

Differential Expression and Function of Toll-like Receptors in Langerhans Cells: Comparison with Splenic Dendritic Cells

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Toll-like receptors are key elements in pathogen recognition by the host immune system. Although the expression pattern and functions of Toll-like receptors have been studied in a variety of cytokine-induced dendritic cells, it remains unknown whether Toll-like receptor stimulation influences maturation and cytokine production of authentic Langerhans cells. We purified murine epidermal Langerhans cells along with splenic dendritic cell using a panning method. Langerhans cells expressed Toll-like receptor 2, 4, and 9 but not 7, the pattern of which suggests Langerhans cells are the closest to one of the murine dendritic cell lineage, $CD11c^{+}11b^{+}8\alpha^{-}4^{-}$. Then we stimulated Toll-like receptor 2, 4, and 9 with the corresponding ligand, *Staphylococcus aureus* Cowan 1, lipopolysaccharide, and CpG, and found that all of these stimuli upregulated expression of B7-1 and B7-2 in splenic dendritic cells but not in Langerhans cells. As in human Langerhans cells, stimulation of murine Langerhans cells with *Staphylococcus aureus* Cowan 1, lipopolysaccharide, and CpG overall resulted in T helper 1-polarizing cytokine production (namely, induction of IL-12p40 and inhibition of TARC (thymus and activation-regulated chemokine)/CCL17). Exceptionally, lipopolysaccharide exhibited no effect on IL-12p40 production by Langerhans cells and inhibited IL-12p40 production by splenic dendritic cells. These results may represent the functional heterogeneity between Langerhans cells and splenic dendritic cells, and are important for better understanding of innate immunity to bacterial infections differentially regulated in the skin and spleen. MeSH terms: Toll-like receptors, Langerhans cells, dendritic cells.

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Toll-like receptors (TLR) represent a family of type I transmembrane proteins that are characterized by an extracellular leucine-rich repeat domain and a cytoplasmic domain similar to interleukin (IL)-1 receptor (Belvin and Anderson, 1996). TLR mediate an innate immune response by directly recognizing pathogen-associated molecular patterns that are common among a large group of pathogens (Aderem and Ulevitch, 2000; Akira *et al*, 2001). For example, TLR2 mediates cellular responses to *Staphylococcus aureus* Cowan 1 (SAC) and peptidoglycan (Heine *et al*, 1999; Schwandner *et al*, 1999; Takeuchi *et al*, 1999; Underhill *et al*, 1999; Yoshimura *et al*, 1999). Similarly, TLR3 is required for the response to viral double-stranded RNA (Alexopoulou *et al*, 2001), TLR4 is for the lipopolysaccharide (LPS) (Poltorak *et al*, 1998; Chow *et al*, 1999; Hoshino *et al*, 1999; Takeuchi *et al*, 1999), TLR7 is for the imiquimod (Hemmi *et al*, 2002), and TLR9 is for the unmethylated CpG motif abundant in bacterial DNA (Hemmi *et al*, 2000). As for TLR4, two additional molecules, CD14 and MD2, are known to be essential in maintaining the functional integrity of this

receptor (Shimazu *et al*, 1999; Akashi *et al*, 2000; Yang *et al*, 2000; da Silva and Ulevitch, 2002). RP105 is another transmembrane protein that, like TLR4, transduces LPS signaling. RP105 has an extracellular leucine-rich repeat but lacks an IL-1 receptor domain, and is physically associated with MD1, an MD2-like molecule (Miyake *et al*, 1998, 2000). Interestingly, most of these microbial ligands for TLR have similar effects on dendritic cells (DC); they recruit an adaptor protein MyD88 to the receptor complex and activate intracellular signaling largely through nuclear factor- κ B (Medzhitov *et al*, 1998). In the case of TLR4 stimulation with LPS, the presence of MyD88-independent signaling pathway that mediates nuclear factor- κ B activation is suggested (Akira *et al*, 2001). Signaling through TLR strongly activates DC to upregulate costimulatory molecules (B7-1 and B7-2) and to induce proinflammatory cytokines, including tumor necrosis factor- α , IL-6, and IL-12 (Brightbill *et al*, 1999; Takeuchi *et al*, 2000a,b; Thoma-Uszynski *et al*, 2000; Edwards *et al*, 2002). The investigation into TLR functions of DC has been of great interest as it remains unclear how cytokine production by DC is regulated and attributed to pathogen recognition.

Abbreviations: ODN, oligodeoxynucleotides; SAC, *Staphylococcus aureus* Cowan 1; TARC, thymus and activation-regulated chemokine; TLR, Toll-like receptors.

