Differential Regulation of GM1 and Asialo-GM1 Expression by T Cells and Natural Killer (NK) Cells in Respiratory Syncytial Virus Infection

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Abstract

We previously reported that respiratory syncytial virus (RSV) infection increases lung CD8⁺ T cell GM1 expression. The related lipid asialo-GM1 (ASGM1) is expressed by T cells in viral infection and by natural killer (NK) cells. The *in vivo* co-expression of GM1 and ASGM1 by immune cells is not defined. Here we analyzed lung lymphocyte GM1 and ASGM1 expression in RSV-infected mice. GM1 and ASGM1 were coordinately up-regulated by activated CD8⁺ T cells in RSV-infected BALB/c and C57BL/6 mice. In contrast, RSV infection had no effect on constitutively high NK cell GM1 expression, while increasing NK cell ASGM1 expression. GM1 and ASGM1 co-localized in lipid raft structures in NK and CD8⁺ T cells sorted from the lungs of RSV-infected mice. Anti-ASGM1 Ab treatment of RSV-infected BALB/c mice depleted GM1/ASGM1-expressing NK cells and GM1/ASGM1-expressing T cells, reduced lung IFN- γ levels, increased viral load, delayed viral clearance, and reduced illness. STAT1^{-/-} mice are more susceptible to RSV replication and disease than wild-type mice. In RSV-infected STAT1^{-/-} mice, anti-ASGM1 Ab altered cytokine levels, but in contrast to BALB/c mice, antibody treatment had no effect on viral load or illness. Taken together, GM1 and ASGM1 expression are differentially regulated by T and NK cells in RSV infection. Also, GM1/ASGM1-expressing cells are important for control of RSV in BALB/c mice, whereas STAT1^{-/-} mice clear RSV by an alternative pathway.

Introduction

Respiratory syncytial virus (RSV) is the chief cause of bronchiolitis and viral pneumonia in infants. In the U.S., RSV-induced bronchiolitis results in >100,000 infant hospitalizations per year (1). In humans and animal models, the cellular immune response is thought to be critical for immunopathogenesis (2). Flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) from infants hospitalized due to RSV infection revealed an increase in natural killer (NK) cells, and RSV stimulation of PBMCs induced a significant percentage of T cells to express IFN- γ , IL-4, and IL-13 (3). NK and T cells likely play important roles in RSV disease.

Virtually all children are infected with RSV by age 2 y (4). The most abundant cytokine in clinical specimens from RSV-infected children is IFN- γ , and most RSV infections likely resolve via a typical antiviral Th-1-type response (2). In BALB/c mice, RSV A2 strain infection causes an increase in the number of IFN- γ -producing NK cells in bronchoalveo-

lar lavage fluid (BALF) that precedes the BALF appearance of abundant IFN- γ -producing CD8 cells and cytotoxic T lymphocytes (CTLs) (5–7). Both CD4⁺ and CD8⁺ T cells exacerbate RSV-induced illness and contribute to virus clearance in primary infection of BALB/c mice (8). RSV-specific CTLs abolish virus load in the lung, induce immunopathology, and IFN- γ plays a role in these processes (9–11).

Approximately 1% of RSV-infected infants in the U.S. develop serious lower respiratory tract disease requiring hospitalization (12). Severe RSV disease in infants has been associated with Th-2-type immune responses (13). Prematurity is the greatest risk factor for severe RSV disease in infants. Premature infants have fewer IFN- α -producing PBMCs than term infants and adults (14,15). STAT1^{-/-} mice are deficient in transcriptional responses to type I (α/β) and type II (γ) IFN and are susceptible to viral disease (16). STAT1 deficiency results in pleiotropic immune defects, including deficient NK and T cell responses (17–19). STAT1^{-/-} mice provide a model of severe RSV pathogenesis. Compared to BALB/c mice,

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STAT1^{-/-} mice exhibit an enhanced Th-2 response with exacerbated illness including pulmonary mucus and airway dysfunction in response to primary RSV A2 strain infection (20,21). In this paper, we study mechanisms of the immune response to RSV in the settings of an antiviral Th-1 response (BALB/c) and a Th-2 response with enhanced disease (STAT1^{-/-}).

Glycosphingolipids (GSLs) are highly diverse components of the outer leaflet of cell membranes. GSLs contain a ceramide backbone anchored in the membrane and a hydrophilic oligosaccharide chain that forms the extracellular headgroup; and more than 300 different GSL headgroup chains have been characterized (22,23). GSLs that contain sialic acid residues in the headgroup, such as GM1, are called gangliosides (22,23). GSLs pack densely together with cholesterol to form lipid microdomains (i.e., lipid rafts or caveolae) because ceramide is highly saturated and because of hydrogen bonding between headgroups (22). Lipid rafts have affinity for signaling proteins (e.g., transmembrane receptors, G proteins, and RhoA) and thus serve as cell surface signaling platforms, "glycosynapses" (24,25). In lymphocytes, lipid rafts cluster to form the immunological synapse (26,27). Signaling proteins can be associated with or excluded from lipid rafts, therefore lipid rafts confer spatial organization to supramolecular cell surface signaling complexes (28). The content and organization of GSLs in membranes is tightly regulated (29,30). GSLs found in lipid rafts include GM1 and asialo-GM1 (ASGM1) (29,31–33).

In addition to being a component of lipid rafts, ASGM1 has been used as a marker for NK cells. Anti-ASGM1 Ab has been used to deplete NK cells in mice (34–44). However, it has been known for some time that ASGM1 can be upregulated on leukocytes other than NK cells in the context of viral infection (45). ASGM1 can be expressed on the surface of T cells, and anti-ASGM1 Ab consequently depletes T cells and reduces CTL activity (46–49).

GM1 and lipid rafts play a central role in RSV biology. At the level of the virus life cycle, RSV replication machinery colocalizes with lipid rafts, and virions are selectively assembled at and likely bud from lipid rafts (50–54). GM1 is incorporated into the RSV viral envelope (55). At the level of the host immune response, RSV-specific and IFN- γ -expressing CD8⁺ T cells have increased GM1 expression *in vivo*, and decreased T-cell GM1 expression in the context of RSV infection was associated with reduced viral clearance and reduced disease severity (56). Defining the role of GSLs in RSV infection and pathogenesis may lead to targeted therapies aimed at reducing viral replication and/or ensuing immunopathology.

We previously showed that activated CD8⁺ T cells in the lungs of RSV-infected mice have increased cell surface GM1 expression (56). We hypothesized that GM1 and ASGM1 are coordinately expressed by NK and T cells. Here we report the cell surface expression and co-localization of the GSLs GM1 and ASGM1 in whole lung lymphocytes isolated from mock-infected and RSV-infected mice. We also investigated the role of GM1/ASGM1-expressing cells in RSV pathogenesis in BALB/c and STAT1^{-/-} mice.

Materials and Methods

Virus and mice

The A2 strain of RSV was provided by R. Chanock (National Institutes of Health, Bethesda, MD). Viral stocks were

grown in HEp-2 cells, and stock titers were determined by plaque assay in HEp-2 cells (57). Female BALB/c mice (Charles River Laboratories, Wilmington, MA) and C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were purchased. STAT1^{-/-} mice on a BALB/c background are maintained in specific pathogen-free conditions (21). Eight- to 10-week-old mice were lightly anaesthetized and infected intranasally with A2 strain RSV or with mock-infected cell culture supernatant in a volume of 0.1 mL of Eagle's MEM (57).

