Immunopathologic Features of Allergic Contact Dermatitis in Humans: Participation of Plasmacytoid Dendritic Cells in the Pathogenesis of the Disease?

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Contrary to our abundant knowledge about the sensitization phase of human contact hypersensitivity, little is known about the cell types orchestrating the effector phase. In order to address this issue, we phenotypically analyzed biopsies from 72 h epicutaneous patch test reactions (n = 10) and normal human skin (n = 5) for the presence of various leukocyte differentiation antigens. The inflammatory infiltrate was dominated by $CD3^+$/$CD4^+$ T cells with approximately 30% of the cells coexpressing CD25 and CTLA-4, a phenotype consistent with either activated effector or regulatory T cells. In our search for professional antigen-presenting cells, we were surprised to find not only sizeable numbers of $CD1a^+$ dendritic cells and $CD1c^+$ dendritic cells, but also of $CD123^+$, $CD45RA^+$, BDCA-2^+, CLA^+, and $CD62L^+$ plasmacytoid dendritic cells. Although virtually absent in normal human skin, these cells were detectable already 6 h after hapten challenge and were often found in close proximity to $CD56^+$ natural killer cells, indicative of a functional interaction between these cell types. The detailed knowledge of the cellular composition of the inflammatory infiltrate in allergic contact dermatitis and its kinetics should form the basis for the investigation of the immunologic and molecular events operative in the perpetuation and resolution of the eczematous response. *Key words: allergic contact dermatitis/dermal dendritic cells/immunofluorescence/Langerhans cells/plasmacytoid dendritic cells/regulatory T cells. J Invest Dermatol 121:1409–1418, 2003*

Allergic contact dermatitis (ACD) is a T cell mediated cutaneous immune response to low-molecular-weight chemicals termed haptens (Belsito, 1999). Upon contact, haptens penetrate the skin and can then be covalently attached to amino acid residues on proteins or to the major histocompatibility complex (MHC) molecule itself (Martin and Weltzien, 1994). Resident antigen-presenting cells, i.e., Langerhans cells or dermal dendritic cells (DDC), internalize the hapten, migrate to regional lymph nodes and present the hapten-bound MHC–peptide complex to naïve T cells. This sensitization phase leads to hapten-specific MHC class I- and/or class II-restricted T cell proliferation.

Re-exposure of a sensitized individual to a relevant hapten elicits the effector phase of contact hypersensitivity (CHS), which clinically results in eczema. Contrary to the ample knowledge about the sensitization phase, only little is known about the mechanisms guiding and governing the effector phase.

It has long been assumed that hapten-specific $CD4^+$ T cells are the critical effector cells in CHS (Sinigaglia *et al*, 1985; Cher and Mosmann, 1987). As these $CD4^+$ T cells were found to produce mainly T1 cytokines (interferon-γ and interleukin (IL)-2), allergic eczema was classified as a T1-mediated response (Sinigaglia *et al*, 1985; Kapsenberg *et al*, 1992). More recent studies in mice and humans, however, yielded controversial data raising the question as to what extent the allergic tissue reaction is propagated by T1 or T2 cells and by CD4 or CD8 T cells (Gocinski and Tigelaar, 1990; Probst *et al*, 1995; Xu *et al*, 1996; Werfel *et al*, 1997; Cavani *et al*, 1998; Wang *et al*, 2000). Particularly intriguing was the observation of nickel-specific $CD8^+$ T1 cells in nickel-allergic individuals only, whereas nickel-specific $CD4^+$ T cells were isolated from the blood of both allergic and nonallergic persons (Probst *et al*, 1995; Cavani *et al*, 1998). This could indicate that a nonallergic state of a given individual does not necessarily result from a lack of antigenic contact, but is rather a consequence of active suppression of hapten-specific effector cells by IL-10-secreting $CD4^+$ regulatory T cells. As these cells have the capacity to block DC-dependent hapten-specific T cell responses, they may be crucial for terminating the eczematous reaction in allergic individuals. (Cavani *et al*, 2000).

The elicitation of ACD presumably requires close interactions between infiltrating T cells and antigen-presenting cells, which either reside in the skin or migrate from blood. The nature of the antigen-presenting cell type supporting T cell mediated tissue inflammation remains elusive. Although Langerhans cells/DDC were thought to be the major players in this process (Romani and Schuler, 1992), other evidence exists that depletion of resident Langerhans cells after sensitization in mice results in an increased, and not a decreased, CHS response (Grabbe *et al*, 1995).

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Abbreviations: ACD, allergic contact dermatitis; CHS, contact hypersensitivity; NS, normal skin; EPT, epicutaneous patch test; DC, dendritic cell; DDC, dermal dendritic cell; pDC, plasmacytoid dendritic cell.

There also exist anecdotal observations on numerical, phenotypic, and/or functional changes in cells/molecules of the innate immune system.

Given all these controversial results concerning the nature of cells being centrally involved in its development and resolution, ACD is probably a much more complex event than previously thought. In this study we immunophenotypically characterized the leukocytic infiltrate of allergic patch test reactions at various stages of development, focusing particularly on DC and T cell subsets as well as on members of the innate immune system. By doing so, we sought to gain useful information about kinetics, quality, and quantity of the inflammatory tissue response operative in allergic eczema.

MATERIALS AND METHODS

Patients and tissue material This study was performed in accordance with the guidelines of the Declaration of Helsinki. After approval of the investigational protocol by the institute and the local Ethics committee of Vienna University's Medical School, 10 patients with positive epicutaneous patch tests (EPT) to nickel (n = 5) or fragrance mix (n = 5) were enrolled in the study after giving their informed consent. The allergens were applied for 6 h, 24 h, 48 h, and 72 h on the patients' upper back via standardized patch tests. After the indicated time points, one 4 mm punch biopsy of the allergic patch test reaction was taken under local anesthesia from each test person. All reactions were classified using the criteria of the International Contact Dermatitis Research Group and ranged between 1 and 3, according to the state of inflammation. Normal skin biopsies (NS) from healthy volunteers (n = 5) were used as the control group.

The biopsies were embedded in OCT Tissue-Tek (Sakura Finetek Europe B.V. Zoeterwoude, The Netherlands), snap-frozen in liquid nitrogen, and stored at –80°C until further processing.

Immunofluorescence staining Frozen tissue was cut into 5 μm thick sections and mounted on capillary gap microscope slides (DAKO, Glostrup, Denmark). The cryostat sections were air-dried for 20 min, fixed in ice-cold acetone for 10 min and either stained immediately or stored at –20°C. To determine phenotype, distribution, and number of the different leukocyte subsets, single or multicolor immunostaining was performed. The monoclonal antibodies used in this study and their source are shown in **Table I**.

Single staining was performed as follows. Purified/biotinylated antibodies were mixed with 2% bovine serum albumin (Sigma-Aldrich, St Louis, Missouri) in phosphate-buffered saline (Gibco, Invitrogen GmbH, Lofer, Austria) and applied to frozen sections in a humid chamber overnight at 4°C. After washing with phosphate-buffered saline, primary antibody binding was visualized by incubating the sections with either streptavidin–fluorescein (1/100; Amersham Pharmacia Biotech UK Limited, Cardiff, UK) or rhodamine (TRITC)-conjugated AffiniPure $F(ab')_2$ fragment goat anti-mouse IgG (H + L) (1/200; Jackson ImmunoResearch Laboratories, West Baltimore, Pennsylvania) for 45 min at room temperature. After washing, slides were placed in 96% ethanol, mounted in Vectashield Mounting Medium (Vector Laboratories, Burlingame, California) and cover-slipped.

