

## Research Article

**Peptide Vaccination in Montanide Adjuvant Induces and GM-CSF Increases CXCR3 and Cutaneous Lymphocyte Antigen Expression by Tumor Antigen-Specific CD8 T Cells**Eleanor Clancy-Thompson<sup>1,2</sup>, Laura K. King<sup>3</sup>, Lenora D. Nunnley<sup>3</sup>, Irene M. Mullins<sup>1</sup>, Craig L. Slingluff Jr<sup>4</sup>, and David W. Mullins<sup>1,2</sup>**Abstract**

T-cell infiltration of melanoma is associated with enhanced clinical efficacy and is a desirable endpoint of immunotherapeutic vaccination. Infiltration is regulated, in part, by chemokine receptors and selectin ligands on the surface of tumor-specific lymphocytes. Therefore, we investigated the expression of two homing molecules, CXC chemokine receptor 3 (CXCR3) and cutaneous lymphocyte antigen (CLA), on vaccine-induced CD8 T cells, in the context of a clinical trial of a melanoma-specific peptide vaccine. Both CXCR3 and CLA have been associated with T-cell infiltration of melanoma. We show that a single subcutaneous/intradermal administration of peptide vaccine in Montanide adjuvant induces tumor-specific CD8 T cells that are predominantly positive for CXCR3, with a subpopulation of CXCR3<sup>+</sup>CLA<sup>+</sup> cells. Addition of granulocyte macrophage colony-stimulating factor (GM-CSF) significantly enhances CXCR3 expression and increases the proportion of CLA-expressing cells. Concurrent with CXCR3 and CLA expression, vaccine-induced CD8 cells express high levels of T-bet, IFN- $\gamma$ , and interleukin-12 receptor (IL-12R $\beta$ 1). Collectively, these studies show that peptide vaccination in adjuvant induces CD8 T cells with a phenotype that may support infiltration of melanoma. *Cancer Immunol Res*; 1(5): 332–9. ©2013 AACR.

**Introduction**

T-cell infiltration of melanoma is required, yet still poorly achieved, for effective immune-mediated tumor control of this aggressive and recalcitrant cancer. T-cell extravasation from circulation and infiltration to tissue is a multistep process that is mediated, in part, by lymphocyte-expressed chemokine receptors (CCR), which recognize chemokines displayed on the luminal surface of vascular endothelium (1, 2). The CXC chemokine receptor 3 (CXCR3) is expressed by Th1-skewed CD4 helper T cells (3, 4) and Tc1-type CD8 effector T cells (5). CXCR3 mediates chemotaxis upon binding its chemokine ligands: CXCL9 (monokine induced by IFN- $\gamma$ , MIG; ref. 6), CXCL10 (IFN-induced protein of 10 kDa, IP-10; ref. 7), and CXCL11 (IFN-inducible T cell alpha chemoattractant, I-TAC; ref. 8). These ligands are strongly induced by type II interferon (IFN- $\gamma$ ) and to a lesser extent by IFN- $\alpha/\beta$  and TNF- $\alpha$  (9).

Strong induction of CXCL9-11 in sites of IFN-driven type-I inflammation leads to the accumulation of Th1 helper cells and Tc1-skewed CTL (10).

Recent studies have shown the relevance of CXCR3 and its chemokines for the effective immune-mediated control of melanoma. Expression of CXCR3 by tumor-specific CD8 T cells has been associated with increased survival in patients with advanced metastatic melanoma (11), suggesting that CXCR3 is a critical mediator of T cell chemotaxis to the tumor microenvironment. Accordingly, the presence of CXCL9 and CXCL10 in primary or metastatic melanomas is associated with robust T-cell infiltrates (12, 13). Thus, the induction of CXCR3<sup>+</sup> tumor antigen-specific CD8 cells is thought to be a desirable outcome of active immunotherapy.

CXCR3 is absent from naïve T cells but upregulated by dendritic cell-induced activation of human T cells *in vitro* (3) and murine T cells *in vivo* (14). Using *in vitro* assay systems, CXCR3 is expressed within 48 to 72 hours following activation of CD8 T cells. Na and colleagues have also reported that adding granulocyte macrophage colony-stimulating factor (GM-CSF) to intradermal/subcutaneous peptide vaccines significantly enhanced CXCR3 expression on CD4<sup>+</sup> T cells specific for the neoantigen keyhole limpet hemocyanin (KLH), suggesting that CXCR3 expression on vaccine-activated T cells can be modulated by addition of cytokine to the vaccine microenvironment (15). However, it remains unknown whether peptide vaccination and adjuvant can induce or increase CXCR3 expression by CD8 T cells

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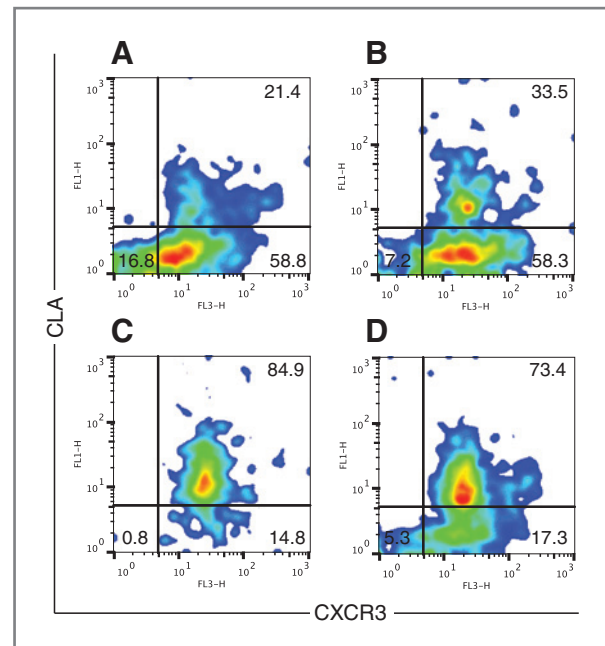
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that recognize and target endogenous melanocyte differentiation protein (MDP)-derived antigens or cancer-testis antigens.

