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TLR2 Engagement on Dendritic Cells Promotes High Frequency Effector and Memory CD4 T Cell Responses[1]

Smita S. Chandran,* David Verhoeven,* John R. Teijaro,* Matthew J. Fenton,[2†] and Donna L. Farber[3]*

Ligation of TLR by distinct pathogen components provides essential signals for T cell priming, although how individual TLR engagement affects primary and memory T cell responses is not well defined. In this study, we demonstrate distinct effects of TLR2 vs TLR4 engagement on primary and memory CD4 T cell responses due to differential effects on APC. Priming of influenza hemagglutinin (HA)-specific naive CD4 T cells with HA peptide and the TLR2 agonist Pam3CysK in vivo resulted in a high frequency of activated HA-specific CD4 T cells that predominantly produced IL-2 and IL-17, whereas priming with HA peptide and the TLR4 agonist LPS yielded a lower frequency of HA-specific CD4 T cells and predominant IFN-γ producers. TLR2 agonist priming depended on TLR2 expression by APC, as wild-type CD4 T cells did not expand in response to peptide and Pam3CysK in TLR2-deficient hosts. TLR2-mediated priming also led to an increased frequency of Ag-specific memory CD4 T cells compared with TLR4 priming and mediated enhanced secondary responses to influenza challenge. Our results show that TLR engagement on APC influences both primary and secondary CD4 T cell responses, and suggest that long-term functional capacities of T cells are set by innate signals during early phases of an infection. *The Journal of Immunology,* 2009, 183: 7832–7841.

L igation of TLR during the early stages of infection by pathogen-associated motifs provides critical costimulatory signals for the initiation of adaptive immune responses. Individual TLR have distinct pathogen-associated motif specificities, with TLR4 recognizing LPS (1), TLR2 binding bacterial lipopeptides (2), while TLR3 and TLR9 bind viral and bacterial nucleic acids, respectively (3–5). The use of noninfectious TLR agonists as vaccine adjuvants is a promising approach to generate pathogen-specific adaptive responses (6, 7); however, the ability of specific TLR engagement to promote primary and secondary immune responses is not clearly resolved.

TLR ligation induces dendritic cell (DC)[4] maturation characterized by up-regulated expression of MHC class II and the CD86/CD80 costimulatory family molecules resulting in enhanced Ag presentation and cytokine secretion (8, 9). Naive CD4 T cells become activated by these mature DC and subsequently differentiate into effector cells that can be divided into four distinct subsets based on their pattern of cytokine production: Th1 cells secrete inflammatory cytokines IFN-γ and TNF-α; Th2 cells produce IL-4

and for B cell help, Th17 cells produce the proinflammatory cytokine IL-17; and induced regulatory T cells (Tregs) produce immunoregulatory cytokines (10–12). A number of studies has demonstrated that engagement of specific TLR can influence CD4 T cell priming and effector differentiation by skewing the Th1-Th2 balance inducing inflammation and/or promoting regulatory activity (13). For example, TLR4 is known to predominantly generate Th1 responses (14, 15) and is upstream of Th17 differentiation (16–18). By contrast, TLR2 engagement was shown to favor Th2 responses in some cases (19), to promote IFN-γ production in other studies (20), and has been shown to promote IL-10 production and enhance Treg activity in vivo (21). TLR2 has been shown to also regulate IL-17 production; however, different infection models have shown either enhancing (22, 23) or inhibitory effects (24, 25), and its direct effect on IL-17 production by Ag-specific T cells is not clear. Therefore, the role of TLR2 engagement in driving the differentiation of CD4 T cells during priming in vivo remains an unresolved issue.

T cell priming and effector differentiation also results in the generation of long-lived memory T cells, with the capacity to mediate efficacious secondary responses at the site of infection (26). The generation of memory CD4 T cells was shown to require TLR signaling, as mice lacking the common signal transducer MyD88 were unable to generate Ag-specific memory T cell responses (27). Antigenic peptide priming in the presence of LPS as a TLR4 agonist can promote memory CD4 T cell generation (28), albeit at a low frequency (29). The ability of other TLR agonists, including those that trigger TLR2 engagement, to generate memory T cell responses and subsequent secondary responses upon rechallenge is not known.

TLR engagement by agonists in vivo not only targets DC, but can also bind to TLR expressed by T cells themselves (30, 31). Notably, memory and regulatory CD4 T cells have been shown to express surface TLR2 (32), and TLR2 engagement can promote Treg function (33). Moreover, engagement of TLR2 on CD8 T cells has been recently demonstrated to facilitate memory T cell development from effector cells (34). Whether TLR2 engagement

in vivo can promote CD4 T cell priming and memory formation via alterations in DC activation or due to direct effects on the T cells has not been investigated.

In this study, we hypothesized that differential TLR engagement during priming may influence T cell effector and memory generation. We investigated how TLR2 engagement in vivo influenced CD4 T cell priming and memory generation compared with the well-characterized TLR4 agonist LPS. We demonstrate that Ag-specific CD4 T cells undergo extensive proliferation in the presence of peptide and TLR agonist, with TLR2 priming resulting in greater expansion compared with TLR4 priming. In addition, TLR2-primed CD4 T cells produced predominantly IL-2 and IL-17 in contrast to TLR4-primed CD4 T cells consisting of IFN-γ and IL-2 producers. The ability of the TLR2 agonist to differentially prime CD4 T cells was due to its engagement on APC, as wild-type CD4 T cells in TLR2-deficient hosts lost the ability to expand or produce IL-2 upon peptide recall. Moreover, TLR2 agonist priming of influenza hemagglutinin (HA)-specific CD4 T cells resulted in a higher frequency of persisting HA-specific memory CD4 T cells which mediated robust secondary responses upon challenge with influenza virus compared with TLR4 agonist-primed cells. Our findings demonstrate that TLR2 engagement of APC promotes a high frequency of effector and memory CD4 T cells in primary and secondary immune responses, with implications for promoting T cell differentiation in vaccines and to pathogens in vivo.

Materials and Methods

Mice

BALB/c and C57BL/6 mice were purchased from the National Cancer Institute Biological Testing Branch (Frederick, MD). BALB/c (Thy-1.1$^+$)-congenic mice, DO11.10 (35), and HA-TCR-transgenic mice (36), OT-II TCR-transgenic mice (37) (obtained from Dr. L. Zhang, University of Maryland, Baltimore, MD), and TLR2-deficient mice (38) (The Jackson Laboratory) were bred and maintained under specific pathogen-free conditions and used at 6–10 wk of age. All animal studies were approved by the University of Maryland, Baltimore Institutional Animal Care and Use Committee.

Abs and Reagents

The following Abs were purchased from Bio X Cell: anti-CD8 (TIB105), anti-CD4 (GK1.5), anti-I-A^d (212.A1), anti-Thy-1 (TIB238). Fluorochrome-conjugated anti-CD44, anti-CD62L, anti-CD127, anti-Vα2 TCR, anti-CD4, anti-active caspase 3, anti-CD80, anti-CD86, and anti-CD11c; anti-MHC class II and anti-IL-2 Abs were purchased from BD Pharmingen; and fluorochrome-conjugated anti-CD90.2, anti-Vβ5, anti-IFN-γ, anti-IL-17, and anti-IL-4 Abs from eBioscience. Highly purified LPS from *Escherichia coli* O111:B4 was obtained from List Biological Laboratories and Pam3Cys-SKKKK (Pam3C) from EMC Microcollections. Peptides corresponding to influenza HA$_{110-118}$ and OVA$_{323-339}$ were synthesized by the Biopolymer facility (University of Maryland).

