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*J Immunol* 2002; 168:5573-5581; doi: 10.4049/jimmunol.168.11.5573

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CD4 Cell Priming and Tolerization Are Differentially Programmed by APCs upon Initial Engagement

Amy D. Higgins, Marianne A. Mihalyo, Patrick W. McGary, and Adam J. Adler

Bone marrow-derived APCs present both parenchymal-self and pathogen-derived Ags in a manner that elicits either T cell tolerization or immunity, respectively. To study the parameters that confer tolerogenic vs immunogenic APC function we used an adoptive transfer system in which naive TCR transgenic hemagglutinin (HA)-specific CD4+ T cells are either tolerized upon encountering HA expressed constitutively as a parenchymal self-Ag (self-HA) or primed to express effector function upon encountering transiently expressed vaccinia-derived HA (viral-HA). When the duration of viral-HA presentation was extended for the period required to elicit tolerization toward self-HA, CD4 cell tolerization to viral-HA did not occur. Furthermore, CD4 cells exhibited both phenotypic as well as functional differences during early stages of tolerization and priming, suggesting that these divergent differentiation processes are programmed soon after the initial APC-CD4 cell interaction. When mice expressing self-HA were infected with an irrelevant vaccinia, CD4 cell tolerization still occurred, indicating that priming vs tolerization cannot be explained by pathogen-induced third parties (i.e., non-APCs) that act directly on CD4 cells. Taken together, these results suggest that CD4 cell tolerization to parenchymal self-Ags and priming to pathogen-derived Ags are initiated by functionally distinct APCs. The Journal of Immunology, 2002, 168: 5573–5581.

Materials and Methods

Mice

Mice were on the B10.D2 background (except where otherwise indicated). C3-HAlo and C3-HAhi transgenic mice both express the influenza HA gene (A/PR/8/34 Mount Sinai strain) under the control of the rat C3 (1) promoter, which directs HA expression to a variety of nonlymphoid organs. Although both transgenic founder lines express HA in the same subset of tissues, HA protein expression in C3-HAhi mice appears to be at least 1000-fold higher than that in C3-HAlo mice (1, 12). 6.5 TCR transgenic mice express a clonotypic TCR that recognizes an I-Ek-restricted HA epitope (15SFERFEEIPKE163) (13).

BM chimeras

BM chimeras were generated as previously described (1) with the following modifications. Parent→F1 chimeras were generated using F1 hosts (B10.D2 × C57BL/6J (H-2b)) that either received 1000 rad ionizing radiation in a single dose or 1300 rad in split doses of 650 rad 4 h apart. One day before allogeneic BM transplantation, hosts were depleted of NK cells by i.p. injection of 15 μl rabbit anti-asialo GM1 gammaglobulin (WAKO Chemicals, Richmond, VA).

Adoptive transfers

Adoptive transfers of 2.5 × 106 naive clonotypic CD4 cells were performed as previously described (1, 12) with the following modifications. For adoptive transfers using CFSE-labeled clonotypic CD4 cells, 6.5 transgenic donors on a B10.D2 background expressing the Thy1.1 congenic

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Received for publication January 22, 2002. Accepted for publication April 1, 2002.

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1 This work was supported by National Institutes of Health Grant AI49813 (to A.J.A.).

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3 Abbreviations used in this paper: BM, bone marrow; DC, dendritic cell; HA, hemagglutinin; iAPC, immunogenic APC; NT, nontransgenic; tAPC, tolerogenic APC.
marker were used. Before labeling with CFSE (Molecular Probes, Eugene, OR) (12), 6.5 LN preparations were depleted of CD8 cells using anti-mouse CD8-conjugated magnetic beads (Dynal, Lake Success, NY). All adoptive transfer recipients expressed the Thy1.2 congenic marker.

**Proliferation assays**

Proliferation assays were performed as previously described (1, 12), with data expressed as counts per minute/cloneotypic CD4 cell (mean ± SEM).

**Recombinant vaccinia**

The recombinant vaccinia virus expressing HA (vacc-HA) has previously been described (14), and the recombinant vaccinia vacc-GH that expresses the CMV GH protein was provided by Drs. J. Shanley and C. Wexler (University of Connecticut Health Center). Both vacc-HA and vacc-GH were constructed by recombining either the HA or GH gene, respectively, into the thymidine kinase gene of wild-type vaccinia. Vaccinia were amplified in HuTK- cells, purified over sucrose, and titrated on HuTK- cells using standard protocols (15). Inoculations were performed by i.p. injection of the indicated dose in 0.2 ml HBSS.

