Inflammation Research (2019) 68:715–722
https://doi.org/10.1007/s00011-019-01254-8

Inflammation Research

ORIGINAL RESEARCH PAPER

Anti-inflammatory activity of extensively hydrolyzed casein is mediated by granzyme B

Xuefei Hu[1] · Yan Zhong[2] · Tim T. Lambers[2] · Wenzheng Jiang[1]

Received: 4 April 2019 / Revised: 23 May 2019 / Accepted: 28 May 2019 / Published online: 5 June 2019

Abstract

Objective Nutritional factors such as extensively hydrolyzed casein (eHC) have been proposed to exert anti-inflammatory activity and affect clinical outcomes such as tolerance development in cow's milk allergy. Granzyme B (GrB) induces apoptosis in target cells and also controls the inflammatory response. Whether eHC could affect the activity of granzyme B and play a role in GrB-mediated inflammatory responses in vitro was unknown.

Methods The activity of GrB was measured using the substrate Ac-IEPD-pNA. Inflammatory responses were induced with GrB in HCT-8 and THP-1 cells, and pro-inflammatory cytokines were determined at the transcriptional and protein level.

Results GrB could induce the expression of IL-1β in HCT-8 cells, and IL-8 and MCP-1 in THP-1 cells, respectively. Interestingly, GrB acted synergistically on LPS-induced inflammation in HCT-8 cells and eHC reduced pro-inflammatory responses in both GrB and LPS-mediated inflammation. Further analyses revealed that eHC could inhibit the biological activities and cytotoxic activities of GrB and then could reduce GrB-mediated inflammatory response.

Conclusion The results from the current study suggest that anti-inflammatory activity of extensively hydrolyzed casein is, to a certain extent, mediated through modulation of granzyme B activity and responses.

Keywords Extensively hydrolyzed casein · Granzyme B · Inflammation · Pro-inflammatory cytokine

Introduction

Granzymes (Gr) belong to the family of serine proteases, which are synthesized by a variety of different cell types (e.g., T lymphocytes, natural killer cells, basophils, mast cells) and stored in secretory granules which are located in effector cells [1]. Granzymes used to be considered affecting exclusively immune-mediated target cell death through a perforin-dependent mechanism. Herewith, perforin facilitates the entry of granzymes into target cells and granzymes induce cell death by cleaving critical intracellular substrates such as lamin, histone, HMG2, SET, and Ape1 [2–6]. Apart from their role in cytotoxicity, recent studies have suggested other non-cytotoxic activity of granzymes in health and diseases, e.g., in the context of inflammation and extracellular matrix remodeling and induction/enforcement/prolonging of pro-inflammatory responses. The increased levels of GrA, GrB and GrK are observed in a wide array of inflammatory diseases including atherosclerosis, arthritis, chronic obstructive pulmonary disease (COPD), Crohn's disease and asthma [7–11].

In humans, five granzymes (GrA, GrB, GrH, GrK, and GrM) have been identified to date with different substrate specificity [12, 13]. Among the granzymes, GrA and GrB are the most abundant and are described to be involved in pathophysiological process. GrB has been found to be elevated in the lungs of patients with asthma after allergen exposure [14, 15]. It was also identified as a novel basophil-derived mediator of allergic inflammation [16]. In addition, IL-13, as a major mediator of allergy and asthma, can induce de novo synthesis of GrB in basophil granulocytes [16]. Increases of IL-13 correlated with increased levels of GrB in bronchoalveolar lavage fluids

Responsible Editor: Bernhard Gibbs.

✉ Wenzheng Jiang
wzjiang@bio.ecnu.edu.cn

[1] Shanghai Key Laboratory of Regulatory Biology, School of Life Sciences, East China Normal University, Shanghai 200241, China

[2] Global Discovery Department, Mead Johnson Pediatric Nutrition Institute, Middenkampweg 2, 6545 CJ Nijmegen, The Netherlands

of patients with allergic asthma after provocation [15]. Bratke et al. also reported an upregulation of GrB in endobronchial pDCs 24 h after allergen challenge, and this was accompanied by enhanced GrB concentrations in bronchoalveolar lavage fluid [16]. GrB may thus play a role in the pathology of allergic diseases. The pivotal role of GrB in the pathogenesis of HDM-induced allergic lung disease was further confirmed by a recent study [17]. Using adoptive transfer of NK cells from GrB-deficient mice, it was found that GrB production by NK cells is required to promote airway inflammation. Granzyme B can regulate inflammation by non-cytotoxic mechanisms, for example, IL-1α, which has undergone proteolysis by granzyme B, can promote allergic airway inflammation [18, 19]. Therefore, the above findings suggest that GrB could be used as a novel therapeutic target for the prevention and treatment of allergic diseases.

Besides allergic inflammation, GrB is involved in the pathogenesis of other chronic inflammatory disease as well, such as inflammatory bowel diseases (IBD), which is characterized by chronic relapses of hyperinflammation of gastrointestinal tract and dysregulated mucosal immune response [20, 21]. Children with IBD can have features of both Crohn's disease and ulcerative colitis. Different factors are involved in the pathology of IBD such as oxidative stress, neutrophil infiltration, overproduction of pro-inflammatory mediators including cytokines, dysfunction of immune systems, and imbalanced microflora [22–26]. A recent study showed that IBD-related inflammation was marked by mucosal accumulation of cytotoxic, GrB-expressing cells, suggesting a role for GrB or GrB-expressing cells in IBD-associated epithelial damage [27].

