

Complex 2B4 Regulation of Mast Cells and Eosinophils in Murine Allergic Inflammation

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The cell surface molecule 2B4 (CD244) is an important regulator of lymphocyte activation, and its role in antiviral immunity and lymphoproliferative disorders is well established. Although it is also expressed on mast cells (MCs) and eosinophils (Eos), the functions of 2B4 on these allergy-orchestrating cells remain unclear. We therefore investigated the role of 2B4 on murine MCs and Eos, particularly how this molecule affects allergic and nonallergic inflammatory processes involving these effector cells. Experiments in bone marrow–derived cultures revealed an inhibitory effect for 2B4 in MC degranulation, but also an opposing stimulatory effect in eosinophil migration and delayed activation. Murine disease models supported the dual 2B4 function: In 2B4 –/– mice with nonallergic peritonitis and mild atopic dermatitis (AD), modest infiltrates of Eos into the peritoneum and skin (respectively) confirmed that 2B4 boosts eosinophil trafficking. In a chronic AD model, 2B4 –/– mice showed overdegranulated MCs, confirming the inhibiting 2B4 effect on MC activation. This multifunctional 2B4 profile unfolded in inflammation resembles a similar mixed effect of 2B4 in natural killer cells. Taken together, our findings provide evidence for physiological 2B4 stimulatory/inhibitory effects in MCs and Eos, pointing to a complex role for 2B4 in allergy.

Journal of Investigative Dermatology (2014) **134**, 2928–2937; doi:10.1038/jid.2014.280; published online 21 August 2014

INTRODUCTION

2B4 (CD244) is a CD2-family surface molecule with multiple activities, and it is typically found on natural killer (NK) cells and T lymphocytes (McNerney *et al.*, 2005; Vaidya and Mathew, 2006; Veillette, 2006; Waggoner and Kumar, 2012). 2B4 was first described as a stimulator of NK cytotoxicity IFN- γ secretion, as discovered by specific antibody (Ab) binding (i.e., cross-linking) of the molecule in mouse (Garni-Wagner *et al.*, 1993; Mathew *et al.*, 1993) and human (Valiante and Trinchieri, 1993; Chuang *et al.*, 2001) NK cultures. The activating role for 2B4 on NK cells surfaced also upon engagement with its natural high-affinity ligand CD48, a CD2-family glycosyl–phosphatidyl–inositol-anchored protein (Yokoyama *et al.*, 1991; Brown *et al.*, 1998; Elishmereni and Levi-Schaffer, 2011), on human (Tangye *et al.*, 2000) and mouse (Bloch-Queyrat *et al.*, 2005; Lee

et al., 2006) target cells. Nevertheless, inhibitory 2B4 phenotypes also exist, predominantly in mice: ligation of 2B4 on murine NK cells by CD48+ cells (e.g., tumor cells, dendritic cells, and activated T cells) hampers the NK killing potential, an inhibitory effect that is lost in 2B4-lacking NK cells (Lee *et al.*, 2004; McNerney *et al.*, 2005; Morandi *et al.*, 2005; Vaidya *et al.*, 2005; Vaidya and Mathew, 2006; Chlewicki *et al.*, 2008; Waggoner *et al.*, 2010).

Despite the growing knowledge on the multiple functions of 2B4 in lymphocytes and NK cells, its particular role in allergy and on its key effector cells, mast cells (MCs) especially in the early phase, and eosinophils (Eos) in the late/chronic stage (Galli *et al.*, 2008; Hogan *et al.*, 2008), is unclear. Indeed, 2B4 expression on human peripheral blood Eos was established in our earlier studies (Munitz *et al.*, 2005). In these human cells, 2B4 triggers Eos degranulation and release of IFN- γ and IL-4 (Munitz *et al.*, 2005), and it boosts Eos viability (Elishmereni *et al.*, 2011). 2B4 has more recently been implicated also in human Eos adhesion and migration (El-Shazly *et al.*, 2011). Human MCs, however, do not express 2B4 (Munitz *et al.*, 2005) but rather its ligand CD48 (Malaviya *et al.*, 1999; Malaviya and Georges, 2002). Interestingly, in the murine setting, 2B4 appears on both MCs and Eos (Kubota, 2002; Elishmereni *et al.*, 2013), yet its functionality in the mouse system has not been properly dissected, neither *in vitro* nor *in vivo*. Given the complex 2B4 pattern in murine/human NK cells (Vaidya and Mathew, 2006; Chlewicki *et al.*, 2008), the question of whether 2B4 is inhibiting and/or activating in murine MCs and Eos, and as a consequence whether 2B4

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Abbreviations: AD, atopic dermatitis; AEU, allergic effector unit; BMMCs, bone marrow mast cells; BMEos, bone marrow eosinophils; c48/80, compound 48/80; DNP, dinitrophenyl; Eos, eosinophils; EPO, Eos peroxidase; NK, natural killer; OVA, ovalbumin; PAF, platelet-activating factor; SEB, staphylococcal enterotoxin B

Received 27 November 2013; revised 29 April 2014; accepted 20 May 2014; accepted article preview online 7 July 2014; published online 21 August 2014

signaling in allergic inflammation is beneficial or detrimental, remains open.

In the present study, we examined the role of 2B4 on MCs and Eos in allergic and nonallergic inflammation. Our comprehensive analysis used murine MC and Eos cultures using Ab ligation and inhibition of the molecule, as well as in cells derived from 2B4-deficient (2B4^{-/-}) mice. The *in vitro* effects were confirmed in nonallergic peritonitis and allergic atopic dermatitis (AD) induced in 2B4^{-/-} mice. Our findings implicate both positive and negative functions for 2B4 in allergic inflammation, aligning with the established multifunctional role of this regulating receptor in the immune system.

