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ORIGINAL ARTICLE

Proliferatory defect of invariant population and accumulation of non-invariant CD1d-restricted natural killer T cells in the joints of RA patients

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Abstract

Objectives. While numerical and functional defects of invariant NKT cells have been demonstrated in rheumatoid arthritis (RA), the detailed characterization of proliferative and secretory responses following CD1d-mediated presentation is lacking; the presence of non-invariant populations has never been assessed in human autoimmunity. We have evaluated both invariant and non-invariant populations in the blood and synovial fluid from patients to assess feasibility of NKT cell-directed manipulations in RA.

Methods. NKT cell populations were quantified by anti-CD4/anti-Vα24 staining and/or CD1d tetramers. Proliferation was measured in cultures of mononuclear cells following stimulations with αGalCer and cytokine secretion determined by multi-bead assay.

Results. We have confirmed a proliferative defect of *i*NKT cells in both peripheral blood and synovial fluid from RA patients, but no changes in baseline frequencies. Moreover, we have detected an enlargement of non-invariant cell pool in synovial fluid samples. In addition, we noted an evident Th2 shift following exposure to αGalCer and pronounced IL-6 secretion.

Conclusions. While RA patients suffer from defective proliferative responses of invariant NKT cells, non-invariant cells accumulate at the site of inflammation. While stimulation with αGalCer results in reduced TNF-α and increased suppressive IL-10, abundantly produced IL-6 could potentially contribute to the induction of Th17 cells in the joints.

Keywords

Autoimmunity, CD1d, Rheumatoid arthritis, αGalCer, NKT cells, Vα24$^+$ cells

History

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Introduction

Well-controlled experiments in a number of animal models of autoimmunity established that invariant NKT cells played an important role in controlling the emergence of autoreactive T cells [1,2]. The disappearance of *i*NKT cells from the circulation just prior to disease onset [3], the ability to prevent or delay clinical disease by the activation or transfer of NKT cells [1] and the accelerated disease progression in NKT cell-depleted animals provide robust evidence for the immunoregulatory role of these cells. This regulatory function of *i*NKT cells appears to be dependent on the secretion of Th2 cytokines, especially IL-4 [4].

Studies in human autoimmune conditions are less conclusive. The relatively low frequency of these cells in the circulation in humans makes their accurate quantification challenging. Nonetheless, many reports have proposed that numerical defects and reduced cytokine production by *i*NKT cells could be a feature of several human autoimmune diseases [5–8]. Of the human diseases, IDDM has been the most extensively investigated. However, initial reports of reduced *i*NKT cell numbers and function in IDDM [9] have subsequently been called into question [10,11].

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Constantly evolving definitions of NKT cells make the interpretation of the data in the literature difficult. The historical definition, a CD3$^+$ T cell expressing the NK cell marker, CD161, was abandoned following the discovery of the limited TCR expression patterns in these cells. In mice NKT cell-associated TCR complexes were found to contain the Vα14Jα chain [12] and the human equivalent was reported to be the Vα24JQ chain [13]; these are paired with a limited range of TCR Vβ chains [14,15]. NKT cells utilizing these TCR complexes were termed invariant NKT cells (*i*NKT). As the relationship between NKT cell subsets identified using the different definitions is not always clear, it is difficult to draw definitive conclusions on the role of NKT cells in the development of human autoimmunity.

In rheumatoid arthritis (RA) four studies have been conducted to date, with the number of RA patients was very limited in some of these. Three groups reported reduced *i*NKT cell frequencies in the peripheral blood [8,16,17] and reduced cytokine production following αGalCer exposure was also detected in two of these studies [16,17]. The presence of *i*NKT cells in the joints was investigated by two groups; both of these detected a reduced *i*NKT cell frequency [17,18]. In contrast, cells in the joints seem to produce a cytokine profile comparable to that seen in the peripheral blood of healthy volunteers [17].

Unfortunately, those RA studies have focused on the CD4 and CD8 negative population [16] and the experiments assessing the proliferative capacity of NKT cells were complex and included

the addition of exogenous cytokines [16,17]. However, it is now known that the addition of various cytokines skews the proliferative responses of NKT cell subsets, making the interpretation of the early RA data difficult [19].

Finally, identification of non-invariant CD1d-restricted NKT cells [20,21] adds complexity to the overall picture, especially as this novel subpopulation possessed some unique characteristics distinguishing them from their Vα24 + counterparts. Interestingly, opposite function has been attributed to them in tumor immunity and autoimmune inflammation in animal studies [22,23]. Surprisingly, however, to date no studies identifying this population in human autoimmunity has been published.

In current study we have evaluated frequencies and proliferative responses of *i*NKT cells in peripheral blood and synovial fluid samples from affected joints. In addition, the pool of non-invariant of CD1d-restricted cells was also assessed both in the blood and in the synovial fluid. Finally, we have conducted an analysis of secretory outcome following CD1d-mediated stimulation.

Materials and methods

Samples

After obtaining informed consent venous blood and/or synovial fluid was withdrawn from RA patients attending an outpatient clinic at Aintree University Hospitals NHS Foundation Trust, Liverpool, who satisfied the ACR criteria. Age and gender-matched control individuals, with no personal or family history of autoimmunity, were recruited from a panel of normal blood donors. Ethics approval for the study was obtained from the Liverpool Research Ethics Committee and the South Sefton Ethics Committee.

Quantification of NKT cells

For *i*NKT cell identification mononuclear cells were stained with anti-CD4 FITC (clone RPA-T4, BD Biosciences) and anti-Vα24 PE (clone C15, Beckman Coulter). For identification of CD1d-restricted population MCs were additionally stained with anti-CD19 PE (clone 4G7, BD Biosciences) and α-galactosyl-ceramide-loaded APC-conjugated CD1d tetramers (Alexis and Proimmune, respectively). Loading was achieved by 24–48 h incubation of the tetramers at 40-fold molar excess of the ligand at 37°C. Samples were acquired on CyAn SL Flow Cytometer (Dako, Glostrup, Denmark) and plots were analyzed with the Summit software (Version 4.3). For the analysis of tetramer staining CD19 + cells were excluded (Supplementary Figure 1 available online at http://informahealthcare.com/doi/abs/10.3109/14397595.2013.844309).

