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# Orchestration of CD4 T Cell Epitope Preferences after Multipeptide Immunization

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# Orchestration of CD4 T Cell Epitope Preferences after Multipeptide Immunization

**Jacqueline Tung and Andrea J. Sant**

**A detailed understanding of the molecular and cellular mechanisms that underlie epitope preferences in T cell priming is important for vaccines designed to elicit a broad T cell response. Protein vaccinations generally elicit CD4 T cell responses that are skewed toward a small fraction of epitopes, a phenomenon known as immunodominance. This characteristic of T cell responses, which limits the diversity of CD4 T cell recognition, is generally attributed to intracellular Ag processing. However, we recently discovered that immunodominance hierarchies persist even after vaccination with synthetic peptides. In this study, we probed the regulatory mechanisms that cause diminished CD4 T cell responses to subdominant peptides after such multipeptide immunization in mice. We have found that the delivery of subdominant and dominant epitopes on separate dendritic cells rescues expansion of less favored CD4 T cells. Furthermore, through the use of genetic models and inhibitors, we have found that selective losses in CD4 T cell responses are mediated by an IFN-γ–induced pathway, involving IDO, and that regulatory T cell activities may also regulate preferences in CD4 T cell specificity. We propose that after multipeptide immunization, the expansion and differentiation of dominant T cells initiate complex regulatory events that determine the final peptide specificity of the elicited CD4 T cell response.** ***The Journal of Immunology*****, 2013, 191: 764–772.**

Recently, there has been tremendous progress in both epitope discovery and the development of predictive algorithms to identify antigenic peptides that can participate in protective T cell responses toward both pathogenic organisms and neoplastic tissue (reviewed in Refs. 1–6). With the steadily increasing number of known pathogen- and cancer-derived epitopes comes great potential for the use of synthetic peptides for vaccination and immunotherapy. Peptide-based immunotherapy has been most commonly explored in the treatment of various forms of cancer (7–10) since the early identification of the melanoma-associated Ag peptide by Boon and colleagues (11). Synthetic peptides have significant advantages over other vaccine modalities, including minimal toxicity, chemical stability, indefinite storage, and easy characterization for purity and composition using well-established technology (reviewed in Ref. 12). Also, peptides are free of risk from bacterial or viral contamination and concerns regarding genetic integration. Synthetic peptides can be used in dendritic cell (DC)–based immunization strategies or in conjunction with adjuvants to elicit more vigorous T cell priming. Although peptide-based vaccines for cancer immunotherapy have focused on stimulating CD8 T cells, more recent efforts have advocated inclusion of CD4 T cell epitopes that promote more robust priming and long-term protective CD8 T cell responses (reviewed in Ref. 13).

In addition to safety and convenience, a theoretical advantage of peptide-based vaccines is that they avoid the complications of Ag processing and the barrier of immunodominance. Protein vaccines and viral vectors require intracellular proteolysis of Ag prior to association of the derived peptides with MHC molecules, unlike synthetic peptides that can bind directly to MHC proteins at the cell surface. The responses to complex Ags are characterized by dramatic asymmetries in the specificity of the elicited T cell repertoire, where only a few dominant specificities are detected (14–19). Typically, cryptic and subdominant peptide epitopes only elicit robust T cell responses when administered as single peptides. Accordingly, T cell hierarchies have been thought to be primarily a consequence of intracellular and presentation events within APCs. Earlier studies by our laboratory identified the parameters that determine immunodominance in CD4 T cell responses to protein Ags and found that the immunogenicity of a peptide can be both predicted and regulated by the kinetic stability of the peptide:MHC class II (pMHC) complex (18, 20). Biochemical studies revealed that dominant peptides persist on class II molecules with a $t_{1/2}$ of 100–200 h, whereas cryptic peptides typically display off-rates of only 2–10 h. Our subsequent studies revealed that DM editing favors export and cell surface presentation of high stability pMHC complexes on APCs, allowing recruitment of a disproportionate fraction of the immune response (21). These and other studies (22–27) support the view that intracellular DM editing selects the peptides that can be presented by class II molecules and can thus elicit CD4 T cells.

The preceding model predicts that Ag in the form of peptides should avoid issues of immunodominance. However, the most recent studies by our group revealed that the preference of CD4 T cell responses to high-stability pMHC complexes persists, even when the Ag is introduced in the form of peptides, which do not require Ag processing or DM editing for presentation (28). After multipeptide immunization, responses to low-stability pMHC complexes were found to initially prime CD4 T cells, but the T cells failed to expand further at later time points. The study reported in this paper

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Abbreviations used in this article: DC, dendritic cell; dLN, draining popliteal lymph node; DT, diphtheria toxin; DTR, diphtheria toxin receptor; IFN-γKO, IFN-γ–deficient; IFN-γRKO, IFN-γR–deficient; iNOS, inducible NO synthase; LN, lymph node; 1-MT, 1-methyl-D-tryptophan; pMHC, peptide:MHC class II; Treg, regulatory T cell; WT, wild-type.

was initiated to identify the mechanisms that favor such CD4 T cell responses to dominant peptides at the expense of simultaneous responses to subdominant peptides. We have identified a network of suppression that involves IFN-γ, IDO, and regulatory T cells (Tregs) that together can reduce CD4 T cell responses to subdominant peptides when immune responses to dominant peptides are occurring simultaneously. The mechanisms revealed in this paper are relevant for both clinical applications of peptide-based vaccines intended to elicit a broad CD4 T cell response and for our understanding of the normal homeostatic mechanisms that regulate CD4 T cell priming in secondary lymphoid tissue.

## Materials and Methods

### *Mice*

BALB/c mice (6–8 wk) were purchased from the National Cancer Institute. C57BL/6J, Foxp3GFP, IFN-γ–deficient (IFN-γKO), and IFN-γR–deficient (IFN-γRKO) mice were purchased from The Jackson Laboratory and maintained in our facility. NOS2KO mice were purchased from Taconic Farms. Foxp3DTR breeders were obtained with a material transfer agreement from Dr. A. Rudensky (Memorial Sloan–Kettering Cancer Center, New York, NY). Thy1.1 mice were gifts from Dr. D. J. Fowell (University of Rochester, Rochester, NY). Animal handling was performed in accordance with the University Committee on Animal Care at the University of Rochester.