Flow cytometry

Lungs were harvested, homogenized through a cell strainer, and mononuclear cells were isolated by Ficoll-Hypaque cushion centrifugation. Cells were counted then treated and stained for cell surface molecules (CD3, CD4, CD8, and CD49b) and intracellular IFN- γ as previously described (56). For intracellular cytokine staining, cells were incubated in RPMI/10% heat-inactivated FBS/1 μ M ionomycin (Sigma, St. Louis, MO), 10 ng/mL PMA (Sigma), 7 μ L/10 mL Golgi Stop (BD Pharmingen, San Jose, CA) for 6 h at 37°C and 5% CO₂. Rabbit polyclonal anti-ASGM1 Ab (Wako Chemicals USA, Richmond, VA) and isotype control Ab (rabbit gamma globulin; Jackson ImmunoResearch, West Grove, PA) were labeled with PE-conjugated anti-rabbit IgG Fab fragments according to the manufacturer's protocol, and 1 μ g of Ab was used per 2 $\times$ 10⁵ cells (Zenon Rabbit IgG Labeling Kit; Molecular Probes, Eugene, OR). We used 0.1 μ g of FITC-labeled cholera toxin B-subunit (CtxB-FITC, Sigma-Aldrich, St. Louis, MO) per 2 $\times$ 10⁵ cells to stain GM1 as previously described (56). RSV-specific CD8⁺ T cells were stained with RSV M2 protein-specific PE-labeled H-2K^d tetramers (RSV-tetramer), or influenza NP-specific PE-labeled H-2K^d tetramers (flu-tetramer; Beckman Coulter, Fullerton, CA) as a negative control as previously described (56). Samples were analyzed using a LSR II flow cytometer (BD Biosciences, San Jose, CA), and 2–5 $\times$ 10⁴ live lymphocytes per lung were analyzed based on forward and side-scatter properties. Data were analyzed using WinMDI or FlowJo software. The total number of cells of a defined population (e.g., CD3⁺CD8⁺) per lung was obtained by multiplying the percentage of total events gated by the number of mononuclear cells counted with a hemacytometer.

FACS and fluorescence microscopy

Whole-lung lymphocytes were isolated from mock-infected and RSV-infected BALB/c mice as described above. Cells were pooled from 4–6 mock-infected and 4–6 RSV-infected mice and stained for 30 min at 4°C with anti-CD49b, anti-CD3, and anti-CD8a. The cells were washed twice and resuspended in 1 $\times$ PBS/3% FBS. NK (CD49b⁺CD3⁻) and C8⁺ T (CD3⁺CD8⁺) cells from mock-infected and RSV-infected mice were sorted into 100% FBS using a FACS Aria cell sorter (BD Biosciences). Post-sort flow cytometric analyses indicated that the purity of sorted NK and CD8⁺ T cells was >99%. The sorted cells were washed with 1 $\times$ PBS/3% FBS, stained with anti-ASGM1 and CtxB-FITC as described above, washed again, and fixed in 1 $\times$ PBS/1.5% FBS/2% paraformaldehyde. The following day, a portion of the cells were analyzed by flow cytometry, and a portion of the cells were analyzed by fluorescence microscopy. For flow cyto-

metric analysis, sorted cells were analyzed with a LSR II flow cytometer (BD Biosciences) equipped with a 532-nm laser. FITC was excited with a 488-nm laser, and PE was excited with the 532-nm laser, resulting in no spectral overlap requiring compensation.

For fluorescence microscopy, the cells were analyzed in solution in 35-mm Petri dishes with 14 mm poly-d-lysine-coated microwells (MatTek, Ashland, MA) using a Zeiss LSM510 META laser-scanning confocal microscope with a 63 $\times$ objective. Controls for fluorescence microscopy included unstained unsorted whole-lung lymphocytes, sorted NK and CD8⁺ T cells that were not stained with either PE-anti-ASGM1 or CtxB-FITC, and sorted NK and CD8⁺ T cells that were stained with either PE-anti-ASGM1 or CtxB-FITC. Spectral overlap between PE and FITC was controlled using the emission fingerprinting technique, whereby a META detector is used to collect images over a series of emission wavelengths (lambda stacks), and the relative contributions of PE and FITC are determined by linear unmixing based their known spectra. For each sample, 9–12 sequential images in the vertical plane (Z-stacks) were recorded for 3–5 fields. Images were analyzed using Zeiss LSM Image Browser software.

In vivo anti-ASGM1 treatment

Mice harvested at 6 days post-infection (d.p.i.) were injected IP with 1.5 mg of anti-ASGM1 or 1.5 mg of control Ab (rabbit gamma globulin) diluted in 0.2 mL of PBS on -2, -1, 0, 2, and 4 d.p.i. Mice harvested at 8 d.p.i. and mice in RSV-induced weight loss experiments also received 1.5 mg of Ab on day 6 p.i.

Quantitation of virus and cytokines in lung tissue

Lungs were removed, and infectious RSV was titrated by plaque assay in HEP-2 cells as previously described (57). The lungs were individually ground with a precooled mortar and pestle and sterile ground glass. Glass and tissue debris were removed from lung homogenates by centrifugation (15 min at 2000 rpm). Supernatants were immediately assayed in triplicate and frozen for later use. IFN- γ and IL-13 were quantified in lung homogenates using ELISA kits (R&D Systems, Minneapolis, MN).

Statistical analysis

Results are expressed as mean $\pm$ standard error of the mean (SEM). *p* values were determined by a two-tailed *t*-test, assuming equal variance. Data shown represent at least three replicate experiments with reproducible findings.

Results

Effect of RSV infection on NK and CD8⁺ T cell ASGM1 and GM1 surface expression

We previously showed that RSV infection increases GM1 expression on the surface of activated lung CD8⁺ T cells at 6 and 8 d.p.i. (56). GM1 and ASGM1 are GSLs associated with lipid rafts and cell activation (30,33,58,59). We hypothesized in the current study that ASGM1 and GM1 are coordinately expressed on NK and CD8⁺ T cells in RSV infection. We first analyzed ASGM1 cell surface expression on

NK and T cells isolated from mock-infected and RSV-infected BALB/c mice. We define NK cells in BALB/c mice as CD49b⁺ (DX5⁺) and CD3⁻ (60). In time course flow cytometric studies (at 3, 4, 5, 6, 7, and 8 d.p.i.), we found that the peak of NK cell numbers in the lungs of RSV-infected mice was 4–6 d.p.i. (data not shown). We chose 6 d.p.i. for our experiments because it represents the NK cell response, the peak day of IFN- γ levels, and the onset of the T-cell response with large numbers of GM1^{high} CD8⁺ T cells in the lung (21,56).

Compared to the isotype control, NK cells in the lungs of mock-infected mice expressed low levels of ASGM1, and T cells in the lungs of mock-infected mice were ASGM1-negative (Fig. 1A, B, and C). RSV infection resulted in a significant increase in the percentage of NK and CD8⁺ T cells that were ASGM1⁺ at 6 d.p.i. (Fig. 1A, B, and D). ASGM1⁺CD8⁺ T cells had threefold higher mean fluorescence intensity (MFI) staining than ASGM1⁺NK cells in RSV-infected mice. RSV infection increased ASGM1 expression on pulmonary NK and CD8⁺ T cells, but not CD4⁺ T cells. (Fig. 1).

We previously showed that RSV infection increases CD8⁺ T-cell GM1 expression (56). RSV infection increased NK and CD8⁺ T-cell ASGM1 expression (Fig. 1). We hypothesized that RSV infection increases NK cell GM1 expression. We analyzed GM1 expression on NK cells in mock-infected and RSV-infected mice at 6 d.p.i. NK cells in mock-infected mice expressed high levels of GM1 (Fig. 2A and B). Among gated lymphocytes from the lungs of mock-infected mice, NK cells had the highest GM1 expression (Fig. 2A). Contrary to our hypothesis, RSV infection had no effect on NK cell surface GM1 levels (Fig. 2A and B).

