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Research paper

Flow cytometric analysis of cytokine expression in short-term allergen-stimulated T cells mirrors the phenotype of proliferating T cells in long-term cultures

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ABSTRACT

Background: Allergen-specific T_H cells play an important role in IgE-mediated disorders as allergies. Since this T_H cell-population only accounts for a small percentage of T_H cells, they are difficult to phenotype without prior selection or expansion.
Methods: Grass-pollen-specific T_H cell profiles were evaluated in 5 allergic and 4 non-allergic individuals using three different approaches: CD154 expression on ex vivo grass-pollen-activated PBMCs (i); CFSE-dilution in grass-pollen-restimulated PBMCs (ii) and T cell lines enriched for allergen-specific T cells (iii).
Results: Relatively low numbers of allergen-specific T_H cells were detected using CD154 expression, limiting the power to detect phenotypic differences between allergic and non-allergic individuals. In contrast, higher frequencies of proliferating T_H cells were detected by loss-of-CFSE intensity in PBMCs and TCLs after grass-pollen-stimulation, resulting in the detection of significantly more IL-4 producing T_H cells in allergic vs non-allergic individuals. In addition, higher numbers of IFNγ producing T_H cells were detected in long-term cultures compared to the CD154 expressing T_H cells.
Conclusion: To detect allergen-specific T_H cells for a common allergen as grass-pollen, expansion is not absolutely necessary, although within 8-day grass-pollen cultures, higher numbers of proliferating T_H cells resulted in increased statistical power to detect phenotypic differences. However, this approach also detects more bystander activated T_H cells. TCLs resulted in comparable percentages of cytokine expressing T cells as 8-day cultures. Therefore enrichment can be necessary for detection of T_H cells specific for a single allergen or allergen-derived peptides, but is dispensable for the detection and phenotyping of allergen-specific T_H cells using crude extracts.

1. Introduction

Allergen-specific T_H cells play an important role in IgE-mediated disorders such as allergies. The production of high amounts of the T_H2 type cytokines IL-4, IL-5, and IL-13, are responsible for the development and maintenance of allergic diseases (Romagnani, 2000). Due to the low precursor frequencies of allergen-specific T_H cells (Givan et al., 1999;

Abbreviations: SIT, Allergen-specific Immunotherapy; FCS, Fetal Calf Serum; PBMC, Peripheral blood mononuclear cell; CFSE, carboxyfluorescein-diacetate–succinimidylester; BrdU, bromodeoxyuridine; CMV, Cytomegalovirus; TCL, T cell line; TCC, T cell clone.

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Rimaniol et al., 2003), they are difficult to phenotype without prior selection or expansion (Thiel et al., 2004). Therefore an easy-to-use, and reproducible method to detect allergen-specific T_H cells is important to study the immunopathogenesis of allergic diseases. Moreover, accurate T-cell phenotyping is important to monitor therapies targeting T-cells in the treatment of allergic diseases, such as allergen-specific immunotherapy (SIT).

MHC-II tetramers bound to a specific peptide, target T_H cells with specific TCR (Casares et al., 2001; Hackett and Sharma, 2002; Van Overtvelt et al., 2008; Wambre et al., 2008; Bonvalet et al., 2011). Despite the high specificity, the applicability is limited by the fact that only a selection of defined HLA alleles can be used, the immunodominant peptides have to be predefined and recombinant proteins produced. Furthermore, in most patients the number of T_H cells specific for a single allergen-derived peptide is extremely low, limiting the possibility for direct characterization. Therefore simultaneous detection of T cells reactive to all immunogenic epitopes in a complex antigen mixture such as allergen-extracts could be advantageous.

Approaches toward the detection of all T_H cells reactive toward a complex allergen-extract, without the need for prior patient HLA typing, are based on T_H cell activation or proliferation after allergen stimulation. One recently described technique is based on the activation marker CD154, which is transiently expressed on T_H cells after allergen-specific stimulation of peripheral blood mononuclear cells (PBMCs) (Campbell et al., 2010; Frentsch et al., 2005). This technique allows allergen-specific T_H cells to be studied without long-term in vitro culturing. Another method uses tracking of allergen-stimulated proliferation of CD4+ T cells by reduced carboxyfluoresceine-diacetate succinimidylester (CFSE) intensity or by DNA incorporation of bromodeoxyuridine (BrdU) (Houck and Loken, 1985; Fazekas de St Groth et al., 1999; Munier et al., 2009). These techniques allow identification of allergen-stimulated cell division, and can be combined with standard intracellular cytokine stainings.

In this study we directly compare the use of the activation marker CD154 and reduced CFSE-intensity to phenotype allergen-specific T_H cells. CD154 is measured, after 16 h of in vitro allergen-specific stimulation, whereas CFSE profiles are measured after restimulation of 8-day PBMC, and T cell line (TCL) cultures in the presence of grass-pollen-extract. Our data shows that, although a lower statistical power, the phenotype of CD154-expressing T_H cells after 16 h ex vivo stimulation faithfully predicts the cytokine profiles of the long-term cultures with a reduced frequency of bystander T_H cell activation.

