

Activation of the D Prostanoid Receptor 1 Regulates Immune and Skin Allergic Responses¹

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The mobilization of Langerhans cells (LCs) from epithelia to the draining lymph nodes is an essential process to initiate primary immune responses. We have recently shown that in mice, PGD₂ is a potent inhibitor of epidermal LC emigration. In this study, we demonstrate that activation of the D prostanoid receptor 1 (DP1) impedes the TNF- α -induced migration of human LCs from skin explants and strongly inhibits the chemotactic responses of human LC precursors and of maturing LCs to CC chemokine ligands 20 and 19, respectively. Using a murine model of atopic dermatitis, a chronic Th2-type allergic inflammatory disease, we demonstrate that the potent DP1 agonist BW245C dramatically decreases the Ag-specific T cell activation in the skin draining lymph nodes and markedly prevents the skin lesions following repeated epicutaneous sensitization with OVA. Interestingly, analysis of the local response indicates that BW245C treatment strongly reduces the recruitment of inflammatory cells into the dermis and disrupts the Th1/Th2 balance, probably through the increased production of the immunoregulatory cytokine IL-10, in the skin of sensitized mice. Taken together, our results suggest a new function for DP1 in the regulation of the immune and inflammatory responses. We propose that DP1 activation by specific agonists may represent a strategy to control cutaneous inflammatory Th2-associated diseases. *The Journal of Immunology*, 2004, 172: 3822–3829.

Dendritic cells (DCs)⁴ are highly specialized professional APCs involved in the induction of tolerance, priming, and chronic inflammation (1, 2). Among them, epidermal Langerhans cells (LCs) play a key role in cutaneous primary immune responses (1). Resident LCs display an immature phenotype characterized by high Ag uptake and processing abilities and poor T cell stimulatory function. During infections, injuries, or eczematous diseases inflammatory stimuli, such as LPS, TNF- α , or IL-1 β , promote the maturation of LCs and their migration via the lymphatic vessels to the regional lymph nodes, in which they localize in the T cell-rich paracortex to generate Ag-specific primary T cell responses (1–3).

In the past few years, a large number of studies have been devoted to elucidate the mechanisms involved in the control of LC emigration. In this process, adhesion molecules (4–6) as well as seven transmembrane-spanning G protein-coupled receptors play key roles (7). For instance, the chemokine receptor CCR7, which is up-regulated during LC-DC maturation (8), coordinates the accumulation and the organization of DCs and T cells in the lymph nodes in response to CC chemokine ligand (CCL)19 (also known as macrophage-inflammatory protein (MIP)-3 β) and CCL21 (secondary lymphoid-tissue chemokine) (9). More recently, it has been suggested that the balance between pro- and anti-inflammatory cytokines (10–12) as well as the local synthesis of lipidic mediators, including eicosanoids, play key regulatory functions in the control of LC mobility. For instance cysteinyl leukotriene C₄ and PGE₂ potentiate chemokine-driven DC migration (13–15), whereas PGD₂ inhibits LC trafficking to the draining lymph nodes (DLNs), at least in mice (16). We have shown that this inhibitory effect is mediated by the activation of the D prostanoid receptor 1 (DP1), but not via DP2 (also known as chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes), a newly described membrane-bound PGD₂ receptor involved in the recruitment of effector cells to inflammatory sites (17, 18). Conversely, to ensure cell homeostasis, migratory LCs/DCs are replaced by cell precursors recruited from the circulation into the skin. Under inflammatory conditions, CCR2, and its ligand CCL2 (monocyte chemoattractant protein 1), participates in the trafficking of LC precursors into the skin (19), whereas CCL20 (MIP-3 α), via CCR6 activation, seems to attract immature DCs into the dermis of inflamed skin (20).

The balance between newly recruited and emigrating LCs/DCs may be disrupted during chronic inflammation such as in atopic dermatitis (AD). Indeed, this Th2-related inflammatory reaction is characterized by an increased number of dermal DCs and also by a predominant skin infiltration of Th2 cells and eosinophils (21,

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⁴ Abbreviations used in this paper: DC, dendritic cell; LC, Langerhans cell; CCL, CC chemokine ligand; MIP, macrophage-inflammatory protein; DLN, draining lymph node; DP, D prostanoid receptor; AD, atopic dermatitis; CXCL, CXC chemokine ligand.

22). Interestingly enough, mature LCs/DCs retained at the inflammatory site may contribute to the recruitment of effector cells by their capacity to produce cytokines and chemokines. For instance, the production of CCL22 (macrophage-derived chemokine) by dermal DCs in AD-like skin lesions of NC/Nga mice (23) and skin lesions from patients with AD (24) was reported to participate in the recruitment of Th2/Tc2 CCR4⁺ lymphocytes (25, 26). Thus, besides their role in the priming of the immune response, LCs/DCs are also involved in skin pathophysiology by maintaining and/or amplifying the local inflammatory reaction (27).

In this study, before investigating the effect of DP1 activation on immune and allergic inflammatory responses in mice, we first aimed at confirming the inhibitory effect of DP1 on LC migration in humans. We show that DP1 activation impairs the TNF- α -induced LC emigration from human skin explants as well as their responsiveness to chemokines. We next analyzed *in vivo* the consequences of DP1 activation in a murine model of AD elicited by repeated sensitization with OVA (21). We show that mice treated topically with BW245C, a potent DP1 agonist, during the sensitization phases developed a dramatic reduction of skin lesions that was associated with an alteration of the local Th1/Th2 balance. We propose that DP1 activation may represent the basis of a new therapeutic approach to control inflammatory Th2-dependent skin diseases.

