

# Galectin-1 Expression in CD8<sup>+</sup> T Lymphocytes Controls Inflammation in Contact Hypersensitivity



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Allergic contact dermatitis, also known as contact hypersensitivity, is a frequent T-cell-mediated inflammatory skin disease characterized by red, itchy, swollen, and cracked skin. It is caused by the direct contact with an allergen and/or irritant hapten. Galectin-1 (Gal-1) is a  $\beta$ -galactoside-binding lectin, which is highly expressed in several types of immune cells. The role of endogenous Gal-1 in contact hypersensitivity is not known. We found that Gal-1-deficient mice display more sustained and prolonged skin inflammation than wild-type mice after oxazolone treatment. Gal-1-deficient mice have increased CD8<sup>+</sup> T cells and neutrophilic infiltration in the skin. After the sensitization phase, Gal-1-depleted mice showed an increased frequency of central memory CD8<sup>+</sup> T cells and IFN- $\gamma$  secretion by CD8<sup>+</sup> T cells. The absence of Gal-1 does not affect the migration of transferred CD4<sup>+</sup> and CD8<sup>+</sup> T cells from the blood to the lymph nodes or to the skin. The depletion of CD4<sup>+</sup> T lymphocytes as well as adoptive transfer experiments demonstrated that endogenous expression of Gal-1 on CD8<sup>+</sup> T lymphocytes exerts a major role in the control of contact hypersensitivity model. These data underscore the protective role of endogenous Gal-1 in CD8<sup>+</sup> but not CD4<sup>+</sup> T cells in the development of allergic contact dermatitis.

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## INTRODUCTION

Contact dermatitis (CD) is a prevalent inflammatory skin disease affecting 15–20% of the general population. Two main different types of CD are (i) irritant CD, a primary skin reaction to chemicals, and (ii) allergic CD (ACD) or contact hypersensitivity (CHS), an immune reaction caused by contact with a hapten (Martin, 2013). A typical CHS reaction displays two phases: (i) sensitization, which occurs after the first contact with the hapten, and induces clonal expansion of specific central memory and effector T cells (Gaide et al., 2015) and (ii) elicitation, which is triggered by re-exposure

to the hapten. This second phase comprises the reactivation and recruitment of specific central memory and effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells at the site of allergen exposure (Allen, 2013; Saint-Mezard et al., 2004; Vocanson et al., 2005).

Galectins (Gals) are  $\beta$ -galactoside-binding animal lectins characterized by shared consensus amino acid sequences in the carbohydrate-recognition domain (Liu and Rabinovich, 2010). Gal-1 was the first described Gal family member and is expressed by many tissues (Camby et al., 2006). Hence, Gal-1 is synthesized and secreted by many different types of cells, such as activated T and B cells, regulatory T cells (Tregs), macrophages, dendritic cells, and  $\gamma\delta$  T cells (Sundblad et al., 2017).

The role of Gal-1 in inflammation has been explored in several in vitro and in vivo experimental models (Auvynet et al., 2013; Corrêa et al., 2017; Iqbal et al., 2011; Rabinovich et al., 2000; Zanon et al., 2015). Exogenous Gal-1 affects the viability, proliferation, and T helper type 1 responses of nonmalignant T cells involved in the progression of cutaneous T-cell lymphoma (Cedeno-Laurent et al., 2012b). Importantly, the administration of exogenous Gal-1 induces IL-10 release by Foxp3<sup>+</sup>CD4<sup>+</sup> T cells, which suppress inflammation (Cedeno-Laurent et al., 2012a, 2010). Moreover, the absence of Gal-1 expression in murine and human CD4<sup>+</sup>CD25<sup>+</sup> T cells reduces their capacity to control the proliferation of effector CD4<sup>+</sup> T cells (Garín et al., 2007). However, the differential role of endogenous Gal-1 in the function of regulatory and effector T lymphocyte subsets in CHS has not been addressed.

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Abbreviations: ACD, allergic contact dermatitis; CD, contact dermatitis; CHS, contact hypersensitivity; Gal, galectin; OXZ, oxazolone; Treg, regulatory T cell

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In this study, we show that Gal-1-deficient (Gal-1<sup>-/-</sup>) mice displayed increased inflammation and CD8<sup>+</sup> T-cells infiltration in the skin after treatment with oxazolone (OXZ). Gal-1 expression does not regulate the migration of lymphocytes to the inflamed skin as well as does not affect the regulation of CD8<sup>+</sup> T-cell proliferation by CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in CHS model. However, the depletion of CD4<sup>+</sup> T cells as well as adoptive transfer experiments demonstrate a major role for Gal-1 in CD8<sup>+</sup> T cells in CHS. Mechanistically, our data indicate that Gal-1 regulates the central memory CD8<sup>+</sup> T-cell compartment and the secretion of IFN- $\gamma$  by effector CD8<sup>+</sup> T cells.

## RESULTS

### Gal-1<sup>-/-</sup> mice showed increased ear swelling and inflammation after CHS

To assess the role of endogenous Gal-1 in the regulation of lymphocyte functions in CHS model, OXZ was administered to wild-type (Gal-1<sup>+/+</sup>) and Gal-1-deficient (Gal-1<sup>-/-</sup>) mice. Gal-1<sup>-/-</sup> mice showed increased ear swelling and sustained inflammation compared with Gal-1<sup>+/+</sup> mice (Figure 1a). Flow cytometry analysis showed that OXZ significantly increased the total numbers of infiltrating CD45<sup>+</sup> cells and myeloid cells such as neutrophils (CD11b<sup>+</sup>Ly6G<sup>+</sup>) and macrophages (CD11b<sup>+</sup>CD64<sup>+</sup>) in Gal-1<sup>-/-</sup> mice compared with those in Gal-1<sup>+/+</sup> mice (Figure 1b). Furthermore, histological analysis revealed that the increase in epidermal and dermal thickness was higher in Gal-1<sup>-/-</sup> mice than in Gal-1<sup>+/+</sup> mice at 48 hours after the elicitation phase (Figure 1c). These results suggest that the presence of Gal-1 prevents the inflammation induced by OXZ.

We analyzed whether the expression of Gal-1 is differently modulated in lymphoid cells, compared with that in blood and skin tissue, on CHS challenge. The expression of Gal-1 among effector T cells (CD4<sup>+</sup>, CD8<sup>+</sup>,  $\gamma\delta$ <sup>+</sup>) and Tregs (CD4<sup>+</sup>Foxp3<sup>+</sup>) populations was higher in blood than in lymph nodes at 48 hours (Figure 1d). However, the highest expression of Gal-1 was detected in T cells in the inflamed tissue (Figure 1d).

We also evaluated Gal-1 expression in the ear sections of Gal-1<sup>+/+</sup> mice treated with OXZ at 48 hours after the second challenge by immunofluorescence. The detection of Gal-1-positive cells corresponds mainly to CD45<sup>+</sup> cells, although the expression of Gal-1 was also observed in vessels but not in keratinocytes (Figure 1e).

### Gal-1 deficiency increases skin effector T lymphocytes after elicitation phase of CHS

We analyzed the lymphoid T-cell subsets in Gal-1<sup>+/+</sup> and Gal-1<sup>-/-</sup> mice expressing IL-17-GFP and Foxp3<sup>+</sup> RFP in steady state. These experiments demonstrated that Gal-1<sup>-/-</sup> mice are similar to Gal-1<sup>+/+</sup> mice in terms of the total number and frequency of T-cells populations (CD8<sup>+</sup>, CD4<sup>+</sup>, and CD4<sup>+</sup>Foxp3<sup>+</sup>) in blood, skin, lymph nodes, and spleen (Supplementary Figure S1a–d). In the thymus, the absence of Gal-1 did not cause any significant difference in the number of CD4<sup>+</sup>CD8<sup>+</sup> T cells, single-positive cells, or natural Tregs (CD4<sup>+</sup>Foxp3<sup>+</sup> cells) (Supplementary Figure S1e).

To analyze the role of Gal-1 in the sensitization phase, Gal-1<sup>+/+</sup> and Gal-1<sup>-/-</sup> mice were treated with OXZ in the abdomen (Supplementary Figure S2a). Immune cell populations were analyzed in the lymph nodes after 48 hours and

5 days. We observed that after the first challenge with the hapten, Gal-1<sup>-/-</sup> mice displayed the same frequency and total number of CD4<sup>+</sup>, CD8<sup>+</sup>, and Tregs as Gal-1<sup>+/+</sup> mice (Supplementary Figure S2b and c). Moreover, the neutrophilic infiltration found in the skin after 48 hours of the first challenge with OXZ was similar between both genotypes (Supplementary Figure S2d). Overall, these results indicated that Gal-1 mainly controls the inflammatory response induced after the second challenge with OXZ.

