Identification of a Novel CD160$^+$CD4$^+$ T-Lymphocyte Subset in the Skin: A Possible Role for CD160 in Skin Inflammation

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CD160 is a glycosylphosphatidylinositol-anchored cell surface molecule expressed by human circulating cytotoxic lymphocytes that correspond to the majority of natural killer cell (NK) expressing CD56^{dim}, TCR$\gamma\delta$ lymphocytes, and to a minor CD8 T-cell subset. CD160 engagement by major histocompatibility complex class I molecules triggers by itself both cytotoxic function and cytokine production in NK lymphocytes, whereas it provides co-activating signals to TCR-induced proliferation in T CD8+ lymphocytes. In this study, we analyzed by immunohistochemistry the phenotype of lymphocytes infiltrating normal skin and inflammatory skin lesions of atopic dermatitis, contact dermatitis, and psoriasis. We identified a minor original subset of CD4$^+$CD160$^+$ T cells infiltrating inflammatory lesions. We found that this lymphocyte subset localization is not restricted to the skin, as we demonstrated that CD160 transcripts could be induced in IL-2 or IL-15-activated CD4$^+$ peripheral blood lymphocytes. Finally, we report that CD160 acts as a co-activator receptor for CD3-induced proliferation of CD4$^+$CD160$^+$ T cells isolated from inflammatory skin lesions. Thus, we hypothesize that the unique CD4$^+$CD160$^+$ lymphocyte subset plays a role in the pathogenesis of skin inflammation.

Journal of Investigative Dermatology (2007) **127,** 1161–1166. doi:10.1038/sj.jid.5700680; published online 11 January 2007

INTRODUCTION

Infiltration of the skin by T cells is a major factor in the pathogenesis of inflammatory skin diseases such as atopic dermatitis (AD), contact dermatitis (CD), and psoriasis (PSO) (Akiba *et al.*, 2002; Gudjonsson *et al.*, 2004; Leung *et al.*, 2004). AD and PSO are chronic inflammatory skin diseases whose pathogenesis is not yet totally understood. A combination of factors has been incriminated in the pathogenesis of AD, such as genetic background, environment triggering factors, skin barrier dysfunction, and abnormal immunologic responses (Leung *et al.*, 2004). AD lesions are characterized by a dermal and epidermal inflammatory infiltrate made essentially of CD4-activated T cells (Lugovic *et al.*, 2001). In PSO, evidence indicates that keratinocyte proliferation, which is the striking feature of the disease, results from specific antigen recognition by T cells (Gudjonsson *et al.*, 2004). T cells also play a key role in the development of CD by a mechanism of allergen sensitization (Lepoittevin and Cribier, 1998). However, molecular pathways recruited by T cells in skin inflammatory lesions are not yet totally understood.

CD160 is a cell surface molecule expressed by human and mouse circulating cytotoxic lymphocytes, as natural killer cells (NK) CD56^{dim}CD16$^+$, most TCR$\gamma\delta$ T cells and cytotoxic effector TCR$\alpha\beta$ CD8T cells (Bensussan *et al.*, 1993; Maïza *et al.*, 1993). It is also expressed by most intestinal intraepithelial lymphocytes. It corresponds to a multimeric glycosylphosphatidylinositol-anchored glycoprotein with a single Ig-like domain, weakly homologous to the KIR2DL4 inhibitory receptors (Anumanthan *et al.*, 1998). Both human and mouse CD160 exhibit a broad specificity for major histocompatibility complex class Ia and Ib molecules (Agrawal *et al.*, 1999; Maeda *et al.*, 2005). The engagement of CD160 by HLA-C is a triggering mechanism of circulating NK-mediated cytotoxicity (Le Bouteiller *et al.*, 2002) and cytokines production (Barakonyi *et al.*, 2004). On another hand, CD160 is a co-receptor of TCR induced proliferation of cytotoxic T cells (Nikolova *et al.*, 2002; Rey *et al.*, 2006). In order to determine whether CD160$^+$ T lymphocytes contributed to the pathogenesis of skin inflammation, we studied their presence among lymphocytes infiltrating inflammatory lesions and whether CD160 triggering provides co-stimulatory effect to anti-CD3 mAb-induced proliferation.

