

IL-33-activated dendritic cells are critical for allergic airway inflammation

Anne-Gaëlle Besnard^{1,2}, Dieudonné Togbe^{1,2,3}, Noëlline Guillou^{1,2},
François Erard^{1,2}, Valérie Quesniaux^{1,2} and Bernhard Ryffel^{1,2}

¹ University of Orleans, Molecular Immunology and Embryology, Orleans, France

² CNRS, UMR 6218, Orleans, France

³ Artimmune SAS, Centre d'Innovation, Orleans, France

IL-33, a new member of the IL-1 family cytokine, is involved in Th2-type responses in a wide range of diseases and signals through the ST2 receptor expressed on many immune cells. Since the effects of IL-33 on DCs remain controversial, we investigated the ability of IL-33 to modulate DC functions *in vitro* and *in vivo*. Here, we report that IL-33 activates myeloid DCs to produce IL-6, IL-1 β , TNF, CCL17 and to express high levels of CD40, CD80 OX40L and CCR7. Importantly, IL-33-activated DCs prime naive lymphocytes to produce the Th2 cytokines IL-5 and IL-13, but not IL-4. *In vivo*, IL-33 exposure induces DC recruitment and activation in the lung. Using an OVA-induced allergic lung inflammation model, we demonstrate that the reduced airway inflammation in ST2-deficient mice correlates with the failure in DC activation and migration to the draining LN. Finally, we show that adoptive transfer of IL-33-activated DCs exacerbates lung inflammation in a DC-driven model of allergic airway inflammation. These data demonstrate for the first time that IL-33 activates DCs during antigen presentation and thereby drives a Th2-type response in allergic lung inflammation.

Keywords: Allergy · Cytokines · DC · IL-33 · Lung inflammation



See accompanying Commentary by Akdis



Supporting Information available online

Introduction

Allergic asthma is a chronic disorder characterized by eosinophilic airway inflammation, mucus hypersecretion, antigen-specific-IgE antibodies, airway remodeling and increased airway hyperreactivity [1, 2]. The process of airway inflammation involves various cells types, such as eosinophils, mast cells, epithelial cells, lymphocytes and DCs. Th2 cells have been shown to play a predominant role in allergic asthma and Th2 cytokines, such as IL-4, IL-5 and IL-13, exacerbate disease severity [3, 4].

IL-33, the recently discovered Th2 cytokine, is found at high levels in the plasma of asthmatic patients [5, 6] and in the lungs of mice during experimental allergic asthma [7, 8].

IL-33 is a member of IL-1 family [9–11]. Like IL-1 β or IL-18, IL-33 is synthesized as a precursor and can be cleaved by caspase-1 and 3 but the cleavage products are biologically less active than the precursor [12, 13]. In contrast to the other IL-1 family members, IL-33 is mainly expressed in non-hematopoietic cells such as fibroblasts, epithelial cells and endothelial cells [10, 14, 15]. Because of its nuclear localization sequence, IL-33 is usually present in the nucleus, where it acts as a potential transcriptional repressor [16]. Recently, IL-33 has been shown to be released from necrotic cells and may act as an alarmin in a similar manner to IL-1 α [17] or high mobility group box1 protein HMGB1 [18, 19].

Correspondence: Bernhard Ryffel
e-mail: bryffel@cnrs-orleans.fr

IL-33 signals through a heterodimeric membrane receptor composed of ST2 and the IL-1R1 accessory protein and activates basophils [20, 21], mast cells [22, 23], eosinophils [24, 25], NK and NKT cells [26], Th2 lymphocytes [5, 27] and macrophages [14]. In accordance with its Th2 functions, administration of IL-33 into naive mice induces severe inflammation in the lung and digestive tract with elevated levels of IL-4, IL-5 and IL-13, splenomegaly and increased serum Ig [10]. In vitro, IL-33 has also been reported to polarize naive CD4⁺ T cells to produce IL-5 and IL-13, but not IL-4 [5]. Polarization of this atypical Th2 population is independent of IL-4, STAT6 and GATA3. On macrophages, IL-33 amplifies IL-13-mediated polarization of alternatively activated macrophages to produce CCL17 and CCL24, which contribute to airway inflammation [14].

Limited information is available on the role of IL-33 in DC function. During mouse bone-marrow derived DC (BMDC) differentiation, IL-33 was shown to promote DC generation in vitro by triggering GM-CSF production by other BM leukocyte populations [28]. Myeloid DCs generated in the presence of IL-33 exhibit a relatively limited T-cell stimulatory capacity compared with conventional DC cultures established with GM-CSF. On the other hand, Rank et al. reported that mature DC treatment with IL-33 upregulated cell-surface expression of MHC class II molecules and the costimulatory molecule CD86 [29]. Furthermore, IL-33-activated DCs could polarize CD4⁺ T cells to produce IL-5 and IL-13, suggesting that IL-33 may play a role in the induction of adaptive immune responses.

Since DCs are crucial for the promotion of allergic asthma, we investigated the role of IL-33 on DC function in the experimental model of allergic airway inflammation. First, we showed that DCs directly respond to IL-33 through ST2 leading to an upregulation of the cell surface costimulatory molecules CD40 and CD80, and an increased production of proinflammatory cytokines and chemokines IL-6, IL-1 β , TNF and CCL17 (TARC). DCs treated with IL-33 prime naive T cells to produce the pro-allergic cytokines IL-5 and IL-13. Reduced DC activation was observed in ST2-deficient mice compared with WT mice after allergen sensitization and challenge. Finally, we demonstrated that adoptive transfer of IL-33-activated DCs into naive recipient mice enhanced lung airway inflammation. Therefore, we showed here that IL-33 plays a critical role during antigen sensitization through DC activation.

Results

IL-33 activates BMDCs in vitro

DCs, the most powerful antigen-presenting cells, are considered the key players for initiating and maintaining an allergic Th2 immune response to inhaled allergens in asthma [30]. Since ST2 is expressed in DCs at both the transcriptional and protein levels [29], we decided to fully characterize effects of IL-33 on DCs. BMDCs from WT and ST2-deficient mice were cultured for

24 h with increasing concentrations of recombinant murine IL-33. We found that 1 ng/mL of IL-33 significantly activated DCs to produce the proinflammatory cytokines IL-6, TNF, IL-1 β and the chemokine CCL17 (Fig. 1A). In addition, IL-33 induced upregulation of CD40, CD80 and OX40L expression on DCs (Fig. 1B), but had no effect on MHC class II molecule or CD86 expression (data not shown). IL-33 effects were dose-dependent and the maximal effect was found at 10 ng/mL. IL-33 treatment had no effect on ST2-deficient BMDCs. These results demonstrate that IL-33 potently activates DCs through ST2, leading to IL-6, IL-1 β , TNF and CCL17 production and costimulatory surface molecule expression.

IL-33-activated DCs promote T-cell proliferation and Th2 polarization

To determine whether IL-33 increased the ability of DCs to stimulate T cells, we performed coculture experiments. DCs were cultured with or without IL-33 for 24 h, then washed twice and cocultured with CFSE-labelled naive CD4⁺ T cells from DO11.10 mice (OVA-TCR transgenic BALB/C mice) in the presence of the OVA-specific peptide. After 6 days of coculture, CD4⁺ T-cell proliferation was assessed by flow cytometry. IL-33-activated DCs increased T-cell proliferation compared with untreated DCs (Fig. 2A). To determine whether IL-33-activated DCs can affect T-cell polarization, we cocultured CD4⁺ T cells from naive WT or ST2-deficient mice with DCs in the presence of increasing doses of IL-33. DCs alone or WT T cells cultured alone were used as controls. Cytokine levels were analysed after 6 days in the culture supernatants. CD4⁺ T cells from WT mice cultured with DCs in the presence of IL-33 produced IL-5 and IL-13 in a dose-dependent manner (Fig. 2B and C), but no IL-4, IL-10 or IFN- γ (data not shown). This is consistent with previous reports, which showed that IL-33 drives an atypical Th2 response [5, 29]. Importantly, when cocultured with DCs and IL-33, naive CD4⁺ T cells from ST2-deficient were unable to produce IL-5 and IL-13 (Fig. 2B and C). Absence of IFN- γ or IL-10 in the coculture supernatant indicates that IL-33 does not favour Th1 or Treg polarization. Importantly, naive CD4⁺ T cells alone do produce cytokines when incubated with IL-33 (data not shown). These results indicate that IL-33 has no direct effect on naive T cells, and the presence of DCs is required for Th2 cells differentiation.