Materials and Methods

Reagents and Abs

All reagents were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France) unless otherwise notified. BW245C and PGD₂ were from Cayman Chemicals (Ann Arbor, MI) and CFSE from Molecular Probes (Eugene, OR). The anti-I-A^d/I-E^d mAbs (clone M5/114, rat IgG2b) was kindly provided by Dr A. Ager (National Institute of Medical Research, London, U.K.). The anti-mouse CD90.2 and the anti-human CD45 FITC-conjugated mAbs were from BD PharMingen (San Diego, CA). PE-conjugated mAbs included anti-human CD14 (BD PharMingen), anti-human CCR6 (R&D Systems, Abingdon, U.K.), and KJ1-26 (clonotypic OVA TCR; Caltag Laboratories, Burlingame, CA). The PerCP-labeled anti-human CD34 and the CyChrome-labeled anti-HLA-DR and CD1a mAbs were from BD Biosciences (Mountain View, CA). The allophycocyanin-labeled anti-murine CD4 was from BD PharMingen. The purified anti-human E-cadherin mAb was purchased from Immunotech (Marseille, France). The following were used as secondary Abs: biotin-conjugated anti-mouse IgM (BD PharMingen), anti-rat IgG whole molecule (Jackson ImmunoResearch Laboratories, West Grove, PA), PE-labeled anti-mouse IgG1, and anti-FITC peroxidase-conjugated (Boehringer Mannheim, Mannheim, Germany). The streptavidin-PE was purchased from Southern Biotechnology Associates (Birmingham, AL). Isotype control mAbs included purified, FITC-, PE-, CyChrome-, and PerCP-labeled mouse IgG1 or IgG2a (BD Biosciences). For immunohistochemical analysis, the biotinylated reagents were detected using ABC complex HRP (DAKO, Carpinteria, CA). Recombinant GM-CSF and recombinant Flt3-ligand were from Novartis (Basel, Switzerland) and Serotec (Cergy Saint-Christophe, France), respectively. Recombinant human TNF- α , CCL19, and CCL20 were obtained from R&D Systems.

Mice

Female BALB/c mice (6- to 8-wk-old) were purchased from Iffa-Credo (L'Arbresle, France) and maintained under special pathogen-free conditions in our animal facilities. The OVA-TCR transgenic mice (DO11-10) (28) were bred at the Erasmus Medical Center (Rotterdam, The Netherlands).

Human skin explant assay

Skin explants were obtained, after informed consent, from individuals undergoing plastic surgery (29). Each explant was trimmed to 1 cm² and four explants were floated, dermal side down, in 4 ml of RPMI 1640 supplemented with 25 mM HEPES, 10% FCS, and gentamicin (50 μ g/ml). Human explants were intradermally injected with 100 μ l of BW245C (10 μ M) or vehicle (DMSO) and 15 min later with 100 μ l of human recombinant TNF- α (50 ng). To determine the number of LCs that emigrated into the culture medium 24 h after incubation at 37°C in a 5% CO₂ incubator, cells were recovered and were triple-stained with anti-CD45-FITC, anti-HLA-DR-CyChrome and anti-CD1a-PE. The percentage of

CD45⁺, HLA-DR⁺, CD1a⁺ was determined on a FACSCalibur flow cytometer (BD Biosciences).

In vitro generation of human LCs and chemotaxis assay

LC precursors and mature LCs were obtained from CD34⁺ cord blood mononuclear cells (CD34 Multisort kit; Miltenyi Biotec, Auburn, CA) as described (30, 31). After 6 (LC precursors) or 14 days (mature LCs) of culture in RPMI 1640 plus 10% FCS, 200 U/ml GM-CSF, 50 U/ml TNF- α , and 50 ng/ml, Flt3-ligand cells were used for chemotaxis assays (31, 32). Cells were resuspended in the migration buffer (HBSS, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.1% BSA) at a density of 2–3 $\times$ 10⁶ cells/ml. Chemokine solution (600 μ l) or buffer alone were added to individual wells of 24-well plates (Costar, Cambridge, MA) on ice. Immediately thereafter, transwell devices with 5- μ m pore size (polycarbonate membranes) were inserted into the wells, and a 100- μ l cell suspension, pretreated or not with increasing doses of PGD₂ or BW245C 15 min before, was layered on top of the membrane. After a 2-h incubation at 37°C, nonmembrane-bound, transmigrated cells were recovered (i.e., 600 μ l) and were pooled with the eluates obtained after two washes of the same well with 200 μ l of HBSS. Thereafter, 10% of the volume (i.e., 100 μ l) of individual samples was subjected to cell enumeration on a FACScan (BD Biosciences), and the cells recovered from the remaining 90% of the volume were double-stained with anti-CD1a-FITC and CD14-PE (cells recovered from day 6 of culture) or with anti-CD1a-FITC and E-cadherin/PE-labeled anti-mouse IgG (cells recovered from days 13 and 14) and analyzed by flow cytometry.