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Abbreviations: AD, atopic dermatitis; CD, contact dermatitis; cDNA, complementary DNA; mRNA, messenger RNA; NK cell, natural killer cell; PB, peripheral blood; PSO, psoriasis

Received 31 May 2006; revised 23 August 2006; accepted 9 September 2006; published online 11 January 2007

RESULTS

In situ immunohistochemical staining of inflammatory and normal skin infiltrating cells revealed the presence of CD160$^+$ T lymphocytes

Single staining with anti-CD3, anti-CD4, anti-CD8, anti-CD56, and anti-CD160 antibodies of four AD, six CD, and one PSO lesion biopsy samples revealed in all cases a marked dermal inflammatory infiltrate essentially made of CD4$^+$ T cells. A representative immunostaining obtained with an AD skin biopsy is shown in Figure 1, right column. Although few CD8$^+$ T cells could be visualized (Figure 1d), no CD56$^+$ lymphocytes were detected in most dermal inflammatory samples except for one AD sample in which very few scattered CD56+ cells could be found (Figure 1h). In contrast, in normal skin samples (Figure 1, left column), only a few T cells infiltrated the dermis (Figure 1a), with a peri-vascular distribution, and both CD4$^+$ (Figure 1c) and CD8$^+$ T-cell (Figure 1e) subsets were equally represented. Interestingly, we found that anti-CD160 antibodies stained a variable proportion of cells within the inflammatory infiltrate of AD (Figure 1j), CD, and PSO lesions (mean number of 16.6 CD160$^+$ cells per high-power field (70 cells/mm^2), range: 2–37 cells/high-power field (8–155 cells/mm^2). Their presence was not restricted to inflammatory skin lesions as CD160$^+$ cells were also present in the dermis of both normal skin samples (mean number of 20 CD160$^+$ cells per high-power field (84 cells/mm^2), range: 15.5–24.3 cells per high-power field (65–102/mm^2) (Figure 1i). Further, double immunohistochemical staining with anti-CD3, anti-CD4, and anti-CD160 mAbs performed with two AD, two CD and one PSO lesions samples revealed the presence of CD160$^+$ CD3$^+$ and CD160$^+$CD4$^+$ cells within the dermal inflammatory infiltrate (Figure 2a and b for AD and PSO lesions, respectively), indicating the skin localization of a T-lymphocyte subset expressing CD160 molecules.

CD160 messenger RNA (mRNA) transcript is isolated from inflammatory and normal skin samples as well from peripheral blood (PB)-CD4$^+$ cultured *ex vivo* with cytokines

In order to further demonstrate that the anti-CD160 mAb was effectively reactive with CD160 molecules expressed by T lymphocytes in the skin as evidenced by immunohistochemical staining, we performed RT-PCR analysis using CD160-specific primers on two AD and two healthy skin samples and found that CD160 mRNA transcripts were present in all tested skin samples as compared to PB-NK cells (Figure 3a). Control purified PB-CD4$^+$ were negative for CD160 mRNA. Next, CD160 transcripts were isolated from both healthy and AD skin for sequencing. The results clearly established that the PCR products corresponded to CD160 mRNA as the amino-acid sequence alignment of healthy and AD CD160 proteins showed that they were identical to the CD160 sequence already described. Interestingly, CD160 sequencing in one AD skin biopsy revealed a novel allelic form with two substituted residues that was also obtained in one normal individual (Figure 3b). Next, as most CD160$^+$ PB lymphocytes correspond to subsets of NK, T CD8$^+$, and TCR$\gamma\delta^+$ lymphocytes, it was important to assess whether PB-CD4$^+$ lymphocytes could be induced to transcribe CD160 under the presence of proinflammatory cytokines. We found that whereas CD160 mRNA transcripts were not detected in freshly isolated PB-CD4$^+$ (Figure 4, lane 1) nor after 6 days of culture with IL-4 (Figure 4, lane 2), they could be detected when these cells were cultured during 6 days in the presence of IL-2 alone (Figure 4, lane 3), IL-2, + IL-4 (Figure 4, lane 4),

