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Absence of the adaptor protein Shb potentiates the T helper type 2 response in a mouse model of atopic dermatitis

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Summary

Aberrant regulation of T helper (Th) cell maturation is associated with a number of autoimmune conditions, including allergic disorders and rheumatoid arthritis. The Src homology domain protein B (Shb) adaptor protein was recently implicated as a regulator of Th cell differentiation. Shb is an integral component of the T-cell receptor (TCR) signalling complex and in the absence of *Shb* the TCR is less responsive to stimulation, resulting in the preferential development of Th2 responses under conditions of *in vitro* stimulation. In the present study, we extend those observations to an *in vivo* situation using a murine model of atopic dermatitis. *Shb* knockout mice develop more pronounced symptoms of atopic dermatitis with increased localized oedema, epidermal hyperplasia and IgE production. Dermal infiltration of mast cells, eosinophils, $CD4^+$ Th cells and $F4/80^+$ macrophages was also significantly increased in *Shb*-deficient mice. This correlated with elevated transcription of the hallmark Th2 cytokines interleukin-4 and interleukin-5. The loss of *Shb* therefore alters TCR signalling ability, thereby favouring the development of Th2-driven inflammation and exacerbating symptoms of allergy.

Keywords: atopic dermatitis; cytokines; Src homology domain protein B; T helper type 2 responses; T-cell receptor signalling.

Introduction

Activity of the immune system is largely regulated through the production of different types of cytokines by T helper (Th) cells. Based on cytokine secretion profiles, several Th cell subsets have been identified.[1–3] Th2 cells partake in the host's defence against extracellular parasites such as helminths,[2,4–7] and are characterized by production of high levels of interleukin-4 (IL-4), IL-5 and IL-13. A meticulous balance between different populations of Th cells is, however, essential for optimal pathogen clearance as well as for proper immune regulation. Several autoimmune conditions are linked to deregulated cytokine profiles.[8–10] Individuals suffering from allergic disorders such as asthma and atopic dermatitis often have an aberrant Th2 response with an elevation in eosinophil and basophil blood counts as well as increased levels of IgE.[11–15]

A wide variety of factors influence naive Th cells as they differentiate toward mature effectors in the periphery. The cytokine microenvironment in combination with antigen avidity and affinity will contribute to the mature cytokine profile of the Th cell.[16–20] T-cell receptor (TCR) signalling also appears to be of great importance since removal of central TCR pathway components has demonstrated that Th cell responses can be altered if the balance in the TCR signalling complex is disrupted.[21–24]

The association between allergic disease and Th2 driven inflammation is well established both in humans and in murine models. Patients suffering from asthma or atopic dermatitis show linkage to specific polymorphisms in the IL-4 receptor (IL4R) gene locus as well as to downstream effectors of the IL-4 signalling chain.[25,26] Additionally, deletion of typical Th2 cytokines results in milder symptoms in murine experimental models of asthma and atopic dermatitis.[27–31] Thymic stromal lymphopoietin (TSLP) has recently emerged as a key regulator of Th2 responses and allergic conditions.[32–34] TSLP acts on a wide variety of immune cells, including dendritic cells, basophils, mast cells and T cells, inducing a potent Th2 promoting microenvironment.[35–38] Epithelial cells secrete TSLP in response

Abbreviations: Ig, immunoglobulin; IL, interleukin; IL4R, interleukin-4 receptor; TCR, T cell receptor; TSLP, thymic stromal lymphopoietin; Shb, Src homology domain protein B; VEGF-A, vascular endothelial growth factor-A; VEGFR-2, vascular endothelial growth factor receptor-2

to danger signals such as mechanical injury or exposure to microbes and allergens.[37–39] TSLP secretion, however, is not only triggered by potentially harmful agents. Epidermal application of vitamin D or vitamin D analogues can elicit robust TSLP expression in skin keratinocytes.[40] Mice subjected to daily exposures of a vitamin D analogue develop skin lesions reminiscent of human atopic dermatitis with dermal hyperplasia and significant infiltration of $CD4^+$ T cells, so providing a convenient model system to study allergic inflammation.[35,40,41]

The Src-homology 2 domain containing adaptor protein Shb is mainly involved in mediating signals from activated tyrosine kinase receptors such as the vascular endothelial growth factor receptor-2, the platelet-derived growth factor receptor as well as the TCR.[42–44] Shb's Src-homology 2 domain preferably binds to phosphotyrosine residues in the cytoplasmic tail of receptor tyrosine kinases and subsequently recruits further signalling mediators.[45] Shb associates with the ζ-chain in the activated CD3-complex of the TCR and engages LAT, Grb2 and SLP76.[44,46] This complex can in turn promote the phosphorylation and activation of several central TCR signalling components including PLC-γ, Vav-1 and nuclear factor of activated T cells.[46,47]

An *Shb* knockout mouse was recently generated and a study on that mouse further confirmed the importance of Shb as a modulator of T-cell activation.[48] *Shb* null $CD4^+$ Th cells are hyperproliferative and display a bias towards a Th2 profile under *in vitro* stimulation. TCR signalling is aberrant in the absence of Shb, with increased basal activation of Vav-1, Cbl and p38 mitogen-activated protein kinase but blunted stimulation-induced phosphorylation of several signalling mediators including PLC-γ.[49] Together, these findings prompted the current investigation of Shb's role in *in vivo* regulation of the immune system under Th2 promoting conditions.

Materials and methods

Animals

The generation of *Shb* knockout mice has been described previously.[48] Since the *Shb* knockout was embryonically lethal on a C57BL/6 background the animals were initially maintained on a mixed genetic background consisting of 129/SvJ, FVB/N and C57Bl/6. To conduct experiments in a Th2 promoting environment the *Shb* knockout was bred on to the BALB/c background, known to be Th2 biased. BALB/c females (Scanbur AB, Sollentuna, Sweden) were mated with *Shb* knockout males and the resulting $Shb^{+/-}$ pups were continuously backcrossed with BALB/c for seven generations. At this point $Shb^{+/-}$ matings were set up to generate $Shb^{-/-}$ and $Shb^{+/+}$ animals. Subsequent experiments were all conducted on these *Shb* knockout (−/−) or wild-type (+/+) animals on the BALB/c background. All experiments were approved by the local animal ethics committee at Uppsala University.

