SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 3: 472-476, 2012

IL-15, in synergy with RAE-1ε, stimulates TCR-independent proliferation and activation of CD8+ T cells

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Received August 17, 2011; Accepted November 18, 2011

DOI: 10.3892/ol.2011.495

Abstract. CD8+ T cells play critical roles in immunosurveillance by killing malignant or virally infected cells. Interleukin 15 (IL-15) is a critical cytokine for promoting proliferation and the effector capacity of CD8+ T cells, and has been used to support the growth of CD8+ T cells in cellular therapies of neoplastic diseases. Recent studies have shown that IL-15, in synergy with other cytokines, such as IL-6, enhances the T-cell receptor (TCR)-independent proliferation and function of CD8+ T cells. The aim of the present study was to investigate the role of BaF3-mb15-RAE cells in stimulating mouse CD8+ T cells. BaF3 cells were cultured and B16F10 cells were grown in DMEM. MTT assay was used to detect the proliferation of CD8+ T cells. Cells were analyzed using flow cytometry. The results showed that IL-15 synergistically acts with another T-cell stimulatory molecule, RAE1ε, to potently promote the proliferation of CD8+ T cells, induce CD8+ T-cell activation and enhance granzyme B and interferon-γ (IFN-γ) production in the absence of signaling via the TCR. Moreover, IL-15 in combination with RAE1ε resulted in a cooperative effect on CD8+ T-cell-mediated cytotoxicity against B16F10 tumor cells. Thus, results of the present study showed that IL-15, in synergy with RAE1ε, enhances the TCR-independent effector function of CD8+ T cells *in vitro*, which may be useful in the cellular immunotherapy of cancer.

Introduction

Cytotoxic CD8+ T cells are effector cells that play a key role in immunosurveillance through the elimination of malignant or virally infected cells. Interleukin 15 (IL-15) is a pivotal cytokine for the activation and proliferation of cytotoxic CD8+ T cells (1-3). As opposed to other soluble cytokines that induce signals upon binding to their respective receptors, IL-15 delivery is relatively unique (4). IL-15 bound to its high-affinity receptor (IL-15R)α-chain may be retained on the cell surface and presented in trans to target cells such as CD8+ T cells, which express only IL-2/15Rβ and γ (5). The receptor-cytokine complex may act as a membrane-bound stimulatory molecule in a contact-dependent manner (6). Numerous studies raised the possibility that the IL-15/IL-15Rα complex is a promising and potent agent for tumor immunotherapy (7-11).

NKG2D is an activating receptor present on the surface of mouse and human NK, γδT, human CD8+ T and activated mouse CD8+ T cells (12,13). The ligands for the NKG2D receptor include RAE1 (RAE1α-RAE1ε), Mult1 and H60 in the mouse, and MICA/B and RAET1, also known as UL-16 binding proteins (ULBPs), in the human (14,15). Results of previous studies showed that NKG2D may function as a costimulatory receptor for both mouse and human CD8+ T cells (16,17). IL-15 selectively upregulates the expression and function of NKG2D (18). In addition, IL-15 confers NK-like activity to effector CD8+ T cells, unveiling the cytotoxic properties of NKG2D (18).

Since NKG2D and IL-15 play significant roles in the activation and/or expansion of CD8+ T cells (as mentioned above), we expressed both membrane-bound forms of IL-15 (the mouse IL15 gene fused to the gene encoding the mouse CD8α transmembrane and cytoplasmic domain) and NKG2D ligand RAE1ε on BaF3 cells (termed BaF3-mb15-RAE) in a previous study (19). In this study, we investigated the role of BaF3-mb15-RAE cells in stimulating mouse CD8+ T cells. CD8+ T cells exhibited an activated phenotype, and increased proliferation, granzyme B and interferon-γ (IFN-γ) release, as well as cytotoxicity when stimulated with BaF3 cells co-expressing RAE1ε and IL-15.