$\gamma\delta$ <sup>+</sup>IL-17<sup>+</sup> T cells are relevant mediators of skin inflammatory diseases such as ACD (Jiang et al., 2017) and psoriasis (Cibrian et al., 2020, 2016). To address the role of Gal-1 in  $\gamma\delta$  T cells in the CHS model, we studied this population using the gating strategy indicated in Figure 2a. The number of dermal  $\gamma\delta$  T cells but not dendritic epidermal  $\gamma\delta$  T cells increased by day 7 after the elicitation phase in Gal-1<sup>-/-</sup> mice in comparison with that in Gal-1<sup>+/+</sup> mice (Figure 2b). Interestingly, kinetic analyses of lymphocyte infiltration of OXZ-treated ears demonstrated that Gal-1 deficiency increased the total number of effector CD8<sup>+</sup> (Figure 2b) and  $\gamma\delta$ <sup>+</sup>IL-17<sup>+</sup> (Figure 2c) T cells at day 7 after treatment. In contrast, Gal-1<sup>-/-</sup> mice were not significantly different from Gal-1<sup>+/+</sup> in terms of the numbers of CD4<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>+</sup> cells over time (Figure 2d). These data indicate that Gal-1<sup>-/-</sup> mice develop a sustained inflammation at day 7 after OXZ treatment compared with Gal-1<sup>+/+</sup> mice, likely owing to an increase of CD8<sup>+</sup> and IL-17-secreting  $\gamma\delta$  T cells.

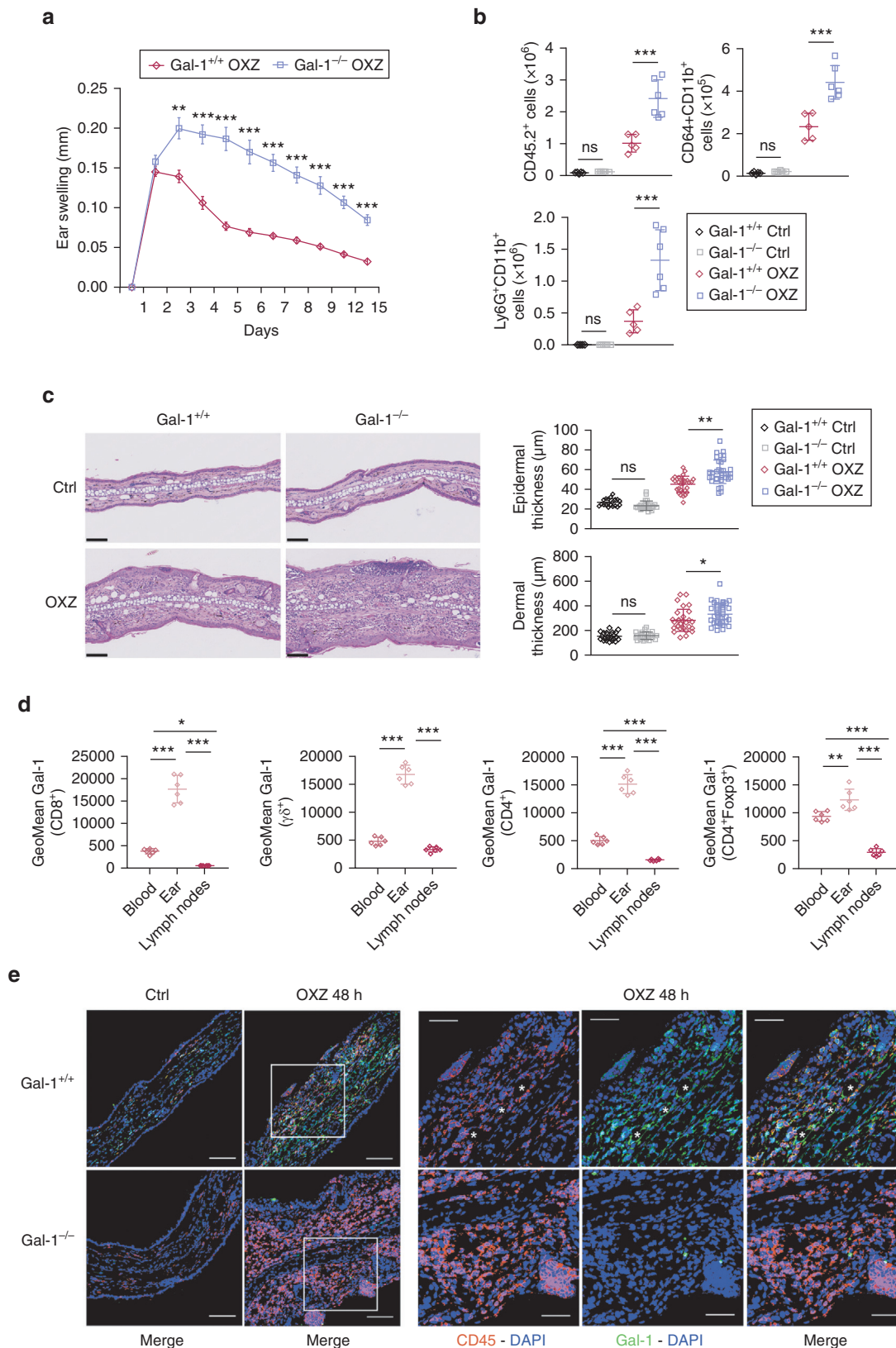
### Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> T cells display a similar migratory ability to inflamed skin

Gal-1 can be found in circulation as a soluble protein as well as can be expressed by endothelial cells (Norling et al., 2009; Thijssen et al., 2008). To study whether the Gal-1-mediated effect in CHS is related to the adhesion and migration of T cells to OXZ-treated skin, we induced CHS in the presence of a blocking anti-ICAM-1 antibody (Kish et al., 2011). We found that Gal-1<sup>-/-</sup> mice still displayed increased inflammation compared with Gal-1<sup>+/+</sup> mice, ruling out the involvement of ICAM-1 in the anti-inflammatory effect of Gal-1 in the CHS response induced by OXZ (Figure 3a).

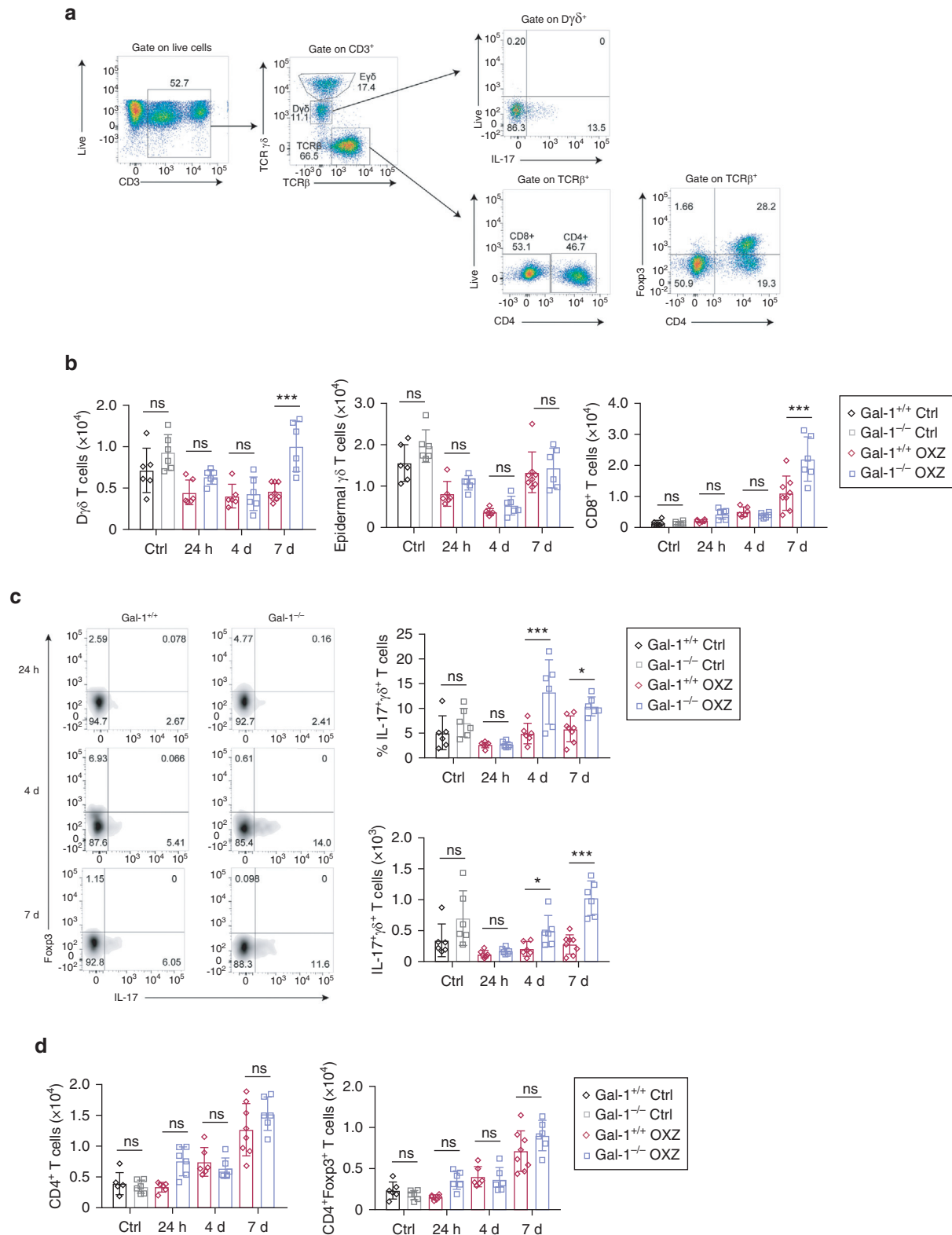
To further assess the role of soluble and endothelial Gal-1 in the migration of immune cells to inflamed skin and lymph nodes, wild-type cells from draining lymph nodes of OXZ-treated mice were intravenously injected in Rag1<sup>-/-</sup>Gal-1<sup>-/-</sup> mice or Rag1<sup>-/-</sup>Gal-1<sup>+/+</sup> mice (Figure 3b). Flow cytometry data revealed that Gal-1<sup>+/+</sup> T cells (CD4<sup>+</sup> and CD8<sup>+</sup>) similarly migrate to the ears of Rag1<sup>-/-</sup>Gal-1<sup>-/-</sup> and Rag1<sup>-/-</sup>Gal-1<sup>+/+</sup>-recipient mice (Figure 3c). Furthermore, circulating or endothelial Gal-1 expression does not play a relevant role in CD4<sup>+</sup> and CD8<sup>+</sup> T-cells entrance or exit to the lymph nodes (Figure 3d).

