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Expression of the Tim3-galectin-9 axis is altered in drug-induced maculopapular exanthema

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Abstract

Background: Drug-induced maculopapular exanthemas (MPEs) are mediated by Th1 CD4⁺ T cells. One of the mechanisms of control of Th1 cells in homeostasis is the interaction between the checkpoint inhibitor Tim3 and its physiological ligand galectin-9 (Gal9). Disorders affecting this axis may be responsible for various autoimmune and immunological diseases. The aim of this study was to determinate the influence of the Tim3-Gal9 axis on the development of MPE induced by drugs.

Methods: Frequencies of different cell subsets and the expression of Tim3 and Gal9 were measured in peripheral blood by flow cytometry and in skin biopsies by immunohistochemistry. Gal9 expression was assessed by RT-qPCR; its release was measured by multiplex assay. The effects of blocking or enhancing the Tim3-Gal9 axis on monocyte-derived dendritic cell (moDC) maturation and T-cell proliferation were determined by flow cytometry.

Results: The expression of Tim3 was significantly reduced in peripheral blood Th1 cells and in the skin of MPE patients vs controls. Gal9 expression and release were significantly reduced in patient peripheral blood and moDCs, respectively. The addition of exogenous Gal9 significantly reduced Tim3⁺ Th1 proliferation, although Treg proliferation increased.

Conclusion: This study showed the involvement of the Tim3-Gal9 axis in MPE. The reduced expression of Tim3 in Th1 cells together with the impaired expression of Gal9 in PBMCs and DCs appears to have a role in the development of the disease. The

Abbreviations: DCs, Dendritic cell; DPT, Drug provocation test; Gal9, Galectin-9; moDCs, Monocyte-derived dendritic cells; MPE, Maculopapular exanthema; NIRs, Nonimmediate reactions; PBMCs, Peripheral blood mononuclear cells; Tim3, T-cell Ig- and mucin domain-containing molecule; Tregs, T regulatory cells.

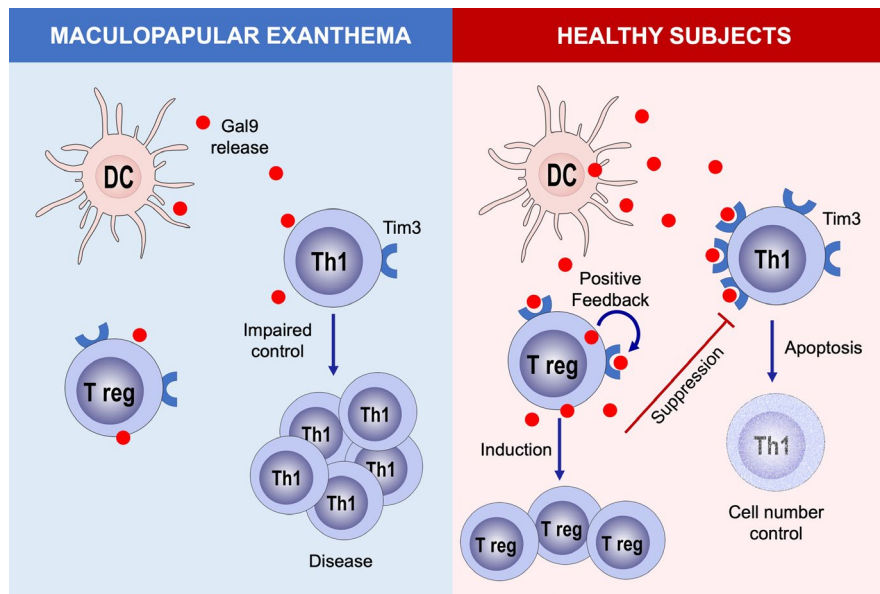
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potential of Gal9 to suppress Th1 and enhance Treg proliferation makes it a promising tool for treating these reactions.

KEYWORDS

checkpoint inhibitor, drug allergy, Galectin-9, maculopapular exanthema, Tim3



GRAPHICAL ABSTRACT

The reduced level of the checkpoint molecule Tim3 in Th1 cells together with the impaired expression of Gal9 in PBMCs and dendritic cells hampers the control of Th1 cell numbers in patients with drug-induced maculopapular exanthema (MPE). The reduced expression of Gal9 in patients with drug-induced MPE could be responsible for the low frequencies of Treg cells in these patients. Exogenous administration of Gal9 could be a potential therapeutic tool able to suppress Th1 and enhance Treg differentiation in patients with drug-induced MPE.

1 | INTRODUCTION

Allergic reactions to drugs can be clinically classified as immediate, occurring within one to six hours after drug intake and mediated by IgE, or nonimmediate reaction (NIR), occurring later than one hour after the administration of the drug, mainly mediated by T lymphocytes.¹ The organ most frequently affected in NIR is the skin, with clinical manifestation ranging from mild, such as maculopapular exanthema (MPE), to more severe reactions, such as Stevens-Johnson syndrome or toxic epidermal necrolysis.² Fortunately, most frequent NIR manifestations are mild reactions,³ which are characterized mechanistically by a predominance of IFN- γ secreting CD4⁺ T with a Th1 pattern (IFN- γ , CXCR3)⁴ and the expression of molecules that facilitate its recruitment to the skin (CLA, CCR4, CCR6 and CCR10).⁵

Other cell subpopulations, such as the Th17 CD4⁺ T cells which produce IL-17 and/or IL-22, can be found in the skin,⁶ and, together with Th1 cells, they have an important role in the development of some cutaneous diseases,⁷ such as psoriasis⁸ and contact dermatitis,⁹ in which an increased expression of IL-17 in skin lesions has been observed. The production of IL-17 in psoriasis, in synergy with IFN- γ , can induce the expression of ICAM-1 by keratinocytes, increasing

T-cell adhesion and favouring Fas-FasL-mediated T-cell cytotoxicity and keratinocyte death.¹⁰ Moreover, IFN- γ can also induce the secretion of IL-1 and IL-23 by antigen-presenting cells, driving polarization towards a Th17 phenotype and enhancing the inflammatory reaction.^{11,12} These pathologies are driven by an uncontrolled number of Th1 and/or Th17 cells, which suggests the impaired function of the mechanisms that control these cell numbers in homeostasis.

The T-cell Ig- and mucin domain-containing molecule (Tim3) is a type I membrane glycoprotein expressed on Th1 cells, but not present in Th2 cells. Moreover, it is also expressed constitutively at high levels on dendritic cells (DCs) and at lower levels on monocytes.^{13,14} This molecule has been identified as an immune checkpoint inhibitor that can regulate the Th1 immune response, inducing the apoptosis of this cellular subtype.¹⁵⁻¹⁷ Tim3 is also present in Th17 cells,^{18,19} where it activates negative regulatory signals inhibiting the production of proinflammatory cytokines and cellular adhesion molecules.²⁰ Moreover, the blocking of Tim3 has been shown to increase IFN- γ and IL-17 production by CD4⁺ T cells.¹⁹ One of the known ligands of Tim3 is galectin-9 (Gal9), a soluble lectin that binds β -galactosides. This molecule has been described as a multifunctional regulator of the immune response

and homeostasis.²¹ Its interaction with Tim3 induces Th1 and Th17 apoptosis and the suppression of its differentiation from Th0.²⁰ Interestingly, IFN- γ can induce the expression of Gal9 by DCs, inducing a negative feedback that regulates the numbers of Th1 and Th17 cells.²²

Thus, the Tim3-Gal9 axis is involved in the control of immune responses produced by Th1 and Th17 CD4⁺ T cells and a dysfunction in this axis can alter immunological homeostasis, inducing a pathological state as it has been described for several autoimmune diseases such as psoriasis²³ or multiple sclerosis.²⁴ In the skin, keratinocytes and fibroblasts are sources of Gal9, and an increased expression of this molecule by fibroblasts has been found in skin lesions of patients with psoriasis compared to nonaffected skin from the same patients.²⁵ However, this increased expression of Gal9 does not seem to be enough to control the disease may be due to the fact that the expression of Tim3 on Th1 and Th17 CD4⁺ T cells is reduced.^{23,24} T regulatory cells (Tregs) are another source of Gal9; in fact, a positive feedback has been described, through which Gal9 can induce the differentiation of Tregs and stimulate FoxP3 expression in experimental models of autoimmune arthritis.²⁰ The Tim3-Gal9 interaction is also important for regulatory mechanisms, and the blocking of this axis has been shown to inhibit the suppressor activity of Tregs *in vitro* and in an allogeneic skin graft model *in vivo*.²⁶

In this work, as we are dealing with Th1-driven diseases, we investigate the importance of the Tim3-Gal9 axis as checkpoint mechanism to control mild NIR such as MPE. Moreover, we investigate whether Th17 CD4⁺ T cells have a role in the development of these diseases, as it occurs in psoriasis, contact dermatitis and other autoimmune diseases.

2 | METHODS

This study included individuals who had experienced drug-induced MPE and attended the Allergy Service at the Hospital Regional Universitario de Malaga (HRUM) during a 1-year period (2014-2015), and who were finally confirmed as allergic.²⁷ As controls, we selected 10 sex- and age-matched subjects with confirmed tolerance to the drugs involved in the reactions. The study was conducted in the Allergy Service and Research Laboratory at the HRUM, according to the Declaration of Helsinki, and was approved by the ethics committee of the hospital. All participants signed informed consent forms before the start of the study.

