Thesis for doctoral degree (Ph.D.) 2009

Thesis for doctoral degree (Ph.D.) 2009

NF-κB IN SKIN IMMUNE HOMEOSTASIS AND CANCER DEVELOPMENT Maria Helena Ulvmar

Thesis for doctoral degree (Ph.D.)
2009

NF-κB IN SKIN IMMUNE HOMEOSTASIS AND CANCER DEVELOPMENT

Maria Helena Ulvmar

Karolinska Institutet

Karolinska Institutet

From The Department of Biosciences and Nutrition
Center for Biosciences
Karolinska Institutet, Stockholm, Sweden

NF-κB IN SKIN IMMUNE HOMEOSTASIS AND CANCER DEVELOPMENT

Maria Helena Ulvmar

Karolinska Institutet

Stockholm 2009

ISBN 978-91-7409-548-7

Printed by
REPROPRINT AB
Stockholm 2009
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

POPULÄRVETENSKAPLIG SVENSK SAMMANFATTNING

Vår hud fungerar som en barriär mellan kroppen och vår yttre värld. Huden utsätts ständigt för påverkan från vår miljö i form av solljus, bakterier, kemiska produkter och direkta skador från mekanisk påfrestning. Våra hudceller är inte bara viktiga som byggstenar för hudens skyddande struktur utan kommunicerar också ständigt med vårt immunförsvar för att det skall kunna eliminera de yttre farorna. Ibland förlorar kroppen kontrollen över den inflammatoriska reaktionen och en kronisk inflammation kan utvecklas. Hudcellernas utsatta läge gör också att de löper en stor risk att ackumulera skador som kan leda till att de kan omvandlas till cancerceller. Skivepitelcancer som bildas från våra hudceller är den näst vanligast cancerformen hos män och den tredje vanligaste cancerformen hos kvinnor. Inflammation kan öka risken för att utveckla cancer, men all typ av inflammation leder inte till cancer. I ett större perspektiv syftar den forskning som ligger till grund för avhandlingen till att förstå hur inflammatoriska processer regleras i vår hud med speciellt fokus på hur kommunikationen mellan hudcellerna och immunförsvaret regleras. Vi vill också förstå vad som utmärker den inflammation som ökar risken för att utveckla cancer. Ju mer vi lär oss om detta desto större möjlighet har vi att hitta nya sätt att bryta de inflammatoriska reaktionerna och därmed hitta nya behandlingsmetoder, både för inflammatoriska sjukdomar och för cancer. Vi använder oss till stor del av musmodeller i vår forskning eftersom kommunikationen mellan huden och immunförsvaret tyvärr inte kan återskapas utanför kroppen.

I de försök som redovisas i avhandlingen har vi studerat funktionen av ett protein som heter NF-κB och som kan reglera hur mycket det produceras av andra proteiner. NF-κB har i tidigare forskning visats öka uttrycket av en rad inflammatoriska proteiner och det har satts likhetstecken mellan ökad aktivitet av NF-κB och ökad inflammation. Vår grupp har visat att rollen av NF-κB är mer komplex än man tidigare trott. Vi har visat att om aktiviteten av NF-κB hämmas i hudceller hos möss ger även det, mot förväntan, upphov till kronisk inflammation. Denna inflammation leder dessutom ofrånkomligen till utveckling av skivepitelcancer. Direkt koppling mellan vår modell och människa finns hos patienter med en medfödd mutation som hämmar NF-κB (Incontinentia Pigmenti patienter). De utvecklar en liknande inflammation i huden som våra möss och kan få hudtumörer, speciellt under naglarna. I avhandlingen visar vi att det finns ett samband mellan inflammationen hos våra möss och ett av kroppens mest centrala inflammatoriska proteiner, TNFα. Om vi tar bort möjligheten för TNFα, att påverka kroppens celler genom att ta bort dess så kallade receptor, TNFR1, så kan vi förhindra både utvecklingen av inflammation och cancer.

Vi har sedan gått vidare med vår modell och visar att den typ av inflammation som mössen utvecklar har mycket speciella egenskaper. Bl.a. finns där tidigt en stor mängd immunceller som kallas regulatoriska T-celler och som hämmar förmågan hos andra celler i immunförsvaret att känna igen och döda de farliga cancercellerna. Vi visar också att hela den inflammatoriska kaskaden enbart drivs av svaret på TNFα från hudcellerna, vilket är förvånande eftersom det är ett protein som i andra sammanhang främst verkar via de inflammatoriska cellerna. I avhandlingen redovisas även att hämning av NF-κB påverkar bildningen av hår och svettkörtlar, men att detta är oberoende av den inflammation och cancer som utvecklas efter födseln. Vi har även

gjort försök där vi hämmar NF-κB i hudceller isolerade från människa, som vi odlar på plattor. Vi har sedan undersökt vilken effekt hämmad NF-κB har på cellernas förmåga att växa och svara på substanser som kan påverka deras tillväxt.

Våra resultat illustrerar vilken viktig roll hudcellerna kan ha i kommunikationen med immunförsvaret. När aktiviteten av NF-κB hämmas i hudcellerna leder detta till att hudens immunologiska balans bryts och en kronisk inflammation utvecklas. En kronisk inflammation som ofrånkomligen leder till cancer. Vi visar att både inflammationen och utvecklingen av cancer är kopplat till signalering från TNFα/TNFR1 i hudcellerna. Resultaten är speciellt spännande eftersom det redan finns godkända anti-TNF medel som idag används för behandling av inflammatoriska sjukdomar hos människa. Att anti-TNF behandling även skulle kunna användas som terapi emot hudcancer är en attraktiv möjlighet.

ABSTRACT

The outermost part of the skin, the epidermis, is built up by cells called keratinocytes. The epidermis provides a vital barrier between the body and the outer world. It is in addition a target for constant physical and microbial injury. Reflecting this, the keratinocytes are not only structural cells but are endowed with a capability to communicate with our immune system in order to eliminate the danger. The constant exposure to the environment also makes the skin vulnerable to carcinogenesis. Squamous Cell Carcinoma (SCC), which originates from the keratinocytes, is the second most common cancer in men, and the third most common in women in Sweden. The transcription factor Nuclear Factor kappa B (NF-κB) plays a crucial role as a coordinator of inflammation, proliferation and apoptosis and is expressed by virtually all cells in our body. Usually, increased NF-κB activity is associated with inflammation and cancer. However, the skin challenges our perspectives on this key player, since both increased and decreased NF-κB activity in the skin, have been linked to the development of inflammatory diseases. Our group has previously shown that inhibition of NF-κB in keratinocytes in mice do not only cause inflammation, but inevitably leads to the development of SCC. Inflammation and rare subungual skin tumours are also seen in the human congenital disease Incontientia Pigmenti (IP). IP is caused by a genetic defect that interferes with NF-κB activation. Our mouse model mimics many aspects of this disease. Inflammation and cancer are intimately linked, and understanding the molecular circuits that set up a pro-tumourigenic inflammatory response is an important task. The work in this thesis aimed to further explore the role of NF-κB in keratinocytes in order to gain new insights to their role in skin immunology and in the development of skin cancer.

Paper I: The proinflammatory cytokine Tumour Necrosis Factor alpha (TNFα) has been implicated in several inflammatory diseases and is thought to be crucial for the promotion of tumourigenesis. In this paper we show that signalling mediated by TNFα downstream of its receptor TNF receptor 1 (TNFR1) is necessary both for the development of inflammation and cancer in response to inhibition of NF-κB in keratinocytes. Moreover, we show that the TNFR1 signalling that initiates the disease occurs in non-immune cells.

Paper II: In this paper, we investigated the role of NF-κB in keratinocytes growth in culture. We show that whereas normal keratinocytes cease to grow when they are exposed to phorbol esters, keratinocytes with inhibited NF-κB continue to grow. Surprisingly, this effect is TNFR1 independent. In addition to the effects on inflammation, this ability may be a contributing factor to the development of skin cancer in response to NF-κB inhibition. We also demonstrate a TNFR1-independent, aberrant response of NF-κB deficient skin to the treatment of phorbol esters *in vivo*.

Paper III: Besides inflammation, inhibition of NF-κB is linked to defects in the formation of ectodermal appendages such as hair follicles and sweat glands. To be able to distinguish the developmental effects of NF-κB inhibition from its postnatal effects on inflammation and to better be able to follow the temporal induction and progression of the disease, we here took advantage of a conditional mouse model. We demonstrate that induction of inflammation after birth is independent from the developmental effects of NF-κB inhibition. Moreover, we show that F4/80+ inflammatory cells (e.g. macrophages) are the first cells to invade the skin after the onset of NF-κB inhibition

and link their infiltration to the cooperative upregulation of three chemokines (MCP-1-3). Furthermore, our data reveal that the macrophages indirectly contribute to the increased angiogenesis in the inflamed skin by producing Vascular Endothelial Growth Factor (VEGFA).

Paper IV: In this paper we demonstrate that TNFR1 expression in keratinocytes alone mediates the inflammatory circuit elicited by inhibition of NF-κB. TNFR1 expression in all other cell types is surprisingly redundant. In addition, we show that TNFR1 expression outside the epithelial compartment in our model of inflammation-driven skin tumourigenesis, is dispensable both for the cellular and molecular signature of the inflammatory response at all stages of tumourigenesis. Our analysis reveals that an inflammatory environment with an immunosuppressive and a tissue remodulatory signature is established early, already in pre-malignant skin. This includes an accumulation of macrophages of an alternative, tumour promoting, M2 type, together with an increase in CD4+CD25+FoxP3+ regulatory T-cells. Unexpectedly, keratinocytes with inhibited NF-κB upregulate the immunosuppressive cytokine Interleukin-10 (IL-10). This may provide a molecular explanation for the early establishment of an immunosuppressive environment in this model.

Taken together, our results shed new light on the unique role of the keratinocytes as messenger cells to our immune system and demonstrate how the NF-κB pathway downstream TNFR1 controls this feature. Our discoveries couple TNFα/TNFR1 to skin cancer progression and opens up the prospective use of anti-TNF in skin cancer treatment.

LIST OF PUBLICATIONS

This thesis is based on the following original articles, which will be referred to in the text by their Roman numerals:

I. **Lind M.H**., Rozell B., Wallin R.P., van Hogerlinden M., Ljunggren H.G., Toftgård R., Sur I. Tumour necrosis factor receptor 1-mediated signalling is required for skin cancer development induced by NF-κB inhibition. (2004) ***Proceedings of the National Academy of Science United States of America.***, 101(14): 4972-7.

II. Sur I., **Ulvmar M**., Jungedal R., Toftgård R. Inhibition of NF-κB signalling interferes with phorbol ester-induced growth arrest of keratinocytes in a TNFR1 independent manner. (2009) ***Journal of Receptors and Signal Transduction***, 29(1): 44-51.

III. **Ulvmar M.H**., Sur I., Mémet S., Toftgård R. Timed NF-κB inhibition in skin reveals dual independent effects on development of HED/EDA and chronic inflammation. (2009) ***Journal of Investigative Dermatology***, Jun 11, Advanced Online Publication.

IV. **Ulvmar M.H**., Wallin R.P., Sur I, Toftgård R. NF-κB inhibition and skin cancer: a viscous circle of chronic inflammation and immunosuppression driven by TNFR1 in kertinocytes. (2009) **Manuscript in preparation**.

CONTENTS

1 INTRODUCTION..... 1
1.1 CANCER..... 1
1.1.1 The definition of cancer..... 1
1.1.2 The interplay between the immune system and the tumour – a seventh hallmark of cancer?..... 1
1.2 THE SKIN..... 4
1.2.1 Squamous Cell Carcinoma (SCC) of the skin..... 5
1.2.1.1 SCC a model of the dual function of the immune system in carcinogenesis..... 6
1.2.1.2 Congenital disorders with increased risk of SCC development..... 6
1.2.1.3 Mutations associated with SCC..... 7
1.2.1.4 Treatments for SCC..... 7
1.3 NUCLEAR FACTOR KAPPA B (NF-κB)..... 8
1.3.1 The NF-κB family..... 8
1.3.2 The IκB family..... 8
1.3.4 Activation of NF-κB..... 9
1.4 TUMOUR NECROSIS FACTOR ALPHA (TNFα) SIGNALLING..... 10
1.5 NF-κB IN THE SKIN..... 12
1.5.1 In ectodermal morphogenesis..... 12
1.5.2 In inflammatory diseases of the skin..... 13
1.5.2.1 Inflammation induced by increased NF-κB activity in the epidermis..... 14
1.5.2.2 Inflammation induced by decreased NF-κB activity in the epidermis..... 14
1.5.3 NF-κB in the growth control of keratinocytes..... 16
1.6 NF-κB IN TUMOURIGENESIS..... 17
1.6.1. NF-κB in skin tumourigenesis..... 18
2 COMMENTS ON METHODOLOGY..... 20
2.1 Transgenic technology targeting the epidermis..... 20
2.2 Conditional transgene expression with the tetracycline controlled systems..... 20
3 AIMS OF THE THESIS..... 21
4 SUMMARY OF THE RESULTS..... 22
4.1.1 Paper I..... 22
4.1.2 Paper II..... 23
4.1.3 Paper III..... 24
4.1.4 Paper IV..... 25
5 DISCUSSION AND FUTURE PERSPECTIVES..... 28
6 ACKNOWLEDGEMENTS..... 33
7 REFERENCES..... 35

LIST OF ABBREVIATIONS

AK	Actinic Keratosis
BHA	Butylated HydroxyAnisole
CDK4	Cyclin-Dependent Kinase 4
CSF-1	Colony Stimulating Factor 1
CYLD	CYLinDromatosis
DD	Death Domain
DMBA	7,12-DiMethylBenz(a)Anthracene
dox	doxycycline
EDAR	EctoDysplasin-A receptor
FADD	Fas-Associated Death Domain protein
FOXP3	FOrkhead boX Protein 3
GCSFR	Granulocyte Colony-Stimulating Factor Receptor
Ha-Ras	Harvey rat sarcoma viral oncogene homologue
HCC	HepatoCellular Carcinoma
IL	Interleukin
IκB	Inhibitor of Nuclear factor kappa B
IKK	IKappaB Kinase
IP	Incontinentia Pigmenti
JNK	c-Jun N-terminal Kinase
MAPK	Mitogen Activated Protein Kinase
MDSC	Myeloid Derived Suppressor Cells
MMP	Matrix MetalloProteinase
NEMO	NF-κB Essential MOdulator (i.e. IKKγ)
NF-κB	Nuclear Factor kappa B
NIK	NF-κB Inducing Kinase
NK	Natural Killer
NLS	Nuclear Localization Signal
NMSC	Non-Melanoma Skin Cancer
PN	Post Natal
RAG	Recombination-Activating Gene
Rel	v-rel avian Reticuloendotheliosis viral oncogene homolog
RHR	Rel Homology Region
RDEB	Recessive Dystrophic Epidermolysis Bullosa
RIP	Receptor-Interacting Protein
ROS	Reactive Oxygen Species
SCC	Squamous Cell Carcinomas
Scid	Severe combined immunodeficiency
Tab2	TAK-1 binding protein
TAD	TransActivation Domain
TAM	Tumour Associated Macrophage
TAK-1	TGFβ Activated Kinase-1
TGFβ	Transforming Growth Factor beta
Th	T helper cell (CD4+)
TNFα	Tumour Necrosis Factor alpha
TNFR	Tumour Necrosis Factor Receptor

TPA	12-0-TetradecanoylPhorbol-13-Acetate
TRAF	TNF Receptor Associated Factor
TRADD	TNF Receptor Associated Death Domain protein
Tregs	Regulatory T-cells
tTA	tetracycline responsive TransActivator
UV	Ultraviolet
VEGFA	Vascular Endothelial Growth Factor A
XP	Xeroderma Pigmentosum

1 INTRODUCTION

1.1 CANCER

1.1.1 The definition of cancer

Cancer is characterized by an atypical, increased growth of cells, giving rise to a tumour literary meaning an abnormal swelling of the flesh. It may also involve the spread of the tumour from one organ to another, a process named metastasis. The Latin term cancer (from the Greek, carcinoma and carcinos) meaning crab fish goes back to observations that were made by the Greek doctor Hippocrates around 460–370 BC. It refers to the swollen veins that can surround solid tumours, resembling legs of a crab sticking out from the shell. Today we know that cancer is caused by genetic and epigenetic changes to our genome. These changes result in the activation of so called proto-oncogenes into oncogenes, promoting carcinogenesis and furthermore in the inactivation of tumour suppressor genes that normally protects the cells against transformation.

In the year 2000, Professor Robert A. Weinberg and Professor Douglas Hanahan presented a seminal model describing six functional capabilities that characterize almost all types of human cancers and which they named “The hallmarks of cancer” (1). These capabilities include: 1. Self-sufficiency in growth signals; 2. Insensitivity to antigrowth signals; 3. Evasion of apoptosis; 4. Possession of limitless replicative potential; 5. Sustainment of angiogenesis, and 6. Facilitation of tissue invasion and metastasis. Importantly, cancer is not one disease but rather a number of different diseases. The characteristics of a particular cancer are governed by the tissue in which it arises and, depending on the origin of the cancer, the pathways to achieve these cancer specific capabilities will differ. Although all cells in our body share the same genome, they differ in epigenetic signature and in their transcriptome (expressed genes). Cells from different tissues and even cells with different functions within a particular tissue, display differential sensitivity to specific mutational changes as well as cell type specific responsiveness to environmental cues (e.g. the oestrogen dependence of some breast cancer tumours is unique for this tumour type and is an effect that directly reflects its origin). Hence, in order to understand the cancer we also have to study and understand the healthy organ in which the cancer arose.

1.1.2 The interplay between the immune system and the tumour – a seventh hallmark of cancer?

Our immune system has a dual role in the biology of cancer: both protecting against cancer development and promoting cancer (2, 3). In 1957 F.M. Burnet, suggested that the immune system can recognize and destroy transformed cells, a process that was later named immunological surveillance (4-6). The immunosurveillance theory has been debated over the years but research during the last decade has provided some supporting evidences (7, 8). This includes experimental data from mice that lack essential immune system components and that show increased susceptibility to the development of spontaneous or chemically-induced tumours. An example is the mice deficient for the Recombination Activating Gene 2 (RAG2), which results in the

absence of T- and B-cells (9). These mice have an increased frequency of both chemically induced sarcomas and spontaneous intestinal neoplasia (10). Additionally, mice that lack the γ-chain of the T-cell receptor, resulting in the absence of γδ T cells, have an increased susceptibility to chemically induced sarcomas as well as chemically induced skin tumours (11). In humans, patients with deficiency in the cytotoxic activity of CD8 T-cells and Natural Killer (NK)-cells, caused by mutations in the perforin gene, are predisposed to the development of lymphomas (12). However, the outcome of the deficiency in specific immune cells and/or in the effector functions of the immune cells appears to vary between different tissues. Hitherto no specific deficiency has been described that gives a general increased cancer susceptibility in all tissues, likely reflecting divergence in the immunology between different tissues. Conversely, it is clear that tumours do arise despite the presence of an intact immune system. This can be explained by a process called tumour escape where the neoplastic cells acquire the capability to escape recognition and/or destruction of the immune cells (7, 8).

In 1863 the German doctor Rudolf Virchow observed that leucocytes were present in the tumours and that cancer can develop from inflammatory hyperplasia (13). Indeed most, if not all, solid malignancies show some degree of immune cell infiltration even without an underlying inflammatory condition. In view of the immunosurveillance theory, this may appear contradictory. However, the critical point is the type of immune response that is elicited in the tumour microenvironment. Together, experimental data from mice and clinical data from humans indicate that a so called type 1 immune response with activation of CD8+ T cells and NK-cells is necessary for the eradication of tumour cells (7, 8).

Type 1 refers to the initial division of CD4+ T-helper (Th) cells into two types: Th1 and Th2 based on their cytokine production (14). For example, Th1 cells produce Interferon gamma (IFNγ) and supports cell-mediated immunity by CD8+ cytotoxic T-cells and NK-cells, whereas Th2 cells produce Interleukin-4 (IL-4) and supports humoral immunity (e.g. production of IgE antibodies by B-cells). Since it has later become clear that other cell types, e.g. macrophages, participate in the polarization of the immune response, by the production of cytokines, the Th1/Th2 concept has sometimes been extended and referred to as type 1 (characterized by e.g. IFNγ, IL-12, IL-2) and type 2 (characterized by e.g. IL-4, IL-5, IL-6) immune responses (15). The immune response in cancer patients often displays features that fits with a type 2 polarization (15-17). However, this is probably a simplification and the Th1/Th2 concept is going through a major revision due to the identification of additional CD4+ T-helper subsets in recent years: the Th17 and the Treg cells. Th17 cells are defined by their expression of IL-17 (18). These cells have a strong pro-inflammatory profile but it is not clear whether they have a dominant function in tumour surveillance or in tumour promotion. The complicated interregulatory circuits between the Th cells where both Th1 and Th2 cells can negatively regulate Th17 development makes the predictions even harder (19, 20). The natural Tregs (regulatory T-cells) are defined by their expression of the Forkhead box Protein 3 (FoxP3) and their ability to repress the proliferation of effector T-cells in a cell-to-cell contact dependent manner *in vitro* (21). *In vivo* their suppressive activities in addition involve the secretion of IL-10 and Transforming Growth Factor beta (TGFβ). FoxP3 negative, regulatory T-cells, named Tr1 and Th3 cells also exist, defined by expression of IL-10 + TGFβ (Tr1) or only TGFβ (Th3). The Tregs have been linked to cancer progression and may be more

critical than Th2 cells (21). The potential role of B-cells in tumourigenesis is largely undefined. Some clues to the role of B-cells were found when it was shown that soluble factors from B-cells (likely to be antibodies) have an important role in a model of HPV16-mediated carcinogenesis in skin by the establishment of a tumour-promoting inflammatory environment with chronic activated innate immune cells (22).

