

Natural Killer Cells Accumulate in Lung-Draining Lymph Nodes and Regulate Airway Eosinophilia in a Murine Model of Asthma

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Received 21 December 2009; Accepted 25 April 2010

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Abstract

Increasing evidence suggests a key role for the innate immune system in asthma development. Although the role of Natural Killer (NK) cells in allergic asthma is poorly known, modifications of the blood NK cell populations have been found in asthmatic and/or allergic patients. Their repartition and activation status in the inflammatory (lungs) and the regulatory (draining lymph nodes) sites of the allergic reaction is unknown. The aim of our study was to monitor NK cell migration pattern and activation status and to investigate the consequences of NK cell depletion during allergic airway reaction in a mouse model. Ovalbumin sensitization and challenges of BALB/cByJ mice had no effect on the total number of lung NK cells but significantly decreased the number of most mature NK cells and increased the level of the activation marker CD86. In the lung-draining mediastinal lymph nodes, ovalbumin sensitization and challenges led to increased number of NK cells, and more precisely, immature NK cells and increased expression of CD86. Ovalbumin-sensitized mice also exhibited increased percentage of proliferating NK cells in lung-draining mediastinal lymph nodes. Anti-ASGM1 antibody treatment depleted most NK cells and decreased bronchoalveolar lavage eosinophilia but did not modify airway responsiveness. Altogether, our study shows that pulmonary allergic sensitization induces modification in the NK cell compartment at the inflammatory and regulatory sites and suggests that NK cells may participate in the regulation of the asthmatic response and, more particularly, to the allergic airway eosinophilia.

Introduction

Allergic asthma is a chronic inflammatory disease associated with airway obstruction, hyperreactivity and inflammation. Mucosal eosinophilic inflammation is one of the distinctive features of asthma, and the particular T helper type 2 (Th2) phenotype of allergic patients favours it. In general, the clinical severity of asthma correlates well with the degree of inflammation [1]. Numerous studies suggest a key role for the innate immune system in asthma development [2]. Nonetheless, whereas the role of the adaptive immune response has been well characterized, the role of the innate immune system in the pathogenesis of asthma is less clear. In particular, the role of natural killer (NK) cells

in asthma is unknown although several studies suggest their potential involvement [3–5].

NK cells are key weapons of the innate immune system and are primarily described for their antimicrobial and antitumoral activities [6]. Their development mainly occurs in bone marrow (BM) and alternatively in lymph nodes and thymus [7–9]. NK cells are widely distributed throughout the body in both humans and mice, with a more important frequency in non-lymphoid organs compared to lymphoid organs [10]. At steady state, NK cells represent about 10% of lung lymphocytes in both human and mouse [10, 11]. In mice, NK cell frequency was found to be the highest in lungs compared to other organs [10]. At steady state, NK cells are also present at a very low frequency (<1%) in the human and mouse

lymph nodes [10, 12–14]. After immunization or infection, mouse NK cells are enriched in the paracortex of draining lymph nodes, where they can influence subsequent adaptive immune response [12, 14, 15]. Murine NK cells can be divided into four subsets according to the level of CD27 and CD11b expression. Highly immature CD27^{low}CD11b^{low}, immature CD27^{high}CD11b^{low}, mature CD27^{high}CD11b^{high} and most mature CD27^{low}CD11b^{high} NK cells develop sequentially. This developmental programme appears to be associated with a progressive acquisition of NK cell effector functions [16, 17]. Defects in NK cell functions were shown in peripheral blood NK cells from asthmatic allergic patients, with increased cytotoxicity and predominance of NK2 cells (producing IL-5 and IL-13) over NK1 cells (producing IFN- γ), compared to healthy non-allergic donors [4, 5]. More recently, the crosstalk between NK cells and dendritic cells was shown to be less efficient with NK cells isolated from allergic patients compared to non-allergic patients. This defect led to reduced capacity of NK cells to produce IFN- γ in response to dendritic cell stimulation [3]. In a mouse model of allergic airway inflammation, NK cell depletion obtained after anti-NK1.1 antibody administration before immunization inhibited lung eosinophilia, CD3⁺ T-cell infiltrate and ovalbumin (OVA)-induced levels of IL-4, IL-5 and IL-12 in bronchoalveolar lavage (BAL) of C57BL/6 mice [18]. However, NK1.1 is not solely expressed by NK cells, but also found on some T cells, especially most Natural Killer T (NKT) lymphocytes, thus making difficult the interpretation of this study. Indeed, NKT cells were found to play a role in the development of experimental asthma [19, 20].

The aim of our study was first to monitor the number and the phenotype of NK cells in the lungs as site of inflammation and the lung-draining mediastinal lymph nodes (MLN) as site of regulation during allergic airway inflammation. We secondly assessed the consequences of NK cell depletion in another mouse model of allergic asthma using a different NK cell-depleting antibody, without effect on NKT cell subset [21]. In a model of OVA sensitization in BALB/cByJ mice, we showed that, in parallel to airway inflammation and airway hyperresponsiveness (AHR), OVA sensitization increased NK cell activation in the lungs and affected NK cell number, subset distribution, activation status and proliferation in MLN. Moreover, NK cell depletion decreased lung eosinophilia, without modifying AHR. Therefore, our results suggest that NK cells may participate in the regulation of the asthmatic response.

