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A conditioned dendritic cell can be a temporal bridge between a CD4$^+$ T-helper and a T-killer cell

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To generate an immune response, antigen-specific T-helper and T-killer cells must find each other and, because they cannot detect each other's presence, they are brought together by an antigen-loaded dendritic cell that displays antigens to both[1–3]. This three-cell interaction, however, seems nearly impossible because all three cell types are rare and migratory. Here we provide a potential solution to this conundrum. We found that the three cells need not meet simultaneously but that the helper cell can first engage and 'condition' the dendritic cell, which then becomes empowered to stimulate a killer cell. The first step (help) can be bypassed by modulation of the surface molecule CD40, or by viral infection of dendritic cells. These results may explain the long-standing paradoxical observation that responses to some viruses are helper-independent, and they evoke the possibility that dendritic cells may take on different functions in response to different conditioning signals.

We began our study to discriminate between two interpretations of this three-cell interaction (Fig. 1). The antigen-presenting cell (APC) has been proposed to have a rather passive relationship with the killer cell (also known as a cytotoxic T lymphocyte) and to function mainly to stimulate the helper cell to produce the interleukin (IL)-2 that the killer needs[1,2] (Fig. 1a). There is no guarantee, however, that a rare helper and an equally rare killer should find the same APC at the same time. As resting killers recognizing antigen become tolerant if there is no help[4–6], many potentially useful killers would founder while, elsewhere, some T helpers would wastefully secrete cytokines into an environment containing no killers to receive them. We therefore suggested a dynamic model (Fig. 1b) in which the T helper stimulates the APC to become able to activate the killer[6].

To discriminate between these possibilities, we studied responses to the male antigen H–Y because, first, killers that recognize H–Y are helper-dependent[6,7]; second, H–Y has no known crossreactive environmental mimics[8]; and third, primary and secondary responses can be easily distinguished because T cells from normal virgin female mice respond *in vitro* only if they were first primed *in vivo* with professional APCs[7,9].

Figure 2a–c shows that help is necessary for generation of killing activity against H–Y and that it can be replaced by soluble factors[1]. Female C57Bl/6 (B6) mice, immunized *in vivo* with male spleen cells, generated good *in vitro* killer-cell responses against male spleen stimulators (Fig. 2a). The responses disappeared if we removed the CD4$^+$ cells just before the culture (Fig. 2b) and reappeared if we added soluble helper factors (concanavalin A supernatant (CAS); Fig. 2c).

In some cases where help is minimal, such as in newborns (which have very few T cells) and in B6.bm12 mice (with mutated major histocompatibility complex (MHC) class II molecules), a killer-cell response can be induced by an injection of activated male dendritic cells[10,11]. We found, however, that activated dendritic cells could not stimulate purified CD8$^+$ killer cells unless we added helper cells, in the form of Marilyn, an H–Y-specific T-helper clone (Fig. 2e). Thus a small number of helpers may go a long way but without them dendritic cells are unable to activate killers against H–Y.

Because activated T helpers express CD40-ligand, which can stimulate CD40 to induce proliferation in B cells[12] and enhance the function of dendritic cells[13,14], we tried replacing T-cell help with antibodies against CD40. We found that overnight crosslinking with anti-CD40 antibodies turned dendritic cells into excellent stimulators (Fig. 2f). To rule out the possibility that the crosslinked dendritic cells were simply stimulating better IL-2 production from a few contaminating CD4$^+$ cells, we tested dendritic cells from MHC-class II-knockout (MHC II KO) mice, which are deficient in MHC class II molecules because of a gene-targeted deletion. Although these dendritic cells cannot present antigen to CD4$^+$ helpers, they became good stimulators for killers (Fig. 2h).

