, especially those patients undergone primary treatment manifested a reduced BTLA expression on $CD8^+$ T cells as compare to HV controls, but no perceptible difference in terms of BTLA expression on $CD8^-$ T cells was noted between ATB patients and HV controls. Moreover, Mtb antigen Ag85B stimulation further attenuated BTLA expression on αβ T cells ($CD8^+$ and $CD8^-$) originated from ATB patients. Surprisingly, Mtb antigen stimulation also decreased BTLA expression on αβ T cells derived from HV individuals. On the contrary, significantly enhanced BTLA expression on $CD8^+$ T cells of ATB patients was noted after 20~40 days of ATD treatment. These data demonstrate that Mtb infection leads to down-regulation of BTLA expression on αβ T cells, especially on $CD8^+$ T cells, while ATD treatment induces BTLA expression on αβ T cells, which is associated with bacterial eradication.

Although Mtb infection attenuates BTLA expression on αβ T cells, $BTLA^{high}$ $CD8^+$ T cells predominantly manifest a central memory or naive phenotype. In sharp contrast, $BTLA^{low}$ $CD8^+$ T cells are characterized by the effector memory/effector memory RA phenotype. However, $BTLA^{high}$ $CD8^-$ T cells predominantly display a TCM phenotype, while $BTLA^{low}$ $CD8^-$ T cells are manifested by a TEM phenotype, but no significant difference for the percentage of TN and TEMRA cells between $BTLA^{high}$ $CD8^-$ T cells and its $BTLA^{low}$ counterparts. These data suggest that BTLA expression may be associated with immune memory function for αβ T cells against Mtb infection. Indeed, this notion was further confirmed by surface expression of CD62L, a surrogate marker for central memory T cells [27, 28].

Central memory T cells are important to elicit a rapid immune response during the course of a particular infectious disease. They possess the capacity to differentiate into effector T cells and to produce inflammatory cytokines, and retain the capability to home to secondary lymphoid tissues. More importantly, a precursor-product relationship between TCM and TEM cells has been suggested by the fact that TCM cells retain longer telomeres than TEM cells,

