

T helper type 2 differentiation and intracellular trafficking of the interleukin 4 receptor- α subunit controlled by the Rac activator Dock2

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The lineage commitment of CD4⁺ T cells is coordinately regulated by signals through the T cell receptor and cytokine receptors, yet how these signals are integrated remains elusive. Here we find that mice lacking Dock2, a Rac activator in lymphocytes, developed allergic disease through a mechanism dependent on CD4⁺ T cells and the interleukin 4 receptor (IL-4R). Dock2-deficient CD4⁺ T cells showed impaired antigen-driven downregulation of IL-4R α surface expression, resulting in sustained IL-4R signaling and excessive T helper type 2 responses. Dock2 was required for T cell receptor-mediated phosphorylation of the microtubule-destabilizing protein stathmin and for lysosomal trafficking and the degradation of IL-4R α . Thus, Dock2 links T cell receptor signals to downregulation of IL-4R α to control the lineage commitment of CD4⁺ T cells.

During immune responses, antigen-stimulated naive CD4⁺ T cells differentiate into functionally distinct subsets of T helper cells^{1,2}. T helper type 1 (T_H1) cells secrete interferon- γ (IFN- γ) and are important in cell-mediated immunity, whereas T_H2 cells produce cytokines such as interleukin 4 (IL-4), IL-5 and IL-13 and are involved in humoral immunity and allergic responses. The polarization of naive CD4⁺ T cells toward the T_H1 or T_H2 lineage is determined by many parameters, including the affinity of the T cell receptor (TCR) for antigen, the concentration of antigen present, the duration of TCR triggering and, in particular, the cytokine milieu^{3–8}. Thus, the lineage commitment of CD4⁺ T cells is coordinately regulated by signals through the TCR and cytokine receptors.

IL-4, by binding to its receptor (IL-4R) expressed on the surfaces of naive CD4⁺ T cells, promotes T_H2 differentiation^{6,8}. IL-4R consists of the IL-4R α -chain (IL-4R α), which binds IL-4 with high affinity, and the common γ -chain, which transmits signals into the cell interior and is also a component of the receptors for IL-2, IL-7, IL-9, IL-15 and IL-21 (refs. 9,10). Signaling through IL-4R results in recruitment of the transcription factor STAT6, which is phosphorylated by the kinases Jak1 and Jak3 (ref. 9). STAT6 then translocates to the nucleus, where it activates expression of IL-4 target genes, including *Il4ra*^{6,11}. Although STAT6 may directly regulate *Il4* expression by binding to the *Il4* promoter and 3' enhancer¹², the most important function of STAT6 in T_H2 differentiation is the initial upregulation of GATA-3, a transcription factor that facilitates *Il4* transcription by functioning

cooperatively with other transcription factors such as c-Maf^{13–15}. In addition, GATA-3 suppresses T_H1 differentiation by inhibiting the expression of IL-12R β 2 and STAT4 (refs. 13,16), which are required for the transmission of T_H1-promoting IL-12 signals. Thus, IL-4R amplifies its own signal transduction cascade and ensures the polarization of CD4⁺ T cells toward the T_H2 cell lineage. Maintaining a balance between T_H1 and T_H2 differentiation may therefore require strict regulation of IL-4R signals during the early stages of helper T cell differentiation.

Rac is a member of the Rho family of GTPases that function as molecular 'switches' by cycling between GDP-bound inactive states and GTP-bound active states¹⁷. Once activated, Rac regulates various cellular functions through remodeling of the actin cytoskeleton. However, evidence suggests that Rac also regulates microtubule dynamics through phosphorylation and inactivation of the microtubule-destabilizing protein stathmin (also called Op18)^{18–21}. Three Rac isoforms exist: Rac1 is expressed ubiquitously, Rac3 is expressed in the brain and Rac2 expression is restricted mainly to hematopoietic cells. Data indicate that a dominant negative Rac mutant or Rac2 deficiency inhibits the production of IFN- γ by CD4⁺ T cells *in vitro*²². In addition, Rac2 is expressed mainly in T_H1 cells²². However, Rac2 deficiency alone does not affect cytokine profiles and disease susceptibility during *Leishmania major* infection²³; the outcome of such infection critically depends on a T_H1-versus-T_H2 balance²⁴. Therefore, although Rac might be involved in the lineage commitment of CD4⁺

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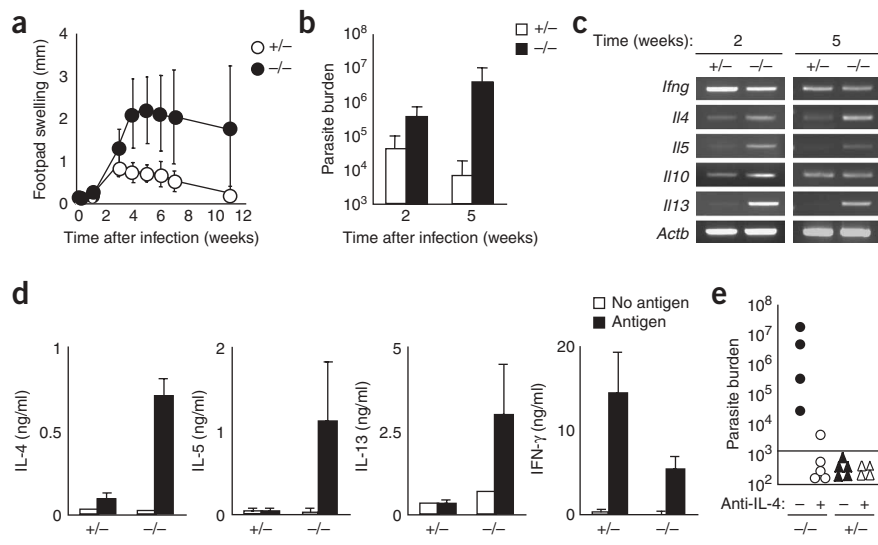


Figure 1 Excessive T_H2 immune responses in $Dock2^{-/-}$ C57BL/6 mice. **(a)** Footpad swelling of $Dock2^{+/-}$ (+/-; $n = 7$ mice) and $Dock2^{-/-}$ (-/-; $n = 6$ mice) littermates after *L. major* infection. **(b)** Parasite burdens of $Dock2^{+/-}$ and $Dock2^{-/-}$ littermates ($n = 3$ mice per group) after *L. major* infection. **(c)** Expression of genes encoding T_H1 or T_H2 cytokines in $CD4^+$ T cells obtained from the popliteal lymph nodes of $Dock2^{+/-}$ and $Dock2^{-/-}$ mice after *L. major* infection. *Actb*, normalization control. **(d)** Cytokine production by $CD4^+$ T cells from *L. major*-infected $Dock2^{+/-}$ and $Dock2^{-/-}$ mice after cultivation with or without *L. major* antigen *in vitro*. **(e)** Parasite burdens of $Dock2^{+/-}$ and $Dock2^{-/-}$ mice at 6 weeks after infection with *L. major*; mice were left untreated (-) or treated with anti-IL-4 (+; $n = 4$ –5 mice per group). Each symbol represents an individual mouse; horizontal line indicates the detection limit of the parasite burden. Data are representative of four **(a)** or two **(b–d)** independent experiments (mean $\pm$ s.d., **a,b**; mean $\pm$ s.d. of triplicate cultures, **d**).

cells, its physiological importance and the mechanistic basis of its functions are poorly understood.

Dock2 is a mammalian homolog of the *Caenorhabditis elegans* protein CED-5 and the *Drosophila melanogaster* protein myoblast city and is expressed mainly by hematopoietic cells²⁵. Although Dock2 does not contain the Dbl homology and pleckstrin homology domains typically present in guanine nucleotide-exchange factors¹⁷, Dock2 binds to nucleotide-free Rac and, through its Docker (also called DHR-2) domain, catalyzes GTP loading^{26,27}. TCR-mediated activation of Rac1 and Rac2 is almost completely abolished in Dock2-deficient ($Dock2^{-/-}$) T cells²⁸. However, Dock2 deficiency does not affect TCR-induced calcium mobilization and activation of the transcription factor NF- κ B²⁸, which promote cytokine gene expression²⁹. Thus, $Dock2^{-/-}$ mice might be useful for determining the importance of Rac activation in the 'quality control' of immune responses. Here we show that Dock2 links TCR signals to IL-4R α downregulation to control the lineage commitment of $CD4^+$ T cells *in vitro* and *in vivo*. Although

Dock2 was not required for internalization of IL-4R α , by influencing Rac activation and microtubule dynamics, Dock2 controlled lysosomal trafficking of the internalized IL-4R α . Our results thus define a regulatory mechanism for helper T cell differentiation and provide insight into how TCR and cytokine receptor signals are integrated during the activation of $CD4^+$ T cells.