To characterize further the infiltrate of lesional skin, we carried out double or triple stainings, respectively. The first step of the multicolor staining procedure was performed as described above. After washing, the slides were incubated for 20 min with normal mouse serum (DAKO) to diminish background problems. Afterwards the secondary antibody, either biotinylated or fluorescein isothiocyanate (FITC) labeled, was applied to the sections for at least 6 h at 4°C. To prevent FITC-labeled antibodies from early fading, another staining step was performed with Oregon Green-labeled goat IgG anti-FITC (1/300; Molecular Probes, Eugene, Oregon) for 30 min at room temperature. Sections stained with biotinylated antibodies were further incubated for 45 min with streptavidin TRITC (1/200; Jackson ImmunoResearch Laboratories) to visualize antibody binding. For triple stainings, incubation with biotinylated monoclonal antibodies together with FITC-labeled monoclonal antibodies for 60 min at room temperature was followed by simultaneous incubation with streptavidin Cy5 (1/250; Jackson ImmunoResearch Laboratories) and Oregon Green-labeled goat IgG anti-FITC fraction for 45 min at 20°C.

In all staining experiments, substitution of the primary antibodies with isotype-matched IgG or IgM (purified, biotinylated, or FITC labeled) and omission of the primary antibody served as negative controls.

Table I. List of monoclonal antibodies used in the study

Antibody specificity	Clone and source of antibodies	Isotype
CD4	SK 3; BD, San Jose, CA	mIgG1
CD4 FITC	RPA-T4; PharMingen, BD Biosciences, Heidelberg, Germany	mIgG1
CD8	C8/144B; DAKO, Glostrup, Denmark	mIgG1
CD3 FITC	SK-7; BD	mIgG1
γ/δ TCR	5.A6.E9; Endogen, Woburn; MA	mIgG1
CD45RA	L48; BD	mIgG1
CD45RA FITC	ALB11; Immunotech, Coulter, Marseille, France	mIgG1
CD25	2A3; BD	mIgG1
CD25 FITC	M-A251; PharMingen	mIgG1
CTLA-4 PE	BNI3; PharMingen	mIgG2a
CD56 (biot)	My31; BD	mIgG1
CD94	HP-3D9; PharMingen	mIgG1
CD15	VIM16; kindly donated by Otto Majdic (Department of Immunology, Vienna Medical School)	rIgM
CD14	MΦP9; BD	mIgG2b
CD1c	M241; Ancell, Bayport, MN	mIgG1
CD1a	M2120; PeliCluster; CLB, the Netherlands	mIgG2b
Langerin	DCGM4; Immunotech	mIgG1
MHC II FITC	L243; BD	mIgG2a
CDw123	9F5; PharMingen	mIgG1
BDCA-2 FITC	AC144; Miltenyi Biotech GmbH, Germany	mIgG1
CD62L	SK11; BD	mIgG2a
CD62L FITC	Dreg 56; PharMingen	mIgG1
PNAd	MECA-79; PharMingen	rIgM
CLA FITC	HECA-452; PharMingen	rIgM
CD83	HB15e; PharMingen	mIgG1
CD83 FITC	HB15a; Immunotech	mIgG2b
CD86 FITC	HA5.2B7; Immunotech	mIgG2b
CD117	95C3; An der Grub GmbH, Austria	mIgG1
ECP	EG1; Kabi Pharmacia Diagnostics, Sweden	mIgG1
FcεRI	15.1 (Ref. Wang *et al*, 1992)	mIgG1
Ki-67	Ki-S5; DAKO	mIgG1
CXCR3	49801.111; R&D Systems GmbH, Biomedica Vienna	mIgG1

Evaluation of immunofluorescence results Biopsy specimens were read in a blinded fashion at 400 × magnification by two independent investigators using a fluorescence microscope (ocular 10 × /25; Axiophot, Zeiss, Oberkochen, Germany) equipped with a filter for double stainings. The mean observer coefficient was under 10%.

Skin-infiltrating leukocytes were quantified separately in the epidermis and dermis, respectively. Labeled cells were enumerated per visual field and expressed as the number of cells (± SEM) per mm/basement membrane epidermis and per mm^2 dermis. Only cells clearly positive for the antigen of interest were counted. Statistical significance was determined by Student's t test. Confocal immunofluorescence microscopy (LSM 510, Zeiss) was used to evaluate triple stainings and to document the sites of preferential expression.

Flow cytometry Punch biopsies (3 × 6 mm) from patients with positive EPT were cut into small pieces and incubated in 50 mL tubes with RPMI 1640 medium for 2 h at 37°C. Cell suspensions were prepared by mincing the skin through a cell strainer. For three-color immunostaining, cells were washed in ice-cold phosphate-buffered saline, resuspended in blocking reagent (Octagam 5 mg per mL; Octapharma, Vienna, Austria) and preincubated at 4°C for 20 min. Cells were then incubated on ice for 15 min with anti-CD123 PE (Becton Dickinson, San Jose, California) combined with anti-MHC II PerCP (Becton Dickinson) and several FITC-labeled antibodies (anti-CD45RA FITC (Becton Dickinson), anti-BDCA-2 FITC (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany), anti-CD62 ligand (anti-CD62L) FITC (PharMingen), anti-cutaneous lymphocyte antigen (anti-CLA) FITC (PharMingen, Heidelberg, Germany), anti-CD83 FITC (Immunotech, Coulter, Marseille; France),

and anti-CD86 FITC (Immunotech). Alternatively, the anti-MHC II PerCP antibody was replaced by anti-CD4 PerCP (Becton Dickinson). Following incubation with the indicated monoclonal antibodies, cells were washed twice with ice-cold phosphate-buffered saline and subjected to flow cytometric analysis using a FACSCalibur flow cytometer (Becton Dickinson). In order to compare characteristics of skin-derived and blood-derived plasmacytoid dendritic cells (pDC), peripheral blood mononuclear cells were obtained simultaneously from the same allergic donors by Ficoll-Paque density gradient centrifugation (Histopaque-1077, Sigma). Peripheral blood mononuclear cells were stained and analyzed as described for cutaneous cell suspensions.

RESULTS

Number and distribution of leukocytes in allergic patch test reactions In this study we aimed at giving a detailed view of the different cell types orchestrating the eczematous reaction in the effector phase of human ACD. Biopsies from positive 72 h EPT reactions (n = 10) and NS (n = 5) were subjected to immunofluorescence analysis using a broad panel of antibodies (**Table I**). The results are shown in **Table II**. When cryostat sections from NS were incubated with the pan-leukocyte marker CD45, relatively few cells were detected in both the epidermis and the dermis. Further stainings revealed that these cells mainly represent $CD1a^+$ DC in the epidermal compartment, as opposed to $CD3^+$ T cells, $CD14^+$ macrophages, $CD1c^+$ DC as well as $CD117^+$ mast cells in the dermal compartment (**Table II**). In 72 h EPT, by contrast, we found a massive leukocytic infiltrate ($CD45^+$ cells) in both layers. As compared with NS, the number of $CD45^+$ cells was nearly doubled in the epidermal compartment, and increased 5-fold in the dermis (**Table II**). In their majority, they represented T cells or belonged to the DC family. Besides, we also noted an increased presence of mast cells, neutrophils ($CD15^+$), and eosinophils ($EG1^+$) when compared with NS.

