# TLR2 Engagement on CD8 T Cells Enables Generation of Functional Memory Cells in Response to a Suboptimal TCR Signal[1]

**Blandine C. Mercier,[2]*†‡ Anne Cottalorda,[2]*†‡ Charles-Antoine Coupet,*†‡ Jacqueline Marvel,*†‡ and Nathalie Bonnefoy-Bérard[3]*†‡**

**TLR are involved in the detection of microbial infection as well as endogenous ligands that signal tissue and cell damage in mammals. This recognition plays an essential role in innate immune response and the initiation of adaptive immune response. We have previously shown that murine CD8 T cells express TLR2, and that costimulation of Ag-activated CD8 T cells with TLR2 ligands enhances their proliferation, survival, and effector functions. We also demonstrated that TLR2 engagement on CD8 T cells significantly reduces their need for costimulatory signals delivered by APC. We show in this study that TLR2 engagement on CD8 T cells lowers the Ag concentration required for optimal activation, and converts a partial activation into a productive process leading to a significant expansion of cells. Using altered peptide ligands, we demonstrate that TLR2 engagement increases CD8 T cell activation and enables the generation of functional memory cells in response to a low TCR signal. This increased activation is associated with an augmented activation of the PI3K. Taken together, our results demonstrate that TLR2 engagement on CD8 T cells lowers their activation threshold for TCR signal strength and enables efficient memory cell generation in response to a weak TCR signal.** ***The Journal of Immunology,*** **2009, 182: 1860–1867.**

The CD8 T cells play a key role in the control of viral or intracellular bacterial infections and subsequent protection against these pathogens, but also in the control of tumor growth. The means of improving CD8 T cell activation and memorization, however, still remain an important challenge in the field of infectious diseases and cancer.

Following TCR stimulation, the fate of naive CD8 T cell and their differentiation into memory cells result from the integration of different parameters, such as the following: 1) TCR signal strength; 2) the presence or absence of costimulatory signals; and 3) the cytokine environment (1). TCR signal strength is partly controlled by the quantity of available peptide presented by MHC molecules, and it has been shown that a T cell response is induced when the number of TCR triggered reaches an appropriate threshold (2). TCR signal strength is also determined by the nature of the TCR ligand, i.e., the peptide-MHC complex. A single amino acid exchange in a given antigenic peptide can alter its affinity for MHC molecules or TCR, and it has been demonstrated that the affinity of altered peptide is correlated with the MHC-peptide-TCR complex $t_{1/2}$, and thus, with the amplitude of the T cell response (3–5). TCR signal strength is thus determined by at least two different parameters: the quantity of available TCR ligands in the environment and peptide affinity toward MHC molecules or TCR.

Furthermore, costimulatory signals are required to induce an efficient T cell activation. Indeed, it has been shown that CD28 engagement on T cells makes them more sensitive to Ag stimulation by decreasing their activation threshold (2). CD28 engagement can also enable full activation in the presence of a partial agonist, suggesting that CD28 acts as an amplifier of the TCR signal (6, 7).

As for CD28, TLR might act as costimulatory receptors for T cells. TLR recognize damage-associated molecular patterns, such as pathogen-associated molecular patterns conserved in different classes of microorganisms (8), but also endogenous alarmins that signal tissue and cell damage (9). TLR were initially found to be involved in the innate immune response and the initiation of adaptive immune response. More recently, we and others have described that T cells express certain TLR (10–14) and that TLR ligands can directly modulate T cell responses. It has been shown that ligands for TLR2, TLR5, and TLR8 modulate the proliferation and suppressive functions of $CD4^+CD25^+$ regulatory T cells (15–18), and that ligands for TLR2, TLR3, TLR4, TLR5, and TLR9 enhance the proliferation and/or the effector functions of conventional T cells (10–14, 19, 20). In particular, we have previously demonstrated that costimulation of Ag-activated murine CD8 T cells with the lipopeptide $Pam_3CysSK_4$ (Pam),[4] a TLR1/2 ligand, enhances the proliferation, survival, and effector functions of these cells. We also reported that TLR2 engagement on CD8 T cells significantly reduces their need for costimulatory signals usually

*Université de Lyon, Lyon, France; †Institut National de la Santé et de la Recherche Médicale, Unité 851, Lyon, France; and ‡Université Lyon1, Lyon, France

Received for publication April 9, 2008. Accepted for publication December 8, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by institutional grants from Institut National de la Santé et de la Recherche Médicale and Université Claude Bernard Lyon I, and additional support from Association pour la Recherche sur le Cancer, Rhône-Alpes Region, and Cancéropole National. B.C.M. and C.-A.C. are supported by a fellowship from the French Education and Research Ministry, and A.C. was supported by a fellowship from the French Education and Research Ministry and currently by a fellowship from the Association pour la Recherche sur le Cancer.

[2] B.C.M. and A.C. made equal contribution to this work.

[3] Address correspondence and reprint requests to Dr. Nathalie Bonnefoy-Bérard, Institut National de la Santé et de la Recherche Médicale, Unité 851, 21 Avenue Tony Garnier, 69365 Lyon Cedex 07, France. E-mail address: nathalie.bonnefoy-berard@inserm.fr

[4] Abbreviations used in this paper: Pam, $Pam_3CysSK_4$; DC, dendritic cell; GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; MALP-2, macrophage-activating lipopeptide-2; NP, nucleoprotein; PKB, protein kinase B; KLRG1: killer cell lectin-like receptor G1.



www.jimmunol.org/cgi/doi/10.4049/jimmunol.0801167

delivered by mature APC (10). Thus, TLR ligands could directly enhance CD8 T cell responses, and therefore facilitate antiviral or antitumoral responses. In this context, TLR ligands offer new perspectives for the immunotherapeutic manipulation of T cell responses. In contrast, they could favor the activation of autoreactive CD8 T cells, leading to the emergence of autoimmune diseases. A precise understanding of their direct effects on T cells, and especially on CD8 T cells, is thus essential to anticipate adverse effects associated with their use in therapeutic protocols.

In this study, we addressed the capacity of TLR2 engagement on CD8 T cells to lower the TCR signal strength required for their activation. We tested whether costimulation with TLR2 ligands could drive CD8 T cell differentiation into memory cells in conditions that would normally lead to partial activation. We first tested whether engagement of TLR2 on CD8 T cells could lead to efficient proliferation, expansion, and differentiation into memory cells in the presence of low doses of antigenic peptide or in the presence of peptide-MHC molecule complexes that engage TCR with a low affinity. To address this question, we used the well-defined $F_5$ TCR transgenic model and analyzed in vitro and in vivo Ag-specific CD8 T cell responses to full agonist peptides or altered peptide ligands. Our results demonstrate that TLR2 engagement on CD8 T cells lowers their activation threshold for TCR signal strength and converts a partial activation into the generation of functional memory cells. This effect is associated with an increased activation of the PI3K/protein kinase B (PKB) signaling pathway.