It is well established that chronic activated innate immune cells play pivotal roles in the progression of cancer (2, 3, 23, 24). Two cell types of this innate arm of the immune system have been put into focus in recent years, namely the Myeloid Derived Suppressor Cells (MDSCs) and the macrophages. MDSCs are immature myeloid cells that can display both granulocytic and monocytic properties (25). They are potent suppressors of both innate and adaptive antitumour functions and have been found to accumulate at the tumour site, in lymphnodes, spleen and blood of tumour-bearing hosts. From the same myeloid cellular linage as MDSCs, macrophages can also be seen accumulating at the tumour sites in several human cancer forms and their presence is generally correlated with poor prognosis (26). These cells are often referred to as Tumour Associated Macrophages (TAMs). Similar to T-cells, macrophages can display type 1 (M1 macrophages) or type 2 (M2 macrophages) polarization (27). Whereas M1-activated macrophages kill tumour cells and promote the activation of cytotoxic T-cells, M2 macrophages suppress cell mediated anti-tumour responses. The general consensus is that a M2 type of polarization prevails in established tumours (28, 29). Figure 1 summarizes the key cellular and molecular features, of the immune responses that are associated with progression and regression of cancer.

The TAMs also exemplify how the innate immune infiltrate sourrounding the tumour, in addition to the inhibition of the cell-mediated anti-tumour responses, promotes several aspects of the tumour development by changing the microenvironement (2, 3, 24). TAMs and other immune cells act directly on the tumour cells by the production of growth factors. In addition, in the process of matrix remodulation, angiogenesis and lymphangiogenesis the innate inflammatory cells take a center stage; especially TAMs, but also other cells from the myeloid lineage including neutrophils and mast cells (23). M2-polarized TAMs produce the pro-angiogenic growth factor Vascular Endothelial Growth Factor A (VEGFA) and Matrix MetalloProteinases (MMPs) and thus are particularly potent in aiding the angiogenesis and matrix remodulation around the developing tumour. Matrix remodelling and angiogenesis is also seen in wound healing and, in parts, the distortion of the stroma surrounding tumours, replicates the responses seen in wound healing but with the crucial difference that the resolution of the response is lacking (24). The latter is an important point, putting focus to the chronic part of the inflammatory response seen in progressing cancers, a phenomenon which cannot be explained by the type of polarization but that reflects a dysregulation of the immune response.

We can envision at least two alternatives for the inflammation associated tumourigenesis. In the first scenario the inflammatory response develops as a consequence of the tumourigenesis – a so called tumour-educated immune response, fostered by the Darwinian selection of transformed cells that first can escape immune recognition and second establish a microenvironment that facilitates tumour progression (7, 8). Interesting in this context, it has been shown that prototypical oncogenes, like members of the Ras family (homologs of Harvey or Kirsten rat sarcoma virus oncogenes) and the oncogene MYC (myelocytomatosis oncogene),

which dysregulate the cell cycle machinery, in addition, induce a tumour promoting inflammatory environment (2, 30-32). In another scenario, the inflammatory environment precedes the transformation of cells, causing an increased mutational pressure by inflammation related oxidative stress (2, 3) and/or by the selection of cells with pre-existing mutations (33). Both evasion of immune recognition (7, 8) and cancer related inflammation (34) has been proposed to be the seventh hallmark of cancer. As described above, the two phenomena are overlapping. Clearly, there is still much to learn about the complex cellular and molecular circuits that foster a tumour-promoting inflammatory response.

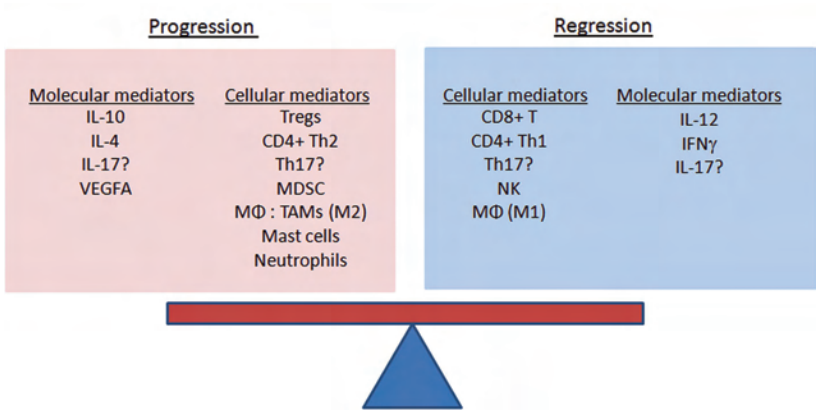

Figure 1: Key cellular and molecular features of the immune response associated with progression and regression of tumours. Inspired by (35).

1.2 THE SKIN

The skin is the largest organ of our body. It forms an anatomical barrier to the external environment, is vital for our regulation of heat in the body and prevents loss of fluids from the body. It is composed of three major layers: an outermost epithelial layer (epidermis) which provides the barrier, a connective tissue layer (dermis) which serves as a location for the appendages of the skin (hair follicles, sebaceous glands and sweat glands), and an adipose layer (hypodermis) that supply the skin with nutrients. The epidermis at the skin surface is a stratified epithelium built up by keratinocytes and it is continually renewed (36). Its proliferative part is located at the innermost basal layer in contact with the basement membrane, which separates the epidermis from the dermis. Periodically keratinocytes in the basal layer withdraw from the cell cycle and start to migrate outward, passing through a series of distinct differentiation stages. The result is seen as the three upper epidermal layers: stratum spinosum, stratum granulosum and the outermost layer stratum corneum, where the terminal differentiated keratinocytes eventually are shed off as dead cornified squames. Whereas human epidermis is multilayered, the dorsal epidermis of the adult mouse encompasses only 1–2 cell layers. However, the basic scheme of differentiation is the same. Mouse tail skin is more similar in structure to that of humans. Histological sections of human and mouse skin

and expression of structural proteins which characterize the different layers of the epidermis are shown in Figure 2 below.

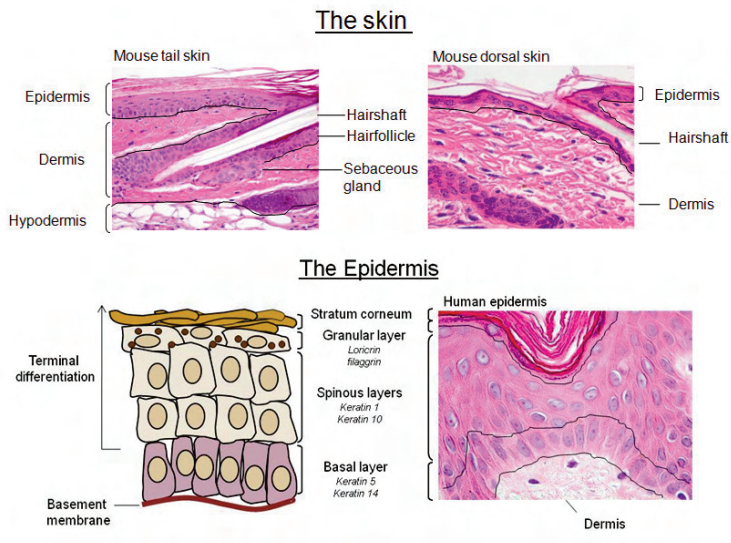

Figure 2. Hematoxylin Eosin (HE) staining of tail and dorsal mouse skin with indicated structures. Below, is a general overview of the structure of the epidermis with a HE staining of human epidermis. The different layers of the epidermis are indicated together with structural proteins characteristic for each layer.

As an interface between the inside and outside the keratinocytes, together with intercalated dendritic cells (langerhans cells) and dendritic epidermal T-cells in mice, are the first cells to encounter invading pathogens. Reflecting this, keratinocytes are endowed with the capability to express multiple cytokines and chemokines as well as antimicrobial peptides (37-39). In addition, they express several receptors of the Toll-like receptor family which recognize pathogen-associated molecules like lipopolysaccaride (LPS), peptidoglycan and CpG oligonucleotides (40, 41). Hence, beside their role as structural cells of the epidermis, the keratinocytes also have the potential to 1. sense a change in the microbial composition of the skin via the TLRs; 2. respond to this by the upregulation of antimicrobial peptide and 3. send out danger signals in the form of chemokines and cytokines that can contribute to the innate and the adaptive immune response against the pathogen or to the response to physical injury.

1.2.1 Squamous Cell Carcinomas (SCC) of the skin

Skin cancer that originates from the keratinocytes is called Non-Melanoma Skin Cancer (NMSC) to distinguish it from malignant melanoma that is derived from the pigment producing melanocytes of the skin. About 80% of all NMSC cases are Basal Cell Carcinomas (BCCs) whereas Squamous Cell Carcinomas (SCCs) stand for about 20% (42). However, whereas BCCs are benign tumours, SCCs, if not detected at an early stage, are potentially aggressive malignant tumours associated with a high risk of

metastasis. The incidence of NMSC (i.e. SCC since BCC has not been included) has been reported since 1958 in the Swedish Cancer Registry, National Board of Health and Welfare. During the last two decades, an average annual increase of 3.2 % has been observed for men and 4.3 % for women (43). It is the second most common cancer in male, third in females and mainly affects the elderly people. Sunlight is the most important risk factor for skin cancer development in general. High chronic cumulative exposure to sunlight appears to be the most important risk factor for the development SCC (44). A benign precursor lesion to SCC is the Aktinic Keratosis (AK). Only, around 1/1000 AK progress to SCC (45).

1.2.1.1 SCC a model of the dual function of the immune system in carcinogenesis

A special high-risk group for development of SCC is the solid Organ Transplantation Recipients (OTRs) that receive immunosuppressive drugs in order to prevent transplant rejection. The OTR patients display an increased risk of NMSC development up to 100 times compared to the control average population (46, 47). Curiously, the risk of malignant melanoma is unchanged and whereas BCCs outnumber SCCs in the general population, SCCs tend to outnumber BCCs in OTR patients. The SCCs in these patients show an aggressive behaviour and it is a major cause of mortality in these patients. It has been suggested that the increase in SCC is a reflection of the repressed immunity in these patients which could interfere with immunosurveillance functions and with anti-viral immunity, although adverse effects by the drugs, directly on the epithelial cells is not fully excluded (46). Whereas a functional immune system seems to be absolutely necessary to protect the body against the development of SCC, the immune system can also foster the development of these tumours as shown by the fact that there is an association between development of SCC and chronical wounds and/or chronic inflammatory disorders. Examples include burn scars (Marjolin´s ulcers), chronic venous stasis ulcers, discoid lupus erythematosus and hidradenitis suppurativa (48-51). SCC in that sense fits very well with the description of tumours as wounds that do not heal (24, 52).

1.2.1.2 Congenital disorders with increased risk of SCC development

Perhaps the most direct connection between a congenital disease and development of SCC comes from patients with Recessive Dystrophic Epidermolysis Bullosa (RDEB) (53). RDEB patients suffer from a blistering skin disease, with development of chronic wounds. It is caused by mutations in the *COL7A1* gene. This gene encodes a keratinocyte-produced type VII collagen which is part of the basement membrane. Although the SCCs in the RDEB patients often are of a highly differentiated type, around half of these patients die due to metastatic SCC. The difference between patients that do develop metastatic SCC and those who do not has been linked to differences in the mutations of the *COL7A1*, which affects the cells ability to anchor itself to the underlying dermis and hence invade the underlying tissue (54). However, the complete aetiology behind the development of SCC in these patients is unknown although the chronic inflammatory disease is likely to contribute. A second group of patients are the Xeroderma Pigmentosum (XP) patients (55). They have defects in nucleotide excision repair or postreplication repair, which makes them very prone to UV-induced DNA damage. The disease is often discovered in early childhood when

they develop severe sunburns in response to very limited sun exposure. The tumour incidence is around 1000 times higher than in the general population. Mouse models of XP indicate that changes in their immunological response to UV may be a contributing factor for the increased cancer formation, with an increased immunosuppression (56). Interestingly, these diseases, again point back to the connection between, chronic inflammation on the one side (RDEB) and immunosuppression on the other side (XP) in the development of SCC.

1.2.1.3 Mutations associated with SCC.

Mutations in the tumour suppressor gene *TP53* (Tumour Protein 53), which encodes the transcription factor p53, have been found both in the benign precursor lesions of SCC, the Actinic Keratoses (AK), and in fully developed SCC (42). However, mutations in *TP53* are also found to a very high frequency in normal human epidermis (57). Moreover, patients with the Li-Fraumeni syndrome caused by a germline mutation in *TP53* (58) and p53 knockout mice do not display increased risk for SCC formation (59). Hence, a mutation in or loss of *TP53* is not solely sufficient to cause AK or SCC and is unlikely to be the initiating factor. P53 could instead be important for later stages of the tumourigenesis since loss of p53 increases the rate of malignant conversion of chemical induced skin cancer in mice (60). This would correspond to the transition between AK to SCC in humans.

In chemical-induced skin cancer in mice, application of the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) induces prototypical mutations in the mouse Ha-Ras gene (61). However mutations in *HRAS* or the closely related *KRAS* and *NRAS* does not appear to be very common in human SCCs and thus can only explain a limited subsets of SCCs (62-64).

Mutations in exon 2 of the CDKN2A locus which encodes both p16/INK4A and p14/ARF have been found in SCC (65, 66). Congenital mutations in this locus have been linked to familial malignant melanoma (67). These patients however do not have an apparent increased risk of SCC development and, like mutations in *TP53*, mutations in *CDKN2A* could be a factor that is involved in later progression, rather than initiation, of the cancerous growth.

1.2.1.4 Treatment for SCC

AKs can be treated with cryotherapy or by topical application of the cytostatic drug 5-Fluoruacil 1%. New methods for treatment of AK also involve the use of topical imiquimod 5% cream (Aldara^{TM}) (68). It has agonistic effect on the Toll-like receptors 7 and 8, and induces both immunological and non-immunological responses. The induction of a Th1 type of immune response is thought to be one important arm of its anti-tumour effects. Treatment with imiquimod on SCCs has been associated with recruitment of CD8+ cytotoxic T-cells into the lesions and to increase effector functions of the T-cells (69, 70). Radical surgery is used for developed SCC to prevent metastasis. Metastatic SCCs have a relatively poor prognosis and treatment may involve radiation, lymph-node dissection and systemic chemotherapy (42).

1.3 NUCLEAR FACTOR KAPPA B (NF-κB)

1.3.1 The NF-κB family

NF-κB was discovered in 1986 as a factor that bound to an enhancer element of the immunoglobulin (Ig)κ light-chain gene (71). It was initially thought to be a B-cell specific factor but it has later become clear that it is expressed in virtually all the cell types. Today, the name NF-κB refers to a whole family of transcription factors that can both induce and repress gene expression (72). NF-κB has been shown to have an essential role in the regulation of immune responses and for protection against apoptosis in the cell (73, 74). Depending on the tissue, it also regulates growth and organogenesis, and the number of NF-κB target genes is an ever growing list (75). In mammalian cells, five NF-κB members have been identified: p50/p105 (*NFKB1*) (76), p52/p100 (*NFKB2*) (77, 78), p65 (*RELA*) (79, 80), RelB (*RELB*) (81) and c-Rel (*REL*) (82). The different members form homo- and hetero-dimeric complexes. Only p65, RelB and c-Rel contains transactivation domains (TADs). If not combined with TAD-containing factors, the p50 and p52 subunits will be transcriptionally repressive. The general DNA consensus sequence for NF-κB binding is 5´GGGRNNYYCC 3´(N= any base; R=purine; W=adenine or thymine; and Y=pyrimidine) (83). All NF-κB proteins share a 300-amino acid Rel Homology Region (RHR), which is composed of two Immunoglobulin (Ig)-like domains. The RHR mediates the dimerization, DNA binding and the interaction with inhibitory proteins of the IκB family.

1.3.2 The IκB family

The IκB family consists of IκBα (*NFKB1*) (84), IκBβ (*NFKBIB*) (85), IκBε (*NFKBIE*) (86), Bcl-3 (*BCL3*) (87), IκBζ (*NFKBIZ*) (88, 89), and the precursors of p50 – p105 and p52 – p100. In addition, in mice an alternative transcript of the *NFKB1* gene encodes IκBγ, which is identical to the C-terminus of p105 (90). All IκBs contain either six or seven ankyrin repeats which are stacked helical domains that can mediate the binding to the RHR domain in NF-κB (72). Only IκBα, IκBβ and IκBε contains N-terminal regulatory regions, which are required for degradation in response to receptor signalling. IκBα was the first IκB member to be cloned and is still the best characterized. It can bind multiple NF-κB dimers containing p65 or c-Rel, but the p65/p50 heterodimer is the primary target in most cells. Binding of IκBα to p65/p50 is responsible for the primarily cytoplasmic retention of p65/p50 in the cytoplasm. However, rather than a static confinement to the cytoplasm, the IκBα/p65/p50 complex is constantly shuttling in and out of the nucleus (91). The explanation for this phenomena is that IκBα masks the NLS in p65 whereas the NLS in p50 remains exposed and the shuttling is the result of a constant competition between the p50 NLS and the Nuclear Export Signal (NES) of IκBα.

In response to a stimulus, IκBα is phosphorylated on the two conserved serines 32 and 36, leading to polyubiquitination which targets IκBα to the degradation by the 26S proteasome and allows the transfer of the NF-κB subunits to the nucleus. IκBα is a target gene of NF-κB which creates an autoregulatory feedback loop that can contribute to the termination of the response (92, 93). IκBβ and IκBε have similar capability as IκBα to bind and retain NF-κB complexes in the cytoplasm but they differ in their temporal degradation and resynthesis (86, 94). To further complicate the picture,

removal of all three (IκBα, IκBβ and IκBε) does not shift the localization of NF-κB but increases basal NF-κB transcriptional activity (95). Thus, the cytoplasmic retention model could be a simplification of how the IκBs exert their inhibition of NF-κB.

The atypical IκB proteins Bcl-3 and IκBζ do not function as negative regulators of NF-κB. Instead, Bcl-3 contains a TAD and can bind p50- or p52- homodimers, which confers transcriptional activity to these otherwise repressive NF-κB dimers (96, 97). IκBζ is more similar to Bcl-3 than to the other IκBs. It associate primarily with p50 homodimers and appears to act as a coactivator, probably through recruitment of additional proteins since it does not contain a TAD (98).

1.3.3 **Activation of NF-κB**

There are a number of pathways that can lead to the activation of NF-κB (72). The typical activation that leads to the phosphorylation of IκBα is referred to as the classical or canonical pathway and is dependent on the activation of the IKappaB Kinase (IKK) complex. The IKK complex is composed of two homologous kinase subunits IKKα/IKK1 (*CHUK*) and IKKβ/IKK2 (*IKBKB*) (99-101) and a regulatory subunit IKKγ/NEMO (NF-κB Essential MOdulator) (*IKBKG*) (102, 103). Although part of the IKK complex, IKKα is redundant for the classical NF-κB signalling, which only requires IKKγ and the kinase activity of IKKβ (104). IKKα instead is responsible for the alternative or non-canonical NF-κB activation, which specifically activates p52/RelB heterodimers (105, 106). This involves the activation of IKKα homodimers by NF-κB Inducing Kinase (NIK). The activated IKKα homodimers then phosphorylate p100 which undergoes proteolytic processing to generate the p52 subunit of NF-κB.

A large number of different stimuli can result in the activation of NF-κB including bacterial and viral products, inflammatory cytokines and physical and chemical stresses (75). The classical pathway is activated by a broad range of receptors whereas the alternative pathway is activated downstream of a more restricted number of receptors. In the nucleus the different NF-κB dimers differ in their affinity to defined NF-κB sites but this is only one layer of specificity. DNA binding, stability and activity is further regulated by post translational modification of the NF-κB proteins, including phoshorylation and acetylation (107). The basic scheme of classical and alternative NF-κB activation is presented in Figure 3.

