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Combined Triggering of Dendritic Cell Receptors Results in Synergistic Activation and Potent Cytotoxic Immunity

James W. Wells,* Christopher J. Cowled,* Farzin Farzaneh,† and Alistair Noble2*

Elimination of malignant cells and intracellular infections involves collaboration between CTLs and Th1 inflammation. Dendritic cells drive this response via costimulation and cytokines. We have defined key signals required for the exponential expansion of specific CD8+ T cells in vivo in mice. Immunization with two or more TLR agonists, anti-CD40, IFN-γ, and surfactant were sufficient to drive unprecedented levels of CD8 response to peptide or protein Ag and highly polarized Th1 CD4 responses. CD40 signaling was required for CD8 expansion but could be provided by a concomitant CD4 Th response in place of anti-CD40. Triggering of these pathways activated migration and activation of myeloid and plasmacytoid dendritic cells and secretion of IL-12. Cross-presentation can thus be exploited to induce potent cytotoxic responses and long-term memory to peptide/protein Ags. When combined with a tumor-associated peptide from tyrosinase-related protein 2, our combined adjuvant approach effectively halted tumor growth in an in vivo melanoma model and was more effective than anti-CD40 and a single TLR agonist. Antitumor immunity was associated with long-lived effector memory CD8 cells specific for the naturally processed and presented tumor Ag, and tumor protection was partially but not entirely dependent on CD8 T cells. This flexible strategy is more effective than existing adjuvants and provides a technological platform for rapid vaccine development. *The Journal of Immunology, 2008, 181: 3422–3431.

The CD8 T cell is required for the eradication of genetically transformed cells, such as those in cancer (1), and intracellular pathogens such as HIV, hepatitis, and influenza (2, 3). Tumor immunotherapies have largely failed because the induction of cytotoxic responses against tumor Ags has been insufficient to eliminate established tumors (4). The unique ability of CD8 T cells to kill targets expressing peptide Ags associated with MHC class I ensures that they are key to cellular immunity. Cell-mediated immunity often requires collaboration between CD8 and Th1 CD4 cells along with Abs elicited from B cells by Th1 interactions (2, 5). CD8 cells are known to undergo larger clonal expansions than their CD4 T cell counterparts (6, 7), and these expansions result in long-lived memory T cell populations that provide lasting immunity (8). Indeed, in experimental models CD8 memory cells were much longer lived than Th1 or Th2-type CD4 cells (9, 10). Because CD8 cells respond to intracellular Ags, it has been widely assumed that the induction of CD8-mediated immunity requires intracellular expression of Ag within professional APCs. DNA vaccines and recombinant viral particles have been used to achieve this in vivo (11, 12). However, it is clear that dendritic cells (DCs),3 macrophages, and activated B cells (13–15) are capable of presenting exogenously acquired Ag in the context of MHC class I to CD8 cells. This cross-presentation pathway is important in maintaining peripheral tolerance to self-Ags (16), but its role in immune responses to extracellular protein or microbes is less clear.

Primary CD8 cell expansion is dependent on the activation of DCs expressing TLRs, which signal through MyD88 (17) and costimulation through CD80/86 and CD137L:CD137 (4-1BB) interaction (18). However, the induction of IL-12 from DCs is known to require additional signals from T cells, i.e., CD40 ligation by CD40 ligand (CD40L) and IFN-γ (19). IL-12 has been described as the “third signal,” in addition to Ag and costimulation, that is required for CD8 cell expansion (20). Signaling through CD40, TLR, and/or IFN-γR in the absence of Ag in vitro has been shown to result in high-level production of IL-12 (21, 22).

Molecular adjuvants targeting receptors on DCs have also been useful in determining the requirements for cellular immunity in vivo (23). Th1-associated Ab responses (24) and CTL induction (25) have been achieved with Ag and TLR agonists. With the discovery of tumor-specific Ags, adjuvants are required to prime the acquired immune system to self/tumor-specific Ags that are poorly immunogenic. Currently, however, few effective and safe CD8-inducing adjuvants are available for human use (26).

In this study we demonstrate that the activation of DCs rather than the endogenous expression of Ag is sufficient to induce maximal levels of Ag-specific CD8 T cell expansion in vivo. We show that a variety of molecular adjuvant combinations used with peptide or protein Ag act synergistically to induce rapid expansion of Ag-specific CD8 clones and potent Th1-associated immunity. Potent cytotoxic immunity directed toward a naturally expressed peptide on tumor cells could be elicited with this approach. Our data

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3 Abbreviations used in this paper: DC, dendritic cell; CASAC, combined adjuvant for synergistic activation of cellular immunity; CD40L, CD40 ligand; i.d., intradermal; KLH, keyhole limpet hemocyanin; LN, lymph node; mDC, myeloid DC; MPL, monophosphoryl lipid A; Pam3CSK4, (S)-[2-[3-bis(palmitoyloxy)propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys]3OH, 3HCl; pDC, plasmacytoid DC; polyIC, polyinosinic-polycytidylic acid; TDM, trehalose 6,6’-dimycolate; TRP-2, tyrosinase-related protein 2; VSV, vesicular stomatitis virus.
provide an ideal strategy for the development of effective subunit vaccines against infectious or malignant disease in humans.

