Differentiation

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J Immunol published online 21 March 2014
http://www.jimmunol.org/content/early/2014/03/21/jimmunol.1302569

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/03/21/jimmunol.1302569.DCSupplemental

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TLR4 Ligands Lipopolysaccharide and Monophosphoryl Lipid A Differentially Regulate Effector and Memory CD8+ T Cell Differentiation

Weiguo Cui,*† Nikhil S. Joshi,* Ying Liu,* Hailong Meng,† Steven H. Kleinstein,†‡ and Susan M. Kaech*§

Vaccines formulated with nonreplicating pathogens require adjuvants to help bolster immunogenicity. The role of adjuvants in Ab production has been well studied, but how they influence memory CD8+ T cell differentiation remains poorly defined. In this study we implemented dendritic cell–mediated immunization to study the effects of commonly used adjuvants, TLR ligands, on effector and memory CD8+ T cell differentiation in mice. Intriguingly, we found that the TLR4 ligand LPS was far more superior to other TLR ligands in generating memory CD8+ T cells upon immunization. LPS boosted clonal expansion similar to the other adjuvants, but fewer of the activated CD8+ T cells died during contraction, generating a larger pool of memory cells. Surprisingly, monophosphoryl lipid A (MPLA), another TLR4 ligand, enhanced clonal expansion of effector CD8+ T cells, but it also promoted their terminal differentiation and contraction; thus, fewer memory CD8+ T cells formed, and MPLA-primed animals were less protected against secondary infection compared with those primed with LPS. Furthermore, gene expression profiling revealed that LPS-primed effector cells displayed a stronger pro-memory gene expression signature, whereas the gene expression profile of MPLA-primed effector cells aligned closer with terminal effector CD8+ T cells. Lastly, we demonstrated that the LPS-TLR4-derived “pro-memory” signals were MyD88, but not Toll/IL-1R domain–containing adapter inducing IFN-b, dependent. This study reveals the influential power of adjuvants on the quantity and quality of CD8+ T cell memory, and that attention to adjuvant selection is crucial because boosting effector cell expansion may not always equate with more memory T cells or greater protection. The Journal of Immunology, 2014, 192: 000–000.
cytotoxic CD8+ T cells (18–22). However, IL-12 and IFN-γ have also been shown to negatively affect memory CD8+ T cell potential in activated CD8+ T cells (14–17, 23). Additionally, recent work has also demonstrated that Wnt signaling and certain cytokines, such as IL-10 and IL-21, provide the positive signals for memory CD8+ T cell differentiation and maturation (24–27). Thus, it is crucial to further investigate not only how commonly used adjuvants enhance the expansion and activation of T cells during vaccination, but also how they augment the inflammatory milieu, which can affect the quantity and quality of memory T cells formed.

In this study, we implemented a well-established dendritic cell (DC)–mediated immunization strategy (14, 15, 28) to compare the effects of different adjuvants on CD8+ T cell memory. Ultimately, we focused on two TLR4 ligands, LPS and monophosphoryl lipid A (MPLA), because we found that they both boosted expansion of Ag-specific CD8+ T cells, but their effects on memory CD8+ T cell numbers differed greatly. That is, a significantly larger fraction of LPS-primed CD8+ T cells populated the memory cell pool compared with those primed with MPLA. Compared to LPS-priming, MPLA priming promoted the formation of a larger frequency of killer cell lectin–like receptor G1 (KLRG1)hiIL-7Rlo terminal effector cells and also negatively affected IL-7Rhi memory precursor cell survival, leading to greater rates of effector T cell contraction. Furthermore, gene expression profiling revealed that the intrinsic properties of IL-7Rhi memory precursor cells generated through LPS and MPLA priming were distinct and that LPS induced a more memory-like pattern of gene expression whereas MPLA induced a more effector-like pattern. Moreover, the inflammatory milieu produced by LPS and MPLA were largely distinct and the “pro-memory” signals derived from LPS-TLR4 signaling were dependent on MyD88, but not Toll/IL-1R domain–containing adapter inducing IFN-β (Trif). These results highlight important ways in which the selection of adjuvants can influence the quantity and the quality of memory T cells that arise during vaccination.

Materials and Methods

Mice

Thy1.1+ P14 TCR transgenic mice have been described previously (29). To make P14 chimeric mice, ∼5 × 10^5 Thy1.1+ P14 CD8+ T cells were transferred into naive Thy1.2+ C57BL/6 (B6) mice that were purchased from the National Cancer Institute/Charles River Laboratories. After transfer, the recipient mice typically contain ∼5 × 10^6 naive P14 CD8+ T cells. IL-6−/− and IL-12Rβ2−/− mice were purchased from Dr. Ruslan Medzhitov at Yale University. All animal experiments were performed under approved Institutional Animal Care and Use Committee protocols.

