

Short communication

Relationships between natural T cells, atopy, IgE levels, and IL-4 production

Background: Th2 cells govern allergic disorders. Mechanisms leading to the Th2 commitment are dominated by the requirement of IL-4. A potential source of this triggering IL-4 could be the CD4+ subset of a small population of T cells, natural T (NT) cells. Indeed, this subset is involved in IgE responses in mice and produces promptly high amounts of IL-4 in both mice and man.

Methods: NT cells were identified in peripheral blood by flow cytometry with antibodies against V α 24 and V β 11, recognizing the T-cell receptor specific for NT cells. Simultaneous staining with anti-CD3, anti-CD4, or anti-CD8 antibodies was performed. The frequency of NT cells in man was studied according to the presence of atopy defined by the positivity of skin tests, according to total IgE levels in serum, and according to IL-4 concentration of whole-blood culture supernatants determined by a flow cytometer microsphere-based assay.

Results: Seventy subjects were included, of whom 30 were atopic. The number of CD4+ NT cells was higher in atopics than in nonatopics ($P=0.009$). This number was correlated to the total IgE levels ($r=0.34$, $P=0.03$). In addition, the number of CD4+ NT cells, but also of CD8+ NT cells, was correlated to the levels of IL-4 ($r=0.71$, $P=0.01$, and $r=0.6$, $P=0.03$, respectively).

Conclusions: These results show that the number of NT cells, particularly the CD4+ subset, is related to atopy, IL-4 production, and IgE levels. Therefore, this population of T cells is likely to play a role in the Th2 commitment initiating atopic diseases.

A. Magnan, L. Mély, S. Prato, D. Vervloet

UPRES 2050, Groupe de Recherche Clinique
"Pathologie respiratoire et cutanée liée à
l'environnement", Service de Pneumo-Allergologie, et
Centre d'Investigations Cliniques, INSERM, Assistance
Publique Hôpitaux de Marseille, Hôpital Ste
Marguerite, Marseilles

F. Romagné, C. Camilla, A. Necker, B. Casano, F. Montero-Jullian, V. Fert

Immunotech, BP 177, Marseilles

B. Malissen

Centre d'immunologie INSERM-CNRS de Marseille-
Luminy, Marseilles

P. Bongrand

INSERM U 387, Hôpital Ste Marguerite, Marseilles,
France

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Dr A. Magnan
Service de Pneumo-Allergologie
Hôpital Ste-Marguerite
270 Bd de Ste Marguerite
13274 Marseille Cedex 09
France

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Many data suggest that atopic diseases are orchestrated by Th2 cells, which mainly produce interleukin (IL)-4, IL-5 (but no or little IL-2), and interferon (IFN)- γ (1). Therefore, a major objective of allergy research is the elucidation of the mechanisms underlying the Th2 commitment. Among these mechanisms, IL-4 itself is a major agent (2). However, the cells producing the IL-4 required for the development of Th2 cells are unknown (3, 4).

A small population of special T cells, called natural T (NT) cells because of characteristics shared with natural killer (NK) cells, was recently identified in mice (5, 6) and man (7). In mice, NT cells express the NK 1.1 antigen and account for 1% of lymph-node and 2% of mature spleen T cells. They all express an invariant

T-cell receptor (TCR) alpha chain. Human NT cells express the NK-specific CD56 antigen and an invariant TCR composed of a V α 24J α Q chain paired with V β 11 (8, 9). The cognate ligand of the NT cell receptor was shown to be CD1 in mice, a major histocompatibility class-I-related antigen (5, 6), and it is CD1d in man (10). Some murine NT cells express CD4, but not CD8. By contrast, the human counterpart can express CD4 or CD8. However, when expressed on human NT cells, CD8 is in the homodimeric form CD8 $\alpha\alpha$, and the CD8 β chain is not detected (8, 9). This phenotype is reminiscent of $\gamma\delta$ T cells, another reactive T-cell population, and of NK cells. Together with the expression on these cells of the IL-2 receptor beta chain and CD44, the expression of CD8 $\alpha\alpha$ suggests a state of

activation of these cells (8). In mice, the CD4⁺ subset of NT cells was responsible for an early and abundant production of IL-4 *in vivo* after intravenous stimulation by an anti-CD3 antibody (11). In man, CD4⁺ NT cells produce both IL-4 and IFN- γ , whereas the CD4⁻ subset secretes only IFN- γ (8). CD4⁺ NT cells were shown to produce much higher amounts of IL-4 than conventional T cells. Therefore, it was proposed that CD4⁺ NT cells, by secreting promptly high amounts of IL-4, could play a pivotal role in the initial priming of T cells toward Th2 differentiation (12).

