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Antigen Persistence Is Required for Dendritic Cell Licensing and CD8$^+$ T Cell Cross-Priming[1]

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It has been demonstrated that CD4$^+$ T cells require Ag persistence to achieve effective priming, whereas CD8$^+$ T cells are on "autopilot" after only a brief exposure. This finding presents a disturbing conundrum as it does not account for situations in which CD8$^+$ T cells require CD4$^+$ T cell help. We used a physiologic in vivo model to study the requirement of Ag persistence for the cross-priming of minor histocompatibility Ag-specific CD8$^+$ T cells. We report inefficient cross-priming in situations in which male cells are rapidly cleared. Strikingly, the failure to achieve robust CD8$^+$ T cell activation is not due to a problem with cross-presentation. In fact, by providing "extra help" in the form of dendritic cells (DCs) loaded with MHC class II peptide, it was possible to achieve robust activation of CD8$^+$ T cells. Our data suggest that the "licensing" of cross-presenting DCs does not occur during their initial encounter with CD4$^+$ T cells, thus accounting for the requirement for Ag persistence and suggesting that DCs make multiple interactions with CD8$^+$ T cells during the priming phase. These findings imply that long-lived Ag is critical for efficient vaccination protocols in which the CD8$^+$ T cell response is helper-dependent. ***The Journal of Immunology,*** **2008, 181: 3067–3076.**

Both in vitro and in vivo experimentation have defined an essential role for Ag-specific CD4$^+$ helper cells in the cross-priming of CD8$^+$ T cells (1–3). This helper activity requires that the Ag be present on the same APC that is engaging the CD8$^+$ T cell, much like the carrier effect that had been previously defined for T cell/B cell cooperation (4). This led to the proposal of three-cell clusters of T cells with APCs, as it could best explain the epitope linkage and noncognate requirements of the in vivo cytolytic response. Due to an awareness that the probability of such interactions would be rare as a result of the low precursor frequency of naive T cells and the scarcity of Ag-loaded APCs, this model gave way to what is commonly referred to as the "serial two-cell" model. Accordingly, CD4$^+$ T cells "license" dendritic cells (DCs)[3] that in turn prime CD8$^+$ T cells. This terminology was first introduced by Lanzavecchia (5), following from three studies that support the role of CD4$^+$ T cells offering activation signals to the APC that permit them to prime CD8$^+$ T cells (2, 3, 6).

It has also been demonstrated that CD8$^+$ T cell priming may occur following a relatively short exposure to Ag. In infection situations such as *Listeria monocytogenes*, effective primary CD8$^+$ T cell responses require less than 24 h of antigenic stimulation (7, 8). Similarly, it has been demonstrated in vitro, using monoclonal T cells that CD8$^+$ T cells given 20 h of stimulus will respond by proliferating extensively and differentiating into effector cells (8, 9). This phenomenon was termed an "autopilot" response by Bevan and Fink (10), and distinguishes CD8$^+$ T cell activation from the regulation of CD4$^+$ T cell priming (11–13). Indeed, recent experiments from Obst et al. (14) demonstrated that CD4$^+$ T cell proliferation requires cognate stimulation throughout their expansion phase. These results raise a paradox regarding the priming of helper-dependent CD8$^+$ T cell responses: what happens if the DC makes contact with an Ag-specific CD8$^+$ T cell before it is "licensed?" The current model suggests that it would be tolerized, even though the cognate helper cell may be present in the repertoire, albeit late in getting to the Ag-loaded DCs. Moreover, the concept that DCs are licensed by CD4$^+$ T cells during their initial encounter does not account for the need to first prime the CD4$^+$ T cell and induce surface expression of CD40L, a critical molecule for DC activation.

To evaluate the requirement for Ag persistence during helper-dependent cross-priming and address the timing of DC licensing, we studied responses in female mice to the male Ag HY uty peptide, which has the minor histocompatibility locus of Y chromosome in ubiquitously transcribed tetratricopeptide repeat gene *uty*. In this model, CD8$^+$ T cells that recognize the uty peptide presented by H-2D^b are dependent on CD4$^+$ T cell help during the priming phase (6, 15). Moreover, cross-priming to this epitope has been reported to be inefficient in models when allo (H2^k→H2^b) or β_2-microglobulin-deficient (β_2m$^{-/-}$) cells are used as the source of male Ag (16, 17). Finally, the HY model is physiologically and pathologically relevant because the minor histocompatibility Ags are normal cellular proteins and have been implicated in the clinical complications associated with graft-versus-host disease. We

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[3] Abbreviations used in this paper: DC, dendritic cell; CFDA-SE, carboxyfluorescein diacetate succinimidyl ester; β_2m, β_2-microglobulin; DLN, draining lymph node; SFC, spot forming cell; WT, wild type.