Flow cytometry analysis

For two- or three-color immunolabeling, cells were washed twice in ice-cold PBS and incubated in 50 μ l of PBS containing the appropriate purified (un)labeled mAbs (2.5 μ g/ml each) for 30 min on ice. For the detection of unlabeled murine anti-human mAbs, cells were further exposed to anti-mouse-PE or anti-biotinylated mAbs followed by streptavidin-PE. At least 10,000 cells were analyzed on a FACSCalibur flow cytometer. For intracellular staining (anti-DP Abs), cells were fixed in PBS plus 4% paraformaldehyde and permeabilized in PBS plus 1% BSA/0.1% saponin. For analysis of CFSE-labeled DO11.10 T cells, lymph node cells were stained with anti-CD4-allophycocyanin and KJ1-26 PE. Propidium iodide was added to every staining combination for exclusion of dead cells, immediately before FACS analysis. Final analysis and graphical output were performed using FlowJo software (Treestar, Costa Mesa, CA).

OVA sensitization and determination of LC density

Epicutaneous sensitization of mice was performed as previously described (21). Briefly, mice have their backs shaved with electric clippers and their abdomens with razor blade. One day later (day 0), 25 μ l of OVA (grade V, 2 mg/ml) in PBS or PBS alone was placed on two patches of sterile gauze (0.7 cm²), which were secured to the abdominal skin with a transparent bio-occlusive dressing. In certain cases, mice were treated topically with 25 μ l of BW245C (50 μ M) or vehicle (DMSO) 15 min before the sensitization. After 24 h, the density of MHC class II⁺ cells in the epidermis was determined as described (16).

Determination of T cell proliferation in the DLNs

Because the frequency of OVA-specific T cells is very low in immunized animal, the primary activation of a naive T cell is difficult to detect. To avoid this problem, naive T cells purified from DO11.10 mice were adoptively transferred into BALB/c mice. Briefly, pooled peripheral lymph nodes (inguinal and mesenteric) and the spleen were harvested from DO11.10 mice, homogenized and after red cell lysis, cell suspensions were labeled with CFSE as previously described (33). Viable cells were enumerated by trypan blue exclusion before their transfer into recipient mice. Two days before the first sensitization, each recipient mouse received 10 $\times$ 10⁶ labeled cells *i.v.* via the lateral tail vein. At day 0 (first sensitization) and day 3 (second sensitization), mice were topically treated with BW245C (50 μ M) or vehicle 15 min before the sensitization with 25 μ l of OVA (2 mg/ml in PBS) or PBS. To determine the proliferation of CFSE-labeled OVA-specific T cells in the DLNs, mice were sacrificed 2 days after the second sensitization.

Elicitation of AD

To elicit AD, the patches were placed for 1 week with one impregnation at day 5 and then removed. Two weeks later, two identical patches were reapplied to the same skin site. Each mouse had a total of three 1-wk exposures to the patch separated from each other by 2-wk intervals. Mice were treated topically with 25 μ l of BW245C (50 μ M) or vehicle (DMSO) 15 min before the sensitizations.

mRNA extraction and RT-PCR

Skin from mice was excised, immediately frozen in dry ice and total RNA was isolated using TRIzol reagent (Invitrogen, Cergy Pontoise, France). cDNA was synthesized from 1 μ g of total RNA with random hexamer primers and Superscript reverse transcriptase (Promega, Madison, WI) using standard procedures. Semiquantitative PCR amplifications were performed with the primer pairs listed in Table I. Amplified products were subjected to 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Histological and immunochemical analysis

Skin biopsies were obtained 24 h after the third sensitization. Specimens were fixed in a formaldehyde-free zinc fixative (ImmunoHistoFix; Interstiles, Brussels, Belgium) for 7 days at 4°C. After dehydration in graded alcohol baths, embedding was performed by three successive immersions in ImmunoHistoWax (Interstiles) at 37°C. Sections of 5- μ m thickness were dewaxed in acetone for 5 min and stained with H&E or May-Grunwald-Giemsa or toluidine blue. For immunohistochemical analysis, sections were immunostained with anti-I-A^d/I-E^d or anti-CD90.2 mAbs as described (16). For each section, 10 random fields were examined at $\times 630$. Cell frequency was converted to cells per square millimeter and results were expressed as mean $\pm$ SD.

Statistical analysis

For all experiments, the statistical significance of differences between experimental groups was calculated using the Student *t* test excepted for the human skin explant assay for which the statistical significance of differences was determined using the Wilcoxon signed rank test for paired groups.

Results

DP1 activation impairs the migration of human LCs

Using an ex vivo approach based on the migration of LCs from skin explants (34), we first investigated whether DP1 activation may affect the motility of human LCs. For this purpose, skin explants from healthy donors were injected intradermally with the specific DP1 agonist BW245C 15 min before TNF- α , a factor known to promote a strong LC departure from the epidermis (35). The number of emigrated LCs (CD45⁺, HLA-DR⁺, CD1a⁺) was then determined 24 h after TNF- α injection by flow cytometry. After a preliminary dose-response study (data not shown), pre-

treatment of skin explants with BW245C (optimal dose, 10 μ M) impaired the TNF- α -induced emigration of LCs ($45 \pm 20\%$ inhibition, mean of five independent experiments) (Fig. 1A). These results confirm that in humans, DP1 activation is efficient in reducing the TNF- α -induced migration of LCs from the epidermis.