We tested whether ASGM1 and GM1 are co-expressed on NK and CD8⁺ T cells in RSV-infected mice. In the live lymphocyte-gated populations, GM1^{high} cells had high or intermediate levels of ASGM1 (Fig. 2C). ASGM1⁺ NK cells in mock-infected and RSV-infected mice were GM1^{high} (Fig. 2C). Strikingly, RSV infection induced the coordinate up-regulation of ASGM1 and GM1 by CD8⁺ T cells (Fig. 2C). RSV infection increased the percentage of NK and CD8⁺ T cells in the lungs that were ASGM1⁺GM1^{high} (Fig. 2D). Thus, in response to RSV infection, NK cells increased ASGM1 expression while maintaining high GM1 expression. CD8⁺ T cells coordinately increased ASGM1 and GM1 expression in response to RSV infection.

GM1 and ASGM1 as activation markers of lung NK and CD8⁺ T cells in RSV infection

We previously showed that IFN- γ -expressing CD8⁺ T cells in the lungs of RSV-infected mice are predominantly GM1^{high} (56). Here we analyzed ASGM1 and GM1 cell surface and intracellular IFN- γ levels in NK and CD8⁺ T cells from the lungs of mock-infected and RSV-infected mice at 6 d.p.i. The majority of IFN- γ -producing NK and CD8⁺ T cells isolated from the lungs of RSV-infected mice were ASGM1⁺ (Fig. 3A and B). We quantified the total number of ASGM1⁺IFN- γ ⁺ NK and ASGM1⁺IFN- γ ⁺ CD8⁺ T cells in the lungs of mock-infected and RSV-infected mice. RSV infection induced the accumulation of large numbers of ASGM1⁺IFN- γ ⁺ NK and ASGM1⁺IFN- γ ⁺ CD8⁺ T cells in the lungs at day 6 p.i. (Fig. 3C). We analyzed GM1 cell surface and intracellular IFN- γ levels in NK cells harvested from

FIG. 1. BALB/c mice were mock-infected ($n = 3$) or infected with 10^6 PFU of RSV ($n = 3$). Lung mononuclear cells were isolated at 6 d.p.i. and stained with anti-CD49b, anti-CD3, anti-CD8, anti-CD4, and either anti-ASGM1 or an isotype control (anti-rabbit IgG). Representative dot plots are shown for gated NK (A), CD8⁺ T (B), and CD4⁺ T (C) cells. The percentage of PE⁺ cells is indicated above the upper-right corner of each plot. The MFI of ASGM1 staining of NK cells in the circular gates is also indicated. (D) Percentage of the indicated gated cell populations that were ASGM1⁺ for mock-infected (open circles) and RSV-infected (filled circles) mice.

mock-infected and RSV-infected mice at 6 d.p.i. The majority of IFN- γ -expressing NK cells were GM1^{high}. Thus, as with ASGM1 (Fig. 3D), GM1 cell surface levels correlated with IFN- γ expression.

RSV-specific CD8⁺ T cells are GM1^{high} (56). As GM1^{high} CD8⁺ T cells are ASGM1⁺ (Fig. 2C), we hypothesized that RSV-specific CD8⁺ T cells are ASGM1⁺ GM1^{high}. Twenty-two percent of gated CD8⁺ T cells isolated from the lungs of RSV-infected mice at 6 d.p.i. were RSV-specific (Fig. 3F). Compared to tetramer-negative CD8⁺ T cells, RSV-specific CD8⁺ T cells coordinately expressed high levels of ASGM1 and GM1 (Fig. 3G and H). Taken together with our previous study, IFN- γ -expressing NK and CD8⁺ T cells in the lungs

of RSV-infected mice, as well as RSV-specific CD8⁺ T cells, are ASGM1⁺ GM1^{high} (Figs. 2 and 3) (56).

NK and CD8⁺ T-cell GM1 and ASGM1 expression in BALB/c and B6 mice

BALB/c mice are typically used for investigating RSV pathogenesis because this strain is relatively permissive to RSV replication (61). However, NK cells in BALB/c mice express a functional *Nkpr1* gene that differs from the B6 allele in that it does not contain the NK1.1 alloantigen and therefore is not recognized by anti-NK1.1 mAb (62). As NK1.1 is a standard NK cell marker in B6 mice and NK cell functions can differ between B6

FIG. 2. BALB/c mice were mock-infected ($n = 5$) or infected with 10^6 PFU RSV ($n = 5$). Lung mononuclear cells were isolated at 6 d.p.i. and stained with anti-CD49b, anti-CD3, anti-CD8, anti-ASGM1, and CtxB-FITC. (A) Representative dot plots of GM1 staining of gated lymphocytes and NK cells from mock-infected and RSV-infected mice. The percentage of GM1^{high} NK cells is indicated in the upper-right quadrant in the upper-right quadrant. (B) Percentage of NK cells that were GM1^{high} for mock-infected (open circles) and RSV-infected (filled circles) mice. (C) Representative dot plots of ASGM1 and GM1 staining of gated NK cells and CD8⁺ T cells from mock-infected and RSV-infected mice. The percentage of ASGM1⁺ GM1^{high} cells is shown in the upper-right quadrant. (D) Percentage of NK and CD8⁺ T cells that were ASGM1⁺ GM1^{high} for mock-infected (open circles) and RSV-infected (filled circles) mice.

and BALB/c mice, we tested whether GM1 and ASGM1 expression by NK and T cells differs between BALB/c and B6 mice. First, we determined the co-expression of CD49b and NK1.1 by NK cells in the lungs of RSV-infected B6 mice at 6 d.p.i. Among gated CD3-negative lymphocytes, 95% of CD49b-expressing cells also expressed NK1.1 (Fig. 4A). Thus, pulmonary NK cells in RSV-infected B6 mice were CD3-CD49b⁺NK1.1⁺. The percentage of NK cells in the lungs of RSV-infected BALB/c and B6 mice was equivalent (Fig. 4B). The pattern of increased GM1 expression by gated lung CD8⁺ T cells, but not NK cells, upon RSV infection was identical between BALB/c and B6 mice (Fig. 4C). The patterns of increased ASGM1 expression by gated lung NK and CD8⁺ T cells upon RSV infection was identical between BALB/c and B6 mice (Fig. 4C). Thus, constitutively high NK cell GM1 expression, increased lung CD8⁺ T-cell GM1 expression, and increased ASGM1 expression by lung NK and CD8⁺ T cells upon RSV infection was the same in BALB/c and B6 mice.

We also compared GM1 and ASGM1 expression by NK and T cells in the lung and spleen in these experiments. The percentage of lymphocytes in the spleens of mock-infected and RSV-infected BALB/c and B6 mice at 6 d.p.i. that were NK cells was 2–5% (data not shown). Splenic NK cells were ASGM1⁺GM1^{high}, and T cells were ASGM1⁻GM1⁻. RSV infection has no effect on the number of NK or T cells in the spleen at 6 d.p.i. or on GM1/ASGM1 expression by these cells in the spleen. These results were not surprising because the spleen is not a site of RSV infection.

