Potential Role of the Chemokine Receptors CXCR3, CCR4, and the Integrin αEβ7 in the Pathogenesis of Psoriasis Vulgaris

James B. Rottman, Tammy L. Smith, Kenneth G. Ganley, Toyoko Kikuchi, and James G. Krueger

Millennium Pharmaceuticals (JBR, TLS, KGG), Cambridge, Massachusetts; and The Rockefeller University (TK, JGK), New York, New York

SUMMARY: Various adhesion molecules have been implicated in T lymphocyte binding to dermal vascular endothelium in psoriasis vulgaris, but the chemotactic signals that promote subsequent homing into the adjacent dermis and overlying epidermis are poorly defined. We studied chemokine receptor (CCR1–CCR5, CXCR1–CXCR3), chemokine (interferon-γ inducible protein 10 [IP-10]), monokine induced by interferon-γ (MIG), thymus and activation-regulated chemokine (TARC), macrophage-derived chemokine (MDC), and adhesion molecule (cutaneous lymphocyte antigen [CLA], E-selectin, lymphocyte function-associated antigen-1 [LFA-1], intercellular adhesion molecule-1 [ICAM-1], very late antigen 4 [VLA-4], vascular cell adhesion molecule-1 [VCAM-1], αEβ7, and E-cadherin) expression in psoriasis by immunohistology, flow cytometry, and molecular techniques. CXCR3 and CCR4 were expressed by dermal CD3+ lymphocytes, and their chemokine ligands, IP-10, MIG, TARC, and MDC, were up-regulated in psoriatic lesions. Keratinocytes stimulated with tumor necrosis factor-α and interferon-γ up-regulated expression of IP-10, MIG, and MDC mRNA, whereas dermal endothelial cells, similarly stimulated, up-regulated expression of IP-10, MDC, and TARC mRNA, suggesting that these cell types were sources of the chemokines detected in biopsies. There was enhanced expression of E-selectin, CLA, LFA-1, ICAM-1, VLA-4, VCAM-1, and αEβ7 in psoriatic lesions versus nonlesional skin. Finally, intra-epidermal CLA+ and αEβ7+ T lymphocytes selectively expressed the chemokine receptor CXCR3. Collectively, these data suggest that CXCR3 and CCR4 may be involved in T lymphocyte trafficking to the psoriatic dermis and that CXCR3 is selectively involved in subsequent T cell homing to the overlying epidermis. (*Lab Invest 2001, 81:335–347*).

*P*soriasis vulgaris, a disease that affects up to 2.0% of the population, is characterized clinically by inflammation and scaling of affected skin areas. Microscopic lesions include epidermal hyperplasia, parakeratosis, intra-epidermal accumulations of neutrophils, and profound infiltration of the dermis and epidermis with mononuclear cells (MNCs), principally lymphocytes. Because the epidermal changes are so prominent, it was assumed for years that psoriasis was a disease of keratinocytes (Bos and De Rie, 1999). However, during the past two decades, convincing evidence has been presented that psoriasis is, in fact, an autoimmune disease mediated by T lymphocytes.

Phenotypic characterization of the T cell infiltrate has demonstrated that the majority of intra-epidermal T cells are CD8+ (Paukkonen et al, 1992), whereas the majority of T cells present within the dermis are CD4+ cells (Bos et al, 1989). Of the two cell types, the CD8+ intra-epidermal population may be the most important for mediating disease. For example, type I (familial) psoriasis has been linked to several major histocom-

patibility complex (MHC) class I haplotypes including human leukocyte antigen (HLA)-Cw6 and HLA-DR7 (Ikaheimo et al, 1996), and clonal expansion of CD8+ cells has been detected in the epidermis (Chang et al, 1994). Furthermore, the T cells frequently exhibit signs of activation including up-regulation of MHC class II molecules and expression of CD25, the interleukin-2 (IL-2) receptor (Ferenczi et al, 2000). It is also interesting to note that CD8+ T cells isolated from lesions predominantly produce TH1 cytokines including interferon-γ as well as IL-2 and tumor necrosis factor-α (TNF-α), but minimal TH2 cytokines such as interleukin-4 (IL-4) or interleukin-10 (IL-10) (Austin et al, 1999). As such, the CD8+ cytotoxic T cells isolated from psoriatic lesions have been called TC1 cells (equivalent to TH1 phenotype of CD4+ cells) (Austin et al, 1999). Collectively, these observations suggest that certain MHC class I molecules may present an epidermal autoantigen to CD8+ T cells resulting in activation and proliferation. To date, however, the putative epidermal autoantigen remains undefined.

There is additional evidence from clinical drug trials that implicates T cells in the pathogenesis of psoriasis. For example, drugs that modulate T cell function, such as cyclosporine (Baker et al, 1987), FK106 (Abu El-magd et al, 1991), or CD4 monoclonal antibodies (Rizova et al, 1994), have produced profound resolution of cutaneous lesions. In addition, treatment of
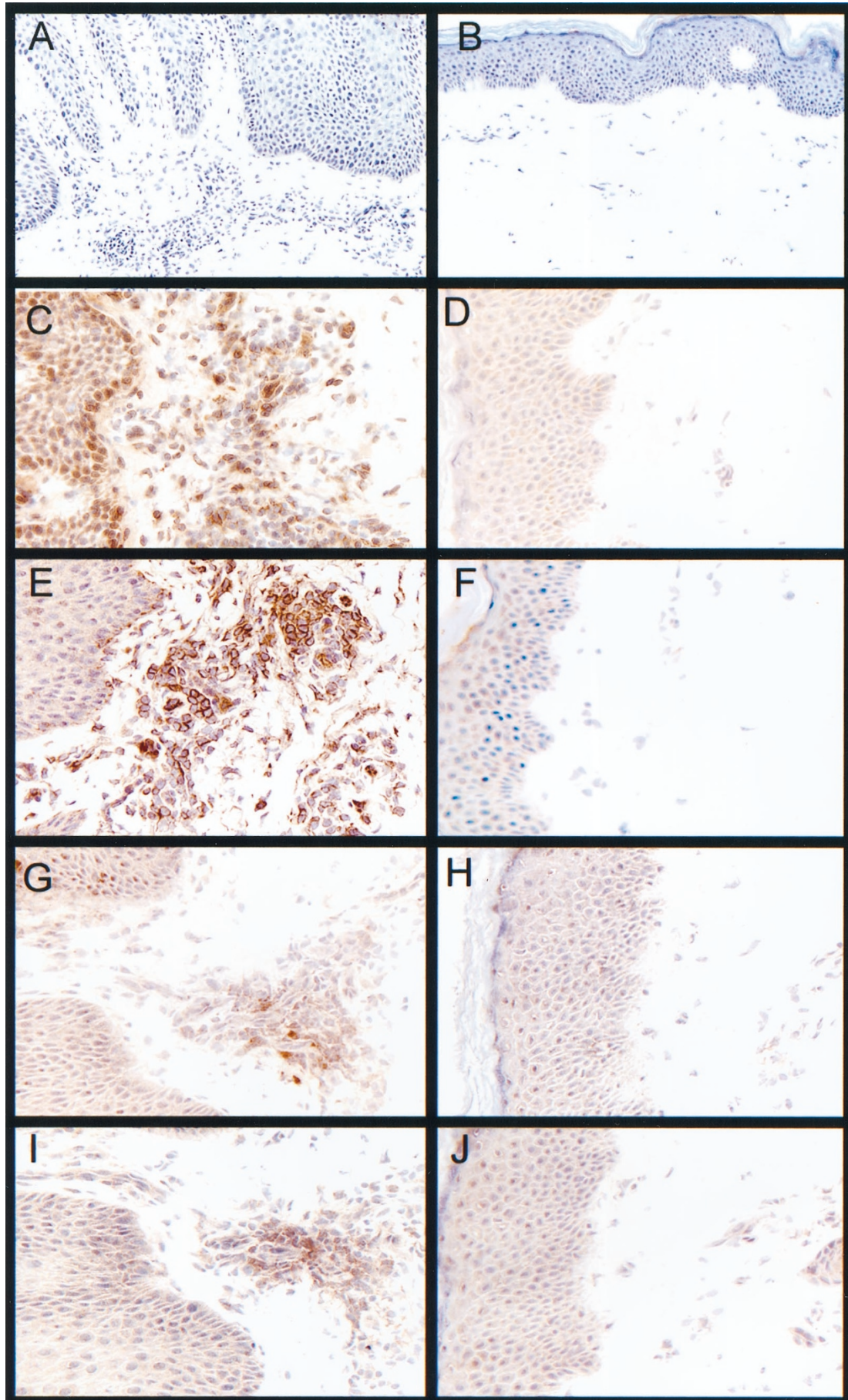
psoriasis patients with a chimeric CTLA-4 immuno-globulin (Ig) resulted in prominent resolution of lesions, thus demonstrating the importance of T cell co-stimulation in psoriasis (Abrams et al, 1999). Finally, patients treated with an IL-2 diphtheria toxin fusion protein (DAB$_{389}$IL-2), which selectively blocks the growth and proliferation of T cells but not keratino-cytes, also had striking clinical improvement, further implicating T cells in the pathogenesis of psoriasis (Gottlieb et al, 1995).

