Università degli Studi di Firenze

Dipartimento di Patologia e Oncologia Sperimentali

Scuola di Dottorato di Ricerca in Oncologia Sperimentale e Clinica

XXII Ciclo

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Tesi di Dottorato di Ricerca

THE ORIGIN OF MELANOMA FROM ACRAL VOLAR SKIN: PUTATIVE ROLE OF INNATE IMMUNITY

Candidato: Dr. Daniele Torchia

Coordinatore del Dottorato:
Prof. Persio Dello Sbarba

Tutor:
Prof. Gabriele Mugnai

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Università degli Studi di Firenze

Dottorato di Ricerca in
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A conclusione del corso triennale del XXII° Ciclo del Dottorato di Ricerca in Oncologia Sperimentale e Clinica, il Collegio dei Docenti, facendo propria la relazione presentata dal Prof. Gabriele Mugnai, in qualità di *tutor*, circa l'attività di ricerca, l'operosità e l'assiduità del Dr. Daniele Torchia, rilascia con parere unanime il seguente attestato da valere come presentazione del candidato ai fini dell'espletamento dell'esame finale.

Il Dr. Daniele Torchia, nato a Firenze il 07/03/1977, si è laureato in Medicina e Chirurgia il 17/10/2002 presso l'Università degli Studi di Firenze, discutendo una tesi dal titolo "Linfociti T, citochine e recettori delle chemochine nelle lesioni cutanee della dermatomiosite" (relatore: Prof. Paolo Fabbri) e riportando la votazione di 110/110 e lode. Dopo la laurea, il Dr. Daniele Torchia ha conseguito la specializzazione in Dermatologia e Venereologia l'11/12/2006 con il massimo dei voti e lode con una tesi dal titolo "Il ruolo dell'apoptosi nelle lesioni cutanee della dermatomiosite" presso l'Università degli Studi di Firenze (relatore: Prof. Paolo Fabbri). In seguito il Dr. Daniele Torchia ha continuato a svolgere attività di ricerca nel Dipartimento di Scienze Dermatologiche (Università degli Studi di Firenze), sotto la supervisione del Prof. Paolo Fabbri, nella Division of Dermatology (University of California, San Diego), sotto la supervisione del Dr.. Richard L. Gallo, e nel Department od Dermatology and Cutaneous Surgery (University of Miami), sotto la supervisione del Dr. Paolo Romanelli e del Dr. Lawrence A. Schachner.

A partire dal 01/01/2007, il Dr. Daniele Torchia è stata ammesso alla frequenza del programma di Dottorato di Ricerca in Oncologia Sperimentale e Clinica (XXII Ciclo), continuando la propria attività di ricerca presso il Dipartimento di Patologia e Oncologia Sperimentale, sotto la supervisione del Prof. Gabriele Mugnai, nell'ambito di un progetto intitolato "The origin of melanoma from acral volar skin: putative role of innate immunity".

L'attività di ricerca svolta dal Dr. Daniele Torchia nel corso del triennio di Dottorato ha avuto come oggetto lesioni melanocitiche acrali e immunità innata della cute. In questo settore, il Dr. Daniele Torchia è autore dei seguenti articoli pubblicati:

1. Palleschi GM, Cipollini EM, Torchia D, Torre E, Urso C. Fibrillar pattern of a plantar acquired melanocytic nevus: correspondence between epiluminescence microscopy and transverse section histology. Clin Exp Dermatol 2006;31:449-51.
2. Palleschi GM, Torchia D. Fibrillar pattern of acral melanocytic naevi may be a dermatoscopic, but not a histologic artefact. Clin Exp Dermatol 2007;32:324.
3. Palleschi GM, Urso C, Torre E, Torchia D. Histopathologic correlates of the parallel-furrow pattern seen in acral melanocytic naevi at dermatoscopy. Dermatology 2008;217:356-8.
4. Torchia D. Transverse-section histology for parallel-ridge pattern. Actas Dermo-Sifiliogr 2010;101:572.
5. Antiga E, Volpi W, Torchia D, Fabbri P, Caproni M. The effects of tacrolimus ointment on Toll-like receptors in atopic dermatitis. Clin Exp Dermatol (2010) doi: 10.1111/j.1365-2230.2010.03948.x

Molti dei risultati di tutti gli studi elencati sono stati anche presentati dal Dr. Daniele

Torchia a congressi nazionali e internazionali.

Durante il corso di dottorato, il Dr. Daniele Torchia ha seguito con il massimo impegno il programma didattico stabilito dal Collegio dei Docenti ed ha portato avanti, con continuità e intelligenza, le ricerche che sono oggetto delle sue pubblicazioni e della sua tesi.

Per quanto sopra, il Collegio dei Docenti unanime ritiene che il Dr. Daniele Torchia, per l'assidua frequenza alle attività didattiche, per l'operosità nel lavoro di ricerca e per i risultati sperimentali ottenuti, possa meritatamente aspirare a conseguire il titolo di Dottore di Ricerca in Oncologia Sperimentale e Clinica.

Firenze, 15 Dicembre 2010

per il Collegio dei Docenti
Il Coordinatore del Corso
Prof. Persio Dello Sbarba

UNIVERSITA' DEGLI STUDI DI FIRENZE

Dipartimento di Patologia e Oncologia Sperimentali

DOTTORATO DI RICERCA IN ONCOLOGIA SPERIMENTALE E CLINICA

XXII Ciclo

Dr. Daniele Torchia

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Nel triennio di dottorato, gli studi del Dr. Daniele Torchia hanno avuto come oggetto lesioni melanocitiche acrali e immunità innata della cute. In questo settore, il Dr. Daniele Torchia è autore dei seguenti articoli pubblicati:

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Durante il triennio di Dottorato, il Dr. Daniele Torchia ha seguito con il massimo impegno il programma didattico stabilito dal Collegio dei Docenti ed ha portato avanti con entusiasmo e determinazione le sue ricerche, dando prova di grande inventiva ed intraprendenza, nonché di notevole capacità nella elaborazione dei dati sperimentali. Nel corso del triennio, il Dr. Daniele Torchia ha maturato una buona cultura di base ed una vasta

esperienza diretta in metodiche sperimentali, cellulari e molecolari, anche molto complesse. La curiosità e la tenacia del Dr. Daniele Torchia ne fanno un ricercatore di notevoli potenzialità, in gran parte già ampiamente espresse.

Firenze, 15 Dicembre 2010

il *tutor*
Prof. Gabriele Mugnai

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Acral melanoma 1

Prologue

No Woman, No Cry was Robert Nesta “Bob” Marley's first international hit outside of Jamaica in 1975. Six years later, he died of complications from metastatic malignant melanoma. Few details are available concerning Marley's primary lesion, although there is a consistent accounting of an injury to his right great toe. In July 1977 while in London, he was diagnosed with acral lentiginous melanoma (ALM). A few months earlier he underwent medical care for a soccer injury to the same region. During the next few months, his nail fell off and a nonhealing wound developed.

After his melanoma diagnosis, citing religious beliefs, Marley refused an amputation. Months later he underwent skin grafting to the hallux, with acceptable results. Years later while jogging in Central Park, New York City, Marley lost consciousness. Shortly after that, on the morning of May 11, 1981, he died at Cedars of Lebanon Hospital in Miami, Florida, from metastatic melanoma to his lungs and brain. Twenty-nine years later, I am typing these words in a small office located in the same facility, now named University of Miami Hospital. *De nobis fabula narratur…*

History and definition

Although ALM was defined relatively recently, its presence was recognized long before it was officially described. In 1886 Hutchinson reported the clinical features of subungual ALM as "melanotic whitlow." He described this entity as “a rare disease of the nailbed that is malignant” and noticed that injury appeared to bring these lesions to light.[1] Gibson et al. expanded upon the term "melanotic whitlow" in 1957 by providing a detailed histopathologic description and outlining its epidemiology.[2] However, ALM still was not recognized as a separate subtype in 1969 when Clark et al. first described the other three subtypes of melanoma: superficial spreading melanoma (SSM), nodular melanoma (NM), and lentigo maligna melanoma (LMM).[3] It was not until 1976 that Reed introduced ALM as a distinct clinicopathologic entity and defined its clinical and histopathologic characteristics.[4] He described ALM as “pigmented lesions on the extremities, particularly on plantar regions, like the palms of the hands and soles of the feet, that are characterized by a lentiginous (radial) growth phase evolving over months or years to a dermal (vertical) invasive stage.”[4] In 1977 Arrington et al. advocated that ALM was a distinct type of melanoma based on its location on the palms, soles, and subungual skin and described its characteristic radial growth phase of lentiginous melanocyte proliferation.[5] They were also the first to note that this type of melanoma was the most common expression of melanoma in blacks and that patients with ALM had a very poor prognosis.[5]

This classification has been widely used both by clinicians and researchers, and was the template for the current World Health Organization classification.[6] Its distinctions, based on the microscopic growth patterns, are associated with clinical features such as anatomic site of primary tumor and patient age.

However, in 1980 Ackerman questioned the existence of biologically distinct melanoma types, and proposed a unifying concept of melanoma.[7] He maintained that all melanomas evolve in a similar way, at first spread horizontally within the epidermis and eventually extend vertically into the dermis, and that the morphological differences are entirely secondary to the anatomic site in which the tumor arises. In case of NM, morphological differences may be a consequence of differences in the pace of tumor evolution; i.e., the vertical growth phase simply supervenes upon a briefer horizontal growth phase. In fact, the impact of Clark's classification on clinical management is limited, because no significant difference in overall survival could be demonstrated between the categories when tumors of equivalent thickness were compared.[8]

Recently, in their effort to integrate clinicopathological features with somatic genetic alterations, Bastian and his colleagues proposed a new classification system of melanoma.[9] They described distinct pattern of chromosomal aberrations and mutations in the oncogenes such as *BRAF*, *NRAS* and *KIT*, which correlated with the site of primary tumor and the degree of chronic sun-induced damage of the surrounding skin. Based on these observations, they classified melanomas into four groups; melanoma on skin with chronic sun-damage (CSD melanoma), melanoma on skin without chronic sun-damage (non-CSD melanoma), melanoma on palms, soles and nail bed (acral melanoma), and melanoma on mucous membranes (mucosal melanoma). Non-CSD melanoma roughly corresponds to SSM in the Clark's classification, CSD melanoma to LMM, and acral melanoma to ALM.

Epidemiology

Cutaneous malignant melanoma (CMM) is the most lethal form of skin cancer and accounts for approximately 78% of all skin cancer deaths. In the United States, the incidence of CMM has

been increasing rapidly, and currently, CMM is the sixth and seventh most commonly diagnosed cancer among men and women, respectively.[10] According to the SEER (Surveillance, Epidemiology, and End Results) Program of the National Cancer Institute, the estimated incidence of CMM in the United States in 1973 was 6.8 per 100,000 person-years, and this rate increased to 20.8 per 100,000 in 2005.[11] This increase in incidence is among the highest in SEER, with the exception of lung cancer in women. Four major histologic subtypes of CMM are currently recognized. SSM is the most common subtype, accounting for approximately 70% of cases, and occurs most often on the trunk. NM accounts for about 15% of melanomas and has only a vertical growth phase. LMM accounts for 13% of melanomas and correlates with long-term sun exposure in fair-skinned older individuals. ALM, occurring on the nail beds, palms, and soles, accounts for 2-3% of all melanomas.[11,12] The overall incidence of CMM in darker-skinned individuals is low compared with whites; however, ALM makes up a much higher proportion of CMM in dark-skinned individuals (i.e., blacks, Asians, Hispanics). Several single-institution case series of ALM have been published.[13-18] However, because this subtype of melanoma is rare, these studies have been limited by small sample sizes and have not been population-based.

A recent study showed that the incidence of ALM increased slightly, from 1.6 to 2.1 cases per 1 000 000 person years from 1992 to 2005.[19] This increase is most likely a result of ALM being recognized as a separate histologic subtype of melanoma in the mid-1980s and represents an overall increase in diagnosis. The incidence rate for ALM was similar in non-Hispanic whites and blacks, but statistically lower in Asian/Pacific Islanders.[19] Interestingly, Hispanic whites had statistically higher incidence rates of ALM.[15] Several studies demonstrated a peak age of onset in the 7th decade of life for ALM lesions.[20,21] Age of onset in the patients with skin type V and VI

was slightly lower compared with the general population.[22,23] The occurrence of ALM in the pediatric population seems to be extremely rare.[24]

In people of color ALM has been shown to have a predilection for plantar regions.[14,25] This predilection for ALM on plantar locations has led many to believe that trauma may be important in the etiology for ALM, because sun exposure has not been shown to be a risk factor for ALM.[15,20] In 2 retrospective ALM case series in which a trauma history was taken, 13% of 119 patients[20] and 25% of 35 patients[14] reported prelesional trauma (i.e., puncture wounds, stone bruises, friction blisters, contact dermatitis). Arguments against the trauma theory include the fact that the hand is exposed to more UV light and acute trauma.[15] Furthermore, no change in incidence of melanoma of the soles was seen when African tribes became urbanized and began to wear shoes.[14,26,27] Another factor that may play a role in the predilection of ALM for plantar locations is the fact that the density of melanocytes is 50% higher there than on the palms.[15]

Clinical features

ALM involves the palms, soles, and subungual regions and accounts for 60-80% of all acral melanocytic malignancies. The remaining non-ALM acral lesions are SMM (15-30%) and NM (3-15%).[17,18] Distribution is 60-63% for palmoplantar and 37-40% for subungual sites.[15,20] It occurs most frequently on the plantar surface of the feet.[22,23] When it involves the digits, the thumb and hallux are most commonly involved.

ALM of the palms and soles, apart from its anatomic location, is clinically very similar to SSM. The features of asymmetry, irregular borders, and color variegation may be present. In later lesions, nodular components may be present, which are typically associated microscopically with a dermal component.[28] Areas of ulceration may or may not be present. Barnhill and Mihm

described the epidermal component of the lesion as typically being of striking black color, whereas the dermal component is often elevated and associated with a dusky blue, black, or amelanotic region.[29]

Differential diagnoses of palmar and plantar ALM include melanocytic lesions such as lentigines and nevi. Nonmelanocytic lesions are also in the differential and include such diverse entities as tinea nigra, traumatic hemorrhage, tattoos with silver nitrate, seborrheic keratosis, pyogenic granuloma, pigmented basal cell carcinoma (BCC), and Kaposi's sarcoma.[29]

Subungual lesions may present with variable clinical findings. One such finding is that of a pigmented nail streak that enlarges in size and varies in color. The lesion may extend onto the proximal or lateral nail fold, a finding referred to as Hutchinson's sign. Other nail changes may indicate ALM. Nail dystrophy, such as thickening and splitting, may occur in early lesions, while ulceration and hemorrhage may appear in late stages. The differential diagnosis of subungual ALM includes traumatic hemorrhage and onychomycosis.[28] A history of trauma, however, does not exclude the diagnosis of melanoma.[18]

Diagnosis

The importance of an early diagnosis of melanoma has been pointed out by many previous studies. The well-known ABCDE rule[30] (Asymmetry, Borders, Color, Diameter, Elevation) aims to improve recognition of suspicious cutaneous pigmented lesions by clinicians. However, this algorithm cannot be applied to acral locations, as palms and soles have a peculiar anatomy which is characterized by marked orthokeratosis and the presence of *sulci* (furrows) and *gyri* (ridges). In fact, the pigmentation follows the skin markings of palms and soles, giving an asymmetrical appearance with an irregular and notched edge to all, even benign, flat pigmented lesions.

Neither can the ABCDE rule be applied to nail unit examination because the continuous outgrowth of the nail plate gives a band-like appearance to early pigmented nail matrix tumours. Other clinical signs of cutaneous malignancy such as bleeding, crusting, itch, fast growth, and nonhealing sore, albeit poorly specific, should be taken into consideration as well.

In vivo epiluminescence microscopy

In vivo epiluminescence microscopy – better known as dermatoscopy – has been demonstrated to be a useful and noninvasive technique for the diagnosis of cutaneous pigmented lesions. It significantly contributes to the improvement of diagnostic accuracy in melanocytic lesions.[31,32] However, pigmented lesions on acral sites, palmoplantar skin as well as the nail unit, do not show the classical pigment network pattern and other classical dermatoscopic features characteristically observed elsewhere on skin. Specific dermatoscopic patterns for lesions located on the palms and soles have been thoroughtly described by Saida and coworkers[33-36] and Malvehy and Puig among others.[37] The following dermatoscopic patterns have been recorded: parallel-ridge pattern (PRP, pigmentation following the ridges); irregular diffuse pigmentation (IDP) with variable shades from tan to black and without parallel disposition of pigment; parallel-furrow pattern (PFP, pigmentation following the furrows); lattice-like pattern (linear pigmentation following and crossing the furrow); fibrillar pattern (filamentous pattern with parallel fine streaks crossing the dermatoglyphs in a slanted direction); globular pattern (nonparallel distribution of globules in an area of diffuse light-brown pigmentation); acral reticular pattern (reticulated pigmentation similar to the pigment network of nonglabrous skin); homogeneous pattern; globulostreak-like pattern; and nontypical pattern when none of the above-mentioned features is present.[38]

Saida and coworkers have demonstrated that PRP is characteristic of the macular portions of acral melanoma and was present in as many as 86% of cases.[35] Sensitivity and specificity for acral melanoma of PRP were 86.4% and 99%, respectively.[35] The second characteristic pattern of ALM was IDP with variable shades from tan to black, which was present in 85% of melanomas in their series.[35] IDP seemed to be more suggestive of invasive acral melanomas. The positive predictive value for melanoma was found to be significantly higher for PRP than for IDP (93.7% vs. 80.7%, $P < 0.01$).[35] These results, obtained out of an Asian population, were grossly confirmed also in whites.[39]

The detection of early ungual melanoma is one of the most challenging diagnoses in clinical dermatology. For ALM of the nail apparatus the dermatoscopic patterns have been reported as follows: brown or greyish coloration of the background, regular or irregular lines in their coloration, spacing and thickness, with or without parallelism disruption, presence of blood spots, linear microhaemorrhages, and onychodystrophy.[40]

Another challenge in diagnosing ALM is the high frequency of amelanotic lesions, which are often misdiagnosed and mistreated. A rate of 27% of hypomelanosis or amelanosis among ALM, against less than 10% in other melanoma subtypes, has been reported.[20] These unpigmented variants were statistically correlated with a greater Breslow thickness and a worse prognosis.[20,41] For achromic lesions, comma-like vessels, arborizing vessels, glomerular vessels, dotted vessels, hairpin vessels, linear irregular vessels, milky-red globules/areas, and multiple vascular patterns (three or more) within the same lesion may be observed.[39] Dermatoscopy is also helpful for the detection of microscopic subtle or 'remnants' of pigmentation, which are nearly constant but can not be seen by the naked eye.