RESULTS

Enhanced T_H2 responses in $Dock2^{-/-}$ mice

To examine the function of Dock2 in immune response *in vivo*, we first injected *L. major* into the hind footpads of $Dock2^{-/-}$ and $Dock2^{+/-}$ mice on a T_H1 -prone C57BL/6 genetic background. Swelling of the footpads of $Dock2^{+/-}$ mice peaked at 3 weeks after inoculation and gradually decreased to basal thickness at 11 weeks after inoculation (Fig. 1a). In contrast, swelling of the footpads of $Dock2^{-/-}$ mice was more severe and sustained even at 11 weeks after inoculation (Fig. 1a). In addition, $Dock2^{-/-}$ mice had 330-fold higher parasite burden

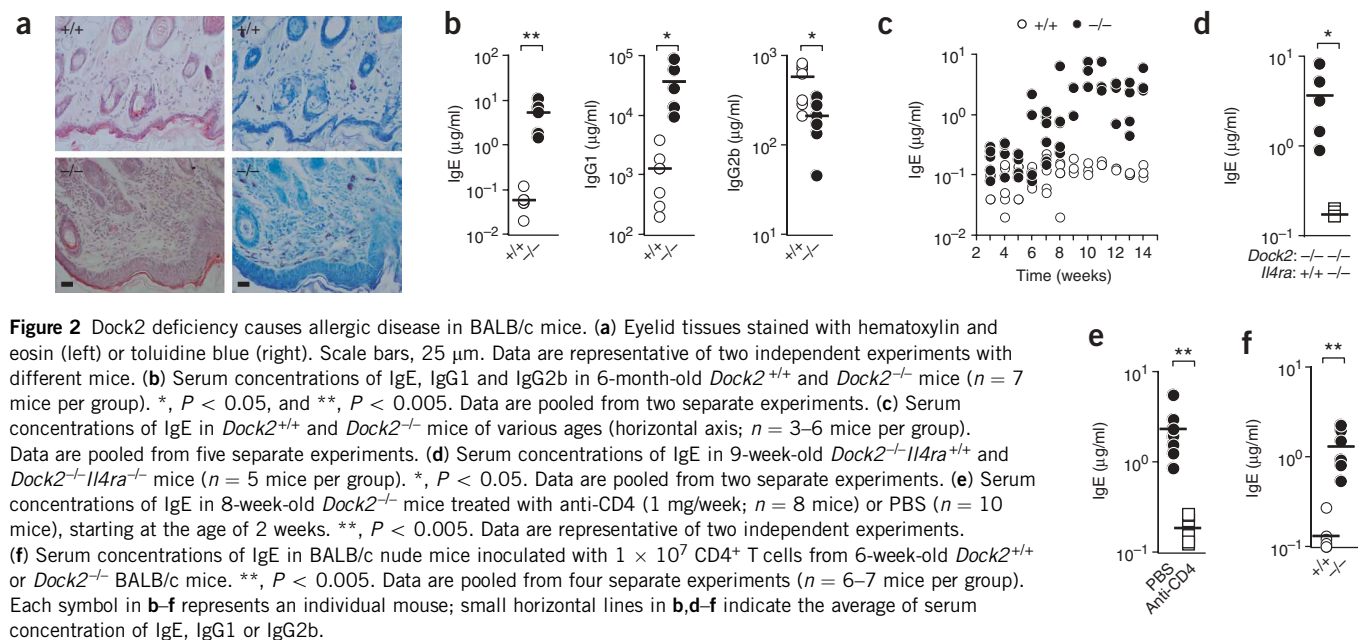


Figure 2 Dock2 deficiency causes allergic disease in BALB/c mice. **(a)** Eyelid tissues stained with hematoxylin and eosin (left) or toluidine blue (right). Scale bars, 25 μ m. Data are representative of two independent experiments with different mice. **(b)** Serum concentrations of IgE, IgG1 and IgG2b in 6-month-old $Dock2^{+/-}$ and $Dock2^{-/-}$ mice ($n = 7$ mice per group). *, $P < 0.05$, and **, $P < 0.005$. Data are pooled from two separate experiments. **(c)** Serum concentrations of IgE in $Dock2^{+/-}$ and $Dock2^{-/-}$ mice of various ages (horizontal axis; $n = 3$ –6 mice per group). Data are pooled from five separate experiments. **(d)** Serum concentrations of IgE in 9-week-old $Dock2^{+/-}Il4ra^{+/-}$ and $Dock2^{-/-}Il4ra^{+/-}$ mice ($n = 5$ mice per group). *, $P < 0.05$. Data are pooled from two separate experiments. **(e)** Serum concentrations of IgE in 8-week-old $Dock2^{-/-}$ mice treated with anti-CD4 (1 mg/week; $n = 8$ mice) or PBS ($n = 10$ mice), starting at the age of 2 weeks. **, $P < 0.005$. Data are representative of two independent experiments. **(f)** Serum concentrations of IgE in BALB/c nude mice inoculated with 1×10^7 $CD4^+$ T cells from 6-week-old $Dock2^{+/-}$ or $Dock2^{-/-}$ BALB/c mice. **, $P < 0.005$. Data are pooled from four separate experiments ($n = 6$ –7 mice per group). Each symbol in **b–f** represents an individual mouse; small horizontal lines in **b,d–f** indicate the average of serum concentration of IgE, IgG1 or IgG2b.

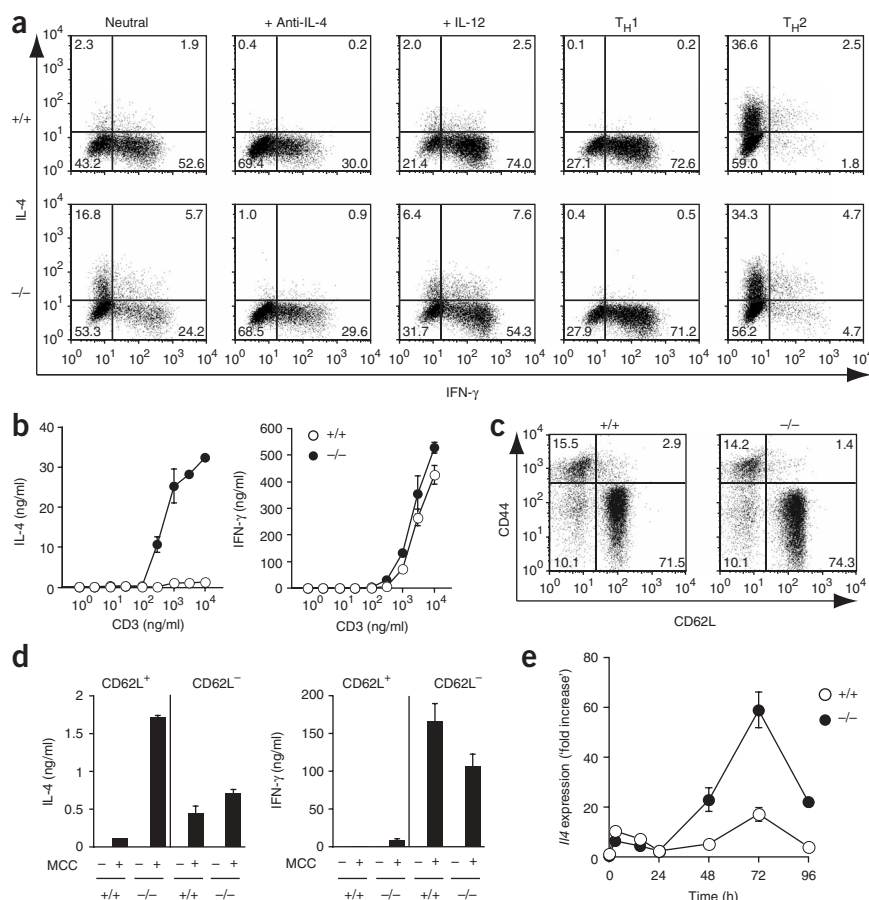


Figure 3 *Dock2* $^{-/-}$ naive CD4 $^+$ T cells produce excessive IL-4 after antigen stimulation. **(a,b)** Production of IFN- γ and IL-4 by *Dock2* $^{+/+}$ 2B4Tg and *Dock2* $^{-/-}$ 2B4Tg CD4 $^+$ T cells stimulated with MCC(88–103) peptide in medium alone (Neutral) or in the presence of anti-IL-4 or IL-12, or anti-IL-4 plus IL-12 (T_H1) or IL-4 plus anti-IL-12 (T_H2). **(a)** Flow cytometry of cells restimulated with immobilized anti-CD3 and anti-CD28 at 4 d after the initial stimulation. Numbers in quadrants indicate percent cells in each. **(b)** Enzyme-linked immunosorbent assay of IL-4 and IFN- γ in CD4 $^+$ T cells initially stimulated in neutral conditions (medium alone) and restimulated for 2 d with immobilized anti-CD3. **(c)** Expression of CD44 and CD62L on CD4 $^+$ T cells from 8-week-old *Dock2* $^{+/+}$ 2B4Tg and *Dock2* $^{-/-}$ 2B4Tg mice. Numbers in quadrants indicate percent cells in each after gating on CD4 $^+$ cells. **(d)** Enzyme-linked immunosorbent assay of IL-4 and IFN- γ in *Dock2* $^{+/+}$ 2B4Tg and *Dock2* $^{-/-}$ 2B4Tg CD62L $^+$ and CD62L $^-$ CD4 $^+$ T cells stimulated for 2 d with irradiated B10.BR spleen cells in the presence (+) or absence (–) of MCC(88–103) (MCC). **(e)** Real-time PCR analysis of *Il4* expression by *Dock2* $^{+/+}$ 2B4Tg and *Dock2* $^{-/-}$ 2B4Tg CD4 $^+$ T cells stimulated for various times (horizontal axis) with irradiated B10.BR spleen cells plus MCC(88–103). Expression ('fold increase') is relative to that of unstimulated samples after normalization to the internal control *Hprt1*. Data are representative of three **(a,c,e)** or two **(b,d)** independent experiments (mean $\pm$ s.d. of triplicate measurements, **b,d,e**).

than *Dock2* $^{+/+}$ mice had at 5 weeks after inoculation ($420 \times 10^4 \pm 96.4 \times 10^4$ parasites versus $1.27 \times 10^4 \pm 0.69 \times 10^4$ parasites, respectively; $P = 0.025$; **Fig. 1b**). CD4 $^+$ T cells in the popliteal lymph nodes of *Dock2* $^{+/+}$ and *Dock2* $^{-/-}$ mice had similar expression of *Ifng*, but *Dock2* $^{-/-}$ CD4 $^+$ T cells had higher expression of *Il4*, *Il5* and *Il13* at 2 and 5 weeks after inoculation (**Fig. 1c**). CD4 $^+$ T cells prepared from the popliteal lymph nodes of *Dock2* $^{-/-}$ mice at 4 weeks after inoculation produced IL-4, IL-5 and IL-13 after challenge with *L. major* antigen *in vitro*, but those from *Dock2* $^{+/+}$ mice did not (**Fig. 1d**). These results collectively indicate that during *L. major* infection, *Dock2* deficiency results in T_H2 immune responses even in T_H1 -prone C57BL/6 mice.

