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Clinical, pathological and immunological features of psoriatic-like lesions affecting keratin 14-vascular endothelial growth factor transgenic mice

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Summary. Up-regulation of vascular endothelial growth factor (VEGF) plays a primary role in the pathogenesis of psoriasis. Transgenic mice over-expressing VEGF under the Keratin 14 (K14) promoter develop an inflammatory skin condition with many of the pathobiological features of human psoriasis.

In this work, the development of spontaneous psoriatic-like dermatitis in K14-VEGF transgenic mice was monitored from week 6 to week 44 and skin lesions were characterized clinically (application of a clinical score system comparable to the human Psoriasis Area and Severity Index), microscopically (histopathology, leukocyte subset and neoangiogensis) and immunologically (evaluation of local and systemic cytokine/chemokine profiles).

Based on PASI score system, three progressive clinical phases were identified: mild acute (8-14 weeks of age), moderate subacute (15-21 weeks of age) and severe chronic-active (22-44 weeks of age) dermatitis. Microscopically, skin lesions consisted of progressive proliferative psoriatic-like dermatitis dominated by dermo-epidermal infiltrates of CD3-positive lymphocytes, an increased number of mast cells and neoangiogenesis. Both local and systemic up-regulation of pro-inflammatory (IL-12, TNF-alpha, IL-6, MCP-1 and IL-8) and regulatory (IL-10) cytokines/chemokines was observed, mainly during the later stages of disease development.

The results obtained in this study further confirm the central role of VEGF over-expression in the development of psoriatic-like dermatitis. Similarly to what is reported for human psoriasis, both the local and systemic immunologic profiles observed in K14-VEGF transgenic mice suggest that a combined Th1 and Th17 response may be implicated in lesion development. The identification of three progressive stages of disease, each with peculiar clinicopathological features, renders the K14-VEGF transgenic mouse a valuable model to study novel immunotherapies for psoriasis.

Key words: Angiogenesis, VEGF, Disease animal models, Psoriasis, Skin

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Introduction

Psoriasis is a common, chronic autoimmune skin condition, affecting approximately 2% of the human population (Nestle et al., 2009). Clinically, the disease is usually manifested as multiple, raised, well-demarcated cutaneous plaques with consistent scaling and variable erythema (Krueger and Bowcock, 2005; Danilanko, 2008). The key histopathological features of psoriasis include epidermal hyperplasia with acanthosis, elongated rete ridges, hypogranulosis, parakeratosis, and leukocytic infiltration of the dermis and epidermis (Nickoloff and Nestle, 2004; Krueger and Bowcock, 2005; Lowes et al., 2007).

The etiology of human psoriasis has not been fully elucidated yet. A genetic basis has been suspected and several different psoriasis susceptibility gene clusters (designated PSORS1, PSORS2, PSORS3, etc.) have been identified, underscoring the heterogeneous nature of this condition (Nickoloff and Nestle, 2004; Krueger and Bowcock, 2005). The pathogenesis of psoriasis is also far from being completely elucidated, but there have been recent advances in the understanding of some of its

complex immunopathologic mechanisms. It has been demonstrated that psoriatic lesions develop in the context of a combined Th1/Th17-polarized immune response generated through a tight autocrine/paracrine interplay among proliferating keratinocytes, infiltrating leukocytes and activated microvascular endothelium (Kauffman et al., 2004; Lee et al., 2004; Park et al., 2005; Krueger and Bowcock, 2005; Blauvelt, 2007; Nickoloff, 2007; Lowes et al., 2007).

Nowadays, mirroring the complexity of mechanisms that underlie psoriasis, there is a multiplicity of genetically engineered mouse (GEM) models in which a psoriatic-like phenotype is the result of specific manipulations targeting critical biomolecular pathways in keratinocytes, leukocytes and/or vascular endothelium (see Danilenko, 2008 for a comprehensive review). Each GEM model has its similarities to psoriasis, as well as its limitations, not least of which are the fundamental morphologic and functional differences between human and murine skin (Schön, 1999; Nickoloff and Nestle, 2004; Lowes et al., 2007). In general, a relevant animal model for human psoriasis should (i) recapitulate the cardinal histopathologic features of human disease, (ii) have similar pathogenetic mechanisms and (iii) respond to therapeutic agents that human psoriasis has been demonstrated to respond to. However, psoriasis is a heterogeneous disease with a complex pathogenesis, therefore so far a single model of psoriasis that completely mirrors all aspects of this stigmatizing disease has not been identified yet (Danilenko, 2008).

One of the key features of psoriasis, besides epidermal hyperproliferation and leukocyte infiltration, is neovascularization (Zollner et al., 2007). Cutaneous blood vessels in psoriasis are found to be enlarged, tortuous and hyperpermeable. This phenomenon appears to be directly associated with up-regulation of vascular endothelial growth factor (VEGF) (Bhushan et al., 1999; Yalin et al., 2007). To study the specific biologic contribution of sustained VEGF expression to the development of psoriasiform skin lesions, Detmar and colleagues generated transgenic mice, using a transgene vector in which the coding sequence of murine VEGF164 was cloned into a human keratin 14 promoter expression cassette (Detmar et al., 1998). These transgenic mice with specific VEGF over-expression in the basal layer of the epidermis were characterized by marked proliferation of tortuous and hyperpermeable dermal capillaries and enhanced leukocyte rolling and transmigration in postcapillary skin venules. In addition, chronic orthotropic over-expression of VEGF in the murine epidermis resulted in a progressive hyperplastic dermatitis mainly affecting auricles and snout and recapitulating most of the pathologic features of human psoriasis. The K14-VEGF transgenic mouse model represented one of the first *in vivo* demonstration of the critical role of VEGF as a major inducer of dermal neoangiogenesis and leukocyte recruitment during progressive skin inflammation (Detmar et al., 1998; Xia et al., 2003).

In this work, a detailed clinical and immunopathological characterization of the K14-VEGF transgenic mouse model of psoriasis has been performed. This resulted in the identification of three progressive stages of disease, each with distinct clinicopathological features and cytokine/chemokine profiles.

Materials and methods

Animals

A total number of 93 K14-VEGF transgenic mice (43 males and 50 females) were generated as previously described at Regeneron Pharmaceutical (New York) using Velocigene Technology (Thurston et al., 1999). The genetic background of the mice was FVB/Ntac (98%) and C57BL/6J (2%). An equal number of Wild Type (WT) littermates were also included in the experiment as controls. Animals were maintained under standard environmental conditions (2±5°C; relative humidity, 30% to 70%; 12:12-h light:dark cycle) and were provided standard chow (Teklad Global Rodent Diet 2018, Harlan Laboratories, Milan, Italy) and tap water ad libitum. All *in vivo* studies were performed according to the European Council Directive 86/609/EEC and the Italian Ministry guidelines for care and use of experimental animals (decree #116/92). All experimental protocols were authorized by the Italian Ministry of Health (decree #111/2004 B).