2. Methods

2.1. Patients

The study was approved by the local Medical and Ethical committee, and all volunteers gave a written informed consent before participation. Blood samples were obtained from grass-pollen-allergic (n = 5) and non-allergic (n = 4) individuals outside the grass-pollen-season, between January and April 2010. Both groups were sex and age matched; 60% males with a mean age of 28.4 for the allergic-individuals, and 75% males with a mean age of 33.3 for the non-allergic individuals. Allergic patients suffered from rhinitis during the grass-pollen season, but not from allergic asthma, or atopic dermatitis. No patients underwent specific-immunotherapy, and no medication was used, at time of blood collection. Allergic sensitization was defined as specific IgE>2 kU/l (Phadia, Uppsala, Sweden).

2.2. 16 h grass-pollen-specific stimulation

1×10^6 PBMCs/well were stimulated in a 96-well U-bottom plate (Greiner Bio-one, Frickenhausen, Germany) for 16 h with grass-pollen-extract (60 μg/ml, ALK-abello, Hørsholm, Denmark, Endotoxin levels <0.05 EU/ml in culture) or SEB (Staphylococcal enterotoxin B) (5 μg/ml, Sigma-Aldrich), in the presence of 1 μg/ml soluble CD28-specific antibody (28.2, BD Pharmingen, Franklin Lakes, USA), which is critically required as costimulatory molecule for antigen-specific T cell activation, and their cytokine expression (Van Neerven et al., 1998). After the first 2 h of stimulation, Brefeldin A (10 μg/ml, Sigma-Aldrich) was added to the cultures. Optimal stimulation conditions were determined based on the expression of CD154 after stimulation with different concentrations (2.5–120 μg/ml) and after different times of stimulation (6–24 h) (data not shown).

2.3. CFSE labeling

10×10^6 cells (PBMCs or TCLs) were stained in 0.5 ml of CFSE solution in a final concentration of 10 μM for 15 min at 37 °C. To stop the reaction, cells were washed 3 times with RPMI 1640 (Bio-Whittaker) supplemented with 10% Fetal Calf Serum (FCS).

2.4. 8-day PBMC cultures

PBMCs labeled with CFSE were cultured in 24 well plates (3524 Costar, Cambridge, Mass, USA) in 500 μl of Ultra Culture Medium (BioWhittaker) supplemented with 2 mM glutamine and 2×10^{-5} M β-mercaptoethanol in the presence of 60 μg/ml of grass-pollen-extract. Wells without grass-pollen-extract served as control. At day 7 cells were restimulated in flat-bottom 96-well plates with plate-bound anti-CD3 (ON 30 μl 5 μg/ml, OKT-3), and soluble anti-CD28 (1 μg/ml, BD biosciences) for 16 h in the presence of 10 μg/ml brefeldin A.

2.5. T cell lines

Oligoclonal TCLs were developed as described earlier by Bohle et al. (2005). In short, PBMCs (1.5×10^6) were stimulated with 60 μg/ml grass-pollen-extract in 24-well plates (Costar), in 500 μl of Ultra Culture Medium (BioWhittaker) supplemented with 2 mM glutamine and 2×10^{-5} M β-mercaptoethanol. After 4 days, 10 U/ml human rIL-2 (Roche, Basel, Switzerland) was added to the cultures. Cultures without grass-pollen-extract served as control. At day 7, T cell blasts were harvested by means of density gradient centrifugation, and unspecifically expanded using irradiated PBMCs and IL-2. Ten days after the last feeding, TCLs were labeled with CFSE, and restimulated with grass-pollen-extract (2.5 μg/ml) in the presence of irradiated (60 Gy) autologous PBMCs at a final concentration

of 1.5×10^6 cells/well. At day 5 these cells were restimulated in flat-bottom 96-well plates with plate-bound anti-CD3 (ON 30 µl 5 µg/ml, OKT-3), and soluble anti-CD28 (1 µg/ml, BD biosciences) for 16 h in the presence of 10 µg/ml brefeldin A.

2.6. Flow cytometry

PBMCs were washed with cold (4 °C) dPBS, and stained for surface markers at 4 °C for 30 min in dark with the following antibodies: CD3-eFluor605 (eBioscience, San Diego,USA), CD4-PerCP, CD8-Alexa-Fluor700, and CD69-APC-Cy7 (all BD-Pharmingen). Subsequently, cells were fixed using 2% formaldehyde (Merck KGaA, Darmstadt, Germany) in PBS during 20 min at RT in dark, and permeabilized using 0.1% saponin (Sigma-Aldrich) and 0.5% BSA (Sigma-Aldrich) in PBS. Intracellular antibodies: IL-4-PE, CD154-PE-Cy5 (BD Pharmingen), IFNγ-PE-Cy7 (eBioscience). Isotype controls as well as non-restimulated cells served as control. Measurements were performed using a BD FacsCanto, or a BD LSR-II flow Cytometer (BD Pharmingen), and analyzed using Flowjo 9.2 software (Tree Star, Inc, Ashland, Ore).