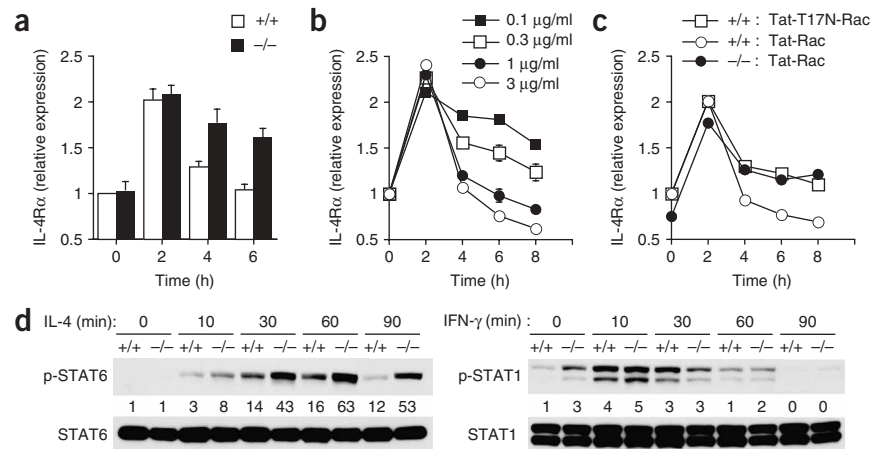
Because *Dock2* regulates lymphocyte motility²⁵, the altered susceptibility of *Dock2* $^{-/-}$ mice to *L. major* may have been due to defective lymphocyte migration. Indeed, lymphocyte accumulation in the popliteal lymph node was impaired in *Dock2* $^{-/-}$ mice during infection (**Supplementary Fig. 1** online). To determine whether the T_H2 immune response contributed to disease susceptibility of *Dock2* $^{-/-}$ mice, we treated *Dock2* $^{-/-}$ mice with antibody to IL-4 (anti-IL-4) at 0 d and 1 d after *L. major* infection. Treatment with anti-IL-4 effectively suppressed the parasite burden in *Dock2* $^{-/-}$ mice analyzed 6 weeks after inoculation (**Fig. 1e**). However, neither the number nor the composition of cells in the popliteal lymph node was affected by treatment with anti-IL-4 (**Supplementary Fig. 1**). These results collectively indicate that *Dock2* deficiency renders C57BL/6 mice susceptible to *L. major* infection mainly by enhancing T_H2 immune responses.

Next we sought to determine how *Dock2* deficiency affects T_H2 -prone mice. For this, we crossed *Dock2* $^{-/-}$ C57BL/6 mice onto

the T_H2 -prone BALB/c genetic background for seven generations. Over 75% of the *Dock2* $^{-/-}$ BALB/c mice spontaneously developed swelling and erythema of the eyelid by the age of 6 months (**Supplementary Fig. 2** online). Blepharitis first appeared 3 months after birth and generally worsened with the age and never developed in *Dock2* $^{+/+}$ or *Dock2* $^{+/+}$ littermate mice. Histological analysis of the eyelid lesion showed dense infiltrates of eosinophils and mast cells in the subepithelial stroma and skin (**Fig. 2a**). Furthermore, *Dock2* $^{-/-}$ mice had much higher serum concentrations of immunoglobulin E (IgE) than *Dock2* $^{+/+}$ control mice had (5.45 ± 3.19 μ g/ml versus 0.06 ± 0.03 μ g/ml, respectively; $P = 0.00076$; **Fig. 2b**). We first detected this in some 6-week-old *Dock2* $^{-/-}$ mice and found it in all 8-week-old *Dock2* $^{-/-}$ mice (**Fig. 2c**). IgG isotype analysis also showed higher serum concentrations of IgG1 and lower serum concentrations of IgG2b in 6-month-old *Dock2* $^{-/-}$ mice (**Fig. 2b**).

IL-4 is essential for the induction of IgE synthesis and enhances class switching to IgG1 (refs. 30,31). To determine whether IL-4 was involved in the development of disease in *Dock2* $^{-/-}$ BALB/c mice, we crossed those mice with IL-4R α -deficient (*Il4ra* $^{-/-}$) BALB/c mice. In contrast to the results obtained with *Dock2* $^{-/-}$ mice, we found neither blepharitis nor higher serum IgE concentrations in *Dock2* $^{-/-}$ *Il4ra* $^{-/-}$ mice (**Fig. 2d**), which indicated that IL-4-mediated signaling is critical for disease development. We then examined the function of CD4 $^+$ T cells by administering anti-CD4 (GK1.5) to *Dock2* $^{-/-}$ mice intraperitoneally every 6–7 d beginning at the age of 2 weeks. Although all untreated *Dock2* $^{-/-}$ mice had more serum IgE than did *Dock2* $^{+/+}$ mice without anti-CD4 treatment at the age of 8 weeks, this phenotype completely disappeared in *Dock2* $^{-/-}$ mice treated with anti-CD4 (2.25 ± 1.30 μ g/ml versus 0.19 ± 0.06 μ g/ml,

Figure 4 Dock2 acts through Rac to promote IL-4R α downregulation. (a–c) Flow cytometry of the cell surface expression of IL-4R α during activation of CD4 $^{+}$ T cells. (a) *Dock2* $^{+/+}$ 2B4Tg and *Dock2* $^{-/-}$ 2B4Tg CD4 $^{+}$ T cells stimulated with immobilized anti-CD3 in the presence of anti-IL-4 (time, horizontal axis). (b) *Dock2* $^{+/+}$ 2B4Tg CD4 $^{+}$ T cells stimulated with various concentrations of anti-CD3. Expression in a,b is relative to that of unstimulated *Dock2* $^{+/+}$ CD4 $^{+}$ T cells. (c) *Dock2* $^{+/+}$ 2B4Tg and *Dock2* $^{-/-}$ 2B4Tg CD4 $^{+}$ T cells incubated with green fluorescent protein-tagged Tat-T17N-Rac or wild-type Rac control construct (Tat-Rac) and stimulated with immobilized anti-CD3. Expression is relative to that of unstimulated *Dock2* $^{+/+}$ CD4 $^{+}$ T cells expressing wild-type Rac control construct. (d) Immunoblot analysis of phosphorylated STAT6 (p-STAT6) and STAT1 (p-STAT1) in lysates of *Dock2* $^{+/+}$ 2B4Tg and *Dock2* $^{-/-}$ 2B4Tg CD4 $^{+}$ T cells stimulated with IL-4 or IFN- γ after being preactivated for 4 h with immobilized anti-CD3 in the presence of anti-IL-4 or anti-IFN- γ , respectively. Numbers below lanes indicate expression of p-STAT6 and p-STAT1 relative to that of unstimulated *Dock2* $^{+/+}$ CD4 $^{+}$ T cells. Data are representative of at least three independent experiments.



respectively; $P = 0.00039$; **Fig. 2e**), which indicated that the disease development involved CD4 $^{+}$ T cells. Adoptive transfer experiments with BALB/c nude mice as recipients confirmed this conclusion. Whereas BALB/c nude mice injected with *Dock2* $^{+/+}$ CD4 $^{+}$ T cells did not show any signs of the disease, inoculation with *Dock2* $^{-/-}$ CD4 $^{+}$ T cells resulted in blepharitis in five of seven BALB/c nude recipients (**Supplementary Fig. 3** online) and increased serum IgE (**Fig. 2f**). These results collectively indicate that the allergic disease of *Dock2*-deficient BALB/c mice requires IL-4R signaling and the presence of CD4 $^{+}$ T cells.

Excess IL-4 from naive *Dock2* $^{-/-}$ CD4 $^{+}$ T cells

To explore the mechanism by which Dock2 regulates the differentiation of helper T cells, we analyzed transgenic mice expressing the 2B4 $\alpha\beta$ TCR, which recognizes the moth cytochrome C peptide of amino acids 88–103 (MCC(88–103)) bound to I-E ^{k} molecules³² in the presence and absence of Dock2 (*Dock2* $^{+/+}$ 2B4Tg and *Dock2* $^{-/-}$ 2B4Tg mice, respectively)²⁸. *Dock2* $^{+/+}$ 2B4Tg and *Dock2* $^{-/-}$ 2B4Tg CD4 $^{+}$ T cells stimulated with MCC(88–103) in conditions promoting T $_H$ 1 or T $_H$ 2 differentiation showed no phenotypic differences (**Fig. 3a**). However, whereas *Dock2* $^{+/+}$ 2B4Tg CD4 $^{+}$ T cells ‘preferentially’ differentiated into T $_H$ 1 cells in neutral conditions, a considerable fraction of *Dock2* $^{-/-}$ 2B4Tg CD4 $^{+}$ T cells (15–20%) produced IL-4 but not IFN- γ after neutral stimulation (**Fig. 3a,b**). This predisposition toward T $_H$ 2 differentiation depended on IL-4, as IL-4-producing *Dock2* $^{-/-}$ 2B4Tg CD4 $^{+}$ T cells disappeared when anti-IL-4 was added to primary neutral cultures (**Fig. 3a**). These results indicate that the excessive T $_H$ 2 differentiation of *Dock2* $^{-/-}$ CD4 $^{+}$ T cells depends on IL-4 produced by these cells early after TCR stimulation.

