

Phenotypic Characterization of Human CD4⁺ Regulatory T Cells Obtained from Cutaneous Dinitrochlorobenzene-Induced Delayed Type Hypersensitivity Reactions

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In this study, we describe the generation and characterization of cloned human CD4⁺ T lymphocyte populations that have infiltrated into cutaneous, 2,4-dinitrochlorobenzene-induced delayed type hypersensitivity reactions in healthy human subjects. It is shown that, in addition to T helper type 1 clones, elevated numbers of regulatory T clones, producing high levels of interleukin-10 and interleukin-5, but no measurable interleukin-4, were isolated from delayed type hypersensitivity reactions in four of six donors. A subsequent challenge with 2,4-dinitrochlorobenzene of two donors from whom only few interleukin-10-producing T cell clones had been generated after primary challenge, resulted in a decrease in the frequency of T helper type 1 clones and a strong increase in the number of interleukin-10-producing T helper type 2 and regulatory T clones. Culture supernatants from the latter cells, activated with anti-CD3 and anti-CD28 monoclonal antibody,

inhibited alloantigen-mediated T cell proliferation which was, partly dependent on interleukin-10, and independent of transforming growth factor- β . In addition, dendritic cells generated *in vitro* in the presence of these culture supernatants were impaired in their ability to induce alloantigen-induced proliferative responses. Differential expression of transcripts for the T1/ST2 molecule enabled a phenotypic distinction between resting regulatory T cells and T helper type 2 cells, but not between regulatory T cells and T helper type 1 cells. This experimental model provides a useful tool to isolate human inflammatory and anti-inflammatory T cell subpopulations and, furthermore, enables the study of the kinetics of their appearance into delayed type hypersensitivity reactions. **Key words:** allergy/cytokines/hapten/human/interleukin-10/skin/T lymphocytes. *J Invest Dermatol* 117:318–325, 2001

Cutaneous sensitization with haptens (small lipophilic chemicals that become antigenic in combination with carrier proteins and peptides present in the groove of major histocompatibility antigens) (Martin *et al*, 1992), results in contact hypersensitivity which, like delayed type hypersensitivity (DTH) reactions, is a local inflammatory immune response mediated by T effector cells. It has been demonstrated in experimental murine models that interferon (IFN)- γ and IFN- γ -promoting cytokines, such as interleukin (IL)-12, play an important part in the induction of DTH reactions following sensitization to haptens, whereas contact hypersensitivity is down-regulated by IL-4 and IL-10, as a result of their strong anti-inflammatory activity (DiIulio *et al*, 1996; Xu *et al*, 1996). Results from studies, using major histocompatibility complex class I and/or class II deficient mice, indicate that major histocompatibility complex class I restricted, CD8⁺ T cells, rather than IFN- γ -producing CD4⁺ Th1 cells, are sufficient for the induction of contact hypersensitivity to the strongly sensitizing hapten dinitro-

fluorobenzene (Bour *et al*, 1995). Although, initially, contact hypersensitivity of humans to haptens such as nickel was thought to be associated with the presence of CD4⁺ Th1 cells, generated from peripheral blood and cutaneous biopsies from nickel-sensitive patients (Sinigaglia *et al*, 1985; Kapsenberg *et al*, 1992), CD8⁺ effector T cell clones (Cavani *et al*, 1998), as well as IL-4-producing nickel-specific Th2 cells (Werfel *et al*, 1997) have been isolated as well. Moreover, it has been reported that allergic responses to nickel in patients with allergic contact dermatitis are associated with a decrease in IL-10 production (Cavani *et al*, 1998), suggesting that the clinical outcome of contact sensitivity, following exposure to hapten is determined by the balance between IFN- γ -producing CD8⁺ T effector cells and anti-inflammatory IL-4- and IL-10-producing CD4⁺ T cells.

Recently, a unique population of CD4⁺ lymphocytes, designated T regulatory type 1 (Tr1) cells has been described (Groux *et al*, 1997a). These cells, which have strong suppressive properties and human Tr1 cells, are characterized by the high production of IL-10 and IL-5, but absence of production of IL-4. In the mouse, CD45RB⁺ Tr cells have been shown to play a crucial part in the normal regulation of intestinal immune responses (reviewed in Groux and Powrie, 1999). In addition, hapten-specific Tr-like suppressor cells, isolated from mice following the application of hapten to ultraviolet-irradiated skin were found to block the induction of Th1-cell-mediated contact sensitivity in adoptive

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Abbreviations: DNCB, 2,4-dinitrochlorobenzene; Tr, T regulatory; DTH, delayed type of hypersensitivity.

transfer experiments (Shreedhar *et al*, 1998). Apart from their cytokine production profile and strong anti-inflammatory properties, little is known about the phenotypic characteristics of Tr cells, in contrast to CD4⁺ Th1 and Th2 cells, which can be identified based on the presence or absence of expression of functional receptors for IFN- γ (Pernis *et al*, 1995; Groux *et al*, 1997b) and IL-12 (Rogge *et al*, 1997; Szabo *et al*, 1997). In addition, an IL-1R-like cell surface molecule, designated T1/ST2 (Klemenz *et al*, 1989; Tominaga, 1989) has been reported to be involved in murine Th2 effector function, but its expression on human Th2 and Tr cells is unknown (Löhning *et al*, 1998; Xu *et al*, 1998).

In this study, we have induced cutaneous DTH reactions in healthy human subjects, following sensitization and challenge with 2,4-dinitrochlorobenzene (DNCB), with the aim of generating and characterizing subpopulations of T cells that have migrated into the site of inflammation. The use of this naive hapten permits the study of the *in vivo* appearance of polarized subpopulations of T cells into DTH reactions, whereas these cells upon cloning and expansion can be used for a detailed functional and phenotypic characterization.