Generation of bone marrow–derived DCs and DC immunization

Bone marrow–derived CD11c+ DCs were generated after 5 d of culture with GM-CSF and matured with LPS (50 ng/ml) overnight as described previously (11, 30). Matured DCs were pulsed with glycoprotein (GP)33–41 peptide (200 ng/ml) for 2 h, washed with PBS, and 1 × 10^6 DC-33 cells were injected i.v. Simultaneously, DC-immunized mice were injected i.p. with either PBS or one of the following TLR ligands: Pam3CSK4 (50 μg), polyinosinic-polycytidylic acid (50 μg), LPS (20 μg), MPLA (30 μg), flagellin (20 μg), lipoteichoic acid (20 μg), R848 (20 μg), CpG-A (50 μg), or CpG-B (50 μg). The dose for each adjuvant was primarily determined by either previous publications (14, 15, 31–34) and/or manufacturer recommendations. Additional titrations were done in some cases to ensure comparable CD8+ T cell responses at the effector phase, as shown in Supplemental Fig. 1, and no perceivable side effects. LPS was purchased from Sigma-Aldrich (St. Louis, MO). CpG-A and CpG-B were synthesized at Yale University Keck Facility, and the rest were purchased from InvivoGen (San Diego, CA).

Abs and surface and intracellular staining

Lymphocyte isolation, GP33–41 peptide stimulations, and surface/intracellular staining were performed as previously described (14). All Abs were purchased from eBioscience (San Diego, CA) except for anti-KLRG1 (2F1) hybridoma, which was a gift from Dr. D. Rauler (University of California, Berkeley, CA) and was conjugated to Alexa Flour 647 (Invitrogen, Eugene, OR). All flow cytometry was analyzed on a FACS-Calibur (BD Biosciences) with FlowJo software (Tree Star, San Carlos, CA).

Gene array analysis

Mice that contain small numbers of P14 CD8+ T cells were immunized with DC-33 either alone or in combination with LPS or MPLA. KLRG1hiIL-7Rhi memory precursor effector cells (MPECs) were purified by FACs sorting, and mRNA isolated from MPECs was subjected to whole-genome expression profiling using an Illumina MouseWG-6 v2.0 Expression BeadChip. Gene expressions of lymphocytic choriomeningitis virus (LCMV)-MPEC and LCMV–short-lived effector cell (SLEC) CD8+ T cells were measured by Affymetrix GeneChip Mouse Genome 430 2.0 Array (GSE86787) (17). Microarray hybridization was carried out at the Yale Keck Microarray Facility. The data analyses were carried out using packages in R (35). Raw MPEC expression data from MLA and LPS groups were normalized using the quanile method provided by the lumi package in R/Bioconductor (36). MLA (LPS) signature genes were defined by two criteria: 1) a log2 (fold change) ≥1 of MLA (LPS) samples relative to LPS (MLA) samples, and 2) a statistically significant change in expression as determined by limma (37) using a Benjamani–Hochberg false discovery rate cutoff of q < 0.05. Raw LCMV-MPEC and LCMV-SLEC microarray data in CEL file format were read in and normalized with the robust multichip average method provided by the Oligo package in R/Bioconductor (38). To test the association of MLA and LPS signature genes with LCMV-MPEC and LCMV-SLEC expression profiles (GSE86787) (17), genes common to the two microarray platforms (according to gene symbols) were ranked based on their r-statistics from the comparison of LCMV-MPEC and LCMV-SLEC gene expression profiles using the limma package in R/Bioconductor. The enrichment of MLA and LPS signature genes was then measured based on their relative positions in the ranked gene list using the geneSetTest in R/Bioconductor (39).

The microarray data of P14 CD8+ T cells immunized with DC-33 either alone or in combination with LPS or MLA have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (40) and are accessible through Gene Expression Omnibus series accession no. GSE50764 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50764).

Statistical analyses

Where indicated, p values were determined by a two-tailed unpaired Student t test. A p value < 0.05 was considered significant. All graphs show averages of the mean ± SEM.

