Codelivery of the Chemokine CCL3 by an Adenovirus-Based Vaccine Improves Protection from Retrovirus Infection

Ruth Lietz,^a Wibke Bayer,^{a,b} Teona Ontikatz,^{a*} Lena Johrden,^a Matthias Tenbusch,^a Michael Storcksdieck genannt Bonsmann,^a Klaus Überla,^a Ulf Dittmer,^b and Oliver Wildner^{a*}

Department of Molecular and Medical Virology, Institute of Microbiology and Hygiene, Ruhr-University Bochum, Bochum, Germany,^a and Institute of Virology, University Hospital Essen, University Duisburg-Essen, Essen, Germany^b

Processing and presentation of vaccine antigens by professional antigen-presenting cells (APCs) is of great importance for the efficient induction of protective immunity. We analyzed whether the efficacy of an adenovirus-based retroviral vaccine can be enhanced by coadministration of adenovirus-encoded chemokines that attract and stimulate APCs. In the Friend retrovirus (FV) mouse model we coexpressed CCL3, CCL20, CCL21, or CXCL14 from adenoviral vectors, together with FV Gag and Env antigens, and then analyzed immune responses and protection from pathogenic FV infection. Although most tested chemokines did not improve protection against FV challenge, mice that received adenoviral vectors encoding CCL3 together with FV antigens showed significantly better control over viral loads and FV-induced disease than mice immunized with the viral antigens only. Improved protection correlated with enhanced virus-specific CD4⁺ T cell responses and higher neutralizing antibody titers. To apply these results to an HIV vaccine, mice were immunized with adenoviral vectors encoding the HIV antigens Env and Gag-Pol and coadministered vectors encoding CCL3. Again, this combination vaccine induced higher virus-specific antibody titers and CD4⁺ T cell responses than did the HIV antigens alone. These results indicate that coexpression of the chemokine CCL3 by adenovirus-based vectors may be a promising tool to improve anti-retroviral vaccination strategies.

Dendritic cells (DCs) are specialized antigen-presenting cells (APCs) that play a central role in the induction of primary cellular immune responses (reviewed in references 1 and 42). After antigen uptake and activation, DCs mature and migrate to lymphoid tissues, where they present antigen-derived peptides on major histocompatibility complex type II (MHC-II) molecules and provide stimulatory signals for antigen-specific T cells. Because of the important role of DCs in the induction of protective immunity, DC targeting of antigens has been a much-pursued strategy in the development of genetic and protein-based vaccines. For this, vaccine antigens were fused to antibodies or ligands of DC surface molecules and delivered directly as a protein vaccine or through encoding DNA in a genetic plasmid- or viral vector-based vaccine regimen (4, 33, 37, 43, 44). A different approach is the coexpression of chemoattractant molecules by a genetic vaccine to recruit APCs to the site of vaccine delivery. This approach has been studied in immunotherapy of tumors (16, 19, 34, 46) and also for vaccination against virus infections (5, 13, 26, 47), but it has not yet been tested in a retrovirus challenge model.

In this vaccination study we sought to increase the presence of DCs at the site of vaccine delivery. For this, we coadministered adenovirus vectors encoding different chemokines along with viral antigens. Chemokines are a group of proinflammatory proteins of 6 to 14 kDa that act as ligands of G-protein-coupled receptors (reviewed in reference 31) expressed on leukocytes. Chemokines induce the migration of these cells and play an important role in both homeostasis and inflammation. For these different processes, some chemokines are expressed continuously in certain tissues, while others are only expressed in response to inflammatory stimuli. Depending on the expression of their respective receptors, chemokines can stimulate multiple cell types. In the present study we studied the effects of the chemokines CCL3, CCL20, CCL21, and CXCL14 on immune responses in-

duced by an adenovirus-based vaccine. All four tested chemokines, while acting on differing ranges of target cells, are known to be chemoattractants for DCs (reviewed in reference 48).

We analyzed the adjuvant effect of chemokines for retroviral immunity using an HIV vaccination mouse model and the Friend retrovirus (FV) model. FV is an immunosuppressive retroviral complex, consisting of the apathogenic Friend murine leukemia virus (F-MuLV) and the replication-defective but pathogenic spleen focus-forming virus, that causes splenomegaly and lethal erythroleukemia in susceptible mice (15). In contrast to the vaccination against HIV proteins, the FV model allows for challenging immunized mice with a pathogenic retrovirus. The FV infection model has offered valuable insights into the role of particular cell types in the immune response to a retroviral infection and into the basic requirements for immune protection. Using attenuated F-MuLV helper virus, it was demonstrated that complete protection from lethal FV challenge requires both humoral and cellular responses, comprising antibodies and CD4⁺ and CD8⁺ T cells (10). Although the correlates of immune protection from HIV

Received 7 September 2011 Accepted 10 November 2011

Published ahead of print 16 November 2011

Address correspondence to Wibke Bayer, wibke.bayer@uni-due.de.

* Present address: T. Ontikatz, Institute of Cell Biology (Cancer Research), Department of Molecular Cell Biology, University Hospital Essen, University Duisburg-Essen, Essen, Germany; O. Wildner, Division of Medical Biotechnology, Paul-Ehrlich-Institut, Langen, Germany.

R. Lietz and W. Bayer contributed equally to this article.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.06244-11

infection are still unclear, it is now widely assumed that complex immunity is required to protect against retroviruses in general.

The delivery of vaccine antigens by adenoviral vectors is a much-pursued strategy in HIV vaccine research. In studies in nonhuman primates, this vaccine approach has resulted in strong immune responses that were shown to confer partial protection from challenge infections (25, 39, 40). In a large phase IIb study, however, no protective effect was seen in vaccinated individuals (9). Thus, it is urgently necessary to further improve these vaccine approaches, and a promising strategy is the enhancement and modulation of vaccine-induced immune responses with genetic adjuvants.

We have previously used the FV model to evaluate adenovirus-based vaccines against retrovirus infections. We demonstrated in those experiments the advantage of heterologous prime-boost combinations (2) and generated a new type of adenoviral vector that displays vaccine antigens on the virus capsid, which induced increased CD4⁺ T cell and neutralizing antibody responses, resulting in improved immune protection (3).

In the present study we evaluated adenoviral vectors encoding F-MuLV antigens combined with vectors encoding the chemokines CCL3, CCL20, CCL21, or CXCL14 for their protective effects against a lethal FV challenge infection and compared the vaccine-induced immune responses. The immune responses to a combination vaccine were then further characterized in mice vaccinated with adenoviral vectors encoding HIV proteins. The results from both models suggest that CCL3 may be a promising agent for the improvement of adenovirus-based vaccines.