Epidermal Langerhans cells are bone marrow-derived, major histocompatibility complex class II antigen-bearing antigen-presenting cells that comprise 1 to 3% of total

epidermal cells (Katz *et al*, 1979). Murine DC subsets have been classified into six populations (Kelsall *et al*, 2002): (1) three ($CD11c^{+}11b^{+}8\alpha^{-}$, $CD11c^{+}11b^{+}8\alpha^{-4+}$, and $CD11c^{+}11b^{-}8\alpha^{+}$) exist in the spleen, peripheral lymph nodes, and Peyer's patches; (2) mesenteric lymph nodes contain two additional populations ($CD11c^{+}11b^{lo}8\alpha^{-}$ and $CD11c^{+}langerin^{+}$); and (3) a plasmacytoid DC precursor ($CD11c^{int}Gr-I^{+}B220^{+}$) exists in lymphoid tissues and blood. In particular, $CD11c^{+}langerin^{+}$ DC subtypes are found only in skin-draining lymph nodes and believed to be the mature form of Langerhans cells (Henri *et al*, 2001). These six subsets show qualitative and quantitative differences in the expression of TLR (Kelsall *et al*, 2002); $CD11c^{+}11b^{+}8\alpha^{-}$ DC express TLR2, TLR4, and TLR9, $CD11c^{+}11b^{+}8\alpha^{-4+}$ DC express TLR7 and TLR9, $CD11c^{+}11b^{-}8\alpha^{+}$ DC express TLR3 and TLR9, and a plasmacytoid DC precursor expresses TLR7 and TLR9; however, little is known about TLR recognition by Langerhans cells because of the paucity of Langerhans cells in the epidermis. In this study we applied a classical panning technique that has been proven as a powerful tool when examining the functions of resident (namely authentic) Langerhans cells (Salgado *et al*, 1999) and compared the expression pattern and functions of TLR between epidermal Langerhans cells and splenic DC. We mainly focused on TLR2, TLR4, TLR7, and TLR9 in terms of, and for the better understanding of, innate immunity to bacterial infections of the skin.

Results

Fresh Langerhans cells express TLR2, TLR4, TLR9, and RP105 mRNA but not TLR7 mRNA mRNA samples were extracted from Langerhans cells (fresh), splenic DC, and keratinocytes and analyzed with reverse transcription-polymerase chain reaction to detect specific TLR transcripts. To minimize contamination, only splenic DC samples with a purity of $\approx 95\%$ (as well as Langerhans cells) were used in this experiment. As shown in Fig 1(a), TLR2 and TLR4 transcripts were present in Langerhans cells, splenic DC, and keratinocytes. In contrast, TLR9 and RP105 mRNA were expressed by Langerhans cells and splenic DC, but not by keratinocytes. TLR7 mRNA was absent in all three types of cells other than splenic DC. The lack of TLR7 expression on Langerhans cells was further confirmed by the preliminary experiment showing that IL-6, IL-12p40, and TARC production by Langerhans cells stayed unchanged regardless of stimulation with imiquimod (data not shown). Interestingly, CD14 mRNA was present in splenic DC but not in Langerhans cells or keratinocytes. MD2 and MyD88 mRNA expression was demonstrated in all three types of cells (data not shown). MD1 mRNA was detected in Langerhans cells and splenic DC but not in keratinocytes (data not shown).

Next, we examined the protein expression of TLR2, TLR4, and RP105 in Langerhans cells (fresh) and splenic DC. The expression of TLR2 was confirmed by immunoblotting (Fig 1b); however, we could not show cell surface expression of TLR2 as the MoAb used for immunoblotting did not work with flow cytometry. The cell surface expression of TLR4 and RP105 was detected on Langer-

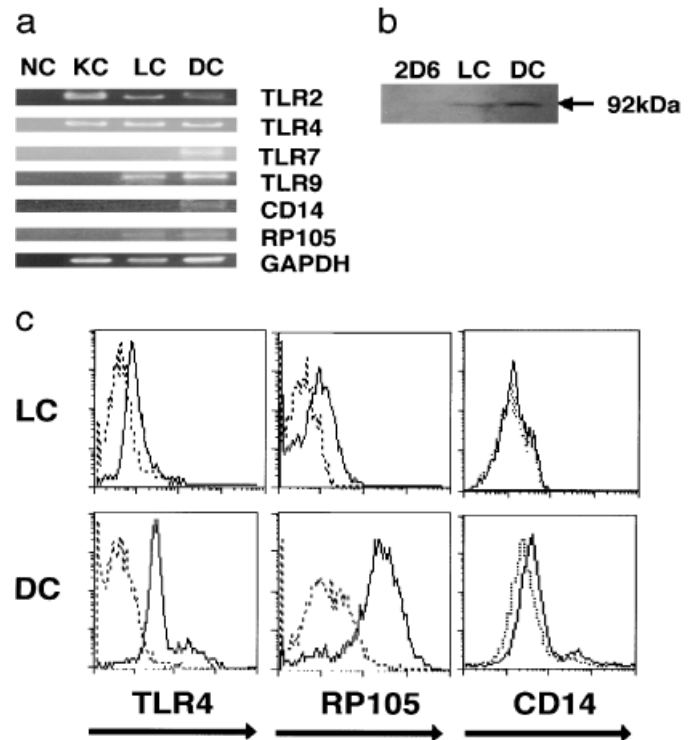


Figure 1
Murine Langerhans cells express TLR2, TLR4, TLR9, and RP105 but not TLR7. (a) mRNA samples from Langerhans cells, splenic DC, and keratinocytes were reverse transcribed. The resultant cDNA was amplified using the primers described in *Materials and Methods*. The size of products is as follows: TLR2, 922 bp; TLR4, 556 bp; TLR7, 656 bp; TLR9, 542 bp; CD14, 1327 bp; RP105, 787 bp; GAPDH, 640 bp. NC denotes negative control. (b) Protein samples from Langerhans cells, splenic DC, and 2D6 were immunolabeled with anti-TLR2 antibody. The apparent size of TLR2 is 92 kDa. (c) Langerhans cells and splenic DC were analyzed for expression of TLR4, RP105, and CD14 (bold lines) by flow cytometry. Thin lines indicate isotype control staining. The results shown in the figure are representative of three independent experiments.

