

# 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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## ABSTRACT

Asparaginase is an important drug for the treatment of leukemias. However, anti-asparaginase antibodies often develop, which can decrease asparaginase drug levels and increase the risk of relapse. The aim of this study is to identify the immunoglobulin isotypes and receptors responsible for asparaginase hypersensitivities. Mice immunized with asparaginase developed anti-asparaginase IgG1 and IgE antibodies, and challenging the sensitized mice with asparaginase induced severe hypersensitivity reactions. Flow cytometry analysis indicated that macrophages/monocytes, neutrophils, and basophils bind asparaginase *ex vivo* through FcγRIII. In contrast, asparaginase binding to basophils was dependent on FcγRIII and IgE. Consistent with the asparaginase binding data, basophil activation by asparaginase occurred via both IgG/FcγRIII and IgE/FcεRI. Depleting >95% of B cells suppressed IgG but not IgE-dependent hypersensitivity, while depleting CD4<sup>+</sup> T cells provided complete protection. Combined treatment with either anti-IgE mAb plus a platelet-activating factor receptor antagonist or anti-FcγRIII mAb plus a H1 receptor antagonist suppressed asparaginase hypersensitivity. The observations indicate that asparaginase hypersensitivity is mediated by antigen-specific IgG and/or IgE through the immunoglobulin receptors FcγRIII and FcεRI, respectively. Provided that these results apply to humans, they emphasize the importance of monitoring both IgE- and IgG-mediated asparaginase hypersensitivities in patients receiving this agent.

## Introduction

L-Asparaginase (ASNase) is given repeatedly during treatment regimens for acute lymphoblastic leukemia (ALL). The non-human enzyme is derived from bacteria and inhibits leukemic cell proliferation by depleting asparagine.<sup>1</sup> The most common adverse reaction of ASNase in children results from the production of anti-ASNase antibodies (seen in up to 70% of patients) and the onset of clinical hypersensitivity reactions during treatment.<sup>2,7</sup> ASNase-mediated hypersensitivity can occur in 30-75% of patients receiving native *E. coli* ASNase<sup>3,8-10</sup> and typically manifest as urticaria, angioedema, bronchospasm, dyspnea, and anaphylaxis.<sup>11</sup> Typically, if a patient develops a hypersensitivity reaction to first-line PEG-ASNase, a substitution with *Erwinia* ASNase is recommended; a subsequent reaction to *Erwinia* ASNase may necessitate discontinuing ASNase therapy.<sup>12</sup> In addition, the development of anti-ASNase antibodies can increase the risk of relapse by neutralizing ASNase *in vivo*.<sup>13</sup>

ASNase-mediated hypersensitivity during ALL treatment is most common upon drug re-exposure,<sup>2</sup> this suggests that patients are sensitized to the agent earlier in therapy. However, the mechanism of ASNase hypersensitivity is not clear, as some patients develop reactions in the absence of detectable anti-ASNase IgG antibody. Moreover, many patients who have circulating anti-ASNase IgG never develop a

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hypersensitivity reaction.<sup>2,7</sup> Interestingly, few studies have assessed anti-ASNase IgE levels in patients or have suggested a role of anti-ASNase IgE in the ASNase hypersensitivity reaction,<sup>14-16</sup> likely due to a lack of methods available to detect anti-ASNase IgE in the presence of high anti-ASNase IgG levels.

Pharmacogenomic studies of the immune response to ASNase have identified risk variants in genes involved in antigen presentation<sup>17</sup> or T-cell activation,<sup>18</sup> supporting the importance to B/T-cell activation during ASNase sensitization and in the production of anti-ASNase antibodies. Anti-ASNase IgG can elicit a hypersensitivity reaction by forming an immune complex with ASNase, binding to the Fcγ receptor of immune cells (e.g., basophils, mast cells, neutrophils, and/or macrophages), and resulting in the release of platelet activating factor (PAF).<sup>19</sup> Alternatively, cells expressing FcεRI, such as mast cells and basophils, can bind anti-ASNase IgE during sensitization and mediate a hypersensitivity upon antigen exposure via the release of histamine.<sup>20</sup> Recently, a murine model of ASNase hypersensitivity has been described that recapitulates many of the features of clinical ASNase-mediated hypersensitivity.<sup>21</sup> Studies using this model indicate that pretreatment with the antihistamine, triprolidine, and the PAF receptor antagonist, CV-6209, can strongly mitigate the onset of ASNase hypersensitivity, suggesting that both histamine and PAF release play a role in the immune response to ASNase.

The current study uses the murine model of ASNase hypersensitivity to identify the immune cells required for ASNase sensitization and the immunoglobulin isotypes and receptors responsible for the onset of hypersensitivity. Our results indicate that anti-ASNase IgE plays an important role in ASNase-induced hypersensitivities and that the binding of ASNase to basophils may be predictive of hypersensitivity. In accordance with the importance of binding to basophils, we show that ASNase activates basophils through both FcγRIII- and IgE-dependent mechanisms. Our results suggest that both anti-ASNase IgG and IgE play important roles in the development of ASNase hypersensitivity.

## Methods

### ASNase sensitization

8-week-old, female, C57BL/6 mice were injected intraperitoneally (IP) with 10 μg of *E. coli* ASNase formulated with 1 mg of aluminum hydroxide adjuvant, on days 0 and 14, as previously described.<sup>21</sup> ASNase hypersensitivity reactions were induced in sensitized mice by challenging with a 100 μg IV dose of *E. coli* ASNase on Day 24 of treatment. All experiments with mice were reviewed and conducted under approved protocol by the University of Pittsburgh Institutional Animal Care and Use Committee.

### Detection of anti-ASNase IgE by flow cytometry

Anti-IgE-biotin (Biolegend, USA) at 1 μg/mL was bound to 3×10<sup>6</sup> streptavidin-coupled 6-8 μm diameter magnetic particles (Spherotech, USA). Plasma samples diluted to 1:100 in PBS were added to anti-IgE-coated beads for 30-60 minutes at room temperature, washed with PBST, and stained with labeled ASNase at 1 IU/mL. The stained samples were analyzed by flow cytometry for ASNase fluorescence.

### Basophilic activation test (BAT)

BAT was performed as previously described.<sup>22,23</sup> Briefly, 50 μL of blood was incubated for 15 min at 37°C and further stimulated with EM-95 at 300 ng/mL, 2.4G2 at 300 ng/mL, ASNase at 1 IU/mL, or medium (as a negative control). Samples were further incubated for 2 h at 37°C in 5% CO<sub>2</sub>, quenched by adding 20 mM EDTA, and incubated on ice for 10 minutes. Cells were blocked with 15% HS in PBS for 30 minutes on ice, washed, and stained with anti-IgE, anti-CD49b, anti-CD200R3, and anti-CD200R1 mAbs for 30-60 minutes at 4°C. The cells were then lysed, washed with 1% BSA in PBS, and analyzed by flow cytometry. The percent change in CD200R1 expression is equal to the mean experimental expression of CD200R1 minus that of the mean expression of the sample stimulated with medium, divided by the mean expression of the sample stimulated with medium. Similarly, the percent change in CD200R3 is the mean expression of the sample stimulated with medium minus the mean experimental expression of CD200R3, divided by the mean expression of the sample stimulated with medium.

### In vivo immune cell depletion

Anti-CD4 mAb or anti-CD19 mAb were injected IP in mice at 200 μg/mouse three days before each sensitization dose of ASNase. Cell depletions were confirmed by flow cytometry, as described above, where different mAb clones targeting CD19 or CD4 were used for cell depletion and staining. Mice were challenged with *E. coli* ASNase on Day 24, as described above.

