



Staphylococcal enterotoxin B increases TIM4 expression in human dendritic cells that drives naïve CD4 T cells to differentiate into Th2 cells

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Received 11 January 2007; received in revised form 18 February 2007; accepted 9 March 2007

Abstract

Aberrant T helper (Th)2 polarization plays a critical role in the pathogenesis of allergic disorders; the etiology remains unclear. Dendritic cells (DCs) express T cell immunoglobulin mucin domain (TIM)4 that ligates TIM1 on CD4 T cells to drive them to become Th2 cells, but the pathogenic source of TIM4 is unknown. Here we report that a significant increase in TIM4 expression in human DCs was observed in response to Staphylococcal enterotoxin B (SEB) stimulation via Toll-like receptor (TLR)2 and nucleotide-binding oligomerization domain (NOD)1 pathway. Coculture SEB-conditioned DCs with naïve CD4 T cells induced Th2 responses that could be abolished using TLR2 or NOD1 or TIM4 or TIM1 with counterpart antibodies or RNA interference. The results demonstrate that *Staphylococcus aureus* derived SEB promotes the TIM4 production in human DCs. The interaction between TIM4 and TIM1 drives naïve CD4 T cells to develop to Th2 cells.

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Keywords: T helper cell; Dendritic cell; Staphylococcal enterotoxin B; Toll-like receptor

1. Introduction

The prevalence of allergy is as high as 15–20% of populations and increased rapidly in recent several decades (Eder et al., 2006). The pathogenesis of allergy has been well studied (Tournoy et al., 2006); however, the etiology of allergy remains unclear (Kowalski, 2007). It is believed that T helper (Th)2 polarization plays a critical role in the pathogenesis of allergy (Tournoy et al., 2006). Upon activation, naïve CD4 T cells differentiate into Th1 or Th2 cells characterizing as secreting cytokine of Th1 [e.g., interferon (IFN)- γ] or Th2 [e.g., interleukin (IL)-4, IL-13] (Umetsu and DeKruyff, 2006). Yet, the mechanism dictating naïve CD4 T cells to differentiate into Th1 or Th2 responses is to be elucidated.

Dendritic cells (DC) are the cells dictating naïve CD4 T cells to develop to either Th1 cells or Th2 cells depending on certain environment (Corthay, 2006). DCs secrete IL-12 in response to microbial stimulation (de Jong et al., 2005). IL-12 is required to induce CD4 T cells to become Th1 cells to produce Th1 cytokine such as IFN- γ (Peluso et al., 2006). Although IL-10 was proposed to play a role in the induction of Th2 response (Mingomataj et al., 2006), further studies indicate that its function focuses more in immune regulatory activities (Akbari et al., 2001; Urry et al., 2006). Recent studies indicate that DCs express T cell immunoglobulin mucin domain (TIM)4 that ligates TIM1 on Th2 cells to promote Th2 cell development (Meyers et al., 2005). The report has shed a new light on the study of the mechanism of allergic diseases. However, the factors in up-regulating TIM4 expression in DCs remain unknown.

Several Toll-like receptors (TLR) such as TLR2, TLR4 are expressed by DCs. TLRs recognize the highly conserved structural motifs called pathogen-associated molecular patterns play

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a critical role in early innate immunity by sensing the presence of microbial pathogens and initiating a response to clear them (Yeh and Chen, 2003; Barton and Medzhitov, 2003). TLR2 can be activated by several bacterial products, including peptidoglycans, lipoproteins, mycobacterial lipoarabinomannan (Takeda et al., 2003) and Staphylococcal enterotoxin B (SEB) (Mandron et al., 2006). TLR signals are involved in production of major histocompatibility complex (MHC) (Reis e Sousa, 2004), costimulatory molecules (Mazzoni and Segal, 2004) and cytokines in DCs (Guillot et al., 2005).

Two intracellular sensors, nucleotide oligodimerization domains 1 and 2 (Nod1 and Nod2) have been described (Inohara et al., 2001). Nod1 and Nod2 proteins are located in the cytosol and recognize mainly peptidoglycan-derived muropeptides (Chamaillard et al., 2003). The signals of NOD1 or NOD2 in response to microbial stimulation induce cell activation through receptor-interacting protein 2, the activation of NF- κ B (Inohara et al., 2000) as well as c-Jun N-terminal kinase (Thome et al., 1998). Although the action of NOD1 and NOD2 is involved in DC's function (Fritz et al., 2005), whether the action is also involved in T helper (Th)2 polarization is not clear.

To understand the etiology and mechanism of allergic diseases, animal models are employed. We developed several animal models in studying the mechanism of intestinal allergic responses in past 10 years (Berin et al., 1997; Cameron et al., 2003; Yang et al., 1999, 2000, 2001, 2006; Yu et al., 2001). A common requirement in these animal models was the concurrent exposure to microbial products (used as adjuvants, such as pertussis toxin) and food antigens. Other investigators and we also reported similar data, such as cholera toxins (Sun et al., 2006), *Staphylococcus aureus* enterotoxin B (SEB) (Yang et al., 2005) and Freud adjuvant (Rivera et al., 2005) were required as adjuvants in the developing animal models of allergic diseases. The role of adjuvants is suggested to speed lymphocyte proliferation (Corthay, 2006; Lavelle et al., 2004). However, the mechanism is not clear. Therefore, in this study, we hypothesized that the microbial derived adjuvants might act on DCs to promote TIM4 expression in DCs and subsequently, drive naïve CD4 T cells to differentiate into Th2 cells. We aimed to investigate if SEB, a common microbial product, increased TIM4 expression in human DCs; if SEB-conditioned DCs drove naïve CD4 T cells to differentiate into Th2 cells and how important of TIM4 was in human Th2 cell development. The results showed that exposure to SEB enhanced the expression of TIM4 in DCs that further facilitated Th2 cell development.

2. Materials and methods

2.1. Human DC and CD4 T cell preparation

The procedures of the experiments in this study were approved by the Research Ethics Committee at Shanxi Medical University. Twenty healthy volunteers were selected as blood donors. Peripheral blood mononuclear cells (PBMCs) were separated using Histopaque (SG-1.077; Sigma) density gradients (Martin et al., 2005). CD11c⁺ DCs and naïve CD4⁺CD45RA⁺ T cells (in short: CD4 T cells) were separated from the peripheral

blood mononuclear cells (PBMCs) using reagent kits (Myltenyi Biotec). As checked by flow cytometry (FACS), the purity of separated DCs and CD4 T cells was over 90%. Separated DCs or CD4 T cells were resuspended in RPMI 1640 medium (Invitrogen Life Technologies) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 25 mM HEPES and cultured at 37 °C in 5% CO₂ environment.