hans cells as determined by flow cytometry analysis (Fig 1c). The mean fluorescence intensity (MFI) of TLR4 and RP105 in splenic DC was ≈ 8 and ≈ 16 times higher than that in Langerhans cells, respectively. Splenic DC but not Langerhans cells was immunoreactive for CD14.

Stimulation of TLR (SAC, LPS, and CpG) drives maturation of splenic DC but not of Langerhans cells As it was reported that maturation of murine splenic DC is driven by TLR stimulation (Edwards *et al*, 2002), we cultured Langerhans cells and splenic DC (positive control) for 18 or 36 h with or without $1:10,000$ diluted SAC, $1 \mu\text{g}$ LPS per mL, $6 \mu\text{g}$ CpG ODN 1668 per mL, or 10 ng GM-CSF per mL (positive control) and compared the MFI of I-A^d, B7-1, and B7-2. GM-CSF upregulated B7-1 and B7-2 (but not I-A^d) expression on Langerhans cells and splenic DC during 18 or 36 h of culture (Fig 2). Addition of SAC, LPS, or CpG ODN 1668 to splenic DC resulted in upregulation of B7-1 and B7-2 (but not of I-A^d), which was consistent with a previous study (Edward *et al*, 2002). The MFI of these major histocompatibility complex class II and costimulatory molecules on Langerhans cells, however, remained unchanged regardless of deliberate stimulation of TLR (Fig 2).

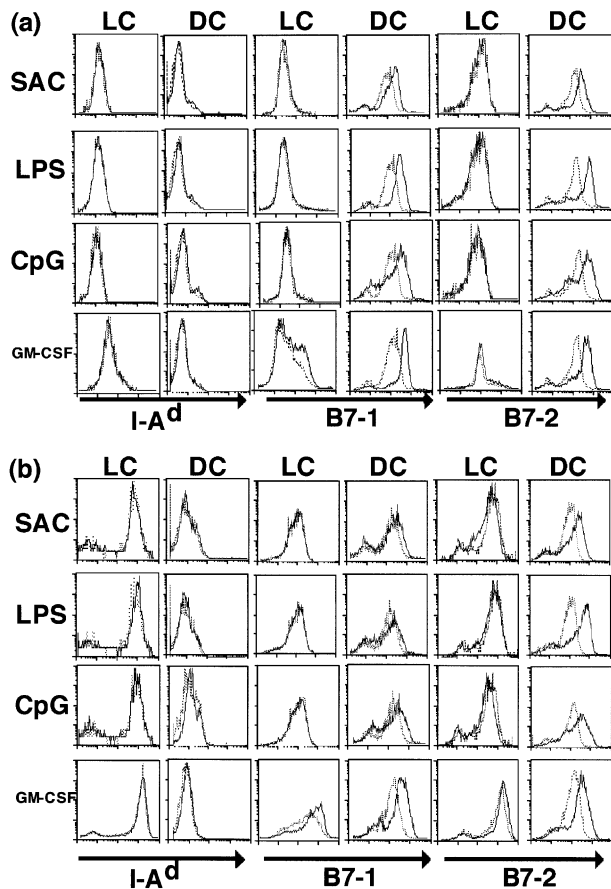


Figure 2
Stimulation of TLR (SAC, LPS, and CpG) drives maturation of splenic DC but not of Langerhans cells. Langerhans cells and splenic DC were cultured for 18 h (a) or 36 h (b) with or without SAC (1:10,000 diluted), LPS (1 μ g per mL), CpG ODN1668 (6 μ g per mL), or GM-CSF (10 ng per mL), and assessed for expression of I-A^d, B7-1, or B7-2 by flow cytometry. Cells cultured with TLR stimulation (bold lines) were compared with cells cultured without TLR stimulation (dotted lines). Stimulation of splenic DC with SAC, LPS, CpG, or GM-CSF (positive control) resulted in upregulation of B7-1, and B7-2 (but not of I-A^d). No upregulation of I-A^d, B7-1, and B7-2 was observed on Langerhans cells. Data shown are representative of three separate experiments.