Clinical evaluation of skin lesions

K14-VEGF transgenic mice were monitored clinically from week 6 to week 44 of age for the full development of spontaneous auricular and facial psoriatic-like dermatitis. Groups of 8-10 randomly selected K14-VEGF transgenic mice were sacrificed at different time points during the study (see next section for further details). For this reason, the number of transgenic mice clinically investigated during the different periods of this experiment decreased progressively as follows: 93 (week 6); 84 (weeks 7-10), 75(weeks 11-14), 66 (weeks 15-16), 58 (weeks 17-18), 50 (weeks 19-20), 42 (weeks 21-22), 34 (weeks 23-25), 26 (weeks 26-27), 18 (weeks 28-33) and 9 (weeks 34-44). For each period, an equal number of Wild Type (WT) littermates were also examined as controls. Multiple clinical parameters were assessed daily. Increased thickness of ear pinna (ear swelling) was evaluated through caliper measurement (Mytutoyo, Urdorf, Switzerland) and it represented the main clinical indicator of skin inflammation. The severity of both auricular and facial skin inflammation was also assessed through a composite clinical score comparable to the Psoriasis Area and Severity Index (PASI) scoring system used in human clinical dermatology (Louden et al., 2004). Based on PASI, the following parameters were evaluated: number of lesions, redness, scaling and

scales. The severity for each parameter was rated on a 0-3 scale (0= no involvement, 1= weak, 2= moderate and 3= severe) and the sum of the values obtained resulted in a final composite clinical score ranging from 0 to 12 (for further details on the adopted criteria see Table 1). Clinical data collected from K14-VEGF transgenic mice were compared to those obtained from WT littermates to reveal significant differences.

Experimental design and sampling procedures

At: 6, 10, 14, 16, 18, 20, 22, 25, 27, 33 and 44 weeks of age, groups of 8-10 K14-VEGF transgenic mice and WT littermates were randomly selected and euthanized with an overdose of isofluorane. Ear pinnae were then removed. Half of the sampled tissue was frozen in liquid nitrogen pending cytokine/chemokine measurements and the other half was fixed in 10% buffered formalin for 24-48 h for histopathology and immunohistochemistry. Blood samples were also collected from each animal at time of euthanasia for systemic cytokine/chemokine measurement.

Table 1. Schematic representation of composite clinical score parameters evaluation.

Clinical parameter /Severity	Description	Score
a Redness		
weak	localized to the basal part of the ear	1
moderate	widespread over the entire ear	2
severe	widespread over the entire ear + presence of lesions and scales	3
b Number of lesions		
weak	1 lesion	1
moderate	>1 but < 5 lesions	2
severe	>5 + scaling and scales	3
c Scaling		
weak	localized to the basal part of the ear	1
moderate	ear + snout	2
severe	ear + snout + lesions and scales	3
d Scales		
weak	1 scale	1
moderate	>1 but < 5 scales	2
severe	>5 bloodiness + scaling	3

a, b, c, d for ear and snout of each the examined mouse, 4 clinical parameters were evaluated: redness, number of lesions, scaling and scales. For each parameter, degree of severity is rated on a 0-3 scale (0= no involvement, 1= weak, 2= moderate, 3= severe). The sum of the values assigned to each parameter give the final composite clinical score ranging from 0 to 12.

Histopathology and immunohistochemistry

Formalin-fixed samples of ear pinnae collected from K14-VEGF transgenic mice and WT littermates were embedded in paraffin blocks. Three to four μm-thick tissue sections were then obtained and routinely processed for histopathological examination (H&E stain) and for the detection of dermal mast cells (Giemsa stain kit Artisan^{TM} code AR164, Dako). Additional serial tissue sections were also processed for indirect immunoperoxidase staining following the procedures described elsewhere (Radaelli et al., 2009). Details concerning primary antibodies, antigen retrievals and detection systems used during immunohistochemistry are reported in Table 2. To document increased dermal angiogenesis in K14-VEGF transgenic mice, the baseline ranges of CD31-positive blood vessels calculated in WT littermates were compared at each time point to the ranges obtained from K14-VEGF transgenic mice. For each animal, CD31-positive dermal blood vessels were counted in five 200x microscopic fields randomly selected within the superficial dermis. Dermis in proximity of ulcerative lesions was not considered because of the marked proliferation of granulation tissue.

Cytokine and chemokine measurements

Pooled samples of frozen ear pinnae collected from either K14-VEGF transgenic mice or WT littermates at each of the different time points were pulverized, dissolved in Lysing Buffer (PBS 1X stored at 4°C, Na3VO4, NaF, NaPir) for 1 h and centrifuged at 13000rpm, 4°C, for 15 minutes. Supernatant was collected and protein quantification was done by Bradford method (Biorad). After protein quantification,

Table 2. Details concerning primary antibodies and correlated immunohistochemical procedures.

Antigen	Source	Code and/ or Clone	Clonality	Antigen retrieval	Working dilution	Condition of incubation	Detection system
Myeloperoxidase (neutrophils)	DAKO	N1578	Rb poly	HIER	RTU	40' at 37°C	EnVision
CD3 (T-cells)	DAKO	A0452	Rb poly	HIER	1/200	40' at 37°C	EnVision
CD45R/B220 (B-cells)	BD-Pharmingen	RA3-6B2	Rat mon	HIER	1/200	40' at 37°C	ABC
F4/80 (histiocytes)	Serotec	MCA497GA	Rat mon	Ezymatic digestion	1/250	o/n	ABC
CD31 (endothelial cells)	Serotec	6430-0758	Rat mon	HIER	1/200	o/n	ABC

Rb poly: rabbit polyclonal; Rat mon: rat monoclonal; HIER: heat-induced epitope retrieval, 0.01 mol/L citrate buffer pH = 6.0; enzymatic digestion: Digest-all™3 Pepsin solution RTU (Zymed, S. Francisco, USA); RTU: ready to use; ABC: Vector Lab, Vectastain Elite ABC kit PK-6100; EnVision: Dako EnVision™ system; o/n: overnight; 1h RT: one hour at room temperature; 40' at 37°C: forty minutes at 37°C

100μg of extract from each pooled sample were used to measure Interleukin-6 (IL-6), Interleukin-10 (IL-10), Monocyte Chemoattractant Protein-1 (MCP-1), Interferon-γ (IFN-γ), Tumor Necrosis Factor (TNF) and Interleukin-12p70 (IL-12p70) levels through Cytometric Bead Assay (CBA) Mouse Inflammation Kit (Becton Dickinson, BD Biosciences/Pharmingen, San Diego, CA). Mouse Th1/Th2 Cytokine CBA Kit (BD) was also applied to measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interferon-γ (IFN-γ) and Tumor Necrosis Factor-α (TNF-α) levels. Measurement of systemic cytokine/chemokine profiles was achieved applying the same CBA kits described above on 25 μL of plasma obtained from pooled blood samples collected from either K14-VEGF transgenic mice or WT littermates at each of the different time points. Measurement of local and systemic cytokine/chemokine profile levels was performed in triplicate. For all the soluble markers included in the panels, detection limit of CBA assays was 20pg/mL.

