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MHC Class II Epitope Nesting Modulates Dendritic Cell Function and Improves Generation of Antigen-Specific CD4 Helper T Cells

Courtney L. Erskine,* Christopher J. Krco,* Karen E. Hedin,* Nancy D. Borson,[†] Kimberly R. Kalli,[‡] Marshall D. Behrens,* Sabrina M. Heman-Ackah,* Eric von Hofe,[§] Peter J. Wettstein,[†] Mansour Mohamadzadeh,[¶] and Keith L. Knutson*

CD4 Th cells are critical to the development of coordinated immune responses to infections and tumors. Th cells are activated through interactions of the TCR with MHC class II complexed with peptide. T cell activation is dependent on the density of MHC peptide complexes as well as the duration of interaction of the TCR with APCs. In this study, we sought to determine whether MHC class II peptides could be modified with amino acid sequences that facilitated uptake and presentation with the goal of improving Th cell activation *in vitro* and *in vivo*. A model epitope derived from the murine folate receptor α , a self- and tumor Ag, was modified at its carboxyl terminus with the invariant chain-derived Ii-Key peptide and at its N terminus with a peptide that enhances uptake of Ag by APC. Modification of a peptide resulted in enhanced generation of high-avidity murine folate receptor α T cells that persisted *in vivo* and homed to sites of Ag deposition. The nesting approach was epitope and species independent and specifically excluded expansion of CD4 regulatory T cells. The resulting Th cells were therapeutic, enhanced *in vivo* helper activity and had an increased ability to resist tolerizing immune microenvironments. In addition to improved immunoadjuvants, this epitope modification strategy may be useful for enhancing *ex vivo* and *in vivo* generation of Th cells for preventing and treating diseases. *The Journal of Immunology*, 2011, 187: 316–324.

Recently, increasing importance is being given to the stimulation of a CD4⁺ Th response in cancer immunotherapy. Th cells are critical to the development of a tumor-specific immune response by activating Ag-specific effector cells and recruiting cells of the innate immune system such as macrophages and dendritic cells (DC) (1). At least three predominant Th cell subtypes exist: Th1, Th2, and Th17 (2). Th1 cells, characterized by secretion of IFN- γ and TNF- α , are primarily responsible for activating and regulating the development and persistence of CTL. In addition, Th1 cells activate APCs and induce limited production of the type of Abs that can enhance the uptake of infected cells or tumor cells into APCs. Th2 cells favor a predominantly humoral response. The role of Th17, a newly described T cell, remains uncertain, but recent evidence suggests that they act as a link between innate and adaptive immune

responses (3). Particularly important during Th differentiation is the cytokine environment at the site of Ag deposition or in the local lymph node (2). Th1 commitment relies on the local production of IL-12, Th2 development is promoted by IL-4 in the absence of IL-12, and Th17 induction requires a combination of TGF- β and IL-6 (2). On the basis of our improved understanding of their role, therapeutic methods to augment Th cell numbers (e.g., vaccines and T cell therapy) for the treatment or prevention of human diseases, such as cancer or infectious diseases, are being developed (1, 4–7). Overcoming tolerance or other regulatory mechanisms (e.g., TCR-binding thresholds) is a key problem for generating high-avidity T cell responses using defined epitopes.

Th cells generally respond to 15 aa peptides presented by class II MHC or HLA molecules (1). T cell activation is only triggered when TCR and MHC interactions reach a specific half-life threshold. Efficient T cell activation is facilitated by high epitope density on APCs (8). In this study, we speculated that increased epitope storage coupled with enhanced MHC class II exchange would result in a higher apparent affinity, assuming a higher K_{on} rate and perhaps a reduced K_{off} rate leading to improved generation of Th cells. To achieve increased exchange of an epitope onto MHC class II, a model epitope derived from the folate receptor α (FR α) was modified at its carboxyl terminus with Ii-Key, a fragment of the invariant chain (Ii, CD74) that is well-known to promote exchange of the peptides in the MHC class II molecules at the site of action of HLA-DM (9). FR α , a self-Ag, is often overexpressed on breast and ovarian cancers but has limited expression in normal healthy tissues and is mainly found in the kidney (10). Animal modeling has shown that modification of MHC class II peptides with Ii-Key enhances *in vivo* immunization (11). These and similar studies (12–14) fostered a recent human clinical trial in which disease-free breast cancer patients were immunized with a HER-2/neu-derived HLA-DR peptide conju-

*Department of Immunology, College of Medicine, Mayo Clinic, Rochester, MN 55905; [†]Department of Surgery, College of Medicine, Mayo Clinic, Rochester, MN 55905; [‡]Department of Oncology, College of Medicine, Mayo Clinic, Rochester, MN 55905; [§]Antigen Express, Inc., Worcester, MA 01606; and [¶]Feinberg School of Medicine, Northwestern University, Chicago, IL 60611

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Address correspondence and reprint requests to Dr. Keith L. Knutson or Dr. Mansour Mohamadzadeh, Mayo Clinic, 200 First Street Southwest, Rochester, MN 55905 (K.L.K.) or Feinberg School of Medicine, Northwestern University, 303 East Chicago Avenue, Chicago, IL 60611 (M.M.). E-mail addresses: knutson.keith@mayo.edu (K.L.K.) and m.zadeh@northwestern.edu (M.M.).

Abbreviations used in this article: B6, C57BL/6; DC, dendritic cell; DCpep, dendritic cell peptide; FR α , folate receptor α ; KC, keratinocyte-derived chemokine; MMC, mouse mammary carcinoma; Treg, regulatory T cell.

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gated to Ii-Key (15). The seminal and unusual observation from that trial was that patients developed HER-2/neu-specific immunity without the use of an adjuvant. They also observed that the T cells generated with the vaccine could persist in patients for at least 6 mo (15).

To enhance storage, the N terminus of the class II epitope was modified with an amino acid sequence discovered by Mohamad-zadeh and colleagues (16) using peptide phage display libraries. In that study, the investigators identified several 12-mer peptides that could bind to the cell surface of mouse and human DC. These peptides were fused to the hepatitis C protein Ag and used to augment the ex vivo expansion of Ag-specific T cells from individuals previously immunized with the native form of Ag. The peptides bind saturably, but it is not known to what receptor molecule they bind. Importantly, binding enhances the uptake and accumulation of Ag within the DC for sustained delivery to MHC molecules. We selected one of the 12-mer peptides called DC peptide (DCpep; FYPSYHSTPQRP) because of its ability to bind to both human and mouse cells. We show in the current article that nesting of MHC class II epitopes between Ii-Key and DCpep results in enhanced generation of Th cells capable of responding to low levels of Ag and maintaining proliferative activity at sites of Ag deposition.

