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Discordant Expression of CD28 Ligands, BB-1, and B7 on Keratinocytes *in Vitro* and Psoriatic Cells *in Vivo*

Brian J. Nickoloff,* Raj S. Mitra,* Kelvin Lee,† Laurence A. Turka,† Jonathan Green,† Craig Thompson,†‡ and Yoji Shimizu§

From the Department of Pathology, Department of Medicine,† Howard Hughes Medical Institute,‡ and Departments of Microbiology/Immunology,§ University of Michigan, Ann Arbor, Michigan*

The process for optimal T-cell activation requires not only engagement of the T-cell receptor/CD3 complex, but also the delivery of additional co-stimulatory signals that synergize with the primary response mediated through the T-cell receptor. Thus, the regulated expression of ligands for such co-stimulatory molecules can be critical in determining whether a cell can effectively activate T cells following the presentation of a foreign antigen. The CD28 antigen has recently been shown to mediate such co-stimulatory signals by interacting with the B7/BB-1 molecule expressed on activated B cells and monocytes. We show in this study that activated keratinocytes, both* in vitro *and* in vivo *display a discordance in expression between B7 and BB-1 based on differential monoclonal antibody (MAb) reactivity. Activated keratinocytes* in vitro*, as well as psoriatic keratinocytes and epithelial cells in the thymus, are reactive with the BB-1 MAb but not anti-B7 MAbs. These BB-1 positive cells fail to express detectable B7 messenger RNA by Northern blot analysis. Furthermore, keratinocytes bind specifically to CD28-transfected COS7 cells, and this binding is inhibited by anti-CD28 and anti-BB-1 but not B7 MAbs. These studies suggest: 1) that the MAb against BB-1 binds a functional epitope on a molecule distinct from B7 as detected on activated keratinocytes* in vitro *and* in vivo *and 2) that keratinocytes in skin and epithelial cells in thymus can express cell-surface molecules that might mediate T-cell co-stimulation via CD28. (Am J Pathol 1993, 142:1029–1040)

Optimal activation of T cells requires not only engagement of the T-cell receptor complex (TCR)/CD3, but also delivery of additional co-stimulatory signals via engagement of other T-cell surface receptors such as CD28. The role of the CD28 receptor in T-cell activation has been a subject of increasing interest among immunologists (reviewed in ref. 1). The CD28 molecule is a member of the immunoglobulin gene superfamily and is expressed by 95% of CD4+ T cells and 50% of CD8+ T cells in human peripheral blood.[2] Stimulation of the CD28 molecule leads to activation of a unique signal transduction pathway capable of synergizing with TCR/CD3 receptor stimulation, leading to optimal cell proliferation and lymphokine production.[1] Further understanding of the role of CD28 in T-cell function has come through the recent identification of the B7 molecule as a CD28 ligand expressed on antigen-presenting cells such as activated B cells.[3] These studies of B7 expression and function have used two monoclonal antibodies (MAbs), anti-B7 and anti-BB-1, each of which binds to the B7 molecule without any significant discordant patterns of expression on the various cultured cell lines tested to date. Comparative analysis of their immunoreactivity in various organs has not been performed.[4, 5] Rather it has been assumed that MAbs directed against BB-1 and B7 recognize identical epitopes on the same CD28 ligand. However, since a complementary (c)DNA for BB-1 has not been cloned, the precise molecular identity and functional relationship between BB-1 and B7 have not been definitively established.

The skin represents one of the major microenvironments where a foreign antigen is initially encountered. Consequently, the regulated expression of mol-

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Address reprint requests to Dr. Brian J. Nickoloff, Department of Pathology, University of Michigan Medical School, M4232 Medical Science I, 1301 Catherine Street, Ann Arbor, Michigan 48109-0602.

ecules on cells in the skin that can facilitate presentation of foreign antigen to T cells is critical to our understanding of T-cell function in the skin. Recently, it has been observed that interferon-γ (IFN-γ) could induce B7/BB-1 on monocytes.[6] Because we have previously observed[7] that IFN-γ could induce keratinocytes to express class II major histocompatibility antigen (HLA-DR) and intercellular adhesion molecule-1 (ICAM-1; CD54), we assessed whether keratinocytes could be induced to express either B7 or BB-1. In this report we describe the discordant expression of B7 and BB-1 epitopes using previously characterized IgG and IgM MAbs, as well as at the messenger (m)RNA level using a B7 cDNA probe, on activated keratinocytes *in vitro* and *in vivo*. The cell-surface expression of BB-1 by keratinocytes can mediate adhesion to CD28-transfected COS cells. Such discordance in expression between B7 and BB-1 and the ability of BB-1 positive, B7-negative cells to bind to CD28-transfected cells suggests potentially important and previously unrecognized complexities in the regulation of CD28 function during T-cell activation.

Materials and Methods

Antibodies

The anti-BB-1 mouse MAb (IgM isotype) was a gift from P. Linsley (Oncogen Division, Bristol-Myers-Squibb Pharmaceutical Research Institute, Seattle, WA).[4] Two different antibodies against B7 were used, including one IgM isotype (kindly provided by L. Nadler, Dana Farber Cancer Institute, Boston, MA),[8] and one IgG1 isotype (B1.1; obtained from RepliGen Corp., Cambridge, MA). Anti-ICAM-1 MAb RR 1/1 (IgG_1 isotype) was obtained as a gift from T. Springer, Blood Research Center, Boston, MA) and anti-HLA-DR MAb, L243, purchased from Becton-Dickinson (Mountainview, CA). Anti-CD28 (9.3) was obtained from J. Ledbetter (Bristol-Myers-Squibb) and anti-LFA-3 (CD58; TS2.9) was obtained from American Type Culture Center, (Rockville, MD). Isotype control MAbs included anti-Leu-2a (IgG_1 Becton-Dickinson) and pooled IgM (#6602402; Coulter Corp., Hialeah, FL). Anti–von Willebrand's factor IgM MAb was obtained from P. Bockenstadt (Univ. of Michigan, Ann Arbor, MI). Optimal staining concentrations were determined for each antibody in preliminary studies. For the BB-1 and B7 antibodies (abs) this was 5 μg/ml, the control abs were used at equivalent concentrations.

Cell Culture

Multipassaged human keratinocytes were obtained from biopsy specimens of normal adult skin (after informed consent and approval by the University of Michigan Human Subjects Committee). Keratinocytes were grown at 37 C in a humidified incubator with 5% CO_2 in a fully supplemented low calcium, serumfree medium (KGM, Clonetics Corp., San Diego, CA) and utilized in passage two to four, as previously described.[7] Recombinant human IFN-γ and tumor necrosis factor-α (TNF-α) were obtained from Genentech Inc., (S. San Francisco, CA). Cycloheximide, 12–0-tetradecanoyl phorbol 13-acetate ester (TPA), and the calcium ionophore, ionomycin, were purchased from Sigma Chemical Corp. (St. Louis, MO) and added to subconfluent cultured keratinocytes. Recombinant human interleukin-4 was purchased from R+D Systems, Inc. (Minneapolis, MN). Keratinocytes were also grown both in 35-mm and 10-cm plastic dishes (Corning Inc., Corning, NY) as well as in 8-well chambers (Nunc Inc., Naperville, IL).