In vivo priming of mice with TLR agonists

CD4 T cells were purified from spleens of HA-TCR and DO11.10 TCR-transgenic mice by negative selection following depletion of CD8 T cells and MHC class II-expressing cells as previously described (39). The resultant CD4 T cells were further fractionated into CD44^{low}, naive CD4 T cells by negative selection using the autoMACS (Miltenyi Biotec) and anti-CD44-FITC and anti-FITC magnetic microbeads as previously described (40), yielding 98% purity. Naive CD4 T cells were labeled with CFSE (Invitrogen) and adoptively transferred (200,000 cells/mouse) into congenic BALB/c (Thy1.1) hosts as described elsewhere (40). One day after transfer, mice were primed i.p. with peptide (HA, 100 μg and OVA, 50 μg) alone or in combination with LPS (30 μg) or Pam3C (30 μg), with the dose of TLR agonists determined from dose-response studies (see Fig. 2). After 1–4 wk, CD4 T cells were purified from the spleen, lung, and mesenteric lymph nodes of the differentially primed mice and were phenotypically and functionally analyzed. For experiments using TLR2$^{-/-}$ and C57BL/6 hosts, naive CD4 T cells were similarly purified from OVA-specific OT-II mice, transferred into TLR2$^{-/-}$ and C57BL/6 hosts, and

primed with OVA plus LPS or OVA plus Pam3C as above and were harvested 1 wk later as above.

Cytokine assays

For cytokine analysis using ELISPOT, CD4 T cells from differentially primed mice were plated (75,000/well) on IL-2-, IFN-γ-, and IL-4 (BD Biosciences)-coated ELISPOT plates with APC (T-depleted splenocytes as previously described (40)) either alone for controls or in the presence of 5 μg/ml HA or 1 μg/ml OVA peptide for 36 h as previously described (41). Spots were enumerated using the Immunospot ELISPOT reader (CTL; BD Biosciences). For intracellular cytokine staining (ICS) analysis, CD4 T cells (10^6/well) were plated in 24-well plates with APC (3 $\times$ 10^6/well), peptide (5 μg/ml HA or 1 μg/ml OVA), and anti-CD28 (1 μg/ml) for 18 h. PMA/ionomycin stimulation served as a positive control and CD4 T cell stimulation with APC and anti-CD28 in the absence of peptide served as a negative control.

In vitro priming of CD4 T cells

CD4 T cells isolated from HA-TCR-transgenic mice (10^6/well) were plated in vitro with APC (3 $\times$ 10^6/well) in the presence of HA peptide alone (5 μg/ml) or in conjunction with LPS (1.5 μg/ml) or Pam3C (1.5 μg/ml) and incubated at 37ºC for 3 days. Activated CD4 T cells were harvested as described elsewhere (40) and adoptively transferred (2 $\times$ 10^6/mouse) into congenic BALB/c recipient mice. Spleen, lung, and mesenteric lymph nodes were harvested from these mice 4 wk after transfer to analyze the generation of memory CD4 T cells.

In vivo analysis of DC activation

BALB/c mice were administered HA peptide alone or in the presence of LPS or Pam3C, and splenocytes were harvested 6, 18, or 30 h later and analyzed for surface phenotype by flow cytometry. Cell death was assessed by ICS with anti-active caspase 3 Ab.

Flow cytometry

Cells were surface and intracellularly stained with fluorochrome-conjugated Abs as previously described (42), fixed, and acquired using an LSRII flow cytometer (BD Biosciences) with a minimum acquisition of 500,000 events. Analysis of acquisition events was accomplished using FACSDiva (BD Biosciences) and FlowJo software (Tree Star).

Influenza virus infection

Influenza virus (A/PR/8/34) was grown in the allantoic fluid of 10-day-old embryonated chicken eggs as previously described (43). Determination of influenza viral titers in lung homogenates was accomplished by the tissue culture infectious dose 50 assay (TCID$_{50}$) as described previously (44), with titers expressed as the reciprocal of the dilution of lung extract that corresponds to 50% virus growth in Madin-Darby canine kidney cells or calculated by the Reed Muench method. For in vivo infection, mice were anesthetized with isoflurane and 20 μl of PR8 influenza virus containing 500 TCID$_{50}$ was administered intranasally. All infected mice were housed in the biocontainment suite at the University of Maryland animal facility, where tissue harvest was also performed. Lung tissue sections were harvested from formalin-perfused lungs, stored in formalin at $-80°$C, and H&E stain was performed by the core facility at the University of Maryland, Baltimore.

Results

TLR priming promotes rapid Ag-driven expansion of CD4 T cells in an in vivo mouse model