2.2. Coculture of CD4 T cells with SEB-conditioned DCs in vitro

Separated DCs were cultured in RPMI 1640 media in the presence of SEB for 48 h at gradient doses (0–100 ng/ml) to make them as “SEB-conditioned DCs”. After washing with fresh culture media for three times, the SEB-conditioned DCs (10⁵ cells/well) were cocultured with CD4 T cells (10⁶ cells/well) for 48 h and then subjected to further analyses.

2.3. RNA interference (RNAi)

The following duplexed RNA oligonucleotides (Stealth RNAi) were synthesized by Invitrogen: TLR2: ggauccacuacau-uagca, NOD2, 5'-uauuguuauucgcgcaauacagagc-3' and control: aaauccggaacgugucacgu. The control sequence was checked with software Blast and did not match any other sequences. Separated DCs were transfected with 15 pmol TLR2siRNA or NOD1siRNA or control siRNA with the reagent set and instruction provided by Invitrogen. At 72 h post-transfection, DCs were exposed to SEB (200 ng/ml) for an addition of 48 h. TIM4 expression in DCs was determined with ELISA. The cells were then subjected to the subsequent experiments with CD4 T cells.

2.4. Flow cytometry

Cells were collected at the end of experiments and fixed with 1% paraformaldehyde for 30 min at 4 °C, washed with 1% BSA/PBS for three times, and blocked for 30 min at 4 °C with 1% BSA. The cells were incubated with indicated antibodies (1 μ g/ml) for 1 h at 4 °C. After antibody incubating, the cells were washed and incubated with a 1% dilution of F(ab')₂-FITC-goat anti-mouse Ab (BD Pharmingen) for 30 min at 4 °C. After three washes, cells were resuspended in 400 μ l of PBS. The mean intensity of fluorescence was determined for 20,000 cells using a FACScan flow cytometer (BD Biosciences). An isotype IgG was used as a negative control. Each experiment was performed at least three times.

2.5. Western blot analysis

Proteins were extracted from cultured DCs using lysis buffer (0.1% Triton X-100, 12.1 mM sodium deoxycholate, 3.5 mM SDS in PBS, pH 7.4) containing a cocktail of protease inhibitors. Protein concentrations were determined with Bio-Rad protein assay. Proteins from cellular lysates (40 μ g/lane) were separated on 10% SDS-PAGE gels (Invitrogen Life Technologies). Following electrotransfer to supported nitrocellulose membranes (Invitrogen Life Technologies), blots were blocked with 5%

nonfat dry milk in TBS, 0.1% Tween 20 for 1 h at room temperature. Blots were then incubated overnight at 4 °C with one of the following antibodies: 1/1000 dilution of primary antibodies [anti-TIM4 (R&D Systems), anti-TLR2 (Serotec) and anti-NOD1 (R&D Systems)]. The peroxidase-conjugated secondary antibodies (1/10,000 dilution) were used to detect specific protein bands with the ECL system (Amersham Pharmacia Biotech).

2.6. ELISA

Levels of IL-4, IL-13, IL-10, IL-12p70 and IFN- γ in culture media were evaluated with ELISA kits (R&D Systems) following the manufacturer's instructions. Levels of TIM4 in culture media were also determined with ELISA (anti-TIM4 antibody was purchased from R&D Systems). The procedures were referred to previous report (Nishimura et al., 2006a,b). Negative control experiments were performed with an isotype IgG instead of the primary antibodies.

2.7. Immunocytochemistry

Cell pellets were fixed by resuspending in 2% paraformaldehyde for 30 min. Cells were blocked by incubating with 2% goat serum for 30 min, and then, incubated with the first antibody (goat anti human TIM4, 100 ng/ml; mouse anti human TLR2, 50 ng/ml; rabbit anti human NOD1, 100 ng/ml) and the fluorescein conjugated second antibodies (1:100–300, DAKO), respectively, for 1 h at room temperatures. Control cells were omitted the first antibody or incubated with an isotype IgG. Cells were washed with phosphate buffered saline (PBS) for 5 min (three times) following each step. The positively stained cells were smeared on a slide and observed with a confocal microscope (LSM510).

2.8. Statistics

Data were expressed as the means $\pm$ S.D. Differences between groups were determined with Student's *t*-test (2 groups) or ANOVA (3 or more groups); a *p* < 0.05 was accepted as the significant criteria.

3. Results

3.1. Human DCs express TIM4 that is enhanced by exposure to SEB

Previous reports indicated that DCs expressed TIM4 that plays a critical role in inducing peripheral Th2 polarization in a murine model (Meyers et al., 2005); but so far the pathogenic source of TIM4 has not been identified. In this study, we first sought to identify if human DCs also express TIM4. The peripheral blood was collected from 20 healthy volunteers and the mononuclear cells were prepared. CD11c⁺ DCs were further separated from the mononuclear cells with MACS. As shown by immunocytochemistry, protein of TIM4 was localized in DCs using confocal microscopy (Fig. 1A). Expression of TIM4