M16B is an Epstein–Barr virus–transformed B cell line obtained from Dr. S. Shaw (National Health Institutes, Bethesda, MD) and grown in RPMI + 10% fetal calf serum (Gibco Inc, Grand Island, NY) at 37 C. Cytospin preparations were made by centrifugation of 200 μl of a 10^6 cells/ml suspension (Shandon Cytospin 3 Instrument, Pittsburgh, PA) onto glass slides, which were fixed at room temperature for 10 minutes with 50:50 mixture of acetone-methanol.

Patient Samples

Six-mm punch biopsies were obtained from either normal healthy individuals (N = 4), clinically symptomless skin of psoriatic patients, or the active advancing margin of untreated psoriatic plaques (after informed consent and approval by the Human Subjects Committee). For each of the six adult psoriatic patients who had active untreated plaques, 2 biopsies were obtained from the lower back and/or buttocks region. One biopsy was from a plaque and the other from clinically symptomless skin at least 10 cm away from a lesion. These biopsy specimens were snap frozen in liquid nitrogen chilled isopentane and stored at −80 C. Thymic tissue was obtained from young patients undergoing cardiothoracic surgery (N = 2).

Immunofluorescence and Immunoperoxidase Staining

Cells were analyzed for cell-surface antigens by indirect immunofluorescence and flow cytometric analysis as described.[7] Briefly, sub-confluent keratinocytes were exposed to either KGM medium alone or with IFN-γ and/or TPA for various time intervals and a single cell suspension prepared using 0.1% trypsin/0.03% ethylenediaminetetraacetic acid (EDTA) (5 minutes, 37 C). This mild trypsinization procedure had no effect on cell-surface expression of either B7 or BB1 on M16B cells (data not shown). Next, the cells were washed with a phosphate-buffered saline/5% bovine serum albumin/0.1% azide and exposed to the primary abs on ice for 30 minutes. After two washes, cells were exposed to a fluorescein isothiocyanate–conjugated goat anti-mouse antigen-binding fragment, $F(ab')_2$, IgG- and IgM-specific secondary ab (Tago Inc., Burlingame, CA). After two additional washings, cells were fixed with 1% paraformaldehyde and analyzed on a fluorescence-activated cell sorter (FACS) flow cytometer (Becton-Dickinson). For some experiments, unfixed cells were immediately analyzed, with no significant differences compared to the fixed cells. For all results, at least three separate experiments were performed with representative results portrayed.

Immunoperoxidase staining of either fixed cytospin slides, 8-well chambers, or 4-micron-thick cryostat sections of skin utilized a highly sensitive avidin-biotin technique as described.[7] The chromogen (red reaction product) 3-amino-4-ethylcarbazole was used, followed by hematoxylin counterstaining.

RNA Blots

RNA was extracted from treated keratinocyte cultures and M16B cells as previously described.[9] Briefly, monolayers of keratinocytes, or M16B cells grown in suspension, were extracted with guanidine hydrochloride (5.7 mol/L), potassium acetate (100 mmol/L, pH 5), sonicated on ice for 60 seconds ethanol precipitated, and centrifuged at 15,000*g* for 30 minutes at 4 C. Ten μg total cellular RNA were then separated, according to molecular weight, on 1% agarose gels containing 8% formaldehyde and electroblotted on nylon membranes (Bio-Rad Laboratories, Richmond, CA). Hybridization with ^{32}P-labeled cDNA inserts, using random primer method to a specific activity of 3×10^8 cpm/μg for ICAM-1 and cyclophilin, was performed as described.[9] The B7 cDNA probe was obtained through polymerase chain reaction of a Raji cell cDNA library, with site-specific oligonucleotide primers synthesized on an Applied Biosystems model 380B DNA synthesizer (Applied Biosystems Inc., Foster City, CA). The 5′ primer (TGT TCA GGT GTT ATC CAC GTG ACC AAG GAA) and 3′ primers (TGG GGC AAA GCA GTA GGT CAG GCA GCA TAT) were used in a 50 μl reaction under polymerase chain reaction conditions of 94 C × 1′15, 72 C × 5′ for 30 cycles. The resulting product was subcloned into Bluescript SK (Stratagene, La Jolla, CA) and verified by sequence analysis. The nylon membranes were hybridized overnight, washed in 0.3 mol/L sodium chloride, 0.03 mol/L sodium citrate and 0.2% sodium dodecyl sulfate at 65 C, and subjected to autoradiography. Determination of the size of the B7 mRNA molecules was accomplished using various molecular weight markers, including radiolabeled fragments generated from Hind III digestion of λ phage DNA.

Adhesion Assay

COS7 cells (kind gift of Dr. R. Larsen) were plated at 8×10^5 cells/100-mm dish 24 hours before use. These cells were subsequently transfected with either 10 μg of a human CD28 expression construct (gift of Dr. B. Seed, Massachusetts General Hospital, Boston, MA) or 10 μg of the vector alone utilizing DEAE-Dextran.[10] Twenty-four hours after transfection, the cells were transferred to 6-well plates and used in adhesion assays 48 hours later. Transfection efficiency was assessed by MAb 9.3 (CD28) staining and flow cytofluorometric analysis on the day of the assay and was always in excess of 50%. The transfected COS7 monolayers were fixed to the plastic with 0.5% paraformaldehyde (10 minutes at 25 C) and subsequently washed with Ca^{2+}/Mg^{2+}free phosphate-buffered saline immediately before use, as previously described.[3]

Unstimulated keratinocytes were released from their dishes with 3 mmol/L EDTA and subsequently fluorescently membrane-labeled with PKH2 dye according to the manufacturer's protocol (Zynaxis Cell Sciences, Malvern, PA). Labeled keratinocytes were >95% viable (by trypan blue exclusion) and fluoresed $>10^2$ to 10^3-fold brighter than unlabeled keratinocytes, as determined by flow cytofluorometry. The keratinocytes were then resuspended at 1×10^6 cells/ml in Hanks' Balanced Salt Solution/5 mm EDTA/5% fetal calf serum. Potential blocking abs

were added at 10 μg/ml immediately before the adhesion assay. Keratinocytes (1×10^6) were then added to each well containing the transfected COS7 monolayers and incubated at 37 C for 1 hour. The wells were then gently washed 4 times with ice-cold media. The remaining adherent cells were then assessed by both light and fluorescence microscopy. Specific binding was calculated as the ratio of cells/high-power field adherent to CD28-transfected COS7 cells compared to the mock-transfected COS7 cell monolayer. At least six high-power fields were evaluated, and the mean number of cells bound to COS7 cells (CD28 versus mock transfected) was calculated. In addition, the COS cells were grown as a monolayer on round, 13-mm tissue culture coverslips (Thermanox, Miles Scientific, Naperville, IL) in the bottom of 24-well plates, and the adhesion assay was performed as above. After co-incubation with keratinocytes, the coverslips were removed, washed, and then fixed by immersion in methanol and stained with toluidine blue or fixed with 50:50 mixture of methanol:acetone and immunostained.