**Flow cytometry**

Surface staining for the clonotypic TCR 6.5, CD4, and CD44 on non-CFSE-labeled cells was performed as previously described (1, 12). Surface staining of CFSE-labeled clonotypic CD4 cells was performed using PerCP-conjugated anti-Thy1.1 and either PE-conjugated anti-CD25 or an isotype control (BD Pharmingen, San Diego, CA). Intracellular cytokine staining was performed by stimulating 1 × 10^6 splenocytes (prepared from adoptive transfer recipients) with 100 μg/ml synthetic HA peptide and 5 μg/ml brefeldin A (Sigma-Aldrich, St. Louis, MO) for 5 h at 37°C in 1 ml CTL medium (1). Cells were then washed once in FACS buffer, fixed in PBS and 2% formaldehyde for 5 min at 37°C, permeabilized by washing twice in FACS buffer containing 0.25% saponin, and then stained with PerCP-conjugated anti-Thy1.1 and PE-conjugated mAbs specific for IL-2, IFN-γ, or an isotype control (BD Pharmingen). All quantitative FACS data are expressed as the mean ± SEM. To allow direct comparison of FACS data collected from separate experiments, all samples were analyzed on the same flow cytometer (FACScan; BD Biosciences, San Jose, CA) using identical settings.

**Results**

*Naive CD4 cells can only recognize parenchymally derived self-epitopes via indirect presentation*

In transgenic mice expressing low levels of the model parenchymal self-Ag HA, adoptively transferred naive clonotypic TCR transgenic CD4 cells that recognize an I-E^d-restricted HA epitope become tolerized upon encountering BM-derived APCs that have acquired and presented parenchymally derived HA. Thus, in parent→F1, BM chimeras in which the HA-expressing parenchyma are genetically capable of presenting the HA epitope (H-2^bxd), but APCs are not because they express a nonrestricting haplotype (i.e., H-2^d), adoptively transferred clonotypic CD4 cells remained naive (1). Since these initial studies used mice expressing low levels of HA (1, 12), it might have been possible that high level HA expression would enable tolerancy to directly present the I-E^d-restricted epitope to naive clonotypic CD4 cells. To test this possibility, we generated parent→F1 chimeras using both the C3-HA^low as well as the C3-HA^high transgenic mice that express HA in the same subset of parenchymal tissues, but differ in their level of HA expression by at least 1000-fold (12). In the first experiment (Fig. 1A), F1 (H-2^bxd) nontransgenic (NT), C3-HA^low and C3-HA^high mice were lethally irradiated with 1000 rad and reconstituted with NT BM expressing either the HA-restricting (H-2^d) or a nonrestricting (H-2^b) haplotype. Consistent with our previous results (1, 12), clonotypic CD4 cells recovered 9 days post-transfer from the spleen and LN of C3-HA^low as well as C3-HA^high chimeras reconstituted with H-2^d BM (d→low and d→high, respectively) expressed levels of the activation marker CD44 that were at least 3-fold higher than those of naive counterparts recovered from control NT chimeras, indicating that the clonotypic CD4 cells had encountered the HA epitope and lost their naive phenotype. Furthermore, clonotypic CD4 cells recovered from C3-HA^low chimeras reconstituted with H-2^b BM (b→low) expressed low levels of CD44, indicating that they retained a naive phenotype and confirming that direct parenchymal presentation of the I-E^d-restricted epitope does not occur in chimeras expressing low levels of parenchymal HA (1). Interestingly, clonotypic CD4 cells recovered from b→high chimeras exhibited a moderate increase in CD44 expression (2-fold over control levels), possibly resulting from either direct parenchymal Ag presentation or radioresistant host-derived APCs (H-2^bxd). We reasoned that if the latter possibility were correct, a more severe myeloablative regimen would eliminate this residual presentation. Consistent with this prediction, when hosts received 1300 rad, clonotypic CD4 cells recovered from b→high chimeras expressed CD44 levels that were equivalently low as those of the NT control chimeras (Fig. 1B). Thus, when the host-derived APCs were efficiently eliminated, the parenchymal cells expressing high levels of HA were revealed to be incapable of presenting the class II-restricted HA epitope to naive clonotypic CD4 cells.

