Sustained antigen presentation can promote an immunogenic T cell response, like dendritic cell activation

Reinhard Obst*†, Hisse-Martien van Santen*‡, Rachel Melamed*, Alice O. Kamphorst§, Christophe Benoist*¶, and Diane Mathis*¶

*Section on Immunology and Immunogenetics, Joslin Diabetes Center; Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02215; †Institute for Immunology, Ludwig Maximilians University, Goethestrasse 31, 80336 Munich, Germany; and §Laboratory of Molecular Immunology and Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10021

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Activation of dendritic cells (DCs) enhances their ability to prime naïve T cells. How activation renders them immunogenic rather than tolerogenic is unclear. Here, we show, using temporally regulated expression of a transgene-encoded neoself antigen in DCs, that either prolonged antigen presentation or DC activation could elicit full expansion, effector cytokine production, and memory-cell differentiation. Microarray analysis of gene expression in T cells showed that all changes linked to DC activation through CD40 could be reproduced by persistent antigen delivery, suggesting that stabilization of antigen presentation is an important consequence of DC activation *in vivo*. In this system, DC activation by CD40 engagement indeed extended their ability to present antigen to CD4$^+$ T cells *in vivo*, although different results were obtained with antigen delivered to DCs by means of endocytosis from the cell surface. These results suggest that antigen persistence may be an important discriminator of immunogenic and tolerogenic antigen exposure.

immune response | immune tolerance | regulated transgene | MHC class II

The interaction of naïve T cells with antigen-presenting DCs is crucial for the initiation of T cell-dependent immune responses but can also result in T cell deletion, anergy, or diversion to a regulatory cell phenotype. Numerous *in vitro* studies have demonstrated that activation of DCs leads to more effective T cell priming, whereas presentation by immature DCs elicits abortive activation or anergy. After activation, DCs undergo a number of phenotypic changes that may explain their stronger stimulatory capacity: increased MHC protein expression, up-regulation of adhesion and costimulatory molecules, and induction of chemo- and cytokine secretion. The relative role of these components for T cell responses, however, remain unclear (1–4).

The importance of sustained signaling via the T cell receptor (TCR) for T cell commitment to expansion and effector gene expression was first shown *in vitro*, by using tumor cells, T cell lines, and TCR-transgenic T cells (5–7). Iezzi *et al.* (8) proposed that the duration of antigen presentation is "the major factor" determining T cell behavior. Using an approach wherein antigen presentation to CD4$^+$ T cells can be controlled by a transgenic switch *in vivo*, we have recently shown that antigen persistence tightly controls the expansion of CD4$^+$ T cells.

Our studies showed, unexpectedly, that presentation of a neoself antigen is effective in the absence of DC activation, if persistent. We now ask whether DC activation modifies these parameters. Previous *in vitro* experiments revealed that MHC class II molecules are stabilized at the cell surface of DCs upon activation, a phenomenon summarily referred to as "antigenic memory" (9–11) and recently found to be regulated by ubiquitination (12–14). By examining the genomic signature of T cells triggered by antigen presented by resting or activated forms of DCs, we find that the programmatic differences elicited in T cells by DC activation can be reproduced by enforcing antigen persistence on the DCs. The results show that antigenic memory in DCs does occur *in vivo*, at least with some modes of epitope delivery to MHC molecules, and suggest that the persistence of peptide/MHC complexes is an important element used by activated DCs for optimal CD4$^+$ T cell priming.

Results

Prolonged Antigen Expression Can Replace DC Activation for Effector Cell Differentiation. To investigate how DC activation might affect the kinetics of MHC class II-restricted antigen presentation *in vivo*, we used a double-transgenic mouse line (hereafter dtg) described previously, wherein DC presentation of a peptide/MHC-II complex to CD4$^+$ T cells can be manipulated over time (8). In the presence of doxycycline (dox), a reverse tetracycline-dependent transactivator (Ii-rTA) induces the transcription of a second transgene that encodes an invariant chain (Ii) cDNA whose CLIP region was replaced by the H2-E^k-binding epitope of moth cytochrome *c*, $(MCC)_{93–103}$ (*TIM*) (15, 16). The tetracycline-inducible invariant chain with MCC (TIM) protein also carries a C-terminal amino acid replacement, such that it effectively shuttles the MCC peptide into the MHC class II processing pathway but does not compete with endogenous Ii (17). *TIM* expression was found almost exclusively in CD11c$^+$ DCs (8) and about equally in the CD8$^-$, CD8$^+$, and CD11c^{int}120G8$^+$ subsets (data not shown). Dtg animals were used as recipients of T cells transferred from the AND TCR transgenic line (18).

