Quiescent and activated mouse granulocytes do not express granzyme A and B or perforin: similarities or differences with human polymorphonuclear leukocytes?

Praxedis Martin, Reinhard Wallich, Julian Pardo, Arno Müllbacher, Markus Munder, Manuel Modolell, and Markus M. Simon

Polymorphonuclear leukocytes have been shown to use a multitude of effector functions to combat pathogens and tumors, including enzymes, defensins, and toxic products such as oxygen radicals and nitrogen oxides. Recent studies provided evidence for the expression of granzymes (gzms) and perforin (perf) within the cytotoxic arsenal of human neutrophils, the validity of which was questioned by 2 subsequent studies. We have now used cytology, intracellular flow cytometry, enzymatic assays, immunoelectron microscopy, and quantitative reverse transcriptase–polymerase chain reaction to obtain evidence of the presence of gzms and/or perf in mouse Gr−1+ granulocyte populations. The data obtained clearly demonstrate that neither in vitro nor in vivo–derived mouse granulocytes synthesize gzmA and gzmB or perf, even following infection/immunization with pathogens or pathogen-derived material. A parallel comparable analysis on the expression of gzmB in human neutrophils from 3 healthy control subjects and 4 patients with diverse diseases failed to detect gzmB expression. The data indicate that polymorphonuclear leukocytes from mice and humans lack the 3 cytotoxic effector molecules, gzmA, gzmB, and perf, generally associated with natural killer and cytotoxic T lymphocytes.

Introduction

Perforin (perf) and granzymes (gzms) are major components of cytoplasmic granules from natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) and are critical cytolytic effector molecules in NK/CTL-mediated apoptosis and are required for the control of intracellular pathogens and tumors. In mice and humans, gzms, in particular gzmA and gzmB, and perf are produced mainly by the majority of NK cells and CTLs, a fraction of CD4+ T cells, and to a lesser extent by related cell types, such as metrial gland cells, but not by polymorphonuclear leukocytes (granulocytes/neutrophils) and monocytes.

Two recent studies provided evidence that gzmA and perf are also produced by human polymorphonuclear neutrophils (PMNs). These reports were intriguing in light of the known biologic effects of gzms and perf in NK/CTL-mediated immune responses and the role of PMNs as a first-line defense against microbial pathogens and tumors. However, the assumption that PMNs use the same set of molecules as NK/CTL to combat pathogens and tumors was challenged by 2 subsequent studies. To date, the discrepancy of the 4 studies, which used similar techniques for the enrichment of PMNs and to analyze the expression of GZH- and PRF1-specific transcripts and/or proteins, has not been resolved.

We have now analyzed in detail the expression of gzmA, gzmB, and perf in in vitro–generated and ex vivo–derived mouse granulocytes by combining cytology, correlative cell surface phenotypic analysis, intracellular flow cytometry, enzyme assays, immunoelectron microscopy (IEM), and reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. In addition we have investigated the expression of gzmB in human PMNs from healthy individuals and a panel of patients with distinct diseases.

Materials and methods

Mouse strains and cell lines

Inbred C57BL/6 (B6) and mouse strains deficient for gzmA (gzmA−/−), gzmB (gzmB−/−), and gzmA × B (gzmA × B−/−) bred on the B6 background were maintained at the Max-Planck-Institut für Immunbiologie, Freiburg, Germany, and analyzed for their genotypes as described. Male mice of 8 to 12 weeks of age were used in all experiments and were conducted in accordance with the ethical guidelines of the European Laboratory Animal Science Association.

The mouse cell lines 1.3E6SN (CTL; perf+, gzmA+, gzmB+), EL4.F15 (thymoma; perf+, gzmA+, gzmB+) were used as control cells for the analysis of perf- and gzm-specific transcripts and proteins.

Cell lines were cultured in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 2-mercapto ethanol (10−4 M) at 7% CO2, as described; for 1.3E6SN cells, medium was supplemented with 10% Concanavalin A supernatant (ConA SN). ConA SN was used as cytokine source for expansion of mouse T cells. To produce ConA SN the SN of rat spleen cells was previously stimulated with ConA (5 μg/mL, 2 days) and subsequently treated with 20 μg/mL α-methyl-D-mannopyranosid to block residual ConA activity.

In vitro–propagated alloreactive T-cell lines (H-2b anti–H-2d) were generated by weekly restimulation with irradiated spleen cells and ConA SN, as described. For analysis of intracellular gzmA/gzmB expression, alloreactive T-cell lines (∼ 85%–90% CD8+) of B6, gzmA−/−, and gzmB−/− mice obtained after the third in vitro stimulation were used.
Isolation of PBMCs and PMNs from human blood

Blood was taken from 4 healthy donors (2 men, 2 women) and 4 patients. The patients were from the University Hospital Heidelberg, Germany. Patient 1 was a 28-year-old woman with Still syndrome, rheumatoid arthritis, acute inflammatory episode with fever, joint pains, and high C-reactive protein (CRP; 150 mg/L). Patient 2 was a 70-year-old woman with chronic lymphatic leukemia, fever, and tumor progression (CRP; 30 mg/L). Patient 3 was a 81-year-old man with B-non-Hodgkin-lymphoma with fever (no CRP increase, no infection). Patient 4 was a 56-year-old woman with acute progressive myeloid leukemia with fever, pneumonia, and sepsis (CRP, 86 mg/L). Cells from one healthy donor were used for cytospin analysis, cells of the other 3 donors were used for fluorescence-activated cell sorting (FACS) staining.

