Oligomerization of CXCL10 Is Necessary for Endothelial Cell Presentation and In Vivo Activity

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The physiological role of chemokine dimerization and oligomerization has not yet been fully established and remains an important question that needs to be answered to fully understand chemokine function. Earlier in vitro studies done with monomeric chemokine mutants suggested that monomers can bind and activate their corresponding chemokine receptors similar to the wild-type protein (22–25). This led to the conclusion that chemokines are biologically active as monomers. Consistent with this view, mutants of IL-8 (CXCL8) with reduced potential to form dimers were found to be fully active in vivo (26), although it is not known whether these mutants could form oligomers on GAGs. However, a more recent study demonstrated that monomeric IL-8 was cleared more rapidly from the lung, suggesting that the ability to dimerize plays a role in the retention of this chemokine in tissue (27). In support of a physiological role for chemokine oligomerization, studies with three CC chemokines, RANTES (CCL5), MCP-1 (CCL1), and MIP-1β (CCL4), demonstrated that monomeric mutants had markedly reduced potential to recruit cells in vivo, although they were active in vitro chemotaxis (28). However, no mechanism for this effect was established. Oligomerization was also important for RANTES-induced CCR1-mediated monocyte arrest in vitro (29). Furthermore, although the heparin binding affinity of some monomeric chemokine variants is only slightly lower than the wild-type proteins, oligomerization was found to increase the avidity for GAGs by positive cooperativity (21), suggesting that oligomerization plays a role in chemokine-GAG interactions.

In prior studies, we demonstrated that IP-10, a CXC chemokine, forms high molecular mass complexes in solution as well as on the plasma membrane (15). However, no data are available on the physiological role of oligomerization for IP-10 or any other CXCR3 ligand. We therefore studied a monomeric variant of IP-10 and retained chemokines on the endothelium and extracellular matrix (15–18). For example, it has recently been demonstrated that endothelial heparan sulfate is required both for in vitro chemokine presentation on endothelial cells and for in vivo chemokine-induced recruitment of leukocytes (19). In addition, we have shown that chemokines can be secreted bound to GAGs as high molecular mass complexes (20). Similarly, it has been demonstrated that chemokines can oligomerize on GAGs (18, 21) at physiological, low nanomolar concentrations, where they would normally be present as monomers.

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The chemokine IFN-γ-inducible protein of 10 kDa (IP-10; CXCL10) regulates the in vivo migration of effector T cells and other effector lymphocytes, such as NK and NKT cells. IP-10 exerts its effect on these cells by binding to the seven-transmembrane, G protein-coupled receptor CXCR3, which it shares with two other ligands, IFN-inducible T cell-α-chemoattractant (I-TAC/CXCL11) and monokine-induced by IFN-γ (Mig/CXCL9). IP-10 is up-regulated in a wide range of human inflammatory diseases, including skin diseases (1–3), atherosclerosis (4), multiple sclerosis (5, 6), allograft rejection (7, 8), viral hepatitis (9), and others. In murine models of human diseases, IP-10 has been shown to play a role in T cell recruitment and disease pathology (10–13).

In addition to binding to its high affinity receptor CXCR3, IP-10, like many other chemokines, also binds to glycosaminoglycans (GAGs) (14, 15). It is generally accepted that GAGs help sequester and retain chemokines on the endothelium and extracellular matrix (15–18). For example, it has recently been demonstrated that endothelial heparan sulfate is required both for in vitro chemokine presentation on endothelial cells and for in vivo chemokine-induced recruitment of leukocytes (19). In addition, we have shown
and compared its in vitro and in vivo activities with those of the wild-type chemokine. We found that oligomerization plays an important role for the in vivo activity of IP-10 and have elucidated the mechanism by which oligomerization is required for the function of IP-10, which may serve as a paradigm for other chemokines that require oligomerization for their in vivo activity.

**Materials and Methods**

**Materials and mice**

Chemically synthesized IP-10 (wild-type or monomeric variant) was obtained from the University of British Columbia (Vancouver, Canada). Recombinant IP-10 and mutants of IP-10 were purified as previously described (14). C57BL/6 mice were purchased from the National Cancer Institute. The OT-1 TCR mice on the C57BL/6 background were obtained from Jackson ImmunoResearch Laboratories. All protocols were approved by the Massachusetts General Hospital Subcommittee on Research and Animal Care.

**Cell culture and transfection**

300-19 cells were maintained in complete RPMI containing 10% FCS and transfected with human or murine CXCX3 as described (30). Human microvascular endothelial cells (HMEC) were maintained in complete Clonetix EGM medium, Chinese hamster ovary (CHO) cells and Beas2B cells, a cell line derived from human bronchial epithelial cells transformed with a hybrid adeno-SV40 virus, in complete F12 medium.