### *IFA immunization*

For peptide/IFA immunizations, mice were immunized in both hind footpads with 50 μl emulsion containing 12.5 nmol subdominant peptides and 2.5 nmol dominant peptides with 20 ng LPS (Sigma-Aldrich) per footpad.

### *Flt3L-secreting cells*

Lung epithelial Line 1 carcinoma cells (29) were transfected with a pHβ Apr-1-neo vector expressing the human Flt3L under the β-actin promoter (data not shown). The subclone with the best Flt3L secretors was maintained with G418 selection until ready for use.

### *DC immunization*

To expand splenic DC in vivo, $5 \times 10^5$ Flt3L-secreting cells were injected intradermally into BALB/c mice on the flank, and the tumor was allowed to grow for 10–14 d before mice were euthanized. Purification of DCs was conducted using serum-free DMEM containing 1% penicillin/streptomycin. Intact spleens were injected with 0.3 mg/ml Liberase DL (Roche) in PBS containing $Ca^{2+}$ and $Mg^{2+}$ and were incubated at 37°C for 20 min before making single-cell suspensions. The spleens were treated with RBC lysis buffer (0.15 M $NH_4Cl$, 1 mM $KHCO_3$, and 0.1 mM $Na_2$ EDTA [pH 7.2]) prior to purification of $CD11c^+$ cells by using a positive selection kit (Miltenyi Biotec). DCs were pulsed with 100 μM subdominant peptides and 10 μM dominant peptides with 0.5 μg/ml LPS at 37°C for 1 h, washed with PBS, and injected into both hind footpads at $1 \times 10^6$ cells in 50 μl PBS/footpad. For CFSE labeling, DCs were resuspended at $3 \times 10^6$ cells/ml in PBS plus 1.5 μM CFSE (Molecular Probes) and incubated at room temperature for 8 min. before washing with serum-free media.

### *Cell isolation*

Unless otherwise indicated, draining popliteal lymph nodes (dLNs) or inguinal lymph nodes were treated with 1 mg/ml collagenase D in PBS containing $Ca^{2+}$ and $Mg^{2+}$ for 20 min at 37°C prior to making single-cell suspensions.

### *ELISPOT assay*

CD4 T cells were purified by a negative selection kit (Miltenyi Biotec) and were plated at several dilutions with $5 \times 10^5$ syngeneic splenic APCs and 10 μM peptides overnight at 37°C. IL-2 and IFN-γ spots were quantified with an Immunospot Reader. The total number of T cell responders per LN was calculated from spots obtained per CD4 T cells plated and by back calculating to the starting LN cell count and percentage CD4 in the LN.

### *Diphtheria toxin treatment*

At the indicated time points, Foxp3DTR mice were administered with 50 μg diphtheria toxin (DT) (Sigma-Aldrich) per kg body weight in 100 μl PBS i.p (30). DT was reconstituted according to the manufacturer's protocol.

### *IDO inhibition*

At the time of immunization and for up to 10 d thereafter, mice were either given pH-balanced, sterile, animal-grade water containing 2 mg/ml 1-methyl-D-tryptophan (1-MT) (Sigma-Aldrich) with aspartame or aspartame-only control water in light-sensitive water bottles (31). The solution was changed every 7 d.

### *Treg suppression assay*

$Foxp3GFP^+$ cells were sorted by flow cytometry from dLNs of immunized Foxp3GFP mice based on expression of GFP. Sorted Tregs were plated at $1 \times 10^5$ and serial 2-fold dilutions in 96-well U-bottom plates with $1 \times 10^5$ $CD4^+$ $CD62L^+CD25^-$ target T cells and $1 \times 10^5$ T cell–depleted splenic APCs from Thy1.1 mice. Target T cells were labeled with 5 μM CFSE (Molecular Probes) at $1 \times 10^7$ cells/ml PBS plus 5% FBS for 5 min at room temperature. Cells were resuspended in RPMI 1640 medium containing glutamine, 1% penicillin/streptomycin, 10% heat-inactivated FBS, and 2-ME. Cocultures were stimulated with 1 μg/ml anti-CD3 mAb (BD Pharmingen; clone 2C11) for 72 h at 37°C.

### *Reagents*

Purified rat anti-mouse IL-2 (JES6-1A12), biotinylated rat anti-mouse IL-2 (JES6-5H4), purified rat anti-mouse IFN-γ (AN-18), biotinylated rat anti-mouse IFN-γ (XMG1.2), purified rat anti-mouse IL-4 (11B11), and biotinylated rat anti-mouse IL-4 (BVD6-24G2) were purchased from BD Biosciences. Peptides were purchased from BioPeptides. The hybridoma-producing mAb J1j.10 was acquired from American Type Culture Collection and was used to complement deplete T cells from spleens. Low-tox guinea pig complement was obtained from Cedarlane.

## Results

### *CD4 T cell responses to subdominant peptides are suppressed when there are simultaneous responses to dominant peptides*

We have previously established that after multipeptide immunization, when there are simultaneous CD4 T cell responses to dominant peptides, CD4 T cells specific for subdominant peptides initiate proliferation but abort expansion midway through the response, typically at day 5 or 6 (28). Fig. 1A shows the kinetics of CD4 T cell responses to a representative subdominant CD4 T cell epitope $OVA_{327–339AS}$ when it is administered as a single peptide or coadministered with dominant CD4 T cell epitopes ($MalE_{102–115}$ and $Myo_{107–118}$). Peptides were emulsified in IFA with added LPS and administered s.c. in BALB/c mice, and expansion of Ag-specific T cells was enumerated by quantifying the number of CD4 T cells that secrete IL-2 or IFN-γ within dLNs over time. Expansion of CD4 T cells specific for subdominant peptides was robust when introduced as a single peptide, but when two dominant peptides were cointroduced, CD4 T cells specific for the subdominant peptide halted expansion early (day 6 postimmunization) (Fig. 1A). In contrast, CD4 T cells specific for the two dominant peptides continued to expand until day 8 and then declined (Fig. 1B, 1C). In the kinetic studies shown in Fig. 1, we noted that expansion of CD4 T cells specific for dominant peptides is detectable at day 4 and peaks with four to six times more Ag-specific T cells at day 6 as compared with the CD4 T cells specific for subdominant epitopes. Data that quantify the number of peptide-reactive cells at day 6 producing IFN-γ or IL-2 under conditions of multipeptide immunization are shown in Fig. 1D. Thus, early in the response, dominant CD4 T cells are more abundant than CD4 T cells specific for subdominant responses. Earlier experiments by our laboratory showed that delivering subdominant peptides in a separate emulsion from dominant peptides bypassed the suppression (28), suggesting that the induced loss in responses required that CD4 T cells of different specificities be in close proximity. We therefore considered that there could be competition between different CD4 T cell specificities for APC interactions (32–35), and that over time, adhesion, costimulatory molecules, or APCs themselves could become increasingly limiting as dominant CD4 T cells expand.

