

An eosinophil immune response characterizes the inflammatory skin disease observed in Tie-2 transgenic mice

Daniel Voskas,^{*,†,1,2} Yael Babichev,^{*,†,1} Ling S. Ling,^{*,†} Jennifer Alami,[†] Yuval Shaked,[†] Robert S. Kerbel,^{*,†,‡} Brian Ciruna,^{§,¶} and Daniel J. Dumont^{*,†,‡,3}

Departments of ^{*}Medical Biophysics and [§]Molecular & Medical Genetics, University of Toronto, Toronto, Ontario, Canada; [†]Division of Molecular and Cellular Biology, Sunnybrook Research Institute, Toronto, Ontario, Canada; [‡]Toronto-Sunnybrook Regional Cancer Centre, Toronto, Ontario, Canada; and [¶]Department of Developmental & Stem Cell Biology, Hospital for Sick Children, Toronto, Ontario, Canada

Abstract: Although mouse models of inflammatory skin diseases such as psoriasis and atopic dermatitis fail to completely phenocopy disease in humans, they provide invaluable tools to examine the molecular and cellular mechanisms responsible for the epidermal hyperplasia, inflammation, and excess angiogenesis observed in human disease. We have previously characterized a tyrosine kinase with Ig and epidermal growth factor homology domain-2 (Tie-2) transgenic mouse model of an inflammatory skin disease exhibiting these features. More specifically, we demonstrated that the inflammatory component consisted of increased infiltration of CD3-positive T lymphocytes and mast cells in the skin. Here, we further characterize the inflammatory component in the blood and skin of Tie-2 transgenic mice at cellular and molecular levels. We observed increased numbers of CD3-positive T lymphocytes in the blood and increased infiltration of eosinophils in the skin. Furthermore, we characterized cytokine protein and gene expression in the blood and skin, respectively, and observed the deregulated expression of cytokines associated with Th1 and eosinophil immune responses. Interestingly, treatment of Tie-2 transgenic mice with anti-CD4 antibody appeared to resolve aspects of inflammation but did not resolve epidermal hyperplasia, suggesting an important role for eosinophils in mediating the inflammatory skin disease observed in Tie-2 transgenic mice. *J. Leukoc. Biol.* 84: 000–000; 2008.

Key Words: angiopoietin · eotaxin · antibody array · blood · inflammation

INTRODUCTION

Inflammatory skin diseases are generally characterized by deregulated epithelial, immune, and vascular systems. Two common inflammatory skin diseases include psoriasis and atopic dermatitis (AD). More recent animal models targeting cells or

molecules specifically within the epithelial, immune, or vascular compartment have allowed us to learn more about the deregulated cellular processes and molecular mechanisms responsible for skin diseases. Previously, we identified a role for tyrosine kinase with Ig and epidermal growth factor homology domain-2 (Tie-2) in mediating an inflammatory skin disease in mice resembling psoriasis [1]. Tie-2 double-transgenic (DT) mice exhibited several features associated with a psoriasis-like phenotype, including epidermal hyperplasia, inflammation, and excess angiogenesis [1].

Tie-2 is a receptor tyrosine kinase predominantly expressed on the surface of endothelial cells and activated by the angiopoietin (Ang) family of ligands, which include Ang-1, Ang-2, and Ang-3 in mice. Importantly, Tie-2 is also expressed in nonendothelial cells, including hematopoietic stem cells (HSCs) [2–6]. In the embryo, Tie-2 plays an essential role in the development and maintenance of the cardiovascular system. Tie-2 knockout mice exhibit lethality by Embryonic Day 10.5 as a result of decreased maintenance of endocardial cells in the heart and endothelial cells in the blood vasculature [7–9]. Not surprisingly, Tie-2 also plays a positive role in the maintenance of endothelial cells in the lymphatic vasculature [10]. Consistent with its role in the maintenance of the cardiovascular system in the embryo, Tie-2 also plays a critical role in the maintenance of the hematopoietic system in the adult [5, 6, 11]. However, unlike its role in the blood and lymphatic vasculature, the role for Tie-2 in the maintenance of hematopoietic cells does not appear to be dependent on survival [10].

Tie-2 additionally modulates inflammation in the adult. Upon activation by Ang-1, Tie-2 down-regulates inflammatory events such as endothelial cell permeability, endothelial and immune cell adhesion, and cytokine expression [12–16]. For example, Ang-1 down-regulates the expression of cell adhesion

¹ These authors contributed equally to this work.

² Current address: Department of Developmental & Stem Cell Biology, Hospital for Sick Children, Toronto, Ontario, Canada.

³ Correspondence: Sunnybrook Research Institute, 2075 Bayview Avenue, Research Building, S-227, Toronto, Ontario, Canada, M4N 3M5. E-mail: dan.dumont@sri.ca

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molecules such as E-selectin, ICAM-1, and VCAM-1 and cytokines such as IL-8 in endothelial cells [15, 16]. In support of an anti-inflammatory role for Ang-1, inflammatory cytokines such as IL-1 β and TNF- α down-regulate the expression of Ang-1 in endothelial cells and/or smooth muscle cells [17–19]. TNF- α may also up-regulate the expression of Ang-1 in stromal cells such as synovial fibroblasts; however, this effect may be specific to pathological situations such as arthritis [20–23]. In contrast, upon activation by Ang-2, Tie-2 up-regulates inflammatory events, such as permeability [24–28]. In support of a proinflammatory role for Ang-2, inflammatory mediators such as TNF- α and thrombin up-regulate the expression of Ang-2 in endothelial cells [20, 29, 30]. Furthermore, unlike Ang-1, which counteracts inflammatory events such as permeability in situations where inflammatory or stress mediators are already present [12–16, 31, 32], Ang-2 potentiates permeability in such situations [26–28]. However, Ang-2 may also counteract permeability in situations such as cardiac allograft vasculopathies [25, 33, 34].

Here, we further characterize the psoriasis-like phenotype described previously in Tie-2 DT mice [1]. We demonstrate that deregulation of immune cells and cytokines in the blood and/or skin of Tie-2 DT mice is characteristic with deregulation of eosinophil and Th1 lymphocyte immune responses.

