



Using 3 TLR ligands as a combination adjuvant induces qualitative changes in T cell responses needed for antiviral protection in mice

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TLR ligands are promising candidates for the development of novel vaccine adjuvants that can elicit protective immunity against emerging infectious diseases. Adjuvants have been used most frequently to increase the quantity of an immune response. However, the quality of a T cell response can be more important than its quantity. Stimulating certain pairs of TLRs induces a synergistic response in terms of activating dendritic cells and eliciting/enhancing T cell responses through clonal expansion, which increases the number of responding T cells. Here, we have found that utilizing ligands for 3 TLRs (TLR2/6, TLR3, and TLR9) greatly increased the protective efficacy of vaccination with an HIV envelope peptide in mice when compared with using ligands for only any 2 of these TLRs; surprisingly, increased protection was induced without a marked increase in the number of peptide-specific T cells. Rather, the combination of these 3 TLR ligands augmented the quality of the T cell responses primarily by amplifying their functional avidity for the antigen, which was necessary for clearance of virus. The triple combination increased production of DC IL-15 along with its receptor, IL-15R α , which contributed to high avidity, and decreased expression of programmed death-ligand 1 and induction of Tregs. Therefore, selective TLR ligand combinations can increase protective efficacy by increasing the quality rather than the quantity of T cell responses.

Introduction

There has been an increasing global threat by the recent emergence of many viral infectious diseases, including HIV/AIDS, avian influenza, SARS, Ebola, and West Nile virus. Vaccination promises to be an effective means to provide protection and control such diseases. Live microorganisms containing protective antigens have been shown to produce high vaccine efficacy, but meanwhile, the organisms used can be harmful to the host, as most of them were originally pathogenic. Since it is the microbial components that boost vaccine responses, using the fewest of them that can generate near equivalent efficacy would be more advantageous and less risky in immune activation.

A host recognizes microorganisms through its pattern recognition receptors by specifically interacting with highly conserved constituent microbial components. TLRs are an important group of these receptors, widely expressed by various immune cells and able to induce immune responses by way of sensing different types of microbial invasion (1, 2). DCs are among the primary sensors in the TLR-mediated pathogen recognition and induction and control of adaptive immune responses against microbial infection (3–5). Development of effective adjuvants for vaccines against infectious diseases relies considerably on a better understanding of the mechanisms by which DCs can boost desired immune responses against microbial invasion (6, 7).

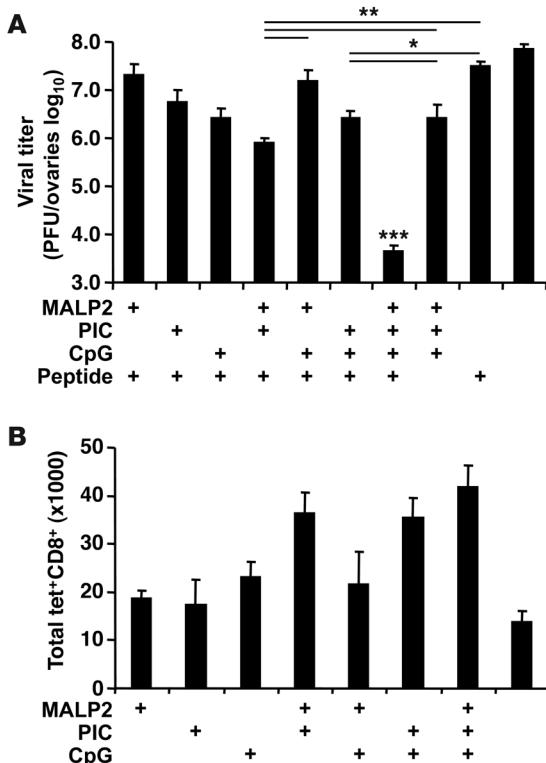
During natural infections, microbially derived TLR ligands do not often occur singly. Some of them together may be recog-

nized as a combinatorial assault and trigger more vigorous host responses, thereby preventing a considerable infection from being established. For example, bacteria may carry ligands for TLR2 (macrophage-activating lipoprotein 2 [MALP2] or lipoteichoic acid), TLR4 (LPS), TLR5 (flagellin), and TLR9 (unmethylated CpG motif-based oligodeoxynucleotide or CpG ODN). We and others have shown that certain TLRs can synergize with each other to enhance T cell-mediated immune responses through synergistic activation of DCs when their ligands are detected in pairs by DCs (5, 8–10). However, an infection does not commonly involve as few as 2 TLRs. It is intriguing to investigate how immune responses are induced by more than 2 TLR ligands and whether there are mechanistic differences between double- and triple-TLR ligand combinations in immune activation.

We previously found that double combinations MALP2+poly(I:C) or CpG+poly(I:C) [where poly(I:C) indicates polyinosinic polycytidylic acid] acted synergistically in activation of DCs and subsequent increases in numbers of activated T cells (5). Here, we demonstrate that, compared with the double-TLR combinations, immunization with an HIV peptide vaccine with the combination of all 3 ligands, MALP2+poly(I:C)+CpG, induced substantially more effective responses against viral challenge. Unlike the double combination that induced IL-12 but little IL-15 production and mostly increased the number of responding T cells, the triple-TLR combination augmented IL-15 transpresentation and induced immune factors favoring enhancement of T cell functionality and avidity, i.e., quality. Our study revealed that, whereas these double combinations of TLR ligands quantitatively expand T cell responses, the triple combination qualitatively strengthens the responses by

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inducing higher-functional avidity T cells and thus more effectively protects against viral challenge.