**APCs are sufficient to induce CD4 cell tolerization**

To determine whether APCs that indirectly present (i.e., cross-present) parenchymal self-Ags are sufficient to induce CD4 cell tolerization, allogeneic chimeras were generated by reconstituting lethally irradiating C3-HA^high mice backcrossed onto a H-2^b background with NT H-2^d BM, so that only APCs could present the HA epitope. As shown in Fig. 2A, 11 days post-transfer naive clonotypic CD4 cells adoptively transferred into C3-HA^high d→b chimeras developed a tolerant phenotype (i.e., diminished proliferative potential to peptide stimulation). Since the clonotypic CD4 types.
cells were prepared from mice on an H-2d background, this experiment might have been complicated by the possibility that they were alloresponsive to residual host-derived APCs (H-2b). However, this did not appear to be the case, as clonotypic CD4 cells recovered from control NT d→b chimeras maintained a naive CD44 expression pattern (Fig. 2B). Additionally, 6.5 clonotypic CD4 cells develop normally on an F1 (H-2bxd) background (data not shown). Perhaps the skewed 6.5 transgenic TCR repertoire precludes the development of alloresponsiveness against the H-2b haplotype. Nonetheless, these results extend our previous finding that APCs are not only necessary for tolerization to parenchymal self-Ags (1), but are also sufficient.

Assay to distinguish CD4 cell tolerization vs priming

Naive clonotypic CD4 cells expressing a congenic Thy1.1 marker were labeled with the fluorescent marker CFSE and adoptively transferred into the following recipients: C3-HAhigh mice on an H-2b background were lethally irradiated and reconstituted with NT H-2d BM (d→b chimeras) and subsequently received adoptive transfers of naive clonotypic CD4 cells (H-2d). A. Eleven days post-transfer, the in vitro proliferative response of clonotypic CD4 cells (counts per minute/clonotypic CD4 cell) recovered from d→b C3-HAhigh (C3-HA) or control d→d NT chimeras were measured in the presence or the absence of synthetic HA peptide (333 μg/ml). n = 2 for each group. The data shown are representative of two independent experiments. B. Representative histogram plots of CD44 expression on clonotypic CD4 cells (H-2d) recovered from NT and C3-HAhigh d→b chimeras 9 days post-transfer, with mean fluorescence intensity (MFI) values shown.

FIGURE 2. Cross-presenting APCs are sufficient to induce CD4 cell tolerization to parenchymal self-Ags. C3-HAhigh mice on an H-2b background were lethally irradiated and reconstituted with NT H-2d BM (d→b chimeras) and subsequently received adoptive transfers of naive clonotypic CD4 cells (H-2d). A. Eleven days post-transfer, the in vitro proliferative response of clonotypic CD4 cells (counts per minute/clonotypic CD4 cell) recovered from d→b C3-HAhigh (C3-HA) or control d→d NT chimeras were measured in the presence or the absence of synthetic HA peptide (333 μg/ml). n = 2 for each group. The data shown are representative of two independent experiments. B. Representative histogram plots of CD44 expression on clonotypic CD4 cells (H-2d) recovered from NT and C3-HAhigh d→b chimeras 9 days post-transfer, with mean fluorescence intensity (MFI) values shown.

FIGURE 3. Intracellular cytokine staining of primed and tolerized clonotypic CD4 cells. Naive CFSE-labeled clonotypic CD4 cells (Thy1.1+) were adoptively transferred into the following recipients: C3-HAhigh, NT infected with vacc-HA, or NT infected with vacc-GH (all recipients were Thy1.2+). Six days later the responses of clonotypic CD4 cells recovered from spleens were analyzed. A. Representative CFSE histogram plots. B. Representative histogram plots of intracellular cytokine staining upon restimulation. The percentage of clonotypic CD4 cells expressing IL-2 or IFN-γ as well as the levels of cytokine expression (mean fluorescence intensity (MFI)) are shown above and below, respectively, the reference bar. C. Quantitative analysis of cytokine expression for C3-HAhigh (S; n = 5) and vacc-HA (V; n = 7) recipient groups. Total cytokine expression (arbitrary units) = % cytokine positive cells × level of cytokine expression per positively expressing cell. Data were pooled from several experiments.
FSC of naïve cells before transfer was 365 ± 5 (n = 10), while the respective values for self-HA and viral-HA on day 6 was 491 ± 11 (n = 5) and 429 ± 5 (n = 8) compared with 739 ± 27 (n = 6) and 806 ± 23 (n = 7) on day 2 when the clonotypic CD4 cells are actively dividing (refer to Fig. 6A).