We next investigated whether DP1 activation may also affect the chemokine-driven motility of human LCs in vitro. After checking that neither PGD₂ nor BW245C exerts chemotactic or chemokinetic effects on LCs (data not shown), we first analyzed the effect of PGD₂ and BW245C on the CCL20-induced migration of LC precursors, as assessed by transwell chemotaxis assay. After a preliminary dose-response experiment (data not shown), we found that PGD₂ and BW245C (10 μ M, optimal dose) inhibited the chemotactic response of LC precursors to CCL20 by 52% and 60%, respectively (compared with vehicle, DMSO) (Fig. 1B). Similarly, the CCL19-driven migration of mature LCs was reduced by PGD₂ (35%) and particularly by BW245C (61%). Flow cytometric analysis revealed that the decreased responsiveness to CCL20 and CCL19 induced by PGD₂ and BW245C was not associated with a reduced expression of the corresponding receptor CCR6 and CCR7, respectively (data not shown). These findings indicate that DP1 activation inhibits the chemotactic response of LC precursors and of mature LCs, suggesting a role for DP1 in the control of human LC homeostasis.

DP1 activation inhibits LC migration and affects the proliferation of Ag-specific T cells in the DLNs after OVA sensitization

Because DP1 activation is important in controlling LC migration, we next hypothesized that it may also impact on the development of the primary immune response elicited by epicutaneous sensitization of OVA in mice. First, we verified that in this model, topical treatment with BW245C inhibited the emigration of LCs from the epidermis. To this end, the frequency of MHC class II⁺ cells was determined in the epidermis 24 h after the sensitization. As seen in Fig. 2A, sensitization with OVA resulted in a decreased number of epidermal LCs. By contrast, treatment of mice with BW245C did

Table I. PCR primers used for amplification^a

Gene	Primer	Sequence	Size (bp)	Cycle
IL-4	5'	5'-GAATGTACCAGGAGCCATATC	384	38
	3'	5'-CTCAGTACTACGAGTAATCCA		
CCL11	5'	5'-ATTCCTGCTGCTCAGGTC	306	28
	3'	5'-GAATCAAGCAGTTCTTAGGC		
CCL22	5'	5'-ATGGCTACCCTGCGTGTCCC	219	32
	3'	5'-CTAGGACAGTTTATGGAGTA		
CCL17	5'	5'-TCCTATCAGGAAGTTGGTGAGCT	306	32
	3'	5'-TGTGTTTCGCTGTAGTGCATAAG		
CCR3	5'	5'-GCTGACAATTGACAGATACC	306	28
	3'	5'-TCAGCAGAGTTTAAATGATTCC		
CCR4	5'	5'-TCTACAGCGGCATCTTCTTCAT	199	28
	3'	5'-CAGTACGTGTGGTTGTGCTCT		
IFN- γ	5'	5'-GTCTGAAGTACTATTTAACTCAAG	278	32
	3'	5'-GTGGGTTGTTTCGACCTCGTAACCTGGC		
CXCL10	5'	5'-CCTATCCTGCCCCAGTGTG	431	28
	3'	5'-CGCACCTCCACATAGCTTACA		
CXCR3	5'	5'-TGCCACCCATTGCCAGTACAAC	489	32
	3'	5'-TCAGTTGTCTCAGACCAGGATGA		
CCR5	5'	5'-ACTGCCAACAGGGAAGAACCACT	551	32
	3'	5'-GAAGCCATCTGGAAGTGTGACT		
IL-10	5'	5'-TCCTTAATGCAGGACTTTAAGGGTTAC	246	34
	3'	5'-GACACCTTGGTCTTGGAGCTTATTA		
β -Actin	5'	5'-GTCGGCGCCCGGACCA	539	28
	3'	5'-CTCCTTAATGTACGCACGATTC		

^a Semiquantitative PCR primers, product sizes, and PCR cycle numbers.

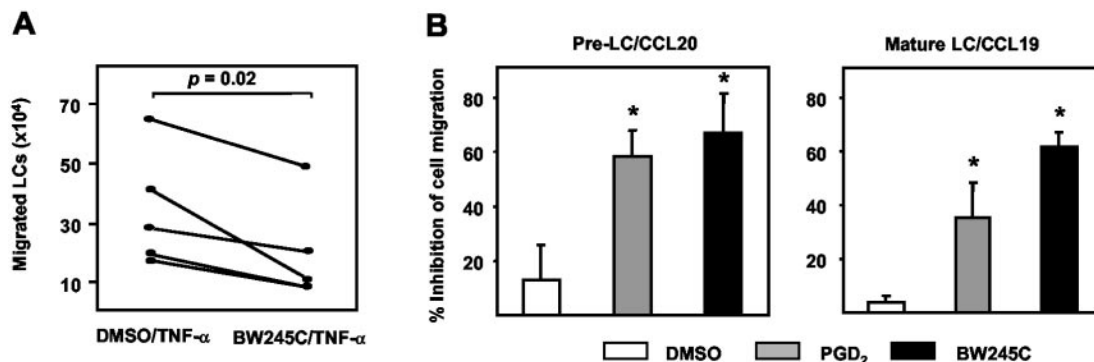


FIGURE 1. A, Inhibition of the TNF- α -induced migration of human LCs from skin explants by BW245C. Explants of human skin were pretreated with 10 μ M BW245C or vehicle. After 15 min, explants were treated with 50 ng of TNF- α and were floated dermal side down in culture medium. The number of LCs that emigrated into the cultured medium after 24 h incubation was quantified by triple-staining (CD45⁺, HLA-DR⁺, CD1a⁺) by FACS analysis. Data points represent values for each individual donor. Significant difference is indicated. B, Impairment of chemotactic responses of LC precursors and mature LCs by DP1 activation. Pre-LCs (CD14⁻, CD1a⁺) or mature LCs (E-cadherin⁺, CD1a⁺) were treated with vehicle (DMSO), PGD₂, or BW245C (10 μ M) for 15 min before being subjected to the chemotaxis assay. After 2 h at 37°C, cells that transmigrated into the lower compartment were harvested and stained with anti-CD1a-FITC and anti-CD14-PE mAbs or anti-CD1a-FITC and anti-E-cadherin followed by anti-mouse IgG-PE mAbs. Results are given as the mean percentage of inhibition of cell migration compared with untreated cells (six experiments performed). Cell viability was not altered by the indicated treatments (data not shown). *, Statistical significance with $p < 0.01$ in Student's t test (compared with DMSO).

not result in a significant reduction in the number of LCs/mm², compared with the control.