NK and CD8⁺ T-cell GM1 and ASGM1 expression in RSV-infected STAT1^{-/-}

RSV-infected STAT1^{-/-} mice exhibit higher viral loads, greater disease severity, and enhanced Th-2-type inflammation compared to RSV-infected wild-type BALB/c mice (21,63). STAT1^{-/-} mice have impaired NK and T-cell cytotoxic functions (17,18). In addition to playing a role in antiviral IFN functions, STAT1 mediates antiproliferative effects on lymphocytes elicited by type I IFN (64–66). We previously showed that RSV-specific CD8⁺ T cells are predominantly GM1^{high} (56). Here, we tested whether STAT1 regulates NK and CD8⁺ T-cell regulation of GM1 and ASGM1 expression in RSV infection. As in BALB/c mice, RSV infection of STAT1^{-/-} mice resulted in increased ASGM1 expression by NK cells, whereas NK cell GM1 expression was constitutively high (Fig. 5). Similarly, RSV infection increased GM1 and ASGM1 expression by CD8⁺ T cells equivalently in BALB/c and STAT1^{-/-} mice (Fig. 5). Thus, increased GM1 and ASGM1 expression by NK and CD8⁺ T cells does not depend on STAT1 signaling.

ASGM1 co-localizes with GM1 in lipid raft-like structures on NK and CD8⁺ T cells sorted from the lungs of RSV-infected mice

Both ASGM1 and GM1 have been shown to accumulate in lipid rafts in the plasma membrane, and lipid rafts have

FIG. 3. BALB/c mice were mock-infected ($n = 5$) or infected with 10⁶ PFU RSV ($n = 5$). Lung mononuclear cells were isolated at 6 days p.i. and stained with anti-CD49b, anti-CD3, anti-CD8, anti-IFN- γ , and anti-ASGM1. (A) Representative dot plots show IFN- γ and ASGM1 staining of gated NK and CD8⁺ T cells. The percentage of cells in the ASGM1⁺IFN- γ ⁺, ASGM1⁺IFN- γ ⁻, and ASGM1⁻IFN- γ ⁺ quadrants is indicated. (B) Percentage of IFN- γ ⁺ NK and IFN- γ ⁺ CD8⁺ T cells that were ASGM1⁺ for mock-infected (open circles) and RSV-infected (filled circles) mice. (C) The total number of ASGM1⁺IFN- γ ⁺ NK and ASGM1⁺IFN- γ ⁺ CD8⁺ T cells in the lungs of mock-infected (open circles) and RSV-infected (filled circles) mice is shown. Each symbol represents an individual mouse. Horizontal lines represent means. * $p < 0.05$ compared to mock-infected mice. (D) Representative dot plots of GM1 and IFN- γ staining of NK cells from mock-infected and RSV-infected mice. BALB/c mice were infected with 10⁶ PFU RSV. Lung mononuclear cells were isolated at 6 days p.i. and stained with anti-CD3, anti-CD8, anti-ASGM1, CtxB-FITC, and either (E) influenza-tetramers or (F, G, and H) RSV-tetramers. (E and F) The percentage of virus-specific CD8⁺ T cells is indicated. (G) The percentage of gated tetramer-negative CD8⁺ T cells that were ASGM1⁺GM1^{high} is indicated. (H) The percentage of gated tetramer-positive CD8⁺ T cells that were ASGM1⁺GM1^{high} is indicated.

FIG. 4. BALB/c and B6 mice were mock-infected or infected with 10^6 PFU RSV ($n = 4$ per group). Lung mononuclear cells were isolated at 6 d.p.i. and stained with anti-CD49b, anti-NK1.1, anti-CD3, anti-CD8, anti-ASGM1, and CtxB-FITC. (A) Representative dot plot of CD49b and NK1.1 expression by gated CD3-negative lung lymphocytes from RSV-infected B6 mice. (B) Percentage of lung lymphocytes that were NK cells (circular gate) in mock-infected and RSV-infected BALB/c and B6 mice is indicated. (C) Representative dot plots showing GM1 and ASGM1 expression by lung NK cells (top row, circular gate from B), and gated CD8⁺ T cells (bottom row) in mock-infected and RSV-infected BALB/c and B6 mice. The percentage of ASGM1⁺GM1^{high} cells is indicated in the upper right quadrant. Data in (C) are transformed with bi-exponential axes in order to show zero and negative values.

been implicated in lymphocyte activation and signaling (26,33). RSV infection increased the number of ASGM1⁺GM1^{high} NK cells and ASGM1⁺GM1^{high} CD8⁺ T cells in the lung (Fig. 2E and F). We therefore hypothesized that ASGM1 and GM1 co-localize in lipid rafts in NK and CD8⁺ T cells in the lungs of RSV-infected mice. We analyzed NK and CD8⁺ T cells sorted from the lungs of mock-infected and RSV-infected mice by flow cytometry and confocal laser scanning microscopy. Flow cytometric analysis of aliquots of cells used for microscopy experiments enabled us to quantify the signals for PE and FITC for each sample and to confirm that the staining in each experiment was comparable to previous results (e.g., Fig. 2E). There is spectral overlap between FITC and PE. Therefore, we used META processing to isolate FITC- and PE-specific signals

(see Materials and Methods section). The signal for FITC was strong. This resulted in significant loss of PE signal. However, we were able to detect ASGM1 on the surface of NK and CD8⁺ T cells in RSV-infected mice (Fig. 6). ASGM1 accumulated in patches on the plasma membrane, and ASGM1 co-localized with GM1 (white arrows in Fig. 6). Since we detected ASGM1 and GM1 co-localization in lipid patches in the plasma membrane of NK and CD8⁺ T cells isolated from the lungs of RSV-infected mice but not mock-infected mice, these data indicate that ASGM1 is involved in signaling active lipid rafts. Interestingly, ASGM1 was more restricted to lipid raft-like structures in NK and CD8⁺ T cells sorted from the lungs of RSV-infected mice than was GM1, which was enriched in ASGM1-containing regions (Fig. 6).

FIG. 5. BALB/c and STAT1^{-/-} mice were mock-infected or infected with 10⁶ PFU RSV (n = 5 per group). Lung mononuclear cells were isolated at 6 d.p.i. and stained with anti-CD49b, anti-CD4, anti-CD3, anti-CD8, anti-ASGM1, and CtxB-FITC. Representative dot plots show GM1 and ASGM1 expression by gated NK (top row) and CD8⁺ T cells (bottom row). The percentage of ASGM1⁺GM1^{high} cells is indicated in the upper right quadrant.

FIG. 6. BALB/c mice were mock-infected (n = 6) or infected with 10⁶ PFU RSV (n = 6). Lung mononuclear cells were isolated at 6 d.p.i. Cells from mock-infected and RSV-infected mice were pooled and stained with anti-CD49b, anti-CD3, and anti-CD8. Gated NK and CD8⁺ T cells from mock-infected and RSV-infected mice were sorted, stained with ASGM1-PE and CtxB-FITC, and fixed. Aliquots of cells were analyzed by flow cytometry (left-hand column) or fluorescence microscopy. The percentage of ASGM1⁺GM1^{high} cells in each dot plot is indicated above the upper right hand quadrant. Fluorescence microscopy images represent one Z stack from three Z-stack series captured from three different fields at 63 $\times$ magnification (see Materials and Methods section). Areas of co-localization of ASGM1 and GM1 are indicated with white arrows.