Materials and methods

Sensitization and airway challenges. Seven-week-old female BALB/cByJ were obtained from Iffa Credo (L'Arbresle,

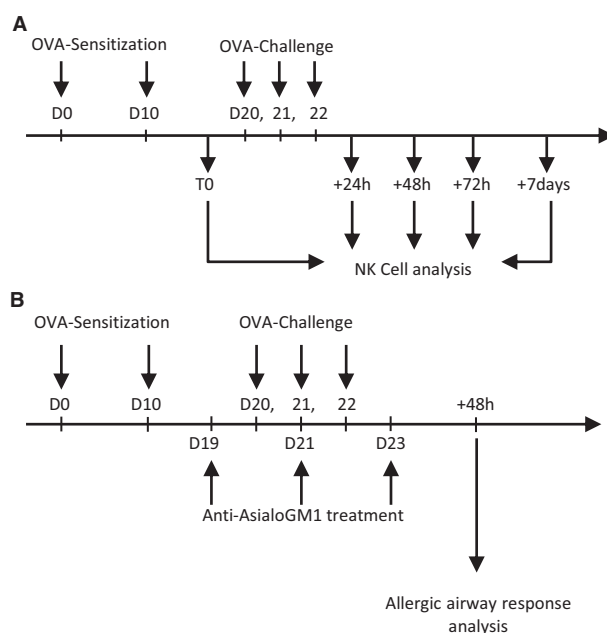


Figure 1 Protocols of mouse sensitization, anti-asialo-GM1 treatment and natural killer (NK) cell analysis. Female BALB/cByJ mice were sensitized to ovalbumin (OVA) by two i.p. injections of OVA-alum, followed by three OVA aerosols. Control mice were administered with phosphate buffer saline instead of OVA. (A) NK cell numbers were measured before (T0) or at various times after the last OVA challenge (24, 48, 72 h or 7 days). Phenotype and proliferation analyses were performed at 48 h. (B) Anti-asialo-GM1 antibody was injected i.v. 1 day before the first OVA challenge and then every other day until analysis. Analysis was performed 48 h after the last OVA aerosol.

France) and were maintained on OVA-free diet. All procedures were approved by regional ethical committee. Mice were sensitized by i.p. injection of 20 μ g OVA (Grade V; Sigma-Aldrich, St Louis, MO, USA) emulsified in 100 μ l of aluminium hydroxide (Reheis, Berkeley Heights, NJ, USA) on days 0 and 10 and challenged with OVA [2% in Phosphate Buffer Saline (PBS)] by aerosol on days 20, 21 and 22 (afterwards called OVA mice) (Fig. 1). The aerosol administrations were performed for 20 min using an ultrasonic nebulizer (Ultramed, Medicalia, Italy). Control group (afterwards called PBS mice) received i.p. injection of PBS emulsified in 100 μ l of aluminium hydroxide and was challenged by PBS aerosols.

Tissue single-cell suspension. Before (T0) or at various time after the last PBS or OVA challenge (24, 48, 72 h or 7 days), lungs, MLN, blood, BM and spleen were harvested (Fig. 1A), and cells were isolated as followed on 4–7 mice per group at each time point.

Lungs were perfused with PBS containing 0.5 mM EDTA, removed and cut into pieces. After incubation in 3 ml of complete RPMI (Lonza, Basel, Switzerland) (RPMI 1640 supplemented with 10% foetal calf serum, 100 IU/ml penicillin, 100 mg/ml streptomycin, 1% Ticarpen and 2 mM L-glutamine) containing type IV

collagenase (0.5 mg/ml; Serlabo, Paris, France) and DNase I (2 UI/ml; Roche Diagnostics, Meylan, France) for 60 min at 37 °C, the digested lungs were shorn with a sterile 20-gauge needle, and the red blood cells were lysed using a buffer containing 155 mM NH₄Cl, 10 mM KHCO₃ and 0.5 mM EDTA diluted in sterile water for 15 min on ice. Pellets were resuspended in Percoll[®] 33% (GE Healthcare, Amersham, UK) at room temperature and centrifuged during 20 min at 2300 g. Cells were resuspended in PBS, filtered through 100- μ m porous nylon mesh (Saati France, Saily Saillisel, France) and counted. An average of 12×10^6 cells was usually obtained from lungs.

MLN and spleens were homogenized in a Tenbroeck tissue grinder. Cell recovery from MLN was 2.5×10^6 and 5×10^6 from PBS and OVA mice, respectively. Spleen cells and blood peripheral cells were obtained after one or two successive lyses of red blood cells, respectively. BM cells were isolated from femurs. For all organs, cells were filtered through 100- μ m porous nylon mesh, resuspended in PBS and counted immediately after separation on hemocytometer.

Surface stainings for flow cytometry analysis. Cells isolated from lungs and MLN were resuspended at a concentration of 1×10^7 cells/ml in PBS containing 2% foetal calf serum. A total of 10^6 cells were incubated with rat anti-mouse CD16/CD32 (2.5 μ g/ml, BD Pharmingen, San Diego, USA) for 10 min on ice. Surface stainings (30 min on ice) were performed using fluorescein-, phycoerythrin-, cyochrome- or allophycocyanin-conjugated monoclonal antibodies (mAbs) against CD3, CD49b, CD27, CD11b, CD69 and CD86 (eBiosciences, Montrouge, France) and the matched IgG isotypes according to the manufacturer's instructions. Stained cells were immediately analysed by flow cytometry (FACScalibur[®]; BD Biosciences, San Diego, CA, USA). NK cells were identified as CD49b⁺CD3⁻ cells. Six mice per group were analysed.

