

Triggering of specific Toll-like receptors and proinflammatory cytokines breaks allergen-specific T-cell tolerance in human tonsils and peripheral blood

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Background: The generation and maintenance of allergen-specific T-cell tolerance is a key step in healthy immune responses to allergens and successful allergen-specific immunotherapy. Breaking of peripheral T-cell tolerance to allergens can lead to the development of allergies, but the mechanisms are not completely understood.

Objective: We sought to identify molecular mechanisms that break allergen-specific T-cell tolerance in human subjects.

Methods: Proliferative responses of allergen-specific T cells from tonsils and peripheral blood were measured by using tritiated thymidine incorporation and carboxyfluorescein succinimidyl ester (CFSE) dilution experiments. Cytokine levels in cell-free supernatants were quantified by using the cytometric bead array,

and mRNA expression of transcription factors and cytokines was determined by using quantitative PCR. Myeloid dendritic cells (DCs) were characterized by using flow cytometry.

Results: In allergic patients the immune profile of the tonsils represents the atopic status of patients, with low expression of the T_H1 cell-specific transcription factor T-bet and the cytokine IFN- γ , as well as IL-10. Human tonsils show very low levels of allergen-induced T-cell proliferation, thus representing a very suitable *in vivo* model to assess mechanisms of breaking allergen-specific T-cell tolerance. Triggering of Toll-like receptor (TLR) 4 or TLR8 and the proinflammatory cytokines IL-1 β or IL-6 break allergen-specific T-cell tolerance in human tonsils and peripheral blood through a mechanism dependent on the adaptor molecule myeloid differentiation primary response gene (88) (MyD88). In particular, myeloid DCs and stimulations that activate them broke the tolerance of allergen-specific CD4⁺ T cells, whereas plasmacytoid DCs and stimulations that activate them, such as TLR7 and TLR9, did not have any effect. Tolerance-breaking conditions induced by different molecular mechanisms were associated with a mixed cytokine profile with a tendency toward increased levels of IL-13 and IL-17, which are T_H2 and T_H17 cytokines, respectively.

Conclusion: Certain innate immune response signals and proinflammatory cytokines break allergen-specific CD4⁺ T-cell tolerance in normally unresponsive subjects, which might lead to the development or exacerbation of allergic diseases after encountering microbes or inflammatory conditions. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Peripheral tolerance, tonsils, proinflammatory cytokines, IL-1 β , IL-6, Toll-like receptors, allergen-specific T cells, myeloid dendritic cells

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The prevalence of allergic diseases has significantly increased over the last decades, but the reasons and underlying mechanisms are not fully understood.^{1,2} Currently, it is well established that the induction of peripheral T-cell tolerance through the generation of allergen-specific regulatory T (Treg) cells is essential in healthy immune responses to allergens and represents a key step in successful allergen-specific immunotherapy.³⁻⁷ Allergy is a T_H2-mediated disease involving the formation of specific IgE antibodies against innocuous environmental allergens.⁷ Several studies demonstrated that allergen-specific effector T and Treg cells recognizing the same T-cell epitopes are present both in healthy and allergic subjects. The delicate balance between the ratios of these T-cell subsets determines the development of a healthy or an allergic immune response to allergens.^{2,8-12} In this context we have recently demonstrated that human tonsils

Abbreviations used

CFSE:	Carboxyfluorescein succinimidyl ester
CT:	Cycle threshold
DC:	Dendritic cell
DC-SIGN:	Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
EF1 α :	Elongation factor 1 α
FOXP3:	Forkhead box protein 3
mDC:	Myeloid dendritic cell
MyD88:	Myeloid differentiation primary response gene (88)
OX40L:	OX40 ligand
pDC:	Plasmacytoid dendritic cell
PLA ₂ :	Phospholipase A ₂
TMC:	Tonsillar mononuclear cell
TLR:	Toll-like receptor
TLR-L:	Toll-like receptor ligand
Treg:	Regulatory T

are organs of immune tolerance in which the generation and maintenance of allergen-specific Treg cells occur.¹³ Supporting these findings, McClory et al¹⁴ have recently reported that in human tonsils extrathymic T-cell development programs are imprinted.¹⁵ Although it is becoming more clear that the observed increment in the incidence of allergic diseases is due to the loss of peripheral T-cell tolerance to allergens, the mechanisms or specific cytokines or innate immune response stimulating factors involved in such processes remain largely unknown.^{2,16,17}

Dendritic cells (DC) play a pivotal role in the orchestration of immune responses by linking innate and adaptive immunity.¹⁸⁻²³ In human subjects circulating DCs can be broadly divided into 2 main groups: myeloid dendritic cells (mDC) and plasmacytoid dendritic cells (pDC).²⁴ Both mDCs and pDCs are equipped with a specific repertoire of Toll-like receptors (TLRs).²⁵ mDCs express TLR2 to TLR6 and TLR8, whereas pDCs express TLR7 and TLR9.^{26,27} DCs can control the suppressive activity of Treg cells in several ways through the release of proinflammatory cytokines, such as IL-1 β or IL-6, after TLR-dependent responses to pathogen-associated molecular patterns.²⁸⁻³² Although recent studies have demonstrated that triggering of TLRs can directly regulate Treg cell functions,^{33,34} there is still scarce information about the role played by specific TLR ligands (TLR-Ls) or proinflammatory cytokines in breaking allergen-specific T-cell tolerance, especially in human subjects.

In this study we identified molecular mechanisms involved in breaking allergen-specific T-cell tolerance. We demonstrated that triggering of TLR4 or TLR8, as well as IL-1 β or IL-6, is able to enhance allergen-specific CD4⁺ T-cell responses in human tonsils and peripheral blood. We showed mDCs as the main DC subset mediating such effects through mechanisms partially dependent on MyD88 and upregulation of costimulatory molecules.

METHODS**Study group**

The study has been reviewed and approved by the ethics committees of the Cantons of Graubünden and Zürich and Satakunta Central Hospital, Pori, Finland. Heparinized peripheral blood samples from well-characterized nonallergic healthy and allergic donors were used in the study. Healthy buffy coats obtained from the Red Cross, Zurich, Switzerland, were used to optimize experimental conditions and general experiments with purified DC subsets. Noninflamed tonsils from patients undergoing tonsillectomy were obtained

from the hospitals of Davos and Chur, Switzerland. Tonsil samples from atopic and nonatopic patients were recruited from Satakunta Central Hospital, Pori, Finland; stabilized; and stored at -80°C in RNeasy RNA Stabilization Reagent (Qiagen, Hilden, Germany) until used for total RNA isolation. Atopic subjects were included according to a well-defined clinical history of allergy, specific IgE in serum, and positive skin prick test responses to at least 3 allergens. For the experiments of breaking allergen-specific T-cell tolerance in peripheral blood, the included nonatopic healthy donors did not report any allergy-related clinical symptoms and showed low specific IgG and IgE levels in serum to the tested allergens.

Study methods

Allergen-specific T-cell proliferation was measured by using tritiated thymidine incorporation and carboxyfluorescein succinimidyl ester (CFSE) dilution experiments. Cytokine levels in cell-free supernatants were quantified by using the cytometric bead array. Quantitative mRNA expression of transcription factors and cytokines was determined by using quantitative PCR. Allergen-specific T-cell tolerance-breaking experiments were performed with different allergens in the presence or absence of cytokines, TLR-Ls, myeloid, and pDCs. A detailed description of all the methods used in this article is given in the **Methods** section in this article's Online Repository at www.jacionline.org.

RESULTS**Characterization of the polyallergic atopic status in human palatine tonsils**

To study mechanisms of breaking allergen-specific T-cell tolerance in human tonsils, we first analyzed whether these tissues reflect the classical features of allergic diseases at the T-cell level. Therefore we compared the mRNA expression levels of T-cell lineage-related cytokines and transcription factors in tonsillar tissues directly excised from a randomly selected population of 24 polyallergic atopic and 24 nonallergic control subjects. Experiments were performed without any stimulation to demonstrate the direct *in vivo* situation. In **Fig 1** the quantification of mRNA levels for different cytokines and transcription factors is displayed. IFN- γ and IL-10 mRNA levels were significantly low in tonsillar tissues of polyallergic atopic compared with nonatopic subjects. IL-13 and TGF- β mRNA levels did not show any significant differences between the 2 groups. The mRNA levels of T-bet were significantly lower in the tonsillar samples of atopic patients compared with those from nonatopic subjects. There was no significant difference in mRNA levels of GATA3 between the 2 groups. The T-bet/GATA3 ratio was significantly higher in nonatopic donors compared with that seen in atopic donors, indicating a T_H2 shift in tonsils from atopic subjects. Although there was not any significant difference in forkhead box protein 3 (FOXP3) mRNA levels between atopic and nonatopic subjects, in both cases relatively high levels of FOXP3 mRNA were detected. We further analyzed functional responses in tonsillar mononuclear cells (TMCs) from 4 atopic and 4 nonatopic subjects after 5 days of Phl p 5a or Bet v 1 allergen stimulation (**Fig 2**). After allergen stimulation, TMCs from atopic subjects showed a tendency to express less T-bet, IFN- γ , and IL-10 mRNA compared with TMCs from nonatopic subjects, but differences were not statistically significant. IL-13 mRNA levels were significantly higher in TMCs from atopic compared with nonatopic subjects after Phl p 5a stimulation (**Fig 2**). The same tendencies were also observed at the secreted cytokine level after 5 days of allergen stimulation of TMCs. IL-4 and IL-5 levels were undetectable. Atopic subjects showed relatively high IL-13 levels, whereas IFN- γ and IL-10 levels did not change (**Fig E1, A**, in this article's

