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Serine Protease Inhibitor 6 Protects iNKT Cells from Self-Inflicted Damage

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The role played by apoptosis in the homeostasis of effector cells of the innate immune system is unclear. Serine protease inhibitor 6 (Spi6) is an inhibitor of granzyme B (GrB) that protects cytotoxic T lymphocytes of the adaptive immune system from apoptosis. To determine whether Spi6 also protects cells of the innate immune system from self-inflicted damage we have examined invariant NKT (iNKT) cells. Spi6-deficient iNKT cells harbored increased levels of GrB after TCR stimulation with the PBS-57 glycolipid Ag and were susceptible to apoptosis. The increased apoptosis of Spi6 knock-out (KO) iNKT cells lead to a complete loss in the production of IL-4 and IFN-γ by Spi6 KO iNKT cells after PBS-57 challenge. The increased activation-induced apoptosis resulted in impaired survival and a decreased clonal burst size of Spi6 KO iNKT cells, which could be corrected by GrB deficiency. However, the clonal burst of Spi6 KO iNKT cells after TCR-independent activation with lymphocytic choriomeningitis virus was not affected. Our findings demonstrate that Spi6 protects cytotoxic cells of the innate immune system from GrB-mediated self-inflicted triggered by the recognition of Ag. The Journal of Immunology, 2010, 185: 000–000.

In addition to an immunomodulatory role, iNKT cells also kill cancer cells through contact-mediated cytotoxicity (2, 7, 16). In this process, the exocytosis of perforin (17, 18) facilitates the entry of serine proteases called granzymes from cytotoxic granules of lymphocytes, such as iNKT cells, into the cytoplasm of target cells where they trigger apoptosis (16). Granzyme B (GrB) is required for the induction of target cell death (19) through the activation of caspase 3 and the induction of nuclear DNA fragmentation (16). Given the effectiveness of the exocytosis CTL pathway, mechanisms for the protection from self-inflicted damage have been proposed for some time (20).

Serine protease inhibitor 6 (Spi6) (SERPINB9) is an intracellular serine protease inhibitor of the OVA family (OVA-serpin) (21) that is specific for GrB and expressed in the cytoplasm of mouse CTL and NK cells (22, 23). Using Spi6 knock-out (KO) mice, we have shown that Spi6 protects CTLs from apoptosis by inactivating GrB that leaks from cytotoxic granules into cytoplasm (24). Spi6 acts a classical OVA-serpin substrate for GrB resulting in the denaturation and destruction of the apoptotic serine protease (24).

Although the cytolytic lymphocytes of the innate (iNKT cells) and adaptive (CTL) immune systems both use the exocytosis pathway for cell killing, there are significant differences in their immunobiology. The most obvious being the rapidity of effector responses after first Ag encounter and the relatively short life span of iNKT cells compared with CTL (2, 3). Therefore, it is not clear whether protection from GrB by intracellular OVA-serpins is likely to be a general homeostatic mechanism shared between CTL of the adaptive immune system and iNKT cells of the innate immune system.

Materials and Methods

Mice

C57 BL/6 Spi6 KO (24), C57BL/6 GrB KO (24), and control wild-type (Wt) (purchased from Charles River Laboratories, Margate, U.K.) were bred and maintained under the appropriate conditions in the Imperial College animal facility. Mice were injected i.p. with 2 μg of PBS-57 and control mice were treated with PBS. Mice were infected with lymphocytic choriomeningitis virus (LCMV) Armstrong (2 × 10³ PFU/mouse i.p.)(24). All studies were approved by the Home Office (London, U.K.). PBS-57 (9) is kindly provided by Paul Savage (Brigham Young University, Provo, UT).
**Cell preparation**

Splenocytes were prepared as described previously (24). Perfused liver was minced, processed through stainless steel mesh, and suspended in RPMI 10 (Invitrogen, Paisley, U.K.) supplemented with 5% FBS (Invitrogen). After washing twice with medium, the pellet was resuspended in 5 ml medium and passed through a 70-μm cell strainer. Cells were washed and resuspended in 40% Percoll (GE Healthcare, Chalfont St. Giles, U.K.) and overlaid on 70% Percoll solution, then centrifuged at 2000 rpm for 20 min at room temperature. Interface layer containing mononuclear cells were removed carefully and washed. Cell pellets were treated with RBC lysis buffer (eBiosciences, San Diego, CA), and washed twice with and resuspended in medium.