We next investigated whether this defected LC migration affects the activation of Ag-specific T cells in the DLNs of mice epicutaneously sensitized with OVA. Because the precursor frequency of Ag-specific T cells is low in naive BALB/c mice, CFSE-labeled CD4⁺ T cells from DO11.10 mice were adoptively transferred into naive syngeneic mice 2 days before the sensitization and their proliferation was studied 5 days after the first sensitization. As expected, flow cytometry analysis (CFSE⁺, CD4⁺, KJ1-26⁺) of inguinal lymph nodes showed that no T cell division had occurred in the absence of sensitization (Fig. 2B). On the contrary, in BW245C- and vehicle-treated sensitized mice, dividing cells were seen and had undergone up to six divisions 5 days after OVA sensitization. However, in BW245C-treated mice, the size of the T cell pool dividing in response to OVA was reduced. Indeed, within the OVA-reactive population (CFSE⁺, CD4⁺, KJ1-26⁺), 52.9 $\pm$

5.7% of cells demonstrated a CFSE profile consistent with at least 1 division in mice that received OVA/DMSO vs 35.5 $\pm$ 4.5% of cells in the group that received OVA/BW245C (33% reduction, $p < 0.01$). These data demonstrate that upon epicutaneous sensitization with OVA, topical application of BW245C reduces the migration of LCs and diminishes the number of OVA-specific activated T cells in the DLNs.

DP1 activation reduces the skin inflammatory response in a model of AD

We next investigated whether the effects exerted by BW245C treatment on T cell priming induced by OVA could affect the pathology in a model of allergic skin inflammation elicited by repeated epicutaneous sensitizations with OVA. For this purpose, BW245C was topically applied during the sensitization phases and the inflammatory response in the skin was examined 24 h after the

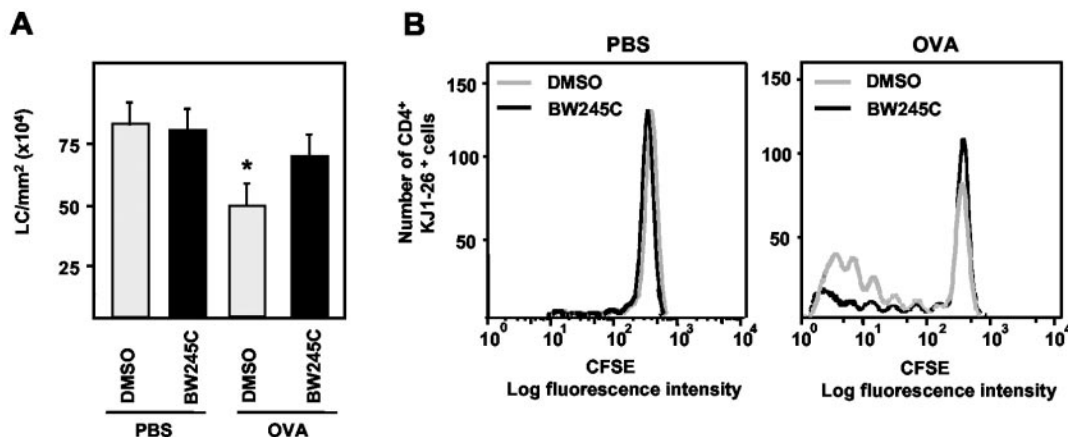


FIGURE 2. Effect of topical treatment with BW245C on the local responses induced by OVA sensitization. A, Epidermal sheets were prepared 24 h after the sensitization and the number of LC/mm² was determined after anti-MHC class II staining. Results are expressed as means $\pm$ SD and are representative of three independent experiments ($n = 4$). *, Significant difference ($p < 0.01$). B, Effect of BW245C on the proliferation of CFSE-labeled OVA-specific T cells following epicutaneous sensitization with OVA. Two days before the sensitization, CFSE-labeled T cells from OVA TCR transgenic mice were adoptively transferred to recipient mice (six mice per group). On days 0 and 3, mice received topical application of BW245C (50 μ M) or vehicle 15 min before each sensitization. Lymph node cells were taken on day 5 and T cells were analyzed by FACS according to their scatter characteristics, expression of CD4, OVA-TCR, and propidium iodide exclusion. Cell division in T cells corresponds to sequential halving of CFSE fluorescent (x-axis) and cell number is reported on y-axis.

