

were used to provide indices of fishing activity. These indices were based on the annual weight of landings of demersal species by Scottish boats to ports in Orkney and Shetland, and to all Scottish ports. Analyses focused on demersal catches because these fisheries were the most likely to produce by-catch and fisheries waste.

Data analyses

Estimates of cohort variation in recruitment rate were based on the 1,281 chicks that were ringed at sites on Eynhallow between 1958 and 1980. The effort put into catching the new recruits to the breeding population varied between years, but should not bias overall estimates of cohort recruitment rate because searching effort for each cohort was integrated over 18 field seasons. The probability of observing breeding adults at the colony was estimated for colour-ringed birds that were confirmed breeders and known to be alive in that year (on the basis of positive sightings in subsequent years). Analyses were based on observations made between 1954 and 1995, using a total sample of 987 colour-ringed breeding adults.

To standardize effort across years, estimates of colony size, hatching success and fledging success excluded data from part of the colony where steep cliffs prevented easy access to nest sites. The sub-area used in this study represented roughly 64% of the whole colony, and these estimates therefore differ slightly from previously published data from all nests²⁴. A highly significant relationship between the number of occupied nests in this sub-area and the whole colony (data from 1950 to 1995: $F_{1,44} = 808$, $P < 0.0001$, $r^2 = 0.95$) suggests that its demographic development was representative.

Because disturbance has been shown previously to influence breeding performance²⁷, we also incorporated this factor into our analysis of variations in reproductive success. Annual estimates of disturbance levels were based on the number of days that researchers were present on the island during the incubation and chick-rearing period. The influence of different climate indices, fishing effort and disturbance on cohort recruitment rate and breeding performance (hatching success and fledging success) was determined by stepwise multiple regression. Initial factors included in each model were (1) colony size; (2) Northern Hemisphere temperature anomaly; (3) winter NAO; (4) annual NAO; (5) year; (6) Scottish demersal catch; (7) Orkney and Shetland demersal catch; and (8) disturbance.

The demographic consequences of observed environmentally induced variation in reproductive success and recruitment were initially analysed using an autoregressive model that estimated the influence of the following variables on subsequent colony size: (1) winter NAO index (which is predicted to influence attendance; Fig. 1); (2) lagged values for colony size; and (3) lagged values for the winter NAO index (which are predicted to influence the number of fledglings produced in previous cohorts; Fig. 2b); and (4) lagged values for the Northern Hemisphere temperatures (which are predicted to influence the subsequent recruitment rate for each cohort of fledglings; Fig. 3). The effect of lagged values for colony size was not significant, and this variable was therefore dropped from the final model. Year was included in each model to account for the strong temporal trend in the data. Values of 1–9 years were used for the time lag in the effects of colony size and climate indices on future colony size, on the basis of biologically reasonable estimates of the delay between fledging and first reproduction¹⁴.

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Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors

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Many pathological processes, including those causing allergies and autoimmune diseases, are associated with the presence of specialized subsets of T helper cells (T_H1 and T_H2) at the site of inflammation^{1–4}. The diversity of T_H1 and T_H2 function is not predetermined but depends on signals that drive the cells towards either subset^{1–4}. Histamine, released from effector cells (mast cells and basophils) during inflammatory reactions can influence immune response^{5–8}. Here we report that histamine enhances T_H1 -type responses by triggering the histamine receptor type 1 (H1R), whereas both T_H1 - and T_H2 -type responses are negatively regulated by H2R through the activation of different biochemical intracellular signals. In mice, deletion of H1R results in suppression of interferon (IFN)- γ and dominant secretion of T_H2 cytokines (interleukin (IL)-4 and IL-13). Mutant mice lacking H2R showed upregulation of both T_H1 and T_H2 cytokines. Relevant to T-cell cytokine profiles, mice lacking H1R displayed increased specific antibody response with increased immunoglobulin- ϵ (IgE) and IgG1, IgG2b and IgG3 compared with mice lacking H2R. These findings account for an important regulatory mechanism in the control of inflammatory functions through effector-cell-derived histamine.

H1R and H2R both belong to the G-protein-coupled receptor family^{9,10}; however, they trigger different intracellular events upon activation^{5,6,11}. We investigated the expression of these receptors on T_H1 and T_H2 cells bearing the CD4 antigen (CD4⁺) and whether histamine stimulation exerts differential effects on these cells. Cells bearing the histamine receptor were stained with fluorescein-labelled histamine; histamine bound significantly more strongly to T_H1 cells than to T_H2 cells (Fig. 1a). The H1R-specific antagonist tripeleennamine inhibited histamine binding in T_H1 but not in T_H2 cells, which indicates predominant H1R expression on T_H1 cells. Neither the H2R antagonist ranitidine nor the H3R antagonist clobenpropit had any effect on histamine binding to T_H1 cells (not shown). IL-3 significantly increased histamine receptor expression on T_H1 but not on T_H2 cells (Fig. 1b, c). Predominant expression of H1R on T_H1 cells and H2R on T_H2 cells is demonstrated by antibodies generated against the H1R and H2R (Fig. 1d, e). Predominant expression of H1R messenger RNA was detected in T_H1 cells compared with T_H2 cells (Fig. 1f). Furthermore, IL-3 enhanced expression of H1R mRNA in T_H1 but not in T_H2 cells. In contrast, H2R mRNA was predominantly expressed in T_H2 cells (Fig. 1g).

