



Brilliant Violet™ Antibody Conjugates
Superior Performance for the Violet Laser



NK Cell-Depleting Anti-Asialo GM1 Ab Exhibits a Lethal Off-Target Effect on Basophils In Vivo

Hideto Nishikado, Kaori Mukai, Yohei Kawano, Yoshiyuki Minegishi and Hajime Karasuyama

This information is current as of April 15, 2011

J Immunol; Prepublished online 13 April 2011;
doi:10.4049/jimmunol.1100370
<http://www.jimmunol.org/content/early/2011/04/12/jimmunol.1100370>

Supplementary Data	http://www.jimmunol.org/content/suppl/2011/04/12/jimmunol.1100370.DC1.html
Subscriptions	Information about subscribing to <i>The Journal of Immunology</i> is online at http://www.jimmunol.org/subscriptions
Permissions	Submit copyright permission requests at http://www.aai.org/ji/copyright.html
Email Alerts	Receive free email-alerts when new articles cite this article. Sign up at http://www.jimmunol.org/etoc/subscriptions.shtml/

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include the digital object identifier (DOIs) and date of initial publication.

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
Copyright ©2011 by The American Association of
Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



NK Cell-Depleting Anti-Asialo GM1 Ab Exhibits a Lethal Off-Target Effect on Basophils In Vivo

Hideto Nishikado,^{*,1} Kaori Mukai,^{*,1,2} Yohei Kawano,^{*} Yoshiyuki Minegishi,^{*,†} and Hajime Karasuyama^{*,†}

NK cells are innate immune lymphocytes and play a key role in both innate and adaptive immunity. Their pivotal functions in vivo have been illustrated in mice by means of their ablation with NK cell-depleting Abs, particularly anti-asialo GM1 (ASGM1). In this study, we show that the whole population of basophils constitutively expresses ASGM1 as well as CD49b (DX5) as does the NK cell population and was ablated in vivo by anti-ASGM1 as efficiently as by a basophil-depleting anti-FcεRIα Ab. Anti-ASGM1-mediated basophil depletion was operative as for NK cell depletion in various mouse strains, irrespective of NK1 allotype and MHC H2 haplotype, including C57BL/6, BALB/c, C3H, and A/J mice. These results identified basophils as a previously unrecognized target of anti-ASGM1-mediated cell depletion and raised concern about possible contribution of basophils, rather than or in addition to NK cells, to some of phenotypes observed in anti-ASGM1-treated mice. Indeed, regardless of the presence or absence of NK cells in mice, anti-ASGM1 treatment abolished the development of IgE-mediated chronic cutaneous allergic inflammation as efficiently as did the treatment with basophil-depleting Ab. Given the fact that basophils have recently been shown to play crucial roles in a variety of immune responses, our finding of the off-target effect on basophils issues a grave warning about the use of anti-ASGM1 and underscores the need for careful interpretation of phenotypes observed in anti-ASGM1-treated mice. *The Journal of Immunology*, 2011, 186: 000–000.

Natural killer cells provide innate defense against viruses and tumor cells by killing target cells and producing immunoregulatory cytokines (1–3). Two types of NK cell-depleting Abs, a polyclonal Ab specific to asialo GM1 (ASGM1) (4–6) and an mAb specific to NK1.1 (7), have commonly been used to elucidate in vivo functions of NK cells in mice. Anti-ASGM1-mediated NK cell depletion is effective in a variety of mouse strains, whereas anti-NK1.1-mediated NK cell depletion works only in certain strains such as C57BL/6 and SJL and not in many other strains lacking the NK1.1 allotype, including BALB/c, C3H, and A/J mice (8). Anti-ASGM1 activity was first identified >30 y ago in antiserum produced by immunizing rabbits with mouse brain tissues and shown to be responsible for antiserum-mediated elimination of NK activity in the

spleen from various mouse strains (4). The expression of ASGM1 is not strictly confined to NK cells among hematopoietic cells and is detected on a subpopulation of NKT, CD8⁺ T, and γδ T cells (9, 10) and some activated form of CD4⁺ T cells, macrophages, and eosinophils under certain experimental conditions (11–13). Nevertheless, anti-ASGM1-mediated NK cell depletion still remains a powerful tool to analyze in vivo functions of NK cells.

Basophils are basophilic granulocytes and represent <1% of peripheral blood leukocytes (14). Interestingly, basophils in mice express typical NK markers CD49b (DX5) and NKR2B4 (2B4) on their surface (15–17) and produce granzyme B-like serine protease, mouse mast cell protease-8 (18, 19), even though cytotoxic activity of basophils remains to be investigated. Basophils also display several characteristics shared by tissue-resident mast cells, including surface expression of the FcεRI, and the release of allergy-related chemical mediators such as histamine in response to various stimuli (14). Because of these mast cell-like phenotypes and their rarity, basophils have often erroneously been considered as minor relatives of mast cells or as blood-circulating precursors of tissue-resident mast cells (20). However, recent studies using basophil-depleting Abs (21–23) and genetically engineered mice deficient only in basophils (24, 25) have illuminated nonredundant roles for basophils in acquired immunity regulation, protective immunity to pathogens, and immunological disorders such as allergy and autoimmunity (26–36).

In the current study, we found that the whole population of basophils in mice homogeneously expresses high levels of ASGM1 and that in vivo administration of anti-ASGM1 readily ablates most of basophils, in addition to NK cells, in a variety of mouse strains, regardless of NK1 allotype and MHC H2 haplotype. These unexpected findings raise concern about the possible contribution of basophils, rather than or in addition to NK cells, to some of phenotypes observed in anti-ASGM1-treated mice. Indeed, treatment of mice with anti-ASGM1 abolished the development of

^{*}Department of Immune Regulation, Tokyo Medical and Dental University Graduate School, Tokyo 113-8519, Japan; and [†]Japan Science and Technology Agency, Core Research for Evolutional Science and Technology, Tokyo Medical and Dental University Graduate School, Tokyo 113-8519, Japan

¹H.N. and K.M. contributed equally to this work.

²Current address: Department of Pathology, Stanford University School of Medicine, Stanford, CA.

Received for publication February 3, 2011. Accepted for publication March 15, 2011.

This work was supported by research grants from the Japan Science and Technology Agency, Core Research for Evolutional Science and Technology, the Japanese Ministry of Education, Culture, Sports, Science and Technology, Takeda Science Foundation, the Mitsubishi Foundation, the Naito Foundation, and the Uehara Memorial Foundation.

Address correspondence and reprint requests to Dr. Hajime Karasuyama, Department of Immune Regulation, Tokyo Medical and Dental University Graduate School, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail address: karasuyama.mhch@tmd.ac.jp

The online version of this article contains supplemental material.