Results

Differential effects of TLR ligands on memory CD8+ T cell generation

To investigate how commonly used adjuvants affect memory T cell formation, we first carried out a comprehensive and systematic analysis of various TLR ligands on memory CD8+ T cell development. To facilitate the detection of Ag-specific CD8+ T cell responses in vivo, P14 chimeric mice were generated in which a small number (1 × 10^5) of naive Thy1.1+ P14 CD8+ T cells, which recognize the DβGP33–41 LCMV epitope, were transferred into wild-type (WT) B6 mice. These mice were immunized with GP33–41 peptide-pulsed bone marrow–derived DCs (referred to as DC-33) either alone or in combination with various TLR ligands. Both the DCs and adjuvants were administered systemically, with the DCs given i.v. and the TLR ligands given i.p. The bone marrow–derived DCs were matured with LPS for 12 h using standard protocols (30) to induce high expression levels of CD11c, MHC class I and II, and costimulatory molecules CD80/86. However, at the time of immunization, these DCs produced only low amounts of proinflammatory cytokines, such as IL-6, IL-12,
and TNF, as their expression peaked at ∼6 h after LPS stimulation (Refs. 14, 28 and data not shown). Thus, this DC immunization system allowed us to vary the inflammatory milieu that the CD8⁺ T cells were exposed to systemically, through the injection of different adjuvants, while keeping antigenic stimulation relatively constant. Without DC immunization, the donor P14 CD8 T cells were undetectable in the recipient mice because of the small numbers of cell transferred, but with DC-33 immunization alone (e.g., in the absence of TLR ligands), P14 effector and memory CD8 T cell responses were readily detected at days 7 and 45 after immunization (Fig. 1A). As expected, DC-33 immunization with the nine selected adjuvants—Pam3CSK4 (TLR1/2), polyinosinic-polycytidylic acid (TLR3), LPS (TLR4), MPLA (TLR4), flagellin (TLR5), lipoteichoic acid (TLR2/6), R848 (TLR7/8), CpG-B (TLR9), and CpG-A (TLR9)—boosted the overall expansion of the P14 effector CD8 T cells, with comparable efficiency, over that of DC immunization alone (Supplemental Fig. 1). However, when analyzed 45 d later, P14 CD8 T cells primed in the presence of LPS generated a significantly larger frequency and number of memory CD8⁺ T cells than did the other adjuvants or DC-33 alone. Surprisingly, MPLA, which is a low-toxicity derivative of LPS and recently approved for use as an adjuvant in humans, generated the smallest number and percentage of splenic memory CD8⁺ T cells (Fig. 1B, 1C). These data suggested that priming with the TLR4 ligand LPS, but not MPLA, provided a unique set of signals that was beneficial for memory CD8⁺ T cell formation and survival.

**Effects of TLR4 ligands LPS and MPLA on effector and memory CD8⁺ T cell differentiation**

It was intriguing that the two TLR ligands LPS and MPLA, which signal through the same receptor, would have such opposing effects on memory CD8⁺ T cell generation. To further dissect how LPS or MPLA affect the development of memory CD8⁺ T cells in vivo, we immunized P14 chimeric mice with DC-33 alone or in conjunction with either LPS or MPLA. We first compared the effects of LPS and MPLA on the number and quality of effector and memory CD8⁺ T cells that formed during DC-33 immunization. The DC-33 vaccination generated ∼1 × 10⁶ P14 effector CD8⁺ T cells at the peak; ∼90% of the effector CD8⁺ T cells induced by DC-33 contracted with ∼10% of them developing into memory cells (Fig. 2A). Remarkably, there was virtually no contraction of the effector CD8⁺ T cells generated by DC-33 plus LPS immunization, and this led to ∼10-fold more memory cells than that formed during DC-33 alone (Fig. 2A). The expansion induced by DC-33 plus MPLA was equivalent to that of LPS; in contrast, however, the numbers of memory CD8⁺ T cells were substantially lower than any other condition (Figs. 1C, 2A). Taken together, these data suggested that MPLA produced signals that could augment activated CD8⁺ T cell expansion, compared with DC-33 alone, but reduced their memory cell potential and long-term survival. In contrast, LPS could produce signals that potently enhanced formation of memory cells and their progenitors, or, alternatively, blocked the activated CD8⁺ T cells from receiving signals that diminish memory cell potential.

Next, we compared the phenotypes of CD8⁺ T cells at the effector phase among DC-33 alone, DC-33 plus LPS, and DC-33 plus MPLA groups. Strikingly, DC-33 plus MPLA immunization induced the formation of effector CD8⁺ T cell subsets that could be distinguished, based on our past work, as KLRG1lo/IL-7Rhi SLECs and KLRG1hi/IL-7Rlo MPECs. Nearly all of the MPLA-induced KLRG1hi/IL-7Rlo cells died between days 7 and 45, leaving behind a predominately KLRG1lo/IL-7Rhi memory CD8⁺ T cell population. This was due to direct apoptosis of KLRG1hi cells rather than conversion of the KLRG1hi → KLRG1lo cells (data not shown). In contrast, both DC-33 alone and DC-33 plus LPS induced a relatively homogenous KLRG1lo/IL-7Rhi population, and the resulting memory CD8⁺ T cells were of a similar phenotype (Fig. 2B). Similar observations were obtained both in spleens and other tissues such as blood, lungs, livers, and lymph nodes (data not shown). Consistent with increased expression of KLRG1, DC-33 plus MPLA–induced effector CD8⁺ T cells also expressed less CD62L, CD27, and CXCR3 (Fig. 2C). Additionally, memory CD8⁺ T cells formed after DC-33 plus MPLA immunization were less “polyfunctional” in comparison with that of DC-33 or DC-33 plus LPS immunization (Fig. 2D, 2E). Overall, immunization with LPS appeared to induce a greater

**FIGURE 1.** TLR ligands differentially affected the number of memory CD8⁺ T cells that form following DC vaccination. B6 mice containing a small number (∼5 × 10⁶/mouse) of naive P14 CD8⁺ T cells were immunized with either DC-33 i.v. alone or in combination with various TLR agonists i.p., as indicated (see Materials and Methods for doses), and an additional group of mice served as untreated controls. (A) Spleens were harvested on day 7 and 45 d postimmunization and the Ag-specific P14 CD8⁺ T cells were plotted in the dot plots. (B) Frequency of CD8⁺ T cells and (C) numbers per spleen of Ag-specific P14 cells on day 45 postimmunization were plotted in the bar graphs. Data shown are representative of at least three independent experiments. "p < 0.05. Note that the baseline number of naive P14 CD8 T cells without DC-33 immunization would be ∼5 × 10⁷ cells/mouse.
fraction of IL-7R<sup>hi</sup> effector cells that could survive and populate the memory pool compared with DC-33 or DC-33 plus MPLA immunization, suggesting that LPS priming instilled the activated CD8<sup>+</sup> T cells with memory cell developmental potential.

