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15 kDa Granulysin Causes Differentiation of Monocytes to Dendritic Cells but Lacks Cytotoxic Activity

Carol Clayberger,* Michael W. Finn,* Tianhong Wang,* Reena Saini,* Christine Wilson,* Valarie A. Barr,* Marianna Sabatino,† Luciano Castiello,† David Stroncek,† and Alan M. Krensky*

Granulysin is expressed as two isoforms by human cytotoxic cells: a single mRNA gives rise to 15 kDa granulysin, a portion of which is cleaved to a 9 kDa protein. Studies with recombinant 9 kDa granulysin have demonstrated its cytolytic and proinflammatory properties, but much less is known about the biologic function of the 15 kDa isoform. In this study, we show that the subcellular localization and functions of 9 and 15 kDa granulysin are largely distinct. Nine kilodalton granulysin is confined to cytolytic granules that are directionally released following target cell recognition. In contrast, 15 kDa granulysin is located in distinct granules that lack perforin and granzyme B and that are released by activated cytolytic cells. Although recombinant 9 kDa granulysin is cytolytic against a variety of tumors and microbes, recombinant 15 kDa granulysin is not. The 15 kDa isoform is a potent inducer of monocyctic differentiation to dendritic cells, but the 9 kDa isomer is not. In vivo, mice expressing granulysin show markedly improved antitumor responses, with increased numbers of activated dendritic cells and cytokine-producing T cells. Thus, the distinct functions of granulysin isoforms have major implications for diagnosis and potential new therapies for human disease. The Journal of Immunology, 2012, 188: 6119–6126.

Cytotoxic cells protect the body from diseases and infection. CTLs and NK cells use a variety of mechanisms to kill target cells, including release of granules containing lytic molecules (e.g., perforin and granzymes) and expression of cell surface molecules such as FasL that trigger apoptosis when bound to cognate receptors on target cells (1–3). In addition, cytotoxic cells release an assortment of soluble molecules (e.g., IFN-γ, TNF, and RANTES) that can further modulate the immune response (4). Granulysin was identified in a search for genes expressed “late” (3–5 d) after activation of human CD8+ T cells (5). A single mRNA is translated into 15 kDa granulysin, some of which is processed at both the amino and carboxy termini to a 9 kDa protein. Granulysin is coexpressed in cytolytic granules with perforin and granzymes and is released via receptor-mediated granule exocytosis. Recombinant 9 kDa granulysin is lytic for tumors and microbes, including Gram-negative and Gram-positive bacteria, Mycobacteria tuberculosis, and Plasmodium falciparum, and acts as a chemoattractant for T lymphocytes, monocytes, and other inflammatory cells (6–9). Expression of granulysin has been broadly associated with good outcomes in cancer and infection (10–13). The 15 kDa isoform of granulysin is less well characterized, but it has been implicated as the causative agent in Stevens–Johnson syndrome and toxic epidermal necrolysis (14, 15).

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; iDC, immature dendritic cell; LAMP-1, lysosomal associated membrane protein-1; mDC, mature dendritic cell; TIL, tumor-infiltrating lymphocyte; WT, wild type.

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Materials and Methods

Cells

Human PBMCs were obtained from healthy donor leukopacs and leukocytes enriched by centrifugation over Ficoll; monocytes were obtained from healthy donors by leukopheresis and elutriation (Transfusion Medicine Institute, National Institutes of Health, 37 Convent Drive, Room 2016, Bethesda, MD 20892; and *Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; and †Cell Processing Section, Warren Grant Magnuson Clinical Center, National Institutes of Health, Bethesda, MD 20892

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†Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; and *Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Institutes of Health, the National Cancer Institute, National Institutes of Health, Bethesda, MD 20892
Granulysin expression

Single-cell suspensions of PBMCs were stained with fluorochrome-conjugated Abs specific for CD3, CD4, CD8, CD56, CD27, and CD45RA (BD Biosciences, San Jose, CA), fixed, and permeabilized (BD Cytofix/Cytoperm, BD Biosciences) and then stained with rabbit anti-granulysin antisemur or preimmune rabbit serum (5, 18). The flow cytometry data were analyzed with FlowJo analysis software (Tree Star, Ashland, OR). CD8+ T and NK cells were prepared from PBMCs using negative selection with magnetic bead purification (StemCell Technologies) (purity was >95%). For activation, 10^6 NK cells or 10^6 CD8+ T cells were cultured in medium with or without 50 ng/ml recombinant human IL-15 (eBioscience, San Diego, CA) for 2 d, and supernatants and pellets were collected following centrifugation. Where indicated, PMA (5 ng/ml) plus ionomycin (500 ng/ml) were added for the final 6 h to induce degranulation. Western blot analysis was conducted as described previously (6).