CD4 $^{+}$ T cells are categorized as naive and memory-like subpopulations depending on their surface expression of CD44 and CD62L. Although it has been reported that CD44 $^{+}$ CD62L $^{-}$ memory-like CD4 $^{+}$ T cells produce IL-4 after antigen stimulation³³, we found the proportion of this population was similar in *Dock2* $^{+/+}$ 2B4Tg and *Dock2* $^{-/-}$ 2B4Tg mice (**Fig. 3c**). To determine which type of CD4 $^{+}$ T cells produced IL-4 in *Dock2* $^{-/-}$ 2B4Tg mice, we purified CD62L $^{+}$ and CD62L $^{-}$ CD4 $^{+}$ T cells and analyzed cytokine production in response to MCC(88–103). CD62L $^{+}$ naive CD4 $^{+}$ T cells from *Dock2* $^{-/-}$ 2B4Tg mice, but not those from *Dock2* $^{+/+}$ 2B4Tg mice, produced abundant IL-4 after stimulation for 2 d with MCC(88–

103) (**Fig. 3d**). CD62L $^{-}$ memory-like CD4 $^{+}$ T cells from *Dock2* $^{-/-}$ 2B4Tg and *Dock2* $^{+/+}$ 2B4Tg mice produced similar amounts of IL-4 after the same treatment. Thus, Dock2 deficiency allows naive CD4 $^{+}$ T cells to produce excessive IL-4 after antigen stimulation.

It has been reported that CD4 $^{+}$ T cells transcribe *Il4* in a STAT6-independent way as early as 1 h after TCR stimulation and before cell division³⁴. To determine precisely the kinetics of *Il4* expression, we measured *Il4* transcripts in CD4 $^{+}$ T cells at various times after stimulation with MCC(88–103). At 3 h after stimulation, *Dock2* $^{-/-}$ 2B4Tg and *Dock2* $^{+/+}$ 2B4Tg CD4 $^{+}$ T cells had similar expression of *Il4* (**Fig. 3e**), indicating that Dock2 deficiency does not affect IL-4R-independent *Il4* transcription. However, *Dock2* $^{-/-}$ 2B4Tg CD4 $^{+}$ T cells transcribed *Il4* more effectively than *Dock2* $^{+/+}$ 2B4Tg CD4 $^{+}$ T cells did after 24 h, and the difference in *Il4* expression reached a maximum at 72 h (**Fig. 3e**). The upregulation of *Il4* expression in *Dock2* $^{-/-}$ 2B4Tg CD4 $^{+}$ T cells was totally abolished by treatment of cells with anti-IL-4 during the first 24 h of culture (**Supplementary Fig. 4** online). This finding, together with the relatively late appearance of excess *Il4* transcripts in *Dock2* $^{-/-}$ 2B4Tg CD4 $^{+}$ T cells, raised the possibility that Dock2 might regulate *Il4* expression induced by IL-4R signals.

Dock2 regulates IL-4R α downregulation

We first compared the surface expression of IL-4R α on antigen-stimulated *Dock2* $^{-/-}$ 2B4Tg and *Dock2* $^{+/+}$ 2B4Tg CD4 $^{+}$ T cells. IL-4R α was transiently upregulated on the surfaces of CD4 $^{+}$ T cells stimulated with immobilized anti-CD3 in the presence of anti-IL-4, regardless of Dock2 expression (**Fig. 4a**). However, whereas IL-4R α expression on the surfaces of *Dock2* $^{+/+}$ 2B4Tg CD4 $^{+}$ T cells returned to basal prestimulation amounts within 4 h, this ‘downregulation’ was impaired in *Dock2* $^{-/-}$ 2B4Tg CD4 $^{+}$ T cells (**Fig. 4a**). The extent of IL-4R α downregulation increased with higher concentrations of immobilized anti-CD3, indicating that this process might depend on the strength of the TCR signals (**Fig. 4b**). In contrast, surface expression of the common γ -chain component of IL-4R remained constant (data not shown).

Next we examined whether Rac activation is involved in IL-4R α downregulation by treating *Dock2* $^{+/+}$ CD4 $^{+}$ T cells with a cell-permeable dominant negative Rac mutant (Tat-T17N-Rac)³⁵. A wild-type Rac control construct did not affect the downregulation

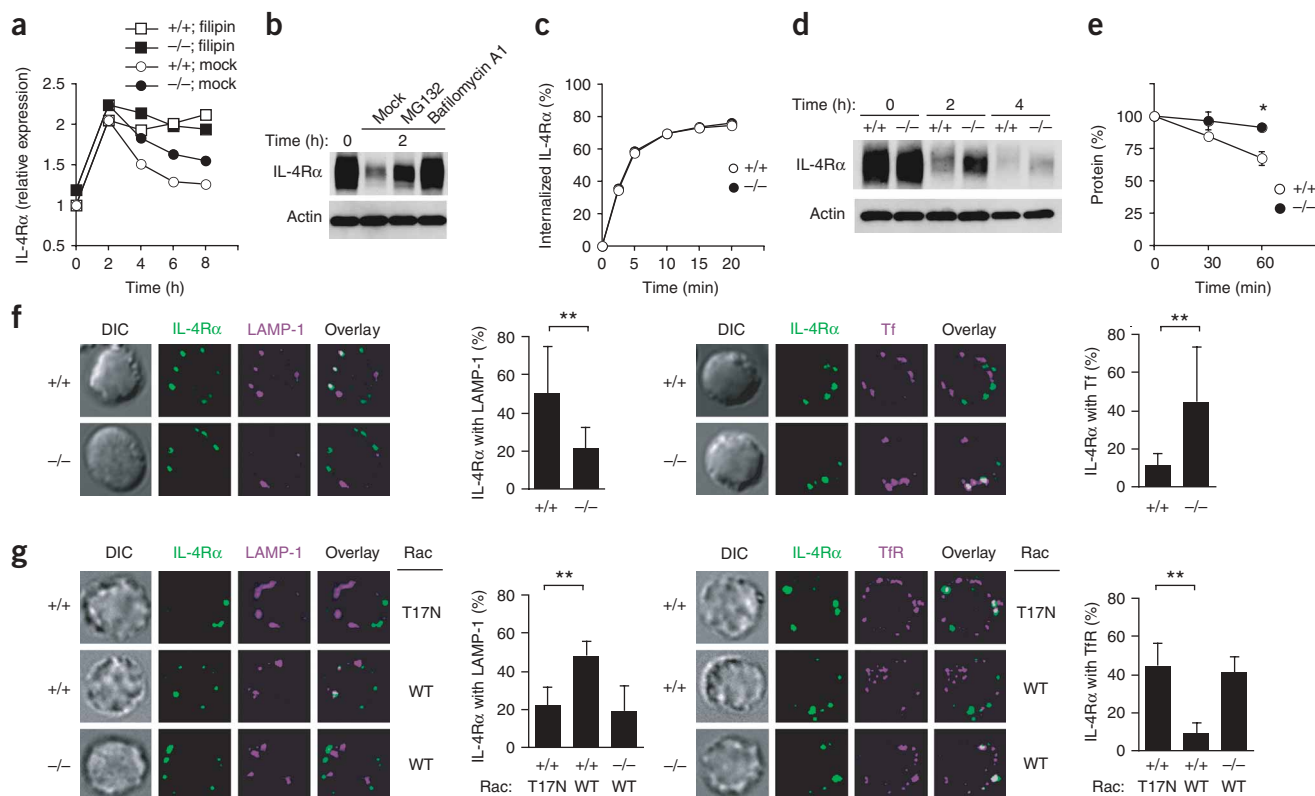


Figure 5 Dock2 regulates the intracellular trafficking of IL-4R α . Analysis of *Dock2*^{+/+}2B4Tg and *Dock2*^{-/-}2B4Tg CD4⁺ T cells stimulated with immobilized anti-CD3 in the presence of anti-IL-4. (a) Flow cytometry of IL-4R α on CD4⁺ T cells treated with filipin (added 90 min after initial priming with anti-CD3) or with vehicle (methanol) alone (Mock). (b) Immunoblot analysis of IL-4R α degradation in *Dock2*^{+/+}2B4Tg CD4⁺ T cells treated with bafilomycin A1, MG132 or vehicle (DMSO; Mock) at 90 min after initial priming with anti-CD3, followed by the addition of cycloheximide 30 min later and analysis at 0 h and 2 h after the addition of cycloheximide. (c) Internalization of IL-4R α during the activation of CD4⁺ T cells primed with anti-CD3; at 2 h after initial priming (time, 0 min), cells were stained with biotinylated anti-IL-4R α , followed by incubation at 37 °C and labeling of the remaining surface IL-4R α with CyChrome-streptavidin. (d) Immunoblot analysis of IL-4R α degradation in lysates of cells treated with cycloheximide 2 h after initial priming with anti-CD3 and assessed 2 h and 4 h later. (e) Pulse-chase analysis of IL-4R α degradation in CD4⁺ T cells labeled with [³⁵S]methionine and stimulated for 2 h with anti-CD3 (time, 0 min); IL-4R α was measured 30 min and 60 min later and is presented as the percent relative to that at 'time 0' (set as 100%). *, *P* < 0.01. (f) Localization of internalized IL-4R α together with LAMP-1 (left) or transferrin (Tf; right) in cells labeled on ice at 2 h after initial priming with anti-CD3 and incubated for 30 min at 37 °C in the presence of bafilomycin A1. DIC, differential interference contrast. **, *P* < 0.001. (g) Localization of internalized IL-4R α together with LAMP-1 (left) or the transferrin receptor (TfR; right) in cells transfected with plasmids encoding V5-tagged wild-type Rac (WT) or T17N-Rac (T17N), analyzed as described in f. Analysis was done with cells stained with anti-V5 (data not shown). **, *P* < 0.001. Original magnification, $\times 60$. Graphs in f,g: percent IL-4R α localized together with LAMP-1, transferrin or the transferrin receptor (mean $\pm$ s.d.; twenty (f) or seven to eight (g) cells per group). Data are representative of three (a–d,f) or two (g) independent experiments or are the mean $\pm$ s.d. of three experiments (e).

of IL-4R α (Fig. 4c), but expression of Tat-T17N-Rac in *Dock2*^{+/+} CD4⁺ T cells inhibited IL-4R α downregulation to the extent found in *Dock2*^{-/-} CD4⁺ T cells (Fig. 4c). These results indicate that during activation of CD4⁺ T cells, Dock2 acts by means of Rac activation to promote the downregulation of IL-4R α .

