The role of granulocyte macrophage -colony stimulating factor in the regulation of anaphylactic reactions

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Directed by Professor Ki Taek Nam

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This certifies that the Master's Thesis
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And all of my friends, thanks for cheering me up in my hard time.

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ABSTRACT

The role of granulocyte macrophage-colony stimulating factor
in the regulation of anaphylactic reactions

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Granulocyte-macrophage colony stimulating factor (GM-CSF) is one of the hematopoietic cytokines that affect differentiation and survival of myeloid lineage cells. Recently, GM-CSF has drawn attention as an inflammatory cytokine because of its prominent role in both autoimmune diseases and allergic diseases. However, the relationship between GM-CSF and anaphylaxis has not been clearly elucidated. To investigate this, I established two ovalbumin (OVA)-induced anaphylaxis models in C57BL/6 mice. One employed alum as an adjuvant, and the other utilized GM-CSF. In the model sensitized with alum, mild anaphylactic reactions were observed after both local or subcutaneous injection of OVA and systemic or intravenous injection of OVA. Meanwhile, in the model sensitized with GM-CSF, anaphylactic reactions were only observed upon systemic OVA challenge but not local injection. These systemic anaphylactic reactions were further aggravated when the number of

sensitization with GM-CSF increased. Along with the intensified anaphylaxis, the levels of total IgE, OVA-specific IgE and OVA –specific IgG were elevated. Then I examined the change of leukocyte populations over time in the spleen and peritoneal cavity following treatment with GM-CSF. Significant basophilia was observed in the spleen, whereas the number of mast cells was not altered in the spleen and peritoneal cavity. Besides, in the peritoneal cavity, the numbers eosinophils and small peritoneal macrophages increased while the number of large peritoneal macrophages decreased. Therefore, for the first time, the distinct anaphylactic features in humoral and cellular aspects of immune responses are revealed by treatment with GM-CSF. All in all, the study suggests that GM-CSF-induced strong basophilia in the spleen might play an important role in the systemic anaphylaxis, which has not been recognized previously.

Key words : GM-CSF, Anaphylaxis, basophil, mast cell, macrophage

Role of Granulocyte Macrophage-Colony Stimulating Factor in The Regulation of Anaphylactic Reactions

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I. INTRODUCTION

Dendritic cells (DCs) are professional antigen presenting cells that initiate and orchestrate immune responses. They capture, process and present antigen to naïve T cells to elicit adaptive immunity. They locate not only in skin and gut where frequently contacts with external environment, but the whole body. They are very heterogeneous population, which functions and expressing markers differ by locating organs and subsets[1,2]. The heterogeneity of DCs originates from the differentiation of hematopoietic stem cells (HSCs) into various subsets by hematopoietic cytokines like FMS-like tyrosine kinase 3 ligand (FLT3L), macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF). In vitro culture of mouse bone marrow cells with FLT3L produces 3 different DC subsets: classical DC1 (cDC1), cDC2, and plasmacytoid DC (pDC). When cultured with GM-CSF, HSCs develop into monocyte-derived dendritic cells (moDCs), which is inflammatory DC[1].

Those DCs have unique functions and surface markers that can distinguish them. cDCs are outstanding sentinels that sense, capture and process cell-associated and environmental-associated antigens. They migrate to secondary lymphoid organs followed by presenting processed antigens to T cells. There are 2 types of cDCs: $XCR1^+$ $CD8^+$ cDC1 and $CD11b^+$ $CD4^+$ cDC2. cDC1s excel in cross-presenting antigens to $CD8^+$ T cells through major histocompatibility complex (MHC) I. They start natural killer cell- and innate lymphoid cell (ILC) 1-associated type I immune responses and polarize naïve T cells to type 1 T helper cells (Th cells). cDC2s induce Th2 cell-, ILC2-related type 2 immune responses and present MHCII-loaded antigen to $CD4^+$ T cells. $B220^+$ $Siglec\text{-}H^+$ pDCs are not phagocytic DCs but when encounter with virus, they secrete type I interferons to clear it. Finally, $CD209\ (DC\text{-}SIGN)^+$ $Ly6C^+$ moDCs, develop in inflammatory site after pathogen infection, eliminate pathogens through secreting inflammatory mediators like tumor necrosis factor (TNF)-α. Fully differentiated moDCs show enhanced antigen presenting and stimulating ability than those of cDC1[1,3].

The fore-mentioned GM-CSF is one of the hematopoietic cytokines that in steady state, epithelial cells, endothelial cells, and fibroblasts in skin, lung and gut secrete small amount of it. It affects differentiation and survival of myeloid lineage cells like granulocytes, monocytes, macrophages and monocyte-derived DCs as well as DC subsets of non-lymphoid organs[4-7]. Not only GM-CSF works as hematopoietic cytokine, also it works as inflammatory cytokine. According to clinical studies, the

level of GM-CSF is elevated in skin tissue of atopic dermatitis patients and synovial joint in rheumatoid arthritis patients[7,8]. Another recent study reported that culture of bone marrow cells with high concentration of GM-CSF showed increase of monocytic myeloid cells which is reproduced in spleen, blood, lung and liver of GM-CSF-over expressing mice. but intriguingly, in culture of bone marrow cells with moderate concentration of GM-CSF, moDCs increased[4]. The other study stated that GM-CSF is a pivotal factor in experimental autoimmune encephalomyelitis, the mouse model version of human inflammatory demyelinating disease and the in vivo model of allergic lung disease[7,9-11]. In inflammatory condition, Th cells, B cells, and air epithelial cells also secrete GM-CSF which triggers migration of myeloid cells such as monocytes, macrophages and neutrophils to inflammatory cite. They secrete reactive oxygen species, inflammatory cytokines and chemokines followed by tissue damage and aggravation of inflammation[9].

Lately, unknown cells were discovered in the spleen of the mouse after subcutaneous injection of GM-CSF. They were $MHCII^{+}$ $CD11c^{+}$ $XCR1^{-}$ $33D1^{-}$ cells that distinguish with $MHCII^{+}$ $CD11c^{+}$ $DEC205^{+}$ $XCR1^{+}$ cDC1s and $MHCII^{+}$ $CD11c^{+}$ $CD172\alpha^{+}$ $33D1^{+}$ cDC2s. Along with representative markers of dendritic cells, MHCII and CD11c, they also highly expressed CD115, markers of monocytes, and CD301b. With those markers, we established gating strategy to distinguish unknown cells from cDC1s and cDC2s. Injection of GM-CSF

increased $CD115^+$ $CD11b^+$ cells which contain $Ly6C^+$ $MHCII^-$ classical monocytes (P.I), $Ly6C^-$ $MHCII^-$ non-classical monocytes and especially $Ly6C^-$ $MHCII^+$ cells (P.X). P.X cells were divided into two populations depending on the expression of CD301b, $CD11c^+$ $CD301b^-$ cells (P.III) and $CD11c^+$ $CD301b^+$ cells (P.IV). To identify the unknown cells, P.III and P.IV, morphology of P.III and P.IV was examined. Interestingly, P.III stretched dendrites while P.IV formed cluster with dendrite, the morphology of dendritic cells. Since P.IV showed morphology of dendritic cells, antigen presenting ability of P.IV was investigated. P.IV efficiently proliferated naïve $CD4^+$ T cells with blood-born antigens whereas P.I, P.II and P.III incompetently or weakly proliferated naïve $CD4^+$ T cells. Base on those data, P.IV was denominated as GM-CSF-induced dendritic cells (GMiDCs) and P.III was denominated as pre-GMiDC since pseudotemporal and RNA velocity analyses showed P.III differentiates into P.IV. In certain conditions with cytokines, dendritic cells can polarize naïve T cells into effector T cells, Th cells and regulatory T cells[3]. In Th2 skewing condition, GMiDCs excel at proliferation and differentiation of Th2 which implies involvement of GMiDCs in allergic diseases.

Under GM-CSF treatment condition, significant changes were observed in the allergic airway mouse model. Eosinophilic and neutrophilic asthma was aggravated in the mice sensitized with GM-CSF and OVA. To verify the engagement of GMiDCs in allergic airway inflammation model, P.I, P.III and P.IV cells from OVA and GM-CSF pre-treated mice were transferred to recipient mice.

Mice received pre-GMiDCs and GMiDC showed aggravated eosinophilic airway inflammation while mice received classical monocytes showed no reactions. This data showed that splenic GMiDCs

Anaphylaxis is another Th2-mediated allergic reaction that accompanies breathing and circulation problems, hypotension, and temperature drop. There are two mechanisms of anaphylaxis: classical pathway and alternative pathway. Classical pathway involves FcεRI-expressing mast cells sensitized by allergen-specific immunoglobulin E (IgE) and involves histamine for primary effector molecule. Alternative pathway entails FcγR-bearing macrophages, monocytes, neutrophils and entails platelet-activating factor (PAF) as shock-inducing molecule[12,13].

Base on those unpublished data, GM-CSF might develop GMiDCs or might affect other DCs that stimulate Th2 responses leads to anaphylaxis-prone condition. Understanding how GM-CSF regulates anaphylaxis may contribute to the treatment of allergic diseases as well as providing extended point of view approaching allergic diseases.