We evaluated the ability of TLR2 and TLR4 agonists to prime naive CD4 T cells using an in vivo transfer model in which small numbers of TCR-transgenic CD4 T cells are transferred into intact adoptive hosts and subsequently primed with their cognate Ag (45). We transferred 200,000 CFSE-labeled purified naive CD44^{low} CD4 T cells from HA-TCR-transgenic mice into BALB/c (Thy1.1) hosts and subsequently primed these hosts with HA peptide alone or in the presence of LPS or Pam3C as TLR4 and TLR2 agonists, respectively. (We determined that 200,000 cells was the minimum number of CD4 T cells needed to visualize Ag-specific priming in our system (data not shown).) After 1 wk, we analyzed in vivo proliferation, expansion, and activation in the spleen as well as migration to multiple tissue sites. We found that
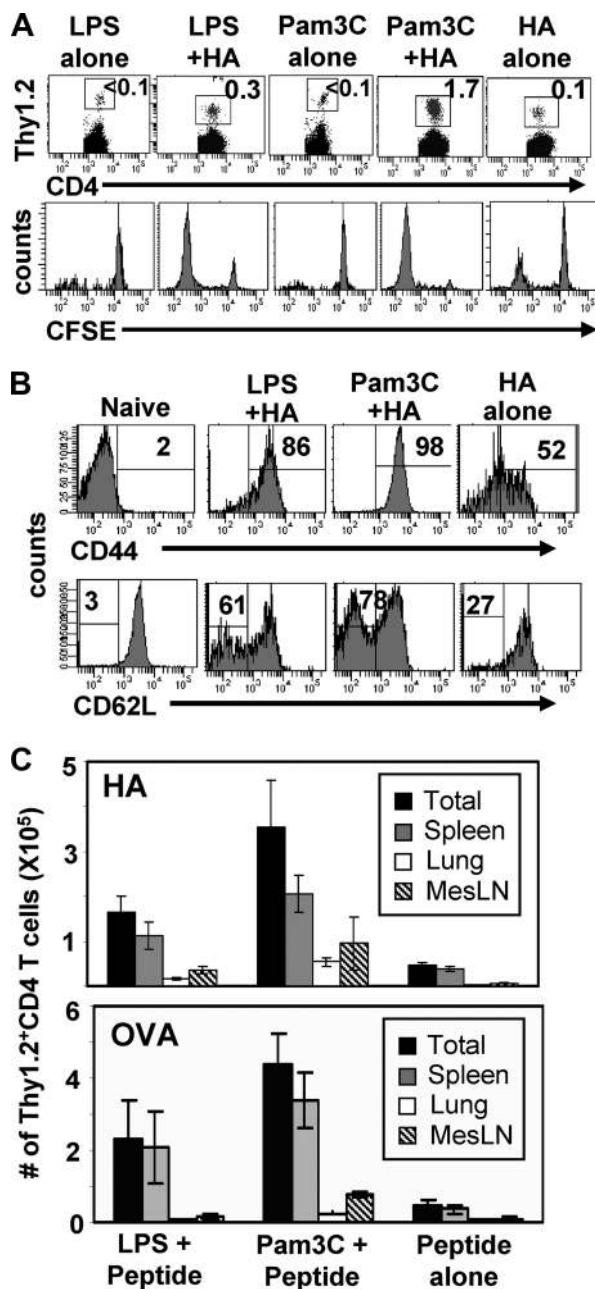
FIGURE 1. TLR2 engagement during CD4 T cell priming results in increased Ag-driven CD4 T cell expansion compared with TLR4 engagement. CFSE-labeled, naive CD4 T cells from HA-TCR-transgenic mice were transferred (2×10^5) into BALB/c (Thy1.1)- congenic hosts and immunized i.p. with LPS alone, LPS + HA peptide, Pam3C alone, Pam3C + HA peptide, or HA peptide alone and analyzed 1 wk later. *A*, Frequency of Thy1.2$^+$ HA-specific CD4 T cells (*top row*) within total recipient CD4 T cells and CFSE dilution profile (*bottom row*) of HA-specific CD4 T cells. Average frequency of primed, Ag-specific CD4 T cells in the total CD4 T cell population primed with LPS + HA, Pam3C + HA, or HA peptide alone was 1.0, 3.2, and 0.2%, respectively, from two experiments with three mice per group. *B*, Phenotypic analysis of CD44 and CD62L expression by input naive and differentially primed HA-specific CD4 T cells, with markers indicating percent CD44^{high} (*upper*) and CD62L^{low} (*lower*). *C*, Yield and tissue distribution of differentially primed HA-specific (*upper panel*) and OVA-specific (*lower panel*) CD4 T cells. Graphs show absolute numbers of Thy1.2$^+$ CD4 T cells harvested from spleen, lung, and mesenteric lymph nodes (MesLN) of differentially primed recipient mice as the mean ± SD from three mice per group; representative of five independent experiments. $p = 0.03$ comparing total number of HA-specific cells resulting from LPS vs Pam3C priming and $p = 0.05$ comparing total number of HA-specific cells resulting from Pam3C vs HA priming.
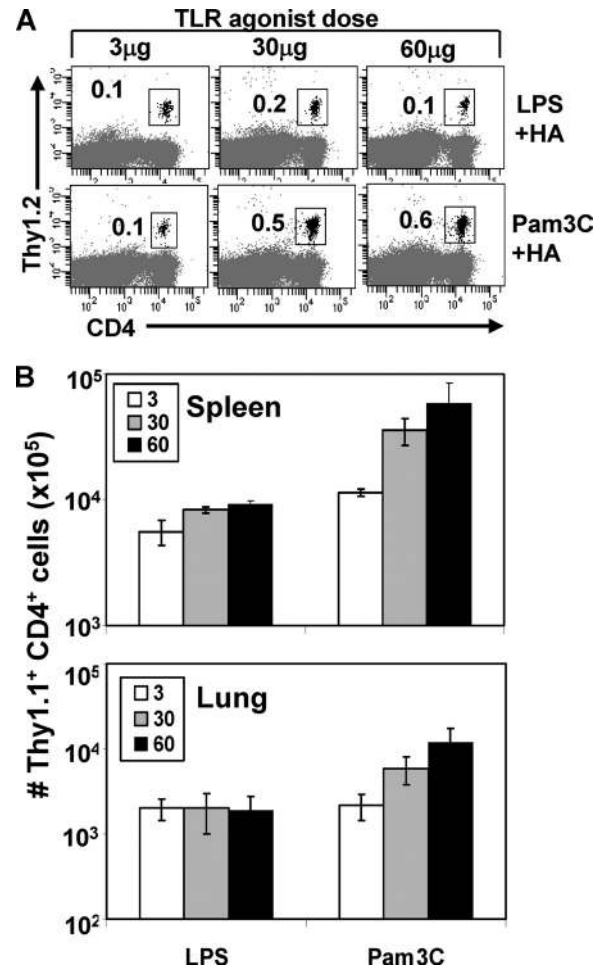
FIGURE 2. Dose response of TLR agonist for antigenic priming of CD4 T cells. Naive HA-specific CD4 T cells were transferred into BALB/c (Thy1.1) hosts as above, primed with 100 μg of HA peptide, and the indicated doses of either LPS or Pam3C, and cells were harvested after 1 wk. *A*, Frequency of HA-specific CD4 T cells in the spleen of differentially primed mice. *B*, Total HA-specific CD4 T cell yield obtained from spleen (*upper*) and lung (*lower*) of mice primed as in *A* with the mean and SD from three to four mice per group. Numbers in the key refer to μg of each TLR agonist.

HA-specific CD4 T cells underwent significant proliferation as assessed by complete CFSE dilution in mice primed with HA peptide in the presence of LPS or Pam3C compared with minimal proliferation in mice primed with LPS or Pam3C alone and some division induced by HA peptide alone (Fig. 1*A, bottom*). Notably, antigenic priming in the presence of the TLR2 agonist Pam3C resulted in the highest frequency of HA-specific (1.7%) CD4 T cells in spleen compared with priming with HA and the TLR4 agonist LPS (0.3%), with minimal frequencies of HA-specific cells detected in mice primed with LPS, Pam3C, or HA peptide alone (Fig. 1*A, top*). These results demonstrate marked differences in the frequencies of Ag-primed cells using different TLR agonists as adjuvants.