αGalCer-driven Vα24$^+$ cells expansion assay with PBMC

Cells were seeded into a 96-well plate in RPMI medium, supplemented with 10% of FCS, 1% Glutamine and 1% Pen/Strep. α-galactosylceramide (αGalCer, Alexis Biochemicals) was added at the concentration of 100 ng/ml. The cells were cultured for 14 days. Aliquots were stained as above on Days 4, 7, 10 and 14 and the supernatant was saved at the same time points. Fold increases were calculated by dividing maximum frequencies reached during 14-day long expansion period by the frequencies seen on Day 0.

Assessment of cytokine secretion

Supernatant samples were collected on Day 4 following αGalCer exposure. The concentration of secreted cytokines was determined using the Th1/Th2 11-Plex multi-bead assay kit (FlowPlex, UK Bio), carried out according to the manufacturer's instructions. Samples were acquired on a CyAn SL Flow Cytometer (Dako), and analyzed with the software provided by UK Bio.

Data analysis

Following testing for sphericity of data, comparison of total *i*NKT cell prevalence between sources was performed using a two-factor mixed factorial ANOVA. Time-series analysis for results of cell proliferation studies used two- and three-factor within subjects ANOVA. Post-hoc Bonferroni comparisons were applied. Comparison of cytokine results used Mann–Whitney U-tests as data were non-normally distributed. SPSS for Windows V14, (SPSS Corp, US) and Graph Pad Prism V4 were used. Significance was accepted at the 5% level throughout.

Results

Baseline iNKT cell frequencies in the peripheral blood and synovial fluid

The prevalence of *i*NKT cells in the blood of healthy volunteers was 0.424% (SD = 0.22) of all cells within the lymphocyte gate. The frequencies for different individuals ranged from 0.19% to 0.99%. Although we observed a trend towards a reduction of the size of the *i*NKT cell pool in RA patients, there was no significant difference between frequencies of all *i*NKT cells, nor those either with or without co-expression of CD4, between the groups. Also, there was no statistically significant difference between cell prevalence in blood and synovial fluid in RA patients (Figure 1A and B; ANOVA comparing all three categories, $F = 1.608$, $P = 0.21$). We found no correlation between the sizes of *i*NKT cell populations and the length of the disease (recorded as from the date of the first diagnosis; data not shown). Prevalence for the three sources of cells studied, by expression profile, is presented in the supplementary resources (Table 1).

Taken together we failed to observe any pronounced numerical defects of population of iNKT cells in both peripheral blood and synovial fluid from RA joints.

iNKT cells show reduced proliferative capacity in the blood and synovial fluid of RA patients

A defect of proliferative responses of *i*NKT cells in autoimmune diseases has been suggested previously, we therefore investigated these in our study groups. Firstly, PBMCs isolated from two healthy individuals were exposed to αGalCer and the frequencies of *i*NKT cells in these cultures were determined by daily enumeration of Vα24$^+$ cells. These preliminary experiments indicated that the earliest time point at which an increase of *i*NKT cell numbers could be detected was on Day 4, the response peaked between Day 7 and Day 10 and the number of Vα24$^+$ cells started to decline by Day 14 (data not shown). Thus, for the analysis of patients and the controls in the study groups *i*NKT cell numbers were determined at these time points.

As shown in Figure 2, *i*NKT cells derived from the blood of healthy volunteers increased in number with time. Although multiple-range testing (Bonferroni) showed that the difference between 0 and 10 days was not significant ($p = 0.12$), the overall time-series change was highly significant (one-way ANOVA $F = 4.24$, $p = 0.016$). The increase in cells with time for the healthy group was also highly significantly different overall compared with that for either source of cells from rheumatoid patients ($p = 0.005$ compared with RA blood and $p = 0.016$ compared with RA synovial fluid (RASF)). Figure 2 represents the mean frequencies of these cells in all healthy controls ($n = 20$), RA blood ($n = 22$) and synovial fluid ($n = 12$) samples.