We then investigated the changes in histamine receptor expression during naive cell differentiation towards T_H1 and T_H2 cells. CD45RA⁺ T cells expressed little or undetectable mRNA and no detectable HRs on the surface (Fig. 1a, h, i). Stimulation of CD45RA⁺ T cells with IL-12 resulted in enhanced H1R expression, whereas IL-4 suppressed H1R mRNA expression (Fig. 1h, i). These results demonstrate that CD45RO⁺ T_H1 and T_H2 cells preferentially but not exclusively express H1R and H2R, respectively, and that H1R and H2R are regulated by cytokines present in the immune environment.

The differential expression of H1R and H2R on T_H1 and T_H2 cells should result in distinct intracellular events triggered upon receptor stimulation. Therefore, we investigated the signals induced by histamine in T_H1 and T_H2 cells. The primary signal from H1R is delivered through calcium-dependent activation of phospholipase C with generation of inositol 1,4,5-triphosphate (IP₃) and 1,2-diacylglycerol (DAG)¹¹. Because H1R is overexpressed on T_H1 cells, stimulation of T_H1 (Fig. 2a) but not T_H2 cells (Fig. 2b) with histamine resulted in significant Ca²⁺ flux. This effect was specifically blocked by tripeleennamine, demonstrating H1R-dominated signalling in T_H1 cells. Activation of H1R can produce signals

via the Gq/11 family of G proteins, which are capable of communicating with signalling mediated by T-cell receptors. The Gq-mediated signal induces translocation of the nuclear factor (NF)-AT to the nucleus, which is involved in activation of the IL-2 gene¹². Stimulation through H2R results in cAMP formation^{5,6,13}. T_H2 cells are characterized by high levels of cyclic AMP¹⁴. The cAMP-inducible transcriptional repressor protein ICER (inducible cAMP early repressor), which inhibits activity of the IL-2 promoter, has been described in T cells¹⁵. Accordingly, only T_H2 cells showed dose-dependent increases in cAMP to histamine stimulation (Fig. 2c). This effect was fully abrogated by the specific H2R antagonist ranitidine but not by tripeleennamine (Fig. 2d), indicating the H2R-dominated signalling in T_H2 cells.

We further investigated whether distinct intracellular events induced in T_H1 and T_H2 cells on stimulation with histamine influence the functions of immune effectors. We analysed the effect of histamine on proliferation and cytokine secretion of human T_H1 and T_H2 cells. Histamine enhanced anti-CD3-induced proliferation of T_H1 cells, whereas T_H2 cells were inhibited (Fig. 3a). Tripeleennamine fully blocked the effect of histamine on T_H1 cells, whereas ranitidine blocked the action of histamine on T_H2 cells. The relevance of these findings was investigated on T_H1- and T_H2-type antigen-specific responses. Peripheral blood mononuclear cells (PBMCs) from sensitized individuals were stimulated with either purified protein derivative of *Mycobacterium bovis* (PPD) or allergens (Der p1, a major house dust mite allergen, and phospholipase A₂ (PLA), a major bee venom allergen). Predominance of T_H1 has been reported in PPD-specific T-cell responses¹⁶, and histamine enhanced proliferation in PPD-stimulated cultures. In contrast, allergen-stimulated cells, which showed T_H2 predominance, from individuals allergic to house dust mites¹⁷ and bee venom¹⁸ were suppressed (Fig. 3b). We also observed that secretion of T_H1 cytokine IFN- γ was enhanced in T_H1 cells, whereas T_H2 cytokines (IL-4, IL-13) were inhibited in T_H2 cells by histamine (Fig. 3c).

To provide direct evidence linking our findings to *in vivo* immunoregulatory processes, we investigated mutant mice lacking H1R and H2R. Both types of mice developed normally and were apparently healthy and fertile^{19,20}. It has been demonstrated that deletion of either H1R or H2R results in abnormalities in the central nervous and gastrointestinal systems^{19–21}. Here we show that H1R- and H2R-deleted mice are characterized by a marked deviation of the immune response. H1R- and H2R-deleted mice showed

