Research Article

Identification of T- and B-Cell Subsets That Expand in the Central and Peripheral Lymphoid Organs during the Establishment of Nut Allergy in an Adjuvant-Free Mouse Model

Babu Gonipeta,1,2 David Duriancik,2 EunJung Kim,1,2,3 Elizabeth Gardner,2 and Venu Gangur1,2

1 Food Allergy and Immunology Laboratory, Michigan State University, East Lansing, MI 48823, USA
2 Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48823, USA
3 Division of Applied Life Science (Bk 21 Program), Gyeongsang National University, Jinju, Republic of Korea

Correspondence should be addressed to Venu Gangur; gangur@msu.edu

Received 25 February 2013; Accepted 15 March 2013

Academic Editors: S. Burastero, B. F. Gibbs, and R. Paganelli

Copyright © 2013 Babu Gonipeta et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Nut allergies are potentially fatal and rarely outgrown for reasons that are not well understood. Phenotype of T- and B-cell subsets that expand during the early stages of nut allergy is largely unknown. Here we studied this problem using a novel mouse model of nut allergy. Mice were rendered hazelnut allergic by a transdermal sensitization/oral elicitation protocol. Using flow cytometry, the T- and B-cell phenotype in the bone marrow (BM), spleen, and the mesenteric lymph node (MLN) of allergic and control mice was analyzed. Nut allergic mice exhibited an expansion of CD4+ CD62L⁺ T cells in BM and spleen; a similar trend was noted in the MLN. There was expansion of CD80+ B cells in BM and spleen and MLN and CD62L− cells in BM and spleen. Interestingly, among CD80+ B cells, significant proportion was CD73− particularly in the MLN. These data demonstrate that during the early establishment of hazelnut allergy there is (i) expansion of CD4+CD62L⁺ T-cell subsets in both the BM and the periphery, (ii) expansion of CD80+ and CD62L− B-cell subsets in BM and the periphery, and (iii) a significant downregulation of CD73 on a subset of B cells in MLN.

1. Introduction

Food allergies such as tree nut allergies are potentially fatal group of immune-mediated disorders [1]. Recent studies demonstrate that both the prevalence and severity of food allergies are escalating for reasons that are not well understood at present [1, 2]. Tree nut allergies, along with peanut allergy, are the leading causes of food-induced systemic anaphylaxis in USA and European countries [3]. Furthermore, once individuals are sensitized, there is a very low potential for outgrowing tree nut allergies [4, 5]. Consequently, they are considered not only serious but also persistent health problems for rest of the life of sensitized subjects [4, 5].

The specific identity of T- and B-cell subsets that expand during the early stages of establishment of life-threatening nut allergies is largely unknown at present. Notably, most of the current knowledge about early expansion and establishment of immune memory cells comes from studies of infectious diseases [6–12]. Knowledge about identity of such immune cells is urgently needed for potential therapeutic targeting in nut allergies.

We have previously reported an adjuvant-free mouse model of tree nut allergy using hazelnut as a model tree nut [13]. This model employs a protocol combining transdermal sensitization followed by oral allergen challenge to elicit systemic anaphylaxis [13–15]. Furthermore, hazelnut allergy, once established, remains persistent even when the allergen is withdrawn for at least eight months in this model; and persistence of clinical sensitivity is associated with robust memory T-cell and memory B-cell responses [15]. Since
this model does not use an external adjuvant, it offers a unique opportunity to determine the phenotype of T- and B-cell subsets that expand during the early stage establishment of nut allergy. Here we report the identification of the phenotype of T- and B-cell subsets that expand in the central and peripheral lymphoid organs during the establishment of nut allergy in this mouse.

2. Material and Methods

2.1. Reagents. Hazelnut protein extract (Greer Labs, Lenoir, NC, USA); protein content of these three protein extracts was measured by Lowry’s method [16]. Normal saline was prepared in our lab (0.85% W/V NaCl solution): streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA, USA). The following fluorochrome-conjugated monoclonal antibodies were purchased from BD Biosciences (San Diego, CA, USA): CD3e (clone 145-2c11)-PerCP-Cy5.5, CD44(IM7)-PE, CD62L(MEL-14)-APC, and B220(RA3-6B2)-AlexaFluor700 were purchased from eBiosciences (San Diego, CA, USA).

