Activation of CXCR4 Triggers Ubiquitination and Down-regulation of Major Histocompatibility Complex Class I (MHC-I) on Epithelioid Carcinoma HeLa Cells*

Ziqing Wang1, Li Zhang2, Aimin Qiao1,†, Kurt Watson1,§, Jingwu Zhang2,3,4, and Guo-Huang Fan1,5,†

From the 1Institute of Health Sciences, Shanghai Institutes for Biological Sciences Chinese Academy of Sciences and Shanghai Jiaotong University School of Medicine, Shanghai 200025, China, the 2Department of Veterans Affairs Medical Center, Nashville, Tennessee 37212, the 3Department of Neurobiology and Neurotoxicology, Meharry Medical College, Nashville, Tennessee 37208, and the 5Department of Pathology, Vanderbilt University School of Medicine, Nashville, Tennessee 37212

The class I major histocompatibility complex (MHC-I)2 molecules, which are constitutively expressed on most types of nucleated cells, are made of a highly polymorphic glycosylated transmembrane heavy chain, associated with β2-microglobulin (β2M), a nonpolymorphic and nonglycosylated light chain. Antigen receptor on cytotoxic T lymphocyte (T-cell receptor) can recognize antigenic peptides only when the latter are presented associated with MHC antigens. Thus, the expression of MHC-I molecules plays a crucial role in determining the susceptibility of target cells to cytotoxic T cells. Interestingly, MHC-I has been found down-regulated in many malignant lesions with a clinical association with histopathological markers of poor prognosis of the disease (1). Remarkably, MHC-I molecules are significantly down-regulated in migrating cancer cells in vitro and in invading cancer cells in vivo (2). Although the underlying mechanisms remain incompletely understood, the reduced level of MHC-I antigens may result in decreased sensitivity to cytotoxic T lymphocyte-mediated lysis, and thereby, may help tumor cells evade the classical T cell-dependent immune surveillance. However, normal MHC class I molecule expression is necessary for the protection from natural killer cell-mediated killing, and thus, the tumor-associated MHC class I inhibition might render them sensitive to natural killer cell-mediated killing. It has recently been proposed that cancer cells have an additional strategy to evade natural killer cell-mediated killing (3).

Recently, increasing lines of evidence have demonstrated that many tumor cells express chemokine receptors, a family of G protein-coupled receptors primarily found to direct the migration of leukocytes to the inflammatory site or to secondary lymphoid organs (4, 5). As the corresponding ligands of these chemokine receptors are found at sites of tumor metastases (6–8), these findings suggest an involvement of chemokine receptors in cancer metastasis. Among these chemokine receptors, CXCR4 has received a great deal of attention because this receptor is a major chemokine receptor expressed by at least 23 epithelial, mesenchymal, and hematopoietic cancers and has been suggested as one of the critical factors for their metastasis (9–11). As MHC-I down-regulation has been considered to be a hallmark of metastatic tumor cells, we attempted to determine whether CXCR4 plays a role in MHC-I down-regulation in tumor cells. Here, we demonstrate that ligand

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1 The abbreviations used are: MHC, major histocompatibility complex; β2M, β2-microglobulin; siRNA, small interfering RNA; GST, glutathione S-transferase; PBMC, peripheral blood mononuclear cells; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; HLA, human leukocyte antigen; RIPA, radioimmune precipitation buffer; FACS, fluorescence-activated cell sorter; MFI, mean fluorescence intensity; FITC, fluorescein isothiocyanate; E3, ubiquitin ligase; HEK, human embryonic kidney; HA, hemagglutinin.

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1 To whom correspondence should be addressed. Tel: 615-327-6363; Fax: 615-327-6757; E-mail: gfan@mmc.edu.

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stimulation of CXCR4 resulted in down-regulation of MHC-I heavy chain from the cell surface of the human epithelioid carcinoma HeLa cells and several other types of tumor cells, and the internalized MHC-I heavy chain proteins were localized in the late endosomes. Activation of CXCR4 induced ubiquitination of MHC-I heavy chain, and mutation of the C-terminal lysine residues on one of the MHC-I alleles, HLA.B7, blocked CXCL12-induced ubiquitination and the subsequent down-regulation of HLA.B7. These findings reveal a novel function of CXCR4 in tumor progression via mediating down-regulation of MHC-I molecules.