T cells represent the predominant population in 72 h EPT specimens The overwhelming majority of the infiltrating cells in the epidermis and dermis of 72 h EPT reactions consist of $CD3^+$ cells. **Figure 1** shows that two-thirds of these T cells belong to the $CD4^+$ and one-third to the $CD8^+$ subset. Only a negligible proportion of T cells was $CD3^+/CD4^-/CD8^-$ (**Fig 1**) and we did not detect any $CD4^+/CD8^+$ T cells (data not shown). As recent findings describe a regulatory role for γδ-T cells in murine CHS (Guan *et al*, 2002), we searched for the presence of such T cells in human ACD and correlated their number to the more frequently occurring αβ-T cell receptor-bearing cells. Although the great majority of infiltrating T cells in the dermis of EPT lesions expressed the αβ-T cell receptor (data not shown), a distinct number of T cells also displayed anti-γδ-T cell receptor reactivity (11.9 per mm^2 dermis) (**Table II**). Double stainings with the proliferation marker Ki-67 and CD3 yielded negative results (data not shown), indicating that the increase in T cells in EPT lesions is due to their influx from the peripheral blood and does not result from local expansion of resident T cells.

Approximately 5% of all dermal $CD4^+$ cells also reacted with anti-CD25 (IL-2Rα-chain) (data not shown). As these molecules are also known to be expressed by activated DC and macrophages, flow cytometric analysis of single cell suspensions from 72 h EPT reactions was performed with non-T cells being excluded by means of a T cell-specific morphogate. These examinations revealed a $CD4^+/CD25^+$ T cell subset (approximately 30%) that coexpresses CTLA-4. $CD4^+/CD25^-$ T cells, by contrast, hardly expressed any CTLA-4 (**Fig 2*A***). Confocal microscopy of immunofluorescence triple stainings confirmed these results by showing sizeable numbers of $CD4^+/CD25^+/CTLA\text{-}4^+$ cells *in situ* (**Fig 2*B***). To learn about the kinetics of infiltrating $CD4^+/CD25^+$ cells, EPT reactions obtained at 0, 24, 48, and 72 h past hapten challenge were comparatively examined (**Fig 2*C***). At 24 h past challenge, we already observed considerable numbers of $CD4^+$ T cells, but only very few of them displayed anti-CD25 reactivity (**Fig 2*C***). After 48 h, however, the relative contribution of $CD25^+$ cells to the entire $CD4^+$ T cell infiltrate had substantially increased (**Fig 2*C***).

Table II. Number of leukocytes occurring in 72 h EPT lesions and NS[a]

		Epidermis (positive cells per mm)		Dermis (positive cells per mm^2)	
Cell type	Antigen(s)	Normal skin	72 h EPT	Normal skin	72 h EPT
Pan-leukocytes	$CD45^+$	14.1 (12.1–17.7)	30.8 (21.5–50.6)	86 (64.7–97.6)	435.3 (240.6–858)
T cells	$CD3^+$	2.7 (0–3.5)	20.9 (3.7–43.2)	39.5 (2.4–58.4)	513.1 (109.3–896)
DN T cells[b]	$CD3^+/CD4^-/CD8^-$	0 (0)	0.4 (0–6.1)	0 (0–1)	14.4 (7.6–24.7)
Helper T cells	$CD3^+/CD4^+$	2.3 (0–2.9)	16.8 (3.2–29.2)	20.4 (2.4–45.2)	396.7 (83–696.8)
Cytotoxic T cells	$CD3^+/CD8^+$	0.6 (0.3–0.9)	6.7 (0.5–13)	8.6 (3.2–44.4)	133.9 (26.3–363.4)
γδT cells	γδ-TCR^+	0. (0)	1 (0–6.4)	2.2 (0–3.7)	11.9 (2.2–36.6)
Teff/Treg[c]	$CD4^+/CD25^+$	0 (0)	2.2 (1.6–11.2)	3.2 (1.3–5.2)	21 (9.7–53.8)
Natural killer T cells	$CD94^+/CD3^+$	0 (0)	0 (0–3.1)	0.7 (0–1.6)	10.1 (3.2–19.4)
Natural killer cells	$CD94^+$	0 (0)	1.9 (0–5.2)	2.4 (0.4–8.2)	39.7 (12.2–217.6)
Natural killer cells	$CD56^+$	0 (0)	0.2 (0–5.3)	7.7 (3.7–19.7)	23.1 (8.1–115.1)
pDC	$CD123^+/CD45RA^+$	0 (0)	3.5 (0–6.5)	1.4 (0–2.8)	39.8 (14.6–85.8)
Langerhans cells	$Langerin^+$	27.7 (18.2–32.8)	18 (13.3–21.5)	1.1 (0.9–3.2)	17.5 (1.1–28.1)
Langerhans cells	$CD1a^+$	18.7 (14.1–24.3)	12.1 (4.1–20.3)	13.5 (10.9–31.4)	44.5 (20.6–95.7)
DDC	$CD1c^+$	4.45 (1.6–7.7)	16.6 (3–26.3)	24 (11.6–49)	97.1 (53–164.5)
IDEC/IDDC[d]	$CD1a^+/CD1c^+$	0.5 (0–2.4)	5.2 (1.4–8.2)	2.3 (1.1–20.4)	18.9 (4.8–43.5)
Mature Langerhans cells	$CD1a^+/CD83^+$	0.7 (0–1.6)	3.5 (1–6.4)	0.9 (0–1.8)	14.9 (9–40.9)
Mature DDC	$CD1c^+/CD83^+$	1 (0.2–2)	1.4 (0.8–2.3)	5.9 (1.6–17.2)	11 (3.7–14.8)
Mast cells	$CD117^+$ (c-Kit)	0 (0)	0 (0)	18.5 (6–40.3)	31.1 (14.7–54.5)
Macrophages	$CD14^+$	0 (0–1)	1.9 (0–3.8)	15.2 (9.7–25.8)	30.6 (14.8–51)
Neutrophils	$CD15^+$	0 (0)	0 (0–6.4)	0 (0)	29.8 (4.8–167.7)
Eosinophils	$EG1^+$	0 (0)	0 (0)	0 (0)	21.5 (1.1–37.6)

[a]Cells were visualized using the indicated markers and evaluated by immunofluorescence analysis. Numbers indicate median and range (described in brackets) of cells counted in 10 EPT reactions and five NS biopsies.
[b]Double-negative T cells.
[c]T effector cells/T regulatory cells.
[d]Inflammatory epidermal/dermal dendritic cells.

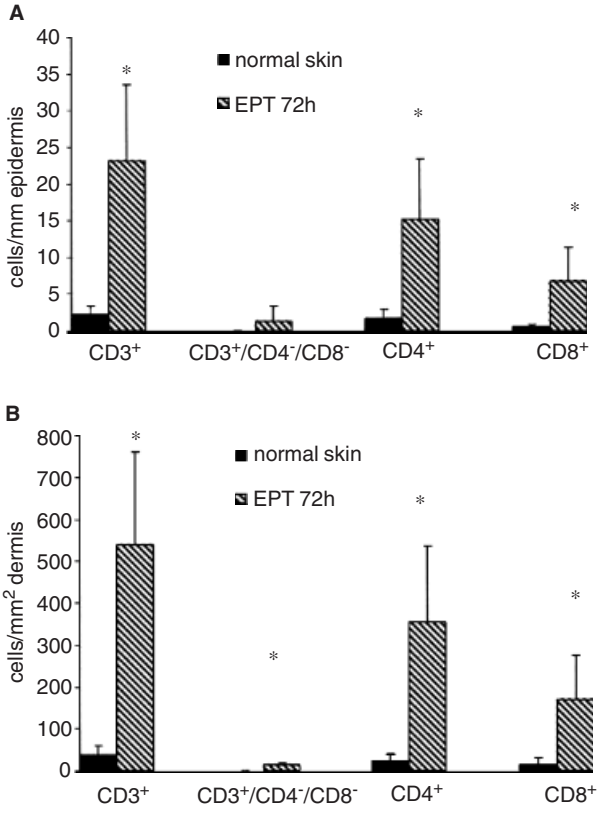

Figure 1. Increase of T cells in the epidermis and dermis of 72 h EPT lesions. Frozen tissue sections of NS and EPT lesions were subjected to immunofluorescence analysis. T cells and their subpopulations in the (*A*) epidermal and (*B*) dermal compartment were characterized with either anti-CD3 alone, anti-CD3 (FITC)/anti-CD4 (TRITC), or anti-CD3 (FITC)/anti-CD8 (TRITC) double stainings. DN T cells were identified with anti-CD4 (TRITC) and anti-CD8 (TRITC) followed by anti-CD3 (FITC). Only cells showing a clear reactivity were scored as positive. Data represent the mean number of T cells ± SEM of 10 EPT reactions and five NS biopsies. Statistical significance was determined by Student's t test (*$p < 0.012$).

