

# 'Educated' dendritic cells act as messengers from memory to naive T helper cells

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**Ingested antigens lead to the generation of effector T cells that secrete interleukin 4 (IL-4) rather than interferon- $\gamma$  (IFN- $\gamma$ ) and are capable of influencing naive T cells in their immediate environment to do the same. Using chimeric mice generated by aggregation of two genotypically different embryos, we found that the conversion of a naive T cell occurs only if it can interact with the same antigen-presenting cell, although not necessarily the same antigen, as the effector T cell. Using a two-step culture system *in vitro*, we found that antigen-presenting dendritic cells can act as 'temporal bridges' to relay information from orally immunized memory CD4 T cells to naive CD4 T cells. The orally immunized T cells use IL-4 and IL-10 (but not CD40 ligand) to 'educate' dendritic cells, which in turn induce naive T cells to produce the same cytokines as those produced by the orally immunized memory T cells.**

At times, the immune response to orally administered antigens is called 'oral vaccination' because the ensuing response protects against a pathogen such as poliovirus<sup>1</sup>. At other times it is called 'oral tolerance' because the ensuing response ameliorates an experimentally induced autoimmune disease<sup>2</sup>. Although the names are different, the responses seem similar, with local production of immunoglobulin A (IgA)<sup>3</sup> and a systemic accumulation of T cell populations, specific for the 'fed antigen' (called 'fed T cells' here), that do not proliferate profusely or produce copious amounts of interferon- $\gamma$  (IFN- $\gamma$ ) but instead produce interleukin 4 (IL-4), transforming growth factor- $\beta$  (TGF- $\beta$ ) and IL-10 (refs. 4,5). The systemic T cells that arise after feeding have been called 'T helper type 3' (T<sub>H</sub>3), because they drive the production of IgA<sup>6</sup>, or 'T regulatory 1' (T<sub>R</sub>1), because they have a strong suppressive effect on the proliferation and IFN- $\gamma$  production of naive T cells<sup>7</sup>. The suppressive effect can be shown by adoptive transfer of T cells from antigen-fed mice into naive recipients followed by immunization with the fed antigen<sup>4</sup>, and it can also be seen in the original antigen-fed mice as a 'bystander effect' on responses to newly encountered antigens. For example, T cells from mice immunized subcutaneously with a pair of antigens in complete Freund's adjuvant (CFA) usually respond by proliferating and secreting IFN- $\gamma$ , but these responses are suppressed in an animal that has previously been fed one of the two antigens. The naive T cells are not totally suppressed, however. They simply switch to the production of the IgA-promoting cytokines, IL-4, IL-10 and TGF- $\beta$ <sup>4,7,8</sup>, an appropriate response for antigens encountered in the gut.

The bystander effect occurs only if the challenge inoculum containing the new antigen also contains the originally fed antigen<sup>9,10</sup>. This indicates that T<sub>H</sub>3 or T<sub>R</sub>1 cells specific for the 'fed' antigen can affect only naive T cells that are in close proximity. As mouse T cells do not express major histocompatibility complex (MHC) class II molecules (I-A and I-E) and

therefore cannot interact directly with each other in an antigen-specific way, the requirement for proximity has led to the suggestion<sup>11</sup> that the memory T cells from antigen-fed mice may influence the neighboring naive T cells by secreting factors such as TGF- $\beta$ , IL-4 or IL-10. Although this has some support from *in vitro* studies with rats in which fed T cells influenced naive T cells even when the two were separated by a membrane<sup>10</sup>, we did not find the same result with mouse T cells. We turned instead to the alternative possibility that memory T cells specific for a fed antigen might act by 'educating' an antigen-presenting cell (APC) in a way similar to the mechanism by which a T helper lends assistance to a T killer cell<sup>12–14</sup>. A T cell specific for a fed antigen might 'educate' the DC presenting that antigen to give a particular set of signals to a naive T cell specific for another antigen.

## RESULTS

### The experimental model

To study the mechanism by which memory T cells from an orally immunized animal might have a bystander effect on naive T cells, we set up an experimental system that uses two pure T cell populations from T cell receptor (TCR)-transgenic mice with known specificities for different antigen-MHC complexes. 5CC7 T cells from B10.A mice are of the H-2<sup>a</sup> haplotype and are specific for pigeon cytochrome c (PCC) in complex with I-E<sup>a</sup> or I-E<sup>k</sup> (' $\alpha$ PCC' T cells), and DO11.10 T cells from B10.D2 mice are of the H-2<sup>d</sup> haplotype and react with ovalbumin (OVA) in complex with I-A<sup>d</sup> (' $\alpha$ OVA' T cells). To generate pure monoclonal T cell populations, and to preclude any influence by antibodies, we bred the TCR transgenes and the appropriate MHC alleles into mice negative for recombination activating gene 2 (*Rag2*). In initial experiments, we found that the pure populations of 5CC7 and DO11.10 T cells did not cross-react with each other's antigens or react in an alloreactive way

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with each other's APCs (Supplementary Fig. 1 online). As expected, when we adoptively transferred the  $\alpha$ PCC T cells into B10.A  $Rag2^{-/-}$  mice ( $A^{-}$  mice; Fig. 1a) and the  $\alpha$ OVA T cells into B10.D2  $Rag2^{-/-}$  mice ( $D^{-}$  mice; Fig. 1b) and subcutaneously immunized them with PCC or OVA in CFA, respectively, they made strong  $T_H1$  responses, showing good T cell proliferation (Fig. 1a,b, left) and IFN- $\gamma$  production (Fig. 1a,b, right). However, when the T cell-reconstituted recipient mice were first fed the appropriate antigen and then subcutaneously immunized, the T cells acted differently. They proliferated less (Fig. 1a,b, left), made much less IFN- $\gamma$  and instead produced IL-4 (Fig. 1a,b, right). Thus, the direct response to oral antigen in this idealized adoptive transfer system mimicked that of normal mice.

To determine whether the bystander effect can also be demonstrated in this system, we transferred a mixture of the two naive transgenic T cell populations into ( $A^{-} \times D^{-}$ )F<sub>1</sub> recipients (which express both MHC haplotypes), fed the mice OVA or PCC, then immunized the mice subcutaneously with a mixture of OVA and PCC in CFA and monitored their responses (Fig. 1c,d). In control recipients that had been fed water without either of the two antigens, immunization with a mixture of OVA and PCC resulted in a classic  $T_H1$  response to both antigens, in which 22% of the  $\alpha$ PCC cells and almost 30% of the  $\alpha$ OVA T cells secreted IFN- $\gamma$  and fewer than 0.5% secreted IL-4. In antigen-fed recipients, however, the responses to both antigens switched. After immunization with the mixture of antigens, there were 10–20 times more IL-4-producing T cells and one tenth the number of IFN- $\gamma$ -producing T cells compared with the responses of unfed mice. A composite of 17 recipient mice from two independent experiments confirmed that the IL-4 response to both antigens predominated in mice that had been fed either PCC or OVA, whereas the IFN- $\gamma$  response predominated in mice that had not been fed antigen (Fig. 1d). Thus, like heterogeneous T cell

populations tested in the past, both of the pure TCR-transgenic T cell populations, when confronted with antigen given orally, generated an IL-4-dominated response that affected not only the memory T cells for the original fed antigen but also the naive T cells for the new antigen given subcutaneously in CFA.

