# Migratory Langerhans Cells in Mouse Lymph Nodes in Steady State and Inflammation

Patrizia Stoitzner,* Christoph H. Tripp,* Patrice Douillard,† Sem Saeland,† and Nikolaus Romani*
*Department of Dermatology, Innsbruck Medical University, Innsbruck, Austria; †Laboratory for Immunological Research, Schering-Plough Corporation, Dardilly, France

**Dendritic cells cells induce immunity or—in the steady state—maintain peripheral tolerance. Little is known in that regard about Langerhans cells. Therefore, we investigated migrating Langerhans cells in the steady-state *versus* inflammation. Increased numbers of Langerhans cells, as determined by immunostaining for Langerin/CD207, appeared in the lymph nodes in response to a contact allergen. Whereas a large proportion of Langerhans cells expressed CD86 in the steady state, CD40, and CD80 were found on a smaller percentage. During inflammation, more CD40$^+$, CD80$^+$, CD274/B7-H1/PD-L1$^+$, and CD273/B7-DC/PD-L2$^+$ Langerhans cells were found in the lymph nodes, and they expressed higher levels of these molecules. CD275/inducible T cell co-stimulator (ICOS) ligand was not detected. Langerhans cells in the nodes of contact allergen-treated mice produced more IL-12p40/70. This correlated with more interferon-γ being produced by activated lymph node T cells. Epicutaneous immunization with ovalbumin under inflammatory conditions led to a more vigorous proliferation of antigen-specific CD4 T cells *in vitro* and *in vivo* as compared with immunization in the steady state. The latter modality, however did not induce strong CD4 T cell tolerance in this model. Thus, the overall phenotype of Langerhans cells is not an indicator for their immunogenic or tolerogenic potential.**

Key words: cell surface molecules/cytokines/dendritic cells/inflammation/skin/steady state
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Dendritic cells (DC) primarily induce immunity. During the last few years, however, it has become apparent that DC also play a role in the maintenance of tolerance (Heath and Carbone, 2001; Steinman *et al*, 2003). How the cells can perform these two opposing roles is still under investigation.

There is evidence that in the steady state DC induce tolerance to specific antigens targeted to the cells via the lectin receptor DEC-205/CD205. Simultaneous injection of a maturation-inducing anti-CD40 monoclonal antibody (mAb) overcame the tolerogenic situation and induced immunity against that same antigen (Hawiger *et al*, 2001; Bonifaz *et al*, 2002). An elegant model with a Cre/LoxP system in which antigen expression could be induced in DC *in vivo* at any point in time was used by Probst and coworkers to further complement these data. When mice were immunized with antigen in the steady state, antigen-specific tolerance could be observed; it was broken when mice were co-injected with a maturation-inducing anti-CD40 mAb (Probst *et al*, 2003). Together, these data suggest that non-activated immature DC in the steady state induce tolerance whereas activated, fully mature DC generate immunity. The properties of Langerhans cells (LC), the epidermal variant of DC (Romani *et al*, 2003; Wilson and Villadangos, 2004), have not yet been specifically studied in that regard.

Several reports have investigated the phenotype of DC in lymph nodes (LN), with special regard to skin-derived populations (Salomon *et al*, 1998; Ruedl *et al*, 2000; Henri *et al*, 2001). In these studies, skin-derived DC in LN were characterized after application of a cell tracer onto the epidermis as large cells expressing DEC-205/CD205, E-cadherin, Langerin/CD207, and co-stimulatory molecules. Ruedl *et al* (2001) and Hawiger *et al* (2001) demonstrated that co-stimulatory molecules could be further upregulated after application of an inflammatory stimulus. Wilson *et al* (2003). reported that the migratory skin-derived LN DC in the steady state display a mature phenotype in that they express high levels of co-stimulatory molecules and major histocompatibility complex- (MHC-) class II in contrast to resident LN DC. In addition, it was demonstrated that LC are critically involved in the transport of self-antigens from the skin to draining LN in the steady state (Hemmi *et al*, 2001). Phenotypical and functional propertiesof such migratory LC, however, were not addressed in these studies.

Thus, we were interested to determine possible differences in phenotype and function of LC in LN during inflammation as compared with a steady-state situation. Langerin is a C-type lectin receptor expressed by LC, which can bind mannan and is involved in Birbeck granule formation (Valladeau *et al*, 2000). Antibodies against murine Langerin al-

Abbreviations: CFSE, 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester; DC, dendritic cells; FITC, fluorescein isothiocyanate; IFN-γ, interferon-γ; IL, interleukin; LC, Langerhans cells; LN, lymph node; MHC, major histocompatibility complex; mAB, monoclonal antibody; OVA, ovalbumin; PE, phycoerythrin; TNCB, 2,4,6-trinitro-1-chlorobenzene; TNF-α, tumor necrosis factor-α

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low the direct and simple identification of LC in skin and in LN (Valladeau *et al*, 2002) and we recently reported that LC can be tracked on the basis of Langerin expression on their way from the epidermis to the LN (Stoitzner *et al*, 2003). In this study, we therefore used anti-Langerin mAb's to investigate changes in LC migrating to the LN after induction of inflammation in the skin.

## Results

**Inflammation in the skin by a contact allergen increases the absolute numbers of LC in draining LN** The single application of the contact allergen TNCB (2,4,6-trinitro-1-chlorobenzene) induced strong inflammation in the treated skin area. The activated LC co-expressed CD40 and CD86 in the epidermis and started to emigrate from the epidermis (data not shown, Weinlich *et al*, 1998). In accordance with the emigration of LC from the TNCB-inflamed skin the draining LN were swollen. When LN cell suspensions were analyzed we observed that cellularity, i.e., total cell numbers, increased 2- to 3-fold. On LN sections it was not possible to visualize the increased immigration of Langerin$^+$ cells as the LC-densities varied between the sections. But when absolute numbers of LC were calculated, based on hemocytometer-derived total cell numbers and flow cytometry derived percentages of Langerin$^+$ cells, we observed higher numbers of LC 48 h after application of contact allergen. With the mAb detecting intracellular Langerin (929F3), we observed about $1 \times 10^4$ LC per LN in control mice as opposed to $2.5 \times 10^4$ LC in mice treated with the contact allergen (Fig 1*A*). When we used a mAb 205C1 against cell surface Langerin we detected only one-third of all LC, indicating that most of the LC had internalized Langerin during migration to the LN. An increase in numbers was observed in the inflammatory situation; however, the difference was not statistically significant (Fig 1*B*). Furthermore, the total number of CD11c$^+$DC was augmented in contact allergen treated mice when compared with untreated mice (Fig 1*C*). It should be mentioned that similar increases were observed when contact allergen-treated mice were compared with vehicle-treated mice.