MATERIALS AND METHODS

Cells and cell culture. The human embryonic kidney cell line 293 (Mirox Biosystems, Toronto, Ontario, Canada) and the human lung carcinoma cell line A549 (ATCC no. CCL-185) were propagated in Dulbecco modified Eagle medium with high glucose. A murine fibroblast cell line from *Mus dunni* (24) was maintained in RPMI medium (Invitrogen/Gibco, Karlsruhe, Germany). Cell culture media were supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen/Gibco) and 50 µg of gentamicin/ml. The cell lines were maintained in a humidified 5% CO₂ atmosphere at 37°C.

Adenoviral vectors. The adenoviral vectors Ad5.env, Ad5F35.env, Ad5.gag, and Ad5F35.gag (2) encode full-length F-MuLV Env or Gag proteins amplified by PCR from F-MuLV clone FB29 (35); vectors were obtained by using the AdEasy system and the vectors pAdTrackCMV, pAdEasy-1, and pAdEasy-1/F35.

For the construction of chemokine-encoding vectors, the coding sequences were subcloned from the expression vectors pORF9-mMIP1α (mCCL3), pORF5-mMIP3α (mCCL20), pORF5-mExodus2 (mCCL21), and pORF-mBRACK (mCXCL14; InvivoGen, San Diego, CA) into the plasmid pShuttle-CMV resulting in pShuttleCCL3, pShuttleCCL20, pShuttleCCL21, and pShuttleCXCL14. Recombinant Ad5- and Ad5F35-based vectors were obtained by homologous recombination of pShuttle constructs with pAdEasy-1 and pAdEasy-1/F35, respectively, and transfection into 293 cells as described previously (2).

The adenoviral vector Ad5.Henv contains a codon-optimized full-length env based on HIV clade C isolate CN54 and was obtained using the AdEasy system after cloning of synthesized DNA into pShuttle-CMV plasmid. Ad5.Hgpsyn contains a codon-optimized HIV gag-pol based on the HIV clade B isolate BH10 that was described earlier (45) and was constructed using the AdEasy system and the vectors pShuttle-CMV and pAdEasy-1. Ad5.Henv and Ad5.Hgpsyn have been described before (3a).

The adenoviral vectors Ad5.Luc and Ad5F35.Luc encoding firefly luciferase have been described (11, 21).

All adenoviral vectors were purified with a Vivapure AdenoPACK 100

kit (Vivascience, Hanover, Germany). The adenovirus particle concentrations were determined by spectrophotometry as described previously (28).

The expression levels of the chemokines by the viruses were verified in the supernatants of infected A549 cells by using mMIP1α and m6Ckine enzyme-linked immunosorbent assay (ELISA) kits (both from Raybiotech, Norcross, GA), an mMIP3α ELISA kit (R&D Systems, Minneapolis, MN), CXCL14 antibody (Aviva Systems Biology, San Diego, CA), and recombinant mCXCL14 (R&D Systems).

Mice. Female CB6F1 hybrid mice (BALB/c × C57BL/6 F1; H-2^{b/d} Fv1^{b/b} Fv2^{r/s} Rfv3^{r/s}) and female BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Mice entered studies when they were between 8 and 9 weeks of age and were treated in accordance with the regulations and guidelines of the institutional animal care and use committee of the Ruhr University, Bochum, Germany.

Immunization. For immunization against FV, CB6F1 mice were immunized with Ad5- and Ad5F35-based vectors using a heterologous prime-boost immunization protocol with a 21-day interval. A total of 10⁹ viral particles (VP) each of F-MuLV Env- and Gag-encoding vectors were mixed with either 10⁹ VP of a chemokine-encoding vector or a luciferase-encoding vector as a control and injected into both hind footpads in 100 µl of phosphate-buffered saline (PBS). Prime immunizations were conducted with Ad5 vectors and boost immunizations with fiber-chimeric Ad5F35 vectors.

In a control experiment, CB6F1 mice were immunized as described above with 10⁹ VP of chemokine-encoding vectors alone, using Ad5 vectors for prime and Ad5F35 vectors for boost immunizations.

To deplete CD4⁺ or CD8⁺ cells around the time of vaccination, mice were injected intraperitoneally with the antibodies 191.1 or 169.4 (8), respectively, on days −3, −1, +1, +3, and +5 around the day when the vaccine was applied. The depletion was performed when mice were both prime and boost immunized.

For immunization against HIV, BALB/c mice were vaccinated with a single or a prime-boost immunization with Ad5-based vectors encoding HIV Env and Gag-Pol with chemokine- or as control luciferase-encoding vectors as described for vaccination against FV (10⁹ VP of each vector). When mice were boost immunized, Ad5-based HIV Env- and Gag-Pol-encoding vectors and Ad5F35-based chemokine- or luciferase-encoding vectors were used.

FV and challenge infection. Uncoloned, lactate dehydrogenase-elevating virus-free FV stock was obtained from BALB/c mouse spleen cell homogenate (10% [wt/vol]) at 14 days postinfection with a B-cell-tropic, polycythemia-inducing FV complex (7). CB6F1 mice were challenged by the intravenous injection of 250 spleen focus-forming units (SFFU). The course of disease was monitored twice a week by palpation of the spleen of each animal under general anesthesia. The spleen size was rated on a scale ranging from 1 (normal spleen size) to 4 (severe splenomegaly), as described previously (18).

Viremia assay. At 10 days postchallenge (p.c.), plasma samples from CB6F1 mice were obtained, and viremia was determined in a focal infectivity assay (41). Serial dilutions of plasma were incubated with *M. dunni* cells for 3 days under standard tissue culture conditions. When the cells reached ~100% confluence, they were fixed with ethanol, labeled with F-MuLV Env-specific MAb720 (36), and then labeled with a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin antibody (Dako, Hamburg, Germany). The assay was developed using aminoethyl carbazole (Sigma-Aldrich, Deisenhofen, Germany) as a substrate to detect foci. The foci were counted, and the focus-forming units (FFU)/ml of plasma were calculated.

Infectious center assay. FV-infected animals were sacrificed by cervical dislocation, the spleens were removed and weighed, and single-cell suspensions were prepared. Serial dilutions of isolated spleen cells were seeded onto *M. dunni* cells and incubated under standard tissue culture conditions for 3 days, fixed with ethanol, and stained as described for the

viremia assay. The resulting foci were counted, and the infectious centers (IC)/spleen were calculated.

Binding antibody ELISA. For the analysis of F-MuLV-binding antibodies, MaxiSorp ELISA plates (Nunc, Roskilde, Denmark) were coated with whole F-MuLV antigen (5 μ g/ml), blocked with fetal calf serum, and incubated with serum dilutions. Binding antibodies were detected using a polyclonal rabbit anti-mouse HRP-coupled anti-IgG antibody and the substrate tetramethylbenzidine (TMB+), both from Dako, Hamburg, Germany. Sera were considered positive if the optical density at 450 nm was 3-fold higher than that obtained with sera from naive mice.