Histopathology

At conventional histology, melanocytes are present as nests and single cells along the dermal epidermal junction. Pagetoid upward migration tends to be extensive and widespread over the breadth of these lesions. Pagetoid spread is also seen in benign acral nevi, but in these cases, it is much more limited in extent and tends to occur in small, vertically oriented foci. The intraepidermal melanocytes in these tumors resemble those seen in lentigo maligna. The melanocytes are most commonly hyperchromatic and somewhat spindled in configuration. Nucleoli are not readily apparent in many cases. Dermal invasion is characterized by a proliferation of spindle shaped, hyperchromatic melanocytes coursing in fascicles, nests and single cells through the dermis. There is a marked tendency to track down eccrine structures, and aggregation around blood vessels is often seen. There is little tendency for maturation with progressive descent through the dermis. Mitotic activity is variable in degree.

When biopsied or resected tissues of acral skin are cut perpendicularly to the skin markings, two different epidermal rete ridges can be recognized; the *crista profunda limitans*, situated under the surface sulcus, and the *crista profunda intermedia* underlying the surface ridge. The latter epidermal rete ridge is passed through by an eccrine duct. When considered in conjunction with the dermoscopic patterns of melanocytic lesions on acral volar skin discussed above, it can be inferred that melanocytes proliferate in the *crista profunda intermedia* in melanoma *in situ*, whereas nevus cells proliferate in the *crista profunda limitans*.[35,36,42-47]

Prognosis

The median 5-year survival rate of patients with ALM varies in the literature from 24%[48] to 82%.[18] In general, ALM is associated with a poor prognosis,[49,50] mainly due to a later diagnosis

when the disease is advanced, misdiagnosis, and not to a more aggressive behaviour of ALM. Acral melanomas are frequently misdiagnosed, which leads to a delay in diagnosis of a minimum of 1 year.[51] Other authors claim a good or excellent prognosis when ALM is detected at an early stage and is treated with complete excision.[16,50]

Disease-specific survival appears to be better for patients with three-dimenstional histology, and worse for patients with high tumour thickness and histological ulceration.[52] Local recurrence plays no part in disease-specific survival.[52] In the literature, tumour thickness played also the main role concerning disease-specific survival of ALM.[18,20,50,53,54] Other prognostic factors often found were level of invasion, male gender, age, high mitotic rate, *c-myc* oncogene expression, ethnicity/race, ulceration, presence of vascular infiltration, microsatellites, location, amelanosis and, of course, a higher stage at time of diagnosis.[52,55]

Education programmes, early diagnosis and awareness of physicians that ALM may mimic different benign or malignant dermatoses may improve the outcome of ALM.[56] Histological examination should always be performed in acral lesions that do not heal.[51]

Tumour thickness and ulceration are independent risk factors for recurrence-free survival.[52] Other studies of cutaneous melanoma showed tumor thickness and type of surgery as independent risk factors for recurrence-free survival.[57] Phan et al. showed median recurrence-free survival of 10.1 years, with Breslow tumour thickness, male gender, amelanosis, mitotic rate, microsatellites and Clark level as independent risk factors for recurrence-free survival in the multivariate analysis.[20,41]

Treatment

Management of ALM is the same of that of melanoma in general. An excisional biopsy is preferred, both to give the dermatopathologist an optimal specimen and to allow evaluation of the excision margins for residual tumor. Incisional biopsies should not be performed when an excisional biopsy is technically possible. A biopsy-proven lesion has to be completely excised surgically with margins varying upon tumor thickness. According to recent guidelines, excision margins should be 5 mm for *in situ* lesions (i.e. confined within the epidermis without trespassing the basement membrane zone); 10 mm for invasive lesions thinner than 2 mm; 20 mm for invasive lesions thicker than 2 mm or thinner than 2 mm but with ulceration or areas of regression; 5 mm for every case treated by Mohs' micrographic surgery.[58] Lately, the tendency is towards preferring smaller excision margins, possibly using Mohs' micrographic surgery.

Since the introduction of the sentinel lymph node biopsy, elective lymph node dissection no longer plays a role in melanoma management except in selected clinical studies. The sentinel lymph node biopsy was developed to make possible the evaluation of the first draining lymph node in the regional lymphatic system. The procedure is appropriate for patients in whom neither palpation nor sonography has suggested the presence of lymph node metastases. The evaluation of the sentinel lymph node is not well standardized, so that the risk of missing a micrometastasis depends heavily on the techniques used (number of sections; hematoxylin&eosin stain; immunohistochemical stains; reverse transcription-polymerase chain reaction for melanoma-associated molecules). The sentinel lymph node biopsy should be performed in patients whose primary melanoma is thicker than 1.0 mm. Sentinel lymph node biopsies should be performed in centers where both the operative and nuclear medicine teams are experienced in the procedure. If there are other unfavorable prognostic parameters (Clark level IV–V; ulceration; regression), a

sentinel lymph node biopsy can be considered for thinner tumors. The sentinel lymph node biopsy is to be considered as a supplementary staging procedure (pathologic staging), not as a therapeutic measure with proven value in increasing survival time. If there is evidence of metastasis/micrometastasis, Radical lymph node dissection is considered standard therapy.

If operable metastases involve the skin or only one internal organ (e.g. lungs or brain), then operative removal of the metastases should be seen as therapy of choice (with brain metastases, stereotactic radiation therapy is equally effective). The possibility of first using neoadjuvant therapy and then excising the metastases should be considered. Many studies show that excision of solitary metastases can be associated with a surprisingly good prognosis for stage IV patients.

In special clinical situations, such as older patients with multiple systemic diseases and/or high surgical risks, primary radiation therapy (either soft radiographs or electron beam) can be considered an alternative to surgery.

Clinical follow-up examinations should be scheduled every 3 months for the first 5 years after the primary excision or the first recurrence and every 6 months for the next 5 years. Technical examinations (chest X-ray, ultrasound of the abdomen and regional lymph nodes) should be performed at least once a year.

Etiology and pathogenesis

The pathogenesis of ALM is still unclear, as classical risk factors for melanoma, e.g. UV, seem to be less relevant in acral lesions. ALM is thought to be less likely to arise from a pre-existing benign lesion, whereas melanoma in nonacral sites occurs from a melanocytic nevus in approximately 10–30% of cases.[59-61] Reports with convincing histopathological proof of association of ALM with a pre-existing lesion are scarce, and no subungual ALM on a pre-

existing nevus has been described yet to the best of current knowledge. Wong et al.[62] reported five cases (two invasive and three *in situ*) arising in association with pre-existing nevi on the foot. Kuchelmeister et al.[18] and Phan et al.[41] reported a rate of 10.4% and 3% of ALM arising on pre-existing nevi, respectively.

Multi-step progression model of melanoma

In addition to the classification system, Clark et al. also proposed a *multi-step progression model* of melanoma.[63] The theory comes from clinical and histopathological observations in sporadic melanomas (mostly non-CSD melanomas) as well as in familiar melanomas in dysplastic nevus syndrome patients,[64] all of which are more prevalent in whites. Several models of the genetic basis of melanoma development and progression are based on this Clark's multi-step model. According to these models, the first phenotypic change in normal melanocytes is the development of *benign melanocytic nevus*. Like melanoma, melanocytic nevi frequently harbor activating *BRAF* mutations,[65] which are thought to be an initial step in melanocytic neoplasms.

The next step toward melanoma evolution is *dysplastic nevus*, which histopathologically shows structural and cytological atypia. Dysplastic nevi may arise from a preexisting melanocytic nevus or as a new lesion. The molecular abnormality at this stage of progression may be disruption of $p16^{INK4a}$-retinoblastoma (Rb) pathway, mostly by the inactivation of *CDKN2A*, a gene encoding $p16^{INK4a}$ and $p19^{ARF}$.[66]

The third step in progression is the *radial growth phase (RGP) melanoma*, which spreads progressively within or just beneath the epidermis. In this phase of progression, neoplastic melanocytes are immortal because of the activation of human telomerase reverse transcriptase (hTERT). Deficiency in the $p16^{INK4a}$-Rb pathway, as well as hTERT activation, appears

necessary for melanocyte immortalization, and $p16^{INK4a}$-deficient human melanocytes showed a high rate of apoptosis that is suppressed by keratinocytes or their products.[67] Thus, it is speculated that RGP melanoma cells require keratinocytes or their products for survival, and can grow only in or near the epidermis.

The final stage in melanoma progression is the *vertical growth phase (VGP) melanoma*, which grows deep in the dermis and is capable to metastatize. For progression to the VGP, mutations repressing apoptosis would be required, which allow cells to survive in the absence of keratinocytes. These include PTEN loss, which would inhibit apoptosis via AKT activation, over-expression of a number of protein kinases or RAS activation, and β-catenin activation.[203] Progression from RGP to VGP is also marked by the loss of E-cadherin, as well as the aberrant expression of N-cadherin and αVβ3 integrin.[68] In addition, interaction between melanoma cells and stromal fibroblasts is important, creating a context that promotes tumor growth, migration, and angiogenesis.[69]

If a melanocytic nevus is a senescent clone of melanocytes that acquire *BRAF* mutations, as suggested by the Clark model of melanoma progression, all nevus cells within the lesions of melanocytic nevus should be *BRAF* mutants; however, this is not the case. In a study by Lin et al., detection rates of *BRAF* mutations in melanocytic nevi were markedly different between direct sequencing and the more sensitive shifted termination assay, suggesting that minor populations of nevus cells with BRAF mutations were admixed with *BRAF* wild-type nevus cells in these lesions.[70] These and other findings clearly indicate the polyclonality of *BRAF* mutations in acquired melanocytic nevi and contradict the current models predicting *BRAF* mutation as a founder even in melanocytic neoplastic progression.

De novo model of melanoma

While the Clark model of melanoma evolution was prevailing, a few investigators have maintained that most primary melanomas arise *de novo*, not associated with melanocytic nevus, because the majority of melanoma emerge in normal skin, not in association with dysplastic nevus, even in patients with familial dysplastic nevus syndrome.[71] From clinical and histopathological observations in acral melanoma, it is believed that this subtype always arises *de novo*.[72] Recently, it is suggested that melanoma arises from divergent pathways, and that many melanomas arise *de novo* whereas some may arise from pre-existing melanocytic nevi.[73] Thus, the current molecular models of melanoma development, based on the Clark's model may not be applied to these *de novo* melanomas.

Michaloglou et al. speculated that the first event in *de novo* melanoma development would be a yet unidentified hit, which may enable melanocytes to escape from oncogene-induced senescence.[74] This initial hit might collaborate with $p16^{INK4a}$-Rb inactivation, which could be achieved by deletion, mutation or promoter methylation of the *CDKN2A* gene, amplification of *CCND1* or *CDK4*, or *Rb* mutation. When a melanocyte already suffering from this hit acquires a proliferative mutation such as $BRAF^{V600E}$, it may fail to undergo oncogene-induced senescence and clonally proliferate. Thus, it is possible that the order of somatic genetic changes may determine whether a melanoma originates from a nevus or *de novo*. To gain immortality, proliferating melanocytes have to overcome replicative senescence by inactivating $p16^{INK4a}$-Rb pathway and by maintaining a minimum telomere length, which can be achieved by activation of hTERT. If inactivation of $p16^{INK4a}$-Rb had occurred earlier, this step could be achieved by hTERT activation alone. Finally, full oncogenic transformation to VGP melanoma may require additional genetic or epigenetic hits. These would include mutations suppressing apoptosis,

changes of adhesion molecule, and up-regulation of growth factors. However, this model of *de novo* melanoma formation is only speculative.

Molecular pathogenesis of acral melanoma

KIT mutations in acral and mucosal melanomas

KIT encodes a receptor tyrosine kinase, whose ligand is a stem cell factor (SCF). The dysregulation of *KIT* is thought to play a role in certain neoplastic disorders, including systemic mastocytosis, acute myelogenous leukemia, germ cell tumors, and gastrointestinal stromal cell tumors.[75] KIT–SCF signaling is also essential for melanocyte development, differentiation, proliferation, survival and migration.[76] While *KIT* expression is found in normal melanocytes, benign melanocytic nevi and *in situ* melanomas, it appears to be down-regulated in invasive and metastatic melanomas.[77,78] Furthermore, activating *KIT* mutation was rare, found in only 3 of 153 melanomas in one large-scale study.[79] Based on these obsevations, *KIT* was formerly dismissed as not acting as a melanoma oncogene.

However, a recent study by Curtin et al. has highlighted the important role of *KIT* in a subset of melanomas.[80] These authors examined 102 primary melanomas excised from various anatomical sites, and found mutations and/or copy number increases of *KIT* in 39% of mucosal melanomas, 36% of acral melanomas and 28% of CSD melanomas. By contrast, none of the non-CSD melanomas, which are the predominant type of melanomas in Caucasians, had *KIT* aberrations. The result indicates that the former ignorance of *KIT* was due to selection bias of only examining particular melanoma types. Several follow-up studies have confirmed rather frequent *KIT* mutations and/or amplifications in acral and mucosal melanomas.[81-84] Furthermore, *in vitro* studies have shown significant growth suppressive effects of small molecular inhibitors targeting

KIT, such as imatinib and sunitinib, in melanoma cell lines harboring *KIT* mutations.[85,86] Because *KIT* activity constitutes the major signaling node involved in growth and survival, treatment of *KIT*-targeting drugs lead to the simultaneous inhibition of the mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K)/Akt, and janus kinase (JAK)/signal transducer and activation of transcription (*STAT*) signaling pathways, as well as cell-cycle arrest and apoptosis.[86] Dramatic clinical effects of imatinib have actually been seen in mucosal melanoma patients with documented *KIT* activating mutations.[87,88] These observations indicate that *KIT* is an important oncogene and a critical target in melanomas arising on acral skin and mucosa.

CCND1 gene amplifications in acral melanoma

Together with MAPK and PI3K/Akt activation, the $p16^{INK4a}$-CCND1-CDK4-Rb pathway is functionally altered in most melanomas.[88] *CCND1* positively regulates the activity of CDKs, leading to phosphorylation of Rb and promoting entry into mitosis. The *CCND1* gene is an oncogene, rearranged and activated in centrocytic lymphoma and parathyroid cancers, and amplified and over-expressed in various human cancers.[89] Although the role of *CCND1* was less well established in melanoma, recent studies have revealed frequent amplifications (~45%) of the *CCND1* gene in acral melanoma.[90-92] Interestingly, while gene amplifications are usually found in association with disease progression in other cancers, *CCND1* gene amplifications in acral melanoma are detected early in the RGP of primary melanoma[90] and in the intraepidermal melanocytes in very early acral melanoma *in situ* lesions, in which only a slight increase of non-atypical melanocytes in the basal cell layer of the epidermis was observed.[93] Furthermore, copy number increase of *CCND1* gene was observed even in normal-looking melanocytes in the epidermis beyond the histopathologically recognizable margin of melanoma.[90,94] These genetically aberrant cells with normal morphology are “field cells”,[95] which represent a latent

progression phase that precedes the stage of melanocyte proliferation in the epidermis. Such field cells in acral melanoma extend from a few to 10 mm beyond the histopathological margin, and the extent does not correlate with tumor depth or diameter.[94] These observations suggest that amplification of the *CCND1* gene might be one of the earliest events in melanoma development in glabrous skin. The presence of amplifications, however, indicates that other aberrations likely cause genomic instability.[94] The progressive increase of the *CCND1* copy number from normal-looking melanocytes to the *in situ* portion to the invasive melanoma was observed, suggesting that the increased CCND1 gene dosage confers a growth advantage during tumor progression;[94] however, this may simply reflect the increase of genomic instability associated with melanoma progression.

Reflecting the characteristic of early melanoma cells, SMYM-PRGP, a cell line from a RGP of acral melanoma, is not tumorigenic *in vivo* and shows growth-factor dependency,[96] especially for endothelin-1, a known potent growth factor for normal melanocytes.[97] While it shows neither *NRAS* nor *BRAF* mutations, SMYM-PRGP harbors focal amplifications of *CCND1* and *hTERT* genes. The CCND1 protein level is about 10 times higher than that of normal melanocytes. Thus, instead of *NRAS* or *BRAF* mutations, *CCND1* amplification may be a crucial genetic change, which induces the proliferation of SMYM-PRGP cells through the endothelin signaling; however, upon stimulation with endothelin-1, although *CCND1* protein levels increased, phosphorylation of Rb protein did not increase, suggesting that over-expression of *CCND1* protein may have little effect on cell-cycle progression but may act as a survival factor in an early stage of acral melanoma evolution.[96] This particular cell line also provides insights into the immortalization of melanocytes. Previous investigations indicate that both deficiency of the $p16^{INK4a}$-Rb pathway and telomere maintenance are required for immortalization of human

melanocytes.[98] While SMYM-PRGP cells harbor amplifications of the *hTERT* gene, and show increased telomerase activity, they express $p16^{INK4a}$ protein at a level equal to that of normal melanocytes.[96] Thus, there is a different mechanism which coordinates with *hTERT* in melanocyte immortalization. This could be the over-expression of *CCND1* protein caused by gene amplification, since it may disrupt the $p16^{INK4a}$-Rb pathway by increasing CCND1-CDK4/6 complexes. It is also suggested that CDK4 amplification may obviate the need for $p16^{INK4a}$ inactivation.[99]

A hypothetical genetic model of acral melanoma has been proposed recently.[98] The earliest genetic alteration so far identified in acral melanoma is the amplification of *CCND1*, which are detected in the very early lesions of acral melanoma *in situ*;[92] however, this could not be the initial genetic alteration, but rather represents genomic instability, because gene amplification requires recurrent double-stranded DNA breaks.[93] Thus, the first genetic aberration affecting normal melanocytes in acral volar skin would be one that disrupts the maintenance of genomic integrity, for instance, failure of the cell-cycle checkpoint. Melanocytes harboring the amplification of *CCND1* may be selected for clonal expansion. While the oncogene amplification would lead to cell proliferation, it may act as a survival factor as well, as suggested in the study of SMYM-PRGP cells.[95] Then, acquiring activating mutations in oncogenes, such as *KIT*,[80] may be a crucial step inducing proliferation of “field melanocytes”. Because inactivation of $p16^{INK4a}$-Rb pathway is already accomplished by the *CCND1* amplification in “field melanocytes”, they could overcome oncogene-induced senescence. The proliferating melanocytes should also be immortal by extending telomeres, which is usually through activation of the expression of telomerase by the up-regulation of *hTERT*. Although RGP melanoma cells are immortal, they are not tumorigenic and are dependent on growth factors

secreted from keratinocyte, such as endothelin-1.[95] The final step from RGP to VGP may be mediated by similar genetic or phenotypic alterations discussed in the Clark model and the *de novo* melanoma model.

A personal hypothesis for ALM development

As reported in the previous chapters, ALM is a recently cathegorized and somehow "neglected" variant of malignant melanoma despite its relative high frequency and mortality. According to recent advances on research in this field, it seems likely that the molecular pathogenesis of ALM follows a multistep pathway which is different from the "classical", more common variants of ultraviolet-induced melanoma arising on hairy skin. However, as highlighted in the previous paragraph, studies conducted so far has failed to identify a definite pathogenic mechanism at the molecular level.