LPS upregulates cell surface expression of TLR4 in splenic DC but not in Langerhans cells As it has been shown that TLR4 expression is upregulated in human and murine DC with LPS treatment (Gatti *et al*, 2000; Muzio *et al*, 2000; Visintin *et al*, 2001; An *et al*, 2002), we examined if it is also the case with murine resident Langerhans cells. We cultured Langerhans cells and splenic DC (positive control) for 36 h in the presence or absence of LPS and measured the MFI of TLR4. The cell surface expression of TLR4 showed relatively modest upregulation in splenic DC by LPS (Fig 3), which confirms the findings of others (Gatti *et al*, 2000; Muzio *et al*, 2000; Visintin *et al*, 2001; An *et al*, 2002). On the contrary, no remarkable change in the TLR4 expression was observed in Langerhans cells regardless of whether LPS were added to the cultures (Fig 3).

SAC induces IL-6 and IL-12p40 production by Langerhans cells and splenic DC, inhibits TARC production by Langerhans cells, and shows no effect on TARC production by splenic DC The production of IL-6 (inflam-

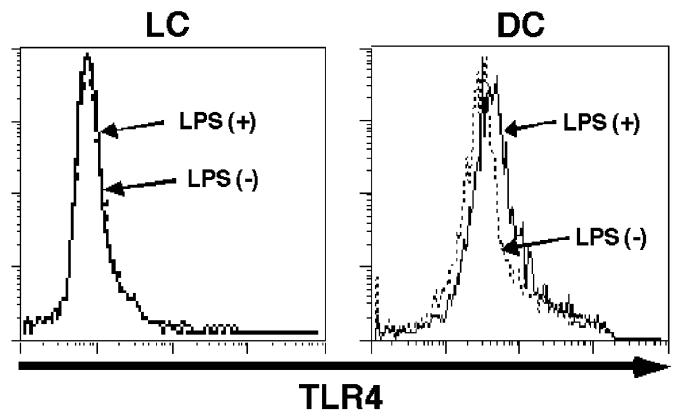


Figure 3
LPS upregulates cell surface expression of TLR4 in splenic DC but not in Langerhans cells. Langerhans cells and splenic DC were cultured for 36 h in the presence or absence of LPS (1 μ g per mL). The cell surface expression of TLR4 was significantly upregulated by LPS in splenic DC, but not in Langerhans cells. Similar results were obtained in three independent experiments.

matory cytokine), IL-12p40 (T helper (Th)1-polarizing cytokine), and TARC (Th2 cytokine) by Langerhans cells and splenic DC was determined by ELISA. Although the purity of splenic DC fluctuated over 80%, we continued to use the positive selection technique because we wanted to isolate Langerhans cells and splenic DC with as similar conditions as possible, namely, using a single MoAb to avoid unnecessary stimulation. Fortunately, cytokine production by DC showed drastic changes with each stimulation to TLR, and there was no significant difference between results obtained by three independent experiments in which splenic DC with respective purities of 82%, 88%, and 94% were used. Thus, we combined the results of three experiments in each graph (Fig 4a–i).

Langerhans cells and splenic DC were cultured with or without 1:10,000 diluted SAC, and culture supernatant was harvested after 36 h. SAC induced IL-6 and IL-12p40 production by Langerhans cells (Fig 4a,b). A similar induction by SAC was also observed in splenic DC (Fig 4a,b). On the other hand, SAC inhibited TARC/CCL17 production by Langerhans cells (Fig 4c), whereas splenic DC showed no significant change in TARC production in the absence or presence of SAC (Fig 4c).

LPS induces IL-6 production by Langerhans cells and splenic DC, inhibits IL-12p40 production by splenic DC and TARC production by Langerhans cells, and shows no effect on IL-12p40 production by Langerhans cells and TARC production by splenic DC Similar to SAC-stimulated Langerhans cells and splenic DC, LPS induced IL-6 production by Langerhans cells and by splenic DC (Fig 4d). Of note, IL-12p40 production by Langerhans cells was unchanged with or without LPS stimulation (Fig 4e). Furthermore, LPS inhibited IL-12p40 production by splenic DC (Fig 4e). As with the case of treatment with SAC and CpG, LPS also inhibited TARC production by Langerhans cells and showed no effect on splenic DC (Fig 4f).

CpG induces IL-6 and IL-12p40 production by Langerhans cells and splenic DC, inhibits TARC production by Langerhans cells, and shows no effect on TARC