2.2. Mice. All mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All animals were females and used at 7-8 weeks of age. All procedures involving mice were in accordance with Michigan State University Animal Use Policies. Mice were acclimated for one week to their new environment before starting the experiment.

2.3. Transdermal Sensitization and Oral Elicitation of Systemic Anaphylaxis. Transdermal sensitization followed by oral allergen challenge protocols was performed using the method described previously [17, 18]. Sensitization was determined by measuring hazelnut-specific IgE antibody levels in the plasma using an ELISA-based method as described. Systemic anaphylaxis upon oral allergen challenge was quantified by rectal thermometry before and at 30 minutes after oral challenge using a digital temperature probe (Thermalert TH-5, Physitemp; NJ, USA; instrument specifications: resolution: 0.1°C; accuracy 0.1°C ± 1 digit; operating range: 25–45°C). Animals were euthanized 1 hr after oral challenge and used for tissue collection.

2.4. Phenotype Analysis of T and B Cells by Flow Cytometry. Following euthanasia after 1 hour after oral challenge and collection of hypothermia data, bone marrow, spleen, and mesenteric lymph nodes were harvested, and single-cell suspensions were prepared using the standard protocols. One million cells were aliquoted for flow cytometry staining. Cells were blocked with anticomouse CD16/32 (2.4G2 prepared in house) on ice for 10 minutes. Subsequently, cells were stained with either a memory T-cell master mix (CD3, CD4, CD8, CD44, and CD62L) or memory B-cell master mix (B220, CD80, CD73, CD44, and CD62L) of monoclonal antibody-fluorochrome conjugates. Each monoclonal antibody-fluorochrome conjugate was used at concentrations recommended by the manufacturer or previous antibody titration. Cells were incubated with the master mix for 30 minutes on ice in the dark, washed twice with FACS buffer, and run on a BD FACS Canto II flow cytometer. Compensation was established using BD Biosciences compensation beads.

Postacquisition flow cytometry analysis was performed using FlowJo software (Tree Star, Inc., Ashland, OR, USA). Live cells were gated based on forward scatter and side scatter for both T- and B-lymphocyte samples. T cells were gated as CD3+ on a histogram, CD4+ or CD8+ based on a dot plot, and subsequently a quadrant was drawn to separate CD44hi and CD62L+ for each T-cell subset. Effector memory CD4 and CD8 T cells were defined as CD44hi/CD62Lneg. Central memory CD4 and CD8 T cells were defined as CD44hi/CD62L [9, 19]. For B-cell analysis, histogram of B220hi+ was drawn out of live cells, and a quadrant gate was used to distinguish CD62L+ and CD80+ cells. Expression of CD73 was analyzed by histogram from each quadrant. Increased expression of CD80, CD62L, and CD73 is indicative of memory B-cell phenotype, and expression of CD73 may indicate isotype switched B cells [20, 21].

2.5. Statistical Analysis. ANOVA and Student’s unpaired tests with Welch correction were used to evaluate significance using a software program (GraphPad software, San Diego, CA, USA). The statistical significance level was set at 0.05.

3. Results

3.1. Hazelnut Allergic Mice Exhibit a Significant Expansion of CD4+ CD62L− T Cells in the Bone Marrow and Spleen. Groups of mice were rendered hazelnut allergic using the transdermal sensitization followed by oral elicitation of systemic anaphylaxis to hazelnut that we have described before. The induction of hazelnut-specific IgE antibody response upon transdermal exposure is shown (Figure 1(a)). Systemic anaphylaxis to oral allergen challenge was confirmed by hypothermia responses (Figure 1(b)).

The gating strategy we used, the phenotype of expanding T- and B-cell subsets, in the central and peripheral lymphoid organs is shown in Figures 2(a) and 2(b). We examined the subsets of CD4 cells for naïve versus memory phenotype using high CD62L expression as a marker of naïve cells and low CD62L expression as a marker of memory phenotype. As evident, there was consistent expansion of CD4+ CD62L− T cells in bone marrow and spleen (Figures 3(a)–3(c)). Although there was a similar trend in the MLN, it was not statistically significant.