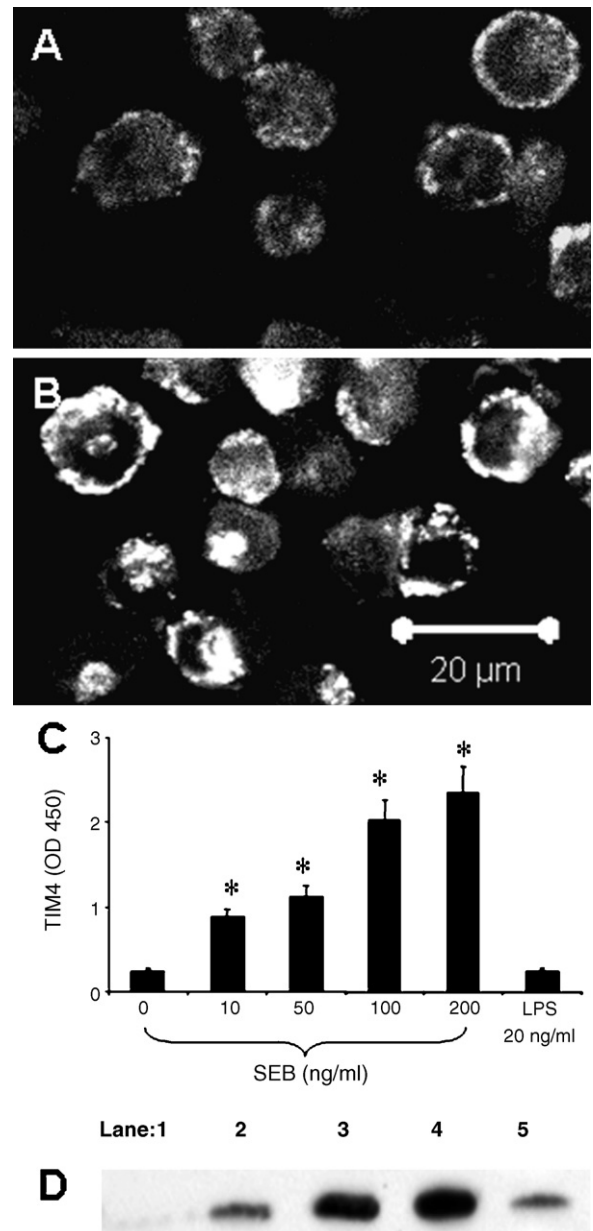


Fig. 1. *Staphylococcus aureus* enterotoxin B (SEB) promotes the expression of TIM4 in human dendritic cells (DCs). DCs were separated from the peripheral blood mononuclear cells and exposed to SEB at gradient doses. (A and B) representative confocal images show the expression of TIM4 (in white color) in DCs. (A) naïve DCs; (B) DCs treated with SEB (200 ng/ml) for 48 h. (C) Data from ELISA show TIM4 expression in extracts of DC (mean $\pm$ S.D.). LPS, the protein was extracted from DCs treated with LPS (20 ng/ml). **p* < 0.05, compared with dose 0. (D) Representative Western blots show TIM4 expression from the extracts from DCs. Lane 1, negative control; lane 2–4, proteins were extracted from DCs exposed to SEB at doses of 0 ng/ml (lane 2), 50 ng/ml (lane 3) and 200 ng/ml (lane 4). Lane 5 shows a blot from LPS treated DCs. Each experiment was repeated at least three times.

in DCs increased in response to SEB stimulation in a dose-dependent manner (Fig. 1B). Expression of TIM4 in human DCs was further confirmed by Western blot (Fig. 1C). Another set of DCs was exposed to LPS to serve as a control. The results showed that LPS did not have any effect in modulating TIM4 expression in DCs (Fig. 1B and C).

3.2. TLR2 and NOD1 are involved in SEB-increased TIM4 expression in DCs

Toll-like receptors and NOD (1 and 2) proteins are involved in sensing microbes and their products (Yeh and Chen, 2003; Inohara et al., 2001). We next sought to identify the role of these two kinds of molecule in SEB-induced TIM4 expression in DCs. Expression of TLR2 in human DCs was examined using immunocytochemistry. The positive stain for TLR2 was localized on the surface of DCs (Fig. 2A). We also examined the expression of NOD1 in human DCs. The results showed that

the NOD1 positive stain was localized inside the cytoplasm (Fig. 2B). The expression of TLR2 and NOD1 in DCs was confirmed by the results of Western blot (Fig. 2E and F). We next observed if human DCs absorbed SEB. As shown in Fig. 2C, positive stain for SEB was localized in the cytoplasm of DCs after incubation with SEB for 2 h. A set of DCs was treated with anti-TLR2 antibody or transfected with TLR2siRNA prior to the exposure to SEB. As expected, the absorption of SEB was completely abrogated (Fig. 2D). Considering NOD1 and NOD2 are the intracellular sensor for microbial products (Inohara et al., 2001), they may be involved in the process of SEB to induce TIM4 expression in DCs. In a preliminary study, we tested to block SEB induced TIM4 expression by pretreatment with anti-NOD1 or anti-NOD2 antibody. The preliminary results showed that the pretreatment with anti-NOD1 antibody significantly inhibited the SEB-induced TIM4 expression in DCs (data not shown); we thus chose anti-NOD1 mechanism in this study. Another set of DCs were pretreated with anti-NOD1 antibody, and then exposed to SEB for 48 h. The results showed that anti-NOD1 efficiently inhibited SEB-enhanced TIM4 expression in DCs (Fig. 2G). To further verify the role of NOD1 in SEB induced TIM4 expression, separated DCs were transfected with NOD1 siRNA and then exposed to SEB. The results showed the expression of TIM4 was abolished by the treatment with NOD1 siRNA, but not by control siRNA (Fig. 2G).

3.3. SEB promotes DC maturation

Mature DCs produce MHC I or II and costimulatory molecules to induce T cell activation. We next sought if the exposure to SEB also facilitated DC maturation. Separated DCs were cultured in the presence of SEB at gradient doses for 48 h. DCs were collected and processed for FACS. The DCs were stained with antibodies for CD80 (Fig. 3A), CD83 (Fig. 3B), CD86 (Fig. 3C) and MHC II (Fig. 3D), respectively. As shown by FACS, exposure to SEB significantly increased the median fluorescence intensity (MFI) of MHC II and costimulatory molecules in the DCs as compared with naïve controls. Pretreatment with antibody of TLR2 or NOD1 or transfected with siRNA of TLR2 or NOD1 abolished the SEB-increased production of MHC II and costimulatory molecules in DCs. LPS treated DCs also showed increases in MHC II and costimulatory molecules. The levels of IL-12p70 in culture media were also determined by ELISA. As shown in Fig. 4, LPS significantly induced IL-12p70 production whereas treatment with a series of doses of SEB did not show any effect on IL-12p70 production in DCs (Fig. 4).