For the analysis of HIV Env-binding antibodies, MaxiSorp ELISA plates were coated with recombinant, His tag purified HIV Env gp120 produced in *Escherichia coli*. After incubation with serum dilutions, binding antibodies were detected using either HRP anti-mouse-IgG1 or HRP anti-mouse-IgG2a antibodies (BD Bioscience, Heidelberg, Germany) and TMB+ substrate as described above.

Complement-dependent F-MuLV-neutralizing antibody assay. To detect F-MuLV-neutralizing antibodies, serial dilutions of plasma in PBS were mixed with purified F-MuLV and guinea pig complement (Institut Virion-Serion, Würzburg, Germany), incubated at 37°C for 60 min, and then added to *M. dunni* cells that had been plated at a density of 7.5×10^3 cells per well in 24-well plates the day before. After 72 h, the cells were stained as described for the viremia assay. Dilutions that resulted in a reduction of foci by 50% or more were considered neutralizing.

Tetramer staining of F-MuLV-specific T cells. Spleens of CB6F1 mice were removed 3 days p.c., and single-cell suspensions were prepared. For analysis of CD4⁺ T cells, spleen cells were stained with a phycoerythrin (PE)-coupled MHC-II tetramer (containing the I-Ab-restricted F-MuLV Env epitope EPLTSLTPRCNTAWNRLKL [22], kindly provided by the MHC Tetramer Core Facility of the National Institutes of Health, National Institute of Allergy and Infectious Disease, Atlanta, GA), peridinin chlorophyll protein (PerCP)-anti-CD4, and fluorescein isothiocyanate (FITC)-labeled anti-CD11b (BD Bioscience). For the detection of virus-specific CD8⁺ T cells, the spleen cells were stained with PE-coupled MHC-I tetramer (containing the H-2D^b-restricted F-MuLV Gag-leader epitope AbuAbuLLAbuLTVFL in which cysteine residues of the original amino acid sequence were replaced by amino-butyric acid to prevent disulfide bonding [6]) and allophycocyanin-labeled anti-CD8 and FITC-labeled anti-CD43. The data were acquired on a flow cytometer (FACSCalibur; Becton Dickinson, Mountain View, CA) and analyzed using CellQuest Pro 4.0.1 (BD Bioscience).

Intracellular cytokine staining. HIV-specific T cells were characterized by intracellular cytokine staining. Two weeks after immunization with vectors encoding HIV Env and Gag-Pol, the spleens were removed, and spleen cells were stimulated for 6 h *in vitro* with several HIV Gag-derived peptides (AMQMLKETI p24₆₅₋₇₃ [12]; SPEVIPMFSALEGA, p24₁₆₅₋₁₇₉, PVGEIYKRWIILGLN, and p24₂₅₇₋₂₇₁ [44]; Metabion, Martinsried, Germany) representing described CD8⁺ and CD4⁺ T cell epitopes. The cells were stained with PE-labeled anti-interferon gamma (IFN- γ), allophycocyanin-labeled anti-interleukin-2 (IL-2), FITC-labeled anti-tumor necrosis factor alpha (TNF- α), and either PerCP-anti-CD8 or PerCP-anti-CD4 (BD Bioscience) and then analyzed by flow cytometry.

Statistical analyses. SigmaStat 3.1 (Systat Software, Erkrath, Germany) was used for a Mann-Whitney rank sum test, or one way analysis of variance (ANOVA) or Kruskal-Wallis one-way ANOVA on ranks, both with the Student-Newman-Keuls multiple-comparison test.

RESULTS

Coadministration of CCL3-encoding vectors resulted in improved vaccine protection against FV-induced disease. To analyze the effect of chemokine coexpression on antiretroviral vaccination, we constructed adenoviral vectors encoding the chemokines CCL3, CCL20, CCL21, and CXCL14. We used a heterologous prime-boost immunization scheme using Ad5-based vectors for prime immunizations and fiber-chimeric Ad5F35 vec-

tors containing the fiber knob and shaft domain of Ad35 for boost immunizations, since this had resulted in enhanced immune protection in previous studies (2). The identity of the encoded transgenes was verified by sequence analysis, and equal expression levels were confirmed by ELISA. When A549 cells were infected at a multiplicity of infection of 100, concentrations of ~ 800 pg of the chemokines/ml could be detected in the supernatants of the infected cells after 48 h.

First, the adjuvant effect of chemokines on vaccine protection was analyzed in the FV model. As a control, we examined whether the treatment with chemokine-encoding vectors alone would influence the course of FV infection. CB6F1 mice received adenoviral vectors encoding CCL3, CCL20, CCL21, or CXCL14 twice, followed by a challenge infection with 250 SFU of FV 3 weeks later. Virus-induced disease was monitored by twice-weekly palpations for splenomegaly, and viral loads were determined in the plasma and spleen. No difference in FV infection was observed between mice treated with chemokine-encoding vectors and control mice (data not shown), indicating that chemokines alone did not affect virus infection and pathogenesis.

To vaccinate against FV, CB6F1 mice were primed with Ad5-based vectors encoding F-MuLV Env and Gag alone or in combination with vectors encoding CCL3, CCL20, CCL21, or CXCL14. For boost immunization, animals received the respective Ad5F35-based vectors 3 weeks later. Mice vaccinated with Env/Gag only received an adenovirus encoding the irrelevant transgene firefly luciferase to ensure that mice in all treatment groups received the same amount of adenoviral particles. Three weeks after the boost immunization mice were challenged with FV, and the disease course was monitored by palpation of the spleens (Fig. 1A). Since the mice had been immunized with the lower dose of 10^9 VP of each vector, i.e., one-fifth of the dose we used in our previous studies (2, 3), control over FV-induced disease conferred by Env/Gag encoding vectors alone was weak, and these mice developed grave splenomegaly only slightly later than unimmunized control mice. The reduced vaccination dose was selected to make adjuvant effects of the coexpressed chemokines readily detectable. While the coadministration of vectors encoding CCL20, CCL21, and CXCL14 did not improve control over FV infection and all mice developed severe splenomegaly, mice coadministered CCL3-encoding vectors exhibited a significantly improved control over FV-induced splenomegaly with only transiently enlarged spleens. When the spleens were removed on day 21 p.c., this observation was confirmed, since only mice vaccinated with Env/Gag/CCL3-encoding vectors had significantly lower spleen weights than unvaccinated or Env/Gag-vaccinated mice, and they were also significantly lower than in the other chemokine groups (Fig. 1B). The mean spleen weights of these mice was 0.545 g, which is still higher than the normal spleen weight of a naive mouse, but it is a common finding in mice of intermediate susceptibility that after recovery from splenomegaly, spleens remain slightly enlarged compared to uninfected mice (18).