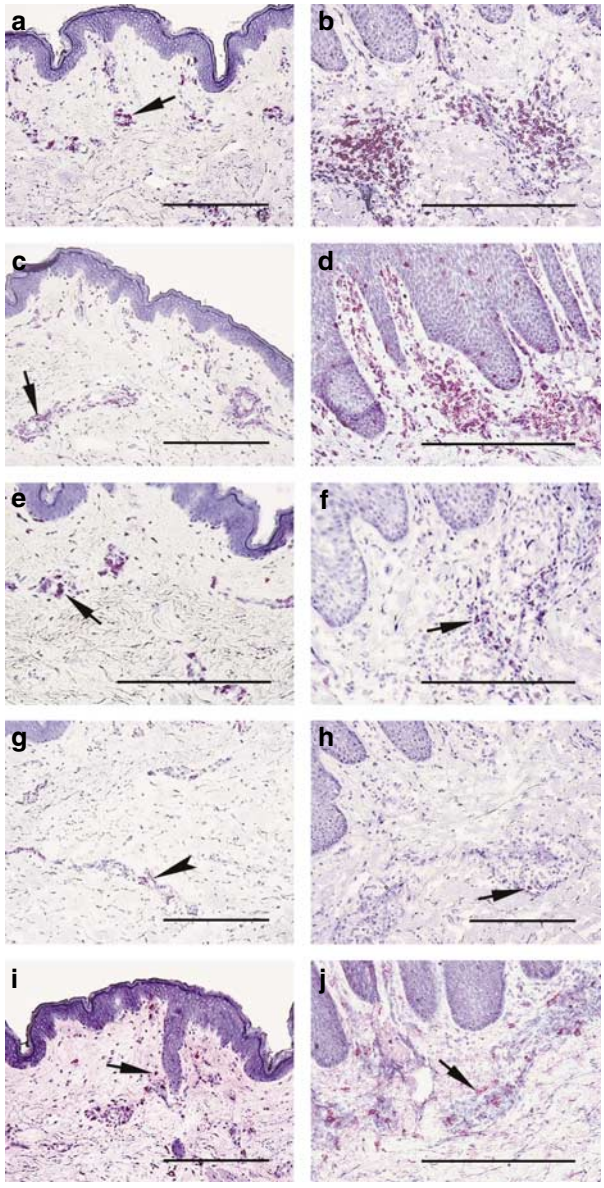

Figure 1. *In situ* phenotype of inflammatory and normal skin cellular infiltrates: detection of a CD160$^+$ cell subset. Phenotype of cellular infiltrates of the skin established by immunohistochemistry (×100) on AD skin (right column) and healthy skin (left column). In normal skin, there was a minor infiltrate of CD3$^+$ (a), CD4$^+$ (c) and CD8$^+$ T cells (e). In AD skin, we found a marked dermal infiltrate made of CD3$^+$ lymphocytes (b), mainly CD4$^+$ T cells (d) accompanied by CD8$^+$ cells (f). There were no NK cells, as CD56 stained only small nervous fibres of the dermis (g and h indicated by arrow heads). CD160$^+$ cells were present both in the inflammatory infiltrate (j) and among resident cells of normal skin (i). Positively stained cells are indicated with arrows. Bar = 0.25 mm.

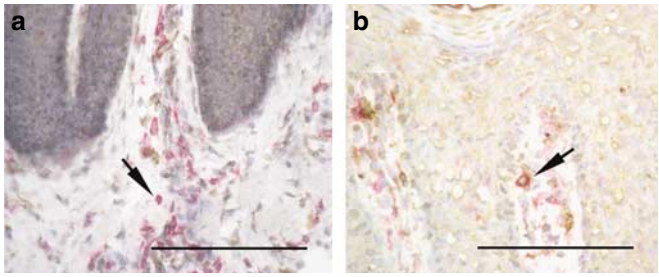

Figure 2. Presence of CD160$^+$ T-cells and CD160$^+$CD4$^+$ cells within the inflammatory skin infiltrates. Demonstrated by immunochemistry double staining (× 100). (**a**) CD160$^+$ T cells infiltrating the dermis in AD. CD3 is stained in red and CD160 in brown. CD160$^+$ T cells are indicated with arrows. (**b**) CD160$^+$CD4$^+$ cells in the papillary dermis of a PSO lesion are indicated with arrows. CD4 is stained in red and CD160 in brown. Bar = 0.1 mm.

