Measurement of CD4 + and CD8 + T-Lymphocyte Cytokine Secretion and Gene Expression Changes in *p*-Phenylenediamine Allergic Patients and Tolerant Individuals

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Factors predisposing to individual susceptibility to contact allergic dermatitis are ill defined. This study was designed to characterize the response of allergic and tolerant individuals' T-lymphocytes after exposure to *p*-phenylenediamine (PPD). Peripheral blood mononuclear cells (PBMCs) from allergic patients proliferated when treated with PPD and Bandrowski's base (BB) and secreted IL-1α, -1β, -4, -5, -6, -8, -10, and -13; IFN-γ; tumor necrosis factor-α; MIP-1α/β; MCP-1 (monocyte chemotactic protein-1); and RANTES. PBMCs from tolerant individuals were stimulated to proliferate only with BB, and they secreted significantly lower levels of Th2 cytokines. Principal component analysis showed that genes are differentially expressed between the patient groups. A network-based analysis of microarray data showed upregulation of T helper type 2 (Th2) gene pathways, including IL-9, in allergic patients, but a regulatory gene profile in tolerant individuals. Real-time PCR confirmed the observed increase in Th2 cytokine gene transcription in allergic patients. Purified CD4 + and CD8 + T cells from allergic patients were stimulated to proliferate and secrete Th2 cytokines following antigen exposure. Only CD4 + T cells from tolerant individuals were stimulated by BB, and levels of Th2 cytokines were 80% lower. The nature of the antigenic determinant stimulating PBMCs and levels of Th2 cytokines, including IL-9, was confirmed in a validation cohort. These studies show increased activity of Th2 cytokines in CD4 + and CD8 + T cells from individuals with allergic contact dermatitis.

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INTRODUCTION

Allergic contact dermatitis is a delayed-type, cell-mediated immune reaction, occurring after cutaneous exposure to certain low-molecular-weight chemicals. Classic studies by Landsteiner and Jacobs (1935) showed that irreversible protein modulation is a prerequisite for immune activation, a hypothesis that is still widely accepted today. Cutaneous dendritic cells capture hapten-modified protein and, after acquiring additional T helper type 1 (Th1) or Th2 polarizing signals (Cumberbatch *et al.*, 2005; Mori *et al.*, 2008), migrate toward draining lymph nodes to present peptides to naive T cells. Subsequent exposure to the same chemical is associated with the cutaneous migration of antigen-specific CD4 + and CD8 + T cells, the destruction of keratinocytes, and the development of contact dermatitis (Kehren *et al.*, 1999; Akiba *et al.*, 2002). Our understanding of the cellular basis of allergic contact dermatitis derives to a large extent from murine models in which CD8 + T cells are effectors, mediating cytotoxicity through FAS- and perforin-dependent pathways (Kehren *et al.*, 1999; Akiba *et al.*, 2002; Saint-Mezard *et al.*, 2004, 2005; Vocanson *et al.*, 2006). In contrast, CD4 + T cells are thought to play a more regulatory role, possibly inhibiting pathogenic responses and the development of contact dermatitis.

In humans, our understanding of the involvement of different T-cell populations in the development of contact dermatitis is less well defined. The contact allergen 2,4-dinitrochlorobenzene stimulates IFN-γ secretion from both blood-derived CD4 + and CD8 + T cells (Pickard *et al.*, 2007) and punch biopsies from delayed-type hypersensitivity reactions (Lecart *et al.*, 2001) in all exposed individuals. Lecart *et al.* (2001) also isolated IL-5- and/or IL-10-secreting

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Abbreviations: BB, Bandrowski's base; PBMC, peripheral blood mononuclear cell; PPD, p*-phenylenediamine*

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T-cell clones and postulated that they may regulate the size of the induced immune response. Similarly, the metal allergen nickel, which complexes with the major histocompatibility complex and specific T-cell receptors (Gamerdinger *et al.*, 2003; Lu *et al.*, 2003; Moulon *et al.*, 2003), stimulates IFN-γ secretion from CD4+ and CD8+ T cells isolated from allergic individuals (Cavani *et al.*, 2000), whereas, in contrast, tolerant individuals' T cells seem to prevent the development of contact dermatitis through multiple regulatory pathways (Cavani *et al.*, 1998, 2003).

Recently, we have used the hair dye component *p*-phenylenediamine (PPD) as a model allergen with which to explore the chemical and cellular bases of contact dermatitis. PPD, a diamine-substituted aromatic ring, is susceptible to sequential oxido-conjugation reactions in aqueous solution and in contact with skin. The ultimate product of these reactions is the trimer Bandrowski's base (BB) (Picardo *et al.*, 1990). T cells from PPD-allergic and -tolerant individuals (Sieben *et al.*, 2002; Coulter *et al.*, 2008), but not cord blood (Coulter *et al.*, 2008), were stimulated to proliferate by BB, implying an acquired immune response that is not translated into an allergic reaction. This hypothesis is supported by the low incidence of patch test–positive reactions to BB in PPD-allergic patients (White *et al.*, 2006). Eilstein *et al.* (2006, 2008) demonstrated that primary quinonediimine intermediates bind to nucleophilic amino acids via a complex series of reaction mechanisms, and, indeed, protein modified with PPD quinonediimine may be an important antigenic determinant (Coulter *et al.*, 2008). In this respect, lymphocytes from allergic patients alone were found to be specifically stimulated with PPD.

In contrast to 2,4-dinitrochlorobenzene and nickel (discussed above), in a murine model of PPD-induced contact sensitization Th2 cytokines, IgE antibodies, and mast cells were found to play a crucial role in the development of tissue pathology (Yokozeki *et al.*, 2003). T-cell clones from allergic human patients secrete predominantly Th2 cytokines (IL-4, -5, and -13) following PPD stimulation (Sieben *et al.*, 2002; Coulter *et al.*, 2008), which is in accordance with the recently described increased expression of IL-4, IL-13, and IL-31 genes in allergic dermatitis (Neis *et al.*, 2006).

The aim of this study was to explore the response of allergic and tolerant individuals' peripheral blood mononuclear cells (PBMCs) and purified CD4+ and CD8+ T cells after PPD and BB exposure. PBMCs and CD4+ and CD8+ T cells from allergic patients were stimulated to proliferate and secrete cytokines, including IL-5 and -13, after treatment with PPD and BB. Tolerant individuals' PBMCs and CD4+, but not CD8+, T cells were also specifically stimulated (but only with BB); however, only low levels of the Th2 cytokines IL-5 and IL-13 were detected. A microarray study established that it was possible to differentiate allergic and tolerant patient samples on the basis of differing gene expression profiles. Furthermore, a network-based analysis of the data identified Th2 and regulatory cytokine signaling pathways in allergic and tolerant individuals, respectively. These data, which were validated in an expanded independent cohort, support the hypothesis that PPD-mediated Th2 cytokine secretion from T cells contributes to the development of contact allergic dermatitis.

RESULTS

Peripheral blood mononuclear cell proliferation with PPD and BB

Peripheral blood mononuclear cells from allergic patients were stimulated to proliferate by both PPD and BB ($n=5$; maximum stimulation index (SI) PPD: 39.0 ± 25.5; BB: 45.1 ± 36.9). PBMCs from tolerant individuals were not specifically stimulated with PPD (SI less than 2); however, BB treatment was associated with a concentration-dependent proliferative response ($n=6$; SI 10.4 ± 7.4; Table 1).

Cytokine and chemokine secretion from PPD- and BB-stimulated PBMCs

Significant concentrations (more than 30 pg/ml) of IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IFN-γ, TNF-α (tumor necrosis factor-α), MCP-1, MIP-1α, MIP-1β, IP-10, and RANTES were detected from allergic patients' PBMCs stimulated with PPD or BB (Table 2). BB stimulation of tolerant patients' PBMCs was associated with similar levels of IL-1α, IL-1β, IFN-γ IL-6, IL-8, IL-10, IP-10, MIP-1β, MCP-1, TNF-α, and RANTES secretion (Table 2; $P>0.05$). Allergic and tolerant patients' PBMCs differed in terms of the extent of IL-4, IL-5, IL-13, and MIP-1α secreted (Figure 1). Levels of IL-4 and IL-5 found in supernatants from tolerant samples stimulated with BB were below the limit of detection, whereas the level of IL-13 secretion was approximately 15% of that found in equivalent samples from allergic patients. BB-stimulated cells from tolerant individuals secreted high levels of IL-8 and IL-10; however, when cells from allergic patients and tolerant individuals were compared, the values did not reach statistical significance (Table 2). Tolerant patients' PBMCs stimulated with PPD secreted only IL-8 and MCP-1.