Statistical analyses

All statistical analyses were performed using analysis of variance (ANOVA) followed by Dunnet or Bonferroni's post-tests. GraphPad Prism4 (San Diego, CA) was used to perform analyses. Data were expressed as mean ± standard error (SEM) and levels of significance were assigned as follows: *p<0.05, **p<0.01, ***p<0.001. Analysis of statistical correlations were performed using nonparametric correlation (Spearman).

Results

Clinical evaluation of skin lesions

Clinically affected K14-VEGF transgenic mice were first detected at 7 weeks of age. The percentage of affected animals progressively increased starting from 8 weeks of age to reach the involvement of the entire K14-VEGF transgenic population at 20 weeks (Fig. 1a). Pruritus with self-inflicted ulcerative skin lesions was occasionally recorded starting from 19 weeks of age.

Compared with the WT littermates, detectable increases in the thickness of auricles of K14-VEGF transgenic mice were first recorded at 6 weeks of age. The degree of ear swelling remained constant from 7 to

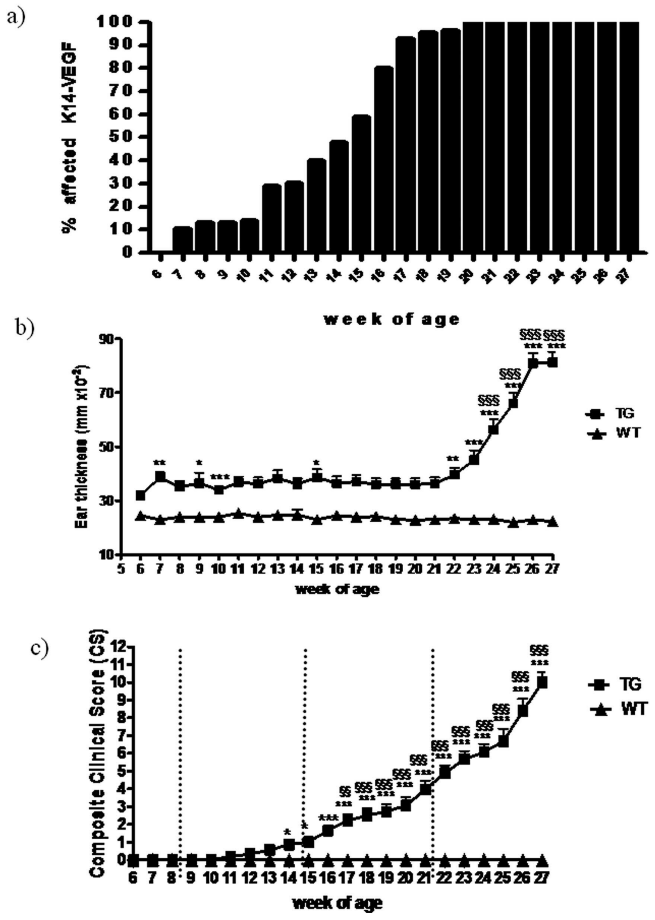

Fig. 1. Results of the clinical evaluation on Keratin 14 (K14)-VEGF transgenic mice monitored daily from week 6 to week 44 of age for the development and progression of psoriatic-like dermatitis. No significant variations in the clinical parameters measured were observed after 27 weeks of age. Numbers of transgenic mice examined during the different periods of the experiment were: 93 (week 6); 84 (weeks 7-10), 75 (weeks 11-14), 66 (weeks 15-16), 58 (weeks 17-18), 50 (weeks 19-20), 42 (weeks 21-22), 34 (weeks 23-25), 26 (weeks 26-27). For each period, an equal number of Wild Type (WT) littermates were also examined as controls. **a.** Representation of the clinical prevalence of psoriatic-like dermatitis in K14-VEGF transgenic mice during the different periods of the experiment. The percentage of clinically affected K14-VEGF transgenic mice progressively increased, starting from week 8 of age to reach the involvement of the entire population at week 20. None of the WT littermates showed clinical signs of psoriatic-like dermatitis. **b.** Evaluation of ear swelling in K14-VEGF transgenic mice compared to WT controls. Ear thickness was measured with a caliper and mean group values (mm x 10^{-2}) are reported. Ear swelling indicates the basal difference in ear thickness between K14-VEGF transgenic mice and WT controls. In K14-VEGF transgenic mice the degree of ear swelling remained constant from week 7 to 21 of age and then progressively increased reaching a peak at 27 weeks. No significant variations in ear thickness were observed in WT littermates during the entire period of the experiment. Statistical analyses were performed using two-way ANOVA followed by Bonferroni's post test (*p<0.05; **p<0.01; *** p<0.001 TG vs WT); one-way ANOVA followed by Tukey's multiple comparison post test ($$$p<0.001 vs week 6). **c.** Application of a composite clinical score system comparable to the human Psoriasis Area and Severity Index (PASI) to assess severity of psoriatic-like dermatitis in K14-VEGF transgenic mice. Composite clinical score pointed out a significant increase of disease severity that started at 8 weeks of age and reached the peak at 27 weeks. Three progressive clinical phases have been identified: (i) mild acute (8-14 weeks of age), (ii) moderate subacute (15-21 weeks of age) and (iii) severe chronic-active (22-27 up to 44 weeks of age). None of the WT littermates showed PASI composite clinical score system > 0. Statistical analyses were performed using two-way ANOVA followed by Bonferroni's post test (*p<0.05; *** p<0.001 TG vs WT); one-way ANOVA followed by Tukey's multiple comparison post test ($$$p<0.001; $$p<0.01) vs week 6.

21 weeks of age and then progressively increased reaching a peak at 27 weeks. Differences in ear thickness between the two groups were statistically significant starting from the 7th week of age (Fig. 1b).