3.4. SEB-conditioned DCs induce Th2 polarization

The interaction of TIM1–TIM4 drives naïve CD4 T cells to become Th2 cells (Meyers et al., 2005); SEB induced TIM4 expression in DCs was observed in this study; CD4 T cells express TIM1 (Meyers et al., 2005); thus, we next sought to determine if SEB-conditioned DCs induced Th2 cell development. Naïve CD4 T cells were separated from healthy human blood mononuclear cells. Separated DCs were cultured in the

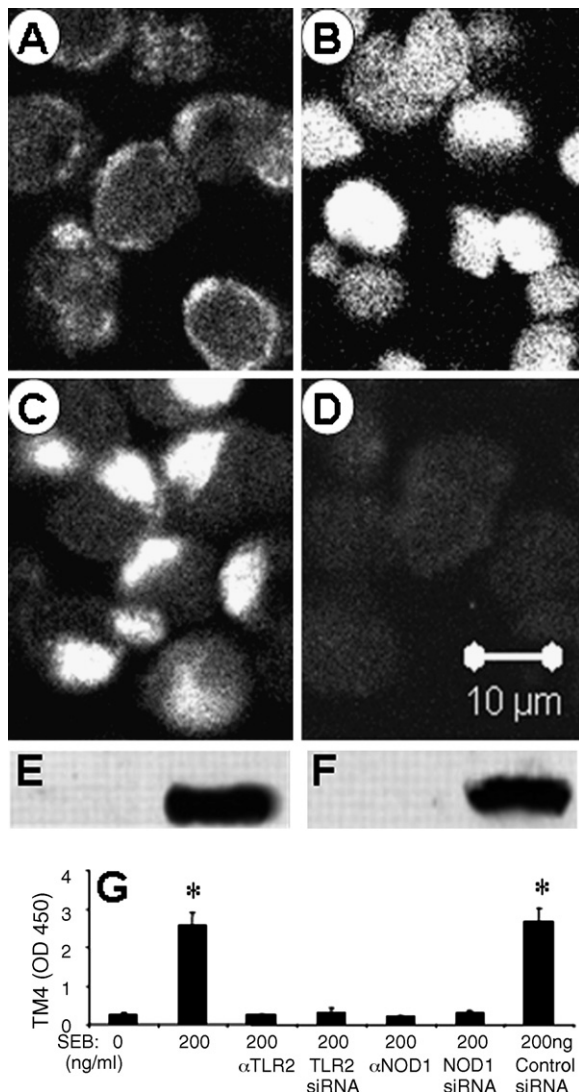


Fig. 2. SEB modulates the expression of TIM4 via TLR2 and NOD1. Human DCs were stained with anti-TLR2 (A) or anti-NOD1 (B) or anti-SEB (C and D) antibody. (A–D) representative confocal images. (A) TLR2 expression (white color) on DCs. (B) NOD1 expression in DCs. (C) SEB was absorbed into DCs (white color). (D) naïve DCs were stained with anti-SEB antibody served as negative controls. (E and F) representative Western blots; (E) TLR2; (F) NOD1. (G) Bars indicate the contents of TIM4 in extracts of DCs (by ELISA). αTLR2, DCs were pretreated with anti-TLR2 antibody. αNOD1, DCs were pretreated with anti-NOD1 antibody. TLRsiRNA and NOD1siRNA, DCs were transfected with siRNA of TLR or NOD1, and then exposed to SEB. * $p < 0.05$, compared with dose 0 (SEB). # $p < 0.05$, compared with the group of cells treated with SEB alone at a dose of 200 ng/ml.