Results

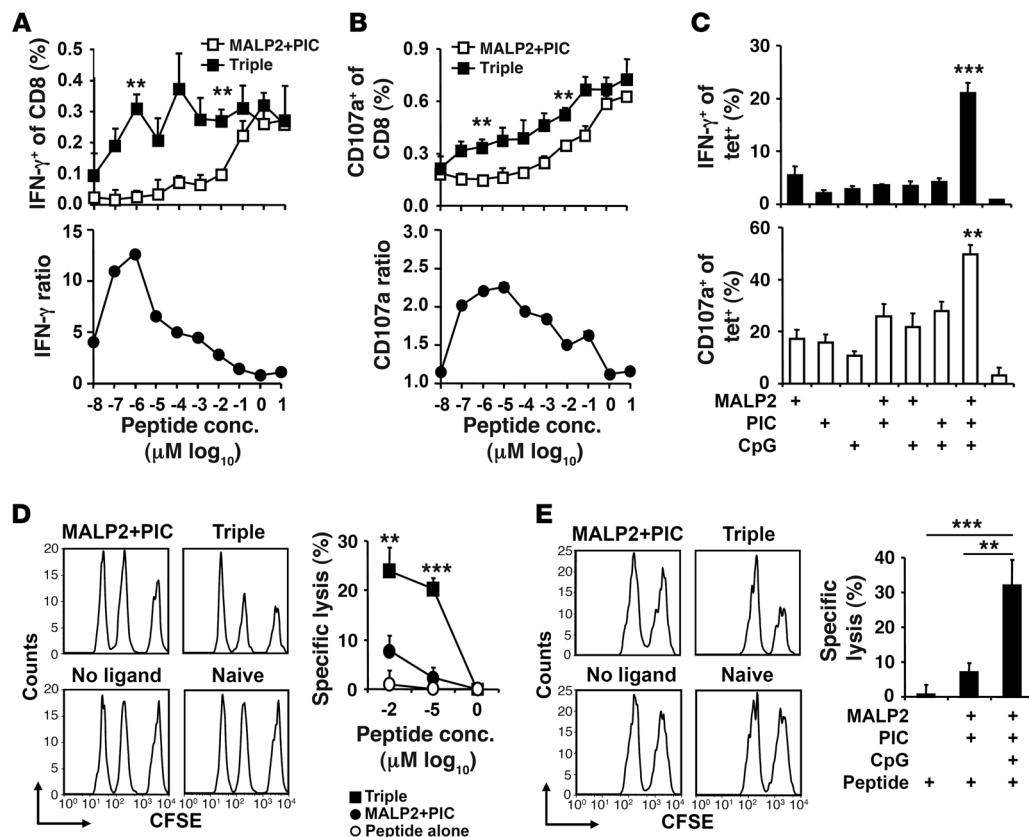
Malp2, poly(I:C), and CpG ODN in triple combination enhance protective immunity against virus challenge. We previously investigated double combinations for Malp2, poly(I:C) (denoted as PIC in figures), and CpG ODN and reported that Malp2+poly(I:C) and poly(I:C)+CpG, but not Malp2+CpG, could induce synergistic activation of DCs and T cell responses (5). Synergy was studied at doses or concentrations found to be suboptimal for each TLR ligand alone to have sufficient window to detect supra-additive responses. The mechanism was found to involve unidirectional amplification through Toll/IL-1 receptor (TIR) domain-containing adapter-inducing IFN (TRIF) of MyD88 signaling pathway-dependent DC activity, resulting in increased production of IL-12, TNF- α , and IL-6, which are essential for induction and enhancement of T cell responses. The T cell responses, particularly those of the CD8⁺ T cells, are essential in controlling viral infections. Here, we asked whether these ligands in combination act as effective immune adjuvants to enhance CD8⁺ T cell protective immunity against viral infection. This hypothesis was tested in an HIV antigen mouse immunization model using a synthetic polypeptide, PCLUS3-18IIIB, containing the HIV Env CD8⁺ CTL epitope peptide P18-I10 (presented by H-2D d) and a CD4 helper epitope comprising a cluster of overlapping helper epitopes (11). The peptides were mixed with TLR ligands in N-[1-(2,3-dioleoyloxy)propyl]-N,N,N trimethylammonium methylsulfate (DOTAP) and given to BALB/c mice intracolorectally (i.c.r.) with a 3-week interval between prime and boost, and mice were challenged with recombinant vaccinia virus vPE16 (expressing the HIV full-length Env) through the same route 4 months later (5, 12). Vaccination

Figure 1

TLR ligands in a triple combination in a peptide vaccine can markedly reduce viral load after virus challenge. **(A)** BALB/c mice were primed and boosted 3 weeks apart with an HIV Env peptide vaccine together with Malp2+poly(I:C)+CpG by the i.c.r. route (see details in Methods). Four months later, immunized animals were challenged i.c.r. with vPE16, and after 6 days, paired ovaries were recovered for virus titration with a plaque forming assay. One of 2 independent experiments with similar results is shown. Asterisk indicates the difference between groups (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $n = 5$). TLR ligand combination group is significantly different from all other groups. **(B)** Induction of antigen-specific (tetramer⁺) T cells in the draining popliteal LNs after s.c. immunization in the footpad with PCLUS3-18IIIB and TLR ligands. LN cells were recovered at 5 days and enumerated by staining for tetramer and intracellular IFN- γ . Results represent 1 of 2 independent experiments. tet, tetramer. Results are shown as mean \pm SEM.

using the combination of Malp2 and poly(I:C) (Malp2+poly[I:C]) resulted in more than a 1 log reduction in virus titers in the ovaries in contrast to vaccination with peptide alone or together with single ligands (Figure 1A) ($P < 0.01$). Poly(I:C)+CpG ODN vaccines induced a limited but statistically significant antiviral effect compared with single ligands, while Malp2+CpG ODN provided almost no improvement. These results suggested that certain double combinations acted as adjuvants to increase T cell numbers, but did not effectively suppress virus replication. However, when immunized with all 3 TLR ligands, namely Malp2+poly(I:C)+CpG ODN, mice suppressed virus replication by greater than 3 logs. Thus, the triple combination induces more effective protective immunity than any double combinations ($P < 0.001$).

The triple combination of TLR ligands increases high-functional avidity T cells. The enhanced protective immunity induced by the triple combination Malp2+poly(I:C)+CpG ODN versus Malp2+poly(I:C) was intriguing and surprising, since we previously showed CpG does not synergize with Malp2 (5). We initially thought that the enhancement could be due to a marked increase in the frequency of antigen-specific CD8⁺ T cells and thus examined P18-I10-specific CD8⁺ T cells after immunization in the footpad by s.c. injection of the PCLUS3-18IIIB peptide and TLR ligands. Despite the better protection, the number of P18-I10 tetramer-positive CD8⁺ T cells in the draining popliteal LNs at 5 days induced by the triple combination was not synergistically increased versus the double-synergistic combinations (Figure 1B). Consistent with the *in vivo* data, the number of either CD4⁺ or CD8⁺ T cells expressing the activation marker CD69 was indeed not substantially increased by the triple combination compared with the double synergistic combinations when T cells purified from naive mouse spleens were cocultured *in vitro* with BM-derived DCs (BM-DCs) pretreated with TLR ligands, as previously described (5) (Supplemental