MATERIALS AND METHODS

Generation, genotyping, and treatment of transgenic mice

Construction of the driver transgene pTek-tTA and the responder transgene pTet^{OS}-Tek has been described previously [35]. CD1 (outbred) transgenic mouse lines were maintained and genotyped as described previously [1]. DT and wild-type (WT) mice were treated with 5 mg per kg mouse of anti-CD4 antibody (GK1.5; eBioscience, San Diego, CA, USA) or PBS (Sigma Chemical Co., St. Louis, MO, USA) by i.p. injection every 2nd day for a period of 14 days. All adult mice used in these experiments were 4–6 months old, and unless otherwise specified, mice were generated, treated, and examined at least in triplicate ($n \geq 3$ per group).

Gross morphology and histology

Mice were photographed using a Cybershot digital camera (Sony, Japan). Spleen and skin tissues, isolated from ears or approximately the same anatomical site on the back of all DT mice and WT littermates, were fixed in 4% paraformaldehyde (pH 7.4) solution for 4 h at room temperature, embedded in paraffin, sectioned (6 μ m) on SuperFrost glass slides, and processed and stained for H&E or Luna using standard techniques. Slides were analyzed on a Zeiss Axioplan 2 light microscope (Carl Zeiss, Thornwood, NY, USA), and photos were processed using Adobe Photoshop v6.0 (Adobe Systems, San Jose, CA, USA).

Quantitative analysis of eosinophil numbers

Eosinophil numbers were measured in sections from equivalent areas of DT and WT adult skin stained with Luna using a Zeiss Axioplan 2 light microscope and Adobe Photoshop v6.0. All measurements were made from three high-powered fields per section, each section represented one animal, and three animals were analyzed per genotype ($n=3$). Statistical significance was assessed using Student's *t*-test; $P < 0.05$.

Flow cytometry

Peripheral blood or spleen samples were obtained from DT mice and WT littermates. Blood cells were treated with 1 $\times$ RBC lysis buffer (eBioscience), washed with flow cytometry staining (FCS) buffer (eBioscience), and incubated

with lineage-specific mAb, including allophycocyanin-conjugated anti-mouse B220, allophycocyanin-conjugated anti-mouse CD3 ϵ , PE-conjugated anti-mouse CD11b (membrane-activated complex 1), PE-conjugated anti-mouse CD41, PE-conjugated anti-mouse Gr-1 (Ly-6G), and PE-conjugated anti-mouse Ter-119 (Ly-76) antibodies (eBioscience) for 30 min at 4°C. Blood cells were then washed, suspended in FCS buffer containing propidium iodide (PI; 1 μ g/mL, Sigma Chemical Co.) to label dead cells, sorted using the FACS-Calibur system (Becton Dickinson, San Jose, CA, USA), and analyzed using CellQuest Pro software (Becton Dickinson). Spleen cells were similarly treated but incubated with PE-conjugated anti-mouse CD4 antibody (eBioscience). For all experiments, at least 5000 events were analyzed, and dead cells were excluded by PI staining and forward-scatter. As negative controls, aliquots were incubated with irrelevant isotype-matched antibodies (eBioscience). Data obtained from the flow cytometry analyses are presented as mean $\pm$ SD. Statistical significance was assessed using Student's *t*-test; $P < 0.05$.

Antibody arrays

Peripheral blood samples were obtained from DT mice and WT littermates, centrifuged at 9300 rpm for 10 min to precipitate insoluble material, and analyzed as described in the TranSignal Mouse Cytokine Antibody Array 2.0 kit (Panomics, Redwood City, CA, USA).

ELISAs

Peripheral blood samples were obtained from DT mice and WT littermates, centrifuged at 9300 rpm for 10 min to precipitate insoluble material, and analyzed for eotaxin (CCL-11), IL-12p40 (IL-12p40), thymus and activation-regulated chemokine (TARC; CCL-17), and soluble TNF receptor I (sTNFRI) as described in the Quantikine M kits (R&D Systems, Minneapolis, MN, USA). ODs were measured at 450 nm using the Bio-Rad Model 550 microplate spectrophotometer (Bio-Rad Laboratories Inc., Hercules, CA, USA). The concentrations were calculated from a standard curve generated by a curve-fitting program. Data obtained from the ELISA assays are presented as mean $\pm$ SD. Statistical significance was assessed using Student's *t*-test; $P < 0.05$.

Gene expression

Skin tissues were obtained from DT mice and WT littermates. Total RNA was isolated using TRI reagent (Sigma Chemical Co.), and RT for PCR was carried out using the first-strand cDNA synthesis kit (Clontech, Palo Alto, CA, USA). PCR was carried out by amplifying cDNA templates that were undiluted or diluted 1 in 10, 1 in 100, or 1 in 1000. Mouse primers for eotaxin-1, IFN- γ , IL-2, IL-4, IL-5, IL-6, IL-12p40, RANTES, TARC, ICAM-1, ICAM-2, PECAM-1, E-selectin, P-selectin, and VCAM-1 have been described previously [36–42]. Primers for *Tie-2* were designed to amplify mouse and human sequences (327 bp regions) and cross intron/exon boundaries; *Tie-2*, forward primer 5'-GAT TTT GGA TTG TCC CGA GGT CAA G-3'; *Tie-2*, reverse primer 5'-CAC CAA TAT CTG GGC AAA TGA TGG-3'. Primers for β -actin were used as controls; β -actin, forward primer 5'-CTG GGC CGC TCT AGG CAC CAA-3'; β -actin, reverse primer 5'-CTC TTT GAT GTC ACG CAC GAT TTC-3'.

