Disruption of Id1 reveals major differences in angiogenesis between transplanted and autochthonous tumors

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Summary

Id **genes regulate tumor angiogenesis and loss of** ***Id1*** **inhibits tumor xenograft growth in mice. Here we evaluate the role of Id1 in a more clinically relevant tumor model system using a two-step chemical carcinogenesis protocol. Remarkably, we find that** $Id1^{-/-}$ **mice are** ***more*** **susceptible to skin tumorigenesis compared to their wild-type counterparts. Cutaneous neoplasms in** $Id1^{-/-}$ **mice show increased proliferation without alterations in tumor angiogenesis; however,** $Id1^{-/-}$ **mice possess 50% fewer cutaneous γδ T cells than their wild-type counterparts due to an intrinsic migration defect associated with loss of expression of the chemokine receptor, CXCR4. We suggest that there are important differences between the mechanisms of angiogenesis in transplanted and autochthonous tumors and that these findings will have significant implications for the potential utility of antiangiogenic therapies in cancer.**

Introduction

In order for tumors to grow beyond a critical size, they must develop an associated vasculature. Since the original proposal that targeting a tumor vasculature could be an efficient form of cancer therapy (Folkman, 1971), much data have accumulated to support the critical role of tumor-associated vessels in tumor growth and progression. Recent studies in genetically altered mice have supported the notion of tumor angiogenesis being initiated by an "angiogenic switch" that tips the balance of angiogenic factors in favor of endothelial growth and vessel formation (reviewed in Hanahan and Folkman, 1996). Over the past several years, data have been mounting to support a central role for the Id family of helix-loop-helix factors in the process of tumor angiogenesis (Benezra et al., 2001). Remarkably, mice lacking from 1-3 Id1 and/or Id3 alleles have been demonstrated to resist growth of tumor explants due to defects in tumor angiogenesis (Lyden et al., 1999). More recently, the angiogenesis inhibitor thrombospondin-1 was demonstrated to be a target of Id1 transcriptional repression and was found to be a significant mediator of the effects of Id1 on tumor angiogenesis in vivo (Volpert et al., 2002). While the above studies supported a role for *Id* genes in promoting tumor vessel growth, the tumor model system evaluated in those studies involved either the intradermal injection of 2×10^7 xenograft tumor cells into host animals (Lyden et al., 1999, 2001) or the use of angiogenic stimuli in matrigel plugs. Since much data suggests that tumor angiogenesis is initiated by an angiogenic switch that shifts the balance of endothelial cell growth and migration toward induction of angiogenesis, (Hanahan and Folkman, 1996), we sought to determine whether Id1 expression affects the angiogenic switch in vivo using a more genetically relevant tumor model system. Here we show that skin tumors initiated by classical two-step chemical carcinogenesis (Yuspa et al., 1996) do not demonstrate altered angiogenesis in Id1 null animals and propose a model for tumor angiogenesis that may occur via Id-dependent or Id-independent mechanisms.

Results

Id1 null mice have an increased susceptibility to skin cancer

De novo tumor formation and associated angiogenesis were induced in $Id1^{-/-}$ mice using classical two-step chemical carci-

SIGNIFICANCE

In order for tumors to develop beyond a limited size, they must develop an associated vasculature. The mechanisms underlying tumor-induced angiogenesis have been a source of great interest since inhibiting tumor vessel growth shows promise as a therapeutic intervention. The Id proteins regulate tumor angiogenesis in xenograft models and mice lacking *Id* genes are resistant to transplanted tumor growth. Here we show that tumor angiogenesis in autochthonous skin tumors does not depend on the expression of Id1. We further show that loss of *Id1* results in loss of expression of chemokine receptors in $Id1^{-/-}$ skin-specific γδ T cells. We suggest that tumor angiogenesis can occur by Id-dependent and Id-independent mechanisms, which may be a function of CXCR4 receptor activity, and that these differences are important for the design of antiangiogenic agents for cancer treatment.