Atopic dermatitis induction

The low calcaemic vitamin D agonist MC903 (Sigma-Aldrich, St Louis, MO) was dissolved in ethanol at a concentration of 50 μM; 1 nmol in a volume of 10 μl was applied to the inner and outer surfaces of the ear as previously described.[40] Ten microlitres of ethanol was used as vehicle control treatment. The animals were monitored daily for the development of symptoms and ear thickness was recorded every third day using digital callipers. MC903 and vehicle-treated mice were killed on day 9, and ears and auricular draining lymph nodes were isolated.

Histology

Upon isolation, ears were cut in half. One half was fixed in 4% formalin overnight and subsequently paraffin embedded whereas the other half was frozen and embedded in section media (Richard Allan Scientific, Thermo Scientific, Waltham, MA). Paraffin-embedded tissue was cut in 5-μm sections and mounted on polylysine microscope slides (Menzel Gläser, Braunschweig, Germany) and stained with haematoxylin & eosin, alkaline 0·5% Congo red or toluidine blue. Frozen tissue was cut into 8-μm sections and mounted on SuperFrost glass slides (Menzel Gläser) and fixed for 10 min in ice cold methanol before immunofluorescence staining.

Immunofluorescence

Frozen sections were stained with primary antibodies directed towards Th cell marker CD4 or macrophage marker F4/80 (both primary antibodies were from eBioscience, Hatfield, UK) in a concentration of 1 : 300. Secondary anti-rat Alexa Fluor 488 antibodies (Invitrogen, Carlsbad, CA) were used in a 1 : 500 dilution. Sections were mounted with Vectashield HardSet mounting medium (Vector Laboratories, Burlingame, CA) containing 4′,6-diamidino-2-phenylindole (DAPI) for staining of nuclei. A Nikon Eclipse TE2000-U fluorescence inverted microscope (Nikon, Tokyo, Japan), with a Nikon D Eclipse C1 camera was used to take photos and NIKON ACT-1C v1.02.10 (Nikon) and IMAGEJ v. 1.45s (NIH, Bethesda, MD) software programs were used for image analysis.

IgE ELISA

Immunoglobulin E was determined on serum samples from five wild-type and five *Shb* knockout mice either subject to MC903 treatment or not for 9 days using an IgE ELISA according to the manufacturer's recommendation (BioLegend, San Diego, CA).

RNA isolation and real-time reverse transcription-PCR

Auricular draining lymph nodes were fixed and preserved in RNALater (Qiagen, Solna, Sweden) immediately after isolation. RNA was extracted using an RNAeasy mini kit (Qiagen) following the instructions provided by the manufacturer and gene expression was determined by one-step real-time reverse transcription PCR using QuantiTect™ SYBR® Green RT-PCR kit (Qiagen). The PCR conditions were as follows; reverse transcription at 50° for 20 min, inactivation at 95° for 15 min, 50 cycles of denaturation at 94° for 15 seconds, annealing for 25 seconds at the various temperatures indicated in Table 1, and extension at 72° for 15 seconds. Primer sequences are listed in the Supporting information, Table S1. All reactions were run on a LightCycler™ real-time PCR machine (Roche Diagnostics, Basel, Switzerland). Cycle threshold (C_T) values were estimated with the LIGHTCYCLER SOFTWARE v 4.1 (Roche, Basel, Switzerland) and transcript levels were normalized by subtracting the corresponding β-actin values.

Miles assay

To determine the effects of acute histamine permeability a modified Miles assay was performed.[50,51] Mice were shaved on the back 2 days before the experiment. On the day of the experiment, the mice were anaesthetized with avertin at a dose of 0·02 ml/g body weight. Then, 1% Evans Blue (Sigma Aldrich, St Louis, MO) dissolved in sterile PBS was injected at 2 µl/g body weight via the tail vein. After 30 min, the mice were intradermally injected with 3 µg histamine in 20 µl on their right side and 20 µl PBS on the left side, serving as a negative control. The animals were killed 30 min later and the affected tissue was collected and weighed. Evans Blue was extracted in 1·5 ml formamide at 70° and the optical density was measured at 630 nm using a FLUOStar Omega spectrophotometer (BMG Labtech, Ortenberg, Germany) to quantify the amount of Evans Blue extracted.

Statistical analysis

All values are presented as mean ± SEM. Comparisons between groups were performed by one-way analysis of variance followed by post hoc analysis with Bonferroni's test. Statistical significance was set to $P < 0{\cdot}05$.

Results

Shb knockout mice develop more severe symptoms in a model of atopic dermatitis

Initial attempts to induce atopic dermatitis in *Shb* knockout mice by ovalbumin exposure to tape-stripped skin

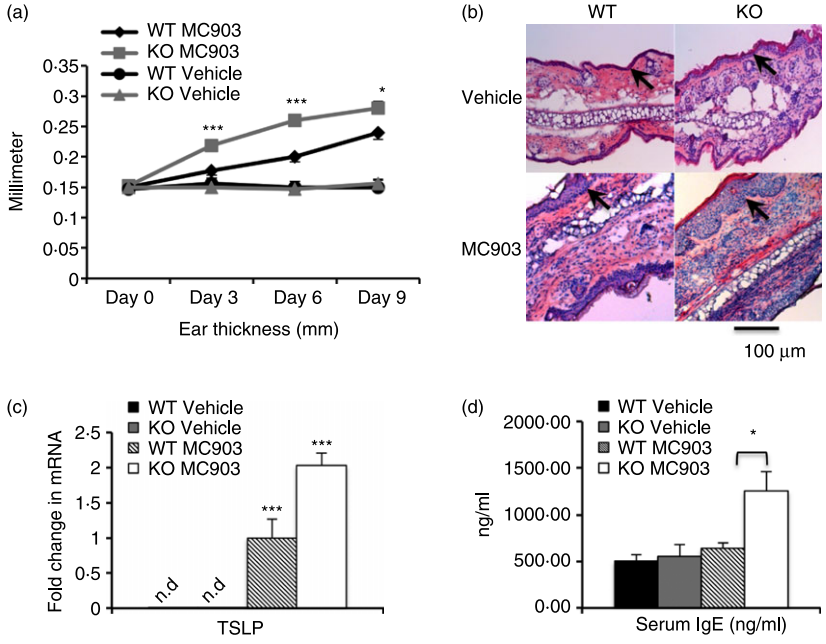

Figure 1. Development of MC903-induced dermatitis. (a) Oedema development was monitored by measurement of ear thickness every third day with a digital calliper. (b) Haematoxylin & eosin-stained sections of ears from untreated and MC903-treated mice. Arrows point to the junction between the epidermis and the dermis. Images were obtained with a 20× objective. Data are presented as mean ± SEM and based on six MC904-treated and three vehicle-treated mice of each genotype. (c) TSLP gene expression in response to MC903 treatment. Values are relative gene expression after normalization for actin in three untreated and five MC903-treated mice each group. n.d. = not detectable. Significant statistical comparisons were against untreated groups. (d) Serum IgE after MC903 treatment for 9 days or no treatment for five mice each group. *$P < 0{\cdot}05$ and ***$P < 0{\cdot}001$ as determined by one-way analysis of variance followed by Bonferroni's test.