### In vivo blocking of ASNase-induced hypersensitivity reactions with anti-IgE or anti-FcγRIIB/III mAb

To prevent IgE- or IgG-mediated hypersensitivities, a single 100 μg dose of anti-IgE (EM-95)<sup>20</sup> or 500 μg of anti-FcγRIIB/III mAb (2.4G2)<sup>24</sup> was administered IP 24 hours before the ASNase challenge. Pretreatment medication, as a single agent or in combination, was given before the ASNase challenge in a total volume of 150 μL per injection. The doses of each drug used are based on previous studies.<sup>21</sup> 66 μg of CV-6209 (PAF receptor antagonist) was given 5 minutes before challenges via IV injection, and 200 μg of antihistamine (triprolidine, an H1 receptor antagonist) was given IP 30 minutes before the ASNase challenge. Additional Methods are included in the *Online Supplementary Material*.

## Results

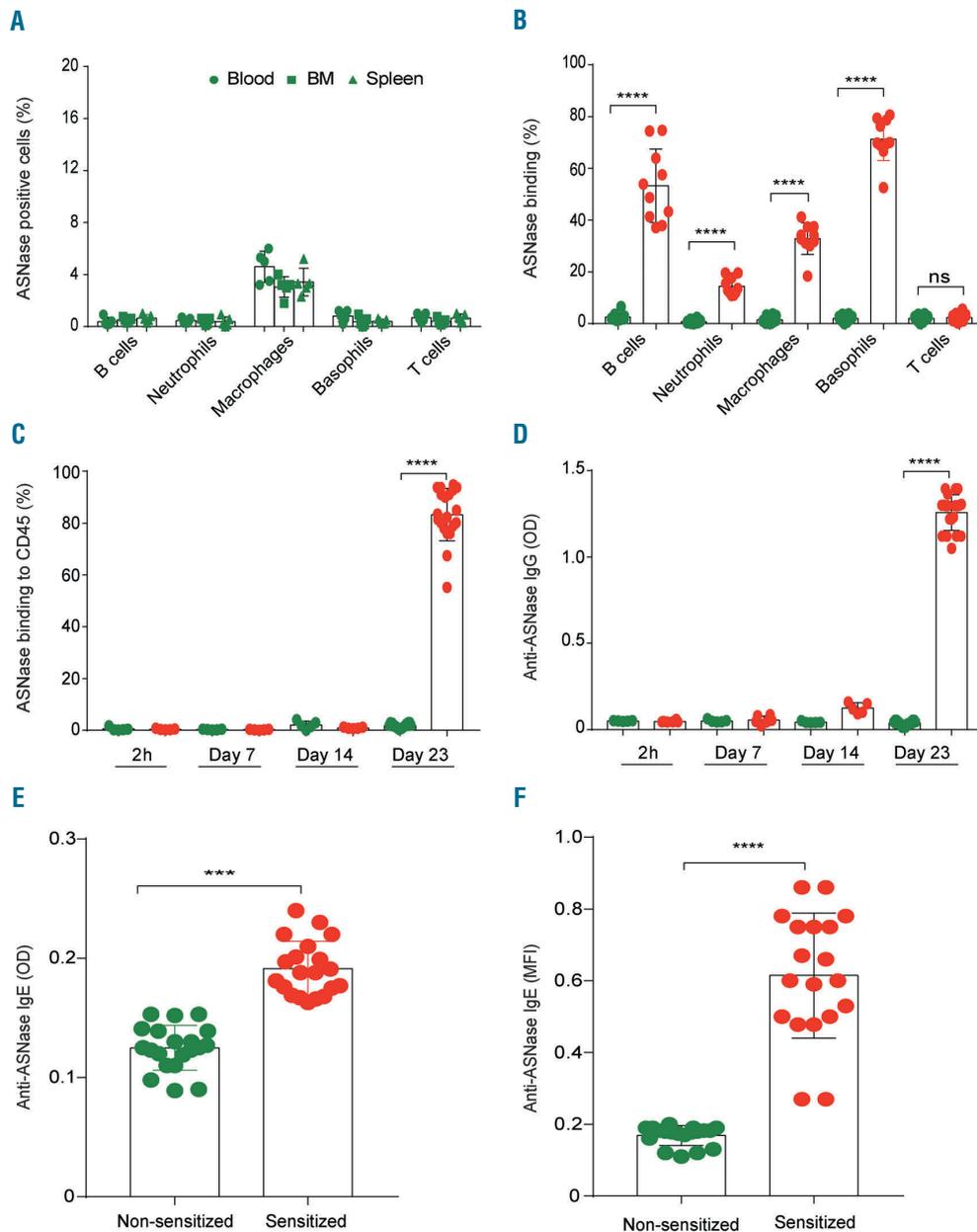
### Anti-ASNase IgE plays a role in ASNase-specific recognition

The onset of ASNase-mediated hypersensitivity requires a humoral immune response to the agent after its initial use during induction therapy and antigen-specific recognition upon drug re-exposure after sensitization. Therefore, as expected, staining peripheral blood cells from naïve mice with fluorochrome-labeled ASNase (Figure 1A) revealed no ASNase positive neutrophils, T cells, basophils, or B cells. Yet, consistent with the perceived mechanism of ASNase clearance involving macrophages of the reticuloendothelial system (RES),<sup>25</sup> a small percentage of macrophages/monocytes were ASNase positive (Figure 1A). Mice were given 100 μg of fluorochrome-labeled ASNase IV to verify its uptake by macrophages/monocytes. The labeled ASNase was prima-

rily detected in cells obtained from the liver (73.6%), blood (11.4%), and spleen (5.0%) 8 h after administration (*Online Supplementary Figure S1A*). Among CD45<sup>+</sup> leukocytes, ASNase accumulates primarily in macrophages/monocytes of the liver and blood (95.1%, and 93.7%, respectively; *Online Supplementary Figure S1A and B*), to a lesser extent in neutrophils (1.1% and 2.1%, respectively), but not in other immune cells. Supporting the role of macrophages in ASNase clearance, the uptake of ASNase was assessed using a murine macrophage cell line (i.e., RAW 264.7 cells). ASNase positive RAW cells were detected after 5 minutes of incubation with ASNase

and the percentage of ASNase<sup>+</sup> RAW cells plateaued after about 2 h of incubation with the drug (*Online Supplementary Figure S2A-B*). Therefore, it is likely that the binding of ASNase to naïve macrophages/monocytes is associated with drug clearance rather than antigen-specific recognition.

After sensitizing mice to ASNase, as described previously<sup>21</sup> (*Online Supplementary Figure S3A*) and staining blood samples with ASNase and immune cell surface markers, we found that the drug was bound *ex vivo* by B cells (53.3%), neutrophils (14.5%), macrophages/monocytes (32.9%), and basophils (69.5%), but not T cells (Figure 1B).



**Figure 1. ASNase is recognized *ex vivo* by B cells, neutrophils, macrophages/monocytes, and basophils after sensitization.** (A) Peripheral blood cells were collected from naïve mice, cultured with labeled ASNase for 30 minutes, and analyzed for ASNase positive immune cells by flow cytometry. (B) The *ex vivo* ASNase-specific recognition by B cells, neutrophils, macrophages/monocytes, basophils, and T cells of the blood were analyzed within CD45<sup>+</sup> populations by flow cytometry of sensitized (red data points) and non-sensitized (green data points) mice on Day 23 of the sensitization protocol. (C) ASNase binding to total leukocytes (CD45<sup>+</sup>) and (D) anti-ASNase IgG antibodies were measured throughout the sensitization protocol. Anti-ASNase IgE antibodies were measured by (E) ELISA and (F) flow cytometry using plasma samples collected on Day 23 of the sensitization protocol. A total of 5 to 20 mice were included in each analysis, as indicated, and P value significance is indicated as \* for  $P < 0.05$ , \*\* for  $P < 0.01$ , \*\*\* for  $P < 1 \times 10^{-3}$ , and \*\*\*\* for  $P < 1 \times 10^{-4}$ .