Molecules other than CCRs are also important for T-cell targeting of inflamed or neoplastic tissues, and recent studies have highlighted the importance of cutaneous lymphocyte antigen (CLA) in the infiltration of melanoma lesions (16). CLA is an inducible carbohydrate modification of P-selectin glycoprotein ligand-1 (PSGL-1; ref. 17) that facilitates binding of T cells to E-selectin, an adhesion molecule expressed on vascular endothelium in inflamed skin (18). E-selectin was reported to be expressed by tumor-infiltrating vasculature in a majority of examined dermal malignant melanomas (19), although largely absent from metastases (20). CLA is expressed on T cells following antigen-specific activation in peripheral lymphoid tissues (21), and CLA expression has been linked to T-cell activation and expression of CXCR3 and interleukin-12 receptor (IL-12R; ref. 22). Most melanoma-specific active immunotherapies are delivered by subcutaneous and/or intradermal injection, resulting in antigen presentation in skin draining lymph nodes, yet it is unknown whether peptide vaccination induces CLA-expressing T cells.

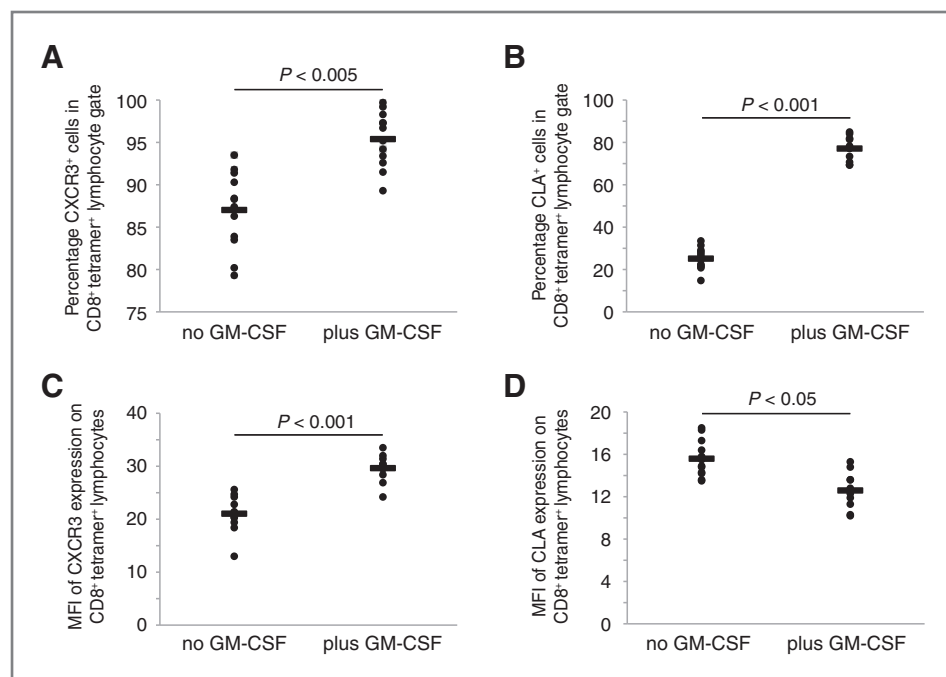
We hypothesized that subcutaneous/intradermal vaccination with peptide antigens in adjuvant may induce, and that GM-CSF may enhance, the expression of CXCR3, CLA, and IL-12R by antigen-specific CD8 T cells. Because the binding partners of CXCR3 and CLA may be present or inducible in melanoma-associated vasculature, the expression of CXCR3 and CLA may define the capacity of vaccine-induced T cells to efficiently infiltrate tumors. In the present study, we evaluated CXCR3 and CLA expression on human tumor-specific CD8 cells isolated from patients



**Figure 1.** Peptide vaccination with MELITAC 12.1 in Montanide adjuvant induces CXCR3 and CLA expression in tetramer-positive CD8<sup>+</sup> T cells. Representative flow cytometry of patient-derived peripheral blood samples are shown. Each panel represents an individual patient. PBMC were collected 1 week after a single vaccination with MELITAC 12.1, either with (A and B) or without (C and D) GM-CSF. All populations are gated on CD8<sup>+</sup> CD45RO<sup>+</sup> tetramer<sup>+</sup> cells and show intracellular CXCR3 and surface CLA staining. Each panel is from an individual patient.

following the administration of a multipptide vaccine and Montanide ISA-51 in the presence or absence of GM-CSF (23).

