NBR1 is a new PB1 signalling adapter in Th2 differentiation and allergic airway inflammation *in vivo*

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Allergic airway inflammation is a disease in which T helper 2 (Th2) cells have a critical function. The molecular mechanisms controlling Th2 differentiation and function are of paramount importance in biology and immunology. Recently, a network of PB1-containing adapters and kinases has been shown to be essential in this process owing to its function in regulating cell polarity and the activation of critical transcription factors. Here, we show *in vivo* data showing that T-cell-specific NBR1-deficient mice show impaired lung inflammation and have defective Th2 differentiation *ex vivo* with alterations in T-cell polarity and the selective inhibition of Gata3 and nuclear factor of activated T cell activation. These results establish NBR1 as a novel PB1 adapter in Th2 differentiation and asthma.

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Introduction

PB1-domain-containing signalling regulators include kinases such as MEK5 α , MEK3 and the atypical PKCs (aPKCs) PKC ζ and PKC λ /i, as well as the signalling adapters p62 and Par-6 (Moscat *et al*, 2006). These latter two molecules serve to locate the aPKCs into the NF- κ B and cell polarity signalling cascades, respectively (Moscat *et al*, 2009). The analysis of gene-knockout (KO) mice deficient in the different PB1 molecules is shedding light on their actual functions *in vivo* and at the cellular level. That is, PKC ζ -deficient mice show impaired T-cell differentiation towards the T helper 2 (Th2) lineage because of the critical function that PKC ζ has in IL-4 signalling *ex vivo* and *in vivo* (Martin *et al*, 2005). Extensive evidence shows a critical function of Th2 cells in the genesis of asthma and other allergic diseases (Paul, 1997; Luster and Tager, 2004). Naive Th cells can differentiate in response to antigen stimulation into different effector lineages, including T helper 1 (Th1) and Th2; these are characterized by the secretion of different sets of cytokines as well as by performing

different regulatory functions in the immune system (Mosmann and Coffman, 1989; Shuai and Liu, 2003). Th1 cells mainly produce IFN- γ and IL-2 and have an essential function in cell-mediated immune responses against intracellular pathogens. On the other hand, Th2 cells produce IL-4, IL-5, IL-10 and IL-13 and are important in the control of humoral immunity and allergy. The differentiation of CD4⁺ T cells along the Th2 lineage is modulated by signals emanating from the T-cell receptor (TCR), in combination with pathways triggered by cytokines generated during polarization, particularly IL-4, which is extensively used for the *in vitro* differentiation of CD4⁺ T cells towards Th2 (Ho and Glimcher, 2002; Murphy and Reiner, 2002). As IL-4 is important for induction and maintenance of differentiated Th2 cells, our data showed that PKC ζ impinges on Th2 differentiation because it is a critical target of IL-4 signalling (Martin *et al*, 2005).

More recent findings showed that the other aPKC, PKC λ /i, is likewise important for Th2 differentiation but, in contrast to PKC ζ , is not involved in IL-4 signalling. Instead, it has a more general function in the control of T-cell polarity (Yang *et al*, 2009), a critical mechanism whereby essential regulators are located at the immunological synapse (IS) and the opposite pole during TCR activation (Ludford-Menting *et al*, 2005; Krummel and Macara, 2006; Chang *et al*, 2007; Yeh *et al*, 2008). Consistent with this, PKC λ /i-deficient mice show impaired responses to allergic airway inflammation, a typical Th2 response, and show diminished induction of Th2 differentiation in *ex vivo* experiments (Yang *et al*, 2009). These observations establish that at least two PB1-containing kinases perform similar cellular functions through different signalling mechanisms. Interestingly, the genetic inactivation of the aPKC-interacting, PB1-containing, signalling adapter p62 also reveals its function in Th2 differentiation in *ex vivo* studies, as well as *in vivo* in the above-mentioned lung inflammatory model (Martin *et al*, 2006).

Recently, another PB1-domain protein has been identified that has a remarkably similar domain organization to that of p62, including zinc-finger and UBA domains (see below). From overexpression and transfection studies, it has been suggested that NBR1 is involved in growth factor trafficking (Mardakheh *et al*, 2009), and/or p62-mediated processes (Lange *et al*, 2005; Kirkin *et al*, 2009). However, its precise *in vivo* function has not been elucidated yet owing to a lack of studies involving gene inactivation at an organismal level. As at least three PB1-containing proteins are involved in Th2 differentiation, PKC ζ , PKC λ /i and p62, we tested whether NBR1 was part of a novel PB1-organized network of signalling molecules controlling T-cell differentiation and allergic airway inflammation *in vivo*. Here, we report the generation and characterization of mice in which NBR1 has been specifically knocked out in activated T cells, and show for the first time, through *ex vivo* and *in vivo* studies, that it is a critical

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mediator of T-cell activation in the control of Th2 differentiation and allergic airway inflammation *ex vivo* and *in vivo*.

Results

Generation of conditional NBR1 KO mice in activated T cells

We generated a conditional NBR1-KO (NBR1^{fl/fl}Cre^{OX40}) mouse line, in which NBR1 is specifically deleted in activated T cells (Supplementary Figure S1). Thus, we bred NBR1^{fl/fl} mice with Cre^{OX40} mice in which the expression of Cre is under the control of the Tnfrsf4 locus (Zhu *et al*, 2004; Klinger *et al*, 2009). OX40 is expressed almost exclusively in activated T cells, especially CD4⁺ cells, upon stimulation (Zhu *et al*, 2004; Klinger *et al*, 2009). By using this strategy, we prevent potential embryonic lethality and possible confounding effects resulting from the deletion of NBR1 during development or in resting cells. This very same approach has been used previously to specifically delete the *GATA3* and *PKC λ /1* genes in activated T cells during Th2 differentiation experiments (Zhu *et al*, 2004; Yang *et al*, 2009). PCR genotyping was used to screen for homozygous conditional NBR1-deficient (NBR1^{fl/fl}Cre^{OX40}) and wild-type (NBR1^{wt/wt}) mice. No Cre-mediated effects were detected when NBR1^{wt/wt} Cre^{OX40} and NBR1^{fl/fl} mice were compared (data not shown). The deletion of NBR1 in activated CD4⁺ T cells was confirmed by western blot of extracts from T cells activated with anti-CD3 plus anti-CD28. Interestingly, we observed a significant upregulation of NBR1 in T cells from wild-type mice, but not in those from conditional NBR1-KO mice (Figure 1A and B). Therefore, NBR1 is normally induced during sustained T-cell stimulation and is effectively deleted in the mutant cells. This implies that NBR1 levels are very low in resting cells, but that it is induced in T cells activated for a relatively long period (Figure 1A). Of note, the NBR1-interacting partner p62 is also induced upon T-cell activation, as is PKC λ /1, which is a partner of p62 (Figure 1A). This is consistent with the previously published data (Martin *et al*, 2006; Yang *et al*, 2009). The loss of NBR1 in the mutant mice does not affect p62 or PKC λ /1 induction (Figure 1B). On the basis of these observations, we hypothesized that NBR1 might be required for the sustained signalling leading to T-cell differentiation.