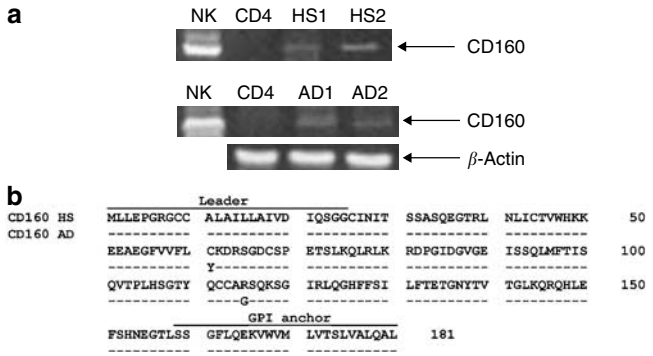

Figure 3. Detection of CD160 mRNA in healthy and inflammatory skin samples. (**a**) CD160 mRNA was identified by PCR analysis after mRNA extraction from frozen samples of healthy skin (HS1 and HS2) and AD lesions (AD1 and AD2). Positive and negative controls consisted respectively of PB-NK and CD4$^+$ T-cells from a healthy subject. Detection of β-actin mRNA also served as a positive control for each experiment. (**b**) CD160 mRNA was identified by cDNA sequence analysis.

or IL-15 (Figure 4, lane 5). These results were obtained with several other healthy donors, (data not shown), demonstrating that CD160 mRNA expression is induced or increased in normal individuals' PB-CD4$^+$ lymphocytes by proinflammatory cytokines (Figure 4), and thus indicating that CD160 expression is not a characteristic of CD4$^+$ skin lymphocytes but is rather a characteristic of proinflammatory cytokine activated CD4$^+$ lymphocytes, as recently reported in mice (Maeda *et al.*, 2005).

CD160 is expressed and is functional in CD4$^+$ lymphocytes isolated from inflammatory skin

To study the function of CD160 expressed by CD4$^+$ lymphocytes isolated from inflammatory skin, biopsy specimens from 3 AD (AD1, AD2, and AD3), 2 CD (CD1 and CD2) and 1 PSO were expanded in culture medium supplemented with cytokines (IL-2 and IL-4) for 1 week. The phenotypic results presented in Table 1 indicated that the growing cells consisted almost exclusively of CD3$^+$ T cells (95–100%), with a vast majority of CD4$^+$ T cells (64–100%)

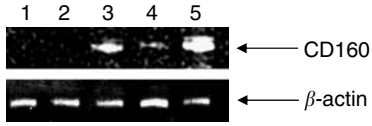

Figure 4. Regulation of the CD160 expression by CD4$^+$ T cells by IL2 and IL15 and not by IL-4. The expression of CD160 mRNA transcript in PB-CD4$^+$ is induced by IL-2 and IL-15, but not IL-4. CD160 mRNA transcript was not detected in freshly isolated (1) PB-CD4$^+$ nor after 6 days of culture in the presence of (2) IL-4, however it could be detected in PB-CD4$^+$ cultured during 6 days in the presence of (3) IL-2, (4) IL-2 + IL-4, or (5) IL-15. Detection of β-actin mRNA served as positive control for each experiment.