LPS, but not MPLA, enhanced memory CD8<sup>+</sup> T cell–mediated protective immunity

To better characterize how LPS and MPLA directly affected the development of memory CD8<sup>+</sup> T cells in vivo, we immunized B6 mice with DC-33 alone or in combination with LPS or MPLA (Fig. 3A). Note, in this system we could monitor the primary peptide-specific CD8<sup>+</sup> T cell responses by MHC class I DbGP33 tetramer staining in the peripheral blood (data not shown); however, at most memory time points the Ag-specific CD8<sup>+</sup> T cells were often below the level of detection in the peripheral blood. To test the adjuvant effects on memory CD8<sup>+</sup> T cell protection, the aforementioned three groups of mice were challenged with lymphocytic choriomeningitis clone 13 strain (LCMV-clone 13) 8 wk after initial immunization, and virus-specific CD8<sup>+</sup> T cells were monitored in the peripheral blood between days 4 and 7 postinfection. Although all immunized groups mounted greater CD8<sup>+</sup> T cell responses than did the naive control group, the kinetics and magnitudes of the response were quite diverse (Fig. 3B). Strikingly, mice immunized with DC-33 plus LPS generated more rapid and robust antiviral CD8<sup>+</sup> T cell responses than did any other groups, both in frequency (Fig. 3B) and number (Fig. 3C). Additionally, the LPS-primed memory CD8<sup>+</sup> T cells demonstrated the greatest control over viral replication compared with the other groups because viral titers in the peripheral blood were ∼5- to 10-fold lower at days 4–6 postinfection and were below the level of detection by day 7 postinfection (Fig. 3D). Interestingly, priming with MPLA failed to improve CD8<sup>+</sup> T cell–mediated immune protection against LCMV infection in comparison with animals immunized with DC-33 alone (Fig. 3B–D). To ensure that the CD8<sup>+</sup> T cell recall response was LCMV DbGP<sub>33-41</sub> peptide–specific, we also examined another LCMV epitope, DbNP<sub>396-404</sub>. This showed that the expansion of DbNP<sub>396-404</sub>-specific primary effector CD8<sup>+</sup> T cells was similar among all groups of mice.
Collectively, these results suggest that the use of LPS as an adjuvant during immunization induced a larger number of memory CD8+ T cells and conferred greater immunological protection against secondary infection than that of MPLA. LPS enhances activated CD8+ T cell survival, but not homeostatic turnover

The different outcomes of the CD8+ T cell responses induced by either DC-33 plus LPS or DC-33 plus MPLA prompted us to further investigate whether the LPS mediated pro-memory effects on CD8+ T cells were due to improved survival of memory precursor cells or increased homeostatic proliferation of memory CD8+ T cells. To test these possibilities, we first measured the magnitude of contraction of KLRG1loIL-7Rhi MPECs among DC-33 alone, DC-33 plus LPS, and DC-33 plus MPLA groups. This demonstrated that ∼33% of KLRG1loIL-7Rhi MPECs formed at day 7 after DC-33 immunization survived and matured into functional memory CD8+ T cells in the spleens. Interestingly, almost 100% of KLRG1loIL-7Rhi MPECs formed following DC-33 plus LPS immunization survived to memory phase without obvious contraction. Conversely, only 9% of MPECs formed following DC-33 plus MPLA immunization survived contraction phase (Fig. 4A), suggesting that MPLA not only promoted SLEC formation but also impeded MPECs from surviving and transitioning to mature memory CD8+ T cells. To further confirm that these distinct contraction rates were CD8+ T cell–intrinsic, we next adoptively transferred the equal number of P14 effector CD8+ T cells that were generated from three groups of immunizations into naive recipients and directly assessed their magnitude of contraction following transfer. In agreement with observations mentioned above, the number of LPS-primed effector cells recovered from the spleen 4 wk later was ~4- and 50-fold more than the DC-33 alone and MPLA-primed CD8+ T cells, respectively (Fig. 3B). A similar trend was observed in the lymph nodes, although the numbers of donor cells recovered between the DC-33 alone and MPLA-primed CD8+ T cells were more comparable (Fig. 4B). Furthermore, we found that the memory CD8+ T cells generated by all three immunizations underwent similar rates of homeostatic proliferation (Fig. 4C). Lastly, we also compared the ability of these different memory CD8+ T cell populations to respond and protect against secondary infection by adoptively transferring equal numbers of P14 memory CD8+ T cells from mice immunized with DC-33 alone, DC-33 plus LPS, or DC-33 plus MPLA 1 d prior into naive mice that were subsequently infected with LCMV-clone 13. These experiments showed that all three groups of memory CD8+ T cells expanded and cleared the virus with very similar kinetics (Supplemental Fig. 3). Collectively, these findings demonstrate that the LPS-mediated augmentation of the numbers of memory CD8+ T cells formed and the protection they confer stems largely from the ability of LPS.
priming to increase the lifespan of activated CD8+ T cells following immunization rather than increasing the rates of memory CD8+ T cell homeostatic turnover or their inherent ability to respond to secondary infection.