Conditional NBR1-deficient mice appeared to develop normally (not shown), and the percentages of T and B cells (CD4⁺CD8⁺ and B220⁺ cells) in the spleens and lymph nodes (LN) of these mice were normal (Supplementary Tables SI and SII). The number of double-positive cells (CD4⁺CD8⁺) and single-positive cells (CD4⁺ or CD8⁺) in the thymus were also identical between conditional NBR1-deficient and wild-type mice (Supplementary Table SIII).

Reduced Th2 cytokine secretion by CD4⁺ T cells from conditional NBR1-deficient mice

In the next series of experiments, we determined whether the conditional deletion of NBR1 in activated T cells would influence T-cell differentiation. For this, purified CD4⁺ T cells from mice of different genotypes were stimulated with anti-CD3 plus anti-CD28 for 1 and 3 days, after which the levels of secreted cytokines were detected by ELISA. Interestingly, we observed reduced secretion of the Th2 cytokine, IL-4, in activated NBR1-deficient cells as compared with wild-type littermate controls (Figure 1C). The secretion of IL-13, another Th2 cytokine, was also reduced in activated NBR1-deficient T cells (Figure 1D). When secretion of IFN- γ , a Th1 cytokine, was measured under the same conditions, it was clear that its production was slightly reduced, but not significantly affected by the lack of NBR1 (Figure 1E). No changes were observed in IL-2 levels when both genotypes were compared (Figure 1F). Consistent with the data of Figure 1C–E, intracellular staining analyses revealed a reduction in the levels of IL-4 and IL-13, but no changes in IFN- γ in NBR1-deficient T cells (Figure 1G–I). Data of Supplementary Figure S2A show that the loss of NBR1 does not affect T-cell proliferation. Likewise, the staining profile of cell surface markers such as CD44, CD62L, CD25 and CD69 was normal in NBR1-deficient T cells (Supplementary Figure S2B–D). In addition, no changes in Treg population or activation was observed upon NBR1 deficiency, although NBR1 is induced in activated Tregs and is efficiently deleted in cells from the OX40-Cre mouse line (Supplementary Figure S3). As these data suggest that NBR1 is critical in the control of the decision point between Th2 and Th1 cytokines, we then determined whether NBR1 would also be required for Th2/Th1 differentiation *in vitro*. To address this possibility, isolated CD4⁺ T cells were cultured for 4 days under non-skewing (Th0), or under Th1- or Th2-skewing conditions. Interestingly, NBR1 levels are selectively increased in Th2 cells (Figure 1J). Differentiated T cells along the three lineages were then washed and re-stimulated with anti-CD3/CD28 for 24 h, and cytokine secretion was determined by ELISA. The loss of NBR1 in activated T cells impaired IL-4 and IL-5 secretion by Th2-polarized T cells, whereas IFN- γ secretion by Th1-polarized T cells was mildly or not at all affected (Figure 1K–M). Collectively, these results indicate that NBR1 is required in activated T cells for tilting the balance of Th2 versus Th1 cytokine production.

NBR1 is an important mediator in OVA-induced allergic airway inflammation

As NBR1 seems to regulate Th2 cytokine production, it is conceivable that there are particular pathways set in motion *in vivo* that require NBR1 for an adequate Th2 response.

Figure 1 NBR1 is upregulated upon T-cell activation and is required for Th2 cytokine secretion. (A, B) Purified splenic CD4⁺ T cells from WT (A) or conditional NBR1-deficient mice (B) were stimulated with anti-CD3/CD28 for 0–48 h. Cells lysates were analysed by western blot for NBR1, p62, PKC λ /1 and actin. The results shown are representative of three independent experiments. (C–F) Purified splenic CD4⁺ T cells from mice of different genotypes were stimulated with anti-CD3/CD28 for 1 and 3 days; supernatants were collected for ELISA assays to detect cytokines. Data (mean $\pm$ s.e.) are from three experiments with measurements for each sample performed in triplicate. (G–I) Cells were stimulated with anti-CD3/CD28 for 1 day with BD GolgiPlugTM in the last 5 h, and collected for intracellular cytokine staining. Percentages of cytokine-positive CD4⁺ T cells are shown. The results are representative of two independent experiments. (J–M) Purified CD4⁺ T cells were also differentiated under Th0, Th1 or Th2 conditions for 4 days and then re-stimulated with plate-bound anti-CD3/CD28 for 1 day. Cells were harvested for NBR1 expression by immunoblotting (J). Supernatants were collected for ELISA assays to detect IL-4 (K), IL-5 (L) or IFN- γ (M). Data (mean $\pm$ s.e.) are from three experiments with measurements for each sample performed in triplicate. ***P* < 0.01.