### Endogenous expression of Gal-1 in T-cell compartment regulates the development of CHS

To ascertain whether the deletion of Gal-1 in bone marrow-derived lymphoid and myeloid cells could recapitulate the enhanced and sustained inflammation observed in the CHS model, we analyzed the inflammation in CD45.1 Gal-1<sup>+/+</sup> mice lethally irradiated and bone marrow transferred with Gal-1<sup>+/+</sup> or Gal-1<sup>-/-</sup> cells (Figure 4a). We found that the absence of Gal-1 in hematopoietic cells recapitulates the

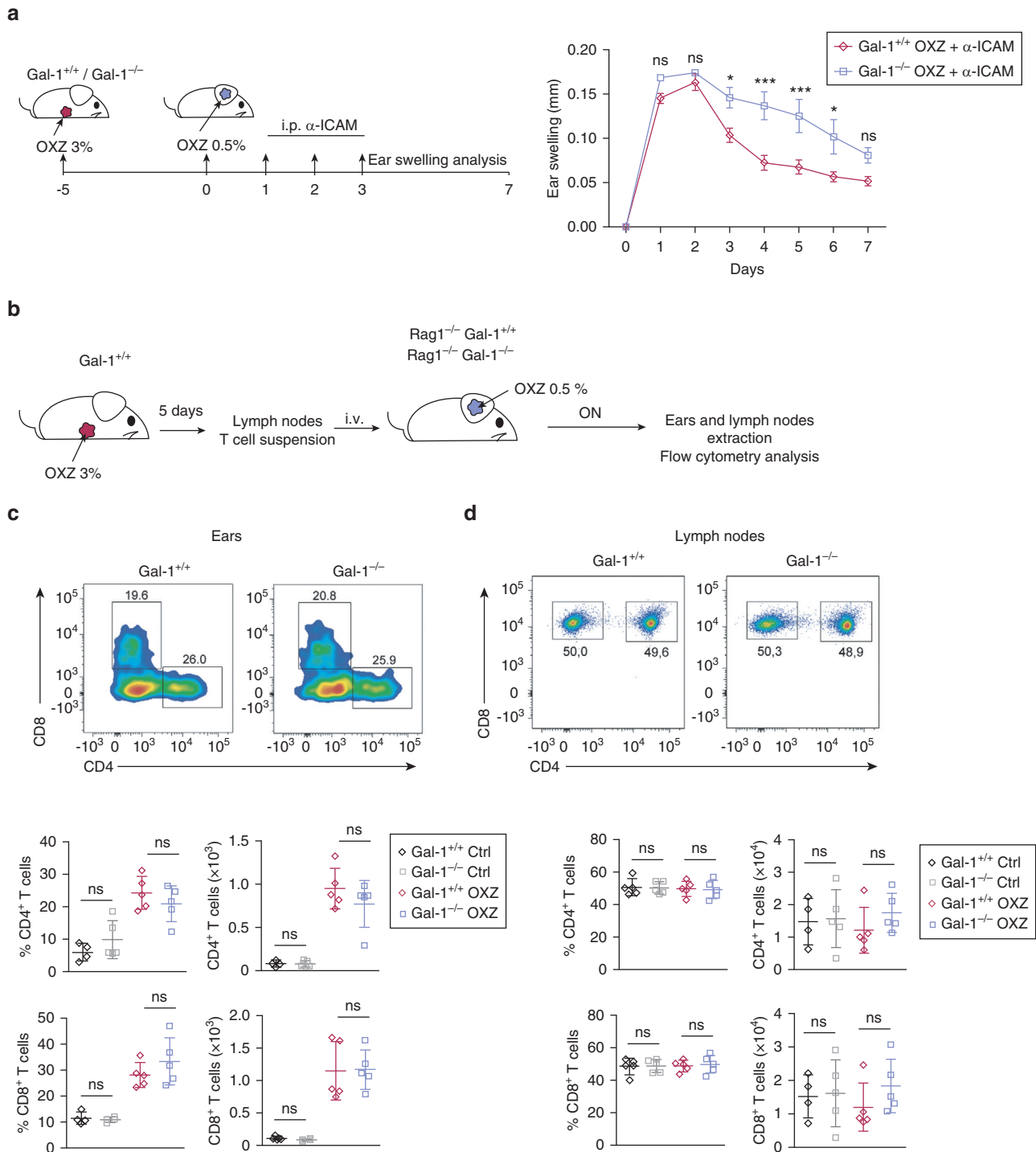


**Figure 1. Gal-1<sup>-/-</sup> mice display increased ear swelling and inflammation after CHS challenge.** (a) Ear swelling in Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice after OXZ. (b) CD45.2<sup>+</sup> cells, neutrophils (Ly6G<sup>+</sup>CD11b<sup>+</sup>), and macrophages (CD64<sup>+</sup>CD11b<sup>+</sup>) abundance. (c) H&E sections of OXZ-treated ears of Gal-1<sup>+/+</sup> and Gal-1<sup>-/-</sup> mice (48 h) (left). Epidermal and/or dermal thickness values (right). (d) Gal-1 expression in CD8<sup>+</sup>, γδ<sup>+</sup>, CD4<sup>+</sup>, and CD4<sup>+</sup>Foxp3<sup>+</sup> T cells after 48 h of OXZ are shown. (e) Immunofluorescence of Gal-1 (green) and CD45 (red) in the ear sections. Nuclei were stained with DAPI (blue). Vessels (white asterisk) and zoom areas (right) (white box) are indicated. Bars = 100 μm and 50 μm. Data (mean ± SD) from one experiment of three are shown. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001; one-way ANOVA was used for **b**, **c**, and **d** or two-way ANOVA for **a** with the Bonferroni post hoc test. CHS, contact hypersensitivity; Ctrl, control; Gal-1, galectin-1; h, hour; ns, not significant; OXZ, oxazolone.



**Figure 2. Increased effector T-cells recruitment in the absence of Gal-1 expression.** (a) Gating strategy applied to skin CD3<sup>+</sup> live-gated cells. (b) The total cell numbers of Dγδ<sup>+</sup>, Eγδ<sup>+</sup>, and CD8<sup>+</sup> T cells detected in the skin of OXZ-treated or -untreated (Ctrl) Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice are shown in the graphs. (c) Density plots (left), frequencies, and total numbers of IL-17<sup>+</sup>γδ<sup>+</sup> T cells quantified in the ears. (d) The total cell numbers of CD4<sup>+</sup> T cells and CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in the skin of Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice are shown. Individual data (mean ± SD) from one representative experiment of three are shown. \**P* < 0.05, \*\*\**P* < 0.001; two-way ANOVA with Bonferroni post hoc test. Ctrl, control; d, day; Dγδ, dermal γδ; Eγδ, epidermal γδ; Gal-1, galectin-1; h, hour; ns, not significant; OXZ, oxazolone.