Although clonotypic CD4 cells proliferated to a similar extent upon initially encountering either self-HA or viral-HA (Figs. 3A and 6A), as we have previously observed (12), upon termination of their respective differentiation processes they exhibit profound functional differences (Fig. 3, B and C). Self-HA-exposed clonotypic CD4 cells are severely impaired in their ability to proliferate upon secondary Ag exposure in comparison with viral-HA-primed counterparts (12). Furthermore, as measured by intracellular staining, viral-HA-primed clonotypic CD4 cells efficiently produce both the T cell growth and differentiation factor IL-2 and the prototypical Th1 effector cytokine IFN-γ upon in vitro restimulation. In contrast, self-HA-exposed counterparts express these cytokines weakly (Fig. 3, B and C). These differences in cytokine expression are manifested in the percentages of clonotypic CD4 cells that express each cytokine as well as the level of cytokine expression within the positively expressing cells. When the products of these two parameters were calculated to assess the total levels of cytokine expression (expressed as arbitrary units), there were 6- and 28-fold differences in the levels of IL-2 and IFN-γ expression, respectively, between viral-HA- and self-HA-exposed clonotypic CD4 cells (Fig. 3C). Thus, self-HA-exposed clonotypic CD4 cells exhibit a cytokine expression pattern consistent with a tolerant phenotype, while that of viral-HA-primed counterparts is consistent with an effector/memory phenotype.

The duration of Ag presentation does not determine tolerogenic vs immunogenic APC activity

To test whether differences in the duration of self-HA vs viral-HA presentation play a role in programming clonotypic CD4 cell tolerization vs priming, respectively, we assessed the duration of viral-HA presentation by infecting NT mice with vacc-HA at various times before adoptively transferring CFSE-labeled naïve clonotypic CD4 cells (Fig. 4). Vacc-HA infections were performed using either 1 × 10^6 PFU (as in Fig. 3) or a higher dose (1 × 10^7 PFU) that induces similar levels of effector function (i.e., ability to produce cytokines; data not shown). Clonotypic CD4 cells transferred into NT recipients that had been infected with either dose of vacc-HA 1 day earlier underwent robust proliferative responses (seven or more divisions at 6 days post-transfer). However, even at the higher inoculation dose, the proliferative response began to diminish when the transfer was given 4 days postinoculation and lessened even further at 6 days postinoculation, suggesting that the level of viral-HA presentation diminishes over the course of 6 days. At the lower inoculation dose the clonotypic CD4 cell proliferative response was somewhat variable even at 2 days postinfection (compare days –2, 1, and 2), suggesting that the duration of viral-HA presentation diminishes even more quickly. Given that the level of viral-HA presentation does not appear to remain high for the time required to induce tolerization to self-HA, it might have been possible that the transient nature of viral-HA presentation plays a role in conferring immunogenicity. To test this possibility, NT adoptive transfer recipients were subjected to multiple vacc-HA inoculations (at 1 × 10^6 PFU/inoculation) throughout a 6-day experiment to maintain a high level of viral-HA presentation. Inoculations were either performed every second day (days –1, 1, 3, and 5), or daily (days –1 through 5). As shown in Fig. 5A, inoculating every second day did not affect the ability of clonotypic CD4 cells to express IL-2 and IFN-γ upon in vitro restimulation (relative to single inoculation controls). Daily inoculations did not effect IL-2 expression; however, there was a modest reduction in both the percentage of clonotypic CD4 cells expressing IFN-γ (25%) and the level of IFN-γ expression (30%). Nonetheless, total IFN-γ expression was still 12-fold greater than that in the self-HA-tolerized group, indicating that while extending the duration of viral Ag presentation might have reduced effector function somewhat, it did not lead to tolerization. To ensure that the initial vaccinia inoculations did not generate neutralizing immunity that prevented productive infections from the later inoculations, CFSE-labeled clonotypic CD4 cells were adoptively transferred into NT mice that had been inoculated daily with vaccinia that does not express HA (vacc-GH) from days –1 through 4, then given a single inoculation of vacc-HA on day 5. If the repetitive vacc-GH inoculations had induced neutralizing immunity (either innate or adaptive), the final vacc-HA inoculation would not have been productive, and the clonotypic CD4 cells not stimulated to proliferate. To the contrary, this regimen did elicit a clonotypic CD4 cell proliferative response equivalent to that of controls receiving a single vacc-HA inoculation (Fig. 5B). This result infers that repetitive vacc-HA inoculations did not induce neutralizing immunity during the previous experiments (Fig. 5A), thus supporting the conclusion that extending the duration of viral-HA presentation does not induce tolerization.