Although the immunomodulatory agents described above resulted in therapeutic benefit for patients, there were often prominent side effects. An alternative therapeutic approach may be to block T cell recruit-ment to the dermis/epidermis with antagonists of adhesion molecules and/or chemokine receptors. Ef-fective therapy of this nature requires an understand-ing of the recruitment pathways involved in lympho-cyte homing to the inflamed skin. This is the first comprehensive analysis of chemokine receptor, che-mokine, and adhesion molecule expression in biop-sies collected from patients with psoriasis. Here we demonstrate by immunohistology, immunofluores-cence, flow cytometry, ribonuclease protection assay, and reverse transcription-polymerase chain reaction (RT-PCR) that there is enhanced expression of the chemokine receptor/ligand pairs CXCR3/interferon-γ inducible protein 10 (IP-10), monokine induced by interferon-γ (MIG), CCR4/thymus and activation-regulated chemokine (TARC), and macrophage-derived chemokine (MDC) in lesional skin, suggesting that these molecules may mediate T cell trafficking to the psoriatic dermis. Furthermore, we demonstrate that intra-epidermal cutaneous lymphocyte antigen+ (CLA+) and αEβ7+ T cells prominently express the chemokine receptor CXCR3, suggesting that this re-ceptor may be responsible for subsequent trafficking of T cells from the dermis to the psoriatic epidermis.

Results

T Cells within the Psoriatic Dermis Express the Chemokine Receptors CXCR3 and CCR4

To identify pathways important for lymphocyte recruit-ment to inflamed skin, we began by studying chemo-kine receptor expression in skin biopsies from psori-asis patients. Frozen sections of lesions and nonlesional skin were stained ($n = 8$) for the receptors CCR1–5 and CXCR1–3. In all cases, lesional skin had microscopic changes consistent with psoriasis, in-cluding parakeratotic hyperkeratosis, prominent perivascular mononuclear cell infiltration, and numer-ous intra-epidermal mononuclear cells insinuated be-

tween keratinocytes (Fig. 1A). In contrast to lesional skin, the nonlesional biopsies had normal epidermal thickness and keratinocyte maturation sequence, and very few mononuclear cells were present in either the dermis or epidermis (Fig. 1B). CXCR3 was expressed by a significant number of infiltrating mononuclear cells in the dermis and a subset within the basal layer of the epidermis of psoriatic lesions (Fig. 1C). By two-color immunofluorescence, the majority of CXCR3+ cells were demonstrated to be CD3+ T cells (Fig. 2). In contrast to lesional skin, rare CXCR3+ cells were present within nonlesional skin (Fig. 1D).

The receptor CCR4 was expressed by many perivascular dermal mononuclear cells within psoriatic lesions (Fig. 1E). By two-color immunofluorescence, CCR4+ cells were shown to consist of a subset of CD3+ lymphocytes and a subset of CD68+ mono-cyte/macrophages (data not shown). Rare CCR4+ lymphocytes were observed within the epidermis of lesional skin. In nonlesional skin, CCR4+ cells were almost never identified (Fig. 1F). In additional serial sections, lymphocytes expressing the receptors CCR2, CCR3, CCR5, CXCR1, or CXCR2 were rarely observed. Thus, of the chemokine receptors studied, the receptors CXCR3 and CCR4 appear to be the most prominently expressed and, hence, may be involved in the movement of lymphocytes from the blood into the psoriatic dermis.

Detection of CXCR3 Ligand (IP-10 and MIG) and CCR4 Ligand (TARC and MDC) Expression in Skin Biopsies, Cultured Keratinocytes, and Dermal Endothelium

Next, we reasoned that if a chemokine receptor is involved in the recruitment of lymphocytes to a site of inflammation, the corresponding chemokine ligands should also be present within the lesion. By immuno-histochemistry, we demonstrated that the CXCR3 ligands IP-10 (Fig. 1G) and MIG (Fig. 1I) are expressed within psoriatic lesions, principally by dermal blood ves-sels and the infiltrating mononuclear cells. Interestingly, in serial sections, IP-10 and MIG-immunoreactive MNCs exactly colocalized with cells expressing CXCR3. In some of the cases reviewed, the epidermis and adnexa of lesional skin were lightly immunoreactive for IP-10 and MIG. In contrast, there was little immunoreactivity of these two chemokines in nonlesional skin (Fig. 1, H and J). Immunohistochemical reagents for TARC and MDC were not available at the time of this writing.

To study expression of chemokine mRNA within psoriatic lesions, we performed semi-quantitative RT-PCR analysis of lesions ($n = 2$) and nonlesional skin ($n = 2$). In nonlesional skin, we detected small

Figure 1.

