Hypersensitivity reactions to asparaginase in mice are mediated by anti-asparaginase IgE and IgG and the immunoglobulin receptors FcεRI and FcγRIII

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SUPPLEMENTAL MATERIAL

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Running Title:
ASNase hypersensitivities are IgG/IgE dependent

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SUPPLEMENTARY METHODS:

Mice

Female C57BL/6 (wild-type) mice were purchased from Jackson Laboratories (USA) and housed at the University of Pittsburgh animal facility.

Antibodies and other reagents

Fluorescent labeled monoclonal or blocking antibodies for flow cytometry analysis include anti-mouse CD45, CD200R3 (Miltenyi Biotec, Germany), IgE, FcεR1α (Biolegend, USA), CD19, Ly6G, CD11b, CD3, CD4, CD8, FoxP3, CD200R1 and CD49b (eBiosciences, USA). 2.4G2 (rat IgG2b anti-mouse FcγRIIB/III mAb) from ATTC (Rockville, MD) and EM-95 (rat IgG2a anti-mouse IgE mAb) from Zelig Eshhar (Rehoveth, Israel) were used to investigate the role of immunoglobulin receptors on antigen-specific recognition. Anti-CD4 and anti-CD19 (Bio X Cell, USA) were used for cell depletion studies. L-ASNase produced from *E. coli* was obtained from BioVendor Laboratory Medicine Inc., USA. Aluminum hydroxide adjuvant (Imject Alum, Thermo Scientific, USA) was used for mouse immunization with ASNase. ASNase was labeled with Alexa Fluor-647 (Alexa Fluor 647 Protein Labeling Kit, Molecular probe, USA). Fixation permeabilization buffer (eBiosciences, USA), horse serum (HS, Thermo Fisher, USA), red blood cell lysis solution (10X) (Miltenyi Biotec, Germany), CV-6209 (Santa Cruz Biotechnology, USA), Triprolidine, Ficoll histopaque, and ethylenediaminetetraacetic acid disodium salt dehydrate-EDTA (Sigma, USA) were used as described below.

ASNase Uptake Experiments

Mice were injected intravenously (IV) with 100 μg of Alexa Fluor 647-labeled ASNase or PBS as a vehicle control. Mice were euthanized by CO_2/O_2 asphyxiation 8 hours (h) after injection. Blood and major organs were harvested and processed for ASNase uptake by flow cytometry. The livers of mice were filtered using a 0.2 μm cell strainer and $1x10^6$ liver or blood cells were stained with immune cell markers (i.e., neutrophils, macrophages/monocytes, T cells, B cells, and basophils), as described below.

ASNase uptake by murine macrophages

The RAW 264.7 murine macrophage cell line (2×10^5 cells) was cultured in DMEM culture medium, containing 10% (v/v) fetal bovine serum, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in a humidified 5% CO_2-95% air atmosphere. RAW cells were cultured with

labeled ASNase at 1 μg/mL for 0 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h or 24 h at 37 °C. ASNase was removed and the cells were washed with PBS and 0.25% trypsin was added to the cells for 5 min at 37 °C. Cells were re-suspended directly with PBS, stained with anti-CD11b for 30 min, washed with 1% BSA in PBS, and re-suspended in 200 μL of FACS running buffer (Miltenyi Biotec, Germany) for cytometry analysis.

Detection of ASNase-induced hypersensitivity and sample collection

Hypersensitivity was detected by monitoring decreases in rectal temperature using a digital thermometer (model BAT-12, Physitemp Instruments, Clifton, NJ, USA). Rectal temperature measurements were performed immediately before (time 0) and at different time points for 2 h following the ASNase challenge. The area under the temperature versus time curve was calculated using the trapezoidal rule. Lower AUC values indicate a larger drop in core body temperature and/or a longer period of reduced body temperature relative to before the ASNase challenge (Figure 4A, 5C, and Supplemental Figure 7). More severe hypersensitivities are indicated by a greater change in core body temperature or as lower AUC values. Differences in the severity of ASNase hypersensitivities among groups were determined based on the extent of hypothermia by comparing the AUC values of different groups (Figure 4A, 5C, and 7). Pre-challenge plasma and blood samples were collected on days 0 (2 hours after immunization and indicated throughout the text as 2 h), and on days 7, 14, and 23 of the treatment protocol by submandibular bleeding, while post-challenge samples were collected by cardiac puncture at the end of the experiment. Results from sensitized or non-sensitized mice are represented as green or red data points, respectively, throughout the manuscript.

