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Murine Epidermal Cell Antigen (Skn)-Directed Autoimmunity Induced by Transfer of CD4$^+$ T Cells

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Abstract. While pathogenic T cells have been identified for several diseases with epithelial cell damage, an autoimmune T cell-mediated response targeted against a known keratinocyte antigen has not been reported. Previously we described an autoimmune response directed to the mouse epidermal cell antigens, Skn. For our murine model, primed Skn-immune lymphocytes are adoptively transferred to recipients, which develop lesions at the site of mild skin trauma. In this study we investigated the nature of the autoimmune component of the Skn response. A time-course study demonstrated a relationship between the number of primed Skn-immune cells injected and the severity of skin lesions in the recipients. Immunohistochemical staining revealed the presence of both CD4$^+$ and CD8$^+$ T cells in lesional skin, with a predominance of CD4$^+$ T cells. To support a role for CD4$^+$ T cells in the initiation of the autoimmune response, Skn-immune donor cells were either enriched or depleted of various subsets prior to transfer into recipients, which showed that CD4$^+$, but not CD8$^+$, T cells were essential for induction of lesions. Analysis of mRNA for T-helper (Th) cell cytokines in lesional skin displayed a Th1 bias, and treatment with cyclosporin A (CsA) or anti-interleukin (IL)-2 antibody controlled the development of lesions. Overall the results clearly show an immunopathogenic profile consistent with a T cell-mediated mechanism. *(received 13 October 2002, accepted 22 December 2002)*

Keywords: CD4 T cells, Th1 cytokines, keratinocytes, autoantigens, autoimmunity

Introduction

Autoimmune disease in humans is estimated to affect at least 2% of the population in the United States. Sometimes, however, immune reactivity can cause tissue damage without signs that the disease is caused by a response to self antigen. We have previously described an autoimmune response directed to the skin in mice that targets the self epidermal cell antigens, Skn [1].

Skn antigens are localized selectively to epidermal cells [2-4] and neuronal cells [2,5]. They are encoded by 2 unlinked genes, *Skn1* and *Skn2* [6], each with alternative alleles expressed as allo-antigens in various mouse strains [7]. Monoclonal antibody produced to *Skn2* identifies a 95 kD protein [5] that is likely to have a human equivalent, since anti-*Skn2* antibody recognizes a similar sized molecule in human epidermal cells (Goldberg and Rees, personal communication).

Previous work indicates that Skn immunization of donors is important for the transfer of disease. Lymphocytes from donors primed to Skn antigens (referred to as Skn-immune cells) cause lesions in recipient mice, while lymphocytes from donors immunized to an irrelevant (non-Skn) antigen do not [1]. Allelic specificity is demonstrated by the finding that recipients of Skn-immune cells, normally tolerant of grafts with either set of Skn alleles, become able to reject grafts that express the priming alleles [1]. This indicates that the anti-Skn response requires Skn-allele-specific autoimmune cells and does not result from any pretreatment procedures per se.

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Because many organ-specific autoimmune diseases of humans are thought to be caused predominantly by T cells, the aim of this study was to evaluate more fully the phenotype of the immune contribution required for initiating Skn autoimmune pathogenesis. We found that $CD4^+$ T cells were essential for the adoptive transfer of Skn autoimmunity and that the Th-cell cytokine profile in lesional skin had a Th1 bias . These results were supported by the finding that treatment with cyclosporin A (CsA) or anti-interleukin (IL)-2, but not anti-IL-4, antibody controlled the development of lesions.

Materials and Methods

The Institutional Animal Care and Use Committee of Marshall University approved all experiments.

Mice. Female C57BL/6J (B6), A/J (A), and B6AF1/J (B6AF1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a conventional animal facility. Sentinel mice were used to screen for various pathogens and these tests were consistently negative.

Donor lymphocytes. Because Skn congenic mice are not available, hematopoietic radiation chimeras were produced and immunized as previously described [1] to induce a specific response to Skn alleles found on A strain mouse skin (*Skn1.1, Skn2.1*) and to avoid a response both to the alternative Skn alleles (*Skn1.2, Skn2.2*) and to major histocompatibility complex (MHC) antigens. Spleen cells from these chimeras were used as the source of donor anti-Skn lymphocytes (Skn-immune cells) for adoptive transfer into B6AF1 recipients.