Immunohistochemical detection of various chemokines and receptors in psoriatic lesions and nonlesional skin. Typical microscopic appearance of a psoriatic lesion characterized by parakeratotic hyperkeratosis and profound perivascular mononuclear cell (MNC) infiltrate (A), and normal microscopic appearance of nonlesional skin (B) (A and B, hematoxylin counterstain; magnification, ×200). There are a significant number of CXCR3+ lymphocytes in the perivascular space and the basal layer of the epidermis in lesional skin (C), whereas rare CXCR3+ cells are present in nonlesional skin (D). Within lesional skin, many of the perivascular MNCs also express CCR4 (E), but CCR4+ cells are rare in nonlesional skin (F). In lesional skin, IP-10 and monokine induced by interferon-γ (MIG) were expressed by dermal blood vessels, infiltrating MNCs (G and I), and there was light immunoreactivity in the epidermis in many cases. In nonlesional skin, there was minimal IP-10 and MIG immunoreactivity (H and J). (C to J: immunoperoxidase technique; hematoxylin counterstain; magnification, ×400).
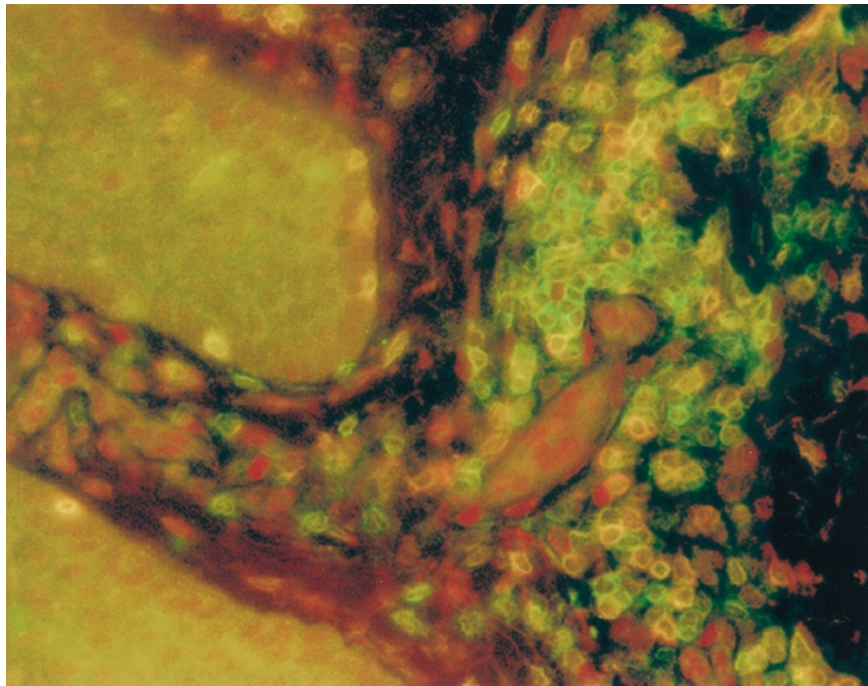
Figure 2.
Immunofluorescent detection of CXCR3 and CD3 within psoriatic lesions. Within lesional skin, many of the infiltrating mononuclear cells were CD3+ T lymphocytes (green). Although a few non-T cells expressed CXCR3 (red), the majority of CXCR3+ MNCs were a subset of CD3+ T cells (yellow). (Two-color immunofluorescence; magnification, ×400).

amounts of MDC expression (Figure 3A, lane 4), but we were unable to detect significant expression of IP-10, MIG, or TARC (Fig. 3A, lanes 1–3). In contrast, relative to the housekeeping gene G3PDH (Fig. 3A, lanes 5 and 10), psoriatic lesions had increased expression of all four chemokine mRNAs (Fig. 3A, lanes 6–9).

To identify the cellular source of the chemokine mRNAs detected in skin biopsies, we performed RT-PCR analysis of cultured keratinocytes and dermal endothelium. We found that cultured keratinocytes constitutively expressed small amounts of IP-10 mRNA (Fig. 3B, lane 1). Following stimulation with TNF-α and interferon-γ (IFN-γ), there was prominent induction of IP-10, MIG, and MDC mRNA (Fig. 3B, lanes 6, 7, and 9). TARC expression was not detected in cultured keratinocytes. In contrast, dermal endothelial cells did not constitutively express any of the aforementioned chemokines (Fig. 3C, lanes 1–4), but upon stimulation with TNF-α and IFN-γ, we were able to detect expression of IP-10, MIG, and TARC (Fig. 3C, lanes 6–8). MDC expression was not detected in cultured dermal endothelial cells.

By RT-PCR analysis, the chemokine mRNA that appeared to be most strongly induced in cultured cells was IP-10. To measure the level of induction, we performed ribonuclease protection analysis of cultured keratinocytes and dermal endothelium. Compared to constitutive expression, stimulation of keratinocytes and dermal endothelium with TNF-α and IFN-γ resulted in a 152-fold induction of IP-10 in keratinocytes and a 190-fold induction of IP-10 in dermal endothelium (Fig. 4). In summary, keratino-

cytes and dermal endothelial cells are both capable of providing chemotactic signals to promote the recruitment of CXCR3+ and CCR4+ T cells to inflamed skin.

CLA and E-Selectin Involvement in Lymphocyte Recruitment to Psoriatic Skin

Leukocyte trafficking to inflamed tissue is a multistep process involving not only chemokines and chemokine receptors, but also certain adhesion molecules (Springer, 1994). To determine which adhesion molecules potentially may be involved in lymphocyte trafficking to psoriatic skin, we performed immunohistochemical analysis of lesional and nonlesional skin. The adhesion molecule E-selectin was prominently upregulated in the superficial dermal endothelium of psoriatic lesions (Fig. 5A) but rarely observed in nonlesional skin (Fig. 5B). The cutaneous lymphocyte antigen (CLA) is specifically expressed on skin-homing lymphocytes (Fuhlbrigge et al, 1997) and an important ligand for E-selectin. CLA was prominently expressed by lymphocytes in the superficial dermis of psoriatic lesions (Fig. 5C) but was not observed in nonlesional skin (Fig. 5D). We next studied CLA expression on peripheral blood and epidermal T cells of psoriasis patients by flow cytometry, and representative data from one patient is presented in Table 1. CLA was expressed on a relatively low percentage of peripheral blood CD4+ and CD8+ T cells (17.9% and 10.8%), but the majority of epidermal CD4+ and CD8+ T cells from the same patient expressed CLA (91.3% and 92.6%) suggesting selective recruitment to the epidermis.
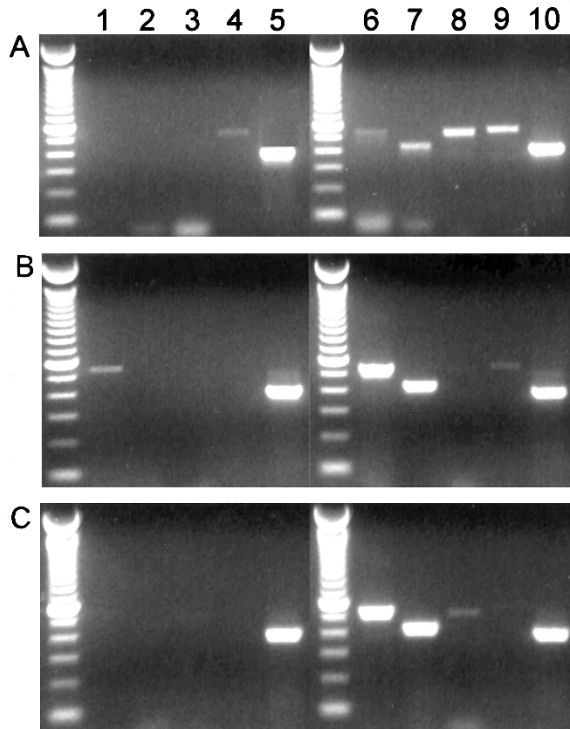
Figure 3.

RT-PCR analysis of various chemokines in skin, cultured keratinocytes, and dermal endothelium. Expression of IP-10 (lanes 1 and 6), MIG (lanes 2 and 7), thymus and activation-regulated chemokine (TARC) (lanes 3 and 8), macrophage-derived chemokine (MDC) (lanes 4 and 9), and G3PDH, (lanes 5 and 10). In nonlesional skin, the only detectable chemokine mRNA was MDC (panel A, lane 4). In contrast, all four chemokines were detectable in lesional skin (panel A, lanes 6–10). In cultured keratinocytes, IP-10 mRNA was constitutively expressed (panel B, lane 1), and upon stimulation with TNF-α and IFN-γ, there was massive up-regulation of IP-10 (panel B, lane 6) and MIG (panel B, lane 7), and slight up-regulation of MDC (panel B, lane 9). In cultured dermal endothelium, none of the chemokine mRNAs were detected (panel C, lanes 1–4). Upon stimulation with TNF-α and IFN-γ, there was massive up-regulation of IP-10 (panel C, lane 6) and MIG (panel C, lane 7), and slight up-regulation of TARC (panel C, lane 8) mRNA.