EXPERIMENTAL PROCEDURES

Plasmids and siRNAs—The pRC/CMV plasmids containing the cDNA of CXCR4 or its truncation mutant CXCR4Δ34 were obtained from Dr. Gang Pei at the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The plasmids for HLA.B7 and HLA.B7-2R mutant were obtained from Dr. Laurent Coscoy in the Department of Microbiology, University of California Medical Center, San Francisco, CA. The PET28 plasmid containing His-tagged β2M was constructed by inserting the polymerase chain reaction-amplified β2M cDNA into the BamH I and Xho I sites of the PET28 vector. The plasmids for enhanced green fluorescence protein (EGFP)-conjugated Rab7 were obtained from Dr. Angela Wandinger-Ness (12). The GST-CXCR4 C terminus plasmid was constructed as described previously (13). The specific β2M siRNA and a control siRNA consisting of a scrambled sequence that will not lead to the specific degradation of any cellular message were purchased from Santa Cruz Biotechnology, Inc.

Cell Culture and Transfection—HeLa cells, U251 cells, MDA-MB 231 cells, SK-N-BE (2) cells, and human embryonic kidney (HEK 293) 293 cells were grown in Dulbecco’s modified Eagle’s medium, containing 10% fetal bovine serum, 100 units/mL penicillin/ml, and 100 μg of streptomycin/mL in 5% CO2, 95% air at 37 °C. Cells were cultured in P-100 dishes or on 22-mm glass coverslips. Transfection was performed using Lipo- fectamine 2000 reagent (Invitrogen). Unless otherwise indicated, ~80% of cells were transfected.

Isolation and Culture of Peripheral Blood Mononuclear Cells (PBMCs)—Venous blood (3 ml from children and 50 ml from adult volunteers) was drawn into EDTA-containing vials. Plasma was separated and PBMCs were prepared using Ficoll-Hypaque according to the previously described methods (14). PBMCs were washed twice with Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10 mM HEPES and 10 mM penicillin-streptomycin.

Confocal Microscopy—To visualize the subcellular localization of MHC-I and EGFP-Rab7, HeLa cells growing on coverslips were transiently transfected with plasmids encoding EGFP-Rab7. Cells were treated with carrier buffer or CXCL12 before being fixed with methanol. Cells were washed with phosphate-buffered saline (PBS) and incubated with a mouse polyclonal antibody against MHC-I heavy chain (HLA-A, -B, and -C) (Antigenex America Inc.) for 30 min followed by a Cy3-conjugated anti-mouse secondary antibody (Molecular Probes, Eugene, OR) for 30 min. Confocal microscopy was performed on an LSM-510 laser scanning microscope (Carl Zeiss, Thornwood, NY) with a 63 × 1.3 numerical aperture oil immersion lens using dual excitation (488 nm for fluorescein isothiocyanate (FITC), 568 nm for Cy3) and emission (515–540 nm for FITC, 590–610 nm for Cy3) filter sets. All digital images were captured at the same settings. Final images were processed using Adobe PhotoShop software.

Co-immunoprecipitation and Western Blot—HEK293 cells stably expressing Myc-CXCR4 were treated with CXCL12 (10 nM) for various time intervals, washed three times with ice-cold PBS, and lysed in 1 ml of RIPA buffer containing PBS (pH 7.0), 0.1% sodium deoxycholate, 0.01% SDS, and 1% Nonidet P-40. The cell debris was removed by centrifugation (15,000 × g, 15 min), and the supernatant was precleared by incubation with 40 μl of protein A/G agarose (Pierce) for 1 h at 4 °C to reduce nonspecific binding. After removing the protein A/G agarose by centrifugation (15,000 × g, 1 min), the cleared supernatant was collected and incubated with 10 μl of mouse monoclonal anti-Myc antibody (Santa Cruz Biotechnology) overnight at 4 °C in the presence of protein A/G (40 μl/ml). The protein A/G-antibody-antigen complex was collected by washing three times with ice-cold RIPA buffer, and the final pellet was resuspended in 40 μl of SDS sample buffer containing 5% β-mercaptoethanol. Forty microliters of this preparation was separated by SDS-PAGE, and the proteins on the gel were transferred to nitrocellulose membranes (Bio-Rad). Western blot analysis was performed by incubating the nitrocellulose membrane with a rabbit polyclonal anti-MHC-I heavy chain antibody followed by a horseradish peroxidase-labeled goat anti-rabbit secondary antibody. The co-immunoprecipitated proteins were detected by chemiluminescence (Pierce).

