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Thymic stromal lymphopoietin plays an adjuvant role in BCG-mediated CD8$^+$ cytotoxic T cell responses through dendritic cell activation

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Abstract Although Bacillus Calmette–Guérin (BCG) has historically emerged as a potent adjuvant in cancer immunization through dendritic cell (DC) activation, the efficacy of its antitumor effect has been limited. Therefore, the strategy of adjuvant therapy using BCG needs to be improved by adding enhancers. Here we found that thymic stromal lymphopoietin (TSLP) acts as an enhancer for the BCG-mediated antitumor effect. While BCG-stimulated DCs induced CD8$^+$ T cell production of IFN-γ without strong cell expansion, TSLP-stimulated DCs induced robust CD8$^+$ T cell expansion without high quantities of IFN-γ production. Notably, DCs stimulated with both BCG and TSLP induced robust expansion of CD8$^+$ T cells that produced a large amount of IFN-γ with a potent cytolytic activity related to granzyme B expression. Our data suggest that TSLP is a good adjuvant to enhance the BCG-mediated cytotoxic T cell effect through DC activation, and provide a functional basis for a novel strategy for antitumor immune-based therapy.

Introduction

For the last two decades, a search has been underway for an effective antitumor immune-based therapy. Immunotherapies with antigen-specific antitumor agents have been tested for several advanced malignancies such as melanoma [1–3], non-Hodgkin's lymphoma [4], and prostate cancer [5], but clinical benefits have rarely been observed. Modest efficacy has been observed with non-specific immune stimulants such as *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) or its cell wall skeleton for superficial bladder cancer [6], renal carcinoma [7], melanoma [8], bladder cancer [9], lung cancer [10], and colon cancer [11]. Meanwhile, the cellular basis underlying the adjuvant effects of BCG is most likely due to CD8$^+$ CTL activity with Th1-type cellular response [12]. Current consensus is that BCG serves as a strong immune adjuvant via

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maturation of dendritic cells (DCs) shown by the upregulation of major histocompatibility complex (MHC) class I/II and CD80/CD86 and production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-12 [13,14]. We have previously reported that human CD11c$^+$ DCs stimulated with BCG instruct naïve CD4$^+$ T cells to differentiate into IFN-γ-producing Th1 cells [15]. DCs can also directly activate CD8$^+$ T cells by cross-priming, which is an essential functional feature for vaccines to induce CTLs from naïve CD8$^+$ T cells. Moreover, it has been reported that BCG-stimulated human DCs have the ability to induce and expand IFN-γ-expressing CD8$^+$ T cells with the killing activity against BCG-infected macrophages [16]. Although much effort has been focused on studies of the cellular mechanism of BCG immunotherapy, the efficacy of its antitumor effect has still been limited [17,18]. Therefore, the strategy of adjuvant therapy using BCG needs to be improved or modified by adding optimal enhancers.

Thymic stromal-derived lymphopoietin (TSLP) is a newly found epithelial cell-derived cytokine that triggers allergic inflammatory cascade by inducing DC-mediated CD4$^+$ inflammatory Th2 cell responses, and has been implicated in atopic dermatitis and asthma [19,20]. TSLP has two unique features in DC-mediated T cell responses: (i) TSLP strongly activates human CD11c$^+$ DCs without inducing Th1-polarizing cytokines IL-12 or pro-inflammatory cytokines such as TNF-α and IL-6; and (ii) these TSLP-activated DCs induce prominent naïve CD4$^+$ T cell expansion, with which cells differentiate into Th2 effector cells that secrete large amounts of IL-4, IL-5, IL-13, and TNF-α. Interestingly, TSLP activates DCs to also induce robust expansion of naïve CD8$^+$ T cells in the presence of CD40 ligand (CD40L) stimulation [21], this ligand being basically derived from activated CD4$^+$ T cells within lymphoid tissues during DC-T cell interaction as a supplementary factor to help CD8$^+$ T cell activation [22–25]. Thus, TSLP may possibly be one of the most potent DC-activation factors, inducing the prominent expansion of not only CD4$^+$ but also CD8$^+$ allogeneic naïve T cells mediated by DCs.