Blood samples and skin biopsies were obtained from each patient within the first 24 h after the initiation of the reaction (acute phase), and, once the patients were confirmed as allergic, another blood sample was obtained at least 60 days after the resolution of symptoms (resolution phase). Peripheral blood mononuclear cells (PBMCs) were isolated using conventional methods. Control skin samples were obtained from the Biobank at the HRUM.

The frequencies of different T-cell subpopulations and the expression of Tim3 were assessed using flow cytometry²⁸ (Figure S1). Immunohistochemical staining was performed in skin biopsies as described.⁵ Gal9 gene expression analysis was performed by RT-qPCR; Gal9 levels were measured in sera and culture supernatants by Luminex Bead-Based Assay (R&D Systems Inc, Minneapolis, MN, USA). Monocyte-derived DC (moDC) maturation and T-cell proliferation assays were performed and analysed by flow cytometry.

Comparisons of quantitative variables without a normal distribution were made using the Mann-Whitney *U* test, the Kruskal-Wallis test and the Wilcoxon test for matched paired samples. All reported

TABLE 1 Clinical characteristics and results of the allergological workup of the patients included in the study

Patients	Sex	Age	Drug	Skin test	DPT
1	Female	31	AX	IDT -	+
2	Female	68	Paracetamol	IDT -	+
3	Female	26	AX	IDT -	+
4	Male	52	Sulphametoxazole	IDT -	+
5	Female	70	Iodixanol	IDT +	+ (Iomeprol)
6	Male	69	Iodixanol	IDT -	+
7	Female	21	AX-clavulanic	IDT -	+
8	Female	51	Metamizole	IDT +	nd
9	Male	77	Iodixanol	IDT -	+
10	Female	63	Enoxaparin	IDT +	nd
11	Female	69	AX	IDT -	+
12	Male	53	Allopurinol	PT -	+
13	Female	32	Sulphametoxazole	PT +	nd
14	Female	26	AX	IDT -	+
15	Female	28	AX-Clavulanic	IDT + (CLV)	- (AX)
16	Female	16	AX	IDT +	- (BP)
17	Female	54	Iodixanol	IDT -	+
18	Female	80	Iodixanol	IDT -	+

Abbreviations: AX, amoxicillin; BP, benzylpenicillin, DPT, drug provocation test; IDT, intradermal test; nd, not done; PT, patch test.

P-values represent two-tailed tests, with values <0.05 considered statistically significant.

Full details of the methods, including manufacture details and primer sequences, are given in Appendix S1.

3 | RESULTS

The study included 18 patients, 14 females and 4 males (mean age: 49.2 years). All the patients developed a confirmed MPE with erythematous macules, often symmetric and typically polymorphous. The drugs involved in the reactions were beta-lactams in 7 cases, iodine contrast media in 5, sulphonamides in 2, nonsteroidal anti-inflammatory drugs in 2, heparins in 1 and allopurinol in 1. The diagnosis was confirmed for 6 cases by skin tests and for 12 by drug provocation tests (DPTs). In all cases, a viral infection was ruled out by viral serology. Table 1 shows the clinical characteristics and the results of the allergological workup for patients included in the study.

3.1 | Th1 (and not Th17) cells are involved in MPE, and these patients present decreased expression of Tim3

The phenotypical analysis of Th1 and Th17 cells in PBMCs from patients showed an increase in the percentage of Th1 cells ($CD4^+CXCR3^+$) for both acute and resolution phases of the reaction, compared to controls ($P = 0.033$ and $P < 0.001$, respectively). Moreover, in patients the percentage of Th1 cells decreased during the resolution phase ($P = 0.001$), although without reaching the levels observed in controls (Figures 1A and S2A). However, there was a decrease in the percentage of those Th1 cells expressing Tim3 receptor in patients compared to controls during both acute and resolution phases ($P < 0.001$ and $P = 0.013$, respectively). This decrease was more pronounced during the acute phase, with levels even lower than those observed in the resolution phase ($P = 0.021$; Figures 1B and S2B). Regarding Th17 cells ($CD4^+ROR\gamma^+IL-17^+$), we did not find any significant difference between patients and controls, or between the acute and resolution phases, in terms of their frequency (Figures 1C and S2C) and in terms of the surface expression of Tim3 (Figures 1D and S2D).

These results in PBMCs were confirmed in skin biopsies obtained from patients and controls. We observed a higher number of Th1 cells ($CXCR3^+$) in skin lesions (Figure 1E), whereas those that express Tim3 were fewer in patients compared to controls ($P < 0.001$ for both; Figure 1F). Again, there were no differences in the number of cells expressing IL-17 (Th17 cells) between patients and controls in the skin (Figure 1G).

3.2 | Patients with MPE show reduced expression of Gal9 in PBMCs during the acute phase of the reaction

We found lower Gal9 mRNA expression in PBMCs from patients during the acute phase of the reaction compared to controls ($P = 0.003$). We also observed significant differences in patients between the acute phase and the resolution phase ($P = 0.005$) with higher levels in the later (Figure 2A).

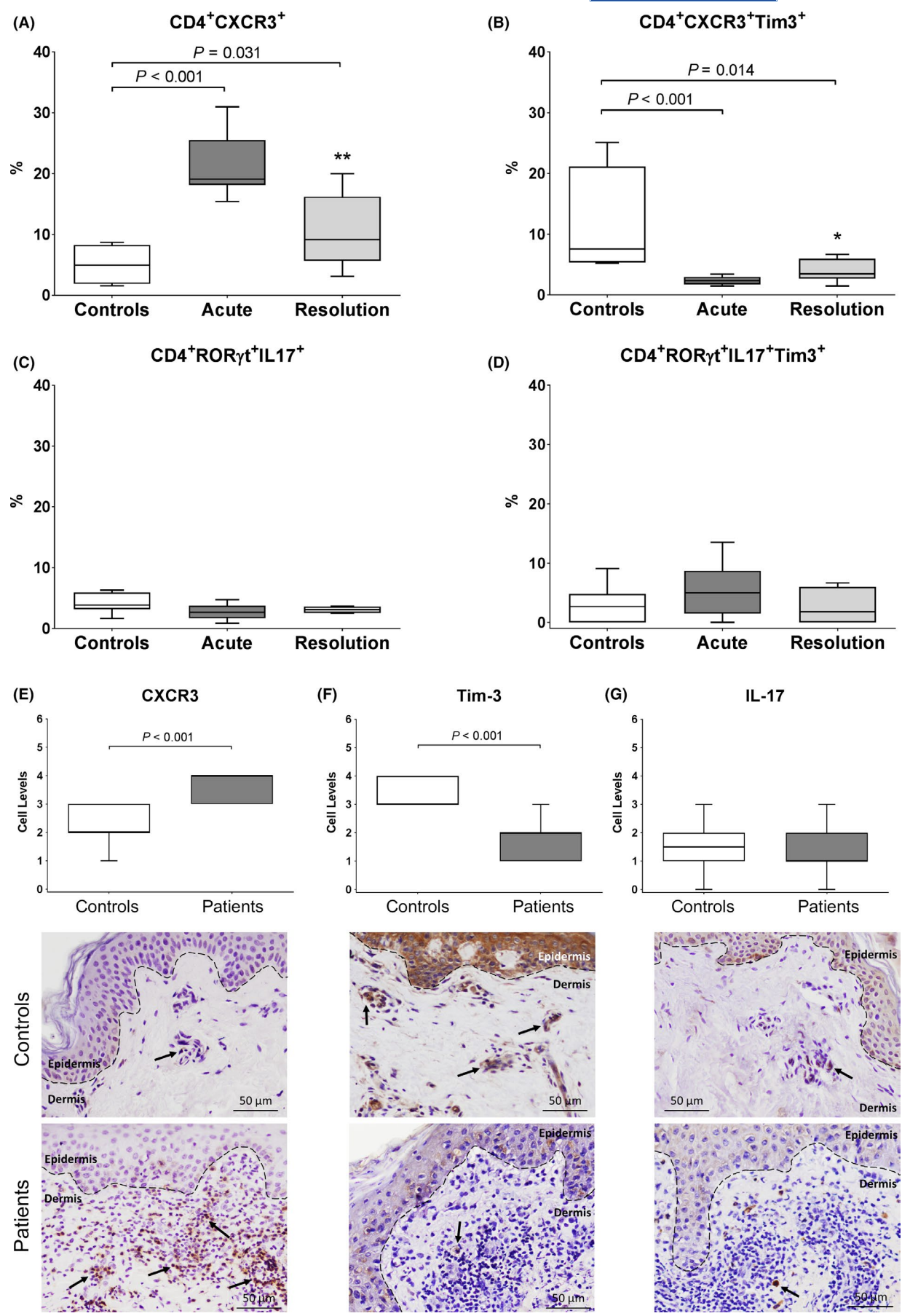
These results contrast with those related to the number of cells producing Gal9 and its secretion. We did not find any significant differences in the levels of Gal9 measured in sera obtained from patients and controls, neither in the acute phase nor in the resolution phase (Figure 2C). The same pattern was found in the skin, where we observed similar levels in skin biopsies obtained from controls and from lesions of the affected skin in patients. However, when we analysed the expression of Gal9 in different skin layers we detected higher levels in keratinocytes for controls, whereas expression in the dermal cell infiltrate was higher in patients (Figure 2B).