**Figure 2.** Peptide vaccine enhances the proportion of tetramer-positive CD8 T cells expressing CXCR3 and CLA. The percentage of tetramer-positive CD45RO<sup>+</sup> CD8<sup>+</sup> cells expressing CXCR3 and CLA is shown. A, CXCR3 expression in tetramer-positive CD8 cells, with and without GM-CSF in vaccine. B, CLA expression in tetramer-positive CD8 cells, with and without GM-CSF in vaccine. C, expression levels [mean fluorescence intensity (MFI)] of CXCR3 on tetramer-positive CD8 cells, with and without GM-CSF in vaccine. D, expression levels (MFI) of CLA on tetramer-positive CD8 cells, with and without GM-CSF in vaccine.



## Materials and Methods

### Vaccination and collection of patient samples

T cells analyzed in this study were collected from patients with advanced (stage III or IV) melanoma who had been vaccinated in an experimental phase II melanoma peptide vaccine trial, which has been reported (University of Virginia trial Mel43; ref. 23). The clinical trial was approved by the University of Virginia Human Investigations Committee/Institutional Review Board (HIC #10524) and the U.S. Food and Drug Administration (BB-IND #9847), and was registered at [clinicaltrials.gov](http://clinicaltrials.gov) (NCT00089193).

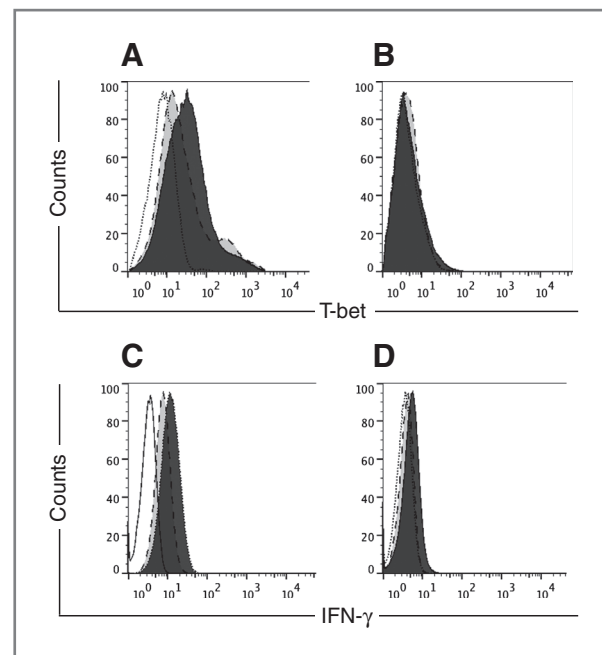
For primary analyses, patients received a vaccine comprising 12 melanoma peptides restricted by HLA-A1, HLA-A2, or HLA-A3 as previously described (23): A1 peptides: DAEKSDICTDEY (Tyrosinase<sub>240-251</sub>, which has a substitution of S for C at residue 244), SSDVIPIGTY (Tyrosinase<sub>146-156</sub>), EADPTGHSY (MAGE-A1<sub>161-169</sub>), and EVDPIGHLY (MAGE-A3<sub>168-176</sub>); A2 peptides: YMDGTMSQV (Tyrosinase<sub>369-377D</sub>), IMDQVPFSV (gp100<sub>209-217</sub>, 209-2M), YLEPGPVTA (gp100<sub>280-288</sub>), and GLYDGMHL (MAGE-A10<sub>254-262</sub>); and A3 peptides: ALLAVGATK (gp100<sub>17-25</sub>), LIYRRRLMK (gp100<sub>614-622</sub>), SLFRAVITK (MAGE-A1<sub>96-104</sub>), and ASGPGGGAPR (NY-ESO-1<sub>53-62</sub>). The tetanus helper peptide used was AQYKANSKFIGITEL. This combination of 12 MHC class I-restricted peptides plus one class II-restricted peptide is called MELITAC 12.1. Each vaccine was 2 mL of a stable water-in-oil emulsion consisting of 100 µg of each of the 12 MHC class I-restricted peptides, 190 µg of the tetanus helper peptide, and 1 mL Montanide ISA-51 adjuvant (Seppic Inc.). For some patients, the emulsion also contained 110 µg GM-CSF (Berlex, now Genzyme). The full emulsion was administered to one extremity, with half of the dose administered subcutaneously and half administered intradermally. Vaccines were administered on days 1, 8, 15, 29, 36, and 43 and then at 3, 6, 9, and 12 months.

To obtain peripheral blood mononuclear cells (PBMC), blood (80–100 mL) was drawn before treatment and 1 week after each vaccination. Lymphocytes were isolated using Ficoll gradient centrifugation and cryopreserved in 10% dimethyl sulfoxide/90% serum.

### Flow cytometric staining and analyses

Enumeration of antigen-specific T-cell responses was done using tetrameric human lymphocyte antigen (HLA) class I/peptide reagents (tetramers; Beckman Coulter), as described previously. HLA-A2 tetramers used were MAGE-A10<sub>254-262</sub> (GLYDGMHL), gp100<sub>209-217</sub> (IMDQVPFSV), gp100<sub>280-288</sub> (YLEPGPVTA), and Tyrosinase<sub>369-377D</sub> (YMDGTMSQV); A3 tetramers used were gp100<sub>17-25</sub> (ALLAVGATK) and gp100<sub>614-622</sub> (LIYRRRLMK). Tetramers for the other six peptides were not studied: two do not recognize functional antigen-reactive T cells (DAEKSDICTDEY and SLFRAVITK), one could not be synthesized (ASGPGGGAPR), two others were not used because of the low rates of T-cell response previously observed in ELISpot assays (EADPTGHSY and SSDVIPIGTY), and one worked but was not evaluated (EVDPIGHLY).