failed because the mice rapidly dismantled the bandage holding the ovalbumin-containing patch.[52,53] However, atopic dermatitis-like symptoms[40] can be induced by exposure to the vitamin D analogue MC903, which via TSLP transcription results in increased levels of Th2 cytokines. MC903 was therefore applied daily to the ears of *Shb* knockout and wild-type mice. A second group of mice treated with ethanol served as a vehicle treatment control group. All MC903-treated mice developed localized oedema by day 6; however, the ear swelling was significantly pronounced in the *Shb*-deficient mice, suggestive of an aggravated reaction (Fig. 1a). The treatment continued over 9 days and the increased oedema persisted in the *Shb* knockout mice at each recorded time-point (Fig. 1a). The vehicle-treated group remained unaffected throughout the course of the experiment (Fig. 1a). Histological examination of the ears on day 9 revealed more epidermal hyperplasia and dermal infiltration of what appeared to be leucocytes in *Shb* null mice (Fig. 1b). MC903 augmented ear TSLP expression (Fig. 1c) and increased serum IgE in *Shb* knockout mice (Fig. 1d). To confirm that the cells infiltrating the dermis of the oedematous ears were leucocytes, frozen ear sections were stained for Th cell marker CD4 and macrophage marker F4/80.[54,55] Wild-type as well as *Shb* knockout MC903-treated ears contained increased numbers of $CD4^+$ Th cells and macrophages compared with the vehicle group (Fig. 2a,b), but the degree of increase was higher in the knockout (Fig. 2a,b). Mast cells are important effector cells in allergic inflammation; ears were therefore stained with toluidine blue to enable identification of mast cells.[56] *Shb*-deficient atopic dermatitis mice also displayed increased numbers of mast cells compared with their wild-type counterparts (Fig. 3a). The minimal increase of mast cell numbers observed in the wild-type situation is likely to reflect a milder disease and that mast cell infiltration is a late event in MC903-induced allergic dermatitis. There was also an increased infiltration of eosinophils in MC903-treated *Shb* knockout ears (Fig. 3b) further reinforcing the view that a more severe inflammatory reaction prevails in the absence of *Shb*. The loss of *Shb* therefore appears to affect

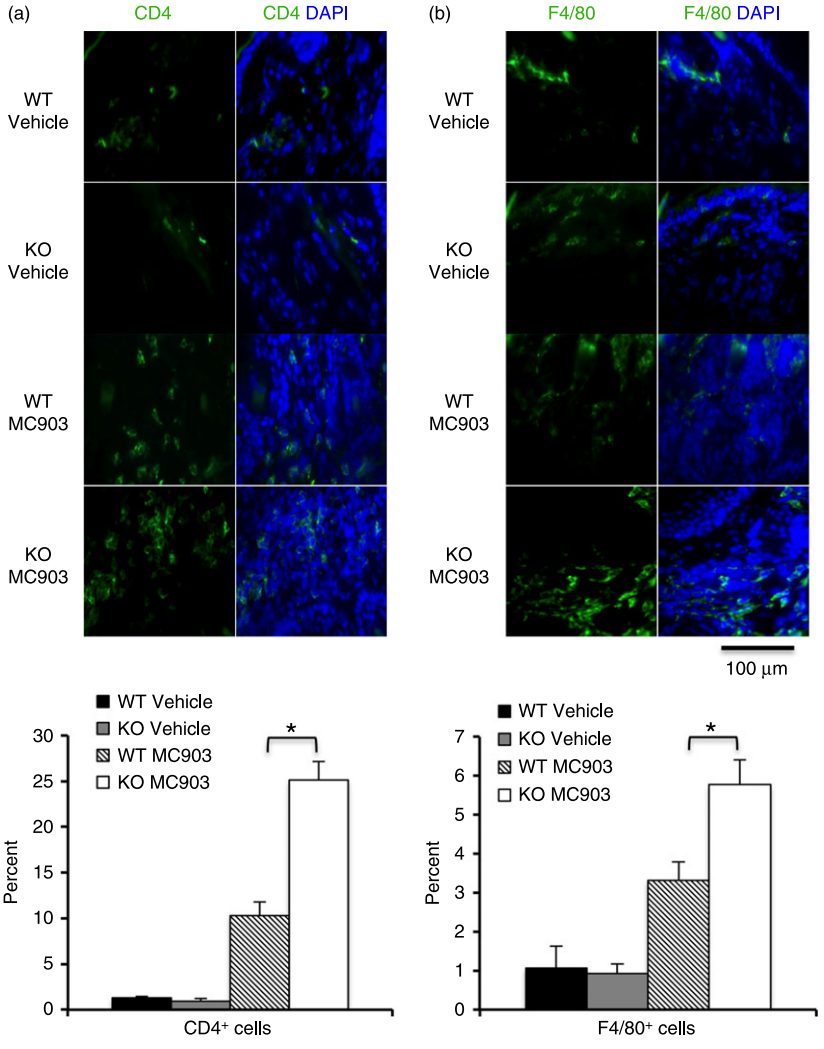

Figure 2. Analysis of T helper (Th) cell and macrophage recruitment. Frozen sections of MC903-treated and vehicle-treated ears were stained for CD4 (a) and F4/80 (b) to evaluate dermal infiltration of Th cells and macrophages, respectively. The proportions of respective cell type were determined by relating the number of marker-positive cells to the total number of DAPI-stained nuclei in the dermis. The results are presented as mean ± SEM and based on six MC904-treated and three vehicle-treated mice of each genotype. All photos were taken at 20× magnification. $*P < 0{\cdot}05$ as determined by one-way analysis o f variance followed by Bonferroni's test.