Results

Immunoreactivity of BB-1 and B7 MAbs on Cultured Cells

Previous studies have shown that both BB-1 and B7 MAbs bind to Epstein–Barr virus–transformed B cell lines. We reconfirmed those initial studies using the Epstein–Barr virus–transformed B cell line M16B by cytospin immunostaining and FACS analysis. Cytospin preparation of M16B cells revealed that the cells had strong positive membrane staining, as well as weak cytoplasmic staining for BB-1 (Figure 1a). Identical immunoreactivity on cytospin preparations of M16B cells stained with B7 (IgG and IgM) was observed in comparison to BB-1 staining (data not shown). FACS analysis (Figure 1b) of M16B cells stained with a panel of MAbs confirms that M16B cells expressed significant cell-surface levels of BB-1 and B7 (IgG and IgM), as well as being constitutively positive for ICAM-1 (CD54).

Despite similar patterns of immunoreactivity of BB-1 and B7 MAbs to M16B cells, there are distinct differences in BB-1 versus B7 reactivity with keratinocytes. Cytospin preparations of keratinocytes grown in the absence of IFN-γ and/or TPA (Figure 2a) displayed cell-associated immunoreactivity with the BB-1 MAb that was primarily in the peripheral cytoplasm near the plasma membrane, but no positive cell staining was observed with either B7 (IgM) or B7 (IgG) MAbs (data not shown). BB-1 immunoreactivity was particularly apparent on the smaller rounded keratinocytes that represent the relatively undifferentiated cells that proliferate in low-calcium, serumfree growth medium. Treatment of keratinocytes with either IFN-γ (200, 500, or 2000 U/ml; 6, 24, 48, hours) or TPA (5 nmol/L) tended to induce differentiation in these cells, which is manifested by their enlargement. Representative cytospins reveal that after IFN-γ (500 U/ml, 24 hours; Figure 2b) or TPA (5 nmol/L, 24 hours; Figure 2c) treatment, there was more heterogeneity in BB-1 immunoreactivity compared to untreated cultures. Within the treated cell populations, the smaller keratinocytes retained BB-1 immunoreactivity (primarily in the peripheral cytoplasm), whereas the larger cells were usually negative, with only rare, occasional cells possessing a diffuse cytoplasmic staining pattern. Under the same treatment conditions, there was complete

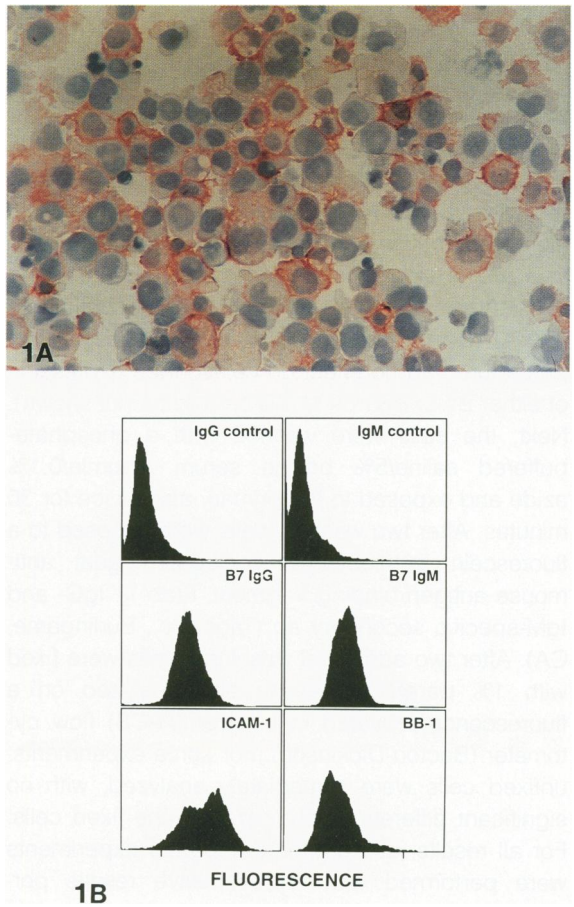

Figure 1. *M16B cells express BB-1 and B7 immunoreactivity. Cytospin preparations reveal strong cell-surface expression of BB-1* (**A**: *immunoperoxidase stain magnification ×150). FACS analysis confirms cell-surface expression of BB-1, B7 (IgG), B7 (IgM), and ICAM-1 (CD-54;* **B**).

