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The Dendritic Cell-Specific Chemokine, Dendritic Cell-Derived CC Chemokine 1, Enhances Protective Cell-Mediated Immunity to Murine Malaria

Oscar Bruna-Romero, John Schmieg, Margarita Del Val, Michael Buschle, and Moriya Tsuji

Cell-mediated immunity plays a crucial role in the control of many infectious diseases, necessitating the need for adjuvants that can augment cellular immune responses elicited by vaccines. It is well established that protection against one such disease, malaria, requires strong CD8+ T cell responses targeted against the liver stages of the causative agent, Plasmodium. In this report we show that the dendritic cell-specific chemokine, dendritic cell-derived CC chemokine 1 (DC-CK1), which is produced in humans and acts on naive lymphocytes, can enhance Ag-specific CD8+ T cell responses when coadministered with either irradiated Plasmodium yoelii sporozoites or a recombinant adenovirus expressing the P. yoelii circumsporozoite protein in mice. We further show that these enhanced T cell responses result in increased protection to malaria in immunized mice challenged with live P. yoelii sporozoites, revealing an adjuvant activity for DC-CK1. DC-CK1 appears to act preferentially on naive mouse lymphocytes, and its adjuvant effect requires IL-12, but not IFN-γ or CD40. Overall, our results show for the first time an in vivo role for DC-CK1 in the establishment of primary T cell responses and indicate the potential of this chemokine as an adjuvant for vaccines against malaria as well as other diseases in which cellular immune responses are important. The Journal of Immunology, 2003, 170: 3195–3203.

Cell-mediated immunity plays a crucial role in the control of many significant infectious diseases, including HIV, tuberculosis, and malaria (1). The development of effective vaccines against these diseases requires the induction of strong CD8+ and/or CD4+ T cell responses; however, current vaccine protocols approved for human use fail to generate strong cellular immune responses (2). Given the need for vaccine preparations that can elicit strong T cell responses, much recent research has been focused on the evaluation of various molecules as possible adjuvants capable of enhancing vaccine-elicited T cell responses. These molecules include a number of cytokines, such as IL-12 and GM-CSF, as well as certain microbial products, such as unmethylated CpG DNA, that are known to stimulate and/or support cell-mediated immune responses (3, 4).

Malaria remains one of the most malignant infectious diseases afflicting humans today, accounting for ~2 million deaths/year. A vaccine continues to be one of the best hopes for ultimate control of the disease, and much research has been performed on the development of a malaria vaccine (5). The feasibility for the development of a malaria vaccine was first suggested when it was shown that immunization with irradiated Plasmodium spp. sporozoites could protect mice, monkeys, and humans from the development of malaria after challenge with live sporozoites (6, 7). Subsequent studies indicated that CD8+ T cells play an important role in this immunity, targeting the liver stages of the disease, and preventing the development of the pathogenic blood stages (8–13). Given the importance of CD8+ T cells in the prevention of Plasmodium liver stages, a number of experimental malaria vaccines have been developed that stimulate strong CD8+ T cell responses in mice. In particular, recombinant viruses expressing specific Plasmodium Ags, such as adenosine (14) and Sindbis virus (15), as single immunizing doses and influenza virus or adenovirus followed by vaccinia virus in prime-boost regimens (16, 17) have been shown to stimulate strong CD8+ T cell responses against malaria liver stages and mediate protection against the disease.

Dendritic cell-derived CC chemokine 1 (DC-CK1; also known as MIP-4/PARC/AMAC-1/CCL18) is a recently identified human CC chemokine produced by dendritic cells. In vitro studies have revealed that DC-CK1 induces both intracellular Ca2+ mobilization and chemotaxis in CD4+ and CD8+ T cells bearing the naive T cell marker CD45RA, but not in T cells bearing the activation marker CD45RO and not in nonlymphocytic cells such as monocytes and granulocytes (18, 19). Another study revealed that DC-CK1 is also chemotactic for CD38-negative mantle zone B cells, indicating that this chemokine acts not only on naive T cells, but on naive B cells as well (20). Given the activity of DC-CK1 on naive lymphocytes and its production by dendritic cells, it is possible that this chemokine is involved in the induction of primary T and B cell responses. This idea is supported by a study that showed a 60-fold increase in DC-CK1 mRNA production in dendritic cells

Abbreviations used in this paper: DC-CK1, dendritic cell-derived CC chemokine 1; AdPyCS, adenovirus expressing the P. yoelii CS protein; CD40L, CD40 ligand; CS, circumsporozoite; IFA, immunofluorescence assay; MCMV, murine CMV; m.o.i., multiplicity of infection; PI3K, phosphatidylinositol 3-kinase; γ-spz, irradiated sporozoite; WT, wild type.
that undergo maturation (21). In addition, DC-CK1 expression has been detected in both germinal centers and T cell areas of lymph nodes, areas where primary B and T cell responses take place (18, 20). Furthermore, a recent in vivo study has shown that naïve T cells cluster in the vicinity of mononuclear cells, producing DC-CK1 mRNA in portal area lymphoid follicles of livers from patients with chronic hepatitis C (22). Taken together, these data strongly suggest a role for DC-CK1 in the initiation and generation of primary T and B cell responses.