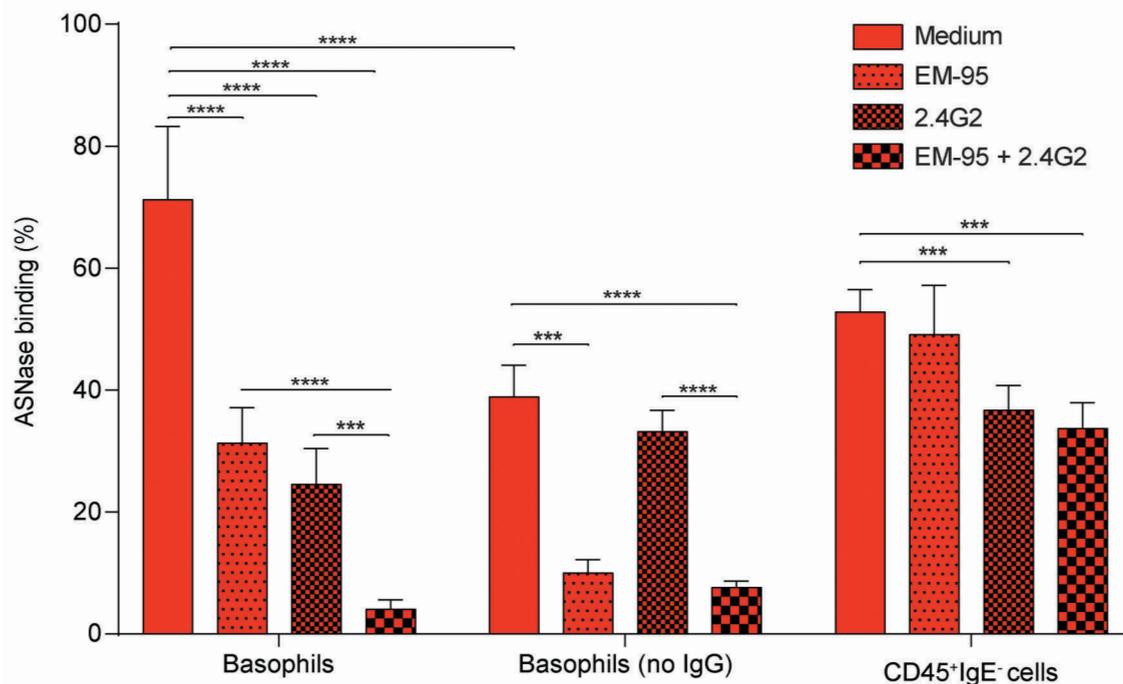
However, the binding of ASNase to immune cells might have depended on anti-ASNase IgG, primarily the anti-ASNase IgG1 subclass (*Online Supplementary Figure S4*), because CD45<sup>+</sup>ASNase<sup>+</sup> cells were solely detected on Day 23 of our protocol (Figure 1C,  $P < 1 \times 10^{-4}$ ), which is when plasma anti-ASNase IgG antibody levels became elevated relative to naïve mice (Figure 1D,  $P < 1 \times 10^{-4}$ ).

Basophils express both the high-affinity IgE receptor, FcεRI, and the low-affinity IgG receptor, FcγRIII, and, therefore, can bind antigen via cell-associated IgE or antigen-specific IgG (i.e., after immune complex formation). Suggesting that anti-ASNase IgE may play a role in the detected binding to basophils, plasma anti-ASNase IgE antibodies were elevated in the plasma samples of sensitized mice on Day 23 relative to controls by ELISA (Figure 1E). Due to the possible interference of anti-ASNase IgG during the detection of anti-ASNase IgE by ELISA,<sup>26</sup> the presence of anti-ASNase IgE in plasma samples was confirmed by capturing IgE using anti-IgE-coated polystyrene beads and staining with labeled ASNase (Figure 1F). Based on the antigen-specific antibody isotypes detected, it is possible that recognition or binding of free ASNase to immune cells after sensitization can occur through cell associated-IgE among cells expressing FcεRI (e.g., basophils, Figure 1B) or via the binding of ASNase-IgG immune complexes to cells expressing FcγRIII (e.g., neutrophils, macrophages/monocytes, basophils, Figure 1B). Supporting this proposed mechanism of recognition, the binding of ASNase to the basophils of sensitized mice is decreased by blocking antibodies targeting the FcγRIII receptor (2.4G2, Figure 2,  $P < 1 \times 10^{-4}$ ) or IgE (EM-95, Figure 2,  $P < 1 \times 10^{-4}$ ). The binding of ASNase to the basophils of

sensitized mice is further decreased when both antibodies are used in combination rather than singly (Figure 2,  $P < 1 \times 10^{-3}$ ). Thus, basophils can bind ASNase immune complexes and free ASNase, and both can occur simultaneously. In contrast, the binding of ASNase to IgE<sup>-</sup> leukocytes of sensitized mice is decreased after blocking with 2.4G2 (Figure 2,  $P < 1 \times 10^{-3}$ ) but binding is not affected by EM-95 (Figure 2,  $P > 0.05$ ). Consistent with requiring immune complex formation prior to ASNase binding to cells expressing the FcγRIII receptor, if soluble IgG is removed during erythrocyte lysing before staining cells, then the binding of ASNase to basophils is decreased relative to non-lysed samples (43% decrease, Figure 2,  $P < 1 \times 10^{-4}$ ). Blocking lysed cells with 2.4G2 has little to no effect on the binding of ASNase to basophils; in contrast, blocking with EM-95 strongly inhibited binding (Figure 2,  $P < 1 \times 10^{-4}$ ). The results suggest that ASNase binding to immune cells depends on the surface expression of FcγRIII and an IgE receptor, most likely, FcεRI.

### The frequencies of blood basophils, B cells, and CD4<sup>+</sup> Tregs increase during sensitization to ASNase

Upon challenge with ASNase, immune cell binding to ASNase in sensitized mice may lead to a hypersensitivity reaction, yet other cells that do not recognize or bind to ASNase likely play a role during sensitization to the drug. To identify other cells that may be involved in sensitization, we measured the frequency of B cells, CD4<sup>+</sup>/8<sup>+</sup> T cells, CD4<sup>+</sup>/8<sup>+</sup> Tregs, neutrophils, macrophages/monocytes, and basophils in blood within the CD45<sup>+</sup> leukocytes population at five different time points during sensitization (Figure 3A-D; *Online Supplementary Figure S5A-D*).



**Figure 2.** *Ex vivo* ASNase binding to basophils is dependent on FcγRIII and FcεRI. *Ex vivo* ASNase binding to basophils after immunoglobulin receptor blocking with 2.4G2 (anti-FcγRIII/III mAb) and/or EM-95 (anti-IgE mAb) suggests that the binding is dependent on both FcγRIII and FcεRI ( $n = 10$ ). Furthermore, lysing cells and removing soluble IgG antibodies before measuring ASNase binding reduces the frequency of ASNase<sup>+</sup> cells relative to non-lysed cells ( $P < 1 \times 10^{-4}$ ). The binding of ASNase to basophils is attenuated by EM-95 but not 2.4G2 after removing soluble IgG. In contrast, ASNase binding to CD45<sup>+</sup> IgE<sup>-</sup> cells decreases after blocking with 2.4G2 but not after blocking with EM-95 ( $n = 10$ ).  $P$  value significance is indicated as \* for  $P < 0.05$ , \*\* for  $P < 0.01$ , \*\*\* for  $P < 1 \times 10^{-3}$ , and \*\*\*\* for  $P < 1 \times 10^{-4}$ .