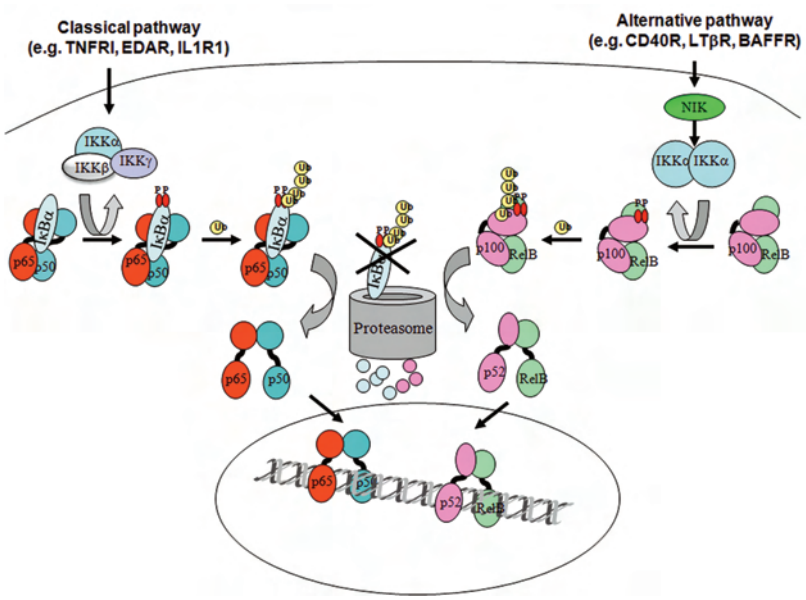

Figure 3: Activation of NF-κB via the classical and the alternative pathway. In the classical activation, receptor-mediated activation of the IKK complex results in phosphorylation, ubiquitination, and degradation of the I*κ*B*α* protein. This allows the translocation of NF-*κ*B dimers into the nucleus. The alternative pathway is regulated by IKK*α* homodimers that can be activated by the upstream kinase NIK. Activation of the IKK*α* homodimers leads to the phosphorylation, ubiquitinylation, and processing of the p100 precursor protein allowing nuclear translocation of p52/RelB complexes.

1.4 TUMOUR NECROSIS FACTOR ALPHA (TNFα) SIGNALLING

Tumour Necrosis Factor alpha (TNFα) signalling is a prototypical example of classical NF-κB signalling and in addition provides an example of the crosstalk between NF-κB and other signalling pathways. TNFα was, as the name implies, discovered as a factor that could induce necrosis of sarcomas (108). However, later research has shown that this proinflammatory, multifunctional cytokine, in most cases, promotes rather than inhibits carcinogenesis. Whereas TNFα cannot be detected in the serum from normal healthy persons it is detected in sera from cancer patients and TNFα production is associated with poor prognosis and cachexia (109). Besides cancer, TNFα has also been coupled to several inflammatory diseases e.g. rheumatoid arthritis and inflammatory bowel disease (110).

TNFα can be produced by multiple cell types; e.g. macrophages, neutrophils, fibroblasts and keratinocytes (109, 110). It is a homo-trimeric type II transmembrane protein and can be active both as a membrane form and as soluble protein after release from the cell surface by proteolytic cleavage. TNFα exerts its effects through two receptors: TNFα receptor I (TNFR1), also known as p55/CD120a/Tnfrsf1a (111, 112), and TNFα receptor 2 (TNFR2) also known as p75/CD120b/Tnfrsf1b, (113); both of which, like TNFα itself, are active as homo-trimers. Whereas the extracellular domains

of the receptors are homologous, the intracellular domains differ. The intracellular cytoplasmic tail of TNFR1 contains so called death domains (DDs) which are lacking in TNFR2 and which allow the interaction with other DD-containing proteins. TNFR1 is thought to be responsible for most of the biological effects by TNFα.

The signalling network downstream of TNFR1 is complex and involve activation of multiple, parallel and inter-regulated signalling pathways (72, 114). As mentioned before, central to this network is the activation of NF-κB. Ligand binding to TNFR1 leads to the recruitment of DD-containing adaptors. Binding of the TNFR-Associated DD protein TRADD allows the subsequent recruitment of Receptor Interacting Protein-1 (RIP-1), a serine/threonine kinase, and TNFR-Associated Factor-2 or -5 (TRAF-2/-5), which are E3 ubiquitin ligases (115, 116). TRAF-2/-5 with RIP-1 allows the recruitment and activation of the IKK complex which leads to the downstream activation of NF-κB (117). The signalling to IKK downstream of TRAF-2/-5/RIP-1 depends on the recruitment of Mitogen-Activated Protein 3 Kinases (MAP3Ks), e.g. the TGFβ-activated kinase (TAK-1). Absence of TAK-1 abolish NF-κB activation downstream TNFR1 (118, 119). Whether TAK-1 directly phosphorylates IKK or acts through other kinases is not clear.

By the recruitment of MAP3Ks, like TAK-1, the TRAF-2/-5/RIP-1 arm of the TNFR1 signalling also leads to MAP2K-activation and downstream activation of c-Jun N-Terminal kinase-1 (JNK-1) and p38 MAPKs (114, 118, 119). Activated JNK-1 phosphorylates several transcription factors including c-Jun, a subunit of the transcription factor Activating Protein-1 (AP-1), which leads to increased AP-1-mediated transcriptional activation.

In parallel to the MAP3K/TAK-1 mediated signalling, TRADD can also recruit Fas-associated DD protein (FADD) (115, 120) with subsequent recruitment and activation of pro-caspase 8 by self-cleavage. Activated caspase-8 initiates a downstream cascade of caspase activation leading to cell death by apoptosis (121). In the presence of NF-κB the induction of apoptosis downstream TNFR1 is blocked by a dual mechanism: 1. NF-κB induces transcription of several inhibitors of the caspases (e.g. cFLIP and the IAPs) (73) preventing the activation of caspases downstream FADD and 2. NF-κB prevents prolonged JNK activation which can contribute to the TNFα-induced cell death in some cases (114). The mechanism behind the inhibition of JNK-1 by NF-κB has not been fully delineated but may involve inhibition of the production of Reactive Oxygen Species (ROS) which otherwise leads to prolonged JNK activation by the inhibition of multiple mitogen-activated protein phosphatases (122, 123). A simplified summary of the key signalling pathways activated downstream TNFR1 and how they are inter-regulated are shown in Figure 4.

TNFα/TNFR1/2 belongs to a large TNF/TNFR superfamily of related ligands and receptors, with important roles in immune-regulation and development (124). Examples includes; BAFF/BAFFR, CD40L/CD40, EDA/EDAR, FAS/FASL and LTα/HVEM. The TNF-related ligands can show overlapping specificities. For example, besides TNFα, TNFR1 and TNFR2 can bind LTα. The receptors of the TNF/TNFR superfamily share intracellular signalling pathways and NF-κB activation is a common theme to most of these members.

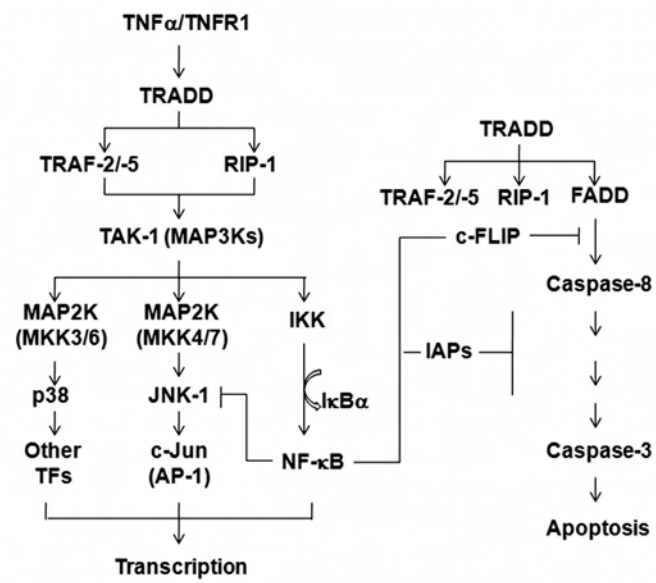

Figure 4: Simplified scheme over the major TNFR1-mediated signalling pathways. Activation of NF-κB plays a central role in the regulation of these signalling circuits, by preventing prolonged JNK-activation and by regulating the expression of inhibitors of apoptosis including c-FLIP and the IAPs. Adapted from (114).

1.5 NF-κB IN THE SKIN

1.5.1 In ectodermal morphogenesis

The ectodermal appendages, including the teeth, hair, nails and the sweat glands arise by an intricate crosstalk between the epithelium (i.e. epidermis) and the underlying mesenchyme during the development of the organism (125). NF-κB has a central role in the ectodermal morphogenesis downstream of the EctoDysplasin-A Receptor (EDAR), which is a member of the TNFR superfamily. Mutations in EDAR *(autosomal inheritance)*, the downstream adaptor protein EDARADD *(autosomal inheritance)* or the ligand Ectodyplasin-A *(X-linked inheritance)*, are associated with congenital Anhidrotic Ectodermal Dysplasia (EDA) and Hypohidrotic Ectodermal Dysplasia (HED) in humans (126-128). Patients with EDA/HED lack or display hypoplasia of distinct exocrine glands including the sweat glands (129).

The mouse homologues for EDAR, EDARADD and Ectodysplasin-A are *downless*, *crinkled* and *tabby* respectively (128, 130-132). Instead of the normal four types of pelage hair (guard/monotrich, awl, zigzag hair and the rare auchenes), which is found in wild type mice, these mice have abnormal awl hairs and completely lack guard hairs and zigzag hairs (133). An area of naked skin is found behind the each ear; due to the fact that zigzags are the only hair type found there in the normal mouse. The tail generally lacks hairs or has reduced numbers of hairs. The *downless/crinkled/tabby* mice also display reduced number of vibrissae and they lack multiple exocrine glands including the sweat glands and meibomian glands of the eyes (134). In addition, they have malformed teeth (135). Overall, the phenotype in these mice including the

reduced hair, the malformed teeth and the defects in glands is very much overlapping with the clinical manifestations of HED/EDA in humans.

NF-κB was genetically linked to EDAR-signalling when it was shown that hypomorphic mutations in IKKγ, which is the regulatory subunit of the IKK complex, cause a special form of X-linked EDA with immunodeficiency (EDA-ID) (136-138). Besides the ectodermal defects, EDA-ID patients display impaired innate immune responses, as a consequence of their NF-κB deficiency. In addition, mutations in IκBα from two individual patients have been associated with an autosomal dominant form of EDA with T-cell deficiency (AD-EDA-ID) (139, 140). The genetic data is further supported by experimental data which reveal that EDAR activates the canonical NF-κB pathway via the IKK complex and IκBα (141).

In mice, the connection between EDAR/(i.e. mouse downless) and NF-κB was demonstrated in 2001 by the expression of a superrepressor form of IκBα from the β-catenin loci ($c^{I\kappa B\alpha\Delta N}$ mice) which gave rise to a similar phenotype as in *downless/crinkled/tabby* mice (142). This include: lack or hypoplastic sweat glands, no meibomian glands and defects in the hair and the teeth. β-catenin is ubiquitously expressed and $c^{I\kappa B\alpha\Delta N}$ mice have multiple immune defects, similar to the EDA-ID and AD-EDA-ID patients . Interestingly, the effect on the hair coat is stronger in $c^{I\kappa B\alpha\Delta N}$ mice, as compared to *downless/crinkled/tabby* mice, indicated by a decrease in the number of hair follicles. Recently, it was shown that additional signals besides Ectodysplasin-A/EDAR appear to regulate NF-κB in the formation of zigzag hairs (142). Candidates for this include the receptors XEDAR and TAJ/TROY (143-145). Both belong to the TNF receptor family and can activate NF-κB.

Further connections between the NF-κB pathway and ectodermal morphogenesis include data from TNFR-associated factor 6 (TRAF6) deficient mice which display defects in hair follicle formation and exocrine glands, similar to *downless/crinkled/tabby* mice (146). TRAF6 has been associated with signalling downstream of both the EDAR and the XEDAR receptor (146, 147). A link down to TAK-1 has also been found via the binding of the protein Tab2 (Tak1-binding-protein 2) (147). Studies of embryonic mouse skin have shown that ablation of both p65 and c-Rel, is required to see the defects in hair follicle and tooth formation (148). In summary, a canonical signalling pathway involving Ectodysplasin-A – EDAR – EDARADD – TRAF6 – Tab2 – Tak-1 – IKK – IκBα –NF-κB/(p65/c-rel) in hair, teeth and gland development can be delineated. The transcriptional targets of NF-κB downstream EDAR is still largely unknown. EDAR/NF-κB has been placed upstream of the induction of the morphogen Sonic Hedgehog (Shh) in the formation of hair follicles (142). It is though not formally proven yet that Shh is a direct target of NF-κB in this setting.

1.5.2 **In inflammatory diseases of the skin**

The prevailing view of the role of NF-κB in inflammation has been that it functions as a pro-inflammatory factor. Indeed, multiple data supports that it is positively involved in the regulation of multiple cytokines and chemokines and increased NF-κB activity is associated with inflammatory diseases. The epidermis of the skin provides an exception from this rule. Both increased and decreased NF-κB activity can cause inflammatory

skin disease and the balance of NF-κB activity in keratinocytes appears to be crucial (149-152).

1.5.2.1 Inflammation induced by increased NF-κB activity in the epidermis.

The first reports of a NF-κB mouse model with inflammation in the skin was published by the group of D. Baltimore in 1995 and C.L. Stewart in 1996 (153, 154). They showed that increasing NF-κB activity by deletion of the IκBα gene in mice, resulted in inflammation that, although IκBα is ubiquitously expressed, only manifested in the skin. The only additional defect in the mice was a dysregulated myelopoiesis. The *IκBα*$^{-/-}$ mice are normal at birth but within 3 days PN the inflammatory skin disease starts to develop. Death occurs within 7–10 days. This can be delayed to an age of 3 weeks if the mice are transferred to a p50 knockout background. It has been possible only recently, with the use of conditional knockout animals to determine which cell types mediates the skin disease in *IκBα*$^{-/-}$ mice.

In 2007 Rebholz *et al.*, showed that deletion of IκBα in keratinocytes alone resulted in hyperplasia and dermal inflammation, which improved by week 3 after birth (149). However, if IκBα was concomitantly deleted in T-cells the infiltration of inflammatory cells extend to involve the epidermis as well and the disease progressed beyond 3 weeks of age. In addition, they demonstrated that deletion of p65 from the keratinocytes completely ameliorated the development of the disease in the mice and that inflammation was dependent on the cytokines of the TNF superfamily: TNFα, LTα, LTβ. Hence, the disease critically depended on a cross talk between keratinocytes and T-cells with increased NF-κB activity which appears to function downstream of the TNFα and LTα/β pathways.

T-cells are also involved in the human inflammatory skin disease psoriasis, which has many common features with the *IκBα*$^{-/-}$ model. Interestingly, increased NF-κB activity (155, 156) or dysregulated NF-κB responses (157, 158) have been reported in samples from psoriatic patients. These reports contain conflicting data, especially concerning the distribution of the NF-κB subunits in normal and psoriatic skin, warranting further studies.

1.5.2.2 Inflammation induced by inhibition of NF-κB activation in the epidermis.

In 1999, R. Toftgårds group demonstrated that overexpression of a non-degradable, mutated form of IκBα in basal keratinocytes of the epidermis (*K5-IκBα* mice) also induces an inflammatory skin disease (159). These data have later been corroborated by epidermal specific knockouts of the IKK subunits IKKβ (152) (designated *IKKβ*$^{\Delta K14/\Delta K14}$) and IKKγ (150) (designated *IKKγ*$^{\Delta K14/\Delta K14}$). All these models have an inhibition of the classical NF-κB pathway specifically in the keratinocytes. The onset of inflammatory disease depends on the genetic background (unpublished data Ulvmar M.H.), but is never seen before day 3 PN, similar as in *IκBα*$^{-/-}$ mice. In *IKKγ*$^{\Delta K14/\Delta K14}$ and *K5-IκBα* mice the inflammation is accompanied by an increased apoptosis (150, 159, 160), which is not seen in *IKKβ*$^{\Delta K14/\Delta K14}$ mice (152). Common to all three models is a massive infiltration of granulocytes and macrophages/monocytes along with a hyperproliferative response of the kertinocytes (150-152, 160). In contrast to IκBα deficient mice, conventional T-cells (i.e. αβ T lymphocytes) are redundant for development of disease in *IKKβ*$^{\Delta K14/\Delta K14}$ mice (152). Further, crossing of the

IKKγ$^{\Delta K14/\Delta K14}$ mice to RAG1-deficient mice (which will delete both αβ T-cells and the γδ T-cells in the epidermis and the B-cells (161)) cause only a short 1-2 day delay in the onset of the disease but does not prevent development of inflammation (150). Instead of T-cells, the myeloid cell linage appears to be required for the phenotype. Stratis *et al*., demonstrated that clodronate liposome treatment, which depletes phagocytotic cells (e.g. macrophages), attenuates the disease in the *IKKβ*$^{\Delta K14/\Delta K14}$ model and that the depletion of phagocytic cells also indirectly reduced the infiltration of granulocytes and T-cells in this model (162).

All mouse models with decreased activity of NF-κB in the skin can be rescued if the mice are crossed to a TNFR1 knockout background (150-152). This abolishes both the hyperproliferation and the inflammation. Hence, although the immunological aetiology is different from the *IκBα*$^{-/-}$ model they share a causative link to TNFα. The role of TNFR1 in the inflammatory disease induced by inhibition of NF-κB in the epidermis forms the basis of this thesis and will be discussed in detail later.

The *IKKγ*$^{\Delta K14/\Delta K14}$ model links the mouse models of epidermal NF-κB inhibition to the congenital human inflammatory skin disease Incontinentia Pigmenti (IP). As described in section 1.5.1, hypomorphic mutations in IKKγ causes HED/EDA in humans (136-138). Loss of function mutations in IKKγ is instead the reason behind development of IP (163, 164). The mutations are lethal in males, which reflect the crucial role of IKKγ in NF-κB activation. Heterozygous females survive and due to the inactivation of one of the two X-chromosomes they are functionally chimeras for the mutated allele. Similar to HED/EDA patients, females with IP display variable abnormalities of the skin, hair, teeth and eyes (165). However, the dermatological pathology also involves hyperpigmentation and development of inflammation (165). Mice with ubiquitous deficiency in IKKγ mimic the human disease (166, 167). It has not been clear to what extent the disease is caused by NF-κB deficiency in the keratincytes but that inflammatory skin lesions can be induced solely by the IKKγ deletion in the epidermis of *IKKγ*$^{\Delta K14/\Delta K14}$ mice (150) infer this is likely to be the underlying cause of the disease in human IP patients.

IKK-IκBα is coupled to the activation of p65 and c-rel containing NF-κB dimers that share the common partner p50. Mice lacking p65 die during embryogenesis due to massive liver apoptosis (168). The liver apoptosis is prevented if the mice are crossed a TNFR1 knockout background (169). As described above, TNFR1 deficiency will ameliorate any effects on the skin since the inflammation and hyperplasia due to NF-κB inhibition in keratinocytes depend on TNFR1. *c-Rel*$^{-/-}$ and *p105/p50*$^{-/-}$ mice survive to adulthood but exhibit severe defects both in innate and adaptive immunity reflecting their essential role for the normal function of B- and T-cells and dendritic cells (170-175). In these mice, no skin phenotype has been reported. However, embryonic skin deficient for both p65 and c-rel and TNFα when transplanted onto immunodeficient mice was shown result in the induction of inflammation (148). The same is not true in similar experiments for skin deficient in p65 alone, which was reported to result in hyperplasia but without inflammation (176). This suggests that c-rel accounts for the immunosuppressive role of NFκB in keratinocytes whereas p65 controls proliferation. The possible role of p65 in keratinocyte proliferation will be discussed further in the next section 1.5.3.

The NF-κB subunit RelB is not activated by the canonical IKK-IκBα pathway but depends on IKKα via the alternative, non-canonical NF-κB pathway (105, 106). RelB deficiency results in multiorgan inflammation, involving also the skin with similarities to human dermatitis (177, 178). The disease is dependent on T-cells (177, 179). At this point it is unknown what contribution RelB deficiency in the keratinocytes has to the phenotype, since no transplantation experiments or skin epidermal deletions have been made. Until it has been tested experimentally, it therefore remains a possibility that both canonical IKK-IκBα-p65/c-rel and non-canonical IKKα-RelB signalling plays a role in skin immune regulation mediated by keratinocytes.