MHC-I Ubiquitination Assay—Cells treated with the specific ligand for either CXCR4 or CXCR2 were lysed in RIPA buffer. MHC-I heavy chain proteins were immunoprecipitated by incubating the cell lysate with an anti-MHC-I heavy chain antibody in the presence of protein A/G agarose as described above. The immunoprecipitates were separated by SDS-PAGE, and the proteins on the gel were transferred to nitrocellulose membranes. Western blot analysis was performed using an ubiquitin antibody. In a parallel experiment, equal amounts of the cell lysates were subjected to SDS-PAGE followed by Western blotting of β-actin to confirm equal loading.

Purification of GST-CXCR4 C Terminus Fusion Protein and His-β2M Fusion Protein—For the purification of GST or GST-CXCR4 C-terminal fusion protein, bacterial strains (DH5α) transformed with plasmids encoding GST or GST-CXCR4 C terminus fusion protein were cultured overnight at 37 °C, and then isopropyl-β-D-thiogalactopyranoside was added, and incubation was continued for another 3 h to induce protein expression. The bacteria were lysed in lysis buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg each of leupeptin and aprotinin and then sonicated on ice for 10 s. The supernatant of the bacterial lysate was incubated with glutathione-Sepharose at 4 °C for 30 min. The GST- or GST fusion protein-bound beads were washed in lysis buffer and resuspended in RIPA buffer. For the purification of the His-tagged β2M fusion proteins, bacteria strain DH5α transformed with plasmids encoding His-β2M were cultured at

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37 °C for 12 h, and then isopropyl β-D-thiogalactopyranoside was added and incubation was continued for another 3 h to induce protein expression. The bacteria were resuspended in TMP buffer containing 50 mM Tris-HCl, pH 8.0, 20 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and then sonicated. The supernatant of the bacterial lysate was applied to equilibrated nickel-nitritriacetic acid-agarose by gravity flow, and His-β2M was eluted with elution buffer containing TMP and 100 mM NaCl, 100 mM imidazole, and 10% glycerol.

**GST Pull-down Assay**—Aliquots of the purified glutathione-Sepharose bound GST or GST CXCR4-C terminus fusion protein were incubated with equal amounts of His-β2M fusion protein at 4 °C for 2 h with rotation. Beads were pelleted by centrifugation (15,000 × g, 2 min) and washed four times with RIPA buffer. Bound proteins were released by boiling in loading buffer containing 5% β-mercaptoethanol for 5 min and were separated by SDS-PAGE. The association of His-β2M with the GST-CXCR4 C terminus was detected by Western blot analysis using an anti-His antibody. The blots were stripped and reprobed with a GST antibody to confirm equal loading.

**Fluorescence-activated Cell Sorting (FACS) Analysis**—The CXCR4-expressing cells were treated with or without CXCL12 (10 nM) for 60 min. Cells were washed in ice-cold medium and incubated with a mouse polyclonal MHC-I heavy chain antibody at 4 °C for 60 min. Cells were washed with ice-cold medium followed by incubation with FITC-labeled goat anti-mouse IgG at 4 °C for 45 min. The control cells were incubated with or without CXCL12 for 60 min. Cells were incubated with a mouse preimmune serum for 60 min 4 °C followed by incubation with a FITC-labeled goat anti-mouse IgG at 4 °C for 45 min. Cells were washed and fixed in 1% formaldehyde in PBS and analyzed in a FACSscan equipped with CellQuest software (BD Biosciences). Data were analyzed using the following equation to determine the specific mean fluorescence intensity (MFI). Specific MFI (cells treated with or without CXCL12) = MFI (stain cells treated with or without CXCL12 with MHC-I antibody) − MFI (stain cells treated with or without CXCL12 with preimmune serum). Since no difference in the isotype MFI between the cells treated with and without CXCL12 was observed in all the cell lines examined, only the basal (isotype control) fluorescence for the cells without CXCL12 treatment was shown in each of the related figures.

