$CD8^+$T cell granzyme B activates keratinocyte endogenous IL-18

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Abstract

IL-18 is a pro-inflammatory cytokine of the IL-1 family involved in Th1/Th2 polarization. IL-18 is produced and stored as an inactive precursor (proIL-18) in several cells including keratinocytes, and thus appropriate processing is required to release its active form. In a previous study using recombinant protein, we demonstrated that granzyme B (GrB) cleaves proIL-18 into its active forms in a similar fashion as caspase-1 and human mast cell chymase. GrB released from cytotoxic T lymphocyte (CTL) and NK cells has roles in apoptosis and cytotoxic activity. In certain inflammatory skin diseases with epidermal cell death, the epidermal keratinocytes are targets of CTL and NK cells. However, IL-18 activation during the direct interaction of CTL/NK with keratinocytes has not been described so far.

We here investigated the interaction between CTL and keratinocytes, and IL-18 processing by CTL derived GrB using cultured CD8+T cells and keratinocyte cell line HaCaT. GrB(+)/caspase-1(-)CD8+T cells cultivated from healthy human PBMC were co-cultured with interferon(IFN)-γ-treated HaCaT cells. The expression of GrB and caspase-1 in HaCaT cells was analyzed by flow cytometry and PCR. The IL-18 concentration in the culture supernatant was measured by specific ELISA. The interaction between HaCaT cells and CTL co-culture increased the number of cytoplasmic GrB-positive HaCaT cells with limited endogenous GrB mRNA expression. The concentration of mature IL-18 levels increased in the co-culture supernatant. GrB from CTLs acts double roles to keratinocytes: a IL-18 converting enzyme and pro-apoptotic factor in the skin inflammatory diseases.

Introduction

IL-18 is not only a pro-inflammatory mediator but also a regulator of the cytotoxic activity of Natural Killer (NK) cells and T cells [1, 8]. Further, IL-18 can support the differentiation and activation of different T helper (Th) cell subsets depending on the surrounding cytokine profile [2]. In the presence of IL-12 during pathological conditions such as chronic eczema or psoriatic skin inflammation, IL-18 favors Th1 responses; but in the absence of IL-18, theTh2 response is enhanced as seen in the acute phase of atopic dermatitis and cutaneous T cell lymphoma [12, 18, 19]. IL-18 is produced by a wide range of cells including Kupffer cells, macrophages, T cells, B cells, osteoblasts, keratinocytes, dendritic cells, astrocytes and microglial cells [11]. IL-18 is produced as a biologically inactive precursor, and thus appropriate processing is required to secrete its active form. In a previous study, we demonstrated that granzyme B (GrB) cleaves proIL-18 into its active forms using a recombinant protein, in a similar fashion as caspase-1 and human mast cell chymase [13, 14]. The inflammatory cytokine activation during the CTL/NK-mediated keratinocyte injury has not been as yet clarified; it is still unclear how proIL-18 is activated in the epidermis during inflammatory conditions. In the current study, we investigated the interaction between CTL and keratinocytes, and the mechanism of IL-18 processing by CTL-derived GrB using a co-culture system of CD8+T cells and a keratinocyte cell line.

Materials and methods

Preparation of CD8 cells

PBMCs from healthy donors were prepared by density gradient centrifugation over Ficoll-Histopaque (Sigma Chemicals, St. Louis, MO, USA). CD8+ T cells were purified with MACS CD8 microbeads (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's instructions, and 1×10^6/ml of cells were cultured with 2×10^7/ml of irradiated autologous PBMCs in complete medium containing RPMI, 10% human AB serum (Gemini bio-products), 2.0 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Nacalai Tesque, Kyoto, Japan), and 10mM Hepes buffer supplemented with 100U/ml of recombinant human IL-2 (Proimmune, Bradenton, FL, USA) and 80 ng/ml of anti-CD3 mAb (Miltenyi Biotec). Half of the medium was replaced by complete medium containing IL-2 (20 U/ml), and this replacement was repeated twice a week [14]. After 2 weeks of culture, CD8+T cells were re-purified by MACS CD8 microbeads and stimulated with Phorbol 12-myristate 13-acetate (PMA), and ionomycin (Sigma-Aldrich, St. Louis, MO, USA) for 4hours.

