Direct and Indirect Control of T-Cell Activation by Keratinocytes

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Keratinocytes can function as antigen-presenting cells/accessory cells and regulate T cells with three distinct outcomes, depending on the nature of the stimulus. In the presence of alloantigen, it appears that a "null" event takes place between T cells and keratinocytes, with neither activation nor induction of tolerance. Using nominal antigen, keratinocytes induce antigen-specific tolerance. In contrast, with bacterial-derived superantigens, phytohemagglutinin, or immobilized CD3 monoclonal antibody, keratinocytes can significantly activate resting autologous T-cell proliferation and cytokine release. To understand these highly divergent responses, we focused on the two-signal model of T-cell activation, with particular emphasis on costimulatory molecules expressed by keratinocytes. Such second signals, as highlighted by the B7 and CD28 receptor families, provide useful insights into the complex interactions involving keratinocytes and T cells. In this review, we summarize recent evidence indicating that keratinocytes regulate T-cell activation in a direct and indirect manner by their mutual expression and responsiveness involving adhesion molecules, cytokines, and costimulatory signals. As investigative momentum continues to grow in the fields of immunology and keratinocyte biology, it is likely that manipulation of CD28:B7 interactions will not only provide a useful model to understand further the complexities of skin immune reactions, but will also serve as the basis for new therapeutic opportunities for numerous T-cell–mediated diseases that involve aberrant reactions with keratinocytes. ***Key words: CD28:B7 costimulation/skin disease/superantigen/cytokines. J Invest Dermatol 105:25S–29S, 1995***

SELECTIVE RECRUITMENT AND LOCAL ACTIVATION OF T CELLS IN THE SKIN

The skin is frequently the site of numerous immune-mediated disease processes that involve prominent infiltration by T lymphocytes. During the past several years, much has been learned regarding the molecular basis for T-cell trafficking in normal and diseased skin, with particular emphasis on the role of adhesion molecules [1]. Indeed, it is currently accepted that numerous ligand-receptor interactions can be operative at multiple sites (reviewed in [2,3]). With the recognition of skin-seeking T-cell subsets [4] and the nonrandom migration pattern of T cells into the skin mediated by adhesion molecules [5], attention was directed at exploring how T cells become activated to proliferate once they enter epidermal and dermal compartments. Our principal contention is that T-cell activation and proliferation occur after selective recruitment of resting T cells within skin, rather than by nonspecific infiltration of T cells activated elsewhere, which passively enter the skin [6]. Based on this premise, we can ask: What is the molecular basis for T-cell activation/proliferation?

To answer this question, we have relied heavily on advances made by cellular immunologists working in areas outside of dermatology. There are many complex signaling mechanisms that regulate T-cell activation and proliferation. This should not be surprising, as many fundamentally important responses are governed by T-cell activation, including induction and maintenance of central and peripheral tolerance and immune responses to infectious agents, exogenous allergens, tumor antigens, etc. [7]. A review of all of these topics cannot be presented herein; rather, we highlight one specific regulatory event involving the role of antigen-presenting cells (APCs) in T-cell activation in normal and diseased skin. In the past, most investigators were involved with defining the number, distribution, and type of lymphocytes (i.e., helper/inducer *versus* cytotoxic/suppressor; CD4 *versus* CD8; naive *versus* memory; TH1 *versus* TH2, etc.) rather than the type of APC that accompanies the T-cell infiltration. We take a more proximal perspective, suggesting that the APC is the key factor that directs and regulates whether and how T cells respond to exogenous or endogenously derived antigens responsible for initiating skin immune reactions.

HISTORIC PERSPECTIVE FROM A DERMATOLOGIC VIEWPOINT ON REGULATION OF T-CELL ACTIVATION

Immunodermatologists initially seized upon the two-step model for T-cell activation by focusing on the role of constitutive human leukocyte antigen (HLA)-DR expression by epidermal Langerhans cells and the role of keratinocyte-derived epidermal thymocyte-activating factor [8]. As most investigators recognized at the time, Langerhans cells were portrayed as acquiring antigen and expressing it to appropriate T cells in the context of HLA-DR. However, it was recognized early that this so-called signal 1 required an additional signal if T-cell proliferation was to follow, and this was portrayed as being delivered in the form of a soluble signal 2 (i.e., interleukin-1 [IL-1] [9]). This two-step model was gratifying

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Abbreviations: APC, antigen-presenting cell; MLR, mixed lymphocyte reaction.