The frequency of macrophages/monocytes increased by approximately 5-10% after each ASNase dose (2 h and Day 14; Figure 3A), possibly reflecting the role of macrophages/monocytes on ASNase clearance; however, no other change in immune cell phenotype was detected among samples collected 2 h after the first sensitization dose or on Days 7 or 14 of the sensitization protocol (*Online Supplementary Figure S5A-D*). In contrast, frequencies of CD4<sup>+</sup> Tregs, B cells, and basophils in the blood increased by Day 23 relative to naïve mice (Figure 3B-D, respectively). The data are consistent with a Th2-mediated response to ASNase and suggest that CD4<sup>+</sup> Tregs may limit sensitization to ASNase.

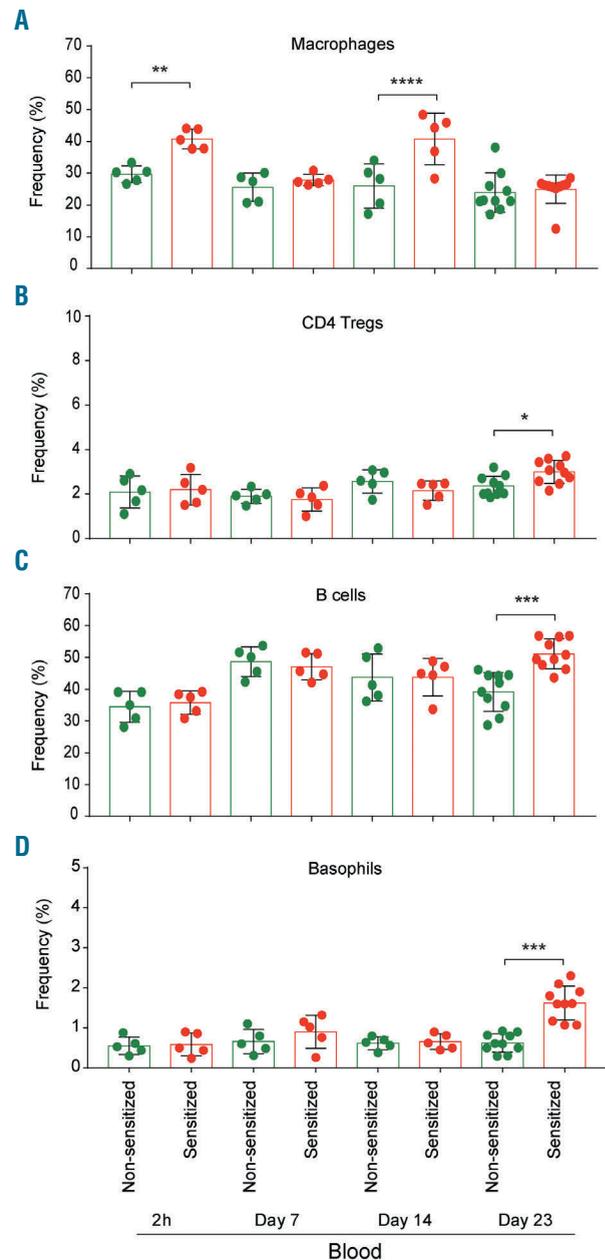
### Depletion of CD4<sup>+</sup> T cells protects against the development of the anti-ASNase antibodies

An immediate drop in rectal temperature was measured in ASNase sensitized mice upon ASNase challenge on Day 24, which was quantified by estimating the area under the temperature *versus* time curve (AUC, Figure 4A). Plasma concentrations of mMCP-1 were elevated ~10 fold 2 h after the challenge relative to controls (Figure 4B), suggesting that mast cell degranulation is playing a role in the reactions. Sensitized mice had much lower blood ASNase enzyme activity than controls (Figure 4C), indicating rapid, presumably antibody-mediated clearance of the drug. Similar to the pre-challenge data, only the frequency of CD4<sup>+</sup> Tregs and basophils of the blood increased relative to naïve controls (*Online Supplementary Figure S6A-H*). Furthermore, ASNase bound *ex vivo* to B cells, neutrophils, macrophages/monocytes and basophils (Figure 4D). However, there was a sharp drop in the binding of ASNase to B cells (~25%) and a modest increase in ASNase<sup>+</sup> basophils (~10%) after the challenge (Figure 4D) relative to one day before the ASNase challenge (Day 23; Figure 1B).

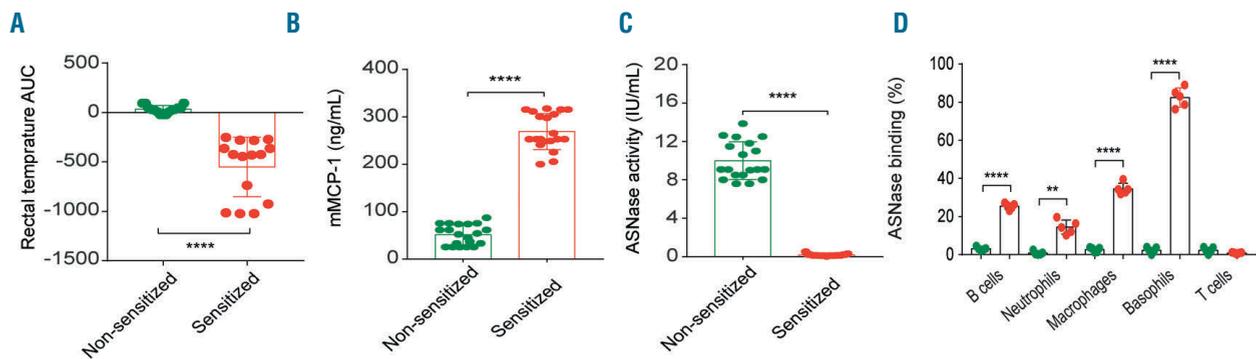
To determine if B-cell or CD4<sup>+</sup> T-cell depletion can prevent ASNase sensitization and the onset of hypersensitivity reactions, we depleted B cells or CD4<sup>+</sup> T cells using anti-CD19 or anti-CD4 mAb, respectively, three days before each immunization dose (*Online Supplementary Figure S3B*). Less than 5% of B or CD4<sup>+</sup> T cells remained after depletion in the blood (Figure 5A-B). Upon ASNase challenge, mice with depleted B or CD4<sup>+</sup> T cells were protected from severe hypersensitivity reactions compared to sensitized mice with no immune cell depletion (Figure 5C; *Online Supplementary Figure S7*;  $P < 0.01$ ). Surprisingly, B-cell depletion did not completely protect mice from hypersensitivity (Figure 5C,  $P < 0.05$ ), whereas CD4<sup>+</sup> T-cell depletion provided full protection (Figure 5C,  $P > 0.05$ ). The stronger effect of the CD4<sup>+</sup> T cell-depleting mAb than the B-cell depleting mAb may reflect the ability of anti-CD4 mAb to suppress the function of CD4<sup>+</sup> T cells that it fails to deplete,<sup>27</sup> while anti-CD19 mAb has a less global suppressive effect on B-cell function.<sup>28</sup>

Both groups failed to develop detectable levels of anti-ASNase IgG (Figure 5D,  $P > 0.05$ ), and consistent with the lower anti-ASNase antibodies measured, both had similar ASNase enzyme activity levels relative to naïve mice (Figure 5E,  $P > 0.05$ ). CD4<sup>+</sup> T-cell depletion led to similar mMCP-1 levels as naïve mice, whereas mice depleted of B cells had elevated mMCP-1 relative to both naïve and T cell-depleted mice (Figure 5E,  $P < 1 \times 10^{-4}$ ), suggesting that hypersensitivity in B cell-depleted mice was mediated by anti-ASNase IgE and mast cell degranulation. Supporting

this interpretation, the plasma anti-ASNase IgE levels after B-cell depletion were similar to sensitized mice and elevated compared to non-sensitized, naïve controls and CD4<sup>+</sup> T cell-depleted mice by ELISA (Figure 5G,  $P < 1 \times 10^{-3}$ ) and by flow cytometry (Figure 5H,  $P < 1 \times 10^{-4}$ ). On Day 24 after the challenge, *ex vivo* binding of ASNase to basophils was detected in mice with depleted B cells (*Online Supplementary Figure S8A*), but not in mice depleted of CD4<sup>+</sup> T cells (*Online Supplementary Figure S8B*). The *ex vivo* binding of ASNase to macrophages/monocytes was significantly higher in sensitized mice after CD4<sup>+</sup> T-cell or B-