Figure 2

The triple-TLR ligands induce high-functional avidity CD8⁺ T cells. (A and B) Mice were immunized s.c. in the footpad with PCLUS3-IIIB and TLR ligands, and the LN cells were recovered at 5 days. Cells were stained with CD107a (B) upon restimulation with P18-I10 at various concentrations and stained for intracellular IFN- γ 5 hours after restimulation (A). (C) Mice were immunized s.c. in the back flank with BM-DCs pretreated with TLR ligands and pulsed with P18-I10. Spleen cells were recovered at 1 month and restimulated with P18-I10 at 10⁻² μ M. IFN- γ and CD107a were measured 5 hours after restimulation. Values represent the percentage of tetramer⁺CD8⁺ T cells with the indicated function. Results represent 1 of 2 independent experiments with similar results. (n = 3). (D) s.c.-immunized mice received naive splenocytes as targets pulsed with different concentrations of P18-I10 at 1 month after immunization. Amounts used in histograms are as follows: left, 0 μ M; middle, 10⁻⁵ μ M; right, 10⁻² μ M. In vivo-specific lysis of transferred targets in the spleen was assayed at 5 hours. (E) Splenocytes isolated from the immune mice were restimulated in vitro with 10⁻² μ M of P18-I10 for 5 days and examined for their ex vivo 4-hour killing activity on vPE-16-infected P815 cells. Results represent 1 of 2 independent experiments and are shown as mean \pm SEM. **P < 0.01; ***P < 0.001.

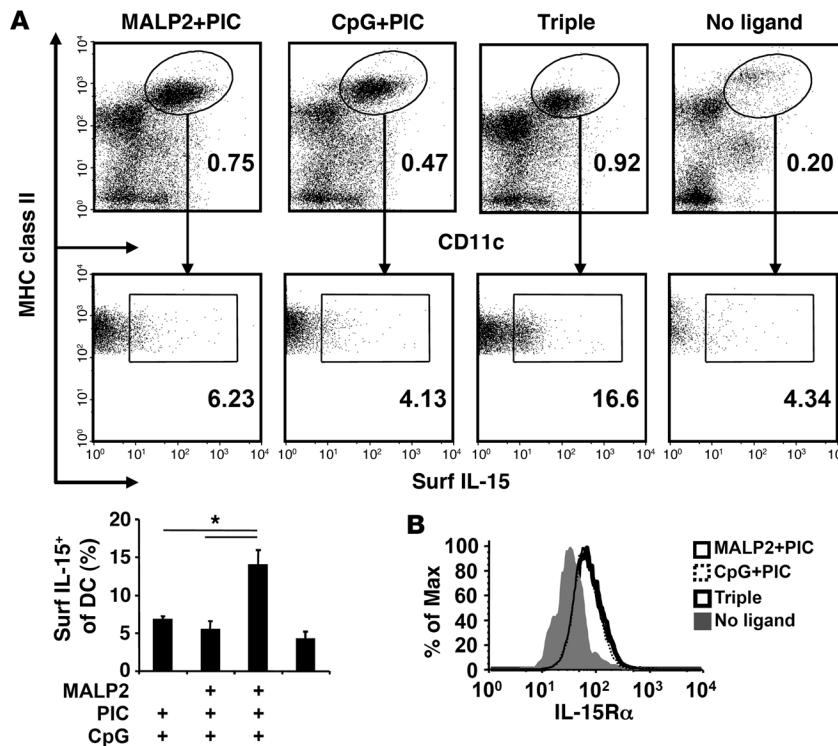
Figure 1; supplemental material available online with this article; doi:10.1172/JCI39293DS1). In fact, the triple-TLR ligand combination did not further increase BM-DC IL-12 production (Supplemental Figure 2), which probably accounted for the limited further increase in the number of activated T cells.

To sort out the contrasting findings between the protective immunity and the number of antigen-specific T cells induced, we asked whether the triple-ligand-stimulated-specific T cells differed in quality and, in particular, had a higher functional avidity than did those induced by other TLR ligand combinations. This question was addressed by measuring the specific T cell responses to a serially diluted antigen peptide vaccine component, for example, in the production of IFN- γ or cytolytic activity (13). T cells isolated at day 5 from mice vaccinated with PCLUS3-18IIB plus the MALP2+poly(I:C) combination responded well to a higher concentration range (10⁻¹ to 10¹ μ M) of the peptide P18-I10 by produc-

ing IFN- γ , but the response quickly decayed when the peptide concentration was decreased to 10⁻² μ M and lower (Figure 2A). In contrast, T cells recovered from mice vaccinated with the triple adjuvant responded not only to higher concentrations but also to lower concentrations of antigen peptide used for stimulation (Figure 2A). The majority of the cells responsive to peptide stimulation at higher concentrations were still responsive to the peptide at as low as 10⁻⁵ and 10⁻⁶ μ M, giving rise to increased ratios of responses to the triple compared with the double combinations at low antigen concentrations, indicating induction of higher-avidity T cells (Figure 2A). Similarly, the triple-ligand vaccination resulted in a greater fraction of T cells showing degranulation in response to stimulation at different peptide concentrations (Figure 2B). Based on our previous study showing that DCs are required for T cell activation by TLR ligands (5), we speculated that induction of higher-functional avidity T cells was probably still mediated through induction of more highly functional DCs by the triple combination of TLR ligands. This speculation was tested by an experiment in which

mice were immunized s.c. with TLR ligand-pretreated, P18-I10-pulsed BM-DCs. The triple-ligand-pretreated DCs induced more high-functional avidity CD8⁺ T cells (measured at 10⁻² μ M) in the spleen than did MALP2+poly(I:C)-pretreated DCs (Figure 2C and Supplemental Figure 3). Therefore, vaccination with the triple-ligand combination induces high-functional avidity CD8⁺ T cells and can achieve this effect through stimulating DCs.

We further investigated whether TLR ligand-induced higher-functional avidity T cells are also more cytotoxic. Splenocytes isolated from cognate naive mice were transferred into immune mice as target cells after being pulsed with different concentrations of peptide P18-I10 and labeled with different concentrations of CFSE. Specific lysis of the target cells pulsed with either a moderate or low concentration of peptides in the mice immunized with the triple combination was nearly equal, while in MALP2+poly(I:C)-immunized mice, targets with the moderate peptide level were less effectively lysed,

**Figure 3**

Production of IL-15 by DCs is enhanced by TLR ligands in the triple combination. Mice were immunized s.c. in the footpad as in Figure 2. At 2 days, popliteal LN cells were recovered for surface staining of IL-15 (surf IL-15) (A) and IL-15R α expression (B) and analyzed based on MHC II hi and CD11c $^{+}$ (top panels). Numbers in the top panel (A) indicate percentage of double-positive DCs (from the gate excluding lymphocytes) out of total recovered LN cells. Results represent 1 of 3 independent experiments with similar results.