Following this line of reason, we sought to determine whether NBR1 is necessary for an optimal Th2 inflammatory response *in vivo*. Therefore, we next determined the requirement for NBR1 in the OVA-induced model of allergic airway

inflammation. Wild-type and conditional NBR1-deficient mice were immunized by intraperitoneal injection of OVA and then challenged three times with aerosolized OVA, or with PBS as a negative control, as previously described (Yang

Figure 2 Function of NBR1 in OVA-induced allergic airway inflammation. Conditional NBR1-deficient mice and their wild-type littermates were immunized with OVA twice and then challenged with aerosolized OVA or PBS as a control. Mice were killed 24 h after the last challenge. (A) The total cells in BAL fluid were counted by using a haemocytometer. (B) Differential cell counts of >300 cells were performed on cytospins stained with Kwik-Diff. The numbers of eosinophils (Eo), macrophages (M ϕ), neutrophils (Neu) and lymphocytes (Lym) in BAL are shown. (C) Representative H&E staining of lung tissue sections. (D–G) Cytokine levels in BAL fluid were determined by ELISA. The results are expressed as mean $\pm$ s.e. from two independent experiments ($n = 10$ per group). * $P < 0.05$; ** $P < 0.01$.

et al, 2009). Twenty-four hours after the last aerosol challenge, mice were killed and subjected to bronchoalveolar lavage (BAL) to determine the recruitment of inflammatory cells. In addition, lungs were fixed and examined histologically by haematoxylin/eosin (H&E) staining to assess cellular infiltration. Interestingly, there was an increase in total numbers of BAL cells in wild-type mice that had been OVA immunized and challenged with aerosolized OVA, as compared with unimmunized naive mice (Figure 2A). Eosinophils accounted for most of this increase in the recruitment of inflammatory cells (Figure 2B). However, conditional NBR1-mutant mice showed a dramatic reduction in these parameters in Figure 2A and B. H&E histological analysis of lung sections from these experiments consistently showed that challenged wild-type mice displayed a prominent inflammatory response with massive perivascular and peribronchial infiltration, whereas the conditional NBR1-mutant mice had a highly reduced response (Figure 2C). Collectively, these data indicate that the *in vitro* experiments showing inhibited Th2 cytokine secretion by T cells derived from these mutant mice would account for the effect of NBR1 loss in airway inflammation *in vivo*. Consistent with this idea, ELISA data on the cytokine levels in BAL from these *in vivo* experiments showed that IL-4, IL-5 and IL-13 were significantly increased in OVA-challenged wild-type mice, but inhibited in identically treated conditional NBR1-mutant mice (Figure 2D–F). IFN- γ was dramatically induced in OVA-challenged wild-type mice to levels comparable with those of NBR1-mutant mice (Figure 2G), consistent with the *in vitro* data of Figures 1E

and H. Interestingly, IL-4, IL-5, IL-13, eotaxin and RANTES mRNAs were upregulated in the lungs of challenge wild-type mice as compared with unimmunized mice, and this upregulation was significantly reduced in conditional NBR1-mutant mice (Figure 3A–E). However, IFN- γ mRNA levels were mildly, or not at all, affected by the loss of NBR1 (Figure 3F). On the other hand, it has been suggested that the calcium chloride-activated channel hCLCA1 (gob-5 in the mouse) increases MUC-5AC gene expression (Busse *et al*, 2005). Both MUC-5AC and gob-5 were dramatically increased in challenged wild-type mice, but over two-thirds of this increase was lost in the conditional NBR1-mutant mice (Figure 3G and H). When the expression of NBR1 was determined in splenic CD4 $^{+}$ or total tracheal LN cells from these activated mice, it was apparent that NBR1, but not p62 or PKC $\lambda/1$ levels were dramatically reduced in the mutant cells (Supplementary Figure S4A and B). Taken together, these results indicate that the NBR1 is a novel and relevant player in Th2 cell differentiation and/or function.

Function of NBR1 in T-cell activation

As NBR1 is important for Th2 cytokine secretion *ex vivo* and *in vivo*, we explored in the next series of experiments the signalling pathways potentially altered by the loss of NBR1 in activated T cells. As Th2 cytokine secretion requires sustained activation of TCR signals, we next investigated the transcription factors reported to be essential for this process in WT and NBR1-deficient cells. The activation of GATA3, which is necessary and sufficient to drive T cells to the Th2 lineage,

Figure 3 Cytokine and mucin glycoprotein mRNA levels. Total RNA from the right lower lobe of the lungs used in Figure 2 was extracted for real-time PCR analysis. The mRNA levels of IL-4 (A), IL-5 (B), IL-13 (C), RANTES (D), Eotaxin (E), IFN- γ (F), Muc-5AC (G) and Gob-5 (H) were determined by RT-PCR and expressed as arbitrary units. All samples were determined in triplicate; the data are normalized to an 18S reference. The results are expressed as mean $\pm$ s.e. from two independent experiments ($n = 10$ per group). * $P < 0.05$.

was determined by confocal microscopy and immunoblotting in cell cultures of wild-type or NBR1-deficient CD4⁺ T cells that had been incubated with anti-CD3 plus anti-CD28 for 14 h. Figure 4A shows a representative image showing that GATA3 was highly expressed and localized to the nucleus in activated wild-type CD4⁺ T cells, but that its levels were reduced in activated NBR1-deficient cells. Figure 4B shows

the percentage of cells displaying GATA3 staining based on counts of 400 cells, and shows that, although around 60% of activated wild-type T lymphocytes had nuclear GATA3, it was only detected in the nuclei of about 30% of the NBR1-deficient T cells. These results show the relevance of NBR1 for the activation of a Th2-specific transcription factor and are consistent with NBR1 being important for Th2 cytokine

Figure 4 *In vivo* deletion of NBR1 in activated T cells regulates Th2 transcription factors. (A) Purified splenic CD4⁺ T cells were activated with anti-CD3/CD28 for 14 h. Cells were harvested and allowed to settle onto and adhere to poly-L-lysine-coated coverslips for immunofluorescence staining. Nuclei were stained by propidium iodide. Cells were analysed by using a confocal microscope. Nuclear translocation of Th2 transcription factors is shown. Images are examples from >300 cells for each staining, and are representative of three independent experiments. (B) Cells with nuclear translocation were quantified by cell counting ($n > 400$) under a microscope. Nuclear (C), cytoplasmic (D) and total (E) extracts of T cells treated as above were analysed by immunoblotting with antibodies for the respective transcription factors and signalling kinases. T cells were pooled from 5 to 10 mice per genotype. (F) Gata3 mRNA levels are shown (arbitrary units). Total RNA was extracted from splenic CD4⁺ T cells differentiated into Th0 and Th2 cells and re-stimulated with anti-CD3/CD28 for 1 day for real-time PCR analysis. All samples were determined by triplicate, the data are normalized to an 18S reference. * $P < 0.05$; ** $P < 0.01$.