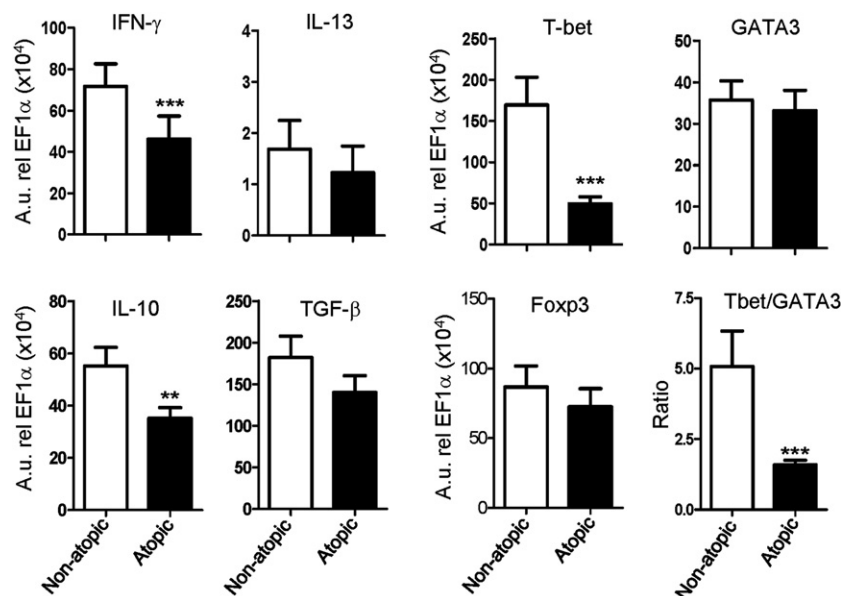


FIG 1. *In vivo* mRNA expression levels of cytokines or T-cell transcription factors in tonsils. The mRNA expression in palatine tonsils of 24 atopic and 24 nonatopic subjects was determined by using quantitative real-time RT-PCR. The Tbet/GATA3 ratio is also shown. Arbitrary units (A.u.) are $2^{-(\Delta CT)}$ values multiplied by 10^4 , with ΔCT defined as the difference between the cycle threshold value for the gene of interest and EF1 α . ** $P < .01$ and *** $P < .001$. Data represent means with SEMs.

Online Repository at www.jacionline.org). Although differences were not statistically significant, purified tonsillar CD4⁺ T cells from atopic donors stimulated for 24 hours with a mixture of anti-CD2/CD3/CD28 mAbs produced higher levels of IL-4, IL-5, and IL-13 compared with those seen in nonatopic donors, suggesting a predominant tonsillar T_H2 profile for atopic donors (see Fig E1, B). These results demonstrate that tonsils are organs in which regulation of immune responses to allergens occurs. In addition, the cytokine profile of tonsils from atopic and nonatopic subjects might represent the allergic status at the T-cell level.

Triggering of TLR8, but not TLR7 or TLR9, breaks allergen-specific T-cell tolerance in tonsils

After analysis of the direct *in vivo* situation in human tonsils, we attempted to identify conditions that break peripheral T-cell tolerance to allergens. TMCs from different atopic and nonatopic donors were stimulated with food allergens and aeroallergens, and proliferative responses were assayed and compared with those observed in PBMCs by using tritiated thymidine incorporation. TMCs from atopic patients displayed significantly lower T-cell proliferation to common aeroallergens (major timothy grass pollen and birch pollen allergens Phl p 5a and Bet v 1, respectively) or to the food allergen Cor a 1 (major allergen from hazelnut) compared with PBMCs, thus suggesting a state of peripheral T-cell tolerance to these common allergens in human tonsils (Fig 3, A). TMCs and PBMCs from nonatopic donors did not show T-cell proliferation after stimulation with the analyzed allergens.

Next, we studied the role of the innate immune response-stimulating factors that trigger specific DC subsets, such as TLR7-L and TLR9-L for pDCs and TLR8-L for mDCs, in breaking allergen-specific T-cell tolerance in human palatine tonsils. CFSE-labeled TMCs from donors showing no proliferative responses to Phl p 5a, Bet v 1, and Cor a 1 (Fig 3, A) were

stimulated with the allergens alone, with TLR-Ls alone, or with the allergens in combination with TLR-Ls. CD4⁺ T cells from TMCs did not show significant proliferative responses to the allergens when compared with those under unstimulated conditions. After stimulation with the allergens in combination with TLR8-L, the proliferation of CD4⁺ T cells in TMCs significantly increased compared with that after stimulation with allergens or TLR8-L alone. TLR7 and TLR9 triggering in combination with the allergens did not induce significant proliferation of CD4⁺ T cells in TMCs (Fig 3, B and C). The proliferative responses induced by specific allergens in the presence of TLR8-L were significantly higher than those with TLR8-L or specific allergens alone, confirming that TLR8, but not TLR7 or TLR9, stimulation breaks allergen-specific T-cell tolerance in human tonsils. TLR7 and TLR9 do not induce proliferation of CD4⁺ T cells. Tritiated thymidine incorporation and CFSE dilution experiments with TMCs demonstrated that the proliferation induced by TLR7-L and TLR9-L is particularly due to CD4⁺ T cells, most likely B cells that represent around 65% of the total TMC composition (see Fig E2 in this article's Online Repository at www.jacionline.org).³⁵

Triggering of TLR4 or TLR8 breaks allergen-specific T-cell tolerance in peripheral blood of healthy subjects

To investigate the potential role of TLR activation in the loss of allergen-specific T-cell tolerance in peripheral blood, PBMCs isolated from nonatopic donors were stimulated with TLR4-L, TLR7-L, TLR8-L, and TLR9-L alone or in combination with allergens. CD4⁺ T cells from stimulated PBMCs did not show proliferative responses to allergens compared with unstimulated conditions (Fig 4, A and B). When PBMCs were simultaneously stimulated with TLR4-L and allergens, the proliferation of

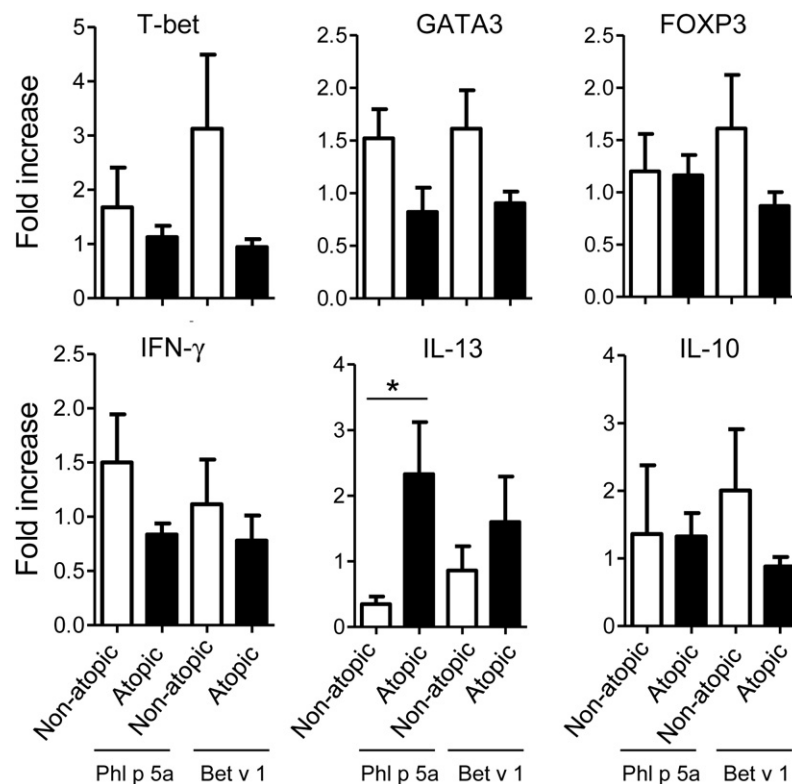


FIG 2. TMCs from atopic and nonatopic subjects respond differently to allergen stimulation. A total of 8 TMCs from atopic ($n = 4$) and nonatopic ($n = 4$) subjects were stimulated with Phl p 5a and Bet v 1 for 5 days. Fold-increased mRNA expression of the indicated genes relative to the corresponding unstimulated condition is shown. Data represent means with SEMs. * $P < .05$.

CD4⁺ T cells significantly increased compared with that after stimulation with allergens or TLR4-L alone (Fig 4, A). The same effect was observed after TLR8-L stimulation (Fig 4, B). Interestingly, stimulation with TLR7-L or TLR9-L in combination with allergens did not induce significant allergen-specific T-cell proliferation compared with stimulation with TLR-Ls or allergens alone (see Fig E3 in this article's Online Repository at www.jacionline.org). These results clearly demonstrate that triggering of TLR4 and TLR8, but not TLR7 or TLR9, breaks allergen-specific T-cell tolerance in human peripheral blood. Stimulation of PBMCs with TLR4-L induced the production of significantly higher amounts of IL-13, IFN- γ , IL-1 β , and IL-17 compared with allergens alone (Fig 4, C). A proinflammatory microenvironment with a mixed T_H1, T_H2, and T_H17-like cytokine profile in the presence of IL-1 β is generated by TLR4-L stimulation, which might contribute to the breaking of allergen-specific tolerance. Similar tendencies in cytokine profile are observed for TLR8-L.

