

ORIGINAL ARTICLE

Tamoxifen counteracts the allergic immune response and improves allergen-induced dermatitis in mice

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Summary

Background Tamoxifen (TX) represents the prototype selective oestrogen receptor modulator. In addition to its use in breast cancer, TX possesses immunomodulatory functions and displays beneficial effects in models of systemic lupus erythematosus. We hypothesized that TX might inhibit type I allergic reactions, which are also characterized by deviations in humoral immunity.

Objective To evaluate the effects of TX on the allergic immune response in appropriate mouse models.

Methods Balb/c mice were sensitized with ovalbumin (OVA)-alum by the intraperitoneal route, and humoral parameters, T cell cytokine patterns and OVA-induced ear swelling responses were determined in a preventive (start of TX treatment before sensitization) and a therapeutic setting (start after sensitization), respectively. In addition, the impact of TX on clinical signs, epidermal thickness and leucocyte infiltration of the skin was investigated in a model of allergen-induced dermatitis.

Results Preventive TX treatment interfered with all aspects of the allergic immune response, leading to a reduction of allergen-specific Ig levels (IgE, IgG1 and IgG2a), a skewing effect in the T cell compartment with the inhibition of IL-4 and an abrogation of ear swelling responses. Interestingly, a therapeutic TX administration was also effective in reducing Ig levels and ear swelling responses. The vigorous systemic effects were additionally mirrored by local changes in allergen-dependent dermatitis with reduced clinical symptoms, diminished epidermal thickness and decreased CD4⁺ and CD8⁺ cell infiltrates.

Conclusion TX inhibits allergic responses when given preventively and also therapeutically, and improves allergen-induced dermatitis. Because of its effectiveness, TX could bear significant therapeutic potential for the treatment of allergies.

Keywords atopic dermatitis, Igs, sex hormones, tamoxifen, Th1/Th2 cytokines

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Introduction

Tamoxifen (TX) represents the prototype selective oestrogen receptor modulator (SERM) that has been used extensively in the therapy of breast cancer and in chemoprevention of high-risk groups [1]. TX has additionally been shown to exert immunomodulatory effects. For example, TX can directly act on B cells, antigen-presenting cells (APC), neutrophils and mast cells (MC), where it elicits complex response patterns [2–8]. Most of TX' action is believed to be mediated by oestrogen

receptors to which TX binds with a high affinity to act either in a pro- or in an anti-oestrogenic manner depending on tissue context [1]; the dominance of one action vs. the other is the consequence of a delicate balance between the two oestrogen receptor species (ER α and ER β) and the equipment with co-activators and co-repressors that form the multi-subunit complexes on the genes in question [1]. In addition, TX can also act in a rapid, non-genomic manner by mechanisms not related to ER signalling or via cell surface ER receptors, resulting in a plethora of changes, including the inhibition of protein kinase C, calmodulin-dependent cAMP phosphodiesterase and ion channels, respectively [8–10].

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In the past years, ligands of nuclear receptors have come into focus as novel regulators of allergic inflammation and atopic dermatitis when a number of laboratories consistently demonstrated their allergy-modulating properties [11–14]. Thus, this large group of nuclear hormone receptor targets appear to display a spectrum of modulating functions in the regulation (and dysregulation) of immune responses, and therefore bears considerable therapeutic potential. We have shown previously that retinoids, hormonally active vitamin D₃, as well as ligands of PPAR γ and LXR can all inhibit the allergic immune response, in part by acting directly on B cells [15–20]. Because oestrogens are also known as potent immunomodulators (summarized in [21]), we hypothesized that the modulation of oestrogen action *in vivo* may have a major impact on the allergic immune response. It is of special interest that TX has been reported to be therapeutically effective in mouse models of systemic lupus erythematosus (SLE) where it acts by reducing anti-DNA-antibodies and/or glomerular complex deposits, and by correcting leucopenia, thrombocytopenia and proteinuria [7, 22, 23]. Because SLE and allergies share a dysregulated humoral immune response, we decided to study the impact of TX in a frequently used mouse allergy model [24]. We report that pretreatment with TX potently inhibits all aspects of the systemic immune reaction to an allergen with decreased allergen-specific Ig production and suppressed T helper type 2 (Th2) cytokine responses to allergen, events which are functionally reflected by an impaired ear swelling response. Interestingly, even a short therapeutic application of TX (i.e. after the allergic sensitization) is likewise effective at reducing Ig levels and ear swelling responses. In addition, TX ameliorates allergen-induced dermatitis and T cell inflammation in the skin, suggesting that the systemic parameters can translate to local environments of a complex skin disease. The considerable effectiveness of TX may not only help to delineate the basic mechanisms of allergic immune responses and atopic dermatitis but also holds promise for novel anti-allergic treatments.

Methods

Mice and treatments

Female BALB/c mice were purchased from Charles River Breeding Laboratories (L'Arbresle, France) or from Harlan Winkelmann (Borchen, Germany) and kept in a specific pathogen-free environment under laboratory conditions (20–22 °C; 50% relative humidity; 12 h light, 12 h dark cycle) with mouse chow (low in phyto-oestrogen) and water was available *ad libitum*. Mice were used at 12 weeks of age. All experimental protocols were approved by the local Animal Ethics Committee and performed in

compliance with protocols approved by the local State Office of Health and Social Affairs.