Measurement of BrdU incorporation. Mice were given one i.p. injection of 1.5 mg BrdU (BD Pharmingen, San Diego, CA, USA) on day 23. Twelve hours after the injection, BM, MLN and blood were harvested and cells were isolated as previously described. Cells were resuspended at a concentration of 1×10^7 cells/ml in PBS containing 2% of serum from foetal calf, horse, rat and mouse, 10 μ g/ml rat anti-mouse CD16/CD32 and 0.09% sodium azide. Surface stainings (30 min on ice) of 10^6 cells were performed using APC-H7, Peridin-Chlorophyll-protein complex, PacificBlue, phycoerythrin, phycoerythrin-cyochrome7 or Alexa Fluor 647-conjugated mAbs against CD19, CD5, Gr-1, CD122, CD49b and NKp46 (Becton Dickinson for anti-CD19 and anti-CD5 mAbs and eBiosciences for the other mAbs). After fixation and permeabilization, cells were treated with DNase and stained with FITC anti-BrdU mAb, according to manufacturer instructions. BrdU incorporation for NK cells was measured by flow cytome-

try (FACSaria; BD Biosciences). NK cell precursors (NKp) were defined as Lineage (Lin)⁻ (CD5⁻CD19⁻Gr1⁻) CD122⁺CD49b⁻NKp46⁻, and NK cells were defined as Lin⁻CD122⁺CD49b⁺NKp46⁺. A total of six or seven mice were analysed in the PBS or OVA group, respectively.

In vivo NK cell depletion. NK cell depletion was performed by i.v. injections of rabbit anti-asialo ganglio-N-tetraosylceramide (ASGM1) antibodies (400 μ g/mouse; Wako, Neuss, Deutschland). The first injection was performed 1 day before the first OVA challenge and every other day until analysis (Fig. 1B). Preliminary experiments showed that this protocol resulted in the best depletion of NK cells, whilst no depletion of F4/80⁺, IA^{d+}/CD11c⁺, CD19⁺ and CD3⁺ cells was observed in lungs. Similar depletion was measured in lungs, MLN and spleen. Therefore, for the following experiments, the depletion was checked for each individual mouse by measuring NK cell number in the spleen. It averaged 72.6% at the time of the analysis ($2.53 \pm 0.27\%$ of NK cells in rabbit Ig-treated mice compared to $0.69 \pm 0.08\%$ in anti-ASGM1 antibody-treated mice). Non-depleted groups received rabbit immunoglobulins (Ig) as control antibodies (400 μ g/mouse; Sigma-Aldrich) by i.v. injections using the same protocol. A total of 9–11 mice per group were analysed.

Lung resistance measurement. Lung resistance was assessed using Flexivent[®] (Scireq[®], Montreal, Canada). Mice were anesthetized [5 ml/kg body weight of 10% medetomidine (Pfizer, Paris, France) and 10% ketamine (Merial, Lyon, France)], paralysed [5 ml/kg pancuronium bromide 1% (Organon)] and immediately intubated with an 18-gauge catheter, followed by mechanical ventilation. Respiratory frequency was set at 150 breaths/min with a tidal volume of 0.2 ml, and a positive-end expiratory pressure of 2 ml H₂O was applied. Mice were exposed to nebulized PBS followed by increasing concentrations of nebulized metacholine (3–25 mg/ml in PBS) (Sigma-Aldrich) using an ultrasonic nebulizer (Aeroneb, Aerogen, Galway, Ireland). For each dose, 10 cycles of nebulization and measurements were performed. Nebulization was conducted during the first cycle and consisted of 20 puffs per 10 s, with each puff of aerosol delivery lasting 10 ms. For each cycle, measurements were obtained for 15 s followed by ventilation for 5 s. Baseline resistance was restored before administration of the subsequent doses of methacholine. For each dose, the maximum resistance value measured was taken.

BAL cellular composition. Forty-eight hours after the last OVA challenge and immediately after the measurement of lung resistance, lungs were lavaged via the tracheal cannula with 1 ml of PBS. Total leucocyte numbers were counted, cytocentrifuged (Shandon cyospin 4; Thermo Fisher Scientific Inc., Waltham, MA, USA) and stained with May Grünwald Giemsa (Diff-Quik[®]; Dade Behring, Newark, DE, USA). Cells were identified as macrophages, eosinophils, neutrophils and lymphocytes by standard

haematological procedures, and at least 200 cells were counted under $\times 400$ magnification.

Measurement of OVA-specific serum Ig. The OVA-specific serum IgG1 and IgG2a were measured by ELISA as previously described [22]. Briefly, 96-well plates (Corning Incorporated, Corning, NY, USA) were coated with OVA (100 $\mu\text{g}/\text{ml}$). After addition of serum samples, OVA-specific IgG1 and IgG2a were detected using horseradish peroxidase-labelled goat anti-IgG1 and anti-IgG2a (Southern Biotechnology, Birmingham, AL, USA). For OVA-specific IgE titration, 96-well plates (Corning Incorporated) were coated with purified rat anti-mouse IgE (2 $\mu\text{g}/\text{ml}$, clone R35-72; BD Pharmingen). After addition of serum samples, OVA-specific IgE were detected by the addition of biotinylated OVA followed by incubation with streptavidine-horseradish peroxidase (Invitrogen, Carlsbad, CA, USA). The OVA-specific Ig titres of the samples were related to pooled standards that were generated in the laboratory and expressed as ELISA units per ml (EU/ml).

Statistical analysis. Values of all measurements were expressed as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using GRAPHPAD Prism 4 software (GraphPad Software, Inc., La Jolla, CA, USA). Non-parametric Mann–Whitney or Kruskal–Wallis tests were used to calculate significance levels for most measurements. For some measurements, values were compared using the two-way ANOVA. When statistical significance was observed, differences were subsequently analysed using the Bonferroni test. Values of $P < 0.05$ were considered statistically significant.