CXCR3 Is the Predominant Chemokine Receptor Expressed by Skin-Homing (CLA+) T Cells Isolated from the Psoriatic Epidermis

To determine which of the chemokine receptors, CXCR3 and/or CCR4, is involved in T cell homing to the inflamed epidermis, we studied receptor expression on peripheral blood and epidermal-derived T cells with a skin-homing (CLA+) phenotype. Representative data from one patient is presented in Table 2. The receptor CCR4 was expressed on relatively similar numbers of CD4+ and CD8+ skin-homing T cells in the blood (4.9% and 2.7%) and the epidermis (8.3% and 3.7%). In contrast, there was a significant difference in CXCR3 expression between skin-homing CD4+ and CD8+ T cells in the blood (14.5% and 3.9%) and epidermis (44.4% and 33.3%). Collectively, our data suggest that although the chemokine receptors CXCR3 and CCR4 are both potentially involved in the homing of T lymphocytes from the blood to the inflamed epidermis, CLA+ skin-homing lymphocytes selectively use CXCR3 to home to the overlying inflamed epidermis.
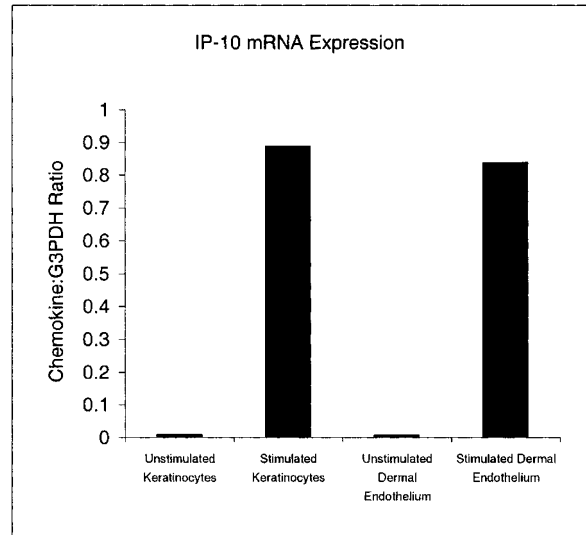

Figure 4.

Ribonuclease protection analysis of cultured keratinocytes and dermal endothelial cells. There was minimal IP-10 mRNA expression in unstimulated keratinocytes, but upon stimulation with TNF-α and IFN-γ, IP-10 mRNA expression was massively increased. Similarly, there was minimal dermal endothelial cell IP-10 mRNA expression, but upon stimulation with TNF-α and IFN-γ, IP-10 mRNA expression was massively increased.

Table 1. CLA Expression on Various T Cell Subsets

	Blood (%)	Epidermis (%)
CD4+	17.9	91.3
CD8+	10.8	92.6

CLA, cutaneous lymphocyte antigen.

Table 2. Chemokine Receptor Expression on Various Subsets of CLA+ T Cells

	Blood (%)	Epidermis (%)
CD4+CCR4+	4.9	8.3
CD8+CCR4+	2.7	3.7
CD4+CXCR3+	14.5	44.4
CD8+CXCR3+	3.9	32.3

Enhanced Expression of LFA-1/ICAM-1 and VLA-4/VCAM-1 by Mononuclear Cells and Dermal Vascular Endothelium within Psoriatic Skin

The integrins, lymphocyte function-associated antigen-1 (LFA-1) (Fig. 5G) and very late antigen 4 (VLA-4) (Fig. 6C), were prominently expressed by mononuclear cells in psoriatic lesions, but few cells expressing these ligands were observed in nonlesional skin (Figs. 5H and 6D). The counter-receptor for LFA-1, intercellular adhesion molecule-1 (ICAM-1), was demonstrated in both lesional (Fig. 5E) and nonlesional skin (Fig. 5F) of psoriatic patients. In both sets of tissues, dermal vascular endothelium and a subpopulation of infiltrating mononuclear cells expressed ICAM-1. Similarly the VLA-4 counter-receptor, vascular cell adhesion molecule (VCAM), was prominently expressed by endothelium and a subset of leukocytes
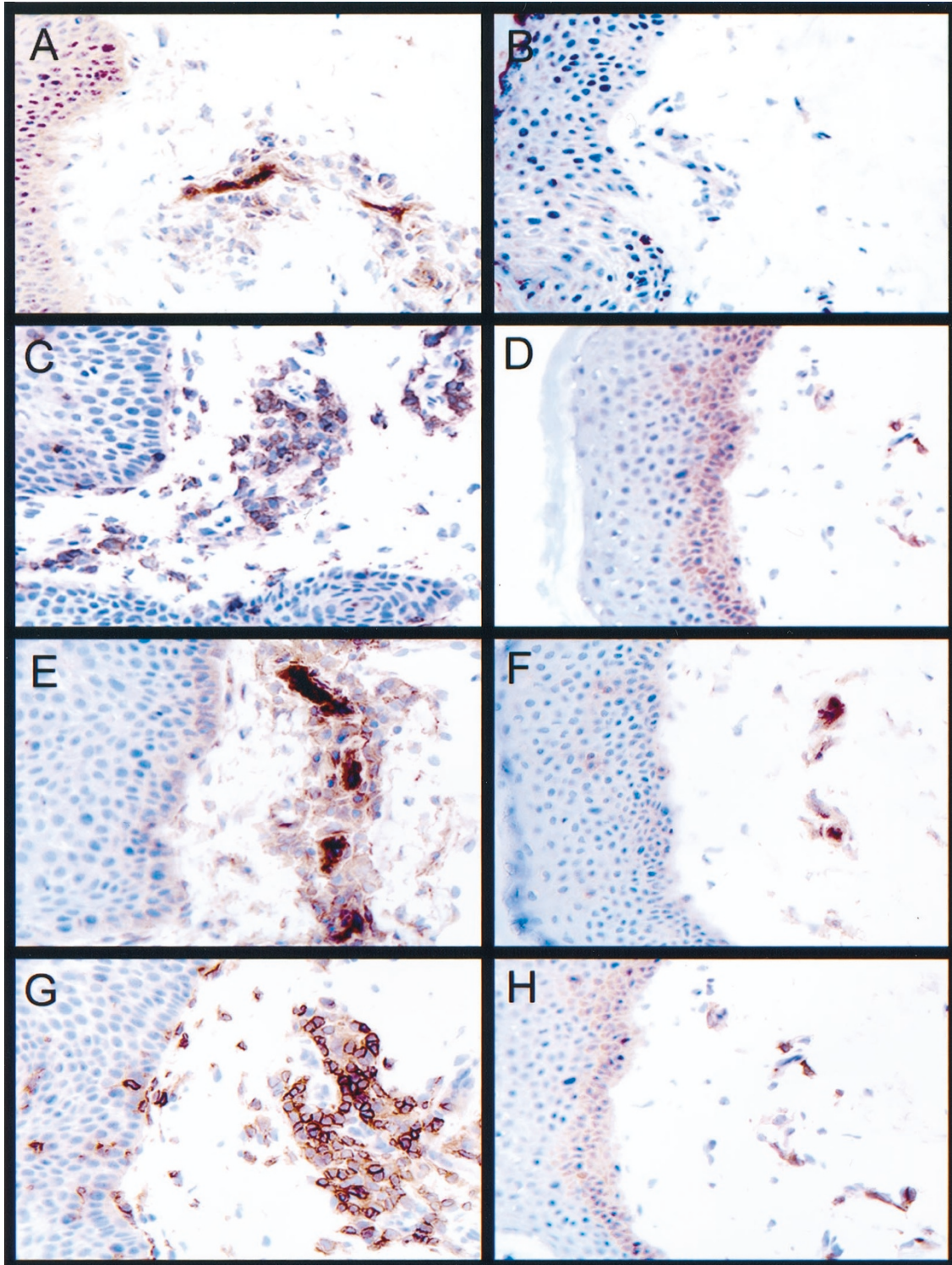
contrast, lymphocytes expressing this integrin were not observed in nonlesional biopsies (Fig. 6H). To confirm specific recruitment of αEβ7+ lymphocytes to the epidermis, we next studied expression on peripheral blood and epidermal T cells of psoriasis patients by flow cytometry, and representative data from one patient is presented in Table 3. αEβ7 was expressed on a relatively low percentage of peripheral blood CD4+ and CD8+ T cells (1.8% and 6.1%), but there was enhanced expression on epidermal CD4+ and CD8+ T cells from the same patient (23.5% and 84.4%), suggesting selective recruitment to the epidermis. Next, by IHC (Fig. 6, E and F), we demonstrated that the αEβ7 counter-receptor E-cadherin was constitutively expressed by keratinocytes in both lesional and nonlesional skin. Finally, by flow cytometry, we demonstrated that E-cadherin was constitutively expressed by cultured keratinocytes and that the level of expression did not increase upon stimulation with TNF-α and IFN-γ (data not shown). Thus, selective accumulation of αEβ7+ lymphocytes within the epidermis implies that the αEβ7/E-cadherin interaction may be important for recruiting and/or retaining lymphocytes within the psoriatic epidermis.