**Flow cytometry**

PBS-57 loaded/CD1d tetramer allophycocyanin was provided by the National Institutes of Health Tetramer Facility (Atlanta, GA). The following Abs were used: anti-mouse FITC or PE TCRβ and PE-NK1.1 from (BD Pharmingen, San Diego, CA), and PE-Cy7-CD3ε, Pacific Blue-CD24, allophycocyanin-CD44, allophycocyanin-NK1.1, PE Cy7, and Pacific Blue-CD69, FITC-CD1d, FITC-CD122 (eBiosciences), anti-human PE-GrB (Caltag Laboratories, Burlingame, CA), brefeldin A, PE-active caspase 3, Annexin V kit, and BrdU detection kit (BD Pharmingen). Cells were stained with CD1d tet and TCRβ, followed by intracellular staining (ICS) with rabbit anti-Spi6 antiserum (1/1000 dilution) as described previously (24). For GrB staining, cells were cultured for 2 h in complete RPMI 1640 medium in the presence of brefeldin A (BD Pharmingen). For ICS of Spi6, GrB, and active caspase 3 we used BD FACs/Permit reagents. Cells were analyzed on a Cyan ADP (DakoCytomation, Carpinteria, CA) flow cytometer and analyzed using FlowJo software (TreeStar, Ashland, OR). For annexin V staining, cells were surface stained with CD1d tet and TCRβ, washed, and labeled with either FITC-conjugated (BD Pharmingen) or Pacific Blue-conjugated (Invitrogen) annexin V, according to manufacturer’s protocol (BD Pharmingen Annexin V kit). For activated iNKT cells, mice were injected i.p. with BrdU in PBS (1 mg, BD Biosciences), then fed BrdU (0.8 mg/ml) drinking water, supplemented with 5% (weight/volume) glucose continuously for 3 d. Mice were fed with BrdU in drinking water for 4 d to study homeostatic proliferation. Cells were surface stained and BrdU staining was performed according to manufacturer’s protocol (BD Pharmingen BrdU Flow Kit).

**ELISA**

Serum was collected by tail vein bleeding, 24 h prior to (prebleed), and 2 h postinjection with PBS-57 and stored at −80˚C for further analysis. Collected samples were then heated to 37˚C for 10 min and centrifuged at 5000 × g for 5 min, and the resulting supernatant extracted for use in a multiplex sandwich ELISA detection for mouse IL-4 and IFN-γ (Luminex, Austin, TX/Invitrogen). This was performed under the manufacturer’s instructions. Serum concentrations were calculated from standard curves with both cytokines. The level of IL-4 in serum from prebleeds was 200 pg/ml (Wt) and <100 pg/ml (Spi6 KO), and for IFN-γ 200 pg/ml (Wt) and <50 pg/ml (Spi6 KO).

**Confocal immunofluorescence microscopy**

Spleens were harvested on day 3 after Ag challenge, stained with CD1d tet and CD3ε and purified on a Dako MoFlo (>90% pure), iNKT cells were subjected to ICS for GrB as cytopsins and examined with a Leica TCS SP5 confocal microscope. To localize nucleus cells were mounted with DAPI-containing medium (Vectashield, Vector Laboratories, Burlingame, CA).

**Statistical analysis**

Data are presented as mean ± SEM. Statistical analysis was performed by Student t test. All analyses were performed using GraphPad Prism 5 software (GraphPad, San Diego, CA).

**Results**

**Normal development of iNKT cells in Spi6 KO mice**

Spi6 is highly expressed by cytotoxic lymphocytes, such as CTL and NK cells, that kill using GrB (22, 25). To determine whether GrB-containing iNKT cells also express Spi6, we performed ICS on Wt and Spi6 KO cells with our anti-Spi6 antiserum (25). We found iNKT cells constitutively express Spi6 (Fig. 1A). The mean fluorescence intensity (MFI) of anti-Spi6 staining was ∼14-fold higher in Wt compared with Spi6 KO iNKT cells (Fig. 1B). To study the role of Spi6 in the development, maturation, and organ distribution

