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A Chemokine-Mediated *In Vivo* T-Cell Recruitment Assay

Gabriele S. V. Campanella and Andrew D. Luster

Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy and Immunology, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts, USA

Abstract

The ability of chemokines to induce the migration of cells expressing their cognate G-protein–coupled receptor is a characteristic property of chemokine function. To study this important function, *in vitro* chemotaxis assays are most often used, which, although useful, lack many components of the complex *in vivo* trafficking process. Reliable *in vivo* recruitment assays have been very difficult to establish. We describe a robust *in vivo* T-cell recruitment assay for adoptively transferred T lymphocytes in mice. Instillation of the CXCR3 chemokine ligands IP-10/CXCL10 or I-TAC/CXCL11 into the airways results in robust recruitment of transferred T lymphocytes. The assay thereby models the natural environment of chemokine function, as chemokines are expressed in the airways during inflammation, inducing selective leukocyte homing. This assay is particularly useful for the analysis of chemokine and chemokine receptor mutants in structure function studies and for testing the *in vivo* efficacy of inhibitory chemokine and chemokine receptor antibodies and small molecule antagonists.

1. Introduction

Trafficking of leukocytes to sites of inflammation is a complex process. Chemokines and other chemoattractants play important roles in multiple aspects of this process. Chemokines presented by endothelial glycosaminoglycans bind to their cognate G-protein–coupled receptors on leukocytes, resulting in the activation of leukocyte integrins, firm arrest, and subsequent leukocyte extravasation through the endothelium into the tissue. Chemokines also contribute to migration, retention, and survival of leukocytes once in the tissue (Luster et al., 2005).

The ability of chemokines to induce migration of leukocytes has been widely studied *in vitro*, but robust *in vivo* recruitment assays to study chemokine functions *in vivo* are rarely used. Although very useful, *in vitro* chemotactic assays are limited in that they lack many components of the complex *in vivo* trafficking process. In the most commonly used *in vitro* chemotaxis assays, exemplified by the Boyden transwell chamber, chemokines and cells are placed on opposite sides of a membrane with a specific pore size. The cells are allowed to migrate through the membrane in response to the chemokine, and their numbers are compared with the numbers of cells migrating without chemokine. These chemotaxis assays clearly lack many of the components of *in vivo* migration, such as a chemokine gradient, chemokine presentation by endothelial cells, and physiologic flow. To overcome some of these limitations, in some *in vitro* chemotaxis assays, the membranes are coated with extracellular matrix proteins, or endothelial or epithelial cells are grown on the membrane, simulating the transmigration process. Furthermore, some chemotactic chambers try to attain a chemotactic gradient along which leukocytes can migrate (Zicha et al., 1991; Zigmond, 1977). Still others model physiologic flow by use of flow chambers (see Chapter 14). However, each of these systems can only partially mimic the complex *in vivo* trafficking process. Therefore, to fully investigate the ability of chemokines to induce leukocyte

trafficking, a robust *in vivo* recruitment assay is required. In this chapter, we describe such an assay for chemokine-mediated recruitment of T cells into the airways of mice.

2. *In Vitro* Activation of T Lymphocytes

The availability of large numbers of a uniform cell population responsive to the chemokine of interest is critical for this recruitment assay. The CXCR3 chemokine ligands IP-10/CXCL10 and I-TAC/CXCL11 mediate migration of activated T cells. Thus, in naïve animals CXCR3 responsive T cells are relatively sparse. Instead of systemic activation of the endogenous immune system by agents like adjuvants, in this assay, T lymphocytes are activated *in vitro* and then adoptively transferred into naïve animals. These adoptively transferred cells can be tracked by markers (e.g., Thy1.1 allele), resulting in high recruitment indices with low backgrounds. The responsiveness of adoptively transferred cells to the chemokine of interest should be tested *in vitro* before the *in vivo* recruitment assay is conducted. For our purposes, we activate CD8⁺ T lymphocyte from T cell receptor–transgenic mice in the C57Bl/6 background specific for the ovalbumin peptide SIINFEKL (OVA_{257–264}) (OT-I mice) (Clarke *et al.*, 2000; Hogquist *et al.*, 1994). This allows for the efficient expansion of CD8 T lymphocytes with the SIINFEKL peptide *in vitro*. The protocol used for *in vitro* culturing of activated CD8 T lymphocytes and their *in vitro* characterization is described in the following.

