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Cross-priming with an epicutaneously introduced soluble protein antigen generates Tc1 cells

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Epicutaneous sensitization with a protein antigen was demonstrated to induce a predominant type 2 CD4 T cell response with high IgE production in mice. On the other hand, its CD8 T cell responses have not been addressed probably partly because of the generally accepted concept that cross-priming of soluble protein is an inefficient process. Here, we used an established patch-applied murine model to demonstrate that cross-priming with an epicutaneously introduced soluble protein antigen, though inefficient, generated mainly Tc1 cells, but not Tc2 cells. In the presence of an irritant or hapten, the efficiency of this cross-priming process could be enhanced and more Tc1 cells were generated. CpG oligonucleotides also promote the generation of Tc1 cells. In contrast, lipopolysaccharide and poly (inosinic-cytidylic) acid [poly (I:C)] have no effect. Together, these results provide supportive evidence of the epicutaneous sensitization of human cutaneous lymphocyte-associated antigen-positive CD8 T cells found in the peripheral blood or tissues of patients. The surprising observation of the type 1 character of the generated CD8 T cells will also help us to better understand the complicated pathogenesis of atopic and cutaneous inflammatory diseases.

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Introduction

While MHC class II molecules associate with peptides derived from exogenous Ag, MHC class I molecules are classically thought to present peptides of endogenous origin. However, exogenous Ag can also enter the MHC class I pathway of APC by a process called "cross-presentation" [1]. The physiologic outcome of cross-presentation can be either positive immunity (cross-priming) or tolerance (cross-tolerance). Cross-priming is thought to be crucial for developing CD8 T cell responses against tumors and viruses. In contrast, cross-tolerance of endogenous Ag helps prevent the development of autoimmune diseases[2]. This pathway is accessible to apoptotic cells, heat-shock protein-peptides, and immune complexes [3–5]. However, it is generally accepted that exogenous soluble protein Ag are cross-presented with substantially lower efficiency [6, 7]. Nevertheless, a transport pathway involving the ER and an ER-associated degradation process was shown to be the mechanism of cross-presentation of soluble protein Ag by DC [8].

The crucial role of Th2 CD4 T cells in the development of atopic diseases is well established. It is now clear that CD8 T cells also have Th1- or Th2-type cytokine patterns, which are designated as Tc1 or Tc2 cells, respectively. However, their roles in the pathogenesis of atopic disease have not been fully elucidated [9].

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Abbreviations: **AD:** atopic dermatitis · **CLA:** cutaneous lymphocyte-associated antigen · **ODN:** oligodeoxynucleotides · **Poly (I: C):** poly (inosinic-cytidylic) acid · **TNBS:** 2,4,6-trinitrobenzenesulfonic acid solution

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In humans with atopic dermatitis (AD), unlike healthy individuals, the number of IFN-γ-producing CD8 T cells was diminished after *in vitro* polyclonal activation of their PBMC [10]. However, symptomatic atopics have greater numbers of allergen-specific CD8 T cells that are capable of producing IFN-γ efficiently [11]. Moreover, the effector role of CD8 T cells was supported by the observation that AD developed in AIDS patients [12]. In animal models, the data are even more ambiguous due to different immunization protocols and means of analysis. In general, CD8 T cells generated by i.p. or s.c. immunization could inhibit IgE production and airway eosinophilia [13, 14]. CD8 T cells, as a source of IL-13, were shown to be required in the effector phase of airway hyperresponsiveness [15, 16]. Furthermore, studies that examined adoptive transfer of allergen-specific, *in vitro* differentiated Tc1 or Tc2 cells showed that Tc1 cells induced a neutrophilic airway inflammation without induction of bronchial hyperresponsiveness. In contrast, Tc2 cells responded to inhaled soluble Ag and produced an inflammatory response qualitatively similar to Th2 cells [17].

The cutaneous lymphocyte-associated antigen (CLA) was originally identified as a homing receptor for T cells with tropism for skin. It is expressed by most infiltrating T cells present in cutaneous inflammatory sites, including contact dermatitis, AD and psoriasis [18]. The contribution of CLA in the complex molecular interactions between lymphocytes and cutaneous vascular endothelium that takes place during the lymphocyte migration to the skin is well documented. However, the evidence supporting the epicutaneous sensitization of these human CLA^+ T cells is circumstantial. This includes that CLA is induced on T cells undergoing naive to memory transition in lymph nodes that drain the skin and CLA^+ T cells respond to Ag preferentially found in the cutaneous microenvironment [19, 20].