Based on PASI composite clinical score system, three progressive clinical phases were identified in K14-VEGF transgenic mice: mild acute (8-14 weeks of age,

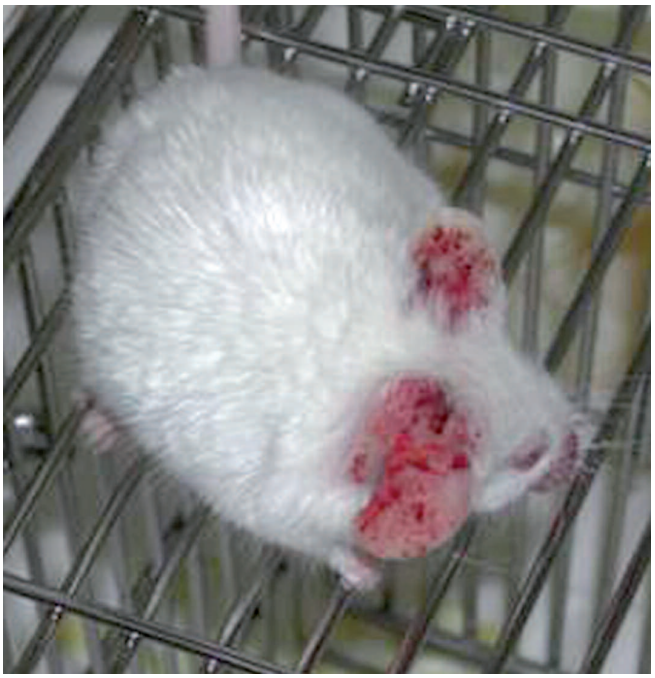

Fig. 2. Clinical picture of a K14-VEGF transgenic mouse at maximal disease severity K14-VEGF transgenic mouse at maximal disease severity (week 27 of age), presenting a composite clinical score of 12. Redness was clearly widespread over the entire ears; presence of more than five lesions were observed; scaling was diffusely present along the ear's pinnae and snout; more than five bloody scales and ulceration have been also identified.

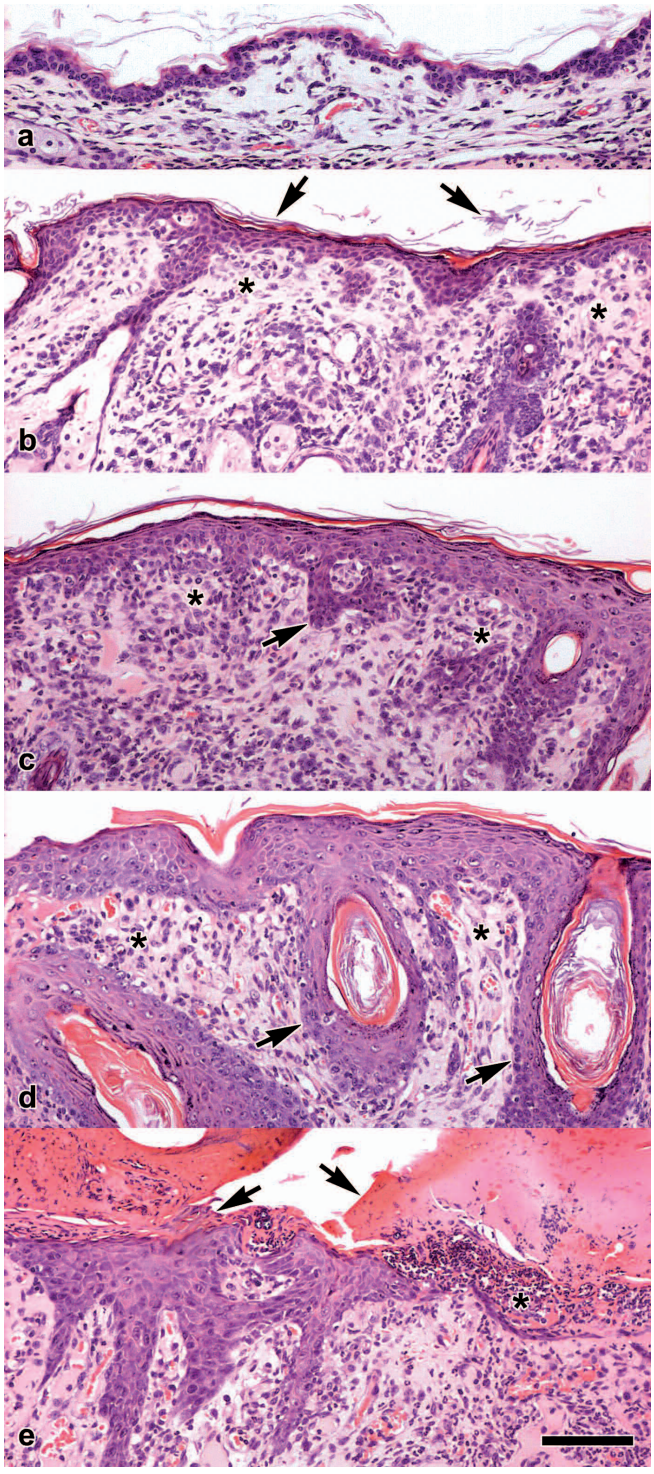

Fig. 3. Histopathological analysis of auricular skin lesions Hematoxilin and Eosin stain in sections of ear pinnae from K14-VEGF transgenic. All pictures have been captured at the same magnification. **a.** At baseline (6 week of age) K14-VEGF transgenic mice do not present any dermo-epidermal change and they are histologically undistinguishable from WT littermates. **b.** In 8 to 14 weeks old K14-VEGF transgenic mice (minimal clinical disease severity / CS= 0-1), the mild multifocal subacute inflammatory changes of the upper dermis (asterisks) are associated with early epidermal hyperplasia and orthokeratotic hyperkeratosis (arrows). **c.** In 15 to 21 weeks old K14-VEGF transgenic mice (intermediate clinical disease severity/ CS= 2-5), the diffuse moderate subacute inflammatory changes of the upper dermis (asterisks) are consistently accompanied by epidermal hyperplasia with anastomosing rete ridge formation (arrow). **d.** In 22 to 27 weeks old K14-VEGF transgenic mice (maximal clinical disease severity/ CS= 6 to 12), the diffuse severe chronic inflammatory and fibrosing changes of the upper dermis are associated with marked neoangiogenesis (asterisks) and prominent hyperplasia and hyperkeratosis affecting both the superficial and infundibular (arrows) epidermis. **e.** Details of superimposed pyotraumatic skin lesion affecting a 25-27 weeks old K14-VEGF transgenic mouse (maximal clinical disease severity/ CS= 6 to 12). Note the intense dermo-epidermal neutrophilic inflammation (asterisk) accompanied by epidermal hyperplasia with anastomosing rete ridge formation and focal skin ulceration with thick serocellular crusts covering the affected area (arrows). Scale bars: 100 μm.

minimal clinical disease severity; weak/ composite clinical score: 0 to 1), moderate subacute (15-21 weeks of age, intermediate clinical disease severity; moderate/composite clinical score: 2 to 5) and severe chronic-active (22-27 up to 44 weeks of age, maximal clinical disease severity; severe/composite clinical score: 6 to 12) skin inflammation (Fig. 1c). Figure 2 shows the clinical picture of a K14-VEGF transgenic mouse at maximal disease severity (week 27 of age/composite clinical score=12).