In the present report we demonstrate that human DC-CK1 enhances Ag-specific primary CD8+ T cell responses when coadministered with malaria vaccines in mice. We further demonstrate an adjuvant effect for DC-CK1. We show that DC-CK1 is chemotactic for DC-CK1-deficient mice with an H-2d background were generated as previously described (23). P. yoelii (17XNL strain) was maintained by alternate cyclical passages in Anopheles stephensi mosquitoes and Swiss-Webster mice as previously described (24). Sporozoites obtained from dissected salivary glands of infected mosquitoes 2 wk after their infective blood meal were used for immunization as well as its possible use as an adjuvant for vaccines where strong CD8+ T cell responses are required.

**Materials and Methods**

**Animals and parasites**

Six- to 8-wk-old female BALB/c mice, purchased from the National Cancer Institute (Bethesda, MD), were used for most experiments. IL-12p40-deficient mice and CD40-deficient mice of BALB/c background were purchased from the Jackson Laboratory (Bar Harbor, ME). IFN-γ receptor-deficient mice with an H-2d background were generated as previously described (23). P. yoelii (17XNL strain) was maintained by alternate cyclical passages in Anopheles stephensi mosquitoes and Swiss-Webster mice as previously described (24). Sporozoites obtained from dissected salivary glands of infected mosquitoes 2 wk after their infective blood meal were used for immunization as well as challenge of the mice.

**Construction and screening of recombinant adenosviruses**

Recombinant adenosvirus expressing the P. yoelii circumsporozoite (CS) protein (AdPyCS) was constructed by first inserting a PCR fragment containing the open reading frame for aa 1–356 of the CS protein into a CMV expression cassette containing the CMV immediate early promoter, followed by transfer of this cassette into the adenoviral shuttle vector pMV60 (25). Recombinant human IFN-γ 5 adenoviruses were generated in 293 cells (ATCC CRL-1573; American Type Culture Collection, Manassas, VA) by homologous recombination of the constructed shuttle plasmid and plasmid pM17 containing the complete Ad5 DE1AE3 genome (26). Individual viral clones were obtained and analyzed for recombinant gene expression by RT-PCR and for recombinant protein expression by Western blotting (27). 293 cells were infected with AdPyCS at a multiplicity of infection (m.o.i.) of 10 for 24 h and then assayed, while for Western blot, 293 cells were infected with AdPyCS at an m.o.i. of 200 for 36 h. The clone expressing the most recombinant protein was then purified via large-scale CsCl purification, dialyzed, and frozen in aliquots for use in immunizing animals.

Recombinant adenovirus Adp89-CD8, homologue to the recombinant vaccinia virus sc-A9A (27), expresses the H-2Ld-restricted nonameric CTL epitope from the immediate early protein 1 (IE1/pp89) of the murine CMV (MCMV) inserted into a modified hepatitis B virus HBeAg in which the original signal peptide of the protein was replaced by the signal peptide of the hemaggulitin protein of influenza virus. A PCR fragment encoding this protein was inserted into the same CMV expression cassette noted above, which was then transferred into shuttle vector pMV60. Replication-deficient, human type 5 adenoviruses were generated as described above, and individual clones were screened for recombinant protein expression by Western blot. For this purpose, BALB/c mouse fibroblasts were infected with Adp89-CD8 at an m.o.i. of 200 for 36 h and then probed with polyclonal goat anti-HBe (27) and HRP-labeled anti-rabbit IgG (Pierce, Rockford, IL). The clone expressing the most recombinant product was then purified via large-scale CsCl purification, dialyzed, and frozen in aliquots for use in immunizing animals.

Recombinant adenovirus AdDC-CK1 was constructed by first PCR cloning cDNA of human DC-CK1 using forward primer 5′-GGTTG GTCTAGAACCATGAAGGCTTGGCATCT-3′ and reverse primer 5′-TCAAATCTAGATCCAGGATTGCAGTTCAGGTC-3′, followed by insertion of the cDNA into the same CMV expression cassette noted above, with individual clones being screened for DC-CK1 mRNA and protein production by RT-PCR and immunofluorescence assay (IFA), respectively. For RT-PCR, 293 cells were infected with AdDC-CK1 at an m.o.i. of 10 for 24 h, and DC-CK1 cDNA was probed using the primers noted above. For IFA, primary cultures of BALB/c mouse fibroblasts were infected with AdDC-CK1 at an m.o.i. of 200 for 36 h, and DC-CK1 protein was probed using a mouse mAb against human DC-CK1 (R&D Systems, Minneapolis, MN) and a FITC-labeled goat Ab against mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The clone expressing the most DC-CK1 was purified by large-scale CsCl purification, dialyzed, and frozen in aliquots for subsequent experiments.

**Immunizations and challenge**

Details of the protocols used for immunization in different experiments are described in the figure legends. In all experiments groups of three to five mice were immunized s.c. with suboptimal doses of immunogen (2 × 103 irradiated sporozoites/mouse, 2 × 105 PFU AdPyCS/mouse, or 5 × 106 PFU Adp89-CD8/mouse) with or without doses or recombinant human DC-CK1 protein (Research Diagnostics, Flanders, NJ) or AdDC-CK1 in a final volume of 100 μl/mouse.