The palmoplantar epidermis is characterized by alternating surface ridges, that include the eccrine pores and overlying *cristae profundae intermediae* (passed through by the intraepidermal eccrine duct), and surface furrows with the overlying *cristae profundae limitantes*. Because of this peculiar anatomy, melanocytic lesions arising on acral volar skin are characterized by unique clinical and dermatoscopic features. As highlighted above, a PRP pattern showing prominent pigmentation on the ridges of the skin markings, is often detected in macular portions of malignant melanoma and melanoma *in situ* upon dermatoscopical examination. In contrast, a PFP and its variants, showing pigmentation along the sulci of the skin markings, are the most prevalent dermatoscopic features observed in benign melanocytic nevi. Recent histopathological studies, some of which carried out by myself, showed that, as expected, the PRP and PFP result mainly from the accumulation of pigmented cells in the *cristae profundae intermediae* and

cristae profundae limitantes, respectively.[42-47] These findings imply that malignant transformation of melanocytes occurs almost exclusively within the *cristae profundae intermediae*.[47] Moreover, it has been shown that melanin is released to the upper epidermal layers only if underlying nests of normal melanocytes, and not melanoma cells, are present.[46,47,100,101] The overmentioned phenomena have been investigated poorly to date and their putative role in ALM pathogenesis has been never considered.

My working hypothesis is that local changes in the innate immune functions may play a role in the pathogenesis of ALM, in accordance to growing evidences which indicate that the innate immune system, namely the Toll-like receptors-related pathways, may play a role in the pathogenesis of cancer (see Chapter 2). Namely, an impairment of innate immune system-related molecules, taking place only in well-circumscribed microanatomical regions of acral volar skin, may contribute to malignant transformation of the melanocytes harbored in those regions (e.g. the *cristae profundae intermedia*). Chapters 2 and 3 will now describe and discuss the general background underlying this hypothesis.

Innate immunity 2

Current understanding of cutaneous immune defense mechanisms has developed into the recognition of two coordinated systems by which our bodies defend against infection and disease: innate and adaptive immunity. Innate immunity involves a collection of preexisting, nonspecific anatomical, secretory, and cellular mechanisms to combat infection.[235] This is in contrast to specific or adaptive immunity that reacts following education by a specific immune stimulus (antigen). Although each system has distinct responsibilities, neither is effective alone and each is influenced by the other.

Innate immunity is an evolutionarily ancient part of our defense system that is common among plants and animals, vertebrates and invertebrates. It provides the first line of defense against infection and typically acts as a broad-spectrum and rapid defense mechanism to clear pathogens or reduce their spread. It works with a limited repertoire of recognition molecules. Adaptive immunity, on the other hand, only exists in vertebrates and provides specific recognition of foreign antigens, leading to immune memory. Only if innate immunity is unable to control the pathogen will the adaptive immune system be fully activated. Because activation of the adaptive immune system can require several days, the innate immune system effectively buys the body time until the slower effector molecules of both the innate and the adaptive system appear to fight.

Historically, innate and adaptive immunity have been studied and considered as separate entities. Today, however, synergistic and interfacing parts of the two systems have been appreciated. There are now several mechanisms known by which the innate immune system instructs and directs adaptive immune responses. Understanding this, and the distinct responsibilities of the innate immune system, is at the forefront of current basic research efforts in innate immunology.

Being the first line of defense against pathogen attack, the innate immune system responds to a microbe in two different ways: 1) it recognizes invading pathogens and distinguishes pathogenic microorganisms from nonpathogens; 2) it starts a coordinated physiological response to the offenders producing substances that can destroy them directly. Innate immunity consists of three major sets of elements: the physical/anatomical barriers, the cellular components, and the chemical/secretory molecules.

The anatomical and physical barriers of innate immunity

The anatomical barrier of an epidermal surface meets the definition of the innate immune system, yet is frequently overlooked by immunologists. In particular, the skin produces sweat and sebum and forms a stratum corneum that guards our inner environment from the outside, yet permits and possibly encourages microbial colonization of nonpathogens. A similar phenomenon occurs with mucosal membranes that cover the surface of gastrointestinal, urogenital, and respiratory tracts and produces saliva, tears, and mucous. Specialized physical elements such as cilia of broncho-pulmonary tree help get rid of microbes where tolerance or the need for symbiotic organisms is less important. Physical factors, such as our normal body temperature, inhibit growth of many microorganisms, whereas the establishment of an acidic pH at the surface provides yet another mechanism to discourage microbial growth. Thus, when considered

together, the anatomical/physical barrier is the first innate immune defensive shield. Recognition of this as a part of the immune system as a whole is now leading to greater understanding of how alteration of structural elements may play a role in modifying inflammatory and other biologic events.

The cellular components of the innate immunity

The cellular compartment of the innate immune system was traditionally believed to consist of only a few cell populations, including neutrophils, macrophages, monocytes, natural killer (NK) cells, and NK T cells (NKT-γδ T cells). We now know that innate immunity also involves many other cell types. All of these cells rapidly differentiate into short-lived effector cells whose main role is to rid the body of infecting organisms.

Neutrophils have classically been known as the most important cells in bacterial destruction. They contain cytoplasmic granules (lysosomes) that carry many secretory components including lysozymes (e.g., myeloperoxidase), proteases (elastase, cathepsin G, etc.), cationic proteins, and defensins. These secretory elements play an integral part in defending our bodies against pathogens. Neutrophils, together with macrophages, are major phagocytic cells in the skin. They have the capacity to migrate to the site of infection, detect the pathogen(s) using their receptors [namely, complement receptors and pattern recognition receptors (PRRs)] and destroy the invading organisms. In addition to phagocytosis, upon activation, these cells also induce several important effector mechanisms such as triggering the production of cytokines.

NK cells, on the other hand, are our major defense against viral infections and malignancies before the adaptive immune responses have had a chance to launch. Simply put, NK cells are lymphoid cells without a specific antigenic receptor that T cells and B cells express. Instead, they

recognize the malignant or virus-infected cells via sugar-lectin interaction or become activated in response to interferons (IFNs) or macrophage-derived cytokines.[102] NK cells do not attack the pathogen itself but rather the cells that have been infected by that pathogen. They are considered to be cytotoxic cells due to the possession of small cytoplasmic granules of perforin, granulysin, or other proteases (granzymes). However, the exact mechanisms of target cell death are not fully known.

The chemical and secretory components of the innate immunity

The soluble/secretory/chemical group of the innate immune system includes the chemical components that are constitutively produced by a number of cells that prevent pathogen colonization and growth. Examples of specialized chemical innate barriers include lactic and fatty acids in skin secretions that provide a low pH (5.6-6.4) and the degradation product of sebum-derived triglycerides that contribute to making a hostile environment for pathogen growth.

Following invasion of the epithelial barrier, the complement system, known long before the concepts of innate and adaptive immunity evolved, provides an essential systemic innate chemical defense system. The complement system is best known for induction of phagocytosis (opsonization) and bacterial lysis. Upon activation by a variety of specific and nonspecific immunologic mechanisms, the circulating proteins of the complement system participate in enzymatic cascades resulting in the destruction of pathogenic organisms. Additionally, the complement system is involved in modulating other parts of the immune system such as mast cell activation, neutrophil recruitment, and inflammation.

Other circulating components important to innate immunity were first described in the setting of their activity on cells participating in adaptive responses. This diverse group of regulators, generally referred to as cytokines, includes the growing list of interleukins (ILs), IFNs, chemokines, the tumor necrosis factor (TNF) family, and growth factors. An excellent example of the role of such molecules in innate immunity is IL-1. IL-1 is secreted by epithelial cells, macrophages, monocytes, and dendritic cells, and binds the IL-1 receptor (IL-1R), which is similar to the toll-like family of receptors known as classical elements of innate immune pattern recognition.[103]

Antimicrobial peptides (AMPs) refer to a group of small peptides, containing less than 100 amino acids, which have an inherent ability to kill a broad range of pathogens, including Gram-negative and Gram-positive bacteria, fungi, and viruses. Their function, once considered to be only antimicrobial, has now been understood to involve many more immunologic activities.[104,105] For example, they act as important regulators by driving cytokine expression in keratinocytes.[106,107] AMPs are produced in part by the epithelial surfaces as a first-line defense system but also by PMNs, macrophages, platelets, lymphocytes, dendritic cells, and mast cells. Some AMPs are present constitutively although they are not necessarily always active and may require processing.[108] Others are inducible by bacterial products, cytokines such as TNF-α or IL-1, or vitamin D.[109] AMP production increases with inflammation and injury, and they also have important roles in host repair and adaptive immune response.

How does innate immunity work?

As previously mentioned, innate immunity provides a shield in defense against invading pathogens. As a constitutive system, several known elements combine for the basic innate

protective system. Skin pH has a key role in fighting bacteria because only few species can proliferate in such low pH. AMPs and proteins can be found at the skin surface and help to hinder microbial proliferation.[110] The stratum corneum barrier consists of several layers of cornified keratinocytes embedded in a lipid matrix that provides a physical barrier. The lipid matrix, composed of ceramides, cholesterol, and free fatty acids, helps form a continuous physical network along the corneocytes and also exerts direct antibacterial activities. Free fatty acids have been described to have antibacterial activity against *Staphylococcus aureus*.[111]

Complementing the constitutive defense system, an inducible innate system also exists to appropriately respond to noxae that are not adequately controlled, as outlined below.

Recognition by the innate immune system

As discussed earlier, the hallmark of the innate immune response is its speed. As a first step, if a microbe is not controlled by the constitutive barrier and poses a threat to the host, this pathogen must be recognized and discriminated from "self".[112] The innate immune system plays an important role in discriminating between "infectious nonself" and "noninfectious self".[113] In addition, a third group, "noninfectious nonself," must also be distinguished at external epithelial interfaces. The innate immune system of the skin must make the first decision about potential danger and whether or not respond. Most skin disease can be categorized as either inappropriate decision about danger or an inappropriate response to danger.

Pathogen recognition by the innate immune system happens through a limited number of germline-encoded receptors. These receptors are special in that they recognize not one specific but many similar molecules; they are less stringent and more promiscuous receptors than their adaptive counterparts. Therefore, they have been referred to, by some authors, as pattern

recognition receptors (PRRs). PRRs gain their specificities from inherited genetic material. They have evolved to recognize patterns that are common among pathogens but not present in the host, called pathogen-associated molecular patterns (PAMPs). A classic example is the recognition of lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, by PRRs present on antigen-presenting cells (APCs) and other cells. Macrophages and dendritic cells of the innate immune system continually survey the environment and recognize PAMPs.[114] This is in contrast to the cells of adaptive immune system that can create a large repertoire of specificity by combination of genetic material. This gives the adaptive immune system a much greater variability in recognition potential that is responsive to education. Because these potential lymphocyte receptors are subject to expansion only in response to the exposure of each individual to the target antigen, each generation must reinvent their own repertoire. In contrast, innate immune receptors are limited in number but are retained in evolution and capable of recognizing more broad structural features of a molecule.[114]

PRRs of the innate immune system are not confined to the cell membrane. They are also present in intracellular compartments, circulating plasma, and tissues.[113,114] Once activated, these receptors participate in phagocytosis, opsonization, activation of complement and coagulation cascades, activation of proinflammatory signaling pathways, and induction of apoptosis.[115-117]

Among soluble PRRs that bind common structures in microbes are mannan-binding lectin and ficolins. Upon stimulation, they activate complement factors C2 and C4, leading to activation of the central molecule of the complement cascade, C3. Complement cascade, in turn, promotes opsonization, phagocytosis, and cytokine and chemokine release. The result is a proinflammatory antimicrobial environment that helps eliminate the pathogen.[115,116]

Another important cellular PRR is the macrophage mannose receptor (MMR) of the C-type lectin family. MMR interacts with a variety of Gram-positive and Gram-negative bacteria and fungal pathogens by stimulating their phagocytosis and subsequent delivery into the lysosomal compartment where they are destroyed by lysosomal enzymes.[118]

Macrophages also express macrophage scavenger receptor (MSR), a phagocytic PRR, of the scavenger receptor type A (SR-A) family. MSR has a broad specificity, which recognizes double-stranded RNA (dsRNA), LPS, and lipoteichoic acid.[119,120]

Other PRRs include the protein kinase receptor ‘and 2′-5′-oligoadenylate synthase (OAS)/RNaseL.

However, among the most well known of all PRRs are the toll-like receptors (TLRs).

TOLL-LIKE RECEPTORS

Introduction

The innate immune system is evolutionally conserved and is the first line of the host for protecting it from invading microbial pathogens.[121,122] Previously, innate immunity was considered only as a series of nonspecific responses that mediate cell killing through phagocytotic cells such as neutrophils and macrophages. However, the discovery of Toll receptor in *Drosophila* broke this traditional concept. The Toll receptor in *Drosophila* was found to play an important role in the induction of antifungal peptide expression against fungal infection, providing the first evidence that *Drosophila* expresses a specific receptor responsible for sensing

fungi infection.[123,124] Subsequently, a human homolog of Toll (hToll) was identified, and showed that this protein had an ability to induce production of inflammatory cytokines and expression of costimulatory molecules. Remarkably, a loss-of-function mutation of mouse homolog of hToll was identified in LPS-hyporesponsive mice,[125] which resulted in the development of Gram-negative sepsis but otherwise leaving most other immune functions intact. All these findings, and many others in other pattern recognition systems, suggest that PRRs are a key component of innate immunity. Here we will focus on describing the roles of TLRs in infectious and inflammatory diseases of the skin.

Toll-like receptor signaling

To date, 13 members of TLRs have been identified in humans and mice. TLRs 1-9 are conserved between these two species, whereas tlr10 is functional only in humans. In mouse the C-terminal half of tlr10 gene is substituted by a non-related sequence. Mouse TLR10 is non-functional, while mouse TLR11 is functional. Furthermore, TLRs 12-13 have only been found in mice.

The TLRs are expressed in a variety of cell types and in two general cellular locations, plasma membrane (TLR1,2,4-6), or intracellular compartments such as the endoplasmic reticulum and endosomes (TLR3, 7-9).[126,127] TLRs recognize and respond to a variety of molecules, such as lipids (TLR1,2,4,6), proteins (TLR5) and nucleic acids (TLR3, 7-9). Accumulating evidence suggests that TLRs are able to recognize both exogenous ligands such as those produced by microbes, and endogenous ligands including damage-associated molecular patterns and extracellular matrix molecules such as hyaluronan,[128] heat-shock proteins (HSPs), and fibronectin.

TLRs are transmembrane proteins with a series of leucine-rich repeats in the N-terminal extracellular domain and a cytoplasmic portion greatly similar in structure to that of IL-1R, hence referred to as the Toll-IL-1 receptor (TIR) homology domain.[129] With IL-1R signaling, TLRs have been shown to activate both nuclear factor (NF)-κB and MAP kinase pathways via myeloid differential factor 88 (MyD88), a common adaptor molecule recruited towards the TIR-domain of TLRs. Furthermore, four additional adaptor proteins involved in MyD88 independent pathways have been identified.[125,127] As a result of TLR stimulation by cognate ligands, the proinflammatory response genes including cytokines such as TNF-α, IL-6 and IL-12, and co-stimulatory molecules are induced via the activation of NF-κB and MAP kinases, while type-1 IFNs and their inducible genes are induced via interferon regulatory factors (IRF) 3 and/or 7.[130,131] Therefore, stimulation of individual TLRs leads to the robust but specific activation of innate immune responses.

The MyD88 dependent pathway

MyD88 is a universal adaptor for almost all TLRs identified with the exception of TLR3. The association of TLRs and MyD88 recruits members of the IRAK family, including IRAK1 and IRAK4. Upon TLR activation by cognate ligands IRAKs subsequently dissociate from MyD88 and the phosphorylated IRAK-1 interacts with TNF receptor-associated factor 6 (TRAF6).[132,133] TRAF6 is the signaling molecule following IRAK. The activation of TRAF6 in turn activates TAK1. TAK1, in combination with TAB1, TAB2 and TAB3, activates two downstream pathways involving the IKK complex and MAP kinase family. Phosphorylation of TAK1 and TAB2 occurs initiating the dissociation of TRAF6/TAK1/TAB1/TAB2 from the membrane to the cytosol, and IRAK-1 is degraded. TAK1 is subsequently active, resulting in phosphorylation

and degradation of IκB, which leads to the translocation of transcription factor NF-κB and, consequently, controlling the expression of different inflammatory cytokine genes.[134] TRAF6 has been shown to have a role in all TLR pathways to NF-κB tested to date, functioning as a central signaling molecule that can dock with multiple effectors and thus lead to NF-κB and MAPK activation by different routes. Moreover, phosphorylation of MAPK activates the transcription factor AP-1 in responses to multiple TLRs activation.

The MyD88 independent/TRIF dependent pathway

TLR3 is unique in that it signals exclusively through the third adapter to be discovered, TRIF. In addition to TLR3, TLR4 signaling independent of MyD88 has also been shown to require both TRIF and the fourth adapter, TRAM. TRIF binds to TLR3 and recruits TRAF6 directly through a TRAF6-binding motif in its N-terminal domain, which leads to TAK1 activation and subsequent NF-κB activation in an IRAK1- and IRAK4-independent manner.[135] TRIF can also activate NF-κB through an alternative pathway. The C-terminal of TRIF possesses a RIP homotypic interaction motif (RHIM), and it associates with receptor interacting protein 1 (RIP1) through homophilic interaction of RHIM domains.[102] A dominant negative form of RIP1 inhibits TRIF-mediated NF-κB activation. Thus, TRAF6 and RIP1 are involved in TRIF-dependent activation of NF-κB. However, there is also a TRAF6-independent NF-κB activation in the TRIF-dependent pathway. TRIF, but not MyD88, activates the type I interferon promoters. It has been shown that the noncanonical IKKs, IKKε and TANK-binding kinase-1(TBK1), mediate activation of IRF3 through interaction with TRIF and TRAM, thereby inducing the IFNβ promoter.[135]

Toll-like receptors in the skin

The skin is an ideal example of the innate immune system at work, providing physical barriers and other cellular rapid innate immune responses. All TLRs are expressed in the variety of cells that reside in the skin, but the expression and function of TLRs differs greatly between individual cell types. Relevant cells in the skin include keratinocytes and Langerhans cells in the epidermis, resident and trafficking bone-marrow-derived cells in the dermis such as macrophages, dendritic cells (DCs), T and B cells, mast cells, endothelial cells of the skin microvasculature, and skin stromal cells such as fibroblasts and adipocytes.[137] As a consequence of its location, human skin is exposed to a myriad of microorganisms. The cutaneous innate immune system selectively alerts the host of the presence of microbial pathogens by sensing PAMPs or endogenous signals of injury through TLRs and other PRRs and responds rapidly by producing cytokines, antimicrobial peptides or antimicrobial intermediates (e.g. radical oxygen species and nitric oxide).[138] When the innate immune system is unable to combat a microbial infection, as is frequently the case, the adaptive immune system then must play a role as a second line of defense. TLR activation also contributes to this process by initiating and shaping the adaptive immune response through the activation of dendritic cell maturation and influencing T and B cell function. Thus, maximal immunity is achieved to clear pathogens when TLRs coordinate the rapid innate response with the slower adaptive response. However, overactivated responses could be dangerous to the host as exemplified by sepsis or autoimmune diseases. Therefore, the responses need to be tightly controlled by associated negative regulators, negative feedback loop and/or by anti-inflammatory factors such as TGF-β, IL-10 and steroid hormones.

