

Intratumoral Induction of CD103 Triggers Tumor-Specific CTL Function and CCR5-Dependent T-Cell Retention

Katarzyna Franciszkiewicz,¹ Audrey Le Floch,¹ Abdelali Jalil,¹ Frédéric Vigant,² Thomas Robert,³ Isabelle Vergnon,¹ Andrzej Mackiewicz,⁴ Karim Benihoud,² Pierre Validire,⁵ Salem Chouaib,¹ Christophe Combadière,⁶ and Fathia Mami-Chouaib¹

¹Institut National de la Santé et de la Recherche Médicale U753, Laboratoire "Immunologie des tumeurs humaines: Interaction Effecteurs cytotoxiques-système tumoral," Institut Fédératif de Recherche-54, Institut de cancérologie Gustave Roussy; ²CNRS UMR 8121 Vectorologie et Transfert de Gènes and ³Unité de génomique fonctionnelle, Institut de cancérologie Gustave Roussy, Villejuif, France; ⁴Department of Cancer Immunology, Poznan University of Medical Sciences and Great Poland Cancer Center, Poznan, Poland; and ⁵Service d'Anatomie-Pathologie, Institut Mutualiste Montsouris; ⁶Institut National de la Santé et de la Recherche Médicale U543, Assistance Publique-Hôpitaux de Paris, Faculté de Médecine, Université Pierre et Marie Curie-Paris 6, Paris, France

Abstract

We have reported previously that the interaction of $\alpha_E(\text{CD103})\beta_7$ integrin, expressed on a CD8^+ tumor-infiltrating lymphocyte (TIL) clone but not on a peripheral blood lymphocyte (PBL) counterpart, with the epithelial marker E-cadherin on human lung tumor cells plays a crucial role in T-cell receptor-mediated cytotoxicity. We show here that both TIL and PBL clones are able to migrate toward autologous tumor cells and that chemokine receptor CCR5 is involved in this process. Adoptive transfer of the PBL clone in the cognate tumor engrafted in nonobese diabetic/severe combined immunodeficient mice and subsequent coengagement of T-cell receptor and transforming growth factor- $\beta 1$ receptor triggers CD103 expression on T-cell surface resulting in strong potentiation of antitumor lytic function. Moreover, interaction of $\alpha_E(\text{CD103})\beta_7$ integrin with E-cadherin, but not lymphocyte function-associated antigen-1 with intercellular adhesion molecule-1, promotes CCR5 recruitment at the immunologic synapse formed between TIL and tumor cells, leading to inhibition of T-cell sensitivity to CCL5 chemotactic gradient. These results provide evidence for a role of tumor microenvironment, namely MHC class I-restricted antigen presentation and transforming growth factor- $\beta 1$ secretion, in regulating the effector phase of tumor-specific CTL response. They also suggest a unique role of CD103 in T-cell retention at the tumor site by a CCR5-dependent mechanism. [Cancer Res 2009;69(15):6249–55]

Introduction

CTL play a crucial role in defense against tumors and viral infections. They exert their lytic function through polarized exocytosis of cytotoxic granules at the immunologic synapse formed between T cells and target cells (1). As a prerequisite, CD8^+ T cells must migrate to sites where the specific antigen is expressed and physically engage their target following T-cell receptor (TCR)-mediated recognition of MHC class I/peptide complex. The

migration of antigen-experienced T cells to peripheral tissues is a multistep process mediated by a complex array of adhesion molecules differentially expressed by T cells and endothelial cells (2, 3). Chemokines and their receptors also play a fundamental role in lymphocyte trafficking. Indeed, adhesion to the vascular endothelium and subsequent transmigration into peripheral tissues is mediated by chemokine receptors triggering on T cells (2–4).

The migration of tumor-reactive T cells from the bloodstream to the tumor site is a key event in the process of antitumor immune response (5). However, the precise mechanisms that regulate lymphocyte recruitment and persistence within the tumor are poorly understood, but chemokines and their receptors most likely play an important role (6, 7). Adhesion molecules, particularly integrins, have been also reported to be crucial for leukocytes homing to specific peripheral tissues (8, 9), but the nature of molecules involved in T-cell infiltration and retention within the tumor needs to be clearly defined (10). In the present study, we compared the migratory potential of two tumor-reactive T-cell clones, derived either from peripheral blood lymphocyte (PBL) or from tumor-infiltrating lymphocyte (TIL) of a lung cancer patient, and characterized the receptors implicated in this process. Although both clones expressed CCR5, CXCR3, and CXCR4 and displayed a CCR5-dependent migratory response to autologous tumor cells, only the TIL clone expressed chemokine receptor CCR6 and integrin $\alpha_E(\text{CD103})\beta_7$. CD103 could be induced on the PBL clone surface following TCR and transforming growth factor (TGF)- $\beta 1$ receptor coengagement within the cognate tumor resulting in increase in antitumor cytotoxicity. Moreover, interaction of CD103 with E-cadherin triggered CCR5 recruitment at the immunologic synapse leading to reduced T-cell responsiveness to CCL5 gradient.

Materials and Methods

Tumor cell lines and T-cell clones. The IGR-Heu tumor cell line and the Heu171 and H32-22 T-cell clones were established from a non-small cell lung carcinoma (NSCLC) patient (11). The IGR-Heu-CCL5 cell line was obtained by infection of IGR-Heu with a lentivirus encoding human CCL5.⁷

Monoclonal antibodies and immunoassays. Monoclonal antibodies (mAb) recognizing CCR5, CCR6, CXCR3, CXCR4, and E-cadherin were purchased from R&D. Anti-CD103 and anti-CD8 mAb were purchased from Immunotech. The PKH67 green fluorescent cell linker was purchased from

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Fathia Mami-Chouaib, Institut National de la Santé et de la Recherche Médicale U753, Institut Gustave Roussy, 39 rue Camille-Desmoulins, F-94805 Villejuif, France. Phone: 33-1-42-11-49-65; Fax: 33-1-42-11-52-88; E-mail: cfathia@igr.fr.