Accordingly, we hypothesized that CD4⁺ NT cells could be involved in the triggering of atopy and related diseases. Therefore, we studied the number of NT cells, CD4⁺ NT cells, and CD8⁺ NT cells in peripheral blood, according to the presence of atopy, the levels of total IgE in serum, and the IL-4 production by whole-blood cells.

Material and methods

Subjects

Seventy healthy volunteers were included in this study. None of them were smokers, and none of them were asthmatic as assessed by clinical history, spirometry, and methacholine challenge test. A blood sample was collected from each patient for IgE assay, blood culture, and flow cytometry studies. The ethics committee of Marseille-I approved the protocol, and each subject gave informed consent.

The diagnosis of atopy was confirmed by the positivity of at least one skin prick test to a common environmental allergen from a battery of extracts (Laboratoire des Stallergènes, Paris, France). In any case, a positive test (codeine phosphate 9%) and a negative test were performed.

Identification and counting of NT cells

Staining of NT cells. NT cells were defined by the expression of the V α 24V β 11 TCR. Immunotech, Marseilles, France, provided all antibodies. For three-color flow cytometry, 100 μ l of whole blood was incubated for 20 min with 10 μ l of the PE-conjugated anti-V β 11 mAb (clone C15, IgG1), of the FITC-conjugated anti-V α 24 mAb (clone C19, IgG2a), and of either a PE-Cy5-conjugated anti-CD3 mAb (clone 13B8-2, IgG1), a PE-Cy5-conjugated anti-CD4 mAb (clone PS1, IgG2A), or a PE-Cy5-conjugated anti-CD8 mAb (clone B9-11, IgG1). Irrelevant FITC-IgG2a (clone U7.27) and PE-IgG1 (clone 679.1 Mc7) isotypic controls were used to assess the specificity of the staining. After a wash, the cells were resuspended in 100 μ l OPTILYSE B (Immunotech) to lyse erythrocytes, and 1 ml of water was added after 10 min. The cells were centrifuged, resuspended in 100 μ l PBS, and analyzed immediately in a Coulter

EPICS XL/MCL flow cytometer (Beckman Coulter, Miami, FL, USA).

Flow cytometry. For each subject, three types of stained samples were analyzed. Samples stained with anti-CD3, anti-V α 24, and anti-V β 11 antibodies were analyzed first. Dead cells, monocytes, and granulocytes were excluded by forward- and side-scatter gating. An amount of 4×10^4 CD3⁺ cells was acquired, and the number of V α 24⁺ V β 11⁺ cells was recorded as the number of NT cells among 4×10^4 T cells. Samples stained with anti-CD4, anti-V α 24, and anti-V β 11 antibodies were then analyzed. The same number of events as for the first sample was acquired in the lymphocyte window, so that the number of CD4⁺ V α 24⁺ V β 11⁺ cells acquired corresponded to the number of CD4⁺ V α 24⁺ V β 11⁺ cells among 4×10^4 CD3⁺ cells. The same procedure was used for the detection of CD8⁺ NT cells. Results were therefore expressed as the number of NT cells among 4×10^4 T cells. After a rinse, samples stained with isotypic controls were analyzed. The same number of events as for specific stainings was acquired in samples stained with isotypic controls.

IgE assay

IgE was assayed in the sera of the patients by the PRIST technique (Pharmacia, Uppsala, Sweden). Values were expressed in UI/ml.

Cell culture

Cell culture was performed at 37°C in an atmosphere containing 5% CO₂. Cells were cultured in six-well plates in a volume of 1 ml of whole blood, in the presence or not of 100 ng/ml of phorbol 12-myristate acetate (PMA) and 2 μ g/ml of ionomycin. After 6 h of culture, cells were harvested and centrifuged, and the supernatants were frozen and kept at -80°C until assay.

Soluble cytokine assay

The flow cytometer microsphere-based assay uses sets of immunoassays performed on fluorescent and antibody-coated microspheres developed by Immunotech (Marseilles, France) (13). Each microsphere set is designed for an individual assay and is coded by the intensity of the green fluorescent emitter. Each cytokine assay involves anti-cytokine antibody-coated microspheres and a complementary biotinylated antibody to capture the cytokine in the sample. Detection is achieved with a red streptavidin-conjugated emitter as reporter molecule. After the assay, the microspheres are individually analyzed in a flow cytometer. Measurements of green (FL1: 525 nm) and red (FL4:

675 nm) fluorescence yield both the specificity of the microsphere sets and the amounts of immune complex formed respectively on their surface.