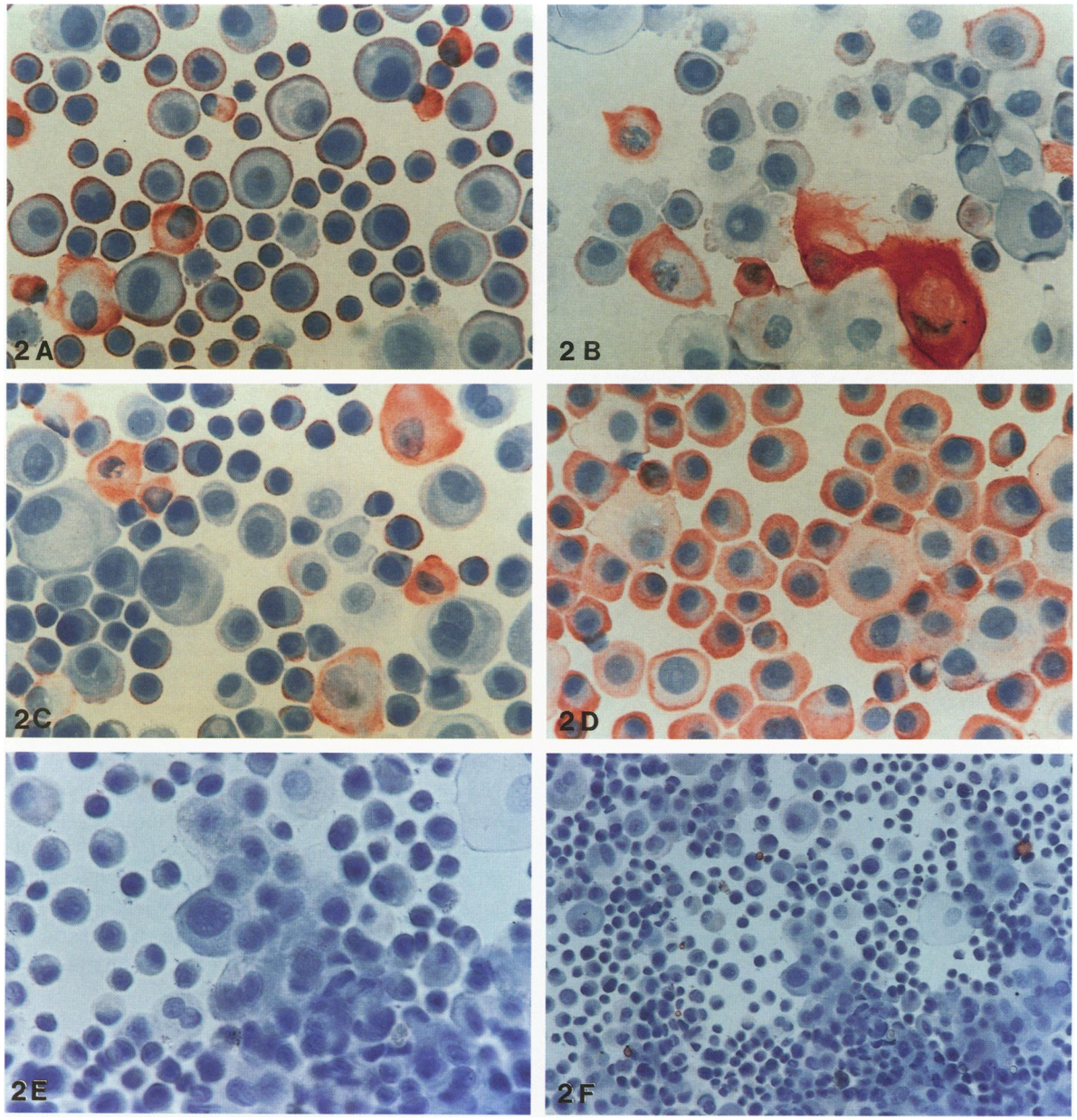

Figure 2. *Cultured keratinocyte cytospin preparations express BB-1 but not B7 immunoreactivity. Untreated keratinocytes display cell-associated immunoreactivity for BB-1 on small rounded cells* (**A**). *Keratinocytes treated with either IFN-γ alone* (**B**) *or TPA alone* (**C**) *reveal peripheral cytoplasmic BB-1 immunoreactivity of the smaller cells with the majority of larger cells being negative and rare cells having a diffuse cytoplasmic reactivity. Keratinocytes simultaneously treated with the combination of IFN-γ plus TPA markedly increase BB-1 immunoreactivity, which is particularly notable on smaller cells* (**D**), *but without any detectable expression using B7 (IgM,* **E**) *or B7 (IgG,* **f**). *Immunoperoxidase stain: magnification ×150.*

negativity for all cells stained with either B7 MAb (IgG or IgM, data not shown). However, keratinocytes co-treated simultaneously with both IFN-γ (500 U/ml) plus TPA (5 nmol/L) for 24 hours displayed a markedly increased BB-1 immunoreactivity on almost all cells (Figure 2d), whereas these cells remained completely negative for either B7 (IgM; Figure 2e) and B7 (IgG; Figure 2f). The combination of IFN-γ plus TPA was chosen because we have previously observed that this treatment produces a synergistic induction of ICAM-1 expression on keratinocytes.[11]

To investigate further this discordant immunoreactivity of BB-1 versus B7 MAbs, intact monolayers of keratinocytes were similarly stained before and after exposure to either KGM alone or after 24 hours of addition of either IFN-γ, TPA, or IFN-γ plus TPA. Figure 3 reveals that untreated keratinocytes pos-

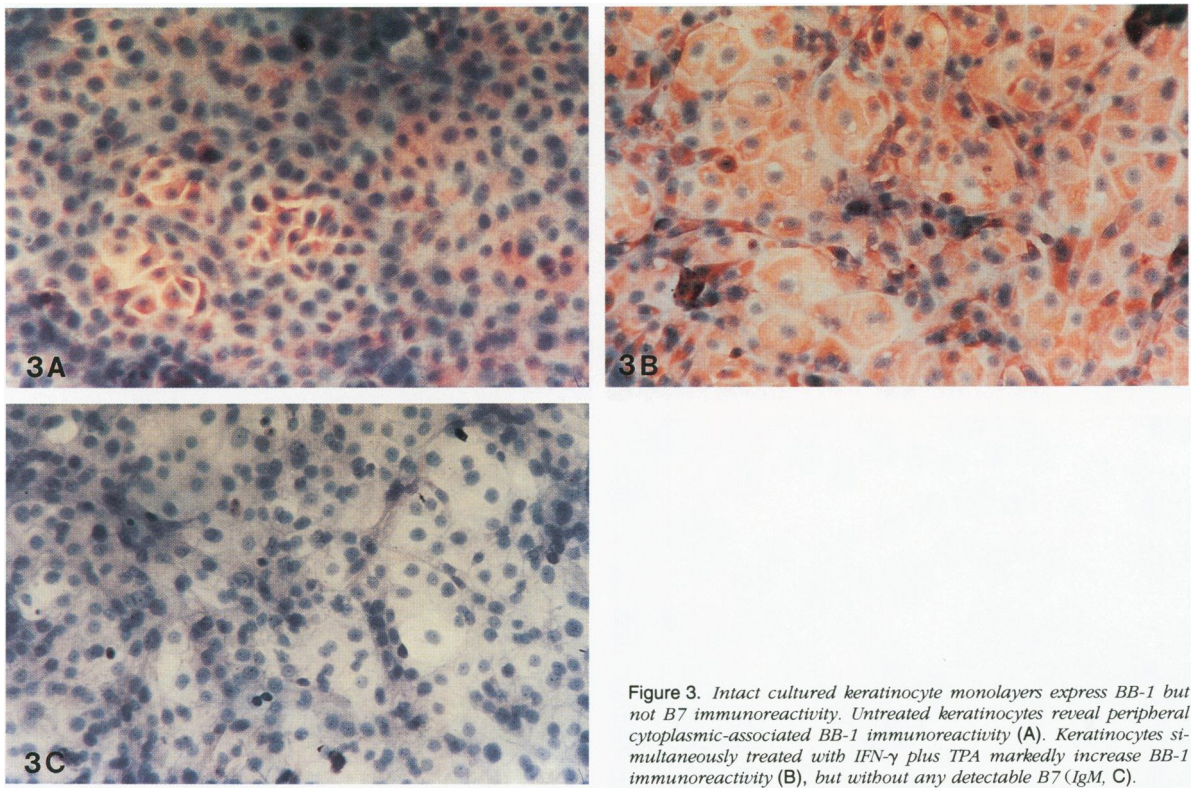

Figure 3. *Intact cultured keratinocyte monolayers express BB-1 but not B7 immunoreactivity. Untreated keratinocytes reveal peripheral cytoplasmic-associated BB-1 immunoreactivity* (A). *Keratinocytes simultaneously treated with IFN-γ plus TPA markedly increase BB-1 immunoreactivity* (B), *but without any detectable B7 (IgM,* C).

sessed BB-1 peripheral cytoplasmic staining, whereas keratinocytes treated with IFN-γ plus TPA exhibited strong and diffuse cellular expression of BB-1 but no B7 (IgM or IgG) reactivity. Keratinocyte monolayers treated with either IFN-γ alone or TPA alone revealed essentially similar patterns as described for the cytospin preparation (data not shown). As it is difficult to assess accurately whether the immunostaining of either the fixed cells in suspension or the monolayer of cells included cell-surface staining, intact, nonfixed cells were subjected to indirect immunofluorescence staining and FACS analysis.