Challenge of mice to determine the inhibition of liver stage development was performed by i.v. injection of 10,000 viable sporozoites into the tail vein 2 wk after immunization. The outcome of the challenge was determined 42–44 h later by measuring the parasite burden in the liver of the mice using a quantitative real-time RT-PCR method.

**Chemotaxis assays**

The capacity of BALB/c splenocytes to migrate in response to human DC-CK1 was assessed in 24-well plates where each well was divided into two chambers by 6.5-mm Transwell inserts containing 5-μm pore size polycarbonate filters (Costar, Cambridge, MA). The filters inserts were filled with 2 × 106 cells, and the lower compartment contained different dilutions of human DC-CK1 protein (Research Diagnostics) or supernatants from AdDC-CK1-infected cells in a final volume of 600 μl. For generation of supernatants of AdDC-CK1-infected cells, 1 × 106 293 cells were infected with the recombinant virus at an m.o.i. of 10 for 36 h. Culture medium was substituted for DMEM without FBS during the last 16 h to avoid interference in the assay of other chemotactic factors with the secreted protein. Migration was allowed to occur for 3 h at 37°C in 5% CO2, after which the cells from the lower compartment were collected and counted at a fixed flow rate (60 μl/min) for 60 s using a FACSCalibur (Becton, Dickinson and Co., San Jose, CA). All determinations were performed in triplicate. Purified populations of lymphocytes were obtained by magnetic bead labeling using FITC-labeled Abs against CD62L, Thy1.2, and CD19 molecules (all from BD PharMingen, San Diego, CA) together with anti-FITC beads and MACS purification columns (Miltenyi Biotec, Auburn, CA). Populations were consistently 85–94% pure.

**Detection and quantification of P. yoelii rRNA sequences by RT and real-time PCR**

Quantification of Plasmodium 18S rRNA sequences was performed using a recently developed real-time RT-PCR technique (28). Briefly, total RNA (2 μg) from the livers of mice challenged with 10,000 viable sporozoites 42–44 h earlier was reverse transcribed, and an aliquot of the resulting cDNA (133 ng) was used for real-time RT-PCR amplification of P. yoelii 18S rRNA sequences. This amplification was performed in a GeneAmp 5700 sequence Detection System (PE Applied Biosystems, Foster City, CA). For this purpose, we used primers 5′-GGGATTTGTTTGATCGTITTTCGCG-3′ (54 nM) and 5′-AACATTAAAAAAGCGGATCATCCCTAT-3′ (60 nM) together with the dsDNA-specific dye SYBR Green I incorporated into the PCR reaction buffer (PE Biosystems, Foster City, CA) to detect the PCR product generated. The temperature profile of the reaction was 95°C for 15 s and annealing/extension at 60°C for 1 min.

Quantification of epiteme-specific CD8+ T cells by ELISPOT assay

The relative number of CS-specific, IFN-γ-secreting CD8+ T cells in the spleens of mice receiving different immunization regimens was determined by direct ex vivo ELISPOT assays, as previously described (29). For these assays, we used MHC-compatible A20.23 target cells coated with the CD8-derived H-2Kb-restricted epitope SYVPSAEQI, which is recognized by CS-specific CD8+ T cells. For quantification of IFN-γ-secreting CD8+ T cells.
cells specific for the CTL epitope of the MCMV immediate early protein 1, we used A20.2J target cells coated with the H-2L<sup>d</sup>-restricted epitope YPHFMPPTNL.

**Indirect IFA**

*P. yoelii* anti-sporozoite Ab titers in the sera of immunized mice were determined using sporozoites air-dried onto multispot glass slides. After 1 h of incubation of these Ag slides with different sera diluted in PBS containing 1% BSA, the slides were washed and incubated with a FITC-labeled goat anti-mouse IgG Ab for another hour (Kirkegaard & Perry Laboratories). After repeated washes, the slides were mounted, and the anti-sporozoite Ab titers were determined as the highest serum dilution producing fluorescence when viewed under an UV microscope.

**Results**

Coadministration of DC-CK1 enhances Ag-specific T cell responses elicited by vaccines

CD8<sup>+</sup> T cells secreting IFN-γ have been shown to be the main mediators of protection against the liver stages of murine malaria (8, 13). Given the probable involvement of DC-CK1 in promoting the generation of primary T cell responses, we sought to determine whether exogenously administered DC-CK1 could enhance primary antimalaria T cell responses. For this purpose we used two sources of exogenous DC-CK1: recombinant DC-CK1 protein (rDC-CK1) and a recombinant adenovirus expressing DC-CK1 (AdDC-CK1). Groups of BALB/c mice were immunized with either irradiated sporozoites (γ-spz) or a recombinant adenovirus expressing the CS protein of *P. yoelii* (AdPyCS) in the presence or the absence of different doses of rDC-CK1 or AdDC-CK1. Two weeks after immunization, the number of CS-specific, IFN-γ-secreting CD8<sup>+</sup> T cells was determined by ELISPOT assay.