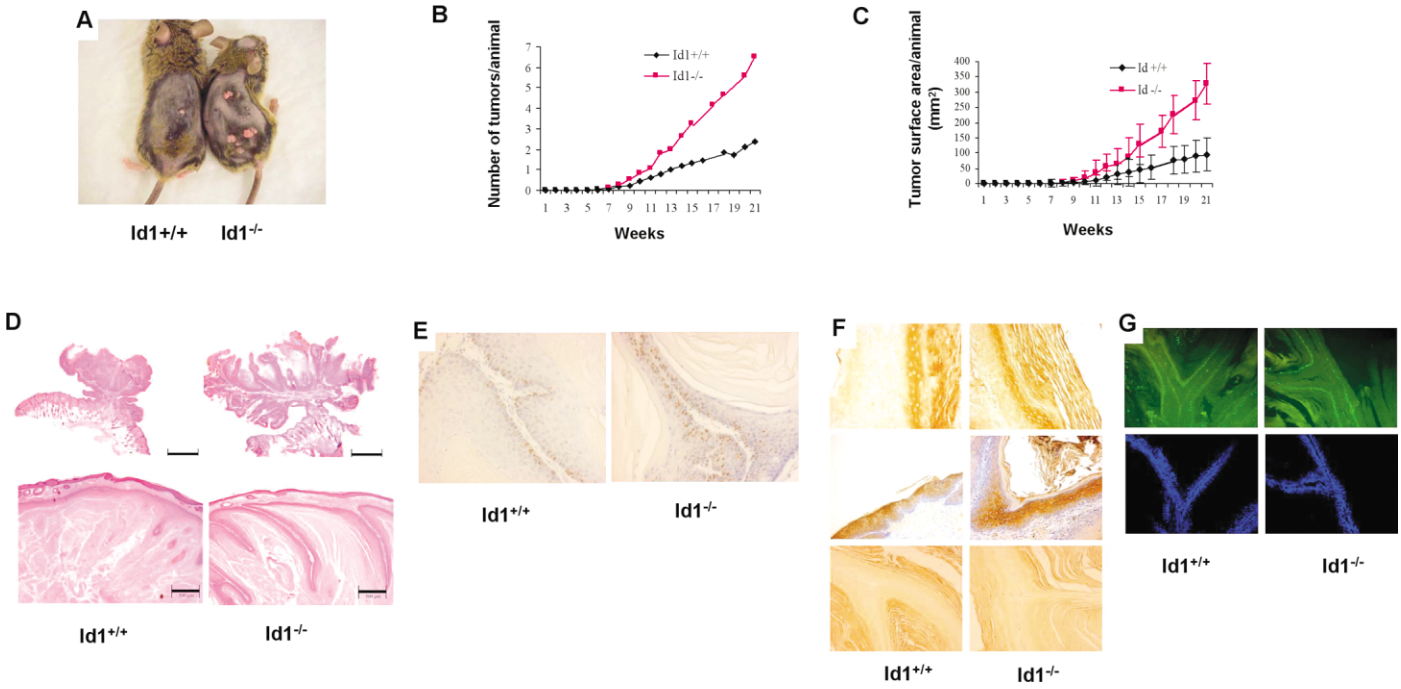

Figure 1. Increased skin tumorigenesis is seen in Id1 null mice

A: Mice treated with DMBA and TPA for 20 weeks.
B: Tumor incidence in *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice throughout course of treatments.
C: Tumor burden in *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice throughout course of treatment (mean tumor surface area ± SD are shown). **D:** Histopathology of skin tumors in *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice treated with DMBA and TPA. Tumors were either in the form of exophytic papillomas (top) or inverted papillomas (bottom).
E: Ki-67 expression in tumors from *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice.
F: Keratin-6 (top), -10 (middle), and -14 (bottom) expression in skin tumors from *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice.
G: Apoptosis as measured by TUNEL staining (top) and DAPI staining (bottom) of skin tumors from *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice.

nogenesis (Yuspa et al., 1996). Mice were initiated with DMBA at birth, treated with TPA twice weekly for 20 weeks, and tumors were evaluated on a weekly basis. Remarkably, we found an *increased* incidence of skin tumors and increased tumor burden in *Id1*$^{-/-}$ mice versus wild-type controls (Figures 1A–1C). Histologic evaluation of tumors demonstrated both exophytic and inverted squamous papillomas with an increase in cellularity in *Id1*$^{-/-}$ tumors (Figure 1D). Immunohistochemical evaluations demonstrated increased cellular proliferation in tumors from *Id1*$^{-/-}$ mice as measured by Ki-67 expression (Figure 1E) but no significant differences in cellular differentiation patterns (Slaga et al., 1996) as determined by keratin-6, keratin-10, and keratin-14 markers (Figure 1F). Interestingly, tumors from *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice were noted to be Id1 negative by in situ hybridization, which differs from the expression seen in human squamous cell carcinomas of the head and neck (Langlands et al., 2000). They also did not express significant levels of p16/INK4a (data not shown) as has been seen previously (Mortier et al., 2002), or significant alterations in apoptosis (Figure 1G).

Tumors in Id1 null mice do not show defects in angiogenesis

Since *Id* genes have been demonstrated to affect tumor angiogenesis, tumors from *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice were evaluated histologically for Factor VIII staining of tumor-associated vessels (Figure 2A), and tumors were quantitatively assessed for vessel caliber and density (Figure 2B). Tumor stroma and parenchyma did not demonstrate significant differences in vessel density or caliber in *Id1*$^{+/+}$ versus *Id1*$^{-/-}$ mice despite an overall increase in angiogenesis in all tumors versus control normal skin.

Id1 null mice lack skin-specific γδ T cells

Given the paradoxical increase in tumor incidence and progression in *Id1*$^{-/-}$ mice, we sought possible mechanisms for differences in tumor growth that might be unrelated to angiogenic defects. *Id* genes have been shown to regulate the development of B lymphocytes (Becker-Herman et al., 2002; Sun, 1994; Yokota et al., 1999), T lymphocytes (Morrow et al., 1999; Rivera et al., 2000), and Langerhans cells (Hacker et al., 2003), and associated E proteins have been shown to participate in V(D)J recombination of the γδ T cell receptor (Bain et al., 1999); however, Id1 null mice have been shown to possess normal lymphoid (CD4^{+}, CD8^{+}, B220^{+}, IgM^{+}) and myeloid (Mac-1^{+}, GR-1^{+}) lineages by fluorescence-activated cell sorter (FACS) analysis (Yan et al., 1997). Since γδ T cells in the skin play an important role in tumor immunosurveillance and mice lacking γδ T cells have an increased susceptibility to skin tumor development (Girardi et al., 2001), we evaluated epidermal sheets from Id1 null mice for the presence of γδ T cells and Langerhans cells. *Id1*$^{-/-}$ mice were found to possess a 50% reduction in skin-specific γδ T cells (Figures 3A and 3B) with a normal complement of Langerhans cells (Figure 3C). Since the absolute