MATERIALS AND METHODS

Sensitization and induction of DNCB-mediated cutaneous DTH reactions Cutaneous DTH reactions were induced in healthy human volunteers (without any form of atopic disease), after an initial sensitization and a subsequent challenge with DNCB; this was done according to a protocol, approved by the ethical commission of the University Hospitals of Montpellier. During sensitization, a petrolatum-backed 11 mm filter, containing 30 μ g DNCB (Sigma/Aldrich Chemicals, St Louis, MO), diluted in 50 μ l acetone was placed within a metal chamber (diameter 12 mm: Finn Chambers, Promédical, La Chaussée St Victor, France) and the assembly was immobilized with hypoallergenic adhesive tape on the left buttock for 48 h. Fourteen days after the initial sensitization, a DTH reaction was elicited; this was following the application of 30 μ g DNCB, diluted in acetone to the inner side of the upper arm, opposite of the sensitization site, using a 7 mm petrolatum-backed filter disk and a 8 mm metal chamber (Finn Chambers). Forty-eight hours after the challenge, the intensity of the DTH reaction was determined, according to the guidelines of the International Contact Dermatitis Group (Adams, 1981), and a 5 mm punch biopsy from DTH reactions with a score >3 was taken from the site of inflammation. Scores were determined as follows: (1) no reaction; (2) mild erythema; (3) moderate erythema, occasionally with papulation; (4) strong erythematous reaction with edematous vesicular changes; and (5) extreme of spreading reaction, including bullous or ulcerative reactions. For kinetics studies, DNCB was applied twice on the inner arm 14 d after the initial sensitization with an interval of 14 d between the first and the second challenge and again a punch biopsy was taken 48 h after the challenge. Punch biopsies were transferred to a 24-well tissue culture plate (Linbro, McLean, VA) in a final volume of 1 ml of a culture medium (described by Yssel *et al*, 1984), supplemented with 1% human AB⁺ serum and 2 ng per ml recombinant (r)IL-2 (a kind gift of Dr Satish Menon, DNAX Research Institute, Palo Alto, CA) and incubated at 37°C in 5% CO₂. After 3 d of culture, fresh medium containing 2 ng per ml rIL-2 was added to the cultures and growing T cells were collected after 7 d of culture for cloning.

T cells Cultures of T cells, grown out of punch biopsies from DNCB-induced DTH reactions, were cloned by limiting dilution in the presence of 5×10^4 irradiated (90 Gy) allogeneic peripheral blood mononuclear cells (PBMC), 5×10^3 irradiated (90) cells of the Epstein-Barr virus-transformed B cell line JY and 0.1 μ g per ml (Murex, Beckenham, U.K.) in round-bottom wells of a 96-well tissue culture plate (Nunc, Roskilde, Denmark), as described previously (Spits *et al*, 1982). After 5 d of culture, rIL-2 was added at a final concentration of 2 ng per ml. Between 10 and 14 d after the cloning procedure, growing T cell clones were analyzed for their cytokine production profile (see below). Selected T cell clones were maintained in culture by weekly stimulation with the feeder cell mixture referred to above (Spits *et al*, 1982). Three to 4 d after each restimulation, the cultures were expanded in Yssel's medium, supplemented with 1% AB⁺ human serum and 2 ng per ml rIL-2 and were used in experiments between 10 and 12 d after the last stimulation with feeder cells.

PBMC were isolated from freshly collected, heparinized peripheral blood (Center de Transfusion sanguine, Montpellier, France) by Ficoll-Hypaque density gradient centrifugation. CD4⁺ peripheral blood T cells were purified (purity >95%) by negative selection from mononuclear cell preparations, using monoclonal antibody (MoAb)-coated magnetic beads (StemCell Technologies, Vancouver, Canada), according to the manufacturer's instructions.

Monocytes and dendritic cells Monocytes (purity >95% CD14⁺) were isolated from adherent PBMC (incubation at a concentration of 10^7 cells per ml at 37°C in Yssel's medium/1% human serum for 60 min in a Petri dish) by negative selection using MoAb-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions.

Immature dendritic cells were generated from monocytes cultured in Iscove's modified Dulbecco's medium (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Boehringer, Mannheim, Germany), 200 ng per ml recombinant granulocyte-monocyte colony-stimulating factor and 20 ng per ml rIL-4 (generous gifts from Drs Gregorio Aversa, Novartis Research Institute, Vienna, Austria and N. Nagabushan, Schering-Plough Research Institute, Kenilworth, NJ, respectively), according to the method of Sallusto and Lanzavecchia (1994). At day 6 of culture, lipopolysaccharide (Sigma) was added at a concentration of 250 ng per ml for 24 h to induce maturation of the cells. The resulting population of dendritic cells was predominantly CD1a⁺, CD14⁻, CD83⁺, HLA-DR⁺ (Results not shown).

Analysis of cytokine production by enzyme-linked immunosorbent assay (ELISA) Two hundred thousand T cell clones were transferred to flat bottom wells of a 96 well culture plate (Nunc) and stimulated with the immobilized, plate-bound, anti-CD3 MoAb SPV-T3b (Spits *et al*, 1983) and anti-CD28 MoAb B-T3 (a kind gift of Dr John Wijdenes, Diaclone, Besançon). MoAb were coated at 4°C for 48 h on to the culture plates at a concentration of 10 μ g per ml in phosphate-buffered saline and plates were washed twice with phosphate-buffered saline and once with culture medium prior to the addition of cells. Control supernatant was prepared following identical treatment in the absence of T cells. After 24 h of culture, supernatants were harvested, spun at $270 \times g$ for 7 min to remove remaining cells, aliquoted, and stored at -20°C prior to cytokine measurements. Cytokine production was analyzed by specific ELISA, as described previously (Pène *et al*, 1998). For ELISA standards, preparations of purified human rIL-4, rIL-5, and rIFN- γ were purchased from R&D Systems Europe (Abingdon, U.K.). Production of IL-10 was measured using a commercial ELISA (Diaclone, Besançon, France), according to the manufacturer's instructions. Standard curves for IL-4 and IFN- γ were calibrated to reference samples from the National Institute for Biological Standards and Control (NIBSC; Hertfordshire, U.K.). The limits of sensitivity of the assays were about 5 pg per ml for each of the cytokines.