Toll-like receptors and inflammatory cytokines and chemokines in the skin

In skin the activation of TLRs regulates gene expression profiles including the production of cytokines such as TNF-α, IL-1, IL-6 and IL-12, chemokines such as IL-8 and MIP2, and upregulation of co-stimulatory molecules such as CD40, CD80, and CD86. Activation of TLR3 results in the production of TNF-α, IL-8, type I IFNs, the monocyte and basophil chemokine CCL2, and the macrophage inflammatory protein 3 (CCL20) in human keratinocytes.[137] Moreover, activation of TLRs 3 and 5 results in an increased production of CCL27, which promotes memory T-cell recruitment specifically to the skin.[137] In monocytes/macrophages the activation of TLRs induces the production of proinflammatory cytokines, such as TNF-α and IL-6, chemokines, and increases the phagocytic ability of macrophages.[138] TLR3 stimulation of Langerhans cell-like DCs but not monocyte-derived DCs increased the production of type I IFNs, suggesting that Langerhans cells can initiate direct antiviral activity after TLR3 activation.[139] Furthermore, major histocompatibility complex (MHC) molecules and co-stimulatory molecules such as CD80 and CD86 on the cell surface were upregulated during the process of myeloid DC maturation by TLRs. Therefore, activated myeloid DCs migrate from the skin to draining lymph nodes where they can express antigen to T cells and elicit cell-mediated immune responses.[140] Activation of myeloid DCs also induced the production of IL-12, which promotes Th1-type immune responses.[140] Taken together, all these data suggest that inflammatory cytokines and chemokines induced by TLRs play a critical role in eliciting distinct host defense mechanisms against invading pathogens in the skin.

Toll-like receptors and antimicrobial peptides

The endogenous antimicrobial peptides are effector molecules of the innate host defense system and are secreted by epithelial and other cell types.[141] AMPs have a broad antimicrobial spectrum and inactivate microorganisms by direct interaction with biomembranes or other organelles. Besides their direct antimicrobial function, it has been suggested that AMPs play multiple roles as mediators of inflammation with impact on epithelial and inflammatory cells influencing diverse processes such as cytokine release, cell proliferation, angiogenesis, wound healing, chemotaxis, immune induction and protease antiprotease balance.[142]

Human skin is exposed to millions of microbial organisms and these microorganisms produce various kinds of TLR ligands. However, the skin is an effective barrier against invading microorganisms both with and without inflammation. This protective function is partly mediated by the presence of AMPs as well as inflammatory cytokines and chemokines after TLRs have been activated in the skin. Several AMPs have been detected in skin keratinocytes, such as β-defensins, cathelicidin and psoriasin.[143] Studies strongly suggest that the TLR2-dependent pathway in keratinocytes is essential for antimicrobial activity.[144] Flagellin from *Escherichia coli* triggers TLR5 in human keratinocytes and strongly induces the expression of S100A7c (psoriasin).[145] Moreover, hormonally active vitamin 1,25-dihydroxyvitamin D(3) seems to act as a signaling molecule in cutaneous immunity by increasing pattern recognition through TLR2, increasing the expression and function of antimicrobial peptide, cathelicidin and killing of intracellular *Mycobacterium tuberculosis*.[145]

Besides the ability to kill microbes, AMPs can mediate both proinflammatory and anti-inflammatory effects in skin to link innate and adaptive immune responses. AMPs stimulate chemokine and cytokine secretion from a variety of cell types and can act through receptor-

dependent mechanisms.[146] In particular, several observations provide evidence that cathelicidin antimicrobial peptides mediate an anti-inflammatory response through TLRs 1, 2 and 4.[147,148]

Toll-like receptors link innate and adaptive immunities

TLR activation not only produces AMPs and proinflammatory cytokines, but also bridges the link between innate and adaptive immunity. This link comes in part through DC maturation. DCs couple TLR-mediated innate immune recognition to the initiation of T cell and B cell activation. Accumulating evidences show that DC maturation, which involves up-regulation of co-stimulatory molecules, is controlled by TLRs of the innate immune system.[149] Langerhans cells and dermal DCs express none and/or very low levels of MHC and co-stimulatory molecules, and are incapable of inducing T cell priming. In case of an infection, microbial presence is detected by TLRs expressed on DCs. Subsequently, DCs degrade antigens into MHC class I and II binding peptides, upregulate co-stimulatory molecule expression and translocate from the site of injury/infection to regional lymph nodes where they interact with naïve T cells.[150] In addition to controlling expression of co-stimulatory molecules, TLRs are also responsible for induction of cytokine and chemokine production by DCs,[151] including IL-12, which subsequently promotes Th1 cell-mediated adaptive immune response. Thus, in responses to microbial infection, TLRs not only produce early inflammatory and antimicrobial responses of the innate immune response but also initiate and subsequent adaptive immune responses.[137]

Toll-like receptor cross-talk

Individual TLR agonists are unique in their response profile in spite of the use of apparently common activation pathways. These TLR agonists can cross-talk to each other to augment or

suppress their responses either directly through ligand interactions or via secondary adaptor molecules. A great number of *in vitro* and *in vivo* studies have demonstrated that TLR stimulation induces different but overlapping Th1-Th2 cytokine-chemokine profiles, suggesting a complex interplay of stimulatory signals between TLRs.[152,153] The induction of type-1 IFN-gamma production is common to both activated endosomal TLRs (TLR3, 7, 7/8, 8, 9) as well as cell surface TLRs (e.g. TLR4).[154] In addition, it is known that the host target cells express discrete but multiple TLRs and an invading pathogen can have multiple TLR agonists.[155] As a result, a pathogen can activate multiple TLRs and in turn allows the immune system to determine the profile of the invading pathogen and to launch an appropriate immune response. Furthermore, the TLR cross-talk also can establish a cytokine-chemokine network and set point of one pathogen that may differ from another which in turn may be detrimental to the survival of one another and may serve as beneficial to the host.[156]

TLR cross talk may also provide a mechanism by which cells that are non- or low-responsive to a particular TLR may switch to a "responsive mode" due to concomitant activation with another TLR agonist or cytokine,[157] and hence drive responses toward preferred T cell polarization. Although this upregulation of TLRs by TLRs often results in augmenting immune responses by engaging more TLRs, the initial stimulating dose and the timing of stimulation by the second TLR agonist can influence the immune response.

Negative regulators in Toll-like receptor overactivation

TLRs are critical for the initiation of inflammatory and immune responses by detecting conserved microbial ligands.[151] These ligands trigger TLRs for the induction of inflammatory cytokines, which is required for effectively clearing pathogens and plays an instructive role in

the development of the adaptive immune response, in particular the Th1 response.[158] As discussed previously, TLR cross-talk can either boost or suppress these responses. However, TLR activation is a double-edged sword. On one hand its activation is essential for provoking the innate response and enhancing adaptive immunity against pathogens and on the other hand TLRs are also involved in the pathogenesis of autoimmune, chronic inflammatory and infectious diseases. Overactivation of TLRs causes persistence of producing proinflammatory cytokines, such as TNF-α and IL-6. Therefore, the inflammatory response must be tightly regulated and indeed, multiple regulatory mechanisms control the extent and duration of TLR-induced inflammation.[159] These include the inhibition of TLR signaling by inducible negative regulators, production of anti-inflammatory cytokines and alterations of the TLR signaling complex.[159]

To limit TLR-induced inflammation several negative regulators of TLR signaling are involved via sequestration of signaling molecules, blockade of their recruitment, degradation of target proteins or inhibition of transcription.[160] This negative regulation is achieved at multiple levels. During acute bacterial infection, soluble TLRs (such as MyD88s) are produced and provide the first line of regulation by functioning as decoy receptors that prevent a direct interaction between TLRs and their microbial ligands. Once TLR and ligand interact, TLR signaling can be further controlled by intracellular regulators, which can inhibit TLR signaling pathway.[160] Furthermore, some negative regulators are also involved in negative feedback of TLR signaling. Overall, TLR signaling is tightly controlled by negative regulators to terminate immune and inflammatory responses, prevent excessive inflammation, and balance the beneficial or detrimental roles of TLRs in host defense.

Toll-like receptors and skin infectious and inflammatory diseases

Upon microbial infection, the innate immune system alerts the host of the presence of microbial pathogens, activates TLR signaling to produce cytokines and antimicrobial intermediates, further orchestrates the adaptive immune response, and thus achieves the maximal immunity to clear pathogens. TLRs thereby play a critical role in infectious and inflammatory diseases. Indeed, the engagement of TLRs appears to be intricately associated with certain skin diseases. However, although the activation of TLRs is necessary for host action against various pathogens, in some cases, microbes also take advantage of the TLR-induced innate responses to develop infections. The following are examples of some skin infectious and inflammatory diseases relevant to TLRs.

***Staphylococcus aureus*-induced infections**

Staphylococcus (S.) aureus is a common type of bacterium that live on the skin and mucous membranes of humans. When crossing the skin barrier, *S. aureus* causes skin abscesses from which the bacteria can disseminate and cause bacteremia, sepsis, endocarditis and keratitis. *S. aureus* infections also often happen to people with some skin diseases, such as atopic dermatitis (AD).

Staphylococci have several TLR inducers. Bacterial lipoproteins and LTA serve as TLR2/6 or TLR2/1 agonists, while peptidoglycan is sensed by NOD2. Studies showed that TLR adapter molecule MyD88 is required in response to all *S. aureus* infection models in mice. In skin abscesses and systemic infection, MyD88 plays a pivotal role in bacterial clearance and limitation of the disease.[161] Furthermore, cytokine production and neutrophil recruitment were proven to be MyD88-dependent in cutaneous and corneal infections. Moreover, polymorphisms

in the TLR2 gene have been demonstrated to fail to clear *S. aureus*.[162] However, whether TLR2 abnormalities are relevant to the susceptibility of skin colonization with *S. aureus* remains unknown.[163]

Vaccinia virus

Vaccinia virus (VV), a member of poxviruses family, has been used extensively as a vaccine vehicle in the clinical application for infectious diseases and cancer. Fetal vaccinia, manifested by skin lesions and organ involvement, often results in fetal or neonatal death. Poxviruses employ many strategies to evade and neutralize the host immune response. VV encodes proteins that antagonize important components of host antiviral defense. A46R and A52R, two VV proteins that share amino acid sequence similarity with the TIR domain, have a key role in innate immunity and inflammation. A46R partially inhibits IL-1-mediated activation of the transcription factor NF-κB, and A52R potently blocked both IL-1- and TLR4-mediated NF-κB activation.[164] In addition to IL-1- and TLR4-mediated NF-κB activation, VV protein A52R also blocks the activation of NF-κB by TLR3. Therefore, A46R and A52R are likely to represent a mechanism used by VV to suppress TIR domain-dependent intracellular signaling. VV elicits innate immune responses also through TLR2 and MyD88, leading to the production of proinflammatory cytokines, suggesting that VV can target multiple TLRs pathways to work together in achieving efficient activation of host defense.

Atopic dermatitis

Atopic dermatitis (AD) is a widespread inflammatory skin condition marked by flares and remissions. Patients with AD, compared to patients with other skin inflammatory diseases,

exhibit defects in innate and acquired immune responses, resulting in a heightened susceptibility to bacterial, fungal and viral infections, most notably colonization by *S. aureus*.[165] A recent study showed the skin from AD patients, compared with normal skin and psoriasis skin lesion, had much decreased production of 2 endogenous antimicrobial peptides, LL-37 and beta-defensin 2.[166] Activation of TLR2 signaling has been shown to induce β-defensin 2.[167] Another hypothesis is that Staphylococcal exacerbation of AD patients may be due to abnormalities/dysfunction in the TLR2 signaling. Although the precise role of TLR2 in AD is not well understood, the dysfunction of TLR2 as a potential mechanism in the pathogenesis of AD seems a rational theory.[163]

Psoriasis

Psoriasis is a chronic inflammatory skin disease considered to be an immune-mediated, organ-specific (skin and/or joints) inflammatory disease, in which intralesional T lymphocytes trigger primed basal stem keratinocytes to proliferate and perpetuate the disease process. A study that compared TLR expression and HSP expression in normal and psoriatic skin showed that HSPs are naturally occurring ligands that can stimulate APCs by way of TLR4,[168] which may be relevant to the pathogenesis of psoriasis because HSPs are overexpressed by keratinocytes in psoriatic lesions. HSPs trigger TLR4 on APCs such as Langerhans cells, leading to maturation, and TNF-α and IL-12 secretion, thus, contributing to psoriasis immunopathology. Moreover, an immunohistochemical analysis of normal and psoriatic skin demonstrated that TLR1 and TLR2 expression was increased in psoriatic keratinocytes in the suprabasal layer, whereas TLR5 was down-regulated by basal keratinocytes in psoriasis compared with normal human skin.[169] Another recent interesting observation is that LL-37 can serve to present self-DNA to activate

IFN-γ release from plasmacytoid dendritic cells.[170] That this event may perpetuate inflammation in psoriatic patients remains to be demonstrated.

Toll-like receptors and cancer

TLRs are very potent activators of gene transcription and translation. The role of TLRs in mammalian cell biology seems overwhelming, and reaches far beyond the simple antimicrobial immune response of the innate immunity. Therefore, it has become even more tempting to scrutinize TLR pathways in human diseases, especially in malignancies. TLRs are believed to be involved in tumor development and growth, with several possible mechanisms, but they have also been found in reactions directed against tumor cells.

Toll-like receptors as negative regulators of cancer[171]

In the 18th century Deidier observed a positive correlation between infection and the remission of malignant disease, making the first inference that microbes could have anticancer properties. At the end of the 19th century, William Coley observed that repeated injections of a mixture of bacterial toxins from the Gram-positive bacterium *Streptococcus pneumoniae* and the Gram-negative bacterium *Serratia marcescens* served as efficient anti-tumour therapeutic agents, providing evidence that microbial products, rather than infection itself, may mediate an anti-tumour effect. It was later discovered by Shear and Turner that LPS was the "haemorrhage producing fraction" of Coley's toxin that accounted for its anti-tumour effect. As LPS stimulates TLR4, these results suggest that the anti-tumour effect of Coley's toxin was a result of TLR activation.

Other microbe-derived therapeutics with anti-tumour activity can be linked to their ability to activate TLR. OK-432, a lyophilized preparation of group A streptococcus that is used in the treatment of cervical, gastric and oral squamous cell carcinoma[172-174] was recently shown to stimulate TLR4.[175]

Mycobacterium bovis bacillus Calmette-Guérin (a potent activator of TLR2- and TLR4-dependent signalling) has been used for 30 years as an effective treatment of bladder cancer through the intravesicular injection of these mycobacteria.[176]

Potent anticancer effects against established tumors in both mice and humans after the administration of purified ligands for TLRs have been demonstrated as a result of local (at the site of the tumor) and systemic delivery. Administration of LPS has been used in phase II clinical trials for the treatment of colorectal and lung cancer[177] and leads to tumor regression when directly injected into adoptively transferred tumors.[178] This effect has also been shown on injection of flagellin.[179] Locally, application of synthetic ligands for TLR7 and TLR8, such as imiquimod, are under investigation for the treatment of skin cancer and chronic lymphocytic leukaemia[180,181] as is the TLR9 ligand, CpG, for the treatment of brain, skin and renal cancer and lymphoma.[182]

TLR agonists might mediate their antitumour activity through a multitude of mechanisms. High doses of TLR agonists, especially those such as poly(I:C) that stimulates TLR3, can lead to apoptosis and have been shown to directly kill both tumor cells and ancillary cells of the tumour microenvironment, such as the vascular endothelium.[183] TLR activation may also cause tumors to regress by increasing vascular permeability and by directly or indirectly recruiting leukocytes, resulting in tumour lysis by NK and cytotoxic T cells.

One of the most appreciated functions of TLRs in cancer therapy is stimulation of the adaptive immune system. In these studies, tolerance to tumor self-antigens is broken, presumably by TLR-mediated upregulation of co-stimulatory signals to the adaptive immune response, a property known as adjuvanticity.

Does activation of TLRs have a physiologic (not only an iatrogenic) role in inhibiting tumorigenesis? In the majority of studies, exogenous TLR agonists have been used to induce anticancer T-cell responses. The responses are often not achieved under physiologic circumstances. However, two recent studies have suggested a more physiological role of TLRs in inducing anti-tumor T-cell responses. In one study, the ability of numerous chemotherapeutic agents to kill established, adoptively transferred tumors was decreased in TLR4- and MyD88-deficient mice.[184] In a second report, C3H/HeJ mice with a loss-of-function mutation in TLR4 that were treated with a carcinogen to induce skin tumors developed more tumors than wild-type mice, perhaps owing to decreased activation of IFN-γ-dependent anti-tumor T-cell responses.[185]

The major physiological role that TLRs have in cancer may be preventing infection by microbial pathogens that are associated with its development. TLRs are important in the recognition of microbial pathogens such as *Epstein–Barr virus,*[186] *hepatitis B* and *C viruses*,[187,188] human papilloma virus[189] and *Helicobacter pylori*,[190] all of which are important etiological agents of human cancer.

Classically, the ability of TLR signaling to activate the adaptive immune system has led to attempts to harness this response against cancer cells through the use exogenous administration of TLR ligands. More research is needed to determine the role of microbial and endogenous TLR ligands in inhibiting tumorigenesis in both infectious and non-infectious situations.

TLR stimulation drives tumorigenesis

The original idea that stimulation of TLRs has a positive role in tumorigenesis came from reports demonstrating that TLR ligands augment the growth of adoptively transferred tumors.[191,192] For example, systemic LPS administration increased migration, invasion and angiogenesis at secondary sites of an intravenously injected metastatic mammary adenocarcinoma cell line.[193] Similarly, increased rates of proliferation and decreased rates of apoptosis were evident in metastatic tumours that were formed after the adoptive transfer of a colonic adenocarcinoma cell line followed 9 days later by an intraperitoneal injection of LPS.[194] Indeed, TLR stimulation in a variety of tumor cell lines leads to increased survival and proliferation *in vitro*. Isolated plasma cells from patients with multiple myeloma were shown to express an increased repertoire of TLR compared with plasma cells from healthy donors.[195,196]

Stimulation of these cells with various TLR ligands led to increased proliferation in part owing to autocrine secretion of IL-6.[197,198] By decreasing endogenous expression of TLRs in tumor cell lines it was demonstrated that TLR4 was required for the optimal growth of adoptively transferred tumor cells lines (even in the absence of exogenous LPS administration)[199] and that intratumoral administration of *Listeria monocytogenes* induced TLR2 signalling in tumor cells and promoted their growth.[191] TLR ligand administration might also act on host cells to enhance the growth of adoptively transferred tumor cells. In a model in which systemic administration of LPS enhanced the growth of adoptively transferred cells, it was shown that TLR4 signalling in the recipient was required for LPS-induced tumor growth. The suggested mechanism involved a host-dependent increase in circulating levels of TNF that led to the upregulation of NF-κB-regulated antiapoptotic factors, such as BCL-XL, cIAP1 and cIAP2, in the tumor cells.[200]

The studies cited above have demonstrated a role of TLRs in promoting tumor survival and growth using adoptive transfer methods. Recently, however, data have indicated that TLRs (and IL-1/IL-18R signalling) have a crucial role in the development of tumors as they arise in their natural microenvironment, thus revealing a previously unknown aspect of tumorigenesis.