When mice were injected s.c. with suboptimal doses of either γ-spz or AdPyCS, together with different doses of rDC-CK1, we found that coadministration of 100 ng of rDC-CK1 resulted in the induction of the highest number of CS-specific T cells elicited by either immunogen (Fig. 1, A and B). This dose of chemokine stimulated a 3- to 4-fold increase in the number of CS-specific CD8<sup>+</sup> T cells secreting IFN-γ compared with that of control mice receiving either immunogen alone. This DC-CK1-elicited increase in the CS-specific T cell response was also observed when rDC-CK1 was coadministered with either immunogen i.m., but not when it was given by a different route as the immunogen or at a different time (data not shown).

A similar result was obtained when we injected groups of mice with a suboptimal dose of AdPyCS together with different doses of AdDC-CK1. The number of CS-specific, IFN-γ-secreting CD8<sup>+</sup> T cells elicited upon coadministration of AdPyCS and AdDC-CK1 depended on the dose of AdDC-CK1 coadministered, with a dose 10<sup>7</sup> PFU of AdDC-CK1 resulting in the greatest enhancement of the CS-specific T cell response (Fig. 1C). This dose of AdDC-CK1 resulted in a 3- to 4-fold increase in the number of CS-specific CD8<sup>+</sup> T cells secreting IFN-γ compared with that of control mice receiving AdPyCS alone. This AdDC-CK1-driven increase in the CS-specific CD8<sup>+</sup> T cell response was due to DC-CK1, and not some other nonspecific effect, because mice coinfected with AdPyCS and a control adenovirus expressing the *Escherichia coli* β-galactosidase gene (AdLacZ) showed no increase in the malaria-specific T cell response (Fig. 1D). Interestingly, when AdDC-CK1 was coadministered with a suboptimal dose of γ-spz, no enhancement of the CS-specific T cell response was observed regardless of the dose of AdDC-CK1 given (data not shown).

In addition to the number of IFN-γ-secreting CD8<sup>+</sup> T cells elicited by DC-CK1, we were interested in determining DC-CK1’s effect on the number of CS-specific CD8<sup>+</sup> T cells secreting IL-4.

For this purpose we immunized mice with γ-spz or AdPyCS with or without AdDC-CK1 or Adpp89-CK1, and 2 wk later determined the number of CS-specific CD8<sup>+</sup> T cells secreting IL-4 by ELISPOT assay. Coadministration of DC-CK1 with either malaria immunogen resulted in no difference in the number of CS-specific, IL-4-secreting CD8<sup>+</sup> T cells compared with mice receiving either immunogen alone (data not shown). Likewise, when we determined the serum titers of anti-sporozoite Abs via an IFA of air-dried sporozoites, we found that coadministration of DC-CK1 with immunogen failed to increase the sporozoite-specific Ab titers in the sera of malaria-immunized mice (data not shown).

Finally, we were interested in determining whether DC-CK1 could enhance T cell responses elicited by vaccines to other microbial infections in which cell-mediated immunity plays an important role. To address this question, we used as an immunogen a recombinant adenovirus expressing a well-characterized, protective, H-2L<sup>d</sup>-restricted CTL epitope of the MCMV pp89 protein (Adpp89-CD8) (30). Groups of mice were immunized s.c. with a suboptimal dose of Adpp89-CD8, with or without coadministration of 100 ng of recombinant DC-CK1 or 10<sup>9</sup> PFU of AdDC-CK1, and the number of epitope-specific CD8<sup>+</sup> T cells secreting IFN-γ was determined 2 wk later by ELISPOT assay. As with AdPyCS, both DC-CK1 protein and AdDC-CK1 strongly enhanced the MCMV-specific CD8<sup>+</sup> T cell response elicited by Adpp89-CD8 immunization (Fig. 2). These results indicate that DC-CK1 can enhance T cell responses regardless of the Ags used. It also indicates that DC-CK1’s ability to enhance vaccine-elicited T cell responses is a phenomenon related not only to the H-2K<sup>d</sup>-restricted CD8<sup>+</sup> T cell epitope of the CS protein, but can also be applied to epitopes restricted to other MHC class I molecules.

**FIGURE 1.** DC-CK1 increases the level of Ag-specific CD8<sup>+</sup> T cell responses elicited by malaria vaccines. Groups of BALB/c mice were immunized s.c. with 2 × 10<sup>4</sup> γ-spz (A) or 2 × 10<sup>7</sup> PFU AdPyCS (B) with or without different doses of rDC-CK1 by the same route, 2 × 10<sup>7</sup> PFU AdPyCS with or without different doses of AdDC-CK1 by the same route (C), or 2 × 10<sup>7</sup> PFU AdPyCS with or without 10<sup>8</sup> PFU AdDC-CK1 or 10<sup>8</sup> PFU of a control adenovirus, AdLacZ, by the same route (D). Two weeks after immunization, splenic lymphocytes were isolated from all mice, and the relative numbers of IFN-γ-secreting, CS-specific CD8<sup>+</sup> T cells were determined by ELISPOT assay. The results are expressed as the average ± SD of triplicate cultures. In all figures the data represent one of two or more experiments with similar results.
Coadministration of DC-CK1 enhances protective antimalaria immunity elicited by malaria immunogens