Another approach to address the potential role of the duration of Ag presentation in conferring tolerogenic vs immunogenic APC function is to compare the responses of clonotypic CD4 cells soon after encountering either self-HA or viral-HA. In Fig. 6, CFSE-labeled clonotypic CD4 cells were recovered for analysis from the spleens of either C3-HA^high or vacc-HA-infected NT recipients only 42 h post-transfer. Although the clonotypic CD4 cells in both groups had undergone an average of three divisions, they exhibited both phenotypic as well as functional differences. While self-HA induced a significant up-regulation in the expression of the high affinity IL-2R (i.e., CD25), viral-HA induced even higher levels. Additionally, clonotypic CD4 cells that had encountered viral-HA
produced much higher levels of IFN-γ upon restimulation than those that had encountered self-HA. IL-2 expression was also higher in viral-HA- relative to self-HA-exposed clonotypic CD4 cells, although this difference was less than that observed for IFN-γ. To assess whether fluctuations in the level of viral-HA presentation might have played a role in conferring increased responses in the viral-HA-primed group, we also included a group that received daily vacc-HA inoculations (days 1, 0, and 1). Interestingly, this group did exhibit a decrease in the level of CD25 expression relative to the group that received a single vacc-HA inoculation on day 1; however, CD25 expression was still greater than that in the self-HA group. Similar to the day 6 analysis (Fig. 5), repetitive vacc-HA inoculations did not alter IL-2 expression, but did reduce total IFN-γ expression by ~50%. Nonetheless, total IFN-γ expression was still 23-fold greater than that in the self-HA group (Fig. 6, A and B). Thus, it appeared that distinct differentiation programs had been established following only brief encounters with self-HA vs viral-HA.

Irrelevant vaccinia infection does not induce priming to self-Ag

To determine whether non-antigenic immunogenic signals associated with vaccinia infection can elicit priming to self-HA, C3-HA<sup>high</sup> mice were infected with the irrelevant vaccinia vacc-GH (1 × 10<sup>6</sup> PFU) 1 day before receiving an adoptive transfer of naive CFSE-labeled clonotypic CD4 cells (Thy1.1<sup>+</sup>) were adoptively transferred into NT recipients (Thy1.2<sup>+</sup>) inoculated with vacc-HA on day 1 or with vacc-GH daily from days 1 through 4, followed by vacc-HA on day 5. Proliferative responses were analyzed 3 days after vacc-HA exposure (experimental design diagrams are shown next to the respective CFSE histogram plots, which are representative of at least two replicates).

FIGURE 5. Extending the duration of viral Ag presentation does not induce tolerization. A, Naive CFSE-labeled clonotypic CD4 cells (Thy1.1<sup>+</sup>) were adoptively transferred into the following Thy1.2<sup>+</sup> recipients: C3-HA<sup>high</sup> (S; n = 2), NT and vacc-HA on day −1 (V; n = 3), NT and vacc-HA on days −1, 1, 3, and 5 (M; n = 4), and NT and vacc-HA daily from days −1 through 5 (M*; n = 3). On day 6, quantitative analysis of cytokine expression was performed as described in Fig. 3. The M and M* datasets were collected from different experiments, each including a V sample to confirm consistency. Experimental design diagrams are shown below. B, Naive CFSE-labeled clonotypic CD4 cells (Thy1.1<sup>+</sup>) were adoptively transferred into NT recipients (Thy1.2<sup>+</sup>) inoculated with vacc-HA on day −1 or with vacc-GH daily from days −1 through 4, followed by vacc-HA on day 5. Proliferative responses were analyzed 3 days after vacc-HA exposure (experimental design diagrams are shown next to the respective CFSE histogram plots, which are representative of at least two replicates).
At 6 days post-transfer, clonotypic CD4 cells exposed to self-HA plus vacc-GH also exhibited weak cytokine responses; total cytokine expression was 2,984/110062,073 (IL-2) and 3,889/110061,120 (IFN-γ) arbitrary units (n=3) compared with 12,089/110061,919 (IL-2) and 41,141/110064,984 (IFN-γ) arbitrary units for vacc-HA-primed cells (n=7; data taken from Fig. 3). Additionally, infection of either C3-HA low or C3-HA high transgenic recipients with 1×107 PFU of wild-type vaccinia (which is at least 10-fold more virulent than recombinant vaccinia (16)) also failed to prevent tolerization (data not shown).

