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Epicutaneous sensitization with a protein antigen induces Th17 cells

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ABSTRACT

Background: Th17 is a newly identified effector T cell lineage which plays a central role in many human inflammatory diseases and experimental animal models. Epicutaneous sensitization with a protein antigen has been proven to induce a Th2-predominant immune response and lead to development of atopic diseases in a murine protein-patch model.

Objective: We sought to assess the generation of Th17 cells in epicutaneous sensitization with a protein antigen and its regulation by environmental elements and genetic background.

Methods: BALB/c, C57BL/6, and DO11.10 mice were epicutaneously immunized by patch application of the following: ovalbumin alone, or co-administration of one of TLR ligands, irritant, hapten or superantigens. IL-17 and IL-22 contents in supernatants of in vitro reactivation culture of lymph nodes cells were determined by ELISA. Frequency of IL-17-secreting CD4 T cells was measured by ELISPOT.

Results: Small but significant amounts of IL-17 and IL-22 could be detected in supernatants of in vitro reactivation culture of lymph nodes cells of mice receiving patch application of ovalbumin. ELISPOT assay for IL-17 also revealed low frequency of IL-17-secreting CD4 T cells in lymph nodes cells in ovalbumin group. All TLR ligands tested including agonists for TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 as well as many environmental elements including irritant, hapten and superantigen could further promote the generation of Th17 cells. In addition, C57BL/6 mice generate less Th17 cells than BALB/c mice in epicutaneous sensitization.

Conclusion: This study demonstrates Th17 generation and its regulation by environmental elements and genetic background to a protein antigen by epicutaneous route.

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1. Introduction

There is no doubt that Th2 cells play a central role in the pathogenesis of atopic diseases. In asthma, Th2 cells are responsible for the development of inflammation and hyperactivity by contributing to the differentiation and recruitment of eosinophils and mast cells, as well as production of IgE [1]. In atopic dermatitis (AD), acute skin lesions have a large number of Th2 cells, but few Th1 cells [2]. In contrast, the role Th1 cells play as effectors of atopic diseases is widely debated. Nevertheless, increasing evidence supports the notion that Th1 cells participate in the propagation of chronic inflammation and pathology of atopic diseases. For example, the frequency of Th1 cells and the level of expression of

IFN- γ from airway secretions of asthmatics appear to be positively related to the severity of disease [3]. Moreover, in a low-level challenge murine model of asthma, administration of a neutralizing Ab to IFN- γ suppresses air hyperreactivity [4]. In AD, a shift to a Th1-type cytokine milieu is always observed in chronic skin lesions [2]. Recently, a new effector T cell lineage, Th17, which produces IL-17 and IL-22, has been identified [5,6]. IL-17 is a potent proinflammatory cytokine that induces the expression of IL-1, IL-6, numerous chemokines, and recruit neutrophils [7]. Th17 is now considered to be the critical T effector subset responsible for mediating pathogenesis of many commonly studied models of autoimmunity and human autoimmune disorders [8]. For allergic reactions, the function of Th17 is less clear. Th17 has been shown to be a negative regulator of established allergic asthma in a murine model [9]. However, IL-17 performs important effector functions during the elicitation of contact hypersensitivity responses [10]. Studies regarding the generation of the Th17 lineage have demonstrated that IL-6 and TGF- β act non-redundantly and cooperatively to achieve Th17 commitment, and IL-23 functions subsequently on the committed Th17 cells to promote their expansion [7,8].

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Abbreviations: LN, lymph node; SDS, sodium dodecyl sulfate; TNBS, 2,4,6-trinitrobenzenesulfonic acid solution; SEB, staphylococcal enterotoxin B; TSLP, thymic stromal lymphopoietin; Poly(I:C), poly(inosinic-cytidylic) acid; ODN, oligodeoxynucleotide.

Efficient immune responses depend on a close interaction between the innate and adaptive immune systems. The innate immune system not only reacts promptly to microbial infection or environmental insult, but also instructs APCs to activate and secrete cytokines in order to polarize T cells toward an appropriate effector phenotype [11]. To date, the best characterized pattern recognition receptors which recognize pathogen-associated molecular patterns shared by many microorganisms are the TLRs. Most TLR agonists, with the exception of TLR2, stimulate the Th1 response [11,12]. In contrast, TLR2 ligands, as well as superantigen produced by microorganisms (e.g., staphylococcal enterotoxin B [SEB]), promote Th2 differentiation [13,14]. Thymic stromal lymphopoietin (TSLP) produced by epithelial and mast cells have also been shown to induce Th2 development [15].