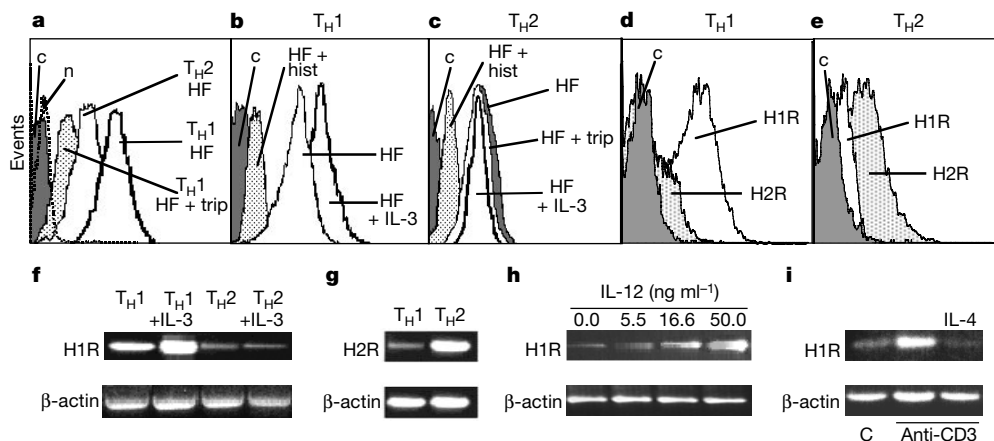


Figure 1 Histamine receptor expression on T_H1 and T_H2 cells. **a**, Inhibition of histamine fluorescein (HF) binding by H1R antagonist tripeleennamine (trip) in T_H1 but not T_H2 cells (c, control FITC; n, CD45RA⁺ T cells). **b**, Specificity of HF cell binding confirmed by inhibition with unlabelled histamine (hist). IL-3 increases H1R expression on T_H1 cells. **c**, HR expression on T_H2 cells is neither enhanced by IL-3 nor inhibited by tripeleennamine. **d**, **e**, Preferential but not exclusive H1R and H2R expression on T_H1 and T_H2 cells,

respectively, by specific antibodies. **f**, T_H1 cells show high H1R mRNA expression. IL-3 stimulation results in enhanced H1R mRNA on T_H1 but not on T_H2 cells. **g**, T_H2 cells express high H2R mRNA. **h**, IL-12 induces increased H1R mRNA in CD45RA⁺ T cells. **i**, IL-4 suppresses H1R mRNA expression in CD45RA⁺ T cells. Results are of six flow cytometric measurements and three mRNA determinations from different donors.

differences in development of T_H1 and T_H2 cells, as demonstrated by frequencies of IL-4- and IFN- γ -producing spleen T cells (Fig. 4a). H1R-deleted mice showed lower, whereas H2R-deleted mice showed higher, percentages of IFN- γ -producing T cells compared with wild-type mice ($P < 0.001$). A tendency for increased frequency of IL-4-producing cells was observed both in H1R- and H2R-deleted mice compared with wild-type mice. After histamine stimulation, consistent with the findings in humans, H1R-deleted mice demonstrated abundant suppression of IFN- γ secretion by T cells (Fig. 4b). In contrast, the T_H2 cytokines (IL-4 and IL-13) were enhanced.

These results demonstrate the importance of H1R-mediated signalling in the induction of T_H1 -type cytokine responses. It was previously reported that the signal generated by histamine through H1R augments immune responses mediated by the antigen receptor, suggesting a cross-talk between H1R and antigen-receptor-mediated signalling⁸. T-cell proliferation and ZAP-70 tyrosine kinase phosphorylation were strongly reduced in H1R-deleted mice⁸. H2R-deleted mice showed significant enhancement of both T_H1 - and T_H2 -type cytokine secretion by histamine pre-incubation (Fig. 4b). This indicates that H2R triggering provides a negative signal for T cells. Activation of H2R on T cells increases intracellular cAMP and thus downregulates both T_H1 - and T_H2 -type cytokines.

The influence of histamine and differential HR expression on T_H1 and T_H2 cells was further analysed in ovalbumin (OVA)-specific, T-cell-dependent antibody production in H1R- and H2R-deleted mice (Fig. 5). H1R-deleted mice produced high OVA-specific IgG1 and IgE compared with wild-type mice ($P < 0.05$). In contrast, H2R-deleted mice showed decreased serum levels of OVA-specific IgG3 and IgE compared with wild-type mice ($P < 0.01$). H1R-deleted mice produced significantly higher amounts of OVA-specific IgE, IgG1, IgG2b and IgG3 compared with H2R-deleted mice ($P < 0.001$). These results demonstrate the dominant role of H1R and related T_H1 response in the suppression of humoral