We have previously reported that epicutaneous exposure of a protein Ag induced a predominant Th2-like response with high IgE production in mice [21]. Spergel *et al* [22] reported supportive evidence that epicutaneous sensitization with protein Ag led to AD-like cutaneous lesions and allergic asthma. This study examines the efficiency of the cross-priming with an epicutaneously introduced soluble protein Ag and the characteristics of the generated CD8 T cells. In conclusion, Tc1 cells were generated by cross-priming with an epicutaneously introduced soluble protein Ag.

Results

Epicutaneous exposure to a soluble protein Ag induces Th2-like CD4 T cells

It is generally accepted that BALB/c and C57BL/6 mice are high- and low-IgE responders, respectively. Because the only known MHC class I peptide sequence of OVA (SIINFEKL) is presented by the K^b MHC locus of the C57BL/6 mice, we first checked whether epicutaneous exposure to OVA induced Th2-like CD4 cells in C57BL/6 mice. A group of mice received a 5-day patch application of the OVA solution for the immunization course. Three weeks later, the cytokine profiles of supernatants collected from *in vitro* Ag reactivation cultures of splenocytes were measured. The IFN-γ and IL-4 contents of culture supernatants were always shown to be below detectable levels. Sometimes, we could detect small amounts of IL-5 and IL-13 secretion in this culture system. But most of the time, it was also below the detectable levels. In contrast, immunized splenocytes always secreted significant amounts of IL-10 after *in vitro* reactivation (Fig. 1A). Then, a group of mice was immunized with two courses of a 5-day patch application separated by a 3-week interval. Only IL-10 secretion could be consistently measured, whereas the IFN-γ, IL-4, IL-5, and IL-13 contents of the supernatants were all undetectable (Fig. 1A). Therefore, we decided to employ the more sensitive ELISPOT technique. Fig. 1B shows that IL-4- or IL-5-secreting CD4 T cells were generated after epicutaneous OVA patch application. Taken together, although C57BL/6 mice are low-IgE responders, the main CD4 T cells generated after epicutaneous exposure to soluble protein Ag are of the Th2 type.

Cross-priming with an epicutaneously introduced soluble protein Ag generates mainly Tc1 cells

For comparison, we employed a group of mice immunized with an intravenous injection of OVA solution at a high concentration. This is the most commonly used experimental model system for the cross-presentation of soluble protein Ag. Groups of mice received either a 5-day patch application or an intravenous injection of the OVA solution. Three weeks later, the cytokine contents of supernatants of *in vitro* reactivation cultures of purified splenic CD8 T cells, stimulated with peptide-pulsed APC, were determined. There were no detectable levels of IFN-γ, IL-4, IL-5, IL-10, or IL-13 secreted in the supernatants of cells cultured from mice that underwent the patch application, whereas, a significant amount of IFN-γ was detected in the i.v. treated mouse group (data not shown). However, ELISPOT assays of splenic CD8 T cells revealed small, but significant, amounts of IFN-γ-

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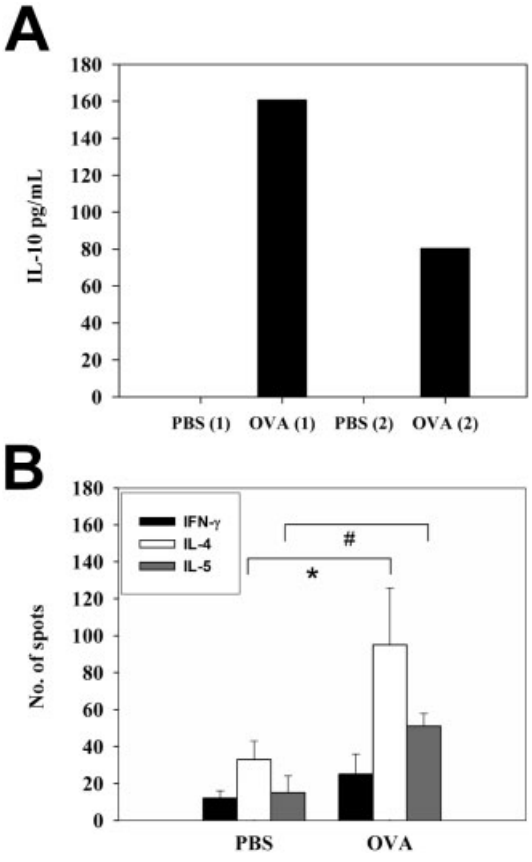