LPS promoted memory signature gene expression and memory T cell maturation

To gain further insights into how LPS and MPLA priming qualitatively impacted memory CD8+ T cell survival and differentiation, we compared the genome-wide gene expression patterns of IL-7Rhi effector cells (i.e., MPECs) generated 7 d after DC-33 alone, DC-33 plus LPS, and DC-33 plus MPLA immunization. We identified 96 genes with highest expression variance (coefficient of variation $> 0.8$) among all three groups. We next generated a heat map that contained these 96 genes and 56 additional effector and memory signature genes derived from previously published studies (17, 41–43) (Fig. 5A), in which 12 genes (i.e., aqp9, bcl2, cdh1, fosb, prss12, xcl1, aurkb, birc5, bab1b, cdea3, cdea8, and kcnj8) were overlapping between these two lists. Manual inspection of this data set suggested that the gene expression pattern of the IL-7R hi effector cells was closely clustered between DC-33 alone and DC-33 plus LPS group, whereas the DC-33 plus MPLA group appeared more distinct from the other two (Fig. 5A). Among all three groups, several well-characterized memory signature genes were expressed at the similar levels, such as tcf7, sell, cxcr3, ccr7, and bcl6. Interestingly, some late-memory signature genes (41), such as, aqp9, prss12, and cdh1, and prosurvival genes, such as bcl2 and srp3a (encodes Sp1-2a), were preferentially expressed in the MPECs of the DC-33 plus LPS group (Fig. 5A). This suggests that LPS may accelerate memory precursor cell maturation and/or promote their long-term survival even at this late effector phase. Conversely, the IL-7R hi effector cells generated by MPLA priming not only had reduced expression of the late-memory genes, but also preferentially upregulated several terminal effector signature genes, such as birc5, cdea3, prc1, and zbtb32 (17, 41–43). To further compare
FIGURE 5. LPS promoted memory signature gene expression and memory T cell maturation. B6 mice containing a small number of naive P14 CD8\(^+\) T cells were immunized with either DC-33 alone or in combination with LPS or MPLA. KLRG1\(^{hi}\)-IL-7R\(^{hi}\) MPECs were purified by FACS sorting and their mRNA was isolated and subjected to whole-genome expression profiling using Illumina MouseWG-6 v2.0 Expression BeadChips. (A) Heat map shows gene expression of 96 probe sets with highest variance (coefficient of variation \(> 0.8\)) and 54 known memory (in red) and effector (in green) signature genes across the CD8\(^+\) T cell populations primed via DC-33 alone, DC-33 plus LPS, and DC-33 plus MPLA. Colors indicate log\(_2\) expression intensities. (B) Barcode plot shows the locations of signature genes of LPS- and MPLA-primed CD8\(^+\) T cells in the full ordered gene list ranked by the \(t\)-statistics to quantify the differential expression in LCMV-MPECs versus LCMV-SLEC. MPLA signature genes (vertical bars in top barcode) are enriched among genes upregulated in SLEC (toward the right) \((p = 8.2 \times 10^{-6})\) whereas LPS signature genes are enriched among genes upregulated in the MPEC samples (toward the left) \((p = 3.4 \times 10^{-13})\). Known memory signature genes are in red, and effector signature genes are in green.
the intrinsic properties of MPECs between LPS and MPLA priming, we took most differentially expressed LPS and MPLA signature genes to examine their enrichment in the full-ordered gene list ranked bidirectionally based on t-statistics from the comparison of LCMV-MPEC and LCMV-SLEC gene expression profiles (17, 41–43). This analysis clearly revealed a significant enrichment of the LCMV-MPEC gene signature in the IL-7Rhi cells formed by LPS priming whereas those primed by MPLA displayed significant enrichment of the LCMV-SLEC signature (Fig. 5B). Taken together, these analyses demonstrate that the differential effects of LPS and MPLA priming on memory precursor cell differentiation involve transcriptional changes that correlate with, and likely direct, the long-term fate of the effector T cells. LPS positively induced several genes associated with the enhanced longevity observed in LCMV-specific IL-7Rhi memory precursor cells, whereas MPLA induced greater expression of genes associated with terminal effector fates.