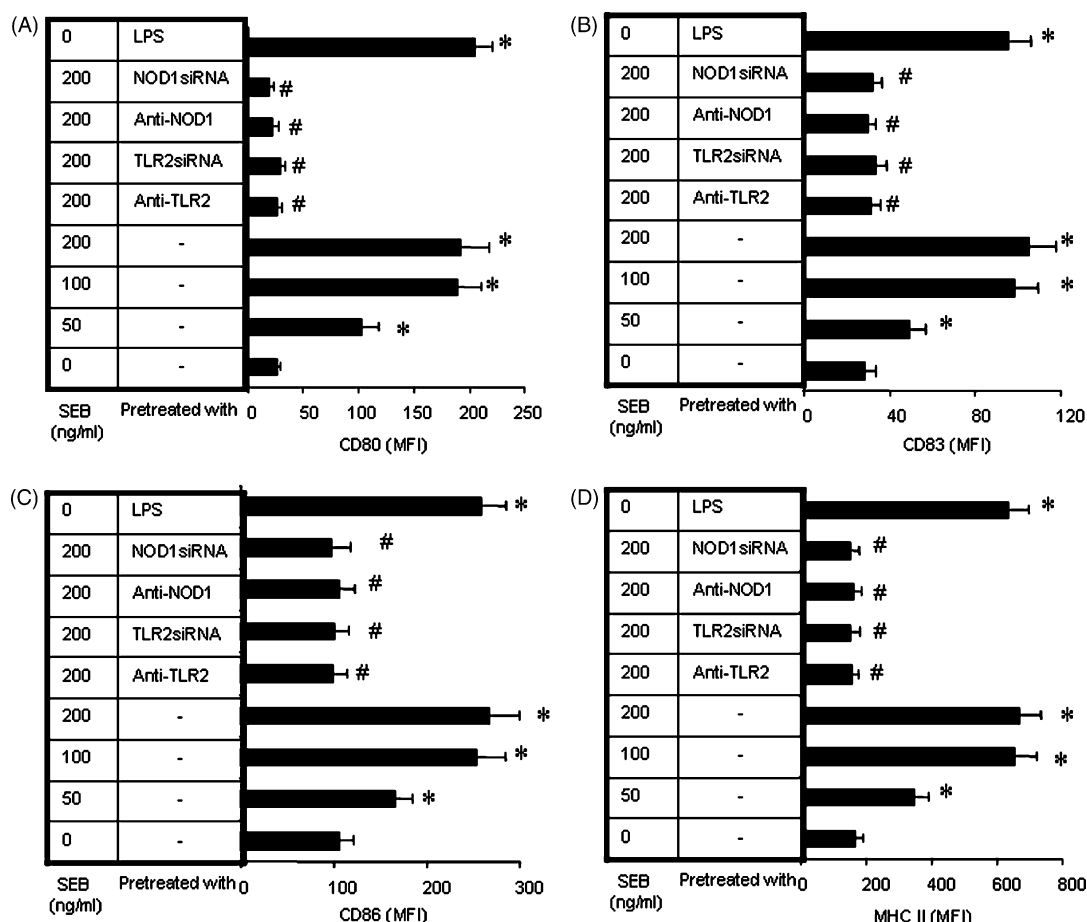


Fig. 3. SEB promotes DC maturation. DCs were cultured in the presence of SEB at gradient doses for 48 h and stained with antibodies of CD80 (A), CD83 (B), CD86 (C) and MHC II (D). Stained cells were analyzed with flow cytometry. Data are expressed as the median fluorescence intensity (MFI, mean $\pm$ S.D.). α TLR2 and α NOD1: the set of cells was treated with anti-TLR2 or anti-NOD1 antibody prior to the exposure to SEB at a dose of 200 ng/ml. LPS: a set of DCs was exposed to LPS at a dose of 20 ng/ml and stained with the same set of antibodies (A–D). * $p < 0.05$, compared with dose 0 (SEB). # $p < 0.05$, compared with the group of cells treated with SEB alone at a dose of 200 ng/ml.

presence of SEB (200 ng/ml) or LPS (20 ng/ml) for 48 h. After washing with fresh media for 3 times, the SEB- or LPS-conditioned DCs were cocultured with naïve CD4 T cells for 48 h. [^{3} H] thymidine was added to the culture for the last 16 h at a dose of 0.5 μ Ci. The results from the β scintillation counter

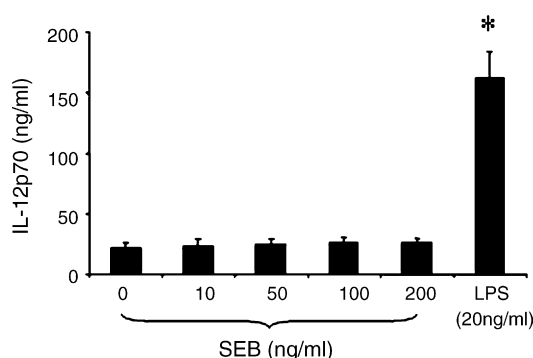


Fig. 4. Expression of IL-12 in DCs. Separated DCs were cultured in the presence of SEB at gradient doses or LPS for 48 h. Contents of IL-12p70 in culture media were evaluated by ELISA. Bars indicate the levels of IL-12p70. Data are expressed as the means $\pm$ S.D. * $p < 0.05$, compared with dose 0.

analysis showed high [^{3} H] thymidine incorporation in the CD4 T cells cocultured with either SEB- or LPS-conditioned DCs (Fig. 5A). To differentiate the increased T cell response was Th1 pattern or Th2 pattern, samples from culture media were analyzed by ELISA with antibodies against cytokines of Th1 (IFN- γ , Fig. 5B) and Th2 (IL-4 and IL-13; Fig. 5C and D). The results showed SEB-conditioned DCs mainly increased the expression of Th2 cytokines (Fig. 5B–D). Those SEB-conditioned DCs pretreated with antibodies or siRNAs against TLR2 or NOD1 did not show any effects on enhancing Th2 response (Fig. 5A–D).

3.5. TIM1–TIM4 interaction plays a crucial role in the Th2 polarization induced by SEB-conditioned DCs

To further clarify the role of human DC-derived TIM4 in Th2 cell differentiation, experiments on TIM1–TIM4 interaction were carried out. Separated human naïve CD4 T cells were cocultured with SEB-conditioned DCs for 48 h in the presence or absence of anti-TIM1 or anti-TIM4 antibodies. The results showed that DCs cultured in the presence of anti-TIM4 and