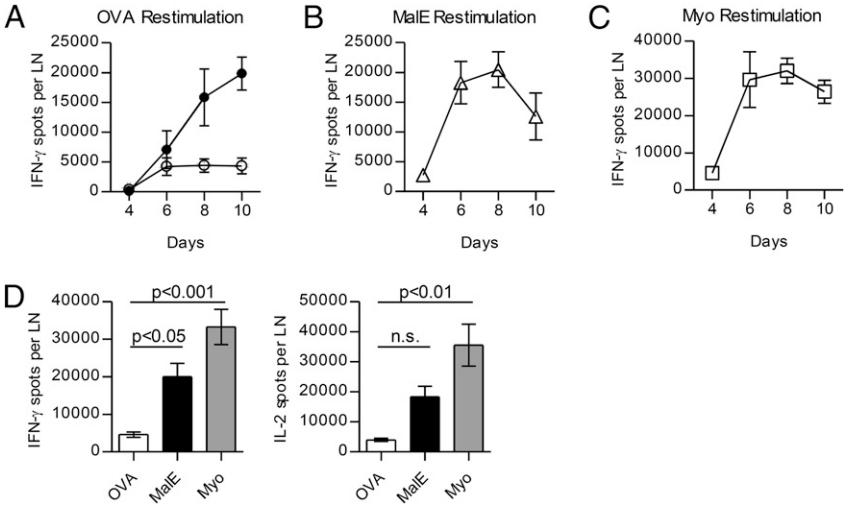


**FIGURE 1.** Expansion of CD4 T cells specific for cryptic peptides fails to progress when there are ongoing CD4 T cell responses to dominant peptides. BALB/c mice were immunized with peptide/IFA emulsions containing OVA peptide alone (filled) or in combination with dominant MalE and Myo peptides (open). CD4 T cells were purified from pooled popliteal LNs of two mice per group and were restimulated with OVA peptide (**A**), MalE peptide (**B**), or Myo peptide (**C**) in IFN-γ ELISPOT assays, with the peptide used for restimulation indicated above each panel. (**D**) CD4 T cells were restimulated with the indicated peptides at day 6 postimmunization, and the total number of cytokine-producing cells for the indicated peptide are shown for IFN-γ (*left panel*) or IL-2 (*right panel*). Data are represented as mean and SD between four independent experiments; *p* values were calculated using one-way ANOVA.

To rigorously evaluate whether suppression required simultaneous presentation of the different epitopes on the same APC, we developed a DC priming regimen, which allowed manipulation of APCs that will present peptides to CD4 T cells. A serum-free method was used to expand and purify DCs from donors to avoid loading DCs with foreign Ags present in FBS. This regimen involved the expansion of splenic DCs in vivo through intradermal injection of Flt3L-secreting carcinoma cells in donor mice. $CD11c^+$ DCs were purified from the spleens of Flt3L-treated mice, pulsed with peptides, and then used to prime naive syngeneic mice. Expansion of CD4 T cell responses was assessed at several time points after immunization (Fig. 2). These experiments confirmed that DC-based priming elicited a similar pattern of response as did emulsion-based immunization: Mice immunized with DCs pulsed with subdominant peptide alone elicited a readily detectable response. However, when dominant peptides were cointroduced on the same DC, although the CD4 T cells specific for the subdominant peptides rapidly initiated a response, expansion failed to progress past day 5 (Fig. 2A). Therefore, under competitive conditions, CD4 T cells specific for the subdominant peptide accumulated to levels only one-third of that achieved when the peptide was introduced alone (Fig. 2D). As before, CD4 T cell responses to dominant peptides attained higher T cell numbers early in the immune response by day 5 (Fig. 2B, 2C). This pattern of loss in responses because of competition was seen at various doses of peptide-pulsed DCs ($5 \times 10^5$–$2 \times 10^6$/footpad; data not shown).

We next tested whether the suppression required the display of the two types of peptides on the same APC by pulsing subdominant and dominant peptides on separate populations of DCs that were then cointroduced s.c. at the same tissue site. These studies, shown in Fig. 2D, revealed that expansion of CD4 T cells specific for subdominant peptides is rescued when subdominant peptides are presented by different DCs (filled bar versus hatched bar). There was no significant difference in the response to the subdominant peptide introduced alone compared with the response elicited when there were bystander responses initiated to dominant peptides on separate cohorts of APCs (Fig. 2D; open bar versus hatched bar). Thus, loss of CD4 T cell responses requires simultaneous engagement of CD4 T cells specific for dominant and subdominant peptides on the same DCs, supporting the hypothesis that suppression is due to highly localized events.

There are at least two nonmutually exclusive possibilities to explain the preceding result. First, it is known that membrane fragments containing surface-bound molecules such as pMHC and costimulatory molecules can be transferred or snatched from the APCs onto T cells after immunological synapse formation and upon cellular detachment, a process known as trogocytosis (reviewed in Refs. 36–38). We speculated that, as the dominant CD4 T cells expand over time, APCs may lose stimulatory properties through loss of pMHC ligands and costimulatory molecules via this process of membrane transfer. This "membrane snatching" by T cells would render the peptide-bearing APCs less able to