Differential cytokine milieus induced by LPS and MPLA modulate effector and memory CD8 T cell differentiation

Given that a large body of evidence has shown that inflammatory cytokines directly influence effector and memory T cell fate decisions (2, 14–17, 26, 27, 44, 45), we attempted to discover the cytokines that may contribute to the different effects of LPS and MPLA on memory CD8 T cell development. We first performed multiplex cytokine arrays on serum samples from mice that were vaccinated with DC-33, DC-33 plus LPS, and DC-33 plus MPLA at 6 and 18 h after immunization. Because previous work has clearly demonstrated that similar to MPLA, CpG-B can induce KLRG1hi terminal effector CD8 T cells (14, 17), we also analyzed mice immunized with DC-33 plus CpG-B as a pro-effector control. Among 22 cytokines and chemokines examined, we found that LPS and CpG-B elicited very different cytokine signatures. CpG-B preferentially induced IL-12 and IFN-γ (Fig. 6A, 6B), whereas LPS preferentially induced IL-6, IL-10, and IL-1β (Fig. 6C–E). Consistent with previous studies (46, 47), MPLA was a poorer trigger of inflammatory cytokines, but modest inductions of IL-12p70, IFN-γ, and IL-1β were observed (Fig. 6A, 6B, 6E).

Furthermore, LPS, MPLA, and CpG-B induced small amounts of common γ-chain cytokines IL-2, IL-7 and IL-15, and moderate, but similar, amounts of several other cytokines and chemokines, such as GM-CSF, IL-4, and IL-8 (data not shown). Collectively, this examination revealed differences in the cytokine milieu produced by different adjuvants that could contribute to their respective effects on the differentiation of different types of effector and memory CD8 T cells.

Although MPLA only triggered modest amounts of IL-12p70, we wondered whether the formation of KLRG1hi terminal effector CD8 T cells following MPLA priming was IL-12-dependent as found previously during CpG-B priming of CD8 T cells (14, 48). To this end, WT mice containing small numbers of either WT or IL-12Rβ2−/− P14 CD8 T cells were immunized with DC-33 plus MPLA, and the formation of KLRG1hi effector CD8 T cells was analyzed 7 d later. This showed that IL-12 signaling was critical for the formation of KLRG1hi terminal effector cells during priming with MPLA (Fig. 6F).

Although IL-6, IL-10, and IL-1β have been reported to promote memory T cell differentiation in certain infection or immunization models (26, 27, 49, 50), we did not observe significantly impaired memory CD8 T cell differentiation in either IL-6−/− or IL-1β converting enzyme −/− mice in comparison with WT mice following DC-33 plus LPS immunization (Supplemental Fig. 4). Because IL-10−/− mice succumbed to even low-dose LPS treatment, we were unable to test whether IL-10 was required for LPS-induced pro-memory effects on CD8 T cells. These data collectively suggested that multiple cytokines elicited by LPS might act cooperatively to regulate memory CD8 T cell differentiation, and it is possible that the ratios of IL-12 to IL-10 or IL-6 are ultimately what influences memory cell potential and development the most during LPS priming.

**LPS promoted memory T cell differentiation in an MyD88-dependent but Trif-independent manner**

Given that the above approach failed to identify a single cytokine that was responsible for the pro-memory effects of LPS priming, we considered the unique features TLR4 signaling downstream of LPS and MPLA. LPS activation of TLR4 activates both MyD88- and Trif-dependent signaling pathways (51, 52). MPLA, alternatively, induces certain conformational changes in TLR4 that predominantly activates the Trif pathway with minimal activation of MyD88 pathway (47, 53). This accounts for why MPLA provokes low levels of proinflammatory cytokines relative to LPS and has been considered a safe adjuvant for vaccines (7, 54). To dissect the roles of MyD88 and Trif in generating pro-memory signals downstream of LPS, we immunized MyD88−/− and Trif−/− mice that contained small numbers of WT P14 cells with DC-33 plus LPS. To our surprise, the development of KLRG1hiIL-7Rhi effector cells was impaired in MyD88−/− mice immunized with LPS, and rather a larger fraction of KLRG1hiIL-7Rlo terminal effector cells formed. In contrast, Trif deficiency had little effect on the formation of KLRG1hiIL-7Rhi MPECs following DC-33 plus LPS immunization (Fig. 7A). Additionally, our results further demonstrated that LPS-mediated survival of effector CD8 T cells during contraction phase was diminished in MyD88−/− mice but not in Trif−/− mice (Fig. 7B). Importantly, note that given that both the DCs and P14 cells used in the aforementioned experiments are derived from WT mice, these results demonstrate that the LPS-induced pro-memory signals are derived from bystander host cells as opposed to the donor Ag-presenting DCs. Taken together, these observations strongly indicate that activation of MyD88, but not Trif, is critical for production of signals that promote IL-7Rhi MPEC formation and long-term survival. These findings reveal previously unappreciated roles for these critical innate signaling pathways, and possibly unique qualities of the adjuvants LPS and MPLA, in generating memory CD8 T cells.