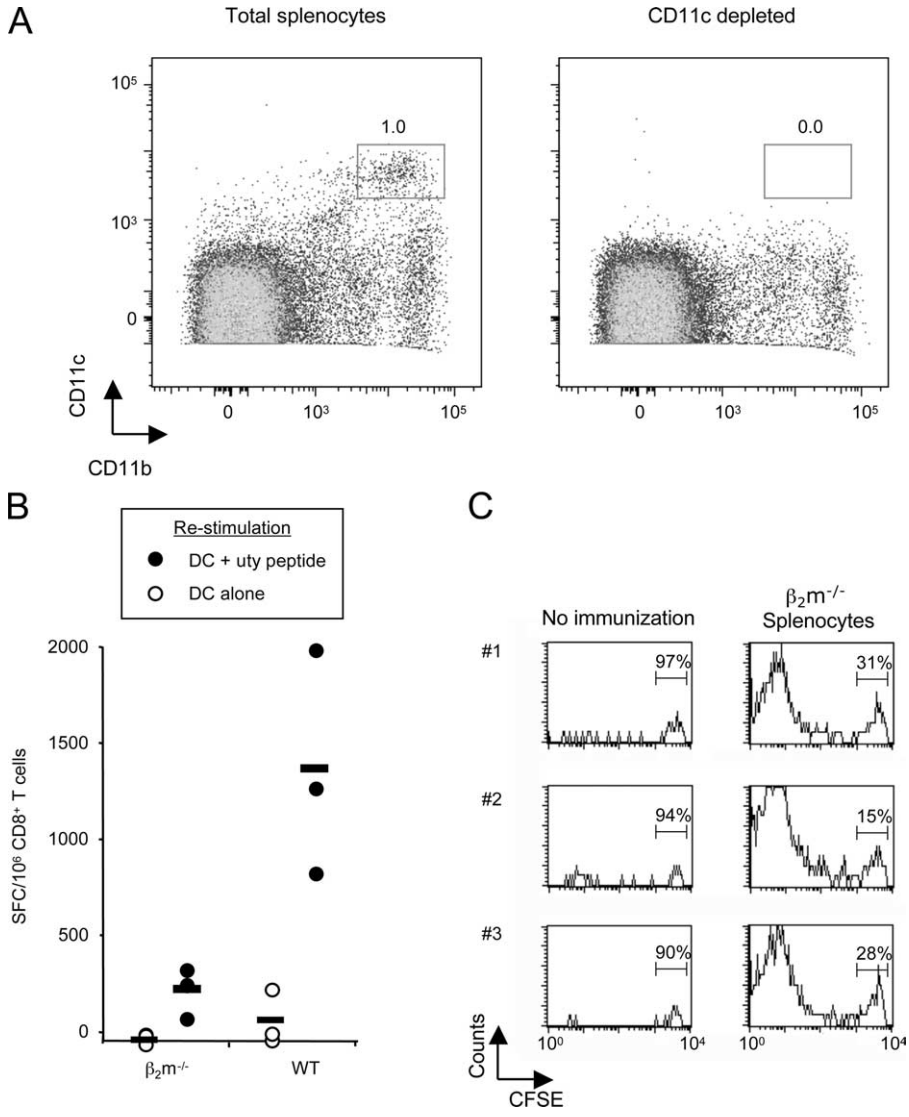

FIGURE 1. Efficient cross-presentation of $\beta_2m^{-/-}$ male cells does not permit effective cross-priming of H-2D^b uty-specific CD8$^+$ T cells. *A*, Female mice were immunized intradermally with 5×10^6 CD11c-depleted splenocytes that had been isolated from WT or $\beta_2m^{-/-}$ male mice. Live, nonclumped, lineage-positive cells were gated. Depletion was confirmed using FACS analysis. *B*, After 12 days, the efficiency of priming uty-specific CD8$^+$ T cells was determined using IFN-γ ELISPOT. Purified CD8$^+$ T cells were restimulated ex vivo for 24 h using female DCs loaded with uty peptide (●). Unpulsed DCs served as a negative control (○). Each mouse is represented and the number of SFC per 10^6 total CD8$^+$ T cells is measured. Horizontal bars indicate the mean. This experiment is representative of eight similar experiments. *C*, To monitor cross-presentation of injected male Ag, the triggering of MataHari CD8$^+$ T cell division was assessed. A total of 2×10^5 CFDA-SE-labeled MataHari CD8$^+$ T cells (CD45.2) was transferred into recipient female mice (CD45.1), and T cell division was followed 3 days after immunization with $\beta_2m^{-/-}$ male cells by gating on CD8β^+CD45.2$^+$ cells. Results are representative of four independent experiments from which 3/3 mice tested (1, 2, 3) are in one such experiment.

report that contrary to the autopilot expansion of CD8$^+$ T cells that has been demonstrated in helper-independent models of CD8$^+$ T cell priming, the priming of HY-reactive CD8$^+$ T cells requires the persistence of Ag.

Materials and Methods

Mice

C57BL/6/J wild-type (WT) mice were obtained from Charles River Breeding Laboratories. Ptprc^{a}Pepc^b/BoyJ (CD45.1), MHC class II$^{-/-}$ (B6.129S-*H2$^{dlAb1\text{-}Ea}$*/J), and $\beta_2m^{-/-}$ (B6.129P2-*B2m^{tm1Unc}*/J) mice were obtained from The Jackson Laboratory. MataHari CD8$^+$ TCR transgenic mice (18) were obtained from CDTA (Centre de Distribution Typage et Archivage animal, Orleans, France). All mice were maintained in a *Helicobacter*-free specific pathogen-free facility, and used under approved protocols.

Abs and reagents

FACS Abs were obtained from BD Biosciences, Invitrogen, or eBioscience. FACS analysis was performed on a FACSCalibur (BD Biosciences) with the exception of the male cell enrichment studies, which were performed on a FACSCanto II cytometer (BD Biosciences). Abs used in the IFN-γ ELISPOT assays were purchased from Mabtech. Anti-NK1.1 (clone PK136; eBioscience) was used for NK depletion. Recombinant mouse TNF-α was obtained from R&D Systems. The immunodominant H2-D^b uty peptide (WMHHNM DLI, accession no. CAA70422, aa 246–254) (19) was provided by NeoMPS. The immunodominant I-A^b dby peptide (NAGFNSNRANSSRSS, accession no. CAA07483, aa 608–622) and OVA peptide (KISQAVHAAHAEINEAG, NP990483, aa 323–339) were provided by Invitrogen Life Technologies. The peptide dby (DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked) is used in priming experiments. Labeling with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) was performed using the Vybrant CFDA-SE cell tracer kit from Molecular Probes.

Priming HY-specific CD8$^+$ T cells

Splenocytes used for immunization were isolated from male mice. CD11c$^+$ cells were depleted using the Miltenyi MACS system. Of note, the removal of DCs from the injectate ensured that uty protein had to gain access to a host DC as the uty epitope is dependent on expression of the immunoproteasome (20). Immunization was conducted via the intradermal route for the experiments shown, but similar results (as shown in Fig. 1*A*) were obtained when using s.c. and i.p. routes for immunization. For NK depletion experiments, we also depleted the injected splenocytes of NK cells to be sure that opsonized cells were not interfering in the priming response (21). NK depletion of mice was achieved by repeated injections of 100 μg of Ab delivered i.p. on the day before and the day of immunization.

Monitoring HY-specific CD8$^+$ T cells

At 12 days after immunization, spleens were harvested, and CD8$^+$ T cells were purified using anti-CD8 microbeads and MS column (Miltenyi Biotec). ELISPOT assays for IFN-γ-producing cells were performed as previously described (22). The ELISPOT plate evaluation was performed in a blinded fashion by an independent evaluation service (Zellnet Consulting) using an automated ELISPOT reader (Carl Zeiss). For tetramer analysis, soluble MHC/peptide tetramers were produced using a modified version of that described by Altman et al. (23). Baseline staining and the threshold for tetramer reactivity were performed on unimmunized female mice.

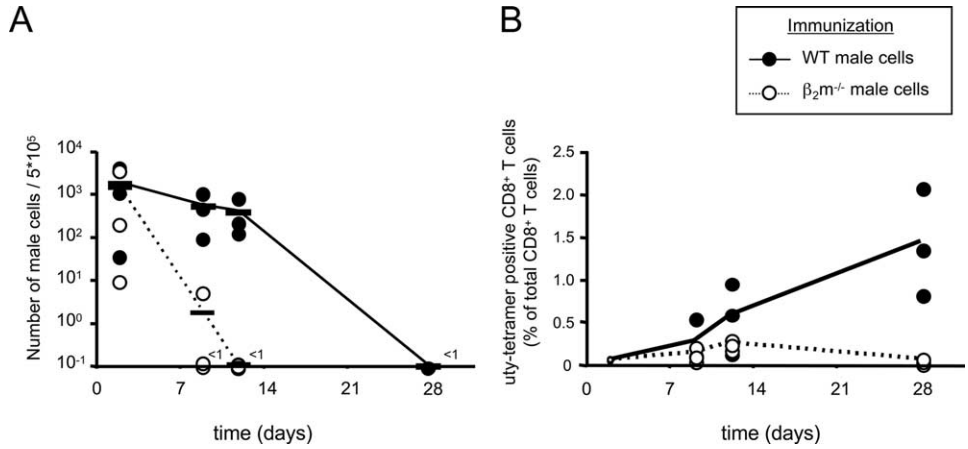

FIGURE 2. $\beta_2m^{-/-}$ males cells are rapidly cleared after immunization. *A*, Clearance of $\beta_2m^{-/-}$ male cells was assessed by quantitative measurement of male Y chromosome-specific genomic DNA. WT or $\beta_2m^{-/-}$ male cells were used to immunize female recipients. After 2, 9, 12, and 28 days, leukocytes were isolated from the DLN (shown in this experiment) and spleen (data not shown) and assayed as described in *Materials and Methods*. A follow-up experiment carefully defined the timing of $\beta_2m^{-/-}$ male cell disappearance as occurring between days 6 and 8 (data not shown). Evaluation of the nondraining lymph node indicated that the $\beta_2m^{-/-}$ male cells were not simply hiding in an alternate lymphoid organ (data not shown). Quantification of the number of male cells was performed and plotted with each individual WT (●, solid line) and $\beta_2m^{-/-}$ (○, dotted line) mouse represented. The limit of detection is a single male cell in 5×10^5 female cells. Lines depicted to connect mean values (horizontal bar). *B*, Using the remaining cells from mice described in *A*, uty-tetramer staining was performed to evaluate the expansion of uty-H2-D^b specific CD8$^+$ T cells.