Pretreatment of recipients before adoptive cell transfer. As previously described [1], B6AF1 recipients (age 4-8 mo) were pretreated with mild epidermal trauma by shaving, and with immunosuppression by an iv injection of cyclophosphamide (200 mg/kg body wt, NEOSAR, Adria Laboratories, Columbus, OH). Epidermal trauma was performed one day (day -1) and immunosuppression was delivered 4 hr before adoptive cell transfer (day 0).

Adoptive cell transfer and evaluation of lesions. As previously described [1], spleen cells from immunized chimeras (Skn-immune cells) were dispersed into a single cell suspension, washed, and adjusted to the appropriate cell number in PBS. B6AF1 recipients received the designated number of Skn-immune cells delivered iv in 0.2 ml PBS (day 0). Control B6AF1 recipients received spleen cells from syngeneic B6AF1 donors (naive spleen cells) or were not injected with cells; both groups of control animals showed identical results.

All recipients were examined daily for gross cutaneous lesions and a clinical score was given on a scale of 0-5 as follows: 1 = shiny patches; 2 = shiny patches with scaling; 3 = crusts and/or erythema, area affected covering <50% of shaved area; 4 = similar to grade 3, area affected >50% but less than total shaved area; 5 = similar to grade 3, area affected covering total shaved area.

Two persons, one of whom was unaware of the specific treatment, examined the mice for lesions. At various times after cell transfer, skin sections were stained with hematoxylin and eosin and histopathology was analyzed in a blinded fashion by a dermatologist and a pathologist, who each evaluated slides from 6-10 experimental mice and 5-6 controls at the specified time points.

Immunohistochemistry. Skin tissue was stained as previously described [8], except that methyl green was used as a counterstain. Primary antibodies were rat anti-CD4 (clone GK1.5) and rat anti-CD8 (clone 53-6.7), purchased from BD Biosciences (San Diego, CA). Tissue sections used as control samples were treated with PBS instead of the primary antibody and gave negative results. Two of the co-authors independently evaluated each slide.

Immunomagnetic separation of T cells. Spleen cells from immunized chimeras were separated into lymphocyte subsets by immunomagnetic separation using streptavidin-coated BioMag beads (Advanced Magnetics, Cambridge, MA). Single cell suspensions were incubated with saturating concentrations of rat anti-CD4 or rat anti-CD8 (clones as above) for 30 min at 4°C, washed, and then incubated with biotinylated-anti-rat IgG (Vector Laboratories,

Burlingame, CA) for 30 min. After washing, the cells were incubated with BioMag beads for 1 hr at 4°C (70 beads/target cell).

For negatively selected cells, the antibody-coated population was removed by exposing the cell suspension to a magnetic field (BioMag Separator, Advanced Magnetics) and the non-bound cells were eluted. For positively selected cells, the bound cells were collected and the BioMag beads were removed by culturing the cells overnight in RPMI 1640 supplemented with 10% fetal calf serum, 1% nonessential amino acids, 20 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.1 mM Na pyruvate (Life Technologies Inc., Rockville, MD).

Purity of depleted or enriched T cell populations was verified by flow cytometry after staining the cells with FITC-conjugated anti-CD8 and PE-conjugated anti-CD4 mAbs (Caltag Laboratories, San Francisco, CA).

RNA extraction and RT-PCR. Tissues were homogenized with a Tekmar SDT Tissumizer (Tekman, Cincinnati, OH) and total cellular RNA was extracted using a Micro-Scale Total RNA Separator Kit (Clontech Laboratories, Palo Alto, CA) or with TRIzol Reagent (Life Technologies Inc.) according to the manufacturer's instructions. RNA was stored at -70°C.

cDNA was synthesized from 1 μg total RNA as previously described [8]. Five microliter aliquots of the cDNA reaction products were amplified by PCR for 35 cycles as previously described [8].

PCR products were analyzed by electrophoresis on 3% NuSieve/1% SeaKem (FMC Bioproducts, Rockland, ME) agarose gels and visualized by ethidium bromide staining. Optical densities of the bands were obtained from scanned prints or directly from stained gels using the EDAS 120 System (Eastman Kodak Co., Rochester, NY). The relative level for each cytokine was calculated as OD_{260} for the cytokine band ÷ OD_{260} for a β-actin band.