These findings may reflect a delayed influx of the $CD4^+/CD25^+$ subset from peripheral blood. Alternatively, $CD4^+/CD25^+$ cells may evolve after 48 h from those $CD4^+$ T cells that had already entered the skin 24 h after challenge.

The $CD4^+/CD25^+$ T cell subset may represent either activated effector or regulatory T cells (Jonuleit *et al*, 2001a) and could thus function either to enhance or limit the allergen-specific inflammation. Unfortunately, limitations in cell numbers did not allow us to investigate the functional properties of this cell population.

Alterations in DC subsets in 72 h EPT lesions Langerin and CD1a were used as markers for Langerhans cell, CD1c for DDC of myeloid origin and CD123/CD45RA for the recently described pDC (Cella *et al*, 1999). Under physiologic circumstances, we found large numbers of $CD1a^+$ and $Langerin^+$ DC (18.7 and 27.7 per mm) in the epidermal and moderate numbers of $CD1c^+$ DC (24 per mm^2) in the dermal compartment of human skin (**Table II** and **Fig 3**). Only few $CD1c^+$ cells (4.5 per mm) could be detected in the epidermis (**Fig 3*A***). In 72 h EPT lesions the situation had changed completely (**Fig 3*B***). The number of dermal $CD1a^+$ cells (44.5 per mm^2) was significantly increased at the expense of their epidermal counterpart (12.1 per mm basement membrane). Nevertheless, the number of $CD1a^+$ cells in total (epidermis and dermis) was larger in EPT, when compared with NS (**Fig 3**). We also noted an immense increase

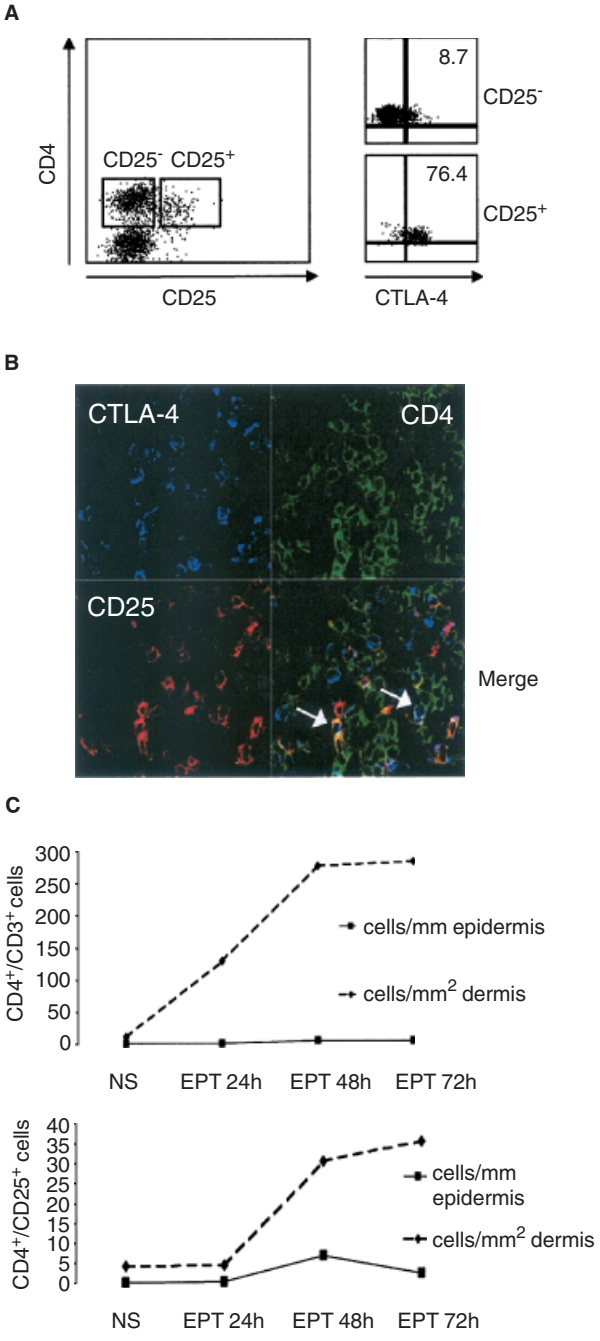

Figure 2. Detection of $CD4^+/CD25^+$ T cells in the inflammatory infiltrate of EPT lesions. (*A*) Epidermal/dermal cell suspensions of biopsies from 72 h EPT lesions were prepared and subjected to FACS analysis. To exclude macrophages or DC from the analysis, T cells were defined in the corresponding morphogate. $CD4^+$ T cells were assessed for positive immunoreactivity with anti-CTLA-4 and anti-CD25. Significant expression of CTLA-4 was only found on $CD25^+$, but not on $CD25^-$ T cells. Data are representative of three experiments. (*B*) *In situ* triple staining of $CD4^+$ (FITC)/$CD25^+$ (TRITC) cells in 72 h patch test reactions. *Arrows* denote cells with triple positive immunoreactivity (whitish). (*C*) Kinetics of infiltrating $CD4^+/CD3^+$ and $CD4^+/CD25^+$ T cells. Biopsies obtained at four different time points past hapten challenge (0 h, 24 h, 48 h, 72 h) were subjected to immunofluorescent analysis. Cells double-positive for either CD4/CD25 or CD4/CD3 were evaluated. $CD4^+/CD3^+$ T cell infiltration into the dermal compartment started early (upper graph), and preceded that of $CD4^+/CD25^+$ cells by 24 h (lower graph).

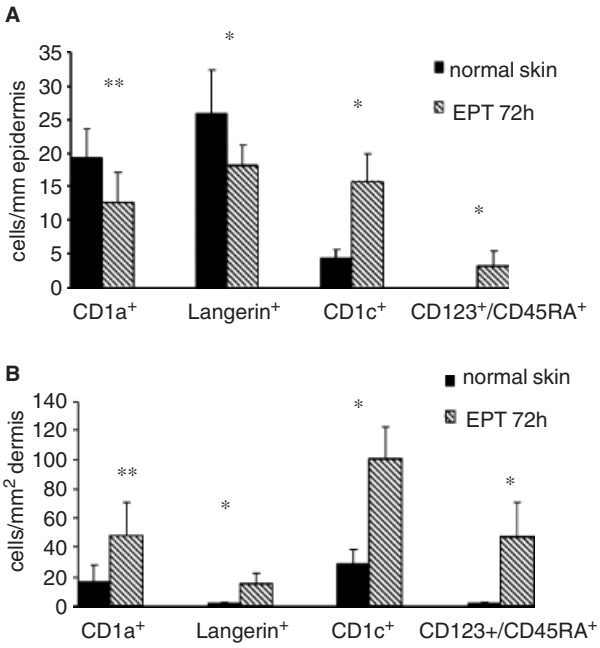

Figure 3. Distribution of Langerhans cells, DDC, and pDC in the epidermis and dermis of 72 h EPT lesions and NS. We subjected frozen sections to immunofluorescence analysis and characterized the various DC subsets. Single stainings were performed to identify Langerhans cells (Langerin and CD1a) and DDC (CD1c), double stainings to visualize pDC (CD123 FITC/CD45RA TRITC) in the epidermis (*A*) and dermis (*B*) of NS and 72 h EPT. Only cells showing a clear immunoreactivity were scored as positive. Statistical significance was determined by Student's t test ($^{*}p < 0.005$; $^{**}p < 0.025$).