Nutritional strategies play an important role in preventing and alleviating inflammatory reactions [28, 29]. For example, mounting evidence shows that nutritional therapies can reduce inflammation in Crohn's disease [30]. Protein hydrolysates show equivalent nutritional value compared with the whole protein; however, particularly extensive hydrolysates do not induce allergic responses in sensitized individual and can, therefore, be applied for the dietary management of cow's milk allergy [31, 32]. Within the dietary management of cow's milk allergy, a particular extensively hydrolyzed casein (eHC)-based formula was demonstrated to accelerate tolerance acquisition, whereas immunological inert amino acid (AA)-based formula lacked such activity [33–35]. Whether eHC could alleviate the inflammatory response by affecting the activity of GrB remains unknown. In the current study, using THP-1 and HCT-8 cell lines as cell models, the inhibitory effect of eHC on GrB-mediated inflammatory response was studied. In addition, effects of GrB-mediated responses and inhibition by dietary factors were compared to LPS-mediated inflammatory responses.

Materials and methods

Reagents

Cell culture medium RPMI 1640 and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA). LPS and the antibiotics (penicillin and streptomycin) were obtained from Sigma (St. Loius, MO, USA). Active human granzyme B (GrB) protein was obtained from Abcam (Cambridge, MS, USA). Ac-IEPD-pNA was obtained from SM Biochemicals LLC (CA, USA). The primers of MCP-1, IL-8, IL-1β, and TNF-α for Q-PCR were synthesized by Shanghai Sangon Biotech Co., Ltd (Shanghai, China). PULSin in vitro Protein Delivery Reagent was bought from Polyplus Transfection Company (NY, USA). RNA extraction kits, reverse transcript kits and Q-PCR kits were obtained from TaKaRa Biotech (Dalian) Co., Ltd. The samples, including extensively hydrolyzed casein (eHC) > 500 Da fraction of eHC (enriched in longer sequences, eHC > 500 Da) and amino acid preparation with similar amino acid composition as eHC (negative control, AA), were provided by Mead Johnson Nutrition [36].

Activity assay of human granzyme B protein

GrB was diluted in 1 × PBS and the activity was determined by hydrolysis of substrates at 37 °C in 96-well culture plates in 0.1 ml volumes with appropriate enzyme reaction buffers. Paranitroanilide substrate, acetyl-Ile-Glu-Pro-Asp-paranitroanilide (Ac-IEPD-pNA), was routinely used at 2 mM in reaction buffer containing 50 mM HEPES (pH 7.5), 10% (w/v) sucrose, 0.05% (w/v) CHAPS and 5 mM DTT. Released chromogenic group pNA was measured as absorbance at 405 nm over a number of time points. To detect the effect of hydrolyzed casein on the activity of granzyme B, the tested samples at different concentration were co-cultured with active granzyme B protein for indicated time, then the activity of GrB was assayed.

Cell culture

THP-1and HCT-8 cell lines were purchased from ATCC (USA). Cells were maintained in RPMI 1640 supplemented with 10% (v/v) FBS, 100 mg/ml penicillin, 100 mg/ml streptomycin, and kept in a humidified atmosphere with 5% CO_2. For THP-1 cells, extra 0.05 mM β-mercaptoethanol is needed in the growth medium. Cells were routinely passaged every 4–5 days. To detect the pro-inflammatory effect of GrB in THP-1 or HCT-8 cells, THP-1 or HCT-8 cells were treated with active granzyme B protein in RPMI 1640 media without FBS for 6 h.

Q-PCR

Total RNAs from THP-1 or HCT-8 cells were isolated with Trizol and purified with phenol and chloroform (phenol: chloroform = 1: 1). Forward and reverse primers for the PCR are listed in Table 1. The following cycling conditions of Q-PCR were used: 95 °C 30 s, 60 °C 30 s, 72 °C 30 s for 40 cycles. Sequence-specific products were identified by generating a melting curve, and the relative quantification of gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method [37].

ELISA

The supernatants of the cultured cells treated by GrB with or without hydrolyzed casein were collected and the level of cytokines was measured by ELISA according to the instruction of ELISA detection kits.

Table 1 The sequence of primers

Primers		Sequences (from 5′ to 3′)
GAPDH	Forward	GGAGCGAGATCCCTCCAAAAT
	Reverse	GGCTGTTGTCATACTTCTCATGG
IL-1β	Forward	ATGATGGCTTATTACAGTGGCAA
	Reverse	GTCGGAGATTCGTAGCTGGA
IL-8	Forward	ACTGAGAGTGATTGAGAGTGGAC
	Reverse	AACCCTCTGCACCCAGTTTTC
TNF-α	Forward	CCTCTCTCTAATCAGCCCTCTG
	Reverse	GAGGACCTGGGAGTAGATGAG
MCP-1	Forward	CAGCCAGATGCAATCAATGCC
	Reverse	TGGAATCCTGAACCCACTTCT

Statistical analysis

The significance of differences between control and test groups was determined by independent *t* test using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, USA) and a *P* value of 0.05 was set as significant threshold.

