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LETTERS

Granulysin is a key mediator for disseminated keratinocyte death in Stevens-Johnson syndrome and toxic epidermal necrolysis

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Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are life-threatening adverse drug reactions characterized by massive epidermal necrosis, in which the specific danger signals involved remain unclear. Here we show that blister cells from skin lesions of SJS-TEN primarily consist of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, and both blister fluids and cells were cytotoxic. Gene expression profiling identified granulysin as the most highly expressed cytotoxic molecule, confirmed by quantitative PCR and immunohistochemistry. Granulysin concentrations in the blister fluids were two to four orders of magnitude higher than perforin, granzyme B or soluble Fas ligand concentrations, and depleting granulysin reduced the cytotoxicity. Granulysin in the blister fluids was a 15-kDa secretory form, and injection of it into mouse skin resulted in features mimicking SJS-TEN. Our findings demonstrate that secretory granulysin is a key molecule responsible for the disseminated keratinocyte death in SJS-TEN and highlight a mechanism for CTL- or NK cell—mediated cytotoxicity that does not require direct cellular contact.

Adverse drug reactions (ADRs) account for 6–7% of all hospital admissions and remain a major clinical problem[1]. Among them, SJS and TEN are two of the most serious and life-threatening cutaneous ADRs and carry a 10–50% mortality rate[1]. These two disorders are considered to be variants of the same disease with different severity; both are characterized by a rapidly developing blistering exanthema of purpuric macules and target-like lesions accompanied by mucosal involvement and skin detachment to a varying extent[2]. SJS is defined as skin detachment of less than 10%, TEN as skin detachment greater than 30%, and overlapping SJS-TEN as 10–30%[3]. Here we use SJS-TEN to include SJS, SJS overlapping TEN and TEN. The histopathology observations of SJS and TEN include marked keratinocyte apoptosis in the epidermis with dermo-epidermal separation and epidermal necrosis, resulting in bullae and extensive mucocutaneous shedding[2–4]. Aside from the severe cutaneous manifestations, SJS-TEN may be accompanied by fever, myocarditis, myocardial infarction, hepatitis and acute renal failure and may compromise the respiratory and gastrointestinal systems. Although the incidence of SJS and TEN is low, these conditions can kill or severely disable previously healthy people. A few cases have prompted pharmaceutical companies' withdrawal of newly released drugs[2].

The pathogenesis of these serious, life-threatening ADRs is not fully understood, but it is believed to be immune mediated, as rechallenging an individual with the same drug typically shortens the incubation period before the disease and results in more severe manifestations[5]. The clinical, histopathological and immunological findings in SJS-TEN support the concept that SJS and TEN are specific drug hypersensitivity reactions initiated by CTLs[2,5]. Previous *in vitro* studies suggest that a major histocompatibility class I–restricted drug presentation leads to a clonal expansion of $CD8^+$ CTLs and induces immune reactions[6,7]. The major histocompatibility complex–restricted presentation of a drug or its metabolite for T cell activation is now supported by our recent findings of strong genetic association between human leukocyte antigen-B alleles and reactions to specific drugs[8,9]. Cytotoxic T cells and NK cells are observed to infiltrate the skin lesions of subjects with SJS-TEN[7]. These observations point to a cutaneous recruitment of antigen-primed CTLs in the pathogenesis of SJS-TEN.

However, the number of infiltrating inflammatory cells in the skin lesions of SJS-TEN is too few to explain the widespread death of keratinocytes; thus, there may be soluble mediators that contribute to keratinocyte death[10]. Two pathways may be involved: granule-mediated

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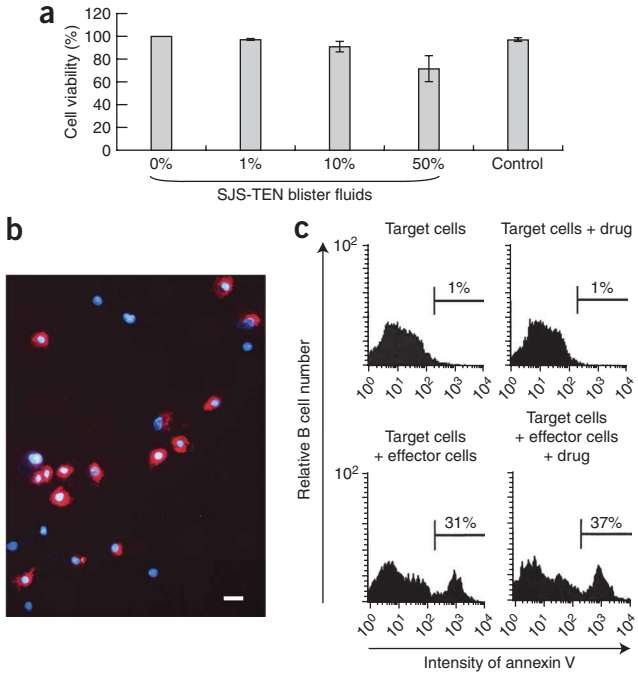

Figure 1 Cytotoxicity of blister fluids and blister cells of subjects with SJS-TEN. (**a**) Cytotoxicity of SJS-TEN blister fluid against the keratinocyte cell line KERTr, as measured by cell viability. Keratinocytes were incubated in culture medium containing 0–50% of blister fluids from the acute stage of SJS-TEN ($n = 9$) for 24 h. Cultures containing 50% blister fluids from burn injuries were used as controls ($n = 5$). Cell viability was measured by MTT assays. Values represent the average of nine SJS-TEN samples ± s.d. (**b**) Fluorescence image showing marked cell apoptosis induced by SJS-TEN blister fluid. Primary keratinocytes were incubated in culture medium containing 20% of blister fluid of SJS-TEN for 4 h. The cellular nuclei were counterstained by DAPI (blue), and apoptotic keratinocytes were revealed by the staining of phycoerythrin (PE)-conjugated annexin V (red). Scale bar, 10 μm. (**c**) Flow cytometry profiles of the cytotoxic activity of blister cells. Blister cells from a subject with carbamazepine-induced SJS were used as effector cells and Epstein-Barr virus–transformed autologous B cells were used as target cells in the presence or absence of culprit drug (carbamazepine 10,11-epoxide) for 4 h at 37 °C. Dead target cells were assessed by flow cytometry with the incubation of PE-conjugated annexin V and FITC-labeled monoclonal antibody to CD20. See **Supplementary Methods** for detailed information.

