T lymphocyte phenotype of contact allergic patients

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T lymphocyte phenotype of contact allergic patients: experience with nickel and *p*-phenylenediamine

Kate Wicks[1], Clare Stretton[1], Amy Popple[1], Lorna Beresford[1], Jason Williams[2], Gavin Maxwell[3], John P. Gosling[4], Ian Kimber[1] and Rebecca J. Dearman[1]

[1]Faculty of Biology, Medicine and Health, University of Manchester, [2]Contact Dermatitis Investigation Unit, Salford Royal NHS Foundation Trust and [3]Unilever Safety and Environmental Assurance Centre, Colworth Science Park, Sharnbrook and [4] School of Mathematics, University of Leeds, Leeds.

Address for correspondence: Dr Kate Wicks, University of Manchester, Faculty of Biology, Medicine and Health, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK. Tel: 441612755368. Email: kate.wicks@manchester.ac.uk

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SUMMARY

Background

There is considerable interest in understanding the immunological variables that have the greatest influence on the effectiveness of sensitization by contact allergens, particularly in the context of developing new paradigms for risk assessment of novel compounds

Objectives

To examine the relationship between patch test score for 3 different contact allergens and the characteristics of T-cell responses.

Patients/Materials/Methods

A total of 192 patients with confirmed nickel, *p*-phenylenediamine (PPD) or methylisothiazolinone allergy were recruited from the Contact Dermatitis Investigation Unit at Salford Royal Hospital. Severity of allergy was scored by patch testing and peripheral blood lymphocytes characterized for T cell phenotype by flow cytometry and proliferative activity by radiolabelled thymidine incorporation. Comparisons were drawn with buffy coat samples from healthy volunteers.

Results

Patch test positivity to nickel, PPD and MI was associated with changes in the phenotype of peripheral blood T-cells: increases in naïve cells, decreases in Treg cell frequency and the $CD4^{+}$:$CD8^{hi}$ ratio and increased expression of the skin homing marker CLA, particularly for those patients with a +++ patch test score.

Conclusions

This increased understanding of the characteristics of the T cell responses to contact allergens may provide parameters to better measure health risks associated with skin sensitization.

KEY WORDS
Lymphocyte transformation test; T-cell phenotype; CD4; CD8; inverted CD4:CD8, skin homing CLA

1. INTRODUCTION

Skin sensitization resulting in allergic contact dermatitis is an important health issue, and it is now clear that many hundreds of chemicals, and possibly in excess of several thousands, have some potential to cause skin sensitization (1). There remains a considerable interest in defining the cellular and molecular mechanisms through which skin sensitization is acquired and allergic contact dermatitis elicited, particularly in the context of developing new paradigms for risk assessment of novel compounds. Of particular relevance is the nature and dynamics of T lymphocyte responses to contact allergens, and contributions made by functional subpopulations and differentiated subsets of T-cells (2-7).

One important tool for characterization of human T-cell responses to contact allergens is the lymphocyte transformation test in which the proliferation of cultured T lymphocytes induced by contact allergens, or by contact allergens conjugated with proteins, is measured (8-15). Experience with the lymphocyte transformation test has been predominantly with metals, and in particular nickel, although somewhat variable results have also been reported with certain organic contact allergens, including: 2,4-dintrochlorobenzene (9), methylisothiazolinone (MI)(7), and *p*-phenylenediamine (PPD) (11, 12).

In the present investigations the lymphocyte transformation test has been used to explore further the characteristics of T lymphocyte responses to nickel (as nickel sulfate), and to PPD, a proven contact allergen in hair dyes and henna tattoos (1)(16), (delivered in culture as a hapten-protein conjugate). Furthermore the relative frequency of discrete subpopulations of T lymphocytes has been measured in subjects sensitized to PPD or nickel. In previous investigations of subjects with allergic contact dermatitis to MI it was found that there existed an association between skin sensitization and the relative frequency of $CD4^+$ and $CD8^+$ naive, memory and effector T lymphocytes (7). Comparable studies have been

conducted here with PPD and nickel to determine whether similar patterns are found in subjects sensitized to these contact allergens and previously published analyses of MI have been extended. Finally, in addition, experiments have been performed to determine whether sensitization to PPD or nickel (and also to MI) is associated with changes in the relative frequency in peripheral blood of regulatory T-cells (Treg) (17) or skin homing (CLA^+) T lymphocytes (18).

2. MATERIALS AND METHODS

2.1 Patient recruitment

A total of 192 individuals were recruited to this study (NRES Ethics Committee North West – Greater Manchester East [12/NW/0602]). All subjects were recruited from patients attending the Contact Dermatology Investigation Unit at Salford Royal Hospital for diagnosis of contact allergy and gave their informed consent to participate in this study. Of the 57 individuals recruited who were allergic to nickel, five were also allergic to PPD. Of the 53 individuals recruited who were allergic to PPD, five also had an allergy to nickel. In addition to the 56 individuals with MI allergy who had been reported in the previous publication (7), a further 27 individuals were recruited, of whom eight also had an allergy to nickel or PPD.

2.2 Healthy volunteers

Blood samples were obtained from generally healthy individuals who met the NHS Blood and Transplant Service screening criteria for blood donation (n=14). Samples were obtained in the form of a 'buffy coat' preparation, which was prepared by centrifugation of whole blood at 18^{o}C within 24 h of blood draw, and received by our laboratory for processing within the following 48 h.

2.3 Patch testing and blood draw

Patch testing was performed on the skin of the upper back using Finn chambers (8mm inner diameter) that contained potential contact allergens at a standard concentration (nickel: 5% (as nickel sulfate); PPD: 1%; MI: 0.2%). After four days, patients' reactions were scored as no reaction (-), weakly sensitized (+), strongly sensitized (++), or extremely sensitized (+++) according to standards set by the International Contact Dermatitis Research Group and up to 30 mL peripheral blood taken into a lithium heparin-coated collection tube (Sigma Aldrich, St Louis, Missouri). Of the patients recruited, six consented to an additional, pre-patch test blood draw of up to 30 mL, which was taken immediately before application of

the Finn chambers. This was in addition to the post-patch test blood sample, which was taken four days after application of the Finn chambers as standard.

2.4 Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation of a maximum of 30 mL whole blood (diluted 1:1 in PBS), layered over an equal volume of Histopaque 1077 (Sigma Aldrich), according to the manufacturer's protocol. An aliquot of $1x10^6$ cells was collected and stored in 10% DMSO/90% human AB serum (Sigma Aldrich) at -80^{o}C for flow cytometric analysis, and the remaining cells cultured for thymidine incorporation assays. Cells for thymidine incorporation assay were cultured as described previously (2) in the presence or absence of cognate contact allergen or carrier control (nickel sulfate: 0.5-50 µg/mL; free PPD: 0.01-1 µg/mL ; PPD conjugated to HSA: 10-100 µg/mL; HSA alone: 100 µg/mL), medium alone.