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**FIGURE 1.** Spi6 KO iNKT cell development and homeostatic proliferation. Naïve Wt and Spi6 KO thy, spl, and liver mononuclear cells were surface stained with PBS-57 loaded/CD1d tetramer and TCRβ. A, Splenocytes were subjected to ICS with rabbit anti-Spi6 antiserum and preimmune serum. The MFI (± SEM) of anti-Spi6 staining is indicated. B, The MFI of anti-Spi6 staining was subtracted for preimmune serum staining and was ∼14 times higher for Wt than for Spi6 KO iNKT cells. n = 5 mice. C, Dot plot showing percentage of CD1d tet+TCRβ+ iNKT cells (indicated top left corner) in the thy, spl, and liv. D, Mean percentage (± SEM) of iNKT cells. E, iNKT cell homeostatic proliferation in BM, thy, spl, and liv. n = 5 mice. Dot plots showing BrdU incorporation during S-phase after 7-AAD staining. F, Mean percentage (± SEM) of BrdU+iNKT cells. n = 5 mice. Data are representative of at least two to three independent experiments. *p < 0.0001. thy, liver; spl, spleen; thy, thymus.
of iNKT cells, a comprehensive phenotypic analysis was performed in Spi6 KO mice. We found that the thymic development and maturation of iNKT cells, as shown by the expression of heat stable Ag (CD24), CD44, and NK1.1, was unaffected in Spi6 KO mice (Supplemental Fig. 1). Moreover, the levels of iNKT cells (CD1d+ TCRβ+) in the in thymus, spleen, and liver were also not affected in Spi6 KO mice (Fig. 1C, 1D). iNKTs display an activated phenotype as indicated by the expression of activation markers, such as CD122, and undergo cell division in the absence of overt antigenic stimulation (2, 5). We found the activation status of iNKT cells, as indicated by CD122 expression, was unaffected in Spi6 KO mice (Supplemental Fig.1D). Furthermore, the proliferation of Spi6 KO iNKT cells, as measured by BrdU incorporation into the DNA of S-phase cells, was also unaffected (Fig. 1E, 1F). We conclude that Spi6 deficiency has no major effect on the development, distribution, and homeostasis of iNKT cells in the absence of overt Ag stimulation.

GrB mediates activation-induced apoptosis of Spi6 KO iNKT cells

We have shown previously that Spi6 protects CTL from apoptosis by binding to and destroying GrB in the cytoplasm after it leaks from cytotoxic granules (24). To determine whether Spi6 similarly protects iNKT cells, we measured the level of GrB in Ag-stimulated Spi6 KO iNKT cells. Wt and Spi6 KO mice were challenged with PBS-57 and the level of intracellular GrB measured in stimulated Spi6 KO iNKT cells. Wt and Spi6 KO mice were challenged with PBS-57 and the level of intracellular GrB measured in iNKT cells after 2 h by ICS with anti-GrB Ab. Flow cytometry revealed that after activation, all Wt iNKT cells stained positive for GrB with ~20% expressing high levels of GrB (GrBhi) (Fig. 2A). There were ~2-fold more GrBhi iNKT cells from Spi6 KO compared with Wt (p = 0.02) (Fig. 2B). Therefore, Ag-stimulated Spi6 KO iNKT cells harbor increased levels of GrB presumably because Spi6 acts to inhibit and destroy misdirected GrB in the cytoplasm (24).

Stimulation in vivo of the TCR with glycolipid Ag or anti-CD3e Ab induces the apoptosis of iNKT cells (10, 11, 26). We detected apoptosis of iNKT cells by measuring the expression of phosphotidyl serine by annexin V binding. After challenge with PBS-57 in both the liver (p = 0.03) and the spleen (p = 0.04) of Spi6 KO mice, we observed a significant increase in the percentage of annexin V+ iNKT cells compared with Wt (Fig. 2C, 2D). Staining for the CD69 activation marker revealed the activation status of Spi6 KO iNKT cells was no different to Wt (Fig. 2E). We conclude that Spi6 KO iNKT cells undergo increased apoptosis after Ag stimulation because of increased levels of GrB.

To determine the functional consequences of the increased apoptosis of Spi6 KO iNKT cells, we measured the production of the cytokines IFN-γ and IL-4 after challenging Spi6 KO mice with PBS-57 (2–7). The levels of both IFN-γ and IL-4 were dramatically reduced to barely detectable levels (>100-fold decrease) in the serum of Spi6 KO mice compared with Wt controls 2 h after PBS-57 challenge (Fig. 2F). In fact, the levels of IL-4 and IFN-γ in Spi6 KO mice were no higher than the levels observed in prebleeds before PBS-57 challenge. Therefore, there was essentially a complete loss in the production of IL-4 and IFN-γ by iNKT cells in Spi6 KO mice after Ag challenge. We conclude that protection from GrB-mediated death by Spi6 is required for immunoregulatory function of iNKT cells.