exocytosis (mainly of perforin and granzyme B) and Fas–Fas ligand (FasL; CD95) interaction[6,7,11,12]. One study proposed that a suicidal interaction between Fas and FasL, which are both expressed by keratinocytes, leads to extensive necrosis of epidermal cells in individuals with SJS-TEN[11]. However, another study argued against that hypothesis, as FasL is hardly detected on skin lesions, and the researchers reported that soluble FasL (sFasL) secreted by peripheral blood mononuclear cells (PBMCs) has a crucial role in the apoptosis and pathomechanism of TEN and SJS[13]. In contrast, a third group reported that perforin and granzyme are the main weapons used by the drug-specific CTLs in SJS-TEN[6,7]. Nevertheless, recent studies yet further argue against the crucial roles of Fas-sFasL or perforin and granzyme in SJS-TEN, as both pathways do not seem to be specific to SJS-TEN, and they are also upregulated in drug-induced skin eruptions, such as maculopapular exanthema (MPE), where massive apoptosis does not occur in the skin[14,15]. In addition, another problem of both proposed pathways is their requirement for cell-to-cell contact to kill the target cell, which cannot explain why few inflammatory cells cause extensive epidermal necrosis in SJS-TEN. Moreover, increased expression of the death receptor ligand, tumor necrosis factor or tumor necrosis factor receptor-1, has been reported in the epidermis, blister mononuclear cells, PBMCs or serum of subjects with SJS-TEN[16]. However, none of these proteins are specific to SJS-TEN. Until now, there was no useful biomarker for diagnosis and monitoring of disease progression of SJS-TEN.

Because the specific danger signal molecules that link the immune reaction to the keratinocyte apoptosis remain controversial, here we studied the global gene expression profiles of blister cells, measured the cytotoxic protein abundance in blister fluids and performed *in vitro* and *in vivo* cytotoxicity studies to identify specific cytotoxic molecules responsible for the disseminated epidermal necrosis in SJS-TEN.

RESULTS

Blister fluids and SJS-TEN cells show cytotoxicity

We determined the immunophenotypes of the cells present in the blister fluids from five subjects with SJS-TEN induced by carbamazepine (subjects 1, 4 and 5), phenytoin (subject 2) or amoxicillin (subject 3). Regardless of the culprit drugs, the majority of the cells in the blister fluids of subjects with SJS-TEN is composed of $CD3^+$ T cells with a predominance of the $CD8^+$ CTL subset (33–70%), $CD56^+$ NK cells (48–100%) and $CD8^+CD56^+$ NKT cells (**Supplementary Table 1** online).

We examined the toxicity of blister fluids against keratinocytes by incubating KERTr cells, a keratinocyte cell line, in culture medium containing different concentrations of blister fluids that were obtained from the acute stage (≤3 d after onset of blister formation) of subjects with SJS-TEN ($n = 9$). Blister fluids from subjects with burn injuries were used as controls ($n = 5$). MTT assays showed that blister fluids from subjects with SJS-TEN were cytotoxic against keratinocytes in a dose-dependent manner, whereas burn blister fluids had no effect (**Fig. 1a**). We observed similar cytotoxicity when we used B cells (data not shown). To investigate the mechanism of the cytotoxicity, we incubated primary keratinocytes in culture medium containing 20% SJS-TEN blister fluid. Marked cell apoptosis was revealed by intense annexin V staining (**Fig. 1b**).

The cytotoxic activity of the blister cells was examined with either Epstein-Barr virus–transformed autologous B cells or keratinocytes as the target cells. When using autologous cells as target cells, in the absence of blister cells, no cell death was seen with or without carbamazepine 10, 11-epoxide, the culprit drug metabolite (**Fig. 1c**). With an effector cell to target cell ratio of 5:1, blister cells triggered 31% of B cell apoptosis, which increased to 37% in the presence of the drug (**Fig. 1c**). Similar results were seen with carbamazepine or with nonautologous B cells (data not shown). Two more subjects (subjects 1 and 2) were studied with the same effector cell to target cell ratio, and the results showed that subject 1 had 44% and 46% cell apoptosis and subject 2 had 32% and 30% cell apoptosis in the absence and the presence of drug, respectively. Co-culturing of blister cells with keratinocytes, the target cells in SJS-TEN, also induced keratinocyte death in the absence of drug (data not shown). These data suggest that the blister cells had already been activated and showed strong cytotoxicity.

Upregulated CTL and NK cell genes in blister cells

The global gene expression profiles of skin blister fluid cells from five subjects with SJS-TEN were compared against those of PBMCs from