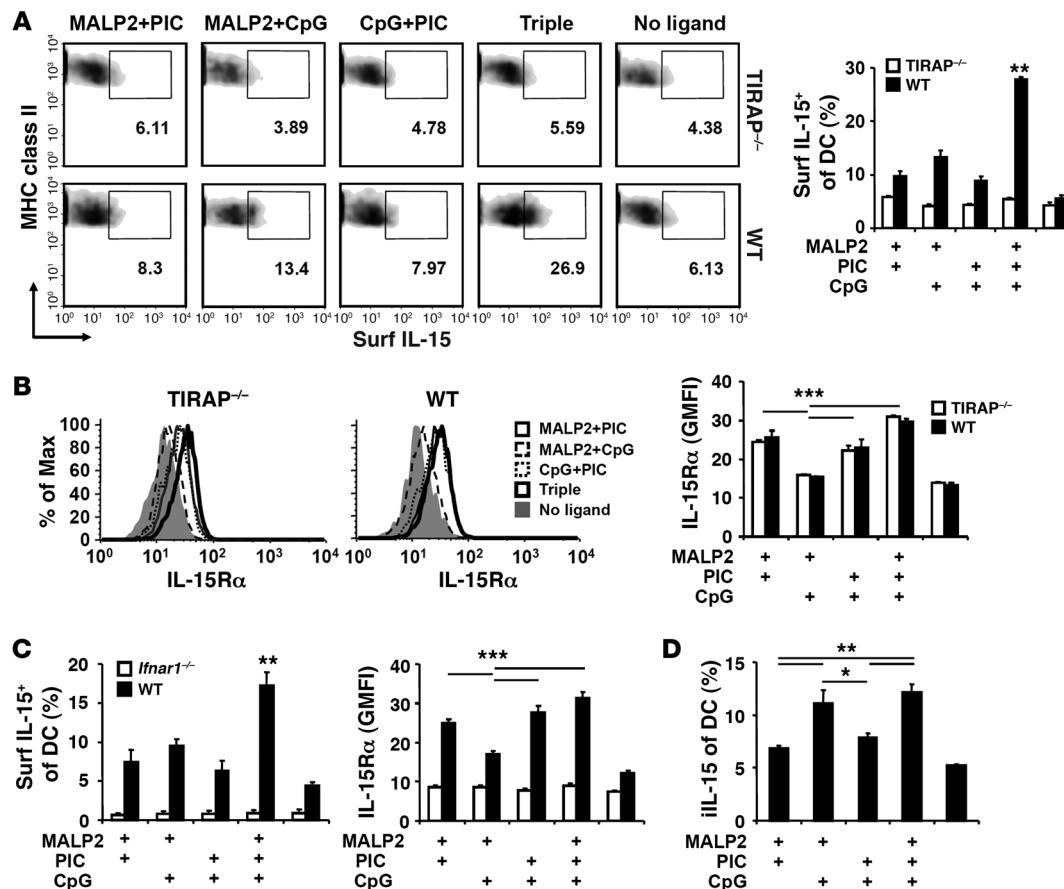
and there was almost no lysis if the peptide level was even lower (Figure 2D). To confirm that activated T cells were able to kill virus-infected targets, splenocytes isolated from the immune mice were restimulated with 0.01 μ M P18-I10 for 5 days and then cocultured in vitro with P815 cells infected with vPE-16 for 4 hours. Splenocytes in the triple-TLR-combination-immunized mice demonstrated higher ex vivo cytotoxic activity on virus-infected cells than those in the double-combination-immunized mice (Figure 2E). Thus, CD8 $^{+}$ T cells with higher cytotoxic activity are also induced after vaccination with the triple-TLR combination. This is consistent with earlier observations that higher avidity cells are more effective at clearing virus infections by several mechanisms (14, 15).

The triple combination of TLR ligands enhances IL-15 expression by DCs. IL-15 at the time of priming has been shown to be effective for the induction of high-functional avidity CD8 $^{+}$ T cells (16). To determine whether greater IL-15 production might account for the increased avidity induced by the triple combination, we measured IL-15 production from DCs, using draining LN DCs gated for CD11c and MHC class II hi expression 2 days after footpad injection with TLR ligands. The triple-ligand treatment resulted in a substantial increase in the surface expression of IL-15 (i.e., IL-15 presented in trans on its receptor, IL-15R α) on the recovered LN DCs, while neither double combination was able to enhance IL-15 as compared with no ligand treatment (Figure 3A). This marked upregulation of IL-15 was due to the cooperation of all 3 different ligands rather than increased quantity of total TLR ligand molecules because replacing CpG ODN with 1 additional dose of MALP2 did not manifest similar activity (data not shown). Further, enhanced IL-15 surface expression was not due to a higher level of IL-15R α availability, since the triple combination did not further enhance the upregulated receptor level induced by a double combination (Figure 3B).

TLRs utilize different intracellular pathways for signal transduction. Both TLR2 and TLR9 signal through MyD88, while TLR3 signals independently of MyD88. In addition, TLR signaling is associated with TIR domain-containing adapter protein (TIRAP, also known as Mal), which mediates a MyD88-independent signaling pathway. We thus investigated whether TIRAP contributes to IL-15 expression by triple-TLR-stimulated DCs. *TIRAP* $^{-/-}$ DCs had impaired IL-15 expression after stimulation with the triple-TLR combination (Figure 4A), although IL-15R α levels were not affected (Figure 4B), suggesting TIRAP is essential for boosting IL-15 presentation by the combinatorial stimulation by TLR ligands.

Conversely, it has been demonstrated that type I IFNs are required for IL-15 α expression (17) and can enhance IL-15 levels by DCs (18). Here, we found that DCs derived from *Ifnar1* $^{-/-}$ mice express neither IL-15 α nor IL-15 when stimulated with TLR ligands in combination (Figure 4C), suggesting the important role of IFN- $\alpha\beta$ and its induction of IL-15R α in facilitating IL-15 expression. As a matter of fact, MALP2 and CpG together induce more intracellular IL-15, the level of which is comparable to that of the triple combination (Figure 4D). Of note, in contrast to other double or the triple combinations, MALP2 and CpG did not upregulate IL-15R α (Figure 4, B and C). The reduced IL-15R α expression correlates with the low IFN- β production from DCs (Supplemental Figure 4A), while IL-12 augmentation was barely impaired (Supplemental Figure 4B).