In K14-VEGF transgenic mice, no consistent changes in clinical manifestations, thickness of auricles or PASI composite clinical scores were observed after 27 weeks of age. None of the WT littermates showed clinical signs of psoriatic-like dermatitis, increased ear thickness or PASI composite clinical score system >0.

Histopathological analysis of auricular skin lesions

Representative microscopic pictures of ear sections from both WT littermates and K14-VEGF transgenic mice at different stages of the disease are reported in Fig. 3 panels a,b,c,d,e.

No dermo-epidermal changes were found in WT littermates throughout the experiment or in K14-VEGF transgenic mice at 6 weeks of age (baseline) (Panel a). In general, a good correlation between the progression of clinical disease, as assessed by PASI scoring system, and the severity of histopathologic changes was observed.

Eight to fourteen week-old K14-VEGF transgenic mice showed a mild multifocal subacute inflammation of the upper dermis (minimal clinical disease severity/ composite clinical score: 0 to 1) characterized by moderate hyperemia of small caliber blood vessels, mild neoangiogenesis, mild perivascular edema and sparse perivascular to interstitial infiltration of lymphocytes and mast cells (Panel b). Mild diffuse epidermal hyperplasia and orthokeratotic hyperkeratosis but no serocellular crust formation or epidermal ulceration were recorded at this stage.

Fifteen to twenty-one week-old K14-VEGF transgenic mice showed diffuse moderate subacute inflammation of the upper dermis (intermediate clinical disease severity /composite clinical score: 2 to 5) characterized by moderate hyperemia and endothelial activation of small caliber blood vessels, marked neoangiogenesis, mild fibroplasia, moderate perivascular edema and marked perivascular to interstitial infiltration of lymphocytes, mast cells and neutrophils. There was also moderate diffuse epidermal hyperplasia focally accompanied by anastomosing rete ridge, compact parakeratotic hyperkeratosis, serocellular crusting and full-thickness epidermal necrosis/ulceration with massive neutrophilic infiltration of the subjacent dermis (interpreted as pyotraumatic lesions) was also observed (Panel c). At this stage, neutrophilic epidermal exocytosis was restricted to the epidermis bordering the ulcerative lesions.

Twenty-two to twenty-seven week-old transgenic mice showed a diffuse severe chronic-active inflammation of the upper dermis (maximal clinical disease severity/composite clinical score: 6 to 12) characterized by moderate hyperemia and endothelial activation of small caliber blood vessels, marked neoangiogenesis, moderate fibroplasias/fibrosis, moderate perivascular edema and extensive perivascular to interstitial infiltration of lymphocytes, neutrophils, mast cells, plasma cells and histiocytes. Cell-poor infiltrates of lymphocytes diffusely obscured the superficial and infundibular dermo-epidermal junction with multifocal exocytosis and hydropic degeneration of basal keratinocytes. There was also marked, diffuse, both superficial and infundibular epidermal hyperplasia, with prominent anastomosing rete ridge, compact parakeratotic hyperkeratosis, serocellular crusting, diffuse epidermal neutrophilic exocytosis with small intracorneal and/or subcorneal pustules and full-thickness epidermal necrosis/ulceration with massive

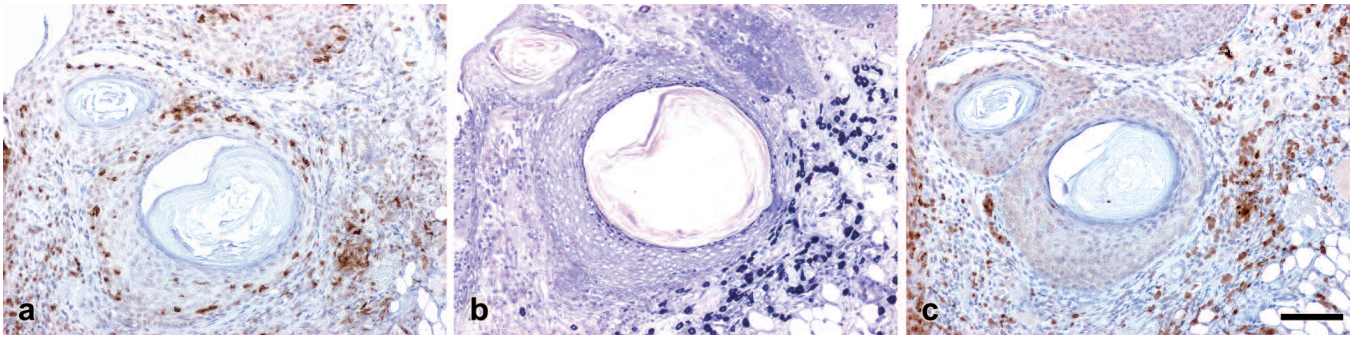

Fig. 4. Leukocyte subset of skin lesions. K14-VEGF transgenic mouse of 27 weeks of age, serial sections of the same representative microscopic field. All pictures have been captured at the same magnification. **a.** CD3 immunohistochemistry. Infiltrates of CD3-positive T cell expand the upper dermis, obscure superficial and infundibular dermo-epidermal junction and display multifocal exocytosis into the hyperplastic epidermis. **b.** Giemsa-stain. Mast cells displaying the typical metachromatic staining properties are markedly increased in upper dermis. **c.** Myeloperoxidase immunohistochemistry. Infiltrates of MPO-positive neutrophils expand the upper dermis and display multifocal epidermal exocytosis into the hyperplastic epidermis. Scale bars: 100 μm.

neutrophilic infiltration of the subjacent dermis (interpreted as pyotraumatic lesions) (Panels d and e).

At week 27, lesions were fully developed reaching the highest histopathological severity. No further relevant changes were observed in terms of histopathology between week 27 and 44.

