Memory CD4 T Cells Enhance Primary CD8 T-Cell Responses\textsuperscript{\textdagger}

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CD4 T-cell help is required for optimal memory CD8 T-cell responses. We have found that engaging preexisting CD4 Th1, but not Th2, memory cells at the time of CD8 T-cell priming results in increased CD8 effector responses to both bacterial and viral pathogens. The enhanced responses are characterized by increased numbers of cytokine-producing, antigen-specific cells. These findings suggest that engaging endogenous memory Th1 cells may increase cellular responses in an immunotherapy or vaccination setting.

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\textsuperscript{\textdagger} MATERIALS AND METHODS

Mice and reagents. C57BL/6 (B6) mice were purchased from Jackson Laboratories and were housed according to animal care guidelines at the University of Pennsylvania. Conjugated antibodies used to detect CD4, CD8, gamma interferon (IFN-\gamma), interleukin-2 (IL-2), and IL-4 for fluorescence-activated cell sorting (FACS) analysis were purchased from Pharmingen. LLO\textsubscript{190-201} (NEKYAQ AYPNVSNV) and Ova\textsubscript{257-264} (SIINFEKL) were purchased from Invitrogen. Ova-specific T cells were detected using MHC-I tetramers (K\textsuperscript{b}/Ova\textsubscript{257-264}). rLMova was prepared as described previously (2, 8) and was grown in brain heart infusion medium for 15 h at 37°C; 3 x 10\textsuperscript{6} CFU per mouse was injected intravenously unless otherwise indicated.

DC culture. Bone marrow DC precursors were differentiated for 8 to 10 days in the presence of 20 ng/ml of granulocyte-macrophage colony-stimulating factor in RPMI containing 10% fetal calf serum, 100 U/ml penicillin/streptomycin, 50 \mu M \beta-mercaptoethanol, and 2 mM l-glutamine. On days 8 to 10 of culture, DCs were pulsed with 18 ng/ml of Propionibacterium acnes antigen and 50 \mu g/ml schistosome egg antigen (SEA) with or without 10 ng/ml LLO\textsubscript{190} peptide. Following incubation with cells, antigens were washed in phosphate-buffered saline, and 5 x 10\textsuperscript{6} DCs were injected intraperitoneally into each mouse.

Ex vivo cytokine production analysis. Splenocytes were harvested and restimulated for 6 h at a concentration of 15 x 10\textsuperscript{6} cells/ml with or without 1 \mu g/ml LLO\textsubscript{190} in the presence of 50 U/ml human IL-2 (Peprotech) and Golgistop (Pharmingen) in Icoce's medium supplemented with 10% fetal calf serum or 5% normal mouse serum, 100 U/ml penicillin/streptomycin, 50 \mu M \beta-mercaptoethanol, and 2 mM l-glutamine. Cytokine production was determined by using a BD Cytometric Bead Array kit according to the manufacturer's protocol. Data were collected using a BD FACSCalibur and LSRII and were analyzed using FlowJo.

Ad construction and preparation. The Ad hu5 adenovirus (Ad) with E1 and E3 deleted was constructed to express green fluorescent protein (GFP) containing the LLO\textsubscript{190} and Ova\textsubscript{257} epitopes (Ad.LLO.Ova). Ad.LLO.Ova was prepared as described previously (3). pADTrackCMV containing LLO.Ova.GFP was cotransfected with pADEasy-1 into BJ5183 bacteria, and pADTrack/pADEasy recombinants were amplified and transfected into 293 cells. Virus was harvested by multiple rounds of freezing and thawing. A purified Ad construct was prepared by serial infection of 1 plate to 5 plates to 20 plates to 50 150-mm plates of cells. The purified Ad construct was purified by two rounds of buoyant density ultracentrifugation using CsCl, and the preparation was desalted using a desalting column. The genome titer was determined by measuring genomic DNA by multiple rounds of freezing and thawing. The purified Ad construct was purified by two rounds of buoyant density ultracentrifugation using CsCl, and the preparation was desalted using a desalting column. The genome titer was determined by measuring the optical density at 260 nm using a spectrophotometer.