Assays of IL-4 were performed. A volume of 50 µl of sample was incubated for 2 h with 10 µl of the mixture of coated microspheres (100 µg/ml) and 50 µl of the mixture of biotinylated antibodies (1 µg/ml) at room temperature under shaking. After two washes, 100 µl (0.5 µg/ml) of streptavidin-PE-Cy5 (PC5) conjugate was incubated with microspheres for 30 min at room temperature under shaking, and washed twice. The microspheres were then analyzed on a Coulter EPICS XL/MCL flow cytometer (Beckman Coulter, Miami, FL, USA). The instrument was carefully set to provide optimal discrimination for FL1-coded microspheres and the optimal range for FL4-binding. Data acquisition was performed at high speed until about 500 microspheres per assay had been analyzed. The resulting FCS files were analyzed by an in-house software program which calculates mean fluorescence for each individual assay. Concentrations were subsequently calculated by standard immunoassay data-processing methods.

Statistical analysis

Numbers of positive cells and cytokine concentrations were compared between groups by Student's *t*-test if the normality test of Kolmogorov-Smirnov allowed the use of parametric tests. In other cases, data were log-transformed in order to obtain a gaussian distribution, and Student's *t*-test was used. Results were expressed as mean $\pm$ SEM for normally shaped data and as geometric mean and 25–75% confidence intervals in other cases. Numbers of NT cells were expressed as number of cells among 4×10^4 T cells. Correlations between IL-4 levels, log IgE, and log NT cell numbers were assessed with the linear regression test.

Table 1. Age, total IgE levels, IL-4 production, and natural T-cell counts in atopic and control subjects

	Controls	Atopics
Age (years) ^a	0.3 $\pm$ 2.4 Range 22–62	37.6 $\pm$ 3.4 Range 18–60
IgE (UI/ml) ^b	20.73 (7–56)	117.5 (41–364)
IL-4 (pg/ml) ^a	293.1 $\pm$ 56.5	503.8 $\pm$ 51
NT cells (cells/ 4×10^4 T cells) ^b	41.6 (22–94)	42.2 (19–96)
CD4 + NT cells (cells/ 4×10^4 T cells) ^b	6.9 (3–20)	13.3 (7.5–34.2)
CD8 + NT cells (cells/ 4×10^4 T cells) ^b	23 (8.5–56.2)	20 (11–46.5)

^aMean $\pm$ standard error of mean.

^bGeometric mean and interquartile ranges (25%/75%).

Results

Characteristics of patients

The characteristics of the patients are given in Table 1. Among the 70 subjects included, 30 were atopic.

Frequency of NT cells in the whole population

NT cells were counted in 70 samples, CD4 + NT cells in 54, and CD8 + NT cells in 21. NT cells were present in all but one blood sample. They were easily identified by flow-cytometry analysis as a discrete subset among the CD3 + population. On NT cells, the level of expression of CD3 was intermediate as compared to mainstream T cells. On CD8 + NT cells, CD8 was always expressed on NT cells at a lower level than on mainstream CD8 + T cells, consistent with the expression of CD8 as an $\alpha\alpha$ homodimer on NT cells. Isotype control stainings were negative.

The mean NT cell count was 42 (20–96) cells/ 4×10^4 T cells, range 0–404 cells/ 4×10^4 T cells (0–1.78% of T cells). The CD4 + NT subpopulation varied from 0 to 65 cells/ 4×10^4 T cells, mean 11 (3–20.25). The CD8 + NT cell population varied from 0 to 186 cells/ 4×10^4 T cells, mean 28 (9–53.75).

The NT cell population correlated with both the CD4 + ($r=0.619$, $P<0.001$) and the CD8 + ($r=0.944$, $P<0.01$) subsets. Furthermore, the CD4 + subpopulation was correlated to the CD8 + subset ($r=0.56$, $P=0.009$).

Frequency of NT cells according to diagnosis

Results according to diagnosis are given in Table 1. The number of total NT cells was not different between atopic and nonatopic subjects, nor was the number of CD8 + NT cells. By contrast, the number of CD4 + NT cells was significantly higher in atopics than controls (13.3 cells/ 4×10^4 T cells vs 6.9 cells/ 4×10^4 T cells; $P=0.009$).