Mice injected with the mutagen diethylnitrosamine develop liver carcinomas that are dependent on both mutagen-induced cell death and compensatory proliferation. It has been suggested that the response of stromal cells, such as tissue-resident macrophages, to hepatocyte death is crucial to the proliferation and expansion of pre-cancerous cells and tumor promotion.[197] This promotion is the result of the NF-κB-dependent production of inflammatory mediators such as IL-6 following recognition of necrotic hepatocytes by the tumor stroma. Recently, it was shown that MyD88 is crucial for the promotion of diethylnitrosamine-induced hepatocellular tumors and that MyD88 signalling was responsible for the activation of NF-κB and for the production of factors such as IL-6 in response to hepatocyte cell death.[197]

MyD88 has also recently been shown to be crucial for tumor promotion in models of spontaneous and carcinogen-induced intestinal tumorigenesis. *ApcMin*/+ mice (heterozygous for a mutant allele of the tumor suppressor adenomatous polyposis coli) that are deficient in MyD88 have both a decreased incidence and size of tumors compared with *ApcMin*/+ wild-type mice.[201] Insight into the mechanism by which MyD88 regulated tumorigenesis came from analysis of positive regulators of tumorigenesis, which demonstrated that MyD88 regulated the expression of COX2, MMP7 and cytosolic phospholipase A2,[201] which are important in many aspects of tumor growth.[202] A recent report demonstrated that MyD88 is a crucial positive regulator of chemically induced tumors of both skin and connective tissue.[202] MyD88-deficient mice formed fewer tumors upon administration of various carcinogens, which led to the development of skin

papillomas and sarcomas.[203] These recent studies indicate that TLR signaling contributes to the growth of tumors in numerous organs and thus may represent a general principle of tumorigenesis. As MyD88 is also activated by IL-1-IL-18R activity, it is possible that this pathway also contributes to tumorigenesis in these models.

Whether TLRs are involved in tumor initiation is not yet clear. The data from the *ApcMin*/+ mice indicate that they are not. However, the *Il10*–/– mouse model of colitis-associated carcinoma shows that colitis in this context is dependent on TLR recognition of the intestinal microflora. Thus, as tumor initiation is secondary to colitis, a role for TLRs seems likely.[204] A formal role of TLRs in initiation with concatenate inflammation is yet to be determined; however, one can envision several possible roles for TLRs in initiation. Chronic and unregulated stimulation of TLRs could lead to damage and mutation of DNA and aberrant chromosomal translocation by inducing free radicals or activation-induced deaminase. Furthermore, induction of factors such as macrophage migration inhibiting factor and BCL-6 by TLRs may impair the DNA damage response through inhibition of p53-mediated growth arrest and apoptosis,[205] thereby driving tumor initiation.

Importantly, the experiments discussed above indicate a link between tissue repair and tumorigenesis at the level of molecular recognition of tissue injury. It remains to be determined whether the TLR-mediated homeostatic response to tissue injury that is associated with tumorigenesis orchestrates processes such as angiogenesis that are ancillary to tumor promotion. TLR activation is known to stimulate angiogenesis *in vitro* through the expression of pro-angiogenic factors such as IL-8, vascular endothelial growth factor and matrix metalloproteinases, and there is *in vivo* evidence that TLRs might regulate the angiogenic

switch.[206] Furthermore, TLR signaling in tumor cells, the vascular endothelium or other cells may aid various stages of progression and metastasis.

Initial evidences suggest that innate immunity and TLRs may play a relevant role also in melanocyte-derived tumors, as outlined in the following chapter.

Pigmentation and immunity 3

Pigmentation

Melanocytes can absorb ultraviolet rays (UV) and survive considerable genotoxic stress. The skin is the main barrier to the external environment, and relies on melanocytes to provide, among other things, photoprotection and thermoregulation by producing melanin.[207] The degree of pigment production manifests as skin phototype and is the most useful predictor of human skin cancer risk in the general population.

The colors we see in feathers, fur and skin are largely determined by melanocytes. In addition to carotenoids and haemoglobin, melanin is the main contributor to pigmentation. There are two main types of melanin — red/yellow pheomelanin and brown/black eumelanin. Melanin-containing granules are known as melanosomes and are exported from melanocytes to adjacent keratinocytes, where most pigment is found. As a result, pigmentation differences can arise from variation in the number, size, composition and distribution of melanosomes, whereas melanocyte numbers typically remain relatively constant.

The migratory pathway of the melanoblast

Melanocyte development from its precursor, neural-crest cells, highlights the unique properties of this cell type. Neural-crest cells are pluripotent cells that arise from the dorsalmost point of the neural tube between the surface ectoderm and the neural plate. In addition to melanocytes,

neural-crest cells give rise to neurons and glial cells, adrenal medulla, cardiac cells and craniofacial tissue.[207] Melanoblasts, the precursors of melanocytes, migrate, proliferate and differentiate *en route* to their eventual destinations in the basal epidermis and hair follicles, although precise distribution of melanocytes varies among species.

Melanocyte development has been well characterized in several species, including the mouse embryo. In mice, melanoblasts differentiate from pluripotent neural-crest cells at about embryonic day (E)8.5, migrating along the dorsolateral pathway and eventually diving ventrally through the dermis. Defects in melanocyte migration typically appear most prominently on the ventral surface as “white spotting”, as this is at the greatest distance (watershed zone) from the dorsum. By E14.5 in mice, melanocytes exit from the overlying dermis and populate the epidermis and developing hair follicles. Melanocytes also reach the choroid of the back of the eye, the iris, the leptomeninges and the stria vascularis of the cochlea (inner ear).

Various signalling pathways and transcription factors tightly regulate melanocyte migration. These proteins and pathways provide and integrate spatial and temporal signals to create the proper environment for normal development and migration. Mutations in genes affecting this process produce hypopigmentation that arises from lack of melanocytes rather than lack of pigment in viable melanocytes, as occurs in albinism. Key genes in this developmental pathway include paired-box 3 (*PAX3*), sex-determining region Y box 10 (*SOX10*), microphthalmia-associated transcription factor (*MITF*), endothelin 3 and endothelin receptor B (*EDNRB*). Disruption of these genes has led to clearer understanding of certain human inherited pigmentation disorders, specifically Waardenburg syndrome, which is characterized by hearing and pigmentary defects. Given the defined molecular and phenotypic relationships among the

genes responsible for these syndromes, they represent an epistatic tree of interacting melanocytic regulatory factors.[207]

MITF is a member of the Myc-related family of basic helix–loop–helix leucine zipper (bHLH-Zip) transcription factors and is conserved in essentially all vertebrate species. In melanocytes, *MITF* alone seems to be the critical family member. Three MiT factors, including *MITF*, have also been identified as human oncogenes and found to be involved in multiple malignancies, including melanoma.[208]

The WNT/β-catenin-signalling pathway is also essential for neural-crest induction and melanocyte development. Mice lacking wild-type *Wnt1* and *Wnt3a* have pigmentation defects.[209] WNT1 and WNT3A trigger a canonical pathway resulting in β-catenin-induced transcription at TCF/LEF (T-cell factor/lymphoid enhancer factor) promoter/enhancer elements. Numerous targets of TCF/LEF have been identified, including *MITF*, *TRP2* and *SOX10* in melanocytes and melanoma cells.[210]

Melanocyte homing to the epidermis and hair follicles

c-Kit is a tyrosinase kinase receptor involved in melanoblast expansion, survival and migration.[211] Activation of c-Kit by Kit-ligand (also stem cell factor, SCF) leads to Ras activation and multiple canonical signalling as well as post-translational modification of MITF.[212] *c-Kit*, *SCF* and *SNAI2* (also known as *SLUG*) mutations have all been identified in human piebaldism, an autosomal dominant ventral depigmentary disorder. The availability of SCF has a crucial role in permitting melanoblast survival and promoting proliferation, both initially in the dorsolateral migration pathway and later from the dermal mesenchyme to colonize the hair follicles and epidermis.[213] *SLUG* encodes a zinc finger transcription factor and seems to

be required for melanoblast migration and/or survival. In a mouse model of melanoma, the inhibition of SLUG by small interfering RNA (siRNA) suppressed metastatic propensity, potentially linking SLUG to both migration and metastasis-related behaviours.[214]

In addition to c-Kit/SCF, other mechanisms are likely to be involved in the late steps of melanocyte migration from the dermis into the epidermis. These include endothelins 1 and 3, hepatocyte GF and basic fibroblast GF (FGF).[215] Cadherins are also implicated: as dermal melanoblasts move through the basement membrane they express E-cadherin, which is then downregulated and replaced by P-cadherin during migration into the hair follicles.[216]

Melanoblast survival

MITF expression is activated early on during the transition from pluripotent neural-crest cells to melanoblasts and is required for the survival of migrating melanoblasts.[217] Complete deficiency of MITF results in absence of melanocytes, suggesting that MITF is essential for lineage survival, for proliferation or to prevent transdifferentiation towards other neural-crest lineages (such as glia and neurons). Evidence that *MITF* remains vital for melanocyte survival throughout the life of an organism comes from the hypomorphic mutant allele $Mitf^{vit}$, which exhibits near-normal melanocyte development, but accelerated age-dependent greying of coat color due to postnatal melanocyte loss.[218]

B-cell leukemia/lymphoma (Bcl)-2, a transcriptional target of MITF and known inhibitor of apoptosis, is also required for melanocyte viability.[219] Cyclin-dependent kinase (Cdk)-2, a cell-cycle regulator, is another MITF target. Melanoma cells have been shown to require Cdk2 expression to maintain their cell cycle and viability.[220] Nevertheless, MITF's interactions with Bcl-2 and Cdk2 do not fully explain melanocytes' requirement for MITF to remain viable during

development, because the *Mitf*-null homozygous phenotype is more severe than either of the single knockouts, and expression of either gene in *Mitf*-knocked-down melanoma cells only partly rescues viability.[220]

Many additional targets of MITF have been identified, including the cytokine receptor c-Met and CDK inhibitors $p16^{INK4a}$ and p21/Cip1. Together, they probably contribute to MITF's dual activities in relation to melanocytic differentiation and survival/proliferation, and some may serve as surrogate drug targets for the MITF oncogenic pathway. The function of MITF-mediated survival should be distinguished from differentiation markers, as MITF is believed to coordinately regulate the expression of pigment genes, although probably in conjunction with other context-dependent factors.

Melanocyte stem cells

Melanocytes of the hair follicle are responsible for hair pigment. The lifecycle of the hair follicle melanocyte is closely linked to that of the rest of the hair follicle, which is typically in growth (anagen), but moves through a brief period of regression (catagen) and finally enters a resting phase (telogen) in which the hair is released and the cycle can begin a new. Multipotent epidermal stem cells exist in the bulge region (at the bottom of the permanent portion of the follicle, just below the sebaceous gland). This is also where melanocyte stem cells reside. These are responsible for restoring the pool of differentiated melanocytes to the hair bulb, where the pigment is incorporated while new hair is being synthesized. Independent validation that the melanocyte stem-cell niche resides in hair follicles comes from patients with vitiligo (absence of epidermal melanocytes) resulting from immunosuppressive treatment, in whom repigmentation is clearly initiated in radial fashion from hair follicles.

Melanogenesis

Melanin production occurs predominantly in a lysosome-like structure known as the melanosome. Pheomelanin and eumelanin differ not only in color but also in the size, shape and packaging of their granules. Both melanins derive from a common tyrosinase-dependent pathway with the same precursor, tyrosine. The obligatory step is hydroxylation of tyrosine to dopaquinone, from which L-DOPA can also be derived. The absence or severe dysfunction of tyrosinase and other key pigment enzymes (including P gene, tyrosinase-related protein[TRP] 1, and membrane-associated transporter protein) results in oculocutaneous albinism, which presents with intact melanocytes but inability to make pigment.

From dopaquinone, the eumelanin and pheomelanin pathways diverge. Two enzymes crucial to eumelanogenesis are TRP1 and TRP2. Pheomelanin is derived from conjugation by thiol-containing cysteine or glutathione. As a result, pheomelanin is more photolabile and can produce, among its by-products, hydrogen peroxide, superoxide and hydroxyl radicals, all known triggers of oxidative stress, which can cause further DNA damage. Individual melanocytes typically synthesize both eumelanins and pheomelanins, with the ratio of the two being determined by a balance of variables, including pigment enzyme expression and the availability of tyrosine and sulphydryl-containing reducing agents in the cell.[221]

Melanin is packaged and delivered to keratinocytes by melanosomes. The formation, maturation and trafficking of melanosomes is crucial to pigmentation, and defects in this process lead to depigmented and dilutionary disorders such as Hermansky-Pudlak syndrome and Chediak–Higashi syndrome. On the keratinocyte side, the protease-activated receptor-2, a seven-transmembrane receptor on keratinocytes, has a central role in melanosome transfer.[222] Once in

keratinocytes, melanosomes are distributed and, in response to UV, positioned strategically over the "sun-exposed" side of nuclei to form cap-like structures resembling umbrellas.

Ultraviolet-induced pigmentation

The most common example of acquired pigmentation is tanning. To the naked eye, the effects of UV are marked by "sunburn" and/or "sun-tan". Sun sensitivity is the degree of cutaneous inflammation (erythema) and pigmentation that results from exposure to UV, which can manifest clinically in several ways. Even at low doses of UV, DNA damage can occur before there is any evidence of change on the skin.[223]

The tanning response is one of the most striking examples of environmental adaptation in humans. It is well known that α-melanocyte-stimulating hormone (MSH) increases skin darkening in humans, a phenomenon traditionally observed in patients with adrenal insufficiency, whose pituitary glands produce compensatory excesses. Pro-opiomelanocortin (POMC) is the precursor for both α-MSH and adrenocorticotropic hormone (ACTH), as well as for other bioactive peptides, including β-endorphin. Although originally identified in the pituitary, POMC production is now known to occur in skin and hair follicles as well.[224,225] After its production from POMC, α-MSH is secreted by both keratinocytes and melanocytes. In humans, mutations in *POMC* result in a red-haired phenotype (like that of melanocortin-1 receptor [*MC1R*] alleles), as well as metabolic abnormalities such as adrenal insufficiency and obesity.[225]

There is evidence that DNA damage in itself might be important to the triggering of pigment production. UV – typically UVB (wavelengths 290-320 nm) – induces thymidine breaks in DNA and the most common signature is cyclobutane dimer formation. The molecular mechanism

might be linked to p53, p21 or proliferating cell nuclear antigen, levels of which are increased after exposure to pTpT in a time frame similar to that seen after UV irradiation.[207]

p38 stress-activated kinase has been suggested to participate in UV-related pigmentation by phosphorylating upstream transcription factor 1, that can bind to the tyrosine promoter. Other pathways implicated include those involving endothelin-1, β-FGF, nitric oxide (NO), p-locus and SCF.[366] Separate physiological processes — such as X-ray irradiation and DNA-damaging chemotherapeutic agents — can also stimulate a tanning response, which potentially involves pathways that overlap with the UV response.[207]

Inability to tan highlights several genetic features that might be instructive with regard to the UV-pigmentation response. Variants of *MC1R*, which produce a weakly functioning receptor either through decreased ligand binding or decreased activation of adenylyl cyclase, exhibit relative inability to tan (for example, red-heads cannot tan). Such individuals tend to freckle but do not produce an even, protective pigment. They are also prone to sunburn, although the triggers for this process remain unclear. Sunburn cells — keratinocytes in which apoptosis is triggered by UV — have been shown by Brash and colleagues to require p53 for their formation,[226] thus identifying a key role for p53 in modulating a physiological response to a common environmental exposure (UV), and implicating p53 as a “guardian of the tissue”. Cui et al. eventually showed that the likely mechanism behind the failure to tan involves the ability of p53 to stimulate transcription of the *POMC* gene in keratinocytes.[227] They posit that the transcription of *POMC* in sun-exposed keratinocytes leads to increased release of α-MSH, activation of the MC1R, on melanocytes, and consequently, increased melanogenesis, melanocytic differentiation, and transfer of melanosomes to keratinocytes — which together are responsible for the tanning response.[227]

Further insight into the UV-tanning response was recently obtained through use of the K14-SCF transgenic mouse.[228] By using this transgenic background, fair-skinned mice (*Mc1r*$^{e/e}$) containing epidermal melanocytes were obtained, and found to be acutely UV-sensitive. Mutant MC1R was, not surprisingly, seen to ablate any detectable tanning response. However, a rescue strategy of topically-delivered forskolin was used to bypass the *Mc1r* mutation and directly activate adenylyl cyclase, and was observed to induce profound skin darkening in genetically fair-skinned mice. The induced pigmentation pattern exhibited normal histological features, such as nuclear "capping" in keratinocytes, and produced significant protection against sunburn cell formation, pyrimidine dimer formation and skin cancers after UV exposure.[228] These results suggest that the dark pigmentation machinery remains available if appropriately stimulated, and ongoing studies are examining whether this is the case in humans.

Response to UV and the risk for skin cancer

How does pigmentation protect skin? Although this is presumed to involve straightforward shielding by melanin, our understanding of the process is far from complete. It is clear that skin pigmentation as well as the capacity to tan portend diminished skin cancer risk. When the protective effect of melanin has been calculated using minimal erythematous dose measurements, protection for even the darkest-skinned of individuals is no more than 10–15 fold that seen in the absence of melanin (presumably signifying a relatively weak sun-protection factor). But the factor of protection in terms of skin cancer risk is 500-1,000, indicating that highly pigmented skin is profoundly protected from carcinogenesis.[207] This discrepancy, although subject to numerous caveats, including quantification estimates and endpoint

surrogates, suggests that pigment's protective mechanisms might vary for different endpoints such as sunburn and skin cancer.

One possible explanation for the high cancer protection afforded by dark pigmentation might involve mechanism(s) of increased risk associated with blonde/red pigments. *MC1R* variants have been shown to confer an increased risk of melanoma and non-melanoma skin cancers, independently of skin pigment (including red-haired phenotype).[229] Pheomelanin might function as a photosensitizer capable of generating reactive oxygen species upon UV irradiation and has been associated with higher rates of apoptotic cells after UV irradiation.[230] Thus, increased pheomelanin production might be a risk factor for melanoma, although the precise underlying mechanism for this process remains to be fully elucidated to ensure that it is not merely a marker of melanoma risk but is directly involved in carcinogenesis.