1.5.3 In the growth control of keratinocytes

Hyperproliferation of the epidermis is a common theme in mice with inhibited NF-κB activity in the keratinocytes. Still, keratinocytes from *K5-IκBα*, *IKKβ*$^{\Delta K14/\Delta K14}$ or *IKKγ*$^{\Delta K14/\Delta K14}$ mice do not show increased proliferation in culture *in vitro* and keratinocytes from *IKKβ*$^{\Delta K14/\Delta K14}$ mice was even reported to have a decreased proliferation compared to wild type cells (150, 152, 180). This would imply that the hyperproliferation seen *in vivo* is not cell autonomous and depend on signals from surrounding cells, very likely inflammatory cells. However, several reports from the lab of Paul A. Khavari suggest that NF-κB could have a cell autonomous effect. Firstly, overexpression of either p65 or p50 was shown to cause growth arrest of keratinocytes *in vitro*, which *in vivo* manifested as hypoplasia of the epidermis (181). Secondly, they reported that transplantation of *p65*$^{-/-}$ embryonic skin result in epidermal hyperplasia but without any inflammatory response (176). Notably, keratinocytes from *p65*$^{-/-}$ deficient mice displayed increased proliferation *in vitro*. Third, human genetically engineered epidermis expressing Non-Degradable mutant IκBα (IκBαND) was likewise reported to display hyperplasia, independent of inflammation, when transplanted to immunodeficient *scid/scid* mice (176, 181). Similar to the *K5-IκBα*, *IKKβ*$^{\Delta K14/\Delta K14}$ or *IKKγ*$^{\Delta K14/\Delta K14}$ mouse models it was demonstrated that the hyperproliferation *in vivo* in both p65 deficient embryonic mouse skin and IκBαND expressing human keratinocytes was dependent on TNFR1 (176). In addition, the response was linked to an activation of JNK1/2 and downstream upregulation of the Cyclin Dependent Kinase 4 (CDK4) (176, 182). Pharmacologic inhibition of JNK or CDK4 ablation abolished the hyperproliferation. Increased JNK activation is also seen in *K5-IκBα* and *IKKγ*$^{\Delta K14/\Delta K14}$ mice but is secondary to the inflammation and upregulation of CDK4 is a late rather than an early event in hyperplastic *K5-IκBα* epidermal keratinocytes (150, 151).

In contrast to the transplanted embryonic *p65* $^{-/-}$ skin, it was recently reported that p65 epidermal specific knockout mice neither display hyperplasia nor inflammation (149). It can be inferred that p65 exerts an inducible role in the growth control of keratinocytes in response to stress such as inflammation or Ras expression (see below section 1.6.1). The grafting of skin onto the *scid/scid* mice could be considered as stress and should involve at least a transient wound response around the graft. *In vitro* stress may be dependent on differences in culture conditions. Challenge of the p65 epidermal specific knockout model would discard or prove this model.

Of note, both *p65*$^{-/-}$*/c-Rel*$^{-/-}$ *Tnfα*$^{-/-}$ and *IKKγ* deficient embryonic skin display decreased proliferation before birth that could be explained by a delay to progress from G1 to S-phase (148, 150). The biology of the embryonic mouse skin compared to the adult differs in many aspects and these data infer that the role of NF-κB may change over time. The transient dermal inflammation during the first 3 weeks of life which later resolves in mice with keratinocyte specific deletion of IκBα, could also be a reflection of this (149).

1.6 NF-κB IN TUMOURIGENESIS

NF-κB has been suggested to be a tumour promoter in several human malignancies. This has been based both on genetic data and on the constitutive NF-κB activity observed in a number of human tumours (183, 184). Since NF-κB targets such a broad range of different genes it can potentially affect all hallmarks of cancer. During recent years, progress in our understanding of the role of NF-κB in cancer has been obtained from the use of transgenic and conditional knockout mouse models in which it is possible to specifically intervene with the pathway in a specific tissue and even in a specific cell type within that tissue. Below are some examples of this strategy.

Deletion of IKKβ in hepatocytes result in an increased sensitivity for chemical induced HeptoCellular Carcinoma (HCC) (185). The response was coupled to an increased JNK activation, formation of ROS and cell death which elicited a cytokine-driven compensatory proliferation of the hepatocytes. Concomitant deletion of IKKβ in Kupffer cells (special type of macrophages in the liver) could decrease the susceptibility to cancer, as could treatment with the antioxidant Butylated Hydroxyanisole (BHA). Deletion of IKKβ in adult hepatocytes does not cause a complete inhibition of NF-κB activation and does not cause any spontaneous liver disease. In contrast, deletion of IKKγ in liver parenchymal cells was recently reported to cause spontaneous development of HCC (186). Again the disease was driven by an increased cell death coupled to compensatory proliferation of the liver cells and again, attenuation in response to BHA was reported. Upregulation of TNFα from the Kupffer cells was suggested to be implicated in both above models of liver cancer but its' role was not formally proven. In an additional liver model, NF-κB inhibition was induced by a non-degradable form of IκBα in a mouse model of spontaneous hepatitis and HCC (*Mdr2* knockout mice) (187). In this case IκBα could not affect tumour formation in early stages of tumourigenesis but did so in late stages by inducing apoptosis. Anti-TNF antibodies or IκBα expression had similar effects. The differences between these models can be explained by differential grade of NF-κB inhibition and by the use of different model systems.

Deletion of intestinal IKKγ has been linked to spontaneous defects in intestinal homeostasis, coupled to an increased apoptosis downstream TNFR1 – no development of cancer was reported in this model (188). Similar to the liver, deletion of IKKβ did not cause spontaneous disease, but has been evaluated for chemical induced colitis associated cancer (189). IKKβ deletion in intestinal cells leads to a decreased cancer incidence, although it did not affect the size of the tumours. This was linked to an increased inflammatory and apoptotic response to the tumour promoter dextran sulfate sodium salt (DSS). Conversely, IKKβ deletion in myeloid cells did not decrease

tumour incidence but decreased the tumour size, probably because it decreased the induction of pro-inflammatory cytokines from these cells.

These models illustrate the difficulty of predicting the effects of NF-κB inhibition in the development of cancer and show that the response may differ both depending on the tissue and depending on the timing. They also tell us that the extent of NF-κB inhibition i.e. IKKβ versus IKKγ can be an important factor.

1.6.1 NF-κB in skin tumourigenesis

As described earlier, mice with epidermal NF-κB inhibition develops a TNFR1-dependent chronic inflammation with hyperplasia of the epidermis (150-152). In mice with IκBα mediated inhibition of NF-κB (*K5-IκBα*) the hyperplasia progresses to Squamous Cell Carcinomas, (SCC), with 100% penetrance (159). Deletion of TNFR1 abolishes the inflammatory reaction, the hyperplasia and the development of cancer in these mice (151). The interplay between NF-κB and TNFR1 in this model is the subject of this thesis and will therefore not been discussed in further detail. IKKβ and IKKγ deficient mice die within 10 days after birth (150, 152). It is therefore unknown if they are prone to spontaneous skin cancer development. However, as described earlier, the IKKγ knockout mouse links these models to the human congenital and X-linked disease IP, which involves inflammation of the skin. It is interesting to note that subungual tumours and cases of keratoacanthomas and SCC have been described as late manifestations of IP (190-197). The subungual tumours often appear after puberty and often with multiple lesions (165). They are usually benign, but can be difficult to classify and are very painful. Due to the limited number of patients with IP and their diverse disease manifestations there is no statistical data available for the associated skin cancer risk. The inflammatory skin lesions in IP usually resolve and the keratinocytes, which express the mutant form of IKKγ are replaced by keratinocytes in which the mutated X-chromosome is inactivated. The latter phenomena likely explain why tumours are rare in IP-patients.

Other clues about the possible role of IκBα mediated NF-κB inhibition in human skin cancer have been obtained from experiment with human keratinocytes transplanted to immunodeficient *scid/scid* mice. Firstly, expression of IκBα could overcome the growth inhibitory effect of oncogenic Ha-Ras (Gly12Val) in human keratinocytes cultured *in vitro* (198). Secondly, when these cells where transplanted to *scid/scid* mice they gave rise to neoplasia that resembled human SCC. Ha-Ras alone resulted in hypoplasia and IκBα alone resulted in only mild hyperplasia. This effect, similar to the *K5-IκBα* model, has been demonstrated to depend on TNFR1. In addition it has been coupled to an IκBα mediated stabilization of the CDK4 protein and an increase in activated JNK mediating activation of the AP-1 (c-Jun) transcription factor (198, 199). The activation of JNK was in this system associated with the MAPK kinase MKK7 (199). Similar to IκBα, expression of MKK7 could restore the levels of CDK4 in response to Ha-Ras induced growth arrest and co-expression of MKK7/Ha-Ras in human keratinocytes resulted in the formation of SCC when transplanted to *scid/scid* mice. It can be inferred that the TNFR1/MKK7/JNK/AP-1 signalling cascade elicits proliferative signals in keratinocytes that are counteracted by NF-κB and that together with Ha-Ras leads to transformation of these cells.

Another link between NF-κB and non-melanoma skin cancer comes from patients with inherited cylindromatosis, which have congenital mutations in the CYLinDromatosis gene (CYLD) (200). These patients develop multiple cylindromas and can in addition display trichoepitheliomas and spiradenomas (201). Cylindromas are slow-growing benign tumours that expand in the dermis. They have been suggested to have either eccrine or apocrine sweat gland origin, but may arise from the hair follicle epithelium as well. CYLD has deubiquitination enzyme activity. Ubiquitination is involved in the targeting of proteins for degradation (K48 linked) and in the regulation of signal transduction (K63 linked), by providing a bridge for the interaction with other proteins. (202). Both K48- and K63-linked ubiquitination serve as important regulatory mechanisms in the NF-κB pathways. Several groups have demonstrated that CYLD deubiquitinates both TRAF2 and TRAF6. This was linked to inhibition of classical NF-κB activation (i.e. activation of p65/p50) (203-205) and to decreased JNK-activation (206) downstream of several stimuli, including TNFα. In addition to these effects, it was demonstrated that CYLD regulates the localization of Bcl-3 in response to UV and in response to the phorbol ester 12-O-TetradecanoylPhorbol-13 Acetate (TPA), but not TNFα (207). In cyld knockout mice, this was coupled to p50/Bcl-3- and p52/Bcl-3-induced proliferation in keratinocytes and an increased incidence of papilloma formation in response to 7,12-DiMethylBenz(a)Anthracene (DMBA)/TPA or UV-induced non-melanoma skin cancer (207).

2 COMMENTS ON METHODOLGY

2.1 TRANSGENIC TECHNOLOGY TARGETING THE EPIDERMIS

The defined expression of different structural proteins to distinct layers of the epidermis is a very useful tool for the targeting of transgene expression to a specific layer. As illustrated earlier in Figure 2, keratin 5 (K5) and keratin 14 (K14) are expressed in the basal layer of the epidermis, whereas keratin 1 (K1) and Keratin 10 (K10) are expressed in the suprabasal layers. In addition to the interfollicular epidermis, K5 and K14 are expressed in the outer root sheath of the hair follicle whereas K1 and K10 are found in the inner root sheath. In this study we have used a construct with 5´sequences of the bovine keratin 5 promoter (pBK5/97) (208) to direct the expression of the transgenes to the basal layer of the epidermis. Besides the epidermis of the skin, this promoter also directs expression to other K5 expressing stratified epithelia and to glands. This includes the salivary glands, basal and suprabasal cells of the oesophagus and the palate, the oral epithelium, the tongue, the nose cavity, the forestomach, thymic epithelial cells, the renal pelvis and the urinary bladder. The extent of expression varies between the different epithelia and is highest in the skin and in the oral cavity (208).

Karolinska Institutet has a core facility for the production of transgenic mice: The Karolinska Center For Transgene Technologies (KCTT). Briefly, the transgenic mice are generated by microinjection of a linearized expression vector to the pronucleus of fertilized mouse eggs (209). The eggs are next implanted to pseudopregnant foster mothers. Transgene integration is easily detected by the Polymerase Chain Reaction (PCR) method. The integration site is random and the position of the transgene can affect both the transgene expression and putative flanking gene sequences. It is therefore important to establish several founder lines to be able to exclude influences on the phenotype caused by the integration site.

2.2 CONDITIONAL TRANSGENE EXPRESSION WITH THE TETRACYCLINE-CONTROLLED SYSTEMS

The tetracycline regulated system is a bi-transgenic system that makes it possible to control both the spatial and the temporal expression of a transgene. It is based on the use of a tetracycline responsive repressor (TetR), originally discovered in *Escherichia coli* that was fused to the VP16 transactivating domain from the Herpes Simplex Virus (210). This protein, named tTA (tetracycline responsive TransActivator) activates transcription from promoters containing the heptameric tet*O* repeat sequence from the tet-operon in a tetracycline-dependent manner. Transactivation by tTA is suppressed by tetracyclines (Tet-off). A mutant form of the tTA protein, rtTA is instead activated by tetracyclines (Tet-on) and the two systems, Tet-off and Tet-on, complement each other (211).

3 AIMS OF THE THESIS

The general aims of the work in this thesis were to elucidate the role of NF-κB in the maintenance of skin immune homoeostasis and in skin cancer development.

Specific aims:

Paper I: To evaluate the contribution of TNFR1 and IL1R1 signalling for the development of inflammation and cancer induced by IκBα mediated inhibition of NF-κB activation in keratinocytes.

Paper II: To explore the role of NF-κB in phorbol-ester induced growth arrest of keratinocytes and the role of TNFα/TNFR1 in this response.

Paper III: To follow the temporal induction and progression of the disease in mice with epidermal specific inhibition of NF-κB and to evaluate as to what extent the developmental role of NF-κB inhibition can be separated from its postnatal role.

Paper IV: 1. To assess in which cell type(s) TNFR1 signalling is important for the initiation of the inflammatory disease and the later development of cancer, in skin with epidermal inhibition of NF-κB. 2. To further understand the nature of the inflammatory response that drives the development of cancer in this setting.

4 SUMMARY OF THE RESULTS

4.1.1 **Paper I:**

The basis for this work was the novel finding by Rune Toftgårds group, published in Cancer Research 1999 (159). Hogerlinden *et al*., showed that IκBα-mediated inhibition of NF-κB in the basal and follicular keratinocytes in mice (*K5-IκBα*) leads to chronic inflammation, hyperplasia of the epidermis and ultimately Squamous Cell Carcinoma (SCC) development with 100% penetrance.

The data indicated that the cytokine TNFα was upregulated in the skin of these mice (159, 160). However, the relevance of this finding was not known. TNFα is a master cytokine in the inflammatory response and it had been shown to play a pivotal role in chemical-induced development of SCC in the skin (212, 213). In the latter model the carcinogenesis is induced by prototypical mutations in the Ha-Ras gene, which is absent in the *K5-IκBα* model (214). Hence, the aetiology of the cancer induced in these systems differs. This merited an evaluation of the role of TNFα-induced signalling in our model system, both for the initiation of the inflammatory disease and in the development of cancer.

K5-IκBα (FVB/N background) mice were crossed to *Tnfr1*$^{-/-}$ (C57Bl6/J background) mice. *K5-IκBα* mice, that were wild type (*Tnfr*$^{+/+}$) or heterozygous (*Tnfr1*$^{+/-}$) for the TNFR1 receptor, developed macroscopic inflammatory skin changes within 3 weeks of age. Due to an increased inflammatory response in the mixed FVB/N-B6 background compared to the original FVB/N background, the mice had to be euthanized within 4 week of age. In contrast, *K5-IκBα/Tnfr1*$^{-/-}$ mice displayed no inflammatory skin changes or cancer. The latter was confirmed by histology and by immunohistochemical analysis of inflammatory markers. These results showed that TNFR1-mediated signalling is central for the development of inflammation in these mice and for the development of cancer. FVB/N is a more cancer-prone background than C57Bl6 (215). To exclude the influence of the genetic background on the results we therefore repeated the experiment with *Tnfr1*$^{-/-}$ mice backcrossed to a FVB/N background. We obtained the same results. In *K5-IκBα/Tnfr1*$^{-/-}$ mice on a FVB/N background both the development of inflammation and the development of SCC is abolished for at least up to 1 year of age.

Cytokine array and PCR analysis revealed that the cytokines, Interleukin-1α and Interleukin-1β (IL-1α and IL-1β) were upregulated together with TNFα in the inflamed skin of *K5-IκBα* mice. Immunohistochemical analysis of IL-1β showed strong expression in the inflammatory infiltrate. IL-1α and IL-1β both target the Interleukin 1 receptor 1 (IL1R1) (216). To evaluate a potential role of the IL-1-mediated inflammatory axis in the inflammation and skin cancer induced by IκBα-mediated inhibition of NF-κB in keratinocytes, we crossed the *K5-IκBα* mice to an *IL1R1*$^{-/-}$ mouse background. In contrast to TNFR1-deficiency, this did not affect the inflammatory response or the development of cancer.

Having excluded the contribution of IL-1α/β, we again turned our focus to the role of TNFα/TNFR1 in this model. The *K5-IκBα/Tnfr1*$^{-/-}$ mice presented higher values of TNFα compared to the *Tnfr1*$^{-/-}$ mice, in this study [*see footnote]. TNFα is associated with the activation of immune cells in inflammation (110). It was therefore an attractive

[*] These results have later been reevaluated since it cannot be detected in a pure FVB/N or pure C57Bl6 background. (Ulvmar M.H. unpublished data).

possibility that TNFR1 acting on immune cells played an initiating role in the inflammatory disease of the *K5-IκBα* mice. To test this hypothesis, we performed bonemarrow transplantation experiment where lethally irradiated *K5-IκBα/Tnfr1*$^{-/-}$ mice were reconstituted with *Tnfr1*$^{+/-}$ or *Tnfr1*$^{-/-}$ bonemarrow from MHC matched littermates. Our data show that TNFR1 on immune cells was not sufficient to reintroduce the inflammatory response or the cancer in these mice. This suggested that the critical TNFR1 signalling occurred in non-immune cells.

Shortly before our data were published, Paul Khavaris group reported that TNFR1 signalling with downstream JNK activation drives hyperproliferation in *p65*$^{-/-}$ skin transplanted to *scid/scid* mice (176). Moreover, they showed that IκBα overcomes Ras-mediated growth arrest in human keratinocytes *in vitro*, and that IκBα in conjunction with oncogenic Ras triggers invasive human neoplasia in human keratinocytes transplanted to *scid/scid* mice (198). In the latter study the effect was associated with a stabilization of the CDK4. As mentioned earlier we had excluded mutations in the Ha-Ras gene in our mouse model (214). By immunohistochemistry we also excluded that we had an activation of the Ras-pathway by staining for activated p42/p44 MAPK (Erk1/Erk2), which are downstream targets in this pathway. Additionally, we showed that increased levels of CDK4 appeared to be a late rather than early event in the progression of disease in our model. CDK4 was assayed both by immunohistochemistry in the skin and in whole cell extracts from keratinocyte isolated from wildtype or *K5-IκBα* mice. However, as described for *p65*$^{-/-}$ skin we did demonstrate a strong upregulation of active phophorylated JNK in the hyperplastic keratinocytes of inflamed skin.

4.1.2 **Paper II:**

As mentioned earlier, inhibition of NF-κB in keratinocytes endow these cells with the capability to overcome growth arrest induced by oncogenic Ras by CDK4 stabilization (198). This response has later been shown to depend on TNFR1 (199). If IκBα-mediated hyperproliferation was unique for Ras-signalling was unknown (198). In the present study we therefore aimed to identify additional growth inhibitory signals that could be modulated by NF-κB. This led us to investigate the effect of IκBα mediated NF-κB inhibition in phorbol ester induced growth arrest of human keratinocytes *in vitro*.

Since human keratinocytes are difficult to transfect we used a methodology of retroviral transduction which resulted in >95% efficiency of gene transfer. Experiments were performed both with primary human keratinocytes and with the telomerase immortalized human keratinocyte cell line N/TERT-1,2G, which retains the growth properties of primary human keratinocytes in culture (217). The keratinocytes were transduced with a mutated non-degradable form of IκBα, here denoted, IκBα Dominant Negative (IκBαDN); or by vector control. Expression of IκBαDN did not alter the growth of the keratinocytes under normal conditions. However, in response to chronic treatment with the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which activated NF-κB and induced cell cycle arrest in control cells, IκBαDN-expressing cells continued to grow. Thorough analysis of the kinetics of the response by flow cytometric cell cycle analysis showed that the IκBαDN-expressing cells initially displayed a normal G0/G1 arrest in response to TPA; surprisingly, this

appeared to be increased compared to control cells. However, by 48 h the IκBαDN cells were released from this block whereas control cells still showed no entrance to early S-phase. Hence, the IκBαDN cells appeared to lower the threshold for release from the G0/G1 arrest induced by TPA.