CXCR3 Is the Predominant Chemokine Receptor Expressed by αEβ7+ T Cells Isolated from the Psoriatic Epidermis

To determine which chemokine receptor(s) αEβ7+ T cells might use to home to the psoriatic epidermis, we studied CXCR3 and CCR4 expression on peripheral blood and epidermal T cells by four-color flow cytometry. Representative data from one patient is presented in Table 4. CCR4 was expressed by a low percentage of αEβ7+CD4+ and αEβ7+CD8+ T cells in the blood (0.3% and 1.1%) and epidermis (1.9% and 7.2%). In contrast, although CXCR3 was expressed by a low percentage of αEβ7+CD4+ and αEβ7+CD8+ T cells in the blood (0.4% and 2.2%), there was significantly increased expression on epidermal αEβ7+CD4+ and αEβ7+CD8+ T cells from patients with psoriasis (16.5% and 49.7%). Thus, CXCR3 may be involved in the recruitment of αEβ7+ T cells, particularly CD8+ T cells, from the dermis to the psoriatic epidermis.

Proposed Lymphocyte Recruitment Pathways in Psoriasis

As Figure 7 demonstrates, we propose the following model of lymphocyte recruitment in psoriasis. CLA+CXCR3+CCR4+ lymphocytes bind to E-selectin expressed by dermal endothelium and are activated by TARC, MIG, and/or IP-10, also expressed by dermal endothelium. The activated lymphocytes then bind avidly to the dermal endothelium through the LFA-1/ICAM-1 and/or VLA-4/VCAM interaction and then use CXCR3 and CCR4 to follow the IP-10, MIG, TARC, and/or MDC gradients to the adjacent dermis. Lymphocytes, especially CD8+ cells, then use CXCR3 to follow the IP-10 and MIG gradients to the epidermis. Once arriving in the epidermis, lymphocytes bind avidly to keratinocytes through the αEβ7+/

E-cadherin interaction. This interaction anchors the cells in the epidermis and the lymphocytes are lost as the associated keratinocytes desquamate.

Discussion

Previously, using the rhesus macaque delayed-type hypersensitivity model of skin inflammation, we demonstrated expression of multiple chemokine receptors and chemokines by infiltrating mononuclear cells within skin lesions (Rottman, 1999). These observations suggested to us that lymphocytes may be able to use more than one pathway to traffic to a site of inflammation. Here, using IHC, immunofluorescence (IF), flow cytometry, RT-PCR, and ribonuclease protection, we have demonstrated that lymphocytes infiltrating psoriatic skin lesions may potentially use a combination of chemokines, chemokine receptors, and adhesion molecules during the journey from dermal blood vessels to the epidermis.

The first step in the pathway would appear to be the binding of lymphocyte surface molecule CLA to E-selectin expressed on the activated dermal endothelium. It is well established that CLA is a marker for skin-homing lymphocytes (Campbell et al, 1999; Fuhlbrigge et al, 1997; Jones et al, 1997) and that E-selectin is up-regulated in dermal blood vessels in psoriatic skin lesions (Wakita et al, 1994). Furthermore, down-regulation of E-selectin expression is associated with lesion resolution (Thomson et al, 1993). In support of previous studies, we demonstrated that in lesional skin, there were abundant CLA+ lymphocytes surrounding vessels that were intensely immunoreactive for E-selectin. In nonlesional skin, CLA+ lymphocytes were rarely seen and E-selectin expression was not prominent.

According to the "area-code" model of leukocyte homing to inflamed tissues (Springer, 1994), the next step in the pathway is activation of leukocytes "tethered" to the endothelium, an event mediated by chemokine binding to a chemokine receptor expressed on the surface of the cells. In this study, we demonstrated that the chemokine receptors CXCR3 and CCR4 are prominently expressed on infiltrating lymphocytes, principally within the dermis. In serial sections, these cells also prominently expressed CLA, suggesting that both receptors may be involved in recruitment of skin-homing (CLA+) T cells to the inflamed dermis.