Blood was taken by venous puncture using EDTA (ethylenediaminetetraacetic acid)-coated tubes (Sarstedt, Nürnberg, Germany), and cells were separated on Ficoll Paque gradient (Amersham Pharmacia Biotech, Freiburg, Germany) according to established protocols. The interphase containing the peripheral blood mononuclear cells (PBMCs) was removed and stored on ice. The pellet containing the PMNs and the erythrocytes were mixed with phosphate-buffered saline (PBS) and 3% Dextran (Amersham Pharmacia Biotech; diluted in PBS) and was added 1:1 and mixed. After sedimentation of erythrocytes at room temperature for 20 minutes the supernatant (SN) was removed, cells were resuspended in buffer containing 155 mM NH4Cl/10 mM KH2CO3/0.1 mM EDTA in H2O, and incubated for 15 minutes on ice to lyse resting erythrocytes. After centrifugation the SN was discarded, and the pellet containing neutrophils was resuspended in RPMI.

Approval was obtained from the Medizinische Klinik 5, University Heidelberg institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki.

Flow cytometry

All monoclonal antibodies (mAbs) for surface staining, except anti-(α) CD4 mAb, oCD8 mAb (both from Coulter, Marnes-la-Coquette, France; used for the healthy individuals 2 and 3), and oCD66b mAb (Coulter, Marnes-la-Coquette, France) were obtained from BD Pharmingen, Heidelberg, Germany. Splenocytes, mouse granulocytes, and human PBMCs and PMNs were washed with FACS-washing buffer (PBS, 5% FCS, 0.1% NaN3) as previously described31 and incubated with 10 μL mAbs for 20 minutes. oMouse (m) mAb used were fluorescein isothiocyanate (FITC)– and phycoerythrin (PE)–labeled oRX220 (clone RA3-682), PE-labeled oCD8 (clone 53-67), FITC- and PE-labeled oGr-1 (clone RB6-8C5), FITC-labeled oMac1 (clone M1/70), FITC- and PE-labeled oNK1.1 (clone PK136), and FITC- and PE-labeled oTh1.2 (clone 53-2.1). All fluorescence-conjugated omAbs were diluted in an oF receptor antibody (clone 2.4G2), 1:50 for FITC-labeled mAb and 1:100 for PE-labeled mAb. oHuman (hu) mAbs used were FITC-labeled oCD4 (clone RPA-T4), PE-labeled oCD8 (clone RPA-T6), and FITC-labeled oCD66b (clone 80H3; Coulter) for all patients and the healthy individual 1; FITC-labeled oCD4 (Coulter), PE-labeled oCD8 (Coulter), and FITC-labeled oCD66b (clone GI0FS) for the healthy individuals 2 and 3. The ohu mAbs were diluted in washing buffer. Stained cells were washed twice and fixed in 100 μL PBS containing 1% paraformaldehyde (PFA), examined in a FACSCalibur (Becton Dickinson), and analyzed with CellQuest software (Becton Dickinson, Franklin Lakes, NJ). For analysis of intracellular gzmA and gzmB, 1.3E6SN, EL4.F15, splenocytes from LCMV-immune mice (day 8 after infection), mouse granulocytes, and human PBMCs or PMNs were stained with surface markers as described in “Flow cytometry” and then fixed with 100 μL PBS containing 2.5% PFA for 15 minutes at 4°C. Subsequently, cells were incubated in 100 μL permeabilizing buffer (PBS, 5% FCS, 0.1% NaN3, 0.1% Saponin; Roth, Karlsruhe, Germany) for 10 minutes at 4°C, then stained for 45 minutes at 4°C with 40 μL rabbit anti-CD3 (BD Pharmingen) for gzmB were used. Stained cells were washed twice in permeabilizing buffer, fixed in 100 μL PBS containing 1% PFA, examined in a FACSCalibur, and analyzed with CellQuest software.

For cell sorting, the enriched granulocytes (BM, day 9, G-CSF) were washed with FACS-washing buffer and incubated with 50 to 100 μL FITC/PE-labeled aGr-1 mAb (diluted 1:500; 1:100 in an Fc receptor SN [clone 2.4G2]) 20 minutes at 4°C. Cells were washed in PBS, homogenized with a separation filter (Miltenyi Biotec, Bergisch Gladbach, Germany), and positively sorted with the cell sorter MoFlo (Cytomation, Freiburg, Germany).