Figure 1. Epicutaneous exposure to a soluble protein Ag induces Th2-like CD4 T cells in B6 mice. (A) Groups of B6 mice ($n = 4$) were immunized by one (1) or two (2) courses of patch application with OVA solution or PBS control. Three weeks later, IL-10 contents of supernatants of *in vitro* reactivation culture of splenocytes were determined. (B) Groups of B6 mice ($n = 4$) were immunized by one course of patch application with OVA solution or PBS. Three weeks later, ELISPOT assays for OVA-specific, IFN-γ-, IL-4- or IL-5-producing purified CD4 T cells were performed. Each value represents the average ± SD. * $p<0.05$, # $p = 0.05$.

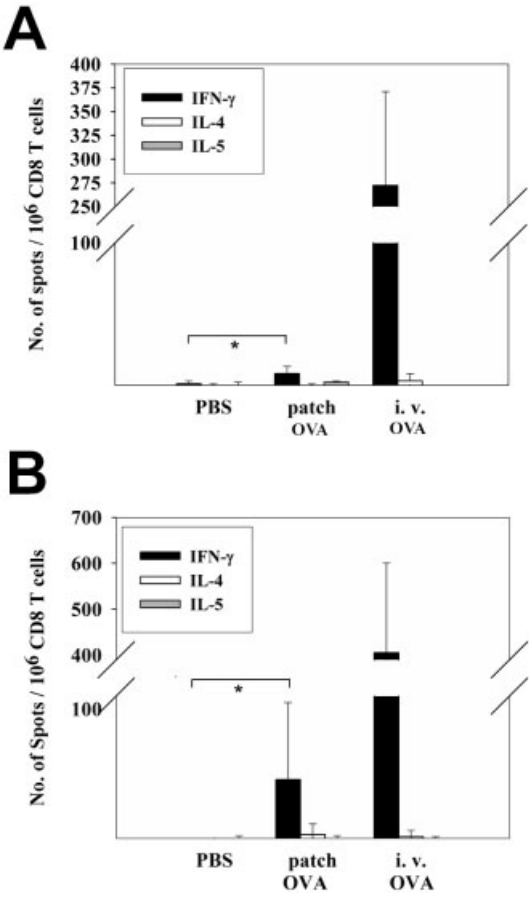

Figure 2. Cross-priming with an epicutaneously introduced soluble protein Ag generates mainly Tc1 cells. Groups of mice ($n = 4$) were immunized with one (A) or two (B) courses of OVA or PBS patch application or intravenous injection of OVA solution. Three weeks after the start of last immunization, ELISPOT assays for OVA-specific, IFN-γ-, IL-4-, or IL-5-producing CD8 T cells for individual mouse were performed. Each value represents the average ± SD. * $p<0.05$.

producing Ag-specific CD8 T cells acquired from mice who received patch applications (Fig. 2A). In contrast, no significant amount of IL-4- or IL-5-producing Ag-specific CD8 T cells was detectable in this latter mouse group (Fig. 2A). The efficiency of cross-priming with epicutaneously introduced OVA was obviously much less than that evident in the i.v. route, particularly when comparing the numbers of generated IFN-γ-producing CD8 T cells (Fig. 2A). When the mice were immunized by two courses of patch application or i.v. injection, both groups showed much increased numbers of the IFN-γ-producing CD8 T cell. However, there were still no or very few IL-4- or IL-5-producing CD8 T cells detectable by the ELISPOT assay (Fig. 2B). Cytokine analyses of *in vitro* reactivation culture supernatants could not detect any significant secretion of IFN-γ, IL-4, IL-5, IL-10, or IL-13 in the mice that underwent patch applications. In contrast, a significant amount of IFN-γ secretion was again detected in the i.v. treated mouse group (data not shown). Collectively, these data demonstrate that cross-priming with an epicutaneously introduced soluble protein Ag, though inefficient when compared with the intravenous route, generates mainly Tc1 cells but not Tc2 cells.

