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Dendritic cells govern induction and reprogramming of polarized tissue-selective homing receptor patterns of T cells: important roles for soluble factors and tissue microenvironments

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Tissue-selective homing is established during naive T cell activation by the tissue microenvironment and tissue-specific dendritic cells (DC). The factors driving induction and maintenance of T cell homing patterns are still largely unknown. Here we show that soluble factors produced during the interaction of T cells with $CD11c^+$ DC isolated from skin- or small intestine-associated tissues differentially modulate expression of the corresponding tissue-selective homing receptors (E-selectin ligands and α4β7 integrin/CCR9, respectively) on murine $CD8^+$ T cells. Injection of tissue-specific DC via different routes induces T cells with homing receptors characteristic of the corresponding local tissue microenvironment, independent of the origin of the DC. These data indicate an important role for signals delivered *in trans*. Moreover, DC can reprogram the homing receptor expression on T cells previously polarized *in vitro* for homing to skin or small intestine. Importantly, skin-homing memory T cells stimulated directly *ex vivo* can also be reprogrammed by intestinal DC to a gut-homing phenotype. Our results show that tissue-selective homing receptor expression on effector and memory T cells is governed by inductive as well as suppressive signals from both DC and tissue microenvironments.

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Introduction

T cell homing to different tissues is highly heterogeneous and is determined by the combination of adhesion molecules and chemotactic receptors on the cell surfaces [1–4]. Best characterized among these homing receptors are α4β7 integrin [5, 6] and the chemokine receptor (CCR)9 [7–9] for lymphocyte homing to the small intestinal lamina propria and the mucosal epithelium. In contrast, T cell trafficking to inflamed skin is mediated by E-selectin ligands (E-lig) and P-selectin ligands (P-lig) [10–12] as well as chemokine receptors such as CCR4 and CCR10 [13–15]. Expression of the correct access code of homing receptors by T cells has been shown to be crucial for efficient tissue-specific immune responses as

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Abbreviations: **BM-DC:** Bone marrow-derived DC · **CCR:** Chemokine receptor · **CM:** Conditioned media · **E-lig:** E-selectin ligands · **FLT3-L:** FMS-like tyrosine kinase 3 ligand · **i.c.:** Intracutaneous · **LC:** Langerhans cell · **MLN:** Mesenteric lymph nodes · **M-CM:** Mesenteric DC conditioned media · **M-DC:** MLN DC · **P-CM:** P-DC conditioned media · **P-DC:** Peripheral lymph node DC · **PLN:** Peripheral lymph nodes · **PP:** Peyer's patch · **PP-DC:** Peyer's patch DC · **S-CM:** S-DC-conditioned media · **S-DC:** Splenic DC

well as autoimmune diseases or allergy [1–4], and interfering with T cell homing proves to be a promising therapeutic strategy [16–18]. It has been established that different T cell trafficking patterns are rapidly imprinted after priming in different secondary lymphoid tissue microenvironments [19–24]. Thus, up-regulation of P-lig or α4β7 and acquisition of CCL25 responsiveness of $CD4^+$ T cells following systemic antigen injection was dependent on the site of priming [19]. Similar results were found for E-lig and α4β7 expression on $CD8^+$ T cells primed with antigen-pulsed bone marrow-derived DC (BM-DC) via different routes [22], and the *in vivo* relevance of the immunization route has been shown for contact hypersensitivity [22] and melanoma [25] in mouse models. Moreover, we and others have demonstrated an education of naive T cells by tissue-specific DC *in vitro* [21–24]. Little is known about the factors that are involved in the polarization of T cell homing, although important roles for cytokines have been suggested from *in vitro* studies [26–29]. However, the *in vivo* relevance of these experiments, which were done in the absence of tissue-specific DC, remains unclear. Moreover, the role of the different cellular constituents of the tissue microenvironment that drive T cell homing (*i.e.* stromal cells, DC and matrix components) and the molecular mechanisms still have to be defined in detail.

In the present study, we therefore investigated whether homing receptor polarization is mediated by soluble factors produced by tissue-specific DC upon interaction with T cells. We observed that conditioned media (CM) generated by co-culture of DC isolated from different tissues with $CD8^+$ P14 T cells [30, 31] was able to polarize the homing patterns of anti-CD3-stimulated T cells. In contrast, the same DC failed to induce their corresponding T cell homing phenotype in an alternative tissue *in vivo* following injection via different routes. These data indicate that signals from tissue-specific DC can be overridden or modified by the local microenvironment. Finally, we tested the ability of DC to reprogram established homing patterns of effector and memory T cells polarized for homing to the skin or small intestine *in vitro* and *in vivo*, respectively. We found a DC-driven switch of homing receptor expression corresponding to the tissue origin of the DC used for stimulation.

