

variance/mean, or by comparing the average MEJC amplitude distribution skewness (Fig. 5a). This indicates that large spontaneous events are not due to the nonrandom coupling of multiple spontaneous release events by gated influx of calcium into the presynaptic terminal. However, we cannot discount the possibility that calcium fluxes from intracellular stores cause the nonrandom spontaneous release of multiple vesicles.

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Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs

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CD8⁺ cytotoxic T lymphocytes (CTLs) mediate resistance to infectious agents and tumours. Classically, CTLs recognize antigens that are localized in the cytoplasm of target cells, processed and presented as peptide complexes with class I molecules of the major histocompatibility complex (MHC)¹. However, there is evidence for an exogenous pathway whereby antigens that are not expected to gain access to the cytoplasm are presented on MHC class I molecules^{2–6}. The most dramatic example is the *in vivo* phenomenon of cross-priming⁷: antigens from donor cells are acquired by bone-marrow-derived host antigen-presenting cells (APCs) and presented on MHC class I molecules. Two unanswered questions concern the identity of this bone-marrow-derived cell and how such antigens are acquired. Here

we show that human dendritic cells, but not macrophages, efficiently present antigen derived from apoptotic cells, stimulating class I-restricted CD8⁺ CTLs. Our findings suggest a mechanism by which potent APCs acquire antigens from tumours, transplants, infected cells, or even self-tissue, for stimulation or tolerization of CTLs.

Influenza A virus establishes a non-toxic infection in human dendritic cells (DCs)^{8,9}. Once infected, these DCs are capable of eliciting virus-specific, recall CTL responses within 7 days. The CTLs generated are class I-restricted and kill virus-infected monocytes and peptide-pulsed target cells^{8,10}. Compared with DCs, influenza-infected monocytes are poor stimulators of CTL responses⁸ and undergo apoptosis^{11,12}. We have exploited these observations to investigate the role of apoptosis in the generation of antigen that could be acquired by uninfected DCs.

DCs were prepared from peripheral blood precursors of HLA-A2.1⁺ donors^{13,14}. Uninfected DCs and syngeneic T cells were cocultured with influenza-infected syngeneic (Fig. 1a) or allogeneic (Fig. 1b) monocytes for 7 days. Influenza-specific CTLs were generated in these cocultures, suggesting that the DCs acquired antigen from the monocytes. The DCs and not the infected monocytes functioned as the antigen-presenting cell (APC), because the latter failed to stimulate CTLs in the absence of DCs, even at a stimulator:responder ratio of 1:2 (Fig. 1a, dashed lines). The CTL responses generated in these cocultures were as potent as those induced by influenza-infected DCs, and as few as 5×10^3 infected monocytes could charge uninfected DCs for the induction of robust CTLs (Fig. 1b). To confirm that antigen is transferred to and expressed directly by DC MHC class I products, we cocultured uninfected DCs and influenza-infected monocytes, and used the DCs as targets for influenza-specific CTLs (Fig. 1c). DCs cocultured with infected monocytes were recognized as efficiently as virus-infected DCs. Collectively, the results in Fig. 1 suggest that influenza antigen from infected monocytes gained access to MHC class I of the DC, that is, antigen from HLA-mismatched monocytes were cross-priming T cells through the DC.

It was important to exclude live virus as the agent responsible for the transfer of antigen to DCs. Influenza-infected monocytes produce only low levels of infectious virus before they undergo apoptosis^{9,11}. Infection of 5×10^4 monocytes with a multiplicity of infection (MOI) of 2.0 would be expected to yield up to 1×10^3 infectious virions after a 24 h culture period. To prevent infection of DCs by monocyte-produced virus, experiments were done in human serum, which contains blocking anti-haemagglutinin antibodies. Indeed, the addition of influenza virus (10^4 infectious virions) directly into cultures containing uninfected DCs and T cells did not yield a CTL response (data not shown). Furthermore, <1 haemagglutination units (HAU) ml⁻¹ was detected in the medium at 12 h, 24 h and 7 days of coculture.