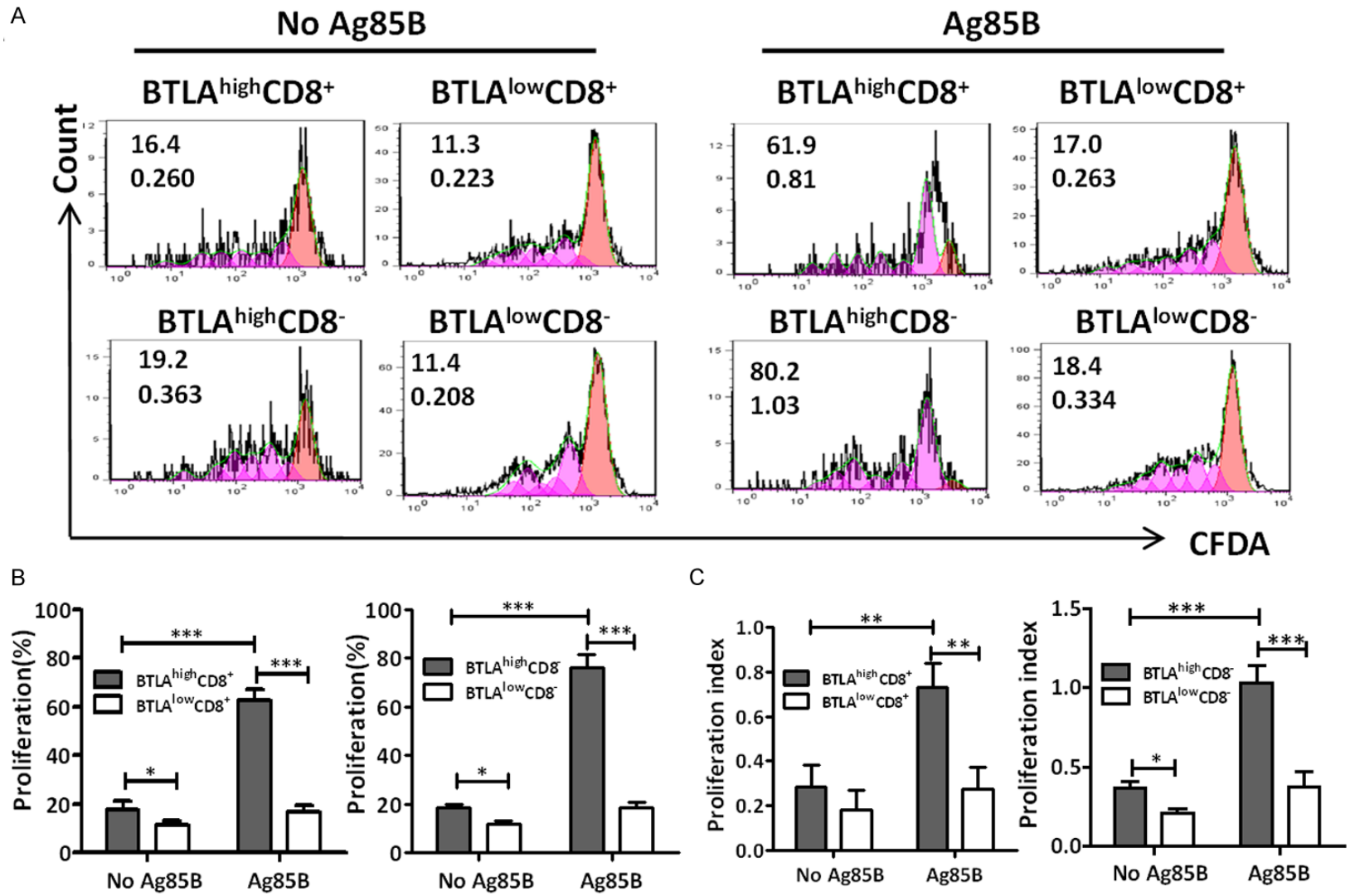
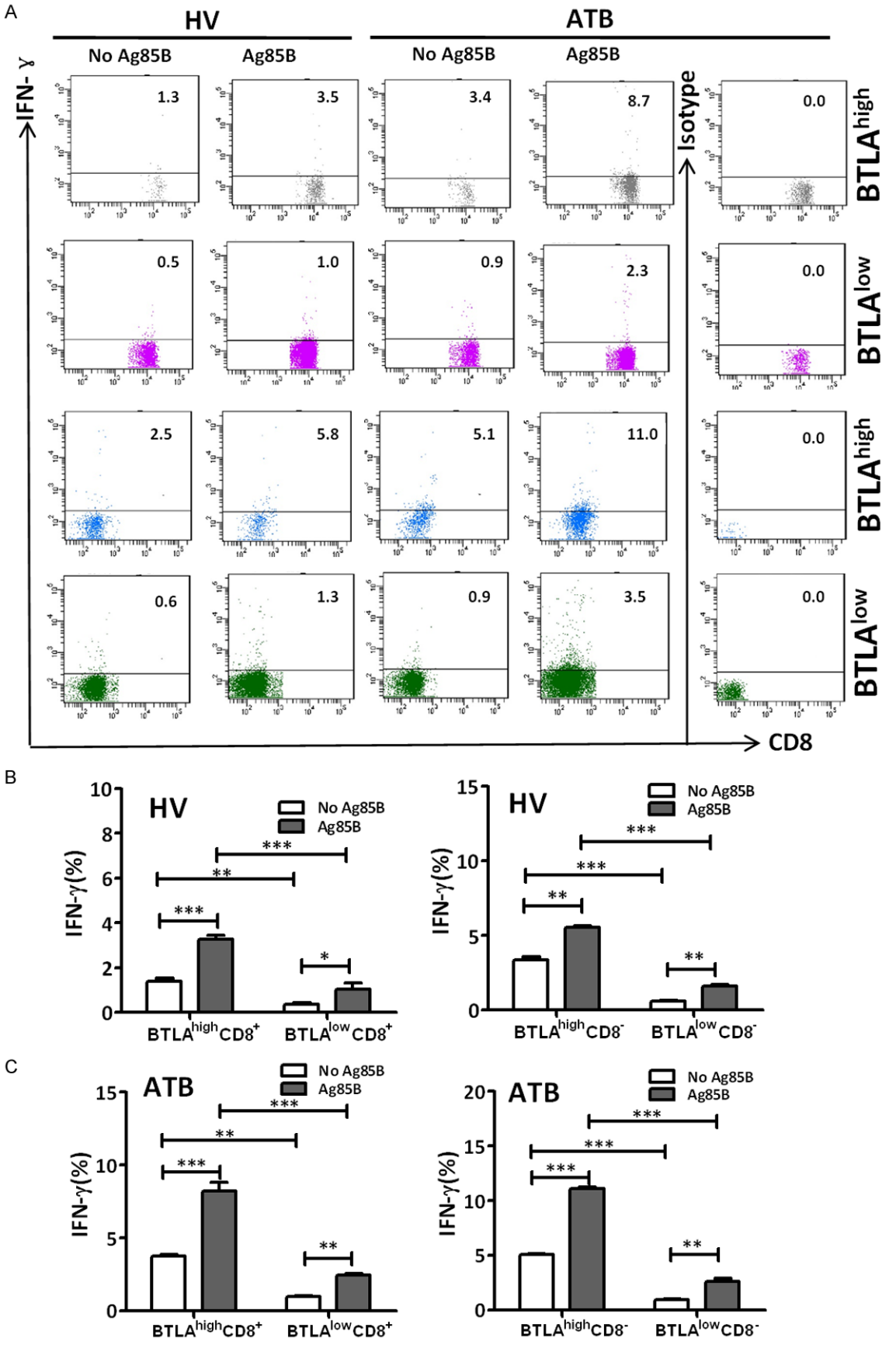