In dtg mice, expression of the MCC/E^k epitope is extinguished with a half-life of ≈1 day after dox removal. In our previous experiments with fluorescein (CFSE)-labeled AND T cells, the early termination of antigen exposure *in vivo* led to incomplete T cell activation (8). We asked whether DC activation, for example by triggering CD40, might be having the same effect as persistent antigen. Thus, we compared CD4$^+$ T cells expanding under brief or long antigen presentation, by DCs that were activated with the stimulatory αCD40 mAb FGK45.5 or not. Sixty hours after transfer,

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Abbreviations: DC, dendritic cell; TCR, T cell receptor; Ii, invariant chain; dox, doxyxycline; MCC, moth cytochrome *c*; TIM, tetracycline-inducible invariant chain with MCC; dtg, double transgenic; FC, fold change; CFSE, carboxyfluorescein diacetate succinimidyl ester.

‡Present address: Centro Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, 28049 Madrid, Spain.

¶To whom correspondence may be addressed at: Section on Immunology and Immunogenetics, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215. E-mail: cbdm@joslin.harvard.edu.

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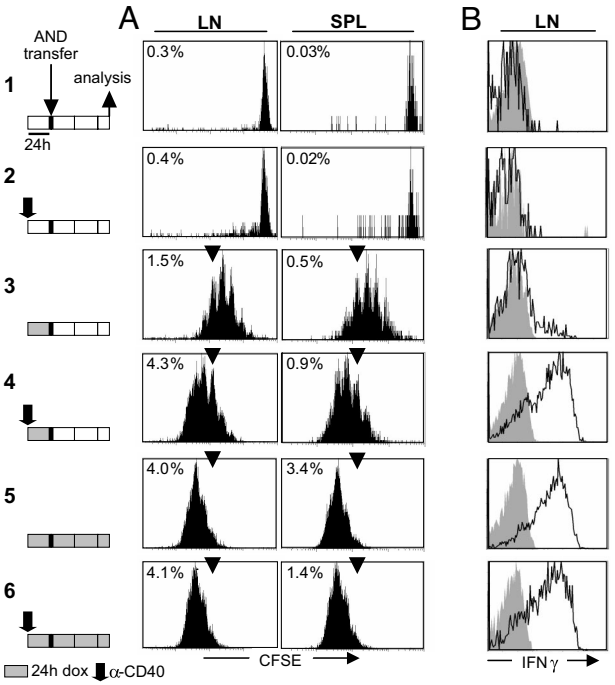

Fig. 1. Comparison of CD4$^+$ T cell responses in animals with transient and persistent TIM expression and activated DCs. (*A*) Lymph node (LN) and spleen (SPL) cells of dtg recipients of CFSE-labeled CD90.1$^+$ AND T cells were analyzed 60 h after transfer. Dtg mice were treated with dox for 1 or 3.5 d (gray boxes), and treated with a stimulatory αCD40 mAb (thick black arrow). Numbers indicate the percentages of donor cells among CD4$^+$ cells. Representative of three experiments. (*B*) For IFNγ analysis, lymph node cells were restimulated for 6 h and stained intracellularly for IFNγ (open histogram) or with an isotype control-Ig (filled histogram). Results are representative of two experiments. Histograms are gated on CD4$^+$CD90.1$^+$ cells.

labeled AND T cells exposed to waning numbers of MCC/E^k complexes divided 3–5 times and stopped, whereas those transferred into cage-mates that had received an αCD40 injection proliferated more extensively (Fig. 1*A*, conditions 3 and 4). AND cells transferred into animals wherein DCs expressed TIM throughout the experiment divided 6–8 times, but αCD40 injection had little effect in this instance (Fig. 1*A*, conditions 5 and 6). IFNγ, an indicator of effector cell differentiation, was found in cells that had divided extensively, resulting from either long exposure to antigen or αCD40 treatment (Fig. 1*B*).

These data suggested that persistent antigen was having the same effect as voluntary DC activation. One caveat was that DCs were being indirectly activated by the antigen-specific T cells to which they were presenting cognate peptide. Two lines of evidence showed that this was not the case. First, DCs from dtg recipients of AND T cells were compared with those from PBS- and αCD40-treated animals [supporting information (SI) Fig. 6*A*]. Although the AND T cells were activated and expanded, no significant up-regulation of activation markers CD80, CD86, and MHC class II could be observed on DCs 24 or 60 h after T cell transfer. Second, and more directly, we asked whether pretransfer of AND T cells into the dtg mice would enhance presentation by DCs to a second wave of AND T cells transferred 60 h later in the same hosts (and distinguishable by the CD90.1 marker). Rather than being enhanced, the proliferation of this second wave of AND cells was actually suppressed (SI Fig. 6*B*). Thus, and in keeping with previous results (19), responding AND T cells do not mimic αCD40 to license them for improved CD4$^+$ T cell priming later on. In addition, not all DC activators behaved similarly, and TLR ligands such as CpG oligonucleotides only poorly extended MCC presentation (data not shown).