To determine whether the cell surface retention of IL-4R α affected signaling events 'downstream' of IL-4R α , we compared IL-4-induced phosphorylation of STAT6 in *Dock2*^{+/+}2B4Tg and *Dock2*^{-/-}2B4Tg CD4⁺ T cells. We preactivated CD4⁺ T cells with immobilized anti-CD3 for 4 h in the presence of anti-IL-4, then collected and washed the cells and stimulated them with IL-4. *Dock2*^{-/-}2B4Tg CD4⁺ T cells had greater and more sustained STAT6 phosphorylation than *Dock2*^{+/+}2B4Tg CD4⁺ T cells had (Fig. 4d). However, we detected no differences in IFN- γ -induced STAT1 phosphorylation in *Dock2*^{-/-}2B4Tg and *Dock2*^{+/+}2B4Tg CD4⁺ T cells analyzed in similar conditions (Fig. 4d). These results indicate that defective IL-4R α downregulation leads to augmented and sustained IL-4R signals in *Dock2*^{-/-}2B4Tg CD4⁺ T cells.

Dock2 in lysosomal trafficking of IL-4R α

To understand better the endocytic pathway traveled by the IL-4R α , we analyzed the internalization and degradation of IL-4R α in the presence of various chemical inhibitors. Filipin is a polyene antifungal agent that disrupts the cholesterol-rich membrane domains often called 'lipid rafts'. Treatment with filipin or with bafilomycin A1, an inhibitor of lysosomal function, completely inhibited the TCR-induced downregulation or degradation of IL-4R α , respectively, in *Dock2*^{+/+}2B4Tg CD4⁺ T cells (Fig. 5a,b). These results indicate that after TCR stimulation, IL-4R α is internalized through lipid rafts and is degraded in lysosomal compartments.

We next compared TCR-induced internalization and degradation of IL-4R α in *Dock2*^{+/+}2B4Tg and *Dock2*^{-/-}2B4Tg CD4⁺ T cells. TCR-induced internalization of IL-4R α occurred to a similar extent in *Dock2*^{+/+} and *Dock2*^{-/-} CD4⁺ T cells (Fig. 5c). However, treatment with cycloheximide, an inhibitor of protein synthesis, showed that degradation of IL-4R α was suppressed considerably in *Dock2*^{-/-} CD4⁺ T cells (Fig. 5d). We obtained similar results with pulse-chase labeling

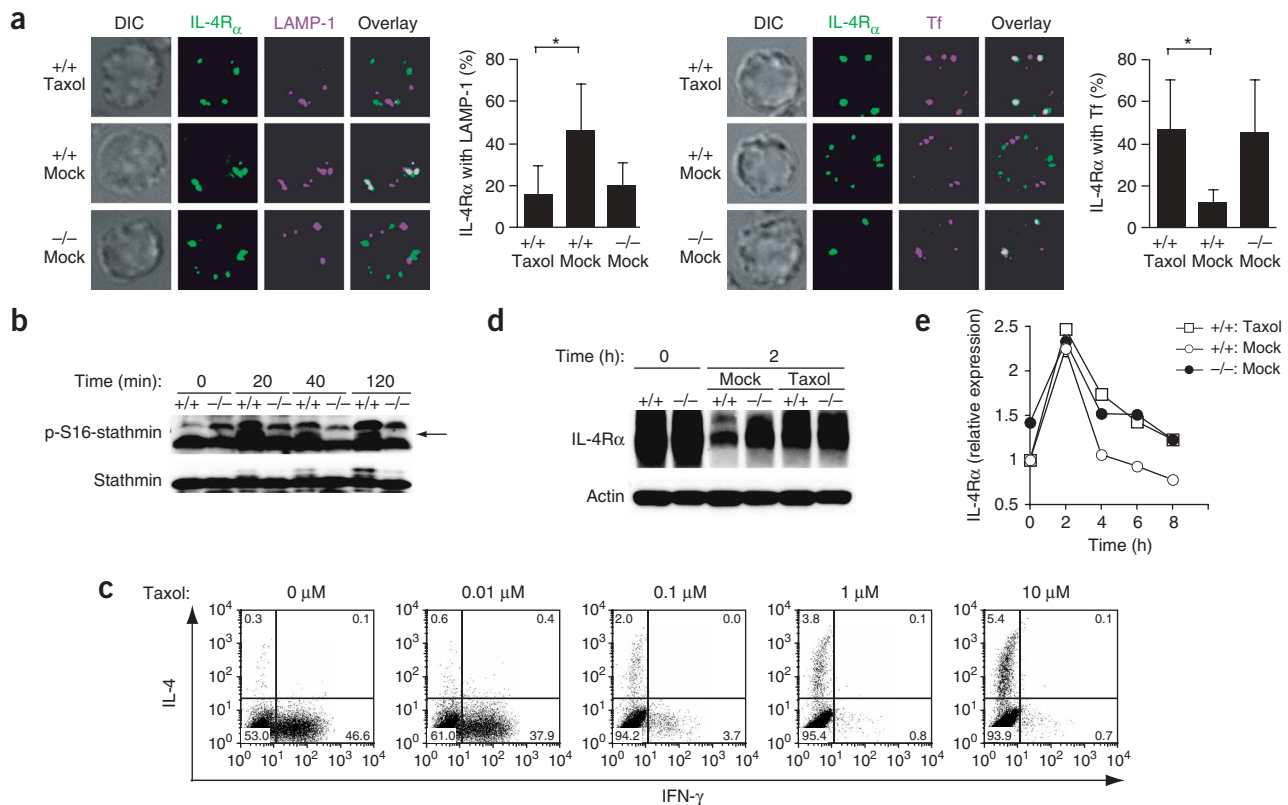


Figure 6 Dock2 downregulates IL-4R α through microtubule dynamics. **(a)** Localization of IL-4R α together with LAMP-1 (left) or transferrin (right) in *Dock2*^{+/+}2B4Tg and *Dock2*^{-/-}2B4Tg CD4⁺ T cells treated for 30 min at 37 °C with taxol or vehicle (DMSO; Mock), then stimulated with anti-CD3 in the presence of anti-IL-4 and taxol. Original magnification, $\times 60$. Right (graphs), percent IL-4R α localized together with LAMP-1 or transferrin (20 cells per group). *, $P < 0.001$. **(b)** Immunoblot analysis of lysates of *Dock2*^{+/+}2B4Tg and *Dock2*^{-/-}2B4Tg CD4⁺ T cells stimulated with immobilized anti-CD3 (time, above lanes). Arrow indicates band corresponding to stathmin phosphorylated at Ser16 (p-S16-stathmin). **(c)** Flow cytometry of *Dock2*^{+/+}2B4Tg CD4⁺ T cells treated for 30 min at 37 °C with taxol before being stimulated with MCC(88–103) peptide in neutral conditions in the presence of the same concentration of taxol, followed by intracellular cytokine staining. Numbers in quadrants indicate percent cells in each. **(d)** Immunoblot analysis of IL-4R α degradation in lysates of *Dock2*^{+/+}2B4Tg and *Dock2*^{-/-}2B4Tg CD4⁺ T cells treated for 30 min at 37 °C with taxol or vehicle (DMSO; Mock) before being stimulated for 2 h with anti-CD3, followed by the addition of cycloheximide and analysis at 2 h after the addition of cycloheximide. **(e)** Flow cytometry of IL-4R α on *Dock2*^{+/+}2B4Tg and *Dock2*^{-/-}2B4Tg CD4⁺ T cells treated for 30 min at 37 °C with taxol or vehicle (DMSO; Mock), then stimulated with immobilized anti-CD3 in the presence of anti-IL-4 and taxol. Expression is relative to that of unstimulated *Dock2*^{+/+} CD4⁺ T cells treated with vehicle (Mock). Data are representative of two **(a,b)**, three **(c,d)** or four **(e)** independent experiments.

with [³⁵S]methionine (Fig. 5e). Thus, Dock2 is essential for the timely degradation of IL-4R α during the activation of CD4⁺ T cells.