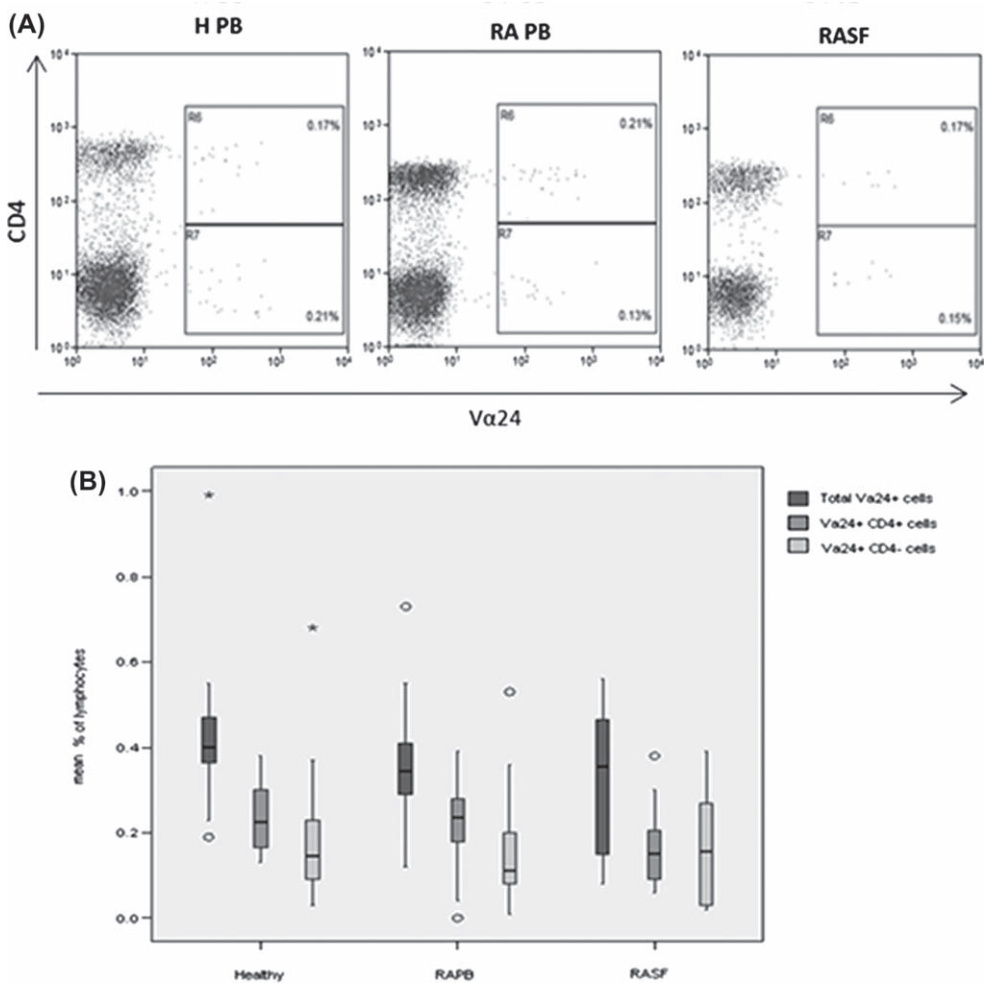

Figure 1. Frequencies of *i*NKT cells in RA blood and synovial fluid. A) Representative plots of CD4/Vα24 staining; B) Mean frequency of *i*NKT cells in peripheral blood of healthy controls (H), RAPB and RASF for the total Vα24$^+$, CD4$^+$ and CD4$^-$ cells (the latter represents a combined figure for both CD4/CD8 DN and rare CD8$^+$*i*NKT cells). Symbols represent outliers as identified by statistical analysis software likely reflecting biological variability.

There was a marked inter-individual variability in observed response within each group. Notably, even in the healthy control group there were two individuals whose *i*NKT cells did not proliferate following αGalCer challenge (Table 2). However, the lack of response of the RA blood and synovial fluid samples shown in Figure 2 was not primarily due to a higher number of individuals who did not respond, but reflected a general reduction in proliferative capacity of *i*NKT cells in these samples.

When CD4$^+$ and CD4$^-$ *i*NKT cells were analyzed separately, there was again a significant increase over time following stimulation in the group derived from healthy volunteers as compared to both RA sources (pairwise comparisons $p = 0.002$ healthy vs. RA blood, $p = 0.007$ healthy vs. RASF), but no difference between RA blood and synovial sources ($p = 1.0$). There was no significant difference in the expansion between CD4$^+$ and CD4$^-$ *i*NKT cells derived from healthy volunteers. Although there was a slight increase in cell numbers in those derived from either synovium or blood of RA patients, this failed to reach significance. There was a suggestion that CD4$^-$ *i*NKT cells derived from the blood, but not synovial fluid, of RA patients did not respond to stimulation in the same way as CD4$^+$ *i*NKT cells from the same patients. However, again, this difference failed to reach significance. At the same time, failure to proliferate was seen more often (50%), in the CD4$^-$ population than in the CD4$^+$ population (25%) in the patient blood samples (Table 2). Finally, the proliferative capacity of both subsets of *i*NKT cells in the synovial fluid was practically identical and the number of non-responders was also the same, 25%.

Table 1. Prevalence of the total and CD4$^{+/-}$ Va24$^+$ cells in the sample sources.

	Descriptive statistics		
Disease and source	Mean	Std. Deviation	*N*
Total Va24			
Healthy PB	0.4245	0.16353	20
RAPB	0.3636	0.13333	22
RASF	0.3300	0.16906	12
Total	0.3787	0.15487	54
CD4$^+$ Va24$^+$			
Healthy PB	0.2415	0.07795	20
RAPB	0.2200	0.09626	22
RASF	0.1658	0.09784	12
Total	0.2159	0.09305	54
CD4$^-$ Va24			
Healthy PB	0.1830	0.14582	20
RAPB	0.1436	0.11862	22
RASF	0.1642	0.12717	12
Total	0.1628	0.12985	54

To summarise, we have observed a pronounced defect of proliferation of *i*NKT cells obtained from peripheral blood and synovial fluid samples from RA patients following stimulation with a potent CD1d ligand.

Increased frequency of non-invariant CD1d-restricted NKT cells in RASF

CD1d tetramer/αGalCer staining revealed much higher frequencies of total NKT cells in synovial fluid obtained from RA patients compared with peripheral blood samples collected from both healthy donors and patients (mean values for percentages of CD1d tetramer + lymphocytes: 0.655; 0.672 and 1.591 for H, RA peripheral blood (RAPB) and RASF, respectively; Figure 3A and B).