Results

Regulation of T cell homing receptor patterns by tissue-specific DC

The tissue homing phenotype of T cells is established by tissue-specific DC during T cell priming *in vitro* [21–24]. In contrast to our previous study [22], here we used tissue-specific DC from mice injected with B16 melanoma cells expressing FMS-like tyrosine kinase 3 ligand (FLT3-L) [32] for *in vitro* priming of $CD8^+$ P14 T cells (Fig. 1A). Similar to our previous results [22], we found a clear polarization towards E-lig expression using Langerhans cells (LC) or peripheral lymph node DC (P-

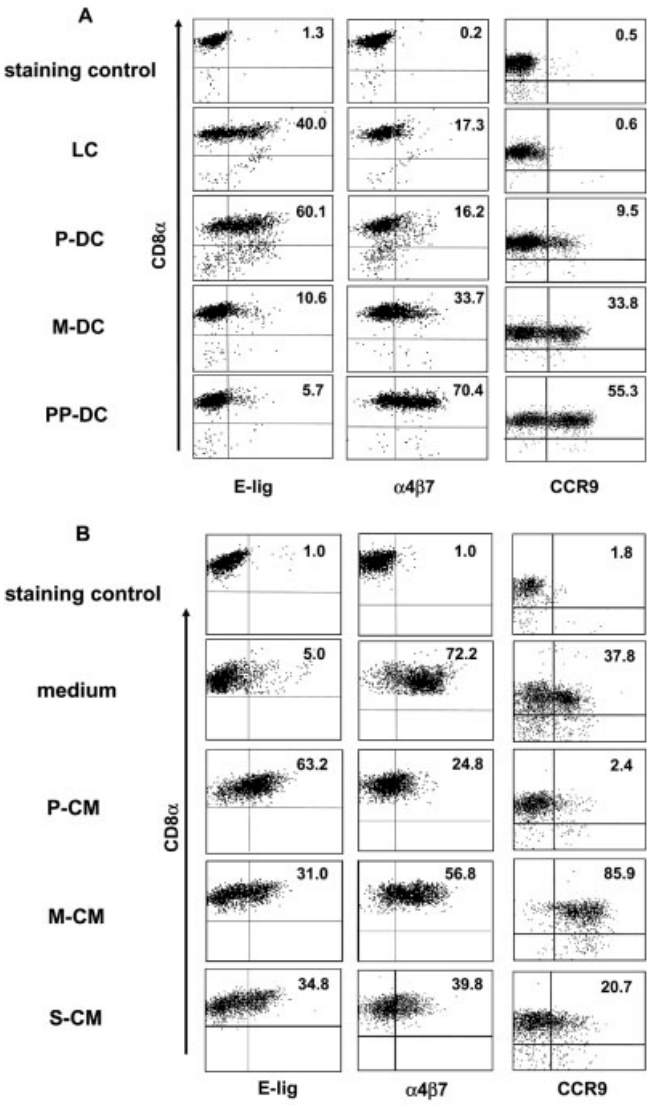

Fig. 1. DC or soluble factors regulate expression of tissue-specific homing receptors. (A) Purified $CD8^+$ P14 T cells were activated *in vitro* with $CD11c^+$ DC isolated from the indicated tissues of C57BL/6 mice treated with B16 melanoma cells expressing FLT3-L. Expression of E-lig, α4β7 integrin and CCR9 was determined by flow cytometry on day 4. (B) $CD8^+$ P14 T cells were activated *in vitro* with anti-CD3 mAb in the absence or presence of CM from 48 h co-cultures of $CD8^+$ P14 T cells with DC isolated from PLN (P-CM), MLN (M-CM) or spleen (S-CM). On day 4, expression of E-lig or α4β7 integrin and CCR9 was measured by flow cytometry. CCR9 measurements in (A) are from a different experiment. Numbers give the percentage of the $CD8^+$/homing receptor-positive cells. Data are representative of eight (A) and three (B) independent experiments. Staining controls were as described in Materials and methods.

DC) and towards α4β7/CCR9 expression with mesenteric lymph node DC (M-DC) and Peyer's patch DC (PP-DC) on day 4. Interestingly, E-lig levels on T cells were lower with M-DC from FLT3-L-treated mice as compared to DC isolated from untreated mice [22], while polarization to a skin-homing phenotype with skin-associated DC was more pronounced.

Soluble factors mediate DC-driven homing receptor polarization on T cells

In order to determine whether soluble factors are involved, naive $CD8^+$ P14 T cells were primed *in vitro* with anti-CD3 mAb in the absence or presence of CM, *i.e.* supernatants from co-cultures of $CD11c^+$ DC from P-DC (P-CM), M-DC (M-CM) or splenic DC (S-DC, S-CM) with $CD8^+$ P14 T cells. Homing receptor expression was determined by flow cytometry on day 4 (Fig. 1B and Table 1). E-lig were not efficiently up-regulated on $CD8^+$ P14 T cells by anti-CD3 in the absence of CM, while α4β7 was strongly expressed. Moderate CCR9 levels were detected. In contrast, E-lig were strongly induced on T cells primed with anti-CD3 mAb in the presence of P-CM, while α4β7 and CCR9 levels were down-regulated in comparison to the medium control and M-CM. M-CM promoted a gut-homing phenotype (Fig. 1B). These results suggest a suppression of α4β7/CCR9 and induction of E-lig by P-CM. M-CM also induced E-lig, however less efficiently. In the presence of S-CM, we observed intermediate E-lig and low α4β7/CCR9 levels, indicating suppressive effects on the gut-homing receptors. Similar results were obtained when phorbol 12-myristate 13-acetate (PMA)/ionomycin was used for T cell stimulation (data not shown).