We also assessed the activation and differentiation state of HA-specific CD4 T cells primed with HA peptide with or without TLR agonists. HA-specific CD4 T cells primed with LPS plus HA or Pam3C plus HA exhibited activated/effector cell phenotypes marked by increased expression of CD44 and decreased expression of CD62L compared with the input naive CD4 T cells which were CD44^{low} and CD62L^{high} (Fig. 1*B*). By contrast, HA peptide priming without TLR agonists did not appreciably alter the naive
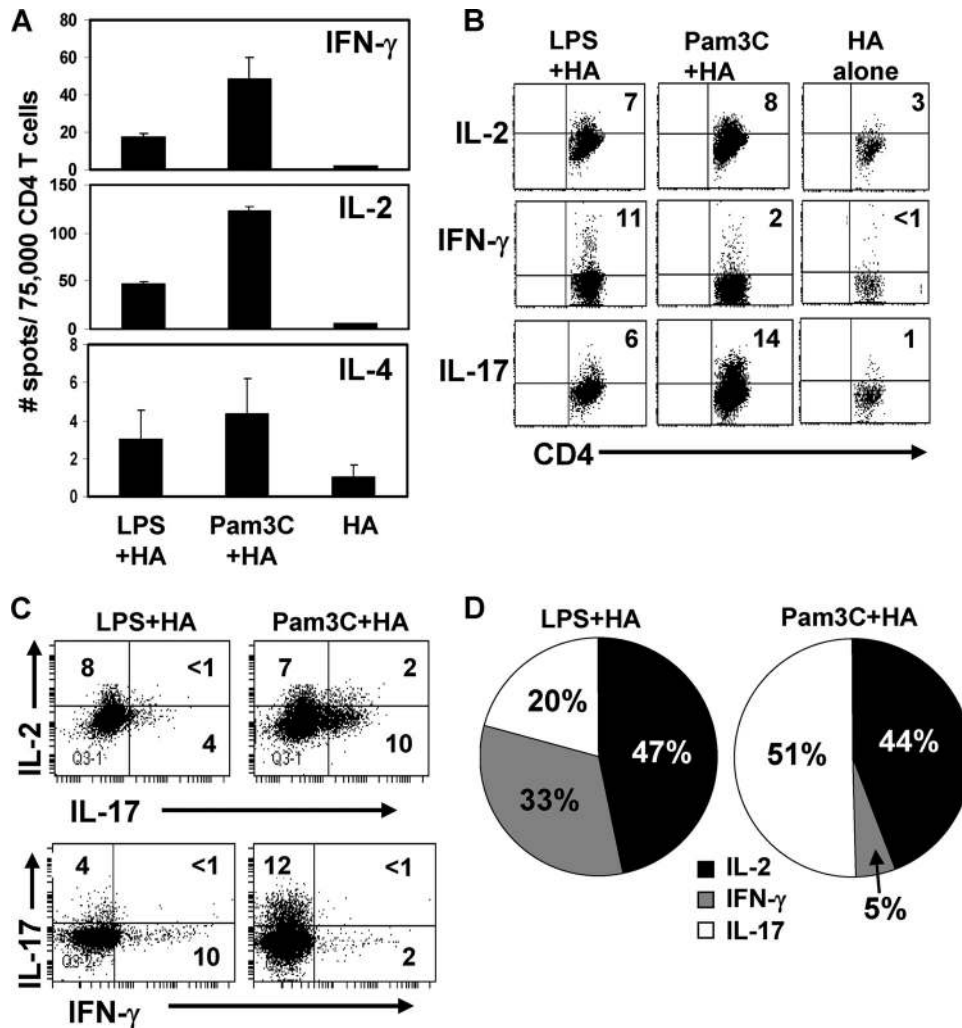
FIGURE 3. Ag-specific CD4 T cells primed in the presence of TLR2 vs TLR4 agonists exhibit distinct cytokine profiles. Naive HA-specific CD4 T cells were transferred into BALB/c (Thy1.1) hosts, primed with HA peptide in the presence of LPS or Pam3C as above, and cells were harvested from spleen and lymph nodes 1 wk after priming. *A,* Cytokine profile from spleens of mice with HA-specific CD4 T cells differentially primed with TLR2 vs TLR4 agonists as determined by ELISPOT analysis following stimulation with HA peptide and APC for 36 h. Values shown represent mean ± SD from triplicate wells subtracted for background for cytokine production in wells containing CD4 T cells, APC, and no peptide. Results are representative of five independent experiments. *B,* Cytokine profile of LPS + HA, Pam3C + HA, and HA-primed HA-specific CD4 T cells recovered 1 wk after priming, stimulated ex vivo for 18 h with HA peptide and APC. Plots show CD4 vs IL-2, IFN-γ, or IL-17 expression as determined by ICS gated on Thy1.2+ Ag-specific CD4 T cells. Results are representative of three mice per group and four independent experiments. *C,* Polyfunctional cytokine profile of HA-specific CD4 T cells primed with LPS + HA or Pam3C + HA stimulated as in *B.* Plots show IL-2 vs IL-17 production, and IL-17 vs IFN-γ production gated on a HA-specific CD4 T cell population. *D,* Summary of cytokine distribution profile in HA-specific CD4 T cells primed with LPS + HA or Pam3C + HA from four experiments.

phenotype of HA-specific CD4 T cells (Fig. 1*B*). These results indicate that antigenic priming in the presence of TLR agonists results in optimal T cell activation/differentiation, with TLR2 engagement leading to more extensive down-regulation of CD62L in vivo.

We also investigated whether antigenic priming with TLR2 and TLR4 agonists resulted in different numbers of Ag-specific cells in other tissue sites. In addition, we asked whether the increased priming of Ag-specific cells seen with Pam3C was also observed in another Ag system using OVA-specific CD4 T cells from DO11.10 TCR-transgenic mice. We primed mouse recipients of either naive HA-specific or OVA-specific CD4 T cells with HA or OVA peptide, respectively, with or without LPS or Pam3C as above. We purified CD4 T cells from spleen, lung, and mesenteric lymph nodes of differentially primed mice and quantitated the absolute numbers of Ag-specific CD4 T cells in each site. As shown in Fig. 1*C*, peptide priming alone resulted in minimal numbers of

Ag-specific CD4 T cells in lymphoid tissue and no cells migrating to the lung. Priming with peptide and Pam3C gave the highest numbers in spleen and lymph nodes compared with priming with peptide and LPS for both HA- and OVA-specific CD4 T cells (Fig. 1*C*). Although the LPS-primed groups yielded negligible numbers of primed T cells in the lung, Pam3C priming resulted in significant numbers of Ag-specific cells in the lung, albeit at much lower levels than those found in lymphoid tissue (Fig. 1*C*). These results indicate that antigenic priming in the presence of a TLR2 agonist results in the highest yield of Ag-primed cells in lymphoid and nonlymphoid tissue, with predominant distribution in lymphoid sites.

The ability of Pam3C vs LPS to prime for a higher frequency of Ag-specific T cells could be due to its increased potency as an adjuvant for T cell activation or a different optimal dose response. We therefore performed a dose titration experiment using different concentrations of LPS or Pam3C with a single optimal Ag dose. At

low doses of LPS or Pam3C, we observed similar frequencies of activated Ag-specific T cells in vivo in both spleen and lung (Fig. 2). With increasing doses of LPS, we observed only slight increases in the frequency of Ag-primed CD4 T cells, with the highest doses resulting in significant morbidity to mice (data not shown), consistent with the well known toxic effects of systemic administration of LPS. By contrast, increasing the dose of Pam3C resulted in substantial increases in the frequency of Ag-specific CD4 T cells in both spleen and lung (Fig. 2), with minimal toxicity observed at the highest dose used (60 μg; data not shown). These results indicate that Pam3C is a more potent adjuvant for Ag-specific T cell priming in vivo, with lower toxicity than LPS.