Detection of whole anti-ASNase IgG, $IgG_{1, 2a, 2b, 2c}$ and IgE antibodies by ELISA

Plasma was used to determine anti-ASNase IgG levels using an ELISA-based method, as described previously.[1-3] Plasma samples from non-sensitized mice were used as negative controls, and rabbit polyclonal anti-ASNase IgG antibodies were used as positive controls (Rockland, USA). Secondary antibodies to whole mouse IgG (Sigma, USA), IgG1, IgG2a, IgG2b (Southern Biotech, USA), IgG2c (Thermo Fisher, USA), and IgE (Thermo Fisher, USA) were used for detection at a 1:1000 dilution. The samples were diluted to 1:400 in blocking buffer (1% dry milk powder in water) for IgG detection and to 1:100 for IgE detection.

Mouse mast cell protease-1 (mMCP-1) ELISA

Mouse MCP-1 concentrations were determined from plasma samples using a commercially available ELISA kit, according to the manufacturer's instructions (Ready-SET-Go, eBioscience, USA).

Asparaginase activity determination

The ASNase activity in plasma samples was determined by monitoring the enzymatically coupled oxidation of reduced nicotinamide adenine dinucleotide (NADH) to NAD^+ in a 96-well format, as described previously.[4]

Inhibiting the *ex vivo* binding of ASNase to leukocytes or basophils using anti-IgE or anti-FcγRIIB/III mAb

Blood from sensitized mice (Day 23) was incubated with EM-95, 2.4G2, both (1μg/mL) or medium (as a negative control) for 1 h. Samples were then stained with labeled ASNase, CD45, CD49b, and IgE for 30 minutes in the dark at 4°C, lysed, washed, and acquired by flow cytometry to determine the binding of ASNase on leukocytes or basophils in the presence and absence of blocking antibodies. To determine the extent of ASNase binding that is independent of soluble anti-ASNase IgG antibody, samples were first washed and lysed to remove soluble antibodies (i.e., anti-ASNase IgG) before staining with ASNase.

Flow cytometric analysis

Freshly collected blood cells in EDTA were stained with fluorochrome-labeled mAbs for 30-60 minutes at 4 °C. Immune cell populations were defined as follows: leukocytes ($CD45^+$), B cells ($CD45^+CD19^+$), neutrophils ($CD45^+CD11b^+Ly6G^+$), macrophages/monocytes ($CD45^+CD11b^+Ly6G^-$), basophils ($CD45^+CD49b^+IgE^+$), T cells ($CD3^+$), $CD4^+$ T cells ($CD3^+CD4^+$), $CD8^+$ T cells ($CD3^+CD8^+$), $CD4^+$ Tregs ($CD3^+CD4^+FoxP3^+$), and $CD8^+$ Treg ($CD3^+CD8^+FoxP3^+$). Fluorochrome-labeled ASNase was used to determine its *ex vivo* binding with immune cell populations. The cell surface markers described above are commonly used for identification of said cells;[5-8] however, other cell types at low frequencies may have also been included in the analysis. For example, neutrophil populations may contain some macrophages/monocytes,[9] macrophages/monocytes populations may include Ly6G low or negative neutrophils,[10] and Treg populations may include some activated T cells that also express FoxP3.[11, 12]

Staining procedure

Whole blood staining (no lyse/no wash): A 25 µL aliquot of blood and 75 µL of 1% BSA in PBS containing fluorochrome-labeled antibodies were mixed together and incubated for 30-60 minutes in the dark at 4°C. After incubation, 1.9 mL of 1% BSA in PBS was added to the sample, which was immediately analyzed by flow cytometry (acquired 100 µL / 2000 µL, with at least 20,000 $CD45^+$ events recorded).