Flow cytometry analysis of MHC class I expression on HaCaT cells

The human keratinocyte cell line, HaCaT cells (Cell Line Service, Eppelheim, Germany) were cultured in Eagle's MEM (Sigma, St Louis, MO, USA) medium supplemented with 5% fetal calf serum in an incubator containing 5% CO2. HaCaT cells were pre-treated with several concentrations of IFN-γ (PEPRO TECH, Rocky Hill, NJ, USA) (0, 25, 50, 75, 150 ng/ml) for 48hours, leading to increased major histocompatibility complex (MHC) class I expression. To confirm the expression of MHC class I, HaCaT cells were stained with PE-conjugated anti-HLA-A2 antibody (MBL, Nagoya, Japan). The fluorescence profile was analyzed by flow cytometry using FACSCalibur (BD Biosciences, San Jose, CA, USA)(n=5).

mRNA expression in CD8+T cells and HaCaT cells

The RNA was extracted from cultured CD8+ T cells using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Briefly, 1 ml of homogenate was mixed with 200 µl of chloroform and then centrifuged. The aqueous phase was separated and mixed with 0.5 ml of 2-propanol (Nacalai Tesque) to precipitate RNA. After centrifugation, the precipitate was washed with 70% ethanol (Nakalai Tesque) and the RNA was suspended in 30µl RNase-free water. The RNA concentration was measured at 260 nm. cDNA was synthesized from 1 µg of RNA using a High Capacity RNA-to-cDNA Kit (ABI, Foster City, CA, USA) according to the manufacturer's protocol. GAPDH was used as an internal control for each cell preparation. The primer sequences were as follows (5'-3'), GrB: TGC AAC CAA TCC TGC TTC TG and GAC ATT TAT GGA GCT TCC CC, caspase-1: AGA GCA CAA GAC CTC TGA CA and TCT TTC AGT GGT GGG CAT CT, human IL-18: TAC TTT GGC AAG CTT GAA TCT AAA T and GTC TTC GTT TTG AAC AGT GAA CAT T, GAPDH: GTG GGG CGC CCC AGG CAC CA and CTC CTT AAT GTC ACG CAC GAT TTC [14]. The fragments of GrB, IL-18 and caspase-1 genes were amplified under the following conditions: 30 cycles of amplification consisting of 30s denaturation (94°C), 45s primer-annealing (56°C), 40s primer-extension (72°C), and 480s final-extension (72°C). The PCR products were then run on 2% agarose gels in triethanolamine buffer (pH 7.4) containing ethidium bromide, and the gels were photographed under UV light. The densities of the DNA bands were determined using the Lane & Spot Analyzer software (Atto, Tokyo, Japan), and the integrated density value of the bands was measured.

Co-culture of GrB(+)/caspase-1(-) human CD8+T cells and IFN-γ-treated HaCaT cells

GrB(+)/caspase-1(-) human CD8+T cells and 25ng/ml of IFN-γ-treated HaCaT cells

were co-cultured for 18hours in RPMI medium.1.6 $\times 10^6$ of HaCaT cells were seeded in plate and CD8+ T cells were supplemented at the ratio of 1:0, 1:2, 1:4, 1:8, 1:16 (HaCaT cells and CD8+ T cells), respectively. After 18hours, the culture plates were examined under microscope. Then, the collected culture supernatant was used to measure cytokines. To collect the cultured cells completely, cells were incubated with 2.5g/l-trypsin/1mmol/l-EDTA for 5min at 37 °C (Nacalai Tesque). Part of the collected cells was washed and analyzed by flow cytometry as described below. The remaining co-cultured cells were used to isolate HaCaT cells from the mixture of cells using MACS CD8 microbeads positive selection. The expressions of the GrB mRNA in HaCaT cells were examined as described above (n=5).

GrB and caspase-1 expression in CD8+ T cells and HaCaT cells

The intracellular cytokine staining of CD8+ T cells and HaCaT cells were performed following the Intracellular Cytokine Staining Protocol (BioLegend). Briefly, for the detection of GrB, after treatment with the fixation buffer and permeabilization wash buffer, the cells were incubated with FITC-conjugated anti-human Granzyme B antibody (BioLegend). For caspase-1staining, the cells were firstly incubated with the rabbit antibody against cleaved caspase-1 and then with FITC conjugated anti-rabbit monoclonal antibody (BioLegend). The fluorescence profiles were analyzed by flow cytometry using FACSCalibur (n=5).

IL-18 level in the culture supernatant

The supernatant from co-culture of HaCaT cells and CD8+ T cells was collected, and the concentration of bio-active IL-18 was measured by ELISA (MBL, Nagoya, Japan) according to the manufacturer's protocol (n=5).

Statistical analysis

Statistical analysis was performed using the Kruskal-Wallis nonparametric ANOVA and Dunn's multiple comparison tests for *post hoc* analysis. A p value of less than 0.05 was considered as statistically significant.

Results

MHC class I expression on HaCaT cells

The expression of MHC class I was upregulated on HaCaT cells with IFN-γ supplementation, reaching a plateau at 20% of positive cells at a concentration of 25 ng/ml IFN-γ (Fig. 1). In this system, the most adequate concentration of IFN-γ for MHC class I expression was 25 ng/ml.