Enzymatic assays

Cell lysates from 1.3E6SN, EL4.F15, positively sorted (oCD8 Microbeads; Miltenyi, Biotec; according to manufacturers instruction) CD8+ LCMV-immune splenocytes (day 8 after infection) and from in vitro–enriched granulocytes (BM, day 9, G-CSF) were prepared in 0.1% Triton X-100/10 mM Tris [tris(hydroxymethyl)aminomethane]–HCl in H2O, pH 7.5 (1 hour on ice) and tested as described31 on the following substrates: Ac-Ile-Glu-Pro-Asp-pNA (gzmB; Bachem, Weil am Rhein, Germany), Suc-Phe-Pro-Phe-pNA (CathepsinG; Bachem). The absorbance was examined in a spectrophotometer SpectraMax 190 (Molecular Devices, Ismaning/Munich, Germany; wavelength 405 nm/690 nm) and analyzed with SOFTMax Pro software (Molecular Devices).
Electron microscopy

In vitro–enriched granulocytes and 1.3E6SN cells were fixed in 4% PFA in 0.2 M PHEM buffer (240 mM PIPIES [Piperazine-1,4-bis(2-ethanesulfonic acid)]/100 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]/40 mM EGTA [ethyleneglycoltetraacetic acid]/8 mM MgCl2, pH 6.), stored in 0.5% PFA in 0.1 M PHEM buffer, and then processed for ultrathin cryosectioning as previously described.12 Cryosections (45 nm) cut at 125°C using diamond knives (Drukker, Cuijk, The Netherlands) in an ultracryomicrotome (Leica Aktiengesellschaft, Vienna, Austria) were transferred with a mixture of sucrose and methylcellulose onto formvar-coated copper grids.33 The grids were placed on 35-mm Petri dishes containing 2% gelatin. For double immunolabeling, a published procedure34 was followed.

Western blot analysis

Intracellular gzmA was determined in cell lysates from magnetic-activated cell sorting (MACS)–sorted LCMV-immune CD8⁺ cells of B6, gzmA⁻/⁻, and gzmB⁻/⁻ mice by Western blotting under nonreducing conditions. The omgzmA mAb (clone 7.1) was generated as described,30 the omβ-actin mAb (clone C-11) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Then the blot was stained with peroxidase-conjugated secondary antibodies, goat or rabbit IgG, and then visualized by chemiluminescence (ECL) from Pierce (Rockford, IL) was used for detection. Purified gzmA protein (50 μg/mL)35 was used as positive control.

Probing for mRNA transcription

Total RNA was extracted from up to 5 × 10⁶ cells, using the QIashredder spin columns, the RNaseasy Mini Kit, and the RNase-free DNase Kit (all from Qiagen, Hilden, Germany) according to the manufacturer’s instructions. mRNA was transcribed by incubating total RNA with random hexamer primer (660 ng/μL; Pharmacia, Freiburg, Germany), RNasin inhibitor (20 U; Promega, Madison, WI), deoxyribonucleoside triphosphate (0.5 mM; Qbiogene, Heidelberg, Germany), and Omnisept reverse transcriptase (4 U; Qiagen) as advised by the manufacturer. Reverse transcription was performed in a Thermocycler PTC-200 (MJ Research, Waltham, MA). The transcription reaction profile was as follows: 37°C for 60 minutes and 70°C for 10 minutes. The resulting cDNA was used as a template for RT-PCR hypoxanthin:guanine phosphoribosyltransferase (Hprt1), Prf1, Gzma, and Gzmb amplification. Polymerase chain reaction (PCR) for Hprt1, Prf1, Gzma, and Gzmb was carried out in a Thermocycler PTC-200 as described in “Probing for mRNA transcription.” The PCR reaction profile for Hprt1, Prf1, Gzma, and Gzmb was as follows: 1 cycle at 94°C for 2.5 minutes as an initial denaturation step; then denaturation at 94°C for 20 seconds; annealing at 56°C for 20 seconds for Hprt1 or 55°C for 20 seconds for Prf1, Gzma, and Gzmb; extension at 72°C for 20 seconds for Hprt1 or 72°C for 30 seconds for Prf1, Gzma, and Gzmb (35 cycles); followed by further incubation for 5 minutes at 72°C (1 cycle). The PCR reaction profile for the cathepsin G gene (Ctsg) was as follows: 1 cycle at 95°C for 1 minute as an initial denaturation step, then denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 70°C for 3 minutes (35 cycles), followed by further incubation for 8 minutes at 70°C. The following primers were used for amplification: Hprt1-specific primers, sense (5′-GCT GGT GAA AAG GAC CTC TCT-3′) and antisense (5′-CAC AGG ACT AGA ACA CCT GC-3′); Hprt1 primers, sense (5′-GAG CCC CTG CAC ACA TTAT CTG GA-3′) and antisense (5′-AAC TTC TCA AAG AGG ATC ATG TCT-3′); Hprt1 primers, sense (5′-GAG CCT GGA CTA CAA CTT GGA CGG-3′) and antisense (5′-ATT GCC GCA GCA GTC CTT ACC A-3′); Hprt1 primers, sense (5′-ATT GCC GCA GCA GTC CTT ACC A-3′) and antisense (5′-ATT GCC GCA GCA GTC CTT ACC A-3′); Hprt1 primers, sense (5′-GAG CCC CTG CAC ACA TTAT CTG GA-3′) and antisense (5′-AAC TTC TCA AAG AGG ATC ATG TCT-3′); Hprt1 primers, sense (5′-GAG CCT GGA CTA CAA CTT GGA CGG-3′) and antisense (5′-ATT GCC GCA GCA GTC CTT ACC A-3′) amplifying a 249-base pair (bp) segment; Prf1-specific primers, sense (5′-GAC TCT GTC TGG TCT TGG ATC GCT-3′) and antisense (5′-ATG GGA ACT CTC CTC CTA CTC C-3′) amplifying a 380-bp segment; Gzma-specific primers, sense (5′-GGG GAT CTA CAA CTT GGA CGG-3′) and antisense (5′-ATT GCC GCA GCA GTC CTT ACC A-3′) amplifying a 291-bp segment; Gzmb-specific primers sense (5′-TCA GGC TGC TGA TCT TGG ATC GCT-3′) and antisense (5′-ATG GGA ACT CTC CTC CTA CTC C-3′) amplifying a 135-bp segment; Ctsg-specific primers, sense (5′-CAT CCA AAT GGG AGA GAG-3′) and antisense (5′-CAT CCA AAT GGG AGA  TGA-3′) amplifying a 272-bp segment. The primers for Hprt1, Gzma, Gzmb, and Prf1 were synthesized by Hermann (Freiburg, Germany), for Ctsg by Thermo Electron (Ulm, Germany). The PCR products were analyzed by gel electrophoresis (2% agarose), stained with ethidium bromide, and analyzed using an UV-transluminator (Vilber Lourmat, New Ark, NJ). As size markers, 1-kilobase (kb) DNA marker was used (BioLabs, Frankfurt/Main, Germany).