**RESULTS**

CXCR4 is a major chemokine receptor involved in metastasis of many cancer types (9–11). As many invasive cancer cells exhibit down-regulated MHC-I molecules, we attempted to determine the possible role of CXCR4 in MHC-I down-regulation. We treated human epithelioid carcinoma HeLa cells, which endogenously express functional CXCR4 (15), with or without CXCL12 (10 nM) for different time intervals, detected the cell surface expression of MHC-I heavy chain by FACS analysis, and calculated the CXCL12-induced reduction of MFI, which reflects down-regulation of MHC-I. We observed a time-dependent down-regulation of MHC-I heavy chain in response to CXCL12 treatment, and maximal down-regulation (>50%) occurred 60 min after CXCL12 treatment (Fig. 1A). It should be noted that CXCL12 treatment did not affect the basal MFI (data not shown).

To examine whether activation of CXCR4 down-regulates MHC-I in other tumor cells or normal PBMCs endogenously expressing CXCR4, we assessed the effect of CXCL12 stimulation on MHC-I expression in human U251 and U87 glioblastoma cells (16), human MDA-MB 231 breast cancer cells (17), human SK-N-BE (2) neuroblastoma cells (18), and isolated normal PBMCs. We observed that CXCL12 stimulation for 60 min significantly down-regulated the MHC-I expression in all these cells, albeit in different extent (Fig. 1B).

Although the subcellular localization of the internalized MHC-I in tumor cells remains unclear, studies in Kaposi sarcoma-associated herpes virus have shown that the virus encodes several proteins that down-regulate the cell surface MHC-I in T-lymphocytes, and this down-regulation results from enhanced endocytosis and subsequent lysosomal degradation of the target proteins (19, 20). Encouraged by these findings, we attempted to determine whether activation of CXCR4 induces sorting of MHC-I into late endosomes/lysosomes for degradation in HeLa cells. We transfected HeLa cells with EGFP-conjugated Rab7, a late endosome marker, treated the cells with or without CXCL12 (10 nM) for 60 min, and visualized the subcellular localization of MHC-I by confocal microscopy. As shown in Fig. 1C, MHC-I molecules were internalized and co-localized with EGFP-Rab7 after CXCL12 treatment for 60 min. These data demonstrate for the first time that activation of CXCR4 induces endocytosis of MHC-I molecules that target to late endosomes/lysosomes for degradation.

A question is raised: whether the CXCL12-induced down-regulation of MHC-I is a specific effect or a nonspecific bystander effect. To address this question, we examined the effect of CXCL12 treatment on the cell surface expression of epithelial growth factor receptor in HeLa and U251 cells. We observed that CXCL12 treatment of these cells for 60 min did not result in down-regulation of the cell surface epithelial growth factor receptors (Fig. 1D). These data suggest a specific effect of CXCL12 on MHC-I down-regulation.

Ubiquitination has been shown to be required for Kaposi sarcoma-associated herpes virus viral protein-induced MHC-I endocytosis in target cells (19, 21). To examine whether activation of CXCR4 results in MHC-I ubiquitination in tumor cells, we treated HeLa with CXCL12 (10 nM) for different time intervals (0, 5, 15, 30 min), immunoprecipitated MHC-I heavy chain from the cell lysate, and detected MHC-I ubiquitination by Western blot analysis using a specific anti-ubiquitin antibody. As shown in Fig. 2A, CXCL12 stimulation induced ubiquitination of MHC-I heavy chain in a time-dependent manner, which peaked at 5 min and lasted for at least 30 min. To assess whether activation of other chemokine receptors similarly induces MHC-I ubiquitination, we determined MHC-I ubiquitination in HEK293 cells stably expressing CXCR2, a chemokine receptor for both CXCL1 and CXCL8. We observed that CXCL8 (10 nM) treatment for 5 min also induced a robust ubiquitination of MHC-I heavy chain.