Materials and methods

Mice and cell culture. C57BL/6J mice, at 5-6 weeks of age, were obtained from Joint Ventures Sipper BK Experimental Animal Co. (Shanghai, China). Animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Animal Welfare Association of Jiangsu province (Jiangsu, China). The study was approved by the Ethics Committee of Yangzhou University. The mouse pro-B lymphocyte cell line BaF3 cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) and 250 pg/ml recombinant mouse IL-3

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Key words: CD8+ T cells, RAE1ε, IL-15, proliferation, activation

(R&D Systems, Minneapolis, MN, USA). Mouse melanoma B16F10 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with a high glucose concentration (4.5 g/l), supplemented with 10% FBS.

Development of BaF3 derivatives. BaF3 derivatives were prepared as previously described (19). Briefly, BaF3 cells were transduced with the construct encoding RAE1ε and the membrane-bound form of IL-15. Cells were cloned by limiting dilution, and a single-cell clone with a high expression of RAE1ε and a surface expression of IL-15 (termed BaF3-mb15-RAE) was expanded. BaF3 cells expressing a membrane-bound form of IL-15 (termed BaF3-mb15) or RAE1ε (termed BaF3-RAE) were produced using a similar procedure.

Isolation of mouse CD8+ T cells and CFSE labeling. Spleen-derived CD8+ cells were enriched using CD8α-conjugated microbeads as recommended by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany), and the cells were then labeled with anti-CD3 and anti-CD8α antibodies (Biolegend, San Diego, CA, USA). The $CD3^+CD8\alpha^+$ cells were sorted by FACSAria (BD Biosciences, San Jose, CA, USA). The purity of the sorted cells was routinely >96%. In all experiments, CD8+ T cells were identified as $CD8\alpha^+CD3^+$ cells. For CFSE labeling, CD8+ T cells were washed with 0.1% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS) and resuspended at a density of $5x10^6$ cells/ml in 0.1% BSA/PBS at a final concentration of 5 μM CFSE (Invitrogen, Karlsruhe, Germany) for 5 min at 37˚C, followed by washing with 2% FBS in PBS.

Proliferation of CD8+ T cells. MTT assay and CFSE dilution were used to detect the proliferation of CD8+ T cells. CD8+ T cells ($1x10^5$) or CFSE-labeled CD8+ T cells were incubated in a 96-well round-bottom plate with or without $1x10^5$ irradiated BaF3-derivative stimulator cells. On days 3 and 6, the cells were restimulated with irradiated BaF3-derivative stimulatory cells. On day 8, CD8+ T cells were analyzed for CFSE dilution and the MTT assay was performed. Dimethyl sulfoxide (DMSO) (Sigma) was used to dissolve the formazan, and the absorbance was measured at 570 nm.

Flow cytometry and IFN-γ assay. The CD8+ T cells were co-cultured with the indicated irradiated stimulatory cells. Following 24 h of culture, the cells were stained with fluorescently labeled monoclonal antibodies (mAbs) specific for CD3, CD8α, CD69, CD25 and NKG2D or isotype controls, and were analyzed using a BD LSR II flow cytometer (BD Biosciences) and FlowJo software (Tree Star, San Carlos, CA, USA). The supernatants were collected for IFN-γ assay (BD Biosciences) by cytometric bead array (CBA) technology, according to the manufacturer's instructions.

Intracellular detection of granzyme B. CD8+ T cells were co-cultured with the indicated irradiated stimulatory cells for 24 h and then stimulated with PMA (50 ng/ml), ionomycin (500 ng/ml) and GolgiStop (BD Pharmingen) for an additional 6 h. The cells were then stained with anti-CD3-FITC, anti-CD8α-PE/Cy7 conjugated mAbs, and fixed and permeabilized with the Cytofix/Cytoperm kit (BD Pharmingen). Permeabilized cells were incubated with anti-mouse granzyme B-PE-conjugated mAb. The cells were analyzed with the BD LSRII flow cytometer (BD Biosciences) and FlowJo software.

Cytotoxicity assay. Lysis of target cells was determined by a flow cytometry-based cytotoxicity assay, as previously described (20). Briefly, CD8+ T cells were co-cultured with a constant number of CFSE-labeled target cells ($2.5x10^4$) at various effector-target (E:T) ratios as indicated in the presence of irradiated BaF3 derivatives. After 24 h, 7 amino-actinomycin D (7-AAD) was added to measure the death of target cells.