RT-PCR analysis of CD8+Tcells and HaCaT cells

CD8+ T cells cultured for 2weeks showed expression of GrB mRNA, but not of caspase-1 or proIL-18 mRNA. On the other hand, HaCaT cells stimulated with IFN-γ showed no expression of GrB mRNA but it was positive for caspase-1 and IL-18 expressions (Fig. 2).

Granzyme B and caspase-1 expression in CD8+ T cells and HaCaT cells after co-culture

GrB positive HaCaT cells were undetectable without stimulation. However, the GrB expression level on HaCaT cells was increased after co-culture with GrB(+)/caspase-1(-) CD8+ T cells (Fig.3A). Around 65% of CD8+ T cells expressed GrB after co-culture. On the other hand, GrB(+)/caspase-1(-) human CD8+T cells and HaCaT cells showed little expression of the mature form of caspase-1 even after co-culture (Fig.3B). Isolated HaCaT cells showed weak expression of GrB mRNA after co-culture, and the density of the band remained at same levels after co-culture with increased numbers of CD8+ T cells. The integrated density value was not elevated with the addition of CD8+ T cells (Fig.3C).

Microscopic findings of HaCaT and CD8+ T cells after co-culture

HaCaT cells were adherent to the dish (Fig.4A); but after co-culture, HaCaT cells

detached from the dish and changed to round shape (Fig.4B). At high magnification, some CD8+ T cells tightly adhered to HaCaT cells (Fig.4C).

Level of mature IL-18 in the co-culture supernatant

The level of mature IL-18 in the supernatant was elevated at significant level after co-culture with GrB(+)/ caspase-1(-) human CD8+T cells (Fig.5).

Discussion

Keratinocytes store several types of inflammatory cytokines including IL-18; under steady-state-conditions IL-18 is stored as an immature form, and is converted and released in mature forms under some stimulated conditions including inflammation [18]. It is well-known that IL-18 is a cytokine playing a key role in the enhancement of immunological polarization. In the presence of IL-12, IL-18 promotes immunological balance to Th1 differentiation. However in the absence of IL-12, IL-18 turns the balance to Th2 dominant conditions. Therefore, the mature form of IL-18 acts as an accelerator of the immune responses. Immunohistopathologic and RT-PCR analysis demonstrated high levels of IL-18 and IL-18 receptors expression in skin lesions associated with cutaneous lupus erythematosus, psoriasis, graft versus host disease, cutaneous T cell lymphoma and atopic dermatitis, but not in the normal skin [6, 7, 12, 15, 17]. Enzyme cleavage by specific enzymes including casapse-1 is required for processing of IL-18 proform to its mature form [3, 10, 16]. However, intracellular caspase-1 is rarely detected in keratinocytes. In the current study, HaCaT cells were found to express caspase-1 mRNA. However, caspase-1 itself also requires posttranslational activation. In fact, functional caspase-1 protein was undetected by flow cytometry in the present study. Using recombinant protein, we have previously demonstrated that GrB cleaves and activates proIL-18 as well as caspase-1, and human mast cell chymase [13, 14]. GrB is a serine protease abundantly stored in secretory granules of CTLs and NK cells. After recognizing the target cells, CTLs and NK cells secrete GrB and other granule proteins including perforin through the immunological synapse: the submicroscopic intercellular clefts. GrB translocated into the cytoplasm of the target cell induces cell death. In the current study, to clarify the interaction between CD8+ T cells and HaCaT cells with the expression of MHC class I by IFN-γ stimulation [1, 9]. To determine the optimal concentration of IFN-γ, HaCaT cells were stimulated with several concentrations of this cytokine. The expression rate reached a plateau of 20 % at a

concentration of 25 ng/ml; therefore, HaCaT cells were then stimulated with 25 ng/ml IFN-γ. As a result, tight entanglement of HaCaT cells and CD8+ T cells was observed during co-culture, in association with an increased number of GrB-positive HaCaT cells.

As the source of GrB in keratinocytes for pro-IL-18 cleavage, we hypothesized exogenous supply from CTL/NKs. However, the endogenous GrB in keratinocytes is another possible candidate for GrB in IL-18 processing. Hernandez-Pigeon reported that UVB induces GrB in human keratinocytes conferring them potent cellular cytotoxicity against various cellular models including immune cells [4, 5]. Without stimulation, GrB is undetectable in HaCat cells, however, co-culture with CD8+ cells is a potent stimuli for endogenous GrB induction as well as UVB. Interestingly, GrB mRNA was induced in HaCaT cells after co-culture. However, GrB mRNA expression levels were weak and remained same levels after increase of co-cultured CD8+ T cell number. In contrast, the GrB-positive HaCaT cell number significantly increased from 0% to more than 60% according to increase of CD8+ T cell number in co-culture. Inducible but steady low levels GrB mRNA expression in HaCaT cells and significant augmentation in GrB in HaCaT after co-culture implicates the main source of GrB in HaCaT is exogenous GrB from CD8+. Thus, GrB from CD8+Tcells may mainly contributes to converting and release of IL-18 from keratinocytes. In addition, increase of lactate dehydrogenase (LDH) in the culture supernatant was not detected (data not shown), which implicates that release of IL-18 from HaCaT cells is not simply resulted from leakage of IL-18 from HaCaT cells injured by CD8+ Tcells.