To visualize the intracellular fate of IL-4R α during the activation of CD4⁺ T cells, we labeled the IL-4R α on the surfaces of preactivated 2B4Tg CD4⁺ T cells and analyzed its intracellular localization in cells treated with bafilomycin A1. At 30 min after TCR-induced internalization in *Dock2*^{+/+} CD4⁺ T cells, IL-4R α was visible in intracellular vesicles; 50.2% $\pm$ 26.6% of these were stained positively for the lysosomal marker LAMP-1 (Fig. 5f, left). In *Dock2*^{-/-} CD4⁺ T cells, however, only 21.1% $\pm$ 13.3% ($P = 0.00009$) of IL-4R α -containing vesicles were stained positively for LAMP-1; a greater proportion of the internalized IL-4R α localized together with transferrin (44.7% $\pm$ 30.2% in *Dock2*^{-/-} CD4⁺ T cells versus 11.7% $\pm$ 7.9% in *Dock2*^{+/+} CD4⁺ T cells; $P = 0.000032$; Fig. 5f, right), which recycles back to the surface through recycling endosomes³⁶. These results suggest that Dock2 deficiency alters the intracellular trafficking pattern of IL-4R α , resulting in recycling rather than lysosomal degradation.

We then determined whether lysosomal trafficking of IL-4R α requires Rac activation. Although wild-type Rac did not affect the localization of IL-4R α in TCR-stimulated *Dock2*^{+/+} CD4⁺ T cells, expression of T17N-Rac in *Dock2*^{+/+} CD4⁺ T cells decreased

TCR-induced lysosomal accumulation of IL-4R α to an extent resembling that in *Dock2*^{-/-} CD4⁺ T cells (48.3% $\pm$ 9.2% for wild-type Rac versus 22.0% $\pm$ 11.2% for T17N-Rac in *Dock2*^{+/+} CD4⁺ T cells; $P = 0.00014$; Fig. 5g, left). Conversely, the proportion of IL-4R α localized together with the transferrin receptor was significantly higher in *Dock2*^{+/+} CD4⁺ T cells expressing T17N-Rac (9.3% $\pm$ 7.0% for wild-type Rac versus 45.0% $\pm$ 13.2% for T17N-Rac; $P = 0.0000092$; Fig. 5g, right). Thus, Dock2 acts through Rac to promote lysosomal degradation of IL-4R α .

Microtubule dynamics in IL-4R α downregulation

Microtubule reorganization is required for the efficient delivery of protein ‘cargo’ to the lysosome^{37,38}. To determine whether lysosomal trafficking of IL-4R α also required microtubule dynamics, we treated CD4⁺ T cells of *Dock2*^{+/+}2B4Tg mice with taxol, a microtubule-targeted drug that inhibits the dynamic instability of microtubules. As with Rac inhibition, taxol treatment ‘rerouted’ internalized IL-4R α from lysosomes to recycling endosomes (Fig. 6a). Evidence suggests that Rac regulates microtubule dynamics through phosphorylation and inactivation of stathmin^{19–21}. Anti-CD3 stimulation resulted in the appearance of a high-molecular-weight form of stathmin in

Dock2^{+/-}2B4Tg CD4⁺ T cells (Fig. 6b). This alteration in molecular weight partially correlated with phosphorylation of the serine residue at position 16 of stathmin (Fig. 6b). However, we did not detect the high-molecular-weight form or the phosphorylated form of stathmin in *Dock2*^{-/-}2B4Tg CD4⁺ T cells (Fig. 6b). These results collectively suggest that Dock2-mediated Rac activation regulates lysosomal trafficking of IL-4R α through microtubule dynamics.

If the defect in lysosomal trafficking of IL-4R α contributes to the abnormal T helper differentiation of *Dock2*^{-/-} CD4⁺ T cells, taxol treatment should alter differentiation of *Dock2*^{+/-} CD4⁺ T cells. To test that idea, we stimulated *Dock2*^{+/-}2B4Tg CD4⁺ T cells with MCC (88–103) in neutral conditions in the presence or absence of taxol. Without taxol, *Dock2*^{+/-} CD4⁺ T cells ‘preferentially’ differentiated into T_H1 cells. However, taxol treatment, like Dock2 deficiency, permitted excessive T_H2 differentiation (Fig. 6c). As expected, degradation and downregulation of IL-4R α were also impaired in CD4⁺ T cells treated with taxol (Fig. 6d,e). Thus, disruption of the microtubule dynamics in *Dock2*^{+/-} CD4⁺ T cells produced T helper differentiation phenotypes resembling those of *Dock2*^{-/-} CD4⁺ T cells.

DISCUSSION

Although downregulation of surface receptors is a key event allowing the modulation of signal transduction in various cell types, its physiological importance in immune response is poorly understood. Here we have provided evidence that Dock2 controls the lineage commitment of CD4⁺ T cells by promoting the downregulation and degradation of IL-4R α . In wild-type CD4⁺ T cells, surface expression of IL-4R α was transiently upregulated after TCR stimulation and returned to basal quantities within several hours. IL-4R α downregulation was impaired in *Dock2*^{-/-} CD4⁺ T cells, resulting in sustained IL-4R signals and overproduction of IL-4. IL-4R α downregulation seems to be important in maintaining homeostasis of the immune system, as *Dock2*^{-/-} BALB/c mice spontaneously developed allergic disease resembling that of transgenic mice overexpressing IL-4 in T cells³⁹. Our results thus define a previously unknown regulatory mechanism controlling the differentiation of helper T cells.

Accumulating evidence indicates that many receptors are downregulated after encounter with cognate ligands. However, ligand-independent downregulation of several receptors, including cytokine receptors and chemokine receptors, has also been documented^{40–43}. We found that during activation of CD4⁺ T cells, IL-4R α was downregulated by a mechanism dependent on the concentration of immobilized anti-CD3. As downregulation occurred even in the presence of anti-IL-4, downregulation was ligand independent but TCR dependent. Although the expression of IL-4R α on the surfaces of CD4⁺ T cells has been analyzed extensively, antigen-driven upregulation and downregulation has not been reported, perhaps because previous studies analyzed the expression of IL-4R α at later time points (beyond 6 h after stimulation)^{44,45}. Like Dock2 deficiency, the dominant negative Rac mutant inhibited the downregulation of IL-4R α during activation of CD4⁺ T cells. Given that Dock2 is critical for TCR-mediated activation of both Rac1 and Rac2, these results indicate that Dock2 links TCR signals to the downregulation of IL-4R α through Rac activation.

It has been shown that slight differences in the recycling efficiency of some receptors can have profound effects on effective ligand concentrations^{46,47}. In *Dock2*^{-/-} CD4⁺ T cells, internalized IL-4R α was ‘preferentially’ sorted to the recycling endosome; as a result, its degradation was inhibited. This change in intracellular trafficking was due to defective Rac activation, as expression of the dominant negative Rac mutant in wild-type CD4⁺ T cells resulted in similar alterations in

the localization of internalized IL-4R α . Rac regulates microtubule dynamics through the phosphorylation and inactivation of stathmin^{19–21}, and here, Dock2 was required for TCR-induced phosphorylation of stathmin. As noted in studies of other surface receptors^{38,48}, taxol-mediated disruption of microtubule dynamics impaired TCR-induced lysosomal trafficking and degradation of internalized IL-4R α . Although the precise mechanism by which the disruption of microtubule dynamics alters the localization of IL-4R α is unknown, it has been reported that the kinetics of transferrin recycling are unaffected by microtubule disruption^{36,38}. This might explain why *Dock2*^{-/-} CD4⁺ T cells and taxol-treated wild-type CD4⁺ T cells accumulated internalized IL-4R α in recycling endosomes. Our results thus indicate that Dock2-Rac signaling controls the lineage commitment of CD4⁺ T cells through intracellular trafficking of IL-4R α .

The lineage commitment of CD4⁺ T cells is influenced not only by cytokine receptor signals but also by the strength of TCR signals. Therefore, how these signals are integrated during activation of CD4⁺ T cells has been a subject of considerable debate^{6,7,29,45}. It has been shown that TCR and IFN- γ receptor 1 polarize together at the immunological synapse; this copolarization is suppressed by IL-4, suggesting that it is critical for T_H1 differentiation⁴⁹. As Dock2 is required for TCR polarization during the formation of immunological synapses²⁸, it might be suggested that a similar mechanism governs the lineage commitment of CD4⁺ T cells in *Dock2*^{-/-} mice. Indeed, we found that polarization of TCR and IFN- γ receptor 1 together was impaired in *Dock2*^{-/-} CD4⁺ T cells (Y.T. and Y.F., unpublished observations). However, it seems unlikely that this defect impeded IFN- γ receptor signals in *Dock2*^{-/-} CD4⁺ T cells, as IFN- γ -induced phosphorylation of STAT1 was not impaired.

Instead, it seems that the differences in expression of IL-4R α on *Dock2*^{+/-} and *Dock2*^{-/-} CD4⁺ T cells after TCR stimulation affected IL-4-induced phosphorylation of STAT6. We found that downregulation of IL-4R α was influenced by the strength of TCR stimulation and was regulated by Dock2-Rac signaling. Our results thus suggest that strong TCR signaling induces full activation of Rac and transiently inhibits IL-4R signaling⁴⁵, whereas weak TCR signaling permits IL-4R to transmit signals in response to IL-4, which can be produced by CD4⁺ T cells after TCR engagement in a STAT6-independent way³⁴. This may explain why low doses of antigen induce ‘preferential’ T_H2 polarization of purified CD4⁺ T cells cultured *in vitro* in neutral conditions^{3,4}.

In conclusion, we have demonstrated here that Dock2 links TCR signals to the degradation and downregulation of IL-4R α through Rac activation and microtubule dynamics and that this process controls T helper lineage commitment during early stages of differentiation. It seems unlikely that the involvement of Dock2 in intracellular trafficking is limited to IL-4R α . Whether and how Dock2-Rac signaling controls the membrane trafficking of other receptors and proteins remains to be determined.