RESULTS AND DISCUSSION

CD4 T-cell help influences the magnitude and quality of the CD8 T-cell response. In some systems, the primary CD8 T-cell responses primed in the absence of CD4 T-cell help are deficient relative to those primed in the presence of T-cell help (1, 4, 12). In other experimental systems, CD4 cells are not required for effective primary CD8 responses (7, 9). However, more profound and consistent deficiencies are observed in memory CD8 T-cell populations when they are generated in the absence of CD4 T-cell help (4, 7, 9, 10). During a primary immune response, CD4 and CD8 T cells expand and differentiate simultaneously, acquiring an effector function 2 to 5 days following antigenic challenge, suggesting that the delivery of help by Th cells may be delayed. In contrast, memory CD4 T cells respond rapidly in response to antigenic challenge, potentially allowing the immediate delivery of help to CD8 T cells, which could influence early events in CD8 T-cell responses. Here we used a system in which CD8 T-cell responses are primed in the presence of T-cell help from preexisting memory CD4 T cells. Specifically, mice primed with dendritic cells (DCs) pulsed with a major histocompatibility complex class II (MHC-II)-restricted peptide, LLO\textsubscript{190} (NEKYAQ AYPNVS), from the listeriolysin O protein of Listeria monocytogenes, which generates an LLO\textsubscript{190} specific Th response, are subsequently challenged with recombinant L. monocytogenes expressing the MHC-I-restricted epitope of ovalbumin (Ova\textsubscript{257-264}, SIINFEKL) (rLMova) (8). This experimental system allows analysis of the primary CD8 T-cell response to Ova in a setting where the LLO\textsubscript{190} specific memory CD4 T-cell population has been engaged. In this study, we showed that engaging memory T helper cells at the time of naïve CD8 T-cell priming enhances CD8 T-cell responses. The enhanced responses are characterized by increased numbers of cytokine-producing, antigen-specific cells. These findings suggest that engaging endogenous memory Th1 cells may increase cellular responses in an immunotherapy or vaccination setting.
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Mice were immunized with DCs pulsed with LLO190 plus heat-killed P. acnes (DC/P. acnes/LLO190) to generate LLO190-specific Th1 CD4 T cells (6). Seven days later, splenocytes were harvested, stimulated with the LLO190 peptide, and stained for intracellular IFN-γ. In line with previous findings (6), DC/P. acnes/LLO190-immunized mice generated Th1 responses as LLO190-specific CD4 T cells produced IFN-γ and IL-2 but not IL-4 (Fig. 1A and data not shown).

To determine whether DC immunization generates functional memory T cells, mice were immunized with DCs pulsed with P. acnes alone or with P. acnes and LLO190 and 60 days later were challenged with rLMova. Seven days following challenge, splenocytes were harvested and stimulated with the LLO190 peptide. Compared to mice immunized with DCs pulsed with P. acnes, mice which had been immunized with DC/P. acnes/LLO190 exhibited a significantly enhanced Th1 response upon challenge with rLMova (Fig. 1B), demonstrating that DC immunization generates functional CD4 memory T cells, which are capable of responding to subsequent L. monocytogenes infection.

To examine the effects of engaging CD4 memory during the CD8 primary response, mice were first immunized with DCs pulsed with P. acnes or with DC/P. acnes/LLO190 and then 60 days later challenged with rLMova. Seven days following challenge, splenocytes were harvested, the numbers of Ova257-specific CD8 T cells were determined by tetramer staining, and IFN-γ- and IL-2-producing cells were identified by intracellular staining (7). We found that the size of the primary CD8 T-cell response, as assessed by each of these parameters, was significantly greater in mice with LLO190-specific memory Th1 cells than in mice that had no CD4 memory for L. monocytogenes.
genes (Fig. 2). These data demonstrate that CD4 memory T cells enhance the primary CD8 T-cell response.

Differentially activated DCs can induce either Th1 or Th2 effector differentiation. DCs pulsed with Toll-like receptor ligands promote differentiation of CD4 T cells to the Th1 lineage (5, 6). Conversely, DCs pulsed with helminth antigens, such as soluble SEA, promote the differentiation of CD4 T cells to the Th2 lineage (6). We took advantage of this system to determine whether the ability of CD4 memory T cells to help the primary CD8 T-cell response was characteristic of CD4 T cells in general or specifically a quality of memory Th1 cells and not Th2 cells. Mice were immunized with DC/PA/LLO190, which generated LLO 190-specific memory Th1 cells, or DCs pulsed with LLO190 plus SEA, which induced LLO190-specific memory Th2 cells. After 60 days, mice were challenged with rLMova. Activating memory Th2 cells did not increase the primary CD8 T-cell response, suggesting that the ability to enhance primary CD8 T-cell responses is a specific property of Th1 memory cells (Fig. 3).