Because it is possible that the precursor frequency of T cells encountering antigen-presenting cells (APCs) impacts their behavior (20, 21), we performed the same transfers with graded cell numbers. The data in SI Fig. 7 show that cell proliferation was similarly enhanced by αCD40 treatment and persistent antigen, irrespective of the number of transferred cells. This indicates that the *TIM* turn-off is detected by T cells at high and low precursor frequencies in a similar way and that competition or crowding effects do not operate in our transfer system.

Gene Expression Analysis Demonstrates Antigen Persistence as a Key Component of DC Activation. There are two possible explanations for the effect of αCD40 in the context of brief antigen expression: that the activated DCs delivered costimulatory signals that allowed the T cells to overcome inadequate antigen exposure, or that activated DCs retained and presented the agonist peptide for longer periods of time. Stated in classical terms, the DCs were providing Signal 2 or were just stabilizing Signal 1. To distinguish between these two possibilities, we assessed the gene-expression profile of AND T cells responding under the four conditions described above (Fig. 2*A*). Should the first explanation apply, we would expect to find a distinct signature of costimulatory influences; explanation 2, on the other hand, would predict similar profiles elicited by long exposure and by short exposure plus DC activation. Congenically marked AND lymph node cells were transferred into control hosts or into dtg recipients whose DCs expressed the *TIM* transgene transiently or continuously and were treated with the αCD40 mAb or not (Fig. 2*A*). An overview of the results is displayed in Fig. 2*B*, where the gene-expression profile for each condition is compared with that of cells transferred into control recipients (condition 1). Widespread differences were observed under conditions 4–6, less extensive changes were seen with the transient *TIM* expression of condition 3, and very few changes were detectable in condition 2.

We first compared in detail the changes that occurred with transient versus sustained expression of antigen, using the ratio or fold change (FC)/FC plots that visualize the FC relative to a control condition (Figs. 2*C* and 3). In such plots, data points crowd around the $x = y$ diagonal if there is no or little difference in gene expression under the two induction regimens. However, most of the induced genes were more strongly induced (or repressed) in AND T cells confronted with antigen for the whole of their expansion phase (condition 5 in Fig. 2*A*) versus with transient exposure to antigen (condition 3). The bulk of the induced genes fell off the ratio plot's diagonal as did most of the repressed genes (Fig. 2*C Upper*), indicating that interruption of antigen presentation dramatically altered the expression of hundreds of genes.

We found that several groups of genes could be distinguished on the basis of their behavior in response to disappearing antigen (Fig. 2*C Lower*; a full listing is presented in SI Tables 1–3): the expression of some genes appeared much more dependent on continuous TCR signals than others. The genes represented by green data points (group A) (Fig. 2*C Lower*) responded essentially the same to the two conditions. Thus, because expression of these genes (including *CD122*, *Stat4*, *Cxcr3*, *Ctla2*, and *Ctla4*) did not require sustained TCR engagement, their regulation departs from that of the bulk of genes induced upon activation of CD4$^+$ T cells. In contrast, the group B transcripts, labeled blue in Fig. 2*C Lower*, were expressed ≈2-to 4-fold higher in T cells continuously triggered by peptide/MHC. Genes implicated in effector T cell differentiation like *Maf*, *Tbx21* (encoding T-bet), and *Icos* lie in this group as well as *IFNγ*, confirming the notion that persistent antigen is necessary for elicitation of effector function, as shown in Fig. 1. Interestingly, this group was also highly enriched in genes related to DNA metabolism and cell cycle progression (SI Table 2), a finding that was confirmed by