Abbreviations used in this article: APCy, allophycocyanin; ASGM1, asialo GM1; BMPC, bone marrow protective cell; IgE-CAI, IgE-mediated chronic allergic inflammation; TNP, 2,4,6-trinitrophenol.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/\$16.00

IgE-mediated chronic cutaneous allergic inflammation as efficiently as did the treatment with basophil-depleting Ab.

Materials and Methods

Mice

C57BL/6, BALB/c, C3H mice (6–10 wk old) were purchased from CLEA Japan (Tokyo, Japan). A/J mice were from Japan SLC (Hamamatsu, Japan), and *rag2*^{-/-} *γc*^{-/-} C57BL/6 mice were from Taconic Farms (Germantown, NY). All animal studies were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Abs and other reagents

FITC-conjugated anti-CD3ε (145-2C11) and anti-rabbit IgG, FITC-conjugated streptavidin, PE-conjugated anti-TCRγδ (GL3), PE-conjugated streptavidin, allophycocyanin (APCy)-conjugated anti-CD8α (53-6.7) and CD49b (HMα2), and APCy-conjugated streptavidin were purchased from BD Pharmingen (San Diego, CA). PE-conjugated anti-FcεRIα (MAR-1) and biotinylated anti-CD3ε (145-2C11) were purchased from eBioscience (San Diego, CA). Unconjugated anti-FcεRIα (MAR-1), APCy-Cy7-conjugated streptavidin, PE-Cy7-conjugated anti-CD3ε (145-2C11), and PE-Cy7-conjugated streptavidin were purchased from Biolegend (San Diego, CA). Anti-CD16/32 (2.4G2) and anti-NK1.1 (PK136) were prepared from hybridoma culture supernatants in our laboratories. Anti-CD200R3 (Ba91) was established as reported previously (21). Rabbit anti-ASGM1 was purchased from Wako Pure Chemicals (Osaka, Japan), and control rabbit serum, mouse IgG2a, monosialoganglioside GM1, and ASGM1 were from Sigma-Aldrich (St. Louis, MO).

Flow cytometric analysis

Cells prepared from the spleen, bone marrow, and peripheral blood were preincubated with anti-CD16/32 mAb and normal rat serum on ice for 15 min prior to incubation with the indicated combination of Abs to prevent the nonspecific binding of irrelevant Abs. For ASGM1 staining, cells were incubated with rabbit anti-ASGM1 (1:500 diluted) on ice for 20 min, followed by FITC-conjugated goat anti-rabbit IgG (1:500 diluted). In the experiments shown in Fig. 3B, anti-ASGM1 was preincubated in 100 μl with 10 μg ASGM1 or control GM1 before cell staining.

In vivo depletion of NK cells and basophils

For NK cell depletion, mice were given an i.p. injection of anti-ASGM1 (10 μl) or anti-NK1.1 (300 μg). For basophil depletion, mice were treated with a total of 30 μg anti-FcεRIα (MAR-1) that was administered i.p. twice a day (5 μg each time) for 3 consecutive d (23). Stained cells were analyzed on a FACSCanto II (BD Biosciences, Palo Alto, CA), and data were analyzed with FACSDiva software (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

IgE-mediated chronic allergic skin inflammation

IgE-mediated chronic allergic skin inflammation was elicited as described (17). In brief, mice were passively sensitized with IgE by an i.v. injection of 300 μg 2,4,6-trinitrophenol (TNP)-specific IgE (IGELb4). A day later, 10 μg TNP-conjugated OVA (Sigma-Aldrich) in 10 μl PBS was injected s.c. into the left ear of the mice under light anesthesia with diethyl ether, and an equal amount of OVA was injected into the right ear using a microsyringe. Ear thickness was measured with a dial thickness gauge (G-1A; Ozaki, Tokyo, Japan) at the indicated time points. The difference in ear thickness (left – right) was calculated at each time point.

Statistical analysis

The statistical significance of differences between groups was calculated with a two-tailed Student *t* test or ANOVA followed by Ryan's test. A *p* value <0.05 was considered statistically significant.

Results

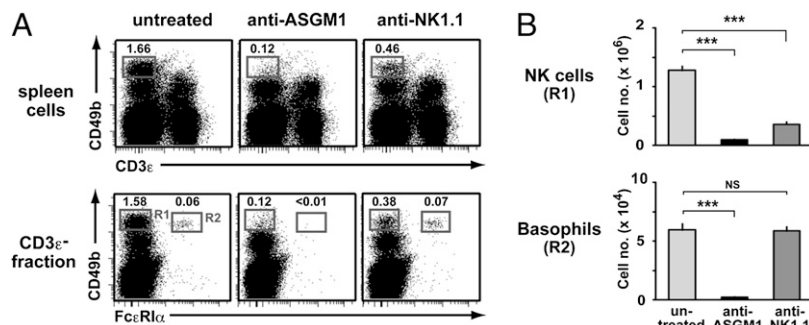
NK cell-depleting anti-ASGM1 but not anti-NK1.1 Ab ablates basophils in vivo

Preparation of a highly purified basophil population for functional analysis is extremely difficult, particularly in mice, due to the rarity of basophils and the lack of murine basophil-specific Ab that leaves basophils unstimulated. Although enrichment of basophils (CD49b⁺FcεRIα⁺) without their activation can be achieved in mice by magnetic sorting using anti-CD49b-bound beads, basophils represent only 5–20% of the CD49b⁺ cells in the spleen, bone marrow, and peripheral blood, whereas the rest of cells are mainly NK and NKT cells. We thought that Ab-mediated depletion of NK/NKT cells in vivo before the cell preparation could increase the efficiency of basophil enrichment. When administered i.p., both anti-ASGM1 and anti-NK1.1 were indeed effective in reducing number of CD49b^{high}CD3ε⁺ cells in the spleen with higher efficacy by anti-ASGM1 than by anti-NK1.1 (Fig. 1A, upper panels). The CD49b^{high}CD3ε⁺ fraction of splenocytes includes FcεRIα⁺ NK cells (90–95%) and FcεRIα⁺ basophils (5–10%). Unexpectedly, anti-ASGM1 but not anti-NK1.1 drastically reduced the number of basophils in the spleen, whereas both anti-ASGM1 and anti-NK1.1 efficiently depleted NK cells (Fig. 1A, lower panels, and summarized in Fig. 1B). As many as 90% of basophils are depleted by the anti-ASGM1 treatment as compared with those in mice treated with control rabbit serum (Fig. 2A). The efficacy of basophil depletion by anti-ASGM1 was comparable to that mediated by a basophil-depleting anti-FcεRIα mAb MAR-1 that did not deplete NK cells, unlike anti-ASGM1 (Fig. 2A). One shot of anti-ASGM1 injection reduced the number of basophils in the spleen, for 4 d after the injection, to less than a quarter of that in the control mice treated with normal rabbit serum (Fig. 2B, upper panel). The basophil number gradually increased thereafter and returned to the normal level by 14 d after the injection. These kinetics of recovery were faster than those of NK cells (Fig. 2B), most likely due to the quicker turnover of basophils than NK cells (37, 38).