Materials and Methods

Adjuvant combinations and vaccination procedures

All animal procedures were performed according to U.K. Home Office and institutional regulations. No inflammatory skin lesions were observed in any immunized mice in this study. We immunized C57BL/6 or BALB/c mice (Harlan) intradermally (i.d.) with combinations of the following reagents in PBS (per mouse): OVA(257–264) (SINFEKL) peptide (100 μg/Peprotec), tyrosinase-related protein 2 (TRP-2)342–350 peptide (Pro-Immune), vesicular stomatitis virus (VSV)G32–40 peptide (100 μg/University of Sheffield, Sheffield, U.K.), gp10025–33 peptide, insulin B14–23 peptide, and the oncologic Ag-derived tumor peptide OVA(155–163) (all from Peptide Protein Research), anti-CD40 (clone 3/23, 25 μg; Sigma-Aldrich), IFN-γ (100 μg; Peprotec), CpG 1826 (25 μg/ml; InvivoGen), Ag85B240–254 peptide (100 μg; Bio), R837 (50 μg/ml; InvivoGen), MPL (50 μg/ml; InvivoGen), OVA(123–139) peptide (100 μg/ml; Mimotopes), Ag85B240–254 peptide (100 μg/ml; InvivoGen). We pulsed splenocytes from unprimed C57BL/6 mice with 10 μg/ml anti-CD8 antibody, and labeled with 3HCl (Pam3-CSK4), synthetic lipopolysaccharide (100 μg/ml; EMC Microcollections), R837 (50 μg/ml; InvivoGen), MPL (50 μg/ml; InvivoGen), OVA(123–139) peptide (100 μg/ml; Mimotopes), Ag85B240–254 peptide (100 μg/ml; InvivoGen). We performed on days 20–22, unless otherwise indicated. In vivo CTL assays were performed on days 19–21 unless otherwise indicated. Mice were primed on day 0 and boosted on days 9–11 with the following formula: killing (%) = 1 – [(no. of targets/no. of control cells in immunized animal)/(no. of targets/no. of control cells in control animal)] × 100.

We harvested inguinal LN 24 h after immunization and digested them with 0.5 mg/ml collagenase type IV (Sigma-Aldrich) for 1 h at 37°C. We added nonlabeled anti-CD16/CD32 (clone CT-17,1/CT-17,2; Caltag Laboratories) to prevent nonspecific staining. LN cells were then incubated with combinations of the following reagents for 15 min at 4°C: TRP-2180–188 (ProImmune), tyrosinase-related protein 2 (TRP-2)342–350 (ProImmune), Ag85B240–254 (MPL) plus trinitrophenyl (TNP)dimycolate (TDM) emulsion (Ribi adjuvant, used as per manufacturer’s instructions; Sigma-Aldrich), gp10025–33 peptide, insulin B14–23 peptide, and the oncologic Ag-derived tumor peptide OVA(155–163) (all from Peptide Protein Research), anti-CD40 (clone 3/23, 25 μg; Sigma-Aldrich), (5)-[2,3-bis(palmito- loxy)-2(R,S)-propyl]-N-palmitoyl-(R)-Cys-(S,S)-Ser-Lys4-OH, 3HCl (Pam3-CSK4), synthetic lipopolysaccharide (100 μg/ml; EMC Microcollections), R837 (50 μg/ml; InvivoGen), MPL (50 μg/ml; InvivoGen), OVA(123–139) peptide (100 μg/ml; Mimotopes), Ag85B240–254 peptide (100 μg/ml; InvivoGen). We pulsed splenocytes from unprimed C57BL/6 mice with 10 μg/ml anti-CD8 antibody and labeled with 3HCl (Pam3-CSK4), synthetic lipopolysaccharide (100 μg/ml; EMC Microcollections), R837 (50 μg/ml; InvivoGen), MPL (50 μg/ml; InvivoGen), OVA(123–139) peptide (100 μg/ml; Mimotopes), Ag85B240–254 peptide (100 μg/ml; InvivoGen). We pulsed splenocytes from unprimed C57BL/6 mice with 10 μg/ml anti-CD8 antibody and labeled with 3HCl (Pam3-CSK4), synthetic lipopolysaccharide (100 μg/ml; EMC Microcollections), R837 (50 μg/ml; InvivoGen), MPL (50 μg/ml; InvivoGen), OVA(123–139) peptide (100 μg/ml; Mimotopes), Ag85B240–254 peptide (100 μg/ml; InvivoGen). We then mixed squalene (containing TDM) with MPL, T LN, and PBS to give a solution consisting of 500 μg/ml MPL, 500 μg/ml TDM, 4.4% squalene, and 0.4% Tween 80. To create an emulsion, the mix was sonicated on ice with a Vibra-Cell VCX-130 sonicator from Sonics & Materials (2 × 5 s at 60% with a 3-mm stepped microtip). The emulsion was then mixed 1:1 with the other vaccine components before injection. We exchanged MPL with other TLR agonists in indicated experiments. Aqueous adjuvant mixtures were prepared in 0.2% TWEEN 80 in PBS.