In the presence of an irritant or hapten, more Tc1 cells are generated by cross-priming

Skin is the outer most organ exposed to irritants and haptens in the environment. We checked the effects of irritant or hapten co-exposure to the cross-priming with an epicutaneously introduced soluble Ag. SDS, a prototypic irritant, was applied simultaneously with OVA by the patch application technique for one immunization course. In the presence of SDS, much higher numbers of Ag-specific CD8 T cells were

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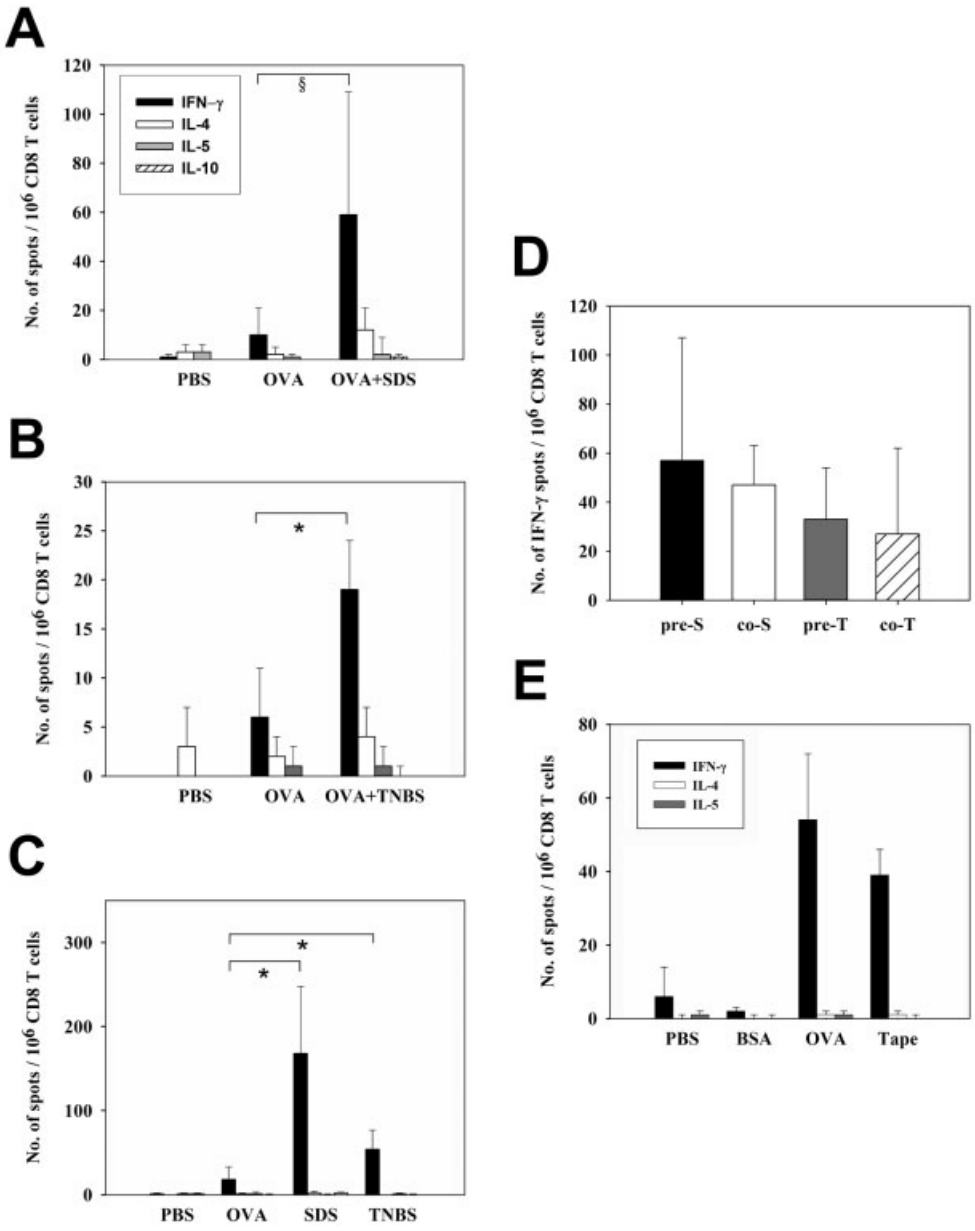