2.5 Preparation of PPD conjugates

PPD conjugates were prepared as previously described (3). Briefly, four separate preparations of PPD-HSA were made by incubating 15 mM PPD (Sigma Aldrich; a 100x molar excess) with 10 mg/mL (150 µM) HSA (Sigma Aldrich) in PBS for 2 days. After dialysis against PBS for three days, the solution was lyophilized for 24 h and stored at -80^{o}C until required. PPD-HSA was dissolved in PBS for cell activation. Only two of the batches of conjugate resulted in cell activation; these batches were used for subsequent experiments. New batches of PPD-BSA were tested in a proliferation assay alongside the existing batch using both patient samples and negative control samples. Only those new batches giving comparable results to the existing batch were used in subsequent assays.

2.6 Thymidine incorporation assays

Thymidine incorporation assays were performed on freshly isolated cells cultured for five days as described above. For the final 16-20 hours of culture, cells were pulsed with 22.2 μBq [^{3}H]-thymidine (specific activity 2Ci/mmol; Amersham International, Amersham, UK) per well. Cells were harvested into scintillation fluid and incorporation of tritiated thymidine measured by βscintillation counting. A positive proliferative response was defined as one that resulted in a stimulation index (SI: mean disintegrations per min of test sample divided by mean disintegrations per min of medium alone control sample) of ≥2.5 as compared with control (medium only) cells(2).

2.7 Flow cytometric analyses

Flow cytometric analyses of T-cell population composition were performed using a standard protocol and one of four antibody panels designed to detect CD4+ve T-cell subsets, CD8+ve T-cell subsets, Tregs, and expression of CLA by $CD4^{+}$ and $CD8^{+}$ cells, respectively. All cells used for flow cytometric analysis had been frozen upon receipt at the laboratory and were thawed prior to staining. Antibody panels were as follows: panel 1: allophycocyanin (APC)-anti-CD4, fluorescein isothiocyanate (FITC)-anti-CD45RA, and phycoerythrin (PE).Cy5-anti-CD27; panel 2: APC-anti-CD8, FITC-anti-CD45RA, and PE.Cy5-anti-CD27; panel 3: FITC-anti-CD4 and APC-anti-CD25 pool, PE-anti-FoxP3; panel 4: FITC-anti-CD4, PE.Cy5-anti-CD8, and eFluor660-anti-CLA. All antibodies were from eBioscience (San Diego, California) and were used at 1/20 dilution with staining carried out in flow cytometry staining buffer for 30 min at 4^{o}C. Single stained controls were used to set compensation settings prior to acquisition and to establish a compensation matrix for data analysis. Flow cytometry was performed on a minimum of 10,000 cells using a FACS-calibur (Becton Dickinson, Mountain View, California). FlowJo (TreeStar, Ashland, Oregon) was used for analysis. Gating was standardized across all samples. Non-viable cells and debris were excluded from analyses by gating on the viable lymphocyte population based on forward and side scatter. Gates were then drawn to delineate the $CD4^{+}$ population and the $CD8^{hi}$ population. For T-cell subset analysis, quadrants were drawn to divide both the $CD4^{+}$ population and the $CD8^{hi}$ population into

$CD27^+$ $CD45RA^+$ (naïve cells), $CD27^+$ $CD45RA^-$ (central memory), $CD27^-$ $CD45RA^-$ (effector memory), and $CD27^-$ $CD45RA^+$ (EMRA) subsets (19). Representative flow plots showing the gating strategy for $CD4^+$ and $CD8^{hi}$ cells are shown in Supplementary Figure 1. CD4:CD8 ratios were then calculated from the percentage of $CD4^+$ cells and the percentage of $CD8^{hi}$ cells of the total viable lymphocytes. CLA^+ cells were defined as those that expressed CD4 or high levels of CD8, and also expressed CLA. Tregs were defined as $CD4^+$ $CD25^+$ cells that also expressed FoxP3.

2.8 Statistical analyses

Statistical analyses were performed using the computing environment R (v3.2.5, R Core Team, 2016). Unless otherwise stated, comparisons were performed between samples taken from the general population ('buffy coat' group) and each of the three sensitized sub-groups (+, ++, and +++). For the ratios of $CD4^+$ to $CD8^{hi}$ cells for nickel-sensitive, PPD-sensitive and MI-sensitive patients, abundance of Tregs, and expression of CLA, the nonparametric Mann–Whitney U test was used. For comparisons of distribution of $CD4^+$ and $CD8^+$ T-cells across the four T-cell subsets, including in samples taken from the same individual pre- and post-patch test, these compositional data were first transformed using an additive log ratio (as described in (20)) to remove linear dependence, and a paired Hotelling's T^2 test (21) was then applied to test for significance of differences across the multivariate means.

3. RESULTS

3.1 Lymphocyte proliferative responses to contact allergen

The vigour of the T-cell response to contact allergen was assessed in individuals identified by patch testing to be weakly (+), strongly (++) or extremely (+++) allergic to nickel or to PPD (a small number were allergic to both as detailed in the methods). PBMCs were cultured for 120 hours with various concentrations of cognate allergen at a variety of concentrations, in the presence of radiolabelled thymidine for the final 16-20 hours of culture. An SI of greater than or equal to 2.5 was regarded as being indicative of a biologically meaningful level of proliferation (7). For nickel sensitive donors, the range of SI values recorded and proportions of positive donors were similar for weakly (5/7) and strongly (7/12) allergic individuals (Fig. 1A). However, only 5/12 extremely allergic (+++) donors achieved a positive SI. Previous reports have demonstrated that despite individuals being strongly allergic to PPD, proliferative responses to free organic PPD (rather than the known metabolite Brandowski's base) were either not detected or only detected in a minority of patients unless they had extremely severe allergic reactions (11, 22-24). Our approach was to prepare water-soluble conjugates of PPD with the carrier protein HSA; interestingly although four batches of conjugate were prepared under identical conditions only two of these conjugates elicited robust proliferative responses (data not shown). Whereas negative or a minority of borderline positive results were recorded in the presence of carrier protein alone, marked proliferative responses were observed when cells were stimulated with PPD-HSA conjugate, particularly in those individuals with +++ patch test scores (8/11) (Fig. 1B). Fewer patients in the ++ and + patch test groups had SI values of >2.5 (3/8 in each group). Interestingly, this +++ cohort of patients also responded to free PPD in greater numbers (14/16) and with greater vigour than did weakly (+; 2/13) or strongly allergic patients (++; 4/17).