Figure 4, a and b, reveals the FACS analysis of untreated and treated keratinocytes. In both treated and untreated keratinocyte cultures, cell-surface expression of BB-1 but not B7 was detectable. As was observed by the immunohistochemical staining of cytospin preparations (Figure 2), there was heterogeneity in the extent of BB-1 expression, as judged by FACS analysis, in all of the keratinocyte cultures tested (N = 4). Whereas the keratinocytes treated with either IFN-γ alone (Figure 4a) or TPA alone (data not shown) possessed slightly increased BB-1 cell-surface immunoreactivity, the combined treatment with IFN-γ plus TPA produced an even greater enhancement of BB-1 cell-surface immunoreactivity. Under these same conditions, there was no detectable B7 immunoreactivity. To explore further the relative effects of IFN-γ on the keratinocytes, the cells were also simultaneously examined for ICAM-1. Figure 4a reveals that IFN-γ induced ICAM-1 cell-surface expression on keratinocytes, which was further increased by the combination of IFN-γ plus TPA as previously described.[7,12] Figure 4b provides a complete FACS profile of keratinocytes treated with the combination of IFN-γ plus TPA.

Northern Blot Analysis of B7 and ICAM-1 on Cultured Cells

To correlate these positive and negative immunostaining results with mRNA expression, Northern blot analysis was performed (Figure 5). As suggested by the positive immunostaining with all abs (i.e., BB-1, B7-IgG, and B7-IgM), M16B cells contained four detectable B7 transcripts with approximate molecular weights of 1.7, 2.9, 4.2, and 10 kb in good agreement with previously published results.[6] M16B cells also contained ICAM-1 and cyclophilin transcripts. However, all treatment regimens of keratinocytes (including cycloheximide, IFN-γ, TPA, TNF-α, IFN-γ plus TNF, TNF-α, IFN-γ plus TPA, TPA plus TNF-α, or interleukin-4), failed to induce any of the four B7 mRNA transcripts de-

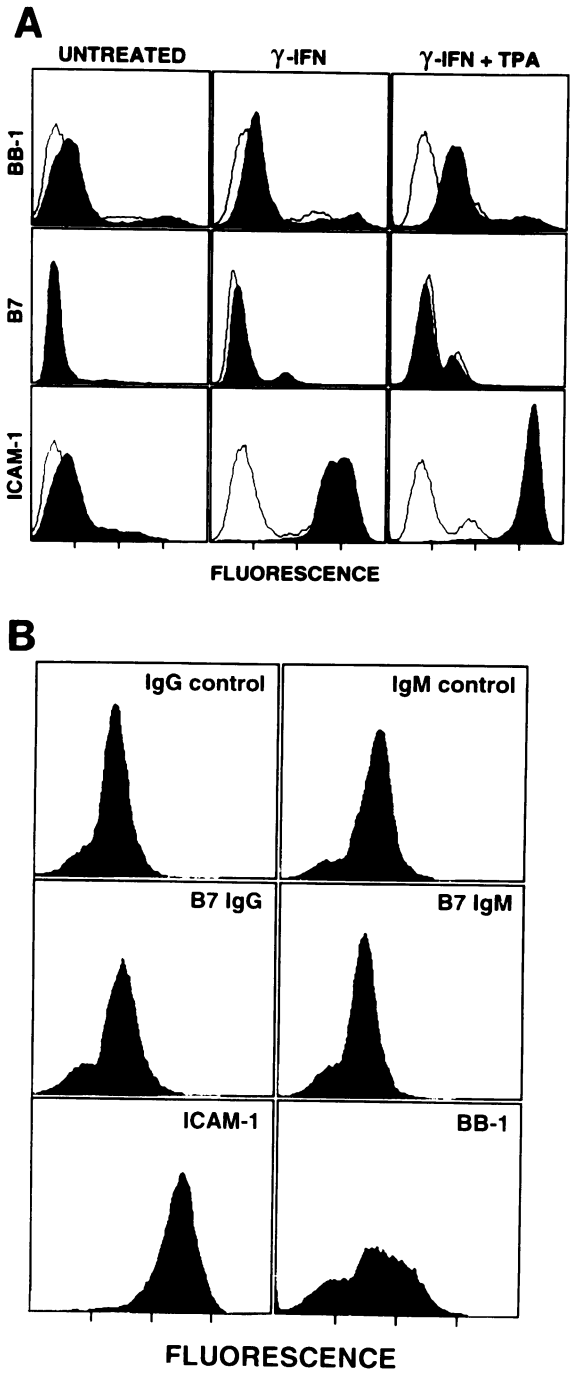

Figure 4. **A:** *Composite FACS profile of cultured keratinocytes demonstrating positive BB-1 but not B7 (IgG) cell-surface expression. Untreated keratinocytes or cells treated with either IFN-γ alone (500 U/ml; 24 hours) reveal relatively weak but detectable cell-surface BB-1 expression, but without B7 expression. Untreated keratinocytes express low level of ICAM-1, which is increased by IFN-γ alone and further increased by combined treatment of IFN-γ plus TPA. Keratinocytes simultaneously treated with both IFN-γ (500 U/ml) and TPA (5 nmol/L; 24 hours) increase BB-1 but not B7 expression. Solid profile represents indicated ab staining pattern, whereas open profile represents isotype-matched control staining.* **B:** *In a separate experiment, a complete FACS profile for keratinocytes treated with the combination of IFN-γ plus TPA is depicted.*

tected in the M16B cells. Failure to detect any of the reported B7 transcripts suggests that absence of B7 surface immunoreactivity is not due to alternative splicing of the B7 transcript. This broad range of treatments was selected based on our past experience with the ability of these reagents to stimulate cultured keratinocyte to express either ICAM-1 or other immunoregulatory molecules such as interleukin-8.[7,9,13] The immunomodulation of the keratinocytes by some of these treatments (i.e., IFN-γ with or without TPA) was verified by positive detection of ICAM-1 transcripts, with the detection of cyclophilin transcripts serving as an internal control of keratinocyte viability and RNA isolation and loading.

Immunoreactivity of BB-1 and B7 on Epithelial Cells in Vivo

Biopsies of normal controls, as well as symptomless and lesional psoriatic skin, were chosen to extend our *in vitro* studies using IFN-γ plus TPA, because hyperkinetic keratinocytes in a psoriatic plaque are associated with elevated IFN-γ levels and activation

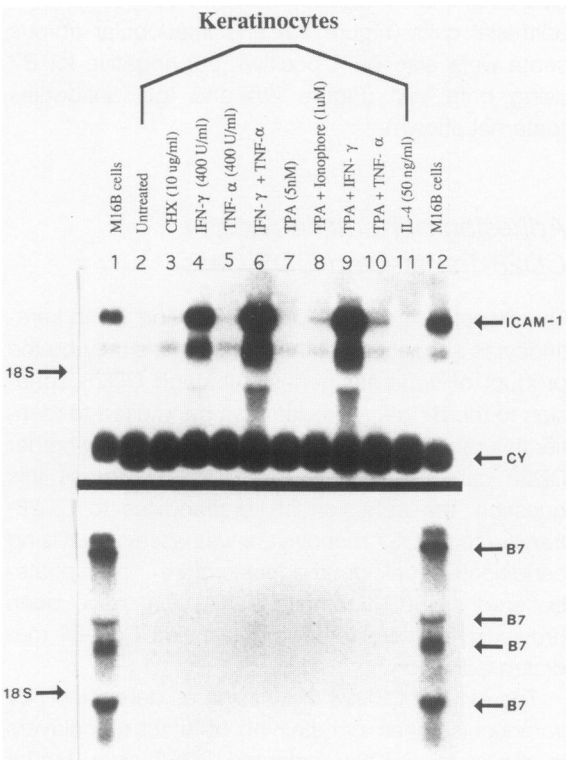

Figure 5. *Northern blot analysis of M6B cells and keratinocytes to detect ICAM-1, cyclophilin, and B7 mRNA transcripts.* **Lanes 1** *and* **12** *represent M16B cells and* **lanes 2** *to* **11** *represent keratinocytes that have either been untreated or treated with the indicated regimens for 18 hours before isolation of RNA.*