RESULTS

Inhibitory signal of 2B4 in murine MCs *in vitro*

To understand how the 2B4 pathway works in murine MCs and Eos, we first dissected the respective influences of this receptor on both effector cells *in vitro*. We examined whether 2B4 activates or inhibits the functions of bone marrow-derived MCs (BMMCs). Ligation of 2B4, through cross-linking by a specific monoclonal Ab, inhibited IgE activation of BMMCs: the cells released lower β -hexosaminidase levels following dinitrophenyl (DNP) stimulation, as compared with control (isotype-containing) cultures (Figure 1a). In tandem, preblocking 2B4 on BMMCs by Abs (to inhibit signaling by its natural ligand, CD48, on neighboring cells) resulted in elevated β -hexosaminidase release in both basal and IgE-activation conditions (Figure 1b). Hence, continuous 2B4 binding in BMMCs (most likely by CD48 on other BMMCs (Elishmereni *et al.*, 2011)) regulates degranulation. This effect was apparent primarily under suboptimal levels of DNP, as 2B4 blocking was unable to further augment the already potent activation in higher (saturating) DNP concentrations (Figure 1b). To assess whether an inherent deficiency in 2B4 expression on BMMCs affects their activation, we compared BMMC phenotypes in 2B4^{-/-} and wild-type (wt) mice. 2B4-lacking BMMCs developed normally, contained regular granular amounts, and expressed the characteristic cKit and Fc ϵ RI markers, CD48, and other typical adhesion molecules (Supplementary Figure S1a online). Nonetheless, the 2B4-knockout BMMCs consistently showed enhanced responses to IgE stimulation as compared with wt BMMCs (Figure 1c). The effect of the 2B4 deficiency on activation was strongest in the saturated DNP concentration (Figure 1c), implying that 2B4-mediated inhibition of BMMCs may be proportional to the degree of their activation.

Stimulatory signal of 2B4 in murine Eos *in vitro*

Next, we studied the role of 2B4 on bone marrow-derived Eos (BMEos). 2B4 stimulation did not modify BMEos degranulation patterns: Eos peroxidase (EPO) release from 2B4-crosslinked cells under basal or platelet-activating factor-induced conditions was comparable to that of control (isotype-treated) cells (Figure 2a). 2B4 blockade by neutralizing Abs (to prevent CD48 signaling from adjacent Eos) was also inconsequential to degranulation (Figure 2b). 2B4 absence does not appear to affect Eos phenotype and maturation: BMEos from 2B4^{-/-}

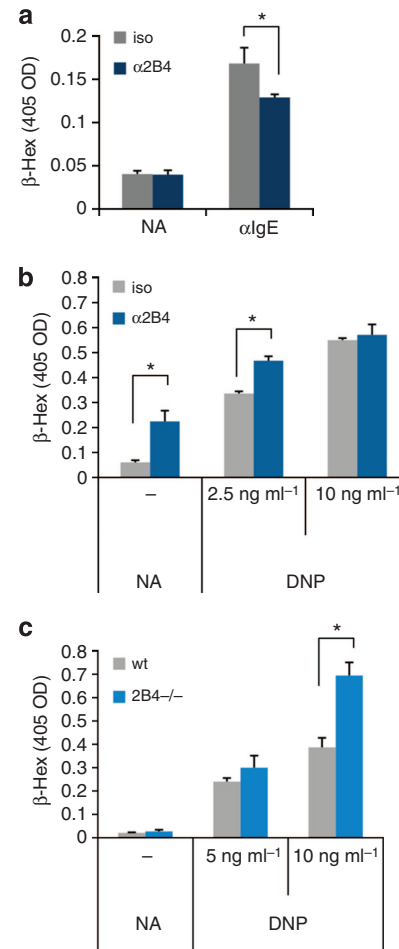


Figure 1. 2B4 inhibition of BMMCs. (a) β -Hexosaminidase (β -Hex) release from resting or IgE (DNP)-activated BMMCs that were also ligated with 2B4 Ab (or control isotype Ab). (b) β -Hex release from resting or IgE (DNP)-activated BMMCs that were first blocked by a 2B4 Ab (or control isotype Ab). (c) β -Hex release from resting or IgE (DNP)-activated BMMCs derived from 2B4^{-/-} mice or their wt counterparts. β -Hex was measured by a chromogenic assay at 405 optical density (OD). All data represent $n = 3$ experiments and are shown as mean \pm SEM ($*P < 0.05$). Ab, antibody; BMMC, bone marrow-derived mast cell; DNP, dinitrophenyl; NA, not activated; wt, wild-type.

mice developed normally and showed typical expression of Siglec-F, CCR3, and CD48 (Supplementary Figure S1b online). Moreover, the 2B4-lacking cells showed intracellular granule content comparable to that of wt mice (data not shown), and normal response to platelet-activating factor-mediated activation (Figure 2c).

To examine the involvement of 2B4 in murine Eos chemotaxis, we assessed the migration ability of BMEos from 2B4^{-/-} mice toward different established stimuli. Compared with wt cells, 2B4-lacking BMEos showed diminished chemotaxis toward DNP-activated BMMCs or mouse Eotaxin-1 (Elishmereni *et al.*, 2013), suggesting that 2B4 facilitates trafficking of Eos (Figure 2d). We also examined whether 2B4 can influence long-term BMEos activation. Indeed, BMEos ligated with the 2B4 Ab for 48 hours increased