### The function of APCs in the bystander effect *in vivo*

To determine if this bystander effect occurs by direct communication between T cells or if APCs are involved, we began by creating chimeric recipient mice that contained two types of APCs, each able to present to only one of the responding T cell populations. For this, we generated allophenic embryo aggregation chimeras by culturing eight-celled embryos of  $D^{-}$  mice (which are H-2<sup>d</sup> and present to  $\alpha$ OVA T cells) together with eight-celled embryos of  $A^{-}$  mice (which are H-2<sup>a</sup> and present to  $\alpha$ PCC T cells). After 24 h, when the cocultured embryos had formed single blastocysts, we implanted them into pseudopregnant surrogate mothers and allowed them to complete development. In such chimeras, most bodily cells do not fuse (with the exception of a few particular cell types, such as striated muscle cells<sup>15</sup>), and thus the  $A^{-}$  +  $D^{-}$  chimeric mice contained two separate populations of APCs (A and D), each able to present to one of the two types of T cells, but no APCs able to present to both (Fig. 2a). As controls, we also made pseudochimeric mice by aggregating two ( $A^{-} \times D^{-}$ )F<sub>1</sub> embryos in which the APCs would be able to present to both types of T cells. When the mice reached adulthood, we reconstituted the chimeric mice with the two types of T cells and fed them OVA or PCC to study the bystander effect. To see if the two T cell populations and their APCs were well mixed in the chimeric recipients or whether they might be separately compartmentalized, we did double-label immunohistochemistry of lymph nodes before and after immunization and found that the two different APCs and T cells were indeed in close proximity to each other (Fig. 2b). If fed memory T cells influence naive T cells directly through secreted cytokines, then close proximity between the T cells should suffice to allow the interaction, and the bystander effect should therefore occur in both ( $A^{-}$  +  $D^{-}$ ) and ( $F_1$  +  $F_1$ ) recipients. If, however, the fed memory T cells influence naive T cells indirectly by educating an APC, then the bystander effect should occur only in the ( $F_1$  +  $F_1$ ) mice and not in the ( $A^{-}$  +  $D^{-}$ ) chimeras, in which the individual APCs can present to only one of the two T cell populations. We assessed the responses by *in vitro* cytokine production (as in Fig. 1) and by *in vivo*

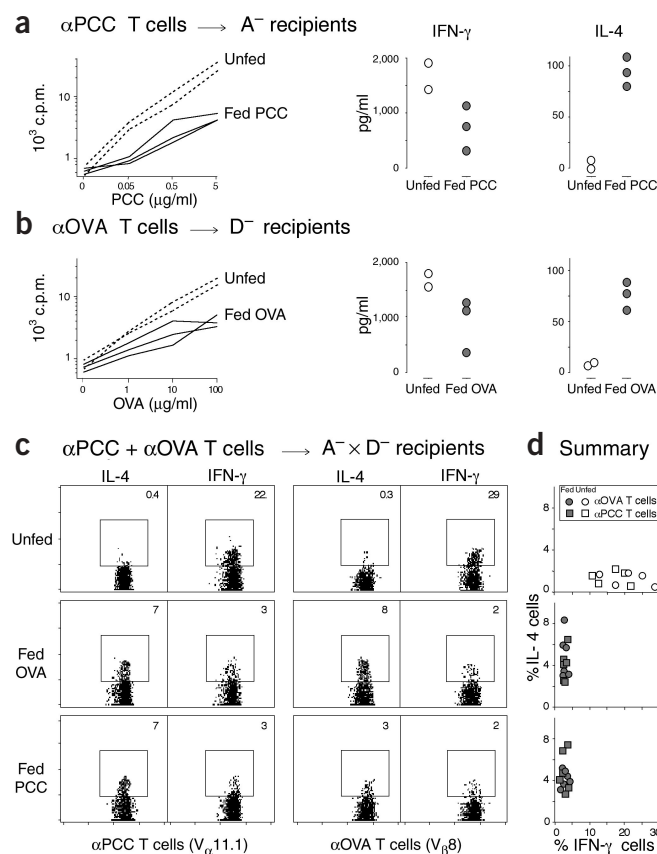


**Figure 1** TCR-transgenic T cells mediate the bystander effect. (a,b) Adoptive transfer of single T cell populations to test the effect of feeding antigen.  $A^{-}$  mice (a) and  $D^{-}$  mice (b) were reconstituted with  $\alpha$ PCC or  $\alpha$ OVA T cells, respectively. From 2 to 16 d later, the mice were fed PCC or OVA, respectively, in their drinking water, or were given plain water (Unfed). On day 18, all mice were immunized with their respective antigens. On day 27, draining lymph node cells were collected and cultured with graded concentrations of PCC or OVA and were tested for proliferation (left) and cytokine production (right). Each line (proliferation) and each symbol (cytokines) represents one mouse. Data represent 10 mice of a total of 31 tested. (c) Adoptive transfer of mixed cell populations to test the bystander effect. ( $A^{-} \times D^{-}$ )F<sub>1</sub> mice were reconstituted with  $\alpha$ PCC and  $\alpha$ OVA T cells, then were fed OVA or PCC or were given plain water (Unfed), as described in a,b, and were immunized with OVA plus PCC emulsified together in CFA on day 18. On day 27, draining lymph node cells were collected and the numbers of cytokine-secreting cells were measured by capture assay. The  $\alpha$ PCC and  $\alpha$ OVA T cells were visualized with mAbs specific for V $\alpha$ 11.1 or V $\beta$ 8, respectively. Numbers in the right upper corners represent the percentage of the gated cells that were positive for the respective cytokine. (d) Cytokine-producing cells in 17 recipient mice from two experiments. Each symbol represents a single mouse, and its placement represents the percent of T cells that were positive for production of IFN- $\gamma$  or IL-4 in the draining lymph nodes.

delayed-type hypersensitivity (DTH), measured as the extent of footpad swelling after an intradermal challenge with the test antigens.

As in normal mice, the T cells in unfed chimeras of both types made strong IFN- $\gamma$  (Fig. 2c) and DTH (Fig. 2d) responses when immunized subcutaneously with a mixture of OVA and PCC in CFA. Thus, in mice not pre-fed antigen, the peripheral responses of both sets of T cells were normal  $T_H1$  responses. In antigen-fed recipients, as expected, the direct responses to oral antigen switched to  $T_H2(3)$ -like responses:  $\alpha$ PCC T cells produced IL-4 and no DTH responses in PCC-fed mice, and  $\alpha$ OVA T cells produced similar responses in OVA-fed mice. Thus the direct responses to subcutaneous and oral antigen seemed normal in both the ( $F_1 + F_1$ ) and the ( $A^- + D^-$ ) reconstituted recipients. In contrast, however, we found the bystander effect only in the ( $F_1 + F_1$ ) and not in the

( $A^- + D^-$ ) chimeras. In OVA-fed ( $F_1 + F_1$ ) chimeras, in which the APCs can present to both populations of T cells, the naive  $\alpha$ PCC T cells responded to immunization with a mixture of antigens by producing IL-4, and in PCC-fed ( $F_1 + F_1$ ) mice, the naive  $\alpha$ OVA T cells responded similarly (Fig. 2c,d). In the ( $A^- + D^-$ ) chimeras, however, each T cell population acted as though the other were not there. In OVA-fed mice, the  $\alpha$ PCC T cells produced strong IFN- $\gamma$  and DTH responses, and in PCC-fed mice, the  $\alpha$ OVA T cells produced similar  $T_H1$ -like responses.

