



Allergen-Specific Low Zone Tolerance Is Independent of MRP8/14-, TLR4-, TLR7-, and TLR9-Mediated Immune Processes

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TO THE EDITOR

Low zone tolerance (LZT) is regarded as a physiological mechanism to regulate and to circumvent allergies to contact allergens. It is induced by epicutaneous applications of low doses of haptens resulting in the generation of IL-10-producing CD4⁺CD25⁺Foxp3⁺ regulatory T cells and CD8⁺ suppressor T cells (Luckey et al., 2011, 2012). LZT induction prevents the development of the Tc1-mediated contact hypersensitivity (CHS), which mimics the allergic contact dermatitis in humans. However, the role of the innate immune system during the early phase of LZT is still elusive.

Toll-like receptors (TLRs) are pattern recognition receptors expressed by cells of the innate immune system that can either recognize pathogen-associated molecular patterns of microbial pathogens or damage-associated molecular patterns, representing endogenous danger signals inducing inflammatory responses (Kawai and Akira, 2010). It has been shown that contact allergens such as nickel or 2,3,6-trinitro-1-chlorobenzene activate mechanisms of the innate immune system, including TLR2-/TLR4 signaling, which play pivotal roles during CHS induction (Esser et al., 2012; Kaplan et al., 2012). In particular, nickel triggered proinflammatory signaling via a direct interaction with TLR4 during allergic contact dermatitis development in men and reconstitution of TLR4 signaling in mice restored nickel-induced CHS development (Schmidt et al., 2010). In contrast, several studies revealed that stimulation of

TLRs such as TLR4, TLR7, or TLR9 can also result in activation of tolerance-promoting immune cells (Caramalho et al., 2003; Forward et al., 2010; Tripathi et al., 2016) or in a hyporesponsive state of TLRs after repeated exposures to the same ligand as shown for TLR4 (lipopolysaccharide/endotoxin tolerance), TLR7, and TLR9 (Biswas and Lopez-Collazo, 2009; Hayashi et al., 2009; Julian et al., 2015). Furthermore, the myeloid-related proteins (MRPs) 8 and 14 (or known as alarmins S100A8 and S100A9), serving as ligands for TLR4, were required for the development of myeloid-derived suppressor cells, leading to a significantly enhanced CHS reaction in the absence of these molecules in MRP8/MRP14 knockout (KO) mice (Petersen et al., 2013).

Taking into account that TLR-related mechanisms are activated by contact allergens and that TLR signaling is also involved in diverse mechanisms of tolerance induction and hyporesponsiveness, we addressed the question whether TLR-related mechanisms may play a critical role during the induction phase of the hapten-specific LZT, resulting in CHS prevention as previously shown (Luckey et al., 2011, 2012). For this purpose, we used TLR4, TLR7, TLR9, and MRP14 KO mice, the latter ones being deficient for the TLR4 ligands MRP8 and MRP14, and C57BL/6 wild-type controls.

To induce an LZT, 8- to 10-week-old mice received 10 epicutaneous applications of low quantities of contact allergens (0.45 or 4.5 µg 2,3,6-trinitro-1-chlorobenzene) onto the skin before sensitization (high amount of

2,3,6-trinitro-1-chlorobenzene [450 µg], abdomen) and subsequent challenge (2,3,6-trinitro-1-chlorobenzene [45 µg]) onto the ear skin. Mice repeatedly treated with solvent followed by CHS induction and challenge served as controls and developed a CD8⁺ Tc1-mediated skin inflammation (demonstrated by an enhanced ear swelling and increased hapten-specific T-cell response). All animal experiments were conducted in accordance with federal guidelines and approved by the Animal Care Committee of Rhineland-Palatinate.

