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*J Immunol* published online 19 January 2011
http://www.jimmunol.org/content/early/2011/01/19/jimmunol.0902157

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/01/19/jimmunol.0902157.DC1

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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: 000–000.

G ranzyme (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 noncystotoxic 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, and IL-5 in vaccination-induced protection (12). In BALB/c mice, Treg suppress defense, whereas IL-4, IL-5, IFN-γ, 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 gzm3 in pathogen-induced inflammation and antihelminth 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 infected mites on the back of the mice (for RT-PCR only), four gzmA ko mice, four wt mice, and three gzmA ko mice sacrificed at days 7, 9, 26, 27, and 35 p.i. Worms were counted after flushing 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 µg OVA (Sigma-Aldrich) in 2 mg alun hydrolxide (Sigma-Aldrich) and boosted after 14 d; control mice received PBS/alum hydrolxide only. Mice were bled and sacrificed at day 24 postimmunization.

Cell isolation and in vitro stimulation

Pleural exudate cells (PEC) 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). Splenins were homogenized through a cell strainer and red cells removed by erythrolysis in ACK buffer. For cytokine analysis by gentamicin). Spleens were homogenized through a cell strainer and red cells removed by erythrolysis in ACK buffer. For cytokine analysis by

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 Cytofix/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 and 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 IFN-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’-tetramethylbenzidine (Roht, Karlsruhe, Germany) as substrate. Blood was obtained by retro-orbital puncture and plasma kept at −20°C. LsAg- and OVA-specific Igs 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-μM (clone LO-MM-9), anti-gIg1 (LO-MG1-2), anti-gIg2c (goat serum), mouse IgG2a (clone LO-MG2b-3), mouse IgG3 (STAR 84P, Serotec), or mouse IgE (STAR 110P, Serotec), or anti-cytokine Abs. Relative ELISA units were calculated by subtracting the negative control (buffer) from the ODtissue 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 1×10⁶ PLEC/mouse group, homogenized in 350 µl RA1 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 array allowing gene expression analysis for 233 genes related to inflammation. The method is based on a system with two identical 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 (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, 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 (RP49), 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 B6 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).

**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 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
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 controls (Fig. 3A). Different to PLEC, naive CD4+ and CD8+ gzmA×B ko than wt PLEC in some (n = 3) but not all experiments. Such ex vivo 7-AAD+ cells were higher in gzmA×B ko than wt spleen 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).

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**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. 4C). 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. 5A). CD4+ T cells were the main source of this IL-2, and their frequencies were significantly higher in wt than gzmA×B ko mice (Fig. 5B). With infection, IL-2 production was restored in gzmA×B ko spleen cells (data not shown) as in wt PLEC at day 9 p.i. (Fig. 5C). Splenic IFN-γ (Fig. 5D), 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.
Granzyme A deficiency early in infection with \textit{L. sigmodontis}.

**Discussion**

Granzymes were originally known to play a role in the defense against intracelluar parasites. To our knowledge, the present study provides the first evidence that gzmA plays a role in resistance to an extracellular and multicellular parasite such as the rodent filaria \textit{L. sigmodontis}, based on the observation that gzmA 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 melting was not affected ex vivo or in culture, and gzmA/B were not detected in worm material (immunohistology, gzmB Western blot, data not shown). However, because filariae require NK cells to develop (15), they might require gzmB from NK or other cells. This would fit with the reduced worm loads in gzmB and double ko mice, but also with the higher worm loads of gzmA ko mice because gzmB is present. But because gzmB is present in gzmA ko and wt mice, which have different worm loads, other mechanisms play a role, as discussed further below. The mechanism how gzmB could facilitate development remains elusive. As an indirect effect, gzmB might facilitate migration of filariae to the pleural cavity by degradation of matrix proteins in the host tissue.