## Materials and Methods

### *Mice*

$F_5$ TCR transgenic mice that express a TCR recognizing an H2-$D^b$-restricted A/NT/60/68 influenza virus nucleoprotein (NP) epitope (NP68) (21) were a gift from D. Kioussis (National Institute for Medical Research, London, U.K.), and TLR2$^{-/-}$ mice were obtained from S. Akira (Research Institute for Microbial Diseases, Osaka, Japan). C57BL/6 mice were purchased from Charles River Laboratories. All mice were bred at the Plateau de Biologie Expérimentale de la Souris (Experimental Murine Biology Platform) in specific pathogen-free conditions. Experimental procedures were submitted for approval of an institutional review board, Regional Animal Experimentation Ethics Committee.

### *Peptides*

The full agonist NP68 peptide (366–374) (ASNENMDAM) was used to activate CD8 T cells from $F_5$ mice (D. Fischeux, Institut de Biologie et Biochimie des Protéines, Lyon, France). Cells were also activated with partial agonist peptides NP3R (ASRENMDAM) and NP4Q (ASNQN MDAM) (D. Fischeux, Institut de Biologie et Biochimie des Protéines, Lyon, France) (22). These altered peptide ligands were generated by mutating the antigenic peptide in amino acid positions predicted to interact with the TCR (NP4Q) or the MHC-I groove (NP3R) (23).

### *Cell preparation*

CD8 T cells from spleens and lymph nodes of $F_5$ TCR Tg mice were first purified by magnetic beads using negative selection, and were then stained with CD8 and CD44 mAbs (BD Pharmingen) and FACS sorted according to CD8 and CD44$^{low}$ expression using FACSVantage SE option Diva (BD Biosciences). The purity of isolated CD8 T cell was typically greater than 99%. Bone marrow cells obtained from TLR2$^{-/-}$ mice were resuspended at $1 \times 10^6$ cells/ml in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 40 $\mu$g/ml gentamicin (Invitrogen Life Technologies), and 50 $\mu$M 2-ME (Sigma-Aldrich), and cultured for 10 days in the presence of 0.2 $\mu$g/ml human rFlt3 ligand (TEBU) at 37°C. Following differentiation, 95% of cells were CD11c$^+$, and among them 80% were B220$^-$ CD11b$^+$. Peptide pulse and dendritic cell (DC) activation were conducted on day 10 of the culture by addition of 100 nM peptide and 250 ng/ml *Salmonella minesota*-derived LPS (Sigma-Aldrich).

### *CFSE labeling*

To follow cell division, cells ($1–2 \times 10^7$ cells/ml) were incubated with 0.75 $\mu$M 5,6 CFSE (Molecular Probes) for 12 min at 37°C. They were then washed twice and resuspended in culture medium or in PBS (Invitrogen Life Technologies).

### *Cell culture*

CD8 T cells from $F_5$ mice were cultured at $1 \times 10^6$ cells/ml in complete RPMI 1640 medium in the presence of a dose range of the full agonist NP68 peptide or partial agonist NP3R and NP4Q peptides, with or without 2 $\mu$g/ml Pam (EMC Tübingen).

Lymphoblasts were obtained by resuspending spleen cells from $F_5$ TCR transgenic mice at $0.67 \times 10^6$ cells/ml in complete DMEM supplemented with 6% FCS and cultured for 5 days in the presence of 10 nM NP68 peptide and 4 ng/ml IL-2 (homemade supernatant). Cells were then washed and maintained for 6 days in the presence of 4 ng/ml IL-2. Following these steps, the purity of CD8 lymphoblasts was greater than 98%. Lymphoblasts were then activated for 30 min or 1 h with a dose range of NP3R or NP68 peptide alone or in combination with Pam (10 $\mu$g/ml), macrophage-activating lipopeptide-2 (MALP-2; 1 $\mu$g/ml; Alexis), or LPS (10 $\mu$g/ml; Sigma-Aldrich).

### *Adoptive transfer of activated CD8 T cells*

CD8 T cells ($2.3 \times 10^5$ cells/ml) were activated with 100 nM NP3R in presence of irradiated TLR2$^{-/-}$ spleen cells ($7 \times 10^5$ cells/ml), with or without Pam. At 72 h postactivation, viable cells were isolated after Ficoll separation and, for each condition, $6 \times 10^6$ activated cells were transferred to C57BL/6 mice by i.v. injection.

### *Adoptive transfer of naive CD8 T cells and immunization protocol*

Following CFSE staining, naive CD8 T cells from $F_5$ TCR transgenic mice were resuspended in PBS, and each mouse i.v. received a total of $2 \times 10^6$ cells. One day after adoptive transfer, 100 nM NP3R peptide was i.v. administrated in 200 $\mu$l of PBS. Controle mice received 200 $\mu$l of PBS only. Mice also received injections of 20 $\mu$g of Pam or PBS over 3 consecutive days. Analyses were performed 3 days after activation.

### *Flow cytometry analysis of in vitro CD8 T cell responses*

Forty-eight hours postactivation, CD8 T cells were stained with PE-cyanine 5- or PerCP-cyanine 5.5-coupled anti-CD8$\alpha$, anti-CD25 PE, anti-CD44 FITC, and anti-CD122 PE (BD Pharmingen) mAbs for 30 min at 4°C. After rinses, the cells were resuspended in 300 $\mu$l of PBS containing 2% BSA (10 g/L) and 0.2% $NaN_3$. Cells were analyzed on a FACSCalibur or a FACSCanto cytometer (BD Biosciences).

### *Flow cytometry analysis of in vivo CD8 T cell responses*

Lymph node cells (pool of inguinal, axillary, and lateral axillary lymph nodes) from C57BL/6 or TLR2$^{-/-}$ recipient mice were collected at different times after immunization. A total of $2 \times 10^6$ cells was stained with anti-CD8 PerCP-cyanine 5.5 in combination with anti-CD44 FITC or allophycocyanin, anti-CD127 PE, and anti-CD62L allophycocyanin and biotinylated anti-CD122, anti-Ly6C, and anti-killer cell lectin-like receptor G1 (KLRG1) mAb, as described above. Biotinylated mAb were revealed by incubating cells with allophycocyanin-coupled streptavidin (BD Pharmingen). For intracellular cytokine detection assays, $1 \times 10^6$ lymph node cells from C57BL/6 recipient or TLR2$^{-/-}$ mice were incubated in 96-well plates with 0.67 $\mu$l/ml GolgiStop (BD Pharmingen) and 10 nM NP68 or NP3R peptide for 5 h. After incubation, surface staining with anti-CD8$\alpha$ PE-cyanine 5 was conducted for 20 min at 4°C. After one wash, the cells were permeabilized for 20 min in Cytofix-Cytoperm (BD Pharmingen) and washed with Perm/Wash Buffer (BD Pharmingen). After one wash, anti-IFN-$\gamma$ PE (BD Pharmingen) was added to the permeabilized cells and left overnight at 4°C. The cells were then washed once with PBS and analyzed by FACS. Memory CD8 T cells were detected using NP68-H-2-$D^b$ dextramer (DakoCytomation). Dextramer staining was conducted for 30 min at 4°C. The number of $F_5$ CD8 T cells was calculated using the percentage of CFSE$^+$ $F_5$ CD8 T cells (primary response) or dextramer-positive $F_5$ CD8 T cells (memory phase). Total cell number found in the collected lymph nodes was determined with the formula (total number of viable cells $\times$ the percentage of CFSE- or dextramer-positive $F_5$ CD8 T cells).