Statistical analysis. Data are presented as the mean ± SD for separate experiments. Statistical significance was determined by the Student's t-test. $P<0.05$ was considered to indicate statistically significant differences.

Results

Stimulation with BaF3-mb15-RAE cells induces CD8+ T-cell activation. We evaluated CD8+ T-cell activation, following stimulation with the BaF3-mb15-RAE cells, by flow cytometry using surface markers known to be upregulated on CD8+ T cells upon activation (18,21). As shown in Fig. 1A and B, the percentage of $NKG2D^+$, $CD25^+$ and $CD69^+$ CD8 T cells increased significantly following BaF3-mb15-RAE stimulation. No significant differences were found among other pairwise comparisons of surface marker expressions obtained with BaF3, BaF3-mb15 and BaF3-RAE cells. Thus, CD8+ T cells acquire an activated status following BaF3-mb15-RAE cell stimulation.

Stimulation with BaF3-mb15-RAE cells promotes CD8+ T-cell proliferation. To evaluate the impact of stimulation with BaF3-mb15-RAE cells on CD8+ T-cell proliferation, irradiated BaF3 derivatives were cultured with CFSE-labeled CD8+ T cells for 8 days. As shown in Fig. 2A and B, the expression of RAE1ε on BaF3 cells, as with IL-15, increased CD8+ T-cell proliferation, but the co-expression of RAE1ε and IL-15 exhibited the greatest effect. Similar results were obtained by the MTT assay (Fig. 2C). These data suggest that the IL-15 and NKG2D signaling pathways have a synergistic effect on CD8+ T-cell proliferation.

Stimulation with BaF3-mb15-RAE cells leads to an increased granzyme B and IFN-γ production of CD8+ T cells. To evaluate the impact of stimulation with BaF3-mb15-RAE cells on the function of CD8+ T cells, we first determined perforin and granzyme B production, an indication of target lysis, by intracellular flow cytometry. The combination of IL-15 and RAE1ε resulted in a marked increase in the amount of granzyme B per CD8+ T cell, as compared with that observed in cells stimulated with IL-15 or RAE1ε alone, as indicated by the mean fluoresece intensity (Fig. 3A). However, no difference in perforin production was detected among CD8+ T cells co-cultured with BaF3-RAE, BaF3-mb15 or BaF3-mb15-RAE (data not shown).

We also evaluated the production of IFN-γ, the cytokine involved in activating immune cells against tumors, using