TLR4 KO mice showed a significantly reduced and MRP14 KO animals an enhanced allergic cutaneous inflammation as previously described by other groups (Esser et al., 2012; Petersen et al., 2013) (Figure 1). In contrast, we did not observe any differences in the CHS reaction comparing mice lacking TLR7 and TLR9 expression with wild-type animals (Figure 2). Intriguingly, in our study, we found that LZT induction resulted in an unaffected tolerance development as demonstrated by a significantly abolished CHS reaction in the absence of TLR4-, TLR7-, TLR9-, or MRP8/14-mediated signaling (Figures 1 and 2). These results were determined by a significantly inhibited cutaneous skin inflammation (ear swelling) in vivo after LZT induction in KO mice, which was comparable with that in tolerized wild-type controls (Figures 1a, b and 2a, b). In addition, the analysis of mice lacking TLR4, TLR7, TLR9, or MRP8/14 revealed a significantly diminished allergen-specific T-cell response after LZT induction (reduced T-cell proliferation, impaired Tc1-cytokine production [IFN-γ, IL-2]) after hapten-specific restimulation in vitro (Figures 1c–f and 2c–f). Because of the reduced CHS reaction in TLR4-deficient mice, the absolute differences between the CHS and LZT

Abbreviations: CHS, contact hypersensitivity; KO, knockout; LZT, low zone tolerance; MRP, myeloid-related protein; TLR, toll-like receptor

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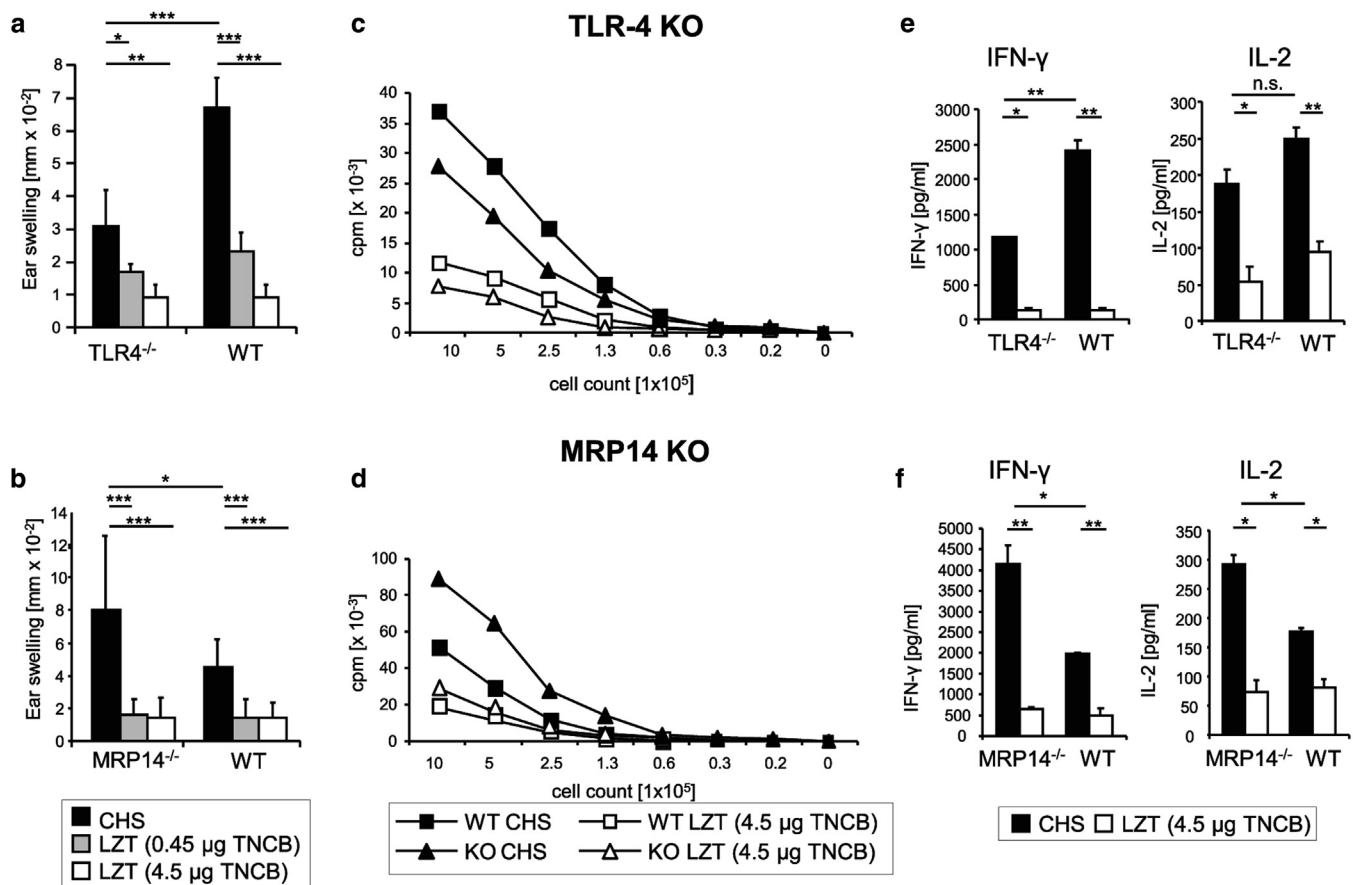