2.1. Purification of CD8 T lymphocytes and preparation of antigen-presenting cells

1. Prepare fresh buffer for bead selection (termed here “MACS buffer”), with PBS without Ca²⁺Mg²⁺, adding 0.5% BSA and 2 mM EDTA. Sterile-filter and degas buffer. This buffer can be stored for up to 10 days at 4 °C.
2. Prepare cell culture medium. We use RPMI with 10% heat-inactivated fetal calf serum (FCS) (Sigma), 10 mM HEPES, 100 U/ml Pen/Strep, 2 mM L-glutamine, 1× nonessential amino acids, 1 mM Na pyruvate. In our experience, the FCS can greatly affect the growth and activity of the cultured effector CD8 T lymphocytes. We recommend testing different types and batches of serum and using the same lot of serum for subsequent experiments.
3. Harvest spleen and peripheral lymph nodes (we normally harvest inguinal, popliteal, axillary, brachial, internal jugular, superficial cervical, and facial lymph nodes, depending on the desired number of CD8 T lymphocytes) from C57Bl/6 OT-I mice. Place in tube with sterile HBSS, kept on ice.
4. Harvest spleen from 1 to 2 wild-type C57Bl/6 mice and place in tube with sterile HBSS kept on ice. These spleens will provide the antigen-presenting cells (APCs) for presenting the Ova peptide to the CD8 T lymphocytes.
5. Prepare lymphatic cell suspension by straining spleens and lymph nodes through a 70-μm cell strainer with HBSS, both for CD8 T lymphocytes from OT-I mouse and for antigen-presenting cells.
6. Spin cells for 10 min at 1500 rpm, remove supernatant.
7. Gently resuspend pellet in 1 ml of red cell lysis buffer (Sigma Aldrich) and incubate for 3 min at room temperature. Add HBSS (15 to 20 ml), shake lightly.
8. Spin for 10 min at 1500 rpm. Decant supernatant, gently resuspend pellet in 10 ml HBSS. Keep antigen-presenting cells for step 19.
9. For cells from OT-I mouse, spin cells for 10 min at 1500 rpm. Decant supernatant.

10. To avoid any cell clumps, which might interfere with the CD8 bead selection, pass cells through a 30- μ m preselection cell strainer (MACS preselection filter, Miltenyi Biotech #130-041-407). Wet preselection cell strainer with 500 μ l MACS buffer. Resuspend OT-I cells in 2 ml MACS buffer and pass through 30- μ m strainer into new tube. Wash cell strainer with 500 μ l MACS buffer three times. Count cells.
11. Spin down cells at 1500 rpm for 5 min and resuspend in 90 μ l per 10^7 cells MACS-buffer. Add 1 μ l anti-CD8 α microbeads (Miltenyi Biotech 130-049-401) per 10^6 total cells.
12. Incubate for 20 min in 12 °C H₂O bath. Add 10 ml MACS-buffer and spin at 300g, 10 min.
13. Meanwhile, place MACS Separation LS+ column (Miltenyi Biotech #130-042-401) inside magnet slot and prerun with 3 ml MACS-buffer.
14. Resuspend cells in 5 ml MACS-buffer. It is very important to avoid any bubbles during resuspension and during the whole bead selection process.
15. Add the CD8 α -bead labeled cells to the column, and collect the flowthrough into a 50-ml tube. Wash column with 3 ml MACS-buffer at least 3 times.
16. When the column is almost empty, remove it from the magnet and place it inside a 15-ml tube. Fill the column again with 5 ml MACS-buffer and apply the plunger into the tube. Most CD8 T lymphocytes will elute in the first drops after the column is removed from the magnet. It is, therefore, very important to place the column into the new tube immediately after being removed from magnet or already having the column under the new tube when removing it from the magnet.
17. Add cell culture media to cell suspension to relieve cells from the MACS buffer. Spin down cells at 1500 rpm for 10 min and resuspend in 5 ml cell culture media, count CD8 T lymphocytes. Adjust cell concentration to 1×10^6 /ml. We normally obtain between 10 and 25 million CD8 T lymphocyte per mouse.
18. Irradiate APCs from step 9 with 3000 rad. Alternately, the cells can be treated with mitomycin-C.
19. Spin APCs at 1500 rpm for 10 min and resuspend in 5 ml cell culture media. Count. Adjust concentration to 10 to 12 million/ml.