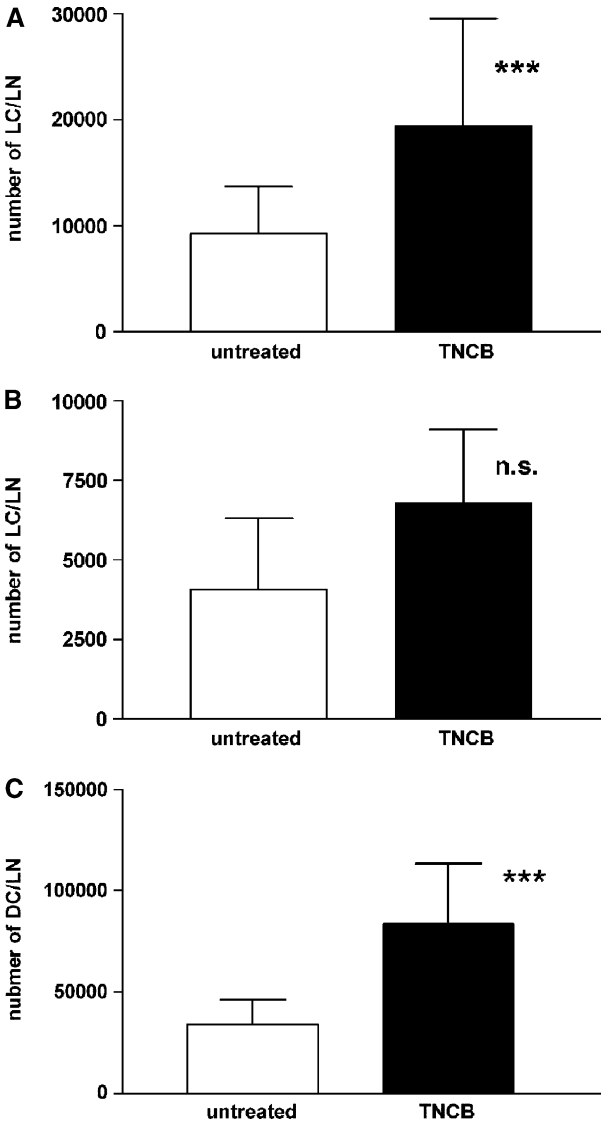


**Figure 1**
**The contact allergen 2,4,6-trinitro-1-chlorobenzene (TNCB) induces migration of Langerhans cells (LC) from the epidermis to the skin-draining lymph node.** Mice were treated with 1% TNCB, and 48 h later draining auricular and inguinal lymph nodes were taken. Cell suspensions were prepared by collagenase digestion and total cell numbers counted in the hemocytometer and stained with anti-Langerin monoclonal antibodies (mAb) to determine percentages of LC among all viable cells. From these data, absolute numbers of LC and dendritic cells (DC) per lymph node were calculated. Numbers of (*A*) LC expressing Langerin intracellularly (mAb 929F3, mean $\pm$ SD, n = 12); (*B*) LC expressing Langerin molecules on the cell surface as detected by the mAb to the extracellular part of Langerin (mAb 205C1, mean $\pm$ SD, n = 6); (*C*) CD11c$^+$DC (mean $\pm$ SD, n = 15). $^{**}p < 0.005$; $^{***}p < 0.001$; N.S. not significant.

**The phenotype of LC in the LN changes in response to inflammation** Current evidence indicates that DC migrating to the LN in an immature or semi-mature (Menges *et al*, 2002) stage may induce tolerance in the steady state. During inflammation, however, DC acquire a fully mature phenotype and can induce an immune response to antigens (Steinman and Nussenzweig, 2002). We therefore asked whether this was similar when specifically addressing migrating LC, rather than DC in general. We examined the expression of co-stimulatory molecules CD40, CD80, CD86, B7-H1 (PD-L1), B7-DC (PD-L2), and ICOS-ligand (inducible T-cell co-stimulator ligand) on Langerin$^+$ cells in LN cell suspensions. In the steady state, a large proportion of LC in the LN, as defined by the expression of intracellular Langerin, co-expressed CD86, but only part of these cells showed CD40, CD80, B7-H1, and B7-DC on their surface. After application of a contact allergen onto the epidermis more LC in the LN expressed CD40, CD86, B7-H1, and B7-DC (Fig 2*A*). Similar results were obtained by immunofluorescence analyses on sections from LN of TNCB-treated and control mice. Langerin$^+$ cells were localized in T cell areas and were mostly absent from B cell follicles as described earlier (Stoitzner *et al*, 2003). Although occasional Langerin-single-positive cells (i.e., immature LC) were visible, a large part of the Langerin$^+$ cells co-expressed CD40 and CD86 (data not shown). Few Langerin$^+$ cells in the LN expressed B7-H1 and B7-DC in the steady state, whereas during inflammation more LC expressing these molecules

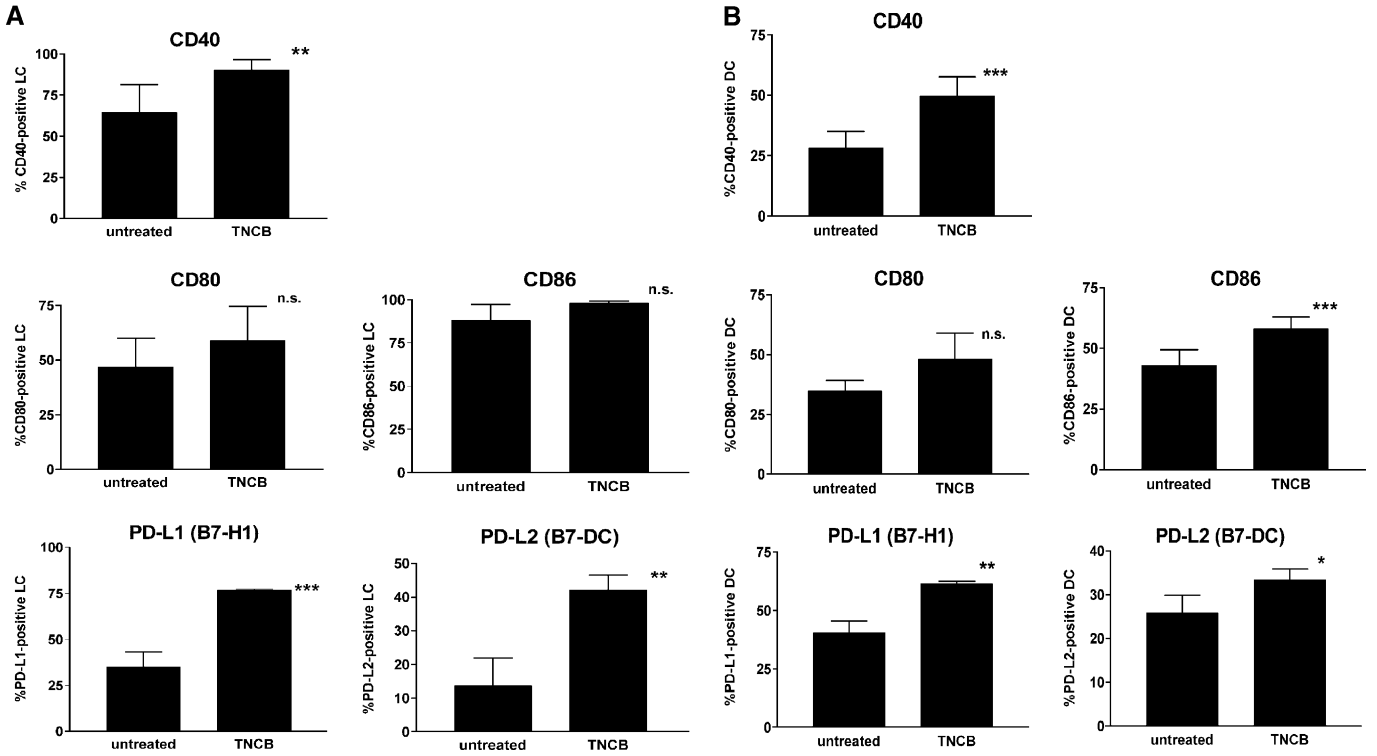


**Figure 2**
**Numbers of mature Langerhans cells (LC) in skin-draining lymph nodes increase during inflammation.** Lymph node cell suspensions were labeled for Langerin (monoclonal antibodies (mAb) 929F3), CD11c, and co-stimulatory molecules CD40, CD80, CD86, B7-H1/PD-L1, B7-DC/PD-L2, and ICOS-ligand (ICOS-L). The percentage of LC ($CD11c^+Langerin^+$ cells, *A*) and resident/dermal DC ($CD11c^+Langerin^-$ cells, *B*) positive for co-stimulators was determined by flow cytometry. Experiments were carried out at least three times. Results are shown as mean ± SD. $^*p<0.05$; $^{**}p<0.005$; $^{***}p<0.001$; N.S. not significant.