**Figure 3. Frequencies of CD4<sup>+</sup> Tregs, B cells, and basophils increase in blood after ASNase sensitization.** The frequency of (A) macrophages/monocytes, (B) CD4<sup>+</sup> Treg cells, (C) B cells, and (D) basophils was measured in the blood of mice at various time points during sensitization. A total of 5 or 10 mice were included in each analysis, as indicated, and *P* value significance is indicated as \* for  $P < 0.05$ , \*\* for  $P < 0.01$ , \*\*\* for  $P < 1 \times 10^{-3}$ , and \*\*\*\* for  $P < 1 \times 10^{-4}$ .



**Figure 4. AS Nase-sensitized mice develop hypersensitivity reactions when challenged.** The rectal temperature of sensitized and non-sensitized mice was monitored for 2 hours after the AS Nase challenge on Day 24. (A) Sensitized mice experienced a drop in rectal temperature that was quantified by estimating the AUC of the rectal temperature versus time curve. (B) Sensitized mice had elevated levels of mMCP-1 and (C) low AS Nase drug levels compared to non-sensitized mice. (D) AS Nase binds to B cells, neutrophils, macrophages/monocytes, basophils, and T cells after challenging with AS Nase on Day 24. A total of 5 or 20 mice were included in each analysis, as indicated, and *P* value significance is indicated as \* for  $P < 0.05$ , \*\* for  $P < 0.01$ , \*\*\* for  $P < 1 \times 10^{-3}$ , and \*\*\*\* for  $P < 1 \times 10^{-4}$ .

cell depletion relative to naïve mice (*Online Supplementary Figure S8A-B*,  $P < 1 \times 10^{-4}$ ), but at about half of the frequency relative to sensitized mice with no cell depletion (Figure 4D). No binding of AS Nase to B cells or neutrophils was detected after CD4<sup>+</sup> T-cell or B-cell depletion (*Online Supplementary Figure S8A-B*,  $P > 0.05$ ). The data suggests that binding of AS Nase to B cells, neutrophils, or macrophages/monocytes may not correlate with the onset of AS Nase hypersensitivity, whereas the binding of AS Nase to basophils may be a useful marker of AS Nase hypersensitivity.

#### A basophil activation test (BAT) can detect IgG and/or IgE AS Nase hypersensitivity

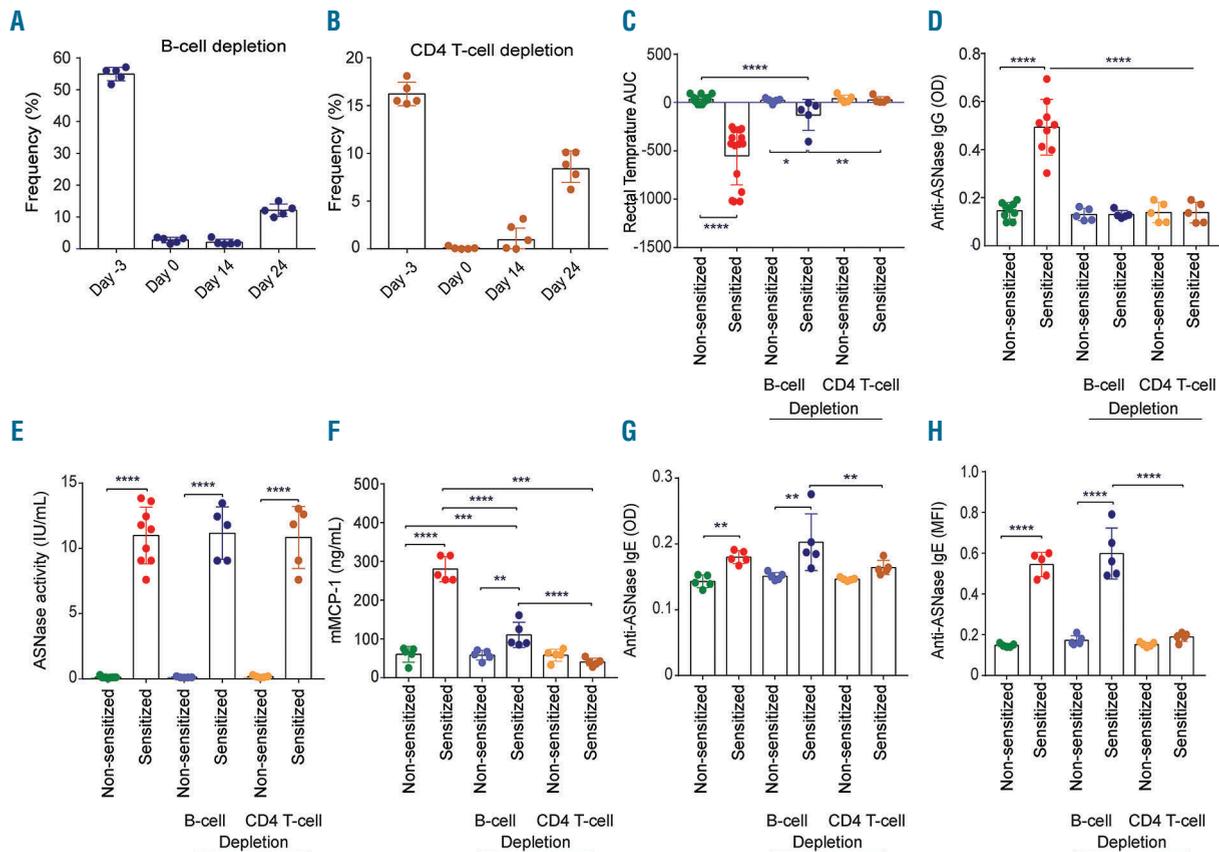
A basophil activation test was used to determine if AS Nase-induced activation can be mediated and detected via both AS Nase immune complex binding to FcγRIII and free AS Nase binding to cell-associated IgE. Blood samples from sensitized and non-sensitized mice were collected on Day 23 of the sensitization protocol (*Online Supplementary Figure S3A*) and incubated with AS Nase, RPMI-medium (negative control), EM-95 (positive control for IgE-mediated activation, Figure 6A) or 2.4G2 (positive control for IgG-mediated activation, Figure 6B). Samples from naïve mice showed no AS Nase-mediated basophil activation (Figure 6C-D); however, AS Nase-sensitized mice showed an upregulation of CD200R1 and a downregulation of CD200R3 relative to naïve mice (Figure 6C-D,  $P < 0.01$ ), suggesting that the activation of basophils and possibly the onset of AS Nase hypersensitivities may be mediated by both pathways of anaphylaxis. We anticipated that anti-AS Nase IgG may interfere with the binding of AS Nase to cell-associated IgE.<sup>26</sup> Consistent with that hypothesis, after washing the samples with mouse plasma and removing anti-AS Nase IgG antibodies, CD200R3 was no longer downregulated after incubation with AS Nase (Figure 6D,  $P > 0.05$ ), whereas a significant upregulation of CD200R1 after removing anti-AS Nase IgG was measured relative to sensitized basophils with no antibody removal and to naïve control samples (Figure 6C,  $P < 0.01$ ).