Mice treatments for the experimental panels are depicted in Fig. 1. Sensitization with ovalbumin (OVA) was as described [19]. In brief, 10 μ g of OVA (Sigma-Aldrich, Taufkirchen, Germany) adsorbed to 2 mg aluminium hydroxide + 2 mg magnesium hydroxide (Imject Alum, Pierce/Perbio, Bonn, Germany) in a total volume of 100 μ L were injected intraperitoneally (i.p.) on days 1, 14 and 21.

Continuous treatment with TX was achieved by the implementation of a slow-release TX pellet (5 mg/pellet, 90-day release; Innovative Research of America, Sarasota, FL, USA), which is within the range of doses typically applied to mice and gives rise to serum levels that are comparable to those achieved in humans on TX therapy [25–27]. Control mice received matched placebo pellets. As depicted in Fig. 1a and b, pellets were implanted either before OVA sensitization (day –7, preventive protocol) or after the three i.p. allergen injections (day 23, therapeutic protocol). For the induction of dermatitis, the epicutaneous allergen application patch test was used, as described in detail elsewhere [19] (depicted in Fig. 1c). In brief, the skin on the mice belly was shaved and OVA was applied by the patch test method. Each mouse had a total of three 1-week exposures to the same skin site, with patch renewal after 3 days, respectively, and a 2-week interval between applications.

Determination of immunoglobulin concentrations

Ig levels in sera were determined using specific ELISA assays, as described [19]. Serum anti-OVA IgE levels were measured using the anti-mouse IgE-specific R35-72 (BD Pharmingen, Heidelberg, Germany) as the capture antibody and biotinylated OVA and horseradish peroxidase-conjugated streptavidin (R&D systems, Wiesbaden, Germany) for detection. For anti-OVA IgG1 and IgG2a detection, an ELISA with OVA-coated plates and a biotinylated anti-mouse IgG1 antibody (A85-1, BD Pharmingen) or anti-mouse IgG2a (R19-15, BD Pharmingen), followed by horseradish peroxidase-conjugated streptavidin were utilized for detection. Plates were developed, stopped and read at 450 nm. The concentrations of OVA-specific Igs were estimated by comparison with a standard curve made from pooled serum of OVA-sensitized mice, and data are expressed as laboratory units/mL [19].

Ex-vivo re-stimulation of splenocytes

Animals were killed by cervical dislocation and spleens were collected aseptically. Spleen cells were isolated by maceration of the tissue and passage of the resulting cell suspension through a 100 μ m nylon mesh followed by a second filtration through a 50 μ m nylon mesh. Red blood

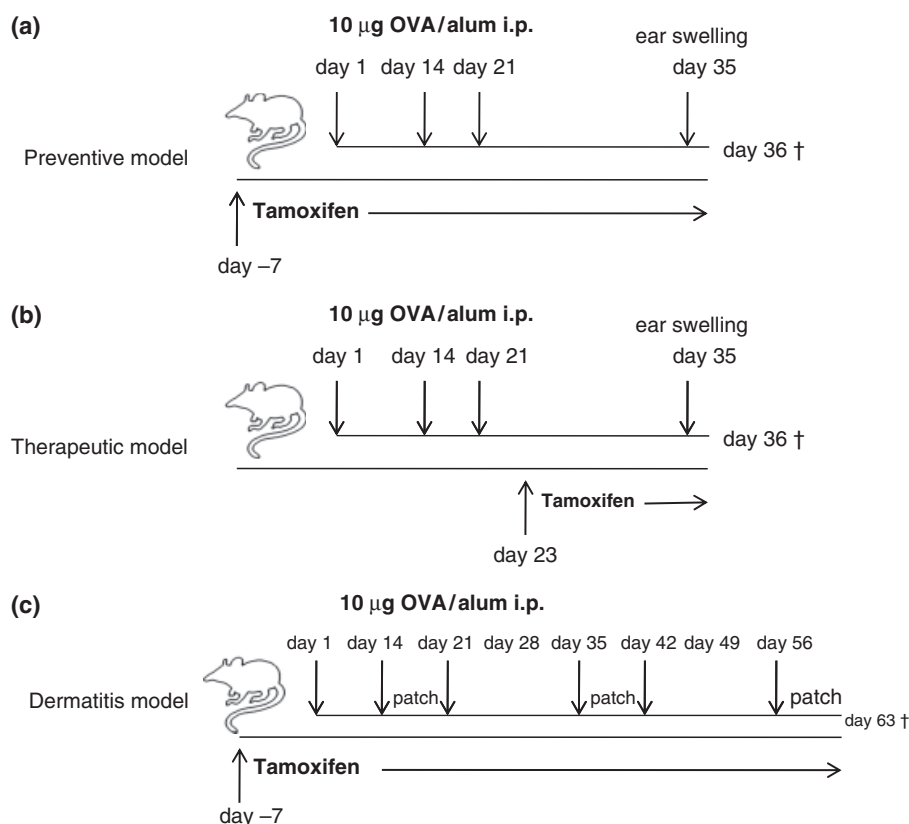


Fig. 1. Experimental design. Eight to 10 mice were used per experimental group. (a) In the preventive model, mice were pretreated with TX (from day -7 onward), then immunized by three intraperitoneal (i. p.) injections of OVA-Alum (days 1, 14, and 21) and challenged on day 35 on the ear. On the following day, mice were killed and spleens were prepared for T cell re-stimulation and cytokine quantification; serum was collected to determine Ig titres. (b) In the therapeutic model, mice were immunized as in (a), but the TX (and placebo) pellets were only implanted after the three sensitization steps, i.e. on day 23; mice were otherwise treated as in (a). (c) In the dermatitis model, mice were pretreated with TX and immunized with OVA-Alum i.p. (as in (a)) and then subjected to three epicutaneous exposures to the allergen to trigger atopic-like dermatitis lesions (as described in "Methods"). After removal of the last patch, skin and blood were taken for the analyses described.