It has been reported that the C-terminal lysine residues on MHC-I molecules are the major sites of viral protein-induced ubiquitination (19, 21). To determine whether these lysine res-
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A

None

Without CXCL12

With CXCL12

B

None

Without CXCL12

With CXCL12

C

MHC-I heavy chain

EGFP-Rab7

Overlay

Selected area

D

None

Without CXCL12

With CXCL12
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idues are similarly required for CXCR4-mediated ubiquitination of MHC-I in HeLa cells, we assessed CXCL12-induced ubiquitination of wild-type HLA.B7 and a mutant of HLA.B7 (HLA.B7-2R) in which two of the C-terminal lysine residues (at positions 332 and 337) are replaced with arginine (18) (Fig. 3A). We observed that CXCL12 stimulation induced a robust ubiquitination of the wild-type HLA.B7 but not the HLA.B7-2R mutant (Fig. 3B), thereby suggesting requirement of the C-terminal lysine residues for CXCR4-mediated ubiquitination of MHC-I molecules.

To determine whether the C-terminal lysine residues on MHC-I are required for CXCR4-mediated MHC-I endocytosis, we treated HeLa cells stably expressing wild-type HLA.B7 or HLA.B7-2R mutant with CXCL12 (10 nm) for 60 min and detected their cell surface expression by FACS analysis. As shown in Fig. 3C, CXCL12 stimulation resulted in a remarkable down-regulation of the wild-type HLA.B7 (MFI reduction: 22.74 ± 4.83%). In contrast, CXCL12 treatment failed to induce a significant down-regulation of HLA.B7-2R (MFI reduction: 4.47 ± 1.25%).

It raises an interesting question. How does activation of CXCR4 induce ubiquitination and down-regulation of MHC-I? One possible mechanism is that MHC-I molecules directly or indirectly interact with CXCR4. Because of the high polymorphism of the MHC-I heavy chain, we were unable to determine the direct interaction between CXCR4 and MHC-I heavy chain. Instead, we examined whether the nonpolymorphic MHC-I light chain, β2M, directly interacts with CXCR4 C-terminal domain that resides intracellularly. The purified GST-conjugated CXCR4 C-terminal fusion proteins were incubated with the purified His-tagged β2M fusion proteins, and the co-precipitated proteins were subjected to SDS-PAGE and Western blot analysis. As shown in Fig. 4A, the

His-β2M was co-precipitated with the GST-CXCR4 C-terminal fusion proteins but not with the GST proteins, thereby suggesting direct interaction between the C terminus of CXCR4 and β2M.

The binding property of β2M with both CXCR4 and MHC-I heavy chain suggests a possible indirect interaction between CXCR4 and MHC-I heavy chain. To test this possibility, we transfected HEK293 cells stably expressing Myc-CXCR4 with a scramble siRNA or a β2M-specific siRNA, treated the cells with or without CXCL12 (10 nm) for 5 min, immunoprecipitated Myc-CXCR4 from the cell lysate, and detected co-precipitated MHC-I heavy chain using an antibody against MHC-I heavy chain. As shown in Fig. 4B, CXCL12 stimulation resulted in a significant increase in the co-immunoprecipitation of MHC-I heavy chain with CXCR4 in the scramble siRNA transfected cells but not in the β2M-specific siRNA transfected cells, which exhibited robust knockdown of β2M expression, thereby sug-

FIGURE 2. CXCR4- or CXCR2-mediated ubiquitination of MHC-I. A, HeLa cells were treated with CXCL12 (10 nm) for indicated time intervals, and MHC-I molecules were immunoprecipitated from the cell lysates. The immunoprecipitates (IP) were subjected to SDS-PAGE followed by Western blot (IB) analysis to detect MHC-I ubiquitination using an anti-ubiquitin (Ub) antibody. In a parallel experiment, an equal amount of the cell lysate was subjected to SDS-PAGE followed by Western blot analysis of β-actin to confirm equal amounts of proteins in the samples for the immunoprecipitation. Data from three independent experiments indicating the fold change of MHC-I ubiquitination are summarized in the bar graph. B, HEK293 cells stably expressing CXCR2 were treated with CXCL8 (10 nm) for 5 min. MHC-I molecules were immunoprecipitated from the cell lysates, and MHC-I ubiquitination was assessed as described above. In a parallel experiment, an equal amount of cell lysate was subjected to SDS-PAGE followed by Western blot analysis of β-actin to confirm equal amounts of proteins in the samples for the immunoprecipitation. Data from three independent experiments indicating the fold change of MHC-I ubiquitination are summarized in the bar graph.
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A