Regulation of pigmentation

The contribution of melanocytes to pigmentation is conserved through many species. In certain species such as fish, pigment is provided by other cell types, known as xanthophores and iridiphores.[207] Despite the identification of more than 100 loci involved in vertebrate pigmentation, the MC1R is consistently a representative locus and major determinant of pigment phenotype. The extension locus ($Mc1r^{e/e}$) was first identified in mice on the basis of altered coat color. The recessive mutants have yellow or pheomelanotic hair, whereas wild-type mice have black/brown eumelanotic hair. This mutation has been conserved in furred animals from mammoths to present-day cats and dogs.[207]

MC1R encodes a seven-transmembrane, G-protein-coupled receptor. Agonist-bound MC1R activates adenylyl cyclase, inducing cyclic (c) AMP production, which leads to phosphorylation

of cAMP responsive-element-binding protein (CREB) transcription factor family members. CREB, in turn, transcriptionally activates various genes, including that encoding MITF. Agonists of human MC1R include α-MSH and ACTH, and these cause an increase in eumelanin production through elevated cAMP levels.[231] The agouti (*Asip*) gene encodes an antagonist of MC1R,[232] which is responsible for the pheomelanotic banding pattern of wild-type mouse fur. An inactivating mutation (nonagouti) at this locus is responsible for the black fur of the C57BL6 mouse strain. Recently, evidence has been reported for an association between a single nucleotide polymorphism in the 3´ *untranslated* region of the human agouti protein and dark hair and brown eyes.[233] The role of *MC1R* in hair pigmentation is striking. The human *MC1R* coding region is highly polymorphic with at least 30 allelic variants, most of which result in a single amino-acid substitution.[207] For instance, certain substitutions, such as R151C, R160W and D294H, are associated with red hair. It may be also possible to have an additive effect among two variant alleles.

Links between pigmentation and immunity

There is considerable evidence that immunity and melanization are genetically, biochemically and functionally linked.[234] Molecular evidence concerning one such link are recent findings about attractin, a protein that appears to have functions in regulating both melanization and immunity (the homolog in the mouse is called the mahogany gene).[235] Attractin, by an unknown mechanism, potentiates the inhibitory reaction of agouti and agouti-related protein against α-MSH. α-MSH itself binds to the MC1R on both melanocytes and macrophages. Relevant is that attractin is also present on numerous different cell types in the immune system and accumulates

rapidly on the surface of activated T cells. Worth noting is the fact that α-MSH itself also has extensive and well-documented immunoregulatory effects.[236-238]

More generally, melanin may play a role in regulating the activity of immunological cytokines. Synthetically derived melanin at non-cytotoxic concentrations reversibly suppressed the production of TNF and inhibited the production of interleukins IL-1β, IL-6 and IL-10 by LPS-stimulated monocytes.[240] A genetic link between immunity and melanization is further demonstrated by a number of clinical conditions that result in albinism and impaired immunity from lysosome deficiency (e.g. Chediak-Higashi, Griscelli, and Hermansky-Pudlak syndromes). This indicates that gene(s) involved in the formation of lysosomes and melanosomes are phylogenetically and functionally linked.[231]

Role of pigmentation in the evolution of skin types in humans

Tracing *MC1R* loci through different skin types and geographic regions has led to different theories on the evolution of human pigmentation. Epidemiological studies suggest that pigmentation is under functional constraint in Africa and that this constraint has been lost in the populations that left the African continent. It is not clear whether the drive for selection was necessitated by UV-induced vitamin D production over protection from the DNA damage caused by UV or as a result of an undiscovered critical pathway. MC1R might be evolutionarily significant for other biological reasons, such as increased κ-opioid analgesia.[231] Despite the apparently strong influence of MC1R on both hair and skin pigmentation, it is clear that other factors are also involved in the control of skin pigmentation, because there are many fair-skinned but dark-haired individuals in whom MC1R alone is unlikely to limit skin pigmentation.

Recently *SLC24A5*, which encodes a putative cation exchanger, was identified as the human homologue of a zebrafish gene that causes the "golden" phenotype.[241] Although its function is unclear, it is plausible that cation chemistry might modulate melanosomal maturation processes. Humans have two primary *SLC24A5* alleles, which differ by a single amino-acid substitution. In almost all Africans and Asians the substitution is alanine, but in 98% of Europeans the allele encodes threonine. The function of *SLC24A5* in human pigmentation remains to be determined, but the correlation of its variants to human populations is striking and suggests that it is important in the control of cutaneous pigmentation.

The distribution of human skin color within a phylogenetic framework indicates that the transitions have been from dark to light colored skin and coincide with the migration of ancestral populations into higher latitudes.[242] However, the "UV hypothesis" of the evolution of human skin color fails to include several issues. For example, the sunprotected skin of the genitalia of humans, including newborns, contains higher concentrations of melanocytes and melanin than on the arms and chest.[231] Melanocytes also occur in other tissues including key immunological sites, such as the throat, nasal and auditory passages.

It has been hypothesized that melanocytes, melanosomes and melanin evolved and function as an integral part of the innate immune defense system against invading microorganisms, in particular fungi and bacteria.[231] Pigmentation levels would be therefore optimized to local parasite pressures and conditions, and positive selective pressure would have been determined by several factors besides UV levels (resistance to infections, sexual appeal, mimetism etc.).

Comparative biology clues on the links between pigmentation and immunity

Melanization in *Drosophila*

Beginning over a decade ago, *Drosophila melanogaster* (the "fruit fly") has been an invaluable model organism for understanding the molecular mechanisms regulating the activation of innate immune responses. The Toll and IMD pathways in particular play major roles in the innate immune system of *Drosophila* by controlling the expression of AMPs and other immune responsive genes.[243] However, it can take a few hours to a few days for the Toll and IMD pathways to induce their immune effectors. In contrast, a more immediate immune response, induced within a few minutes after infection, is melanization. During melanization, phenols are oxidized to quinones, which then polymerize to form melanin. Melanin is deposited around intruding microorganisms to help sequester them at wound sites. The quinone substances and other reactive oxygen intermediates generated during melanization are thought to be directly toxic to microorganisms. In addition, the melanization reaction has also been shown to cooperate with other immune responses such as blood coagulation, wound healing, phagocytosis and AMP expression.[244]

Activation of the melanization reaction has to be strictly regulated. Uncontrolled melanization generates excessive toxic intermediates that could kill the host, as demonstrated by the lethality resulting from mutations of serpins that inhibit melanization. Indeed, phenol oxidase (PO), a key enzyme in melanin biosynthesis during melanization, is usually synthesized as an inactive zymogen called pro-phenoloxidase (PPO). Recognition of pathogens and/or injury leads to the activation of a serine protease cascade that culminates in proteolytic cleavage of inactive PPO to active PO. Serine protease inhibitors called serpins add another level of regulation by inhibiting

members of the serine protease cascade such that melanization is strictly localized at the site of injury or infection.

In *Drosophila*, the molecular mechanism involved in triggering PO activation remains poorly understood. However, PRRs that trigger immune responses upon pathogen recognition, specifically of the peptidoglycan recognition protein family, have been shown to activate PO in *ex vivo* assays involving large insects such as the silkworm *Bombys mori*.[245]

Pigmentation and immunity in lower vertebrates

A class of melanin-containing macrophage called melanomacrophages occurs in a diverse range of species including reptiles, amphibians and fishes.[231] In these animals melanomacrophages are recognized as an important component of the innate immune system against protozoan and bacterial pathogens. The cells are found in a diverse range of organs other than skin, especially the liver. Since they are mobile and accumulate at sites of infection an antimicrobial defense role may be more obvious than it would be for stationary cells such as melanocytes and keratinocytes. Bacteria inducible melanization of fish skin was demonstrated in aquarium-reared cichlids (*Oreochromis mossambicus*) naturally infected with the bacterium *Mycobacterium marinum*.[435] Cutaneous injuries on frogs become deeply melanized in response to injury, as is characteristic of many invertebrates.[231]

Evidences that melanocytes function as part of the innate immune defense system

In mammals the primary phagocytic cells include polymorphonuclears, macrophages, microglial cells of the brain, dendritic cells, and Kupffer cells of the liver. In the latter intracellular lysosomes in lower vertebrates are in fact melanosomes.[247] It is clear that melanosomes are lysosomal structures.[248] In addition to melanin, melanosomes contain a rich list of lysosomal

enzymes and other enzymes that participate in the degradation of bacterial and other tissue.[249] Several studies also showed that melanosomes and lysosomes share common functions.[250,251] Moreover, the formation of melanosomes and lysosomes appear to be under partly the same genetic regulation and several different mutant genes in humans, mice and *Drosophila*, most of which were identified initially on the basis of reduced pigmentation, have been associated with defects of melanosomes, lysosomes and granules. Normal human melanocytes also function as phagocytes against microorganisms.[252]

Human melanocytes produce NO in response to LPS and melanin synthesis is increased in cultured human melanocytes in response to increased levels of NO.[253,254] The expression of the NO was modulated by α-MSH and mediated intracellularly by inducible nitric oxide synthase.

A number of other well-recognized mediators of the inflammatory defense system, including histamine and arachidonic acid, also stimulate melanogenesis. Yoshida et al. demonstrated that melanocytes express the cell surface histamine receptor H2 and that the stimulatory effects of histamine on melanocytes were as potent as that of other melanogens including α-MSH.[254]

Other studies have also shown that melanocytes produce numerous immunologic makers and signals, including IL-1 and IL-6.[255]

Expression and function of TLRs in normal and neoplastic melanocytes

A few studies have been performed to analyze the expression and function on TLRs in human melanocytes. Ahn et al. showed that TLR4 and its adaptor molecule CD14 and MyD88 were constitutively expressed in cultured human melanocytes.[256] Co-staining of histological human skin sections with TLR4 and a melanocyte marker, gp100, confirmed the expression of TLR4 in melanocytes under physiological conditions. LPS upregulated the expression of TLR4 and

MyD88 and induced NF-κB nuclear translocation in melanocytes. Treatment of LPS increased pigmentation of human melanocytes, thus suggesting that TLR4 may play a role in microbial-induced melanogenesis.[257]

Kang et al. demonstrated that normal human melanocytes express TLR7.[258] This receptor may be functional, as stimulation of melanocytes with imiquimod lead to decreased tyrosinase and MITF expression and was accompanied by decreased pigmentation. Imiquimod also inhibited the growth of melanocytes. These effects appeared to be TLR-dependent, as siRNA TLR7 reversed the inhibitory effects of imiquimod on melanogenesis and cell proliferation.[258]

The above findings were confirmed and expanded by Yu et al. by showing that human melanocytes constitutively express TLRs 1–4, 6, 7 and 9 mRNA.[259] Ample amounts of TLRs 2-4, 7 and 9 were confirmed at protein level. Stimulation of melanocytes with TLRs ligands resulted in the release of cytokines (IL-8 and IL-6) and the mRNA accumulation of chemokines (CCL2, CCL3 and CCL5). Triggering of TLRs in melanocytes resulted in the up-regulation of phosphorylated IκBα and in the nucleus translocation of NF-κBp65.[259]

Similarly, only a few studies are available concerning the expression and role of TLRs in melanoma. Goto et al. found TLR2, TLR3, and TLR4 to be highly expressed.[260] By PCR array analysis, specific stimulation of TLRs 2-4 on melanoma cells showed significant activation of the adaptor protein MyD88, as well as downstream signal transduction factors NF-κB and inflammatory response-related factors. Specific ligand activation of TLR2, TLR3, and TLR4 was shown to induce cell migration. The expression and functionality of TLR3 was confirmed by other studies.[261-264] Voelcker et al. showed that melanoma invasiveness is dependent from TLR4, which induced MMP and cytokine expression.[265] Other studies provided preliminary evidence in mice that several TLR agonists may help to treat murine melanoma.[266,267]

Aim of the study 4

As reported in the previous chapters, acral lentiginous melanoma (ALM) is a recently cathegorized and somehow “neglected” variant of malignant melanoma despite its relative high frequency and mortality. ALM is characterized by peculiar epidemiological, clinical, histopathological and dermatoscopical features. Also, according to recent advances on research in this field, it seems likely that the molecular pathogenesis of ALM follows a multistep pathway which is different from the “classical”, more common variants of ultraviolet (UV)-induced melanoma arising on hairy skin.

The palmoplantar epidermis is characterized by alternating surface ridges, that include the eccrine pores and overlying *cristae profundae intermediae* (passed through the intraepidermal eccrine duct), and surface furrows with the overlying *cristae profundae limitantes*. Because of this peculiar anatomy, melanocytic lesions arising on acral volar skin are characterized by unique clinical and dermatoscopic features. At dermatoscopy, a parallel ridge pattern (PRP), showing prominent pigmentation on the ridges of the skin markings, is often detected in macular portions of malignant melanoma and melanoma *in situ*. In contrast, a parallel furrow pattern (PFP), showing pigmentation along the sulci of the skin markings and its variant, the lattice-like pattern, are the most prevalent dermatoscopic features observed in benign melanocytic nevi.

Several recent histopathological studies, some of which carried out by the candidate, showed that, as expected, the PRP and PFP result mainly from the accumulation of pigmented cells in the

cristae profundae intermediae and *cristae profundae limitantes*, respectively.[448-453] These findings imply that malignant transformation of melanocytes occurs almost exclusively within the *cristae profundae intermediae*.[449,454] Moreover, the same studies highlighted that melanin is released to the upper epidermal layers only if underlying nests of normal melanocytes, and not melanoma cells, are present.[455-457]

The overmentioned phenomena have been investigated poorly to date. Further research is therefore warranted as ALM represents a peculiar *in vivo* model of carcinogenesis and tumor microenvironment.

New advancements and lines of research indicate that the innate immune system, namely the Toll-like receptors (TLRs)-related pathways, may play a role in the pathogenesis of cancer (see Chapter 2). The innate immune system plays an important role in discriminating between "infectious nonself" and "noninfectious self". Pathogen recognition by the innate immune system happens through a limited number of germline-encoded receptors. These receptors are special in that they recognize not one specific but many similar molecules; they are less stringent and more promiscuous receptors than their adaptive counterparts. Therefore, they have been referred to, by some authors, as pattern recognition receptors (PRRs). Among the most well known of all PRRs are the TLRs, which bind certain antigenic ligands of infectious agents and activate distinct signaling pathways resulting in a similar but not identical pattern of mediators, including inflammatory cytokynes such as interleukin (IL)-1, -6 and -12, tumor necrosis factor (TNF)-α and IFN-β. Most of the cell types harbored in the skin (keratinocytes, dendritic cells, macrophages, mast cells) or homing into it (lymphocytes, neutrophils, natural killer cells) contribute to form the cellular compartment of the cutaneous innate immune system.

The current evidences demonstrate that TLRs are expressed and functional in neoplastic cells, especially epithelium-derived ones. TLR expression may promote malignant transformation of cells. Moreover, engagement of TLRs increases tumor growth and tumor immune escape, and induces apoptosis resistance and chemoresistance.

The biology and physiology of melanocytes have long been studied because of their function as unique professional melanin-producing cells in human skin. Nevertheless, growing experimental evidence raises the possibility that melanocytes are a component of the skin immune defense system. The pigment production itself is a conserved mechanism of defense, and several molecules generated during such biosynthetic pathway feature strong antimicrobial activity.

Specific aims

Our *long-term goal* is to contribute understanding the pathogenic mechanisms leading to ALM. The *objective here*, which is our next step in pursuit of that goal, was to preliminarily assess whether the innate immune system, in particular TLRs, are expressed and functional in melanocytic cells and may play a role in ALM. The *central hypothesis* is that disturbances of TLR-related functions are significantly involved in the malignant transformation of melanocytes harbored in the acral volar skin. The *rationale* of the proposed research was that ALM occurs in a nonrandom microscopic distribution within the acral epidermis, a finding which may underlie an altered immune function only in certain portions of the epidermis itself.

We tested our central hypothesis by pursuing the following *specific aims*:

1. ***Evaluate the expression and function of TLRs in normal human melanocytes and human melanoma cells in vitro.***

   In particular, the primary endpoints were:

- Expression of mRNA coding for TLR-1, -2, -3, -4, -5, -6, -7, -8, and -9 by means of real time-polymerase chain reaction (RT-PCR) and the expression of selected TLRs in cells by means of immunocytochemistry.
- Expression of mRNA coding for TNF-α, IFN-β, IL-1 and IL-6 by means of RT-PCR and the production of the same cytokines after stimulation with TLR agonists by means of enzyme-linked immunosorbent assay (ELISA).
- Expression of mRNA coding for tyrosinase by means of RT-PCR after stimulation with selected TLR agonists.
- Production of melanin after stimulation with selected TLR agonists.

2. ***Evaluate the expression of selected TLRs and related molecules in acral melanoma.***

   In particular, the primary endpoint was:

   - Expression of TLR-2, -3, -4, -9, and selected cytokines by means of immunohistochemistry on lesions of ALM and acral melanocytic nevus.

To sum up, the present study represents an explorative step in investigating the role of innate immunity in melanocyte physiology and pathology with particular focus on ALM.

Materials and Methods 5

Cell culture and stimuli

Cell lines

A375 human melanoma cells (originally transfered *in vitro* from an acral lentiginous melanoma [ALM] lesion) were obtained from the American Type Culture Collection (Rockville, MD). The cells were routinely maintained in Dulbecco's Modified Eagle's Medium (Sigma Aldrich, St. Louis, MO), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen Corporation, Carlsbad, CA), at 37 °C in a humidified atmosphere containing 5% CO_2. They were passaged twice a week at the ratio of 1:5 to keep an exponentially growing state. The substratum was cell culture grade plasticware (Costar, Cambridge, MA).

Normal human melanocytes were isolated from foreskin belonging to light-, medium- and dark-skinned volunteers and were cultured in 254 medium (Cascade Biologics, Portland, OR), supplemented with human melanocyte growth supplement (Cascade Biologics) and penicillin/streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2. They were passaged twice a week at the ratio of 1:5 to keep an exponentially growing state. The substratum was cell culture grade plasticware (Costar, Cambridge, MA) (75 mL bottles, 6- or 12-well plates).

Cell stimuli

Cells at 70-80% confluence were stimulated in fresh medium. Stimuli included Toll-like receptor (TLR)1/2 ligand (the synthetic tripalmitoylated lipopeptide PAM3CSK4) (10 μg/ml; InvivoGen, San Diego, CA), TLR2 ligand lipoteichoic acid (LTA) (2 μM; InvivoGen), TLR3 ligand polyinosinic:polycytidylic acid (poly[I:C]) (0.1-100 μg/mL InvivoGen), TLR4 ligand lipopolysaccharide (LPS) (1 μg/ml; Sigma-Aldrich), TLR5 ligand flagellin (50 ng/ml; Alexis Biochemicals), TLR6 ligand macrophage-activating lipopeptide 2 kDa (Malp-2) (0.001-0.5 μg/mL; Alexis Biochemicals), TLR7 ligand imiquimod (2 μg/mL; Invivogen) and TLR9 ligand CpG-oligodeoxynucleotide (ODN) DNA (10 μg/ml; Sigma-Aldrich). When appropriate and/or possible, cells were also stimulated or co-stimulated with p38 mitogen-activated protein (MAP) (and TLR-3) inhibitor inhibitor SB-202190 (Selleck Chemicals, Houston, TX) at 1 μg/mL and TLR-9 inhibitor LL-37 (Invivogen) at 2 μg/mL. Unless otherwise specified, plates were incubated for 24 hours. In order to obtain time-dependent curves, plates were also incubated for 6, 12 hours, 48 and/or 72 hours where deemed necessary. When necessary, cell cultured media were stored at −20 °C until analysis.