Samples for homing receptor analysis were selected on the basis of previous demonstration of immunogenic response to



**Figure 3.** Peptide vaccine and adjuvant enhances T-bet (TBX21) and IFN- $\gamma$  production in CXCR3<sup>+</sup>, but not CXCR3<sup>-</sup>, tetramer-positive CD8 T cells. Open histogram, unstained control; light gray histogram, no GM-CSF in vaccine; dark gray histogram, GM-CSF in vaccine. A, representative T-bet staining in CXCR3<sup>+</sup> tetramer-positive cells, with or without GM-CSF in vaccine. B, representative T-bet staining in CXCR3<sup>-</sup> tetramer-positive cells, with or without GM-CSF in vaccine. C, representative IFN- $\gamma$  staining in CXCR3<sup>+</sup> tetramer-positive cells, with or without GM-CSF in vaccine. D, representative IFN- $\gamma$  staining in CXCR3<sup>-</sup> tetramer-positive cells, with or without GM-CSF in vaccine.

at least one antigen, as assessed by ELISpot assays (23). After thawing, PBMCs were enriched for CD8<sup>+</sup> cells (Miltenyi Biotec), washed twice, suspended in PBS/2% fetal calf serum (FCS; Sigma-Aldrich), and incubated with tetramer for 15 minutes at room temperature before adding a mixture of anti-CD8 (clone: SFC121Thy2D3; Beckman Coulter), anti-CD45RO (clone UCHL1; BD Biosciences), and anti-CLA (clone HECA-452; BD Biosciences). Some samples were then stained for surface 12R $\beta$ 1 (clone 69319; R&D Systems). For intracellular staining, following tetramer staining, T cells were washed and fixed with 1% paraformaldehyde in PBS at room temperature for 20 minutes (24). Intracellular CXCR3 was assessed using anti-CXCR3 (clone 1C6; BD Biosciences); intracellular T-bet protein was assessed using a monoclonal antibody (1 µg/10<sup>6</sup> cells; eBioscience clone 4B10); and intracellular IFN- $\gamma$  protein was assessed using a monoclonal antibody (1 µg/10<sup>6</sup> cells; eBioscience clone 4S.B3). For all experiments, data were acquired on a Becton-Dickinson LSRFortessa and analyzed with FlowJo (TreeStar) software.

### Statistical analysis

The Student *t* test or Mann-Whitney *U* test was used to determine whether there were statistically significant differences in the percentage of chemokine receptor-positive cells in pre- and postvaccine samples. Statistical analyses were conducted using MiniTab 16 software.

## Results

### CXCR3 and CLA expression is increased on tumor antigen-specific CD8 T cells *ex vivo*

To assess whether peptide vaccination induces the expression of selected tissue homing markers, we evaluated PBMC samples of 24 patients who had received repeated immunizations with 12 class I-restricted MDP- and cancer testis antigen-derived peptides and a class II-restricted tetanus peptide (MELITAC 12.1) either with ( $n = 12$ ) or without ( $n = 12$ ) GM-CSF (23). Cryopreserved patient-derived PBMCs were thawed and then enriched for CD8 cells, which were then stained with a pooled set of HLA-A2-restricted melanoma antigen/MHC tetramers and monoclonal antibodies (mAb) for homing markers. As CXCR3 is internalized upon ligand binding and may not be available for surface staining (25), we used a modified protocol of Dimopoulos and colleagues (24) for intracellular staining of CXCR3 (representative data, Supplementary Fig. S2).

Following a single vaccination with MELITAC 12.1, the majority of tetramer-positive CD8 cells ( $87.0\% \pm 4.5\%$ ) expressed intracellular CXCR3, and a subset ( $25.1\% \pm 5.2\%$ ) expressed surface CLA (representative data from 4 patients in Fig. 1). Our data for CXCR3, assessed by intracellular staining, suggest a higher proportion of CXCR3-expressing cells following vaccination than previously estimated using surface staining in similar patient isolates (26). Addition of GM-CSF to the vaccine modestly but significantly ( $P < 0.005$ ) increased the proportion of tetramer-positive cells expressing CXCR3 ( $95.4\% \pm 3.2\%$ ; Fig. 2A) and significantly ( $P < 0.001$ ) enhanced the proportion of cells expressing CLA ( $77.1\% \pm 5.4\%$ ; Fig. 2B), but vaccination did not induce, and GM-CSF did not enhance ( $P > 0.05$ ), CXCR3 or CLA expression in non-antigen-specific (tetramer-negative) cells (Supplementary Fig. S3).