Quantitative analysis of Y chromosome DNA persistence by real-time PCR

Draining lymph nodes (DLN), nondraining lymph nodes, and splenocytes were harvested and genomic DNA was extracted. Mouse Y chromosome-specific DNA was quantified relative to β-actin using the following primers: HY (forward) 5′-AGACAAGTTTTGGGACTGGTGAC-3′ and (reverse) 5′-AGCCCTCCGATGAGGCTGATA-3′; and β-actin (forward) 5′-AGACAAGTTTTGGGACTGGTGAC-3′ and (reverse) 5′-AGCCCTC CGATGAGGCTGATA-3′. Quantitative PCR was performed on genomic DNA using the SYBR Green JumpStart *Taq* ReadyMix according to the manufacturer's instructions (Sigma-Aldrich). The reactions were run on a PTC200 equipped with a Chromo4 detector (MJ Research). All the measures were performed in duplicate and validated when the difference in threshold cycle (Ct) between the two measures was less than 0.3. Amplification and dissociation curves displayed a single peak, ruling out the presence of primer dimers (data not shown). The ratio of gene of interest to housekeeping gene was calculated according to the formula ratio = $2^{-\Delta Ct}$, in which ΔCT is mean threshold cycle of gene − mean threshold cycle of housekeeping gene. Ratio was normalized to a standard curve, consisting of 10^2, 10^3, 10^4, 2×10^4, 10^5, and 5×10^5 male splenocytes in a total of 5×10^6 female splenocytes.

Quantitative analysis of male cells by enrichment and cytometric analysis

For these experiments, CD45.2 male cells were injected into CD45.1 female hosts. After 2 or 12 days, leukocytes were harvested from 13 lymph nodes (2 inguinal, 2 axillary, 2 brachial, 4 cervical (deep and superficial), 2 para-aortic, and the mesenteric chain) and the spleen, yielding a total of over 1.5×10^8 cells. Anti-CD45.2-PE Ab (eBioscience) followed by labeling with anti-PE Ab linked to iron particles (Miltenyi Biotec) were used to label and enrich the male cells using a MACS column (Miltenyi Biotec). Bound and unbound cells were labeled with anti-CD45.1-FITC (BD Biosciences), a mix of Abs to lineage markers (CD3, CD11b, CD11c, NK1.1, B220, and F4/80) all PacBlue-conjugated (eBioscience). Just before analysis, propidium iodide was added as a marker for dead cells. Approximately 1.5×10^6 cells were recovered from the bound fraction, and all cells were analyzed using a FACSCanto II (BD Biosciences). Live, nonclumped, lineage-positive cells were gated, and the CD45.2$^+$ male cells were enumerated. Total cell numbers were used to determine the absolute number of male cells that persisted in female hosts. Note, this protocol was adapted from the tetramer enrichment studies of Moon and colleagues (24, 25).

Determining persistence of H2-D^b/uty-MHC peptide complexes within female recipients

MataHari (transgenic animals on a CD45.2 C57BL/6 background) CD8$^+$ T cells were isolated from spleen and lymph nodes and labeled with 5 μM CFDA-SE in PBS. After extensive washing in ice-cold PBS, 5×10^5 MataHari CD8$^+$ T cells were injected i.v. into CD45.1 immunized female recipients. At 3 days after injection, the DLN (regional to the site of immunization) and the spleen were collected. Organs were processed independently, and cells were labeled with CD8β–PE and CD45.2-allophycocyanin Abs allowing for the identification of the transferred MataHari CD8$^+$ T cells and the determination of CFDA-SE intensity.

Results

Efficient cross-presentation does not permit effective cross-priming of H-2^b uty-specific CD8$^+$ T cells

We hypothesized that the failure to achieve efficient cross-priming of male Ag was due to rapid clearance of the male $\beta_2m^{-/-}$ cells after transfer into female hosts due to activation of NK cells (17). To test this possibility and to determine the requirements for cross-priming CD8$^+$ T cells, we established assays to discriminate the priming of H2-D^b-restricted HY uty-specific (referred to in our study as uty) CD8$^+$ T cells and the presentation of the uty peptide. To study CD8$^+$ T cell priming, we injected by intradermal route 5×10^6 male cells into the flank of female hosts. Male splenocytes were depleted of CD11c$^+$ cells to prevent direct presentation of the uty epitope to female CD8$^+$ T cells. Depletion of DCs was confirmed by FACS analysis (Fig. 1*A*). It also bears mentioning that the protein under investigation requires processing by the immunoproteosome and thus under sterile, noninflammatory conditions none of the injected cells may themselves generate H-2D^b-uty complexes (20). At 12 days after immunization, CD8$^+$ T cells were isolated (>98% purity) and restimulated using uty peptide-loaded DCs. IFN-γ ELISPOT assays were performed as a measure of T cell priming, and data are reported as spot forming cells (SFC) per 10^6 CD8$^+$ T cells. As previously shown, immunization of female hosts with $\beta_2m^{-/-}$ male splenocytes resulted in inefficient cross-priming as compared with the injection of WT male cells, which induced a robust response (Fig. 1*B*).