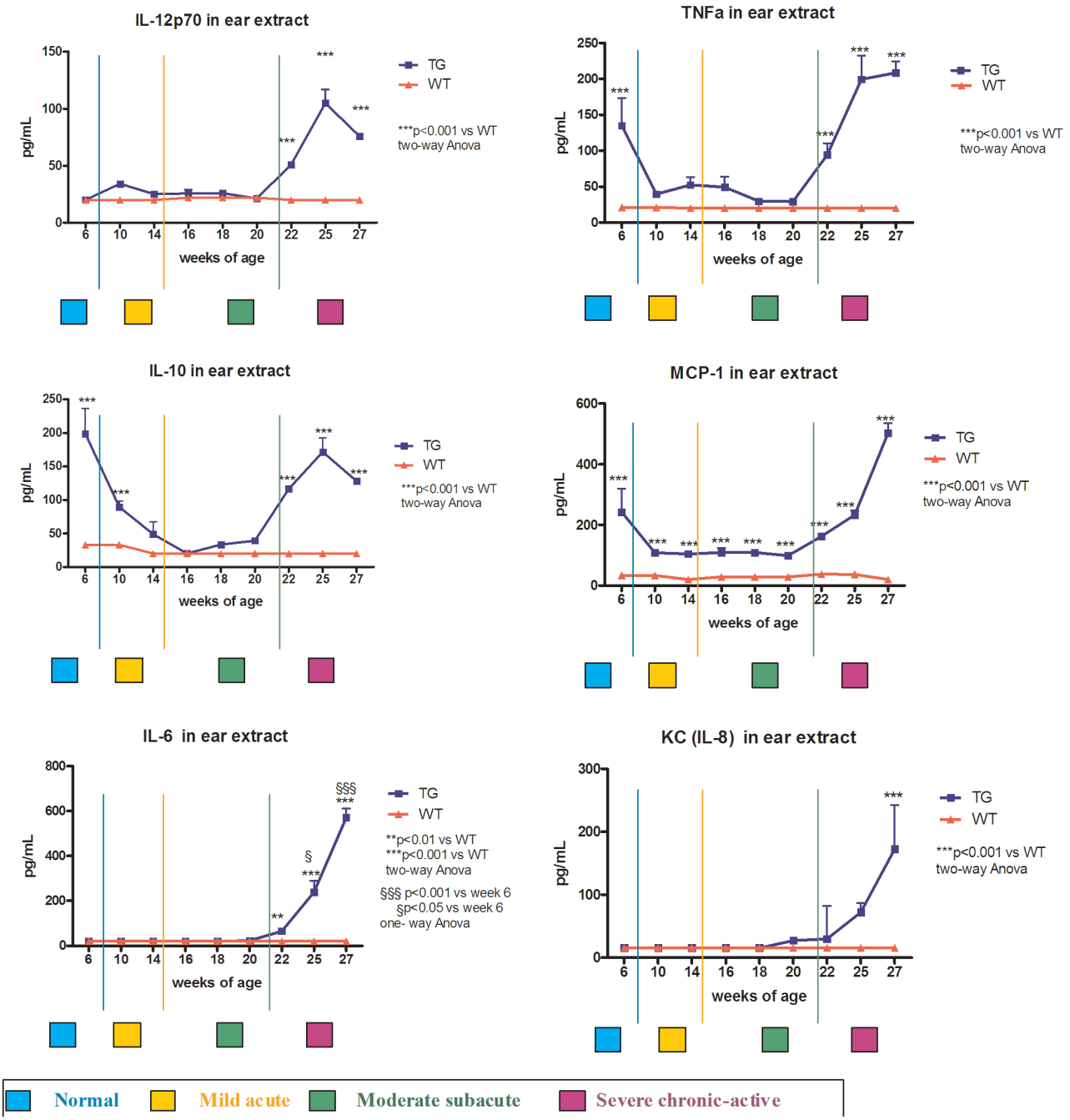

Fig. 5. Cytokine and Chemokine profiles in ear extract CBA assay was performed on ear extracts of K14-VEGF spontaneously developing the disease (n=8 per group). Detection limit of the assay is 20pg/mL. Results are given as mean ± SEM of n=8 mice per group. Statistical analyses were performed using two-way ANOVA followed by Bonferroni's post test (TG vs WT) and one-way ANOVA followed by Tukey's multiple comparison post test (vs week 6). An overall up-regulation of pro-inflammatory cytokines/chemokines (MCP-1/TNFa) was observed in ear extract. Up regulation of all soluble factors at week 25 of age was detected (***p<0.001 vs WT; two-way Anova was performed/ *p<0.05 vs week 6; one-way Anova was performed). IL-10 was already significantly up-regulated at week 6 of age (***p<0.001 vs WT; two-way Anova was performed). Baseline levels of IL-4, IL-2, IL-5 and IFN-γ were found in ear extracts.