Primers were purchased from Clontech Lab or synthesized at our institution's DNA Core Facility.

In vivo administration of monoclonal antibody (mAb) or cyclosporin A (CsA). The mAbs to cytokines were delivered by ip injection at 20 μg/day in PBS on days 1 to 6 after the adoptive transfer of Skn-immune cells. The total dose given to each animal was chosen from a study that reported an enhanced T cell-mediated response in a time period similar to the anti-Skn response [9]. Control animals received an equivalent volume of PBS.

CsA was prepared in olive oil and injected ip (10 mg/kg body wt/day) on the specified days. Control mice received an equal volume of olive oil. Because all control reagents gave similar results, the data have been combined. Purified rat anti-mouse IL-2 and IL-4 mAbs (clone S4B6 or 11B11, respectively, without stabilizers or preservatives) were purchased from BD Biosciences/PharMingen Co (San Diego, CA). Cyclosporin A was a product of Novartis Pharmaceuticals Corp (East Hanover, NJ).

Statistics. Data for cytokine mRNA were analyzed by the Mann-Whitney rank sum test or the paired t-test. In vivo treatments were compared by the non-paired t-test. Significance was defined as $p < 0.05$.

Results

Donor Skn-immune cells have a controlling role in the development of autoimmune skin lesions. To demonstrate that Skn-immune cells have a role in directing the autoimmune response, various doses of transferred Skn-immune lymphocytes were injected and the time-course of lesion development and severity was followed. As shown in Fig. 1, the increasing mean clinical scores over time generally correlated with the number of autoimmune cells transferred, while animals injected with 100 $x10^6$ naive B6AF1 spleen cells did not form lesions (data not shown).

On days 10 and 13 post transfer, clinical scores were significantly different for animals receiving the highest and lowest transfer inocula. For subsequent experiments, a dose of 50 $x10^6$ Skn-immune cells was used, because, with minor exceptions (<1%), all mice developed lesions in the shaved area of skin and the daily progression of lesion formation could easily be monitored. In these animals, scaly lesions appeared by days 4 to 6 post-transfer, with erythema and crusts around days 8 to 10 (Fig. 2a).

Microscopic examination of lesional skin revealed various epidermal abnormalities, including hyperkeratosis, parakeratosis, and progressive acanthosis. Most mice with lesions that scored ≥ 3+ had pusular scale crusts and/or areas of ulceration, a thickened dermal layer, and the presence of leukocytic infiltrates mainly in the dermis and subcutis (Fig. 2b). The infiltrates consisted of polymorphonuclear and mononuclear cells. In contrast, cutaneous alteration and a cellular infiltrate were not observed in controls that had undergone the pretreatment conditions of shaving plus single-dose immunosuppression with cyclophosphamide, but were not injected with Skn-immune cells (Fig. 2c).

CD4⁺ T cells predominate in skin lesions. Immunohistochemical analysis was performed to determine the presence and phenotype of T cells in the cellular

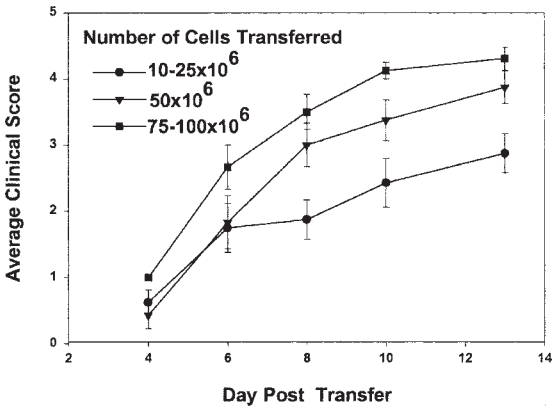

Fig. 1. Severity of lesions correlates with the number of Skn-immune cells transferred. Recipient mice pretreated by shaving and immunosuppression with cyclophosphamide were injected iv with different doses of Skn-immune cells. The clinical scores of recipients were recorded for the days indicated. Results are reported as mean ± SE of groups of 6-13 mice per time point and are combined data from 5 experiments