II. MATERIALS AND METHODS

1. Mice

Age and sex matched 6- to 9-week-old female C57BL/6 mice were used in the experiments. The mice were purchased from Orient Bio (Seongnam, Republic of Korea). All animals were maintained in specific pathogen-free (SPF) facilities in the Yonsei University College of Medicine. Animal care and all experimental protocols were approved and performed according to the guidelines of the Institutional Animal Care and Use Committee of the Yonsei University College of Medicine.

2. Induction of local and systemic anaphylaxis

A. Local and ayatemic anaphylaxis model of mice sensitized with alum

Mice were intraperitoneally inoculated with 1mg of Alhydrogel (alum) or alum mixed with 100 μg of ovalbumin (OVA) once a week for 3 weeks. 13 days after last sensitization, peripheral blood was collected. Next day, the left ears of mice were administered 10 μl of PBS and the right ears of mice were administered 50 μg of OVA using 3/10cc insulin syringe capped with 31G needle.

A week after ear challenge, 100 μg of OVA was injected intravenously to check systemic anaphylaxis. Another week later, the left ears of mice were intradermally injected 10 μl of PBS and the right ears of mice were injected 50 μg of OVA using 3/10cc insulin syringe capped with 31G needle. 30 minutes after challenge, 0.5% Evans blue dye in 200 μl of PBS was injected into tail vein of the mouse. An hour

later, each side of the ear was harvested in E-tube containing 500 μl of formamide and incubated overnight in the heat block set to 63℃. Absorbance of supernatant was measured sing spectrophotometer at 620nm.

B. Local and systemic anaphylaxis model of mice sensitized with GM-CSF

Mice were subcutaneously injected 10 μg of home-made mouse GM-CSF (mGM-CSF) once a day for 3 days in a row. On day 3, mice were intravenously sensitized with 100 μg of OVA. Depend on experiments, same procedure was repeated at a week intervals. 13 days after last sensitization, peripheral blood was collected. Next day, 100 μg of OVA was injected intravenously to check systemic anaphylaxis.

A week after systemic challenge, the left ears of mice were administered intradermally 10 μl of PBS, and their right ears were administered 50 μg of OVA using 3/10cc insulin syringe capped with 31G needle.

Another week later, the left ears of mice were challenged intradermally 10 μl of PBS, and their right ears were challenged 50 μg of OVA. 30 minutes after challenge, 0.5% Evans blue dye in 200 μl of PBS was injected into the tail vein of the mouse. An hour later, each side of the ear was harvested in E-tube containing 500 μl of formamide and incubated overnight in the heat block set to 63℃. Absorbance of supernatant was measured using spectrophotometer at 620nm.

The schematic protocols for the above experiments are shown in figure 1.

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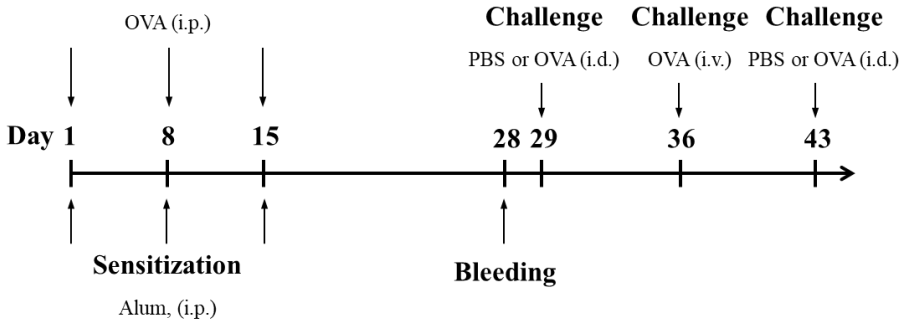

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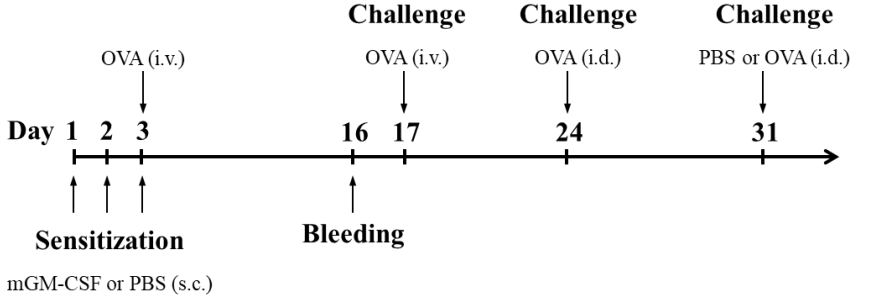

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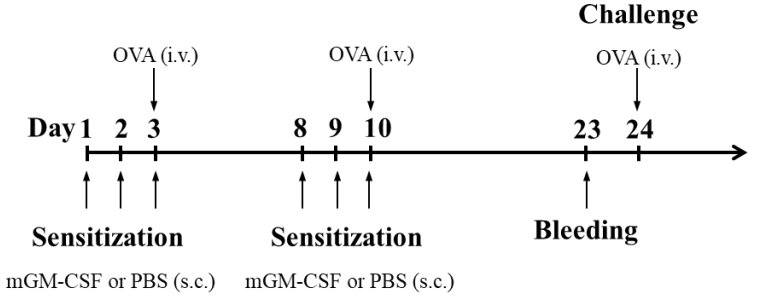

Figure1. Schematic protocols of local and systemic anaphylaxis. (A) Local and systemic anaphylaxis model of mice sensitized with alum (B) Local and systemic anaphylaxis model of mice sensitized with 3-daily GM-CSF injection and (C) Local and systemic anaphylaxis model of mice sensitized with repeated 3-daily GM-CSF injection.

3. Evaluation of local and systemic anaphylaxis

For the local anaphylaxis, ear thickness and optical density of Evans dye were measured. A caliper was used to measure ear thickness prior to challenge, 12 hours post -challenge, 24 hours post-challenge. For systemic anaphylaxis, core body temperature was measured at 10 minutes intervals over an hour. Temperature below 32℃ was calculated as 31.5℃. Also, symptoms of systemic anaphylaxis were evaluated from 0 to 5 as described previously[14,15]: 0, no symptoms; 1, scratching and rubbing around nose and head; 2, puffiness around eyes and mouth, diarrhea, pillar erect, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, and cyanosis around mouth and tail; 4, no activity after prodding or tremor and convulsion; 5, death.

4. ELISA

For measurement of immunoglobulin levels, 96-well immunoplates were coated with OVA (2.5 μg) or anti-mouse IgE (0.1 μg) in 50 μl PBS overnight at 4 ℃. The plates were washed with PBS-Tween 20 (0.05%) (PBST) and blocked with 5% normal goat serum in PBST (blocking solution). Serially diluted serum were added to each well and incubated for 1 hour at 37 ℃ followed by incubation of HRP-conjugated goat anti-mouse IgG or IgE diluted in blocking buffer. After final wash with PBST, 100 μl of 1-Step™ ultra TMB-ELISA solution was added and incubated for 20 minutes. The reaction was stopped by adding 50 μl of 4M sulfuric acid and measured using microplate spectrophotometer at 450nm.

5. Cell isolation

Spleen and peritoneal cavity cells were harvested in mouse. Spleens were grinded on 100 μm cell strainer using syringe plunge to get single cell. The erythrocytes were lysed by 1x RBC lysis buffer. For peritoneal cavity cells, ice cold DPBS containing 1% of FBS (isolation buffer) were flushed into peritoneal cavity using 26G needle and abdomen of mouse was massaged. Suspended peritoneal cavity cells were harvested using syringe capped with 22G needle.

6. Flow cytometry

Single cell suspensions were incubated in the culture supernatant of 2.4G2 hybridoma cells to block Fc receptor for 20 minutes at 4°C followed by wash with FACS buffer composed of DPBS containing 2% FBS, 0.1% sodium azide, and 2mM EDTA. Then, cells were incubated with cocktails of fluorochrome- or biotin-conjugated monoclonal antibodies in 96-well v-bottom plate at 4°C for 30 minutes. Multiparameter analysis of each sample was performed on FACSVerse™ and LSRFortessa™ flow cytometers (BD Biosciences, San Jose, CA) at the Flow Cytometry Core Facility of the Yonsei University College of Medicine. Collected data were analyzed with FlowJo software (BD Biosciences).