©2009 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-08-3571

⁷ <http://www.tronolab.unige.ch/>

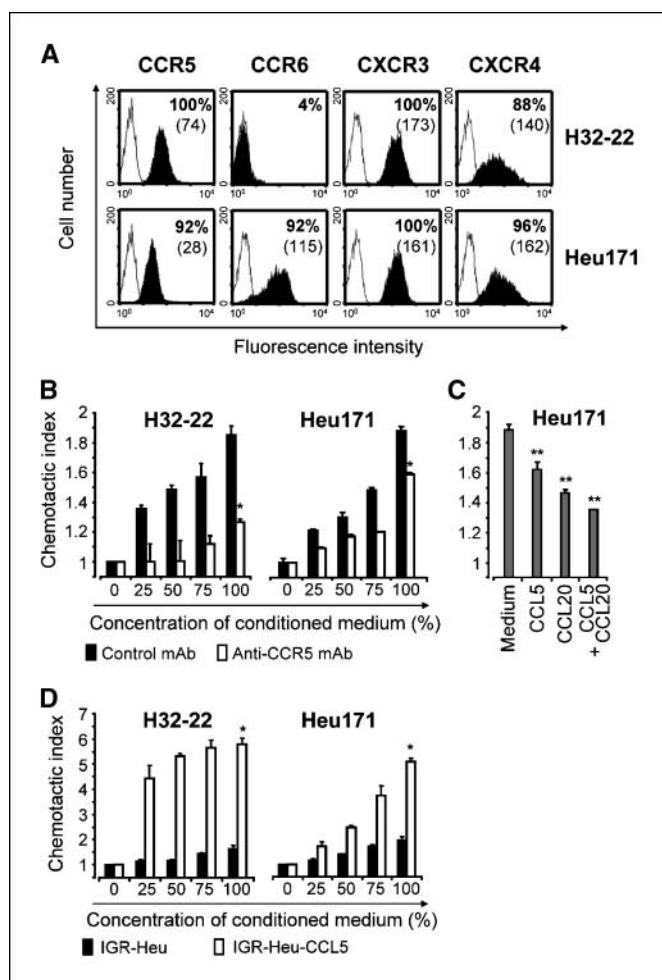


Figure 1. A, chemokine receptor expression on T-cell clones. Expression of CCR5, CCR6, CXCR3, and CXCR4 on the H32-22 and Heu171 clones was assessed by immunofluorescence staining using specific (filled histograms) and isotype-matched control (open histograms) mAb. Percentages of positive cells are indicated; mean fluorescence intensity values are in parentheses. B, CCR5 is involved in H32-22 and Heu171 migration toward IGR-Heu supernatant. T cells, preincubated for 30 min with neutralizing anti-CCR5 or control mAb, were seeded in the top chambers of Transwell plates and then exposed to a gradient of IGR-Heu supernatant dilutions loaded in the bottom chambers. The number of T cells that had migrated into the bottom chambers was determined. C, TIL clone chemotaxis toward IGR-Heu supernatant is CCR5 and CCR6 dependent. Heu171 cells were preincubated or not for 15 min with desensitizing dose of rCCL5, rCCL20, or a combination of both chemokines and then assessed for migration toward undiluted supernatant. D, CCL5 overexpression by IGR-Heu potentiates the H32-22 and the Heu171 migratory response. T-cell migration toward IGR-Heu or CCL5-transduced IGR-Heu supernatant dilutions was determined. All results are represented as mean \pm SD chemotaxis index of triplicate samples. Representative of three independent experiments. *, $P < 0.01$ (Wilcoxon test); **, $P < 0.01$ (Mann-Whitney U test).

Sigma-Aldrich. Phenotypic analyses were done using a FACSCalibur flow cytometer (Becton Dickinson).

Chemokine secretion was assessed by intracytoplasmic immunofluorescence staining using specific mAb for CCL3, CCL4, CXCL9, and CXCL10 (R&D Systems) and CCL5 (BD Biosciences), by Multiplex Bead Immunoassay for CCL3, CCL4, and CCL5 (Invitrogen), or by ELISA for CCL20 and CXCL12 (R&D Systems).

Cytotoxicity assay. Cytotoxic activity was measured by a conventional 4 h ^{51}Cr -release assay (12).

Chemotaxis assay. T cells (2×10^5) were seeded in top chambers of 5 μm pore size polycarbonate 96-well Transwell plates (Corning Life Sciences) in serum-free medium. Bottom chambers contained serial

dilutions of IGR-Heu conditioned medium. After 2 h of incubation at 37°C, the number of cells that had migrated into the bottom chambers was determined by flow cytometry. Chemokine receptor activity was abrogated with neutralizing mAb or with recombinant chemokines (1 $\mu\text{g}/\text{mL}$). Responsiveness of lymphocytes engaged by tumor cells to chemokines was assessed after preincubation of T cells with target cells in the top Transwell chambers at a 1:5 effector-to-target ratio for 15 min for conjugate formation. rCCL5 (25 nmol/L) or rCXCL12 (50 nmol/L) was then added to the bottom chambers to elicit T-cell migration. Results were expressed as chemotaxis index corresponding to a fold change in the number of T cells migrating in response to a chemotactic agent relative to the number of cells spontaneously migrating to medium.

In vivo experiments. Human lung tumors, Heu-n, Bla-n, and Mau-n, were established as described (13). Briefly, tumor cells (IGR-Heu and IGR-B2) or tumor specimens (Mau) were engrafted subcutaneously into the flank of nude mice. After tumor growth, mice were sacrificed and tumor specimens were transplanted subcutaneously into the flank of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. For T-cell homing, NOD/SCID mice bearing palpable tumors were injected intravenously with 5×10^6 PKH67-labeled H32-22 cells. Recombinant interleukin-15 (3 $\mu\text{g}/\text{mouse}/\text{d}$; Amgen) was then administered intraperitoneally to promote T-cell survival. At indicated time points, tumors were removed, dissociated, and passed through a cell strainer (Becton Dickinson). Single-cell suspensions were analyzed by flow cytometry for fluorescent T-cell infiltration. Six mice per group were included in each experiment; two tumors from each group were analyzed at a time point.

To follow-up lymphocyte behavior within the tumor, H32-22 cells were injected intratumorally into either autologous Heu-n or allogenic Bla-n (HLA-A2) and Mau-n (HLA-A1) tumors established in NOD/SCID mice. Eight days later, tumors were dissociated and analyzed by flow cytometry for CD8/CD103 expression. For further experiments, H32-22 cells from Heu-n tumors were isolated using Ficoll-Hypaque (Pharmacia Fine Chemicals) density gradient and expanded *in vitro* (11). For TGF- β 1 neutralization, mice were inoculated intravenously with 10^{11} plaque-forming units of AdTGF- β RII-Fc 4 days before T-cell injection. AdCO1 empty adenovirus was used as a negative control. Plasma soluble TGF- β 1 receptor levels were measured (14).