Compelling evidence from clinical studies support the notion that epicutaneous sensitization is one of the important immunization routes for atopic diseases [16]. Our research group, and others have demonstrated in a murine model that epicutaneous sensitization with a protein antigen by patch application induces a predominant Th2 response with high IgE production and leads to the development of allergic asthma [17,18]. Epicutaneous sensitization with protein antigen has also been proven to be an important route for allergic rhinitis, and eosinophilic esophagitis in animal model [19,20]. Moreover, it also induces non-specific suppressor T cells to inhibit contact hypersensitivity [21]. In this study, we used a similar model to determine if Th17 cells could be induced by epicutaneous sensitization and regulated by innate elements. Moreover, the difference in Th17 induction and regulation between Th1-prone C57BL/6 mice and Th2-prone BALB/c mice was also explored.

2. Materials and methods

2.1. Mice and reagents

Eight- to twelve-week-old female BALB/c, C57BL/6, and DO11.10 (OVA-specific TCR transgenic) mice were obtained from the Animal Center of the National Taiwan University College of Medicine and housed in a specific pathogen-free environment. All animal experiments were approved by the Animal Care Committee of the Medical College of National Taiwan University. Ovalbumin (OVA; grade V), 2,4,6-trinitrobenzene sulfonic acid solution (TNBS), and LPS (derived from *Escherichia coli*) were purchased from Sigma (St. Louis, MO, USA). Pam3CSK4, flagellin, and imiquimod (R837) were purchased from InvivoGen. Sodium dodecyl sulfate (SDS), poly(inosinic-cytidylic) acid (poly(I:C)), and thymic stromal lymphopoietin (TSLP) were purchased from Calbiochem, Amersham Bioscience, and R&D Systems, respectively. Thioate-modified CpG-oligodeoxynucleotide (ODN-1826) was custom-synthesized by MDBio (Taipei, Taiwan). Capture and biotin-conjugated detecting Abs for IFN- γ , IL-4, and IL-5 were purchased from PharMingen. The mouse IL-13 ELISA development kit (Peprotech), IL-17A ELISA set (eBioscience), and IL-22 ELISA construction kit (Antigenix America) were used for detection of IL-13, IL-17, and IL-22, respectively.

2.2. Epicutaneous immunization

Mice were immunized as previously described [22]. Briefly, 20 μ l of 100 mg/ml OVA solution was placed on the disc of a Finn chamber (Epitest). 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 5. For groups of mice ($n = 5-7$) receiving immunization with OVA in the presence of each TLR ligand, 10 μ l of Pam3CSK4 (0.5 μ g/ μ l), poly(I:C) (2 μ g/ μ l), LPS (2 μ g/ μ l), flagellin (0.1 μ g/ μ l), imiquimod (2 μ g/ μ l), and CpG-ODN (2 μ g/ μ l) were added to each Finn chamber disc. For

groups of mice ($n = 5-7$) receiving immunization with OVA in the presence of irritant, hapten, SEB, and TSLP, 10 μ l of 5% SDS, 10 μ l of 1% TNBS, 10 μ l of 0.4 μ g/ μ l SEB, and 10 μ l of 0.01 μ g/ μ l TSLP were added, respectively.

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

Ten days after beginning the immunization course, mice were sacrificed and LN cells were pooled. 5×10^5 LN cells were cultured in the absence or presence of 100 μ g/ml OVA. Supernatants were harvested 48 h later and were stored at -80°C . IFN- γ , IL-5, IL-13, IL-17, and IL-22 content of supernatants were measured by a standard sandwich ELISA. The limits of detection for IL-5, IL-13, IL-17, and IL-22 were all 10 pg/ml, whereas the limit of detection for IFN- γ was 50 pg/ml.

