Chemokine receptor-mediated delivery directs self-tumor antigen efficiently into the class II processing pathway in vitro and induces protective immunity in vivo

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Nonimmunogenic antigens can be efficiently rendered immunogenic by targeting them to antigen-presenting cells via differentially expressed chemokine receptors. For example, self-tumor or HIV antigens genetically fused with proinflammatory chemokotransactants elicit potent immune responses and protective antitumor immunity in mice. Herein we demonstrate that the mechanism by which chemokine fusions elicit responses is efficient uptake, processing, and presentation of antigens via the major histocompatibility complex class II pathway. Experiments with inhibitors of intracellular trafficking suggest that chemokotransactant fusion proteins, but not antigen alone, were processed and presented through early/late endosomal and Golgi compartments and stimulated antigen-specific CD4+ T cells both in vitro and in vivo. Chemokine fusion also facilitated the presentation of antigen by dendritic cells to an autologous human tumor-specific CD4+ T-cell line. Taking advantage of chemokine redundancy, viral chemokine fusions were equally potent in inducing protective immunity in vivo, providing a possible strategy to circumvent hypothetical vaccine-induced antibody autoimmunity, for example, by use of viral chemokotransactants in humans. (Blood. 2004;104:1961-1969)

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Introduction

Cell trafficking is regulated by differential expression of heterotrimeric G protein coupled 7-transmembrane–domain chemokine receptors (GPCRs).1 Sentinel antigen-presenting cells (APCs), the immature dendritic cells (DCs), preferentially express CCR1, CCR2, CCR5, and CCR6.2-4 Upon ligand binding the receptor is phosphorylated and endocytosed through clathrin-coated vesicles using β-arrestin adaptors,5-7 although some viral chemokine receptors, such as US28, are endocytosed independently of β-arrestins.8 The internalized receptors may then be dephosphorylated and recycled back to the cell surface or targeted for degradation.5,9 CCR5 is transported to early endosomes and subsequently recycled to the cell surface, bypassing the Golgi apparatus and late endosomes, and this process does not involve protein synthesis.5 Upon chemokine receptor binding, the chemokine ligand is also internalized although its fate is not known and presumed to be degraded. Moreover, the fate of the internalized receptor and the bound ligand may be regulated by the strength of the ligand-induced signaling or the nature of the ligand itself. For example, CCR5 is endocytosed through clathrin-coated vesicles on binding to RANTES or AOP-RANTES (aminooxypentane regulated-on-activation normal T-expressed and secreted), although the latter drives CCR5 to a degradation pathway, whereas RANTES-bound CCR5 is recycled to the cell surface.5,10 The internalized receptors are degraded by proteosomes, which are considered as major regulators of cytokine receptor expression.11-13

Active immunotherapy based on the targeting of idiotypic antigen (Id), expressed by malignant B cells, is one of the most promising human cancer vaccine approaches.14 Recently, we have demonstrated that effective adaptive immunity against weakly immunogenic tumor antigens could be induced by targeted delivery of such antigens to chemokine receptors on professional APCs by linkage to their chemokotransactant ligands (β-defensins or chemokines). Mice immunized with chemokotransactants fused with nonimmunogenic lymphoma Id or sFv elicited potent anti-idiotypic responses and were protected from challenge with a lethal dose of syngeneic lymphoma cells.15,16 Moreover, we demonstrated that protective and therapeutic antitumor immunity depended on the ability of the vaccine to target immature, but not mature DCs, in vivo. The vaccine did not require use of any adjuvants; immune responses were elicited from injections of recombinant proteins alone or DNA constructs encoding fusion proteins. However, it was essential that tumor antigen was fused physically with a functionally active chemokine because immunizations with unlinked free chemokine plus antigen did not induce immune responses.
Moreover, fusion constructs lacking the ability to bind chemokine receptors were unable to elicit any immune responses in vivo.\textsuperscript{15} These data suggested that antigens are efficiently taken up, processed, and presented by APCs when they are delivered to chemokine receptors via chemotractant carriers.