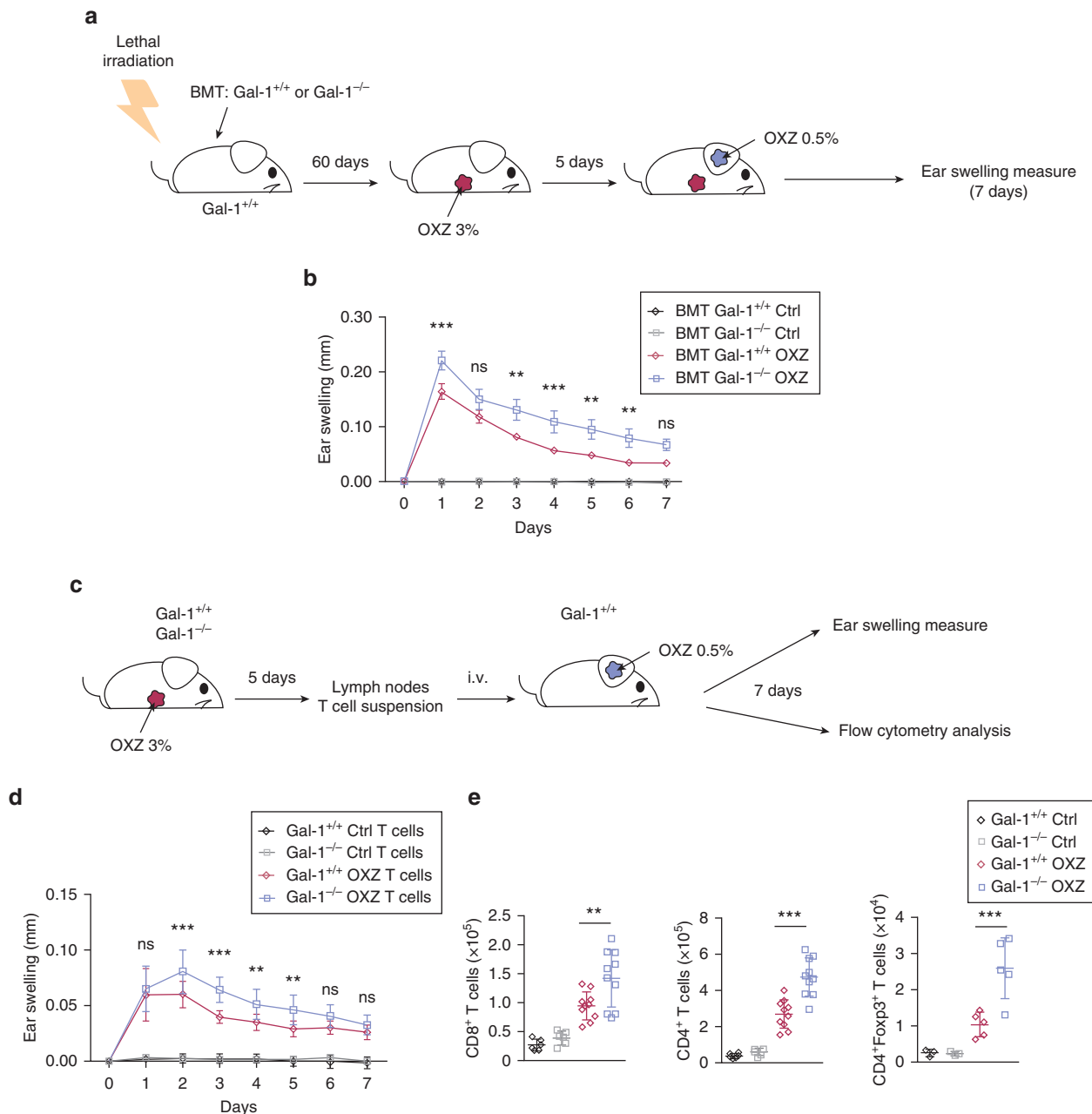


**Figure 3. Gal-1 expression does not determine differential migration of T cells to inflamed skin.** (a) Ear thickness of Gal-1<sup>+/+</sup> and Gal-1<sup>-/-</sup> mice treated with OXZ and anti-ICAM-1 antibody. (b) Rag1<sup>-/-</sup> Gal-1<sup>-/-</sup> and Rag1<sup>-/-</sup> Gal-1<sup>+/+</sup> mice were sensitized by applying 0.5% OXZ on the right ear (both sides) and were injected with Gal-1<sup>+/+</sup> T-cell suspension isolated from OXZ-treated mice. Representative plots of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells populations of (c) ears and (d) lymph nodes from Rag1<sup>-/-</sup> Gal-1<sup>-/-</sup> and Rag1<sup>-/-</sup> Gal-1<sup>+/+</sup>-recipient mice. Individual values of frequencies (left) and total cell numbers (right) are shown in the graphs. Individual data (mean  $\pm$  SD) from one representative experiment of three are shown. \* $P < 0.05$ , \*\*\* $P < 0.001$ ; one-way ANOVA was used for c and d and two-way ANOVA was used for a with Bonferroni post hoc test. Ctrl, control; Gal-1, galectin-1; i.p., intraperitoneal; i.v., intravenous; ns, not significant; ON, overnight; OXZ, oxazolone.

sustained inflammation observed in Gal-1 full-deficient mice (Figure 4b).

To directly assess whether endogenous Gal-1 expression in T cells mediates CHS, we transferred CD45.2<sup>+</sup> T lymphocytes of OXZ-treated Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice into CD45.1<sup>+</sup>Gal-

1<sup>+/+</sup>-recipient mice (Figure 4c). After the OXZ challenge, mice receiving Gal-1<sup>-/-</sup> T cells displayed more sustained inflammation than those transferred with Gal-1<sup>+/+</sup> T cells, indicating that endogenous expression of Gal-1 in T cells but not in the myeloid compartment is sufficient to control CHS (Figure 4d).



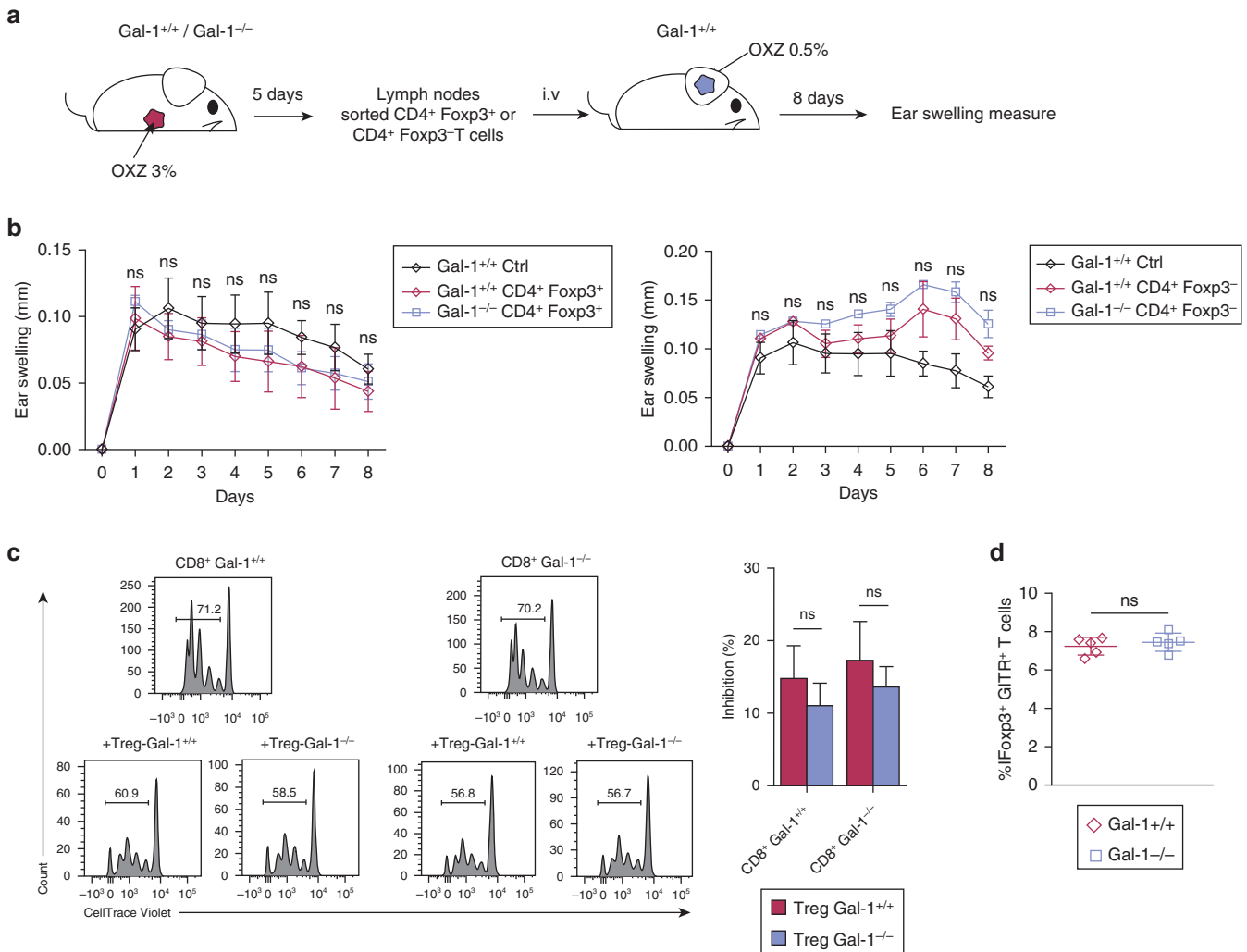
**Figure 4. Gal-1 expression plays a major role in T-lymphoid compartment in CHS.** (a) Wild-type-recipient CD45.1 mice were lethally irradiated and BMT with Gal-1<sup>+/+</sup> and Gal-1<sup>-/-</sup> cells. CHS response was assessed in chimeric mice after 60 days. (b) Increase of ear thickness at different time points. (c) Wild-type-recipient mice injected with T cells from OXZ-challenged Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice were treated with OXZ. (d) Ear thickness increase is represented. (e) The total numbers of CD8<sup>+</sup>, CD4<sup>+</sup>, and CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in lymph nodes are shown. Individual data (mean ± SD) from one experiment of three are shown. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001; one-way ANOVA was used for **e** and two-way ANOVA was used for **b** and **d** with Bonferroni post hoc test. BMT, bone marrow transplanted; CHS, contact hypersensitivity; Ctrl, control; Gal-1, galectin-1; i.v., intravenous; ns, not significant; OXZ, oxazolone.