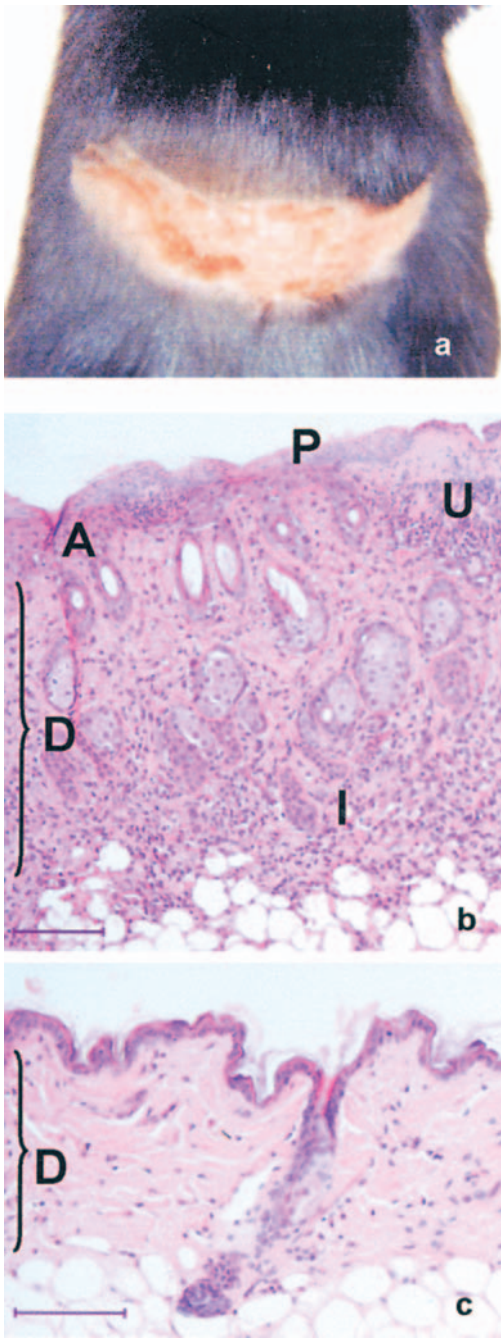

Fig. 2. Lesions in mice receiving Skn-immune cells. Recipient mice, pretreated by shaving and immunosuppression with cyclophosphamide and injected with $50x10^6$ Skn-immune cells, develop lesions confined to the shaved portion of skin. (**Panel a**) A representative mouse at clinical score 4 depicts the erythema and crusts that develop in the shaved area. (**Panel b**) H&E-stained section of clinical score 4 shows epidermal alterations (A, acanthosis; P, parakeratosis; U, ulcer), thickened dermis (D), and inflammatory infiltrate (I). (**Panel c**) The skin of mice pretreated but not injected with Skn-immune cells was normal. Scale bar = 50 μm.