Results

Phenotype of BM-derived and in vitro–propagated mouse granulocytes

Granulocytes were generated from mouse BM by in vitro cultivation with G-CSF (day 9) and further enriched by FACS sorting with mAb to TAG CTG CAC-3’) amplifying a 291-bp segment; the primers for Hprt1, Gzma, Gzmb, and Prf1 were synthesized by Hermann (Freiburg, Germany), for Ctsg by Thermo Electron (Ulm, Germany). The PCR products were analyzed by gel electrophoresis (2% agarose), stained with ethidium bromide, and analyzed using an UV-transluminator (Vilber Lourmat, New Ark, NJ). As size markers, 1-kilobase (kb) DNA marker was used (BioLabs, Frankfurt/Main, Germany).

Figure 1. Phenotypic analysis of in vitro–generated and positively selected granulocytes from B6 BM cells. (A) B6 granulocytes were generated from B6 BM cells by cultivation with G-CSF for 9 days. Enriched granulocytes were further enriched by positive selection via FACS using umGr-1 mAb. All 3 populations were stained with mAb specific for Gr-1, NK1.1, B220, Thyl.2, and Mac-1 and analyzed with the FACSCalibur. SSC indicates side scatter. (B) Freshly isolated B6 BM cells and in vitro–propagated and FACS-sorted (umGr-1 mAb) granulocytes were centrifuged on microscope slides and stained with May–Grünewald/Giemsa as described in “Materials and methods.” Arrows indicate polymorphonuclear cells; arrowheads, mononuclear cells. (C) Not activated B6 spleen cells and B6 spleen cells cultured in vitro in the presence of either ConA (5 μg/mL) or ConA/ConA SN (5 μg/mL; 10% final) were subjected to FACS analysis (FACSCalibur), using mAb to Gr-1, NK1.1, B220, and/or Thyl.2. FSC indicates forward scatter. Numbers in graphs indicate the percentages of gated cells.
Gr-1. This protocol leads to a highly purified granulocyte population (Figure 1A): freshly isolated BM cells consisted of approximately 50% Gr-1**/H11001**, 3% NK1.1**/H11001**, 44% B220**/H11001**, 6% Thy1.2**/H11001**, and 48% Mac-1**/H11001** cells (Figure 1Ai), with approximately 94% of Gr-1**/H11001** cells staining in addition for Mac-1 and approximately 2% to 3% for NK1.1, B220, or Thy1.2. The cell population propagated in G-CSF consisted of greater than 93% Gr-1**/H11001** Mac-1**/H11001** (Figure 1Aii) and on further enrichment by FACS of greater than 99% Gr-1**/H11001** Mac-1**/H11001** with only a minority (<1%) also staining for NK1.1, B220, or Thy1.2. Cytospin analysis revealed that the majority of Gr-1–sorted cells are granulocytes (Figure 1B, BM-derived versus in vitro–propagated and FACS-sorted cells). The majority of granulocytes generated under these conditions are Gr-1**/H11001** Mac-1**/H11001**, with only few, if any, expressing markers for NK, B, and T cells. However, the concern that the Gr-1 marker is not restricted to mouse granulocytes36,37 (and information provided by manufacturer) is supported by phenotypic data obtained with ex vivo–derived or in vitro mitogen-stimulated spleen cells. From the approximately 7% of Gr-1**/H11001** cells detected in ex vivo–stimulated spleen cells (day 8 after infection), approximately 16% to 20% also stained for NK1.1 or B220 and greater than 75% for Thy1.2 (Figure 1C). Furthermore, spleen cell populations sensitized with ConA in vitro to enrich for activated T cells contained approximately 15% Gr-1**/H11001** cells, of which approximately 2%, approximately 35%, and approximately 60% also stained for NK1.1, B220, and Thy1.2, respectively. When the same cell population was cultured in the presence of ConA plus ConA SN, supporting polyclonal T-cell expansion, from the approximately 32% Gr-1**/H11001** spleen cells recovered, approximately 17% and approximately 90% also stained for B220 and