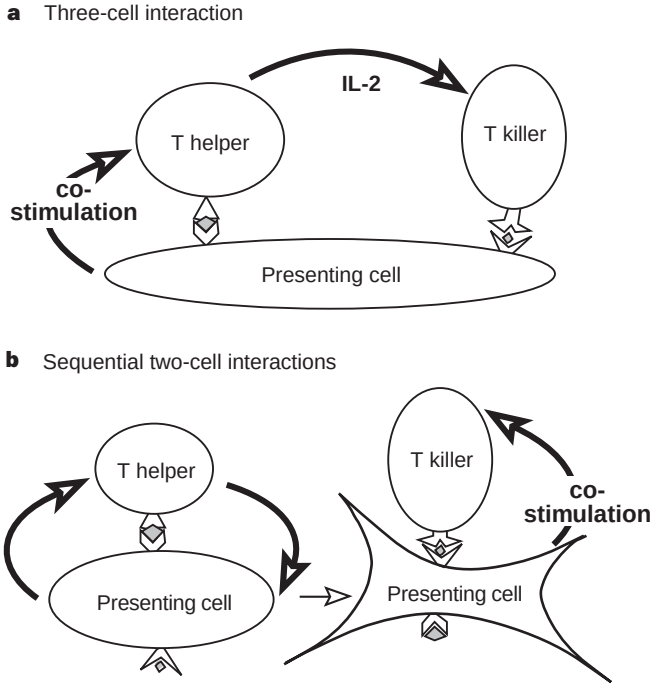

Figure 1 Two models of the delivery of help to CD8$^+$ killers. **a**, The 'passive' model in which the dendritic (presenting) cell presents antigen to both the T helper and the killer but delivers co-stimulatory signals only to the helper, which is thereby stimulated to produce IL-2 for use by the nearby killer. **b**, The 'dynamic' model in which the dendritic cell offers co-stimulatory signals to both cells. It initially stimulates the T helper (left), which, in turn, stimulates and 'conditions' the dendritic cell to differentiate to a state (right) where it can now directly co-stimulate the killer.

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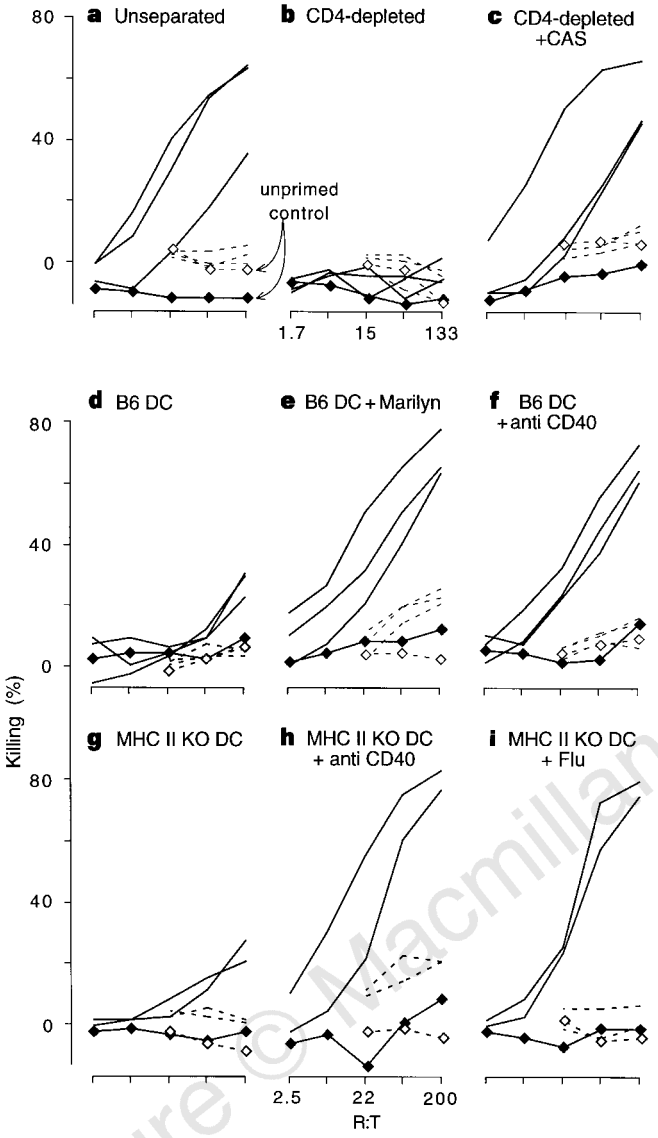