### *In vivo cytotoxic assay*

This assay was conducted as previously described (24). NP68- or NP3R-pulsed mature DC were transferred to C57BL/6 mice that had received activated $F_5$ CD8 T cells 30 days previously. Twenty-four hours later, targets were prepared from a suspension of C57BL/6 spleen cells that was divided into two populations. One was pulsed with 1 $\mu$M NP68 or NP3R

peptide for 90 min at 37°C, washed, and labeled with a high CFSE concentration (10 $\mu$M). The control population was cultured without peptide for 90 min at 37°C, washed, and labeled with a low CFSE concentration (1 $\mu$M). After this, $10^7$ peptide-pulsed $CFSE^{high}$ and $10^7$ $CFSE^{low}$ cells were mixed and injected i.v. into the recipient C57BL/6 mice. After 18 h, spleen cells were collected and analyzed by flow cytometry to determine the proportion of CFSE-labeled target cells. The percentage of specific lysis was calculated according to the following formula: 1 − (ratio control/ratio transferred) × 100. Ratio control = percentage of $CFSE^{low}$/percentage of $CFSE^{high}$ cells remaining in mice that had only received target cells, and ratio transferred = percentage of $CFSE^{low}$/percentage of $CFSE^{high}$ cells remaining in mice that had been transferred with naive or activated cells.

### *Western blot analysis*

Whole-cell lysates were prepared by lysing 10–20 × $10^6$ cells in 50 $\mu$l of Nonidet P-40 lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, and 1% Nonidet P-40) supplemented with protease inhibitor mixture (Sigma-Aldrich) and phosphatase inhibitors (300 mM NaF and 200 mM $Na_3VO_4$). Protein extracts (60–100 $\mu$g) were separated on a NuPage 4–12% Bis-Tris gel (Invitrogen) and transferred to nitrocellulose membrane (Invitrogen). Protein expression was analyzed by Western blotting using specific Abs against phosphorylated or total PKB rabbit mAbs (Cell Signaling Technology) and actin mAb (Sigma-Aldrich). Protein-Ab complexes were visualized by chemiluminescence (Western Lighting Chemiluminescence Reagent Plus; PerkinElmer).

### *Statistical analysis*

We used Student's two-tailed unpaired *t* test to analyze the significance of the phenomena described in the different experimental conditions indicated in the figures.

## Results

### *TLR2 engagement on CD8 T cells lowers the quantity of Ag required for their activation*

We previously reported that TLR2 engagement on CD8 T cells enables CD25 expression and expansion of cells that have been activated with nonmatured DC (10). We also observed that engagement of TLR2 on CD8 T cells reduces the quantity of mature DC required for a given response by 80% (our unpublished results). These results strongly support the hypothesis that TLR2 engagement on CD8 T cells reduces their need for costimulatory signals usually delivered by mature DC. Mature DC, however, generate numerous signals that participate in CD8 T cell activation, and variations in the DC:T ratio correspond not only to variable levels of costimulation and cytokine production, but also to variable quantities of peptide-MHC complexes presented to CD8 T cells.

To better characterize the role of TLR2 in CD8 T cell activation, we analyzed in this study whether TLR2 engagement on CD8 T cells could modulate the TCR signal strength required for their activation. To this end, we used a model in which antigenic peptides are mutually self-presented by CD8 T cells (25). This enables a precise control of signals that are delivered to CD8 T cells. Naive CD8 T cells were FACS sorted from $F_5$ TCR transgenic mice and activated with a dose range of the full agonist NP68 peptide. Without any peptide, CD8 T cells neither proliferated nor expressed CD25 (Fig. 1, *A* and *B*). In the presence of NP68 alone, the percentage of cells undergoing cell division increased with peptide concentration, varying from 73% with 1 nM NP68 to 91% with 1000 nM NP68, at 48 h. Regardless of the concentration, however, NP68 peptide alone induced neither high CD25 expression nor effective cellular expansion (Fig. 1, *B* and *C*). Therefore, in the absence of any additional signal, stimulation with peptide alone induced a partial activation. In contrast, addition of the TLR2 ligand Pam to cell cultures led to the proliferation of more than 95% of cells, even in the presence of the lowest NP68 dose tested (1 nM) (Fig. 1*A*). Moreover, the presence of TLR2 ligands not only induced proliferation of all CD8 T cells, but also led to an efficient

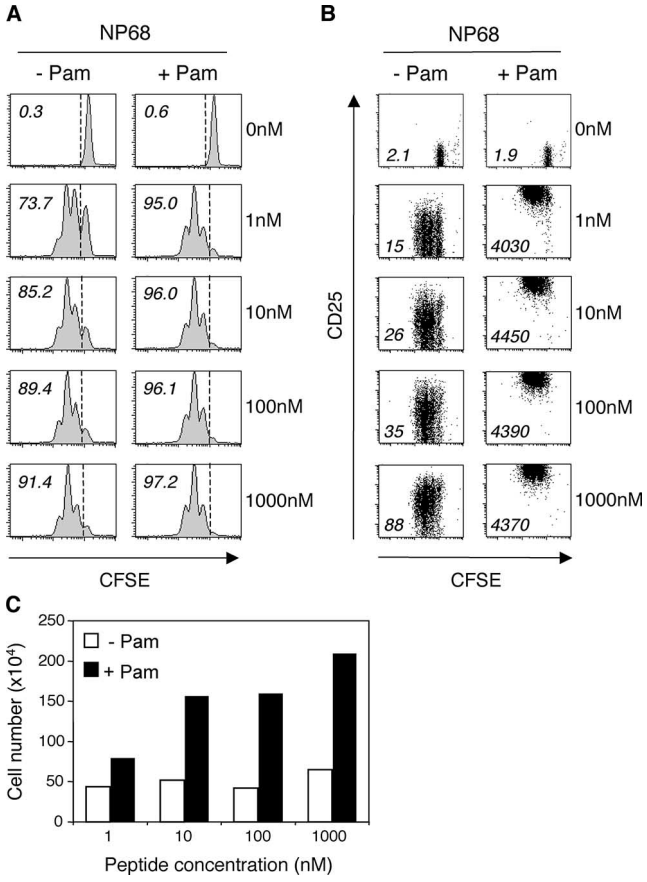


**FIGURE 1.** TLR2 engagement on CD8 T cells lowers the activation threshold for Ag concentration. Naive CD8 T cells from $F_5$ TCR transgenic mice were kept unstimulated or were activated with a dose range of NP68 peptide alone or in combination with Pam. Cells were labeled with CFSE before culture and, at 48 h, CFSE staining (*A*) and CD25 expression (*B*) were analyzed by flow cytometry. Numbers in the histograms indicate the percentage of cells that underwent cell division, and numbers in dot plots indicate the geometric mean fluorescence intensity for CD25. *C*, Viable cell numbers were measured by flow cytometry using Calibrite allophycocyanin beads as standard. Results are representative of three independent experiments.

activation, with high CD25 expression and significant cell accumulation (Fig. 1, *B* and *C*). Thus, TLR2 engagement on CD8 T cells decreases the Ag concentration required for their activation and converts an unproductive proliferation into an efficient expansion.