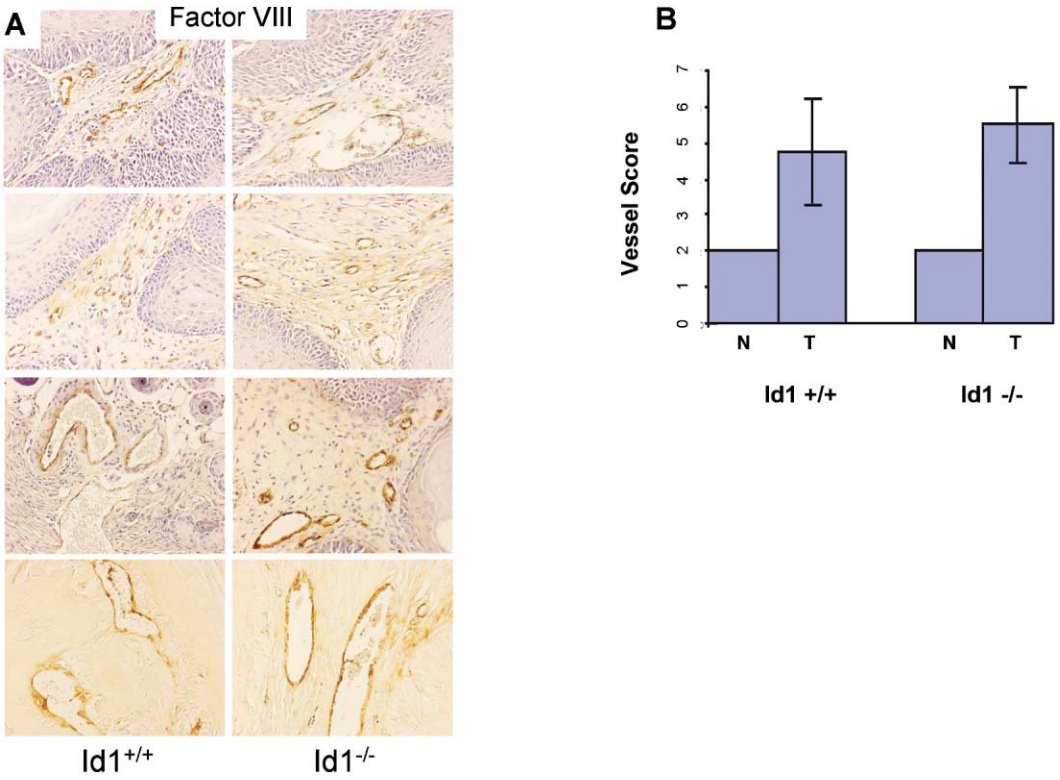

Figure 2. Angiogenesis is unaltered in skin tumors from *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice

A: Factor VIII staining of blood vessels in skin tumors from *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice (200× magnification).
B: Quantitative analysis of blood vessel density and caliber in *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice ± stardard deviation.

numbers of skin-specific γδ T cells were decreased in *Id1*$^{-/-}$ mice, we evaluated whether this was due to altered thymic differentiation, altered homing to the skin, or increased destruction of cells within the skin. Thymuses were obtained from E17.5 *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mouse embryos and analyzed for skin-specific γδ T cells by FACS analysis (Vγ3 TCR). We found a normal complement of Vγ3 TCR^{+} cells in E17.5 *Id1*$^{-/-}$ thymuses versus wild-type controls, suggesting normal T cell differentiation (Figure 3D). In addition, evaluation of epidermal γδ T cells at 1 week and 2 weeks of age showed a 50% reduction of γδ T cells in *Id1*$^{-/-}$ mice versus wild-type controls, similar to that seen in adult animals (data not shown). We therefore conclude that loss of γδ T cells in the skin of *Id1*$^{-/-}$ mice is due to defects in homing of γδ T cells to the skin and suggest that this reduced number of skin-specific dendritic cells may account for the increased tumor incidence in *Id1*$^{-/-}$ mice due to associated defects in immune surveillance.

Recent studies noted an increased incidence of skin tumors in mice null for γδ T cells and that γδ T cells could kill skin tumor cells by engaging the NGK2d receptor (Girardi et al., 2001). We suggest that even a 50% decrease in γδ T cells can lead to increased skin tumorigenesis due to a loss of immune surveillance. We further propose that the decrease in epidermal Vγ3^{+} T cells is due to a defect in homing of these cells to the skin. Since we recently identified a series of *Id1* target genes that include thrombospondin-1, extracellular matrix proteins, and integrin receptor subunits (Volpert et al., 2002), we sought to evaluate whether altered expression of such proteins may be involved in the defective homing of Vγ3^{+} T cells to the skin in Id1 null mice or whether the cells themselves possess an intrinsic homing defect to the skin.

Id1 null γδ T cells have a cell-intrinsic homing defect

In order to determine the nature of the homing defect of *Id1*$^{-/-}$ Vγ3^{+} T cells, we performed an in vitro cell migration assay of sorted Vγ3^{+} T cells from *Id1*$^{+/+}$ and *Id1*$^{-/-}$ embryonic thymocytes. Cells were evaluated for migration to the universal T cell chemokine receptor ligand, CXCL12, at varying concentrations, and migrated cells were evaluated under fluorescence microscopy (Figure 4A). We noted a significant difference in Vγ3^{+} T cell migration from *Id1*$^{+/+}$ versus *Id1*$^{-/-}$ cells at all concentrations of CXCL12 tested. We sought in vivo confirmation of this cell-autonomous defect in migration of *Id1*$^{-/-}$ Vγ3^{+} T cells by performing transplants of *Id1*$^{+/+}$ and *Id1*$^{-/-}$ embryonic thymocytes into RAG$^{-/-}$ mice. Vγ3^{+} T cells were evaluated in the skin 6 weeks post transplantation using fluorescence microscopy (Figure 4B). We found that *Id1*$^{+/+}$ Vγ3^{+} T cells were present in the skin of transplanted RAG$^{-/-}$ mice on either an SV129 or a BALB/C background; however, *Id1*$^{-/-}$ Vγ3^{+} T cells failed to migrate to the skin of transplanted RAG$^{-/-}$ mice to an appreciable extent, thus confirming the cell-autonomous defect in homing of *Id1*$^{-/-}$ Vγ3^{+} T cells to the skin.

Id1 null γδ T cells have reduced expression of the chemokine receptor CXCR4

Since we had established a defect in homing of Vγ3^{+} T cells to the skin in Id1 null cells, we evaluated the expression of