Herein, we report that chemokine receptors can indeed facilitate uptake and processing of tumor antigens to elicit major histocompatibility complex (MHC) class II–restricted antigen presentation. We demonstrate that APCs incubated with functionally active, but not mutant, chemotractants fused with model lymphoma antigens, single-chain antibody, and V\textsubscript{\textbeta} chain of MOPC315 tumor, or human lymphoma-derived Id induce efficient antigen-specific cellular responses both in vitro and in vivo. Our data suggest that chemokine receptors targeted with chemotractant fusion proteins were internalized to early endocytic compartments and used the MHC class II antigen-processing pathway. Furthermore, the approach not only is potent and does not require any adjuvants, but also xenogeneic chemokines, such as the viral broad-range chemokine antagonist viral macrophage inflammatory protein II (\(\nabla\text{MIP-2}\)), which binds to multiple chemokine receptors, can be used to reduce the possibility of vaccine-induced antihost chemokine autoimmunity.

Materials and methods

Fusion gene cloning and plasmid construction

Cloning of sFv from 38C13 has been reported previously.\textsuperscript{15} The same strategy was applied to clone V\textsubscript{\textbeta} and V\textsubscript{\textgamma} fragments from MOPC315 plasmacytoma (American Type Culture Collection [ATCC], Manassas, VA) and arrange them as sFv315 using the following primers: for V\textsubscript{\textbeta} chain, PRMOPC315VH-1, AAACATATGCTGGAGAAGTCGGACTGTCAGGAGTCTC, and PRMOPC315VH-R1, TTGCGAGCGCGCCGCAGGAGAACCACCCACCTGAGGAGACTGTGAGAGT; for VL chain, PRMOPC315VL-2, AAACCTGAAGTGGCGCGGCGGAGCCACGTGTTGTGACTCAGGAA, and PRMOPC315VL-R2, ATAAGAATCTTCCGCGGCTAGAACTGACCTGGTGTGACTCAGGAA, and PRMOPC315VL-R1, TTGCGAGCGCGCCGCAGGAGAACCACCCACCTGAGGAGACTGTGAGAGT. For bacterial expression, the different V\textsubscript{\textbeta} and V\textsubscript{\textgamma} genes were generated by PCR replacing the first cysteine with a serine to generate not mutant chemoattractants fused with model lymphoma antigens, single-chain antibody, and V\textsubscript{\textbeta} chain of MOPC315 tumor, or human lymphoma-derived Id induced efficient antigen-specific cellular responses both in vitro and in vivo.

Generation of human DCs

Monocyte-derived iDCs were generated from cryopreserved peripheral blood mononuclear cells (PBMCs), as previously described,\textsuperscript{22} with some modifications. Briefly, PBMCs were enriched for monocytes by depleting of T cells with CD3 microbeads over a magnetic column (Miltenyi Biotec, Auburn, CA) using the manufacturer’s protocol. The T cell–depleted PBMCs were plated in serum-free AIM-V medium (Invitrogen, Carlsbad, CA) using the manufacturer’s recommendations.

In vitro chemotaxis assay

The migration of DCs was assessed using a 48-well microchemotaxis chamber (Neuro Probe, Cabin John, MD) with a 5-μm polycarbonate filter (Osmonics, Livermore, CA) as described.\textsuperscript{28,29} Cells were incubated at 37°C with 5% CO\textsubscript{2} for 1.5 hours. DCs migrating across the filter were counted using a Bioquant semiautomated counting system (Bioquant Image Analysis, Nashville, TN). The results (as the mean ± SE of triplicate samples) were presented as chemotactic index (CI) defined as the fold increase in the number of migrating cells in the presence of test factors over the spontaneous cell migration (in the absence of test factors). MIP-3\textalpha and MIP-3\textbeta were from PeproTech.
serum albumin (BSA)) were incubated with 1 ng/mL radioiodinated chemokine (New England Biosciences, Boston, MA) in the presence of increasing concentrations of unlabeled fusion proteins or human MIP-1β (PeproTech) for 20 minutes at room temperature. The cells were filtered through a 10% sucrose/phosphate-buffered saline (PBS) cushion and then were measured for γ emission. The rate of inhibition of binding was calculated by the formula: % inhibition = 1 - (cpm in the presence of unlabeled ligands/cpm in the presence of radiolabeled ligand alone) × 100%.