Discussion

In C3-HA<sup>low</sup> transgenics, APCs that indirectly present parenchymal-HA are the only initiators of tolerization for naive clonotypic CD4 cells (1). Since some parenchymal cell types express class II MHC molecules under noninflammatory conditions (17) and can efficiently interact with naive T cells in vivo (18), it might have been possible that direct parenchymal presentation of self-HA to cognate naive CD4 cells could occur in our system when HA expression is high. Nonetheless, in parent→F<sub>1</sub> C3-HA<sup>high</sup> chimeras
reconstituted with NT BM expressing a nonrestricting haplotype for the HA epitope (H-2^{b}), adoptively transferred naive clonotypic CD4 cells remained in a naive state, indicating that indirect presentation is the exclusive pathway regardless of the level of parenchymal HA expression. Interestingly, the clonotypic CD4 cells were only able to retain their naive phenotype in b→C3-HA^{high} chimeras that had received high dose irradiation (1300 rad), while in b→C3-HA^{low} chimeras the clonotypic CD4 cells remained naive in hosts that had received either 1000 or 1300 rad. Thus, radioresistant host-derived APCs (H-2^{b+low}) that can engage naive clonotypic CD4 cells are present in b→C3-HA^{high}, but not in b→C3-HA^{low}, chimeras that have received low dose irradiation. This observation suggests that when APCs present greater numbers of MHC-self-peptide complexes, fewer APCs are required to induce tolerization.

While the above-mentioned experiments demonstrate that HA-expressing parenchyma in C3-HA transgenics cannot initiate CD4 cell tolerization, they did not rule out a role for these parenchyma during later stages of tolerization. When naive CD4 cells receive a tolerizing stimulus in the form of a bolus injection of soluble peptide, they undergo an initial expansion phase in secondary lymphoid tissues, followed by the migration of a significant fraction into nonlymphoid tissues such as lung, etc. Subsequently, all the CD4 cells develop a tolerant phenotype (19). Since the C3-HA transgenic mice express HA in the lung (1, 12), it might have been possible that after having encountered APCs indirectly presenting parenchymally derived HA in the spleen or LN, the clonotypic CD4 cells migrated into the lung (and possibly other HA-expressing tissues) where parenchymal HA presentation provides a second tolerogenic signal. In d→b C3-HA^{high} chimeras (in which only APCs were capable of presenting the HA epitope) naive clonotypic CD4 cells became fully tolerant. While this result does not necessarily infer that CD4 cells undergoing tolerization cannot interact with parenchyma expressing cognate self-Ag, if this interaction does take place, it is not essential for tolerization. Thus, APCs that indirectly present parenchymal self-Ags are not only necessary for CD4 cell tolerization, but they are also sufficient.