**Heparin Sepharose and S Sepharose chromatography**

Aliquots of 20 μg of IP-10 were loaded on a 1-ml Heparin HiTrap or S Sepharose FF (cationic exchange) column (both from Pharmacia) equilibrated in 50 mM Tris, pH 7.5, on an AKTA machine (Pharmacia). The mutants were eluted with a 20-ml gradient of 0–2 M NaCl in 50 mM Tris, pH 7.5, and their elution time were measured by OD214.

**Superdex 200 gel filtration**

Aliquots of 20 μg of IP-10 were loaded on a Superdex 200 column (Amersham Biosciences) equilibrated in either PBS or 25 mM sodium phosphate buffer, pH 7.5, with 0.5 M NaCl on an AKTA machine (Pharmacia). The proteins were eluted in the same buffer over 1.5 column volumes (30 ml), and protein elution was measured by OD214.

**Sizing gels**

For native gels, 1 μg of IP-10 was loaded onto a 4–20% Ready PAGE gel (Bio-Rad), and run with reversed charge at 140 V, without the addition of SDS or DTT. Cytochrome c (30 μg; Sigma-Aldrich; 15 kDa; pI 10.5) was used as a dye front to determine how far the gel had run. For cross-linking experiments, IP-10 was diluted into PBS and incubated for 30 min before the addition of a 50-fold molar excess of bis(sulfosuccinimidyl)suberate (BS)2 or sulfo-ethylene glycolbis(sulfosuccinimidyl) succinate (Sulfo-EGS; Pierce). After 60 min of incubation at 37°C, the reaction was terminated by the addition of 50 mM Tris. Samples were spun for 10 min to remove any precipitate and run on a 4–20% SDS-PAGE gel with DTT. Proteins were visualized with Silver-Stain ProteoPlus (Sigma-Aldrich).

**Receptor binding assay**

Binding assays were performed as previously described (14).

**Chemotaxis**

Chemotaxis assays were performed as described (14). Briefly, chemokine dilutions were added to the bottom well of a 96-well chemotaxis plate (NeuroProbe). Activated OT-1 cells, days 8–11 in culture with IL-2, were added on top of the membrane (2.5 × 104 cells) and allowed to migrate at 37°C for 2 h, after which cells in the bottom wells were counted under a microscope. For transendothelial migration, HMEC cells were grown to confluency on the bottom side of the chemotaxis filter. HMEC were washed twice with cold RPMI 1640, and, where indicated, chemokines were added to the cells in chemotaxis media and incubated for 60 min at 37°C. Nonbound chemokines were removed by washing the cells four times with cold RPMI 1640 before proceeding with the chemotaxis assay as described above.

**Internalization of CXCX3**

Internalization of murine CXCX3 on 300-19 cells was measured as previously described (31). Internalization experiments with human CXCX3-transfected cells yielded similar results (data not shown).

**In vivo OT-1 recruitment.** OT-1 cells were prepared as previously described (32, 33) and harvested on day 6 with Lympholyte (Cedarlane Laboratories). OT-1 cells (5 × 105) were injected i.p. into male C57BL/6 mice. After 48 h, 0.5–50 μg of IP-10 in 50 μl PBS were injected intratracheally. After 18 h, lungs were lavaged with six aliquots of 0.5 ml of PBS containing 0.6 mM EDTA. RBC were lysed with RBC lysis buffer after which total cells in the bronchoalveolar lavage (BAL) were counted. Cells were incubated for 10 min with 2.4G2 anti-FcγIII/II receptor (BD Pharmingen) and were then stained with FITC-conjugated anti-murine CD3, PE-conjugated anti-murine CD4, and allophyocyanin-conjugated anti-murine CD8 at 4°C for 20 min. Cells were fixed with 1% paraformaldehyde, and cytfluorimetry was performed using a FACSCalibur cytometer (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

**ELISA**

Concentrations of IP-10 in the BAL were measured using a human IP-10 ELISA kit (R&D Systems) according to the manufacturer’s instruction.

**Imaging**

Wild-type and monomeric IP-10 were conjugated to diethylamidine-pentaacetic (DTPA) dihydride (Sigma-Aldrich) at a 1:50 molar ratio in 50 mM sodium phosphate buffer, pH 7.4 for 30 min on ice, and diazoyzed extensively to remove unbound DTPA. For radiolabeling, DTPA-IP-10 (100–150 μg) was incubated with 1–2 μCi of 111InCl3 at pH of 6.5 for 30 min. After separation of remaining free 111In by size exclusion, the radio-labeled IP-10 was immediately injected i.p.