Based on these findings, we hypothesized that TSLP could enhance the effect of classical adjuvants such as BCG, and thus we studied the adjuvant activities and DC-mediated therapeutic potential using a combination of BCG and TSLP. We here demonstrate that, through DC activation, human TSLP amplified the BCG-mediated expansion and functions of CTLs that produce IFN-γ and exhibit cytolytic activity related to the expression of intracellular granzyme B. Thus, our results identify a specialized role of TSLP as a potent adjuvant for the expansion of CTL responses induced by BCG in the DC-mediated immune process.

Materials and methods

Isolation and culture of blood DCs

CD11c$^+$ DCs were isolated from PBMCs from HLA-A2-positive healthy adult donors, as described [26]. Briefly, the DC-enriched population (CD4$^+$/CD3$^-$/CD14$^-$ cells) was obtained from PBMCs by negative and subsequent positive immunoselections [26]. The CD11c$^+$/lin$^-$/BDCA4$^-$/CD4$^+$ cells (CD11c$^+$ myeloid DCs) were sorted by a FACS Aria® (BD Biosciences) by using PE-labeled or allophycocyanin (APC)-labeled anti-CD11c (BD Biosciences), APC-labeled or PE-labeled anti-CD304 (BDCA-4/Neuropilin-1) (Miltenyi Biotec), a mixture of FITC-labeled mAbs against lineage markers, CD3 (BD Biosciences), CD11b (CALTAG), CD14 (BD Biosciences), CD15 (BD Biosciences), CD16 (Exalpha), CD19 (BD Biosciences) and CD56 (BD Biosciences); and PE-Cy5.5-labeled anti-CD4 (CALTAG) to reach >99% purity. The CD11c$^+$ DCs were cultured in RPMI 1640 and supplemented with 2% human AB serum, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin G, and streptomycin in flat-bottomed 96-well plates in the presence of TSLP (15 ng/ml) and/or BCG (1 MOI: EMD Biosciences) at 5×10^4 cells in 200 μl of medium/well for 24 h. In some experiments, DCs were cultured on the pre-irradiated and pre-seeded CD40L-transfected L cells (at 4×10^4 cells/well). The TSLP was purchased from R&D Systems and it should be noted that, according to the accompanying data sheet, the endotoxin level was less than 1.0 EU per 1 μg of the protein, as measured using the LAL method.

Analyses of DCs

To analyze the expression of surface markers, the cultured DCs were collected and stained with FITC-labeled CD86 (BD Biosciences), PE-labeled anti-4-1BBL (BD Biosciences), PE-labeled anti-GITRL (eBioscience), PE-labeled anti-IL-7Rα (CD127) (Beckman Coulter), or APC-labeled anti-TSLPR (BioLegend), or with an isotype-matched control mAb, and then analyzed by a FACScalibur® (BD Biosciences). The production of cytokines in the culture supernatants after 24 h was determined by Cytometric Beads Array (BD CBA FLEX sets for IL-12p70, TNF-α, and IL-6 were purchased from BD Biosciences).

Ex vivo DC vaccination into PBMCs

2×10^5 PBMCs were cultured with 2×10^4 freshly purified allogeneic CD11c$^+$ DCs in 96-well round bottomed culture plates in 200 μl of medium/well for 7 days in the presence or absence of TSLP (15 ng/ml) and/or BCG (1 MOI for DC numbers). The cultured cells were collected and stained with anti-CD3, anti-CD4 and anti-CD8 to detect the percentage of CD8$^+$ T cells (CD3$^+$CD4$^-$CD8$^+$ cells) in total PBMCs.

Naïve CD8$^+$ T cell purification

CD8$^+$ T cells were enriched from PBMCs from HLA-A2-negative healthy adult donors using CD8$^+$ T cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. Enriched CD8$^+$ T cells were stained with an FITC-labeled lineage cocktail (CD4, CD14, CD16, CD19, CD56, CD45RO, TCRγ/δ and Glycophorin A), PE-labeled anti-CD27 (BD Biosciences), and allophycocyanin-labeled anti CD45RA (BD Biosciences). Naïve CD8$^+$ T cells were sorted by fractions of lineage$^-$CD45RA$^+$CD27$^+$ cells (purity>98%).

Stimulation of CD8$^+$ T cells

2×10^4 freshly purified naïve CD8$^+$ T cells were cocultured with purified allogeneic CD11c$^+$ DCs pre-cultured under different conditions (DC to T ratio, 1:4) in 96-well round bottomed culture plates for 7 days. In some experiments, purified naïve