Quantitative real-time polymerase chain reaction

RNA extraction

RNA was extracted from adherent cells after supernatant collection using TRIzol (Invitrogen). In particular, supernatant was removed from 6- or 12-well plates and TRIzol added (1mL/3.5 cm diameter). Homogeneization was performed by pipetting for 5 minutes at room temperature (RT). Phase separation was achieved by: 1) addition of chloroform (20% of TRIzol volume: 0.2 to 1 mL); 2) 15 second shaking; 3) 5 minute incubation at RT; 4) 15 minute spin at 10500 g at

4°C; 5) collection of supernatant. RNA precipitation was achieved by: 1) addition of isopropanol (50% of TRIzol volume); 2) 10 minute incubation at RT; 3) 15 minute spin at 10500 g at 4°C; 4) supernatant removal. RNA wash was achieved by: 1) pellet wash with 70 ethanol prepared with RNase-free water; 2) 10 minute spin at 10500 g at 4°C; 3) 10 minute pellet air drying; 4) eppendorf tube transfer; 5) addition of 50-100 μm filtered water; 6) 5 minute spin at 10500 g at 4°C; 7) pipetting up and down until complete dissolution. Samples were then further processed right away or stored at –70°C.

cDNA synthesis

cDNA was synthesized from RNA by the iScript cDNA Synthesis Kit (BioRad, Hercules, CA) as described by the manufacturer's protocol. Namely, 1 μg RNA was placed immediately on ice and nuclease-free water added to 15 μL. 5x iScript Reaction Mix (4 μL) and iScript reversal transcriptase (1 μL) were also added for an overall volume of 20 μL. The complete reaction mix was then incubated at the following temperatures: 1) 5 minutes at 25ºC; 2) 30 minutes at 42ºC; 3) 5 minutes at 85ºC. Samples were eventually used right away or stored at –20ºC.

Quantitative real-time polymerase chain reaction amplification

TaqMan™ Gene Expression Assays (Applied Biosystems ABI, Foster City, CA) were used to analyze gene expression as described by the manufacturer's instruction. Namely, reaction plates were prepared no more than 4 hours in advance and a 96-well fast setup was used (20 μL reaction). PCR reaction mixes were prepared separately for each sample. The mix was composed by TaqMan Gene Expression Assay (20X), cDNA template and TaqMan Fast Universal PCR Master Mix using 5.0, 45.0 and 50.0 μL, respectively, for 4 10-μL reactions. Then it was

proceeded as follows: 1) tubes were capped and solutions mixed by gentle inversion; 2) tubes were briefly centrifuged to spin down the contents and eliminate any air bubbles from the solutions; 3) the appropriate volume of each reaction mixture (20 μL) was transfered to wells of an optical reaction plate; 4) the plate was covered with an optical adhesive cover; 5) tubes were briefly centrifuged to spin down the contents and eliminate any air bubbles from the solutions; 6) the reaction plate was placed in the sequence detector; 7) the thermal cycling conditions were set at 20 seconds at 95°C (denature), 40 cycles of 1 second at 95°C (denature) and 20 seconds at 60°C (anneal/ext end), hold at 4°C; 8) the run was started.

Predeveloped Taqman assay probes (Applied Biosystems) were used for analyzing the expression of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, IL-1, IL-6, IFN-β and TNF-α. Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) mRNA was used as an internal control to validate RNA for each sample.

Analysis of results

All analyses were performed in triplicate from 2-3 independent experiments in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Fold induction relative to the vehicle treated control in in vitro experiments was calculated using the comparative threshold cycle (Ct) method, where ΔCt is ΔCtstimulant – ΔCtvehicle, ΔCt is Ctgene – CtGAPDH, and Ct is the cycle at which an arbitrary detection threshold is crossed. For quantification of transcript abundance in tissue samples, target gene expression was normalized to GAPDH and, when appropriate, compared with nonstimulated or other controls. All fold change values were substracted of a unit (1.0) in order to set the negative controls (normally fold change = 1.0) at zero.

Immunocytochemistry

Cells were plated in a 8-chamber glass slide and grown until cultures were subconfluent. Media were poured off from the wells, each chamber was washed twice quickly with 0.5 mL phosphate-buffered saline (PBS) and the cells were fixed by adding 0.5 mL of 4% paraformaldehyde to each chamber and incubating for 15 min. Cells were washed three times in 0.5 mL 0.05% Tween-20 in PBS (PBT) (per chamber), 5 min each, and then incubated for 30 min. in 0.5 mL of 1% bovine serum albumin (BSA). Cells were washed three times in 0.5 mL PBT as previously described, then incubated in 0.2 mL of the working concentration of the primary antibody (1:200), diluted in 0.1% BSA, for 60 minutes at room temperature. Cells were washed three times in 0.5 mL PBT (per chamber), 5 min each. Cells were incubated in 0.2 mL of the working concentration of the second antibody (about 1:500), diluted in 0.1% BSA, for 40 minutes at room temperature. Cells were washed three times in 0.5 mL PBT (per chamber), 5 min each. Cells were incubated with streptavidin-horse radish peroxydase (HRP) (three drops) for 40 minutes at room temperature. Cells were washed three times in 0.5 mL PBT (per chamber), 5 min each. Cells were incubated in in 0.2 mL of BSA for 1 minutes at room temperature. Cells were washed three times in 0.5 mL PBT (per chamber), 5 min each. Cells were once again fixed for 15 min. in 0.5 mL of 4% PFA. The plastic chamber piece and sealer holding it in place completely were removed. Finally one drop of mounting solution (Permount) on each sheet of cells and coverslips were added to each sheet. Anti-TLR2 antibodies (clone TL2.1; Abcam, Cambridge, MA) and anti-TLR-9 antibodies (clone 5G5, Abcam) were used.

Enzyme-linked immunosorbent assay

ELISA's were performed according to the manufacturer's instructions. Despite slight differences between the various kits, the following general procedure was used: 1) the wells of a polyvynilchloride microtiter plate were coated with the capture antibody at the suggested concentration in carbonate/bicarbonate buffer (pH 7.4); 2) the plate was covered with an adhesive plastic and incubated overnight at 4°C; 3) the coating solution was removed and the plate washed twice by filling the wells with 200 μL PBS; 4) the washes were removed by flicking the plate over a sink and the remaining drops were removed by patting the plate nn a paper towel; 5) the remaining protein-binding sites in the coated wells were blocked by adding 200 μL of blocking buffer (5% non fat milk in PBS) per well; 6) the plate was covered with an adhesive plastic and incubated for at least 1-2 h at room temperature; 7) 100 μL of appropriately diluted samples to each well were added in triplicates (together with the standards also in triplicates) and incubated for 90 minutes at 37°C; 8) the samples were removed and the plate washed twice by filling the wells with 200 μL PBS; 9) 100 μL of diluted detection antibody were added to each well; 10) the plate was covered with an adhesive plastic and incubated for 2 h at room temperature; 11) the plate was washed four times with PBS; 12) 100 μL of secondary conjugated antibody, diluted at the optimal concentration (according to the manufacturer) was added in blocking buffer immediately before use; 13) the plate was covered with an adhesive plastic and incubated for 1-2 h at room temperature; 14) 50-100 μL of HRP substrate solution (H_2O_2 and 3,3',5,5'-tetramethylbenzidine per well) was added to each well and incubated for 15-30 minutes; 15) an equal volume of stopping solution (2 M H_2SO_4) was added; 16) optical density was read at the spectrophotometer (450 nm).

Antibodies were directed against IL-1β (R&D Systems, Minneapolis, MN), IL-6 (R&D Systems), and TNF-α (Abcam, Cambridge, MA). No anti-IFN-β antibody suitable for ELISA was found in commerce.

Melanin measurement

After removal of the supernatant, 6-well plates were trypsinized (2% trypsin for 5 minutes at room temperature). Both the supernatant and trypsinized material were then centrifugated (5000 g for 10 minutes). After removal of the supernatant pellets were incubated in NaOH 1M for 2 hours at 60°C in order to dissolve melanosomes. Melanin content was then analyzed at the spectrophotometer (405 nm). Readings of a standard protein solution (bovine serum albumin) were subtracted from the sample readings. Dilutions of 99% pure synthetic melanin (Sigma-Aldrich) were used as standard solution. Samples were analyzed in triplicates.

Skin samples

After approval by the local Institutional Review Board, the following skin samples were retrospectively obtained for immunohistochemical analysis: 1) 3 samples of ALM; 2) 2 samples of acquired acral melanocytic nevus; 2) 2 samples of normally-appearing acral skin. The main subjects' data are outlined in **Table 1** (see Appendix). All specimens were obtained from 3-mm punch biopsies retrieving acral skin samples which included the epidermis and dermis till the reticular portion.

A preliminary examination on routinary hematoxylin&eosin slides confirmed retrospectively the pathological diagnoses. **Table 2** (see Appendix) details such findings in each considered

specimen. For explicative purposes, **Figure 1** (see Appendix) shows some sections of the histopathologic patterns belonging to the lesions considered.

Immunohistochemistry

Formalin fixed pieces of skin were processed according to standard histology protocol. Five µm-thick sections obtained from the paraffin embedded blocks were attached to positively charged slides. Following drying, the slides were placed in a section drying oven for 30 minutes at 58°C, were de-paraffinized in xylene and re-hydrated through graded alcohols and briefly washed in de-ionized water and then placed in PBS) The slides were then incubated in a 3% solution of H_2O_2 in PBS to block the staining of intrinsic endogenous peroxidase. The sections were then sequentially incubated with horse normal serum (Vector Laboratories, Burlingame, CA), polyclonal antibody and monoclonal antibody for one hour at room temperature then washed in PBS for 2 x 5 minutes. The sections were then incubated for 30 minutes with either anti-rabbit IgG or anti-mouse IgG biotinylated antibody (Vector Laboratories, Burlingame, CA), washed for 5 minutes in PBS and further incubated for 30 minutes with Avidin Biotin Complex (Vector Laboratories, Burlingame, CA). Following 2 x 5 minute washes in PBS, the sections were exposed to a freshly prepared solution of H_2O_2 and 3,3' diaminobenzidine to visualize the antigen-antibody site. The sections were then counterstained in Mayers Hematoxylin for 1 minute, blued for 5 minutes in running water, then dehydrated through graded alcohols, cleared in xylene and coverslipped. Omission of the primary antibody served as a negative control and known positive and reactive cells in the skin served as positive controls. Monoclonal antibodies included those against TLR-2 (dilution 1:150; clone TL2.1; Imgenex, San Diego, CA), TLR-3 (dilution 1:500; clone 713E4.06; Imgenex), TLR-4 (dilution 1:50; clone unknown; Imgenex),

TLR-9 (dilution 1:50; clone unknown; Imgenex) and TNF-α (dilution 1:500; clone unknown; Gene Tex Inc., San Antonio, TX).

Statistics

Wherever appropriate, differences between groups were compared using 2-tailed Student's *t* test or Mann-Whitney test. A *P* value less than 0.05 was considered significant.

Results 6

Melanoma cells and normal human melanocytes express TLRs in vitro

As detailed in **Figure 2** (see Appendix), nonstimulated A375 melanoma cells constitutively expressed all mRNAs coding for Toll-like receptors (TLRs) with the exception of TLR-8. Nonstimulated normal human melanocytes constitutively expressed all mRNAs coding for TLRs with the exception of TLR-6 and TLR-8, TLR-9 being the most expressed one.

The expression of selected TLRs *in vitro* was confirmed by means of immunocytochemistry in both A375 melanoma cells and normal human melanocytes. In particular, TLR-2 and TLR-4 were found to be expressed mainly on the cell membrane surface, while TLR-3 and TLR-9 were mainly detected in intracellular structures (photomicrographs not shown).

TLR agonists trigger the production of cytokines in melanoma cells and normal human melanocytes in vitro

After stimulation with TLR agonists, A375 melanoma cells and normal human melanocytes were induced to produce innate immune-related cytokines. In particular, as shown in **Figures 3** to **6** (see Appendix), *TLR-1 agonist PAM3CSK4* induced the production of interleukin (IL)-1β in A375 melanoma cells, but not in normal human melanocytes. No synthesis of tumor necrosis

factor (TNF)-α, IL-6 nor interferon (IFN)-β was highlighted in both cell types; only, IL-6 was secreted by normal human melanocytes, although this finding was not confirmed by the presence of specific mRNA. *TLR-2 agonist lipoteichoic acid* (LTA) did not show any effect on the production of the cytokines under investigation in neither cell type. *TLR-3 agonist polyinosinic:polycytidylic acid* (poly[I:C]) induced the intense production of TNF-α, IL-1β, IL-6 and IFN-β in normal human melanocytes, and to a much lesser extent, by A375 melanoma cells. *TLR-4 agonist lipopolysaccharide* (LPS) triggered the synthesis and release of IL-1β by A375 melanoma cells. All other tested cytokines were not detected; only, a weak production of mRNA coding for IL-6 and IL-1β was detected in A375 melanoma cells and normal human melanocytes, respectively. *TLR-5 agonist flagellin* induced the production of IL-1β and IL-6, but not of TNF-α and IFN-β, in A375 melanoma cells, while normal human melanocytes did not respond to such a stimulus. *TLR-6 agonist macrophage-activating lipopeptide 2 kDa* (Malp-2) triggered the production, of TNF-α, IL-1β and IL-6 in A375 melanoma cells (although to a modest extent the former two), but not the synthesis of mRNA coding for IFN-β. No effect was instead detected in normal human melanocytes. The stimulation with *TLR-7 agonist imiquimod* was not followed by a detectable response in normal human melanocytes but for the production of small quantities of mRNA coding for IFN-β, and translated in only a barely detectable production of mRNA coding for IL-1β and IL-6 in A375 melanoma cells. Because of the lack of expression of TLR-8, experiments with *TLR-8 agonist CL075* were not carried out; in fact, a preliminar check confirmed the absence of CL075 stimulation on normal human melanocytes. *TLR-9 agonist CpG-oligodeoxynucleotide* (CpG-ODN) failed to trigger any detectable stimulation on all cells but on normal human melanocytes, which were stimulated to synthetize mRNA coding for IFN-β.

Dose- and time-dependent curves

Dose- and time-dependent responses of A375 melanoma cells and normal human melanocytes to selected TLR agonists were obtained by means of enzyme-linked immunosorbent assay (ELISA) for TNF-α, IL-1β and IL-6 using TLR-3 agonist poly(I:C) and TLR-6 agonist Malp-2. As shown in **Figure 7** (see Appendix), normal human melanocytes started responding to poly(I:C) administered at concentrations as low as ten times less than that used for the experiments carried out as described in the previous paragraph, while at higher doses the response amount seemed to adjust to a plateau pattern. The response of A375 melanoma cells to poly(I:C) was much weaker as no response was detected al low concentrations of the stimulus, the same plateau effect being observed at higher doses. With regard to the dose-response curve for TLR-6 agonist Malp-2, A375 melanoma cells, the threshold for a detectable response by A375 melanoma cells was at the second last lower 1:10 dilution for IL-1β and IL-6, while it proved to be much higher for TNF-α. In all tested cytokines, their increased production was not proportional to the increase of Malp-2 and a more or less evident plateau effect could be foreseen. Normal human melanocytes confirmed not to be affected by the stimulation with Malp-2, not even at the highest dose.

Figure 8 (see Appendix) shows the time-dependent curves. A375 melanoma cells produced a detectable amount of IL-1β and IL-6 as early as 6 hours after the start of incubation with poly(I:C) or Malp-2, while the synthesis and release of TNF-α begun after 24 and 12 hours, respectively. Normal human melanocytes started secreting all three cytokines after 6 hours of incubation with poly(I:C), but they confirmed not to be able to respond to Malp-2. In most cases actually responding to the stimuli, the total amount of cytokine found in the supernatant increased with time as expected, however the secretion rate seemed to slower already after 12

hours of incubation in some instances (e.g. poly(I:C)-induced IL-1β by normal human melanocytes and Malp-2-induced TNF-α by A375 melanoma cells).

TLR agonists trigger melanogenesis in melanoma cells and normal human melanocytes in vitro

As anticipated in the Chapters 4 and 5, normal human melanocytes and A375 melanoma cells were also tested for their capacity to induce melanogenesis upon stimulation with selected TLR agonists. Because of funding limitations and based upon the results previously achieved, agonists of intracellular TLRs (poly(I:C) for TLR-3 and CpG-ODN for TLR-9) agonist were chosen as stimuli, aw well as the TLR-6 agonist MALP-2.

As shown in **Figure 9** (see Appendix), *TLR-3 agonist poly(I:C)* induced a significantly higher amount of mRNA coding for tyrosinase in both normal human melanocytes and A375 melanoma cells than in controls (lack of any stimulus or stimulation/co-stimulation with SB-202190, a molecule capable of inhibiting TLR-3 pathways). Also, poly(I:C) induced a significantly higher melanin production in normal human melanocytes, while such an increase in A375 melanoma cells did not reach statistical significance. Further experiments showed that both A375 melanoma cells and normal human melanocytes produced melanin in a slight poly(I:C) dose-dependent and time-dependent fashion. The minimum poly(I:C) concentration at which cells started to respond were 10 times lower than that used for the experiments detailed above and the earliest detectable responses were obtained as early as 12 hours after stimulation (data not shown). SB-202190 was able to inhibit almost completely melanin production (data not shown).

TLR-6 agonist MALP-2 triggered an increased synthesis of tyrosinase in A375 melanoma cells but not, as expected, in normal human melanocytes. Malp-2 also increased melanin production in A375 melanoma cells in a statistically significant fashion with respect to unstimulated controls. Unfortunately, no TLR-6 inhibitors were made available. Also, no dose- and time-dependent curves were performed for Malp-2 related melanin synthesis.

TLR-9 agonist CpG-ODN was not able to trigger – as expected – any increased production of mRNA coding for tyrosinase in A375 melanoma cells. Similarly, normal human melanocytes failed to transcript any increased amounts of mRNA tyrosinase. However, stimulation with LL-37 alone (an antimicrobial peptide capable of inhibiting TLR-3 pathways) induced a significantly higher amount of mRNA coding for tyrosinase (**Figure 9**, see Appendix). On the other hand, melanin content in CpG-ODN stimulated plates proved significantly lower than controls in normal human melanocytes, while no significant changes were observed in A375 melanoma cells. Further experiments showed that normal human melanocytes were inhibited to produce melanin in a sharp CpG-ODN dose-dependent and time-dependent fashion. The minimum CpG-ODN concentration at which cells started to respond were 10 times lower than that used for the experiments detailed above and the earliest detectable responses were obtained as early as 12 hours after stimulation (data not shown). Co-stimulation of CpG-ODN and LL-37 failed to inhibit melanin production (data not shown).

Toll-like receptors are expressed in acral lentiginous melanoma and acral melanocytic nevi

Immunohistochemistry for ***TLR-2*** highlighted an intense staining on all keratinocyte layers and the visible eccrine ducts, while only a faint background was detected in the *stratum corneum* and

the papillary dermis (**Figure 10**, see Appendix). In the two *in situ* acral lentiginous melanomas (ALMs) considered, the staining of junctional and intraepidermal (pagetoid) melanocytes, arranged singularly or in small nests, was comparable to that of keratinocytes. In the dermis, fibroblasts stained weakly but scattered cells (probably dendritic in nature) appeared to be strongly positive. Large dermal nests of atypical and densely packed melanocytes, expressing TLR2 similarly to the overlying epidermis, were the main finding in the invasive ALM examined. In acral melanocytic nevi the staining intensity appeared intense, basically overlapping with that of the nearby epidermis. The healthy skin specimens featured only a strong staining for TLR2 in the epidermis with particular accentuation in the basal layer. In negative controls (obtained as specified in Chapter 6 by omission of the primary antibody) the background was almost colorless in all portions of the specimens.