It has been shown that both γ-spz and AdPyCS are capable of conferring sterile immunity to malaria in a significant number of immunized mice subsequently challenged with live sporozoites (31). This sterile immunity was found to be due to the immunogens’ ability to induce strong, specific T cell responses that target and suppress the development of *Plasmodium* liver stages. Since we found that DC-CK1 enhances malaria-specific CD8+ T cell responses induced by γ-spz and AdPyCS, we sought to determine whether DC-CK1 could enhance the levels of antimalaria protection elicited by these two immunogens as well. For this purpose we immunized mice with suboptimal doses of either γ-spz or AdPyCS, with or without coadministration of DC-CK1. Two weeks later these mice along with unimmunized controls were challenged i.v. with 10,000 live sporozoites, and 42–44 h later the livers of the mice were obtained. Total RNA from the livers was then used in a highly sensitive, real-time RT-PCR assay to quantify the amounts of parasite-specific 18S rRNA present in mouse livers. The amounts of parasite-specific rRNA in the livers served as an indication of the degree to which *Plasmodium* liver stages were able to develop in the differentially immunized mice.

When we immunized mice with a suboptimal dose of γ-spz together with 100 ng of rDC-CK1, we found that the amount of parasite-specific 18S rRNA in the livers was less than that found in mice receiving the immunogen alone (Fig. 3A). This enhanced protection against the development of *Plasmodium* liver stages was also observed in mice immunized with a suboptimal dose of AdPyCS together with 100 ng of rDC-CK1 (Fig. 3B). These results mirror the aforementioned data showing that DC-CK1 can enhance malaria-specific CD8+ T cell responses elicited by γ-spz and AdPyCS, suggesting that such T cell responses are responsible for the inhibited development of *Plasmodium* liver stages.

Finally, we found that coadministration of a suboptimal dose of AdPyCS along with 10^8 PFU of AdDC-CK1 also results in the appearance of less parasite-specific 18S rRNA in the livers of mice compared with those receiving AdPyCS alone (Fig. 3C). Interestingly, coadministration of 10^7 or 10^9 PFU of AdDC-CK1 with AdPyCS did not result in enhanced protection against malaria liver stages. As shown earlier, these two doses of AdDC-CK1 fail to enhance malaria-specific CD8+ T cell responses elicited by AdPyCS. Thus, as with rDC-CK1, the ability of AdDC-CK1 to augment antimalaria protection appears to be due to the enhanced T cell responses stimulated by DC-CK1.

**DC-CK1 stimulates chemotaxis in murine lymphocytes**

To clarify the mechanism by which DC-CK1 exerts its adjuvant activity, we first sought to determine what cell types in mice respond to the chemokine. Although the mouse homologue of DC-CK1 has not yet been identified, a previous study showed that i.p. injection of synthetic human DC-CK1 into mice resulted in the accumulation of CD4+ and CD8+ T cells in the peritoneal cavity, but not monocytes or granulocytes. Thus, in mice, as in humans, DC-CK1 appears to act preferentially on lymphocytes (32). To confirm this, we performed in vitro chemotaxis assays, which measure the ability of cells to migrate from an upper chamber through a porous membrane into a lower chamber containing chemokine. First, we checked the ability of DC-CK1 to stimulate chemotaxis in unfractionated murine splenocytes. As sources of DC-CK1 we used both rDC-CK1 as well as supernatants from cells infected with AdDC-CK1. Recombinant DC-CK1 protein was able to stimulate chemotaxis in murine splenocytes in a dose-dependent manner. Increasing concentrations of DC-CK1 protein resulted in increasingly larger numbers of splenocytes migrating from the upper chamber into the DC-CK1-filled lower chamber (Fig. 4A). Similarly, supernatants collected from cell cultures infected with AdDC-CK1 also stimulated chemotaxis in murine splenocytes. This activity was due to DC-CK1 present in the supernatant, because supernatants from cells infected with the control adenovirus, AdLacZ, did not induce significant chemotaxis in the same cell population (Fig. 4B). Overall, these data indicate that DC-CK1 can act on mouse cells and stimulate chemotaxis.

To characterize the murine cell types responsive to DC-CK1, we purified various cell populations from unfractionated splenocytes and performed chemotaxis assays to identify the cell types that respond to the chemokine. First, we purified B and T cells by way

![FIGURE 2. DC-CK1 increases the level of the Ag-specific CD8+ T cell response elicited by a recombinant adenovirus expressing an MCMV CD8 epitope. Groups of three BALB/c mice were immunized with 5 × 10^6 PFU AdPyCS-CDC with or without 100 ng of rDC-CK1 or 10^6 PFU AdDC-CK1 by the same route. Two weeks later splenic lymphocytes were isolated, and the relative numbers of IFN-γ-secreting, pp-89-specific CD8+ T cells were determined by ELISPOT assay. The results are expressed as the average ± SD of triplicate cultures.](https://www.jimmunol.org/content/189/4/3198/F2.large.jpg)