Results

Increase in $\text{CD11b}^{\text{low}}$ NK cells in the MLN after OVA sensitization and challenges

We first investigated the kinetics of NK cell numbers in the different compartments where the allergic reaction occurs: BAL, lungs and lung-draining MLN, at different time points during the treatment with OVA. No NK cells were detected in BAL of OVA or PBS mice at any time (data not shown). OVA sensitization and airway challenges did not modify the number of NK cells in lungs (Fig. 2A) whilst decreasing the NK cell percentage significantly at 7 days ($8.0 \pm 0.4\%$ in OVA mice and $10.6 \pm 0.9\%$ in PBS mice) compared to the PBS group. In contrast to the lungs, the number of NK cells in the MLN significantly increased 48 h after the last OVA challenge ($26.5 \pm 3.9 \times 10^3$ compared to $4.4 \pm 1.0 \times 10^3$ in the PBS mice) (Fig. 2B). This increase was specific to MLN, related to numbers but not percentages and was not observed in the mesenteric lymph nodes (data not shown).

NK cell subsets, characterized according to their level of CD27 and CD11b expression, were identified in lungs and MLN 48 h after the last OVA challenge, time corre-

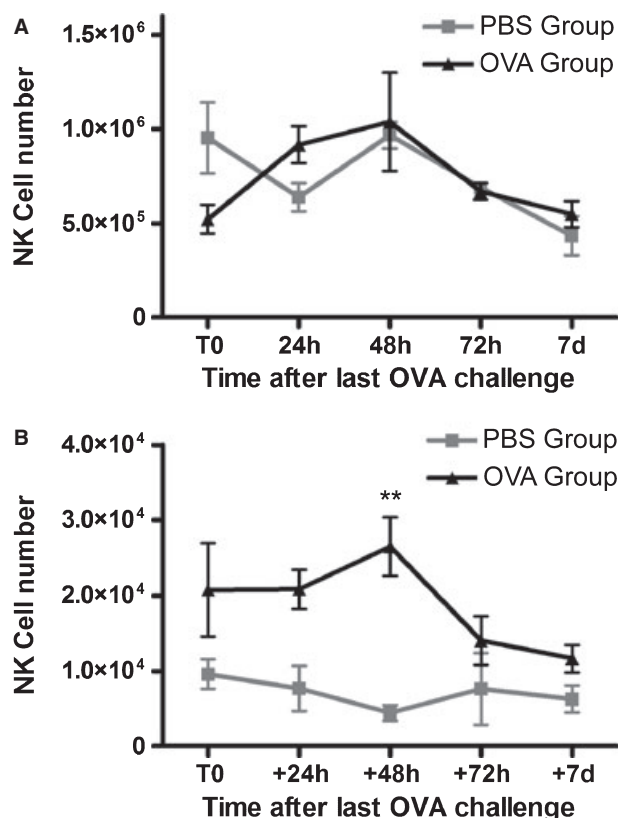


Figure 2 Effect of ovalbumin (OVA) sensitization and OVA challenges on natural killer (NK) cell numbers in lungs and mediastinal lymph nodes (MLN). Before (T0) or at various times after the last OVA challenge (24, 48, 72 h or 7 days), cell suspension was obtained from lungs (A) and MLN (B) and stained using anti-CD49b (anti-DX5) and anti-CD3 antibodies. Kinetics of NK cell numbers $\pm$ SEM is shown. $n = 4$ –7 mice per group.

sponding to the increase in total NK cell number in MLN. In the lungs, $\text{CD27}^{\text{low}}\text{CD11b}^{\text{high}}$ NK cells were found to be the main subset in PBS and OVA mice. OVA sensitization and challenges significantly decreased their number ($6.3 \pm 0.9 \times 10^5$ compared to $8.0 \pm 0.5 \times 10^5$ in PBS mice) but did not modify their percentage (Fig. 3A, B). The percentage of CD86-expressing lung NK cells ($25.1 \pm 1.8\%$ compared to $18.8 \pm 1.0\%$ in PBS mice) and CD86 expression (2.3 ± 0.3 compared to 1.5 ± 0.1 ΔMFI in PBS mice) from OVA mice was significantly increased (Fig. 3C), and a trend towards an increased expression of the early activation marker CD69 was observed (Fig. 3D). These results suggest that OVA sensitization and challenges led to NK cell activation in the lungs.

In the MLN, OVA sensitization and challenges led to a significant increased number of the two subsets of immature NK cells ($\text{CD27}^{\text{low}}\text{CD11b}^{\text{low}}$: $8.0 \pm 0.6 \times 10^3$ and $13.4 \pm 2.1 \times 10^3$, $\text{CD27}^{\text{high}}\text{CD11b}^{\text{low}}$: $7.4 \pm 2.0 \times 10^3$ and $17.4 \pm 6.7 \times 10^3$ in PBS and OVA mice, respectively). No significant modification of mature NK