7. Antibodies and reagents

LIVE/DEAD™ Fixable Yellow Dead Cell Stain Kits were purchased from ThermoFisher Scientific Korea (Seoul, Republic of Korea) and used following the instructions. FlorOvalbumin (Grade V, Sigma-Aldrich, St. Louis, MO), Alhydrogel (InvivoGen, San Diego, CA), anti-mouse IgE (Biolegend, SanDiego, CA, USA), anti-mouse goat IgE and IgG (SouthernBiotech, Birmingham, AL), RBC lysis buffer (BioLegend, San Diego, CA) and 1-Step™ ultra TMB-ELISA solution (Thermo Fisher Scientific) were purchased respectively. Chinese hamster ovary (CHO) cells were cultured in DMEM containing L-glutamine, high glucose, and pyruvate (Cytiva, Logan, UT, USA) supplemented with 3% or 7% of fetal bovine serum (FBS, Gibco, USA), 100x non-essential amino acid (Gibco) and 100x antibiotics-antimycotic (Gibco). Mouse GM-CSF was produced and purified in house as described previously[16]. Anti-mouse/human CD11b Percp/Cy5.5 (cloneM1/70), Anti-mouse CD11c BV421 (clone N418), Anti-mouse CD45.2 A700 (clone:104), Anti-mouse CD115 biotin (clone:AFS98), Anti-mouse CD117 A647 (clone:2B8), Anti-mouse F4/80 PE/CY7 (clone:BM8), Anti-mouse FcεRIα FITC (clone:MAR-1), Anti-mouse Ly6G PE/CY7 (clone:1A8), Streptavidin PE (clone:RA3-6B) were purchased from Biolegend.

9. Statistical analysis

Statistical analyses were performed by using Prism v.5 (GraphPad Software). Data are presented as the mean ± standard error of the mean (SEM) from at least two independent experiments. Unpaired Student's t-test, one-way analysis of variance (ANOVA) and two-way ANOVA using Bonferroni's correction were used to calculate statistical significance. Statistical significances were indicated below. *$p \leq 0.5$, ** $p \leq 0.01$, *** $p \leq 0.001$: ns, means not significant.

III. RESULTS

1. Local and systemic anaphylaxis elicit in anaphylaxis model in mouse sensitized with alum

To check the tendency of general anaphylaxis, I set up active local and systemic anaphylaxis in vivo model using type 2 helper T cell-promoting adjuvant, alum and OVA as antigen. I sensitized mice with adjuvant or antigen mixed with adjuvant once a week for 3 weeks. 14 days after last sensitization, I challenged mice via two ways: intradermal challenge for local anaphylaxis and intravenous challenge for systemic anaphylaxis. Local anaphylaxis is evaluated through ear thickness and ear vascular leakage while systemic anaphylaxis is evaluated through core temperature drop and clinical symptoms. To quantify vascular leakage, I injected Evans blue dye intravenously after local challenge and measured amount of extravasated dye (Figure 2A). I also measured degree of ear swelling using a caliper (Figure 2B). Mice sensitized with OVA mixed with alum showed strong acute local anaphylaxis which peaked in 12 hours post challenge compared with mice only given adjuvant. To confirm if alum-induced anaphylaxis model also shows systemic anaphylaxis, I challenged mice via tail vein and observed for an hour. During observation, core temperature of mice was measured every 10 minutes and maximal clinical score was recorded. From 10 minutes post-challenge, temperature drop started and peaked at 50 minutes post-challenge, followed by recovery. Combined with descending temperature, mice scored 4 out

of 5, which represent severe systemic anaphylaxis (Figure 3). I also analyzed level of total immunoglobulin E (IgE), OVA-specific IgE and OVA-specific IgG in the serum. Mice sensitized with alum and OVA showed slightly elevated level of total IgE and OVA-specific IgE while OVA-specific IgG was drastically increased. There was little difference of the immunoglobulins between mice only given alum and naïve mice (Figure 4).

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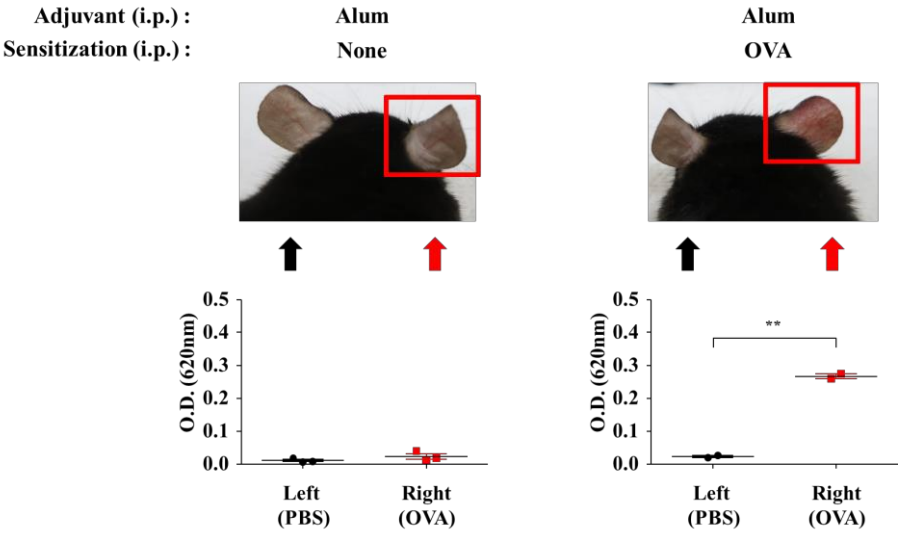

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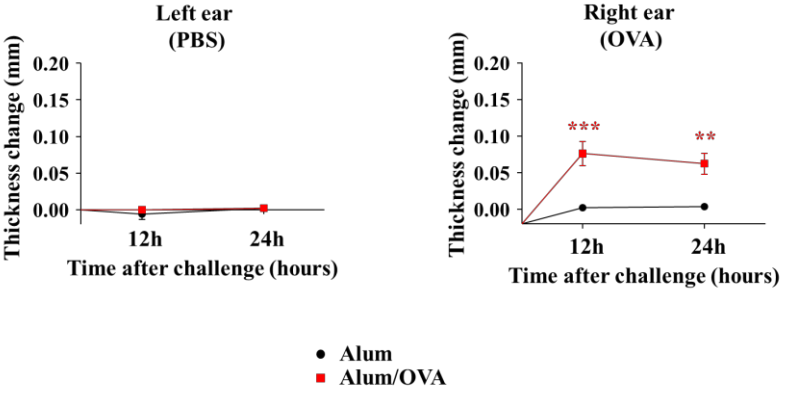

Figure 2. Acute local anaphylaxis is elicited in anaphylaxis model in mouse sensitized with alum. Mice were intraperitoneally injected with alum or alum mixed with OVA once a week for 3 weeks. 2 weeks after last sensitization, PBS or OVA were injected in each side of mice ears. (A) Picture of 12 hours post-intradermal challenge and optical density of extravasted Evans blue dye in alum or alum (+OVA) sensitized mice. (B) The graph of ear thickness change which was measured in 12 hours intervals after challenge.

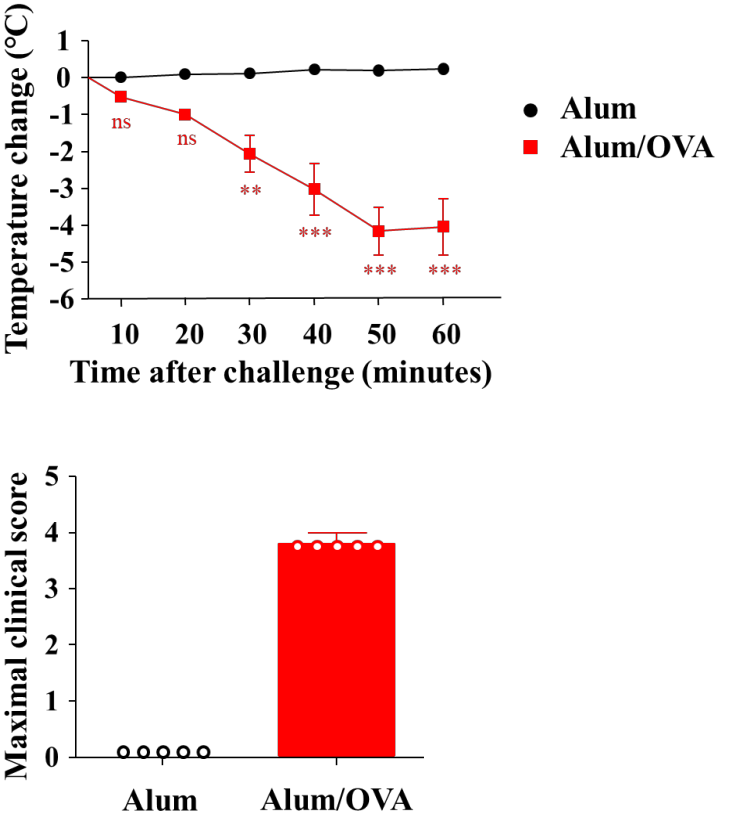

Figure 3. Systemic anaphylaxis is elicited in anaphylaxis model in mouse sensitized with alum. Mice were sensitized with alum or alum mixed with antigen OVA were challenged via tail vein and observed for an hour. Core temperature was measured at 10 minutes intervals over an hour and maximal clinical symptoms were evaluated.