3.2. Hazelnut Allergic Mice Exhibit a Modest Increase in CD4+ CD44+ T Cells in the Central and the Peripheral Immune Compartments. We also examined the subsets of CD4 cells for naïve versus memory phenotype using high CD44 expression as a marker of memory cells and low CD44 expression as a marker of naïve phenotype. As evident, there was a modest increase in the number of CD4+ CD44+...
3.3. Hazelnut Allergic Mice Exhibit a Modest Increase in CD80+ and CD62L− B Cells in Both Central and Peripheral Lymphoid Organs. Then we examined the B cells for naïve versus memory phenotype using antibodies to CD80 and CD62L as markers. As evident, there was a modest increase in the proportion of CD80+ B cells in the BM, spleen, and MLN. A similar trend was noticed for CD62L− B cells in BM and spleen but not in MLN (Table 2).

3.4. Hazelnut Allergic Mice Exhibit a Significant Downregulation of CD73 on CD80+ B Cells in the MLN. We surprisingly found a significant reduction in CD73 expressing cells among B cells with both CD80+ CD62L+ and CD80+ CD62L− phenotypes in the mesenteric lymph nodes but in bone marrow or the spleen (Figures 4(a)–4(c)).

4. Discussion
This study was undertaken to identify the phenotype of T- and B-cell subsets that expand during the early stages of establishment of hazelnut allergy in an adjuvant-free mouse model that we have described and characterized before [13–15, 22]. We studied the immune cell expansion in the bone marrow as a central lymphoid organ, spleen as a systemic peripheral lymphoid organ, and the mesenteric lymph node as representative draining lymphoid organ of the gut. There are three novel and important findings from this study: (i) we report a significant expansion of CD4+ CD62L− T cells in both the BM and the periphery; (ii) an increase in CD80+ and CD62L− B cells in BM and the periphery; (iii) a significant downregulation of CD73 expression on a subset of B cells particularly in the MLN.

We have previously reported that hazelnut allergy once established in model remains persistent for up to 8 months despite withdrawing allergen exposure [15]. However, the specific phenotype of immune subsets that expand at early stages of the disease establishment in the allergic mice

Table 1: Analysis of CD4+ CD44+ memory T cells in hazelnut allergic and nonallergic control mice.

| Group                      | Percentage of CD4+ CD44+ T cells |
|----------------------------|----------------------------------|
|                            | Bone marrow | Spleen | Mesenteric lymph node |
| Control mice (n = 5)       | 21.18 ± 2.1 | 15.19 ± 0.4 | 7.31 ± 0.5 |
| Hazelnut allergic mice (n = 6–7) | 24.09 ± 2.3 | 18.92 ± 2.4 | 5.51 ± 1.1 |

T cells in bone marrow and spleen, but this was not statistically significant (Table 1).
Figure 2: Gating strategy used to identify T- and B-lymphocyte subsets. Shown is one representative spleen sample. (a) Gating of memory T cells. Based on forward scatter, and side scatter live cells were gated and a histogram of CD3 (a(i)) was used to gate CD3+ cells. Subsequently, CD3+ cells were separated into CD4+ and CD8+ cells based on dot plot (a(ii)). Central memory T cells were gated as CD62L<sup>hi</sup>/CD44<sup>hi</sup> and effector memory T cells were gated as CD62L<sup>neg</sup>/CD44<sup>hi</sup> out of both CD4+ (a(iv)) and CD8+ T cells (a(iii)). (b) Gating of memory B cells. Based on forward scatter and side scatter, live cells were gated and a histogram of B220 (b(i)) was used to gate B220+ cells. In the bone marrow, only B220<sup>hi</sup> cells were gated (data not shown). Subsequently, B cells were gated in the dot plot based on CD62L and CD80 expression (b(ii)). The expression of CD73 was assessed by histogram (b(iii)); only CD80+/CD62L<sup>neg</sup> quadrant is shown as a representative example.
Figure 3: ((a)–(c)) Analysis of central and peripheral phenotype of T lymphocytes from hazelnut allergic and nonallergic control mice. Groups of BALB/c ($n = 5$ to 7 per group) were sensitized with hazelnut protein (0.5 mg/mouse) via transdermal exposure and then confirmed for allergy by oral challenge. After 1 hour, bone marrow (a), spleen (b), and mesenteric lymph nodes (MLN) (c) were harvested and single-cell suspension prepared. Cells were stained with antibodies against CD3, CD4, and CD62L markers and analyzed by flow cytometry. Differences between allergic and nonallergic mice data were compared by Student's $t$-test for significance ($P < 0.05$).

