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A Novel and Divergent Role of Granzyme A and B in Resistance to Helminth Infection

Wiebke Hartmann,† Benjamin J. Marsland,‡ Benjamin Otto,§ Jens Urny,¶ Bernhard Fleischer,* and Simone Korten*†‡

Granzyme (gzm) A and B, proteases of NK cells and T killer cells, mediate cell death, but also cleave extracellular matrices, inactivate intracellular pathogens, and induce cytokines. Moreover, macrophages, TH2 cells, regulatory T cells, mast cells, and B cells can express gzm. We recently reported gzm induction in human filarial infection. In this study, we show that in rodent filarial infection with Litomosoides sigmodontis, worm loads were significantly reduced in gzmA×B and gzmB knockout mice during the whole course of infection, but enhanced only early in gzmA knockout compared with wild-type mice. GzmA/B deficiency was associated with a defense-promoting Th2 cytokine and Ab shift, enhanced early inflammatory gene expression, and a trend of reduced alternatively activated macrophage induction, whereas gzmA deficiency was linked with reduced inflammation and a trend toward increased alternatively activated macrophages. This suggests a novel and divergent role for gzmA in helminth infection, with gzmA contributing to resistance and gzmB promoting susceptibility. The Journal of Immunology, 2011, 186: 2472–2481.

Granzyme (gzm) A and B are major cytotoxic serine proteases of NK cells and cytotoxic T cells, well known to play a key role in killing tumor cells, virally infected cells, or other intracellular pathogens (1). GzmB expression is increased in rat hookworm infection (2), but the mechanisms are hardly defined. GzmA and B are best characterized from the 5 human granzymes (A, B, H, K, and M) and the 11 murine gzm (A–G, K–N) (3). GzmA and B were long thought to mainly induce apoptosis in target cells via different signaling cascades (1), but recent evidence reveals induction of necrosis by gzmA (4) and noncytotoxic intra- and extracellular proteolytic activities: gzmA and B inactivate intracellular viruses, cleave surface receptors and extracellular matrix proteins such as collagen IV facilitating leukocyte migration, and induce cell death by cellular detachment (5). Recently, gzmA/B expression was discovered in mast cells (6), macrophages (7), regulatory T cells (Treg) (8), and human B cells (9). These studies point at novel potent immunoregulatory and proinflammatory roles such as gzmB-mediated effector cell killing by Treg and gzmA-induced production of proinflammatory cytokines and phagocytosis by monocytes (4, 10).

Recently, we reported a new role for granzymes in human helminth infection (11): gzmA/B expression in the local response to Onchocerca volvulus was associated with immunosuppression, Treg, and Ag release from treatment-induced dying filariae. Filarial nematodes like O. volvulus, Wuchereria, and Brugia spp. cause considerable morbidity in tropical countries. The aim of the current study was to elucidate gzmA and B-dependent mechanisms in vivo in helminth infection employing the murine infection with the rodent filaria Litomosoides sigmodontis in resistant B6 mice (12). Immature adult worms are degraded in contrast to fully patent (microfilaremic) susceptible BALB/c mice. In the complex life cycle, infectious third-stage larvae (L3) are transmitted by tropical mites (Ornithonyssus bacoti), migrate via the lymphatics and lung to the pleural cavity within 3–5 d, and molt at day 8/9 postinfection (p.i.) into fourth-stage larvae (L4). After the fourth molt around days 26–29 p.i., young adult worms are degraded by granuloma formation within 1 to 2 wk. Known mechanisms of B6 resistance involve a mixed Th1/Th2 response with IL-4, proinflammatory cytokines, strong pleural leukocytosis, eotaxin, and IL-5 in vaccination-induced protection (12). In BALB/c mice, Treg suppress defense, whereas IL-4, IL-5, IL-6, INF-γ, B, and CD4+ T cells promote it (13). Resistance is also influenced by the infectious dose and immunomodulatory capacities of the worms (13, 14).

In the current study, we analyzed whether gzmA and B deficiency of resistant wt B6 mice affects worm development/survival and host immune mechanisms of resistance in natural infections with L. sigmodontis. This was based on reports that human gzmB critically affects growth and molting of Caenorhabditis elegans in vitro (15) and that NK cells provide unknown immune signals for early Brugia development in mice (16), but also for late defense against adult L. sigmodontis (17). However, we found no effects on worm development but hyperresistance in mice lacking gzmA/B or gzmB alone and, in contrast, a higher worm load and shorter worms...
in gzmA knockout (ko) mice. Hyperresistance in gzmA×B ko mice was mainly associated with a Th2 cytokine/Ab shift, enhanced early inflammatory gene expression, and a trend toward a reduced induction of alternatively activated macrophages (AAMs). Susceptibility in gzmA ko mice was associated with a trend toward increased differentiation of AAM and reduced proinflammation. Therefore, we show a novel and divergent role of gzm in pathogen-induced inflammation and anthelminth resistance in vivo.

Materials and Methods
Mice and infection with L. sigmodontis
Wild-type (wt) B6, gzmA×B, gzmA, and gzmB ko mice on the resistant B6 background were provided from the Max-Planck-Institute for Immunobiology Freiburg, Freiburg, Germany, and bred at the Bernhard Nocht Institute (BNI) animal facilities under specific pathogen free conditions in microisolator cages. Natural infections of mice with L. sigmodontis were performed using infectious mites kept at the BNI as described previously (18). To achieve a high infectious dose of at least, roughly estimated, 200 L3, yielding ~60 adult worms (14), mites in similar amounts of sawdust were fed on cotton rats with an optimal microfilariaem of 800–1500 microfilariae/μl blood, and the whole sawdust kept in one glass flask for the maturation of L3 was used to infect mice. Sawdust with mites infected with two rats was taken when only cotton rats with <800 mfl/μl were available. In each experiment, four to six wt and four to six ko mice (one strain; in one experiment, two strains) were placed anesthetized and mixed in the same cage on the sawdust of one tank. All experiments were performed in accordance with the local ethical animal regulations (project no. 82/07, 61/04).