### *TLR2 engagement on CD8 T cells leads to effective activation in presence of a weak TCR signal*

Peptide affinity for the MHC groove or for the TCR is another important parameter that determines TCR signal strength. We therefore asked whether TLR2 engagement could also impact on the response of CD8 T cells following their stimulation with low-affinity peptides. We previously used the full agonist NP68 peptide, but in the following experiments, we also used the altered peptide ligands NP4Q and NP3R, which have been mutated at amino acid positions predicted to interact with the $F_5$ TCR (NP4Q) or the MHC-I groove (NP3R) (23). Thus, NP4Q and NP3R peptides form unstable interactions with the TCR and MHC molecules. We observed in this study that equal concentrations of NP4Q and NP3R peptides alone led to lower proliferation rates than those observed with the full agonist NP68 peptide. Interestingly, we did not detect any daughter cell in the presence of 100

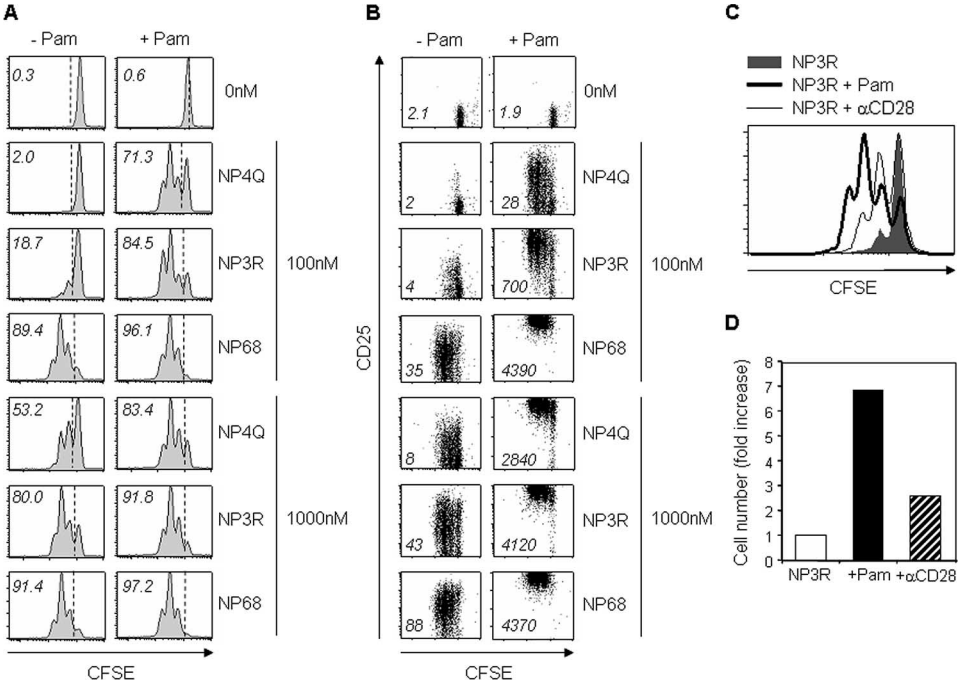


**FIGURE 2.** TLR2 engagement on CD8 T cells enables an effective activation in presence of a weak TCR signal. Naive CD8 T cells from $F_5$ TCR transgenic mice were kept unstimulated or were activated in the presence of altered peptide ligands NP3R or NP4Q (100 and 1000 nM) alone or in combination with Pam or CD28 (2 $\mu$g/ml). Cells were labeled with CFSE before culture, and CFSE staining (*A* and *C*), CD25 expression (*B*), and viable cell numbers (*D*) were analyzed by flow cytometry at 48 h. Numbers in histograms indicate the percentage of cells that underwent cell division, and numbers in dot plots indicate the geometric mean fluorescence intensity for CD25. Results are representative of three independent experiments.

nM NP4Q peptide alone, whereas, in the presence of NP4Q plus Pam, more than 70% of the cells had undergone cell division. Similarly, the addition of Pam strongly increased the percentage of daughter cells following stimulation with 100 nM NP3R peptide (18.7 vs 84.5). Moreover, we observed that CD8 T cell proliferation induced by 1000 nM NP3R in the presence of Pam was similar to that observed with 100 or 1000 nM NP68 alone, with more than 90% of the cells undergoing cell division (Fig. 2*A*). These experiments also confirmed that TLR2 engagement on CD8 T cells strongly increased CD25 expression (Fig. 2*B*).

We previously demonstrated that costimulatory signals delivered by TLR2 were at least as efficient as those delivered by CD28 for driving the expansion and effector functions of NP68-activated CD8 T cells (10). Furthermore, it was shown that CD28 engagement allows lymphocyte activation in the presence of weak agonists (6, 7). This led us to compare how engagement of these two receptors affects cellular responses following NP3R peptide stimulation. As expected, CD28 engagement increased the percentage of cells that had undergone cell division and led to a more efficient accumulation of cells in comparison with stimulation with NP3R alone. We also observed that TLR2 engagement resulted in a higher cell proliferation and expansion than CD28 costimulation (Fig. 2, *C* and *D*). Taken together, these data demonstrate that TLR2 engagement, like CD28 engagement, leads to an effective activation in response to a weak TCR signal.

*TLR2 engagement on CD8 T cells induces PKB phosphorylation*

PI3K plays a key role in the signaling cascade leading to T cell activation, downstream from both the TCR and CD28 molecules (26). Recently, it was clearly demonstrated that the costimulatory effect of TLR9 ligands on murine CD4 T cells is mediated through a MyD88-dependent PI3K signaling pathway (27). Whether the TLR2 costimulatory effect on CD8 T cells could also be associated with molecular events linked with the PI3K signaling pathway remained, however, to be determined. PI3K had already been demonstrated to be recruited and activated following triggering of human TLR2 in THP-1 cell line (28). We therefore questioned whether TLR2 engagement on CD8 T cells could activate the PI3K pathway. Because PKB is a primary target of PI3K, we first analyzed the effect of TLR2 engagement on the phosphorylation status of PKB in peptide-activated CD8 T cells. We observed no PKB phosphorylation in FACS-sorted naive CD8 T cells, whereas it was induced within 30 min following activation with NP68 peptide alone and was increased further in the presence of Pam (Fig. 3*A*). These results were confirmed using an Ab specific for phospho-PKB substrates (RXRXXS/T) (our unpublished results). To obtain higher quantities of cell lysate material to conduct dose-response experiments, we used lymphoblasts derived from $F_5$ TCR transgenic mice. In agreement with the above observations in naive CD8 T cells, the

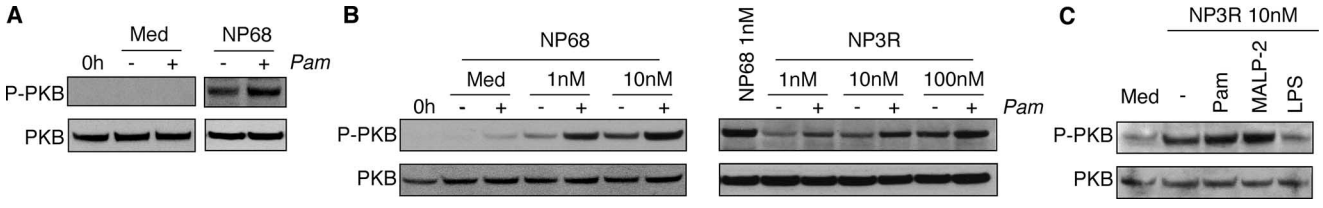


**FIGURE 3.** TLR2 engagement on CD8 T cells increases activation of the PI3K/PKB signaling pathway. *A*, FACS-sorted naive CD8 T cells were activated with or without 10 nM NP68, alone or in the presence of Pam. *B*, Lymphoblasts were obtained, as described in *Materials and Methods*, and were stimulated by a dose range of NP68 or NP3R, with or without Pam (10 $\mu$g/ml), MALP-2 (1 $\mu$g/ml), or LPS (10 $\mu$g/ml). *B* and *C*, After 30 min, phospho-PKB (P-PKB) and total PKB expressions were determined by Western blot. Results are representative of two (*A* and *C*) or three (*B*) independent experiments.

