

Altered Peripheral Invariant Natural Killer T Cells in Atopic Dermatitis

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Running head: *iNKT Cells in Atopic Dermatitis*

ABSTRACT

Background

Conflicting data exist on the number of invariant NKT (iNKT) cells in atopic dermatitis (AD); furthermore, no data have been published on their functional capacity.

Methods

The frequency and number of circulating 6B11+CD3+ iNKT cells and their CD4+ and CD4- subpopulations were evaluated in peripheral blood obtained from 41 patients with AD by four-color flow cytometry. Likewise, functional properties of iNKT cells were measured by five-color intracellular cytokine staining.

Results

The number and percentage of total iNKT cells and their CD4/CD8 subpopulations were significantly lower than the controls. Of further importance, the CD4-CD8- (double negative: DN) iNKT subgroup showed the strongest positive correlation with total iNKT cells. In addition, the DN subgroup exhibited the most pronounced functional alteration with significantly decreased levels of intracellular IFN γ and significantly increased levels of intracellular IL-4 in AD patients compared with the controls.

Conclusion

The significantly altered number and cytokine production of iNKT cells from AD patients suggests that these cells may play an important role in the pathogenesis of AD.

Key words

Atopic dermatitis-invariant natural killer T cell-cytokine-flow cytometry

INTRODUCTION

Atopic dermatitis (AD) is a chronic, pruritic inflammatory skin disease with a prevalence of 10-20% in children and 1-3% in adults, which significantly impairs quality of life of affected patients [1]. The interplay of several factors including susceptibility genes, environmental triggers and immunologic responses results in the development of the clinical phenotype of AD [2]. There are certain theories proposing that sequential helper T (Th) lymphocyte activation plays a role in the pathogenesis of AD. According to these theories, the predominance of Th2 lymphocytes in acute skin lesions of the initial phase is replaced by Th1 cell subsets during the persisting chronic phase of the disease [3]. Investigations of new T cell subsets in patients with AD revealed the involvement of naturally occurring CD4+CD25+ regulatory T cells; moreover, a possible role for Th17 and Th22 cells in AD was also suggested [4-6]. In addition to adaptive immune system alterations, recent data suggest important roles for both innate immune responses and skin barrier defects in the pathogenesis of AD [7, 8].

In the last decade, despite their small number in the peripheral blood, a heterogeneous subpopulation of mature T cells, i.e., human invariant natural killer T (iNKT) cells, have entered into the focus of immunological investigations.

These cells express both the semi-invariant T cell receptor (TCR) and the NK cell receptor NKR1A (CD161) [9-11]. The majority of human iNKT cells express TCRs consisting of the V α 24/J α 18 α chain paired preferentially with the V β 11 β chain [12]. They are divided into four subsets [CD4+, CD8+, double negative (DN), and double positive (DP)] and are activated by glycolipid antigens presented by CD1d on antigen presenting cells [13, 14].

iNKT cells play important effector and immune regulatory roles through their potent cytotoxicity and rapid secretion of cytokines [15]. They form a bridge between innate and

adaptive immune responses, and of further importance, iNKT cells have been implicated in numerous human diseases [16, 17]. Namely, a selective reduction in iNKT cell number has been shown in various autoimmune syndromes such as systemic sclerosis, systemic lupus erythematosus, and type I diabetes mellitus [18-22]. These cells have also been reported to play an active role in various skin diseases, such as allergic contact dermatitis, but the relationship between AD and iNKT cells has not been thoroughly investigated [23, 24].

The unique ability of iNKT cells to produce high amounts of both Th1-type (IFN γ) and Th2-type (IL-4) cytokines suggested the possibility that they may have a pathogenic role in AD [11]. Indeed, few and conflicting results have been published on iNKT cell frequencies and numbers in AD to date; however, the functional properties of iNKT cells have not been discovered [25, 26]. Hence, the aim of our present study was to elucidate whether the frequency and/or the absolute number of peripheral iNKT cells and their different subsets are altered in AD patients; furthermore, their effector functions were investigated by examining intracellular IFN γ and IL-4 cytokine production.