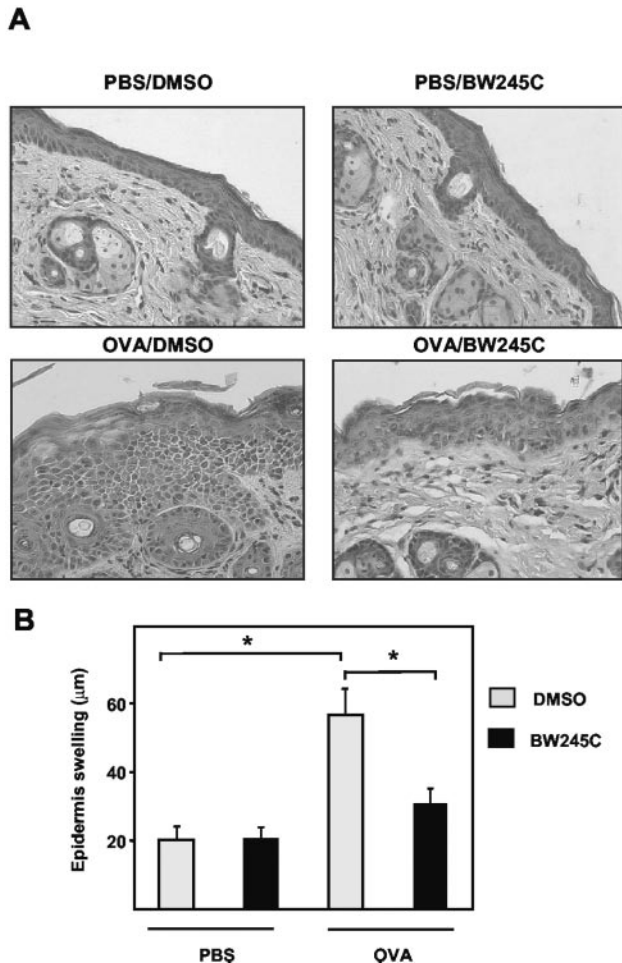


FIGURE 3. Improvement of AD skin lesions in BW245C-treated OVA-sensitized mice. Fifteen minutes before each sensitization, mice received topical application of BW245C (50 μ M) or DMSO. Skin biopsies were taken 24 h after the last sensitization after which skin sections were stained with H&E (magnification, $\times 200$) for histopathological analysis (A) and epidermal thickness measurement (B). These data are representative of three experiments (15 mice per group). *, Significant differences ($p < 0.01$).

last sensitization. In vehicle-treated sensitized mice, the histopathologic analysis revealed a dramatic hyperkeratosis accompanied by desquamation and marked spongiosis, typical of AD lesions, which was absent in unsensitized mice (Fig. 3A). Compared with unsensitized mice, epidermal thickening at the inflammatory site was increased 2.8-fold in vehicle-treated sensitized mice. In sharp contrast, these symptoms were considerably less pronounced in BW245C-treated OVA-sensitized mice in which the epidermal thickening was reduced by 72% ($p < 0.01$) (Fig.

3B). This shows that topical treatment with BW245C exerts marked inhibitory effect on inflammatory symptoms induced by OVA sensitization. Histological study also revealed that this effect was accompanied by a reduced cellular infiltration into the dermis. As shown in Table II, compared with unsensitized mice, OVA sensitization led to an increased number of mast cells, eosinophils, CD90.2⁺ cells (T cells), and I-A^d/I-Ed^{d+} cells. Strikingly, the infiltration of mast cells and eosinophils in the dermis was reduced by 40% and 48%, respectively, in BW245C-treated mice. In addition, BW245C also decreased the number of recruited T cells (by 43%) and I-A^d/I-Ed^{d+} cells (by 77%) in sensitized mice. These data indicate that the topical application of BW245C during the sensitization phases strongly limits the cellular infiltrate elicited by OVA and dramatically improves skin lesions present in chronic AD.

DP1 activation decreases the expression of mRNAs for Th2-related molecules and increases those for Th1 markers in the skin of OVA-sensitized mice

The local production of Th2-type cytokines, chemokines, and chemokine receptors in the skin actively participates in the pathology in AD. We first examined by RT-PCR the presence of mRNAs for Th2-associated molecules in the skin of sensitized mice. As shown in Fig. 4, an increase in IL-4, CCL11 (eotaxin), CCL22, and CCL17 (thymus and activation-regulated chemokine, TARC) mRNA levels was detected in the skin from vehicle-treated OVA-sensitized mice compared with unsensitized mice. By contrast, a marked down-regulation of these molecules was observed in BW245C-treated OVA-sensitized mice. These results show that BW245C decreases the expression of IL-4, a cytokine involved in the development of AD, and chemokines known to be important in the recruitment of effector cells. Similarly, the levels of mRNAs for CCR3 (the CCL11 receptor) and CCR4 (the CCL17 and CCL22 receptor), some chemokine receptors known to be preferentially expressed on type 2 cells, were reduced after BW245C treatment, in comparison with vehicle-treated sensitized mice. Furthermore, analysis of Th1-associated molecules revealed a weak expression of IFN- γ and CXC chemokine ligand (CXCL)10 (IFN- γ -induced protein 10) mRNAs in the skin of vehicle-treated OVA-sensitized mice. Interestingly, the expression of these markers was markedly up-regulated by BW245C treatment in OVA-sensitized mice. In addition, compared with vehicle-treated OVA-sensitized mice, a strong increase was also observed for CXCR3 (the receptor for CXCL10) and CCR5, both chemokine receptors known to be preferentially expressed on Th1 cells. These data strongly suggest that BW245C improves the AD skin lesions by altering locally the Th1/Th2 balance. Finally, we investigated whether this phenomenon was associated with the expression of the immunoregulatory cytokine IL-10. Interestingly, IL-10 mRNA expression was strongly increased in sensitized mice following BW245C treatment.