2.2. Culturing of CD8 T lymphocytes

We usually culture 5×10^6 purified CD8 T lymphocytes with 5 to 6×10^7 APCs in T75 tissue culture flasks, as described in the following.

1. Incubate antigen-presenting cells with SIINFELK Ova peptide for 5 min. We resuspend the SIINFELK peptide at 200 μ g/ml and add 105 μ l per 50 to 60 million APC used per T75 flask.
2. Place peptide pulsed APCs (50 to 60 million total), CD8 T lymphocytes (5 million total) in T75 flask, adding culture medium to a total volume of 30 ml.
3. Add anti-CD28 (BD Pharmingen, #553294, 2 μ g/ml final), IL-2 (Peprotech, #212-12, 10 ng/ml final), IL-12 (R&D, #419-ML-010, 10 ng/ml final). Culture cells in 37 °C, 5 % CO₂ in a humidified incubator.
4. On day 3 after purification, split cells in half using cell culture media supplemented with 10 ng/ml IL-2. We do not spin down the cells but add 30 ml new media with IL-2 to each 30-ml culture. After gently mixing, 30 ml of cells are transferred to a

new T75 flask. By day 3, if the cells are growing well, they should form little cell clumps.

5. The activated CD8 effector T lymphocytes can be used after 5 or 6 days of *in vitro* activation. If cells are used after 6 days, on day 5, we feed the cells by adding 15 ml of culture medium supplemented with IL-2 per 30 ml culture. For CXCR3 ligand induced migration, we found that CD8 T lymphocyte activated for 6-day *in vitro* display the highest *in vivo* recruitment activity (Campanella *et al.*, 2008).
6. Harvest cells: We harvest the effector CD8 T lymphocytes with Lympholyte M (Cedarlane) to remove dead cells (in particular APCs). For this, cells are spun down and the cell pellet from each T75 flask resuspended in 5 ml room temperature HBSS. The cells are transferred into 15-ml Falcon tube and 5 ml of Lympholyte M are added to the bottom of the tube. The cells are spun for 25 min at room temperature at 1000g.
7. Carefully collect the lymphocyte fraction at the interface. Wash the CD8 T lymphocytes three times in HBSS. Count cells.
8. The effector CD8 T lymphocytes can now be used for the *in vivo* recruitment assay (see below).
9. The effector CD8 T lymphocytes can be further cultured to test their responsiveness to chemokines at the time of chemokine instillation. We plate the cells at 0.5×10^6 /ml in cell culture medium supplemented with IL-2. Depending on cell growth, the cells are fed every 2 days with fresh media.

2.3. *In Vitro* characterization of CD8 T lymphocytes

The activation and purity of the effector cells can be evaluated by flow cytometry. We usually use the activation markers CD25 and CD62L to test the activation status of the cells. As shown in Fig. 18.1, before culturing (day 0), the CD8 bead-purified T cells express high levels of CD62L and low levels of CD25. After 6 days of *in vitro* activation (day 6), the cells express lower levels of CD62L and very high levels of CD25.

Furthermore, we analyze the expression of the chemokine receptor of interest by flow cytometry. In this case, we analyzed the expression of CXCR3 with a directly conjugated anti-CXCR3 antibody from R&D systems. Interestingly, we found that the expression of CXCR3 after 6 days of *in vitro* activation was very low on the effector CD8 T lymphocytes (Fig. 18.1B). After further *in vitro* culturing in the presence of IL-2, CXCR3 expression increased and peaked at day 8. This indicates that on the day when the cells are adoptively transferred into mice, they are not yet responsive to the CXCR3 chemokines IP-10 and I-TAC. It is critical to evaluate the chemokine receptor expression of the transferred cells *in vivo* at the time they are required to migrate in response to the chemokines. We, therefore, evaluated CXCR3 expression of the transferred cells in the spleen on the day of harvest, with the Thy1.1 marker on the transferred cells, after injection into Thy1.2 mice. As seen in Fig. 18.1C, CXCR3 expression was high on Thy1.1⁺ transferred cells on the day of harvest, a critical prerequisite for the cells to be responsive to CXCR3 ligands.