Quantification of parasite burden
Mice were sacrificed at days 7, 9, 26/27, and 35 p.i. Worms were counted after homogenization of vital female worms as described previously (18). Worms were counted after thawing the thoracic cavity with PBS. Worms were fixed in 70% hot ethanol, and length was measured (stereomicroscope). The measurements of length were pooled at each time point irrespective of the female and male sex, because the sex is not distinguishable at days 7–9 p.i., and not all worms molt together into adult worms at the same time. At the fourth molt, worms molt between days 26 and 29 p.i., and proportions of female and male worms vary usually between experiments. L. sigmodontis Ag (LsAg) was prepared by homogenization of vital female worms as described previously (18).

Immunization of mice with OVA
The wt and gzmA×B ko mice were immunized i.p. with 100 μl 10 μg OVA (Sigma-Aldrich) precipitated in 2 mg alum hydroxide (Sigma-Aldrich) and boosted after 14 d; control mice received PBS/alum hydroxide (Sigma-Aldrich) precipitated in 2 mg alum hydroxide (Sigma-Aldrich). Intracellular cytokines were measured after stimulation of 106 spleen cells with 10 ng/ml PMA and 500 ng/ml ionomycin or Con A for 6 h.

Cell isolation and in vitro stimulation
Plural exudate cells (PLEC) were centrifuged after removal of L4 or adult worms, washed twice with cold PBS, and resuspended in 1 ml RPMI 1640 medium (supplemented with 10% FCS, 2 mM l-glutamine, and 50 μg/ml gentamicin). Spleens were homogenized through a cell strainer and red cells removed by erythrolysis in ACK buffer. For cytokine analysis by ELISA, 5 x 103 or 1 x 106 spleen cells or 5 x 105 PLEC were cultivated in 96-well plates for 72 h with either medium alone or with 2 μg/ml Con A, 12.5 μg/ml LsAg, or 100 μg/ml OVA. Supernatants were stored at −20°C. A total of 4 x 105 PLEC/well was stimulated for [3H]thymidine incorporation. Intracellular cytokines were measured after stimulation of 1 x 106 cells with 10 ng/ml PMA and 500 ng/ml ionomycin or Con A for 6 h. Monensin (GolgiStop; BD Biosciences) was added for the last 4 h before the staining procedure. CD69 expression was measured after stimulation with medium, LsAg, or Con A for 6 h. For analysis of activation-induced cell death (AICD), total PLEC were restimulated with medium, Con A, LPS (10 μg/ml), or anti-CD3 mAb (10 ng/ml) for 2 or 6 h.

Reagents and Abs
The following Abs and secondary reagents were used for FACS analysis: 1) from BD Biosciences Pharmingen (San Diego, CA): anti-CD4–allophycocyanin or -FITC (clone RM4-5), -CD8–PerCP Cy5.5 (55-6.7), -CD19–FITC/PE (RPA-T1), -CD3–FITC (clone 145-2C11), -CD4–PE (RM4-5), -CD8–PE (53-6.7), -CD49b–PE (DX5), -IgM–FITC, -IgG1–PE, and -IgG2b–PE (clone XMG1.2), anti-CD8–PerCP Cy5.5 (53-6.7), -CD69–PE (XMG1.2), streptavidin-PerCP, Fixation/Permeabilization Solution Kit with BD Biosciences GolgiStop, Annexin V-FITC Apoptosis Detection Kit I, 7-aminoactinomycin D (7-AAD) staining (Via Probe), anti-IL-5–allophycocyanin (TRFK5), -IL-2–allophycocyanin (C15.6), and appropriate isotype controls such as rat IgG2a–PE/allophycocyanin, mouse IgG1–PE, rat IgG1–allophycocyanin, and rat IgG2b–allophycocyanin (2) from ebiosis (San Diego, CA); anti-CD8–allophycocyanin (53-6.7), -CD49b–PE/allophycocyanin (DX5), biotinylated anti-mβ TCR (H57-957), and anti-mouse/ anti-rat Foxp3–PE/allophycocyanin staining set (FKJ-16s); 3) from Santa Cruz Biotechnology (Santa Cruz, CA): mouse IgG2b–PE, and 4) CFSE.

Flow cytometry
Surface staining of spleen cells and PLEC was performed as described previously (17) using the above mAbs. For intracellular cytokine staining (ICS), the Cytotox/Cytoperm Plus kit (BD Biosciences, San Diego, CA) was applied according to the manufacturer’s instructions. Proliferation was also measured by CFSE incorporation and cell death by propidium iodide incorporation, Annexin V, or 7-AAD staining. Samples were analyzed on a FACSCalibur (BD Biosciences), and 20,000–100,000 gated events were collected in forward and sideward scatter for analysis by CellQuest software (BD Biosciences).