IL-33 exposure induces DC recruitment and activation in vivo

Recently, IL-33 was shown to directly activate conventional and alternative alveolar macrophages in vivo [5, 14]. We sought to determine the effect of IL-33 in vivo on DCs. We administered recombinant murine IL-33 (1 μ g/mouse) or saline to WT mice. After 24 h, lungs were digested and analysed for infiltrated lung cell differentiation and activation. IL-33 induced cell recruitment

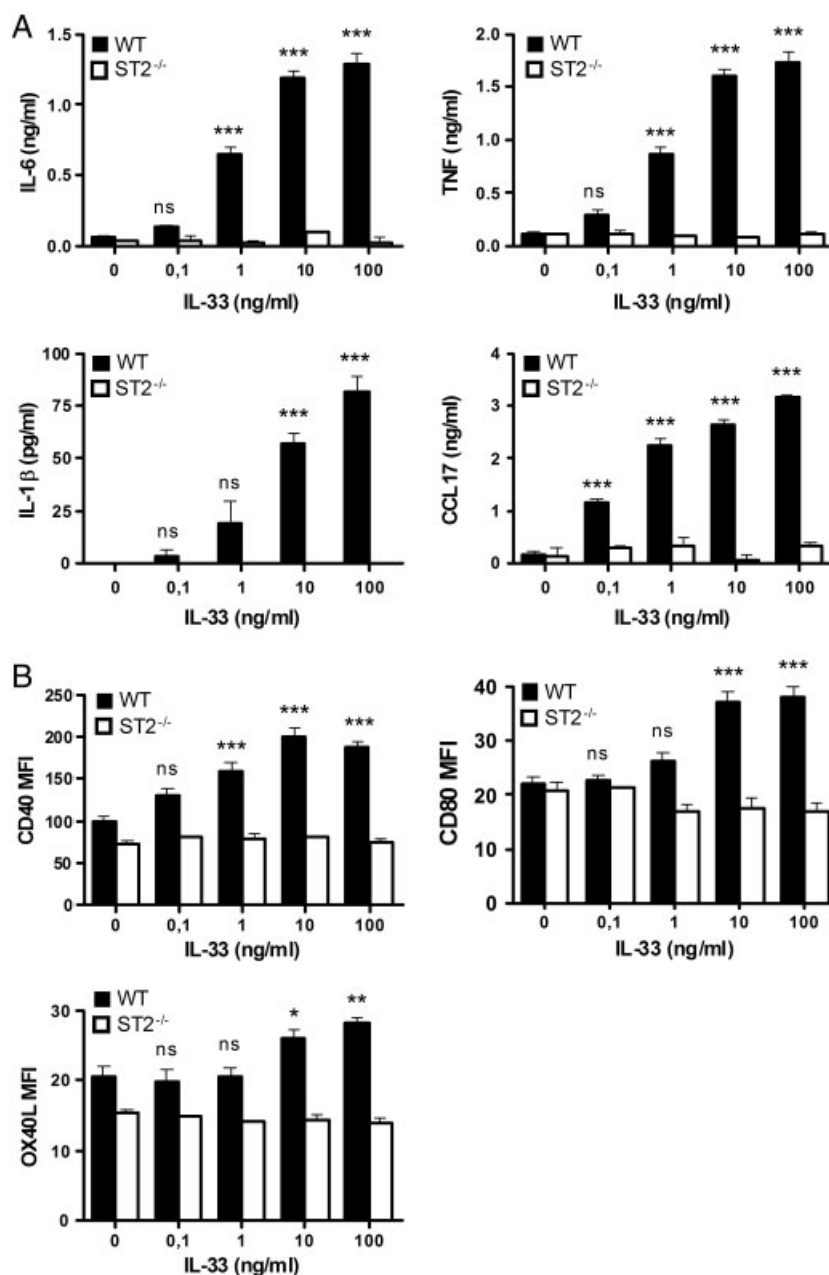


Figure 1. IL-33 activates BMDCs in vitro through the ST2 receptor. BMDCs from WT or ST2-deficient mice were cultured for 24 h with medium or recombinant murine IL-33 (0.1–100 ng/mL). (A) Supernatants were analysed for cytokines and chemokines by ELISA. (B) MFI of CD40, CD80 and OX40L were examined by flow cytometry on BMDCs after 24 h IL-33 stimulation. Data are presented as mean ± SEM and are representative of three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant. Statistical evaluation of differences between the experimental groups was determined by using one-way ANOVA followed by a Bonferroni post test.

into the lung (Fig. 3A), mainly CD11c⁺ cells, and there was no significant difference in T- and B-cell populations in IL-33-treated mice compared with PBS-control mice (Fig. 3B). Expression of CD40, CD80 and OX40L was increased on IL-33-treated CD11c⁺ cells compared with vehicle-treated control mice (Fig. 3C and D). In addition, DCs from IL-33-treated mice expressed higher levels of CCR7, which is known to be involved in DC migration to draining LNs (Fig. 3C and D). Thus, IL-33 activates directly in vivo DCs to express costimulatory molecules.

Reduced DC activation in ST2-deficient mice after OVA sensitization

To determine whether the observed properties of IL-33 on DCs had physiological significance, the immune response induced after OVA sensitization was assessed in WT and ST2-deficient mice. Mice were subcutaneously immunized twice with OVA or saline on days 0 and 7. DC activation in LNs was analysed a week after the last immunization. As expected, CD11c⁺ DCs from LNs

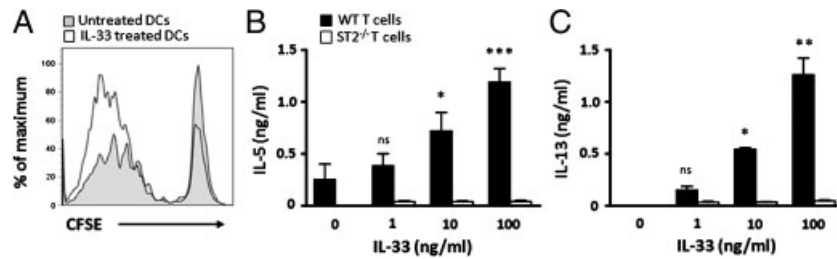


Figure 2. IL-33-activated DCs promote T-cell proliferation and Th2 polarization of naive CD4⁺ T cells. (A) CD4⁺ T cells purified from naive DO11.10 mice were labelled with CFSE and cocultured with untreated or IL-33-treated DCs at a ratio of 1:10 (T cells:DCs) in the presence of OVA peptide (5 µg/mL). T cells proliferation was analysed after 6 days of coculture by flow cytometry. Data are representative of two independent experiments (n = 5). (B, C) CD4⁺ T cells extracted from naive WT or ST2-deficient mice were cocultured with DCs with increased doses of recombinant murine IL-33. (B) IL-5 and (C) IL-13 levels were determined by ELISA in the supernatant after 6 days of coculture. Data are presented as mean ± SEM and are representative of two independent experiments. *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Statistical evaluation of differences between the experimental groups was determined by using one-way ANOVA followed by a Bonferroni post test.