MATERIALS AND METHODS

Patients and Controls

Forty-one patients with AD (16 female and 25 male, mean age 18.59 ± 8.9 years) and 16 healthy non-atopic controls (HCs) (11 female and 5 male, mean age 18.9 ± 5.9 years) were selected. AD patients fulfilled the diagnostic criteria established by Hanifin and Rajka [27]. All participants gave written informed consent according to the Declaration of Helsinki Principles. The severity of the disease was determined by the SCORAD (SCORe Atopic Dermatitis) index [28, 29]. The mean objective SCORAD was 26.7 ± 13.1 . When the subjective scores were also added, the mean SCORAD was 41.2 ± 14.6 . The median serum total IgE was 584.3 kU/L with a range of 251.3–1245.0 kU/L, and the median serum lactate dehydrogenase (LDH) was 475 U/L with a range of 268.1–732.4 U/L, reference: 230–460 U/L. Patients had not received any oral glucocorticosteroids, or other systemic immunomodulatory agents, for at least 4 weeks and had not been treated with antihistamines or topical corticosteroids for at least 5 days before the collection of blood samples.

Isolation of peripheral blood mononuclear cells (PBMC)

Heparin anti-coagulated blood samples were layered over Histopaque-1077 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and centrifuged at $700 \times g$ for 30 minutes, and the mononuclear cells were isolated from the plasma-Ficoll interface.

Determination of frequency and number of iNKT cells and CD4/CD8 iNKT cell subsets by flow cytometry

The number and frequency of iNKT cells were determined by flow cytometry on PBMCs from 41 patients with AD using anti-CD3 and 6B11 (specific for the CDR3 loop on the $V\alpha 24$

chain of the TCR) monoclonal antibodies (mAbs). Cells (1×10^6) were preincubated with 100 $\mu\text{g}/\text{mL}$ purified human IgG (Sigma) for 20 minutes on ice to prevent nonspecific binding to Fc receptors. After two washes in staining buffer (phosphate buffered saline, 5% fetal calf serum, and 1% bovine serum albumin, all from Sigma), cells were then incubated with anti-CD3-fluorescein isothiocyanate (FITC), 6B11-phycoerythrin (PE) (BD Pharmingen, San Jose, CA, USA), anti-CD4-PE-Texas Red (ECD) and anti-CD8-PE-Cyanine7 (PC7) (Immunotech, Marseille, France) mAbs for 45 minutes on ice. Cells were then washed and analyzed by four color flow cytometry on a Beckman Coulter FC500 flow cytometer and CXP software. For all staining experiments, appropriate isotype-matched controls were included. The absolute cell numbers were calculated by applying the two platform method using lymphocyte counts.

Determination of functional properties of iNKT cells and their CD4/CD8 subgroups by intracellular cytokine analysis

PBMCs of 10 AD patients and 10 HCs were investigated. Intracellular cytokine levels of iNKT cells and their CD4/CD8 subsets were determined by five-color flow cytometry as described [30]. Briefly, after stimulating PBMCs with 25 ng/mL phorbol-12-myristate 13-acetate (PMA) and 1 $\mu\text{g}/\text{mL}$ ionomycin (Io) in the presence of 10 $\mu\text{g}/\text{mL}$ Golgistop Brefeldin A (all from Sigma), the cells were washed, and surface staining of the cells was performed with appropriate mAbs to CD3 (FITC-labeled for IL-4-allophycocyanin (APC) staining and APC-labeled for IFN γ -FITC staining, both from BD Pharmingen), 6B11, CD4 and CD8. The cells were then fixed and permeabilized with IntraprepTM permeabilization reagent (Immunotech) according to the manufacturer's instructions, and intracellular cytokines were stained with IFN γ -FITC or IL-4-APC mAbs (both from BD Pharmingen) for 30 minutes. After two washes with staining buffer, the cells were analyzed on a Beckman Coulter FC500 flow cytometer and CXP

software. At least 300,000 cells were counted from each sample. Appropriate isotypic controls were included in all experiments. The specific response of the cells to PMA/Ionomycin is calculated by subtracting the percentage of positive events in the unstimulated sample from the percentage of positive events in the activated sample. Results were expressed as net percentage of cytokine positive cells.