3.2 Characterization of T lymphocyte naïve and memory populations in peripheral blood of contact allergic individuals

We have shown recently that the composition of the T-cell compartment in the peripheral blood of patients with allergy to MI was significantly altered compared to healthy (buffy coat) controls, with increases in the proportion of naïve $CD4^+$ and $CD8^+$ T-cells and a corresponding decrease in central memory, effector memory, and EMRA cells (7). For comparison we have now characterized the T-cell populations in nickel and PPD allergic patients by flow cytometric analysis of the proportions of $CD4^+$ and $CD8^+$ T-cells in each of four T-cell subsets: naïve, central memory, EMRA, and effector memory, defined on the basis of expression of CD27 and CD45RA (19). The $CD4^+$ or the $CD8^{hi}$ population was gated (Suppl. Fig. 1), and then the proportion of cells in each of four subpopulations determined in comparison with healthy (buffy coat) control samples. The patient cohort for each contact allergen was subdivided based on degree of allergy (+, ++, +++) as described previously. In nickel-allergic individuals, an increase in naïve $CD4^+$ T-cells and a corresponding decrease in EMRA and effector memory cells was observed across all three patient cohorts compared to the healthy controls, but this only reached statistical significance in the severely allergic patients (+++) (P <0.01; Fig. 2A). The same analysis of the $CD4^+$ T-cell compartment of PBMCs from PPD-allergic patients showed the same trend of increases in the proportion of the naïve cell subpopulation with corresponding decreases in EMRA and effector memory cells but such did not reach statistical significance (Fig. 2B). The composition of the $CD8^{hi}$ T-cell compartment was then analysed for the cohorts of nickel- and PPD-allergic patients. The $CD8^{hi}$ population in both cohorts showed a trend for an increased proportion of naïve cells, and a contraction in the EMRA and effector memory populations compared to the healthy controls. This change in population composition was statistically significant in strongly nickel-allergic individuals (++) (P <0.05; Fig. 2C) and in both the strongly (++) and extremely (+++) PPD-allergic cohort (P <0.001 and P <0.05, respectively; Fig. 2D).

Given that the blood sampling in these studies is performed four days after application of diagnostic patch tests, a potential explanation for the expansion of the naïve T-cell population and a contraction of memory populations in the allergic patients is that there is recruitment of allergen-specific memory T-cells to the site of application, which depletes the numbers detectable in peripheral blood. To examine this possibility, blood samples were taken from individuals with either a weak (+) or a strong (++) allergy (n=3 for both groups) immediately prior to application of the suspected contact allergen for patch testing, and at the standard time point (4 days after initiation of patch testing). The proportions of $CD4^+$ and $CD8^{hi}$ T-cells in the naïve, central memory, EMRA and effector memory subsets were then analysed by flow cytometry as described previously (Fig. 3). Although there was some inter-individual variation in the relative contribution of the different subsets, it was notable that there were no significant changes in either CD4 or CD8 cell phenotype with time, even for those individuals that had the highest proportion of naïve cells at the day 4 time point. This supports the hypothesis that individuals with allergic contact dermatitis may have an expanded pool of naïve T-cells and this is independent of whether there is an ongoing skin reaction.

Finally, the ratio of $CD4^+$ to $CD8^{hi}$ cells was also calculated for the same samples. For the buffy coat control donors, a mean $CD4^+$: $CD8^{hi}$ value of approximately 2.6 was recorded, although there was considerable inter-individual variation. This is consistent with previous studies of healthy donors where ratios of 2.0 and 2.3 were observed (25,26). In the nickel-allergic cohort, a significant decrease in the $CD4^+$:$CD8^{hi}$ ratio was demonstrated in strongly allergic individuals (++; $P<0.05$), whilst in the PPD-allergic donors, there was a marked decrease in the $CD4^+$ to $CD8^{hi}$ ratio in weakly, strongly and extremely allergic cohorts (combined population compared with buffy coat samples; $P<0.001$). To complement the previous studies of T-cell dynamics in allergy to MI, the ratio of $CD4^+$ to $CD8^{hi}$ cells was measured for an additional cohort of 27 MI-allergic individuals (+=14; ++=7; +++=6). Although the mean ratio of $CD4^+$ to $CD8^{hi}$ cells in the extremely allergic cohort was

considerably lower than the healthy controls (approximately 1.5 compared to 2.6), the difference was not statistically significant.

3.3 Further phenotypic characterization of the T lymphocyte population: contribution of Treg and CLA positive cells

Additional flow cytometric analyses were then performed in order to investigate further the phenotype of $CD4^+$ and $CD8^{hi}$ T-cells in contact allergic individuals. These analyses also incorporated a number of newly recruited MI-allergic patients. Regulatory T-cells (Tregs) are a subset of the $CD4^+$ population that in humans can be defined as $CD25^+$ $FoxP3^+$ $CD4^+$ cells (17). Their role is to dampen inflammation, and defective or reduced numbers of Treg cells have been associated with inflammatory and autoimmune diseases including multiple sclerosis, systemic lupus erythematosus and atopic dermatitis (27,28). We therefore hypothesized that individuals with more severe nickel, PPD or MI allergy would have reduced numbers of Tregs in peripheral blood. Flow cytometry analysis was performed in which $CD4^+$ cells from allergic individuals or buffy coat controls were then gated for expression of both CD25 and FoxP3 (Fig. 4A, representative gating strategy). The abundance of Tregs as a percentage of the $CD4^+$ population was then assessed for weakly (n=22), strongly (n=15) and extremely (n=12) allergic individuals (pooled across the allergens nickel, PPD and MI), as well as nine buffy coat controls. For the buffy coat controls, Tregs were ~1.1% of the total $CD4^+$ population, somewhat lower than previous reports where 3.2% of the CD4 population was identified as Treg cells (29) (Fig. 4B). Despite the relatively low control levels, a significant decrease in the percentage of $CD4^+$ cells that were Treg cells was recorded for extremely allergic individuals (Fig. 4B, $P<0.05$).

Finally, expression of the skin-homing marker cutaneous lymphocyte antigen (CLA) (18) by $CD4^+$ and $CD8^{hi}$ cells was measured by flow cytometry, to determine whether contact allergy was associated with an increase in CLA expression in peripheral blood T-cells.

Representative flow cytometry plots for (A) $CD4^+$ and (B) $CD8^{hi}$ cells are shown in Figure 5. Approximately 14% of healthy control (buffy coat) $CD4^+$ cells expressed CLA, consistent with previous publications which also reported ~14% CLA^+ $CD4^+$ cells in healthy controls (30). A marked increase in the percentage of CLA^+ $CD4^+$ cells was recorded for all 3 categories of patch test positive individuals (+, P <0.05; ++, P <0.001; +++, P<0.05; pooled for PPD, MI or nickel allergic donors). For the $CD8^{hi}$ population, there was little change in the number of CLA^+ cells (~7%), but the mean level of CLA expression (mean fluorescence intensity) was significantly elevated in individuals strongly (++; P <0.001) or extremely (+++; P <0.05) allergic to PPD, MI or nickel. This indicates that increased CLA expression may be a hallmark of allergic contact dermatitis, particularly in the case of severe allergies.