METHODS

Mice. *Dock2*^{-/-} mice (H-2^b) and *Dock2*^{+/-}2B4Tg and *Dock2*^{-/-}2B4Tg mice (H-2^k) have been described^{25,28}. All animals were maintained in specific pathogen-free conditions in the animal facility of Kyushu University. Mouse breeding is described in the **Supplementary Methods** online. All experiments were done in accordance with the guidelines of the committee of Ethics of Animal Experiments, Faculty of Medical Sciences, Kyushu University.

***L. major* infection and immune response.** *L. major* were maintained *in vivo* and grown *in vitro* in Medium 119 (Invitrogen) supplemented with 10% (vol/vol) FCS. For infection, mice were subcutaneously inoculated in the right hind footpad with 1×10^7 stationary-phase promastigotes. Antigen-specific immune

responses were analyzed by cultivation of popliteal lymph node CD4⁺ T cells (2×10^5 cells per well) for 66 h with irradiated C57BL/6 mouse spleen cells (1×10^6 cells per well) in the presence or absence of *L. major* antigens (equivalent to 5×10^5 promastigotes).

RT-PCR. RNA samples were treated with RNase-free DNase I (Invitrogen), were reverse-transcribed with oligo(dT) and SuperScript™ II reverse transcriptase (Invitrogen) and were amplified by PCR with specific primers (Supplementary Table 1 online). Expression of the gene encoding β -actin (*Actb*) was evaluated first, and cDNA amounts were adjusted accordingly for subsequent PCR. In some experiments, the SYBR Green real-time PCR assay (PE Applied Biosystems) and specific primers (Supplementary Table 2 online) were used for RT-PCR. 'Multiplex' reactions were run in triplicate and expression was normalized to expression of the internal control gene encoding hypoxanthine guanine phosphoribosyl transferase (*Hprt1*). Sequence-detection software supplied with the instrument was used for analysis.

Isolation and differentiation of CD4⁺ T cells. CD4⁺ T cells were isolated by magnetic sorting with Dynabeads Mouse CD4 followed by treatment with DETACHaBEAD Mouse CD4 (both from Dynal). In some experiments, CD62L⁺ and CD62L⁻ CD4⁺ T cells were separated with anti-CD62L beads and magnetized columns (Miltenyi Biotech). For antigen stimulation, CD4⁺ T cells (3×10^5 cells per well) from 2B4Tg mice were cultured in a 24-well plate with T cell-depleted, irradiated B10.BR spleen cells (5×10^6 cells per well) in a total volume of 2 ml in the presence of MCC(88–103) (ANERADLIAYLK QATK; 0.33 μ g/ml). TH1 cells were generated by the addition of recombinant IL-12 (10 U/ml; Peprotech) and anti-IL-4 (11B11; 10 μ g/ml; BD Pharmingen), and TH2 polarization was initiated by the addition of recombinant IL-4 (100 U/ml; Peprotech) and anti-IL-12 (C17.8; 10 μ g/ml; BD Pharmingen). For analysis of the effect of taxol on helper T cell differentiation, CD4⁺ T cells pretreated for 30 min at 37 °C with taxol (Sigma) were stimulated in neutral conditions in the presence of taxol.

Expression and internalization of IL-4R α . CD4⁺ T cells were stimulated with plate-bound anti-CD3 (2C11; 0.1–3 μ g/ml) in the presence of anti-IL-4 (1 μ g/ml). For analysis of the surface expression of IL-4R α , cells were recovered at various time points, were washed with PBS containing 0.5% (wt/vol) BSA, were treated with anti-FcR (2.4G2; BD Pharmingen) and were stained with phycoerythrin-conjugated anti-IL-4R α (mIL4R-M1; BD Pharmingen). After being stained, cells were fixed in 4% (wt/vol) paraformaldehyde and were analyzed with a FACSCalibur (BD). In some experiments, CD4⁺ T cells were cultured with inhibitors such as filipin (50 μ g/ml; Sigma) and taxol (10 μ M; Sigma) or were treated with bacterially produced Tat-T17N-Rac or wild-type Rac construct (200 nM each) before stimulation. For internalization assays, cells were collected at 2 h and were stained for 30 min on ice with biotinylated anti-IL-4R α in ice-cold PBS containing 0.5% (wt/vol) BSA. Cells were then extensively washed with PBS containing 0.5% (wt/vol) BSA for the removal of unbound antibody and were incubated for various times at 37 °C. After incubation, surface-associated biotinylated anti-IL-4R α was visualized by labeling with CyChrome-streptavidin. Internalized IL-4R α was calculated according to the following equation: $100 \times [1 - (a - c) / (b - c)]$, where *a* is the mean fluorescence intensity of the sample, *b* is the mean fluorescence intensity of the sample at 0 min and *c* is the mean fluorescence intensity of a sample stained with CyChrome-streptavidin alone.

Immunoblot analysis. For analysis of the activation of STAT6 or STAT1, CD4⁺ T cells were stimulated with recombinant IL-4 (1 U/ml) or IFN- γ (250 U/ml; Peprotech) for various times after being preactivated for 4 h with plate-bound anti-CD3 (1 μ g/ml) in the presence of anti-IL-4 (1 μ g/ml) or anti-IFN- γ (XMG1.2; 1 μ g/ml; BD Pharmingen), respectively. Cell lysates were analyzed by immunoblot with anti-STAT6 (23; BD Biosciences), antibody to STAT6 phosphorylated at Tyr641 (9361; Cell Signaling), anti-STAT1 (9172; Cell Signaling) and antibody to STAT1 phosphorylated at Tyr701 (9170; Cell Signaling). For analysis of the degradation of IL-4R α , CD4⁺ T cells were stimulated with immobilized anti-CD3 (1 μ g/ml) in the presence of anti-IL-4 (1 μ g/ml). After 2 h, cycloheximide (50 μ g/ml; Sigma) was added to the cultures and cell lysates were prepared at various times for immunoblot analysis with anti-IL-4R α (S-20) and anti-actin (I-19; both from Santa Cruz). In some

experiments, inhibitors such as bafilomycin A1 (100 nM; Wako Pure Chemical Industries), MG132 (10 μ M; Peptide Institute) or taxol (10 μ M) were added to the culture at 30 min before the addition of cycloheximide. TCR-mediated phosphorylation of stathmin was analyzed with anti-stathmin (O0138; Sigma) and polyclonal rabbit antiserum to stathmin phosphorylated at Ser16 (ref. 19).

Immunofluorescence microscopy. CD4⁺ T cells were stimulated for 2 h at 37 °C with plate-bound anti-CD3 (1 μ g/ml) in the presence of anti-IL-4 (1 μ g/ml) with the addition of bafilomycin A1 (100 nM) during the final 30 min of the culture. In some experiments, cells were pulsed for 60 min at 37 °C with Alexa Fluor 546–transferrin (50 μ g/ml; Molecular Probes) for visualization of transferrin localization. After being stimulated, cells were stained on ice with biotinylated anti-IL-4R α (mIL4R-M1) followed by Alexa Fluor 488–streptavidin (Molecular Probes). Cells were then transferred into poly-D-lysine-coated glass-bottomed culture dishes (MatTek), were incubated for 30 min at 37 °C in the presence of bafilomycin A1 (100 nM) and were fixed with 4% (wt/vol) paraformaldehyde. Fixed cells were made permeable for 5 min with 0.1% (vol/vol) Triton-X-100 and were incubated with anti-LAMP-1 (1D4B) or anti-transferrin receptor (C2; both from BD Biosciences) followed by Alexa Fluor 546–conjugated anti-rat IgG (Molecular Probes). In some experiments, CD4⁺ T cells were transfected by electroporation with plasmid encoding V5-tagged wild-type Rac or T17N-Rac with the Mouse T Cell Nucleofector kit (Amaxa Biosystems) at 3 h before stimulation. The DeltaVision Restoration Microscopy system (Applied Precision) with a 60 $\times$ objective attached to a cooled charge-coupled device camera was used for microscopy. Images were acquired with the DeltaVision SoftWorx Resolve 3D capture program and were collected as a stack of 0.2- μ m increments in the *z* axis. After deconvolution, images were viewed as a single section on the *z* axis. The area of IL-4R α and that of IL-4R α localized together with transferrin, the transferrin receptor or LAMP-1 was calculated in each cell with the MetaMorph imaging system (Universal Imaging) and the percentage of colocalization was determined by dividing the area of IL-4R α localized together with transferrin, the transferrin receptor or LAMP-1 by the area of IL-4R α .

Antibody treatment and adoptive transfer, enzyme-linked immunosorbent assay, intracellular cytokine staining and pulse-chase metabolic labeling. These procedures are described in the Supplementary Methods.

Statistical analysis. Standard deviation was calculated with the Microsoft Excel program. Statistical significance was determined with the one-tailed Student's *t*-test.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

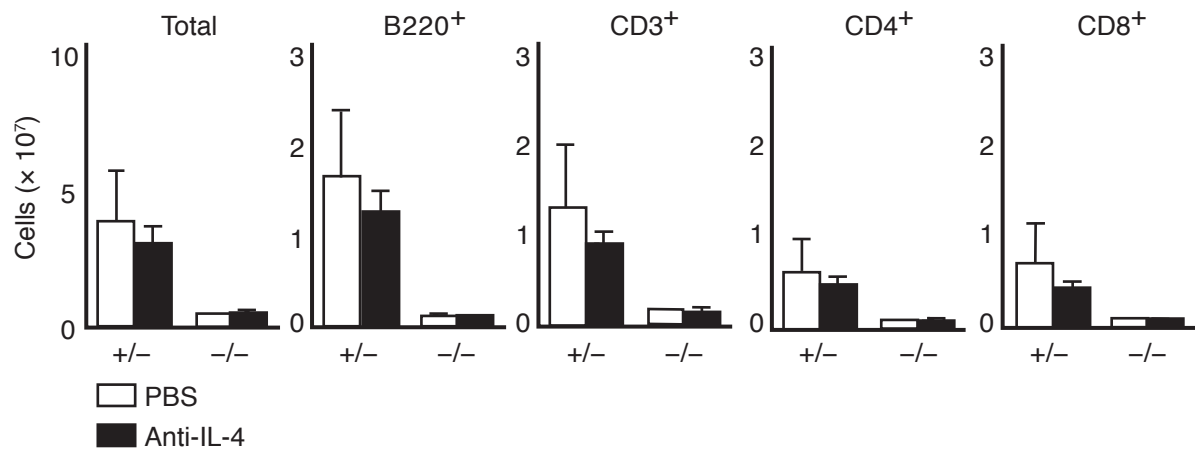
Y.T. did experiments, analyzed data and contributed to manuscript preparation; S.H. did experiments and analyzed data; K.G., Y.M., Y.K., A.N., R.T., M.K. and A.I. did experiments; S.M., K.H. and T.S. interpreted data; and Y.F. designed experiments, analyzed and interpreted data and wrote the manuscript.