of protein kinase C.[9,14] Moreover, our previous hypothesis regarding the cytokine network in psoriasis pointed toward the possible involvement of keratinocyte-derived surface molecules in mediating T-cell activation.[15] Figure 6 reveals that there is no immunoreactivity of epidermal keratinocytes with MAb BB-1 in normal skin and only weak staining of clinically symptomless skin of a psoriatic patient. The collagenous stroma of the dermis in all skin specimens was weakly positive in a diffuse, nonspecific pattern. However, there is strong, focal BB-1 immunoreactivity of keratinocytes in the psoriatic lesion, particularly in the epidermal zone at the tips of the dermal papillae. This so-called squirting papilla is where epidermal T-cell trafficking occurs and is accompanied by keratinocyte ICAM-1 expression.[16] Note that intraepidermal T cells that stain positive for CD28 co-localize to this area of keratinocyte BB-1 positivity. Immunostaining of serial sections of these same biopsies failed to reveal any positive keratinocyte reaction with either B7 antibodies (i.e., IgM or IgG). In two of the psoriatic plaques, focal B7 (IgM) staining of epidermal dendritic cells was observed. Finally, because we had previously reported that thymic epithelial cells and interlobular fibrous septa were BB-1 positive,[17] we also examined a new set of thymic tissues. Thymic epithelial cells (Figure 7a) and interlobular fibrous septa were also BB-1 positive, but negative for B7 using both IgM (Figure 7c) and IgG antibodies (data not shown).

Adhesion of Keratinocytes to CD28-Transfected COS Cells

The discordant expression of BB-1 and B7 in keratinocytes suggests that these molecules may be the product of separate genes. Although CD28 adhesion to the B7 gene product has been used to identify this receptor-ligand pair, it is not known whether CD28 can also adhere to BB-1. To address this question, the adhesion of keratinocytes to CD28-transfected COS7 monolayers was assayed utilizing conditions (calcium/magnesiumfree phosphate-buffered saline, 5 mmol/L EDTA) that have been previously demonstrated to maximize CD28–B7 mediated adhesion.[3]

The extent of CD28 expression as determined by immunohistochemical staining of intact monolayers is shown for CD28-transfected COS7 cells (Figure 8a) and its absence in mock-transfected COS7 cells (Figure 8b). As shown in Figure 9, adhesion of keratinocytes to the CD28-transfected COS7 monolayer was significantly higher (greater than 12-fold) than mock-transfected monolayers. In addition, keratinocytes often adhered in rosettes around CD28-transfected COS7 cells, whereas this pattern was rarely seen with the mock-transfected COS7 cells. Figure 8c reveals the light microscopic appearance of small, round keratinocytes binding to the underlying CD28-transfected COS7 cell monolayer. Figure 8d reveals a representative immunostained preparation demonstrating BB-1-positive rounded keratinocytes binding to underlying CD28-transfected COS7 cells. Further specificity of this adhesion was demonstrated through the use of blocking antibodies (Figure 9). Antibodies directed against BB-1 or CD28 significantly reduced keratinocyte binding to CD28-transfected COS7 monolayers down to background levels, whereas antibodies against B7, von Willebrand's factor (isotype matched IgM), and leukocyte function antigen-3 (expressed on the surface of keratinocytes and capable of mediating adhesion of epithelial cells[18]) did not inhibit adhesion (data not shown). These data demonstrate that keratinocytes can specifically adhere to CD28-transfected COS7 cells and this adhesion can be blocked with antibodies directed against BB-1 or CD28 but not with the other abs utilized.

Discussion

Recent studies have shown one CD28 ligand to be the B7 molecule; the ability of BB-1 specific MAbs to bind to B7 transfectants and to block CD28/B7 interactions have previously implied that B7 and BB-1 MAbs recognize the same molecule. The results presented in this study suggest two potential roles for CD28-mediated T-cell interactions in the skin. First, we provide evidence that a molecule recognized by the BB-1 MAb is expressed on activated keratinocytes *in vitro* and *in vivo* and that this molecule is a ligand for CD28. From a functional perspective, the ability of keratinocyte BB-1 to serve as a ligand for CD28 was clearly demonstrated by the ability of keratinocytes to bind to CD28-transfected COS7 cells, which was inhibited by antibodies against either BB-1 or CD28 (Figure 9). This suggests that a molecule critical to T-cell activation can be expressed by nonlymphoid cells in the skin and can also be induced to co-express HLA class II antigens. Second, we demonstrated that activated keratinocytes *in vitro* and *in vivo* express an epitope recognized by anti-BB-1 MAb that is not recognized by two anti-B7 MAbs. This lack of