Figure 4. BTLA^{high} αβ T cells exhibited greater proliferation functions than BTLA^{low} counterparts. PBMC were labeled with CFDA and then cultured ex vivo in presence or absence of Ag85B for 10 days, stained for fluorochrome-conjugated mAbs and analyzed by polychromatic flow cytometry. BTLA^{high} CD8$^+$, BTLA^{low} CD8$^+$, BTLA^{high} CD8$^-$, and BTLA^{low} CD8$^+$ T cell subpopulations,were indcated to detect cell proliferation. (A) Shows representative flow cytometric maps under the conditions with or without ex vivo stimulation with Ag85B. The value (inside) for the percentage of cells that divided at least once (top left corner) and the average number of cell divisions (bottom left corner) are indicated for each groups. (B) Showed the percentage of cells that divided at least once, (C) Showed the average number of cell divisions of BTLA$^{high/low}$ CD8$^+$ and BTLA$^{high/low}$ CD8$^-$ T cells, respectively. Groups were compared by t test with GraphPad Prism Version 5.0. Significant differences are indicated by *P<0.05, **P<0.01, ***P<0.001.


A
HV
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1.3
3.5
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Figure 5. $BTLA^{high}$ αβ T cells exhibited greater memory functions for IFN-γ production than $BTLA^{low}$ counterparts. PBMC derived from ATB patients (n = 10) and HV (n = 10) were cultured ex vivo in presence or absence of Ag85B, then stained with fluorochrome-conjugated anti-IFN-γ intracellularly and analyzed by polychromatic flow cytometry. $BTLA^{high}$ $CD8^+$, $BTLA^{low}$ $CD8^+$, $BTLA^{high}$ $CD8^-$ and $BTLA^{low}$ $CD8^-$ T cell subpopulations were indcated to examine IFN-γ production. (A) Shows representative flow cytometric dot plots derived from HV and ATB patient, indicating percentages of IFN-γ producing cells. (B) Show bar graph data from HV and demonstrate the percentages of IFN-γ producing T cells within $BTLA^{high/low}$ $CD8^+$ and $BTLA^{high/low}$ $CD8^-$ T-cell subsets, respectively. (C) Show bar graph data from ATB patients, and disply each IFN-γ producing T cell subsets. Groups were compared by *t* test with GraphPad Prism Version 5.0. Significant differences are indicated by $*P<0.05$, $**P<0.01$, $***P<0.001$.