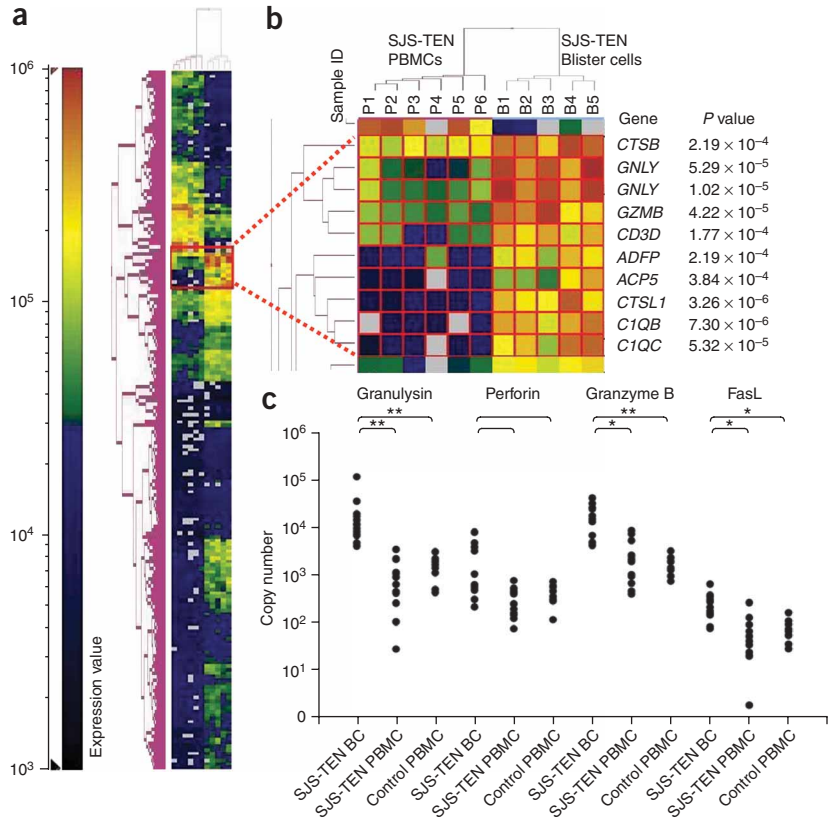

Figure 2 Gene expression analyses of blister cells (BCs) and PBMCs of subjects with SJS-TEN. (**a**) Cluster image of microarray profiles of the 200 most differentially expressed genes. The genes were clustered hierarchically into groups with Genedata Expressionist software on the basis of the similarity of their expression profiles. The expression pattern of each gene is displayed as a horizontal strip. (**b**) A cluster with the most significant overexpression in the blister cells (B1 to B5) compared to PBMCs (P1 to P6) in subjects with SJS-TEN. The *P* values represent the significant difference between the signal intensity of gene(s) in the blister cells and the PBMCs of subjects with SJS-TEN. There were two oligonucleotide probes representing granulysin transcript in the array; therefore, there are two values for that transcript. (**c**) mRNA copy numbers of granulysin, granzyme B, perforin and FasL in SJS-TEN blister cells, as determined by real-time PCR. The respective mRNA levels were compared among the paired blister cells ($n = 13$) and PBMCs ($n = 13$) from subjects with SJS-TEN and control PBMCs from healthy persons ($n = 10$). $^{*}P < 0.001$; $^{**}P < 0.00001$.

six subjects (five were paired samples). All samples were taken within 3 d of disease onset and before treatment. We performed analyses for each group on U133 Plus 2.0 gene chips (Affymetrix) with individual RNA samples.

By comparing the global gene expression profiles of blister cells and PBMCs, we identified the 200 most differentially expressed genes (**Supplementary Table 2** online). They were clustered hierarchically and are displayed as a horizontal strip (**Fig. 2a**). We looked for the genes that were most upregulated in the blister cells as compared to the PBMCs of subjects with SJS-TEN and found that the most significant cluster contained genes associated with CTLs and NK cells, including those encoding granulysin, granzyme B, CD3D antigen, cathepsin B, cathepsin L and complement protein 1q (**Fig. 2b**). The signal intensity of granulysin in the blister cells was 10–20–fold higher than that found in the PBMCs of subjects with SJS-TEN ($P = 1.02 \times 10^{-5}$ by *t*-test; **Supplementary Table 2**). The corresponding values for granzyme B and perforin transcripts were eightfold and threefold higher ($P = 4.22 \times 10^{-5}$ and $P = 0.017$, respectively, by *t*-test); however, it was only twofold higher for FasL and was statistically not significant ($P > 0.05$). Of interest to us was that, among the cytotoxic proteins, perforin and FasL were not among the 200 significant differentially expressed genes (**Supplementary Table 2**).

To verify the initial screening data, we performed real-time PCR and absolute quantification of messenger RNA levels of granulysin, granzyme B, perforin and FasL in the blister cells and compared their respective levels to the PBMCs from subjects with SJS-TEN ($n = 13$) or PBMCs from healthy subjects ($n = 10$). The mRNA copy numbers (means ± s.d.) of granulysin were 20,350 ± 28,758 in the blister cells and 975 ± 975 in the PBMCs of subjects with SJS-TEN and 1,703 ± 865 in the PBMCs of healthy controls; for perforin, they were 2,426 ± 2,709 in the blister cells and 318 ± 192 in the PBMCs of subjects with SJS-TEN and 371 ± 185 in the PBMCs of healthy controls; for granzyme B, they were 16,029 ± 11,430 in the blister cells and 2,717 ± 2,510 in the PBMCs of subjects with SJS-TEN and 1,613 ± 684 in the PBMCs of healthy controls; and for FasL, they were 249 ± 145 in the blister cells and 63 ± 64 in the PBMCs of subjects with SJS-TEN and 72 ± 36 in the PBMCs of healthy controls (**Fig. 2c**). Thus, granulysin transcript levels were the highest among the four cytotoxic proteins in the blister cells, and the fold increase in the blister cells over that in the PBMCs was also higher than the granzyme B, perforin and FasL levels.

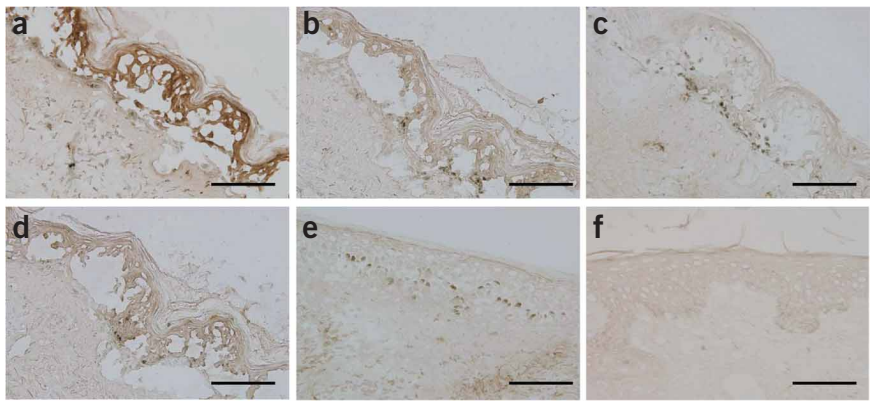

Figure 3 Immunohistochemistry staining of cytotoxic proteins in skin biopsies. (**a**–**d**) Serial sections of a skin biopsy from a subject with TEN were stained with antibodies against granulysin (**a**), granzyme B (**b**), perforin (**c**) and FasL (**d**). (**e**,**f**) Skin biopsies from a subject with maculopapular exanthema (**e**) and a healthy person (**f**) were stained with RC-8 antibody against granulysin. Scale bars, 200 μm.