were detectable on immunolabeled LN sections (data not shown). Flow cytometry analyses revealed that LN LC still expressing Langerin on the cell surface showed a more mature phenotype with regard to the expression of co-stimulatory molecules (Figure S1). When we investigated the expression of co-stimulatory molecules on the cell surface of resident and interstitium (dermis)-derived DC (i.e., $Langerin^-$ DC) we observed their partial expression on these subsets of DC, and some of these markers were up-regulated during inflammation (Fig 2*B*). Extending these observations, FACS analyses of LN cell suspensions showed that not only the numbers of positive cells increased but also expression levels of all co-stimulatory molecules on LC in LN were higher during TNCB-induced inflammation (Fig 3*A*). The tolerance-inducing co-stimulatory molecule ICOS-L was, however, never detected on LC and only on a small fraction of the $Langerin^-$ DC. This up-regulation of expression levels was similar on the LC expressing Langerin either intra- or extracellularly (data not shown). In contrast, when we examined the expression levels of co-stimulatory molecules on the surface of $Langerin^-$ DC we observed only upregulation on part of the cells, possibly dermal DC (Fig 3*B*).

**More LC producing pro-inflammatory cytokines are found in skin-draining LN during inflammation** Next, we studied cytokine expression patterns of LC in the LN in the steady state *versus* inflammation. DC were isolated from LN of TNCB-treated or control mice and examined by flow cytometry for production of the cytokines interleukin (IL)-12, IL-10, and tumor necrosis factor-α (TNF-α). DC populations were stimulated with lipopolysaccharide (LPS) plus an agonistic mAb against CD40 to increase cytokine production. Without any stimulation of DC *in vitro*, some IL-12p40/70 was detected in resident and interstitium (dermis)-derived DC (i.e., $Langerin^-$ DC) as well as in LC (i.e., $Langerin^+$ DC) in the draining LN. Proportionally more LC than $Langerin^-$ DC produced IL-12. After application of the contact allergen TNCB onto the epidermis, LC in skin-draining LN showed a significantly higher expression of IL-12p40/70 as compared with the steady state and this was not the case for $Langerin^-$ DC (Fig 4*A*, Figure S2*A*). This was also true when TNCB-treated mice were compared with vehicle-treated mice. The pro-inflammatory cytokine TNF-α was also examined. Only few LC as well as resident/dermal DC produced TNF-α (0.9%–12% $TNF\text{-}\alpha^+$ DC/LC per LN). During inflammation, TNF-α production was up-regulated only in resident/dermal DC but not in LC of the skin-draining LN. These differences, however, were not statistically significant (Fig 4*B*, Figure S2*B*). The anti-inflammatory cytokine IL-10 was also produced by resident/dermal DC and LC in the skin-draining LN, again by small numbers of cells (0.2%–7% $IL\text{-}10^+$ DC/LC per LN). This cytokine appeared not to be regulated during inflammation (Fig 4*C*, Figure S2*C*).

**T cells in skin-draining LN are activated and more T cells produce interferon-γ (IFN-γ) during inflamma-**

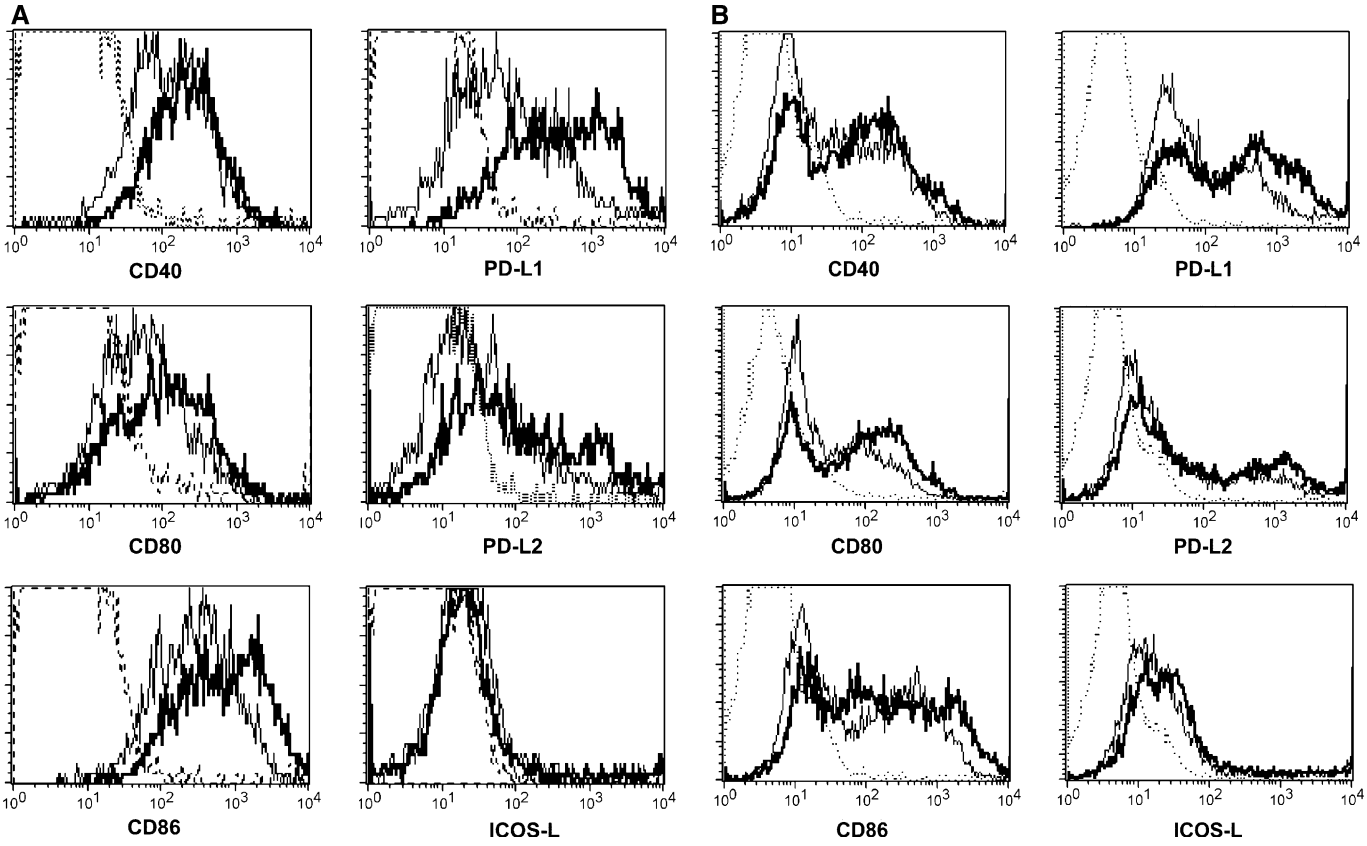


**Figure 3**
**Expression levels of co-stimulatory molecules increase in Langerhans cells during inflammation.** Lymph node cell suspensions were prepared 48 h after epicutaneous application of contact allergen and dendritic cells were enriched on a Nycodenz gradient. Cells were labeled for co-stimulatory molecules, CD11c and Langerin (mAb 929F3), and gated on Langerin$^+$ (*A*) or Langerin$^-$ (*B*) cells. Expression levels of co-stimulatory molecules in steady state (i.e., untreated mice) and during 2,4,6-trinitro-1-chlorobenzene (TNCB)-induced inflammation are shown. Bold line, TNCB-treated mice; thin line, untreated control mice; dotted line, isotype control. Experiments were carried out at least three times.