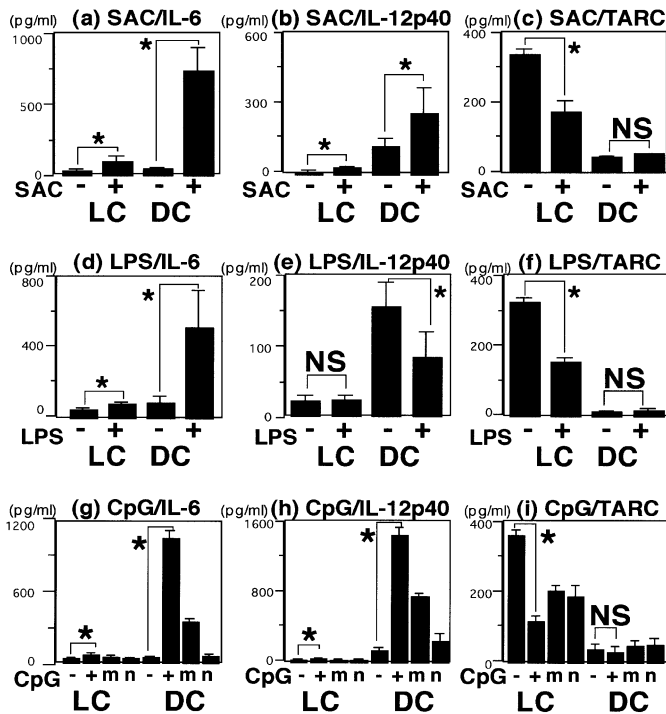


Figure 4

SAC, LPS, and CpG induce IL-6 and IL-12p40 production by murine resident Langerhans cells and inhibit TARC/CCL17 production, with the only exception is that LPS shows no effect on IL-12p40 production. Langerhans cells and splenic DC were cultured with or without SAC (1:10,000 diluted), LPS (1 μ g per mL), or CpG ODN 1668 (6 μ g per mL). Cytokine levels in the supernatants were measured after 36 h. SAC induced IL-6 and IL-12p40 production by Langerhans cells or by splenic DC (a,b). SAC, however, inhibited TARC production by Langerhans cells, whereas splenic DC did not show any significant change in TARC production in the absence or presence of SAC (c). LPS induced IL-6 production by Langerhans cells or by splenic DC (d). Of note, IL-12p40 production by Langerhans cells stayed unchanged with or without LPS stimulation (e). Furthermore, LPS inhibited IL-12p40 production by splenic DC (e). LPS inhibited TARC production by Langerhans cells and showed no effect on splenic DC (f). The IL-6 and IL-12p40 production by Langerhans cells or by splenic DC were enhanced in the presence of CpG ODN 1668, but not of CpG ODN 1668m (6 μ g per mL) or ODN 1720 (6 μ g per mL) (g, h). CpG impaired TARC production by Langerhans cells (i). TARC production by splenic DC stayed unchanged with the stimulation of CpG (i). All values are expressed as mean \pm SD (n=3 in each group). Significance of differences was determined by paired two-tailed Student's t-test (*p<0.05). NS stands for not significant. In g-i (+) denotes stimulation with CpG ODN 1668 (m) with 1668m, and (n) with 1720, respectively.

production by splenic DC Basically, regulation of cytokine production by Langerhans cells and splenic DC in response to CpG ODN was the same as that in response to SAC. The IL-6 and IL-12p40 production by Langerhans cells were enhanced in the presence of 6 μ g CpG ODN 1668 per mL (Fig 4g,h). This enhancement was less obvious when using CpG ODN 1668m, and production of these two cytokines returned to around the baseline with CpG ODN 1720 (Fig 4g,h). Using splenic DC, the change in IL-6 and IL-12p40 production exhibited the same tendency (Fig 4g,h). A series of CpG treatment experiments indicated that the unmethylated, specific CpG motif is crucial for the induction of IL-6 and IL-12p40 by Langerhans cells. TARC production by Langerhans cells was impaired by bacterial CpG motif (Fig 4i). TARC production by splenic DC stayed unchanged with the stimulation of CpG ODN1668, 1668m, and 1720

compared with that without stimulation (Fig 4i). All these data suggest that signaling to TLR is differentially processed between Langerhans cells and splenic DC in terms of IL12p40 and TARC production.

Discussion

DC are professional antigen-presenting cells that have an ability to mediate specific acquired immunity by stimulating naive T cells or by maintaining peripheral T cell tolerance (Liu, 2001; Mellman and Steinman, 2001). It was recently shown that human and murine DC express functional TLR and play an important part in innate immunity. For example, TLR2 and TLR4 interaction with the corresponding ligand is essential for bacterial-induced maturation and cytokine secretion of murine DC (Michelsen *et al*, 2001). TLR9 is another molecule that is expressed on murine DC and involved in bacterial infection (An *et al*, 2002). In this study, we focused on the role of TLR2, TLR4, and TLR9 using epidermal Langerhans cells and splenic DC, both are resident cells isolated and purified with the classical panning method, in order to compare their functional differences mediated through TLR. Almost all of DC studies have been performed with cytokine DC induced from CD34⁺ precursor cells or mononuclear cells by GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994). It has not fully been proven, however, that such cytokine-induced DC are absolutely equal to the resident DC and that CD11c⁺ langerin⁺ DC can be regarded as identical to epidermal Langerhans cells. In fact, most monocyte-derived DC are different from Langerhans cells in many respects, including lack of Birbeck granules, expression of chemokine receptors, and expression of mannose receptors (Banchereau and Steinman, 1998; Grassi *et al*, 1998; Sozzani *et al*, 1999). We constantly achieved more than 95% purity of murine Langerhans cells and used them in the series of experiments.