Table 1. Phenotypic analysis of *ex vivo* cultured lymphocytes isolated from inflammatory skin biopsies

	CD3 (%)	CD4 (%)	CD160 (%)
AD1	96	64	<1
AD2	98	97	<1
AD3	Not tested	93	77
CD1	Not tested	90	15
CD2	100	100	9
PSO	NT	24	9

AD, atopic dermatitis; CD, contact dermatitis; PSO, psoriasis.
Skin biopsies were obtained from patients with atopic dermatitis (AD1, AD2, and AD3), contact dermatitis (CD1 and CD2), and psoriasis (PSO).

except for the PSO sample (24% CD4+). CD160$^+$ cells were present in lower proportions than those found *in situ* by immunochemistry analysis on frozen samples obtained concomitantly for the same patients (Table 1). This is not surprising as biopsy samples are cultured in the presence of IL-2 and Il-4, which enabled cell migration out from the skin and cell proliferation, and that we have demonstrated with TCRγδ T-cell clones (Nikolova *et al.*, 2002) and with PB-NK lymphocytes (Le Bouteiller *et al.*, 2002) that surface expression of CD160 is down-regulated by IL-2, whereas CD160 transcripts are still highly detectable (Giustiniani *et al.*, in press). Nevertheless, cultured lymphocytes from AD3 (Figure 5a) CD1, CD2, and PSO skin biopsies still expressed detectable surface expression of CD160 on CD4$^+$ cells. A representative CD4 and CD160 double staining result obtained with AD3 cells is shown (Figure 5b). To study the function of CD160 expressed on these cultured CD4$^+$ skin lymphocytes, we performed proliferative assays of AD3 cells with anti-CD3 mAb in the presence or absence of anti-CD160 mAb. Noted, CD160 was mostly expressed by CD4$^+$ and not by CD4$^-$ cells obtained from AD3 (Figure 5b). We observed a significant increase in the CD3-induced proliferation in the presence of anti-CD160 mAb as compared to isotype-matched control mAb (Figure 5c). Thus, these results obtained with CD4$^+$ skin lymphocytes confirm previous reports that revealed that CD160 engagement triggers co-activator signals to CD4$^-$ T Lymphocyte proliferation induced by anti-CD3 mAb or HIV peptides (Nikolova *et al.*, 2002, 2005). Also in agreement with previous results the

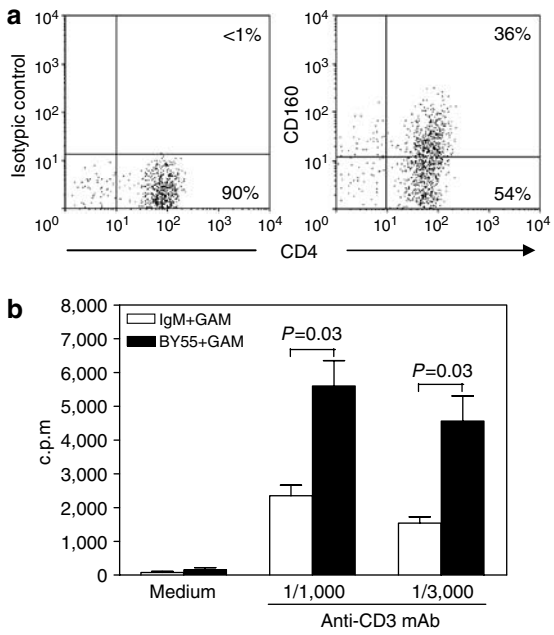

Figure 5. Expression and Function of CD160 on lymphocytes extracted from CD. (**a**) *In vitro* identification of the CD160$^+$ CD4$^+$ T-cell subset. Flow cytometry phenotype analysis of cells obtained from AD3 AD biopsy after cell expansion *in vitro* showed that CD160 was mostly expressed by CD4$^+$ lymphocytes (**b**) CD160 acts as a co-receptor of CD3 induced proliferation of *ex vivo* cultured CD4$^+$CD160$^+$ T cells (AD3). Proliferation assay showing increased CD3-induced proliferation after CD160 stimulation as compared to controls (isotype-matched mAb IgM and goat anti-mouse antiboby (GAM)). BY55 is IgM anti-CD160 mAb.

stimulation of CD160 alone by anti-CD160 mAb did not induce cell proliferation (data not shown).

DISCUSSION

In this study, we identified a subset of CD160$^+$ T cells infiltrating inflammatory skin lesions of AD, PSO, and CD, as shown by immunohistochemistry, tissue mRNA extraction, and complementary DNA (cDNA) sequence analysis results. Interestingly, we detected a particular minor subset of CD160$^+$CD4$^+$ T cells in inflammatory skin lesions, as shown by *in situ* immunohistochemistry experiments.