Cytokine and Ig ELISA
Concentrations of IL-2, IL-4, IL-10, and INF-γ were determined in culture supernatant (SN) from spleen cells and PLEC by sandwich ELISA (R&D Systems, Wiesbaden, Germany) according to the manufacturer’s instructions and using 3,5,3’5’ tetramethylenediamine (Rotth, Karlsruhe, Germany) as substrate. Blood was obtained by retro-orbital puncture and plasma kept at −20°C. LsAg- and OVA-specific Iggs was determined by indirect ELISA in the plasma from infected or from OVA/alum-immunized mice. Ninety-six-well ELISA plates were coated with 4 μg/ml LsAg or 1 μg/ml OVA or cytokine capture Abs in carbonate buffer (pH 9.6) at 4°C overnight. After blocking with 5% BSA/PBS, plates were incubated with serially diluted individual mouse plasma or SN in duplicate. Bound Abs were detected using HRP-conjugated anti-mouse Abs, (Zymed, Karlsruhe, Germany): anti-IgM (clone LO-MM-9), -IgG1 (LO-MG1-2), -IgG2c (goat serum), -mouse IgG2b (clone MO-MG2b-3), -mouse IgG3 (STAR 84P, Serotec), -mouse IgE (STAR 110P, Serotec), or anti-cytokine Abs. Relative ELISA units were calculated by subtracting the negative control (buffer) from the OD540 of each sample on each ELISA plate. Ig titers were determined from serial plasma dilutions and defined as positive when greater than the double-negative control.

RNA isolation, microarray analysis, and real-time PCR
PLEC were isolated from six mice/group (gzmA×B ko, gzmA ko, wt mice) at day 7 p.i., and two mice were pooled per group, yielding nine samples for microarray analysis and real-time PCR (RT-PCR). In a second experiment (for RT-PCR only), four gzmA ko mice, four wt mice, and three gzmA×B ko mice were analyzed individually, and the RT-PCR data were pooled from both experiments. Total RNA was isolated from 1 x 106 PLEC/mice (gzmA×B ko, gzmA ko, wt mice) per group, homogenized in 350 μl RAl buffer using the NucleoSpin RNA II Kit (Macherey-Nagel, Berlin, Germany), kept at −20°C, and processed after thawing according to the manufacturer’s protocol. RNA quality was checked with a Bioanalyzer (Agilent Technologies). A total of 5 μg was used for retrotranscription in the presence of biotin-11-dCTP and biotin-11-dATP (PerkinElmer) and Superscript II Reverse Transcriptase (Invitrogen) in the DualChip mouse inflammation microarray (Eppendorf), a low-density DNA array allowing gene expression analysis for 233 genes related to inflammation. The method is based on a system with two internal arrays on a glass slide and three technical replicate spots per gene for each array. Hybridizations on the arrays were carried out as described by the manufacturer. Detection was performed with a gold-conjugated IgG anti-biotin Ab (Jackson ImmunoResearch Laboratories) using the Silverquant detection kit from Eppendorf. The arrays were scanned with a Silverquant scanner (Eppendorf) at a resolution of 12 μm, quantified, and compared with the Silverquant analysis software. Statistical testing was performed with a Welch t test and significant genes identified by a p value <0.05 and a minimal fold change of 2 (and the same trend in two or three samples). Samples were normalized by several internal controls.

Further, gene expression of FIZZ, YM-1, arginase, Aicam, and inducible NO synthase (iNOS) was also quantified by RT-PCR (1-cycler; Bio-Rad) as described by Marsland et al. (19), using the same RNA as above. Samples were normalized to expression levels of the housekeeping gene RNA polymerase II (RPII), shown as quantity ratios.

Statistical analysis
Calculation of means (± SE), Student t, and Mann–Whitney tests were performed with Prism software (GraphPad, San Diego, CA) and p values <0.05 considered statistically significant.
Results

Granzyme A×B double deficiency renders C57BL/6 mice hyperresistant to L. sigmodontis

To define the role of gzmA and B in resistance to L. sigmodontis, we compared worm burdens from naturally infected gzmA×B, gzmA, and gzmB ko mice with those from wt B6 mice at three time points (Fig. 1): 1) the L3/L4 stage at days 8/9 p.i.; 2) the early adult stage at days 26/27 p.i.; and 3) the advanced adult stage at day 35 p.i. In the L3/L4 and early adult stage, significantly lower numbers of worms were recovered from the pleural cavities of gzmA×B ko than wt mice (Fig. 1A). The early difference was obtained with a roughly defined high mite transmitted infectious dose (14), yielding a medium (50–100 worms) or high (100–300 worms) worm load in the pleural cavity. At days 26/27 p.i., this difference was significant for high, but not medium worm loads, when analyzed separately (data not shown). At day 35 p.i., most worms were dead, and worm numbers were lower in both strains, but still slightly reduced in gzmA×B ko compared with wt mice (p = 0.17, Fig. 1A). Worm growth was not affected, as their length was similar in both strains (Fig. 1A).

Therefore, the main result was that gzmA×B deficiency rendered a more resistant (hyperresistant) phenotype of B6 mice, which occurred early in infection and was sustained until the young adult worm stage depending on the infectious dose.

Opposing effects of single granzyme A and B deficiency: gzmA ko mice were more susceptible, whereas gzmB ko mice were hyperresistant

We further dissected the role of gzmA versus gzmB in this hyperresistant phenotype by infecting single ko mice. In contrast to gzmA×B ko mice, gzmA ko mice were ~2-fold more susceptible to high-dose infections than wt mice at days 8/9 p.i., even when only a medium worm load was achieved in wt mice in several experiments (Fig. 1B). This effect was confined to the early stage different to the double deficiency (Fig. 1B). However, worms from gzmA ko mice were significantly smaller at the later time point (Fig. 1B). The opposite effect was seen in gzmB ko mice in the early and later phase: they were as hyperresistant as gzmA×B ko mice in response to a high (Fig. 1C) infectious dose compared with wt mice; worm growth was not affected (Fig. 1C). This was significant for the early phase (p = 0.025) and highly significant for the late phase (p = 0.0047) when a high worm load was achieved (121.8 ± 21.2 worms in wt mice on day 9 p.i. and 136.1 ± 28 worms on day 27 p.i.). Only a tendency was observed at day 9 p.i., but a significant difference at day 27, when medium and high worm loads were pooled (Fig. 1C).