![FIGURE 3. DC-CK1 enhances protective antimalaria immunity induced by irradiated sporozoites and a recombinant adenovirus expressing a plasmodial Ag. Groups of five BALB/c mice were immunized s.c. with 2 × 10^7 γ-spz (A) or 2 × 10^7 PFU AdPyCS (B) with or without 100 ng rDC-CK1 or with 2 × 10^7 PFU AdPyCS with or without different doses of AdDC-CK1 (C). Two weeks after immunization, all mice along with unimmunized controls were challenged i.v. with 10,000 live sporozoites, and the amounts of parasite-specific 18S rRNA in the livers were determined by real-time RT-PCR assay. Results are expressed as the average ± SD of five mice.](https://www.jimmunol.org/content/189/4/3198/F3.large.jpg)
of positive selection and performed chemotaxis assays on both cell types. After fractionation, we performed chemotaxis assays on each cell population and found that a higher number of CD62L<sup>+</sup> cells responded to DC-CK1, suggesting that both cell populations are equally responsive to the chemokine. Next, we wanted to determine whether DC-CK1, as in humans, preferentially acts on naive lymphocytes in mice (Fig. 4C). Despite this observed preference of DC-CK1 for CD62L<sup>+</sup> cells, a certain number of CD62L<sup>−</sup> cells did respond to the chemokine, suggesting that DC-CK1 does not act exclusively on naive lymphocytes in mice.

**FIGURE 4.** DC-CK1 stimulates chemotaxis in mouse splenocytes. Unfractionated splenocytes from BALB/c mice were exposed to different concentrations of rDC-CK1 (A) or culture supernatants (B) of cells infected with control medium (CM), AdDC-CK1, or AdLacZ and analyzed for migration through 5-μm pore size filters via a chemotaxis assay. Lymphocyte subsets purified from unfractionated splenocytes representing T (Thy1.2<sup>+</sup>) and B (CD19<sup>+</sup>) cells (C) along with naive (CD62L<sup>+</sup>) and effector/memory (CD62L<sup>−</sup>) cells (D) along with the corresponding controls were exposed to either 300 ng/ml rDC-CK1 (+) or control medium lacking rDC-CK1 (−) and then analyzed for migration using a chemotaxis assay. Values were determined by flow cytometry during a fixed period of time and a fixed flow rate. Results are expressed as the average ± SD of triplicate cultures.

**FIGURE 5.** The adjuvant activity of DC-CK1 does not require IFN-γ. Groups of three WT control mice (A) or IFN-γR-KO mice (B) were immunized s.c. with 2 × 10<sup>8</sup> PFU AdPyCS with or without 100 ng rDC-CK1. Two weeks later, the relative numbers of IFN-γ-secreting, CS-specific CD8<sup>+</sup> T cells were determined by ELISPOT assay. Groups of three WT control mice (C) or IL-12-knockout mice (D) were immunized with 2 × 10<sup>8</sup> PFU AdPyCS with or without rDC-CK1 by the same route, and the relative numbers of IFN-γ-secreting, CS-specific CD8<sup>+</sup> T cells were determined 2 wk later by ELISPOT. Results are expressed as the average ± SD of triplicate cultures.

**FIGURE 6.** The adjuvant effect of DC-CK1 requires IL-12. Groups of three WT control mice (A) or IL-12-knockout mice (B) were immunized s.c. with 2 × 10<sup>8</sup> PFU AdPyCS with or without 100 ng rDC-CK1. Two weeks later, the relative numbers of IFN-γ-secreting, CS-specific CD8<sup>+</sup> T cells were determined by ELISPOT assay. Groups of three WT control mice (C) or IL-12-knockout mice (D) were immunized with 2 × 10<sup>8</sup> PFU AdPyCS with or without rDC-CK1 by the same route, and the relative numbers of IFN-γ-secreting, CS-specific CD8<sup>+</sup> T cells were determined 2 wk later by ELISPOT. Results are expressed as the average ± SD of triplicate cultures.

CD8<sup>+</sup> T cell responses in the absence of IL-12

IFN-γ and IL-12 are two cytokines important in the induction and maintenance of Th1-polarized T cell responses, which are characterized by IFN-γ-producing T cells (33–36). Since DC-CK1 enhances such responses in malaria vaccine-immunized mice in vivo, we wanted to learn whether either one of these molecules is involved in the adjuvant effect of DC-CK1. For this purpose, we immunized mice deficient in either IFN-γ receptor or IL-12p40 along with wild-type (WT) control mice of the same genetic background with AdPyCS with or without rDC-CK1 or AdDC-CK1. Two weeks after immunization we measured the CS-specific, CD8<sup>+</sup> T cell response by ELISPOT. In IFN-γ receptor-deficient mice, AdDC-CK1 was able to enhance the CS-specific, CD8<sup>+</sup> T cell response elicited by AdPyCS to the same degree as WT controls, indicating that the adjuvant effect of DC-CK1 is independent of IFN-γ (Fig. 5). In contrast, DC-CK1 failed to enhance the CS-specific CD8<sup>+</sup> T cell response elicited by AdPyCS in mice lacking IL-12, but not in WT controls (Fig. 6). This IL-12 dependence was observed in AdPyCS-immunized mice receiving either rDC-CK1 (Fig. 6, A and B) or AdDC-CK1 (Fig. 6, C and D), indicating that the adjuvant activity of DC-CK1 requires IL-12.