**MOPC315 Id-specific T-cell line stimulation**

The BALB/c mouse CD4+ T-cell clone 7A10B2 specifically recognizes an idiotypic peptide from the light chain of the murine plasmacytoma MOPC315 immunoglobulin (amino acids 91–101), presented by the MHC class II molecule I-Eκ. BALB/c bone marrow dendritic cells (BMDCs) were incubated with endotoxin-free fusion proteins overnight, washed extensively with cold PBS, irradiated (2000 rad), and placed in culture with 7A10B2 cells (PeproTech) for 20 minutes at room temperature. The cells were chemokine (New England Biosciences, Boston, MA) in the presence of serum albumin (BSA) were incubated with 1 ng/mL radioiodinated 7A10B2 cells in 96-well round-bottom plates at a 1:1 ratio (2 × 10^5 cells each) for 48 hours. Supernatants were assessed for interferon-γ (IFN-γ) by enzyme-linked immunosorbent assay (ELISA). Control DCs were matured by overnight treatment with lipopolysaccharide (LPS, 10 ng/mL) before peptide pulsing with 0.2 μg/mL specific 91-101 peptide, or 10 μg/mL of an irrelevant peptide. The second control iDCs, were also pulsed with 10 μg/mL of the 91-101 peptide at the same time DCs were treated with fusion proteins.

**Human idiotypic specific T-cell line**

An idiototype-specific T-cell line was generated by repeated stimulation and rest cycles as described elsewhere10 from a patient with follicular lymphoma who had received Id-KLH vaccine.4 Briefly, after vaccination, PBMCs from patient LE were first stimulated in vitro with autologous Id protein (100 μg/mL). During subsequent restimulations, irradiated (3300 rad) autologous prevaccine PBMCs were used as APCs. The Id-specific T-cell line, LE-1, consisted of more than 99% CD3+ CD4+ T cells and they recognized autologous Id in an HLA class II–associated manner.41 The T cells were generally used between 10 and 15 days following previous antigen stimulation.

**Cytokine induction assay for human idiotypic specific T-cell line**

iDCs were irradiated to 2000 rad and plated in triplicate at 1 × 10^4 cells/100 μL/well in a 96-well U-bottom plate. DCs were cultured for 4 hours in the presence or absence of autologous idiotypic protein (100 μg/mL), irrelevant idiotypic protein (100 μg/mL), hMIP3s3Fv38 (10/100/1000 ng/mL), MIP3s3Fv38 (10/100/1000 ng/mL), sFvLF (100/1000 ng/mL), or LPS (10 ng/mL). The antigen was removed after 4 hours of incubation by washing the DCs twice with complete medium, and Id-specific LE-1 T cells (1 × 10^5/well) were added to the DCs in 200 μL complete medium. Supernatants were harvested and pooled from replicate wells after 72 hours of incubation. Cytokine production (IFN-γ and GM-SCF) was measured by ELISA using Quantikine kits (R&D Systems, Minneapolis, MN).

**Intracellular trafficking and processing of fusion proteins**

BALB/c mouse splenocytes were incubated transfected HEK293 cells in a dose-dependent manner (Figure 2A). No chemotaxis was detected in fusions that contained a point mutation of the first cysteine residue in the chemokine moiety (not shown), or when Id (sFv) was fused with the natural, inactive form of β-defensin, pro-β-defensin 2 (proDF2βsFv38; Figure 2A). Fusion proteins with wild-type chemokine moieties were also able to bind the respective chemokine receptors. For example, viral chemokine antagonist peptide vMIP-2 fusion protein (vMIP2sFv38; Figure 2B) efficiently competed with human MIP-1β (hMIP-1β; Figure 2B) for CCR5. Control proteins with mutated (m) vMIP-2 or macrophage-derived chemokine (MDC) moieties were not able to bind CCR5 (vMIP2M-sFv38 and hMDCM-sFv38, respectively; Figure 2B). Thus, these data and our previous report15 suggest that the fusion proteins retain chemotactic function and are able to bind the respective chemokine receptors.