Table II. Quantification of the cellular infiltration in the dermis^a

Cells/mm ²	PBS/DMSO	PBS/BW245C	OVA/DMSO ^b	OVA/BW245C ^c
Mast cells	72.9 $\pm$ 14.8	79.3 $\pm$ 11.7	136.8 $\pm$ 14.3	117.4 $\pm$ 19.3
Eosinophils	61.1 $\pm$ 9.5	67.4 $\pm$ 5.5	190.8 $\pm$ 39	133.8 $\pm$ 26.6
CD90.2 ⁺ cells	67.4 $\pm$ 5.5	87.2 $\pm$ 11.2	280.8 $\pm$ 17.8	206.3 $\pm$ 20.7
I-A ^d /I-Ed ^{d+} cells	400.8 $\pm$ 39.3	484.2 $\pm$ 20.2	808.1 $\pm$ 62.3	574.4 $\pm$ 20.3

^a Mean values are cells per mm² from 10 random fields ($\pm$ SD). Biopsies were taken 24 h after the last sensitization. These data are representative of three experiments ($n = 15$).

^b $p < 0.001$ (PBS-sensitized vs OVA-sensitized).

^c $p < 0.005$ (DMSO-treated OVA-sensitized vs BW245C-treated OVA-sensitized).

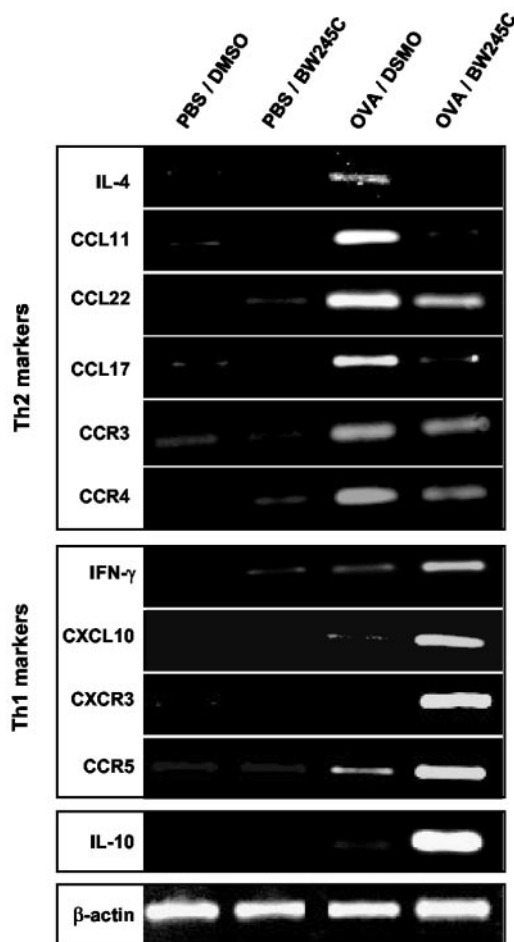


FIGURE 4. RT-PCR analysis of mRNAs specific for cytokines, chemokines, and chemokine receptors in the skin. Skin biopsies were taken 24 h after the last sensitization, total RNA was extracted, and RT-PCR was conducted using the primers shown in Table I. Representative results of three independent experiments are shown.

Discussion

The polarization of the primary immune response toward a Th1 or Th2 profile is important in conferring protection against infectious pathogens or tumors but may also lead to immunopathology (1, 2). For example, allergic diseases such as asthma or AD are associated with a predominant Th2 response to environmental allergens. This response is characterized by the infiltration of activated Th2 cells, monocyte-derived DCs, eosinophils, and mast cells in the inflamed tissue and by the production of Th2-associated isotype Abs, including IgE. Accumulating evidence suggests that DCs play a pivotal role not only in the induction but also in the maintenance of Th2 allergic responses (36). Therefore, agents able to alter DC functions, for instance their migratory properties, might impact on the outcome of chronic inflammatory responses associated with allergic diseases.

We have previously shown that DP1 activation impedes, under inflammatory conditions, the emigration of murine LCs from the epidermis to the DLNs (16). More recently, we have also demonstrated that DP1 is important in the control of airway DC migration in steady-state conditions (37), indicating that this inhibitory effect is not only operative in the skin but also in other epithelial/mucosal sites, including the lungs. However, the role of DP1 in human LCs/DCs migration has not been investigated so far. In this study, we first demonstrate that DP1 activation induces a severe reduction of the TNF- α -induced migration of human LCs from cultured skin

explants. Moreover, the chemotactic response of maturing CD34⁺-derived human LCs to CCL19 is profoundly inhibited by the DP1 agonist BW245C *in vitro*. This suggests that in humans, DP1 activation, by down-regulating the TNF- α induced and the chemokine-driven migration of LCs, may play an important regulatory role in primary immune responses. We next investigated, in mice, whether DP1 activation could affect the Ag-specific T cell priming in the skin DLNs, after epicutaneous sensitization with OVA. Indeed, alteration in DC migration may induce abnormal primary immune response by affecting the kinetic and/or the intensity of the response (38, 39). Although Ag-specific T cells that were activated in the DLNs from BW245C-treated OVA-sensitized mice underwent similar rounds of division than those from vehicle-treated sensitized mice, the number of those activated CD4⁺, KJ1-26⁺ T cells to proliferate was markedly decreased in BW245C-treated mice. This was neither due to enhanced migration of divided effector cells to the periphery nor to a direct effect of BW245C on T cell division, as assessed by *in vitro* experiments (data not shown). This indicates that DP1 activation at early time points after sensitization decreased the magnitude of OVA-specific naive T cell activation in the DLNs. Unfortunately enough, the lack of sensitivity of the model did not allow us to investigate the impact of BW245C treatment on the synthesis of Th1/Th2 cytokines by OVA-specific T cells in the DLNs, for instance by intracellular cytokine staining of KJ1-26⁺ cells. There are several possible explanations for this decreased activation of T cells in the DLNs. First, the defect in LC migration from the periphery to the DLNs may lead to a reduced intensity of Ag presentation that has been demonstrated to affect the magnitude of T cell activation (40). Second, DP1 activation may influence the type of effector and memory T cells generated by affecting the kinetics of DC arrival in the site of Ag presentation, as suggested by Langenkamp et al. (41). Finally, we cannot rule out the possibility that the effect observed on T cell activation is also due to an alteration of the APC function of DCs. The recent finding that BW245C modulates the synthesis of costimulatory molecules and immunoregulatory cytokines by maturing DCs supports this assumption (42).