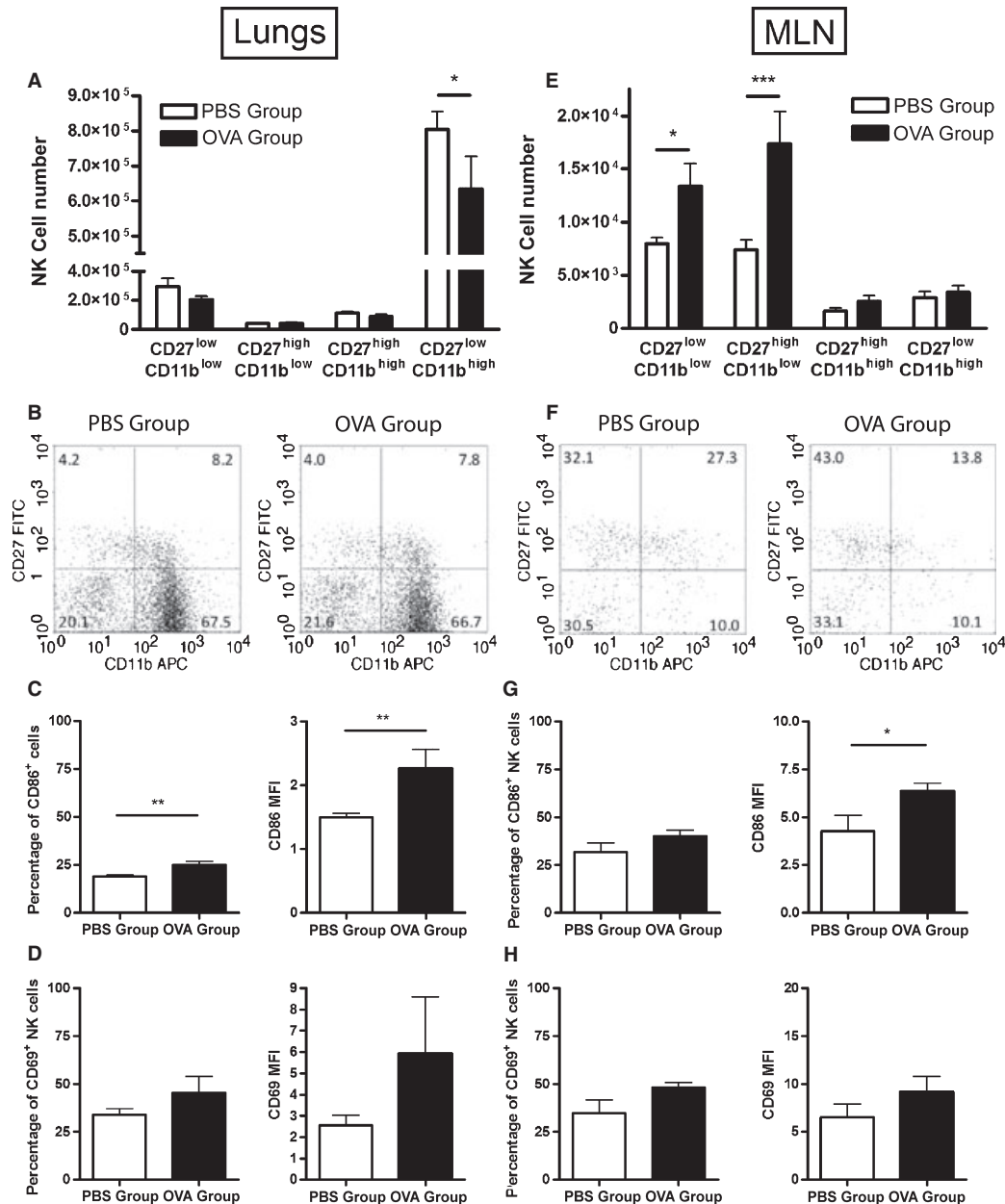


Figure 3 Effect of ovalbumin (OVA) sensitization and OVA challenges on natural killer (NK) cell subsets and activation in lungs and mediastinal lymph nodes (MLN). Cell suspension was obtained from lungs (A–D) and MLN (E–H) and stained using anti-CD49b and anti-CD3 antibodies. (A, B, E, F) Distribution of NK cell subsets according to the expression levels of CD27 and CD11b in the CD49b⁺CD3⁺ gate at 48 h ($n = 6$ per group). Results are shown as the mean cell number according to the subset $\pm$ SEM (A, E) and as representative dot plots with the percentage of each subset in quadrant (B, F). (C, D, G, H) Mean percentage and Δ MFI (Mean Fluorescence Intensity for mAb of interest–Mean Fluorescence Intensity for control mAb) $\pm$ SEM of CD86 and CD69 expression (C and G, D and H, respectively) on CD3⁺CD49b⁺ NK cells ($n = 6$ per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

cell number was observed (Fig. 3E). These results were concordant with a significant increase in the percentage of immature CD27^{high}CD11b^{low} NK cells ($43.2 \pm 3.1\%$ compared to $31.3 \pm 2.3\%$ in PBS mice) and a decrease in the percentage of mature CD27^{high}CD11b^{high} NK cells ($14.3 \pm 1.0\%$ compared to $21.1 \pm 2.1\%$ in PBS mice) (Fig. 3F). OVA sensitization and challenges did not mod-

ify the percentages of CD86- and CD69-expressing NK cells, nor the expression of CD69, but significantly increased the level of CD86 expression (Δ MFI = 6.4 ± 0.4 compared to 4.3 ± 0.8 in PBS mice), sign of recent activation (Fig. 3G–H).

Altogether, these data show that the number of immature NK cells increased in the MLN during the allergic