2.7. Statistics

Differences between the T_H cell responses from the two groups are detected using non-parametric Mann–Whitney Tests, and results are expressed as median (range). When groups were normally distributed, Student's T test was used, and results expressed as mean ± SEM. A p-value <0.05 was considered to be statistically significant (GraphPad Prism 4 for Mac; Inc, La Jolla, USA). To determine the power of the different methods, Cliff's delta was calculated to determine the effect size of non-parametrically distributed data sets. The effect size measures the strength of a relationship between two variables in a statistical population, and is a simple way of quantifying the difference between two groups. In our case, the effect size represents the average difference taking into account the variation, between the allergic and non-allergic individuals detected using the different methods.

3. Results

3.1. Direct detection of allergen-specific T cells after 16 h of grass-pollen-stimulation

Detection of ex-vivo allergen-activated T-cells after 16 h of stimulation with grass-pollen-extract and anti-CD28 was based on the expression of activation markers CD69 and CD154 within the T_H cell population (Fig. 1A). PBMCs cultured in the presence of medium alone, or medium supplemented with anti-CD28 did not express CD154 in combination with CD69 (Median (range): 0.11% (0.09–0.13) and 0.09% (0.09–0.12) respectively). After grass-pollen-stimulation, both allergic (n = 5) and nonallergic (n = 4) individuals expressed CD69 and CD154, with no difference detected between allergic and non-allergic individuals (Table 1).

The expression of activation markers CD69 and CD154 on T_H cells cultured in the presence of SEB served as a positive control (Fig. 1A). Significantly more T_H cells expressed CD69 and CD154 (7.2% ± 1.4) after SEB stimulation compared to grass-pollen-stimulated PBMCs in both allergic and non-allergic individuals (0.57% ± 0.18, $p < 0.0001$). Again no difference was observed in the number of activated T_H cells between allergic (8.0% (7.1–12.8)) and non-allergic (5.4%

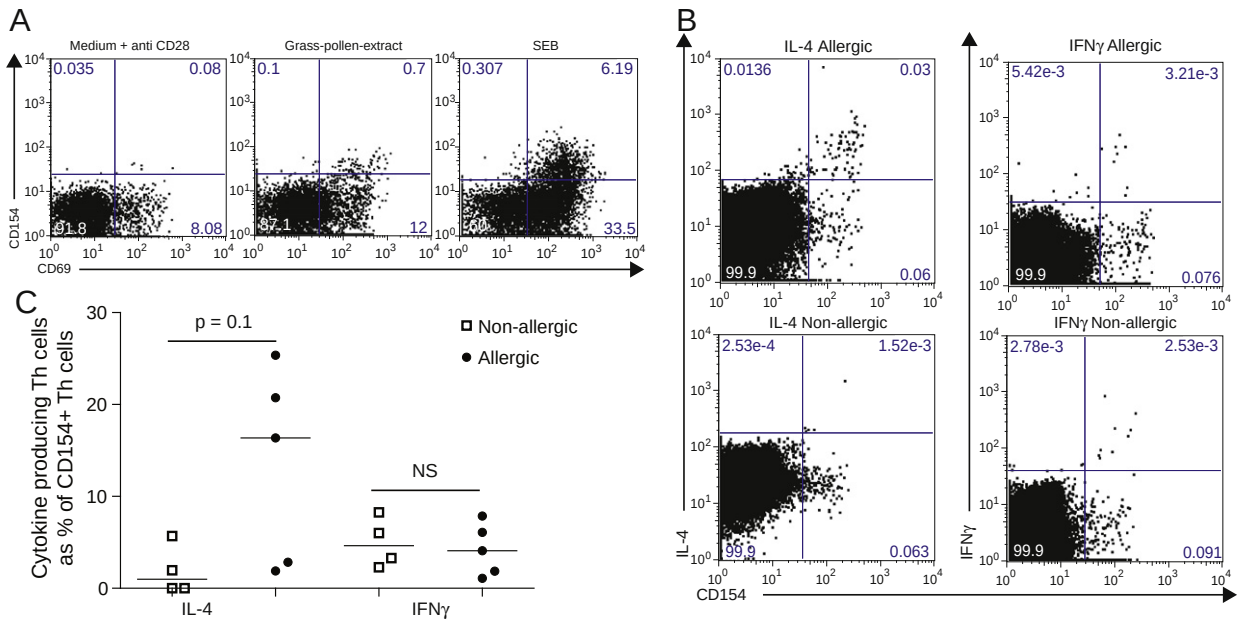

Fig. 1. Flow cytometric assessment and phenotyping of grass-pollen-specific T_H cells according to intracellular antigen induced CD154 expression. (A) Comparison of CD154 expression in CD3+ CD8− T cells cultured for 16 h in the presence of medium, grass-pollen or SEB. (B) T_H cells from a representative allergic and non-allergic individual stimulated with grass-pollen-extract for 16 h. Intracellular IL-4 and IFNγ versus CD154 expression of CD3+ CD8− T_H cells are analyzed. (C) The percentages of IL-4 and IFNγ producing cells within the CD154 expressing T_H cell population of 4 nonallergic (open squares) and 5 allergic (solid rounds) individuals. The symbols represent individual patients, and the line indicates the mean value.