Both our current as well as previous studies (1) have indicated that parenchymal self-HA is indirectly presented by tAPCs; however, we do not know the pathway by which vaccinia-derived HA is presented to induce priming. Although priming is initiated by APCs presenting vaccinia-derived HA (20), it is unknown whether APCs indirectly present HA acquired from vaccinia-infected parenchyma, if vaccinia-infected APCs present endogenously synthesized HA, or if both pathways operate. Nonetheless, given that APCs perform the dual function of inducing both T cell tolerance and immunity, it is important to understand the parameters that confer these different functions. As a first step in understanding the relationship between tAPCs and iAPCs, we simply asked whether they differed functionally. While functionally distinct APCs might induce either tolerance or immunity depending on qualitative differences in the signals that they deliver to cognate naive T cells, it is also possible that functionally equivalent APCs induce either tolerance or immunity depending upon extrinsic parameters. While we do not know the relative levels of self-HA vs viral-HA epitope presentation in our system (although they induce similar kinetics of naive clonotypic CD4 cell proliferation), it is unlikely that this parameter determines immunogenicity vs tolerogenicity. Thus, when naive clonotypic CD4 cells are transferred into C3-HA transgenic mice that differ in their level of HA expression by at least 1000-fold, the time course of the responses differ, but the final outcome (i.e., tolerization) does not (12) (data not shown). A similar relationship has been observed for CD8 cell tolerization (21). Along similar lines, varying the dose of vacc-HA used to prime clonotypic CD4 cells in our system did not alter functional differentiation (12) (data not shown). Similarly for CD8 cell responses, priming to Listeria monocytogenes Ags does not differ qualitatively over a range of infection doses (22). Although high dose lymphocytic choriomeningitis virus infection leads to the phenomenon of high zone tolerance in which cognate CD8 cells undergo accelerated priming, followed by complete exhaustion of function over a 2-wk period (10, 23), high zone tolerance appears to be qualitatively different from the tolerization observed in our system that develops toward self-Ag, in that the former seems to represent a strong immunogenic response that cannot sustain itself, while the latter response is programmed to be tolerogenic from the outset (refer to Fig. 6). In addition to the above-mentioned empirical evidence, it seems logical that the immune system is programmed to be nonresponsive to parenchymal self-Ags regardless of their expression levels.

Functionally equivalent APCs could potentially induce either tolerization or immunity through differential kinetics of Ag presentation. Thus, naive T cells encountering APCs presenting either cognate self or pathogen-derived epitopes would initially undergo priming; however, due to the constitutive expression and presentation of self-Ags, self-reactive T cells would become overstimulated and driven into a tolerant state. In the case of pathogen-derived Ags, as the pathogen is cleared, the level of APC presented Ag would diminish below the threshold required to induce tolerization. If this clonal exhaustion model were correct, two predictions could be made. The first is that at early stages of tolerization and priming, cognate CD4 cells should exhibit similar responses and should only begin to differ at later stages when the level of pathogen-derived Ag presentation declines. Second, extending the duration of pathogen-derived Ag presentation for the time required to induce tolerization toward self-Ag should result in tolerization. Contrary to the first prediction, clear differences were observed between CD4 cells undergoing tolerization vs priming after only 42 h. Despite having both undergone an average of three divisions (indicating that these two responses develop at similar rates) clonotypic CD4 cells that had encountered viral-HA expressed higher levels of the high affinity IL-2R (CD25) and were also able to produce higher levels of both IFN-γ and IL-2 upon in vitro re-stimulation than counterparts exposed to self-HA. Comparable results have been observed between naive CD8 cells encountering cognate self vs viral Ag (24). The decreased IL-2R expression and ability to produce IL-2 in CD4 cells undergoing an early stage of tolerization (relative to primed counterparts) are consistent with previous studies suggesting that suboptimal IL-2 signaling plays a role in this process (25, 26). Further investigation, however, will be required to determine whether suboptimal IL-2 signaling in our system actually contributes to or is simply a result of tolerization. Nonetheless, the observation that CD4 cells undergoing tolerization vs priming exhibit different responses soon after initial Ag encounter suggests that naive CD4 cells become programmed to undergo either tolerogenic or immunogenic differentiation long before the potential effects of chronic vs transient Ag presentation come into play. When the duration of viral-HA presentation was extended for the 6 days required to induce tolerization toward self-HA, clonotypic CD4 cells were not tolerized, although they did exhibit a slight reduction in their level of effector function, raising the possibility that extending viral-HA presentation over much longer periods could eventually lead to tolerization. In fact, chronic lymphocytic choriomeningitis virus infection does result in cognate CD4 cell tolerization, but only after 6 wk (11). Along similar lines, CTL primed by admixture of soluble peptide and a CD40 agonist become tolerized if peptide presentation persists for several weeks (27). Taken together, the processes of T cell priming
to pathogen-derived Ags and tolerization to parenchymal self-Ags appear to be programmed soon after initial Ag encounter, and while the duration of Ag presentation does not appear play a role in establishing these basic programs, it might exert an effect over the long term.