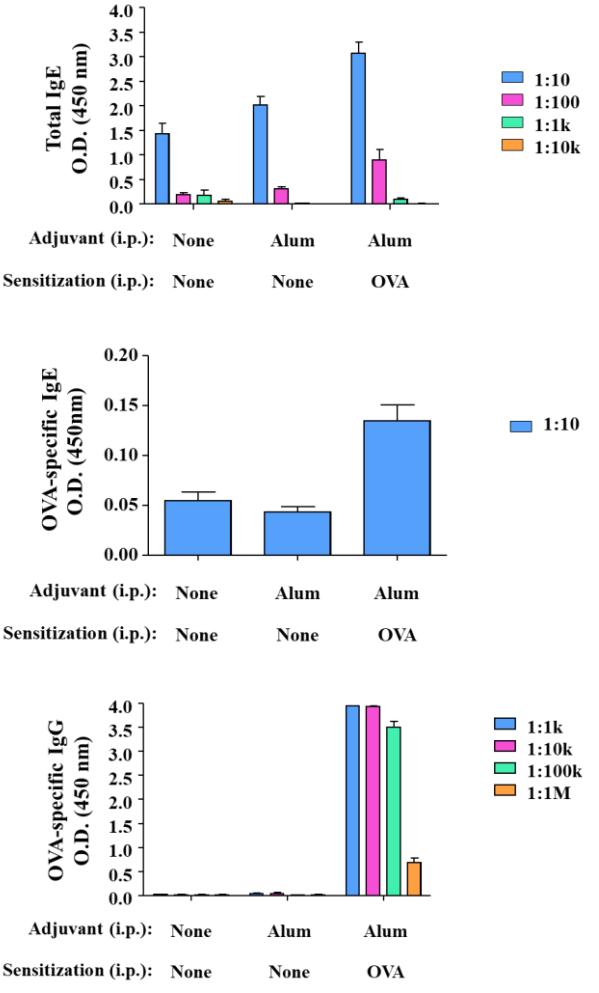

Figure 4. Serum level of total IgE, OVA-specific IgE and OVA-specific IgG in anaphylaxis model in mouse sensitized with alum. 13 days after last injection with alum or alum mixed with OVA, peripheral bloods of mice were harvested and sera were isolated. Serum level of total IgE, OVA-specific IgE and OVA-specific IgG were analyzed using ELISA.

2. Mice sensitized with GM-CSF elicit systemic anaphylaxis but not local anaphylaxis

To investigate whether GM-CSF could elicit local or systemic anaphylaxis, I divided mice into 3 groups with different immunization: one is unsensitized group, another immunized via intravenous injection of OVA under 3-daily subcutaneous injection of PBS and the other sensitized via intravenous injection of OVA under 3-daily subcutaneous injection of GM-CSF which is same condition with allergic airway model mentioned in the introduction. 14 days after last sensitization, I challenged mice as mentioned above (Figure 1B). There was no vascular leakage (Figure 5A, 5B) or ear thickening (Figure 5C) in all of three different groups of mice, including GM-CSF and OVA sensitized group. However, mice sensitized with GM-CSF and OVA showed systemic anaphylaxis with moderate core temperature drop and clinical scores with quick recovery while the others, unsensitized mice and mice sensitized with blood-born antigen OVA under subcutaneous injection of PBS showed no differences in temperature and clinical symptoms (Figure 6). To clarify whether the systemic anaphylaxis is antigen specific reaction, I sensitized mice with or without intravenous injection of OVA under 3-daily subcutaneous injection of GM-CSF. Only mice receiving OVA showed temperature drop and symptoms of systemic anaphylaxis after same antigen administration (Figure 6).

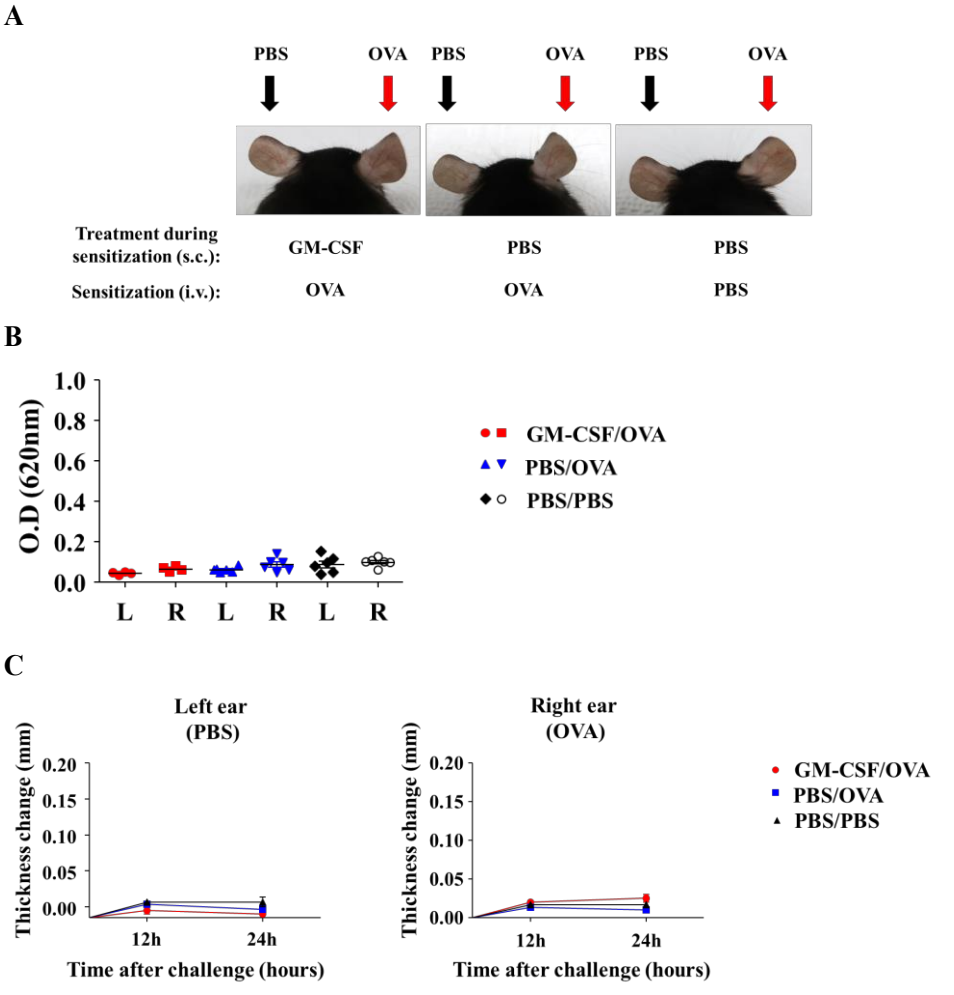

Figure 5. Mice sensitized with GM-CSF show no local anaphylactic reactions. (A) Ear image of 12 hours post-challenged mice which were immunized via subcutaneous injection of GM-CSF or PBS with intravenous injection of OVA or PBS described in figure 1B. (B) Optical density of exudated Evans blue dye and (C) quantified data of ear swelling of three different groups of mice mentioned above. L, left ear: R, right ear.

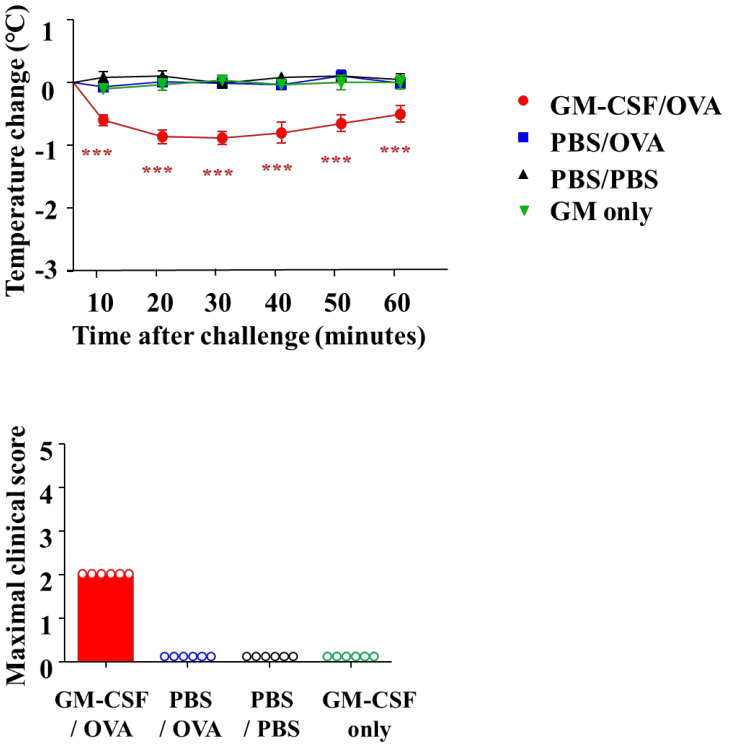

Figure 6. Mice sensitized with GM-CSF show moderate systemic anaphylaxis. Unsensitized mice or mice intravenously sensitized with OVA along with subcutaneous injection of GM-CSF or PBS are challenged via tail vein and observed for an hour. Core temperature was measured at 10 minute intervals and maximal clinical symptoms were evaluated

3. Mice sensitized with GM-CSF shows aggravated systemic anaphylaxis depending on the number of sensitization

As anaphylaxis model in mice sensitized with GM-CSF showed mild response, I immunized mice with 3-daily subcutaneous injection of GM-CSF or PBS with intravenous injection of OVA once a week for 2 weeks to verify intensified anaphylaxis (figure 1C). GM-CSF and OVA sensitized mice showed acute systemic anaphylactic reaction that all of the mice were dead during 1 hour observation while the others showed no responses. Combined with severe hypothermia, GM-CSF sensitized group showed maximum clinical score which reaction is much severe than alum-sensitized anaphylaxis model sensitized three times (Figure 7). Figure 6 and figure 7 indicate that antigen specific systemic anaphylaxis exacerbates as the number of sensitization using GM-CSF increases.