**Figure 2.** Mouse Gr-1**/H11001** granulocytes do not express gzmA or gzmB intracellularly. All indicated cell populations were stained, intracellularly, with either rabbit αgzmA IS followed by FITC-labeled rabbit IgG (1:500) or with APC-labeled αgzmB mAb; rabbit IgG and mouse IgG were used as isotype control, as described in “Flow cytometry.” Analysis was performed using FACSCompB. (A) 1.3E6SN, EL4.F15:αgzmA IS (1:1000), αgzmB mAb (1:100). (B) Ex vivo–derived LCMV-immune (day 8 after infection) spleen cells from B6, gzmA**−/−**, and gzmB**−/−** mice were stained with αCD8 mAb (1:100) and, subsequently, with either αgzmA IS (1:100), or αgzmB mAb (1:1000) mAb for intracellular expression of gzs and analyzed as described in “Flow cytometry.” (Bi) In vitro–propagated alloreactive (H-2b anti–H-2d; third stimulation) T cells from B6, gzmA**−/−**, and gzmB**−/−** mice were treated as in panel Bi. (Ci) In vitro–generated granulocytes (BM cells, day 9, G-CSF) from either B6, gzmA**−/−**, or gzmB**−/−** mice were stained with αGr-1 mAb and, subsequently, with either αgzmA IS (1:100), or αgzmB mAb (1:10) mAb for intracellular expression of gzs, as described in “Flow cytometry.” (C) Alternatively, in vitro–enriched B6 granulocytes were sensitized to either LPS (1 μg/mL) or Lip-OspA (10 μg/mL) for 8 hours, prior to phenotypic analysis, using αGr-1 mAb and either αgzmA IS (1:100) or αgzmB mAb (1:10) mAb for intracellular expression of gzs as described above. Numbers in graphs indicate the percentages of gated cells.

**Figure 3.** In vitro–propagated and zymosan-activated B6 granulocytes do not express gzmA or gzmB. In vitro–generated B6 granulocytes were incubated with zymosan (50 μg/mL; 10^5 cells; 90 minutes) and subsequently analyzed following staining with αGr-1 mAb and either αgzmA IS (1:100) followed by FITC-labeled rabbit IgG (1:500) or αgzmB mAb (1:10) as described in “Flow cytometry”; rabbit IgG and mouse IgG were used as isotype control, as described in “Materials and methods.” Numbers in graphs indicate the percentages of gated cells.
Thy1.2, respectively. These observations are critical for a meaningful evaluation of data regarding expression of gzms and perf in mouse granulocytes and underscore the necessity for a correlative phenotypic analysis of the Gr-1^+ cells, in particular for NK and T-cell–associated cell surface markers, so as to exclude gzm/perf-expressing contaminating cells.

In vitro–derived mouse granulocytes do not express gzmA or gzmB intracellularly

In vitro–enriched BM-derived granulocytes were analyzed by FACS for intracellular expression of gzmA and gzmB by using omgzmA IS or ohuugzmB mAb, the latter is known to crossreact with mouse gzmB. The specificity of both antibodies for the respective gzm was verified by using the gzmA/gzmB-positive CTL line, 1.3E6SN, and the gzmA/gzmB-negative thymoma line, EL4.F15, as well as ex vivo–derived LCMV-immune spleen cells (day 8 after infection) from B6, gzmA^−/−, and gzmB^−/− mice. As expected, only 1.3E6SN, but not EL4.F15, cells stained with omgzmA IS and ohuugzmB mAb (Figure 2A). In addition, omgzmA IS stained a fraction of CD8^+ T cells from LCMV-immune B6 and gzmB^−/− but not gzmA^−/− mice and, conversely, ohuugzmB mAb stained a fraction of CD8^+ T cells from LCMV-immune B6 and gzmA^−/− but not gzmB^−/− mice (Figure 2Bi). The lower intensities seen with T cells stained for gzmB compared with gzmA may be due to the fact that a monospecific mAb rather than a polyspecific IS was used in the former case. However, using in vitro–propagated alloreactive T-cell lines, known to express much higher levels of both gzms, it is clearly shown that both, omgzmA IS or ohuugzmB mAb, are specific and differentially stain the respective cell populations from B6, gzmA^−/−, and gzmB^−/− mice (Figure 2Bii). Most importantly, none of the in vitro–enriched granulocyte populations from B6, gzmA^−/−, and gzmB^−/− mice stained with omgzmA IS and/or ohuugzmB mAb (Figure 2Ci), even after earlier activation with either LPS or the lipoprotein Lip-OspA (Figure 2Cii; only shown for B6 granulocytes). Similar results were obtained with in vitro–enriched B6 granulocytes previously stimulated (90 minutes) with zymosan (Figure 3). The combined data indicate that in vitro–generated mouse granulocytes do not express gzmA and gzmB, even after activation and expansion.