Figure 4: ((a)–(c)) Analysis of central and peripheral phenotype of B lymphocytes from hazelnut allergic and nonallergic control mice. Groups of BALB/c ($n = 5$ to 7 per group) were sensitized with hazelnut protein and then confirmed for allergy by oral challenge. After 1 hour, bone marrow (a), spleen (b), and mesenteric lymph nodes (MLN) (c) were harvested and single-cell suspension prepared. Cells were stained with antibodies against surface markers CD80, CD62L, and CD73 and analyzed by flow cytometry. Differences between allergic and nonallergic mice data were compared by Student's $t$-test for significance ($P < 0.05$).
CD62L as a marker to distinguish naïve versus memory T cells, and CD80, CD62L, and CD73 as markers of memory cells. We, however, acknowledge that the cells and CD80, CD62L, and CD73 as markers of memory cells.

B cells that we hypothesize as a potential subset of memory B cells. An unusual subset of cells might represent a subset of quiescent CD73 expression in allergic mice. This suggests that this fact that memory cells are known to be and expected to be heterogeneous, and choosing only a few selected markers is a limitation of our study. Also cell analyses were done in mice after they were orally challenged with the allergen to induce shock. Therefore, shock-induced redistribution of cells (if at all occurred in this short time of 1 hour, which is very unlikely) also possibly contributed to the observations in this study.

These data demonstrate for the first time that during the early stages of establishment of hazelnut allergy there is (i) expansion of CD4+ CD62L− T cell subset in both the BM and the periphery, (ii) expansion of CD80+ and CD62L− B-cell subset in BM and the periphery, and (iii) a significant downregulation of CD73 on a subset of B cells particularly in MLN.

Conflict of Interests

Authors do not have any competing or conflict of interests or financial interest to disclose with any of the companies mentioned in this paper.

Acknowledgments

This work was supported by funding from US EPA STAR Grants RD833133 and RD8348220; EJ Kim is supported by a scholarship from the Brain Korea 21 Program, the Ministry of Education, Science, and Technology, Republic of Korea. The authors thank Dr. Radhakrishna Para for meticulous assistance with paper preparation.

References

[1] A. Nowak-Wegrzyn and H. A. Sampson, “Future therapies for food allergies,” *Journal of Allergy and Clinical Immunology*, vol. 127, no. 3, pp. 558–573, 2011.
[2] A. M. Branum and S. L. Lukacs, “Food allergy among children in the United States,” *Pediatrics*, vol. 124, no. 6, pp. 1549–1555, 2009.
[3] S. A. Bock, A. Muñoz-Furlong, and H. A. Sampson, “Further fatalities caused by anaphylactic reactions to food, 2001–2006,” *Journal of Allergy and Clinical Immunology*, vol. 119, no. 4, pp. 1016–1018, 2007.
[4] R. S. Kagan, “Food allergy: an overview,” *Environmental Health Perspectives*, vol. 111, no. 2, pp. 223–225, 2003.
[5] J. Wang and H. A. Sampson, “Food allergy: recent advances in pathophysiology and treatment,” *Allergy, Asthma and Immunology Research*, vol. 1, no. 1, pp. 19–29, 2009.
[6] M. J. Bevan, “Understand memory, design better vaccines,” *Nature Immunology*, vol. 12, no. 6, pp. 463–465, 2011.
[7] K. Tokoyoda, A. E. Hauser, T. Nakayama, and A. Radbruch, “Organization of immunological memory by bone marrow stroma,” Nature Reviews Immunology, vol. 10, no. 3, pp. 193–200, 2010.

[8] M. A. Daniels and E. Teixeiro, “The persistence of T cell memory,” Cellular and Molecular Life Sciences, vol. 67, no. 17, pp. 2863–2878, 2010.

[9] K. Tokoyoda, S. Zehentmeier, A. N. Hegazy et al., “Professional memory CD4^+ T lymphocytes preferentially reside and rest in the bone marrow,” Immunity, vol. 30, no. 5, pp. 721–730, 2009.

[10] H. Li, J. Liu, A. Carville et al., “Durable mucosal simian immunodeficiency virus-specific effector memory T lymphocyte responses elicited by recombinant adenovirus vectors in rhesus monkeys,” Journal of Virology, vol. 85, no. 21, pp. 11007–11015, 2011.