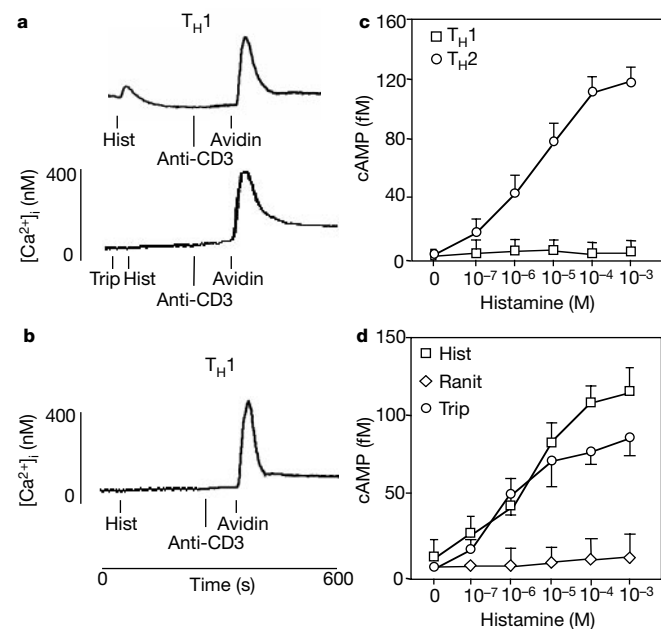


Figure 2 Histamine-induced intracellular calcium $[Ca^{2+}]_i$ flux and cAMP in T_H1 and T_H2 cells. **a**, $[Ca^{2+}]_i$ flux in histamine (hist) stimulated T_H1 cells and blockage by tripelennamine (trip). Crosslinking of the CD3 molecule was used as control. **b**, Histamine does not induce $[Ca^{2+}]_i$ flux in T_H2 cells. **c**, Histamine-induced intracellular cAMP levels in T_H2 but not in T_H1 cells. **d**, Histamine-induced cAMP in T_H2 cells was inhibited by ranitidine (ranit) but not tripelennamine. Mean $\pm$ s.d. of triplicates are shown. The data are representative of four separate experiments performed under identical conditions.

immune response. Accordingly, IgG2a, an isotype dependent on the T_H1 cytokine IFN- γ , did not show any suppression in H2R-deleted mice. H2R-deleted mice showed significantly decreased OVA-specific IgE production compared with wild-type and H1R-deleted mice. Although T cells of H2R-deleted mice secreted increased IL-4 and IL-13, OVA-specific IgE was suppressed in the presence of highly increased IFN- γ , supporting the dominant role of IFN- γ on the suppression of IgE production²².

Collectively, our results indicate that histamine can modulate T-cell-mediated immune response. Mast cells and basophils, which are considered mainly as effector cells for IgE-mediated reactions, can modulate the immune response through secreted histamine^{23,24}. IL-3 is important in the regulation of mast cell and basophil development by T cells²⁵. The increase in H1R expression on T_H1 cells by IL-3 may account for negative-feedback regulation due to an anti-allergic role of T_H1 cells. We conclude that histamine secreted from effector cells of inflammation potently

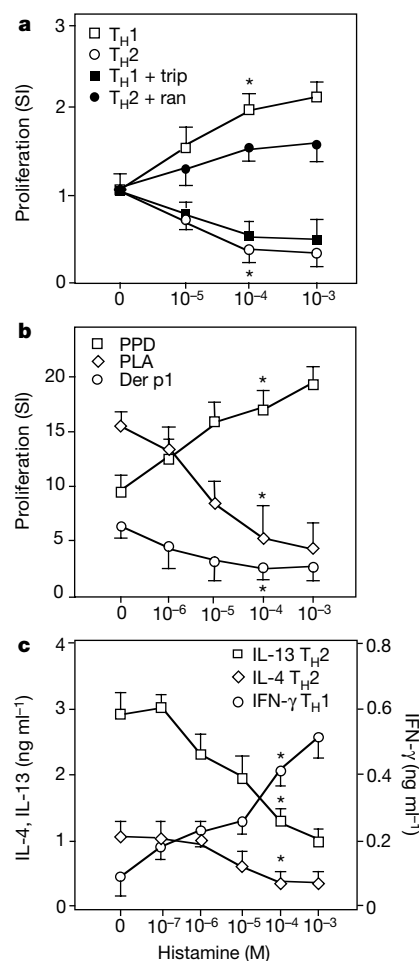


Figure 3 Regulation of T_H1 - and T_H2 -type responses by histamine. **a**, Influence of histamine on anti-CD3-induced proliferation of T_H1 and T_H2 cells; blockage by tripelennamine (trip) and ranitidine (ran). Stimulation index (SI) of 1 representing proliferation with anti-CD3 in the absence of histamine corresponds to 51,000 c.p.m. in T_H1 cells and 35,000 c.p.m. in T_H2 cells. **b**, The influence of histamine on T_H1 - and T_H2 -type proliferative responses to antigens in PBMC cultures. SI of 1 representing background proliferation of unstimulated cells was 1,600 c.p.m. in PPD, 1,100 c.p.m. in PLA and 1,300 c.p.m. in Der-p1-stimulated cultures. **c**, Effect of histamine on cytokine secretion in anti-CD3-stimulated T_H1 and T_H2 cells. Results shown are representative of six experiments performed under identical conditions. Mean $\pm$ s.d. of triplicates are shown (asterisk, $P < 0.001$, paired t -test between 10^{-4} M histamine dose and without histamine; the median effective dose ED_{50} was 5×10^6 M).