RESULTS

Tie-2 DT mice exhibit splenomegaly and increased numbers of CD3-positive T lymphocytes in the blood

Recently, we demonstrated that the inflammatory skin disease observed in Tie-2 DT mice is characterized by epidermal hyperplasia, inflammation, and excess angiogenesis [1]. At a gross morphological level, this phenotype was observed as an erythema of the skin around the ears, eyes, snout, head, neck, and tail. At a cellular level, the inflammation was observed as an increase in staining for CD3-positive T cells and toluidine blue-stained mast cells in the skin. Consistent with a proinflammatory response, Tie-2 DT mice also exhibit splenomegaly (**Fig. 1a**). Low magnification images of H&E-stained spleen

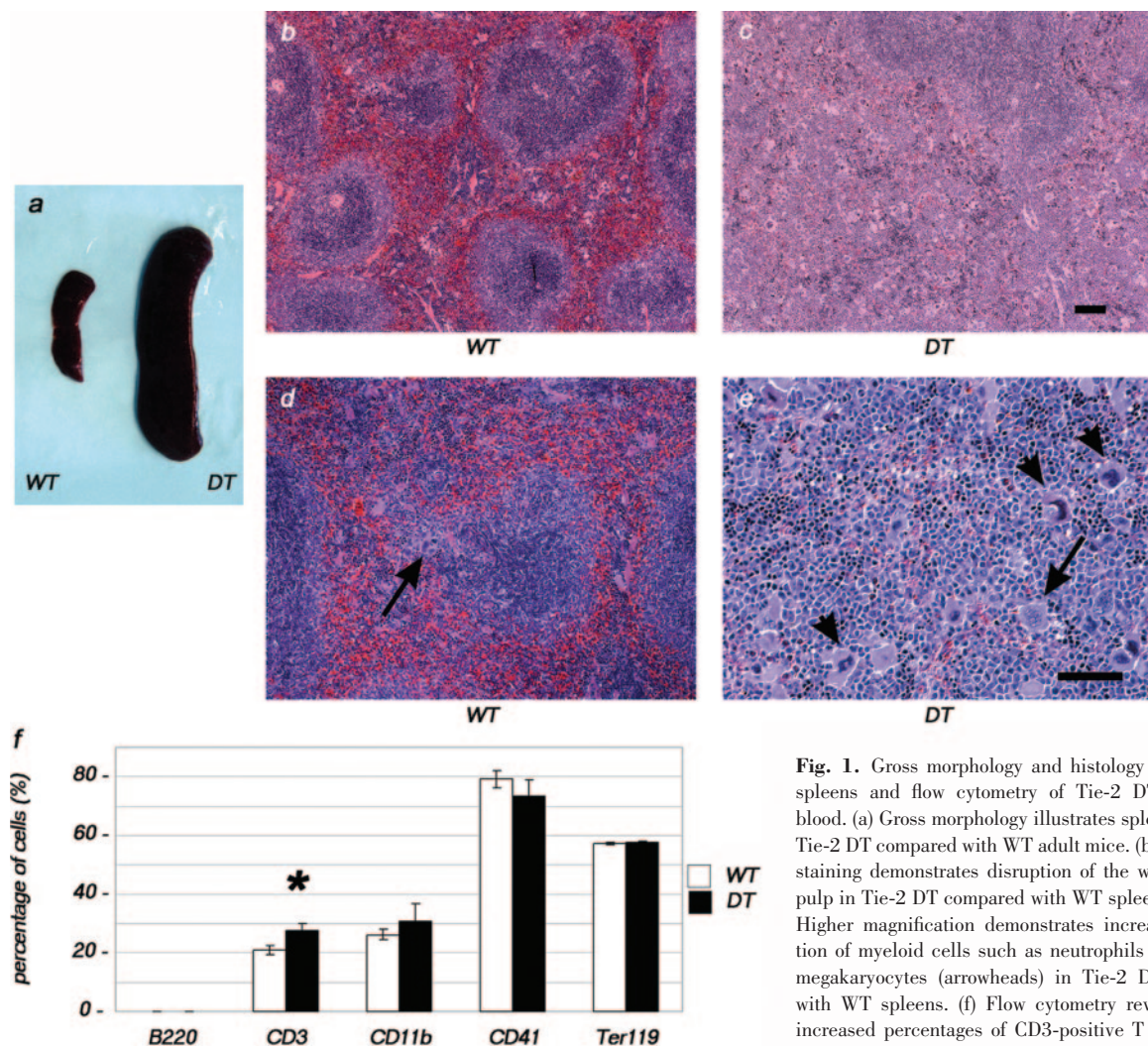


Fig. 1. Gross morphology and histology of Tie-2 DT spleens and flow cytometry of Tie-2 DT peripheral blood. (a) Gross morphology illustrates splenomegaly in Tie-2 DT compared with WT adult mice. (b and c) H&E staining demonstrates disruption of the white and red pulp in Tie-2 DT compared with WT spleens. (d and e) Higher magnification demonstrates increased infiltration of myeloid cells such as neutrophils (arrows) and megakaryocytes (arrowheads) in Tie-2 DT compared with WT spleens. (f) Flow cytometry reveals slightly increased percentages of CD3-positive T lymphocytes but unchanged percentages of B220-positive B lymphocytes.

cytes, CD11b-positive myeloid cells, CD41-positive megakaryocytes, and Ter-119-positive RBCs in the blood of Tie-2 DT mice compared with WT littermates (*, $P < 0.05$, Student's *t*-test). Original scale bar, 100 μ m.

sections revealed disruption of the normal red and white pulp architecture in Tie-2 DT compared with WT mice (Fig. 1, b and c). High magnification images revealed increased infiltration of white blood cells such as myeloid cells and megakaryocytes in the red pulp of DT compared with WT mice (Fig. 1, d and e). To further characterize the inflammatory response observed in Tie-2 DT mice, we profiled numbers of immune cells in Tie-2 DT and WT peripheral blood by flow cytometry. We used anti-B220, -CD3, -CD11b, -CD41, and -Ter-119 antibodies as markers of B lymphocytes, T lymphocytes, myeloid cells, megakaryocytes, and erythrocytes, respectively. Consistent with increased numbers of CD3-positive T cells in the skin [1], we observed moderately increased numbers of CD3-positive T cells in the blood of Tie-2 DT mice compared with WT littermates (Fig. 1f). Surprisingly, numbers of other immune cells did not change significantly in the blood.