DNCB-induced and allo-antigen-induced proliferative responses For the measurement of DNCB-specific responses, 10^5 T cell clones were stimulated with 90 g per ml DNCB sulfate (Sigma/Aldrich) in the presence of 10^5 autologous irradiated (50 Gy) PBMC. For alloantigen-induced proliferative responses, 10^5 purified CD4⁺ peripheral blood T cells were incubated with 2.5×10^4 purified irradiated (50 Gy) monocytes from an HLA-DR mismatched healthy donor. Cultures were carried out in 96 well round-bottom culture plates (Nunc), in the absence or presence of different dilutions of culture supernatants generated from activated T cell clones and neutralizing anti-IL10 MoAb (B-N10, Diaclone Research) and/or the neutralizing anti-transforming growth factor (TGF) - β MoAb 2G7 (a kind gift of Dr. Cees Melief, Leiden University, the Netherlands), respectively, both at a final concentration of 10 μ g per ml. As a control MoAb the anti-IL-5 MoAb 39D10 (kindly provided by Dr. John Abrams, DNAX Research Institute, Palo Alto, CA) was used. To remove residual anti-CD3 and CD28 MoAb, culture supernatants were incubated for 30 min at 4°C with goat anti-mouse coated magnetic beads (Dyna), which were subsequently removed using a magnetic separator.

Alternatively, CD4⁺ T cells were stimulated with dendritic cells generated *in vitro* in the presence or absence of culture supernatants and neutralizing anticytokine antibodies. Recombinant IL-10 (a generous gift of Dr Nagabushan, Schering Plough Research Institute, Kenilworth, NJ) was used as a positive control at a concentration of 10 ng per ml. After 5 d of incubation at 37°C and 5% CO₂, 37 kBq (1 μ Ci) of tritiated thymidine (Amersham France, Les Ulis, France) was added to the cultures. After another 8 h of culture, the cells were harvested on to glass fiber sheets, using an automated cell harvester (Tomtec, Orange, CT) and

Table I. List of primers used for RT-PCR

Gene	Direction location	Size	cDNA	Sequence	Annealing temperature	Cycles
β actin	5'	310	231	GCT GCT GAC CGA GGC CCC CCT GAA C	60	25
	3'		541	CTC CTT AAT GTC ACG CAC GAT TTC	60	
IL-4	5'	300	199	ACT CTG TGC ACC GAC TTG ACCGTA A	60	35
	3'		499	TCT CAT GAT CGT CTT TAG CCT TTC C	60	
IFN- γ	5'	494	109	ATG AAA TAT ACA AGT TAT ATC TTG GCT TT	60	35
	3'		603	GAT GCT CTT CGA CCT CGA AAC AGC AT	60	
IFN- γ R	5'	321	848	TTA AAT ACA CCG ACA GTA AAT GGT T	48	35
β chain	3'		1164	AAA GGC CGT GGA GGT ATC AGC GAT G	48	
IL-12R	5'	214	992	ATC TTC GTT GGT GTT GC	50	35
β_2 chain	3'		1189	GGG GTG AGG TTG ATT CC	50	
ST2L	5'	393	-17	CTT GAT TGA TAA ACA GAA TG	50	35
	3'		376	CTG ATC CAG ATA CTG TTG AA	50	

Table II. Number of T-cell clones obtained and phenotype from biopsies of healthy donors

Donors	Total Nb of clones obtained	Number of clones / % of total ^a				
		Phenotype ^b				
		Tr	Th1	Th2		Th0
				IL-10 ⁻	IL-10 ⁺	
1	123	17	15	3	64	37
		12.5	11.0	2.2	47.1	27.2
2	21	7	5	1	1	7
		33.3	23.8	4.8	4.8	33.3
3	182	69	111	0	0	2
		37.9	61.0	0.0	0.0	1.1
4	210	148	32	1	0	29
		70.5	15.2	0.5	0.0	13.8
5	168	9	48	23	14	74
		5.4	28.6	13.7	8.3	44.0
6	135	5	97	0	1	32
		3.7	71.9	0.0	0.7	24.7

^aNumbers and frequencies of T cell clones of each subpopulation indicated in plain and bold/underlined numbers, respectively.

^bProduction levels of T cell clones with a Tr phenotype defined as IL-10 ≥ 100 pg per ml, IL-4 < 10 pg per ml, IL-5 production > 1 ng per ml; a Th1 phenotype defined as IL-10 < 100 pg per ml, IL-5 < 10 pg per ml or IL-4 < pg per ml or IFN- γ /IL-4 ratio \geq and a Th2 phenotype defined as IL-5 production > 1 ng per ml, IFN- γ < 20 pg per ml or IL-4/IFN- γ ratio ≥ 5 ; IL-10⁺ ≥ 100 pg per ml; IL-10⁺ ≥ 100 pg per ml; IL-10⁻ < 100 pg per ml.

radioactivity was measured, using a scintillation counter (Wallac, Turku, Finland). Results are expressed as mean \pm SD of triplicate cultures.