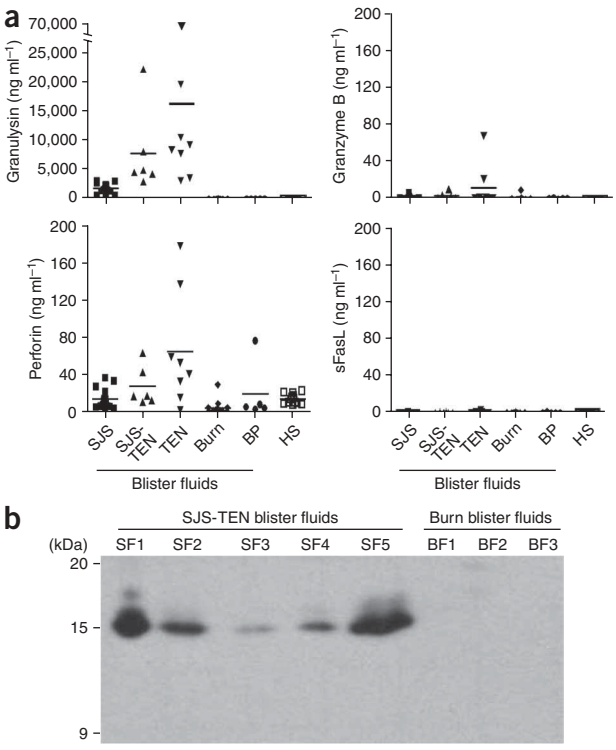

Figure 4 Analyses of cytotoxic proteins in the blister fluids. (**a**) Amounts of granulysin, granzyme B, perforin and sFasL in the blister fluids of subjects with SJS, overlapping SJS-TEN, TEN, burn injuries or bullous pemphigoid (BP), and in the serum of healthy subjects (HS), as measured by ELISA. The correlation between granulysin levels in blister fluids and the clinical severity of SJS, overlapping SJS-TEN and TEN was examined by one-way analysis of variance ($P = 0.02$). (**b**) Western blot analysis of granulysin protein abundance in blister fluids probed with the monoclonal antibody (RF-10) to granulysin. Each lane contained 10 μl of blister fluids from subjects with SJS-TEN (SF1 to SF5) or burn (BF1 to BF3).

Highly expressed granulysin protein in skin lesions

To further verify the mRNA results, we performed immunohistochemistry to examine the expression of granulysin and other cytotoxic proteins in the skin lesions of subjects with SJS-TEN ($n = 5$) and MPE ($n = 3$; **Fig. 3**). A representative skin biopsy from a subject with TEN showed intense staining of granulysin around the detached necrotic area of the epidermis (**Fig. 3a**). By contrast, granulysin was weakly stained in the skin of a subject with MPE (**Fig. 3e**) and undetectable in the skin of a healthy control (**Fig. 3f**). In the TEN skin biopsy, the expression of cytotoxic proteins (granzyme B, perforin and FasL) was less than that of granulysin (**Fig. 3b–d**).

To test the specificity of granulysin expression to certain skin disorders, we found that granulysin was highly expressed in the SJS and TEN skin lesions, weakly expressed in graft-versus-host disease (GVHD) and lichen planus and not detectable in bullous pemphigoid, lupus and psoriasis (**Supplementary Fig. 1** online).

With flow cytometry, we found that granulysin was expressed primarily in the $CD8^+$ and $CD56^+$ cells (CTLs, NK cells, and NKT cells) of the blisters of subjects with SJS-TEN. No $CD4^+$ cells were found to express granulysin (**Supplementary Table 3** online).

Granulysin protein levels correlate with clinical severity

We measured the concentrations of granulysin and other cytotoxic proteins in the blister fluids of subjects with SJS-TEN by ELISA. Blister fluids from subjects with burn injuries or bullous pemphigoid were used as controls. Blister fluids of subjects with SJS-TEN ($n = 29$) showed high amounts of granulysin (6,920.6 ± 12,050.2 ng ml^{-1};