The absence of the bystander effect in the ( $A^- + D^-$ ) chimeras might have been due to the lower frequencies of each APC type, as antigen dose can influence the effector class<sup>16,17</sup>. To test this, we generated a new set of chimeric recipients, using [ $D^- + (A^- \times D^-)F_1$ ] embryos, in which 100% of the APCs (both the D and the  $F_1$  APCs)

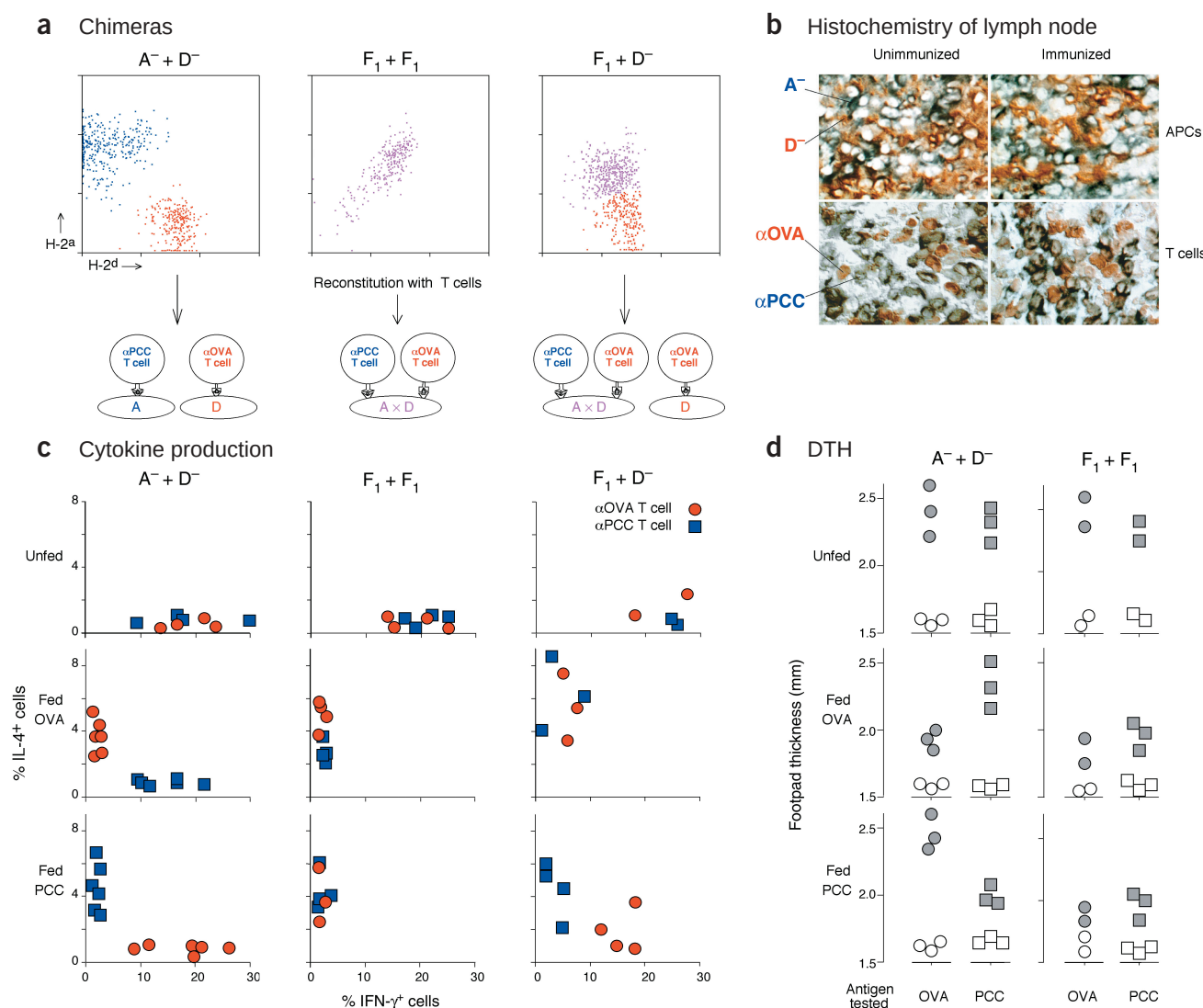


**Figure 2** The bystander effect does not occur in chimeric mice that carry a mixture of APCs. **(a)** Aggregation chimeras were generated by aggregation of  $A^-$  with  $D^-$  embryos and also ( $A^- \times D^-$ ) $F_1$  embryos with each other. Blood from each resulting mouse was analyzed by flow cytometry with antibodies to H-2<sup>a</sup> and H-2<sup>d</sup>. Mice with balanced numbers of each haplotype were then reconstituted with  $\alpha$ PCC and  $\alpha$ OVA T cells ( $\alpha$ PCC T and  $\alpha$ OVA T). **(b–d)** Reconstituted mice were fed, immunized and tested as described in Figure 1. **(b)** Inguinal lymph nodes were collected before or after immunization, cryosectioned, stained with biotinylated antibodies to V $\alpha$ 11.1 ( $\alpha$ PCC), I-E<sup>k</sup> (B10.A), V $\beta$ 8 ( $\alpha$ OVA) or I-A<sup>d</sup> (B10.D2) and incubated with avidin-biotin complex–horseradish peroxidase. Color was generated by final staining with 3,3'-diaminobenzidine (brown for  $\alpha$ OVA T cells and  $D^-$  APCs) and NiCl<sub>2</sub> (black for  $\alpha$ PCC T cells and  $A^-$  APCs). **(c)** Each symbol represents the percent T cells from the popliteal nodes of a single mouse that were positive for production of IFN- $\gamma$  or IL-4. **(d)** Seven days after immunization, an intradermal injection of either OVA or PCC was given to the left hind footpad and swelling was measured at 48 h. Each mouse is represented by two symbols: filled, injected left foot; open, uninjected right foot.

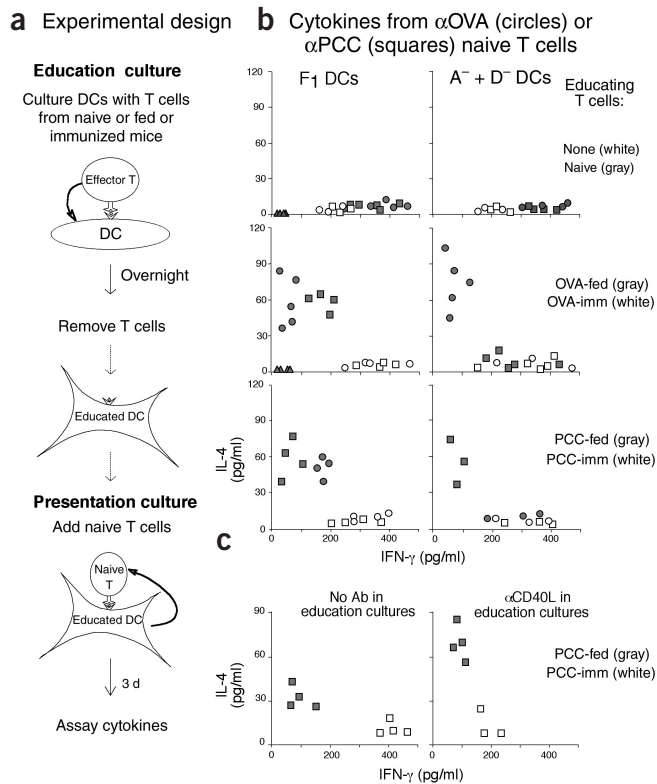


**Figure 3** Educating DCs with T cells. **(a)** Experimental design. For education cultures, CD11c-enriched splenic DCs from (A<sup>-</sup> × D<sup>-</sup>)F<sub>1</sub> mice or a 50:50 mixture of splenic DCs from A<sup>-</sup> and D<sup>-</sup> mice were cultured overnight in the presence of 10  $\mu$ g/ml PCC and 100  $\mu$ g/ml OVA either alone or with  $\alpha$ PCC or  $\alpha$ OVA T cells from spleens of mice that had been fed water (naive T cells) or PCC or OVA ('fed' T cells), or with T cells from the draining popliteal lymph nodes of mice that had been immunized with PCC or OVA in CFA ('imm' T cells). For presentation cultures, cells from the education culture were treated with anti-T cell plus complement to remove the T cells, and the remaining DCs were cultured in the presence of 10  $\mu$ g/ml of PCC and 100  $\mu$ g/ml of OVA either alone (gray triangles) or with new naive  $\alpha$ PCC (squares) or  $\alpha$ OVA (circles) T cells. Cytokines in the supernatants were measured on day 3. **(b)** Cytokines made by naive  $\alpha$ PCC (circles) or  $\alpha$ OVA (squares) T cells stimulated by DCs educated in various ways. **(c)** Effect of treatment with anti-CD40L ( $\alpha$ CD40L) on the education culture. Ab, antibody.