production. Interestingly, we measured the activation of nuclear factor of activated T (NFAT) c1, NF- κ B (p65) and Stat6, three transcription factors important for this process, and it was clear that the nuclear levels of NFATc1 and Stat6 were severely reduced by the loss of NBR1 (Figure 4A and B). However, NF- κ B (p65) was readily detected in the nuclei of wild-type and NBR1-deficient CD4⁺ T cells activated under identical conditions (Figure 4A and B). NFATc1 stimulation is linked to the activation of the TCR (Schulze-Luehrmann and Ghosh, 2006), whereas activation of Stat6 is likely because of the secretion of IL-4 in activated T cells. IL-4 production is reduced in activated NBR1-deficient T cells (Figure 1C, G and K), and this is likely to account for the reduced nuclear Stat6 levels detected in NBR1-mutant T cells.

Immunoblot analysis in a similar experiment showed the dramatic inhibition of nuclear GATA3, NFATc1 and Stat6, and unchanged p65 and p50, in the activated NBR1-deficient T cells (Figure 4C). Interestingly, the nuclear levels of the Th1 transcription factor Stat1 was not affected by the loss of NBR1 in the above experiments (Figure 4C). Consistent with a lack of effect of NBR1 in NF- κ B activation, NBR1 deficiency did not affect phospho-I κ B α , or total levels of I κ B α and I κ B β (Figure 4D). Likewise, phospho-AKT and phospho-ERK levels were not influenced by the lack of NBR1 in activated T cells (Figure 4D). Interestingly, total cytosolic levels of NFATc1 were also reduced in the mutant cells (Figure 4D), indicating that NBR1 controls NFATc1 nuclear translocation likely by controlling its expression. In keeping with this notion,

Figure 5 Regulation of NFATc1 by NBR1 is critical for Gata3 activation in T cells. **(A)** Purified splenic CD4⁺ T cells were activated with anti-CD3/CD28 for 14 h without or with rIL-4 (15 ng/ml). Nuclear and cytosol extracts were analysed by western blot. **(B, C)** Activated CD4⁺ T cells were retrovirally infected with a GFP-tagged constitutively active mutant of NFATc1. Two days after infection, cells were stimulated with anti-CD3/CD28 for 14 h and harvested for immunofluorescence staining. Gata3 nuclear translocation is shown in GFP-positive cells. Images are examples from >300 cells **(B)**. Cells with nuclear translocation were quantified by cell counting ($n > 400$) under a microscope **(C)**. T cells were pooled from 3 to 5 mice per genotype. The results shown are representative of two independent experiments. **(D)** Cells were transfected with the luciferase reporter pGL3-NFAT along with different doses of HA-NBR1 (50, 100, 500 ng) and a *Renilla* control plasmid. Luciferase activity was determined 2 days after transfection and normalized for *Renilla*. NBR1 expression levels were analysed by immunoblotting with anti-HA antibody. The results are the means $\pm$ s.d. for triplicates.

NFATc1 mRNA levels were dramatically reduced in NBR1-deficient Th2 cells, as compared with wild-type Th2 cells (not shown). In contrast, the lack of NBR1 did not affect NFATc2 nuclear translocation (not shown). This is not surprising because NFATc2 induction is an earlier event than that of NFATc1, and NBR1 is induced in the later part of the T-cell activation pathway (Figure 1A). Of note, the knockdown of NBR1, as well as that of PKC λ/ι , in activated Jurkat T cells produced a dramatic inhibition of NFATc1 nuclear levels (Supplementary Figure S5). Taken together, these results indicate that NBR1 is a critical element in the TCR activation of NFATc1 transduction, which is important for GATA3 activation and the synthesis of IL-4, thus influencing the stimulation of Stat6. Consistent with this notion, total GATA3 protein and mRNA levels were reduced in NBR1-deficient-activated T cells (Figure 4E and F). The activation of p38 was not detected at this late time point and was not affected by the lack of NBR1 (Figure 4E). We deduce from all these results that NBR1 is not only induced during Th2 differentiation, but that is also required for the activation of important transcription factors for this process.

Even though NBR1 is required for IL-4 synthesis, which could account for the defective stimulation of Stat6 in the experiments of Figure 4, Th2 differentiation induction that heavily relies on exogenous IL-4 addition is also severely impaired in NBR1-deficient mice (Figure 1K and L), suggesting a function of NBR1 not only upstream but also downstream of IL-4 actions. Interestingly, this is a very similar phenotype to that reported for NFATc1-deficient mice (Ranger *et al*, 1998; Yoshida *et al*, 1998). Consistent with the NBR1 function in NFATc1 production as an important event in this

phenotype, the exogenous addition of IL-4, although totally reverts the defect in Stat6 activation in the NBR1-deficient T cells, is unable to rescue the inhibition in GATA3 production observed in the mutant cells (Figure 5A). In this regard, the expression of an NFATc1 permanently active mutant described previously (Monticelli and Rao, 2002) is able to rescue GATA3 activation defects in the NBR1-deficient T cells (Figure 5B and C). Furthermore, according to the data of Figure 5D, the simple overexpression of NBR1 is sufficient to promote the activity of an NFATc1-dependent luciferase promoter, supporting the notion that NBR1 is a critical mediator in that pathway.

On the other hand, NBR1 has been shown to regulate autophagy (Kirkin *et al*, 2009; Moscat and Diaz-Meco, 2009). Our results in T-cell activation, however, show that the loss of NBR1 has no effect on autophagy as determined by LC3 immunofluorescence and immunoblot analysis (Supplementary Figure S6). Consistent with this, we found no changes in the surface expression of CD3/CD28 between T cells of the two different genotypes (Supplementary Figure S6).