In vivo anti-ASGM1 Ab treatment of RSV-infected mice depletes ASGM1⁺GM1^{high} NK and ASGM1⁺GM1^{high} CD8⁺ T cells from the lung

RSV infection of BALB/c mice resulted in the pulmonary accumulation of IFN- γ -producing ASGM1⁺GM1^{high} NK cells and IFN- γ -producing ASGM1⁺GM1^{high} CD8⁺ T cells in the lung (Figs. 1 and 2). Therefore, we hypothesized that *in vivo* anti-ASGM1 treatment will deplete ASGM1⁺GM1^{high} NK cells and ASGM1⁺GM1^{high} CD8⁺ T cells. Anti-ASGM1 Ab administration to RSV-infected BALB/c mice resulted in depletion of ASGM1⁺ NK cells and ASGM1⁺ CD8⁺ T cells (Fig. 7A and 7B). Likewise, anti-ASGM1 Ab treatment resulted in depletion of GM1^{high} NK cells and GM1^{high} CD8⁺ T cells (Fig. 7C and 7D). As ASGM1⁺ NK and CD8⁺ T cells

are also GM1^{high} (Figs. 2 and 3), these data indicate that anti-ASGM1 Ab depletes ASGM1⁺GM1^{high} NK cells and ASGM1⁺GM1^{high} CD8⁺ T cells from the lungs of RSV-infected mice.

We enumerated by flow cytometry the total number and the number of IFN- γ -expressing NK cells and CD8⁺ T cells in the lungs of RSV-infected BALB/c and STAT1^{-/-} mice that were treated at -2, -1, 0, 2, and 4 d.p.i. with anti-ASGM1 or control Ab. Anti-ASGM1 treatment decreased the number of total and the number of IFN- γ -expressing NK cells in the lungs of RSV-infected BALB/c and STAT1^{-/-} mice at 6 d.p.i. (Fig. 7E). Anti-ASGM1 treatment decreased the number of total and the number of IFN- γ -expressing CD8⁺ T cells in the lungs of RSV-infected BALB/c and STAT1^{-/-} mice at 6 d.p.i. (Fig. 7E). Similar results were observed at 8 d.p.i.

FIG. 7. BALB/c mice infected with 10^6 PFU of RSV were treated with anti-ASGM1 ($n = 4$) or control Ab ($n = 4$) as described in the Materials and Methods section. Lung mononuclear cells were isolated at 6 d.p.i. and stained with anti-CD49b, anti-CD3, anti-CD8, anti-CD4, and anti-ASGM1. (A) Representative dot plots of ASGM1 staining of gated NK and CD8⁺ T cells from anti-ASGM1 Ab-treated and control Ab-treated mice. The percentage of ASGM1⁺ cells is indicated in the upper right hand corner. (B) The percentage of gated NK and CD8⁺ T cells that were ASGM1⁺. RSV-infected mice (10^6 PFU) were treated with anti-ASGM1 ($n = 4$) or control Ab ($n = 4$). Lung mononuclear cells were isolated at 6 d.p.i. and stained with anti-CD49b, anti-CD3, anti-CD8, and CtxB-FITC. (C) Representative dot plots of GM1 staining of gated NK and CD8⁺ T cells from anti-ASGM1 Ab-treated and control Ab-treated mice. The percentage of GM1^{high} cells is indicated in the upper right hand corner. (D) Percentage of gated NK and CD8⁺ T cells that were GM1^{high}. (E) RSV-infected BALB/c and STAT1^{-/-} mice (10^6 PFU) were treated with control Ab or anti-ASGM1 Ab (five mice per group). Lung mononuclear cells were isolated at 6 d.p.i. and stained with anti-CD49b, anti-CD3, anti-CD8, and anti-IFN- γ . The total number of cells per lung of the indicated gated population $\pm$ SEM is shown. * $p < 0.05$ compared to isotype control Ab treatment.

(data not shown). Thus, anti-ASGM1 Ab depletes ASGM1/GM1-expressing NK and CD8⁺ T cells in RSV-infected BALB/c and STAT1^{-/-} mice.

Effect of in vivo anti-ASGM1 treatment of RSV-infected BALB/c and STAT1^{-/-} mice

We showed that oseltamivir treatment of RSV-infected mice reduces NK and CD8⁺ T-cell GM1 expression (56). We also showed that oseltamivir treatment of RSV-infected mice results in delayed viral clearance and reduced illness (56). These results suggest that GM1 plays an important role in the immune response to RSV. Here we tested the hypothesis that ASGM1/GM1-expressing cells play an important role in RSV pathogenesis by depleting these cells using anti-ASGM1 Ab.

We assessed the role of ASGM1⁺GM1^{high} cells in RSV pathogenesis using weight loss, a surrogate for RSV-induced illness. ASGM1⁺GM1^{high} cell depletion significantly reduced weight loss in RSV-infected BALB/c mice compared to control Ab-treated mice ($p < 0.005$ on days 7 through 10; Fig. 8A). Thus, ASGM1⁺GM1^{high} cells contribute to RSV-induced illness in BALB/c mice. In contrast, depletion of ASGM1⁺GM1^{high} cells in RSV-infected STAT1^{-/-} mice did not reduce illness severity (Fig. 8A-B). We found that depletion of ASGM1⁺GM1^{high} cells in RSV-infected BALB/c mice resulted in significantly increased lung viral load at 6 d.p.i. (Fig. 8C), and we found that infectious virus was detectable in anti-ASGM1 Ab-treated but not control Ab-treated BALB/c mice at 8 d.p.i. (Fig. 8D). ASGM1⁺GM1^{high} cell depletion had no effect on viral load at 6 and 8 d.p.i. in RSV-infected STAT1^{-/-} mice (Fig. 8C and D). Therefore, unlike in BALB/c mice, ASGM1/GM1-expressing

cells did not play a role in RSV clearance in STAT1^{-/-} mice. *In vivo* anti-ASGM1 treatment had no effect on the levels of infectious RSV in the lung in BALB/c or STAT1^{-/-} mice at 4 d.p.i. (data not shown). These data show that depletion of ASGM1⁺GM1^{high} cells results in elevated viral load and delayed viral clearance in RSV-infected BALB/c but not STAT1^{-/-} mice.

We quantified by ELISA cytokine levels in the lungs of RSV-infected BALB/c mice that were treated with anti-ASGM1 Ab or control Ab. Anti-ASGM1 treatment reduced the level of IFN- γ by fivefold in the lungs of RSV-infected BALB/c mice at 6 d.p.i. compared to control Ab treatment ($p < 0.05$; Fig. 9). It has been shown that RSV-infected STAT1^{-/-} mice have higher lung IFN- γ levels than RSV-infected BALB/c mice at 6 d.p.i. (63). Similarly, we found that IFN- γ levels were sixfold higher in control Ab-treated STAT1^{-/-} mice than control Ab-treated BALB/c mice (Fig. 9). Anti-ASGM1 treatment reduced by fourfold the level of IFN- γ in the lungs of RSV-infected STAT1^{-/-} mice compared to control Ab treatment (Fig. 9). While IL-13 was not detected in RSV-infected BALB/c mice, anti-ASGM1 treatment elevated lung IL-13 levels in RSV-infected STAT1^{-/-} mice at 6 d.p.i. (Fig. 9) and 8 d.p.i. (data not shown). Thus, ASGM1/GM1-expressing cells regulate the lung cytokine response to RSV infection in BALB/c and STAT1^{-/-} mice.

Discussion

In this study, we investigated the role of lipid-raft-associated GSLs in the host immune response to RSV infection. ASGM1 and GM1 were co-expressed by IFN- γ -expressing NK and CD8⁺ T cells in RSV-infected mice. ASGM1 was detected

FIG. 8. Weight loss $\pm$ SD in BALB/c and STAT1^{-/-} mice infected with 10⁶ PFU (A) and 10⁵ PFU (B) of RSV and treated with control Ab or anti-ASGM1 Ab ($n = 10$ per group). * $p < 0.005$. Survival of STAT1^{-/-} mice with 10⁶ PFU was $<100\%$ after 7 d.p.i. Infectious virus was titrated (C) at 6 d.p.i. and (D) at 8 d.p.i. from the lungs of RSV-infected mice treated with control Ab or anti-ASGM1 Ab. Each symbol represents an individual mouse. The short horizontal lines represent mean titers. The dotted line at 3.3×10^3 represents the lower limit of detection of the assay. ‡ $p < 0.05$.