cells in spleens were lysed with Tris-NH₄Cl. After repeated washing with phosphate-buffered saline (PBS)-ethylene diamine tetra acetic acid (EDTA), the number of viable spleen cells was adjusted to 2×10^6 /mL in RPMI 1640 medium supplemented with 10% FCS and antibiotics (all from Biochrom, Berlin, Germany). Suspensions were stimulated with OVA at 500 and 10 µg/mL of an anti-CD28 antibody (clone 37.51, eBioscience, San Diego, CA, USA) for 96 h. After centrifugation, supernatants were collected and stored frozen at -80 °C.

Quantification of cytokines

The amounts of IL-4, IL-5, IL-10 and IFN γ in cell culture supernatants were determined using a Flow Cytometry-based Fluorescent Bead Immunoassay (Bender MedSystems, Vienna, Austria), exactly as described by the manufacturer. Concentrations of each cytokine were calculated relative to the respective standard curves.

Ear swelling test

The right ear pinnae was injected intradermally with 100 µg OVA in a volume of 20 µL of saline. The left ear received 20 µL sterile PBS alone. Ear thickness was measured with a digimatic micrometre (Mitutoyo, Kawasaki, Japan) in triplicate under mild anaesthesia.

Changes in ear thickness were recorded at the times indicated, when both ears were measured. Results are expressed as follows: allergen-specific ear swelling = (Xh measurement - 0h measurement) of the right ear - (Xh measurement - 0h measurement) of the left ear.

Assessment of dermatitis

Erythema, oedema, excoriation, dryness and extension were used as typical hallmarks of human atopic dermatitis to evaluate clinical severity, as described [19]. Each parameter was evaluated independently and blindly by three investigators. Severity was rated according to scores

as follows: 0, no reaction observed; 1, mild symptoms; 2, intermediate symptoms; 3, severe symptoms. The total skin score was taken as an index of severity.

Epidermal thickness was determined by Axiovisions measuring tools on the Axioplan light microscope (Zeiss, Berlin, Germany) at $\times 100$ magnification. Ten to 20 measurements were obtained per mouse.

Immunohistochemistry

Sections of 4 μm were mounted on Superfrost Plus glass slides and used for staining by the streptavidin–biotin complex system. Sections were blocked with 10% normal goat serum (Dako, Hamburg, Germany) and the avidin/biotin blocking kit (Vector, Peterborough, Cambridgeshire, UK) and then incubated with rat-anti mouse CD4 (RM4-5, at 1 $\mu\text{g}/\text{mL}$), CD8 (53-6.7, at 1 $\mu\text{g}/\text{mL}$), followed by incubation with biotinylated goat anti-rat IgG (all from BD Pharmingen). CD11c⁺ cells were detected using purified hamster anti-mouse antibody (HL3, at 10 $\mu\text{g}/\text{mL}$) and biotinylated antihamster IgG cocktail (BD Pharmingen). Negative controls were run in parallel omitting either the primary or the secondary antibody. Signals were detected by the alkaline phosphatase/red detection kit (Dako) and haematoxylin counter staining. MC were detected upon staining with acidic toluidine blue (0.1% w/v in 0.5 N HCl) for 1 h at room temperature. In all cases, positively stained cells were counted in 6–12 fields of 100 $\times$ 200 μm , respectively, and are expressed as mean cell number (cells/0.1 mm²) per mouse.

Data analysis

Data were analysed using the statistical program InstatP (GraphPad Software) by an appropriate test (unpaired Student's *t*-test or Mann–Whitney test when data were not normally distributed). Results are shown as mean $\pm$ SEM or as the median with range, as appropriate. Significance between experimental and control mice was assumed at $P < 0.05$ for both tests.

Results

Preventive tamoxifen treatment counteracts the allergen-specific immune response

To assess the effects of TX on the humoral response to allergen, TX pretreated and control mice were sensitized i.p. with OVA (as depicted in Fig. 1a), an extensively used mouse allergy model [24], and OVA-specific Ig concentrations in the sera were determined on day 36. TX led to a profound inhibition of the allergen-specific IgE response (Fig. 2a, left), nearly abolished the production of OVA-specific IgG1 (Fig. 2a, middle) and decreased allergen-

specific IgG2a, albeit not quite significantly (Fig. 2a, right).

Considering the profound effects of TX on allergen-specific Ig production, we next determined whether TX may modulate OVA-dependent T cell immune reactions. The data are summarized in Fig. 2b. *In vivo* TX treatment skewed the Th1/Th2 cytokine pattern upon subsequent *in vitro* restimulation of spleen cells, where IL-4 was vigorously suppressed down to $\sim 20\%$ of control levels, while IFN- γ was concomitantly increased. A down-regulation of IL-10 in the TX panel was noted, as well, but did not reach statistical significance.