4. DISCUSSION

In the current study T lymphocyte responses have been characterized in individuals with contact allergy to nickel, which despite Europe wide attempts to reduce exposure, remains one of the most common skin sensitizers (31,32) and to PPD, a potent allergen which is a major cause of adverse reactions to hair dyes (33, 34). Consistent with previous analyses of PBMC derived from MI sensitized individuals (7), patch test positivity to both nickel and PPD was associated with increases in both naïve $CD4^+$ and naïve $CD8^+$ lymphocytes, with corresponding decreases in the EMRA and effector memory cell populations. It should, however, be noted that statistical significance was only seen in severely allergic patients. The healthy control comparator population of PBMCs utilized in the study reported herein was isolated from buffy coat samples (n=14). It could be argued that this was an inappropriate control as there are differences in the storage and isolation procedure compared with the patch test donor samples. Whilst patch test donor PBMCs are isolated from blood within hours of blood being drawn and then snap frozen prior to phenotypic analyses, buffy coats are prepared within 24 hours of venepuncture, received in the laboratory for PBMC preparation within 48 hours and then snap frozen. However, there are several studies which have compared directly freshly isolated and buffy coat PBMC and these have concluded that there are no significant differences in cell viability and purity and CD4/CD8 phenotype between the two preparation methods (35, 36). The parameter that is most likely to impact on cell phenotype is freeze/thawing (37); given that both patch test positive patient samples and buffy coats are frozen once PBMCs have been isolated this should not be a cause for concern. Reference data for the distribution of naive, central memory, effector memory and EMRA T cell subsets in peripheral whole blood anti-coagulated with EDTA are available from a cohort of 253 healthy adults between the ages of 19 and 67 (38). The distribution of subsets amongst $CD4^+$ cells was similar to the buffy coat data reported herein (mean values naïve: 43.1% [versus ~32%], central memory: 32.8% [versus ~38%], effector memory: 16.7% [versus ~20%]) with the exception of the EMRA

subset, which was somewhat larger in the buffy coat samples, although still the smallest subset (~10% versus 1.6%). For $CD8^+$ cell subsets, taking into account the markers that have been used in the two different studies, there were more differences between these control data and the buffy coat data, but values were broadly similar (mean values naïve: 44.6% [versus ~35%], effector memory: 4.7% [versus ~14%], EMRA: 9.9% [versus ~16%], central memory: 28.5% [versus 35%]). Taken together, these data suggest that the buffy coat sample not only represent an appropriate control for the patch test positive donor samples, but also that the patch donor samples display a different distribution to healthy controls. Had it been possible to obtain such samples, a more appropriate negative control would have been individuals who were patch-test negative for all patch tests applied; however, during the course of the study, only one such individual was identified.

One additional technical issue regarding the study design was that in order to be confident of the identity of the allergen to which the patch test positive donors were allergic, blood samples were drawn four days after the initial application of the patch tests, on the day of patch test reading. In other studies using peripheral blood samples taken from patients with contact allergy, blood sampling has tended to be conducted before patch testing, as authors were concerned that exposure to contact allergen might activate the cells (15, 39). Alternatively, donors have been drawn from a pool of previously patch-test positive or negative individuals (40). Given that significant differences were found between the phenotype of the healthy control population and the nickel and PPD positive donors in our study, particularly with respect to increases in the proportion of naïve $CD4^+$ and $CD8^{hi}$ cells, it is possible that the observed differences were due to cellular recruitment of skin homing lymphocytes to the patch test site. For a subset of donors we therefore took blood samples before and 4 days after patch test application. There were no significant differences observed in the phenotype of $CD4^+$ or $CD8^{hi}$ T-cells in samples taken prior to and after patch test readings, suggesting that individuals with allergic contact dermatitis do indeed have an

expanded pool of naïve T-cells and reduced effector memory and EMRA numbers regardless of an ongoing skin patch test response. Due to low sample numbers, it was not possible to determine whether ongoing patch test inflammation was contributing to the alteration in Treg cell numbers or CLA expression.

It should be noted that peripheral blood T-cell phenotypes show considerable inter- (and intra-) individual variation and can be influenced by factors such as age and disease, with for example cytomegalovirus infection resulting in decreasing numbers of naïve $CD4^+$ T-cells (41) and declines in both CD4 and CD8 naïve cells reported with age (42, 43). However, it is of interest that an expanded pool of naïve T-cells was recorded in patients with allergy to nickel and PPD, and in a previous publication, to MI (7), suggesting that this might be a common feature of contact allergy. Increased levels of naive T-cells could be as a result of the release from the bone marrow and thymus of increased numbers of naive T-cells and T-cell precursors, or due to increased survival of these cells. It is known that the half life of naive human T-cells is ~50 days, and that survival of naive T lymphocytes requires signalling from interleukin (IL) -7 (44).It is possible, therefore, that the general systemic cytokine milieu that is a result of contact allergy provides an environment for improved naïve T-cell survival, through increased levels of cytokines such as IL-7.

The ability of PBMC from allergic individuals to proliferate *in vitro* in response to challenge with contact allergen was also examined using a lymphocyte transformation test. There is considerable interest in developing such *in vitro* measures of allergen specific responses in order to supplement, or perhaps even to replace, patch testing where there are concerns about severe reactions or unintended sensitization (8-15). In the absence of cytokine supplements (IL-2 or IL-7 for example)(11, 39) used to enhance proliferative responses, the overall positive rate for nickel was 17/31, which is lower than many other reported studies

(16/18; 62/74; 13/14) (15, 39, 40) although similar to the frequency of 13/19 reported in 2013 by Pacheco et al for patients referred by local orthopedists for evaluation for possible nickel sensitization who were patch test positive (45). The reasons for this lower rate of positivity in our study are not clear, although it is noted that minor technical differences in the lymphocyte transformation test protocol can have marked effects, such as the use of autologous serum (46), or the length of time in culture (5 days versus 6 or 7 days)(39, 40, 47). The fewest number of responders (5/12) was recorded for the extreme (+++) sensitized group, although mean proliferation was similar between ++ and +++ groups. This inverse dose response may indiate that the culture conditions were not optimal, rather than a true negative correlation of proliferation with patch test sensitivity. In general concerns regarding nickel and the measurement of proliferative responses tend to be regarding nonspecific responses in negative control groups (false positives) so alternative end points are being investigated. Cytokine expression profiling including the ratio of IL-5 to IL-8 production (47) or secretion of interferon-γ are being explored currently (48). Experience with PPD is rather more variable, with some groups reporting vigorous proliferative responses to free PPD (6/9 +++ patch test donors; 7/8 ++ or +++ patch test donors and 10/11 donors said to have a strong history of sensitization to PPD) (11, 12, 24). However, there are other studies that report that free PPD stimulated proliferative responses in a minority of donors (1/6), despite having ++ or +++ scores in patch tests (49), or that 0/9 sensitized donors tested positive with free PPD although 7/9 responded to the metabolite Brandowski's base (22). We have previously observed with a different cohort of PPD patch test positive donors that PPD alone gave variable responses in a minority of individuals and that PPD-HSA conjugates were more successful at stimulating proliferative responses, with most vigorous effects observed amongst the +++ patch test group (6). In the current study, free PPD has been more effective in the +++ patch test positive group, but the conjugate was more effective at provoking significant levels of thymidine incorporation in the more weakly sensitized groups. Given the level of sensitivity, the number of false negatives and inter-laboratory variation, the lymphocyte transformation test seems very unlikely to be a

replacement for patch testing, but does have the advantages that additional endpoints such as identity of the proliferating cells (CD4 or CD8) or nature of cytokine production can be incorporated to further mechanistic understanding (7, 47, 48).