Single-photon emission computed tomography (SPECT) data were acquired on a combined small animal SPECT-computed tomography (CT) scanner (XSPECT; Gamma Medica) with a submillimeter resolution. After the SPECT acquisition (radius of rotation 3 cm, 32 projections, 60 s/projection) a CT scan was acquired (256 projections, 50 kV, 500 mA) and coregistered with the SPECT dataset for image fusion and exact three-dimensional anatomical localization of the tracer signal. Imaging took place after injection and after 4, 24, 48, and 120 h. After 24 h, some mice were sacrificed for conventional biodistribution analysis by harvesting the indicated organs and measuring their activity in a Wallac 1480 Wizard gamma counter (PerkinElmer). SPECT data analysis was performed to obtain the number of counts per region in the lung.

**Binding of IP-10 to endothelial and epithelial cells**

Wild-type or mutant IP-10 was biotinylated with EZ-Link Sulfo-NHS-Biotin (Pierce) at a 1:5 molar ratio in 50 mM sodium phosphate buffer overnight at pH 6.5 to preferentially label the N terminus free amine group. The reaction was quenched by the addition of 50 mM Tris, pH 7.0. Coupling of the wild-type and monomeric IP-10 to biotin was similar as determined by ELISA. IP-10 was added to cell suspensions (1.5 × 105) in complete medium for 1 h at 37°C. Cells were washed with cold PBS and stained with Streptavidin-allophycocyanin. For control experiments, cells were preincubated with anti-CXCR3 Ab (clone 1C6 from BD Biosciences or clone Streptavidin-allophycocyanin. For control experiments, cells were preincubated with anti-CXCR3 Ab (clone 1C6 from BD Biosciences or clone 1C6 from BD Biosciences or clone L27NMe III/II receptor (BD Pharmingen). After wash, cells were incubated with IP-10 biotinylated IP-10 and stained with Streptavidin-allophycocyanin. For control experiments, cells were preincubated with anti-CXCR3 Ab (clone 1C6 from BD Biosciences or clone 49801 from R&D Systems) or control IgG Ab for 10 min at 10 μg/ml before addition of IP-10. In some experiments, HMEC (5 × 104) were loaded onto a 1-ml Heparin HiTrap or S Sepharose FF (cationic exchange) column (both from Pharmacia) equilibrated in 50 mM Tris, pH 7.5, with 0.5 M NaCl on an AKTA machine (Pharmacia). The proteins were eluted in the same buffer over 1.5 column volumes (30 ml), and protein elution was measured by OD214.

**Results**

**In vitro characterization of monomeric IP-10**

To analyze the aggregation state of wild-type or monomeric IP-10, we loaded the protein on a Superdex 200 gel filtration column. Under physiological salt conditions, wild-type IP-10 did not elute from the gel filtration column, suggesting that it forms obligers that are unable to elute from the column. A synthetic obliger monomer of IP-10, with the mutation L27NMe, that was designed to solve the nuclear magnetic resonance structure of IP-10 (34), eluted at the expected elution volume (Fig. 1A). In contrast, the previously described mutant R22E, which has reduced heparin and CXCR3 binding affinity, behaved like wild-type IP-10. At a higher
were run on a 4–20% SDS-PAGE gel. Abs (214 nm), OD214.

These results do not preclude the existence of even higher molecular mass oligomers that higher ionic strength interrupts oligomer formation. On a native gel, monomeric IP-10 ran at a lower molecular mass than the monomeric variant. Because native gels do not allow size determination, cross-linking experiments were performed, and cross-linked proteins were run on an SDS-PAGE gel, which allows estimation of molecular mass. Wild-type and R22E IP-10 displayed monomeric, dimeric, and tetrameric forms on an SDS-PAGE gel after cross-linking with Sulfo-EGS or BS3 (Fig. 1D), and trimeric and tetrameric forms were also occasionally observed after cross-linking with BS3 (data not shown). These results do not preclude the existence of even higher molecular mass oligomers for wild-type IP-10, which might not form stable complexes with the cross-linkers used. In contrast, monomeric IP-10 displayed only the monomeric form (Fig. 1D).

Next, we analyzed the heparin binding affinity of wild-type and monomeric IP-10 by elution from a heparin-Sepharose column. In contrast, monomeric IP-10 eluted at 0.59 M NaCl, only 0.6 M less than from the heparin column. These data indicate that monomeric IP-10 has lost most of its specific binding to heparin.