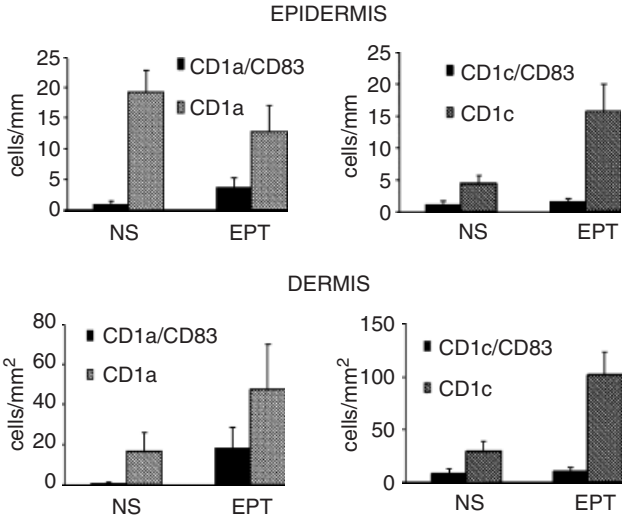

Figure 4. Proportion of CD83$^+$ cells in CD1a$^+$ and CD1c$^+$ populations in the skin. The maturation/activation of Langerhans cells and DDC in healthy skin and in 72 h EPT reactions was investigated by visualizing CD1a$^+$/CD83$^+$ and CD1c$^+$/CD83$^+$ cells by immunofluorescence. Left graphs: In EPT lesions there is a significant increase in the number of dermal CD1a$^+$ cells (grey bars) at the expense of their epidermal counterpart in EPT lesions. In contrast to NS, which contains only a small percentage of CD1a$^+$/CD83$^+$ cells (black bars) in the epidermal (upper graph) and dermal compartment (lower graph), a substantial proportion of CD1a$^+$/CD83$^+$ cells (black bars) was found in the epidermis and dermis of EPT lesions. Right graphs: The number of CD1c$^+$ DDC (grey bars) is increased in the epidermis (upper graph) and dermis (lower graph) of EPT reactions compared with NS. In contrast to Langerhans cells, the majority of DDC remains negative for CD83 after-hapten challenge (black bars).

in the amount of CD1c$^+$ cells compared with NS, not only in the dermis (97.1 per mm^2), but also in the epidermis (16.6 per mm) (**Fig 3**). Closer investigation revealed coexpression of CD1a in approximately 25% of both epidermal and dermal CD1c$^+$ cells (**Table II**). These cells could either represent inflammatory dendritic epidermal cells, or a subpopulation of Langerhans cells (Lenz *et al*, 1993; Wollenberg *et al*, 1996).

To distinguish between immature and mature DC present in inflamed skin, we performed *in situ* double stainings with the activation/maturation marker CD83. As opposed to NS with only few CD1a$^+$ cells expressing CD83 (3.6% of epidermal and 4.3% of dermal CD1a$^+$ cells), we found a substantial proportion of mature Langerhans cells present in EPT lesions (28% of epidermal and 38.1% of dermal CD1a$^+$ cells) (**Fig 4**). In contrast, the vast majority of CD1c$^+$ cells remained CD83$^-$ (91% of epidermal and 90% of dermal CD1c$^+$ cells) (**Fig 4**).

We also detected a small number of the newly defined CD123$^+$/CD45RA$^+$pDC population in the dermal, but not in the epidermal compartment of NS (1.4 per mm^2). Interestingly, these cells represented a substantial proportion of the entire DC infiltrate (39.8 per mm^2) in EPT lesions (**Fig 3**).

Phenotypic features of pDC in ACD lesions In humans, pDC can be identified by the coexpression of CD123 and CD45RA, BDCA-2, CD68, CXCR3, and CD62L (Cella *et al*, 1999; Dzionek *et al*, 2001; Farkas *et al*, 2001; Wollenberg *et al*, 2002). To learn more about the phenotypic characteristics of pDC in NS and EPT lesions, we performed double and triple stainings with the indicated markers (**Figs 5, 6, and 7*A–C***). In NS, pDC could barely be detected. The epidermis was devoid of pDC, and in the dermis we found a few scattered cells with positive immunoreactivity for CD123/CD45RA, CD123/BDCA-2, and CD123/CD68 (**Fig 5**). Lesional skin, in contrast, contained pDC in both the epidermal and dermal compartment (**Fig 5**). Whereas CD123$^+$ cells showed a comparable coexpression of CD45RA (median (m) = 3.5 per mm epidermis and 39.8 per mm^2 dermis), BDCA-2 (m = 1.6 per mm and 32.8 per mm^2), and CD68 (m = 2.3 per mm and 24 per mm^2), CD62L (L-selectin) was merely present on one-third of the pDC population (m = 0.4 per mm and 8.5 per mm^2) (**Figs 5 and 7*A,B***). As local proliferation of pDC was excluded by double stainings with BDCA-2 and the proliferation marker Ki-67 (data not shown), these findings may reflect a cutaneous influx of blood-derived pDC that downregulate CD62L after their entry into skin. To characterize skin-derived pDC better, we performed flow cytometric analysis of single cell suspensions from biopsies of 72 h EPT lesions and compared them with peripheral blood-derived pDC of the same patient (**Fig 6**). Approximately 90% of the gated CD123$^+$/CD45RA$^+$ cell population in both the skin and the blood were positive for CD4 and/or MHC II (**Fig 6*A***). When gating on CD123$^+$/MHC II$^+$ cells, the population showed homogeneous surface expression of BDCA-2 in the skin and in the blood (**Fig 6*B***). Blood-derived CD123$^+$/MHC II$^+$ cells did not react with the maturation-defining anti-CD83 and anti-CD86 antibodies (Kohrgruber *et al*, 1999). In contrast, we found approximately 50% mature CD83- and CD86-bearing cells within the pDC population infiltrating the skin (**Fig 6*B***).