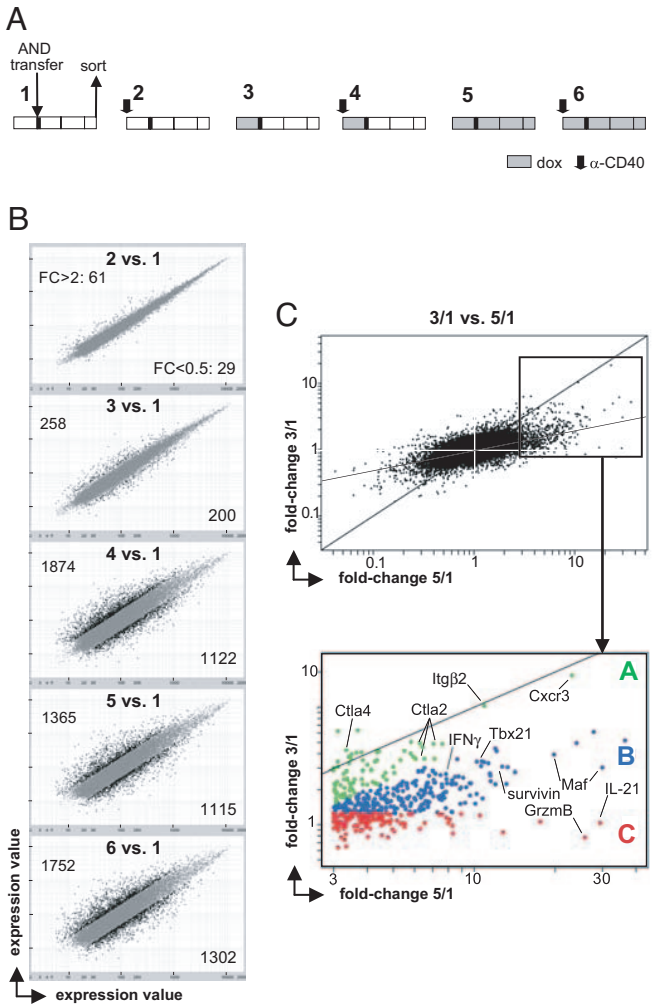

Fig. 2. Gene expression in CD4$^+$ T cells in response to transient or persistent antigen presented by steady-state or activated DCs. (*A*) Experimental outline. Host mice were treated as indicated, transferred with unlabeled CD90.1$^+$AND T cells. These were sorted 60 h later and their RNA analyzed with microarrays. (*B*) Gene expression in AND T cells primed as indicated in *A*, compared with untreated control animal 1. Genes up- or down-regulated relative to control by >2-fold are depicted in black, and their numbers are indicated. (*C*) The kinetics of antigen presentation regulates gene expression in CD4$^+$ effector cells. (*Upper*) The FC/FC plot compares expression values of treatments 3 and 5 relative to the control condition 1. (*Lower*) Magnified view of the *Inset* in *Upper* on activation response transcripts (5/1 FC >3). Color-coding distinguishes three sets of activation-induced transcripts with different response modes and is detailed in *SI Methods*.

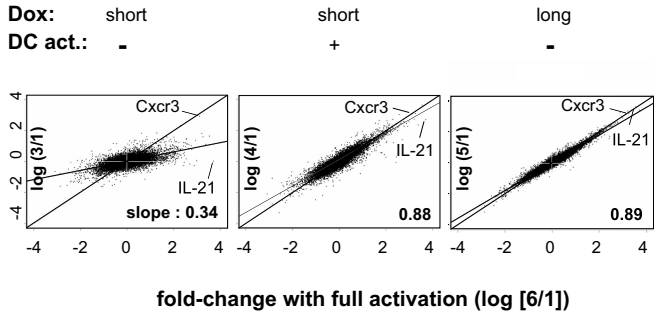

Fig. 3. Effector CD4$^+$ T cell gene expression is shaped by the kinetics of antigen presentation. FC/FC plots compare changes elicited by transient antigen without (*Left*) and with DC activation via CD40 (*Center*) and by continuous antigen without DC activation (*Right*) relative to responses to persistent antigen presentation by activated DCs (*x* axes). Correlation coefficients of r^2 = 0.34, 0.84, 0.94 (*Left*, *Center*, and *Right*, respectively); the slopes of the linear regression trend lines are shown. Data and conditions are as in Fig. 2.

statistical analysis of Gene Ontology identifiers (SI Table 4) and that is consistent with the dependence of CD4$^+$ T cell proliferation on continued TCR engagement shown before (Fig. 1). Group C genes, depicted as red in Fig. 2*C Lower*, completely depended on persistent antigen. Here, genes involved in DNA replication and cell cycle control were significantly enriched (SI Table 3). *IL-21* and the *granzyme A*, *B*, and *K* genes were in this group. In summary, genes involved in CD4$^+$ T cell priming were responsive to waning TCR signals with different kinetics, and it is tempting to speculate that these groups of transcripts are regulated by separate mechanisms.

Next, we compared the gene-induction patterns of the conditions 3–5 depicted in Fig. 2*A* to the fully stimulatory treatment with αCD40 and dox given throughout, condition 6 (Fig. 3). In the comparison between gene induction to transient or persistent antigen presentation (conditions 3 and 5), the slope of a linear regression trend line (0.34) indicated an ≈3-fold-lower level of gene expression resulting from antigen removal (Fig. 2*C Upper*). When αCD40 was given with persistent antigen, the data plotted similarly (conditions 3 and 6, Fig. 3 *Left*). However, DC activation by αCD40 had a strong effect on this "dampening" of the activation signature when TIM was turned on only transiently: in the FC/FC plots comparing conditions 4 and 6 with the control, most of the data points lined up around the $x = y$ diagonal and, e.g., *Cxcr3* and *IL-21* were now expressed as they were in cells persistently stimulated with antigen. The trend line of the data points now had a slope of 0.88 (Fig. 3 *Center*). This high degree of similarity in gene-expression profiles suggests that persistent display of peptide/MHC complexes by DCs and their activation by αCD40 (coupled with transient expression of *TIM*) had the same consequence on a T cell response. This interpretation was confirmed when antigen presentation was maintained by dox treatment and αCD40 was omitted, most of the data points crowded tightly around the trend line with the slope of 0.89, i.e., DC activation did not induce additional genes (Fig. 3 *Right*).