Confocal microscopy

Freshly isolated or IL-15-activated NK cells or the NK-like cell line NK92 were immobilized on poly (l-lysine)-coated slides, fixed in 2% paraformaldehyde, and permeabilized (0.01% saponin and 0.1% Triton X-100). Cells were stained with the following Abs: rabbit anti-granulysin antisemur (5), monoclonal anti-15 kDa granulysin (clone RF10; MBL International, Woburn, MA), perforin (clone SG9; BD Biosciences), granzyme B (clone GB11; Gen-Tex, Irvine, CA), lysosomal-associated membrane protein-1 (LAMP-1) (clone H4A3; BD Biosciences), and RANTES (clone VL1; Invitrogen, Carlsbad, CA) and counterstained with fluorochrome-conjugated Abs specific for CD3, CD4, CD8, CD56, CD27, and CD45RA (Molecular Probes, Carlsbad, CA). Images from fixed cells were collected with a Zeiss 510 LSM, using a x63, 1.4 NA objective (Carl Zeiss, Thornton, NY). Z stacks of complete cells were then filtered with Nyquist sampling frequency for colocalization analysis. Imaris 7.2.3 (Bitplane; Andor) was used for most image processing and for colocalization analysis. Briefly, one channel was used to produce a surface that included the entire cell to be analyzed, and this surface was used to make a channel where all pixels outside the cell were assigned a value of 0. This new channel was used to define a region of interest for the Imaris colocalization analysis. Thresholds were set for each channel by selecting the dimmest pixel to be included in the analysis. Data from 25 to 40 independent cells were collected for each Ab. Adobe Photoshop and Illustrator (Adobe Systems, San Jose, CA) were used to prepare composite figures.

Cytotoxicity of 9 and 15 kDa granulysin

Apoptosis of U937 cells and killing of Salmonella typhimurium were measured as described previously (7, 8).

Generation and analysis of DCs

Human monocytes were cultured at 2 × 10^6 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Hyclone, Ogden, UT), 2 mM l-glutamine, and 100 U/ml penicillin–streptomycin (complete media). Recombinant 9 and 15 kDa granulysin were prepared as described previously (5, 19). Monocytes were cultured with 15 kDa granulysin (10 nM) or with recombinant human GM-CSF (10 ng/ml) (BD Biosciences) and recombinant human IL-4 (10 ng/ml) (R&D Systems) for 3–4 d to produce iDCs. Cell surface Ags were assessed by FACS using a panel of fluorochrome-conjugated Abs from BD Biosciences and eBioscience.

Mice

C57BL/6 granulysin transgenic mice (GNY^+) (17) were backcrossed to BALB/c mice for >10 generations. Mice were bred at the National Cancer Institute, and mice >6 wk of age were used in all experiments. The Animal Care and Use Committee of the National Cancer Institute approved all animal experiments. GNY^+ and wild-type (WT) littermates were injected in the right flank with 1.5 × 10^6 CT26 tumor cells. After 12–14 d, tumors were removed and weighed. TILs and lymphocytes from the draining inguinal and popliteal lymph nodes were analyzed by flow cytometry.

Results

Expression of granulysin by human PBMCs

Buffy coats were obtained from 10 healthy donors, and granulysin expression was determined by intracellular staining and FACS. PBMCs were stained with various combinations of Abs to identify CD4+ or CD8+ naive, effector, and memory cells as well as NK and NKT cells. Cells were then fixed, permeabilized, and stained with rabbit anti-granulysin antisemur or preimmune serum. After gating on lymphocytes, expression of CD27 and CD45RA was used to subdivide CD4+ or CD8+ cells. As previously described, naive cells are CD27+CD45RA+ (Q2), effector cells are CD27−CD45RA− (Q1), and memory cells are CD27+CD45RA− (Q3) (20). Table I summarizes the results from all 10 donors, and results from a typical donor are shown in Fig. 1A. Granulysin expression is rare in CD4+ or CD8+ memory or naive T cells. In contrast, 17% of CD4+ and 75% of CD8+ effector T cells express granulysin. Essentially all CD56+ NK cells and the majority of CD56−CD3+ NK T cells express granulysin (Fig. 1B, Table I).