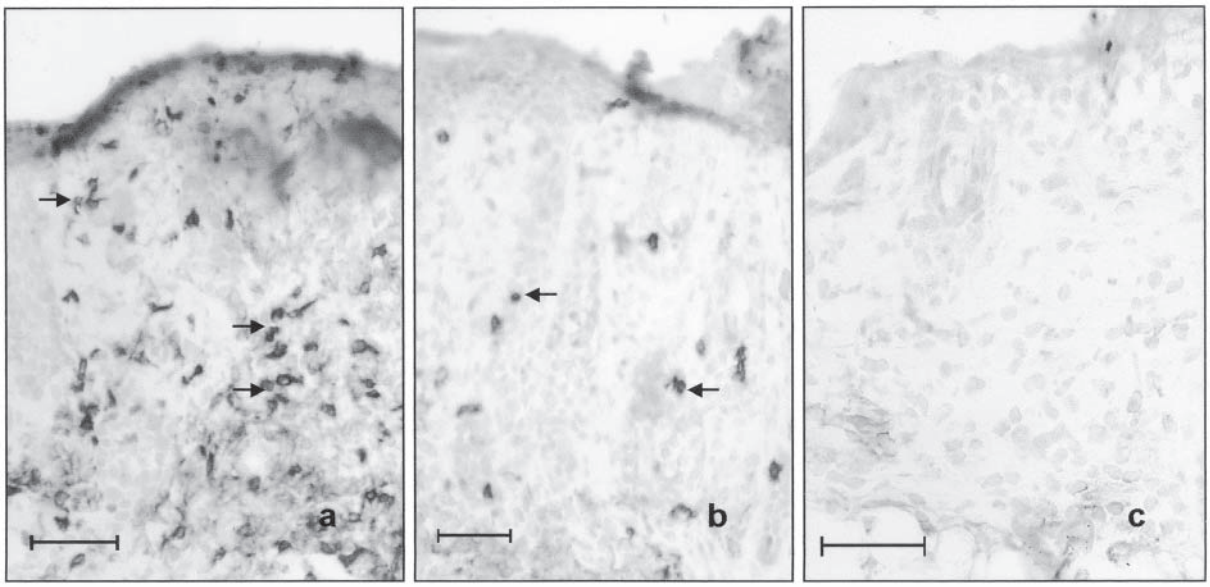

Fig. 3. $CD4^+$ T cells predominate in Skn-induced skin lesions. Cryostat sections of lesional skin from mice treated as described in Fig. 2 were immunostained for the localization of $CD4^+$ (**Panel a**) or $CD8^+$ (**Panel b**) T cells, which are identified as darkly stained cells. Arrows point to representative cells. (**Panel c**) $CD4^+$ T cells were not found in mice pretreated by shaving and cyclophosphamide, but not injected with Skn-immune cells. Images are representative of 6 mice from 3 experiments at days 5-11 after cell transfer. Counterstained with methyl green, which stains cell nuclei. Scale bar = 50 μm.

infiltrate of mice with lesions. Areas of shaved skin, stained with antibody directed to CD4 or CD8 antigens, showed that the T cell population in the epidermis and dermis consisted mainly of $CD4^+$ T cells (Fig. 3a). In comparison, fewer $CD8^+$ T cells were found in the infiltrate (Fig. 3b). Neither $CD4^+$ (Fig. 3c) nor $CD8^+$ (not shown) T cells were observed in the skin of controls that did not receive Skn-immune cells. The large number of infiltrating $CD4^+$ T cells suggested that $CD4^+$ T cells have a major role in Skn-induced autoimmunity.

$CD4^+$ T Skn-immune lymphocytes are able to adoptively transfer anti-Skn autoimmunity. To better define the T cell phenotype(s) that were essential for the adoptive transfer of anti-Skn immunity, Skn-immune spleen cells were depleted of, or enriched for, $CD4^+$ or $CD8^+$ T cells. Depletion of $CD4^+$ T cells from the Skn-immune donor population eliminated the ability of the remaining cells to transfer Skn immunity (Table 1). In contrast, depletion of $CD8^+$ T cells did not abrogate the development of lesions. Likewise enriched $CD4^+$, but not $CD8^+$, T cells were capable of transferring disease (Table 1). The mean scores of lesions found for unfractionated versus CD8-depleted or CD4-enriched T cells were not statistically different. In general, these results implied that $CD4^+$ T cells are the main effector cells for the induction of Skn autoimmunity. The role for $CD8^+$ T cells is presently unknown.

T-helper-1 cell cytokine mRNA profiles in skin lesions. The local immune response resulting from the adoptive transfer of Skn-immune cells was analyzed for cytokine transcripts using RNA isolated

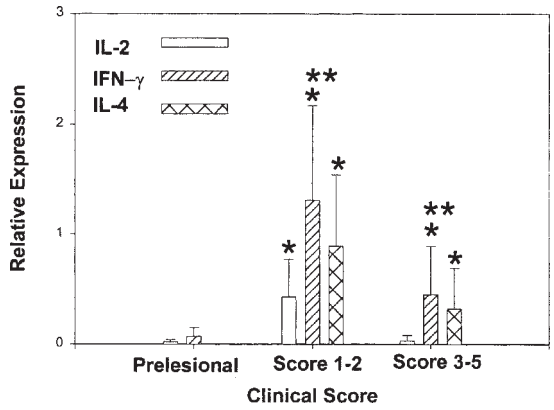

Fig. 4. Cytokine mRNA expression in lesional skin shows a Th1 bias. Mice were treated as described in Fig. 2. Amplified products from RT-PCR assays for IL-2, IFN-γ, IL-4, and β-actin from prelesional (n = 4) and lesional skin (n = 6 or 7) were analyzed by densitometric scanning. Data are shown as relative amounts of cytokine mRNA by using β-actin levels for normalization among animals. The values are means ± SD. * $p < 0.05$ versus prelesional level of cytokine; ** $p < 0.05$ versus paired IL-4 level from individual animals. Data are representative of 2 or 3 independent experiments.