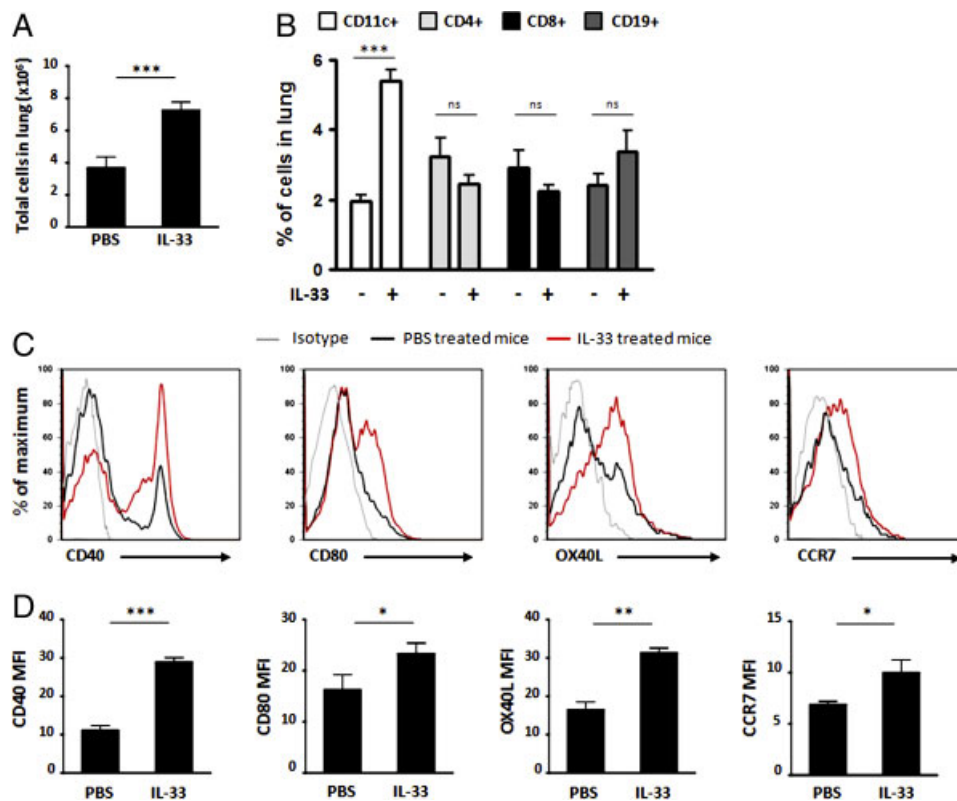


Figure 3. IL-33 directly activates DCs in vivo. Lungs from PBS or IL-33-treated WT mice were digested with DNase/collagenase. Lung mononuclear cells were stained for extracellular FACS analysis. (A) Total cell numbers and (B) percentage of CD11c⁺, CD4⁺, CD8⁺ and CD19⁺ cells are shown. (C) Representative histograms and (D) MFI of CD40, CD80 and OX40L and CCR7 are shown. Data are presented as mean ± SEM of six mice per group. Data are presented as mean ± SEM and are representative of two independent experiments. *p<0.05; **p<0.01; ***p<0.001; ns, not significant. Statistical evaluation of differences between the experimental groups was determined by using one-way ANOVA followed by a Bonferroni post test.

of OVA-sensitized mice displayed increased expression of the maturation molecules CD40 and CD80 compared with saline control mice (Fig. 4A). In contrast, CD40 and CD80 expression on DCs was significantly lower in OVA-immunized ST2-deficient mice compared with WT mice. MHC class II expression was similar in the two groups of mice (data not shown).

Cells isolated from LNs of WT or ST2-deficient mice were restimulated in vitro with anti-CD3 and anti-CD28 antibodies for

48 h to assess cytokines production. High levels of IL-4, IL-5 and IL-13 were found in cell culture supernatants from WT mice after OVA sensitization (Fig. 4B). By contrast, IL-4, IL-5 and IL-13 levels were significantly reduced in cell culture supernatant from OVA-treated ST2-deficient mice compared with WT mice. IFN-γ levels were similar in cell culture supernatant from WT and ST2-deficient mice. These data suggest that IL-33/ST2 pathway is involved in Th2 cells priming by DCs in vivo after antigen exposure.

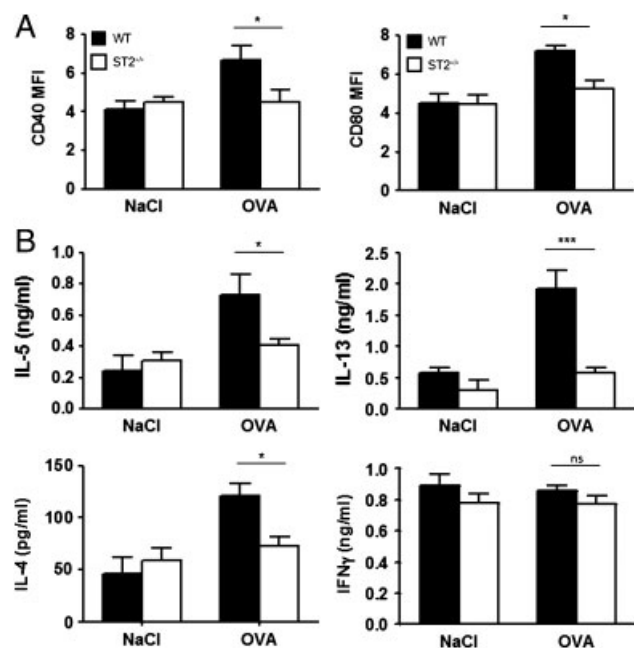


Figure 4. Reduced activation of DCs in ST2-deficient mice in LNs after OVA sensitization (A) WT or ST2-deficient mice were twice immunized with 10 μ g OVA or saline at days 0 and 7. CD11c⁺ cells from LNs were analysed for CD40 and CD80 expression at day 14 by flow cytometry. Data are presented as the mean $\pm$ SEM of MFI ($n = 6$). (B) Cells from LNs are restimulated in vitro with anti-CD3 and anti-CD28 for 48 h at 5×10^6 cells/mL. Cytokine expression levels were analysed in the supernatants by ELISA. * $p \leq 0.05$; *** $p \leq 0.001$; ns, not significant. Statistical evaluation of differences between the experimental groups was determined by using one-way ANOVA followed by a Bonferroni post test.

ST2-deficient mice develop attenuated allergic airway inflammation

Since we observed that DC activation and Th2 cytokines were reduced in ST2-deficient mice after allergen sensitization, we next compared the immune response of WT and ST2-deficient mice after OVA sensitization and challenge. WT and ST2-deficient mice were sensitized twice with OVA in the absence of aluminium adjuvant on days 0 and 7, and challenged intranasally with OVA on days 14–16. Control mice received saline during antigen challenge. Twenty-four hours after the last challenge, the severity of pulmonary inflammation was assessed. OVA-sensitized and challenged WT mice showed a significant increase of allergic lung inflammation with eosinophil influx in the BAL and lung (Fig. 5A and B), increased levels of IL-5 in the BAL (Fig. 5C), IL-1 β , IL-33, CCL5 (RANTES), CCL11 (eotaxin) and CCL17 (TARC) in the lung (Fig. 5E–I). The serum OVA-specific IgE level was also enhanced in WT mice challenged with OVA compared with saline mice (Fig. 5D). By contrast, all these parameters were reduced in OVA-sensitized and challenged ST2-deficient mice (Fig. 5A–I). Thus, lung IL-33 levels increased during effector phase but IL-33 overexpression was clearly less pronounced in ST2-deficient mice. In addition, the frequency and absolute number of IL-13-producing CD4⁺ T cells were reduced in OVA-sensitized and

challenged ST2-deficient mice compared with WT mice (Supporting Information Fig. S1 A and B). Histological analysis of the lung of asthmatic mice revealed peribronchial and perivascular cell infiltrations after OVA challenge, accompanied by goblet cells hyperplasia and mucus hypersecretion in WT mice (Fig. 5J and K). ST2-deficient mice exhibited reduced lung pathology after antigen challenge, with less inflammatory cell infiltration and mucus secretion than in WT mice (Fig. 5J and K).

DC activation and migration are impaired in ST2-deficient mice

To determine whether DC activation and migration were altered in ST2-deficient mice, we analysed the DC phenotype in lung and mediastinal LN (mLN) 24 h after the last challenge. The absolute number of DCs was significantly enhanced in the lungs and in mLN of OVA-sensitized and challenged mice compared with saline-challenged mice (Fig. 6A). DC numbers in the lung of ST2-deficient and WT mice was similar, indicating that innate immunity was not altered in the absence of ST2. However, CD40 and CD80 expression on lung DCs was significantly reduced in ST2-deficient mice after OVA challenge. Furthermore, DC numbers were markedly reduced in mLN of ST2-deficient mice compared with WT mice (Fig. 6B). CD40 and CD80 expression was also decreased in the absence of ST2. This indicates that in the absence of IL-33 signalling, pulmonary DC activation is impaired, leading to reduced migration into mLN.