Materials and Methods

Animals

FVB/N breeding mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 (B6) mice were obtained from an internal Mayo Clinic breeding colony. Only female mice 8–12 wk old from the B6 strain were used for experimentation. The adoptive T cell experiments used C57BL/6J mice obtained from The Jackson Laboratory. Only female mice 6–7 wk old were used for these experiments. All experiments were performed in accordance with Institutional Animal Care and Use Committee guidelines.

Media

All experiments and cell culture were carried out using the following media formula, hereafter referred to as complete media: RPMI 1640 medium supplemented with 10% FBS, 25 mM HEPES, 1.5 g/l sodium bicarbonate, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 mM 2-ME (Invitrogen, Carlsbad, CA). All other additives were obtained from Mediatech (Herndon, VA).

Tumor cell culture and lysate

A mouse mammary carcinoma (MMC) cell line was generated from mechanical processing of a spontaneous tumor harvested from neu-transgenic mice as described previously (17). MMC lysate was produced by freezing and thawing pellets of MMC cells five times.

Peptide synthesis

For most experiments, a naturally processed CD4 T cell-activating peptide, FR74 (aa 74–88, KDISYLYRFNWNHCG) derived from mouse FR α , was modified at one or both termini with one of two unique peptides, Ii-Key (LRMK) (18) or DCpep (FYPSYHSTPQRP) (16). The peptides and linkers used are shown in Fig. 1A. All peptides, except the native FR74, were acetylated at the N terminus to prevent degradation. FR74.1 is the native peptide with N-terminal acetylation. Other MHC class II peptides used included a human FR α epitope, FR56 (QCRPWRKNACCSTNT) (19), and the OVA I-A^b epitope OVA 323 (ISQAVHAAHAEINEAGR). These peptides were modified in exactly the same manner as FR74. As an irrelevant control, a peptide derived from human collagen II, HII.71 (PPGLTGPAGEPGRQSGPGAD), was synthesized in parallel and used throughout. The H2-Kb peptide FR161 (SSGHNECPV, aa 161–169) was used to test for the ability of high-avidity Th cells to enhance CD8 T cell immunity. All peptides were synthesized by the Mayo Clinic peptide synthesis core (Rochester, MN).

T cell isolation and activation

To isolate naive CD4⁺ T cells from single-cell suspensions of lymphoid organs, all non-CD4⁺ T cells were magnetically labeled with a mixture of

biotin-conjugated Abs and then depleted using an AutoMacs separator (Miltenyi Biotec, Bergisch Gladbach, Germany). Naive T cells were enriched to ~97% purity using this strategy (data not shown). T cells were activated using peptide-pulsed bone marrow-derived DC. For DC generation, bone marrow was harvested from mouse long bones and seeded in 12-well plates (2×10^6 /well) for adoptive T cell experiments and 6-well plates (3×10^6 /well) for all other experiments. The DC were plated in complete media supplemented with murine GM-CSF (10 ng/ml) and IL-4 (1 ng/ml). Media and cytokines were replaced after 3 d. Following media and cytokine replacement on day 3, cytosine phosphorothiate guanine oligonucleotides were added to the DC cultures at 1 μ g/ml on day 5. The DC were pulsed with peptide at 37°C for 4 h on day 6. After being pulsed with peptide, CD4⁺ T cells were added and incubated at 37°C. On days 8 and 10, IL-2 (50 U/ml) was added (eBioscience, San Diego, CA). On day 15, the cells were assayed. Anti-mouse IL-4R Ab, anti-mouse CXCL10, anti-mouse keratinocyte-derived chemokine (KC), and murine IFN- γ used in cell culture were obtained from R&D Systems (Minneapolis, MN).

Adoptive T cell therapy

Following media and cytokine replacement on day 3, the DC were prepared for T cell induction on day 6. The tissue culture-generated DC were pulsed with 10 μ g/ml FR74 peptide or its modification, FR74.4, at 37°C for 4 h. Next, the peptide-loaded DCs were washed with media, and CD4⁺ T cells, generated as previously described, were added at 1×10^6 /well and incubated at 37°C. On days 8 and 10, IL-2 (50 U/ml) was added. On day 15, cells were expanded with CD3/CD28 beads (Invitrogen). The expanded cells received another dose of IL-2 on day 19 and were then injected i.p. into C57BL/6J mice at ratios of 1×10^5 , 1×10^6 , or 1×10^7 T cells/mouse on day 24. On day 54, mouse spleens and tumors were harvested. A total of 5×10^6 ID8 ovarian tumor cells, derived from murine ovarian epithelial cells, were injected i.p. into C57BL/6J mice 10 d before T cell injection (day 9). Cyclophosphamide (1 mg) (Sigma-Aldrich, St. Louis, MO) was also injected i.p. 5 d (day 14) before the T cell injection.

Proliferation evaluation of CD4 T cell growth by [³H]thymidine assay

To determine the number of T cells that proliferate in the presence of their Ag-specific peptide, splenocytes were seeded at 1×10^6 cells/well with 10 μ g/ml peptide in a 96-well plate and incubated for 24 h at 37°C. [³H]thymidine was then added at 1 μ Ci/well and incubated for 24 h. After incubation, the cells were harvested on a filtermate harvester machine (PerkinElmer, Boston, MA). The unfilter-96 filter membrane was dried, and scintillation fluid was added. Finally, the amount of radioactivity was counted on a Top Count NXT scintillation counter. Results are presented as cpm following subtraction of backgrounds.

IFN- γ ELISPOT assay

ELISPOT plates (Millipore, Billerica, MA) were coated with IFN- γ capture Ab (MabTech, Mariemont, OH) and incubated overnight. The culture plates were then washed with PBS and blocked with media for 2 h. Next, MHC-matched splenocytes pulsed with peptide or tumor lysates were added (1×10^5 /well). T cells (5×10^4 /well) were added and incubated at 37°C for 24 h. After washing with PBS containing 0.05% Tween 20, a biotinylated anti-IFN- γ secondary Ab (MabTech) was added, and the plates were incubated for 2 h at room temperature, followed by another wash cycle. Next, streptavidin-HRP (BD Pharmingen, San Diego, CA) was added, and the plates were incubated for 1 h. Finally, plates were washed with PBS containing 0.05% Tween 20, followed by PBS, and plates were then developed using a 3-amino-9-ethyl-carbazole substrate (Sigma-Aldrich); the reaction was stopped with water. After drying overnight, the plates were read on an AID ELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany).

Multiplexed microsphere cytokine immunoassay

Supernatants were removed from wells containing stimulated or unstimulated T cells. Cytokines and chemokines were measured using multiplex microspheres per the manufacturer's direction (Bio-Rad, San Diego, CA), as we have published previously (20).