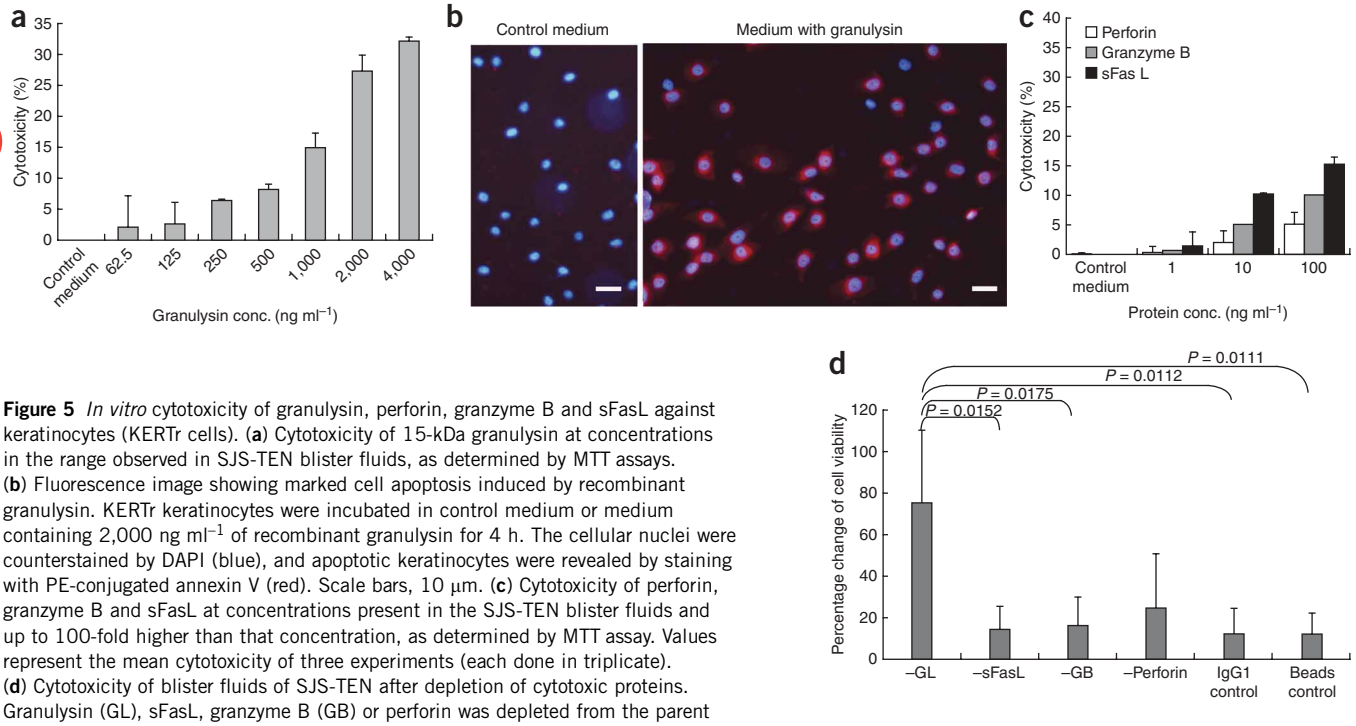

Figure 5 *In vitro* cytotoxicity of granulysin, perforin, granzyme B and sFasL against keratinocytes (KERTr cells). (**a**) Cytotoxicity of 15-kDa granulysin at concentrations in the range observed in SJS-TEN blister fluids, as determined by MTT assays. (**b**) Fluorescence image showing marked cell apoptosis induced by recombinant granulysin. KERTr keratinocytes were incubated in control medium or medium containing 2,000 ng ml^{-1} of recombinant granulysin for 4 h. The cellular nuclei were counterstained by DAPI (blue), and apoptotic keratinocytes were revealed by staining with PE-conjugated annexin V (red). Scale bars, 10 μm. (**c**) Cytotoxicity of perforin, granzyme B and sFasL at concentrations present in the SJS-TEN blister fluids and up to 100-fold higher than that concentration, as determined by MTT assay. Values represent the mean cytotoxicity of three experiments (each done in triplicate). (**d**) Cytotoxicity of blister fluids of SJS-TEN after depletion of cytotoxic proteins. Granulysin (GL), sFasL, granzyme B (GB) or perforin was depleted from the parent blister fluids with monoclonal antibodies conjugated to agarose beads. Cell viability was measured by MTT assays using KERTr keratinocytes as target cells and expressed as percentage change over the parent fluids (without depletion). Mouse IgG1 monoclonal antibody and agarose bead treatment alone were used as controls. Values represent the mean of four SJS-TEN samples (each done in triplicate). *P* values of *t*-test are shown.

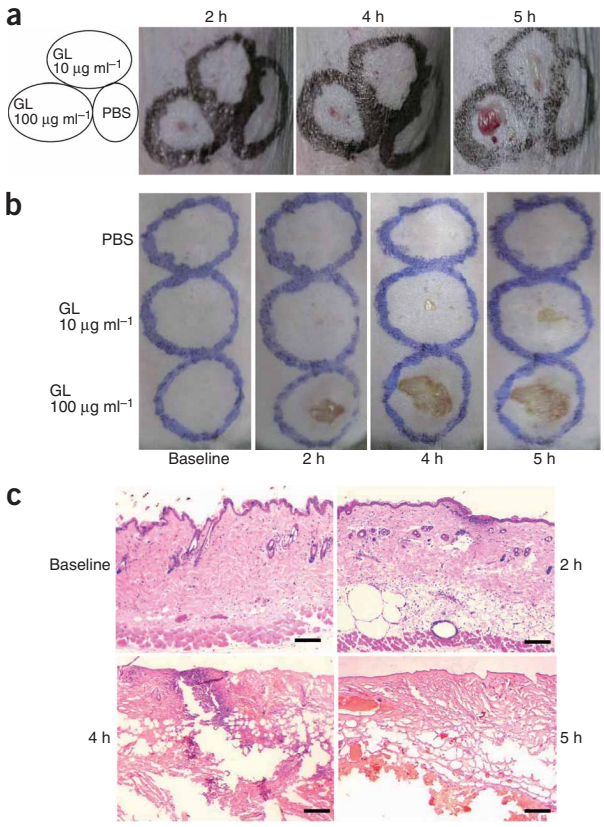

Figure 6 Intradermal injection of 15-kDa granulysin in mice. (**a**,**b**) Photographs of the skin lesions at different time points of a nude mouse (**a**) and a shaved C3H mouse (**b**), both of which received intradermal injections of purified 15-kDa granulysin (10 or 100 μg ml^{-1} in a total volume of 100 μl injected every hour for a total of five injections) or control PBS only. (**c**) Histopathology of the skin lesions at the site injected with granulysin (10 or 100 μg ml^{-1}), stained with H&E at baseline, early stage (2 h), and at late stages (4 h and 5 h), showing increasing inflammation and necrosis. Scale bars, 200 μm. The source of the granulysin was the recombinant N-terminal (histidine)$_6$-tagged 15-kDa expressed in the cell-free system.

range, 633.3–63,392.3 ng ml^{-1}), which was two to four orders of magnitude higher than the amounts of other cytotoxic proteins present in the fluids (perforin, 31.74 ± 39.44 ng ml^{-1}; granzyme B, 4.69 ± 12.81 ng ml^{-1}; sFasL, 0.41 ± 0.5ng ml^{-1}; **Fig. 4a**). Moreover, the abundance of granulysin protein in the blister fluids of subjects with SJS-TEN was 311-fold higher than the amount present in burn lesions (22.3 ± 24.8 ng ml^{-1}; $n = 15$) and 313-fold higher than the amount in the subjects with bullous pemphigoid (22.1 ± 12.7 ng ml^{-1}; $n = 5$), indicating the specificity of granulysin to SJS-TEN (**Fig. 4a**). Sera from healthy subjects had very low concentrations of granulysin (7.2 ± 1.4 ng ml^{-1}; $n = 13$).