In addition to sensitization being associated with changes in the profile of naïve and effector/central memory and EMRA cells, we also observed that particularly for extremely allergic individuals, the Treg cell frequency was decreased, as was the $CD4^+$:$CD8^{hi}$ ratio, whereas CLA expression was increased. These findings appeared to be a feature of sensitization to each of the 3 different allergens, despite the fact that they have very different chemistries. It has been demonstrated previously that PBMCs from nickel allergic individuals express lower levels of the Treg cytokines IL-10 and transforming growth factor β (39) and their Tregs had limited suppressive capacity compared with nickel non-allergic individuals (49). There has been one previous report that allergic contact dermatitis is associated with a decreased frequency of Treg cells. Compared with healthy controls (4.06% Tregs), significantly lower levels of Tregs were recorded (1.6%) in 8 individuals with skin sensitization to unspecified allergens, despite the fact that the average length of time since diagnosis was only 54 days (27). Perhaps the most interesting report comes from a study of melanoma patients who received an *in vivo* infusion of a recombinant IL-2-diphteria toxin fusion protein which depleted Treg cells from ~3% (3.5-2%) to ~2% (2.5-1%) of $CD4^+$ cells. Prior to depletion only weak contact allergic responses were provoked by sensitization with the potent contact allergen diphencyprone, whereas after depletion very vigorous elicitation reactions to this allergen were recorded (50). If a reduced frequency of Treg cells is a feature of contact allergy, particularly severe contact allergy, then this could be a factor in polysensitisation, as those individuals with +++ patch test scores, and potentially low Treg numbers, have a higher risk of becoming polysensitised (51). In this study, individuals with +++ patch test scores to nickel and MI also tended to have patch positive reactions to other allergens.

There are reports that suggest that $CD4^+$ cells may be more prevalent in skin lesions (52), however, it is generally accepted that both $CD4^+$ and $CD8^+$ cells producing interferon-γ, IL-17 and IL-22 are involved in contact allergic responses (1, 53-55). To our knowledge there are no previous reports that have demonstrated that contact allergy is associated with increased numbers of total $CD8^+$ cells and a reduced frequency of total $CD4^+$ cells. This is also not a feature of other forms of allergic disease such as allergic asthma, where even severe asthmatics had $CD4^+/CD8^+$ ratios similar to those of healthy controls (56). There are other situations in which decreased CD4:CD8 cell ratios have been recorded; in early menopause with inadequate oestrogen levels, for example, where it can be restored with hormone replacement therapy (from 1.4% +/- 0.16 to 2.4% +/- 0.3) (57) or with immune senescence due to ageing (58). Viral infection, particularly cytomegalovirus infection, is also associated with a similar increase in CD4:CD8 ratio through an expansion of CD8 cells, although these tend to be in the EM and EMRA T-cell compartments rather than naïve T-cells (59). Other chronic infections such as tuberculosis, dimorphic fungi, toxoplasmosis, and leishmaniasis also lead to CD8 expansion with altered ratios (60). It seems likely therefore that the inverted CD4:CD8 ratio observed in highly sensitized donors is due to an expanded $CD8^+$ T-cell population.

The final feature of T-cells isolated from sensitized individuals was increased numbers of skin-homing marker CLA^+ CD4 cells and increased expression of this marker on CD8 cells. It is well accepted that skin resident T-cells are CLA^+, indeed, it has been calculated from comparisons of the frequency of these cells in skin and blood and the relative volume/surface area of blood and skin, that 98% of CLA effector memory T-cells are found in the skin (61-63). In peripheral blood this marker has been reported to be expressed on 17% CD4 cells and 11% CD8 cells (64), primarily on central and effector memory cells (65). To our knowledge this marker has not been examined systematically in peripheral blood of contact allergic patients, or previously correlated with the severity of allergy. It may be that

this increase in CLA expression reflects an activation of T-cells as a result of the patch test applied 4 days prior to the blood draw or could represent a bystander effect with cytokines produced by allergen specific T-cells nonspecifically activating other T lymphocytes (66).

In conclusion, these studies have revealed that patch test positivity to nickel, PPD and MI was associated with changes in the phenotype of peripheral blood T-cells : increases in naïve cells, decreases in Treg cell frequency and the $CD4^{+}$:$CD8^{hi}$ ratio and increased expression of the skin homing marker CLA. This increased understanding of the characteristics of the T-cell response to contact allergens may provide insight into useful parameters to consider in the development of health risk assessment models for chemical skin sensitizers.

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FIGURE LEGENDS

Figure 1. Proliferative responses of PBMCs isolated from nickel- or PPD-allergic individuals. PBMCs were isolated by density gradient centrifugation from nickel (A) or PPD (B) patch test-positive donors (n=31 and n=46), grouped according to allergy severity. Cells were cultured in 96-well flat-bottomed plates, at 1×10^5 cells/well, and stimulated with allergen or cultured in the presence of medium alone, and incubated at 37∘C (≥triplicate wells) for 120 h. Cells were pulsed with [^{3}H] thymidine for the final 16-20h of culture, then harvested and processed for β-scintillation counting. PBMCs from nickel allergic patients (A) were stimulated with 0.5-50 μg/mL nickel sulfate (+ n=7, ++ n=12, +++ n=12). PBMCs from PPD-allergic patients (B) were incubated with 0.01-1 μg/mL free PPD (+ n=13, ++ n=17, +++ n=16) and a subset was also stimulated with 10-100 μg/mL of one of two PPD-HSA conjugates or with 100 μg/mL HSA (+ n=8, ++ n= 8, +++ n= 11). For the allergen stimulation, the data from the concentration which gave maximal proliferation are shown. Data are shown as a stimulation index (SI) [fold change in proliferation above baseline (medium alone)] for individual patients (●) and mean and SEM per group; an SI of ≥2.5 was regarded as being indicative of cellular proliferation (illustrated with horizontal dotted line).

Figure 2. T lymphocyte phenotype of freshly isolated PBMCs of nickel and PPD allergic patients: comparisons with buffy coat donors. PBMCs were isolated from nickel (A, C; n=56) or PPD (B, D; n=30) patch test-positive donors four days after initiation of patch testing or from buffy coat donors (n=14) by density gradient centrifugation. Cells were stained with anti-human CD4–APC, CD8–APC, CD45RA–FITC and CD27–PE-Cy5 antibodies. Data are shown as percentage of total viable $CD4^+$ or $CD8^{hi}$ cells in each phenotype [naïve ($CD45RA^+$ $CD27^+$) (i), central memory ($CD45RA^-$ $CD27^+$) (ii), effector memory RA ($CD45RA^+$ $CD27^-$) (iii) and effector memory ($CD45RA^-$ $CD27^-$) (iv)] within the $CD4^+$ (A, B) or $CD8^{hi}$ (C, D)

T lymphocyte populations. Donors were segregated according to patch test status [buffy coat donors (n=14), + donors (nickel n=14, PPD n=8), ++ donors (nickel n=27, PPD n=11), and +++ donors (nickel n=15, PPD n=11)], and data are shown as group mean (horizontal line) and SEM and for each individual donor (●). Statistically significant shifts (Hotelling's T^2 test after transformation by additive log ratio) compared with buffy coat controls were seen in $CD4^+$ population composition in patients with severe nickel allergy (P <0.01) and in $CD8^{hi}$ population composition in patients with moderate nickel allergy (P <0.05), and in patients with moderate or severe PPD allergy (P <0.001 and P <0.05 respectively*)*.