Up-regulation of α4β7 in late skin DC/P14 T cell co-cultures

The gut-homing integrin α4β7 is up-regulated *in vitro* by M-DC or PP-DC around day 4 of culture [22–24], whereas levels stay low with skin-associated P-DC [22, 23]. Monitoring of α4β7 expression in priming cultures beyond day 6 revealed that P14 T cells activated with P-DC or LC also became $\alpha4\beta7^+$ as observed for co-culture with M-DC and PP-DC (Fig. 2A). Interestingly, α4β7 up-regulation was less pronounced in cultures primed by LC. This further underlines the strongest skin-specific polarization of T cells by LC [22]. We also observed such a general up-regulation of α4β7 upon priming of $CD4^+$ DO11.10 cells with P-DC, M-DC or PP-DC from untreated BALB/c mice (Fig. 2B) or $CD11c^+$ DC from C57BL/6 × BALB/c F1 mice treated with FLT3-L expressing melanoma cells [32] (data not shown). Tissue-specific

Table 1. Homing receptor polarization by soluble factors[a)]

	E-lig	α4β7	CCR9
Medium	3.2±3.4	83.4±9.7	35.9±14.3
P-CM	51.6±15.5	16.7±12.8	4.7±2.4
M-CM	20.3±9.3	64.5±6.9	62.7±27.9
S-CM	32.9±2.5	22.0±17.0	16.0±13.1

[a)] Experiments were performed as described in Fig. 1B. Numbers indicate the mean percentage of homing receptor-positive, $Thy1.1^+$ cells ± SD from three independent experiments. Differences in the mean values among groups are statistically significant at $p<0.001$ for E-lig and α4β7 and $p=0.014$ for CCR9.

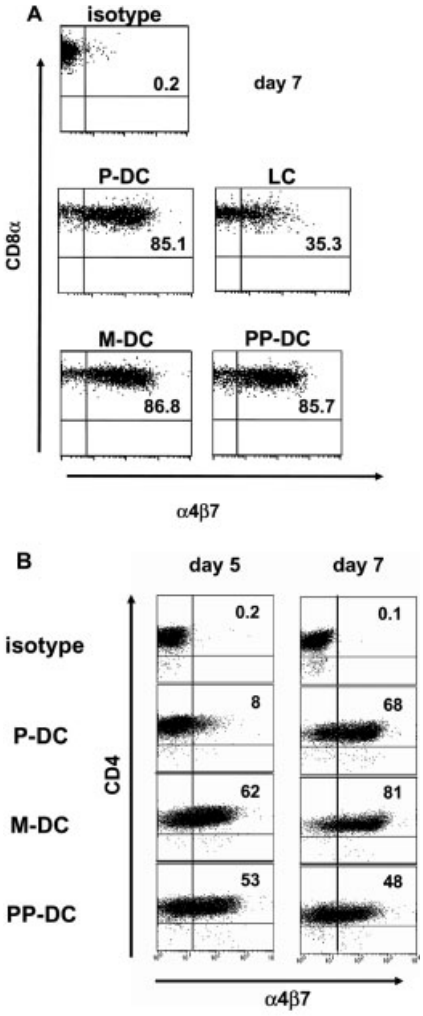

Fig. 2. Late up-regulation of α4β7. (A) $CD8^+$ P14 T cells were primed with the indicated tissue-specific DC as described in Fig. 1A. Flow cytometry was performed on day 7 of culture. (B) $CD4^+$ DO11.10 T cells were activated *in vitro* with DC isolated from the indicated tissues of untreated BALB/c mice. On days 5 and 7 of antigen-specific activation, expression of α4β7 was measured by flow cytometry. Numbers give the percentage of $CD8^+/\alpha4\beta7^+$ (A) or $CD4^+/\alpha4\beta7^+$ (B) cells. Data are representative of five (A) and two (B) independent experiments. Isotype control was as described in Materials and methods.

differences could still be observed on day 5, whereas the expression of α4β7 was similarly induced in all cultures on day 7. These results may be explained by an active suppression of α4β7 by skin-associated DC, which is absent following DC apoptosis around day 4 [24].