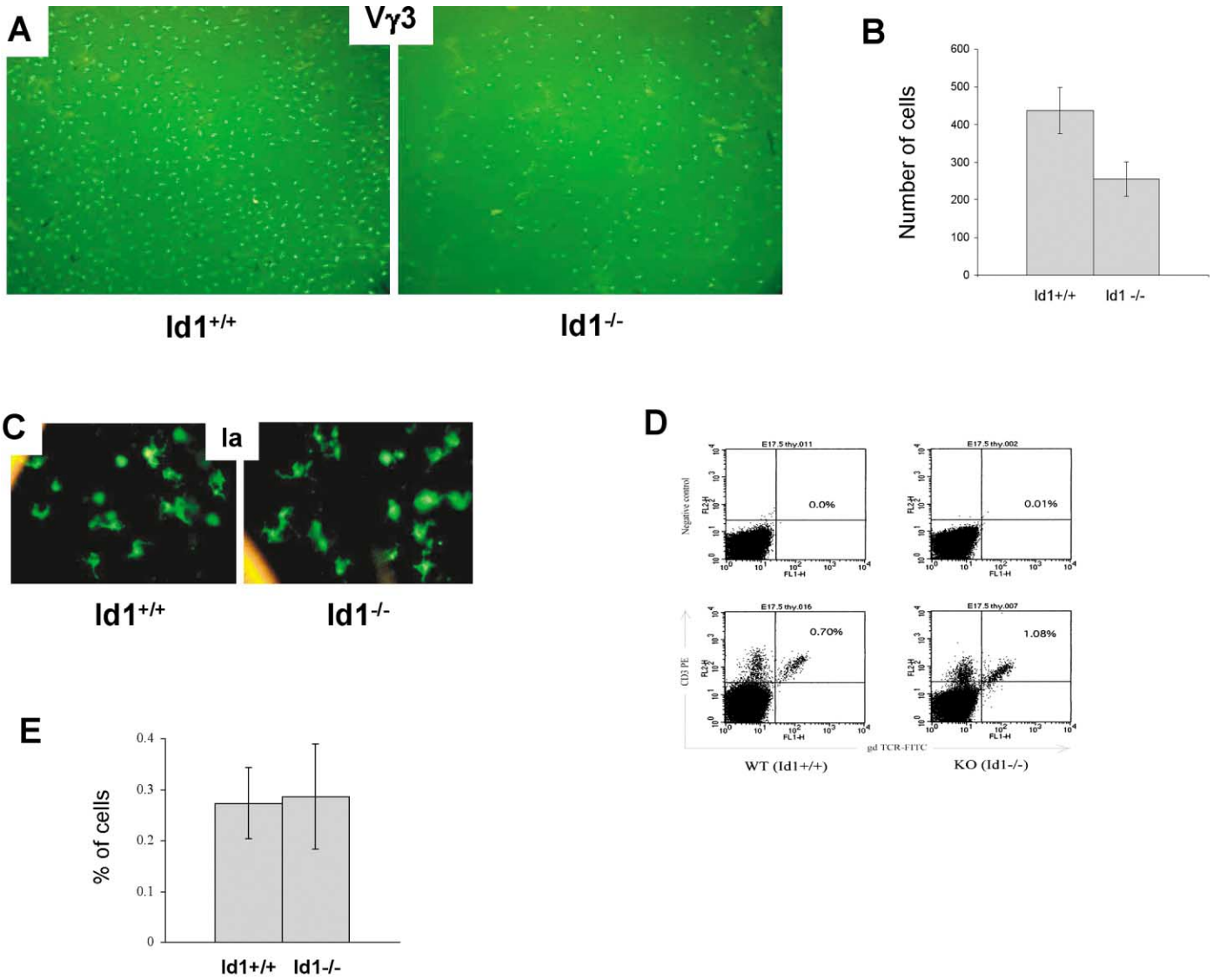

Figure 3. Id1 null mice show a loss of epidermal γδ T cells due to defective homing to the skin

A: Immunostaining of Vγ3$^+$ T cells in the epidermis of adult *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice.
B: Quantitative analysis of Vγ3$^+$ T cells in the epidermis of adult *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice.
C: Immunostaining of epidermal Langerhans cells in *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice.
D: FACS analysis of Vγ3$^+$ T cells from E17.5 day embryonic thymuses of *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice.
E: Quantitative analysis of Vγ3$^+$ T cells from E17.5 day embryonic thymuses of *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice.

chemokine receptors in *Id1*$^{+/+}$ and *Id1*$^{-/-}$ Vγ3$^+$ T cells to determine whether homing might be altered due to loss of chemokine receptor expression. Id1 expression was evaluated by RT-PCR in *Id1*$^{+/+}$ and *Id1*$^{-/-}$ Vγ3$^+$ T cells to assure that any cell-autonomous defect in homing was due to loss of endogenous Id1 expression. *Id1*$^{+/+}$ Vγ3$^+$ T cells demonstrated high-level expression of Id1 while *Id1*$^{-/-}$ cells did not express any endogenous Id1 (Figure 5A). Quantitative RT-PCR evaluation of chemokine receptor expression in *Id1*$^{+/+}$ and *Id1*$^{-/-}$ Vγ3$^+$ T cells revealed mild alterations in expression of various chemokine receptors in *Id1*$^{-/-}$ cells with the exception of the chemokine receptor CXCR4, which showed >90% loss of expression versus *Id1*$^{+/+}$ cells (Figure 5B).

Discussion

Id genes have been implicated in regulating tumor angiogenesis since Id null mice show defects in vascularization of tumor xenografts (Lyden et al., 1999, 2001); however, the true contribution of *Id* genes to the stepwise development of autochthonous tumors has not been evaluated until now. In the above studies, we use classical two-step chemical carcinogenesis to define the role of Id1 in regulating the angiogenic switch in vivo. Remarkably, contrary to data seen with xenografts in Id null mice, skin tumors induced by chemical carcinogens were *increased* in onset and progression in Id1 null mice versus wild-type controls. Histologic evaluation of skin tumors in *Id1*$^{+/+}$ and *Id1*$^{-/-}$ mice