The data obtained by FACS analysis were verified by IEM (Figure 4, only shown for gzmA; omgzmA mAb; 7.1). Only specimens of 1.3E6SN but not EL4.F15 (data not shown) were heavily stained with omgzmA mAb (7.1) in particular in cytoplasmic granules. In contrast, no or only few grains were seen in sections of in vitro–enriched mouse neutrophils or eosinophils. The specificity of the omgzmA mAb (7.1) has been described before and was verified again by WB analysis, using cell lysates from MACS-sorted CD8^+ LCMV-immune cells from B6, gzmA^−/−, and gzmB^−/− mice as well as purified gzmA (Figure 4B).

Cell lysates from in vitro–enriched mouse granulocytes as well as from 1.3E6SN and EL4.F15 cell lines were tested for proteolytic activities of gzmB and cathepsin G, a granulocyte-specific protease, by using appropriate chromogenic substrates. 1.3E6SN lysates contained gzmB– (19 U/10^6 cells) but not cathepsin

![Figure 4. Ultrastructural localization of gzmA in 1.3E6SN but not in B6 neutrophils and eosinophils by immunogold electron microscopy.](image)
G–related activity (0 U/10⁶ cells), whereas EL4.F15 lysates did not harbor either of the 2 enzymatic activities (0 U/10⁶ cells). In addition, lysates from ex vivo–derived and enriched LCMV-immune CD8⁺ T cells from B6 (3 U/10⁶ cells) but not gzmAxB⁻/⁻ mice (0 U/10⁶ cells) expressed gzmB activity, although at much lower levels (3 U versus 19 U/10⁶ cells, as expected from previous studies; M.M.S. unpublished data, February 2004) when compared with the long-term cultured T-cell line, L3E6SN. Both ex vivo–derived T-cell populations expressed low but significant levels of cathespin G or cathespin G–like activity (1.3 U/10⁶ cells). In contrast, lysates from in vitro–enriched granulocytes, unstimulated or LPS-stimulated, expressed only cathespin G–related (17 U/10⁶ cells), but not gzmB-related (0 U/10⁶ cells), enzymatic activities.

**Ex vivo–derived mouse granulocytes do not express gzmB**

B6 mice were injected intraperitoneally with LPS or infected intraperitoneally with *L. monocytogenes*. Twenty hours and 48 hours after infection, respectively, cells were recovered from the peritoneal cavity and analyzed by FACS for surface phenotype and the expression of intracellular gzmB. Approximately 45% of cells from LPS-challenged mice stained Gr-1⁻, although with differential intensity. Only approximately 3% to 4% of cells expressing Gr-1 were also positive for NK1.1 and Thy1.2, and mainly in the Gr-1⁻/H11011 cell population. When tested for the expression of gzmB, only few Gr-1⁺ but significantly more Gr-1⁻/H11011 cells stained for gzmB. In contrast to Gr-1⁺ cells, a much higher proportion from Thy1.2⁺ cells (~13%, total) and NK1.1⁺ cells (~3%, total), expressed gzmB (~10% and 20%, respectively) (Figure 5Ai). As expected, none of the ex vivo–derived LPS-sensitized Gr-1⁺ cells from gzmB⁻/⁻ mice expressed gzmB (Figure 5Aii).

The peritoneal cell population from *L. monocytogenes*–infected mice contained approximately 34% Gr-1⁺ cells, with approximately 74% and approximately 6% being also positive for NK1.1 and Thy1.2, respectively (Figure 5B). However, none of the Gr-1⁺ cells expressed gzmB, whereas approximately 4% of the NK1.1⁺ cells (~43%, total) and approximately 9% of the Thy1.2⁺ cells (~22%, total) were positive for gzmB. Similar results were obtained for gzmA (data not shown). Together, these data indicate that ex vivo–derived Gr-1⁺ mouse granulocytes do not express gzmA and gzmB, even during infection or on challenge with pathogen-derived agents (LPS).

**Mouse BM-derived granulocytes do not express transcripts for gzmA, gzmB, and perf**

In vitro–enriched and FACS-sorted Gr-1⁺ BM-derived granulocyte populations from B6 and gzmB⁻/⁻ mice were assayed by RT-PCR for the expression of *Gzma, Gzmb*, and *Prf1*–specific transcripts. None of the 2 cell populations expressed *Gzma, Gzmb*, or *Prf1*–specific mRNA, irrespective of whether they were stimulated with LPS or left untreated (Figure 6A). As expected, mRNA from L3E6SN, but not EL4.F15, cells expressed transcripts for *Gzma, Gzmb*, and *Prf1*. In contrast, when in vitro–enriched but unsorted granulocyte populations were used, *Gzmb*–specific transcripts were detectable, the level of which was enhanced on pretreatment with LPS (Figure 6B; lanes 2 and 3). No *Gzma* or *Prf1*–specific transcripts were seen in in vitro–generated and FACS-enriched Gr-1⁺ cells (Figure 6B; lane 1). In contrast, specific bands for the granulocyte–specific *Ctsa* were only detected in samples of granulocytes, but not in L3E6SN and EL4.F15 (Figure 6B). Similar results were obtained when mRNA preparations were subjected to quantitative RT-PCR using the light cycler system (data not shown).