Flow cytometry

Staining for cell surface molecules and Foxp3 and flow cytometry were performed as described by Knutson et al. (21). For flow cytometric analysis, 10,000–400,000 events were collected for all groups. Abs against CTLA-4, MHC class I, and Foxp3 were obtained from eBioscience. Abs against murine CD80, CD62L, CD4, CD44, and CD86 were obtained from BD Pharmingen.

TCR-based repertoire analysis

RNA isolation and real-time PCR analysis of the TCR repertoire of FR74-specific T cell lines were carried out as described by Wettstein et al. (22).

Vaccinations

B6 mice were immunized with MHC class I epitope, FR161 with or without FR74, or FR74.4 three times 3 d apart over the course of a single week. CFA was used with the first vaccine, whereas the second and third vaccines were mixed with IFA. Seven days following the final immunization, splenocytes were harvested as described previously (20).

Statistical analyses

Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad, San Diego, CA) (<http://www.graphpad.com>). Student *t* test or one-way ANOVA test (with Tukey post test) was performed to determine statistically significant difference. A *p* value <0.05 was considered as significant.

Results

Nesting of MHC class II epitopes enhances generation of high avidity CD4 T cells

The MHC class II peptide used as a model, which was termed FR74, constitutes aa 74–88 of murine FR α (Fig. 1A). FR74 is immunogenic when administered as a vaccine with adjuvant, activating predominantly CD4 T cells (Fig. 1B). T cell lines generated against FR74 recognized DC pulsed with lysates derived from an FR α -expressing breast cancer cell line, MMC (Fig. 1C). The N terminus of FR74 was modified with the four-amino acid Ii-Key peptide (LRMK) using 5-aminovaleric acid, a flexible polymethylene spacer to yield FR74.2 (Fig. 1A). The C terminus of FR74 was modified with DCpep using a furin-sensitive linker to yield FR74.3 (Fig. 1A) (7). FR74 with both modifications was designated FR74.4.

Either modified peptide (FR74.2 and FR74.3), when used alone without the other, was effective at increasing the numbers of FR74-specific T cells (Fig. 1D). Importantly, the modifications unmasked a population of high-affinity T cells that recognized Ag in ELISPOT assays at concentrations as low as 10^{-9} g/ml native peptide. Treatment of purified CD4 T cells with dual-modified nested peptide (FR74.4) resulted in a higher number of cells measured at all concentrations of native peptide, except 10^{-9} g/ml. Similar results were obtained from the assessment of the proliferative response to native FR74 (Fig. 1E). The notable exception was that only the nested FR74.4 peptide was able to induce FR74-specific T cells that were able to respond with proliferation to Ag at 10^{-9} g/ml. FR74-specific CD4 T cells generated with FR74.4 recognized naturally processed Ag from MMC lysates (data not shown). Overall, these results are consistent with Ohlen et al. (23) who showed that tolerance to self-Ag is maintained at the level of expansion rather than function.

A potential source of variance between the comparisons described above is the differing masses of the peptides. To avoid variance in outcomes related to these differences, the experiments described thus far were performed using equal masses of peptide. Because this results in differences (albeit, minor) in the molarity of the MHC class II epitope, differences in the T cell activation when T cells were incubated under equal molar amounts of peptides was also examined. As shown in Fig. 1F, FR74.4 demonstrated superior ex vivo generation of FR74-specific T cells relative to the native peptide. Therefore, regardless of molarity or mass, nesting results in superior T cell generation. The capability of the nested epitopes to elicit Ag-specific T cells from purified naive pools was also examined. Remarkably, as shown in Fig. 1G, the nested epitope resulted in the generation of Ag-specific T cells from naive pools of T cells, whereas no specific reactivity could be elicited with the native epitope.

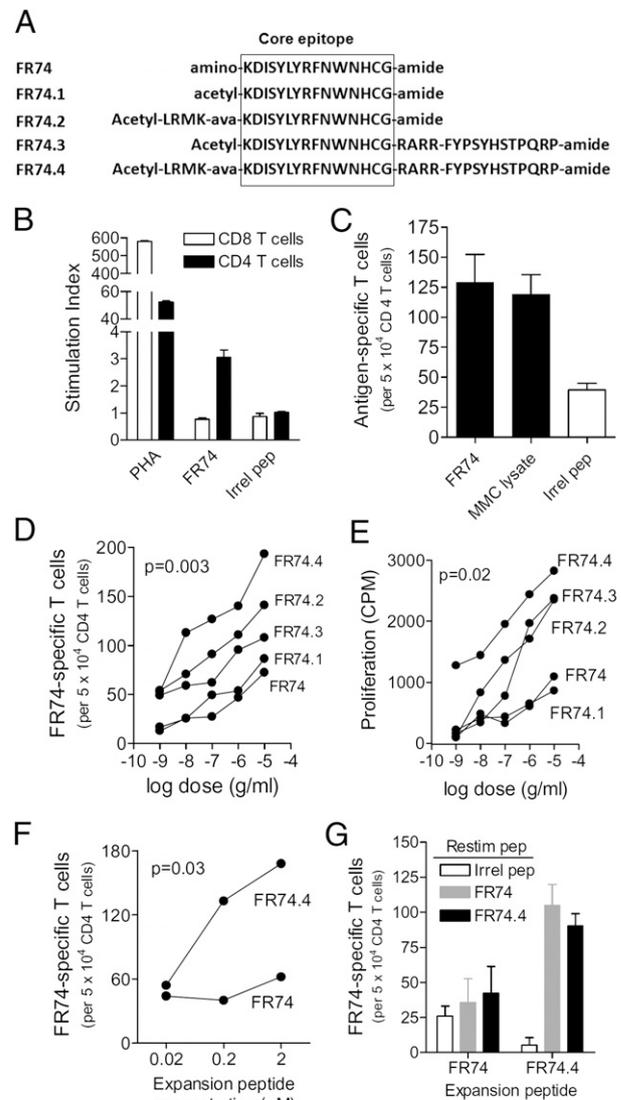
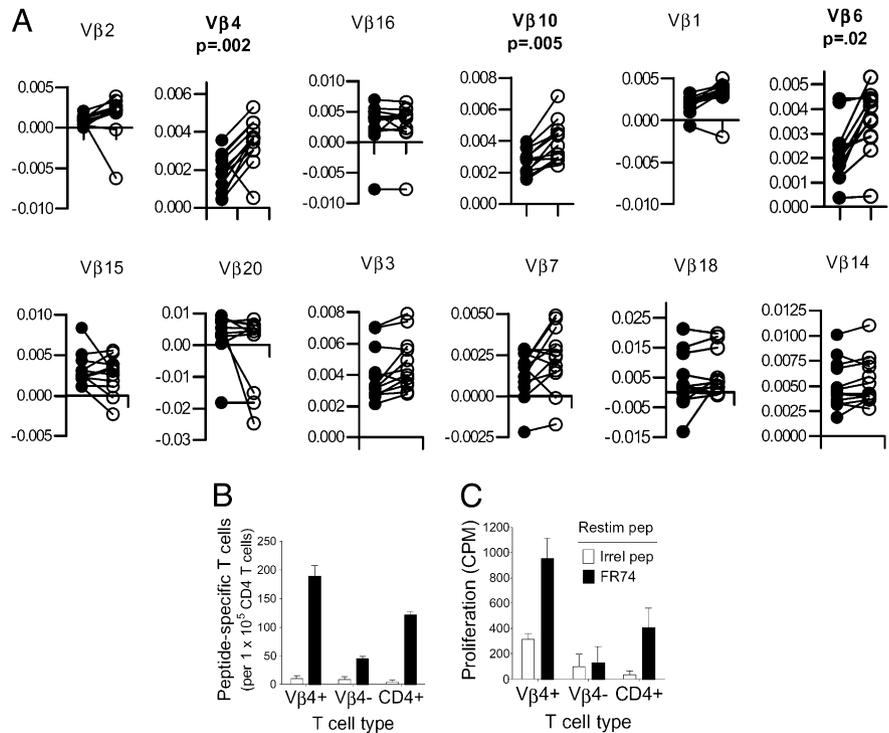