The distribution of CD1d-restricted cell frequencies varied in all three groups. The RASF group had the highest numbers (up

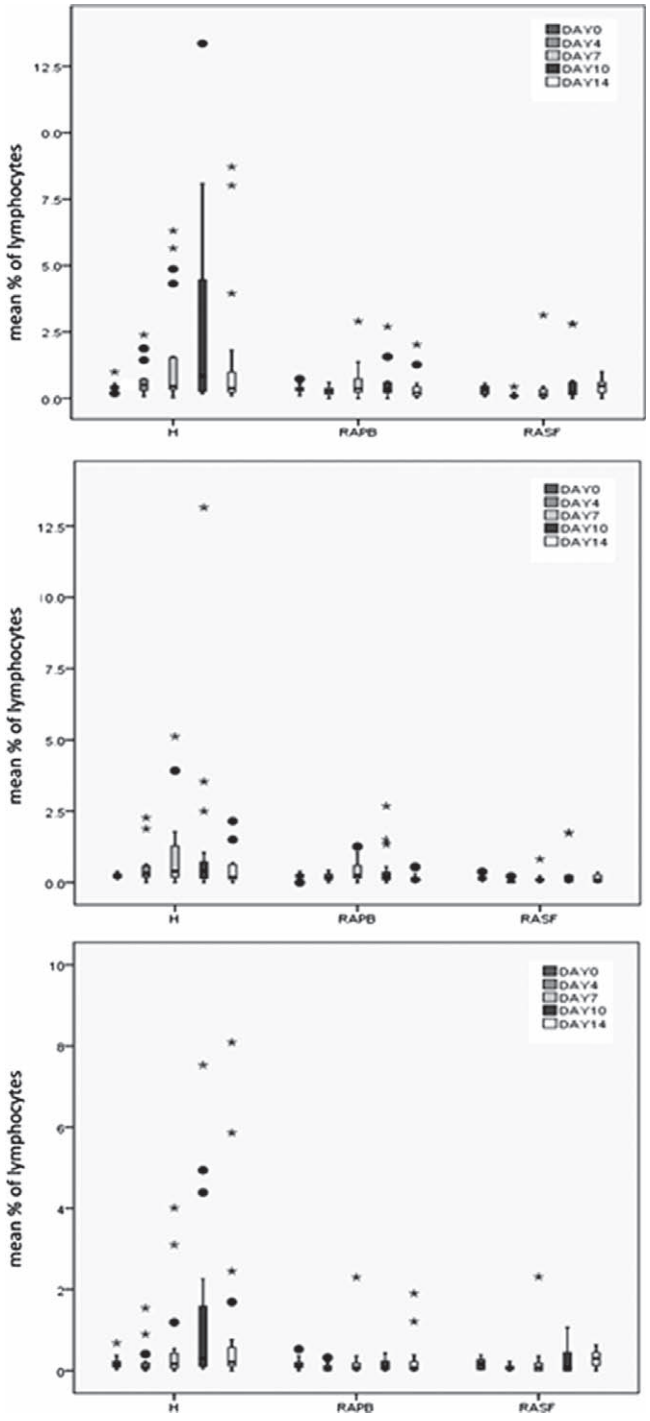

Figure 2. The time course of total *i*NKT expansion after αGalCer exposure. Mean frequencies of total invariant NKT cells observed in cultures of peripheral blood mononuclear cells of healthy donors and RA patients and SFMC from patients. The proliferative responses on Days 4, 7, 10 and 14 are shown; horizontal lines represent median frequencies. The overall time-series change one-way ANOVA $p = 0.016$. The increase in cell enrichment with time healthy versus RA blood and RASF were significantly different, $p = 0.005$ and $p = 0.016$, respectively. Symbols represent outliers as identified by statistical analysis software which reflect biological variability. Mean values for the H, RAPB and RASF groups: 7.59, 2.70, 4.97 and 12.7, 4.0, 4.97, $CD4^+$ and $CD4^-$ subset, respectively.

to 2.4% of all lymphocytes), intermediate numbers were seen in RAPB samples (1.39%) and healthy control PB samples contained only up to 1.05% of NKT cells within the lymphocyte population. The observed differences were statistically very highly significant between control blood and synovial fluid samples ($p < 0.0001$) and also between PB samples from RA patients and SF samples

Table 2. Fold increases in *i*NKT cell frequencies in αGalCer-stimulated mononuclear cell cultures.

	Fold increase			
	Min	Max	Mean	% of non-responders
$CD4^+$				
Healthy	1.14	39.85	7.59	10
RAPB	1.05	8.12	2.70 *	25
RASF	1.47	13.38	4.97	25
$CD4^-$				
Healthy	1.11	84	12.7	5
RAPB	1.05	15.8	4.0 **	50
RASF	1.62	10.66	4.97 ***	25

The percentage of cultures showing no evidence of *i*NKT cell proliferation is also indicated. *H vs. RAPB ($p = 0.03$), **H vs. RAPB ($p = 0.003$), ***RAPB vs. RASF ($p = 0.035$).

($p = 0.0002$). There was no correlation between the length of the disease since the initial diagnosis and CD1d-restricted population (data not shown).

Since there is ample evidence in the literature that CD4 + and CD4- NKT cells show differences in proliferative responses, cytokine secretion patterns and functional relevance [24–29], we used an anti-CD4 antibody to discriminate between these subpopulations. The frequency of CD4 + CD1d tetramer/α-GalCer+ cells among lymphocytes was increased for both RASF and peripheral blood compared with blood samples from healthy controls (mean values: 0.829; 0.412 and 0.274, SD = 0.49, 0.22 and 0.1, respectively; Figure 3B). The differences reached statistical significance between healthy donors' PB and synovial fluid samples from RA patients ($p = 0.002$) and between patients' peripheral blood samples and synovial fluid samples ($p = 0.02$).

A slightly different picture emerged for CD4- subpopulations; these cells were significantly decreased in the blood of the patients compared with the blood of healthy donors while in the synovial fluid an increase was seen with mean values of 0.381, 0.26 and 0.762 for healthy PB, RAPB and RASF, respectively (Figure 3B). Again, there was relatively large inter-individual variability (respective SD: 0.37, 0.1 and 0.37). However, significance was high with $p = 0.05$ between the blood of the patients and controls, $p = 0.007$ between control blood and RA SF and $p = 0.0005$ between blood and synovial fluid from RA patients. The abundance of CD4- CD1d-restricted NKT cells observed in the synovial fluid of patients was almost three times that of the blood of patients. The relative proportions of the CD4 + and CD4- subpopulations differed between patients and healthy donors. While in healthy individuals 40% of CD1d-restricted cells were CD4+, the proportion of these cells increased to 60% and 50% in the blood and synovial fluid of the patients, respectively (not shown). Again, we found no correlation between the sizes of these populations and the length of the disease since diagnosis (data not shown).