To address the ability of DCs to induce a Th2 response in an antigen-specific manner, cells from mLN were ex vivo restimulated with OVA for 5 days. Increased Th2 cytokine production was found in mLN cell culture supernatant from OVA-sensitized and challenged WT mice. In contrast, mLN cell culture supernatants from ST2-deficient mice showed a significant reduction of IL-4, IL-5 and IL-13 (Fig. 6C). Collectively, these results demonstrated that Th2 cell response induced by DCs is impaired in ST2-deficient mice during allergic airway inflammation.

IL-33-activated and OVA-pulsed DCs enhance airway inflammation

To directly demonstrate the involvement of IL-33-activated DCs during antigen sensitization, we transferred OVA-pulsed DCs (2×10^6 cells), pre-treated or not with IL-33 into naive recipient mice. Recipient mice were challenged with OVA 10 days after cell transfer (at days 10–12) to induce allergic airway inflammation. Twenty-four hours after the last challenge, the severity of inflammation was assessed. Mice receiving OVA-pulsed-DCs exhibited massive eosinophil recruitment in the BAL compared with mice receiving unpulsed DCs (Fig. 7A). Interestingly, eosinophil recruitment was higher in mice transferred with IL-33-treated OVA-pulsed DCs. Eosinophil peroxidase (EPO) activity was significantly increased in lung homogenates of mice

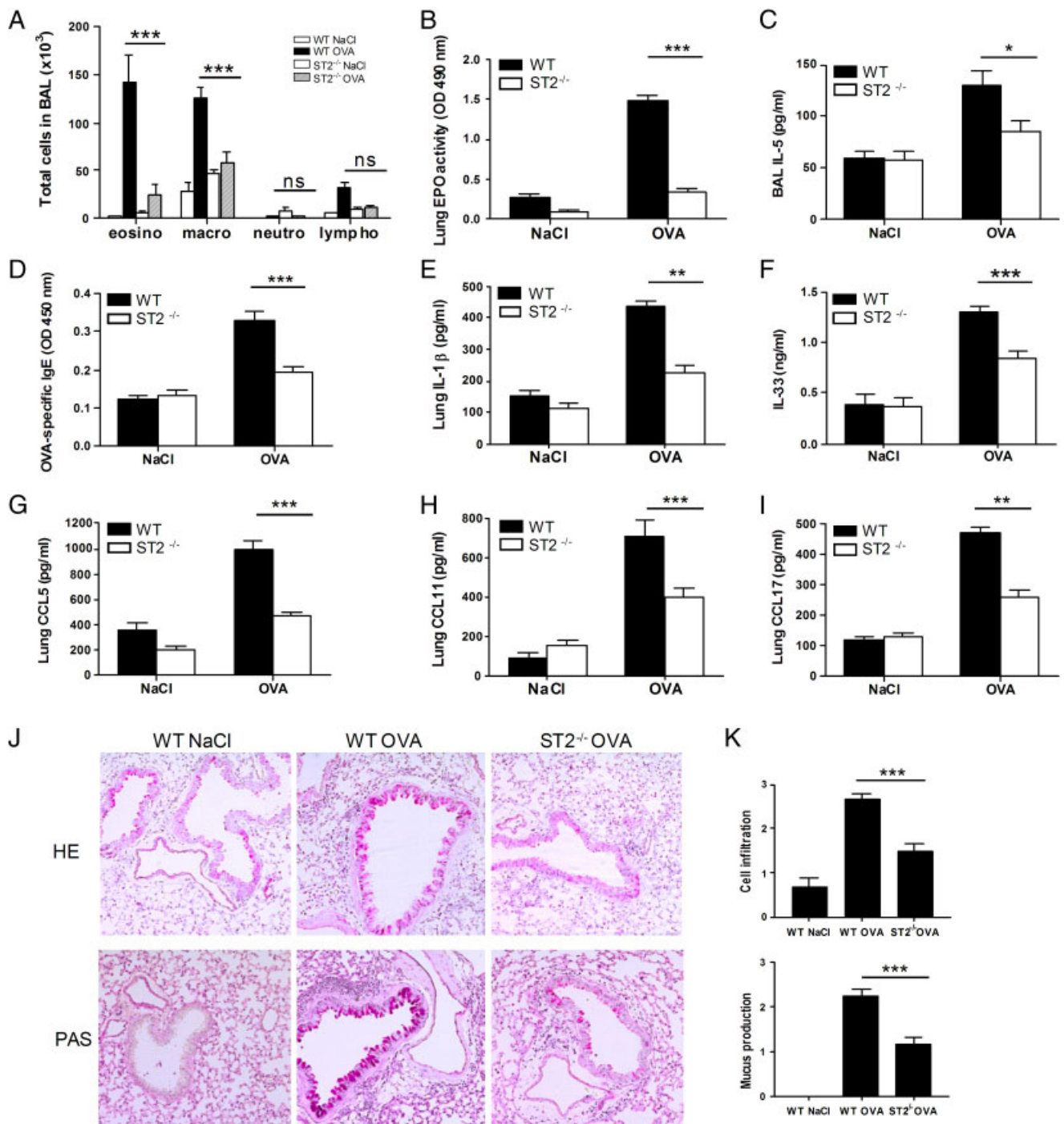


Figure 5. ST2-deficient mice develop attenuated allergic airway inflammation. OVA-sensitized WT and ST2-deficient mice were challenged three times with OVA instillation. (A and B) Twenty-four hours after the third challenge, eosinophil numbers were determined in the BAL and in lung tissues (EPO activity). (C and E–I) Cytokines and chemokine levels were measured by ELISA in lung homogenate or BAL. (D) OVA-specific IgE concentrations were assayed in sera by ELISA. (J) Lung sections from saline or OVA-challenged mice were stained with H&E or PAS. Representative section of each group is shown. Magnification is ×400, and scale bar is 10 μm. (K) The extent of cell infiltrate and mucus hypersecretion was scored by two independent observers (0–3). Data are presented as mean±SEM of six mice per group and are representative of at least three experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant. Statistical evaluation of differences between the experimental groups was determined by using one-way ANOVA followed by a Bonferroni post test.