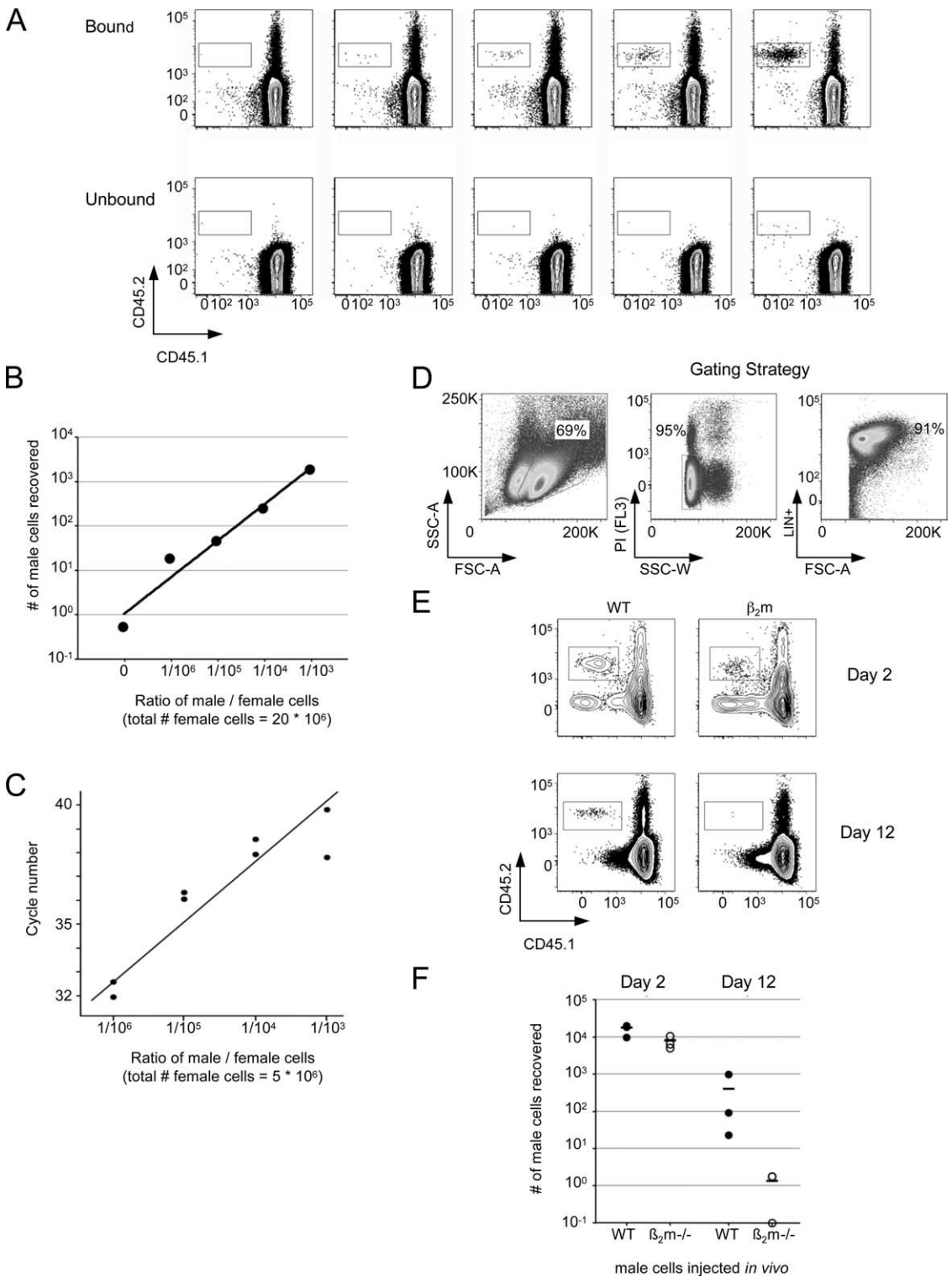

FIGURE 3. Enumeration of male cells in female mice. An in vitro mixture of CD45.2$^+$ male and CD45.1$^+$ female cells were used to validate our ability to enrich a small number of male cells in the context of female hosts. 0, 20, 200, 2000, or 20,000 male cells were added to 2×10^7 female cells. Samples were split and analyzed separately. *A*, In the first group, the cell mixture was incubated with anti-CD45.2-PE Ab followed by an anti-PE Ab coupled to an iron particle (Miltenyi Biotec). Enrichment of the CD45.2$^+$ cells was performed using magnetic columns and both the bound and unbound cells were stained using Abs specific for CD45.1 and lineage markers (mixed in a single channel). In addition, propidium iodide was added to exclude dead cells. Live, nonclumped lineage-positive cells are shown, and the gate represents the CD45.2$^+$ cells recovered. *B*, Data from *A* are graphically represented, plotting the number of male cells recovered against the input ratio (male cells to female cells) used. The limit of detection was determined to be <1 male cell in 10^6 female cells. *C*, Quantitative PCR analysis of the other fraction was performed using the methods described in *Materials and Methods*. *D–F*, For in vivo analysis of male cells persisting in female hosts, 13 lymph nodes and the spleen were harvested 2 or 12 days postimmunization with 5×10^6 WT or $\beta_2m^{-/-}$ cells. The gating strategy is shown in *D* and follows from what is described. *E*, Representative contour plots are shown for individual mice and gated cells indicate the male cells recovered. *F*, Data from 3/3 mice per group are plotted with the absolute number of male cells persisting in female mice reported. Individual WT male cells (●) or $\beta_2m^{-/-}$ cells (○) are indicated; bar indicates the average of the population. Data are representative of five experiments.

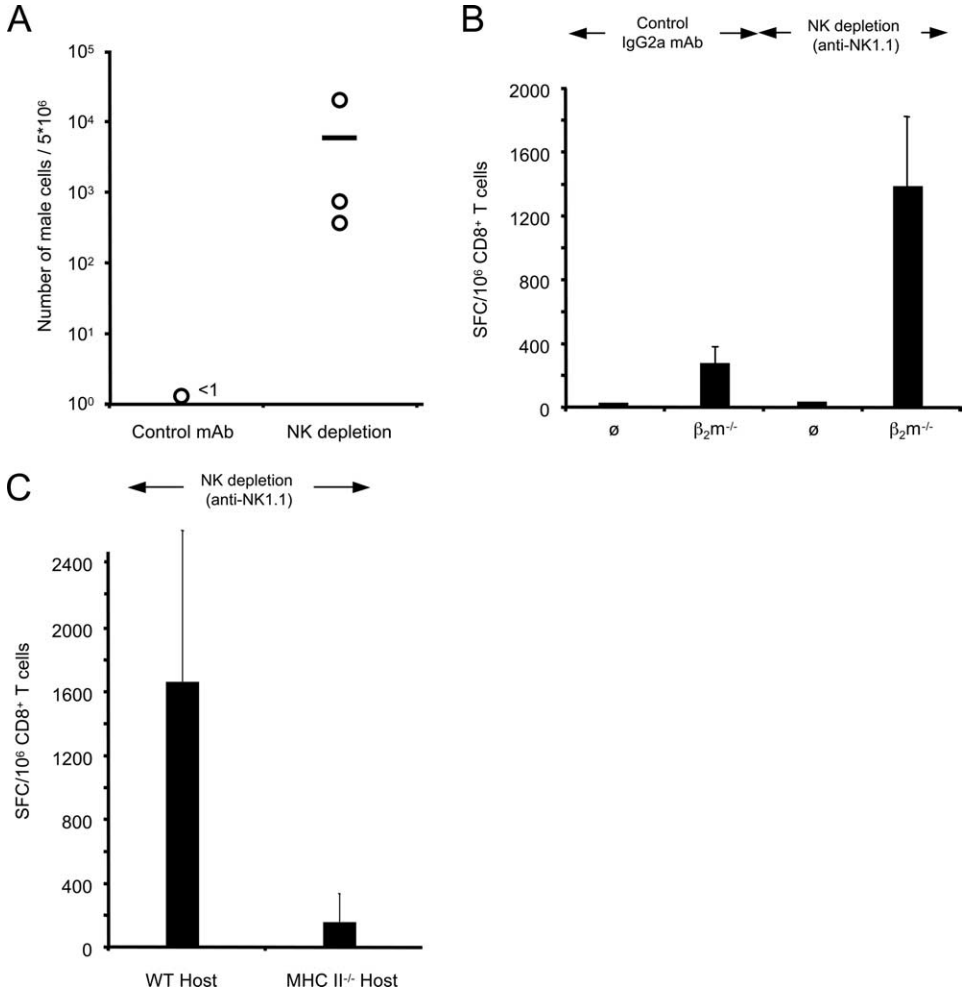

FIGURE 4. Ag persistence restores efficient cross-priming. To evaluate the effects of delaying Ag clearance, female recipients were depleted of their NK cells by repeated injection of 100 μg of anti-NK1.1 Ab or as a control, injected with similar doses of an isotype matched Ab. WT (*A* and *B*) or MHC class II$^{-/-}$ (*C*) female mice were immunized with β_2m$^{-/-}$ male cells. After 12 days, persistent male cells were enumerated (*A*) and an IFN-γ ELISPOT was performed (*B* and *C*). The number of SFC per 10^6 CD8$^+$ T cells is reported for restimulation with female DCs pulsed with uty peptide. Control wells with unpulsed DCs resulted in fewer than 10 SFC/10^6 CD8$^+$ T cells and are not shown (*B* and *C*). Mean value of three mice is shown, and error bar indicates the SEM. Data are representative of three independent experiments.