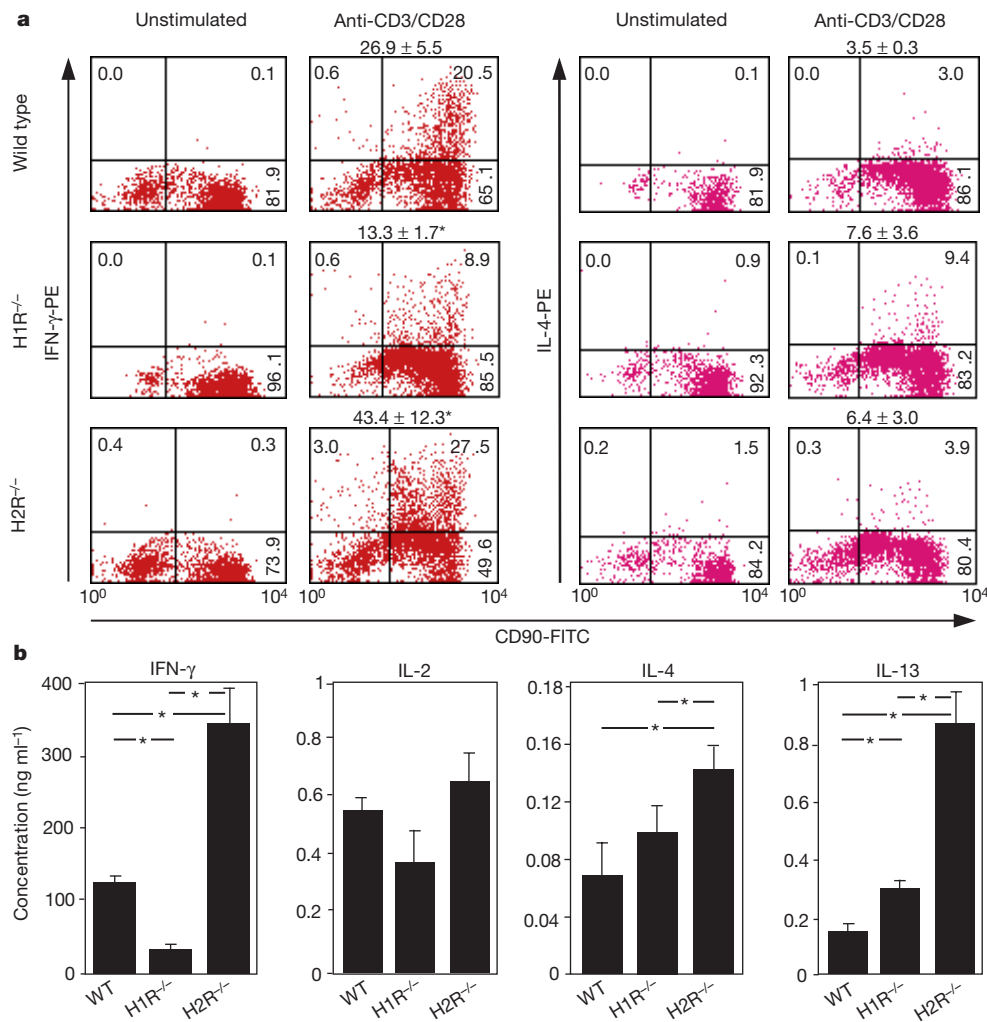
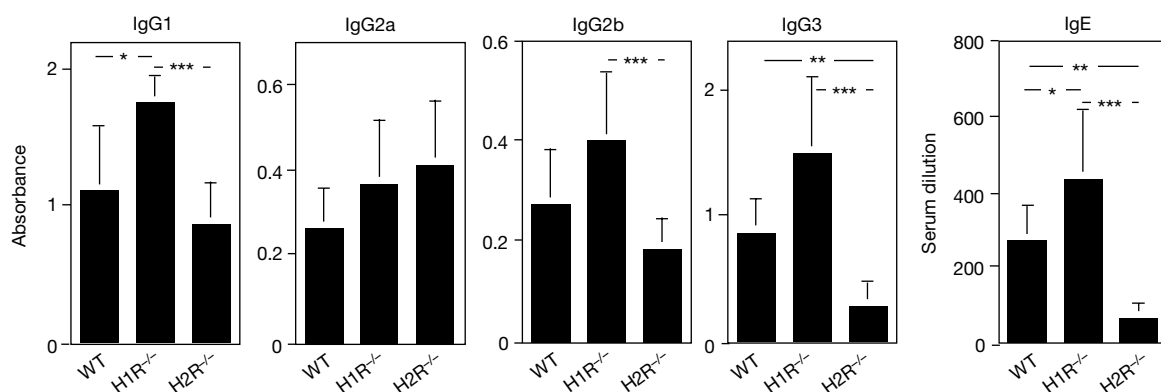


Figure 4 T-cell cytokine profiles in wild-type (WT), H1R-deleted and H2R-deleted mice. **a**, Spleen T cells were stimulated with anti-CD3 and anti-CD28 antibodies and intracytoplasmic IL-4 and IFN-γ were detected after 48 h. Cells were counter-stained with anti-CD90 antibody. The percentage of positive cells is shown on each quadrant. Data are representative of six mice in each group. Mean ± s.d. of cytokine-positive T cells is depicted over each histogram. The same changes in cytokine profiles were observed after

stimulation for 8 h and 24 h (data not shown). **b**, Histamine-pretreated spleen cells were stimulated with anti-CD3 antibodies and cytokines were detected from supernatants collected after 72 h. The results shown are mean ± s.d. of triplicate cultures from three mice of each type. The same results were obtained in six experiments (asterisk, $P < 0.001$).