Differential cytokine profile of TLR2- vs TLR4-primed CD4 T cells

We evaluated the cytokine profile of CD4 T cells differentially primed by Ag in the presence of TLR2 and TLR4 agonists. ELISPOT assays (Fig. 3A) show higher overall numbers of cytokine producers in TLR2-primed mice compared with TLR4-primed mice, consistent with increased expansion seen in TLR2 priming. Low numbers of IL-4 producers were detected from both TLR2- and TLR4- primed CD4 T cells (Fig. 3A). In addition, HA-specific cells derived from mice primed with HA peptide alone did not produce significant levels of any cytokine at this time point (Fig. 3A). To closely examine the cytokine profile of differentially primed cells, we performed ICS (Fig. 3B). We found that LPS plus HA- primed CD4 T cells produced IFN-γ, IL-2, and some IL-17, whereas Pam3C plus HA-primed CD4 T cells produced predominantly IL-2 and IL-17, with very low levels of IFN-γ (Fig. 3B). To examine whether differentially primed CD4 T cells were polyfunctional, we costained for IL-2, IL-17, and IFN-γ and observed that Pam3C-primed CD4 T cells were predominantly single IL-17 producers with a small population of IL-2$^+$IL-17$^+$ double producers while IFN-γ and IL-17 producers were mutually exclusive (Fig. 3C). Of the total cytokine- producing population, TLR2 engagement primed for IL-2 and IL-17 single producers with only a small fraction of IFN-γ producers, whereas TLR4 agonists promoted differentiation of IFN-γ producers as well as IL-2 and IL-17 producers (Fig. 3D). We found similar results with both HA- and OVA-specific systems (data not shown). We also analyzed whether TLR engagement promoted Treg differentiation, given its known regulatory effects in vivo (33, 46), but we did not observe up-regulation of FoxP3 expression by Ag-specific CD4 T cells primed with Ag in the presence of LPS or Pam3C (data not shown). These results indicate that TLR2 agonist priming promotes expansion of CD4 T cells producing IL-2 and IL-17, while TLR4 agonist priming results in Th1 effector generation.

Differential priming of CD4 T cells occurs via TLR engagement on APC

TLR2 is expressed not only by multiple APC types, but also on activated T lymphocytes (30, 47, 48). To determine whether the increased expansion seen with Ag priming in the presence of Pam3C is due to its effects on APC or CD4 T cells, we transferred OVA-specific wild-type CD4 T cells into TLR2-deficient hosts and assessed DC maturation and CD4 T cell expansion and function. To verify that DC maturation in TLR2-deficient mice could not occur in response to Pam3C, we measured CD86 up-regulation on splenic DC after in vivo administration of TLR agonists. In wild-type mice, we observed CD86 up-regulation on CD11c$^+$ DC in vivo following administration of LPS or Pam3C compared with control PBS; however, in TLR2-deficient mice, CD86 up-regulation was only observed following LPS administration and was not observed following Pam3C or PBS treatment (Fig. 4A). These re-
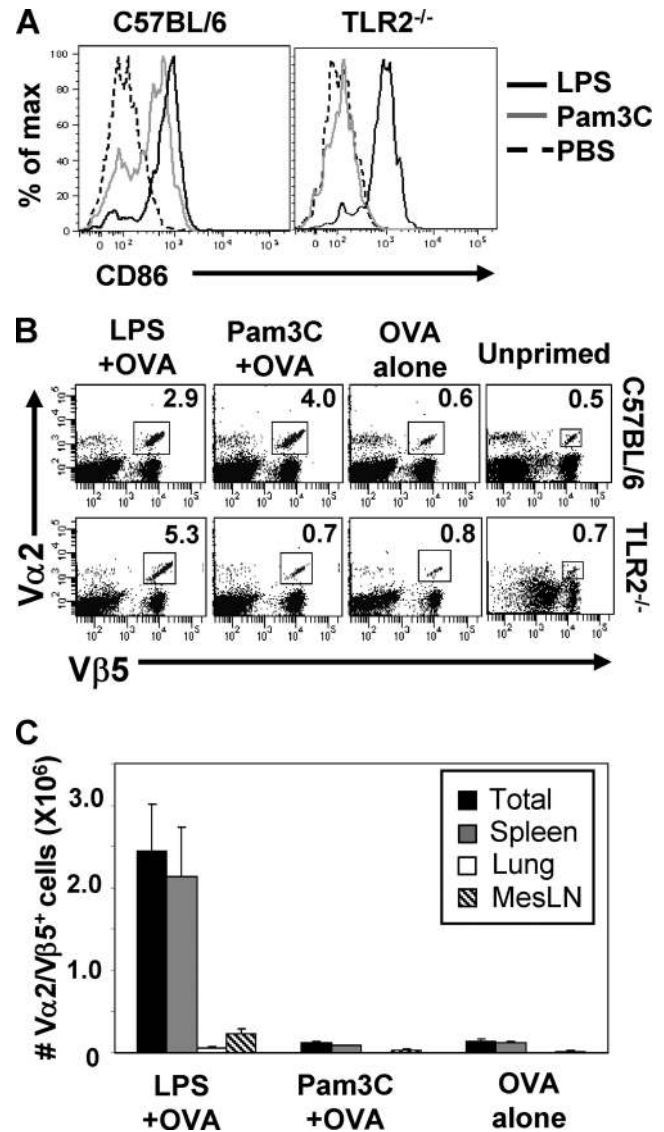

FIGURE 4. Expansion of Ag-specific CD4 T cells primed with Pam3C is due to TLR2 engagement on APC. *A*, DC activation in wild-type and TLR2$^{-/-}$ mice. C57BL/6 and TLR2$^{-/-}$ mice were administered LPS, Pam3C, or PBS and 18 h later, splenic DC were analyzed for up-regulation of costimulatory ligand expression by flow cytometry. Plots show CD86 expression of CD11c$^+$ DC from PBS control, LPS-treated, or Pam3C-treated mice. *B*, Ag-specific priming in TLR2-deficient hosts. Naive CD4 T cells from OT-II TCR-transgenic mice were transferred into C57BL/6 or syngeneic TLR2$^{-/-}$ hosts, primed with LPS + OVA, Pam3C + OVA, or OVA peptide alone, and CD4 T cells were harvested from different tissues 1 wk after priming. Flow cytometry plots show frequencies of OT-II CD4 T cells (Vα2$^+$Vβ5$^+$) within total splenic CD4 T cells in C57BL/6 and TLR2$^{-/-}$ hosts primed with OVA peptide with or without TLR agonist and endogenous Vα2$^+$Vβ5$^+$ CD4 T cells from unprimed mice. The frequency of Vα2$^+$Vβ5$^+$ CD4 T cells resulting from LPS + OVA priming ranged from 2 to 3% in B6 hosts and 1–9% in TLR2$^{-/-}$ hosts. *C*, Yield of OT-II CD4 T cells harvested from spleen, lung, and mesenteric lymph nodes (MesLN) of differentially primed TLR2-deficient mice from three mice per group. p = 0.05 comparing total number of OVA-specific cells resulting from LPS vs Pam3C priming in TLR2-deficient hosts. Results are representative of three independent experiments.

sults confirm the TLR2 dependence of Pam3C-mediated DC activation.

To determine whether TLR engagement on APC was leading to the differential effects of priming by TLR2 vs TLR4 agonists, we