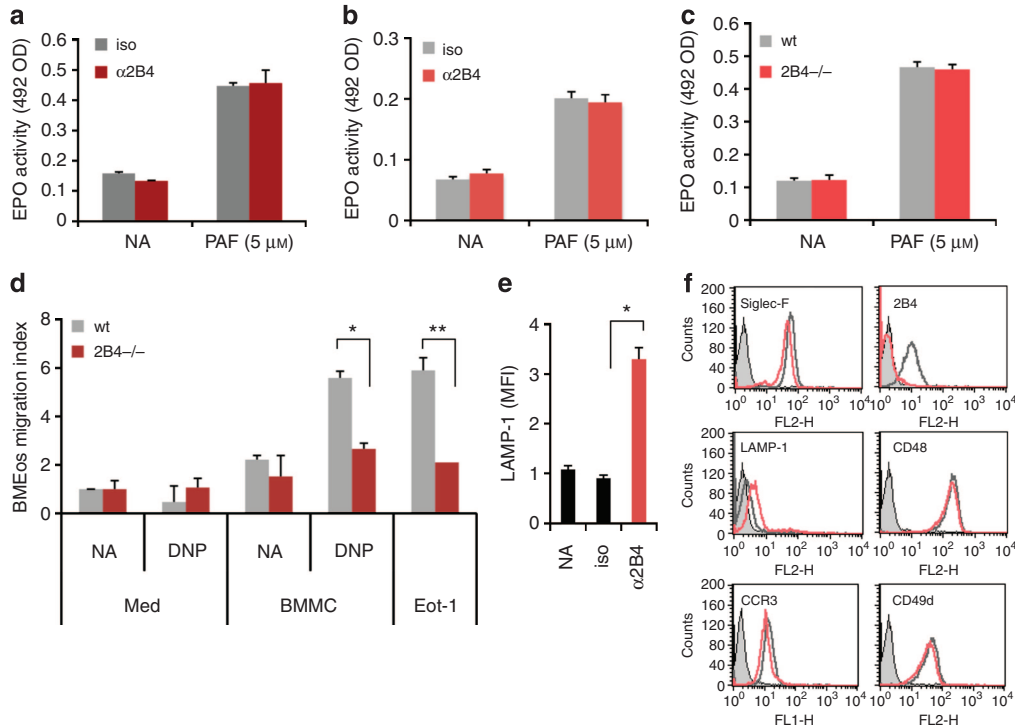


Figure 2. Late 2B4 activation of BMEos. EPO release from platelet-activating factor (PAF)-activated (vs. resting) BMEos after (a) ligation or (b) blockade by 2B4 or isotype (iso) Abs. (c) EPO release in 2B4^{-/-} BMEos under PAF activation, or in resting conditions. (d) wt and 2B4^{-/-} BMEos migration toward resting and IgE (DNP)-activated BMMCs, and eotaxin-1 (Eot-1). Controls contain medium (Med) ± DNP. Chemotaxis is expressed as a migration index relative to baseline migration. (e) Mean fluorescence intensity (MFI) of lysosomal-associated membrane protein-1 expression in wt BMEos ligated with a 2B4 (or isotype) Ab for 48 hours, vs. resting BMEos. (f) Expression of surface molecules in wt BMEos ligated for 48 hours with a 2B4 Ab (pink curves) or isotype Ab (gray curves). Gray-filled histograms mark isotype staining. All data represent *n* = 3 experiments and are shown as mean ± SEM (**P* < 0.05, ***P* < 0.005). Ab, antibody; BMEos, bone marrow–derived Eos; BMMC, bone marrow–derived mast cells; DNP, dinitrophenyl; EPO, Eos peroxidase; NA: not activated; wt, wild-type.

the expression of the Eos activation marker lysosomal-associated membrane protein-1 (LAMP-1; Figures 2e and f), indicating prolonged stimulation. The Ab binding of 2B4 on these BMEos was confirmed as steady even after 48 hours, as 2B4 could not be stained by a second PE-conjugated 2B4 Ab (Figure 2f). The 2B4-induced increase in LAMP-1 was specific, as other surface markers (Siglec-F, CD48, CCR3, and CD49d) on these BMEos were unchanged under similar constant 2B4 ligation (Figure 2f). Thus, 2B4 signaling facilitates a persisting activated phenotype in murine Eos.

2B4 effects in nonallergic peritonitis

We tested whether the above *in vitro*-dissected 2B4 stimulatory/inhibitory effects occur also *in vivo*. Peritonitis was induced in 2B4^{-/-} mice by i.p. challenge with compound 48/80 (c48/80), a MC degranulator causing rapid inflammation independently of IgE (Das *et al.*, 1997; Ribeiro *et al.*, 2000). Peritoneal lavages post-challenge showed a minor (yet insignificant) rise in granulocytes/neutrophils and reduction in monocytes/lymphocytes (with no differences between wt and 2B4^{-/-} mice; Supplementary Figure S2 online), pointing to a general inflammatory response. Peritoneal MCs (cKit+ mononuclear-derived cells) were also reduced after challenge (Figure 3a), likely owing to c48/80-induced degranulation

and/or depletion of MCs (Das *et al.*, 1997; Ribeiro *et al.*, 2000). MCs were lower even in control 2B4^{-/-} mice as compared with wt mice, implying that 2B4 absence causes baseline depletion or overdegranulation of MCs, and possibly indicating inhibitory 2B4 signals in MCs (as found *in vitro*). Challenge also increased the infiltration of Eos (Siglec-F+ cells) in wt mice (Figure 3b), as expected in view of the strong MC activation (Das *et al.*, 1997). Interestingly, however, this did not occur in diseased 2B4^{-/-} mice, where low peritoneal Eos were found despite highly degranulating MCs. Hence, concomitant with culture experiments, a 2B4 stimulatory effect on Eos trafficking may exist.

2B4 effects in mild (short-term) AD

To explore the 2B4 duality in allergic inflammation, we used experimental AD, a disease accompanied by substantial skin infiltration of MCs and Eos (Savinko *et al.*, 2005). AD was induced in 2B4^{-/-} and wt mice by repeated epicutaneous sensitization with ovalbumin (OVA) and streptococcal enterotoxin B (SEB) in a mild vs. chronic model (see (Savinko *et al.*, 2005; Wang *et al.*, 2007) and Supplementary Materials and Methods online).

In the mild model (Figure 4a), both mice displayed characteristic skin remodeling following OVA/SEB treatment.

This was shown by thickening of the dermis and more so of the epidermis, although the latter effect was slightly milder in 2B4^{-/-} mice (Figure 4b). Serum total IgE was increased in diseased wt mice, but not in 2B4^{-/-} mice (Figure 4c). It is noteworthy that total IgE was higher in control 2B4^{-/-} mice compared with wt, implying a role for 2B4 in regulating IgE production. The overall milder inflammation in 2B4^{-/-} mice was observed in other parameters: OVA-specific IgE was increased in both strains, yet more prominently in wt mice (Figure 4c). Serum tumor necrosis factor- α (Supplementary Figure S3a online) and skin-draining lymph node cellular content (Supplementary Figure S3b online) were also enhanced in challenged wt (but not 2B4^{-/-}) mice. Similar trends were observed in serum IFN- γ , IL-13, and IL-5, although not significantly, owing to high variance between individual mice (Supplementary Figure S3a online; IL-4 levels were undetectable). Similarly, skin infiltrates of CD3⁺, CD4⁺, and Foxp3⁺ (regulatory) lymphocytes were higher in diseased wt, but not in 2B4^{-/-} mice (Figure 4d), with a constant ratio between T effector cells and T regulatory cells as recognized in this model (Fyhrquist *et al.*, 2012). However, other cells such as CD8⁺ cells and activated (degranulated, non-dense) MCs were increased in both mouse types under challenge (Figures 4d and e). Of note, the rise in activated MCs was probably not due to trafficking, considering the comparable total MC counts in all groups (Figure 4e). Infiltration of skin Eos also appeared in both diseased groups (Figure 4e), yet the Eos level in 2B4-lacking mice was lower (Figure 4e). Collectively, most inflammation markers and particularly Eos responses are hampered under absent 2B4 signaling.