Dominance of tissue microenvironment over DC signals

The contribution of tissue microenvironments at the site of T cell priming, which beside the antigen-presenting DC might play a role in the induction of homing patterns, had not yet been investigated. We were interested in whether tissue-derived DC retain the ability to confer their specific homing information to T cells when placed in a 'foreign' tissue microenvironment. Therefore, we injected GP33-loaded P-DC or M-DC i.v., intracutaneously (i.c.) or i.p. and measured the homing receptor polarization on adoptively transferred P14 T cells after isolation from the corresponding secondary lymphoid tissues (Fig. 3, Table 2) where priming occurred. Analysis of T cells on day 3.5 revealed that up-regulation of E-lig was restricted to skin-draining peripheral lymph nodes (PLN) after i.c. injection of P-DC or M-DC, with greater efficiency upon P-DC injection (Fig. 3A), while high α4β7 expression was only observed in mesenteric lymph nodes (MLN) upon i.p. injection (Fig. 3B), irrespective of the tissue origin of the injected DC. Interestingly, efficient up-regulation of neither α4β7 nor E-lig was observed in the spleen, where most of the injected DC will be found after i.v. injection. These data demonstrate a general functional dominance of the secondary lymphoid tissue microenvironment over DC signals for the programming of homing phenotypes.

DC reprogram *in vitro*-polarized homing receptor expression

The role of DC in the imprinting of T cells for tissue-selective trafficking has been established [4, 21–24]. However, it is not known if effector T cells are still sensitive to this process or whether they are terminally imprinted. Therefore, we analyzed whether $CD8^+$ effector T cells can switch homing receptor patterns in response to new tissue-specific signals. P14 T cells were primed *in vitro* with LC or PP-DC as the strongest skin- or gut-polarizing DC, respectively. On day 6, cells were restimulated with DC from the same or the other tissue. Flow cytometry on day 3 after restimulation revealed an adaptation of E-lig and α4β7 expression levels to the new tissue-specific DC signals (Fig. 4). P14 T cells primed with LC down-regulated E-lig and up-regulated α4β7 integrin upon restimulation with PP-DC. Similarly, PP-DC-primed P14 T cells lost α4β7 expression and up-regulated E-lig following restimulation with LC. Similar results were obtained with P-DC and M-DC (data not shown). We conclude that tissue-specific DC can

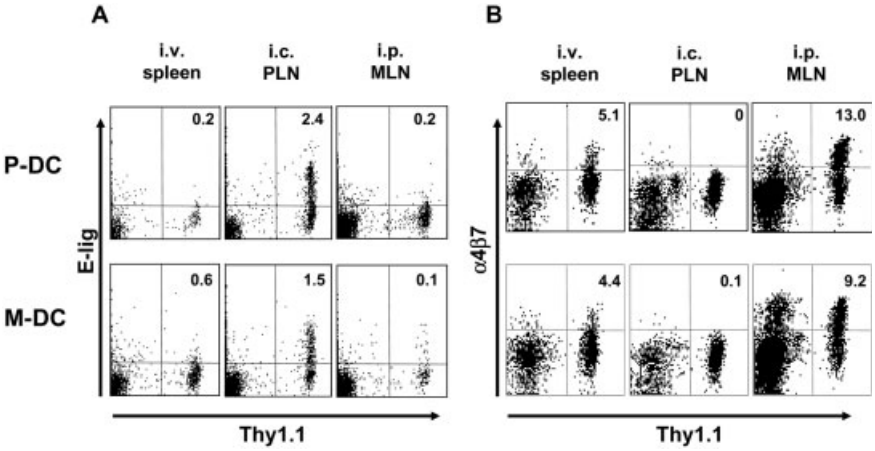

Fig. 3. Secondary lymphoid tissue microenvironment is dominant over tissue-specific DC. $CD8^+$ P14 T cells were adoptively transferred and activated *in vivo* with P-DC or M-DC administered via i.v., i.c. or i.p. injection. On day 3.5, expression of E-lig (A) and α4β7 (B) was measured on $Thy1.1^+$ cells from the indicated secondary lymphoid tissue by flow cytometry. Numbers give the percentage of the indicated cell population gated on $CD8^+$ cells. Data are representative of three independent experiments. Staining controls were as described in Materials and methods.

Table 2. Dominance of lymphoid tissue microenvironment over tissue-specific DC[a)]

	E-lig			α4β7		
	i.v. spleen	i.c. PLN	i.p. MLN	i.v. spleen	i.c. PLN	i.p. MLN
P-DC	3.1±1.54	62.8±10.8	5.9±2.4	17.6±5.1	2.3±1.0	49.2±14.6
M-DC	3.5±2.3	51.8±16.5	4.6±1.5	16.9±5.1	3.6±1.3	51.6±12.2

[a)] Experiments were performed as described in Fig. 3. Numbers indicate the mean percentage of homing receptor-positive, $Thy1.1^+$ cells ± SD from three independent experiments. Differences in the mean values among groups are statistically significant at $p<0.001$.