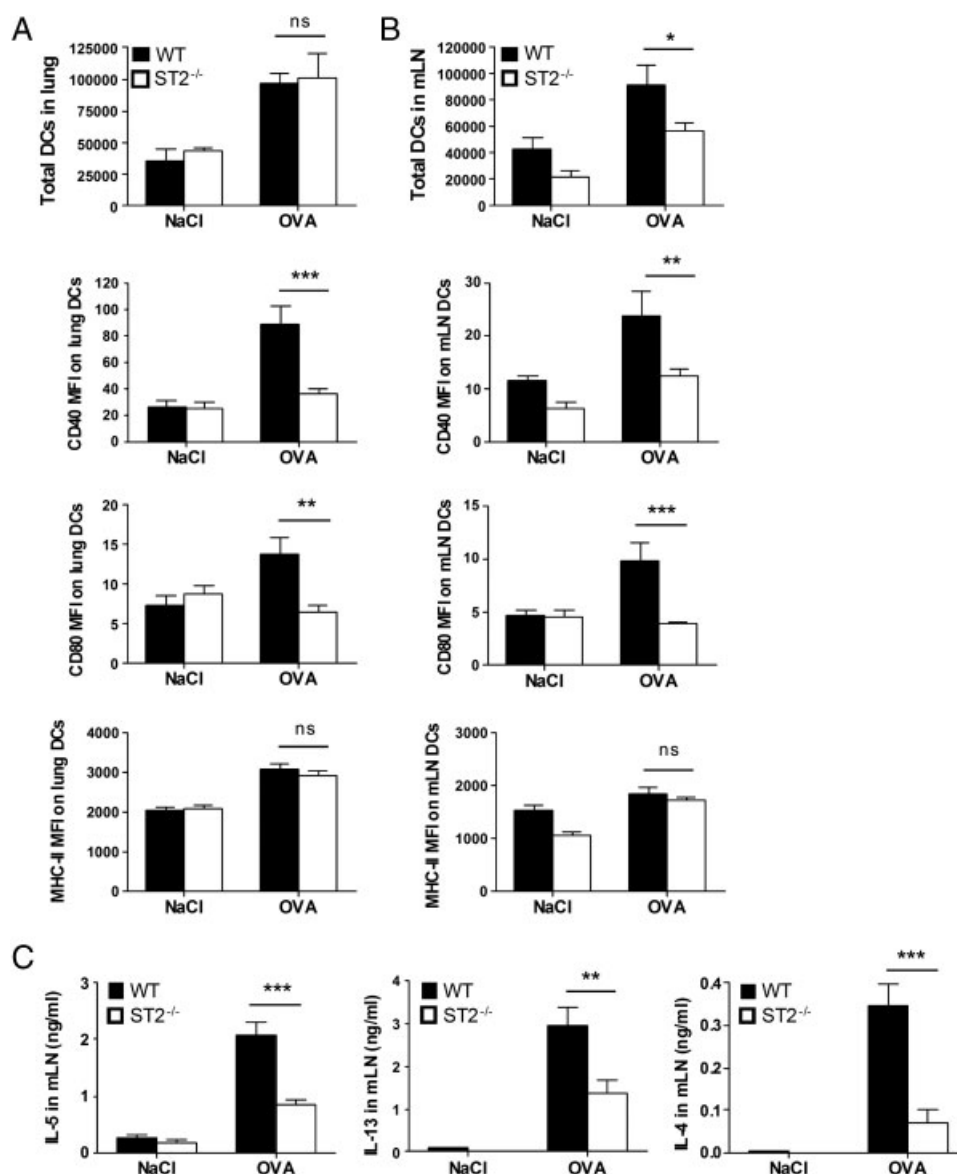


Figure 6. DCs activation and migration to draining LNs are reduced in ST2-deficient mice. Lungs from OVA-sensitized and challenged WT and ST2-deficient mice were digested with DNase/collagenase. (A) Lung mononuclear cells and (B) mLN cells were stained for extracellular FACS analysis. Absolute numbers of CD11b⁺CD11c⁺ cells and MFI of CD40, CD80 and MHC-II are shown. Data are presented as mean+SEM of six mice per group and are representative of two independent experiments. (C) Cells from mLNs of OVA-sensitized and challenged mice were in vitro restimulated with OVA for 5 days (5×10^6 cells/mL). Cytokine expression levels were analysed in the supernatants by ELISA. Data are presented as mean+SEM of six mice per group and are representative of three independent experiments. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; ns, not significant. Statistical evaluation of differences between the experimental groups was determined by using one-way ANOVA followed by a Bonferroni post test.

transferred with IL-33-treated OVA pulsed-DCs as compared with mice transferred with control OVA-pulsed DCs (Fig. 7B). The peribronchial eosinophil and lymphocyte recruitment, together with mucus hypersecretion, seen in lung tissue of mice transferred with OVA-pulsed DCs were increased in the lung of mice transferred with IL-33-treated OVA-pulsed DCs. The ex vivo treatment of OVA-pulsed DCs with IL-33 significantly increased the potential of these cells to induce eosinophilic airway inflammation in mice (Fig. 7C-D). These data demonstrate that IL-33 has the ability to significantly affect DC maturation, thereby leading to Th2 priming and allergic airway inflammation.

Discussion

At the crossroad of innate and adaptive immunity, DCs have an important role in determining how allergic immune responses are initiated and modulated. Located alongside epithelial cells in the skin and the airways, DCs have the ability to capture allergens and subsequently to present them to naive lymphocytes in draining LNs. The outcome of DC-induced T helper cell polarization depends of the type of captured antigen, the route of exposure and the microenvironment. Here, we report a critical role for IL-33 on DC activation, maturation and initiation of Th2-

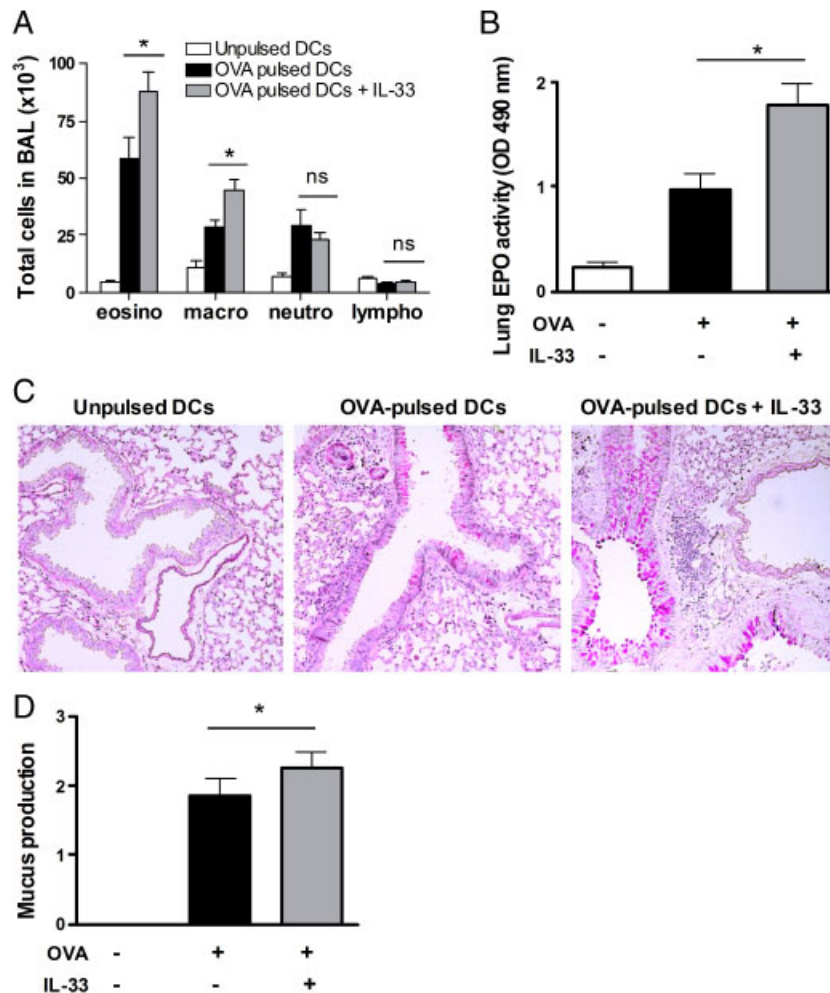


Figure 7. IL-33 enhances allergic airway inflammation induced by OVA-pulsed DCs. On day 0, naive BALB/c mice were injected intranasally with 2×10^6 unpulsed DCs, OVA-pulsed DCs or OVA-pulsed DCs in the presence of IL-33. From days 10–12, mice were intranasally challenged with OVA. Twenty-four hours after the last challenge, the severity of the airway inflammation was evaluated. (A) BAL was subjected to total differential cell counts. (B) The EPO activity was quantified in lung homogenate. (C) Lung sections from saline or OVA-challenged mice were stained with PAS. A representative section of each group is shown. Magnification is $\times 400$, and scale bar is 10 μm . (D) The extent of mucus hypersecretion was scored by two independent observers (0–3). Data are presented as mean $\pm$ SEM of eight mice per group and are representative of three independent experiments. * $p < 0.05$; ns, not significant. Statistical evaluation of differences between the experimental groups was determined by using one-way ANOVA followed by a Bonferroni post test (which allows comparing all pairs of the group).

mediated allergic inflammation. In vitro, we observed that IL-33 potently activates BMDCs to produce proinflammatory cytokines IL-6, TNF, IL-1 β and the chemokine CCL17, but not IL-12p40 or IL-23p19 (data not shown). IL-6 and CCL17 production by DCs was related to T cell differentiation into Th2 type [7, 31, 32]. We also found that IL-33 treatment upregulates cell surface expression of CD40, CD80 and OX40L on DCs. It has been shown that interaction between membrane OX40L on DCs and OX40 (CD134) on naive T cells results in the induction of IL-4 production by T cells followed by their subsequent polarization into the Th2 subset [33, 34]. Taken together, our results suggest that IL-33 directly activates DCs to produce pro-Th2 cytokines/chemokines and costimulatory surface molecules. It was reported that IL-33 upregulated MHC-II and CD86 expression on DCs [29]. We did not observe any significant change in the expression of these surface molecules. This discrepancy may be explained by

technical differences in the approach such as the composition of the medium or the purity of CD11c⁺ cells.