Figure 2 Four ways to help a killer. **a**–**c**, Role of helper cells and helper factors. Unseparated (**a**) or CD4-depleted (**b**, **c**) spleen cells from immunized female B6 mice were stimulated *in vitro* with B6 male spleen cells with (**c**) or without (**b**) CAS. **d**–**i**, Modulation of dendritic cells. CD4-depleted spleen cells from immunized female B6 mice were stimulated *in vitro* with male B6 dendritic cells (DC) (**d**), DC + Marilyn (**e**), CD40-modulated DC (**f**), unconditioned DC from MHC II KO mice (**g**), CD40-modulated DC from MHC II KO mice (**h**), or MHC II KO DC infected with influenza (Flu) (**i**). Solid lines, killing of male targets; dashed lines, killing of female targets; diamonds, unprimed controls. R:T, responder-to-target ratio.

Many other antibodies to dendritic cells did not have this effect, indicating that the CD40 molecule, rather than Fc receptors or other nonspecific changes, was responsible (not shown). We also found that conditioned dendritic cells can stimulate $CD8^+$ killers from a RAG (recombination-activating gene) KO, H–Y-specific, TCR (T-cell antigen receptor) transgenic mouse that cannot generate any other T-cell subsets (not shown), thus excluding the possibility that the dendritic cells function simply by eliciting help from unconventional T-helper cells, such as the newly described NK1 subset[15].

Some viruses can elicit helper-independent killer responses in CD4-deficient mice[16,17], whereas others, such as influenza, induce diminished but still potent responses[18,19]. We reasoned that infected dendritic cells might undergo a change similar to that induced by T-helper cells[6], and found that influenza-infected dendritic cells from male MHC II KO mice were indeed excellent stimulators of anti-H–Y killer cells (Fig. 2i).

Figure 3 summarizes 117 tests, showing the range and variation of responses in normal, CD4-depleted, and reconstituted cultures. Memory killers were stimulated by B6 or MHC II KO dendritic cells that were conditioned by $CD4^+$ helpers, anti-CD40 modulation, or virus infection. Dendritic cells that were cultured overnight with Marilyn and then sorted by fluorescence-activated cell sorting (FACS) to remove the helper cells were as stimulatory as those in which the helpers remained. Thus a helper T cell need not linger to communicate directly with the responding killers. It can stimulate a dendritic cell and leave. What might be the relevant change in the dendritic cell?

In many cell types, modulation of CD40 induces upregulation of the B7 co-stimulatory molecules[13,20,21]. However, our dendritic cells