Serum Ab determination by ELISA

We analyzed serum samples for OVA-specific IgG1 or IgG2a by coating microtiter plates with 50 μg/ml OVA overnight. Standard curves used positive serum given a value in arbitrary units. Sera diluted in PBS, 0.5% FBS, and 0.5% Tween 20 were added overnight. Plates were washed and incubated for 2 h with anti-IgG1–alcaline phosphatase or anti-IgG2a–alkaline phosphatase (Serotec) in PBS, 0.5% FCS, and 0.05% Tween 20. After washing, p-nitrophenyl phosphate in 1 M diethanolamine was added for 1 h and measured at OD405.

To determine the anti-DNA IgG2a autoantibody, we coated microtiter plates with 5 μg/ml calf thymus DNA (Sigma-Aldrich) (boiled for 10 min and cooled on ice). Sera diluted in PBS, 0.5% FBS, and 0.5% Tween 20 were added overnight. Sera derived from mice with induced graft-vs-host disease were used as positive control (27). Plates were incubated with anti-IgG2a–alkaline phosphatase and developed as described above.

Serum IL-12 p70 was measured using a commercial ELISA kit (R&D Systems) according to the manufacturer’s instructions.

B16 melanoma model

B16-A5 (H-2Kb) melanoma cells (European Collection of Cell Cultures) cultured in DMEM, 10% FCS, and 50 μM 2-ME were washed and resuspended in PBS and 106 cells in 200 μl were injected s.c. into the dorsa of C57BL/6 mice on day 0. On day 3 the mice were immunized with 400 μg of TRP-2180–188 in CASAC (anti-CD40, CpG, polyIC, and IFN-γ in squalene/Tween 80), anti-CD40 plus CpG alone i.p., or control PBS at two separate sites. One group of immunized mice received 75 μg of anti-CD88 i.p. on day 2, and the other group received control rat IgG. Vaccination was repeated on days 11 and 19. Tumor growth was measured every 3 days using vernier calipers and animals were killed when tumors reached 15 mm. Blood was taken at day 22 and stained with TRP-2180–188/H-2Kb pentamer (Promimmune) or with the SINFEKL/H-2Kb pentamer as a negative control. All mice were killed on day 72 and LN/spleen cells were stained for IFN-γ production after 12 h of stimulation with 5 μg/ml TRP-2180–188 or gp10025–33 as above. In some experiments B16 cells were labeled with 2.5 μM CFSE and 2 × 106 cells were transferred i.v. into preimmunized or control mice (two doses of vaccine as above at 8-day intervals; cells were transferred on day 16). Three days later lung and
spleen cells were harvested by collagenase digestion or dissociation, respectively. Total CFSE¹ events in each sample were counted by flow cytometry.

Statistical analysis
All error bars represent SEM. Comparisons of mean values were performed using unpaired Student’s t tests as indicated: *, p < 0.05; **, p < 0.005; and ***, p < 0.0001. INSTAT software was used; no significant deviations from normality were found.

Scores for peptide:MHC interaction
Parker binding scores, which estimate peptide:MHC dissociation rates, were obtained online (bimas.dct.nih.gov/molbio/hla_bind/). SYFPFEITHI scores, which predict peptide:MHC binding affinity, were also obtained online (www.sfypeithi.de/).

Results
Requirements for peptide-specific CD8 clonal expansion
To determine whether triggering multiple receptors on DCs could synergistically induce CD8 cell expansion, we immunized mice i.d. with combinations of agonists for pathways linked to DC activation and with OVA-derived, MHC class I-restricted OVA₂₅₇₋₂₆₄ (SIINFEKL) peptide. MHC pentamer staining was used to track SIINFEKL-specific CD8 cell expansion in the blood (Fig. 1a). The highest levels of clonal expansion were induced when CpG, anti-CD40 Ab, IFN-γ, SIINFEKL peptide, and MPL plus TDM emulsion were used in combination. Omission of CpG, anti-CD40, or MPL plus TDM emulsion led to substantially weaker CD8 responses. The presence of a small quantity (100 ng) of IFN-γ in the adjuvant significantly enhanced the response (Fig. 1b). The highest response we observed was 80.1% of total CD8 cells that were SIINFEKL-specific following two vaccinations containing the combined agonists (Fig. 1c), indicating massive clonal expansion of specific cells from initial background levels. For simplicity, this adjuvant combination is referred to as the combined adjuvant for synergistic activation of cellular immunity or CASAC. Simultaneous triggering of multiple signaling pathways was therefore required to induce optimal CD8 cell expansion.