To assess the contribution of *i*NKT cells to the CD1d-restricted cell pool staining with anti-Vα24 antibodies was also carried out. The frequency of non-invariant NKT cells was calculated by subtracting Vα24 + cells from the total CD1d tetramer + cell population. This revealed a large increase of the non-invariant cell fraction in the joints, but not in peripheral blood, of RA patients (H vs. RASF $p < 0.001$, $p < 0.001$, $p = 0.003$ for the unfractionated, CD4 + and CD4- cell populations, respectively; Figure 4). Further comparison of relevant contribution of these subsets indicated that in healthy individuals Vα24 + NKT cells comprised around 84% of CD4 + and 48% of CD4- CD1d-restricted cells. Respective values in the blood of patients were 56% versus 67%. However, the striking differences were observed in the synovial fluid where only 22.5% of the CD4 + and 20% of the CD4- NKT cells expressed the Vα24 chain (data not shown).

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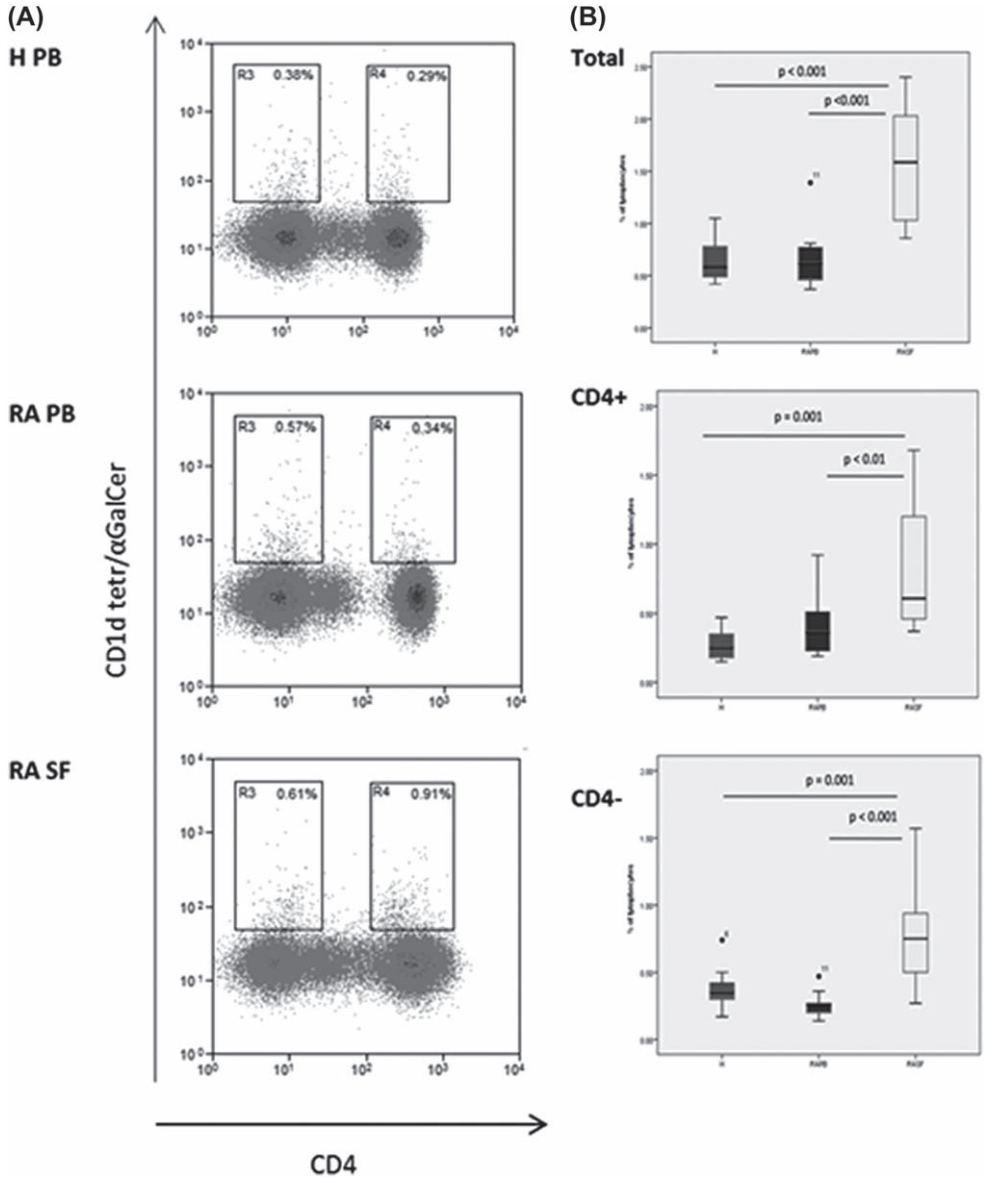

Figure 3. CD1d-restricted cells in the RAPB and RASF patients. A) Representative plots of CD4/CD1d tetramer staining; B) Mean frequencies of CD1d tetramer + NKT cells within lymphocyte populations in healthy controls (H), RAPB and RASF are shown. Upper panel: total CD1d-restricted cells; middle panel: CD4 + CD1d-restricted NKT cells; lower panel: CD4- CD1d-restricted cells. Symbols represent outliers as identified by statistical analysis software likely to reflect biological variability.

To conclude, we have observed a striking numerical increase of total CD1d-restricted cell population RASF as compared with peripheral blood samples from both patients and controls. This observed increase was a result of accumulation of non-invariant CD1d-restricted cells.

Cytokine production by CD1d-restricted NKT cells in RA

The lack of immunoregulatory role of *i*NKT cells, regulating the activation/proliferation of autoreactive T cells, has been attributed to deficiencies in cytokine production by these cells. While IL-4 and IFN-γ are generally measured in NKT cell studies, the secretion of other cytokines received very little attention. In the present study we therefore assessed 11 different cytokines by multi-bead approach. Summarized results of these experiments are shown in Figure 5.