Basophils express ASGM1 on their surface

We next examined whether the basophil depletion by the anti-ASGM1 polyclonal Ab stems from the expression of ASGM1 on the surface of basophils or due to an ASGM1-independent, off-target effect of the polyclonal Ab. Flow cytometric analysis revealed that virtually all basophils in the spleen, peripheral blood, and bone marrow were homogeneously stained with anti-ASGM1 but not anti-NK1.1 (Fig. 3A). The ASGM1 staining of basophils

FIGURE 1. Anti-ASGM1 but not anti-NK1.1 Ab ablates basophils in vivo. *A* and *B*, C57BL/6 mice were left untreated or treated with i.p. injection of anti-ASGM1 (10 μl) or anti-NK1.1 (300 μg). One day later, spleen cells were isolated and stained for CD49b, CD3ε, and FcεRIα. Representative staining profiles are shown in *A*. The numeral in each panel shows the percentage of gated cells among whole spleen cells. The numbers of NK cells (CD49b^{high}CD3ε⁺FcεRIα⁺) and basophils (CD49b^{high}CD3ε⁺FcεRIα⁺) in the spleen of each group are shown in *B* (mean ± SEM, *n* = 4 each). Data shown in *A* and *B* are representative of four repeated experiments. ****p* < 0.001.



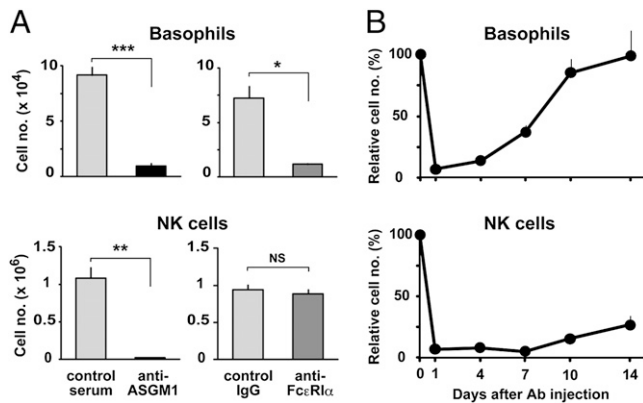


FIGURE 2. Anti-ASGM1 depletes basophils as efficiently as does anti-FcεRIα. *A* and *B*, C57BL/6 mice were treated with 10 μl anti-ASGM1 (or control rabbit serum) or 30 μg anti-FcεRIα (or control hamster IgG) as described in *Materials and Methods*. In *A*, spleen cells were isolated 1 d after anti-ASGM1 injection or after the last injection of anti-FcεRIα and stained as in Fig. 1*A* to identify basophils and NK cells. Basophils in anti-FcεRIα-treated mice are defined as CD49b⁺CD200R3⁺ cells. Data shown are the numbers of basophils and NK cells in the spleen of each group (mean ± SEM, *n* = 4 each) and are representative of three repeated experiments. In *B*, the relative number of basophils and NK cells in the spleen of anti-ASGM1-treated mice, as compared with those of mice treated with control serum, at the indicated time points after the Ab injection is shown (mean ± SEM, *n* = 4 each) and is representative three repeated experiments. The cell number in mice treated with control serum is set as 100% at each time point. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

was blocked by preincubation of anti-ASGM1 with ASGM1 but not GM1, as observed in the staining of NK cells (Fig. 3*B*), demonstrating the specificity of the Ab and expression of ASGM1 on basophils. The expression of ASGM1 was also detected on NKT cells, CD8⁺ T cells, and γδ T cells, but the expression was confined to a subpopulation of them and heterogeneous in its level even among the same cell lineage (Fig. 3*A*), in accordance with previous reports (9, 10). No significant expression of ASGM1 was

detected on other hematopoietic cells, including neutrophils and eosinophils in the spleen, peripheral blood, and bone marrow as well as mast cells in the peritoneum (data not shown).

Anti-ASGM1 treatment ablates basophils in various mouse strains irrespective of NK1.1 allotype and MHC H2 haplotype

The anti-NK1.1-mediated NK cell depletion, unlike the anti-ASGM1-mediated one, can be applied to only certain mouse strains with NK1.1 allotype, such as C57BL/6 (H2^b) (8). For this reason, anti-ASGM1 is commonly used for the investigation of in vivo NK cell function in NK1.1[−] mouse strains. Accordingly, we examined whether basophils express ASGM1 and are depleted by anti-ASGM1 treatment in NK1.1[−] mouse strains BALB/c (H2^d), C3H (H2^k), and A/J (H2^a). Basophils as well as NK cells from all the mouse strains expressed ASGM1 (Fig. 4*A*), and the anti-ASGM1 treatment drastically reduced the number of both cell types in these mice, irrespective of NK1 allotype and MHC H2 haplotype (Fig. 4*B*). This finding raised concern about the possible contribution of basophils, rather than or in addition to NK cells, to some of phenotypes that have been reported in anti-ASGM1-treated mice with various genetic backgrounds.

Anti-ASGM1 treatment abolishes basophil function in vivo

To address the issue of whether a certain phenotype observed in anti-ASGM1-treated mice could indeed be attributed to basophil depletion, we examined the effect of anti-ASGM1 treatment on IgE-mediated chronic allergic inflammation (IgE-CAI) in the skin. IgE-CAI is elicited by passive sensitization of mice with hapten TNP-specific IgE and subsequent challenge with s.c. injection of TNP-conjugated OVA, and basophils play an essential role in the development of IgE-CAI (17, 21, 25). Treatment of C57BL/6 mice with anti-ASGM1 but not control rabbit serum 1 d after the Ag challenge completely abolished the development of IgE-CAI as judged by the inhibition of ear swelling (Fig. 5*A*, left panel), as observed in mice treated with basophil-depleting Ab (21). In contrast, treatment with anti-NK1.1 showed no significant effect on IgE-CAI (Fig. 5*A*, right panel). Furthermore, treatment with anti-ASGM1 but not control rabbit serum abolished the development

FIGURE 3. ASGM1 but not NK1.1 is expressed on the surface of basophils. *A*, Flow cytometry profiles of the indicated cell lineages of C57BL/6 mice that were stained with anti-ASGM1 (upper panels) or anti-NK1.1 (lower panels) are shown as representative five repeated experiments. Basophils and NK cells were defined as in Fig. 1*A*. NKT, CD8⁺ T, and γδ T cells were defined as CD49b⁺CD3e⁺, CD8⁺CD3e⁺, and TCRγδ⁺CD3e⁺ cells, respectively. Shaded histograms show the staining with control Abs (normal rabbit serum or mouse IgG2a). *B*, Spleen cells from C57BL/6 mice were stained with anti-ASGM1 alone (upper panels) or anti-ASGM1 that had been preincubated with ASGM1 (middle panels) or GM1 (lower panels). Staining profiles of basophils and NK cells are shown as representative of three repeated experiments. Shaded histograms show the staining with control rabbit serum.