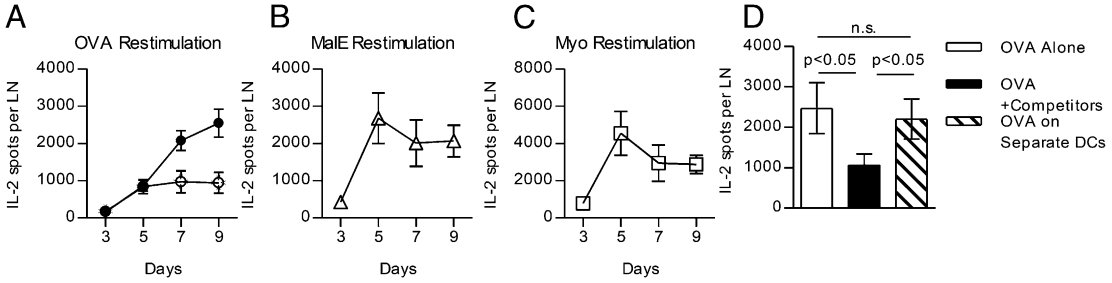


**FIGURE 2.** Use of DC-based priming regimen to evaluate the requirement for simultaneous presentation of subdominant and dominant peptides by the same DC. (**A**–**C**) BALB/c mice were immunized with DCs loaded with OVA peptide alone (filled) or in combination with dominant MalE and Myo peptides (open). CD4 T cells were purified from pooled popliteal LNs of three to five mice per group and were restimulated with OVA peptide (A), MalE peptide (B), or Myo peptide (C) in IL-2 ELISPOT assays at the indicated time points postimmunization, with the peptide used for restimulation indicated above each panel. (**D**) Mice were immunized with DCs loaded with either OVA peptide alone (open bar), OVA in combination with dominant peptides (closed bar), or OVA on separate DCs (hatched bar). CD4 T cells were purified from pooled popliteal LNs of three to five mice per group at day 9 and restimulated with OVA peptide in IL-2 ELISPOT assays. Data are represented as the mean and SEM from four to five independent experiments; statistical analysis was conducted using one-way ANOVA.

sustain continued engagement of CD4 T cells specific for the subdominant peptide, halting their progression program. The second possibility is that APC contact with dominant CD4 T cells induces local suppressive mechanisms that act toward CD4 T cells specific for the subdominant peptides. We have evaluated both of these possibilities.

We first explored whether trogocytosis of molecules on the peptide-bearing APCs contributed to the loss in continued expansion of CD4 T cells specific for subdominant peptides. Although trogocytosis is Ag specific and TCRs have to initially engage cognate pMHC ligands, T cells may also snatch noncognate pMHC ligands if these molecules are present within the transferred membrane fragment. This process would be compounded as dominant daughter CD4 T cells expand and interact with APCs. Under these conditions, requisite cell surface molecules on APCs that are required for continued expansion of CD4 T cells specific for subdominant peptides may become limiting. This idea is supported by published data that suggest that noncognate and endogenous pMHC molecules accumulate within the immunological synapse (39). To evaluate trogocytosis, the level of several cell surface molecules on peptide-loaded DCs was quantified over time within dLNs over the course of the immune response. The expression of MHC class II I-$A^d$ and ICAM-1 molecules on the cell surface of DCs was monitored ex vivo, and we compared three separate immunization conditions: 1) DCs with no peptide; 2) DCs pulsed with subdominant peptide; and 3) DCs pulsed with both subdominant and dominant peptides on the same DCs. Peptide-pulsed DCs were labeled with CFSE prior to immunization to facilitate their identification. dLNs were isolated from recipient mice at several time points after immunization and analyzed by flow cytometry at various time points (Fig. 3). The expression level of MHC class II and ICAM-1 was indistinguishable over time among the three experimental groups, indicating that the loss of molecules from the cell surface over time occurs independently of Ag-specific CD4 T cell interaction. The same was observed for CD80 and CD86 (data not shown). These results suggest that trogocytosis does not account for the selective loss in responses to less favored peptides during competitive T cell responses. We also assessed whether the absolute numbers of peptide-bearing DCs suffered greater losses when there are ongoing responses to dominant peptides. The frequency and absolute number of CFSE-labeled DCs were compared between peptide–pulse conditions in the dLN over time. These studies revealed that the number of DCs accumulating and retained in the LN was independent of peptide, as were the rates of loss of DCs in LNs (Fig. 3B). We have also analyzed trogocytosis in APCs that have migrated from the tissue site after IFA-based immunizations by labeling the emulsion with CFSE, which labeled DCs that emigrated from the site of immunization to the dLN and found the same results (data not shown). Altogether, increased loss of DCs or key cell surface molecules on DCs does not detectably contribute to diminished CD4 T cell responses to subdominant peptides when responses to dominant peptides are ongoing.

*Treg activity is increased under competitive conditions*

One alternate explanation for the loss in CD4 T cell responses to subdominant peptides is that CD4 Tregs are more suppressive when simultaneous responses to dominant peptides are ongoing. Various mechanisms of Treg suppression, either mediated by cell contact–dependent or soluble mechanisms, have been proposed, and the specific mechanism in vivo may depend on the organ and inflammatory context (reviewed in Refs. 40–44). Tregs can suppress target CD4 T cells by inhibiting T cell proliferation and T effector cytokine production, inducing T cell cytolysis, physically blocking DCs, and downregulating costimulatory molecules on DCs (41). We therefore assessed whether Treg activity is increased numerically or functionally after multipeptide immunization compared with single peptide immunization. Foxp3GFP reporter mice were used to quantify Tregs after peptide immunization and to easily sort them for functional assays. By tracking the expansion of Tregs based on expression of Foxp3 within the CD4 T cell compartment, we found that both the frequency (Fig. 4A) and absolute number of Tregs (Fig. 4B) were similar between mice immunized with either subdominant peptide alone or coadministered with dominant peptides at all time points assessed. Because there was no significant increase in Treg numbers in dLNs, we examined whether the function of Tregs changes when multiple peptide epitopes are introduced. An in vitro suppression assay was used to assess Treg-suppressive activity (45, 46). Foxp3GFP mice were immunized with subdominant peptides alone or with the addition of dominant peptides, and at day 6 postimmunization, flow cytometry was used to isolate Tregs from dLNs. Serial dilutions of Tregs were cocultured with CFSE-labeled polyclonal naive target T cells and irradiated splenic APCs. After stimulation of cocultures with anti-CD3 for 72 h, proliferation of CFSE-labeled target CD4 T cells was assessed. Fig. 5A shows that, although modest and variable from experiment to experiment, the suppressive activity of Tregs was enhanced by the presence of dominant peptides in the immunogen. Therefore, Tregs appear to have somewhat more functional activity on a per cell basis in LNs where immune responses to multiple epitopes take place. On the basis of this result, we asked whether suppression of T cell responses to subdominant peptides would be alleviated if Tregs were depleted. Mice expressing the human DTR