Searching for mechanisms involved in pDC adhesion to endothelial cells and migration from peripheral blood to skin, we found CLA expression on approximately 80% of skin and 95% of blood-derived pDC (**Fig 6*B***). Adhesion of CLA$^+$ pDC could be mediated by binding to E-selectin, as this corresponding ligand is known to be strongly upregulated on venules in inflammatory cutaneous lesions such as CHS (Picker *et al*, 1991; Harari *et al*, 1999). Circulating pDC gain access into regional

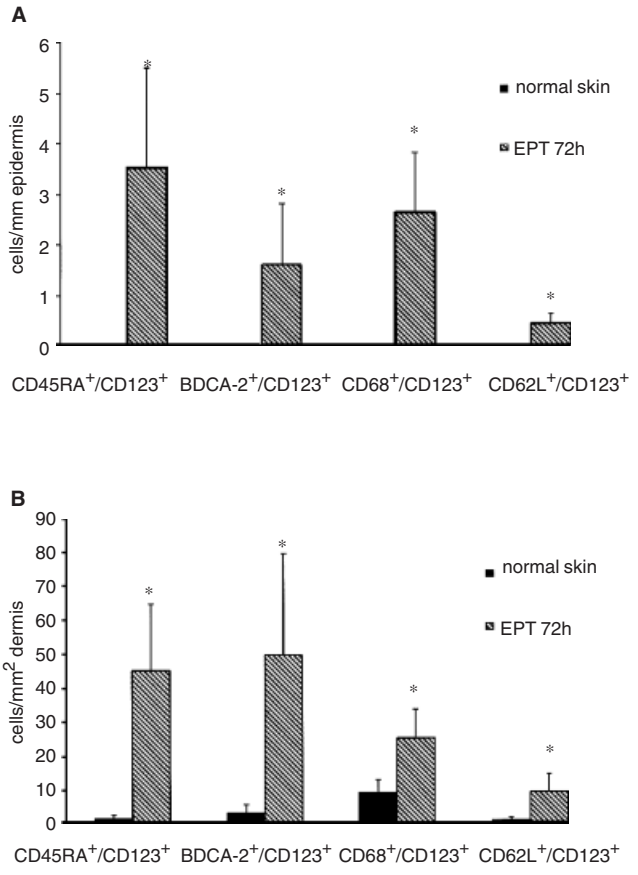

Figure 5. Phenotypic characterization of pDC. NS and 72 h EPT lesions were phenotypically analyzed by double stainings for CD123/CD45RA, CD123/BDCA-2, CD123/CD68, and CD123/CD62L. Contrary to EPT, the epidermis of NS is devoid of pDC. Pronounced increase in the number of dermal $CD123^+/CD45RA^+$, $CD123^+/BDCA\text{-}2^+$, and $CD123^+/CD68^+$ cells in ACD when compared with NS. Note that only few $CD123^+$ cells express CD62L. Statistical significance was determined by Student's t test ($^*p < 0.01$).

lymph nodes via ligation of CD62L to the peripheral lymph node vascular addressin (PNAd), expressed on endothelial cells of high endothelial venules (HEV) (Cella *et al*, 1999). As expected, almost 100% of blood-derived pDC were strongly $CD62L^+$, whereas the expression level on skin-derived pDC varied from low to intermediate (**Fig 6*B***). The downregulation of CD62L on skin-derived pDC is in accordance with our *in situ* findings (**Fig 5**). To investigate whether pDC could indeed enter EPT lesions via CD62L, we searched for the presence of PNAd on vascular endothelium (Lechleitner *et al*, 1999) and found a small proportion of endothelial cells displaying positive MECA-79 reactivity in 72 h EPT lesions (data not shown). A subset of $CD123^+$ pDC also coexpressed the chemokine receptor CXCR3 (**Fig 7*C***) and may therefore be attracted by activated skin cells secreting CXCR3 ligands (Sebastiani *et al*, 2002). In summary, these results are indicative of a substantial influx of $CD62L^+/CLA^+$ pDC into 72 h EPT lesions, partially undergoing maturation in inflamed skin.

Emergence kinetics of pDC To investigate the emergence and disappearance kinetics of infiltrating pDC, time course experiments were performed. The evaluation of $CD123^+/CD45RA^+$ cells in EPT reactions revealed an increase in cell number at 24 h past challenge (0.7 per mm epidermis and 17.4 per mm^2 dermis), which peaked at 48 h (1.2 per mm epidermis and 28.2 per mm^2 dermis), and then decreased again (**Fig 8*A***). Examinations of skin biopsies at even earlier time points past challenge (6 h and 12 h) also showed a clear increase in the number of pDC compared with unchallenged skin (data not shown).

Proximity of pDC and natural killer cells in EPT lesions The early appearance of this cell type in the elicitation phase of ACD, the fact that pDC play a part in linking innate and adaptive immunity (Colonna *et al*, 2002), and recent findings on the role of cells of the innate immune system in murine CHS (Tsuji *et al*, 2000) prompted us to search for the presence of other prominent members of innate immunity, namely natural killer and natural killer T cells. We identified natural killer cells in EPT lesions by using antibodies against CD56 and CD94 (**Table II**) and were surprised to observe a higher number of

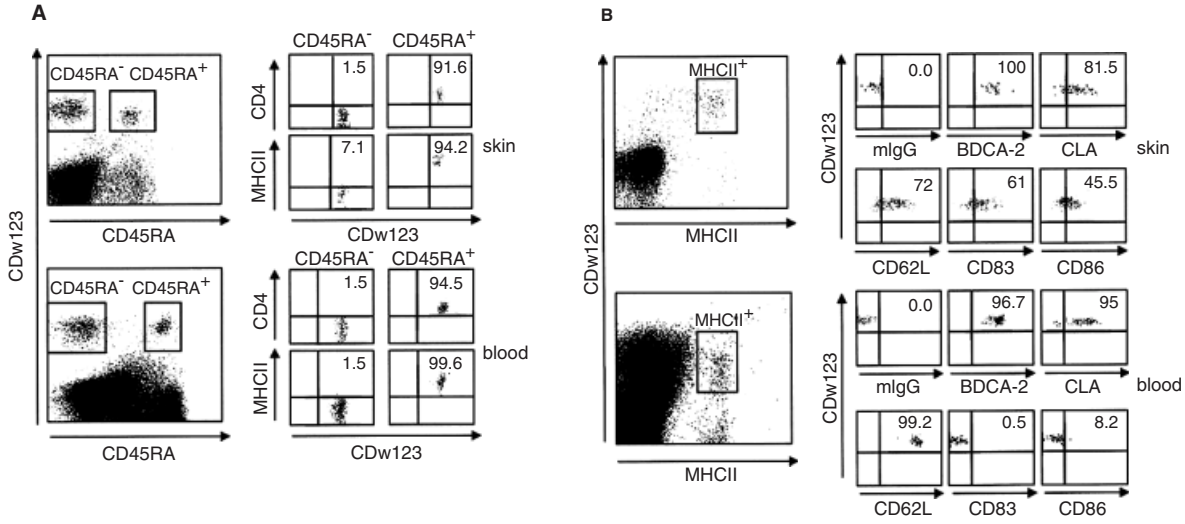

Figure 6. Flow cytometric analysis of cell suspensions of EPT lesions and peripheral blood mononuclear cells. (*A*) Skin- and blood-derived pDC display CD123/CD45RA/CD4/MHC II on their surface. $CD123^+/CD45RA^-$ and $CD123^+/CD45RA^+$ cell populations were gated and analyzed for the expression of CD4 and MHC II. (*B*) Comparison of the expression of adhesion molecules and maturation markers on $CD123^+/MHC\ II^+$ cells in skin and blood. Whereas CLA is displayed on skin- and blood-derived pDC in a comparable fashion, we find that approximately 30% of skin-derived pDC have lost CD62L on their surface. In contrast to blood-derived pDC there is abundant expression of the maturation markers CD83 and CD86 on skin-derived pDC.

$CD94^+$ cells when compared with $CD56^+$ cells. Characterizing natural killer T cells, we found that approximately one-fourth of all $CD94^+$ cells bore the T cell receptor (**Table II**), whereas $CD56^+$ cells remained $CD3^-$ (data not shown). This $CD94^+/CD3^+/CD56^-$ natural killer T cell subset may account for the numerical difference between the $CD56^+$ and the $CD94^+$ cells.