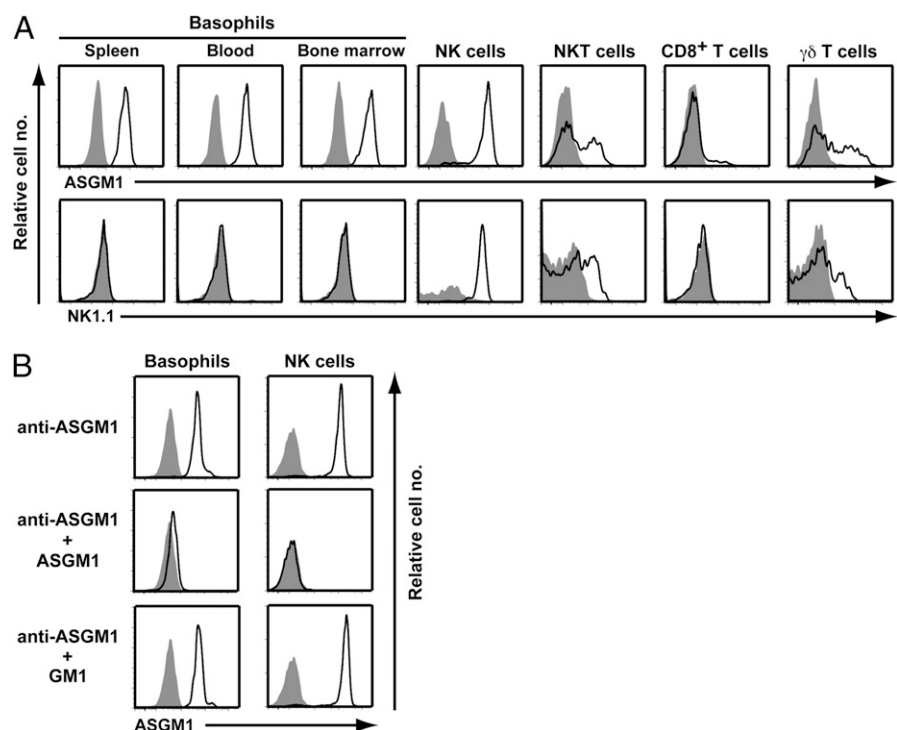
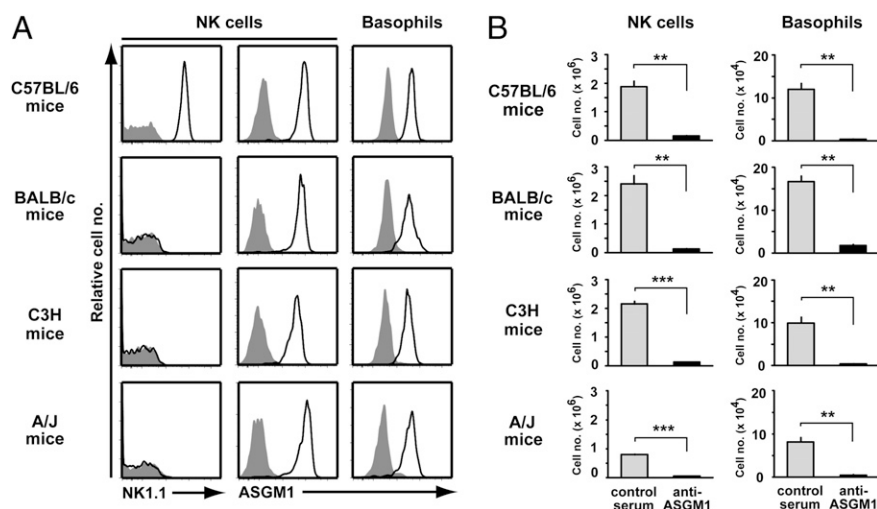


FIGURE 4. Anti-ASGM1 ablates basophils in various mouse strains irrespective of NK1 allotype and MHC H2 haplotype. **A**, NK1.1 and ASGM1 staining profiles of NK cells and basophils from the spleen of the indicated mouse strains are shown, as representative of three repeated experiments. **B**, The indicated mouse strains were treated with i.p. injection of anti-ASGM1 or control rabbit serum (10 μ l for C57BL/6 and 50 μ l for BALB/c, C3H, and A/J), and 1 d later, spleen cells were analyzed as in Fig. 2A. Data shown are the numbers of NK cells and basophils in the spleen of each group (mean $\pm$ SEM, $n = 4$ each) and are representative of three repeated experiments. Basophils in A/J mice are defined as Fc ϵ RI $^{+}$ CD200R3 $^{+}$ cells because of their lack of CD49b expression. ** $p < 0.01$, *** $p < 0.001$.



of IgE-CAI in rag2/ γ c doubly deficient mice that lack NK and NKT cells as well as T and B cells (Fig. 5B). These results demonstrated that the inhibitory effect of anti-ASGM1 on IgE-CAI was not due to the secondary effect of NK/NKT cell depletion. Thus, the anti-ASGM1 treatment abolishes basophil function in vivo.

Discussion

Anti-ASGM1 is commonly used as an NK cell-depleting Ab in mice for study on NK cell functions in diverse immune responses. In the current study, we revealed a previously unrecognized and lethal off-target effect of anti-ASGM1 on basophils. Among hematopoietic cells examined, only NK cells and basophils, at least under homeostatic conditions, show constitutive and homogeneous expression of ASGM1 at high levels. Although ASGM1 expression is also detected on NKT, CD8 $^{+}$ T, and $\gamma\delta$ T cells, the expression is confined to a subpopulation of them and is heterogeneous even among the same cell lineage. In accordance with this, in vivo administration of anti-ASGM1 ablated the vast majority of both NK cells and basophils with no significant reduction in the number of other lineage cells, including NKT, CD8 $^{+}$ T, and $\gamma\delta$ T cells, as far as examined (Supplemental Fig. 1).