plus complement (**Supplementary Fig. 2** online) and used the educated DCs in step two (the 'presentation' culture) as APCs for a new set of naive  $\alpha$ PCC or  $\alpha$ OVA T cells, along with more of the antigen mixture, and assessed the cytokine response of the new T cells (**Fig. 3b**). The bystander effect seemed to have similar characteristics *in vitro* and *in vivo*. Whereas (A<sup>-</sup> × D<sup>-</sup>)F<sub>1</sub> DCs that had been cultured alone induced weak IFN- $\gamma$  responses, DCs that had been educated overnight by fed memory T cells subsequently elicited IL-4, and DCs that had been educated overnight by naive or subcutaneously immunized T cells subsequently offered stronger IFN- $\gamma$ -inducing signals (**Fig. 3b**, left). The educated IL-4-inducing DCs instructed both naive  $\alpha$ PCC and  $\alpha$ OVA T cells to produce IL-4, regardless of the specificity of the educating T effector cell. Thus if an APC displays more than one antigen, education by a T cell specific for one of the antigens can influence the subsequent presentation to a naive T cell specific for the second.

To begin to decipher the mechanism by which T helper cells from fed mice educate DCs to become IL-4 inducers, we asked if cell contact was obligatory and whether soluble factors are involved. Having found that T cells from antigen-fed mice could not educate APCs when the two were separated by distance and a membrane (**Supplementary Fig. 3** online), we sought to determine if cell contact was an absolute necessity or whether closer proximity might allow for bystander education. We repeated the two-step education and presentation study using a mixture of (A<sup>-</sup> + D<sup>-</sup>) APCs (**Fig. 3b**, right) and found that the education process was limited to the DCs that had been directly in contact with the effector T cells. When the mixture of DCs was educated by T cells from OVA-fed mice (which interact with DCs of the D<sup>-</sup> type), only the D<sup>-</sup> DCs became IL-4 inducers, and when educated by fed  $\alpha$ PCC cells (which interact with DCs of the A<sup>-</sup> type), only the A<sup>-</sup> DCs became IL-4 inducers. In both cases, the neighboring DCs in the mixture, which had not had an opportunity to interact with a T cell during the education culture, gave IFN- $\gamma$ -inducing signals to new naive T cells in the presentation culture. Thus, under *in vitro* conditions, as *in vivo*, T cells can educate only those DCs with which they come in direct contact. If there are soluble factors involved, they must act at extremely short range.

Because CD40 and its ligand (CD40L) are involved in interactions between immunized T helper cells and DCs (for example, to 'license' them to activate cytotoxic T lymphocytes (CTLs)<sup>12–14</sup>) and B cells (to induce antibody<sup>18</sup>), we tested whether CD40L interactions might also be important in DC education by fed T helper cells. We added monoclonal antibody specific for CD40L to the overnight education cultures and measured the cytokines subsequently elicited in the 'presentation' cultures by the educated DCs. As expected, this treatment decreased the IFN- $\gamma$ -enhancing educational effect of conventionally primed T helper cells (**Fig. 3c**). To our surprise, however, the treatment actually

would express H-2<sup>d</sup> and only a fraction (the F<sub>1</sub> APCs) would express H-2<sup>a</sup> (**Fig. 2a**, right). We chose chimeras in which the frequencies of F<sub>1</sub> APCs were 20–30% of the total, reconstituted them with both  $\alpha$ PCC and  $\alpha$ OVA T cells, fed them either PCC or OVA and immunized them with a mixture of PCC and OVA in CFA. Even with this low number of F<sub>1</sub> APCs, the bystander effect was intact. OVA-fed (D<sup>-</sup> + F<sub>1</sub>) mice responded essentially like (F<sub>1</sub> + F<sub>1</sub>) mice (**Fig. 2c**), showing that the OVA-fed T cells were able to affect the activity of the  $\alpha$ PCC T cells through the F<sub>1</sub> APCs they have in common. In contrast, responses of PCC-fed (D<sup>-</sup> + F<sub>1</sub>) mice tended to be between the extreme T<sub>H</sub>1 or T<sub>H</sub>2 profiles, with intermediate frequencies of cytokine-producing cells. Our interpretation is that the individual naive  $\alpha$ OVA T cell that finds an F<sub>1</sub> APC (which it shares with the fed  $\alpha$ PCC T cells) tends to make IL-4, whereas the naive  $\alpha$ OVA T cell that finds a D<sup>-</sup> APC (which is not affected by fed  $\alpha$ PCC T cells) tends to make IFN- $\gamma$ . These results suggest that the two types of response are independent, determined by the 'conversation' that occurs between each individual naive T cell and its APC.

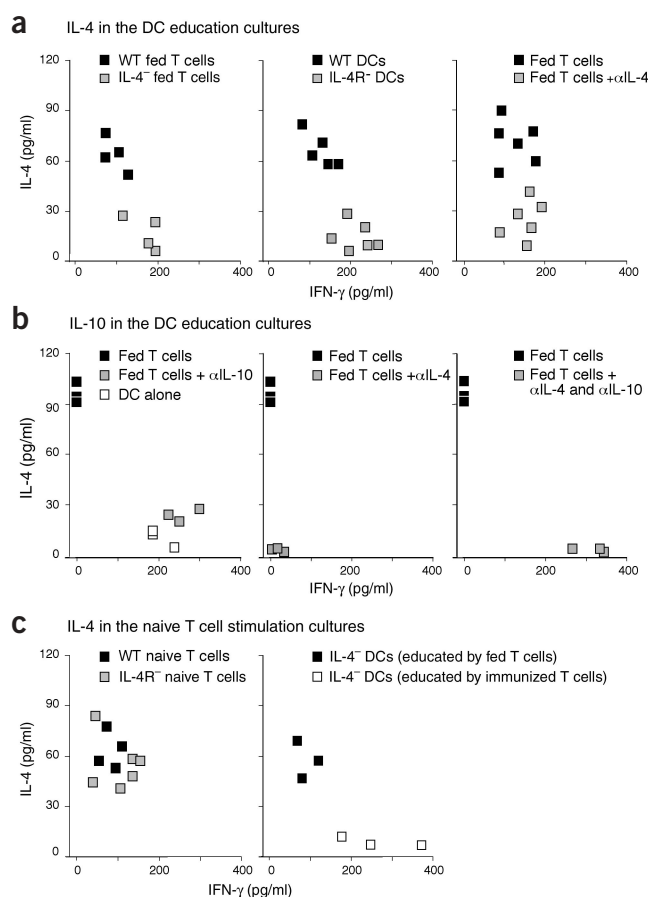
### The education of a dendritic cell

The results from the embryo fusion chimeras left two possibilities. Either the fed memory T cells act directly on naive T cells by secreting cytokines that can function only across extremely short distances, or they act indirectly by inducing changes in the APC that in turn induce naive T cells to switch their effector class. To discriminate between these two possibilities, we set up a two-step *in vitro* culture system in which we could dissociate the T cell–APC interaction from the T cell–T cell interaction (**Fig. 3a**). In step one (the 'education' culture), we cultured freshly isolated splenic dendritic cells (DCs) overnight with a mixture of PCC plus OVA, either alone or together with memory T cells from mice that had been either subcutaneously immunized or fed PCC or OVA. We then removed the T cells by lysis with antibody