The triple combination of TLR ligands prolongs DC survival. IL-15 as a pleiotropic cytokine has multifaceted functions, including enhancement of DC survival (19, 20). We thus investigated whether the triple-TLR ligands promote DC survival and protect DCs from death. BM-DCs stimulated with TLR ligands for different days in vitro were enumerated. At day 5, the number of DCs dra-


Figure 4

Enhanced IL-15 expression of the triple-TLR combination is associated with TIRAP and upregulated IL-15R α . BM-DCs were treated with the TLR ligands in different combinations for 20 hours and measured for IL-15 and IL-15R α production by MHC class II hi and CD11c $^{+}$ DCs. (A and B) Surface staining of IL-15 (A) and IL-15R α (B) on the DCs from *Tirap*^{-/-} mice in comparison with WT. Numbers indicate percentages of DCs positive for surface IL-15. (C) Surface staining of IL-15 and IL-15R α on *Ifnar1*^{-/-} DCs versus WT DCs. (D) Staining of intracellular IL-15 (iIL-15) on WT DCs. *P < 0.05; **P < 0.01; ***P < 0.001. Results are shown as mean \pm SEM.

matically declined in all other groups except for the triple-combination group, in which the change in cell numbers was marginal (Figure 5A). Of note, MALP2+poly(I:C) induced cell loss earlier (at day 3) than other groups (Figure 5A), and demonstrated markedly upregulated activated caspase-3, which was at the highest level compared with other groups (Figure 5B). In contrast, there was barely any upregulation of activated caspase-3 in the triple-ligand-treated DCs, and levels of caspase-3 were not even higher than they were in the DCs not treated with ligands (Figure 5B).

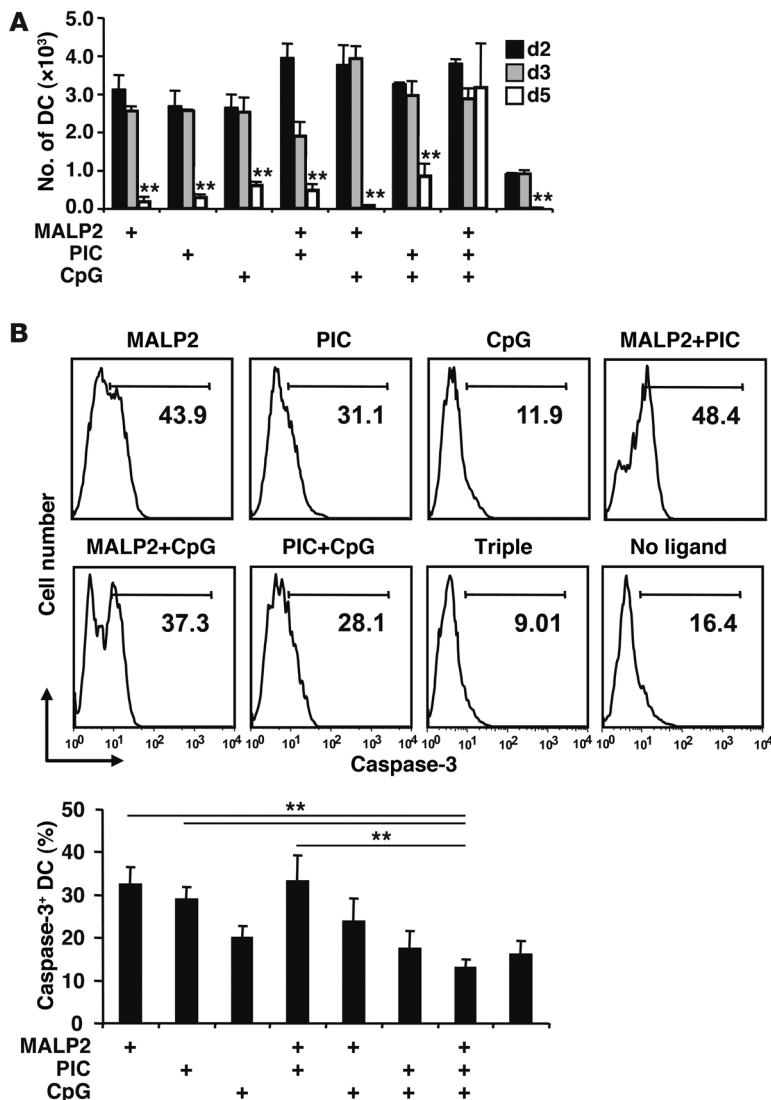
The triple-TLR combination reduces induction of Foxp3-expressing T cells. It has been reported that, while stimulating antigen-specific effector T cells, Tregs may be expanded to regulate effector T cells (21, 22). We investigated whether the combinations of TLR ligands studied above also significantly expanded Tregs. BM-DCs pretreated with TLR ligands were used to stimulate purified naive T cells. At 24 hours, single-TLR ligand-treated DCs were able to increase the number of forkhead box p3-expressing (Foxp3-expressing) CD4 $^{+}$ T cells during coculture compared with unpretreated controls (Figure 6A). The 2 double combinations, MALP2+CpG and poly(I:C)+CpG, were less potent to increase Foxp3 $^{+}$ CD4 $^{+}$ T cells, while MALP2+poly(I:C) as well as the triple combination had almost no effect. The number

of Foxp3 $^{+}$ CD4 $^{+}$ T cells fell at 48 hours in all groups, but the levels in the triple-combination group were still the lowest. The data suggest that the triple combination of TLR ligands might prevent expansion of Foxp3 $^{+}$ Treg cells and thereby improve T cell responses.

DCs treated with the triple combination downregulate expression of programmed death-ligand 1. DCs can inhibit T cell responses through the inhibitory costimulatory molecule programmed death-ligand 1 (PD-L1). (23). We further investigated the differences in PD-L1 expression between the double- and triple-TLR ligand stimulations. Whereas there were no differences in CD86 expression between DCs treated with either the double MALP2+poly(I:C) combination and the triple MALP2+poly(I:C)+CpG (Figure 6B), PD-L1 was significantly upregulated by the double-combination treatment in contrast to the triple-combination treatment, which barely stimulated PD-L1 (Figure 6B). Therefore, in comparison with the double combinations, the triple combination also prevents PD-L1 expression on DCs.