Figure 3. Influence of patch test application on CD4 and CD8 cell phenotype. PBMCs were isolated and stained as described in the legend to figure 2 for the identification of naïve, central memory, EMRA and effector memory $CD4^+$ (A) and $CD8^{hi}$ (B) subsets. Blood samples were drawn immediately before patch testing (black bars) and at day 4 after a positive patch test (white bars) was recorded for nickel, MI, or PPD for 3 individuals with a weak (+) and 3 individuals with a strong (++) allergy. Results are displayed as mean and SEM. There were no statistically significant changes in subset composition with time (Hotelling's one-sample T^2 test after additive-log-ratio transformation).

Figure 4. Influence of allergy on CD4 to CD8 cell ratio PBMCs were isolated and stained as described in the legend to figure 2 for the identification of $CD4^+$ and $CD8^{hi}$ subsets. The percentage of $CD4^+$ and $CD8^{hi}$ cells in each PBMC sample was calculated as a function of the total viable cell population and is displayed as a ratio of $CD4^+$ to $CD8^{hi}$ cells. PBMCs from MI (n=27), nickel (n=56), and PPD (n=30) allergic patients were compared with healthy (buffy coat) donors (n=14). Patients were segregated according to patch test status: + donors (MI n=14, nickel n=14, PPD n=9), ++ donors (MI n=7, nickel n=27, PPD n=10), and +++ donors (MI n=6, nickel n=15, PPD n=11), and data are shown as group mean (horizontal line) and SEM

and for each individual donor (●). Statistically significant differences versus buffy coat donors were assessed by Mann-Whitney U test. *P <0.05; ***P <0.001.

Figure 5. Association of severe contact allergy with decreased Treg cell numbers PBMCs were isolated from individuals allergic to nickel (n=30), PPD (n=5), or MI (n=14) or from buffy coat samples (n=9) and stained with anti-human CD4/CD25-FITC/APC antibody cocktail, and anti-human FoxP3-PE for flow cytometric analysis. Data are shown as the proportion of $CD4^+$ PBMCs co-expressing CD25 and FoxP3 and therefore classed as regulatory T cells (Tregs). Individuals analyzed were a subset of the total allergic population and were mildly (+), moderately (++), or strongly (+++) allergic to nickel (closed circle; + n=11; ++ n=10; +++ n=9), PPD (open circle; + n=3; ++ n=1; +++ n=1), or MI (half-filled circle; + n=8; ++ n=4; +++ n=2). Statistically significant differences versus buffy coat donors (n=9) were assessed by Mann-Whitney U test; *P <0.05. Line and error bars indicate mean and SEM.

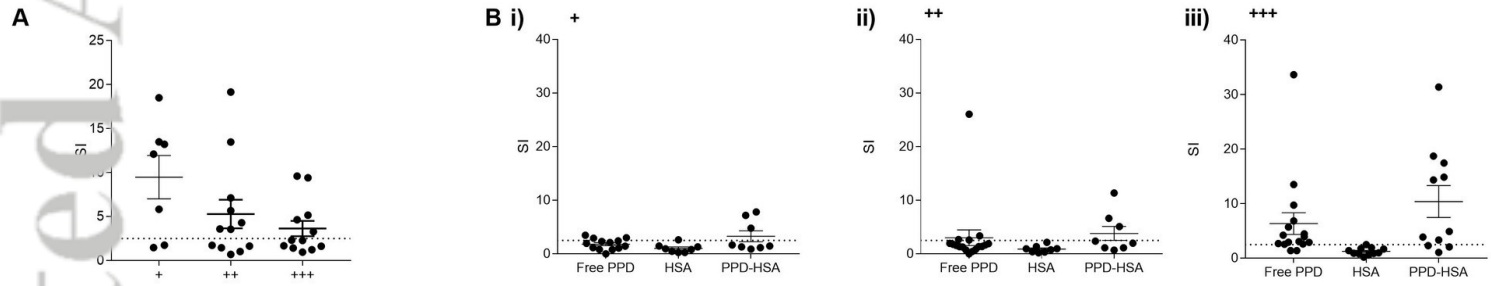
Figure 6. Expression of CLA on CD4 and CD8 cells: comparisons with buffy coat donors

PBMCs were isolated from individuals allergic to nickel (n=28), PPD (n=6), or MI (n=9), or from 'buffy coat' samples representative of the general population and stained with anti-human CD4-FITC, CD8-PE-Cy5, and CLA-eFluor660. A representative gating strategy is shown in (A) and (B) for analysis by flow cytometry of CLA expression by (C) the $CD4^+$ and (D) the $CD8^{hi}$ PBMC subsets. For each of (A) and (B), panel (i) indicates expression of CLA by buffy coat samples, and panel (ii) indicates CLA expression by patient-derived cells. For the $CD4^+$ subset, the proportion of CD4+ cells expressing CLA is shown. For $CD8^{hi}$ cells, the mean fluorescence intensity of CLA-eFluor660 as a proxy for surface levels of CLA is shown. Data are from a pool of individuals weakly (+), strongly (++), or extremely (+++) allergic to nickel (half-filled circle; + n=9; ++ n=10; +++ n=9), PPD (filled circle; + n=4; ++ n=1; +++ n=1), or MI

(open circle; + n=3; ++ n=4; +++ n=2), and buffy coat controls (n=10). Significant differences versus the buffy coat population were analyzed by Mann-Whitney U test. *P<0.05; ***P<0.001. Error bars indicate SEM.

Supplementary Figure 1. Gating strategy for CD4 and CD8 cells.

Representative flow cytometry for standardized gating of PBMCs into (A) $CD4^+$ and (B) $CD8^{hi}$ subsets and naïve, central memory, EMRA and effector memory cells is shown. Flow cytometry was performed on a minimum of 10000 cells using a FACS-calibur. Dead cells and cellular debris were excluded based on forward and side scatter to give a viable lymphocyte gate (i). Cells were stained for (A) CD4 or (B) CD8 and then gated for CD4 (Aii) and $CD8^{hi}$ (Bii) expression, then respective $CD4^+$ (Aiii) and $CD8^{hi}$ (Biii) populations were separated on the basis of CD27 and CD45RA expression into 4 quadrants: $CD27^+$ $CD45RA^+$ (naïve cells), $CD27^+$ $CD45RA^-$ (central memory), $CD27^-$ $CD45RA^-$ (effector memory), and $CD27^-$ $CD45RA^+$ (EMRA) subsets.