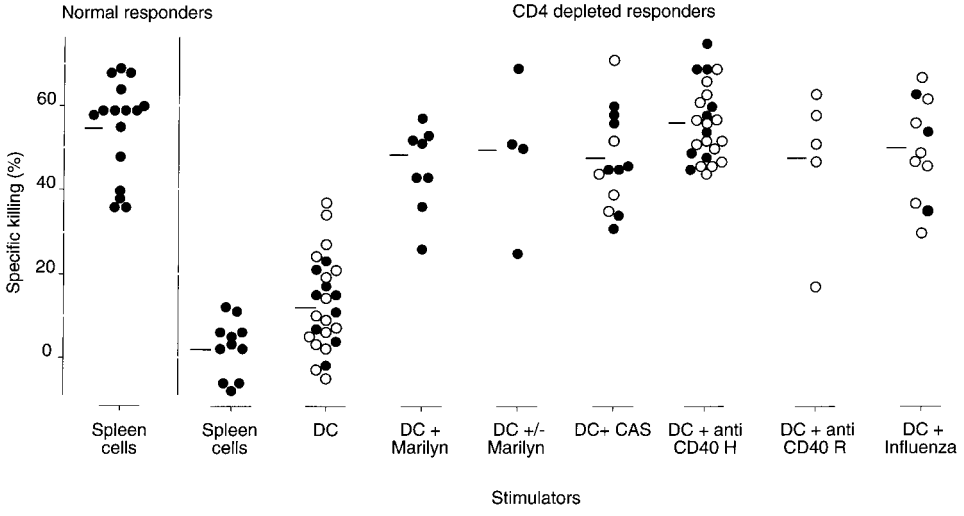

Figure 3 Summary of 117 tests showing five ways to help a killer. Spleen cells from anti-H-Y-primed female B6 mice were or were not depleted of $CD4^+$ T cells and were then stimulated with spleen or dendritic cells from normal (filled circles) or MHC II KO (open circles) mice. Reading left to right: spleen cells, dendritic cells (DC), DC incubated overnight with Marilyn (+ Marilyn), DC incubated overnight with Marilyn and then sorted to remove the T cells (+/– Marilyn), DC plus 10% CAS, DC modulated with a hamster (H) or a rat (R) anti-CD40 monoclonal antibody, DC infected with influenza virus. Each point represents the killing from a single culture at an R:T ratio at which killing shown by cultures from control mice drops off the plateau. Background killing of female targets is subtracted. Horizontal lines indicate the group average.

express very high levels of both B7.1 and B7.2 and these levels do not change with CD40 conditioning. Nevertheless, the recombinant mouse protein CTLA-4–Ig (which binds both B7 molecules) blocks their ability to stimulate killer cells, both when there is help available to the killers (Fig. 4a) and when the killers are stimulated directly (Fig. 4b). The control antibody, Ly5.2, does not block stimulation, and antibodies against B7.1 or B7.2 block only when used together, suggesting that T-killer cells may be able to use either of the two co-stimulatory molecules interchangeably. Thus, though B7 molecules are not sufficient for the stimulation of killers, they are nevertheless necessary.

Although CD40-modulated dendritic cells produce IL-6 and IL-12, these cytokines did not substitute for T-cell help (not shown). It is most likely that conditioned dendritic cells co-stimulate $CD8^+$ killers directly, as they do helpers, and that this results in the production of IL-2 by the killer cell itself[22]. In support of this, we found that CD4-depleted memory cells were able to produce a small amount of IL-2 when stimulated by conditioned dendritic cells from MHC II KO mice (data not shown). This would explain why we can bypass help in two ways: by supplying the necessary IL-2 or by empowering the killer cell to make its own.

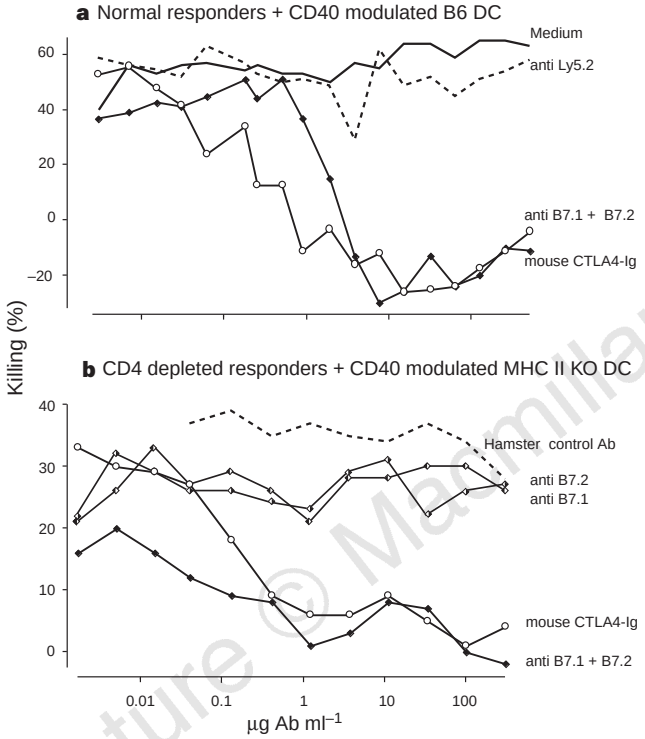

Figure 4 B7.1 and B7.2 are involved in stimulation of killer cells by conditioned dendritic cells. **a**, Unseparated, or **b**, CD4-depleted responding cells were stimulated with CD40-modulated dendritic cells (DC) from (**a**) B6 mice or (**b**) MHC II KO mice, in the presence of titrated amounts of various blocking reagents. Ab, antibody.