Induction of potent cytotoxic immunity and long-term memory
To determine whether CD8 cells induced with CASAC represent functional CTL, in vivo CTL assays were performed (Fig. 1d). Immunized mice were consistently observed to have >99% reduction of the peptide-pulsed target population compared with the unpulsed control population, demonstrating potent peptide-specific CTL activity.

We monitored SIINFEKL-specific CD8 cells for up to 107 days following immunizations (on days 0 and 9; Fig. 1e). After an initial expansion and contraction phase, the numbers of SIINFEKL-specific cells fell to ~5% (after day 100). When challenged with peptide in the absence of CASAC on day 107, we observed a strong recall response in the immunized group (Fig. 1e), demonstrating that a long-lived memory CD8 cell population had been induced. In a similar experiment, we performed an in vivo CTL assay 17 days after rechallenge with peptide alone and demonstrated functional memory CTL activity (Fig. 1f).

The ligation of CD137 (4-1BB) during priming has been shown to enhance the development of CD8 memory populations (28). We investigated whether agonistic anti-CD137 Ab could inhibit the contraction phase of CD8 responses and enhance long-term memory. Injection of anti-CD137 into the original injection site 7 days after the second immunization resulted in a significantly higher recall response after rechallenge with peptide, compared with control Ab (Fig. 1g). There was no significant enhancement in the size of the long-term memory cell pool after the ligation of CD137, but a higher proportion of cells were competent to respond to future challenge.

CD8 responses are enhanced by direct CD40 ligation or via CD4 T cell help
Our data indicated that direct ligation of CD40 on DC with anti-CD40 provided them with crucial signals for CTL priming. To determine whether signaling via CD40 could be indirectly provided through CD40L expression on activated CD4 T cells, we exchanged anti-CD40 for an MHC class II peptide (OVA₃₂₁₋₃₃₉).
High level expansion of SIINFEKL-specific CD8 cells was seen, similar to that induced by anti-CD40 (Fig. 2a); thus, a related class II peptide can substitute for anti-CD40 by providing CD4 “help.” Furthermore, Ag85B240–254, an un-related class II peptide derived from Mycobacterium tuberculosis (29), could also provide help (Fig. 2b) in a linked bystander fashion. CD8 help could also be provided by the immunogenetic protein KLH (Fig. 2c).

We then examined whether a whole protein Ag would be cross-presented to drive a MHc class I-restricted CD8 cell response, assisted by CD4 help from class II presentation. Mice were immunized with whole OVA protein combined with CpG, IFN-γ, and MPL plus TDM emulsion (Fig. 2d). Again, CD8 cells expanded strongly, but not to the same extent as with anti-CD40 or class II peptide. This may have been due to the expansion of several class I restricted clones, reducing SIINFEKL-specific expansion through competition. The data show that exogenous proteins are efficiently processed via class I and can induce strong CD8 cell responses when DCs receive sufficient stimuli.

Using polyIC (TLR3 agonist) and Pam3CSK4 (TLR1/2 agonist), we determined whether TLR9 signaling was critical to induct CD8 cell expansion. Mice were immunized with either CpG, polyIC, or Pam3CSK4 incorporated into CASAC. This did not affect the degree of CD8 cell expansion (Fig. 2e). Because MPL, present in MPL plus TDM emulsion, is a TLR4 agonist, it appeared that coligation of distinct TLRs was a critical factor in adjuvanticity as reflected by a 50% reduction in response when a second TLR agonist was omitted (Fig. 1, a and e). We determined critical components of the commercial MPL plus TDM emulsion (Ribi adjuvant) by using its constituent reagents (MPL, TDM, squalene oil, and Tween 80 detergent; Fig. 2f). Absence of squalene and TDM did not reduce adjuvanticity; however, absence of MPL or Tween 80 significantly reduced responses. To test whether TLR4 was critical, mice were immunized with SIINFEKL, IFN-γ, anti-CD40, and Tween 80 in conjunction with TLR agonists in aqueous solution. The combination of polyIC (TLR3 agonist) and CpG (TLR9 agonist), or polyIC with Pam3CSK4 (TLR1/2 agonist), resulted in a similar degree of adjuvanticity as pairing MPL with CpG (TLRs 4 and 9, respectively; Fig. 2g).