2B4 effects in chronic (long-term) AD

In chronic AD (Figure 5a), stronger skin remodeling was observed in both strains, as dermal and epidermal thickening increased similarly (Figure 5b). Both challenged strains also showed higher total and OVA-specific IgE, even though the rise in OVA-specific IgE in 2B4^{-/-} mice was more minor (Figure 5c). (Notably, as in mild AD, total IgE in control groups differed between wt and 2B4^{-/-}, yet with greater levels in wt mice, in contrast to Figure 4c.) Challenged wt mice also showed higher serum IFN- γ and IL-13 levels than 2B4^{-/-} mice (Supplementary Figure S3c online; levels of tumor necrosis factor- α , IL-4, and IL-5 were undetectable). Similarly, although skin CD3⁺ and CD4⁺ cells were normally increased in both challenged wt and 2B4^{-/-} mice (Supplementary Figure S4 online; a, b), the 2B4 knockouts displayed a more modest rise in CD8⁺ cells, and the increased Foxp3⁺ cells observed in wt mice were lacking in 2B4^{-/-} mice (Supplementary Figure S4 online; a, b). Hence, the absence of 2B4 partially impairs the inflammatory response also in chronic AD.

Interestingly, activated (but not total) MCs were increased after OVA/SEB challenge in both mouse strains, yet 2B4^{-/-} mice exhibited significantly higher numbers of these MCs (Figure 6a). Unlike in mild AD, Eos infiltrates were comparable in both mouse types under challenge (Figure 6a). MCs physically contact Eos to form couples (allergic effector units; AEU), especially under activated settings, and this

contact occurs partly through 2B4 interactions with CD48 (Elishmereni *et al.*, 2011; Elishmereni *et al.*, 2013). We thus hypothesized that MC-Eos coupling would be impaired in 2B4-lacking mice under inflammation. The MC-Eos pairing found in OVA/SEB-challenged wt mice (but not in controls) was in fact much less abundant in the challenged 2B4^{-/-} mice (Figures 6b and c), even though total MCs and Eos were similar in both groups, implying that 2B4 is needed for AEU formation *in vivo*.

DISCUSSION

The effects of 2B4 have been widely explored in NK cells, particularly in the context of viral infections, lymphoproliferative disorders, and antitumor responses (Chlewicki *et al.*, 2008; Waggoner and Kumar, 2012), but not yet in inflammation and allergic hypersensitivity. We presently demonstrate the multifaceted role of 2B4 on the main allergic inflammation effectors MCs and Eos in mice, and expand on the known functions of 2B4 in human Eos (as summarized in Supplementary Figure S5 online), using a comprehensive approach integrating *in vitro* and *in vivo* models.

We revealed a 'time-dependent' stimulatory role for 2B4 in murine Eos: 2B4 ligation did not induce BMEos degranulation, but rather a 'late activation' phenotype in BMEos (as reflected by selectively increased LAMP-1). The notion that 2B4 signaling affects delayed Eos functions is supported also by our previous study, where human Eos were more viable following a 3-day 2B4 activation (Elishmereni *et al.*, 2011). It is noteworthy that 2B4 ligation did induce rapid degranulation of human peripheral blood Eos in our earlier study (Munitz *et al.*, 2005). However, this effect may not have been observed in BMEos, because they are not *in vitro* primed or derived from atopic individuals (in contrast to human Eos; (Munitz *et al.*, 2005)). It is also plausible that human and murine Eos 2B4 effects do not coincide, especially as 2B4 functions in mouse and human NK cells are known to differ (Vaidya and Mathew, 2006; Chlewicki *et al.*, 2008).

The chemotactic ability of Eos was also dependent on 2B4: BMEos from 2B4^{-/-} mice showed significantly reduced migration capacity *in vitro*, consistent with the observed poor Eos infiltration in 2B4^{-/-} mice with peritonitis. The fact that similar outcomes were found in both disease models indicates a robust 2B4 effect, likely independent of IgE processes. This is also supported by the recent finding that 2B4-cross-linking on human peripheral blood Eos, and on a human Eos cell line, causes cell adhesion to collagen type IV and increased migration through tyrosine kinase phosphorylation (El-Shazly *et al.*, 2011). A similar tyrosine-dependent mechanism may control 2B4-stimulated Eos chemotaxis also in mice. Further, it is tempting to speculate that CD48 on endothelial cells (Khan *et al.*, 2007) may be the trigger for this migration, by binding to 2B4 on Eos.

At the other end, 2B4 downregulates murine MCs: BMMCs that were 2B4-blocked, as well as BMMCs from 2B4^{-/-} mice, were more strongly activated. The finding that 2B4^{-/-} mice have normal MC/Eos properties (in concert with normal hematopoietic phenotypes that these mice present with; (Lee *et al.*, 2004; McNerney *et al.*, 2005)) suggests that the

heightened degranulation in 2B4-deficient BMMCs is a direct result of the missing 2B4 signal, and not a by-product of anomalous functioning of cells. Low peritoneal MC levels in 2B4^{-/-} mice also support a regulating 2B4 signal in these cells. An inhibitory effect of 2B4 in murine MCs is akin to its role in murine NK cells (which show lower cytotoxicity toward CD48⁺ targets, whereas 2B4-deficient or 2B4-blocked NK cells present augmented cytotoxicity toward the

same cells (Lee *et al.*, 2004; Vaidya and Mathew, 2006; Chlewicki *et al.*, 2008)).