**tion** Skin-draining LN swelled 48 h after application of the contact allergen TNCB and total cell numbers increased 2–3-fold. Some of the CD3$^+$ T cells expressed the IL-2 receptor CD25 (3%–10% of all T cells) and the early activation marker CD69, thus indicating the presence of activated T cells. After application of a contact allergen onto the skin absolute numbers of CD25$^+$ as well as CD69$^+$ T cells increased markedly in the LN draining the inflamed skin (Fig 5*A*). For examination of cytokine production, T cells were additionally stimulated with anti-CD3 plus anti-CD28 mAb. After stimulation, most of the T cells expressed the very early antigen CD69 as well as CD25 (80%–90% of all T cells, data not shown) and IFN-$\gamma$ became clearly detectable. When TNCB was applied onto the epidermis, significantly more T cells produced IFN-$\gamma$ in the LN draining the inflamed skin as compared with control mice in the steady state. This increase in IFN-$\gamma$ expression was observed both in CD4$^+$ and in CD8$^+$ T cells. The frequencies of CD8$^+$ T cells producing IFN-$\gamma$ were higher than those of their CD4$^+$ counterparts (Fig 5B, Fig S3). IL-4- or IL-10-producing T cells were never detectable, neither in LN from control nor TNCB-treated mice (data not shown).

**Antigen-specific T cell proliferation is increased during inflammation** The difference in cytokine expression patterns and the upregulation of co-stimulatory molecules on LC in LN suggested an altered T cell stimulatory capacity of LC in the inflammatory situation. Direct isolation of LC from skin-draining LN was impossible as the Langerin mAb 929F3 only detects the intracellular molecule, and the mAb 205C1 against the extracellular Langerin would have allowed sorting of only a part of the LC as the molecule disappears from the cell surface on a large portion of LC during their migration to the LN. Therefore, we sorted all CD11c$^+$ DC, including LC, from skin-draining LN with immunomagnetic beads and co-cultured them with allogeneic T cells. No difference was observed in stimulation of allogeneic T cells between DC isolated from LN draining inflamed and normal skin (data not shown). To further investigate possible differences in T cell stimulatory capacity we used an antigen-specific model to perform both *in vitro* and *in vivo* experiments. To this end, ovalbumin (OVA) protein was applied topically onto either untreated or TNCB-treated skin to target LC in the epidermis. For this purpose, the protein was dissolved in phosphate-buffered saline (PBS) and then mixed 1:1 with a creme to improve penetration into the skin. This method enabled us to apply antigen onto undisturbed skin. The draining auricular LN were collected 48 h later, digested and CD11c$^+$DC sorted with magnetic beads. DC were co-cultured with OVA-specific T cells from OT-II mice for 24–72 h to examine induction of T cell proliferation. DC in the skin-draining LN were able to present peptides on

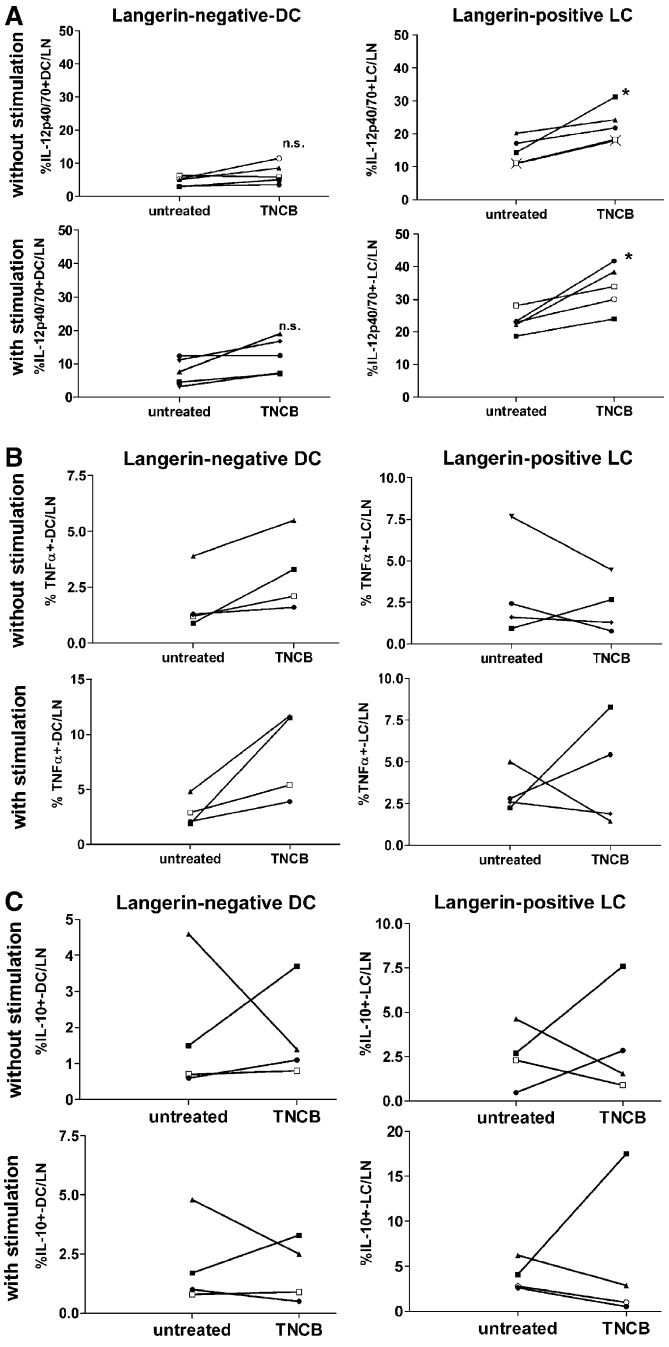


**Figure 4**
**Langerhans cells (LC) in the skin-draining lymph nodes produce more interleukin (IL)-12 in an inflammatory situation.** Lymph nodes were digested with collagenase 48 h after epicutaneous application of the contact allergen 2,4,6-trinitro-1-chlorobenzene (TNCB) and dendritic cells (DC) were enriched on a Nycodenz gradient. DC were either stimulated for 4 h with 100 ng per mL lipopolysaccharide (LPS) plus 5 μg per mL anti-CD40 monoclonal antibodies (mAb) or left unstimulated in medium in the presence of Brefeldin A. Cells were stained with anti-CD11c-APC (allophycocyanin) and anti-Langerin-fluorescein isothiocyanate (mAb 929F3) to identify LC and further examined for production of the cytokines IL-12p40/70 (*A,* n = 5), tumor necrosis factor-α (TNF-α) (n = 4), and IL-10 (*C,* n = 4). LC are defined as $CD11c^+$/$Langerin^+$ cells; resident/dermal DC as $CD11c^+$/$Langerin^-$ cells. Lines connect data points from individual experiments. N.S. $^*p < 0.05$.