In summary, gzmA promoted early defense against the worms, and its deficiency impaired worm growth, whereas gzmB was associated with persistent susceptibility.

GzmA×B deficiency altered early total leukocyte and NK cell frequencies in L. sigmodontis-infected pleural cavities

To elucidate the mechanisms responsible for the hyperresistant and susceptible phenotypes, we tested whether the extracellular matrix-degrading properties of gzmA and B could facilitate leukocyte migration to the pleural cavity, altering leukocyte subset frequencies. Early total PLEC numbers were reduced in gzmA×B ko compared with wt mice at day 7 p.i., but not thereafter (Fig. 2A). Total pleural lymphocyte, macrophage, and granulocyte counts (data not shown) and frequencies were not affected by gzmA×B deficiency throughout infection (neither in the spleen, data not shown), but changed over time (Fig. 2B, 2C): granulocytes constituted 10–30% of PLEC peaking at day 9 p.i. Lymphocytes initially constituted almost two thirds, but decreased substantially (Fig. 2C), whereas macrophages increased steadily to 40% (Fig. 2D). A detailed analysis of pleural lymphocyte subsets revealed that NK cells were significantly reduced in gzmA×B ko mice compared with wt mice at day 9 p.i. (Fig. 2E), whereas CD4+ T cell (including Foxp3+/CD25+ Treg; Fig. 2F), CD8+ T cell, and CD19+ B cell frequencies were similar (data not shown). B cells were the main lymphocyte subset in both strains (40–50%, data not shown).

In summary, the presence of both gzmA and B enhanced very early leukocytosis and NK cell frequencies in filaria-infected pleural cavities.

Local and temporary systemic proliferative hyporesponsiveness

To determine whether gzm deficiency affected pleural and spleen cell proliferation influencing leukocytosis, [3H]thymidine incorporation was analyzed. Hyporesponsiveness of spleen and lymph node cells is typical for L. sigmodontis-infected B6 mice (12). Both wt and gzmA×B ko PLEC, isolated at day 9 p.i., hardly proliferated spontaneously ex vivo (Fig. 3A). Proliferation was even more depressed in both strains in response to LsAg or Con A compared with

FIGURE 1. Divergent effects of gzmA×B, gzmB, and gzmA deficiency on resistance of B6 mice to infection with L. sigmodontis. A. Number of worms (worm load, left panel) in pleural cavities of gzmA×B ko mice (days 8/9; n = 26; days 26/27; n = 34; day 35; n = 3) and wt B6 mice (n = days 8/9; n = 27; days 26/27; n = 35, day 35; n = 15) after natural high-dose infections (analyzed together), three to seven separate experiments with four to six mice/group. Worm length (right panel) measured after hot ethanol fixation. Each dot represents one worm. B. Worm loads and length (days 26/27 p.i.) in gzmA ko (day 9: n = 22; day 26: n = 14) and B6 mice (day 9: n = 29, day 26: n = 19) after natural high-dose infections, four to five separate experiments. C. Worm loads and length (days 26/27 p.i.) in gzmB ko mice (day 9: n = 18; day 26: n = 21) and wt mice (day 9: n = 18; day 26: n = 19) after natural high-dose infections, three to four separate experiments. *p < 0.05, **p < 0.01, ***p < 0.001 by Student t test.
medium controls (Fig. 3A). Different to PLEC, naive CD4+ and CD8+ gzmA×B ko spleen cells proliferated more weakly to Con A than wt cells (Fig. 3B), whereas NK and B cells were unaffected (data not shown). However, gzmA×B ko spleen cell proliferation adjusted to substantial levels similar to wt mice in response to LsAg and Con A within the first 9 d of infection (Fig. 3C).  

GzmA and B mediate spontaneous cell death and AICD of wt lymphocytes at the main infection site  

We then tested whether gzmA and B could induce spontaneous death or AICD of pleural lymphocytes during infection. Numbers of early apoptotic (Annexin V+) and late apoptotic or dead (Annexin V+/propidium iodide+, trypan blue+, data not shown; 7-AAD+, Fig. 3F) cells were lower in freshly isolated and in medium alone cultured gzmA×B ko than wt PLEC in some (n = 3) but not all experiments. Such ex vivo 7-AAD+ cells were CD4+, CD8+, or CD19+ (Fig. 3F). Slightly higher spontaneous death of wt than gzmA×B ko NK cells was observed (data not shown). In summary, lower spontaneous ex vivo cell death rates and nonspecifically stimulated AICD rates of pleural T and B cells as well as of B cells occurred in the absence of gzmA and B.  

B cells were preferentially activated in L. sigmodontis-infected pleural cavities  

In line with this, ~30–40% of CD19+ pleural B cells from both wt and gzmA×B ko mice spontaneously expressed the early activation marker CD69 ex vivo at day 9 p.i. (Fig. 4A, 4B). CD69 expression was further increased on B cells after stimulation with LsAg or Con A (Fig. 4A, 4B), but again, did not differ between gzmA×B ko and wt mice. Only ~15% of CD4+ and CD8+ T cells were CD69+ and therefore less preactivated (data not shown).  