The CTL responses were also not due to free peptide released by the dying monocytes, thereby charging class I molecules on DCs. Media from wells containing the infected monocytes were collected after an overnight culture and transferred to fresh wells containing T cells and uninfected DCs. Virus-specific CTLs were generated after a 7 day culture period (Fig. 2a, transfer). This CTL activity was abrogated if the medium was passed through a filter (0.45 µm pore size) before being added to T-cell–DC cultures (Fig. 2a, filter), suggesting that the antigenic material was neither live virus nor free peptide (as both would have passed through the filter). This was confirmed by sedimentation experiments (Fig. 2b). Media from wells containing infected monocytes were removed and spun at 250g. The antigenic material could be localized in the pellet, but not in the supernatant fraction. Notably, the pelleted material (which lacked detectable haemagglutinating activity) fully accounted for the CTL activity generated in direct transfer. These results are most consistent with the source of antigen being material derived from apoptotic cells.

The role of apoptosis in the transfer of antigen to the uninfected DC was therefore assessed. We first extended prior work¹¹, showing that influenza-infected monocytes undergo apoptosis as detected by annexin V binding, an early marker for programmed cell death (Fig. 3a, middle panel). In contrast, heat-inactivated (HI) influenza virus, which reliably reduces viral replication, failed to induce apoptosis in monocytes (Fig. 3a, bottom panel). However, DCs that are infected with HI influenza still stimulate potent influenza-specific CTL responses¹⁰. We employed this replication-deficient virus to probe the requirement for apoptosis in cross-priming. When media from wells containing the monocytes infected with HI influenza were collected after an overnight culture and transferred to fresh wells containing T cells and uninfected DCs, no influenza-specific CTLs were generated (Fig. 3b, 10 h transfer, HI Flu). In contrast, DCs exposed to media derived from live virus-infected cultures, which contained apoptotic cells, did elicit a CTL response (Fig. 3b, 10 h transfer, Live Flu). However, if the monocytes infected with HI influenza were allowed to undergo apoptosis spontaneously, as occurs during 7 days of culture¹⁵, antigenic material was generated and virus-specific CTLs were induced (Fig. 3b, coculture, HI Flu). This data also argues against the possibility that live virus within the apoptotic cell is responsible for the transfer of antigenic material to the DCs.

To establish that apoptosis is the critical trigger for cross-priming, we used Z-VAD-CHO, an irreversible peptide inhibitor of caspase

activity. Apoptosis of influenza-infected monocytes was blocked (determined by TUNEL), without affecting the expression of viral proteins (data not shown). Influenza-infected monocytes were cultured overnight in the presence of varying concentrations of Z-VAD. Media from these wells were then added to fresh wells containing T cells and uninfected DCs. At concentrations of Z-VAD which inhibited apoptosis of the infected monocytes, antigenic material was not transferred to the uninfected DCs, and virus-specific CTLs were not generated (Fig. 3c). We next compared apoptotic death to necrotic death for the generation of antigenic material. Influenza-infected 293 cells express influenza proteins after 10 h of infection but, unlike monocytes, do not undergo apoptosis. Apoptosis or necrosis was induced by ultraviolet B irradiation and hypotonic shock, respectively, and the cells were then cocultured with uninfected DCs and T cells for 7 days. CTL activity was measured using matrix peptide-pulsed T2 targets. Only the apoptotic cells and not the necrotic cells were capable of charging DCs with antigen (Fig. 3d). Apoptotic versus necrotic influenza-infected monocytes gave similar results (data not shown).

It was important to investigate the cellular requirements for generating CTLs through this pathway. When CD4⁺ and CD8⁺ subpopulations were purified at the end of the 7 day culture period, CTL activity was detected only in the CD8⁺ fraction (data not shown). We next compared DCs to macrophages as potential mediators of this exogenous pathway. Uninfected HLA-A2.1⁺