There was also significant correlation of granulysin levels in blister fluids with clinical severity (**Fig. 4a**). The mean protein concentrations of granulysin in blister fluids were highest in cases of TEN (15,751.2 ng m^{-1}; $n = 8$) followed by SJS overlapping TEN (7,867.8 ng ml^{-1}; $n = 6$) and SJS (1,832.1 ng ml^{-1}; $n = 15$; $P = 0.02$, one-way analysis of variance).

Western blot analysis detected granulysin precursor protein with a molecular weight of 15 kDa in blister fluids of subjects with SJS-TEN ($n = 5$; **Fig. 4b**), and no 9-kDa mature processed form was detectable, even though the antibodies used in the study included RF-10 and DH4, which both recognize the 15- and 9-kDa forms. Furthermore, no granulysin was detected in the burn injuries ($n = 3$; **Fig. 4b**). In contrast, both the precursor 15-kDa form and the 9-kDa mature form were present in the blister cells (**Supplementary Fig. 2** online).

In vitro cytotoxicity of the 15-kDa granulysin protein

Using the keratinocytes as the target cells, we determined the cytotoxicity of 15-kDa granulysin protein *in vitro*. Purified recombinant 15-kDa granulysin showed cytotoxicity in a dose-dependent manner, and we observed substantial cytotoxicity already when the culture medium contained granulysin at concentrations below its mean concentration in the blister fluids of subjects with SJS-TEN (32.14% cytotoxicity with 4,000 ng ml^{-1}; **Fig. 5a**). To determine whether the cytotoxicity was mediated through apoptosis, we incubated KERTr cells in the culture medium containing 2,000 ng ml^{-1} of recombinant granulysin and stained the cells with annexin V. The majority of the cells were undergoing apoptosis (**Fig. 5b**). By comparison, the recombinant sFasL, perforin, or granzyme B proteins at their physical concentrations in the blister fluids of subjects with SJS-TEN showed little cytotoxicity (**Fig. 5c**).

Cytotoxic proteins in the SJS-TEN blister fluids were further depleted with monoclonal antibodies to granulysin, FasL, granzyme B or perforin conjugated onto protein A/G-agarose beads. Only granulysin depletion showed a significant increase of cell viability over the controls (**Fig. 5d**; $P = 0.0112$ and $P = 0.111$ compared to IgG and bead controls, respectively, by *t*-test). Depleting granulysin increased keratinocyte viability to a much greater extent than did depletion of other cytotoxic proteins present in the fluids, such as sFasL, granzyme B or perforin (mean increase in cell viability of 75.4% achieved by depleting granulysin versus 14.7% ($P = 0.015$ by *t*-test), 16.4% ($P = 0.017$) and 24.7% ($P = 0.19$) achieved by depleting sFasL, granzyme B and perforin, respectively (**Fig. 5d**)), and the effect of depleting the latter protein (perforin) was not statistically significant.

In vivo injection of granulysin induces epidermal necrosis

The *in vivo* effect of 15-kDa granulysin was evaluated by direct intradermal injections of purified recombinant granulysin (10–100 μg ml^{-1} in a total volume of 100 μl injected every hour for a total of five injections) into the skin of nude mice (**Fig. 6a**) and shaved C3H mice (**Fig. 6b**). Within 5 h, we observed considerable skin necrosis and blisters mimicking the clinical features of SJS-TEN at the injection sites of granulysin at both low and high dosages, whereas the skin at control sites of injection, which received only vehicle, had no effect (**Fig. 6a**,**b**). Histopathology of the granulysin injected sites showed extensive epidermal and dermal necrosis with inflammatory cell infiltration (**Fig. 6c**). In contrast, injection of the lysozyme protein (a basic protein like granulysin) at the same concentrations used for the granulysin experiments and injection of granzyme B (also a cytotoxic protein) at concentrations of 10 ng ml^{-1} and 100 ng ml^{-1}, which are higher than the granzyme B concentrations present in the blister fluids (~4 ng ml^{-1}), did not induce blistering or cause marked tissue damage as seen with granulysin (data not shown).

DISCUSSION

In this study, we showed that SJS-TEN blister cells were mainly composed of CD8$^+$ CTLs and CD56$^+$ NK and NKT cells, and these effector cells showed cytotoxicity against target cells. Similar results have been previously reported[6,7]; however, unlike those in previous studies, the effector cells in the blisters in this study were already fully activated and did not require the addition of the culprit drugs to induce cytotoxicity. The difference is probably due to the fact

that fresh blister cells were used here, whereas frozen cells were used in the previous reports. Supporting this notion was our observation of decreased cytotoxicity when we used frozen cells as compared to the fresh cells from the same individuals. Also different from previous studies, we show that secretory 15-kDa granulysin, not granzyme B, perforin, or sFasL as previously implicated, is a key molecule responsible for the disseminated keratinocyte death. The massive amounts of secretory granulysin produced by the fully activated CTLs, NK cells and NKT cells leads to undesired apoptosis and tissue damage and results in the unique clinical presentation of SJS-TEN.