Maintenance of the skin disease in Tie-2 DT mice is not CD4-positive T lymphocyte-dependent

Previously, we also demonstrated that the inflammatory skin disease observed in Tie-2 DT mice was completely Tie-2-

dependent [1]. Treatment of Tie-2 DT mice with doxycycline (Dox), an inhibitor of Tie-2 transgene expression, resolved the epidermal hyperplasia, inflammation, and excess angiogenesis observed in the skin. Tie-2 expression and phosphorylation were decreased in Dox-treated mice. Surprisingly, cyclosporine A (CsA), a known suppressor of T cell activity, also resolved the epidermal hyperplasia, inflammation, and excess angiogenesis. Although Tie-2 expression was unaltered, Tie-2 phosphorylation was decreased in CsA-treated mice. Roles for CsA have previously been established in suppressing inflammatory responses; however, roles have also been described in inhibiting angiogenesis [43–45]. To more strictly assess the contribution of T cells to the development or maintenance of the skin disease observed in Tie-2 DT mice, we used an anti-CD4 antibody (GK1.5) known to deplete numbers of CD4-positive T cells in vivo [46]. CD4-positive T cells are known to contribute to the maintenance of inflammatory skin diseases such as psoriasis and AD [47, 48]. After 2 weeks of treatment with anti-CD4 antibody, erythema of the skin around the ears, eyes, snout, head, neck, and tail improved in treated compared with untreated Tie-2 DT mice (Fig. 2, a and b). However, epidermal hyperplasia did not resolve in treated compared with

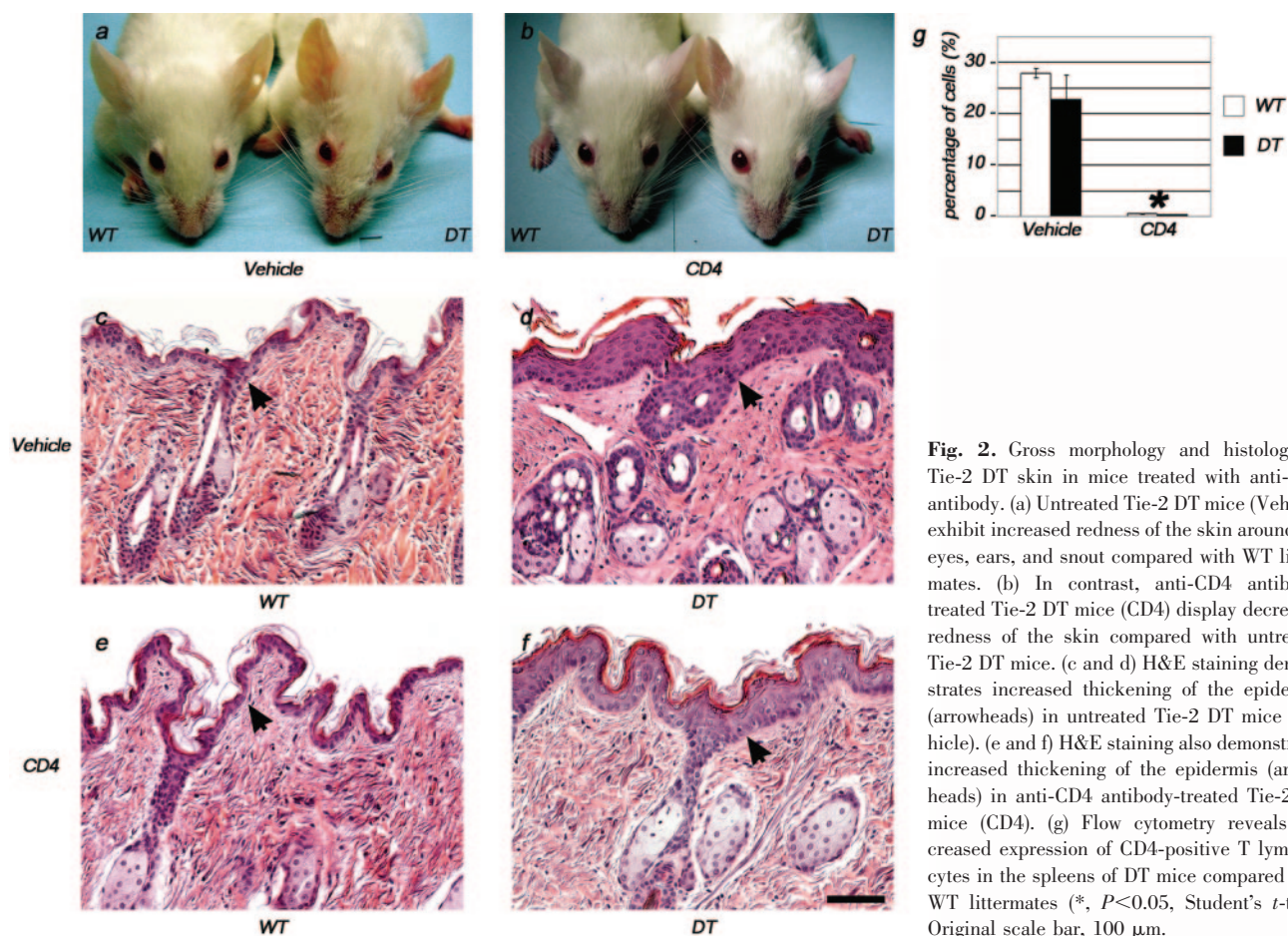


Fig. 2. Gross morphology and histology of Tie-2 DT skin in mice treated with anti-CD4 antibody. (a) Untreated Tie-2 DT mice (Vehicle) exhibit increased redness of the skin around the eyes, ears, and snout compared with WT littermates. (b) In contrast, anti-CD4 antibody-treated Tie-2 DT mice (CD4) display decreased redness of the skin compared with untreated Tie-2 DT mice. (c and d) H&E staining demonstrates increased thickening of the epidermis (arrowheads) in untreated Tie-2 DT mice (Vehicle). (e and f) H&E staining also demonstrates increased thickening of the epidermis (arrowheads) in anti-CD4 antibody-treated Tie-2 DT mice (CD4). (g) Flow cytometry reveals decreased expression of CD4-positive T lymphocytes in the spleens of DT mice compared with WT littermates (*, $P < 0.05$, Student's t -test). Original scale bar, 100 μm .

untreated Tie-2 DT mice (Fig. 2, d and f). We confirmed reduced numbers of CD4-positive T lymphocytes in treated Tie-2 DT mice and WT littermates by flow cytometry (Fig. 2g).