2.4. ELISPOT assays

A standard IL-17-specific ELISPOT assay was used. CD4 T cells were purified from LN cells by positive selection using CD4 microbeads (Miltenyi Biotec, Germany). The LN purity was $>95\%$. 1.5×10^5 (1×10^4 for DO11.10) purified CD4 T cells obtained from individual mice were cultured with 3×10^5 (2×10^4 for DO11.10) irradiated splenocytes from naïve mice in the absence or presence of OVA (100 μ g/ml) for 48 h. The spots were read by an automatic immunospot analyzer (BD ParMingen). The net spot number (spot number in the absence of OVA subtracted from the spot number in the presence of OVA) was calculated.

2.5. Quantitative real-time PCR

OVA-TCR transgenic mice (DO11.10; three per group) were patched with PBS or OVA for 5 days. Draining LNs were obtained 10 days later. *In vitro* OVA reactivation cultures were grown for 48 h. The harvested cells were purified for CD4 T cells by microbeads. Total RNA extraction, cDNA preparation, and quantitative real-time PCR were performed according to the manufacturer's instructions. The relative mRNA expression levels of each sample were normalized according to β -actin expression.

2.6. Statistical analysis

For each experiment, increased folds of IL-17 and IL-22 contents in mice immunized by patch application with OVA plus one of TLR ligands or environmental elements compared with mice receiving patch application with OVA only were calculated. The mean and standard deviation of increased folds were calculated from 4 to 6 independent repeated experiments. Wilcoxon rank sum test was performed for statistical analysis. $P < 0.05$ was set to be significant.

3. Results

3.1. Epicutaneous sensitization with a protein Ag induces a small, but significant number of Th17 cells

We have previously shown that epicutaneous sensitization with a protein Ag induces a predominant Th2 immune response in BALB/c mice [17]. To investigate whether Th17 cells were also induced, the IL-17 and IL-22 content in supernatants of *in vitro* reactivation cultures of LN cells were determined. Fig. 1A shows that small, but significant amounts of IL-17 and IL-22 could be detected in mice receiving a patch application of OVA. ELISPOT assay for IL-17 also revealed a low frequency of IL-17-secreting CD4 T cells in LN cells in the OVA group (Fig. 1B). However, we did not detect IL-17-secreting CD4 T cells by intracellular staining, possibly due to the low

Fig. 1. Induction of IL-17 and IL-22 production and ROR γ t expression by epicutaneous sensitization with OVA. Groups of BALB/c (A and B) or DO11.10 (C–E) mice were immunized by patch application with OVA solution or PBS control. Ten days later, draining LNs were obtained. (A and C) The IL-17 and IL-22 content of supernatants of *in vitro* reactivation cultures of the pooled draining LN cells ($n = 5-7$) were determined by ELISA. Net concentration (concentration in the absence of OVA subtracted from concentration in the presence of OVA) was presented. More than ten times independent experiments were obtained with similar results. Data from one representative experiment was shown. (B and D) CD4 T cells were purified and frequency of IL-17-secreting CD4 cells was determined by ELISPOT assay. Net frequency (frequency in the absence of OVA subtracted from frequency in the presence of OVA) was presented. (E) CD4 T cells were purified after 48 h in *in vitro* reactivation cultures, then quantitative real-time PCR was performed. The RT-PCR data shown were normalized to β -actin levels and the level of expression of OVA reactivation culture of PBS group was set at 1.0.

frequency (data not shown). To further confirm the induction of Th17 cells by epicutaneous sensitization, OVA-specific TCR transgenic mice (DO11.10) were used. Fig. 1C shows that OVA patch application induced LN cells to secrete large amounts of IL-17 and IL-22 upon *in vitro* restimulation with OVA. The ELISPOT assay also showed a significant frequency of IL-17-secreting CD4 T cells in LN cells (Fig. 1D). Moreover, increased mRNA levels of IL-17, IL-22, and Retinoid-related orphan receptor gamma t (ROR γ t), a Th17-specific transcription factor, were observed in DO11.10 with OVA patch applications (Fig. 1E). Since IL-6, TGF- β , and IL-23 act cooperatively to prime and maintain Th17 development, we next analyzed the mRNA expression of these cytokines in draining LN of BALB/c mice by quantitative real-time PCR. We could not detect the expression of IL-6 and IL-23 in naïve mice and mice receiving OVA application (data not shown). TGF- β expression could be detected and showed no increase after epicutaneous sensitization with OVA (data not shown). Collectively, these data demonstrate that epicutaneous sensitization with a protein Ag induces a small, but significant number of Th17 cells.