In summary, these results clearly demonstrate that mouse granulocytes, even after in vitro activation or on encounter with pathogens in vivo, do not express gzmA, gzmB, or perf. The present data obtained by cytologic, intracellular flow cytometric, enzymatic, IEM, and semiquantitative RT-PCR analyses, support earlier studies, indicating that in mice the 3 cytolytic effector molecules, gzmA, gzmB, and perf, are produced mainly by NK and CTL,¹²,¹⁵,¹²⁸ and to some extend also by myeloid cells,¹⁴ but not by other cells of the lymphoid or myeloid lineages.

**Human granulocytes from patients with various disease symptoms and healthy individuals do not express gzmB**

In light of the published conflicting results on the expression of gzmA, gzmB, and perf in human neutrophils,¹⁸,¹⁹,²⁶,²⁷ we tested a panel of PBMC and PMN populations from 4 healthy individuals (cells of 1 individual were used for cytospin, cells of the other 3 individuals were obtained when mRNA preparations were subjected to quantitative RT-PCR using the light cycler system (data not shown).
were stained intracellularly) and 4 patients with either an autoimmune disorder or B-cell chronic lymphatic leukemia, B-cell non-Hodgkin lymphoma, or acute myelogenous leukemia. PBMCs and PMNs were separated by standard methods and subsequently applied to cytospin and FACS analysis. Both cell populations were stained for surface markers CD4, CD8, and CD66b as well as intracellularly for gzmB using mAbs (GB12). PBMCs from the healthy individual consisted of approximately 44% CD4+ and approximately 20% CD8+ T cells, whereas PBMCs from the 4 patients had CD4+/CD8+ T-cell ratios, ranging from approximately 74%:6% (patient 4) to approximately 30%:30% (patient 3), respectively (Figure 7A). PBMCs from all individuals consisted of greater than 98% CD66b+ cells with no contaminants of CD4+ or CD8+ T cells. The polymorphonuclear nature of CD66b-enriched cells of a healthy individual was also revealed by cytospin analysis in an independent experiment (Figure 7B). When tested for the intracellular expression of gzmB, a significant number of CD8+ PBMCs (ranging from 16% to 71%) but also a fraction of CD4+ PBMCs (ranging from 0.5% to 17%) stained positive for the enzyme. In contrast, none of the PBMC populations from the healthy individuals expressed gzmB.

Our findings that only human PBMCs, in particular fractions of CD4+ and CD8+ T cells, but none of the enriched PMN populations from the healthy individuals and 4 patients with various disease symptoms expressed gzmB are in line with 2 recent studies, but challenge 2 others. Final proof whether human PMNs are able to produce gzms and/or perf under certain conditions is still pending. A cautionary note regarding proof of principle needs to be added to avoid erroneous conclusions. This pertains, in particular, to enrichment of granulocytes, via mAb. We found that the majority of cytologically defined mouse granulocytes are Gr-1+, but that they may differentially express NK- and T-cell–specific markers, depending on their activation state. However, fractions of ex vivo–derived spleen cells or spleen cells, previously activated in vitro with ConA or ConA plus ConA SN were double positive for either NK1.1 and Gr-1 or Thy1.2 and Gr-1 (Figure 1C).

Phenotype analysis of human PBMCs and PMNs led to similar observations. Thus, highly enriched PBMC populations contained small fractions of CD56+ or CD3+ cells, which also expressed CD66b and, vice versa, highly enriched PMNs (> 95% CD66b) contained a small fraction of cells, which expressed either CD56 or CD3.

<table>
<thead>
<tr>
<th>B6</th>
<th>gzmA+/-</th>
<th>gzmB+/+</th>
<th>perf</th>
<th>HPRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>sorted</td>
<td>+ LPS</td>
<td>4</td>
<td>+ LPS</td>
</tr>
<tr>
<td>2</td>
<td>sorted</td>
<td>+ LPS</td>
<td>5</td>
<td>sorted</td>
</tr>
<tr>
<td>3</td>
<td>not stimulated</td>
<td>+ LPS</td>
<td>7</td>
<td>1.3E6SN</td>
</tr>
<tr>
<td>4</td>
<td>not stimulated</td>
<td>+ LPS</td>
<td>8</td>
<td>1.3E6SN</td>
</tr>
<tr>
<td>5</td>
<td>not stimulated</td>
<td>+ LPS</td>
<td>9</td>
<td>1.3E6SN</td>
</tr>
<tr>
<td>6</td>
<td>not stimulated</td>
<td>+ LPS</td>
<td>10</td>
<td>1.3E6SN</td>
</tr>
<tr>
<td>7</td>
<td>not stimulated</td>
<td>+ LPS</td>
<td>11</td>
<td>1.3E6SN</td>
</tr>
<tr>
<td>8</td>
<td>not stimulated</td>
<td>+ LPS</td>
<td>12</td>
<td>1.3E6SN</td>
</tr>
<tr>
<td>9</td>
<td>not stimulated</td>
<td>+ LPS</td>
<td>13</td>
<td>1.3E6SN</td>
</tr>
<tr>
<td>10</td>
<td>not stimulated</td>
<td>+ LPS</td>
<td>14</td>
<td>1.3E6SN</td>
</tr>
</tbody>
</table>