IL-1 β or IL-6, but not IL-17 or IL-23, breaks peripheral allergen-specific T-cell tolerance

To identify proinflammatory cytokines that are able to break allergen-specific T-cell tolerance, PBMCs from nonatopic healthy donors were cultured with the cytokines IL-1 β , IL-6, IL-17, or IL-23 alone or in combination with certain allergens, and T-cell proliferation was assayed by using CFSE dilution and tritiated thymidine incorporation methods. As shown in Fig 5, PBMCs stimulated with the indicated allergens alone did not

show any proliferative responses, demonstrating an allergen-specific T-cell tolerance state. IL-1 β alone was able to slightly induce nonspecific CD4⁺ T-cell proliferation when compared with that seen in unstimulated PBMCs. Interestingly, stimulation of PBMCs with IL-1 β or IL-6 in combination with allergens significantly increased CD4⁺ T-cell proliferative responses, demonstrating that both cytokines are capable of breaking peripheral allergen-specific T-cell tolerance (Fig 5, A and B). The profile of cytokines secreted by CD4⁺ T cells after stimulation with IL-1 β or IL-6 is displayed in Fig E4 in this article's Online Repository at www.jacionline.org. After stimulation of PBMCs with IL-1 β or IL-6, increases in the production of proinflammatory cytokines were observed in the same direction as stimulations through TLR4 and TLR8. IL-1 β was more potent to induce a mixed proinflammatory cytokine pattern, whereas IL-6 alone did not upregulate the cytokines efficiently, suggesting a different molecular mechanism of action in the breaking of T-cell tolerance by IL-6. When allergen-specific T-cell tolerance-breaking conditions were compared with non-tolerance-breaking conditions, the increase in IL-13 and IL-17 levels was particularly observed in tolerance-breaking stimulations, whereas both IL-13 and IL-17 levels did not show any increase with TLR7 and TLR9 in the presence of allergens in conditions that did not break tolerance (Figs 4 and 6 and see Fig E4). The combination of IL-1 β and IL-6 triggered a strong nonspecific proliferation of PBMCs, and tolerance-breaking effects were not observed when both cytokines were used together in combination with allergens (see Fig E5 in this article's Online Repository at www.jacionline.org). In

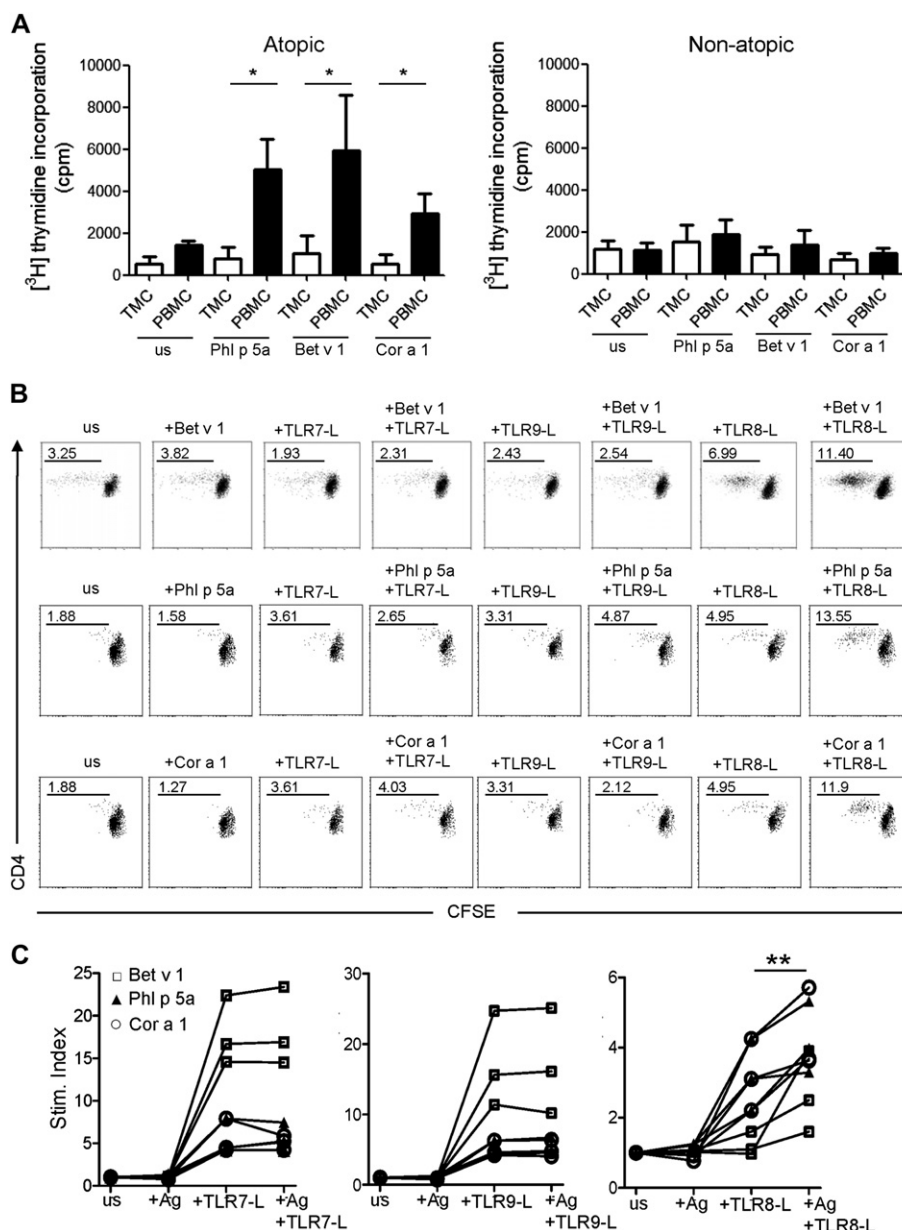


FIG 3. TLR8 triggering breaks allergen-specific unresponsiveness in human TMCs. **A**, TMCs ($n = 4$) and PBMCs ($n = 7$) from nonatopic subjects and TMCs ($n = 3$) and PBMCs ($n = 3$) from atopic subjects were cultured alone (*us*) or with the indicated allergens. Data represent means with SEMs. $*P < .05$. **B**, Proliferation of CFSE-labeled $CD4^+$ TMCs after 5 days of activation with the indicated stimulus. The mean percentage of proliferating cells of at least 2 independent experiments is shown. **C**, TMCs ($n = 9$) from 3 different donors were cultured with the indicated stimulus for 5 days. Proliferative responses were measured by using tritiated thymidine incorporation. Background of proliferation ranged between 102 and 460 cpm. *Stim. Index*, Stimulation index. $**P < .01$.

addition, neither IL-17 nor IL-23 was capable of breaking allergen-specific T-cell proliferation alone or in the presence of allergens (see Fig E5).

To shed light into the potential mechanisms involved in breaking allergen-specific tolerance, we performed experiments using a specific peptide inhibitor of MyD88, which is a key adapter molecule for TLR and IL-1 β signaling pathways. Our data indicated that MyD88 is an essential downstream molecule in the breaking of $CD4^+$ T-cell tolerance to allergens mediated by TLR4 and TLR8-Ls, as well as by IL-1 β (Fig 5, C, and see Fig E6

in this article's Online Repository at www.jacionline.org). In all these conditions, blocking of MyD88 signaling did not allow the breaking of allergen-specific tolerance.

mDCs, but not pDCs, can break allergen-specific $CD4^+$ T-cell tolerance

Because specific mDC-stimulating, but not pDC-stimulating, TLR triggers break allergen-specific tolerance, we investigated the potential role of specific DC subsets in loss of peripheral T-cell