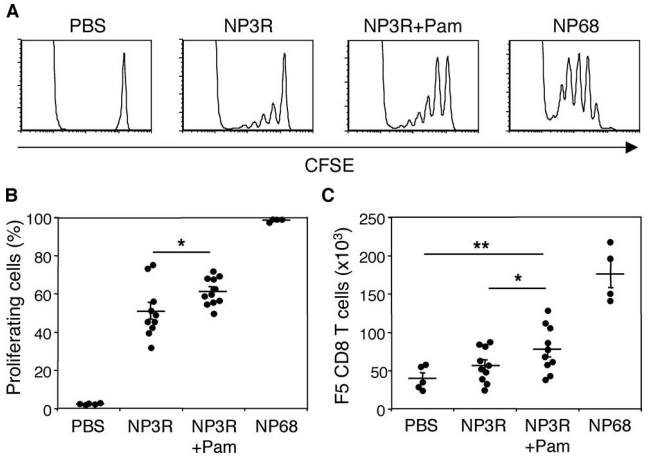


**FIGURE 4.** TLR2 acts as a costimulatory receptor for CD8 T cells in vivo. Naive CD8 T cells from $F_5$ TCR transgenic mice were FACS sorted and labeled with CFSE. A total of $2 \times 10^6$ cells was then adoptively transferred to TLR2$^{-/-}$ recipient mice. One day later, mice were immunized with 100 nM NP3R or NP68. A group of control mice received 100 $\mu$l of saline instead of peptide. Immunized mice also received 20 $\mu$g of Pam or PBS 3 consecutive days. Three days after immunization, lymph node cells were isolated and CFSE staining was analyzed by flow cytometry. *A*, CFSE histograms are gated on total CD8$^+$ cells. *B*, The percentage of proliferating cells corresponds to the percentage of cells that underwent cell divisions. Dots represent individual mice, and bars indicate arithmetic means. *C*, The number of $F_5$ CD8 T cells was calculated, as described in *Materials and Methods.* Results were obtained from two independent experiments. *, $p < 0.05$; **, $p < 0.01$.

addition of Pam strongly increased PKB phosphorylation in lymphoblasts, for all NP68 peptide concentration used (Fig. 3*B*, *left panel*). Similar observations were made after stimulation with the NP3R peptide (Fig. 3*B*, *right panel*). The effect of TLR2 engagement on PKB phosphorylation was confirmed using MALP-2, another TLR2 ligand (Fig. 3*C*). All of these data clearly demonstrate that TLR2 engagement on CD8 T cells increases PKB phosphorylation, suggesting that at the molecular level, the costimulatory effect of TLR2 ligands on CD8 T cells might be linked to an increased activation of the PI3K/PKB signaling pathway.

### *TLR2 engagement on CD8 T cells increases their activation in vivo*

We next addressed the direct effects of TLR2 engagement on CD8 T cells in vivo. Freshly isolated and CFSE-labeled naive $F_5$ CD8 T cells were adoptively transferred to TLR2$^{-/-}$ mice. This ensured that transferred $F_5$ CD8 T cells were the only TLR2 ligand-responsive cells. Recipient mice were subsequently challenged i.p. with NP68 or NP3R peptides alone or with Pam. Mice from the control group received one i.p. injection of PBS instead of the challenge, and all mice received injection of Pam or PBS on 3 consecutive days. Three days after priming with the full agonist NP68 peptide, we observed that 100% of the Ag-specific CD8 T cells had proliferated, whereas ~50% had proliferated in NP3R-immunized mice. Interestingly, the injection of Pam led to increased cellular proliferation of Ag-specific CD8 T cells in NP3R-immunized mice (Fig. 4, *A* and *B*). In addition, priming with NP3R peptide plus Pam induced a significant cellular expansion compared with control mice or mice immunized with NP3R alone (Fig. 4*C*). The effect of Pam required TCR engagement, because no cell accumulation was observed in mice receiving only Pam (data not shown). These results indicate that immunization with NP3R peptide led to a partial CD8 T cell activation without any cell accumulation, whereas TLR2 engagement during priming induced a significant clonal expansion. In contrast to what we observed in vitro, expansion was not similar to that observed with the full agonist NP68 peptide (Fig. 4*C*). This can be explained by the complexity of immune signals in vivo or by a reduced TLR2 ligand or peptide bioavailability. Nevertheless, these results clearly demonstrate that TCR signal and TLR2 triggering can cooperate in vivo to induce CD8 T cell proliferation and expansion.

### *TLR2 engagement on CD8 T cells improves the generation of functional memory cells*

The above experiments demonstrate that the presence of TLR2 ligands improves CD8 T cell responses induced by weak TCR signals. Even so, it remained to be shown that the TLR2 signal could drive CD8 T cell differentiation into memory cell in response to a suboptimal TCR activation. In the above in vivo model, which consists in a single injection of peptide, activation signals received by T cells enable their proliferation and expansion, but do not lead to the generation of memory cells, even in the presence of the full agonist NP68 peptide (our unpublished data). Therefore, we decided to use another model previously described by von Andrian and colleagues (29) to generate memory cells and which allows a precise control of activation conditions. According to this model, we activated $F_5$ CD8 T cells in vitro with NP3R in the presence or absence of TLR2 ligands. After 3 days of activation, cells were extensively washed to remove any residual Pam or peptide, and a constant number of activated CD8 T cells was adoptively transferred to recipient mice. At that time, all peptide-activated CD8 T cells showed an effector phenotype because they expressed high levels of CD44 and heterogeneous levels of Ly6C and CD62L. They also expressed CD122 and CD127 (Fig. 5*A*). Thirty days after the adoptive transfer, activation of cells with the NP3R peptide alone resulted in the recovery of a small number of Ag-specific cells. These cells expressed CD127, heterogeneous levels of CD44 and Ly6C, and no CD122 (Fig. 5*A*). In contrast, when cells were activated in the presence of NP3R and Pam, a 5-fold higher number of Ag-specific cells was recovered (Fig. 5*B*). They presented a memory cell phenotype characterized by CD127 expression, high levels of CD44 and Ly6C, and low level of CD122 (Fig. 5*A*). Furthermore, we did not detect any KLRG1 expression, and CD62L, which is down-regulated by effector cells and effector memory cells, was expressed (Fig. 5*A*). Thus, cells recovered after activation in presence of NP3R and TLR2 ligands were memory cells rather than long-lived effector cells. As control, we also transferred naive $F_5$ CD8 T cells to recipient mice. As expected, 30 days after transfer, a small number of these cells was recovered (Fig. 5*B*) and they presented a naive phenotype (our unpublished data).