To determine which isoforms of granulysin are present in human NK and T cells, CD56+ or CD8+ cells were purified by negative selection using magnetic beads. Cells were then cultured in medium alone or medium supplemented with 50 ng/ml recombinant human IL-15 for 2 d, and the supernatants and cell pellets were analyzed for granulysin isoforms by Western blot. Both 15 and 9 kDa granulysins were present in the cell pellets of CD8+ T and NK cells cultured in medium or IL-15 (Fig. 1C). IL-15 caused a much greater increase in granulysin levels in NK cells than in CD8+ T cells. CD8+ T cells activated with IL-15 for 2 d did not secrete detectable levels of either 9 or 15 kDa granulysin. NK cells incubated in medium alone did not secrete any granulysin, but NK cells cultured with IL-15–secreted 15 kDa, but not 9 kDa, granulysin (Fig. 1C). NK cells cultured with IL-15 and then stimulated with PMA and ionomycin for the final 6 h of culture secreted both 9 and 15 kDa granulysin, although the amount of 15 kDa granulysin was much higher.

Confocal microscopy was used to further characterize the subcellular localization of 9 and 15 kDa granulysin in NK cells isolated from PBMCs. We used two Abs to visualize granulysin: 1) a polyclonal Ab raised against recombinant 9 kDa granulysin that recognizes both the 9 and 15 kDa forms in Western blot analysis (5) and 2) a mAb that recognizes an epitope on the 15 kDa but not the 9 kDa isoform (21). Cells were also stained with Abs specific for perforin, granzyme B, and LAMP-1, proteins located in cytolytic granules. Staining was compared in freshly isolated NK cells (Supplemental Fig. 1) and in NK cells activated by a 5-d culture with IL-15 (Fig. 2). Both resting and IL-15–activated NK cells stained with the polyclonal and monoclonal anti-granulysin Abs.

Table I. Expression of granulysin in freshly isolated PBMCs

<table>
<thead>
<tr>
<th>% Granulysin Positive</th>
<th>Range (% Granulysin Positive)</th>
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<tbody>
<tr>
<td>CD4^+ naive T cells</td>
<td>0.7</td>
</tr>
<tr>
<td>CD4^+ memory T cells</td>
<td>0.7</td>
</tr>
<tr>
<td>CD4^+ effector T cells</td>
<td>17</td>
</tr>
<tr>
<td>CD8^+ naive T cells</td>
<td>5</td>
</tr>
<tr>
<td>CD8^+ memory T cells</td>
<td>7</td>
</tr>
<tr>
<td>CD8^+ effector T cells</td>
<td>75</td>
</tr>
<tr>
<td>CD56^−CD3^+</td>
<td>94</td>
</tr>
<tr>
<td>CD56^+CD3^+</td>
<td>54</td>
</tr>
</tbody>
</table>

*Mean of 10 donors.

CD27^+CD45RA^+.

CD27^+CD45A^+.

CD27^+CD45RA^−.
Although the polyclonal Ab recognizes both isoforms under denaturing conditions (see Fig. 1C), there was minimal overlap in staining with the monoclonal 15 kDa-specific Ab, regardless of whether these Abs were applied simultaneously or sequentially (Fig. 2A). Because the 9 and 15 kDa isoforms share a common core that contains the epitopes recognized by the polyclonal Ab, these epitopes must be inaccessible in the native 15 kDa granulysin molecule in vivo. NK cells were then stained with the polyclonal and monoclonal granulysin Abs as well as a LAMP-1–specific Ab (Fig. 2B). Resting NK cells contain very little LAMP-1, and there is little if any colocalization of LAMP-1 with granulysin. IL-15–activated cells express much more LAMP-1 and the majority of it colocalizes with 9 kDa granulysin; minimal colocalization of 15 kDa granulysin and LAMP-1 was observed. Results were similar for perforin and granzyme B; both were expressed at low levels in resting NK cells, expression increased after culture with IL-15, and the 9 kDa, but not the 15 kDa, isoform colocalized with perforin and granzyme B (Fig. 2C, 2D). We performed a similar analysis using the NK-like cell line NK92 (Supplemental Figs. 2, 3). In agreement with our findings in activated NK cells, 9 but not 15 kDa granulysin colocalized with perforin and granzyme B but not with the chemokine RANTES, which has been shown to be secreted in a different type of granule (22). A quantitative summary of these findings is shown in Table II.