To determine if the BAT can distinguish between sensitized and non-sensitized samples from mice with AS Nase exposure, we collected blood samples after a single sensi-

zation dose of AS Nase on Day 9 and challenged the mice with IV AS Nase on Day 10 (*Online Supplementary Figure S3C*). We detected no basophil activation by BAT (*Online Supplementary Figure S9A-B*) or *ex vivo* AS Nase binding to basophils (*Online Supplementary Figure S9C*). Consistent with these results, mice did not develop hypersensitivity reactions upon AS Nase challenge (*Online Supplementary Figure S9D*). Taken together, our data suggest that the binding of AS Nase to basophils and AS Nase-mediated basophil activation correlate with the onset of AS Nase hypersensitivity.

#### *In vivo* blocking with EM-95 or 2.4G2 suggests multiple mechanisms of AS Nase-induced hypersensitivity

*In vivo* blocking experiments with EM-95 or 2.4G2 were performed to determine whether AS Nase hypersensitivity occurs via both the FcεRI/IgE- and FcγRIII/IgG-mediated pathways as suggested by our BAT data. Mice were pretreated with either mAb on Day 23 and challenged the next day with AS Nase. Mice receiving EM-95 or 2.4G2 were partially protected from hypersensitivity compared to sensitized mice (Figure 7,  $P < 1 \times 10^{-3}$ ). The results suggest that AS Nase hypersensitivity occurs via IgG- and IgE-mediated mechanisms and that hypersensitivity after pretreatment with EM-95 was due to PAF release and that after 2.4G2 was due to histamine release.<sup>20,24</sup> We, therefore, tested this hypothesis by pretreating mice with CV-6209 or triprolidine after EM-95 or 2.4G2 administration, respectively, or with each receptor antagonist alone or in combination with no blocking antibody. Consistent with our hypothesis, mice receiving EM-95 or 2.4G2 as well as the appropriate pretreatment medication were completely protected from AS Nase hypersensitivity (Figure 7,  $P > 0.05$ ). Pretreating with CV-6209 or triprolidine alone yielded similar results as either blocking antibody (Figure 7,  $P > 0.05$ ), and AS Nase hypersensitivity was completely suppressed in sensitized mice pretreated with CV-6209 and triprolidine (Figure 7,  $P > 0.05$ ). Our results indicate that both pathways of hypersensitivity simultaneously play a role in AS Nase-mediated reactions and that receptor antagonists of both histamine and PAF are required to completely block the severity of the hypersensitivity reaction in our mouse model.



**Figure 5. The *in vivo* depletion of CD4<sup>+</sup> T cells but not B cells prevents ASNase-mediated hypersensitivity reactions.** (A) B cells or (B) CD4<sup>+</sup> T cells were depleted *in vivo* using anti-CD19 or anti-CD4 mAb three days prior to each ASNase sensitization dose. Less than 5% of B or CD4<sup>+</sup> T cells remained after depletion in the blood. (C) The rectal temperature AUC after B or CD4<sup>+</sup> T-cell depletion was determined after challenging with ASNase. (D) Anti-ASNase IgG levels, (E) ASNase activity, (F) mMCP-1 concentrations, and anti-ASNase IgE levels, as assessed by (G) ELISA and (H) flow cytometry, were measured from the plasma samples of mice collected after the challenge. A total of 5 or 10 mice were included in each analysis, as indicated, and *P* value significance is indicated as \* for *P*<0.05, \*\* for *P*<0.01, \*\*\* for *P*<1×10<sup>-3</sup>, and \*\*\*\* for *P*<1×10<sup>-4</sup>.

## Discussion

ASNase is an essential component of ALL treatment, but the ASNase-neutralizing antibody response to this chemotherapeutic agent can increase the risk of leukemia relapse.<sup>13</sup> Little is known about the mechanism of the immune response to ASNase, and currently no clinical test is available to accurately predict which patients will develop hypersensitivity to subsequent doses of ASNase. The results of our study suggest that a possible explanation for the lack of predictive biomarkers is that multiple mechanisms of ASNase hypersensitivity can occur separately or simultaneously. Our data indicate that hypersensitivity to ASNase can be mediated by both anti-ASNase IgG and IgE through the immunoglobulin receptors FcγRIII and FcεRI, respectively, which is consistent with the heterogeneity observed in the clinical immune response to the chemotherapeutic agent.<sup>2</sup> We provide the following evidence supporting our proposed mechanisms of hypersensitivity: (1) ASNase binds *ex vivo* to sensitized cells expressing FcγRIII and/or FcεRI (Figure 1B). Among IgE-leukocytes, ASNase binding is inhibited by antibodies targeting FcγRIII but not IgE (Figure 2), whereas, in contrast, the *ex vivo* binding of ASNase to basophils, which express both FcγRIII and FcεRI, is inhibited by antibodies targeting IgE and FcγRIII (Figure 2). (2) B cell-depleted mice devel-

oped IgE-mediated hypersensitivity reactions; that is, B cell-depleted mice have no detectable anti-ASNase IgG (Figure 5D), but develop elevated levels of anti-ASNase IgE (Figure 5G and H), experience hypersensitivity reactions when challenged with ASNase (Figure 5C) and have basophils that bind ASNase (*Online Supplementary Figure S8*). This is consistent with previous evidence that partial Rag deficiency (Omenn syndrome), which leads to B and T-cell oligoclonality, is associated with relative enrichment of IgE responses.<sup>29</sup> (3) Markers of ASNase basophil activation also support multiple mechanisms of ASNase hypersensitivity in mice (Figure 6C-D). (4) Pretreating sensitized mice with anti-IgE (EM-95) or anti-FcγRIII/III (2.4G2) demonstrates that each pathway is playing a substantial role in hypersensitivity reactions (Figure 7). These results are consistent with our current and previous study demonstrating that receptor antagonists of histamine and PAF are required to completely mitigate the severity of the ASNase immune response (Figure 7),<sup>21</sup> as well as a previous study of hypersensitivity in mice treated with insulin-derived peptides.<sup>24</sup>