A shift toward a Th2-specific humoral response in gzmA×B-deficient mice  

As CD19+ B cells were activated in the main pleural lymphocyte subset, we compared the plasma Ab subclass responses between wt and gzmA×B ko mice at day 26 p.i. (Fig. 3C): LsAg-specific IgM and IgG1 were the dominant Ig isotypes released in both strains. A titration of the plasma yielded a significantly higher LsAg-specific IgM titer in gzmA×B ko than wt mice, whereas IgG1 titers were not different and much lower (Fig. 4E). Additionally, LsAg-specific levels of the Th1-type subclass IgG2b were significantly reduced in gzmA×B ko mice in comparison with wt mice (Fig. 4D). Th1-type IgG2c and IgG3 levels tend to be also reduced in ko mice (Fig. 4C). Furthermore, OVA immunization of naive mice revealed increased OVA-specific IgG1 (mainly Th2-inducible) (Fig. 4F), but reduced IgG2b (data not shown) in gzmA×B ko mice similar to LsAg-specific IgG2b.  

In summary, the presence of gzmA and B in wt mice promoted a stronger Th1-type Ag-specific IgG subclass response, whereas Ag-specific IgM release was reduced.  

A partial Th2 cytokine bias in gzmA×B ko mice was preceded by a temporary deficit in IL-2 production by naive mice  

We further tested whether altered Th1/Th2 cytokine patterns were associated with defense, measuring IL-2 and IFN-γ (Th1) as well as IL-4, IL-5, and IL-10 (Th2) intracellularly and/or in cell-culture supernatants from naive, infected, or OVA-immunized mice. Naive gzmA×B ko spleen cells secreted significantly less IL-2 than wt spleen cells in response to Con A (Fig. 4D). IL-2 production was restored in gzmA×B ko spleen cells (data not shown) as in wt PLEC at day 9 p.i. (Fig. 3C). Splenic IFN-γ (Fig. 4D), IL-4, and IL-10 as well as pleural IL-4 responses were similar in Con A or LsAg-stimulated naive wt and gzmA×B ko mice and at days 8/9 p.i. in both strains; spontaneous IL-4 or IL-10 release was hardly detectable in both strains, and IL-4 was also absent in stimulated naive spleen cells. IL-10 was 10 times more strongly induced by Con A early in infection compared with naive mice, but equally in gzmA×B ko and wt mice (data not shown). Strikingly, at the same time, day 8/9 p.i., splenic IFN-γ release upon Con A stimulation was by tendency lower in gzmA×B ko than in wt mice (p = 0.08) and significantly reduced in Con A-stimulated gzmA×B ko PLEC compared with wt PLEC (p = 0.025) (Fig. 5D). By ICS, CD4+ T cells (Fig. 5E), equally CD8+ T cells (8 to 9% of lymphocytes, data not shown) and NK cells (5 to 6%, Fig. 5E) were the main pleural IFN-γ producers at day 7 p.i., unaffected by gzmA×B deficiency in contrast to the lower IFN-γ release in culture (Fig. 5D). Frequencies of IFN-γ+
NK and CD4+ T cells were largely reduced by ∼90% in PLEC of both strains isolated 2 d later (day 9), indicating release associated with the third molt (Fig. 5E), whereas frequencies of IFN-γ+CD8+ T cells significantly increased in inguinal lymph nodes of infected wt mice on days 8 to 9 p.i. compared with naive mice (data not shown). At day 26 p.i., IFN-γ secretion was not detectable any more in ex vivo Con A-stimulated PLEC of both strains and very low in spleen cells with a trend of lower levels in gzmA3Bk o than wt mice (p = 0.0771, data not shown). In contrast, IL-5 expression was higher in pleural gzmA3B ko than wt cells in the late infection phase at days 26/27 p.i. (Fig. 5F). In OVA-immunized mice, Con A-induced splenic IL-10 was higher in gzmA3B ko than wt mice (Fig. 5G), as was spontaneous and OVA-specific IL-5, whereas nonspecific IL-2 was reduced similar to infected gzmA3B ko mice (data not shown).

In summary, a reduced Th1 and partially Th2-biased cytokine response was demonstrated in naive stimulated cells, during infection and after OVA immunization in the absence of both granzyme A and B.

Gene expression analysis of gzmA×B, gzmA ko, and wt B6 mice: early effects on inflammatory markers and alternatively activated macrophages

To further define early gzm-induced modifications of the inflammatory response to infection, we studied inflammatory gene expression profiles of whole PLEC from gzmA×B ko, gzmA ko, and wt B6 mice at day 7 p.i. using the Eppendorf DualChip mouse inflammation one-color microarray (Fig. 6A) (Eppendorf). Surprisingly, no differences in the transcription of genes for the classical Th1/2-markers IFN-γ, IL-4, IL-5, or IL-10 were detected.
Granzymes were originally known to play a role in the defense against intracellular parasites. To our knowledge, the present study provides the first evidence that gzms play a role in resistance to an extracellular and multicellular parasite such as the rodent filaria *L. sigmodontis*, based on the observation that gzmA×B and gzmB ko mice were hyperresistant, whereas gzmA deficiency temporarily enhanced the early worm load. We discuss the impact of primary gzm-mediated effects, infectious dose and worm loads, cytokine and humoral response, gzm-mediated cell death, macrophage function, and proinflammatory gene expression to explain the phenotype. Therefore, we divided the discussion into seven subsections.

**No direct gzm effects on worms**

The reduced worm loads in the absence of gzmB rules out a direct killing effect of gzmB and rather suggests a dominant and sustained defense-suppressive role, counterbalanced by gzmA, which promotes proinflammation. GzmA had no direct effect either, as worm vitality or molting was not affected ex vivo or in culture, and gzmA/B B-deficiency early in infection with *L. sigmodontis* underscores the primary role of gzmA in proinflammation (4).