Surface staining: A 25 µL aliquot of blood was depleted of erythrocytes by adding 1 mL of 1X RBC lysis solution (Miltenyi Biotech, Germany) for 5-7 minutes at 4°C. Cells were washed by adding 1 mL of 1% BSA in PBS and centrifuged at 1600 rpm for 5 minutes. Surface marker mAb mixtures were added and the cells were incubated for 30-60 minutes in the dark at 4°C, and subsequently washed before flow cytometry analysis or intracellular staining.

Intracellular staining (IC): After surface staining, cells were washed, re-suspended in 1 mL of cold, freshly prepared fixation/permeabilization solution (eBiosciences, USA), and incubated for 30 minutes in the dark at 4°C. The cells were washed and re-suspended in 50-100 µL of permeabilization buffer. IC FoxP3 mAb was then added and the cells were incubated in the dark at 4°C for 30-60 minutes. After incubation, the cells were washed, re-suspended in 200 µL of 1% BSA, and immediately analyzed. All flow cytometry data were analyzed using FlowJo_V10 (TreeStar, USA).

Statistics

Statistical analysis was performed with two-tailed Student's t test between two groups and one-way analysis of variance (ANOVA) for multiple groups, followed by Tukey's post-hoc test. A P value of less than 0.05 was considered significant. If not stated otherwise, data are represented as means ± standard deviation. All statistical analysis was performed with the GraphPad Prism 7 statistical software.

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. ASNase accumulates in the liver and blood of naïve mice. (A) Naïve mice were injected intravenously with 100 μg of labeled ASNase or PBS as control and samples were harvested 8 h after the dose. The ASNase uptake of different tissues and organs was determined by flow cytometry. (B) ASNase uptake by immune cells within gated $CD45^+$ leukocytes was determined from the liver and blood cells of naïve mice (n=3).

Supplemental Figure 2. Macrophage uptake of ASNase was determined by flow cytometry. (A) The kinetics of labeled ASNase uptake by RAW 647.7 cells after 0 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h or 24 h of incubation were determined by flow cytometry. (B) The FACS plots show representative data of ASNase-negative (upper panel) and positive (lower panel) CD11b positive RAW cells after 0.5 hours of incubation.

Supplemental Figure 3. Protocol for native *E. coli* asparaginase sensitization, immune cell depletion, and sample collection. (A) Mice received intraperitoneal injections of *E. coli* ASNase (10 μg per mouse) or PBS formulated with aluminum hydroxide adjuvant (Alum, 1 mg) on Day 0 and Day 14 of the immunization schedule to sensitize mice. On Day 24, mice were challenged with an intravenous dose of ASNase (100 μg per mouse) to induce hypersensitivity reactions. (B) B cells or $CD4^+$T cells were depleted on Days -3 and 11 using monoclonal antibodies targeting CD19 or CD4, respectively. (C) A subset of mice were challenged after a single sensitization dose on Day 10 of the protocol.

Supplemental Figure 4. Mice developed an anti-ASNase IgG1 response after sensitization. Sensitized mice develop an anti-ASNase IgG1 subclass response, whereas IgG2a/b/c were not detected relative to controls among samples collected on Day 23 by ELISA (n = 20).

Supplemental Figure 5. No changes in the frequency of $CD4/8^+$ T cells, $CD8^+$ Tregs, or neutrophils were detected in the blood during sensitization. (A-D) No changes in the frequency of $CD4/8^+$ T cells, $CD8^+$ Tregs, or neutrophils were detected in the blood of sensitized mice (red data points) at Day 0 (2 h after immunization), Day 7, Day 14, and Day 23 of the protocol relative to controls (green data points). A total of 5 or 10 mice were included in each analysis, as indicated.