Table 1
(a) % of allergen-specific T cells detected within the total cell population. % of IL-4 (b) and IFNγ (c) producing allergen-specific T_H cells. NS: non significant, *: $p<0.05$.

	(a) Allergen-specific T_H cells %			(b) % IL-4$^+$ from a			(c) % IFNγ$^+$ from a		
	A	NA	S	A	NA	S	A	NA	S
CD154	0.5 (0.3–0.8)	0.3 (0.07–0.9)	NS	16.4 (1.9–25.4)	1 (0–5.7)	NS	4.1 (1–7.9)	4.6 (2.3–8.3)	NS
8-day PBMC	6.9 (4.8–11.1)	5.8 (2.2–14.2)	NS	3.3 (1.9–10.4)	0.5 (0–0.6)	*	20.1 (15.6–30.3)	18.6 (5.8–26.6)	NS
TCL	34.7 (22.3–65.9)	42.3 (34.5–56.1)	NS	6.9 (2–11.3)	1.3 (0–2.2)	*	6.9 (3.8–27.2)	12.1 (3.5–12.9)	NS

(3.0–7.9)) individuals ($p=0.34$). All CD154 expressing cells co-expressed CD69 (Fig. 1A), therefore CD154 can be used as a single marker to detect recently activated T_H cells.

To characterize the T_H1 or T_H2 type cytokine profile of CD154$^+$ T_H cells from grass-pollen-allergic and non-allergic individuals, we evaluated their intracellular IL-4 and IFNγ expression. Fig. 1B, shows representative dot plots of the intracellular cytokine staining of CD154-positive T_H cells after grass-pollen-stimulation from one allergic and one non-allergic individual. Higher numbers of IL-4 producing cells were found in the CD154 expressing T_H cells from allergic compared to non-allergic individuals, though reached no statistical significance (Table 1, $p=0.1$). The number of IFNγ producing cells within the CD154 expressing T_H cells was similar in allergic and non-allergic individuals (Fig. 1C, and Table 1). Therefore a higher number of T_H2 cells was detected in allergic individuals, while in non-allergic individuals a clear T_H1 response was detected.

To compare the power to detect statistical differences in the number of IL-4 producing CD154$^+$ T_H cells between allergic and non-allergic individuals, the effect size (ES) was calculated ($ES=0.7$) using Cliff's delta for nonparametric measurements.

Within these short-term cultures, an allergen-specific population was detected using the activation marker CD154, with a trend toward increased numbers of IL-4 producing cells in allergic individuals.

3.2. Detection of allergen-specific T_H cells by CFSE dilution after 8-day PBMC cultures in the presence of grass-pollen-extract

After 8-day cultures of CFSE-labeled PBMCs in the presence of grass-pollen-extract (60 μg/ml) or medium alone, PBMCs were stained for CD3 and CD4, to select the T_H cell population. Within the wells cultured with grass-pollen-extract, proliferating T_H cells could be detected by reduced CFSE-intensity, which were not observed in the PBMCs cultured in medium alone (Fig. 2A). The allergen-specific T_H cell subpopulation, detected after culturing with grass-pollen-extract, constituted a larger fraction of the total T_H cell population compared to the ex vivo analysis using CD154 expression (Fig. 2B, Table 1). To characterize the T_H1 or T_H2 type cytokine profile of these allergen-specific T_H cells identified by reduced CFSE-intensity, the cultures were restimulated for 16 h using plate bound anti-CD3, and soluble anti-CD28. In Fig. 2C, representative dot plots from the same individuals as in Fig. 1B show the IL-4, and IFNγ stainings for the CFSE-low T_H cells.

We found that in allergic individuals ($n=5$), significantly more CFSE-low T_H cells produced IL-4, compared to non-allergic individuals ($n=4$) ($p=0.01$), while the percentages of IFNγ producing cells were comparable between allergic and non-allergic individuals (Fig. 2D, Table 1). Remarkably the percentage of IFNγ producing allergen-specific T_H cells detected after 8-day culture was much higher compared to percentage within the CD154 expressing T_H cells after 16 h of ex-vivo grass-pollen-restimulation. The statistical power of this method to detect differences in the percentage of CFSE-low PBMCs from allergic and non-allergic individuals producing IL-4 was 1 (Cliff's delta).