Tie-2 DT mice exhibit increased protein expression of cytokines associated with eosinophil immune responses in the blood

Complete resolution of the skin disease in CsA-treated Tie-2 DT mice but lack of resolution in anti-CD4 antibody-treated Tie-2 DT mice suggest that another CsA-sensitive immune cell may be responsible for development of the skin disease. As mentioned above, we observed increased infiltration of toluidine blue-stained mast cells in the skin of Tie-2 DT mice compared with WT littermates. This led us to believe that mast cells or perhaps other immune cells such as eosinophils or neutrophils may be responsible for the disease. In an attempt to further characterize the inflammatory component by identifying potential mediators of the skin disease, we profiled cytokine protein expression in the blood of Tie-2 DT and WT mice by antibody array analysis (Fig. 3a). We observed markedly increased expression of eotaxin-1 (CCL11), IL-12p40, and sTNFRI (Fig. 3, b and c), moderately increased expression of KC (CXCL1), MCP-1 (CCL2), MCP-5 (CCL12), MIP-1 α (CCL3), MIP-2 (CXCL2), MIP-3 β (CCL19), RANTES (CCL5), TIMP-1, and TPO, and slightly increased expression of IL-4, IL-9, IL-12p70, leptin, TARC (CCL17), and VEGF (Fig. 3, d and e). To confirm increased expression of the markedly regulated cytokines, we profiled the expression of

eotaxin-1, IL-12p40, and sTNFRI by ELISAs. As increased expression of eotaxin-1 and TARC strongly correlate with inflammatory skin diseases such as AD [49–54], we also profiled the expression of TARC by ELISA. We observed significantly increased expression of eotaxin-1, IL-12p40, and TARC but not sTNFRI in the blood of Tie-2 DT mice compared with WT littermates (Fig. 3f).

Tie-2 DT mice exhibit increased mRNA expression of cytokines associated with eosinophil and Th1 lymphocyte immune responses in the skin

To further characterize the contribution of eosinophils, T lymphocytes, and endothelial cells to the development of the skin disease observed in Tie-2 DT mice, we profiled mRNA expression of several cytokines associated with eosinophil, Th1, and Th2 immune responses and molecules associated with endothelial and immune cell adhesion in the skin by semi-quantitative (sq)PCR. In agreement with antibody array and/or ELISA data, we observed increased gene expression of chemokines associated with eosinophil responses in Tie-2 DT compared with WT skin, including *eotaxin-1* and *RANTES* (Fig. 4a). Also in agreement with antibody array and ELISA data, we observed increased gene expression of TARC (Fig. 4a). Interestingly, we observed increased gene expression of Th1-type cytokines (*IFN- γ* , *IL-2*, and *IL-12-p40*) but not Th2-type cytokines (*IL-4*, *IL-5*, and *IL-6*) in Tie-2 DT compared with WT