This *in vivo* recruitment assay relies on the presence of a large population of adoptively transferred cells, which are responsive to the chemokine of interest. The responsiveness to the chemokine of interest should be validated during the establishment of the *in vivo* assay. We routinely use two different *in vitro* assays to evaluate the effector T lymphocytes for their ability to respond to chemokines. First, we conduct receptor internalization assays, in which the ability of chemokines to induce receptor internalization is measured. The method and characteristics of CXCR3 internalization have been described in detail elsewhere (Sauty

et al., 2001). Activated CD8⁺ T cells were cultured with IL-2 as described previously for a total of 8 or 9 days. The cells were washed and resuspended in culture media at a concentration of 0.5×10^6 /ml. Different concentrations of I-TAC or IP-10 (usually 10 to 1000 ng/ml) were added to the cells and incubated for 30 min at 37 °C. The cells were washed and stained with anti-mCXCR3 antibody conjugated to PE (R&D Systems) or an IgG control and analyzed by FACS. As can be seen in Fig. 18.2A, the addition of both 100 ng/ml IP-10 or I-TAC induced robust internalization of CXCR3, with I-TAC having a stronger effect than IP-10, as previously described (Colvin *et al.*, 2004; Sauty *et al.*, 2001).

Second, we test the *in vitro* chemotactic activity of the effector cells (Fig. 18.2B). Different methods of *in vitro* chemotaxis can be used. In our case, IP-10 or I-TAC were diluted in RPMI media supplemented with 1% low endotoxin BSA (Sigma Aldrich) and added to the bottom well of a 96-well chemotaxis plate (Neuroprobe). Activated effector T cells (days 8 to 9 in culture with IL-2) were washed and resuspended in the same buffer at a concentration of 0.5×10^6 cells/ml and 50 μ l of cells were added on top of the membrane (5- μ m pore size, polycarbonate filters). The chemotaxis plate was incubated at 37 °C for 2 h and transferred to 4 °C for 10 min before removing the membrane. The cells that had migrated to the bottom wells were counted under a microscope. Chemotactic indices were calculated by dividing the number of cells in the bottom well in response to the chemokine by the average number of cells in the bottom well without the addition of chemokines. As seen in Fig. 18.2B, both IP-10 and I-TAC induced strong chemotaxis of the effector T lymphocytes, confirming that these cells were responsive to our chemokines of interest.

3. In Vivo Chemokine-Mediated Recruitment

The *in vivo* recruitment assay consists of three main steps: (1) adoptive transfer of *in vitro* activated effector T lymphocytes; (2) instillation of chemokine into a well-defined tissue compartment; and (3) harvest and subsequent analysis of T lymphocytes that have trafficked in response to the instilled chemokine.

3.1. Adoptive transfer of T lymphocytes

To provide a large target cell population responsive to the chemokine of interest, effector T lymphocytes are activated *in vitro* as previously described. In our case, effector CD8 T lymphocytes are activated for 6 days in culture. Similarly, activated CD4 T lymphocytes can also be used as we have previously described (Campanella *et al.*, 2008). In our experience, adoptive transfer of 5 million to 15 million effector cells results in good recruitment indices. Injection of higher numbers of effector cells resulted in higher numbers of recruited cells but also increased the background number of effector cells present in the anatomic site chosen for chemokine instillation (Campanella *et al.*, 2008). Therefore, we usually adoptively transfer 7×10^6 effector CD8 T lymphocytes in 500 μ l HBSS. We transfer the effector cells by intraperitoneal injection into mice and instill the chemokine 48 h after cell transfer. Alternately, the effector cells could be transferred by tail vein injection, in which case the chemokines may need to be instilled earlier (e.g., 24 h) because i.v.-injected cells enter the bloodstream faster than i.p.-injected cells.

3.2. Intratracheal instillation of chemokines

Two days after intraperitoneal transfer of effector cells, the chemokines are injected into mice. Different anatomic locations can be chosen for chemokine instillation. We first tried injection of chemokines into the peritoneum (after i.v. transfer of effector cells) but did not obtain robust and specific recruitment indices after peritoneal washes. As an alternative, we chose to instill chemokines into the airways as a defined anatomic compartment. IP-10 and I-TAC are expressed by bronchial epithelial cells during various inflammatory diseases, as

shown for tuberculosis (Sauty *et al.*, 1999), chronic obstructive pulmonary disease (COPD) (Saetta *et al.*, 2002), and rhinovirus infections (Spurrell *et al.*, 2005), resulting in the recruitment of CXCR3⁺ lymphocytes into the airways. Intratracheal injections, therefore, model the natural environment in which chemokines are expressed during inflammation. Furthermore, the airways are an anatomic site, where few T lymphocytes are present in the absence of chemokine instillation and few adoptively transferred effector T lymphocytes migrate into the airways without a stimulus.