It is known that optimal IL-12 production by APCs during the induction of Th1-polarized T cell responses requires engagement of the APC’s CD40 molecules by the CD40 ligand (CD40L) molecules of activated T cells (37–40). To determine whether the adjuvant activity of DC-CK1 requires such signaling, we immunized mice deficient in CD40 along with WT controls with AdPyCS with or without AdDC-CK1, and 2 wk later we measured the CS-specific, CD8<sup>+</sup> T cell response by ELISPOT. CD40 knockout mice receiving both AdPyCS and AdDC-CK1 developed a 2- to 3-fold increase in the number of CS-specific, CD8<sup>+</sup> T cells secreting
IFN-γ compared with knockout mice receiving AdPyCS alone (Fig. 7B). Similarly, WT control mice receiving both AdPyCS and AdDC-CK1 developed a 2- to 3-fold enhancement of the CS-specific, CD8⁺ T cell response compared with WT mice receiving AdPyCS alone (Fig. 7A). Thus, it appears that the IL-12-dependent adjuvant activity of DC-CK1 does not operate through the CD40-CD40L signaling pathway, but, instead, involves other factors.

**Discussion**

In the current report we assessed the ability of the recently discovered human chemokine DC-CK1 to modulate acquired antimalaria immunity in mice. We found that coadministration of DC-CK1, as either a recombinant protein (rDC-CK1) or a recombinant adenovirus (AdDC-CK1), to mice immunized with either a suboptimal dose of adjuvant or a recombinant adenovirus expressing the protective CD8⁺ T cell epitope of the MCMV pp89 protein (Adpp89-CD8). Whether as rDC-CK1 or AdDC-CK1, chemokine coadministration with Adpp89-CD8 enhanced the MCMV-specific CD8⁺ T cell response to a degree comparable to that of γ-spz and AdPyCS. This result indicates that the adjuvant activity of DC-CK1 is not specific only for malaria Ags, but works for other pathogens as well.

It is noteworthy that the administration of a high dose of either rDC-CK1 (300 ng) or AdDC-CK1 (10⁸ PFU) with malaria vaccine failed to induce an optimal increase in the CS-specific CD8⁺ T cell response. It is possible that giving too much DC-CK1, as either a recombinant protein or a recombinant adenovirus, results in an overstimulation of T cells, which may lead to inhibitory effects that partially cancel out the stimulatory effects of the chemokine. As discussed below, DC-CK1’s adjuvant activity requires the presence of IL-12. The primary effect of IL-12 on activated T cells is the stimulation of IFN-γ production by these cells (33–36, 40). It is well established that one effect of IFN-γ on activated T cells is enhanced apoptosis (41–43). Thus, too much DC-CK1 may, via IL-12, cause an overproduction of IFN-γ, which could result in enhanced apoptosis of recently activated CS-specific CD8⁺ T cells, thereby lowering the number of such cells elicited by vaccination and canceling out some of the stimulatory effects of the chemokine.

Alternatively, injecting too high a dose of DC-CK1 could result in a divergence of responsive cell types away from the anatomical locales where T cell activation takes place. As discussed below, DC-CK1’s adjuvant effect appears to result from its interaction with naive T cells in the cortical regions of draining lymph nodes. It is possible that injecting too high a dose of DC-CK1, either as a recombinant protein or a recombinant adenovirus, results in a more widespread distribution of the chemokine to parts of the body other than the draining lymph nodes. Such a broader distribution of the chemokine could conceivably result in a diversion of circulating naive T cells to these other sites, preventing them from reaching the appropriate lymph node areas where Ag presentation and T cell activation occur. As a result, fewer numbers of naive, Ag-specific T cells would interact with APCs, thereby counteracting any stimulatory effect the chemokine has on those naive T cells that do.

In the case of rDC-CK1, another possibility is that administration of too high a concentration results in aggregation of the chemokine. Due to their hydrophobic character, chemokines have a tendency to self-aggregate under physiological conditions, especially at higher concentrations, resulting in an inability of the chemokine to interact with its cognate receptor (44). If such a phenomenon is occurring at a disproportionate level with the higher doses of rDC-CK1, it could conceivably result in an effective neutralization of much of the chemokine and, as discussed below, lesser amounts migrating to the appropriate anatomical compartment, where it can interact with responsive cell types.