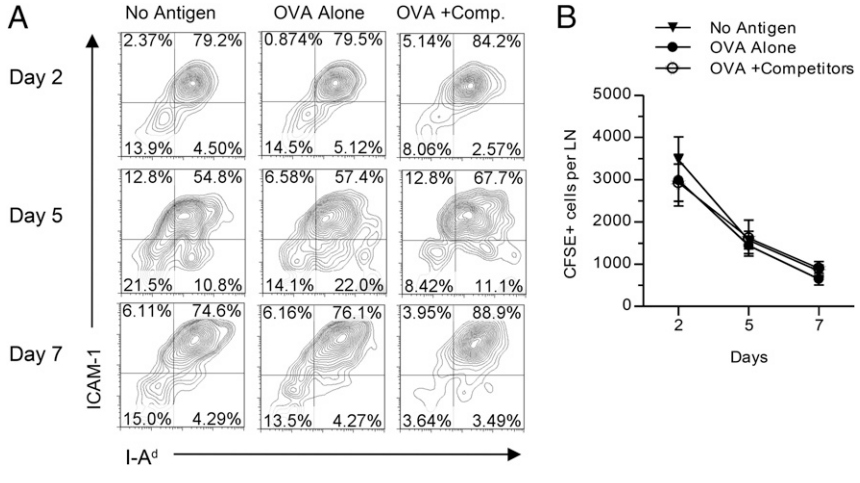


**FIGURE 3.** Cell surface expression of MHC class II and ICAM-1 on DCs is not further reduced after multiple peptide immunization. BALB/c mice (four to five mice per group) were immunized with CFSE-labeled DCs loaded with no Ag, OVA peptide alone, or OVA peptide in combination with dominant MalE and Myo peptides. (**A**) Expression of ICAM-1 and I-$A^d$ on gated $CFSE^+$ cells in pooled dLNs. (**B**) Absolute number of CFSE bright cells per LN represented as the mean and SEM between four independent experiments.

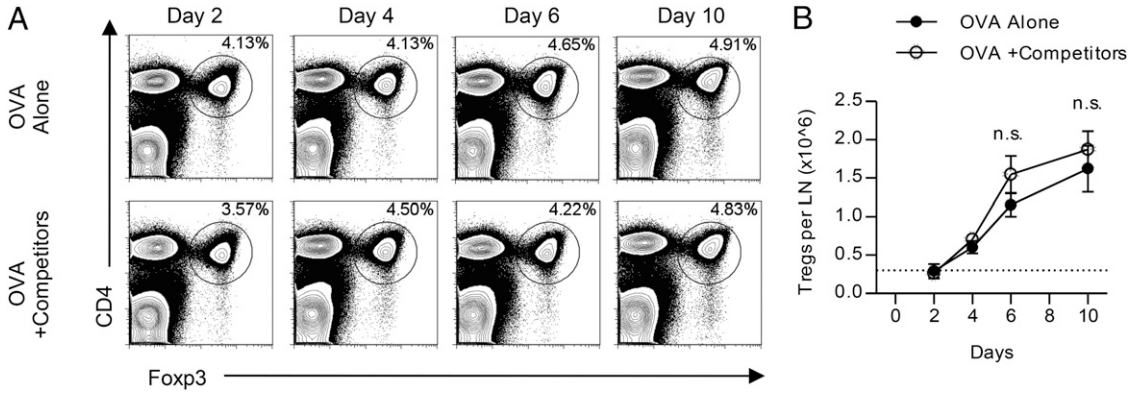


**FIGURE 4.** Multiple peptide immunization does not selectively increase Treg expansion. Foxp3GFP mice were immunized with peptide/IFA emulsions containing OVA peptide alone or in combination with dominant MalE and Myo peptides (+Competitors). (**A**) Frequency of Foxp3$^+$ CD4 T cells in unfractionated popliteal LNs from a representative mouse of two is shown for each group over time. (**B**) Absolute number of Tregs per popliteal LN was quantified from the experiment in (A). Data are representative of three independent experiments. Error bars represent the mean and SD between two individual mice per group; statistical analysis was conducted using two-way ANOVA.

under the control of the Foxp3 promoter (Foxp3DTR) were immunized, and DT was administered at days 3 and 4 postimmunization. DT administration at days 3 and 4 depleted Tregs for at least 6 d, as indicated by the lack of Foxp3DTR-GFP expression within the CD4 T cell compartment at day 10 (Supplemental Fig. 1). We compared T cell responses to subdominant peptides at the peak of the immune response. The experiments displayed in Fig. 5B show that, at day 10, CD4 T cell responses to subdominant peptides were partially rescued when immunized Foxp3DTR mice were depleted of Tregs by treatment with DT. Collectively, these data suggest that when the immune system is confronted with multiple epitopes, more suppressive Tregs are elicited, and these act to selectively diminish expansion of some peptide specificities.

### *IFN-γ mediates suppression of CD4 T cell responses to subdominant peptides*

Because depletion of Tregs did not lead to complete rescue of responses to subdominant peptides, we explored other potential mechanisms responsible for the loss of responses. We considered the possibility that as CD4 T cells specific for dominant peptides expand and differentiate, they produce cytokines that downregulate the ongoing response in the same dLN. The main cytokines detected under the conditions of priming used in this study are IL-2 and IFN-γ (Ref. 28 and this study). Although IFN-γ is historically considered a proinflammatory cytokine, there is also evidence that it can have suppressive effects (47–50). To test the potential involvement of IFN-γ in the loss of responses to subdominant peptides, a genetic approach was adopted. CD4 T cell responses were compared in wild-type (WT) mice and mice deficient in IFN-γ (Fig. 6). These studies revealed that the magnitude of the CD4 T cell responses specific for subdominant peptides in competitive conditions is partially recovered in mice lacking IFN-γ (Fig. 6A). Responses to dominant peptides were not detectably altered by the presence of IFN-γ in the host (Fig. 6B, 6C).