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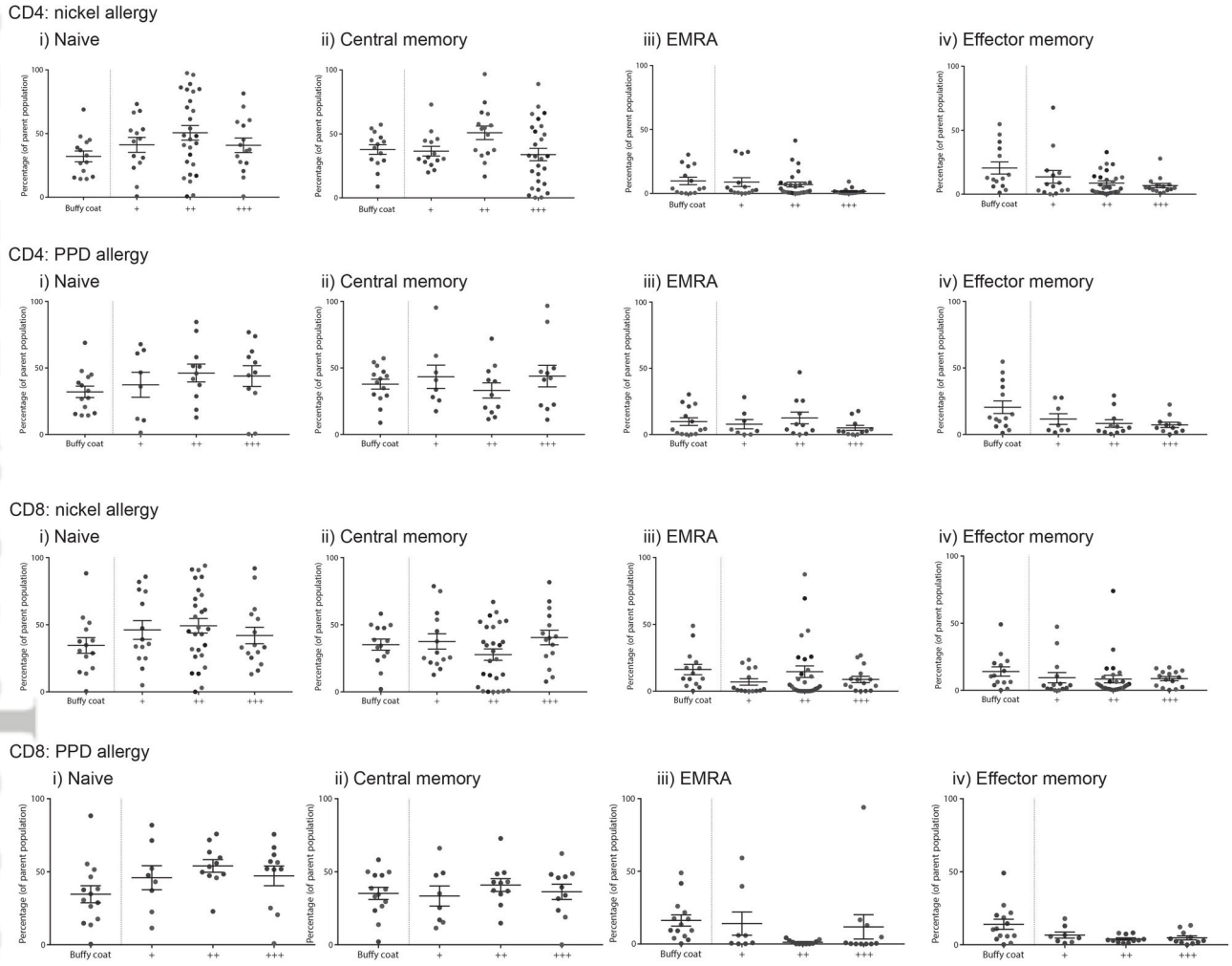

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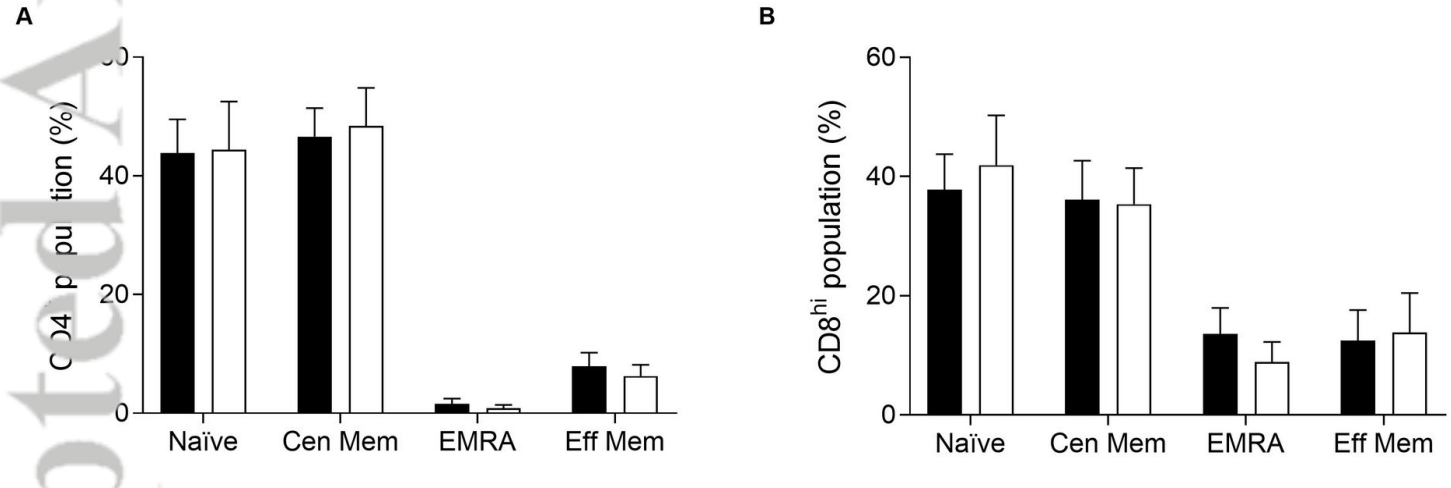