Confocal microscopy. Tumor and effector cells were plated on poly-L-lysine-coated coverslips (Sigma-Aldrich) at a 2:1 effector-to-target ratio and incubated for 15 min at 37°C for conjugates formation. Cells were then fixed, permeabilized, and stained with anti-CCR5 mAb followed by a secondary Alexa Fluor 488-coupled mAb (Molecular Probes) as described (12). Coverslips were mounted with Vectashield (Vector Laboratories) and analyzed by a fluorescence microscope (Carl Zeiss LSM-510). The percentage of conjugates with CCR5 accumulation at the immunologic synapse was calculated. Silencing of E-cadherin expression in IGR-Heu was done using specific small interfering RNA (siRNA)-E2 (12). Luciferase siRNA (siRNA-Luc) was included as a negative control.

Statistical analysis. Data were compared using the two-tailed Student's t test, Mann-Whitney U test, or Wilcoxon test. Two groups were considered as significantly different if $P < 0.05$.

Results

Chemokine receptor expression by TIL- and PBL-derived T-cell clones. We isolated previously, from PBL and TIL of a NSCLC patient, two tumor-reactive T-cell clones, H32-22 and Heu171, respectively (11). Although both clones expressed a unique TCR and displayed similar lytic potential, only the TIL clone elicited a strong cytotoxic activity toward the cognate IGR-Heu tumor cell line [E-cadherin⁺/intercellular adhesion molecule-1 (ICAM-1)⁻], which was dependent on the expression of $\alpha_E\beta_7$ integrin on the TIL clone surface (12). In the present study, we investigated whether the PBL clone is able to migrate to the tumor site and whether it can acquire a specific lytic activity once recruited into the tumor. To identify mechanisms involved in T-cell

migration, we first compared chemokine receptor repertoires of H32-22 and Heu171 by microarray analysis⁸ (12). Results depicted in Supplementary Table S1 indicate that the two clones displayed similar chemokine receptor transcription profiles, except for CCR6 and CCR7, which were 15- and 6-fold stronger expressed in Heu171 than in H32-22, respectively. In both clones, high levels of CCR5, CXCR3, and CXCR4 mRNA were detected.

We then assessed expression of chemokine receptors by immunofluorescence analysis. Results summarized in Supplementary Table S2 and shown in Fig. 1A indicate that CCR5, CXCR3, and CXCR4 were expressed on both clones, whereas CCR6 was present only on the TIL clone. Contrary to microarray data, both clones failed to express CCR7 (11). It is worth noting that CCR6 was also

expressed on several TIL-derived clones and on a subset of CD8⁺ uncultured TIL but to a lesser extent on CD8⁺ fraction of peripheral blood mononuclear cells and PBL-derived T-cell clones (Table 1).

Regulation of T-cell clone migration toward tumor cells.

Next, experiments were done to analyze the migratory potential of TIL and PBL clones toward autologous tumor cells and chemokine receptors involved in this process. For this purpose, we first tested the functionality of chemokine receptors expressed on T-cell clones in *in vitro* migration assays using recombinant chemokines. Results indicated that both clones migrated toward the CCR5 ligand CCL5 (RANTES), the CXCR3 ligand CXCL11 (I-TAC), and the CXCR4 ligand CXCL12 (SDF-1). As expected, only the TIL clone migrated toward the CCR6 ligand CCL20 (MIP-3 α ; Supplementary Fig. S1). We then measured expression levels of chemokines of interest in IGR-Heu cells or in IGR-Heu supernatant. Results summarized in Table 2 indicate that tumor cells expressed CCR5 ligand CCL3, lower levels of CCR5 ligands CCL4 (MIP-1 β) and CCL5, and CCR6 ligand CCL20. In contrast, they failed to express CXCR3 ligands CXCL9 (MIG) and CXCL10 (IP-10) and CXCR4 ligand CXCL12.

To identify chemokine receptors involved in CTL migration toward the cognate tumor, we tested the migratory response of H32-22 and Heu171 to IGR-Heu supernatant in the presence or absence of specific neutralizing mAb or recombinant chemokines. Results indicated that both clones were able to migrate toward tumor cell supernatant in a dose-dependent manner and that this migration was inhibited by anti-CCR5 mAb (Fig. 1B). Figure 1B also indicates that the PBL clone was more sensitive to the CCR5 blockade than the TIL clone most likely because of CCR6 expression on the latter cells, which may also promote T-cell migration. Indeed, migration of Heu171 toward IGR-Heu supernatant was significantly ($P < 0.01$) inhibited by rCCL20, combined or not with rCCL5 (Fig. 1C). In contrast, anti-CXCR3 and anti-CXCR4 mAb had no effect (data not shown). To emphasize the involvement of CCR5 in T-cell migration, we transduced IGR-Heu with CCL5 and tested its capacity to attract Heu171 and H32-22. Results depicted in Fig. 1D indicate that T-cell migration was promptly increased and that the PBL clone was more sensitive to the chemokine gradient than the TIL clone. These results suggest a role for CCR5 in promoting T-cell homing to the tumor site.

T-cell recruitment in the cognate tumor promotes CD103 expression and TCR-mediated lysis. To assess whether H32-22 was also able to migrate to the tumor site *in vivo*, and to follow up its behavior *in situ*, we used the NOD/SCID mouse model transplanted with IGR-Heu (Heu-n; ref. 13) and transferred intravenously with PKH67-labeled clone. Although this model has some limitations, it may reflect antigen-specific T-cell behavior within human tumors. Kinetic studies showed increasing T-cell recruitment within the tumor starting from day 8 (Fig. 2A). We then investigated the effect of tumor microenvironment on recruited cells by injection of the clone into the cognate tumor and its follow-up at different time points. Results indicated an induction of CD103 on a subpopulation of the clone starting from day 8 (Fig. 2B). For further studies, we amplified *in vitro* the generated T cells, thereafter named H32-22-TIL. Immunofluorescence staining showed high expression levels of CD103 and CCR6 on H32-22-TIL (Fig. 2C), which were maintained throughout cell cultures. Furthermore, cytotoxicity experiments

Table 1. CCR6 distribution among TIL- and PBL-derived T-cell clones and uncultured TIL and peripheral blood mononuclear cells