Statistical Analysis

Results are presented as median, interquartile ranges (IQR: 25th-75th percentiles), and ranges (5th-95th percentiles). Statistical analysis was performed with SigmaPot v. 11.0 software (SyStat Software Inc, USA). A non-parametric Mann-Whitney Rank Sum Test or Student's *t*-test was used. Correlation analysis was performed using a Spearman's rank test. A *p* value less than 0.05 was considered statistically significant.

RESULTS

Percentage and number of total iNKT cells in AD patients

Circulating iNKT cells were identified as CD3+6B11+ cells among the lymphocytes of PBMCs obtained from peripheral blood of AD patients and HCs. The flow cytometry gating strategy is shown in Fig. 1a-c. Notably, the frequency of CD3+6B11+ iNKT cells was significantly reduced ($p=0.003$) in AD patients (0.07%; IQR: 0.040-0.155%) compared with HCs (0.295%; IQR: 0.125-0.430%) (Fig. 1d). In addition, the median circulating number of iNKT cells was also significantly diminished ($p<0.001$) in AD patients (0.394 cells/ μ L; IQR: 0-2.029 cells/ μ L) compared with the controls (4.921 cells/ μ L; IQR: 2.856-11.076 cells/ μ L) (Fig. 1e).

Because alteration in the T cell numbers may affect the iNKT cell count, we determined the percentage of the CD3+ T cell, CD4+, CD8+ T cell subsets and the CD4/CD8 ratio in PBMC of both AD patients and HCs, and in this sense the AD population did not differ significantly from the investigated controls (data not shown). No correlations were found between the frequency or number of iNKT cells and the age or sex of the patients, disease severity (measured by the SCORAD index), or the serum levels of total IgE and LDH.

Subgroup analysis of iNKT cells in AD patients

Among the gated CD3/6B11 double positive total iNKT cells, the four iNKT subsets were identified on the basis of their CD4 and CD8 expression (Fig. 1c). Analysis of the CD4/CD8 phenotype of iNKT cells revealed a significant decrease in the percentage of CD4- subpopulations (DN and CD4-CD8+) ($p=0.016$ for DN; $p=0.03$ for CD4-CD8+) in AD patients compared with HCs. When the absolute numbers were counted, the results showed an even more pronounced decrease in the CD4/CD8 iNKT subpopulations of AD patients compared with HCs

($p < 0.001$ for all cases). The detailed results of the iNKT subset analysis are summarized in Table I.

Positive correlations were found between the frequency of total iNKT cells and the frequencies of their subsets in AD patients ($p < 0.01$ for all cases) except for DP iNKT cells (Fig. 1f), and these correlations were more pronounced ($p < 0.001$) when the absolute cell counts were analyzed (Fig. 1g). Presumably, the very low number of cells in the DP iNKT subset accounts for the absence of the aforementioned relationships and data for this subset are not presented in Fig. 1f, g. The correlation coefficients (r) for percentage of iNKT subsets were $r_{DN} = 0.741$; $r_{CD4+CD8-} = 0.631$; $r_{CD4-CD8+} = 0.452$; and $r_{DP} = n.s.$, and for absolute counts were $r_{DN} = 0.929$; $r_{CD4+CD8-} = 0.922$; $r_{CD4-CD8+} = 0.856$; and $r_{DP} = 0.392$. For both the frequencies and the absolute numbers, the strongest correlation was found between the values of the total iNKT group and the DN iNKT subgroup.

Functional analysis of iNKT cells in AD patients

To assess the effector functions of iNKT cells in AD patients, the intracellular IFN γ and IL-4 cytokine content of total iNKT cells and subgroups was determined. After comparing cells from 10 AD patients and 10 HCs, the intracellular IFN γ expression was found to be decreased (Fig. 2a, b), whereas that of IL-4 was increased (Fig. 3a, b) in the total iNKT cells from AD patients compared with those from HCs; however, these alterations did not reach the limit of statistical significance. Next, the functional properties of the different subsets of iNKT cells were analyzed. When the intracellular cytokine levels of iNKT subgroups from AD patients and HCs were compared, the DN iNKT subgroup from AD patients exhibited significantly lower IFN γ levels ($p < 0.05$; Fig. 2c) and significantly increased intracellular IL-4 levels ($p < 0.01$; Fig. 3c). With respect to the other three iNKT subgroups, no significant differences were detected in the intracellular IFN γ and IL-4 cytokine levels between AD patients and controls.