**Chemotactant fusion proteins can be taken up, processed, and presented to antigen-specific T cells**

Previously, we reported that immunizations with chemokines fused with Id, a weakly immunogenic lymphoma antigen, elicited effector CD8+ T-cell–dependent antitumor immunity13 and hypothesized that the mechanism was chemokine receptor-mediated uptake. To elucidate this, we tested whether antigen uptake by APCs would be augmented by chemokine fusion, and, if so, whether the internalized antigens would be efficiently processed

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Results

Chemotactant fusion proteins retain their functional activity

First, we produced a number of chemotactant fusion proteins with idiotypic (Id) fragments isolated from MOPC315 plasmacytoma cells (Figure 1) by purifying them from bacterial inclusion bodies using cobalt affinity columns under denaturing conditions, followed by a refolding process and heparin- Sepharose affinity chromatography.16 Purity was on average above 95%, and endo- toxin content was reduced (≤ 0.1 EU/μg protein) using Acierce etox resins (Sterogene Bioseparations). Chemotactant fusion proteins retained functional activity after being fused with various immunoglobulin fragments, namely, single-chain antibody fragments (sFv) or VH from MOPC315 IgA chain, such as MIP-3αVL315 (murine MIP-3α fused with VL315) or DEF2βsFv315 (murine β-defensin 2 fused with sFv315) chemotactacted CCR6 transfected HEK293 cells in a dose-dependent manner (Figure 2A).

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and presented to T cells. To this end, the CD4+ T-cell clone 7A10B2, which specifically recognizes an Id peptide from the λ chain of the murine plasmacytoma MOPC315 immunoglobulin (amino acids 91-101), presented by the MHC class II molecule I-Ekd,24 was stimulated with irradiated immature BMDCs or splenocytes from BALB/c mice, pretreated with titrated amounts of fusion proteins overnight, and thoroughly washed. Compared with unfused sFv, significant IFN-γ production was detected after 48 hours of incubation only in groups treated with as little as 100 ng/mL MIP-3α or β-defensin 2 fused with MOPC315 Id fragments (MIP-3αsFv315 and DF2βsFv315, respectively; Figure 3A). All 3 types of APCs tested, splenocytes (Figure 3A,C-D), iDCs (Figure 3B), and an epidermis-derived immature DC cell line XSS52 (not shown), were able to efficiently stimulate 7A10B2 cells. Viral chemokine antagonists also induced internalization of chemokine receptors and may be used to target antigen. For example, DCs treated with a viral antagonist MC148 fusion protein stimulated 7A10B2 cells (MC148VL315; Figure 3B). The process optimally required chemokine receptor engagement because DCs or splenocytes incubated with sFv315 alone (Figure 3A-D) or unlinked mixtures of free sFv315 with MIP-3α (MIP-3αsFv38 + sFv315; Figure 3C), β-defensin 2 (Figure 3A), or chemokine antagonist MC148 (not shown) stimulated T cells only weakly. Moreover, fusion proteins containing mutated chemokines (MC148MVL315; Figure 3B), which were unable to bind its respective receptor and elicit chemotaxis,16 failed to stimulate T cells compared with antigen alone. The DCs used had an immature phenotype and expressed low levels of MHC class II. Such DCs directly pulsed with MOPC315 IgA peptide (amino acids 91-101) at concentrations up to 10 μg/mL inefficiently stimulated 7A10B2 T cells (Figure 3B), unless they were preactivated and matured. For example, DCs matured by overnight treatment with LPS before pulsing stimulated 7A10B2 T cells with as little as 0.2 μg/mL of the peptide (Figure 3B). Taken together, these data suggest that uptake of Id antigen is facilitated by binding chemokine receptors expressed on BMDCs and splenic APCs and that such receptor-targeted antigens are processed and presented to specific CD4 T cells.