	CCR6 expression, % (mean fluorescence intensity)
TIL clones (CD8⁺)	
Heu171 ($\alpha_E\beta_7$)	90 (42)
Heu127 ($\alpha_E\beta_7$)	80 (26)
Heu161 ($\alpha_E\beta_7$)	64 (23)
PBL clones (CD8⁺)	
H32-22 ($\alpha_E\beta_7$)	4
H32-8 ($\alpha_E\beta_7$)	10 (50)
H32-25 ($\alpha_E\beta_7$)	7 (22)
CCR6 expression on CD8⁺ fraction, % (mean fluorescence intensity)	
Uncultured TIL	
Patient 2	28 (274)
Patient 4	39 (96)
Patient 5	34 (135)
Patient 6	49 (299)
Patient 7	47 (300)
	Mean, 39 \pm 9
Uncultured peripheral blood mononuclear cells	
Patient 1 (Heu)	1
Patient 5	15 (129)
Patient 6	1
Patient 7	10 (141)
Patient 8	20 (55)
Patient 9	3
	Mean, 8 \pm 8
Uncultured peripheral blood mononuclear cells	
Donor 1	18 (113)
Donor 2	12 (87)
Donor 3	14 (51)
Donor 4	8 (57)
Donor 5	14 (104)
Donor 6	14 (85)
Donor 7	2
	Mean, 11 \pm 5

NOTE: Mean percentage of CCR6-expressing CD8⁺ cells was significantly higher in TIL than in peripheral blood mononuclear cells from NSCLC patients ($P = 0.000057$) and healthy donors ($P = 0.0000109$). Statistical analyses were done using the two-tailed Student's *t* test.

⁸ <http://www.ebi.ac.uk/arrayexpress/>

Table 2. Chemokine production by IGR-Heu cells

	IGR-Heu cells	IGR-Heu supernatant (ng/mL)
CCR5 ligands		
CCL3	95 (24)	53 ± 23
CCL4	12 (57)	14 ± 7
CCL5	7 (54)	1 ± 0.2
CXCR3 ligands		
CXCL9 (MIG)	0.1	ND
CXCL10 (IP-10)	0.1	ND
CCR6 ligand		
CCL20	ND	6.5 ± 3
CXCR4 ligand		
CXCL12	ND	0.01 ± 0.005

NOTE: Percentages of positive cells are indicated; mean fluorescence intensity values are in parentheses. ND, not done.

showed that induction of CD103 resulted in strong potentiation of T-cell-mediated lysis of IGR-Heu, which was abrogated with neutralizing anti-CD103 mAb (Fig. 2D). These results show that tumor-reactive CD8⁺ T cells can acquire specific effector function following CD103 induction at the tumor site.

To investigate mechanisms involved in intratumoral CD103 induction, we also injected H32-22 into allogenic tumors transplanted into NOD/SCID mice. Contrary to results obtained with Heu-n, transfer of the clone into Bla-n and Mau-n tumors did not result in CD103 expression. This was not correlated with *in vivo* expansion of the transferred cells, because they were unable to proliferate in either autologous or allogeneic tumors in response to murine cytokines (Supplementary Fig. S2). Moreover, IGR-Heu and H32-22 cells failed to express FasL and PD1, respectively, excluding their contribution in CD103 induction (data not shown). However, inoculation of autologous tumor-bearing mice with an adenovirus encoding human soluble TGF- β 1 receptor II (AdTGF- β 1RII-Fc; ref. 14) before T-cell transfer resulted in decreased CD103 expression on H32-22 injected in Heu-n tumor (Fig. 2B). AdCO1 empty adenovirus, used as a negative control, had a marginal effect. These results emphasize the role of TCR engagement, through MHC class I/antigen complex recognition, and TGF- β 1 production by tumor cells (15) in CD103 induction on tumor-reactive CD8⁺ T cells.

Interaction of CD103 with E-cadherin triggers CCR5 recruitment at the immunologic synapse. It has been reported that chemokine receptors can be recruited at the immunologic synapse formed between T cells and antigen-presenting cells (16). To determine whether CCR5, CCR6, CXCR3, and CXCR4 might be engaged at the immunologic synapse, we incubated IGR-Heu with either H32-22 or Heu171 and analyzed the formed conjugates by confocal microscopy. Results showed that CCR5 was recruited at the immunologic synapse formed between tumor cells and the TIL clone but not that formed with the PBL clone (Fig. 3A). CXCR3, CXCR4, and CCR6 were not redistributed at the immunologic synapse in any case (data not shown). Notably, clustering of CCR5 was also observed at the immunologic synapse formed between H32-22-TIL and IGR-Heu (Fig. 3A). This was found in 88% of conjugates formed between IGR-Heu and either Heu171 or H32-22-TIL but only in 14% of conjugates formed with H32-22 (Fig. 3C). These results suggest that CCR5 is selectively recruited at the immunologic synapse and that CD103 can be required.