Granulysin is a cationic cytolytic protein released primarily by CTLs and NK cells[17]. Previous studies on granulysin focus on the 9-kDa form, which is the processed form of the 15-kDa precursor[18,19]. Similar to other granular cytolytic proteins, the 9-kDa granulysin is released into the granule via a calcium-dependent pathway into the intercellular space between effector and target cells. The 9-kDa granulysin has homology to other cytolytic molecules of the saposin-like protein family and shows cytolytic activity against a variety of microbes and tumors through its binding to the target cell surface on the basis of charge, resulting in ion flux[17,20,21]. The ion flux induces mitochondrial damage and the release of cytochrome C and an apoptosis-inducing factor, leading to programmed cell death[22]. In addition, granulysin has been reported to be a chemoattractant and proinflammatory activator[23]. These multiple functions suggest granulysin has a crucial role in cellular immunity.

However, the granulysin detected in the blister fluids in this study was predominately the 15-kDa form; the 9-kDa form was not detected, even though the antibodies used in the study (RF-10 and DH4) recognize both forms[19] (W.-H.C., S.-I.H. and Y.-T.C., unpublished data). The 15-kDa granulysin has been shown to be secreted extracellularly by NK cells and CTLs via a nongranule exocytotic pathway, and its concentrations are elevated after T cell activation[24,25]. Furthermore, 15-kDa granulysin can be found in serum, and its levels are elevated in acute viral infections and in GVHD[25,26]. However, 15-kDa granulysin is generally believed not to be cytotoxic, and its full function is not yet known[19,24,25]. Our data show that 15-kDa granulysin at the concentrations observed in the SJS-TEN blisters possesses a similar potent cytotoxicity to that of the 9-kDa form *in vitro*, and, similarly to the 9-kDa form, the cytotoxicity of the 15-kDa form is also mediated through apoptosis. Furthermore, the injection of purified 15-kDa granulysin into mouse skin induced blistering and considerable epidermal and dermal necrosis. All of these findings indicate that 15-kDa granulysin is not a nonspecific, unprocessed product; instead, the high amounts of extracellular secretory 15-kDa granulysin in necrotic or blistering skin lesions are probably the cause of rapidly developing extensive epidermal apoptosis and necrosis in SJS-TEN. It is noteworthy that the relative amounts of granulysin were low in SJS lesions, which are generally rich in mononuclear cells, whereas in the paucicellular TEN lesions granulysin concentrations were high. Our data could explain the histopathology observed in SJS-TEN showing that infiltration of sparse dermal mononuclear cells results in extensive epidermal necrosis[10].

At the protein level, the abundance of granulysin in the blister fluids of subjects with SJS-TEN was much higher than that of granzyme B, yet the copy numbers of granulysin mRNA in the blister cells were only 1.3-fold higher than those of granzyme B. Because the granulysin detected in the blister fluids was primarily the 15-kDa form, the form known to be secreted extracellularly by NK cells and CTL cells, we hypothesized that the continuous secretion of granulysin by the NK cells and NKT cells in the blister fluids, via a nongranule exocytotic pathway, resulted in high concentrations of granulysin in the fluids.

If potency is compared using equimolar concentrations, sFasL may be more potent than granulysin, granzyme B or perforin. However, at the concentrations present in the blister fluids, only granulysin and not granzyme B, perforin or sFasL showed marked cytotoxicity. Furthermore, depleting granulysin from the SJS-TEN blister fluids reduced blister fluid cytotoxicity to a much greater extent than did depleting sFasL, granzyme B or perforin, indicating the essential role of granulysin in causing massive skin cell death in SJS-TEN. However, it is possible that the other cytotoxic proteins enhance the granulysin-mediated cell killing and further augment the cell death. Such synergistic effects of perforin have been reported in granulysin-induced lysis of bacteria[18].

Cytotoxic lymphocytes (NK cells and NKT cells) may have some mechanisms to protect themselves from self destruction after producing cytotoxic proteins. It has previously been reported that surface cathepsin B protects cytotoxic lymphocytes from self destruction after degranulation[27]. In our study, we found that cathepsin B (and cathepsin L) transcript levels were highly increased in the blister cells, as shown in the gene expression profile of SJS-TEN blister cells. We propose that high cathepsin levels in the CTLs, NK cells and NKT cells of the blister fluids may render them more resistant to granulysin-induced cytotoxicity.

In our study, the amounts of granulysin in the blister fluids were positively correlated with the clinical severity of the disease. This suggests that granulysin may be used to monitor the disease progression. In addition, our findings showed that granulysin was highly elevated only in SJS-TEN and not in other bullous skin diseases such as bullous pemphigoid, which suggests that measurement of granulysin may be helpful in differential diagnosis of bullous skin diseases, thus making the skin biopsy unnecessary.

Current therapy for SJS-TEN is not effective. The present treatments emphasize immunosuppression; however, a large dose of immunosuppressant often results in a secondary infection, among other complications. The efficacy of therapy using pooled human intravenous immunoglobulin is still controversial. Despite treatments, the disease still carries substantial morbidity and mortality. Our data suggests that granulysin could be used as a specific therapeutic target to develop a more effective therapy for SJS-TEN.

The usefulness of our study goes beyond SJS-TEN. First, GVHD, a frequent complication of allogenic bone marrow transplantation caused by the donor's immune cells attacking the body of the recipient, has skin and other internal organ manifestations that resemble SJS-TEN[28], and in this study we found that granulysin was also expressed in the skin of subjects with GVHD. Second, the current overemphasis of perforin and granzyme as the main pathway of granule-mediated cell death should be revisited, as our data suggest that granulysin secreted by the CTLs and NK cells may also have a crucial role. This mechanism probably represents the last line of host defense, as secretory granulysin kills the invading organism but leads to unfavorable, unwanted host cell apoptosis and tissue damage.

In conclusion, we show that secretory granulysin, not sFasL, granzyme B or perforin, as previously implicated, is a key molecule responsible for the disseminated keratinocyte apoptosis in SJS-TEN. This study provides a pathogenetic mechanism for SJS-TEN and a new role for granulysin, as well as a new diagnostic and therapeutic target for SJS-TEN.