and therefore, TEM cells can be generated from TCM cells [29]. Although controversial results had been reported in terms of the role for memory T cells in tuberculosis, a recent study revealed that bifunctional $IFN\gamma^+$ and $TNF\alpha^+$ producing $CD4^+$ T cells manifest an effector memory phenotype associated with active TB infection, while Mtb antigen responsive T cells in cured TB patients are characterized by a central memory phenotype [30]. Some studies also revealed a higher proportion of Ag-specific effector memory TEM cells and a decreased frequency of TCM $CD4^+$ T cells in patients with active TB [31]. Interestingly, we observed in 5 followed-up patients that ATD treatment for one month induced both $BTLA^{high}$ $CD8^+$ and $CD8^-$ T cells to manifest a TCN phenotype. Therefore, BTLA expression is likely essential for retaining the central memory function of αβ T cells against Mtb. Interestingly, $BTLA^{high}$ αβ T cells (either $CD8^+$ or $CD8^-$) exhibited greater proliferation capacity than $BTLA^{low}$ counterparts in ATB patients. Furthermore, Mtb antigen Ag85B stimulation significantly enhanced the proliferation capacity for $BTLA^{high}$ αβ T cells, but without discernable impact on the $BTLA^{low}$ counterparts, indicating that BTLA expression is required for specific antigen induced proliferation.

It is worthy of note, we provided evidence demonstrating that BTLA expression on αβ T cells is associated enhanced cytokine IFN-γ and perforin production. Both $BTLA^{high}$ $CD8^+$ T cell or $BTLA^{high}$ $CD8^-$ T cells showed higher IFN-γ and perforin production as compare with that of $BTLA^{low}$ counterparts with or without Mtb antigen stimulation. Interestingly, BTLA expression has also been found to be associated with higher IFN-γ and perforin production in HV controls. These data are somehow contradictory to the previous reported data, in which BTLA signaling inhibits IFN-γ production [32]. Nevertheless, recent studies also revealed that BTLA acts as a ligand to cross-link with its receptor HVEM, and by which it promotes $CD8^+$ cell survival and memory T cell generation in the setting of bacterial and viral infection [5, 12]. Indeed, we found that PBMCs from ATB patients express high levels of BTLA along with HVEM (data not shown). Therefore, BTLA may mediate transsignal through HVEM to promote cytokine production.

In summary, we demonstrated a previously unappreciated role for BTLA in αβ T cells in ATB patients. Mtb infection attenuates BTLA expression on αβ T cells, while ATD treatment induces BTLA expression along with bacterial clearance. BTLA expressing αβ T cells display a central memory phenotype to combat Mtb infection as manifested by the higher capacity for proliferation and capability for cytokine secretion. Therefore, BTLA expression on αβ T cells is associated with protective immune memory in ATB patients against Mtb infection.

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Disclosure of conflict of interest

None to declare.