CD160$^+$ cells were also identified within the cellular infiltrate of normal skin. A low-density lymphocyte infiltrate is usually present in the dermis of normal skin. It is mostly composed of activated CD4 and CD8 T cells in similar proportions, expressing HLA-DR, and the α chain-IL-2 receptor (CD25) (Bos *et al.*, 1987), and these cells are thought to play a role of immune surveillance of the skin (Streilein, 1989).

There is evidence for a role of cytotoxic T cells in the development of lesions in AD, PSO, and CD. Both CD4$^+$ and CD8$^+$ T lymphocytes expressing granzyme B and perforin are present within the inflammatory infiltrate of AD and PSO lesions (Yawalkar *et al.*, 2001). Furthermore, Fas-induced keratinocyte apoptosis mediated by skin infiltrating lymphocytes is considered as a major event in the development of AD lesions (Trautmann *et al.*, 2000). Skin inflammation during CD depends on the early recruitment of CD8 cytotoxic T cells inducing keratinocyte apoptosis (Akiba *et al.*, 2002). We previously reported that BY55/CD160 receptor expression is restricted to functional circulating cytotoxic lymphocytes that are mostly NK lymphocytes and a minor subset of CD8$^+$ T cells (Bensussan *et al.*, 1994). The engagement of CD160 triggers NK-mediated cytotoxicity (Le Bouteiller *et al.*, 2002) and contributes to the enhancement of T-cell proliferation induced by anti-CD3 mAb stimulation (Nikolova *et al.*, 2002) and to the enhancement of anti-CD3 mAb redirected cytotoxicity (Rey *et al.*, 2006). As we found that CD160 is expressed by a subset of CD4$^+$ T cells in inflammatory skin infiltrates, it suggests that its engagement with the physiological ligands (major histocompatibility complex class I molecules) provides *in situ* co-stimulatory signals for CD4$^+$ T-cell proliferation, proinflammatory cytokine production and for killer cell activities. Cytotoxicity was initially attributed to NK and CD8$^+$ T cells, but it is now recognized that CD4$^+$ T cells can also exhibit cytotoxic activity using granzyme B/perforin or Fas/Fas-L-mediated pathways (Vergelli *et al.*, 1997). However, triggering mechanisms of CD4$^+$ T-cell-mediated cytotoxicity are still not completely understood. Here, using *ex vivo* cultured T lymphocytes infiltrating inflammatory skin lesions we demonstrated that the engagement of CD160 enhances the CD4$^+$CD160$^+$ cells proliferation induced by the stimulation of CD3. In addition, we observed by flow cytometry analysis that these cells co-expressed granzyme B (data not shown). Thus, for the first time we report a unique lymphocyte population with a CD4$^+$CD160$^+$ phenotype that could be isolated and cultured from inflammatory skin lesions. Further studies are needed for evidencing that engagement of CD160 could be part of the triggering signal leading to CD4$^+$ cytotoxicity, as we recently reported for the circulating CD8$^+$ lymphocyte population expressing CD160 (Rey *et al.*, 2006).

Interestingly, we demonstrated that IL-2 or IL-15 induces CD160 mRNA expression in PB-CD4$^+$ T-cells, indicating that CD160$^+$CD4$^+$ cells is not a characteristic of skin-derived lymphocytes. On another hand, we have previously demonstrated that IL-2 downregulates the membrane expression of CD160 by peripheral circulating NK lymphocytes (Maïza *et al.*, 1993; Le Bouteiller *et al.*, 2002) and T-cell clones (Nikolova *et al.*, 2002). Thus, IL-2 expressed in inflammatory skin lesions of AD (Grewe *et al.*, 1995), PSO (Bata-Csorgo *et al.*, 1995), and CD (Lepoittevin and Cribier, 1998) could regulate *in situ* the expression of CD160 by CD4$^+$ T cells infiltrating the lesions.

In conclusion, our results suggest that CD160 expression on infiltrating T lymphocytes plays a role in the development of skin inflammation, but its exact function *in situ* remains to be elucidated by further experiments.