100,000-fold for IgG1, 500-fold for IgG2a, 50,000-fold for IgG2b and 500-fold for IgG3. IgE was determined by passive cutaneous anaphylaxis. Results demonstrate mean ± s.d. of two experiments each with six mice. Triple asterisk, $P < 0.001$; double asterisk, $P < 0.01$; single asterisk, $P < 0.05$.

influences T_H1 and T_H2 responses as well as antibody isotypes as a regulatory loop in inflammatory reactions. □

Methods

H1R- and H2R-deleted mice

H1R- and H2R-deleted mice were generated and the mutant mice were backcrossed to C57BL/6 mice six and eight times, respectively, and maintained under pathogen-free conditions with proper aeration, light and temperature^{19,20}.

Differentiation of human T_H1 and T_H2 cells

T_H1 and T_H2 cells were obtained by differentiation of CD45RA⁺ T cells derived from cord blood, as described before³. Cytokine production by T_H1 and T_H2 cells revealed distinct profiles. T_H1 cells (10^6 per ml) secreted 5.6 ng ml⁻¹ of IFN- γ , <0.1 ng ml⁻¹ of IL-4, <0.1 ng ml⁻¹ of IL-5 and <0.2 ng ml⁻¹ of IL-13. T_H2 cells (10^6 per ml) secreted 4.6 ng ml⁻¹ of IL-4, 7.2 ng ml⁻¹ of IL-5, 9.2 ng ml⁻¹ of IL-13, and <0.1 ng ml⁻¹ of IFN- γ when given $10 \mu\text{g ml}^{-1}$ plate-bound anti-CD3 stimulation in 48 h.

Flow cytometry and mRNA expression

For flow cytometric analysis, 5×10^4 cells were stained either with fluorescein-conjugated histamine (Molecular Probes) alone or together with histamine dihydrochloride, H1R antagonist triplenamine hydrochloride, H2R antagonist ranitidine dihydrochloride or H3R antagonist clobenpropit dihydrobromide (RBI Research Biochemicals). Mouse IgG was used as a negative control. Fluorescein-conjugated histamine (10^{-3} M), histamine (10^{-3} M), triplenamine (10^{-6} M), ranitidine (6.3×10^{-3} M) and clobenpropit (1.25×10^{-7} M) were used at optimized concentrations. The concentrations of triplenamine, ranitidine and clobenpropit were 1,000 times the pA_2 value, that is, the negative logarithm of the concentration required to get 50% reduction of the efficacy of histamine¹¹. The specificity of histamine receptor staining was further demonstrated by rabbit polyclonal antibodies generated against the amino-terminal peptides of H1R (SCLEDKMCEN) and H2R (SFCLDSTACKIT).

For the semiquantitative analysis by polymerase chain reaction with reverse transcription (RT-PCR) of H1R and H2R, 2×10^6 T_H1 , T_H2 or purified CD45RA⁺ T cells were cultured in RPMI-1640 medium^{26,27} with or without 25 ng ml⁻¹ IL-3 (PharMingen), 25 ng ml⁻¹ IL-4 (Novartis), or 5.5, 16.6 or 50.0 ng ml⁻¹ IL-12 (PharMingen). Solid-phase bound anti-CD3 monoclonal antibody (Ortho Diagnostics) was used at $10 \mu\text{g ml}^{-1}$. Total RNA was isolated using Trizol reagent (Life Technologies) following the instructions of the manufacturer. One step RT-PCR was performed using the One Tube Titan RT-PCR System (Roche). Primers used to amplify specific gene products were as follows: H1R forward primer, 5'-ttt ctc tct ttt ctg tgg gtt att c-3'; H1R reverse primer, 5'-ctt ggg ggt ttg gga tgg tga ctt c-3'; H2R forward primer, 5'-ttc atc gtg tcc ttg gct atc-3'; H2R reverse primer, 5'-ctg aga ggc gtt gga cct c-3'; β -actin forward primer, 5'-atc tgc cac cac acc ttc tac aat gag ctg cg-3'; β -actin reverse primer, 5'-cgt cat act cct gct tgc tga tcc aca tct gc-3'. H1R PCR and H2R PCR products were 387 base pairs (bp) and 804 bp, respectively. β -actin was used as the housekeeping gene. Primers amplified a 838-bp PCR product. The reverse transcription of RNA was performed, 50°C for 30 min, followed by 94°C for 2 min. Conditions for PCR were 10 cycles of 94°C for 30 s, 55°C for H1R (62°C for H2R) for 30 s (annealing), 68°C for 45 s (elongation), followed by 25 cycles of 94°C for 30 s and 55°C for H1R (62°C for H2R) for 30 s (annealing) and 68°C for 45 s plus 5 s per step (elongation). The elongation time was prolonged in one cycle to 7 min (68°C). PCR products were identified by 1% agarose gel electrophoresis.