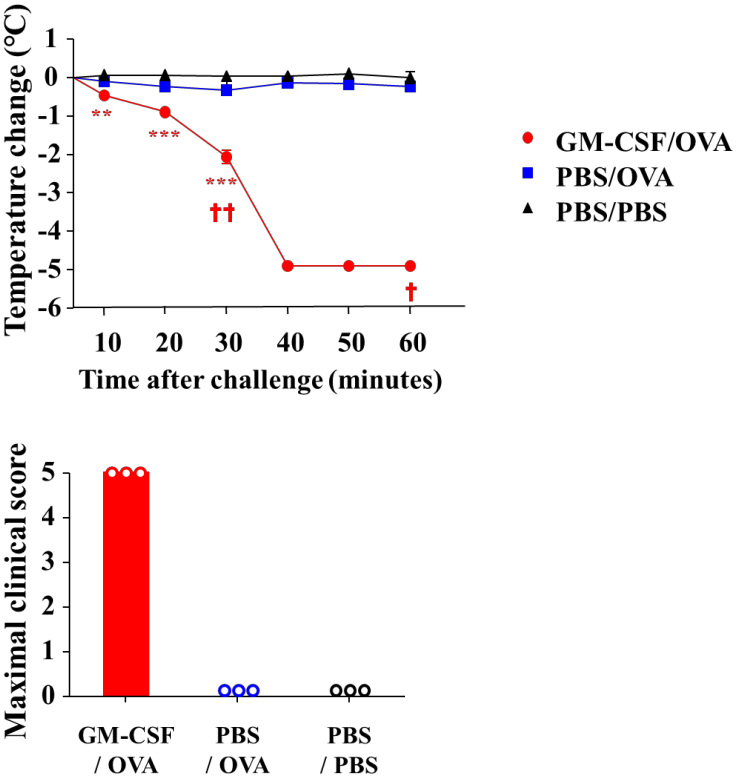

Figure 7. Repeated inoculation of GM-CSF provokes lethal anaphylaxis. Core temperature change and maximal clinical score were evaluated after intravenous challenge of OVA into mice sensitized with repeated 3-daily injection of GM-CSF or PBS along with OVA sensitization in 1 week interval or unsensitized mice. †, time of mortality. Two-way ANOVA was used to calculate statistical significance until 30 minutes post-challenge.

4. GM-CSF elevates titer of total IgE, antigen-specific IgE and antigen-specific IgG

Since GM-CSF-induced anaphylaxis model showed different intense of anaphylactic reaction depending on the number of sensitization, I analyzed the serum level of immunoglobulin isotypes including IgE and IgG, each indicating severity and tolerance of allergic reaction[17,18], in 3 different groups of mice in the GM-CSF-induced anaphylaxis models sensitized once or twice by ELISA. GM-CSF and OVA sensitized group showed highest titer of total IgE (Figure 8A), OVA-specific IgE (Figure 8B) and OVA-specific IgG (Figure 8C) which is slightly elevated after repeated inoculation of GM-CSF and OVA. This data suggests that there might be other factor in aggravated anaphylactic reaction.

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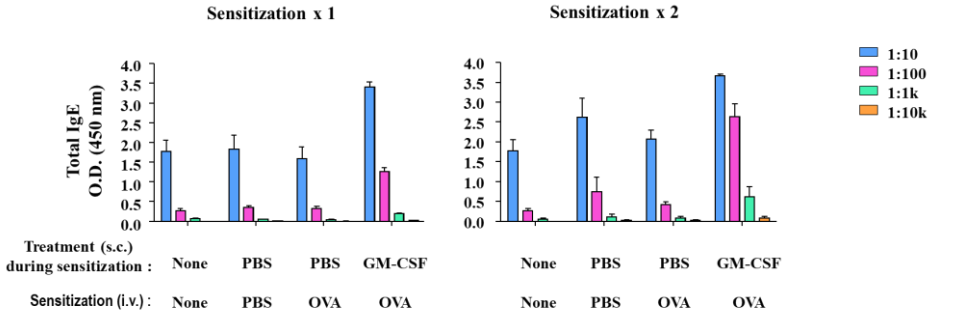

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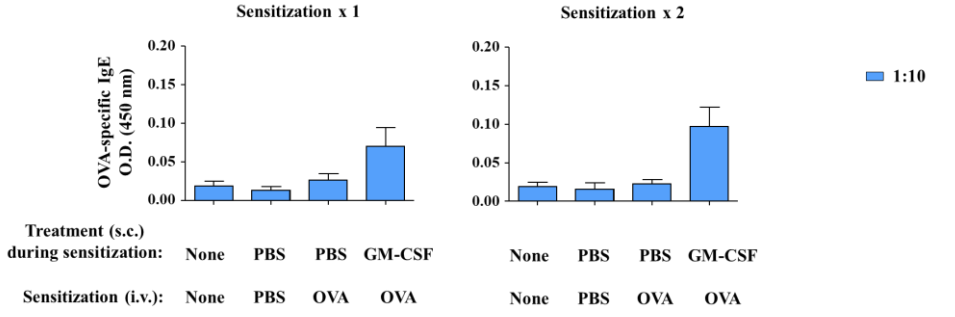

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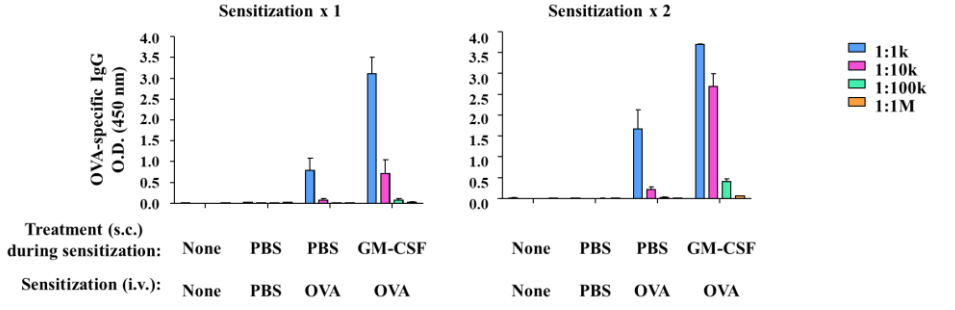

Figure 8. Serum level of immunoglobulin isotypes of anaphylaxis model in mice sensitized with GM-CSF with different number of sensitization. 13 days after last sensitization, peripheral bloods were collected and sera were isolated from GM-CSF-induced anaphylaxis models sensitized once or twice. Serum level of (A) total IgE, (B) OVA-specific IgE, and (C) OVA-specific IgG were measured by ELISA.

5. GM-CSF treatment induces basophilia in the spleen

It has been identified that GM-CSF aggravates antigen-specific systemic anaphylaxis depending on the number of exposure which slightly elevates titer of IgE. To figure out major contributor of aggravated anaphylaxis reaction, I examined whether GM-CSF changes proportion of mast cells. Mast cells are major effector cells in IgE-mediated anaphylaxis expressing high affinity IgE receptor, FcεRI[12]. Mast cells distribute in the peripheral tissues like skin, gut and peritoneal cavity[19,20]. So I investigated mast cells and basophils in the spleen, the filter of blood-born antigen and peritoneal cavity of GM-CSF or PBS injected mice. Mice were subcutaneously injected with GM-CSF or PBS once a day for three days in a row. To maximize the effect of GM-CSF, I repeated the 3-daily injection a week later. Two weeks after last immunization, spleen and peritoneal exudate were harvested from the mice and single cell suspensions were made following the mentioned methods. Splenocytes and peritoneal exudate cells were stained with surface markers of basophils and mast cells[21-24] and analyzed by flow cytometry. Gating strategy is shown in Figure 9A and Figure 10A. In the spleen, only basophils could be found which is significantly increased after GM-CSF treatment (Figure 9B, 9C). In the peritoneal cavity, both mast cells and basophils exist where mast cells were a majority. Interestingly, neither mast cells nor basophils were expanded after GM-CSF injection (Figure 10B, 10C).

Collectively, those data indicates that basophils, the least abundant lymphocytes in the circulation, but not mast cells locate in peripheral tissues, increases in the spleen after GM-CSF treatment might play a important role in the systemic anaphylaxis.