Principal component analysis of the gene expression data deriving from PPD- and BB-stimulated PBMCs

To identify in more detail the global differences between allergic and tolerant individuals, principal component analysis was applied randomly to the microarray data, with no preformed opinions. From the two components showing greatest variance (18.71 and 12.49%, respectively), it was possible to draw a clear division and separate the data points from allergic and tolerant individuals, irrespective of antigen treatment (Figure 2a).

Allergic and tolerant patient gene expression profiles and biologically related ontologies

Lists showing gene expression altered as a result of PBMC stimulation with PPD and BB for 24 hours indicated that allergic patients' cells expressed genes related to positive regulation of cell proliferation, apoptosis, inflammatory response, humoral immune response, and lymphocyte activation. Tolerant individuals' samples stimulated with BB underwent similar biological processes, with the following exceptions: (1) antigen stimulation was associated with negative regulation of cell

Table 1. Concentration-dependent proliferation of lymphocytes from allergic and tolerant individuals

	Lymphocyte proliferation (SI)											
		PPD (μM)						BB (μM)					
	TT	0.1	0.5	1	2	5	10	0.1	0.5	1	2	5	10
Allergic patients													
1[1]	84.9	36.3	52.0	9.2	1.1	0.5	0.3	72.0	111.5	88.1	77.1	20.2	0.5
2	41.9	34.9	55.5	55.1	49.1	32.5	0.3	26.0	37.1	48.0	48.0	66.1	11.3
3	18.9	4.7	6.4	8.6	2.0	0.1	0.2	10.9	14.7	13.6	14.7	3.9	1.7
4	87.8	12.5	34.4	42.2	54.2	74.1	37.9	12.7	13.0	20.4	19.7	5.1	1.5
5	58.0	8.4	15.1	5.5	4.1	6.8	1.0	6.4	19.5	25.0	29.5	7.1	0.4
Tolerant patients													
1	25.8	<2	<2	<2	<2	<2	<2	1.4	3.6	3.6	4.2	7.6	1.6
2	8.1	<2	<2	<2	<2	<2	<2	1.6	2.8	3.7	4.0	5.2	2.1
3	59.2	<2	<2	<2	<2	<2	<2	3.9	7.0	9.3	9.9	12.9	5.3
4	10.3	<2	<2	<2	<2	<2	<2	1.7	3.3	5.2	5.9	3.2	0.2
5	40.7	<2	<2	<2	<2	<2	<2	1.9	8.7	14.3	16.4	4.5	2.0
6	8.1	<2	<2	<2	<2	<2	<2	1.2	4.5	4.5	5.7	6.1	0.4

BB, Bandrowski's base; PPD, *p*-phenylenediamine.
[1]PPD- and BB-specific stimulation of lymphocytes from allergic patients. Patients 1–3 were described previously (Coulter *et al.*, 2008). Current data are derived from an additional blood donation obtained 12 months later (Coulter *et al.*, 2008).

Table 2. Cytokine and chemokine secretion from PPD- and BB-stimulated lymphocytes

		Cytokine/chemokine (pg/ml)[1]							
Patients	**Antigen**	**IL-1α**	**IL-1β**	**IL-4**	**IL-5**	**IL-6**	**IL-8**	**IL-10**	**IL-13**
Allergic (*n*=5)	PPD	167.4[2]	138.7	109.5	117	19.5	97.0	50.9	1342.1
	BB	222.0	108.5	112.9	120	1,196	97.0	148.3	1,697.6
Tolerant (*n*=6)	PPD	0	0.1	0.6	1.1	10.5	429.6	0	18.2
	BB	248.6	57.4	4.9	17.4	1,120.5	361.4	230.9	252.6

		Cytokine/chemokine (pg/ml)						
Patients	**Antigen**	**IFN-γ**	**IP-10**	**MCP-1**	**MIP-1α**	**MIP-1β**	**RANTES**	**TNF-α**
Allergic (*n*=5)	PPD	36.3	85.7	111.9	96.5	451.0	515.0	60.9
	BB	138.8	74.9	290.5	1,009.1	540.2	694.5	189.0
Tolerant (*n*=6)	PPD	6.1	30.4	352.6	0	0	0	3.2
	BB	38.5	169.1	281.9	128.9	540.2	635.2	108.1

BB, Bandrowski's base; PPD, *p*-phenylenediamine; TNF-α, tumor necrosis factor-α.
[1]Levels of the cytokines and chemokines TGF-α, IL-12, IL-15, IL-17, and eotaxin in PPD- or BB-treated samples were not significantly different from those in solvent-treated controls.
[2]Data represent average cytokine data from allergic patients and tolerant individuals with vehicle control levels (without antigen; less than 10 pg/ml cytokine, with the exception of IL-13 (50 pg/ml) and IL-6 and IL-8 (up to 1 ng/ml)) subtracted.

proliferation and (2) genes relating to the humoral immune response were not regulated. PPD stimulation of tolerant patients' PBMCs was not associated with biological processes relating to an immune response (Figure 2b).

Individual genes of interest showing an increased expression after antigen stimulation of allergic and tolerant patients' PBMCs are listed in Figure 3. Increased expression of the Th2 cytokines (IL-5, IL-9, and IL-13) and chemokines (CCL17 and

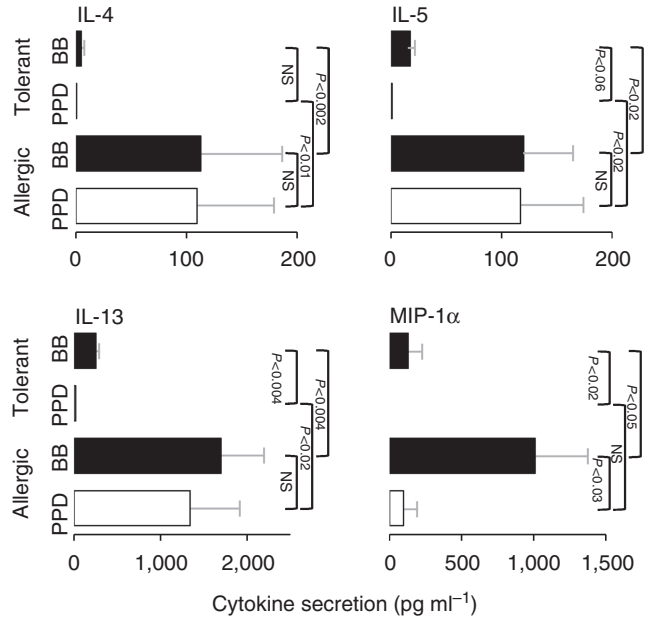
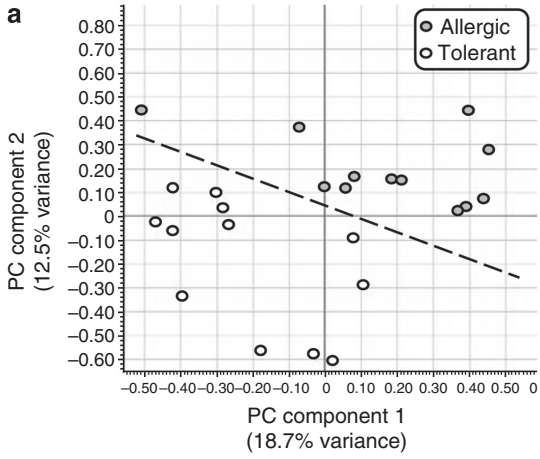


Figure 1. Cytokine secretion from p-phenylenediamine and Bandrowski's base stimulated lymphocytes. IL-4, IL-5, IL-13, and MIP-1α secretion averaged from allergic and tolerant patients' peripheral blood mononuclear cells (PBMCs) in response to maximal stimulatory concentrations of *p*-phenylenediamine and Bandrowski's base.Data are presented as mean ± SD, with values from unstimulated PBMCs (less than 10 ng ml^{-1} (50 ng ml^{-1} for IL-13)) subtracted.



b

Allergic patients	
BB stimulation	PPD stimulation
Positive regulation of proliferation	Inflammatory response
Inflammatory response	Positive regulation of proliferation
Apoptosis	Apoptosis
Humoral immune response	Cellular protein metabolism
Lymphocyte activation	Chemotaxis
	Cell growth
	Humoral immune response
	GPCR protein signaling pathway
	Lymphocyte activation

Tolerant patients	
BB stimulation	PPD stimulation
Inflammatory response	GPCR protein signaling pathway
Chemotaxis	
Negative regulation of proliferation	
Apoptosis	
Regulation of programed cell death	
Regulation of progression through cell cycle	
GPCR protein signaling pathway	

Figure 2. Gene changes associated with antigen stimulation of allergic and tolerant patients' lymphocytes. (**a**) Principal component analysis to detect global variation between samples from allergic and tolerant patients. (**b**) Gene lists common across allergic patients' samples with respect to treatment, generated and analyzed using FatiGOPlus software focusing on the identification of key gene ontology biological processes.