To determine whether conditioned dendritic cells can prime naive killers in the absence of T-cell help, we used the dendritic cells to prime killers in MHC II KO mice, which respond to some viruses[17,18] but which had not been previously tested with helper-dependent antigens. These mice did not reject syngeneic male skin grafts (not shown) nor did they respond to injections of male spleen or dendritic cells (Fig. 5), but they did respond when primed by two injections of conditioned dendritic cells. Thus both naive and memory H–Y-specific $CD8^+$ killers need help and the help can be delivered by a dendritic cell that has been conditioned by interaction with a helper, or by a virus infection.

Although there are other bodily systems in which two cells communicate through a third, most of the interactions are either mediated by soluble factors that traverse the necessary distances (such as the hypothalamic–pituitary–adrenal axis) or facilitated by stable linkages between cells (in the nervous system). The cells of the immune system use the fourth dimension, time, to transform a nearly impossible interaction between three rare, migratory and transient cells into a sequential pair of manageable two-cell engagements. This solves several problems at once. Helpers need not wastefully secrete IL-2 into their surroundings and killers need not wait for help after binding to antigen, because APCs present antigen and co-stimulatory signals simultaneously to killers, as they also do to helpers. There is also the bonus that the activity of a few helpers can be amplified, as a single T helper can 'arm' several APCs which can then, in their turn, activate a multitude of killers.

These results explain some of the contrasting findings from studies of help for killer cells. As infection by a virus can directly empower a dendritic cell to stimulate killers, some antiviral responses will depend more heavily on T helpers than others. For example, the MHC II KO mice respond normally to Sendai virus but less well to influenza. For these viruses, two types of dendritic cell may present viral antigens to $CD8^+$ killers. Some dendritic cells are themselves infected and consequently directly empowered to activate killers. Others may instead be stimulated, by danger signals at the infected site[23], to pick up viral antigens. As they are not actually infected, they need helpers to become empowered and thus, in a MHC II KO mouse, will be unable to stimulate $CD8^+$ killers. A prediction from this reasoning is that the helper independence of a killer response to a virus should correlate directly with that virus's ability to infect and condition the APCs.