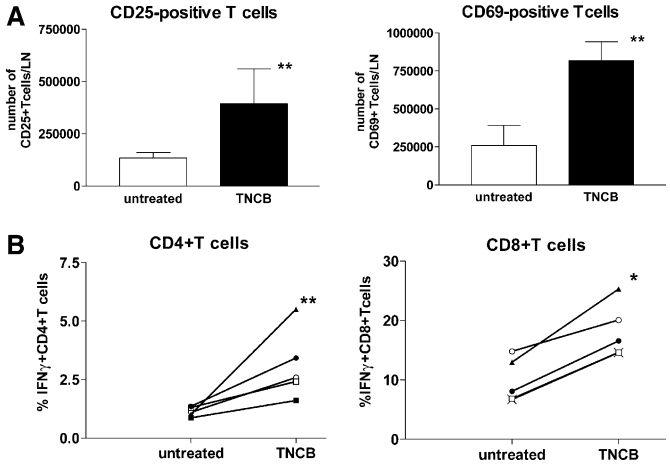


**Figure 5**
**More activated T cells from lymph nodes draining inflamed skin produce interferon-γ (IFN-γ).** Lymph node cell suspensions were prepared 48 h after application of contact allergen. (*A*) T cells were retrieved from the pellet after Nycodenz-gradient centrifugation and immediately stained with monoclonal antibodies (mAb) against the activation markers CD25 and CD69. After fluorescence activated cell sorting analysis the absolute numbers of activated T cells per lymph node were calculated. n = 3; $^{**}p < 0.005$. (*B*) For detection of cytokines T cells were stimulated overnight with 3 μg per mL immobilized anti-CD3 and 3 μg per mL soluble anti-CD28 mAb in the presence of 30 U per mL IL-2. Brefeldin A was present during the last 4 h of stimulation. CD4 T cells and CD8 T cells were gated and examined for IFN-γ production. Lines connect data points from individual experiments. All five experiments are shown. $^*p < 0.05$; $^{**}p < 0.005$.

MHC-class II to antigen-specific T cells *in vitro*. Induction of inflammation in the skin with contact allergen resulted in markedly higher proliferation of antigen-specific T cells *in vitro*. Notably, some antigen-specific proliferation of T cells was also observed with DC from epicutaneously immunized but otherwise untreated mice (Fig 6*A*). Different concentrations of OVA (100 μg–1 mg) gave similar results (data not shown). As some activation of DC by the isolation procedure itself could not be ruled out, *in vivo* proliferation assays were performed. CFSE-labeled antigen-specific OT-II T cells were transferred into mice 24 h prior to application of OVA protein onto the epidermis, which was either untreated or TNCB-treated. After 3 d, the skin-draining LN were examined for *in vivo* proliferation of antigen-specific T cells. Following application of PBS, i.e., in the absence of antigen and inflammation, we never observed proliferation of T cells in the LN. T cells in control mice without skin inflammation showed some proliferation in response to OVA indicating an inefficient presentation of peptides on MHC-class II by DC in the draining LN. In contrast, application of the contact allergen prior to OVA resulted in a dramatically increased T cell proliferation *in vivo*, as most of the $CFSE^+$ T cells had started to proliferate and some had divided up to six times (Fig 6*B*, *C*). The application of lower concentrations of OVA (500 μg) gave similar results (data not shown).

**Antigen-specific T cells respond to rechallenge with OVA** In the next step, we wanted to know whether we would observe signs of tolerance when applying OVA in the absence of an inflammatory stimulus, i.e., in the steady state. Mice were injected with antigen-specific OT-II T cells and 24 h later OVA was applied onto the epidermis of these mice with or without contact allergen pre-treatment. We determined the numbers of transgenic T cells in the LN up to 21 d after the first antigen application. As expected, the

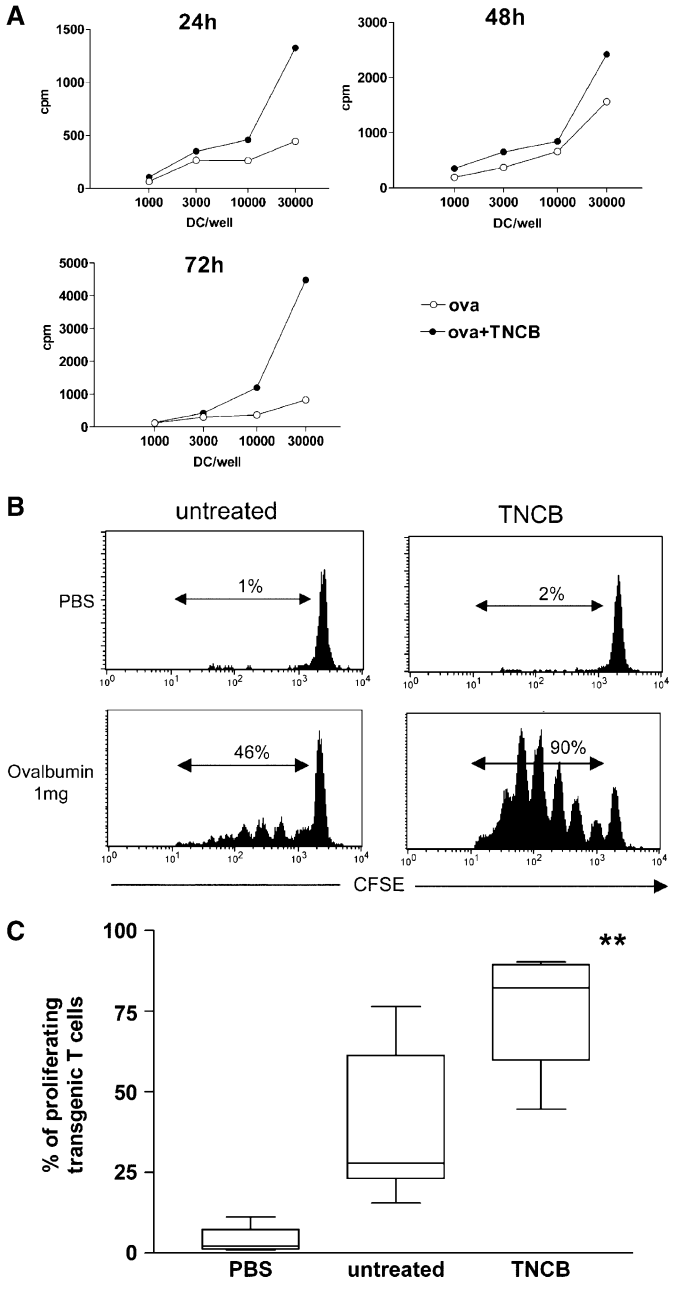


**Figure 6**
**Lymph node dendritic cells (DC) stimulate stronger antigen-specific proliferation of T cells when the skin is inflamed.** (*A*) One milligram of ovalbumin (OVA) was applied onto the epidermis of untreated or 2,4,6-trinitro-1-chlorobenzene (TNCB)-treated (1 h prior to antigen application) mice and lymph node DC were sorted 48 h later with anti-CD11c-MACS beads. Graded doses of DC were co-cultured with $1 \times 10^5$–$2 \times 10^5$ transgenic T cells isolated from OT-II mice for 24–72 h. During the last 16 h, cells were pulsed with $H^3$-thymidine to measure proliferation of T cells. One experiment is shown out of three. (*B*) T cells from antigen-specific OT-II mice were isolated and labeled with 2 μM CFSE prior to intravenous injection. Twenty-four hours later 1 mg OVA in creme was applied onto the ear skin of either untreated or contact allergen-treated mice (1 h prior to antigen application). Cell suspensions from the draining lymph nodes were prepared 3 d after antigen application and examined for T cell proliferation, as measured by a decrease/dilution of CFSE intensity. One representative experiment is shown out of 4. (*C*) Same protocol as above but all four experiments (representing data from eight individually analyzed mice per experimental group) are summarized. $^{**}p < 0.005$.