Discussion

Both human and animal studies have indicated that induction of T cell responses is pivotal in the control of many viral infections

**Figure 5**

DCs activated with the triple-TLR ligands have prolonged survival and reduced caspase-3. **(A)** BM-DCs were treated in triplicate with TLR ligands for various times as indicated. Cells were enumerated with trypan blue exclusion. Asterisks indicate a significant difference in the number of remaining DCs between day 5 and day 3 or day 2. **P < 0.01. One representative result out of 3 similar experiments is shown. **(B)** After 2 days of treatment with TLR ligands, caspase-3 was stained in BM-DCs. Numbers indicate percentage of DCs positive for caspase-3. **P < 0.02. Results represent 1 of 2 independent experiments with similar results and are shown as mean \pm SEM.

ity T cells can kill cells sooner after they are infected, when they express less viral protein but also have made less virus progeny, so killing at this early stage is more effective at aborting a virus infection; and second, that high-avidity T cells kill target cells more rapidly (15). Therefore, induction of IL-15 may result in improved CTL quality and thus account for effective vaccination with these triple-TLR ligands, enhancing protective immunity against virus challenge, in contrast to double combinations inducing less IL-15 and only limited protective effects. In addition, the presence of IL-15 during the priming phase is found to facilitate memory T cell responses (18, 31, 34, 35). This may provide an explanation for protection seen at least 3 months after immunization.

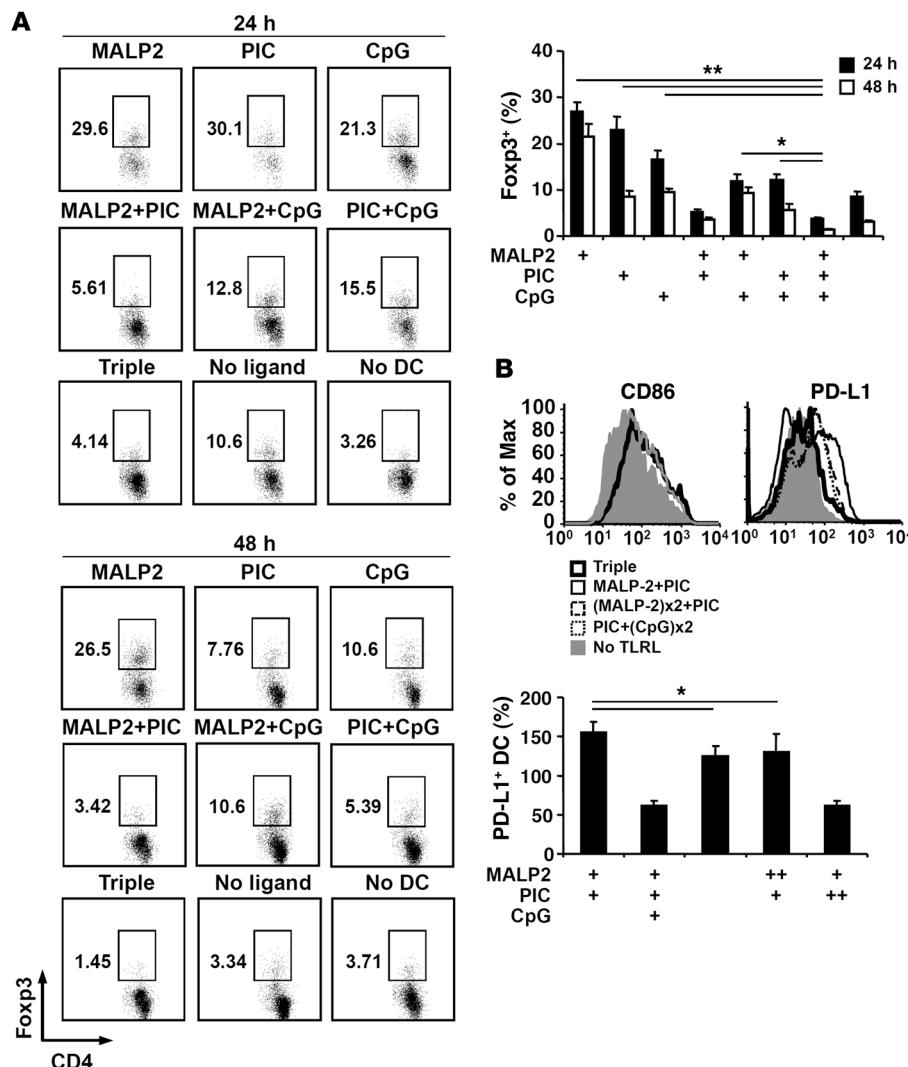
Whereas TLR2 and TLR9 utilize MyD88 for signal transduction, TLR2 (as well as TLR4), but not TLR9, also signals through TIRAP (36, 37). It has been shown that TIRAP is as essential as MyD88 for IL-15 gene activation via TLR2 stimulation (38). Here, we show that, in the absence of TIRAP, TLR-induced surface IL-15 on DCs was abolished, while IL-15R α expression was unaffected. Several lines of evidence have suggested that TIRAP differs from MyD88 in signal transduction. TIRAP mediates NF- κ B nuclear translocation independently of MyD88 in response to

(13, 24–29). The present study describes what we believe are novel T cell activation mechanisms by which the quality of immune response is enhanced by a combination of the 3 different TLRs, ligands MALP2+poly(I:C)+CpG, as opposed to double combinations that increase quantity but not quality. The triple combination induced higher-functional avidity CD8 $^{+}$ T cells against antigens as well as higher IL-15 production. IL-15 induces high-avidity T cells during the priming phase (16).

In the current study, markedly increased IL-15 expression by DCs was detected in the draining LNs at 2 days after immunization with the triple-TLR ligands, suggesting that IL-15 could be induced promptly by the triple-TLR ligands in the draining LNs for priming of memory T cells. Previous work has shown that IL-15 is important for the induction of high-functional avidity T cell responses (16, 30). It has also been convincingly shown that vaccination with IL-15 enhances CD8 $^{+}$ T cell-mediated immunity against infection (31, 32). We and others have previously demonstrated that CTLs with high functional avidity more effectively clear a virus infection *in vivo* (14, 15, 33). Two complementary mechanisms we discovered were as follows: first, that high-avid-

TLR2 and TLR4 (36, 39). TIRAP has a TNF receptor-associated factor 6-binding (TRAF6-binding) domain, which is lacking in MyD88, that can recruit TRAF6 directly for downstream signaling (39). TIRAP phosphorylation by Bruton's tyrosine kinase is an important mechanism in TLR2 (and TLR4) signaling (40). TIRAP also interacts with and is cleaved by caspase-1 for NF- κ B activation (41). Since TIRAP does not participate in TLR9 signaling, the MyD88-dependent TLR9 signaling pathway may coordinate with the MyD88-independent, TIRAP-mediated TLR2 signaling pathway to potentiate IL-15 production. Further investigation is needed to dissect their intracellular crosstalk.