Finally, converted mature IL-18 was abundantly released from HaCaT cells. These findings clearly demonstrate that skin infiltrating T cells interact directly to keratinocytes, inducing keratinocytes apoptosis and mature IL-18 release using GrB pathway. In brief, these results provide more insights in the mechanism of keratinocyte injury and overproduction of IL-18 that may develop in skin disorders associated with

epidermal cell injury including toxic epidermal necrosis and Stevens-Johnson syndrome. The roles of CD8+ T cells is not limited in classical perforin/GrB cytotoxic pathway, but CD8+ T cells have additional profile as an immunomodulator releasing inflammatory cytokine IL-18.

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Figure legend

Fig.1 Expression of MHC class I on HaCaT cells. HaCaT cells were pre-treated with IFN-γ (0,25,50,75,150 ng/ml) for 48hours. The MHC class I expression was up-regulated by treatment with 25ng/ml of IFN-γ, reaching a plateau at 20 % of positive cells. In this system, adequate concentration of IFN-γ for MHC class I expression was determined as 25 ng/ml.

Fig.2 RT-PCR analysis in CD8+T cells and HaCaT cells. CD8+ cells express granzyme B mRNA, but not caspase-1 or IL-18 mRNA. HaCaT cells stimulated with IFN-γ express caspase-1 and IL-18 mRNA, but not granzyme B mRNA.

Fig.3 Granzyme B and caspase-1 expression in HaCaT cells and CD8+T cells. (A) After co-culture with GrB(+)/caspase-1(-) CD8+ T cells, GrB expression increased in HaCaT cells. GrB expression in CD8+ T cells was strongly detected (65%), and remained same levels after co-culture. **(B)** Immunoreactive caspase-1 in HaCaT cells and CD8+ T cell was scarcely detected after co-culture. **(C)** GrB mRNA in the isolated HaCaT cells after co-culture was weakly detected, and its expression levels was unchanged after co-culture with increased number of CD8+ T cells. The integrated density value was not elevated with the addition of CD8+ T cells.

Fig.4 Microscopic findings of HaCaT cells and CD8+ T cells. (A) HaCaT cells cultured in the regular medium well adhered to the dish (x40). **(B)** After co-culture with GrB(+)/caspase-1(-) CD8+T cells, HaCaT cells detached from the dish, and separated in round shape (x40). **(C)** Some CD8+ T cells tightly bind to HaCaT cells at high magnification (x400).

Fig.5 Mature IL-18 in the co-culture supernatant of HaCaT cells and CD8+ T cells. The concentration of mature IL-18 elevated in the co-culture supernatant from HaCaT and GrB(+)/ caspase-1(-) human CD8+T cell. The mature IL-18 concentration increased co-cultured CD8+ T cell number dependently.