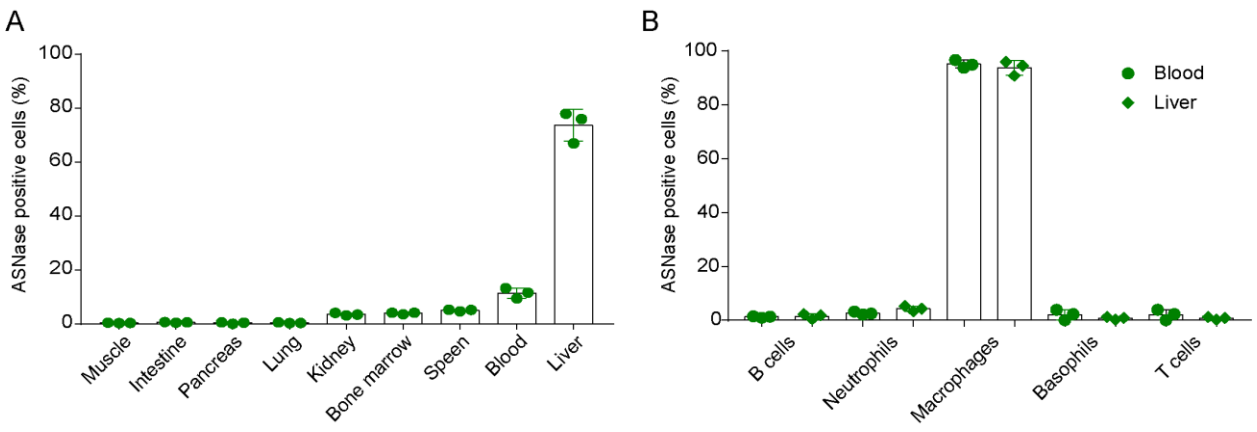
Supplemental Figure 6. The frequency of $CD4^+$ Tregs and basophils increases after challenging with ASNase in the blood. (A-B) The frequency of $CD4^+$ Tregs and basophils were higher in sensitized mice on Day 24 after the ASNase challenge relative to controls. (C-H) No changes in the frequency of $CD4^+/8^+$ T cells, B cells, neutrophils, $CD8^+$ Tregs, or macrophages were detected in sensitized mice (red data points) on Day 24 after the ASNase challenge relative to controls (green data points, n = 10).

Supplemental Figure 7. *In vivo* depletion of $CD4^+$ T cells prevents ASNase-mediated hypersensitivity reactions. $CD4^+$ T cells or B cells were depleted throughout the sensitization protocol. The rectal temperatures of non-sensitized controls, sensitized controls, and sensitized mice with $CD4^+$ T cell or B cell depletion were measured after challenging with ASNase (n = 5).

Supplemental Figure 8. ASNase binding to basophils correlates with the onset of ASNase hypersensitivities. The binding of ASNase to B cells, basophils, macrophages, or neutrophils was determined after (A) B cell or (B) $CD4^+$ T cell depletion and challenging with ASNase. The basophils of mice after B cell depletion bound to ASNase *ex vivo*, whereas the basophils of mice after $CD4^+$ T cell depletion showed no binding to ASNase (n = 5). P value significance is indicated as * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 1x10^{-3}$, and **** for $P < 1x10^{-4}$.

Supplemental Figure 9. A single dose of ASNase is not sufficient to induce basophil activation, demonstrate antigen-specific recognition by basophils, or induce ASNase hypersensitivities. The basophils of mice receiving a single dose of ASNase during sensitization were not (A-B) activated and (C) did not show ASNase-specific recognition when ASNase was added *ex vivo* to blood samples collected on Day 9 of sensitization. (D) Consistent with a lack of basophil activation and ASNase binding, the mice did not develop hypersensitivities when challenged with ASNase on Day 10.

Supplemental Figure 1.