Long-Term CD4$^+$ T Cell Survival Is Influenced by Antigen Persistence, but Not Exclusively. We asked next whether αCD40 and/or persistent antigen promoted long-term survival and memory formation. Congenically marked AND T cells were transferred and followed over time by serial removal of s.c. lymph nodes between days 3 and 31. After the last time point, the transgene was turned back on to challenge the remaining AND T cells (Fig. 4). Irrespective of DC activation, AND cells transferred into control animals not expressing TIM did not persist long term, in agreement with a recent report suggesting that self-peptide/MHC complexes are limiting for the survival of naïve TCR-transgenic T cells (21). In all dtg mice induced to express cytochrome peptide the AND cells had expanded by day 3. However, in hosts where the cells were exposed to antigen only transiently in the absence of DC activation (condition 3), the cells had essentially disappeared by day 10, and there was no response to the secondary challenge. In contrast, a persistent memory T cell population was detected under all three conditions of longer antigen exposure, whether elicited by αCD40 treatment or by 60 h of TIM induction alone (Fig. 4*B*). Such cells could expand and produce IFNγ in response to re-expressed antigen (Fig. 4*A*). In hosts that had been initially exposed to persistent antigen and treated with αCD40, the number of memory cells was higher than in the absence of DC activation (Fig. 4 *A* and *B*, compare animals 5 and 6) indicating a synergistic effect of maintained antigen presentation and costimulation for memory-cell formation. Note that the T cells transferred into