FIGURE 5. In vivo kinetics of DC maturation in response to Pam3C or LPS stimulation. BALB/c mice were treated with LPS + HA, Pam3C + HA, HA alone, or PBS, and splenic CD11c$^+$ DC were isolated after 6, 18, and 30 h. *A*, Absolute numbers of DC in the spleen at 6–30 h following TLR agonist administration represented as the mean ± SD numbers of CD11c$^+$ splenic DC from three mice per group. $p = 0.05$ comparing absolute numbers of CD11c$^+$ DC at 6 h vs 18 h following LPS administration. *B*, Expression of active caspase 3 in CD11c$^+$B220$^-$ DC 18 h postpriming following TLR agonist administration. Results are shown as the average percentage of caspase 3$^+$ DC from three independent experiments with three mice per group. *C*, Kinetics of costimulatory ligand up-regulation by DC in response to TLR2 and TLR4 engagement. Graph shows mean fluorescence intensity (MFI) of CD86 and CD80 expression by CD11c$^+$ DC harvested 6–30 h after TLR agonist administration. $p = 0.05$ comparing CD86 levels between LPS-primed and Pam3C-primed DC 6 h after administration. Results are representative of four independent experiments.
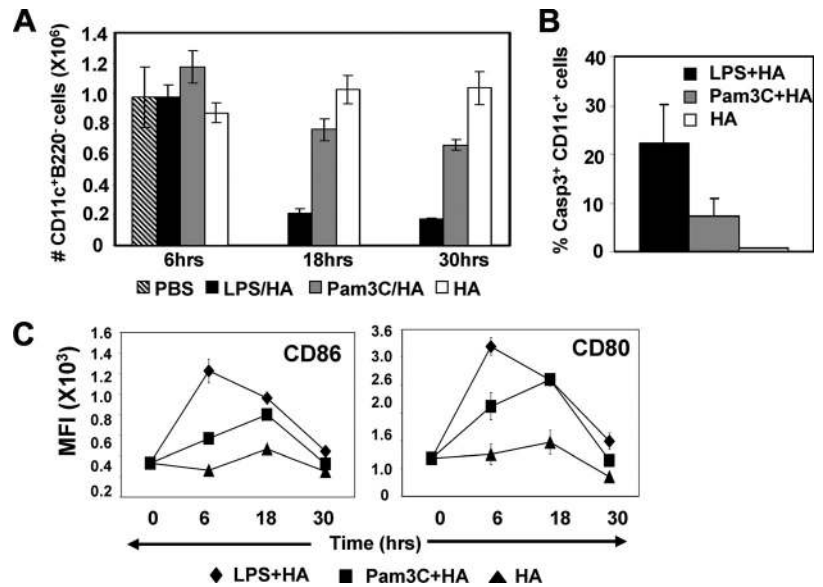
transferred naive CD4 T cells from OT-II- transgenic mice into either wild-type C57BL/6 or TLR2$^{-/-}$ hosts primed with OVA alone or in the presence of LPS or Pam3C as above and harvested spleen, lung, and mesenteric lymph nodes 1 wk later. We found an increased frequency of OVA-specific Vα2$^+$Vβ5$^+$ CD4 T cells in LPS plus OVA and Pam3C plus OVA-primed mice compared with OVA alone in wild-type hosts and to the endogenous frequency of Vα2$^+$Vβ5$^+$ CD4 T cells in unprimed hosts (Fig. 4*B*). Similar to our results in BALB/c hosts (Fig. 1), we observed a higher frequency of Ag-specific CD4 T cells following antigenic priming in the presence of Pam3C compared with LPS in C57BL/6 hosts (Fig. 4*B, top row*). By contrast, in TLR2-deficient hosts, both the frequency (Fig. 4*B, bottom row*) and absolute numbers (Fig. 4*C*) of Vα2$^+$Vβ5$^+$ CD4 T cells were comparably low in OVA alone and Pam3C plus OVA-primed mice compared with the increased frequency and numbers observed with LPS plus OVA priming (Fig. 4, *B* and *C*). These results demonstrate that the ability of Pam3C to stimulate robust expansion of Ag-primed CD4 T cells is due to engagement of TLR2 on APC.

TLR2 and TLR4 priming leads to differential kinetics of APC maturation and persistence

We further investigated potential differences in DC activation in vivo in response to TLR2 vs TLR4 engagement. Analysis of the absolute numbers of DC in the spleen after priming showed a dramatic reduction in DC numbers in LPS-primed mice that was not observed in mice that received Pam3C or peptide (Fig. 5*A*). This loss of splenic DC correlated with enhanced apoptosis of the remaining DC in LPS-treated mice as seen by up-regulation of active caspase 3 expression in DC 18 h after LPS priming (Fig. 5*B*). By contrast, in Pam3C-primed mice, DC numbers in the spleen were maintained and the proportion of apoptotic DC was significantly lower than in LPS-primed mice. These results indicate that use of Pam3C as a TLR agonist for CD4 T cell priming allows maintenance of mature DC numbers in the spleen, whereas LPS administration results in cellular toxicity manifested by increased DC apoptosis and loss of DC numbers.

Both TLR agonists stimulated up-regulation of CD80/CD86 in vivo early after TLR agonist treatment (6 h) which decreased to control levels by 30 h, although the kinetics of activation differed in LPS vs Pam3C-treated mice. Costimulatory ligand expression was higher at 6 h in LPS vs Pam3C-primed DC but reached com-

parable levels by 18 h after priming and, by 30 h, both groups down-regulated costimulatory molecule expression (Fig. 5*C*). In contrast, mice that received peptide alone did not up-regulate CD80/CD86, confirming the importance of TLR agonist stimulation in APC maturation. These results indicate that although the in vivo effects of TLR agonists on DC activation are very rapid, LPS stimulates a high level of DC activation and promotes DC apoptosis, whereas TLR2 engagement by Pam3C results in slower kinetics of DC activation yet decreased apoptosis.

TLR2 engagement enhances memory CD4 T cell generation

We asked whether the different priming efficiencies resulting from TLR2 vs TLR4 agonist priming led to differences in the generation of memory CD4 T cells. We primed mouse recipients of naive HA-specific CD4 T cells as above and recovered persisting Ag-specific memory CD4 T cells from different tissue sites 4 wk after priming. In Pam3C plus HA-primed mice, we observed an increased frequency (Fig. 6*A, upper panel*) and absolute number (Fig. 6*A, lower panel*) of persisting HA-specific memory CD4 T cells compared with LPS plus HA-primed mice, with the majority of memory CD4 T cells in spleen and lymph nodes and lower numbers in the lung. Negligible numbers of HA-specific memory CD4 T cells resulted from priming with HA peptide alone (Fig. 6*A*). Furthermore, the persisting splenic HA-specific cells resulting from LPS plus HA or Pam3C plus HA priming exhibited a similar CD44^{high}CD62L^{low}CD127$^+$ profile characteristic of a memory CD4 T cell phenotype (Fig. 6*B*). These results demonstrate that TLR engagement during peptide Ag priming is necessary for memory CD4 T cell generation and that the TLR2 agonist Pam3C promotes a high frequency of persisting memory CD4 T cells.