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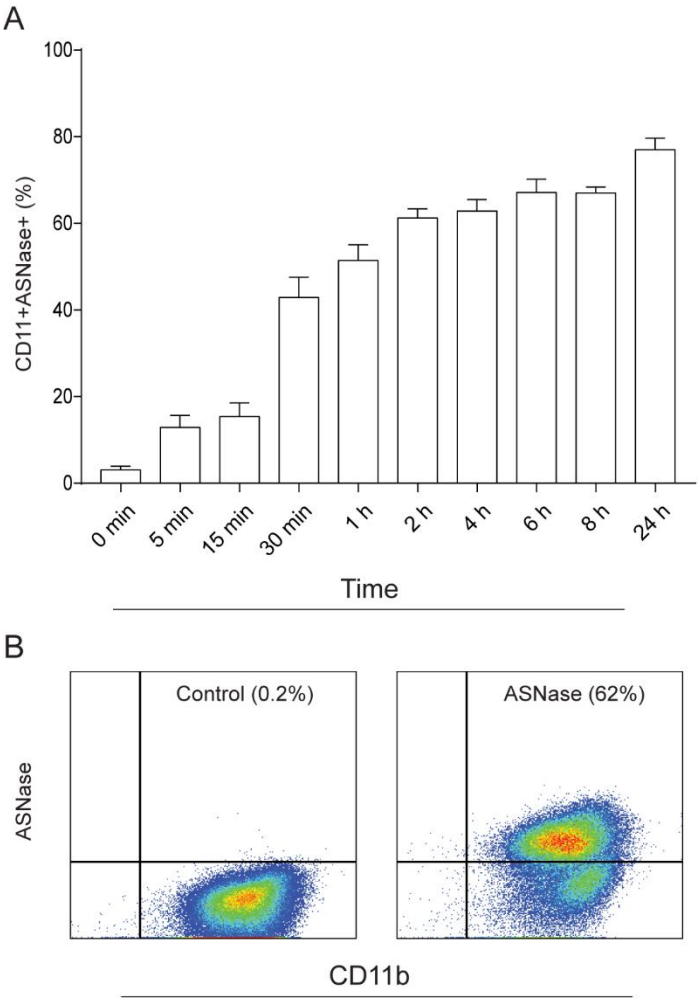

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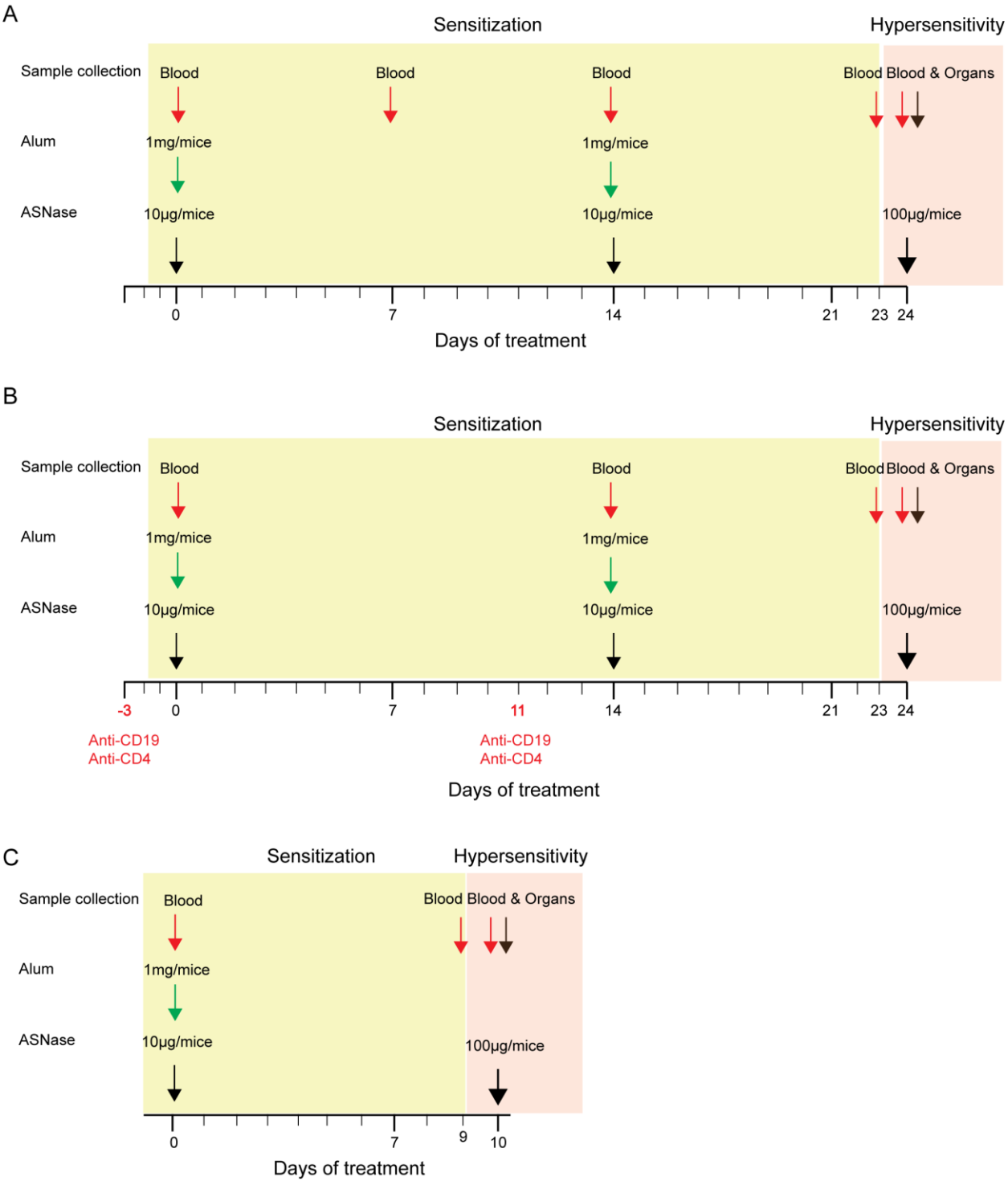