FIGURE 1. Nesting of MHC class II epitopes enhances generation of high-avidity CD4 T cells. **A**, The five FR α -derived MHC class II peptides with or without modifications (see text). **B**, Stimulation indices (mean \pm SE; *n* = 3) from a proliferation assay in which CD4 and CD8 T cells from FR74-immunized mice were tested for response to nonspecific stimulation (PHA), FR74 peptide, or an irrelevant peptide (Irrel pep). **C**, IFN- γ ELISPOT analysis of FR74-specific T cell lines stimulated in vitro with DC pulsed with FR74, FR α -overexpressing tumor cell lysate (MMC), or an irrelevant peptide Ag. Bar is mean \pm SE (*n* = 3). **D** and **E**, CD4 T cells were activated, ex vivo, with DC pulsed with one of the five peptides in **A** for 14 d. IFN- γ ELISPOT and proliferation analysis of the T cells, following expansion with peptides depicted in **A**, over a distribution of concentrations of native FR74 peptide. Each bar is mean \pm SE (*n* = 3). **F**, IFN- γ ELISPOT results of FR74-specific and FR74.4-specific T cell cultures generated with various (*x*-axis) concentrations of peptide. Cells were subsequently restimulated with native FR74 peptide. **G**, The results of IFN- γ ELISPOT analysis of FR74-specific T cells expanded from purified naive CD4 T cells with FR74 and FR74.4. Each culture was tested for reactivity by restimulating with FR74, FR74.4, an irrelevant peptide. Each bar is mean \pm SE (*n* = 3). The *p* values were calculated using one-way ANOVA.

Epitope nesting focuses the TCR repertoire

Collectively, the results demonstrated that nesting of the epitopes results in recruitment of high-avidity T cells that would not ordinarily be activated using native peptide alone. This conclusion

FIGURE 2. Epitope nesting focuses the TCR repertoire. *A*, Comparisons in the V β usage, respectively, of FR74 (closed symbols)- and FR74.4 (open symbols)-generated cultures. Changes are calculated from levels in CD4 T cells cultured in the absence of peptides, and the *x*-axis of each plot represents baseline TCR usage in naive CD4 T cells. Values above or below the axis show higher or lower usage levels relative to naive T cells, respectively. *p* values were calculated using a paired *t* test. Each data point is a unique V β and J β pair. *B* and *C*, ELISPOT and proliferation analysis, respectively, of FR74.4-expanded V β 4⁺ and V β 4⁻ or whole unfractionated CD4 T cells restimulated with irrelevant peptide or native FR74. Each bar is mean \pm SE (*n* = 3).



was confirmed through the assessment of TCR V β usage, which showed that FR74.4 resulted in preferential recruitment of T cells that used V β 4, V β 10, and V β 6 when compared with FR74 (Fig. 2A). Because the percent increase in V β 4 (184% increase) tran-

scripts was greater than either V β 6 (158%) or V β 10 (150%), we suspected that the majority of the FR74-specific T cell immunity was in the V β 4 TCR compartment. This was confirmed by purification and exposure of V β 4-expressing naive CD4 T cells to