This observation was further confirmed using human fusion proteins; specifically, chemokine fusion proteins facilitated the uptake and presentation of a human lymphoma sFv and stimulated Id-specific T cells from the same patient. For example, patient DCs, treated with human MIP-3α fused with the patient’s sFv (hMIP-3αsFvLF, 1 μg/mL), and mixed with an autologous T-cell line (Figure 4A). In contrast, a much higher concentration of intact chemokine fusion protein with an irrelevant sFv (MIP-3αsFv38) failed to stimulate T cells compared with the patient’s sFv. Moreover, fusion proteins containing mutated chemokines (vMIP2-MUC1 and vMIP2-MUC3) were unable to bind its respective receptor and elicit chemotaxis,16 failed to stimulate T cells compared with antigen alone. The DCs used had an immature phenotype and expressed low levels of MHC class II. Such DCs directly pulsed with MOPC315 IgA peptide (amino acids 91-101) at concentrations up to 10 μg/mL inefficiently stimulated 7A10B2 T cells (Figure 3B), unless they were preactivated and matured. For example, DCs matured by overnight treatment with LPS before pulsing stimulated 7A10B2 T cells with as little as 0.2 μg/mL of the peptide (Figure 3B). Taken together, these data suggest that uptake of Id antigen is facilitated by binding chemokine receptors expressed on BMDCs and splenic APCs and that such receptor-targeted antigens are processed and presented to specific CD4 T cells.

**Chemok attractant fusion proteins are also taken up, processed, and presented to T cells in vivo**

Next, we tested whether APCs could uptake and present Id when targeted with chemok attractant fusion proteins in vivo. The 7A10B2 T cells were mixed with irradiated draining lymph node cells from BALB/c mice removed 10 and 48 hours after subcutaneous injection with 25 μg fusion proteins. Significant IFN-γ secretion was observed from T cells stimulated with lymph node cells from mice given injections 10 hours previously with MIP-3α or β-defensin.
fusion proteins (MIP-3αVL315 and DF2βsFv315, respectively; Figure 4B). The response was much less apparent with lymph node cells obtained 48 hours after injection. By contrast, lymph node cells removed from control mice injected with sFv315 alone failed to stimulate T cells (Figure 4B). These results suggest that APCs can uptake, process, and present antigen to T cells in vivo, when their chemokine receptors are targeted with chemokine-fused proteins.

Mechanism of T-cell processing of chemotactant fusion proteins

To further elucidate the mechanism of chemokine receptor-mediated antigen processing, various inhibitors of intracellular trafficking and processing were tested. Brefeldin A is a fungal metabolite that disassembles the Golgi apparatus and inhibits vesicle transport of newly synthesized MHC class II molecules between endoplasmic reticulum (ER) and Golgi32. Monensin is a sodium/potassium/proton ionophore that blocks Golgi transport and prevents acidification of intracellular compartments and inter-

Figure 2. Integrity of chemokine-fused proteins. (A) MIP3αVL315 and DF2βsFv315, but not proDF2βsFv38, fusion proteins induce chemotaxis of murine CCR6-transfected HEK293 cells. Protein concentration used is shown in μg/mL. Representative data from 6 independent experiments are presented as chemotactic index (CI = SEM of triplicate samples), defined by the fold increase in the number of migrating cells in the presence of test factors over the spontaneous cell migration. Murine MIP-3α (mMIP3α; PeproTech) was used as control. (B) vMIP2sFv38, but not vMIP2M-sFv38 or MDCC-sFv38, binds to CCR5. Titrated amounts of proteins (0-100 μg/mL) were used to inhibit binding of 0 to 200 ng/mL human radiolabeled human MIP-1β (PeproTech) to hCCR5-transfected HEK293 cells. Unlabeled human MIP-1β (hMIP1β; PeproTech) was used as control. Data are from 2 independent experiments.