FIG. 9. IFN- γ and IL-13 levels at 6 d.p.i. were quantified by ELISA in lung homogenates of RSV-infected mice (10^6 PFU) treated with control Ab ($n = 5$) or anti-ASGM1 Ab ($n = 5$) mice. Data $\pm$ SEM are shown. * $p < 0.05$.

mostly in lipid raft structures in NK and CD8⁺ T cells sorted from the lungs of RSV-infected mice but not mock-infected mice, and GM1 was enriched in regions of the plasma membrane containing ASGM1. Depletion of ASGM1⁺GM1^{high} cells in RSV-infected BALB/c mice resulted in decreased numbers of total and IFN- γ -producing NK and CD8⁺ T cells in the lung, decreased lung IFN- γ levels, increased peak viral titers, delayed viral clearance, and reduced illness in RSV-infected mice. Depletion of ASGM1⁺GM1^{high} cells in RSV-infected STAT1^{-/-} mice decreased IFN- γ levels and increased IL-13 levels in the lung, but had no effect on viral load or illness severity. Our results suggest that ASGM1 plays a role in signaling-active lipid rafts in NK and CD8⁺ T cells *in vivo*, and show that ASGM1⁺GM1^{high} cells control RSV in BALB/c mice but not STAT1-deficient mice.

Our observations that RSV infection increased ASGM1 levels but not GM1 levels on NK cells, whereas RSV infection increased both ASGM1 and GM1 levels on CD8⁺ T cells, show that these cell types differentially regulate the expression of distinct lipid raft-associated GSLs. However, little is known about the regulation of GSL synthesis in lymphocytes or the role of distinct GSLs in cellular processes. In our experiments, NK cells constitutively expressed high levels of GM1, and NK cells had the highest GM1 expression level of gated lung lymphocytes. As GM1 is the GSL most associated with active signaling via the immunological synapse, we speculate that constitutively high NK cell GM1 expression may contribute to the "natural" ability of these cells to engage target cells and express cytokines without prior sensitization (67). GM3 is another lipid raft-associated GSL (29). Chen *et al.* found that treatment of T cells with GM3 inhibits the production of IL-4, IL-5, and IL-13, while having no effect on IL-2 or IFN- γ synthesis (46,68). These authors hypothesized that GSL metabolism plays a role

in modulating T-cell cytokine expression (68). Cell type-specific regulation of distinct GSLs may be an important mechanism of immune signaling modulation.

Fluorescence microscopy and flow cytometry are good experimental approaches for studying the role of GSLs and lipid rafts in pathogenesis. Initial reports identified lipid rafts as detergent-insoluble membrane complexes that float in low-density gradient centrifugation (28,58,69). However, the observation that detergent can induce the formation of lipid rafts hampers these biochemical approaches (70,71). Microscopic analyses of *in vitro* anti-CD3-activated T cells and T-cell:APC conjugates have provided much insight into the structure of the immunological synapse (72). Here we quantified the expression of and visualized ASGM1 and GM1 in NK and CD8⁺ T cells isolated from the lungs of RSV-infected mice. In interpreting our results, it must be recognized that these cells were not in contact with target cells (e.g., RSV-infected cells) when analyzed. Thus, although our results suggest that ASGM1 is partitioned into lipid rafts in activated IFN- γ -producing ASGM1⁺GM1^{high} NK and CD8⁺ T cells, it is not known whether ASGM1 partitions into lipid rafts in these cells while in direct contact with target cells. Also it is not known whether the NK or CD8⁺ T cells we purified had been in contact with target cells in the lung parenchyma at the time of isolation, or whether increased GSL expression in NK and CD8⁺ T cells occurs concomitantly with homing to sites of inflammation.

CtxB is known to be specific for GM1 (32,73). However, CtxB can bind with low avidity to high concentrations of ASGM1 in synthetic phospholipid membranes (74). It has been argued that potential low-avidity interactions between CtxB and other ligands may confound the use of CtxB as a reagent to detect cell surface GM1 (71). Since RSV infection increased ASGM1 levels but not GM1 levels on the surface of NK cells, as measured by flow cytometry, we conclude that anti-ASGM1 Ab and CtxB do not have significant cross-reactivity in our system.

The anti-ASGM1 Ab has been used to assess the *in vivo* role of NK cells in several viral models (34–36,38,41,44). Our results agree with those of Slifka *et al.*, who showed that anti-ASGM1 Ab depletes activated T cells in a mouse model of viral infection (48). We found that ASGM1 is an activation marker of NK and CD8⁺ T cells. In both BALB/c and C57BL/6 mice, IFN- γ -expressing CD8⁺ T cells in the lungs of RSV-infected mice expressed high levels of ASGM1. NK cells in mock-infected mice had expressed higher levels of ASGM1 than T cells in mock-infected mice. Thus, NK cells constitutively expressed higher lipid raft-associated GSL (GM1 and ASGM1) levels than T cells in mock-infected mice, whereas IFN- γ -expressing CD8⁺ T cells expressed higher GSL levels than NK cells in RSV-infected mice.

The phenotype of ASGM1⁺GM1^{high} cell depletion in RSV-infected BALB/c mice is similar to the phenotype of RSV-infected mice depleted of CD8⁺ T cells (8). This is not surprising because ASGM1⁺GM1^{high} CD8⁺ T cells are IFN- γ -expressing (this report) and RSV-specific (56). Thus our data suggest that ASGM1⁺GM1^{high} cells (NK and CD8⁺ T cells) play a dominant role in RSV-induced T-cell immunopathology in BALB/c mice.

In contrast to BALB/c mice, ASGM1⁺GM1^{high} cells in RSV-infected STAT1^{-/-} mice did not contribute to illness or limit viral replication. Despite exhibiting profound weight

loss and mortality, anti-ASGM1-Ab and control Ab-treated RSV-infected STAT1^{-/-} mice were able to clear infectious RSV to below plaque assay detection levels. STAT1^{-/-} mice exhibit Th-2-type inflammation in response to RSV infection that includes eosinophilia and neutrophilia (63). We speculate that cell types other than activated NK and T cells (e.g., neutrophils or eosinophils) may contribute to control of RSV and play a role in severe immunopathology in STAT1^{-/-} mice. Depletion of ASGM1⁺GM1^{high} cells in RSV-infected STAT1^{-/-} mice elevated lung IL-13 levels. Thus ASGM1⁺GM1^{high} cells, which include IFN- γ -expressing NK and CD8⁺ T cells, suppress IL-13 expression by a STAT1-independent mechanism. We speculate that IFN- γ suppresses IL-13 by a STAT1-independent mechanism. IFN- γ induces the expression of many genes via a STAT1-independent mechanism, including IFN- β , which, in turn, has been shown to downregulate IL-13 expression *in vitro* (75,76).