MATERIALS AND METHODS

Subjects and skin specimens

After obtaining written informed consent and institutional approval of experiments, we performed punch biopsies of 3–4 mm from lesions of 11 subjects affected with inflammatory skin conditions

(four women and seven men, mean age of 47 years, range: 15–77 years): four AD diagnosed according to Hanifin and Rajka (1980) criteria, six CD and one PSO. In addition, two normal skin samples were obtained from abdominal and breast plastic surgery (two women of 39 and 60 years, mean age of 49.5 years). The Declaration of Helsinki Principles were followed. Skin samples were snap-frozen without tissue embedding medium using liquid nitrogen and stored at −80°C. These samples were used for immunohistochemistry, and immunoflurescence staining, and for tissue RNA extraction. A second concomitant sample was obtained from four of these patients (two patients with AD (AD1 and AD2) and two patients with CD (CD1 and CD2)) and from two additional patients (37-year-old male with AD (AD3) and a 36-year-old female with PSO) for *ex vivo* cell cultures.

Lymphocytes isolation and cultures

As previously described (Bang *et al.*, 2001), inflammatory skin samples from three patients with AD (AD1, AD2, and AD3), two patients with CD (CD1 and CD2), and one patient with PSO were placed in a 24-well plate with culture medium consisting of Rosewell Park Memorial Institute 1640 (Gibco, Paisley, UK), 10% heat-inactivated human serum sodium pyruvate (1 mmol per liter), L-glutamine 2 mmol per liter), streptomycin (100 μg per ml), penicillin (100 U per ml, fungizone (1 μg per ml), IL-2 (200 U per ml; gifted from Sanofi-Aventis, Labege, France) and IL-4 (50 ng per ml; PeproTech, Levallois-Perret, France), at 37°C, which enabled after 1 week cell migration out from the skin, followed by marked proliferation. These cells were then used for phenotypical studies by flow cytometry and proliferation assays.

Peripheral blood CD4$^+$ (PB-CD4$^+$) T cells were obtained by positive selection from freshly isolated peripheral blood mononuclear cells from a healthy donor using magnetic bead sorting (MAC/Miltenyl Biotec, Bergisch Gladbach, Germany). Freshly sorted PB-CD4$^+$ were separated in different groups of 5×10^6 cells each for 6 days of culture in medium containing different cytokines including 200 U per ml IL-2 alone or with 50 ng per ml IL-4, 50 ng per ml IL-4 alone, or 10 ng/ml IL-15 alone (PeproTech).

Immunohistochemistry

Single staining for CD3, CD4, CD8, CD56, and CD160 was performed using the avidin–biotin complex/alkaline phosphatase method. Primary monoclonal antibodies anti-CD3 (IgG), anti-CD4 (IgG), and anti-CD8 (IgG) were purchased from Dako, Copenhagen, Denmark, and used diluted 1/50. Anti-CD56 was obtained from Coulter/Beckman, Marseille, France, and used diluted 1/50. Anti-CD160 was produced in our laboratory and used diluted 1/20. Substitution of the primary antibody with isotype-matched Ig served as negative control. After fixation for 5 minutes with acetone and brief air drying, slides were incubated for 10 minutes with blocking serum (Vectastain/Vector, Burlingame, CA), then for 1 hour with the primary mAb at room temperature followed by a biotinylated secondary anti-mouse antibody (Vectastain/Vector) and thereafter with avidin-alkaline phosphatase (Vectastain/Vector). Finally, all sections were developed in naphtol-fast red (Sigma-Aldrich, Saint Quentin Fallavier, France) and counterstained with hematoxylin. Double staining was performed in inflammatory skin samples by combining CD3 or CD4 staining using anti-IgG conjugated with alkaline phosphatase with CD160 staining using the avidin–biotin complex/peroxydase method. After fixation for 5 minutes with acetone and brief air drying, slides were incubated for 10 minutes with blocking serum (Vectastain/Vector), then for 1 hour with the primary mouse mAb (anti-CD3 or anti-CD4, IgG) at room temperature, followed by a secondary anti-IgG antibody conjugated with alkaline phosphatase (Sigma-Aldrich). Slides were developed in naphtol-fast red (Sigma-Aldrich). Slides were then incubated with BSA for 20 minutes in order to block any possible cross-reaction. Endogenous peroxydase was quenched with peroxydase inhibitor (Dako). Afterwards, slides were incubated with anti-CD160 IgM mouse mAb, followed by a biotinylated secondary anti-mouse IgM antibody (Vectastain/Vector) and thereafter with avidin-peroxidase (Vectastain/Vector). Slides were developed in 3′-3′-diaminobenzidine (Vectastain/Vector) and counterstained with hematoxylin.