3.2. TLR ligands and other environmental elements promote the induction of Th17 cells in epicutaneous sensitization

Skin is the outermost organ exposed to many microorganisms in the environment, which are recognized as TLRs by the innate

immune system of the host. Thus, we checked the effect of TLR ligands on the induction of Th17 cells in epicutaneous sensitization. We chose poly(I:C), LPS, flagellin, and CpG-ODN as representative agonists for TLR3, TLR4, TLR5, and TLR9, respectively. For TLR2 agonist, preliminary experiments using different TLR2 agonists, including purified lipoteichoic acid, zymosan, peptidoglycan, and Pam3CSK4 revealed that Pam3CSK4 was the most effective TLR2 agonist among the agonists tested (data not shown). For the TLR7 agonists, imiquimod (R873) showed a stronger effect than *E. coli* RNA/LyoVec in the preliminary studies. Thus, Pam3CSK4 and imiquimod were used in the following experiments for TLR2 and TLR7 agonists, respectively. As shown in Fig. 2A, BALB/c mice co-administered Pam3CSK4, poly(I:C), LPS, flagellin, imiquimod, or CpG-ODN with OVA generally showed a trend of increased secretion of IL-17 and IL-22 when the LN cells were *in vitro*-reactivated with OVA, relative to BALB/c mice receiving an OVA application only. Among all the TLR ligands tested, CpG-ODN had the strongest capability to promote IL-17 and IL-22 production. The increase reached statistical significance in Pam3CSK4, flagellin and CpG-ODN groups. There are also many kinds of environmental elements, including irritants, haptens, and superantigens secreted by microorganisms, as well as products of epithelium, which could influence Th effector production through innate immunity. We chose SDS, TNBS, SEB, and TSLP as representatives. Fig. 2B shows that mice receiving epicutaneous

Fig. 2. Induction of Th17 cells in epicutaneous sensitization is further enhanced by TLR ligands and other environmental elements. Groups of BALB/c mice were immunized by patch application with OVA only or adding one of the followings: Pam3CSK4, poly(I:C), LPS, flagellin, imiquimod, and CpG-ODN (A), SDS, TNBS, SEB, and TSLP (B). Ten days later, cytokine contents of *in vitro* reactivation cultures of draining LN cells were determined by ELISA. Increased folds of IL-17 and IL-22 content compared with patch application with OVA only were shown. Pooled data were obtained from four to six independent experiments. Wilcoxon rank sum test was used for biostatistical analysis. $P < 0.05$ (*) was set to be significant.

sensitization by co-administration of SDS, TNBS, or SEB with OVA also showed a trend of higher contents of IL-17 and IL-22 in supernatants of LN cell reactivation culture than mice receiving OVA only. The increase reached statistical significance in SDS and SEB groups. In contrast, mice receiving epicutaneous sensitization by co-administration of TSLP with OVA showed similar levels of IL-17 and IL-22 secretion with the OVA only group. The increase of IL-17 and IL-22 production is not due to the direct stimulation of TLR ligands or environment elements because mice receiving patch application of either TLR ligand alone or environmental element alone showed comparable IL-17 and IL-22 levels with PBS control (data not shown). Taken together, these data suggest that many TLR ligands and environmental elements could further promote the induction of Th17 cells in epicutaneous sensitization.