There is additional evidence to implicate CXCR3 in the pathogenesis of psoriasis. CXCR3 is expressed on activated memory cells of TH1 phenotype and is associated with "TH1" diseases such as rheumatoid arthritis and multiple sclerosis (Annunziato et al, 1999; Balashov et al, 1999; Qin et al, 1998). In this study, by RT-PCR we were unable to demonstrate IP-10 and MIG mRNA expression in nonlesional skin; however, there was significant up-regulation of both chemokines in lesional skin. Furthermore, by IHC and ribonuclease protection assay (RPA), we demonstrated that dermal endothelium and keratinocytes were potent sources of both chemokines. Interestingly, in serial IHC sections, lymphocytes expressing CXCR3
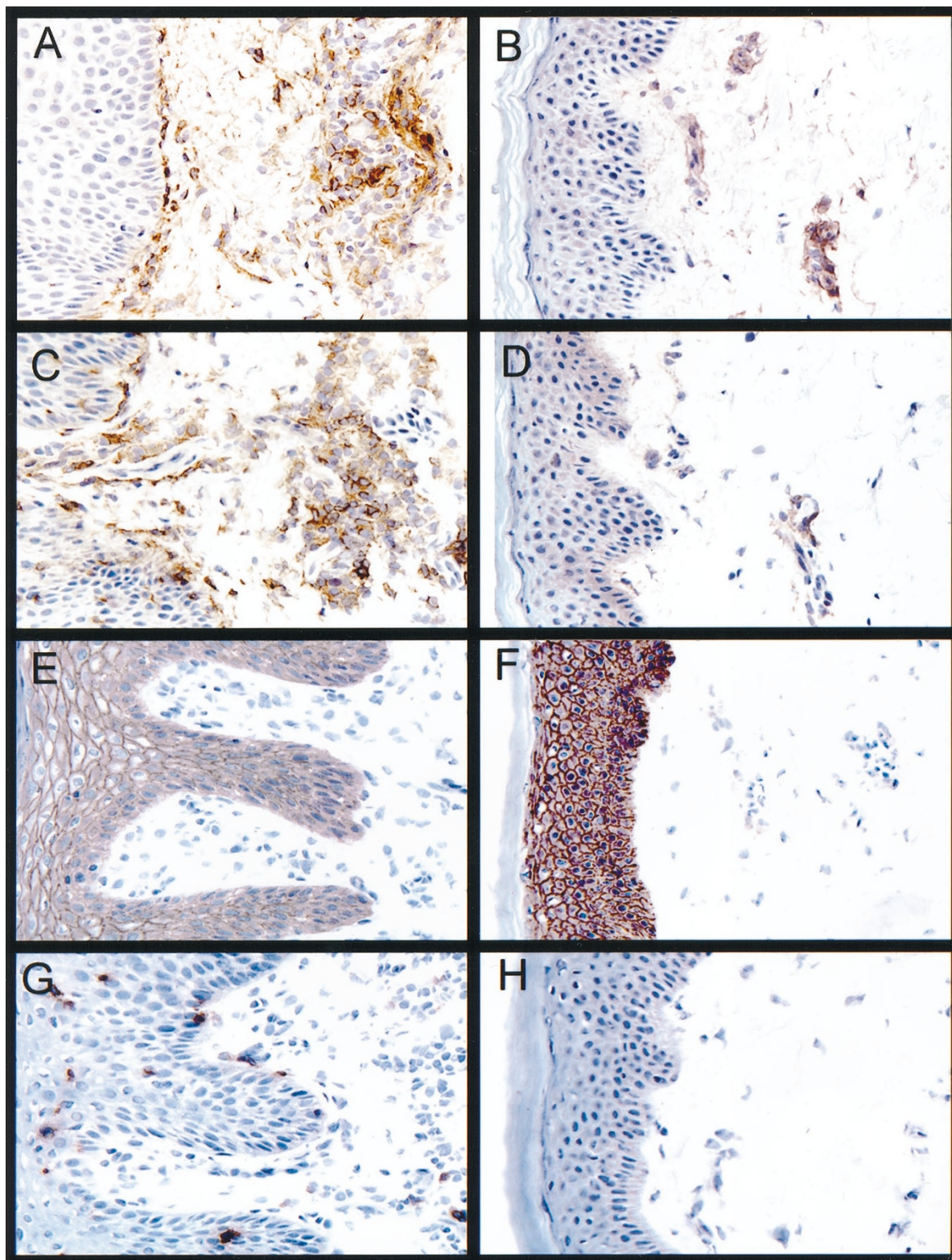
Figure 6.

Expression of various adhesion molecules in psoriatic lesions and nonlesional skin. There was abundant vascular cell adhesion molecule-1 (VCAM-1) expression by dermal endothelium and a subset of perivascular MNCs in psoriatic lesions (A) and patchy expression by endothelium within nonlesional skin (B). The VCAM-1 ligand VLA-4 was expressed by a significant number of perivascular MNCs within psoriatic lesions (C), and rare VLA-4 immunoreactive cells were present within nonlesional skin (D). E-cadherin was constitutively expressed in both psoriatic lesions and nonlesional skin, principally at the level of intracellular junctions within the epidermis (E and F). The adhesion molecule $\alpha E \beta 7$ was expressed specifically by intra-epidermal lymphocytes and rare dermal cells within lesional skin (G). In contrast, few $\alpha E \beta 7$ immunoreactive cells were present within nonlesional skin (H). (Immunoperoxidase technique; hematoxylin counterstain; magnification, $\times 400$).

Table 3. αEβ7 Expression on Various T Cell Subsets

	Blood (%)	Epidermis (%)
CD4+	1.8	23.5
CD8+	6.1	84.4

Table 4. Chemokine Receptor Expression on Various Subsets of αEβ7 + T Cells

	Blood (%)	Epidermis (%)
CD4+CCR4+	0.3	1.9
CD8+CCR4+	1.1	7.2
CD4+CXCR3+	0.4	16.5
CD8+CXCR3+	2.2	49.7

and IP-10/MIG exactly colocalized, suggesting that the infiltrating population can perpetuate its own recruitment. Furthermore, previous studies have demonstrated enhanced IP-10 expression in psoriasis (Gottlieb et al, 1988; Luster et al, 1998). Finally, we demonstrated that CXCR3 is selectively expressed on skin-homing CLA+ lymphocytes isolated from the psoriatic epidermis. Collectively, these data suggest that CXCR3 and the ligands IP-10 and MIG are involved in T cell trafficking, initially to the psoriatic dermis and ultimately to the inflamed epidermis.

CCR4 and the ligands TARC and MDC may also play an important role in lymphocyte trafficking to psoriatic skin. In a previous study, we used IHC to localize TARC expression to the dermal endothelium in inflamed skin (Campbell et al, 1999). In the present study, by RT-PCR we demonstrated that TARC and MDC mRNA were up-regulated in psoriatic tissue. Interestingly, by RT-PCR, TARC was principally expressed by dermal endothelial cells, but not keratinocytes, whereas MDC demonstrated the opposite pattern of expression. Thus, TARC may be important to activate CCR4+ lymphocytes tethered to the dermal blood vessels and mediate trans-endothelial migration, and MDC may direct CCR4+ lymphocytes to the dermis. In contrast to CXCR3, skin-homing CLA+ T cells isolated from psoriatic epidermis did not express CCR4 at high levels. Collectively, these data suggest that CCR4 and ligands may be involved in initial T cell recruitment to the inflamed dermis, but not the epidermis. Another chemokine/receptor pair recently reported to promote lymphocyte homing to psoriatic skin is CCR6/MIP-3α (Homey et al, 2000).

After chemokine activation, lymphocyte integrins promote firm cellular adherence to vascular endothelium. The integrin/counter-receptor pairs, LFA-1/ICAM-1 and VLA-4/VCAM, have been proposed as important pathways for T cell recruitment to psoriatic skin (Onuma, 1994; Thomson et al, 1993; Wakita and Takigawa, 1994). In this study, LFA-1 was prominently expressed on perivascular lymphocytes within superficial dermal infiltrates. Interestingly, the counter-receptor ICAM-1 was expressed in both lesional and nonlesional skin, as was reported previously (de Boer et al, 1994). In nonlesional skin, ICAM-1 was ex-

pressed principally on dermal endothelium. It is likely that up-regulation of ICAM-1 in nonlesional skin is due to the systemic effects of mediators that are produced locally within lesional skin, such as TNF-α and/or IL-6, but that are also detectable in the serum of psoriasis patients (Bonifati et al, 1994).

Likewise, the integrin VLA-4 was also prominently expressed on infiltrating leukocytes, and the counter-receptor VCAM-1 was diffusely expressed by endothelium and a subset of leukocytes in lesional skin. VCAM-1 expression was previously described on dermal endothelium of psoriatic lesions (Groves et al, 1993). In contrast to a previous report in which VCAM-1 was not observed in nonlesional skin (de Boer et al, 1994), we did observe patchy VCAM-1 expression in nonlesional skin. It is possible that our "nonlesional" skin biopsies were collected closer to the inflamed skin than those of the previous study or that our IHC detection technique was more sensitive. Finally, in another report, the adhesion molecule vascular adhesion protein-1 (VAP-1) was prominently expressed in psoriatic lesions, and anti-VAP-1 antibodies inhibited lymphocyte binding to frozen skin sections by 60% (Arvilommi et al, 1996). Thus, once activated by a chemokine, lymphocytes infiltrating psoriatic skin may use at least three different adhesion molecule pathways to cross the endothelium and enter the dermis.