To analyze vaccine-mediated control over FV replication, viral loads in plasma were determined on day 10 p.c. (Fig. 2A). In all immunized mice mean viral loads were significantly reduced compared to unvaccinated control mice, but only mice that received Env/Gag/CCL3 or Env/Gag/CCL20 encoding vectors had significantly lower mean FV plasma levels than mice vaccinated with Env/Gag encoding vectors alone, with a tendency to the lowest viral loads in the Env/Gag/CCL3 group. When spleen infec-

FIG 1 Vaccine-induced control over FV-induced disease. CB6F1 mice were immunized twice with adenoviral vectors encoding FV Env and Gag in combination with adenoviral vectors encoding the murine chemokines CCL3, CCL20, CCL21, or CXCL14. For prime immunization, Ad5-based vectors were used, while Ad5F35-based vectors were used for boost immunization. To ensure that all mice received equal amounts of viral particles, mice immunized with Env/Gag alone were coadministered an adenoviral vector encoding luciferase as an irrelevant transgene. (A) Three weeks after the boost immunization, the mice were challenged with 250 SFU of FV and the development of splenomegaly as a surrogate marker for disease progression was monitored. (B) On day 21 p.c., the spleens were removed and weighed. The graphs show mean values and standard errors of the means for eight mice per group. Statistically significant differences ($P < 0.05$, Kruskal-Wallis ANOVA on ranks with a Student-Newman-Keuls multiple-comparison test) compared to the unvaccinated control group or to mice immunized with Env/Gag alone are indicated by “*” and “#”, respectively; significant differences of the other chemokine groups to Env/Gag/CCL3 are indicated by “^”. The results were obtained in two independent experiments.

tious centers (IC) were analyzed on day 21 p.c., the mean viral loads of all immunized mice, with the exception of mice vaccinated with Env/Gag/CCL21, were significantly lower than in unvaccinated control mice (Fig. 2B). In mice immunized with Env/

Gag-encoding adenoviral vectors alone, the mean viral load was reduced ~7.5-fold compared to unvaccinated control mice (6.54×10^5 versus 4.94×10^6 IC/spleen), demonstrating a low degree of protection conferred by the reduced dose of Env/Gag

FIG 2 Reduction of virus load in the plasma and spleen in vaccinated mice. CB6F1 mice were prime and boost immunized with Ad5- and Ad5F35-based vectors, respectively, encoding FV Env and Gag and coadministered vectors encoding the chemokine CCL3, CCL20, CCL21, or CXCL14 and challenged with FV 3 weeks after the boost immunization. The viral loads in plasma (A) and spleen (B) samples were analyzed at days 10 and 21 p.c., respectively. The graphs show the mean values and standard errors of the means for six mice per group. Statistically significant differences ($P < 0.05$, one-way ANOVA [A] and Kruskal-Wallis ANOVA on ranks [B] with Student-Newman-Keuls multiple-comparison test) compared to the unvaccinated control group or to mice immunized with Env/Gag alone are indicated by “*” and “#”, respectively; significant differences of the other chemokine groups to Env/Gag/CCL3 are indicated by “^”. The results were obtained in two independent experiments.

FIG 3 Induction of FV-specific CD4⁺ T cell responses by MHC-II tetramer staining. For the analysis of CD4⁺ T cell responses, CB6F1 mice were immunized twice with Ad5 and Ad5F35 vectors, respectively, encoding FV Env and Gag and coadministered vectors encoding CCL3, CCL20, CCL21, or CXCL14. To enhance immune responses, mice were challenged with FV and T cell responses were analyzed 3 days later by MHC-II tetramer staining of spleen cells. (A) The graph shows mean values with standard errors of the means of four mice per group. Statistically significant differences ($P < 0.05$, Kruskal-Wallis ANOVA on ranks with Student-Newman-Keuls multiple-comparison test) compared to the unvaccinated control group or to mice immunized with Env/Gag alone are indicated by “*” and “#”, respectively; significant differences of the other chemokine groups to Env/Gag/CCL3 are indicated by “^”. The results are representative of two independent experiments. (B) Dot plots from representative mice showing the percentage of MHC II tetramer⁺ cells after gating on the lymphocyte population in a forward- versus side-scatter plot and on CD4⁺ CD11b[−] cells.

vectors. At this time point the mean viral loads in Env/Gag/CCL3-immunized mice and also in Env/Gag/CCL20- and Env/Gag/CXCL14-immunized mice were significantly reduced compared to mice immunized with Env/Gag-encoding vectors alone, with the significantly lowest viral loads in the Env/Gag/CCL3 group. The reduction in viral loads induced by coadministration of CCL20 and CXCL14 was not strong enough to prevent severe FV-induced splenomegaly (Fig. 1), and only coadministration of CCL3 reduced viral loads and protected from severe disease.

Enhanced FV-specific CD4⁺ T cell responses after coadministration of adenoviral vectors encoding CCL3 and CCL20. To elucidate the mechanisms underlying the improved protection in mice receiving chemokine-encoding vectors, FV-specific cellular immune responses were analyzed by tetramer staining. For this, mice were immunized twice and challenged with FV as described above, and spleen cells were isolated 3 days p.c. To detect FV specific CD8⁺ T cells, spleen cells were stained with an MHC-I tetramer presenting the F-MuLV Gag-leader epitope (6), which is the only known cytotoxic T-lymphocyte (CTL) epitope for FV. However, no tetramer⁺ CD8⁺ T cells could be detected in any of the immunized mice, which is a finding we have observed before for Env/Gag-immunized mice and attributed to incorrect or insufficient processing of the leader-Gag protein expressed by the adenoviral vectors (3).

For the detection of FV-specific CD4⁺ T cells, spleen cells were stained with MHC-II tetramers presenting an F-MuLV gp70 derived CD4⁺ T cell epitope (22). Although no induction of FV-specific CD4⁺ T cells was detectable at 3 days p.c. in unvaccinated control mice or mice vaccinated with Env/Gag alone, mice immunized with Env/Gag/CCL3 or Env/Gag/CCL20 had significant levels of MHC-II tetramer⁺ CD4⁺ T cells (Fig. 3A), with the significantly highest percentage of MHC-II tetramer⁺ cells in the Env/Gag/CCL3 group. Representative dot plots are shown in Fig. 3B. Thus, the induction of virus-specific CD4⁺ T cell responses by CCL3 correlated with its positive effect on vaccine protection.