**Figure 4** Molecules involved in message delivery to and from DCs. Education and presentation cultures were as described in **Figure 3a** with the exceptions noted below. Each symbol represents the amount of IL-4 and IFN- $\gamma$  produced by the pooled triplicate presentation cultures, as measured by ELISA. **(a)** Education cultures consisted of wild-type (WT) DCs with fed wild-type or IL-4-deficient (IL-4<sup>-</sup>) T cells (left); wild-type or IL-4 receptor-deficient (IL-4R<sup>-</sup>) DCs with wild-type fed T cells (middle); and wild-type DCs with wild-type fed T cells in the presence or absence of 10  $\mu$ g/ml of antibody to IL-4 ( $\alpha$ IL-4; right). **(b)** Education cultures that consisted of DCs alone or with fed T cells in the presence or absence of antibody to IL-10 ( $\alpha$ IL-10; left) or antibody to IL-4 (middle) or antibodies to both IL-4 and IL-10 (right). **(c)** Presentation cultures consisted of DCs (which had been educated by fed T cells) plus naive wild-type or naive IL-4 receptor-deficient T cells (left); or IL-4-deficient DCs (which had been educated by fed or immunized T cells) plus naive wild-type T cells (right).

increased the IL-4-enhancing effect of fed T helper cells (**Fig. 3c**). Thus, unlike the education of an antibody-secreting B cell, or an IFN- $\gamma$ - and CTL-inducing DC, the education of an IL-4-inducing DC does not seem to require CD40L interactions. If anything, blocking CD40L increased the switch to IL-4, suggesting that CD40L may not be the universal DC activator that it has been thought to be<sup>19</sup> but instead may be involved in promoting only certain classes of immunity<sup>20,21</sup>.

To determine if soluble factors were involved in the education of the IL-4-inducing DC, we used a series of gene-targeted deficient mice as well as antibody-blocking studies. We first tested the requirement for IL-4, as there is some evidence that it can have an effect on DCs<sup>22</sup>, and found that IL-4 is indeed involved in the education of an IL-4-inducing DC (**Fig. 4a**). If the educating T cells were unable to produce IL-4 (IL-4-deficient T cells from antigen-fed mice), or if the naive DC were deficient for the IL-4 receptor (IL-4 receptor-deficient DCs) or if the activity of IL-4 was blocked in the education culture by monoclonal antibody specific for IL-4, the educated DCs no longer elicited strong IL-4 production in the presentation cultures. Thus, the fed T cell uses IL-4 to educate the DC. IL-4 cannot account for the entire effect, however, as a lack of IL-4 did not restore the DCs' ability to elicit IFN- $\gamma$ , suggesting that the educational signals for the two cytokines might be different. As the presence of IL-10 can inhibit the production of IFN- $\gamma$ <sup>23</sup> and enhance the production of IL-4 (ref. 24), we sought to determine if the T cells from antigen-fed mice might use IL-10 to suppress IFN- $\gamma$ -inducing signals. Blocking IL-10 in the education culture enhanced the DCs' ability to elicit IFN- $\gamma$  and lowered the induction of IL-4 (**Fig. 4b**, left). Blocking IL-4 lowered the induction of IL-4 but had no effect on the production of IFN- $\gamma$  (**Fig. 4b**, middle).



Blocking both cytokines had an even stronger effect, lowering to nearly zero the DCs' ability to elicit IL-4 and causing a 300-fold increase in the IFN- $\gamma$ -inducing ability of the educated DCs. Thus, the fed T cell seems to use both IL-4 and IL-10 to instruct the DC.

It has been well established, *in vivo* and *in vitro*, that IL-4 production by T cells depends on the prior presence of early IL-4 (refs. 25,26). Although it is generally thought that the early IL-4 acts on the naive T cells to influence their subsequent cytokine production profile<sup>27</sup>, our results with the education of DCs suggested that the early IL-4 might be signaling DCs rather than T cells. To test this, we used DCs deficient in IL-4 production (IL-4-deficient splenic DCs) or (in the presentation culture) naive T cells that were deficient for the IL-4 receptor.

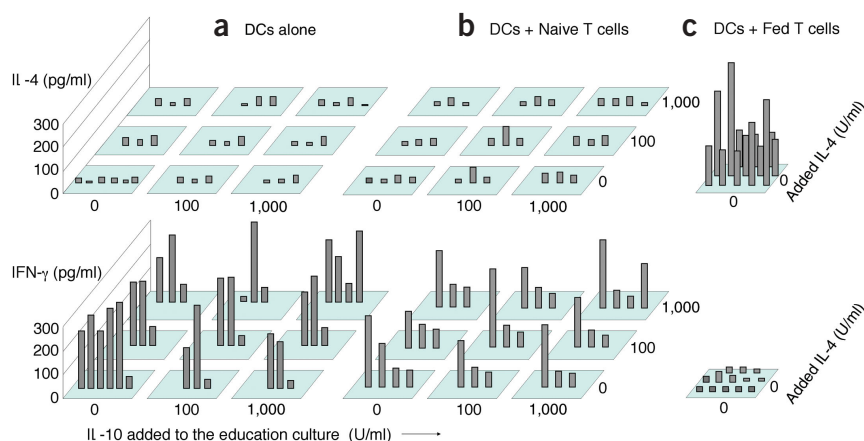


**Figure 5** Exogenously added IL-4 and IL-10, without **(a)** or with **(b)** naive T cells, does not substitute for a fed T cell **(c)** in the education culture. For education cultures, we isolated splenic DCs from B10.A-*Rag2*<sup>-/-</sup> mice, cultured them overnight with fed T cells or with various concentrations of exogenous IL-4 and IL-10 with or without naive T cells, washed them to remove exogenous cytokines and treated them with antibody to T cell plus complement to remove T cells. For presentation cultures, we then offered the DCs to naive anti-PCC T cells in the presence of 10  $\mu$ g/ml of PCC *in vitro*. Data are a composite of three independent experiments.

Neither of these mutations had any effect on the production of IL-4 by the naive T cells (Fig. 4c). As long as the DCs were able to receive an IL-4 signal from fed T cells in the education culture, there was no further need for IL-4 in the presentation culture. Although it is not always possible to extrapolate from *in vitro* results to *in vivo* reality, these data suggest that the early IL-4 *in vivo* may be required for education of the DC rather than of the naive T cell.

To determine if IL-4 and IL-10 were the only signals used by fed T cells to educate DCs, we cultured a naive population of splenic DCs with the two cytokines over a wide range of doses and found that no combination of the two was sufficient to mimic the instructions given by fed T cells (Fig. 5a). We also tried culturing the DCs with a combination of naive T cells (to supply cell surface interactions from a T cell) plus various concentrations of the two cytokines (Fig. 5b) and again found that none of these combinations was able to mimic the DC-educating ability of fed T cells (Fig. 5c). T cells generated in antigen-fed mice must therefore offer other surface interactions not offered by naive T cells or perhaps secrete another cytokine in addition to IL-4 and IL-10.