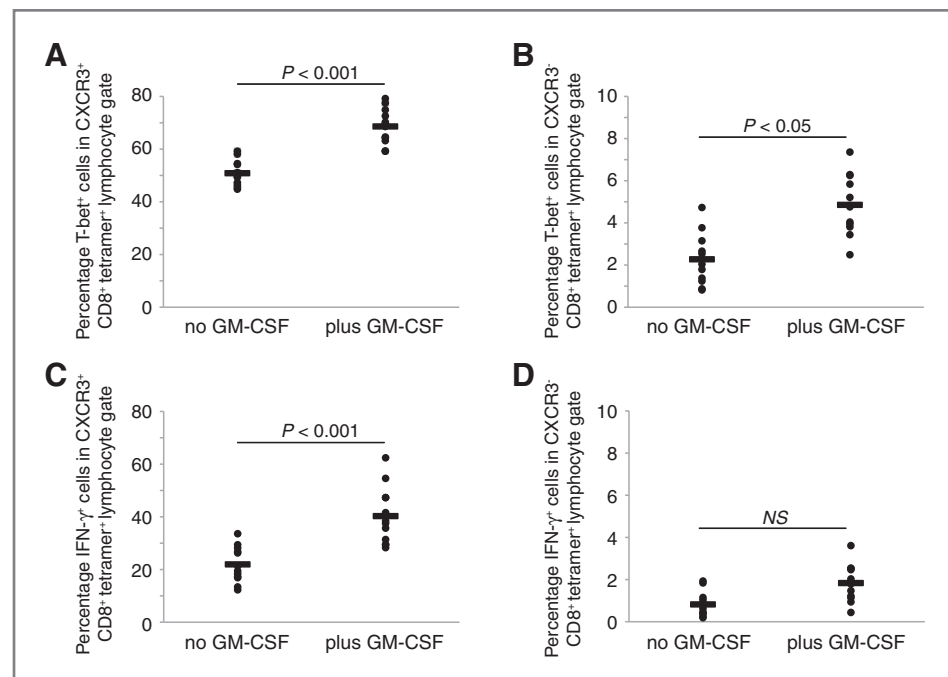
All CD8 cells expressing CLA coexpressed CXCR3. Interestingly, the mean fluorescence intensity of CXCR3 expression was significantly ( $P < 0.001$ ) higher in GM-CSF-treated cells (Fig. 2C), whereas CLA expression was modestly but significantly ( $P < 0.05$ ) lower. Therefore, a peptide vaccine composed of multiple class I-restricted antigens and delivered subcutaneously and intradermally in Montanide adjuvant can induce tumor-specific CD8 T-cell populations that express the pleiotropic homing receptor CXCR3 and the skin-homing glycoprotein CLA, and the addition of GM-CSF enhances the expression of homing receptors on vaccine-induced CD8 cells.

### Vaccination with peptide in adjuvant induces the expression of T-bet (TBX21) and IFN- $\gamma$ in tetramer-positive CD8 cells

Because CXCR3 expression is regulated by T-box transcription factor T-bet (TBX21; ref. 27), we assessed intracellular T-bet following vaccination with MELITAC 12.1, either with or without GM-CSF. T-bet staining was observed in the majority (>50%) of tetramer-positive CXCR3<sup>+</sup> CD8 cells (Figs. 3A and 4A) but unchanged relative to prevaccine status in tetramer-negative populations (data not shown). Addition of GM-CSF increased ( $P < 0.001$ ) the proportion of T-bet-expressing cells, consistent with the observed GM-CSF-associated increase in CXCR3 expression (Figs. 3A and 4A). In the CXCR3-negative, tetramer-positive subset of cells, T-bet was observed in only a small proportion of cells (<5%; Figs. 3B and 4B).

To assess the functionality of vaccine-induced T cells, we measured intracellular IFN- $\gamma$ . IFN- $\gamma$  staining was observed in a subset (~22%) of tetramer-positive CXCR3<sup>+</sup> CD8 cells (Figs. 3C and 4C), and addition of GM-CSF increased ( $P < 0.001$ ) the proportion of IFN- $\gamma$ -expressing cells (>40%, Figs. 3C and 4C).

**Figure 4.** The addition of peptide vaccine to adjuvant enhances the proportion of tetramer-positive CD8 T cells expressing the transcription factor T-bet (TBX21) and IFN- $\gamma$  in CXCR3<sup>+</sup>, but not CXCR3<sup>-</sup>, cells. A, T-bet expression in CXCR3<sup>+</sup> tetramer-positive cells, with or without GM-CSF in vaccine. B, T-bet expression in CXCR3<sup>-</sup> tetramer-positive cells, with or without GM-CSF in vaccine. C, IFN- $\gamma$  expression in CXCR3<sup>+</sup> tetramer-positive cells, with or without GM-CSF in vaccine. D, IFN- $\gamma$  expression in CXCR3<sup>-</sup> tetramer-positive cells, with or without GM-CSF in vaccine.



In tetramer-positive, CXCR3-negative cells, IFN- $\gamma$  staining is largely absent (<2% of cells, regardless of GM-CSF). IFN- $\gamma$  staining was unchanged by vaccination, regardless of the addition of GM-CSF, in tetramer-negative populations (data not shown).