Function of NBR1 in T-cell polarity and recruitment to the immunological synapse

The aPKCs have been implicated in the control of cell polarity in several mammalian *in vitro* cell culture experiments (Goldstein and Macara, 2007). More recently, it has been suggested that cell polarity has a function in T-cell activation (Krummel and Macara, 2006; Chang *et al*, 2007). In agreement with this, our previous data showed that PKC λ/ι is required for proper polarization of T cells during chronic activation through the TCR (Yang *et al*, 2009), which is

Figure 6 NBR1 recruitment to the immunological synapse. Purified splenic CD4⁺ T cells from mice with different genotypes were incubated for 14 h with latex beads coated with anti-CD3/CD28. Cells with different genotypes were treated for immunofluorescence staining as in Figure 4. Recruitment of NBR1 (A, C, D), p62 (A, B, C) and PKCλ/ι (B, D) to the IS is shown in their corresponding knockout and wild-type (WT) mouse cells. Images are representative of >300 cells for each staining. Cells with polarity were quantified by cell counting (right panels, *n* > 400) under a microscope. CD4⁺ T cells were pooled from five conditional KO mice and their wild-type littermates.

characterized by the recruitment of PKCλ/ι and other polarity proteins, such as talin and scribble, to the IS (Yang *et al*, 2009). To determine whether NBR1, like PKCλ/ι, is recruited to the IS, we incubated CD4⁺ T cells, from either

wild-type or NBR1-mutant mice, with latex beads coated with anti-CD3 plus anti-CD28. The results of these experiments show that NBR1 is clearly translocated to the IS (see Figure 6A as an example) in at least 80% of T cells upon

Figure 7 NBR1 and p62 control T-cell polarity. Purified splenic CD4⁺ T cells from conditional NBR1-deficient (A) or p62^{-/-} (B) mice and their wild-type (WT) littermates were activated with anti-CD3/CD28 for 14 h. Cells were treated for immunofluorescence staining of talin and scribble (Scrib). Images are representative of >300 cells for each staining. Cells with polarity were quantified by cell counting (right panels, $n > 400$) under a microscope. The results are representative of two independent experiments with identical results. Jurkat cells were stimulated with PMA plus ionomycin (C, D), or anti-CD3/CD28 (E) for 16 h, and lysates were immunoprecipitated with anti-p62 (C, E), anti-PKCλⁱ (D) or IgG control antibodies. Immunoprecipitates were analysed by western blotting with the different antibodies as shown. The results are representative of three independent experiments with identical results.

activation with anti-CD3/CD28-coated latex beads. We then used confocal immunofluorescence to determine whether the loss of NBR1 impaired the localization of PKCλⁱ. Figure 6B shows that the loss of NBR1 had no effect on the recruitment of PKCλⁱ to the IS. Interestingly, the genetic inactivation of PKCλⁱ, by using the OX40-CRE system as previously reported (Yang *et al*, 2009), did not affect the IS translocation of NBR1 (Figure 6C). Together, these results indicate that the translocation of NBR1 and PKCλⁱ are mutually independent. As p62 binds PKCλⁱ and NBR1 (Lange *et al*, 2005), our next experiments were to determine whether p62 is translocated to the IS upon CD3/CD28 stimulation. Figure 6A shows that this is, in fact, the case in >80% of activated cells. Interestingly, this depends on the presence of NBR1, but is independent of PKCλⁱ (Figure 6B and C). Surprisingly, the translocation of PKCλⁱ is independent of p62, but that of NBR1 is not (Figure 6D). Together, these results show that the likely interaction between p62 and NBR1 is required for their translocation to the IS, whereas the translocation of PKCλⁱ is independent of both adapters, which, likewise, translocates independently of PKCλⁱ.

We next determined the effect of NBR1 deficiency on T-cell polarity by monitoring the ability of the polarity markers talin and scribble to be recruited to the IS in T cells activated as above. The results shown in Figure 7A and B show that the lack of NBR1 or p62 during T-cell activation leads to a significant reduction in the recruitment of these two polarity markers to the IS. Collectively, these results indicate that,

upon T-cell activation, NBR1 is normally translocated to the IS, independently of PKCλⁱ, but probably in conjunction with p62. They also suggest that NBR1 or p62 deficiency leads to impaired polarity during late T-cell activation.

NBR1-mediated interactions during T-cell activation

The fact that both NBR1 and PKCλⁱ are recruited to the IS upon T-cell activation is consistent with their functions in this process, especially to the Th2 lineage. Their independent recruitment suggests that they are anchored to different adapters at the IS, but that, once at the IS, they might contact through their PB1 domains, although they might interact independently of IS recruitment. To determine whether this is in fact the case, we stimulated Jurkat T cells with PMA plus ionomycin, or left them untreated, for 16 h, after which NBR1 was immunoprecipitated from cell extracts and the associated PKCλⁱ was detected by immunoblotting. Conversely, these extracts were also immunoprecipitated with anti-PKCλⁱ and the associated NBR1 was determined as above. We consistently found a robust interaction between PKCλⁱ and p62, as well as between p62 and NBR1 under these conditions (Figure 7C). We were unable to detect a reproducible PKCλⁱ-NBR1 interaction in these experiments (Figure 7D). The activation of Jurkat T cells with anti-CD3/anti-CD28 also led to a detectable and reproducible p62-NBR1 and p62-PKCλⁱ interaction (Figure 7E). Therefore, although NBR1 and PKCλⁱ are able to interact with their common partner, p62, upon T-cell activation, they do not make direct contact

with each other, even though both are located in the IS, and both are critical for normal Th2 function.