We next evaluated whether the failure to cross-prime CD8$^+$ T cells following injection of the β_2m$^{-/-}$ male splenocytes was due to inefficient cross-presentation. Using CFDA-SE-labeled uty-specific TCR transgenic CD8$^+$ T cells (called Matahari) (18), we demonstrate that in fact cross-presentation of uty Ag results in the vigorous expansion of Ag-specific CD8$^+$ T cells (Fig. 1*C*). These data indicate that although the cross-presentation of cell-associated uty is efficient and initial T cell encounter is intact, the indirect pathway does not provide a robust means of priming the endogenous repertoire of female mice.

To evaluate the clearance of male Ag in these priming conditions, we established a quantitative assay to measure the number of male cells that persist in female hosts. Amplification of genomic DNA unique to the Y chromosome allowed for the detection of one male cell within 5×10^5 female cells (data not shown). As expected, WT male cells survived throughout the period of time required for primary expansion and differentiation of the uty-specific CD8$^+$ T cells; however, the β_2m$^{-/-}$ male splenocytes were cleared within 6–8 days (Fig. 2 and data not shown). In the experiment shown, we did not observe contraction of the CD8$^+$ T cells after WT male cell immunization, but based on other kinetic studies this phase of the response seems to occur between 21 and 45 days postimmunization (data not shown).

To confirm our quantitative PCR-based results using a cell-based methodology, we used congenic markers on the male cells and magnetic enrichment before FACS analysis, which together allowed us to detect 10 male cells in a mixture of 20×10^6 female cells (Fig. 3, *A* and *B*). This assay was in concordance with our PCR analysis (Fig. 3*C*). For in vivo analysis following immunization with male cells, we harvested the spleen and 13 lymph nodes yielding a total of $\sim 1.5 \times 10^8$ cells. Using this enrichment method, we concentrated the male cells in a total of $\sim 1.5 \times 10^6$ cells and analyzed all cells using a FACSCanto II cytometer. Live, nonclumped cells were identified based on size, scatter, and their negative result for propidium iodide incorporation (Fig. 3*D*). From these cells, the lineage-positive cells were positively gated (Fig. 3*D*). Female mice injected with 5×10^6 males cells were assayed at days 2 and 12. Results from representative mice are shown (Fig. 3*E*) and the absolute number for three mice per group has been plotted for the two time points (Fig. 3*F*). A similar absolute number of WT and β_2m$^{-/-}$ males cells could be found at day 2; and as described on day 12, we observed the persistence of male cells in the WT group, but not in the mice that had received β_2m$^{-/-}$ male cells.

We next wanted a direct test of the observed correlation; in other words it was important to determine whether the lack of Ag persistence accounted for the inefficient priming when β_2m$^{-/-}$ male cells were used. As NK cells were the likely cause of death for the β_2m$^{-/-}$ splenocytes, we tested the effect of depleting female hosts of NK cells using anti-NK1.1 Ab. NK cell depletion was confirmed by cytometry (data not shown). To avoid potential opsonization by the depleting Ab used for NK depletion, NK cells were removed from the injected male cells. At 12 days postimmunization with β_2m$^{-/-}$ male splenocytes, the female host mice were sacrificed and as described, the number of male cells and the frequency of IFN-γ-producing HY-specific T cells were measured.

Indeed, NK depletion slowed the clearance of $\beta_2m^{-/-}$ male cells (Fig. 4*A*), thus allowing for efficient priming of uty-specific $CD8^+$ T cells (Fig. 4*B*). In addition, we confirmed that cross-priming in NK-depleted hosts required $CD4^+$ T cell help (Fig. 4*C*). Of note, we have shown that NK depletion of the host does not alter the priming efficiency of $CD8^+$ T cells when using WT male cells for the immunization (data not shown). Together, Figs. 1–4 demonstrate that cross-priming of uty-specific $CD8^+$ T cells is critically dependent on Ag persistence as well as the presence of $CD4^+$ T cell help.

Cross-priming requires Ag persistence due to the intrinsic need for CD4$^+$ T cell help

Given the data that $CD8^+$ T cells may be primed after only a short contact with cognate MHC class I-peptide complexes (10), it was important to determine whether the need for persistent Ag was in fact functionally linked to the requirement for $CD4^+$ T cell help. In other words, we evaluated whether uty-specific $CD8^+$ T cell cross-priming fails due to the inability to generate a strong HY-specific $CD4^+$ T cell response. WT mice (harboring only the endogenous repertoire) were immunized with $\beta_2m^{-/-}$ male splenocytes while providing sustained stimulation for the HY-specific $CD4^+$ T cells. To achieve this priming condition, we coadministered WT DCs loaded with the immunodominant MHC class II epitope derived from the male Ag HY-dby (DC/dby). As a control, DCs were loaded with an irrelevant MHC class II peptide derived from OVA (DC/ova). The coadministered DCs were i.v. injected to decrease the possibility that they capture the intradermally injected male cells. Notably, the additional stimulation for $CD4^+$ T cells permitted efficient cross-priming of uty-specific $CD8^+$ T cells (Fig. 5*A*). Interestingly, the DC/dby but not the DC/ova were capable of providing this "extra help," suggesting that this effect was dependent on the activation of a population of helper cells that has the ability to engage APC-presenting Ag derived from the $\beta_2m^{-/-}$ male splenocytes. As additional controls for this experiment, we confirmed that the immunization with DC/dby did not alter the kinetics of clearance of the $\beta_2m^{-/-}$ male cells (Fig. 5B and data not shown), and we demonstrated that the effect was dependent on $CD4^+$ T cells as priming did not occur in MHC class II-deficient mice (data not shown).

Availability of cross-presented MHC class I-peptide complexes in the presence of extra help

To explain how the $CD4^+$ T cells that are primed by the DC/dby influence the cross-priming of $CD8^+$ T cells, we tested whether the injection of DC/dby acts in *trans*, by increasing the survival of cross-presenting host APCs. To test this possibility, we established an in vivo assay that evaluates the persistence of uty-H2-D^b complexes on host APCs, measured as a function of MataHari cell division. As described, female mice were immunized intradermally with either WT or $\beta_2m^{-/-}$ male splenocytes at day 0 of the experiment. CFDA-SE-labeled Matahari were adoptively transferred on the day before immunization (day −1), day 7, or day 11 postimmunization (Fig. 6*A*). MataHari proliferation in the DLN was measured on days 3, 10, or 14, respectively, and the percentage of divided MataHari (as a fraction of the total MataHari detected in recipient mice) is reported. As expected, 3 days postimmunization, MataHari proliferation was observed in the DLN for both conditions (Fig. 6*B*). By the time of the second MataHari cohort (days 7–10), we observed differences between the WT and $\beta_2m^{-/-}$ conditions, and this difference became much more evident at the third time point (days 11–14). Together with the quantitative analysis of surviving male cells (Fig. 2*A*), we conclude that the $\beta_2m^{-/-}$ splenocytes are cleared from female mice by day 8, and