DC are heterogeneous in terms of ontogeny, phenotype, and function (Banchereau and Steinman, 1998). As delineated in the introduction, murine DC subsets are classified into six populations so far (Kelsall *et al*, 2002). Interestingly, these six subsets show qualitative and quantitative differences in the expression of TLR (Kelsall *et al*, 2002): CD11c⁺11b⁺8 α ⁻4⁻ DC express TLR2, TLR4, and TLR9, CD11c⁺11b⁺8 α ⁻4⁺ DC express TLR7 and TLR9, CD11c⁺11b⁻8 α ⁺ DC express TLR3 and TLR9, and a plasmacytoid DC precursor expresses TLR7 and TLR9. The expression pattern of TLR on some populations, including Langerhans cells (CD11c⁺ langerin⁺), has yet to be determined, however. Resident Langerhans cells showed mRNA expression of TLR2, TLR4, and TLR9 but not TLR7, and protein expression was further confirmed as to TLR2 and TLR4. This expression pattern of Langerhans cells was the same as that of CD11c⁺11b⁺8 α ⁻4⁻ DC, suggesting that Langerhans cells are the closest to this splenic DC population from the perspective of the TLR expression. With regard to human DC, it has been shown that myeloid DC express TLR2 and TLR4, whereas plasmacytoid DC express TLR7 and TLR9 (Jarrossay *et al*, 2001; Kadowaki *et al*, 2001; Krug *et al*, 2001). Our data

reveal that TLR expression pattern of murine DC is different from that of human DC.

Several lines of evidence indicate that freshly purified murine epidermal Langerhans cells resemble "immature Langerhans cells" and cultured Langerhans cells can be regarded as "mature Langerhans cells" in terms of cell structure and function. While migrating to the lymph nodes, Langerhans cells start to express and augment I-A^d antigen, costimulatory/accessory molecules, including B7-1, B7-2, and CD40 for efficient T cell activation (Schuler and Steinman, 1985; Winzler *et al*, 1997; Gallucci *et al*, 1999). This "maturation" process is considered to be supported by keratinocyte-derived cytokines, such as GM-CSF, IL-1, and tumor necrosis factor- α (Witmer-Pack *et al*, 1987; Heufler *et al*, 1988; Koch *et al*, 1990; Ozawa *et al*, 1996). Previous data demonstrated that cell surface expression of I-A^d, B7-1, B7-2, and CD40 on murine Langerhans cells cultured for 2 to 3 d with those cytokines was upregulated when compared with that of Langerhans cells cultured without cytokines (Chang *et al*, 1994, 1995; Salgado *et al*, 1999). Recently, it was shown that maturation of murine splenic DC is driven by TLR stimulation (Edwards *et al*, 2002), and we could also confirm that SAC, LPS, and CpG ODN1668 upregulate B7-1 and B7-2 (but not of I-A^d) expression on splenic DC. Surprisingly, and interestingly, all three stimuli to TLR did not upregulate cell surface expression of B7-1 and B7-2 on murine Langerhans cells. We concluded that the part of maturation process (as to upregulation of B7-1 and B7-2) modulated and mediated through TLR is differentially regulated between Langerhans cells and splenic DC, which might represent functional diversity of antigen presentation in various organs. Similarly, our additional experiment demonstrated that murine epidermal Langerhans cells was unresponsive to LPS in terms of cell surface expression of TLR4, which was sharply contradictory to the finding that TLR4 expression is upregulated in human and murine DC with LPS treatment (Gatti *et al*, 2000; Muzio *et al*, 2000; Visintin *et al*, 2001; An *et al*, 2002).

DC govern the development of T cell-mediated immune response into either Th1 or Th2 response, depending on the types of stimulation and pathogens (d'Ostiani *et al*, 2000; Whelan *et al*, 2000). As IL-12 has been implicated as an important factor from DC that direct Th1 response (Banchereau and Steinman, 1998), we focused on the production of IL-12 (Th1-polarizing cytokine) and TARC (Th2 cytokine) as well as IL-6 (proinflammatory cytokine) induced by stimulation to TLR2, TLR4, and TLR9. TLR stimulation activates human DC and induces production of IL-6 and IL-12 (Thoma-Uszynski *et al*, 2000; Kadowaki *et al*, 2001; Krug *et al*, 2001). In the murine system, LPS and CpG stimulation have been reported to augment tumor necrosis factor- α production by cytokine-induced DC (An *et al*, 2002). In this study, TLR2, TLR4, and TLR9 interaction with the corresponding ligand (SAC, LPS, and CpG) induced IL-6 and IL-12p40 production by murine Langerhans cells and inhibited TARC production; the only exception was that LPS showed no effect on IL-12p40 production, as demonstrated by others using splenic DC (Pulendran *et al*, 2001; Boonstra *et al*, 2003). Regarding Th1/Th2 balance, almost all three stimuli resulted in a Th1-mediated immune response both in Langerhans cells and splenic DC, as shown in human DC by

others. In order to determine whether cytokine production is directly mediated through TLR4, we added anti-TLR4 antibody that was used for immunoblotting to culture media, but the antibody did not cancel the effect of LPS on cytokine production (data not shown), probably because the antibody possessed no blocking effect. Alternatively, signaling induced by LPS might have been mediated through RP105. To deny the latter possibility, we compared Langerhans cells and splenic DC from C3H/HeN and C3H/HeJ mice, which are closely related strains but different in their ability to respond to LPS. A functional TLR4 is present in C3H/HeN but absent in C3H/HeJ mice (Eden *et al*, 1988; Poltorak *et al*, 1998; Qureshi *et al*, 1999). LPS induced IL-6 production by Langerhans cells and splenic DC from C3H/HeN mice but not by Langerhans cells and splenic DC from C3H/HeJ mice, suggesting LPS activates TLR4 signaling both in Langerhans cells and splenic DC (data not shown).