3.3 | DCs from patients with MPE show reduced expression of Tim3 and Gal9

Analysis of Tim3 expression on DCs from peripheral blood showed reduced levels of this receptor in the resolution phase compared to controls ($P < 0.001$). However, during the acute phase the expression of Tim3 increased significantly compared to the resolution phase ($P < 0.001$), showing similar levels to controls (Figures 3A and S3). We assessed changes in the release of Gal9 in moDCs incubated with the responsible drug for the reaction, using cells taken from controls and patients during the resolution phase. Data showed a lower release of Gal9 for unstimulated moDCs from patients than for moDCs from controls ($P = 0.002$). However, the drug-stimulated moDCs showed, only for patient cells, increased secretion of Gal9 ($P = 0.002$) although levels were still lower than those observed for controls ($P = 0.034$) (Figure 3B).

In addition, we investigated the effect of blocking the Tim3 receptor on drug-induced moDC maturation. After stimulation with the drug, only the moDCs from patients showed positive maturation ($MI > 2$), with an increased expression of all the assessed markers: CCR7, CD80, CD83 and CD86. The maturation index was always significantly higher than was observed in controls ($P = 0.026$ for CCR7; $P = 0.027$ for CD80; $P = 0.028$ for CD83; and $P = 0.028$ for CD86). Furthermore, the maturation process was reduced by the addition of Tim3 blocking antibodies to the culture, which resulted in a significant decrease for all markers ($P = 0.001$ for CCR7; $P = 0.008$ for CD80; $P = 0.002$ for CD83; and $P = 0.007$ for CD86) only in patients (Figure 3C).

FIGURE 1 Th1, Th17 and Tim3 frequencies in total CD3 lymphocytes in: peripheral blood A, Th1 cells ($CD4^+CXCR3^+$); B, Tim3 in Th1 cells; C, Th17 cells ($CD4^+ROR\gamma^+IL-17^+$); and D, Tim3 in Th17 cells, in patients, during acute and resolution phase, and in controls; and in E, $CXCR3^+$ cells; F, $Tim3^+$ cells; and G, $IL-17^+$ cells in skin lesions from patients and healthy skin from controls (100x magnification, positive cells are labelled in brown and some pointed with arrows). Boxes represent medians $\pm$ interquartile range. Comparisons between controls and patients have been performed using Kruskal-Wallis (for the three groups) and Mann-Whitney *U* tests. Comparisons between acute and resolution phase have been performed using Wilcoxon tests (* $P < 0.05$; ** $P < 0.01$)



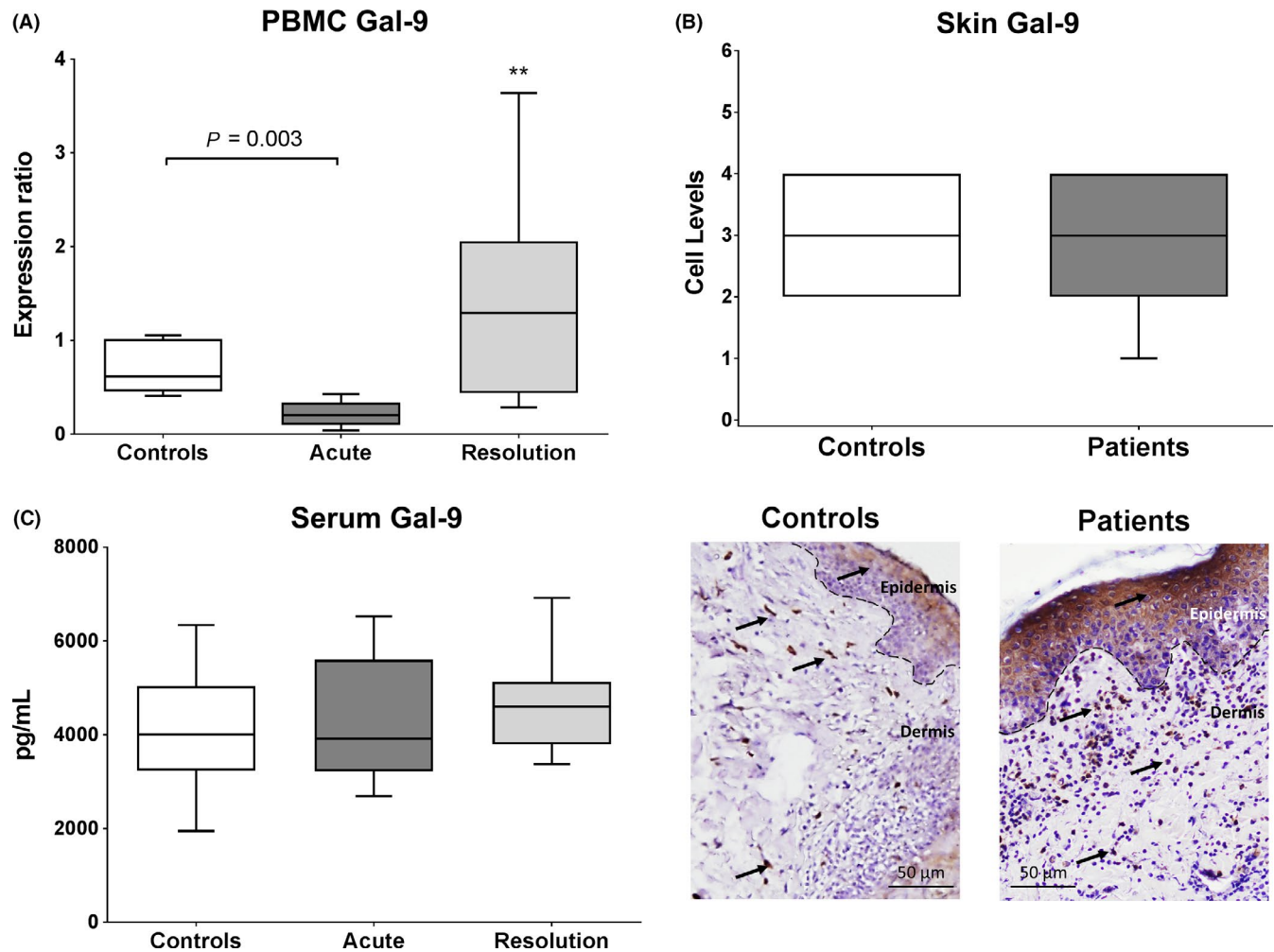


FIGURE 2 Gal 9 expression: A, mRNA expression in PBMCs from patients, during acute and resolution phase, and controls; B, frequencies of Gal9⁺ cells in the skin of patients during the acute phase and controls (100x magnification); C, levels of Gal9 in the sera of patients, during acute and resolution phase, and controls. Comparisons between controls and patients have been performed using Kruskal-Wallis (for the three groups) and Mann-Whitney *U* tests. Comparisons between acute and resolution phase have been performed using Wilcoxon tests (**P* < 0.05; ***P* < 0.01)

3.4 | There is a reduced frequency of IL-10- and Gal9-producing Tregs in patients with MPE

Although we did not find differences in the percentage of Tregs (CD4⁺CD25^{high}FoxP3⁺) between patients and controls, both during the acute and resolution phases (Figures 4A and S4A), the frequency of IL-10-producing Tregs was significantly lower in patients compared to controls in both acute and resolution phases (*P* = 0.024 and *P* = 0.014, respectively; Figures 4B and S4B). Moreover, the frequency of Tregs that produce Gal9 was also reduced in patients compared to controls, being significant at the resolution phase (*P* = 0.010; Figures 4C and S4A).

3.5 | Addition of Gal9 to the cell cultures reduces the proliferation of Th1 cells and induces differentiation of Tregs in patients with MPE

We assessed the changes in the drug-specific proliferative response in PBMCs by blocking or enhancing the Tim3-Gal9 axis through the

addition of α Tim3 blocking antibodies or hGal9 recombinant proteins, respectively. We did not observe any effect in the proliferation of PBMCs in controls for all T-cell subpopulations analysed (Th1, Th1 expressing Tim3 and Tregs), either with or without drug stimulation (Figures 5 and S5). On the contrary, for allergic patients, we observed a positive proliferation of Th1 and Tim3⁺ Th1 cells after stimulation with the culprit drug (16 and 14 positive cases, respectively; Figure 5C), with values significantly higher than those observed in controls (*P* < 0.001 and *P* = 0.002, respectively; Figure 5A,B). In contrast, the proliferation of Tregs was very low, although values were still higher than for controls (*P* = 0.002; Figure 5D).

Blocking the Tim3 receptor did not affect this proliferation in any of the cell subpopulations, and a difference compared to controls was also observed (*P* = 0.003 for Th1 and *P* < 0.001 for Tim3⁺ Th1; Figure 5). Nevertheless, this blocking increased the number of positive cases when Tim3⁺ Th1 was analysed (from 14 to 18 positive cases), but without significant differences in positivity or in the proliferation index mean values (Figure 5B,C).