Discussion

Emerging data suggest that PB1-containing adaptors and kinases are involved in the control of cell signalling specificity and diversity in several systems, including T cells (Etienne-Manneville and Hall, 2003; Moscat *et al*, 2006, 2007; Kirkin and Dikic, 2007; Sumimoto *et al*, 2007; Kirkin *et al*, 2009; Lamark *et al*, 2009; Moscat and Diaz-Meco, 2009). In T lymphocytes, the PB1 kinase PKC ζ regulates IL-4 signalling to control Th2 differentiation and allergic airway inflammation at the level of Jak1 activation (Martin *et al*, 2005). In this same system, the other PB1 kinase, PKC λ/ι , which is highly homologous to PKC ζ , also has a relevant function in Th2 differentiation and asthma, but, surprisingly, through a different mechanism (Yang *et al*, 2009). Thus, PKC λ/ι regulates the recruitment of several polarity proteins to the IS and is critical for the activation of NF- κ B (Yang *et al*, 2009). In contrast, we show here that the genetic inactivation of NBR1, which also harbours a PB1 domain and interacts with the aPKC adapter p62, has no effect on NF- κ B. Instead, it severely impairs the activation of NFATc1, a critical transcription factor in Th2 differentiation (Ranger *et al*, 1998; Yoshida *et al*, 1998). NBR1 deficiency also affects cell polarity and, like PKC λ/ι , NBR1 is recruited to the IS. This recruitment is independent of PKC λ/ι and, likewise, the recruitment of PKC λ/ι is independent of NBR1. Interestingly, p62, another PB1 adapter that is involved in Th2 function (Martin *et al*, 2006) and has a domain organization that is strikingly similar to NBR1 (Lamark *et al*, 2003), is also recruited to the IS. In this case, the translocation of p62 and NBR1 are mutually dependent, but, like p62, NBR1's translocation is independent of PKC λ/ι . Therefore, taking all these data together, a model emerges whereby both PKC λ/ι and NBR1 are recruited to the IS separately, likely through different and still-to-be-defined 'anchors', whereas the recruitment of p62 and NBR1 are interdependent.

Interestingly, we show here that, upon the activation of T cells, p62 binds to PKC λ/ι and to NBR1, but that p62 must be present in more than one complex. We can surmise this because we have been unable to co-immunoprecipitate NBR1 and PKC λ/ι from extracts of activated T cells. At the IS, the two complexes must control different aspects of the NFATc1 signalling cascade. Considering that PKC λ/ι is responsible for the nuclear translocation of NF- κ B (Yang *et al*, 2009), which is important for transcriptional induction of the increase in total NFATc1 levels (Fukushima *et al*, 2008), this could explain why the loss of PKC λ/ι in T cells leads to the inhibition of both NF- κ B and NFATc1 activation. However, NBR1 appears to be responsible for orchestrating, likely through an as-yet-undiscovered pathway, the specific activation of NFATc1 in an NF- κ B-independent manner. Interestingly, our previous data show that p62 is also important in the activation of NF- κ B and NFATc1 (Duran *et al*, 2004). The fact that these PB1 proteins regulate NFATc1 is consistent with their functions, and those of the NFAT proteins, in Th2 functions as all of them are required for optimal activation of Th2 differentiation *ex vivo* and *in vivo* (Ranger *et al*, 1998; Yoshida *et al*, 1998; Martin *et al*, 2005, 2006; Yang *et al*, 2009).

Interestingly, PKC λ/ι , NBR1 and p62 are not only translocated to the IS, but they also regulate the recruitment of cell polarity proteins such as scribble and talin to that supramolecular structure. It has been suggested that the control of T-cell polarity is important in T-cell activation (Ludford-Menting *et al*, 2005; Krummel and Macara, 2006; Chang *et al*, 2007; Yeh *et al*, 2008), and, accordingly, our results show *in vivo* correlations between the loss of PKC λ/ι (Yang *et al*, 2009), p62 (Martin *et al*, 2006) or NBR1, with defective polarity, impaired Th2 differentiation of T cells, and asthma. However, the precise relationship between impaired polarity and defects in the different signalling pathways is not clear. That is, the deletion of PKC λ/ι , p62 or NBR1 similarly impairs the recruitment of talin and scribble to the IS. However, PKC λ/ι and p62 regulate NF- κ B activation (Martin *et al*, 2006; Yang *et al*, 2009), whereas NBR1 does not, but, instead, modulates NFATc1 nuclear translocation. Therefore, a common link between cell polarity defects, at least with regard to talin and scribble, and T-cell signalling is not readily apparent. In addition, whereas the loss of p62 inhibits NF- κ B activation (Martin *et al*, 2006), the lack of NBR1, which impairs p62 recruitment to the IS, does not affect NF- κ B. These results suggest that although p62 is necessary for NF- κ B, its translocation to the IS is not.

These findings should be considered in the light of the fact that the actual function of IS formation in T-cell activation has not been completely clarified, despite intensive study. In this regard, how PKC λ/ι , p62 and NBR1 are recruited; how they control the recruitment of scribble and talin and the relationship between these functions and the regulation of cell signalling still need to be clarified. It is even possible that cell polarity and cell signalling are actually separate events that have different functions in T-cell biology. In fact, the genetic inactivation of the adapter Crtam, which, like PKC λ/ι , p62 and NBR1, is involved in long-term cell polarity, leads to the hyperactivation of T cells and impaired production of Th1 cytokines (Yeh *et al*, 2008), a phenotype opposite to that of PKC λ/ι -, p62- or NBR1-KO T cells. Therefore, it seems that inhibiting polarity by the inactivation of two different types of proteins (PKC λ/ι , p62 and Nbr1 versus Crtam) lead to opposite phenotypes. This can be interpreted to mean that cell polarity is not a determinant event in T-cell activation and/or signalling. However, another logical interpretation is that impaired translocation of talin and scribble is, by itself, too coarse an indicator of impaired polarity, and that more detailed correlative and loss-of-function studies are necessary to identify the polarity proteins that link T-cell activation to distinct cell signalling molecules. In this regard, recent studies are beginning to establish biochemical connections between different polarity proteins and cell signalling molecules (Zhan *et al*, 2008). In any case, what we show here is that there is clearly a function for the novel signalling adapter, NBR1, and that it is part of a larger PB1-dominated network in T-cell signalling and differentiation towards the Th2 lineage *in vitro* and *in vivo*.