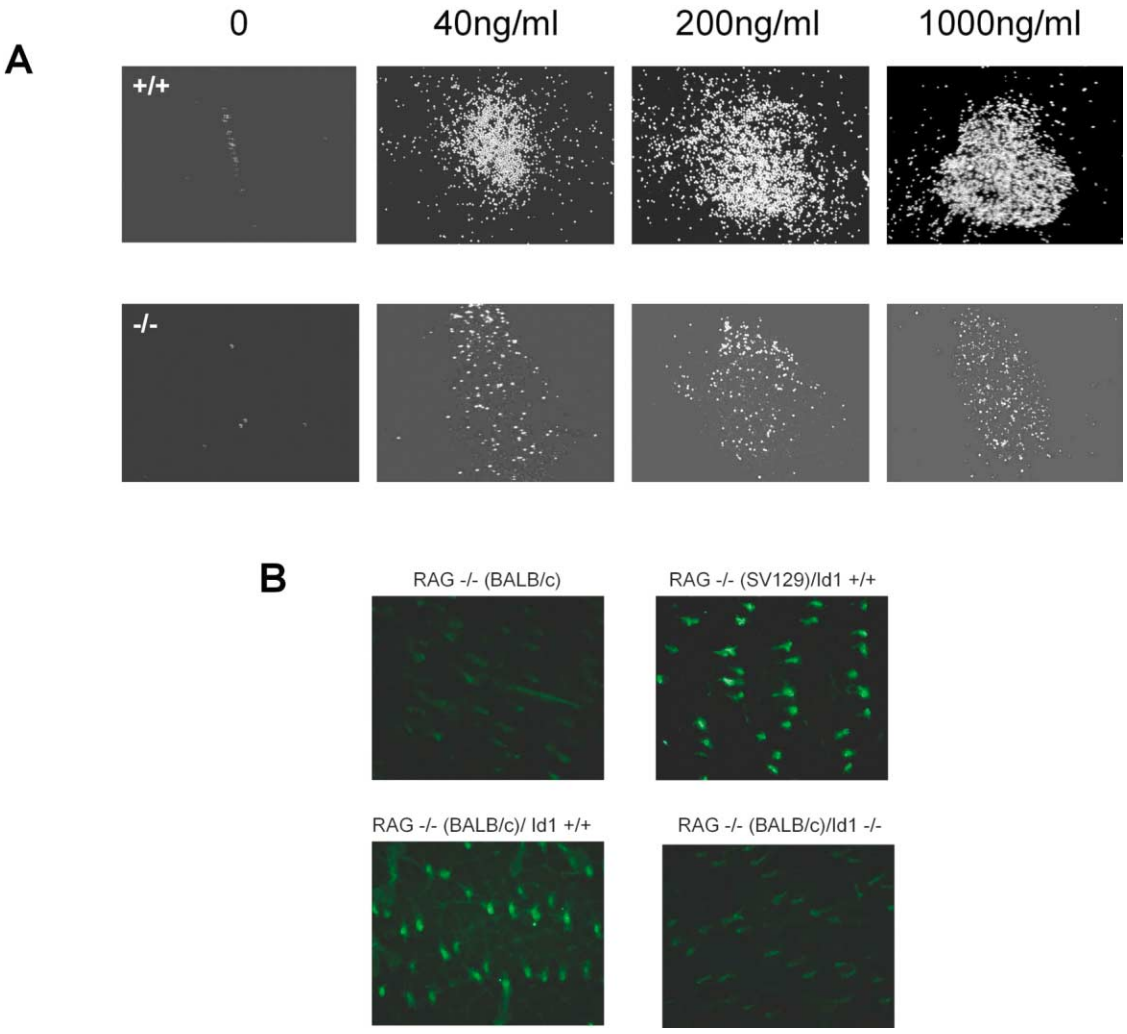

Figure 4. Id1 null γδ T cells show an autonomous defect in homing to chemokines

A: In vitro cell migration assay of *Id1*$^{+/+}$ and *Id1*$^{-/-}$ skin-specific γδ T cells toward varying concentrations (0, 40, 200, 1000 ng/ml) of CXCL12.
B: Embryonic T cell transplants into RAG$^{-/-}$ mice. Genotype to the left is that of recipient mouse, genotype to the right is that of donor thymocytes.

failed to demonstrate significant differences in angiogenesis by either vessel density or vessel caliber despite demonstrated onset of the angiogenic switch in all tumors versus control normal skin. This is in contrast to the defects in angiogenesis noted in tumor xenografts and matrigel assays in *Id1*$^{-/-}$ mice seen by us and others (Lyden et al., 2001; Volpert et al., 2002). We therefore suggest that tumor angiogenesis that occurs through stepwise genetic events and is associated with the "angiogenic switch" is not dependent on *Id* gene expression, whereas angiogenesis that occurs in tumor xenografts or matrigel plugs through the abrupt introduction of angiogenic factors into a severely hypoxic environment is highly dependent on *Id* gene expression. We believe that the latter situation may be analogous to what occurs during tumor metastasis (reviewed in Folkman, 2002) when highly proliferative tumor cells migrate to a novel matrix and must be vascularized (Figure 6).

We found that loss of Id1 resulted in loss of expression of the chemokine receptor CXCR4 in Vγ3^{+} T cells. Since CXCR4 has been shown to play a critical role in T cell trafficking (reviewed in Baird et al., 1999) and chemokines have been shown to mediate T cell homing to the skin (Hwang, 2001), we suggest that loss of expression of CXCR4 on *Id1*$^{-/-}$ Vγ3^{+} T cells results in defects in homing to the skin. Interestingly, CXCR4 has been shown to be the major chemokine receptor expressed on endothelial cells and its expression is induced by VEGF (Salcedo and Oppenheim, 2003). Furthermore, CXCR4 knockout mice die perinatally due to defects in cardiac development and organ-associated angiogenesis (Tachibana et al., 1998). In addition, CXCR4 expression has been demonstrated to be induced in invasive breast cancers and melanomas and is associated with poor prognosis and tumor invasion/metastasis (Muller et al., 2001; Murakami et al., 2002) and has been shown to be down-regulated by the von Hippel-Lindau tumor suppressor, pVHL (Staller et al., 2003). Whether *Id* genes also regulate CXCR4 expression in other cell lineages remains to be determined.

Our data suggest a specific role for Id proteins in regulating angiogenesis associated with conditions seen in tumor xenografts including severe hypoxia, acidosis, and hypoglycemia,