CCL26) and IL17F was observed following stimulation of allergic, but not tolerant, patient PBMCs.

Network-based analysis of allergic and tolerant patients' gene expression profiles

Ingenuity Pathway Assist was used to organize the differentially expressed genes into networks of interacting genes and to find modules of functionally related genes that correspond to known pathways. In our analysis, the top-scoring network (containing a maximum of 35 genes) from antigen-stimulated allergic and tolerant individuals' PBMCs was considered. Genes associated with PPD-stimulated allergic patients' PBMCs were related to the immune response, cellular growth, and proliferation. Several important proinflammatory cytokine and chemokine genes were upregulated, such as IL-17, CCL17, and CCL26, and these proinflammatory genes included a subnetwork of known relevance to Th2 responses (Figure 4a). Genes associated with allergic patients' PBMCs treated with BB formed a profile similar to that of those treated with PPD (Figure 4b). Several important proinflammatory and Th2-associated genes were upregulated.

The top function associated with BB stimulation of tolerant patients' PBMCs was associated with immune response (Figure 4d); however, within this network, the expression of several proinflammatory genes was reduced, whereas the expression of immunoregulatory genes such as IL-10 was increased. Similarly, there was a downregulation of signaling components, such as IFNAR2 (involved in STAT1 and 2 signaling), CXXC5 (involved in activating NF-κB and MAPK signaling pathways), and CABLES1 (cyclin-dependent kinase (CDK)-binding protein that has a role in proliferation and/or differentiation), which could be attributed to the lack of clinical symptoms in tolerant individuals. Genes from tolerant individuals' cells treated with PPD, although associated with the immune response, were also different from those seen in allergic patients (Figure 4c). Importantly, there was no evidence of increased expression of the cytokine genes associated with the development of a Th2 response.

Comparison of IL-5 and IL-13 gene expression and secretion from PPD- and BB-stimulated allergic and tolerant patients' PBMCs

Antigen-stimulated tolerant patients' samples secreted lower levels of IL-5 and IL-13, when allergic and tolerant patients

Allergic patients (PPD stimulation)

Gene name	Symbol	Biological process	Expression ratio		
			1	2	3
Ectonucleotide pyrophosphate	ENPP3	Nucleotide metabolism / phosphate metabolism	2.0	5.1	4.1
Interleukin-2	IL2	Positive regulation of cell proliferation / apoptosis / humoral immune response	5.6	100	149
Interleukin-2 receptor, α	IL2RA	Apoptosis	3.5	7.0	4.0
Interleukin-3	IL3	Positive regulation of proliferation	10.2	197	76.8
Interleukin-5	IL5	Inflammatory response / positive regulation of cell proliferation	1.0	9.7	2.4
Interleukin-9	IL9	Inflammatory response / positive regulation of cell proliferation	12.0	10.5	19.7
Interleukin-13	IL13	Inflammatory response / humoral immune response	6.0	12.5	17.9
Interleukin-17F	IL17F	Inflammatory response / cell protein metabolism / cellular defence response	3.0	2.6	3.8
Interleukin-17 receptor B	IL17RB	Cell growth	1.6	4.5	4.1
Interferon-γ	IFN-γ	Cell growth	2.1	2.4	1.0
Chemokine (C-C) ligand 17	CCL17	Inflammatory response / chemotaxis	2.1	40.0	13.7
Chemokine (C-C) ligand 26	CCL26	Inflammatory response / chemotaxis	6.9	6.3	17.0
Colony-stimulating factor-2	CSF2	Cellular defence response	6.4	27.3	2.7
Chemokine (C-X-C) ligand 7	CXCL7	Chemotaxis / regulation of progression through cell cycle	3.3	2.0	2.5
CD69 antigen	CD69		1.2	2.1	2.2
Cytokine-inducible SH2-containing protein	CISH	Cell growth	1.8	3.5	3.3
Dipeptidyl-peptidays 4	DPP4	Cell protein metabolism	2.0	3.2	4.0
Endothelin 1	EDN1	Positive regulation of cell proliferation / vasoconstriction	2.1	2.4	–1.2
Mal	MAL	Positive regulation of programed cell death / apoptosis	1.0	2.1	2.6
Suppressor of cytokine signaling 1	SOSC1	Protein kinase cascade / cell growth / negative regulation of JAK/STAT cascade	1.2	3.1	2.1
signaling lymphocytic-activation molecule 1	SLAMF1	Humoral response / positive regulation of cell proliferation	1.7	2.4	2.0

Allergic patients (BB stimulation)

Gene name	Symbol	Biological process	Expression ratio		
			1	2	3
Interleukin-2	IL2	Positive regulation of cell proliferation / apoptosis / humoral immune response	6.9	83.7	84.5
Interleukin-2 receptor, α	IL2RA	Apoptosis	2.8	7.8	3.2
Interleukin-3	IL3	Positive regulation of cell proliferation	9.3	177	37.1
Interleukin-5	IL5	Inflammatory response / positive regulation of cell proliferatioin	1.2	8.7	1.9
Interleukin-9	IL9	Inflammatory response / positive regulation of cell proliferatioin	13.8	9.0	11.7
Interleukin-13	IL13	Inflammatory response / humoral immune response	5.0	111	15.2
Interleukin-17 receptor B	IL17RB	Cell growth	5.5	4.5	2.4
Interleukin-17 F	IL17F	Inflammatory response / cell protein metabolism / cellular defence reponse	3.7	2.3	6.8
Interleukin-22	IL22	Inflammatory response	2.4	1.2	2.3
Interferon-γ	IFN-γ	Cell growth	4.1	2.3	1.8
Chemokine (C-C) ligand 17	CCL17	Inflammatory response / chemotaxis	14	38.8	3.5
Chemokine (C-C) ligand 26	CCL26	Inflammatory response / chemotaxis	3.0	5.2	5.7
Cytokine-inducible SH2-containing protein	CISH	Cell growth	1.3	3.9	1.9
Colony-stimulating factor-2	CSF2	Cellular defence response	9.0	25.6	3.9
Chemokine (C-X-C) ligand 2	CXCL2	CHemotaxis / inflammatory response / GPCR Protein signaling pathway	2.2	0.9	2.2

Tolerant patients (PPD stimulation)

Gene name	Symbol	Biological process	Expression ratio		
			1	2	3
Chemokine (C-X-C) ligand 1	CXCL1	Chemotaxis / inflammatory response / negative regulation of cell proliferation	3.2	1.5	3.2
Chemokine (C-X-C) ligand 2	CXCL2	Chemotaxis / inflammatory response / GPCR protein signaling	3.1	2.1	1.2
Chemokine (C-X-C) ligand 3	CXCL3	Chemotaxis / inflammatory response / GPCR protein signaling	3.5	2.2	1.8
Chemokine (C-X-C) ligand 6	CXCL6	Chemotaxis / inflammatory response	3.3	1.9	2.6
Chemokine (C-X-C) ligand 7	CXCL7	Chemotaxis / regulation through cell-cycle progression	1.9	2.0	2.2
Inhibin, β A	INHBA	Apoptosis / regulation through cell-cycle progression / regulation of immune response	5.3	1.1	6.4
Interleukine-1, α	IL1A	Positive regulation of cell proliferation / inflammatory response / apoptosis	9.6	2.2	2.5
Interleukine-6	IL6	Positive regulation of cell proliferation / lymphocyte activation	3.3	1.2	1.4
G-protein-coupled receptor 109B	HM74	GPCR protein signalling pathway	7.6	2.7	4.4
Prostoglandin-endoperoxide synthesis 2	PTGS2	inflammatory response / regulation of immune and inflammatory response / Cellular lipid metabolism	11.0	1.7	2.1