## DISCUSSION

The results presented here demonstrate that memory and/or effector T cells induced by oral administration of antigen can educate DCs (and perhaps other APCs) and in this way influence the subsequent responses of naive T cells. The education signals that result in an IL-4-inducing DC seem to pass through cell-cell interactions between the fed T cell and the DC, through the cytokines IL-4 and IL-10, and at least one other not-yet-known surface or secreted molecule. As shown before for the T cells that license DCs to activate T killers<sup>12–14</sup>, both the *in vitro* and *in vivo* data suggest that the fed T helper cell that educates the IL-4-inducing DC, and the immunized T helper cell that educates an IFN- $\gamma$ -inducing DC, need not recognize the same antigen as the naive T cell to which the DC relays the message. The *in vitro* data also suggest that the educating T cells and the naive T cells need not encounter the APC at the same time. Thus a small number of memory and/or effector T helper cells could find and educate a large number of APCs and in turn influence a large number of naive T cells.

Many other conversations between T cells, resulting in either enhancing or suppressive effects, might also occur through APC education. *In vitro*, for example, PCC-specific T<sub>H</sub>2 cell lines were able to induce naive OVA-specific T cells to make IL-4, but only if both T cells were able to interact with the same APC<sup>11</sup>. Although this could have been a T cell–T cell interaction via extremely short-range cytokines, our data suggest that it was most likely due to APC education. It was also found that CD8 T cells can decrease a DC's ability to promote T cell proliferation<sup>28</sup>. In that case, however, it was not shown whether the DCs were permanently changed or whether the CD8 T cells needed to be continuously present. Nor was it demonstrated whether the suppression of proliferation represented a global suppression or whether other T cell functions were increased. How many cases of 'suppression' have in fact actually been switches to an effector class that was not monitored? In oral 'tolerance', for example, orally immunized T cells do not proliferate profusely, do not secrete copious amounts of IFN- $\gamma$ , do not produce DTH responses (ref. 4 and data shown here) and do not cause autoimmune<sup>6</sup> or alloreactive<sup>29</sup> tissue destruction, and have therefore been called 'tolerant'. In addition, they can cause naive T cells to act in a similar way and have therefore been called 'suppressor T cells' or 'regulatory T cells'. However, although IFN- $\gamma$ , DTH and proliferation are often used as standard measures of T cell activity, they are not the complete picture of immunity. As soon as additional features of immunity are measured, it becomes

apparent that oral immunization does not induce 'tolerance'. Whether induced by the polio vaccine<sup>1</sup>, myelin basic protein<sup>5</sup> or other protein antigens (ref. 4 and data shown here), oral delivery of antigen stimulates the production of IgA (Supplementary Fig. 4 online), by generating T cells that produce IL-4, IL-10 and TGF- $\beta$  (and most likely other, as-yet-unmeasured mediators). At the same time, the T cells turn off the IFN- $\gamma$  and DTH responses that would be inappropriate or even damaging to the gut. Analyzed at the level of B cells and antibody production, this would be called a 'class switch' to IgA production. Analyzed at the level of T cells, it can be called a class switch from an IFN- $\gamma$  and/or DTH response to an IgA-promoting response or, for simplicity, a switch from T<sub>H</sub>1 to T<sub>H</sub>3 (or perhaps 'T<sub>H</sub>2.3'?), the sort of response that is appropriate for the gut environment.

Our findings that orally immunized T cells use cell surface interactions plus IL-4 and IL-10 to educate DCs and that DCs might be the target of the early IL-4 needed for further IL-4 production, together with published data showing that DC function can be influenced by local soluble factors<sup>30,31</sup>, provide a different view of the function of DCs. Are they, as previously thought<sup>32</sup>, the ultimate controllers of immunity? DCs relay messages from damaged tissues to T cells, thereby initiating responses<sup>33</sup>. They relay messages from tissues to T cells that influence the effector class of the response<sup>34–37</sup>. They relay messages from T helper cells to T killer cells<sup>12–14</sup> and (as presented here) from memory T helper cells to naive T helper cells, thereby maintaining consistency in the class of response. Rather than being viewed as 'nature's adjuvant'<sup>33</sup>, perhaps DCs should be viewed as the immune system's ultimate messengers, allowing two cells to communicate with each other across time and space barriers by engaging a third cell.

T cell education of DCs seems to be carefully targeted. The DC seems to receive its instructions from a T cell through cell surface molecules and/or soluble factors that are delivered in such a way that they do not influence neighboring DCs. The most likely scenario is that the factors are delivered across the 'immunological synapse'<sup>38</sup>. Just as neurons can act as relay stations to pass both excitatory and inhibitory information from one neuron to another<sup>39</sup>, the DC seems to be able to relay both excitatory and inhibitory information from one cell to another, in more than one situation and between several types of cells. This allows for a great deal of complexity, as information relayed by cells may be able to endure greater delays, may be able to carry greater mixtures of instructions and may be far more accurately pinpointed than information relayed by soluble factors such as hormones.

## METHODS

**Mice.** B10.A (which are H-2<sup>a</sup> and express H-2E<sup>k</sup>), B10.A Rag2<sup>−/−</sup>, B10.D2 Rag2<sup>−/−</sup> (which are H-2<sup>d</sup>), (A<sup>−</sup> × D<sup>−</sup>)F<sub>1</sub>, B10.A[transgenic]TCR-Cyt-5C7 mice (which are H-2<sup>a</sup> and specific for PCC), B10.D2[transgenic]TCR-OVA-DO11.10 mice (which are H-2<sup>d</sup> and specific for OVA), B10.A *Il4*<sup>−/−</sup> and BALB/c Rag2<sup>−/−</sup>*Il4ra*<sup>−/−</sup> doubly deficient mice were bred at the National Institutes of Health contract at Taconic. The mice were housed at the National Institute of Allergy and Infectious Diseases facility, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The studies were approved under National Institute of Allergy and Infectious Diseases Animal Care and Use Committee Animal Study Protocol LCMI-7.

**Embryo aggregation mice.** Noncompacted eight-celled morulae from (A<sup>−</sup> × A<sup>−</sup>), (D<sup>−</sup> × D<sup>−</sup>) and (A<sup>−</sup> × D<sup>−</sup>) matings were collected and aggregated in appropriate combinations essentially as described<sup>40</sup>. The aggregated embryos were cultured overnight in M16 medium (modified to contain 0.47 g NaCl per 100 ml) and were transferred into pseudopregnant (C57BL/6 × C3H/He)F<sub>1</sub> females. When adult A<sup>−</sup> + D<sup>−</sup> chimeric recipients were chosen (Fig. 2), only mice showing a balanced ratio of the two MHC types (at least 30% hematopoietic chimerism, determined by analysis of peripheral blood) were used.

**T cells.** To obtain pure naive T cell populations from 5CC7 and DO11.10 mice, we collected lymph node cells from the respective TCR-transgenic *Rag2*<sup>-/-</sup> mice, labeled them with magnetic beads coated with antibody to MHC class II (anti-MHC class II), anti-CD11b and anti-CD11c, passed the cells through Midi-MACS columns three times (Miltenyi Biotech) and collected the nonadherent effluent. We purified effector T cells, as described above from the inguinal lymph node cells draining the immunization site of immunized mice (7 d after immunization) or from the spleens of fed mice (2 d after the last feeding).

**DCs.** To obtain splenic DCs, we labeled mechanically dissociated spleen cells from various strains of *Rag2*<sup>-/-</sup> mice with anti-CD11c magnetic beads, passed the cells through Midi-MACS columns three times (Miltenyi Biotech) and collected the adherent fraction, which consisted of more than 96% CD11c<sup>+</sup> cells.