Wt HLA.B7 ..... VVIGAVVAVMCRRKSSGGKGGSYSQAACSDSAQQGSDLTA
HLA.B7-2R ..... VVIGAVVAVMCRRRSSGGRGGSYSQAACSDSAQQGSDLTA

B

| CXCL12 (10 nM) | HLA.B7-2R | - | + |
|---------------|-----------|---|---|
| HA-HLA.B7     | +         | - | + |

IP: HA
IB: Ub
Cell lysate
IB: β-actin

C

None
Without CXCL12
With CXCL12

Wt HLA.B7

HLA.B7-2R

MFI reduction (% of total)

FIGURE 3. Role of the C-terminal lysine residues in CXCR4-evoked ubiquitination and internalization of HLA.B7. A, schematic of the C termini of wild-type (Wt) HLA.B7 and mutant HLA.B7 (HLA.B7-2R). The indicated lysine residues were changed to arginine (bold). B, HeLa cells stably expressing HA-tagged HLA.B7 or HLA.B7-2R were treated with or without CXCL12 (10 nM) for 5 min. The HA-tagged HLA.B7 molecules were immunoprecipitated (IP) from the cell lysates using a mouse monoclonal anti-HA antibody, and ubiquitination of the wild-type or mutant HLA.B7 was assessed by Western blot (IB) analysis using an anti-ubiquitin antibody as described above. In a parallel experiment, an equal amount of cell lysate was subjected to SDS-PAGE followed by Western blot analysis of β-actin to confirm equal amounts of proteins in the samples for the immunoprecipitation. C, HeLa cells stably expressing HA-tagged HLA.B7 or HLA.B7-2R were treated with or without CXCL12 (10 nM) for 5 min. Cells were incubated with a mouse monoclonal antibody against HA at 4 °C for 60 min followed by incubation with a FITC-labeled goat anti-mouse IgG at 4 °C for 45 min. The cell surface expression of the HA-tagged HLA.B7 or HLA.B7-2R was analyzed in a FACScan equipped with CellQuest software (BD Biosciences). Data from three independent experiments indicating the reduction of MFI (mean ± S.E.) are summarized in the lower bar graph. *, p < 0.05, when compared with the wild-type HLA.B7-expressing cells.

suggesting critical role of β2M in the interaction between CXCR4 and MHC-I heavy chain.

Based on the data for the involvement of the C-terminal domain of CXCR4 in the receptor interaction with β2M, we propose that the C-terminal domain of CXCR4 might be required for the receptor-mediated MHC-I endocytosis. To
test this hypothesis, we determined CXCL12-induced MHC-I endocytosis in HEK293 cells stably expressing full-length CXCR4 or a C-terminal truncation mutant of CXCR4 (CXCR4Δ34) (Fig. 5A) (22). As shown in Fig. 5B, CXCL12 stimulation induced a significant down-modulation of MHC-I proteins (MFI reduction: 53.29 ± 5.24%) in the full-length CXCR4-expressing cells. In contrast, CXCL12-induced MHC-I heavy chain down-regulation was significantly reduced in the CXCR4Δ34-expressing cells (26.05 ± 3.79% MFI reduction). These data suggest that interaction between the CXCR4 C terminus and β2M is important for the receptor-mediated down-regulation of MHC-I. Moreover, as truncation of the CXCR4 C-terminal domain has been shown to inhibit the receptor endocytosis (22), it is likely that the internalization of CXCR4 plays a role in the receptor-mediated MHC-I down-regulation.

Finally, we examined the effect of β2M knockdown in CXCR4-evoked ubiquitination of MHC-I heavy chain in HeLa cells. Cells transfected with a scramble siRNA (control) or a β2M-specific siRNA were treated with CXCL12 (10 nM) for 5 min, MHC-I heavy chain proteins were immunoprecipitated from the cell lysate, and ubiquitination of MHC-I heavy chain was assessed as described above. As shown in Fig. 6A, cells transfected with β2M siRNA, which resulted in significant knockdown of β2M expression, exhibited a significantly smaller extent of CXCL12-induced MHC-I heavy chain ubiquitination than that in the scramble siRNA transfected cells. To examine the effect of β2M knockdown on CXCR4-mediated MHC-I heavy chain down-regulation, HeLa cells transfected with a scramble siRNA or a β2M-specific siRNA were incubated with or without CXCL12 (10 nM), and the cell surface expression of MHC-I heavy chain was assessed by FACS analysis. We noticed that the cell surface expression of MHC-I heavy chain was reduced in the cells transfected with the β2M siRNA as revealed in Fig. 6B by the left shift of the curve when compared with the cells transfected with the scramble siRNA, suggesting requirement of
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DISCUSSION