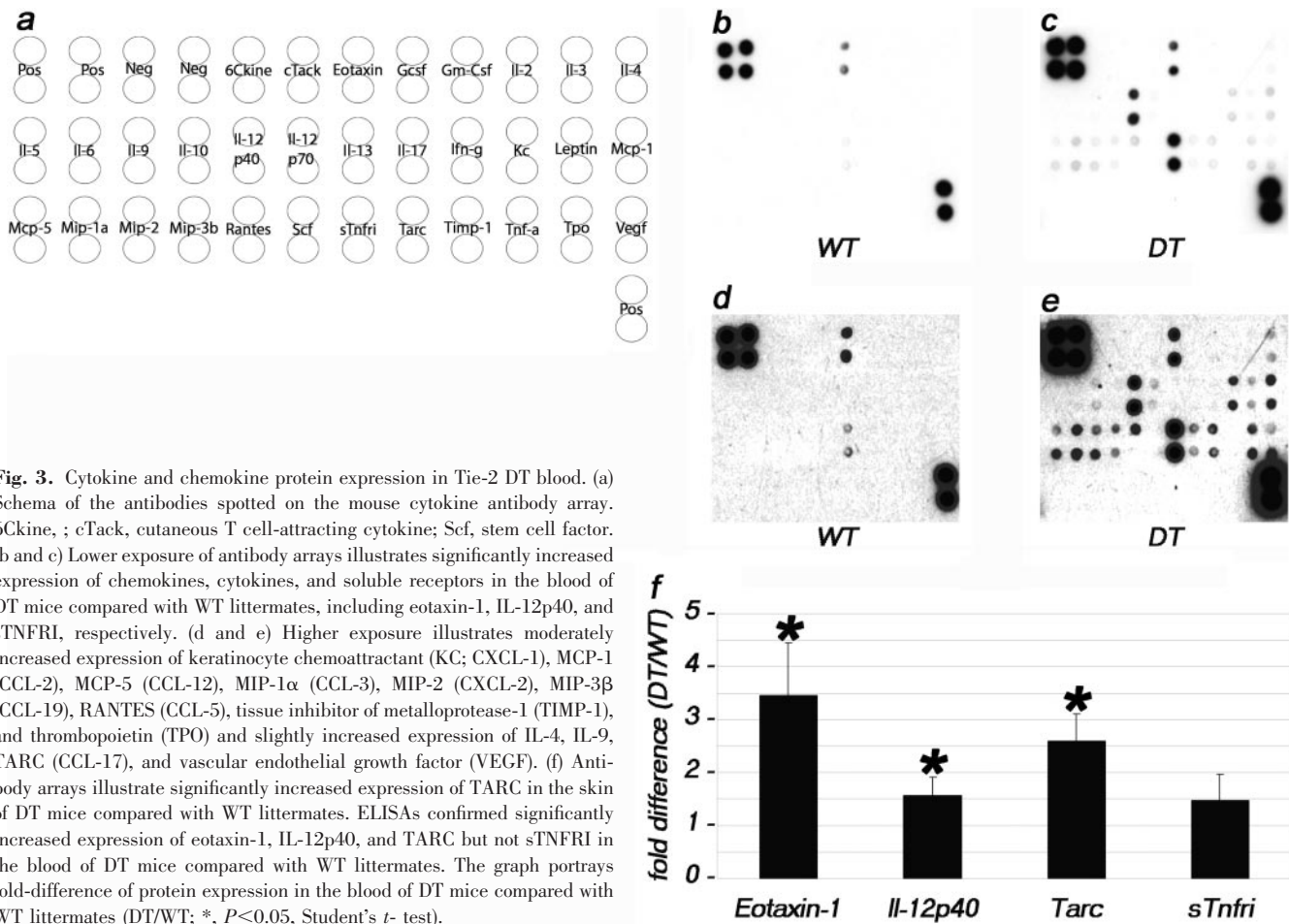


Fig. 3. Cytokine and chemokine protein expression in Tie-2 DT blood. (a) Schema of the antibodies spotted on the mouse cytokine antibody array. 6Ckine, ; cTack, cutaneous T cell-attracting cytokine; Scl, stem cell factor. (b and c) Lower exposure of antibody arrays illustrates significantly increased expression of chemokines, cytokines, and soluble receptors in the blood of DT mice compared with WT littermates, including eotaxin-1, IL-12p40, and sTNFRI, respectively. (d and e) Higher exposure illustrates moderately increased expression of keratinocyte chemoattractant (KC; CXCL-1), MCP-1 (CCL-2), MCP-5 (CCL-12), MIP-1 α (CCL-3), MIP-2 (CXCL-2), MIP-3 β (CCL-19), RANTES (CCL-5), tissue inhibitor of metalloproteinase-1 (TIMP-1), and thrombopoietin (TPO) and slightly increased expression of IL-4, IL-9, TARC (CCL-17), and vascular endothelial growth factor (VEGF). (f) Antibody arrays illustrate significantly increased expression of TARC in the skin of DT mice compared with WT littermates. ELISAs confirmed significantly increased expression of eotaxin-1, IL-12p40, and TARC but not sTNFRI in the blood of DT mice compared with WT littermates. The graph portrays fold-difference of protein expression in the blood of DT mice compared with WT littermates (DT/WT; *, $P < 0.05$, Student's t -test).

skin (Fig. 4, b and c). Furthermore, we observed increased gene expression of molecules associated with endothelial and immune cell adhesion, including *ICAM-1*, *ICAM-2*, *PECAM-1*, *E-selectin*, *P-selectin*, and *VCAM-1* (Fig. 4d).

Tie-2 DT mice exhibit increased numbers of eosinophils in the skin

Given the increased expression of chemokines associated with eosinophil immune responses in the blood and skin of Tie-2 DT mice, we performed and analyzed eosinophil staining in the skin of Tie-2 DT mice. Increased infiltration of Luna-stained eosinophils was observed in the dermis of skin isolated from the back (Fig. 5, a and b) and ear (Fig. 5, c and d) of Tie-2 DT mice compared with WT littermates. Luna-stained eosinophils display a pink cytoplasm. Quantitative analysis of eosinophil numbers revealed significantly increased numbers in Tie-2 DT skin compared with WT littermates (Fig. 5e).

DISCUSSION

In this report, we further characterize deregulation of the immune system in Tie-2 DT mice, which exhibit an inflammatory skin disease. In a previous report, we demonstrated increased infiltration of CD3-positive T cells and toluidine blue-stained mast cells in the skin of Tie-2 DT mice [1]. Here, we further demonstrate increased numbers of CD3-positive T cells in the blood, increased

protein expression of chemokines associated with eosinophil responses in the blood, increased gene expression of chemokines associated with eosinophil and Th1 responses in the skin, and finally, increased infiltration of Luna-stained eosinophils in the skin of Tie-2 DT mice. Collectively, these immune responses provide evidence for an eosinophil-mediated inflammatory skin disease in Tie-2 DT mice.

Roles for eosinophils and mast cells have been well described in allergic inflammatory diseases such as asthma [55, 56]; however, roles are less well defined in inflammatory skin diseases such as AD. Eosinophils and mast cells are granulocytes, which upon activation, may mediate innate or adaptive immune responses in the skin. In mediating innate responses, eosinophils and mast cells secrete granule-derived mediators such as major basic protein and lipid-derived mediators such as leukotrienes and platelet activating factor [55, 56]. In mediating adaptive responses, eosinophils and mast cells secrete various cytokines and chemokines known to attract and/or activate endothelial cells, monocytes, and lymphocytes [55, 56]. Several cytokines and chemokines known to act up- or down-stream of eosinophils are increased in Tie-2 DT mice. For example, eotaxin-1 and RANTES were up-regulated in Tie-2 DT blood and/or skin. Notably, eotaxin-1 and RANTES are also up-regulated in AD patients [49, 50, 57]. Eotaxin-1 and RANTES are secreted by T lymphocytes, dermal fibroblasts, and/or epidermal keratinocytes and act to recruit eosinophils to the skin [49, 58, 59]. Upon activation, eosinophils