In competitive binding assays using 300-19 B cells expressing CXCR3, monomeric IP-10 competed for the binding of 125I-labeled IP-10 with an IC50 of 1.3 nM, whereas wild-type IP-10 had an IC50 of 0.16 nM (Fig. 2B). This 10-fold reduction of binding affinity to the high affinity, seven-transmembrane receptor is similar to what has been described for other monomeric or heparin-binding reduced mutant chemokines (28). In 300-19 cells expressing CXCR3, monomeric IP-10 was able to induce comparable CXCR3 internalization as wild-type IP-10, but only at 10-fold higher concentrations (Fig. 2C). Monomeric IP-10 was also able to induce chemotaxis of activated OT-I CD8+ T cells expressing CXCR3 but again, a 10-fold higher concentration of the monomer was needed to achieve chemotactic indices similar to those induced by wild-type IP-10 (Fig. 2D). However, monomeric IP-10 was as efficacious at its peak concentration as the wild-type protein.

A novel in vivo recruitment assay for IP-10

To test the in vivo activity of IP-10, we developed a new, physiologically relevant in vivo recruitment assay. For this, we purified CD8+ T cells from OT-I mice, which are transgenic for the TCR specific for the OVA peptide SIINFEKL bound to class I MHC (32, 33). After 6 days in culture with IL-2 and IL-12, CD8+ T cells were adoptively transferred by i.p. injection into C57BL/6 mice. Two days after injection of CD8+ T cells, IP-10 (wild-type or mutant) was injected intratracheally into the mice. The following day, lungs were lavaged, and CD8+ T cells were counted by flow cytometry. As seen in Fig. 3A, intratracheal injection of PBS, a very low number of CD8+ or CD4+ T cells was observed in the BAL. However, after injection of 5 μg of wild-type IP-10, a large influx of CD8+, but not CD4+, T cells

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was observed in the BAL. The preferential recruitment of the adoptively transferred CD8$^+$ T cells was further demonstrated by use of the Thy 1.1 marker (G. S. V. Campanella, A. D. Luster, manuscript in preparation). IP-10 has been shown to be expressed by a dose of 5 g of IP-10, cells were recovered from the BAL, and the percentage of CD3$^+$CD8$^+$ T cells was determined by flow cytometry. A. Flow analysis of T lymphocytes in the BAL. After intratracheal injection of PBS or 5 \( \mu \)g of wild-type IP-10, cells were recovered from the BAL, and the percentage of CD3$^+$CD8$^+$ and CD3$^+$CD4$^+$ T cells was determined by flow cytometry. B. Quantification of CD8$^+$ T lymphocytes recruited into the BAL. Total number of CD8$^+$ T lymphocytes recruited into the BAL by indicated doses of wild-type or monomeric IP-10. C. Dose response of IP-10 mutants. IP-10 mutants at the indicated doses were injected intratracheally after adoptive transfer of activated CD8$^+$ T cells 48 h prior. The BAL was harvested 18 h later, and CD8$^+$ T cells were analyzed by flow cytometry. The recruitment index was calculated in comparison with in vivo recruitment of activated OT-I CD8$^+$ T cells in vivo. IP-10 (wild type or mutant) was injected intratracheally at the indicated concentration after adoptive transfer of activated OT-I CD8$^+$ T cells 48 h previously. The BAL was harvested 18 h later, and CD8$^+$ T cells were analyzed by flow cytometry. A. Flow analysis of T lymphocytes in the BAL. After intratracheal injection of PBS or 5 \( \mu \)g of wild-type IP-10, cells were recovered from the BAL, and the percentage of CD3$^+$CD8$^+$ and CD3$^+$CD4$^+$ T cells was determined by flow cytometry. A. Flow analysis of T lymphocytes in the BAL. After intratracheal injection of PBS or 5 \( \mu \)g of wild-type IP-10, cells were recovered from the BAL, and the percentage of CD3$^+$CD8$^+$ T lymphocytes recruited into the BAL. Total number of CD8$^+$ T lymphocytes recruited into the BAL by indicated doses of wild-type or monomeric IP-10. C. Dose response of IP-10 mutants. IP-10 mutants at the indicated doses were injected intratracheally after adoptive transfer of activated CD8$^+$ T cells 48 h prior. The BAL was harvested 18 h later, and CD8$^+$ T cells were analyzed by flow cytometry. The recruitment index was calculated in comparison with intratracheal injection of PBS. D. Coinjection of wild-type and monomeric IP-10. Wild-type or monomeric IP-10 (5 \( \mu \)g) were either injected intratracheally individually, or premixed for 30 min in PBS before injection. The rest of the experiment was performed as described in C. Experiments were performed with four mice per group; one representative experiment out of at least two is shown.