Nine kilodalton, but not 15 kDa, granulysin is cytolytic, whereas 15 kDa, but not 9 kDa, granulysin activates human monocytes

To investigate the biologic role of 15 kDa granulysin, it was expressed in insect cells and purified by fast protein liquid chromatography using sequential heparin and cation exchange columns (19). Because recombinant 9 kDa granulysin exhibits lytic activity against a variety of microbes as well as mammalian tumor cells (7, 8), we compared the ability of recombinant 15 and 9 kDa granulysin to kill S. typhimurium and human tumor cells. Recombinant 15 kDa granulysin was not cytolytic at any concentration tested, whereas 9 kDa granulysin was lytic against both targets (Fig. 3). Similar results were obtained using other target cells including Jurkat, HUT-78, Escherichia coli, and Staphylococcus aureus (data not shown).

We next asked whether either isoform affected freshly isolated human PBMCs. Within a few hours, PBMCs treated with the 15 kDa isoform formed aggregates, whereas those treated with the 9 kDa isoform did not (data not shown). Cell surface staining and FACS analysis of these cultures revealed that monocytes were responsive to 15 kDa granulysin. Using elutriated CD14+ monocytes, we compared the effects of 15 kDa granulysin, 9 kDa granulysin, and GM-CSF, a well characterized activator of monocytes (23). Within 6 h, monocytes cultured with 10 nM 15 kDa granulysin, but not with 9 kDa granulysin (tested over a range of 10–10,000 nM) or GM-CSF (10 ng/ml), formed aggregates (Fig. 4A). Both 15 kDa granulysin and GM-CSF caused a rapid increase in cell size and upregulation of adhesion molecules including CD11b, CD11c, and CD54, as well as molecules associated with differentiation to iDCs, including CD40, CD80, CD86, and HLA-DR (Fig. 4B). Fifteen kilodalton granulysin, but not GM-CSF, promoted increased expression of CD83, whereas GM-CSF, but not 15 kDa granulysin, caused increased expression of CD1a and CD1c.

Previously, we used commercial granulysin to show that 15 kDa granulysin could also promote the differentiation of iDCs to mature DCs (mDCs) (16). We reported that this process required quite high concentrations (2–10 μg/ml) of commercial 15 kDa granulysin. To determine the efficacy of our recombinant 15 kDa granulysin prepared from insect cells on the maturation of iDCs,
we incubated human monocytes with GM-CSF plus IL-4 for 6 d and then added insect-produced 15 kDa granulysin at 10 ng/ml for an additional 24 h. Cell surface analysis of these cells shows that they exhibit a typical mDC phenotype with low expression of CD14 and high expression of CD40, CD80, CD83, CD86, and HLA-DR (Fig. 4C). This observation is in agreement with our previously published finding that our 15 kDa recombinant granulysin is more potent than 15 kDa granulysin obtained from two different commercial sources (19).

Fifteen kilodalton granulysin and GM-CSF activate distinct genes in monocytes

Previously, we analyzed the gene expression of monocytes cultured with either 15 kDa granulysin or GM-CSF. We found that GM-CSF altered the expression of genes involved in cell differentiation, whereas 15 kDa granulysin induced expression of proinflammatory cytokines (23). To extend this observation, we selected a subset of immune-related genes whose expression was increased by 15 kDa granulysin in the microarray analysis by at least 5-fold at 4 h and confirmed their expression by real-time quantitative PCR (Table III). Monocytes from five donors were cultured with medium, 10 nM 15 kDa granulysin, or 10 ng/ml GM-CSF, and aliquots were removed at 4, 12, and 24 h. At the 4-h time point, mRNA for all these genes was increased by 15 kDa granulysin over levels in monocytes cultured in medium alone, while at the same time point, only CD274 and CD80 mRNA were slightly upregulated in monocytes cultured with GM-CSF. At 4 and 12 h, expression of all genes was higher in cells treated with 15 kDa granulysin than in cells treated with GM-CSF. At the 24-h time point, mRNA levels of all genes, except CCL2 and CCL7, were higher in cells treated with 15 kDa granulysin than in cells treated with GM-CSF. These data indicate that 15 kDa granulysin affects monocytes differently from GM-CSF, suggesting that 15 kDa granulysin may induce APCs that functionally differ from those induced with GM-CSF.