Tolerant patients (BB stimulation)

Gene name	Symbol	Biological process	Expression ratio		
			1	2	3
Interleukin-1 α	ILA	Positive regulation of cell proliferation / inflammatory response / apoptosis	15.2	4.9	7.3
Interleukin-1 receptor antagonist	IL1RN	inflammatory response	14.4	3.0	8.3
Interleukin-2 receptor α	IL2BA	Apoptosis	2.1	3.1	2.4
Interleukin-6	IL6	Positive regulation of cell proliferation / lymphocyte activation	1.8	1.9	1.8
Chemokine (C-C) ligand 3	CCL3	Inflammatory response / chemotaxis / GPCR protein signalling pathway	17.5	3.4	3.0
Chemokine (C-C) ligand 20	CCL20	Inflammatory response / humoral immune response / chemotaxis	4.0	3.4	5.6
Chemokine (C-X-C) ligand-2	CXCL2	Chemotaxis / inflammatory respnse / GPCR protein signaling	5.1	2.9	2.1
Colony-stimulating factor-2	CSF2	Cellular defence response 2	10.9	4.1	3.2
Colony-stimulating factor-3	CSF3	Cellular defence response 3	188	4.7	7.8
G-protein-coupled receptor109B	GPR109B	GPCR protein signaling pathway	7.9	3.4	7.8
Inhibin, β A	INHBA	Apoptosis / regulation through cell-cycle progression / regulation of immune response	9.4	3.0	17.1
LIM domain kinase 2	LIMK2	Cellular protein metabolism	3.5	3.1	2.3
Pleckstrin homology-like domain family A, member 2	PHHDA2	Apoptosis	5.2	2.6	2.4
Protein phosphate 1, regulatory (inhibitor) subunit 15A	PPP1R15A	Apoptosis / regulation of progression through cell cycle	2.5	2.4	2.3
Prostaglandin-endoperoxidase synthase-2	PTGS2	Inflammatory response / regulation of immune and inflammatory response / cellular lipid metabolism	15.7	3.1	12.0

Figure 3. Genes of interest (Gene Ontology: Biological Process—Level 6) from allergic and tolerant patients' lymphocytes that showed increased expression in response to treatment with maximal stimulatory concentrations of *p*-phenylenediamine or Bandrowski's base.

were compared (Figure 1). Lower levels of cytokine secretion were associated with decreased cytokine gene transcription (Figure 5). Real-time-PCR analysis performed using fresh samples from allergic patients and tolerant individuals, 24 hours after PPD or BB stimulation, confirmed the microarray data.

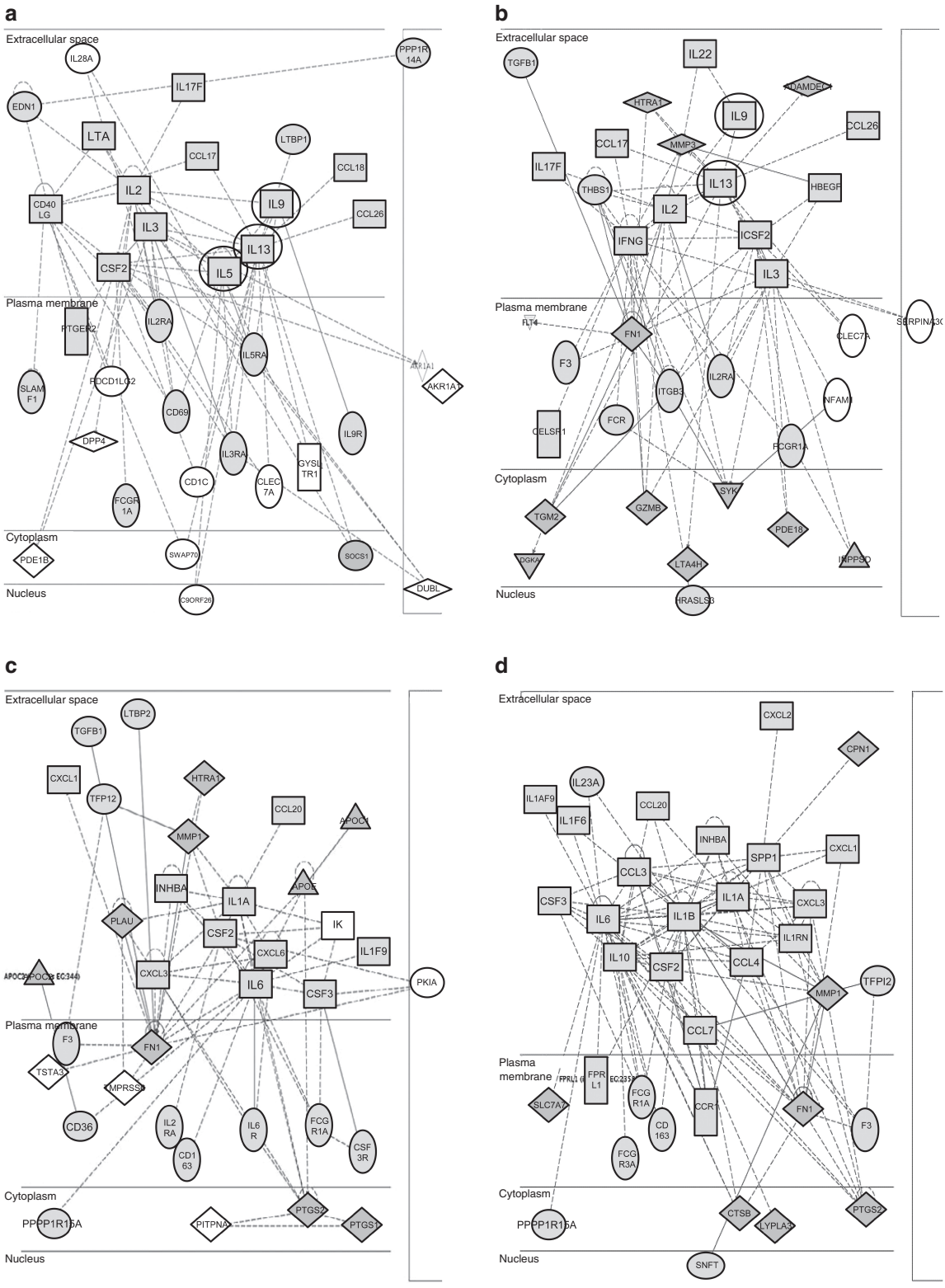


Figure 4. Network representation of interactions between differentially expressed genes from the analysis of antigen-treated lymphocytes from allergic and tolerant individuals. Genes colored gray represent the regulated genes' input into the network; unshaded genes represent genes in the system database associated with relevant networks. Circles highlight regulated T helper type 2 cytokine genes. (**a**) Allergic patients: PPD stimulation; (**b**) allergic patients: BB stimulation; (**c**) tolerant patients: PPD stimulation; (**d**) tolerant patients: BB stimulation.