Materials and methods

Mice

NBR1^{fl/fl} mice were generated in our laboratory by a targeting strategy designed to delete Nbr1 exon 5 (Supplementary Figure S1). Approximately 11.7 kb of mouse genomic DNA encompassing the

murine Nbr1 gene region surrounding exon 5 was isolated by PCR from the 129Sv/Pas genetic background. Nbr1 exon 5 was flanked at the 5' end by a validated *loxP*-FRT-neomycin-FRT cassette and by a single *loxP* site in the 3' direction. The linearized targeting construct was transfected into ES cells by standard electroporation procedures. Positive selection with G418 was started 48 h after electroporation. The recombinant clones identified by PCR were verified by Southern blot analysis. Positive ES clones were injected into C57BL/6J blastocysts, which were then re-implanted into OF1 pseudopregnant females. To generate mice heterozygous for the floxed allele, one highly chimeric male was mated with two C57BL/6J Flp-deleter females to achieve germline excision of the neomycin-selection cassette. The complete excision of the neomycin cassette was detected by two PCR-based screenings and positives were further analysed by Southern blot. Cre^{OX40} mice were generated in Dr Killeen's laboratory (Zhu *et al*, 2004; Klinger *et al*, 2009). Mice with conditional NBR1 deficiency in activated T cells were generated by crossing NBR1^{fl/fl} mice with Cre^{OX40} mice. Mouse genotyping was performed by PCR using primers for NBR1^{fl/fl}, OX40-Cre and NBR1 deletion to screen for homozygous conditional NBR1-deficient (NBR1^{fl/fl} Cre^{OX40}) and wild-type (NBR1^{fl/fl} or NBR1^{wt/wt} Cre^{OX40}) mice. Age (9–12 weeks) and sex-matched mice were used in each experiment.

Antibodies and reagents

Purified Abs recognizing murine CD3e (145-2C11), CD44 (KM114), CD28 (37.51), CD16/CD32 (2.4G2) and PKC λ (41); and FITC-, PE-, PerCP- or APC-conjugated monoclonal antibodies (mAbs) to CD4⁺ (GK1.5), CD8 α (53-6.7), TCR- β (H57-597), CD62L (MEL-14), CD44 (IM7), B220 (RA3-6B2), OX40 (OX86), CD25 (PC61), CD69 (H1.2F3), IL-4 (11B11) and IFN- γ (XMG1.2) were from BD PharMingen (San Diego, CA). IL-13-PE (eBio13A) was from e-Bioscience (San Diego, CA). Antibodies against NBR1 (4BR), Stat1 (E-23), Stat6 (S-20), GATA3 (HG3-31), NF- κ B p65 (C-20), NF- κ B p50 (E-10), NFATc1 (7A6), scribble (K-21), ERK (K-23), phospho-ERK (E-4), I κ B α (C-21), I κ B β (C-20) and actin (I-19) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Akt (C67E7), phospho-Akt (D9E), phospho-I κ B α (14D4), phospho-p38 MAPK (3D7), phospho-ATF-2 and LC3B (D11) were from Cell Signaling (Danvers, MA). The antibody against talin (8D4) was from Sigma (St Louis, MO). Polyclonal Ab to p62 (C-terminal specific, PG62) was from Progen Biotechnik (Heidelberg, Germany). Recombinant murine IL-4, IL-6, IL-12, as well as anti-IFN- γ and anti-IL-4 antibodies, were from R&D Systems (Minneapolis, MN).

OVA-induced allergic airway inflammation

Allergic airway inflammation was induced as described in our previous report (Martin *et al*, 2006; Yang *et al*, 2009). Conditional NBR1-deficient and wild-type mice (10–12-week-old) were immunized i.p. with 50 μ g of OVA (Grade V; Sigma) in 100 μ l (2 mg) of alum (Imject Alum; Pierce, IL) on days 0 and 7. On day 14, mice were challenged two times (60 min each delivered 4 h apart) with aerosolized 1% OVA dissolved in PBS by an Omron NE-C28 Nebulizer (Omron Healthcare, Bannockburn, IL). On day 15, mice were challenged one more time. Control animals were challenged with aerosolized PBS. Mice were killed 24 h after the last challenge. BAL was performed by injecting 1 ml PBS into the lungs through a tracheal cannula and then gently aspirating the fluid. This was repeated three times. The BAL fluid was centrifuged and total cells in the pellet were counted by using a haemocytometer. Differential cell counts on 300 cells were performed on cytopins stained with Shandon Kwik-Diff Stain kit (Thermo Scientific). The BAL fluid from each mouse was concentrated to 0.5 ml by centrifugation with an Amicon Ultra-4 filter unit (Millipore, Billerica, MA) for determination of cytokines by ELISA. For lung histology, the upper lobe of the left lung was fixed with 4% paraformaldehyde overnight, dehydrated, embedded in paraffin, cut into 4 μ m sections and processed for H&E staining. For analysis of lung mRNA, lung tissue was homogenized in Buffer RLT for extraction of total RNA with the RNeasy Mini kit (Qiagen). Serum levels of various OVA-specific antibodies were measured by ELISA with the use of biotinylated goat anti-mouse IgE, IgM, IgG, IgG1, IgG2a and streptavidin HRP (Southern Biotech, Birmingham, AL).

CD4⁺ T-cell isolation and differentiation

Splenocytes were prepared by disrupting spleens of 9- to 12-week-old mice. CD4⁺ T cells were enriched with a Mouse CD4⁺ Isolation Kit used with an AutoMACS Pro (Miltenyi Biotec, Auburn, CA). The purity (>95%) and naive status of the isolated CD4⁺ T cells were confirmed by FACS staining with FITC-, PE-, PerCP- or APC-conjugated mAbs against CD4, CD8, B220, CD44 and CD62L. Naive CD4⁺ T cells (10⁶/ml) were activated with plate-bound anti-CD3 (10 μ g/ml) plus soluble anti-CD28 (2 μ g/ml), or differentiated into Th0, Th1 or Th2 cells (Martin *et al*, 2005). The culture supernatants were collected at different times after activation to assess cytokines by ELISA.