The idea that 2B4 has contradicting effects in murine MCs and Eos is entirely plausible, given the mixed inhibitory/activatory profiles in NK cells in mice and humans (Vaidya and Mathew, 2006; Veillette, 2006; Chlewicki *et al.*, 2008). At least two 2B4 isoforms with distinct functions exist in both humans (Mathew *et al.*, 2009) and B6 mice (Stepp *et al.*, 1999). In a rat NK line, inhibitory effects associate with 2B4-L, the long isoform with four tyrosine motifs in the cytoplasmic domain (and the equivalent of the human 2B4 isoform (Veillette, 2006)), whereas 2B4-S (the short one-tyrosine-motif form) links to activation (Vaidya and Mathew, 2006; Chlewicki *et al.*, 2008). The 2B4 isoform in human or murine Eos remains uncharacterized. Interestingly, BMMCs contain a 2B4S isoform (Kubota, 2002), yet the latter differs structurally from the NK isoform. BMMCs may also contain an additional undefined 2B4 isoform (Kubota, 2002). However, given that the 2B4L isoform has opposing functions in human and murine NK cells, it is unlikely that the 2B4 isoform alone determines outcome. Rather, the actual phenotype will depend also on the 2B4 expression density, glycosylation, and degree of cross-linking (Chlewicki *et al.*, 2008). Indeed, BMMCs and BMEos express different 2B4 levels (Supplementary Figure S1 online), and cytokines/chemokines may alter this expression (El-Shazly *et al.*, 2011). Diverse adaptor recruitment downstream 2B4 also controls outcome: NK activation depends on 2B4 binding to SAP (also expressed in human Eos (Veillette, 2006)), whereas NK inhibition involves EAT-2, ERT, SHP-1, and SHIP (Vaidya and Mathew, 2006) (the latter two phosphatases also present in BMMCs; (Gilfillan and Rivera, 2009)). Future studies should characterize the exact 2B4 sequence variations and signaling molecules underlying 2B4 inhibition of BMMCs and stimulation of BMEos.

Mouse AD mimicking the features of human dermatitis (skin remodeling, cell hyperplasia, immune infiltration, and so on) is best induced by repeated topical OVA sensitization, often together with SEB (Spergel *et al.*, 1998; Savinko *et al.*, 2005; Wang *et al.*, 2007; Jin *et al.*, 2009). The OVA/SEB model is MC and Eos-abundant (Savinko *et al.*, 2005), and allergen/SEB dermatitis models appear to be highly dependent on MCs (Ando *et al.*, 2013). We were able to induce AD in 2B4^{-/-} mice with significant remodeling and inflammation; however, the 2B4 deficiency impaired these effects: in the mild model, systemic inflammatory markers (total and OVA-specific IgE, cytokines, lymph node content) and local inflammatory markers (CD3⁺, CD4⁺, and Foxp3⁺ cells; Eos) were diminished in 2B4^{-/-} mice. However, in chronic AD, some of these markers (total IgE, CD3⁺ and CD4⁺ cells,

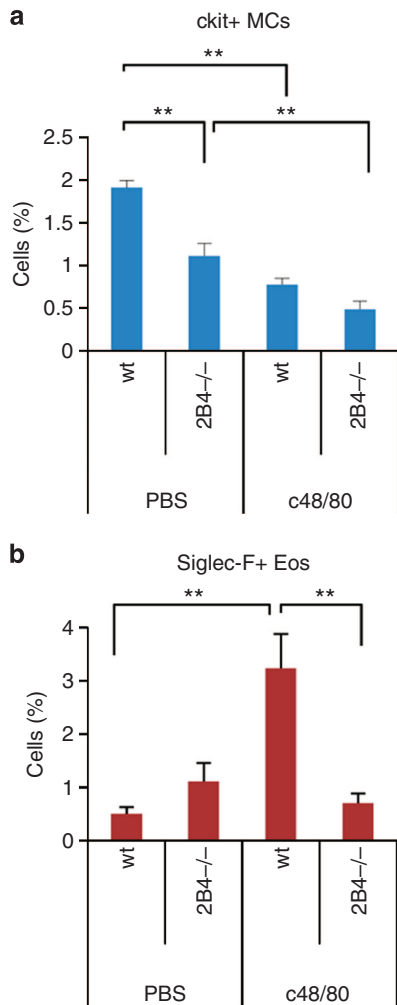
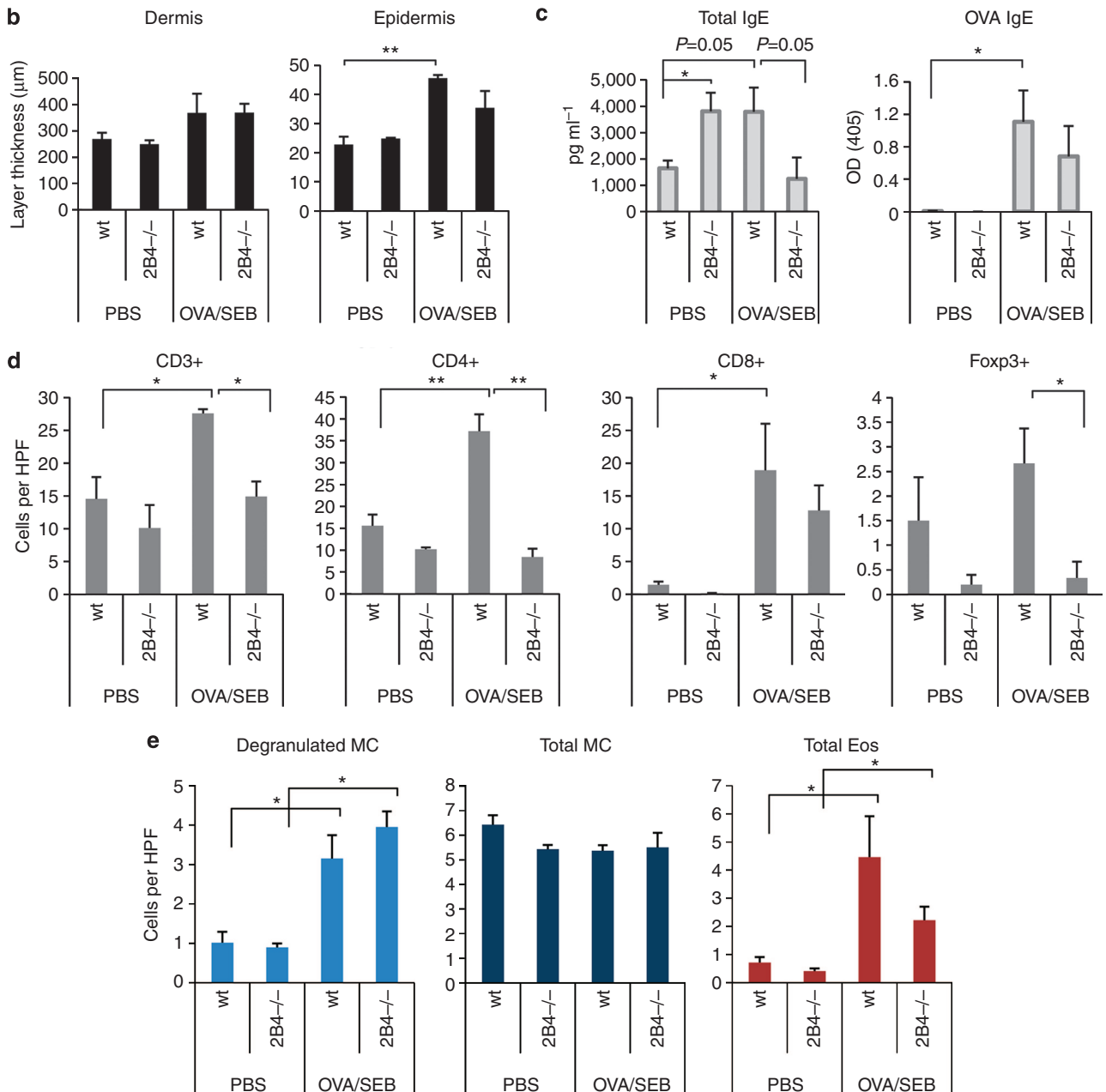
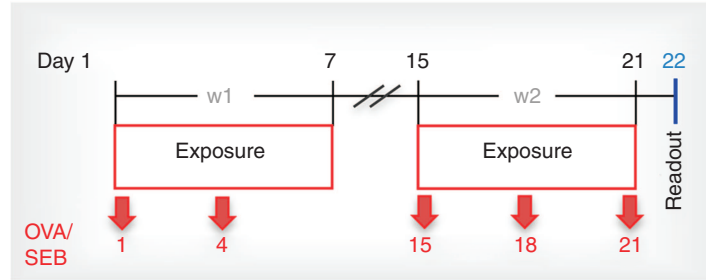