3.3. C57BL/6 mice generate fewer Th17 cells than BALB/c mice in epicutaneous sensitization

It is generally accepted that C57BL/6 and BALB/c mice are Th1- and Th2-prone mice, respectively. To explore the potential difference of Th17 generation in epicutaneous sensitization between BALB/c and C57BL/6, both strains of mice were immunized and examined simultaneously. Fig. 3A shows that OVA-patched C57BL/6 mice always generated less IL-17 and IL-22 than OVA-patched BALB/c mice when their LN cells were *in vitro*-reactivated. Fig. 3B shows that the frequency of IL-17-secreting CD4 T cells in LN cells of OVA-patched C57BL/6 mice was also lower than that of OVA-patched BALB/c mice. We next checked the effects of TLR agonists and other environmental elements on Th17 generation in C57BL/6 mice. As shown in Fig. 3C, mice receiving co-administration of Pam3CSK4, poly(I:C), LPS, flagellin, imiquimod, or CpG-ODN usually produced larger amounts of IL-17 and IL-22 when compared with mice receiving OVA only. Similar to the observation in BALB/c mice, CpG-ODN was also the strongest enhancer among TLR ligands for IL-17 and IL-22 production in C57BL/6 mice. The increase fold reached statistical significance in LPS and CpG-ODN groups. Co-administration of other environmental elements, represented by SDS, TNBS, SEB, or TSLP also showed a trend of increased production of IL-17 and IL-22 in LN cell reactivation cultures (Fig. 3D). The increase fold reached statistical significance in SDS group. Collectively, these results demonstrate that Th1-prone C57BL/6 mice generate fewer Th17 cells in epicutaneous sensitization with protein antigens than Th2-prone BALB/c mice.

4. Discussion

In this study, we demonstrated the induction of Th17 cells by epicutaneous sensitization with protein antigens in mice. This

Th17 induction is further enhanced by many environmental elements, including products of microorganisms, irritants, and haptens. Furthermore, the difference of Th17 induction in epicutaneous sensitization between Th1-prone C57BL/6 and Th2-prone BALB/c mice was also demonstrated.

Th17 is a newly identified effector of T cell lineage which is involved in the pathophysiology of many inflammatory autoimmune or allergic diseases [7]. The differentiation of Th17 cells was intensively investigated recently and studies revealed the crucial roles of TGF- β and IL-6 in its development, and the antagonizing effects of products of Th1 and Th2 lineages. Most of these data were obtained from the *in vitro* systems. There are far fewer reports discussing the development of Th17 cells in *in vivo* systems. Albanesi et al. [23] and He et al. [10] reported that epicutaneous hapten sensitization induces the development of CD4 and CD8 T cells that produce IL-17, respectively. Th17 cells could be demonstrated in a conventional EAE model by subcutaneous immunization of peptides in emulsion of CFA with intravenous injection of pertussis toxin, which has been shown to promote Th17 generation [24,25]. For microorganisms, infection with *Mycobacterium tuberculosis* by the aerosol route and with *Candida albicans* by the intravenous route has been reported to induce Th17 development [26,27]. Very recently, He et al. reported Th17 generation by epicutaneous sensitization in BALB/c mouse [28]. The induced Th17 response could drive airway inflammation after inhalation challenge. Their experimental protocol was different from ours in two ways. Firstly, they sensitized mice for 3 courses. Their mice had a total of three 1-week exposure to patch separated from each other by 2-week interval. In contrast, our mice received patch application for 5 successive days only. Secondly, they performed tape stripping to remove keratin before patch application but we did not. Thus, this report which shows Th17 generation by epicutaneous sensitization with protein antigens, without the help of any adjuvant or manipulations is a pioneer demonstration of Th17 induction under physiological conditions. Considering the enhancing effects of environment elements on Th17 generation demonstrated in our current study, it is reasonable that the Th17 response induced in He's study was much stronger than ours.

In the current study, IL-17-secreting T cells were detected by intracellular staining only by *in vitro* PMA activation in DO11.10 transgenic mice (data not shown). IL-17-secreting T cells could not be detected by intracellular staining by *in vitro* OVA activation in DO11.10 transgenic mice or *in vitro* by PMA or OVA activation in BALB/c mice (data not shown). The most likely reason is the low frequency of IL-17-secreting T cells which was estimated to be around 0.01% by ELISPOT results in BALB/c mice. TSLP has a weak enhancing effect on Th17 induction in C57BL/6 mice, but not in BALB/c mice. The reason is obscure at present. TSLP is generally

Fig. 3. The generation and regulation of Th17 cells by epicutaneous sensitization in C57BL/6 mice. (A and B) Groups of BALB/c and C57BL/6 mice were simultaneously immunized by patch application with OVA and PBS control. Ten days later, draining LNs were obtained. The IL-17 and IL-22 content of supernatants of *in vitro* reactivation cultures were determined by ELISA. Net concentration was presented. Three independent experiments were performed with similar results. Data from one representative experiment was shown (A). CD4 T cells were purified from draining LN cells and the frequency of IL-17-secreting CD4 T cells was determined by ELISPOT assay (B). (C and D) C57BL/6 mice received similar immunization and analysis as in Fig. 2. Pooled data from four to six independent experiments were shown. Wilcoxon rank sum test was used for biostatistical analysis. $P < 0.05$ (*) was set to be significant.

believed to be a Th2-enhancer. Thus, the Th1- and Th2-prone innate character of C57BL/6 and BALB/c mice might be a possible mechanism to explain the differential effect of TSLP in these two mouse strains.

