

3508 AB Utrecht
The Netherlands

^bJulius Center for Health Sciences and Primary Care
University Medical Center Utrecht
Utrecht, The Netherlands

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Type 2 natural killer cells in asthma?

To the Editor:

I read the article by Wei et al, "Involvement of human natural killer cells in asthma pathogenesis: Natural killer 2 cells in type 2 cytokine predominance,"¹ with great interest. My concerns in this study have to do with the techniques used for studying intracellular cytokines in natural killer (NK) cells using flow cytometry (FCM). I also want to discuss recent information about the quite new NK2 cells and their role in asthma and atopic diseases.

The FCM histograms in Figs 1 and 4 do not explain differences between groups (healthy/asthmatic and no treatment/IFN- γ +anti-IL-4, respectively). Freezing and thawing procedures might have played a major role in discrepancies noted between the FCM and RT-PCR data in this study. FCM is the most widely used method for evaluation of lymphocyte subsets and cytokines. Nevertheless, many technical factors, including cryopreservation, might influence the assessment of lymphocyte subsets and their functions.^{2,3} NK and lymphokine-activated killer cells exhibit reduced activity after thawing, suggesting a change in their cytokine profile.^{4,5} Cryopreservation and exposure to the cryoprotectant dimethyl sulfoxide was also shown to modify chronic lymphocytic leukemia, non-Hodgkin's lymphoma, and acute myeloid leukemia phenotypes.^{6,7} In 5 of 7 histo-

grams in Fig 1, A, there are no distinct populations showing IL-4 expression undoubtedly in the asthmatic group compared with the histograms below showing apparent IFN- γ expressions of the healthy group. Basically, the experimental histograms in Fig 1, A, seem to be not significantly different from the controls. As an FCM user, I am not sure how the authors determined that the differences between the healthy and asthmatic subject histograms were significant. Moreover, in the histograms of Fig 4, they show an increase from 4.13% to 8.33% in IFN- γ expression and a decrease from 5.22% to 1.62% in IL-4 expression. First of all, a 3% to 4% difference in the best histograms with FCM is a minimal and insignificant finding and could be easily an artifact. Second, the authors did not keep the same gating in their histograms for evaluating IL-4 expression. The vertical axis line of histogram 1 is between 10^1 and 10^2 but greater than 10^2 in the second histogram. This ignores the standard rule in FCM, which states that the same gate should be maintained for all. Evaluations of histograms with this quality of FCM work made me question the results of this study.

I also came across many writing errors in the text, such as "IFN γ " instead of "IFN- γ " in Figs 1, 2, and 3 and the misplacement of "IFN- γ " and "IL-4" in Fig 5, and there was no explanation for the abbreviation "LT" in Fig 2 and elsewhere.

In addition to eosinophils and mast cells, although the role of the type 2 CD8⁺ cell and its contribution to inflammation in asthma⁸ has been known for a while, that of NK2 cells and their role in atopic diseases and asthma are quite new. However, this is indeed not the first article indicating a possible role of NK2 cells in asthma. In 2003, Wingett and Nielson⁹ described an NK2 subset with the potential to increase asthma exacerbation in human subjects, reversing the cyclic AMP inhibitory effects on T-cell CD40 ligand. Moreover, increased IL-4 and low IL-12 levels in asthmatic patients are thought to be responsible for this NK2 bias.¹⁰ Just recently, NK2 cells were also found to be increased in eczema, with significantly high IL-5/IL-13-producing NK cells in atopic patients.¹¹ However, whether NK2 cells represent a truly distinct subset of NK cells or an immature stage of NK cell differentiation is not certain. Even though some believe these are 2 different populations, some consider these phenotypes to be reflections of multiple changing of faces of an adapting and developing cell.^{12,13} Although the roles of these cells are also uncertain, mostly they seem to be capable of regulating T-cell function and T_H2 cytokine production. For instance, asthmatic NK cells were demonstrated to possess unique regulatory attributes affecting CD40 ligand expression.⁹ Furthermore, NK1 cells were demonstrated to inhibit IgE production by B cells through IFN- γ ¹¹ and reduce lung eosinophilia in respiratory syncytial virus infection.¹⁴ In brief, the association of NK2 bias with asthma exacerbation might indicate that immunologic interventions preventing such an NK bias might benefit patients with asthma.

Recent increasing data in the literature show new anti-inflammatory effects of macrolides, suggesting that their

effects are underappreciated.¹⁵ The authors referred to the use of erythromycin in a study in which no differences were found between atopic and nonatopic patients.¹⁶ This might indicate that the improvement in PC₂₀ might have been a consequence of the known anti-inflammatory effects of erythromycin rather than its effect on NK2 cells. Although this study is somewhat important in suggesting a role for NK2 cells in asthma, further studies are needed for clarification and verification.

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Öner Özdemir, MD
Department of Pediatrics
Division of Allergy/Immunology
1542 Tulane Avenue, T8-1
LSUHSC-NO
New Orleans, LA 70112

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Reply

To the Editor:

We thank Dr   zdemir for his interest and insightful comments¹ on our report.² Summarily, he has 3 points, which we will respond to one by one.

Cryopreserved and thawed lymphocytes are widely used instead of fresh lymphocytes to avoid test-to-test variability in studies of fluctuation as a function of time. We agree that more attention should be paid to the techniques used for studying intracellular cytokines in the freezing-thawing of lymphocytes using flow cytometry (FCM). Although there are a few reports to indicate that the freezing-thawing procedure might influence the assessment of lymphocyte subsets and their functions, as commented on by Dr   zdemir,¹ the well-validated freezing-thawing procedure is used by many laboratories worldwide.³⁻⁶ The cryopreserved dendritic cells, lymphocytes, and macrophages from fresh PBMCs have been used for subset assessments, functional studies (including intracellular cytokine measurement), and adoptive transfer for more than 2 decades.⁷⁻⁹ To maximally exclude the influence of thawing on lymphocytes, as shown in our report, we froze the PBMCs from both asthmatic patients and healthy individuals at each sample harvesting, which might reduce the effect of this artifact potential to its lowest rate.

Dr   zdemir also questioned the differences between groups (healthy/asthmatic, and no treatment/IFN- γ + anti-IL-4, respectively). We do not think that his comment that a 3% to 4% difference in the best histograms with FCM is a minimal and insignificant finding and could be easily an artifact is correct. On the one hand, the difference in our study came from the statistics between groups. On the other hand, in some conditions, even 0.1% to 0.5% differences are significant in FCM analysis, depending on the content and change of the observed cell population in whole cells. We agree that the same gating in histograms is critical for evaluating IL-4 expression. The reason why the vertical axis line of histogram 1 is between 10¹ and 10² but greater than 10² in the second histogram in our Fig 4 is that we think regulated gating clearly separates an independent cell population (IL-4 expression) rather than establishing an artificial cutoff into 2 population. This manipulation is sometimes used in FCM work in other reports.

We thank Dr   zdemir for his generally positive evaluation of our study's importance. However, we do not