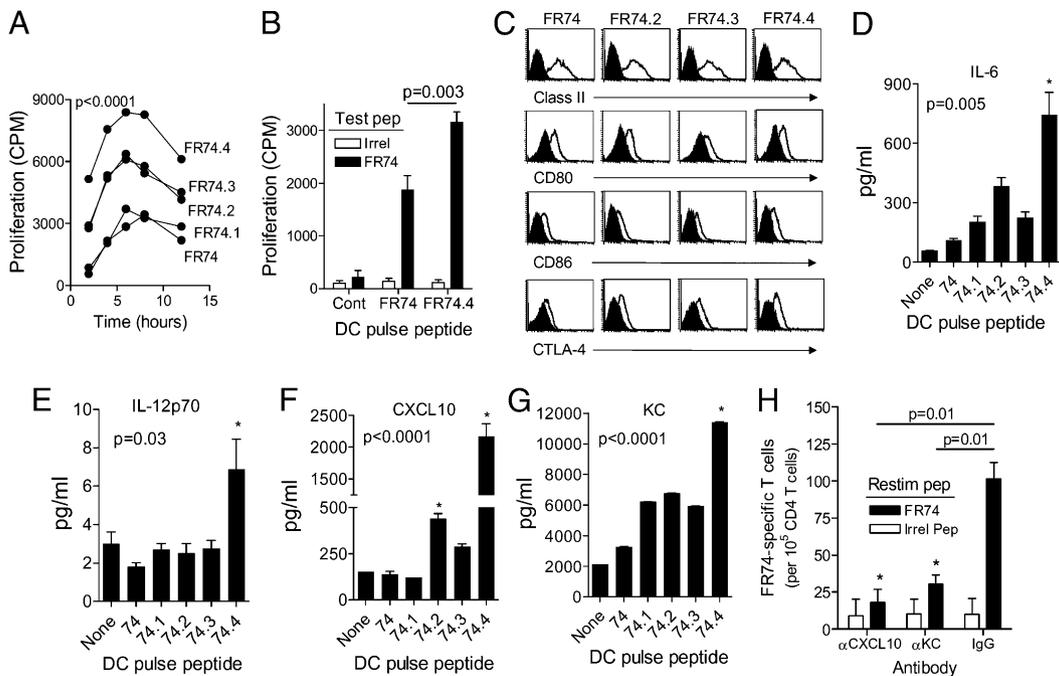


FIGURE 3. Epitope nesting results in sustained high-level Ag presentation from DC, activating T cells in a chemokine-dependent manner. *A*, DC were pulsed with peptides (2 h) shown in Fig. 1A over a time course of 2–12 h. At each time point, the DC were then used to stimulate a proliferation response in an FR74-specific T cell line. Each data point is the mean \pm SE (*n* = 2). A repeat experiment gave similar results. *B*, Proliferation of splenocytes from untreated mice (cont) or mice immunized with DC pulsed with either FR74 or FR74.4. Each bar shows mean (\pm SE) proliferation response of three mice in response to test peptides (test pep) FR74 or irrelevant (irrel) peptide. *C*, Flow cytometry histograms of DC incubated with native peptide FR74 or modified peptides (FR74.2, FR74.3, or FR74.4). Filled and open histograms are cells stained with isotype- or Ag-specific Ab, respectively. *D–G*, Cytokine/chemokine concentrations in supernatants derived from DC pulsed with media alone (none) or one of the five peptides. Each bar is the mean (\pm SE) calculated from duplicate samples. Representative of two separate experiments. *H*, IFN- γ ELISPOT analysis of FR74-specific T cells expanded with FR74.4 in the presence of anti-CXCL10, anti-KC, or nonspecific (IgG) Abs. Each bar is the mean (\pm SE) of three replicates of T cells restimulated (restim pep) with FR74 or irrelevant peptide. Representative of two experiments. **p* < 0.05 compared to native FR74 peptide.

FR74.4 using both proliferation and ELISPOT. Note that although the V β 4-negative subset showed some IFN- γ -secreting T cells (Fig. 2B), virtually all of the proliferative activity to FR74 was detected in the V β 4-positive T cells, which is likely because of better assay sensitivity of the ELISPOT (Fig. 2C).

Epitope nesting results in sustained high level Ag presentation from DC, activating T cells in a chemokine-dependent manner

Consistent with the initial hypothesis and the results on ex vivo CD4 T cell activation, DC pulsed with the FR74.4-modified peptides activated a FR74-specific T cell line to higher levels at all time points measured following pulsing as compared with DC pulsed with native peptide or singly modified epitopes (Fig. 3A). In vivo immunization studies showed that the DC pulsed with the nested epitopes were more immunogenic than native peptide when delivered as a vaccine (Fig. 3B).

Neither increased levels of MHC class II, CD80, and CD86 nor decreased levels of CTLA-4 were able to explain the effects of the epitope nesting (Fig. 3C). Because there was no increase in MHC class II or costimulatory molecules despite increased immunogenicity, cytokine and chemokine release were also examined. As shown in Fig. 3D–G, epitope nesting resulted in increased DC release of cytokines known to be involved in Ag-specific T cell activation, maturation, and growth (i.e., IL-6 and IL-12p70). In-

creased IL-6 production, observed in DC pulsed with FR74.4, was due in part to the Ii-Key motif because the single modified peptide FR74.2 also showed elevated levels (Fig. 3D). In contrast, increased IL-12p70 levels were only observed when both modifications were present (Fig. 3E). Similarly, pulsing of DC with the nested epitope also promoted release of chemokines CXCL10 and KC (i.e., CXCL1) (Fig. 3F, 3G). CXCL10 release was attributed to each modification because FR74.2 and FR74.3 resulted in elevated levels. Notably, however, nesting the epitope resulted in a synergistic effect on CXCL10 release. Elevated KC was observed with acetylation alone (i.e., FR74.1), suggesting a nonspecific interaction of the acetyl group or a role for peptide stability in inducing KC. Like IL-12p70, despite no apparent increase in KC with the individually modified peptides, the combination resulted in enhanced levels. In addition to the previously described functions of Ii-Key (i.e., peptide exchange) and DCpep (binding to DC), these results indicate there are other effects that impact the biology of the DC. Because the epitope nesting resulted in higher chemokine levels, we hypothesized that they may have a role in

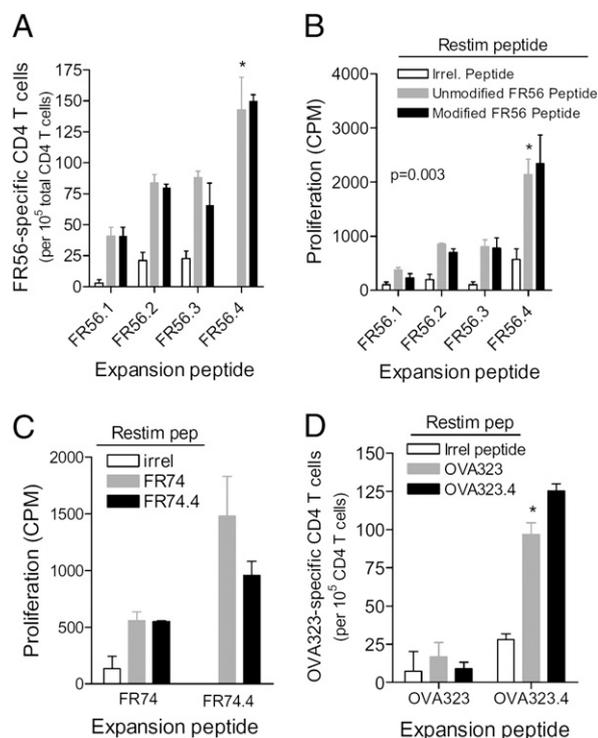


FIGURE 4. Epitope nesting results in CD4 T cell activation in a peptide- and species-independent manner. *A* and *B*, Human CD4 T cells were expanded with DC pulsed with native FR56 (FR56.1) or modified epitopes (FR56.2–FR56.4; same format as Fig. 1A). After 14 d, the cells were assessed with proliferation and IFN- γ ELISPOT by restimulating (restim peptide) with modified, unmodified, and irrelevant peptide. Each bar is the mean \pm SE; $n = 3$. *C*, Purified CD4 T cells from B6 (I-A^b) mice were expanded with FR74 or FR74.4 peptides for 14 d and subsequently tested for FR74 specificity in a proliferation assay (mean \pm SE; $n = 2$) by restimulation (restim pep) with FR74, FR74.4, or irrelevant peptide. Results are representative of two experiments. *D*, IFN- γ ELISPOT results (mean \pm SE; $n = 3$) of CD4 T cells (derived from B6 mice) expanded with native OVA323 epitope or dual-modified OVA323.4 and subsequently restimulated OVA323, OVA323.4, or irrelevant peptide. * $p < 0.05$ compared to native unmodified peptide.