IFN-γ might have negative effects on either APC function or neighboring CD4 T cells. Therefore, we explored the cell type responsive to IFN-γ. To determine whether expression of IFN-γRs on DCs could account for the suppression, we used the DC-based priming strategy. WT and IFN-γRKO DCs were expanded in vivo by using Flt3L as before, and isolated DCs were pulsed with peptide and used to prime WT mice (Fig. 7). Strikingly, CD4 T cell responses initiated by IFN-γRKO DCs were no longer affected by

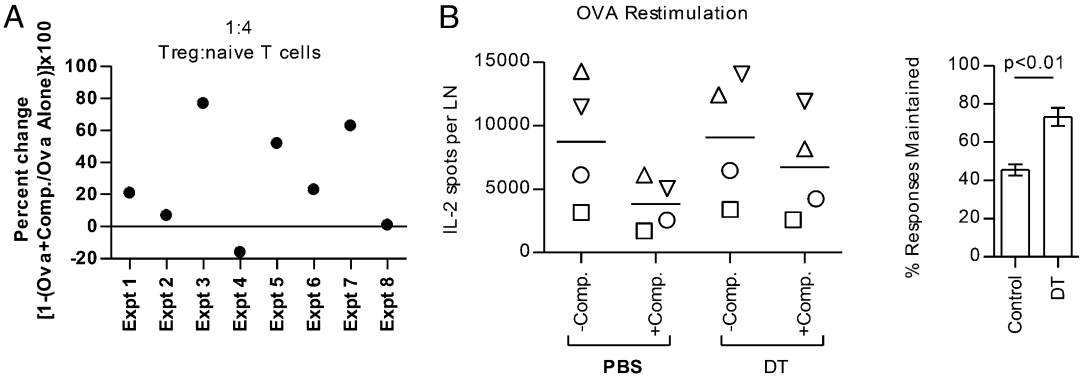


**FIGURE 5.** Treg activity is enhanced by the presence of dominant peptides. (**A**) Tregs were flow sorted from pooled popliteal LNs of Foxp3GFP mice (two mice per group) that were immunized 6 d prior. Tregs were cocultured with irradiated APCs and CFSE-labeled target T cells (Thy1.1) in the presence of 1 μg/ml anti-CD3 for 72 h. Shown is the change in the frequency of Thy1.1$^+$ responder CD4 T cells that have proliferated in cocultures with Tregs sorted from the OVA + Competitors group compared with cocultures with Tregs sorted from the OVA alone group. Data from eight experiments are shown. (**B**) Foxp3DTR mice (two to three mice per group) were immunized with peptide/IFA emulsions containing OVA peptide alone or OVA peptide in combination with MalE, $NP_{261–274}$, and $NP_{310–325}$ competitor peptides. Mice were administered DT or PBS control at days 3 and 4 postimmunization. At day 10, CD4 T cells were purified from pooled inguinal and popliteal LNs and restimulated with OVA peptide. Data from four independent experiments are shown, with each experiment represented by a different symbol (B; *left panel*). The percentage of the OVA-specific response maintained in the presence of the competitor peptides relative to no competition with and without DT treatment is displayed in the bar graph (B; *right panel*).

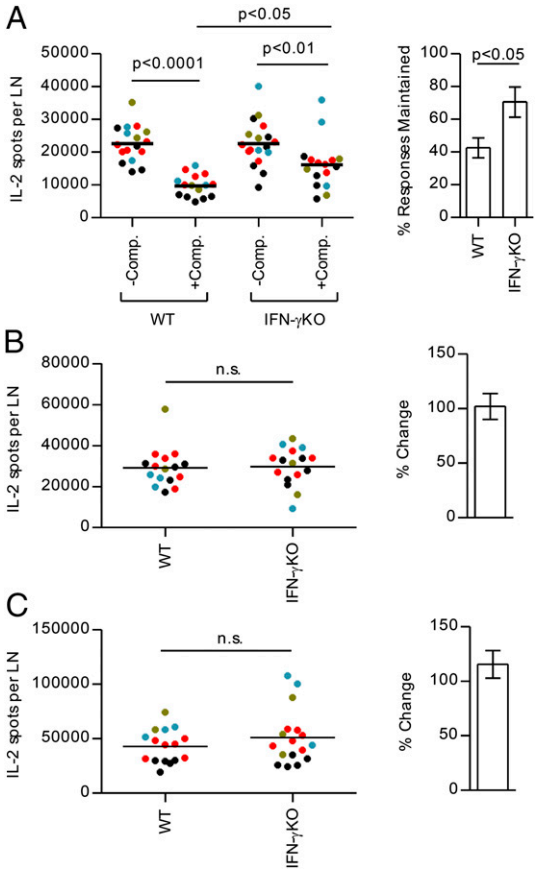


**FIGURE 6.** IFN-γ mediates the loss of cryptic CD4 T cell responses after multiple peptide immunization. WT BALB/c and IFN-γKO mice (three to five mice per group) were immunized with peptide/IFA emulsions containing OVA peptide alone (−Comp.) or OVA peptide in combination with dominant MalE and Myo peptides (+Comp.). At day 10 post-immunization, CD4 T cells were purified from popliteal LNs of individual mice and were restimulated with OVA peptide (**A**), MalE peptide (**B**), or Myo peptide (**C**) in IL-2 ELISPOT assays. The *right panel* of (A) shows the percentage of the OVA peptide–specific response maintained when in competition among the four experiments in WT or IFN-γKO mice. The *right panels* of (B) and (C) show the relative response to dominant peptides of IFN-γKO compared with WT mice. Data from four independent experiments were overlaid (each represented by a different color). The *p* values in the *left panel* of (A) were calculated using one-way ANOVA, followed by a Bonferroni posttest. The *p* values in the *right panel* of (A) and the *left panels* of (B) and (C) were calculated from a Student *t* test.