Rapid Th1 polarization in vivo is induced by CASAC

To evaluate polarization of the CD4 response toward a Th1 or Th2 phenotype, mice were immunized with class II peptides in combination with SIINFEKL plus CASAC or with whole OVA protein in combination with CpG, IFN-γ, and MPL plus TDM emulsion. Draining LN CD4 cells were analyzed for IL-4 and IFN-γ synthesis (Fig. 3a). High proportions of IFN-γ+ CD4 cells, but no IL-4+ cells, were detected, indicating strong polarization toward a Th1 phenotype. Mice immunized with whole OVA protein produced fewer OVA232–339-induced Th1 cells, possibly due to competition between several class II-restricted clones. We then determined which constituents of CASAC were required for Th1 polarization (Fig. 3b). Mice given whole OVA with TLR agonists alone raised weak Th1 responses, whereas the addition of both Tween 80 and IFN-γ to the aqueous adjuvant contributed to more highly polarized Th1 responses in both draining LN and spleen.

To confirm that CASAC was inducing genuine cross-processing of whole OVA and presentation of the SIINFEKL epitope, we stained blood from OVA/CASAC immunized mice with the H-2Kb/SIINFEKL pentamer and a control H-Kb pentamer staining in the blood are shown. Numbers represent the percentage of total CD8 T cells that are peptide specific. Data are representative of two independent experiments with groups of three animals. e. Multiple distinct TLRs can provide equivalent adjuvanticity. Representative responses of mice immunized with MPL plus TDM emulsion and additional TLR agonists as indicated are shown. (n = 3, similar data seen in two experiments).

f. Influence of the individual components of MPL plus TDM emulsion as determined by pentamer staining of blood (n = 6). g. Induction of CD8 responses by pairs of TLR agonists in aqueous solution. Responses induced by combinations of the TLR agonists listed combined with SIINFEKL, anti-CD40, Tween 80, and IFN-γ in PBS (n = 6) are shown.

FIGURE 2. Enhancement of CD8 responses can be provided by anti-CD40 or indirect “help” and via a variety of TLRs. a–d. Comparison of anti-CD40 (a–CD40) with OVA232–339 (a), Ag85B240–254 (b), KLH (c), and OVA (d) in the adjuvant combination. Plots of H-2Kb/SIINFEKL pentamer staining in the blood are shown. Numbers represent the percentage of total CD8 T cells that are peptide specific. Data are representative of two independent experiments with groups of three animals. e. Multiple distinct TLRs can provide equivalent adjuvanticity. Representative responses of mice immunized with MPL plus TDM emulsion and additional TLR agonists as indicated are shown. (n = 3, similar data seen in two experiments).

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Activation of DC subsets and IL-12 secretion in vivo

We studied draining inguinal LN of mice 24 h following i.d. injection of individual CASAC constituents by flow cytometry (Fig. 4). The presence of peptide Ag had no effect on DC number, activation marker expression, or IL-12 secretion and is therefore omitted. Myeloid DCs (mDCs), defined by CD11c and MHC class II coexpression, were subclassified based on CD8 and CD205 expression as described (12). Plasmacytoid DCs (pDCs) were identified as CD11c<sup>-</sup>/H<sub>11001</sub>MPDCA1<sup>-</sup>/H<sub>11001</sub>B220<sup>-</sup>. Numbers of LN-resident CD8<sup>-</sup>/H<sub>11002</sub>CD205<sup>-</sup>mDCs and skin-derived CD8<sup>-</sup>/H<sub>11002</sub>CD205<sup>-</sup>mDCs were unchanged by any adjuvant combination (Fig. 4<sup>a</sup>), whereas the numbers of LN-resident CD8<sup>-</sup>/H<sub>11002</sub>CD205<sup>-</sup>mDCs were significantly increased by IFN-γ injection but not by TLR agonists. By contrast, expression of the DC activation markers CD86 and CD40 was up-regulated by TLR agonists, especially in combination (Fig. 4<sup>b</sup>). This increased expression of costimulatory molecules was most dramatic on CD8<sup>-</sup>/H<sub>11002</sub>CD205<sup>-</sup>mDCs. pDCs were also activated by TLR agonists, although the levels of CD86 and CD40 on this subset were much lower than those on mDCs. IFN-γ (Fig 4<sup>b</sup>) and anti-CD40 Ab (not shown) had little effect on DC activation markers. MHC class II expression levels were not consistently changed on any of the subsets. The data suggest that CASAC induces DC migration from the skin to the draining LNs and activates multiple LN-resident DC subsets.

Because DC secretion of IL-12 is crucial for cell-mediated immunity, we measured the bioactive IL-12 p70 heterodimer in the serum of mice injected as above (Fig. 4<sup>c</sup>). In contrast to the CD86/CD40 data, IL-12 p70 was induced by anti-CD40 Ab (not shown) had little effect on DC activation markers. MHC class II expression levels were not consistently changed on any of the subsets. The data suggest that CASAC induces DC migration from the skin to the draining LNs and activates multiple LN-resident DC subsets.