Basophils are as sensitive as NK cells to anti-ASGM1-mediated ablation, and treatment of C57BL/6 mice with as little as 2.5 μ l anti-ASGM1 still eliminates three quarters of basophils (Supplemental Fig. 2). Although anti-ASGM1-mediated basophil depletion is transient, repeated administration of anti-ASGM1 can

induce a long-term depletion of basophils (Supplemental Fig. 3). Although both NK cells and basophils are depleted in vivo by anti-ASGM1, the mechanism underlying the Ab-mediated depletion appears to differ between them. Treatment of IgFcR-deficient *Fcrg* $^{-/-}$ mice with anti-ASGM1 resulted in depletion of NK cells but not basophils (Supplemental Fig. 4). Anti-ASGM1-mediated basophil depletion was observed even in the absence of NK cells (Fig. 5B), ruling out the depletion through the Ab-dependent cellular cytotoxicity by NK cells. Basophil depletion by anti-CD200R3 is also defective in *Fcrg* $^{-/-}$ mice (21), suggesting that Ab-mediated basophil depletion is dependent on FcRs, probably through FcR-mediated phagocytosis.

NK cells and basophils share CD49b (DX5) expression in addition to ASGM1 expression. NK cell depletion by anti-ASGM1 treatment has often been verified by the disappearance of the CD49b $^{+}$ CD3 $^{-}$ population, particularly in NK1.1 $^{-}$ mouse strains. As long as CD49b is considered as a selective marker of NK cells, the presence of basophils among this population would never be noticed. Moreover, in flow cytometric analysis, basophils, like NK cells, display the side and forward scatter profile closer to that of lymphoid cells rather than myeloid cells. These might be reasons among others why the important off-target effect of anti-ASGM1 treatment on basophils has long been overlooked by researchers. Our findings underscore the need to carefully interpret data obtained from experiments with anti-ASGM1-mediated NK cell depletion. The phenotype observed in anti-ASGM1-treated mice may be attributed to NK cell depletion, the off-target effect on

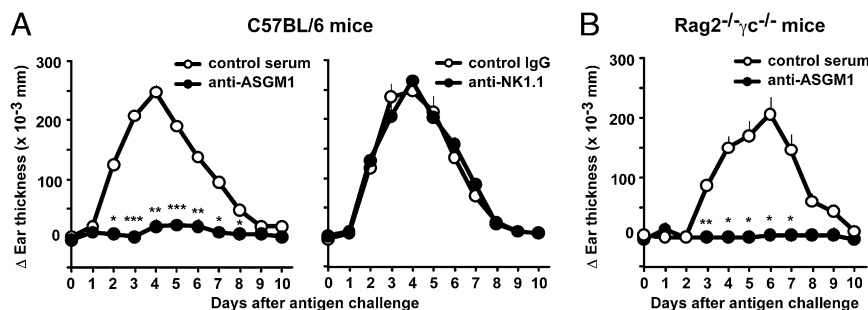


FIGURE 5. Anti-ASGM1 treatment abolishes the development of IgE-CAI. **A**, C57BL/6 mice were passively sensitized with TNP-specific IgE and challenged with s.c. injection of TNP-OVA into the left ear and control OVA into the right ear. One day after Ag challenge, mice were i.p. treated with 10 μ l anti-ASGM1 or control rabbit serum (left panel) or 300 μ g anti-NK1.1 or isotype-matched control (right panel). The thickness of the left and right ears was measured at the indicated time points. The value of Δ Ear thickness, the difference in ear thickness (left – right) at each time point, is plotted (mean $\pm$ SEM, $n = 4$ each). **B**, rag2 $^{-/-}$ γ c $^{-/-}$ C57BL/6 mice were manipulated and analyzed as in A. The value of Δ Ear thickness is plotted (mean $\pm$ SEM, $n = 3$ each). Data shown in A and B are representative of three and two repeated experiments, respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

basophils, or both. Indeed, the impaired IgE-CAI response in anti-ASGM1-treated mice was attributed to the depletion of basophils but not NK cells (Fig. 5B).

Recent studies have illuminated previously unappreciated roles for basophils in various immune responses (26–36). Basophils play an important role in the initiation of Th2-type immune responses by providing IL-4 (22) and even by functioning as APCs under certain conditions (39–41). They also enhance humoral memory responses by promoting B cell activation and differentiation (23, 42). Basophils play crucial roles in protective immunity to parasitic infections (24, 25) and also contribute to the development of immunological disorders, including allergy and autoimmunity (17, 43, 44). We also appreciate that NK cells play a key role in the immune response to certain infections and malignancies by direct cytotoxicity of infected or transformed cells and by secretion of potent immune mediators (1–3). NK cell-derived cytokines, such as IFN- γ and TNF, enhance the innate immune response and shape the subsequent adaptive immune response. Thus, anti-ASGM1-mediated depletion of both NK cells and basophils could have greater impact on various immune responses compared with the depletion of either NK cells or basophils alone. Accordingly, it is important to dissect the contribution of basophils and NK cells to the immunological phenotype observed in anti-ASGM1-treated mice.

NK cells have been suggested to suppress the development of protective immunity against mycoplasma infection in a mouse model in that IFN- γ promotes the protection, whereas IL-4 inhibits it (45, 46). Intriguingly, treatment of BALB/c mice with anti-ASGM1 before immunization with mycoplasma Ags resulted in enhancement rather than suppression of the protection from subsequent mycoplasma infection. Lymphocytes isolated from draining lymph nodes of anti-ASGM1-treated immunized mice produced higher IFN- γ and lower IL-4 compared with those from untreated immunized mice, suggesting a shift toward a more Th1-dominant response in the anti-ASGM1-treated mice. These observations are quite puzzling, given that anti-ASGM1 treatment depleted NK cells alone, as the authors judged on the basis of the finding that all ASGM1⁺ cells expressed a typical NK cell marker CD49b. However, this puzzle might be solved when we take basophils into account in this type of immune response. Basophils might contribute to Th2 differentiation, and therefore anti-ASGM1-mediated basophil depletion results in a shift toward a more Th1-dominant response that is favorable for protection from mycoplasma infection.

A peculiar Th1^{du}CD49b⁺ cell population in the bone marrow, distinct from NK, NKT, and T cells, was identified as a cell type that displays the ability to protect immature B cells from apoptosis induced by BCR engagement and to redirect them toward receptor editing (47, 48). This population, designated as bone marrow protective cells (BMPCs), constitutes only 0.5–1% of the bone marrow, and homogeneously expresses ASGM1. Treatment of mice with anti-ASGM1 eliminates most of this population, leading to the loss of B cell-protective activity in the bone marrow. These features of BMPCs are all shared by basophils. Basophils produce B cell-stimulating molecules such as IL-4, IL-6, and BAFF (23, 42, 44). Therefore, BMPCs probably represent basophils.