The experiments in this study demonstrated that LPS enhanced IL-6 production much more by splenic DC than by Langerhans cells, which might be elucidated by the fact that MFI of TLR4, RP105, and CD14 was higher in splenic DC than in Langerhans cells. Also, SAC and CpG induced IL-6 production much more by splenic DC than by Langerhans cells. We speculate that the significant differences in responsiveness to LPS and CpG might represent biologic significance, along with the fact that stimulation with SAC, LPS, and CpG induced maturation of splenic DC (but not of Langerhans cells), which might be a deliberately built-in system in order to prevent Langerhans cells from unnecessary excessive response to ubiquitous bacteria from the external environment, most of them lack pathogenesis. Our preliminary *in vivo* functional experiments, however, showed that there was no difference in the intensity of contact hypersensitivity between C3H/HeN and C3H/HeJ mice when LPS in a single dose of 100 mg was injected intraperitoneally 36 or 18 h before and after sensitization (data not shown). When elucidating the whole output of innate immune reactions to LPS in the skin, all of the components, their functions, and complicated interactions between them should be taken into account. It is highly possible that keratinocytes, a major component of the epidermis, might play a supplementary role in the cutaneous immune system and cancel out the differences in responsiveness to LPS between Langerhans cells from C3H/HeN and Langerhans cells from C3H/HeJ mice, as mRNA expression for TLR2 and TLR4 was demonstrated in this study, and others have reported that human keratinocytes express functional TLR4 (Song *et al*, 2002).

Collectively, we demonstrated TLR2, TLR4, and TLR9 expression both in Langerhans cells and splenic DC and their functional heterogeneity. TLR play an essential part in the recognition of bacterial components, and the differences in responsiveness to microorganisms between Langerhans cells and splenic DC might be for exerting a different immune response appropriate for each organ. We anticipate that analysis of the functional diversity of TLR will contribute to further understanding of "organ-specific" immunity, manipulating immune responses associated with bacterial infection of the skin, and therefore allow development of novel therapeutic modalities for various infectious and inflammatory skin diseases.

Materials and Methods

Animals BALB/C female mice were purchased from Japan SLC Co. (Hamamatsu, Japan), and maintained under specific pathogen-free conditions at the University of Tokyo Animal Facilities until use at the age of 8 to 12 wk.

Antibodies and reagents The following monoclonal antibodies (MoAb) and immunoglobulin isotypes were used for immunostaining. Fluorescein isothiocyanate-conjugated mouse anti-mouse I-A^d, mouse anti-mouse CD11c⁺, mouse IgG2b, k (isotype control), hamster anti-mouse B7-1, rat anti-mouse CD14, rat IgG1,2 (isotype control), rat anti-mouse B7-2, and rat IgG2b, k (isotype control) were obtained from Pharmingen (San Diego, California). Phycoerythrin-conjugated rat anti-mouse TLR4, rat anti-mouse RP105, and rat IgG2a,k (isotype control) were obtained from eBioscience (San Diego, California). Recombinant murine granulocyte-macrophage colony stimulating factor (GM-CSF) (Pepro-tech, Rocky Hill, New Jersey) was diluted and stored according to the manufacturer's guidelines. SAC (pansorbin) was purchased from Calbiochem (San Diego, California), and LPS (serotype 0111: B4) was from Sigma-Aldrich (St Louis, Missouri). CpG oligodeoxynucleotides (ODN) 1668 (containing the CpG motif), 1668m (methylated derivative), and 1720 (with an inverted CpG motif) were synthesized by QIAGEN (Tokyo, Japan) as follows: 1668 5'-TCC ATG ACG TTC CTG ATG CT-3', 1668m 5'-TCC ATG AXG TTC CTG ATG CT-3' (X, 5-methyl-deoxycytidine) 1720 5'-TCC ATG AGC TTC CTG ATG CT-3' (Deng *et al*, 1999).