One impediment in vaccination research is the difficulty of eliciting the magnitude of response that is necessary to establish protection. For example, the immunogenicity of nonreplicating Ad can be compromised by preexisting immunity to Ad or the toxicity of high viral loads (11). To determine whether primary CD8 T-cell responses to Ad could be enhanced by existing memory CD4 T cells, mice immunized with DCs pulsed with P. acnes or with DC/PA/LLO190 > 60 days previously were vaccinated with Ad/P. acnes/LLO. The number of Ova257-specific CD8 T cells induced by Ad/LLO,Ova was increased approximately 3-fold when LLO 190-specific memory CD4 T cells were present at the time of vaccination (Fig. 4A and B). To examine whether preexisting immunity elicited by infection might be utilized to enhance vaccine efficacy, mice were infected with L. monocytogenes and 60 days later were vaccinated with Ad/LLO,Ova. Similarly, when LLO190-specific memory CD4 T cells were generated by L. monocytogenes infection, the primary Ova257-specific CD8 T-cell responses were >5-fold greater than those in mice that had never been infected with L. monocytogenes (Fig. 4C). Importantly, in response to immunization with lower doses of Ad, the CD8 T-cell responses in the presence of preexisting Th1 help were comparable to those elicited by a 10-fold-greater Ad titer, indicating that priming CD8 T cells in the presence of Th1 memory could significantly reduce the viral load required to

FIG. 2. CD4 memory T cells enhance the CD8 primary response. Mice were immunized with DCs pulsed with P. acnes with or without LLO190 and challenged 60 days later with rLMova. Splenocytes were analyzed for Ova antigen-specific cells by Kb/Ova tetramer staining. (A) Cells gated on live CD8 splenocytes. The numbers indicate the percentages of gated cells which are tetrramer positive (top panels) or produce the cytokines indicated (bottom panels). (B) Percentages of CD8 splenocytes which are Ova specific or produce IFN-γ or IFN-γ and IL-2. The results for three mice from one of three experiments are shown. An asterisk indicates that the P value is <0.05 as determined by Student’s t test. IMM, immunization; CHG, challenge; PA, P. acnes.

FIG. 3. Th2 memory CD4 T cells do not enhance the primary CD8 response. Mice were immunized with DCs pulsed with P. acnes with or without SEA and LLO190 and challenged 60 days later with rLMova. Splenocytes were analyzed for Ova antigen-specific cells by Kb/Ova tetramer staining. (A) Cells gated on live CD8 splenocytes. The numbers indicate the percentages of gated cells which are tetramer positive. (B) Total number of Ova-specific CD8 T cells. The data are the data for three mice from one of three independent experiments. DC/PA/LLO values differed significantly (P < 0.05, Student’s t test) from DC/PA or DC/SEA/LLO values. IMM, immunization; CHG, challenge; PA, P. acnes.
generate productive CD8 T-cell responses (Fig. 4C and data not shown).

Lastly, we asked whether the enhanced primary CD8 T-cell response elicited in mice that have preexisting pathogen-specific Th1 cells could confer a protective advantage during challenge infection. Mice were immunized with DCs pulsed with P. acnes or with DC/P. acnes/LLO\textsubscript{190} or were infected with L. monocytogenes and >60 days later were challenged with L. monocytogenes. The bacterial numbers in the spleens of mice immunized with DC/P. acnes/LLO\textsubscript{190} were significantly lower than those in the spleens of mice that did not have preexisting Th1 memory (Fig. 5). As expected, mice preinfected with L. monocytogenes were completely protected against challenge infection.

CD4 T-cell responses are known to influence the magnitude and quality of CD8 T-cell responses. Here we show that engaging CD4 memory T cells at the time of naïve CD8 T-cell activation enhances the number of antigen-specific CD8 cells capable of producing IFN-γ and IL-2. Interestingly, Th1, but not Th2, memory cells are capable of providing this help to CD8 cells. Our data suggest that preexisting Th1 immunity resulting from vaccination or infection could be harnessed to enhance vaccine-induced CD8 T-cell immunity in an immunotherapy setting or against pathogens for which defined MHC-I-restricted, but not MHC-II-restricted, epitopes are available.

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FIG. 5. Enhanced CD8 T-cell responses are protective. Mice were immunized with DCs pulsed with P. acnes with or without LLO\textsubscript{190} and challenged 60 days later with a 50% lethal dose of rLMova (1 × 10\textsuperscript{9} CFU). Four days following challenge, spleens were harvested, and the numbers of bacteria in the spleens were determined by serial dilution. The double line indicates the limit of detection. Each data point represents the total number of CFU/spleen for one mouse (three mice per group). The data are representative of three independent experiments. The asterisk indicates that the P value is <0.05 as determined by Student’s t test. n.d., not detected; IMM, immunization; PA, P. acnes.

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