DISCUSSION

Although considerable evidence suggests a role for iNKT cells in autoimmune and infectious disorders, there are scarce data on their distribution and function in atopic diseases. The involvement of iNKT cells in bronchial asthma has been extensively investigated by studies utilizing animal models of allergen-induced asthma, ozone exposure, viral infection or bacterial components, which conclude that iNKT cells function either in concert with Th2 cells or independently of adaptive immunity to cause airway hyperreactivity [31]. On the other hand, data on the role of iNKT cells in human asthma are conflicting, since some authors found iNKT cells in low numbers in the airways of subjects with asthma, while others reported opposite results [32, 33].

iNKT cells are able to produce large amounts of Th1 and Th2 type cytokines upon activation, which suggests that they may play a role in the pathogenesis of another atopic disease, such as AD. So far, there have been few and conflicting data published on iNKT cells in AD; the contrary findings can be explained by the different sensitivities and specificities of the distinct methods that were used for the detection of these cells. Currently, CD1d multimers or invariant TCR α chain specific 6B11 monoclonal antibodies are regarded as the most accurate markers for the identification of iNKT cells by flow cytometry [30, 34, 35]. In this study, by using an anti-CD3/6B11 mAb combination to characterize iNKT cells, we were able to show that both the percentage and the number of iNKT cells were significantly decreased in the peripheral blood of patients with AD compared with HCs. In our study the number of iNKT cells in the HCs was higher than usually observed by other investigators. The mean age was about 18 years for HCs and we believe that the higher number of iNKT cells in this population can partly be explained by the younger age [36, 37]. On the other hand a high inter-individual variation in the frequency

of iNKT cells can also be considered [38, 39]. There was no alteration found in the frequency of the total T cells and the CD4/CD8 subsets in AD patients, presuming that the reduction in the prevalence of iNKT cells was not resulted from differences in T cell populations.

In agreement with our results, four previous publications demonstrated diminished numbers of iNKT cells in AD patients; conversely, two groups found increased numbers of these cells [25, 40-43, 26]. These contradictory results suggest that further investigations are needed, as it is possible that not only differences in the applied methods but also differences in the patient selection (extrinsic-intrinsic AD, mild-moderate-severe AD) may alter the iNKT cell number. It is important to emphasize that among the above-mentioned studies only one employed a method similar to ours.

Using phenotypic analysis of the CD4/CD8 subsets of iNKT cells, we investigated which subpopulation was mainly responsible for the decreased iNKT number in AD patients. In HCs, we found that the CD4+CD8- and DN subpopulations of iNKT cells were the most frequent subpopulations and both of them represented a similar percentage of iNKT cells, the CD8+CD4- iNKT cells had an intermediate frequency, and the DP iNKT cells had the lowest frequency. Montoya *et al.* demonstrated similar prevalence of iNKT subsets in healthy individuals, while various frequencies of these subsets have also been reported [30, 44, 45]. In the peripheral blood of patients with AD, the most pronounced alteration was observed in the DN iNKT subset. Although the numbers of all iNKT subsets were found to be decreased, only CD4- (DN and CD4-CD8+) iNKT subsets showed significantly diminished percentages in AD patients. The strongest positive correlation was found between the DN iNKT subset and total iNKT cells. These findings are consistent with the results of previous studies. Takahashi *et al.* detected a decreased tendency of CD4-CD8+ and DN iNKT subsets, while Oishi *et al.* found a greatly

diminished DN iNKT cell number in patients with asthma and with AD [25, 41]. In contrast to these results, some authors reported significantly reduced CD4⁺ iNKT subset in the peripheral blood of patients with AD [46]. The cause for the varied results of the different reports on iNKT subsets are unknown, but may arise from a distinct patient population selection or a difference in gating on CD4⁺ and CD8⁺ iNKT subsets, namely, whether dimly positive cells were involved in these studies.