Impact of CD4⁺ and CD8⁺ T cells on enhanced vaccine protection by CCL3. To analyze whether priming of CD4⁺ and/or CD8⁺ T cells was essential for the enhanced vaccine protection in Env/Gag/CCL3-immunized mice, T cell depletion experiments were performed. Mice were immunized with Env/Gag or Env/Gag/CCL3 vectors as described above, and two different groups of Env/Gag/CCL3 vaccinated mice were injected with CD4⁺ or CD8⁺ cell depleting antibodies, respectively, on days −3, −1, +1, +3, and +5 of the day of adenoviral vector injection. Efficient depletion (>98%) of CD4⁺ or CD8⁺ cells was verified by flow cytometry analysis of blood samples (data not shown). At the time of FV challenge 3 weeks after the boost immunization, the T cell numbers had reached normal levels

FIG 4 Effect of depletion of CD4⁺ or CD8⁺ T cells during vaccination on vaccine-mediated protection from FV infection. To determine the influence of CD4⁺ and CD8⁺ T cells on vaccine-mediated protection from FV infection, mice were depleted of the respective T cell subsets around the time of vaccination, and challenged with FV as before. FV infection was monitored by palpation of the spleens (A) and by analysis of FV loads in plasma on day 10 p.c. (B) and in spleen cells on day 21 p.c. (C). The graphs show mean values with standard errors of the means of four mice per group. Statistically significant differences ($P < 0.05$, Kruskal-Wallis ANOVA on ranks with Student-Newman-Keuls multiple-comparison test [A], one-way ANOVA with Student-Newman-Keuls multiple-comparison test [B and C]) compared to the unvaccinated control group, to mice immunized with Env/Gag alone or to the Env/Gag/CCL3 group are indicated by “*”, “#”, and “^”, respectively, significant differences between CD4- and CD8-depleted mice are indicated by “°”.

again (data not shown). After FV challenge, splenomegaly and viral loads were monitored (Fig. 4). While the immunization with Env/Gag/CCL3 again resulted in efficient control over FV-induced splenomegaly, this protection was completely abolished when mice were depleted of CD4⁺ T cells at the time of vaccination (Fig. 4A). Also, the depletion of CD8⁺ T cells at the time of vaccination resulted in reduced control over splenomegaly, but the effect was less pronounced than after CD4⁺ cell depletion. Mean viral loads in plasma and spleen on day 10 and 21 p.c. were also significantly higher in mice depleted of either CD4⁺ or CD8⁺ cells at the time of vaccination compared to nondepleted mice immunized with Env/Gag/CCL3 (Fig. 4B and C), with the strongest increase in mean viral loads found in the CD4⁺ cell depleted group, which was significantly higher than in the mice depleted of CD8⁺ cells. These findings emphasize the important role of CD4⁺ T cells in CCL3-enhanced vaccine protection, but they also hint at a role of CD8⁺ T cells, either with specificity to unknown FV epitopes or with GagL epitope specificity at an undetectable frequency.

Antibody responses were enhanced by coadministration of chemokine-encoding vectors. To analyze FV-specific antibody

responses, blood samples were collected 2 weeks after boost immunization or 10 days after FV challenge, and binding as well as neutralizing antibody titers were evaluated. Two weeks after boost immunization with Env/Gag-encoding vectors alone, low titers of FV-specific binding antibodies were only detected in some of the mice, whereas all mice immunized with Env/Gag in combination with any chemokine-encoding vector had significantly higher mean titers of binding antibodies at this time point (Fig. 5A). However, no neutralizing antibody responses were detectable in any of the immunized mice after boost immunization (data not shown). To analyze the anamnestic antibody response after virus challenge, neutralizing antibodies were measured at 10 days p.c. F-MuLV neutralizing antibodies were detected in the plasma of almost all immunized mice, whereas no neutralizing antibodies were detected in unvaccinated FV-infected control mice ($P < 0.05$; Fig. 5B), indicating the immune priming by the vaccine. However, only the group of mice immunized with Env/Gag/CCL3 had a mean neutralizing antibody titer that was significantly higher than that of mice immunized with Env/Gag alone or in combination with CCL20, CCL21, and CXCL14. The data show

FIG 5 Antibody response to adenovirus-based vaccination. CB6F1 mice were prime and boost immunized with Ad5- and Ad5F35-based vectors, respectively, encoding FV Env and Gag and coadministered vectors encoding the chemokines CCL3, CCL20, CCL21, or CXCL14. The binding antibody titers of vaccinated mice were analyzed 2 weeks after boost immunization (A), and neutralizing antibody titers were analyzed 10 days after FV challenge infection (B). The dots represent values of individual mice, the lines indicate median values. Statistically significant differences ($P < 0.05$, Kruskal-Wallis ANOVA on ranks with Student-Newman-Keuls multiple-comparison test) compared to the unvaccinated control group or to mice immunized with Env/Gag alone are indicated by “*” and “#”, respectively, significant differences of the other chemokine groups to Env/Gag/CCL3 are indicated by “^”. The data were obtained in two independent experiments.

that CCL3 was the only tested chemokine that could enhance anamnestic neutralizing antibody responses after FV challenge.

CCL3 codelivery by an adenovirus vector also enhanced vaccine-induced HIV-1-specific immune responses. Since CCL3 was the only tested chemokine that efficiently improved vaccine-induced protection and immunity in the FV model, it was of great interest whether it would also enhance HIV-1-specific immunity induced by adenoviral vectors.

Therefore, mice were immunized twice with Ad5-based vectors encoding HIV Env and Gag-Pol with or without coadministration of a vector encoding CCL3. Three weeks after the boost immunization sera were collected from vaccinated mice and binding IgG1 and IgG2a antibody titers were analyzed by an Env-specific ELISA. In both groups of mice IgG1 and IgG2a antibody titers were quite high. There was a trend to higher mean IgG1 antibody titers in the HIV Env/Gag-Pol/CCL3 group than in the control group, but no differences were found for the HIV Env-specific IgG2a titers (Fig. 6).

To analyze the influence of CCL3 codelivery on T cell responses, mice were immunized twice as described above or by a single injection of HIV Env/Gag-Pol vectors with or without a CCL3-encoding vector. Two weeks later cellular immune responses were characterized by intracellular staining of IFN- γ , TNF- α , and IL-2 production by CD4⁺ and CD8⁺ T cells after *in vitro* restimulation with Gag-derived peptides (12, 44). Although the tendencies were the same in both experimental settings, the differences in immune responses between the groups were more pronounced after a single immunization than after prime-boost immunization (Fig. 7). In contrast to unvaccinated controls, a significant induction of IFN- γ , TNF- α , or IL-2-producing CD4⁺ T cells was detected in all vaccinated mice (Fig. 7A to C). For IFN- γ and TNF- α the mean percentage of positive T cells was significantly enhanced by CCL3 coadministration. This shows again that the chemokine CCL3 can augment vaccine-induced retroviral immunity. However, coadministration of CCL3 had no significant effect on vaccine-induced HIV-specific CD8⁺ T cell responses. Vaccination with HIV Env/Gag-Pol vectors induced

considerably higher mean percentages of CD8⁺ T cells producing IFN- γ , TNF- α , or IL-2 compared to CD4⁺ T cells (Fig. 7D to F), but no significant increase in these responses was found when mice were coimmunized with CCL3. These findings confirm our results from the FV model, in which we demonstrated a major influence of CCL3 codelivery on CD4⁺ T cell responses but not CD8⁺ T cell responses.