In this study, we also demonstrated by immunohistology and flow cytometry that the integrin αEβ7 is expressed by a significant population of CD8+CXCR3+ intra-epidermal lymphocytes, but by very few lymphocytes within the dermis. This pattern of expression (lymphocytes within epithelium, but not necessarily within supporting connective tissue) is characteristic of αEβ7 expression in other tissues (Kilshaw, 1999) and suggests that αEβ7 is rapidly up-regulated as lymphocytes transit from the perivascular space to the epidermis. A likely candidate for mediating αEβ7 up-regulation on lymphocytes during transit to the epidermis is transforming growth factor-β (TGF-β), a cytokine expressed principally by skin fibroblasts (Le Poole and Boyce, 1999) that induces expression of αEβ7 on cultured lymphocytes (Kilshaw, 1999). Previous studies have demonstrated that TGF-β is expressed in the subepidermal region of normal and psoriatic skin (Wataya Kaneda et al, 1996). Up-regulation of αEβ7 may be important to anchor the lymphocytes within the epidermis by binding to E-cadherin, a molecule expressed on the surface of keratinocytes, particularly in the region of the desmosomes (Furukawa et al, 1994, 1997). We have demonstrated by IHC, that there was no significant difference in E-cadherin staining between psoriatic lesions and nonlesional skin. Furthermore, flow cytometric analysis of cultured keratinocytes revealed that there was no difference in expression between unstimulated cells and cells stimulated with TNF-α and IFN-γ (data not shown), further proving that E-cadherin is constitutively expressed. Thus, an αEβ7+ lymphocyte potentially can localize in the epidermis of both psoriatic skin and noninflamed skin. The reason more αEβ7+
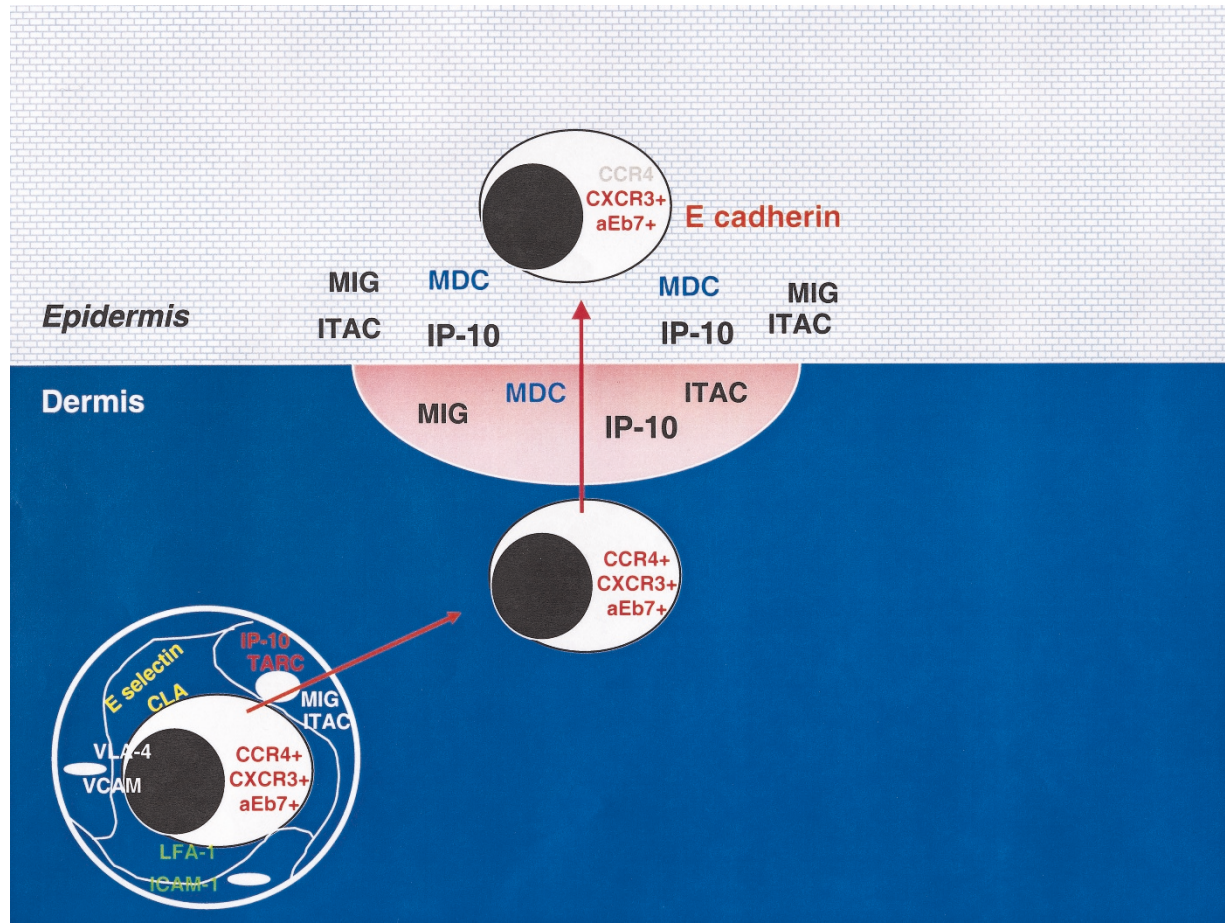
Figure 7.
Model of lymphocyte homing to the skin. CLA + lymphocytes bind to E-selectin expressed on the dermal endothelium within psoriatic lesions. The CXCR3+CCR4+ lymphocytes then are exposed to the chemokines IP-10, MIG, and TARC expressed by the endothelium. This activates the cells and causes them to bind firmly to the endothelium through the LFA-1/ICAM-1 and/or VLA-4/VCAM-1 interaction. CXCR3+CCR4+ lymphocytes then follow the IP-10, MIG, and/or MDC gradients to the dermis. Next, CXCR3+ lymphocytes follow the IP-10/MIG gradient to the epidermis, and during their migration, the cells up-regulate $\alpha E\beta 7$ and bind avidly to E-cadherin expressed by keratinocytes. The cells remain firmly adherent until they are lost with detaching squames.

lymphocytes are not present in nonlesional skin is that the appropriate cascade of adhesion molecules and chemokines is not present to attract skin-homing lymphocytes to the epidermis.

In summary, we have demonstrated that two chemokine receptors, CXCR3 and CCR4, are likely involved in selective T lymphocyte homing to the skin. We propose that both receptors are involved in trans-endothelial migration of T cells into the psoriatic dermis and that CXCR3 is the receptor that ultimately mediates T cell trafficking to the overlying epidermis. Furthermore, we propose that the integrin $\alpha E\beta 7$ is likely involved in anchoring recruited lymphocytes to the epidermis. Although our comprehensive analysis focused specifically on skin biopsies from patients with psoriasis, we acknowledge that the proposed pathways may be generalized mechanisms for T cell recruitment to inflamed skin. As such, the blockade of molecules involved in these recruitment pathways with specific antagonists may be useful in the treatment of psoriasis vulgaris and other T cell-mediated skin diseases.

Materials and Methods

Skin Biopsies

The protocol for harvesting skin biopsies from psoriasis patients was approved by The Rockefeller University Institutional Review Board. Patients signed an informed-consent form before biopsy collection. Six-millimeter punch biopsies were collected from both psoriatic lesions and nonlesional skin and either snap frozen in liquid nitrogen (for immunohistology or RNA isolation) or placed into tissue culture (for flow cytometric analysis).