Differentiating between iNKT subsets is crucially important because distinct subsets may have different functions [34, 47, 48]; furthermore, a cross-regulation between the various iNKT subsets markedly influences the immune response to self and foreign antigens [49]. In healthy individuals, the circulating CD4⁺ iNKT subset can produce both Th1 and Th2 cytokines (IFN γ , IL-4 and IL-13), whereas the DN iNKT subset secretes predominantly Th1 cytokines (IFN γ and TNF α) [10, 34]. Elimination of pathogens and tumour rejection are generally correlated with Th1 responses, whereas tolerogenic mechanisms, such as suppression of graft rejection and inhibitory effects, are usually associated with Th2 responses by iNKT cells [50].

Because data on the number of iNKT cells or even iNKT subgroups are limited if functional properties are not analyzed, intracellular IFN γ and IL-4 cytokine production were also measured. We have found that the overall IFN γ production by iNKT cells was decreased, whereas the IL-4 production was increased, in AD patients; however, these alterations were not significant. On the other hand, these functional alterations were significant when the DN subgroup was investigated. The DN iNKT cells from AD patients produced significantly less IFN γ and significantly more IL-4 compared with HCs. According to our best knowledge, this is the first demonstration of the altered intracellular cytokine pattern in the DN iNKT subgroup of AD patients.

The significantly decreased number and altered function of iNKT cells demonstrated in our results are in good agreement with previous studies, which found that increased plasma IL-18 level in AD patients correlated with the decreased number and dysfunction of iNKT cell in these patients [46]. Lind *et al.* found that elevated plasma levels of IL-18 exerted an inhibitory effect on iNKT cells and that IL-18-mediated dysregulation of iNKT cells may play a role in the pathogenesis of AD.

It is a yet unsolved question whether changes in the number and cytokine expression of total iNKT cells or iNKT subgroups may act as primary pathogenic factors in the development of AD, or whether the disease is a consequence of elevated Th2-type cytokine levels in the peripheral blood of these patients. The decreased number and altered cytokine production of iNKT cells may lead to an impaired immuno-regulatory capacity of these cells, which could contribute to the development of the characteristic cytokine milieu and chronic inflammation observed in AD patients. Aberrations of the adaptive immune system in the peripheral blood and skin of AD patients are well studied, but less is known about the alterations of the innate immune system [3, 51]. Presently, a great deal of evidence suggests that there are various defects in the different components of the innate immune system, such as a reduction in antimicrobial peptide production, lower expression of toll-like receptors, disruption of the epithelial barrier and diminished recruitment of innate immune cells to the skin [7]. Hence, the present study also highlights the possible role of the innate immune system in a chronic inflammatory skin disease, which was previously considered to be influenced mainly by the components of the adaptive immune system.

In conclusion, the significantly altered number and cytokine production of iNKT cells of AD patients suggest that these cells may play an important role in the pathogenesis of AD.

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Table I. iNKT cells and iNKT subsets in AD patients

iNKT SUBSET	FREQUENCY (%)					ABSOLUTE NUMBER (cells/uL)				
	AD		HCs		<i>p</i> -value	AD		HCs		<i>p</i> -value
	Median	IQR	Median	IQR		Median	IQR	Median	IQR	
Total	0.070	0.040-0.155	0.295	0.125-0.430	0.003	0.394	0-2.029	4.921	2.156-11.076	<0.001
CD4+CD8-	0.030	0.020-0.085	0.090	0.020-0.150	0.090	0.181	0-0.722	2.120	0.875-3.508	<0.001
DN	0.030	0.010-0.070	0.090	0.060-0.190	0.016	0.163	0-0.943	1.533	0.892-5.111	<0.010
CD4-CD8+	0.010	0-0.035	0.070	0.020-0.100	0.030	0	0-0.495	1.330	0.632-1.882	<0.001
DP	0	0-0.015	0.010	0.008-0.020	0.052	0	0-0	0.325	0-0.444	<0.001

Median and interquartile ranges (IQR) of frequency and absolute number of iNKT cells and their subsets are presented for 41 AD patients and 16 HCs. *AD* atopic dermatitis, *HCs* healthy controls, *DN* double negative, *DP* double positive. ^aDifference between study groups (Mann Whitney U-test or Student's t-test).

FIGURE LEGENDS

Figure 1. Flow cytometric analysis of iNKT cells and subsets from AD patients and HCs.