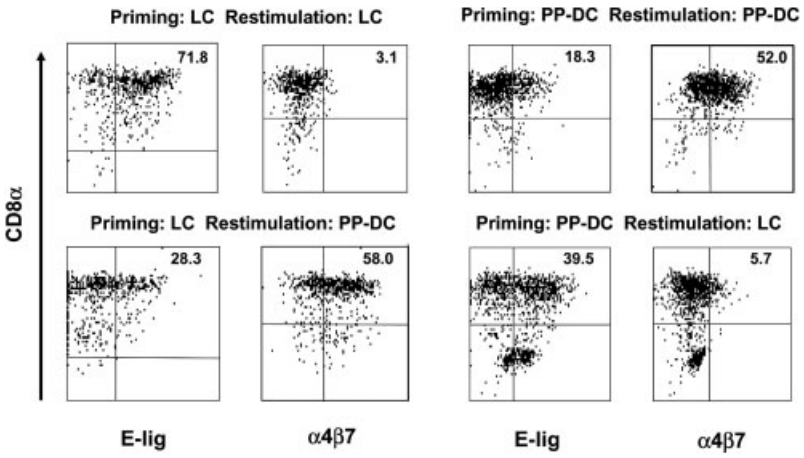

Fig. 4. Reprogramming of homing receptor profiles by tissue-specific DC *in vitro*. $CD8^+$ T cells isolated from P14 spleen were primed with LC isolated from skin or with $CD11c^+$ PP-DC. On day 6, T cells were restimulated with either the same or the alternative DC population. On day 3 after restimulation, expression of E-lig and α4β7 integrin was measured by flow cytometry. Numbers give the percentage of the $CD8^+$/homing receptor-positive cells. Data are representative of four independent experiments.

modulate T cell trafficking patterns not only during priming of naive T cells but also on effector T cells during an ongoing immune response.

Reprogramming of *in vivo*-polarized, skin-homing T cells by M-DC

We were interested in whether the flexibility of homing receptor expression as observed for effector T cells polarized *in vitro* holds true for *in vivo*-polarized skin-homing memory T cells. We injected GP33-loaded BM-DC twice i.c. as previously described for the adoptive P14 transfer system [22]. On day 10, mice were boosted by another i.c. injection of DC, and P14 T cells were isolated from PLN 3 weeks later. The T cells displayed a strongly polarized skin-homing phenotype and were mainly E-lig^{high} and α4β7/$CCR9^{low}$ (Fig. 5) by FACS in comparison to naive P14 T cells. Interestingly, a significant T cell population expressed CCR9. After *in vitro* restimulation of purified $CD8^+$ P14 T cells with LC isolated from skin or $CD11c^+$ DC from PLN, MLN or Peyer's patches (PP), we analyzed homing receptors. The skin-homing phenotype remained stable following restimulation with LC or P-DC (Fig. 5, Table 3). In contrast, restimulation with M-DC efficiently down-regulated E-lig levels and strongly induced α4β7/CCR9 (Fig. 5). Similar results were obtained with PP-DC (data not shown). These results reveal a flexibility of *in vivo*-polarized skin-tropic memory T cells.

Table 3. Reprogramming of homing receptor patterns on memory T cells by DC[a)]

	E-lig	α4β7	CCR9
Naive P14	0.7±0.5	3.6±1.1	86.0±7.7
Memory P14 *ex vivo*	52.3±27.5	4.6±2.0	28.4±4.3
LC	71.5±10.0	8.7±1.4	14.0±7.8
P-DC	54.8±10.7	15.7±7.6	16.6±4.6
M-DC	17.9±7.1	52.2±9.3	65.9±12.0

[a)] Experiments were performed as described in Fig. 5. Numbers indicate the mean percentage of homing receptor-positive, $Thy1.1^+$ cells ± SD from three independent experiments. Differences in the mean values among groups are statistically significant at $p<0.001$.

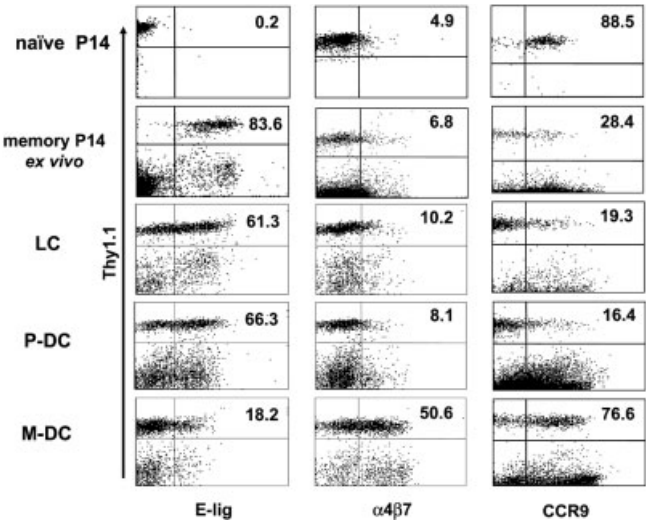

Fig. 5. Reprogramming of *in vivo*-induced skin-homing receptor profile by M-DC *in vitro*. Skin-homing $CD8^+$ P14 memory T cells were generated by adoptive transfer and i.c. injection of peptide-pulsed BM-DC on days 1, 3 and 10. On day 30, $CD8^+$ T cells were isolated from PLN by positive magnetic bead separation, and homing receptor expression on *ex vivo* cells was monitored by flow cytometry in comparison to naive P14 T cells. Restimulation was done with LC or $CD11c^+$ DC isolated from PLN (P-DC) or MLN (M-DC). On day 4, expression of E-lig, α4β7 integrin, and CCR9 on $Thy1.1^+$ T cells was measured by flow cytometry. Expression of CCR9 was measured in a different experiment. Gate was on $CD8^+$ T cells. Numbers give the percentage of total $Thy1.1^+$ cells expressing the indicated homing receptors. Data are representative of four (E-lig and α4β7) and three (CCR9) independent experiments.