[11] R. Ahmed and R. S. Akondy, “Insights into human CD8^+ T-cell memory using the yellow fever and smallpox vaccines,” Immunology and Cell Biology, vol. 89, no. 3, pp. 340–345, 2011.

[12] R. Stephens and J. Langhorne, “Priming of CD4^+ T cells and development of CD4^+ T cell memory; lessons for malaria,” Parasite Immunology, vol. 28, no. 1-2, pp. 25–30, 2006.

[13] N. P. Birmingham, S. Parvataneni, H. M. A. Hassan et al., “An adjuvant-free mouse model of tree nut allergy using hazelnut as a model tree nut,” International Archives of Allergy and Immunology, vol. 144, no. 3, pp. 203–210, 2007.

[14] S. Parvataneni, B. Gonipeta, R. I. Tempelman, and V. Gangur, “Development of an adjuvant-free cashew nut allergy mouse model,” International Archives of Allergy and Immunology, vol. 149, no. 4, pp. 299–304, 2009.

[15] B. Gonipeta, S. Parvataneni, P. Paruchuri, and V. Gangur, “Long-term characteristics of hazelnut allergy in an adjuvant-free mouse model,” International Archives of Allergy and Immunology, vol. 152, no. 3, pp. 219–225, 2010.

[16] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, “Protein measurement with the Folin phenol reagent,” The Journal of Biological Chemistry, vol. 193, no. 1, pp. 265–275, 1951.

[17] L. Navuluri, S. Parvataneni, H. Hassan, N. P. Birmingham, C. Kelly, and V. Gangur, “Allergic and anaphylactic response to sesame seeds in mice: identification of Ses i 3 and basic subunit of 11s globulins as allergens,” International Archives of Allergy and Immunology, vol. 140, no. 3, pp. 270–276, 2006.

[18] X. M. Li, D. Serebrisky, S. Y. Lee et al., “A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses,” Journal of Allergy and Clinical Immunology, vol. 106, no. 1, pp. 150–158, 2000.

[19] P. W. Price and J. Cerny, “Characterization of CD4^+ T cells in mouse bone marrow—I. Increased activated/memory phenotype and altered TCR V\(\beta\) repertoire,” European Journal of Immunology, vol. 29, no. 3, pp. 1051–1056, 1999.

[20] Y. Yamashita, S. W. Hooker, H. Jiang et al., “CD73 expression and fn5-dependent signaling on murine lymphocytes,” European Journal of Immunology, vol. 28, no. 10, pp. 2981–2990, 1998.

[21] S. M. Anderson, M. M. Tomayko, A. Ahuja, A. M. Haberman, and M. J. Shlomchik, “New markers for murine memory B cells that define mutated and unmutated subsets,” Journal of Experimental Medicine, vol. 204, no. 9, pp. 2103–2114, 2007.

[22] B. Gonipeta, S. Parvataneni, R. J. Tempelman, and V. Gangur, “An adjuvant-free mouse model to evaluate the allergenicity of milk whey protein,” Journal of Dairy Science, vol. 92, no. 10, pp. 4738–4744, 2009.

[23] M. K. Slifka and R. Ahmed, “Long-term antibody production is sustained by antibody-secreting cells in the bone marrow following acute viral infection,” Annals of the New York Academy of Sciences, vol. 797, pp. 166–176, 1996.

[24] M. K. Slifka, M. Matloubian, and R. Ahmed, “Bone marrow is a major site of long-term antibody production after acute viral infection,” Journal of Virology, vol. 69, no. 3, pp. 1895–1902, 1995.

[25] F. Sallusto, D. Lenig, R. Förster, M. Lipp, and A. Lanzavecchia, “Two subsets of memory T lymphocytes with distinct homing potentials and effector functions,” Nature, vol. 401, no. 6754, pp. 708–712, 1999.

[26] N. Mojtabavi, G. Dekan, G. Stingl, and M. M. Epstein, “Long-lived Th2 memory in experimental allergic asthma,” Journal of Immunology, vol. 169, no. 9, pp. 4788–4796, 2002.

[27] L. F. Thompson, “Ecto-5'-nucleotidase can provide the total purine requirements of mitogen-stimulated human T cells and rapidly dividing human B lymphoblastoid cells,” Journal of Immunology, vol. 134, no. 6, pp. 3794–3797, 1985.