Viral chemokine antagonist fusions as candidate vaccines for clinical development

Protein or DNA immunizations with lymphoma-derived Id and its fragments alone, particularly from 38C13 lymphoma, fail to induce immunity in syngeneic mice. However, as we reported recently, this nonimmunogenic antigen can be rendered immunogenic by vaccinating with fusion constructs with various syngeneic chemokines. However, use of host chemokine carriers may elicit antihost chemokine autoimmunity, which may hamper their future clinical use. Therefore, to circumvent this potential problem we tested whether xenogenic ligand, viral chemokine antagonists vMIP-2 and MC148, would elicit anti-Id responses. Syngeneic mice were immunized with plasmids encoding vMIP-2 or MC148 fused with sFv38 (pvMIP2sFv38 and MC148sFv38, respectively) and challenged with a lethal dose of 38C13 lymphoma. Control mice were immunized with DNA constructs encoding sFv fused with mutated chemokines (pvMIP2M-sFv38) or prototypic protein vaccine consisting of lymphoma-derived Id chemically crosslinked with KLH (Ig38-KLH), currently being tested in a phase 3 clinical trial. No survival was observed in control groups of mice immunized with PBS (Figure 5A, E) or plasmids encoding sFv38 fused with inactive mutant viral chemokine constructs pvMIP2M-sFv38 (Figure 5A) or pMC148M-sFv38 (not shown). In contrast, significant protective immunity was elicited in mice immunized with both fusion constructs pvMIP2sFv38 or pMC148sFv38 (log-rank P = .0001 compared with pvMIP2sFv38; Figure 5A). The protection elicited with both constructs was comparable to that induced by Ig38-KLH (Figure 5A). DNA vaccinations with fusion constructs with viral chemokines generated mostly significant levels of anti-Id IgG1 antibodies (Figure 5B, C). In contrast, no antibodies were produced in mice immunized with mutant constructs pvMIP2M-sFv38 and pMC148M-sFv38 (not shown), which were unable to bind the respective chemokine receptors. Similarly, as we reported for other host chemokines, no antibody was generated in mice immunized with DNA expressing a mixture of plasmids containing linked Id and vMIP-2 (not shown), suggesting the importance of physical linkage between chemotaxant and antigen.

Next, we tested whether preexisting antichemokine immunity would affect anti-Id responses elicited by pvMIP2sFv38. Ten mice per group were first immunized twice with vMIP-2 constructs fused with an irrelevant antigen, human breast cancer antigen Muc1 (pvMIP2-Muc1). Then mice were immunized 3 more times...
with pvMIP2sFv38. Mice preimmunized with pvMIP2-Muc1 generated significant levels of anti-vMIP2 IgG antibody (pvMIP2-Muc1/pvMIP2-Muc1 and pvMIP2-Muc1/pvMIP2sFv38; Figure 5D). Nevertheless, these same mice that generated anti-vMIP2 antibody also produced idiotype-specific antibody when they were immunized with the specific pvMIP2sFv38 construct ((pvMIP2Muc1)pvMIP2sFv38; Figure 5B,C). Both groups of mice, naive or anti-vMIP2 antibody producer, immunized with pvMIP2sFv38 generated comparable levels of anti-Id antibody (P > .9; Figure 5C). However, only naïve mice immunized with pvMIP2sFv38 were clearly protected from tumor challenge (log-rank P < .03 compared with PBS; Figure 5E). In contrast, pvMIP2sFv38 immunizations of mice with existing anti-vMIP2 antibody elicited lower tumor protection in 2 of 2 experiments with 10 mice/group (Figure 5E, although not statistically significant). Tumor protection was not due to nonspecific effects of chemokine carriers because mice immunized with vMIP-2 fused to the irrelevant antigen (pvMIP2-Muc1; Figure 5E) were not protected.