On the other hand, spleen cellularity was not affected by TX treatment and splenocyte composition (i.e. the numbers of B cells, T cells and monocytes/macrophages) remained largely stable, although there was a slight tendency of the T cell population to decline (data not illustrated). The effect of TX was, therefore, not accompanied by gross changes of lymphopoiesis/haematopoiesis, leucocyte recruitment to the spleen and/or the rate of apoptosis.

Taken together, mice develop a strong deviation of the allergen-dependent cytokine response upon TX pretreatment with a reduced IL-4 and an increased IFN- γ production being the most prominent consequences.

Having established a clear-cut role for TX in the modulation of the response to OVA, we attempted to study the functional relevance of these findings. Therefore, the allergen-induced ear swelling test was applied. As shown in Fig. 2c, OVA triggered a profound ear swelling response in control mice with a pronounced late-phase response starting at 4 h. In mice that had been exposed to TX, the ear swelling reaction was abolished (Fig. 2c), in line with the data on allergen-specific antibody concentrations (Fig. 2a).

Therapeutic tamoxifen treatment interferes with humoral immune responses

Having established that preventive TX administration had a profound impact on the allergic immune response, we were interested to clarify whether TX may suppress established Ig responses, and thus be a possible therapeutic candidate. In order to evaluate the potential of TX in a therapeutic setting, TX treatment was initiated only after the three i.p. sensitization steps, i.e. on day 23 (as depicted in Fig. 1b). To make results comparable with the preventive setting, the protocol remained otherwise the same and mice were subjected to the ear swelling test on day 35 (Fig. 1b). As shown in Fig. 3a, the delayed administration of TX was able to tolerize ongoing Ig responses. IgE levels were decreased to 57.5% (not quite significant), whereas both IgG1 and IgG2a experienced significant down-regulation to 28.5% and 31.8% of the placebo control, respectively. In addition, the ear swelling response was significantly