Figure 3. Peritonitis in 2B4^{-/-} mice. (a) cKit⁺ MCs and (b) Siglec-F⁺ Eos counted in peritoneal lavages of wt and 2B4^{-/-} mice after 24 hours of c48/80 challenge (vs. phosphate-buffered saline control). Cells were gated in flow cytometry by their specific surface markers, and shown as % of all counted cells. Data represent one of the three experiments (n=5 mice per group) and are shown as mean ± SEM (**P<0.005). Eos, eosinophils; MC, mast cell; wt, wild-type.

Figure 4. Mild AD in 2B4^{-/-} mice. (a) AD induced by 2-week epicutaneous challenge of wt and 2B4^{-/-} mice with OVA/SEB (or phosphate-buffered saline in controls), separated by a 1-week resting period. Arrows denote days of sensitization. (b) Dermal/epidermal thickness measured in H&E-stained skin sections in each group. (c) Serum total/OVA-specific IgE measured in each mouse group by ELISA. (d) CD3⁺, CD4⁺, CD8⁺, and Foxp3⁺ cells enumerated in H&E-stained skin sections in each group. (e) Total MCs, degranulated (non-dense) MCs, and infiltrating Eos enumerated in CTB- and H&E-stained skin sections in each group. Skin parameters were evaluated in ×10–100 magnified high-power fields. Data are representative of two experiments (n=4 mice per group) and shown as mean ± SEM (*P<0.05, **P<0.005). AD, atopic dermatitis; CTB, congo-red toluidine blue; Eos, eosinophils; H&E, hematoxylin and eosin; HPF, high-power field; MC, mast cell; OVA, ovalbumin; SEB, streptococcal enterotoxin B; wt, wild-type.