Within 8-day PBMC cultures, a larger allergen-specific population was detected compared to the CD154 expressing T_H cells after 16 h of grass-pollen-stimulation, with a significantly higher number of IL-4 producing cells in allergic individuals.

3.3. Detection of allergen-specific T_H cells in TCLs enriched for grass-pollen-specific T cells

Subsequently TCLs enriched for grass-pollen-specific T cells were generated. TCLs were labeled with CFSE, and after 6 days of grass-pollen-extract restimulation, TCLs were stained for CD3 and CD4 for T_H cell selection. Grass-pollen-extract stimulated TCL cultures showed more proliferating cells compared to TCLs cultured in medium alone, although background proliferation was higher compared to 8-day PBMC cultures (Fig. 3A). Using loss-of-CFSE to define allergen-specific T cells, we observed an increased allergen-specific subpopulation within the total T_H cell population in TCLs after 6 days of grass-pollen-restimulation (after correction for background proliferation), compared to the 8-day PBMC cultures, indicating an enrichment for allergen-specific T_H cells due to blast cell enrichment and expansion (Fig. 3B, Table 1).

To study the cytokine expression in the CFSE-low TCL cells, we restimulated the cultures using plate bound anti-CD3, and soluble anti-CD28. Fig. 3C shows representative dot plots of IL-4 and IFNγ staining on CFSE-low TCLs from the same individuals as shown in Figs. 1B and 2C. Within the CFSE-low T_H cells from the TCLs, higher numbers of IL-4 producing cells were detected within allergic compared to non-allergic individuals ($p=0.03$), while the percentages of IFNγ producing cells were comparable between allergic and non-allergic individuals (Fig. 3D, Table 1). The statistical power of this method to detect differences in the percentage of divided TCL T_H cells from allergic and non-allergic individuals producing IL-4 was 0.9 (Cliff's delta).

We find that TCLs show a higher subpopulation of allergen-specific T_H cells compared to 8-day PBMC cultures. Also within this subpopulation significantly more IL-4 producing T_H cells were observed in allergic compared to non-allergic individuals.