Figure 3. In the presence of an irritant or hapten, more Tc1 cells were generated by cross-priming. Groups of mice (n = 4) were immunized similarly with one (A, B) or two (C) courses of OVA or PBS patch application except that 5% SDS (A, C) or 1% TNBS (B, C) was added to each patch. Three weeks after the start of last immunization, ELISPOT assays were performed. (D) Groups of mice (n = 4) were immunized similarly as in (A, B) (co) or pretreated with SDS or TNBS for 5 successive days followed by another 5-day OVA patch application (pre). Three weeks later, IFN-γ ELISPOT assay was performed. (E) Groups of mice (n = 4) were similarly immunized with PBS, BSA or OVA patch application. One group of them (Tape) received tape stripping pretreatment immediately before each OVA patch application. Three weeks later, ELISPOT assays were performed. * $p<0.05$, § $p = 0.057$.

generated. Most of them secreted IFN-γ as determined by the ELISPOT assay. A small number of cells secreted IL-4 and none of these cells secreted IL-5 (Fig. 3A). In the presence of 2,4,6-trinitrobenzenesulfonic acid solution (TNBS), a commonly used hapten in experimental systems, the quantity of Ag-specific CD8 T cells generated by cross-priming was larger than OVA alone, but smaller than OVA plus SDS. All of the cells secreted IFN-γ as determined by an ELISPOT assay (Fig. 3B). We could not detect any significant secretion of IFN-γ, IL-4, IL-5, IL-10, or IL-13 in supernatants of *in vitro* reactivation cultures by an ELISA assay. The groups of mice that received two courses of patch-application of OVA in the presence of SDS or TNBS were studied. As shown in Fig. 3C, much higher numbers of IFN-γ-producing CD8 T cells were generated, whereas no IL-4- or IL-5-producing CD8 T cells could be detected. Moreover, ELISA-based cytokine profiles of *in vitro* reactivation

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cultures of purified CD8 T cells revealed significant secretion of IFN-γ (data not shown). To evaluate the effects of pre-exposure to irritant or hapten, mice were pretreated with SDS or TNBS for 5 successive days followed by OVA patch application or received co-administration of OVA plus SDS or TNBS by patch application. Fig. 3D showed that pretreatment or co-administration generated similar amounts of IFN-γ-producing Ag-specific CD8 T cells. Skin is also subject to many kinds of injuries resulting in skin barrier defect. To evaluate this, mice received tape stripping treatment immediately before each patch application to induce barrier defect. It seems that pretreatment with tape stripping not only can't enhance the efficiency of cross-priming but also has no effects on phenotype of the generated CD8 T cells (Fig. 3E). Taken together, these results demonstrate an enhancing effect of environmental irritants or haptens in the cross-priming of soluble protein Ag by the epicutaneous route.

CpG oligodeoxynucleotides promote cross-priming with an epicutaneously introduced soluble protein

Skin is also exposed to many kinds of microorganisms in the environment. Some of the identified agonists of the pattern recognition receptors of innate immunity for these microorganisms have been reported to promote cross-priming of soluble protein Ag [23]. We next checked the effects of CpG oligodeoxynucleotides (ODN), LPS and poly (inosinic-cytidylic) acid [poly (I:C)] on the cross-priming of epicutaneously introduced soluble protein Ag. As shown in Fig. 4A, in the presence of CpG ODN but not non-CpG ODN, more Tc1 cells were generated by cross-priming whereas there were still no IL-4-, IL-5- or IL-10-secreting CD8 T cells detectable. In contrast, LPS and poly (I:C) have no detectable enhancing effect on the generation of Tc1 or Tc2 cells (Fig. 4B). The effect of pretreatment of CpG ODN was also evaluated. Fig. 4C shows that pretreatment of CpG-ODN for 5 days has a stronger enhancing effect on cross-priming when compared with co-administration of CpG ODN with protein Ag. Collectively, these results show that microorganism by the mediators of their CpG DNA can promote the generation of Tc1 cells by the epicutaneous routes.