Fig.1

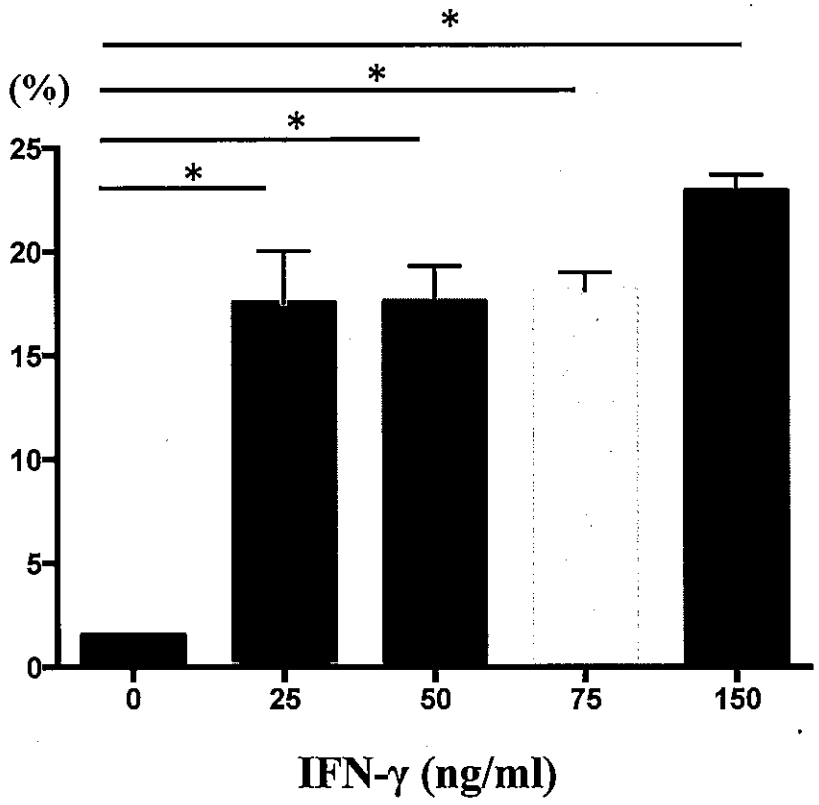

Fig.2

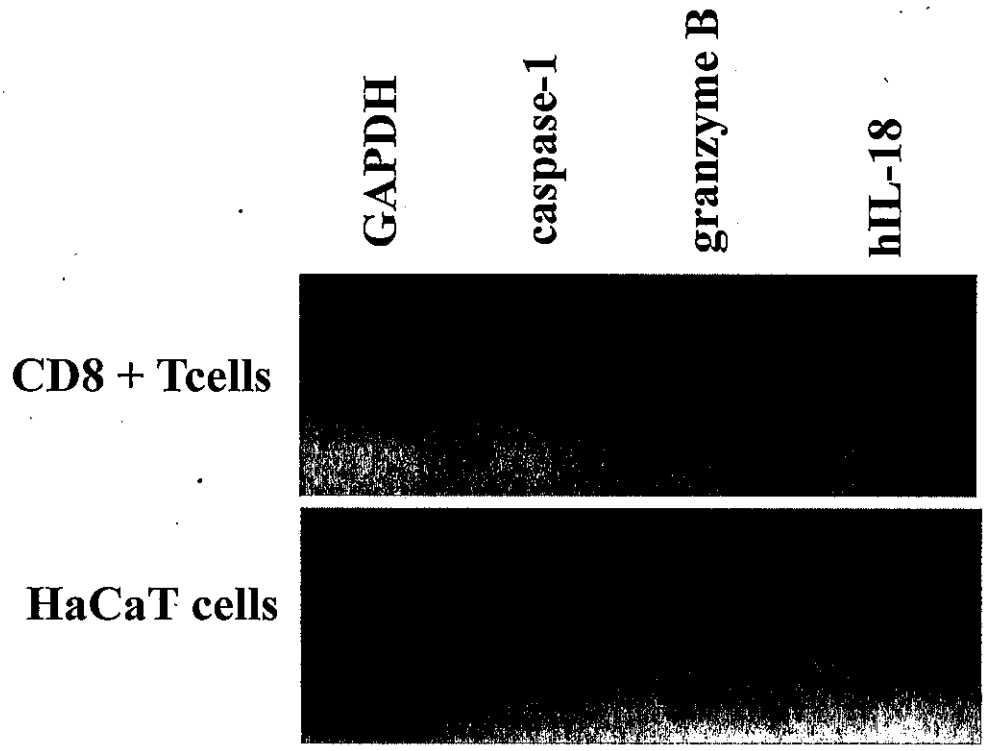

Fig.3A