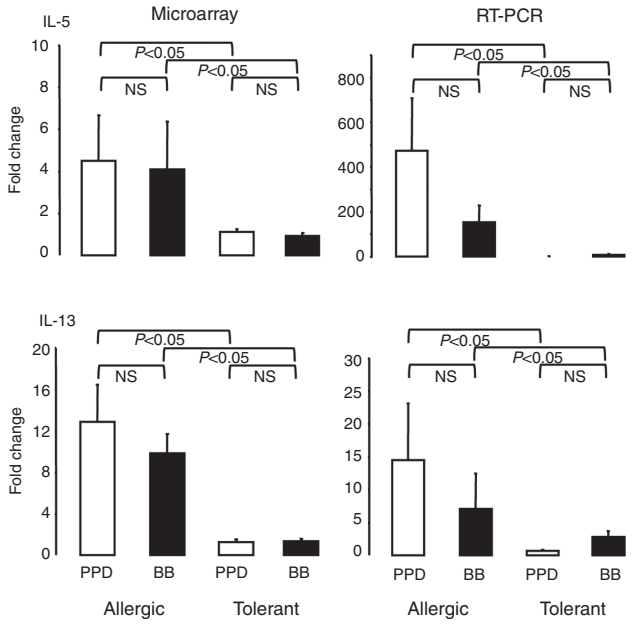


Figure 5. Changes in cytokine gene expression levels using microarray and real-time PCR analysis. Peripheral blood mononuclear cells from allergic and tolerant individuals were treated with maximal stimulatory concentrations of *p*-phenylenediamine and Bandrowski's base for 24 hours. Data are presented as mean ± SD.

Purified CD4+ and CD8+ T-lymphocyte proliferation with PPD and BB

To define the CD phenotype of antigen-stimulated T cells from allergic patients and tolerant individuals, purified CD4+ and CD8+ T cells were cultured with autologous, irradiated monocyte-derived dendritic cells and with either PPD or BB. CD4+ and CD8+ T cells from allergic patients were stimulated to proliferate by both PPD and BB (Figure 6a). In contrast, specific proliferation of tolerant individuals' cells was detected only for purified CD4+ T cells and only in the presence of BB (Figure 6a).

Comparison of IL-5 and IL-13 secretion from PPD- and BB-stimulated allergic and tolerant patients' CD4+ and CD8+ T-lymphocytes

High levels of IL-5 and IL-13, but not IFN-γ, were detected in supernatants from PPD- and BB-stimulated CD4+ and CD8+ T cells from allergic patients. IL-5 and IL-13 were also secreted from BB-stimulated tolerant individuals' CD4+ T cells, but at levels 80% lower than those seen in equivalent samples from allergic patients (Figure 6b).

Comparison of PBMC proliferation and cytokine secretion in an independent cohort of 10 allergic patients and tolerant individuals

Using a new and expanded patient cohort ($n=10$ allergic patients and tolerant individuals), PBMC proliferation and cytokine secretion were measured to determine whether Th2 cytokine secretion is a potential biomarker for discriminating between PPD allergic patients and tolerant individuals. In agreement with our previous findings, blood mononuclear cells from allergic patients and tolerant volunteers proliferated with BB, but only allergic patients' cells responded to PPD (Figure 7). Significantly higher levels of IL-5 ($P<0.01$, PPD; $P<0.05$, BB) and IL-13 ($P<0.01$, PPD; $P<0.01$, BB) were secreted from allergic patients' blood mononuclear cells after antigen stimulation, when allergic and tolerant individuals' cells were compared (Figure 7). In agreement with the original cohort, differences in the levels of IL-1α and IL-6 were not detected on comparison of allergic and tolerant individuals.

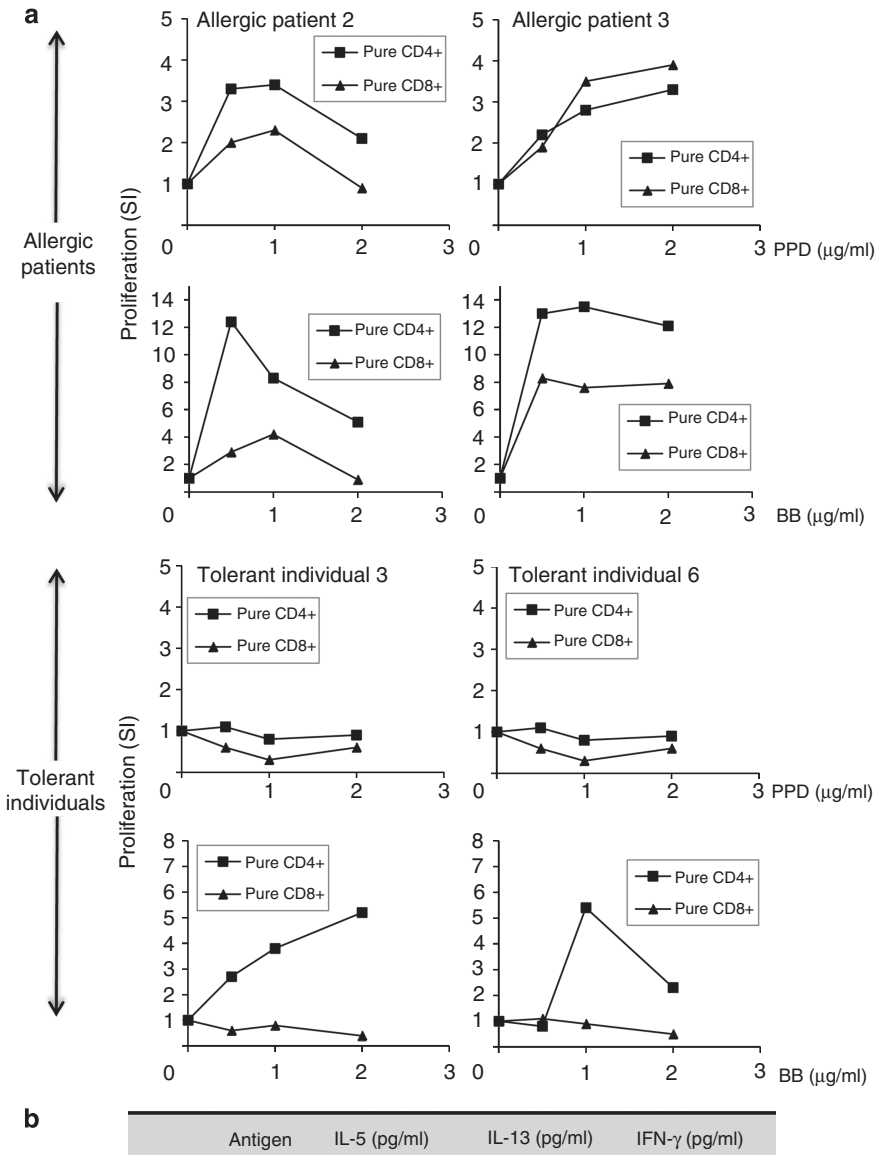
Because the expression of the IL-9 gene was upregulated in allergic patients' PBMCs stimulated with PPD and BB in the microarray study, IL-9 secretion from allergic and tolerant individuals' PBMCs was measured in the expanded patient cohort. IL-9 secretion was detected from allergic patients' PBMCs stimulated with PPD and BB and from BB-stimulated tolerant individuals' PBMCs (Figure 7).

DISCUSSION

p-Phenylenediamine is one of the most common chemical allergens associated with the development of contact dermatitis (Schnuch *et al.*, 1997), a prototype hypersensitivity reaction with an asymptomatic sensitization phase, an effector, and a resolution phase (Grabbe and Schwarz, 1998). Antigen-specific T cells are believed to be involved in the pathogenesis; however, the reason that certain individuals are particularly susceptible to PPD sensitization remains largely unexplored.

We recently established the presence of antigen-specific T cells in the circulation of allergic and most nonallergic individuals (with or without previous hair dye exposure, referred to herein as tolerant individuals (Coulter *et al.*, 2008)). PBMCs from both patient groups were stimulated with BB; their presence seems to reflect an acquired immune response that is not translated into an allergic reaction. This theory is supported by human studies with cord blood, which is not stimulated by BB (Coulter *et al.*, 2008), and mouse studies in which BB-specific T-cell responses were detectable only after *in vivo* exposure (Farrell *et al.*, 2009). In addition, PBMCs from allergic patients were specifically stimulated with PPD, which represents an important laboratory-based discrimination between allergic and tolerant patient groups. This difference between allergic and tolerant patients' PBMCs was reproduced in this study (Table 1).