Intratracheal injections are fast and easy to perform, but at the beginning this should be practiced to ensure that they could be performed reproducibly. For those with no experience in intratracheal injection, a dye can first be used for practice. Successful intratracheal injection of the dye can then be confirmed by opening the chest cavity of the mice to observe whether the dye has reached the lungs. Below is a detailed description of intratracheal chemokine instillation.

1. Sedate mice with ketamine/xylazine (100 mg/kg and 12 mg/kg) given by i. p. injection.
2. Extend neck of mice, and swap neck with 70% ethanol.
3. After a small 0.5-cm incision along the midline of the neck, gently expose the trachea.
4. Under direct visualization of the trachea, inject chemokine in 50 μ l PBS, or PBS only as a control, with a 28-gauge bent needle.
5. Close the neck with suture or wound staples. Allow mice to recover on a warming bed for 30 min.
6. The mice generally tolerate this procedure well without respiratory compromise.

3.3. Bronchial alveolar lavage and T lymphocyte analysis

Recruitment of T lymphocytes to the airways is analyzed by bronchial alveolar lavage (BAL). We obtained maximal specific recruitment of T lymphocytes in response to chemokines 18 h after intratracheal injection (Campanella *et al.*, 2008), but this might vary for other chemokines and other cell types. After BAL, the cells in the BAL fluid are analyzed by flow cytometry. Following is a detailed protocol for these procedures.

1. Sedate mice with ketamine/ xylazine (100 mg/kg and 12 mg/kg) given by i. p. injection.
2. Exsanguinate mice by cutting renal artery.
3. Extend neck and cut open the wound of intratracheal injection.
4. Expose trachea. Guide a suture thread underneath the trachea.
5. Make small incision near the top of the trachea.
6. Insert into the trachea a thin tubing attached to three-way stopcock attached to one empty 3-ml syringe and one 3-ml syringe filled with PBS, without Ca²⁺ and Mg²⁺, supplemented with 2 mM EDTA.
7. Use thread passed under trachea to tighten the tubing in the trachea.
8. Wash the airways with six sequential 0.5-ml washes.
9. Spin down BAL fluid, 10 min at 1500 rpm. The supernatant can be retained for analysis of chemokine or cytokine levels if desired.

10. (Optional) Resuspend cell pellet in 0.5 ml RBC lysis buffer, incubate for 2 min at room temperature. Add 10 ml of HBSS and spin down cells.
11. Resuspend cells in 200 μ l PBS + 0.5% FCS. Count cells.
12. Add 2.4G2 anti-Fc γ III/II receptor (BD Pharmingen), incubate for 10 min on ice.
13. Stain cells with desired antibodies for flow cytometry. In our case, we stain with FITC-conjugated anti-murine CD3, PE-conjugated anti-murine CD4, or PE-conjugated anti-murine CD90.1 and APC-conjugated anti-murine CD8 (all from BD Pharmingen) at 4 °C for 20 min.
14. Analyze cells by cytofluorimetry.

As seen in Fig. 18.3, after instillation of PBS into the airways, very few T lymphocytes are present in the BAL fluid. After instillation of 5 μ g I-TAC, large numbers of T lymphocytes infiltrate into the airways. Use of the Thy1.1 marker clearly reveals that most of the infiltrated T lymphocytes are the adoptively transferred effector CD8 T lymphocytes. Instillation of different concentration of IP-10 or I-TAC shows that CD8 T lymphocyte recruitment depends on chemokine concentration. The lowest amount of chemokine used, 0.5 μ g, induces statistically significant recruitment indices of 3.7 and 5.8 for IP-10 and I-TAC, respectively. Instillation of 5 μ g results in increased recruitment indices of 15.7 and 12.5, whereas instillation of 50 μ g IP-10 or I-TAC yields recruitment indices of 53.6 and 58.8, respectively (Fig. 18.4).

4. Use of *In Vivo* Recruitment Assay for Chemokine Studies

This *in vivo* recruitment assay can be used to evaluate the *in vivo* biology of chemokines. It is especially useful for chemokine structure function studies, in which the functions of different chemokine mutants or chemokine receptor mutants are analyzed. In most studies to date, the biologic effect of mutations is only tested with *in vitro* assays of chemokine function. However, for some mutations the true biologic significance will only become apparent by use of an *in vivo* assay like the one described here. Furthermore, testing the *in vivo* efficacy of blocking antibodies or antagonists can only be done with a robust *in vivo* recruitment assay like the one described here.