Results

Recombinant GrB protein exerts good biological activities

To assess activity of purified GrB, the substrate of GrB, Ac-IEPD-pNA, was used. The activity of GrB was both dose dependent (Fig. 1a) and time dependent (Fig. 1b) with significant activity. These results showed that GrB is biologically active.

GrB displays pro-inflammatory activity in HCT-8 cells and can enhance LPS-mediated responses

To study the pro-inflammatory effect of GrB in HCT-8 cells, we added 15 nM GrB into the culture media of HCT-8. Then, HCT-8 was cultured in RPMI 1640 media without FBS to exclude the effect of components in FBS. 6 h later, the relative mRNA level of several pro-inflammatory cytokines was analyzed by real-time quantitative PCR (Q-PCR). The data showed that GrB could promote the production of IL-1β in HCT-8 significantly (Fig. 2a), although it has no effect on the production of IL-8, TNF-α and MCP-1 (Fig. 2b–d). These results demonstrated that GrB alone had pro-inflammatory activity and could increase the production of IL-1β in HCT-8 cells.

To further investigate whether GrB could affect the pro-inflammatory effect of LPS, we compared the

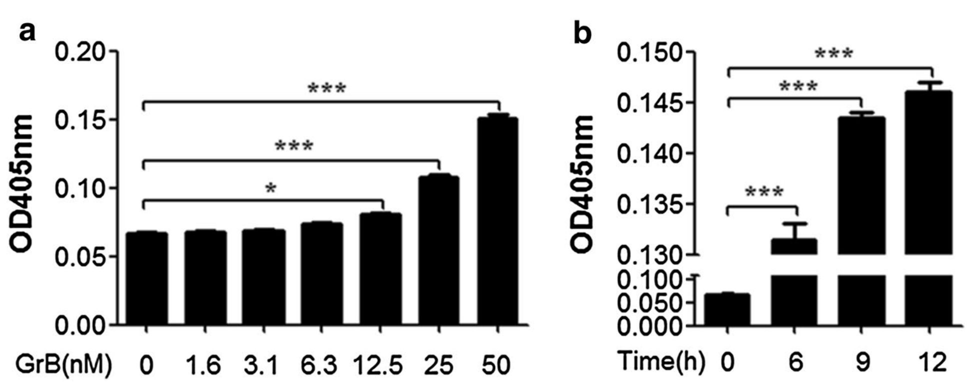

Fig. 1 The detection of the activity of GrB. **a** 0 ~ 50 nM of GrB was incubated with Ac-IEPD-pNA at 37 °C for 12 h. Then, the activity of Granzyme B was measured by absorbance at 405 nm. **b** 50 nM of GrB was incubated with Ac-IEPD-pNA at 37 °C for 0 h, 6 h, 9 h, and 12 h, then the activity of GrB was measured by absorbance at 405 nm. *$p < 0.05$, ***$p < 0.001$. Data represent mean ± SEM of three independent experiments