cDNA synthesis and reverse transcription-polymerase chain reaction (reverse transcription-PCR) analysis For analysis of IL-4, IFN- γ , IFN- γ R β -chain, IL-12R β_2 -chain, and T1/ST2 mRNA expression, 10^6 T cells were either cultured in medium alone or stimulated with immobilized, plate-bound anti-CD3 MoAb and soluble anti-CD28 MoAb for 6 h at 37°C, 5% CO₂. At the end of each culture period, the cells were harvested and spun for 5 min at 270 \times g. After removal of supernatant, the cell pellet was lysed in RNAPlus (Quantum, Illkirch, France), according to the manufacturer's instructions, and total RNA was extracted using chloroform, precipitated with ethidium bromide and quantitated by optical density readings at 260 nm. Reverse transcription and amplification of cDNA by PCR was performed as described previously (Yssel and Cottrez, 1998). PCR cycles were 30 s at 94°C, 30 s at 60°C (48°C and 50°C for T1/ST2 and IL-12R β_2 -chain, respectively), 30 s at 72°C, with 25 cycles for β -actin and 35 cycles for IFN- γ , IL-4, IFN- γ R β -chain, IL-12R β_2 -chain, and T1/ST2. Primer

sequences used in this study are listed in **Table I**. PCR products were analyzed on an ethidium bromide gel and scanned with a densitometer.

Immunofluorescence and flow cytometry Immunofluorescence and flow cytometry procedures were carried out using the method of Lanier and Recktenwald (1991). Cell surface expression of CD4 was analyzed using the phycoerythrin-conjugated Leu-3 MoAb (Becton-Dickinson, San Jose, CA). Cell surface staining on dendritic cells was carried out using the following MoAb: anti-CD1a (Immunotech, Lumigny, France), anti-CD14, anti-CD83, and anti-HLA-DR (Becton Dickinson). Immunofluorescence was analyzed on a FASCcalibur flow cytometer using Cellquest software (Becton Dickinson).

Statistical analysis Statistical analysis was performed by the Mann-Whitney U test was used for intergroup analysis.

RESULTS

Induction of a cutaneous DTH response in healthy donors following sensitization and rechallenge with DNCB Healthy human volunteers were sensitized following the application of a fixed dose of 30 μ g of DNCB on to the skin and were rechallenged with the same amount of DNCB 14 d later. Forty-eight hours after the rechallenge, a local cutaneous inflammation was observed in six of six patients, which was characterized by an intensity score > 3. Punch biopsies, taken from the DTH reactions, were cultured in the presence of 2 ng rIL-2 per ml. Generally, between 5×10^5 and 10^6 T lymphocytes were obtained after 7–10 d of culture.

Cytokine production profiles of T cell clones obtained from DNCB-induced DTH reactions Following cloning by limiting dilution and expansion *in vitro*, the cytokine production profile of T cell clones obtained from DNCB-induced DTH reactions was analyzed. As is shown in **Table II**, a total of 839 CD4⁺ T cell clones obtained from six different donors were analyzed for their capacity to produce IL-4, IL-5, IL-10, and IFN- γ , following stimulation of the cells with anti-CD3 and anti-CD28 MoAb. As expected, Th1 cells, producing IFN- γ , but no IL-4, IL-5, and secreting very low levels of IL-10 (< 100 pg per 10^6 cells), could be isolated from the site of inflammation, although their frequency varied among the different donors (**Table II**). The frequency of Th2 cells, isolated from punch biopsies of four donors, was very low and in one instance only (donor 1), a large number of T cell clones expressing a Th2-like cytokine production profile was generated, characterized by high levels of IL-4, IL-5, and IL-10 production. In addition, from the biopsies of three of six donors, a significant number of T cell clones were generated that produced high levels of IL-10, but no detectable levels of IL-4; this resembled a previously reported subpopulation of Tr cells (Groux and Powrie, 1999) with about 70% of T cell clones among the nearly 200 T cell clones isolated from the skin biopsy of donor 4 expressing a Tr-like

Table III. Phenotype of skin-derived T cell clones is stable during prolonged culture

T cell clones	Sub population	Cytokine	Cytokine production (ng per ml)		
			Number of weeks in culture		
			5	7	9
HAT-B. 180	Tr	IFN	0.9	4.1	7.0
		IL-4	<0.01	<0.01	<0.01
		IL-5	<0.01	<0.01	<0.01
		IL-10	2.6	5.9	2.3
BOY-JF.161	Tr	IFN	20.2	27.4	47.6
		IL-4	<0.01	<0.01	<0.01
		IL-5	4.6	34.1	17.2
		IL-10	37.6	28.7	49.3
PUE.F3.46	Tr	IFN	1.3	5.6	6.3
		IL-4	<10	<10	<10
		IL-5	15.7	18.2	16.8
		IL-10	6.5	4.5	7.9
BOY-JF.157	Th2	IFN	0.08	0.04	0.1
		IL-4	13.4	8.5	11.8
		IL-5	218.0	77.0	120.0
		IL-10	3.7	2.4	2.2
PUE.F3.9	Th2	IFN	0.02	1.2	3.5
		IL-4	10.9	15.4	13.9
		IL-5	15.6	12.0	9.8
		IL-10	5.7	6.6	4.5

cytokine production profile. Moreover, in keeping with results in the literature (Bacchetta *et al*, 1990; Groux and Powrie, 1999), Tr cells produced high levels of IL-5 (**Table III**, and results not shown).

CD4⁺ Th0 clones, producing IL-4, IL-5, IFN- γ , and variable amounts of IL-10, were generated from four of six donors, with a frequency between 14 and 40% of the total number of T cell clones derived from DNCB-induced DTH reactions. Cloning efficiencies of the limiting dilution assays were comparable (52–74%) with the exception of one donor (donor 2) whose punch biopsy yielded only 22 T cell clones with a cloning efficiency of 29%. Among the T cell clones obtained from donors 2, 3, 4, and 6, tested for reactivity with DNCB sulfate, 16%, 25%, 7%, and 3%, respectively, proliferated in response to stimulation with DNCB sulfate in the presence of autologous irradiated PBMC.