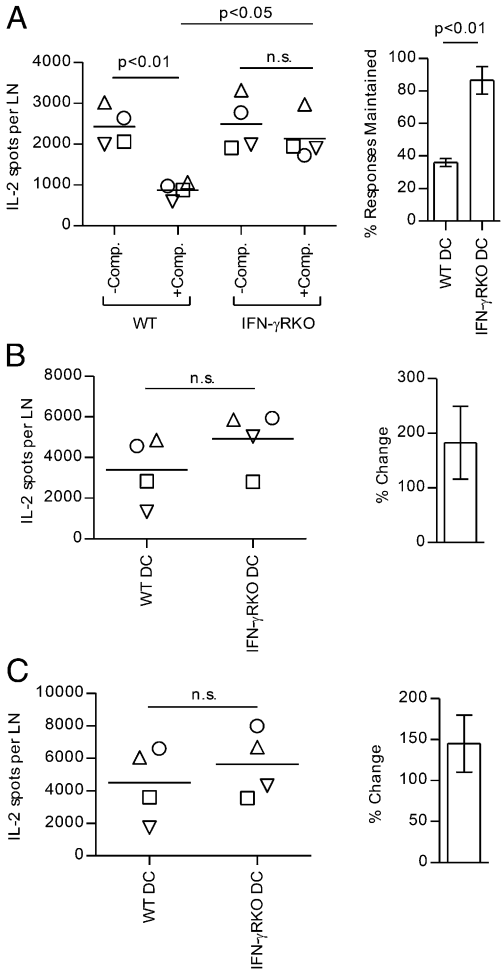


**FIGURE 7.** IFN-γ responsiveness in DCs is necessary for the suppression of subdominant CD4 T cell responses. In four independent experiments (represented by different symbols), WT BALB/c mice (three to five pooled mice per group) were immunized with WT or IFN-γRKO DCs loaded with OVA peptide alone (−Comp.) or OVA peptide in combination with dominant MalE and Myo peptides (+Comp.). CD4 T cells were purified from pooled popliteal LNs at day 9 and tested for reactivity to OVA peptide (**A**), MalE peptide (**B**), or Myo peptide (**C**). The *left panels* show the number of peptide-reactive cells per LN. In (A), the *right panel* shows the percentage of the OVA response maintained when in competition. The *right panels* of (B) and (C) show percent change in T cell responses to the dominant peptides in mice immunized with IFN-γRKO DCs compared with mice immunized with WT DCs. The *p* values were calculated using one-way ANOVA in the *left panel* of (A) and Student *t* test in the *right panels* of (A), (B), and (C).

bystander responses to the dominant peptides. When IFN-γRKO DCs were presenting the antigenic peptides, subdominant peptides were as capable of recruiting and expanding CD4 T cells in the presence of dominant CD4 T cells as in their absence. These experiments suggest that suppression of CD4 T cell responses to subdominant peptides by ongoing CD4 T cell responses can be accounted for by the action of IFN-γ on Ag-bearing DCs.

We next sought to identify potentially suppressive molecules that may be induced in DCs, in response to IFN-γ signaling. It is known that IFN-γ can suppress CD4 T cell responses by activating a tryptophan-catabolizing enzyme, IDO (51–56). IDO catalyzes the rate-limiting step in degradation of the essential amino acid tryptophan. The depletion of tryptophan and accumulation of tryptophan metabolites can promote tolerogenic DC function, proliferative arrest in T cells, and induction of Tregs (56–59). This pathway of regulation is thought to ordinarily function as part of a negative feedback loop that regulates uncontrolled activation of the adaptive immune response (60, 61). To explore the participation of this pathway of regulation, IDO enzymatic activity was blocked throughout the immune response by administering 1-MT in the drinking water (60). As before, at day 10, dLNs were isolated, and peptide-specific cells were quantified by cytokine ELISPOT assays. These studies, shown in Fig. 8, revealed that CD4 T cell responses to subdominant peptides, when in the presence of bystander responses, partially recovered when an IDO inhibitor was present. These results suggested that CD4 T cell responses to subdominant peptides are suppressed by an IDO-dependent mechanism. We have also combined treatments of Treg depletion and 1-MT treatment (Supplemental Fig. 2), as well as IFN-γKO and 1-MT treatment (Supplemental Fig. 3), and did not see additive effects, suggesting that these mediators are part of the same pathway of regulation.

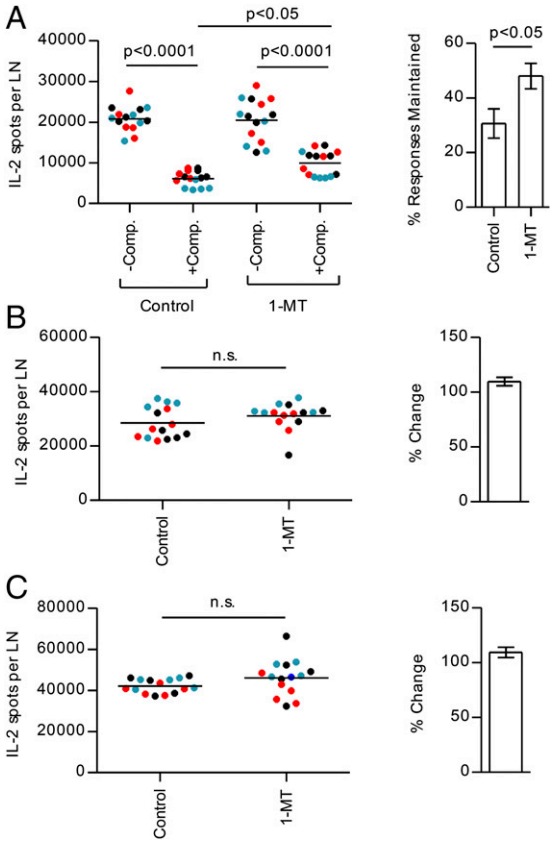


**FIGURE 8.** Involvement of the IDO pathway in regulating the selectivity of CD4 T cell responses. In three independent experiments (each represented by a different color), BALB/c mice (five mice per group) were immunized with peptide/IFA emulsions containing OVA peptide alone (−Comp.) or OVA peptide in combination with dominant MalE and Myo peptides (+Comp.) and were orally administered 1-MT or control. At day 10 postimmunization, CD4 T cells were purified from popliteal LNs of individual mice and were restimulated with OVA peptide (**A**), MalE peptide (**B**), or Myo peptide (**C**). The *left panels* show the total number of peptide-specific T cells. The *right panels* show the percentage of the OVA-specific response maintained when in competition (A) or the percent change in T cell responses in mice treated with 1-MT compared with control (B, C); *p* values shown were calculated from one-way ANOVA, followed by Bonferroni posttest in the *left panel* of (A) or Student *t* test in the *right panel* of (A) and for (B) and (C).

## Discussion

There is a great deal of interest in identifying the epitopes that CD4 T cells focus on during immune responses and the forces that shape preferences in T cell priming in response to pathogen infection and vaccination. Progress in our understanding of this issue would allow the use of these epitopes in vaccines to elicit broadly reactive CD4 T cells. The current study addressed the mechanisms that underlie peptide epitope preferences of CD4 T cell responses to multipeptide vaccination. Our studies revealed that CD4 T cell responses to less favored pMHC complexes can fully expand if subdominant and dominant peptides are loaded on separate DCs. We considered the possibility that expansion of dominant CD4 T cells would diminish the potency of APCs through trogocytosis, thus limiting the amount of molecules available for priming subdominant CD4 T cells. However, we found that the expression level of key costimulatory molecules was not affected by the number of epitopes contained in the immunogen. This raised the possibility that dominant T cell responses actively suppress cryptic or subdominant CD4 T cell responses after multipeptide immunization, an issue not experimentally addressed in the field.