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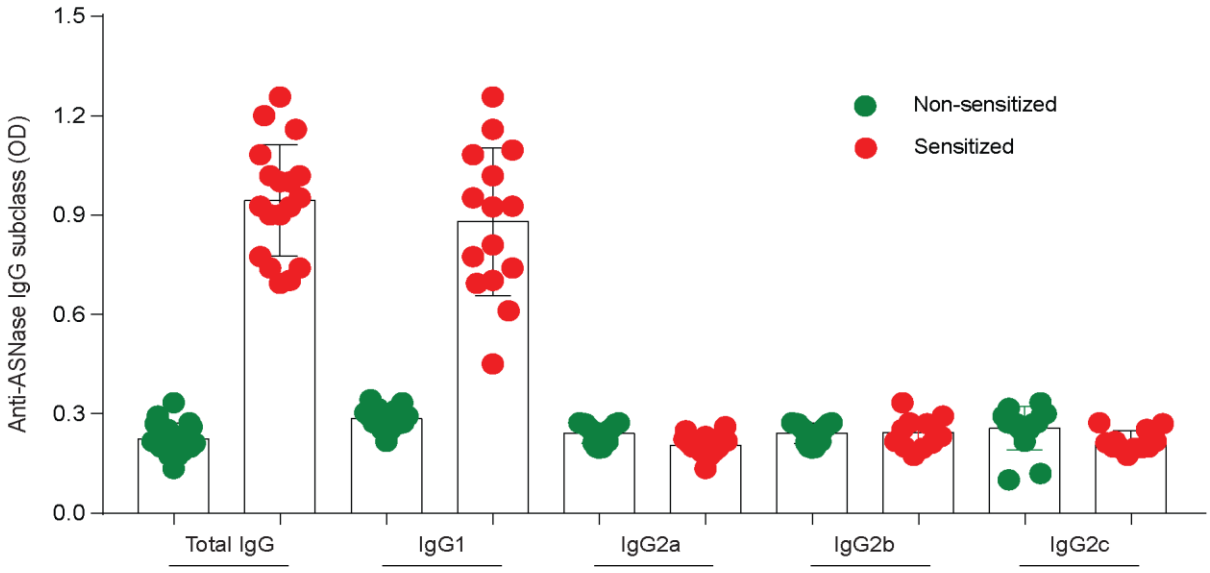