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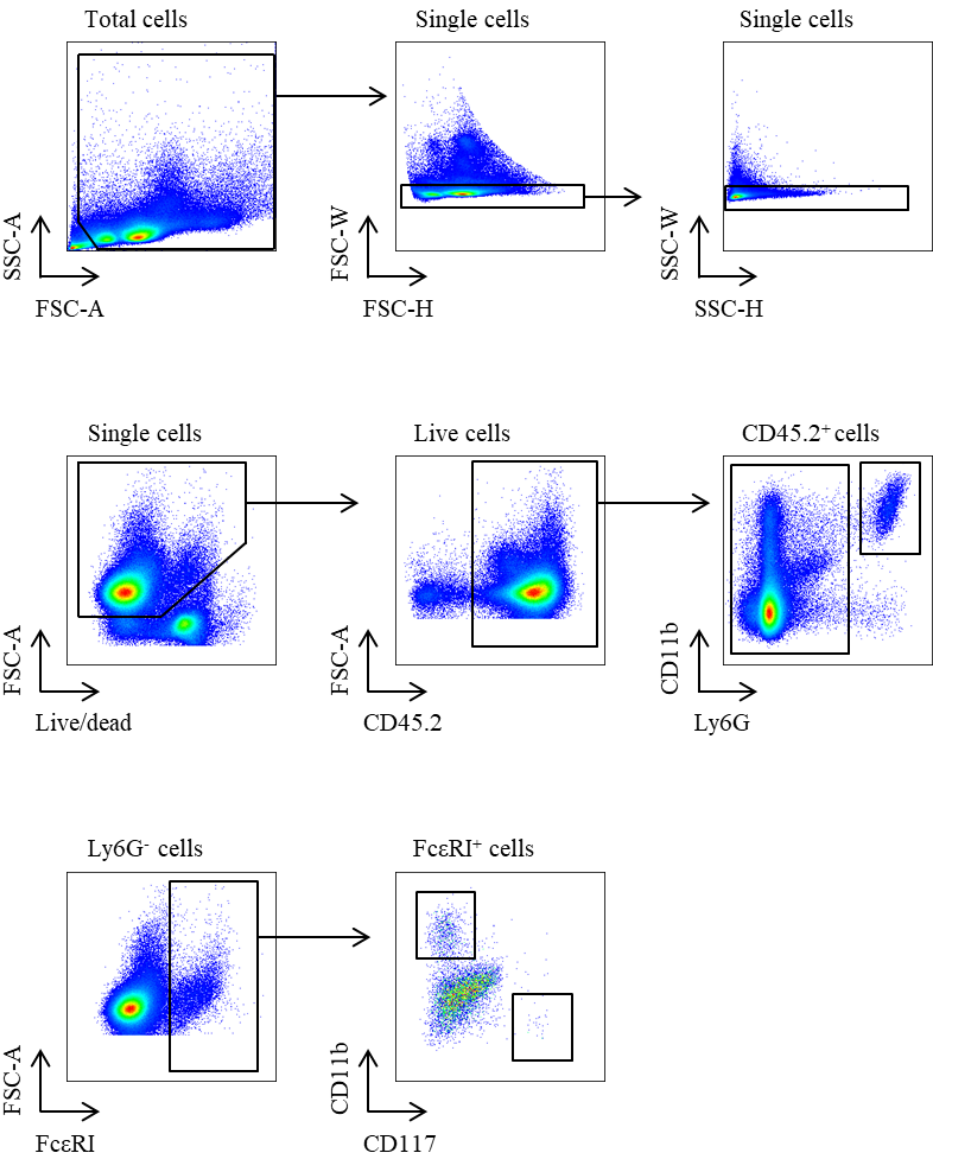

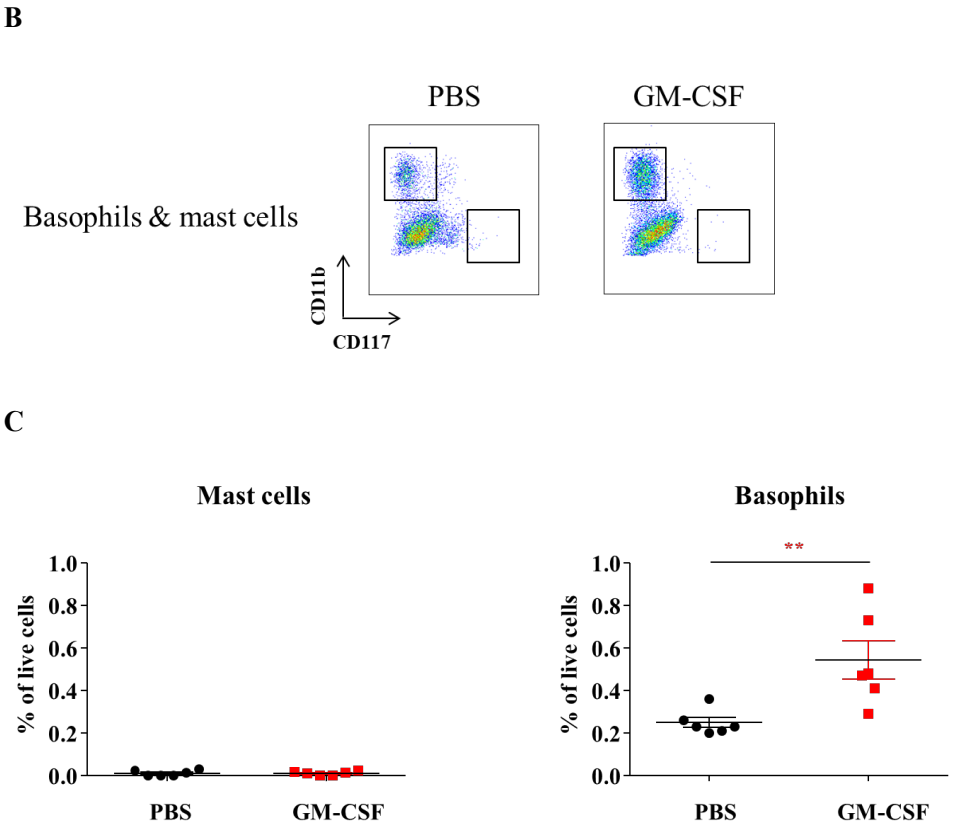

Figure 9. Basophilia is induced by GM-CSF in the spleen. Two weeks after last treatment of GM-CSF or PBS, spleens of mice were harvested and analyzed by flow cytometry. (A) The gating strategies used to divide mast cells and basophils in the spleen. $CD11b^+$ $Ly6G^+$ neutrophils were excluded before dividing $Fc\varepsilon RI^+$ $CD117^+$ mast cells and $Fc\varepsilon RI^+$ $CD11b^+$ basophils. (B) Representative FACS data of basophils and mast cells and (C) percentage of live cells of basophils and mast cells in the spleen.

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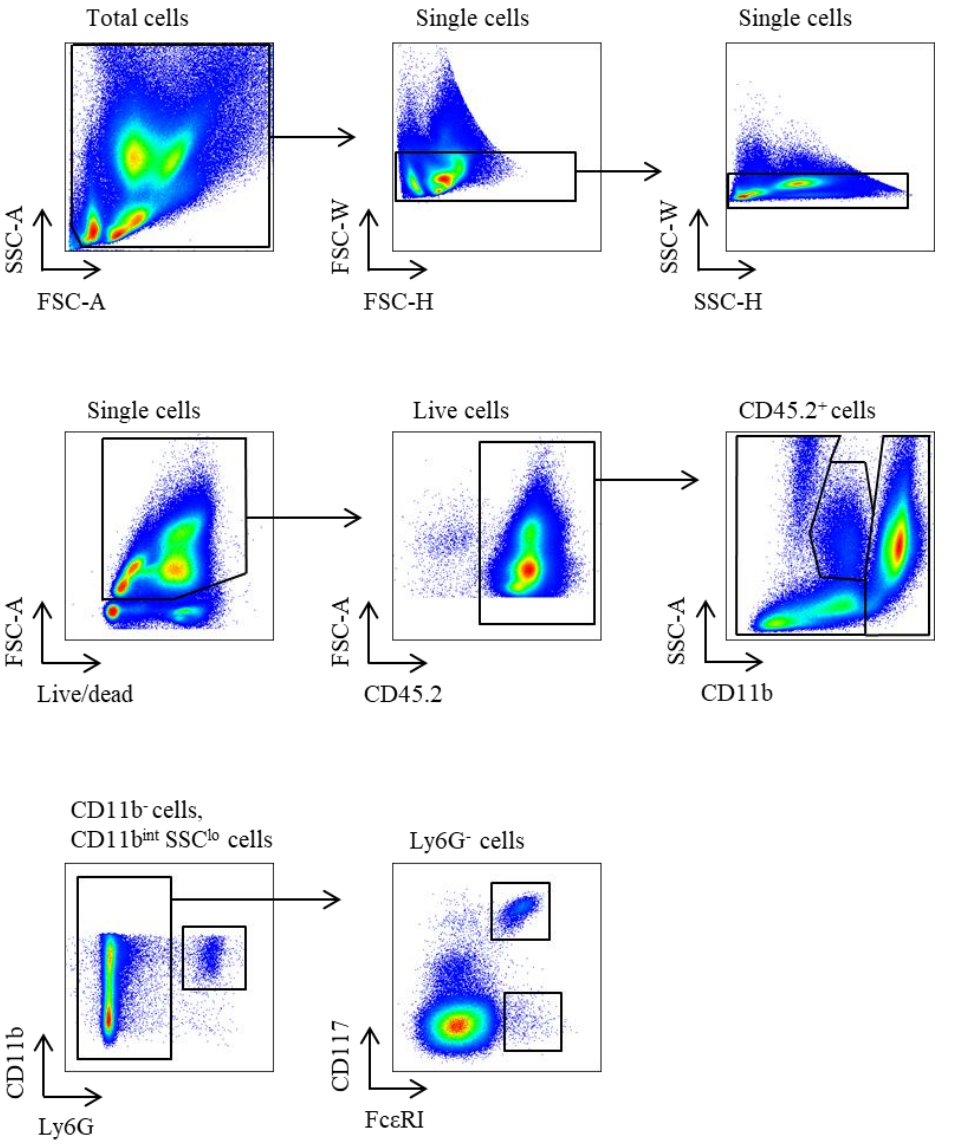