To better characterize the recovered cells, we tested their effector functions. After a short restimulation ex vivo with the full agonist NP68 peptide, we observed that 80% of the cells activated in the presence of Pam secreted IFN-$\gamma$, as opposed to 20% when cells had been activated with NP3R alone and 8% when naive cells had been transferred (Fig. 5*C*). To determine the cytotoxic activity of transferred cells, mice were injected with NP68-pulsed mature DC and, 24 h later, a mixture of control and NP68-pulsed target cells was injected. We observed that Pam-stimulated cells had acquired higher cytolytic functions than unstimulated cells or cells primed with NP3R alone (Fig. 5*D*). Of note, when NP3R peptide was used, instead of NP68 peptide, to reveal effector functions, we also observed that cells that had been activated in the presence of

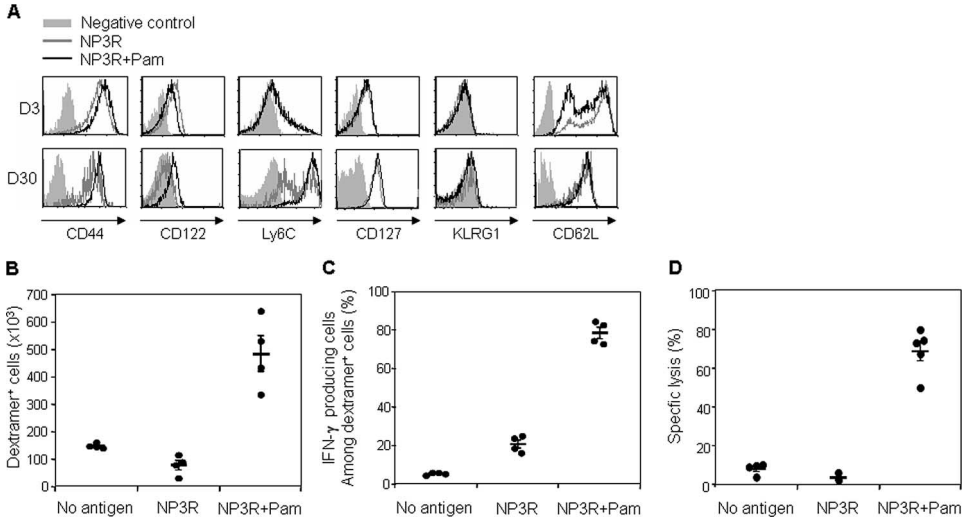


**FIGURE 5.** TLR2 engagement on naive CD8 T improves the generation of functional memory CD8 T cells. Naive CD8 T cells from $F_5$ TCR transgenic mice were activated in vitro for 3 days in the presence of irradiated $TLR2^{-/-}$ spleen cells and 100 nM NP3R peptide, with or without Pam. Viable cells were then isolated, and $6 \times 10^6$ activated CD8 T cells were transferred to C57BL/6 recipient mice. Unstimulated cells (No Ag) were also transferred to recipient mice as controls. Thirty days after activation, lymph node cells were analyzed by flow cytometry. *A*, Transferred cells were identified with NP68/H2D$^b$ multimer staining. Expressions of CD44, CD122, Ly6C, CD127, KLRG1, and CD62L on the surface of NP68/H2D$^b$ multimer-positive cells before transfer (D3) and from lymph nodes 30 days after activation (D30) are shown. Negative controls consist in unlabeled cells (for directly conjugated mAb) or cells treated with streptavidin alone (for biotinylated mAb). *B*, Total number of $F_5$ CD8 T cells collected was obtained, as described in *Materials and Methods*. *C*, For IFN-$\gamma$ staining, cells were stimulated for 5 h with NP68 peptide in the presence of monensin. NP68/H2D$^b$ multimer staining and IFN-$\gamma$ expression were analyzed by flow cytometry. *D*, Cytotoxic function was assayed in vivo 30 days after activation by the transfer of NP68-pulsed mature DC, followed 24 h later by the injection of CFSE-stained peptide-pulsed or nonpulsed target spleen cells from C57BL/6 mice. Mice transferred with unstimulated cells received only target spleen cells. Specific lysis was calculated, as explained in *Materials and Methods*. Data are representative of three independent experiments. **, $p < 0.01$.

Pam presented higher IFN-$\gamma$ secretion (supplementary Fig. 1*A*)[5] and cytolytic functions (supplementary Fig. 1*B*)[5] than cells activated with NP3R alone. These effector responses were, however, weaker than those observed when NP68 peptide was used for the secondary stimulation. These data demonstrate that memory cells generated following stimulation with NP3R and Pam are able to develop effector functions in response to a secondary stimulation with the partial agonist NP3R peptide as well as with the full agonist NP68 peptide.

Collectively, these results demonstrate that costimulation of CD8 T cells via TLR2 results in the generation of functional memory cells, even in the presence of a suboptimal TCR signal.

## Discussion

It is now clearly established that certain TLR are expressed on T cells and that their respective ligands can directly modulate T cell functions (11–14, 19, 20). We previously reported that TLR2 stimulation lowers CD8 T cell dependency for costimulatory signals normally delivered by mature APC (10). We have shown in this study that TLR2 engagement on CD8 T cells can also directly lower the TCR signal strength required for T cell activation. Indeed, we have shown that TLR2 engagement on naive CD8 T cells can convert a partial activation in response to a suboptimal TCR signal into an efficient activation characterized by a strong proliferative response with high levels of CD25 expression, by the expansion of Ag-stimulated cells, and by the generation of functional memory CD8 T cells. We have also shown for the first time that TCR and TLR2 signals cooperate in vivo to induce naive CD8 T cell proliferation and expansion in response to altered peptide ligands. Together with the recent report of Marsland et al. (30) demonstrating that TLR9 engagement in CD4 T cells is sufficient to restore proliferation of anergic protein kinase C-$\theta$-deficient T cells, our results indicate that the threshold for TCR-mediated signaling can be directly modulated by TLR2 and TLR9 engagement in T cells. At a molecular level, we have shown in this study that TLR2 engagement on CD8 T cells increases PKB phosphorylation induced after TCR stimulation with low doses of peptide or with altered peptide ligands. In addition, the involvement of the PI3K/PKB pathway downstream from TLR9 engagement in CD4 T cells was clearly established by Gelman et al. (27). They reported that the comitogenic effect of CpG DNA on $CD4^+$ T cells is associated with the recruitment of the p85 subunit of PI3K to the MyD88 adaptor molecule and with an increased phosphorylation of PKB and glycogen synthetase kinase-3. All of these results strongly suggest that the capacity of TLR ligands to decrease the threshold for TCR-mediated signaling might be linked to an increased or more sustained activation of the PI3K/PKB signaling pathway.