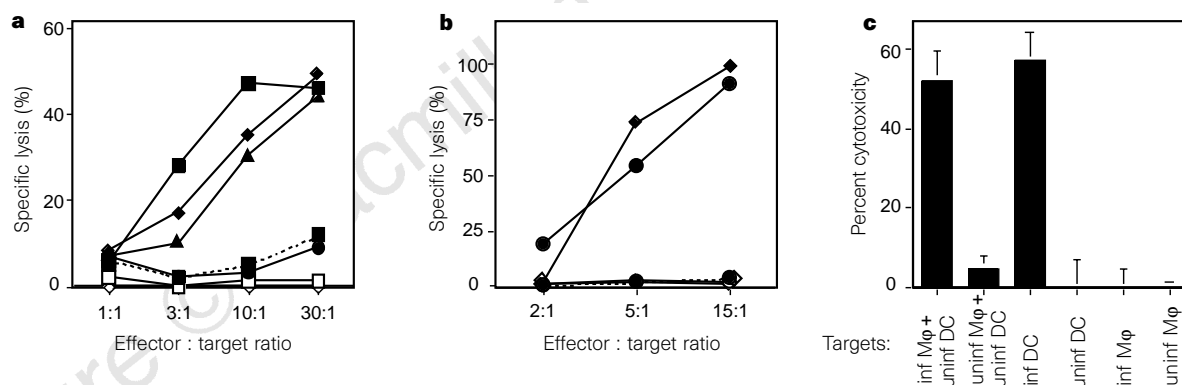


Figure 1 Dendritic cells acquire antigen from influenza-infected cells and induce class I-restricted CTLs. **a**, **b**, 8 to 10 h after infection with influenza virus, varying doses of syngeneic HLA-A2.1⁺ monocytes (**a**) or allogeneic HLA-A2.1⁺ monocytes (**b**) were added to cocultures of DCs and T cells. On day 7, cytolytic activity was tested using syngeneic influenza-infected macrophages (**a**) or T2 cells (a Tap^{-/-}, HLA-A2.1⁺ cell line) pulsed with the immunodominant influenza matrix peptide²⁶ (**b**) as targets. Symbols: filled squares plus solid line 5 × 10⁵ infected monocytes (inf Mφ) + uninfected dendritic cells (uninf DCs); open squares 5 × 10⁵ uninf Mφ + uninf DCs; filled squares plus dotted line, 5 × 10⁵ inf Mφ, no DCs; filled triangles,

5 × 10⁴ inf Mφ + uninf DCs; filled circles plus solid line, 5 × 10⁵ inf Mφ + uninf DCs; open circles, 5 × 10⁵ uninf Mφ + uninf DCs; filled circles plus dotted line, 5 × 10⁵ inf Mφ, no DCs; filled diamonds, inf DCs; open diamonds, uninf DCs. **c**, Uninfected syngeneic DCs are cocultured with influenza-infected or uninfected allogeneic monocytes for 2 days before being used as targets for CTLs. Control targets included influenza-infected syngeneic DCs and influenza-infected allogeneic monocytes. Effector:target ratio = 45:1. Results are representative of 8 experiments and the values shown represent the mean from triplicate wells.

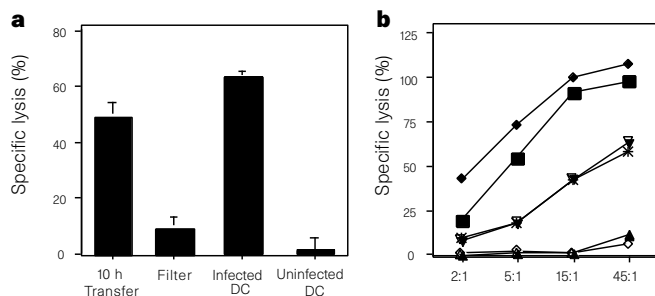


Figure 2 Antigen transfer is not due to live influenza virus or free peptide. **a**, 5 × 10⁴ infected allogeneic HLA-A2.1⁺ monocytes were cultured for 10 h, after which the media from these wells was collected. Media was either directly added to fresh wells containing HLA-A2.1⁺ T-cells and DCs (10 h transfer) or first passed through a 0.45 μm (filter). Effector:target ratio = 30:1. **b**, 5 × 10³ infected allogeneic monocytes were cultured for 10 h, after which T cells and DCs were added to the wells (squares). Alternatively, media from wells containing infected monocytes was removed and transferred to fresh wells containing T cells and DCs (asterisks). Other cultures were established in which the medium was first spun at 250g in a GH-3.8 Beckman rotor for 10 min. The resulting supernatant fraction (triangles) versus the pellet (inverted triangles) was then added to DC-T cell cocultures. Controls included T cells cultured with infected (filled diamonds) and uninfected (open diamonds) DCs. After 7 days, cytolytic activities were determined on T2 cells pulsed with matrix peptide. Results are representative of 3 experiments.