In conclusion, the current study identified basophils as a previously unrecognized target of anti-ASGM1-mediated cell depletion. This finding raises concern about the possibility that certain functions of basophils might have been erroneously interpreted as those of NK cells in studies using anti-ASGM1 for NK cell depletion. Therefore, in the functional study of NK cells with use of anti-ASGM1, it is essential to dissect the role of NK cells and basophils, in conjunction with the use of more special-

ized tools, including anti-NK1.1 and basophil-depleting Abs (21–23), engineered mice deficient only in NK cells (49, 50), and those deficient only in basophils (24, 25).

Acknowledgments

We thank the members of the Karasuyama laboratory for helpful discussions and Michiko Miki for administrative and secretarial assistance.

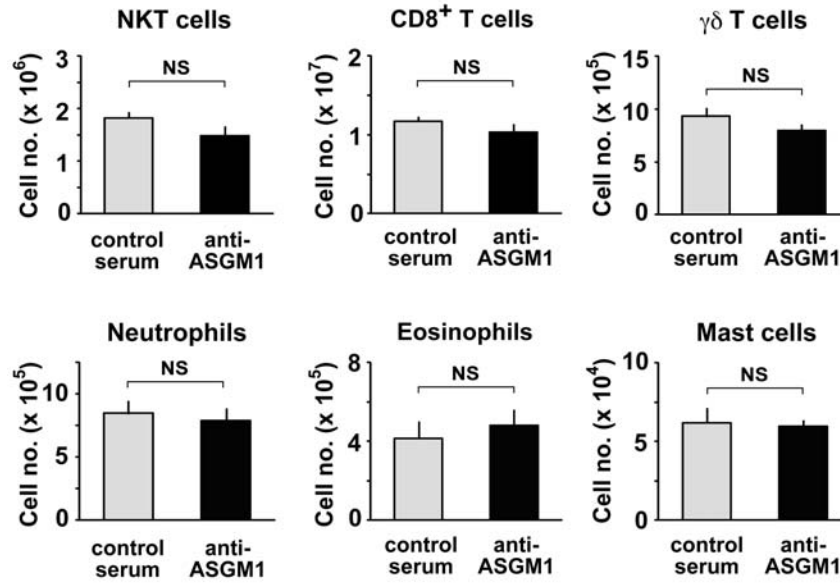
Disclosures

The authors have no financial conflicts of interest.

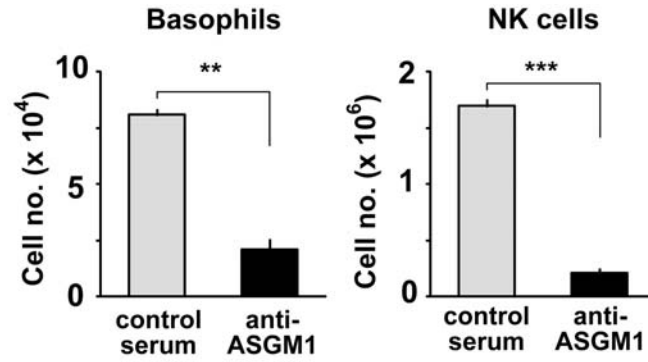
References

- Cooper, M. A., M. Colonna, and W. M. Yokoyama. 2009. Hidden talents of natural killers: NK cells in innate and adaptive immunity. *EMBO Rep.* 10: 1103–1110.
- Orr, M. T., and L. L. Lanier. 2010. Natural killer cell education and tolerance. *Cell* 142: 847–856.
- Vivier, E., D. H. Raulet, A. Moretta, M. A. Caligiuri, L. Zitvogel, L. L. Lanier, W. M. Yokoyama, and S. Ugolini. 2011. Innate or adaptive immunity? The example of natural killer cells. *Science* 331: 44–49.
- Kasai, M., M. Iwamori, Y. Nagai, K. Okumura, and T. Tada. 1980. A glycolipid on the surface of mouse natural killer cells. *Eur. J. Immunol.* 10: 175–180.
- Young, W. W., Jr., S. I. Hakomori, J. M. Durdik, and C. S. Henney. 1980. Identification of ganglio-N-tetraosylceramide as a new cell surface marker for murine natural killer (NK) cells. *J. Immunol.* 124: 199–201.
- Kasai, M., T. Yoneda, S. Habu, Y. Maruyama, K. Okumura, and T. Tokunaga. 1981. In vivo effect of anti-asialo GM1 antibody on natural killer activity. *Nature* 291: 334–335.
- Peace, D. J., and M. A. Cheever. 1989. Toxicity and therapeutic efficacy of high-dose interleukin 2. In vivo infusion of antibody to NK-1.1 attenuates toxicity without compromising efficacy against murine leukemia. *J. Exp. Med.* 169: 161–173.
- Carlyle, J. R., A. Mesci, B. Ljutic, S. Belanger, L. H. Tai, E. Rousselle, A. D. Troke, M. F. Proteau, and A. P. Makrigiannis. 2006. Molecular and genetic basis for strain-dependent NK1.1 alloreactivity of mouse NK cells. *J. Immunol.* 176: 7511–7524.
- Lee, U., K. Santa, S. Habu, and T. Nishimura. 1996. Murine asialo GM1+CD8+ T cells as novel interleukin-12-responsive killer T cell precursors. *Jpn. J. Cancer Res.* 87: 429–432.
- Trambley, J., A. W. Bingaman, A. Lin, E. T. Elwood, S. Y. Waitze, J. Ha, M. M. Durham, M. Corbascio, S. R. Cowan, T. C. Pearson, and C. P. Larsen. 1999. Asialo GM1(+) CD8(+) T cells play a critical role in costimulation blockade-resistant allograft rejection. *J. Clin. Invest.* 104: 1715–1722.
- Wiltrout, R. H., A. Santoni, E. S. Peterson, D. C. Knott, W. R. Overton, R. B. Herberman, and H. T. Holden. 1985. Reactivity of anti-asialo GM1 serum with tumoricidal and non-tumoricidal mouse macrophages. *J. Leukoc. Biol.* 37: 597–614.
- Slifka, M. K., R. R. Pagarigan, and J. L. Whitton. 2000. NK markers are expressed on a high percentage of virus-specific CD8+ and CD4+ T cells. *J. Immunol.* 164: 2009–2015.
- Kataoka, S., Y. Konishi, Y. Nishio, K. Fujikawa-Adachi, and A. Tominaga. 2004. Antitumor activity of eosinophils activated by IL-5 and eotaxin against hepatocellular carcinoma. *DNA Cell Biol.* 23: 549–560.
- Galli, S. J. 2000. Mast cells and basophils. *Curr. Opin. Hematol.* 7: 32–39.
- Min, B., M. Prout, J. Hu-Li, J. Zhu, D. Jankovic, E. S. Morgan, J. F. Urban, Jr., A. M. Dvorak, F. D. Finkelman, G. LeGros, and W. E. Paul. 2004. Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J. Exp. Med.* 200: 507–517.
- Voehringer, D., K. Shinkai, and R. M. Locksley. 2004. Type 2 immunity reflects orchestrated recruitment of cells committed to IL-4 production. *Immunity* 20: 267–277.
- Mukai, K., K. Matsuoka, C. Taya, H. Suzuki, H. Yokozeki, K. Nishioka, K. Hirokawa, M. Etori, M. Yamashita, T. Kubota, et al. 2005. Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunity* 23: 191–202.
- Poorafshar, M., H. Helmbj, M. Troye-Blomberg, and L. Hellman. 2000. MMCP-8, the first lineage-specific differentiation marker for mouse basophils. Elevated numbers of potent IL-4-producing and MMCP-8-positive cells in spleens of malaria-infected mice. *Eur. J. Immunol.* 30: 2660–2668.
- Ugajin, T., T. Kojima, K. Mukai, K. Obata, Y. Kawano, Y. Minegishi, Y. Eishi, H. Yokozeki, and H. Karasuyama. 2009. Basophils preferentially express mouse Mast Cell Protease 11 among the mast cell tryptase family in contrast to mast cells. *J. Leukoc. Biol.* 86: 1417–1425.
- Falcone, F. H., H. Haas, and B. F. Gibbs. 2000. The human basophil: a new appreciation of its role in immune responses. *Blood* 96: 4028–4038.
- Obata, K., K. Mukai, Y. Tsujimura, K. Ishiwata, Y. Kawano, Y. Minegishi, N. Watanabe, and H. Karasuyama. 2007. Basophils are essential initiators of a novel type of chronic allergic inflammation. *Blood* 110: 913–920.
- Sokol, C. L., G. M. Barton, A. G. Farr, and R. Medzhitov. 2008. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat. Immunol.* 9: 310–318.