Intracellular calcium and cAMP measurements

The intracellular ionized calcium concentration ($[Ca^{2+}]_i$) was measured by a spectrofluorometric assay as previously described²⁸. T_H1 or T_H2 cells (4×10^6), labelled with fura-2/AM, were stimulated with histamine 5×10^{-3} M or with biotinylated monoclonal antibody to CD3 (Sigma) ($7 \mu\text{g ml}^{-1}$) followed by crosslinking with Avidin (Sigma) ($1 \mu\text{g ml}^{-1}$). Triplenamine and ranitidine were used at 10^{-6} M. Intracellular cAMP levels were determined using the Biotrak cAMP enzyme immunoassay system (Amersham Pharmacia) according to the manufacturer's instructions.

Cell cultures and cytokine detection

Human T_H1 and T_H2 cells were incubated for 60 min with histamine either alone or with H1R antagonist triplenamine (10^{-6} M) or with H2R antagonist ranitidine (10^{-6} M). PBMCs were incubated with increasing concentrations of histamine for 2 h. Thereafter 10% FCS was supplemented and the cells were stimulated with $10 \mu\text{g ml}^{-1}$ solid-phase bound anti-CD3 monoclonal antibodies in triplicate. Cytokine levels were measured by ELISA after 48 h^{26,27}. Incorporation of [³H]-thymidine was determined after 48 h^{26,27}. PBMCs were stimulated with either Der p1 ($10 \mu\text{g ml}^{-1}$), PLA ($10 \mu\text{g ml}^{-1}$) or PPD ($2 \mu\text{g ml}^{-1}$)²³. Incorporation of [³H]-thymidine was measured in antigen-specific cultures after 5 d.

Spleen T cells from 8-week-old wild-type, H1R-deleted and H2R-deleted mice were purified by passing them through a nylon wool column, and red blood cells were lysed with NH₄Cl lysis buffer. The cells were then suspended in RPMI-1640 medium. Intracytoplasmic IL-4 and IFN- γ were determined after 8, 24 and 48 h of stimulation with $10 \mu\text{g ml}^{-1}$ immobilized anti-CD3e and $10 \mu\text{g ml}^{-1}$ soluble anti-CD28 antibody (both from PharMingen). Monensin (2 μM , Sigma) was added 4 h before the end of cultures. Cells were fixed, permeabilized, stained with PE-labelled anti-IFN- γ and PE-labelled anti-IL-4 (ref. 27). Because CD3 was downregulated during anti-CD3 stimulation, anti-CD90

labelled with fluorescein isothiocyanate (FITC; Thy1.2, PharMingen) was used as a pan T-cell marker for counter stain. Spleen T cells were incubated with 10 mM histamine in the absence of FCS at 37°C for 2 h. After being supplemented with 10% FCS, T cells (2×10^6 per ml) were stimulated with $10 \mu\text{g ml}^{-1}$ solid-phase bound anti-CD3e antibody in triplicate in 24-well plates. Cytokine levels in the 3-d supernatants were determined by ELISA (Amersham Pharmacia).

Determination of OVA-specific antibody production

Eight- to nine-week-old wild-type, H1R-deleted and H2R-deleted mice were immunized intraperitoneally with 100 μg OVA adsorbed to aluminium hydroxide (both from Sigma) at days 0 and 21. Serum was collected at day 28 and OVA-specific IgG1, IgG2a, IgG2b and IgG3 were determined in serial dilutions in duplicates by ELISA⁸. OVA-specific IgE was determined by passive cutaneous anaphylaxis as described²⁹.

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Auxin transport inhibitors block PIN1 cycling and vesicle trafficking

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Polar transport of the phytohormone auxin mediates various processes in plant growth and development, such as apical dominance, tropisms, vascular patterning and axis formation^{1,2}. This view is based largely on the effects of polar auxin transport inhibitors. These compounds disrupt auxin efflux from the cell but their mode of action is unknown³. It is thought that polar auxin flux is caused by the asymmetric distribution of efflux carriers acting at the plasma membrane⁴. The polar localization of efflux carrier candidate PIN1 supports this model⁴. Here we show that the seemingly static localization of PIN1 results from rapid actin-dependent cycling between the plasma membrane and endosomal compartments. Auxin transport inhibitors block PIN1 cycling and inhibit trafficking of membrane proteins that are unrelated to auxin transport. Our data suggest that PIN1 cycling is of central importance for auxin transport and that auxin transport inhibitors affect efflux by generally interfering with membrane-trafficking processes. In support of our conclusion, the vesicle-trafficking inhibitor brefeldin A mimics physiological effects of auxin transport inhibitors.