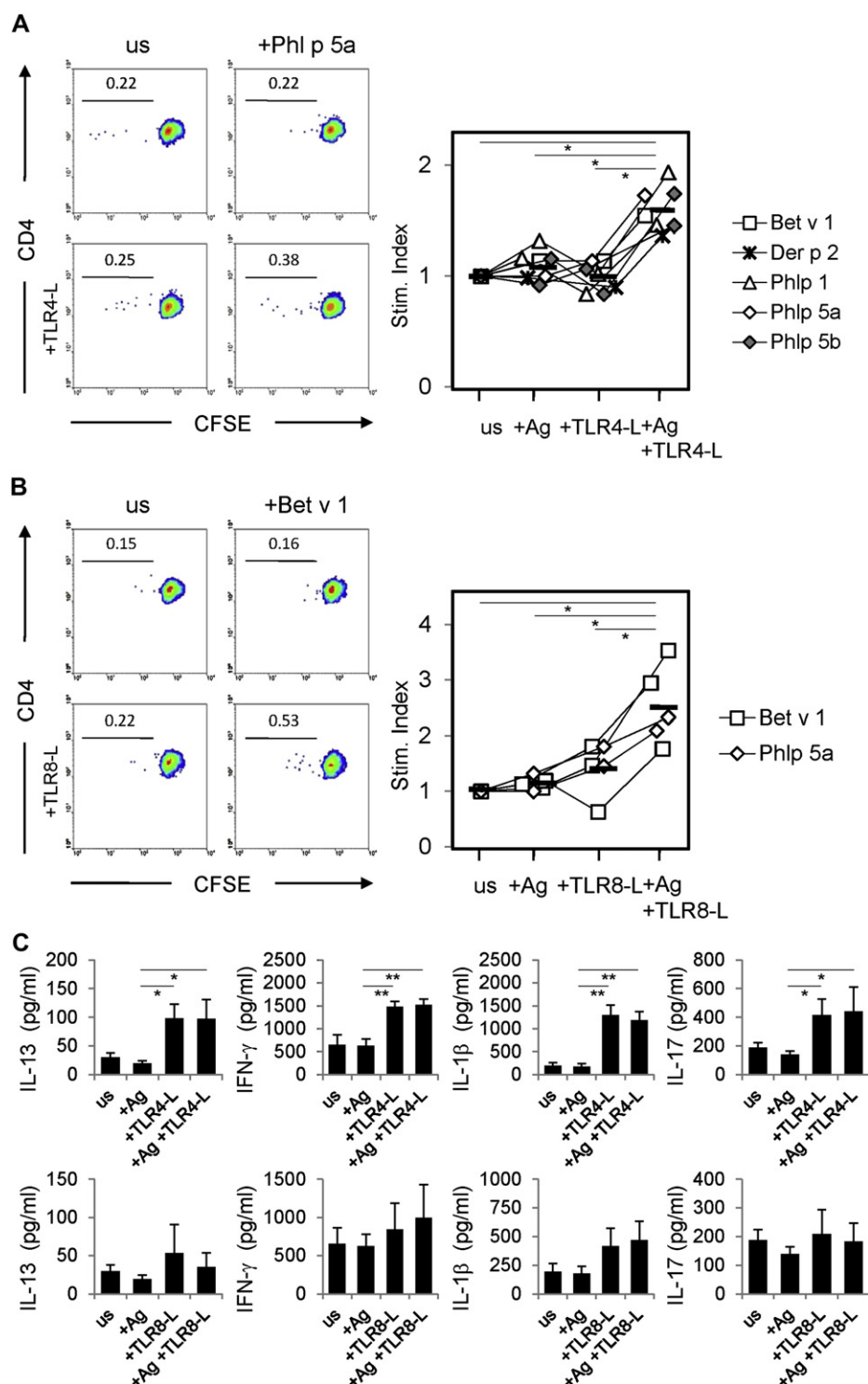


FIG 4. Triggering of TLR4 and TLR8 breaks peripheral allergen-specific CD4⁺ T-cell tolerance. **A**, Proliferation of CFSE-labeled CD4⁺ PBMCs after 5 days of stimulation with TLR4-L alone or in combination with Phl p 5a. **B**, Stimulation with TLR8-L alone or in combination with Bet v 1. One representative example of 4 with similar results is shown. The *graphs* show a minimum of 5 proliferative experiments by using tritiated thymidine incorporation. Background of proliferation was less than 2000 cpm. **P* < .05. Stim. Index, Stimulation index. **C**, PBMCs (*n* = 5) were stimulated with the indicated stimulus for 5 days. IL-13, IFN- γ , IL-1 β , and IL-17 levels in supernatants were quantified. **P* < .05, ***P* < .01. Ag, Antigen; us, unstimulated.

tolerance to allergens in experiments with freshly isolated mDCs or pDCs. PBMCs from healthy donors showing no significant T-cell proliferation to different allergens were cultured with

different allergens in the presence of 2.5×10^4 autologous freshly isolated mDCs. As shown in Fig 6, A, stimulation of PBMCs with allergens in the presence of autologous mDCs induced significant

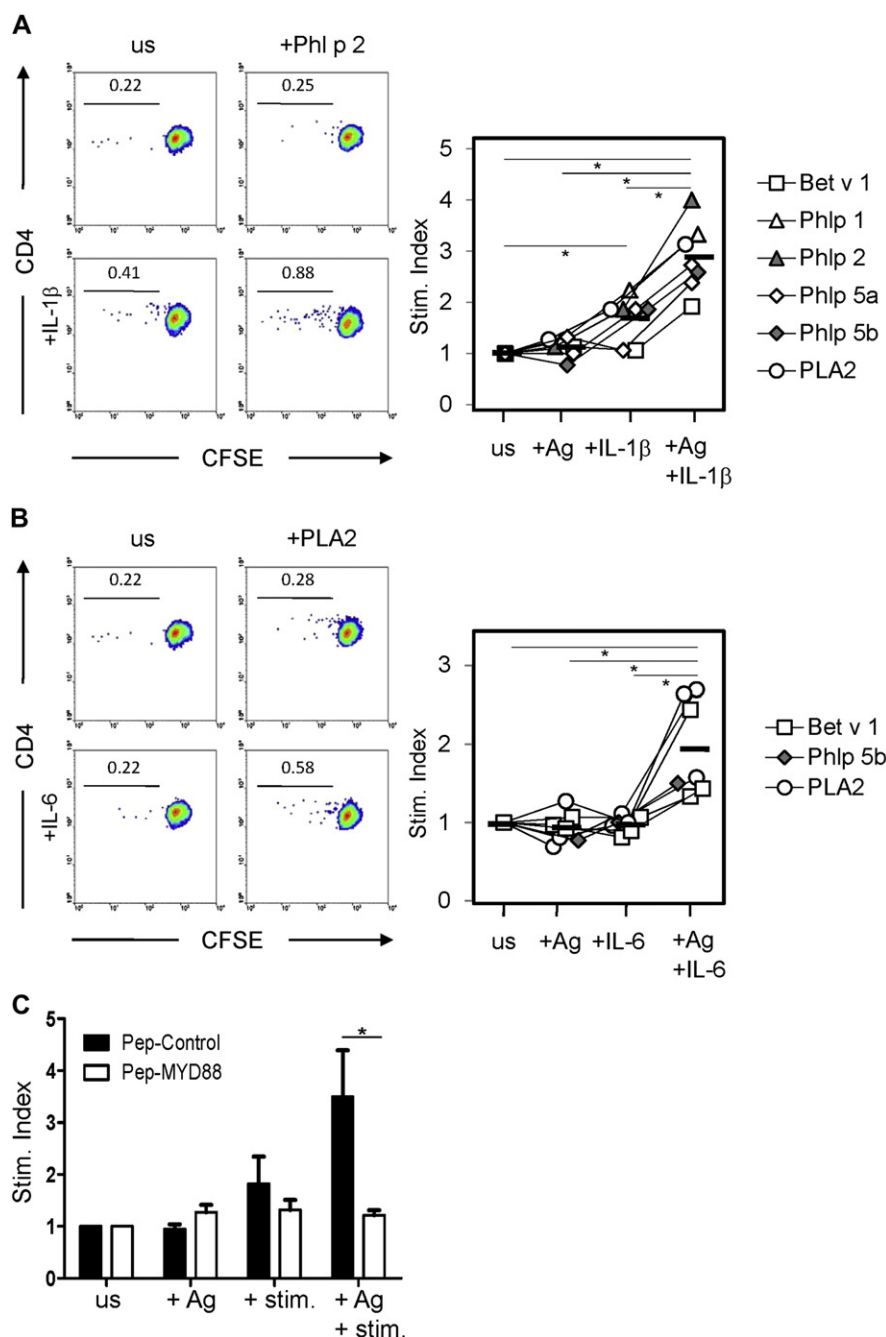


FIG 5. Stimulation with IL-1 β or IL-6 breaks allergen-specific CD4⁺ T-cell tolerance. **A**, Proliferation of CFSE-labeled CD4⁺ PBMCs after 5 days of stimulation with IL-1 β alone or in combination with Phl p 2. **B**, Stimulation with IL-6 alone or in combination with PLA₂. One representative example of 4 with similar results. The graphs show 7 individual proliferative experiments using tritiated thymidine incorporation. Background of proliferation was less than 2000 cpm. **P* < .05. **C**, Proliferative responses of PBMCs preincubated with a peptide inhibitor of MYD88 (*Pep-MYD88*) or the corresponding peptide control (*Pep-Control*) and stimulated as indicated were measured by using tritiated thymidine incorporation. The data represent the means with SEMs of 4 independent experiments. *Stim. Index*, Stimulation index. **P* < .05. *Ag*, Antigen; *us*, unstimulated.

allergen-specific CD4⁺ T-cell proliferation compared with that seen in PBMCs with allergens alone or PBMCs enriched with mDCs alone, thus indicating that mDCs play a role in the breaking of allergen-specific T-cell tolerance. In contrast, when PBMCs from healthy unresponsive donors were stimulated with allergens in the presence of equivalent numbers of pDCs (2.5×10^4 cells), a tolerance-breaking effect was not observed (Fig 6, B). The profile

of cytokines secreted by CD4⁺ T cells after stimulation with mDCs or pDCs is shown in Fig 6, C. Interestingly, after stimulation of PBMCs with mDCs in the presence of allergens, a significantly greater production of the proinflammatory cytokine IL-1 β was observed compared with that seen after stimulation with mDCs alone, which is probably due to the contribution of the few proliferating allergen-specific CD4⁺ T cells. We observed