Purification and culture of Langerhans cells and splenic dendritic cells Langerhans cells were isolated and purified using a previously described panning method (Salgado *et al*, 1999). Briefly, murine skin was treated with dispase (3000 U per mL, Godo Shusei Co., Tokyo, Japan) in RPMI1640 supplemented with 10% fetal calf serum (RPMI10) for 3 h at 37°C. Epidermis was separated from dermis and incubated in RPMI10 containing 0.025% DNase I (Sigma-Aldrich) for 20 min at room temperature. Epidermal cell suspension was obtained by vigorous pipetting of the resultant epidermal sheets, and then reacted with 1:600 diluted mouse anti-mouse I-A^d MoAb (Cedarlane Co., Ontario, Canada) in RPMI10 for 45 min on ice. The cells were incubated in plates coated with 1:100 diluted goat anti-mouse IgG (Fc) (Organon Teknika, Durham, North Carolina) for 45 min at 4°C. After washing out floating cells, adherent cells were collected and designated as purified Langerhans cells. The purity of Langerhans cells was consistently over 95% as determined by flow cytometry using fluorescein isothiocyanate-conjugated mouse anti-mouse I-A^d MoAb (data not shown). The freshly isolated Langerhans cells demonstrated Birbeck granules in their cytoplasm by electron microscopy, and showed marked cell surface expression of CD40, B7-1, and B7-2 molecules upon stimulation (data not shown and Salgado *et al*, 1999), which indicated that the cells are not completely activated. The floating cells were collected before washing and designated as keratinocytes.

DC were purified from murine spleen. Single cell suspension was obtained by gentle teasing with forceps and rubbers, and filtered through a nylon mesh. Erythrocytes were lysed by NH₄Cl treatment. From the remaining unfractionated cell populations, CD11c⁺ cells were separated by positive magnetic selection using microbead-conjugated hamster anti-mouse CD11c MoAb (Mitenyl Biotec, Belgisch Gladbach, Germany) and a magnetic cell separator according to the manufacturer's instructions. The purity of CD11c⁺ cells was consistently over 80% as determined by flow cytometry using fluorescein isothiocyanate-conjugated mouse anti-mouse CD11c MoAb (data not shown) and designated as splenic DC.

In the culture experiment, purified Langerhans cells and splenic DC were in 96-well flat-bottom plates at density of 1.0×10^6 cells per mL for 18 or 36 h. Cultures were incubated with or without different stimuli, including SAC, LPS, and CpG ODN in RPMI10 containing 100 U per mL penicillin G sodium, 100 µg streptomycin

sulfate per mL, and 0.25 µg amphotericin B per mL. The viability of cultured Langerhans cells and splenic DC was carefully checked in each experiment. None of the TLR stimulations used influenced cell viability (data not shown).

Reverse transcription-polymerase chain reaction Purified Langerhans cells, splenic DC, and keratinocytes were lysed and mRNA was extracted using the Quickprep Micro mRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden). About 100 ng of mRNA was obtained from 1.0×10^6 cells. cDNA was synthesized using First-Strand cDNA Synthesis Kit (Pharmacia Biotech) and then amplified by 35 cycles of polymerase chain reaction (94°C for 45 s, 60°C for 1 min, and 72°C for 1 min) with primers for G3PDH sense 5'-CAG GAG CGA GAC CCC ACT AA-3' and anti-sense 5'-GGC ATC GAA GGT GGA AGA GT-3'. The primers used for amplification of TLR2 (Underhill *et al*, 1999), TLR4 (Akashi *et al*, 2000), MD2 (Akashi *et al*, 2000), TLR7 (Bruno *et al*, 2001), TLR9 (Bruno *et al*, 2001), MyD88 (Harroch *et al*, 1995), RP105 (Miyake *et al*, 1995), CD14 (Matsuura *et al*, 1992), and MD-1 (Miyake *et al*, 1998) were described elsewhere. Products were fractionated by 1% agarose gel electrophoresis, stained with ethidium bromide and viewed on an ultraviolet transilluminator.

Flow cytometry analysis Cells were washed and stained in phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% NaN₃ (fluorescence-activated cell sorter buffer). Cells were incubated with 1:50 diluted fluorescein isothiocyanate-conjugated anti-TLR4, anti-RP105, anti-CD14, anti-I-A^d, anti-B7-1, or anti-B7-2 MoAb for 30 min at 4°C. Propidium iodide was added to exclude dead cells. Cell acquisition was performed on FACSCalibur flow cytometer (Becton Dickinson, San Diego, California), and data were analyzed using CELLQuest software (Becton Dickinson).

Immunoblotting For detecting TLR2 expression, cells were lysed with buffer containing 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 5 mM EDTA, and electrophoresed in 7.5% sodium dodecyl sulfate-polyacrylamide gels. The proteins in the gels were electrotransferred on to PVDF membranes (Millipore, Bedford, Massachusetts) and probed with 1:200 diluted goat anti-mouse TLR2 polyclonal IgG antibody (Santa Cruz, California). 2D6 T cell clone was used as negative control of TLR2 (a kind gift from Dr Fujiwara, Biomedical Research Center, Osaka University Medical School, Osaka, Japan) (Maruo *et al*, 1997).

Measurement of IL-6, IL-12p40, and TARC/CCL17 by ELISA Culture supernatants of Langerhans cells and splenic DC were subjected to the quantification of IL-6, IL-12p40, and thymus and activation-regulated chemokine (TARC)/CCL17 by ELISA using commercially available mouse IL-6, IL-12p40, and TARC immunoassay kits (R&D Systems, Minneapolis, Minnesota) according to the manufacturer's instructions. Each sample was tested in duplicate.

Statistical analysis The paired two-tailed Student's t-test was used to analyze the results and a p-value less than 0.05 was considered significant.

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