Consistent with a previous report, we observed that IL-33-activated DCs primed naive CD4⁺ T cells to produce the pro-allergic cytokines IL-5 and IL-13, but no IL-4 [5]. Importantly, we found that naive CD4⁺ T cells did not respond to IL-33 in the absence of DCs. This observation is consistent with the fact that naive T cells do not express ST2 and that IL-33 did not induce Th2 cytokines in non-polarized CD4⁺ T cells [10, 35, 36]. The cytokine expression pattern of T cells polarized by IL-33-activated DCs differs from classical Th2 cells by the absence of IL-4 production. An atypical Th2 subset upon IL-33 activation has been reported, which is independent of IL-4, GATA3 and TCR engagement [26, 29].

The contribution of IL-33 on asthma symptoms by its direct action on mast cells, basophils, eosinophils and Th2 cells has been

well documented [20, 25]. Recently, it was shown that the resolution of allergic inflammation and airway hyperresponsiveness depend on the disruption of ST2/IL-33 pathway [8] and that IL-33-neutralizing antibody administration inhibits airway inflammation in mice [37]. Using ST2-deficient mice, we demonstrated here that IL-33 signalling is necessary upon antigen sensitization to mount an efficient Th2 response. ST2-deficient mice showed a reduced activation of DCs in LNs after antigen sensitization. In vitro restimulation of LN cells from ST2-deficient mice displayed a reduced Th2 response, with a reduction of IL-4, IL-5 and IL-13 production in the supernatant. This indicates that Th2 cell priming by DCs is impaired in the absence of IL-33 signalling during sensitization phase and that IL-4 may in some extent also be dependent on IL-33.

As described in previous studies, ST2-deficient mice showed attenuated allergic airway inflammation with a reduction of eosinophil recruitment in the BAL, associated with a decrease in proinflammatory cytokines and chemokines compared with WT mice. Interestingly, we observed that cells from mLNs of ST2-deficient mice produced less Th2 cytokines, IL-4, IL-5 and IL-13 after antigen in vitro restimulation than cells isolated from WT mice. This observation contrasts with those of Kurowska et al. which showed that absence of IL-33 signalling does not affect IL-4 production [5, 38]. This discrepancy can be explained by the difference in the asthma protocols or the doses of antigen administered during immunization [38–40]. Our model, using two sensitizations with low dose of OVA (10 µg/mouse) without adjuvant, followed by three OVA challenges (10 µg/mouse), seems to favour the development of classical Th2 cells that produce IL-4, IL-5 and IL-13, since we showed a reduction of all these cytokines in ST2-deficient mice.

We next found reduced DC activation in the lung of ST2-deficient mice after OVA challenges compared to WT mice. Local antigen administration during challenge probably results in the release by epithelial cells of inflammatory molecules such as IL-33, which could modulate DC functions in vivo. In the absence of ST2, lung DCs appear to be less activated than in WT mice. Interestingly, absolute numbers of DCs are similar in WT and ST2-deficient mice, indicating that DCs are normally present in the lung, but fail to upregulate costimulatory molecules after antigen challenges. In contrast, a reduced number of DCs was found in mLNs of ST2-deficient mice compared to WT mice. This finding suggests that in the absence IL-33 signalling, migration of lung DCs into mLNs is impaired leading to a reduced Th2 response. DC transfer experiments confirmed that IL-33 increases the immunostimulatory properties of DCs since IL-33-treated DCs enhance lung eosinophilic inflammation and mucus hyperproduction. Together, these data support the notion that IL-33 plays a critical role in the initiation of lung allergic inflammation through DC activation.

In summary, our results reveal a novel role of IL-33 in enhancing DC capacity to induce Th2 priming in the context of allergic airway inflammation. We provide evidence that IL-33 signalling plays a critical role in DC maturation and functions upon antigen sensitization, as in experimental allergic asthma. The role of epithelial cells in the control of Th2 sensitization and asthma development is emerging. Further studies are required to

define precisely how IL-33 and other alarmins regulate adaptive immunity by altering DC functions.

Material and methods

Mice

Female BALB/C and ST2-deficient mice (8–10 wk old) were bred in our specific pathogen-free animal facility at CNRS (UPS 44, Transgenose institute, Orléans, France). ST2-deficient mice were originally provided by Andrew N. J. McKenzie (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK) [41]. Mice were maintained in a temperature-controlled (23°C) facility with a strict 12 h light/dark cycles and were given free access to food and water. Animal experiments were performed according to the French Institutional Committee.

Generation and stimulation of BM-derived DCs

BM cells from WT or ST2-deficient mice were cultured in RPMI 1640 medium, supplemented with 10% FCS (Hyclone), L-glutamine (2 µM, Invitrogen), β-Mercaptoethanol (50 nM, Invitrogen), penicillin (100 U/mL, Invitrogen), streptomycin (100 µg/mL, Invitrogen) and 5% of J558L cells supernatant (GM-CSF). On days 3, 6 and 8 the medium was refreshed and GM-CSF was added. At day 10, nonadherent cells were harvested. The purity of BMDCs was greater than 90%. At day 10, BMDCs were counted and plated in a round-bottomed 96-well plate (5×10^6 cells/well) and stimulated with increasing doses of recombinant murine IL-33 (0.1, 1, 10 and 100 ng/mL, R&D Systems). Culture supernatants were collected at 24 h and analysed by ELISA for IL-1β, IL-6, IL-12p40, IL-23p19, IFN-γ, TNF and CCL17. Cells were collected and stained for flow cytometry analysis as described below.

T-cell proliferation assay

CD4⁺ T cells were purified from LNs of DO11.10 mice (OVA-specific TCR transgenic mice) by CD4⁺ magnetic cell sorting (Miltenyi Biotec) and stained with CFSE (Molecular Probe, Invitrogen). In total, 2×10^5 CD4⁺ T cells were cocultured with 2×10^4 BMDCs (ratio 1/10) in a round-bottomed 96-well plate in the presence of OVA peptide (5 µg/mL). BMDCs were previously treated or not with 10 ng/mL IL-33 for 24 h and washed twice with PBS before the coculture. After 6 days of coculture, T-cell proliferation was assessed by flow cytometry.

DC/T-cell coculture

CD4⁺ T cells were purified from LNs of naive WT mice by CD4⁺ magnetic cell sorting (Miltenyi Biotec). In total, 2×10^4 BMDCs

were seeded in a round-bottomed 96-well plate and cultured with 2×10^5 CD4⁺ purified T cells in the presence of medium alone or IL-33. Wells with DCs alone or T cells alone served as controls. Supernatants were collected after 6 days of culture and assayed for IL-4, IL-5, IL-10, IL-13 and IFN- γ by ELISA.

OVA-induced allergic airway inflammation

WT or ST2-deficient mice were subcutaneously sensitized on days 0 and 7 with 10 μ g of OVA (grade V, Sigma-Aldrich) without alum adjuvant. On days 14, 15 and 16, mice were challenged intranasally with 10 μ g of OVA or saline. Twenty-four hours after the last OVA exposure, mice were killed and BAL, lung and serum were collected. BAL fluid was analysed for cellular composition and cytokine levels. Half of the lung was stored at -80°C for EPO enzymes and cytokines/chemokines analysis, and the other half was fixed overnight in 4% formaldehyde solution for histological analysis. For some experiments, the whole lung was harvested and digested with DNase (1 mg/mL, Sigma) and Type IV collagenase (0.5 mg/mL, Gibco) for 45 min at 37°C . Mononuclear cells were isolated by centrifugation on discontinuous Percoll 35/70% gradient (Amersham, Biosciences) before flow cytometric analysis. mLNs were removed and dissociated in 100 μ m cell strainers for FACS analysis or in vitro restimulation. To study the involvement of IL-33 during sensitization phase, mice were killed at day 14, prior to allergen challenge, and LNs (inguinal, brachial, axillary and mediastinal) were harvested and analysed for cellular composition or cytokine production after 48 h anti-CD3/anti-CD28 *ex vivo* restimulation. Experiments were performed at least twice using groups of six animals.

Allergic airway inflammation induced by intranasal transfer of OVA-pulsed BMDCs

BMDCs were pulsed overnight with OVA 10 μ g/mL (Grade V, Sigma) in the presence or absence of 10 ng/mL recombinant IL-33 (R&D systems). As a control, BMDCs were cultured overnight with medium without OVA. After antigen pulse, nonadherent cells were collected, washed twice with PBS to remove OVA and IL-33, and resuspended in PBS. Naive recipient mice received intranasally 2×10^6 unpulsed DCs, OVA-pulsed DCs or OVA-pulsed in the presence of IL-33 DCs. On days 10–12, mice were challenged with 10 μ g OVA intranasally. Twenty-four hours after the last challenge, the intensity of the airway inflammation was assessed in the BAL and lung homogenate.