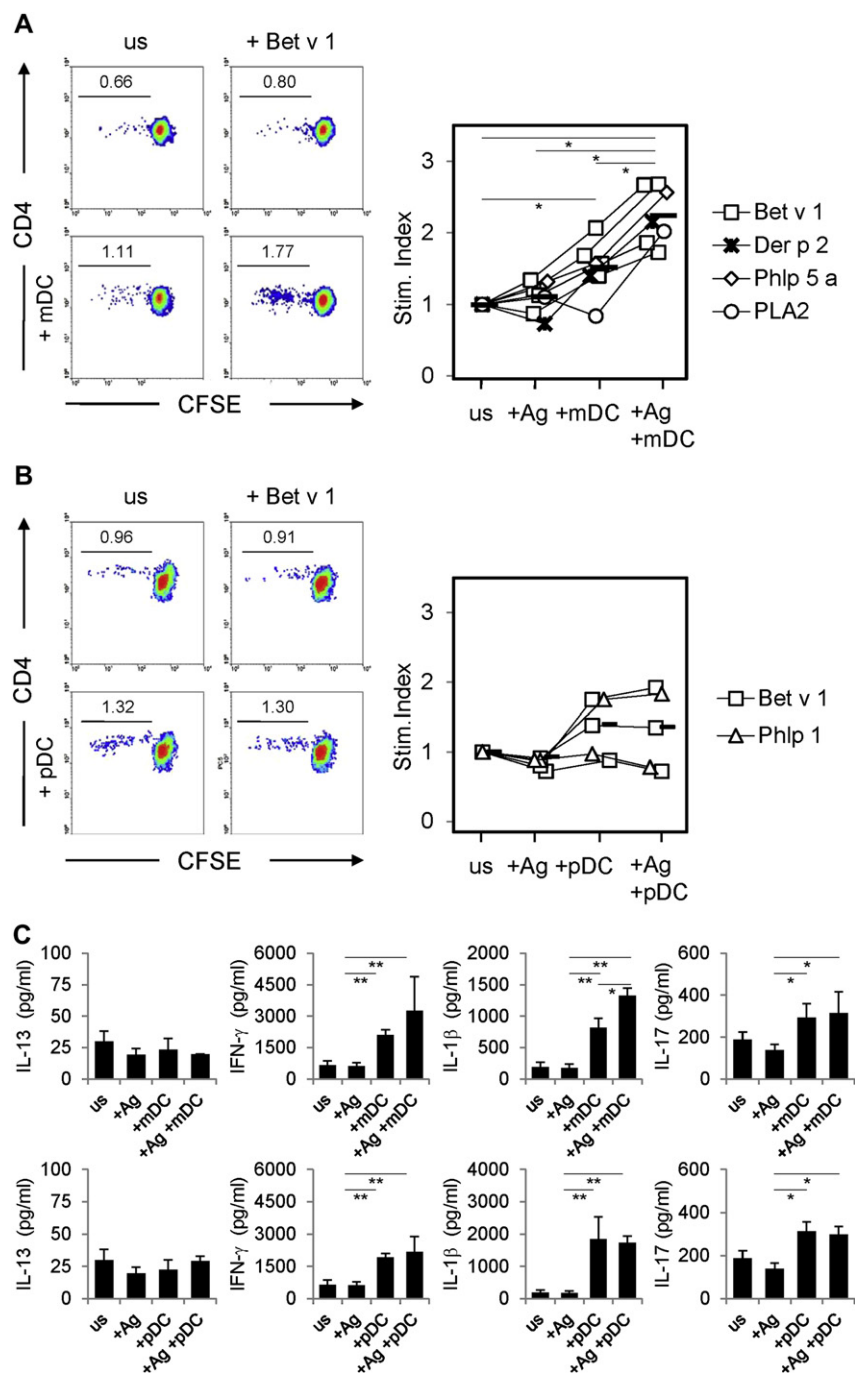


FIG 6. mDCs are potent in the breaking of peripheral allergen-specific CD4⁺ T-cell tolerance. **A**, Proliferation of CFSE-labeled CD4⁺ PBMCs from nonallergic but sensitized subjects after 5 days of stimulation with Bet v 1 alone or in the presence of an additional 2.5×10^4 purified autologous mDCs. One representative example of 4 with similar results. The graph shows 7 individual proliferative experiments using tritiated thymidine incorporation. * $P < .05$. **B**, The same type of experiments in the presence of an equivalent number of pDCs ($n = 5$). Stim. Index, Stimulation index. **C**, PBMCs ($n = 5$) were unstimulated as indicated for 5 days. IL-13, IFN- γ , IL-1 β , and IL-17 levels in supernatants were quantified. * $P < .05$, ** $P < .01$. Ag, Antigen; us, unstimulated.

that pDCs do not break allergen tolerance; however, they do induce a general nonspecific CD4⁺ T-cell proliferation that does not further increase in allergen-stimulated conditions.

To analyze the direct effects of proinflammatory cytokines or TLR-Ls breaking allergen-specific T-cell tolerance on mDCs,

freshly purified mDCs were stimulated with IL-1 β , IL-6, or TLR8-L, and the expression of the specific surface markers HLA-DR, OX40 ligand (OX40L), CD80, and dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) were analyzed by means of flow cytometry. TLR8-L

was the most potent activator of mDCs, significantly enhancing the expression of HLA-DR and slightly increasing the expression of OX40L on mDCs (see Fig E7, A, in this article's Online Repository at www.jacionline.org). In addition, the expression of the costimulatory molecule CD80 was significantly increased on mDCs only after TLR8-L stimulation. In contrast, DC-SIGN was significantly downregulated on mDCs after stimulation with TLR8-L. Interestingly, the HLA-DR/DC-SIGN or CD80/DC-SIGN ratio on mDCs increased after activation with TLR8-L but not after stimulation with IL-1 β or IL-6 (see Fig E7, B).

To analyze potential synergistic effects between the proinflammatory cytokines IL-1 β or IL-6 and mDCs in breaking allergen-specific CD4⁺ T-cell tolerance, CFSE-labeled PBMCs from a well-characterized healthy subject sensitized to the bee venom major allergen phospholipase A₂ (PLA₂) were stimulated with PLA₂ allergen alone, IL-1 β or IL-6 alone, or IL-1 β or IL-6 in combination with PLA₂ allergen with or without mDC enrichment (see Fig E8 in this article's Online Repository at www.jacionline.org). As expected, CD4⁺ T cells did not proliferate after stimulation with PLA₂, IL-1 β , or IL-6 alone. Interestingly, the tolerance-breaking capacity, as demonstrated by IL-1 β and IL-6, was significantly potentiated in the presence of autologous mDCs, suggesting a synergistic effect between mDCs and IL-1 β or IL-6.

DISCUSSION

Our results show that tonsils are organs in which regulation of immune responses to allergens take place that show an essential difference in atopic patients, thus representing a very suitable *in vivo* model to assess mechanisms of breaking allergen-specific T-cell tolerance. We demonstrate how peripheral T-cell tolerance to major environmental allergens can be broken by triggering of TLR4 or TLR8 and by the proinflammatory cytokines IL-1 β or IL-6 in human tonsils and peripheral blood. We also show that relatively low numbers of matured mDCs, but not pDCs, are able to induce proliferation of tolerized allergen-specific CD4⁺ T cells and that this DC subset mediates these effects through mechanisms that partially depend on the upregulation of HLA-DR and the costimulatory molecules OX40L and CD80.

In this study we used human tonsils, as well as peripheral blood, from healthy donors sensitized to the allergens of interest with specific IgG but not proliferative responses to the studied allergens to assess the capacity of danger signals (TLR4-L, TLR7-L, TLR8-L, and TLR9-L) or the cytokines (IL-1 β , IL-6, IL-17, and IL-23) in the breaking of allergen-specific peripheral T-cell tolerance. We have previously shown that human tonsillar T cells do not show significant proliferative responses to common allergens, which is functionally dependent on the presence of large numbers of allergen-specific FOXP3⁺ Treg cells.¹³ Therefore we hypothesized that human tonsils might well represent a very suitable model to study mechanisms of breaking allergen-specific T-cell tolerance. First, we wanted to know whether human tonsils reflect the classical features of peripheral immune responses to allergens in atopic and healthy subjects *in vivo*. The direct analysis of tonsillar tissue mRNA expression indicated that the T_H1/T_H2 balance has shifted toward a T_H2 phenotype, with low levels of T-bet and IFN- γ in the tonsils of atopic subjects. In addition, the IL-10 expression was significantly less in tonsils from atopic subjects. The same tendencies were demonstrated in functional experiments after stimulation of freshly isolated TMCs or purified tonsillar CD4⁺ T cells from atopic and nonatopic

subjects with the major grass and birch pollen allergens Phl p 5a and Bet v 1 or with a polyclonal T-cell activator, such as anti-CD2/CD3/CD28 mAbs. These results indicated that tonsils reflect the classical features of peripheral immune responses to allergens in atopic subjects, with low expression of the T_H1 cell-specific transcription factor T-bet and the cytokine IFN- γ , as well as the immune tolerance-related IL-10.^{17,36}

Next, we evaluated whether innate inflammatory signals specifically triggering pDCs or mDCs affect T-cell tolerance because viral and bacterial infections have been repeatedly proposed to initiate and exacerbate allergic diseases. We demonstrated that triggering of TLR8, but not TLR7 and TLR9, breaks allergen-specific tolerance in tonsils. Considering that pDCs selectively express TLR7 and TLR9 and that mDCs express TLR8,³⁷ these results are in line with our previous data demonstrating the important role of pDCs in the induction and maintenance of tolerance to food and aeroallergens in human palatine tonsils.¹³ Recently, McClory et al¹⁴ have demonstrated human tonsils as organs in which the development of T cells out of the thymus can occur *in vivo*, supporting human tonsils as a very suitable target for immune interventions.¹⁵ In addition, stimulation of PBMCs with ligands for TLR4 or TLR8, but not TLR7 or TLR9, in combination with allergens also leads to increased allergen-specific CD4⁺ T-cell proliferation, suggesting a general role for mDCs in breaking allergen-specific T-cell tolerance.³⁸