Figure 1. TLR4 and MRP14 are involved in the development of CHS but are not required for low zone tolerance to contact allergens. C57BL/6 WT, TLR4, and MRP14 knockout mice were tolerized with 0.45 or 4.5 µg TNCB or solvent treated (for CHS controls) for 10 times, respectively. Subsequently, CHS induction was performed. (a, b) Pooled data of ear swelling of two independent experiments are depicted (mean $\pm$ SD). (c, d) T cells were hapten-specific (TNBS) restimulated in vitro. The hapten-specific T-cell proliferation in vitro was demonstrated (mean value of triplicates in cpm, one representative out of two independent experiments). (e, f) Pooled data of the hapten-specific T-cell cytokine production (IFN- γ , IL-2) of two independent experiments are shown (mean $\pm$ SD) (n = 6–8 mice per experiment). * P < 0.05, ** P < 0.01, *** P < 0.001, n.s. = not significant. CHS, contact hypersensitivity; KO, knockout; LYT, low zone tolerance; MRP, myeloid-related protein; SD, standard deviation; TLR, toll-like receptor; TNBS, 2,4,6-Trinitrobenzenesulfonic acid; TNCB, 2,3,6-trinitro-1-chlorobenzene; WT, wild-type.

data were less pronounced in TLR4 KO compared with wild-type mice, but nevertheless demonstrated a proper and significant LYT development (Figure 1a, c, and e).

Our results are in line with previous findings concerning the role of MRP8/14- as negative and TLR4-related processes as positive regulators in CHS (Esser et al., 2012; Petersen et al., 2013). However, as compared with high doses of the hapten used for CHS induction in both studies, LYT induction by low amounts was not affected in the absence of TLR4, suggesting a dose-dependent effect of TLR4 triggering in contact allergen-specific immune reactions. In contrast, oral tolerance induced by feeding of the allergen DNFB and UV-induced suppression of CHS were dependent on TLR4 signaling

(Ahmad et al., 2014; Hacini-Rachinel et al., 2017; Lewis et al., 2011). In addition, MRP8/14-facilitated myeloid-derived suppressor cell generation (Petersen et al., 2013) did apparently not contribute to LYT induction as we did not detect an altered LYT reaction in MRP14 KO mice. As demonstrated for other inflammatory disease models, repeated activation by TLR ligands can result in an inhibited receptor-mediated signaling (e.g., TLR7, TLR9, TLR4) toward a subsequent exposition to the corresponding ligand (Biswas and Lopez-Collazo, 2009; Hayashi et al., 2009, 2012; Julian et al., 2015; Kawai and Akira, 2010) or in active tolerance induction, for example, by activation of regulatory T cells, immunosuppressive dendritic cells, or natural killer cells (as shown for TLR4, TLR7, TLR9)

(Caramalho et al., 2003; Forward et al., 2010; Kang et al., 2007; Tripathi et al., 2016). However, the absence of TLR4, TLR7, or TLR9 did not influence the LYT development, excluding that the effect of frequently applied low doses of the contact allergen during LYT induction is regulated by TLR4-, TLR7-, or TLR9-mediated tolerance induction.