Relationships between NT cells and IL-4 and IgE levels

IgE levels were assayed in sera from 62 subjects. As expected, IgE levels were higher in atopics than controls (Table 1). The number of CD4 + NT cells was correlated to IgE levels ($r=0.34$, $P=0.03$) (Fig. 1). The number of total NT cells and of CD8 + NT cells was not related to IgE levels.

IL-4 was assayed in whole-blood culture supernatants from 24 subjects. The IL-4 production was higher in atopics than controls ($P=0.015$) (Table 1). There was a high correlation between CD4 + NT cells and IL-4 production ($r=0.71$, $P=0.01$) (Fig. 2A), but also between total NT cells and IL-4 production ($r=0.72$, $P=0.008$, Fig. 2B), and between CD8 + NT cells and IL-4 production ($r=0.6$, $P=0.02$) (Fig. 2C).

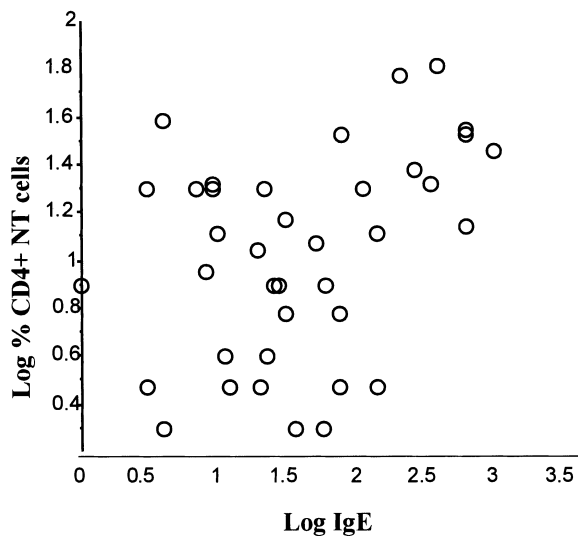


Figure 1. Correlations between CD4+ natural T cells and IgE levels. Volume of 100 μ l of whole blood was stained by PE-Cy 5-anti-CD4, FITC-anti-V α 24, and PE-anti-V β 11. Red cells were specifically lysed, and three-color analysis was performed with FACScan. Number of cells corresponding to 4×10^4 CD3 T cells, determined by previous CD3 staining, was acquired, and number of CD4+ cells among 4×10^4 T cells was determined. IgE levels were determined in patients' sera by PRIST assay.

Discussion

In this study, we assessed the NT cell populations and their relevance to the Th2 commitment in man. The number of NT cells accounted for 0–1.72% of T cells, and was of the same order in man as that described in mice, but with a high interindividual variability. V α 24V β 11 T cells represented about 33% of the V α 24 T cells (data not shown). This demonstrates the relative quantitative importance of this clonotypic population, and suggests that, although very small, the NT cell population may have a peculiar function among T cells. To characterize this function, we first made an attempt to detect the NT cell cytokine production by flow cytometry, but the low number of NT cells and the downregulation of the TCR expression after stimulation rendered the data uninterpretable. Therefore, we made use of features of achieved Th2 differentiation (IL-4 production by whole-blood cells, total IgE levels, and atopic status) to show the relationships between NT cells and the Th2 commitment.

The CD4+ NT cell subset was related to atopy, levels of IgE, and whole-blood cell IL-4 production. These results are consistent with several data suggesting a role for NT cells in the Th2 commitment. In mice, CD4+ NT cells promptly produce high amounts of IL-4 *in vivo* upon anti-CD3 stimulation (11). In V α 14J α 281 transgenic mice, a boosted production of IL-4 and an increase of basal levels of IgE were observed (14). In β_2 -microglobulin-deficient mice, the number of NK1.1 T cells is drastically reduced, and both the IL-