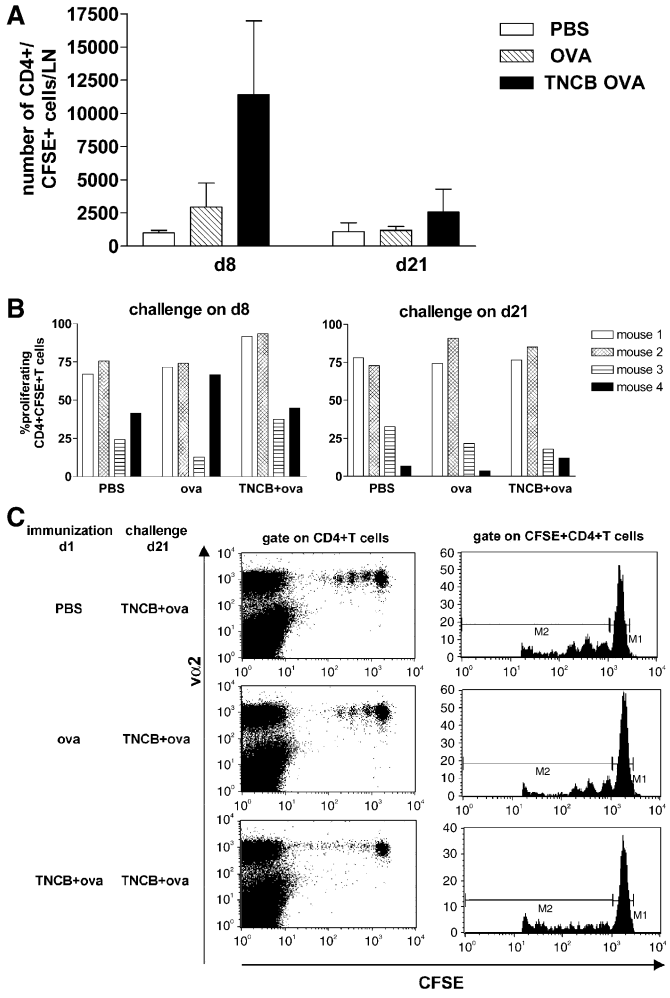


**Figure 7**
**Topical application of ovalbumin (OVA) in the absence of an inflammatory stimulus does not induce tolerance.** We injected $CFSE^+$ transgenic $CD4^+$ T cells intravenously into mice and 24 h later we applied either phosphate-buffered saline (PBS) or 1 mg OVA protein with or without contact allergen 2,4,6-trinitro-1-chlorobenzene (TNCB) (1 h prior to antigen application) epicutaneously. (*A*) On d8 or d21 numbers of remaining transgenic T cells ($CFSE^+CD4^+$) in the lymph nodes were determined in these mice. Absolute numbers of transgenic T cells per lymph node are shown. n = 3. (*B*) On d8 or d21 mice were re-immunized topically with 1 mg OVA and TNCB, and *in vivo* proliferation of T cells was assessed by dilution of CFSE intensity 3 d later. Summary of two experiments is shown; Each bar represents one individual mouse. (*C*) One representative experiment is shown out of three.

numbers of T cells increased after induction of inflammation (i.e., in response to application of TNCB) in the skin and dropped to low numbers within 21 d. The numbers of transgenic T cells, however, in the mice immunized with OVA in the steady state (i.e., without TNCB pre-treatment) remained similar and did not decrease markedly, indicating that these T cells were not deleted (Fig 7*A*). When we re-challenged the mice with OVA plus contact allergen on day 8 or day 21 after the first immunization we observed similar proliferation of transgenic T cells regardless of whether the initial immunization was performed with or without contact allergen. Thus, immunization with OVA in the steady state, i.e., in the absence of an inflammatory stimulus, did not prevent proliferation of transgenic T cells indicating that the transgenic T cells did not become anergic in this experimental setting (Fig 7*B*,*C*).

## Discussion

As opposed to non-epidermal types of DC, the *in vivo* role of LC in the generation of immunity as well as the maintenance of peripheral tolerance is largely unknown (Steinman *et al*, 2003). Anti-Langerin/CD207 antibodies allowed us for the first time to selectively study LC, rather than a mixed population of LC and dermal DC, in the induction of immune responses or tolerance via the skin. We contribute here a number of observations that may help elucidate the role of LC. The LC that migrate from the epidermis in the steady state and can be identified in the draining LN do not express a phenotype that would clearly distinguish them from immunity-inducing LC that migrate under inflammatory conditions as provoked by the contact allergen TNCB. They do, however, display different cytokine-production profiles (less IL-12) and they induce less antigen-specific T cell proliferation *in vitro* and *in vivo*.

**Profiles of co-stimulatory and maturation-associated molecules on LC** CD40 and most of the B7 family members were already expressed on a part of the LC in the steady-state LN. The inflammatory stimulus led to higher absolute numbers of LC expressing higher levels of these molecules on the surface. Some recent publications are in line with these findings. We have previously observed the maturation-associated molecule 2A1 on most LC in steady-state skin-draining LN *in situ* (Stoitzner *et al*, 2003). Ruedl *et al* (2000) and Henri *et al* (2001) demonstrated further that the putative LC population in the steady-state skin-draining LN expresses high levels of CD40, thus not representing true immature DC. Wilson *et al* (2003) recently reported that the skin-derived DC population (dermal DC and LC) in the LN displays features of maturation as determined by phenotype and function in contrast to resident LN DC. Finally, it was shown that also in the steady state migrating DC, in general, and LC, in particular need CCR7, a receptor commonly associated with maturation (Ohl *et al*, 2004). These data indicate that LC start maturing during migration to the LN in the steady state and that migration is coupled with several but not all features of maturation (Randolph, 2002). Our data complement and extend the cited observations in that—to the best of our knowledge—we have analyzed the phenotype specifically of LC for the first time and we have expanded the panel of co-stimulators analyzed.

Our observations with LC are also in accordance with data by Hawiger *et al*, who induced tolerance by targeting antigen to DC in the steady state. The tolerogenic DC expressed substantial levels of CD40 and CD86. Tolerance was overcome by simultaneous injection of anti-CD40 mAb and was accompanied by a slight upregulation of CD40 and CD86 (Hawiger *et al*, 2001), similar to our observations with LC. In summary, these observations emphasize that maturation of DC in general and LC in particular is a complex process. Therefore, the mere presence or absence of some phenotypical maturation markers such as CD40, CD86, or 2A1 does not necessarily mean that such DC/LC are immunogenic or tolerogenic, respectively.

Data from Geissman *et al*, who reported that Langerin$^+$ cells in the LN of patients with dermatopathic lymphadenitis were phenotypically immature expressing no maturation markers like CD83, CD86, and CD208/DC-LAMP (Geissmann *et al*, 2002), appear to be at variance with the observations in the mouse. But explanations for this discrepancy could be a species difference or, more likely, the possibility that the Langerin$^+$ cells in the samples from patients with chronic skin inflammation are different from the steady-state situation.