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because it suggested an immunologic dialogue involving three different cell types that was often seen in anatomic juxtaposition: Langerhans cells:keratinocytes:T cells. From this vantage, keratinocytes were promoted from their previously unappreciated immunocytic function as a passive/inert target of immune-mediated diseases to an active participant producing the critical cytokine IL-1.

Even though T-cell antigen receptor (TCR) occupancy is necessary, T-cell proliferation will not occur in the vast majority of normal T cells unless a costimulus is also present. For dermatologists interested in studying T-cell activation, the situation is frustrating if one dwells on signal 1 only because the nature of the antigen in many T-cell–mediated diseases is unknown. However, by focusing on costimulatory signals, investigators gained several valuable new insights with implications beyond skin disease. Before delving into these new discoveries, we will briefly review the roles of cytokines and non-cytokines in providing costimulation. It has been established that IL-1 can function as a costimulatory molecule (signal 2) in some murine IL-4–producing CD4 T-cell clones [10]. However, it is becoming clear that IL-1 or other keratinocyte-derived cytokines cannot provide signal 2 for many different types of IL-2–producing T-cell clones [11]. Such TH-1–type cells (which have been implicated in psoriasis, for example [12,13]) require something different from a soluble cytokine to be present when signal 1 is delivered to the CD3/TCR to the resting T lymphocytes. This alternate signal 2 is present in the form of a cell-surface adhesion molecule on an APC, rather than as a secreted soluble cytokine. The best-characterized costimulatory system involved in APC:T-cell interaction is mediated by B7:CD28 (reviewed in [14]). We are not suggesting that cytokines are not important, but they probably act in a more distal fashion, and together with more proximal signaling events involving surface molecules, provide the most comprehensive way of understanding the complex multistep process for optimal T-cell activation.

The CD28 molecule is a member of the immunoglobulin gene superfamily that is expressed by 95% of CD4+ T lymphocytes and 52% of CD8+ T lymphocytes obtained from human peripheral blood [15]. When the CD28 molecule becomes engaged, a unique signal-transduction pathway synergistically interacts with TCR/CD3 receptor stimulation to produce maximal cell proliferation and lymphokine secretion [14]. An important advance in understanding the role of CD28 in T-cell function occurred when CD28 ligand was identified [16]. Two antibodies were initially used to detect the CD28 ligand (i.e., B7 and BB-1), and were widely believed to bind to an identical epitope [17,18]. However, as described in the next section, it is now appreciated that there are multiple CD28 ligands, referred to as B7-1 (previously known as B7; CD80), B7-2 (CD86), and B7-3 (previously labeled BB-1) [19,20]. It has been suggested that B7-2 may be a more relevant stimulatory ligand for CD28 than is B7-1 [21–23].

STUDIES OF KERATINOCYTES AS APCs REVEAL DISCORDANT PATTERNS OF EXPRESSION OF B7-1 AND B7-3

Our interest in the APC function of keratinocytes actually dates back a decade to the original observation that although interferon-gamma (IFN-γ) could induce keratinocytes to express HLA-DR and HLA-DQ, these same keratinocytes failed to stimulate a significant allogeneic T-cell proliferative response [24]. This surprising negative result was confirmed by several other groups and suggested that keratinocytes may lack the capacity to provide an appropriate second signal to complement the initial delivery of alloantigen signal 1 in the context of IFN-γ–inducible major histocompatibility complex (MHC) class II molecules. Thus, we obtained a full-length cDNA probe for B7-1 (provided by Dr. Lee Nadler, Boston, MA) and used Northern blot hybridization to examine cultured keratinocytes that had been activated by a wide variety of cytokines and a tumor promoter, 12-0-tetradecanoylphorbol-13 acetate (TPA), without induction of a B7-1 transcript [25]. As part of these studies, we used antibodies to detect the proteins B7-1 and B7-3. Using several different antibodies against B7-1, we found no significant expression by keratinocytes, either *in vitro* or *in vivo*, confirming the mRNA analysis [25]. However, using the anti–BB-1 antibody, we noted several differences in the pattern of B7-3 expression. First, cultured keratinocytes exposed to IFN-γ alone increased B7-3 expression, with further enhancement seen by combining IFN-γ plus TPA, as detected by flow cytometry. Second, *in vivo* immunostaining revealed that normal human skin epidermal keratinocytes did not stain, but psoriatic plaques contained B7-3–staining keratinocytes. Third, thymic epithelial cells also stained for B7-3. These latter results suggested that B7-3 was different from B7-1 and raised the question as to whether BB-1 keratinocytes were capable of recognizing or mediating binding *via* CD28.