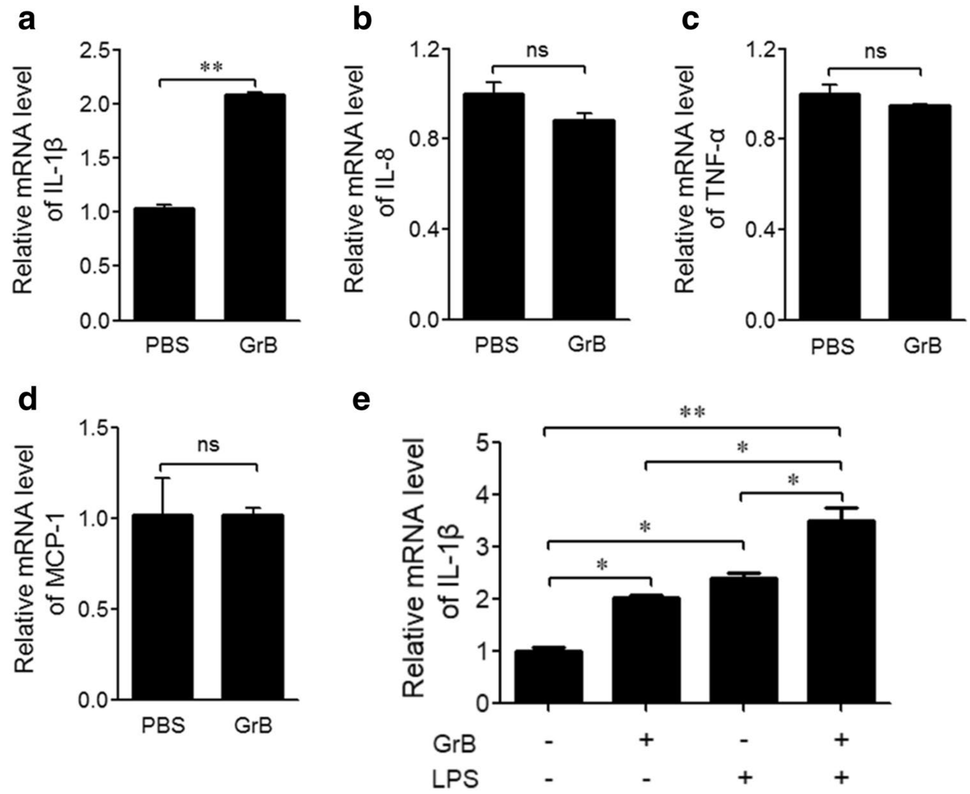

Fig. 2 The pro-inflammatory effect of granzyme B in HCT-8. HCT-8 cells were cultured with RPMI 1640 media without FBS for 6 h and then incubated with 15 nM of GrB or PBS. 6 h later, the relative mRNA level of IL-1β (**a**), IL-8 (**b**), TNF-α (**c**) and MCP-1 (**d**) was analyzed by Q-PCR. **e** HCT-8 cells were treated with DMEM media without FBS for 6 h and then incubated with 100 ng/ml of LPS at the absence or presence of 15 nM of GrB. 6 h later, the relative mRNA level of IL-1β was analyzed by Q-PCR. $*p<0.05$, $**p<0.01$, ns: not significant. Data represent mean ± SEM of three independent experiments

pro-inflammatory effect of LPS with or without GrB in HCT-8 cells; LPS could induce the expression of IL-1β and to our surprise the addition of GrB further increased the production of IL-1β (Fig. 2e). The data indicated that GrB can enhance LPS-induced pro-inflammatory cytokine responses in HCT-8 cells.

GrB displays pro-inflammatory activity in THP-1 cells but does not further increase LPS-mediated responses

To detect the pro-inflammatory effect of GrB in THP-1, GrB was added into the culture media of THP-1 and the cells were cultured in RPMI 1640 media without FBS for 6 h, then the relative mRNA level of several pro-inflammatory cytokines were analyzed by real-time quantitative PCR (Q-PCR). The data showed that GrB could promote the production of IL-8 and MCP-1 in THP-1 cells in a dose-dependent manner (Fig. 3a, b). These data showed that GrB alone had pro-inflammatory activity.

To understand whether GrB and LPS acted synergistically in THP-1 cells, the pro-inflammatory effect of LPS with or without GrB in THP-1 cells was analyzed by Q-PCR. Interestingly, LPS could induce the expression of cytokines such as IL-8 and MCP-1 significantly, but the addition of GrB did not result in an increased expression of IL-8 (Fig. 3c) and MCP-1 (Fig. 3d).

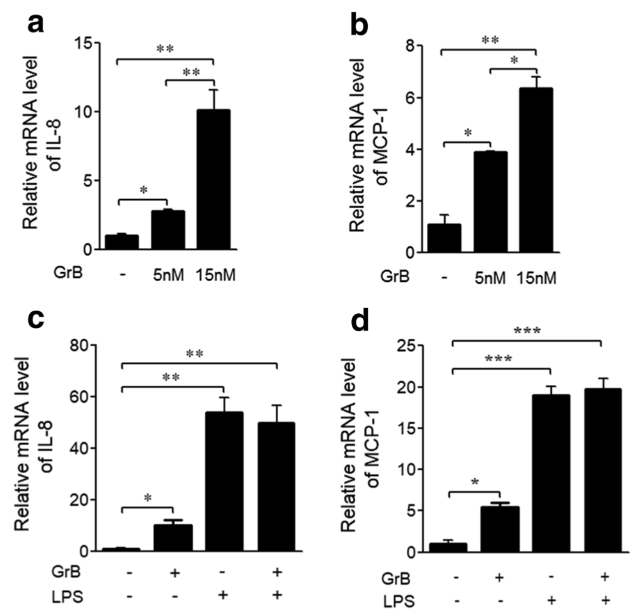

Fig. 3 The pro-inflammatory effect of GrB in THP-1 cells. THP-1 cells were cultured with RPMI 1640 media without FBS for 6 h and then incubated with indicated concentration of GrB or PBS. 6 h later, the relative mRNA level of IL-8 (**a**) and MCP-1 (**b**) was analyzed by Q-PCR. THP-1 cells were cultured with RPMI 1640 media without FBS for 6 h and then incubated with 100 ng/ml of LPS or LPS and 15 nM of GrB together. 6 h later, the relative mRNA level of IL-8 (**c**) and MCP-1 (**d**) was analyzed by Q-PCR. $*p<0.05$, $**p<0.01$, ns: not significant. Data represent mean ± SEM of three independent experiments

A > 500 Da fraction of extensively hydrolyzed casein inhibits GrB-mediated inflammatory responses in HCT-8 and THP-1 cells

eHC, a fraction enriched with longer peptide sequences from eHC (eHC > 500 Da) or an amino acid control having a similar composition as eHC (immunological inert control, AA) was used to investigate a possible inhibitory activity of eHC on GrB-mediated inflammation. GrB was incubated with the eHC preparations and control at 37 °C for 6 h and subsequently the mixture was added to the culture media of HCT-8 or THP-1 cells. GrB alone or PBS was added as additional controls. To investigate a possible inhibitory effect of the tested ingredients on GrB-mediated pro-inflammatory function, the relative mRNA levels of IL-1β in HCT-8 or IL-8 in THP-1 were detected by Q-PCR. Results showed that the expression of IL-1β in HCT-8 (Fig. 4a) and IL-8 in THP-1 (Fig. 4b) decreased significantly when GrB was pro-incubated with eHC > 500 Da. However, eHC itself and the amino acid control did not affect the expression of pro-inflammatory cytokines induced by GrB.