from shaved skin at various times after the transfer process. Shaved skin was utilized since all recipients of Skn-immune cells eventually develop gross lesions at this location [1]. The mRNAs for cytokines representative of Th1 (IL-2 and interferon (IFN)-γ) and Th2 (IL-4) $CD4^+$ T cells were analyzed as well as the mRNA for β-actin, which was used to normalize the results among animals.

In prelesional mice, IL-2 and IFN-γ mRNAs, albeit at extremely low levels, were observed in recipients of Skn-immune cells while IL-4 mRNA was not detected in most animals (Fig. 4). When clinical scores of 1-2 were found, mRNA expression for all cytokines was significantly increased. With mice exhibiting lesion scores of 3-5, IFN-γ and IL-4 mRNA continued to be significantly increased relative to prelesional skin with negligible IL-2 mRNA observed (Fig. 4). While the expression of Th1 versus Th2 cytokine mRNA profiles was not mutually exclusive in skin exhibiting gross lesions, when comparisons were made in individual mice, IFN-γ levels were significantly higher than IL-4 levels ($p < 0.05$) with IFN-γ/IL-4 ratios averaging 2.0.

The data suggested that the cytokine environment in lesional skin had a Th1 bias. With the exception of very weak detection of IFN-γ mRNA in 2 mice, there was no expression of Th cell cytokine mRNA in the skin of control mice, indicating that

Table 1. Characterization of effector T cell populations for adoptively transferred Skn autoimmunity in B6AF1 recipients.[a]

Treatment	Cell population transferred	Number of recipient mice	Number with skin lesions	Clinical score on days 7-12
T cell depletion	Unfractionated	7	7	4.3
	CD4 depleted	11	0	
	CD8 depleted	12	12	3.6
T cell enrichment	Unfractionated	5	5	3.3
	CD4 enriched	4	4	2.9
	CD8 enriched	7	0	

[a] Recipients were shaved, pretreated with 200 mg/kg cyclophosphamide, and given 50 $x10^6$ unfractionated or depleted, or 10 $x10^6$ enriched, Skn-immune cells iv. The results are combined data from 2 or 3 independent experiments, except for the CD4-enriched data, where all mice were from 1 experiment.

the pretreatment regimen alone was not conducive to the appearance of Th cytokine mRNA (data not shown).

Treatment with cyclosporin A (CsA) or anti-IL-2 antibody improves disease course. Because both Th1 and Th2 mRNA were found early in the course of Skn-induced autoimmune disease, the effect of treating recipients of Skn-immune cells with reagents that would interfere with the expression or function of representative cytokines was investigated. CsA or anti-cytokine antibody was used for these experiments. Recipients were pre-treated in the usual manner and injected with Skn-immune cells. CsA treatment by daily ip injection was administered either from day 1 to 14 post-transfer of Skn-immune cells or was started at the onset of gross lesions and continued thereafter for 10 days. When the results of both methods of treatment with CsA were combined, these treatment significantly reduced the disease severity in that 5 of 6 recipients did not develop lesions with a clinical score >1 (Table 2; $p < 0.05$ versus control reagents). However, because the number of animals was small and one mouse did not respond to CsA treatment, these data were interpreted cautiously. Likewise, anti-IL-2 treatment, where antibody was delivered from day 1 to 6 post-transfer of Skn-immune cells, also caused a notable abatement in lesion severity in all recipients (Table 2), with lesion scores limited to 1 ($p < 0.05$ versus control reagents). However, recipients that received anti-IL-4 antibody developed lesions comparable to those of mice injected with control reagents.

The persistence of lesions with anti-IL-4 treatment also served to show that antibody treatment per se could not affect lesion control. Animals were observed for 25 days and in no case did any recipients develop lesions of increased severity after the termination of CsA or anti-IL-2 treatment. Although the number of animals in these experiments was minimal, the data support the $CD4^+$ and Th1 bias found in lesional skin.