Stability of T cell subpopulations obtained from cutaneous DNCB-induced DTH reactions A number of the T helper and Tr clones were selected and the stability of their cytokine production profile upon prolonged culture was determined. As is shown in **Table III**, none of the Tr clones produced IL-4, whereas the production of IL-5 and IL-10 remained stable upon long-term culture; however, the production of IFN- γ in each of the Tr clones increased over time. This phenomenon was also observed in some of the strongly polarized Th2 clones that produced increasing amounts of IFN- γ , following repeated stimulation with feeder cells, required for their maintenance in culture. This notion was confirmed by the analysis of IFN- γ transcripts, which were expressed at low levels in freshly generated, activated, Th2 and Tr clones, but increasingly expressed by these cells following long-term culture *in vitro* (**Fig 3**). In contrast, the cytokine production profile of Th1 clones, obtained from cutaneous DNCB-induced DTH reactions, remained stable and no induction of IL-10, IL-4, or IL-5 was observed during prolonged culture of these cells (Results not shown).

Repeated exposure to DNCB increases the number of IL-10-producing Th2 and Tr cells in cutaneous DTH reactions Because of the high frequency of IL-10-producing

Table IV. Evolution of T cell clone cytokine production phenotype from 2 successive biopsies from the same donor

Biopsy (Donor 5)	Total Nb of clones obtained	Number of clones /		% of total ^a		
		Tr	Th1	Phenotype ^b		
				Th2		
				IL-10 ⁻	IL-10 ⁺	Th0
1	168	9	48	23	14	74
		5.4	28.6	13.7	8.3	44.0
2	153	32	17	13	50	41
		20.9	11.1	8.3	32.7	26.8

^aNumbers and frequencies of T cell clones of each subpopulation indicated in plain and bold/underlined numbers, respectively.

^bProduction levels of T cell clones with a Tr phenotype defined as IL-10 ≥ 100 pg per ml, IL-4 < 10 pg per ml, IL-5 production > 1 ng per ml; a Th1 phenotype defined as IL-10 < 100 pg per ml, IL-5 < 10 pg per ml, IL-4 < pg per ml or IFN- γ /IL-4 ratio \geq and a Th2 phenotype defined as IL-5 production > 1 ng per ml, IFN- γ < 20 pg per ml or IL-4/IFN- γ ratio ≥ 5 ; IL-10⁺ ≥ 100 pg per ml; IL-10⁻ ≥ 100 pg per ml; IL-10⁻ < 100 pg per ml.

Tr cells present in DTH reactions in donors that had been sensitized and rechallenged only once with DNCB, we investigated whether repeated exposure to this hapten specifically enhanced the production of anti-inflammatory cytokines. To this purpose, donors 5 and 6, from whom only 13.7% and 4.4%, respectively, of Tr and IL-10-producing Th2 cells had been isolated, were rechallenged with DNCB and the cytokine production profile of a series of T cell clones isolated from a punch biopsy was analyzed and compared with that of the first series of clones. Analysis of the cytokine production profile of individual T cell clones obtained from donor 5, showed that the frequency of both Tr and IL-10-producing Th2 cell clones had increased about 4-fold, whereas the number of Th1 clones had decreased by 50% (**Table IV**). In addition, a decrease of about 30% in the number of non-IL-10-producing Th2 clones was observed as well. A similar increase in the frequency of IL-10-producing Th2 (3.5-fold) and Tr (4.2-fold) cells and a concomitant decrease in the frequency of Th1 cells (2-fold decrease) generated from donor 6 was observed (Not shown).

Moreover, levels of IL-4 production by T cell clones generated from the second biopsy from donor 5 were enhanced (644 ± 463), as compared with those following the first challenge (218 ± 360) (Mann–Whitney, $p < 0.001$), whereas levels of IL-5 production were only slightly affected (877 ± 298 *vs* 655 ± 432) (Mann–Whitney, $p < 0.001$) (**Fig 1**); however, production levels of IFN- γ (1078 ± 1470 *vs* 209 ± 545) (Mann–Whitney, $p < 0.001$) and in particular those of IL-10 (1070 ± 913 *vs* 143 ± 309) (Mann–Whitney, $p < 0.001$) were strongly increased, as a result of an additional challenge with DNCB.

Together, these results indicate that sensitization with DNCB results in the induction of a DTH reaction, characterized not only by the presence of inflammatory, IFN- γ -producing Th1 cells, but also of anti-inflammatory, IL-10 producing Tr and Th2 cells. Moreover, although both the production of all cytokines increased, following subsequent challenge with the same hapten, the most significant increase was observed with respect to the production of IL-10 by IL-10-producing Th2 and Tr cells.

Culture supernatants from activated cutaneous DTH-derived IL-10-producing Tr cells block alloantigen-induced responses and inhibit *in vitro* differentiation of dendritic cells To determine whether Tr clones, isolated from DNCB-induced DTH reactions, were functionally active, culture supernatants from these cells, activated for 24 h with immobilized anti-CD3 and anti-CD28 MoAb, were tested for their capacity to inhibit an alloantigen-induced proliferative response through their