The supernatants of culture media were collected and measured by ELISA. Less IL-8 protein was detected in THP-1 cells which were treated with GrB pre-incubated with eHC > 500 Da (Fig. 4c). Unfortunately, the protein of IL-1β has not been detected successfully. The possible reason was that the expression of IL-1β was too low to be detectable in HCT-8 cells.

Evaluation of the inhibitory effect of tested samples on the activity of GrB

To understand why hydrolyzed casein could affect the pro-inflammatory effect of GrB, GrB (50 nM) was incubated with eHC, eHC > 500 Da or AA (10 mg/ml) for indicated time, respectively; then the activity of granzyme B was measured. PBS and the samples alone were used as the controls. After being treated with eHC > 500 Da, the activity of GrB decreased (Fig. 5a). However, there was no significant difference for the activity of GrB at the absence or presence of eHC (Fig. 5b) or AA (Fig. 5c). The data showed that eHC > 500 Da had obvious inhibitory effect on the activity of GrB.

GrB is serine protease and can induce the apoptosis when it enters the target cells. To further determine whether hydrolyzed casein can inhibit the activity of GrB, GrB was transferred into the HCT-8 by protein transfection reagent PULSin and the apoptosis of HCT-8 was detected by FACS. The data showed that GrB could induce the apoptosis when it was transfected with PULSin together. Otherwise, the apoptosis decreased when GrB was pre-incubated with eHC > 500 Da but not eHC or AA (Fig. 6).

Discussion

Granzyme B is a protease typically found in the cytotoxic granules of natural killer (NK) and cytotoxic T cells (CTLs), and it can promotes apoptosis of target cells upon delivery into the cytoplasm of virus-infected target cells or tumor cells. However, many reports have showed that the level of granzyme B was elevated in allergic inflammation or chronic inflammatory diseases such as asthma, IBD, and rheumatoid arthritis (RA) [27, 38, 39]. These studies strongly suggested that granzyme B may have pro-inflammatory effect, but the exact mechanisms remain to be defined. Afonina et al. have reported that IL-1α is a substrate for granzyme B and the proteolysis of

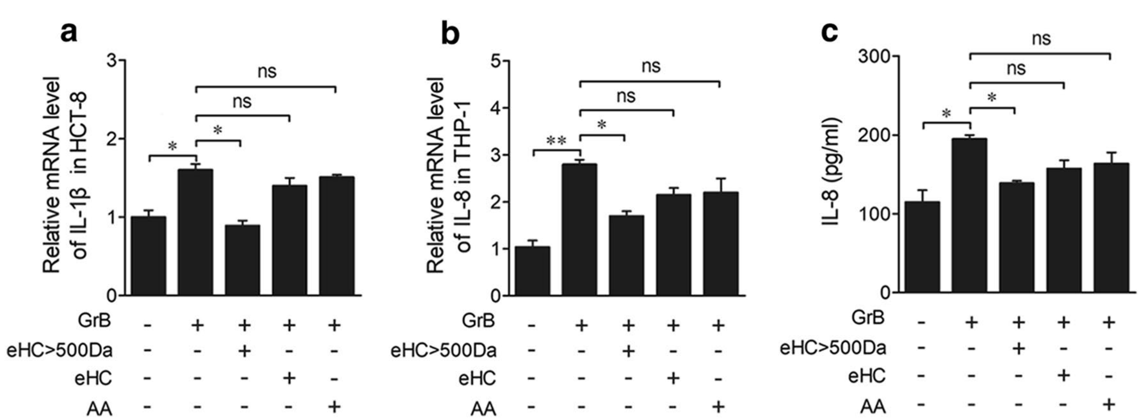

Fig. 4 The effect of hydrolyzed casein on the pro-inflammatory function induced by GrB. **a** HCT-8 cells were treated with GrB (50 nM) pre-incubated with eHC, eHC > 500 Da or AA (10 mg/ml) for 6 h at 37 °C. The relative mRNA level of IL-1β was measured by Q-PCR. **b** THP-1 cells were treated with GrB pre-incubated with eHC, eHC > 500 Da or AA for 6 h at 37 °C. The relative mRNA level of IL-8 was analyzed by Q-PCR. **c** The supernatants of the treated THP-1 cells were collected and the protein level of IL-8 was detected by ELISA. $*p < 0.05$, $**p < 0.01$, ns: not significant. Data represent mean ± SEM of three independent experiments

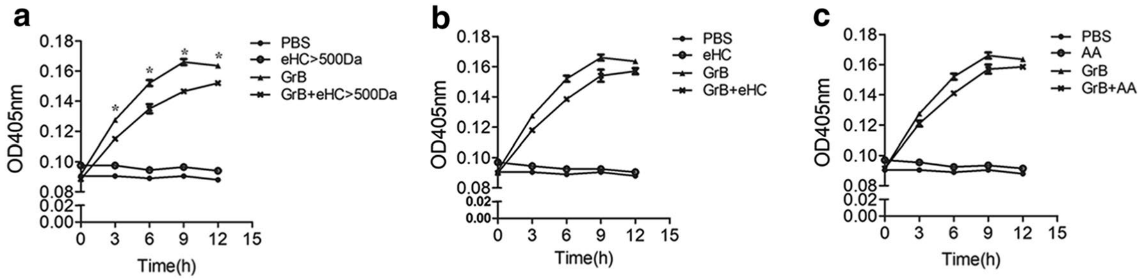

Fig. 5 The detection of the inhibitory of hydrolyzed casein on the activity of GrB. GrB (50 nM) was first incubated with 10 mg/ml of eHC > 500 Da (**a**), eHC (**b**) or AA (**c**) for 6 h at 37 °C, respectively. Then, the activity of GrB was measured by absorbance at 405 nm. PBS and the samples alone were served as controls. **p* < 0.05. Data represent mean ± SEM of three independent experiments