Overall, these data suggest that viral chemokine antagonists vMIP-2 and MCI148 can be used to render a nonimmunogenic tumor antigen immunogenic and elicit protective antitumor immunity, even for a very aggressive lymphoma, 38C13, which kills all control mice within 20 days after challenge. To further test the idea that the immune response was a chemokine receptor-mediated process, we tried to inhibit immunity by coinjection of the competing ligand. Mice were immunized with either pvMIP2sFv38 mixed with DNA encoding an irrelevant chemokine (pMCP3-Muc1), or antigen (pMucS), or with vMIP-2 fused with irrelevant antigen (pvMIP2-Muc1; Figure 5F). Sera of mice immunized with pvMIP2sFv38 together with an irrelevant chemokine or antigen-expressing plasmid contained an amount of idiotype-specific IgG antibodies ranging between 100 and 150 μg/mL (pMCP3-Muc1 +
immunogenic by efficiently delivering antigen to APCs via chemokine receptors. These vaccines require chemokine receptor signaling because Id-specific antibody and protective antitumor immunity were elicited only in mice immunized with sFv physically linked with functionally active chemoattractant moieties. Moreover, immunizations with fusion constructs encoding mutant chemoattractants, which could not bind to respective chemokine receptors, failed to elicit any immune responses. Furthermore, similarly to other receptor-mediated phenomena,16 the immune responses elicited by viral chemokine-fused antigens can be efficiently abrogated by coinjection of a competing ligand. For example, both antibody (Figure 5F) and antitumor protection from pMIP2sFv38 vaccine was abrogated by coinjection of vMIP2–expressing constructs, but not an irrelevant chemokine MCP-3 or antigen. Thus, these data further support the idea that the viral chemokine-based vaccines also require chemokine receptor targeting to deliver and render fused antigens immunogenic and that immunity was not due to generation of an immunogenic neoantigen. The induction of local chemotaxis to the vaccine site alone is not sufficient to break nonresponsiveness to self-tumor antigens, probably due to inefficient antigen uptake by infiltrating cells.16

Our in vitro data support the hypothesis that chemokine-fused antigens are efficiently taken up and processed in early endocytic compartments of the APCs because T-cell stimulation by APCs treated with MIP-3α fusion antigens was significantly reduced by coinoculation with leupeptin or chloroquine, agents that affect antigen processing within endosomal-lysosomal compartments. Furthermore, the antigen was transported to late endosomes and Golgi because monensin, an inhibitor of acidification of intraacellular compartments and pathways dependent on clathrin-coated pits, and brefeldin A, which blocks transport between ER and Golgi, also suppressed T-cell stimulation (Figure 3C). Thus, these data suggest that chemokine fusion antigens are taken up via chemokine receptors, processed in early/late endocytic compartments and presented in the context of MHC class II, although precise data on colocalization and trafficking will require use of more direct techniques.

At present, we do not know whether chemokine-fused antigens can also use the MHC class I presentation pathway. However, we have previously reported data that mice immunized with HIV Env fused with MCP-3 or β-defensin elicited efficient systemic and mucosal CD8 cytotoxic T-lymphocyte (CTL) responses,34 and antitumor therapeutic and protective immunity induced by DNA vaccinations with proinflammatory chemokine fusion antigens was dependent on effector CD8 cells.15 These data suggest indirectly that chemokine may also deliver antigens for MHC class I presentation. Moreover, proteosomes, an essential part of MHC class I processing and presentation,35 play an important role in internalization and down-regulation of several cell surface receptors, including cytokine receptors.11-13 It has been also reported that chemokine receptor CCR5 is constitutively associated with the ζ subunit of proteosome,36 and CD4 receptor degradation induced with HIV Env also depends on proteosomes.37 However, a specific proteosomal inhibitor, lactacytin,38 did not affect MIP-3α-mediated antigen presentation (Figure 3D), despite the fact that it completely inhibits MIP-1β or stromal cell-derived factor 1 (SDF-1α)–induced down-regulation of CCR5 and CXCR4, respectively.37 Therefore, these data suggest that internalization of MIP-3αVL315 mediated by CCR5 may use an alternative proteosome-independent pathway. It is tempting to hypothesize that chemokine fusion proteins, once internalized, are processed or degraded using both MHC class I and class II pathways. The

**Discussion**

Herein, we expand our previous observation that chemokines are able to render a model self-tumor antigen, lymphoma idiotype,15

![Figure 4. Chemokine fusion improves protein uptake in vivo and in vitro.](image)