Discussion

Using a mouse model, we previously reported an autoimmune response to the epidermal cell antigens, Skn [1], and in this report have supported the anti-self specificity of the response by demonstrating that the severity of the clinical response generally correlated with the injected dose of primed Skn immune cells transferred to recipients. We then set out to evaluate the role of $CD4^+$ T cells in the Skn response.

Table 2. Effect of in vivo treatments with CsA, anti-IL-2, or anti-IL-4 antibody on the development of Skn-induced lesions.[a]

		Clinical score of lesions[b]					
Number of Mice	Reagent	no lesion	1	2	3	4	5
6	CsA[c]	0	**5**[d,e]	0	0	**1**[d]	0
4	Anti-IL-2[c]	**1**	**3**	0	0	0	0
4	Anti-IL-4	0	0	**1**	**1**	**2**	0
4	Control reagent [f]	0	0	0	**2**	**2**	0

[a] B6AF1 recipients were shaved, injected with cyclophosphamide, and given 50×10^6 Skn-immune spleen cells.
[b] At days 12-14 post-transfer.
[c] $p < 0.05$ versus control reagents.
[d] Delivered day 1-14 post transfer of Skn-immune cells, n = 3.
[e] Delivered at lesion onset and thereafter for 10 days, n = 3.
[f] Control reagents were either PBS (diluent for antibodies) or olive oil (vehicle for CsA).

In our model, staining of lesional skin with antibody for CD4 and CD8 revealed the presence of both types of T cells with the former as the predominant phenotype. The large number of $CD4^+$ cells present implied that these cells probably have a role in initiating the pathogenic process. This inference was substantiated, in that transferred Skn-immune cells enriched for CD4 but not CD8 could elicit lesions in recipient mice. Likewise, when $CD4^+$ T cells were removed from the transferred inoculum, lesions did not develop.

Several dermatological diseases with chronic etiology appear to have a cell-mediated autoimmune component, although the inciting self antigens have not yet been identified [10]. Mixed CD4/CD8 infiltrates are reported in lesions from patients with lichen planus [11-13], alopecia areata [14], vitiligo [15,16], and psoriasis [17-19]. While lesional appearance of defined cell types does not necessarily imply disease-activating characteristics, a pivitol role for $CD4^+$ T cells has been demonstrated for pathogenesis in animal models of vitiligo [20], psoriasis [21,22], and alopecia aerata [23]. Our results are consistent with the findings from putative autoimmune human skin diseases and strongly suggest that $CD4^+$ cells are preferentially required for initiation of Skn-targeted autoreactivity, although the data do not exclude the possibility that $CD8^+$ cells contribute to the process.

When autoimmune disease was studied in the context of T-helper (Th) cytokines, it was shown that organ-specific autoimmunity is often associated with cytokines that favor the inflammatory Th1 type [24]. Skn skin lesions were evaluated for prototypic cytokine mRNA that defined Th1 (IL-2, IFN-γ) and Th2 (IL-4) subsets, revealing a bias toward a Th1 environment. Moreover, treatment with anti-IL-2, but not anti-IL-4, antibody significantly improved the autoimmune anti-Skn disease outcome, which reinforced the role of Th1 cytokines in the pathogenic process. A similar partiality toward Th1 is observed in active lesions from patients with alopecia areata [25] and vitiligo [26]. Psoriatic patients also show a predominance toward the Th1 cytokine pattern [27-30], although some studies have indicated random Th profiles [31,32] similar to that reported for lichen planus [33].

Immunosuppressive drugs are often used in the treatment of diseases with chronic immune-based pathology. Skn-lesion severity was clearly reduced by cyclosporin therapy and is consistent with this drug's effect against cell-mediated immune responses.

In summary, we have shown that the pathology induced to self epidermal cell antigens has the immune characteristics associated with a $CD4^+$ T cell-mediated response. However, the presence of autoantigen together with autoreactive lymphocytes is often insufficient to precipitate clinical disease, indicating that other factors contribute to pathognensis. In our model, lesions form only in areas stressed by shaving, suggestive of the Koebner phenomenon, a condition in certain skin disorders wherein lesions appear at the site of trauma. We are currently evaluating what additional conditions within the skin and the skin's resident immune system contribute to the local tissue environment that is conducive to lesion formation.

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