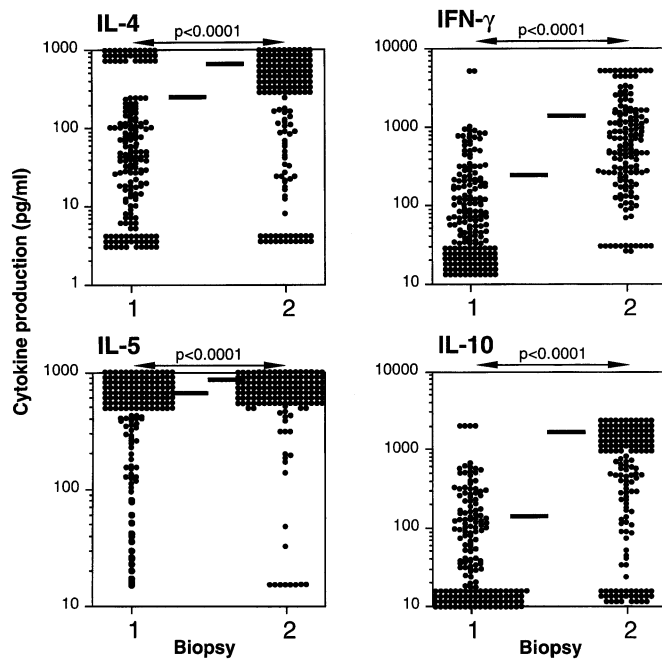


Figure 1. Repeated exposure to DNCB increases the presence of IL-10-producing Th2 and Tr cells in cutaneous DTH reactions. Production of IL-4, IL-5, IFN- γ and IL-10 was determined by ELISA in culture supernatants of activated T cell clones, isolated from cutaneous DTH reactions, following a single (1) or repeated (2) challenge of a healthy donor with DNCB, which had been stimulated with immobilized anti-CD3 and anti-CD28 MoAb for 24 h, as described in *Materials and Methods*. $p < 0.0001$ (Mann-Whitney) increase in cytokine production levels following (2) as compared with (1).

production of IL-10 (Bejarano *et al*, 1992). Stimulation of T cells with irradiated allogeneic PBMC induced a strong proliferative response, measured after 5 d of culture, which was strongly inhibited by the addition of Tr cell-derived culture supernatants (Fig 2A). Addition of a neutralizing anti-IL-10 MoAb to the cultures partly restored the proliferative response, whereas the addition of a neutralizing anti-TGF- β MoAb, either alone, or in combination with the anti-IL-10 MoAb had little effect. A culture supernatant from an activated IL-10-producing Th2 clone also inhibited the alloantigen-induced response (results not shown), whereas culture supernatants from an activated Th1 clone were ineffective (Fig 2A).

Moreover, the addition of culture supernatants from an activated Tr clone affected the differentiation *in vitro* of dendritic cells from peripheral blood monocytes by rIL-4 and recombinant granulocyte-monocyte colony-stimulating factor, as indicated by a lower expression levels of CD1a and CD83 (Results not shown). Dendritic cells derived in the presence of culture supernatants from activated Tr cells showed a strongly diminished capacity to induce an antigen driven proliferative response (Fig 2B), suggesting that the latter cells, via the production of soluble factors, interfere with this process.

IL-10-producing Tr clones express transcripts for cytokine receptors, but do not express T1/ST2 mRNA The expression of transcripts for the IFN- γ R β -chain, the IL-12R β_2 -chain and the IL-1R-homolog T1/ST2 was analyzed by reverse transcription-PCR, using representative samples of T helper and Tr cell clones, in a resting state, as well as following stimulation of the cells for 6 h with anti-CD3 and anti-CD28 MoAb. Transcripts for the IFN- γ R β -chain could be detected in activated and resting Th2 cell clones, but were only very weakly expressed in resting Tr and Th1 clones

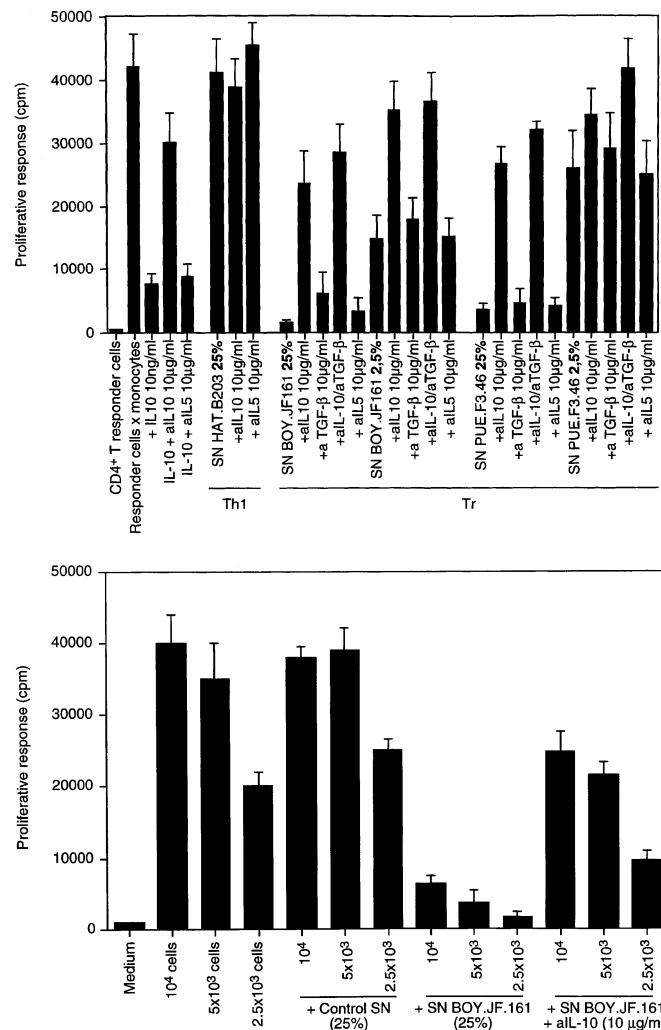


Figure 2. Cutaneous DTH-derived IL-10-producing T helper and Tr cell clones are functionally active. One hundred thousand purified CD4+ T lymphocytes were stimulated with 2.5×10^4 purified allogeneic monocytes, in the presence or absence of either rIL-10, culture supernatants from activated T cell clones (A) or with various numbers of *in vitro* generated allogeneic dendritic cells (B), in the absence or presence of neutralizing anti-IL-10, anti-TGF- β , or anti-IL-5 MoAb, as described in *Materials and Methods*. Control supernatant was used to verify whether all residual anti-CD3 and anti-CD28 MoAb had been removed from the culture supernatants. Proliferative responses were measured after 5 d by tritiated thymidine incorporation and expressed as cpm \pm SD. Results of two independent experiments.