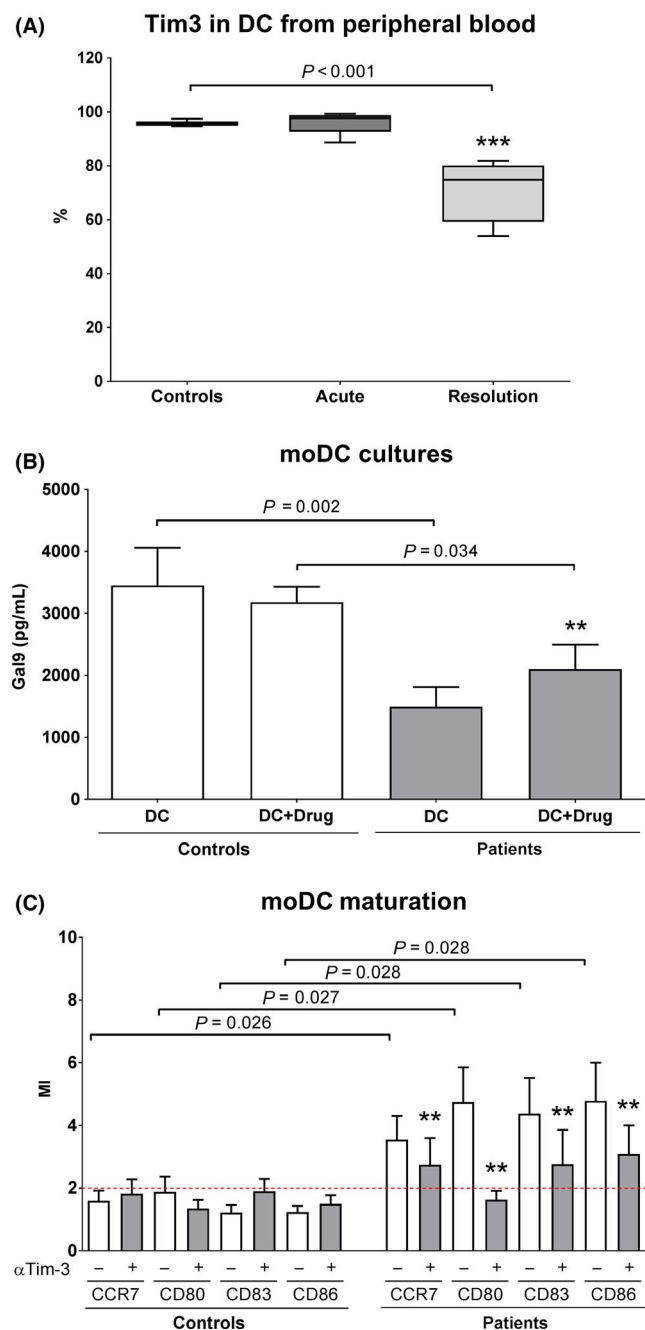


FIGURE 3 The Tim3-Gal9 axis in dendritic cells: A, Frequencies of Tim3⁺ DCs in peripheral blood from patients, during the acute and the resolution phase, and controls; B, levels of released Gal9 to the culture medium by monocyte-derived DCs from patients and controls, cultured in the presence of the culprit drug; C, expression of maturation markers in monocyte-derived DCs from patients and controls, cultured in the presence of the culprit drug. Maturation Index (MI) was calculated as the ratio between the percentages of cells expressing the maturation marker on the drug-stimulated DCs compared to the nonstimulated DCs. Comparisons between controls and patients have been performed using Kruskal-Wallis (for the three groups) and Mann-Whitney *U* tests. Comparisons between acute and resolution phase and with/without treatment have been performed using Wilcoxon tests (***P* < 0.01; ****P* < 0.001). Bars represent means + SEM [Color figure can be viewed at wileyonlinelibrary.com]

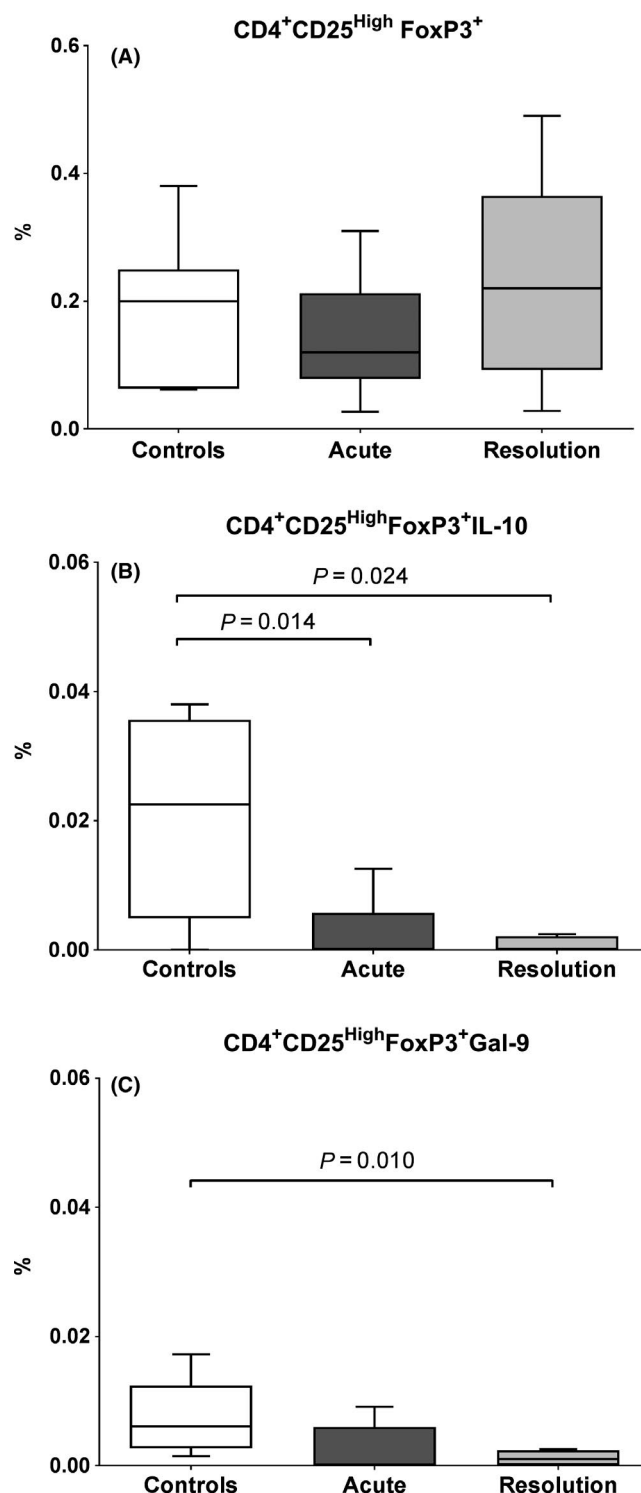


FIGURE 4 Treg frequencies in peripheral blood from patients, during the acute and resolution phase, and controls in total CD3 lymphocytes: A, CD4⁺CD25^{high}FoxP3⁺ cells; B, CD4⁺CD25^{high}FoxP3⁺ cells producing IL-10; C, CD4⁺CD25^{high}FoxP3⁺ cells producing Gal9. Comparisons between controls and patients have been performed using Kruskal-Wallis (for the three groups) and Mann-Whitney *U* tests. Comparisons between acute and resolution phase have been performed using Wilcoxon tests (***P* < 0.01; ****P* < 0.001)