During the past 10 years, our understanding of the mechanisms underlying a tumor-specific immune response and tumor escape from the immune surveillance has substantially increased. Distinct molecular mechanisms underlying the various abnormal MHC-I antigen phenotypes, including total MHC-I antigen loss, selective MHC-I allospecificity loss, MHC-I haplotype loss, total MHC-I antigen down-regulation, and selective MHC-I down-regulation, have been identified (1). Among them, MHC-I down-regulation is the most frequent phenotype in malignant tumor cells (1). In some instances, the down-regulation of MHC-I antigen expression can be corrected by in vitro administration of cytokines, such as interferon-γ and tumor-necrosis factor-α (23). However, mechanisms underlying MHC-I down-regulation in tumor cells remain elusive. In the present study, we demonstrate that ligand stimulation of CXCR4, a major chemokine receptor on many cancer cells, induces down-regulation of MHC-I heavy chain from the cell surface of HeLa cells, human U251 and U87 glioblastoma cells, human MDA-MB 231 breast cancer cells, and human SK-N-NE (2) neuroblastoma cells. Activation of CXCR4 also resulted in down-regulation of MHC-I in human PBMCs. We showed that the internalized MHC-I molecules target to the late endosomes for degradation in HeLa cells, human U251 and U87 glioblastoma cells, human MDA-MB 231 breast cancer cells, and human SK-N-NE (2) neuroblastoma cells. Activation of CXCR4-mediated ubiquitination of MHC-I at the C-terminal lysine residues is required for CXCR4-evoked down-regulation of MHC-I.

Ubiquitination is a process that occurs in a series of three reactions involving an activating enzyme (E1), an ubiquitin conjugating enzyme (E2), and an ubiquitin ligase (E3). The family of E3 ligases, which are classified into three subfamilies, RING, HECT, and PHD (24–27), are believed to be key molecules in numerous physiological and pathological processes as these enzymes determine the substrate specificity for ubiquitination. Previous studies have shown that MHC-I molecules are

β2M for the stable expression of MHC-I heavy chain on the cell surface. CXCL12 stimulation resulted in a significant down-regulation of MHC-I heavy chain in the scramble siRNA transfected cells but not in the β2M siRNA transfected cells (Fig. 6B).

FIGURE 4. In vitro and in vivo interaction of CXCR4 with the MHC-I light chain and heavy chain. A, glutathione- Sepharose bound GST (lane 1) or GST-CXCR4 C terminus were incubated with the purified His-tagged β2M. Co- precipitated proteins were subjected to 12% SDS-PAGE, and His-β2M was detected by Western blotting using a specific His antibody. B, HEK293 cells stably expressing Myc-CXCR4 were transiently transfected with a scramble siRNA (control) or a specific His antibody. Coprecipitated proteins were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane, and associated MHC-I heavy chain proteins were detected by Western blotting (IB). In a parallel experiment, aliquots of cell lysates from the above treated cells were subjected to SDS-PAGE, and Western blot was assessed using an antibody against MHC-I heavy chain (IB: MHC-1 HC). In a parallel experiment, aliquots of cell lysates from the above treated cells were subjected to SDS-PAGE, and Western blot was assessed using an antibody against β2M to verify knockdown of β2M. The blots were stripped and reprobed with a specific anti-β-actin antibody to confirm equal loading.

FIGURE 5. Wild-type (WT) or mutant CXCR4-evoked MHC-I down-regulation in HEK293 cells. A, schematic of the full-length or truncated C terminus of CXCR4. B, HEK293 cells stably expressing wild-type CXCR4 or a C-terminal truncation mutant of CXCR4 (CXCR4 Δ34) were incubated with or without CXCL12 (10 nM) for 60 min. Cells were incubated with a mouse polyclonal MHC-I heavy chain antibody at 4 °C for 60 min followed by incubation with a FITC-labeled goat anti-mouse IgG at 4 °C for 45 min. Cells were washed and fixed in 1% formaldehyde in PBS and analyzed in a FACScan equipped with CellQuest software (BD Biosciences). Data from three independent experiments indicating the reduction of MFI (mean ± S.E.) are summarized in the lower bar graph. * p < 0.05, when compared with the wild-type CXCR4-expressing cells.