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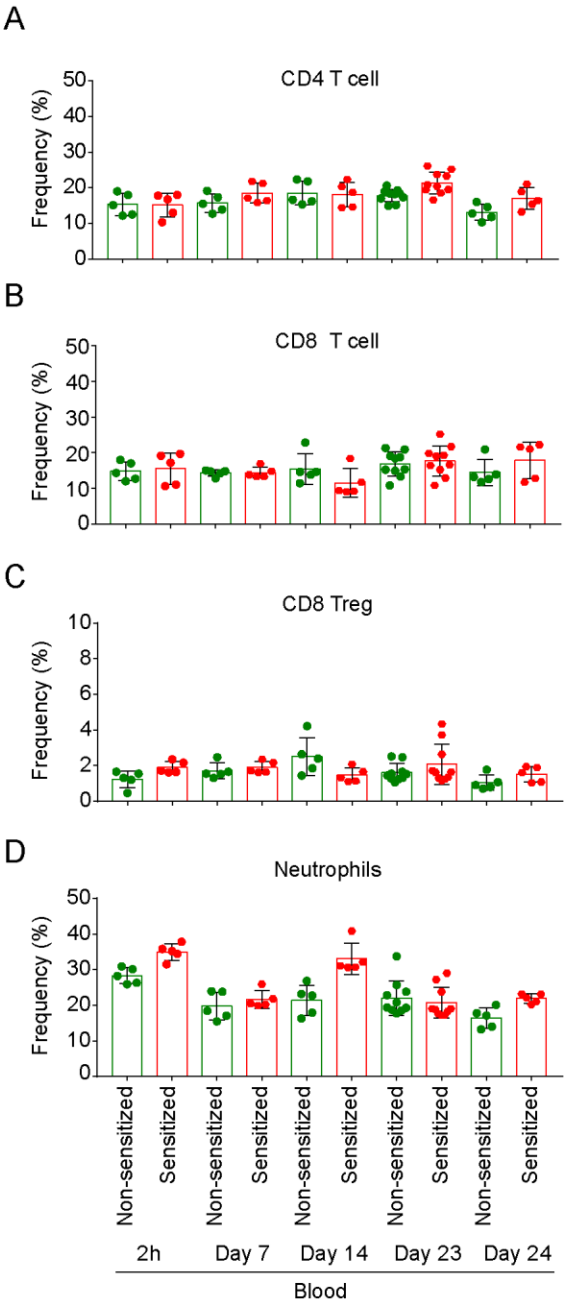