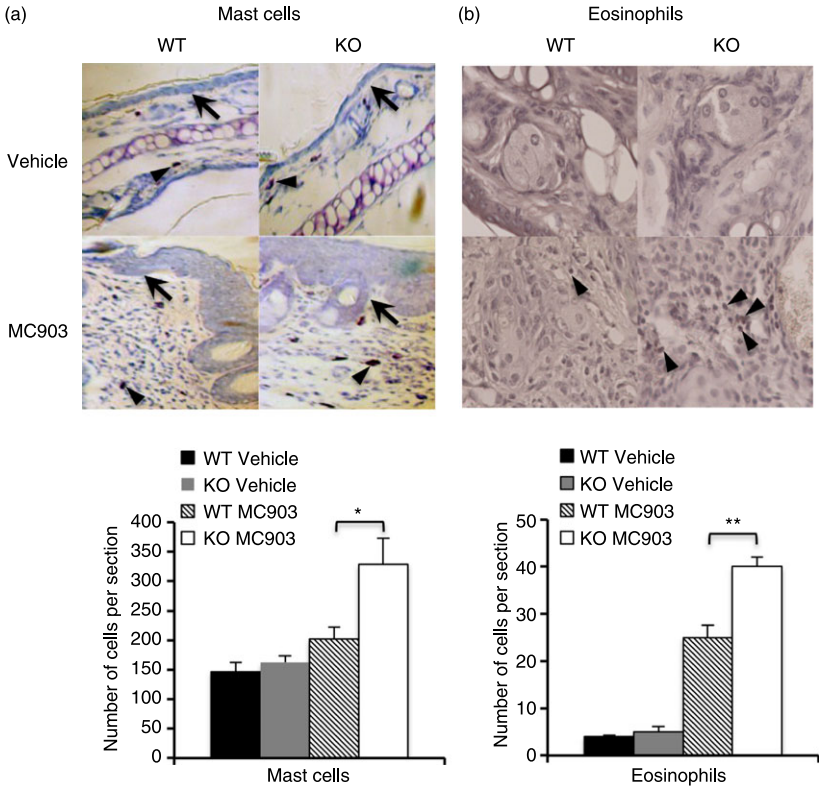

Figure 3. Evaluation of mast cell and eosinophil infiltration. (a) Mast cell numbers in ear dermis were determined in toluidine blue-stained sections of vehicle or MC903-treated ears. Arrows point to the junction between the epidermis and the dermis and the arrowheads point to mast cells. Original magnification was 20×. Values are presented as mean ± SEM with six animals in each group from three independent experiments. (b) Eosinophil infiltration after MC903 treatment for 9 days. Ear sections were stained with Congo red and positive cells were counted per section. Arrowheads point to examples of eosinophils. Values are based on five mice for each genotype after MC903 treatment and three mice each for vehicle control. *$P < 0{\cdot}05$ and **$P < 0{\cdot}01$, as determined by one-way analysis of variance followed by Bonferroni's test. Both MC903-treated groups were significantly elevated compared with vehicle controls.

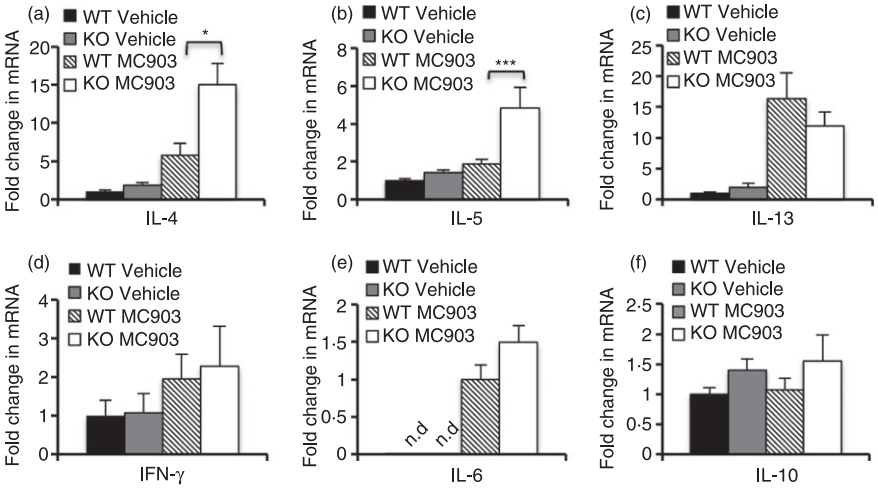

Figure 4. Analysis of cytokine expression patterns in auricular draining lymph nodes. The transcript levels of typical T helper type 2 (Th2) cytokines interleukin-4 (IL-4) (a), IL-5 (b), IL-13 (c), interferon-γ (IFN-γ) (d), IL-6 (e) and IL-10 (f) were evaluated by semi-quantitative real-time RT-PCR. All values were normalized to β-actin and subsequently related to the vehicle-treated wild-type. Means are presented as $2^{-\Delta C_t}$ ± SEM to demonstrate fold change in mRNA content. The results are representative of three independent experiments with six mice in each treatment group. ***$P < 0{\cdot}001$ and *$P < 0{\cdot}05$ as determined by one-way analysis of variance followed by Bonferroni's test.

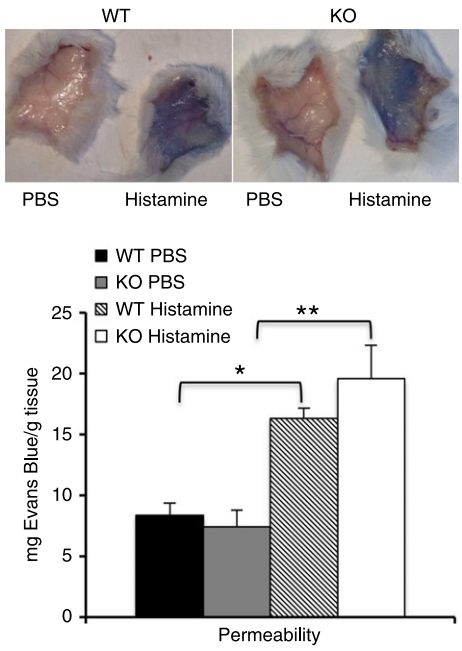

Figure 5. Assessment of histamine-induced vascular permeability. Evans Blue was delivered by a tail vein injection into wild-type and *Shb* knockout mice followed by an intradermal challenge with histamine in the right flank and sterile PBS in the left flank. Vascular permeability was quantified by estimation of micrograms of Evans Blue per gram skin tissue. The values are representative of three independent experiments and based on four mice per genotype. Data are presented as mean ± SEM. $^{**}P < 0{\cdot}01$ and $^{*}P < 0{\cdot}05$ estimated by one-way analysis of variance followed by Bonferroni's test.

the immune response under Th2 inflammatory conditions by enhancing the infiltration of various immune system effector cells.