Our study demonstrates that CASAC is a potent adjuvant for inducing both DC migration and activation, which are essential for the induction of efficient T cell responses. The combination of TLR agonists and IFN-γ further enhances DC activation, leading to increased IL-12 secretion. This synergy provides a promising approach for improving vaccine efficacy.
Table I. Summary of peptide sequences tested in combination with CASAC

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a Oncofetal Ag-derived tumor peptide.

act on distinct DC functions, resulting in synergistic effects only at the level of T cell responses.

Induction of immunity to poorly immunogenic and tumor-associated peptides

It was not clear how antigenic immunogenicity contributed to the level of response, so we immunized mice with a number of less immunogenic peptides. A vesicular stomatitis virus peptide (VSV52–59) plus CASAC induced fewer IFN-γ-positive cells in the spleen than SIINFEKL. (Fig. 5a). However, potent peptide-specific cytotoxicity was induced (Fig. 5b). Furthermore, a tumor-associated, weakly immunogenic, melanoma-derived self-peptide, TRP-2180–188 (30), induced strong T cytotoxicity 1 (Tc1)-type responses when combined with CASAC. This was highly dependent on Ag dose (Fig. 5c). Increasing the dose of TRP-2 peptide also increased cytotoxicity up to ~97% lysis (Fig. 5d). The TRP-2 peptide vaccine also induced long-lived memory cells in both LN and spleen, with specific IFN-γ-producing cells being readily detectable 70 days after immunization (Fig. 5e). Cutting of these cells with the CD44 memory marker and the CD62L LN homing receptor revealed that the memory cells were of the effector memory subset.

We also tested CASAC combined with a range of self/tumor-derived peptide Ags for CD8 IFN-γ responses (Table I). Responses to another melanoma-derived peptide, gp10025–33, were poor, with only one of nine immunized mice displaying a detectable CD8 response. No responses were observed to an insulin-derived self-peptide (31), B15–23, or an oncofetal Ag-derived tumor peptide (32), OFA155–163. Levels of CD8 response failed to demonstrate any correlation with the Parker score (a prediction of peptide:MHC dissociation rate), yet correlated well with SYFPEITHI MHC:peptide binding scores. This suggested that peptide:MHC binding affinity is an important determinant of immunogenicity and that DC activation can only amplify responses to self-Ags if they are inherently immunogenic.

Because the TRP-2180–188 epitope is expressed on B16 melanoma cells (33), we determined whether the CD8 response we had induced with the TRP-2/CASAC vaccine was capable of recognizing and killing B16 cells in vivo. We immunized mice with TRP-2 peptide plus CASAC, transferred CFSE-labeled B16 cells i.v., then counted CFSE+ cells in lung tissue cells 3 days later (Fig. 5f). No tumor cells were detected in the lungs of immunized mice, indicating efficient killing.

Finally we determined the efficacy of CASAC for therapy of B16 melanoma. B16 tumors were induced by s.c. injection of 10⁶ cells; 3, 11 and 19 days later, mice were immunized with TRP-2 peptide plus CASAC or PBS (Fig. 6, a and b). To determine the contribution of CD8 T cells to protection, one group of TRP-2/CASAC-immunized mice received anti-CD8-depleting Ab one day before the first vaccination, the other received control IgG. We also directly compared CASAC to the most effective adjuvant combination currently in development for cancer vaccines,
anti-CD40 plus CpG. Tumors grew rapidly in mice not receiving vaccine. Mice were killed when tumor diameter reached >15 mm, and all control mice were killed by day 30. TRP-2/CASAC immunization completely prevented the appearance of tumors until day 45, when only one of eight mice developed a tumor. Anti-CD40 plus CpG alone induced significantly weaker protection, with only one of six mice surviving to day 72. CD8 depletion significantly reduced protection induced by TRP-2/CASAC, although the appearance of tumors was much delayed even in the absence of CD8 T cells. Surviving TRP-2/CASAC-immunized animals were resistant to further tumor cell challenge with twice as many B16 cells (not shown). We also detected specific staining of CD8 cells in the blood of these mice with a TRP-2180–188/H-2Kb pentamer (Fig. 6c). Pentamer staining cells were only detected in TRP-2/CASAC-immunized mice and were not significantly altered by the challenge with live B16 cells. TRP-2-specific CD8s were undetectable in anti-CD40/CpG-immunized or CD8-depleted animals.