(Fig 3). Activation of both Th1 and Tr clones for 6 h with anti-CD3 and anti-CD28 MoAb, however, resulted in an induction of IFN- γ R β -chain mRNA expression. Resting and activated Th1 clones expressed transcripts for the IL-12R β_2 -chain. Although transcripts for this receptor component were low to undetectable in resting Th2 and Tr clones, activated cells all expressed IL-12R β_2 -chain mRNA. By contrast, expression of T1/ST2 transcripts was not detectable in any of the resting T cell clones from the three subpopulations. T1/ST2 mRNA was only expressed in activated Th2 clones (Fig 3).

As expected, expression of IL-4 transcripts was only detected in activated Th2 clones, but was undetectable in activated Th1 and Tr1 clones (Fig 3). Reminiscent of results obtained by analyzing cytokine production, activated Th1, Th2, and Tr clones expressed variable levels of IFN- γ transcripts, depending on the duration of their time in culture.

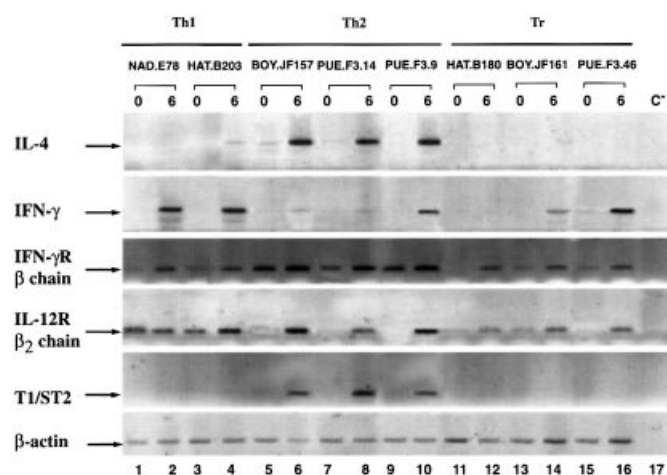


Figure 3. Expression of mRNA encoding IL-4, IFN- γ , IFN- γ R β -chain, and IL-12R β -chain in cutaneous DTH-derived Th1, Th2, and Tr clones. T cell clones were cultured in medium or stimulated with immobilized anti-CD3 and anti-CD28 MoAb for 6 h and the expression of specific transcripts was analyzed by reverse transcription-PCR, as described in *Materials and Methods*. T cell clones were used after eight restimulations with feeder cells, with the exception of PUE.F3.9 (six restimulations) and HAT.B180 (four restimulations). Representative results of three independent experiments. Primer sequences and size of the amplified products are shown in **Table I**.

DISCUSSION

Results reported in the literature, obtained in experimental mouse and human models of inflammation, have indicated that hapten-induced contact sensitivity reactions, are characterized by the presence and the local effects of populations of T helper cells and Tr cells with inflammatory and anti-inflammatory properties, respectively. This study supports this notion by showing that, although sensitization with the hapten DNCB induces strong cutaneous DTH reactions, reportedly as a result of the recruitment of IFN- γ -producing CD8⁺ (Bour *et al*, 1995; Cavani *et al*, 1998; Kehren *et al*, 1999), and Th1 effector cells (Sinigaglia *et al*, 1985; Kapsenberg *et al*, 1992), IL-10-producing CD4⁺ Th2 and Tr cell populations are recruited to the site of inflammation as well.

Most studies on hapten-specific recruitment of T lymphocyte populations in humans have been carried out in allergic patients with contact sensitivity to nickel. The use of a naive hapten, such as DNCB, however, which, in contrast to nickel, also induces strong DTH reactions in healthy individuals, permits the study of kinetics of appearance of T cell subpopulations in sites of cutaneous inflammation, as a result of sensitization followed by a single or multiple challenge (s) with the same hapten. As shown in this study, IL-10 producing Tr cells could be isolated from cutaneous punch biopsies, following sensitization and a single challenge with DNCB, whereas a subsequent challenge resulted in an increase in the frequency of these cells, as well as of IL-10-producing Th2 cells. Our results support those of Cavani *et al* (1998, 2000) who reported that IL-10-producing nickel-specific T cell clones can be isolated from the peripheral blood of allergic and nonallergic individuals and the skin of patients allergic to nickel (Cavani *et al*, 2000). In addition, the latter group showed that these cells produced higher levels of IL-10 and lower levels of IFN- γ , as compared with those isolated from patients with allergic contact dermatitis to nickel (Cavani *et al*, 1998); this suggests that the lack of contact sensitivity against nickel in nonallergic individuals correlates with the presence of increased levels of IL-10.

Tr cells isolated from cutaneous inflammatory reactions were functionally active through the production of IL-10 and possibly other cytokines with immunoregulatory activity. Surprisingly, the

addition of a neutralizing anti-TGF- β MoAb did not interfere with the suppressive effects of Tr-derived culture supernatants. TGF- β reportedly is involved in the activity of (IL-4-producing) regulatory Th3 cells (Chen *et al*, 1994), but it does not seem to be the major suppressive cytokine produced by the (non-IL-4-producing) Tr cells, described in our study that are reminiscent of those reported by Groux *et al* (1997a). Culture supernatants from activated Tr clones strongly decreased the capacity of *in vitro* generated dendritic cells to induce allo-specific proliferative responses; however, this was not due to a decrease in the expression of HLA-DR on these cells as, unexpectedly, Tr cell-derived culture supernatants were found to upregulate HLA class II expression (H. Yssel, unpublished data), confirming an earlier observation made by Cavani *et al* (2000). It seems, therefore, that the suppressive effects exerted by the Tr cells are mediated via downregulation of costimulatory molecules on dendritic cells, rather than as a result of downregulation of HLA class II molecules (de Waal Malefyt *et al*, 1991).