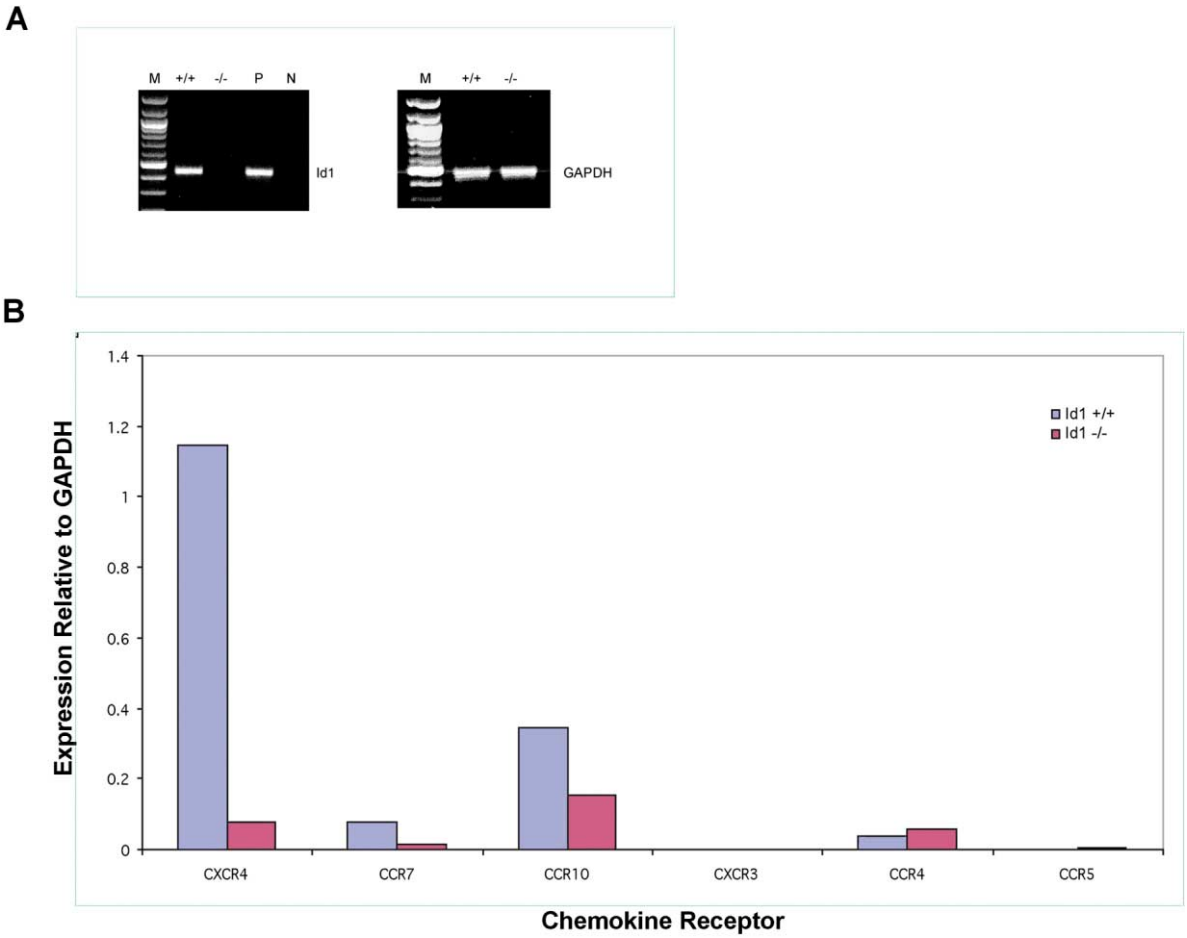

Figure 5. *Id1*$^{-/-}$ skin-specific γδ T cells show loss of expression of the chemokine receptor CXCR4

A: RT-PCR analysis of Id1 expression in *Id1*$^{+/+}$ (+/+) and *Id1*$^{-/-}$ (−/−) skin-specific γδ T cells. M = size marker, P = pos. control, N = neg. control (H_20).
B: Quantitative RT-PCR analysis of chemokine receptor expression in *Id1*$^{+/+}$ and *Id1*$^{-/-}$ skin-specific γδ T cells.

which are often seen in metastatic disease. Since many of the current angiogenesis inhibitors under clinical investigation were identified by their ability to inhibit angiogenesis and ultimately growth of transplanted tumors (reviewed in Kerbel and Folkman, 2002) and early trials of these angiogenesis inhibitors in autochthonous tumors in mice (Bergers et al., 1999) and man (Herbst et al., 2002) have been less effective than anticipated, we propose that the utility of such antiangiogenic therapeutics in human cancers will depend on their ability to inhibit both the angiogenesis that occurs by the "switch" mechanism supporting autochthonous tumor growth and the Id-dependent angiogenesis seen in the xenograft model. We therefore await the identification of classes of antiangiogenic therapies for cancer that are able to efficiently target both Id-dependent and Id-independent tumor-associated angiogenesis.

Experimental procedures

Chemical carcinogenesis

Id1$^{-/-}$ mice were established as previously outlined (Yan et al., 1997), bred into a mixed C57Bl6/Sv129 background, and genotyped as previously noted (Lyden et al., 1999). Wild-type mice were obtained as littermates from heterozygote breedings and bred into a mixed C57Bl6/Sv129 background for at least ten generations. For tumor initiation, a single dose of dimethylbenz(*a*)anthracene (DMBA) (50 μg in 10 μl of acetone) was applied topically at the backs of mice on the day of birth. For tumor promotion, mice were shaved at the backs and treated topically twice per week with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (5 μg in 50 μl acetone) starting one week after DMBA initiation and continuing for 20 weeks. Tumor onset was noted in all mice and tumors counted on a weekly basis. Tumor size was measured on a weekly basis once tumors reached 1.5 mm in diameter. At week 21, all mice were euthanized and tumors were surgically removed for histologic analysis. Thirty mice from each genotype (*Id1*$^{+/+}$ and *Id1*$^{-/-}$) were used for DMBA + TPA treatment. An additional five mice from each genotype were used for each set of control treatments (acetone, DMBA alone, and TPA alone). No tumors resulted from any of the control populations.

Histologic analysis, immunohistochemistry, and in situ hybridization

Tumors were fixed in 10% buffered formalin overnight and embedded in paraffin. Thin sections (4–5 μm) were cut and slides