Fifteen kilodalton granulysin is a chemoattractant for murine leukocytes in vivo but does not activate murine monocytes in vitro

To further investigate a role for 15 kDa granulysin in clinical situations, we turned to a mouse model. Because mice do not have a granulysin homolog, we generated mice transgenic for human granulysin (17). To determine the role of secreted 15 kDa granulysin in vivo, we activated splenocytes from WT and GNLY+/− mice.

<table>
<thead>
<tr>
<th>Ab A</th>
<th>Ab B</th>
<th>% Ab A Colocalized with Ab B</th>
<th>% Ab A Colocalized with Ab B</th>
<th>% Ab A Colocalized with Ab B</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 kDa Granulysin</td>
<td>15 kDa Granulysin</td>
<td>25.55 ± 4.79</td>
<td>12.47 ± 3.18</td>
<td>3.2 ± 0.2</td>
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<tr>
<td>9 kDa Granulysin</td>
<td>Perforin</td>
<td>17.26 ± 3.28*</td>
<td>50.86 ± 3.57*</td>
<td>69.5 ± 11.5*</td>
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<tr>
<td>15 kDa Granulysin</td>
<td>Perforin</td>
<td>6.65 ± 1.71*</td>
<td>29.50 ± 4.98*</td>
<td>12.65 ± 7.1*</td>
</tr>
<tr>
<td>9 kDa Granulysin</td>
<td>Granzyme B</td>
<td>32.19 ± 5.93*</td>
<td>44.79 ± 6.71*</td>
<td>49.0 ± 17.4*</td>
</tr>
<tr>
<td>15 kDa Granulysin</td>
<td>Granzyme B</td>
<td>7.77 ± 2.48*</td>
<td>8.53 ± 3.05*</td>
<td>9.35 ± 14.5*</td>
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<td>9 kDa Granulysin</td>
<td>LAMP1</td>
<td>24.26 ± 3.21*</td>
<td>47.62 ± 7.25</td>
<td>ND</td>
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<tr>
<td>15 kDa Granulysin</td>
<td>LAMP1</td>
<td>11.02 ± 2.23*</td>
<td>28.87 ± 7.9</td>
<td>ND</td>
</tr>
<tr>
<td>9 kDa Granulysin</td>
<td>RANTES</td>
<td>ND</td>
<td>ND</td>
<td>21.8 ± 9.5</td>
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<tr>
<td>15 kDa Granulysin</td>
<td>RANTES</td>
<td>ND</td>
<td>ND</td>
<td>30.5 ± 11.7</td>
</tr>
</tbody>
</table>

*p < 0.01.

FIGURE 4. Fifteen kilodalton granulysin activates human monocytes. (A) Purified CD14+ monocytes were cultured in medium alone or in medium supplemented with 50 μM 9 kDa granulysin, 10 nM 15 kDa granulysin, or 10 ng/ml GM-CSF. Photographs were taken (magnification ×10) 6 h later. (B) Expression of a panel of cell surface molecules by monocytes cultured with 15 kDa granulysin (10 nM) or GM-CSF (10 ng/ml). Purified monocytes were incubated with medium (gray), 10 nM 15 kDa granulysin (blue), or 10 ng/ml GM-CSF (orange) for 24 h and then stained with fluorescent Abs and analyzed by FACS. (C) iDCs were incubated with 10 nM 15 kDa granulysin (black line) or medium (gray) for an additional 24 h and then stained with the indicated fluorescent Abs and analyzed by FACS. Results are representative of four to six similar experiments.
animals with IL-15 for 7 d, isolated NK cells using magnetic beads, and injected 10^6 WT or GNLY^{+/−} transgenic NK cells into the peritoneum of WT BALB/c mice. Cells were collected 24 h later, and both the absolute number and the variety of cell types present were determined (Fig. 5A, 5B). Activated GNLY^{+/−} NK cells caused the influx of approximately three times as many cells as did WT NK cells. However, the percentage of neutrophils, macrophages, T cells, B cells, and NK cells in the infiltrating cells was similar when WT or GNLY^{+/−} transgenic NK cells were injected.