**Profiles of potentially T cell-inhibitory molecules on LC** Some members of the B7 family of co-stimulatory molecules such as B7-H1/PD-L1 and B7-DC/PD-L2 (Freeman *et al*, 2000; Latchman *et al*, 2001), as well as ICOS-ligand/B7-H2 (Witsch *et al*, 2002) are involved in T cell inhibition and tolerance. We hypothesized that these molecules might be upregulated in the steady state and therefore examined their expression and regulation on LC in skin-draining LN. The inverse was found to be the case. Rather than being expressed at higher levels in the steady state, the two PD-L molecules were distinctly upregulated during inflammation. This is in accordance with data published by Freeman *et al* (2000) and Liang *et al* (2003) showing that activated DC increase expression of PD-L molecules suggesting a "counterbalancing" role in the downregulation of ongoing immune responses. Furthermore, ICOS-L was never expressed on LC in the LN. Witsch *et al* reported that ICOS-L is expressed on human LC *in situ* allowing them to induce production of IL-10 in T cells (Witsch *et al*, 2002). We observed low ICOS-L expression on DC in the LN, but these few cells were clearly Langerin$^-$. In summary, it may be concluded from our experiments that LC in the steady-state LN display no distinct "tolerogenic" phenotype in that they would express higher levels of T cell inhibitory co-stimulator molecules or IL-10-inducing molecules.

**Functional properties of LC-cytokine production** As the phenotypical differences between LC in LN in the steady state and inflammation turned out to be subtle we further examined these cells at a functional level. Cytokine production by LC in LN has not been studied so far. The major observation was that during inflammation, LC in the LN produced more IL-12 than in the steady state, and that proportionally more LC than Langerin$^-$ DC in the LN produced IL-12. Consequently, the corresponding T cells in LN draining inflamed skin expressed more of the activation markers CD25 and CD69, and they produced more IFN-$\gamma$. IL-12 indeed plays a role in the induction of contact sensitivity *in vivo*, that is, in the very model used in this study. The mRNA for the IL-12p40 chain was detected in LN 12–14 h after application of 1% TNCB onto the skin, and it was shown by *in situ* hybridization that DC were the producers in the LN (Müller *et al*, 1995). Hochrein *et al* (2001) reported that CD8$\alpha+$ DC from skin-draining LN produce IL-12p70 upon stimulation with CD40 ligand and LPS, whereas DC expressing low amounts of CD8$\alpha$ produce less IL-12p70. Furthermore, Curtsinger *et al* (2003) demonstrated that IL-12 as signal 3 determines the discrimination between tolerance or full activation of CD8$+$ T cells. Thus, the presence or absence of high levels of IL-12 may be a crucial factor in the DC's decision between induction of immunity or tolerance.

The pro-inflammatory cytokine TNF-$\alpha$ was produced by Langerin$^-$ DC and LC in skin-draining LN in small amounts.

TNF-α was upregulated during inflammation in Langerin$^-$ DC only and not in LC leading to an overall increase in pro-inflammatory cytokines in the skin-draining LN during inflammation.

IL-10, a cytokine involved in tolerance (Steinman *et al*, 2003), was only detectable in small amounts in the LN and IL-10 production by DC appeared not to be regulated during inflammation. Furthermore, the T cells in the skin-draining LN in the steady state produced no IL-4 and no IL-10. Thus, we could not detect a "tolerizing cytokine mileu" in the steady-state skin-draining LN as observed in the lungs and intestines (Iwasaki and Kelsall, 1999; Akbari *et al*, 2001).

**Functional properties of LC T cell-stimulation** The altered cytokine production implicates a difference in T cell stimulation in skin-draining LN. It is not possible to sort the LC from LN because of the lack of specific surface markers. Langerin is internalized quickly into maturing LC and therefore does not allow isolation of the entire population of LN LC. Proliferation of antigen-specific CD4$^+$T cells *in vitro* was found to be clearly enhanced when the immunized skin was inflamed. These *in vitro* results were confirmed in *in vivo* proliferation assays in which stronger and more prolonged antigen-specific T cell proliferation was observed during inflammation, too. The assistance of dermis-derived DC in antigen presentation cannot be excluded as it is not known to what extent the contact allergen activates dermal DC as well. Streilein (1989) and Tse and Cooper (1990) reported that dermal DC can induce contact sensitivity as well as LC; thus, a possible involvement of dermal DC must be considered.

Other groups demonstrated that epicutaneous immunization with MHC-class I-restricted peptides induced development of cytotoxic T cells. Inflammation of the skin, such as tape stripping, which leads to disruption of the stratum corneum barrier, was essential. LC isolated from tape-stripped skin expressed higher levels of MHC-class I molecules and co-stimulators and thus were stronger in induction of CTL *in vitro* than LC from intact skin (Seo *et al*, 2000). Consequently, epicutaneous immunization through intact, non-inflamed skin did not lead to immunity. A possible development of tolerance was not tested. Kahlon *et al*. observed similar CTL activity after immunization with an MHC-class I-restricted peptide or with OVA protein onto tape-stripped skin. Co-administration of cholera toxin further increased the immune response (Kahlon *et al*, 2003). Thus, epicutaneous immunization induces responses in T cells in the draining LN, but for an optimal response inflammation in the skin is necessary. These data together with our results suggest that most likely these responses are induced by epidermal LC. It remains to be determined whether dermal DC contribute to this activity.

**Tolerance induction by LC** Using a transgenic model and melanin as a self-antigen it was clearly shown that LC carry self-antigens from the skin to the LN *in vivo* in the steady state (Hemmi *et al*, 2001). Likewise, we detected LC carrying melanin or apoptotic bodies in dermal lymph vessels in a skin explant model (Stoitzner *et al*, 2003). Thus, self-antigens are transported to LN by LC from skin both in inflammation and in the steady state. It was not studied, however, if in the steady state these antigens are presented to T cells in the LN in a tolerogenic fashion. In our setting, transgenic T cells in mice immunized with OVA protein without an inflammatory stimulus were not deleted when we followed them up until 21 d. Furthermore, when mice were rechallenged with OVA and contact allergen we observed similar CD4 T cell proliferation regardless of whether initial immunization was carried out in the absence or presence of inflammation. This indicates that in this model, the T cells do not become anergic and can respond to the antigen in an unimpaired fashion when they encounter it for a second time, at least in the context of another strong inflammatory stimulus. The likely interference of dermal DC in these experiments, however, precludes the conclusion as to the inability of migratory LC in the steady state to induce or maintain tolerance. Further studies need to be performed with antigens expressed in the skin such as in the following two recent reports. In a model where an antigenic OVA peptide is expressed under the K14 promoter, LC were not able to induce tolerance in adoptively transferred antigen-specific CD8$^+$T cells (Mayerova *et al*, 2004). Likewise, in mice in which the whole OVA protein was expressed in the epidermis adoptively transferred CD8$^+$T cells induced a GvH-like disease rather than being silenced by antigen-carrying LC (Shibaki *et al*, 2004). But if these same mice were immunized with OVA into the skin, no delayed type hypersensitivity reaction developed indicating that the mice were rendered tolerant, presumably by LC migrating in the steady state and cross-presenting the antigen to the endogenous antigen-specific T cells (Shibaki *et al*, 2004).

Shortman and Heath (2001) discuss that the completion of the maturation process ("more of the same") may be the determining factor for induction of tolerance *versus* immunity: short-lived, incompletely mature DC, rather than immature DC would induce tolerance and fully activated DC immunity. The virtual absence of immature LC in skin-draining LN would support this notion, suggesting that not fully mature LC with moderate levels of costimulators and low cytokine production induce weak T cell proliferation or even tolerance. In contrast, fully mature LC with maximum levels of co-stimuli and high cytokine production stimulate strong T cell proliferation and thus immunity.

## Materials and Methods

**Mice** Mice of inbred strains C57BL/6, BALB/c, and OT-II were purchased from Charles River Germany, Sulzfeld, Germany, and used at 2–12 mo of age. CD4$^+$T cells from OT-II mice express a transgenic Vα2Vβ5 TCR specific for the OVA peptide 323–339 in the context of I-A$^b$ (Barnden *et al*, 1998). All animal experiments were carried out according to the Institutional Review Board.