Interestingly, IL-1 β or IL-6, but not IL-17 or IL-23, enables CD4⁺ T-cell proliferation after allergen stimulation of PBMCs from tolerized subjects. The combination of IL-1 β and IL-6 did not synergistically enhance allergen-specific CD4⁺ T-cell responses, suggesting different mechanisms of action.³¹ The observed tolerance-breaking effects might be associated with the proinflammatory milieu formed on IL-1 β stimulation because this cytokine displays very potent proinflammatory properties.^{31,39,40} In the same way different studies showed that IL-6 impairs Treg cell function by inhibiting their suppressive capacity.^{30,31,41-46} Our results showing that IL-6 or TLR8-L stimulation significantly enhances allergen-specific T-cell proliferation support the existence of a potential TLR8/IL-6 axis playing a key role in breaking allergen-specific T-cell tolerance. In this sense Pasare and Medzhitov³⁰ previously demonstrated that in response to TLR8 triggering, increased levels of IL-6 block the suppressive capacity of CD4⁺CD25⁺ Treg cells, which in turn leads to the induction of specific adaptive immune responses, breaking the unresponsiveness of T cells. To gain insight into the potential mechanisms involved in breaking tolerance to allergens, we further investigated the role of MyD88, a key intracellular adaptor molecule mediating TLR and IL-1 β signaling. Our data show that MyD88 is an important downstream molecule after IL-1 β , TLR4, or TLR8 triggering, leading to breaking of tolerance to allergens. Although MyD88 is an adaptor molecule also involved in TLR7 and TLR9 downstream signaling,⁴⁷ the activation of alternative pathways specific for such TLRs and the role of other specific molecules, such as cytokines, might contribute to defining the final outcome related to the tolerance state to allergens. In this sense our cytokine results indicate that under tolerance-breaking conditions, a significant proinflammatory cytokine microenvironment is generated with mixed T_H1, T_H2, and T_H17 responses in the presence of IL-1 β that might favor the proliferation of relatively low populations of allergen-specific CD4⁺ T cells that could in turn also contribute to the production of proinflammatory cytokines. As observed in the CFSE dilution experiments after 5 or

6 divisions, the percentage of proliferating cells was in the range of 0.3% to 2.1%. Probably for this reason, although CD4⁺ T cells started to proliferate and tolerance was broken, we did not see additional significant changes in cytokine profiles. This is because tolerance-breaking conditions already induced a high level of cytokine release, which was not affected by proliferating a small quantity of the allergen-specific T cells. An increase in cytokine production with the addition of allergen was especially visible when PBMCs were stimulated with mDCs in combination with allergens at the IL-1 β production level. An increase in IL-13 and IL-17 levels was seen during allergen-specific T-cell tolerance-breaking conditions, whereas neither IL-13 nor IL-17 levels showed any increase with TLR7 and TLR9 in the presence of allergens under conditions that did not break tolerance.

To further analyze the role of mDCs in breaking allergen-specific T-cell tolerance, we purified this DC subset from peripheral blood to quantify expression levels of the costimulatory molecules HLA-DR, OX-40L, CD80, and DC-SIGN after activation with agents that are able to break peripheral allergen-specific T-cell tolerance. Our results showed an increase in HLA-DR, CD80, and OX40L expression on mDCs in response to TLR8-L but not after stimulation with IL-1 β or IL-6. This finding supports the idea that the tolerance-breaking effects mediated by TLR8 triggering occur through mechanisms enhancing the antigen-presenting capacity of mDCs. TLR8-L downregulated the expression of DC-SIGN on mDCs, which might be explained by the negative feedback loop reported between C-type lectin receptors and TLRs.^{48,49} The dysregulation of the balance between the expression levels of TLRs and C-type lectin receptors in favor of the former determines the induction of adaptive immune responses versus tolerance.

In conclusion, our findings demonstrate that specific stimuli that are able to activate innate immunity also condition specific adaptive immune responses to allergens, suggesting a mechanism that explains how healthy subjects can have allergic diseases after encountering microbes or inflammatory conditions. The identification of the factors leading to the loss of peripheral allergen-specific T-cell tolerance and the better understanding of the pathways involved in such mechanisms will allow the design of alternative strategies aimed at preventing the development of allergic diseases, as well as improving the current treatment modalities.

Clinical implications: The identification of factors and mechanisms involved in breaking allergen-specific T-cell tolerance will allow the design of novel strategies aiming at preventing the development of allergic diseases and improving current treatments.

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METHODS

Antigens and reagents

rBet v 1 of birch pollen (*Betula verrucosa*), rDer p 1 and rDer p 2 of house dust mite (*Dermatophagoides pteronyssinus*), rCor a 1 of hazelnut (*Corylus avellana*), and rPhl p 1, rPhl p 2, rPhl p 5a, and rPhl p 5b of grass pollen allergens were used (Allergopharma, Joachim Ganzer KG, Reinbek, Germany). All of the allergens were greater than 99% pure and did not contain detectable amounts of LPS using the Limulus assay (Cambrex Bio Science, Walkersville, Md). The optimum dose of allergen has been determined in dose-titrated preliminary experiments to be 0.6 $\mu\text{mol/L}$. TLR4-L LPS from *Escherichia coli* 055:B5 (100 ng/mL; Sigma-Aldrich, St Louis, Mo), TLR7-L Gardiquimoid (1 $\mu\text{mol/L}$; InvivoGen, San Diego, Calif), TLR8-L CL075 (3M002; 1 $\mu\text{mol/L}$; InvivoGen), and TLR9-L type B CpG2006 (1 $\mu\text{mol/L}$; Mycrosynth, Balgach, Switzerland) were used. IL-1 β (R&D Systems, Minneapolis, Minn) and IL-6 (PeproTech, Rocky Hills, NJ) were used at a concentration of 5 ng/mL, whereas IL-17a (PeproTech) and IL-23 (Alexis Corporation, Lausen, Switzerland) were used at a concentration of 10 ng/mL, all after preliminary titration experiments for optimal dose.

RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR

Tissues that had been previously stabilized in RNAlater were homogenized in grinding tubes containing CK28 ceramic balls (Bertin, Montigny-le Bretonneux, France) by using a Precellys 24 homogenizer (Bertin) at 6000 rpm $\times$ 2 $\times$ 50 seconds to isolate total RNA directly from palatine tonsils. Total RNA from cell samples was isolated by using the RNeasy mini kit (Qiagen).

Reverse transcription was performed with the Revert Aid M-MuLV Reverse Transcriptase (Fermentas, St Leon-Rot, Germany) using random hexamer primers according to the manufacturer's protocol. Gene expression was analyzed by means of quantitative real-time PCR with the iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories, Hercules, Calif) on a 7900HT Fast Real-Time PCR instrument (Applied Biosystems, Foster City, Calif). PCR primers were designed based on sequences reported in GenBank with the Primer Express Software version 1.2 (Applied Biosystems). The sequences of primer pairs were as follows: elongation factor 1 α (EF1 α : forward, CTGAACCATCCAAT; reverse, GCCGTGTGGCAATCCAAT), FOXP3 (forward, GAAACAGCACATTCCAGAGTTC; reverse, ATGGC CCAGCGGATGAG), T-bet (forward, GATGCGCCAGGAAGTTTCAT; reverse, GCACAATCATCTGGGTACATT), GATA3 (forward, GCGG GCTCTATCAGAAAATGA; reverse, GCTCTCTGGCTGCAGACAGC), IFN- γ (forward, TCTCGGAAACGATGAAATATACAAGTTAT; reverse, GTAACAGCCAAGAGAACCCAAA), IL-10 (forward, GTGATGCCC CAAGCTGAGA; reverse, CACGGCCTTGCTTGTGTTT), IL-13 (forward, GCCCTGGAATCCCTGATCA; reverse, GCTCAGATCCTCTGGGTC TT), and TGF- β (forward, AAATTGAGGGCTTTCGCCTTA; reverse, GAACCCGTTGATGTCCACTTG). PCR conditions were 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 90°C and 1 minute at 60°C. Relative quantification was performed by using the comparative $\Delta\Delta$ cycle threshold (CT) method, as previously described.^{E1} All the amplifications were conducted in triplicates, and the housekeeping gene EF1 α was used for normalization. Arbitrary units show the $2^{-(\Delta\text{CT})}$ values multiplied by 10^4 , where ΔCT corresponds to the difference between the CT value for the gene of interest and EF1 α .

Purification of cells

PBMCs were isolated by means of Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation from heparinized peripheral venous blood and resuspended in supplemented RPMI-1640 (cRPMI) medium, as described previously.^{E2} TMCs were isolated from freshly excised palatine tonsils, as previously described.^{E3} mDCs or pDCs were purified from PBMCs by using positive selection of CD1c⁺ or CD304⁺ cells with the BDCA-1⁺ or BDCA-4/Neuropilin-1 DC isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively, according to the manufacturer's protocol.

CFSE labeling

PBMCs were labeled with CFSE to measure T-cell proliferation in response to stimulation. A total of 2×10^7 cells were labeled with CFSE (5 $\mu\text{mol/L}$;

Molecular Probes Europe, BV, Leiden, The Netherlands) for 6 minutes at 4°C and washed twice with cRPMI. Cells were resuspended in cRPMI and counted before use.