We asked whether the increased frequency of memory CD4 T cells generated with antigenic priming with Pam3C derived from the increased number of primed cells already present in Pam3C plus HA-primed mice or an intrinsic ability of TLR2 agonist-primed cells to develop into memory. To address this issue, we primed HA-specific CD4 T cells in vitro with HA peptide and APC in the presence of LPS or Pam3C, adoptively transferred equivalent numbers of these differentially primed CD4 T cells into congenic BALB/c hosts, and analyzed the frequency and numbers of persisting memory CD4 T cells after 4 wk. We observed increased frequencies (Fig. 6*C*) and absolute numbers (Fig. 6*D*) of persisting memory CD4 T cells in mice that had received Pam3C-primed
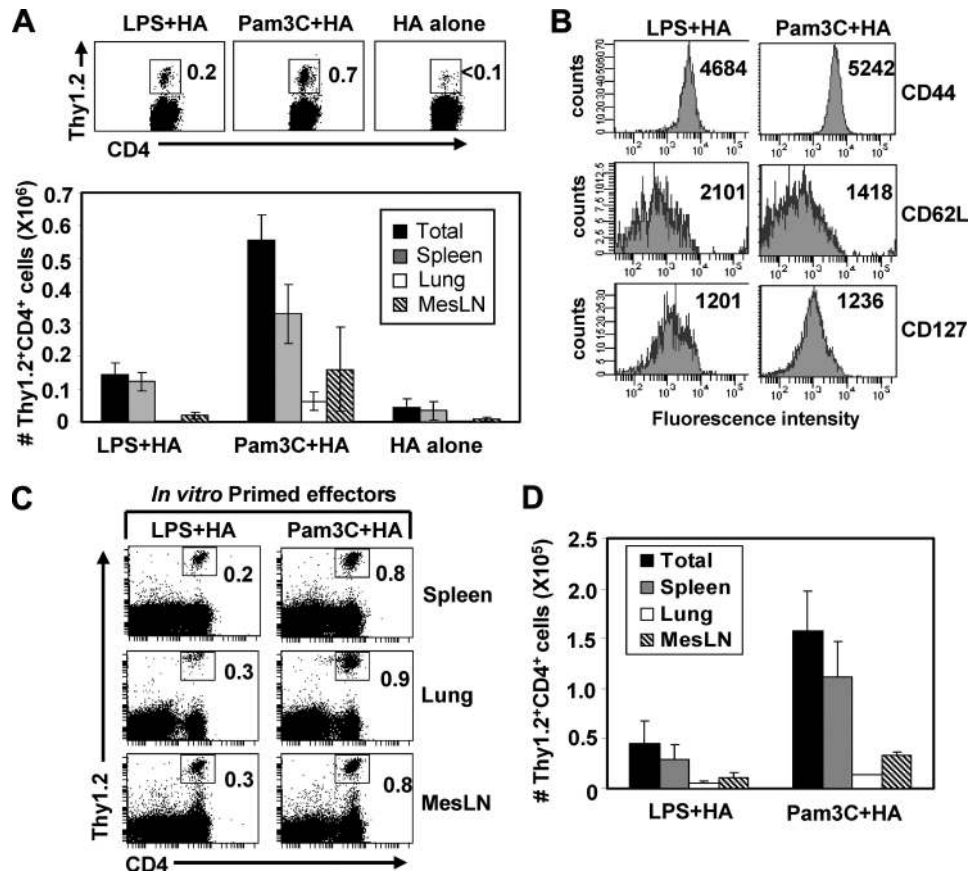
FIGURE 6. TLR2 agonist priming enhances Ag-specific memory CD4 T cell persistence compared with TLR4 agonist priming. Naive HA-specific CD4 T cells were primed in vivo in adoptive hosts as in Fig. 1, and HA-specific memory CD4 T cells were harvested from different tissue sites 4 wk after priming. *A*, Frequency in spleen (*upper panel*) and absolute numbers (*lower panel*) of HA-specific CD4 T cells harvested from the spleen, lung, and mesenteric lymph nodes of differentially primed mice expressed as the mean ± SD of HA-specific cells in each tissue averaged from five mice per group. Average frequency of memory CD4 T cells in the total CD4 T cell population from mice primed with LPS + HA, Pam3C + HA, or HA peptide alone was 0.2, 0.7, and 0.1% respectively, averaged from three independent experiments with a total of nine mice per group. $p = 0.05$ comparing total yield of Ag-specific CD4 T cells obtained from Pam3C-primed vs LPS-primed mice. *B*, Differentially primed HA-specific CD4 T cells express memory phenotypes. Histograms show CD44, CD62L, and CD127 expression on Thy1.2$^+$ HA-specific memory CD4 T cells generated from LPS + HA or Pam3C + HA priming. Results are representative of four experiments. Number in each plot represents mean fluorescence intensity. *C*, Enhanced generation of memory CD4 T cells from TLR2-primed effectors. HA-specific CD4 T cells were primed in vitro for 72 h with HA peptide and APC in the presence of LPS or Pam3C (1.5 μg/ml), transferred into congenic BALB/c hosts, and persisting memory CD4 T cells were recovered 4 wk after transfer. MesLN, Mesenteric lymph node. Plots show frequency and absolute numbers (*D*) of HA-specific memory CD4 T cells from different tissue sites.

compared with LPS-primed CD4 T cells, despite having transferred an equivalent number of cells. These results establish that memory T cell generation occurs with higher frequency from CD4 T cells primed in the presence of a TLR2 agonist.

TLR2-primed memory CD4 T cells mediate robust secondary responses to influenza challenge

We asked whether persisting memory CD4 T cells generated from antigenic priming with TLR2 or TLR4 agonists could generate robust secondary responses to pathogen challenge. We therefore challenged mouse hosts of differentially primed HA-specific memory CD4 T cells with a sublethal dose of PR8 influenza (containing the HA peptide epitope) intranasally and harvested spleen and lung 6 days after infection. Control mice received transferred naive HA-specific CD4 T cells. Although LPS-primed HA-specific memory CD4 T cells and, to a lesser extent, formerly naive HA-specific CD4 T cells were present in significant fractions in the spleen, only TLR2- primed CD4 T cells accumulated to significant extents at the site of infection in the lung (Fig. 7), which was apparent with both frequency (Fig. 7*A*) and absolute numbers (Fig. 7*B*). Moreover, lungs from flu-challenged mice primed with Pam3C plus HA

exhibited more extensive immune cell infiltrates in the lung compared with naive and LPS plus HA-primed mice (Fig. 7*C*). The immune infiltrates in Pam3C plus HA-primed mice consisted of both mononuclear and polymorphonuclear cells (Fig. 7*C*). This result suggests that TLR2-primed memory CD4 T cells mediated more efficient secondary responses at the site of infection compared with naive or TLR4-primed memory CD4 T cells.