We have observed statistically significant differences in secretion of six cytokines; levels of one of the cytokines, TNF-β, did not exceed the detection threshold in any of the cultures (results not shown). The two cytokines that were present at the highest concentrations in the culture supernatants were IL-8 and IL-6; of those secretion of IL-6 was increased from synovial fluid mononuclear cells (SFMC). IL-1β and IL-4 were reduced in the cultures of peripheral blood PBMCs of the patients compared with other sample sources tested. The production of IL-10 by synovial fluid NKT cells was higher than in the peripheral blood of RA patients, while a reduced IL-2 production was detected in the SFMC cultures. Finally, a strikingly reduced TNF-α secretion characterized NKT cells derived from both blood and synovial fluid of the patients.

Overall we have observed an interesting pattern of cytokine secretion evoked by CD1d-mediated antigen presentation, characterized by a reduction of TNF-α levels and increased IL-10 levels in the cultures of synovial fluid mononuclear cells; as well as increased IL-6 secretion.

Finally, we have also calculated the IL-4/IFN-γ ratios for all the secretory outcomes as this is often used to determine the bias of the response. However, while we have observed a trend towards a reduction of those ratios in the blood of RA patients, it was not statistically significant ($p = 0.056$, not shown). However, when we analyzed the ratio between TNF-α and IL-10, the cytokines that were interestingly changed in cultures of cells obtained from the patients, we observed a dramatic reduction in the disease (mean = 22.62; 8.64 and 0.78, for the healthy, RAPB and RASF groups, respectively). These results were highly statistically significant ($p < 0.01$ and $p < 0.00$, respectively),

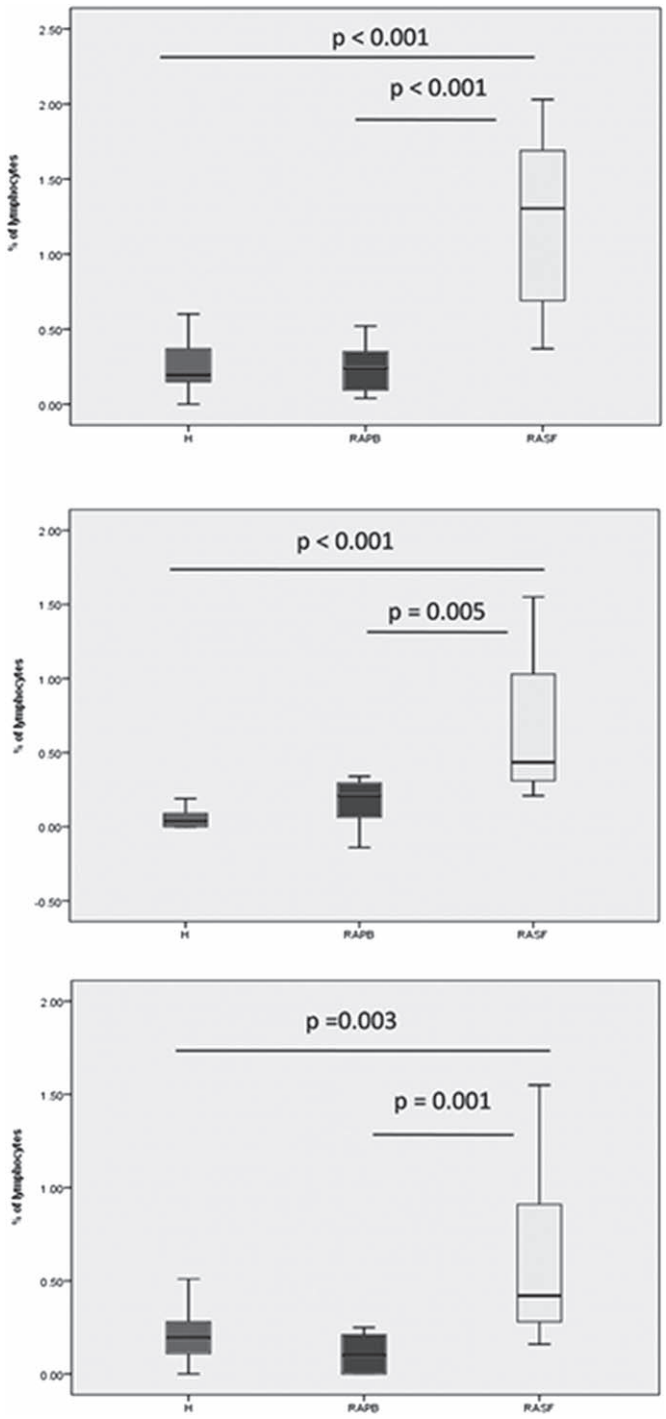

Figure 4. Frequencies of non-invariant CD1d-restricted NKT cells in RA. Non-invariant CD1d-restricted cells observed in the RAPB and RASF patients. The frequencies were calculated by subtraction of *i*NKT cell counts from the total frequencies of CD1d tetramer + NKT cells within lymphocyte populations in healthy controls (H), RAPB and RASF are shown. Upper panel: total non-invariant CD1d-restricted cells; middle panel: CD4 + non-invariant NKT cells; lower panel: CD4- non-invariant NKT cells.

suggesting that the use of TNF-α/IL-10 ratio could be more indicative of the outcome after than IL-4/IFN-γ ratio.

Discussion

NKT cells represent a subset of T lymphocytes that recognize antigens presented via the non-classical Class I molecule, CD1d [30] and respond by rapidly secreting large amounts of cytokines [27,29, 31, 32]. Experiments in animal models have shown that NKT cells participate in the rejection of tumors [33] and the control of bacterial antigens [34]. However, these cells received the most attention for their proposed role in controlling the emergence of self-reactive T cells [2,3,35–37].