A dose response of wild-type IP-10 showed that 0.5 \( \mu \)g of IP-10 caused a statistically significant recruitment of CD8$^+$ T cells, with a mean recruitment index of 3.7, whereas 5 and 50 \( \mu \)g each induced even more recruitment, with mean recruitment indices of 15.7 and 53.6, respectively. However, intratracheal injections of 0.5–50 \( \mu \)g of monomeric IP-10 induced no recruitment of CD8$^+$ T cells into the airways (Fig. 3B).

**FIGURE 3.** Monomeric (Mono) IP-10 does not induce recruitment of activated OT-I CD8$^+$ T cells in vivo. IP-10 (wild type or mutant) was injected intratracheally at the indicated concentration after adoptive transfer of activated OT-I CD8$^+$ T cells 48 h previously. The BAL was harvested 18 h later, and CD8$^+$ T cells were analyzed by flow cytometry. A. Flow analysis of T lymphocytes in the BAL. After intratracheal injection of PBS or 5 \( \mu \)g of wild-type IP-10, cells were recovered from the BAL, and the percentage of CD3$^+$CD8$^+$ T cells was determined by flow cytometry. B. Quantification of CD8$^+$ T lymphocytes recruited into the BAL. Total number of CD8$^+$ T lymphocytes recruited into the BAL by indicated doses of wild-type or monomeric IP-10. C. Dose response of IP-10 mutants. IP-10 mutants at the indicated doses were injected intratracheally after adoptive transfer of activated CD8$^+$ T cells 48 h prior. The BAL was harvested 18 h later, and CD8$^+$ T cells were analyzed by flow cytometry. The recruitment index was calculated in comparison with intratracheal injection of PBS. D. Coinjection of wild-type and monomeric IP-10. Wild-type or monomeric IP-10 (5 \( \mu \)g) were either injected intratracheally individually, or premixed for 30 min in PBS before injection. The rest of the experiment was performed as described in C. Experiments were performed with four mice per group; one representative experiment out of at least two is shown.

**Monomeric IP-10 is unable to cause in vivo recruitment**

A dose response of wild-type IP-10 showed that 0.5 \( \mu \)g of IP-10 caused a statistically significant recruitment of CD8$^+$ T cells, with a mean recruitment index of 3.7, whereas 5 and 50 \( \mu \)g each induced even more recruitment, with mean recruitment indices of 15.7 and 53.6, respectively. However, intratracheal injections of 0.5–50 \( \mu \)g of monomeric IP-10 induced no recruitment of CD8$^+$ T cells into the airways (Fig. 3B).

**Oligomerization rather than heparin binding is important for the in vivo activity of IP-10**

To understand why monomeric IP-10 is unable to induce recruitment of CD8$^+$ T cells into the airways, we studied two of our previously published IP-10 mutants in the in vivo recruitment model. One of them, mutant R84A, has the same heparin-binding affinity and oligomerization pattern as wild-type IP-10 but has a 60-fold reduced CXCR3 binding affinity and no chemotactic in vitro activity (Fig. 2D and Ref. 14). As expected, R84A was unable to induce recruitment of CD8$^+$ cells in vivo (Fig. 3C) and confirmed that the recruitment of the CD8$^+$ T cells was a CXCR3-dependent process. Of particular interest was mutant R22E, which had similarly reduced heparin-binding affinity (elution at 0.61 M NaCl from a heparin-Sepharose column; Ref. 14) as monomeric IP-10. The CXCR3-binding affinity of R22E was also similar to that of monomeric IP-10 (IC$_{50}$ 3.4 nM; Ref. 14), but R22E was not as efficacious as wild-type or monomeric IP-10 in chemotaxis (Fig. 2D). However, mutant R22E oligomerizes like wild-type IP-10 (Fig. 1). In the in vivo recruitment assay, mutant R22E induced a dose-dependent, statistically significant influx of CD8$^+$ T cells at a dose of 5 \( \mu \)g (mean recruitment index, 4.7) and 50 \( \mu \)g (mean recruitment index, 25.5; Fig. 3C). Although this mutant was clearly less potent at lower doses than wild-type IP-10, higher concentrations of mutant R22E could overcome its reduced CXCR3- and heparin-binding affinity. In contrast, monomeric IP-10 was unable to induce any recruitment of T cells even at a dose of 50 \( \mu \)g. This demonstrates that the reduced heparin- and CXCR3-binding affinity of monomeric IP-10 is not the main reason for its inability to induce T cell recruitment in vivo. Instead, it suggests that the ability to oligomerize is an essential requirement for the in vivo activity of IP-10.