Increased production of certain Th2 cytokines in Shb knockout mice with atopic dermatitis

Type 2 cytokines IL-4, IL-5 and IL-13 are mediators of many of the symptoms seen in allergic inflammation. To determine cytokine expression, auricular draining lymph nodes were isolated and RNA was extracted. Both IL-4 and IL-5 were expressed at significantly increased levels in the *Shb* knockout MC903-treated animals (Fig. 4a,b), whereas there was no major difference in the transcription of IL-13 between wild-type and knockout samples (Fig. 4c). Although Th2 cytokines are essential for the establishment of atopic dermatitis, type 1 cytokines like IL-6 and interferon-γ also play an important role in the development of the chronic phase of disease.[57] The transcript levels of these factors was examined, revealing similar expression levels in wild-type and *Shb* knockout atopic dermatitis mice (Fig. 4d,e), further supporting the notion that the absence of *Shb* results in a Th2 skewed response. Deficits in the regulatory T (Treg) cell population have been reported to contribute to the development of excessive Th2 responses.[58] To this end, IL-10 expression was determined. No differences were, however, observed between wild-type and *Shb* knockout samples with regards to IL-10 levels (Fig. 4f). This suggests that the Th2 bias is the result of a Th cell inherent defect. In conclusion, *Shb* deficiency appears to confer a more aggressive development of atopic dermatitis with increased leucocyte infiltration, possibly caused by the elevated expression levels of IL-4 and IL-5.

Histamine-induced vascular permeability is not affected in the absence of Shb

The increased infiltration of macrophages and Th cells displayed by the *Shb* knockout in the atopic dermatitis model might not be dependent on the local cytokine milieu but could merely be a consequence of increased blood vessel permeability. Several previous reports have established that the *Shb* knockout exhibits alterations in the microvasculature and in the permeability responses to stimulation with vascular endothelial growth factor A.[59–61] In Th2-driven inflammation the principal mediator of vessel permeability is histamine and a modified Miles assay with histamine as the permeability-inducing agent was therefore performed.[50] As suggested by the amount of extravasated Evans Blue, wild-type and knockout mice responded with increased vascular permeability to histamine stimulation (Fig. 5). However, there was no difference in the magnitude of the histamine response between the two groups (Fig. 5). The enhanced leucocyte recruitment observed in *Shb* knockout mice suffering from atopic dermatitis is therefore probably not a consequence of increased histamine-induced vascular permeability, but more likely a reflection of the increased production of Th2 cytokines.

Discussion

T helper cell differentiation has previously been demonstrated to be influenced by Shb, as naive *Shb*-deficient Th cells preferentially adopt a Th2 fate upon *in vitro* stimulation. In the present study we expand those findings by evaluating the effect of Shb-dependent signalling on Th cells in an *in vivo* context and consequently reinforce the previously published *in vitro* data because *Shb* knockout mice were found to develop more severe symptoms in a murine model of atopic dermatitis, a disease characterized by an abnormal Th2 response.

The signals that trigger Th cell development have been extensively studied, resulting in a complex model involving factors influencing the fate of the naive Th cell. Signalling via the antigen-recognizing TCR synergizes with the signalling cues from the local cytokine environment.[16,18,19] *Shb* null mice suffering from atopic dermatitis displayed significant changes in their cytokine

expression pattern at the site of inflammation with increased transcript levels of IL-4 and IL-5 in the auricular draining lymph nodes. Both IL-4 and IL-5 facilitate the infiltration of leucocytes by inducing the secretion of leucocyte-attracting chemokines and by enhancing the leucocyte responses to chemoattractants.[62–66] Interleukin-4 can also directly influence leucocyte recruitment by increasing the expression of vascular cell adhesion molecule 1 on endothelial cells.[67] In line with this, *Shb* knockout MC903-treated mice exhibited an exaggerated local recruitment of $CD4^+$ Th cells, macrophages and mast cells.

Notably, IL-13 was found to be expressed at similar levels in *Shb* knockout and wild-type mice after atopic dermatitis induction. Up-regulation of IL-4 and IL-5 often occurs concomitantly with increased expression of IL-13. Th2 cells are considered to be the major source of IL-13; however, other immune cells, including macrophages, mast cells, eosinophils and basophils, are capable of producing IL-13.[68–71] Since the presently employed *Shb* knockout is global, it cannot be excluded that defects in other immune cells contribute to the observed *Shb*-deficient atopic dermatitis phenotype.

Although other cell types may influence the immune responses elicited in the absence of *Shb* expression, defective Th2 regulation is likely to be a major factor in the currently observed aggravated atopic dermatitis symptoms. Our previous report demonstrating a Th2 skewing in *Shb* knockout T cells was based on *in vitro* experiments where purified $CD62L^{Hi}$ $CD44^{Lo}$ $CD4^+$ naive Th cells were stimulated with CD28 and CD3 antibodies.[72,73] These results are suggestive of a Th cell intrinsic defect in cytokine regulation. Previous results have also confirmed that Shb partakes in the signalling complex that assembles after TCR activation and that TCR signalling is disrupted in the absence of *Shb*.[44,46,47,49] TCR signalling strength has been implicated as a key determinant of Th cell differentiation.[74] Low concentrations of antigen that lead to weak TCR activation result in a preferential production of IL-4.[75] Similarly, altered peptide ligands with reduced TCR affinity also elicit robust Th2 responses.[16] The suboptimal TCR activation appears to alter the regulation of transcription factors that are essential for Th2 cell differentiation such as GATA3, AP-1 and nuclear factor of activated T cells.[76–78] Moreover, point mutations in the central TCR signalling mediators ZAP70 and CARMA1, which do not abolish signalling but weaken it, alter the balance in favour of Th2 responses.[58,79,80] In our previously published report, TCR cross-linking of *Shb*-deficient Th cells revealed decreased stimulation-induced tyrosine phosphorylation, suggestive of defective TCR signal transduction. The reduced TCR responsiveness displayed in the absence of *Shb* is likely to result in a Th cell inherent predisposition to promote the transcription of typical Th2 cytokines, so providing a mechanistic explanation for the more severe atopic dermatitis phenotype seen in the *Shb* knockout.