Phorbol esters, like TPA, are known to target the Protein Kinase C (PKC) enzyme family since they are agonists to the endogenous PKC activator, diacylglycerol (218). Other targets for TPA also exist. Treatment with the pan PKC inhibitor bisindolylmaleimide abrogated the growth arrest which proves a PKC dependency in this response. Next we explored the possible connection to CDK4 and TNFR1, which is associated with growth arrest induced by activated Ras (198, 199). CDK4 was reduced after treatment with TPA in normal cells. However, IκBαDN expressing cells retained high levels of CDK4. This is similar to the interference of IκBαDN in growth arrest induced by activated Ras. However, in this case the response was independent of TNFα and TNFR1, assayed by anti-TNFα and anti-TNFR1 treatment.

The TNFR1-independence of this response prompted us to investigate the response to chronic TPA treatment in *K5-IκBα/Tnfr1*$^{-/-}$ animals. In contrast to the growth inhibitory response in kertinocytes grown in culture, TPA elicits an inflammatory hyperproliferative response when applied to skin *in vivo*. This response was increased in *K5-IκBα/Tnfr1*$^{-/-}$ compared to *Tnfr1*$^{-/-}$ mice. Notably, formation of big follicular cysts was observed in *K5-IκBα/Tnfr1*$^{-/-}$ mice.

4.1.3 **Paper III:**

Besides inflammation, inhibition of NF-κB in the skin has been coupled to the developmental defects in ectodermally-derived structures, like the teeth, the hair follicles and the sweat glands (136-138, 142). To be able to follow the temporal induction and progression of the inflammatory disease and to separate the developmental role of NF-κB inhibition from its postnatal role in the epidermis, we developed a conditional IκBα mouse model, *K5-tTA/TRE-IκBα*, where the expression of the IκBα transgene can be controlled by the administration of doxycycline (dox).

When transgenic IκBα was expressed during development the mice developed inflammation and hyperplasia that started within 4 days after birth. The mice died within 10 days but could be rescued by repression of the transgenic IκBα expression. This shows that inflammation induced by transgenic expression of IκBα requires sustained epidermal NF-κB inhibition in order to maintain the inflammatory response. Moreover, the possibility to repress the post-natal inflammatory response allowed us to examine the developmental effects of epidermal NF-κB inhibition. These included a very sparse hair coat, no hair on the tail or behind the ears and multiple glandular defects, i.e. absence or hypoplastic sweat glands, absence of meibomian glands, absence or hypoplasia of the serous and mucus glands of the tongue. These features are consistent with the human congenital diseases HED/EDA and the corresponding mouse mutants *tabby/crinkled/downless*. Identical features were found when the *K5-tTA/TRE-IκBα* mice were transferred to a TNFR1 knockout background that repressed development of inflammation, similar to our original *K5-IκBα* model. These experiments illustrated that the developmental defects downstream of EDAR/(mouse downless), and the post-natal induction of inflammation dependent on TNFR1 in

response to inhibition of epidermal NF-κB, are two completely independent phenomena.

If the transgene expression was induced after birth, between days 2–3 PN, this resulted in inflammation and hyperplasia that started to develop between days 21–23 PN. The delayed onset of the inflammatory disease was due to a repression of transgene expression on the mRNA level, which was independent of dox since induction (i.e. removal of dox from the drinking water) day 2 or day 16 PN both gave the same consistent onset of transgenic IκBα expression and onset of inflammatory changes only on days 21–23 PN. Due to the mixed genetic background of the *K5-tTA/TRE-IκBα* mice (F1 C57B6/FVB/N) the progression of inflammatory disease was fast, and the mice had to be euthanized within 37 days after birth, why we focused our analysis on the pathology related to the inflammation and hyperplasia but not SCC development.

Onset of hyperplasia was associated with a redistribution of the NF-κB subunit p50 in the epidermis from a nuclear to a more cytoplasmic localization. Similarly to the original *K5-IκBα* model, the inflammation could be characterized by an early infiltration of neutrophils, and an upregulation of phosphorylated JNK in the epidermis. The first changes in the epidermis after onset of transgene expression day 21 was seen as small hyperplastic foci defined by the upregulation of the stress related keratin 6 (K6). Immunohistochemical examination revealed that F4/80 + cells (mainly monocytes and macrophages) were the first inflammatory cells to invade these inflammatory foci. They were later followed by neutrophils, assayed by Calgranulin A/S100A8 (CalA/S100A8) expression. The macrophages were shown to provide VEGFA in this system and were indirectly linked to the increased angiogenesis in the skin since treatment of the mice with VEGF-Trap attenuated the angiogenic response.

The early influx of macrophages prompted us to turn our focus on the factors that may induce the infiltration of macrophages. Real time PCR analysis revealed that transcriptional upregulation of TNFα was a relatively late event in the inflammatory cascade. Instead, we observed an epidermal upregulation of multiple chemokines of CC-family (i.e. MCP-1, -2 and -3), which all are associated with the recruitment of monocytes (219). Transfer of the mice to a MCP-1 deficient background did not reduce the influx of F4/80+ cells. An attractive possibility is that the infiltration of macrophages is induced as a cooperative response to multiple chemokines (e.g. MCP-1-3). Neither of these chemokines were upregulated in isolated keratinocytes from *K5-IκBα* mice in culture, hence the epidermal expression could depend on the crosstalk between the altered keratinocytes and their surroundings. Of note, we here also demonstrated that the growth of *K5-IκBα* expressing keratinocytes *in vitro* is normal compared to wild type, in contrast to the hyperproliferative phenotype seen *in vivo*.

4.1.4 **Paper IV:**

The data from paper I indicated that TNFR1 signalling on immune cells was not sufficient for reintroduction of the inflammation or tumour development in a *K5-IκBα/Tnfr1*$^{-/-}$ mice. However, we could not exclude a requirement of TNFR1 in keratinocytes together with immunecells or on cells other than keratinocytes (e.g. dermal fibroblasts).

To formally test if a TNFR1 response in keratinocytes with IκBα mediated inhibition of NF-κB is sufficient to drive the inflammation, hyperplasia and

development of SCC, we created *K5-Tnfr1/Tnfr1*$^{-/-}$ mice. In these mice expression of TNFR1 was targeted to exactly the same cells that were expressing the IκBα transgene. *K5-Tnfr1/Tnfr1*$^{-/-}$ mice were crossed to *K5-IκBα/Tnfr1*$^{-/-}$, all on a FVB/N background. The resulting *K5-IκBα/K5-Tnfr1/Tnfr1*$^{-/-}$ mice appeared normal at birth but started to develop inflammatory changes within 3–4 days PN. Most mice died within 10 days, with inflammatory changes involving the whole skin. Hence, the phenotype was much stronger than in the original *K5-IκBα* model probably due the increased expression of TNFR1. The mRNA levels of Tnfr1 was 3–4 times higher in epidermis from *K5-Tnfr1/Tnfr1*$^{-/-}$ mice than in epidermis from wild type mice. In a very low frequency we did get mice that survived beyond 10 days PN and in all these mice we could confirm either dysplastic changes (n=1) or development of SCC (n=3).

Comparative analysis of the cellular infiltration in *K5-IκBα/K5-Tnfr1/Tnfr1*$^{-/-}$ and our original *K5-IκBα* model showed a striking overlapping pattern with massive infiltration of F4/80+ cells and CalA+ neutrophils. We showed in paper III that F4/80+ cells (i.e. monocytes/macrophages and a subset of dendritic cells) are the first cells to invade the skin after onset of NF-κB inhibition. Macrophages had earlier been shown to be decisive for the inflammatory reaction in a reciprocal model of epidermal NF-κB inhibition induced by IKKβ deletion (162). To further understand the role of these cells we analyzed their activation based on the M1-M2 division (27). Analysis of two typical M2 markers – the mannose receptor CD206/MRC1 and the β-glucan receptor dectin-1 – revealed an upregulation in the inflamed skin, which suggested that these macrophages display characteristics of a M2 type of activation. Of note, the M2 activation preceded malignancy and was independent of TNFR1 on the immune cells and other cells outside the epithelial compartment, since a similar pattern is seen in *K5-IκBα* and *K5-IκBα/K5-Tnfr1/Tnfr1*$^{-/-}$ mice.

The presence of M2 macrophages prompted us to analyze the T-cell infiltrate. The transcription factor FoxP3 and the α chain of the IL-2 receptor (CD25) are both markers for regulatory T-cells which are important for peripheral tolerance but that also have the capability to inhibit tumour rejection (21). To analyze these populations we performed flow cytometric analysis of the infiltrate in tumours, premalignant inflamed skin and wild type skin. We made two important observations, namely: 1. CD8+ cells are virtually absent in the inflamed skin; and 2. The proportion of FoxP3+CD25+ cells is increased both in tumours and in premalignant skin. Again, these features were independent of TNFR1 on the immune cells since a similar pattern is seen in *K5-IκBα* and *K5-IκBα/K5-Tnfr1/Tnfr1*$^{-/-}$ mice.

The striking cellular phenocopy of the inflammatory infiltrate between *K5-IκBα* and *K5-IκBα/K5-Tnfr1/Tnfr1*$^{-/-}$ mice urged us to investigate as to what extent the molecular environment in inflamed skin could be set up by keratinocyte-dependent TNFR1 signalling only. Real time PCR analysis of a number of cytokines (TNFα, IL-1α, IL-1β, IL-10, IL-6, VEGFA) the chemokine MCP-2 and the MMPs (MMP-9 and MMP-13) revealed a striking similar pattern in SCC and inflamed skin from *K5-IκBα/K5-Tnfr1/Tnfr1*$^{-/-}$ and from *K5-IκBα* mice. The molecular environment, to a large degree, appeared to be established already at the onset of inflammation in day 4 *K5-IκBα/K5-Tnfr1/Tnfr1*$^{-/-}$ mice. Immunohistochemical staining for Cyclooxygenase-1 and -2 (COX-1 and COX-2), both enzymes involved in the production of prostaglandins, that can increase inflammation and suppress cellular adaptive immune responses presented the same basic picture, with an increase by day 4 in *K5-IκBα/K5-Tnfr1/Tnfr1*$^{-/-}$ mice.

Of the cytokines analyzed, IL-10 evoked our interest since it is a master cytokine in the establishment of an immunosuppressive environment. Immunofluorescence staining revealed that it was highly expressed in the F4/80+ population, which further confirmed that they are of a M2 type. Interestingly, expression of IL-10 could be observed in keratinocytes as well. Isolation of *K5-IκBα* keratinocytes revealed that IL-10 was upregulated in transgenic cells grown *in vitro* in culture. Hence, although not formally proven, it is an appealing possibility that keratinocyte derived IL-10 might be at least a contributing factor to the establishment of an immunosuppressive environment in the skin of *K5-IκBα* mice.

5 DISCUSSION AND FUTURE PERSPECTIVES

The work that is presented in this thesis started with the original finding published in paper I, 2004, in which we established the link between TNFR1 singling and the development of chronic inflammation and SCC in *K5-IκBα* mice. At that time we had the hypothesis that an upregulation of TNFα from the keratinocytes could be the initiating force of the disease recruiting inflammatory cells. In the same paper, we reported that reintroduction of TNFR1 postive bonemarrow could not reintroduce the inflammatory disease in *K5-IκBα/Tnfr1*$^{-/-}$ mice. This seeded the thought of a direct role of TNFR1 on keratinocytes that formed the basis for the last paper IV, in which we by genetic means demonstrated that the TNFR1 response in keratinocytes alone is sufficient, not only for the initiation of the inflammatory disease, but also for the development of cancer.

Our data from paper III and IV revealed that the upregulation of TNFα surprisingly is a late event in the inflammatory cascade induced by inhibition of epidermal NF-κB. Further, the cytokine Lymfotoxin alpha (LTα), which is the alternative ligand for TNFR1, is not detectable in the inflamed skin (Unpublish data Ulvmar M.H.). Hence, it could be concluded that the inflammatory cascade is initiated as a consequence of a selective, IκBα mediated break in the basal levels of TNFR1 signalling in the skin. However, we cannot rule out the possibility that TNFα is upregulated at the protein level by a transcription-independent mechanism. We have unfortunately not been able to find a reliable TNFα antibody that is suitable for immunohistochemistry analysis on skin sections. A related and relevant question has been if this response stems from an autocrine TNFα/TNFR1 loop in the keratinocytes. Probably this is not the case.

In parallel with our studies of the *K5-IκBα* mice, the group of S. Gerondakis reported that transplantation of *p65*$^{-/-}$*/c-Rel*$^{-/-}$*/TNFα*$^{-/-}$ embryonic skin to *Rag1*$^{-/-}$ mice resulted in inflammation (148). Hence, the source of TNFα is in these experiments unlikely to be the keratinocytes themselves. Our data in paper IV which shows that inhibition of NF-κB decreases rather than increases the levels of TNFα mRNA in isolated keratinocytes *in vitro* indirectly supports this notion. The data from the *p65*$^{-/-}$*/c-Rel*$^{-/-}$*/TNFα*$^{-/-}$ transplanted skin would also exclude the additional skin resident cells as a source of TNFα. So what is the source of TNFα in this skin disease? There are two possibilities. We would either have to assume that infiltration of immune cells precedes the development of hyperplasia and that TNFα is provided by the recruited immune cells in a second step. Another explanation would be that TNFα is induced in distant cells and transferred to the skin by the circulation.

Both mice with epidermal deletion of IKKβ and IKKγ develop inflammatory skin diseases with several similarities to *K5-IκBα* mice (150, 152). All three models have demonstrated the essential role of TNFR1 for the induction of the disease. The inflammatory disease in *IKKβ*$^{\Delta K14/\Delta K14}$ mice was tested for the contribution of granulocytes by crossing them to a *CD18* mutant background (hypomorphic mutation in β2-integrin which reduce extravasion of neutrophils (220)) (162). This prevented the influx of neutrophils to the skin but did not affect development of hyperproliferation or the influx of other immune cells. We have made several attempts to evaluate the role of the specific immune cells in the disease of *K5-IκBα* mice. Cross to a CD18 mutant background does not inhibit the infiltration of neutrophils in *K5-IκBα* mice

(unpublished data Ulvmar M.H.). We have in addition used *Gcsfr*$^{-/-}$ mice. GCSFR deficient mice have reduced numbers of neutrophils both in the bone marrow and in the blood (221). *K5-IκBα/Gcsfr*$^{-/-}$ mice developed tumours to the same extent as *K5-IκBα* mice (unpublished data Ulvmar MH). Neutrophils could still be found in the skin but, notably, there were areas completely devoid of neutrophilic infiltration (Figure 5a). In these areas the hyperproliferation was not attenuated (Figure 5a and unpublished data Ulvmar MH). Further, the F4/80+ infiltrate remained high in these areas. Hence, neutrophils are unlikely to play an important role in the inflammatory reaction or in the progression to cancer.

In paper III, we reported that F4/80+ cells were the first cells to infiltrate the skin after onset of epidermal NF-κB inhibition. Hence these cells are likely to play an important role. As described earlier, this notion is supported by the result from the *IKKβ*$^{\Delta K14/\Delta K14}$ mice where they show that treatment with clodronate, which leads to a depletion of phagocytosing cells, could locally ameliorate the inflammatory disease (162). This treatment mainly reduced the macrophages and did not affect the dendritic cells. To evaluate the role of macrophages in *K5-IκBα* mice we have transferred them to a *Csf1*^{op}*/Csf1*^{op} background.*Csf1*^{op}*/Csf1*^{op} mice have a point mutation in the Csf-1 gene and to a large extent lack macrophages and osteoclasts and suffers from severe osteopetrosis (op stands for osteopetrosis mutation) (222). This mouse model has been very useful for the study of macrophages in breast cancer (223). However, the mutation did not reduce the influx of F4/80+ cells to the skin of *K5-IκBα* mice (data not shown or Figure 5b). It has been reported earlier that whereas *Csf1*^{op}*/Csf1*^{op} mice have a severe reduction in dermal F4/80+ cells the epidermal F4/80+ langerhans cells are unaffected (224). These experimental data raise questions about the nature of the F4/80+ infiltrate in *K5-IκBα* mice and we are currently performing a thorough analysis of the F4/80+ population in *K5-IκBα* mice.

The *IKKβ*$^{\Delta K14/\Delta K14}$ mouse model has been suggested to model the human inflammatory disease psoriasis. Our data from *K5-IκBα* mice tells a completely different story. Psoriasis is considered a Th1/Th17 disease with a mixed CD8+ CD4+ T-cell infiltrate that very rarely progress to cancer (225-227). Indeed, there are

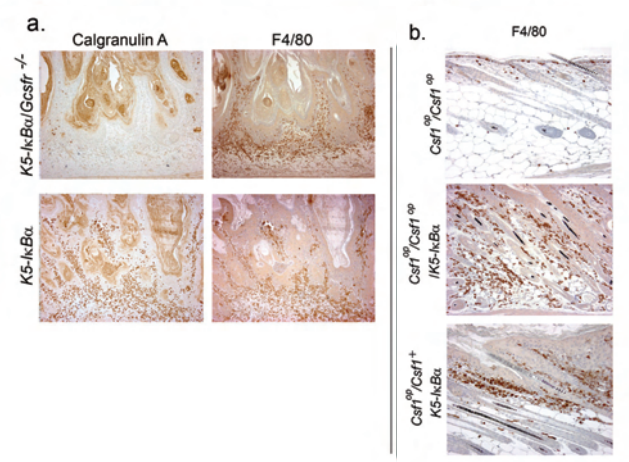

Figure 5: a) Immunohistochemical staining for neutrophils (Calgranulin A) and F4/80+ cells (macrophages, dendritic cells) in 2 month old *K5-IκBα* mice in a wild type or *Gcsfr*$^{-/-}$ background. **b.**) Immunohistochemical staining for F4/80+ cells in *Csf1*^{op}*/Csf1*^{op}(21 days PN), *K5-IκBα/Csf1*^{op}*/Csf1*^{op} (10 days PN) and *K5-IκBα/Csf1*^{op}*/Csf1*$^{+}$ mice (10 days PN).

evidences of an increased resistance for malignant transformation in psoriatic plaques compared to normal skin (228, 229). Our data from the *K5-IκBα* mice and from the *K5-IκBα/K5-Tnfr1/Tnfr1*$^{-/-}$ mice presented in paper IV reveal a immunological signature with increased IL-10, accumulation of M2 macrophages and CD4+FoxP3+ regulatory T-cells with an absence of IL-12 and CD8+ T-cells, that is very different from psoriasis. Notably this is already at the onset of the disease in our model. We propose that the TNFR1/NF-κB axis in keratinocytes is part of the immune surveillance system of the skin. It is interesting to note that *IκBα*$^{\Delta K14/\Delta K14}$ mice and *K5-IκBα* mice show an immunological response that diverges to completely different directions (149). In that sense, NF-κB appears like a sensor in the skin and perturbation in the balance of epidermal NF-κB in either direction evokes inflammation that forces the immune response to adopt alternative fates depending on the NF-κB status in epidermal cells. It remains to be seen if this hypothesis can be validated. As described earlier, inhibition of NF-κB activation due to loss of function mutations in IKKγ is the genetic cause of the inflammatory skin disease Incontientia Pigmenti (IP). The propensity of patients suffering from IP to develop rare subungual skin tumours strengthens the notion that inhibition of NF-κB in keratinocytes could promote human skin tumourigenesis and it would be interesting to look at the inflammatory environment in the skin and in the tumours of these patients.

The data from paper IV can also be interpreted from a broader perspective. TNFα has been associated with tumour progression in several experimental tumour systems as well as in human cancer patients (109). The tumour promoting effects of TNFα is thought to be dependent on its ability to govern the inflammatory response, although a direct effect on DNA damage has been shown in some cellular systems (230). TNFR1 is broadly expressed and it has been largely unknown where the critical TNFα response takes place in the tumour environment. Our data clearly show that at least in this setting of skin tumourigenesis, the only cells in which TNFR1 is needed for establishment of a tumour-permissive, inflammatory environment and for tumour progression are the epithelial cells themselves. Whether this is unique to our model of skin tumourigenesis remains an open question and is a base for further research. Of interest it was recently reported that human SCC cell lines displayed a TNFR1 dependent growth *in vitro* (199). Hence, this supports that the TNFα/TNFR1 axis have a role in human SCC tumour cell maintenance.

Could inflammation alone explain the fast tumour development in *K5-IκBα* mice? Probably not. Several questions still remain on the cell-intrinsic, tumour promoting, effects of NF-κB inhibition in keratinocytes, but very likely it involves an ability to overcome growth arrest. This has been shown in the *in vitro* response to Ras-induced growth arrest (198) and here in paper II in response to the phorbol ester TPA. Notably, activation of PKC in response to TPA can lead both to the activation of the Ras-pathway and to activation of NF-κB (218). Hence, it may be that the results of both this reports stems from the activation of overlapping signalling pathways. However, in our case the response was independent of TNFR1, which is a difference to the TNFR1 dependence in Ras-induced growth arrest. The growth arrest induced by TPA in paper II was not affected by a PCKα specific inhibitor. Other interesting candidates are the PKCδ and PKCη isoforms, which induce growth arrest in human keratinocytes if overexpressed (231) and which have been linked to TPA induced G1 arrest *in vitro*

(232). PKCη is downstream PKCδ and they can both mediate activation of the IKK complex leading to the activation of NF-κB.