#### Expression of CXCR3 and CLA is associated with the expression of IL-12R $\alpha$

Because CXCR3 and CLA expression are associated with the IL-12R (22, 28), we evaluated CD8<sup>+</sup> T cells for expression of IL-12R $\beta$ 1. Receptor staining was observed in a subset (~65%) of tetramer-positive CXCR3<sup>+</sup> CD8 cells, and addition of GM-CSF to the vaccine increased ( $P < 0.001$ ) the proportion of IL-12R $\beta$ 1-expressing cells (>93%; Fig. 5A and C). In the CXCR3-negative, tetramer-positive subpopulation, IL-12R $\beta$ 1 staining was detected in a subset (~26%) of cells and significantly

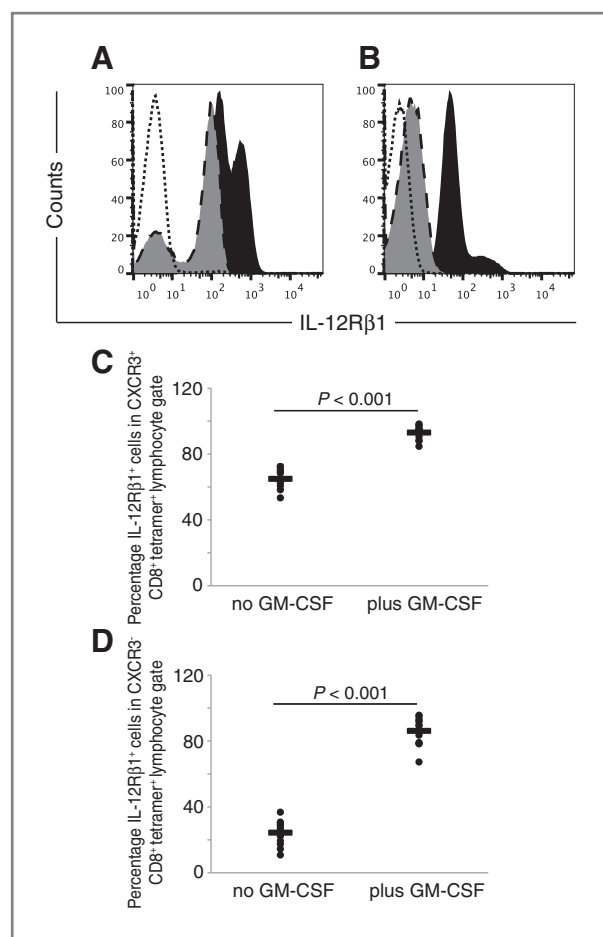
( $P < 0.001$ ) increased with the addition of GM-CSF (Fig. 5B and D). IL-12R $\beta$ 1 staining was unchanged by vaccination, regardless of the addition of GM-CSF, in tetramer-negative populations (data not shown). Thus, addition of GM-CSF to vaccination significantly enhanced antigen-specific CD8 T-cell expression of IL-12R $\beta$ 1, regardless of CXCR3 expression status.

#### Multiple vaccinations with peptide in adjuvant increase the proportion of tetramer-positive CD8 cells expressing CXCR3 and CLA

We next assessed whether multiple vaccinations enhanced the proportions of CXCR3- or CLA-expressing, tetramer-positive CD8 cells in circulation. We assessed CXCR3 and CLA expression, as earlier, before vaccination and after each of 6 weekly vaccinations, and we assessed memory populations (12 weeks after the final vaccination; representative data in Fig. 6A–H). The proportion of tetramer-positive CD8 cells expressing CXCR3 remained relatively stable (92%–98%) throughout the course of vaccination and remained high in the memory compartment (>88%; Fig. 7A). Addition of GM-CSF to the vaccine slightly increased the proportion of CXCR3-expressing cells (Fig. 7A). In contrast with the constant pattern of CXCR3 expression, CLA expression varied over the course of multiple vaccines. Without GM-CSF, the proportion of tetramer-positive CD8 cells expressing CLA reached a maximum (~81% expressing CLA; Fig. 7B) after five vaccinations, then modestly decreased in subsequent weeks; CLA expression in the memory compartment was significantly lower (~16%), even though the cells remained predominantly positive for CXCR3. Addition of GM-CSF to the vaccine increased the proportion of cells expressing CLA (Fig. 7B, Supplementary Table S1), and CLA expression in memory cells was significantly higher than in cells from vaccinated patients without GM-CSF. Thus, sequential peptide vaccination in Montanide adjuvant induces populations of CXCR3- and CLA-expressing tumor antigen-specific cells that persist after the discontinuation of immunization, and the addition of GM-CSF enhances and maintains CLA expression in tumor antigen-specific CD8<sup>+</sup> T cells.