4.1. Chemokine mutant analysis

One example of the clear need for *in vivo* analysis of chemokine mutants was revealed by our investigation into the role of IP-10 oligomerization (Campanella *et al.*, 2006). We found that an obligate IP-10 monomer, which contains the synthetic mutation L27NMe, was able to induce chemotaxis of effector T cells with the same efficacy as wild-type IP-10, although it required 10-fold higher concentrations (Fig. 18.5A). However, in the *in vivo* recruitment assay described here, monomeric IP-10 was not able to induce any homing of T lymphocytes even at the highest concentration tested (Fig. 18.5B). This was in contrast to another IP-10 mutant, R22E, which had similarly reduced CXCR3 and heparin binding affinity as monomeric IP-10 and similar *in vitro* chemotactic potential. Mutant R22E was able to induce *in vivo* recruitment of effector T cells at higher concentrations, clearly demonstrating that IP-10 oligomerization is required for the induction of *in vivo* T-cell trafficking. This conclusion only became apparent with an *in vivo* recruitment assay. A third mutant, R8A, which induced no *in vitro* T-cell chemotaxis even at the highest concentration tested, also did not cause any *in vivo* homing. This recruitment assay, therefore, is very useful to evaluate the *in vivo* effect of chemokine mutations.

4.2. Chemokine receptor mutant analysis

This recruitment assay is also very useful for the investigation of the *in vivo* effects of chemokine receptor mutations, which so far have only been analyzed *in vitro*. We have started this process by comparing the recruitment of wild-type and CXCR3-deficient (CXCR3 KO) effector T cells in response to IP-10. To directly compare the recruitment of both cell types in the same mouse, we used Thy1.1 wild-type OT-I effector T cells and Thy1.2 CXCR3 KO OT-I effector T cells and adoptively cotransferred them into Thy1.1×Thy1.2 mice. Two days later, 5 μ g IP-10 or PBS was instilled into the airways as described previously, and 18 h later the numbers of wild-type and CXCR3 KO effector cells in the spleen and airways were evaluated. Cotransfer of both wild-type and KO or mutant effector cells into the same mouse allows for the calculation of homing ratios as the ratio of wild-type: KO effector cells recovered from each anatomic site. As seen in Fig. 18.6, the homing ratio in the spleen was close to 1 and did not change after chemokine instillation into the airways. However, IP-10 instillation in the airways resulted in trafficking of wild-type (Thy1.1⁺) effector T cells into the airways, but not of CXCR3 KO (Thy1.2⁺) effector T cells. In a similar manner, the *in vivo* effect of chemokine receptor mutations could be analyzed. For this, chemokine receptor mutants could be transfected either into cell lines or into CXCR3 KO primary cells. These transfected cells could then be adoptively transferred into mice to determine their trafficking potential to different chemokines.

4.3. *In Vivo* testing of inhibitory antibodies and small molecule antagonists

A robust *in vivo* recruitment assay is also very useful to test the potency of inhibitory antibodies or small-molecule antagonists against chemokines and chemokine receptors. To confirm the activity of a monoclonal anti-IP-10 antibody produced in our laboratory (1F11) (Khan *et al.*, 2000), we intraperitoneally injected the antibody 4 h before cell transfer and 2 h after intratracheal IP-10 instillation. Injection of 1 mg anti-IP-10 antibody, corresponding to a 10-fold molar excess compared with IP-10, reduced the recruitment index from 9.1 for administration of control antibody to 3.2, a statistically significant reduction (Fig. 18.7). Injection of 0.1 mg anti-IP-10 antibody only slightly reduced the recruitment index to 5.2, which was not statistically significant. The *in vivo* inhibitory activity of our anti-IP-10 antibody was thereby confirmed with this *in vivo* recruitment assay.

5. Conclusion

We have developed a robust, reproducible *in vivo* chemokine-mediated T-cell recruitment assay. This assay uses the adoptive transfer of *in vitro*-generated antigen-specific effector T cells into the peritoneum of naïve mice followed by the intratracheal injection of chemokine into the airways. BAL allows for the recovery of T cells that are recruited into the airway. With the airway, this assay results in high recruitment indices with low background. We have also illustrated that this assay can be effectively used to study the *in vivo* activity of chemokine and chemokine receptor mutants as well as the *in vivo* efficacy of inhibitory antibodies and small-molecule antagonists. We believe that *in vivo* recruitment assays should be more widely used to study the biologic activity of chemokines. The availability of assays such as the one described here will help in this regard.