Because expression of MMP12 was reduced in gzmA×B ko mice in comparison with wt mice (Fig. 6B, Supplemental Table II, Table I) and is associated with AAMs (19), we performed RT-PCR analysis of four AAM markers in PLEC using the same RNA batches as for the microarray and RNA from an additional experiment (only pooled data shown): arginase, FIZZ, YM-1 (Fig. 6C), and AMCcase (data not shown) relative to the housekeeping gene RPII. For FIZZ and arginase, a trend of reduced expression was observed in gzmA×B ko mice compared with gzmA ko mice (Fig. 6C), correlating with the lowest worm load (Fig. 6B). For YM1 and iNOS, no trend was observed (Fig. 6C). AAMs preferentially express arginase over, hardly detectable, iNOS (19). This was observed in all three strains (iNOS <0.1) (Fig. 6C).

In summary, we describe a novel pattern of inflammatory marker genes as well as AAMs to be affected by gzmA/B and gzmA deficiency early in infection with *L. sigmodontis*.

**Discussion**

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**Discussion**

Granzymes were originally known to play a role in the defense against intracellular parasites. To our knowledge, the present study provides the first evidence that gzms play a role in resistance to an extracellular and multicellular parasite such as the rodent filaria *L. sigmodontis*, based on the observation that gzmA×B and gzmB ko mice were hyperresistant, whereas gzmA deficiency temporarily enhanced the early worm load. We discuss the impact of primary gzm-mediated effects, infectious dose and worm loads, cytokine and humoral response, gzm-mediated cell death, macrophage function, and proinflammatory gene expression to explain the phenotype. Therefore, we divided the discussion into seven subsections.

**No direct gzm effects on worms**

The reduced worm loads in the absence of gzmB rules out a direct killing effect of gzmB and rather suggests a dominant and sustained defense-suppressive role, counterbalanced by gzmA, which promotes proinflammation. GzmA had no direct effect either, as worm vitality or molting was not affected ex vivo or in culture, and gzmA/B B-deficiency early in infection with *L. sigmodontis*.

Because expression of MMP12 was reduced in gzmA×B ko mice in comparison with wt mice (Fig. 6B, Supplemental Table II, Table I) and is associated with AAMs (19), we performed RT-PCR analysis of four AAM markers in PLEC using the same RNA batches as for the microarray and RNA from an additional experiment (only pooled data shown): arginase, FIZZ, YM-1 (Fig. 6C), and AMCcase (data not shown) relative to the housekeeping gene RPII. For FIZZ and arginase, a trend of reduced expression was observed in gzmA×B ko mice compared with gzmA ko mice (Fig. 6C), correlating with the lowest worm load (Fig. 6B). For YM1 and iNOS, no trend was observed (Fig. 6C). AAMs preferentially express arginase over, hardly detectable, iNOS (19). This was observed in all three strains (iNOS <0.1) (Fig. 6C).

In summary, we describe a novel pattern of inflammatory marker genes as well as AAMs to be affected by gzmA/B and gzmA deficiency early in infection with *L. sigmodontis*.

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role, as effects were stronger at the late than the early time point. Because at day 35 p.i. worm loads of double ko and wt were similarly reduced, effects of both gzm deficiencies are overruled by other B6 defense mechanisms. Unfortunately, no gzm ko mice were available on the susceptible BALB/c background, which might reveal more insights into gzm-mediated effects on susceptibility.

**Influence of gzm on the Th1/2 immune balance and NK cells**

A divergent immunological role of gzmA and B was recently reported for tumor clearance in mice (8). In line with this, the analysis of major immune parameters in gzm ko and wt mice demonstrated an early reduced Th1 response and a later partial Th2-biased cytokine and an Ig subclass shift in gzmA ko mice. demonstrated an early reduced Th1 response and a later partial Th2-biased cytokine and an Ig subclass shift in gzmA ko mice. NK cells release IFN-γ-mediated Th1 response elicited by invading L3 in gzmA ko mice. NK cells release IFN-γ upon stimulation by Brugia L3 (21), shown in this study for the molting phase from L3 to L4 between day 7 and day 9 of infection, also for CD4+ and CD8+ cells, which was independent from gzmA ko mice.

A very early Th1-biased proinflammatory cytokine production by NK and/or T cells is probably stimulated by LPS-like molecules (22) from the filarial endosymbiont Wolbachia, present in L3 and known to induce proinflammatory cytokines (13).

**Humoral response by B cells**

Such endobacterial LPS-like molecules could also have upregulated CD69 expression on CD19+ B cells in response to infection and female LsAg stimulation representing activation of B cells via TLRs, because endobacterial LPS-like molecules can activate T cells via TLRs (13), and TLR can mediate Ig secretion in LPS-stimulated B cells (23). B cells are also activated by filarial phosphorylcholine to produce IgM (18). More late plasma LsAg-specific IgM, less IgG2b/c and IgG3 in infected gzmAXB ko mice, and more OVA-specific IgG1 in naive gzmAXB ko mice argue in favor of a role of gzm for B cell function altering class switching. The Th2 cytokine bias in gzmAXB ko mice might have...
promoted this, as IL-4 promotes IgG1, but blocks class switching to IgG2b in response to LPS (24). NK cells, which were enhanced early in wt mice, could also have promoted IgG2c production in wt mice (25).