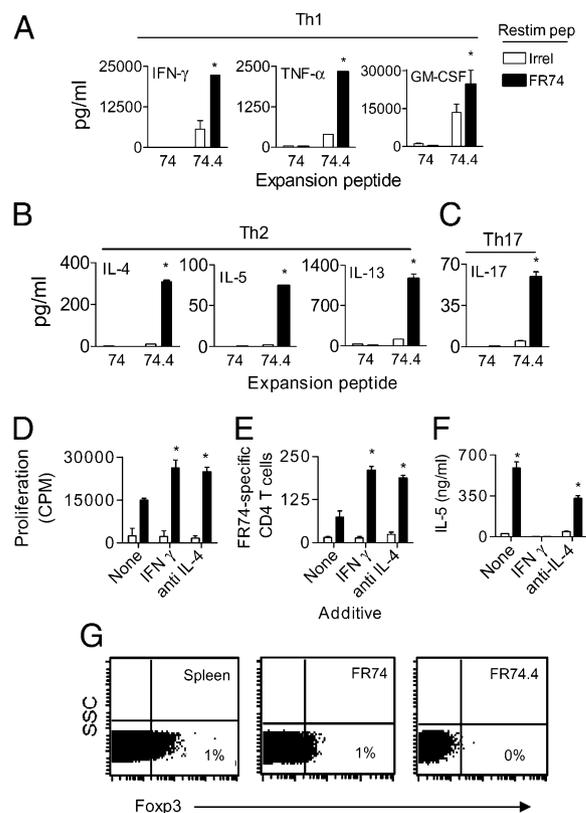


FIGURE 5. Epitope nesting generates CD4 helper T cells. *A–C*, Cytokine/chemokine concentrations (mean \pm SE; $n = 2$) in supernatants collected after 48 h from CD4 T cell lines derived from FVB/N mice) expanded in either FR74.4 (74.4) or FR74 (74) and restimulated with (restim peptide) either irrelevant peptide (\square) or FR74 (\blacksquare). Representative of two similar experiments. *D–F*, Proliferation, IFN- γ ELISPOT, and IL-5 ELISA (of supernatant) results, respectively, for FR74.4-specific CD4 T cells expanded without or with IFN- γ or anti-IL-4. Each bar is mean \pm SE of 3 (proliferation and ELISPOT) or 2 (ELISA) replicates of expanded T cells restimulated with either native FR74 (\blacksquare) or irrelevant peptide (\square). Each experiment was performed two or more times. ELISPOT; number of FR74-specific T cells/ 10^5 total T cells. *G*, Flow cytometry dot plots of naive splenocytes (400,000 total events), FR74-expanded (54,000 total events), and FR74.4-expanded (215,000 total events) CD4 T cell lines evaluated for Foxp3 expression. Quadrants set at isotype signal; figure shown in lower right quadrant is the percentage of total cells in the lymphocyte gate. * $p < 0.05$ compared to FR74-expanded T cells.

enhanced CD4 T cell generation. Thus, these chemokines were blocked with specific Abs during the course of T cell culture. As shown in Fig. 3H, both were critical because the chemokine-specific Abs abrogated the Ag-specific T cell recruitment.

Epitope nesting results in CD4 T cell activation in a peptide- and species-independent manner

Because this strategy is based on peptide extension rather than anchor or TCR-binding residue modification, it was hypothesized that the system should work across different peptides and species. To demonstrate this, a number of distinct systems were used. First, a human FR α epitope, previously discovered in our laboratory, FR56 (see *Materials and Methods* for sequence), was modified in the exact manner as the FR74 series of peptides. As shown in Fig. 4A and 4B, nesting of this human MHC class II epitope resulted in enhanced Ag-specific T cell activation, relative to control or epitopes with only one of the modifications.

The nesting also appears to be useful across mouse strains as indicated by two separate experiments. In the first experiment, the same FR74.4 used in the FVB/N mice can also be used to generate responses in B6 mice that carry I-A^b rather than I-A^d (Fig. 4C). In

the second, the known I-A^b binding peptide OVA323 could also be nested for enhanced OVA-specific CD4 T cell generation (Fig. 4D). Collectively, the results in Fig. 4 show that epitope nesting is effective regardless of the MHC class II subtype and the amino acid sequence.

Epitope nesting generates CD4 helper T cells

It was also observed in a series of experiments that epitope nesting did not exclude specific T cell effector subsets (e.g., Th1, Th2, and Th17). To test this, cytokines were measured in FR74- or FR74.4-generated cultures that were rested and restimulated with native Ag. As shown in Fig. 5A–C, all effector subsets were elicited with FR74.4. Cultures stimulated with FR74 were largely devoid of any Ag-specific cytokine responses, likely because of considerably lower numbers of Ag-specific T cells as shown in Fig. 1D. Despite the apparent lack of specificity for the type of effector T cell, the cultures could be supplemented with cytokines and Abs to preferentially expand one subset over another. For example, as shown in Fig. 5D–F, the inclusion of either IFN- γ or anti-IL-4, which are

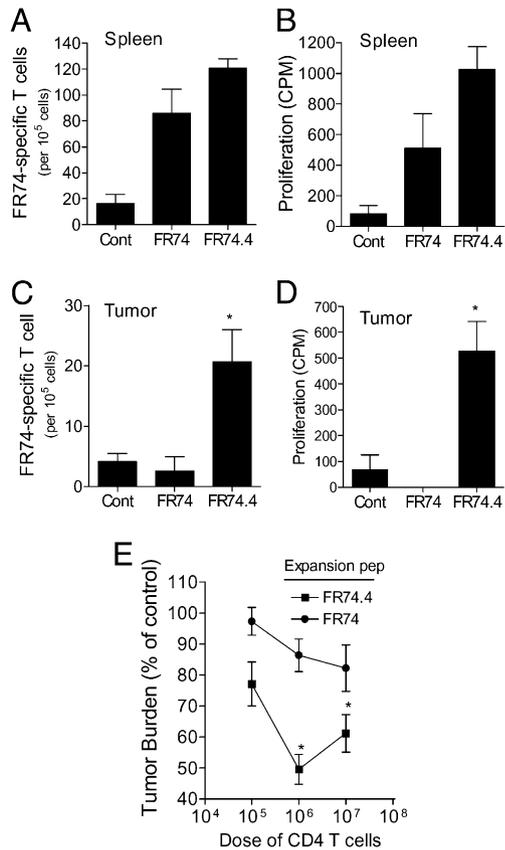


FIGURE 6. Epitope nesting produces CD4 T cells capable of infiltrating into sites of Ag production. A–D, The results of IFN- γ ELISPOT analysis of FR74-specific T cells isolated from the spleen or ovarian tumors derived from mice infused with T cells, expanded with either FR74 or FR74.4 peptides, and transferred (1×10^6) into mice bearing ID8 ovarian tumors. Control mice received 1×10^6 naive CD4 T cells. * $p < 0.05$ compared with FR74 cultured T cells. Each bar is the mean \pm SE of triplicate values. A repeat experiment yielded similar results. *x*-axes labels indicate expansion peptide. E, The mean \pm SE tumor weights expressed as a percentage of control (i.e., tumor-bearing animals that received naive CD4 T cells) in tumor-bearing animals that received varying concentrations of FR74- or FR74.4-expanded T cells. * $p < 0.05$ compared with control tumor-bearing mice.