We next asked whether recombinant 15 kDa granulysin activates murine monocytes in vitro. Both peripheral blood and bone marrow were used as a source of monocytes. Although GM-CSF induced the differentiation of peripheral blood leukocytes into CD11b^+CD11c^+ dendritic-like cells, 15 kDa granulysin did not induce a significant change in the number of CD11b^+CD11c^+ cells (Fig. 5C). Bone marrow is a source of monocyte progenitors, and culture of bone marrow CD11b^+Ly-6C^− cells with GM-CSF for 8 d caused an increase in cells expressing CD80 and CD86 (Fig. 5D). However, the culture of bone marrow CD11b^+Ly-6C^− cells with 15 kDa granulysin for 8 d caused only a slight increase in the number of cells expressing CD80 and CD86. Last, we tested the ability of 15 kDa granulysin to induce maturation of immature mouse DCs. Bone marrow CD11b^+Ly-6C^− cells were cultured for 8 d with GM-CSF and 15 kDa granulysin. Fifteen kilodalton granulysin caused a significant increase in the number of cells expressing higher levels of CD80, CD86, and CD40 (Fig. 5E), indicating that 15 kDa granulysin can activate immature mouse DCs but not mouse monocytes.

GNLY^{+/−} mice exhibit increased numbers of DCs, activated T cells, and enhanced antitumor responses

Syngeneic CT26 colon carcinoma cells (24) were injected into the flanks of WT and GNLY^{+/−} transgenic mice, and 13 d later, tumors and draining lymph nodes were removed. The tumors were weighed, and tumor-infiltrating lymphocytes (TILs) were prepared. Tumor nodules from GNLY^{+/−} mice were significantly smaller than those from WT mice (Fig. 6A). Although the number of TILs recovered from the WT mice was consistently higher than from GNLY^{+/−} animals, the percentages of CD3^+, CD8^+, and DX5^+ cells in the TIL were similar from both GNLY^{+/−} and WT mice (data not shown). Both 9 and 15 kDa isoforms of granulysin were detected by Western blot analysis in TILs from GNLY^{+/−} mice (data not shown). Both 9 and 15 kDa isoforms of granulysin from GNLY^{+/−} or WT mice were injected into the peritoneum of WT mice, and after 24 h, infiltrating cells were obtained. (A) The total number of cells in the infiltrates was determined. Each point represents one animal. *p < 0.01. (B) Infiltrating cells were stained with a panel of Abs and analyzed by FACs. Bars represent the percentage of total cells for the indicated subsets. (C) Peripheral blood leukocytes from BALB/c mice were cultured in medium alone or supplemented with GM-CSF (10 ng/ml) or recombinant 15 kDa granulysin (10 nM). After 24 h, cells were stained for expression of CD11b and CD11c and analyzed by FACs. (D) Bone marrow CD11b^+Ly-6C^− cells from BALB/c mice were cultured for 24 h with medium (solid gray), 10 nM 15 kDa granulysin (blue line), or 10 ng/ml GM-CSF (blue line), and expression of CD80 and CD86 was determined by FACs. (E) Bone marrow cells from BALB/c mice were cultured with GM-CSF (10 ng/ml) plus IL-4 (10 ng/ml) for 8 d, and the resulting iDCs were then cultured with medium (solid gray) or 10 nM 15 kDa granulysin (blue line). Expression of CD80, CD86, and CD40 was determined by FACs. Results are representative of three similar experiments.

Table III. Immune related gene expression in monocytes activated with granulysin or GM-CSF

<table>
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<tr>
<th>Gene</th>
<th>15 kDa Granulysin</th>
<th>GM-CSF</th>
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<tbody>
<tr>
<td></td>
<td>4 h</td>
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<tr>
<td>IL-6</td>
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<tr>
<td>CD274</td>
<td>319</td>
<td>102</td>
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Monocytes were cultured for 4, 12, or 24 h with 15 kDa granulysin (10 nM) or GM-CSF (10 ng/ml) and RNA isolated. Results are expressed as the fold increase relative to the monocytes cultured in medium alone for the indicated time. Results are representative of one of five similar experiments.
to produce granulysin correlates with enhanced costimulatory/coactivating properties of APCs in vivo.