PBMCs were stained with anti-CD3-FITC and 6B11-PE mAbs. **a** Lymphocytes were gated on a forward scatter (FS) – side scatter (SS) dot plot. **b** In the lymphocyte gate, iNKT cells were identified as CD3+6B11+ double positive cells. **c** Phenotypic analysis of iNKT cells was performed by CD4/CD8 staining with anti-CD4-ECD and anti-CD8-PC7 mAbs. **d** Percentage and **e** absolute number of iNKT cells detected in the lymphocyte gate are shown. Significantly reduced percentage ($p=0.003$) and absolute number ($p<0.001$) of iNKT cells were found in AD patients ($n=41$) compared with HCs ($n=16$). Results are presented as medians, IQRs, and ranges (5th and 95th percentiles). Statistical significance was calculated using a Mann-Whitney U-test. **f** and **g** These figures illustrate that the alteration of the CD4/CD8 iNKT subsets (y axis) show correlation with the decrease in the total iNKT cell (x axis) percentage (**f**) and the absolute numbers (**g**). The strongest correlation was detected between the total iNKT cells and the DN subset.

The upper long dash-line with empty circle symbols illustrates DN iNKT vs. total iNKT cells; the middle solid line with filled circle symbols represents CD4+CD8- vs. total iNKT cells; the lowest short dashed-double dotted line with diamond semi-filled bottom symbols illustrates CD4-CD8+iNKT vs. total iNKT cells (due to their low number, DP iNKT cells are not presented). Correlation coefficients (r) and p values for significance can be found at the end of the regression lines on the right side of the plots. The Spearman correlation was significant ($p<0.05$), except for the percentage of DP iNKT. *: $p<0.05$; **: $p<0.001$; ***: $p<0.0001$. Correlation coefficients (r) for percentages/absolute numbers of iNKT subsets: $r_{DN}=0.741/0,929$; $r_{CD4+CD8-}=0.631/0,922$; $r_{CD4-CD8-}=0.452/0,856$; $r_{DP}=n.s./0.392$.

Figure 2. Intracellular IFN γ expression of iNKT cells.

After stimulation of PBMCs obtained from AD patients and HCs with PMA/ionomycin for four hours, the cells were stained with CD4-ECD, 6B11-PE, CD8-PC7 and CD3-APC surface markers, followed by intracellular IFN γ -FITC staining. Cells were analyzed on a Coulter FC500 flow cytometer. **a** Representative scatter plots displaying lymphocyte (Ly) gates and iNKT gates of unstimulated and PMA/Ionomycin (Io) activated samples from an AD patient and HCs are presented. **b** Frequency of intracellular IFN γ positive cells was diminished in total CD3+6B11+ iNKT cells from AD patients compared with HCs. **c** Frequency of intracellular IFN γ in CD4+CD8 $^{-}$, DN (CD4 $^{-}$ CD8 $^{-}$) and CD4 $^{-}$ CD8 $^{+}$ iNKT subsets in AD patients and HCs. Compared to HCs, patients with AD displayed significant reduction in the percentage of DN iNKT cells ($p < 0.05$; Mann-Whitney U-test). The net percentage of IFN γ positive cells was calculated by subtracting the percentage of positive events in the unstimulated sample from the percentage of positive events in the activated sample. Boxes show net percentages of IFN γ positive cells, median, IQR, and ranges (5th and 95th percentiles), *: $p < 0.05$.

Figure 3. Intracellular IL-4 expression of iNKT cells

After stimulation of PBMCs obtained from AD patients and HCs with PMA/ionomycin for four hours, the cells were stained with CD4-ECD, 6B11-PE, CD8-PC7 and CD3-FITC surface markers, followed by intracellular IL-4-APC staining. The cells were analyzed on a Coulter FC500 flow cytometer. **a** Representative scatter plots displaying lymphocyte (Ly) gates and iNKT gates of unstimulated and PMA/Ionomycin (Io) activated samples from one AD patient and one control are presented. **b** Frequency of intracellular IL-4 positive cells was elevated in total CD3+6B11+ iNKT cells in AD patients compared with HCs. **c** Frequency of intracellular IL-4 in CD4+CD8 $^{-}$, DN (CD4 $^{-}$ CD8 $^{-}$) and CD4 $^{-}$ CD8 $^{+}$ iNKT subsets in AD patients and HCs.