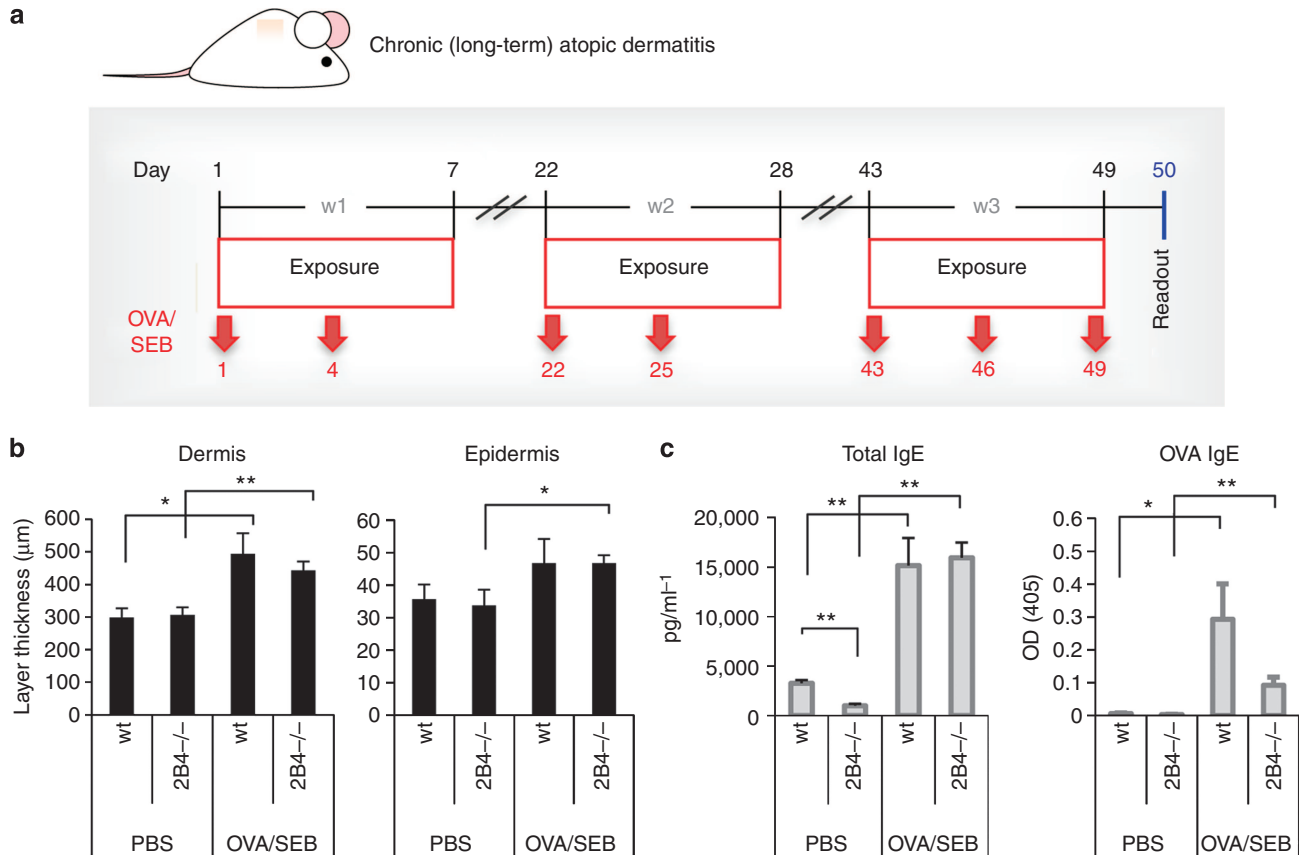


Figure 5. Chronic AD in 2B4^{-/-} mice. (a) AD induced by 3-week epicutaneous challenge of wt and 2B4^{-/-} mice with OVA/SEB (or phosphate-buffered saline in controls), separated by 1-week resting periods. Arrows denote days of sensitization. (b) Dermal/epidermal thickness measured in H&E-stained skin sections in each group. (c) Serum total/OVA-specific IgE measured in each group by ELISA. Skin parameters were evaluated in $\times 10$ –100 magnified high-power fields. Data are representative of two experiments ($n = 7$ mice per group), and shown as mean \pm SEM (* $P < 0.05$, ** $P < 0.005$). AD, atopic dermatitis; H&E, hematoxylin and eosin; OVA, ovalbumin; SEB, streptococcal enterotoxin B; wt, wild-type.

Eos) were no longer impaired in the absence of 2B4, whereas other markers (OVA-specific IgE, cytokines, CD8⁺, and Foxp3⁺ cells) were still hampered. This suggests that 2B4 acts separately on each molecular and cellular component of inflammation, and the dynamics of its effect differ between components. Moreover, the 2B4 transcriptional pathway could also be important for regulation of IgE kinetics, production, and decay, given that the lack of 2B4 causes increased IgE levels in mild AD, but lower levels in chronic AD. Overall, the exact role of 2B4 in AD seems to depend on the disease stage and severity.

The finding that in mild AD Eos infiltration was hampered in 2B4^{-/-} mice, particularly despite the abundance of activated MCs (which are primary chemoattractants for Eos; (Das et al., 1998)), provides additional evidence for a stimulating 2B4 signal for Eos trafficking. Strikingly, the 2B4 absence did not inhibit Eos infiltration in chronic AD, but did elevate activated MCs, offering support for the 2B4 inhibitory effect on MC activation. Notably, this overt MC stimulation in 2B4^{-/-} mice may explain the restored Eos influx, considering that MCs are known inducers of Eos chemotaxis (Das et al., 1998; Hirai et al., 2001; Shakoory et al., 2004).

Thus, the *in vivo* findings of 2B4-stimulated Eos functions and 2B4-inhibited MC functions concur with the *in vitro* understanding of the dual 2B4 effect.

We recently found that, in humans, direct Eos 2B4 binding with its cognate ligand CD48 on MCs participates in MC-induced boosting of Eos viability and in Eos-augmented MC degranulation (Elishmereni et al., 2011; Elishmereni et al., 2013). On the basis of this, the 2B4-CD48 axis was nominated as a prominent component of the AEU, a functional MC–Eos communication system. Interestingly, the 2B4-CD48 axis is probably conserved in humans and mice, as 2B4 engages CD48 at similar affinities and docking topologies in both species (Chlewicki et al., 2008). Indeed, 2B4-mediated contacts between MCs and Eos also appear in mice (Elishmereni et al., 2011; Elishmereni et al., 2013). It is therefore not surprising that MC/Eos pairing in chronic AD is reduced in 2B4-lacking mice, where the 2B4/CD48-binding platform is missing. We rule out the possibility that this reduced pairing stems from different cell quantities, as MC and Eos numbers were uncompromised (Figure 6a). These findings thus support our prior hypothesis (Elishmereni et al., 2011; Elishmereni et al., 2013) (i) that AEU formation