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References

- Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, and Anderson LJ: Bronchiolitis-associated hospitalizations among U.S. children, 1980–1996. *JAMA* 1999;282:1440–1446.
- Moore ML, and Peebles RS Jr: Respiratory syncytial virus disease mechanisms implicated by human, animal model, and *in vitro* data facilitate vaccine strategies and new therapeutics. *Pharmacol Ther* 2006;112:405–424.
- Tripp RA, Moore D, Barskey A, Jones L, Moscattiello C, Keyserling H, and Anderson LJ: Peripheral blood mononuclear cells from infants hospitalized because of respiratory syncytial virus infection express T helper-1 and T helper-2 cytokines and CC chemokine messenger RNA. *J Infect Dis* 2002;185:1388–1394.
- Glezen WP, Taber LH, Frank AL, and Kasel JA: Risk of primary infection and reinfection with respiratory syncytial virus. *Am J Dis Child* 1986;140:543–546.
- Anderson JJ, Norden J, Saunders D, Toms GL, and Scott R: Analysis of the local and systemic immune responses induced in BALB/c mice by experimental respiratory syncytial virus infection. *J Gen Virol* 1990;71(Pt 7):1561–1570.
- Graham BS, Johnson TR, and Peebles RS: Immune-mediated disease pathogenesis in respiratory syncytial virus infection. *Immunopharmacology* 2000;48:237–247.
- Hussell T, and Openshaw PJ: Intracellular IFN- γ expression in natural killer cells precedes lung CD8⁺ T cell recruitment during respiratory syncytial virus infection. *J Gen Virol* 1998;79(Pt 11):2593–2601.
- Graham BS, Bunton LA, Wright PF, and Karzon DT: Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. *J Clin Invest* 1991;88:1026–1033.
- Bangham CR, Cannon MJ, Karzon DT, and Askonas BA: Cytotoxic T-cell response to respiratory syncytial virus in mice. *J Virol* 1985;56:55–59.
- Cannon M J, Openshaw PJ, and Askonas BA: Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. *J Exp Med* 1988;168:1163–1168.
- Ostler T, Davidson W, and Ehl S: Virus clearance and immunopathology by CD8(+) T cells during infection with respiratory syncytial virus are mediated by IFN- γ . *Eur J Immunol* 2002;32:2117–2123.
- Hall CB: Respiratory syncytial virus and parainfluenza virus. *N Engl J Med* 2001;344:1917–1928.
- Legg JP, Hussain IR, Warner JA, Johnston SL, and Warner JO: Type 1 and type 2 cytokine imbalance in acute respiratory syncytial virus bronchiolitis. *Am J Respir Crit Care Med* 2003;168:633–639.
- Cederblad B, Riesenfeld T, and Alm GV: Deficient herpes simplex virus-induced interferon-alpha production by blood leukocytes of preterm and term newborn infants. *Pediatr Res* 1990;27:7–10.
- Neustock P, Kruse A, Bock S, St Pierre B, and Kirchner H: Deficient interferon-alpha response of newborns in comparison to adults. *Lymphokine Cytokine Res* 1993;12:109–114.
- Durbin JE, Hackenmiller R, Simon MC, and Levy DE: Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* 1996;84:443–450.
- Fallarino F, and Gajewski TF: Cutting edge: differentiation of antitumor CTL *in vivo* requires host expression of Stat1. *J Immunol* 1999;163:4109–4113.
- Lee CK, Rao DT, Gertner R, Gimeno R, Frey AB, and Levy DE: Distinct requirements for IFNs and STAT1 in NK cell function. *J Immunol* 2000;165:3571–3577.
- Nishibori T, Tanabe Y, Su L, and David M: Impaired development of CD4⁺ CD25⁺ regulatory T cells in the absence of STAT1: Increased susceptibility to autoimmune disease. *J Exp Med* 2004;199:25–34.
- Durbin JE, Fernandez-Sesma A, Lee CK, et al.: Type I IFN modulates innate and specific antiviral immunity. *J Immunol* 2000;164:4220–4228.
- Hashimoto K, Durbin JE, Zhou W, et al.: Respiratory syncytial virus infection in the absence of STAT 1 results in airway dysfunction, airway mucus, and augmented IL-17 levels. *J Allergy Clin Immunol* 2005;116:550–557.
- Degroote S, Wolthoorn J, and van Meer G: The cell biology of glycosphingolipids. *Semin Cell Dev Biol* 2004;15:375–387.
- Proia RL: Glycosphingolipid functions: insights from engineered mouse models. *Philos Trans R Soc Lond B Biol Sci* 2003;358:879–883.
- Allende ML, and Proia RL: Lubricating cell signaling pathways with gangliosides. *Curr Opin Struct Biol* 2002;12:587–592.
- Hakomori SI: Inaugural article: The glycosynapse. *Proc Natl Acad Sci USA* 2002;99:225–232.
- Dykstra M, Cherukuri A, Sohn HW, Tzeng SJ, and Pierce SK: Location is everything: lipid rafts and immune cell signaling. *Annu Rev Immunol* 2003;21:457–481.
- Simons K, and Toomre D: Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* 2000;1:31–39.
- Brown DA, and Rose JK: Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* 1992;68:533–544.
- Gomez-Mouton C, Abad JL, Mira E, et al.: Segregation of leading-edge and uropod components into specific lipid