RING, HECT, and PHD (24–27), are believed to be key molecules in numerous physiological and pathological processes as these enzymes determine the substrate specificity for ubiquitination. Previous studies have shown that MHC-I molecules are
ubiquitinated by several novel proteins encoded by Kaposi sarcoma-associated herpes virus and murine-herpesvirus 68 (MHV-68), which have been designated as modulator of immune recognition (MIR) 1 and 2 and mK3, respectively (28–35). These viral proteins form a novel family of E3 ligases that are membrane-bound and possess a variant type RING domain (28–35). Ubiquitination of MHC-I molecules by these viral proteins has been shown to be required for MHC-I down-regulation (28, 29). Similarly, we demonstrate that CXCR4-evoked ubiquitination is required for MHC-I down-regulation. However, little is known about the endogenous E3 ligase that is involved in CXCR4-mediated ubiquitination of MHC-I.

Based on the data that mutation of the C-terminal two lysine residues (at positions 332 and 337) on HLA.B7 blocked CXCL12-induced ubiquitination, we propose that CXCR4-evoked ubiquitination of HLA.B7 occurs at the lysine residues. However, we cannot exclude the possibility that some nonlysine residues are involved in the ubiquitination of other MHC-I alleles. Because of the involvement of several nonlysine residues, including cysteine, serine, and threonine, in viral E3 ligase-induced ubiquitination of MHC-I molecules (21, 36), it would be of interest to investigate the possible involvement of these nonlysine residues in CXCR4-evoked ubiquitination of other MHC-I alleles.

In our in vitro protein binding study, we showed that the C-terminal domain of CXCR4 directly interacted with the MHC-I light chain, β2M. This direct interaction appears to be critical for the receptor-mediated MHC-I ubiquitination and the subsequent down-regulation as evidenced by our data showing that knockdown of β2M remarkably reduced CXCR4-mediated HMC-I heavy chain ubiquitination and down-regulation and that truncation of the C-terminal 34 amino acid residues on CXCR4 significantly reduced the receptor-mediated HMC-I heavy chain down-regulation from the cell surface. However, we have noticed that knockdown of β2M resulted in remarkable reduction of the cell surface expression of HMC-I heavy chain, which is consistent with the previous notion that β2M plays a role in the stability of MHC-I complex (37).
technically it is difficult to define the role of β2M deficiency in CXCR4-evoked ubiquitination of MHC-I heavy chain. We also noticed that truncation of the C-terminal 34 amino acid residues on CXCR4 did not result in complete blockade of MHC-I down-regulation. This is most likely due to the incomplete truncation of the whole C terminus of CXCR4 (Fig. 5A) so that the remaining part of the C terminus is still able to interact with β2M and induces partial MHC-I down-regulation. As truncation of the whole CXCR4 C terminus results in failure of cell surface expression of CXCR4 (data not shown), it is technically difficult to determine whether truncation of the whole CXCR4 C terminus completely blocks the receptor-mediated MHC-I heavy chain down-regulation. Nevertheless, based on the in vitro and in vivo results for the interaction between CXCR4 and MHC-I molecules, we propose that the ligand-activated CXCR4 undergoes a conformational change, which facilitates the interaction between CXCR4 and MHC-I molecules and the recruitment of an unknown E3 ligase to the C terminus of MHC-I, thereby resulting in MHC-I ubiquitination and subsequent internalization.

Taken together, the present study demonstrates for the first time that activation of CXCR4 triggers down-regulation of MHC-I in several cancer cell types and that CXCR4-evoked ubiquitination is critical for the down-regulation of MHC-I heavy chain. These findings reveal a new function of CXCR4 in cancer progression via facilitating the tumor evasion of immune surveillance. It is suggested that agents that down-regulate CXCR4 may block MHC-I down-regulation and thereby may resume immune surveillance. Although CXCR4 inhibitors or siRNAs have been shown to limit the growth of certain cancer cells (38, 39), it would of interest to determine whether blockade of CXCR4-mediated MHC-I down-regulation is one of the mechanisms.

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