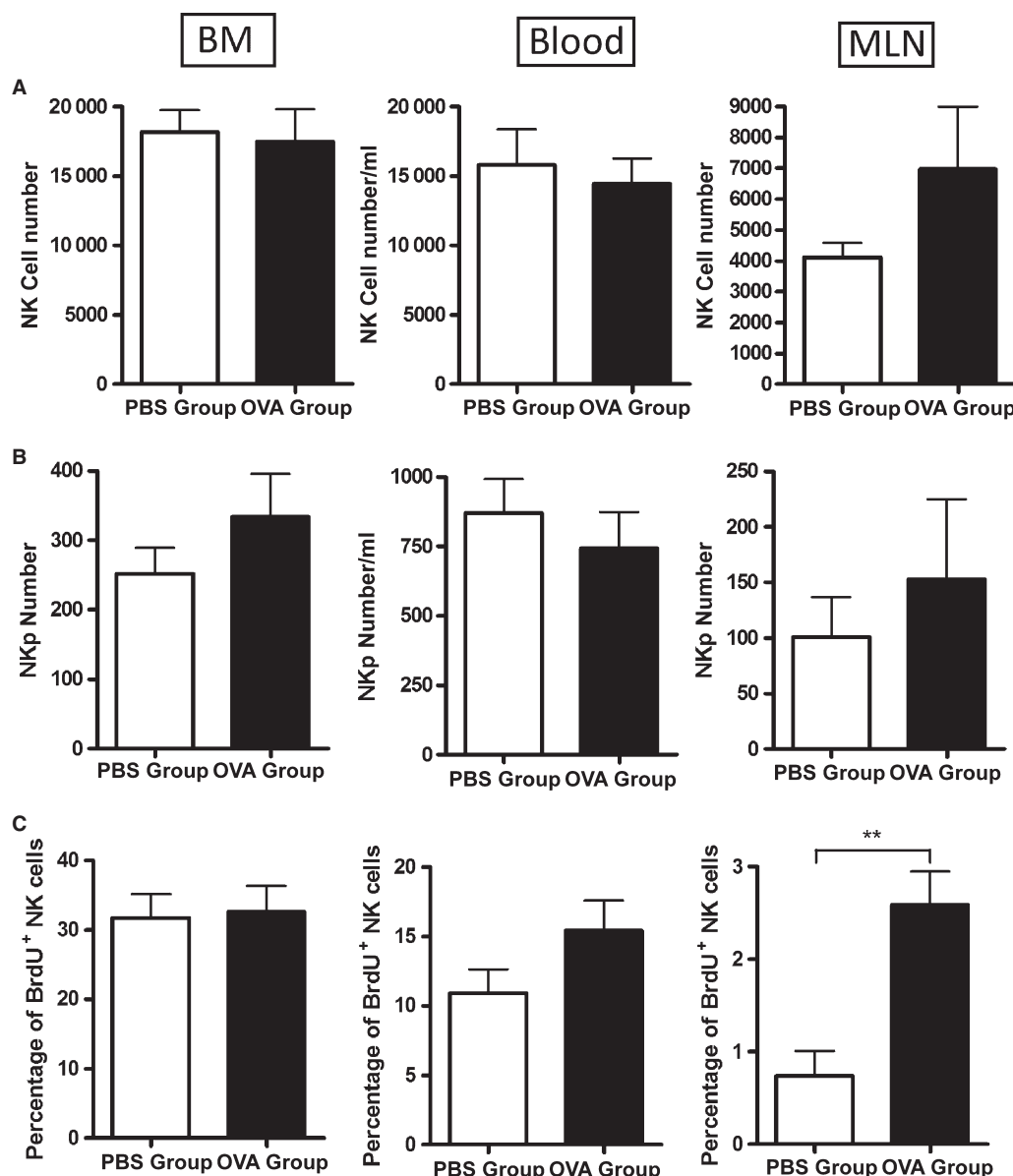


Figure 4 Effect of ovalbumin (OVA) sensitization and challenges on natural killer (NK) cell, NKp and proliferating NK cell number or percentage in bone marrow (BM), blood and mediastinal lymph nodes (MLN). Flow cytometry measurements were performed on cells isolated from phosphate buffer saline ($n = 6$) and OVA mice ($n = 7$) at 48 h. NK cells were defined as Lineage (Lin)[−] (CD5[−]CD19[−]Gr1[−]) CD122⁺CD49b⁺NKp46⁺ (A, C), and NKp as Lin[−]CD122⁺CD49b[−]NKp46[−] (B). Twelve hours after i.p. administration, BrdU incorporation was measured in BM, blood and MLN NK cells by flow cytometry. Results are expressed as cell numbers (A, B) or percentage of BrdU⁺ NK cells (C) $\pm$ SEM. ** $P < 0.01$. NK, natural killer.

reaction and that some NK cells showed signs of recent activation.

Increased percentage of proliferating NK cells in MLN upon OVA-induced airway inflammation

Most NK cells are believed to develop in the BM from NKp, but they may also develop in lymph nodes [7]. Hence, the increase in NK cells observed in MLN upon

airway inflammation may be attributable to *in situ* development and proliferation. Alternatively, they may be recruited from the circulation. To address these different possibilities, we compared the numbers of NK cells (Fig. 4A) and NKp (Fig. 4B) and the percentage of proliferating NK cells (analysed by BrdU incorporation) (Fig. 4C) in the BM, blood and MLN upon OVA-induced airway inflammation. Although NK cell number was increased in the MLN of OVA mice compared to

PBS mice, no modification in BM and blood was observed (Fig. 4A), suggesting that NK cells did not come from BM through blood. Although NKp number tended to increase in BM, no significant modifications were seen in blood and MLN (Fig. 4B), suggesting that increased NK cell number in the MLN was not caused by increased NK cell differentiation from NKp. Finally, the percentage of BrdU⁺ NK cells significantly increased in the MLN upon airway inflammation ($2.6 \pm 0.4\%$ compared to $0.7 \pm 0.3\%$ in PBS mice). Whilst no modification was observed in BM, the percentage of BrdU⁺ NK cells tended to increase in blood ($15.5 \pm 2.2\%$ compared to $10.9 \pm 1.7\%$ in PBS mice) (Fig. 4C), suggesting that proliferating NK cells may originate from MLN and/or blood.

Altogether, these results suggest that increased number of NK cells in MLN after allergic sensitization may result from NK cell proliferation or the recruitment of blood-proliferating NK cells.