**Discussion**

Granulysin was originally identified in a search for genes expressed “late” (3–5 d) after T cell activation (25). Two granulysin isoforms, 9 and 15 kDa, were detected, and subsequent studies showed that the 9 kDa isoform resulted from posttranslational processing of 15 kDa granulysin (5, 6). Recombinant 9 kDa granulysin has been well characterized: it causes apoptosis of tumor cells, kills many pathogens, and activates iDCs (7, 8, 16). However, the function of 15 kDa granulysin is less well understood. In this study, we compare the expression, intracellular localization, and function of 9 and 15 kDa granulysin. Both isoforms are found in CD8+ effector T cells, NK cells, and NKT cells in freshly isolated PBMCs, but only the 15 kDa isoform is detected in supernatants from IL-15–activated cytotoxic cells. Nine and 15 kDa granulysin are located in different compartments in cytolytic cells: the 9 kDa isoform colocalizes with perforin, granzyme B, and LAMP-1 whereas the 15 kDa form does not. In contrast to the 9 kDa isoform, recombinant 15 kDa granulysin is not cytotoxic. Moreover, 15 kDa granulysin, but not 9 kDa granulysin, activates human monocytes to differentiate into DCs. Fifteen kilodalton granulysin is a potent attractant of immune cells in vivo, and transgenic mice expressing granulysin show enhanced ability to reject tumors.

Granulysin is expressed by essentially all NK cells, a majority of NKT and CD8+ effector T cells, but by few CD4+ effector cells in PBMCs from healthy donors. Granulysin expression in CD4+ naive and memory T cells is negligible and quite low in CD8+ naive and memory T cells. These findings confirm and extend those of Ogawa et al. (21) who reported granulysin expression in CD56+ cells as well as in an unidentified portion of CD3+ cells. Both the 9 and 15 kDa isoforms of granulysin were present in purified NK or CD8+ T cells, but only the 15 kDa isoform was detected in supernatants of NK cells activated for 48 h with IL-15. No granulysin was detected in the supernatants from purified CD8+ T cells cultured for 48 h with IL-15, suggesting that activated NK cells are the major source of serum granulysin in vivo. Surprisingly, we were unable to show by confocal microscopy that the polyclonal Ab raised against recombinant 9 kDa granulysin recognizes the 15 kDa isoform in cells. Possible explanations for this observation are that the 15 kDa molecule adopts a tertiary conformation that masks the 9 kDa epitopes or that 15 kDa granulysin is associated in vivo with molecules that block access to the 9 kDa epitopes.

We were unable to detect any cytotoxicity of the recombinant 15 kDa granulysin against a variety of tumor and bacterial targets in a side-by-side comparison with cytolytic 9 kDa recombinant granulysin. In contrast, Chung et al. (14) reported that 15 kDa granulysin causes keratinocyte death. These authors used a recombinant 15 kDa granulysin containing a 6-histidine tag, which may

**FIGURE 6.** GNLY+/- mice exhibit enhanced resistance to tumors, numbers of APCs, and T cell responses. WT or GNLY+/- mice were injected with CT26 tumor cells in the left flank, and the tumor and draining lymph nodes were removed after 12–14 d. (A) Weight of excised tumors. Each point represents one animal. (B) TILs were stimulated in vitro with PMA/ionomycin, and intracellular expression of TNF-α and IFN-γ was measured by flow cytometry. (C) GNLY+/- animals have increased numbers of CD40+ and CD86+ cells in the tumor and draining lymph nodes. *p < 0.01. Results are representative of three similar experiments.
explain our different findings. As detailed by Finn et al. (19), commercially available 15 kDa granulysin preparations contain recombinant tags that may affect function. The preparation from R&D Systems contains a 10-histidine tag at the C terminus, whereas the Novus Biologicals material includes an intact GST tag at the N terminus. We showed that our untagged insect produced 15 kDa granulysin behaves differently from the commercially available tagged forms in a monocyte activation bioassay (19). Furthermore, we show in this study that our recombinant 15 kDa granulysin is more potent than commercial 15 kDa granulysin for inducing the maturation of iDCs. Our 15 kDa granulysin can be used at 10 nM to induce iDCs to become mDCs, whereas Tewary et al. (16) reported that they needed ~100 times as much commercial recombinant 15 kDa granulysin.

NK cells, as with monocytes, are recruited to sites of inflammation or tissue damage. A number of groups have reported that activated NK cells induce maturation of GM-CSF-derived iDC (26–28) or activate resting monocytes (29–31). Zhang et al. (29) found that IL-15 activated CD3−CD68(+) NK cells triggered CD14+ monocytes to differentiate into TH1-promoting DCs. This process required direct contact of monocytes with NK cells and was mediated by GM-CSF and CD154 (29). Our findings suggest that release of 15 kDa granulysin represents yet another mechanism whereby NK cells can activate monocytes.