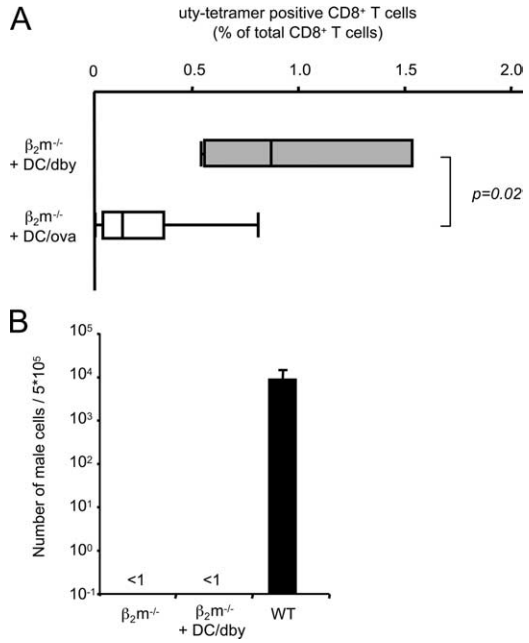

FIGURE 5. Efficient cross-priming may be achieved by providing extra help. *A*, Female mice were immunized with both $\beta_2m^{-/-}$ male splenocytes (delivered intradermally) and 5×10^5 DCs pulsed with the I-A^b-restricted epitope derived from the HY protein dby (i.v. administered). As a test for the requirement of cognate Ag, we also used DCs pulsed with the I-A^b-restricted epitope derived from OVA. Tetramer staining was performed to monitor the expansion of uty-specific $CD8^+$ T cells. Box and Whisker plots are shown and a Mann-Whitney *U* test was perfomed to calculate the *p* value. *B*, Quantification of the number of male cells was performed and average values from three mice are shown with the immunization with male WT splenocytes serving as a positive control.

the presentation of uty-H2-D^b complexes decays soon after, with insufficient levels of expression by day 11 to initiate robust proliferation of MataHari $CD8^+$ T cells.

Using this assay system, we compared the availability of uty-H2-D^b complexes in mice that received $\beta_2m^{-/-}$ male splenocytes, injected alone or in combination with DC/dby. At day 9, a time point in which the cross-presented uty-H2-D^b complexes were beginning to wane, we find that the coadministration of DC/dby could preserve Ag presentation as indicated by the increased percentage of divided MataHari cells (Fig. 6*C*). In an independent experiment ($n = 5$ mice per group), we compared the availability of uty-H2-D^b complexes in mice that received $\beta_2m^{-/-}$ coadministered with DC/dby vs mice that were immunized with WT male cells. Importantly, the extra help provided by DC/dby offered the necessary signals to achieve a level of uty-H2-D^b persistence that was equivalent to what is seen with the injection of WT male cells (data not shown). Based on these results and on the ability of extra help to restore effective $CD8^+$ T cell priming, we conclude that $CD4^+$ T cells act in *trans* to delay the disappearance of MHC class I-peptide complexes generated by Ag cross-presentation.

These experiments evaluate the question of whether Ag-specific cross-priming depends on the continued engagement of the TCR, or on the conditions in which the Ag is initially encountered. As a final control, we returned to the observation that cross-presentation of uty-derived $\beta_2m^{-/-}$ male cells is robust (Fig. 1*B*). In considering this result, we had to clarify why TCR engagement of Ag-specific $CD8^+$ T cells did not result in an autopilot response (10). One important caveat could account for our results: due to an insufficient number of H-2D^b-uty complexes, it may not be possible