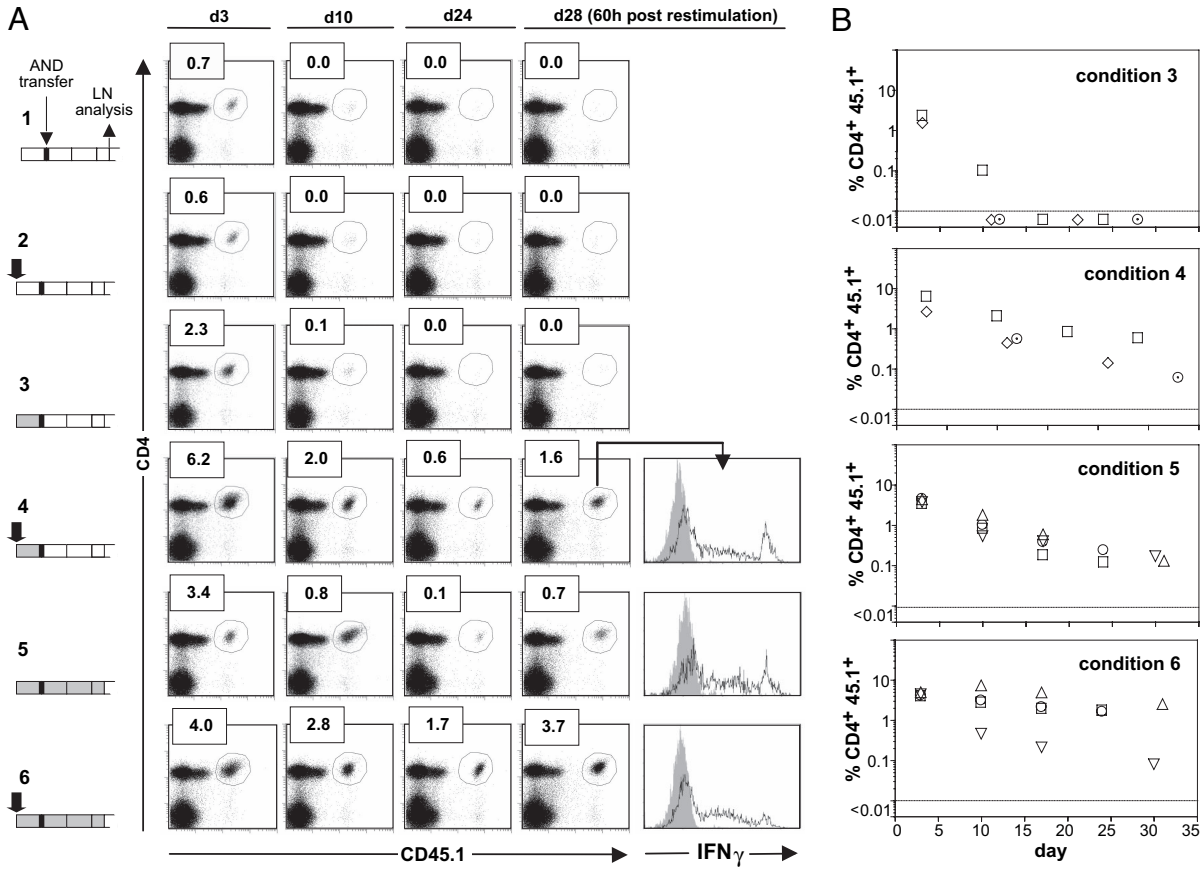

Fig. 4. Deletion versus memory-cell development depends on both antigen persistence and additional features of DC activation. (*A*) CD45.1$^+$ AND LN cells were transferred into dtg mice. At 3, 10, and 24 days later, s.c. LNs were surgically removed and analyzed by flow cytometry. On day 25, all animals were challenged by antigen reexpression (together with anti-CD40 to maximize responses), and LN cells were analyzed 3 d later. Numbers indicate the proportion of donor CD4$^+$ T cells. Splenocytes were also restimulated *in vitro* and stained for IFNγ (gray histograms) or a control mAb (open histograpms). (*B*) Compilation of several experiments. Each point depicts the percentage of CD4$^+$CD45.1$^+$ T cells in s.c. LNs; each symbol represents an independent experiment.

animals 5 and 6 were indistinguishable at 60 h, including in their mRNA profiles, but generated memory cells with a different efficiency. This quantitative difference might be explained by changes in the T cells themselves, like very subtle changes in gene expression, or changes in lipid, carbohydrate, or protein components. Alternatively, later steps of memory formation may be affected by the αCD40 treatment.

DC Activation Extends the Window of Effective *in Vivo* Presentation of Some MHC Class II-Restricted Antigens. Thus far, our results are in agreement with the idea that DC activation stabilizes peptide/MHC complexes *in vivo*. To test this more directly, we transferred labeled AND T cells into dtg recipients 3 days after dox removal. The cells hardly proliferated (Fig. 5*A*, ctrl) unless the recipients were treated with αCD40 at the time of *TIM* turn-on (Fig. 5*A*, condition 1), suggesting that DC triggering via αCD40 was inducing the persistence of stimulatory peptide/MHC complexes. The counterinterpretation that this might be due to costimulatory effects was ruled out by the administration of αCD40 mAb just before T cell transfer, which did not enhance the response (Fig. 5*A*, condition 2). CD40 triggering did not induce aberrant TIM expression, as judged by RT-PCR (data not shown). Thus, DC activation through CD40 engagement extends in this system the duration of antigen presentation *in vivo*.

To determine how long the stimulatory capability of activated DCs persists, we performed time-course experiments in which labeled AND cells were transferred at different times after *TIM* turn-off (Fig. 5*B*). Although a strong drop in stimulatory capacity was observed beyond 3 days, a clear effect of αCD40 was still detectable at 9 days. We also found that the injection of αCD40 coincident with switching on *TIM* was effective (Fig. 5*C*, condition 1), but injection 3 days before resulted in little enhancement of T cell activation (condition 2). This finding indicates that the mAb's effects on the population as a whole are quite transient, which is paralleled by waning activation markers on DCs such as CD86 (SI Fig. 6). Together with the results of Fig. 5*A*, these data suggest that the engagement of CD40 functionally prolongs the window during which the peptide/MHC complexes present on DCs at the time can stimulate a T cell response.

We then asked whether this effect might be observed directly by visualizing peptide/MHC complexes. An independent system of transient antigen exposure was used, where cognate peptides from hen egg lyzozyme (HEL) are targeted to the endocytic pathway of DCs *in vivo*, fused to the 33D1 antibody as chimeric molecules (22). Here, HEL_{48-62}/A^k complexes can be detected directly on the surface of splenic CD8$^-$ DCs with the Aw3.18 mAb, even after 4 d. However, no effect of αCD40 was seen, as the HEL_{48-62}/A^k epitope decayed similarly, with or without αCD40 (Fig. 5*D*). Although the reason for this is not obvious and possible mechanisms will be discussed, this finding poses the caveat that the findings from experiments done *in vitro* as well as in the dtg mice cannot necessarily be generalized to other ways of antigen delivery.