Polar auxin transport inhibitors are important tools in assessing the role of polar auxin transport in plant development^{5–7}. They disrupt auxin efflux from the cell without interacting with the efflux carrier itself^{8,9}. However, their mechanism of action has remained elusive despite considerable efforts towards the biochemical characterization of binding sites^{10–12} and several screens for auxin-transport-inhibitor mutants^{13–16}. *In situ* visualization of *Arabidopsis* putative auxin efflux carriers PIN1 and PIN2 correlated polar auxin transport with their asymmetric distribution in the plasma membrane^{17,18}. This coordinated polar localization of PIN1 is not established in *gnom* mutant embryos. *GNOM* encodes a brefeldin A (BFA)-sensitive regulator of vesicle trafficking¹⁹. Consistent with this, the vesicle-trafficking inhibitor BFA affects auxin efflux from the cell^{8,9}, abolishes polar localization of PIN1 and induces its intracellular accumulation¹⁹.

BFA-induced PIN1 accumulation in two large intracellular compartments was completely and rapidly reversible (Fig. 1a–c). To determine whether this reversible BFA effect reflects protein syn-

thesis and degradation or continuous cycling between the plasma membrane and endosomal compartments, we inhibited protein synthesis by cycloheximide (CHX). Incubation of roots in 50 μ M CHX for 30 min reduced ³⁵S-labelled methionine incorporation into proteins to below 10% of the control value (data not shown). However, treatment with 50 μ M CHX for 4 h had no detectable effect on the amount of labelled PIN1 at the plasma membrane (Fig. 1d), suggesting that PIN1 protein is turned over slowly. CHX did not interfere with the reversible BFA effect as PIN1 still accumulated in endomembrane compartments (Fig. 1e) and, on withdrawal of BFA, reappeared at the plasma membrane (Fig. 1f). Thus, BFA-induced intracellular accumulation of PIN1 resulted from blocking exocytosis of a steady-state pool of PIN1 that rapidly cycles between the plasma membrane and some endosomal compartment.

In animal cells, BFA alters structure and function of endomembrane compartments, especially the Golgi apparatus, which fuses with other endomembranes^{20–22}. BFA seems to have different effects in plant cells. In BFA-treated maize root cells, Golgi stack systems progressively disappear and aggregates of vesicles accumulate in their vicinity, with Golgi markers localizing to the newly formed

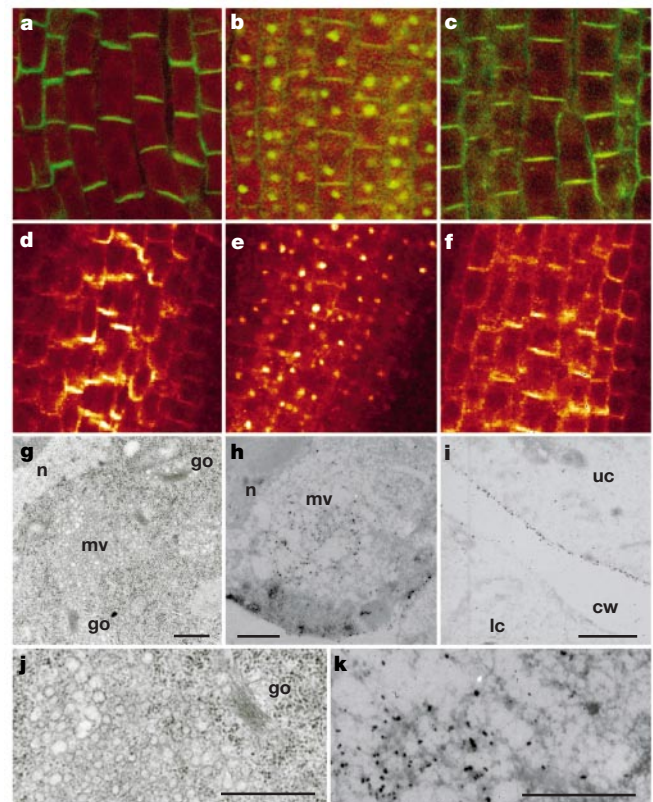


Figure 1 Reversible inhibition of PIN1 recycling by BFA treatment. **a–f**, *Arabidopsis* seedling roots treated with BFA or cycloheximide (CHX) as indicated. **a**, Two-hour buffer control. **b**, Treatment with 50 μ M BFA for 2 h. **c**, Treatment with 50 μ M BFA for 2 h followed by 2 h washing out. **d**, Treatment with 50 μ M CHX for 2 h. **e**, Pre-treatment with 50 μ M CHX for 30 min, then 50 μ M BFA and 50 μ M CHX for 90 min. **f**, Treatment with 50 μ M BFA for 90 min, then 50 μ M BFA and 50 μ M CHX for 30 min followed by 2 h washing out with 50 μ M CHX. **g–k**, Electron micrographs of root cells after treatment with 50 μ M BFA for 30 min (**g**, **h**) and untreated control (**i**). **g**, Ultrastructure. **h**, **i**, Anti-PIN1 immunogold labelling. Note aggregate of membrane vesicles (mv) in ribosome-free cytoplasm near nucleus (n) and Golgi structures (go) of treated cells (**g**, **h**). **i**, Untreated control. Note PIN1 label at the plasma membrane of the upper cell (uc) but not the lower cell (lc). cw, cell wall. **j**, Higher magnification of **g**. **k**, Higher magnification of **h**. Scale bars, 1 μ m.

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