In conclusion, the loss of *Shb* expression disrupts TCR signalling, resulting in aberrant Th cell differentiation and development of exacerbated atopic dermatitis symptoms. The knowledge of how TCR signalling contributes to Th cell differentiation is still limited and mostly based on *in vitro* experiments. Further evaluation of Shb activity in human allergic subjects could therefore provide useful insights into the qualitative and quantitative differences in TCR signal transduction that underlies the development of allergic disorders.

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KG contributed to study conception and design, data collection, data analysis and interpretation, and manuscript writing. EW collected data. MW contributed to conception and design, data analysis and interpretation, manuscript writing and financial support. The study was supported by the Swedish Cancer Foundation (120831), Swedish Research Council (54x-10822), Swedish Diabetes fund (DIA 2012) and Family Ernfors fund.

Disclosures

The authors declare no financial or commercial conflict of interest.

References

1 Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT. Interleukin 17-producing $CD4^+$ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005; **6**:1123–32.

2 Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986; **136**:2348–57.

3 Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; **155**:1151–64.

4 McKenzie AN, Culpepper JA, de Waal Malefyt R *et al.* Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proc Natl Acad Sci USA* 1993; **90**:3735–9.

5 Coffman RL, Seymour BW, Hudak S, Jackson J, Rennick D. Antibody to interleukin-5 inhibits helminth-induced eosinophilia in mice. *Science* 1989; **245**:308–10.

6 Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Kohler G. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* 1993; **362**:245–8.

7 Else KJ, Finkelman FD, Maliszewski CR, Grencis RK. Cytokine-mediated regulation of chronic intestinal helminth infection. *J Exp Med* 1994; **179**:347–51.

8 Hirota K, Yoshitomi H, Hashimoto M *et al.* Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J Exp Med* 2007; **204**:2803–12.

9 Robinson DS, Hamid Q, Ying S *et al.* Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992; **326**:298–304.

10 Annunziato F, Cosmi L, Santarlasci V *et al.* Phenotypic and functional features of human Th17 cells. *J Exp Med* 2007; **204**:1849–61.

11 Macfarlane AJ, Kon OM, Smith SJ *et al.* Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin. *J Allergy Clin Immunol* 2000; **105**:99–107.

12 Reilly KM, Yap PL, Dawes J, Barnetson RS, MacKenzie F, Allan TL. Circulating basophil counts in atopic individuals. *Int Arch Allergy Appl Immunol* 1987; **84**:424–6.

13 Bousquet J, Chanez P, Lacoste JY *et al.* Eosinophilic inflammation in asthma. *N Engl J Med* 1990; **323**:1033–9.

14 Mudde GC, van Reijsen FC, Bruijnzeel-Koomen CA. IgE-positive Langerhans cells and Th2 allergen-specific T cells in atopic dermatitis. *J Invest Dermatol* 1992; **99**:103S.

15 Leiferman KM, Ackerman SJ, Sampson HA, Haugen HS, Venencie PY, Gleich GJ. Dermal deposition of eosinophil-granule major basic protein in atopic dermatitis. Comparison with onchocerciasis. *N Engl J Med* 1985; **313**:282–5.

16 Tao X, Grant C, Constant S, Bottomly K. Induction of IL-4-producing $CD4^+$ T cells by antigenic peptides altered for TCR binding. *J Immunol* 1997; **158**:4237–44.

17 Hosken NA, Shibuya K, Heath AW, Murphy KM, O'Garra A. The effect of antigen dose on $CD4^+$ T helper cell phenotype development in a T cell receptor-$\alpha\beta$-transgenic model. *J Exp Med* 1995; **182**:1579–84.

18 Seder RA, Gazzinelli R, Sher A, Paul WE. Interleukin 12 acts directly on $CD4^+$ T cells to enhance priming for interferon γ production and diminishes interleukin 4 inhibition of such priming. *Proc Natl Acad Sci USA* 1993; **90**:10188–92.

19 Seder RA, Paul WE, Davis MM, Fazekas de St Groth B. The presence of interleukin 4 during *in vitro* priming determines the lymphokine-producing potential of $CD4^+$ T cells from T cell receptor transgenic mice. *J Exp Med* 1992; **176**:1091–8.

20 Pfeiffer C, Stein J, Southwood S, Ketelaar H, Sette A, Bottomly K. Altered peptide ligands can control CD4 T lymphocyte differentiation *in vivo*. *J Exp Med* 1995; **181**:1569–74.

21 Tamura T, Igarashi O, Hino A, Yamane H, Aizawa S, Kato T, Nariuchi H. Impairment in the expression and activity of Fyn during differentiation of naive $CD4^+$ T cells into the Th2 subset. *J Immunol* 2001; **167**:1962–9.

22 Yamashita M, Kimura M, Kubo M, Shimizu C, Tada T, Perlmutter RM, Nakayama T. T cell antigen receptor-mediated activation of the Ras/mitogen-activated protein kinase pathway controls interleukin 4 receptor function and type-2 helper T cell differentiation. *Proc Natl Acad Sci USA* 1999; **96**:1024–9.

23 Blonska M, Joo D, Zweidler-McKay PA, Zhao Q, Lin X. CARMA1 controls Th2 cell-specific cytokine expression through regulating JunB and GATA3 transcription factors. *J Immunol* 2012; **188**:3160–8.

24 Mingueneau M, Roncagalli R, Gregoire C *et al.* Loss of the LAT adaptor converts antigen-responsive T cells into pathogenic effectors that function independently of the T cell receptor. *Immunity* 2009; **31**:197–208.

25 Hershey GK, Friedrich MF, Esswein LA, Thomas ML, Chatila TA. The association of atopy with a gain-of-function mutation in the α subunit of the interleukin-4 receptor. *N Engl J Med* 1997; **337**:1720–5.