Flow cytometry staining

BAL cells were stained for 20 min with FITC-labelled anti-CD3/anti-B220 (lymphocytes), PE-labelled anti-Siglec F (eosinophils), PE-Cy7-labelled anti-GR1 (neutrophils), Peridinin chlorophyll protein-labelled anti-CD11c and APC-labelled anti-MHC class II (macro-

phages/DCs) antibodies in PBS containing 3% FCS and 0.01% azide. Differential cells counts were analysed by flow cytometry as previously described [42]. For the determination of DC activation in lung or LNs, cell suspensions were stained for 20 min with APC-labelled anti-CD80, PE-labelled anti-OX40L, PE-labelled anti-CD40, APC-labelled anti-CD80, APC-labelled anti-CCR7, FITC-labelled anti-MHC class II antibodies. In some experiments, cell suspensions from lung were restimulated in vitro with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (750 ng/mL; both from Sigma-Aldrich) in the presence of Brefeldin A (GolgiPlug, BD Biosciences, France). After 4 h, cells were stained with V450-labelled CD4 (clone RMA-5) and APC-Cy7-labelled CD8a (clone 53–6.7). Cells were permeabilized for 20 min with cytofix/cytoperm kit (BD Biosciences) and stained with Alexa 647-labelled IL-13 (clone Ebio13A, eBioscience). Fluorescence was acquired on a BD CANTO II flow cytometer using DIVA software (BD Biosciences). Fluorescence data were analysed using FlowJO software (Treestar).

Lung histology

Lungs were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Lung sections of 3 μ m were stained with H&E or with periodic acid Schiff (PAS) reagent. Cell infiltration and mucus secretion were assessed by a semi-quantitative score (0–3) using a Leica microscope.

Pulmonary EPO activity

EPO activity was determined in order to estimate the recruitment of eosinophils to the lung parenchym. After BAL and perfusion, lungs were excised, stored frozen at -80°C and homogenized for 30 s in 1 mL of 0.05 M Tris/HCl buffer, pH 8.0. The homogenate was centrifuged for 15 min at 4°C at $10\,000 \times g$. EPO activity in the supernatant was determined as estimated from the oxidation of *O*-phenylenediamine (Sigma-Aldrich) by EPO in the presence of hydrogen peroxide using the protocol by Van Oosterhout et al. [43]. The substrate solution consisted of 10 mM *O*-phenylenediamine in 0.05 M Tris/HCl-buffer (pH = 8) and 4 mM H₂O₂ (BDH, Poole, UK). Substrate solution was added to samples in a 96-well microplate (Greiner) and incubated at 37°C for 30 min. Duplicate incubations were carried out in the absence and presence of the EPO inhibitor 3-amino-1,2,4-triazole (2 mmol/L, Sigma-Aldrich). The absorbance was then measured at 490 nm (Flow Labs, Irvine, UK). Results are expressed as O.D. 490 nm and were corrected for the activity of other peroxidases, which were not inhibited by 3-amino-1,2,4-triazole.

Cytokines, chemokines and OVA-specific IgE measurement

IL-1 β , IL-4, IL-5, IL-6, IL-10, IL-13, IL-33, IFN- γ , TNF- α , CCL5, CCL11, CCL17 were quantified using commercial ELISA kits from

R&D Systems. Serum anti-OVA IgE level was measured by ELISA using anti-IgE (BD Pharmingen) as previously described [44]. Biotinylated OVA and HRP-conjugated streptavidin (Vector Labs) were used for detection.

Statistical analysis

Data are presented as mean+SEM. The difference between groups was calculated with a two-way ANOVA test using Prism software. A *p*-value less than 0.05 was considered to be significant. All experiments were repeated at least twice, with *n* ≥ 6 animals in each experimental group.

Acknowledgements: We thank Professor Eddy Liew for critical reading and comments. We are grateful to the EFIS for financial support allowing a fruitful collaboration with Eddy Liew's group. This work was supported by the "Agence Nationale pour la Recherche" (ANR 2007 MIME-103-02), the "Fondation pour la Recherche Médicale" (FRM allergy DAL 2007 0822007), the "Fond européen de développement régional" (FEDER Asthme 1575-32168).

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

- Shum, B. O., Rolph, M. S. and Sewell, W. A., Mechanisms in allergic airway inflammation - lessons from studies in the mouse. *Exp. Rev. Mol. Med.* 2008. **10**: e15.
- Nakajima, H. and Takatsu, K., Role of cytokines in allergic airway inflammation. *Int. Arch. Allergy Immunol.* 2007. **142**: 265–273.
- Finkelman, F. D., Hogan, S. P., Hershey, G. K., Rothenberg, M. E. and Wills-Karp, M., Importance of cytokines in murine allergic airway disease and human asthma. *J. Immunol.* 2010. **184**: 1663–1674.
- Hamid, Q. and Tulic, M., Immunobiology of asthma. *Annu. Rev. Physiol.* 2009. **71**: 489–507.
- Kurowska-Stolarska, M., Kewin, P., Murphy, G., Russo, R. C., Stolarski, B., Garcia, C. C., Komai-Koma, M. et al., IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *J. Immunol.* 2008. **181**: 4780–4790.
- Prefontaine, D., Lajoie-Kadoch, S., Foley, S., Audusseau, S., Olivenstein, R., Halayko, A. J., Lemiere, C. et al., Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. *J. Immunol.* 2009. **183**: 5094–5103.
- Allakhverdi, Z., Comeau, M. R., Smith, D. E., Toy, D., Endam, L. M., Desrosiers, M., Liu, Y. J. et al., CD34+ hemopoietic progenitor cells are potent effectors of allergic inflammation. *J. Allergy Clin. Immunol.* 2009. **123**: 472–478.
- Kearley, J., Buckland, K. F., Mathie, S. A. and Lloyd, C. M., Resolution of allergic inflammation and airway hyperreactivity is dependent upon disruption of the T1/ST2-IL-33 pathway. *Am. J. Respir. Crit. Care Med.* 2009. **179**: 772–781.
- Smith, D. E., IL-33: a tissue derived cytokine pathway involved in allergic inflammation and asthma. *Clin. Exp. Allergy* 2009. **40**: 200–208.
- Schmitz, J., Owyang, A., Oldham, E., Song, Y., Murphy, E., McClanahan, T. K., Zurawski, G. et al., IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005. **23**: 479–490.
- Sims, J. E. and Smith, D. E., The IL-1 family: regulators of immunity. *Nat. Rev. Immunol.* 2010. **10**: 89–102.
- Cayrol, C. and Girard, J. P., The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. *Proc. Natl. Acad. Sci. USA* 2009. **106**: 9021–9026.
- Luthi, A. U., Cullen, S. P., McNeela, E. A., Duriez, P. J., Afonina, I. S., Sheridan, C., Brumatti, G. et al., Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity* 2009. **31**: 84–98.
- Kurowska-Stolarska, M., Stolarski, B., Kewin, P., Murphy, G., Corrigan, C. J., Ying, S., Pitman, N. et al., IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *J. Immunol.* 2009. **183**: 6469–6477.
- Moussion, C., Ortega, N. and Girard, J. P., The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel "alarmin"? *PLoS One* 2008. **3**: e3331.
- Carriere, V., Roussel, L., Ortega, N., Lacorre, D. A., Americh, L., Aguilar, L., Bouche, G. and Girard, J. P., IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc. Natl. Acad. Sci. USA* 2007. **104**: 282–287.
- Eigenbrod, T., Park, J. H., Harder, J., Iwakura, Y. and Nunez, G., Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells. *J. Immunol.* 2008. **181**: 8194–8198.
- Kazama, H., Ricci, J. E., Herndon, J. M., Hoppe, G., Green, D. R. and Ferguson, T. A., Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity* 2008. **29**: 21–32.
- Scaffidi, P., Misteli, T. and Bianchi, M. E., Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002. **418**: 191–195.
- Pecaric-Petkovic, T., Didichenko, S. A., Kaempfer, S., Spiegl, N. and Dahinden, C. A., Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* 2009. **113**: 1526–1534.
- Suzukawa, M., Iikura, M., Koketsu, R., Nagase, H., Tamura, C., Komiya, A., Nakae, S. et al., An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor. *J. Immunol.* 2008. **181**: 5981–5989.
- Ali, S., Huber, M., Kollewe, C., Bischoff, S. C., Falk, W. and Martin, M. U., IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells. *Proc. Natl. Acad. Sci. USA* 2007. **104**: 18660–18665.
- Moulin, D., Donze, O., Talabot-Ayer, D., Mezin, F., Palmer, G. and Gabay, C., Interleukin (IL)-33 induces the release of pro-inflammatory mediators by mast cells. *Cytokine* 2007. **40**: 216–225.
- Cherry, W. B., Yoon, J., Bartemes, K. R., Iijima, K. and Kita, H., A novel IL-1 family cytokine, IL-33, potentially activates human eosinophils. *J. Allergy Clin. Immunol.* 2008. **121**: 1484–1490.
- Suzukawa, M., Koketsu, R., Iikura, M., Nakae, S., Matsumoto, K., Nagase, H., Saito, H. et al., Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils. *Lab Invest.* 2008. **88**: 1245–1253.