Given these results, it can be considered that the capacity of TLR to lower the signal strength required for T cell responses is a potential mechanism for tolerance breaking in response to danger signals, thus favoring the emergence of autoimmune diseases. The association of danger signals with the onset of autoimmunity is usually attributed to molecular mimicry as well as an inflammatory cytokine-rich context allowing presentation of self-Ag by APC (31). Tolerance breaking could also be induced by endogenous signals and, as an example, available data indicate that the development of autoimmune disorders can be elicited by defects in the clearance of dead cells (32). Because TLR recognize not only microbial products, but also self-derived ligands, new insights on the development of autoimmune diseases are provided by the demonstration that their ligands can directly decrease the threshold for T cell activation and also transiently revert regulatory T cell functions (16–18). In support of this hypothesis, it has been demonstrated, using a model of lyme arthritis, an autoimmune disease

[5] The online version of this article contains supplemental material.

associated with an infection by *Borrelia burgdorferi*, that the outer surface protein A of this bacterium stimulates TLR2 and could directly trigger the release of proinflammatory cytokines from effector T cells (20). Evidence for a direct role of TLR on effector T cells in the development of autoimmune diseases has also been recently suggested in a model of experimental autoimmune myocarditis induced by immunizing protein kinase C-$\theta$-deficient mice with myosin peptide in CFA (30). In those mice, systemic administration of CpG in addition to immunization with myosin H chain peptide is required to develop an autoimmune myocarditis. The authors demonstrated that CpG could act directly on $CD4^+$ T cells in vitro to reverse their anergic status and restore proliferation and survival of these cells. Yet, exogenous IL-6 and TGF-$\beta$ were still required for further Th17 differentiation and consequent disease development. Finally, TLR have also been implicated in the conversion of T cell autoreactivity into autoimmune disease in a model of diabetes induction in receptor interacting glycoprotein (GP) mice following immunization with lymphocytic choriomeningitis virus (LCMV) peptide (33). In this model, and in contrast to LCMV infection that induces diabetes, immunizing with LCMV-GP-derived peptide induced a large number of autoreactive cytotoxic CD8 T cells, but no diabetes. The coadministration of TLR3 or TLR7 ligands, however, elicited overt autoimmune diabetes. To explain that phenomenon, Lang et al. (33) proposed a role of TLR on the organ itself, with an up-regulation of MHC-I expression by $\beta$ cells. Nevertheless, a direct role of TLR ligands on cytotoxic CD8 T cells was not excluded because the authors reported a delayed expansion of autoreactive CD8 T cells after LCMV infection in receptor interacting protein-GP mice deficient for the common TLR adaptor molecule MyD88.

The decrease of the CD8 T cell activation threshold and, more importantly, the improvement of functional memory CD8 T cell generation also present interesting perspectives in the field of vaccination. Many studies have in fact underlined the preponderant role of CD8 T cell responses toward intracellular bacteria, such as *Listeria monocytogenes* and viruses, such as hepatitis C virus or HIV (34, 35). These studies point out the necessity of testing new immunization routes or of designing new adjuvants to induce not only humoral, but also CTL responses to vaccines. At the moment, some synthetic TLR ligands are being examined in clinical trials, based on their effects on APC and especially on DC (36). According to recent results, including ours, prospects for new adjuvants should take into account the fact that TLR agonists could also be used for their direct effects on CD8 T lymphocytes. The capacity of TLR2 ligands, such as lipopeptides, to potentiate CD8 T cell effector response and memory generation in response to poorly immunogenic Ag or to low dose of Ag could in fact improve the efficacy and safety of vaccine candidates. To support this hypothesis, it has been clearly established for many years that the conjugation of a peptide with a lipid moiety strongly enhances immune responses (37). Moreover, HIV lipopeptide vaccines have been recently shown to induce HIV-specific CD4 and CD8 responses, at least in non-HIV-infected volunteers (38). Finally, studies in mice in vivo have reported that lipidated, but not nonlipidated vaccines induce protection against influenza virus or the intracellular bacterium *L. monocytogenes* (39). These effects are TLR2 dependent. However, the direct contribution of TLR2 on T cells has never been investigated.

Our results show that TLR2 engagement on CD8 T cells induces a potent costimulatory signal and promotes an effective immune response that leads to the generation of efficient memory cells, even in the presence of a suboptimal TCR signal. These results open interesting perspectives for the design of new vaccine adjuvants. Molecules that modify the lymphocyte activation threshold, however, should be carefully used because of their potential to induce autoreactivity.

## Acknowledgments

We thank the personnel of the Plateforme de Cytometry en Flux and the Plateau de Biologie Expérimentale de La Souris. We thank Chantal Bella, Odette de Bouteiller, and Catherine Deschildre for cell sorting, and Patrick Manas for murine breeding.

## Disclosures

The authors have no financial conflict of interest.