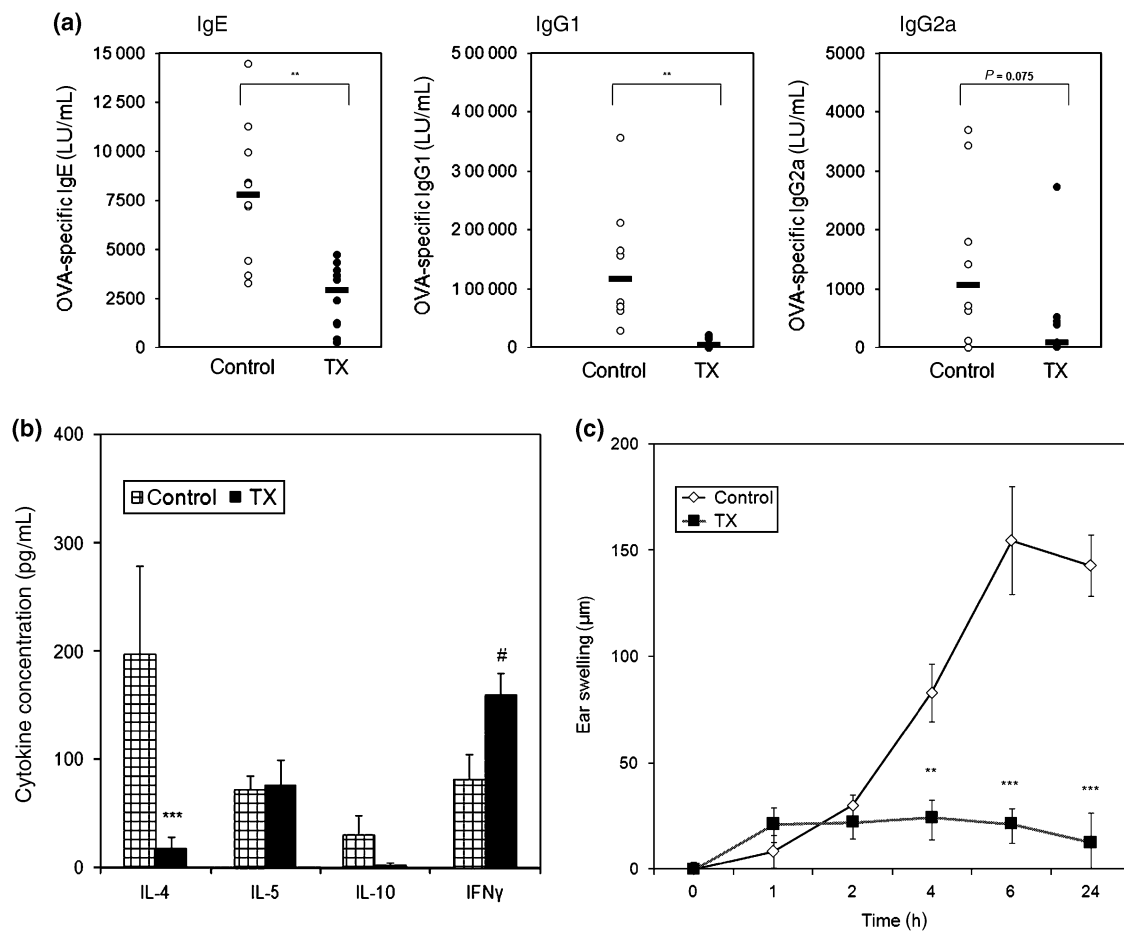


Fig. 2. Preventive tamoxifen (TX) treatment dampens the systemic immune response and abrogates allergen-induced ear swelling. (a) Preventive TX treatment, initiated before the systemic allergic sensitization (as depicted in Fig. 1a), dampens the production of allergen-specific Igs. OVA-specific IgE, IgG1 and IgG2a were determined in mouse sera. (b) TX increases IFN γ but suppresses IL-4 production. Splenocytes from TX-treated and control mice were stimulated as described in "Methods." Results are from 8–10 mice per group and are given as mean $\pm$ SEM. (c) TX abolishes the ear-swelling response. OVA was injected into the right ear, while the control left ear was injected with PBS only. The thickness of each ear lobe was measured immediately before and at various time periods after injection. Results are from 10 mice per group and are given as mean $\pm$ SEM. ** P < 0.01; *** P < 0.001 significantly lower than control. # P < 0.05 significantly higher than control.

inhibited (Fig. 3b), although suppression was clearly lower in potency as in the preventive setting. This difference might be due to the relative longevity of cell-bound Igs, so that their levels in the circulation unlikely mirror exactly those bound in the tissue, especially after such a short period of treatment.

Taken together, even after the allergic sensitization has been established, TX profoundly interferes with the ongoing humoral immune response.

TX ameliorates allergen-induced dermatitis

In view of the potent effects of TX on the systemic reaction to OVA, we analysed the therapeutic role of TX in a model of OVA-triggered dermatitis [19]. According to the experimental design (Fig. 1c), the skin of TX-treated and control mice were assessed after repeated OVA patch-

ing. OVA-specific IgE and IgG1 levels were also measured and confirmed (at this later timepoint) to decrease upon TX treatment (OVA-IgE control: median 66 050, range 23 850–181 667; OVA-IgE TX: median 39 500, range 9492–55 900; P = 0.0379/OVA-IgG1 control: median 139 750, range 57 400–282 600; and OVA-IgG1 TX: median 57 327, range 28 600–98 600; P = 0.0104).

In the skin, the development of dermatitis was clearly inhibited in TX pre-treated animals, as evidenced by a reduced skin score (Fig. 4a). Virtually, all of the typical eczematous signs and symptoms were suppressed in a significant manner. The values for the individual parameters were as follows: erythema control: 2.0 ± 0.5 , TX: 0.71 ± 0.49 , P < 0.001; oedema/papules control: 2.5 ± 0.2 , TX: 1.4 ± 0.8 , P = 0.012; excoriation/crusts control: 2.4 ± 0.5 , TX: 1.4 ± 1.0 , P = 0.041; dryness control: 1.6 ± 0.6 , TX: 1.4 ± 0.8 , P = 0.7; and extension control:

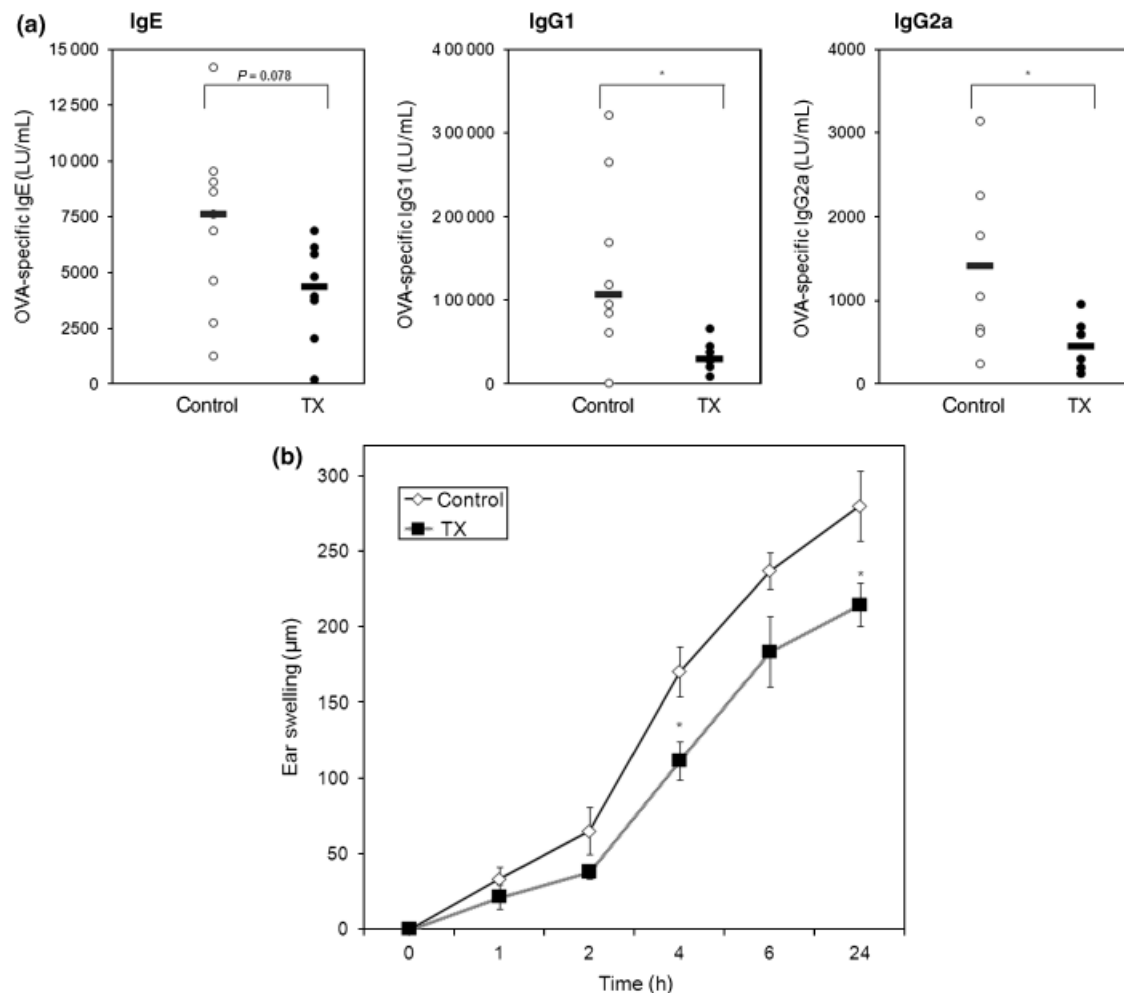


Fig. 3. Therapeutic tamoxifen (TX) treatment dampens the humoral immune response and decreases the allergen-induced ear swelling. (a) TX treatment, initiated after the allergic sensitization (as depicted in Fig. 1b), decreases the production of allergen-specific Igs. OVA-specific IgE, IgG1 and IgG2a were determined in mouse sera. (b) TX decreases the ear-swelling response. The ear swelling test was performed as in Fig. 2c. Results are from eight mice per group and are given as mean $\pm$ SEM. * $P < 0.05$ significantly lower than control.

2.3 ± 0.5 , TX: 1.4 ± 0.8 , $P = 0.034$. Together, erythema was most strongly decreased by TX treatment, while dryness was obviously not affected in a statistically significant fashion, whereas oedema, excoriation and extension were down-regulated less strongly than erythema, yet significantly. This substantiates that the different signs of atopic dermatitis rely on partially distinct mechanisms, at least to a certain extent. In addition, epidermal thickness clearly ameliorated on TX treatment (Fig. 4b; see also the photos in Fig. 4c).

Immunohistochemical staining of skin specimens from the affected areas revealed reduced infiltration of both $CD4^+$ and $CD8^+$ cells in TX-treated mice (Fig. 4c and d). In contrast, the numbers of $CD11c^+$ cells (in the epidermis and dermis, respectively), and MC (in the dermis) did not change, implying that TX mainly acted on the T cell compartment, selectively reducing their recruitment to the skin and/or survival. Both $CD4^+$

and $CD8^+$ cells have been shown before to increase strongly in OVA-triggered dermatitis (3–4-fold for $CD8^+$ and 4–7-fold for $CD4^+$ cells, as compared with the PBS control), whereas MC and $CD11c^+$ cells show slighter increases in allergen-induced skin lesions [19]. The latter two may not significantly contribute to allergen-induced dermatitis in this model, experience functional alterations instead of changes in density under TX treatment or it may be sufficient that other infiltrating cells (in particular T cells) are reduced for the lesions to improve. In fact, MC were shown previously to be dispensable for the extent of allergen-induced dermatitis [28].

Taken together, *in vivo* TX treatment is effective at reducing the signs and symptoms of dermatitis in a murine patch test model, likely owing, at least in part, to a reduction of T cell-mediated inflammation in the affected skin area.