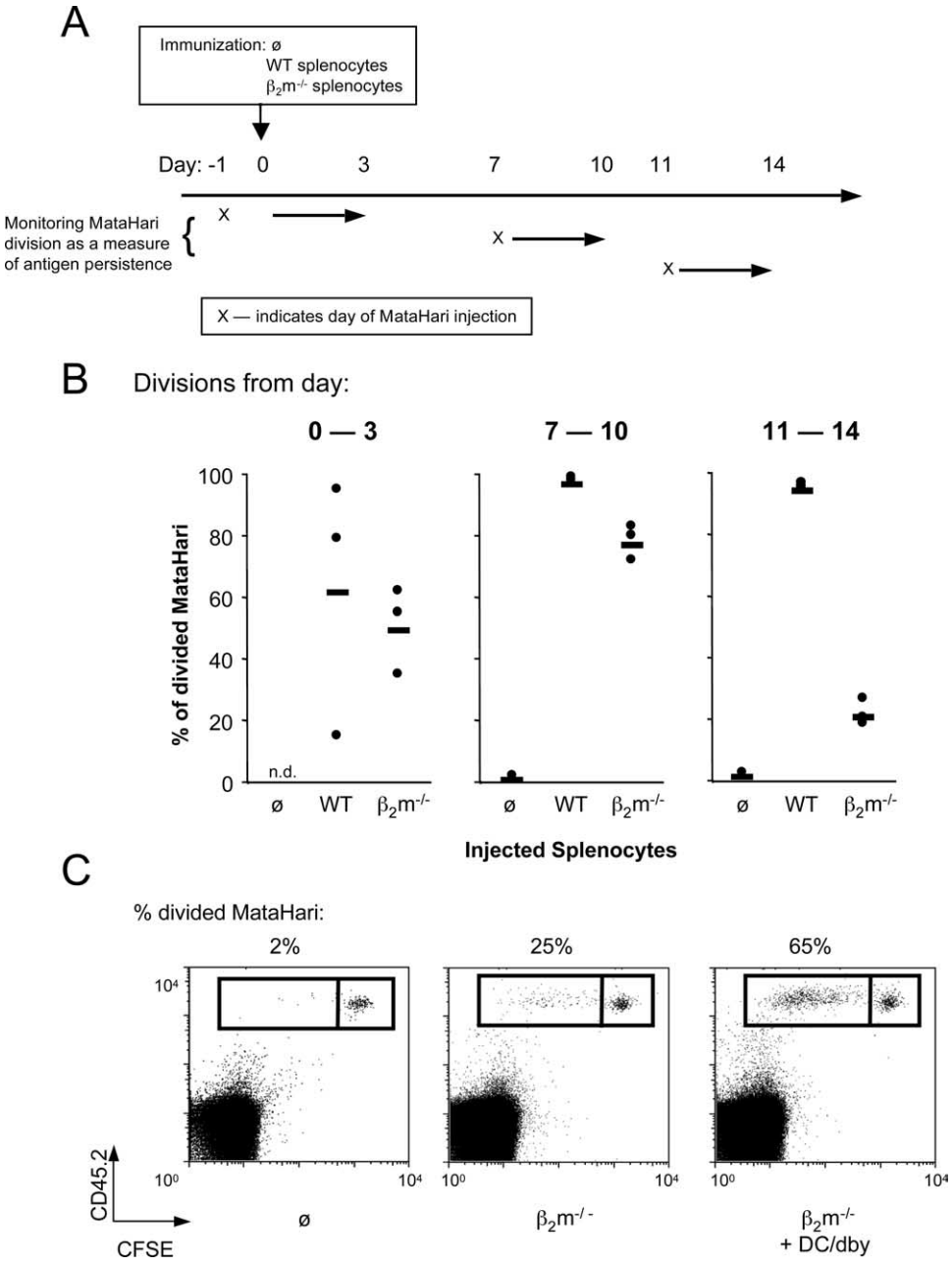

FIGURE 6. Persistence of cross-presented class I Ag is extended by extra help. *A* and *B*, At day 0, female C57BL/6 mice were immunized intradermally with WT or $\beta_2m^{-/-}$ male CD11c-depleted splenocytes (5×10^6 cells/mouse). Schematic representation of the experiment shows three independent cohorts of CFDA-SE-labeled MataHari T cells were injected i.v. to determine the availability of H-2D^b-uty complexes. On days 1, 7, and 11 and 3 days later, the dilution of CFDA-SE in the dividing MataHari T cells was evaluated by FACS. Results are expressed as a percentage of divided MataHari T cells over the total MataHari T cells in the DLN. Each individual mouse is represented, and the horizontal bar represents the mean. *C*, Using the approach detailed in Fig. 5, we monitored the division of MaraHari T cells in female mice coimmunized with $\beta_2m^{-/-}$ male splenocytes (delivered intradermally) and 5×10^5 DCs pulsed with dby (i.v. administered). Data from the interval 9–12 days after immunization are shown. The percentage of divided MataHari is reported as a percentage of the total MataHari CD8$^+$ T cells. Data from one of two representative experiments are shown. FACS plots are a composite representation of $n = 5$ mice, normalized to the absolute number of CD8β^+ T cells in the DLN.

to trigger an autopilot response in the HY system. Again, using adoptive transfer of naive MataHari CD8$^+$ T cells, it was possible to exclude this possibility. It has been shown that by adoptive transfer of a high number of monoclonal T cells, CD8$^+$ T cells are able to help themselves (26, 27). Indeed, this result is true for the HY model as well (Fig. 7*A*). As $\beta_2m^{-/-}$ male splenocytes were injected into

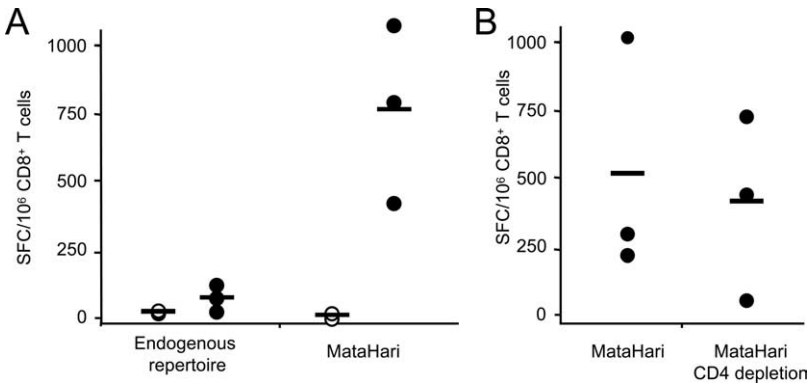

FIGURE 7. High precursor frequency of HY CD8$^+$ T cells obviates the need for persistent Ag and renders cross-priming helper-independent. *A*, A total of 10^5 MataHari T cells were adoptively transferred into female mice. Female C57BL/6 mice were immunized intradermally with WT or $\beta_2m^{-/-}$ male CD11c-depleted splenocytes (5×10^6) as described in Fig. 6. IFN-γ ELISPOT was performed to assess the efficiency of cross-priming. *B*, Depletion of CD4$^+$ T cells was performed 1 day before the immunization, and CD8$^+$ T cell priming was assessed. Restimulation in the ELISPOT was performed with uty-pulsed DCs (●) and DCs alone (○).

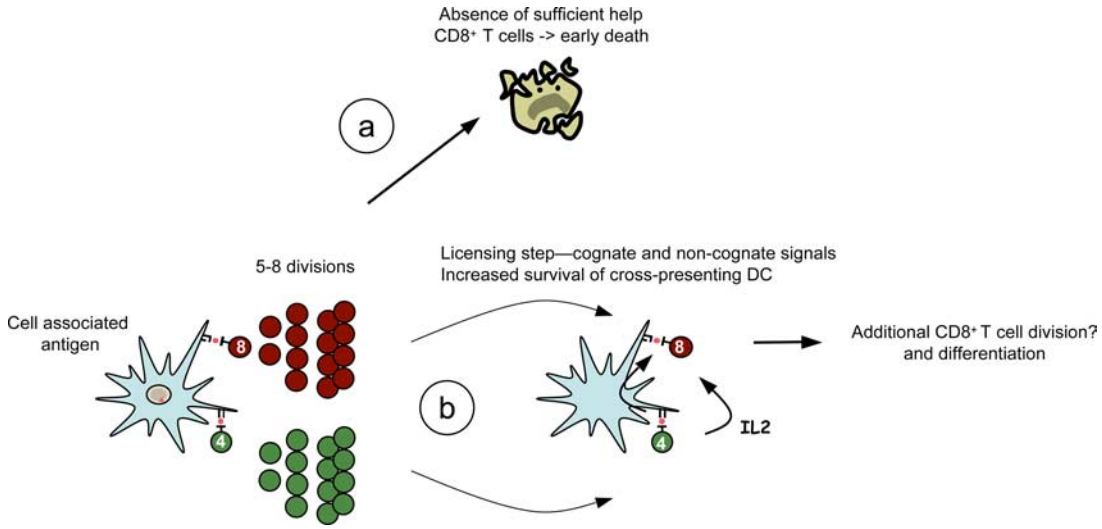

FIGURE 8. Cross-priming $CD8^+$ T cells requires extra help. During physiologic situations of priming, DCs present captured Ag to $CD8^+$ and $CD4^+$ T cells. This mechanism results in several rounds of divisions, and for $CD8^+$ T cells, full expansion in the absence of additional TCR engagement is possible. However, in the absence of a licensing signal, this results in an abortive response as the $CD8^+$ T cells undergo apoptosis (a). We demonstrate that for helper-dependent responses, Ag persistence is required, not necessarily for the $CD8^+$ T cells, but also for the priming and maintenance of $CD4^+$ Th cells that serve to license DCs to cross-prime. We propose that this licensing decision is made after an initial round of $CD8^+$ T cell divisions (b), and as such, there may also be a requirement for $CD8^+$ T cells to re-encounter DCs that are harboring cognate Ag. This work challenges the current interpretation of the autopilot response and resolves several conflicting aspects of DC and $CD8^+$ T cell engagement by discriminating initial engagement of the TCR from the licensing step, which is critically dependent on Ag persistence in physiologic responses.

female mice that had received 10^5 MataHari $CD8^+$ T cells, it was in fact possible to achieve efficient cross-priming. $CD4^+$ T cell depletion confirmed that with an increased precursor frequency of $CD8^+$ T cells, the priming is helper-independent (Fig. 7*B*). Therefore, we conclude that for the endogenous repertoire, in which Ag reactive T cells are rare, cross-priming requires persistent Ag. The persistence is actually playing a dual role: first for the priming of $CD4^+$ Th cells, which in turn prolonged presentation of the MHC class I epitope. Together, these results suggest that DC licensing is a delayed event, thus providing a refined model for how helper-dependent $CD8^+$ T cell priming occurs.