## References

1. Henrickson, S. E., and U. H. von Andrian. 2007. Single-cell dynamics of T-cell priming. *Curr. Opin. Immunol.* 19: 249–258.
2. Viola, A., and A. Lanzavecchia. 1996. T cell activation determined by T cell receptor number and tunable thresholds. *Science* 273: 104–106.
3. Sloan-Lancaster, J., and P. M. Allen. 1996. Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology. *Annu. Rev. Immunol.* 14: 1–27.
4. Lyons, D. S., S. A. Lieberman, J. Hampl, J. J. Boniface, Y. Chien, L. J. Berg, and M. M. Davis. 1996. A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. *Immunity* 5: 53–61.
5. Jameson, S. C., and M. J. Bevan. 1995. T cell receptor antagonists and partial agonists. *Immunity* 2: 1–11.
6. Manickasingham, S. P., S. M. Anderton, C. Burkhart, and D. C. Wraith. 1998. Qualitative and quantitative effects of CD28/B7-mediated costimulation on naive T cells in vitro. *J. Immunol.* 161: 3827–3835.
7. Acuto, O., and F. Michel. 2003. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat. Rev.* 3: 939–951.
8. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21: 335–376.
9. Bianchi, M. E. 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. *J. Leukocyte Biol.* 81: 1–5.
10. Cottalorda, A., C. Verschelde, A. Marcais, M. Tomkowiak, P. Musette, S. Uematsu, S. Akira, J. Marvel, and N. Bonnefoy-Berard. 2006. TLR2 engagement on CD8 T cells lowers the threshold for optimal antigen-induced T cell activation. *Eur. J. Immunol.* 36: 1684–1693.
11. Komai-Koma, M., L. Jones, G. S. Ogg, D. Xu, and F. Y. Liew. 2004. TLR2 is expressed on activated T cells as a costimulatory receptor. *Proc. Natl. Acad. Sci. USA* 101: 3029–3034.
12. Gelman, A. E., J. Zhang, Y. Choi, and L. A. Turka. 2004. Toll-like receptor ligands directly promote activated $CD4^+$ T cell survival. *J. Immunol.* 172: 6065–6073.
13. Caron, G., D. Duluc, I. Fremaux, P. Jeannin, C. David, H. Gascan, and Y. Delneste. 2005. Direct stimulation of human T cells via TLR5 and TLR7/8: flagellin and R-848 up-regulate proliferation and IFN-$\gamma$ production by memory $CD4^+$ T cells. *J. Immunol.* 175: 1551–1557.
14. Tabiasco, J., E. Devevre, N. Rufer, B. Salaun, J. C. Cerottini, D. Speiser, and P. Romero. 2006. Human effector $CD8^+$ T lymphocytes express TLR3 as a functional coreceptor. *J. Immunol.* 177: 8708–8713.
15. Crellin, N. K., R. V. Garcia, O. Hadisfar, S. E. Allan, T. S. Steiner, and M. K. Levings. 2005. Human $CD4^+$ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in $CD4^+CD25^+$ T regulatory cells. *J. Immunol.* 175: 8051–8059.
16. Liu, H., M. Komai-Koma, D. Xu, and F. Y. Liew. 2006. Toll-like receptor 2 signaling modulates the functions of $CD4^+$ $CD25^+$ regulatory T cells. *Proc. Natl. Acad. Sci. USA* 103: 7048–7053.
17. Sutmuller, R. P., M. H. den Brok, M. Kramer, E. J. Bennink, L. W. Toonen, B. J. Kullberg, L. A. Joosten, S. Akira, M. G. Netea, and G. J. Adema. 2006. Toll-like receptor 2 controls expansion and function of regulatory T cells. *J. Clin. Invest.* 116: 485–494.
18. Peng, G., Z. Guo, Y. Kiniwa, K. S. Voo, W. Peng, T. Fu, D. Y. Wang, Y. Li, H. Y. Wang, and R. F. Wang. 2005. Toll-like receptor 8-mediated reversal of $CD4^+$ regulatory T cell function. *Science* 309: 1380–1384.
19. Bendigs, S., U. Salzer, G. B. Lipford, H. Wagner, and K. Heeg. 1999. CpG-oligodeoxynucleotides co-stimulate primary T cells in the absence of antigen-presenting cells. *Eur. J. Immunol.* 29: 1209–1218.
20. Sobek, V., N. Birkner, I. Falk, A. Wurch, C. J. Kirschning, H. Wagner, R. Wallich, M. C. Lamers, and M. M. Simon. 2004. Direct Toll-like receptor 2 mediated co-stimulation of T cells in the mouse system as a basis for chronic inflammatory joint disease. *Arthritis Res. Ther.* 6: R433–R446.
21. Mamalaki, C., T. Norton, Y. Tanaka, A. R. Townsend, P. Chandler, E. Simpson, and D. Kioussis. 1992. Thymic depletion and peripheral activation of class I major histocompatibility complex-restricted T cells by soluble peptide in T-cell receptor transgenic mice. *Proc. Natl. Acad. Sci. USA* 89: 11342–11346.
22. Smyth, L. A., O. Williams, R. D. Huby, T. Norton, O. Acuto, S. C. Ley, and D. Kioussis. 1998. Altered peptide ligands induce quantitatively but not qualitatively different intracellular signals in primary thymocytes. *Proc. Natl. Acad. Sci. USA* 95: 8193–8198.
23. Young, A. C., W. Zhang, J. C. Sacchettini, and S. G. Nathenson. 1994. The three-dimensional structure of H-2Db at 2.4 A resolution: implications for antigen-determinant selection. *Cell* 76: 39–50.

24. Angelov, G. S., M. Tomkowiak, A. Marcais, Y. Leverrier, and J. Marvel. 2005. Flt3 ligand-generated murine plasmacytoid and conventional dendritic cells differ in their capacity to prime naive CD8 T cells and to generate memory cells in vivo. *J. Immunol.* 175: 189–195.
25. Schott, E., N. Bertho, Q. Ge, M. M. Maurice, and H. L. Ploegh. 2002. Class I negative CD8 T cells reveal the confounding role of peptide-transfer onto CD8 T cells stimulated with soluble H2-Kb molecules. *Proc. Natl. Acad. Sci. USA* 99: 13735–13740.
26. Okkenhaug, K., and B. Vanhaesebroeck. 2003. PI3K in lymphocyte development, differentiation and activation. *Nat. Rev.* 3: 317–330.
27. Gelman, A. E., D. F. LaRosa, J. Zhang, P. T. Walsh, Y. Choi, J. O. Sunyer, and L. A. Turka. 2006. The adaptor molecule MyD88 activates PI-3 kinase signaling in $CD4^+$ T cells and enables CpG oligodeoxynucleotide-mediated costimulation. *Immunity* 25: 783–793.
28. Arbibe, L., J. P. Mira, N. Teusch, L. Kline, M. Guha, N. Mackman, P. J. Godowski, R. J. Ulevitch, and U. G. Knaus. 2000. Toll-like receptor 2-mediated NF-$\kappa$B activation requires a Rac1-dependent pathway. *Nat. Immunol.* 1: 533–540.
29. Manjunath, N., P. Shankar, J. Wan, W. Weninger, M. A. Crowley, K. Hieshima, T. A. Springer, X. Fan, H. Shen, J. Lieberman, and U. H. von Andrian. 2001. Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes. *J. Clin. Invest.* 108: 871–878.
30. Marsland, B. J., C. Nembrini, K. Grun, R. Reissmann, M. Kurrer, C. Leipner, and M. Kopf. 2007. TLR ligands act directly upon T cells to restore proliferation in the absence of protein kinase C-$\theta$ signaling and promote autoimmune myocarditis. *J. Immunol.* 178: 3466–3473.
31. Benoist, C., and D. Mathis. 1998. Autoimmunity: the pathogen connection. *Nature* 394: 227–228.
32. Nagata, S. 2007. Autoimmune diseases caused by defects in clearing dead cells and nuclei expelled from erythroid precursors. *Immunol. Rev.* 220: 237–250.
33. Lang, K. S., M. Recher, T. Junt, A. A. Navarini, N. L. Harris, S. Freigang, B. Odermatt, C. Conrad, L. M. Ittner, S. Bauer, et al. 2005. Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. *Nat. Med.* 11: 138–145.
34. Houghton, M., and S. Abrignani. 2005. Prospects for a vaccine against the hepatitis C virus. *Nature* 436: 961–966.
35. Ladel, C. H., I. E. Flesch, J. Arnoldi, and S. H. Kaufmann. 1994. Studies with MHC-deficient knock-out mice reveal impact of both MHC I- and MHC II-dependent T cell responses on *Listeria monocytogenes* infection. *J. Immunol.* 153: 3116–3122.
36. Kanzler, H., F. J. Barrat, E. M. Hessel, and R. L. Coffman. 2007. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. *Nat. Med.* 13: 552–559.
37. BenMohamed, L., S. L. Wechsler, and A. B. Nesburn. 2002. Lipopeptide vaccines: yesterday, today, and tomorrow. *Lancet* 2: 425–431.
38. Pialoux, G., H. Gahery-Segard, S. Sermet, H. Poncelet, S. Fournier, L. Gerard, A. Tartar, H. Gras-Masse, J. P. Levy, and J. G. Guillet. 2001. Lipopeptides induce cell-mediated anti-HIV immune responses in seronegative volunteers. *AIDS* 15: 1239–1249.
39. Jackson, D. C., Y. F. Lau, T. Le, A. Suhrbier, G. Deliyannis, C. Cheers, C. Smith, W. Zeng, and L. E. Brown. 2004. A totally synthetic vaccine of generic structure that targets Toll-like receptor 2 on dendritic cells and promotes antibody or cytotoxic T cell responses. *Proc. Natl. Acad. Sci. USA* 101: 15440–15445.