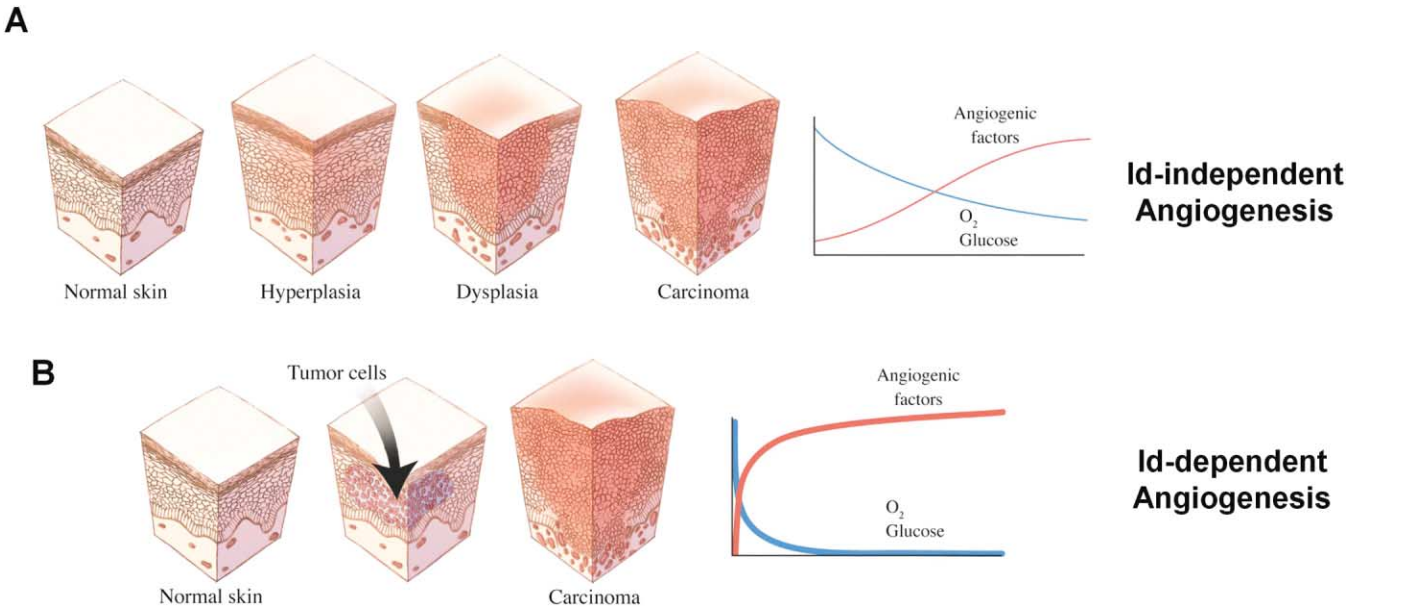

Figure 6. Model for *Id* gene function in tumor angiogenesis

The development of a vasculature in autochthonous tumors with slow onset of increased expression of proangiogenic factors, hypoxia, hypoglycemia, acidosis (**A**) is not highly dependent on *Id* gene expression. Tumors formed in this manner do not demonstrate appreciable alterations in angiogenesis in Id1 wild-type and Id1 null mice. The development of a vasculature in transplanted tumors that grow with the rapid introduction of high levels of proangiogenic factors, extreme hypoxia, hypoglycemia, and acidosis (**B**) is highly dependent on *Id* gene expression. Mice lacking 1–3 Id1 and/or Id3 alleles fail to maintain the growth of tumors initiated by this process.

were prepared for H&E and immunohistochemical staining. Tumor sections were deparaffinized in xylene, rehydrated through washing in a graded series of ethanol and water, and microwaved in 10 mM citrate buffer (pH 6.0) for target retrieval. After blocking endogenous peroxidase (5 min) and 1 hr blocking with serum (from secondary antibody host), slides were stained with primary antibodies. For primary rabbit antibodies (Ki-67, Dianova, cat# dia 550-67, 1:50 diluted; Factor VIII, DAKO, cat# A 0082, 1:200 diluted; keratin 6, clone K6-gen from P. Coulombe lab, 1:200 diluted; keratin 14, clone 199 from P. Coulombe lab, 1:400 diluted), experiments were carried out with Dako EnVision+System rabbit kit (Dako, code# K4010) according to manufacturer's supplied protocol. For primary mouse antibodies (keratin 10, Sigma cat# c-7284, 1:100 diluted; p16/ink4a, Santa Cruz Biotech. cat# sc-1661, 1:25 diluted), slides were incubated with primary antibodies for 1 hr and with secondary antibody (biotinylated goat anti-mouse IgG from CalBiochem, cat# OS02B, 1:50 diluted) for an additional hour. Samples were incubated with streptavidin-peroxidase for 15 min (from DAKO ARK kit, code# K3954), followed by incubation with DAB+ Chromogen (from DAKO ARK kit, code# K3954). Counterstaining was performed with Mayer's hematoxylin (DAKO, code# S3309) and 37 mM ammonia. Slides were then coverslipped on an automated film coverslipper and examined using light microscopy.

For in situ hybridization, total RNA was made from NIH 3T3 cells using RNeasy Mini Kit (Qiagen, cat# 74104) followed by cDNA preparation. Two DNA fragments (471 bp and 460 bp) incorporating T7 promoters into the primers were generated by PCR amplification.

PCR primers for 471 bp DIG riboprobe:
5′: GGATCCTAATACGACTCACTATAGGGAGAGTTCTCTTCCCA CACTCTGTTC
3′: CGCCGTTCAGGGTGCTGAGCGGGGCGC.

PCR primers for 460 bp DIG riboprobe:
5′: GGATCCTAATACGACTCACTATAGGGAGAGCATGTGTTCC AGCCGACGATC
3′: CTAAAGTGTTTGTTTAATAACAACAA

Two DIG-labeled RNA probes were made by in vitro transcription of the above cDNA templates using DIG RNA labeling reagents and T7 RNA polymerase according to the manufacturer's instructions (Boehringer Mannheim). Paraformaldehyde-fixed paraffin-embedded samples were deparaffinized in xylene, rehydrated in a graded series of ethanol and water, treated with 1% hydrogen peroxide, permeabilized with 20 ug/ml freshly prepared Proteinase K (Gibco BRL, cat# 25530-015), and incubated with RNA probes (100 ng/ml) overnight at 65°C. After washing twice in 2× SSC, slides were incubated at 37°C with RNase A/T1 cocktail (Ambion cat # 2288) diluted 1:35 in 2× SSC. Slides were stringently washed twice in 2× SSC, 50% formamide DI (Sigma, cat# F-7503, deionized by mixed bed resins) and then once with 0.08× SSC at 60°C. Before immunodetection, tissues were blocked with 1% blocking reagent (Boehringer Mannheim) containing 1:20 diluted purified, nonspecific rabbit immunoglobulins (DAKO). Sections were then incubated with rabbit HRP-anti-DIG (Dako) diluted 1:100 in blocking buffer for 30 min at RT. After washing with 1× TBST, one drop of ready-to-use biotinyl-tyramide (DAKO) was added directly to slides and incubated in the dark for 15 min at RT. After three washes with 1× TBST, slides were incubated with secondary streptavidin (from DAKO GenPoint Kit) for 15 min at RT. After three washes with 1× TBST, slides were developed with DAB

(DAKO). Cells were counterstained with Mayers Hematoxylin and 37 mM ammonia, coverslipped in permanent mount media (Dako), and observed using light microscopy.