Furthermore, flow cytometry analysis demonstrated higher numbers of CD45.2<sup>+</sup>Gal-1<sup>-/-</sup>CD4<sup>+</sup>, CD8<sup>+</sup>, and Tregs in lymph nodes (Figure 4e). These results confirm that the absence of endogenous Gal-1 increases the reactivation of T lymphocytes after the second challenge with OXZ, which promotes the development of the disease.

#### CD4<sup>+</sup>Foxp3<sup>+</sup> T cells are functional in the absence of Gal-1 expression

To address whether the expression of Gal-1 in CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs accounts for the phenotype observed in Gal-1<sup>-/-</sup>

mice, adoptive transfer of CD4<sup>+</sup>Foxp3<sup>+</sup> or CD4<sup>+</sup>Foxp3<sup>-</sup> T lymphocytes isolated from OXZ-treated Gal-1<sup>-/-</sup> and -Gal-1<sup>+/+</sup> mice into Gal-1<sup>+/+</sup>-recipient mice were conducted (Figure 5a). After the OXZ challenge, mice receiving CD4<sup>+</sup>Foxp3<sup>+</sup> T cells from both genotypes showed a similarly reduced inflammatory response, suggesting that the regulatory capacity of CD4<sup>+</sup>Foxp3<sup>+</sup> cells is not affected by the deletion of Gal-1 in CHS model. Besides, mice receiving effector CD4<sup>+</sup>Foxp3<sup>-</sup>Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> T cells displayed a similarly increased inflammatory response (Figure 5b).



**Figure 5. CD4<sup>+</sup> Foxp3<sup>+</sup> T cells are functional in the absence of Gal-1 expression.** (a) Wild-type-recipient mice transferred with sorted CD4<sup>+</sup>Foxp3<sup>+</sup> or CD4<sup>+</sup>Foxp3<sup>-</sup> T cells from OXZ-challenged Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice were treated with OXZ. (b) Ear thickness increase is shown. (c) Histograms (upper) of Gal-1<sup>+/+</sup> or Gal-1<sup>-/-</sup> CD8<sup>+</sup> T-cell proliferation alone or cocultured with Gal-1<sup>-/-</sup> or Gal-1<sup>+/+</sup> Treg are shown. The percentage of inhibition is shown in the graph (bottom). (d) The frequency of Foxp3<sup>+</sup>GITR<sup>+</sup>CD4<sup>+</sup> T lymphocytes in Gal-1<sup>+/+</sup> or Gal-1<sup>-/-</sup> mice after sensitization is represented in the graph. Individual data (mean  $\pm$  SD) from one representative experiment of three are shown. One-way ANOVA was used for **c** and **d** and two-way ANOVA was used for **b** with the Bonferroni post hoc test. Ctrl, control; Gal-1, galectin-1; GITR, glucocorticoid-induced TNF receptor; i.v., intravenous; ns, not significant; OXZ, oxazolone; Treg, regulatory T cell.

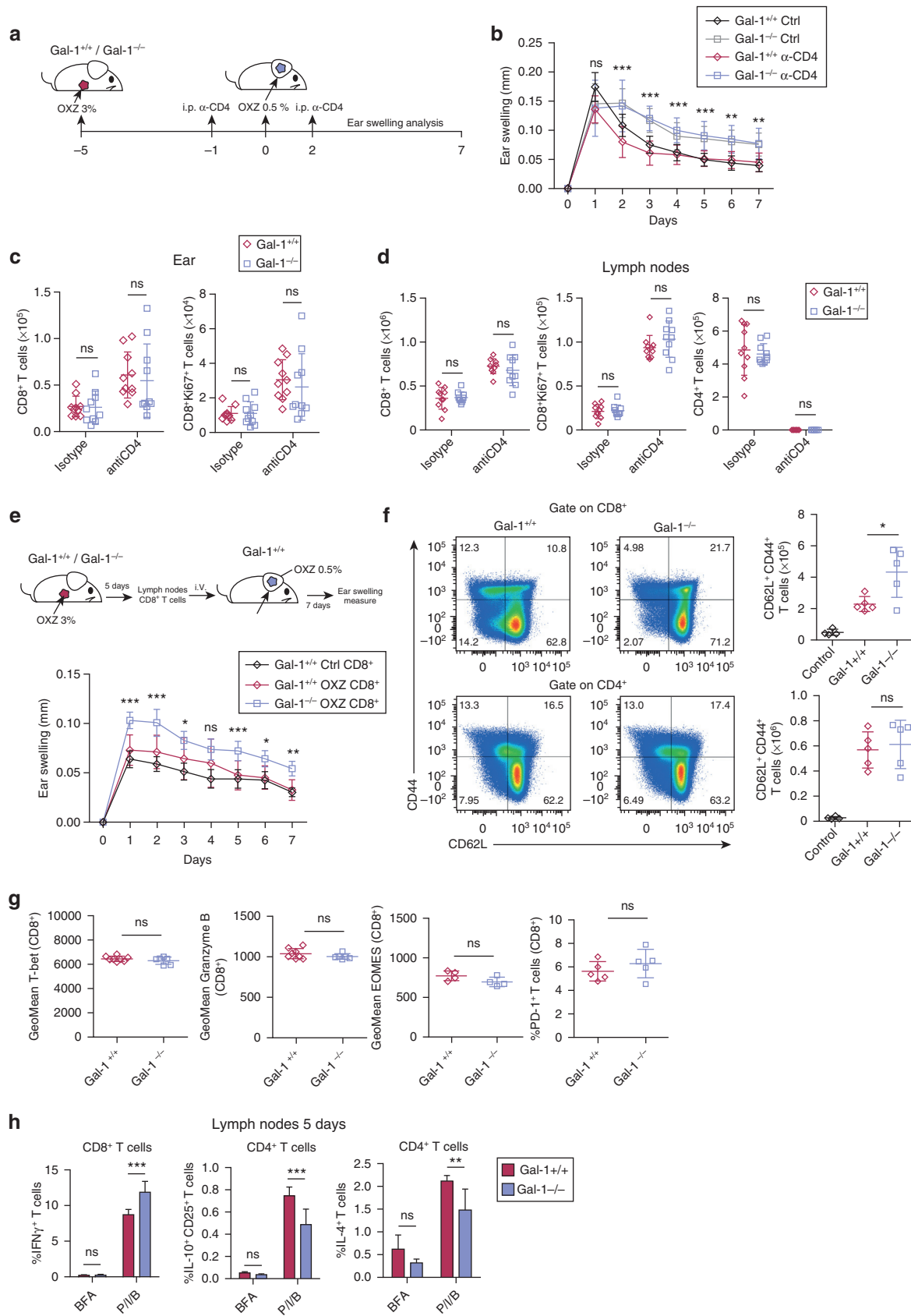
We evaluated the ability of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells derived from OXZ-treated Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice to control CD8<sup>+</sup> T-cell proliferation ex vivo. Inhibition of CD8<sup>+</sup> T-cell proliferation was similarly mediated by CD4<sup>+</sup>Foxp3<sup>+</sup> T cells from Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice (Figure 5c). In addition, the absence of Gal-1 did not seem to modify the expression of glucocorticoid-induced TNF receptor in CD4<sup>+</sup>Foxp3<sup>+</sup> T cells (Figure 5d). Overall, these results indicate that the deletion of Gal-1 in CD4<sup>+</sup> T cells, either effector or regulatory cell, does not affect the CHS response induced by OXZ.

#### Gal-1-mediated control of CHS response is restricted to CD8<sup>+</sup> T-cell compartment

To ascertain the relevance of Gal-1 in CD4<sup>+</sup> T cells versus that in CD8<sup>+</sup> T lymphocytes, in vivo CD4<sup>+</sup> T-cell depletion was performed in Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice (Figure 6a). The differential inflammatory responses detected between both genotypes were maintained in the absence of CD4<sup>+</sup> T cells (Figure 6b), indicating a major role for Gal-1 in CD8<sup>+</sup>

T-cell compartment. Besides, the total number of CD8<sup>+</sup> T cells and their proliferation response in skin tissue (Figure 6c) and lymph node (Figure 6d) was similar in Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice regarding the presence or absence of CD4<sup>+</sup> T cells.