- 26 Smithgall, M. D., Comeau, M. R., Yoon, B. R., Kaufman, D., Armitage, R. and Smith, D. E., IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int. Immunol.* 2008. **20**: 1019–1030.
- 27 Komai-Koma, M., Xu, D., Li, Y., McKenzie, A. N., McInnes, I. B. and Liew, F. Y., IL-33 is a chemoattractant for human Th2 cells. *Eur. J. Immunol.* 2007. **37**: 2779–2786.
- 28 Turnquist, H. R. and Thomson, A. W., IL-33 broadens its repertoire to affect DC. *Eur. J. Immunol.* 2009. **39**: 3292–3295.
- 29 Rank, M. A., Kobayashi, T., Kozaki, H., Bartemes, K. R., Squillace, D. L. and Kita, H., IL-33-activated dendritic cells induce an atypical TH2-type response. *J. Allergy Clin. Immunol.* 2009. **123**: 1047–1054.
- 30 Idzko, M., Hammad, H., van Nimwegen, M., Kool, M., Vos, N., Hoogsteden, H. C. and Lambrecht, B. N., Inhaled iloprost suppresses the cardinal features of asthma via inhibition of airway dendritic cell function. *J. Clin. Invest.* 2007. **117**: 464–472.
- 31 Rincon, M., Anguita, J., Nakamura, T., Fikrig, E. and Flavell, R. A., Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4⁺ T cells. *J. Exp. Med.* 1997. **185**: 461–469.
- 32 Homey, B. and Zlotnik, A., Chemokines in allergy. *Curr. Opin. Immunol.* 1999. **11**: 626–634.
- 33 Hammad, H., Lambrecht, B. N., Pochard, P., Gosset, P., Marquillies, P., Tonnel, A. B. and Pestel, J., Monocyte-derived dendritic cells induce a house dust mite-specific Th2 allergic inflammation in the lung of humanized SCID mice: involvement of CCR7. *J. Immunol.* 2002. **169**: 1524–1534.
- 34 Ito, T., Wang, Y. H., Duramad, O., Hori, T., Delespesse, G. J., Watanabe, N., Qin, F. X. et al., TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J. Exp. Med.* 2005. **202**: 1213–1223.
- 35 Xu, D., Chan, W. L., Leung, B. P., Huang, F., Wheeler, R., Piedrafitra, D., Robinson, J. H. and Liew, F. Y., Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J. Exp. Med.* 1998. **187**: 787–794.
- 36 Kondo, Y., Yoshimoto, T., Yasuda, K., Futatsugi-Yumikura, S., Morimoto, M., Hayashi, N., Hoshino, T. et al., Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. *Int. Immunol.* 2008. **20**: 791–800.
- 37 Liu, X., Li, M., Wu, Y., Zhou, Y., Zeng, L. and Huang, T., Anti-IL-33 antibody treatment inhibits airway inflammation in a murine model of allergic asthma. *Biochem. Biophys. Res. Commun.* 2009. **386**: 181–185.
- 38 Hoshino, K., Kashiwamura, S., Kuribayashi, K., Kodama, T., Tsujimura, T., Nakanishi, K., Matsuyama, T. et al., The absence of interleukin 1 receptor-related T1/ST2 does not affect T helper cell type 2 development and its effector function. *J. Exp. Med.* 1999. **190**: 1541–1548.
- 39 Coyle, A. J., Lloyd, C., Tian, J., Nguyen, T., Eriksson, C., Wang, L., Ottoson, P. et al., Crucial role of the interleukin 1 receptor family member T1/ST2 in T helper cell type 2-mediated lung mucosal immune responses. *J. Exp. Med.* 1999. **190**: 895–902.
- 40 Mangan, N. E., Dasvarma, A., McKenzie, A. N. and Fallon, P. G., T1/ST2 expression on Th2 cells negatively regulates allergic pulmonary inflammation. *Eur. J. Immunol.* 2007. **37**: 1302–1312.
- 41 Townsend, M. J., Fallon, P. G., Matthews, D. J., Jolin, H. E. and McKenzie, A. N., T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J. Exp. Med.* 2000. **191**: 1069–1076.
- 42 van Rijt, L. S., Kuipers, H., Vos, N., Hijdra, D., Hoogsteden, H. C. and Lambrecht, B. N., A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma. *J. Immunol. Methods* 2004. **288**: 111–121.
- 43 Van Oosterhout, A. J., Hofstra, C. L., Shields, R., Chan, B., Van Ark, I., Jardieu, P. M. and Nijkamp, F. P., Murine CTLA4-IgG treatment inhibits airway eosinophilia and hyperresponsiveness and attenuates IgE upregulation in a murine model of allergic asthma. *Am. J. Respir. Cell Mol. Biol.* 1997. **17**: 386–392.
- 44 Woerly, G., Honda, K., Loyens, M., Papin, J. P., Auwerx, J., Staels, B., Capron, M. and Dombrowicz, D., Peroxisome proliferator-activated receptors alpha and gamma down-regulate allergic inflammation and eosinophil activation. *J. Exp. Med.* 2003. **198**: 411–421.

Abbreviations: BMDC: bone-marrow derived dendritic cells · EPO: eosinophil peroxidase · mLN: mediastinal LN · PAS: periodic acid Schiff

Full correspondence: Bernhard Ryffel, Université d'Orléans and CNRS, UMR6218, Molecular Immunology and Embryology, 3B Rue de la Férollerie, 45071 Orléans Cedex 2, France
Fax: +33-238-25-7979
e-mail: bryffel@cnrs-orleans.fr

See accompanying Commentary:
<http://dx.doi.org/10.1002/eji.201141668>

Received: 8/9/2010

Revised: 15/2/2011

Accepted: 15/3/2011

Accepted article online: 21/3/2011

European Journal of Immunology

Supporting Information

for

DOI 10.1002/eji.201041033

IL-33-activated dendritic cells are critical for allergic airway inflammation

Anne-Gaëlle Besnard, Dieudonné Togbe, Noëlline Guillou, François Erard,
Valérie Quesniaux and Bernhard Ryffel

IL-33-activated dendritic cells are critical for allergic airway inflammation

**Anne-Gaëlle Besnard¹, Dieudonnée Togbe^{1,2}, Noëlline Guillou¹, François Erard¹,
Valérie Quesniaux¹ and Bernhard Ryffel¹**

ONLINE SUPPLEMENTARY INFORMATION

Supplementary figure legend

Figure.S1 . Reduced IL-13 production in ST2^{-/-} mice after OVA exposure

OVA sensitized WT and ST2^{-/-} mice were challenged three times with OVA. 24 hours after the third challenge, lung mononuclear cells were isolated and restimulated with PMA (50 ng/ml) and ionomycin (750 ng/ml) in presence of Brefeldin A followed by extracellular staining of CD4 and intracellular staining of IL-13. Representative dot plots (A), frequency and absolute cell numbers of CD4⁺ IL-13⁺ (B) and CD4⁻ IL-13⁺ (C) cell populations are given. These experiments were performed 3 times. Values are the mean $\pm$ SEM of 6 mice per group. * $P \leq 0.05$; ** $P \leq 0.001$.

Supplementary figure S1