Discussion

Activated DCs up-regulate MHC and costimulatory molecules, undergo changes in antigen processing and loading pathways,

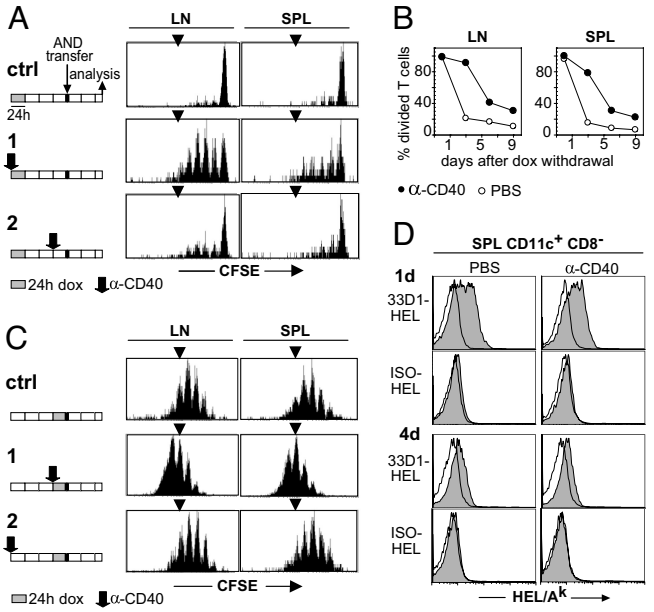

Fig. 5. *In vivo* DC activation extends MHC class II-restricted antigen presentation in dtg mice, but not after mAb-mediated antigen targeting. (*A*) Dtg mice were treated as depicted before the transfer of CFSE-labeled cells from CD90.1$^+$ AND TCR-tg mice. CFSE profiles of CD4$^+$CD90.1$^+$ LN and SPL cells were analyzed 60 h later. Control mice received PBS or irrelevant rat IgG2a mAb. Data are representative of three experiments. (*B*) MCC/E^k complexes disappear from activated DCs ≈6 d after the turn-off. Experiment is as in *A*, with the time between 24-h dox feeding and the AND cell transfer varied from 0 to 9 d. Recipients were injected with PBS (open circles) or αCD40 (filled squares) at the initiation of dox exposure. (*C*) The effect of αCD40 vanishes within 3 days. Experiment performed as in *A*, except that dtg animals were treated with dox at different times relative to αCD40. Representative of three experiments. (*D*) Extended antigen presentation with mAb-mediated antigen targeting to DCs is not detectable. B10.BR animals were injected with hen egg lysozyme coupled to 33D1 or an isotype control and 6 h later with αCD40. Spleen CD11c$^+$CD8$^-$ DCs were analyzed 1 and 4 d later with Aw3.18 (filled histograms) or a control mAb (open histograms). Data are representative of two experiments.

secrete new cytokines and chemokines, and are far more effective as stimulatory APCs. The lack of appropriate *in vivo* systems has impeded our understanding of how much and when these elements contribute to T cell stimulation. This is especially true for the stabilization of MHC class II molecules on activated DCs, which has been amply demonstrated *in vitro* (9–14). Relying on a genetic switch to express an MHC class II-restricted antigen reversibly in DCs, the results reported here lead to two major conclusions: that DCs do show antigenic memory *in vivo*, their activation ostensibly extending the lifetime of peptide/MHC complexes and thereby permitting longer APC function and that this stabilization is an important element accounting for the increased activation potential, leading to full effector function and to memory differentiation.

Prolongation of the effective half-life of peptide/MHC complexes could theoretically involve stabilizing either the DCs, the complexes they present, or stores of intracellular antigen. Enhancement of the survival of DCs by antiapoptotic transgenes has been shown to augment T cell stimulation and might theoretically contribute to persistence of peptide/MHC complexes (23–25). However, we propose that the peptide/MHC turnover we observe in the steady state, and upon DC activation, reflects primarily the molecular turnover of these complexes: disappearance of the MCC/E^k complexes follows similar kinetics in spleen and s.c. lymph nodes (Fig. 5*B*), whereas the reported half-lives of bulk DCs themselves differ in the two organs, being ≈2 and 10 days, respectively (26). Thus, stabilization of MHC class II molecules is likely to be involved, beyond a simple effect on DC survival.

What physiological event does DC activation through an agonistic αCD40 mAb represent? We found no evidence that expanding AND CD4$^+$ T cells can induce MHC class II-restricted antigen persistence (8). Innate lymphocytes might engage CD40 on DCs and be responsible for MHC class II antigenic memory. Indeed, it has been shown recently that NK (27), NKT, and γδ T cells can activate DCs (28). We therefore speculate that lymphocytes of the innate immune system can extend the half-life of peptide/MHC complexes on DCs and thereby enhance acquired immunity. Short antigen exposure might correspond to situations where the offense is cleared rapidly enough by the innate immune system, to the point that it is no longer worth mounting an adaptive immune response.