To address this issue, we performed adhesion assays in which keratinocytes expressing BB-1 were overlaid on COS7 cells that had been either mock transfected or transfected with CD28. The results were clear: keratinocytes would bind only to the CD28-positive COS7 cells (and not to the mock transfectants), and this binding was inhibited only by antibodies against CD28 or BB-1, but not against B7-1. Thus, we concluded that B7-1 and B7-3 were not identical, as had been thought previously, and that keratinocytes could express a cell-surface molecule capable of recognizing CD28. On one hand, it appeared that the lack of allostimulating capacity of keratinocytes could be related to their lack of B7-1, but on the other hand, the immunologic significance of B7-3 expression was unclear. The next series of experiments was initiated to reexamine the APC function of keratinocytes with a particular emphasis on the CD28:B7 pathway. In the following two sections, we review the results for each set of experiments, beginning with the immunologic consequences of B7-1 expression by keratinocytes and in the second section examining the APC capacity of keratinocytes.

TRANSFECTION OF B7-1 INTO KERATINOCYTES

To prove that the lack of alloantigen reactivity of keratinocytes was due to an absence of signal 2 (i.e., B7-1), we transfected an immortalized keratinocyte cell line (HaCat) with B7-1 and determined the allostimulatory capacity of these cells before and after IFN-γ exposure.† Cells were transfected with a B7-1 vector, and the B7-1–expressing cells were sorted by flow cytometry after a 2-d incubation with IFN-γ **(Fig 1)**. Thus, the cells of interest were B7-1 positive and expressed class II MHC antigens and intercellular adhesion molecule-1 (ICAM-1). Compared with mock-transfected cells, B7-1–positive cells provoked an allogeneic T-cell response, but this reaction was not particularly dramatic when compared with other mixed lymphocyte reactions (MLRs) using monocytes/macrophages or dendritic cells. A typical result is shown in **Fig 2**. It should be noted that very similar results were observed independently with different cell lines [26]. We have continued to try to determine why these B7-1–positive keratinocytes were not particularly potent in MLRs and proposed that they may have possessed even more potent allostimulatory capacity if we could have done the following: 1) neutralize the IL-10 they produce, which inhibits the MLR; 2) transfect in the more potent CD28 ligand B7-2 rather than B7-1; 3) transfect in the invariant chain (CD74) used for processing of class II MHC-associated peptides [27,28]; and 4) further enhance their class II MHC levels, as a previous report described relatively low levels of HLA-DR expressed after IFN-γ treatment of keratinocytes compared with professional APCs [29]. We now turn from the issue of lack of alloreactivity to autologous keratinocyte:T-cell interactions.

KERATINOCYTES CAN FUNCTION AS APCs USING SEVERAL DIFFERENT T-CELL MITOGENS

To examine the possible APC role for B7-3 on keratinocytes, we asked whether they could serve as accessory cells, in which T-cell

† Nickoloff BJ, Mitra RS, Turka LA: Transfection of CD28 ligand B7-1 and anti-IL-10 mAb are important for allogeneic T cell response to IFN-γ treated keratinocytes (abstr). *J Invest Dermatol* 102:583, 1994.