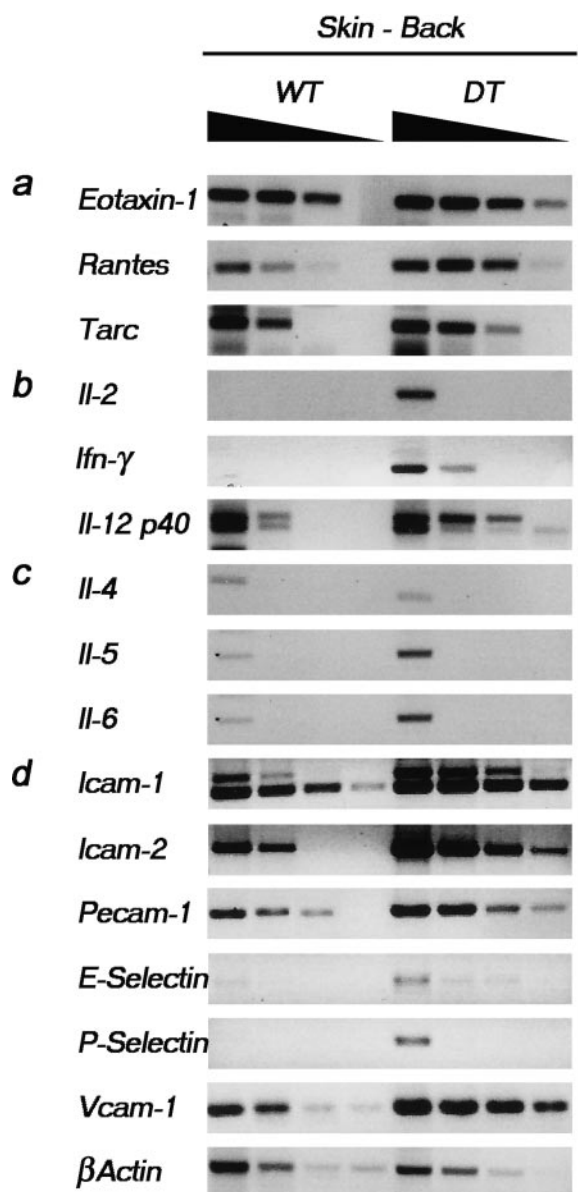


Fig. 4. Cytokine and chemokine gene expression in Tie-2 DT skin. (a and b) sqPCR reveals increased gene expression of Th1-specific cytokines (*IFN- γ* , *IL-2*, and *IL-12-p40*) but not Th2-type cytokines (*IL-4*, *IL-5*, and *IL-6*) in the skin of Tie-2 DT mice compared with WT littermates. (c and d) sqPCRs also reveals increased gene expression of eosinophil-specific chemokines (*eotaxin-1*, *RANTES*, and *TARC*) and endothelial-specific adhesion molecules (*ICAM-1*, *ICAM-2*, *PECAM-1*, *E-selectin*, *P-selectin*, and *VCAM-1*). Gene expression of β -actin demonstrates the presence of amplifiable, total RNA in all samples. cDNA templates for PCR were undiluted or diluted 1/10, 1/100, and 1/1000, as indicated by the wedge.

may secrete Th2- or Th1-type cytokines; however, the secretion of these cytokines is influenced by signals from cells in the surrounding environment [56]. Interestingly, previous studies have demonstrated a biphasic, CD4-positive Th response in AD patients, where Th2-type cytokines *IL-4*, *IL-5*, and *IL-13* predominate in the skin during an earlier acute phase of the disease, and Th1-type cytokines *IFN- γ* , *IL-5*, and *IL-12* predominate during a later chronic phase [60–64]. Subsequent studies have suggested a role for eosinophils in mediating this switch; however, it remains unclear whether this effect is

mediated by eosinophils in the blood or skin [62, 65]. Similar to the Th1 response observed in the skin of AD patients, *IFN- γ* and *IL-12p40* were up-regulated in Tie-2 DT skin. Although the possibility of a biphasic, CD4-positive Th lymphocyte response in Tie-2 DT mice is intriguing, different CD4-positive T cell subsets must be analyzed in the blood and skin of Tie-2 DT mice at early and late stages of disease to validate this hypothesis. Furthermore, protein expression of eotaxin-1 and *IL-12p40* was confirmed in the blood, and mRNA expression was confirmed in the skin; however, protein expression also needs to be confirmed in the skin. Finally, future studies targeting eosinophils are required to assess the role of these cells on the development and/or maintenance of the inflammatory skin disease in Tie-2 DT mice.

Surprisingly, Tie-2 expression was demonstrated recently in eosinophils isolated from healthy human donors [66]. In addition, Ang-1 and Ang-2 mediate Tie-2-dependent eosinophil chemotaxis in vitro [66]. Thus, the receptor may be cell-autonomously responsible for the eosinophil response observed in Tie-2 DT mice. Moreover, Ang-1 and Ang-2 may mediate eosinophil recruitment and/or activation in the skin of these mice. Future studies analyzing eosinophil responses to Ang-1 and/or Ang-2 in vitro and in vivo are required to assess the potential pro- or anti-inflammatory effects of these ligands in disease. Protein analyses, for example, may reveal cytokine profiles that support Th2 and/or Th1 lymphocyte responses.

Important roles for T lymphocytes have been demonstrated in inflammatory skin diseases such as psoriasis and AD [47, 48]. For example, CD8-positive T cytotoxic 1 and CD4-positive Th1 lymphocytes in the skin characterize psoriasis, whereas CD4-positive Th2 lymphocytes in the blood and Th1 lymphocytes in the skin characterize chronic stages of AD [47, 48]. Although the suppression of CD4-positive T did not resolve all aspects of the skin disease observed in Tie-2 DT mice, it appeared to partially resolve inflammation. Thus, CD4-positive lymphocytes are important at least for the maintenance of inflammation. Several cytokines and chemokines known to act up- or down-stream of CD4-positive lymphocytes are increased in Tie-2 DT mice. For example, TARC was up-regulated in Tie-2 DT blood and skin, and *IFN- γ* , *IL-2*, and *IL-12p40* were up-regulated in Tie-2 DT skin. Not surprisingly, TARC and *IL-12p40* are up-regulated in AD patients, and TARC strongly correlates with severity of disease [49–54]. Th2-type chemokines such as macrophage-derived chemokine and TARC are secreted by many cells, including dendritic cells (DC) [67, 68], eosinophils [69], keratinocytes [70, 71], fibroblasts [72], and endothelial cells [73], and act to recruit Th2 lymphocytes to the skin [52–54]. Upon activation, Th2 lymphocytes may subsequently secrete chemokines such as eotaxin-1, RANTES, and MCP-4, which recruit and activate immune cells such as eosinophils and DC [49–51]. As discussed above, in addition to the differing temporal roles for Th2 and Th1 lymphocytes in the skin of AD patients during acute and chronic stages of disease, there appear to be differing spatial roles for Th2 and Th1 lymphocytes in the blood and skin of patients during the chronic stage of disease [74]. Interestingly, protein and gene expression profiles of cytokines in the blood and skin, respectively, suggest that different CD4-positive T cell subtypes may play different roles in the blood and skin of Tie-2 DT mice. For