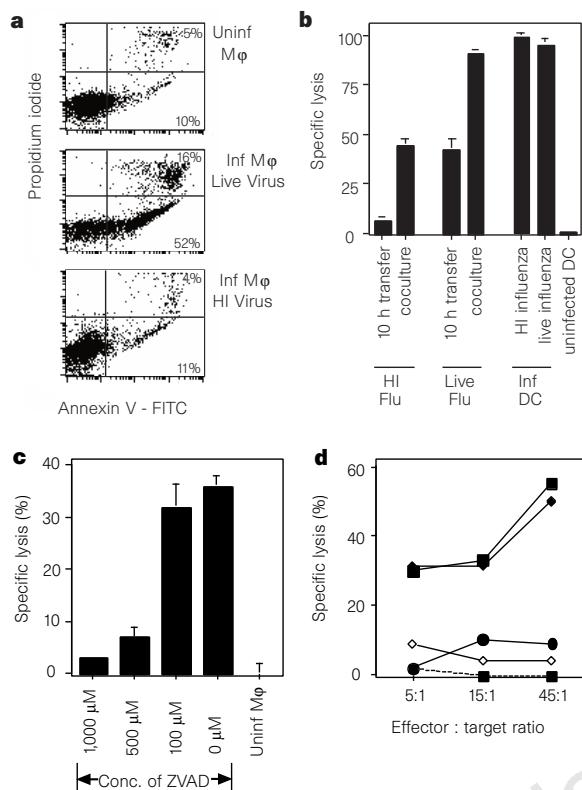


Figure 3 Apoptosis is required for delivery of antigen to DCs. **a**, Monocytes were infected with live or heat-inactivated influenza virus, cultured for 10 h and stained with annexin V – FITC (Kayima Biomedical Company, Seattle, WA) and propidium iodide. Early apoptotic cells are defined by the annexin V⁺, propidium iodide⁺ population. **b**, Allogeneic HLA-A2.1⁺ monocytes were infected with live or heat-inactivated virus. After 10 h, media from wells containing infected monocytes was removed and added to fresh wells containing T cells and uninfected DCs (10 h transfer). Alternatively, the infected monocytes were directly cocultured with the T-cells and DCs (coculture). Heat-inactivated influenza and live influenza-infected DCs served as the positive controls for the experiment. After 7 days CTL activity was measured on matrix peptide-pulsed targets. **c**, Apoptosis of influenza-infected monocytes was inhibited using Z-VAD-CHO (Kayima Biomedical Company). 1×10^4 infected monocytes were exposed to varying doses of Z-VAD for 1 h, washed and cultured for 10 h. Media from these wells was then transferred to fresh wells containing 2×10^5 T cells and 6.67×10^3 DCs. Cytolytic activity was determined as in **b**. **d**, 293 cells, a human kidney epithelial cell line, were infected with influenza virus, and cultured for 10 h. Apoptosis was induced by UVB irradiation for 2 min²⁸. Necrosis was achieved by incubating the 293 cells in a hypotonic solution for 30 min at 37°C, after which all the cells incorporated trypan blue. Apoptotic or necrotic 293 cells were cocultured with T cells and uninfected DCs for 7 days. Cytolytic activity was determined as in **b**. Uninfected 293 cells failed to induce CTL activity (data not shown). Symbols: squares plus solid line, apoptotic inf 293 cells + uninf DCs; circles, necrotic inf 293 cells + uninf DCs; squares plus dotted line, apoptotic inf 293 cells, no DCs; filled diamonds, inf DCs; open diamonds, uninf DCs. The results are representative of 9 experiments.

macrophages or DCs and syngeneic T cells were cocultured with infected HLA-A2.1⁺ monocytes for 7 days, after which cytolytic activity was measured. DCs but not macrophages were capable of stimulating influenza-specific CTLs (Fig. 4). Additionally, as increasing doses of syngeneic uninfected macrophages were introduced into cocultures containing uninfected DCs, T cells and infected allogeneic monocytes, the CTL activity was abrogated. Presumably, the macrophages act to sequester antigen from the DCs by efficiently engulfing the apoptotic cells and degrading the antigen.