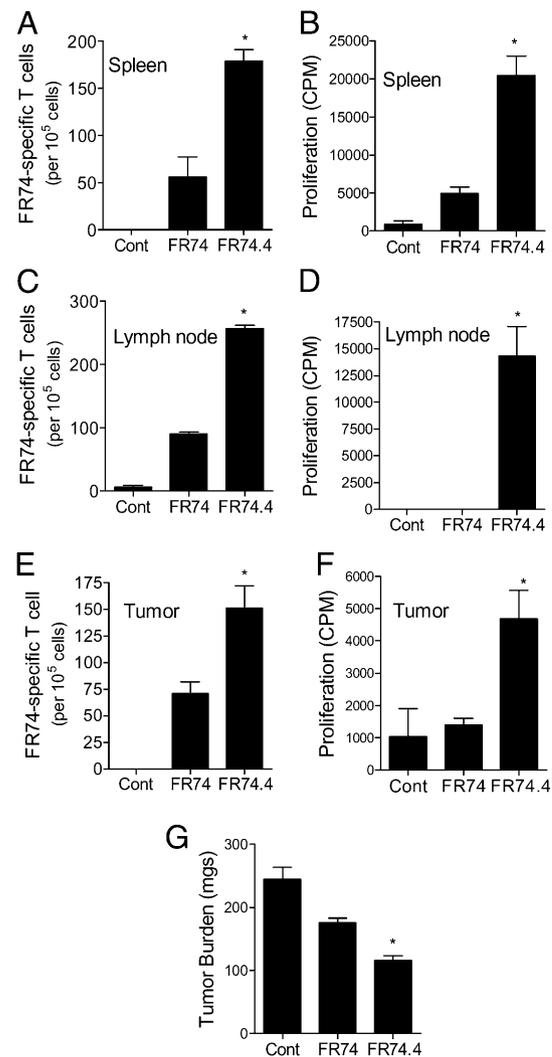


FIGURE 7. Epitope nesting imparts increased immunogenicity to MHC class II epitopes. A–F, The results of IFN- γ ELISPOT (A, C, E) or proliferation (B, D, F) analysis of T cells (restimulated with FR74) isolated from the spleen, lymph nodes, or ovarian tumors derived from tumor-bearing mice vaccinated with CFA/IFA adjuvant alone (cont) or FR74 or FR74.4 peptides. A repeat experiment yielded similar results. Each bar is the mean \pm SE of triplicate values. G, The mean \pm SE tumor weights in FR74.4-vaccinated mice. * $p < 0.05$ compared with FR74 vaccination.

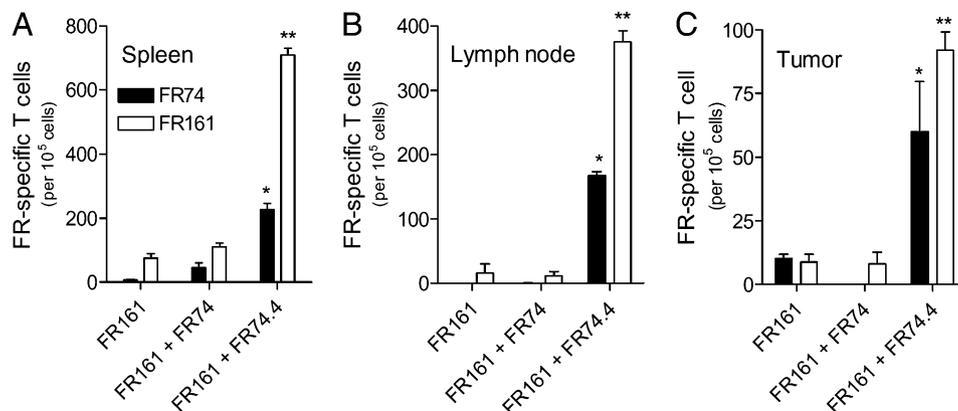


FIGURE 8. Epitope nesting imparts increased helper activity resulting in enhanced CD8 T cell responses. A–C, The results of IFN- γ ELISPOT analysis (restimulation in response to either FR161 peptide or FR74 peptide) of T cells isolated from the spleen, lymph nodes, or ovarian tumors derived from tumor-bearing mice vaccinated MHC class I epitope FR161 alone or a combination with either FR74 or FR74.4 peptides (labels on x-axis show vaccine peptides). Each bar is the mean \pm SE of triplicate values. * $p < 0.05$ compared with FR74-specific response in FR161 + FR74-vaccinated mice, ** $p < 0.05$ compared with FR161-specific response in FR161 + FR74-vaccinated mice.

both known to favor generation of Th1 effectors, greatly enhanced both the numbers of FR74-specific effectors as well as the proliferative response of the cultures. The figure also shows that the IL-5 response was either completely or partially blocked, indicating blockade of Th2 T cell expansion. In contrast to these results, FR74.4 did not favor outgrowth of Foxp3 T cells, suggesting that the effects of nesting were limited to effector T cells (Fig. 5G).

Epitope nesting produces CD4 T cells capable of infiltrating into sites of Ag production

T cell transfer experiments in a mouse ovarian cancer model were done to determine whether T cells generated with nested epitopes retained the ability to accumulate at sites where Ag is expressed and to mediate therapeutic activity. On day 0, the mice were injected with ID8 tumor cells. On day 10, mice received varying concentrations of T cells that were previously elicited with irrelevant peptide (controls), FR74, or FR74.4. The spleens, lymph nodes, and tumors of these mice were excised 30 d after injection and assessed. Fig. 6A and 6B show that after 40 d, FR74-specific T cells could be detected in the spleen regardless of the antigenic challenge (native or nested). Furthermore, the level of FR74-specific T cells in the spleen was independent of the ex vivo Ag. Despite this, however, only FR74-specific T cells generated with FR74.4 could localize to tumor (Fig. 6C, 6D). T cells activated by the nested FR74.4 epitope effectively reduced tumor burden ($p < 0.05$) at doses of 10^6 and 10^7 cells, whereas tumor burden in mice receiving T cells generated with native FR74 was not reduced significantly at any dose level.

Epitope nesting imparts increased immunogenicity to MHC class II epitopes resulting in increased in vivo helper activity

Thus far, the experimental results presented above show that nested epitopes can generate an enhanced number of Ag-specific T cells under controlled cell culture conditions. To determine whether the behavior of epitope nesting is maintained in complex immune microenvironments, tumor-bearing mice were directly immunized with free peptide three times over the course of 1 wk. Analysis of FR74-specific T cells and FR74-specific proliferation were assessed 30 d after the last vaccination. As shown in Fig. 7A–D, immunization with the nested epitope resulted in higher levels of systemic immunity to FR74. Importantly, nesting resulted in enhanced accumulation of FR74-specific T cells that retained proliferative capability at the tumor site, resulting in enhanced

antitumor activity (Fig. 7F, 7G). Last, to determine whether nesting resulted in enhanced helper activity in vivo, mice were immunized with a FR α -derived MHC class I epitope, FR161, with or without either FR74 or FR74.4. Levels of FR161-specific T cells were significantly increased in the spleen, lymph nodes, and tumors by coimmunization with FR74.4 but not FR74 ($p < 0.05$; Fig. 8).