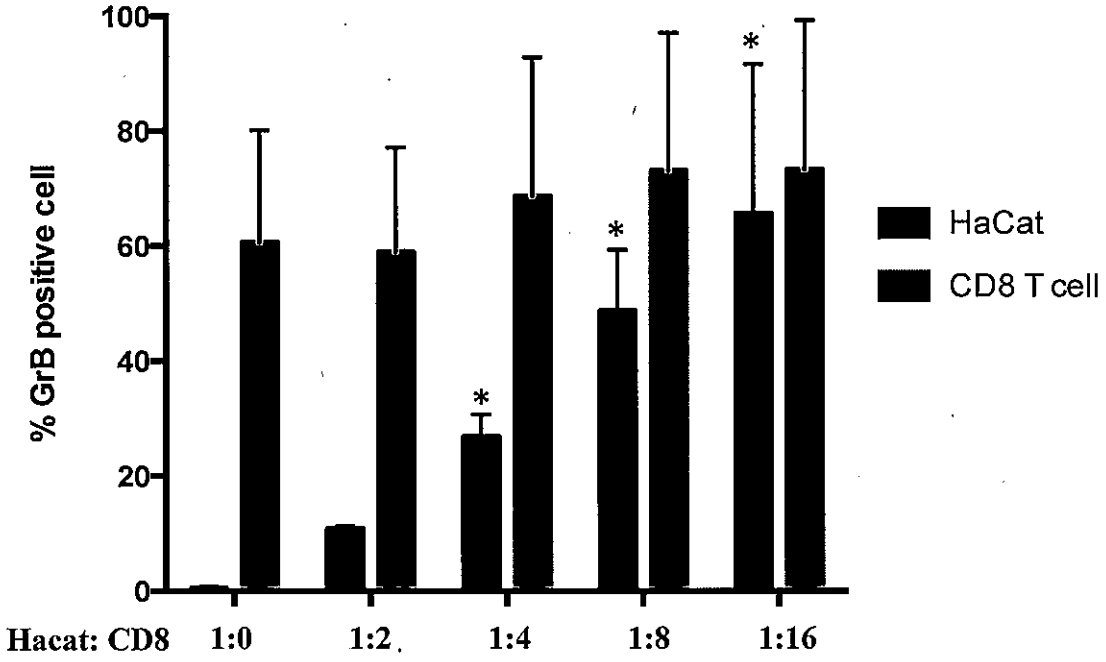
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Fig.3B