FIG 6 Humoral immune response to adenovirus-based immunization against HIV proteins. BALB/c mice were immunized twice with adenoviral vectors encoding HIV Env and Gag-Pol, sera were collected 3 weeks after the boost immunization, and the titers of Env-binding IgG1 and IgG2a antibodies were analyzed. Each dot represents an individual mouse, the bar marks the median value. No significant differences were detected in a Mann-Whitney rank sum test ($P > 0.05$). n.s., not significant.

FIG 7 Cellular immune responses to adenovirus-based vaccination against HIV proteins. Cellular immune responses were analyzed after a single immunization of BALB/c mice with adenoviral vectors encoding HIV Env and Gag-Pol proteins with or without codelivery of CCL3. Two weeks after immunization the spleen cells were removed, and the expression of IFN- γ , TNF- α , and IL-2 after restimulation with Gag-derived peptides *in vitro* was analyzed by flow cytometry. The graphs show the percentages of IFN- γ -, TNF- α -, and IL-2-producing CD4 $^+$ (A to C) and CD8 $^+$ T cells (D to F). The graphs show the mean values and standard errors of the means of eight mice per group. The data were acquired in two independent experiments. Statistically significant differences ($P < 0.05$, Kruskal-Wallis ANOVA on ranks with Student-Newman-Keuls multiple-comparison test) compared to the unvaccinated control group or to mice immunized with HIV Env and Gag-Pol alone are indicated by “*” and “#”, respectively.

DISCUSSION

After classic approaches toward an HIV vaccine failed, novel vaccine strategies had to be considered. A very popular strategy is the application of genetic vaccines such as DNA plasmids or various viral vectors. Because of their relatively low immunogenicity, plasmid-based vaccines are often combined with chemical or genetic adjuvants. The use of immunomodulatory substances can also be of interest when viral vectors are used in order to modify or enhance specific components of the immune response. The adenoviral vectors that we used here are highly immunogenic by themselves, but we could show that coexpression of chemokines resulted, to various degrees, in improved immune responses to the adenovirus-based vaccine and enhanced protection from FV challenge. Although the protection from FV infection was still incomplete, with detectable viral loads at both analyzed time points, mice coadministered CCL3 exhibited strongly improved control over FV infection and disease compared to vaccination with Env/Gag vectors alone. The reduced dose of adenoviral particles used for the immunization resulted in little protection if Env/Gag vectors were used alone; we deliberately chose this reduced dose to make potential adjuvant effects more easily detectable, and the

adjuvant effect should not be assumed to be exclusive for this particular vector dose.

The most pronounced effect on vaccine protection was seen for the chemokine CCL3, whereas the coexpression of CCL20 and CXCL14 had only slight effects on vaccine protection and CCL21 did not improve protection at all. The improvement of vaccine-mediated control over FV-induced disease and viral replication by CCL3 coexpression correlated well with the improved induction of F-MuLV Env gp70-specific CD4 $^+$ T cells and an enhanced anamnestic neutralizing antibody response after FV challenge. Although the binding antibody levels were equally enhanced in all groups of mice receiving chemokines, the neutralizing antibody levels were only enhanced in the Env/Gag/CCL3 group, suggesting that an augmented CD4 $^+$ T cell response was crucial for efficient maturation of virus-specific antibodies after challenge. Although the FV challenge infection seems to be necessary for neutralizing antibody maturation, we believe that the high binding antibody titers induced by the immunization allow for a rather rapid formation of a neutralizing response which contributes to the vaccine-mediated protection.

We did not detect any CD8 $^+$ T cells specific for the GagL

epitope, which is the only known CD8⁺ T cell epitope of FV but seems not to be processed, or only very inefficiently, when the F-MuLV Gag protein is expressed from an adenoviral vector (2). However, the depletion of CD8⁺ T cells at the time of vaccination did result in enhanced viral loads and reduced control over FV-induced splenomegaly. Even if this loss of protection was less pronounced than after CD4⁺ T cell depletion, this finding suggests that either CD8⁺ T cells with unknown epitope specificity or GagL-specific CD8⁺ T cells at undetectable levels played a role in vaccine-mediated protection from FV challenge and that those CD8⁺ T cell responses may have been enhanced by CCL3 coadministration.

Similar to our findings in the FV model, in mice vaccinated against HIV the most pronounced effect of CCL3 codelivery was also on the CD4⁺ T cell responses. The virus-specific production of IFN- γ and TNF- α was significantly enhanced and also the percentage of IL-2-producing cells and polyfunctional T cells expressing all three cytokines (data not shown) was increased. The numbers of cytokine-producing CD8⁺ T cells were not significantly altered, although we found a tendency to slightly more polyfunctional cells (data not shown). These findings resemble the results from the FV model in which we found a strong increase in the CD4⁺ T cell response, which played a crucial role in vaccine protection against FV infection.

It has been demonstrated in detail for FV that for complete immune protection from infection, a complex immune response comprised of CD4⁺ and CD8⁺ T cells and B cells is required (10). However, in mice immunized with T cell epitope peptides (23), vaccinia vectors (17, 30), or adenoviral vectors (2, 3), it could be shown that partial protection can also be achieved if not all three components are induced equally strongly. It was noted, interestingly, that the presence of CD8⁺ T cells at the time of FV challenge was dispensable for the control if the T helper and B cell response was strong enough (17, 23). The studies on immunization with T helper cell epitope peptides (23, 29) emphasized the role of CD4⁺ T cells; this immunization led to the control of FV-induced disease also in highly susceptible mice after low-dose challenge, and the authors reported faster induction of neutralizing antibody titers and immunoglobulin class switching after FV infection. Our results are in accordance with these earlier reports, since we saw a strong correlation of CD4⁺ T cell responses and neutralizing antibody titers with the vaccine-mediated protection, with only a marginal impact of CD8⁺ T cell responses on vaccine protection.