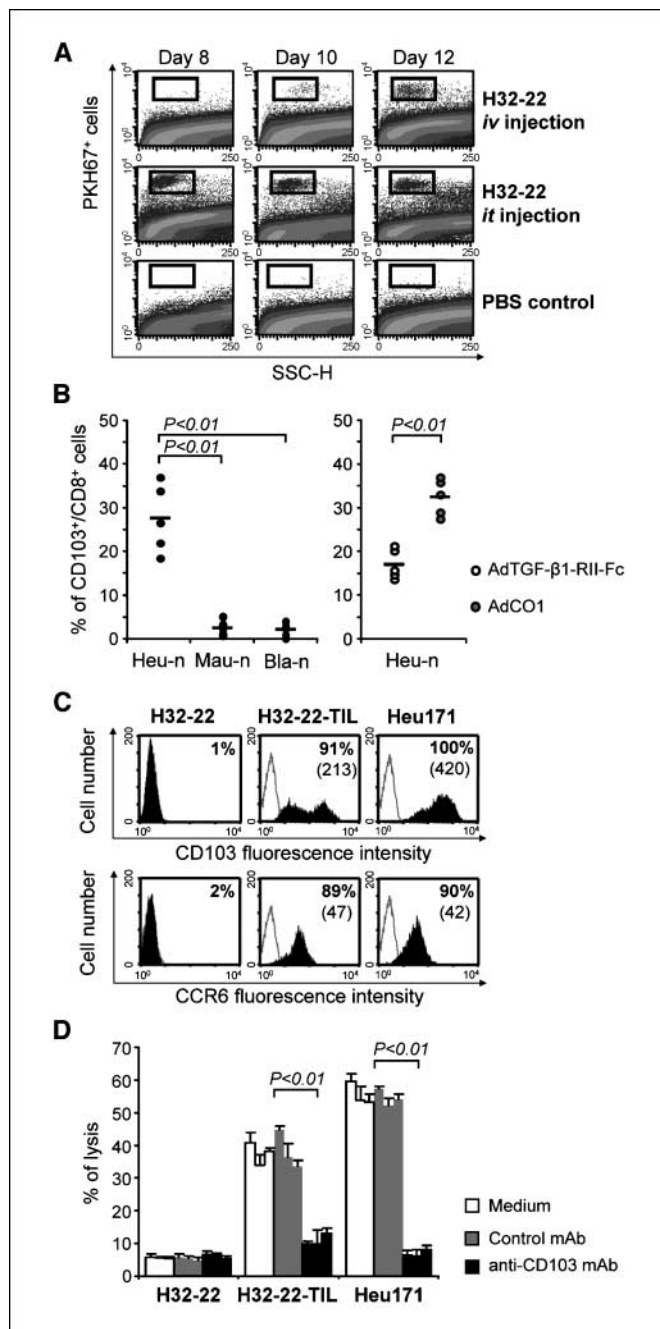


Figure 2. Homing and intratumoral adaptation of the PBL clone. *A*, NOD/SCID mice bearing the IGR-Heu tumor (Heu-n) were injected intravenously (*iv*) with PKH67-labeled H32-22 cells. At indicated time points, tumors were dissociated and single-cell suspensions were analyzed by flow cytometry (*top*). Tumors from mice injected intratumorally (*it*) with H32-22 (*middle*) or PBS (*bottom*) served as controls. *B*, *left*, H32-22 cells were injected intratumorally into either autologous Heu-n or allogenic Bla-n and Mau-n tumors engrafted in NOD/SCID mice. Eight days later, tumors were dissociated and the percentage of CD8⁺ cells expressing CD103 was determined. *Right*, NOD/SCID mice were engrafted with Heu-n tumor. Four days before intratumoral injection of H32-22 cells, mice were inoculated intravenously with AdTGF- β 1RII-Fc or AdCO1 empty vector negative control. Eight days later, the percentage of CD8⁺/CD103⁺ cells was determined. *Horizontal bars*, mean ($n = 5$). $P < 0.01$ (Mann-Whitney *U* test). *C*, surface expression of CD103 and CCR6 on H32-22-TIL. Heu171 and H32-22 were used as controls. Percentages of positive cells are indicated; mean fluorescence intensity values are in parentheses. *D*, H32-22-TIL cytotoxicity toward IGR-Heu. Heu171 and H32-22 cells were used as controls; effector-to-target ratio was 10:1. For blocking experiments, effector cells were preincubated for 1 h with neutralizing anti-CD103 or control mAb. *Bars*, SD of triplicate samples. Three experiments are shown. $P < 0.01$ (Mann-Whitney *U* test).

To test this hypothesis, we knocked down the $\alpha_E\beta_7$ ligand E-cadherin in IGR-Heu using specific siRNA. Silencing of E-cadherin did not alter formation of conjugates between Heu171 and IGR-Heu but resulted in strong inhibition of CCR5 clustering at the immunologic synapse (Fig. 3A). Indeed, only 20% of conjugates formed with siRNA-E2-treated IGR-Heu displayed polarized CCR5 (Fig. 3C). In contrast, control siRNA had no effect, because 86% of conjugates formed with siRNA-Luc-treated IGR-Heu exhibited CCR5 relocalization. These results indicate that interaction of CD103 with E-cadherin promotes recruitment of CCR5 at the immunologic synapse.

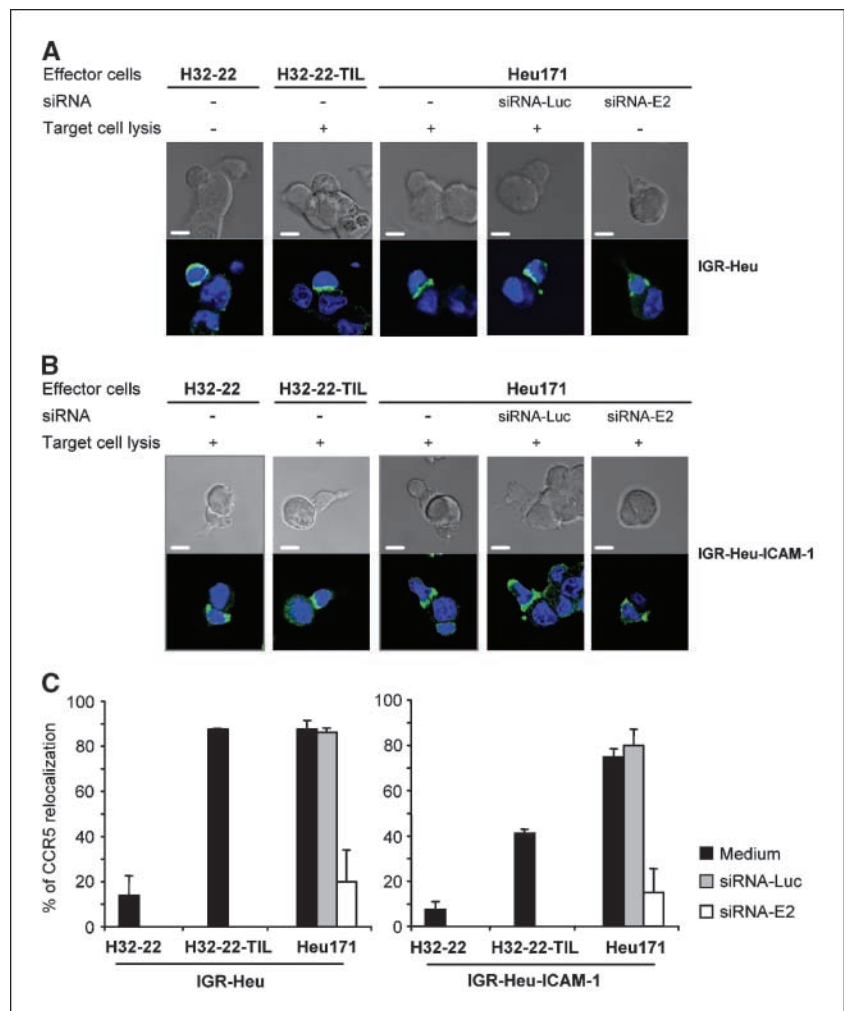
Maturation of the immunologic synapse following lymphocyte function-associated antigen-1-ICAM-1 interaction does not induce CCR5 clustering. Next, we questioned whether CCR5 clustering is subsequent to maturation of the secretory immunologic synapse and whether the interaction of lymphocyte function-associated antigen-1 (LFA-1; $\alpha_L\beta_2$) with ICAM-1 induces the same effect. Thus, we stably transfected IGR-Heu with ICAM-1 and analyzed conjugates formed with either TIL ($\alpha_E\beta_7/\alpha_L\beta_2^+$) or PBL ($\alpha_E\beta_7/\alpha_L\beta_2^-$) clones. As expected, CCR5 was recruited at the immunologic synapse formed with Heu171, but only marginal CCR5 clustering was detected in conjugates formed with H32-22 (Fig. 3B). This was observed in 75% of conjugates formed between IGR-Heu-ICAM-1 and Heu171 and in 8% of conjugates formed with H32-22 (Fig. 3C). Notably, recruitment of CCR5 at the immunologic

synapse formed between H32-22-TIL and IGR-Heu-ICAM-1 was decreased (41%) compared with parental cells.