Discussion

Accumulating data provide evidence that DC play a pivotal role in the regulation of T cell homing patterns. Modulation of $CD8^+$ T cell-homing properties has been established *in vitro* for DC isolated from skin, PLN, MLN and PP [21–24]. *In vitro* priming of naive $CD8^+$ T cells with DC isolated from different tissues revealed that the induction of E-lig for skin-homing [22] or the small intestinal-homing receptors α4β7 and CCR9 [21–24] was crucially dependent on the tissue origin of the DC, as was the ability of $CD8^+$ T cells primed *in vitro* with PP-DC to migrate to the small intestine [24]. However, the signals that control homing receptor expression are largely unknown. We found an unexpected up-regulation of α4β7 on $CD4^+$ as well as $CD8^+$ T cells in late T cell priming cultures beyond day 6, even with P-DC or LC. Similar observations were made with T cells stimulated with anti-CD3 in the presence of P-DC [23]. These data suggest an active suppression of α4β7 by skin-associated DC and a release from suppression when DC undergo apoptosis around day 4 [24]. Persisting lower levels of α4β7 in cultures with LC, which induce the strongest skin-tropic polarization, support this notion. In line with this hypothesis, *in vitro*-generated, 'tissue-neutral' BM-DC do not suppress α4β7 expression [22]. Further evidence for the existence of suppressive signals, in addition to inductive signals, for homing receptor polarization has been previously provided [33, 34]. Low E-lig levels were observed on human $CD4^+$ T cells stimulated with PHA in the presence of DC in serum-containing medium compared to serum-free medium. In contrast, serum factors were crucial for the induction of β7 integrin [33]. Our study demonstrates that tissue-specific DC modulate homing receptors by delivery of soluble factors. Thus, CM from DC/T cell co-cultures are able to induce their corresponding homing receptors and suppress the homing receptors for the alternative tissue on anti-CD3 mAb-stimulated $CD8^+$ P14 T cells. This shows that soluble factors are sufficient *in vitro*.

Interestingly, tissue-specific DC did not induce their corresponding T cell homing phenotype in a 'foreign' microenvironment. α4β7 integrin was up-regulated in MLN upon i.p. DC injection, E-lig in PLN upon i.c. DC injection, regardless of the tissue origin of the DC. We did not observe α4β7 up-regulation following M-DC injection in PLN or spleen. Similarly, E-lig was not significantly up-regulated in MLN or spleen upon injection of P-DC. These data support our previous findings using BM-DC [22] and demonstrate a functional dominance of the secondary lymphoid tissue microenvironment over the origin of the injected DC. Tissue-resident DC and stromal cells may release soluble factors that overrule the direct influences of the transferred antigen-presenting DC population *in trans*. Alternatively, the injected DC might adapt functional characteristics of the new tissue microenvironment [4]. This hypothesis has gained support through the observation that even mature DC, as used in our experiments, are able to differentiate upon interaction with stromal cells [35]. We have previously ruled out efficient antigen cross-presentation by host DC to P14 T cells by injection of heat-killed DC [22]. Importantly, the *in vivo* results also gave evidence for inhibitory factors suppressing E-lig in spleen and MLN, which had not been detected *in vitro* with M-DC.

Our results reveal that DC-driven polarization of T cell homing patterns is mediated, at least in part, by soluble factors. E-lig and α4β7 up-regulation may be a default program induced upon activation as suggested by previous studies [33, 36, 37]. Obviously, microenvironmental signals can efficiently polarize towards skin- or gut-homing receptors, supported by the detection of non-overlapping skin- or gut-tropic memory T cell subsets in blood [10, 38–43]. The soluble factors that are responsible for homing receptor polarization in our study have to be identified in the future. Iwata et al. reported recently that retinoic acid can enhance expression of the small intestine-homing receptors α4β7 and CCR9, while the same factor suppressed skin-homing receptor and fucosyltransferase VII expression under steady-state conditions [44]. Interestingly, small intestine-associated but not skin-associated DC expressed the enzymes required to generate retinoic acid from retinol, an observation that is a first hint to explain some of the tissue-specific characteristics of DC isolated from different tissues [21–24]. It is conceivable that differential expression of genes such as the retinal dehydrogenase genes [44] is the basis for these DC properties.