Depletion of NK cells decreased BAL eosinophilia induced by OVA challenges

The earlier described modifications of NK cell subsets, migration and activation in the context of an allergic reaction suggest that NK may play a role in the allergic reaction. However, the previously published paper on NK cell depletion using NK1-1⁺ antibody remains inconclusive because of its concomitant effect on some T cell subsets including NKT cells; therefore, we sought to evaluate the role of NK cells in the pulmonary allergic reaction using an anti-ASGM1 antibody, which deplete NK cells without depleting NKT cells [21]. OVA sensitization led to a significant increase in airway responsiveness and BAL eosinophilia and an increase in serum OVA-specific IgE, IgG1 and IgG2a (Fig. 5). Baseline lung resistances were similar amongst all groups. Anti-ASGM1-treated OVA mice developed AHR similar to control antibody-treated OVA mice (Fig. 5A). However, NK cell depletion significantly decreased BAL eosinophilia ($0.8 \pm 0.3 \times 10^4$ in anti-ASGM1-treated OVA mice compared to $7.1 \pm 2.6 \times 10^4$ in control Ig-treated OVA mice) without modifying macrophage and lymphocyte numbers (Fig. 5B). No modification in the levels of serum OVA-specific IgE, IgG1 and IgG2a was found (Fig. 5C). Thus, these data suggest that NK cells may regulate the airway inflammation induced by priming and challenges with OVA.

Discussion

There is growing interest in the links between innate and adaptive immunity in allergic asthma. In this context, the information about the role of NK cells, a major component of innate immunity, is very scarce in the

pathophysiology of allergic asthma. Hitherto, no study has evaluated the kinetics and migratory behaviour of NK cells in allergic asthma. In our study, we observed that OVA sensitization and challenges increased the number of immature CD27^{low}CD11b^{low} and CD27^{high}CD11b^{low} NK cells in the MLN. Increased number of murine NK cells in draining lymph nodes has been previously shown after immunization or infection [12, 14, 15] but with a different distribution of NK cell subsets. In particular, immunization with lipopolysaccharide-pulsed dendritic cells led to an increase in all NK cell subsets in the draining lymph nodes at 48 h, with a preferential increase in mature CD27^{high}CD11b^{high} NK cells. After 6 days, immature CD27^{high}CD11b^{low} NK cells were predominant again [15]. The differences between the two studies may be explained in particular by the nature of the stimulus, infectious versus allergic, suggesting that NK cell subsets may be differentially activated by dendritic cells according to the initial stimulus.

NK cell number increase in the MLN may result from recruitment from the inflammatory site (lungs) or from BM through the blood. However, we found that, in lungs, only the number of the most mature CD27^{low}CD11b^{high} NK cells decreased, whereas in MLN, the increased NK cell number was associated with increased immature NK cell number. Moreover, no increase in NK cell number was observed in BM and blood. These results suggest that NK cells in MLN do not originate from lungs or BM. No significant increase in NKp number in MLN was observed, suggesting that increased number of immature NK cells in MLN do not differentiate from NKp in MLN. In contrast, proliferating NK cells were shown to significantly increase in MLN whilst they tend to increase in blood, supporting the hypothesis that OVA sensitization and challenges may induce NK cell proliferation in MLN. The recruitment of proliferating NK cells towards MLN cannot be excluded either.

The role of NK cells in the MLN during airway sensitization shall be further characterized. However, we can presently hypothesize that they may interact either with dendritic cells, as previously showed in other models [12, 14, 23], or directly with T cells [24]. We found that MLN NK cells from OVA mice expressed higher levels of CD86, molecule involved in the crosstalk between activated NK cells and CD4⁺ T cells [24]. We measured low levels of CD86 expression, which is in accordance with previous results showing low level of CD86 expression on IFN- γ producing killer dendritic cells, later demonstrated to be activated NK cells [25, 26]. Moreover, MLN of OVA mice exhibited increased number of immature NK cells, previously shown to express several transcripts associated with T cell activation [16].