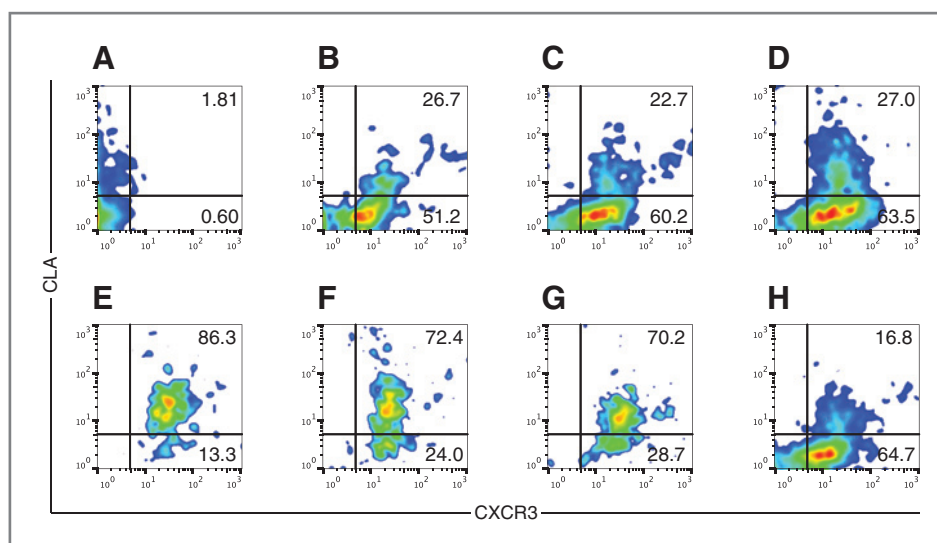
#### Discussion

Vaccination with melanoma peptides in Montanide adjuvant is immunogenic (29, 30), but the expression of tissue-homing receptors by vaccine-induced CD8<sup>+</sup> T cells has not been fully characterized. In the present study, we evaluated whether peptide vaccine in Montanide ISA-51 adjuvant can influence the expression of the chemokine receptor CXCR3 and the glycoprotein ligand CLA on vaccine-activated CD8 T cells. We also investigated whether inclusion of GM-CSF in the vaccine would enhance CXCR3 and CLA expression. Subcutaneous and intradermal administration of class I-restricted tetramer-positive antigens in Montanide ISA-51 induced antigen-specific CD8 cells, and the majority of the vaccine-induced cells expressed both CXCR3 and CLA. A previous report from Na and colleagues (15) showed that addition of GM-CSF to tyrosinase peptide in KLH induced CXCR3 expression by vaccine-induced CD4 T cells (31). We report that the majority of vaccine-induced CD8 cells express CXCR3, based



**Figure 5.** The addition of peptide vaccine to adjuvant enhances the proportion of tetramer-positive CD8 T cells expressing the transcription factor IL-12R $\beta$ 1. Open histogram, unstained control; light gray histogram, no GM-CSF in vaccine; dark gray histogram, GM-CSF in vaccine. A, representative IL-12R $\beta$ 1 staining in CXCR3<sup>+</sup> tetramer-positive cells, with or without GM-CSF in vaccine. B, representative IL-12R $\beta$ 1 staining in CXCR3<sup>-</sup> tetramer-positive cells, with or without GM-CSF in vaccine. C, IL-12R $\beta$ 1 expression in CXCR3<sup>+</sup> tetramer-positive cells, with or without GM-CSF in vaccine. D, IL-12R $\beta$ 1 expression in CXCR3<sup>-</sup> tetramer-positive cells, with or without GM-CSF in vaccine.

**Figure 6.** CLA, but not CXCR3, expression fluctuates over the course of peptide vaccination in adjuvant. The percentage of tetramer-positive CD45RO<sup>+</sup> CD8<sup>+</sup> cells expressing the indicated homing molecule over the course of vaccination is shown. A, before vaccine. B, 1 week after first vaccine. C, 1 week after second vaccine. D, 1 week after third vaccine. E, 1 week after fourth vaccine. F, 1 week after fifth vaccine. G, 1 week after sixth vaccine. H, 12 weeks after sixth vaccine.



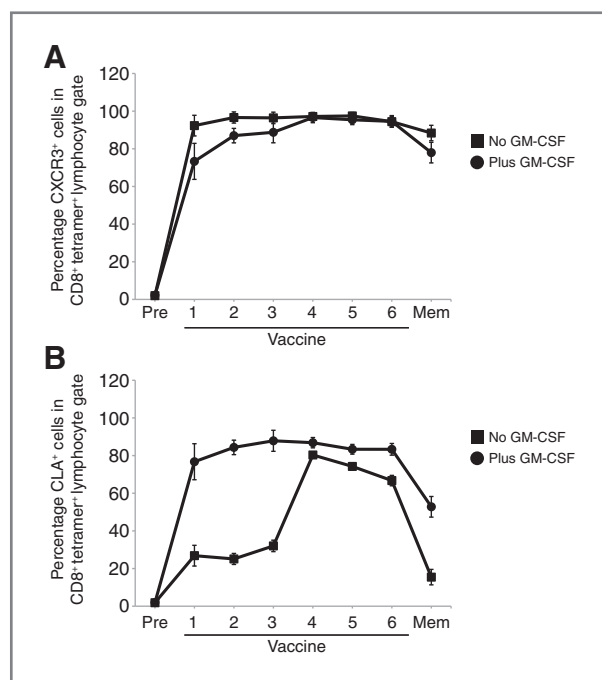
on intracellular staining, and that GM-CSF increases CXCR3 expression by CD8 T cells. Furthermore, we show that GM-CSF drives early expression of CLA in vaccine-induced CD8 cells.

Consistent with the expression of CXCR3, we observed that vaccine-induced T cells expressed IFN- $\gamma$  and T-bet. Interestingly, we observed significant expression of IFN- $\gamma$  and T-bet in cells that were isolated from patient PBMCs and directly stained (after cryopreservation), raising the possibility that

tetramers are sufficient to cross-link and activate T cells for cytokine production as previously reported in mouse models (32). However, in our studies, an analysis of a subset of samples revealed similar proportions of IFN- $\gamma$  and T-bet staining in bulk CD8 populations regardless of the inclusion of tetramer reagents in the staining reaction (data not shown). Furthermore, using samples from the same clinical trial, direct ELISpot assessment with individual peptide components of the MELITAC 12.1 vaccine showed that 7% to 50% of patient samples contained IFN- $\gamma$ -producing CD8 T cells (23). In the present study, we used samples that were previously shown to contain vaccine-induced CD8 T cells, based on direct and restimulated ELISpot analyses. The incubation of our T cells with tetramer was also for a short time period (15 minutes). Thus, we conclude that the observed expression of IFN- $\gamma$  and T-bet is consistent with previous data and reflects the sustained induction of these factors in antigen-specific CD8 T cells following vaccination with MELITAC 12.1.