Figure 6. In vitro–generated and FACS-sorted Gr-1+ mouse granulocytes do not express transcripts for Gzma, Gzmb, and Prf1. (A) mRNA was isolated from in vitro–generated and FACS-enriched (Gr-1+) unstimulated or LPS-sensitized (1 μg/mL; 8 hours) B6 and gzmB+/+ granulocytes. Gzma, Gzmb, Prf1, or Hprt1 transcripts were analyzed with specific primer pairs for Gzma, Gzmb, Prf1, and Hprt1 by RT-PCR. As positive and negative controls, 1.3E6SN (lanes 8, 10), and EL4.F15 (lanes 9, 11) were used. Size of amplified fragments was as follows: Gzma, 291 bp; Gzmb, 135 bp; Prf1, 380 bp; and Hprt1, 249 bp. (B) mRNA was isolated from in vitro–generated unstimulated or LPS-sensitized (1 μg/mL; 8 hours) (lanes 2 and 6,7, respectively) or in addition FACS-enriched (Gr-1+) (lanes 1 and 5) B6, and gzmA+/- granulocytes and analyzed as described in “Probing for mRNA transcription,” with specific primer pairs for Gzma, Gzmb, Prf1, and Hprt1 by RT-PCR. Size of amplified Gztg fragments was as follows: 272 bp; as positive and negative controls, 1.3E6SN (lanes 8, 10), and EL4.F15 (lanes 9, 11) were used.

Figure 7. GzmB is only expressed in fractions of CD4+ and CD8+ PBMCs, but not in CD66b+CD4-CD8- PMNs of 4 patients and 3 healthy individuals. (A) PBMCs and PMNs were isolated from 4 patients (1-4) and 3 healthy individuals and analyzed (FACSCalibur) by staining with either mAb to CD4 and CD8 (PBMCs and PMNs), or mAb to CD66b and CD4+ of CD8+ PBMCs (PMNs), and subsequently with ηhugzmB mAb (1:50) as described in “Materials and methods.” Numbers in graphs indicate the percentages of gated cells. (B) Similarly prepared PBMCs and PMNs from another individual showing a comparable phenotype as the healthy individuals mentioned in panel A were centrifuged on microscope slides and stained with May-Grunwald/Giemsa as described in “Materials and methods.” Arrowheads indicate mononuclear cells; arrows, polymorphonuclear leukocytes.
CD3 in addition to CD66b. This also holds true for another marker, CD15, mainly associated with human PMNs,18,26 was applied (data not shown). Thus, granulocyte populations/PMNs selected solely by Gr-1 (mouse) or CD66b/CD15 (humans) may either contain granulocyte/neutrophil subpopulations expressing NK/T-cell markers or may be contaminated with cells from other lineages, in particular NK, NKT (natural killer T), and T cells, which are the main producers of gzms and perf.

Discussion

The data presented here provide strong supportive evidence that mouse granulocytes do not express gzmA, gzmB, and perf under physiologic or pathophysiologic conditions. This is supported by the lack of Pyrf- and Gzna-Gznb-specific mRNA and the respective proteins in in vitro–generated or ex vivo–derived granulocyte populations, as revealed by cytolytic, intracellular flow cytometry, EIM, and RT-PCR analyses. Gzms and perf could not be detected in granulocytes previously activated either in vitro and/or in vivo, by L-monoyctogony or by pathogen-derived material, such as LPS, Lip-OsPa, or the yeast product, zymosan. This indicates that mouse granulocytes do not use the known NK/CTL-associated cytotoxic effectors molecules, gzmA, gzmB, and perf, as defense against pathogens and tumors. Our finding that fractions of CD4+ and CD8+ T cells, but not highly purified PMNs from 4 patients and 3 healthy individuals, expressed gzmB is in support of recent work from 2 independent groups,26,27 but contrasts with other related studies.18,19 Thus, it remains to be verified whether the discrepant results obtained using human PMNs are merely due to technical differences and whether mouse and/or human PMNs or fractions thereof are able to produce gzms and/or perf, but only under stringent conditions unlike the expression in NK/CTLs.

Acknowledgments

We thank Jero Calafat from The Netherlands Cancer Institute, Division of Cell Biology, Amsterdam, for performing the IEM experiments and for her critical comments, and Verena Weber, Claudia Luckner, Christiane Brenner, and Ayun Ekcikler for expert technical assistance.

References

Quiescent and activated mouse granulocytes do not express granzyme A and B or perforin: similarities or differences with human polymorphonuclear leukocytes?

Praxedis Martin, Reinhard Wallich, Julian Pardo, Arno Müllbacher, Markus Munder, Manuel Modolell and Markus M. Simon