No information is available at present as to whether the magnitude of DNCB-induced contact sensitivity reactions is correlated with the presence or absence of IL-10-producing cells. As the immunosuppressive effects of Tr cells reportedly are antigen-specific (Groux *et al*, 1997a; Akdis *et al*, 1998), the answer to this question can only be resolved by studying cutaneous immune responses, following multiple challenges with this hapten. It is important to note, however, that, unlike nickel, DNCB is able to induce strong cutaneous inflammatory reactions, even in the presence of IL-10 producing cells, as demonstrated in our study. Although only carried out in two individuals, a subsequent challenge with DNCB was found to result in an increase in production levels of all cytokines analyzed (the strongest being observed in the production of IL-10), as well as an increase in the frequency of IL-10-producing T helper and Tr cells. Whether continued exposure to this hapten will result in a segregation of individuals, able to mobilize increased numbers of Tr cells that will suppress contact sensitivity reactions, and those who will eventually become allergic to this hapten is difficult to address due to ethical constraints and, therefore, falls outside the scope of this study.

It has been reported that *in vitro*-generated Tr cells are difficult to maintain in culture (Groux *et al*, 1997a), which has been attributed to the strong immunosuppressive properties of IL-10, as well as to its ability to induce a state of nonresponsiveness in T cells (Groux *et al*, 1996). Whereas levels of IL-10 production varied among the Tr clones, a number of those maintained in culture produced high levels of this cytokine, raising the possibility that they are insensitive to the immunosuppressive effects of IL-10. Whether or not this is due to the absence of a functional IL-10R remains to be determined. Preliminary results indicate that IL-10-producing cells can be detected by ELISPOT among skin-infiltrating lymphocytes, following short-term culture in the presence of rIL-2 (J. Pène, unpublished observations) and their generation *in vitro*, therefore, does not seem to be due to a preferential outgrowth, although it cannot be excluded that their presence may have growth-inhibiting effects on other T cell populations.

Although the cytokine production profile of the Tr and Th2 clones, isolated from DNCB-induced DTH reactions, was relatively stable, most of these cells started to produce increasing amounts of IFN- γ upon prolonged culture. This is most likely to be due to the culture conditions used to expand the cells, as the cytokine environment, containing IL-12, IFN- α , IL-18 (Abbas *et al*, 1996), and IL-23 (Oppmann *et al*, 2000), produced by peripheral blood mononuclear cells used as feeders, exerts IFN- γ -inducing effects, to which activated T cell clones are responsive; this is because of their likely expression of a functional receptor for IL-12 in an activated state (see below). In this respect, it is important to use skin-derived cloned Tr and T effector cells as early as possible for functional studies, following their generation from skin biopsies, at the time that they still display a polarized cytokine production profile.

It has been reported previously that human IL-12R β -chain mRNA expression is strongly downregulated following Th2

differentiation (Rogge *et al*, 1997); however, in the latter study the effect of TCR/CD3 complex-mediated activation on the expression of this receptor component was not addressed. Surprisingly, IL-12R β_2 -chain transcripts were not only detected in Th1, but also in activated Th2 and Tr clones analyzed in this study. It must be stressed, however, that the expression of transcripts for this receptor and the IFN- γ R β -chain has not been analyzed in a quantitative way in our study and that detection of each of these molecules at the cell surface is tedious because of their low expression levels (Novelli *et al*, 1997; Rogge *et al*, 1999). Nevertheless, the results presented here suggest that activated Th2 cells express a functional IL-12R, as, both IL-12R β_1 (results not shown; Rogge *et al*, 1997) and IL-12R β_2 -chain mRNA is detected in these cells, whereas the observed induction of IFN- γ production during prolonged culture of the cells suggest that they are indeed responsive to the IFN- γ production-inducing effects of IL-12. Taken together, it seems that the dynamic and transient expression profile of the IFN- γ R and IL-12R components is likely to interfere with the usefulness of these surface molecules as phenotypic markers.

Recently, it was reported that T1/ST2, an IL-1R receptor-like molecule of the immunoglobulin superfamily (Klemenz *et al*, 1989; Tominaga, 1989) is stably expressed on mouse Th2, but not on Th1 cells and it has been suggested that the T1/ST2 molecule is likely to play an important part in Th2 effector function (Löhning *et al*, 1998; Xu *et al*, 1998). Transcripts encoding the T1/ST2 molecule could only be detected in human Th2 clones. None of the Th1 and Tr clones expressed T1/ST2 mRNA, irrespective of their state of activation and, therefore, do not express this molecule at their cell surface. As at present no information is available on the function and expression of human T1/ST2, the functional implications of this observation cannot be evaluated.

As Th2 cells also produce IL-10, following antigen-specific activation, there is a priori no precedence for a role of Tr cells as a unique cell type producing this cytokine and it is unlikely that Th2-derived IL-10 has different functional capacities as IL-10 produced by Tr cells. Indeed, culture supernatants from IL-10-producing Th2 clones effectively inhibited allo-antigen-induced proliferative responses. Interestingly, however, Tr cells have been found to localize specifically in the target organs, even in the absence of antigenic activation (Groux, H., unpublished results), where they suppress inflammation in the local microenvironment (Chen *et al*, 1994). It is, therefore, to be expected that the latter cells might have a different make-up of adhesion molecules (and possibly chemokine receptors) at their cell surface as compared with those on Th2 cells, which might suggest that IL-10-producing Th2 cells are less effective in exerting anti-inflammatory responses at the site of inflammation.

In conclusion, the model described here, using a strong DTH-inducing hapten to induce the migration of Tr cells into inflamed skin, is useful to isolate this T lymphocyte subpopulation and enables further functional and phenotypic characterization to understand their role in the control of inflammatory immune responses.

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