In the presented work *i*NKT cells were defined as $CD4^+$ or $CD4^-$ T cells expressing the TCR Vα24 chain. A TCR Vβ11 chain-specific antibody was deliberately not used as this could have resulted in not detecting some of the CD1d-restricted *i*NKT cells [15] due to excessively restricted definitions. Using this approach we found that there was a trend towards reduced *i*NKT cell numbers in both the peripheral blood and synovial fluid of the patients. However, the only change that reached statistical significance was the reduction of $CD4^+$ *i*NKTs in the synovial fluid. In previous studies the reduction in NKT cell frequency in the peripheral blood of the patients seemed more pronounced [16,17]. The differences may be due to differences in the ethnicity of the investigated populations and the more permissive definition of *i*NKT cells used in the present study.

Proliferation following αGalCer challenge indicated a generalized reduction of *i*NKT cell responses after the antigen exposure. Previous reports allocated RA patients into a group of αGalCer-responders or non-responders [16,17]. However, these experiments used exogenous cytokine sources and introduced a re-stimulation step, which could result in altered both proliferative and secretory responses. In addition, these prior studies used end point analysis, giving a less-detailed picture. While the failure to proliferate after antigenic challenge was pronounced in the RA patients, it needs to be pointed out that the lack of the response did not absolutely discriminate the healthy controls and the patients; a subset of healthy individuals also showed relatively poor or no proliferation. Thus, it would appear that the dampened proliferative response under these circumstances does not necessarily predispose an individual to autoimmunity. We cannot exclude, however, that those were individuals prone to autoimmunity. This is also supported by the striking finding that there was not a single RA patient in whom the frequency of the proliferating *i*NKT cells exceeded 3.5%. This raises the strong possibility that defective CD1d-mediated antigen presenting pathway may be integral to the development of the immunological abnormalities seen in RA.

Additionally, while there is ample evidence suggesting a regulatory role for the invariant cell subset, non-invariant CD1d-restricted NKT cells [20,21] received very little attention and the frequency of these cells in human autoimmunity has not been investigated previously; in that regards accumulation of non-invariant NKT cells in the RA joints we observed was a very unexpected finding. To our knowledge this is the first study that has described such an increase of the non-invariant CD1d-restricted cell population at the site of autoimmune inflammation. At present our understanding of the functional relevance of non-$V\alpha24^+$ NKT cells is limited. Based on previous studies, it is widely accepted that a reduction in *i*NKT cells contributes to the development of autoreactivity. However, our observation on the accumulation of non-invariant NKT cells at the site of inflammation in RA suggests that this view may be overly simplistic.

Based on the current data it is not possible to state whether selective recruitment or in situ proliferation leads to the predominance of non-invariant NKT cells in the affected joints of RA patients. However, experimental evidence indicates that self-antigens presented by mature autologous dendritic cells are sufficient to induce the expansion of NKT cells in vitro [38]. Thus, an antigen-driven expansion of these cells in the joints is a distinct possibility. Our preliminary data, suggesting the expression of CD1d by synovial tissues in affected joints in RA (Gutowska-Owsiak et al., unpublished) raise the tantalizing possibility that CD1d-mediated antigen presentation could cause the expansion of non-invariant

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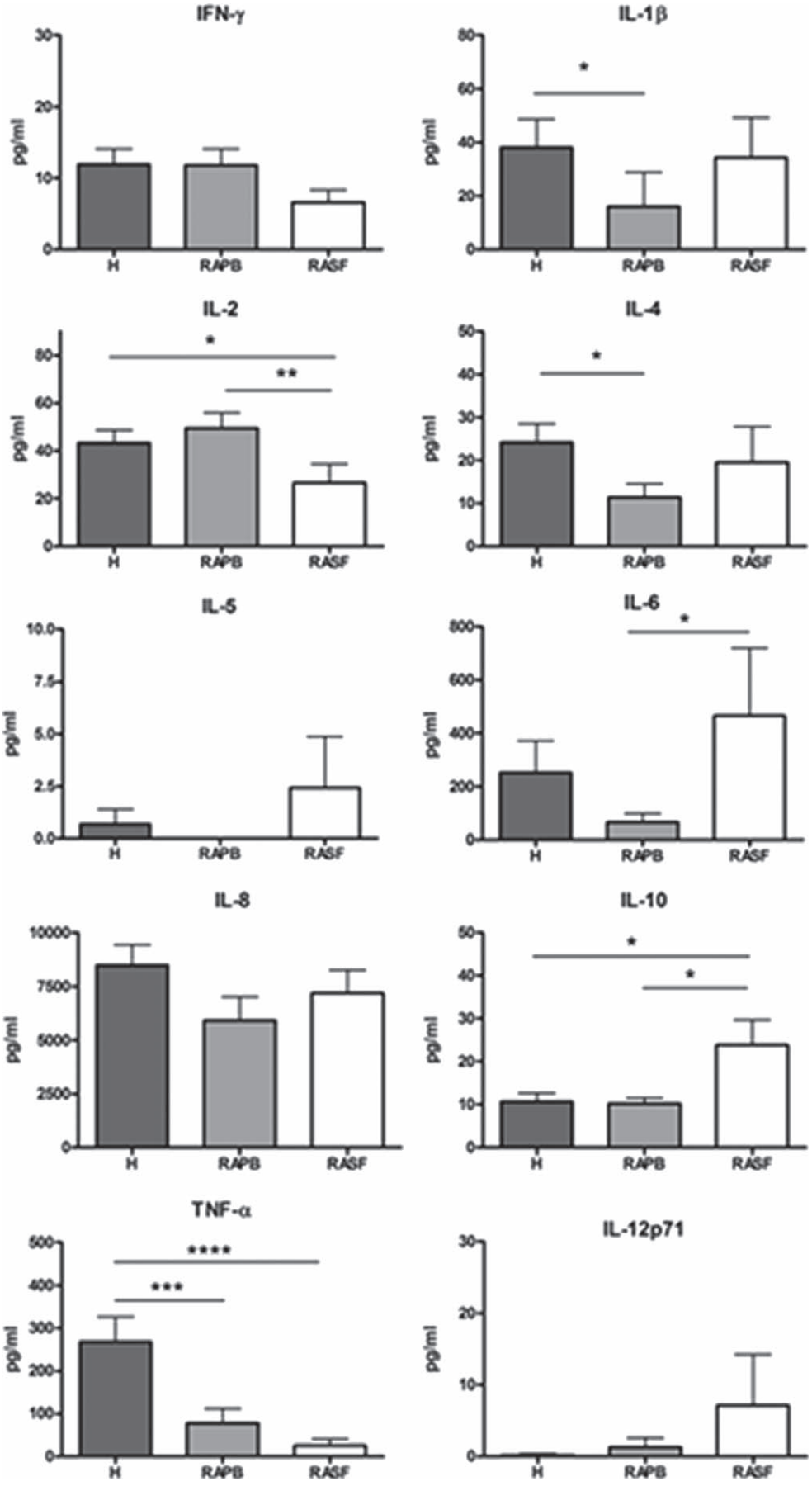