Cell cultures

PBMCs or TMCs were cultured with IL-1 β , IL-6, IL-17, IL-23, TLR4-L, TLR7-L, TLR8-L, or TLR9-L alone or in combination with the allergens mentioned above. Cells (6×10^5) were stimulated in 48-well culture plates at 37°C in a 5% CO₂ atmosphere for 5 days, and proliferative responses were analyzed by using flow cytometry after staining of CFSE-labeled cells with PC5-conjugated anti-CD4 (Beckman Coulter, Fullerton, Calif). Stimulation indices were calculated as the ratio of the percentage of proliferating cells after stimulation and under unstimulated conditions. Alternatively, proliferation of unlabelled cells was measured by adding 1 μCi of tritiated thymidine (Hartmann Analytic, Braunschweig, Germany) during the last 8 hours of culture. For tritiated thymidine incorporation experiments, stimulation indices were calculated as the ratio between the counts per minute value obtained after antigen or other stimulus and the counts per minute value obtained for the corresponding unstimulated condition. Freshly isolated TMCs (5×10^5 cells in 500 μL) from atopic and nonatopic subjects were stimulated with 1 $\mu\text{g/mL}$ Phl p 5a or Bet v 1 for 5 days or left unstimulated. At day 5, supernatants were collected for cytokine determinations by using cytometric bead arrays (Bio-Rad Laboratories), and cell pellets were used to isolate total RNA for gene expression analysis by using quantitative PCR. Tonsillar CD4⁺ T cells were isolated from TMCs of atopic and nonatopic donors by using the CD4⁺ T-cell isolation kit II, according to the manufacturer's indications with slight modifications. The cells were stimulated with anti-CD2/CD3/CD28 mAbs for 24 hours, and cytokine levels in cell-free supernatants were determined. For MyD88 inhibition experiments, PBMCs were preincubated with a specific peptide inhibitor of MyD88 linked to a membrane-targeting peptide or the corresponding peptide control (Pep-MYD and Pep-Control from InvivoGen) for 3 hours, washed, and stimulated with allergens alone; with IL-1 β , TLR4-L, or TLR8-L alone; or with allergens in the presence of the indicated stimulus for 5 days. Proliferative responses were measured by adding 1 μCi of tritiated thymidine during the last 8 hours of culture.

In the same experiments purified autologous mDCs or pDCs were preincubated with IL-1 β , IL-6, TLR4-L, TLR7-L, TLR8-L, or TLR9-L alone or in combination with the allergens. After 2 hours of preincubation, 6×10^5 CFSE-labeled or unlabeled autologous PBMCs were added and cocultured with mDCs or pDCs for 5 days. For assessment of surface molecule expression, mDCs were cultured with IL-1 β , IL-6, or TLR8 for 48 hours and then directly stained with fluorescein isothiocyanate (FITC)-conjugated anti-HLA-DR or anti-CD80, phycoerythrin-conjugated anti-OX40L or anti-DC-SIGN (all from PharMingen, San Diego, Calif), or PC5-conjugated anti-CD83 (Beckman Coulter) and analyzed by using flow cytometry. Analysis was performed with a Beckman Coulter Epics XL flow cytometer running Cell Quest software. WinMDI 2.9 software was used for data demonstration.

Statistical analysis

Data are expressed as means $\pm$ SEMs. Paired *t* tests and Wilcoxon signed-rank tests were used for assessing statistical significance with SPSS 17.0 software (SPSS, Chicago, Ill). The grade of statistical significance is displayed in the legend to the figures, and a *P* value of less than .05 was accepted as statistical significance. The stimulation index was calculated as the ratio of cell proliferation in stimulated conditions to proliferation in unstimulated cells.

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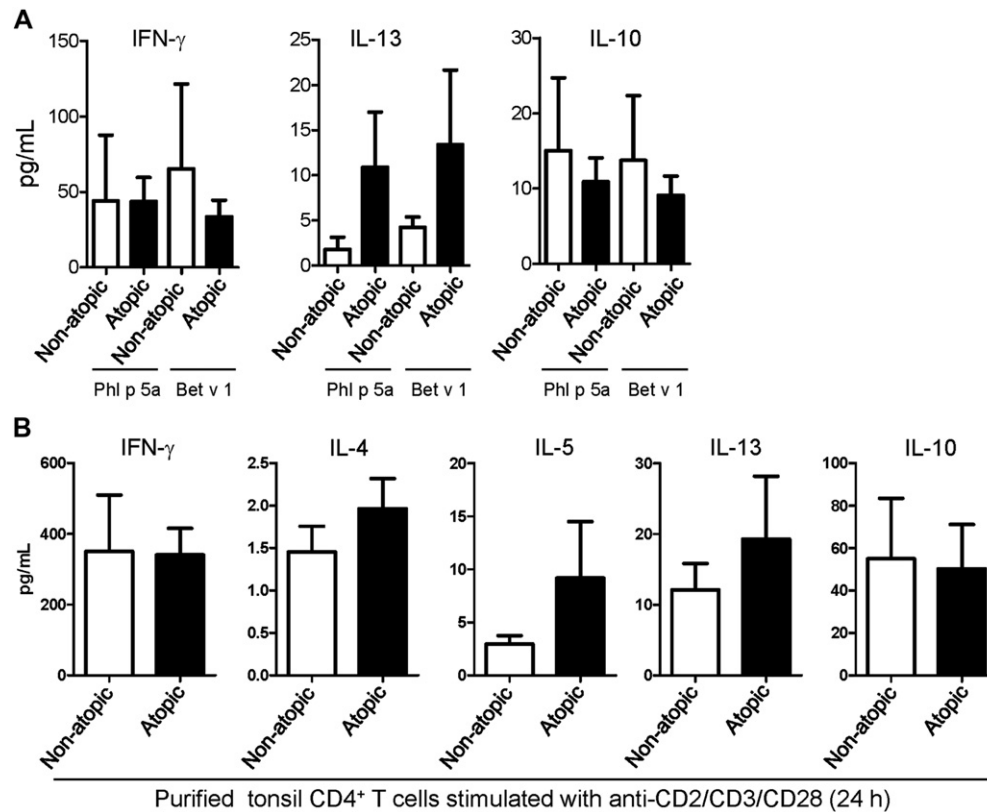


FIG E1. Cytokine profile of tonsillar T cells from atopic and nonatopic donors. **A**, TMCs from atopic ($n = 4$) and nonatopic ($n = 4$) subjects were stimulated with Phl p 5a or Bet v 1 for 5 days. **B**, Purified tonsillar CD4⁺ T cells from atopic ($n = 4$) and nonatopic ($n = 4$) subjects were stimulated with anti-CD2/CD3/CD28 for 24 hours. The levels of the shown cytokines in cell-free supernatants were quantified by using the cytometric bead array. All data are means with SEMs.

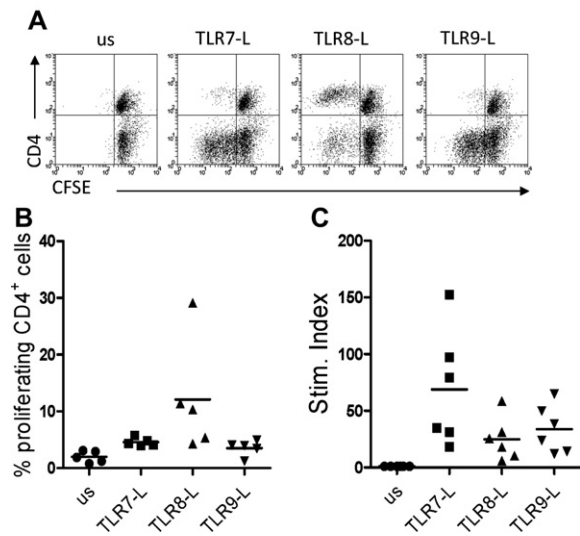


FIG E2. TMC proliferation after stimulation with TLR-Ls. **A**, CFSE-labeled TMCs were stimulated with TLR7-L, TLR8-L, or TLR9-L or left unstimulated (*us*) for 5 days. Proliferating CD4⁺ T cells were visualized by using flow cytometry. One representative example of at least 5 experiments with similar results is displayed. **B**, Quantification of the percentage of proliferating CD4⁺ T cells referred to unstimulated conditions ($n = 5$). **C**, Proliferative responses were also measured by using tritiated thymidine incorporation ($n = 6$). Background proliferation of unstimulated cells was less than 965 cpm. *Stim. Index*, Stimulation index. *us*, unstimulated.

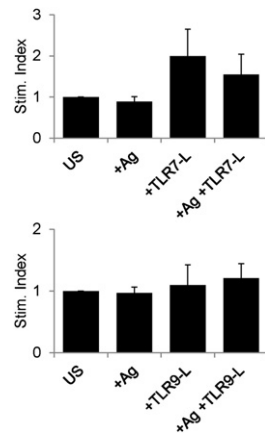


FIG E3. Stimulation of PBMCs with TLR7-L or TLR9-L does not break allergen-specific T-cell tolerance. Results of proliferative experiments with PBMCs after stimulation with allergens alone (+Ag) or in combination with TLR7-L ($n = 7$) or TLR9-L ($n = 9$) for 5 days by using tritiated thymidine incorporation are shown. The graphs show stimulation indices (*Stim. Index*). Background proliferation of unstimulated cells was less than 2000 cpm. All data are means with SEMs. US, Unstimulated.