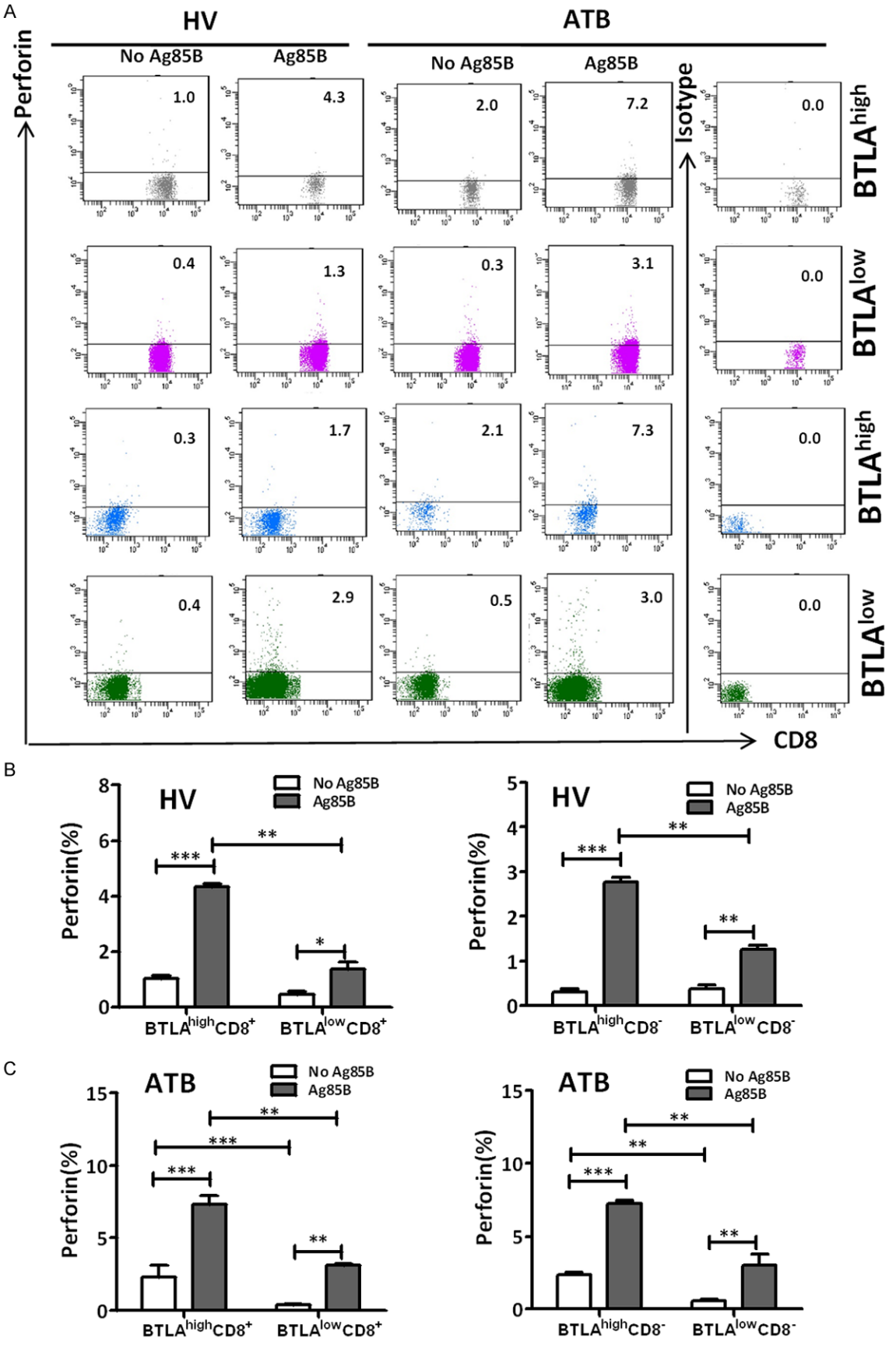
A
HV
ATB
No Ag85B
Ag85B
No Ag85B
Ag85B
Perforin
Isotype
CD8
BTLA^high
BTLA^low
BTLA^high
BTLA^low
1.0
4.3
2.0
7.2
0.0
0.4
1.3
0.3
3.1
0.0
0.3
1.7
2.1
7.3
0.0
0.4
2.9
0.5
3.0
0.0
B
HV
No Ag85B
Ag85B
Perforin(%)
BTLA^high CD8+
BTLA^low CD8+
BTLA^high CD8-
BTLA^low CD8-
C
ATB
No Ag85B
Ag85B
Perforin(%)
BTLA^high CD8+
BTLA^low CD8+
BTLA^high CD8-
BTLA^low CD8-

Figure 6. $BTLA^{high}$ αβ T cells exhibited greater memory functions for cytotoxic molecule perforin production than $BTLA^{low}$ counterparts. PBMC derived from ATB patients (n = 10) and HV (n = 10) were cultured ex vivo in presence or absence of Ag85B, then stained with fluorochrome-conjugated anti-perforin intracellularly and analyzed by polychromatic flow cytometry. $BTLA^{high}$ $CD8^+$, $BTLA^{low}$ $CD8^+$, $BTLA^{high}$ $CD8^-$ and $BTLA^{low}$ $CD8^-$ T cell subpopulations were indcated to examine IFN-γ production. (A) Shows representative flow cytometric dot plots derived from HV and ATB patient, indicating percentages of perforin producing cells. (B) Show bar graph data from HV and demonstrate the percentages of perforin producing T cells within $BTLA^{high/low}$ $CD8^+$ and $BTLA^{high/low}$ $CD8^-$ T-cell subsets, respectively. (C) Show bar graph data from ATB patients, and disply each perforin producing T cell subsets. Groups were compared by *t* test with GraphPad Prism Version 5.0. Significant differences are indicated by $*P<0.05$, $**P<0.01$, $***P<0.001$.