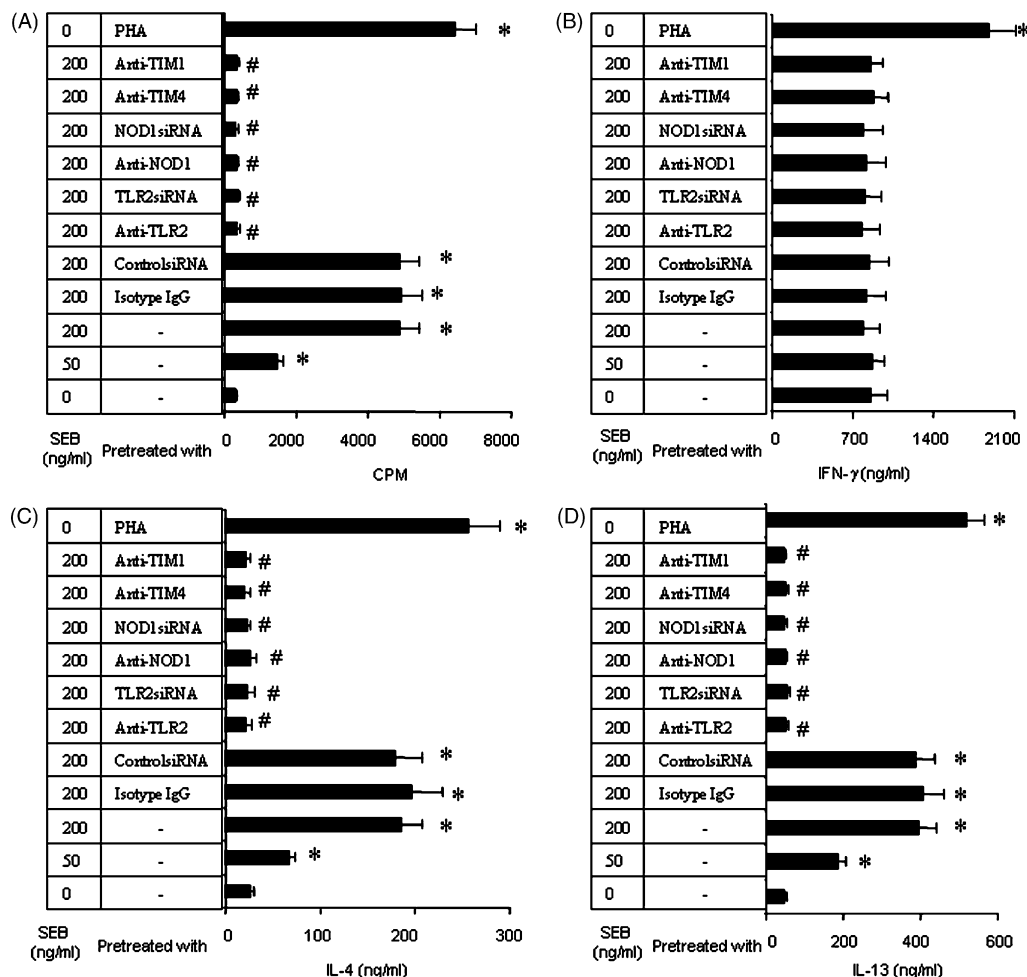


Fig. 5. SEB-conditioned DCs induce Th2 polarization. Separated CD4 T cells were cultured with SEB-conditioned DCs and various treatments. (A) Bars indicate the ratio of [^{3} H] incorporation in CD4 T cells. (B–D), bars indicate the contents of IFN- γ (B) or IL-4 (C) or IL-13 (D) in culture media. Data are expressed as means $\pm$ S.D. * p < 0.05, compared with dose 0 (SEB). # p < 0.05, compared with the group of cells treated with SEB alone at a dose of 200 ng/ml. α TLR2 or TLR2siRNA: the group of DCs was pretreated with anti-TLR2 antibody or transfected with TLR2siRNA; α NOD1 or NOD1siRNA: the group of DCs was pretreated with anti-NOD1 antibody or NOD1siRNA; these two groups of DCs then were exposed to SEB at a dose of 200 ng/ml for 48 h, and then cocultured with naïve CD4 T cells. α TIM4: the group of DCs was treated with both SEB (200 ng/ml) and anti-TIM4 antibody, and then cocultured with naïve CD4 T cells. α TIM1: naïve CD4 T cells were treated with anti-TIM1 antibody prior to the presence of SEB-conditioned DCs. Each experiment was repeated for three times.

SEB or CD4 T cells pretreatment with anti-TIM1 antibody prior to the exposure to SEB-conditioned DCs significantly inhibited the expression of IL-4 and IL-13 (Fig. 5A–D).

4. Discussion

To understand the mechanism by which naïve CD4 T cells develop to Th2 cells and how this process is out control is a critical point to elucidate the mechanism of allergy. The present data show that microbial product SEB significantly increases the expression of TIM4 in DCs and subsequently increases naïve CD4 T cells to become Th2 cells. The interaction of TIM4–TIM1 plays a critical role in the Th2 cell development.

Recent reports indicate that the interaction of TIM1–TIM4 plays a critical role in allergic pathogenesis (Meyers et al., 2005; Kuchroo et al., 2006). Application of antagonists of TIM1 resulted in significant suppression in T cell responses (Mesri et al., 2006) and airway allergic inflammation (Encinas et al., 2005). The ligand of TIM1 is TIM4 that is expressed

in DCs as observed in the present study and reported by others (Meyers et al., 2005). Our data further demonstrated that the expression of TIM4 in DCs could be up-regulated by the exposure to SEB, one of the microbial products commonly existing in natural environment (Lee et al., 2006). The results implicate that microbial infection may be one of the factors induce TIM4 expression in DCs. Because of DCs are localized at the interface of the epithelium and the environment (Figdor et al., 2002; Niess and Reinecker, 2006), a scenario can be envisaged that DCs may often encounter invaded microbes and microbial products. These microbes and their products are quite possibly to be captured by DCs; the expression of TIM4 in DCs may be up-regulated consequently.

In this study, we took insight into the mechanism of TIM4 up-regulation in DCs. The present data indicate that TLR2 and NOD1 are involved in SEB-induced TIM4 enhancement in human DCs. As other investigators reported (Wang et al., 2006; Kaisho and Akira, 2003), we also identified that DCs expressed TLR2; the expression of TLR2 was on the surface

of DCs, the specific area to sense microbial products immediately. The important aspect of our findings is that we have identified that TLR2 mediates the absorption of SEB into DCs. Application of antagonism of TLR2 by using anti-TLR2 antibody or TLR2siRNA efficiently abolished SEB-absorption. This finding is agreement with Mandron's finding that SEB promoted NF- κ activation in HEK293 cells mediated by TLR2 activation (Mandron et al., 2006).

It is proposed that NOD1 is involved in sensing microbial product peptidoglycan (Wolfert et al., 2007). We postulated that NOD1 might sense SEB in DCs as well. We then identified that DCs did express NOD1 inside the cytoplasm. Since the pretreatment with anti-NOD1 antibody or NOD1siRNA showed completely abolished SEB-induced TIM4 expression in DCs, we realized that SEB interacted with NOD1 first and then induced the expression of TIM4 in DCs. The results showed that the expression of TIM4 in DCs could be inhibited by pretreatment with either anti-TLR2 or anti-NOD1 antibody. Whether these two molecules enhance the expression of TIM4 via different pathways or act synergistically is an interested topic. We propose that TLR2 senses SEB first, and then transports SEB to the cytoplasm. The postulation is based on two points: first, anatomically, we observed TLR2 expression mainly on the surface of DCs where TLR2 has the opportunity to detect substances around cells; second, DCs absorbed SEB into the cytoplasm that was inhibited by pretreatment with anti-TLR2 antibody or TLR2siRNA. After absorbed into the cytoplasm, SEB interacts with NOD1 and then initiates the expression of TIM4 in DCs. Yet, the mechanism needs to be further studied.

To initiate immune reactions in CD4 T cells, DC derived MHC II and costimulatory molecules are required (Dai et al., 2005). Exogenous antigens and microbial products are strong stimuli to induce DC maturation indicated by increasing the production of costimulatory molecules. The present data showed that both SEB and LPS are able to increase the expression of costimulatory molecules in separated DCs that is in consistent with previous studies (Jelinek et al., 2007; Higgins et al., 2006; Mandron et al., 2006). The results also showed that although both SEB and LPS induced DC maturation, only SEB was able to induce the TIM4 expression in DCs indicating these two molecules activate DCs via different mechanisms. We postulated that TIM4 might play a role in altering DCs' function. However, the pretreatment with anti-TIM4 antibody did not have any effect on the production of costimulatory molecules in DCs (data not shown). This event indicates that SEB-induced increases in the expression of TIM4 do not interfere with the production of costimulatory molecules in DCs.