Furthermore, an inductive role for IL-12, IL-4 and TGF-β1 in E-lig expression by regulation of fucosyltransferase VII [26–28] and for TGF-β1 in the regulation of β7 integrins [29] has been demonstrated *in vitro*. In our hands, addition of recombinant IL-4 to priming cultures with P-DC or priming with BM-DC from IL-12p35/p40 knockout mice strongly reduced E-lig induction on P14 T cells (data not shown). The unexpected *in vitro* induction of the skin-homing receptor E-lig by gut-associated and S-DC and the corresponding CM may therefore result from accumulation of unphysiologically high DC-derived IL-12 levels in culture. The *in vivo* relevance of these cytokines remains to be determined. Interestingly, recent studies showed that acquisition of skin- vs. gut-homing phenotypes by human $CD4^+$ T cells occurred independently of the Th1 or Th2 subset polarization [33, 37, 45].

Several reports suggest the existence of stable, mutually exclusive tissue-selective memory T cell sub-

sets [10, 38–43]. Memory of the site of antigen encounter may be epigenetically imprinted in these cells by DNA methylation and histone modification [46] as described for cytokine memory of T cell subsets [47] and recently for the regulation of selectin ligands [48]. Here we describe for the first time a reprogramming of polarized E-lig or α4β7/CCR9 expression: tissue-specific DC are not only able to imprint naive T cells but also revert the homing profile of already polarized tissue-selective P14 T cells induced *in vitro*. Even more striking, skin-homing memory T cells polarized *in vivo* could also be reverted into a gut-homing phenotype. This raises the question of whether imprinting of homing properties is indeed permanently fixed or whether a certain degree of flexibility is preserved in polarized memory T cells. An epigenetic memory and integration of previous homing patterns may be the basis for such flexibility and could be reflected by simultaneous DNA modification of loci involved in the regulation of homing receptors for different tissues such as skin and gut. Flexible trafficking patterns are essential for the immune system to cope efficiently with disseminating infections and metastasizing tumors.

With regard to the proposed dichotomy of central vs. effector memory T cells with different access to secondary lymphoid tissues [3, 49], central memory T cells may be the subset that can adapt to different tissue microenvironments, whereas effector memory T cells may keep a topographical memory of the first site of antigen encounter. Although this model seems to be more complex than initially thought [45, 50], it may explain how the immune system can keep and integrate several tissue-specific T cell homing patterns.

In summary, our results show that DC govern tissue-selective T cell homing not only during priming, but also in the effector and memory response by reprogramming of established homing patterns. Soluble factors play an important role. Furthermore, the tissue microenvironment crucially influences homing receptor polarization. Therapeutic modulation of tissue-selective trafficking patterns of effector and memory T cells will allow tissue targeting in order to generate efficient immune responses or therapeutic re-routing of pathogenic T cells in organ-specific autoimmune and inflammatory diseases.

Materials and methods

Mice

C57BL/6 and TCR-transgenic Thy1.1 congenic P14 mice [30, 31] expressing a TCR specific for the lymphocytic choriomeningitis virus (LCMV)-derived peptide GP33 [51] were provided by the breeding facility of the University of Freiburg, Germany. OVA-specific TCR-transgenic DO11.10 mice [52] and BALB/c mice were bred in the BfR (Bundesanstalt für Risikobewertung, Berlin, Germany). All of the experimental procedures were in accordance with institutional, state and federal guidelines on animal welfare.

Peptides

Synthetic H-2D^b-binding peptide GP33 from the glycoprotein of LCMV [51] and I-A^d-binding chicken ovalbumin peptide $OVA_{323–339}$ have been described before [52] and were purchased from BioChip Technologies GmbH (Freiburg, Germany) and the Department of Biochemistry (Humboldt-University, Berlin, Germany), respectively.

Media and chemicals

RP-10 consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 25 mM Hepes buffer, 50 μg/ml penicillin-streptomycin (all from Gibco, Invitrogen Corporation, Karlsruhe, Germany) and 10 μM 2-mercaptoethanol (Sigma, Deisenhofen, Germany).

Antibodies and flow cytometry

Antibodies were from BD Biosciences (Heidelberg, Germany) and used as FITC, PE or biotin conjugates. The latter were revealed with Streptavidin-Cy-Chrome®. mAb were used at 0.1–1 μg/1×10^6 cells in 100 μl HBSS/0.3% BSA. Staining for α4β7 integrin and E-lig with E-selectin/human IgG-Fc-Chimera (R&D Systems, Wiesbaden, Germany) was performed as described [22]. Control staining for E-lig was done with secondary Ab only, while corresponding isotype control Ab was used as a control for α4β7 and CCR9. Ex vivo cells were washed with 5 mM EDTA/PBS before staining. Rat anti-CCR9 Ab has been described elsewhere [53] and was used as B cell hybridoma supernatant at a dilution of 1:4. Secondary FITC-labeled anti-human IgG and biotinylated anti-rat IgG were from DAKO (Hamburg, Germany). Secondary FITC- or PE-labeled anti-rabbit antibodies (Serotec, Eching, Germany) were diluted 1:50–1:100. Data were acquired and analyzed on a FACScan instrument using CellQuest software (BD Biosciences).