(A) pMIP3sFvLF, pMIP3sFv38, or sFvLF fusion proteins at 2 different concentrations (1 μg/mL and 0.1 μg/mL) were incubated for 4 hours with the patient’s PBMC-derived DCs. Then, DCs were thoroughly washed, irradiated, and mixed with the patient-derived Id-specific T-cell lines. After 72 hours, IFN-γ, GM-CSF, and TNF-α production was assayed in culture supernatants. Control DCs were incubated with 100 μg/mL and 1 μg/mL of the patient B-cell lymphoma-derived IgM (Id-LF) or LPS (10 ng/mL). Representative of 2 experiments performed in duplicate wells. (B) BALB/c mice (3/group) were immunized subcutaneously with 25 μg endotoxin-free MIP3sVL315, DF2sFv315, or sFv315 each, or mock injected with PBS. After 10 and 48 hours, lymph node (LN) cells had been produced, irradiated (2000 rad), and mixed (directly without any additional protein stimulation) with 7A10B2 T cells in 96-well round-bottom plates at a 1:1 ratio (2 x 10⁴ cells each) for 48 hours. IFN-γ production was assessed by ELISA in culture supernatants. Representative of 2 consecutive experiments performed in duplicate wells are shown. Error bars depict standard error of the mean of 3 mice per group.
existence of an alternative mechanism of internalization is indirectly supported by the fact that the same IL-8 regulates differentially the internalization of CXCR1 and CXCR2 receptors, and endocytosis of viral chemokine receptor US28 occurs via a clathrin-mediated and β-arrestin independent mechanism. Activated CCR5 is transported to endosomes via clathrin-coated pits and then either is recycled back to the cell surface or transported about 100-fold smaller amounts of Id (hMIP3α) into the lysosomes and degraded in endosomes and lysosomes.41,42

Similarly, T cells from a patient with B-cell lymphoma required only approximately 100-fold smaller amounts of Id (hMIP3α) when compared with those from healthy donors. In vitro, Id alone or fused with mutant and inactive chemokine analogues, an important consideration in clinical trials to circumvent possible autoimmunity against host chemokines. Although, vMIP2-based vaccines elicited protective antitumor immunity against syngeneic B-cell lymphoma, not every viral chemokine is a carrier, because we could not get any immune responses in mice immunized with constructs expressing HHV-6-derived chemokine agonist U83 despite its chemotactic properties for the monocytic cell line, THP-1 (pU83SFSFv38, not shown). It is not clear whether its ineffectiveness is due to the receptor’s inability to be internalized or poor stability of the fusion protein. The fate of the internalized receptor is thought to be controlled by the nature of the ligand and the strength of signaling, because it was observed during cross-desensitization of CXCR1 and CCR1, CXCR4 and CCR5, and inhibition of HIV-1 entry, and preferential inhibition of CCR5 recycling by AOP-RANTES. Selection of separate chemokines also enabled controlled induction of humoral or cellular immune responses. Then the mice were immunized with various constructs expressing HHV-6 chemokine (pMucS/MIP-3α) or irrelevant chemokine plasmid (pMCP3-Muc1) or anti-vMIP2 antibody (pvMIP2-Muc1). The serum levels of anti-id38 IgG (B, C) or anti-vMIP2 IgG (D) tested 2 weeks after the last vaccination. (E) A survival plot of representative 4 independent experiments with 10 mice/group, challenged intraperitoneally with a lethal dose of 38C13 tumor cells. The log-rank P value is for comparison of pMIP2sFv38 with PBS. (F) Chemokine coadministration reduced levels of anti-id38 IgG. Data from pooled sera from 5 mice per group vaccinated with pMIP2sFv38 vaccine together with competing chemokine construct (pvMIP2-Muc1), or irrelevant chemokine plasmid (pMCP3-Muc1) or anti-vMIP2 (pvMIP2-Muc1). The P value is a comparison between groups pMucS + pMIP2sFv38 and pvMIP2-Muc1 + pMIP2sFv38. Error bars depict the standard error of the mean of 5 mice per group.

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References


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