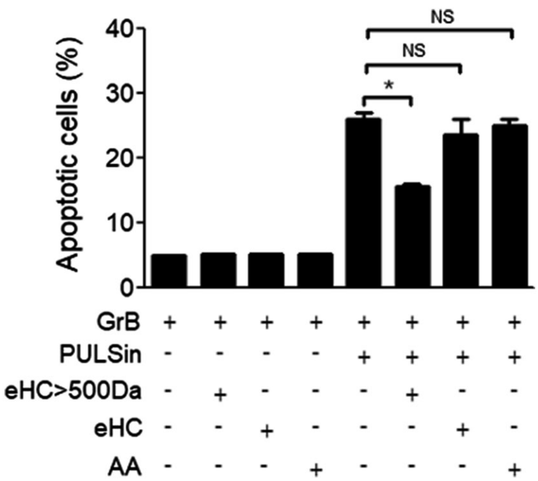

Fig. 6 The detection of apoptosis of HCT-8. GrB was pre-incubated with eHC > 500 Da, eHC or AA and transferred into HCT-8 by PULSin; the apoptosis of HCT-8 cells was analyzed by FACS. PBS, PULSin or the samples alone were used as the controls. **p* < 0.05. ns: not significant. Data represent mean ± SEM of three independent experiments

this cytokine by granzyme B can significantly increase its biological potency. Therefore, granzyme B can enhance the inflammatory responses by processing and activating IL-1α [18]. Furthermore, accumulating evidence suggests that granzymes may also influence the production of pro-inflammatory cytokines [40, 41]. To define whether GrB induces the production of pro-inflammatory cytokines in HCT-8, human colon epithelial cell lines, or THP-1, human monocytic leukemia cell line, HCT-8 or THP-1 cells were treated with GrB and the pro-inflammatory cytokines such as IL-1β, IL-8, TNF-α and MCP-1 were analyzed at the transcriptional or protein level. The results showed that GrB could induce the expression of IL-1β in HCT-8 cells. Furthermore, GrB could synergistically potentiate LPS-induced IL-1β responses in HCT-8 cells. Unfortunately, IL-1β protein levels were too low to detect in HCT-8 cells. However, our data suggest that GrB has pro-inflammatory effects in HCT-8 cells at the level of mRNA expression of this cytokine.

THP-1 was human monocytic leukemia cell line and also was used as the cell model to evaluate the pro-inflammatory effect of GrB in the study. The result of Q-PCR indicated that GrB could promote the expression of IL-8 and MCP-1 in THP-1 in a dose-dependent way. These data strongly support that GrB alone had pro-inflammatory effect in THP-1 cells. However, GrB could not enhance the cytokine expression induced by LPS synergistically in THP-1. The high level of expression of cytokines induced by LPS in THP-1 may be contributed to the result.

Casein hydrolysates have been demonstrated to have anti-inflammatory activity [36]. To understand whether hydrolyzed casein had inhibitory effect on the activity of GrB and GrB-mediated inflammatory response, GrB was incubated with eHC, eHC > 500 Da or AA for 6 h at 37 °C. The results of activity assay showed that eHC > 500 Da, which was composed of eHC fraction (> 500 Da) and was enriched in longer sequences, could reduce the activity of GrB, whereas eHC or AA had no obvious inhibitory effect in current study. These results suggest that only the longer eHC sequences, enriched in the 500-Da fraction display activity and the smaller peptides in this preparation do not affect activity. This particular casein hydrolysate indeed is dominated by smaller (< 500 Da) sequences which is typical for hydrolysates applied for the dietary management of cow's milk allergy [36]. To investigate whether hydrolyzed casein affected GrB-mediated pro-inflammatory response, hydrolyzed casein was pre-incubated with GrB and then added to HCT-8 cells or THP-1 cells, and the expression of pro-inflammatory cytokines was detected. The data suggested that hydrolyzed casein could inhibit GrB-mediated pro-inflammatory response. The mechanisms of anti-inflammatory effect of eHC > 500 Da need to be elucidated and the inhibitory effect on the activities of GrB was one of the possible reasons. The study strongly suggested that hydrolyzed casein containing longer peptide sequences such as eHC > 500 Da had inhibitory effect on the activities of

GrB; otherwise, eHC which only contained about 10% of the longer sequences and the control amino acid (AA) did not affect the activities of GrB.

In summary, GrB had pro-inflammatory effect in HCT-8 cells and THP-1 cells, and could be used as a novel therapeutic target for the prevention and treatment of inflammatory diseases. Hydrolyzed casein could inhibit the activity of GrB to a certain extent and exhibit the anti-inflammatory effect in GrB-mediated pro-inflammatory response in both HCT-8 and THP-1 cells. These data strongly suggest that hydrolyzed casein may be a promising anti-inflammatory peptide to reduce intestinal inflammation.