The physiologic and pathologic significance of induction of Th17 cells by epicutaneous sensitization with protein antigens are still unclear. IL-17-producing CD4 and CD8 T cells have been shown to be important effectors for sensitization and the elicitation phase of contact hypersensitivity which was a prototype animal model of human contact dermatitis [29]. IL-17 and IL-22 cooperatively enhances expression of antimicrobial peptides by keratinocytes, thus regulating cutaneous host defense [30]. Moreover, IL-22 was recently reported to mediate IL-23-induced dermal inflammation and epidermal acanthosis in psoriasis, a common chronic cutaneous inflammatory disorder [31]. For AD, IL-17 expression is enhanced in acute, but not chronic lesions [32]. Very recently, Koga et al. reported that the number of Th17 cells is increased in the peripheral blood as well as acute lesional skin and correlated with severity of AD [33]. It was suggested that Th17 cells participate in the development of AD as an enhancer, but not an immune-polarizer, of AD [33]. Taken together, Th17 cells with their cytokine products may play important roles in the pathogenesis of many cutaneous inflammatory diseases.

Information regarding the reciprocal interaction between Th1/Th17 and Th2/Th17 was obtained from both *in vitro* and *in vivo* systems. In *in vitro* culture systems, antibodies to IFN- γ and IL-4 should be added in order to skew T cells to Th17 differentiation, thus leading to the conclusion that products of Th1 and Th2 lineage antagonize the development of Th17. For *in vivo* animal model systems, most investigators reported similar observations. For example, Ab to IFN- γ worsened EAE and adjuvant arthritis which Th17 cells play a central role in pathogenesis, whereas IFN- γ administration protected the mice from disease development [8]. T-bet-deficient mice were also

reported to show increased Th17 differentiation and increased neutrophilic airway inflammation [34]. Notably, to our knowledge, there is still no report discussing Th17 development in Th2-lineage-deficient mice. For the effect of Th17 on Th1 and Th2 development, Nakae et al. [29] reported that contact and delayed-type hypersensitivities were both significantly reduced in IL-17-deficient mice due to impairment of sensitization of antigen-specific Th1 and Th2 cells. As to airway hypersensitivity response, they showed that OVA/alum-induced airway hypersensitivity response developed normally in IL-17-deficient mice, although IL-4 and IL-5 production by T cells and Ab production to OVA were reduced. They attributed it to an excess Th2 response induced by alum adjuvant because airway hypersensitivity response induced by OVA/PBS inhalation was suppressed in IL-17^{-/-} DO11.10 Tg mice [29]. For immune response modifiers, PGE₂, a conventional Th2 enhancer, was shown to promote Th17 development in experimental inflammatory bowel disease and in *Bordetella* infection of macrophages [35,36]. Dectin-1 agonist was recently demonstrated to be an adjuvant for both Th1 and Th17 priming [24]. Epicutaneous sensitization with protein antigens induces a predominant Th2 immune response in both BALB/c and C57BL/6 mice [17]. However the Th2 immune response induced in C57BL/6 mice is always weaker when compared with BALB/c mice. The induction of Th17 cells in BALB/c and B6 mice follows similar trend. Moreover, all TLR ligands tested and many environmental elements, with either Th1 or Th2 enhancing capability, promote Th17 cells as well as Th2 immune response in our model system (data not shown). Thus, it is likely that the amplitude of Th17 cells induced by epicutaneous sensitization is determined by the intensity of the entire immune response, but not by the balance between Th1/Th2/Th17 development. In conclusion, the interaction of Th1/Th17 and Th2/Th17 is much more complicated than simple antagonism and needs further observation and investigation.

In summary, the demonstration of Th17 induction and its regulation in epicutaneous sensitization with a protein antigen highlights its importance as an allergen sensitization route for atopic diseases, and provides crucial information for prevention of allergen sensitization and vaccine development.

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