Because it was possible that the potency of CASAC could possibly induce epitope spreading for the recognition of other self-Ags present in surrounding tissue or dying tumor cells, anti-DNA IgG2a autoantibodies were measured in TRP-2/CASAC-immunized mice (Fig. 6d). Serum from mice in which chronic graft-vs-host disease had been induced was used as a positive control (27). We did not observe significant anti-DNA IgG2a in TRP-2/CASAC-immunized mice, indicating that CASAC does not trigger generalized autoimmunity. However, low but significant levels of anti-DNA IgG2a were detected in B16 tumor-bearing mice immunized with TRP-2180–188 self-peptide. We therefore tested whether responses to the gp100 epitope expressed by B16 cells had occurred by using IFN-γ analysis (Fig. 6e). The results showed that gp100 stimulation induced no detectable IFN-γ+ cells in the spleen; the response was TRP-2180–188-specific and confined to the effector memory (CD44highCD62L−) compartment. Furthermore, we observed white hairs at the injection sites of TRP-2/CASAC-immunized mice but not elsewhere (not shown), suggesting bystander killing of melanin-producing cells where vaccine was injected, but without widespread autoimmunity.

Discussion
Development of subunit vaccines consisting of defined Ags derived from infectious organisms or tumors has been greatly hampered by the lack of effective CD8 T cell and Th1-inducing adjuvants. These responses collaborate to mediate effective cell-mediated immunity, resulting in the elimination of infected or malignant host cells. Indeed, most vaccines in current use still consist of live attenuated organisms, which can be difficult to manufacture and have potential safety and storage issues. Adjuvants such as mineral oil, mycobacteria, and alum are frequently required to amplify acquired immunity. The most effective is generally considered to be CFA, which can only be used in animals and can cause damaging skin inflammation. In experimental murine studies with molecular adjuvants, the combination of a peptide with anti-CD40 Ab and a TLR agonist is currently the most effective published strategy, with CD8 responses to SIINFEKL reported to be as high as 16% of total CD8 cells in C57BL/6 mice (34).

In this report we have described a novel approach that is much more effective than CFA in the models studied, consists only of defined molecules that have been tested in humans, and can be formulated without oil, making it suitable for use as an aqueous mucosal adjuvant. The levels of Ag-specific CD8 cell responses we induced were higher than those reported in any previous work to our knowledge, and these large clonal expansions consisted of fully functional T cells as indicated by rapid IFN-γ production and CTL activity. Furthermore, we showed that responses could be
induced to single MHC class I-restricted peptides in the absence of CD4 responses if anti-CD40 was included. Anti-CD40 may not be desirable, particularly in prophylactic vaccines, due to its potential to activate B cells (35) or self-reactive CD8 cells (36). Therefore we showed that irrelevant MHC class II-restricted peptides could be used to provide an equivalent signal through provision of CD4 "help," a pathway thought to be mediated via CD40:CD40L interaction and IL-2 (5, 37, 38). By using whole proteins containing MHC class II-restricted epitopes, it was possible to omit anti-CD40. This system would therefore be useful for all types of Ag, ranging from crude microbial extracts to single synthesized peptides predicted from genomic sequences.

Many strategies for the induction of CD8 immunity have used endogenous expression, targeting Ag into the MHC class I pathway. For example, DNA vaccines have been extensively tested but may cause synthesis of Ag within smooth muscle cells rather than targeting DCs (11). Our work shows that potent class I-restricted responses can be induced to exogenous peptide or protein Ag if DCs pick up the Ag and receive sufficient activation signals. The ability of DCs to cross-present exogenous Ag into the class I pathway is dependent on two pathways, one involving Ag transport from the endosomal compartment to the cytosol before entry into the classical class I pathway, and the second most likely involving endosomal digestion and loading of class I molecules (39). Our data suggest that DC activation, and not processing and presentation of Ag, is the limiting factor in the induction of such immunity. DC activation occurs in response to "danger" signals, which include natural ligands of TLRs present on pathogens, inflammation, cytokine release, and cell injury (40). Using this knowledge, we demonstrated that activation via multiple TLRs, CD40, and IFN-γ receptors were all required for a maximal response. Because these signals are also known to cooperate in the induction of IL-12 secretion, it is likely that triggering DC cytokine release in addition to costimulation was critical in obtaining the unrestricted clonal proliferation of CD8 cells. Because the effect of CASAC components on IL-12 secretion (Fig. 4c) was additive rather than synergistic, it is likely that other cytokines or ligands are involved. Increased expression of costimulatory molecules on DC was mainly due to the TLR agonists, whereas increased numbers of the CD8+ CD205+ DC subset in draining LNs was due to the IFN-γ component (Fig. 4). CD40 ligation had no effect on these parameters but contributed to IL-12 secretion. Consistent with this, all components of CASAC were shown to be required for maximal Th1 polarization (Fig. 3b). Therefore the synergistic effects of CASAC components are seen at the level of CD4 and CD8 T cell responses.

pDCs are thought to be particularly involved in the induction of CTLs through their secretion of IFN-α (41), and these were also activated via TLRs. By injecting vaccines i.d., we believe that the Langerhans cell (DC) network in the epidermis (42) was targeted and that signals also reached draining LN-resident DCs such as pDC via lymphatic fluid. Subcutaneous injection induced slightly weaker responses (not shown). Activation of both mDC and pDC may synergize by providing IL-12 (from mDC) in addition to pDC signals (43). mDCs, especially the CD8+ subset, are also more efficient at cross-presentation of protein Ag (39). All of these subsets were activated by TLR agonists. Because TLR expression differs between these lineages (44), this may explain the additional responses seen when two or more agonists, each of which signal through Myd88, are combined. However, although mDCs and pDCs selectively express TLR4 and TLR9, respectively (45), combined TLR4 and TLR9 agonists were no more effective than TLR4/TLR3 or TLR2/TLR3 triggering.