**Coinjection of monomeric IP-10 does not inhibit wild-type IP-10**

It has been reported for the CC chemokines RANTES (36) and MCP-3 (37), that monomeric or heparin-binding reduced mutants were able to inhibit the in vivo activity of the wild-type protein. To test the ability of monomeric IP-10 to inhibit wild-type IP-10, we coinjected 5 \( \mu \)g of both wild-type and monomer IP-10 intratracheally into the same mouse. As shown in Fig. 3D, there was no significant inhibition of wild-type IP-10 by coinjected monomeric IP-10, suggesting that monomeric IP-10 does not act as a dominant negative inhibitor of IP-10. In addition, monomeric IP-10 did not form heterodimers with wild-type IP-10 as determined by gel filtration (data not shown).

**Biodistribution of intratracheally injected IP-10**

To investigate the biodistribution and retention of wild-type and monomeric IP-10 after intratracheal instillation, we labeled both proteins with $^{111}$In. Labeling IP-10 for biodistribution studies did not change its IC$_{50}$ values in the competitive CXCR3 binding assay, its heparin-binding affinity, its oligomerization state as measured by gel filtration, or its in vitro chemotaxis dose response (data not shown). $^{111}$In-labeled wild-type or monomeric IP-10 was injected intratracheally into mice and subsequently imaged by SPECT-CT after 0, 4, 24, 48, and 120 h postinjection. As seen in Fig. 4, A–D, after instillation, most radioactivity was found in the lung for both wild-type and monomeric IP-10. Interestingly, even at later time points, most of the radioactivity in the animal was still found in the lung for both wild-type and monomeric IP-10, up to the last time point studied (120 h). Analysis of the imaging matrix demonstrated that both wild-type and monomeric IP-10 had 70–90% of the radioactivity in the animal localized in the lungs (Fig. 4E). To confirm these results, in some experiments, mice were sacrificed after 24 h, and the radioactivity in the organs was measured (Fig. 4F). As seen in the SPECT-CT, most of the labeled wild-type and monomeric IP-10 was found in the lung, with a small amount in the kidneys and liver. This small amount of radioactivity in the kidneys and liver probably reflects a low level of...
IP-10 excretion. It is estimated that after 24 h ~75% of the injected IP-10 was still in the animal. These imaging experiments clearly demonstrate that the retention of monomeric IP-10 in the lung is similar to wild-type IP-10.

To determine whether the IP-10 was in the airway fluid or in the lung parenchyma, we measured the concentration of wild-type and monomeric IP-10 in the BAL by ELISA. We found only a very small fraction of injected wild-type (0.14%) or monomeric IP-10 (0.08%) was recoverable from the airways after 18 h (Fig. 5). In contrast, in control experiments, most of the IP-10 was recovered when the lavage was done ten minutes after intratracheal injection (46.7% for wild-type and 61.8% for monomeric IP-10). Additionally, increasing the salt concentration of the lavage fluid to 1 M NaCl to release chemokine potentially bound to GAGs on bronchial epithelial cells did not release much more IP-10 and yielded no difference between wild-type and monomeric IP-10. This suggests that within 18 h both wild-type and monomeric IP-10 moved out of the airways into the lung parenchyma.

Reduced endothelial cell binding of monomeric IP-10

To further explore the mechanisms underlying monomeric IP-10’s inability to induce in vivo recruitment, we studied the in vitro binding of wild-type and monomeric IP-10 to endothelial and epithelial cells. For this purpose, we labeled the chemokines with biotin, which did not influence their in vitro activity or oligomerization state (data not shown). Analysis of IP-10 binding to HMEC by flow cytometry demonstrated dose-dependent binding of wild-type IP-10, whereas monomeric IP-10 failed to show significant binding even at a concentration of 10,000 ng/ml (Fig. 6, A and B). There was a low level of background binding of the monomer to the cells; however, it was not concentration dependent. In contrast, biotin-labeled mutant R22E bound to HMEC at higher concentrations, although markedly reduced compared with wild-type IP-10. To exclude that CXCR3, which has been shown by several groups to be expressed on HMEC under certain conditions (e.g., Refs. 38 and 39), is involved in the binding of IP-10 to HMEC, two Abs
that block CXCR3A and B and detect CXCR3 on HMEC were used during the binding studies, and did not inhibit the binding of wild-type or monomeric IP-10 to HMEC (Fig. 6C). To investigate the mechanism of IP-10 binding to HMECs, the cells were digested with glycosidases before performing the binding assay. Wild-type IP-10 binding was reduced by 63% after glycosidase treatment, demonstrating that wild-type IP-10 binding to endothelial cells is mainly dependent on glycosaminoglycans (Fig. 6D). In contrast, the low level binding observed for monomeric IP-10 was not affected by glycosidase treatment.