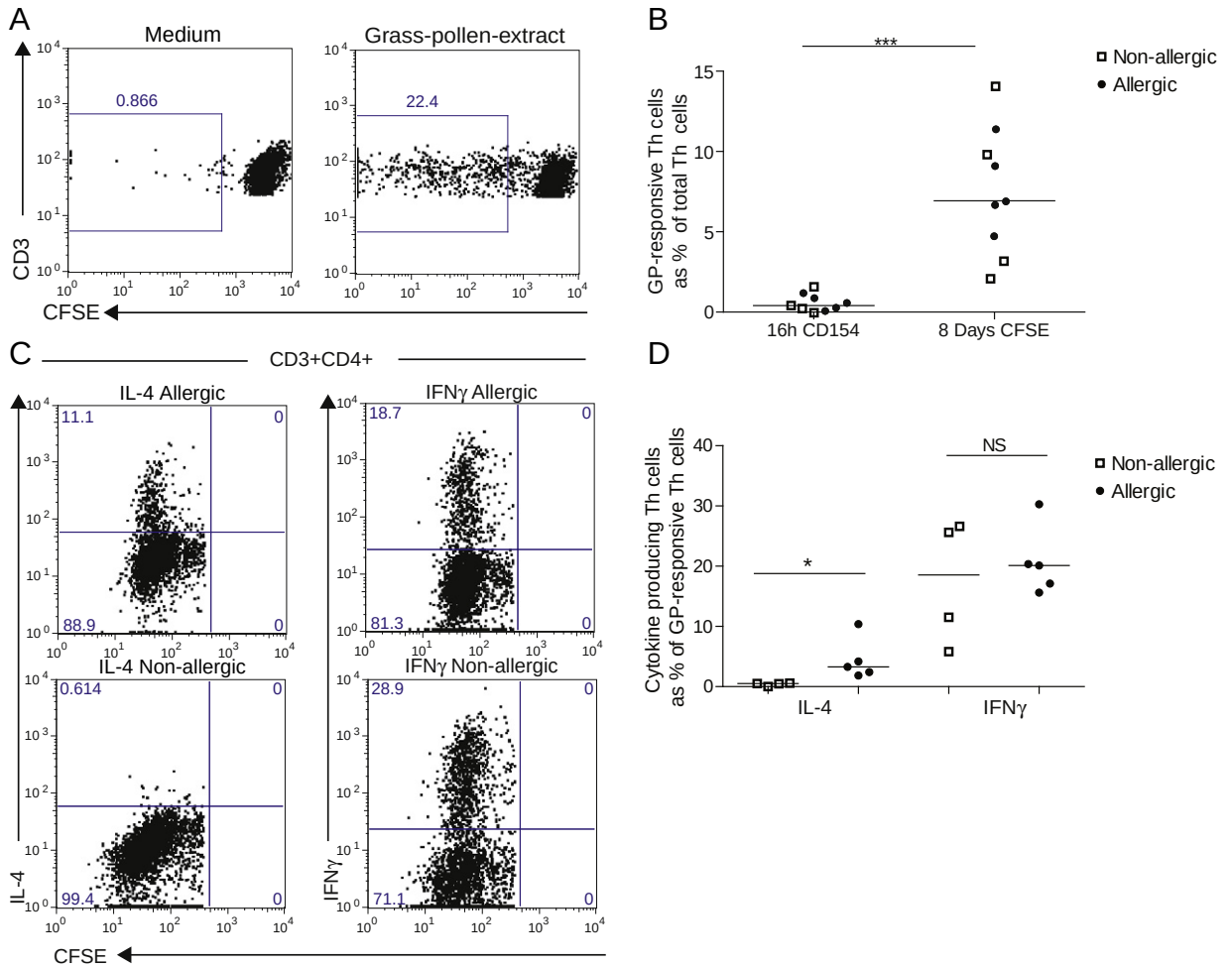

Fig. 2. In vitro tracking of dividing T_H cells labeled with CFSE, cultured in the presence of grass-pollen-extract (60 μg/ml). (A) Representative flow cytometric dot plots of CD3+ CD4+ T cell proliferation. Left plot shows the background CFSE-dilution, right plot the CFSE-dilution in response to grass-pollen-extract (60 μg/ml) after 8 days of PBMC culturing. (B) Percentages of T_H cells detectable for the analysis of cytokine expression using the expression of CD154 or proliferation by CFSE-dilution as different selection methods. (C) Representative flow cytometric dot plots of IL-4 (left) and IFNγ producing CD3+ CD4+ T cells from a representative allergic (top) and non-allergic (bottom) donor. PBMCs were stained with CFSE, cultured in the presence of grass-pollen-extract during 8 days. Cytokine expression is shown as a function of cell division (CFSE) after restimulation with plate bound anti-CD3 and soluble anti-CD28. (D) The percentages of IL-4 and IFNγ producing cells within the CFSE-diluted T_H cell population of 4 non-allergic (open squares) and 5 allergic (solid rounds) individuals. The symbols represent individual patients, and the line indicates the mean value.

4. Discussion

In the present study, we show comparative results between different methods to detect intracellular cytokine profiles of allergen-specific T_H cells using flow cytometry. We used the expression of activation marker CD154 to detect ex-vivo allergen-activated T_H cells, and compared it to loss-of-CFSE-intensity after 8-day PBMC cultures in the presence of grass-pollen-extract or after enrichment of grass-pollen-specific T cells in TCLs, as alternative approaches to detect the same cells. Irrespective of the method used, more IL-4 producing T_H cells were detected in allergic compared to non-allergic individuals.

After 16 h of grass-pollen-stimulation, approximately 0.5% of T_H cells expressed CD154, representative for the number of T_H cells responsive to the grass-pollen-extract. These results are in line with different studies determining the percentage of antigen-specific CD4+ T cells by limited dilution analysis using CFSE dilution, or tritiated thymidine incorporation (Givan et al., 1999; Rimaniol et al., 2003) as well as with other studies using CD154 to detect allergen-specific T-cells (Campbell et al., 2010; Frentsch et al., 2005). Due to the low precursor frequencies, the number of T_H cells expressing CD154 upon allergen-specific stimulation is limited, resulting in low numbers of events and increased variation. Allergen-exposure e.g. pollen seasons, have shown to increase the number of allergen-specific T cells, and their cytokine expression (Wambre et al., 2008). Therefore, the number of CD154 expressing T_H cells can vary depending on the exposure to the allergen. Nevertheless the value of this method is the absence of phenotypic deviation due to the short-term of the culture.

To increase the low number of allergen-specific T_H cells, PBMCs were cultured in the presence of grass-pollen-extract, resulting in the proliferation, and expansion of the allergen-specific population. Moreover using CFSE-labeled PBMCs,