Our data demonstrate that pattern recognition receptor-mediated processes in the form of TLR4-, TLR9-, TLR7-, and MRP8/14-induced mechanisms of the innate immune system are not required for the induction of LYT to haptens and thus are not essential for prevention of allergic contact dermatitis. Further investigation on this topic will lead to the identification of specific targets to induce or modulate tolerance processes and to the development of

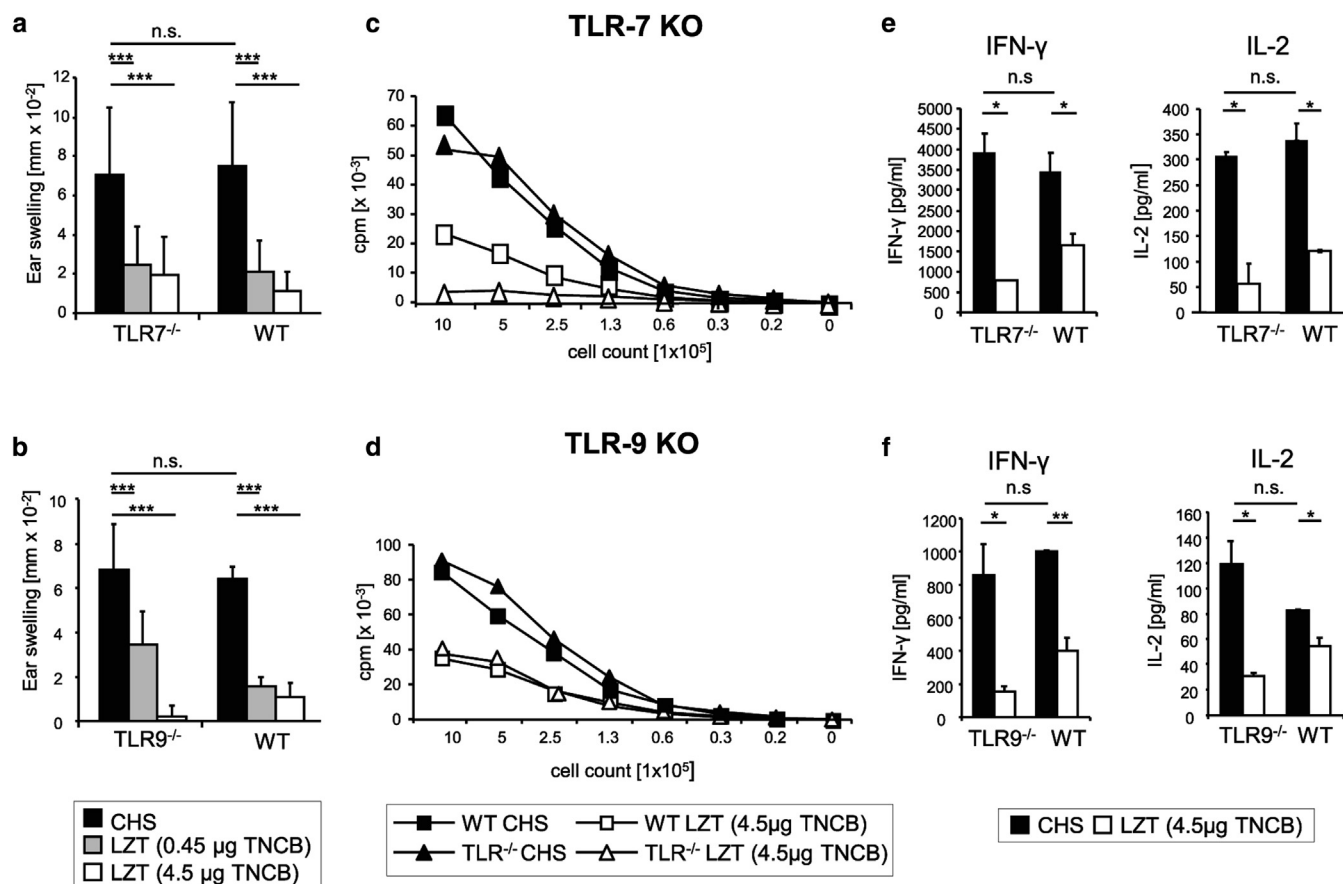


Figure 2. TLR7- and TLR9-mediated signaling does not contribute to the development of LZT and CHS. TLR7 and TLR9 knockout and wild-type animals were tolerized (0.45 µg TNCB or 4.5 µg TNCB) or solvent treated, respectively. (a, b) Absolute increase in ear thickness was assessed after sensitization and challenge (mean ± SD). (c, d) T-cell proliferation after hapten-specific restimulation in vitro is shown (mean values of triplicates in cpm). One representative result of two experiments is demonstrated. (e, f) Hapten-specific T-cell cytokine production (IFN-γ, IL-2) after hapten-specific restimulation in vitro is depicted (pooled data of two independent experiments, mean ± SD) (n = 6–8 mice per experiment). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, n.s. = not significant. CHS, contact hypersensitivity; KO, knockout; LZT, low zone tolerance; SD, standard deviation; TLR, toll-like receptor; TNCB, 2,3,6-trinitro-1-chlorobenzene; WT, wild-type.

protocols that can be used to modulate or inhibit unwanted allergic immune reactions in men.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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How Effective Is Tacrolimus in the Imiquimod-Induced Mouse Model of Psoriasis?

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TO THE EDITOR

The imiquimod (IMQ)-induced mouse model has become a widely used standard to model human psoriasis since its introduction in 2009 and seems to mirror psoriasis in many pathogenetic, clinical, and histological features (van der Fits et al., 2009). Because of various advantages, the number of publications based on this model has increased exponentially in the past 7 years (Hawkes et al., 2017). However, van der Fits et al. already stated that the model's response to antipsoriatic drugs still needs to be shown. A review article in this journal addressed a lack of validation of the model resembling human psoriasis and thus its applicability for therapeutic testing (Hawkes et al., 2017).

The immunomodulatory drug tacrolimus (TAC) is commonly used for the topical treatment of dermatitis because it lacks important side effects of corticosteroids (e.g., skin atrophy). However, the efficacy of topically applied TAC has not yet been achieved in the most common plaque-type psoriasis, in contrast to facial and inguinal psoriasis, and its efficacy after systemic administration (Scheinfeld, 2004).