Tissue RNA extraction, cell RNA extraction, RT-PCR, and cDNA sequence analysis

RNA was extracted from frozen skin samples of two AD lesions and two normal skin samples. Ten cryostat sections of 50 μm from each frozen sample were homogenized in TRIzol reagent (Invitrogen, Cergy Pontoise, France) and RNA was extracted under conditions recommended by the manufacturer. Similar RNA extractions were performed from CD4$^+$ T cells obtained by positive selection from freshly isolated peripheral blood mononuclear cells from a healthy donor and from the same CD4$^+$ T cells further cultured with cytokines. For PCR amplification of the CD160 transcript, 2–5 μg of RNA were reverse transcribed into cDNA (BD Biosciences, Palo Alto, CA). One microgram of cDNA was used for PCR analysis (Invitrogen, Carlsbad, CA) with synthetic oligonucleotide primers (forward: TGCAGGATGCTGTTGGAACCC, reverse: CCTGTGCCCT GTTGCATTCTTC). After an initial hot start, amplification of CD160 consisted of 35 cycles of 30 seconds at 95°C, 20 seconds at 60°C, and 1 minute at 72°C. The following controls were used: PB-CD4$^+$ cDNA from a healthy donor as a negative control, circulating NK cDNA from a healthy donor as a positive control. PCR products were separated by electrophoresis in 5% agarose gel, purified (Qiaex II/Qiagen GmbH, Germany), sequenced with the same oligonucleotide primers and analyzed with an automated fluorescent DNA sequencer (ABI Prism/Applied Biosystem, Foster City, CA).

Flow cytometry analysis

Phenotype of cultured cells obtained from biopsy samples of three AD (AD1, AD2, and AD3), two CD (CD1 and CD2), and one PSO were analyzed by flow cytometry. Expression of cell surface molecules was determined by direct (anti-CD3, anti-CD4, anti-CD8) or indirect (anti-CD160) labeling. Isotype-matched antibodies were used as negative controls. All antibodies were purchased from coulter/Beckman. Phycoerythrin-goat-anti mouse IgM was used as secondary reagent for anti-CD160 antibodies. Samples were labeled as previously reported (Poszepczynska-Guigne *et al.*, 2004) and flow cytometry analysis was carried out using the EPICS XL (Beckman-Coulter, Miami, FL).

Proliferation assays

T CD4$^+$ cells obtained from a biopsy sample of AD (AD3) after an *in vitro* expansion with IL-2 and IL-4 were used in proliferation assays. After extensive washing the cells were plated at 50×10^3 cells/well in 96-well microtiter plates (Flow laboratories, McLean, VA),

previously coated with goat-anti-mouse antibody, in the presence of anti-CD160 and anti-CD3 mAbs or in the presence of isotype-matched control and anti-CD3 mAbs as previously reported (Nikolova, Int Immunol). Anti-CD3 mAb was used at two different concentrations. Each culture was performed in triplicate. Cells were cultured 72 hours, followed by an 8 hours pulse with 1 μ Ci [^{3}H]thymidine. Radioactive incorporation was measured in a liquid scintillation counter (Topcount, Packard, Meriden, CT). The experiment was performed twice.

Statistical analysis

Data were analyzed using GraphPad Prism program. Experimental differences over the controls were analysed by two-tailed unpaired *t*-test. Data were expressed as mean $\pm$ SD, and significant differences were set at $P<0.05$. In all cases at least three independent experiments were conducted.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by grants from INSERM, Paris XII University, Société Française de Dermatologie, Société de Recherche Dermatologique, and ARC (to M.B. and A.B.).

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