We then knocked down E-cadherin in IGR-Heu-ICAM-1 using siRNA-E2 and monitored CCR5 clustering at the immunologic synapse formed with the TIL clone. Similar to results obtained with IGR-Heu, only marginal CCR5 clustering was observed (Fig. 3B). This was found in 15% of conjugates formed between Heu171 and IGR-Heu-ICAM-1-siRNA-E2 and in 80% of those formed with IGR-Heu-ICAM-1-siRNA-Luc control (Fig. 3C). It is noteworthy that CCR5 was also recruited at the immunologic synapse formed between Heu171 (but not H32-22) and the peptide-pulsed IGR-B2 allogeneic NSCLC (E-cadherin⁺/ICAM-1⁺) but not that formed between H32-22 or Heu171 and peptide-pulsed autologous EBV-transformed (EBV-B) cells (E-cadherin⁻/ICAM-1⁺), although this latter target expressed CCL3 (70% of cells), was sensitive to T-cell-mediated lysis (12), and triggered IFN- γ production by both PBL (9 ng/mL) and TIL (5 ng/mL) clones. These results show that the formation of a productive immunologic synapse was required, but not sufficient, to induce CCR5 clustering and argue for a unique functional role of CD103 that cannot be provided by LFA-1.

CD103 and CCR5 coengagement at the immunologic synapse inhibits CCL5-mediated T-cell migration. Previous reports suggested that chemokine receptor engagement at the immunologic synapse impedes “distraction” of engaged T cells by other chemokine sources (16). To evaluate the functional

Figure 3. Engagement of CD103, but not LFA-1, at the immunologic synapse triggers CCR5 recruitment. *A, left*, conjugates formed between IGR-Heu and either H32-22, H32-22-TIL, or Heu171 were analyzed by confocal microscopy. CCR5 recruitment at the immunologic synapse was followed using anti-CCR5 mAb (green). Nuclei were stained with TO-PRO-3 iodide (blue). *Right*, IGR-Heu cells were pretreated with siRNA targeting E-cadherin or siRNA-Luc control. Conjugates formed with the Heu171 clone were then analyzed. *B, left*, conjugates formed between T cells and ICAM-1-transduced IGR-Heu cells were analyzed for CCR5 recruitment. *Right*, IGR-Heu-ICAM-1 cells were pretreated with siRNA-E2 or siRNA-Luc. Conjugates formed with Heu171 were then analyzed. *C*, percentages of conjugates displaying CCR5 recruitment at the immunologic synapse formed between T cells and either IGR-Heu (*left*) or IGR-Heu-ICAM-1 (*right*), pretreated or not with siRNA. Mean \pm SD of two of five representative experiments ($n = 80$).



Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/15/6253/2867859/6253.pdf by guest on 30 November 2023

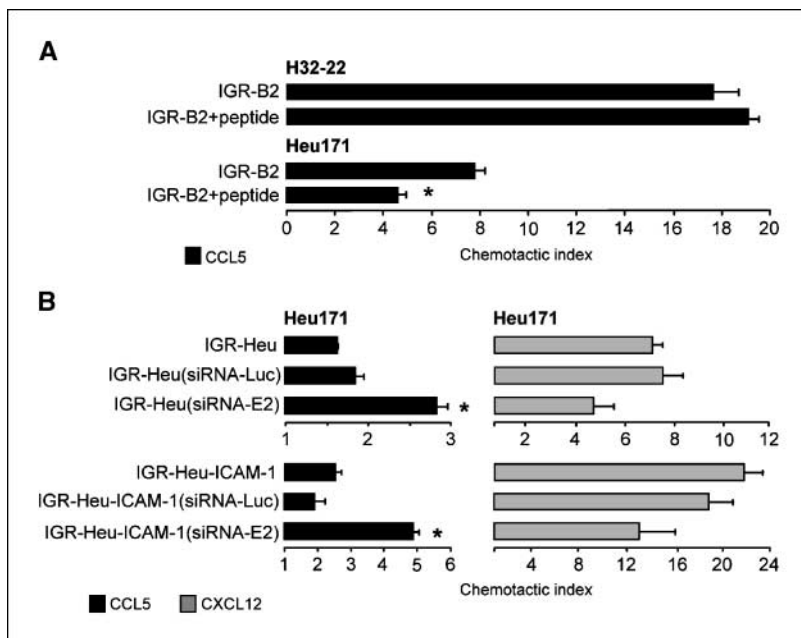


Figure 4. CCR5 clustering at the immunologic synapse reduces T-lymphocyte responsiveness to CCL5. **A**, H32-22 and Heu171 clones were preincubated in the top chambers of Transwell plates with allogeneic IGR-B2 tumor cells, pulsed or not with the antigenic peptide. Conjugates were exposed to CCL5 loaded in the bottom chambers and transmigrating cells were counted. **B**, E-cadherin knockdown reestablishes TIL clone responsiveness to CCL5. The Heu171 clone was preincubated with IGR-Heu or IGR-Heu-ICAM-1, pretreated or not with siRNA, in Transwell top chambers. Conjugates were then exposed to CCL5 or CXCL12 loaded in the bottom chambers, and transmigrating cells were counted. Bars, SD of triplicate samples. Representative of three independent experiments. *, $P < 0.01$ (Mann-Whitney U test).

consequence of CCR5 recruitment at the immunologic synapse on T-cell movement, we measured the ability of chemokine gradients to attract the clones engaged by either autologous or allogeneic tumor cells, pulsed or not with the antigenic peptide. When cocultures of Heu171 with unpulsed IGR-B2 were exposed to CCL5, T cells efficiently migrated toward the chemokine. In contrast, Heu171 conjugated with peptide-pulsed IGR-B2 was less responsive to CCL5 and migrated less efficiently than unengaged clone (Fig. 4A). With regard to H32-22 cocultured with IGR-B2, pulsed or not with the peptide, it likewise migrated toward the chemokine. These results suggest that coengagement of CD103 and CCR5 at the immunologic synapse regulates CCR5-dependent T-cell migration within the tumor and that LFA-1 is ineffective for this function.