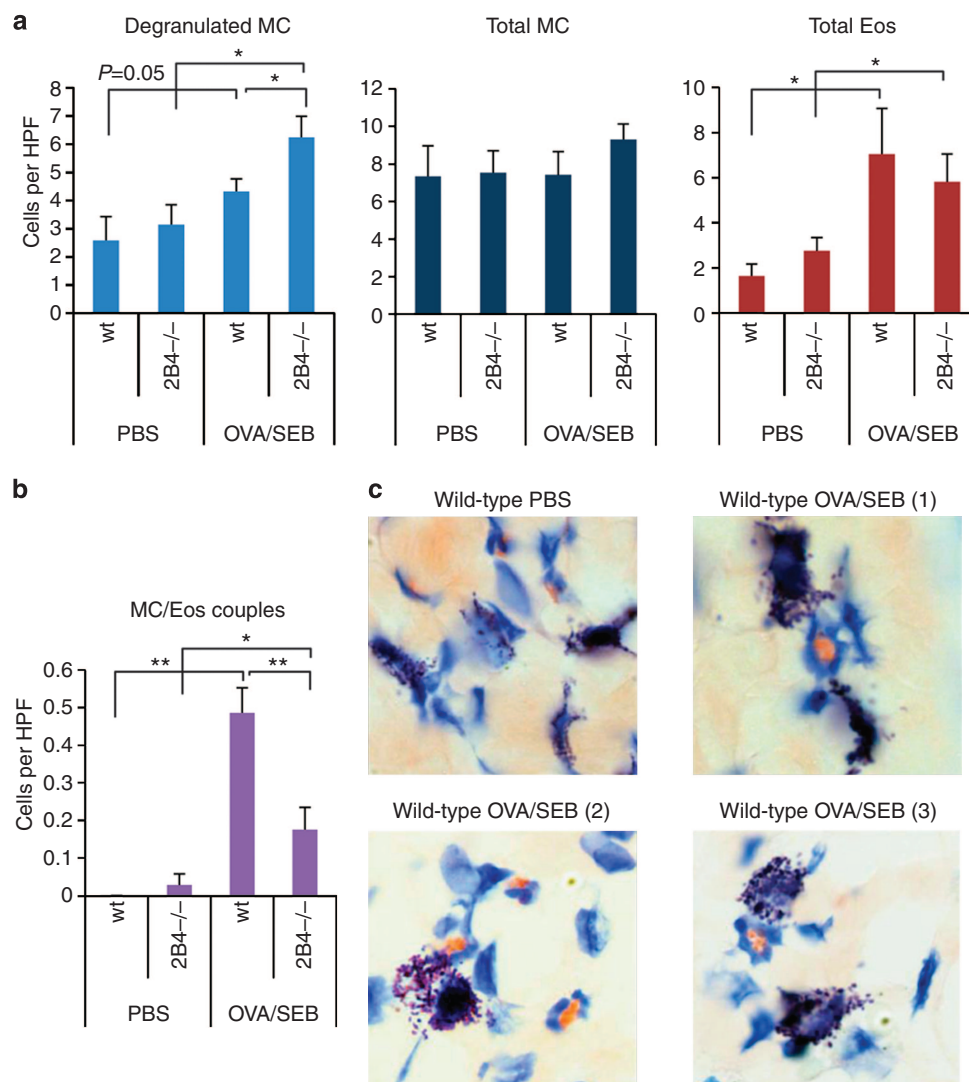


Figure 6. Chronic AD in 2B4^{-/-} mice: MC/Eos coupling. (a) Total and degranulating MCs, and infiltrating Eos, and (b) MC–Eos couples, in CTB- and H&E-stained skin sections of wt and 2B4^{-/-} mice challenged with OVA/SEB (or phosphate-buffered saline in controls) under the chronic AD model. (c) CTB-stained skin sections showing MC–Eos coupling in wt mice under OVA/SEB challenge (vs. phosphate-buffered saline control). Cells were evaluated in $\times 10$ –100 magnified high-power fields. Data represent two experiments ($n=7$ mice per group) and are shown as mean \pm SEM (* $P<0.05$, ** $P<0.005$). AD, atopic dermatitis; CTB, congo-red toluidine blue; H&E, hematoxylin and eosin; HPF, high-power field; MC/Eos, mast cells/eosinophils; OVA, ovalbumin; SEB, streptococcal enterotoxin B; wt, wild-type.

correlates with disease severity and (ii) that AEU occurrence depends, at least partially, on 2B4-mediated binding. Of note, in mice, a two-way CD48–2B4 communication between MCs and Eos is plausible, as both receptors appear on both cells (Elishmereni *et al.*, 2013). The existence of a bi-directional 2B4–CD48 interaction involving both stimulating and inhibiting signals in MCs/Eos immensely complicates the delineation of the CD48–2B4 role in inflammation. Future work in CD48-deficient mice will provide a ‘mirror’ system for corroborating and complementing our present conclusions.

Over the past few years, there has been increasing interest in inhibitory MC/Eos molecules as therapeutic targets for regulating allergy (Munitz and Levi-Schaffer, 2007; Karra and Levi-Schaffer, 2011; Migalovich-Sheikhet *et al.*, 2012; Nissim Ben Efraim *et al.*, 2013). Inhibiting functions revealed

for 2B4 suggest its addition to this arsenal. However, further studies must clarify whether the complex 2B4 activation/inhibition system displayed herein is paralleled in the human setting (in either allergic, or other, immune effector cells). In this case, optimal use of 2B4-targeting agents will need to contemplate inhibitory effects while preventing overstimulation.

MATERIALS AND METHODS

Mice

2B4^{-/-} mice, generated on the C57BL/6 (B6) background (Lee *et al.*, 2004), were obtained as a generous gift from the laboratory of Professor V Kumar (University of Chicago, IL, USA). Age and gender-matched wt B6 mice were purchased from Harlan Laboratories (Jerusalem, IL). Mice were housed and bred in the Hebrew

University of Jerusalem Animal Facilities, in specific pathogen-free conditions. All murine experiments were approved by the Animal Experimentation Ethics Committee of the Hebrew University of Jerusalem.

Cell culture experiments

MCs and Eos were cultured from bone marrow progenitors of female 2B4 $-/-$ and wt mice, and examined for maturity and phenotype after 4 and 2 weeks (respectively). In culture experiments, BMMCs were activated by an IgE/DNP system, under 2B4 Ab-ligating or blocking conditions (in wt), or under normal conditions (in 2B4 $-/-$), and after 1h β -hexosaminidase release was evaluated in a chromogenic assay. BMEos were activated by platelet activating factor under 2B4 Ab-ligation or blockade (in wt) or under normal conditions (in 2B4 $-/-$); EPO release was measured after 1 hour by an enzymatic assay, and surface molecule expression was evaluated after 48 hours by flow cytometry. See complete procedures in Supplementary Materials and Methods online.

In vivo inflammation models

Peritonitis was induced in male 10-week-old wt and 2B4 $-/-$ mice by i.p. injecting c48/80 or phosphate-buffered saline in control groups. After 24 hours, peritoneal lavages were recovered for examination of cellular content by flow cytometry. AD was induced in female 7-week-old mice (wt and 2B4 $-/-$) by repeated epicutaneous sensitization (Savinko *et al.*, 2005; Wang *et al.*, 2007), within a 'mild' (short-term) 22-day model, and in a 'chronic' (long-term) 50-day model. For allergen exposure, mouse back skins were shaved, tape-stripped, and a patch soaked in OVA/SEB or phosphate-buffered saline (in controls) was applied, and later removed during resting periods. At end points, mice were killed and skin samples were processed for histology and immunohistochemistry. Skin-draining lymph node content was assessed by flow cytometry, and sera were examined for cytokines and IgE by ELISA and Luminex. See Supplementary Materials and Methods online for full details.

Statistical analysis

All experiments were carried out at least three times, and data were analyzed by two-tailed paired Student's *t*-test assuming equal variance, where *P*-values <0.05 were considered significant. Data are reported as mean \pm SEMs.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank the Levi-Schaffer lab members for helpful discussions, and Sauli Savukoski and Sirpa Hyttinen in the Alenius lab for technical assistance. This work was supported by the Israel Science Foundation (grant 213/05), the MAARS EU 7th framework (grant no. HEALTH-F2-2011-261366), the Aimwell Charitable Trust (London, UK), and the Adolph and Klara Brettler Center for Research in Molecular Pharmacology and Therapeutics at the Hebrew University of Jerusalem.

Author contributions

ME designed the research, performed all experiments, analyzed data and wrote the manuscript; RSG performed peritonitis experiments; NF, SL, and HA helped set up the two atopic dermatitis models, analyzed sera IgE levels and skin cell infiltrates, and provided helpful guidance and discussions. FL-S designed the research, received the grant support, provided overall supervision, and edited the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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