The key objective of this study was to characterize the functionality of antigen-specific PBMCs and purified CD4+ and CD8+ T cells, focusing on whether the nature of the induced cytokine secretion profile differs between allergic and tolerant patient groups and whether such a difference might be related to the development of contact dermatitis. Supernatant taken from control and maximal stimulatory concentrations of PPD and BB was collected from each participant for the analysis of cytokine secretion. PPD and BB stimulation of allergic patients' PBMCs was associated with the secretion of similar cytokines and chemokines (Table 2). Of particular importance was the detection of the Th2 cytokines IL-4, IL-5, and IL-13, as well as IFN-γ and TNF-α. These findings are consistent with previous studies using



b

	Antigen	IL-5 (pg/ml)		IL-13 (pg/ml)		IFN-γ (pg/ml)	
		CD4+	CD8+	CD4+	CD8+	CD4+	CD8+
Allergic	PPD	914	768	1,583	849	<10	<10
	BB	1,757	757	3,444	1,137	<10	<10
Tolerant	PPD	62	87	<10	42	<10	<10
	BB	393	203	993	127	<10	<10

Figure 6. Stimulation of CD4+ and CD8+ T cells from allergic patients and tolerant individuals with *p*-phenylenediamine and BB. (**a**) Antigen-specific proliferation of CD4+ and CD8+ T cells in the presence of autologous dendritic cells as antigen-presenting cells. Proliferation was determined by the incorporation of [^{3}H]thymidine. Coefficient of variation was consistently less than 20%. (**b**) IL-5, IL-13, and IFN-γ secretion from allergic patients' and tolerant individuals' CD4+ and CD8+ T cells in response to maximal stimulatory concentrations of *p*-phenylenediamine and Bandrowski's base.

T-cell clones from PPD-allergic patients (Sieben *et al.*, 2002; Coulter *et al.*, 2008) as well as with the detection of high levels of Th2 cytokines in skin from PPD-challenged mice (Yokozeki *et al.*, 2003) and the recently described association between TNFA-308 G/A polymorphism and sensitization to PPD (Blomeke *et al.*, 2009). The cytokines IL-1 and IL-6 were also detected in the supernatant of antigen-stimulated PBMC cultures. IL-1 has been detected in individuals with psoriatic keratinocytes and in chronically inflamed skin and shown to be related to a general cutaneous alarm that triggers the release of chemokines such as MIP-3α and IL-8 (Spiekstra *et al.*, 2005). IL-6 is produced by a variety of cells, including keratinocytes, dendritic cells, and T cells, and is known as the principal endogenous circulating pyrogen. Correia *et al.*

	Age	Sex[1]	Dye use	Months since reaction	LTT (SI) PPD	LTT (SI) BB
Allergic patients						
1	37	F	Yes	252	2.3	20.5
2	43	F	Yes	17	10.7	34.4
3	77	F	Yes	18	11.02	14.1
4	51	F	Yes	24	3.3	15.7
5	33	F	Yes	22	12.2	32.6
6	68	F	Yes	44	5.5	24.9
7	52	F	Yes	14	12.3	39.4
8	70	F	Yes	11	17.8	22.2
9	56	F	Yes	22	15.3	17.4
10	43	F	Yes	96	34.9	80.1
Tolerant individuals						
1	26	F	Yes	Na[2]	–[3]	38.6
2	28	M	No	Na	–	19.1
3	24	F	Yes	Na	–	12.3
4	24	F	Yes	Na	–	10.2
5	24	F	Yes	Na	–	36.5
6	28	F	Yes	Na	–	11.5
7	37	M	No	Na	–	31.4
8	23	F	Yes	Na	–	21.9
9	27	M	No	Na	–	2.2
10	27	M	No	Na	–	13.8

[1]M, male ; F, Female; [2]Na, not applicable; [3]SI less than 2.

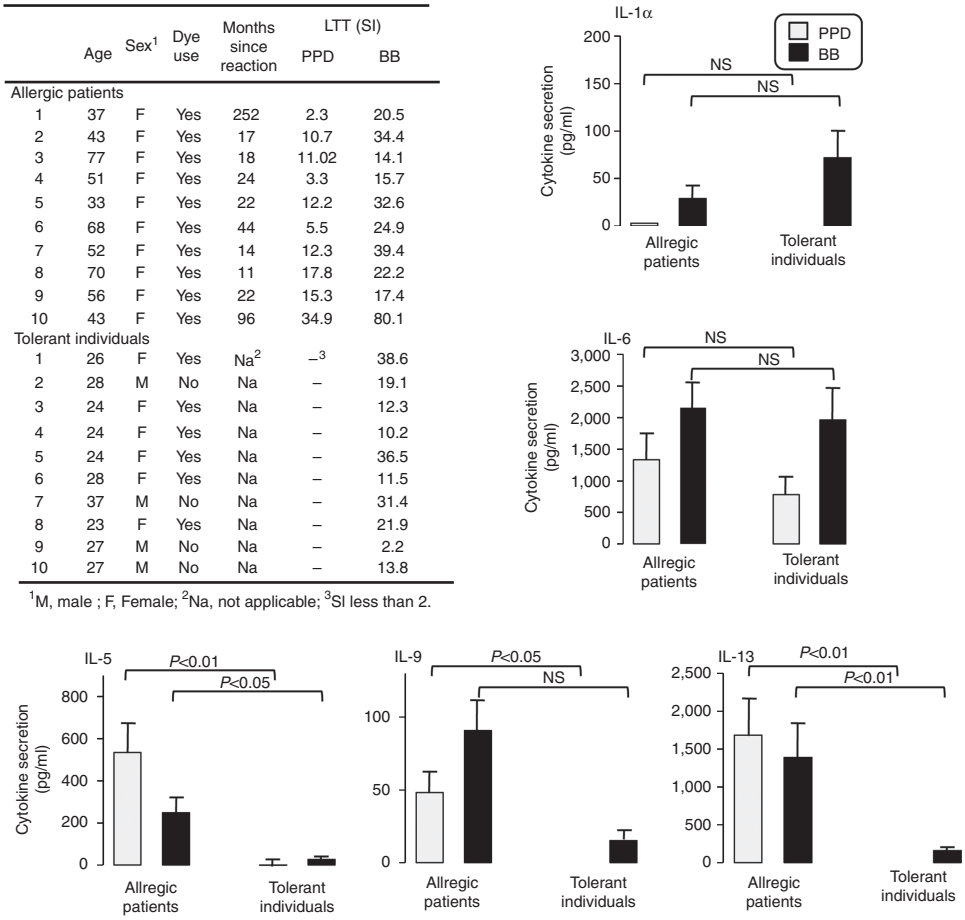


Figure 7. Antigen-specific proliferation and cytokine secretion by lymphocytes from a validation patient cohort. (**a**) Clinical details of the second expanded, independent cohort of *p*-phenylenediamine (PPD)-allergic patients and tolerant individuals, and comparison of maximum proliferation of peripheral blood mononuclear cells (PBMCs) after PPD or Bandrowski's base (BB) stimulation. Proliferation was determined by the incorporation of [^{3}H]thymidine. (**b**) Cytokine secretion from PBMCs stimulated with PPD and BB. Data are presented as mean ± SD, with values from unstimulated PBMCs (less than 10 ng ml^{-1} (50 ng ml^{-1} for IL-13)) subtracted. IL-4 secretion was not detected in either allergic or tolerant individuals.

(2002) recently detected high IL-6 levels in the blister fluid and serum of patients with drug-induced toxic epidermal necrolysis.

A comparison of cytokine secretion profiles from allergic and tolerant patients' PBMCs reveals several important features. First, tolerant patients' PBMCs incubated with BB, which stimulates a proliferative response, secreted significantly lower levels of the Th2 cytokines IL-4, IL-5, and IL-13 (4.3, 14.5, and 14.8%, respectively, that seen with BB-stimulated PBMCs from allergic patients) and the chemokine MIP-1α. Second, a trend toward higher levels of IL-8 and IL-10 secretion was detected with tolerant patients' PBMCs. Third, PPD treatment of PBMCs from tolerant individuals, which is not associated with a proliferative response, stimulated secretion of the chemotactic protein IL-8 and MCP-1, providing preliminary evidence that tolerant patients' PBMCs respond to PPD, but in a manner different from that with allergic patients.