Compared with HCs, patients with AD displayed significant enhancement in the percentage of DN iNKT cells expressing IL-4 ($p < 0.01$; Mann-Whitney U-test). The net percentage of IL-4 positive cells was calculated by subtracting the percentage of positive events in the unstimulated sample from the percentage of positive events in the activated sample. Boxes show net percentages of IL-4 positive cells, median, IQR, and ranges (5th and 95th percentiles), **: $p < 0.01$.

Fig. 1.

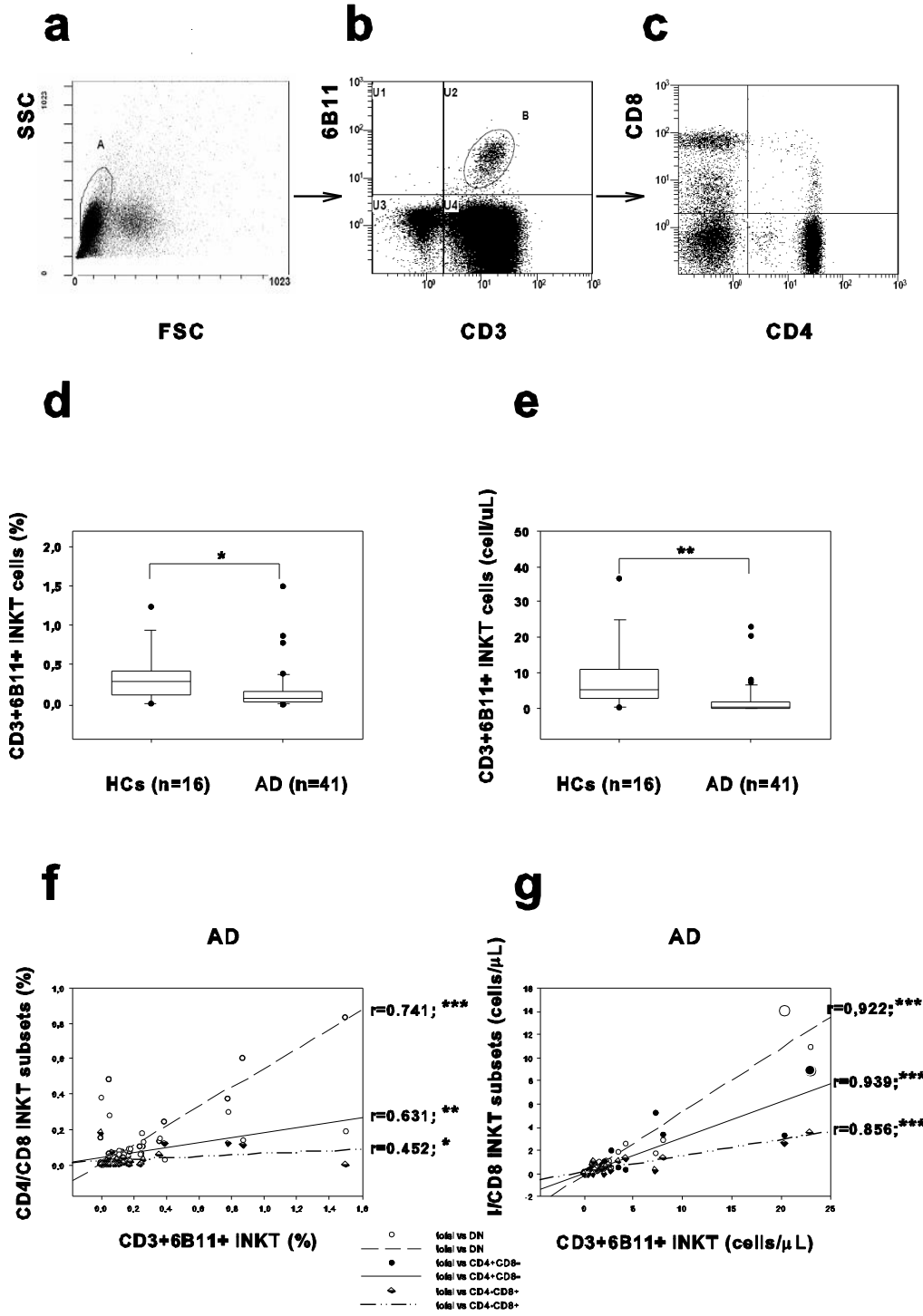


Fig. 2.