To demonstrate that antigen was being processed intracellularly, DCs were pretreated with inhibitors of antigen presentation and

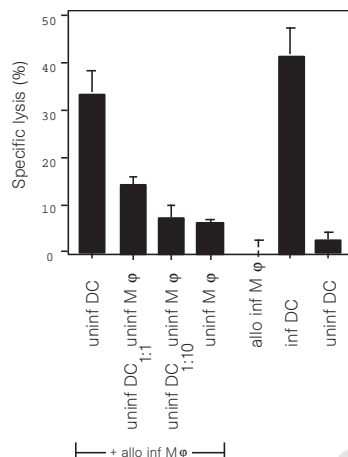


Figure 4 Dendritic cells and not macrophages are capable of cross-presenting apoptotic antigenic material. Uninfected HLA-A2.1⁺ monocytes or DCs and mixtures of both were used as APCs, and cocultured with bulk syngeneic T cells and influenza-infected HLA-A2.1⁺ DCs and infected HLA-A2.1⁺ monocytes served as positive and negative controls, respectively. Cytolytic responses were measured after 7 days. Effector:target ratio = 30:1.

used as targets for influenza-specific CTLs. Both ammonium chloride and Brefeldin A completely inhibited the DC's ability to present antigenic material derived from apoptotic monocytes (data not shown). Lactacystin, an irreversible inhibitor of the 26S proteasome¹⁶, only partially blocked antigen presentation by the uninfected DCs (data not shown), suggesting that both classical and non-classical class I pathways are utilized for the presentation of exogenous antigen derived from apoptotic cells. We next documented the uptake of apoptotic cells by immunofluorescence and electron microscopy. Ten to twenty percent of the DCs contained fragmented or intact cell bodies in Fig. 5a. DCs were identified by the DC-restricted marker, p55, and apoptotic material was identified by the intense DAPI staining of pyknotic nuclei. This was confirmed by electron microscopy (Fig. 5b). About 20–25% of the DCs (identified by expression of CD83, data not shown) had vesicles containing apoptotic corpses with apparently intact plasma membranes. A recent report indicates that DCs associate with apoptotic cells through the vitronectin receptor $\alpha v \beta 3$, but fail to associate with opsonized particles and necrotic cells¹⁷. Further studies will be required to determine if DCs are actually phagocytosing and presenting apoptotic cells through this receptor.

Previous studies have shown that murine DCs have the capacity to present soluble antigens through an exogenous pathway, leading to the induction of MHC class I-restricted CTLs^{5,18–20}. Following intravenous injection of allogeneic cells into rats, interdigitating DCs within lymph nodes were found to contain whole cells and cell fragments²¹. Here we present a physiologically relevant system which demonstrates that human DCs can acquire relevant antigens and stimulate MHC class I-restricted CTLs by phagocytosing apoptotic cells. Our studies provide the first evidence that apoptosis (but not necrosis) is required for the generation and packaging of immunogenic material for delivery to the DC. We believe this pathway accounts for the *in vivo* phenomenon of 'cross-priming', whereby antigens derived from tumour cells²² or transplants²³ are presented by host APCs. Tolerance to tissue-restricted self antigens^{24,25} may also depend upon apoptotic cell death (as occurs during development and normal cell turnover) followed by antigen presentation by DCs. Possibly, heat shock proteins within the apoptotic cells direct antigen into the class I MHC presentation pathway⁴. As a consequence of apoptosis, antigens within cells that lack costimulatory function for T cells can gain access to the potent DC system, thereby eliciting stimulatory or tolerogenic responses. This apoptosis-dependent pathway has the potential to be therapeutically manipulated to induce CTL responses *in vivo* to a variety of antigens including tumour and microbial antigens, and possibly to modulate autoimmune responses.