It is possible that these antigen-driven differences between patient groups may be a function of the individual (for example, decreased Th2 cytokine secretion *per se* in tolerant patients' PBMCs) and not directly attributable to the chemical interaction with cells from allergic and tolerant patients. This explanation is difficult to accept, however, because allergic and tolerant patients' PBMCs secreted similar levels of most cytokines/chemokines following PPD and BB stimulation (Table 2), and stimulation with the protein antigen tetanus toxoid was associated with IL-4, IL-5, and IL-13 secretion in both patient groups (results not shown). It is also possible that decreased cytokine secretion in PBMCs from BB-treated volunteers might be an indication that only one T-cell population is responding and that perhaps certain Th2-producing T cells are missing. To address this question, purified CD4+ and CD8+ T cells from allergic patients and tolerant individuals were stimulated with PPD and BB, and proliferation and cytokine secretion were assessed. CD4+ and CD8+ T cells from allergic patients proliferated and secreted high levels of the cytokines IL-5 and IL-13 after PPD and BB stimulation. In contrast, in tolerant individuals, proliferation and low levels of cytokine secretion were

detected only in the CD4+ T-cell population. The detection of antigen-specific, cytokine-secreting CD8+ T cells from allergic patients alone provides an explanation for our observed results with PBMCs and supports the hypothesis that CD8+ T cells are important effectors in contact allergic dermatitis (Vocanson *et al.*, 2006, 2008). Somewhat surprisingly, the upregulation of cytotoxic molecules such as perforin, which are secreted from antigen-specific CD8+ Tcells and thought to be involved in the pathogenesis of allergic contact dermatitis in the mouse (Kehren *et al.*, 1999), was not detected in our study. In future experiments, we aim to characterize the functionality of PPD-specific polyclonal and monoclonal T cells from allergic patients, focusing specifically on their ability to kill autologous target cells and mechanisms of cytotoxicity. Finally, antigen concentration and/or length of antigen exposure might have an effect on T-cell cytokine production—the current study utilized maximal stimulatory antigen concentrations and one time point for the analysis.To address this issue, a detailed kinetic study using titrated antigen concentrations is in progress.

To explore whether differences in cytokine secretion from PPD- and BB-stimulated allergic and tolerant individuals are related to altered gene transcription, patients were recalled and PBMC proliferation was assessed, together with an analysis of gene expression using two methodologies: microarray and real-time PCR. High levels of IL-5 and IL-13 cytokine secretion from antigen-stimulated samples from allergic patients were associated with increased cytokine gene expression (Figure 5). In contrast, tolerant patients' PBMCs expressed low levels of Th2 cytokine genes after stimulation. These data support previous studies in humans and experimental animals indicating that PPD-specific T cells preferentially secrete Th2 cytokines (Sieben *et al.*, 2002; Yokozeki *et al.*, 2003; Coulter *et al.*, 2008) and suggest that the ability to produce a polarized Th2 response after antigen stimulation may relate to susceptibility to the development of contact dermatitis. A similar phenomenon has been observed after intranasal or cutaneous exposure to the strong respiratory sensitizer trimellitic anhydride and in atopic dermatitis. Sensitization to trimellitic anhydride is associated with IgE antibody production driven by antigen-specific T-cell activation and secretion of IL-4, IL-5, and IL-13 (Grammer *et al.*, 2000; Dearman *et al.*, 2002; Farraj *et al.*, 2006). In atopic dermatitis, a T-cell-mediated skin disease associated with IgE production following exposure to environmental protein antigens, the picture is more complex. In humans, some CD4+ and CD8+ T cells have been found to secrete Th2 cytokines, which induce IgE production, and play a role in the development of tissue destruction (Akdis *et al.*, 2000, 2002, 2003; Teraki *et al.*, 2000). We have recently shown that PPD binds preferentially to serum protein in culture (Coulter *et al.*, 2008), generating a T-cell-stimulatory hapten–protein complex (unpublished data). Interestingly, using a panel of directly reactive chemical allergens (excluding PPD), extracellular (serum) adduct formation was found to be associated with the production of Th2 cytokines in a mouse model (Hopkins *et al.*, 2005). Therefore, it is possible that the cellular pathophysiology of PPD-mediated contact dermatitis and atopic dermatitis relates to the nature of the antigenic determinant formed and the manner in which it is handled by immune cells. Future studies should be aimed at delineating the specific role of Th2-secreting CD4+ and CD8+ T cells (and possibly IgE antibodies) in PPD-mediated allergic contact dermatitis and the point in time at which they begin to act as mediators in the immunopathology of the disease.

The factors underlying antigen-driven differences in cytokine secretion between allergic and tolerant patients are unknown. Thus, microarray analysis was used to relate cellular function to the expression levels of families of related genes, with an overall aim of improving our understanding of the cellular mechanisms of allergic contact dermatitis. Differences between samples from allergic and tolerant patients were immediately apparent when principal component analysis was applied to the microarray data. It was possible to separate data points on the basis of subject group (Figure 2a), irrespective of the nature of antigen stimulation (negative control, PPD, BB, or tetanus toxoid).

Although many biological processes associated with antigen stimulation of allergic and tolerant patients' samples were similar (Figure 2b), regulated genes relating positive regulation of cell proliferation and the humoral immune response were absent in tolerant individuals. These data suggest that differential gene regulation might explain why only certain individuals develop clinical manifestations of contact dermatitis despite the presence of antigen-specific immunity in both patient groups. This hypothesis is supported by the construction of functionally interlinking network diagrams using the Ingenuity Pathway Assist (Mountain View, CA) software program. Gene patterns associated with the development of a Th2 cellular immune response were evident in allergic patients' samples, but absent in those from tolerant individuals (Figure 4). Networks deriving from allergic patients' samples also contained the cytokine IL-9, a signaling molecule secreted from memory T cells (Kajiyama *et al.*, 2007) that promotes IgE production by B cells and has been implicated in allergic diseases such as asthma and food allergy (Ying *et al.*, 2002; Forbes *et al.*, 2008). Recently, IL-9-producing T cells were found to be a distinct T-cell population, provisionally termed Th9 cells, deriving from Th2 cells following programming in the presence of TGF-β (Veldhoen *et al.*, 2008). IL-9 secretion from PPD-stimulated PBMCs was measured in the validation patient cohort (see below). Several individual genes were also identified as being unique among allergic individuals, including the Th2 cytokines discussed above; CCL17 and CCL26, which are known to be associated with Th2-type T-cell recruitment (Garcia *et al.*, 2005); and IL-17F and IL-17 receptor B (although stimulated PBMCs did not secrete IL-17) (Figure 4). Genes regulated in the top-scoring network from tolerant individuals treated with PPD and BB, although associated with immunological functions, displayed a much more regulatory profile involving TGF-β and IL-10 (Figure 4). Both TGF-β and IL-10 are known to have anti-inflammatory properties and are involved in the regulation of allergic rhinitis and allergic reactions in the skin (Cavani *et al.*, 1998;

Benson *et al.*, 2006). A network approach to analyzing microarray data has previously been used to define late-phase allergic reactions of the skin (Benson *et al.*, 2006).

An independent, expanded cohort of allergic patients and tolerant individuals ($n=10$ per group) was recruited to validate the major findings reported herein, namely, the nature of the antigen stimulating a T-cell response (PPD and BB with allergic patients' PBMCs, BB with tolerant individuals' PBMCs) and the lower levels of Th2 cytokines secreted from tolerant individuals' PBMCs (Figure 7). The validation study also permitted an analysis of IL-9 secretion by PBMCs stimulated with PPD and BB. IL-9 secretion was detected from allergic patients' PBMCs stimulated with PPD and BB (Figure 7) and tolerant individuals' PBMCs stimulated with BB, at low levels. Further experiments are planned to define the source of IL-9 secretion and the interplay between IL-9 and classic TH2-secreting cells in patients with allergic contact dermatitis.

In conclusion, these data reveal several important functional differences between PPD-stimulated PBMCs from allergic and tolerant patients. In particular, PPD and BB treatment of allergic patients' samples was associated with (i) the stimulation of CD4+ and CD8+ T cells and (ii) significantly higher levels of Th2 cytokine secretion and increased gene expression, which suggests that the ability to produce cytokines such as IL-4, IL-5, IL-9, and IL-13 following antigen stimulation may be an important factor in the development of contact dermatitis. Because the results were validated in an independent patient cohort, the degree of Th2 cytokine secretion seems to be a potential biomarker for discriminating between individuals who are allergic to PPD and those who are tolerant. The question of whether a Th2 cytokine secretion profile is peculiar to PPD or is prevalent in most contact allergic reactions requires further investigation.