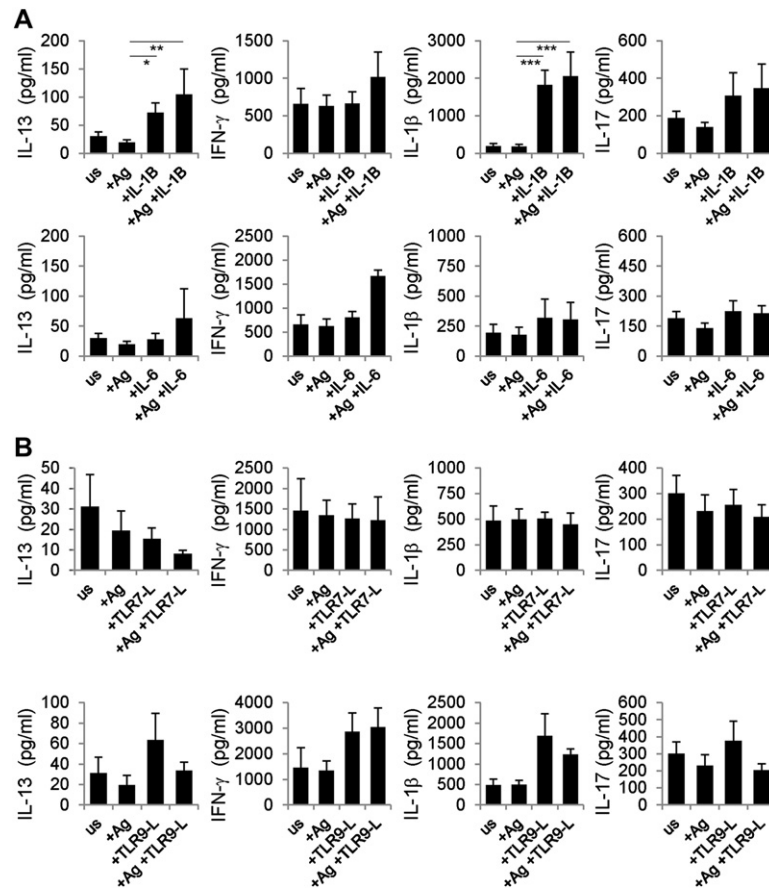


FIG E4. Cytokine profile of PBMCs after allergen stimulation in the presence of IL-1 β or IL-6. PBMCs were unstimulated or stimulated with allergens alone (+Ag), with IL-1 β (n = 5) or IL-6 (n = 5) alone, or with the allergens in the presence of the indicated cytokines for 5 days. The IL-13, IFN- γ , IL-1 β , and IL-17 levels in cell-free supernatants were quantified by using the cytometric bead array. * P < .05, ** P < .01, *** P < .001. us, Unstimulated.

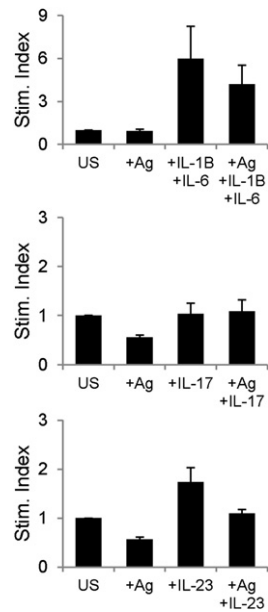


FIG E5. Simultaneous activation of PBMCs with IL-1 β and IL-6 induces nonspecific proliferation. IL-17 or IL-23 stimulation did not have tolerance-breaking effects. Proliferative experiments of PBMCs stimulated with allergens alone (+Ag) or in combination with IL-1 β and IL-6 simultaneously ($n = 4$) or IL-17 ($n = 4$) or IL-23 ($n = 4$) for 5 days by using tritiated thymidine incorporation. The *graphs* show stimulation indices (*Stim. Index*). Background proliferation of unstimulated cells was less than 2000 cpm. All data are means with SEMs. *US*, Unstimulated.

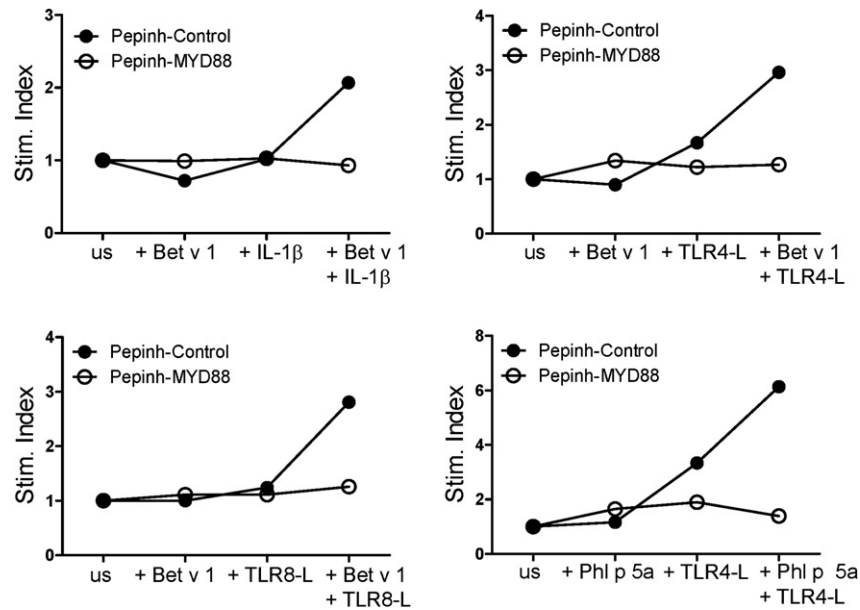


FIG E6. Role of MyD88 in breaking tolerance to allergens by IL-1 β , TLR4-L, and TLR8-L. PBMCs were preincubated for 3 hours with a peptide inhibitor of MyD88 (*Pepinh-MYD88*) or with the corresponding peptide control (*Pepinh-Control*), washed, and stimulated with the indicated allergens alone; with IL-1 β , TLR4-L, or TLR8-L alone; or with the allergens in the presence of the shown stimulus. Proliferative responses by tritiated thymidine incorporation of 4 independent experiments are shown. *Stim. Index*, Stimulation index. *us*, unstimulated.

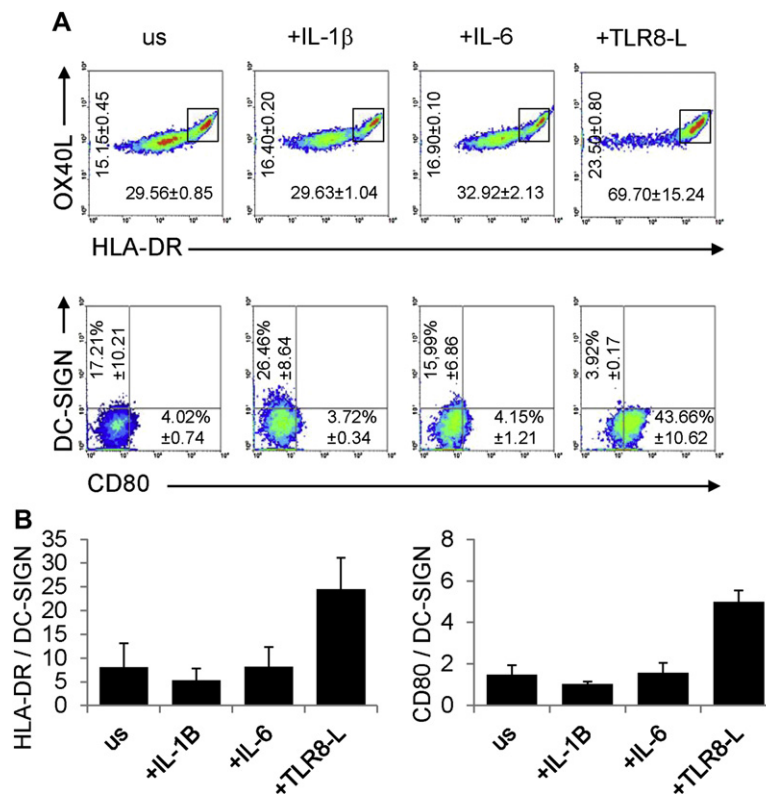


FIG E7. Characterization of mDCs breaking allergen-specific T-cell tolerance. **A**, Flow cytometric representative dot plots of HLA-DR/OX40L or DC-SIGN/CD80 in purified mDCs stimulated with IL-1 β , IL-6, or TLR8-L for 48 hours. One representative example of 3 with similar results is shown. **B**, The mean fluorescence intensity ratio of HLA-DR/DC-SIGN and CD80/DC-SIGN in purified mDCs after 48 hours of activation with IL-1 β , IL-6, or TLR8-L. All data are means with SEMs. us, Unstimulated.

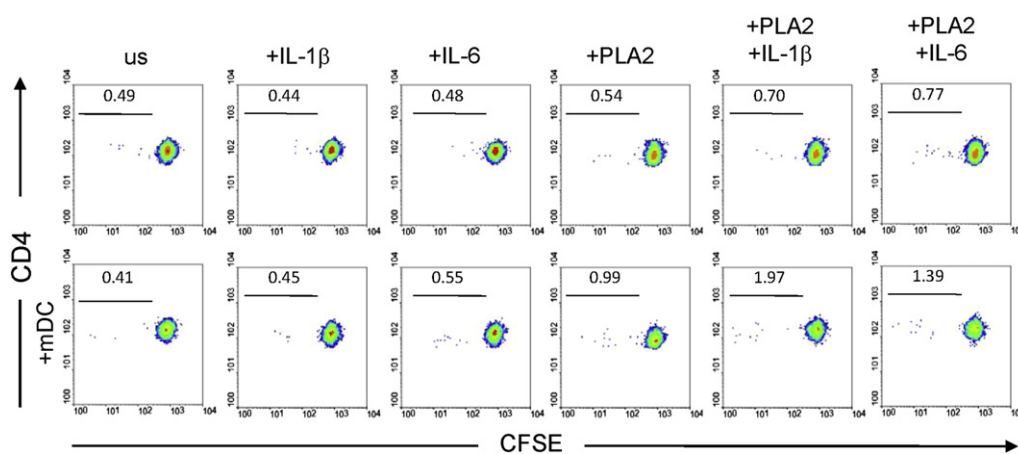


FIG E8. mDCs synergize with IL-1 β or IL-6 to break allergen-specific T-cell tolerance. Representative *dot plots* for the proliferation of CFSE-labeled CD4⁺ PBMCs from a nonallergic subject sensitized to PLA₂ after 5 days of stimulation with IL-1 β , IL-6 or PLA₂ allergen alone, IL-1 β and PLA₂ or IL-6 and PLA₂. The same proliferation assay was also performed in the presence of additional purified autologous mDCs.