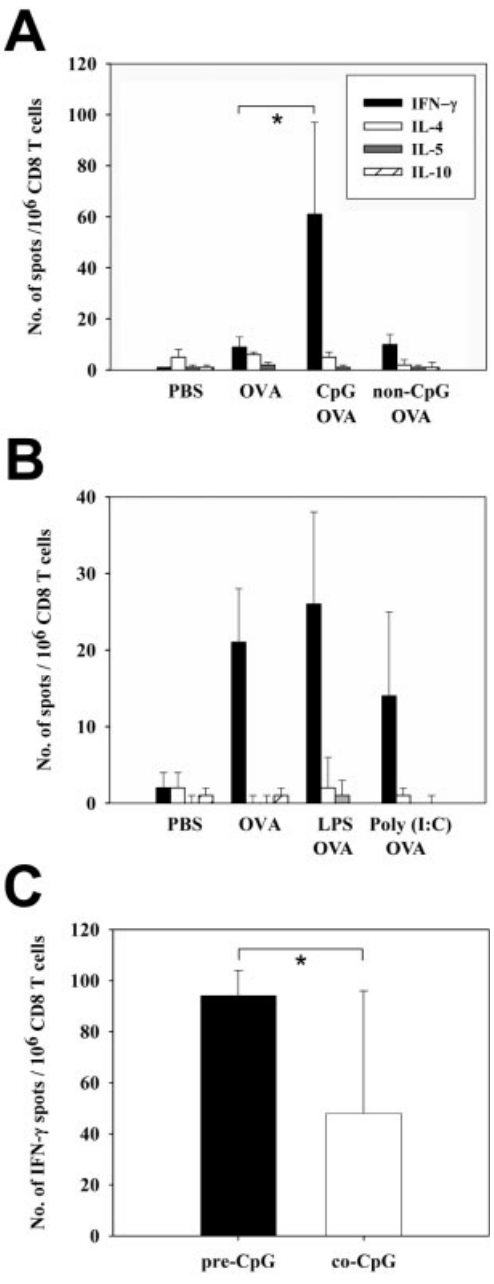

Figure 4. In the presence of CpG ODN, but not LPS or poly (I:C), more Tc1 cells were generated by cross-priming. Groups of mice (n = 4) were immunized similarly with one course of OVA or PBS patch application except that CpG ODN, non-CpG ODN (A), LPS or poly (I:C) (B) were added to each patch. Three weeks after immunization, ELISPOT assays were performed. (C) Groups of mice (n = 4) were immunized similarly as in (A) (co) or pretreated with CpG-ODN for 5 successive days followed by another 5-day OVA patch application (pre). Three weeks later, ELISPOT assay for IFN-γ was performed. * $p<0.05$.

Discussion

In this study, we examined the efficiency of cross-priming with an epicutaneously introduced soluble protein Ag and the characteristics of the generated CD8 T cells. We provided evidence demonstrating that, although relatively inefficient when compared with intravenous route, the cross-priming process generated CD8 T cells that were mainly IFN-γ-producing Tc1 cells. This occurred in contrast to the simultaneously induced Th2 CD4 T cells. Moreover, the presence of an irritant, hapten, or CpG ODN could enhance the efficiency of

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cross-priming and thereby generate more Tc1 cells. To the best of our knowledge, this is the first report demonstrating the efficiency and characteristics of cross-priming with soluble protein Ag by the epicutaneous route.

In this experimental system, there are several points worth emphasizing. First, we always examined the characteristics of the splenocytes 3 weeks after the last immunization. This should avoid the ambiguity associated with the contraction phase of the CD8 T cell responses if no or minimal inflammation were present [24]. We believe that the CD8 T cells detected by the ELISPOT assay at this time point were memory T cells. Next, in our model system, the OVA solution was applied to the skin by a patch application without an adjuvant. In this manner, our experimental system more closely simulates the "physiological condition." In the experiments reported by Dutz's group [25, 26], cholera toxin and CpG ODN can promote the generation of CTL after epicutaneous application of a peptide or a protein Ag. However, in their system, the mouse skin was tape-stripped ten times and wiped once with acetone before applying the Ag. This procedure would not only disrupt the epidermal barrier but would also modulate or deviate the normal physiological immune responses. Finally, irritant, hapten or microorganism are commonly present in our environment and accessible to our skin. Thus, the cross-priming with an epicutaneous soluble protein Ag in the presence of these factors, designed in our experimental system, can be viewed as mimicking a "specialized physiological condition". Taken together, the physiological character of the sensitization procedure and readout time point of our model system should be emphasized.