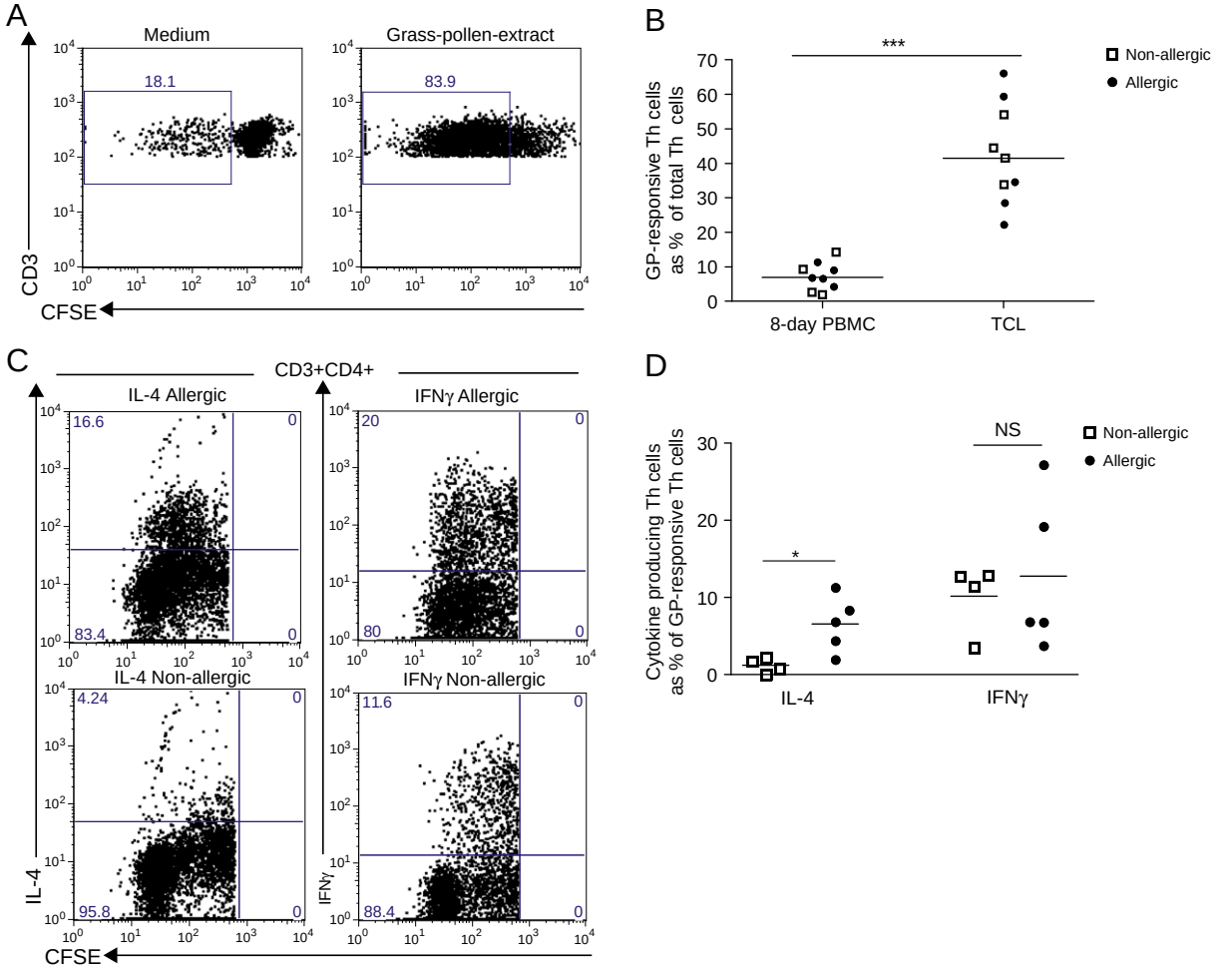

Fig. 3. In vitro tracking of dividing TCLs, labeled with CFSE cultured for 6 days in the presence of grass-pollen-extract (2.5 μg/ml) and irradiated autologous PBMCs. (A) Representative flow cytometric dot plots of CD3+ CD4+ T cell proliferation of TCLs enriched for grass-pollen-specific T cells. Left plot shows the background CFSE-dilution in the presence of autologous feeders alone, right plot the CFSE-dilution in response to autologous feeders in the presence of grass-pollen-extract (2.5 μg/ml) after 6 days of culturing. (B) Percentages of T_H cells proliferating after 8 days of PBMC and 6 days of TCL culturing in the presence of grass-pollen-extract. Each symbol represents an individual patient, and the horizontal line indicates the mean value. (C) Representative flow cytometric dot plots of IL-4 (left) and IFNγ producing CD3+ CD4+ T cells from the TCL dividing upon the 6 day culture in the presence of the grass-pollen-extract. Cytokine expression is shown as a function of cell division (CFSE) after restimulation with plate bound anti-CD3 and soluble anti-CD28. Dot plots from a representative allergic (top) and non-allergic (bottom) donor are shown. (D) Mean ± SEM of the number of IL-4 and IFNγ producing cells within the CFSE-diluted T_H cells after enrichment for allergen-specific T cells are shown for 4 non-allergic (open) and 5 allergic (solid) individuals.