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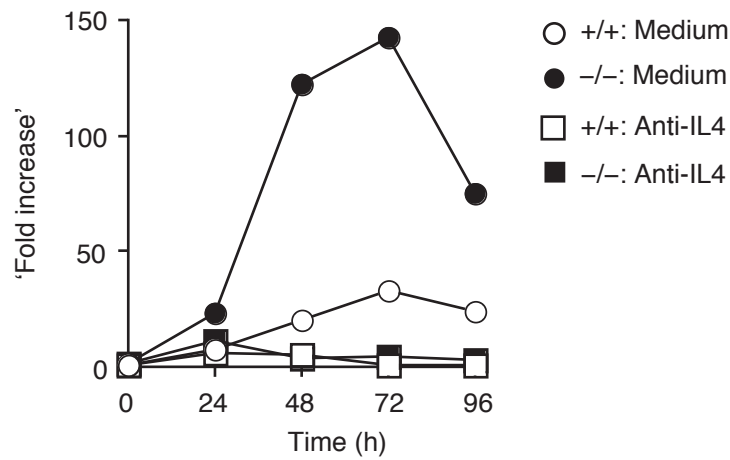
Supplementary Figure 1. The effect of anti-IL-4 treatment on lymphocyte accumulation in *Dock2*^{-/-} mice during *L. major* infection. Mice were inoculated in the right hind footpad with 1×10^7 stationary phase promastigotes. Mice were intraperitoneally treated with 1 mg of anti-IL-4 (11B11) or PBS on day 0 and day 1. At 2 weeks after inoculation, the number of total leukocytes and each subset of lymphocytes in the right popliteal LN were compared between *Dock2*^{+/-} and *Dock2*^{-/-} littermates with or without anti-IL-4 treatment. Data are from an experiment using 3 mice per group.



Supplementary Figure 2. Spontaneous development of blepharitis in *Dock2*^{-/-} BALB/c mice. A photograph of 6-month-old *Dock2*^{+/+} (left) and *Dock2*^{-/-} (right) littermates is shown.



Supplementary Figure 3. Development of blepharitis in BALB/c nude mice inoculated with *Dock2*^{-/-} CD4⁺ T cells. CD4⁺ T cells (1×10^7 cells) from 6-week-old *Dock2*^{+/+} (left) or *Dock2*^{-/-} (right) BALB/c mice were intravenously injected into 5-week-old BALB/c nude mice. A photograph was taken 5 weeks after transfer.



Supplementary Figure 4. Inhibition by anti-IL-4 of *Il4* expression in antigen-stimulated *Dock2*^{-/-} CD4⁺ T cells. *Dock2*^{+/+}2B4Tg and *Dock2*^{-/-}2B4Tg CD4⁺ T cells (3×10^5 cells per well) were stimulated with irradiated B10.BR spleen cells plus MCC88-103 peptide (0.33 μ g/ml) in the presence or absence of anti-IL-4 (10 μ g/ml) for the first 24 h. cDNA samples were prepared at the indicated time points, and *Il4* expression was analyzed with real-time PCR. 'Fold increase' was determined by comparison with unstimulated samples after normalization to the internal control *Hprt*. Data are mean $\pm$ s.d. of triplicate measurements and are representative of two independent experiments.

Supplementary Table 1. PCR primers used to detect cytokine gene expression

Gene name	Primer sequences
<i>Il4</i>	Forward; 5'-CGAAGAACACCACAGAGAGTGAGCT-3' Reverse; 5'-GACTCATTCATGGTGCAGCTTATCG-3'
<i>Il5</i>	Forward; 5'-CTCTAGTAAGCCCACTTCTA-3' Reverse; 5'-TGATACCTGAATAACATCCC-3'
<i>Il10</i>	Forward; 5'-TACCTGGTAGAAGTGATGCC-3' Reverse; 5'-CATCATGTATGCTTCTATGC-3'
<i>Il13</i>	Forward; 5'-CTCCCTCTGACCCTTAAGGAG-3' Reverse; 5'-GAAGGGGCCGTGGCGAAACAG-3'
<i>Ifng</i>	Forward; 5'-AGCGGCTGACTGAACTCAGATTGTAG-3' Reverse; 5'-GTCACAGTTTTTCAGCTGTATAGGG-3'
<i>Actb</i>	Forward; 5'-TGGAATCCTGTGGCATCCATGAAAC-3' Reverse; 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'

Supplementary Table 2. The primers used for real-time PCR analysis of *Il4* expression

Gene name	Primer sequences
<i>Il4</i>	Forward; 5'-CGAAGAACACCACAGAGAGTGAGCT-3' Reverse; 5'-GACTCATTCATGGTGCAGCTTATCG-3'
<i>Hprt</i>	Forward; 5'-CTGGTGAAAAGGACCTCTCG-3' Reverse; 5'-TGAAGTACTCATTATAGTCAAGGGCA-3'

Supplementary Methods

Mouse breeding. *Dock2*^{-/-} mice were backcrossed onto a C57BL/6 background for more than eight generations, and *Dock2*^{-/-} and *Dock2*^{+/-} littermates used for *L. major* infection were obtained by intercrossing *Dock2*^{-/-} males and *Dock2*^{+/-} females. In some experiments, *Dock2*^{-/-} C57BL/6 mice were backcrossed onto a BALB/c background for seven generations before use. To obtain *Dock2*^{-/-} *Il4ra*^{-/-} BALB/c mice, male *Il4ra*^{-/-} BALB/c mice were mated with female *Dock2*^{+/-} BALB/c mice, and the resulting *Dock2*^{+/-} *Il4ra*^{+/-} BALB/c mice were intercrossed.

Antibody treatment and adoptive transfer. *Dock2*^{-/-} and *Dock2*^{+/-} C57BL/6 mice were intraperitoneally injected with 1 mg of anti-IL-4 (11B11) or PBS on day 0 and day 1 during *L. major* infection. *Dock2*^{-/-} BALB/c mice were intraperitoneally injected with anti-CD4 (GK1.5; 1mg per week) or PBS every 6-7 days, starting at the age of 2 weeks. The loss of CD4⁺ T cells in mice treated with anti-CD4 was confirmed by flow cytometry. For adoptive transfer, CD4⁺ T cells (1 × 10⁷ cells in 200 µl PBS) from 6-week-old *Dock2*^{+/+} or *Dock2*^{-/-} BALB/c mice were intravenously injected into 5-week-old BALB/c nude mice.

ELISA. IL-4, IL-5, IL-13 and IFN-γ in supernatants were measured with a cytokine ELISA kit (Pierce). Serum samples obtained from mice were serially diluted and placed in 96-well plates coated with goat anti-mouse IgE or goat anti-mouse Ig (IgM + IgG + IgA, H+L) antibody (SouthernBiotech). After 1 h incubation, wells were washed with PBS and incubated with alkaline phosphatase-conjugated rat anti-mouse IgE, goat anti-mouse IgG1 or goat anti-mouse IgG2b (SouthernBiotech).

Intracellular cytokine staining. CD4⁺ T cells recovered from cultures were restimulated with plate-bound anti-CD3 (2C11; 1 µg/ml) and anti-CD28 (37.51; 1 µg/ml) (both from BD Pharmingen) for 8 h in the presence of brefeldin A (10 µg/ml; Sigma) during the final 2 h of the culture. Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized with PBS containing 0.5% saponin plus 0.5% BSA, and stained with FITC-anti-IFN-γ (XMG1.2) and PE-anti-IL-4 (11B11) (both from BD Pharmingen) for 30 min. Cells were then washed twice with PBS containing 0.5% saponin and 0.5% BSA, resuspended in 0.5% BSA in PBS, and analyzed on a FACSCalibur (BD Biosciences).

Pulse-chase metabolic labeling. CD4⁺ T cells were incubated in methionine-free RPMI 1640 (Sigma) with 10% dialyzed FCS (Biosciences) for 1 h, and resuspended in methionine-free RPMI containing 10% dialyzed FCS plus 100 µCi/ml [³⁵S]-methionine (Amersham Pharmacia Biotech). Cells were then stimulated with anti-CD3 (1 µg/ml) in the presence of anti-IL-4 (1 µg/ml) for 2 h at 37 °C, washed with PBS, and cultured in methionine-containing RPMI with 10% FCS. Cell lysates were prepared at 30 and 60 min after stimulation and subjected to immunoprecipitation with anti-IL-4Rα (mIL4R-M2; BD Pharmingen) for SDS-PAGE. Gels were fixed, dried, and exposed on an imaging plate (Fuji Film). The intensity of radioactive IL-4Rα bands was quantified with BAS2500 (Fuji Film).