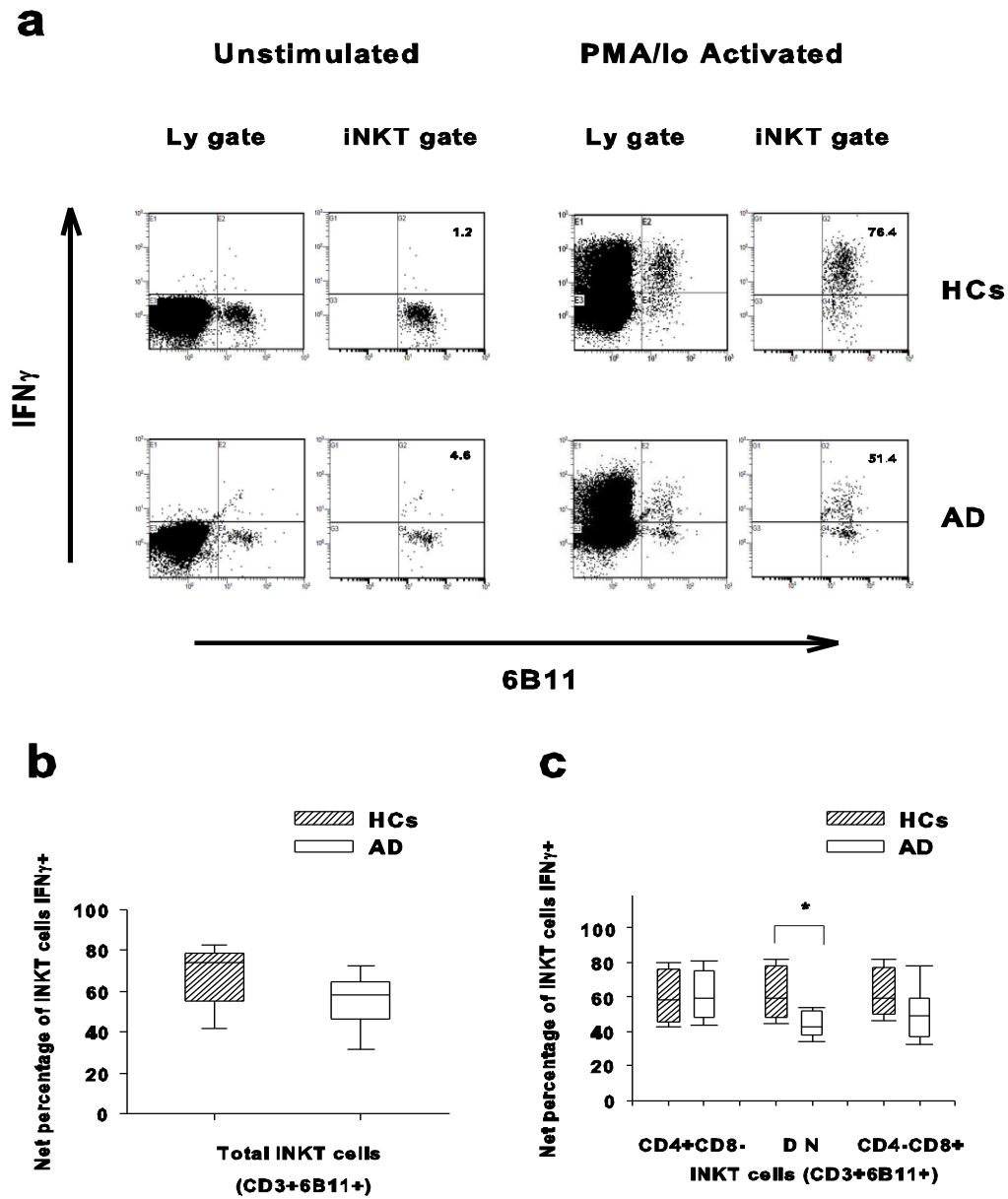


Fig. 3.