26 Tamura K, Arakawa H, Suzuki M, Kobayashi Y, Mochizuki H, Kato M, Tokuyama K. Novel dinucleotide repeat polymorphism in the first exon of the STAT-6 gene is associated with allergic diseases. *Clin Exp Allergy* 2001; **31**:1509–14.

27 Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 1996; **183**:195–201.

28 Walter DM, McIntire JJ, Berry G, McKenzie AN, Donaldson DD, DeKruyff RH, Umetsu DT. Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. *J Immunol* 2001; **167**:4668–75.

29 Herrick CA, Xu L, McKenzie AN, Tigelaar RE, Bottomly K. IL-13 is necessary, not simply sufficient, for epicutaneously induced Th2 responses to soluble protein antigen. *J Immunol* 2003; **170**:2488–95.

30 Spergel JM, Mizoguchi E, Oettgen H, Bhan AK, Geha RS. Roles of TH1 and TH2 cytokines in a murine model of allergic dermatitis. *J Clin Invest* 1999; **103**:1103–11.

31 Brusselle G, Kips J, Joos G, Bluethmann H, Pauwels R. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am J Respir Cell Mol Biol* 1995; **12**:254–9.

32 Harada M, Hirota T, Jodo AI *et al.* Thymic stromal lymphopoietin gene promoter polymorphisms are associated with susceptibility to bronchial asthma. *Am J Respir Cell Mol Biol* 2011; **44**:787–93.

33 Hunninghake GM, Soto-Quiros ME, Avila L *et al.* TSLP polymorphisms are associated with asthma in a sex-specific fashion. *Allergy* 2010; **65**:1566–75.

34 Ying S, O'Connor B, Ratoff J *et al.* Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *J Immunol* 2005; **174**:8183–90.

35 Siracusa MC, Saenz SA, Hill DA *et al.* TSLP promotes interleukin-3-independent basophil haematopoiesis and type 2 inflammation. *Nature* 2011; **477**:229–33.

36 He R, Oyoshi MK, Garibyan L, Kumar L, Ziegler SF, Geha RS. TSLP acts on infiltrating effector T cells to drive allergic skin inflammation. *Proc Natl Acad Sci USA* 2008; **105**:11875–80.

37 Soumelis V, Reche PA, Kanzler H *et al.* Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002; **3**:673–80.

38 Allakhverdi Z, Comeau MR, Jessup HK *et al.* Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. *J Exp Med* 2007; **204**:253–8.

39 Kouzaki H, O'Grady SM, Lawrence CB, Kita H. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J Immunol* 2009; **183**:1427–34.

40 Li M, Hener P, Zhang Z, Kato S, Metzger D, Chambon P. Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitis. *Proc Natl Acad Sci USA* 2006; **103**:11736–41.

41 Knosp CA, Carroll HP, Elliott J *et al.* SOCS2 regulates T helper type 2 differentiation and the generation of type 2 allergic responses. *J Exp Med* 2011; **208**:1523–31.

42 Holmqvist K, Cross MJ, Rolny C, Hagerkvist R, Rahimi N, Matsumoto T, Claesson-Welsh L, Welsh M. The adaptor protein shb binds to tyrosine 1175 in vascular endothelial growth factor (VEGF) receptor-2 and regulates VEGF-dependent cellular migration. *J Biol Chem* 2004; **279**:22267–75.

43 Hooshmand-Rad R, Lu L, Heldin CH, Claesson-Welsh L, Welsh M. Platelet-derived growth factor-mediated signaling through the Shb adaptor protein: effects on cytoskeletal organization. *Exp Cell Res* 2000; **257**:245–54.

44 Welsh M, Songyang Z, Frantz JD *et al.* Stimulation through the T cell receptor leads to interactions between SHB and several signaling proteins. *Oncogene* 1998; **16**:891–901.

45 Karlsson T, Songyang Z, Landgren E *et al.* Molecular interactions of the Src homology 2 domain protein Shb with phosphotyrosine residues, tyrosine kinase receptors and Src homology 3 domain proteins. *Oncogene* 1995; **10**:1475–83.

46 Lindholm CK, Henriksson ML, Hallberg B, Welsh M. Shb links SLP-76 and Vav with the CD3 complex in Jurkat T cells. *Eur J Biochem* 2002; **269**:3279–88.

47 Lindholm CK, Gylfe E, Zhang W, Samelson LE, Welsh M. Requirement of the Src homology 2 domain protein Shb for T cell receptor-dependent activation of the interleukin-2 gene nuclear factor for activation of T cells element in Jurkat T cells. *J Biol Chem* 1999; **274**:28050–7.

48 Kriz V, Mares J, Wentzel P *et al.* Shb null allele is inherited with a transmission ratio distortion and causes reduced viability *in utero*. *Dev Dyn* 2007; **236**:2485–92.

49 Gustafsson K, Calounova G, Hjelm F, Kriz V, Heyman B, Gronvik KO, Mostoslavsky G, Welsh M. Shb deficient mice display an augmented TH2 response in peripheral $CD4^+$ T cells. *BMC Immunol* 2011; **12**:3.

50 Miles AA, Miles EM. Vascular reactions to histamine, histamine-liberator and leukotaxine in the skin of guinea-pigs. *J Physiol* 1952; **118**:228–57.

51 Galaup A, Cazes A, Le Jan S *et al.* Angiopoietin-like 4 prevents metastasis through inhibition of vascular permeability and tumor cell motility and invasiveness. *Proc Natl Acad Sci USA* 2006; **103**:18721–6.

52 Spergel JM, Mizoguchi E, Brewer JP, Martin TR, Bhan AK, Geha RS. Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. *J Clin Invest* 1998; **101**:1614–22.

53 Dahten A, Koch C, Ernst D, Schnoller C, Hartmann S, Worm M. Systemic PPARγ ligation inhibits allergic immune response in the skin. *J Invest Dermatol* 2008; **128**:2211–8.

54 Dialynas DP, Quan ZS, Wall KA, Pierres A, Quintans J, Loken MR, Pierres M, Fitch FW. Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. *J Immunol* 1983; **131**:2445–51.