allergen-reactive T_H cells could be readily detected after 8-days of culturing, which represented a strongly increased subpopulation of allergen-specific T_H cells, compared to 16 h of ex-vivo grass-pollen-stimulation. Remarkably after 8 days of culture the detection of CD154 on restimulated cells lost its specificity (data not shown), which is in line with a recent study comparing CD154 expression with MHC class-II tetramer complexes after 2 weeks of in vitro culturing (Bonvalet et al., 2011). Within the TCLs, 5 times more proliferating T cells were detected compared to the 8-day cultures, indicating an expansion of the allergen-specific subpopulation. The higher number of IL-4 producing T_H cells in allergic compared to non-allergic individuals did not reach statistical significance after 16 h of ex vivo grass-pollen-stimulation, although in 8-day PBMC or TCL cultures the number of IL-4 producing T_H cells was significantly increased within allergic compared to non-allergic individuals. Power calculation based on Cliff's delta for non-parametrically distributed data, showed the higher power to detect differences in the percentage of IL-4 producing T_H cells between allergic and non-allergic individuals in the 8-day PBMC cultures (ES = 1). The TCL cultures showed a slightly lower power of 0.9, while within the CD154 expressing cells after 16 h of grass-pollen-restimulation the power was the smallest (0.7).

Immune deviation within the blood of allergic individuals toward a dominant T_H2 type response is widely accepted and clearly demonstrated [7, 8, 16, 17, 21, 22], however the role of IFNγ producing T cells is less clear. Our results show no impairment of allergen-specific T cells from allergic individuals to produce IFNγ. In agreement with our results, no differences in IFNγ production were found after 6 h of polyclonal (Magnan et al., 2000), or 18 h of allergen-specific stimulation (Akdis et al., 2004) between atopic and non-atopic individuals. Moreover, no differences in IFNγ-production were observed

after 6–8 days allergen co-cultures (Moverare et al., 2000; Bullens et al., 2004; Lindstedt et al., 2005). In contrast it is hypothesized that allergen induced T_H1 cytokine production is down regulated in allergic vs non-allergic individuals. This is demonstrated by Van Overtvelt et al., who showed increased percentages of IFNγ producing Bet v 1-specific CD4+ T cells in non-allergic individuals using MHC Class II Peptide tetramers in Bet v 1 T cell cultures (Van Overtvelt et al., 2008; Wambre et al., 2008). Also earlier studies using allergen-specific T cell clones (TCCs) showed more IFNγ producing TCCs in healthy compared to allergic individuals (Wierenga et al., 1991; Ebner et al., 1995).

The disadvantage of activation or proliferation markers to detect antigen-specific T_H cells is the risk of bystander T cell detection, activated in a non-allergen-specific fashion, or phenotype skewing during culture. Compared to the 16 h stimulations, 8-day PBMC and TCL cultures were characterized by increased percentages of IFNγ producing T_H cells. Similar numbers of IFNγ producing T_H cells were observed after Bet v 1-specific cultures, where the authors demonstrated, by combining CFSE and MHC-II labeling, that most of the IFNγ producing T_H cells were not allergen-specific (Wambre et al., 2008). It can therefore be assumed that also in our case, the IFNγ producing cells are not allergen-specific. Moreover outgrowth of specific T_H cell subpopulations can occur due to preferential proliferation rates, or apoptosis (de Waal Malefyt et al., 1993; Akdis et al., 2003), and non-dividing or anergic allergen-specific T_H cells, that may produce (regulatory) cytokines, will not be detected. Factors present during culturing, as anti-CD28 or the CFSE used for PBMC-labeling, may skew the T_H cell phenotype, or also activate nonspecific T_H cells. As a result, not all detected T_H cells will be allergen-specific, and they will not necessarily include all allergen-specific T_H cells.

Interestingly, the cells producing the highest levels of cytokines after 8 days of PBMC culture are those that proliferated most strongly. This phenomenon was not observed in the proliferating T_H cells from the TCLs. T_H cells are known to acquire the ability to synthesize and secrete cytokines in a division dependent manner, thereby evolving into a more effector T_H cell phenotype (Bird et al., 1998; Gett and Hodgkin, 1998), which may offer an explanation for the difference between the primary culture and the TCLs. After T cell blast enrichment, proliferating T cells are selected, resulting in effector-like phenotypes. Our observations are in line with Fazekas de St Groth et al. who also noticed the highest levels of cytokine production by the strongest proliferating cells within primary PBMC cultures (Fazekas de St Groth et al., 1999).

To conclude, our data suggests that by using CD154 expression, a relatively low number of allergen-specific T_H cells are detected, nevertheless the phenotype observed by this method faithfully reflects the phenotype in long-term cultures. Within 8-day cultures of CFSE-labeled PBMCs, higher numbers of proliferating T_H cells resulted in a higher statistical power, although more bystander activated T_H cells were detected. This enrichment is important to detect phenotypic differences in T_H cells specific for allergens, to which a lower number of precursor T_H cells are present, as recombinant allergens. Even more specific T_H cells could be detected within TCLs, advantaging the detection of T cells specific for a particular part of an allergen, as peptides. Therefore to detect allergen-specific T_H cells for a very common allergen, as grass-pollen, expansion is not absolutely necessary, and short-term stimulation is advantageous over longer cultures to avoid the risk of phenotype skewing due to bystander activation.

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