Acknowledgements This work was supported by National Natural Science Foundation of China (81771306, 81072459), National Key Research and Development Program of China (2016YFC1200400), Mead Johnson Nutrition (MJN).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Lieberman J. The ABCs of granule-mediated cytotoxicity: new weapons in the barsenal. Nat Rev Immunol. 2003;3:361–70.
2. Zhang D, Beresford PJ, Greenberg AH, Lieberman J. Granzymes A and B directly cleave lamins and disrupt the nuclear lamina during granule-mediated cytolysis. Proc Natl Acad Sci USA. 2001;98(10):5746–51.
3. Zhang D, Pasternack MS, Beresford PJ, Wagner L, Greenberg AH, Lieberman J. Induction of rapid histone degradation by the cytotoxic T lymphocyte protease granzyme A. J Biol Chem. 2001;276(5):3683–90.
4. Fan Z, Beresford PJ, Oh DY, Zhang D, Lieberman J. Tumor suppressor NM23–H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. Cell. 2003;112:659–72.
5. Fan Z, Beresford PJ, Zhang D, Xu Z, Novina CD, Yoshida A, Pommier Y, Lieberman J. Cleaving the oxidative repair protein Ape1 enhances cell death mediated by granzyme A. Nat Immunol. 2003;4:145–53.
6. Fan Z, Beresford PJ, Zhang D, Lieberman J. HMG2 interacts with the nucleosome assembly protein SET and is a target of the cytotoxic T lymphocyte protease granzyme A. Mol Cell Biol. 2002;22:2810–20.
7. Bratke K, Klug A, Julius P, Kuepper M, Lommatzsch M, Sparmann G, Luttmann W, Virchow JC. Granzyme K: a novel mediator in acute airway inflammation. Thorax. 2008;63(11):1006–11.
8. Ngan DA, Vickerman SV, Granville DJ, Man SF, Sin DD. The possible role of granzyme B in the pathogenesis of chronic obstructive pulmonary disease. Ther Adv Respir Dis. 2009;3(3):113–29.
9. Saito Y, Kondo H, Hojo Y. Granzyme B as a novel factor involved in cardiovascular diseases. J Cardiol. 2011;57(2):141–7.
10. Kim WJ, Kim H, Suk K, Lee WH. Macrophages express granzyme B in the lesion areas of atherosclerosis and rheumatoid arthritis. Immunol Lett. 2007;111(1):57–65.
11. Choy JC, McDonald PC, Suarez AC, Hung VH, Wilson JE, McManus BM, Granville DJ. Granzyme B in atherosclerosis and transplant vascular disease: association with cell death and atherosclerotic disease severity. Mod Pathol. 2003;16(5):460–70.
12. Lieberman J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nat Rev Immunol. 2003;3:361–70.
13. Trapani JA. Granzymes: a family of lymphocyte granule serine proteases. Genome Biol. 2001;2:1–7.
14. Bratke K, Bottcher B, Leeder K, Schmidt S, Küpper M, Virchow JC Jr, Luttmann W. Increase in granzyme B+lymphocytes and soluble granzyme B in bronchoalveolar lavage of allergen challenged patients with atopic asthma. Clin Exp Immunol. 2004;136:542–8.
15. Simpson JL, Gibson PG, Yang IA, Upham J, James A, Reynolds PN, Hodge S. Altered sputum granzyme B and granzyme B/proteinase inhibitor-9 in patients with non-eosinophilic asthma. Respirology. 2014;19(2):280–7.
16. Tschopp CM, Spiegl N, Didichenko S, Lutmann W, Julius P, Virchow JC, Hack CE, Dahinden CA. Granzyme B, a novel mediator of allergic inflammation: its induction and release in blood basophils and human asthma. Blood. 2006;108:2290–9.
17. Farhadi N, Lambert L, Triulzi C, Openshaw PJ, Guerra N, Culley FJ. Natural killer cell NKG2D and granzyme B are critical for allergic pulmonary inflammation. J Allergy Clin Immunol. 2014;133(3):827–35.
18. Afonina IS, Tynan GA, Logue SE, Cullen SP, Bots M, Lüthi AU, Reeves EP, McElvaney NG, Medema JP, Lavelle EC, Martin SJ. Granzyme B-dependent proteolysis acts as a switch to enhance the pro-inflammatory activity of IL-1α. Mol Cell. 2011;44(2):265–78.
19. Bratke K, Nielsen J, Manig F, Klein C, Kuepper M, Geyer S, Julius P, Lommatzsch M, Virchow JC. Functional expression of granzyme B in human plasmacytoid dendritic cells: a role in allergic inflammation. Clin Exp Allergy. 2010;40(7):1015–24.
20. Müller S, Lory J, Corazza N, Griffiths GM, Z'graggen K, Mazzucchelli L, Kappeler A, Mueller C. Activated CD4+ and CD8+ cytotoxic cells are present in increased numbers in the intestinal mucosa from patients with active inflammatory bowel disease. Am J Pathol. 1998;152(1):261–8.
21. Kim TJ, Koo JS, Kim SJ, Hong SN, Kim YS, Yang SK, Kim YH. Role of IL-1ra and Granzyme B as biomarkers in active Crohn's disease patients. Biomarkers. 2018;23(2):161–6.
22. León AJ, Gómez E, Garrote JA, Bernardo D, Barrera A, Marcos JL, Fernández-Salazar L, Velayos B, Blanco-Quirós A, Arranz E. High levels of proinflammatory cytokines, but not markers of tissue injury, in unaffected intestinal areas from patients with IBD. Mediat Inflamm. 2009;2009:580450.
23. Wang Z, Zhu M, Luo C, Zhen Y, Mu J, Zhang W, Ouyang Q, Zhang H. High level of IgG4 as a biomarker for a new subset of inflammatory bowel disease. Sci Rep. 2018;8(1):10018.
24. Shohan M, Sabzevary-Ghahfarokhi M, Bagheri N, Shirzad H, Rahimian G, Soltani A, Ghatreh-Samani M, Deris F, Tahmasbi K, Shahverdi E, Fathollahi F. Intensified Th9 response is associated with the immunopathogenesis of active ulcerative colitis. Immunol Invest. 2018;47(7):700–11.
25. Frick A, Khare V, Paul G, Lang M, Ferk F, Knasmüller S, Beer A, Oberhuber G, Gasche C. Overt increase of oxidative stress and DNA damage in murine and human colitis and colitis-associated neoplasia. Mol Cancer Res. 2018;16(4):634–42.
26. Pereira C, Coelho R, Grácio D, Dias C, Silva M, Peixoto A, Lopes P, Costa C, Teixeira JP, Macedo G, Magro F. DNA damage and oxidative DNA damage in inflammatory bowel disease. J Crohns Colitis. 2016;10(11):1316–23.
27. Cupi ML, Sarra M, Marafini I, Monteleone I, Franzè E, Ortenzi A, Colantoni A, Sica G, Sileri P, Rosado MM, Carsetti R, MacDonald TT, Pallone F, Monteleone G. Plasma cells in the mucosa of patients with inflammatory bowel disease produce granzyme B and possess cytotoxic activities. J Immunol. 2014;192(12):6083–91.