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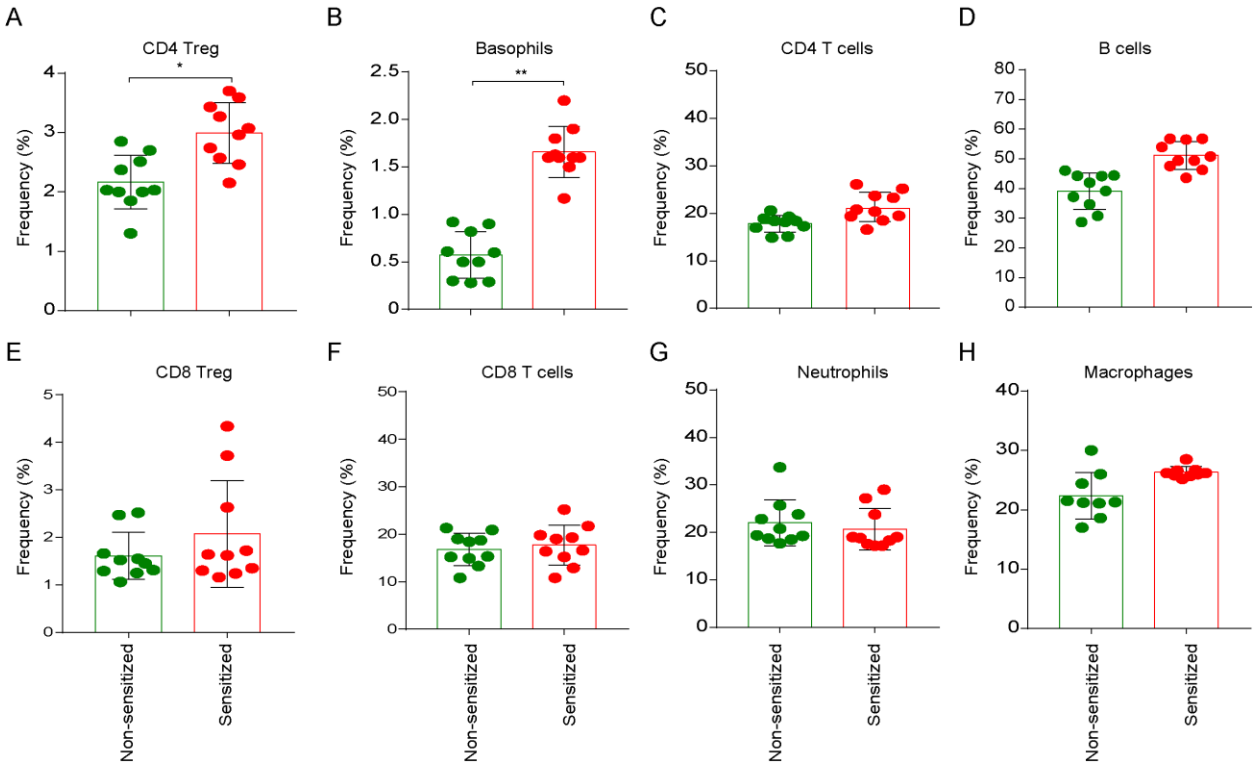
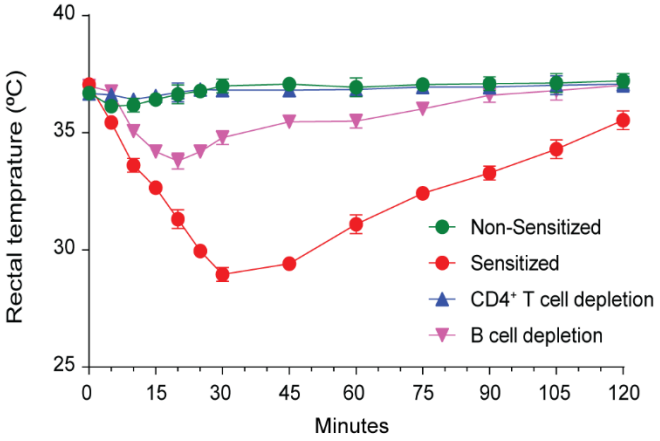
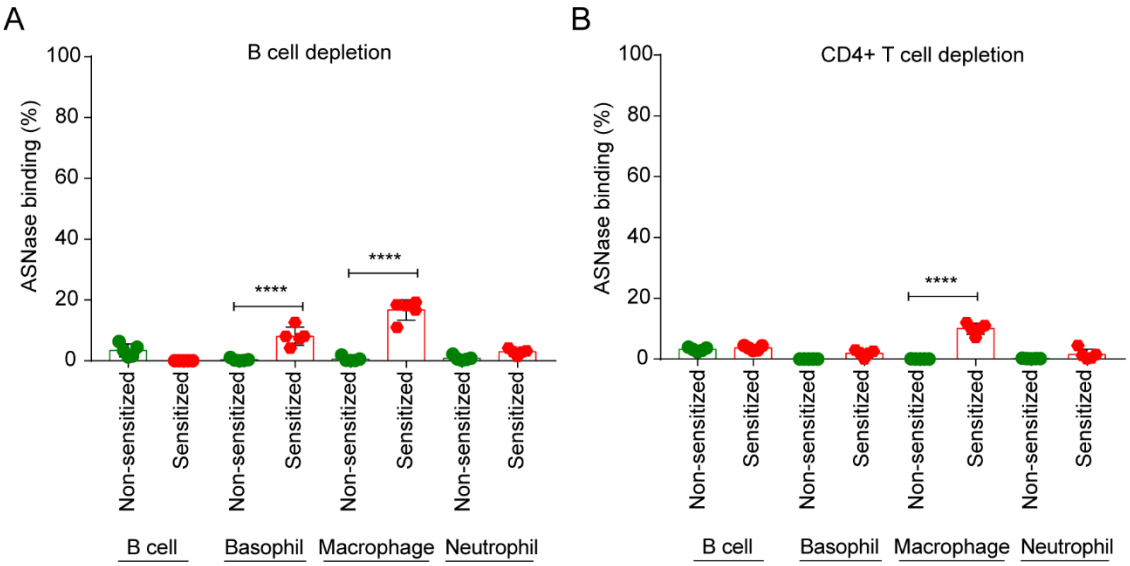


Supplemental Figure 7



Supplemental Figure 8



Supplemental Figure 9