**Feeding and immunizations.** To generate fed memory and/or effector T cells, we added either OVA or PCC to the drinking water at a concentration of 0.02 mg/ml, replenishing the water every 2 d for a total of 14 d. Because mice drink about 5 ml/day, this results in ingestion of about 100 µg of OVA or PCC per day per mouse. At 2 d after the last OVA feeding, we immunized the mice subcutaneously with 50 µg OVA emulsified in CFA in a total of 50 µl, dividing the dose equally between the base of the tail and one footpad, except that for DTH experiments we immunized only at the base of the tail. Control immunized mice were immunized without feeding, at the same time and in the same way as the corresponding fed mice in the same experiment.

**Adoptive transfers.** We injected  $3 \times 10^6$   $\alpha$ OVA T cells and  $5 \times 10^6$   $\alpha$ PCC T cells into the peritoneal cavities of B10.A *Rag2*<sup>-/-</sup> (A<sup>-</sup>) or B10.D2 *Rag2*<sup>-/-</sup> (D<sup>-</sup>) mice, or into (A<sup>-</sup> × D<sup>-</sup>)F<sub>1</sub> mice, or into allophenic embryo fusion chimeras (described below) of type (A<sup>-</sup> × D<sup>-</sup>) or [(A<sup>-</sup> × D<sup>-</sup>)F<sub>1</sub> + (A<sup>-</sup> × D<sup>-</sup>)F<sub>1</sub>]. Starting 2 d after the reconstitution, we fed and immunized the recipient mice as described above.

**DTH.** We injected 50 µg OVA or PCC intradermally into one hind footpad of mice that had been immunized 9 d previously at the base of the tail, and measured the thickness of both hind footpads 48 h later.

**Cytokine assays.** For enzyme-linked immunosorbent assay (ELISA), we collected culture supernatants from antigen-stimulated cells at 48 h and measured IL-4 and IFN-γ by specific sandwich ELISA (Endogen). For capture assay, we collected draining lymph node cells (inguinal, para-aortic and the popliteal node draining the immunized footpad) 9 d after immunization, cultured the cells ( $2 \times 10^5$  cells/well) overnight in the presence of 100 µg/ml of OVA and/or 10 µg/ml of PCC and counted cytokine-secreting cells by capturing cytokines on the surface of the cells with a divalent antibody specific for both CD45 and the cytokine being assayed (Miltenyi). The 5CC7 (αPCC) and DO11.10 (αOVA) T cells were identified with fluorescein isothiocyanate-labeled monoclonal antibodies specific for V $\alpha$ 11.1 or V $\beta$ 8, respectively. Cytokine staining was done with anti-IL-4 and anti-IFN-γ phycoerythrin-labeled antibodies (Miltenyi).

**Proliferation assays.** We incubated  $5 \times 10^5$  lymph node cells in triplicate in 96-well round-bottomed microtiter plates, each well containing a total of 200 µl of culture medium (Iscove's modified Dulbecco's medium supplemented with 10% FCS, 50 U/ml of penicillin, 50 µg/ml of streptomycin sulfate, 50 µg/ml of gentamycin sulfate, 4 mM glutamine and 50 µM 2-β-mercaptoethanol) with or without 'graded' doses of OVA or PCC. The cells remained in culture for 3 d and were pulsed with [<sup>3</sup>H]thymidine for the last 8 h.

**Immunohistochemistry.** We incubated frozen thin sections (3–5 µm in thickness) of lymph nodes with biotinylated antibodies to V $\alpha$ 11.1, V $\beta$ 8, I-E<sup>k</sup> or I-A<sup>d</sup> for 1 h at 26 °C. We then washed the tissue sections in PBS and incubated them for 30 min with avidin-biotin complex linked to horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories) prepared according to the manufacturer's instructions. After a 15-minute wash, tissue sections were incubated with 0.05% (weight/volume) 3,3'-diaminobenzidine (Sigma) and 0.03% H<sub>2</sub>O<sub>2</sub> and were examined under a light microscope for brown color. For double staining, the protocol described above was repeated on the same slide, this time with 3,3'-diaminobenzidine and 0.05% NiCl<sub>2</sub>, for development of black color.

**Two-step education and presentation cultures.** For the effector T cells for step one (the education culture), 5CC7 *Rag2*<sup>-/-</sup> (αPCC) and DO11.10 *Rag2*<sup>-/-</sup> (αOVA) mice were fed 0.02 mg/ml of PCC or OVA, respectively, in the drinking water for 2 weeks. As naive and immunized controls, we used unfed mice or mice immunized with PCC or OVA in CFA. Splenic T cells from fed mice or T cells from the draining nodes of immunized mice were collected on the last day of feeding and samples were enriched by negative selection with magnetic columns (described above). For naive T cells for step two (the presentation culture), naive T cells were purified by negative selection (described above) from the spleens of unmanipulated αPCC or αOVA mice. Dendritic cells were purified by positive selection with anti-CD11c microbeads as described above from mechanically dissociated B10.A *Rag2*<sup>-/-</sup>, B10.D2 *Rag2*<sup>-/-</sup> or F<sub>1</sub> *Rag2*<sup>-/-</sup> spleens. For tests of mechanism, DCs were also isolated as described above from the spleens of B10.A-*Il4*<sup>-/-</sup> mice or of BALB/c *Rag2*<sup>-/-</sup>*Il4*<sup>-/-</sup> doubly mutant mice.

For step one cultures (education culture),  $1.5 \times 10^5$  T cells per 0.2-ml well were cultured overnight with  $4 \times 10^3$  enriched DCs in the presence of 10 µg of PCC, 100 µg of OVA or both. After overnight culture, we removed the T cells either by sorting for cells that were positive for CD11c and negative for CD2 or by lysing the T cells with three rounds of antibody (J11.10 monoclonal antibody to Thy-1) and complement (Low-Tox-M Rabbit Complement; Cedarlane Laboratories); each round was 40 min at 37 °C. In some experiments, 30 µg/ml of MR1 monoclonal antibody (or control hamster antibody) was added to the education culture to block CD40L, 10 µg/ml of 11B11 monoclonal antibody was added to block IL-4, or 4 µg/ml of AF-417-NA (R&D Systems) was added to block IL-10. In some cultures, we added various amounts of IL-4 and/or IL-10 with and without naive T cells ( $1 \times 10^5$  DCs/well). IL-4 and IL-10 (Peprotech) had activities of more than  $1 \times 10^7$  units/mg and more than  $5 \times 10^5$  units/mg, respectively. For cultures for step two (presentation culture),  $2 \times 10^3$  T cell-depleted educated DC samples were then offered to  $7 \times 10^5$  naive αPCC or αOVA T cells in 0.2-ml cultures in the presence of 10 µg/ml of PCC and 100 µg/ml of OVA. IL-4 and IFN-γ were measured in the supernatants on day 3.