Discussion

The effects of specific TLR ligation on the quality and frequency of effector and memory T cell responses is not well understood. In this study, we took an in vivo approach to investigate the effects of TLR2 compared with TLR4 engagement on the generation, function, and recall of effector and memory CD4 T cell responses. We found that antigenic priming in the presence of the TLR2 agonist Pam3C resulted in a higher frequency of Ag-specific CD4 T cells, compared with priming in the presence of the TLR4 agonist LPS, with TLR2-primed CD4 T cells producing more IL-2 and IL-17 and less IFN-γ than TLR4-primed CD4 T cells. The ability of Pam3C to prime for high frequencies of Ag-specific CD4 T cells was due to engagement of TLR2 on DC. TLR2 engagement during
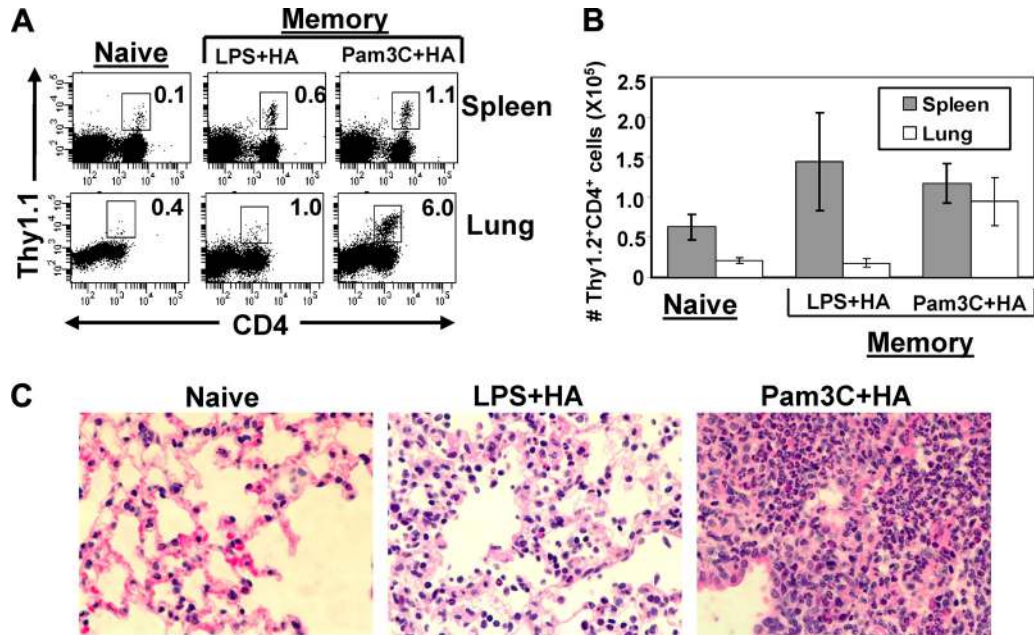
FIGURE 7. TLR2-primed memory CD4 T cells exhibit robust secondary responses following influenza challenge. Mouse hosts of naive HA-specific CD4 T cells or HA-specific memory CD4 T cells generated by LPS + HA or Pam3C + HA priming (see Fig. 6) were challenged intranasally with 500TCID$_{50}$ PR8 influenza, and HA-specific CD4 T cells were recovered from spleen and lung 6 days after infection. *A*, Frequency of HA-specific CD4 T cells in the spleen and lung following influenza challenge of mice with naive HA-specific CD4 T cells or mice with memory CD4 T cells generated by LPS + HA or Pam3C + HA priming. *B*, Absolute numbers of recovered HA-specific CD4 T cells from groups in *A* representing the mean ± SD yield from five mice per group; representative of four experiments. $p = 0.02$ comparing absolute numbers of Ag-specific CD4 T cells in the lung in LPS-treated mice vs Pam3C-treated mice. *C*, H&E staining of lung sections from influenza-infected mouse hosts of naive HA-specific CD4 T cells (*top*) or memory CD4 T cells generated by LPS + HA priming (*middle*) or Pam3C + HA priming (*right*) 6 days after infection.

Ag priming also resulted in an increased frequency of memory CD4 T cells compared with TLR4 engagement, which also mediated enhanced site-specific secondary responses to a respiratory virus infection. These results indicate that TLR engagement at the early phases of T cell activation in vivo can influence the quantity and quality of the primary effector, memory, and subsequent secondary responses, with TLR2 engagement particularly efficacious at generating high-frequency primary and memory T cell responses.

Differential TLR engagement has been shown to have variable effects on the priming and differentiation of CD4 T cells that can be influenced by the type of Ag, DC, location, and dose of TLR agonist (48–50). Previous studies showed that TLR2 ligation promoted Th2 generation compared with TLR4 ligation which is known to promote IFN-γ production and Th1 generation (51, 52). However, other studies found antagonistic effects of TLR2 ligation on Th2 effector cells (53). We also found that antigenic priming with the TLR2 agonist Pam3C resulted in predominantly IL-2 and IL-17 producers with a low number of IFN-γ- and IL-4-producing cells. TLR2 expression was also found to promote IL-17 production in human CD4 T cells (22), consistent with our results here. However, in infection models, TLR2 deficiency has been associated with increased IL-17 production in inflammatory sites (24, 25), suggesting that other influences of inflammation and infection may affect how different TLR control T cell responses. TLR2 engagement has also been associated with increased Treg expansion in vivo (20, 33, 46, 54), although this TLR2-mediated effect was due to direct ligation of TLR2 on Tregs (20, 33). In our system, where TLR2-mediated effects were due to ligation of TLR2 on APC, we did not observe up-regulation of FoxP3 by naive Ag-specific CD4 T cells following antigenic priming with the TLR2 agonist (data not shown). These results indicated that TLR2 engagement in vivo did not trigger conversion of non-Tregs to Tregs in the model system used here.

TLR agonists can act directly on the APC and/or the T cells themselves as certain TLR such as TLR2 are expressed by activated T cells and T cell subsets (20, 33, 47). In this study, we found that the differential effects of TLR2 vs TLR4 engagement during antigenic priming was due to TLR2 ligation on APC and not on T cell populations, as wild-type CD4 T cells were not primed by Pam3C and peptide in TLR2-deficient hosts. We further show that although both LPS and Pam3C triggered up-regulation of costimulatory ligands on DC in vivo, LPS triggered increased DC apoptosis and a concomitant decrease in DC numbers in the spleen, whereas Pam3C did not stimulate a high level of DC apoptosis or affect DC numbers. These findings are consistent with a previous report showing that TLR4 and not TLR2 can facilitate accelerated DC death in a partial caspase-dependent manner (55). Together, these results point to adjuvant effects of both LPS and Pam3C, but Pam3C exhibited less toxic effects in vivo compared with LPS. Furthermore, although use of high-dose LPS for in vivo priming is associated with morbidity, use of higher doses of Pam3C resulted in an enhanced frequency of activated T cells without no associated toxicity. We also investigated whether the increased IL-17 production by Pam3C plus HA-primed CD4 T cells was due to different levels of proinflammatory cytokines produced by differentially stimulated DC; however, Pam3C- vs LPS-activated DC exhibited similar up-regulation of IL-6, TGF-β, and IL-10 production (data not shown), consistent with findings by others (19, 22). It is possible that the enhanced frequency of CD4 T cell priming resulting from using Pam3C compared with LPS as an adjuvant may be due to increased availability of activated DC and their associated proinflammatory cytokines during initiation of T cell activation.

We demonstrate that use of specific TLR agonists as innate immune triggers for antigenic priming can have pleiotropic effects on primary and secondary responses. Because our model system controlled for the host, peptide dose, and T cell precursor frequency, our results indicate that the pathway of CD4 T cell differentiation is significantly affected by exposure of DC to different TLR ligands. We found enhanced memory CD4 T cell survival from TLR2 compared with TLR4 agonist priming, supporting a model that innate immune signals may determine memory vs effector T cell development at very early stages in vivo (56), and that memory T cell fate is determined at the very earliest stages of T cell activation (57).

We show here that TLR2-primed memory CD4 T cells mediated more robust secondary responses to influenza challenge at the site of infection in the lung compared with TLR4-primed memory CD4 T cells. We further observed a more extensive immune infiltrate in the lung in the presence of TLR2-primed memory CD4 T cells, indicating that functional capacities of memory CD4 T cells and their potential to coordinate secondary immune responses are determined at the earlier priming stage. Memory CD8 T cell differentiation and secondary responses to viruses has been shown to be programmed early during priming by factors such as CD4 T cell help and the presence of IL-2 (58–61). We show here that TLR2 agonist-primed CD4 T cells produced predominantly IL-2 and led to greater numbers of memory CD4 T cells, which were also present at greater frequency following secondary challenge, suggesting that early IL-2 production programmed the development of memory CD4 T cells with enhanced capacities for secondary expansion. These results have important implications for vaccine development, where use of specific TLR agonists can determine the long-term outcome of persisting immunity, and suggest that targeting TLR2 for initial priming could be efficacious for promoting memory T cell development.

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

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