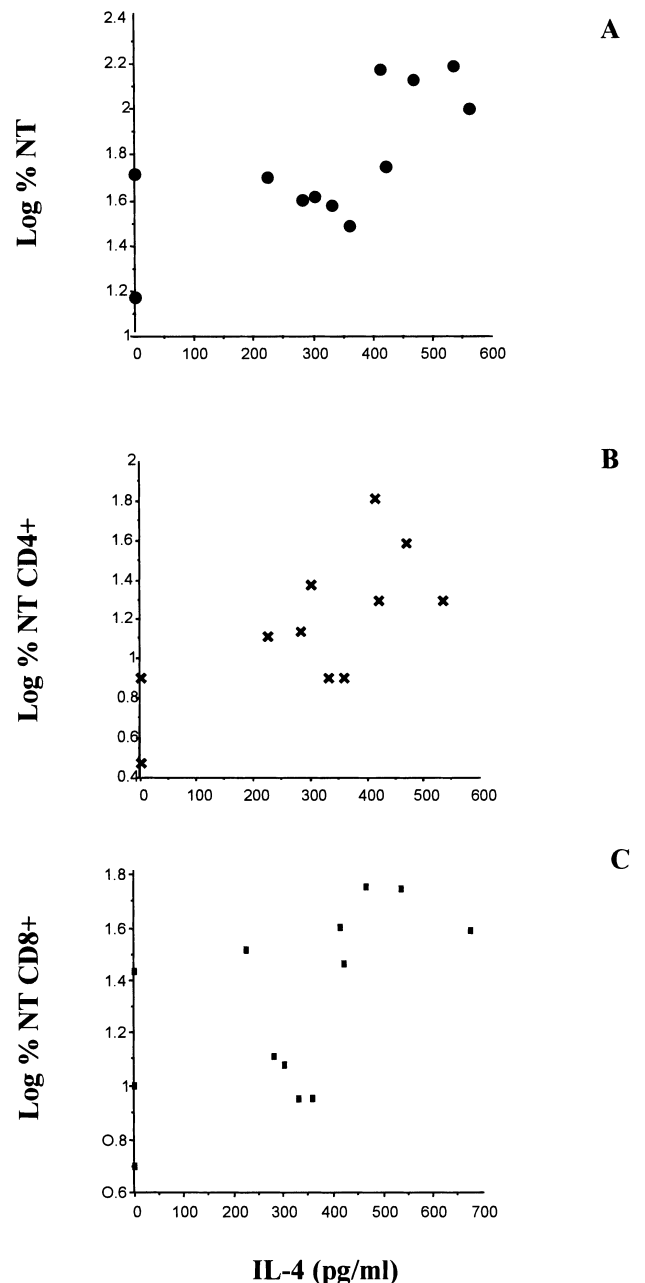


Figure 2. Correlations between NT cell populations and IL-4 production. Numbers of NT cells (A) and of CD4+ (B) and CD8+ (C) subsets were determined as indicated in Fig. 1. Cells were cultured in whole blood for 6 h in presence of PMA and ionomycin, supernatant was harvested, and IL-4 was assayed by microsphere-based method. IL-4 levels are expressed in pg/ml.

4 production upon anti-CD3 stimulation and the IgE response to anti-IgD are abolished (15). The transfer in these mice of NK1.1 T cells and CD1-expressing splenocytes restores these responses. More recently, it was shown that CD4+ NK1.1 T cells can produce high amounts of IL-4 without exogenous IL-4 stimulation. In man, NT cells produce both IFN- γ and IL-4, the latter being produced only by the CD4+ subset (8). In addition, the proportion of IL-4-producing NT cells

was found to be sixfold higher than the proportion of IL-4-producing mainstream T cells.

Surprisingly, not only the CD4+ subset, but also the whole NT cell population and the CD8+ subset were related to IL-4 production. However, these populations were not related to atopy or to IgE levels. This result is probably the indirect consequence of a higher number of CD4+ NT cells in patients with more total and CD8+ NT cells.

However, most atopic subjects have a number of circulating CD4+ NT cells similar to that of non-atopics. This shows that NT cells do not explain the inception of atopy by themselves. Indeed, some data suggest that NT cells are not necessary for the induction of Th2 responses. In CD1-deficient mice, NT cells are not positively selected and the IL-4 production upon anti-CD3 stimulation is abolished, but the IgE response to anti-IgD is conserved (16). In β_2 -microglobulin-deficient mice, Th2 responses to various stimuli are conserved (17). In addition, NT cells can produce

IFN- γ in mice and man, notably upon IL-12 stimulation (18, 19). This shows that, although hyperreactive, NT cells can boost inflammatory reactions in a Th1 as well as in a Th2 pathway. Other small reactive cell populations such as $\gamma\delta$ T cells (20), NK cells (21), or even $\gamma\delta$ NT cells (22) are capable of inducing Th2 responses in some murine models. Such cells could be relevant in some atopic patients with low numbers of CD4+ NT cells, the initiating factors of atopy being different from a patient to another.

In conclusion, we have shown that NT cells, and notably the CD4+ subset, are related to atopy and total IgE levels, probably via the production of IL-4 by mainstream T cells. These results support the hypothesis of a role for human NT cells in the inception of atopy and allergic diseases.

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