METHODS

Clinical samples. The study was approved by the institutional review board of Chang Gung Memorial Hospital and Academia Sinica, and informed consent

was obtained from all participants. We collected blister cells and fluids from subects with SJS-TEN, burn injuries or bullous pemphigoid. We obtained skin biopsies from 15 subjects with SJS-TEN, 5 subjects with drug-induced maculopapular exanthema and healthy subjects. We isolated the PBMCs from the whole-blood samples, and we established B cell lines by Epstein-Barr virus transformation of PBMCs.

***In vitro* cytotoxicity assays.** We examined the cytotoxic activity of blister cells by incubating the fresh isolated blister cells with Epstein-Barr virus–transformed autologous B cells, primary keratinocytes or the KERTr keratinocyte cell line (CRL-2310, American Type Culture Collection) in the presence or absence of the culprit drug, carbamazepine or carbamazepine 10,11-epoxide. We examined the cytotoxic activity of blister fluids and purified recombinant proteins by incubating the keratinocytes in keratinocyte serum free culture medium Invitrogen containing different concentrations of blister fluids (0%, 1%, 10%, or 50%), or recombinant proteins (granulysin, granzyme B, perforin or sFasL). We assessed the percentages of apoptotic target cells either by fluorescence microscopy or by flow cytometry, and we measured t keratinocyte viability by a modified MTT assay.

Affymetrix expression microarray and analysis. We isolated total RNA from blister cells or from PBMCs of subjects with SJS-TEN and subjected it to reverse transcription, and we then hybridized the cleaned complementary RNA to an Affymetrix human genome U133 plus 2.0 array. We analyzed the data with Genedata software (Genedata Expressionist, Genedata).

Quantitative RT-PCR and copy number determination of mRNA. We isolated total RNA and obtained cDNA by reverse transcription. We quantified the amounts of complementary DNA (cDNA) in a sample with LightCycler-based, quantitative real-time PCR (Roche Molecular Biochemicals). For copy number determination, we cloned the cDNA inserts of granulysin, perforin, granzyme B, FasL and β-actin individually into the plasmid pGEM-T Easy (Promega). We calculated the absolute cDNAs amounts of of granulysin, perforin, granzyme B and FasL in each sample.

Immunoblots and immunohistochemistry staining. We probed immunoblots with either the RF-10 monoclonal antibody (MBL) or the DH4 monoclonal antibody (a gift from A.M. Krensky). We performed immunohistochemical staining on paraffin sections of the skin biopsies with monoclonal antibodies to granulysin (clone RC-8; MBL), granzyme B (clone GB7, Abcam), perforin (clone 5B10, Kamiya Biomedical) or FasL (clone Mike-1, Alexis). We used secondary antibodies conjugated to peroxidase and the DAB Detection Kit (Invitrogen) for linking and staining.

Enzyme-linked immunosorbent assay. We measured the concentrations of granzyme B, perforin and sFasL in samples were with ELISA kits for granzyme B (Mabtech), perforin (Mabtech) and sFasL (R&D Systems), with the assay sensitivity of $\sim$10 pg ml^{-1}. For determining the concentrations of granulysin protein, we coated the plates (Nunc) with 2 μg ml^{-1} RB-1 monoclonal antibody (MBL) and serially reacted them with the following materials, with washing steps between each reaction: samples or standards, 1 μg ml^{-1} of biotinylated RC-8 monocloncal (recognizing granulysin epitopes), 2 μg ml^{-1} of horseradish peroxidase–conjugated streptavidin (R&D Systems) and the substrate solution containing H_2O_2 and tetramethylbenzidine (R&D Systems). The assay sensitivity for granulysin was 2.5 ng ml^{-1}.

Expression and purification of the recombinant granulysin protein. We purchased the purified FasL, perforin and granzyme B proteins from Alexis and the N-terminal GST-tagged granulysin proteins of both 15-kDa and 9-kDa forms from Abnova. In addition, we expressed a recombinant N-terminal $(histidine)_6$-tagged 15-kDa granulysin in the Expressway Cell-Free *Escherichia coli* Expression System (Invitrogen). We assessed the identity, purity and concentration of the recombinant granulysin protein in the preparations by Coomassie blue protein assay (Bio-Rad), ELISA Western blotting and Coomassie staining of 17% SDS-PAGE gels.

Depletion of granulysin and other cytotoxic proteins from SJS-TEN blister fluids. We depleted the cytotoxic proteins granulysin, sFasL, granzyme B and perforin from SJS-TEN blister fluids separately with monoclonal antibodies to granulysin (RB1), Fas-L (5G51, Alexis), granzyme B (B18.1, MBL) or perforin (δG9, Bio-Vision) conjugated to protein A/G plus-agarose immunoprecipitation beads (Santa Cruz Biotechnology). We used mouse IgG1 monoclonal antibody (MOPC-21, Sigma) as a control. We defined the percentage change of cell viability in the presence of 50% blister fluids as [(cell viability with the depleted blister fluids) (cell viability with the parent blister fluids) / cell viability with the parent blister fluids] × 100%.

Intradermal injections of granulysin protein into mouse skin. Mouse studies were approved by the Institutional Animal Care and Use Committee of Academia Sinica. We injected the purified 15-kDa granulysin protein (10 or 100 μg ml^{-1}) into the skin of nude mice or shaved C3H/HeNCrNarl mice in a total volume of 100 μl, every hour, five times. After 5 h, we photographed the injected areas, obtained skin specimens and subjected them to H&E staining. We also injected PBS, chicken lysozyme protein (Sigma), or human granzyme B recombinant protein (Alexis) with the same protocol as granulysin.

Additional methods. Detailed methodology is described in **Supplementary Methods** online.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

W.-H.C. and S.-I.H. designed and conducted the experiments and data analysis and wrote the manuscript. J.-Y.Y., C.-C.C., S.-C.C., H.-C.H., C.-H.Y. and C.-F.L. cared for the involved human subjects and provided clinical samples. S.-C.S., S.-P.H., C.-Y.W. and S.-W.C. performed experiments. J.-Y.W. and Y.-D.L. helped design the expression of protein. Y.-T.C. supervised the entire project and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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