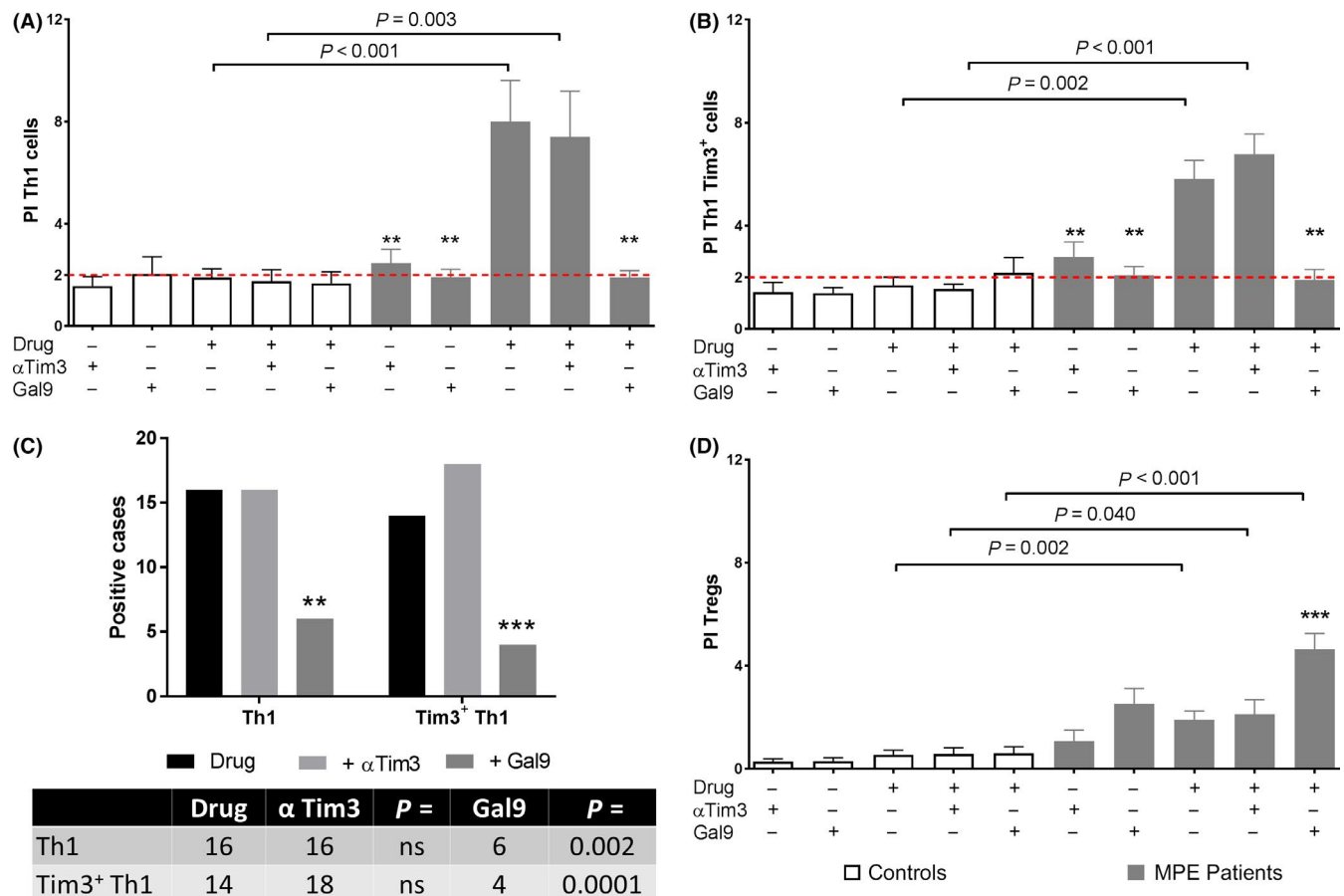


FIGURE 5 Proliferation assays of PBMCs from patients and controls cultured in the presence and absence of the culprit drug and anti-Tim3 blocking antibodies or exogenous Gal9 at different combinations: A, proliferation of Th1 cells; B, proliferation of Tim3⁺ Th1 cells; C, percentage of patients with positive proliferation of Th1 and Tim3⁺ Th1 cells. Results of the chi-squared test between drug stimulation alone or together with αTim3 or Gal9 are shown in the table; D, frequency of Treg cells. Proliferation index (PI) was calculated as the ratio between the % CFSElow drug-stimulated PBMCs and the % CFSElow unstimulated PBMCs. Comparisons between controls and patients have been performed using Kruskal–Wallis (for the three groups) and Mann–Whitney *U* tests. Comparisons between stimulation with the drug and the different treatments have been performed using Wilcoxon tests (***P* < 0.01; ****P* < 0.001) [Color figure can be viewed at wileyonlinelibrary.com]

More interestingly, the addition of exogenous Gal9 significantly decreased drug-induced proliferation in Th1 and Tim3⁺ Th1 for patients (*P* = 0.001 for both), reducing the positivity from 16 to 6 cases for Th1 (*P* = 0.002) and from 14 to 4 cases for Tim3⁺ Th1 (*P* < 0.001; Figure 5A–C). On the contrary, the addition of Gal9 increased the drug-stimulated proliferation of Tregs in patients (*P* < 0.001; Figure 5D).

4 | DISCUSSION

In recent years, inhibitory checkpoint molecules such as Tim3 have received attention due to their critical role in the regulation of both adaptive and innate immune mechanisms.²⁹ The interaction of this molecule with one of its ligands, Gal9, has been shown to have a crucial role in controlling Th1 and Th17 subpopulations in autoimmune diseases, especially for those with skin involvement.^{22,23} In this study, we investigated the role of the Tim3–Gal9 axis in the development of mild drug-induced NIR such as MPE, which are driven

by an increased number of Th1 cells in the affected organ, the skin.⁵ However, the role of Th17 cells, involved in the pathogenesis of other cutaneous diseases such as psoriasis⁸ and contact dermatitis,⁹ has not been investigated for NIR. Interestingly, in this study we observed a significant increase in the frequency of Th1 cells in our patients, especially during the acute phase of the reaction, in both peripheral blood and skin, confirming previous results.⁵ However, we found low levels of Th17 cells in these patients, which were similar to, or in some cases lower than, those observed in healthy subjects with good tolerance to the drugs responsible for the reactions. This suggests a limited contribution of these cells in the development of these reactions, contrary to other skin diseases.^{8,9}

Previous studies have shown that Tim3 is present in Th1 cells and plays a crucial role in their homeostasis^{16,17} by inducing apoptosis through a negative feedback mechanism driven by the production of IFN-γ.²² Here, we observed a dramatic reduction of Tim3 for the Th1 cells of patients with drug-induced MPE, which is strongest during the acute phase of the reaction. We observed this reduction in both skin and peripheral blood cells, and as such, it may be responsible for

the increased number of Th1 cells and therefore one of the causes of the pathogenesis of the drug reaction. This reduced expression of Tim3 has also been observed in skin lesions of patients with psoriasis.²³ Moreover, we observed an increase in Tim3 during the resolution phase; this may be a result of efforts to control the reaction, although the levels were still lower than for controls.

The inhibitory effect of Tim3 on the proliferation of Th1 cells seems to be driven by the interaction with Gal9, one of its ligands.²⁰ Interestingly, here we found that gene expression of Gal9 is reduced in PBMCs during the acute phase of the reaction. This agrees with the negative correlation found between Gal9 mRNA levels in PBMCs of patients with rheumatoid arthritis and the disease activity.³⁰ However, we found serum and skin levels to be similar for both patients and controls; this may be due to the protein being produced before the reduction of the mRNA expression.

Interestingly, the Tim3-Gal9 interaction does not always lead to the suppression of immune responses, showing a contrasting role in the innate immune system.³¹ They possess either negative or positive function in various settings, depending on the cell type and physiological or pathological context. Moreover, we must take into account that other ligands for Tim3 exist and that Gal9 can bind to other receptors on the cell surface as well.²⁹ Thus, more actors not included in this study, may be involved in the impaired immune regulation showed in MPE. Both molecules, Tim3 and Gal9, are expressed by DCs,²² and this axis can synergize with Toll-like receptor (TLR) signalling, inducing maturation and promoting Th1 immunity in viral infections.^{13,32} In this study, we observed a high expression of Tim3 in patient DCs during the acute phase, after the drug stimulation *in vivo*, similar to healthy controls. However, these levels were lower during the resolution phase. Tim3 has been described as an activation and maturation marker of natural killer³³ and DCs³⁴; thus, this increase suggests that the drug is able to selectively activate DCs in allergic patients. On the other hand, the production of Gal9 by moDCs *in vitro* is reduced in patients compared to controls and, although drug stimulation increased its release, its levels were still lower than those observed in healthy subjects. Moreover, in agreement with the increased expression of Tim3, maturation of DCs after drug stimulation is observed only in allergic patients and was inhibited after blocking the Tim3 receptor, indicating the important role of the Tim3-Gal9 axis in the maturation of DCs. This suggests that not only Tim3 expression is affected in these patients, but also the production of Gal9, at least for some cell subtypes. Nevertheless, this did not seem to affect moDC maturation in patients, important for drug presentation and activation of Th1 cells.³⁵ Maturation of DCs and activation of Th1 cells, together with the decreased expression of Tim3 that hampers the control of Th1 numbers, are likely to be responsible for uncontrolled inflammation.

Other important cell subtypes in disease control are Tregs. Although they tend to increase in number during the acute phase of the reaction, we observed a significantly reduced frequency of IL-10-producing Tregs in patients, probably impeding a possible control mechanism for Th1 cells and the inflammatory reaction.³⁶ It has been demonstrated that Gal9 is able to induce Treg differentiation

and stimulate FoxP3 expression during immunization in a model of collagen-induced arthritis, controlling the development of the disease.²⁰ However, we found a reduced percentage of Gal9-producing Tregs in allergic patients during the resolution phase, although its frequency increased during the acute phase, being more similar to healthy controls. Thus, the defect in the Tim3-Gal9 axis could also be responsible for the impaired differentiation of Tregs in allergic patients.

Finally, we have explored the influence of blocking or enhancing the Tim3-Gal9 axis, analysing the drug-induced proliferation of Th1 and the frequency of Treg. After blocking Tim3, we did not find differences in the proliferation of Th1 cells, although we saw a slight increase in the proliferation of those cells that express Tim3, observing an increment in the number of patients that showed a positive proliferation of this cell subpopulation (from 14 to 18 positive results). These increases could confirm the effect of Tim3 in controlling Th1 proliferation in these reactions as it has been published for other diseases.^{16,17,20,22,23} Regarding Tregs, we did not find any effect on its frequency when these cells were cultured with the drug or with Tim3 blocking antibodies.