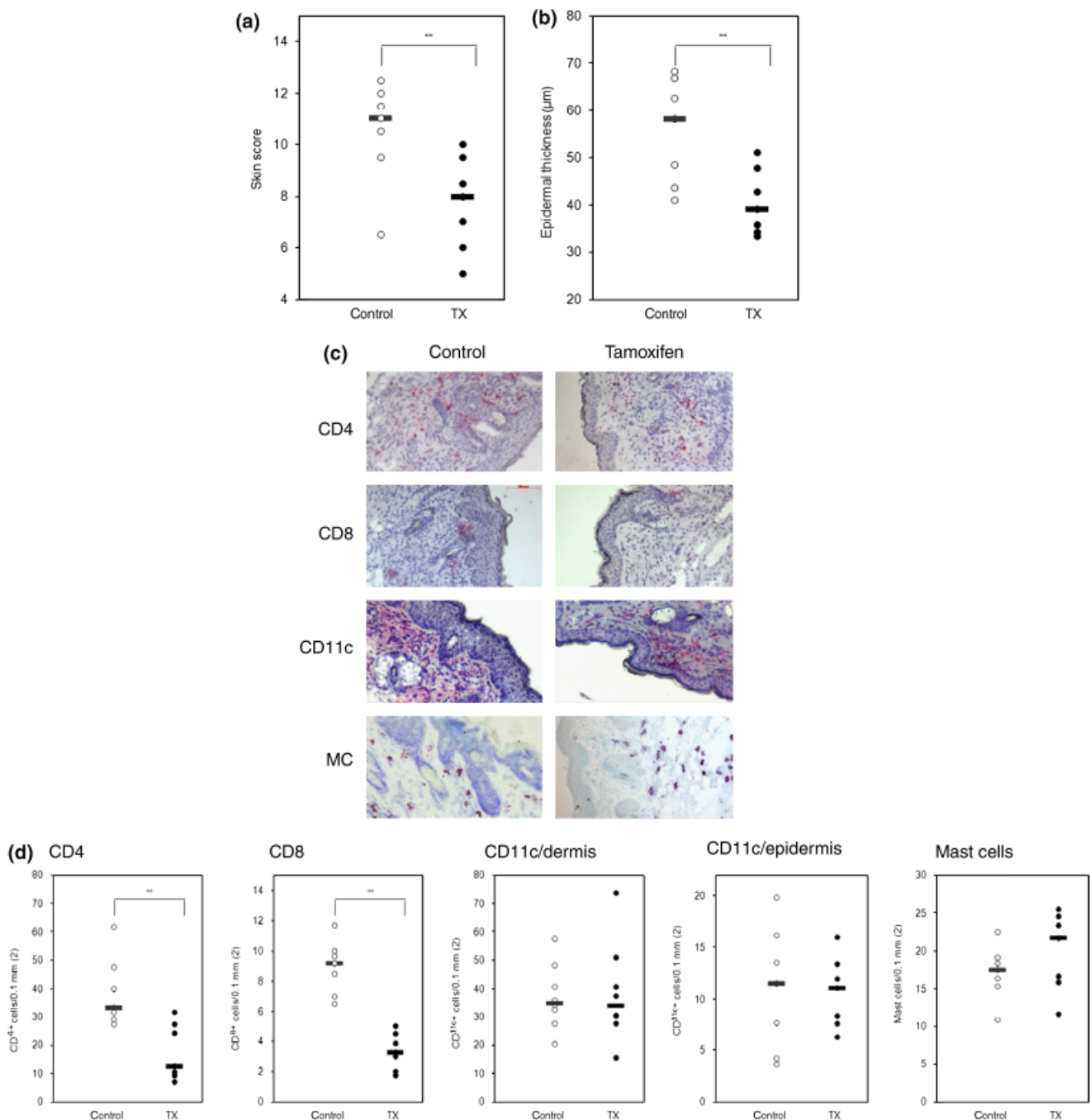


Fig. 4. Tamoxifen (TX) treatment ameliorates allergen-induced dermatitis. (a) TX decreases the clinical signs and symptoms of allergen-triggered dermatitis. Symptoms were evaluated, as described in "Methods." Note that a score of 4 is produced by the patch itself in the absence of allergen, and thus corresponds to an allergen unspecific, mild dermatitis. (b) TX ameliorates epidermal thickness. Epidermal thickness was measured from stratum basale to stratum granulosum using 10–20 sections per mouse. (c) Representative photographs upon immunohistochemical staining of skin lesions. (d) Quantification of CD4⁺ cells, CD8⁺ cells (both in the dermis), CD11c⁺ cells (in the epidermis and dermis, respectively) and MC (in the dermis) in the lesions of control and TX-treated mice. ***P* < 0.01 significantly lower than control.

Discussion

Over the last years, the prototype SERM TX has come into focus as a 'universal' drug beneficial not only in the context of breast cancer but also in various other settings

(summarized in [29]). For example, TX has been found to be cardioprotective, to restore bone mineral density and to be potentially beneficial in the context of neurodegenerative disorders [29]. The pleiotropic effects of a single substance are likely related to its action as an oestrogen

in certain tissues (e.g. bone, skin and brain [29, 30]) but as an anti-oestrogen at various other target sites, a combination, which obviously creates utmost efficiency. So far, it has remained unexplored, however, whether TX might possess positive effects for the large group of allergic diseases.

We report here that TX acts in an anti-allergic manner and, simultaneously improves allergen-induced dermatitis. TX has a very remarkable effect on Ig production. In fact, Ig levels were strongly suppressed in the three models utilized, i.e. in the preventive setting, in the therapeutic protocol and in the model of allergen-triggered dermatitis (outlined in Fig. 1). Oestrogens have long been known to stimulate Ig production by a combination of direct and indirect mechanisms, some of which are reversed by TX (summarized in [21]). For example, TX was shown to inhibit Ig production *in vitro* by acting on B cells [31]. Another study reported that oestrogen-stimulated Ig production is partially mediated by increased IL-10 levels [32]. In our study, IL-10 levels were reduced by TX, thereby potentially reversing the positive impact from oestrogens. Other studies have shown that oestrogen-stimulated Ig responses were the result of relieved CD8⁺ cell-mediated suppression [33, 34], whereas others reported that oestrogen-driven autoimmune responses can be counteracted by TX and that this is due to an interference with B cell/plasma blast maturation [7]. A recent publication reported on a direct inhibition of activation-induced deaminase (AID) expression in B cells by TX through a reversal of oestrogen-mediated AID induction [35]. Therefore, TX appears to impact various aspects of B cell function by direct effects on B cells as well as through indirect effects on other cells. It is of interest in this regard that APCs are also potently influenced by TX and appear to remain in an immature state [4–6], a finding that might potentially explain the shifts in T cell cytokine production in our study (Fig. 2b).

Because of the complexity and combinatorial effects on various cells, it will be challenging in the future to clearly dissect all of the cellular and molecular events that occur simultaneously under TX treatment *in vivo* in an unmanipulated endogenous hormone environment, i.e. under defined and kinetically oscillating oestrogen levels, as encountered in the female gender. Likely, it is precisely this capacity of TX to modulate various key events at the same time to make it such an attractive drug also in the context of autoimmunity and allergy.