Although 15 kDa granulysin activates human monocytes, it has little if any effect on mouse monocytes. In contrast, 15 kDa granulysin activates both human and murine iDCs (16). In humans, ~95% of blood monocytes are CD14(+)CD16− and 5% are CD14(+)CD16+. In mice, monocytes express CD115 (M-CSF receptor), CD11b, F4/80, and they can be further subdivided into Ly-6C+CCR2+CD11c+ and Ly-6C−/CCR2−CD11c− populations. Ingersoll et al. (32) recently used microarray gene profiling to investigate the similarities between human and mouse monocyte subsets. They found that expression of many genes is similar in monocyte subsets of both species but that there are a number of genes whose expression patterns are not conserved. We showed previously that TLR4 is required for activation of murine and human iDCs by granulysin (16). Because mouse monocytes express TLR4 (33) but are not activated by granulysin, we hypothesize that granulysin activates monocytes in a manner analogous to LPS (34, 35). LPS, in the presence of LPS-binding protein, binds to CD14, a GPI-anchored protein that is incapable of initiating transmembrane signaling. LPS is then transferred to MD2, which is associated with TLR4, and TLR4 transduces an activating signal. We hypothesize that 15 kDa granulysin binds to a receptor expressed by human but not mouse monocytes that subsequently interacts with TLR4 to activate monocytes.

DCs are potent APCs that initiate primary T cell responses by presenting antigenic peptides in association with MHC molecules. Because DCs are rare in peripheral blood, clinical protocols have been designed for the in vitro expansion of these potent APCs from bone marrow or peripheral blood monocytes. The most widely used protocols involve culturing peripheral blood monocytes with GM-CSF and other agents, such as IL-1, IFN-γ, or LPS, to mature DCs to augment immune responses in vivo in humans (24, 36, 37). The majority of >100 Investigational New Drug applications using this approach are aimed at tumor therapy (38), but similar principles apply for vaccines against infectious agents and perhaps for therapies to alleviate autoimmunity. Despite the popularity of this approach, results have been mixed, with <10% overall clinical success rate. DCs generated from monocytes in vitro using 15 kDa granulysin differ in some ways from DCs generated with GM-CSF (23). In general, 15 kDa granulysin induced pathways related to costimulation of T cell activation and Th1 development. In contrast, GM-CSF downregulated cytokine production, lymphocyte-mediated immunity, and humoral immune responses at late time points.

Granulysin is a member of the alarmin family, a group of structurally diverse endogenous mediators of innate immunity (39). Alarmins share several features: they are rapidly released in response to infection of tissue damage; they have both chemotactic and activating effects on the immune system; and they exhibit potent in vivo immunoactivating activity. Granulysin is expressed by both NK cells and CD8+ T cells, positioning it as a link between innate and adaptive immunity. It is important in this regard to clearly separate the expression and release of granulysin isoforms. Fifteen kilodalton granulysin is released by activated cytotoxic cells, whereas 9 kDa granulysin is packaged in cytolytic granules and requires receptor-mediated granule exocytosis for release. Thus, release of the 9 kDa cytolytic isoform is tightly regulated while release of the 15 kDa proinflammatory isoform is constitutive by activated NK cells.

Disclosures

A.M.K. and C.C. hold patents on granulysin. The other authors have no financial conflicts of interest.

References


Supplemental Figure 2
Supplemental Figure 3
Supplementary Figure Legends

Supplementary Figure 1. 9 kDa granulysin co-localizes with LAMP-1, granzyme B and perforin while 15 kDa granulysin is present in a distinct compartment in human NK cells. A-D, Freshly isolated NK cells were stained and analyzed by confocal microscopy. Bar indicates 1 μ. Results are representative of 3 similar experiments.

Supplementary Figure 2. NK92 cells were stained with antibodies recognizing 9 kDa granulysin (GNLY), granzyme B (GzmB), Perforin, and RANTES and analyzed by confocal microscopy. Bar indicates 1 u. Results are representative of 3 similar experiments.

Supplementary Figure 3. NK92 cells were stained with antibodies recognizing 15 kDa granulysin (GNLY), granzyme B (GzmB), Perforin, and RANTES and analyzed by confocal microscopy. Bar indicates 1 u. Results are representative of 3 similar experiments.