To summarize, and to look forward, the future challenges in this NF-κB skin field will be to delineate the underlying mechanisms that determine the type of immunity that is elicited either in response to increased NF-κB activity (*IκBα*$^{\Delta K14/\Delta K14}$) or in response to decreased NF-κB activity in the basal epidermal cells of the skin (*K5-IκBα*, *IKKβ*$^{\Delta K14/\Delta K14}$ and *IKKγ*$^{\Delta K14/\Delta K14}$ mice). For the *IκBα*$^{\Delta K14/\Delta K14}$ model we already know that the disease is dependent on concomitant increased NF-κB activity in T-cells but the molecular mechanisms in the crosstalk between the epidermal cells and the T-cells are unknown. We have provided some candidates both for the recruitment of F4/80+ cells into the skin of *K5-IκBα* mice (i.e. MCP-1,-2 and -3 – paper III) and to the polarization of the immune response (i.e. IL-10 – paper IV). Future research will show if these are of importance.

The data presented in this thesis should be of importance for how we view the role of keratinocytes and the interplay between TNFR1 and NF-κB in inflammatory diseases of the skin in general. Concerning the role of TNFα/TNFR1 in cancer our model opens up questions that go beyond the skin. For the understanding of cancer and for SCC in particular, we have a unique tool to elucidate the molecular and cellular mechanism in TNFR1 dependent, inflammation driven cancer. The *K5-IκBα* mice models the dual role of the immune system in cancer development with chronic inflammation on the one side and immunosuppression on the other, and it could be a useful tool both to develop and to test new treatment strategies. Figure 6 presents the current model for inflammation and cancer induced by epidermal NF-κB inhibition, based on the interpretation of the data presented in this thesis.

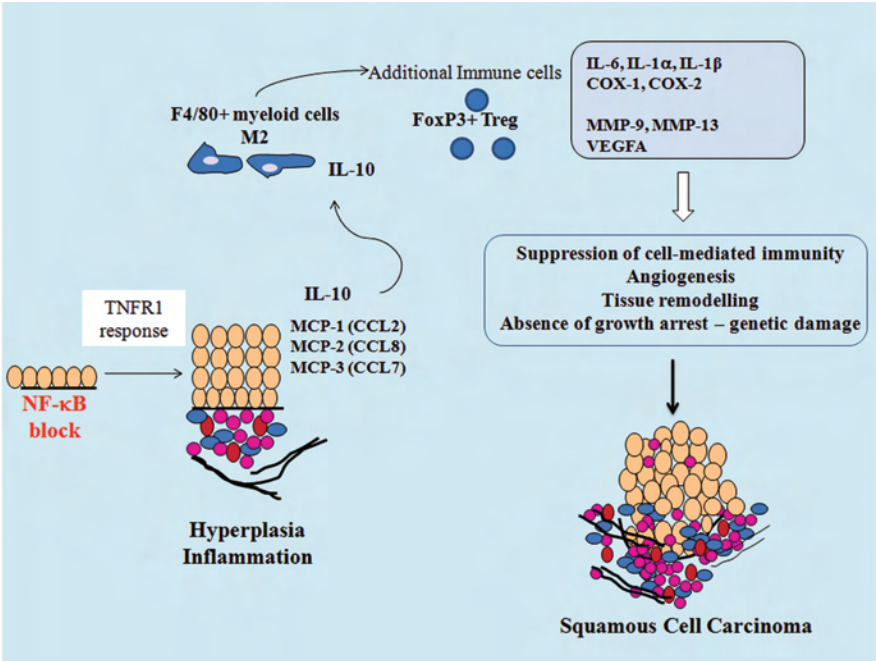

Figure 6. Model for SCC development induced by IκBα mediated NF-κB block in keratinocytes, based on the work presented in this thesis. In the first step, inhibition of NF-kB in the skin interfers with the TNFR1 signalling in the keratinocytes. This causes hyperproliferation and an inflammatory response with upregulation of multiple chemokines (MCP-1-3), and upregulation of IL-10. Our model posits that, the keratinocytes are both recruiting and shaping the inflammatory infiltrate. F4/80+ cells (macrophages, monocytes and a subset of dendritic cells) are the first cells to invade the skin and they will adopt an alternative M2 activation profile, which further boosts the expression of IL-10. The infiltration of F4/80+ cells is followed by additional immune cells, including FoxP3+ regulatory T-cells. The molecular changes in the skin involve upregulation of IL-1α, IL-1β, IL-6, COX1/COX2, multiple MMPs and VEGFA. All these changes are induced before malignancy occurs and will together lead to additional growth stimulation; inhibition of cell mediated immunity, increased angiogenesis and increased tissue remodelling. The keratinocytes with inhibited NF-κB also displays defects in growth arrest, which potentially could allow accumulation of genetic damage in these cells. This viscous circle of inflammation and immuosuppression inescapable leads to the development of SCC.

6 ACKNOWLEDGEMENTS

The path to a doctoral thesis involves many challenges. I would like to express my sincere appreciation to everyone who directly and indirectly helped and guided me on the way towards my goal. Especially I would like to thank:

My supervisor **Rune Toftgård**, for your support and for giving me the freedom to follow my own ideas. Your experience and your never ending optimism are something to reach for as a scientist.

My co-supervisor **Inderpreet Sur**, for always having the time to listen and for sharing your knowledge and ideas. Your help has been invaluable.

Max van Hogerlinden, for forming the basis of my work by making the *K5-IκBα* mice.

Collaborators:
Björn Rozell, for learning me so much about mouse pathology and for the collaborations in paper I and III. **Hans-Gustaf Ljunggren** and **Robert (Robban) Wallin**, for the collaboration in paper I and for many interesting discussions. **Robban**, for your continued interest and willingness to collaborate. I think we will get a really nice paper in the end. **Alf Grandien,** for giving me some ampho cells to use for the virus-production in paper II. **Sylvie Mémet**, for the collaboration in paper III. **Meit Björndahl –** it never worked out with the project but it was a lot of fun to work with you.

The RTO group:
The former PhD girls: **Fahimeh** and **Jessica**. I have missed you both.
The remaining PhD-girls: **Ulrika, Karin** and **Marie**. Thank you for sharing the ups and downs of being both a scientist and a parent. I wish you all the luck with your thesis work and with your future careers. It will be interesting to see where we all end up. I will miss you a lot. You are great friends. **Ramesh** – the only guy among all the girls – good luck with your defence and with starting up your school in India! I am sure you will succeed.

Åsa B., for your technical assistance and advice during the years – I wish I had your skilled hands – and thank you for keeping our lab in such excellent order. Thanks also to **Elin,** who have done many tail clippings and PCR:s for me.

Maria K., for interesting discussions. I admire your inspirational scientific thinking. **Viljar,** for always taking the time to change some words. You really have contributed to a nice scientific atmosphere at the RTO lab. **Åsa K.,** for nice discussions both about the kids and the science world, for help with my abstracts and for advices about the post-doctoral work. **Csaba**, for never giving up organizing our journal clubs. **Takashi**, for interesting stories about life as a scientist in Japan and exotic candy at Fika times.

All the other present and former members of the RTO group who have helped to create a nice environment – no one forgotten.

"My students" who taught me how fun it is to tutor and who contributed to parts of my work – **Yuping Sun** and **Sofia Friberg**.

The ES-cell lab, for nice company at the lunch table. **Britt-Marie** and **Gunnel,** a special thanks for our talks about dogs. **Stephan,** for your advices and help.

Friends from the Biomedical Research School for our nice lunches together – **Patrik, Maria E, Peter, Julian** and **Hanna.**

Göran, my wonderful teacher in Mathematics and Physics at komvux in Norrtälje, who introduced me to the world of knowledge, challenged me and showed me how fun it is to think. You believed in my capability like no one else and have been a mentor in my heart during times of trouble.

My family:
Michaels mother **Gunnel** and his aunt **Margareta,** who have helped us so much with our children and by that have given me more time for my research.

My **Father** and **Lisa** - you are always there for me.
My **Mother** – nothing is too much work for you.
My little brother **Erik** and his wife **Menzi** – you have a special place in my heart
My aunt **Gittan** who has helped us with babysitting when needed.

Michael – the love of my life – thank you for your unconditional love and support. I would never have reached this goal without you.
David, **Nathalie** and **Theo** – our wonderful children – you are my great inspiration. I love you endlessly. ♥♥♥

This work was supported by the Swedish Cancer Society, the Swedish Research Council, NIH Program Project Grant AR47898 and NIH/NCI MMHCC Grant U01 CA105491.

7 REFERENCES

1. Hanahan, D. and Weinberg, R. A. The hallmarks of cancer. Cell, *100:* 57-70, 2000.
2. Mantovani, A., Allavena, P., Sica, A., and Balkwill, F. Cancer-related inflammation. Nature, *454:* 436-444, 2008.
3. de Visser, K. E., Eichten, A., and Coussens, L. M. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer, *6:* 24-37, 2006.
4. Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J., and Schreiber, R. D. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol, *3:* 991-998, 2002.
5. Burnet, F. M. The concept of immunological surveillance. Prog Exp Tumor Res, *13:* 1-27, 1970.
6. Burnet, M. Cancer; a biological approach. I. The processes of control. Br Med J, *1:* 779-786, 1957.
7. Zitvogel, L., Tesniere, A., and Kroemer, G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol, *6:* 715-727, 2006.
8. Dunn, G. P., Old, L. J., and Schreiber, R. D. The three Es of cancer immunoediting. Annu Rev Immunol, *22:* 329-360, 2004.
9. Shinkai, Y., Rathbun, G., Lam, K. P., Oltz, E. M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A. M., and et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell, *68:* 855-867, 1992.
10. Shankaran, V., Ikeda, H., Bruce, A. T., White, J. M., Swanson, P. E., Old, L. J., and Schreiber, R. D. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature, *410:* 1107-1111, 2001.
11. Girardi, M., Oppenheim, D. E., Steele, C. R., Lewis, J. M., Glusac, E., Filler, R., Hobby, P., Sutton, B., Tigelaar, R. E., and Hayday, A. C. Regulation of cutaneous malignancy by gammadelta T cells. Science, *294:* 605-609, 2001.
12. Clementi, R., Dagna, L., Dianzani, U., Dupre, L., Dianzani, I., Ponzoni, M., Cometa, A., Chiocchetti, A., Sabbadini, M. G., Rugarli, C., Ciceri, F., Maccario, R., Locatelli, F., Danesino, C., Ferrarini, M., and Bregni, M. Inherited perforin and Fas mutations in a patient with autoimmune lymphoproliferative syndrome and lymphoma. N Engl J Med, *351:* 1419-1424, 2004.
13. Schmidt, A. and Weber, O. F. In memoriam of Rudolf virchow: a historical retrospective including aspects of inflammation, infection and neoplasia. Contrib Microbiol, *13:* 1-15, 2006.
14. Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol, *136:* 2348-2357, 1986.
15. Lucey, D. R., Clerici, M., and Shearer, G. M. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. Clin Microbiol Rev, *9:* 532-562, 1996.
16. Elsasser-Beile, U., Kolble, N., Grussenmeyer, T., Schultze-Seemann, W., Wetterauer, U., Gallati, H., Schulte Monting, J., and von Kleist, S. Th1 and Th2 cytokine response patterns in leukocyte cultures of patients with urinary bladder, renal cell and prostate carcinomas. Tumour Biol, *19:* 470-476, 1998.
17. Pellegrini, P., Berghella, A. M., Del Beato, T., Cicia, S., Adorno, D., and Casciani, C. U. Disregulation in TH1 and TH2 subsets of CD4+ T cells in peripheral blood of colorectal cancer patients and involvement in cancer establishment and progression. Cancer Immunol Immunother, *42:* 1-8, 1996.

18. Martinez, G. J., Nurieva, R. I., Yang, X. O., and Dong, C. Regulation and function of proinflammatory TH17 cells. Ann N Y Acad Sci, *1143:* 188-211, 2008.
19. Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M., and Weaver, C. T. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol, *6:* 1123-1132, 2005.
20. Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., and Dong, C. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol, *6:* 1133-1141, 2005.
21. Zou, W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol, *6:* 295-307, 2006.
22. de Visser, K. E., Korets, L. V., and Coussens, L. M. De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. Cancer Cell, *7:* 411-423, 2005.
23. Murdoch, C., Muthana, M., Coffelt, S. B., and Lewis, C. E. The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer, *8:* 618-631, 2008.
24. Schafer, M. and Werner, S. Cancer as an overhealing wound: an old hypothesis revisited. Nat Rev Mol Cell Biol, *9:* 628-638, 2008.
25. Gabrilovich, D. I. and Nagaraj, S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol, *9:* 162-174, 2009.
26. Lewis, C. E. and Pollard, J. W. Distinct role of macrophages in different tumor microenvironments. Cancer Res, *66:* 605-612, 2006.
27. Martinez, F. O., Sica, A., Mantovani, A., and Locati, M. Macrophage activation and polarization. Front Biosci, *13:* 453-461, 2008.
28. Van Ginderachter, J. A., Movahedi, K., Hassanzadeh Ghassabeh, G., Meerschaut, S., Beschin, A., Raes, G., and De Baetselier, P. Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. Immunobiology, *211:* 487-501, 2006.
29. Sica, A., Larghi, P., Mancino, A., Rubino, L., Porta, C., Totaro, M. G., Rimoldi, M., Biswas, S. K., Allavena, P., and Mantovani, A. Macrophage polarization in tumour progression. Semin Cancer Biol, *18:* 349-355, 2008.
30. Shchors, K., Shchors, E., Rostker, F., Lawlor, E. R., Brown-Swigart, L., and Evan, G. I. The Myc-dependent angiogenic switch in tumors is mediated by interleukin 1beta. Genes Dev, *20:* 2527-2538, 2006.
31. Ancrile, B., Lim, K. H., and Counter, C. M. Oncogenic Ras-induced secretion of IL6 is required for tumorigenesis. Genes Dev, *21:* 1714-1719, 2007.
32. Sparmann, A. and Bar-Sagi, D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. Cancer Cell, *6:* 447-458, 2004.
33. Thilly, W. G. Have environmental mutagens caused oncomutations in people? Nat Genet, *34:* 255-259, 2003.
34. Mantovani, A. Cancer: Inflaming metastasis. Nature, *457:* 36-37, 2009.
35. Ostrand-Rosenberg, S. Immune surveillance: a balance between protumor and antitumor immunity. Curr Opin Genet Dev, *18:* 11-18, 2008.
36. Fuchs, E. and Byrne, C. The epidermis: rising to the surface. Curr Opin Genet Dev, *4:* 725-736, 1994.
37. Braff, M. H., Bardan, A., Nizet, V., and Gallo, R. L. Cutaneous defense mechanisms by antimicrobial peptides. J Invest Dermatol, *125:* 9-13, 2005.
38. Grone, A. Keratinocytes and cytokines. Vet Immunol Immunopathol, *88:* 1-12, 2002.
39. Schroder, J. M. and Harder, J. Antimicrobial skin peptides and proteins. Cell Mol Life Sci, *63:* 469-486, 2006.
40. Lebre, M. C., van der Aar, A. M., van Baarsen, L., van Capel, T. M., Schuitemaker, J. H., Kapsenberg, M. L., and de Jong, E. C. Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9. J Invest Dermatol, *127:* 331-341, 2007.

41. Kollisch, G., Kalali, B. N., Voelcker, V., Wallich, R., Behrendt, H., Ring, J., Bauer, S., Jakob, T., Mempel, M., and Ollert, M. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. Immunology, *114:* 531-541, 2005.
42. Alam, M. and Ratner, D. Cutaneous squamous-cell carcinoma. N Engl J Med, *344:* 975-983, 2001.
43. Swedish Cancer Registry, C. f. e. Cancer Incidence in Sweden 2007. 2008.
44. Osterlind, A. Etiology and epidemiology of melanoma and skin neoplasms. Curr Opin Oncol, *3:* 355-359, 1991.
45. Marks, R. Squamous cell carcinoma. Lancet, *347:* 735-738, 1996.
46. Berg, D. and Otley, C. C. Skin cancer in organ transplant recipients: Epidemiology, pathogenesis, and management. J Am Acad Dermatol, *47:* 1-17; quiz 18-20, 2002.
47. Lindelof, B., Sigurgeirsson, B., Gabel, H., and Stern, R. S. Incidence of skin cancer in 5356 patients following organ transplantation. Br J Dermatol, *143:* 513-519, 2000.
48. Copcu, E., Aktas, A., Sisman, N., and Oztan, Y. Thirty-one cases of Marjolin's ulcer. Clin Exp Dermatol, *28:* 138-141, 2003.
49. Baldursson, B., Sigurgeirsson, B., and Lindelof, B. Venous leg ulcers and squamous cell carcinoma: a large-scale epidemiological study. Br J Dermatol, *133:* 571-574, 1995.
50. Sulica, V. I. and Kao, G. F. Squamous-cell carcinoma of the scalp arising in lesions of discoid lupus erythematosus. Am J Dermatopathol, *10:* 137-141, 1988.
51. Katz, R. D. and Goldberg, N. H. Marjolin ulcer arising within hidradenitis: a case report and literature review. Ann Plast Surg, *62:* 173-174, 2009.
52. Dvorak, H. F. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med, *315:* 1650-1659, 1986.
53. Mallipeddi, R. Epidermolysis bullosa and cancer. Clin Exp Dermatol, *27:* 616-623, 2002.
54. Ortiz-Urda, S., Garcia, J., Green, C. L., Chen, L., Lin, Q., Veitch, D. P., Sakai, L. Y., Lee, H., Marinkovich, M. P., and Khavari, P. A. Type VII collagen is required for Ras-driven human epidermal tumorigenesis. Science, *307:* 1773-1776, 2005.
55. Norgauer, J., Idzko, M., Panther, E., Hellstern, O., and Herouy, Y. Xeroderma pigmentosum. Eur J Dermatol, *13:* 4-9, 2003.
56. Miyauchi-Hashimoto, H., Okamoto, H., Tanaka, K., and Horio, T. Ultraviolet radiation-induced suppression of natural killer cell activity is enhanced in xeroderma pigmentosum group A (XPA) model mice. J Invest Dermatol, *112:* 965-970, 1999.
57. Ling, G., Persson, A., Berne, B., Uhlen, M., Lundeberg, J., and Ponten, F. Persistent p53 mutations in single cells from normal human skin. Am J Pathol, *159:* 1247-1253, 2001.
58. Malkin, D., Li, F. P., Strong, L. C., Fraumeni, J. F., Jr., Nelson, C. E., Kim, D. H., Kassel, J., Gryka, M. A., Bischoff, F. Z., Tainsky, M. A., and et al. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science, *250:* 1233-1238, 1990.
59. Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature, *356:* 215-221, 1992.
60. Kemp, C. J., Donehower, L. A., Bradley, A., and Balmain, A. Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumors. Cell, *74:* 813-822, 1993.
61. Quintanilla, M., Brown, K., Ramsden, M., and Balmain, A. Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. Nature, *322:* 78-80, 1986.