Our data show that the extended antigen presentation capability induced by DC activation can be an important element sustaining the expansion, differentiation, and memory-cell formation of CD4$^+$ T lymphocytes. This situation contrasts with CD8$^+$ T cells, which rely on a shorter window of antigen presentation for successful priming (29, 30). This difference appears to be reflected in the class-specific biochemistry of MHC molecules on DCs: In fact, the initial *in vitro* observation of DC antigenic memory was made for MHC class II molecules, and class I molecules were found not to be stabilized by DC activation (9, 11).

How the time element controls the outcome of TCR engagement and when exactly the decisive cues are given to CD4$^+$ T cells is unclear. Recent *in vivo* time-lapse microscopy lead to the conclusion that CD4$^+$ T cells are not committed to a state of immunity or tolerance within the first 24 h (31) but can interact tightly with DCs for at least 48 h (32). Our data support the possibility that commitment is a progressive process for CD4$^+$ T cells. Responding cells have to receive TCR signals long enough, perhaps to accumulate transcription or other regulatory factors, or effect epigenetic chromatin modifications, or to dilute out inhibitory molecules. On the other hand, it is possible that CD4$^+$ T cells must be targeted to antigen-presenting DCs long enough to receive late costimulatory signals, such as mediated by the OX40/OX40L- or PD-1/PD-L1-pairs, that can then make the difference between deletion and memory-cell formation. The up-regulation of such costimulation might explain the clearly enhanced differentiation of memory cells by CD40-triggered DCs, especially when T cells are tethered to DCs by persistent antigen.

Although the results from dtg mice agree with *in vitro* studies of MHC class II stabilization on DCs, we could not detect this effect when HEL was targeted to DCs via the mAb 33D1, which is consistent with the fact that memory formation in responding T cells always required DC activation after exposure to antigen ferried by 33D1 or DEC205, even when the stimulatory complexes persisted for several days (22, 33, 34). Overall, the T cell-detectable MCC/E^k complexes in dtg mice seem to disappear faster after turn-off than HEL/A^k after HEL-33D1 injection, where the complex can still be visualized after 4 d by using a mAb (Fig. 5). The discrepancy between the two systems may be due to different biochemical features of these two peptide/MHC complexes, MHC alleles, the particular processing pathways used by DCs for Ii-embedded and 33D1-linked antigens, the biological half-life of the mAb-HEL proteins in mouse serum, the effective dose of antigen, or the fact that different DC subsets may be addressed by internal expression or by mAb targeting. It will be important to dissect the root of the differences, but the present results indicate that persistent antigen presentation in the absence of any additional trigger can be fully competent for full T cell activation. This notion might prompt a caveat on the interpretation of experiments where antigens were delivered with immature DCs or with apoptotic spleen or tumor

cells (e.g., refs. 35–37): the abortive/tolerogenic responses may have been due to rapid clearance, leading to too short a pulse of antigen rather than to intrinsically tolerogenic abilities of these DCs or forms of antigen (38). Our results also suggest that time-controlled antigen exposure may be a means for vaccine optimization and therapeutic tolerance induction. In that sense the present results hark back to a central property of adjuvants: not only do good adjuvants provide activators for innate recognition receptors, but they also serve to release antigen slowly (39, 40), both aspects ensuring that $CD4^+$ T cells can take their time.

Methods

For additional details, see *SI Methods*.

Mice and Reagents. The AND TCR-, *Ii-rTA*- and *TIM*-transgenic mouse lines were described (8, 16). Experimental animals were injected i.p. with 50 μg of FGK45.5 or control mAb in endotoxin-free PBS or PBS alone. Control animals were single- or non-transgenic littermates.

Cell Transfer and Flow Cytometry. The equivalent of 2×10^6 AND T cells were transferred except where indicated otherwise. For DC stainings, organs were digested with 100 units/ml collagenase D (Roche, Indianapolis, IN) in DMEM for 30 min at 37°C. Flow cytometry was performed as described (8).

Cell Sorting, RNA microarray analysis. Lymph node cells from AND TCR-transgenic mice were transferred into recipients and sorted 60 h later on a MoFlo cell sorter (DakoCytomation, Fort Collins, CO). Three independent experiments were performed, except for the conditions 2 and 4 depicted in Fig. 2, for which two independent sorts were done. Sorted cells were lysed in TRIzol, total RNA was prepared, amplified, and biotinylated. Five to 15 μg of aRNA was hybridized to the Affymetrix Mouse Genome 430Av2.0 GeneChip (Affymetrix, Santa Clara, CA). Data preprocessing and analysis were performed within the GenePattern package, v2.0.1 (www.broad.mit.edu/genepattern/). Affymetrix .cel files were collectively normalized and unexpressed and Y-chromosomal genes removed from the analysis with the GenePattern Multiplot module (R.M., unpublished work) and S-Plus 6.2 (Insightful) for linear regression. The microarray data have been deposited in GEO data bank with accession no. GSE5245.

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