**Gzm-mediated T and B cell death**

The Th2 shift might also be strengthened by the lower gzmA and/or B-mediated, autologous spontaneous and activation-induced ex vivo cell death rates of gzmA×B ko mice than wt B cells and CD4+ or CD8+ T cells in line with Devadas et al. (20). Devadas et al. (20) showed that gzmB mediates autologous Th2 cell death, leading to a stronger Th1 skewing in mice expressing gzmB, protecting them from allergen-induced asthma, or B-mediated, autologous spontaneous and activation-induced ex vivo cell death rates of gzmA×B ko mice than wt B cells and CD4+ or CD8+ T cells in line with Devadas et al. (20). Devadas et al. (20) showed that gzmB mediates autologous Th2 cell death, leading to a stronger Th1 skewing in mice expressing gzmB, protecting them from allergen-induced asthma, compared with gzmB ko mice. Our data adds CD8+ T cells and B cells, which can express gzmB, to this concept. We assume that gzmB and not gzmA mediates autologous cell death in this study, because gzmB ko/gzmA−/− mice had reduced worm loads like gzmA×B ko mice. In addition, granzyme-induced apoptosis of target cells, most likely by NK cells or T cells, could also account for the observed cell death. The different death rates of freshly isolated PLEC and PLEC cultured in medium might be due to the varying worm loads causing in vivo preactivation by worm Ags. It is known that PLEC are activated in the pleural cavity by filarial and/or endobacterial molecules, leading to cytokine release into the pleural exudate fluid (13, 26). We therefore assume that AICD of strongly activated cells also occurs in vivo, but does not reduce the early total PLEC numbers (Fig. 2A), because the surface markers such as CD3, CD4, CD8, or CD19 can still be detected on apoptotic cells (27). Because apoptotic cells do not produce mediators any more, AICD alters the humoral and cytokine balance, influencing defense. In weakly but not strongly preactivated cells, the in vitro stimulation with Con A would provide a stronger cell death-inducing signal. The difference in ex vivo responsiveness of wt and ko cells to nonspecific stimuli therefore also indicates differences in the preactivation status in vivo. This could lead to differences in cytokine or Ab production prior to apoptosis. CD19+ B cells, but not CD4+ or CD8+ T cells, were even refractory for further ex vivo stimulation in one experiment, indicating that B cells could be more strongly preactivated in vivo than T cells. CD19 potentiates Th2 cytokine production by B cells (28), which are increasingly recognized as immune modulators (e.g., inducing Th2 CD4+ responses) (23, 29). GzmA×B deficiency could enhance the survival of activated, cytokine-producing CD19+ B cells and T cells, leading to stronger Th2 responses in ko mice as reported for gzmB-deficient Th2 cells (20, 30).

**Gzm effects on macrophages**

GzmB-induced apoptotic cells, when phagocytosed by macrophages, can induce the production of TGF-β by these macrophages and thereby influence the Th1/Th2/Th3 cytokine and Ig balance (31). TGF-β is known to play a regulatory role in onchocerciasis (13), locally and abundantly induced in lymphocytes, macrophages, and other cells by the filariae (32). This notion is supported by our finding of a trend of reduced expression of the Th2-/inducible AAM markers arginase and FIZZ in the absence of gzmB in gzmA×B ko mice compared with gzmA ko (gzmB-expressing) mice, because TGF-β is produced by AAMs (33). This weaker AAM induction in gzmA×B ko mice is supported by their lower MMP12 gene expression, as MMP12 is also expressed by AAMs (e.g., in murine infection with the hookworm *Nippostrongylus*) (19). Therefore, our observation of altered AAM induction supports the novel immunomodulatory role of granzymes in helminth infection. We conclude that gzmB and not gzmA drives AAM differentiation in gzmB-expressing gzmA ko and wt mice, as AAM induction was linked with downregulated inflammatory gene expression and a higher worm load in both gzmA ko and wt mice compared with the double ko mice. In wt mice, the presence of gzmA can counterbalance gzmB, which could explain their intermediate phenotype with regard to worm load and inflammatory gene expression. GzmA is known to induce classically activated macrophages (10). As gzmA ko mice cannot properly respond to LPS (4), possibly also to endobacterial LPS-like molecules, less proinflammation is induced. All three major helminth classes induce the AAM markers FIZZ, YM1, and arginase (34), of which arginase is considered to have the most macrophage-restricted expression profile (35). AAM functions range from facilitating worm installation or defense, tissue repair, to protection against
exacerbated inflammation. Our study supports the anti-inflammationary and antidefensive role of AAMs in filarial infections, as gzmA ko mice had higher worm loads and trend toward upregulated AAM markers. Therefore, to our knowledge, these data show for the first time that gzmA influence AAM-induction in helminth infection. This and the gzm-independent decrease of Foxp3- Treg in B6 mice with the course of infection in contrast to sustained levels in BALB/c mice (data not shown) suggest a Treg-independent way of immune modulation by gzmA/B and via macrophages.

Proinflammatory gene expression

Gene expression analysis revealed other inflammatory markers to be involved and expressed than those known for this infection model and described in the Results section (IL-2, IL-4, IL-5, IL-10, AND IFN-γ) and that these are differentially influenced by gzmA and B. Such incongruity between protein and gene expression levels has also been observed by other groups, but cannot fully be explained yet (36). In line with Hedeler et al. (36), we also present up- and downregulated genes expressed below the expression threshold to elucidate the immunomodulatory pattern. We propose that due to the early (day 7) time point p.i. other early inflammatory markers than IL-4, IL-5, or IFN-γ, as described below, initiate the shift at day 7 p.i., which then affects the production of the classical Th1/Th2 cytokines IFN-γ and IL-5. Another indication for this might be that IFN-γ-expressing CD8+ T cells were increased in inguinal lymph nodes by infection at days 8 to 9, but not yet on day 7 (data not shown). Our analysis shows that hyperresistance in gzmA×B ko mice was associated with an early upregulation of some Th1 markers (STAT4, CXCR4, CXCL1), but primarily of Th2-related genes (IL-3, VEGFA, CCL2, SOCS2), whereas IL-1RL1, the Th2 cell receptor for the Th2 cytokine IL-33 (33) was most markedly downregulated, followed by CCR3 (Th2) and CCR6 (Th17). This pattern could mediate the Th2 cytokine and Ab shift in gzmA×B ko mice. VEGF enhances Th2-mediated lung inflammation (37) and the A subtype pathology in lymphatic filariasis (38). Enhanced CXCR4 expression could reflect less stromal cell-derived factor-1α engagement and explain the temporary lower NK cell numbers. The increased SOCS2 and IL-3 expression suits with the ability of SOCS2 to enhance IL-2 and IL-3 phosphorylation, thus proliferation, cytokine stimulation, and Th lineage differentiation, possibly compensating for the IL-2 deficit in naive gzmA×B ko spleen cells. The strong reduction of the LPS-inducible IL-1RL1 might indicate enhanced engagement by IL-33, which is enhanced early in parasitic infections (33). Further, higher pleural procollagen expression, a marker for wound healing, might reflect and counterbalance gzmA-induced damage. GzmA can detach epithelial cells and is upregulated together with gzmB/K in Schistosoma egg-induced lung damage in Th1-polarized mice (39), because gzmA is inducible by proinflammatory cytokines (40). This could have facilitated early pleural leukocyte extravasation, leukocytosis, and NK cell infiltration in the Th1-biased L. sigmodontis-infected wt mice. The profound difference in early inflammatory gene expression associated with the resistance phenotypes in gzm ko and wt mice underlines our finding of a novel and divergent immunomodulatory role of gzmA and B in helminth infection.