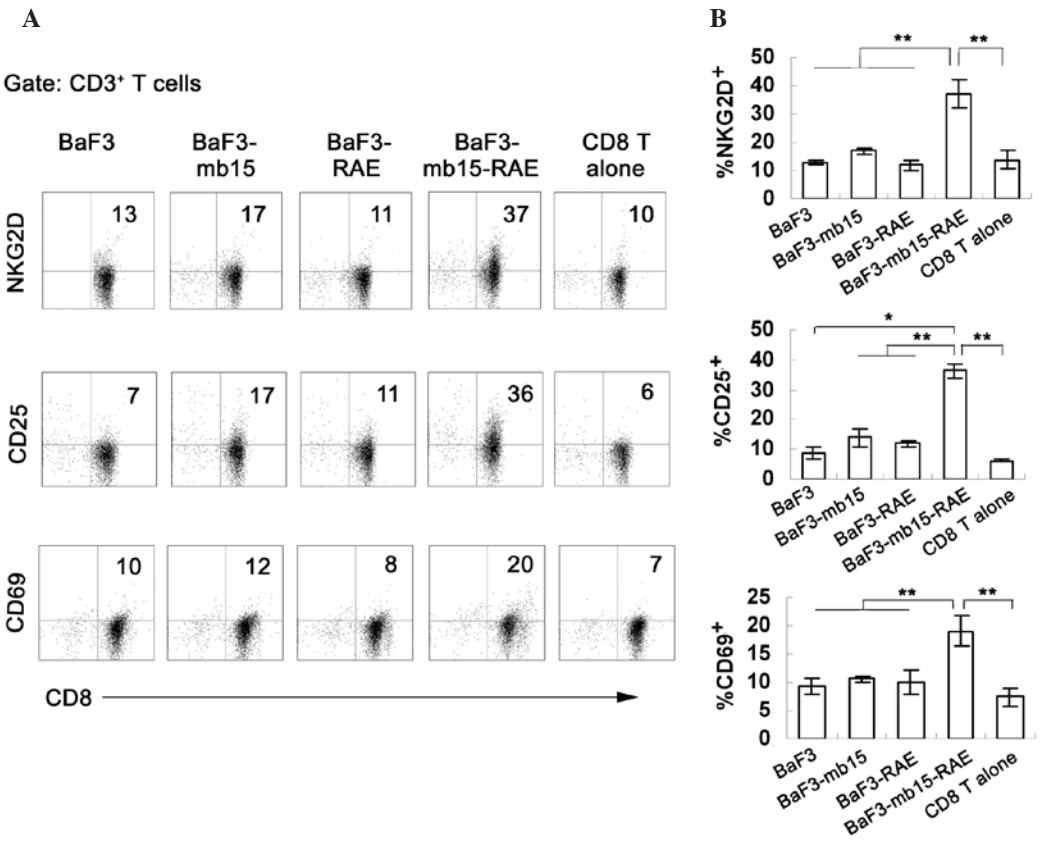

Figure 1. CD8+ T cells were activated following stimulation with the BaF3-mb15-RAE cells. Freshly isolated CD8+ T cells were cultured with various preparations of BaF3 at a 1:1 ratio for 24 h, and the expression of NKG2D, CD25 and CD69 on CD8+ T cells was detected by flow cytometry. (A) Representative dot plot showing NKG2D, CD25 and CD69 expression on CD8+ T cells. Numbers indicate percentages. (B) The percentages of NKG2D+, CD25+ and CD69+ CD8+ T cells were calculated. *P<0.05, **P<0.01 indicated stastistically significant differences. Data are presented as the means ± SD.

CBA technology. As shown in Fig. 3B, CD8+ T cells stimulated with BaF3-mb15-RAE cells were capable of producing higher levels of IFN-γ compared to CD8+ T cells stimulated with BaF3-mb15 or BaF3-RAE cells. Thus, stimulation with BaF3-mb15-RAE cells promotes the function of CD8+ T cells, manifested as induced granzyme B and IFN-γ production.

Stimulation with BaF3-mb15-RAE cells generates CD8+ T cells with enhanced cytotoxicity. We evaluated the impact of stimulation with BaF3-mb15-RAE cells on CD8+ T-cell cytotoxicity. Cytotoxicity of CD8+ T cells against tumor cells (B16F10) was tested using a flow cytometry-based cytotoxicity assay. CD8+ T cells exhibited increased cytotoxicity following stimulation with BaF3-mb15-RAE cells compared with both BaF3-stimulated and -unstimulated controls (Fig. 4). These data suggest that stimulation with BaF3-mb15-RAE cells generates CD8+ T cells with enhanced cytotoxicity.

Discussion

Results of the present study have shown that the expression of both RAE1ε and a membrane-bound form of IL-15 on BaF3 cells increased CD8+ T-cell activation, proliferation, cytotoxicity, granzyme B and IFN-γ secretion in the absence of signaling via the TCR.

Activation of T cells by antigen requires two types of signaling; one signal delivered via the TCR and the other