The use of Gal9 for the treatment of other nonallergic immune diseases has been explored. It has been described that Gal9 exhibits therapeutic potential against autoimmune diabetes,³⁷ psoriasis,³⁸ autoimmune arthritis,²⁰ allograft rejection,^{39,40} graft vs host disease,⁴¹ autoimmune encephalomyelitis,⁴² lupus⁴³ and cancer.⁴⁴ Moreover, the exogenous administration of Gal9 has also been shown to control levels of Th1 and Th17 cells in allergic contact dermatitis³⁸ and mast cell degranulation in allergic asthma.⁴⁵ Interestingly, the addition of exogenous Gal9 to the cell cultures induced a significant reduction of Th1 and Tim3⁺ Th1 cell proliferation, whereas there was a significant increase in Treg frequency. These results add weight to the critical role of the Tim3-Gal9 axis in controlling the homeostasis of effector cells and disease progression. Moreover, they suggest the potential role of Gal9 as a therapeutic target. However, this effect of Gal9 could not be mediated by Tim3, and by other Gal9 receptors. Further studies are needed to identify through which receptor Gal9 is acting.

In conclusion, we have described for the first time the involvement of the Tim3-Gal9 axis in the development of MPE. The reduced level of Tim3 in Th1 cells together with the impaired expression of Gal9 in PBMCs and dendritic cells hampers the control of Th1 numbers and the differentiation of Treg cells in these patients. The capability of Gal9 to suppress Th1 and enhance Treg differentiation makes it a potential therapeutic tool for the treatment of NIR reactions to drugs, although further research in other NIR clinical entities could be needed.

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CONFLICTS OF INTEREST

The author declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

RFS, FP, ARN and MCPS performed experiments and analysed data. MS, ID and GB phenotyped the patients and controls. AA and CM critically read the manuscript and provided insightful information and discussion valuable for design and interpretation of data. MJT and TDF designed the study, analysed data and wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Title: Expression of the Tim3-Galectin 9 axis is altered in drug-induced maculopapular exanthema.

Short Title: Tim3-Galectin 9 axis in drug-induced MPE

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Online Supporting Information

Methods:

Patients and controls

The study included individuals who had experienced maculopapular exanthema, who attended the Allergy Service during a 1-year period (2014-2015), and who were finally confirmed as allergic. The allergological work-up was performed as recommended by the European Network for Drug Allergy (1), including a clinical history, skin testing (intradermal and/or a patch-test, depending on the drug solubility) and, if negative, a drug provocation test. As controls, we selected 10 sex- and age-matched subjects with confirmed tolerance to the drugs involved in the reactions. None of the controls had a history of ADRs nor did they have any cutaneous or immunological diseases at the time of selection. The study was conducted at the Allergy Service and Research Laboratory at the Hospital Regional Universitario de Málaga (HRUM), according to the Declaration of Helsinki and was approved by the ethics committee of the hospital. All participants signed informed consent forms before the start of the study.

Sample collection

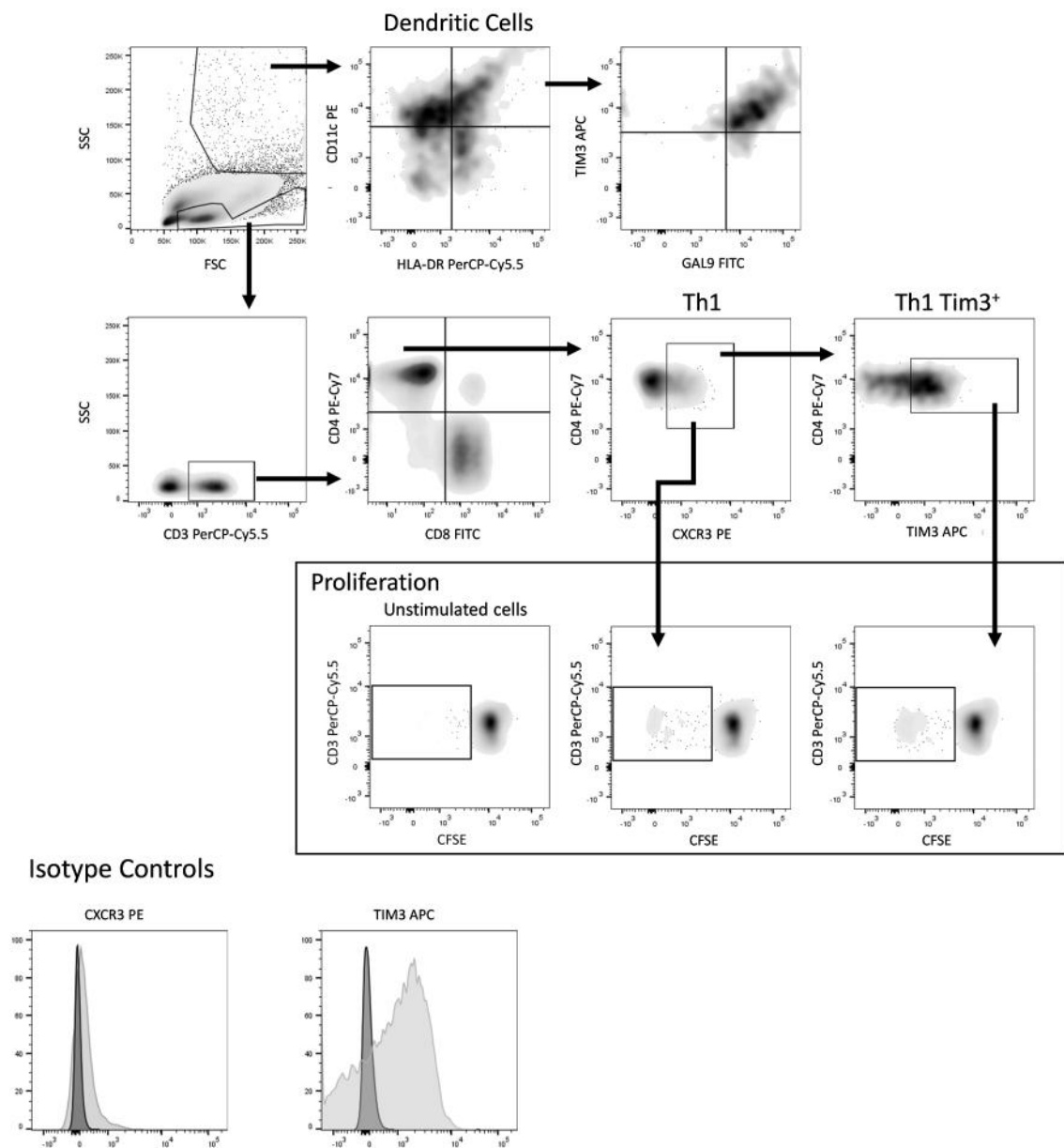
Blood samples and skin biopsies were obtained from each patient within the first 24 h after the initiation of the reaction (acute phase) and, once the patients were confirmed as allergic, another blood sample was obtained at least 60 days after the resolution of symptoms (resolution phase). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient (Rafer S.L Zaragoza, Spain), frozen and stored in liquid nitrogen at the BIOBANK of the HRUM, for future studies. Serum samples for mediator determinations were also obtained during both phases and stored at -20°C until they were analysed. 4 mm punch skin biopsies were fixed in 10% formalin, routinely processed, and paraffin-embedded until the corresponding determination. Skin control samples were obtained from the BIOBANK of the HRUM.

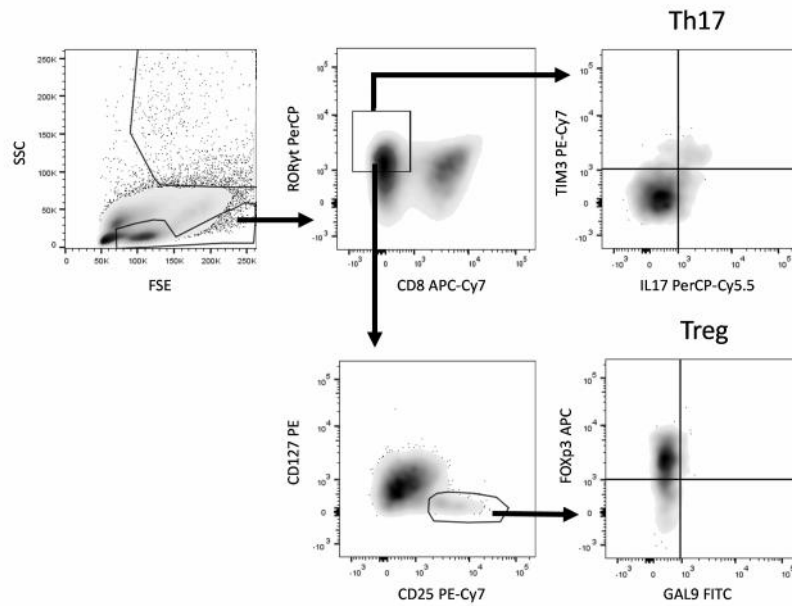
Subpopulation phenotypical analysis

The frequencies of different T cell subpopulations, Th1 (CD4⁺CXCR3⁺) (2), Th17 (CD4⁺RORγt⁺IL17⁺) (3), dendritic cells (DCs; HLA-DR⁺CD11c⁺), and the expression of Tim3 in these populations; and the frequencies of Tregs (CD4⁺CD25^{high}FoxP3⁺) and the expression of IL10 and Gal9 in these populations, were determined in PBMCs during the acute and resolution phases of the reaction (4). Surface and intracellular characterization were carried out with specific moAbs (CD3 PerCP-Cy5.5, CD4 PE-Cy7, CXCR3 PE, IL17 PerCP Cy5.5, CD8 APC Cy7, HLA-DR PerCP Cy5.5, CD4 APC-H7, CD25 PE-Cy7, CD127 PE, FoxP3 AF647, IL10 PE from BD™ Bioscience, Milpitas, CA, USA; CD11c PE, Tim3 APC / PE Cy-7, Gal9 FITC from BioLegend, San