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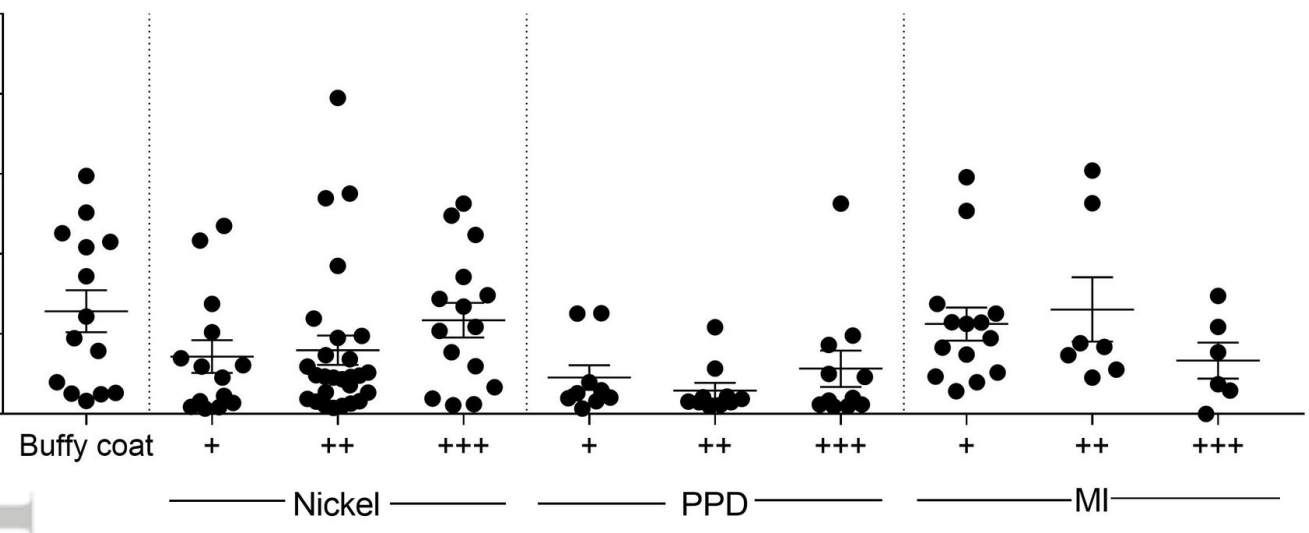

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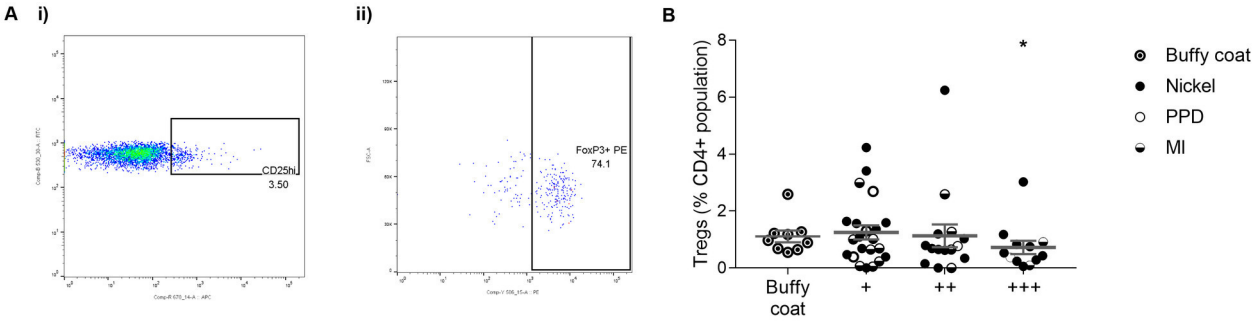
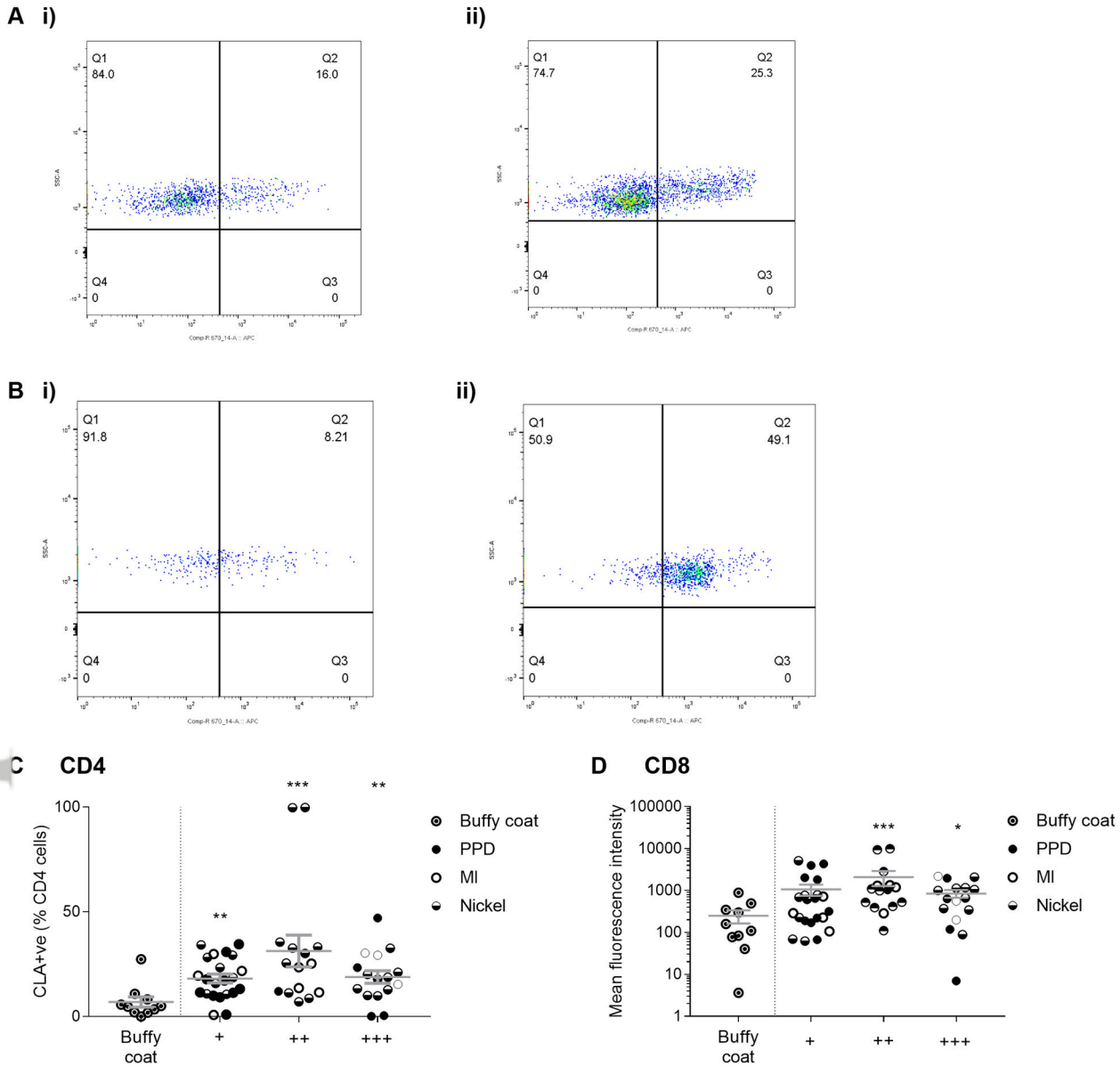


COD_13246_Fig 5 Tregs with schematic.jpg



COD_13246_Fig 6 CLA expression.jpg