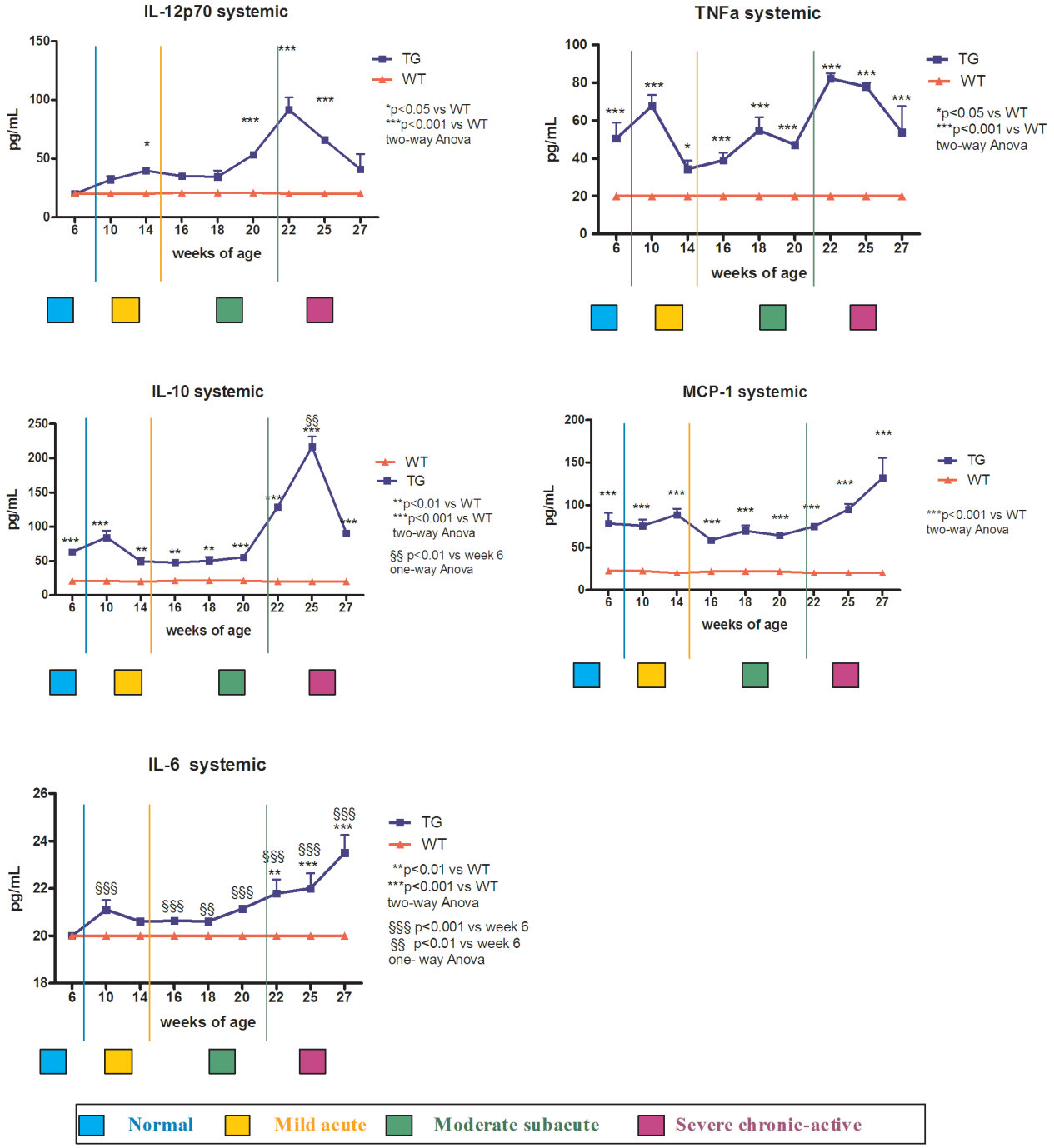

Fig. 6. Cytokine and Chemokine profiles in plasma CBA assay was performed on 25uL of plasma of K14-VEGF spontaneously developing the disease (n=8 per group). Detection limit of the assay is 20pg/mL. Results are given as mean ± SEM of n=8 mice per group. Statistical analyses were performed using two-way ANOVA followed by Bonferroni's post test (TG vs WT) and one-way ANOVA followed by Tukey's multiple comparison post test (vs week 6). A significant up-regulation of pro-inflammatory systemic cytokines and chemokines (TNF-α, MCP-1, IL-12p70, IL-6) was observed during disease development. Up-regulation of IL-10 was observed during disease development (***p<0.001 vs WT; two-way Anova was performed/**p<0.01 vs week 6; one-way Anova was performed). Mild significant increase of IL-6 up to week 27 of age (***p<0.001 vs WT; two-way Anova was performed/ ***p<0.001 vs week 6; one way Anova was performed). Baseline levels of IL-4, IL-2, IL-5, IFN-γ and KC (IL-8) were found in plasma.

Leukocyte subset of auricular skin lesions

Representative microscopic pictures of CD3 and Myeloperoxidase (MPO) immunohistochemistry and Giemsa stain on ear sections from 27 week-old K14-VEGF transgenic mice are shown in Fig. 4, panels a-c.

No CD3-positive T-cells were observed in WT littermates or 6 week-old K14-VEGF transgenic mice. Starting from 10 weeks of age, K14-VEGF transgenic mice showed a progressively increased number of lesional CD3-positive T cells with a peak at 27 weeks. CD3-positive T cells were the most prevalent inflammatory component in the perivascular to interstitial infiltrates of the upper dermis and at the dermo-epidermal junction.

Scattered perivascular Giemsa-positive mast cells were observed in WT littermates and 6 to 14 week-old K14-VEGF transgenic mice. Starting from 16 weeks of age, K14-VEGF transgenic mice showed a progressively increased number of lesional mast cells that peaked at 27 weeks. Giemsa-positive mast cells were the second most prevalent inflammatory component in the perivascular to interstitial infiltrates of the upper dermis.

MPO-positive neutrophils were virtually absent in WT littermates and 6 week-old K14-VEGF transgenic mice. Starting from 10 weeks of age K14-VEGF transgenic mice showed a progressively increased number of lesional MPO-positive neutrophils that peaked at 27 weeks. MPO-positive neutrophils were the most prevalent inflammatory component in association with skin ulcers, serocellular crust and pustules. Scattered neutrophils were also present in the perivascular to interstitial dermal infiltrates.

F4/80-positive macrophages and CD45R/B220-positive B-cells were virtually absent in WT littermates and K14-VEGF transgenic mice with weak or moderate disease severity (8 to 14 weeks and 15 to 21 weeks respectively). Few activated dermal macrophages and scattered B-cells mainly associated with skin ulceration were observed in K14-VEGF transgenic mice that reached the maximal clinical score (after 21 weeks).

No relevant changes in number or distribution of the different inflammatory cell populations were observed in K14-VEGF transgenic mice between week 27 and 44.

Altered angiogenesis in auricular skin lesions

During the study period (from 6 to 44 weeks of age), significantly increased dermal angiogenesis was observed in K14-VEGF transgenic mice when compared to WT littermates (*$p < 0.05$). The baseline range of CD31-positive vascular outlines calculated in WT littermates attested between 4-12 with a mean value of 7.1. The range calculated in K14-VEGF transgenic mice attested between 15 and 26 with a mean value of 17.3.

Cytokine and chemokine profiles

Local and systemic cytokine and chemokine profiles in 6 to 27 week old K14-VEGF transgenic mice and WT littermates are summarized in Figs. 5, 6.

Compared to the baseline levels measured in WT littermates, an overall up-regulation in pro-inflammatory (TNF-alpha, IL-12, IL-6, MCP-1 and IL-8) and, to a lesser degree, regulatory (IL-10) cytokines/chemokines was observed in K14-VEGF transgenic mice at local and systemic level.

A significant increase of local TNF-alpha, IL-10, IL-12, IL-6, and IL-8 levels was observed in the later phase of the disease (from 22 up to 27 weeks of age; maximal clinical disease severity). Local levels of TNF-alpha and IL-10 were also significantly increased early in the disease development (6 week of age). Systemic levels of TNF-alpha, IL-10, IL-12, IL-6 and both local and systemic levels of MCP-1 were significantly elevated during the entire clinical course of the disease.

No detectable elevations over the baseline values were observed for the local levels of IL-4, IL-2, IL-5 and IFN-γ or for the systemic levels of IL-4, IL-2, IL-5, IFN-γ, and IL-8.

Discussion

In our study, the detailed clinical, pathological and immunological characterization of spontaneous psoriatic-like lesions affecting K14-VEGF transgenic mice led to the identification of three well-defined developmental stages of disease, each with peculiar features. The definition of these progressive stages of disease may be particularly useful in the context of preclinical studies aimed at assessing the efficacy of novel immunotherapies for psoriasis. Based on the preventive and/or curative target of the tested protocols, specific time points for pharmacological intervention could be easily identified and clinical parameters monitored to allow a reliable measurement of the therapeutic response. In particular, the application of PASI score system to determine the clinical severity of skin lesions proved to be a feasible and accurate monitoring method, reflecting the effective progression of the disease in terms of modifications of local cytokine/chemokine profiles and evolution inflammation as assessed by histopathology and immunohistochemistry.