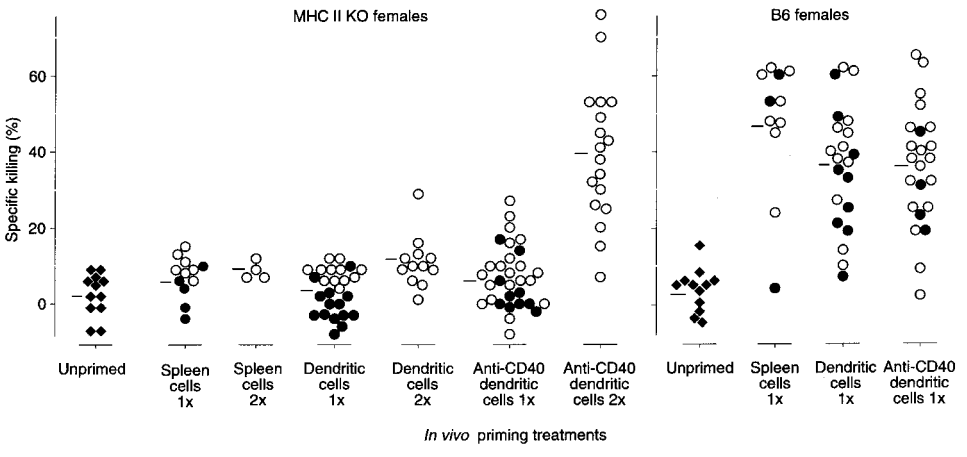

Figure 5 Virgin killers can be primed *in vivo* by CD40-modulated dendritic cells. 173 B6 or MHC II KO female mice were left untreated (diamonds) or injected once or twice with spleen or dendritic cells from B6 (filled circles) or MHC II KO (open circles) male mice. The dendritic cells were either untreated or modulated with a hamster anti-CD40 monoclonal antibody. The *in vitro* cultures contained 10% CAS, which substitutes for help and allows us to determine whether the mouse killer cells were primed *in vivo*. All mice generated killer cells against third-party CBA/J targets. Representation of killing activity is as in Fig. 3.

Conditioning by viruses may also affect responses to non-viral antigens. In an earlier study, we found helper-independent killers against the antigen Qa-1 during a hepatitis virus infection of the mouse colony[6]. For a few months of the current study, CD4-depleted mice generated moderate responses to H–Y that disappeared when we switched source colonies (though the first colony had no known pathogens). Thus, depending on the health status of a mouse, various responses may appear to be dependent or independent of T-cell help.

Finally, the existence of two different functional states of dendritic cells opens the possibility that there may be more, in analogy to the multiplicity of activation states seen with other cells of the immune system. For example, signals from functionally different T-helper cells can persuade B cells to switch to the production of different classes of antibody: T_H1 cells induce production of IgG2a; T_H2 cells elicit production of IgE and IgG1; and T_H3 cells signal the production of IgA[24–26]. Dendritic cells may be similarly conditioned, by the signals they receive, to enter different operational states, each one initiating a different class of response. We showed here that T_H1 cells or a virus infection can empower dendritic cells to activate $CD8^+$ killers. There is also evidence that IL-10 (ref. 27) or fluid from the eye[28] can condition APCs to become inducers of T_H2 rather than T_H1 responses. For us, the dendritic cell is beginning to look like a cell that responds to its environment in several ways and, in turn, influences several aspects of an immune response. First, it is activated by endogenous[23] or exogenous[29] danger signals to capture, process, and present antigen along with co-stimulatory signals and thus initiate an immune response. It is also influenced by the cells, cytokines and other signals in its environment to modify that response so that it is appropriate for both the pathogen that it is directed against and the location in which it unfolds. □

Methods

Mice and immunizations. C57Bl/6 (B6) mice were purchased from Taconic farms, NY. MHC class II-knockout (MHC II KO) mice, backcrossed to B6 (N13) or C57Bl/10 (N11) mice, were from the NIAID breeding contract at Taconic. Some mice were primed by an intraperitoneal injection of 3×10^6 male spleen cells or 5×10^5 dendritic cells in 200 μl of sterile phosphate-buffered saline (PBS). Two weeks later, some of these received a second, similar injection.

Cells. For CD4 purification, spleen cells were depleted using a Midi MACs (Miltenyi, Germany) and an anti-mouse-CD4 monoclonal antibody, GK1.5, yielding less than 0.2% remaining CD4 cells by FACS analysis with the non-competing anti-CD4 monoclonal antibody RM4-4 (Pharmingen, CA).

Dendritic cells were isolated as described[10].

For CD40 crosslinking, dendritic cells were incubated on ice for 10 min in PBS plus 10% mouse serum, for 20 mins with hamster (HM40-3, 5 μg ml^{-1}) or IgG2a rat (3/23, 3.5 μg ml^{-1}; Pharmingen) anti-mouse-CD40 monoclonal antibodies, and then overnight at 37 °C with goat anti-hamster or goat anti-rat antibodies (Caltag, CA) in Iscove's medium plus 10% fetal calf serum (IF10) plus 2 ng ml^{-1} GM-CSF and 200 units ml^{-1} IL-4. The cells were washed between and after the incubations, then injected intraperitoneally or irradiated (1,500 Rads) and used *in vitro*.