**Impact of infectious dose and worm loads**

We found that the magnitude of infectious dose and pleural worm loads influenced the outcome of gzmA deficiency, because a significant difference occurred already with medium pleural worm loads of wt mice when compared with gzmA ko mice, but not when compared with gzmA/B ko or gzmB ko mice. In the double ko and gzmB ko mice, significance was achieved with high worm loads only or pooled high and medium loads. The fact that a difference was only observed in the early infection phase in gzmA ko underscores the primary role of gzmA in proinflammation (4). However, we cannot exclude late effects with high worm loads. In contrast, gzmB plays an initial, but also more long-term regulatory function, and proinflammatory gene expression to explain the phenotype.
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 gzms 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 gzms 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. This shift was confirmed by the Th2-biased OVA-immunization responses. An altered cytokine/chemokine environment and gzm-induced tissue damage (see below) could have led to the temporary lower early total PLEC and NK cell frequencies in infected gzmA/B ko mice. Further, the early IL-2 deficit likely reduced Con A-stimulated proliferation of naive gzmA/B ko compared with wt spleen cells. As IL-2 promotes NK/Th1 cell proliferation and IFN-γ production, this could have weakened the very early NK cell-mediated Th1 response elicited by invading L3 in gzmA/B 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/B deficiency.

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 L4 (21), linked with a reduced worm burden and therefore stronger defense. This shift was confirmed by the Th2-biased OVA-immunization responses. An altered cytokine/chemokine environment and gzm-induced tissue damage (see below) could have led to the temporary lower early total PLEC and NK cell frequencies in infected gzmA/B ko mice. Further, the early IL-2 deficit likely reduced Con A-stimulated proliferation of naive gzmA/B ko compared with wt spleen cells. As IL-2 promotes NK/Th1 cell proliferation and IFN-γ production, this could have weakened the very early NK cell-mediated Th1 response elicited by invading L3 in gzmA/B 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/B deficiency.

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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 response (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
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 Groups†</th>
<th>p Value** (≥ n-fold = Log Ratio)</th>
<th>Normalized Ratio††</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>−2.2</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>+2.6</td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 1, NM_008176.1</td>
<td>CCL2</td>
<td>A×B ko versus A ko</td>
<td>+3.5</td>
<td></td>
</tr>
<tr>
<td>IL-1 receptor-like 1 (T1/ST2), IL-33R, NM_010743.1</td>
<td>IL-1RL1</td>
<td>A×B ko versus A ko</td>
<td>−3.0</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>+2.4</td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-C motif) receptor 6, NM_009835.2</td>
<td>CCR6</td>
<td>A×B ko versus wt; A×B ko versus A ko</td>
<td>−7.1; −5.2</td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 11, NM_011330.1</td>
<td>CCL11</td>
<td>A×B ko versus wt</td>
<td>−3.4</td>
<td></td>
</tr>
<tr>
<td>Matrix metalloproteinase 12, NM_006050.1</td>
<td>MMP12</td>
<td>A×B ko versus wt</td>
<td>−2.55</td>
<td></td>
</tr>
<tr>
<td>CD247 Ag, L03353.1</td>
<td>CD247</td>
<td>A×B ko versus wt</td>
<td>−3.0</td>
<td></td>
</tr>
<tr>
<td>Prokollagen, Typ I, α 1/α 2, NM_007742.2</td>
<td>Coll1/2</td>
<td>A×B ko versus wt; A ko versus wt</td>
<td>−2.5 (~2.7)</td>
<td></td>
</tr>
<tr>
<td>NM_007743.1</td>
<td></td>
<td>A×B ko versus A ko</td>
<td>+2.2 (+2.2)</td>
<td></td>
</tr>
</tbody>
</table>

*†‡** gzmAxB (A×B) ko, gzmA (A) ko.

†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.

††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.

exacerbated inflammation. Our study supports the anti-inflammatory 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 gzsms 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 incongruency between protein and gene expression level 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.
Acknowledgments
We thank Markus M. Simon (Metschikoff Laboratory, Max-Planck-Institute for Immunobiology Freiburg, Freiburg, Germany) for collaboration (gzm ko mice, material, and helpful discussions), A. Ali and colleagues for animal husbandry (Bernhard Nocht Institute for Tropical Medicine), O. Bain (Muséum National d’Histoire Naturelle, Paris, France), and S. Specht (University of Bonn, Bonn, Germany) for additional supplies of nites.

Disclosures
The authors have no financial conflicts of interest.

References