Discussion

In summary, in this study, we found that nesting of helper epitopes results in the recruitment of latent pools of naive high-affinity/avidity FR α -specific T cells. These cells had the ability to home to tumors expressing FR α and to reduce tumor burden compared with animals vaccinated with unmodified peptides. Cytokine expression was enhanced with use of the nested peptides and showed that effector cells rather than regulatory cells were activated. Similar proliferative responses were detected using a nested epitope from human FR α as well as an OVA peptide in the murine system.

The experimental results indicate that the nesting is able to alter more than just the biology of epitope storage and MHC exchange within DC. A novel finding is that the Ii-Key motif is able to stimulate the production of cytokines and chemokines, such as IL-6 and CXCL10, which is also enhanced by the addition of the DCpep motif. Although the exchange feature of Ii-Key is clearly established (14, 18, 24), it can only be speculated as to how the motif might enhance cytokine production. One possibility is that the Ii-Key motif antagonizes Ii or CLIP binding to MHC class II, resulting in augmented levels of biologically active unbound Ii and CLIP, which could modify DC function (25). This is supported by recent studies that have demonstrated that Ii or its proteolytic fragments have significant biologic effects that may be independent of class II presentation. For example, Ii is important in B cell differentiation through NF- κ B, a transcription factor well-known to modulate cytokine release from DC (25–27). An Ii or CLIP displacement mechanism would agree with the finding in the current study that the DCpep causes a synergistic increase in cytokine release. DCpep, by virtue of its binding and increased storage, would increase the concentration of Ii-Key within the cell and potentially result in elevated free Ii or CLIP. The novel finding of increased DC-derived cytokines may provide an explanation as to why MHC class II peptide epitopes linked to Ii-Key are potent immunogens in the absence of immunoadjuvants as observed in human clinical studies (15).

The unmasking of high-avidity T cells against specific self-Ags is an important goal in tumor immunology. Ercolini et al. (28) showed, in the neu-transgenic mouse, that high-affinity neu-specific CD8 T cells are not eliminated by central tolerance and exist in the periphery. Importantly, their studies showed that the activation of these high-affinity T cells, but not low-avidity T cells, is regulated by regulatory T cells (Tregs), such that depletion of Tregs with cyclophosphamide results in rapid recruitment. Our group confirmed this observation using the Treg-depleting immunotoxin Denileukin diftitox (21). Therefore, one possible explanation for the ability of the epitope nesting to recruit latent high-affinity/avidity FR α -specific Th cells is that it prevents expansion of Tregs, which seemed to be confirmed by the demonstration that Th1, Th2, and Th17 cells, but not Tregs, were preferentially elicited with the modified but not native peptides. Studies have suggested that high-avidity T cells are inherently more susceptible to the suppressive actions of Tregs (29). Thus, eliminating Tregs may be one reason for enhanced unmasking of high-avidity FR α -specific T cells. Importantly, our observations raise the question as to how nesting blocks Treg expansion. One likely possibility is the effect of nesting on IL-6 induction in the DC. Several recent reports show that IL-6 suppresses expansion of Tregs and reduces Foxp3 expression and suppressive functions in existing Tregs (30–32).

Alternatively, the activation of high-avidity T cells may be related to the duration of Ag presentation and augmented TCR interactions. González et al. (8) showed that T cells with high-avidity/affinity for MHC:peptide complexes are most efficiently activated when the Ag density is high. In contrast, activation of high-avidity T cells is impaired at low Ag densities because of suboptimal TCR:MHC dwell times. It could be speculated that nesting results in higher densities of MHC:peptide at the surface of the DC. This hypothesis is consistent with our results showing that DC pulsed with nested epitopes persistently present higher levels of Ag compared with DC pulse with only native peptide. Such higher levels of Ag presentation could lead to dwell times that exceed the threshold for priming (33).

Last, another reason for enhanced T cell activation and recruitment of high-avidity T cells is an increase in receptor ligand interactions independent of the TCR:MHC interaction. Of several chemokines analyzed in the current study, two of them, CXCL10 (IP-10) and KC (CXCL1), were significantly upregulated by the dual modifications. Neutralization of either chemokine resulted in abolishing the generation of Ag-specific T cells. There are several potential reasons that could explain the requirement for these chemokines. The first, and perhaps the most obvious reason, is that the enhanced chemokines resulted in better recruitment of CD4 T cells into the proximity of the DC (34–36). Although CXCL10 is a well-known T cell attractant, KC is not generally associated with T cell function but rather neutrophil recruitment (37). Alternatively, the chemokines may enhance TCR-mediated T cell activation and proliferation. Although a role for CXCL10 and KC in T cell signaling is unknown, recent studies from one of the coauthors has shown that CXCL12 ligation of CXCR4 at the time of TCR stimulation can result in substantial costimulation, leading to enhanced T cell activation and cytokine release (38).

Another potentially important finding is that nesting results in the generation of Ag-reactive Th cells capable of accumulating and proliferating in the tumor microenvironment. In contrast, Ag-specific Th cells generated with unmodified peptide, although capable of circulating for several weeks and populating the spleen and lymph nodes, failed to maintain proliferative capability at tumor site. These findings suggest that the modified peptides are better equipped to resist the tolerance inducing tumor microen-

vironment. Indeed, studies in recent years suggest that T cells can acquire resistance to tolerogenic immune microenvironments. For example, Chen and colleagues (39, 40) showed in a recent study that upregulation of the costimulatory molecule TNFR2 induces resistance of conventional T cells to local immune suppressive actions of Tregs. Further studies comparing tetramer-purified Ag-specific T cells generated with or without nested epitopes could potentially reveal novel pathways of resistance to tolerance.

In conclusion, we found that nesting of MHC class II epitopes between a peptide that enhances DC uptake and one that augments exchange of the epitope onto MHC results in effective and consistent activation of high-avidity Ag-specific CD4 T cells. This strategy works across multiple platforms and appears to be useful for recruiting T cells from naive pools. Thus, it is possible that the modification of peptide Ags may be a strategy for improving vaccine or adoptive T cell strategies independent of or in combination with immunoadjuvants.

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Disclosures

E.v.H. is the president and chief executive officer of Antigen Express, a company involved in developing therapies based on the Ii-Key motif described in this article.

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