We then evaluated the possibility that suppressive mechanisms might be induced by robust responses to dominant peptides and found strong evidence for a network of regulation that involves IFN-γ, IDO, and Tregs. Our studies revealed that expression of IFN-γ by the host is necessary for restricting the CD4 T cell responses to only dominant peptides. This possibility is further supported by our results that, when peptide-pulsed APCs are deficient in the receptor for IFN-γ, CD4 T cell responses to subdominant peptides were restored. Although Treg numbers were similar, their suppressive activity was slightly increased when dominant peptides were coadministered. Furthermore, the depletion of Tregs enhanced responses to subdominant peptides. These results collectively suggest that continued expansion of CD4 T cell responses to cryptic or subdominant peptides is inhibited by suppressive cells and molecules that arise when there are stronger responses to cointroduced dominant peptides.

The key findings made in this study suggest a complex pathway of immunoregulation (illustrated in Fig. 9). CD4 T cells reactive to dominant peptides that bind with high stability to the MHC class II molecule expand rapidly, differentiate, and produce IFN-γ. Under the conditions of priming used in this study, typical of pathogens that ligate TLR4, expansion of CD4 T cells is associated with differentiation into IFN-γ–producing cells. During the same time frame, expansion of CD4 T cells for subdominant peptides is initiated but lags behind the dominant T cells, possibly because of lower TcR signaling by unstable pMHC ligands. By day 4–6 after immunization, dominant CD4 T cells have completed their expansion and secrete IFN-γ in the local environment. IFN-γ binds to IFN-γRs on peptide-bearing DCs leading to induction of IDO and kynurenines, activation of Tregs, and attenuation of DC stimulatory capacity. Under these conditions, expansion of CD4 T cells specific for subdominant and cryptic peptides discontinues. Inflammation in the dLN may induce IFN-γ production by NK cells or CD8 T cells. However, we did not observe a difference in T cell responses when NK cell depletion experiments were conducted, and we have determined that our antigenic

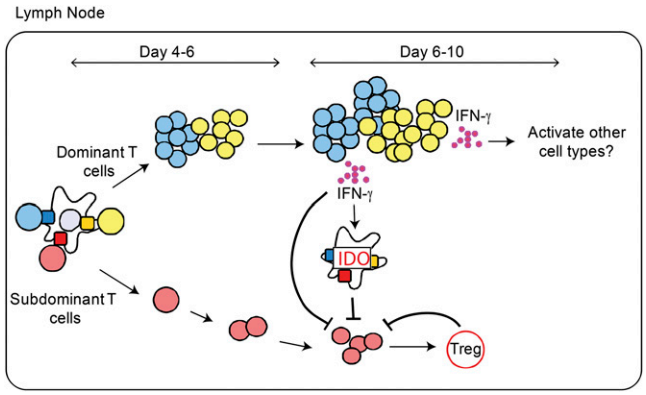


**FIGURE 9.** Ongoing CD4 T cell responses to dominant peptides induce suppressive activities in the local microenvironment that reduce the abundance of CD4 T cells specific for subdominant peptides. Peptides introduced either s.c. or on autologous DCs initially activate all peptide-specific CD4 T cells equally whether in the presence or absence of other antigenic peptides. Within the first few days of DC arrival, CD4 T cells specific for dominant peptides expand more quickly than CD4 T cells specific for subdominant peptides. As dominant CD4 T cells expand, they differentiate into effector cells and produce IFN-γ. The presence of IFN-γ in the local environment alters the function of DCs that are coengaging CD4 T cells specific for subdominant peptides. In response to IFN-γ, DCs rapidly activate IDO, which in turn increases tryptophan catabolism and production of kynurenines that can arrest cell division of subdominant CD4 T cells and induce the generation of Ag-specific Tregs.

peptides do not initiate Ag-specific CD8 T cell responses (data not shown).

There is likely to be at least one more yet unidentified participating cell or molecule that is induced by IFN-γ in DCs because we were not able to completely replicate the rescue of subdominant responses in experiments in which IDO inhibitors were used or when Tregs were depleted, as seen with immunization with IFN-γRKO DCs. One candidate molecule is inducible NO synthase (iNOS). IFN-γ has also been shown to induce iNOS expression in DCs and nonhematopoietic stromal cells of lymphoid organs that can negatively regulate T cell survival and proliferation (62–64). To test the possible involvement of induced iNOS activity in the suppression of subdominant T cell responses after peptide immunization, we compared competitive T cell responses in NOS2KO and WT mice (data not shown). These experiments revealed that deficiency in iNOS had no effect on T cell responses, suggesting that iNOS either may not be optimally expressed in cells during our experimental priming conditions or that its activity was not sufficient to suppress T cell expansion.

A critical issue to understand is why CD4 T cell responses to subdominant peptides are selectively sensitive to suppression by this immunoregulatory network. There are several interesting possibilities. First, in vivo, there is likely to be a finite proliferative program for most CD4 T cells. In our experimental system, the expansion and differentiation of CD4 T cell responses to dominant peptides is completed prior to the induction of suppressive pathways. Sensitivity to the suppression may thus be determined by generation time. If subdominant CD4 T cells are relatively slower to expand, they would immediately be susceptible to inhibitory pathways initiated prematurely by ongoing dominant responses. An alternative possibility is that protection from suppression is dependent on productive TCR signaling, and diminishing epitope density of cryptic and subdominant peptides over time leaves T cells vulnerable to this suppression. In this way, halted expansion of T cells to subdominant peptides may be a consequence of their inefficient signaling. This would potentially be a mechanism to "weed out" less useful T cells, those that are specific for rapidly decaying pMHC complexes.

The current study has clinical implications for DC-based cancer immunotherapy approaches. DC-based approaches are currently used clinically to induce T cell responses to tumor-associated Ags (reviewed in Ref. 65). Our findings suggest that DC immunization strategies can be successful if subdominant peptides are loaded on separate DCs from dominant peptides, which allows the unperturbed expansion of subdominant CD4 T cells and sequesters subdominant CD4 T cells from the effects of ongoing dominant CD4 T cell responses.

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## Disclosures

The authors have no financial conflicts of interest.

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