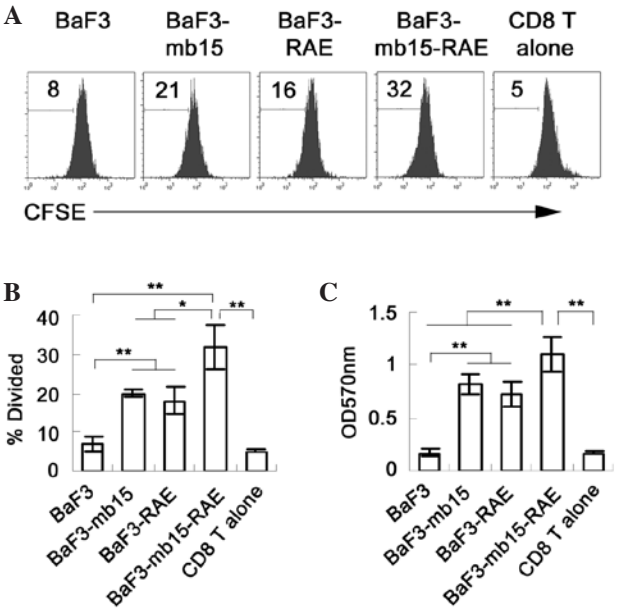

Figure 2. BaF3-mb15-RAE cells promoted proliferation of CD8+ T cells. CD8+ T or CFSE-labeled CD8+ T cells were incubated with irradiated BaF3-derivative stimulatory cells. On days 3 and 6, the cells were restimulated with irradiated BaF3-derivative stimulatory cells. (A and B) On day 8, CD8+ T cells were analyzed for CFSE dilution. (A) Representative histograms showing CD8+ T-cell proliferation. (B) Numbers indicate percentages of divided cells. The percentages of divided cells were calculated. (C) On day 8, the proliferation of CD8+ T cells was measured by MTT assay. *P<0.05, **P<0.01 indicated stastistically significant differences. Data are presented as the means ± SD.

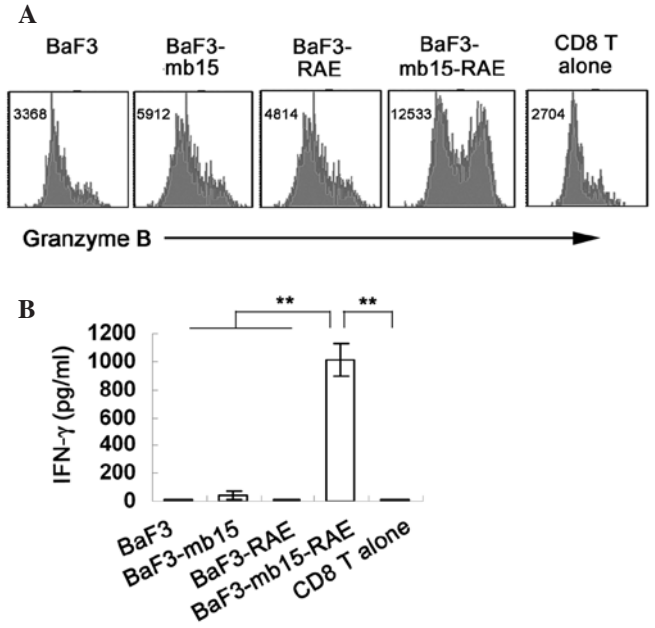

Figure 3. BaF3-mb15-RAE cells promoted granzyme B and IFN-γ secretion of CD8+ T cells. Freshly isolated CD8+ T cells were co-cultured with the indicated irradiated stimulatory cells for 24 h. (A) Intracellular granzyme B expression in CD8+ T cells was analyzed by flow cytometry. (B) The level of IFN-γ in the supernatant was detected by CBA technology. Numbers in histogram A indicate MFI of test samples. Data are presented as the means ± SD. **P<0.01. CBA, cytometric bead array.