The transfer of CD8<sup>+</sup> T cells derived from OXZ-treated Gal-1<sup>-/-</sup> mice was sufficient to recapitulate the phenotype of full Gal-1<sup>-/-</sup> mice (Figure 6e). Interestingly, an increased fraction of CD44<sup>+</sup>CD62L<sup>+</sup>CD8<sup>+</sup> T cells but not CD44<sup>+</sup>CD62L<sup>+</sup>CD4<sup>+</sup> T cells corresponding to the central memory compartment was detected on Gal-1<sup>-/-</sup> mice after the sensitization phase (Figure 6f). Although the expression of T-bet, Granzyme B, EOMES, and PD-1 (Figure 6g) is similar in activated CD8<sup>+</sup> T cells from Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice, a significant increase of IFN- $\gamma$  is detected in CD8<sup>+</sup> T cells deficient for Gal-1 (Figure 6h). Moreover, a reduced frequency of IL-10<sup>+</sup> and IL-4<sup>+</sup>CD4<sup>+</sup> T cells was detected in Gal-1-deficient mice compared with those in Gal-1<sup>+/+</sup> mice.



**Figure 6. The exacerbated inflammation induced by the absence of Gal-1 is mediated by increased CD8<sup>+</sup> T-cell response.** (a) In vivo anti-CD4 treatment. (b) Ear thickness, (c) CD8<sup>+</sup> and CD8<sup>+</sup>Ki67<sup>+</sup> T cells in the ears, and (d) lymph nodes were assessed. (e) Mice transferred with CD8<sup>+</sup> T cells from OXZ-challenged Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice received OXZ (upper). Ear swelling is shown (bottom). (f) The representative plots and total number of CD8<sup>+</sup>CD62L<sup>+</sup>CD44<sup>+</sup> and CD4<sup>+</sup>CD62L<sup>+</sup>CD44<sup>+</sup> T-cell populations. (g) T-bet, granzyme B, EOMES, and PD-1 expression in CD8<sup>+</sup> T cells in the lymph nodes. (h) The frequency of



These results indicate that Gal-1 deletion increases the development of central memory and IFN- $\gamma$ -secreting effector CD8<sup>+</sup>T cells, which induce an exacerbated CHS response.

## DISCUSSION

ACD is a common cutaneous pathology induced by an inflammatory reaction in the skin. Frequently, topical and systemic glucocorticosteroids and oral antihistamines are used as a pharmacologic treatment to control the disease (Kaplan et al., 2012). CHS model is a reliable model of ACD and allows to investigate the molecular and cellular mechanisms involved in this pathology. Understanding these mechanisms may lead to developing new treatments for ACD (Kaplan et al., 2012; Martin, 2013).

Pharmacologic interventions with recombinant Gal-1 protein plays an essential immunoregulatory role by inhibiting CD4<sup>+</sup> T-cell effector functions in different pathologies such as Crohn's disease (Santucci et al., 2003), multiple sclerosis (Toscano et al., 2007), or asthma (Lv et al., 2019). Previous studies have shown that the interaction between Gal-1 and CD69 modulates T helper type 17 effector cell differentiation and function (de la Fuente et al., 2014). Regarding inflammatory skin disease, the administration of recombinant Gal-1 has been proved to be effective in the control of inflammation (Cedeno-Laurent et al., 2010; Corrêa et al., 2017). However, it is unknown whether endogenous expression of Gal-1 directly influences T-cell subpopulations function and whether Gal-1 from the nonlymphoid compartment contributes to CHS. In this study, we directly demonstrate that the endogenous expression of Gal-1 in effector CD8<sup>+</sup> T cells but not in CD4<sup>+</sup> T cells plays an important role in the development of CHS model. The development of central memory CD8<sup>+</sup> T cells as well as the secretion of IFN- $\gamma$  by effector CD8<sup>+</sup> T cells is increased in the absence of Gal-1. In contrast, the regulatory capacity of Tregs does not appear to be affected in CHS by the genetic deletion of Gal-1.

The relevance of Gal-1 on cellular recruitment and adhesion has been characterized in polymorphonuclear cells in several in vitro and in vivo experimental models (Auvynet et al., 2013; Gil et al., 2010; La et al., 2003). However, whether Gal-1 expression directly affects the migration of activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells to lymph nodes or inflamed tissue has not been ruled out. Our observations indicate that Gal-1 expressed by endothelial cells does not participate in the modulation of T-cell migration because CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressing Gal-1 are similarly recruited to the CHS site in Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup>-recipient mice. The interaction of LFA-1 and ICAM-1 is important to regulate T-cell migration to inflamed tissues (Smith et al., 2007; Verma and Kelleher, 2017). ICAM-1 blockade dampens not only T-cell crawling, adhesion, and transmigration in vitro but also reduces T-cell migration from

inflamed ears to the drained lymph nodes in CHS model (Teijera et al., 2017). However, neutralization of ICAM-1 does not prevent the enhanced inflammation mediated by the absence of Gal-1.

Our data showed that Gal-1 function in T-lymphocyte-dependent inflammatory response after OXZ treatment is confined to the elicitation phase. In the development of CD, not only  $\alpha\beta$  T cells but also  $\gamma\delta$  T cells and NK T cells play relevant roles (Askenase, 2001). The role of  $\gamma\delta$  T cells in CHS is controversial because some studies demonstrate that TCR $\delta$ <sup>-/-</sup> mice develop more inflammation than wild-type mice (Guan et al., 2002), but other authors observed less ear swelling in the absence of  $\gamma\delta$  T cells (Jiang et al., 2017). IL-17-secreting  $\gamma\delta$  T cells are increased in Gal-1-deficient mice after the elicitation phase, indicating its potential role in the inflammatory response. However, dermal and epidermal  $\gamma\delta$  T cells are radioresistant populations in chimeric mice transplanted with bone marrow cells from Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice (Jiang et al., 2017), where the phenotype of full deficient mice is recapitulated. These data demonstrate that the expression of Gal-1 on  $\gamma\delta$  T cells does not exert a key role in the development of CHS model. Moreover, chimeric mice experiments using Gal-1<sup>+/+</sup>-recipient mice also ruled out the potential effect of circulating soluble Gal-1 protein in the mechanism of control of skin inflammation.

Memory CD8<sup>+</sup> T cells are antigen-experienced cells that show an improved response to the second challenge in comparison with naive cells. The relationship between central memory CD8<sup>+</sup> T cells and effector CD8<sup>+</sup> T cells detected in the skin during inflammation has been established in CHS model as well as in viral infection (Gaide et al., 2015; Mbitikon-Kobo et al., 2009; Osborn et al., 2019). Our data demonstrate that the expression of Gal-1 controls the generation of central memory CD8<sup>+</sup> T cells and the secretion of IFN- $\gamma$ , which account for the exacerbated skin inflammation. Hence, this work highlights the protective role of endogenous Gal-1 expression in CD8<sup>+</sup> T lymphocytes in the control of CHS-induced skin inflammation.

## MATERIALS AND METHODS

### Mice

Mice expressing IL-17-GFP and Foxp3-RFP proteins (kindly provided by Richard A. Flavell's Laboratory, Yale University, New Haven, CT) were backcrossed with Gal-1<sup>+/+</sup> or Gal-1<sup>-/-</sup> mice (C57BL/6 background) (Jackson Laboratory, Bar Harbor, ME). Rag1<sup>-/-</sup> (C57BL/6 background) (Jackson Laboratory) mice were backcrossed with Gal-1<sup>+/+</sup> and Gal-1<sup>-/-</sup> mice. CD45.1 C57BL/6 mice were purchased (Jackson Laboratory). Male and female, age-matched mice (aged 8–12 weeks) were used. All mice were kept in pathogen-free conditions at the animal facility of Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain. Experimental procedures were approved by the local Committee for Research Ethics and were in accordance with Spanish and European guidelines.

CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup>, CD4<sup>+</sup>IL-10<sup>+</sup>CD25<sup>+</sup>, and CD4<sup>+</sup>IL-4<sup>+</sup> T cells in the lymph nodes. Data (mean  $\pm$  SD) from one experiment out of three are shown. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; two-tailed unpaired Student *t*-test was used for **g**, one-way ANOVA was used for **c**, **d**, **f**, and **h**, and two-way ANOVA was used for **b** and **e** with the Bonferroni post hoc test. BFA, brefeldin A; Ctrl, control; Gal-1, galectin-1; i.p., intraperitoneal; i.v., intravenous; ns, not significant; OXZ, oxazolone; P/I/B, phorbol 12-myristate 13-acetate, ionomycin, and brefeldin A mix.

### CHS model

The shaved abdomen of mice was treated with 200 µl of OXZ (Sigma-Aldrich, St. Louis, MO) solution (3%) dissolved in ethanol on day 1. On day 5, a second challenge was induced using 20 µl OXZ (0.5%) on both sides of the ear. As a control, mice were painted with 20 µl vehicle alone (ethanol). Ear swelling, measured as an increase of ear thickness, was assessed daily.

To carry out *in vivo* ICAM-1 blockade experiments, mice were treated with 150 µg of an anti-CD54 antibody or the isotype control (Bio X Cell, Lebanon, NH) 3 days after elicitation challenge.