IL-15 transpresentation with IL-15R α is crucial for the cytokine to be steadily presented and exert its full bioactivity on effector cells (17, 42, 43). In this study, IL-15R α expression was abrogated on *Ifnar1* $^{-/-}$ DCs stimulated with TLRs, and its expression levels correlated with IFN- β production by the stimulated DCs, suggesting IL-15R α expression is secondary to and dependent on type I IFN. Since intracellular IL-15 production by TLR stimulation is not entirely dependent on type I IFN (44), the diminished surface IL-15 found on *Ifnar1* $^{-/-}$ DCs may be attributed to the abrogation


Figure 6

The triple-TLR ligand-treated DCs prevent expansion of Treg cells and exhibit minimal upregulation of PD-L1. BM-DCs were treated with TLR ligands for 20 hours, and excess TLR ligands were removed. (A and B) Freshly isolated syngeneic splenic T cells were cocultured with TLR ligand-pretreated DCs. Foxp3⁺CD4⁺ cells were evaluated at 24 hours and 48 hours, respectively. Numbers indicate percentage of CD4⁺ T cells positive for Foxp3. (B) BM-DCs were treated with TLR ligands for 20 hours and stained with either anti-CD86 or anti-PD-L1 mAbs to measure levels of the costimulatory molecules. The experiments were repeated twice with similar results. *P < 0.05; **P < 0.01. Results are shown as mean \pm SEM.

of IL-15R α . Thus, the type I IFN to IL-15R α axis likely accounts for the inability of costimulating TLR2 and TLR9 to elevate surface IL-15, even though this combination induces IL-15 intracellularly. However, stimulation of TLR3 with either TLR2 or TLR9 could upregulate IL-15R α expression through amplification of IFN- β production. Therefore, the combinatorial stimulation of all 3 TLRs can augment not only IL-15 but also IL-15R α , enabling the cytokine to be expressed with its receptor at a greater level, inducing more high-functional avidity T cells, whereas none of the pairs of TLR ligands sufficiently upregulate both IL-15 and its receptor. High-functional avidity CTLs eliminate virus-infected cells more efficiently than low-avidity CTLs through rapid antigen recognition and target cell lysis (15) and thus are essential for the control of virus dissemination (14, 45, 46).

We found that treatment of DCs with the triple-TLR ligands prevented the DCs themselves from undergoing apoptosis. The anti-apoptotic effect may result from enhanced production of IL-15, which in turn maintains DC viability in an autocrine fashion and thereby sustains cell survival (20). Our data further indicate that the triple-TLR ligand stimulation enables DCs to limit numbers of Foxp3⁺ cells. As Foxp3⁺ Tregs can disable DC antigen presentation function (47, 48), the decrease in Foxp3⁺ cells may prevent reduc-

tion in the ability of DCs to prime high-avidity T cells. Although activation-induced Foxp3 on CD4⁺ T cells may not necessarily be an indication of prompt inhibition of effector T cells to be stimulated (49), we think that DCs stimulated with the triple-TLR ligands may limit Foxp3⁺ cells (probably their ability to expand) in order to preclude the possibility of Treg-mediated immune suppression in the first place, probably through providing IL-15 (50). Indeed, CD4⁺ T cell activation was not affected because there was no reduction in CD69 expression. Since IL-15 can abolish the PD-1/PD-L1 (where Pd-1 indicates programmed death 1) pathway-mediated inhibition of T cells (51), IL-15 may strengthen the T cell activation by restraining PD-L1 expression found in this study. Altogether, the immune system is likely to take multiple measures to ensure prolonged antigen presentation as well as sustained T cell priming to enhance desired immune responses.

We believe that there exists a hierarchical immune activation system that determines how to respond to microbial invasion during early events of infection, when pathogen-associated molecular pattern (PAMP) levels are low. A host may recognize low doses of single or some paired TLR ligands, probably as an innocuous intrusion, and may not induce robust immune responses (level 1). However, it considers certain paired TLR ligands as inimi-



cal attacks and allows expansion of antigen-specific T cells against them through activation of DCs. Such a T cell response is associated with lower functionality and diminishes rapidly over time, suggesting that this level of immune responses (level 2) is used to deal with infections likely to be quickly controlled by the host. Excessive invasion detected by recognition of a triple-TLR ligand (or greater) combination may signify a serious or worsening infection, and a higher response level (level 3) may be required for the control. However, probably due to the homeostatic limitation or a limited capacity for DCs to activate additional T cells, the effectors do not further expand at level 3. Instead, the quality of the response as measured by T cell avidity is enhanced by a mechanism that appears to involve increased DC-derived IL-15. Further, the immune system is likely to take additional measures by limiting suppressive effects to ensure prolonged antigen presentation as well as sustained T cell priming to enhance desired immune responses, thereby enhancing DC activity in immune activation. Taken together, the initial immune response, elicited immediately after infection when PAMP levels have not become elevated, might be bolstered by recognition of multiple TLR ligands. In our system, bacteria may present ligands for TLR2 (e.g., lipoteichoic acid, or MALP2), TLR4 (LPS), TLR5 (flagellin), and TLR9 (CpG), but addition to that mix of a TLR3 ligand, double-stranded RNA, may signify a combined bacterial and viral infection as in some pneumonias, calling for a more effective immune response.

In our recently conducted rhesus macaque vaccine trial for SIV (unpublished data), the triple-TLR ligand combination, together with IL-15, significantly reduced viral load after challenge with pathogenic SIV, and addition of IL-15 enhanced the protective immunity, indicating that furnishing the immune system with IL-15 is beneficial to control of virus infection by high-avidity T cells (45). The strategy might be used in vaccines for emerging infectious diseases such as SARS, avian influenza, and West Nile virus, since there is need of a CTL response to control these diseases (52–55).