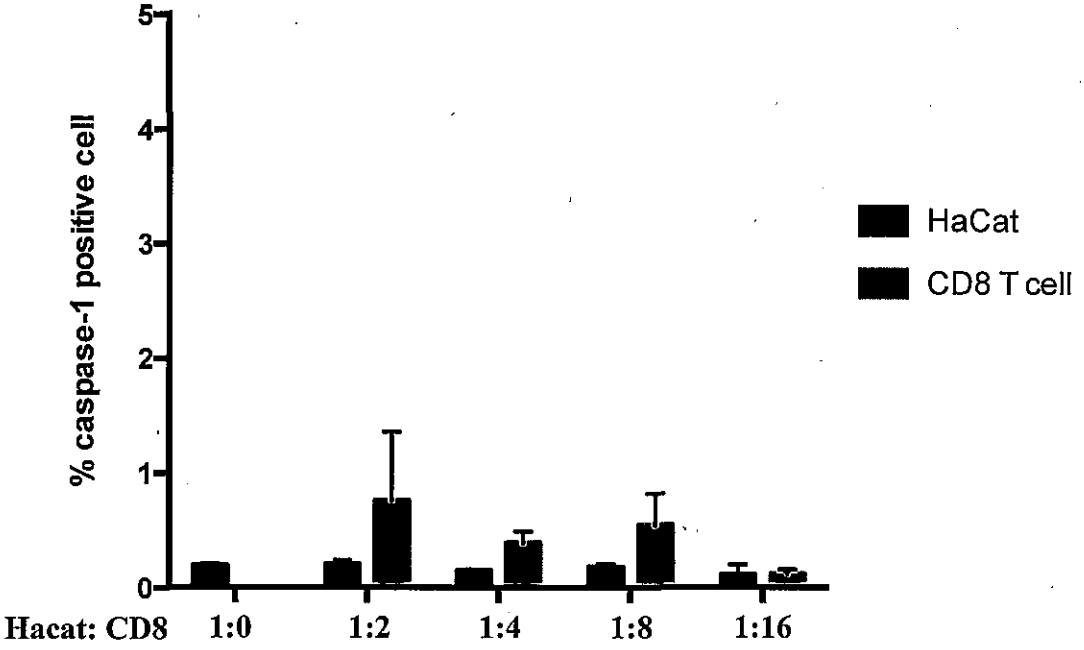
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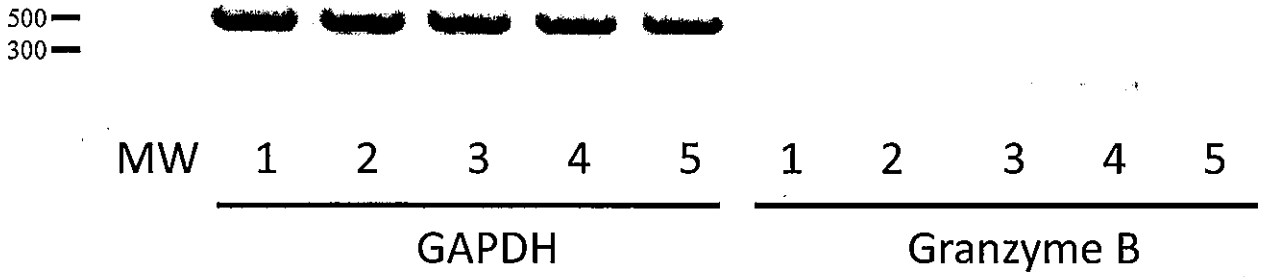
Fig.3C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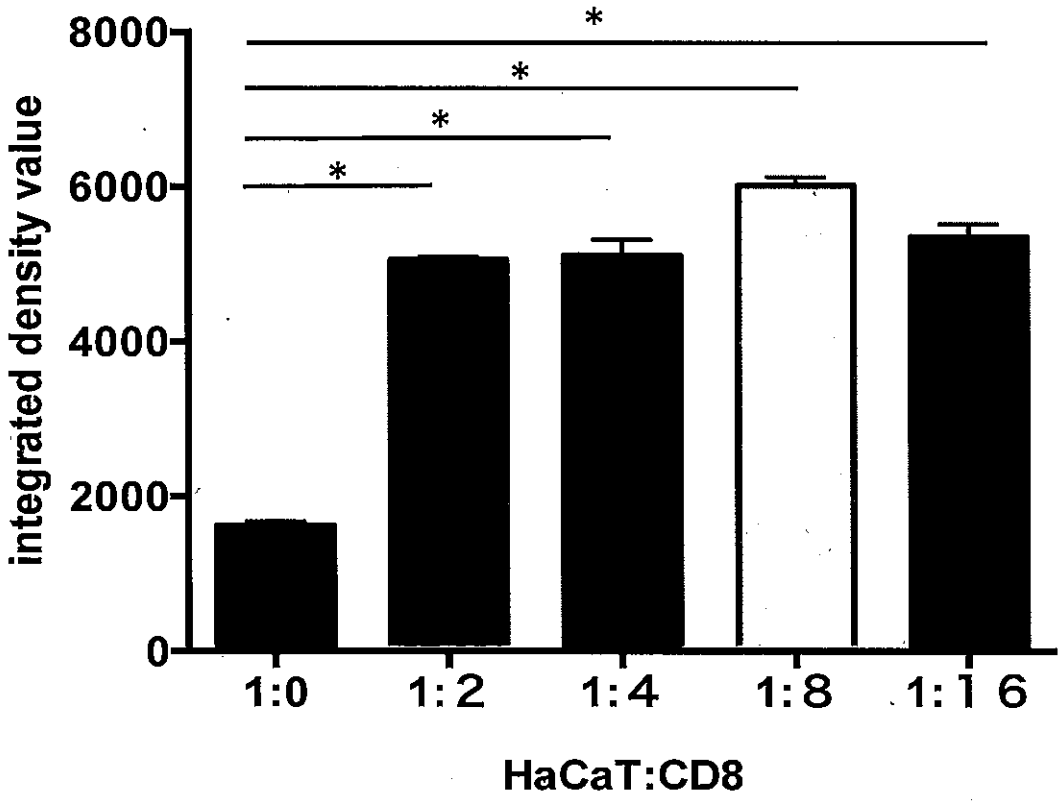
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Fig.4A