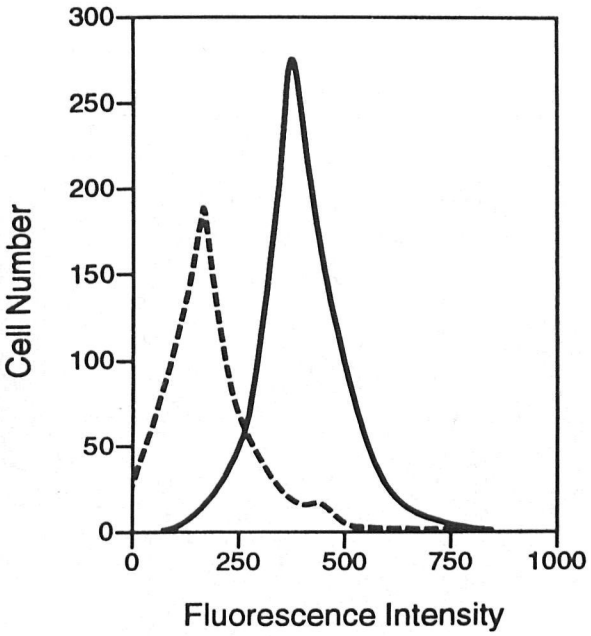

Figure 1. Keratinocytes can be transfected to express B7-1. Flow cytometric profile of IFN-γ–treated (500 U/ml; 48 h) keratinocytes analyzed for B7-1 expression. Before transfection (*dashed line*), keratinocytes are devoid of B7-1 cell-surface expression. However, after cotransfection using a full-length cDNA of B7-1 as well as a neomycin resistance gene, and positive selection, a uniform population of B7-1–positive keratinocytes (*solid line*) was obtained. These IFN-γ–treated, B7-1–transfected cells were also strongly positive for HLA-DR and ICAM-1 (data not shown).

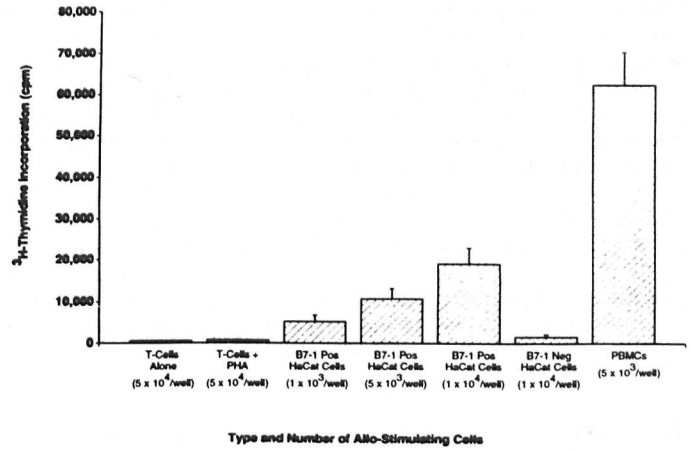

Figure 2. B7-1–transfected keratinocytes stimulate an allogeneic T-cell proliferative response that is greater than that of mock-transfected B7-1–negative keratinocytes. T cells, 5×10^4, were combined with either PHA (to confirm purity of negative-selection procedure with removal of all accessory cells) or with various numbers of allogeneic stimulator cells, as described previously [30]. IFN-γ–treated HaCat cells (500 U/ml; 48 h) were positively sorted using an Epics V flow cytometer (see **Fig 1**) and combined with responder T cells. Note that the B7-1–transfected keratinocytes significantly stimulated ($p < 0.01$) allogeneic T cells to proliferate (6-d coculture; 1 μCi/well ^{3}H-thymidine added for final 6 h before harvesting and liquid scintillation counting) when used at either 1×10^3, 5×10^3, or 1×10^4 cells/well. In contrast, no significant ($p > 0.05$) allogeneic T-cell proliferation was seen using the IFN-γ–treated, B7-1–negative HaCat cells. Note that when 4×10^3 irradiated allogeneic Ficoll-Hypaque interface peripheral blood mononuclear cells (PBMCs) were used as stimulators, a much stronger MLR was observed compared with the keratinocyte reactions. T-cell proliferation data (±SD) are representative of three independent experiments.

signal 1 was provided by either phytohemagglutinin (PHA), bacterial-derived superantigens, or immobilized CD3 monoclonal antibody (MoAb) [30]. Compared with the inability of allogeneic IFN-γ–treated keratinocytes to provoke a strong allostimulatory reaction and the tolerogenic capacity of keratinocytes with nominal antigen [31,32], there was vigorous T-cell proliferation when T cells were incubated with either PHA, superantigens, or immobilized anti-CD3 MoAb in the presence of IFN-γ–treated keratinocytes. Our primary focus was staphylococcal enterotoxins A and B because of the association between bacterial infection and psoriasis [33]. Such bacterial-derived superantigens provided a plausible association between infection in the pharynx or at other extracutaneous sites and skin eruptions mediated by T cells. Superantigens by themselves cannot activate T cells, but when IFN-γ–treated keratinocytes are added, T-cell proliferation and cytokine release ensue [30,34]. Using various blocking reagents, it was determined that this T-cell stimulation was mediated primarily by LFA-1:ICAM-1 interaction. In fact, none of the T-cell proliferation observed using keratinocytes as accessory cells was influenced by inhibitory reagents targeting the B7-1:CD28 molecules. This led us to suggest that the B7-3 expressed by keratinocytes may either play no role or may provide a nonstimulatory signal that is overcome by the positive signal generated *via* either LFA-1:ICAM-1 or LFA-2:LFA-3 interactions.