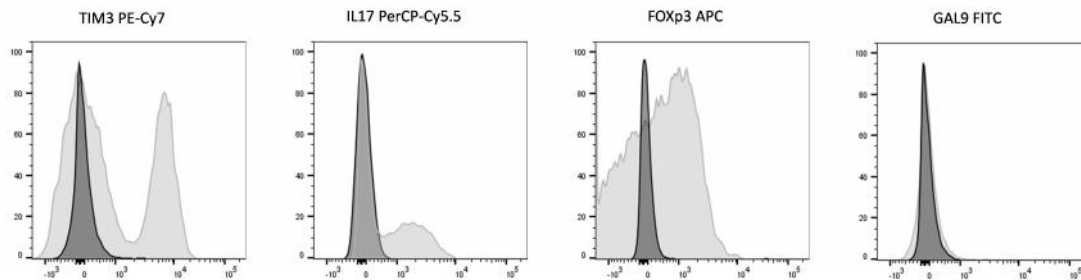
Diego, CA, USA; RORyt PerCP from LifeSpan Bioscience, Seattle, WA USA) and proper buffers following the manufacturer's instructions. For intracellular staining, cells were fixed using the BD Cytofix/CytoPerm Fixation/Permeabilization solution kit with BD GolgiStop (BD™ Bioscience, Milpitas, CA, USA). Cells were washed and immediately phenotyped with a BD™ FACSCanto II flow cytometer (BD Biosciences, Milpitas, CA, USA) and the results analysed with FlowJo® software (Tree Star, Inc. USA). For Tregs staining, human FoxP3 buffer (BD™ PharMingen, San Diego, CA, USA) was used following the manufacturer's instructions.

Figure S1. Gating strategy to select the different cell subpopulations.





Isotype Controls



Immunohistochemistry

A 4 mm punch skin biopsy was obtained during the acute phase of the reaction and fixed in 10% formalin, routinely processed and paraffin-embedded. Microtome sections (8 μ m) were processed for hematoxylin-eosin and immunohistochemical staining, as described (2) with the following polyclonal antibodies: anti-CXCR3, anti-IL-17, anti-Gal9 (Abcam, Cambridge, UK) and anti-Tim3 (LifeSpan BioSciences, Seattle, WA, USA). The binding of these primary antibodies (rabbit and goat IgG) was detected using a broad-spectrum super picture HRP polymer conjugate (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). The visualization of the signal was carried out by using 3-3'-diaminobenzidine substrate kit (Sigma, St Louis, MI, USA) in an optical microscope. The number of cells stained in each tissue section, after having discarded the background, was categorized into different groups: [0] when no stained cells were found in the tissue section, [1] one or two stained cells, [2] fewer than 20 stained cells, [3] between 20 and 100 stained cells, and [4] more than 100 stained cells. When the marker was expressed in dermis and epidermis, both layers were analyzed, the level of expression qualified separately and results expressed as a mean of the values assigned to both layers.

Gal9 Gene Expression Analysis by RT-qPCR

Total RNA from one million PBMCs from controls and patients for the acute and resolution phases, was isolated using RNeasy Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Purity and RNA concentration were measured by the absorbance ratio at 260 and 280 in a Thermo Scientific Nano Drop TM 2000 Spectrophotometer. RNA (10 ng) was reverse-transcribed using a commercially available set of High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The resulting cDNA (20 ng) was amplified on microAmp optical 96-well reaction plate in an EcoTM Real Time PCR System (Illumina, San Diego, CA, United States), using TaqMan Fast Advanced Master Mix (Thermo Fisher, Waltham, MA) and a specific primer for GAL-9 (FW: TAGTGGGTGTGAAAGGAGC; RV: CAGAAAAGGGGACAGCTGGA) and beta2-microglobulin (B2m) as reference gene (FW: CTGCTACGTAACACAGTTCCACCC; RV: CATGATGCTTGATCACATGTCTCG). The 2- Δ Ct method was used to normalize expression results. The values of the housekeeping β 2m gene were used to normalize the values obtained for the gene under study. Relative gene expression was calculated by means of the 2- Δ Ct formula and expressed as fold-change compared with the control.

Monocytes derived DCs cultures

Monocytes from patients and controls were purified from PBMCs with anti-CD14 microbeads following the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). CD14⁺ cells were differentiated into immature monocyte derived DCs (moDCs) by incubating with rhGM-CSF (200ng/mL) and rhIL-4 (100ng/mL) (both from R&D Systems Inc, Minneapolis, MN, USA) for 5 days at 37°C (5). imDCs were after incubated in complete medium at 10⁵ cells/mL in 96-well plates (Nunc AS, Roskilde, Denmark) with the culprit drug (amoxicillin, sulphometoxazol, paracetamol, metamizol, heparin and allopurinol at 250 μ g/mL; iodixanol at 5 mg/mL). LPS at 1 mg/mL (Sigma Aldrich, Saint Louis, USA) was used as the positive control and culture media as the negative control. After 72 h of stimulation at 37°C in 5% CO₂, the DCs were harvested and their maturation state was assessed by determining the upregulation of CCR7, CD80, CD83, and CD86 using fluorescently labelled monoclonal antibodies (BD Pharmigen, San Diego, California) in a FACSCanto II cytometer (BD Biosciences, Milpitas, California). To analyse the effect of blocking Tim3 receptors, the cells were pre-incubated for 1-hour with 10 μ g/ml of α -Tim3 antibodies (6, 7) in different combinations. The data were processed with FlowJo (TreeStar Inc., Switzerland). The results were considered positive when the maturation index (MI), calculated as the ratio between the percentages of the drug-stimulated DCs and the non-stimulated DCs, was greater than two. Supernatants were also harvested and frozen for further studies.

Gal9 determination

Determination of Gal9 was performed in sera from patients (acute and resolution phase) and controls and in DCs cultured supernatants using a Luminex Bead-Based Assay (R&D Systems Inc, Minneapolis, MN, USA) following the manufacturer's instructions. Results were read in a Bioplex 200 reader (Bio-Rad, Hercules, CA, USA) and expressed as pg/mL.

Lymphocyte proliferation

Carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA)-labeled PBMCs (1.5×10^5) from patients at the resolution phase and controls were cultured at a final volume of 250 μ L of complete medium, in 96-well plates with or without stimulus for 6 days at 37°C and 5% CO₂. Cells were stimulated with the culprit drug (amoxicillin, sulphamethoxazol, paracetamol, metamizol, heparin and allopurinol at 250 μ g/mL; iodixanol at 5 mg/mL). Stimulation with Phytohemagglutinin (PHA) at 10 μ g/mL (Sigma Aldrich, Saint Louis, USA) was used as positive control and unstimulated PMBCs as negative control. The proliferation of different lymphocyte subsets Th1, Th17 and Tregs was assessed by flow cytometry, analysing the percentage from them that expresses CFSE^{low}. The results were considered positive when the proliferation index (PI) calculated for each subset as (% CFSE^{low} drug-stimulated PBMCs / % CFSE^{low} unstimulated PBMCs) was greater than 2. To analyse the functional changes induced by the Tim3-Gal9 axis, the cells were pre-incubated for 1-hour with 10 μ g/ml of α -Tim3 antibodies (6, 7) or recombinant human Gal9 (8) (both from Biolegend, San Diego, CA, USA).

Statistical analysis

Comparisons of quantitative variables without a normal distribution were performed using the Mann-Whitney U test, the Kruskal-Wallis test or the Wilcoxon test for matched paired samples. All reported P-values represent two-tailed tests, with values <0.05 considered statistically significant.

Figure S2. Histograms showing the frequencies of (A) Th1; (B) the expression of Tim3 on Th1; (C) Th17; (D) the expression of Tim3 on Th17, comparing controls and patients during the acute and the resolution phase.