To confirm this hypothesis, we used IGR-Heu or IGR-Heu-ICAM-1, treated or not with siRNA-E2, and evaluated the ability of chemoattractant gradients to "distract" the TIL clone from interaction with its target. Results indicated that knockdown of E-cadherin in both targets increased responsiveness of the clone to CCL5 (Fig. 4B). In contrast, the siRNA-Luc control had only a marginal effect. Parallel chemotaxis assays done with CXCL12 or CCL20, the receptors of which were not recruited at the immunologic synapse, showed that Heu171 cells efficiently migrated toward these chemokines regardless of the target cells they previously engaged (Fig. 4B; data not shown). These data are concordant with confocal microscopy analyses and argue that CCR5 clustering at the immunologic synapse leads to a decrease in T-cell responsiveness to CCR5 ligands.

Discussion

Several solid tumors are spontaneously infiltrated by T cells (17, 18), and a correlation between the frequency of TIL and survival of cancer patients has been reported (18, 19). However, the role of adhesion molecules and chemokines in regulating T-cell migration and persistence at the tumor site remains poorly understood. Here, we show that TIL- and PBL-derived tumor-reactive clones display similar capacity to migrate toward

autologous tumor cells and that CCR5 is involved in this process. A role for CCR5 in T-cell migration at the tumor site has been documented (20, 21), and local production of CCL5 or CCL3 induced selective recruitment of CD8⁺ T cells (22–25). Our results indicate that CCL3, produced by human NSCLC, attracts tumor-specific CCR5⁺/CD8⁺ T cells and that transduction of tumor cells with CCL5 enhances this function.

$\alpha_E(\text{CD}103)\beta_7$ integrin is expressed on a large proportion of CD8⁺ T cells infiltrating epithelial tumors (12, 26, 27). It has been assumed that CD103 may serve to locate T cells in epithelial tissues by engaging E-cadherin. Our results indicate that both $\alpha_E\beta_7$ and $\alpha_E\beta_7^-$ clones migrate toward tumor cells, suggesting that CD103 may not be involved in T-cell homing to epithelial tumors. These data are consistent with previous reports documenting normal trafficking of CD103⁻ effectors from blood to peripheral sites (28–31). Our data also indicate that CD103 was induced on tumor-specific PBL-derived T cells following entry into the tumor by a mechanism involving TCR engagement and local TGF- β 1 production. These data are concordant with *in vitro* studies indicating that coengagement of TCR and TGF- β 1 receptor synergistically enhances CD103 expression on tumor-reactive T cells (12, 32).

The function of $\alpha_E\beta_7$ is not fully understood. We have shown previously that its interaction with E-cadherin triggers cytolytic granule polarization and exocytosis resulting in tumor cell lysis (12). Accordingly, we show here that intratumoral induction of CD103 triggers TCR-mediated target cell lysis. Although a balance of evidence also favors a role for CD103 in retention of lymphocyte populations in epithelial tissues (33, 34), the real contribution of this integrin to T-cell accumulation in epithelial tumors remains unknown. We show here that interaction of $\alpha_E\beta_7$ on the TIL clone with E-cadherin on autologous CCL3-producing tumor cells triggers CCR5 recruitment at the immunologic synapse. This recruitment was dependent on $\alpha_E\beta_7$ engagement and was abolished by E-cadherin silencing in tumor cells. As a consequence of CCR5 recruitment at the immunologic synapse, our transmigration experiments indicated that tumor-reactive CTL lose part of their responsiveness to CCL5, which was restored by knockdown of

E-cadherin in target cells. These results suggest a functional role for CD103 in retention of intraepithelial CD8⁺ T cells by a mechanism involving, at least in part, CCR5 clustering at the immunologic synapse.

Our results also indicate that CCR6 was more frequently expressed on CD8⁺ TIL than PBL and might be induced on tumor-specific PBL-derived T cells following arrival at the tumor site. CCR6 expression has been previously reported for a subset of effector T cells (35), associated with T-cell trafficking to mucosal tissues (36), and implicated in arrest of memory T cells (37, 38). In our model, CCR6 did not appear to play a role in T-cell recruitment into NSCLC, but it may play a role locally. Our data also show that, although CCL20 was produced by tumor cells, CCR6 was not recruited at the immunologic synapse formed between TIL and the cognate target.

Taken together, our results emphasize that the interaction of $\alpha_E\beta_7$ on TIL with E-cadherin on epithelial tumors provides a unique integrin/ligand pair that may serve as a fundamental mechanism not only for CTL effector functions but also for arrest of tumor-specific T cells within epithelial cancers. By controlling retention of tumor-reactive T cells and their cytotoxic activity, CD103 can contribute to the outcome of antitumor immune

response. Loss of E-cadherin expression by tumor cells may not only disrupt the potential for adhesive interaction with TIL, resulting in failure of the local antitumor response, but also inhibit accumulation of tumor-specific T cells within the tumor microenvironment. A better understanding of CTL trafficking and retention at the tumor site may offer new opportunities for optimizing the design of immunotherapy strategies in epithelial cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 9/12/08; revised 4/20/09; accepted 6/4/09; published OnlineFirst 7/28/09.

Grant support: Institut National de la Santé et de la Recherche Médicale, Association pour la Recherche sur le Cancer, Ligue contre le Cancer, Cancéropôle IDF, INCa, and ANR. K. Franciszkiewicz was supported by a fellowship from the Association pour la Recherche sur le Cancer.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank A. Trautmann for critically reading the article and P. Dessen and V. Lazar for help with microarray analyses.