The experimental use of chemokines has previously been described for immunotherapy in cancer. It could be demonstrated that the direct administration of chemokines (19) or the injection of chemokine encoding adenoviral vectors (16, 34) into tumor tissue resulted in efficient recruitment of immune cells into the tumor and the induction of tumor-specific CD8⁺ T cells. Other studies reported on the use of chemokines for the modulation of immune responses against viral antigens, where it was demonstrated that the coexpression of CCL3 from a DNA vaccine resulted in improved cellular and humoral immune responses against HIV proteins, with a predominant Th1 immunity (26). Interestingly, another report that compared different chemokines as genetic adjuvants for DNA-based immunization against pseudorabies virus showed a bias to Th2 after codelivery of CCL3 (47), suggesting that the predominance of either Th1 or Th2 is not strictly determined by the codelivered chemokine. One publication reports on the coexpression of CCL20 from a modified vac-

cinia virus Ankara-based vector and shows enhanced cellular and binding antibody responses compared to vaccination with antigen alone, which is in accordance with our results, but no data on vaccine protection after a challenge infection were shown (5).

Interestingly, in our immunization study, only the codelivery of CCL3 had a strong beneficial effect on vaccine-mediated protection resulting in control over FV-induced disease, while the coexpression of CCL20 and CXCL14 only had significant effects on FV load at a late time point, and no effect at all was observed for CCL21. Although all four tested chemokines are known chemoattractants for DCs, the two receptors for CCL3, namely, CCR1 and CCR5, are expressed on multiple cell types, including DCs, T cells, B cells, NK cells, and macrophages (reviewed in reference 48). The more ubiquitous expression of the receptors might play a beneficial role in protection since CCL3 may have a broader function on several immune cell populations compared to the other chemokines. It is also interesting that the receptors of the investigated chemokines are thought to play different roles in the establishment of T cell memory. The receptor for CCL21 (CCR7) is mainly expressed on central memory CD4⁺ T cells, whereas the receptors for CCL3 (CCR1, CCR5) and CCL20 (CCR6) are expressed on effector memory CD4⁺ T cells (reviewed in reference 38). Since we administered the chemokines at the same time as the viral antigens, they likely modulate effector memory responses but not the later-appearing central memory cell responses.

It also has to be taken into consideration that, unlike plasmid DNA, adenoviral vectors are highly immunogenic (20, 32), which is probably the reason why not all immunostimulatory agents can augment an immune response induced by adenoviral vaccine vectors, as we have also seen before for the coexpression of cytokines that had no effect on vaccine protection (2).

For a possible translation of our findings into a vaccination strategy for humans, it is interesting that murine and human CCL3 exhibit a high degree of similarity (reviewed in reference 27). As its murine counterpart, the human CCL3 also signals through the two receptors CCR1 and CCR5 and has been shown to act on multiple immune cell types, including T cells, NK cells, monocytes, and immature DCs. Thus, the coexpression of human CCL3 might possibly have a similar beneficial effect on immune responses and should be considered in human vaccine development. Since the effect of CCL3 on vaccine-induced T cells seems to be more pronounced on CD4⁺ T cells, it is probable that CCL3 coexpression is more promising for prophylactic than therapeutic vaccination, especially when thinking of a vaccine against HIV, where the CD4⁺ T cell population is gravely affected by the infection.

In summary, we demonstrated in the FV model that the codelivery of CCL3 with an adenovirus-based vaccine resulted in enhanced immune responses and improved protection from FV challenge. Since we could also demonstrate that cellular immune responses to an adenovirus-based vaccine against HIV were enhanced by coexpression of CCL3, our data indicate that CCL3 codelivery is a promising approach to improve adenovirus-based antiretroviral vaccination.

ACKNOWLEDGMENTS

This study was supported by the Deutsche Forschungsgemeinschaft (DFG grant GRK 1045 to K.Ü., U.D., and O.W.).

We thank Xiaolong Fan, Lund University, Lund, Sweden, for provid-

ing the plasmid pAdEasy-1/F35, and we thank the NIH tetramer facility for providing the MHC-II tetramers.