62. Campbell, C., Quinn, A. G., and Rees, J. L. Codon 12 Harvey-ras mutations are rare events in non-melanoma human skin cancer. Br J Dermatol, *128:* 111-114, 1993.
63. Rumsby, G., Carter, R. L., and Gusterson, B. A. Low incidence of ras oncogene activation in human squamous cell carcinomas. Br J Cancer, *61:* 365-368, 1990.
64. Pierceall, W. E., Goldberg, L. H., Tainsky, M. A., Mukhopadhyay, T., and Ananthaswamy, H. N. Ras gene mutation and amplification in human nonmelanoma skin cancers. Mol Carcinog, *4:* 196-202, 1991.
65. Kubo, Y., Urano, Y., Matsumoto, K., Ahsan, K., and Arase, S. Mutations of the INK4a locus in squamous cell carcinomas of human skin. Biochem Biophys Res Commun, *232:* 38-41, 1997.
66. Soufir, N., Moles, J. P., Vilmer, C., Moch, C., Verola, O., Rivet, J., Tesniere, A., Dubertret, L., and Basset-Seguin, N. P16 UV mutations in human skin epithelial tumors. Oncogene, *18:* 5477-5481, 1999.
67. Harland, M., Meloni, R., Gruis, N., Pinney, E., Brookes, S., Spurr, N. K., Frischauf, A. M., Bataille, V., Peters, G., Cuzick, J., Selby, P., Bishop, D. T., and Bishop, J. N. Germline mutations of the CDKN2 gene in UK melanoma families. Hum Mol Genet, *6:* 2061-2067, 1997.
68. Wagstaff, A. J. and Perry, C. M. Topical imiquimod: a review of its use in the management of anogenital warts, actinic keratoses, basal cell carcinoma and other skin lesions. Drugs, *67:* 2187-2210, 2007.
69. Huang, S. J., Hijnen, D., Murphy, G. F., Kupper, T. S., Calarese, A. W., Mollet, I. G., Schanbacher, C. F., Miller, D. M., Schmults, C. D., and Clark, R. A. Imiquimod Enhances IFN-gamma Production and Effector Function of T Cells Infiltrating Human Squamous Cell Carcinomas of the Skin. J Invest Dermatol, 2009.
70. Clark, R., Huang, S., Murphy, G., Mollet, I., Hijnen, D., Muthukuru, M., Schanbacher, C., Edwards, V., Miller, D., Kim, J., Lambert, J., and Kupper, T. Human squamous cell carcinomas evade the immune response by down-regulation of vascular E-selectin and recruitment of regulatory T cells. *205:* 2221-2234, 2008.
71. Sen, R. and Baltimore, D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell, *46:* 705-716, 1986.
72. Hayden, M. S. and Ghosh, S. Shared principles in NF-kappaB signaling. Cell, *132:* 344-362, 2008.
73. Kucharczak, J., Simmons, M. J., Fan, Y., and Gelinas, C. To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis. Oncogene, *22:* 8961-8982, 2003.
74. Hayden, M. S., West, A. P., and Ghosh, S. NF-kappaB and the immune response. Oncogene, *25:* 6758-6780, 2006.
75. Gilmore, T. D. http://people.bu.edu/gilmore/nf-kb/. 2009.
76. Ghosh, S., Gifford, A. M., Riviere, L. R., Tempst, P., Nolan, G. P., and Baltimore, D. Cloning of the p50 DNA binding subunit of NF-kappa B: homology to rel and dorsal. Cell, *62:* 1019-1029, 1990.
77. Mercurio, F., Didonato, J., Rosette, C., and Karin, M. Molecular cloning and characterization of a novel Rel/NF-kappa B family member displaying structural and functional homology to NF-kappa B p50/p105. DNA Cell Biol, *11:* 523-537, 1992.
78. Bours, V., Burd, P. R., Brown, K., Villalobos, J., Park, S., Ryseck, R. P., Bravo, R., Kelly, K., and Siebenlist, U. A novel mitogen-inducible gene product related to p50/p105-NF-kappa B participates in transactivation through a kappa B site. Mol Cell Biol, *12:* 685-695, 1992.
79. Ruben, S. M., Dillon, P. J., Schreck, R., Henkel, T., Chen, C. H., Maher, M., Baeuerle, P. A., and Rosen, C. A. Isolation of a rel-related human cDNA that potentially encodes the 65-kD subunit of NF-kappa B. Science, *254:* 11, 1991.
80. Nolan, G. P., Ghosh, S., Liou, H. C., Tempst, P., and Baltimore, D. DNA binding and I kappa B inhibition of the cloned p65 subunit of NF-kappa B, a rel-related polypeptide. Cell, *64:* 961-969, 1991.

81. Ryseck, R. P., Bull, P., Takamiya, M., Bours, V., Siebenlist, U., Dobrzanski, P., and Bravo, R. RelB, a new Rel family transcription activator that can interact with p50-NF-kappa B. Mol Cell Biol, *12:* 674-684, 1992.
82. Bours, V., Villalobos, J., Burd, P. R., Kelly, K., and Siebenlist, U. Cloning of a mitogen-inducible gene encoding a kappa B DNA-binding protein with homology to the rel oncogene and to cell-cycle motifs. Nature, *348:* 76-80, 1990.
83. Baeuerle, P. A. The inducible transcription activator NF-kappa B: regulation by distinct protein subunits. Biochim Biophys Acta, *1072:* 63-80, 1991.
84. Haskill, S., Beg, A. A., Tompkins, S. M., Morris, J. S., Yurochko, A. D., Sampson-Johannes, A., Mondal, K., Ralph, P., and Baldwin, A. S., Jr. Characterization of an immediate-early gene induced in adherent monocytes that encodes I kappa B-like activity. Cell, *65:* 1281-1289, 1991.
85. Davis, N., Ghosh, S., Simmons, D. L., Tempst, P., Liou, H. C., Baltimore, D., and Bose, H. R., Jr. Rel-associated pp40: an inhibitor of the rel family of transcription factors. Science, *253:* 1268-1271, 1991.
86. Li, Z. and Nabel, G. J. A new member of the I kappaB protein family, I kappaB epsilon, inhibits RelA (p65)-mediated NF-kappaB transcription. Mol Cell Biol, *17:* 6184-6190, 1997.
87. McKeithan, T. W., Ohno, H., and Diaz, M. O. Identification of a transcriptional unit adjacent to the breakpoint in the 14;19 translocation of chronic lymphocytic leukemia. Genes Chromosomes Cancer, *1:* 247-255, 1990.
88. Haruta, H., Kato, A., and Todokoro, K. Isolation of a novel interleukin-1-inducible nuclear protein bearing ankyrin-repeat motifs. J Biol Chem, *276:* 12485-12488, 2001.
89. Yamazaki, S., Muta, T., and Takeshige, K. A novel IkappaB protein, IkappaB-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappaB in the nuclei. J Biol Chem, *276:* 27657-27662, 2001.
90. Inoue, J., Kerr, L. D., Kakizuka, A., and Verma, I. M. I kappa B gamma, a 70 kd protein identical to the C-terminal half of p110 NF-kappa B: a new member of the I kappa B family. Cell, *68:* 1109-1120, 1992.
91. Malek, S., Chen, Y., Huxford, T., and Ghosh, G. IkappaBbeta, but not IkappaBalpha, functions as a classical cytoplasmic inhibitor of NF-kappaB dimers by masking both NF-kappaB nuclear localization sequences in resting cells. J Biol Chem, *276:* 45225-45235, 2001.
92. Sun, S. C., Ganchi, P. A., Ballard, D. W., and Greene, W. C. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. Science, *259:* 1912-1915, 1993.
93. Le Bail, O., Schmidt-Ullrich, R., and Israel, A. Promoter analysis of the gene encoding the I kappa B-alpha/MAD3 inhibitor of NF-kappa B: positive regulation by members of the rel/NF-kappa B family. Embo J, *12:* 5043-5049, 1993.
94. Tran, K., Merika, M., and Thanos, D. Distinct functional properties of IkappaB alpha and IkappaB beta. Mol Cell Biol, *17:* 5386-5399, 1997.
95. Tergaonkar, V., Correa, R. G., Ikawa, M., and Verma, I. M. Distinct roles of IkappaB proteins in regulating constitutive NF-kappaB activity. Nat Cell Biol, *7:* 921-923, 2005.
96. Bours, V., Franzoso, G., Azarenko, V., Park, S., Kanno, T., Brown, K., and Siebenlist, U. The oncoprotein Bcl-3 directly transactivates through kappa B motifs via association with DNA-binding p50B homodimers. Cell, *72:* 729-739, 1993.
97. Fujita, T., Nolan, G. P., Liou, H. C., Scott, M. L., and Baltimore, D. The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-kappa B p50 homodimers. Genes Dev, *7:* 1354-1363, 1993.
98. Yamamoto, M., Yamazaki, S., Uematsu, S., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Kuwata, H., Takeuchi, O., Takeshige, K., Saitoh, T., Yamaoka, S., Yamamoto, N., Yamamoto, S., Muta, T., Takeda, K., and Akira, S. Regulation

of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. Nature, *430:* 218-222, 2004.
99. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature, *388:* 548-554, 1997.
100. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. Science, *278:* 860-866, 1997.
101. Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell, *91:* 243-252, 1997.
102. Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. Cell, *93:* 1231-1240, 1998.
103. Rothwarf, D. M., Zandi, E., Natoli, G., and Karin, M. IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. Nature, *395:* 297-300, 1998.
104. Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. Science, *284:* 316-320, 1999.
105. Senftleben, U., Cao, Y., Xiao, G., Greten, F. R., Krahn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S. C., and Karin, M. Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. Science, *293:* 1495-1499, 2001.
106. Yilmaz, Z. B., Weih, D. S., Sivakumar, V., and Weih, F. RelB is required for Peyer's patch development: differential regulation of p52-RelB by lymphotoxin and TNF. Embo J, *22:* 121-130, 2003.
107. Perkins, N. D. Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. Oncogene, *25:* 6717-6730, 2006.
108. Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., and Williamson, B. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci U S A, *72:* 3666-3670, 1975.
109. Balkwill, F. TNF-alpha in promotion and progression of cancer. Cancer Metastasis Rev, *25:* 409-416, 2006.
110. Bradley, J. R. TNF-mediated inflammatory disease. J Pathol, *214:* 149-160, 2008.
111. Schall, T. J., Lewis, M., Koller, K. J., Lee, A., Rice, G. C., Wong, G. H., Gatanaga, T., Granger, G. A., Lentz, R., Raab, H., and et al. Molecular cloning and expression of a receptor for human tumor necrosis factor. Cell, *61:* 361-370, 1990.
112. Loetscher, H., Pan, Y. C., Lahm, H. W., Gentz, R., Brockhaus, M., Tabuchi, H., and Lesslauer, W. Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. Cell, *61:* 351-359, 1990.
113. Smith, C. A., Davis, T., Anderson, D., Solam, L., Beckmann, M. P., Jerzy, R., Dower, S. K., Cosman, D., and Goodwin, R. G. A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. Science, *248:* 1019-1023, 1990.
114. Liu, J. and Lin, A. Wiring the cell signaling circuitry by the NF-kappa B and JNK1 crosstalk and its applications in human diseases. Oncogene, *26:* 3267-3278, 2007.
115. Hsu, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell, *84:* 299-308, 1996.

116. Hsu, H., Huang, J., Shu, H. B., Baichwal, V., and Goeddel, D. V. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity, *4:* 387-396, 1996.
117. Devin, A., Cook, A., Lin, Y., Rodriguez, Y., Kelliher, M., and Liu, Z. The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. Immunity, *12:* 419-429, 2000.
118. Sato, S., Sanjo, H., Takeda, K., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T., Matsumoto, K., Takeuchi, O., and Akira, S. Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat Immunol, *6:* 1087-1095, 2005.
119. Thiefes, A., Wolter, S., Mushinski, J. F., Hoffmann, E., Dittrich-Breiholz, O., Graue, N., Dorrie, A., Schneider, H., Wirth, D., Luckow, B., Resch, K., and Kracht, M. Simultaneous blockade of NFkappaB, JNK, and p38 MAPK by a kinase-inactive mutant of the protein kinase TAK1 sensitizes cells to apoptosis and affects a distinct spectrum of tumor necrosis factor [corrected] target genes. J Biol Chem, *280:* 27728-27741, 2005.
120. Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell, *81:* 505-512, 1995.
121. Festjens, N., Cornelis, S., Lamkanfi, M., and Vandenabeele, P. Caspase-containing complexes in the regulation of cell death and inflammation. Biol Chem, *387:* 1005-1016, 2006.
122. Sakon, S., Xue, X., Takekawa, M., Sasazuki, T., Okazaki, T., Kojima, Y., Piao, J. H., Yagita, H., Okumura, K., Doi, T., and Nakano, H. NF-kappaB inhibits TNF-induced accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death. Embo J, *22:* 3898-3909, 2003.
123. Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., and Karin, M. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell, *120:* 649-661, 2005.
124. Hehlgans, T. and Pfeffer, K. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. Immunology, *115:* 1-20, 2005.
125. Pispa, J. and Thesleff, I. Mechanisms of ectodermal organogenesis. Dev Biol, *262:* 195-205, 2003.
126. Kere, J., Srivastava, A. K., Montonen, O., Zonana, J., Thomas, N., Ferguson, B., Munoz, F., Morgan, D., Clarke, A., Baybayan, P., Chen, E. Y., Ezer, S., Saarialho-Kere, U., de la Chapelle, A., and Schlessinger, D. X-linked anhidrotic (hypohidrotic) ectodermal dysplasia is caused by mutation in a novel transmembrane protein. Nat Genet, *13:* 409-416, 1996.
127. Monreal, A. W., Ferguson, B. M., Headon, D. J., Street, S. L., Overbeek, P. A., and Zonana, J. Mutations in the human homologue of mouse dl cause autosomal recessive and dominant hypohidrotic ectodermal dysplasia. Nat Genet, *22:* 366-369, 1999.
128. Headon, D. J., Emmal, S. A., Ferguson, B. M., Tucker, A. S., Justice, M. J., Sharpe, P. T., Zonana, J., and Overbeek, P. A. Gene defect in ectodermal dysplasia implicates a death domain adapter in development. Nature, *414:* 913-916, 2001.
129. Pinheiro, M. and Freire-Maia, N. Ectodermal dysplasias: a clinical classification and a causal review. Am J Med Genet, *53:* 153-162, 1994.
130. Srivastava, A. K., Pispa, J., Hartung, A. J., Du, Y., Ezer, S., Jenks, T., Shimada, T., Pekkanen, M., Mikkola, M. L., Ko, M. S., Thesleff, I., Kere, J., and Schlessinger, D. The Tabby phenotype is caused by mutation in a mouse homologue of the EDA gene that reveals novel mouse and human exons and encodes a protein (ectodysplasin-A) with collagenous domains. Proc Natl Acad Sci U S A, *94:* 13069-13074, 1997.
131. Yan, M., Zhang, Z., Brady, J. R., Schilbach, S., Fairbrother, W. J., and Dixit, V. M. Identification of a novel death domain-containing adaptor molecule for

ectodysplasin-A receptor that is mutated in crinkled mice. Curr Biol, *12:* 409-413, 2002.
132. Headon, D. J. and Overbeek, P. A. Involvement of a novel Tnf receptor homologue in hair follicle induction. Nat Genet, *22:* 370-374, 1999.
133. Gruneberg, H. More about the tabby mouse and about the Lyon hypothesis. J Embryol Exp Morphol, *16:* 569-590, 1966.
134. Gruneberg, H. The glandular aspects of the tabby syndrome in the mouse. J Embryol Exp Morphol, *25:* 1-19, 1971.
135. Gruneberg, H. Genes and genotypes affecting the teeth of the mouse. J Embryol Exp Morphol, *14:* 137-159, 1965.
136. Zonana, J., Elder, M. E., Schneider, L. C., Orlow, S. J., Moss, C., Golabi, M., Shapira, S. K., Farndon, P. A., Wara, D. W., Emmal, S. A., and Ferguson, B. M. A novel X-linked disorder of immune deficiency and hypohidrotic ectodermal dysplasia is allelic to incontinentia pigmenti and due to mutations in IKK-gamma (NEMO). Am J Hum Genet, *67:* 1555-1562, 2000.
137. Doffinger, R., Smahi, A., Bessia, C., Geissmann, F., Feinberg, J., Durandy, A., Bodemer, C., Kenwrick, S., Dupuis-Girod, S., Blanche, S., Wood, P., Rabia, S. H., Headon, D. J., Overbeek, P. A., Le Deist, F., Holland, S. M., Belani, K., Kumararatne, D. S., Fischer, A., Shapiro, R., Conley, M. E., Reimund, E., Kalhoff, H., Abinun, M., Munnich, A., Israel, A., Courtois, G., and Casanova, J. L. X-linked anhidrotic ectodermal dysplasia with immunodeficiency is caused by impaired NF-kappaB signaling. Nat Genet, *27:* 277-285, 2001.
138. Jain, A., Ma, C. A., Liu, S., Brown, M., Cohen, J., and Strober, W. Specific missense mutations in NEMO result in hyper-IgM syndrome with hypohydrotic ectodermal dysplasia. Nat Immunol, *2:* 223-228, 2001.
139. Courtois, G., Smahi, A., Reichenbach, J., Doffinger, R., Cancrini, C., Bonnet, M., Puel, A., Chable-Bessia, C., Yamaoka, S., Feinberg, J., Dupuis-Girod, S., Bodemer, C., Livadiotti, S., Novelli, F., Rossi, P., Fischer, A., Israel, A., Munnich, A., Le Deist, F., and Casanova, J. L. A hypermorphic IkappaBalpha mutation is associated with autosomal dominant anhidrotic ectodermal dysplasia and T cell immunodeficiency. J Clin Invest, *112:* 1108-1115, 2003.
140. Janssen, R., van Wengen, A., Hoeve, M. A., ten Dam, M., van der Burg, M., van Dongen, J., van de Vosse, E., van Tol, M., Bredius, R., Ottenhoff, T. H., Weemaes, C., van Dissel, J. T., and Lankester, A. The same IkappaBalpha mutation in two related individuals leads to completely different clinical syndromes. J Exp Med, *200:* 559-568, 2004.
141. Kumar, A., Eby, M. T., Sinha, S., Jasmin, A., and Chaudhary, P. M. The ectodermal dysplasia receptor activates the nuclear factor-kappaB, JNK, and cell death pathways and binds to ectodysplasin A. J Biol Chem, *276:* 2668-2677, 2001.
142. Schmidt-Ullrich, R., Aebischer, T., Hulsken, J., Birchmeier, W., Klemm, U., and Scheidereit, C. Requirement of NF-kappaB/Rel for the development of hair follicles and other epidermal appendices. Development, *128:* 3843-3853, 2001.
143. Yan, M., Wang, L. C., Hymowitz, S. G., Schilbach, S., Lee, J., Goddard, A., de Vos, A. M., Gao, W. Q., and Dixit, V. M. Two-amino acid molecular switch in an epithelial morphogen that regulates binding to two distinct receptors. Science, *290:* 523-527, 2000.
144. Kojima, T., Morikawa, Y., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Senba, E., and Kitamura, T. TROY, a newly identified member of the tumor necrosis factor receptor superfamily, exhibits a homology with Edar and is expressed in embryonic skin and hair follicles. J Biol Chem, *275:* 20742-20747, 2000.
145. Eby, M. T., Jasmin, A., Kumar, A., Sharma, K., and Chaudhary, P. M. TAJ, a novel member of the tumor necrosis factor receptor family, activates the c-Jun N-terminal kinase pathway and mediates caspase-independent cell death. J Biol Chem, *275:* 15336-15342, 2000.