Ligation of CD137 on T cells is thought to be a critical costimulatory event for CD8 but not CD4 responses (18), and anti-CD137 treatment can sustain levels of Ag-specific CD8 cells (28). The ability of vaccines to induce long-lived memory T cell populations is critical to vaccine efficacy, because responses decline rapidly in the absence of Ag (Fig. 1e). Our data (Fig. 1g) indicate that CD137 ligation does not prevent the deletion of expanded cells or increase the percentages of Ag-specific cells that survive long term. However, anti-CD137 did improve the quality of memory induced, as indicated by an improved recall response to peptide in the absence of adjuvant, and is therefore a useful component of secondary immunizations.

Our finding that squalene oil is not required in CASAC suggests that Ag "deposits" at sites of injection are not critical for immunogenicity. By contrast, Tween 80 detergent, which might facilitate the entry of TLR agonists into the intracytoplasmic compartment where TLRs are predominantly expressed (46), was beneficial. The potential for the development of aqueous phase vaccines rather than emulsions is advantageous for preparation and storage, and they might be used as mucosal adjuvants applied topically or by inhalation. This could provide superior immunity focused at relevant points of entry for infection (47), because DCs are able to program T cells for migration to relevant tissues (48, 49).

Many Ags are not fully characterized and do not have class I and class II epitopes identified in multiple MHC backgrounds. Use of anti-CD40 or an unrelated helper Ag may therefore be useful in such vaccines. KLH, a highly immunogenic protein tested in humans, was less effective than anti-CD40 Ab or a class II peptide. An attractive strategy for human peptide immunization might be to use recall peptide Ags to provide CD4 help, because human populations have preestablished immune memory due to vaccination programs.

The intensity of the CD8 responses we induced with CASAC led us to test whether bystander responses to autoantigens could be triggered by such potent DC activation. Although we could induce potent immunity to the tumor-associated self-Ag TRP-2 (30), responses to other self-peptides were weak or absent (Table I). The strength of the response appeared related to peptide:MHC binding affinity. We conclude that CASAC amplifies responses to immunogenic peptides, including tumor-associated and viral Ags, without interfering with self-tolerance mechanisms. The potency of CASAC makes it particularly suitable for tumor vaccines as an attractive alternative to DC therapy. This was demonstrated in a tumor treatment model with the B16 melanoma, a highly aggressive metastatic tumor (33).

The use of CASAC with a single tumor-associated peptide Ag (TRP-2100-108) was extremely effective in halting tumor growth (Fig. 6) and resulted in immunity to further challenge. Only CASAC was able to induce detectable TRP-2 MHC pentamer staining cells (Fig. 6c) and long-lived memory responses (Fig. 6e). This memory was mainly detected within the effector memory CD8 pool of the spleen (Fig. 5e). These responses were specific and there was no evidence of epitope spreading, because no responses to the gp100 Ag were detectable in mice rejecting tumor cells. However, low levels of anti-DNA autoantibodies were detectable in these mice, suggesting a degree of bystander response induced by the vaccine. Proportions of IFN-γ+ cells detected after peptide restimulation were higher than those staining with the MHC pentamer. It has been recently shown (50) that TRP-2 peptide immunization can result in low affinity T cells that produce IFN-γ but do not stain with MHC tetramer and are not protective, presumably due to the poor presentation of the peptide by tumor cells. It therefore seems likely that our vaccine induced a mixture...
of low and high affinity MHC class I molecules. However, we observed a good correlation between IFN-γ production and cytotoxicity (Figs. 5, c and d). Work from other laboratories suggests that use of multiple peptide targets would further enhance the effectiveness of such an anticancer vaccine (33). We observed that CD8 T cell depletion failed to completely abolish protection from tumor development, even though the CD8 populations had not recovered by day 22 (Fig. 6, a–c). It is therefore likely that CASAC induced some non-CD8 T cell-mediated protection through the release of cytokines and the activation of NK or NKT cells. Thus, one advantage of our combined adjuvant approach may be the enhanced recruitment of innate immune mechanisms. The absence of live organisms/vectors in CASAC would facilitate translation toward use in immunocompromised individuals, whereas the use of helper peptides rather than anti-CD40 would be ideal for general prophylactic vaccines.

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Disclosures
King’s College London has applied for a patent related to this work. The inventors are J. W. Wells and A. Noble.

References