REFERENCES

- Banchereau J, Steinman RM. 1998. Dendritic cells and the control of immunity. *Nature* 392:245–252.
- Bayer W, Schimmer S, Hoffmann D, Dittmer U, Wildner O. 2008. Evaluation of the Friend virus model for the development of improved adenovirus-vectored anti-retroviral vaccination strategies. *Vaccine* 26: 716–726.
- Bayer W, et al. 2010. Vaccination with an adenoviral vector that encodes and displays a retroviral antigen induces improved neutralizing antibody and CD4⁺ T-cell responses and confers enhanced protection. *J. Virol.* 84:1967–1976.
- 3a. Bayer W, et al. 2011. Improved vaccine protection against retrovirus protection after co-administration of adenoviral vectors encoding viral antigens and type I interferon subtypes. *Retrovirology* 8:75.
- Bonifaz LC, et al. 2004. In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J. Exp. Med.* 199:815–824.
- Chavan R, Marfatia KA, An IC, Garber DA, Feinberg MB. 2006. Expression of CCL20 and granulocyte-macrophage colony-stimulating factor, but not Flt3-L, from modified vaccinia virus Ankara enhances antiviral cellular and humoral immune responses. *J. Virol.* 80:7676–7687.
- Chen W, Qin H, Chesebro B, Cheever MA. 1996. Identification of a gag-encoded cytotoxic T-lymphocyte epitope from FBL-3 leukemia shared by Friend, Moloney, and Rauscher murine leukemia virus-induced tumors. *J. Virol.* 70:7773–7782.
- Chesebro B, Wehrly K, Stimpfling J. 1974. Host genetic control of recovery from Friend leukemia virus-induced splenomegaly: mapping of a gene within the major histocompatibility complex. *J. Exp. Med.* 140: 1457–1467.
- Cobbold SP, Jayasuriya A, Nash A, Prospero TD, Waldmann H. 1984. Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. *Nature* 312:548–551.
- Cohen J. 2003. Public health. AIDS vaccine trial produces disappointment and confusion. *Science* 299:1290–1291.
- Dittmer U, Brooks DM, Hasenkrug KJ. 1999. Requirement for multiple lymphocyte subsets in protection by a live attenuated vaccine against retroviral infection. *Nat. Med.* 5:189–193.
- Dmitriev I, et al. 1998. An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J. Virol.* 72: 9706–9713.
- Doe B, Selby M, Barnett S, Baenziger J, Walker CM. 1996. Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells. *Proc. Natl. Acad. Sci. U. S. A.* 93:8578–8583.
- Flesch IE, Stober D, Schirmbeck R, Reimann J. 2000. Monocyte inflammatory protein-1 α facilitates priming of CD8⁺ T cell responses to exogenous viral antigen. *Int. Immunol.* 12:1365–1370.
- Reference deleted.
- Friend C. 1957. Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. *J. Exp. Med.* 105:307–318.
- Fushimi T, Kojima A, Moore MA, Crystal RG. 2000. Macrophage inflammatory protein 3 α transgene attracts dendritic cells to established murine tumors and suppresses tumor growth. *J. Clin. Invest.* 105: 1383–1393.
- Hasenkrug KJ, Brooks DM, Nishio J, Chesebro B. 1996. Differing T-cell requirements for recombinant retrovirus vaccines. *J. Virol.* 70:368–372.
- Hasenkrug KJ, Brooks DM, Robertson MN, Srinivas RV, Chesebro B. 1998. Immunoprotective determinants in friend murine leukemia virus envelope protein. *Virology* 248:66–73.
- He S, Wang L, Wu Y, Li D, Zhang Y. 2010. CCL3 and CCL20-recruited dendritic cells modified by melanoma antigen gene-1 induce anti-tumor immunity against gastric cancer ex vivo and in vivo. *J. Exp. Clin. Cancer Res.* 29:37.
- Higginbotham JN, Seth P, Blaese RM, Ramsey WJ. 2002. The release of inflammatory cytokines from human peripheral blood mononuclear cells in vitro following exposure to adenovirus variants and capsid. *Hum. Gene Ther.* 13:129–141.
- Hoffmann D, et al. 2008. Evaluation of twenty-one human adenovirus types and one infectivity-enhanced adenovirus for the treatment of malignant melanoma. *J. Invest. Dermatol.* 128:988–998.
- Iwashiro M, et al. 1993. Multiplicity of virus-encoded helper T-cell epitopes expressed on FBL-3 tumor cells. *J. Virol.* 67:4533–4542.
- Kawabata H, et al. 2006. Peptide-induced immune protection of CD8⁺ T cell-deficient mice against Friend retrovirus-induced disease. *Int. Immunol.* 18:183–198.
- Lander MR, Chattopadhyay SK. 1984. A Mus dunni cell line that lacks sequences closely related to endogenous murine leukemia viruses and can be infected by ecotropic, amphotropic, xenotropic, and mink cell focus-forming viruses. *J. Virol.* 52:695–698.
- Letvin NL, et al. 2004. Heterologous envelope immunogens contribute to AIDS vaccine protection in rhesus monkeys. *J. Virol.* 78:7490–7497.
- Lu Y, et al. 1999. Macrophage inflammatory protein-1 α (MIP-1 α) expression plasmid enhances DNA vaccine-induced immune response against HIV-1. *Clin. Exp. Immunol.* 115:335–341.
- Menten P, Wuyts A, Van DJ. 2002. Macrophage inflammatory protein-1. *Cytokine Growth Factor Rev.* 13:455–481.
- Mittereder N, March KL, Trapnell BC. 1996. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J. Virol.* 70:7498–7509.
- Miyazawa M, et al. 1995. Immunization with a single T helper cell epitope abrogates Friend virus-induced early erythroid proliferation and prevents late leukemia development. *J. Immunol.* 155:748–758.
- Miyazawa M, Nishio J, Chesebro B. 1992. Protection against Friend retrovirus-induced leukemia by recombinant vaccinia viruses expressing the gag gene. *J. Virol.* 66:4497–4507.
- Murdoch C, Finn A. 2000. Chemokine receptors and their role in inflammation and infectious diseases. *Blood* 95:3032–3043.
- Muruve DA, Barnes MJ, Stillman IE, Libermann TA. 1999. Adenoviral gene therapy leads to rapid induction of multiple chemokines and acute neutrophil-dependent hepatic injury in vivo. *Hum. Gene Ther.* 10: 965–976.
- Nchinda G, et al. 2008. The efficacy of DNA vaccination is enhanced in mice by targeting the encoded protein to dendritic cells. *J. Clin. Invest.* 118:1427–1436.
- Okada N, et al. 2006. Tumor suppressive efficacy through augmentation of tumor-infiltrating immune cells by intratumoral injection of chemokine-expressing adenoviral vector. *Cancer Gene Ther.* 13: 393–405.
- Perryman S, Nishio J, Chesebro B. 1991. Complete nucleotide sequence of Friend murine leukemia virus, strain FB29. *Nucleic Acids Res.* 19:6950.
- Robertson MN, et al. 1991. Production of monoclonal antibodies reactive with a denatured form of the Friend murine leukemia virus gp70 envelope protein: use in a focal infectivity assay, immunohistochemical studies, electron microscopy and Western blotting. *J. Virol. Methods* 34:255–271.
- Ruffini PA, Grodeland G, Fredriksen AB, Bogen B. 2010. Human chemokine MIP1 α increases efficiency of targeted DNA fusion vaccines. *Vaccine* 29:191–199.
- Sallusto F, Lanzavecchia A. 2000. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. *Immunol. Rev.* 177:134–140.
- Seaman MS, et al. 2005. Multiclude human immunodeficiency virus type 1 envelope immunogens elicit broad cellular and humoral immunity in rhesus monkeys. *J. Virol.* 79:2956–2963.
- Shiver JW, et al. 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* 415: 331–335.
- Sitbon M, Nishio J, Wehrly K, Lodmell D, Chesebro B. 1985. Use of a focal immunofluorescence assay on live cells for quantitation of retroviruses: distinction of host range classes in virus mixtures and biological cloning of dual-tropic murine leukemia viruses. *Virology* 141: 110–118.
- Steinman RM, Banchereau J. 2007. Taking dendritic cells into medicine. *Nature* 449:419–426.
- Tan MC, et al. 1997. Mannose receptor-mediated uptake of antigens strongly enhances HLA class II-restricted antigen presentation by cultured dendritic cells. *Eur. J. Immunol.* 27:2426–2435.
- Trumpfheller C, et al. 2006. Intensified and protective CD4⁺ T cell immunity in mice with anti-dendritic cell HIV gag fusion antibody vaccine. *J. Exp. Med.* 203:607–617.
- Wagner R, et al. 2000. Rev.-independent expression of synthetic gag-pol

- genes of human immunodeficiency virus type 1 and simian immunodeficiency virus: implications for the safety of lentiviral vectors. *Hum. Gene Ther.* 11:2403–2413.
46. Yamano T, Kaneda Y, Huang S, Hiramatsu SH, Hoon DS. 2006. Enhancement of immunity by a DNA melanoma vaccine against TRP2 with CCL21 as an adjuvant. *Mol. Ther.* 13:194–202.
47. Yoon HA, Eo SK. 2007. Differential polarization of immune responses by genetic cotransfer of chemokines changes the protective immunity of DNA vaccine against pseudorabies virus. *Immunology* 120:182–191.
48. Zlotnik A, Yoshie O. 2000. Chemokines: a new classification system and their role in immunity. *Immunity* 12:121–127.