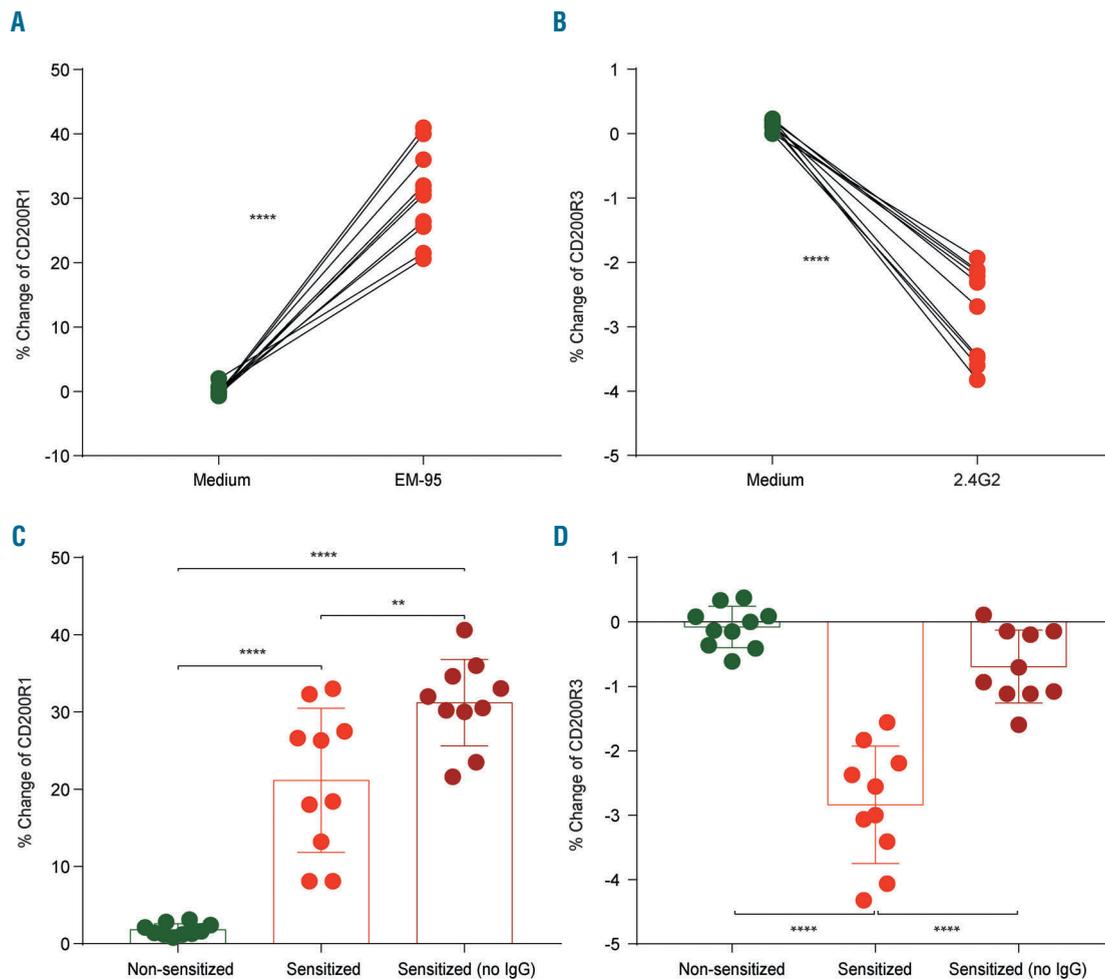
Few clinical studies of the immune response to ASNase have investigated the role of anti-ASNase IgE on the development of hypersensitivity reactions.<sup>14-16</sup> Based on our results demonstrating detectable plasma levels of anti-ASNase IgE (Figure 1E and F), we believe that antigen-spe-

cific recognition and hypersensitivity reactions may be mediated in part by cell-associated IgE. In support of a role for anti-ASNase IgE in the binding or recognition of free ASNase and not ASNase immune complexes, we found that blocking IgE with EM-95 decreases the binding of ASNase to sensitized basophils (Figure 2). Furthermore, removing soluble IgG before assessing *ex vivo* binding to ASNase demonstrates that free ASNase binding to basophils depends on cell-associated IgE and not the FcγRIII receptor (Figure 2). These data are in agreement with our BAT experiments, which demonstrate that basophils activation is IgE- but not FcγRIII-dependent after removing soluble anti-ASNase antibody (Figure 6C-D).

Anti-ASNase IgG antibodies are typically measured during ASNase therapy and higher levels associate with the onset of ASNase hypersensitivity in pediatric patients.<sup>2</sup> The *ex vivo* binding of IgE-leukocytes to ASNase (Figure 2) and the downregulation of CD200R3 basophil expression (Figure 6) were dependent on soluble IgG, indicating that the formation of ASNase immune complexes is required to induce IgG-mediated hypersensitivity. Similarly, and

consistent with a murine IgG1 response (*Online Supplementary Figure S4*),<sup>30</sup> IgG-mediated ASNase hypersensitivity was FcγRIII-dependent (Figure 7). Interestingly, the binding of ASNase to B cells and basophils, but not macrophages/monocytes or neutrophils, increased after challenging sensitized mice with ASNase (Figure 1B and 4D). Although this change in ASNase binding is likely due to the presence of ASNase immune complexes that formed during the challenge, it is not clear why the binding to macrophages/monocytes or neutrophils was unaffected, or why the binding to basophils was increased. A possible explanation for the increased *ex vivo* binding of ASNase to basophils may be that adding additional ASNase after the challenge allowed for free ASNase to bind to cell-associated IgE, whereas before the challenge anti-ASNase IgG may have neutralized the drug before it could bind to basophil-associated anti-ASNase IgE.

Cell depletion studies indicate that depletion of CD4<sup>+</sup> T cells or B cells can block the development of anti-ASNase IgG (Figure 5D) and drug neutralization (Figure 5E). However, CD4<sup>+</sup> T-cell but not B-cell depletion (both



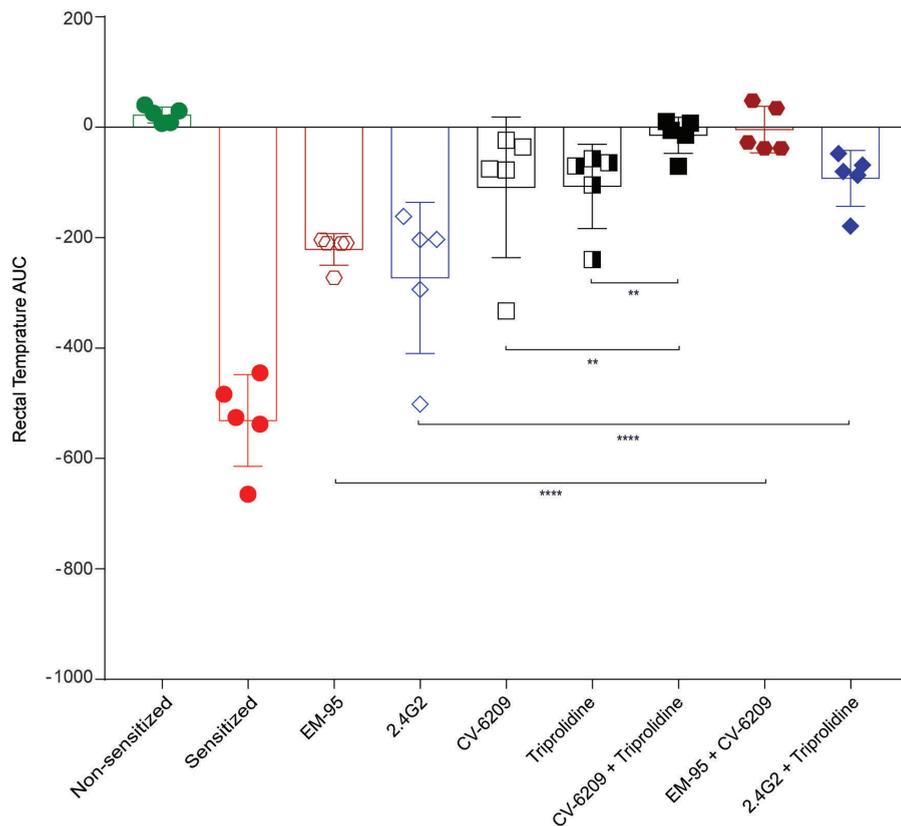
**Figure 6.** *Ex vivo* ASNase activates basophils in an IgG- and IgE-dependent manner. (A) IgE-mediated basophil activation by EM-95 (anti-IgE mAb) upregulates CD200R1 basophil expression relative to media control. (B) IgG-mediated basophil activation by 2.4G2 (anti-FcγRIIB/III mAb) downregulates CD200R3 basophil expression relative to media. (C) ASNase exposure among sensitized peripheral blood cells upregulates the CD200R1 expression of basophils in the presence and absence of anti-ASNase IgG (no IgG). (D) Downregulation of basophil CD200R3 expression in response to ASNase in ASNase-sensitized mice requires anti-ASNase IgG. A total of 10 mice were included in each analysis, as indicated, and *P* value significance is indicated as \* for *P*<0.05, \*\* for *P*<0.01, \*\*\* for *P*<1×10<sup>-3</sup>, and \*\*\*\* for *P*<1×10<sup>-4</sup>.

depleted to below 5%) is sufficient to completely protect mice from the development of anti-ASNase IgE antibodies and the onset of ASNase hypersensitivity (Figure 5C). The development of ASNase hypersensitivity after B-cell depletion is consistent with previous clinical data indicating that patients are depleted of B cells but not T cells during leukemia treatment, but yet develop hypersensitivity reactions to ASNase.<sup>31</sup> The presence of anti-ASNase IgE may explain why some patients develop ASNase hypersensitivity in the absence of detectable anti-ASNase IgG,<sup>2</sup> suggesting that ASNase hypersensitivity reactions probably also occur via multiple pathways in humans. Regarding the prevention of ASNase-mediated immune responses, our results suggest that although treatment with rituximab during ALL induction therapy may protect against the inactivation and accelerated clearance of ASNase by antigen-specific IgG antibodies, rituximab-pretreated patients may not necessarily be protected against ASNase hypersensitivity. It is possible that pretreating patients before ASNase administration with rituximab and antihistamine or an agent that can attenuate T-cell activation may mask or mitigate hypersensitivity reactions while allowing ASNase therapeutic drug levels to be achieved.