Figure 5. Cytokine profiles after CD1d-mediated stimulation. Cytokine secretion profile of mononuclear cell cultures on the fourth day following αGalCer exposure. Bars represent the mean cytokine concentrations in the culture supernatant measured using a multi-bead assay. As there was no detectable IL-5 and TNFβ production, these results are not shown. $^{*}p < 0.05$, $^{**}p < 0.02$, $^{***}p = 0.005$, $^{****}p = 0.0001$.

NKT cells. In previous work, we showed that there was selective recruitment or local proliferation of $CD56^{bright}KIR^{-}$ NK cells in the RA joints [39].

Finally, the analysis of the cytokine production following αGalCer exposure was more comprehensive in the present study than in previous publications. Obvious sources of those cytokines are invariant and non-invariant NKT cell populations that respond to αGalCer; some bystander activation would be also expected. While identification of cytokines source would be certainly interesting, we believe that the secretory pattern in the culture of mononuclear cells is more likely to indicate biologically relevant direction of responses than measuring secretion by purified cells alone.

The previously published data that reported reduction in IL-4 production [17] in the blood of patients and preserved IL-4 secretion by synovial fluid NKT cells were reproduced here. The calculation of IL-4/IFN-γ ratio did not prove to be helpful in the identification of RA patients; high IL-10 secretion combined with a low TNF-α response from synovial fluid NKT cells seems to be a better indicator of the disease. The altered cytokine secretion of these two cytokines alone would be considered inflammation-dampening and highly beneficial to the RA patients. However, we also observed very high secretion of IL-6, especially in SFMC cultures. Interestingly, along with IL-8, this cytokine was by far the most abundantly produced in all the cultures, including PBMC isolated from an RA patient who presented in the clinic with exceptionally enlarged *i*NKT cell population (totaling 7.5% of all lymphocytes; Gutowska-Owsiak et al., manuscript in preparation). Expression pattern of IL-6 and TNF-α by cells in our setting and is a rather surprising finding, since these proinflammatory cytokines are usually observed together. While a reduction of TNF-α would be beneficial, the identification of IL-6 upregulation which could consist a potentially Th17-promoting component of the response, makes αGalCer a less promising therapeutic agent. However, this does not necessarily disqualify all CD1d-presented antigens as medications for RA or other autoimmune diseases, since the outcome of the presentation vary depending on the ligand used [40–42]. In addition, since it has been also reported that IL-10 negatively regulates IL-17 production, including in an autoimmune setting [43–45], it is also likely that the fine balance between pro-arthrogenic and anti-arthrogenic factors could be still more beneficial under the stimulation that with no treatment.

The selective changes of cytokine production by synovial fluid-derived NKT cells raise the question whether cells accumulating there are different from those in the blood. Provided that their cytokine production pattern was sufficiently diverse, an enrichment of non-invariant NKT cells in the joints could explain the unique cytokine profile observed. CD1d-restricted cell subsets represent opposite roles in murine models of lymphoma and autoimmune hepatitis [22,23]. While existence of functional divergence in human pathology remains to be formally proven, the accumulation of the non-invariant cells at the site of active autoimmune inflammation would explain some pathologic events observed in RA, such as neutrophil influx into the joints.

Finally, it is important to remember that data from mice studies is still unclear. While NKT stimulation seems to be beneficial in some of the settings [40,46–48], other animal models suggested that a particular kind of the stimulation may be important [49–51]. Furthermore, important differences between humans and mice regarding CD1 system expression and function do not enable for direct comparisons. Therefore further human studies, perhaps with additional CD1d ligand, are required to dissect the patterns of the responses observed and select a suitable compound for treatment in patients. In summary, our study largely confirms the initial reports on defective *i*NKT cell population in autoimmunity; the data presented in this study suggest that while numerical *i*NKT cell defect may not be as pronounced as reported before, the proliferatory responses observed after antigen challenge are dramatically reduced in RA patients and may, therefore, play an important role in disease pathogenesis. In addition, the assessment of non-invariant NKT cell subset revealed unexpected accumulation of these cells at the site of inflammation; this phenomenon would require further investigation. Importantly, while it seems that secretory responses after CD1d-mediated stimulation have potential to shift the detrimental Th1 into Th2 secretory pattern that would dampen the inflammation, abundant expression of IL-6 could result in pathology via delayed induction of Th17 responses. Thus, we believe there is a strong need for additional studies in this field to fully understand the involvement of NKT cells in RA, especially in the context of design of novel treatment strategies that include CD1d ligands as therapeutic agents.

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Conflicts of interest

Authors declare no conflicts of interests.

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Supplementary material available online

Supplementary Figure 1.