**Antibodies for phenotypical studies** For detection of LC we used two different antibodies against mouse Langerin: a mAb recognizing Langerin in its intracellular conformation (929F3-fluorescein isothiocyanate (FITC), rat immunoglobulin G1 (IgG1) (Stoitzner *et al*, 2003) and another mAb binding to an extracellular part of Langerin exposed at the cell surface (205C1-FITC, mouse IgM, Douillard *et al*, manuscript in preparation). For detection of intracellular Langerin, the cells were permeabilized with the Perm/Wash kit from BD-Pharmingen (San Diego, California). The following antibodies were purchased from BD-Pharmingen: CD40-FITC/-phycoerythrin (PE) (clone 3/23), CD86-FITC/-PE (clone

GL-1), CD80-PE (clone 16–10A1), CD11c-APC (HL3), MHC-class II-biotinylated/-FITC/-PE (clones 2G9 and M5/115), IL-12p40/70-PE (clone C15.6), IL-10-PE (clone JES5-16E3), CD4-FITC/-APC (clone L3T4), CD8α-FITC/-APC (clone Ly-2), IL-4-PE (clone BVD4-24G2), TNFα-PE (MP6-XT22), IFN-γ-APC (clone XMG1.2), CD69-biotinylated (clone H1.2F3), CD25-PE (clone 3C7), Vα2 TCR-PE (clone B20.1), Vβ5.1/5.2 TCR-biotinylated (MR9-4), goat anti-rat Ig-PE, and Streptavidin-PerCP. Other mAb against B7-family members were purchased from eBioscience (San Diego, California): B7-DC-PE (clone TY25), B7-H1-PE (clone MIH5), ICOS-L-PE (clone HK5.3).

**Induction of inflammation in the skin with a contact allergen** Mice were anesthetized with an intraperitoneal injection of vetanarcol (Veterinaria AG, Zürich, Switzerland). Abdominal skin was shaved with an electric razor followed by the application of 250 μL of 1% TNCB (picryl-chloride) (Kodak Eastman, Rochester, New York). Murine ear skin was treated with 15 μL 1% TNCB on both sides (dorsal and ventral) resulting in 30 μL per one ear. The contact allergen TNCB was dissolved in acetone:olive oil (4:1).

**Isolation of LN DC** Skin-draining LN (auricular and inguinal) were excised 48 h after application of the contact allergen. Cell suspensions from LN were prepared by digestion with 0.5 mg per mL collagenase P (Roche, Mannheim, Germany) for 30 min at 37°C and subsequent pressing of the tissue through cell strainers (70 μm, Falcon Labware, Oxnard, California), essentially as described recently for spleen cell suspensions (McLellan *et al*, 2002). For most of the experiments, DC were enriched by Nycodenz gradient centrifugation (Sigma-Aldrich, St Louis, Missouri) as described (McLellan *et al*, 1995). DC were stained with the above-mentioned mAb to investigate differences in phenotype between contact allergen-treated and untreated control mice.

**Detection of intracellular cytokines** DC and T cells were isolated from lymph nodes by collagenase digestion. DC were further enriched with a Nycodenz gradient and stimulated for 4 h with 100 ng per mL LPS (Sigma-Aldrich) and 5 μg per mL anti-CD40 mAb (clone H3/23, BD-Pharmingen) to augment cytokine production of IL-12, IL-10, and TNF-α. To prevent secretion of cytokines 1 μg per mL Brefeldin A (BD-Pharmingen) was added during the whole stimulation period. Lymph node T cells were stimulated by overnight culture with immobilized anti-CD3 mAb (3 μg per mL, clone 17A2, BD-Pharmingen) and soluble anti-CD28 (3 μg per mL, clone 37.51, BD-Pharmingen). To improve survival of the T cells during overnight culture, the medium was supplemented with 30 U per mL IL-2. Brefeldin A at 1 μg per mL was added for the last 4 h of the culture to stop cytokine secretion. For fluoro cytometry analyses, cells were permeabilized with a cell permeabilization kit (An der Grub, Vienna, Austria) and stained with the above-mentioned mAb to detect cytokines in DC and T cells. In cell suspensions from lymph nodes, LC were identified by double labeling with anti-CD11-APC and anti-Langerin–FITC mAb (clone 929F3).

***In vitro* OVA-specific T cell proliferation assay** We applied 1 mg OVA in 20 μL aqueous creme (hydrophilic oil in water emulsion, Ultrasicc, Hecht Pharma GmbH, Stinstedt, Germany) onto the ear skin of either untreated or TNCB-treated mice (treated 1 h prior to antigen application). DC were isolated 48 h later from draining auricular lymph nodes by collagenase digestion of the tissue. DC were further enriched on a Nycodenz-gradient followed by CD11c-magnetic bead sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). DC purity ranged between 70% and 90% after MACS sorting. T cells were isolated from spleens and lymph nodes of OT-II transgenic mice and further purified by negative selection. Cell suspensions were incubated with a lineage cocktail consisting of mAb against anti-MHC II (clone M5.114), anti-CD45RA (clone B220), anti-Ly-6C (clone Rb6), anti-heat stable antigen (clone M1/69), anti-NK1.1 (clone 4D11), and anti-CD8 (clone Lyt-2) followed by anti-rat/anti-mouse IgG coupled to Biomag-beads (Qiagen, Hilden, Germany). Negative fractions were collected and contained over 90% $CD4^+$ T cells. Graded doses of DC were incubated with $2 \times 10^5$ OVA-specific transgenic OT-II T cells for 24–72 h. Proliferation of T cells was measured by incorporation of tritiated thymidine within the last 16 h.

***In vivo* proliferation assays** As described above, T cells were isolated from TCR transgenic OT-II mice and labeled with 2 μM CFSE (Molecular Probes, Leiden, the Netherlands). C57BL/6 mice were injected intravenously with $2 \times 10^6$–$3 \times 10^6$ CFSE-labeled OT-II T cells. Twenty-four hours thereafter, 1 mg OVA, dissolved in 20 μL of aqueous creme was applied topically onto the epidermis of either untreated or contact-allergen-treated mice (treated 1 h prior to antigen application). Cell suspensions were prepared 72 h thereafter by pressing the auricular lymph nodes through cell strainers with the plunger of a syringe. Four separate experiments were performed. In each of these experiments, two mice were used per experimental condition. Draining auricular lymph nodes of each mouse were pooled. The proliferation of antigen-specific $CFSE^+$ OT-II T cells was detected by the progressive dilution of CFSE fluorescence intensity as described earlier (Lyons, 2000).

In some experiments, we challenged mice on day 8 or day 21 after first application of antigen and lymph node cells were examined for T cell proliferation 3 d later.

**Statistical analysis** Appropriate statistical tests (either *t* test or Mann–Whitney *U* test) were used to analyze the data.

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## Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23757/JID23757.htm

**Figure S1** Langerhans cells still expressing Langerin extracellularly in the lymph node show a mature phenotype.

**Figure S2** Langerhans cells in the skin-draining lymph nodes produce more IL-12 in an inflammatory situation.

**Figure S3** More activated T cells from lymph nodes draining inflamed skin produce IFNγ.

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Address correspondence to: Patrizia Stoitzner, PhD, Malaghan Institute of Medical Research, PO Box 7060, Wellington South, New Zealand. Email: patrizia.stoitzner@uibk.ac.at or Sem Saeland, semsaeland@yahoo.fr

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