Semiquantitative assessments of angiogenesis

Representative histological sections of tumors were stained by immunohistochemistry for von Willebrand Factor. Sections were then scored blinded to group by two experienced persons independently (R.M.A., D.L.H.). Scoring was done on a 0 (absent) to 4 (numerous) scale for number of vessels in the tumor and on a 0 (absent) to 4 (large) scale for average size of the vessels to determine a total score. Conflicting results were reviewed until a consensus was reached and the results were then unblinded as to genotype.

Apoptosis assay

Formalin-fixed paraffin-embedded tumor sections were treated in xylene and ethanol, rehydrated, and treated with proteinase K for target retrieval. Samples were then incubated with TUNEL (TdT-mediated dUTP nick end labeling) reaction mixture (Roche Molecular Biochemicals) for 1 hr at 37°C followed by nuclear staining with DAPI. Slides were then observed using fluorescence microscopy.

Epidermal sheet preparation and immunostaining

After euthanasia, mice were shaved and remaining hair was removed with chemical hair remover (Nair). Dorsal skin was cut into small pieces and the epidermal side was gently pressed on a glass slide that was prepared with double-sided tape. After incubation at 37°C in Hank's BSS containing 20 mM EDTA (3 hr) dermis was peeled away gently, leaving the epidermis attached to the slide. Epidermal sheets were fixed with freshly prepared 4% paraformaldehyde in PBS for 10 min at RT and blocked with 1% BSA in PBS for 15 min. Slides were stained with 1:100 diluted FITC-conjugated Vγ3 antibody (BD Pharmingen, cat#553229) for 1 hr at RT and observed using fluorescence microscopy. For Langerhans cell staining, epidermal sheets were prepared by cutting and splitting the ears into two parts (dorsal and ventral), incubating them in 1% dispase for 1 hr at 37°C, peeling the epidermal sheets from the dermis, and fixing in acetone for 10 min at 4°C. Prepared epidermal sheets were then blocked with normal goat serum (10%) for 20 min and incubated overnight with 1:100 diluted primary antibody against Langerhans cells (Rat anti-mouse Ia (eBioscience, cat# 14-5321) at 4°C. After washing, samples were incubated with 1:50 diluted goat anti-rat IgG-biotin (Cedarlane, cat# CLCC40015) at RT for 1 hr followed by another hour of incubation with 1:50 diluted FITC-conjugated streptavidin (Cedarlane, cat# CLCSA1001) at RT. Samples were then coverslipped with mount media and observed using fluorescence microscopy.

Embryonic T cell transplants

E16.5 day pregnant mice (*Id1*$^{+/+}$ and *Id1*$^{-/-}$) were sacrificed and fetal thymocytes were isolated from the thymic lobes of their embryos. 2×10^6 of above thymocytes (0.5 ml volume) were then transplanted through eye-vein injection to RAG1$^{-/-}$ (Balb/c; Jackson Laboratories) or RAG2$^{-/-}$ (SV129; Taconic) recipient mice (three recipient mice for each of the donor cell genotypes) that had had their back hair plucked 48 hr prior to transplantation. After six weeks, recipient mice were sacrificed and their skin was analyzed for the presence of Vγ3$^+$ T cells as described.

FACS analysis of γδ T cells

Thymocytes were harvested from thymus of adult mice (6–8 weeks) or embryonic day 17.5 fetuses. Cells were stained with 1:2000 diluted FITC-conjugated Vγ3 (BD Pharmingen, cat#553229) and 1:800 diluted PE-conjugated CD3 (BD Pharmingen, cat# 553063). Stained cells were analyzed in a fluorescence-activated cell sorter (FACS).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from sorted Vγ3$^+$ T cells using the RNeasy Mini kit (Qiagen) according to manufacturer's protocol and cDNA prepared by reverse transcription. Murine Id1 cDNA was amplified using the following primers:

Forward primer: 5′-ATGAAGGTCGCCAGTGGC-3′
Reverse primer: 5′-CCTCAGCGACACAAGATGCGATCG-3′

Quantitative RT-PCR was performed as previously described (Wiley et al., 2001) using the following primer pairs:

M-CCR10(R432): 5′-TCG TGC GAT GGC CAC AT-3′
M-CCR10(F372): 5′-CCA CGC TGG CTT CCT CTT C-3′

MCCR5(842F): 5′-GCC ATG CAG GCA ACA GAG A-3′
MCCR5(915R); 5′-TCT CCA ACA AAG GCA TAG ATG ACA-3′

MCCR4(847F); 5′-AAC AGA GCA GTG CGC ATG AT-3′
MCCR4(913R): 5′-CGT TGT ACG GCG TCC AGA A-3′

MCXCR4(R644): 5′-AGA CCC ACC ATT ATA TGC TGG AA-3′
MCXCR4(F568); 5′-AGG TAC ATC TGT GAC CGC CTT T-3′

MCCR7(F615); 5′-GGA CAC GCT GAG ATG CTC ACT-3′
MCCR7(R685): 5′-CCA TCT GGG CCA CTT GGA T-3′

MCXCR3(R103): 5′-CGC TCT CGT TTT CCC ATA A-3′
MCXCR3(F30): 5′-GCT AGA TGC TCG GAC TTT GC-3′

Results were normalized to expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH): forward, 5′-ACC CAC TCC TCC ACC TTT GA-3′; reverse, 5′-CAT ACC AGG AAA TGA GCT TGA CAA -3′) and then multiplied by 10^7. Results are shown as means of duplicate samples, which showed $<7\%$ variation between replicated specimens.

In vitro γδ T cell migration assay

Embryonic thymocytes were harvested at day E16.5, stained with Vγ3-FITC antibody, and sorted by FACS. Sorted cells were stained with Calcein (Molecular Probes cat# 3100) at 37°C for 30 min. The chemokine receptor ligand SDF-1/CXCL12 was diluted to 40–1000 ng/ml for use in migration assays and placed in the bottom chamber of a chemotaxis assay plate. Cells were added to the migration filter (3.2 mm diameter; 8 μm pore size; ChemoTx System, Neuro Probe) and incubated for 3 hr at 37°C. Following migration, cells were visualized using fluorescence microscopy and photographed for analysis.

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