Our study is therefore consistent with the concept that a very early bias in the Th1 and proinflammatory response increases survival of L. sigmodontis and that early and late Th2 responses as in gzmA×B ko mice promote defense (41). The dependency of hyperresistance in gzmA×B ko mice on higher infectious doses reinforces this Th2 concept, because high L3 numbers trigger Th2 cytokines in susceptible BALB/c mice (14). We therefore conclude that proinflammatory gzmA and anti-inflammatory gzmB are novel modulators of the Th1/2 balance and defense in helminth infection.

Table I. Comparison of inflammation gene expression in PLEC among gzmAxB ko, gzmA ko, and wt mice at day 7 p.i. determined by the Eppendorf DualChip mouse inflammation one-color microarray

<table>
<thead>
<tr>
<th>Gene Product, Gene Bank Accession No.</th>
<th>Gene Symbol</th>
<th>Compared Groupsa</th>
<th>p Valueb (± n-fold = Log Ratio)</th>
<th>Normalized Ratio c</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3, NM_010556.2</td>
<td>IL-3</td>
<td>A×B ko versus A ko; A ko versus wt</td>
<td>0.0003 (+0.6); 0.0117 (~0.4)</td>
<td>-</td>
</tr>
<tr>
<td>Signal transducer and activator of transcription 4, NM_011487.1</td>
<td>STAT4</td>
<td>A×B ko versus A ko; A ko versus wt</td>
<td>0.0060 (+0.7); 0.0199 (~0.8)</td>
<td>-</td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) receptor 4, NM_009911.2</td>
<td>CXCR4</td>
<td>A×B ko versus A ko; A ko versus wt</td>
<td>0.0120 (+0.3); 0.0183 (~0.2)</td>
<td>-</td>
</tr>
<tr>
<td>Vascular endothelial growth factor A, NM_009505.2</td>
<td>VEGFA</td>
<td>A×B ko versus A ko</td>
<td>0.0145 (+1.3); 0.0099 (~1.5)</td>
<td>-</td>
</tr>
<tr>
<td>Suppressor of cytokine signaling 2, NM_007706.1</td>
<td>SOCS2</td>
<td>A×B ko versus A ko; A ko versus wt</td>
<td>0.0389 (+1.5)</td>
<td>+2.6</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 2, NM_011333.1</td>
<td>CCL2</td>
<td>A×B ko versus A ko</td>
<td>0.0061 (~9.2); 0.0411 (~5.9)</td>
<td>-</td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) ligand 1, NM_008176.1</td>
<td>CXCL1</td>
<td>A×B ko versus A ko</td>
<td>+2.4</td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 1, IL-1 receptor-like 1 (T1ST2), IL-33R, NM_010743.1</td>
<td>IL-1RL1</td>
<td>A×B ko versus A ko; A ko versus wt</td>
<td>-7.1; ~5.2</td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-C motif) receptor 3, NM_009914.2</td>
<td>CCR3</td>
<td>A×B ko versus A ko</td>
<td>+2.5; ~2.7</td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-C motif) receptor 6, NM_009835.2</td>
<td>CCR6</td>
<td>A×B ko versus A ko; A ko versus wt</td>
<td>~0.0004 (~0.5)</td>
<td>-</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 11, NM_011330.1</td>
<td>CCL11</td>
<td>A ko versus wt</td>
<td>-3.4</td>
<td></td>
</tr>
<tr>
<td>Matrix metalloproteinase 12, NM_008605.1</td>
<td>MMP12</td>
<td>A×B ko versus A ko</td>
<td>~2.55</td>
<td></td>
</tr>
<tr>
<td>CD247 Ag, L03353.1</td>
<td>MMP12</td>
<td>A×B ko versus A ko</td>
<td>~3.0</td>
<td></td>
</tr>
<tr>
<td>Procollagen, Typ I α 1/3, 2, NM_007742.2/ NM_007743.1</td>
<td>Col1a1/2</td>
<td>A×B ko versus A ko</td>
<td>~2.5 (~2.7)</td>
<td></td>
</tr>
</tbody>
</table>

a gzmAxB (A×B) ko, gzmA (A) ko.

b p values <0.05 by Student t test (with Welch correction) indicated a significant increase (+) or reduction (−) of gene expression, ranked downward; the 12 most significant values were selected; log ratios >1.5 were considered as marked change.

c Normalized ratios ≥2 with at least two/three samples showing the same tendency were considered as indicative of an enhancement (+) or reduction (−) of gene expression.