Another mechanism by which functionally equivalent APCs could induce either tolerization or priming would be for pathogens to induce the activity of a third-party cell (i.e., non-APC) that produces an immunogenic factor(s) that acts directly on T cells to redirect them to undergo priming rather than tolerization (tolerance being the default response). Precedence that immunogenic signals can alter naive T cell responses in an APC-independent manner comes from studies demonstrating that IL-1 can enhance the proliferation of naive CD4 cells stimulated by immunobilized cognate MHC-peptide complexes (28). Since this third-party model predicts that Ag and the immunogenic signal(s) do not need to be linked, we tested it by providing the immunogenic signals in trans by infecting C3-HA\textsuperscript{high} transgenics with a vaccinia that does not express HA. Since this vaccinia expresses all the immunogenic elements required for clonotypic CD4 cell priming (minus HA), the activity of the Ag nonspecific third-party should have been activated, and naive clonotypic CD4 cells encountering APCs presenting self-HA would have been primed rather than tolerized. Tolerization was not converted to priming, however, indicating that self-HA plus irrelevant vaccinia does not equal vacc-HA, a result inconsistent with the third-party model. This result was actually somewhat surprising given that numerous immunogenic agents that are known to enhance APC function can elicit T cell priming to otherwise tolerogenic Ags. These agents include LPS (29–31), poly(IC) (27, 30), IL-1 (32), IL-12 (33), Flt3 ligand (34), CD40 agonists (35–39), as well as Ag-unrelated pathogens (30). Nonetheless, our current result is consistent with a previous study that found Ag-irrelevant vaccinia to be comparatively ineffective in eliciting T cell priming to a self-Ag relative to a variety of infectious and noninfectious immunogenic agents (i.e., Ag-irrelevant vesicular stomatitis virus and L. monocytogenes, LPS, and poly(IC)) (30) and suggests that the inability of vaccinia to elicit priming to self-Ag may underlie a distinct mechanism by which its confers immunogenicity upon linked Ags. Thus, while our data do not preclude the possibility that the third-party model operates for other pathogens, this model cannot explain tolerogenicity vs immunogenicity in our system.

In attempting to ascertain the critical parameter(s) that determines tolerogenic vs immunogenic APC function, our studies have argued against models that use functionally similar APCs, thus suggesting that qualitative differences in the signals delivered by APCs are the primary determinants. These differential signals might be purely nonantigenic in the form of costimulatory ligands and/or cytokines. Alternatively, as proposed by Mueller et al. (40), the ability of APCs to prime or tolerize might depend on the ratio of their ability to induce TCR ligation, which is presumed to be tolerogenic, vs their ability to deliver counteracting costimulatory signals.

The inference that tAPCs and iAPCs are functionally distinct does not necessarily imply that they derive from distinct cell lineages. While it might be possible that distinct lineages are inherently tolerogenic or immunogenic (e.g., macrophages might be tolerogenic (41–43) in contrast to dendritic cells (DCs), whose immunogenic properties are well documented (4), or different subpopulations of DCs might have different activities (44, 45)), the same APC might develop either tolerogenic or immunogenic activity depending upon the environment in which it acquires Ag. Thus, Ag acquisition in the absence of inflammatory signals (as would be the case for self-Ags) would activate a default tolerogenic activity, while APCs exposed to inflammatory signals (i.e., cytokines, LPS, etc.) during Ag acquisition would become immunogenic (46, 47). The possibility that DCs perform this dual function has been supported by two elegant studies demonstrating that Ig-mediated targeting of exogenous Ag to DCs in vivo results in tolerization of cognate T cells, while admixture of inflammatory signals induces immunity (32, 39). Thus, while it is not clear whether the same or different APC populations present parenchymal self and pathogen-derived Ags to elicit T cell tolerization and immunity, respectively, it appears that these divergent processes are induced by functionally distinct APCs.

Acknowledgments
We thank John Stanley and Carol Wu for providing vacc-GH, and Leonardo Aguila, Sreyashi Basu, Marissa Caudill, Ephraim Fuchs, Leo Lefrancois, Zihai Li, and Anthony Vella for helpful discussions and critical reading of the manuscript.

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