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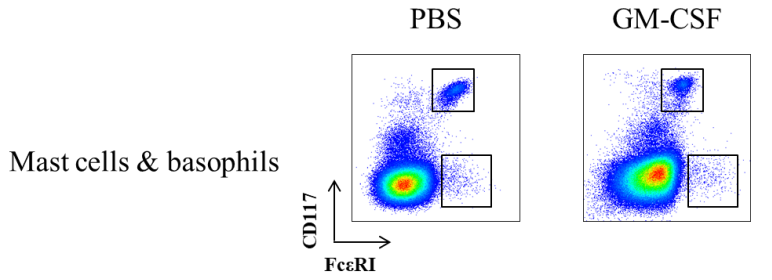

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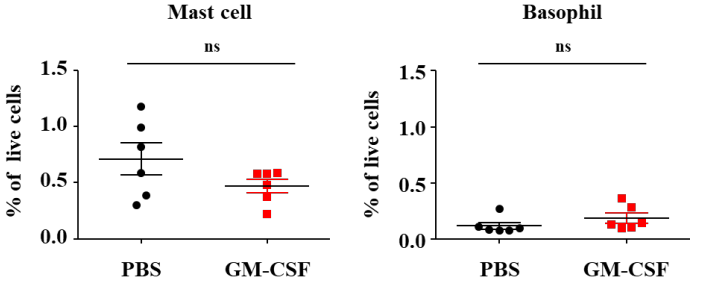

Figure 10. Peritoneal mast cells and basophils were not influence by GM-CSF.

Two weeks after last injection of GM-CSF or PBS, peritoneal exudates of the mice were harvested and analyzed by flow cytometry. (A) Gating strategy used to classify $CD117^{+}$ $Fc\varepsilon RI^{+}$ peritoneal mast cells and $CD117^{-}$ $Fc\varepsilon RI^{+}$ basophils. $CD11b^{hi}$ macrophages and $CD11b^{int}$ SSC^{int} eosinophils were excluded in $CD45.2^{+}$ cells. Then, $CD11b^{+}$ $Ly6G^{+}$ neutrophils were eliminated. $CD117^{+}$ $Fc\varepsilon RI^{+}$ cells were designated as mast cells and $CD117^{-}$ $Fc\varepsilon RI^{+}$ cells were designated as basophils. Representative FACS data (B) and percentage of live cells (C)of mast cells and basophils in the peritoneal cavity.

6. Increment of eosinophils and small peritoneal macrophages and decrement of large peritoneal macrophages in the peritoneal cavity were induced after GM-CSF treatment

Although indistinction of mast cells and basophils were observed in the peritoneal cavity after GM-CSF treatment, other lymphocytes in the peritoneum including eosinophils and macrophages showed dramatic changes. As effect of GM-CSF, eosinophils were quadrupled (Figure 11B), small peritoneal macrophages (SPMs) were doubled and large peritoneal macrophages (LPMs) were halved in the peritoneal cavity (Figure 11C). This data suggest that along with mast cells and basophils, the major effector cells of IgE-mediated anaphylaxis, macrophages and eosinophils might also involve in systemic anaphylaxis induced by GM-CSF.

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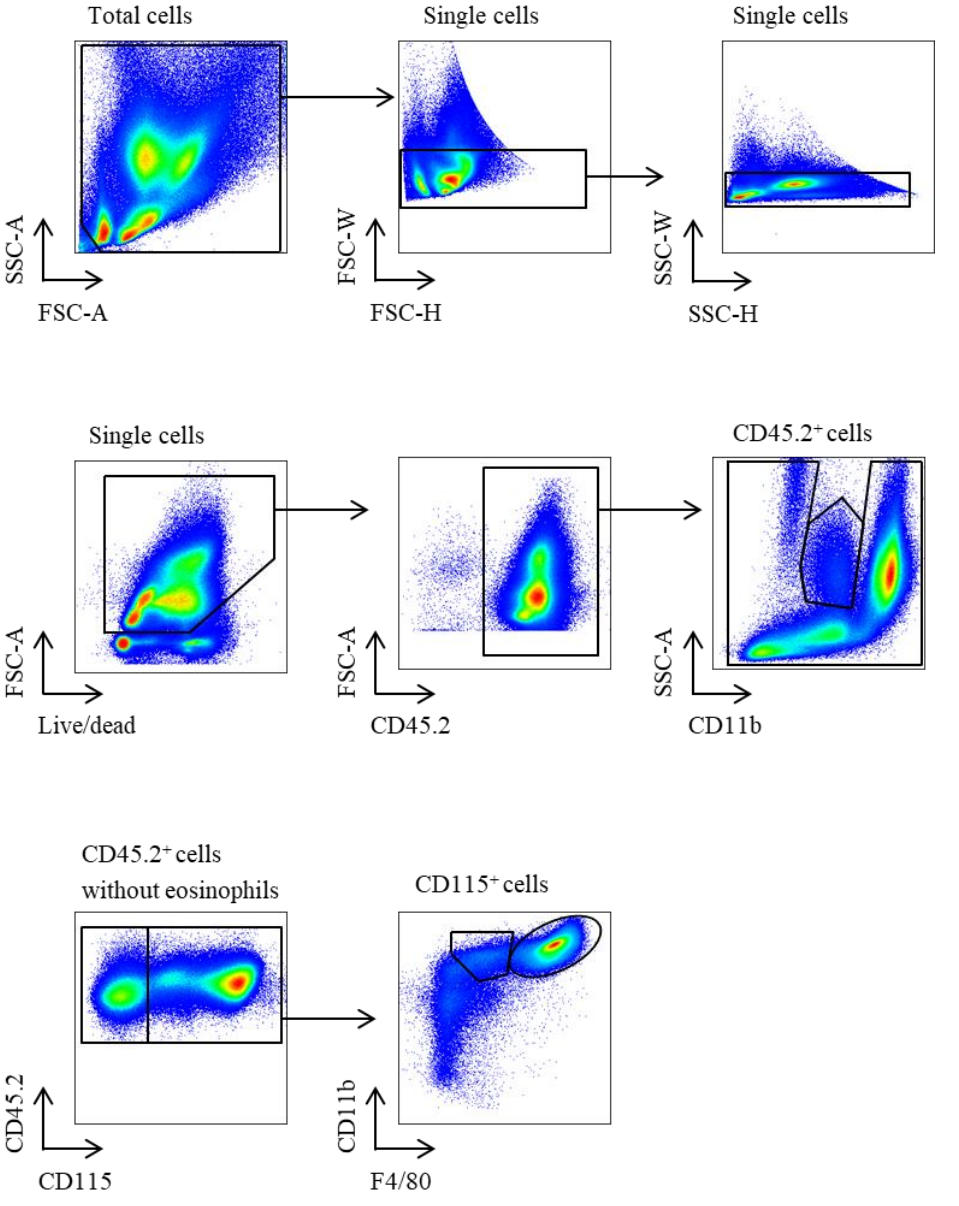

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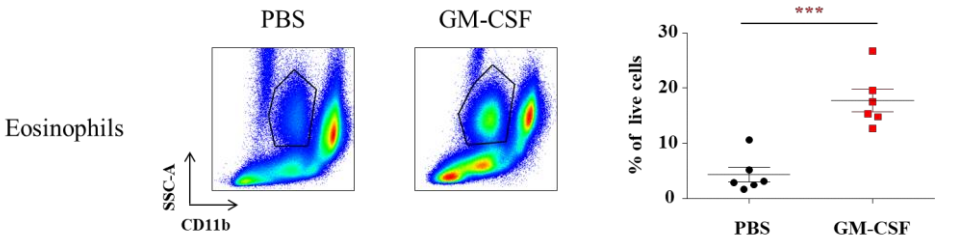