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

#### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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- Kaul, D. & Ogra, P.L. Mucosal responses to parenteral and mucosal vaccines. *Dev. Biol. Stand.* **95**, 141–146 (1998).
- Inobe, J. *et al.* IL-4 is a differentiation factor for transforming growth factor-β secreting Th3 cells and oral administration of IL-4 enhances oral tolerance in experimental allergic encephalomyelitis. *Eur. J. Immunol.* **28**, 2780–2790 (1998).
- Richman, L.K., Graeff, A.S., Yarchoan, R. & Strober, W. Simultaneous induction of antigen-specific IgA helper T cells and IgG suppressor T cells in the murine Peyer's patch after protein feeding. *J. Immunol.* **126**, 2079–2083 (1981).
- Alpan, O., Rudomen, G. & Matzinger, P. The role of dendritic cells, B cells, and M cells in gut-oriented immune responses. *J. Immunol.* **166**, 4843–4852 (2001).
- Chen, Y., Kuchroo, V.J., Inobe, J., Hafler, D.A. & Weiner, H.L. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* **12**, 1327–1340 (1994).
- Weiner, H.L. Oral tolerance: immune mechanisms and treatment of autoimmune diseases. *Immunol. Today* **7**, 335–344 (1997).
- Austin, A.S. *et al.* Identification and characterization of a novel regulatory factor: IgA-inducing protein. *J. Immunol.* **171**, 1336–1342 (2003).
- Cazac, B.B. & Roes, J. TGF-β receptor controls B cell responsiveness and induction of IgA *in vivo*. *Immunity* **13**, 443–451 (2000).
- Dahlman-Hoglund, A. *et al.* Bystander suppression of the immune response to human serum albumin in rats fed ovalbumin. *Immunology* **86**, 128–133 (1995).
- Miller, A., Lider, O. & Weiner, H.L. Antigen-driven bystander suppression after oral administration of antigens. *J. Exp. Med.* **174**, 791–798 (1991).
- Schuhbauer, D.M., Mitchison, N.A. & Mueller, B. Interaction within clusters of dendritic cells and helper T cells during initial Th1/Th2 commitment. *Eur. J. Immunol.* **30**, 1255–1262 (2000).
- Ridge, J.P., Di Rosa, F. & Matzinger, P. A conditioned dendritic cell can be a temporal bridge between a CD4<sup>+</sup> T-helper and a T-killer cell. *Nature* **393**, 474–478 (1998).
- Schoenberger, S.P. *et al.* T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. *Nature* **393**, 480–483 (1998).
- Bennett, S.R. *et al.* Help for cytotoxic-T-cell responses is mediated by CD40 signaling. *Nature* **393**, 478–480 (1998).
- Gearhart, J.D. & Mintz, B. Clonal origins of somites and their muscle derivatives:

- evidence from allophenic mice. *Dev. Biol.* **29**, 27–37 (1972).
16. Parish C.R. & Liew F.Y. Immune response to chemically modified flagellin. 3. Enhanced cell-mediated immunity during high and low zone antibody tolerance to flagellin. *J. Exp. Med.* **135**, 298–311 (1972).
  17. Constant, S., Pfeiffer, C., Woodard, A., Pasqualini, T. & Bottomly, K. Extent of T cell receptor ligation can determine the functional differentiation of naive CD4<sup>+</sup> T cells. *J. Exp. Med.* **182**, 1591–1596 (1995).
  18. Lanzavecchia, A. Antigen-specific interaction between T and B cells. *Nature* **314**, 537–539 (1984).
  19. van Kooten, C. & Banchereau, J. CD40-CD40 ligand. *J. Leukoc. Biol.* **67**, 2–17 (2000).
  20. Stueber, E., Strober, W. & Neurath, M. Blocking the CD40L-CD40 interaction in vivo specifically prevents the priming of T helper 1 cells through the inhibition of interleukin 12 secretion. *J. Exp. Med.* **183**, 693–698 (1996).
  21. Sangster, M.Y. *et al.* An early CD4<sup>+</sup> T cell-dependent immunoglobulin A response to influenza infection in the absence of key cognate T-B interactions. *J. Exp. Med.* **198**, 1011–1021 (2003).
  22. Lardon, F. *et al.* Generation of dendritic cells from bone marrow progenitors using GM-CSF, TNF- $\alpha$ , and additional cytokines: antagonistic effects of IL-4 and IFN- $\gamma$  and selective involvement of TNF- $\alpha$  receptor-1. *Immunology* **91**, 553–559 (1997).
  23. Qi, H., Denning, T.L. & Soong, L. Differential induction of interleukin-10 and interleukin-12 in dendritic cells by microbial toll-like receptor activators and skewing of T-cell cytokine profiles. *Infect. Immun.* **71**, 3337–3342 (2003).
  24. Liu, L., Rich, B.E., Inobe, J., Chen, W. & Weiner, H.L. A potential pathway of Th2 development during primary immune response: IL-10 pretreated dendritic cells can prime naive CD4<sup>+</sup> T cells to secrete IL-4. *Adv. Exp. Med. Biol.* **417**, 375–381 (1997).
  25. Noben-Trauth, N., Hu-Li, J. & Paul, W.E. Conventional, naive CD4<sup>+</sup> T cells provide an initial source of IL-4 during Th2 differentiation. *J. Immunol.* **165**, 3620–3625 (2000).
  26. Noben-Trauth, N., Hu-Li, J. & Paul, W.E. IL-4 secreted from individual naive CD4<sup>+</sup> T cells acts in an autocrine manner to induce Th2 differentiation. *Eur. J. Immunol.* **32**, 1428–1433 (2002).
  27. Nelms, K., Keegan, A.D., Zamorano, J., Ryan, J.J. & Paul, W.E. The IL-4 receptor: signaling mechanisms and biologic functions. *Annu. Rev. Immunol.* **17**, 701–738 (1999).
  28. Chang, C.C. *et al.* Tolerization of dendritic cells by T<sub>S</sub> cells: the crucial role of inhibitory receptors ILT3 and ILT4. *Nat. Immunol.* **3**, 237–243 (2002).
  29. Sayegh, M.H., Khoury, S.J., Hancock, W.W., Weiner, H.L. & Carpenter, C.B. Mechanisms of oral tolerance by MHC peptides. *Ann. NY Acad. Sci.* **778**, 338–345 (1996).
  30. Hochrein, H. *et al.* Interleukin 4 is a major regulatory cytokine governing bioactive IL-12 production by mouse and human dendritic cells. *J. Exp. Med.* **192**, 823–833 (2000).
  31. Kapsenberg, M.L. *et al.* The role of accessory cell products in the regulation of T cell cytokine production. *Adv. Exp. Med. Biol.* **409**, 305–308 (1996).
  32. Kelsall, B.L., Biron, C.A., Sharma, O. & Kaye, P.M. Dendritic cells at the host-pathogen interface. *Nat. Immunol.* **8**, 699–702 (2002).
  33. Gallucci, S., Lolkema, M. & Matzinger, P. Natural adjuvants: endogenous activators of dendritic cells. *Nat. Med.* **11**, 1249–1255 (1999).
  34. Iwasaki, A. & Kelsall, B.L. Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. *J. Exp. Med.* **190**, 229–233 (1999).
  35. Everson, M.P. *et al.* Dendritic cells from Peyer's patch and spleen induce different T helper cell responses. *J. Interferon Cytokine Res.* **18**, 103–115 (1998).
  36. Taylor, A.W., Streilein, J.W. & Cousins, S.W. Immunoreactive vasoactive intestinal peptide contributes to the immunosuppressive activity of normal aqueous humor. *J. Immunol.* **153**, 1080–1086 (1994).
  37. Mora, J.R. *et al.* Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* **424**, 88–93 (2003).
  38. van Der Merwe, P.A. & Davis, S.J. Immunology. The immunological synapse—a multi-tasking system. *Science* **295**, 1479–1480 (1998).
  39. Squire, L.R. *et al.* in *Fundamental Neuroscience* (ed. Haines, D.E.) 32 (Academic, New York, 2002).
  40. Hogan, B., Beddington, R., Costantini, F. & Lacy, E. in *Manipulating The Mouse Embryo. A Laboratory Manual* (ed. Hogan, B., Beddington, R., Costantini, F. & Lacy, E.) 363 (Cold Spring Harbor Laboratory Press, New York, 1994).