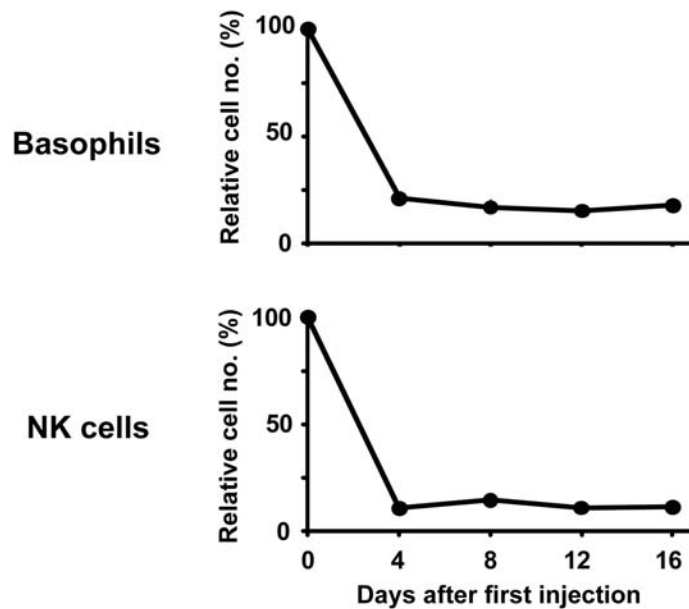
23. Denzel, A., U. A. Maus, M. Rodriguez Gomez, C. Moll, M. Niedermeier, C. Winter, R. Maus, S. Hollingshead, D. E. Briles, L. A. Kunz-Schughart, et al. 2008. Basophils enhance immunological memory responses. *Nat. Immunol.* 9: 733–742.
24. Wada, T., K. Ishiwata, H. Koseki, T. Ishikura, T. Ugajin, N. Ohnuma, K. Obata, R. Ishikawa, S. Yoshikawa, K. Mukai, et al. 2010. Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks. *J. Clin. Invest.* 120: 2867–2875.
25. Ohnmacht, C., C. Schwartz, M. Panzer, I. Schiedewitz, R. Naumann, and D. Voehringer. 2010. Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. *Immunity* 33: 364–374.
26. Min, B. 2008. Basophils: what they ‘can do’ versus what they ‘actually do’. *Nat. Immunol.* 9: 1333–1339.
27. Karasuyama, H., K. Mukai, Y. Tsujimura, and K. Obata. 2009. Newly discovered roles for basophils: a neglected minority gains new respect. *Nat. Rev. Immunol.* 9: 9–13.
28. Sullivan, B. M., and R. M. Locksley. 2009. Basophils: a nonredundant contributor to host immunity. *Immunity* 30: 12–20.
29. Schroeder, J. T. 2009. Basophils beyond effector cells of allergic inflammation. *Adv. Immunol.* 101: 123–161.
30. Gibbs, B. F., C. Streatfield, and F. H. Falcone. 2009. Basophils as critical orchestrators of Th2-type immune responses. *Expert Rev. Clin. Immunol.* 5: 725–734.
31. Sokol, C. L., and R. Medzhitov. 2010. Emerging functions of basophils in protective and allergic immune responses. *Mucosal Immunol.* 3: 129–137.
32. Siracusa, M. C., J. G. Perrigoue, M. R. Comeau, and D. Artis. 2010. New paradigms in basophil development, regulation and function. *Immunol. Cell Biol.* 88: 275–284.
33. Schneider, E., N. Thieblemont, M. L. De Moraes, and M. Dy. 2010. Basophils: new players in the cytokine network. *Eur. Cytokine Netw.* 21: 142–153.
34. Nakanishi, K. 2010. Basophils as APC in Th2 response in allergic inflammation and parasite infection. *Curr. Opin. Immunol.* 22: 814–820.
35. Karasuyama, H., K. Mukai, K. Obata, Y. Tsujimura, and T. Wada. 2010. Non-redundant Roles of Basophils in Immunity. *Annu. Rev. Immunol.* 29: 45–69.
36. Karasuyama, H., T. Wada, S. Yoshikawa, and K. Obata. 2011. Emerging roles of basophils in protective immunity against parasites. *Trends Immunol.* 32: 125–130.
37. Ohnmacht, C., and D. Voehringer. 2009. Basophil effector function and homeostasis during helminth infection. *Blood* 113: 2816–2825.
38. Jamieson, A. M., P. Isnard, J. R. Dorfman, M. C. Coles, and D. H. Raulet. 2004. Turnover and proliferation of NK cells in steady state and lymphopenic conditions. *J. Immunol.* 172: 864–870.
39. Sokol, C. L., N. Q. Chu, S. Yu, S. A. Nish, T. M. Laufer, and R. Medzhitov. 2009. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat. Immunol.* 10: 713–720.
40. Perrigoue, J. G., S. A. Saenz, M. C. Siracusa, E. J. Allenspach, B. C. Taylor, P. R. Giacomini, M. G. Nair, Y. Du, C. Zaph, N. van Rooijen, et al. 2009. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nat. Immunol.* 10: 697–705.
41. Yoshimoto, T., K. Yasuda, H. Tanaka, M. Nakahira, Y. Imai, Y. Fujimori, and K. Nakanishi. 2009. Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat. Immunol.* 10: 706–712.
42. Chen, K., W. Xu, M. Wilson, B. He, N. W. Miller, E. Bengtén, E. S. Edholm, P. A. Santini, P. Rath, A. Chiu, et al. 2009. Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell-stimulating programs in basophils. *Nat. Immunol.* 10: 889–898.
43. Tsujimura, Y., K. Obata, K. Mukai, H. Shindou, M. Yoshida, H. Nishikado, Y. Kawano, Y. Minegishi, T. Shimizu, and H. Karasuyama. 2008. Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. *Immunity* 28: 581–589.
44. Charles, N., D. Hardwick, E. Daugas, G. G. Illei, and J. Rivera. 2010. Basophils and the T helper 2 environment can promote the development of lupus nephritis. *Nat. Med.* 16: 701–707.
45. Bodhankar, S., M. D. Woolard, X. Sun, and J. W. Simecka. 2009. NK cells interfere with the generation of resistance against mycoplasma respiratory infection following nasal-pulmonary immunization. *J. Immunol.* 183: 2622–2631.
46. Bodhankar, S., X. Sun, M. D. Woolard, and J. W. Simecka. 2010. Interferon gamma and interleukin 4 have contrasting effects on immunopathology and the development of protective adaptive immunity against mycoplasma respiratory disease. *J. Infect. Dis.* 202: 39–51.
47. Sandel, P. C., and J. G. Monroe. 1999. Negative selection of immature B cells by receptor editing or deletion is determined by site of antigen encounter. *Immunity* 10: 289–299.
48. Sandel, P. C., M. Gendelman, G. Kelsoe, and J. G. Monroe. 2001. Definition of a novel cellular constituent of the bone marrow that regulates the response of immature B cells to B cell antigen receptor engagement. *J. Immunol.* 166: 5935–5944.
49. Walzer, T., M. Bléry, J. Chaix, N. Fuseri, L. Chasson, S. H. Robbins, S. Jaeger, P. André, L. Gauthier, L. Daniel, et al. 2007. Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. *Proc. Natl. Acad. Sci. USA* 104: 3384–3389.
50. Gascoyne, D. M., E. Long, H. Veiga-Fernandes, J. de Boer, O. Williams, B. Seddon, M. Coles, D. Kioussis, and H. J. Brady. 2009. The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. *Nat. Immunol.* 10: 1118–1124.