For stimulation with Marilyn, 1×10^6 male B6 dendritic cells were incubated overnight with 1.5×10^5 Marilyn, a $CD4^+$ T_H1 clone specific for $H–Y/A^b$ that was isolated from a B6 × CBA/N female mouse. These dendritic cells were irradiated and used as *in vitro* stimulators. In some experiments we removed the Marilyns, by FACS sorting with anti-$H–2^k$, before using the dendritic cells as stimulators. We tested for the efficiency of depletion by staining for CD4, Thy1 and T-cell antigen receptor (TCR) and by culturing an aliquot of the (unirradiated) sorted dendritic cell populations and testing the cells for proliferation. There was no evidence of contaminating Marilyn cells. For example, in two experiments the average of duplicate counts were as follows: male dendritic cells alone, experiment 1: 1,334 c.p.m., experiment 2: 1,853 c.p.m.; dendritic cells plus Marilyn, experiment 1: 25,335 c.p.m., experiment 2: 53,457 c.p.m.; dendritic cells with and then depleted of Marilyn, experiment 1: 1,114 c.p.m., experiment 2: 587 c.p.m.

For infection with influenza, dendritic cells were infected with influenza virus A/PR/8 as described[30], then irradiated and used as *in vitro* stimulators.

***In vitro* cultures.** For standard cultures, 2 weeks to 1 year after *in vivo* immunization, 4×10^6 untreated or CD4-depleted spleen cells were restimulated in 2-ml cultures with 2×10^6 irradiated male spleen cells or 1.5×10^5 dendritic cells, with or without an exogenous source of mouse IL-2 (10% rat Con A supernatant, depleted of Con A, Collaborative Biomedical Products, Bedford, MA). Six days later, T-cell killing of male and female targets was tested using the JAM Test[10].

For antibody-blocking cultures, anti-mouse B7.1 (17A10, hamster), anti-mouse B7.2 (2D10, rat IgG2b), CTLA-4–Ig (recombinant mouse CTLA-4–Ig fusion protein), and control antibodies anti-mouse Ly5.2 (A20.1.7, rat IgG2b, Fig. 3a) and anti-mouse MHC II (M5/114, rat IgG2b; Fig. 3b) were titrated by 1:2 in IF10, starting at 1 mg or 600 μg ml^{-1} in 100-μl volumes in 96-well round-bottomed plates. 50 μl containing 1×10^5 primed unseparated or CD4-depleted B6 female spleen cells plus 50 μl containing 5×10^4 conditioned B6 or MHC II KO male dendritic cells were added to give a final volume of 200 μl. Seven days later, the medium was removed and replaced with 200 μl medium containing 10^4 target cells for the JAM Test. We also used hamster antibody UC3 and human recombinant CTLA-4–Ig. They did not block and are not reported here for clarity.

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Help for cytotoxic-T-cell responses is mediated by CD40 signalling

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Cytotoxic T lymphocytes (CTLs) which carry the CD8 antigen recognize antigens that are presented on target cells by the class I major histocompatibility complex. CTLs are responsible for the killing of antigen-bearing target cells, such as virus-infected cells. Although CTL effectors can act alone when killing target cells, their differentiation from naive CD8-positive T cells is often dependent on 'help' from CD4-positive helper T (T_H) cells[1–4]. Furthermore, for effective CTL priming, this help must be provided in a cognate manner, such that both the T_H cell and the CTL recognize antigen on the same antigen-presenting cell[2,4]. One explanation for this requirement is that T_H cells are needed to convert the antigen-presenting cell into a cell that is fully competent to prime CTL[5]. Here we show that signalling through CD40 on the antigen-presenting cells can replace the requirement for T_H cells, indicating that T-cell 'help', at least for generation of CTLs by cross-priming, is mediated by signalling through CD40 on the antigen-presenting cell.