Clonal burst of Spi6 KO iNKT cells is impaired after glycolipid Ag activation

We next examined the effect of the increase in self-inflicted damage on the response of iNKT cells to Ag stimulation in vivo. Challenge of Wt mice with PBS-57 resulted in the rapid expansion of iNKT cells with a peak of response (clonal burst) on day 3, followed by a steep decline (contraction) in the frequency of iNKT cells before returning to the original level on day 6 in both the spleen (Fig. 3A) and the liver (Fig. 3B) (11–13). In both the spleen and the liver, we observed a >2-fold decrease in the frequency and absolute number of iNKT cells in Spi6 KO mice at the clonal burst on day 3 (Fig. 3A, 3B). Therefore, Spi6 KO mice have an impaired clonal burst of iNKT cells.

To determine the cause of the impaired clonal burst, we examined GrB-mediated apoptosis of Spi6 KO iNKT cells on day 3 after PBS-57 challenge. As we observed for 2 h after activation...
there was about a 2-fold increase in the percentage of GrBhi Spi6 KO iNKT cells during the clonal burst on day 3 after Ag challenge (Fig. 3C). We next examined the activity of caspase 3, which is a key mediator of apoptosis activated by GrB (16), using fluorogenic substrates and flow cytometry. The activity of caspase 3 was ∼2-fold higher in iNKT cells from Spi6 KO mice compared with Wt mice (Fig. 3D). Therefore, increased apoptosis is a likely cause of the decreased clonal burst size of Spi6 KO iNKT cells.

To rule out the possibility of defective proliferation as a reason for impaired iNKT cell expansion, we performed BrdU labeling to measure proliferation on day 3 after challenge with PBS-57. The proportion of proliferating iNKT cells in Spi6 KO mice was no different to Wt mice (Fig. 3E). Consistent with its specificity for the TCR of iNKTs (9), PBS-57 did not drive the expansion of either CD8 T cells or NK cells, which in turn were not affected in challenged Spi6 KO mice (Supplemental Fig. 2D). In addition, the level of the CD1d molecule, which presents Ag to iNKT, was the same as Wt on Spi6 KO bone marrow (BM) cells, thymocytes, and splenocytes (Supplemental Fig. 1D). Therefore, impaired Ag-presentation is unlikely to be responsible for the diminished expansion of Spi6 KO iNKT cells. We conclude that an increase in GrB-mediated apoptosis was the cause of the reduced clonal burst of Ag-stimulated Spi6 KO iNKT cells.

Clonal burst of Spi6 KO iNKTs is not impaired after TCR-independent activation

To further investigate the stimulation requirements for GrB-mediated loss of iNKTs, we infected Spi6 KO mice with LCMV to examine bystander activation (27–30). As others have reported (28, 29), we observed a decrease in the percentage of iNKTs cells in the spleens of Wt mice after acute infection with LCMV (Fig. 4A, B). However, when we examined absolute cell number in multiple mice, we observed an increase on day 8 of not only LCMV-specific CD8 T cells but also iNKT cells of Wt and Spi6 KO mice.

**FIGURE 3.** Clonal burst of Spi6 KO iNKT after glycolipid Ag challenge in vivo. Wt and Spi6 KO mice were injected i.p. with PBS-57 (2 μg) and iNKT cells harvested on day 0, day 3, and day 6. n = 3–5 mice, n = 4–5 mice, and n = 2–4 mice, respectively. Percentage of CD1d tet+ iNKT cells in spl (A) and liv (B) is indicated by the number in the FACS plots. Mean percentage and absolute number of iNKT cells are shown in A and B. C, Mean percentage of GrBhi and (D) active caspase 3+iNKT cells (CD1d tet+) from day 3 after PBS-57 challenge. E, Ag-challenged iNKT cell proliferation on day 3. n = 3–5 mice. FACS plot shows the S-phase DNA as shown by BrdU labeling. Mean percentage of BrdU incorporation in unstimulated and PBS-57-stimulated BM, thy, spl, and liv iNKT cells of Wt and Spi6 KO mice. *p < 0.05; **p < 0.001. liv, liver; spl, spleen; thy, thymus.