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Figure 18.1.

Characterization of *in vitro* activated CD8⁺ T lymphocytes. (A) Expression of activation markers CD62L and CD25 on day 0 and 6 of culture, as determined by Flow cytometry. (B) Expression of CXCR3 on different days of *in vitro* culture. (C) Expression of CXCR3 on Thy1.1⁺ T lymphocytes in the spleen 3 days after adoptive transfer of cells. Reprinted with permission from Campanella *et al.* (2008).

Figure 18.2.

in vitro chemokine-mediated activity of effector cells. (A) CXCR3 internalization induced by 100 ng/ml I-TAC or IP-10. (B) Chemotaxis induced by I-TAC and IP-10. Each bar presents mean chemotactic index STD. Experiments were performed with activated CD8⁺ T lymphocytes after 8 days in culture. Reprinted with permission from Campanella *et al.* (2008).

Figure 18.3.

Flowcytometric analysis of CD8⁺ T lymphocyte recruitment into the airways induced by I-TAC. I-TAC (5 μ g) was injected intratracheally after adoptive transfer of activated CD8⁺ T lymphocytes 48 h prior. The BAL was harvested 18 h later, and CD8⁺ T lymphocytes were analyzed by flow cytometry after gating on the lymphocyte subpopulation. The percentages of CD8⁺/CD3⁺ T cells of total events are shown in the upper right corners. Reprinted with permission from Campanella *et al.* (2008).

Figure 18.4.

Dose response of I-TAC- and IP-10-induced recruitment of activated CD8⁺ T lymphocytes *in vivo*. PBS, I-TAC, or IP-10 was injected intratracheally at the indicated amount. The BAL was harvested 18 h later, and CD8⁺ T lymphocytes were analyzed by flow cytometry. (A) Total CD8⁺ T lymphocytes in BAL. (B) Recruitment index was calculated in comparison to intratracheal injection of PBS. * $p < 0.05$, ** $p < 0.001$ compared with PBS injection. Reprinted with permission from Campanella *et al.* (2008).

Figure 18.5.

Analysis of IP-10 mutants by *in vitro* and *in vivo* recruitment assays. (A) *in vitro* chemotaxis. Chemotaxis of activated OT-I CD8⁺ Tcells in response to IP-10 (wild type or mutants) was performed in duplicate with a Neuroprobe chamber. One representative assay out of three experiments is shown. (B) *in vivo* recruitment assay. IP-10 (wild type or mutant) was injected intratracheally at the indicated concentration after adoptive transfer of activated OT-I CD8⁺ cells 48 h prior. The BAL was harvested 18 h later, and CD8⁺ Tcells were analyzed by flow cytometry. The recruitment index was calculated in comparison with intratracheal injection of PBS. Reprinted with permission from Campanella *et al.* (2006).

Figure 18.6.

Cotransfer of wt- and CXCR3 KO-activated CD8⁺ T cells. Activated wt (Thy1.1) and CXCR3 KO (Thy1.2) OT-I cells (5×10^6 each) were adoptively transferred into the same Thy1.1 $\times$ Thy1.2 mice. Forty-eight hours later, PBS or 5 μ g IP-10 was injected intratracheally. The BAL and spleen were harvested 18 h later, and Thy1.1 and Thy1.2 single positive cells were analyzed by flow cytometry as shown after gating on CD3⁺/CD8⁺ T lymphocytes. The homing ratio was calculated as wt/CXCR3 KO OT-I CD8⁺ T lymphocytes. * $p < 0.05$ compared with PBS injection. Reprinted with permission from Campanella *et al.* (2008).

Figure 18.7.

Testing the in vivo efficacy of a monoclonal antibody against IP-10. Anti-IP-10 monoclonal (1F11) (1 mg or 0.1 mg) or isotype control monoclonal antibody (IgG) (1 mg) was injected i. p. into mice 4 h before adoptive transfer of CD8⁺ T lymphocytes and 2 h after intratracheal injection of PBS or IP-10 (5 μ g). The assay was then performed as described in Fig. 18.4. * p < 0.01 compared with PBS injection, ** p < 0.02 compared with IP-10 instillation. Reprinted with permission from Campanella *et al.* (2008).