References

- Bossi G, Trambas C, Booth S, Clark R, Stinchcombe J, Griffiths GM. The secretory synapse: the secrets of a serial killer. *Immunol Rev* 2002;189:152–60.
- Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. *Science* 1996;272:60–6.
- von Andrian UH, Mackay CR. T-cell function and migration. Two sides of the same coin. *N Engl J Med* 2000;343:1020–34.
- Kunkel EJ, Butcher EC. Chemokines and the tissue-specific migration of lymphocytes. *Immunity* 2002;16:1–4.
- Balkwill F. Chemokine biology in cancer. *Semin Immunol* 2003;15:49–55.
- Frederick MJ, Clayman GL. Chemokines in cancer. *Expert Rev Mol Med* 2001;3:1–18.
- Homey B, Muller A, Zlotnik A. Chemokines: agents for the immunotherapy of cancer? *Nat Rev Immunol* 2002;2:175–84.
- Shimaoka M, Takagi J, Springer TA. Conformational regulation of integrin structure and function. *Annu Rev Biophys Biomol Struct* 2002;31:485–516.
- Carman CV, Springer TA. Integrin avidity regulation: are changes in affinity and conformation underemphasized? *Curr Opin Cell Biol* 2003;15:547–56.
- Palmer DC, Balasubramanian S, Hanada K, et al. Vaccine-stimulated, adoptively transferred CD8⁺ T cells traffic indiscriminately and ubiquitously while mediating specific tumor destruction. *J Immunol* 2004;173:7209–16.
- Dorothee G, Vergnon I, El Hage F, et al. *In situ* sensory adaptation of tumor-infiltrating T lymphocytes to peptide-MHC levels elicits strong antitumor reactivity. *J Immunol* 2005;174:6888–97.
- Le Floch A, Jalil A, Vergnon I, et al. $\alpha_E\beta_7$ integrin interaction with E-cadherin promotes antitumor CTL activity by triggering lytic granule polarization and exocytosis. *J Exp Med* 2007;204:559–70.
- Asselin-Paturel C, Megherat S, Vergnon I, et al. Differential effect of high doses versus low doses of interleukin-12 on the adoptive transfer of human specific cytotoxic T lymphocyte in autologous lung tumors engrafted into severe combined immunodeficiency disease-nonobese diabetic mice: relation with interleukin-10 induction. *Cancer* 2001;91:113–22.
- Gastinne T, Vigant F, Lavenu-Bombled C, et al. Adenoviral-mediated TGF- β 1 inhibition in a mouse model of myelofibrosis inhibit bone marrow fibrosis development. *Exp Hematol* 2007;35:64–74.
- Asselin-Paturel C, Echchakir H, Carayol G, et al. Quantitative analysis of Th1, Th2 and TGF- β 1 cytokine expression in tumor, TIL and PBL of non-small cell lung cancer patients. *Int J Cancer* 1998;77:7–12.
- Molon B, Gri G, Bettella M, et al. T cell costimulation by chemokine receptors. *Nat Immunol* 2005;6:465–71.
- Echchakir H, Vergnon I, Dorothee G, Grunenwald D, Chouaib S, Mami-Chouaib F. Evidence for *in situ* expansion of diverse antitumor-specific cytotoxic T lymphocyte clones in a human large cell carcinoma of the lung. *Int Immunol* 2000;12:537–46.
- Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006;313:1960–4.
- Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* 2003;348:203–13.
- Uekusa Y, Yu WG, Mukai T, et al. A pivotal role for CC chemokine receptor 5 in T-cell migration to tumor sites induced by interleukin 12 treatment in tumor-bearing mice. *Cancer Res* 2002;62:3751–8.
- Musha H, Ohtani H, Mizoi T, et al. Selective infiltration of CCR5(+)CXCR3(+) T lymphocytes in human colorectal carcinoma. *Int J Cancer* 2005;116:949–56.
- Mule JJ, Custer M, Averbook B, et al. RANTES secretion by gene-modified tumor cells results in loss of tumorigenicity *in vivo*: role of immune cell subpopulations. *Hum Gene Ther* 1996;7:1545–53.
- Lavergne E, Combadiere C, Iga M, et al. Intratumoral CC chemokine ligand 5 overexpression delays tumor growth and increases tumor cell infiltration. *J Immunol* 2004;173:3755–62.
- Maric M, Liu Y. Strong cytotoxic T lymphocyte responses to a macrophage inflammatory protein 1 α -expressing tumor: linkage between inflammation and specific immunity. *Cancer Res* 1999;59:5549–53.
- Gough M, Crittenden M, Thanarajasingam U, et al. Gene therapy to manipulate effector T cell trafficking to tumors for immunotherapy. *J Immunol* 2005;174:5766–73.
- French JJ, Cresswell J, Wong WK, Seymour K, Charnley RM, Kirby JA. T cell adhesion and cytolysis of pancreatic cancer cells: a role for E-cadherin in immunotherapy? *Br J Cancer* 2002;87:1034–41.
- Quinn E, Hawkins N, Yip YL, Suter C, Ward R. CD103⁺ intraepithelial lymphocytes—a unique population in microsatellite unstable sporadic colorectal cancer. *Eur J Cancer* 2003;39:469–75.
- Kilshaw PJ, Baker KC. A unique surface antigen on intraepithelial lymphocytes in the mouse. *Immunol Lett* 1988;18:149–54.
- Lefrancois L, Parker CM, Olson S, et al. The role of β_7 integrins in CD8 T cell trafficking during an antiviral immune response. *J Exp Med* 1999;189:1631–8.
- Feng Y, Wang D, Yuan R, Parker CM, Farber DL, Hadley GA. CD103 expression is required for destruction of pancreatic islet allografts by CD8(+) T cells. *J Exp Med* 2002;196:877–86.
- Masson F, Calzascia T, Di Berardino-Besson W, de Tribolet N, Dietrich PY, Walker PR. Brain microenvironment promotes the final functional maturation of tumor-specific effector CD8⁺ T cells. *J Immunol* 2007;179:845–53.
- Ling KL, Dulphy N, Bahl P, et al. Modulation of CD103 expression on human colon carcinoma-specific CTL. *J Immunol* 2007;178:2908–15.
- Wagner N, Lohler J, Kunkel EJ, et al. Critical role for β_7 integrins in formation of the gut-associated lymphoid tissue. *Nature* 1996;382:366–70.
- Suffia I, Reckling SK, Salay G, Belkaid Y. A role for CD103 in the retention of CD4⁺CD25⁺Treg and control of *Leishmania* major infection. *J Immunol* 2005;174:5444–55.
- Liao F, Rabin RL, Smith CS, Sharma G, Nutman TB, Farber JM. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 α . *J Immunol* 1999;162:186–94.
- Kondo T, Takata H, Takiguchi M. Functional expression of chemokine receptor CCR6 on human effector memory CD8⁺ T cells. *Eur J Immunol* 2007;37:54–65.
- Fushimi T, Kojima A, Moore MA, Crystal RG. Macrophage inflammatory protein 3 α transgene attracts dendritic cells to established murine tumors and suppresses tumor growth. *J Clin Invest* 2000;105:1383–93.
- Lecureuil C, Combadiere B, Mazoyer E, et al. Trapping and apoptosis of novel subsets of memory T lymphocytes expressing CCR6 in the spleen of HIV-infected patients. *Blood* 2007;109:3649–57.