28. Thurnham DI. Interactions between nutrition and immune function: using inflammation biomarkers to interpret micronutrient status. Proc Nutr Soc. 2014;73(1):1–8.
29. Thurnham DI, Northrop-Clewes CA. Inflammation and biomarkers of micronutrient status. Curr Opin Clin Nutr Metab Care. 2016;19(6):458–63.
30. Altomare R, Damiano G, Abruzzo A, Palumbo VD, Tomasello G, Buscemi S, Lo Monte AI. Enteral nutrition support to treat malnutrition in inflammatory bowel disease. Nutrients. 2015;7(4):2125–33.
31. Savino F, Liguori SA, Fissore MF. Oggero R breast milk hormones and their protective effect on obesity. Int J Pediatr Endocrinol. 2009;2009:327505.
32. Lillefosse HH, Tastesen HS, Du ZY, Ditlev DB, Thorsen FA, Madsen L, Kristiansen K, Liaset B. Hydrolyzed casein reduces diet-induced obesity in male c57bl/6j mice. J Nutr. 2013;143:1367–75.
33. Cabana MD. The role of hydrolyzed formula in allergy prevention. Ann Nutr Metab. 2017;70(Suppl 2):38–45.
34. Guest JF, Yang AC, Oba J, Rodrigues M, Caetano R, Polster L. Relative cost-effectiveness of using an extensively hydrolyzed casein formula in managing infants with cow's milk allergy in Brazil. Clinicoecon Outcomes Res. 2016;8:629–39.
35. Dupont C, Bradatan E, Soulaines P, Nocerino R, Berni-Canani R. Tolerance and growth in children with cow's milk allergy fed a thickened extensively hydrolyzed casein-based formula. BMC Pediatr. 2016;16:96.
36. Lambers TT, Gloerich J, van Hoffen E, Alkema W, Hondmann DH, van Tol EA. Clustering analyses in peptidomics revealed that peptide profiles of infant formulae are descriptive. Food Sci Nutr. 2015;3(1):81–90.
37. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (−delta delta C(T)) method. Methods. 2001;25:402–8.
38. Annoni R, Silva LF, Nussbaumer-Ochsner Y, van Schadewijk A, Mauad T, Hiemstra PS, Rabe KF. Increased expression of granzymes A and B in fatal asthma. Eur Respir J. 2015;45(5):1485–8.
39. Tak PP, Spaeny-Dekking L, Kraan MC, Breedveld FC, Froelich CJ, Hack CE. The levels of soluble granzyme A and B are elevated in plasma and synovial fluid of patients with rheumatoid arthritis (RA). Clin Exp Immunol. 1999;116(2):366–70.
40. Joeckel LT, Wallich R, Martin P, Sanchez-Martinez D, Weber FC, Martin SF, Borner C, Pardo J, Froelich C, Simon MM. Mouse granzyme K has pro-inflammatory potential. Cell Death Differ. 2011;18(7):1112–9.
41. Metkar SS, Menaa C, Pardo J, Wang B, Wallich R, Freudenberg M, Kim S, Raja SM, Shi L, Simon MM, Froelich CJ. Human and mouse granzyme A induce a proinflammatory cytokine response. Immunity. 2008;29(5):720–33.