Although peptide vaccines in Montanide adjuvant drive the expression of CXCR3 and CLA in CD8 cells, the molecular mechanisms remain undefined. Schaefer and colleagues (33) reported that repeated injections with Montanide induce the formation of tertiary lymphoid organs with a Th2-dominant microenvironment, which would not be expected to support the induction or maintenance of CXCR3 expression. However, mature dendritic cells were aggregated around superficial vessels and surrounded by lymphocytes. Thus, Montanide may polarize dendritic cells to a type I phenotype, which is highly supportive of CXCR3 expression by T cells (34); this is consistent with the observed immunogenicity of peptide/Montanide vaccines, and this possibility is under investigation. Other studies have shown the induction of CLA on CD4 and CD8 T cells by plasmacytoid dendritic cells (35, 36), and the possibility that type I dendritic cells may induce T-cell expression of CLA remains under study.

Addition of GM-CSF to the vaccine significantly increases the expression of CXCR3 and the proportion of CLA-expressing CD8 cells. The molecular and cellular mechanisms of



**Figure 7.** GM-CSF in vaccine-enhanced CLA expression on tetramer-positive CD8 T cells. The percentage of tetramer-positive CD45RO<sup>+</sup> CD8<sup>+</sup> cells expressing the indicated homing molecule over the course of vaccination is shown. A, CXCR3 expression over the course of vaccination. B, CLA expression over the course of vaccination.

GM-CSF activity remain undefined and are the subject of active investigation. GM-CSF is known to enhance the recruitment of dendritic cells to the injection site and may promote dendritic cell maturation and lymph node migration (37), thereby promoting T-cell activation and expression of CXCR3 and CLA on tetramer-positive T cells. Vaccine-induced memory cells retain CXCR3 and CLA expression approximately 5 months after immunization, suggesting that the T cells may have been activated in the presence of IL-12; previous studies show that CD4 T cells activated by IL-12-producing dendritic cells can maintain CXCR3 expression, whereas T cells activated in the absence of IL-12 can express CXCR3 only in a transient manner (38). Montanide and GM-CSF may both promote the activation of dendritic cells to produce IL-12 and promote type I T-cell responses. This is consistent with the report from Mortarini and colleagues (16), who showed that administration of IL-12 leads to a peripheral burst of CLA-expressing T cells, and consistent with our demonstration that vaccine-induced CD8 cells express surface IL-12R. The specific mechanisms of GM-CSF-mediated upregulation of CXCR3 and CLA remain under investigation.

Limited T-cell infiltration of metastatic melanoma remains a major impediment to effective immune therapy (39). A subset of melanomas express CXCR3 ligands (13), and expression of these ligands can be induced by exposure to exogenous interferons (40). We have also observed that intratumoral administration of IFN- $\gamma$  can induce CXCL10 expression in dermal metastases (Mullins and Slingluff, unpublished data). Because CXCR3 expression by tumor antigen-specific CD8 cells has been associated with enhanced survival in patients with advanced melanoma (11), the induction and maintenance of CXCR3 expression by vaccine-induced CD8 cells may significantly enhance the antitumor efficacy of vaccination. Conversely, recent work from Hailemichael and colleagues (41) suggests that CXCR3<sup>+</sup> CD8 cells may be preferentially retained in the Montanide-containing vaccine site, and thus the consequences of enhanced CXCR3 expression for tumor-specific trafficking remain to be determined.

The utility of CLA expression by tumor antigen-specific CD8 cells in the treatment of metastatic melanoma is debatable. Although vasculature in dermal melanomas often expresses the CLA-binding partner E-selectin (19), vasculature in metastatic melanoma is usually devoid of E-selectin (20). However, the Toll-like receptor agonist imiquimod has been shown to induce E-selectin expression in the vasculature of squamous cell tumors (42), leading to the possibility that it will induce E-selectin in melanomas (a possibility that is currently under

investigation). Thus, vaccination that drives CLA expression may be useful for the treatment of dermal metastases and potentially for other melanomas.

In sum, these data highlight novel phenotypic aspects of CD8 T cells induced by vaccination with peptide antigens in Montanide ISA-51 in the presence or absence of GM-CSF. Induction of CXCR3 and CLA may be useful for enhancing T-cell migration to specific tissue compartments, especially in the context of combinatorial therapies to induce specific homing receptor ligands in the tumor microenvironment, and addition of GM-CSF to vaccine increases CXCR3 and CLA expression.

#### Disclosure of Potential Conflicts of Interest

C.L. Slingluff Jr received a commercial research grant from GlaxoSmithKline, has ownership interest, including patents on peptides used in vaccines, and is a consultant/advisory board member of Polynoma, Immatics, and Curetech. D.W. Mullins received a commercial research grant and is a consultant/advisory board member of Qu Biologics. No potential conflicts of interest were disclosed by the other authors.

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# Cancer Immunology Research

## Peptide Vaccination in Montanide Adjuvant Induces and GM-CSF Increases CXCR3 and Cutaneous Lymphocyte Antigen Expression by Tumor Antigen –Specific CD8 T Cells

Eleanor Clancy-Thompson, Laura K. King, Lenora D. Nunnley, et al.

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