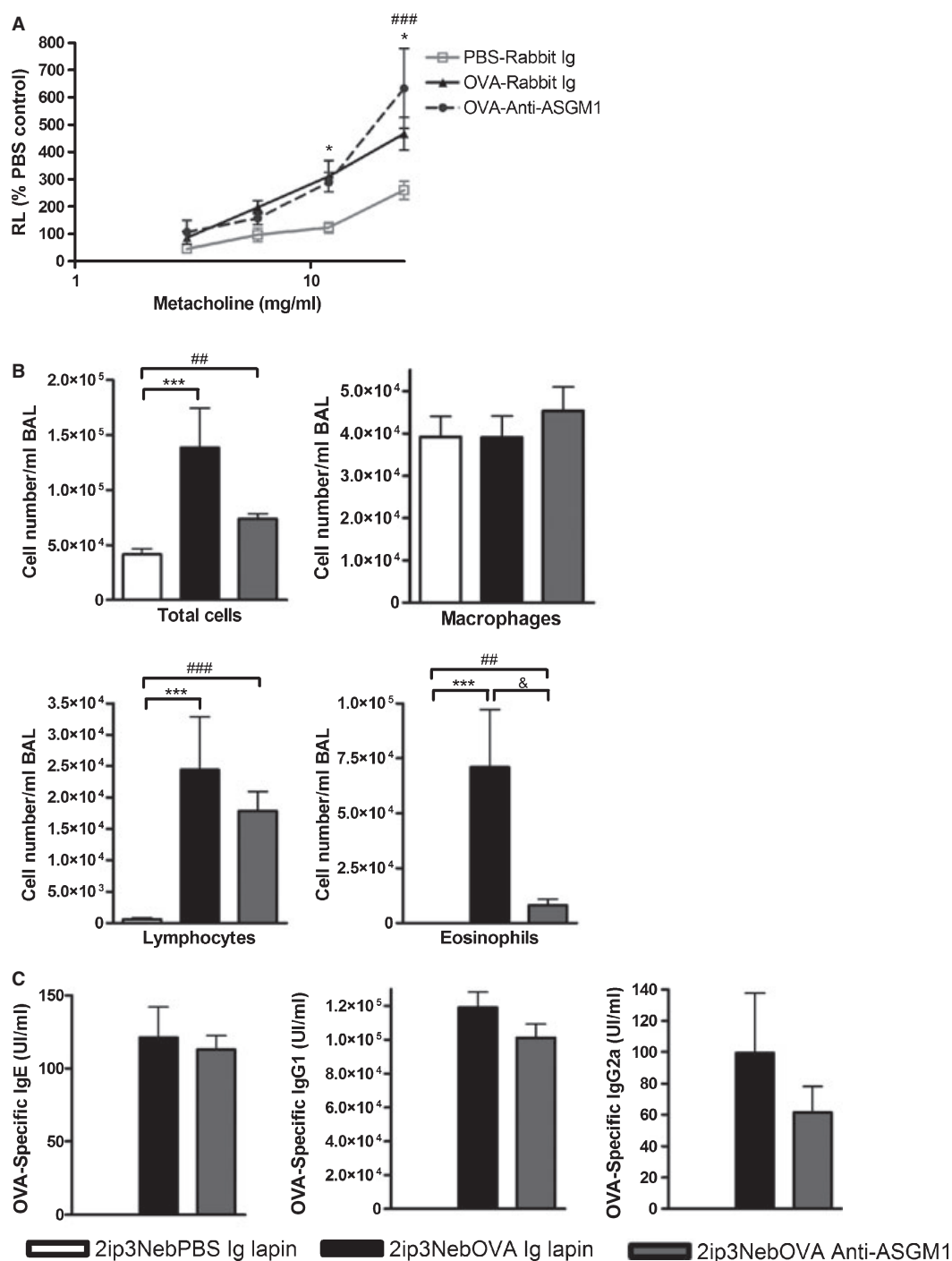


Figure 5 Anti-ASGM1 treatment does not modify AHR nor IgE, IgG1 and IgG2a but decreases BAL eosinophilia. Ovalbumin (OVA) or phosphate buffer saline (PBS) mice were treated with anti-ASGM1 or Rabbit Ig as described in Fig. 1. (A) Lung resistance (R_L) was measured after nebulization of increasing doses of metacholine for Ig-treated PBS mice (open square; $n = 11$), Ig-treated OVA mice (filled triangle; $n = 11$) and anti-ASGM1-treated OVA mice (filled circle; $n = 9$). Results are expressed as mean $\pm$ SEM of percentage of maximum R_L increase above PBS-induced R_L . (B) Total cell, macrophage, lymphocyte and eosinophil numbers in BAL were microscopically identified and counted after May Grünwald staining in Ig-treated PBS mice ($n = 13$), Ig-treated OVA mice ($n = 15$) and anti-ASGM1-treated OVA mice ($n = 12$). Results are expressed as mean $\pm$ SEM of cell number/ml. (C) OVA-specific IgE, IgG1 and IgG2a were measured by ELISA in Ig-treated PBS mice ($n = 11$ – 13), Ig-treated OVA mice ($n = 11$ – 15) and anti-ASGM1-treated OVA mice ($n = 9$ – 13). Results are expressed as mean $\pm$ SEM EU/ml. * $P < 0.05$ and *** $P < 0.001$ between Ig-treated PBS mice and Ig-treated OVA mice; & $P < 0.05$ between Ig-treated OVA mice and anti-ASGM1-treated OVA mice; ## $P < 0.01$ and ### $P < 0.001$ between Ig-treated PBS mice and anti-ASGM1-treated OVA mice.

In the lungs of OVA mice, NK cells were found to be activated as suggested by the increased expression of CD86. We can speculate that such activated NK cells may increase Th2 cytokine production and granzyme B secretion as observed in asthmatic patients [5, 27].

To address the role of NK cells in asthma, we performed depletion studies with a NK-cell-depleting, non-NKT-cell-depleting antibody. We showed that NK cells were involved in the regulation of lung eosinophilia in OVA-induced airway allergic reaction. Similar results regarding lung eosinophilia were found in C57BL/6 mice treated with anti-NK1.1 antibody [18] altogether suggesting that NK cells do play a major role in this effect. After IgE activation through Fc γ RIII, NK cells have been shown to be able to express TNF- α and CCL3 mRNA, two mediators involved in allergic asthma [28]. Thus, CCL3 production by NK cells may contribute to eosinophil recruitment in lungs in our model of allergic asthma. Anti-ASGM1 antibody was found to be unable to deplete eosinophils directly. Indeed, anti-ASGM1 antibody depletes blood NK cells but not eosinophils in IL-5 transgenic mice (data not shown). However, AHR was not affected by the antibody treatment. One explanation is that development of AHR may be dissociated from airway eosinophilia as previously shown in BALB/c mice [29, 30] or linked to the incomplete depletion of NK cells. IgE and IgG levels were not affected either by NK cell depletion, which suggests that NK cells may not regulate an already established systemic Th2 response.

In summary, our results suggest that NK cells may participate in airway eosinophilia in a mouse model of asthma and regulate the immune response in the mediastinal lymph nodes. A better understanding of NK cell role in mice will provide new insights into the control of human allergic reaction.

Acknowledgment

This work was supported by INSERM. C. Ple was supported by a grant from Ministère de l'Éducation et de la Recherche. M. Barrier was supported by Région Nord-pas-de Calais. C. Duez was supported by UCB Institute of Allergy and Société Française d'Allergologie et d'Immunologie Clinique.

Author contributions

CP and MB equally performed the experiments and analysed the data. LA and PM performed some experiments (tissue harvesting and cell preparations). JB provided expertise for flow cytometry analysis and participated in the data analysis. AT, TW and PL contributed to the design of the study and reviewed the article. CD designed and coordinated the study, performed some experiments and wrote the article with CP.

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