Cytokine assay

Cytokines in the supernatants and BAL fluid were measured by ELISA. IL-4, IL-5, IL-6 and IFN- γ were measured with OptEIA kits (BD PharMingen); and IL-13 and eotaxin were measured with DuoSet ELISA kits (R&D Systems). TGF- β 1 was assayed by TGF- β 1 E_{max} ImmunoAssay System (Promega, Madison, WI). ELISA plates were developed with TMB substrate (BD PharMingen), and read with a microplate reader (model 550, Bio-Rad, Hercules, CA). Cytokine mRNA levels were measured by real-time quantitative PCR.

FACS

Spleen and LN cells or purified CD4⁺ cells were incubated with anti-CD16/32 (2.4G2) to block Fc γ II/III, and then stained with various conjugated mAbs. BD Cytotfix/Cytoperm kit (BD PharMingen) was used for intracellular cytokine and NBR1 staining. Treg staining kit from e-Bioscience was used for Tregs following the manufacturer's instructions. CD4⁺ T-cell proliferation was assayed *in vitro* by measuring BrdU incorporation with a BrdU Flow kit (BD PharMingen). Stained cells were analysed by FACSCalibur with CellQuest software (Becton Dickinson, Becton Mountain View, CA).

T-cell-bead conjugation

Polystyrene beads (4.5 μ m, Polysciences, Warrington, PA) were coated with 20 μ g/ml anti-CD3 and anti-CD28 in PBS (pH 7.2) overnight at 4°C with rotation, and then washed with PBS containing 1% BSA. CD4⁺ T cells (4 $\times$ 10⁵) were incubated with 2 $\times$ 10⁶-coated beads in 0.5 ml medium at 37°C for 14 h in 48-well plates with poly-Y-L-lysine-coated coverslips at the bottom of each well. The coverslips were washed, and then processed for immunofluorescence staining.

Retroviral transduction

The Phoenix ecotropic packaging cells were transiently transfected with constitutively active NFATc1 (CA-NFATc1)-GFP retroviral vector (Plasmid #11102, Aaddgene, Cambridge, MA) (Monticelli and Rao, 2002). Virus-containing supernatant was collected 48 and 72 h post-transfection, supplemented with IL-2 (20 ng/ml) and 8 μ g/ml polybrene, and used immediately for spin infection (45 min at 20°C) of anti-CD3/CD28-activated CD4⁺ T cells. Infection was performed on 2 consecutive days. Two days after last infection, cells were re-stimulated with anti-CD3/CD28 for 14 h and harvested for immunofluorescence staining.

Immunofluorescence staining

Purified naive CD4⁺ T cells were cultured with plate-bound anti-CD3 plus anti-CD28 for 14 h. Cells were harvested and allowed to adhere to poly-Y-L-lysine (Sigma)-coated coverslips for 30 min at room temperature, after which non-adherent cells were washed off with PBS. Adherent cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and incubated with different mAbs. Signals were amplified by using the Tyramide Signal Amplification kit (Molecular Probes, Eugene, OR). For nuclear staining, cells were incubated with propidium iodide (Sigma). Coverslips were mounted on Mowiol and examined with a Zeiss LSM-510 Meta Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging, Thornwood, NY).

Western blot

CD4⁺ T and LN cells were either untreated or stimulated with anti-CD3 in the absence or presence of anti-CD28 for 24–72 h. For whole-cell lysates, cells were extracted with RIPA lysis buffer (1 $\times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenyl methyl sulphonyl fluoride and protease inhibitors). For

cytosol/nuclear extracts, cells were lysed with hypotonic buffer (10 mM Tris-HCl pH 7.8, 3 mM MgCl₂, 10 mM NaCl, 0.5% Triton X-100 and protease inhibitors) for 2 min and spun briefly. The supernatants were kept as the cytosol fractions. The cell pellets were washed twice, and lysed with Buffer N/C RIPA (10 mM Tris-HCl pH 7.8, 3 mM MgCl₂, 300 mM NaCl, 0.5% Igepal, 0.1% SDS, 0.5% deoxycholate and protease inhibitors), for 30 min at 4°C with continuous rotation. After centrifugation, these supernatants were collected as the nuclear fraction. Lysates were resolved by SDS-PAGE and then electrophoretically transferred onto a nitrocellulose membrane (GE Healthcare, Piscataway, NJ) and incubated with the appropriate antibodies. The bands were visualized with the enhanced chemiluminescence system (Thermo Scientific, Rockford, IL).

Immunoprecipitation

Jurkat cells were stimulated with PMA (50 ng/ml) plus ionomycin (1000 ng/ml), or plate-bound anti-human CD3 (10 µg/ml) and anti-human CD28 (2 µg/ml) for 16 h and washed twice with ice-cold PBS and then lysed in Sigma-3 buffer (25 mM Tris-HCl pH 8, 100 mM NaCl, 1% Triton X-100, 10% glycerol and protease inhibitor mixture) for 30 min on ice. The lysates were centrifuged at 12 000 g for 15 min at 4°C. The supernatant fractions (adjusted to 1 mg protein) were precleared by incubation with protein G- or protein A-Sepharose (GE Healthcare Bio-Science, Uppsala, Sweden) for 30 min at 4°C with continuous rotation. Immunoprecipitations were performed by overnight rotation of the precleared supernatants with 1–2 µg of the primary Ab indicated, in each case at 4°C. The Ag-Ab complexes were harvested by incubation with protein G- or protein A-Sepharose for 1 h at 4°C. After extensive washing with ice-cold Sigma-3 lysis buffer, the beads were boiled for 5 min in 2 × SDS-PAGE sample buffer to dissociate the immunoprecipitated

proteins. These fractions were analysed by electrophoresis on SDS-PAGE and immunoblot analyses as described.

Real-time PCR

Total RNA was extracted from lung tissues or from cultured cells with the RNeasy Mini kit (Qiagen, Valencia, CA), and cDNA was prepared by using the Omniscript Reverse Transcription kit (Qiagen). Quantitative PCR was performed with the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a Mastercycler ep realplex4 apparatus (Eppendorf, Westbury, NY). The data were normalized to the 18S reference. Primers for IL-4, IL-5, IL-13, eotaxin, MUC-5AC and Gob-5 were designed with OLIG 4.0 software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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