Methods

Generation of mononuclear subsets. Peripheral blood mononuclear cells

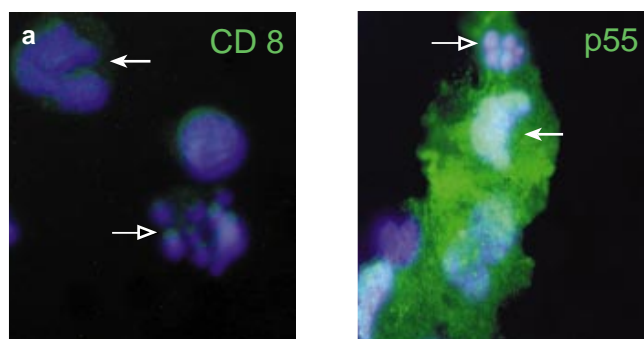
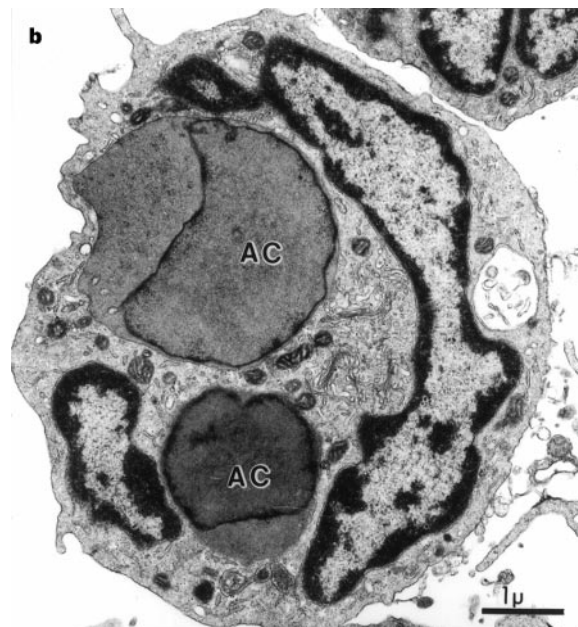


Figure 5 Dendritic cells engulf apoptotic monocytes. Influenza-infected monocytes and uninfected DCs were cocultured for up to 10 h. **a**, Cells were fixed with acetone and stained with anti-CD8 (isotype control) or anti-p55 followed by goat anti-mouse-FITC and incubation with DAPI (Sigma). The nuclei of the DCs (closed arrows) are lobulated and euchromatic as compared with the pyknotic, fragmented nucleus of the apoptotic cells (open arrows). DAPI⁺ material from an apoptotic cell appears to be within the cytoplasm of a p55⁺ DC. **b**, Electron microscopy revealed apoptotic material and apoptotic cells (AC) within the cytoplasm of glutaraldehyde-fixed DCs²⁹.



(PBMcs), T cells, and macrophages were prepared as previously described⁸. Dendritic cells were prepared from T-cell depleted PBMcs by culturing cells for 7 days in the presence of granulocyte and macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), followed by 4 days in monocyte-conditioned medium^{13,14}.

Induction and detection of apoptosis. Monocytes were infected with influenza virus in serum-free RPMI. Cell death was assayed using the Early Apoptosis Detection kit (Kayima Biomedical). Briefly, cells are stained with Annexin V-FITC (Ann V) and propidium iodide (PI). Early apoptosis is defined by Ann V⁺/PI⁻ staining as determined by FACSscan (Becton Dickinson). Cells from the 293 cell line were triggered to undergo apoptosis using a 60UVB lamp (Derma Control Inc.), calibrated to provide 2 mJ cm⁻² s⁻¹.

Coculture of DCs with apoptotic cells. Monocytes from HLA-A2.1⁻ donors were infected with live or heat-inactivated influenza virus. Live influenza virus (Spafas Inc.) was added at a final concentration of 250 HAU ml⁻¹ (MOI of 0.5) for 1 h at 37 °C (ref. 8). Virus was heat-inactivated by treatment for 30 min at 56 °C before use¹⁰. After washing, cells were added to 24-well plates in varying doses. After 1 h, contaminating non-adherent cells were removed and fresh media was added. Following a 10 h incubation at 37 °C, 3.3 × 10³ uninfected DCs and 1 × 10⁶ T cells were added to the wells.

Assay for virus-specific CTLs. After 7 days of culture, T cells were assayed for cytolytic activity using a conventional Na⁵¹CrO₄ release assay⁸. The targets were either influenza-infected syngeneic monocytes or T2 cells (a TAP^{-/-}, HLA-A2.1⁺, class II⁻ cell line) pulsed for 1 h with 1 μM of the immunodominant influenza matrix peptide, GILGFVFTL^{26,27}. Specific lysis was determined by subtracting the per cent killing of uninfected monocytes or unpulsed T2 cells. Influenza-infected DCs served as controls in all experiments in order to measure the donor's CTL responsiveness to influenza. Responses varied as a function of the individual's prior exposure to influenza. Background lysis ranged from 0–5% for the uninfected monocytes and 0–20% for the unpulsed T2 cells. Additionally, syngeneic DCs were used as targets where noted.

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