Another currently unresolved issue involves the nature of the second signal provided by the keratinocytes when T cells have signal 1 delivered *via* PHA, immobilized anti-CD3 MoAb, or superantigens. The lack of involvement of the CD28:B7 family of molecules implies the existence of additional costimulatory pathways, and we are currently characterizing such molecular interactions. Because keratinocytes can mediate antigen-specific tolerance [31,32], it is possible that modifications are needed to the current dogma that long-standing unresponsiveness to nominal antigen is due to delivery of signal 1 without signal 2 [10,11]. Our modification would include consideration of a model in which signal 1 (being nominal antigen) is followed by an inappropriate or nonactivating signal 2 (i.e., *via* B7-3 rather than B7-1 or B7-2), which would result in a functional state of anergy or tolerance. Recent unpublished observations by Dr. Lee Nadler at the Dana Farber Cancer Institute (Boston, MA) have demonstrated the existence of a non–B7-1 or –B7-2 molecule that can actually trigger apoptosis in an antigen-specific manner [35]. Work is currently in progress to determine whether keratinocyte B7-3 can mediate programmed cell death of specific subsets of T cells. In any event, it should be clear that new insights into T-cell activation have been derived from studies designed to address costimulatory signaling pathways involving keratinocyte:T-cell interactions [28].

In studies of bacterial-derived superantigens and keratinocytes, the T-cell response was found to resemble most closely a TH-2–type cytokine production profile, with high levels of IL-4, IL-5, and IL-10, but not IFN-γ [34]. With recognition of the importance of IL-12 in generating a TH-1–type response [36], it was necessary to determine whether keratinocytes could produce IL-12. By reverse transcriptase-polymerase chain reaction analysis, resting or activated cells were found to express the p35 mRNA species of IL-12 but not the inducible p40 mRNA species, and no functional protein was detected in the supernatant by a sensitive enzyme-linked immunosorbent assay [34]. However, if as little as 1 ng/ml of recombinant human IL-12 was added to the superantigen-driven T-cell response in the presence of keratinocytes, approximately 2–9-fold higher levels of IFN-γ were produced. Moreover, when professional APCs (i.e., dermal dendritic cells or blood-derived macrophages) were used rather than the nonprofessional APCs (i.e., keratinocytes), with the same superantigens and the same responder population of T cells, high levels of IFN-γ were produced with little or no IL-4, IL-5, or IL-10 [37]. Another group has observed that the presence or absence of B7 on the APCs dictated whether a TH-1–type or TH-2–type cell response was produced

[38]. Thus, the qualitative nature of the T-cell response (TH-1 *versus* TH-2) was significantly influenced by the presence or absence of IL-12 and by the type of APC used in providing costimulating signals [28]. Cultured dermal dendritic cells can produce IL-12 mRNA, as detected by reverse transcriptase-polymerase chain reaction (Nestle FO, Nickoloff BJ, unpublished results), and apparently so can epidermal Langerhans cells.‡ IL-12 is also important because it can synergize with B7:CD28 interactions in the promotion of T-cell proliferation and cytokine release [39,40]. All of the available data support the view that proximal molecular events involve direct cell-cell interaction between T cells and APCs, mediated by CD3/TCR and costimulatory pathways, followed by the modulatory and more distal effects of cytokines that are present in the local microenvironment.

SUMMARY

Once a resting blood-derived T lymphocyte enters the skin, it comes within the immunologic sphere of influence [41] of several endogenous and recruited cell types, including keratinocytes, dendritic cells, and macrophages. Each of these APCs can regulate or determine whether the T cell becomes activated or tolerogenized by distinctive expression of various cell-surface molecules and cytokines. Given the available techniques for isolation and characterization of each individual APC:T-cell reaction *in vitro*, we can begin to determine hierarchic patterns of costimulatory molecule expression and cytokine production profiles. Although our current understanding of cutaneous T-cell interactions is incomplete, it is clear that keratinocytes function quite differently from professional APCs in their interaction with T lymphocytes [28]. Such interactions are dependent on several variables, including the nature of the antigen (alloantigen, nominal, or superantigen), the presence or absence of cytokines such as IL-10 or IL-12, and the nature of the costimulatory molecular pathway used to deliver signal 2 (CD28 interaction with either B7-1, B7-2, or B7-3). In ongoing studies, it appears that even within professional APC subsets such as macrophages and dendritic cells, important differences can be detected in relative levels of expression of costimulatory molecule and cytokine release. For example, keratinocytes express B7-3, but not B7-1 or B7-2, and they can produce IL-10 but not IL-12. Dermal dendritic cells produce high levels of B7-2 with lower levels of B7-1, and these CD28 ligands are differentially regulated in both a positive and negative fashion by various cytokines, such as tumor necrosis factor-α, granulocyte macrophage–colony-stimulating factor, IL-4, and IL-10 [42]. On the other hand, macrophages express much lower levels of B7-2 compared with dermal dendritic cells and also produce IL-10. **Figure 3** summarizes the key differences with respect to costimulatory molecule expression and cytokine production profile.