**Discussion**

Adjuvants have long been of great interest to vaccine developers. Better understanding of the molecular mechanisms of how existing adjuvants work and how they might be improved will guide better vaccine design. Although a number of TLR agonists are currently under clinical trials for use as adjuvants in vaccine development, how they influence memory CD8 T cell development has not been well studied. In this study, we used a simple vaccination approach to study particularly how different TLR ligands affect the differentiation of effector and memory CD8 T cells. Interestingly, we found that two TLR4 agonists, LPS and MPLA, had distinct effects on effector CD8 T cell differentiation and memory CD8 T cell development. LPS as an adjuvant promoted the formation of long-lived MPECs, whereas MPLA was a more potent adjuvant for stimulating the development of terminal effector cells that died following immunization. The difference between the two adjuvants could be explained in part by their intrinsic effects on the longevity of the KLRG1hiIL-7Rhi effector cells that formed following immunization. Moreover, the ability of LPS to induce greater memory cell potential in the effector cells was directly associated with changes in the transcriptome of KLRG1hiIL-7Rhi effector cells. LPS accentuated the expression of pro-memory
genetic signature in these cells, whereas MPLA promoted the expression of genes associated with terminal effector cells. These differences likely stem from variations in the inflammatory milieus produced by the two adjuvants downstream of TLR4: MyD88-dependent signals promote formation and long-term survival of MPECs, whereas Trif-dependent signals appear to promote the formation of SLECs.

The primary rationale for using adjuvants in vaccine formulation is to boost the magnitude of adaptive immunity. Recent studies have shown that larger numbers of memory CD8+ T cells provide better protection against infections, such as HIV and malaria (55, 56). One way to augment the number of memory CD8+ T cells is to increase CD8+ T cell expansion. This is based on multiple studies showing that the size of the memory CD8+ T cell pool is typically

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**FIGURE 6.** Distinct cytokine milieus produced by LPS, MPLA, and CpG-B may differentially regulate effector and memory CD8 T cell differentiation. (A–E) B6 mice containing a small number of naive P14 CD8+ T cells were immunized with DC-33 alone or in combination with LPS, MPLA, or CpG-B. Serum samples were collected 6 and 18 h postimmunization. Cytokine and chemokine production was measured by a Luminex multiplex assay. Bar graphs show the amounts of IL-12, IFN-γ, IL-6, IL-10, and IL-1β produced after immunization. (F) B6 mice containing a small number of WT or IL-12Rβ2−/− P14 CD8+ T cells were immunized with DC-33 in combination with MPLA. At day 7, the frequency of splenic Thy1.1+ P14 CD8+ T cells and their expression of KLRG1 and IL-7R were analyzed. Data shown are representative of two independent experiments.
during the contraction phase. The effect of LPS on memory CD8+ Th1.1+ P14 CD8+ T cells and their expression of KLRG1 and IL-7R A should be achieved through increased survival of effector CD8+ T cells and prolong their survival, in agreement with previous studies. 

FIGURE 7. MyD88-dependent signals downstream of LPS-TLR4 promote memory CD8 T cell formation. B6 (WT), Trif−/−, and MyD88−/− mice that contained a small number of P14 CD8+ T cells and their expression of KLRG1 and IL-7R were analyzed. (A) Line graph shows the numbers of splenic P14 CD8+ T cells at days 7 and 40 postimmunization from the four different groups of animals. Data shown are representative of two independent experiments.

proportional to the size of the clonal burst of effector T cells. Conceptually, larger numbers of memory CD8+ T cells may also be achieved through increased survival of effector CD8+ T cells during the contraction phase. The effect of LPS on memory CD8+ T cell formation appeared to both improve the expansion of effector CD8+ T cells and prolong their survival, in agreement with another previous study (57). Although LPS itself is not a candidate adjuvant for vaccines because of its ability to induce septic shock, it would be of interest to uncover the pro-memory signals derived from LPS as candidates for future vaccine design.

Given that MPLA is a low-toxicity derivative of LPS with adequate immunostimulatory properties (47), it is an attractive adjuvant for human vaccines (7, 47, 54). MPLA and LPS both signal through TLR4, but MPLA binding induces conformational changes that bias activation of the Trif-dependent pathway, whereas LPS signals through both MyD88- and Trif-dependent pathways (47). Our data show that optimal CD8 T cell memory is generated when both MyD88 and Trif are engaged simultaneously, but this concept should be extended to highlight that vaccines that incorporate multiple adjuvants to trigger a combination of innate-sensing pathways will likely yield more protective and longer lasting immunity. For example, one study illustrated that vaccination with the adjuvants alum + MPLA provided greater CD8 T cell-mediated protective immunity against influenza virus infection than vaccination with either adjuvant alone (58). Although alum may not be able to directly activate the MyD88 pathway, it can trigger the inflammasome to produce IL-1β, which could subsequently activate the IL-1R/MyD88 pathway (59–61). Our results showing reduced CD8 T cell survival after MPLA priming might also help explain why immunization with MPLA alone, without alum, provided poorer protection against influenza, despite that MPLA induced adequate primary response and CTL differentiation (58). In a separate study, vaccination with glucopyranosyl lipid adjuvant–stable emulsion, a synthetic TLR4 agonist, required both MyD88 and Trif for generating protective Th1 responses against Mycobacterium tuberculosis (62). Lastly, a third study reported that nanoparticle-based vaccines complexed with the adjuvants MPLA (TLR4) and R837 (TLR7) induced more potent and longer lived Ab responses than those containing MPLA or R837 alone, and the synergistic effects of MPLA plus R837 were dependent on simultaneous MyD88 and Trif signaling in the Ag-specific B cells (63). Taken together, these reports along with ours demonstrate that vaccines that stimulate multiple PAMP-sensing pathways in innate immune cells as well as B cells can lead to more protective long-term humoral and cellular immunity. Thus, the ground remains quite fertile for testing how various adjuvant combinations, as well as the inflammatory milieu they generate, affect both the number and quality of memory T and B cells.