Here, we investigated how effective topical TAC treatment is in the IMQ-induced mouse model of psoriasis.

The model was induced successfully according to the standard regime as reported by van der Fits et al. by daily topical administration of IMQ to the right ear and flank (Supplementary Figure S1, left column, online) in BALB/c mice. Of note, the model could not be induced in hairless SKH1 mice, which are widely used in dermatology research (Supplementary Figure S1, right column). Detailed information on materials and methods is provided in the Supplementary Material online. Experiments were approved by the State Office of Health and Social Affairs, Berlin, Germany.

To evaluate the therapeutic efficacy of TAC in the IMQ model, psoriasis-like dermatitis was induced in 33 mice. Mice were treated topically with TAC and various control substances, following three different treatment protocols (Figure 1a–c). Clinical parameters were quantified daily, including transepidermal water loss, erythema, skin hydration, and ear thickness. Additionally, skin lesions were clinically scored using a modified psoriasis area and severity index. As

final readout parameters, skin was examined histologically, epidermal thickness was measured, and infiltrating T-lymphocytes were counted in a randomized, blinded manner.

In the first treatment protocol, which has been routinely used in this model (Doppalapudi et al., 2017; Jain et al., 2016), mice were treated daily starting on day 3 for 5 consecutive days (Figure 1a). TAC was applied on six mice, in two different concentrations (0.03%, 0.003%) at the dermatological standard dose of 2 mg ointment/cm². However, no effects on any of the parameters measured were achieved (Figure 1, left column; Supplementary Figure S2 online). By contrast, six mice topically treated with dexamethasone developed significant improvements in ear thickness and less infiltrating T cells. However, no differences in other parameters measured on the flank were observed. The phenomenon of skin region-dependent treatment success has been observed before (Schaper et al., 2013).

The possible causes of lack of TAC efficacy include too low dose, too short treatment period, and too late onset of intervention. To address these possibilities, we repeated the experiment using a second treatment protocol with a higher amount of TAC ointment and the highest commercially available concentration for topical treatment (0.1%, 10–40 mg/cm², n = 5). We also

Abbreviations: IMQ; imiquimod; TAC; tacrolimus

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