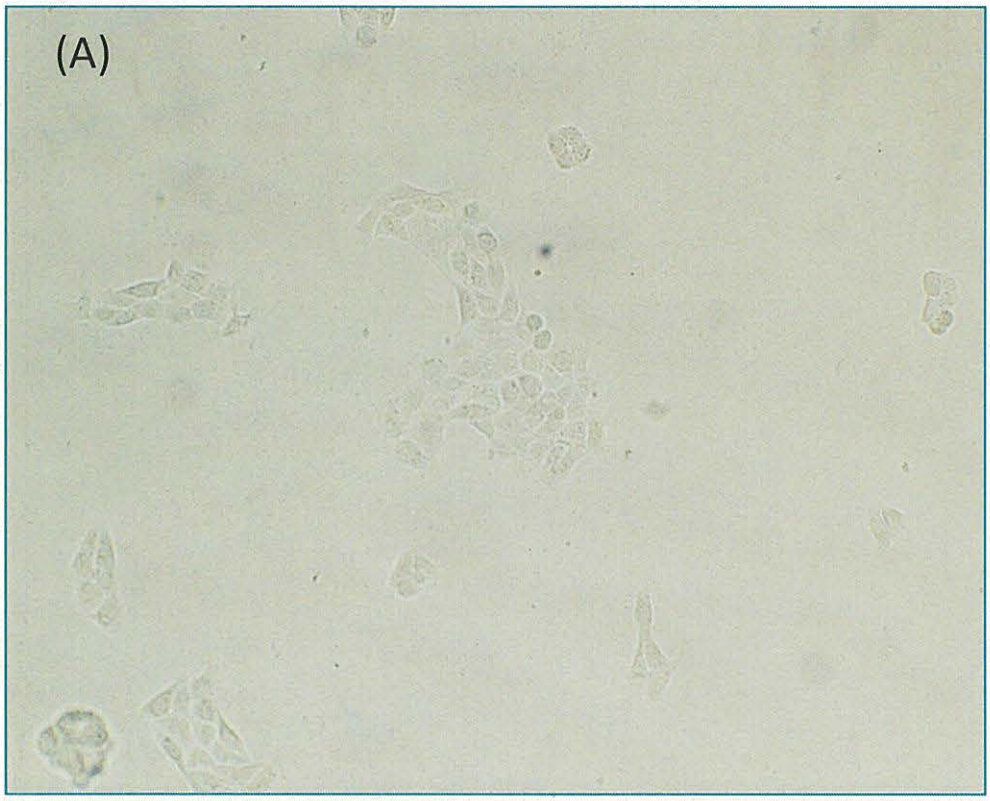

Fig.4B

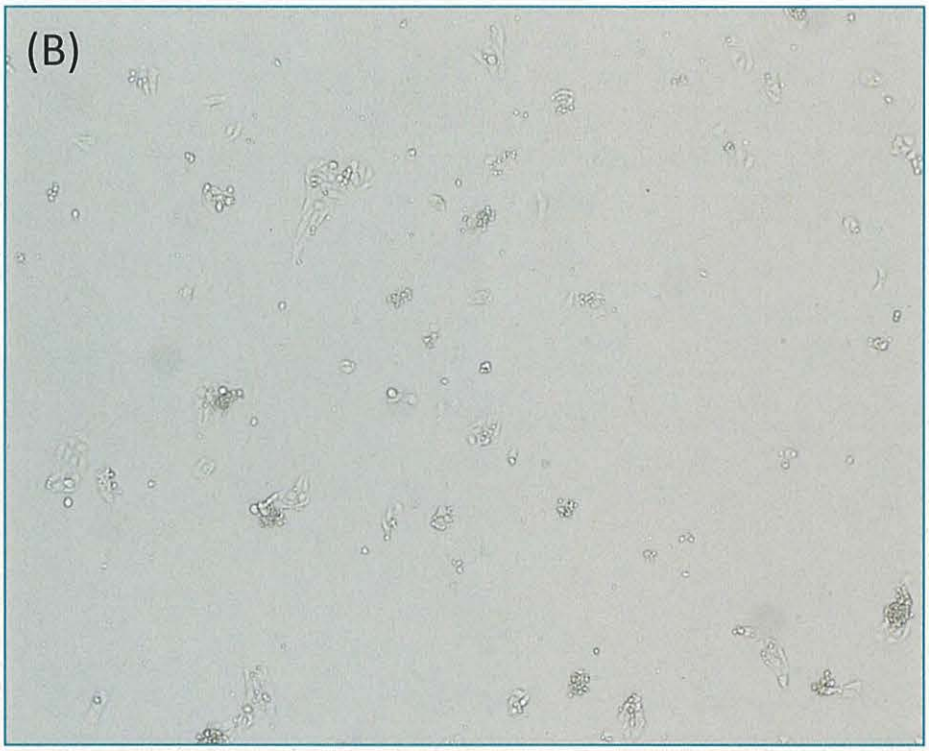

Fig.4C

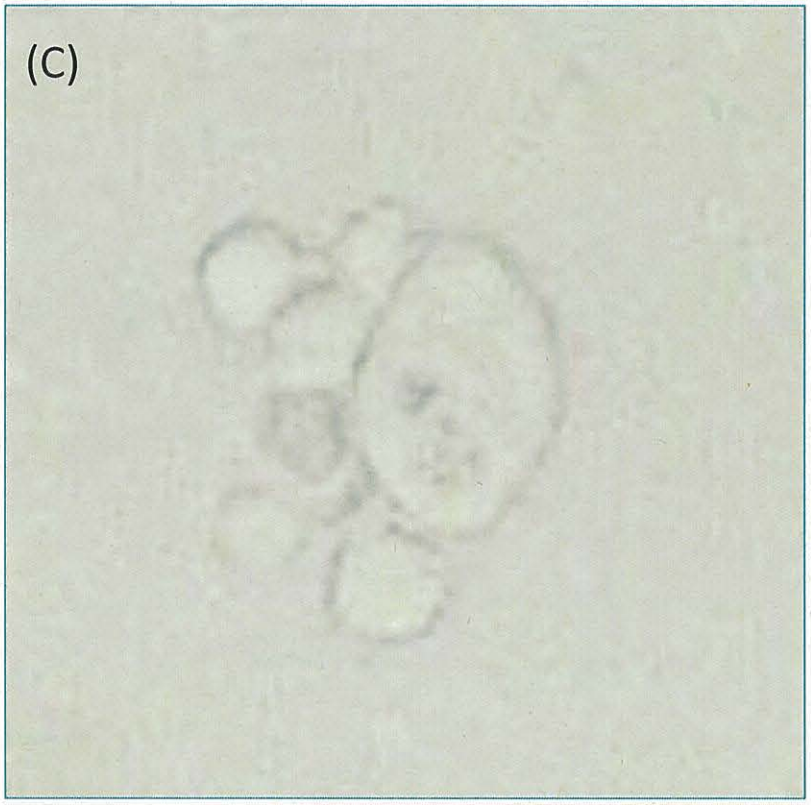

Fig.5