Wild-type IP-10 also bound strongly to epithelial cells, both to human bronchial epithelial cell-derived Beas2B cells (Fig. 6E) as well as CHO cells (Fig. 6F), whereas monomeric IP-10 did not show significant binding to either cell line. Similarly to what was seen with the glycosidase-treated endothelial cells, binding of IP-10 to CHO 745 cells, which are deficient in GAGs, was drastically reduced, demonstrating that epithelial cell binding was also dependent on GAGs (Fig. 6G). Indeed, it appears as if binding of wild-type IP-10 to CHO 745 cells was reduced to the level of monomeric IP-10. Binding to CHO cells was also analyzed by immunofluorescence and clearly illustrated binding of wild-type IP-10 to epithelial cell surfaces (Fig. 6H), whereas there was no significant binding of monomeric IP-10 to CHO cells (Fig. 6I), implying that IP-10 oligomerization is needed for binding to endothelial and epithelial cells.

**Monomeric IP-10 does not immobilize on endothelial cells to induce transendothelial migration**

Leukocytes must traverse the endothelium to enter tissue from the blood. We therefore studied the ability of monomeric IP-10 to induce in vitro migration through an endothelial cell layer. HMEC were grown to confluency on the bottom side of Neuroprobe chemotaxis filters. In some wells, wild-type or monomeric IP-10 was added to the endothelial cells for 1 h at 37°C and allowed to immobilize on the endothelial cell layer. Nonbound IP-10 was washed off the endothelial cells, after which the chemotaxis chamber was assembled without the addition of further IP-10 in the bottom chamber. In other wells, wild-type or monomeric IP-10 was added in solution to the bottom chamber before adding the filter with confluent HMEC. Activated CD8+ T cells were added to the top of the chemotaxis filter and allowed to migrate for 2 h in response to IP-10. Monomeric IP-10 added in solution to the lower chamber was able to induce chemotaxis of T cells through the endothelial cell layer similarly as through bare filters (Fig. 7). In contrast, when IP-10 was bound to the HMEC and washed off before addition of T cells, monomeric IP-10 was unable to induce T cells transendothelial migration, whereas wild-type IP-10 was able to induce chemotaxis of T cells through the HMEC layer. This

*FIGURE 5.* IP-10 recovery in BAL 10 min or 18 h after intratracheal injection. Wild-type or monomeric IP-10 (5 μg) was injected intratracheally into mice. BAL was performed 10 min or 18 h after instillation. After the 18-h BAL, lungs were lavaged with 1 ml of 1 M NaCl buffer. IP-10 concentration was measured by ELISA. Experiments were performed with three mice per group and repeated twice. Results are means ± SD.

*FIGURE 6.* Binding of wild-type (wt), monomeric, and R22E IP-10 to endothelial and epithelial cells. Cells (HMEC, Beas2B, and CHO) were incubated for 1 h at 37°C with the indicated concentration of biotinylated wild-type, monomeric, or R22E IP-10. After the cells were washed, binding was measured by flow cytometry using Streptavidin-allophycocyanin (Strep-APC; A–G) or by immunofluorescence using Streptavidin-FITC (H and I). A. Flow cytometry. Binding of wild-type, monomeric, and R22E IP-10 to HMEC. Representative flow diagram at 10,000 ng/ml IP-10. B. Quantification of binding to HMEC. Mean fluorescent intensity (MFI) at indicated concentrations of IP-10 is shown. C. CXCR3 Ab blocking. Mean fluorescent intensity at 5000 ng/ml IP-10 binding to HMEC after pretreatment with Abs. D. Glycosidase-treated HMEC. Mean fluorescent intensity at 5000 ng/ml IP-10 binding to HMEC after glycosidase treatment. E and F. Flow cytometry. Binding of 5000 ng/ml wild-type or monomeric IP-10 to Beas2B (E). CHO wild-type (E), or CHO 745 cells (F). G. Immunofluorescence of wild-type IP-10 binding to CHO cells. H. Immunofluorescence of monomeric IP-10 binding to CHO cells. Wild-type IP-10 or monomeric IP-10 (5000 ng/ml) binding to CHO wild-type cells (original magnification, ×600). One representative experiment of at least two is shown.
suggestions that monomeric IP-10 is unable to be retained on the endothelial cell layer, whereas wild-type IP-10 can be immobilized on endothelial cells, which is required for transendothelial cell migration of CXCR3-expressing lymphocytes.

**Discussion**

The physiological role of chemokine oligomerization is still unclear. Here, we show that oligomerization is essential for the in vivo activity of IP-10; moreover, we identify a novel mechanism for this effect, which may be applicable to other chemokines.