**FIGURE 4.** Response of Spi6 KO iNKT cells to acute LCMV infection in vivo. Wt and Spi6 KO mice were infected with LCMV. Splenocytes were harvested at indicated time and stained for iNKT and CD8 T cells using specific Abs. A, Percentage of iNKT and CD8 T cell population is shown in FACS plot on days 0, 3, and 8. B, Mean percentage and absolute number of iNKT and CD8 T cells are shown. Data shown as mean percentage (± SEM) and absolute number. n = 5 mice. **p < 0.05; ***p < 0.001.
but also of bystander-activated iNKTs (Fig. 4D, 4E). We think the decrease in the percentage of splenic iNKTs is likely due to dilution by LCMV-specific CD8+ T cells. As we reported previously (24, 25), we observed a severe reduction in the size of the CD8+ T cell clonal burst in Spi6 KO mice on day 8 after LCMV infection (Fig. 4C, 4E). However, there was no difference in the number of iNKT cells in Spi6 KO mice after LCMV infection (Fig. 4D). Thus, TCR-independent stimulation of NKT cells did not result in the reduced survival of Spi6 KO iNKTs.

**Leakage of GrB from cytotoxic granules of Spi6 KO iNKT cells**

Spi6 not only protects CTL from GrB-mediated apoptosis but also ensures the integrity of cytotoxic granules (24). To determine whether Spi6 also protects iNKT cells in this way, iNKT cells were purified from Spi6 KO mice by FACS after PBS-57 challenge. Spi6 KO iNKT cells were then stained for GrB and examined by confocal immunofluorescence microscopy (CIM). We observed the characteristic punctate pattern of Ab staining indicative of distribution of GrB to cytotoxic granules in Wt iNKT cells (Fig. 5). In Spi6 KO iNKT cells, we observed a more diffuse pattern of Ab staining indicative of increased distribution of GrB to the cytoplasm. Therefore, Spi6 prevents the leakage of GrB into the cytoplasm of iNKT cells.

**Clonal burst deficit of Spi6 KO iNKT cells is rescued by GrB deficiency**

We determined whether inhibition of GrB was a physiological mechanism by which Spi6 protected Ag-stimulated iNKT cells from apoptosis. To do this, we examined the effect of GrB deficiency on iNKT cells in Spi6 KO×GrB cluster deficient (GrB KO) mice (19, 24). In Spi6 KO×GrB KO mice, the size of the clonal burst of iNKT cells after PBS-57 challenge was rescued to the Wt level (Fig. 6A, 6B). The proportion of iNKT cells undergoing apoptosis in Spi6 KO×GrB KO mice, as evidenced by the expression of activated caspase 3 was correspondingly reduced (Fig. 6C). In addition, the levels of iNKT cells (Supplemental Fig. 3) were also corrected in single GrB KO mice compared with Spi6 KO mice. Thus, the absence of GrB corrected the deficit in iNKT cell survival caused by Spi6 deficiency and returned the level of iNKT cells to Wt levels. Therefore, we conclude that protection from GrB is at least in part a physiological mechanism by which Spi6 ensures the survival of iNKT cells after Ag-stimulation.

**Discussion**

iNKT cells share many effector properties with peptide/MHC-specific classical T cells. However, compared with both CD8+ and CD4+ effector T cells, the role of apoptosis in the homeostasis of activated iNKT cells is poorly understood. We provide evidence for a physiological role for Spi6 for protecting iNKT cells from self-inflicted apoptosis caused by GrB. Our findings support a new mechanism in which the size of the clonal burst of iNKT cells is determined by protection from GrB-mediated apoptosis.

We have demonstrated previously that Spi6 protects classical CTL from GrB-mediated apoptosis and thereby determines the size of the clonal burst postinfection with either LCMV or *Listeria monocytogenes* (24). Proteinase inhibitor 9 (PI9) is a human homolog of Spi6.
that, as well as being a potent inhibitor of intracellular GrB, is also expressed in cytotoxic lymphocytes (31). A previous study demonstrated GrB-mediated activation-induced cell death of human NK cells but did not directly demonstrate a physiological requirement for PI9 in cell survival (32). Using Spi6 KO mice, we are able to directly demonstrate a physiological requirement of GrB inhibition by Spi6 in determining the viability of a cytotoxic cell of the innate immune system.

iNKT cells perform important immunoregulatory function through the rapid production of cytokines, such as IFN-γ and IL-4, after recognizing glycolipid Ag (2–7). Through this activity, iNKT cells are thought to provide an important link between innate Ag recognition and the recruitment of Th1 (IFN-γ) and Th2 (IL-4) arms of adaptive immunity. Therefore, the dramatic impairment in the ability of Spi6 KO mice to produce IFN-γ and IL-4 after PBS-57 challenge (Fig. 2F) strongly suggests that Spi6 is required for iNKT cell function by ensuring their viability.