146. Naito, A., Yoshida, H., Nishioka, E., Satoh, M., Azuma, S., Yamamoto, T., Nishikawa, S., and Inoue, J. TRAF6-deficient mice display hypohidrotic ectodermal dysplasia. Proc Natl Acad Sci U S A, *99:* 8766-8771, 2002.
147. Morlon, A., Munnich, A., and Smahi, A. TAB2, TRAF6 and TAK1 are involved in NF-kappaB activation induced by the TNF-receptor, Edar and its adaptator Edaradd. Hum Mol Genet, *14:* 3751-3757, 2005.
148. Gugasyan, R., Voss, A., Varigos, G., Thomas, T., Grumont, R. J., Kaur, P., Grigoriadis, G., and Gerondakis, S. The transcription factors c-rel and RelA control epidermal development and homeostasis in embryonic and adult skin via distinct mechanisms. Mol Cell Biol, *24:* 5733-5745, 2004.
149. Rebholz, B., Haase, I., Eckelt, B., Paxian, S., Flaig, M. J., Ghoreschi, K., Nedospasov, S. A., Mailhammer, R., Debey-Pascher, S., Schultze, J. L., Weindl, G., Forster, I., Huss, R., Stratis, A., Ruzicka, T., Rocken, M., Pfeffer, K., Schmid, R. M., and Rupec, R. A. Crosstalk between keratinocytes and adaptive immune cells in an IkappaBalpha protein-mediated inflammatory disease of the skin. Immunity, *27:* 296-307, 2007.
150. Nenci, A., Huth, M., Funteh, A., Schmidt-Supprian, M., Bloch, W., Metzger, D., Chambon, P., Rajewsky, K., Krieg, T., Haase, I., and Pasparakis, M. Skin lesion development in a mouse model of incontinentia pigmenti is triggered by NEMO deficiency in epidermal keratinocytes and requires TNF signaling. Hum Mol Genet, *15:* 531-542, 2006.
151. Lind, M. H., Rozell, B., Wallin, R. P., van Hogerlinden, M., Ljunggren, H. G., Toftgard, R., and Sur, I. Tumor necrosis factor receptor 1-mediated signaling is required for skin cancer development induced by NF-kappaB inhibition. Proc Natl Acad Sci U S A, *101:* 4972-4977, 2004.
152. Pasparakis, M., Courtois, G., Hafner, M., Schmidt-Supprian, M., Nenci, A., Toksoy, A., Krampert, M., Goebeler, M., Gillitzer, R., Israel, A., Krieg, T., Rajewsky, K., and Haase, I. TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. Nature, *417:* 861-866, 2002.
153. Klement, J. F., Rice, N. R., Car, B. D., Abbondanzo, S. J., Powers, G. D., Bhatt, P. H., Chen, C. H., Rosen, C. A., and Stewart, C. L. IkappaBalpha deficiency results in a sustained NF-kappaB response and severe widespread dermatitis in mice. Mol Cell Biol, *16:* 2341-2349, 1996.
154. Beg, A. A., Sha, W. C., Bronson, R. T., and Baltimore, D. Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice. Genes Dev, *9:* 2736-2746, 1995.
155. Lizzul, P. F., Aphale, A., Malaviya, R., Sun, Y., Masud, S., Dombrovskiy, V., and Gottlieb, A. B. Differential expression of phosphorylated NF-kappaB/RelA in normal and psoriatic epidermis and downregulation of NF-kappaB in response to treatment with etanercept. J Invest Dermatol, *124:* 1275-1283, 2005.
156. Abdou, A. G. and Hanout, H. M. Evaluation of survivin and NF-kappaB in psoriasis, an immunohistochemical study. J Cutan Pathol, *35:* 445-451, 2008.
157. Johansen, C., Flindt, E., Kragballe, K., Henningsen, J., Westergaard, M., Kristiansen, K., and Iversen, L. Inverse regulation of the nuclear factor-kappaB binding to the p53 and interleukin-8 kappaB response elements in lesional psoriatic skin. J Invest Dermatol, *124:* 1284-1292, 2005.
158. Westergaard, M., Henningsen, J., Johansen, C., Rasmussen, S., Svendsen, M. L., Jensen, U. B., Schroder, H. D., Staels, B., Iversen, L., Bolund, L., Kragballe, K., and Kristiansen, K. Expression and localization of peroxisome proliferator-activated receptors and nuclear factor kappaB in normal and lesional psoriatic skin. J Invest Dermatol, *121:* 1104-1117, 2003.
159. van Hogerlinden, M., Rozell, B. L., Ahrlund-Richter, L., and Toftgard, R. Squamous cell carcinomas and increased apoptosis in skin with inhibited Rel/nuclear factor-kappaB signaling. Cancer Res, *59:* 3299-3303, 1999.
160. van Hogerlinden, M., Rozell, B. L., Toftgard, R., and Sundberg, J. P. Characterization of the progressive skin disease and inflammatory cell infiltrate

in mice with inhibited NF-kappaB signaling. J Invest Dermatol, *123:* 101-108, 2004.
161. Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S., and Papaioannou, V. E. RAG-1-deficient mice have no mature B and T lymphocytes. Cell, *68:* 869-877, 1992.
162. Stratis, A., Pasparakis, M., Rupec, R. A., Markur, D., Hartmann, K., Scharffetter-Kochanek, K., Peters, T., van Rooijen, N., Krieg, T., and Haase, I. Pathogenic role for skin macrophages in a mouse model of keratinocyte-induced psoriasis-like skin inflammation. J Clin Invest, *116:* 2094-2104, 2006.
163. Smahi, A., Courtois, G., Vabres, P., Yamaoka, S., Heuertz, S., Munnich, A., Israel, A., Heiss, N. S., Klauck, S. M., Kioschis, P., Wiemann, S., Poustka, A., Esposito, T., Bardaro, T., Gianfrancesco, F., Ciccodicola, A., D'Urso, M., Woffendin, H., Jakins, T., Donnai, D., Stewart, H., Kenwrick, S. J., Aradhya, S., Yamagata, T., Levy, M., Lewis, R. A., and Nelson, D. L. Genomic rearrangement in NEMO impairs NF-kappaB activation and is a cause of incontinentia pigmenti. The International Incontinentia Pigmenti (IP) Consortium. Nature, *405:* 466-472, 2000.
164. Aradhya, S., Woffendin, H., Jakins, T., Bardaro, T., Esposito, T., Smahi, A., Shaw, C., Levy, M., Munnich, A., D'Urso, M., Lewis, R. A., Kenwrick, S., and Nelson, D. L. A recurrent deletion in the ubiquitously expressed NEMO (IKK-gamma) gene accounts for the vast majority of incontinentia pigmenti mutations. Hum Mol Genet, *10:* 2171-2179, 2001.
165. Berlin, A. L., Paller, A. S., and Chan, L. S. Incontinentia pigmenti: a review and update on the molecular basis of pathophysiology. J Am Acad Dermatol, *47:* 169-187; quiz 188-190, 2002.
166. Makris, C., Godfrey, V. L., Krahn-Senftleben, G., Takahashi, T., Roberts, J. L., Schwarz, T., Feng, L., Johnson, R. S., and Karin, M. Female mice heterozygous for IKK gamma/NEMO deficiencies develop a dermatopathy similar to the human X-linked disorder incontinentia pigmenti. Mol Cell, *5:* 969-979, 2000.
167. Schmidt-Supprian, M., Bloch, W., Courtois, G., Addicks, K., Israel, A., Rajewsky, K., and Pasparakis, M. NEMO/IKK gamma-deficient mice model incontinentia pigmenti. Mol Cell, *5:* 981-992, 2000.
168. Beg, A. A., Sha, W. C., Bronson, R. T., Ghosh, S., and Baltimore, D. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. Nature, *376:* 167-170, 1995.
169. Rosenfeld, M. E., Prichard, L., Shiojiri, N., and Fausto, N. Prevention of hepatic apoptosis and embryonic lethality in RelA/TNFR-1 double knockout mice. Am J Pathol, *156:* 997-1007, 2000.
170. Grumont, R. J., Rourke, I. J., O'Reilly, L. A., Strasser, A., Miyake, K., Sha, W., and Gerondakis, S. B lymphocytes differentially use the Rel and nuclear factor kappaB1 (NF-kappaB1) transcription factors to regulate cell cycle progression and apoptosis in quiescent and mitogen-activated cells. J Exp Med, *187:* 663-674, 1998.
171. Pohl, T., Gugasyan, R., Grumont, R. J., Strasser, A., Metcalf, D., Tarlinton, D., Sha, W., Baltimore, D., and Gerondakis, S. The combined absence of NF-kappa B1 and c-Rel reveals that overlapping roles for these transcription factors in the B cell lineage are restricted to the activation and function of mature cells. Proc Natl Acad Sci U S A, *99:* 4514-4519, 2002.
172. Gerondakis, S., Strasser, A., Metcalf, D., Grigoriadis, G., Scheerlinck, J. Y., and Grumont, R. J. Rel-deficient T cells exhibit defects in production of interleukin 3 and granulocyte-macrophage colony-stimulating factor. Proc Natl Acad Sci U S A, *93:* 3405-3409, 1996.
173. Grumont, R., Hochrein, H., O'Keeffe, M., Gugasyan, R., White, C., Caminschi, I., Cook, W., and Gerondakis, S. c-Rel regulates interleukin 12 p70 expression in CD8(+) dendritic cells by specifically inducing p35 gene transcription. J Exp Med, *194:* 1021-1032, 2001.

174. Sha, W. C., Liou, H. C., Tuomanen, E. I., and Baltimore, D. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell, *80:* 321-330, 1995.
175. Das, J., Chen, C. H., Yang, L., Cohn, L., Ray, P., and Ray, A. A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. Nat Immunol, *2:* 45-50, 2001.
176. Zhang, J. Y., Green, C. L., Tao, S., and Khavari, P. A. NF-kappaB RelA opposes epidermal proliferation driven by TNFR1 and JNK. Genes Dev, *18:* 17-22, 2004.
177. Barton, D., HogenEsch, H., and Weih, F. Mice lacking the transcription factor RelB develop T cell-dependent skin lesions similar to human atopic dermatitis. Eur J Immunol, *30:* 2323-2332, 2000.
178. Weih, F., Carrasco, D., Durham, S. K., Barton, D. S., Rizzo, C. A., Ryseck, R. P., Lira, S. A., and Bravo, R. Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kappa B/Rel family. Cell, *80:* 331-340, 1995.
179. Weih, F., Durham, S. K., Barton, D. S., Sha, W. C., Baltimore, D., and Bravo, R. Both multiorgan inflammation and myeloid hyperplasia in RelB-deficient mice are T cell dependent. J Immunol, *157:* 3974-3979, 1996.
180. Ulvmar, M. H., Sur, I., Memet, S., and Toftgard, R. Timed NF-kappaB Inhibition in Skin Reveals Dual Independent Effects on Development of HED/EDA and Chronic Inflammation. J Invest Dermatol, 2009.
181. Seitz, C. S., Lin, Q., Deng, H., and Khavari, P. A. Alterations in NF-kappaB function in transgenic epithelial tissue demonstrate a growth inhibitory role for NF-kappaB. Proc Natl Acad Sci U S A, *95:* 2307-2312, 1998.
182. Zhang, J. Y., Tao, S., Kimmel, R., and Khavari, P. A. CDK4 regulation by TNFR1 and JNK is required for NF-kappaB-mediated epidermal growth control. J Cell Biol, *168:* 561-566, 2005.
183. Basseres, D. S. and Baldwin, A. S. Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. Oncogene, *25:* 6817-6830, 2006.
184. Baud, V. and Karin, M. Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. Nat Rev Drug Discov, *8:* 33-40, 2009.
185. Maeda, S., Kamata, H., Luo, J. L., Leffert, H., and Karin, M. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. Cell, *121:* 977-990, 2005.
186. Luedde, T., Beraza, N., Kotsikoris, V., van Loo, G., Nenci, A., De Vos, R., Roskams, T., Trautwein, C., and Pasparakis, M. Deletion of NEMO/IKKgamma in liver parenchymal cells causes steatohepatitis and hepatocellular carcinoma. Cancer Cell, *11:* 119-132, 2007.
187. Pikarsky, E., Porat, R. M., Stein, I., Abramovitch, R., Amit, S., Kasem, S., Gutkovich-Pyest, E., Urieli-Shoval, S., Galun, E., and Ben-Neriah, Y. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature, *431:* 461-466, 2004.
188. Nenci, A., Becker, C., Wullaert, A., Gareus, R., van Loo, G., Danese, S., Huth, M., Nikolaev, A., Neufert, C., Madison, B., Gumucio, D., Neurath, M. F., and Pasparakis, M. Epithelial NEMO links innate immunity to chronic intestinal inflammation. Nature, *446:* 557-561, 2007.
189. Greten, F. R., Eckmann, L., Greten, T. F., Park, J. M., Li, Z. W., Egan, L. J., Kagnoff, M. F., and Karin, M. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell, *118:* 285-296, 2004.
190. Montes, C. M., Maize, J. C., and Guerry-Force, M. L. Incontinentia pigmenti with painful subungual tumors: a two-generation study. J Am Acad Dermatol, *50:* S45-52, 2004.
191. Sakai, H., Minami, M., Satoh, E., Matsuo, S., and Iizuka, H. Keratoacanthoma developing on a pigmented patch in incontinentia pigmenti. Dermatology, *200:* 258-261, 2000.

192. Abimelec, P., Rybojad, M., Cambiaghi, S., Moraillon, I., Cavelier-Balloy, B., Marx, C., and Morel, P. Late, painful, subungual hyperkeratosis in incontinentia pigmenti. Pediatr Dermatol, *12:* 340-342, 1995.
193. Adeniran, A., Townsend, P. L., and Peachey, R. D. Incontinentia pigmenti (Bloch-Sulzberger syndrome) manifesting as painful periungual and subungual tumours. J Hand Surg [Br], *18:* 667-669, 1993.
194. Korstanje, M. J. and Bessems, P. J. Incontinentia pigmenti with hyperkeratotic lesions in adulthood and possible squamous cell carcinoma. Dermatologica, *183:* 234-236, 1991.
195. Simmons, D. A., Kegel, M. F., Scher, R. K., and Hines, Y. C. Subungual tumors in incontinentia pigmenti. Arch Dermatol, *122:* 1431-1434, 1986.
196. Mascaro, J. M., Palou, J., and Vives, P. Painful subungual keratotic tumors in incontinentia pigmenti. J Am Acad Dermatol, *13:* 913-918, 1985.
197. Hartman, D. L. Incontinentia pigmenti associated with subungual tumors. Arch Dermatol, *94:* 632-635, 1966.
198. Dajee, M., Lazarov, M., Zhang, J. Y., Cai, T., Green, C. L., Russell, A. J., Marinkovich, M. P., Tao, S., Lin, Q., Kubo, Y., and Khavari, P. A. NF-kappaB blockade and oncogenic Ras trigger invasive human epidermal neoplasia. Nature, *421:* 639-643, 2003.
199. Zhang, J. Y., Adams, A. E., Ridky, T. W., Tao, S., and Khavari, P. A. Tumor necrosis factor receptor 1/c-Jun-NH2-kinase signaling promotes human neoplasia. Cancer Res, *67:* 3827-3834, 2007.
200. Bignell, G. R., Warren, W., Seal, S., Takahashi, M., Rapley, E., Barfoot, R., Green, H., Brown, C., Biggs, P. J., Lakhani, S. R., Jones, C., Hansen, J., Blair, E., Hofmann, B., Siebert, R., Turner, G., Evans, D. G., Schrander-Stumpel, C., Beemer, F. A., van Den Ouweland, A., Halley, D., Delpech, B., Cleveland, M. G., Leigh, I., Leisti, J., and Rasmussen, S. Identification of the familial cylindromatosis tumour-suppressor gene. Nat Genet, *25:* 160-165, 2000.
201. van Balkom, I. D. and Hennekam, R. C. Dermal eccrine cylindromatosis. J Med Genet, *31:* 321-324, 1994.
202. Skaug, B., Jiang, X., and Chen, Z. J. The role of ubiquitin in NF-kappaB regulatory pathways. Annu Rev Biochem, *78:* 769-796, 2009.
203. Kovalenko, A., Chable-Bessia, C., Cantarella, G., Israel, A., Wallach, D., and Courtois, G. The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. Nature, *424:* 801-805, 2003.
204. Brummelkamp, T. R., Nijman, S. M., Dirac, A. M., and Bernards, R. Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. Nature, *424:* 797-801, 2003.
205. Trompouki, E., Hatzivassiliou, E., Tsichritzis, T., Farmer, H., Ashworth, A., and Mosialos, G. CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. Nature, *424:* 793-796, 2003.
206. Reiley, W., Zhang, M., and Sun, S. C. Negative regulation of JNK signaling by the tumor suppressor CYLD. J Biol Chem, *279:* 55161-55167, 2004.
207. Massoumi, R., Chmielarska, K., Hennecke, K., Pfeifer, A., and Fassler, R. Cyld inhibits tumor cell proliferation by blocking Bcl-3-dependent NF-kappaB signaling. Cell, *125:* 665-677, 2006.
208. Ramirez, A., Bravo, A., Jorcano, J. L., and Vidal, M. Sequences 5' of the bovine keratin 5 gene direct tissue- and cell-type-specific expression of a lacZ gene in the adult and during development. Differentiation, *58:* 53-64, 1994.
209. Gordon, J. W., Scangos, G. A., Plotkin, D. J., Barbosa, J. A., and Ruddle, F. H. Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci U S A, *77:* 7380-7384, 1980.
210. Gossen, M. and Bujard, H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A, *89:* 5547-5551, 1992.
211. Kistner, A., Gossen, M., Zimmermann, F., Jerecic, J., Ullmer, C., Lubbert, H., and Bujard, H. Doxycycline-mediated quantitative and tissue-specific control of

gene expression in transgenic mice. Proc Natl Acad Sci U S A, *93:* 10933-10938, 1996.
212. Moore, R. J., Owens, D. M., Stamp, G., Arnott, C., Burke, F., East, N., Holdsworth, H., Turner, L., Rollins, B., Pasparakis, M., Kollias, G., and Balkwill, F. Mice deficient in tumor necrosis factor-alpha are resistant to skin carcinogenesis. Nat Med, *5:* 828-831, 1999.
213. Suganuma, M., Okabe, S., Marino, M. W., Sakai, A., Sueoka, E., and Fujiki, H. Essential role of tumor necrosis factor alpha (TNF-alpha) in tumor promotion as revealed by TNF-alpha-deficient mice. Cancer Res, *59:* 4516-4518, 1999.
214. van Hogerlinden, M., Auer, G., and Toftgard, R. Inhibition of Rel/Nuclear Factor-kappaB signaling in skin results in defective DNA damage-induced cell cycle arrest and Ha-ras- and p53-independent tumor development. Oncogene, *21:* 4969-4977, 2002.
215. Hennings, H., Glick, A. B., Lowry, D. T., Krsmanovic, L. S., Sly, L. M., and Yuspa, S. H. FVB/N mice: an inbred strain sensitive to the chemical induction of squamous cell carcinomas in the skin. Carcinogenesis, *14:* 2353-2358, 1993.
216. Sims, J. E., Gayle, M. A., Slack, J. L., Alderson, M. R., Bird, T. A., Giri, J. G., Colotta, F., Re, F., Mantovani, A., Shanebeck, K., and et al. Interleukin 1 signaling occurs exclusively via the type I receptor. Proc Natl Acad Sci U S A, *90:* 6155-6159, 1993.
217. Dickson, M. A., Hahn, W. C., Ino, Y., Ronfard, V., Wu, J. Y., Weinberg, R. A., Louis, D. N., Li, F. P., and Rheinwald, J. G. Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. Mol Cell Biol, *20:* 1436-1447, 2000.
218. Griner, E. M. and Kazanietz, M. G. Protein kinase C and other diacylglycerol effectors in cancer. Nat Rev Cancer, *7:* 281-294, 2007.
219. Rollins, B. J. Chemokines. Blood, *90:* 909-928, 1997.
220. Wilson, R. W., Ballantyne, C. M., Smith, C. W., Montgomery, C., Bradley, A., O'Brien, W. E., and Beaudet, A. L. Gene targeting yields a CD18-mutant mouse for study of inflammation. J Immunol, *151:* 1571-1578, 1993.
221. Hermans, M. H., van de Geijn, G. J., Antonissen, C., Gits, J., van Leeuwen, D., Ward, A. C., and Touw, I. P. Signaling mechanisms coupled to tyrosines in the granulocyte colony-stimulating factor receptor orchestrate G-CSF-induced expansion of myeloid progenitor cells. Blood, *101:* 2584-2590, 2003.
222. Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., Shultz, L. D., and Nishikawa, S. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature, *345:* 442-444, 1990.
223. Lin, E. Y., Nguyen, A. V., Russell, R. G., and Pollard, J. W. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med, *193:* 727-740, 2001.
224. Cecchini, M. G., Dominguez, M. G., Mocci, S., Wetterwald, A., Felix, R., Fleisch, H., Chisholm, O., Hofstetter, W., Pollard, J. W., and Stanley, E. R. Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. Development, *120:* 1357-1372, 1994.
225. Zaba, L. C., Fuentes-Duculan, J., Eungdamrong, N. J., Abello, M. V., Novitskaya, I., Pierson, K. C., Gonzalez, J., Krueger, J. G., and Lowes, M. A. Psoriasis is characterized by accumulation of immunostimulatory and Th1/Th17 cell-polarizing myeloid dendritic cells. J Invest Dermatol, *129:* 79-88, 2009.
226. Ghoreschi, K., Weigert, C., and Rocken, M. Immunopathogenesis and role of T cells in psoriasis. Clin Dermatol, *25:* 574-580, 2007.
227. Nickoloff, B. J., Bonish, B. K., Marble, D. J., Schriedel, K. A., DiPietro, L. A., Gordon, K. B., and Lingen, M. W. Lessons learned from psoriatic plaques concerning mechanisms of tissue repair, remodeling, and inflammation. J Investig Dermatol Symp Proc, *11:* 16-29, 2006.

228. Nickoloff, B. J. The skin cancer paradox of psoriasis: a matter of life and death decisions in the epidermis. Arch Dermatol, *140:* 873-875, 2004.
229. Nickoloff, B. J. Creation of psoriatic plaques: the ultimate tumor suppressor pathway. A new model for an ancient T-cell-mediated skin disease. Viewpoint. J Cutan Pathol, *28:* 57-64, 2001.
230. Yan, B., Wang, H., Rabbani, Z. N., Zhao, Y., Li, W., Yuan, Y., Li, F., Dewhirst, M. W., and Li, C. Y. Tumor necrosis factor-alpha is a potent endogenous mutagen that promotes cellular transformation. Cancer Res, *66:* 11565-11570, 2006.
231. Ohba, M., Ishino, K., Kashiwagi, M., Kawabe, S., Chida, K., Huh, N. H., and Kuroki, T. Induction of differentiation in normal human keratinocytes by adenovirus-mediated introduction of the eta and delta isoforms of protein kinase C. Mol Cell Biol, *18:* 5199-5207, 1998.
232. Ishino, K., Ohba, M., Kashiwagi, M., Kawabe, S., Chida, K., and Kuroki, T. Phorbol ester-induced G1 arrest in BALB/MK-2 mouse keratinocytes is mediated by delta and eta isoforms of protein kinase C. Jpn J Cancer Res, *89:* 1126-1133, 1998.