MATERIALS AND METHODS

Donor characteristics

Peripheral blood mononuclear cells (PBMCs) were isolated from PPD-allergic, patch test–positive patients ($n=5$ in patient cohort 1, $n=10$ in patient cohort 2) and hair dye–exposed volunteers ($n=6$ in volunteer cohort 1, $n=10$ in volunteer cohort 2, from this point referred to as PPD-tolerant (Table 3 and Figure 7). Approval for the study was obtained from the local Liverpool research ethics committee, and informed consent was obtained from all participants. Experiments were conducted in accordance with the Declaration of Helsinki Principles.

Determination of PBMC proliferation

Proliferation of PBMCs from allergic and tolerant individuals was measured using the lymphocyte transformation test, as described previously (Naisbitt *et al.*, 2003). PBMCs were cultured with PPD and BB (final concentrations ranged from 0.1 to 20 μM), and absence of the two chemicals was used as a negative control.

Measurement of PBMC cytokine/chemokine secretion

Supernatants (100 μl) were collected from the lymphocyte transformation test, before the addition of [^{3}H]thymidine, for the analysis of cytokine/chemokine secretion, using a LINCOplex multiplex assay kit (LINCO Research, Hampshire, UK). Protein (IL-1α, IL-1β, IL-4, IL-5, IL-6, TGF-α, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, TNF-α, eotaxin (CCL11), MCP-1 (CCL2), sCD40L, MIP-1α (CCL3), MIP-1β (CCL4), IP-10 (CXCL10), and RANTES (CCL5)) content was measured

Table 3. Clinical details of allergic and tolerant patients

	Age	Sex[1]	Previous hair dye use	PPD allergy	Other allergy	Months since reaction	Patch test result	LTT (SI) PPD	LTT (SI) BB
Allergic patients									
1	53	M	Yes	Yes	Red, orange, and blue dye, potassium dichromate	39	++	52.0	111.5
2	42	F	Yes	Yes		43	++	55.1	66.1
3	66	F	Yes	Yes	Nickel, orange dye	87	++	8.6	21.6
4	46	F	Yes	Yes		37	++	74.1	20.4
5	67	F	Yes	Yes		Unknown	++	15.1	29.5
Tolerant patients									
1	25	F	Yes (M)[2]	No			Np[3]	<2	7.6
2	49	F	Yes (M)	No			Np	<2	5.2
3	27	F	Yes (M)	No			Np	<2	12.9
4	35	M	Yes (M)	No			Np	<2	5.9
5	24	M	No	No			Np	<2	24.5
6	35	M	Yes (M)	No			Np	<2	6.1

[1]M, male; F, female.
[2]M, multiple hair dye exposures.
[3]Np, not performed because of ethical restrictions.

using a Liquichip 100 workstation (Qiagen, West Sussex, UK) with LiquiChip IS 2.3 software.

Microarray analysis

Peripheral blood mononuclear cells (PBMCs) (5×10^6) were cultured with stimulatory concentrations of PPD and BB, as well as with a DMSO (0.1% v/v) control. After 24 hours, cells were washed and total RNA was isolated using Trizol reagent. The quality of RNA was assessed by electrophoretic separation on an RNA Nano lab chip in a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA); total RNA samples with a 28S/18S band ratio greater than 1.6 were used for target labeling. CodeLink human whole-genome arrays (http://www.appliedmicroarrays.com) were scanned for the expression of approximately 53,485 genes using a GenePix 4000B Array Scanner (Molecular Devices, Sunnyvale, CA), optimized for laser scanning at a wavelength of 635 nm. A detailed description of the methods used and the quality control criteria fulfilled is given in the Supplementary Materials and Methods.

For analysis, the data were imported into GeneSpring 7.3 GX software (Agilent Technologies). Stringent data reduction was achieved using filters: controls were removed within the chip; filtering was applied on probe type accepting only discovery genes; and filters were applied to discovery genes on the basis of flags present in at least 8 of 24 samples. Identification of differential genes was subsequently carried out by filtering upon fold change, accepting genes whose expression was altered by greater than twofold either up or down in the treated sample compared with the corresponding negative control sample. By constructing gene lists, it was possible to determine potential differences and/or similarities between allergic and tolerant individuals. Genes unique to PPD and BB treatment for each individual were analyzed using FatiGOPlus (fast assignment and transference of information; Al-Shahrour *et al.*, 2005) software (http://www.babelomics.org), with a focus on identifying key gene ontology biological processes. In addition, the Ingenuity Pathway Assist application was used to organize differentially expressed genes into networks of interacting genes and to find modules of functionally related genes that also corresponded to pathways.

All primary data from this work are available from the ArrayExpress database (http://www.ebi.ac.uk/microarray-as/ae) under accession number E-MEXP-1812.

IL-5 and IL-13 gene expression analysis

Real-time PCR primers and probes for IL-5, IL-13, and GAPDH were purchased from Applied Biosystems (Foster City, CA). Quantitative PCR was performed using Taqman probe assays in the Platinum Quantitative PCR Supermix-UDG kit (Invitrogen, Paisley, UK, cat no. 11730-017). IL-5 and IL-13 expression was measured using a Bio-Rad icycler (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) and programmed for amplification as follows: 50°C for 2 minutes hold, 95°C for 2 minutes hold, then 45 cycles of 95°C for 15 seconds to denature and 60–65°C for 30–45 seconds to anneal. Averaged C_t values were normalized back to the averaged C_t values of a control gene (GAPDH). Adjusted average C_t values were used to calculate the average of the biological replicates, and ΔC_t was then calculated between the control and the treated groups. Relative expression levels of the same gene between two samples were determined according to ΔC_t values; a ΔC_t value <1 was considered to signify no change.

Determination of purified CD4+ and CD8+ T-cell proliferation

To measure the proliferation of purified CD4+ and CD8+ T cells from allergic patients and tolerant volunteers, PBMCs were isolated on a density gradient of Lymphoprep and separated into two aliquots. To generate monocyte-derived dendritic cells as a source of antigen-presenting cells in the proliferation assay, the first aliquot of PBMCs was cultured in 24-well plates for 4 hours, followed by repeated washing to remove nonadherent cells. Adherent cells were then cultured in medium, supplemented with IL-4 (800 U/ml) and GM-CSF (800 U/ml). Half of the medium was replaced with fresh complete medium on days 1, 3, and 5, and immature dendritic cells were ready for use on day 6. The second PBMC aliquot was frozen, then thawed on day 6, and CD4+ and CD8+ T cells were positively selected by incubating PBMCs with immunomagnetic microbeads coated with anti-CD4+ or anti-CD8+ antibodies and magnetic cell sorting (Miltenyi Biotec, Bisley, UK). The purity of the sorted T cells was determined using flow cytometry (Coulter EPICS XL-MCL flow cytometer; Beckman Coulter, Fullerton, CA) with conjugated CD3+, CD4+, and CD8+ antibodies (BD Biosciences, Oxford, UK).

To test the antigen specificity of the purified CD4+ and CD8+ T cells, T cells (1×10^5; total volume, 0.2 ml) were incubated with autologous irradiated (60 Gy) dendritic cells (0.2×10^5) and PPD or BB (0.5–2 μg/ml). After 5 days, [^{3}H]thymidine was added and proliferation was measured by scintillation counting.

Supernatant was collected and stored from antigen-stimulated PBMCs and purified T-cell cultures before the addition of [^{3}H]thymidine for the analysis of IL-5, IL-13, and IFN-γ secretion, using the methods outlined above.

Statistical analysis

Increases in cytokine secretion and cytokine gene expression resulting from treatment with the test chemicals, and evaluated using microarray and real-time PCR, were compared between patient groups, as well as between PPD and BB treatment parameters, using the Mann–Whitney test for nonparametric/non-normally distributed data. To identify global differences between allergic and tolerant individuals, principal component analysis was applied to the microarray data. Such analysis is able to identify patterns in data of high dimension and express them in such a way as to highlight their similarities and differences. Principal component analysis was applied randomly, with no preformed opinions as to how the data should be split.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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