We show that Spi6 is a physiological inhibitor of GrB in iNKT cells (Figs. 2, 3, 5). As we observed for CTL, Spi6 ensured the integrity of cytotoxic granules and prevented GrB leakage into the cytoplasm of iNKT cells (Fig. 5). Interestingly, classical Wt CTLs appear to selectively leak GrB, but not granzyme A or perforin, from their granules (24, 33). Given that Spi6 is specific for GrB, this may explain the nonredundant role for Spi6 in protecting CTL and iNKT cells from self-inflicted damage caused by the break- down of cytotoxic granules. However, how the increased activity of cytoplasmic GrB causes the breakdown of cytotoxic granules remains to be determined.

Spi6 is not a physiological inhibitor of Fas-induced apoptosis (22) and so the increase in apoptosis of Spi6 KO iNKT cells is likely to be exclusively through increased GrB levels and activity. This is confirmed by the observations that both the decreased clonal burst of iNKT cells in Spi6 KO mice is rescued by GrB deficiency in Spi6 KO×GrB KO mice (Fig. 6). Therefore, the GrB pathway of iNKT cell homeostasis is therefore likely to be distinct to the Fas–FasL pathway of iNKT activation-induced cell death (15).

iNKT cell can be activated in a TCR-independent fashion by microbes, such as viruses, through inflammatory cytokines, such as type I IFN, IL-12, IL-18, and IFN-γ (34). However, our findings support the view that stimulation through the TCR is needed to induce GrB-mediated death of iNKT cells. This is because we did not observe any decrease in the level of iNKT cells after TCR-independent stimulation with LCMV (Fig. 4). It was only after direct stimulation through the TCR by PBS-57 did we observe an increase in GrB-mediated apoptosis of Spi6 KO iNKT cells (Figs. 2, 3, 6). Our results suggest that TCR-mediated mobilization of GrB is required for death through misdirected GrB. Although it is not clear how GrB reaches the cytoplasm of iNKT cells, our CSM studies indicate that the breakdown of cytotoxic granules on TCR activation is probably involved (Fig. 5). In contrast, classical NK cells, which constitutively express GrB, appear to undergo apoptosis in the absence of overt Ag stimulation (32).

Spi6 was not required for the development or survival on iNKT cells in the absence of Ag stimulation. This is in contrast to the recent finding that the Id2 transcriptional regulator determines the level of unstimulated iNKT cells by ensuring survival in the liver (35). Spi6 is transcriptional target of Id2 and so it is possible that Id2 (36) also protects iNKT cells from activation-induced apoptosis. Deficiency in the proapoptotic member of the Bcl-2 family, Bim could correct the defect in functional maturation and survival of Id2-deficient iNKT cells (35). In addition, Bim deficiency can correct activation induced apoptosis of iNKT cells (11). Therefore, it would seem that Bim is involved in the apoptosis of iNKT cells during development and after Ag stimulation, whereas, GrB exclusively induces apoptosis after TCR stimulation. The PI9 human homolog of Spi6 is a potent inhibitor of intracellular GrB and is also expressed in cytotoxic lymphocytes (31). Therefore, the expansion of iNKT cells in clinically relevant diseases may also be controlled by the protection from self-inflicted damage by natural endogenous inhibitors of GrB.

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Disclosures
The authors have no financial conflicts of interest.

References


**iNKT cell development and maturation.** Naive wt and Spi6 KO thymic iNKT cells were gated for expression of CD24 (heat stable antigen) and CD44 and NK1.1 (upper panel, B). (A) Histogram showing % of CD24+ iNKT cells in thymus. (B) Dot plot and mean percentage showing different stages of thymic iNKT cell development. Stage 1, 2 and 3 of developing iNKT cells based on CD44 and NK1.1. Middle and lower panel represent frequency of matured (CD44+ NK1.1+) iNKT in spleen and liver. Mean percentage of CD44+ NK1.1+ iNKT cells of spleen and liver is shown in B. C, Expression CD122 (IL-2Rβ) expression on iNKT cell gated. (D). Levels of glycolipid antigen presenting molecule CD1d expression on BM, thy, spleen and liver cells.
Levels of CD8⁺ T cells and NK cell after stimulation with PBS-57.

PBS-57 stimulated splenic CD8 T cell and NK cell expansion at peak of response (day 3). Mean percentage (± sem, n=3-5 mice).
Levels of iNKT cell after stimulation with PBS-57.

PBS-57 stimulated iNKT cell expansion at peak of response (day 3). Mean absolute number (± sem, n=5 mice).