55 Austyn JM, Gordon S. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol* 1981; **11**:805–15.

56 Hershko AY, Charles N, Olivera A, Alvarez-Errico D, Rivera J. Cutting edge: persistence of increased mast cell numbers in tissues links dermatitis to enhanced airway disease in a mouse model of atopy. *J Immunol* 2012; **188**:531–5.

57 Grewe M, Walther S, Gyufko K, Czech W, Schopf E, Krutmann J. Analysis of the cytokine pattern expressed *in situ* in inhalant allergen patch test reactions of atopic dermatitis patients. *J Invest Dermatol* 1995; **105**:407–10.

58 Altin JA, Tian L, Liston A, Bertram EM, Goodnow CC, Cook MC. Decreased T-cell receptor signaling through CARD11 differentially compromises forkhead box protein 3-positive regulatory versus T_H2 effector cells to cause allergy. *J Allergy Clin Immunol* 2011; **127**:1277–85 e5.

59 Christoffersson G, Zang G, Zhuang ZW, Vagesjo E, Simons M, Phillipson M, Welsh M. Vascular adaptation to a dysfunctional endothelium as a consequence of Shb deficiency. *Angiogenesis* 2012; **15**:469–80.

60 Funa NS, Kriz V, Zang G *et al.* Dysfunctional microvasculature as a consequence of shb gene inactivation causes impaired tumor growth. *Cancer Res* 2009; **69**:2141–8.

61 Zang G, Christoffersson G, Tian G *et al.* Aberrant association between vascular endothelial growth factor receptor-2 and VE-cadherin in response to vascular endothelial growth factor-α in Shb-deficient lung endothelial cells. *Cell Signal* 2013; **25**:85–92.

62 Mochizuki M, Bartels J, Mallet AI, Christophers E, Schroder JM. IL-4 induces eotaxin: a possible mechanism of selective eosinophil recruitment in helminth infection and atopy. *J Immunol* 1998; **160**:60–8.

63 Mathew A, MacLean JA, DeHaan E, Tager AM, Green FH, Luster AD. Signal transducer and activator of transcription 6 controls chemokine production and T helper cell type 2 cell trafficking in allergic pulmonary inflammation. *J Exp Med* 2001; **193**:1087–96.

64 Xiao T, Fujita H, Saeki H *et al.* Thymus and activation-regulated chemokine (TARC/CCL17) produced by mouse epidermal Langerhans cells is upregulated by TNF-α and IL-4 and downregulated by IFN-γ. *Cytokine* 2003; **23**:126–32.
65 Bonecchi R, Sozzani S, Stine JT, Luini W, D'Amico G, Allavena P, Chantry D, Mantovani A. Divergent effects of interleukin-4 and interferon-γ on macrophage-derived chemokine production: an amplification circuit of polarized T helper 2 responses. *Blood* 1998; **92**:2668–71.
66 Mould AW, Matthaei KI, Young IG, Foster PS. Relationship between interleukin-5 and eotaxin in regulating blood and tissue eosinophilia in mice. *J Clin Invest* 1997; **99**:1064–71.
67 Hickey MJ, Granger DN, Kubes P. Molecular mechanisms underlying IL-4-induced leukocyte recruitment *in vivo*: a critical role for the α4 integrin. *J Immunol* 1999; **163**:3441–8.
68 Burd PR, Thompson WC, Max EE, Mills FC. Activated mast cells produce interleukin 13. *J Exp Med* 1995; **181**:1373–80.
69 Cui P, Sharmin S, Okumura Y, Yamada H, Yano M, Mizuno D, Kido H. Endothelin-1 peptides and IL-5 synergistically increase the expression of IL-13 in eosinophils. *Biochem Biophys Res Commun* 2004; **315**:782–7.
70 Walsh ER, Thakar J, Stokes K, Huang F, Albert R, August A. Computational and experimental analysis reveals a requirement for eosinophil-derived IL-13 for the development of allergic airway responses in C57BL/6 mice. *J Immunol* 2011; **186**:2936–49.
71 Gessner A, Mohrs K, Mohrs M. Mast cells, basophils, and eosinophils acquire constitutive IL-4 and IL-13 transcripts during lineage differentiation that are sufficient for rapid cytokine production. *J Immunol* 2005; **174**:1063–72.
72 Bradley LM, Atkins GG, Swain SL. Long-term $CD4^+$ memory T cells from the spleen lack MEL-14, the lymph node homing receptor. *J Immunol* 1992; **148**:324–31.
73 Budd RC, Cerottini JC, Horvath C, Bron C, Pedrazzini T, Howe RC *et al.* Distinction of virgin and memory T lymphocytes. Stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. *J Immunol* 1987; **138**:3120–9.
74 Yamane H, Paul WE. Early signaling events that underlie fate decisions of naive $CD4^+$ T cells toward distinct T-helper cell subsets. *Immunol Rev* 2013; **252**:12–23.
75 Constant S, Sant'Angelo D, Pasqualini T, Taylor T, Levin D, Flavell R, Bottomly K. Peptide and protein antigens require distinct antigen-presenting cell subsets for the priming of $CD4^+$ T cells. *J Immunol* 1995; **154**:4915–23.
76 Yamane H, Zhu J, Paul WE. Independent roles for IL-2 and GATA-3 in stimulating naive $CD4^+$ T cells to generate a Th2-inducing cytokine environment. *J Exp Med* 2005; **202**:793–804.
77 Jorritsma PJ, Brogdon JL, Bottomly K. Role of TCR-induced extracellular signal-regulated kinase activation in the regulation of early IL-4 expression in naive $CD4^+$ T cells. *J Immunol* 2003; **170**:2427–34.
78 Brogdon JL, Leitenberg D, Bottomly K. The potency of TCR signaling differentially regulates NFATc/p activity and early IL-4 transcription in naive $CD4^+$ T cells. *J Immunol* 2002; **168**:3825–32.
79 Jun JE, Wilson LE, Vinuesa CG *et al.* Identifying the MAGUK protein Carma-1 as a central regulator of humoral immune responses and atopy by genome-wide mouse mutagenesis. *Immunity* 2003; **18**:751–62.
80 Siggs OM, Miosge LA, Yates AL *et al.* Opposing functions of the T cell receptor kinase ZAP-70 in immunity and tolerance differentially titrate in response to nucleotide substitutions. *Immunity* 2007; **27**:912–26.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of primers and T_m used for the real-time RT-PCR.