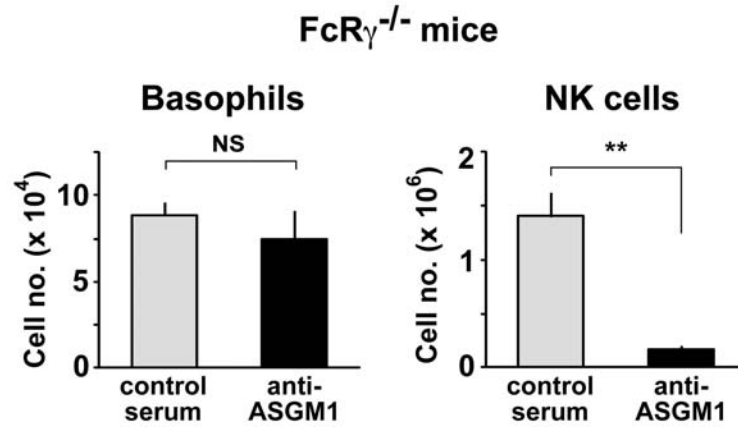
Supplemental Figure 1. No significant effect of anti-ASGM1 treatment on the number of cell lineages other than NK cells and basophils. C57BL/6 mice were treated with 10 μ l of anti-ASGM1 or control rabbit serum. One day later, spleen and peritoneal cells were isolated and subjected to flow cytometric analysis. Data shown are the numbers of NKT cells (CD49b⁺CD3 ϵ ⁺), CD8⁺ T cells (CD8⁺CD3 ϵ ⁺), $\gamma\delta$ T cells (TCR $\gamma\delta$ ⁺CD3 ϵ ⁺), neutrophils (Gr-1^{high}Siglec-F^{low}), eosinophils (Gr-1^{int}Siglec-F⁺) in the spleen, and peritoneal mast cells (c-Kit⁺Fc ϵ R1 α ⁺) of each group (mean $\pm$ SEM, $n=4$ each), and are representative of three repeated experiments. NS, not significant.



Supplemental Figure 2. Treatment of mice with as little as 2.5 μ l of anti-ASGM1 is sufficient to significantly reduce the number of basophils and NK cells. C57BL/6 mice were treated with 2.5 μ l of anti-ASGM1 or control rabbit serum. One day later, spleen cells were analyzed as in Fig. 2A. Data shown are the numbers of basophils and NK cells in the spleen of each group (mean $\pm$ SEM, $n=4$ each), and are representative of three repeated experiments. *** $p<0.001$; ** $p<0.01$.



Supplemental Figure 3. Repeated treatment of mice with anti-ASGM1 leads to a longer-term depletion of basophils. C57BL/6 mice were treated 4 times (on day 0, 4, 8 and 12) with 10 μ l of anti-ASGM1 or control rabbit serum each time. The relative number of basophils and NK cells in the spleen of anti-ASGM1-treated mice, as compared with those of mice treated with control serum, at the indicated time points (4 days after each Ab injection) is shown (mean $\pm$ SEM, $n=4$ each), and is representative of two repeated experiments. The cell number in mice treated with control serum is set as 100% at each time points.



Supplemental Figure 4. Anti-ASGM1-mediated basophil depletion is dependent on FcRs. *Fcrg*^{-/-} C57BL/6 mice were treated with 10 μ l of anti-ASGM1 or control rabbit serum, and one day later spleen cells were analyzed as in Fig. 2A. Data shown are the numbers of basophils and NK cells in the spleen of each group (mean $\pm$ SEM, $n=4$ each), and are representative of two repeated experiments. ** $p<0.01$; NS, not significant.