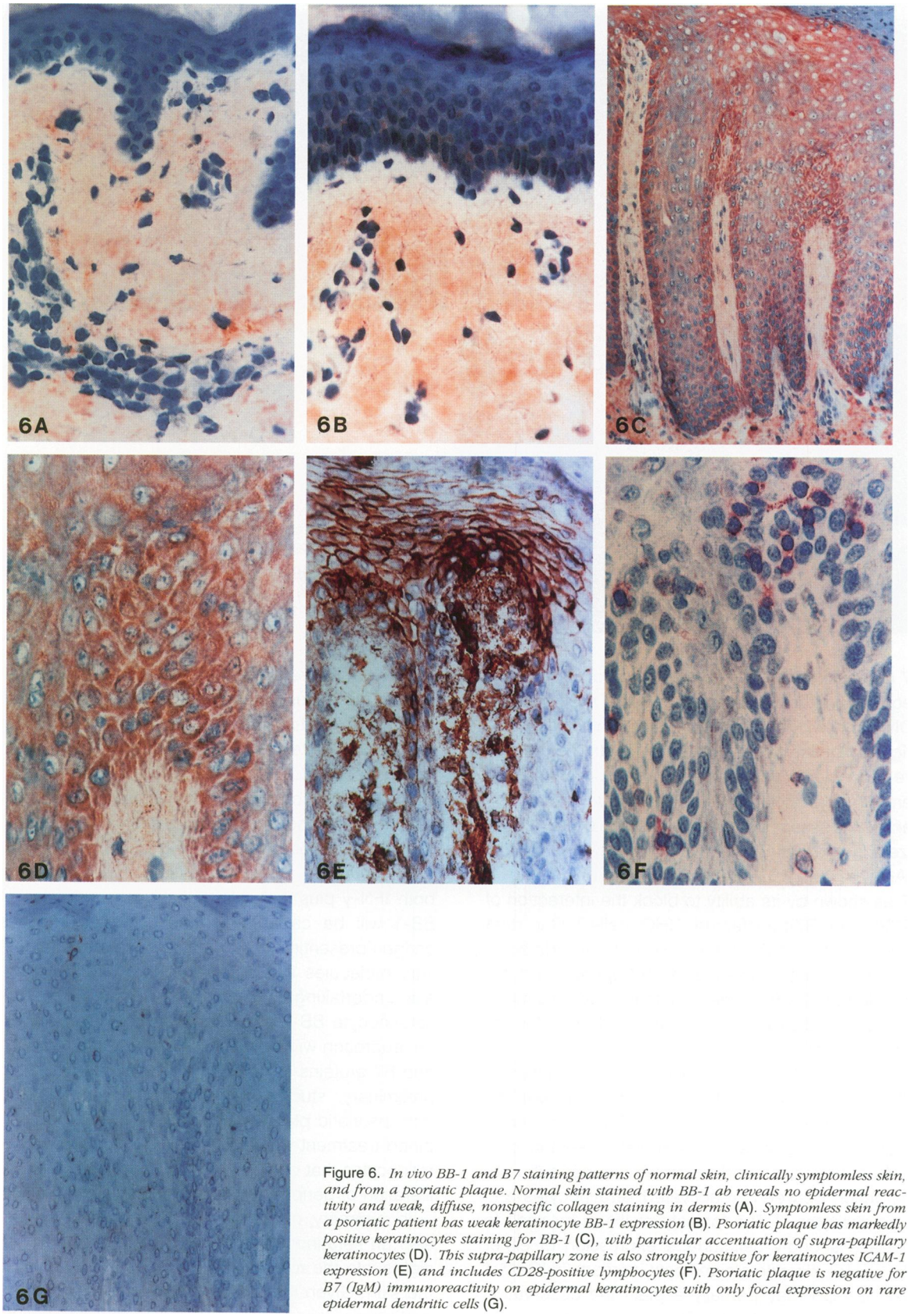

Figure 6. *In vivo BB-1 and B7 staining patterns of normal skin, clinically symptomless skin, and from a psoriatic plaque. Normal skin stained with BB-1 ab reveals no epidermal reactivity and weak, diffuse, nonspecific collagen staining in dermis* (A). *Symptomless skin from a psoriatic patient has weak keratinocyte BB-1 expression* (B). *Psoriatic plaque has markedly positive keratinocytes staining for BB-1* (C), *with particular accentuation of supra-papillary keratinocytes* (D). *This supra-papillary zone is also strongly positive for keratinocytes ICAM-1 expression* (E) *and includes CD28-positive lymphocytes* (F). *Psoriatic plaque is negative for B7 (IgM) immunoreactivity on epidermal keratinocytes with only focal expression on rare epidermal dendritic cells* (G).

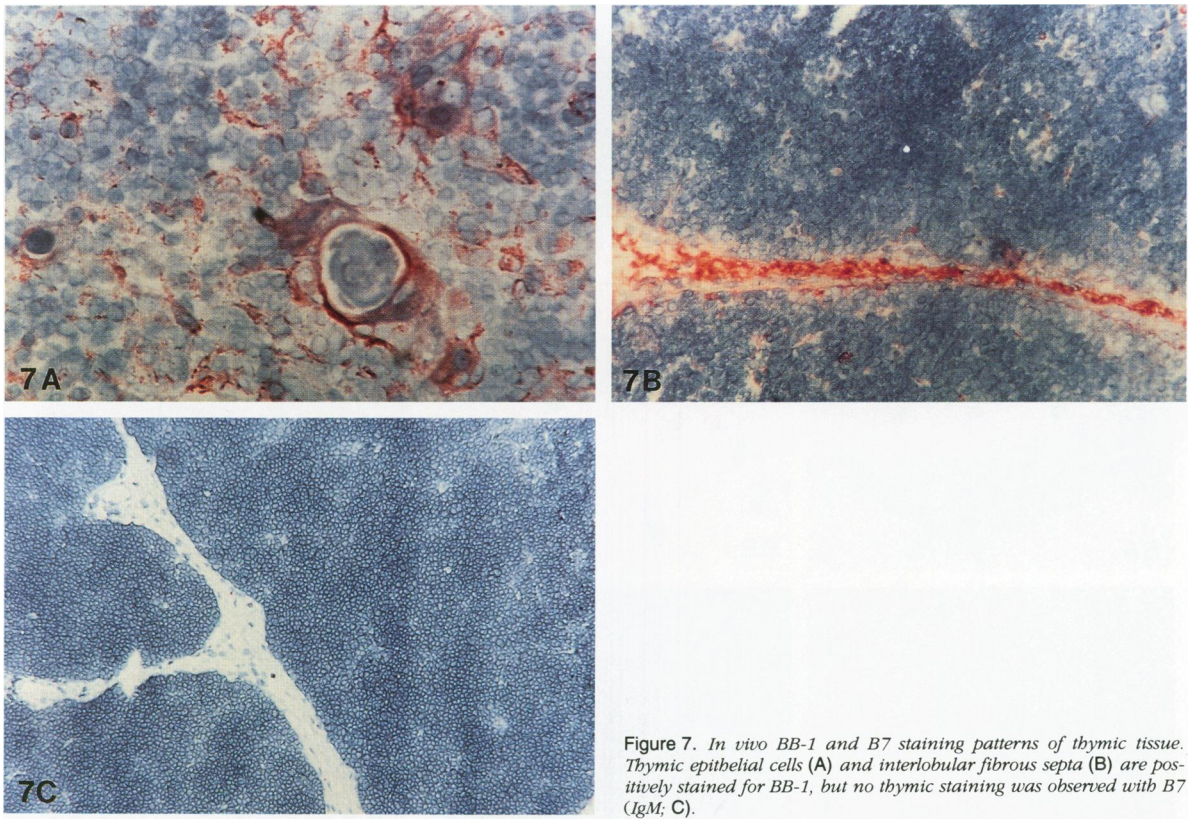

Figure 7. *In vivo BB-1 and B7 staining patterns of thymic tissue. Thymic epithelial cells* (A) *and interlobular fibrous septa* (B) *are positively stained for BB-1, but no thymic staining was observed with B7 (IgM;* C).

B7 expression by keratinocytes was confirmed by a negative Northern blot analysis using a B7-specific cDNA probe. The B7 and BB-1 molecules were originally defined by their reactivity with the respective monoclonal antibodies.[3–5] The work presented here demonstrates that there are antigenic determinants expressed by keratinocytes that are recognized by the anti-BB-1 MAb but not by the anti-B7 MAb. The anti-BB-1 MAb is capable of binding to B7 as shown by its ability to block the interaction of CD28 with B7-transfected CHO cells.[3] Our data suggest that anti-BB-1 MAb binds to an additional molecule(s) not containing the B7-epitope and not encoded by the B7 gene. Whether or not this molecule is related structurally or functionally to B7 is under investigation.