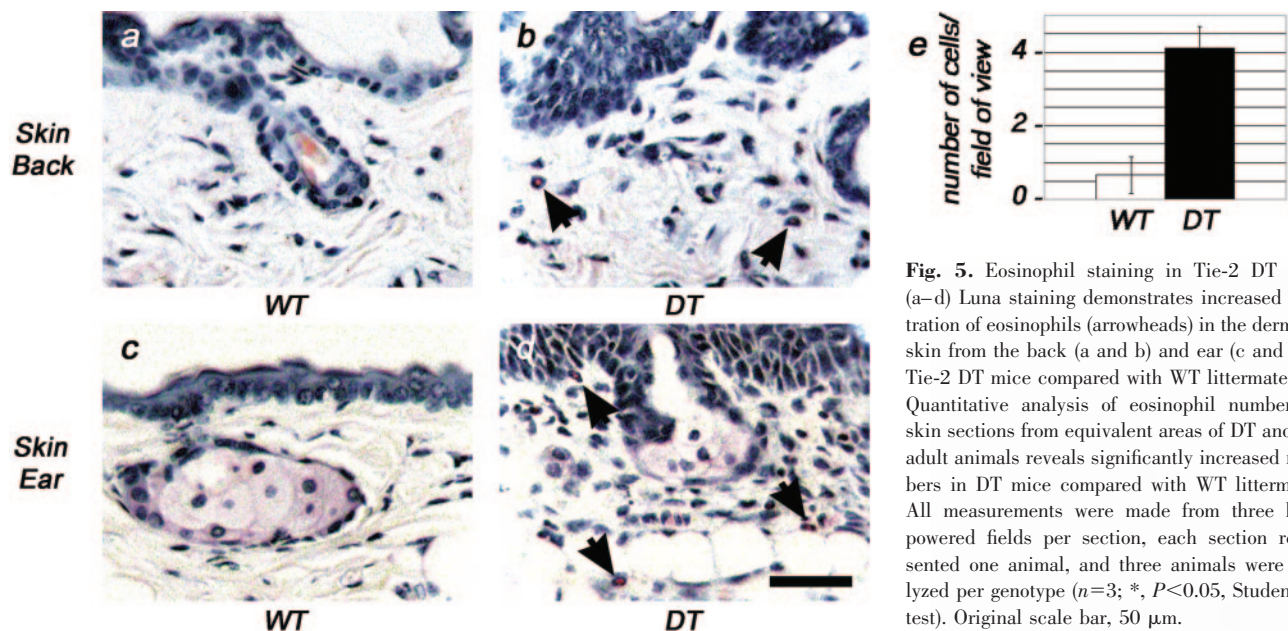


Fig. 5. Eosinophil staining in Tie-2 DT skin. (a–d) Luna staining demonstrates increased infiltration of eosinophils (arrowheads) in the dermis of skin from the back (a and b) and ear (c and d) of Tie-2 DT mice compared with WT littermates. (e) Quantitative analysis of eosinophil numbers in skin sections from equivalent areas of DT and WT adult animals reveals significantly increased numbers in DT mice compared with WT littermates. All measurements were made from three high-powered fields per section, each section represented one animal, and three animals were analyzed per genotype ($n=3$; *, $P<0.05$, Student's t -test). Original scale bar, 50 μm .

example, the expression of Th1-type cytokines such as IL-2, IFN- γ , and TNF- α appeared unaltered, whereas the expression of Th2-type cytokines such as IL-4 and IL-9 appeared slightly elevated in the blood of DT mice according to antibody array data. In contrast, the expression of IL-2, IFN- γ , and TNF- α appeared elevated, whereas the expression of IL-4, IL-5, and IL-6 appeared unaltered in the skin of DT mice according to sqPCR data. As discussed above, different CD4-positive T cell subsets need to be analyzed in the blood and skin of Tie-2 DT mice at late stages of disease to validate this hypothesis, and the expression data need to be confirmed by more specific protein analyses such as ELISAs for proteins in the blood and westerns for proteins in the skin; however, this predominant misexpression of Th2-type cytokines in the blood and Th1-type cytokines in skin of Tie-2 DT mice may be similar to the T lymphocyte responses observed in AD patients.

Roles for myeloid cells such as DC and macrophages have also been described in inflammatory skin diseases [75–77]. DC and macrophages are professional APCs, which express chemokine receptors such as CCR-2, CCR-5, and CCR-6 and are recruited by chemokines such as MCP-1 (CCL-2), MCP-4 (CCL-13), and MCP-5 (CCL-12) and MIP-1 α (CCL-3), MIP-1 β (CCL-4), MIP-2 (CXCL-2), MIP-3 α (CCL-20), MIP-3 β (CCL-19), and RANTES (CCL-5) to sites of inflammation [78]. In contrast to its role as a negative modulator of inflammatory processes such as endothelial cell permeability [12–16, 31, 32], Ang-1 mediates increased gene expression of cytokines such as IL-1 β and TNF- α and chemokines such as KC, MCP-1, MIP-1 α , MIP-1 β , MIP-2, MIP-3 α , and MIP-3 β in a rat aortic ring culture system [79]. Previously, we demonstrated increased Ang-1 protein expression in the skin of Tie-2 DT mice [1] and currently demonstrate moderately increased protein expression of several of these chemokines in the blood of Tie-2 DT mice, including KC, MCP-1, MCP-5, MIP-1 α , MIP-2, MIP-3 β , and RANTES. Not surprisingly, Ang-1 and Ang-2 mediate well-described Tie-2-dependent effects in monocytes [80–83] and neutrophils [84–86]. Thus, future studies targeting monocytes and neutrophils are also required to assess

the role of these cells on the development and/or maintenance of the inflammatory skin disease in Tie-2 DT mice.

In this work, we further characterize the cytokine and chemokine profile in the blood and skin of Tie-2 DT adult mice and demonstrate the molecular deregulation of eosinophil and Th1 immune responses. We have described previously increased T cell and mast cell infiltration in the skin of Tie-2 DT mice and here, additionally describe increased eosinophil infiltration. We also demonstrate increased endothelial and immune cell adhesion molecule expression in the skin of Tie-2 DT mice, suggesting an important role for activated endothelial cells in mediating immune cell infiltration. Future studies will be required to assess the importance of particular immune cell types in the development and/or maintenance of the inflammatory skin disease in Tie-2 DT mice. For example, targeting eosinophils may be more effective in resolving inflammation and epidermal hyperplasia than CD4-positive lymphocytes. Alternatively, targeting eosinophils and CD4-positive lymphocytes may be necessary. Furthermore, as Tie-2 is expressed in KCs [1], HSCs [2, 4–6], and several differentiated hematopoietic cell types, including eosinophils [66], mast cells [87], monocytes [80, 82, 83], and neutrophils [84–86], the role for this receptor in mediating the inflammatory skin disease observed in Tie-2 DT mice may be complex. In fact, targeting expression or activity of the receptor in multiple cell populations such as epidermal, immune, and/or endothelial cells may be more effective and ultimately, provide more insight into the potentially diverse roles Tie-2 plays in mediating this and other diseases.

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