The development of the K14-VEGF transgenic mouse model by Detmar and colleagues back in 1998 stood as one of the first biological proves of the critical role of VEGF in the pathogenesis of psoriasis. In this context, keratinocyte-derived VEGF represents one of the earliest inflammatory signals targeting dermal microvasculature and inducing increased permeability, migration and proliferation of endothelial cells, over-expression of adhesion molecules, including E-selectin, ICAM-1 and VCAM-1 and chemotaxis and progressive infiltration of leukocytes (Creamer et al., 2002; Heidenreich et al., 2008). These effects are dramatically amplified by the synergism of VEGF with potent pro-inflammatory cytokines and chemokines such as TNF-α,

IL-6 and IL-8 (Gillitzer and Goebeler, 2001; Numasaki et al., 2003; Johansen et al., 2006). Based on our observations, all these critical mediators have been found to be consistently increased in psoriatic-like lesions affecting K14-VEGF transgenic mice where they have likely contributed to the promotion of dermal inflammation and neoangiogenesis. This is particularly true for TNF-α, which was found to be up-regulated early during disease development. Besides their stimulation of dermal microvasculature, VEGF, TNF-α and IL-8 over-expressed by psoriatic keratinocytes also induce an autocrine loop that promotes unregulated epidermal proliferation (Duan et al., 2001; Man et al., 2006; Elias et al., 2008). A similar event likely contributed to the progressive hyperplastic epidermal changes observed in K14-VEGF transgenic mice.

The pathobiology of psoriatic-like lesions in K14-VEGF transgenic mice is entirely driven by the over-expression of VEGF164 isoform (homologous of human VEGF165). This could represent a potential limitation for this animal model as recent investigations clearly pointed out that, in addition to VEGF165, VEGF121 is also implicated in the pathogenesis of human psoriasis and each of these isoforms accounts for different and often complementary effects on dermal microvasculature (Zhang et al., 2005, 2008). New GEM models are therefore needed to elucidate the combined contribution of VEGF121 and VEGF165 to the development of psoriatic skin phenotype.

Skin lesions in K14-VEGF transgenic mice are characterized by a prominent infiltration of T cells in the upper dermis (mainly CD4+ T cells) dermo-epidermal junction and overlying hyperplastic epidermis (mainly CD8+ T cells) (Pauls et al., 2001; Xia et al., 2003). This peculiar pattern of distribution closely recapitulates that observed for psoriatic skin lesions in humans where T-lymphocytes represent one of the main components of the heterogeneous inflammatory infiltrate and play a critical role in orchestrating the progression of the disease (Ghoreschi et al., 2007).

In accordance with Detmar et al. (1998) and Xia et al. (2003), our study also confirms that psoriatic-like lesions in K14-VEGF transgenic mice display a prominent mast cells infiltrate. The contribution of mast cells to the pathogenesis of psoriasis has recently been emphasized (Harvima et al., 2008; Heidenreich et al., 2009; Loffredo et al., 2009; Theoharides et al., 2010). Mast cells display a series of pro-inflammatory activities (e. g. angiogenesis, extracellular matrix remodelling, increased vascular permeability, antigen presentation, chemotaxis and leukocyte activation) that are critical for the full development of psoriatic lesions. The mechanism responsible for the increased number of mast cells in psoriatic lesions are not entirely clear. Proinflammatory cytokines, including TNFα, IL-6, IL-8 and cellular events, such as the activation of microvascular endothelium, have been reported to promote survival, activation and migration of mast cells (Harvima et al., 2008). This evidence suggests that consistent up-regulation of TNFα, IL-6 and IL-8 and persistent VEGF-mediated activation of endothelial cells may be similarly responsible for the heavy mast cell infiltration observed in K14-VEGF transgenic mice.

The immunopathology of psoriasis is driven by a complex combination of Th1- and Th17-biased cytokine responses, whereas Th2 mediators are generally down-regulated (Gudjonsson et al., 2004; Arican et al., 2005; Borska et al., 2008). In a similar manner, the psoriatic-like phenotype analyzed in our model showed significant elevations of cytokines priming the Th1 response (i. e. IL-12 and TNFα) and low (baseline) levels of cytokines promoting or resulting from the Th2 response (i.e. IL-4 and IL-5). These observations may suggest that skin lesions in K14-VEGF transgenic mice rely on a predominant Th1-polarized immune response. However, unexpectedly, these mice failed to produce significant elevations of other key Th1 cytokines, most notably IFNγ and IL-2, which are known to be up-regulated in human psoriasis. Although there is no clear explanation for this specific finding, similar results have already been reported by recent investigations where K14-VEGF transgenic mice displayed a progressive shift from a Th1- to a Th17-polarized immune response with low levels of IFNγ (Hvid et al., 2008; Teige et al., 2009). A detailed analysis of Th17-related cytokines was not provided in our study. However, significant elevations of IL-6, one of the main cytokines initiating the Th17 phenotype, and the concurrent over-expression of chemokines (i. e. IL-8 and MCP-1) that are known to be up-regulated upon IL-17 stimulation, suggest that the Th17 response likely contributed to the development of the skin lesions examined (Bettelli et al., 2008; Heidenreich et al., 2009).

In psoriatic skin, the immunomodulatory activity of CD4+ CD25 regulatory T cells is mainly mediated through IL-10 signaling (Shehata and Elghandour, 2007). Recent studies have demonstrated that skin lesions in K14-VEGF transgenic mice are also characterized by a consistent number of regulatory T cells (Teige et al., 2009). Based on these observations, the local biphasic elevation of IL-10 described in our study is likely determined by the transient activation of this subset of regulatory T cells.

Similarly to what has been reported for psoriatic patients, our study also demonstrated that K14-VEGF transgenic mice have an overall increase of systemic pro-inflammatory cytokines/chemokines, including TNF-α, IL-12p70, IL-6, MCP-1 and IL-8 (Arican et al., 2005; Deeva et al., 2010). This evidence suggests that in this model, evaluation of serum cytokines/chemokines levels may be effectively used as a means to monitor therapy responsiveness.

In conclusion, the results obtained in this study further confirm the central role of VEGF signaling in the development of psoriatic-like dermatitis affecting K14-VEGF transgenic mice. A combined Th1 and Th17 response is likely implicated in the skin lesions observed in this model. The application of PASI clinical score

system, to determine the degree of disease severity, proved to be a feasible and accurate monitoring method reflecting the progressive histopathologic changes and the immunologic response. Based on the numerous pathobiological analogies between human psoriasis and psoriatic-like dermatitis described in this study, the K14-VEGF transgenic mouse model may be proposed as a predictive and reliable tool in preclinical studies aimed at assessing the efficacy of novel preventive or curative immunotherapies for psoriasis.

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Competing interests. The present work is part of MC's PhD thesis/PhD programme at the University of Torino, Italy, in close collaboration with MerckSerono International S.A, which is involved in the discovery and commercialization of therapeutics for prevention and treatment of human disease. K14-VEGF construct was previously published and described in the literature. Therefore no patents have been requested and/or used for this work. None of the authors is employed by MerckSerono International S.A.

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