In agreement with a previous report (Mandron et al., 2006), we also observed that SEB-conditioned DCs promoted naïve CD4 T cells to differentiate into Th2 cells. Furthermore, our findings indicate that the induced Th2 cell development was TIM4-dependent since the pretreatment with anti-TIM4 antibody could completely blocked the Th2 polarization although the concurrent existence of costimulatory molecules in the same environment at the same period. On the other hand, pretreatment with anti-TIM1 antibody also could block the induced Th2 polarization by SEB-conditioned DCs, which confirms the interaction

between TIM1 and TIM4 plays a critical role in the induction of Th2 polarization.

In summary, separated human DCs express a low level of TIM4 that can be up-regulated by the treatment with SEB via TLR2 and NOD1 pathways. SEB-conditioned DCs are capable of inducing naïve CD4 T cells to differentiate into Th2 cells, which is TIM4-dependent. Application with anti-TIM4 or anti-TIM1 or anti-TLR2 or anti-NOD1 antibody can block the induced Th2 polarization proposing a potential value in adjusting aberrant Th2 polarization.

Acknowledgements

The authors thank Dr. M.H. Perdue for discussion on this paper. This study was supported by a grant from the Canadian Institute for Health Research and a grant from the Shanxi Natural Science Foundation.

References

- Akbari, O., DeKruyff, R.H., Umetsu, D.T., 2001. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* 2, 725–731.
- Barton, G.M., Medzhitov, R., 2003. Toll-like receptor signaling pathways. *Science* 300, 1524–1525.
- Berin, M.C., Kiliaan, A.J., Yang, P.C., Groot, J.A., Taminiau, J.A., Perdue, M.H., 1997. Rapid transepithelial antigen transport in rat jejunum: impact of sensitization and the hypersensitivity reaction. *Gastroenterology* 113, 856–864.
- Cameron, H.L., Yang, P.C., Perdue, M.H., 2003. Glucagon-like peptide-2-enhanced barrier function reduces pathophysiology in a model of food allergy. *Am. J. Physiol. Gastrointest. Liver Physiol.* 284, G905–G912.
- Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., Valvano, M., Foster, S., Mak, T., Nunez, G., Inohara, N., 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat. Immunol.* 4, 652–654.
- Corthay, A., 2006. A three-cell model for activation of naive T helper cells. *Scand. J. Immunol.* 64, 93–96.
- Dai, S.Y., Nakagawa, R., Itoh, A., Murakami, H., Kashio, Y., Abe, H., Katoh, S., Kontani, K., Kihara, M., Zhang, S.L., Hata, T., Nakamura, T., Yamauchi, A., Hirashima, M., 2005. Galectin-9 induces maturation of human monocyte-derived dendritic cells. *J. Immunol.* 175, 2974–2981.
- de Jong, E.C., Smits, H.H., Kapsenberg, M.L., 2005. Dendritic cell-mediated T cell polarization. *Springer Semin. Immunopathol.* 26, 289–307.
- Eder, W., Ege, M.J., von Mutius, E., 2006. The asthma epidemic. *N. Engl. J. Med.* 355, 2226–2235.
- Encinas, J.A., Janssen, E.M., Weiner, D.B., Calarota, S.A., Nieto, D., Moll, T., Carlo, D.J., Moss, R.B., 2005. Anti-T-cell Ig and mucin domain-containing protein 1 antibody decreases TH2 airway inflammation in a mouse model of asthma. *J. Allergy Clin. Immunol.* 116, 1343–1349.
- Figdor, C.G., van Kooyk, Y., Adema, G.J., 2002. C-type lectin receptors on dendritic cells and Langerhans cells. *Nat. Rev. Immunol.* 2, 77–84.
- Fritz, J.H., Girardin, S.E., Fitting, C., Werts, C., Mengin-Lecreux, D., Caroff, M., Cavaillon, J.M., Philpott, D.J., Adib-Conquy, M., 2005. Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. *Eur. J. Immunol.* 35, 2459–2470.
- Guillot, L., Le, G.R., Bloch, S., Escriviou, N., Akira, S., Chignard, M., Si-Tahar, M., 2005. Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J. Biol. Chem.* 280, 5571–5580.
- Higgins, S.C., Jamicki, A.G., Lavelle, E.C., Mills, K.H., 2006. TLR4 mediates vaccine-induced protective cellular immunity to *Bordetella pertussis*: role of IL-17-producing T cells. *J. Immunol.* 177, 7980–7989.