CD4<sup>+</sup> T cells were depleted *in vivo* using rat anti-mouse CD4 antibody (clone GK1.5; rlgG2b; Bio X cell) (1 mg per mouse, intraperitoneal) injected at 24 hours before and 48 hours after the second challenge. Control mice were treated with rlgG2b isotype control (Bio X cell).

### Adoptive transfer experiments

Gal-1<sup>+/+</sup> mice treated with OXZ (3%) were killed on day 5, and T-cell suspension was obtained from lymph nodes. Rag1<sup>-/-</sup>Gal-1<sup>-/-</sup> or Rag1<sup>-/-</sup>Gal-1<sup>+/+</sup> mice treated with OXZ in the ears were injected (intravenously) with the T-cell suspension (one donor to one recipient). Ears and lymph nodes were analyzed after 24 hours. Similarly, Gal-1<sup>+/+</sup> and Gal-1<sup>-/-</sup> mice were treated with OXZ, and T-cell suspension from lymph nodes was obtained and injected intravenously in CD45.1<sup>+</sup>Gal-1<sup>+/+</sup> mice. Control mice were treated with vehicle.

CD4<sup>+</sup>Foxp3<sup>+</sup> (2.5 × 10<sup>6</sup> cells per mouse) and CD4<sup>+</sup>Foxp3<sup>-</sup> (50 × 10<sup>6</sup> cells per mouse) T cells and CD8<sup>+</sup> cells (40 × 10<sup>6</sup> cells per mouse) were purified (Stemcell Technologies, Vancouver, Canada) and sorted for Treg separation (FACS Aria Cell Sorter, BD Biosciences, San Jose, CA). Purified cells from OXZ-treated Gal-1<sup>+/+</sup> and Gal-1<sup>-/-</sup> mice were injected (intravenously) to CD45.1<sup>+</sup>Gal-1<sup>+/+</sup> mice treated with OXZ in the ears. Control mice were injected with an equal number of T cells from nonsensitized mice.

### Chimeric mice

CD45.1 mice (Gal-1<sup>+/+</sup>) were lethally irradiated and transplanted with 5 × 10<sup>6</sup> Gal-1<sup>+/+</sup> or Gal-1<sup>-/-</sup> bone marrow cells. After reconstitution (60 days), mice were challenged with OXZ as described earlier.

### Flow cytometry

Tissues were dissected and grated through a nylon mesh (70 µm; BD Biosciences) to obtain single-cell suspensions. Ears were digested with the following mix: Liberase TM Research grade (Sigma-Aldrich) (0.08 mg/ml), collagenase IV (0.5 mg/ml), and DNase (Sigma-Aldrich) (100 µg/ml) in RPMI medium supplemented with fetal bovine serum (1%) for 35 minutes at 37 °C. The tissue was mechanically disrupted using 7 mm of stainless-steel beads (Life Technologies, Grand Island, NY) in a TissueLyser LT (Qiagen, Hilden, Germany) one 3-minute cycle (20 oscillations/s). Cell suspensions were incubated with anti-FcR2/3 (clone 2.4G2) before staining with specific antibodies (Supplementary Table S1). Stimulation of T cells were performed with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich) in the presence of brefeldin A (GolgiStop, 1 µg/ml; BD Biosciences), by at least 4 hours. Intracellular stainings were conducted with the Fixation/Permeabilization Solution Kit (BD Biosciences). Dead cell staining was done with Fixable Yellow Viability Dye (Molecular Probes, Eugene, OR). Absolute count of cells was conducted using BD TruCount Tubes (BD

Biosciences). Cell samples were acquired in a FACSCanto and LSRFortessa Flow Cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Woodburn, OR).

### Skin histology and immunofluorescence staining

Samples were fixed in formaldehyde and embedded in paraffin. Slices were stained with H&E and digitalized. Epidermis and dermis thickness were measured every 100 µm using NDP Viewer software (Hamamatsu, Japan).

Deparaffinized sections were boiled in Tris-EDTA buffer: 10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0. After blocking (PBS-5%-chicken serum), sections were incubated with primary antibodies (Supplementary Table S1) followed by secondary antibodies: chicken anti-rabbit AlexaFluor488 and chicken anti-goat AlexaFluor647. Nuclei were counterstained with DAPI. Images were captured in a Zeiss LSM 700 Confocal microscope and analyzed with LSM image browser software (Zeiss, Jena, Germany).

### In vitro assay of Treg function

Mouse cells were cultured in RPMI medium supplemented with fetal bovine serum (5%), 25 mmol/l of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, antibiotics, sodium pyruvate, and β-mercaptoethanol. Single-cell suspensions of lymph nodes from OXZ-treated Gal-1<sup>+/+</sup> and Gal-1<sup>-/-</sup> mice were obtained. Tregs were isolated and purified as CD25<sup>+</sup> T cells using EasySep Release Mouse Biotin Positive Selection Kit (Stemcell Technologies). From the negative fraction (CD25<sup>-</sup> T cells), the CD8<sup>+</sup> T cells were purified (Stemcell Technologies) and labeled using CellTrace Violet Cell Proliferation Kit (Thermo Fisher Scientific, Waltham, MA). Cells were seeded (10 Treg:1 CD8) in plate coated with anti-CD3 (2 µg/ml) and anti-CD28 (0.5 µg/ml) (Tonbo Biosciences, San Diego, CA). CD8<sup>+</sup> T-cells proliferation was assessed at 60 hours.

### Statistical analysis

Statistical significance was assessed by two-tailed unpaired Student *t*-test, one-way ANOVA, or two-way ANOVA with Bonferroni multiple comparisons post hoc test, as required. All analyses were performed with GraphPad software (San Diego, CA).

### Data availability statement

No datasets were generated during this study.

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### CONFLICT OF INTEREST

The authors state no conflict of interest.

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## AUTHOR CONTRIBUTIONS

Conceptualization: FSM, MF; Formal Analysis: RCG, DC, NFG, MRH, MLS; Funding Acquisition: FSM; Investigation: RCG, DC, NFG, MRH, MLS; Methodology: RCG, DC; Resources: HDLF, MNN, MF; Visualization: RCG, DC; Writing - Original Draft Preparation: RCG, DC, FSM; Writing - Review and Editing: RCG, DC, FSM, HDLF, MNN, MF

## SUPPLEMENTARY MATERIAL

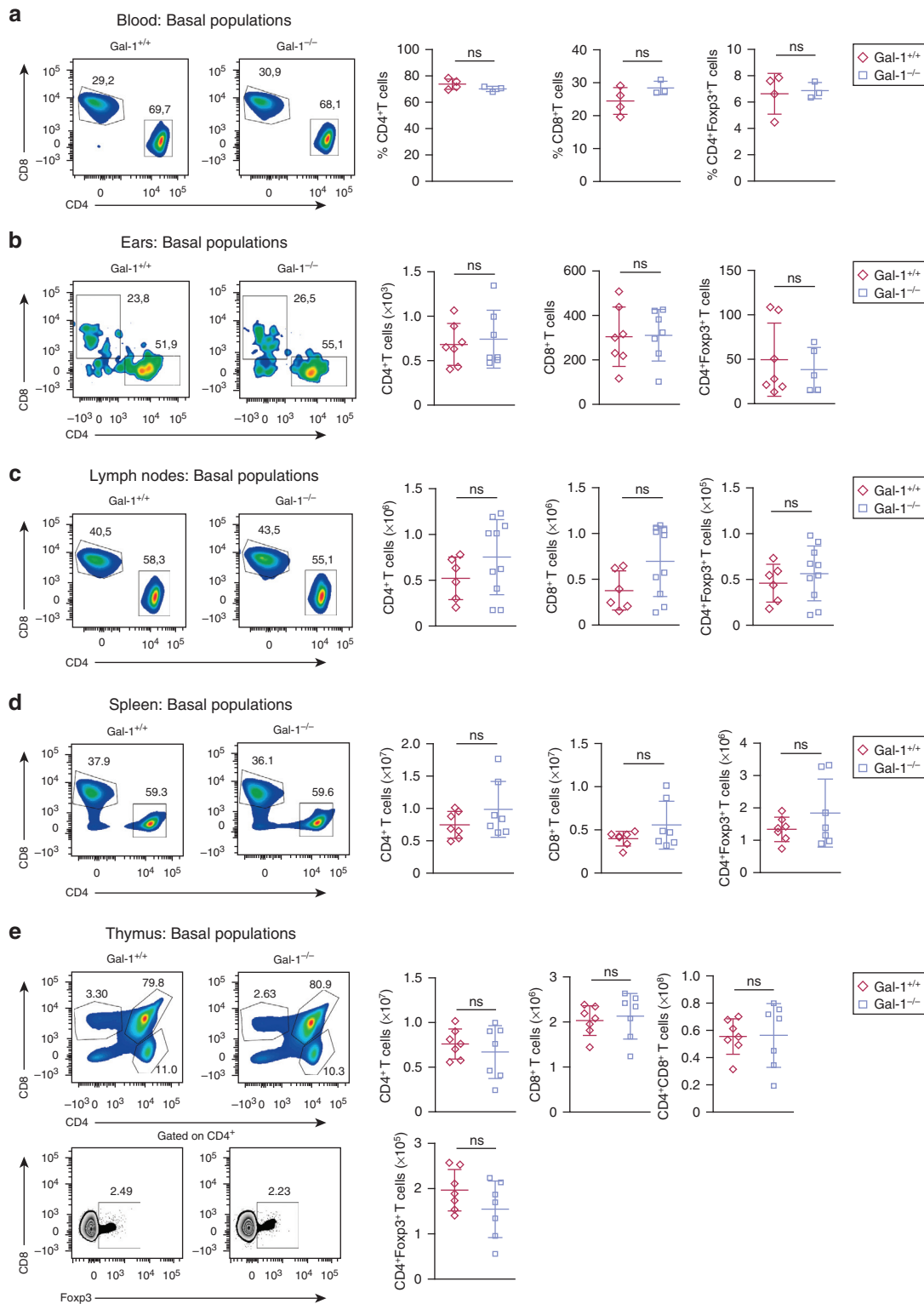
Supplementary material is linked to the online version of the paper at [www.jidonline.org](http://www.jidonline.org), and at <https://doi.org/10.1016/j.jid.2020.10.020>.