The most effective treatment for the induction of BB-1 expression by cultured keratinocytes was the combination of IFN-γ plus TPA. This same combination synergistically up-regulates keratinocyte ICAM-1 expression and promotes a super-adhesive interaction between so-activated keratinocytes and T lymphocytes.[11] The inability of IFN-γ by itself to increase strongly cell-surface expression of either BB-1 or B7, despite a significant increase in ICAM-1 and HLA-DR may partially explain the observation that IFN-γ-treated keratinocytes induce tolerance with several different antigen-specific T cell clones.[19,20] Thus, the failure of IFN-γ to induce prominent keratinocyte cell–surface expression of B7/BB-1, despite the induction of two other potent immunomodulatory molecules (i.e., ICAM-1 and HLA-DR), may contribute to the failure to activate effectively resting T cells via a critically important co-stimulatory pathway involving CD28 and B7/BB-1.[21] Whether or not keratinocytes jointly exposed to both IFN-γ plus TPA with subsequent induction of BB-1 will be capable of functioning as effective antigen-presenting cells mediated via these accessory molecules is currently under study.[22] We are also undertaking the cloning and sequencing of the keratinocyte BB-1 gene. Such a molecular biological approach will definitively establish that the BB-1 and B7 proteins are products of separate genes. In preliminary studies using keratinocytes derived from psoriatic plaques, we observed that the combined treatment with IFN-γ plus TPA resulted in keratinocytes that could significantly stimulate the proliferation of peripheral blood mononuclear cells.[23]

In summary, our studies of BB-1 and B7 expression on keratinocytes provide several important insights into the role of CD28 and T-cell activation in the skin. Given the growing body of evidence supporting an important role for CD28 in T-cell activa-

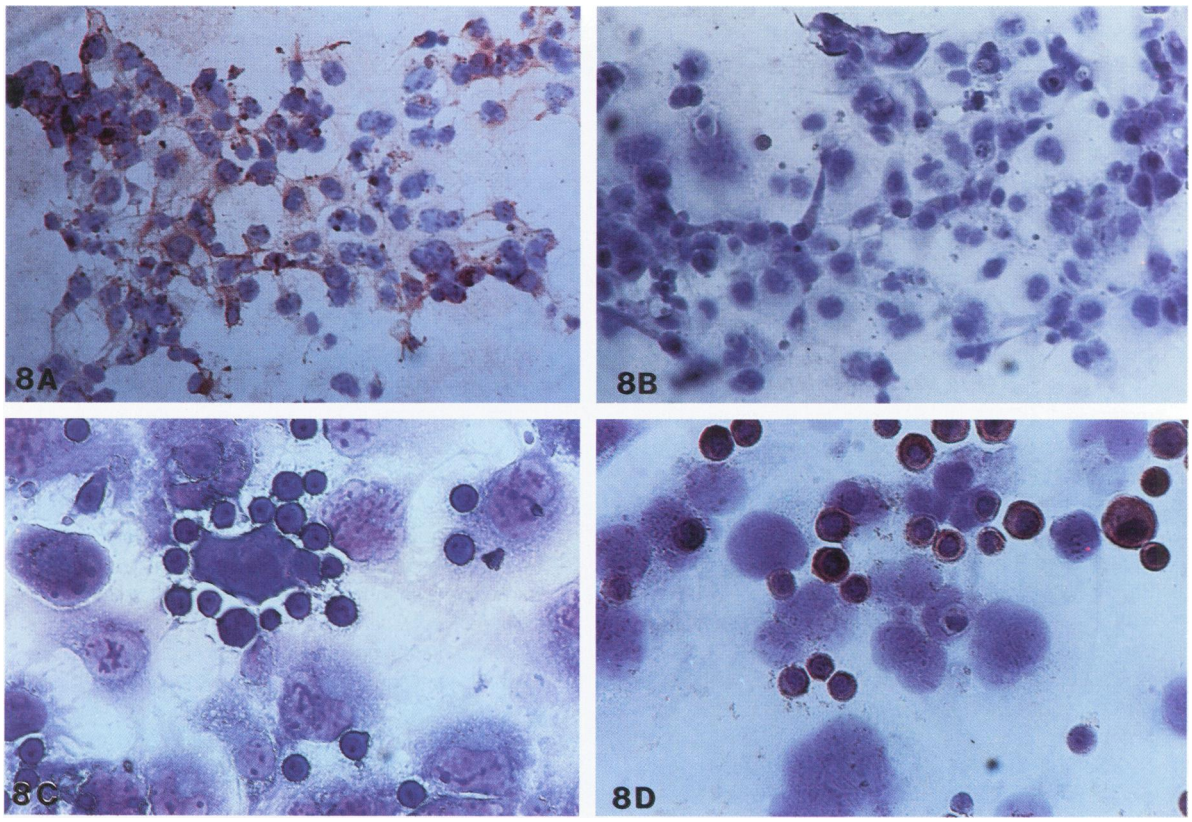

Figure 8. *Adhesion assay demonstrating BB-1-positive keratinocytes binding to CD28-positive COS7 cells. The extent of CD28 expression following successful transfection is reflected by positive immunostaining in the CD28-positive COS7 cell monolayer* **(A)**, *compared to the negative staining in the mock-transfected cells* **(B)**. *Representative adhesion results, in which small round keratinocytes bind to CD28-transfected COS7 cells* **(C)**. *By immunostaining, BB-1-positive rounded keratinocytes bind to BB-1-negative CD28-transfected COS7 cells* **(D)**.

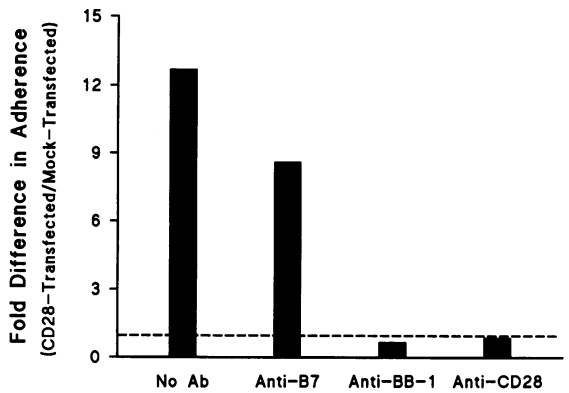

Figure 9. *Ratio of binding of keratinocytes to CD28-transfected COS7 cells compared to mock-transfected COS7 cells in the presence of antibodies (10 μg/ml) as indicated on the x axis. A representative experiment (one of three) is shown; (----) indicates a ratio of 1, the position at which there is no observed difference in the adherence of CD28-transfected COS7 cells and mock-transfected cells.*

tion[1] and the clearly discordant expression of BB-1 and B7 both *in vitro* and *in vivo* in tissues, including the thymus and cutaneous psoriatic plaques, a re-examination of the assumption that BB-1 and B7 are identical is clearly warranted. Such studies may provide a new appreciation for the immunological repertoire of the epithelial cell (i.e., keratinocyte). Because the keratinocytes within the skin provide a major protective barrier, greater understanding of how these cells can either induce antigen-specific tolerance/paralysis or activation of T cells will have enormous therapeutic implications not only for dermatologists, but also for those investigators interested in systemic T-cell-mediated disease processes.

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