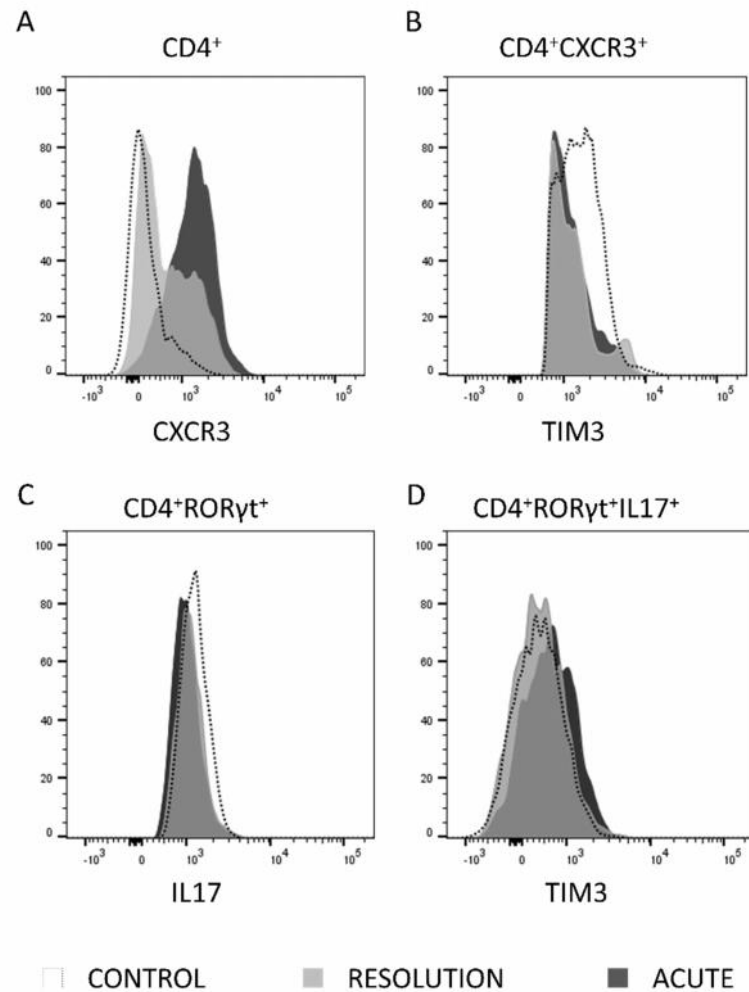


Figure S3. Histograms showing the frequencies of the expression of Tim3 on Dendritic cells, comparing controls and patients during the acute and the resolution phase.

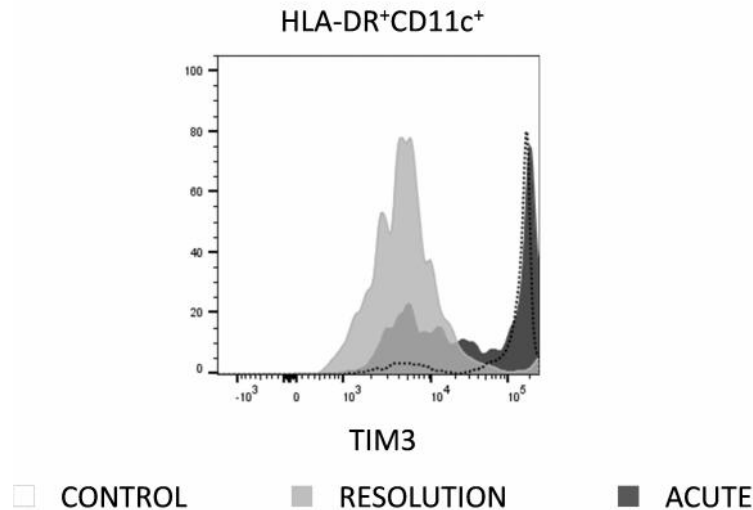


Figure S4. Histograms showing the frequencies of (A) Treg (left) and the expression of Gal9 on Treg (right); (B) the expression of IL10 on Tregs comparing controls and patients during the acute and the resolution phase.

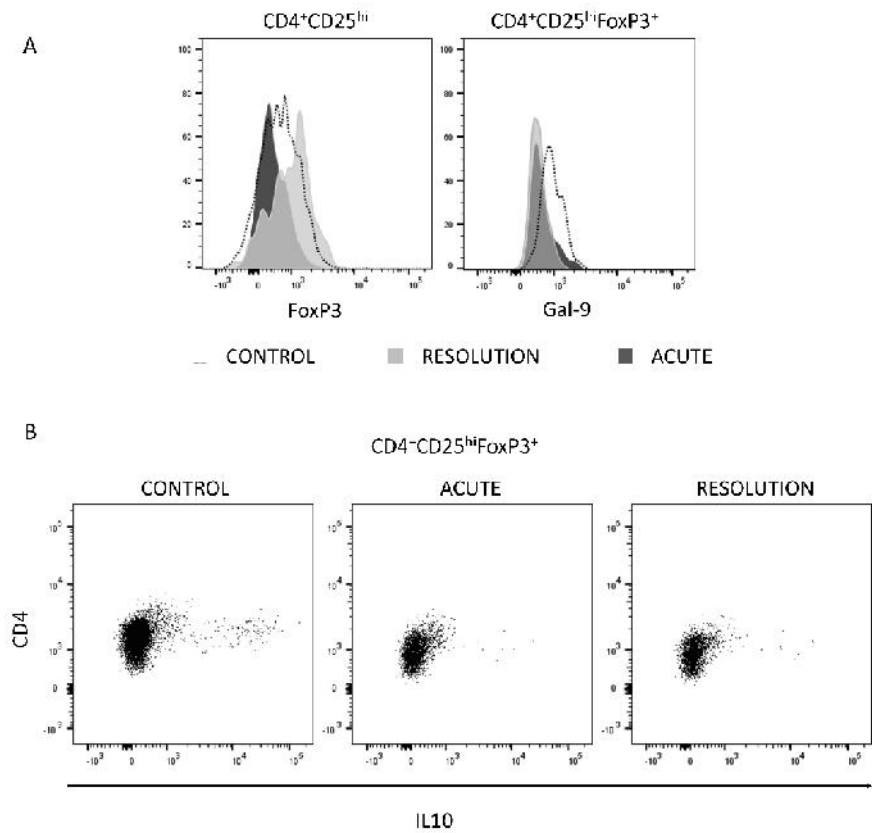
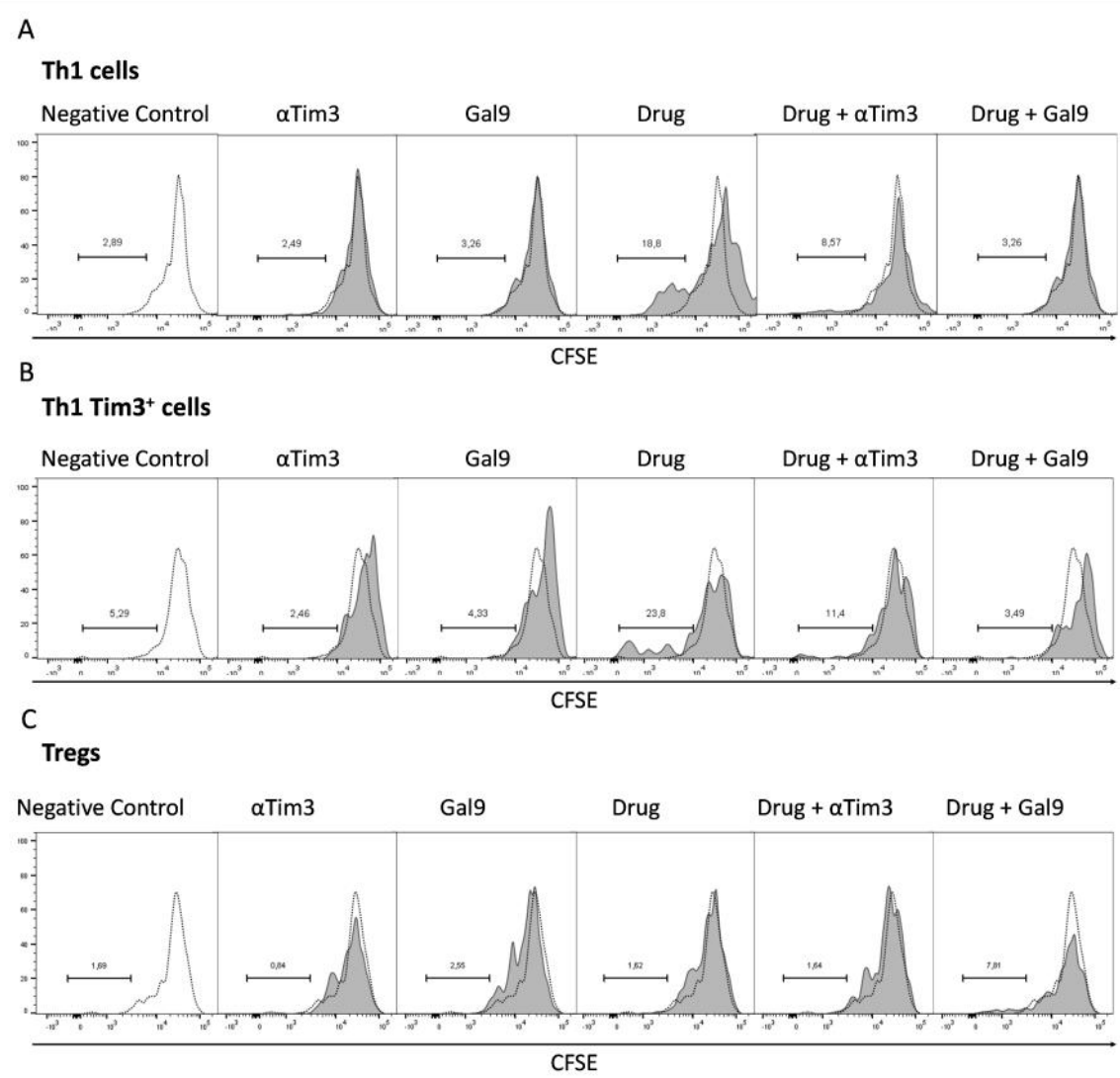


Figure S5. Histograms showing proliferation as the frequency of CFSE^{low} cells in (A) Th1 cells; (B) Th1 Tim3⁺ cells; and (C) Tregs, comparing the different culture conditions.



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