- Inohara, N., Koseki, T., Lin, J., del Peso, L., Lucas, P.C., Chen, F.F., Ogura, Y., Nunez, G., 2000. An induced proximity model for NF- κ B activation in the Nod1/RICK and RIP signaling pathways. *J. Biol. Chem.* 275, 27823–27831.
- Inohara, N., Ogura, Y., Chen, F.F., Muto, A., Nunez, G., 2001. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. *J. Biol. Chem.* 276, 2551–2554.
- Jelinek, I., Laszlo, V., Buzas, E., Pallinger, E., Hangya, B., Horvath, Z., Falus, A., 2007. Increased antigen presentation and Th1 polarization in genetically histamine-free mice. *Int. Immunol.* 19, 51–58.
- Kaisho, T., Akira, S., 2003. Regulation of dendritic cell function through Toll-like receptors. *Curr. Mol. Med.* 3, 373–385.
- Kowalski, M.L., 2007. Aspirin-sensitive rhinosinusitis and asthma. *Clin. Allergy Immunol.* 19, 147–175.
- Kuchroo, V.K., Meyers, J.H., Umetsu, D.T., DeKruyff, R.H., 2006. TIM family of genes in immunity and tolerance. *Adv. Immunol.* 91, 227–249.
- Lavelle, E.C., Jarnicki, A., McNeela, E., Armstrong, M.E., Higgins, S.C., Leavy, O., Mills, K.H., 2004. Effects of cholera toxin on innate and adaptive immunity and its application as an immunomodulatory agent. *J. Leukoc. Biol.* 75, 756–763.
- Lee, J.Y., Kim, H.M., Ye, Y.M., Bahn, J.W., Suh, C.H., Nahm, D., Lee, H.R., Park, H.S., 2006. Role of staphylococcal superantigen-specific IgE antibodies in aspirin-intolerant asthma. *Allergy Asthma Proc.* 27, 341–346.
- Mandron, M., Aries, M.F., Brehm, R.D., Tranter, H.S., Acharya, K.R., Charveron, M., Davrinche, C., 2006. Human dendritic cells conditioned with *Staphylococcus aureus* enterotoxin B promote TH2 cell polarization. *J. Allergy Clin. Immunol.* 117, 1141–1147.
- Martin, M., Rehani, K., Joep, R.S., Michalek, S.M., 2005. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat. Immunol.* 6, 777–784.
- Mazzoni, A., Segal, D.M., 2004. Controlling the Toll road to dendritic cell polarization. *J. Leukoc. Biol.* 75, 721–730.
- Mesri, M., Smithson, G., Ghatpande, A., Chapoval, A., Shenoy, S., Boldog, F., Hackett, C., Pena, C.E., Burgess, C., Bendele, A., Shimkets, R.A., Starling, G.C., 2006. Inhibition of in vitro and in vivo T cell responses by recombinant human Tim-1 extracellular domain proteins. *Int. Immunol.* 18, 473–484.
- Meyers, J.H., Sabatos, C.A., Chakravarti, S., Kuchroo, V.K., 2005. The TIM gene family regulates autoimmune and allergic diseases. *Trends Mol. Med.* 11, 362–369.
- Mingomataj, E.C., Xhixha, F., Gjata, E., 2006. Helminths can protect themselves against rejection inhibiting hostile respiratory allergy symptoms. *Allergy* 61, 400–406.
- Niess, J.H., Reinecker, H.C., 2006. Dendritic cells: the commanders-in-chief of mucosal immune defenses. *Curr. Opin. Gastroenterol.* 22, 354–360.
- Nishimura, M., Funaoka, H., Hosoe, H., Ohkaru, Y., Yakuo, I., Hayakawa, S., Ito, K., 2006a. Development and evaluation of a direct sandwich enzyme-linked immunosorbent assay for the quantification of guinea-pig immunoglobulin e. *J. Pharmacol. Sci.* 101, 58–65.
- Nishimura, M., Iwanaga, T., Ohkaru, Y., Takagi, A., Ikeda, Y., 2006b. Change in immunoreactive human hepatic triglyceride lipase (HTGL) mass and the shelf-life of the HTGL ELISA kit in long-term storage. *J. Immunoassay Immunochem.* 27, 89–102.
- Peluso, I., Pallone, F., Monteleone, G., 2006. Interleukin-12 and Th1 immune response in Crohn's disease: pathogenetic relevance and therapeutic implication. *World J. Gastroenterol.* 12, 5606–5610.
- Reis e Sousa, C., 2004. Toll-like receptors and dendritic cells: for whom the bug tolls. *Semin. Immunol.* 16, 27–34.
- Rivera, E., Ekholm, P.F., Inganas, M., Paulie, S., Gronvik, K.O., 2005. The Rb1 fraction of ginseng elicits a balanced Th1 and Th2 immune response. *Vaccine* 23, 5411–5419.
- Sun, J.B., Raghavan, S., Sjoling, A., Lundin, S., Holmgren, J., 2006. Oral tolerance induction with antigen conjugated to cholera toxin B subunit generates both Foxp3+ CD25+ and Foxp3– CD25– CD4+ regulatory T cells. *J. Immunol.* 177, 7634–7644.
- Takeda, K., Kaisho, T., Akira, S., 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21, 335–376.
- Thome, M., Hofmann, K., Burns, K., Martinon, F., Bodmer, J.-L., Mattmann, C., Tschoep, J., 1998. Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. *Curr. Biol.* 8, 885–888.
- Tournay, K.G., Provoost, S., Van Hove, C., Joos, G., 2006. The role of immune tolerance in asthma pathogenesis. *Curr. Allergy Asthma Rep.* 6, 437–443.
- Umetsu, D.T., DeKruyff, R.H., 2006. The regulation of allergy and asthma. *Immunol. Rev.* 212, 238–255.
- Urry, Z., Xystrakis, E., Hawrylowicz, C.M., 2006. Interleukin-10-secreting regulatory T cells in allergy and asthma. *Curr. Allergy Asthma Rep.* 6, 363–371.
- Wang, Q., McLoughlin, R.M., Cobb, B.A., Charrel-Dennis, M., Zaleski, K.J., Golenbock, D., Tzianabos, A.O., Kasper, D.L., 2006. A bacterial carbohydrate links innate and adaptive responses through Toll-like receptor 2. *J. Exp. Med.* 203, 2853–2863.
- Wolfert, M.A., Roychowdhury, A., Boons, G.J., 2007. Modification of the structure of peptidoglycan is a strategy to avoid detection by nucleotide-binding oligomerization domain protein 1. *Infect Immun.* 75, 706–713.
- Yang, P.C., Berin, M.C., Perdue, M.H., 1999. Enhanced antigen transport across rat tracheal epithelium induced by sensitization and mast cell activation. *J. Immunol.* 163, 2769–2776.
- Yang, P.C., Berin, M.C., Yu, L.C., Conrad, D.H., Perdue, M.H., 2000. Enhanced intestinal transepithelial antigen transport in allergic rats is mediated by IgE and CD23 (Fc ϵ R2). *J. Clin. Invest.* 106, 879–886.
- Yang, P.C., Berin, M.C., Yu, L., Perdue, M.H., 2001. Mucosal pathophysiology and inflammatory changes in the late phase of the intestinal allergic reaction in the rat. *Am. J. Pathol.* 158, 681–690.
- Yang, P.C., Wang, C.S., An, Z.Y., 2005. A murine model of ulcerative colitis: induced with sinusitis-derived superantigen and food allergen. *B.M.C. Gastroenterol.* 5, 6.
- Yang, P.C., Jury, J., Soderholm, J.D., Sherman, P.M., McKay, D.M., Perdue, M.H., 2006. Chronic psychological stress in rats induces intestinal sensitization to luminal antigens. *Am. J. Pathol.* 168, 104–114.
- Yeh, W.C., Chen, N.J., 2003. Immunology: another toll road. *Nature* 424, 736–737.
- Yu, L.C., Yang, P.C., Berin, M.C., Di Leo, V., Conrad, D.H., McKay, D.M., Satoskar, A.R., Perdue, M.H., 2001. Enhanced transepithelial antigen transport in intestine of allergic mice is mediated by IgE/CD23 and regulated by interleukin-4. *Gastroenterology* 121, 370–381.