To investigate the role of IP-10 oligomerization, we utilized a synthetic mutant variant of IP-10 with an additional N-methyl group at position L27, which interrupts the main chain hydrogen bond between residues L27 and I29 on opposing chains. Gel filtration studies as well as sizing gels with or without cross-linkers clearly demonstrated that the mutant variant L27/NMe IP-10 is an obligate monomer, whereas wild-type IP-10 forms higher order complexes.

Although monomeric IP-10 had the ability to induce in vitro chemotaxis and CXCR3 internalization with efficacy comparable with that of wild-type IP-10, monomeric IP-10 was unable to induce recruitment of activated CD8+ T cells into the airways of mice even at concentrations 100-fold higher than the effective wild-type IP-10 concentration. Interestingly, mutant R22E, which had reduced heparin and CXCR3 binding similar to that of monomeric IP-10, yet oligomerized like wild-type IP-10, induced robust recruitment of T cells in vivo at 10-fold higher concentration compared with wild-type IP-10. We were therefore able to differentiate between heparin binding and oligomerization and demonstrate clearly that the inability of monomeric IP-10 to induce in vivo recruitment is due to its inability to oligomerize and not simply due to its reduced heparin or CXCR3 binding affinity.

The biodistribution of chemokines after release into the body is not well understood, and we are reporting here one of the first molecular imaging studies of a chemokine. We found that both wild-type and monomeric IP-10 were retained for >5 days in the lung after intratracheal instillation. Therefore, the difference in in vivo activity of wild-type and monomeric IP-10 was not due to the difference in retention at the site of injection. In addition, our biodistribution data showed a similar proportion of injected 111In-labeled wild-type and monomeric IP-10 in the kidneys and liver. Because clearance through the liver and kidney most likely occurs through the blood stream, these findings suggest that both wild-type and monomeric IP-10 were similarly transported across the epithelium and endothelium. Transport of proteins across the alveolar epithelial barrier has been well documented for various proteins (reviewed by Hastings et al. in Ref. 40 and Kim and Malik in Ref. 41) and occurs mainly through paracellular diffusion or transcytosis across the epithelium. The mechanism by which intratracheally instilled IP-10 crosses the epithelial and endothelial barrier is not known. The results of the BAL ELISA suggest that the vast majority of intratracheally injected wild-type and monomeric IP-10 had left the airways after 18 h. However, the imaging studies demonstrated that most of the IP-10 was retained in the lung, whereas a small proportion was transported into the blood stream. This suggests that both wild-type and monomeric IP-10 crossed the epithelial barrier and were retained in the lung, from where a small fraction of the proteins moved across the endothelium.

To understand the role of oligomerization for binding to endothelial and epithelial cells, we studied the binding of wild-type and monomeric IP-10 to both cells types in vitro. Wild-type IP-10 bound strongly to epithelial cells in a GAG-dependent manner. In contrast, monomeric IP-10 did not bind well to epithelial cells. Therefore, the process by which IP-10 leaves the airways does not seem to require strong, GAG-dependent binding, as both wild-type and monomeric IP-10 had left the airways after 18 h. However, wild-type IP-10 also bound strongly to HMECs, whereas monomeric IP-10 bound very weakly to HMECs, and not in a dose-dependent manner. Together with the biodistribution data showing that both wild-type and monomeric IP-10 are transported into the blood stream equally, this suggests that only wild-type IP-10 is presented on the pulmonary endothelium under physiological flow conditions to establish a haptotactic gradient on the endothelium, which induces T cell recruitment.

To confirm this theory, the ability of wild-type and monomeric IP-10 to induce transendothelial migration was investigated. The continued presence of soluble monomeric IP-10 was able to induce transendothelial migration of T cells similar to that induced by wild-type IP-10. However, when only endothelial cell bound IP-10 was assayed by washing away unbound IP-10, wild-type IP-10 was able to induce transendothelial chemotaxis, but monomeric IP-10 was not. This further demonstrates that monomeric IP-10 is unable to be immobilized on endothelial cells and suggests that presentation of IP-10 on endothelial cells is required for its activity in vivo.

In summary, we found that after intratracheal injection, wild-type oligomeric IP-10 crosses the epithelial barrier, is retained in the lung, and is presented on the endothelium. This binding to endothelial cells is dependent on oligomerization, and establishes a haptotactic gradient, which induces recruitment of effector T cells into the airways (Fig. 8). Monomeric IP-10 also crosses the epithelial barrier and is retained in the lung after intratracheal instillation. However, in the absence of oligomerization, monomeric...
IP-10 is not retained on the endothelium and therefore cannot establish a haematopoietic gradient to recruit CXCR3-expressing lymphocytes into the airspaces. We propose a novel mechanism of chemokine regulation that requires oligomerization for presentation on endothelial cells, which is necessary for chemokine activity in vivo.

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Disclosures

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