Discussion

The experiments described in this study evaluate the question of whether helper-dependent cross-priming requires the persistence of Ag. We used the weakly immunogenic HY model Ag in which priming of uty-specific $CD8^+$ T cells is dependent on $CD4^+$ T cell help during the priming phase (28). Quantitative PCR was used as a highly sensitive method for the detection of male genomic DNA (Fig. 2*A* and data not shown). In addition, we used magnetic enrichment of transferred cells as a means of measuring Ag persistence in its cellular form (Fig. 3). These systems allowed us to dissect the role of Ag as a limiting resource; and we demonstrate that indeed the lack of Ag availability accounts for the inefficient cross-priming of minor histocompatibility Ag-specific T cells (Figs. 1–4). In studying this problem further, we demonstrated that Ag is required for generating an effective helper cell compartment: coadministration of DCs presenting only MHC class II-peptide complexes provided extra help and permitted effective cross-priming of Ag derived from the short-lived $\beta_2m^{-/-}$ splenocytes (Fig. 5). At first look, however, this result did not fit with the data that $CD4^+$ T cells interact with the same APC as the $CD8^+$ T cell, thus breaking with the idea of epitope linkage and upsetting the notion of cognate help. The real surprise, and the solution to this paradox, came when we began tracking the MHC class I-peptide complex on the cross-presenting APC. The presence of an additional stimulus for the $CD4^+$ Th cells resulted in an increased survival of the cross-presenting APC (Fig. 6). These data are supported by the recent observation that CD40 engagement on DCs results in increased survival (29). In this study, exogenous stimulation of CD40 was equated to sustained expression of endogenous Ag. Our work advances this observation and we have integrated their findings into observations that concern helper-dependent $CD8^+$ T cell cross-priming. An alternate, though not favored hypothesis, is that the availability of $CD4^+$ T cells permits serial cross-presentation as this could also account for an increase in half-life of the MHC class I-uty complexes. Thus, we conclude that $CD8^+$ T cells require Ag persistence, not simply for the maintenance of $CD4^+$ Th cells that are serving to license DCs, but perhaps also to complete their own differentiation process, executed by multiple contacts with DCs (Fig. 8).

These data have pushed us to re-evaluate the requirements for naive $CD8^+$ T cells to set in motion a developmental program that includes multiple rounds of division and the acquisition of effector function. Three related studies form the bedrock of the $CD8^+$ T cell autopilot model. In two of the studies, injection of high CFU counts of *L. monocytogenes* resulted in recruitment of Ag-specific naive $CD8^+$ T cells during the first 24 h (7, 11). Antibiotics were used to kill off the Ag and still, in the absence of further TCR engagement, the responding T cells were capable of dividing and differentiating into effector cytolytic T cells (7). In the third study, using an engineered APC, thus permitting control over the kinetics of antigenic stimulation, the time required to achieve $CD8^+$ T cell programming was carefully determined. As little as 20 h of exposure to APCs can trigger subsequent divisions and differentiation (9). From these studies, it was concluded that after initial encounter with Ag, the priming of $CD8^+$ T cells does not rely on additional TCR engagement nor on $CD4^+$ T cell help (10). These data, however, must be considered in the face of more recent work, as our understanding of helper-dependent $CD8^+$ activation has been radically modified. $CD8^+$ T cells that are primed in the absence of help, fail to respond during secondary restimulation (30, 31). The

mechanism for this helper effect may act via the APC, resulting in the programming of naive T cells as supported by Janssen et al. (30). Alternatively, other models support a role for Ag nonspecific $CD4^+$ helper cells that provide survival signals for the maintenance of a high precursor frequency of memory $CD8^+$ T cells (32). What is clear is the need to differentiate the signals involved in $CD8^+$ T cell proliferation from those that govern the acquisition of effector functions. Additionally, these results point out that much of our understanding about the priming of naive T cells was contingent on either the use of TCR transgenic $CD8^+$ T cells or assays that evaluated secondary restimulation.

Two prior studies have concluded that Ag persistence is required for efficient $CD8^+$ T cell priming. Curtsinger et al. (33) evaluated the requirement for priming OVA-specific TCR transgenic T cells. In their in vivo experiments, they adoptively transfer $>10^6$ OT-I and prime with peptide as a source of $OVA_{257–264}$. Although their work offers important information about competition for Ag when the precursor frequency of $CD8^+$ T cells is high, it does not bear on the requirements for priming endogenous T cell responses. In the second study, Storni et al. (34) compared p33 lymphocytic choriomeningitis virus peptide immunization with viral-like particles expressing the p33 peptide fused to the C terminus of hepatitis B c Ag. The results demonstrate that peptide plus CpG fails to maintain robust p14 TCR transgenic T cell proliferation, whereas viral-like particles plus CpG increases $CD8^+$ T cell priming and enhances protection from subsequent vaccinia virus challenge. Based on the short half-life of peptide for the stimulation of $CD8^+$ T cells, the researchers conclude that Ag persistence is an important component for efficient priming. Again, this second model is $CD4^+$ Th cell-independent during the primary $CD8^+$ T cell stimulation (35, 36), and does not address cross-priming. Finally, there is the new study from Tyznik and Bevan (37) concerning the priming of male-specific responses. The report indicates that male $CD8^+$ T cells within the inoculate suppress the priming of female anti-HY $CD8^+$ T cell responses. This result is observed, however, only at high numbers of injected cells (i.v. route); in comparison, we use 4-fold fewer cells (injected intradermally) for immunization and thus do not see evidence for veto suppression in our model system.

So how does our newfound recognition of the importance of $CD4^+$ T cells alter the interpretation of the autopilot model for $CD8^+$ T cell activation? Drawing from the conclusions of our study on helper-dependent cross-priming, it would follow that in the absence of persistent Ag but the presence of a high precursor frequency of responding $CD8^+$ T cells (as was the case for the autopilot studies (7, 9, 11)), $CD8^+$ T cells would divide and differentiate into effector cells; however, secondary engagement would result in an abortive response due to the absence of $CD4^+$ T cells during the priming phase. We conclude that for helper-dependent priming, such as the cross-priming of minor histocompatibility Ag-specific T cells, persistent Ag is required to support the differentiation of effector $CD8^+$ T cells. For the recent study by Prlic et al. (38), we see no conflict because their model system does not have a strict requirement for $CD4^+$ T cell help.

In summary, we propose that under physiologic conditions, $CD4^+$ T cells provide help in a cognate manner and as such, support the creation of a local microenvironment. In particular, the helper cells facilitate the persistence of MHC class I-peptide complexes that provide "signal 1" for cross-priming of $CD8^+$ T cells. Such a model would predict that during physiologic situations of priming, the decision between activation and tolerance may be made after the initial round of $CD8^+$ T cell divisions (Fig. 8). This revised model clarifies the distinct outcomes of the initial contact between DCs and $CD8^+$ T cells. When the outcome is cross-tolerant, we know that $CD8^+$ T cells engage DC cross-presenting tissue-derived Ag, and they undergo several rounds of divisions and proceed to die (1, 26) (Fig. 8). If during this window time period, DCs are licensed by $CD4^+$ T cells, the response may be converted with activation signals being provided to the $CD8^+$ T cells, rescuing them from programmed cell death. By delaying the immunologic decision until a secondary encounter, we offer a revised interpretation for the autopilot response, and with an expanded population of responder T cells, our data may also account for how a rare three-cell interaction is possible under physiologic conditions (39, 40). These results will impact our understanding of graft-versus-host disease and influence vaccination strategies that are aimed at priming $CD8^+$ T cell responses.

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Disclosures

The authors have no financial conflict of interest.

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