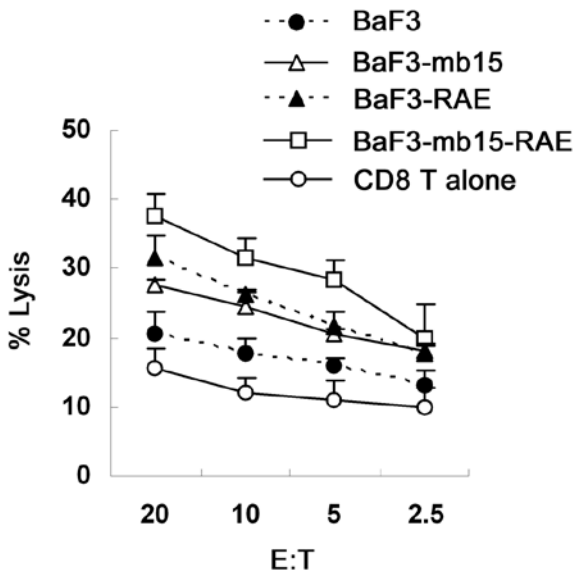

Figure 4. Lysis activity of BaF3-mb15-RAE-stimulated CD8+ T cells. CFSE-labeled target cells were co-incubated with CD8+ T cells at E:T ratios of 2.5:1 to 20:1 in the presence of the indicated irradiated stimulatory cells. The specific lysis was determined by a flow cytometry-based cytotoxicity assay. Results are presented as the means ± SD of triplicates. E:T, effector-target ratio.

signal through engagement of co-stimulatory molecules. However, in response to homeostatic pressure, T cells undergo cytokine-driven proliferation without overt antigen stimulation (22). This hypothesis is supported by previous findings that IL-15 is capable of synergizing with IL-21 or IL-6 to induce proliferation and effector functions of naïve CD8+ T cells, without concomitant stimulation via the TCR (23-25).

IL-15 belongs to the IL-2 family of cytokines, which require a common receptor γ-chain to transduce cell activation signals (26). Findings of previous studies have demonstrated that IL-15 is not usually secreted in a soluble form but presented in trans as a surface complex with IL-15Rα by dendritic cells (DCs) and monocytes (4,5). Cell-bound IL-15 then stimulates the IL-2/IL-15βγc receptor, which is expressed primarily by NK and CD8+ T cells (5,27-30). Therefore, these observations suggest that IL-15, associated with the cell surface, signals in a cell contact-dependent manner (31).

Considering the following two factors, we expressed a membrane-bound form of IL-15, rather than a soluble form, in BaF3 cells. First, the membrane-bound form of IL-15 may impair IL-15 internalization by CD8+ T cells and thereby strengthen signaling through the IL-2/15Rβ and γ (27). This theory deserves consideration since the internalization of certain cytokines serves to attenuate receptor signaling (27,32). Second, the membrane-bound form of IL-15 may be more stimulatory than soluble IL-15. This hypothesis is in agreement with reports that IL-15 has greater biological activity when presented to NK cells bound to IL-15Rα on the cell membrane of stimulatory cells, rather than in its soluble form (5,28,33).

Since IL-15 was reported to act in agreement with other cytokines to enhance T-cell function, we tested whether the IL-15-stimulatory capacity was capable of being increased through an enforced expression of an additional stimulatory molecule. The molecule we focused on is RAE1ε, the ligand of NKG2D. NKG2D is one of the best characterized activating receptors expressed on almost all NK cells, subsets of γδT cells, as well as all human CD8+ T cells and activated mouse CD8+ T cells (12,13). As noted earlier, the binding of NKG2D to its ligands is a potent costimulator of TCR-mediated effector functions but does not appear to function independently of TCR signaling (12,34). IL-15 was found to elicit a coordinated series of biochemical changes in the NKG2D signaling pathway, ultimately arming TCR-independent NK-like killing through NKG2D, and effectively converting effector CD8+ T cells into 'lymphokine-activated killer' (LAK) cells (35).

In the present study, we have shown that a membrane-bound form of IL-15, is capable of synergizing with the NKG2D ligand, RAE1ε, to stimulate the activation of CD8+ T cells in a TCR-independent manner. It has been shown that a number of tumors naturally express appreciable levels of IL-15/IL-15Rα (36) and that NKG2D ligands are upregulated on numerous tumor cells. Our results raise the possibility that the co-expression of IL-15/IL-15Rα and NKG2D ligands in the same cell may render the emerging tumor cells sensitive to NKG2D-dependent elimination during tumorigenesis. *In vitro* activation and proliferation of CD8+ T cells by IL-15, in synergy with NKG2D ligands, may be used to generate tumor-specific cells with ideal properties for cellular immunotherapy of cancer.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (No.81001308) and the Natural Science Fund of Jiangsu Province (BK2010315).

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