Newly generated Ag-specific effector/memory T cells leave the DLNs to reach the site in which foreign Ag was originally encountered. At this site, depending on the environment, activated T cells can amplify the local inflammation through cytokine production. Having demonstrated that DP1 activation decreases OVA-specific T cell activation, we assessed whether this event could, at least in part, impact on the development of allergic skin inflammation elicited by repeated OVA sensitizations. We show that BW245C treatment during the sensitization phase strongly prevents the skin disorders, 24 h after the last sensitization. As assessed by histological analysis, this beneficial effect of BW245C on the pathology was accompanied by a decreased cellular recruitment in the skin. For instance, the number of dermal eosinophils and mast cells, two major effector cell populations involved in the inflammatory response of AD, was reduced. Moreover, compared with controls, the number of I-A^d/I-Ed^d cells was diminished in BW245C-treated mice. Of note, as suggested by Fig. 2A, this later phenomenon may be due to the effect of BW245C on the decreased responsiveness of blood-derived DC precursors to chemokines, such as CCL20. Finally, the infiltration of dermal T cells was strongly impaired in BW245C-treated mice. Although further studies are warranted to determine their phenotype, the decreased expression of mRNAs for CCL11, CCL17, and CCL22, some chemokines preferentially associated to Th2 lymphocyte infiltration (24, 43, 44), in the skin lesions of BW245-treated mice suggests a reduced recruitment of Th2 cells in these mice. The reduced levels for CCR3, CCR4, and IL-4 mRNAs in skin lesions from BW245C-treated mice support this later hypothesis. Conversely, DP1 activation appeared to promote the

recruitment of Th1 cells, as suggested by the increased expression of mRNAs for IFN- γ , CCR5, CXCR3 and its cognate ligand, CXCL10 in the skin of BW245C-treated mice. As previously stated, this difference in the Th1/Th2 balance in the skin may be due to an altered T cell priming in the DLNs ("primary polarization"). It may also be due to a direct effect of BW245C on the local production of chemokines implicated in T cell trafficking by resident or newly recruited skin cells, including DC themselves, which are known to be good producers of CCL22, CCL17, and CXCL10 (24, 45–48). Moreover, we cannot exclude the possibility that through DP1 activation, LCs/DCs may impact on the pattern of cytokines produced by memory T cells in the skin ("secondary polarization"). Altogether, improvement of skin lesions in this model of AD following BW245C treatment is coincident with a disruption of the local Th1/Th2 balance. Additionally, a moderate albeit significant decrease of the Th2 humoral response (namely serum OVA-specific IgG1 and IgE) was also observed in BW245C-treated mice (data not shown). Interestingly, the dramatic increase of mRNA corresponding to the immunoregulatory cytokine IL-10 may explain this Th2 to Th1 shift of the immune response. Although keratinocytes may be involved in its synthesis (49), it is possible that newly recruited T cells, particularly T regulatory cells, may play a part in IL-10 production (50). T regulatory cells, by restoring the balance of the immune system, are considered to be important players in the prevention and treatment of allergic diseases (51, 52). Future experiments will be aimed at more fully appreciating the role of IL-10 in the reduced skin inflammation induced by BW245C as well as at determining the involvement of T regulatory cells in these processes.

Collectively, our results demonstrate that mice locally treated with BW245C during the sensitization phases develop reduced skin disorders in this model of AD. These data differ from that provided by Matsuoka et al. (53), who recently showed in a model of murine asthma that DP1 is rather implicated in the promotion of inflammatory reactions. This apparently conflicting result can be explained by differences in the sensitization protocols (epicutaneous application vs i.p. injection), in the approaches used to study the role of DP1 (local activation by a specific agonist vs systemic deactivation by a gene deletion), and in differences of targeted organs (skin vs lungs). Our data however support the concept that certain PGs, including PGD₂, possess important regulatory functions during inflammation (54–58).

In summary, we show that after epicutaneous sensitization with OVA, DP1 activation inhibits the migration of LCs and affects the priming of Ag-specific T cells in the DLNs. Moreover, after repeated epicutaneous sensitizations with OVA (AD model), DP1 activation reduces the infiltration of inflammatory cells and modifies the Th1/Th2 balance in the skin, these effects being associated with a striking improvement in the skin pathology. To our knowledge, this report is the first to show that topical treatment with an agent capable to inhibit LC emigration can profoundly prevent the development of chronic Th2-type inflammatory disease. Because DP1 is also functional in human LCs, these findings may have important therapeutic consequences to control skin allergic disorders.

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