- rafts during T cell polarization. *Proc Natl Acad Sci USA* 2001;98:9642–9647.
30. Tani-ichi S, Maruyama K, Kondo N, et al.: Structure and function of lipid rafts in human activated T cells. *Int Immunol* 2005;17:749–758.
 31. Mori T, Kiyokawa N, Katagiri YU, et al.: Globotriaosyl ceramide (CD77/Gb3) in the glycolipid-enriched membrane domain participates in B-cell receptor-mediated apoptosis by regulating lyn kinase activity in human B cells. *Exp Hematol* 2000;28:1260–1268.
 32. Smith DC, Lord JM, Roberts LM, and Johannes L: Glycosphingolipids as toxin receptors. *Semin Cell Dev Biol* 2004;15:397–408.
 33. Soong G, Reddy B, Sokol S, Adamo R, and Prince A: TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells. *J Clin Invest* 2004;113:1482–1489.
 34. Bukowski JF, Woda BA, Habu S, Okumura K, and Welsh RM: Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis *in vivo*. *J Immunol* 1983;131:1531–1538.
 35. Bukowski JF, Woda BA, and Welsh RM: Pathogenesis of murine cytomegalovirus infection in natural killer cell-depleted mice. *J Virol* 1964;52:119–128.
 36. Bukowski JF, and Welsh RM: The role of natural killer cells and interferon in resistance to acute infection of mice with herpes simplex virus type 1. *J Immunol* 1986;136:3481–3485.
 37. Byrne P, McGuirk P, Todryk S, and Mills KH: Depletion of NK cells results in disseminating lethal infection with *Bordetella pertussis* associated with a reduction of antigen-specific Th1 and enhancement of Th2, but not Tr1 cells. *Eur J Immunol* 2004;34:2579–2588.
 38. Godeny EK, and Gauntt CJ: Involvement of natural killer cells in coxsackievirus B3-induced murine myocarditis. *J Immunol* 1986;137:1695–1702.
 39. Iwanami N, Niwa A, Yasutomi Y, Tabata N, and Miyazawa M: Role of natural killer cells in resistance against friend retrovirus-induced leukemia. *J Virol* 2001;75:3152–3163.
 40. Junqueira-Kipnis AP, Kipnis A, Jamieson A, et al.: NK cells respond to pulmonary infection with *Mycobacterium tuberculosis*, but play a minimal role in protection. *J Immunol* 2003;171:6039–6045.
 41. Liu ZX, Govindarajan S, Okamoto S, and Dennert G: NK cells cause liver injury and facilitate the induction of T cell-mediated immunity to a viral liver infection. *J Immunol* 2000;164:6480–6486.
 42. Muhlen KA, Schumann J, Wittke F, Stenger S, Van Rooijen N, Van Kaer L, and Tiegs G: NK cells, but not NKT cells, are involved in *Pseudomonas aeruginosa* exotoxin A-induced hepatotoxicity in mice. *J Immunol* 2004;172:3034–3041.
 43. Stein-Streilein J, and Guffee J: *In vivo* treatment of mice and hamsters with antibodies to asialo GM1 increases morbidity and mortality to pulmonary influenza infection. *J Immunol* 1988;136:1435–1441.
 44. Warfield KL, Perkins JG, Swenson DL, et al.: Role of natural killer cells in innate protection against lethal Ebola virus infection. *J Exp Med* 2004;200:169–179.
 45. Yang H, Yogeeswaran G, Bukowski JF, and Welsh RM: Expression of asialo GM1 and other antigens and glycolipids on natural killer cells and spleen leukocytes in virus-infected mice. *Nat Immun Cell Growth Regul* 1985;4:21–39.
 46. Chen XP, Enioutina EY, and Daynes RA: The control of IL-4 gene expression in activated murine T lymphocytes: a novel role for neu-1 sialidase. *J Immunol* 1997;158:3070–3080.
 47. Scalzo AA, Farrell HE, and Karupiah G: Techniques for studying murine natural killer cells in defense against viral infection, in: *Methods in Molecular Biology, Vol. 121: Natural Killer Cell Protocols: Cellular and Molecular Methods*. (KS Campbell and M Colonna), (eds.). Humana Press, Totowa, NJ, 2000, pp. 163–177.
 48. Slifka MK, Pagarigan RR, and Whitton JL: NK markers are expressed on a high percentage of virus-specific CD8+ and CD4+ T cells. *J Immunol* 2000;164:2009–2015.
 49. Stitz L, Baenziger J, Pircher H, Hengartner H, and Zinkernagel RM: Effect of rabbit anti-asialo GM1 treatment *in vivo* or with anti-asialo GM1 plus complement *in vitro* on cytotoxic T cell activities. *J Immunol* 1986;136:4674–4680.
 50. Brown G, Rixon HW, and Sugrue RJ: Respiratory syncytial virus assembly occurs in GM1-rich regions of the host-cell membrane and alters the cellular distribution of tyrosine phosphorylated caveolin-1. *J Gen Virol* 2002;83:1841–1850.
 51. Brown G, Jeffree CE, McDonald T, Rixon HW, Aitken JD, and Sugrue RJ: Analysis of the interaction between respiratory syncytial virus and lipid-rafts in Hep2 cells during infection. *Virology* 2004;327:175–185.
 52. Brown G, Rixon HW, Steel J, McDonald TP, Pitt AR, Graham S, and Sugrue RJ: Evidence for an association between heat shock protein 70 and the respiratory syncytial virus polymerase complex within lipid-raft membranes during virus infection. *Virology* 2005;338:69–80.
 53. McCurdy LH, and Graham BS: Role of plasma membrane lipid microdomains in respiratory syncytial virus filament formation. *J Virol* 2005;77:1747–1756.
 54. McDonald TP, Pitt AR, Brown G, Rixon HW, and Sugrue RJ: Evidence that the respiratory syncytial virus polymerase complex associates with lipid rafts in virus-infected cells: a proteomic analysis. *Virology* 2004;330:147–157.
 55. Jeffree CE, Rixon HW, Brown G, Aitken J, and Sugrue RJ: Distribution of the attachment (G) glycoprotein and GM1 within the envelope of mature respiratory syncytial virus filaments revealed using field emission scanning electron microscopy. *Virology* 2003;306:254–267.
 56. Moore ML, Chi MH, Zhou W, Goleniewska K, O'Neal JF, Higginbotham JN, and Peebles RS Jr: Cutting edge: Oseltamivir decreases T cell GM1 expression and inhibits clearance of respiratory syncytial virus: potential role of endogenous sialidase in antiviral immunity. *J Immunol* 2007;178:2651–2654.
 57. Graham BS, Perkins MD, Wright PF, and Karzon DT: Primary respiratory syncytial virus infection in mice. *J Med Virol* 1988;26:153–162.
 58. Thomas S, Preda-Pais A, Casares S, and Brumeanu TD: Analysis of lipid rafts in T cells. *Mol Immunol* 2004;41:399–409.
 59. Yang H, Yogeeswaran G, Bukowski JF, and Welsh RM: Expression of asialo GM1 and other antigens and glycolipids on natural killer cells and spleen leukocytes in virus-infected mice. *Nat Immun Cell Growth Regul* 1985;4:21–39.
 60. Arase H, Saito T, Phillips JH, and Lanier LL: Cutting edge: the mouse NK cell-associated antigen recognized by DX5 monoclonal antibody is CD49b (alpha 2 integrin, very late antigen-2). *J Immunol* 2001;167:1141–1144.
 61. Prince GA, Horswood RL, Berndt J, Suffin SC, and Chanock RM: Respiratory syncytial virus infection in inbred mice. *Infect Immun* 1979;26:764–766.
 62. Carlyle JR, Mesci A, Ljutic B, et al.: Molecular and genetic basis for strain-dependent NK1.1 alloreactivity of mouse NK cells. *J Immunol* 2006;176:7511–7524.

63. Durbin JE, Johnson TR, Durbin RK, Mertz SE, Morotti RA, Peebles RS, and Graham BS: The role of IFN in respiratory syncytial virus pathogenesis. *J Immunol* 2002;168:2944–2952.
64. Gil MP, Salomon R, Louten J, and Biron CA: Modulation of STAT1 protein levels: a mechanism shaping CD8 T-cell responses *in vivo*. *Blood* 2006;107:987–993.
65. Gimeno R, Lee CK, Schindler C, and Levy DE: Stat1 and Stat2 but not Stat3 arbitrate contradictory growth signals elicited by alpha/beta interferon in T lymphocytes. *Mol Cell Biol* 2005;25:5456–5465.
66. Lee CK, Smith E, Gimeno R, Gertner R, and Levy DE: STAT1 affects lymphocyte survival and proliferation partially independent of its role downstream of IFN-gamma. *J Immunol* 2000;164:1286–1292.
67. Yokoyama WM, Kim S, and French AR: The dynamic life of natural killer cells. *Annu Rev Immunol* 2004;22:405–429.
68. Chen XP, Ding X, and Daynes RA: Ganglioside control over IL-4 priming and cytokine production in activated T cells. *Cytokine* 2000;12:972–985.
69. Yu J, Fischman DA, and Steck TL: Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. *J Supramol Struct* 1973;1:233–248.
70. Heerklotz H: Triton promotes domain formation in lipid raft mixtures. *Biophys J* 2002;83:2693–2701.
71. Munro S: Lipid rafts: elusive or illusive? *Cell* 2003;115:377–388.
72. Potter TA, Grebe K, Freiberg B, and Kupfer A: Formation of supramolecular activation clusters on fresh *ex vivo* CD8+ T cells after engagement of the T cell antigen receptor and CD8 by antigen-presenting cells. *Proc Natl Acad Sci USA* 2001;98:12624–12629.
73. MacKenzie CR, Hiram T, Lee KK, Altman E, and Young NM: Quantitative analysis of bacterial toxin affinity and specificity for glycolipid receptors by surface plasmon resonance. *J Biol Chem* 1997;272:5533–5538.
74. Lauer S, Goldstein B, Nolan RL, and Nolan JP: Analysis of cholera toxin-ganglioside interactions by flow cytometry. *Biochemistry* 2002;41:1742–1751.
75. Pacocha SE, Oriente A, Lichtenstein LM, Huang SK, and Essayan DM: Interferon-beta induces selective enhancement of antigen-specific T cell responses. *J Interferon Cytokine Res* 2000;20:383–389.
76. Ramana CV, Gil MP, Han Y, Ransohoff RM, Schreiber RD, and Stark GR: Stat1-independent regulation of gene expression in response to IFN-gamma. *Proc Natl Acad Sci USA* 2001;98:6674–6679.

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