Finally, for AdDC-CK1, which only reveals an adjuvant effect when coadministered with AdPyCS, the lack of a dose-dependent enhancement of the CS-specific CD8⁺ T cell response is probably due to in vivo competition of the two viruses for cells that can support infection. In vivo, there is only a limited supply of cells bearing a limited number of receptors necessary for adenovirus uptake by dendritic cells.
infection. Injecting an excessive amount of AdDC-CK1 relative to AdPyCS would result in a disproportionate infection of cells by AdDC-CK1, and the blocking of optimal AdPyCS infection in vivo. Such interference would lead to less overall expression of the CS protein, which is the antigenic target of the antimalarial immune response, and thus to a lower CS-specific CD8^+ T cell response. In contrast, injecting too little AdDC-CK1 with AdPyCS would result in insufficient AdDC-CK1 infection in vivo to ensure adequate production of DC-CK1 protein required for an adjuvant effect. Only when AdPyCS and AdDC-CK1 are injected in the proper proportion will both viruses infect enough cells to bring about adequate production of both Ag and chemokine required for an adjuvant effect. With our fixed suboptimal dose of AdPyCS (2 × 10^7 PFU), such a balance only occurs with a dose of 10^8 PFU AdDC-CK1, not more or less.

In humans, DC-CK1 acts exclusively on naive T and B cells (18–20). Although a murine homologue of DC-CK1 has not yet been identified, a recent study by Guan et al. (32) showed that in response to an i.p. injection of synthetic human DC-CK1 in mice, CD4^+ and CD8^+ T cells accumulated in the peritoneal cavity, while monocytes and granulocytes did not; B cells were not examined. This result suggests that DC-CK1 acts on similar types of cells in mice as in humans. To test this idea, we performed in vitro chemotaxis assays on mouse splenocytes to determine what types of murine cells respond best to DC-CK1. Our results showed that T and B cells respond equally well to DC-CK1. In addition, our results indicate that T and B cells expressing the naive lymphocyte marker CD62L respond better to DC-CK1 than such cells not expressing this marker (i.e., effector/memory cells). Taken together, these data suggest that in mice, as in humans, DC-CK1 preferentially acts on naive lymphocytes.

The chemotaxis assay results also suggest that the adjuvant activity of DC-CK1 is mediated by events occurring in naive lymphocytes after interaction with the chemokine. Since naive lymphocytes primarily reside in the cortical regions of lymph nodes, and DC-CK1 only displays an adjuvant effect when injected at the same time and place as the immunogen, the question arises as to how a peripherally deposited chemokine such as DC-CK1 acts on naive lymphocytes. While such an increase in sensitivity might occur by way of a mediated signal transduction pathway and the IL-12-mediated signal pathway, which up-regulates the expression of cell cycle-related molecules such as cyclin D3 and anti-apoptotic molecules such as Bel-2 and down-regulates proapoptotic molecules such as caspase-3. In addition, the authors show that this signaling pathway is not involved in IFN-γ induction by IL-12. Since DC-CK1 acts on naive T cells, a possible explanation for its IL-12-dependent adjuvant effect is that it renders these cells more sensitive to IL-12-induced proliferation and survival after activation by APCs. While such an increase in sensitivity might occur by way of a DC-CK1-mediated up-regulation of the IL-12R in T cells, it is more likely due to a synergistic interaction of the DC-CK1-mediated signal transduction pathway and the IL-12-mediated signal transduction pathway in T cells. Although DC-CK1’s receptor has not yet been identified in humans or mice, it appears that it, like all chemokines identified to date, signals through a seven-transmembrane domain receptor coupled to a pertussis toxin-sensitive heterotrimeric G_{i/o} protein (20, 51, 52). It is known that a number of chemokine receptors, after interaction with their cognate ligand, stimulate both PI3K and Akt via the βγ subunit of their heterotrimeric G_{i/o} protein and that both molecules are required for the stimulation of proper chemotaxis as well as the proliferation and prevention of apoptosis in target cells (52, 53). If DC-CK1’s receptor also stimulates PI3K and Akt as part of its signal transduction cascade, then it would necessarily intersect with the IL-12-stimulated cascade. Moreover, naive T cell interaction with DC-CK1 would stimulate these important downstream messengers involved in IL-12 signaling before IL-12 exposure, potentially amplifying the IL-12 signal to the T cells after activation. Since PI3K and Akt are involved in transmitting proliferative and antiapoptotic signals...
to T cells by IL-12, the enhancement of these activities by DC-CK1 signaling would probably result in an augmented clonal expansion of Ag-specific T cells in response to immunization. In addition, because the IL-12-induced PI3K/Akt signaling pathway is not involved in IFN-γ induction, the adjuvant effect of DC-CK1 would not be expected to use IFN-γ, which is consistent with our results. Unfortunately, DC-CK1’s receptor is not known, making a more thorough analysis of the signaling pathways involved in its adjuvant effect difficult. Whatever the mechanism, the permissive effect of DC-CK1 on IL-12 signaling is intriguing in light of much recent evidence indicating a down-regulatory effect of G protein-coupled receptor signaling on IL-12 production and signaling (54–57).

Overall, our report indicates the potential of DC-CK1 as a vaccine adjuvant for diseases in which strong cellular immune responses are needed. In addition, it adds to the relatively small number of studies showing the promise of chemokines as vaccine adjuvants (58, 59). More importantly, we provide the first evidence that DC-CK1, a chemokine produced by dendritic cells, active on naive lymphocytes, and presumably involved in the orchestration of adaptive immune responses, actually participates in the induction of primary T cell responses in vivo. Furthermore, we show that this participation is IL-12 dependent, thereby shedding new light on the role of chemokines in regulating T cell differentiation.

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References