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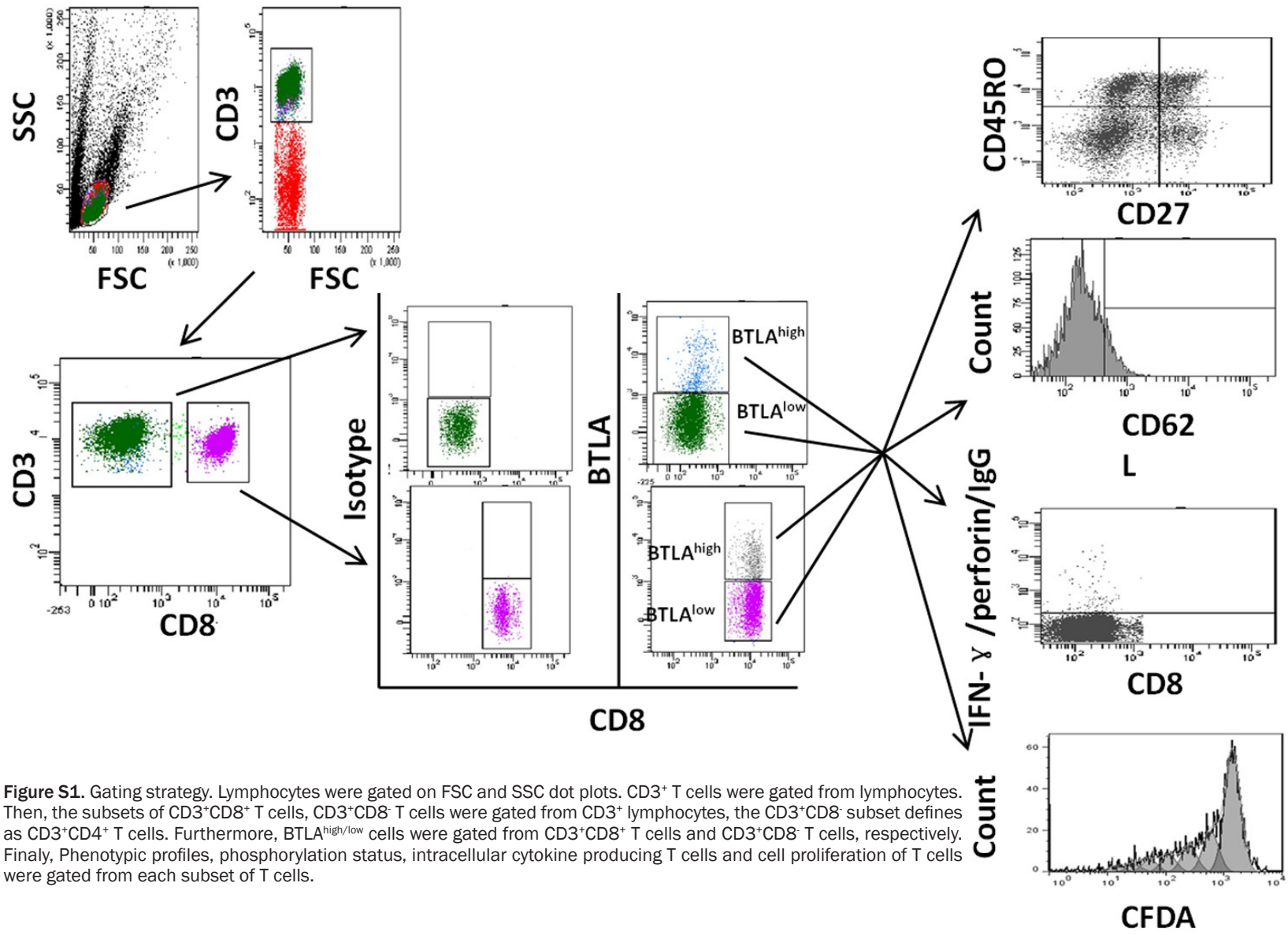

Figure S1. Gating strategy. Lymphocytes were gated on FSC and SSC dot plots. $CD3^+$ T cells were gated from lymphocytes. Then, the subsets of $CD3^+CD8^+$ T cells, $CD3^+CD8^-$ T cells were gated from $CD3^+$ lymphocytes, the $CD3^+CD8^-$ subset defines as $CD3^+CD4^+$ T cells. Furthermore, $BTLA^{high/low}$ cells were gated from $CD3^+CD8^+$ T cells and $CD3^+CD8^-$ T cells, respectively. Finaly, Phenotypic profiles, phosphorylation status, intracellular cytokine producing T cells and cell proliferation of T cells were gated from each subset of T cells.