Identifying pro-memory signals and understanding how they influence the types of memory T and B cells formed is a critical aspect of adjuvant research. Our results suggest that multiple cytokines downstream of the TLR4/MyD88 pathway, such as IL-6, IL-10, and IL-1β, might work in a complementary manner to promote memory CD8 T cell differentiation. Consistent with this idea, our recent work has shown that IL-10 and IL-21 cooperatively sustain memory cell potential and foster their functional maturation in a STAT3-dependent fashion during an acute viral infection (26). IL-10 can induce SOCS3 that can help insulate the CD8 T cells from signaling by “pro-effector” cytokines such as IL-12 (26). Interestingly, IL-6 also signals through STAT3, and IL-6 and IL-10 are necessary for optimal memory CD8 T cell differentiation following a noninfectious immunization or Listeria infection, respectively (26, 27, 49). Therefore, it is likely that multiple signals during LPS priming confer its pro-memory effects, and the ratio of pro-memory versus pro-effector signals (such as IL-10 versus IL-12) may play a more important role than the absolute amounts of individual cytokines in memory CD8 T cell formation. This model helps to explain why even low amounts of IL-12 can induce KLRG1th terminal effector T cell differentiation during MPLA priming when the MyD88-dependent IL-10 production is impaired.

Proper selection of adjuvants or viral vectors used to deliver the vaccine are likely to be as important as the Ags one chooses to express because the inflammatory environment induced by these different factors can vary considerably and influence the types of memory CD8+ T cells that form. For instance, generating memory T cells that home preferentially to the lung, genital or gastrointestinal tracts, or liver is likely to provide the greatest protection against influenza, HIV, or hepatitis C virus, respectively, and therefore identifying the signals that govern the differentiation and homing of these types of memory T cells and how adjuvants or viral vectors may be exploited to enhance this process is a necessary aspect that needs to be included in modern vaccine research. Additionally, although this study primarily examines the systemic effects of adjuvants on memory T cell formation, most vaccines are administrated at specific sites (e.g., oral, i.m. or intranasal). By extension of this study, future work should examine how different types of adjuvants when administered through these routes influence the microenvironment and types of effector and memory T cells generated. Despite the success of currently approved adjuvants for generating immunity to viral and bacterial infections, a tremendous need and challenge remains for improved...
adjuvants that can enhance vaccines aimed directly at generating potent T cell immunity for diseases such as HIV, hepatitis C virus, and malaria (7). By highlighting the disparate effects of adjuvants on the quality of memory CD8 T cells and long-term immunity that forms during vaccination, this study emphasizes the importance of adjuvant selection and analysis of the types of memory T cells that form because boosting effector cell expansion may not always equate with better immunological memory.

Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. TLR ligands boost effector CD8 T cell clonal expansion. Mice that contain small number of P14 CD8 T cells were immunized with DC-33 either alone or in combination with various TLR agonists. Spleens were harvested at day 7 after immunization, and antigen-specific P14 CD8 T cells were enumerated and plotted in the bar graphs.
Supplementary Figure 2. LCMV-specific primary CD8 T cell response. C57/B6 mice were immunized with DC-33 alone or in combination with LPS or MPLA. Eight weeks later, these three groups of mice along with a group of naïve mice were infected with LCMV-Clone13 (2x10^6 pfu/mouse), virus-specific D^bNP_{396-404} tetramer positive CD8 T cells at day 7 post infection was enumerated and plotted in the bar graphs.
Supplementary Figure 3. LCMV-specific CD8 T cell recall responses. Equal number of memory P14 cells (50,000/mouse) from DC-33, DC-33+LPS and DC-33+MPLA immunized mice (6 weeks post immunization) were adoptively transferred into three groups of naïve C57/B6 mice. These three groups of chimeric mice along with a group of naïve mice were infected with LCMV-Clone13 (2x10^6 pfu/mouse), virus-specific P14 cells at day 7 post infection was enumerated and plotted in the bar graphs (A). Viral titers in the serum samples were examined at day 7 post infection and plotted in the bar graphs (B).
Supplementary Figure 4. Normal memory CD8 T cell generation following DC-33+LPS immunization in absence of either IL-6 or IL-1β. Wild type, IL-6⁻/⁻ (A) or ICE⁻/⁻ (B) mice that contained a small number of P14 cells were immunized with DC-33 alone or in combination with LPS. Antigen-specific CD8 T cells from spleens at day 40 post immunization were examined, enumerated and plotted in the bar graphs.