Immunohistology

Skin biopsies were embedded in OCT (Sakura, Finetek, Torrance, California), cut at 6 μm, allowed to dry for 2 hours at room temperature, and then fixed in either 2% paraformaldehyde and PBS (pH 7.2) or 100% acetone for 10 minutes at 4° C. Following a wash with PBS and 1% gelatin (PBS-G), sections were blocked with PBS, 10% normal goat serum, and 5%

human AB serum for 15 minutes at room temperature and the primary antibody added (Table 5). After an overnight incubation at 4° C, slides were washed twice in PBS-G, and biotinylated goat-anti-mouse antibody was added (diluted 1:100 in PBS and 5% human AB serum; Vector, Burlingame, California). Following a 30-minute incubation at room temperature, slides were washed, and then avidin-biotin-peroxidase complexes (Vector) were added. After a final wash, slides were developed with diaminobenzidine (DAB) (Sigma, St. Louis, Missouri) and counterstained with hematoxylin.

Two-Color Immunofluorescence

Skin biopsies were embedded, sectioned, dried, and fixed as above. Following a wash with PBS and 1% gelatin (PBS-G), sections were blocked with PBS, 10% normal goat serum, and 5% human AB serum for 15 minutes at room temperature and the primary antibodies added (goat anti-human CD3 and either mouse anti-CCR4 or mouse anti-CXCR3). After an overnight incubation at 4° C, slides were washed twice in PBS-G and the antibodies, donkey-anti-mouse tetramethylrhodamine isothiocyanate (TRITC) and donkey-anti-goat FITC, were added (diluted 1:200 in PBS and 5% human AB serum; Jackson Immuneresearch, West Grove, Pennsylvania). After a 30-minute incubation at room temperature, slides were washed again and coverslipped with Vectashield (Vector).

Skin Biopsy Tissue Culture

The procedures for obtaining and analyzing epidermal cell suspensions have been described in detail (Fe-

Table 5. Antibody Clones and Sources

Target	Clone	Supplier	Location
CCR1	2D4	Millennium	Cambridge, MA
CCR2	1D9	Millennium	Cambridge, MA
CCR3	7B11	Millennium	Cambridge, MA
CCR4	1G1	Millennium	Cambridge, MA
CCR5	3A9	Millennium	Cambridge, MA
CXCR1	5A12	Millennium	Cambridge, MA
CXCR2	6C6	Millennium	Cambridge, MA
CXCR3	1C6	Millennium	Cambridge, MA
IP-10	6G10	Millennium	Cambridge, MA
MIG	4G10	Millennium	Cambridge, MA
E-Selectin	68-5H11	Pharmigen	San Diego, CA
CLA	HECA452	Pharmigen	San Diego, CA
ICAM	HA58	Pharmigen	San Diego, CA
LFA-1	HI111	Pharmigen	San Diego, CA
VCAM	51-10C9	Pharmigen	San Diego, CA
VLA-4	9F10	Pharmigen	San Diego, CA
$\alpha E\beta 7$	E7.1	Millennium	Cambridge, MA
E-Cadherin	67A4	Immunotech	Westbrook, ME
CD3-ε	1127	Santa Cruz	Santa Cruz, CA
CD68	EBM11	Dako	Carpinteria, CA

IP-10, interferon-γ inducible protein 10; MIG, monokine induced by interferon-γ; CLA, cutaneous lymphocyte antigen; ICAM, intercellular adhesion molecule; LFA, lymphocyte function-associated antigen; VCAM, vascular cell adhesion molecule; VLA, very late antigen.

renczi et al, 2000). Briefly, the epidermal layer was removed and teased into a cell suspension after brief trypsinization with 0.25% trypsin/EDTA (Clonetics Corporation, Walkersville, Maryland) at 37° C. The reaction was stopped with the addition of an equal volume of medium. The cell suspension was centrifuged and washed with medium and incubated overnight at 37° C in petri dishes. Cells were centrifuged and washed with PBS before staining for flow cytometry analysis.

Flow Cytometric Analysis of Skin Biopsies

Cells obtained from the epidermis were resuspended in PBS and aliquoted at ~10^4 cells/100 μl/well of a 96-well, round-bottomed culture plate. Biotinylated antibodies to CCR4 and CXCR3 (Millennium Pharmaceuticals, Cambridge, Massachusetts) were added to the cells and allowed to bind for 30 minutes at room temperature. Cells were subsequently washed once with PBS, 0.1% sodium azide, and 2% FBS (FACS-Wash) and stained for 15 minutes at room temperature with Strepavidin-PE, CD8-PerCP, CD4-APC (Becton Dickinson, San Jose, California) and HECA452-FITC (Heca452 was a generous gift of Dr. Louis Picker of Oregon Health Sciences University, Portland, Oregon, and was FITC-conjugated by our lab) or CD103-FITC (Pharmingen, San Diego, California). Cells were washed, resuspended in FACSWash, and analyzed by four-color flow cytometry using a FACSCalibur instrument with CellQuest software (Becton Dickinson, San Jose, California).

Culture and Stimulation of Keratinocytes and Dermal Endothelial Cells

Normal human epidermal keratinocytes (NHEK) and normal human dermal endothelial cells (HMVEC) were purchased and cultured according to the manufacturer's instructions (Clonetics/Bio-Whittaker, Walkersville, Maryland). At about 80% confluence, the cells were split and stimulated with 10 ng/mL TNFα and 100 ng/mL IFN-γ (R&D Systems, Minneapolis, Minnesota). Following a 16-hour incubation, total RNA was isolated from all cultures using an acid guanidinium-phenol-chloroform method (TRIzol Reagent; Gibco BRL Life Technologies, Grand Island, New York) and resuspended in RNAse-free water.

RT-PCR Analysis of IP-10, MIG, TARC, MDC, and G3PDH Expression

Total RNA isolated from skin biopsies, cultured keratinocytes, or dermal endothelial cells was reverse transcribed according to the manufacturer's instructions (Superscript II; Gibco BRL Life Technologies, Rockville, Maryland). Aliquots of cDNA were subsequently amplified with *Taq* DNA polymerase (Gibco BRL Life Technologies) according to the manufacturer's instructions on a Perkin Elmer Gene Amp PCR System 9600 (Perkin Elmer Applied Biosystems, Foster City, California). Oligonucleotide primer sequences for target molecules included the following: **IP-10**: (a)

5' GAG ACA TTC CTC AAT TGC TTA GAC-3' (b) 5'CCT TCC TAC AGG AGT AGT AGC AG-3'; **MIG:** (a) 5'CCT CTT GGG CAT CAT CTT GCT G-3' (b) 5'GCC ATC CTC CTT TGG AAT GAT AGC-3'; **TARC:** (a) 5'CCC TGA GCA GAG GGA CCT GC-3' (b) 5'CCT TTG TGC CCA TGG CTC CAG-3'; **MDC:** (a) 5'CAG GAC AGA GCA TGG CTC GC-3' (b) 5'GCA GGG GAA TCG CTG ATG GG-3'; **G3PDH:** (a) 5'CCT TCA TTG ACC TCA ACT ACA T-3' (b) 5'CCA AAG TTG TCA TGG ATG ACC-3'. Thermal cycle conditions included denaturation at 94° C for 60 seconds, annealing at 55° C for 90 seconds, and extension at 72° C for 2 minutes for 35 cycles. PCR products were resolved by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

Ribonuclease Protection Assay

IP-10 mRNA expression was assessed in the keratinocyte and dermal endothelial cell cultures by RPA according to the manufacturer's instructions (Pharmingen). Briefly, 20-μg aliquots of RNA were hybridized with ^{32}P-UTP labeled riboprobes complementary to IP-10 and the housekeeping gene G3PDH. After hybridization, digestion with RNAse, electrophoresis, and phosphorimaging, mRNA were quantitated by ImageQuaNT (Molecular Dynamics, Sunnyvale, California). RNA loading was controlled by expressing the data as a fraction of G3PDH signal.

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