It is not routine practice to evaluate anti-ASNase IgE antibody levels during ASNase therapy,<sup>2,32,33</sup> and while some studies have suggested a role of anti-ASNase IgE during hypersensitivity reactions to ASNase,<sup>14-16</sup> the presence or absence of plasma or serum antigen-specific IgE may not necessarily be predictive of FcεRI receptor-bound antigen-specific IgE.<sup>34</sup> Likewise, performing skin prick tests,<sup>35</sup> basophil activation tests that solely incorporate markers of IgE-mediated hypersensitivity,<sup>36</sup> or anti-

ASNase IgG antibody measurements<sup>2</sup> cannot accurately explain or predict clinical reactions to ASNase. Therefore, investigating receptors or immune cells that are directly involved in hypersensitivity reactions, rather than relying on biomarkers of sensitization for prediction, may be more useful. We hypothesize that a cell-based approach will distinguish when antigen and antibody levels are sufficient to form immune complexes capable of binding the Fcγ receptor, and also be able to detect antigen binding to cell-associated IgE. Consistent with our hypothesis, the ASNase basophil activation test can detect activation via both the classical and alternative pathway of anaphylaxis (Figure 6C-D). Although the changes in CD200R3 measured were modest (but statistically significant), they are similar to those we observed using a positive control (i.e., 2.4G2) and to those that have been previously described.<sup>22,23</sup>

ASNase *ex vivo* binding was simultaneously detected in an FcγRIII- and IgE-dependent mechanism of hypersensitivity (Figure 2), and due to the simultaneous upregulation of CD200R1 and downregulation of CD200R3 by the BAT, we hypothesized that ASNase hypersensitivity was both FcγRIII- and IgE-dependent. Supporting our hypothesis, blocking IgE-mediated hypersensitivity using EM-95 or antihistamine only partially mitigated ASNase hypersensitivity (Figure 7). Similar results were obtained when blocking IgG-mediated hypersensitivity reactions using 2.4G2 or PAF receptor antagonist (Figure 7). However, ASNase hypersensitivity reactions were completely inhibited when both pathways of anaphylaxis were simultaneously blocked using EM-95 and PAF receptor antagonist, 2.4G2 and antihistamine, or PAF receptor antagonist and antihistamine. It is not yet known whether both path-



**Figure 7. FcεRI and FcγRIII play a role in ASNase hypersensitivity.** Sensitized mice were pretreated with EM-95 (anti-IgE mAb), 2.4G2 (anti-FcγRIIB/III mAb), CV-6209 (PAF receptor antagonist), triprolidine (antihistamine), EM-95 and CV-6209, 2.4G2 and triprolidine, or triprolidine and CV-6209 and challenged with ASNase to induce hypersensitivities. The AUC of the temperature vs. time curve was estimated. All mice receiving any pretreatment medication had a significant reduction in the severity of ASNase hypersensitivities relative to sensitized, non-pretreated mice ( $P < 1 \times 10^{-4}$ ). A total of 5 mice were included in each analysis, as indicated, and  $P$  value significance is indicated as \* for  $P < 0.05$ , \*\* for  $P < 0.01$ , \*\*\* for  $P < 1 \times 10^{-3}$ , and \*\*\*\* for  $P < 1 \times 10^{-4}$ .

ways of ASNase-induced hypersensitivity are clinically relevant in humans. Furthermore, while our results suggest that ASNase hypersensitivities can be masked by pretreatment with antihistamine and a PAF receptor antagonist, any pretreatment medication to mitigate ASNase hypersensitivity in patients must ensure that adequate drug levels are achieved to avoid the possibility of masking the hypersensitivity without achieving therapeutic ASNase drug levels.

The onset of ASNase hypersensitivities in our model requires two ASNase doses to sensitize mice before hypersensitivity reactions can be induced (*Online Supplementary Figure S9D*), likely due to the non-detectable levels of anti-ASNase IgG or IgE antibodies measured through Day 23 of our protocol (Figure 1D). Nevertheless, ASNase hypersensitivities were induced 10 days after the last sensitization dose (Day 24, Figure 4A), which correlates with the detection of high anti-ASNase antibody levels (Figure 1D).<sup>21</sup> A similar correlation exists between anti-ASNase IgG levels and the onset of clinical ASNase hypersensitivities.<sup>2</sup> Murine hypersensitivity reactions can be monitored via a decrease in core body temperature due to the increased permeability of vascular endothelium that is induced by histamine and other mast cell-produced vasoactive mediators.<sup>20,26,37-39</sup> Increased endothelial permeability results in vascular fluid leak and hypovolemia, which causes shock that is most easily detected as hypothermia.<sup>40,41</sup> Other markers of anaphylaxis include decreased physical activity, increased plasma levels of degranulation products (e.g., mMCP-1), and hemoconcentration (increased hematocrit levels) due to vascular leakage.<sup>20,42</sup> Our study detected hypersensitivities via the development of hypothermia and the release of mMCP-1. Previous studies on ASNase hypersensitivity have demonstrated a correlation between the dose of ASNase, the severity of ASNase hypersensitivity, and the levels of mMCP-1 released, supporting that our methods accurately

measure the onset, severity, and extent of hypersensitivity.<sup>21</sup>

Our data support a hypothesis that the mechanism of ASNase-mediated hypersensitivity involves antigen-specific IgG and/or IgE and the immunoglobulin receptors FcγRIII and/or FcεRI. Our results also indicate that both mechanisms can simultaneously or independently contribute to the onset and extent of ASNase hypersensitivity. We show that cells expressing FcγRIII and FcεRI can bind ASNase *ex vivo* and, therefore, it is likely that multiple cells play a role during the onset of hypersensitivity reactions, including mast cells, which are not present in systemic circulation. Our study has several possible clinical implications regarding predicting, overcoming, and preventing ASNase hypersensitivities. The *ex vivo* binding of ASNase to basophils correlates with the onset of ASNase hypersensitivity reactions, suggesting that ASNase binding to basophils may be a useful biomarker of ASNase hypersensitivity regardless of whether anaphylaxis is mediated by the classical and/or alternative pathway. Similarly, the ASNase BAT can detect IgE/FcεRI- or IgG/FcγRIII-dependent basophil activation in our study; however, human markers of IgG/FcγR-mediated basophil activation are not available. In addition, our results suggest that both PAF and histamine are important mediators of ASNase hypersensitivity and that receptor antagonists of these molecules may be able to block the clinical manifestation of ASNase hypersensitivity. Future research will attempt to demonstrate the specificity and sensitivity of both basophil binding and basophil activation by ASNase for predicting ASNase hypersensitivity and verify the possibility of the alternative pathway of ASNase hypersensitivity in humans.

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