In our preventive protocol, IgG1 was most strongly affected, followed by IgE and IgG2a. Several mechanisms may contribute to the precise response pattern of the distinct Ig isotypes. Firstly, because both IgE and IgG1 were affected in the preventive setting, this was indicative of an impaired Th2 response, as both subclasses require IL-4 for class switch recombination (CSR) (summarized in [36]). In fact, when allergen-specific cytokine production

of spleen cells was investigated, a profound decrease of IL-4 was observed, corresponding to the data on Ig production. Secondly, because AID is required for class switch recombination to all isotypes, the finding by Pauklin *et al.* [35] may have contributed substantially to the decrease of all Ig subtypes, including IgG2a. The fact that IgG2a was less profoundly down-regulated in the preventive setting, might be associated with a counter-regulatory effect from IFN γ , a cytokine that promotes CSR to IgG2a [36], and which was positively influenced by TX (Fig. 2b). The robustness of effects in the preventive setting made it interesting to determine whether TX might also impact ongoing allergic responses when administered after antigenic sensitization. We surprisingly found that TX was able to reduce Ig levels even in the therapeutic protocol. Although somewhat less pronounced than in the preventive schedule, suppression was still very clear, especially if considering that the period with TX was only 13 days (vs. 43 in the preventive protocol) and taking into account the half-life of already produced Igs before contact with TX (e.g. 6–8 days for IgG1 [37]). Ear swelling responses were also significantly decreased by TX, although not abolished as in the preventive protocol. The more pronounced difference may be explained by the relatively longer survival of cell-bound Igs vs. those in the circulation [38] and by effects of TX not only on CSR but also on somatic hypermutation (leading to Igs of higher affinity), as both processes depend on AID [39] and are likely to be more potently affected in the preventive protocol.

The vigorous effects on Ig titres further prompted us to investigate the effectiveness of TX in a disease model of allergen-dependent dermatitis. We have recently optimized a model that gives rise to allergen-dependent skin lesions with epidermal thickening and immune cell infiltration into the skin [19]. Here, we show that a modulation of systemic responses towards allergen by TX is accompanied by a reduced severity of skin lesions. An amelioration of nearly all of the typical dermatitis features was observed, along with a reduced epidermal thickness and a reduced T cell infiltration, a hallmark of the disease (Fig. 4). Our data are perfectly in line with a recent publication, which showed that atopic-like dermatitis in the patch test model strongly depends on Ig responses, in particular those signalling through receptors containing the common Fc γ chain, i.e. Fc ϵ RI and Fc γ RIII to which IgE and IgG1 bind, respectively [40].

There is a sex difference in the incidence of allergic diseases that changes with puberty when girls become more strongly affected [41–43], suggesting either a protective role of male hormones, an exacerbating function of female hormones, or a combination of both in the establishment and/or maintenance of allergies. In line with this, female mice are more readily sensitized to an allergen than male mice (own unpublished observations). Because the majority of epidemiological data points

towards an allergy-promoting role of oestrogens and given that TX can act as an oestrogen receptor antagonist, our data might be explained, at least in part, by a reversed oestrogen action. While there are only few experimental studies that have addressed the impact of oestrogens on allergic inflammation, those that have been carried out, support this notion. For example, in models of allergic asthma, preceding ovariectomy (OVX) reduced lung eosinophilia and IL-5 production [44, 45].

It is likely, however, that the effects presented here are specifically associated with TX and do not simply reflect the loss of central oestrogen production (that would be likewise achieved with OVX), even if an anti-oestrogenic action in the immune system is required to bring about the effectiveness of TX. This can be inferred from the study of Riffo-Vasquez et al. [45] in comparison with our own data. They found that OVX was only protective in allergic inflammation when carried out before the immunization, whereas it had no beneficial effect in a therapeutic setting, in clear contrast with TX. In addition, OVX was not associated with reduced Ig levels in their study, whereas TX is very potent in this regard, as demonstrated here. Also, in their setting, IL-5 was strongly affected, whereas in our study, it was IL-4. In summary, TX administration not only before but also after immunization effectively decreases allergen-specific Ig production, thereby making TX a possible strategy even in ongoing allergic responses.

We used female mice for the study because the allergy models we used are most commonly carried out with female animals. In addition, TX used as a therapeutic strategy is also most frequently tested in females. It was suggested recently, however, that TX may also be therapeutically useful in males, at least in certain settings [29]. Oestrogens are also found in males (albeit at lower concentrations) and males possess likewise oestrogen receptors. If we assume that the lower oestrogen levels found in males are sufficient to favour allergic immune responses, then TX might be able to interfere with allergies even in the male gender. However, effects should be less pronounced, as allergic immune responses are decreased even at baseline in male vs. female mice (own unpublished data), possibly reflecting in part lower oestrogen levels. Future studies will be required to dissect directly a possible anti-allergic function of TX in males.

In conclusion, our data imply that TX has a profound anti-allergic effect, not only in preventing immediate-type hypersensitivity but also in suppressing established allergic sensitization. The efficacy and intensity of TX effects make it highly attractive to clarify in the future the combinatorial mechanisms underlying this efficiency. Our study lays an important basis to such questions.

Although too early to draw any definitive conclusions, our data also imply that TX or related compounds may well serve as a future therapeutic strategy for the

treatment of allergic diseases, perhaps in a gender-tailored fashion.

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