Generation of bone marrow-derived DC

Bone marrow cells were cultured at 7×10^5 cells/ml in the presence of 40 ng/ml GM-CSF (supernatant from producer line X63-Ag8 [54]) and 10 ng/ml recombinant IL-4 (Promocell, Heidelberg, Germany) in 10 ml medium in 10 cm Petri dishes (Greiner, Nürtingen, Germany). On day 3, 10 ml fresh medium containing 40 ng/ml GM-CSF was added. On days 5 and 7, 10 ml medium was replaced with fresh GM-CSF medium. DC were used on days 7–9. Purity was routinely about 90%.

Adoptive P14 T cell transfer and injection of tissue-specific DC

P14 spleen cells (3×10^6 in 200 μl PBS) were injected i.v. into C57BL/6 mice. One day later, DC from mice treated with B16

melanoma cells expressing FLT3-L [32] were incubated for 30 min at 37°C in RP-10 with GP33 peptide (1 μM) and LPS (0.3 μg/ml) (Sigma). After three washes with PBS, cells were used for injection. DC (3×10^6 in 200 μl PBS) were injected i.c. at three sites on the shaved abdomen. The i.v. and i.p. injections were performed with 1×10^6 DC in 200 μl PBS. On day 3.5, mice were killed and lymphoid tissue cells prepared for flow cytometry by gentle teasing of the tissue through a steel mesh and filtration of the suspension through a cell strainer (70 μm, BD Falcon, Heidelberg, Germany).

Isolation of tissue-specific DC and *in vitro* priming

C57BL/6 mice received 1×10^6 B16 melanoma cells producing FLT3-L [32] s.c. in 200 μl PBS. Mice were killed 14–17 days later, and $CD11c^+$ DC were isolated from skin-draining PLN, MLN, PP and spleen by CD11c MicroBeads using AutoMACS (Miltenyi Biotec, Bergisch-Gladbach, Germany) as described [22]. Langerhans cells were isolated from ear sheets as described [22]. Ears were split into dorsal and ventral sheets using forceps. Sheets were incubated for approximately 30 min in 1% trypsin/PBS solution (Gibco) at 37°C until the epidermal layer could be removed by rubbing with forceps. Single-cell suspensions were prepared by extensive up-and-down pipetting of the epidermal sheets, followed by cell strainer filtration (70 μm, BD Falcon) and 16% Nycodenz (Sigma) gradient centrifugation. The purity of the isolated DC was routinely about 90%.

DC were incubated with GP33 peptide (1 μM) for 30 min at 37°C and washed three times. DC (5×10^3/well) and P14 spleen cells (4×10^4/well) were co-cultured in 96-well round-bottom plates (Corning Life Sciences, Wiesbaden, Germany) in 200 μl RP-10. In some experiments $CD8^+$ P14 T cells were isolated with CD8 MicroBeads (Miltenyi), and similar results were obtained.

OVA-specific naive $CD4^+$ T cells were isolated from pooled lymph nodes and spleens of DO11.10 mice using CD4-FITC and anti-FITC MultiSort MicroBeads (Miltenyi), followed by release of the magnetic label and subsequent isolation of naive $CD4^+$ T cells with CD62L MicroBeads. Naive $CD4^+$ DO11.10 T cells (1×10^5) were co-cultured with 1×10^4 DC in the presence of $OVA_{323–339}$ peptide (1 μg/ml) in 96-well plates in 200 μl RP-10.

Preparation of CM and P14 T cell priming

DC (6×10^6) isolated from PLN (P-DC), MLN (M-DC) or spleen (S-DC) were pulsed with peptide GP33 as described above and co-cultured with P14 spleen cells (1.2×10^6) or purified $CD8^+$ spleen cells (4×10^5) in 12-well plates (Corning Life Sciences) in 2 ml RP-10. Supernatants were harvested 40 h later by centrifugation and stored at –40°C. $CD8^+$ T cells were isolated from P14 spleens using CD8 MicroBeads (Miltenyi), and 1×10^4 cells were cultured in 100 μl RP-10 plus 150 μl RP-10 or CM from tissue-specific DC/T cell co-cultures (see above) in 96-well round-bottom plates (Corning Life Sciences). Soluble anti-CD3ε (145–2C11, BD Biosciences) was added to cultures at 10 μg/ml.

In vivo generation of skin-homing P14 T cells and reprogramming of T cells by DC

In vitro co-cultures of tissue-specific DC with P14 T cells were set up in 96-well plates as described above. Cells were harvested on day 6. After washing in RP-10, cell suspensions were diluted 1:4 and added to fresh 96-well plates together with 1×10^4 DC (isolated from different tissues) per well. For in vivo generation of skin-homing T cells, P14 T cells were injected i.v., and mice received 3×10^6 GP33-pulsed and LPS-activated BM-DC i.c. on days 1, 3 and 10. On day 30, $CD8^+$ cells were purified from PLN using CD8 MicroBeads (Miltenyi) and restimulated with tissue-specific DC. T cells (1×10^4) and GP33-pulsed DC (1×10^4) were co-cultured in 200 μl RP-10/2% RCAS in 96-well plates.

Statistical analysis

Statistical analysis of the data was performed by comparison of treatment groups using one-way ANOVA.

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