In conclusion, whereas double combinations boost T cell responses by amplifying numbers, unexpectedly, a specific triple combination of TLR ligands improves T cell responses by increasing the important qualities of high functionality and avidity and long-lasting memory, which account for more effective protection against infections. In this case, quality of the T cell response is more important than the quantity of the responses to clear viral infections, and quality and quantity can be separately manipulated through selective use of TLR ligand combinations. Whether any other combinations of multiple TLR ligands also enhance the quality of T cell responses remains to be studied. Immune factors in addition to IL-15 and their possible effects on T cell differentiation also merit further investigation.

Methods

Animals and cells. Female BALB/c mice (6–8 weeks) were purchased from the Frederick Cancer Research Center (Frederick, Maryland, USA) or Taconic and housed in pathogen-free conditions in the National Cancer Institute Animal Facility. *TIRAP*^{−/−} and *Ifnar1*^{−/−} mice were generated by Shizuo Akira (37) and Daniel Portnoy (56), respectively. All animal experiments were approved by the Animal Care and Use Committee of the National Cancer Institute.

CV-1 cell lines were obtained from ATCC. Isolated mouse cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. BM cells were cultured at 7 × 10⁵/ml for 6 days in the presence of GM-CSF

(Peprotech) at a concentration of 15 ng/ml. DCs were obtained from suspension cells (5). When these cells were cocultured with purified T cells, the ratio was 1:2.5 (DC/T cells).

Vaccines, reagents, and immunization. PCLUS3-18IIIB (KQIINMWQEVG-KAMYAPPISGQIRRIQRGPGRAFVTIGK), P18-I10 (RGPGRAFVTI), and OVA_{257–264} (SIINFEKL) were synthesized by NeoMPS. TLR ligands including MALP2 and poly(I:C) were purchased from Invitrogen. Equimolar mixtures of the phosphorothioate CpG ODNs 1555 and 1466 were used as previously described (5). For in vitro experiments, MALP2, poly(I:C), and CpG ODN were dosed at 0.1 µg/ml, 25 µg/ml, and 2 µg/ml, respectively. For s.c. immunization, these ligands were mixed as 0.1 µg, 25 µg, and 2 µg per dose. For i.c.r. immunization, their doses were 0.2 µg, 50 µg, and 4 µg per injection. 20 µg or 100 µg of PCLUS3-18IIIB were used for s.c. or i.c.r. immunization, respectively. For immunization with DCs, 2 × 10⁵ DCs were stimulated with TLR ligands in vitro for 20 hours and pulsed with peptide (5 µM of P18-I10) for 2 hours. At least 3 animals or samples were included in each group and for each time point.

i.c.r. immunization was performed as previously described with modifications (29). Briefly, peptide and TLR ligands were mixed with 20 µg of DOTAP liposomal transfection reagent (Roche Diagnostic Corp.) and delivered at days 0, 1, and 2 with a polished pipette tip through the anal canal. Three weeks later, mice were boosted with the same vaccines for 3 days in a row. Recombinant replication-competent vaccinia virus vPE16 at a dose of 2 × 10⁷ PFU was used for i.c.r. challenge (29, 57).

Cell isolation and purification. Popliteal LN cells were isolated after footpad immunization. For T cell purification, spleens were removed from naive mice. Total T cells were separated by negative separation (to avoid perturbation) on an autoMACS Separator (Miltenyi Biotec) using a cocktail of antibodies against CD45R, CD49b, CD11b, and Ter-119. The purity of sorted cell populations was at least 97%.

Flow cytometry and cytokine measurements. Antibodies for flow cytometry were purchased from eBioscience or BD Biosciences. To measure intracellular IFN-γ in T cells, cells were stimulated for 5 hours at 37°C with peptide P18-I10 at various concentrations (10¹–10⁸ µM with a 10-fold serial dilution) in the presence of 1 µg/ml of brefeldin A. Anti-CD107a mAbs were added along with the peptide upon restimulation.

To measure intracellular cytokine in in vitro-cultured DCs, cells were stimulated with TLR ligands for 20 hours before staining. LN cells isolated 48 hours after peptide immunization were assayed ex vivo for intracellular cytokines. Following surface staining, cells were fixed and permeabilized and then incubated with antibodies against cytokines. Sample data were acquired on a FACSCalibur or LSR II (BD) and analyzed with FlowJo software (TreeStar Inc.).

To determine secreted cytokines and chemokines from DCs, culture supernatants were collected and measured with LINCOPlex Kits (Linc Research Inc.) on a Bio-Plex System using Luminex xMAP Technology according to the manufacturer's instructions. Supernatants were incubated with capture antibodies for 2 hours at room temperature with shaking.

In vivo and ex vivo CTL assay. The in vivo CTL assay was conducted as previously described (58) with modification. In brief, splenocytes from naive mice were pulsed with peptide at different molar concentrations (10^{−2} or 10^{−4}) or without peptide. Each of the cell populations was then labeled with 5 µM, 0.5 µM, or 0.05 µM CFSE (Invitrogen), respectively. 5 × 10⁶ cells from each target were mixed in 200 µl of PBS per recipient and transferred by i.v. injection. Transferred target cells were collected from the spleen 5 hours later for flow analysis. For the ex vivo CTL assay, splenocytes were isolated from immune mice and restimulated with 0.01 µM of P18-I10 for 5 days prior to the assay. Target cells previously infected with vPE-16 at MOI of 20 for 16 hours were labeled with 0.5 µM CFSE to differ from uninfected targets, which were labeled with 0.05 µM CFSE. Effectors and targets were



mixed at a ratio of 50:1 and incubated at 37°C for 4 hours before cytometry. The percentage of specific killing of the target cells was calculated as follows: 100 - [((% pulsed in immunized/% unpulsed in immunized)/(% peptide pulsed in naive/% unpulsed in naive)) × 100].

Virus titration. The vPE16 replicating vaccinia virus (59), a gift of P. Earl and B. Moss (National Institute of Allergy and Infectious Diseases, NIH), was recovered from paired ovaries 6 days after challenge. Tissues were homogenized in PBS with a homogenizer (POLYTRON; Kinematica Inc.). Plaque-forming assays were performed on CV-1 for 48 hours followed by counterstaining with 5% w/v crystal violet. Virus presence was expressed as total PFUs (\log_{10} PFUs)/organ.

Statistics. Comparisons between groups were analyzed by 2-tailed Student's *t* test. Analyses were performed with SPSS for Windows (SPSS). *P* of values less than 0.05 were considered statistically significant.

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