Interestingly, the infiltration kinetics of $CD56^+$ natural killer cells resembled those of pDC (**Fig 8*B***) with an increase in natural killer cells to 13.7 cells per mm^2 in the dermal compartment at 24 h, a peak at 48 h (21.8 per mm^2) and a decrease at 72 h after challenge. Only a negligible number of $CD56^+$ cells was encountered in the normal dermis (3.9 per mm^2) (**Fig 8*B***, **Table II**). Triple stainings of EPT lesions not infrequently revealed a close proximity of $CD123^+$/MHC II^+ pDC and $CD56^+$ cells in the perivascular infiltrate of the dermis (**Fig 7*D***).

DISCUSSION

Whereas abundant knowledge exists about the cellular and molecular events operative in the sensitization phase of CHS, the understanding of the mechanisms guiding the elicitation phase remains incomplete (Sinigaglia *et al*, 1985; Probst *et al*, 1995). To learn more about antigen-presenting cells directing T cell responses in this process, we have phenotypically analyzed the leukocytic infiltrate of EPT reactions, with special emphasis on DC and T cell subsets. In this context, we describe the occurrence of pDC in EPT lesions and correlate their number and level of activation with that of Langerhans cells and DDC.

Notwithstanding their decisive function in the induction phase of CHS (Romani and Schuler, 1992), the role, if any, of Langerhans cells in the effector phase is a matter of controversy (Grabbe *et al*, 1995). On the basis of CD1a and Langerin expression we compared the numbers of Langerhans cells residing in NS and EPT lesions and found a significant decrease of epidermal and an increase of dermal Langerhans cells in lesional skin. The majority of these Langerhans cells expressed the maturation marker CD83, possibly as a consequence of hapten challenge. It should be emphasized that the number of $CD1a^+$ cells exceeded that of $Langerin^+$ cells in the epidermis and dermis of ACD lesions, raising the question as to the nature of these cells. Three possibilities can be entertained to explain this phenomenon. They could either represent peripheral blood-derived $CD1a^+/Langerin^-$ precursor cells, $CD1a^+/CD1c^+$ inflammatory dendritic epidermal cells, or Langerhans cells migrating to the draining lymph nodes after hapten challenge. (Wollenberg *et al*, 1996). Antigen could also be captured and presented by DDC, a subset of DC with myeloid origin displaying positive reactivity for CD1c (Lenz *et al*, 1993; Cella *et al*, 1997). We found a dense dermal infiltrate of $CD1c^+$ cells with the majority resting in an immature state ($CD1c^+/CD83^-$). Based on these findings we hypothesize that mature Langerhans cells present antigen to hapten-specific effector T cells and thereby initiate and propagate the allergic response. Immature DDC, in contrast, may possibly activate regulatory T cells (Jonuleit *et al*, 2001b), which, in a delayed fashion, would then be responsible for the resolution of the eczematous reaction.

Importantly, we demonstrated the existence of a significant number of $CD123^+/CD45RA^+$ cells in the dermis of human ACD lesions. Their additional expression of high levels of CD68, MHC II, and BDCA-2 qualified them clearly as pDC. Based on their negative immunoreactivity for Ki-67, we would assume that pDC or their precursors enter the skin upon hapten challenge and do not proliferate locally. A sizeable number of pDC could already be observed in 6 h EPT lesions. Searching for possible mechanisms involved in pDC adhesion to the endothelium and migration from blood to skin, we investigated surface expression of CLA, CD62L, and CXCR3. Notably, pDC displayed almost uniform expression of the skin-homing molecule CLA before (95%) and after (80%) skin entry. In addition, almost 100% of the circulating pDC isolated from nickel-allergic individuals were highly $CD62L^+$, yet the expression level of this adhesion molecule was significantly reduced after entry into skin. Similar observations were made in human lymph nodes, where CD62L is cleaved completely from the surface of pDC after entry via HEV (Cella *et al*, 1999). We hypothesize that pDC may adhere to endothelial cells via CLA/E-selectin and CD62L/PNAd interactions (Picker *et al*, 1991; Lechleitner *et al*, 1999), as we found vessels in EPT lesions to be positive for these addressins (data not shown). Our findings of CXCR3 on a subset of lesional pDC suggest that the ligands of this chemokine receptor, i.e., CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (ITAC), are involved in attracting pDC to the skin. This is in line with recent data showing that pDC have the capacity to migrate in response to CXCR3/CXCR4 ligands (Krug *et al*, 2002).

Similar to Langerhans cells and DDC, pDC are capable of stimulating antigen-specific T cell proliferation (Kohrgruber *et al*, 1999; Cella *et al*, 2000). As evidence exists that the state of DC activation determines the type of T cell response (proliferation/anergy) (Steinman *et al*, 2003), we were interested in the maturation profile of skin-derived pDC. Flow cytometric analysis of single cell suspensions revealed reactivity for the maturation markers CD83 and CD86 in approximately 50% of skin-derived pDC, but not blood-derived pDC, indicating that after leaving the blood, pDC undergo, at least partly, a process of maturation and acquire the ability to activate skin-infiltrating T cells. Human *in vitro* studies have shown that, dependent on the type of maturation stimuli, i.e., viral products or IL-3/CD40 ligand (CD40L), pDC have the capacity to prime either for a T helper type 1 or 2 biased immune response (Rissoan *et al*, 1999; Kadowaki *et al*, 2000). In EPT lesions IL-3 may be secreted by activated mast cells (Plaut *et al*, 1989), whereas CD40L could conceivably be provided by infiltrating T cells. In this context, it should be noted that CD40L-activated murine pDC also have the capacity to induce a $CD8^+$ T regulatory cell differentiation (Gilliet and Liu, 2002). The exact function of immature and mature pDC in EPT reactions is currently under investigation.

We found the massive T cell infiltrate in 72 h EPT to be dominated by $CD4^+$ T cells ($CD4^+/CD8^+$ T cells ratio 2:1), which, according to other studies, mainly express αβ-T cell receptors (Sterry *et al*, 1990; Brasch *et al*, 1992). Only few T cells showed a positive reactivity for γδ-T cell receptor. Interestingly, we detected a $CD4^+$ T cell subset coexpressing CD25 and CTLA-4 within lesional, but not NS. The late occurrence of this $CD4^+/CD25^+$ T cell population (48 h past challenge), when compared with $CD4^+/CD25^-$ T cells (24 h past challenge), may either reflect its delayed influx from the peripheral blood or the evolvement from $CD4^+/CD25^-$ T cells that had entered the skin earlier. Whether the $CD4^+/CD25^+$ T cell subset represents activated effector, allergen-specific regulatory T cells (Tr cells) (Cavani *et al*, 1998) or nonspecific suppressor T cells (Jonuleit *et al*, 2001a; Shevach, 2002) and, thus, functions to either enhance or limit the allergen-specific inflammation, remains to be determined.