There have been a few reports addressing the effect of TLR-agonists on cross-priming of soluble protein. Datta *et al*. [27] used an *in vitro* cross-presentation system to demonstrate that CpG ODN and poly (I:C), but not other TLR ligands, induce cross-presentation. Durand *et al*. [28] investigated *in vivo* the effect of different TLR stimuli on the induction of CD8 T cell cross-priming after intramuscular immunization with soluble protein antigen. They showed that CpG ODN, poly (I:C) and LPS, but not other TLR stimuli, induced functional CD8 T cell responses. Our results showed that CpG ODN, but not LPS or poly (I:C), have enhancing effect of cross-priming with an epicutaneously introduced soluble protein Ag. In all of these studies, CpG ODN showed consistent promoting effect, whereas LPS and poly (I:C) showed variable effects in different experimental systems. The reasons for the discrepancy are at present unknown. Variation of regional immune responses is one of the possibilities.

Minimal data are available about the CD8 T cell responses to the introduction of soluble protein Ag by different routes in experimental animal systems. Chung *et al*. [29], and Limmer *et al* [30]. reported cross-tolerance to intestinal Ag and hepatic Ag, respectively. However, for the inhalation route, Holt's group and Gelfand's group [31, 32] both showed that continued exposure of animals to aerosolized OVA generated IFN-γ-producing CD8 T cells. Recently, Brimnes *et al*. [33] also reported the induction of IFN-γ-producing Tc1 cells by exposure to an inhaled protein in the presence of the influenza virus. Our present study showed that cross-priming with a soluble protein Ag generated mainly Tc1 cells more efficiently by the intravenous route than the epicutaneous route.

Some of the implications of our results are the following: First, our results provide direct supportive evidence for the epicutaneous sensitization of CLA^+ CD8 T cells isolated from cutaneous lesions or peripheral blood of patients. Secondly, there are biphasic T cell responses in the lesions of patients with AD [34]. Th2 cells are predominantly found in the acute lesions, while Th1 cells are more often found in the chronic lesions. In the past, this switch was thought to be initiated by the local production of IL-12 from infiltrating eosinophils and inflammatory dendritic epidermal cells. Our results, which showed that cross-priming with epicutaneously introduced soluble protein Ag generated Tc1 cells and was promoted by physiologically relevant agents including irritants, hapten and CpG ODN from bacteria or virus, provide evidence for another pathway that may contribute to a type 1 T cell response-prone microenvironment. Although we did not perform experiment inducing skin damage in the presence of bacteria and virus, their presence in the damaged skin could be expected to further promote type 1 microenvironment. Furthermore, Boasen's recent report [35], which demonstrated that house dust extracts elicit TLR-dependent DC responses through TLR signaling pathway raise the possibility that house dust itself may have enhancing effect on cross-priming by the epicutaneous route. Next, IFN-γ-producing Tc1 cells may exacerbate the spongiosis development found in AD lesions. Keratinocytes normally express low levels of Fas, which can be substantially enhanced by the presence of IFN-γ. Activated infiltrating T cells that express FasL can thus induce keratinocyte apoptosis and result in spongiosis development [36]. Finally, it has been repeatedly demonstrated that CD8 T cells are required in the airway hyperresponsiveness of murine models of asthma [15, 16]. Since epicutaneous sensitization is one possible sensitization route for asthma, cross-priming with an epicutaneously introduced protein Ag may contribute to the generation of the effector CD8 T cells of asthma [22].

In summary, we have demonstrated that the cross-priming with an epicutaneously introduced soluble protein Ag, although inefficient, generates mainly Tc1

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cells. Further, the cross-priming process is promoted in the presence of irritant, hapten or CpG ODN from microorganism. These data contribute to our understanding of the complicated pathogenesis of atopic and cutaneous diseases.