CD8-positive CTLs are responsible for the lysis of antigen-bearing target cells. These CTLs recognize peptide antigens presented by class I molecules encoded within the major histocompatibility complex (MHC). Generation of effective CTL responses often requires help from a second subset of T lymphocytes, the CD4-positive helper T (T_H) cells[1–4], but this is not always the case[6,7]. In response to the soluble protein ovalbumin (OVA), both T_H-cell-dependent and T_H-cell-independent CTL immunity can be induced[4,8]. When the K^b-restricted OVA peptide determinant spanning residues λ57 to 264 (OVAp) was emulsified in complete Freund's adjuvant (CFA) and injected subcutaneously, CTLs could be generated in mice lacking CD4-positive T cells (Fig. 1a)[4]. In contrast, priming by intravenous injection of irradiated spleen cells loaded with OVA by osmotic shock (OVA-loaded spleen cells) required the presence of CD4-positive T cells (Fig. 1b). This latter form of immunization occurs by cross-priming[4,9], requiring representation of antigen by host bone-marrow-derived antigen-presenting cells (APCs). Dissection of the cellular interactions involved in this response[4] revealed that, like the induction of CTLs that are specific for the Qa1 antigen[1], the T_H and CTL populations must recognize OVA on the same APC for effective CTL priming. This could be explained in two ways: either the T_H cells need to closely associate with the CTL to deliver short-range signals such as interleukin(IL)-2 (ref. 1), or they are required to modify the APC, converting it into a stimulatory cell for CTL priming[5].

There is evidence that CD40 and CD40 ligand (CD154) are important in both the humoral and cellular immune responses (reviewed in refs 10, 11). These molecules have been implicated in the generation of CTL responses to adenovirus[12] and in the estab-

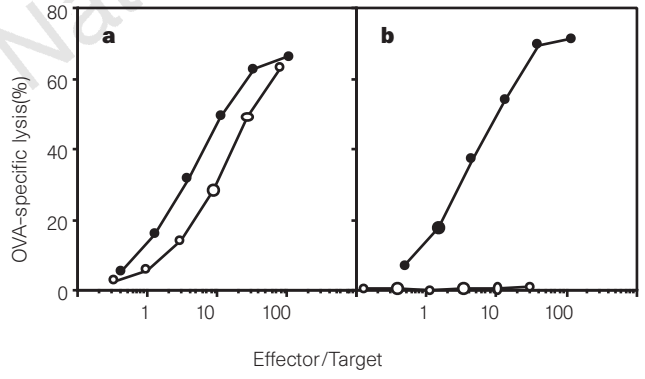

Figure 1 OVA-specific CTLs can be generated in CD4-independent and CD4-dependent ways. Normal B6 mice (filled circles) or B6 mice depleted of CD4-positive T cells by twice-weekly intraperitoneal injection of 100 μl GK1.5 ascites[4] (open circles) were injected either **a**, subcutaneously with 20 μg OVAp in 200 μl CFA, or **b**, intravenously with irradiated B6 OVA-loaded spleen cells as described[4]. After 8 days, spleen cells from each mouse were restimulated for 6 days *in vitro* as described[4]. On the day of assay, effector cells were examined for their ability to lyse ^{51}Cr-labelled EL4 targets that were or were not pulsed with OVAp. Non-specific EL4 lysis was <10%.

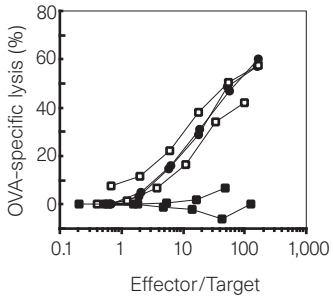

Figure 2 Treatment with a monoclonal antibody against CD40 replaces CD4-positive T-cell help in generating OVA-specific CTLs. Normal B6 mice (circles) and H-2A^b-deficient B6 mice (squares), were injected intravenously with irradiated, OVA-loaded bm1 spleen cells[4]. They were then left untreated (circles), or were injected intravenously daily for 4 days with either 0.1 mg of a CD40-specific monoclonal antibody, FGK45 (ref. 17) (open squares) or an IgG2a isotype control, KT50, specific for Vα8 (filled squares). Eight days after priming, the spleen cells from each mouse were restimulated *in vitro* for six days. We then examined their ability to lyse ^{51}Cr-labelled EL4 targets that were or were not pulsed with OVAp. Nonspecific EL4 lysis was <7%.