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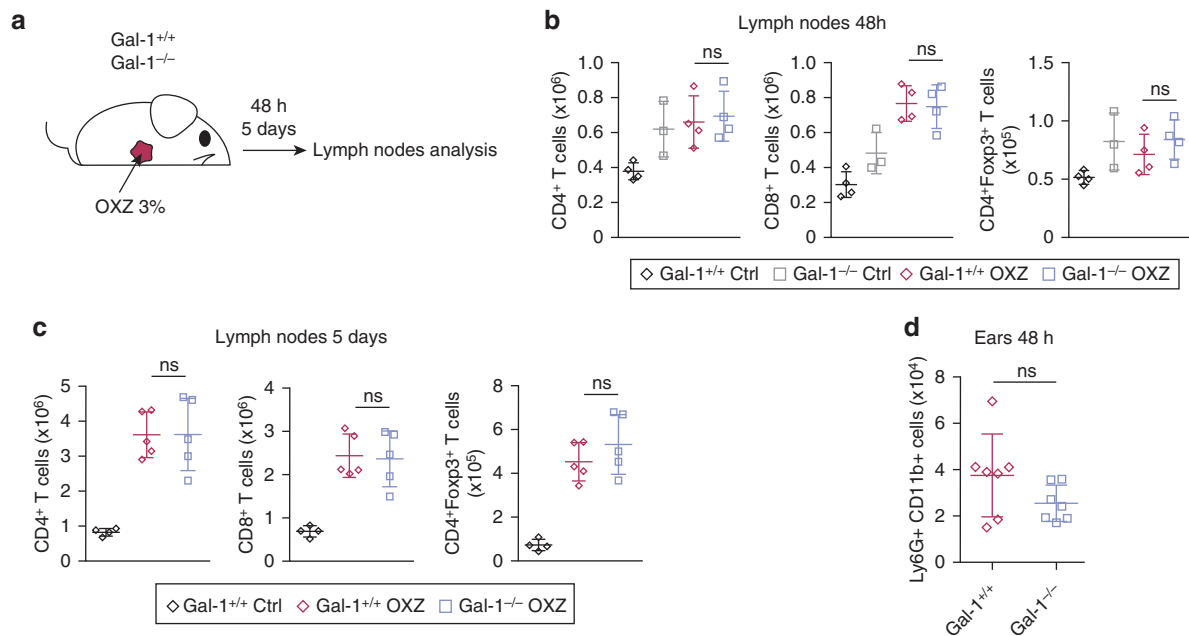


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**Supplementary Figure S1. Gal-1 genetic deletion does not alter the populations of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in homeostasis.** (a) Density plots (left) and frequency (right) of CD4<sup>+</sup>, CD8<sup>+</sup>, and CD4<sup>+</sup>Foxp3<sup>+</sup> cells from TCR $\alpha\beta$ <sup>+</sup> gated cells in the blood. The density plots (left) and total numbers of CD4<sup>+</sup>, CD8<sup>+</sup>, and CD4<sup>+</sup>Foxp3<sup>+</sup> (right) from TCR $\alpha\beta$ <sup>+</sup> cells in the (b) ears, (c) lymph nodes, and (d) spleen in homeostasis in Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice. (e) The density plots (left) and total cell numbers of the populations described earlier and the CD4<sup>+</sup>CD8<sup>+</sup> cells (right) from CD45<sup>+</sup> cells in the thymus are shown in the graphs. Data from one representative experiment of three are shown (mean  $\pm$  SD). Unpaired *t*-test was used for analysis. Gal-1, galectin-1; ns, not significant.





**Supplementary Figure S2. Gal-1 does not regulate the sensitization phase of CHS.** (a) Gal-1<sup>-/-</sup> and Gal-1<sup>+/+</sup> mice were treated with OXZ in the abdomen and killed after 48 h and 5 days. Flow cytometry analysis of the total number of CD4<sup>+</sup>, CD8<sup>+</sup>, and CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in lymph nodes at (b) 48 h and (c) 5 days after OXZ treatment. (d) The total numbers of neutrophils (CD11b<sup>+</sup>Ly6G<sup>+</sup>) detected in the skin at 48 h after the sensitization phase are shown. Individual data (mean ± SD) from one representative experiment of three are shown. One-way ANOVA with Bonferroni post hoc test was used for b–c; unpaired *t*-test was used for the analysis in d. CHS, contact hypersensitivity; Ctrl, control; Gal-1, galectin-1; h, hour; ns, not significant; OXZ, oxazolone.



**Supplementary Table S1. List of Used Antibodies**

Specificity	Reactivity	Clone	Fluorochrome	Origin	Dilution	Catalog No
CD3e	Mouse	145-2C11	APC/BV421/purified	BD Biosciences, San Jose, CA/Tonbo Biosciences, San Diego, CA	1:200/1:250	553066/561416/70-0031
CD4	Mouse	RM4-5	APC/BV421/biotin	Tonbo Biosciences, San Diego, CA/BD Biosciences, San Jose, CA	1:200	20-0042/740007/553045
CD8	Mouse	53-6.7	APC Fire750/Pe-Cy7	BioLegend, San Diego, CA/Tonbo Biosciences, San Diego, CA	1:200	100766/60-0081
CD25	Mouse	PC61.5	Biotin	Tonbo Biosciences, San Diego, CA	1:200	30-0251
CD28	Mouse	37.51	Purified	Tonbo Biosciences, San Diego, CA	1/1,000	70-0281
CD45	Mouse	Polyclonal Goat IgG	Purified	Research and Diagnostic Systems, Minneapolis, MN	1:40	AF114-SP
CD45.2	Mouse	104	Pe-Cy7	eBiocience, San Diego, CA	1:200	560696
CD11b	Mouse	M1/70	FITC/biotin	BD Biosciences, San Jose, CA	1:200	553310/553309
CD11c	Mouse	HL3	PE/biotin	BD Biosciences, San Jose, CA	1:200	557401/553800
CD64	Mouse	X54-5/7.1	APC	BioLegend, San Diego, CA	1:200	139311
EOMES	Mouse	Dan11mag	PE	eBiocience, San Diego, CA	1:200	12-4875-80
FcRII/III (CD16/CD32)	-	2.4G2	Purified	Tonbo Biosciences, San Diego, CA	1:100	70-0161
Gal-1	Mouse	D608T/Polyclonal Rabbit IgG	Biotinylated/purified, IHC formulated	Research and Diagnostic Systems, Minneapolis, MN/Cell Signaling, Danvers, MA	1:100	BAF1245/13888
GITR	Mouse	DTA-1	Pe-Cy7	BioLegend, San Diego, CA	1:200	126317
Granzyme B	Mouse	NGZB	Pe-Cy7	eBiocience, San Diego, CA	1:200	25-8898-80
IFN- $\gamma$	Mouse	XMG1.2	FITC/APC	eBiocience, San Diego, CA	1:200	11-7311-82/17-7311-82
IL-4	Mouse	11B11	PE	BioLegend, San Diego, CA	1:200	504104
IL-10	Mouse	JES5-16E3	BV421	BioLegend, San Diego, CA	1:200	505022
Ly6G	Mouse	1A8	PE	BD Biosciences, San Jose, CA	1:200	551461
PD-1	Mouse	29F.1A12	BV421	BioLegend, San Diego, CA	1:200	135217
$\gamma\delta$ TCR	Mouse	GL3	PerCP/Cy5.5/biotin	eBiocience, San Diego, CA	1:200	560696/13-5711-82
TCR $\alpha\beta$	Mouse	H57-597	APC-Cy7	BioLegend, San Diego, CA	1:200	109220
T-bet	Mouse	4B10	PerCP/Cy5.5	eBiocience, San Diego, CA	1:200	45-5825-82

Abbreviations: APC, allophycocyanin; BV, brilliant violet; Cy, cyanine dye; FITC, fluorescein isothiocyanate; Gal-1, galectin-1; IHC, immunohistochemically; No, number; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.