In conclusion, it is clear that keratinocytes should be added to the list of potentially important APC/accessory cells resident in the skin. Besides dendritic cells and macrophages, which are present in both epidermal and dermal compartments [43], keratinocytes can be distinguished by their expression of various adhesion molecules, costimulatory molecules, and patterns of cytokines produced upon activation. Keratinocytes can therefore directly and indirectly regulate resting T-cell proliferation and activation, with both positive and negative consequences. Compared with professional APCs such as Langerhans cells, keratinocytes express lower levels of HLA-DR and costimulatory molecules and cannot process antigens, thus indicating that in some circumstances they may be less efficient APCs than Langerhans cells. However, given the much greater number of keratinocytes compared with Langerhans cells, it remains to be determined which APC contributes the most effective T-cell–stimulatory signals and the net result of local T-cell activation. Our challenge for future studies will be to try to manipulate these different APC types in a specific manner to help us further understand the immunoregulatory circuits that exist to restore homeostasis and thereby benefit our patients with T-cell–mediated skin disorders.

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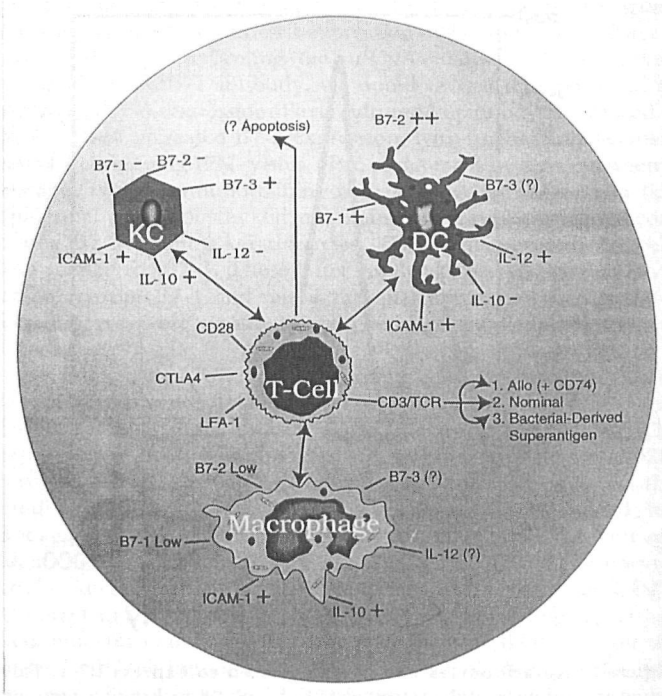

Figure 3. T-cell interactions with APCs in the skin are complex, multifactorial, and bidirectional. The cutaneous sphere of immunologic influence of T lymphocytes by keratinocytes, dendritic cells, and macrophages is mediated *via* differential expression of specific costimulatory molecules and cytokines. Once a resting skin-seeking T lymphocyte enters the dermis or epidermis, it may become activated by reciprocal interactions with various types of APC. Each APC can differentially influence the T cell after the engagement of the CD3/TCR complex (signal 1) by providing distinctive costimulatory molecules (signal 2) and contributing locally produced cytokines. Depending on the nature of the stimulus, the type of APC, and the local cytokine milieu, there are several different potential immunologic outcomes that reflect whether the T cell becomes activated, anergized, and/or begins producing TH-1– and TH-2–type cytokines itself. The most important signaling pathways are portrayed to emphasize the multiplicity of adhesion molecules, costimulatory molecules, and cytokines that have been characterized for these cellular constituents of the dermal and epidermal immune systems.

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