Besides characterizing members of the adaptive immune system, we also describe the occurrence of cells of the innate immune system such as natural killer cells, neutrophils, mast cells, macrophages, and eosinophils in the dermal compartment of 72 h EPT lesions. These cells may be attracted and activated by proinflammatory cytokines produced by keratinocytes in response to hapten challenge as a line of first defense (Luger and Schwarz, 1990; Barker *et al*, 1991). Investigation of the kinetics of natural killer cells and their location within the leukocytic infiltrate revealed a migration profile very similar to that of pDC and, additionally, a close proximity to pDC. It is tempting to speculate about a cross-talk between pDC and natural killer cells in the effector phase of ACD, similar to what has been described for atopic dermatitis (Buentke *et al*, 2002). By producing large amounts of interferon-α, pDC may not only provide an autocrine survival factor (Kadowaki *et al*, 2000), but also contribute to stimulation

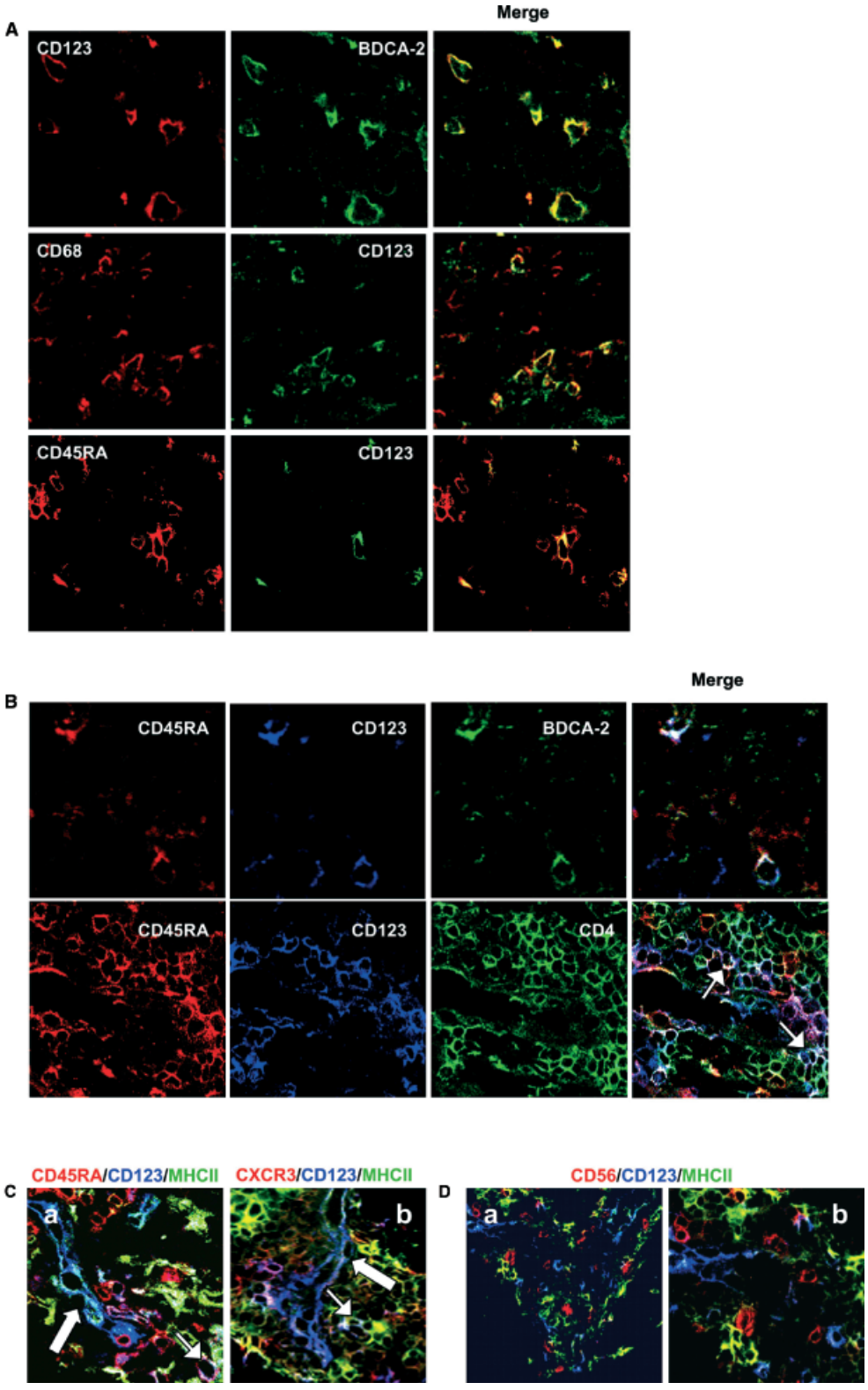

Figure 7. Immunofluorescence double- and triple stainings with pDC-defining markers. (*A*) Visualization of pDC in the papillary dermis by using antibodies against CD123 (red)/BDCA-2 (green) (original magnification × 1000), CD68 (red)/CD123 (green) (original magnification × 600) as well as CD45RA (red)/CD123 (green) (original magnification × 600). The merge of double-stained cells appears in yellow. (*B*) We confirmed the presence of pDC in lesional skin by triple stainings for either CD45RA (red), CD123 (blue), and BDCA-2 (green) (original magnification × 1000) or CD45RA (red), CD123 (blue), and CD4 (green) (original magnification × 400). Merging of three-colored cells results in a whitish color (*small arrows*). (*C*) Triple stainings for CD45RA (red), CD123 (blue) and MHC II (green) (original magnification × 600) (*a*) and for CXCR3 (red), CD123 (blue) and MHC II (green) (original magnification × 400) (*b*). Both pictures show triple-positive cells within the perivascular infiltrate in EPT lesions. *Small arrows* delineate triple stained cells, *big arrows* denote $CD123^+$ vessels. (*D*) Natural killer cells (red) (visualized by CD56) and pDC (green/blue) (visualized by CD123/MHC II) are located in close proximity to each other. Original magnification: (*a*) × 400; (*b*) × 800. Pictures are representative of all evaluated biopsies.

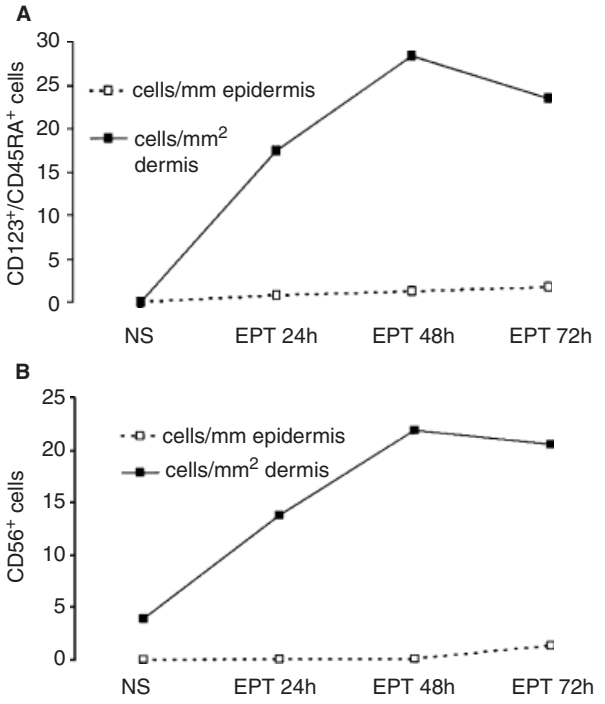

Figure 8. Infiltration kinetics of CD123$^+$/CD45RA$^+$ and CD56$^+$ cells in 72 h EPT lesions. Biopsies obtained at four different time points past hapten challenge (0 h, 24 h, 48 h, 72 h) were subjected to immunofluorescent analysis. (*A*) Simultaneous increase in the number of CD123$^+$/CD45RA$^+$ pDC, and (*B*) CD56$^+$ cells early after challenge with a peak at 48 h.

and subsequent interferon-γ production of natural killer cells propagating a T1-biased response.

Application of nickel to the skin of nonallergic individuals did not change its cellular composition, indicating that the mere hapten contact is not enough to elicit the occurrence of, for example, pDC, DDC, and natural killer cells (data not shown). The initiation of the infiltrate required inflammatory signals that could either be provided by the hapten challenge of a sensitized individual or the cutaneous application of contact irritants such as sodium dodecyl sulfate (data not shown).

To the best of our knowledge, this analysis is one of the first to provide a detailed and synoptic view about the cellular composition of the inflammatory infiltrate in ACD and its kinetics. This understanding should now enable us to examine the relative contribution of the various players in the emergence and resolution of the disease.

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