Materials and methods

Mice and reagents

Six- to eight-week-old female C57BL/6J mice were purchased from the animal center of National Taiwan University College of Medicine and kept in a specific pathogen-free environment. All animal experiments were approved by the animal care committee of the Medical College of University of National Taiwan University. Ovalbumin (Grade V), TNBS, LPS and 4-nitrophenyl phosphate disodium salt solution (pNPP) were purchased from Sigma (St. Louis, MO). SDS was purchased from Calbiochem (San Diego, CA). Poly (I:C) was purchased from Amersham Bioscience (Piscataway, NJ). Thioate-modified ODN were custom synthesized by MDBio InC (Taipei, Taiwan). The CpG ODN (ODN-1826) consisted of 20 bases containing two CpG motifs (TCCATGACGTTCCTGACGTT). The control non-CpG ODN (ODN-1982) was identical except that the CpG motifs were rearranged (TCCATGAGCTTCCTGAGCTT). Capture and biotin-conjugated detecting Ab for IFN-γ, IL-4, IL-5, and IL-10 used in ELISA and ELISPOT assays were all purchased from PharMingen (San Diego, CA). Streptavidin-alkaline phosphatase (AP) was purchased from Southern Biotechnology (Birmingham, AL). BCIP/NBT phosphatase substrate was purchased from KPL (Gaithersburg, MD). The murine IL-13 ELISA development kit (Peprotech, NJ) was used for detection of the IL-13 content of supernatants. The peptide SIINFEKL (OVA peptide 257–264) was a kind gift from Dr. Wolfgang Leitner (NIH, USA).

Immunization

Mice were immunized as previously described with some modifications [21]. Briefly, 20 μL of 100 mg/mL OVA solution was placed on the disc of a Finn chamber (Epitest, Finland). This was then applied to an area of shaved skin on the back of a mouse. For each course of immunization, freshly prepared patches were applied daily from day 1 to day 5. The second course of immunization was similar to the first one and started on day 22. For groups of mice receiving immunization with OVA in the presence of an irritant or hapten, 5% SDS and 1% TNBS were added to each Finn chamber disc. For groups of mice receiving immunization with OVA in the presence of CpG ODN, non-CpG ODN, LPS or poly (I:C), 20 μg was added to each Finn chamber disc. For the i.v. group, 500 μL OVA solution, at a concentration of 4 mg/mL, was injected i.v. on days 1 and 22. For the pretreated with SDS or TNBS, 5% SDS or 1% TNBS patch applications were performed for 5 successive days and followed by another 5-day OVA application. For mice receiving tape stripping, adhesive tape was applied to the patch site for ten times immediately before each patch application.

Purification of CD4 or CD8 T cells

CD4 or CD8 T cells were purified by positive selection using CD4 or CD8α microbeads (Miltenyi Biotec, Germany) following the manufacturer's protocol. The purity after the cell isolation was greater than 95% for CD4 T cells and greater than 93% for CD8 T cells.

The measurement of cytokines in the supernatant of *in vitro* reactivation cultures

Three weeks after the start of the last immunization course, mice ($n = 4$) were sacrificed. Their spleen cells were pooled into a single-spleen cell suspension. For CD4 T cells, 5×10^5 splenocytes per well were cultured in flat bottom 96-well tissue culture plates in the presence or absence of 100 μg/mL OVA. For CD8 T cells, 5 X 10^5 purified CD8 T cells per well were cocultured with peptide-pulsed or sham-pulsed irradiated naive splenocytes at a concentration of 2×10^4 cells per well. Supernatants were harvested 48 h later and were stored at –80° IFN-γ, IL-4, IL-5, IL-10, and IL-13 contents of supernatants were measured by a standard sandwich ELISA. The limits of detection for IL-4, IL-5, IL-10, and IL-13 were all 10 pg/mL, whereas that for IFN-γ was 50 pg/mL.

ELISPOT assays

A standard cytokine-specific ELISPOT assay was used. For CD4 T cells, 5×10^5 (IL-4, IL-5) or 1×10^5 (IFN-γ) purified CD4 T cells per well, acquired from an individual mouse, were cultured with 5×10^5 (IL-4, IL-5) or 2×10^5 (IFN-γ) irradiated splenocytes in the presence or absence of OVA (100 μg/mL). For CD8 T cells, 1 X 10^6 purified CD8 T cells acquired from an individual mouse were cocultured with 2×10^4 peptide-pulsed, or sham-pulsed, irradiated naive splenocytes. The incubation period was 48 h for IL-5 and IL-10 and 24 h for both IFN-γ and IL-4. The spots were read by an automatic immunospot analyzer (BD PharMingen). For statistical calculation, we conducted the nonparametric Wilcoxon rank sum test for testing the means of two independent samples. The level of significance was set at 0.05.

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