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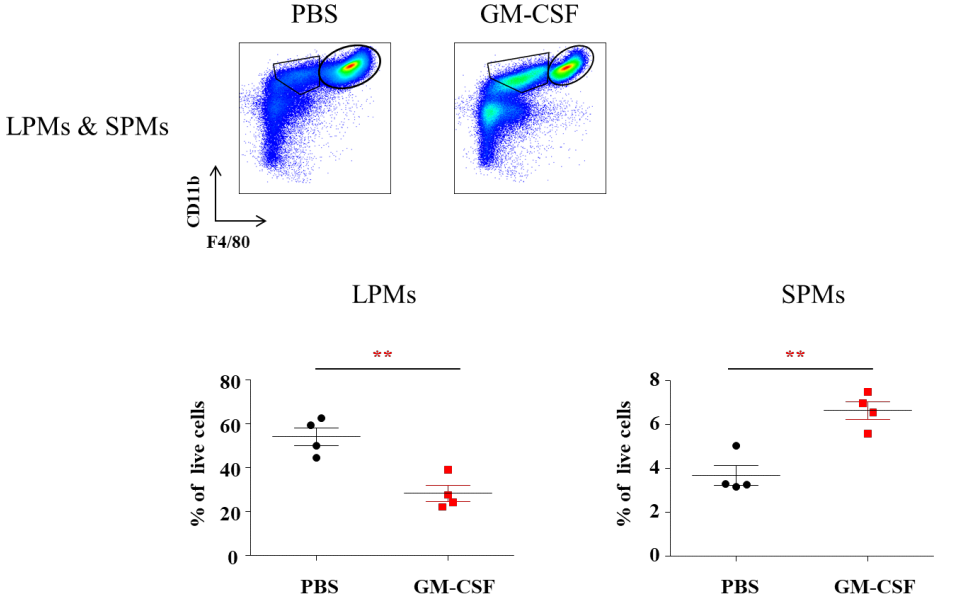

Figure 11. Eosinophilia and proportion change between LPM and SPM are elicit by GM-CSF in the peritoneum. Peritoneal cavity cells of PBS or GM-CSF injected mice were harvested and analyzed by flow cytometry. (A) Gating strategy classifying $CD11b^{int}$ SSC^{int} eosinophils in $CD45.2^{+}$ cells, $CD11b^{+}$ $F4/80^{hi}$ large peritoneal macrophages and $CD11b^{+}$ $F4/80^{int}$ small peritoneal macrophages in $CD115^{+}$ cells. FACS analysis and percentage of live cells of (B) eosinophils and (C) LPMs and SPMs in the peritoneal cavity.

IV. DISCUSSION

Originally, GM-CSF is widely known as the hematopoietic cytokine[9]. In addition to myelopoiesis, the current pre-clinical models and clinical trials showed that GM-CSF also play as the inflammatory cytokine in allergic diseases and autoimmune diseases[7,8,10]. However, whether GM-CSF also regulates anaphylaxis, one of the life-threatening allergic responses, is remain elusive. In this study, I demonstrated that GM-CSF aggravates anaphylaxis via elevating total IgE, antigen-specific IgE and antigen-specific IgG levels. Treatment of GM-CS also increased only basophils in the spleen while mast cells in the spleen and peritoneal cavity showed no difference. Additionally, eosinophils and small peritoneal macrophages were increased while large peritoneal macrophages were decreased. Until now, it has been suggested that mast cells play a key role in IgE-mediated anaphylaxis by secreting mediators like histamine and tryptase. However, patients experiencing anaphylaxis often showed normal serum level of tryptase along with elevated serum level of histamine[25]. This postulates that basophils which also express FcεRI and secrete histamine might be engaged in IgE-mediated anaphylaxis. Current clinical study showed role of basophils in anaphylaxis. Patients with acute allergic anaphylaxis reaction showed significantly decreased number of basophils in the blood while there was no difference in absolute number of polymorphonuclear leukocyte with lowered mRNA level of FCERIA, carboxypeptidase A3, and L-histidine decarboxylase in the whole blood.

Interestingly, the patients showed significantly escalated level of C-C Motif Chemokine Ligand 2, the major basophil chemotactic factor. This indicates that basophil may be recruited to inflamed tissues from circulation. This data correlates with my result, basophilia in the spleen under GM-CSF treatment. My study might present new mechanism of anaphylaxis and mouse model of IgE-mediated anaphylaxis differs from existing IgG-mediated mouse anaphylaxis model. Interestingly, recent study reported that fate of different myeloid lineage cells were determined by quantity of GM-CSF[4]. Bone marrow cells cultured with low dose of GM-CSF differentiated into $Ly6G^{hi}$ $CD11b^{+}$ granulocytes whereas high dose of GM-CSF give rise to $Ly6G^{lo}$ $CD11b^{+}$ monocytic myeloid cells. The latter phenomenon is reproduced in spleen of GM-CSF overexpressing mice. Although previous studies showed both expansion of granulocytes and monocytic myeloid cells[26,27], rather than granulocytes, expansion of monocyte-derived dendritic cells expressing CD11b, CCR2, CD11c and MHCII is observed. Expansion of the monocyte-derived dendritic cells was influenced by A1, the anti-apoptotic protein induced by GM-CSF, with highest differentiation in intermediate dose of GM-CSF. It is not known that basophils in spleen, eosinophils and small peritoneal macrophages in the peritoneal cavity differentiate in low dose of GM-CSF. But this recent report might explain the sequencial increase of newly discovered GMiDCs, mentioned in introduction, to basophil, mast cells and macrophages in spleen and peritoneal cavity under GM-CSF condition. Another intriguing recent study described interaction between eosinophils and mast cells[28]. They synthesized

peptide fragments of major basic protein (MBP) and eosinophil cationic protein (ECP), part of eosinophil granules. In vitro culture of human cord blood-derived mast cells (HCMCs) with these residues resulted degranulation on dose dependent manner. Treating Mas-related G-protein coupled receptor family member X2 (MRGPRX2) antagonist significantly reduced degranulation of HCMCs induced by MBP and ECP residues. Whether peritoneal eosinophils amplified after GM-CSF treatment activates mast cells which causes anaphylaxis is unknown. However, combined with these recent reports, my study might present a new mechanism of anaphylaxis which is regulated by GM-CSF.

V. CONCLUSION

In this study, the effect of GM-CSF in anaphylaxis is investigated. Compared to general anaphylaxis induced by treatment of Th2 promoting adjuvant, alum, the treatment of GM-CSF induced acute systemic anaphylaxis effectively. The anaphylactic reactions were intensified as the number of sensitization with GM-CSF increased. Similarly, the titers of total IgE, allergen-specific IgE and allergen-specific IgG augmented as the number of sensitization wirh GM-CSF increased. Meanwhile, it was uncovered that basophils, but not mast cells, increased in the spleen gradually after sensitization with GM-CSF, suggesting the previously unrecognized role of basophils in the systemic anaphylaxis. For the first time, this study characterizes the features of humoral and cellular immune responses in the anaphylactic reactions induced by sensitization with GM-CSF.

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ABSTRACT(IN KOREAN)

아나필락시스 반응을 조절하는 granulocyte macrophage-colony stimulating factor 의 기능

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박세정

Granulocyte macrophage-colony stimulating factor (GM-CSF)는 골수계통 세포의 분화와 생존에 영향을 미치는 조혈 사이토카인 중 하나이다. 최근 자가면역 질환과 알러지 질환 모두에서 GM-CSF가 중요한 역할을 하는 것으로 알려지면서 염증성 사이토카인으로서의 역할이 두드러지고 있다. 그러나 GM-CSF와 아나필락시스 사이의 관계는 명확하게 밝혀진 바가 없다. 이를 알아보기 위해 C57BL/6 마우스에서 두 개의 OVA 유도 아나필락시스 모델을 확립하였다. 하나는 면역 증강제로 명반을 사용하였고 다른 하나는 GM-CSF를 사용하였다. 명반으로 감작된 모델에서 OVA의 국소 또는 피하 주사와 OVA의 전신 또는 정맥 주사 후 경미한 아나필락시스 반응이 관찰되었다. 하지만 GM-CSF로 감작된 모델에서 전신적인 아나필락시스

반응만 관찰되었고 국소적인 반응은 관찰되지 않았다. 이런 전신적인 아나필락시스 반응은 GM-CSF에 의한 감작 수가 증가할수록 악화되었다. 악회된 아나필락시스 반응과 함께 총 IgE, OVA 특이적 IgE 및 IgG가 증가한 것을 알 수 있었다. 이후, GM-CSF를 주사한 마우스의 비장과 복강에서 시간 경과에 따른 면역세포 변화를 조사하였다. 비장에서 호염구가 유의미하게 증가하였으나 비장 및 복강 내 비만세포는 증가하지 않았다. 또한 비장 및 복강에서 호산구와 작은 복막 세포의 수가 증가한 반면 큰 복막 대식세포의 수는 감소한 것을 알 수 있었다. 이 연구를 통해 처음으로 GM-CSF에 의해 일어난 새로운 아나필락시스의 체액성, 세포성 측면이 밝혀졌다. 이 연구를 통해 GM-CSF에 의해 유도된 비장의 호염구 증가가 이전에는 인식되지 않았던 전신 아나필락시스에 중요한 역할을 할 수 있음이 시사되었다.

핵심되는 말 : GM-CSF, 아나필락시스, 호염구, 비만세포, 대식세포

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