TLR3 was expressed along the full thickness of the epidermal and adnexal keratinocytes in all examined specimens (**Figure 11**, see Appendix), while only a faint background was detected in the *stratum corneum* and the papillary dermis. In both *in situ* and invasive melanomas, expression of TLR3 in neoplastic cells appeared to be weaker than that observed in keratinocytes but stronger than that in most dermal connective cells such as fibroblasts. In acral melanocytic nevi some nevus cells, particularly those belonging to junctional nests, seemed to express stronger amounts of TLR3, while in other melanocytes the staining intensity overlapped with that observed in the surrounding epidermis. The control healthy samples showed only a TLR3 staining in the whole epidermis with the exception of the horny layer. Such staining was absent in negative controls.

Immunostaining for ***TLR4*** highlighted that most keratinocytes and scattered dermal cells proved positive (**Figure 12**, see Appendix) while only a faint background was detected in the *stratum*

corneum and the papillary dermis. In *in situ* ALMs and junctional melanocytic nevi the expression of TLR-4 was comparable to that observed in surrounding epidermal layers, while in the invasive ALM such a staining seemed to be weaker with respect to the same internal control. The control healthy samples showed only a TLR3 staining in the whole epidermis with the exception of the horny layer. Such staining was absent in negative controls.

TLR9 was expressed by the vast majority of epidermal and adnexal keratinocytes in all examined specimens, while no background was detected in the *stratum corneum* and the papillary dermis. In both *in situ* and invasive melanomas, expression of TLR9 in neoplastic cells appeared to be weaker than that observed in keratinocytes. In one out of the two acral melanocytic nevi many nevus cells, particularly those belonging to junctional nests, seemed to express stronger amounts of TLR9 than the surrounding epidermis, while the staining intensity between melanocytes and keratinocytes was similar in the other acral melanocytic nevus. The control healthy samples showed only a TLR9 staining in the whole epidermis with the exception of the horny layer. Such staining was absent in negative controls.

Immunostaining for ***TNF-α*** proved to be completely negative in invasive ALM and control healthy acral skin (**Figure 14**, see Appendix). However in *in situ* melanomas a few dermal infiltrating cells showed to be strongly positive for TNF-α, while all other components of the lesion were negative. In one out of the two acral melanocytic nevi examined, several melanocyte nests showed a faint but detectable staining for TNF-α in a diffuse fashion (figure not available), while the same finding was not confirmed in the other nevus sample. TNF-α staining was negative in negative controls as well.

Discussion 7

As highlighted in previous Chapters, this study was carried out in order to preliminarly evaluate the role of innate immunity in the etiopathogenesis of acral lentiginous melanoma (ALM). Given the vastness of the issue, the study was narrowed to some biological models and mechanisms. In fact, innate immunity represents a key system of living beings including humans, and ranges from macroscopical, purely physical barriers to finely regulated and fairly specific molecular mechanisms. In recent years a growing amount of experimental evidences unveiled a previously unrecognized set of properties of the innate immune system which far exceed the bare, aspecific protection of the body from external harms. Among these features, the regulation of cell growth and therefore a possible role in carcinogenesis have emerged as a possible further mechanism which innate immune-related molecules contribute to regulate. Given some promising data from the literature, Toll-like receptors (TLRs) and some of their related molecules were chosen to be studied. Human melanoma cells were chosen as the *in vitro* model with the normal counterparts as controls. ALM was then chosen as a tissue model. In fact, this variant is the less studied and understood among the main histopathologic types of malignant melanoma and is the only one in which an environmental factor (ultraviolet rays) is not thought to play a role in carcinogenesis. Besides, the particular microanatomical structure of acral volar skin, together with some unique histological features of acral pigmented lesions (e.g. distribution of malignant cells only below the skin ridges, melanin release towards the epidermis taking place only above the neoplastic

nests) allow to hypothesize that the neoplastic transformation of melanocytes at the dermoepidermal junction of acral skin is at least in part dependent upon the differential expression and function of local, nonsoluble factors, such as membrane receptors like TLRs.

The present study attempted first to assess the expression and function of TLRs in ALM-derived human melanoma cells and normal human melanocytes *in vitro*. Both cell types expressed mRNA coding for several TLRs, namely all TLRs but TLR-8 in melanoma cells and all TLRs but TLR-6 and TLR-8 in normal human melanocytes. These findings, confirmed at the protein level by immunocytochemistry for some TLR2, -3, -4 and -9, partially confirm the ones reported in very recent studies, which showed that human melanoma cells express TLR-2, TLR-3 and TLR4,[260] while normal human melanocytes express mRNA coding for TLR-1 to -4, TLR-6, TLR-7 and TLR-9 and the actual translational products TLR-2, TLR-4, TLR-7 and TLR-9.[256-259] TLR functioning was then assessed by testing whether human melanoma cells and normal human melanocytes were able to produce innate immune mediators upon stimulation with specific TLR agonists. These findings are further discussed below.

Toll-like receptors recognizing bacterial lipopeptides or proteins may be involved in melanoma carcinogenesis

TLR-2 was expressed by both the cells lines under investigation at least at the mRNA level. However, TLR-2 agonist lipoteichoic acid (LTA) was not able to trigger any response in terms of cytokine production. These findings are in partial accordance with those by Yu et al., who found that TLR2 stimulation of normal human melanocytes triggered the expression of several mediators, including one also included in our study, i.e. IL-6,[259] and with those by Goto et al.,

who demonstrated that TLR-2 stimulation on melanoma cells induced the activation of the adaptor protein MyD88.[260] However, it should be pointed out that such studies used TLR2 agonists other than LTA, i.e. peptidoglycan or zymosan. Our findings do not exclude a possible role of TLR2 in melanocyte biology and pathophysiology as well since, as previously mentioned, TLR2 forms heterodimers with TLR1 or TLR6, which were both shown to induce responses in our melanoma cell line.

In particular, TLR-1 agonist PAM3CSK4 induced only the production of IL-1β in melanoma cells. The expression and functionality of TLR-1 represent a novel finding for melanoma cells and has been seldom reported for other malignant cells (e.g. choriocarcinoma, lymphoma lines).[268,269]

According to the fact that melanoma cells, but not normal melanocytes, expressed TLR6, the former cells, when stimulated with TLR6 agonist macrophage-activating lipopeptide 2 kDa (Malp-2), produced TNF-α, IL-1β and IL-6 in a dose- and time-dependent fashion. The expression and function of TLR6 in melanoma cells, but not in normal melanocytes, is a quite interesting finding. Although the role of TLR-6 in carcinogenesis has been poorly investigated so far, some recent studies highlighted that some genetic variation of TLR6 gene are associated with malignancies such as prostate cancer, non-Hodgkin lymphoma and chronic lymphocytic leukaemia.[269-271] Whether the TLR2-TLR6 heterodimer-mediated pathways may promote malignant transformation remains to be determined. However, preliminary *in vitro* reports showed that TLR6 activation promotes the expression of costimulatory molecules on leukemia cells and protect such cells from apoptosis.[271]

Lipopolysaccharide (LPS) and flagellin are bacterial antigens recognized by TLR4 and TLR5, respectively. They mainly triggered the production of IL-1β and IL-6 in A375 melanoma cells.

Even if the possible *in vivo* role of such an increased proinflammatory cytokine production by neoplastic cells upon bacterial stimulation remains to be elucidated, and albeit both IL-1β and IL-6 are known to promote apoptosis, these cytokines may possibly favour tumor growth and invasiveness given their effects on the synthesis of lytic enzymes (e.g. collagenases) and angiogenesis.

Intracellular Toll-like receptors may play a role in melanocyte physiology, and TLR3 may be also involved in melanoma carcinogenesis

TLR3 agonist polyinosinic:polycytidylic acid induced cytokine production in both normal and neoplastic melanocytes. Normal human melanocytes produced higher amounts of TNF-α, IL-1β and IL-6 (the latter confirming the results of Yu et al.[259]) than melanoma cells – both in a dose- and time-dependent fashion – while the former were the only cell line to synthetize mRNA coding for IFN-β. These findings allow to conclude that TLR3 function is somehow reduced in neoplastic melanocytes, and to speculate that decreased/absent levels of the cytokines under investigation, particularly TNF-α, may contribute to create a microenvironment promoting tumor growth *in vivo*. On the other hand, the strong action of TLR3 agonists in normal melanocytes may allegedly play a role in the skin innate immune system, including control of infectious agents and tumor surveillance together with other cell types such as keratinocytes.

Imiquimod, an agonist of intracellular TLR7, induced very scarce responses on cytokine production on the tested cells, and only at the mRNA level. These findings are in accordance with those obtained in other studies, according to which, if imiquimod had some inhibitory effect

on pigmentation,[258] it was not able to induce the production of IL-6 at least in normal human melanocytes.[259]

Since TLR-8 was not expressed in neither type of the studied cell types, as already demonstrated previously on normal human melanocytes,[259] there is no evidence to consider such TLR involved in melanocyte physiology and pathology.

The only detected effect of TLR9 agonist CpG-ODN during the experiments was the strong induction of IFN-β on normal human melanocytes at least at the mRNA level. This finding can be preliminarly considered important as it can be speculated that melanocytes are able to respond to internalized antigens of viral nature (mimicked by CpG-ODN) with a significant anti-viral effect.

Toll-like receptors-3, -6 and -9 may play a regulatory role on melanogenesis

Previous reports showed that some TLRs are able to influence the biosynthetic processes leading to the production of melanin. Namely, TLR4 and TLR7 were shown to increase and decrease melanin synthesis *in vitro*, respectively.[256,258]

The present experiments showed for the first time that TLR3 and TLR6 stimulation increased melanin synthesis and that this phenomenon was at least in part to be ascribed to enhanced activity of the enzyme tyrosinase. Such an increase was observed, as expected on the basis of previous findings, in both melanoma cells and normal melanocytes for TLR3 but only in melanoma cells for TLR6. On the other side, TLR9 stimulation triggered a decreased production of melanin in normal melanocytes, dependent upon the inhibition of tyrosinase activity. At least

for TLR3 and TLR9 such an influence on melanogenesis was specific as specific TLR inhibitors reversed the effects of the agonists.

Unfortunately, it was not possible to investigate further the intracellular processes connecting the stimulation of TLRs with melanin biosynthesis. It is likely that the final amount of melanin production is the result of a finely-tuned balance of many cellular and molecular factors which influence more or less directly the activity of tyrosinase in different ways. Under this view, it can be indeed hypothesized that TLR stimulation participates in the regulation of melanogenesis. Taking into considerations our experiments, TLRs may activate nuclear factor (NF)-κB which may in turn promote the synthesis of innate immune-related cytokines. Such molecules may then bind to their receptors on melanocyte surface and eventually modulate the function of proteins, such as microphthalmia-associated transcription factor (MITF), tyrosinase-related protein (TRP)-1 and p53, known to regulate tyrosinase activity. Namely, the reduced production of TNF-α in melanoma cells with respect to human melanocytes upon TLR3 stimulation may attenuate the known inhibitory effect of TNF-α itself on melanogenesis.[272] The overall hyperpigmentation effects of TLR6 may instead depend on the balance between a low TNF-α concentration and IL-6, which supposedly exerts an hypopigmentary effect.[273] Instead, while IL-1α seems to induce hypopigmentation,[274] there are no data concerning the effects of IL-1β on melanogenesis. The inhibitory effects of TLR9 on melanogenesis in normal melanocytes may be at least in part ascribed to the secretion of IFN-β, which, although not known to have any effect on pigmentation, may slower melanocyte proliferation and therefore yield an overall lesser production of melanin.[275]

Toll-like receptors-2, -3, -4 and -9 may influence the pathophysiology of malignant and benign acral melanocytic lesions

In accordance to the second main aim of the study, the expression of selected TLRs was evaluated in malignant and benign acral melanocytic lesions. As detailed in Chapter 6, all TLRs taken into consideration (TLR2, -3, -4 and -9) were expressed in all the specimens considered, which included lesions belonging to in situ ALM, invasive ALM, acral acquired melanocytic nevus (junctional tipe) and clinically healthy acral skin. Since positive controls for the stainings of such TLRs were not made available, internal controls (i.e. already present in the specimens themselves) were considered to evaluate the staining intensity of the cell types and microanatomical structures under specific consideration. In particular, several studies have shown that epidermal keratinocytes strongly express TLR2, -3, -4 and -9 under physiological conditions, at least in non-acral, hairy skin.[268,269] In fact, not surprisingly all epidermal portions of all our specimens showed a strong TLR staining as well and were therefore considered as appropriate internal positive controls.

TLR2, -3, -4 and -9 were expressed on tumoral tissue, that is, both in melanocytic nevi and melanomas. These findings are novel in that no report on the expression of TLRs in melanocyte-derived proliferations is available in the literature. In addition, some differential levels of expression were detected between acquired melanocytic nevi and ALM.

In fact, TLR2 and TLR4 expression appeared to be lower in invasive ALM than in *in situ* ALM and acquired melanocytic nevus. On the basis of these preliminary findings, it is possible to speculate that dysregulation of TLR-2 and TLR-4 expression may belong to the complex series

of events leading ALM cells to acquire an invasive phenotype, and may be therefore interpreted as a sign of de-differentiation. This appears to be somehow in contrast with a previous *in vitro* study showing that melanoma cell invasiveness is at least in part responsible of TLR4 hyperexpression and function.[264] However, it should be considered that these phenomena were observed on cells derived from non-acral hairy skin, where melanoma likely follows different pathogenic pathways. Taking into consideration the present and previous *in vitro* results on the putative effect of TLR-induced cytokine on tumor growth (see Discussion above), no specific correlation can be drawn from decreased TLR2 and TLR4 tissue expression. On the other side, TLR2 and TLR4 may play a yet-to-be determined role in the physiology and pathophysiology of benign acral melanocytic nevi.

While TLR3 did not show any differential levels of expression between the various lesions, TLR9 staining was stronger in melanocytic nevi than in both ALM types. These findings basically confirm the *in vitro* results, which showed that both cell type are expressed on normal and neoplastic melanocytes, TLR9 levels being the the highest at least at a transcriptional level (see above). The role of these intracellular receptors on melanocytes belonging to acral pigmented lesions remains to be further investigated. However, at least two points deserve to be discussed at greater detail. First, as reported in previous Chapters the release of melanin towards the upper layers of acral skin epidermis is a specific – and puzzling – feature of benign pigmented lesions but not of malignant ones (at least at early stages of progression). Since the expression of TLR9 seems to be diminished in ALM but not in acral melanocytic nevi, it can be speculated that such a molecule may somehow influence the transfer of melanosomes from benign nevus cells to the surrounding keratinocytes, provided that other mechanism(s) may also be implied as well. Second, the *in vitro* study showed that normal melanocytes, but not

melanoma cells, produce IFN-β in response to a TLR9 agonist (see above). Speculating that the same happens also *in vivo*, the resulting decreased tissue levels of IFN-β, known as a cytokine with remarkable antitumoral functions, may represent a factor promoting melanoma growth in acral sites.

Finally, the tissue expression of TNF-α, an innate immune system-related molecule which production is known to be triggered by TLRs, was evaluated in acral pigmented lesions. Such cytokine was expressed within benign nevi but not within ALM. This finding is in partial contrast with those shown in pigmented lesions belonging to nonacral hairy skin (where TNF-α is expressed faintly in melanocytic nevi and strongly in melanoma[278]) and allows to hypothesize that TNF-α may exert an inhibitory effect on cells growth in benign lesions. Despite the present study showed that TLR3 and TLR6 agonists are able to stimulate the production of TNF-α in vitro and that TLR3 was actually expressed in acral melanocytic lesions, whether this mechanism is active and significant also *in vivo* remains questionable.

Limitations of the study

The present study was affected by several limitations. In general, logistical issues prevented to assess fully all the facets of the research topic as anticipated in both the initial conception and later setup of the specific aims. Also, limitations were intrinsic to the study as well and included lack of some proper controls, lack of additional investigation techniques, and incomplete panel of markers to be assessed. In particular, the *in vitro* branch of the research 1) did not consider as controls cell types (e.g. normal human melanocyte and benign nevus cells) belonging to acral skin as well as melanoma cells not belonging to acral skin; 2) did not confirm at the protein level

many of the molecules investigated; 3) did not evaluate the intracellular pathways related to TLR activation and the effect on some effector molecules (e.g. the cytokines considered) on function and growth of normal and neoplastic melanocytes; and 4) did not carry out all the procedures on all the molecules in the panel. On the other side, the *ex vivo* branch of the research 1) used a very low number of specimens and markers; 2) did not consider several useful controls (e.g. positive controls and lesions not belonging to acral skin); 3) did not carry out double immunostainings in order to increase the specificity of the results; and 4) did not investigate the pathways leading to melanin production in the lesional tissue.

Conclusions

Despite the overmentioned limitations, and as discussed above, this research project achieved some significant and novel results. It was shown that ALM cells express TLRs both *in vivo* and *ex vivo* and that such receptors are able to trigger the production of innate immune cytokines at least *in vitro*. Moreover, at least some TLRs seem to have a regulatory effect on melanogenesis. This findings add up to the growing bulk of clinical and experimental data concerning both the pathophysiology of melanocytes with respect to their immune function and pathogenesis of ALM. In particular, the latter disease still represents the least investigated of all types of cutaneous melanoma and probably features a peculiar and complex molecular pathogenesis in which the role of innate immune-related mechanisms may be of remarkable importance. Further studies in these exciting fields are therefore warranted.

Abbreviations

ALM	Acral lentiginous melanoma
ACTH	Adrenocorticotropic hormone
APC	Antigen-presenting cells
AMP	Antimicrobial peptide
Bcl	B-cell leukemia/lymphoma
BSA	Bovine serum albumin
CSD	Chronic sun-damage
CMM	Cutaneous malignant melanoma
CDK	Cyclin-dependent kinase
DC	Dendritic cells
ELISA	Enzyme-linked-immunosorbent assay
FGF	Fibroblast GF
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HSP	Heat-shock protein
HRP	Horse radish peroxydase
IFN	Interferon
IRF	Interferon regulatory factors
IL	Interleukin

IL1R	IL-1 receptor
IDP	Irregular diffuse pigmentation
LMM	Lentigo maligna melanoma
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
Malp-2	Macrophage-activating lipopeptide 2 kDa
MAP	Mitogen-activated protein
MIF	Macrophage migration inhibitory factor
MHC	Major histocompatibility complex
MC1R	Melanocortin-1 receptor
MITF	Microphthalmia-associated transcription factor
MyD88	Myeloid differential factor 88
NK	Natural killer
NM	Nodular melanoma
NF	Nuclear factor
ODN	Oligodeoxynucleotide
PFP	Parallel-furrow pattern
PRP	Parallel-ridge pattern
PAMP	Pathogen-associated molecular patterns
PRP	Pattern recognition receptor
PO	Phenol oxidase
PBS	Phosphate-buffered saline
PBT	Tween-20 in PBS

Poly(I:C)	Polyinosinic:polycytidylic acid
POMC	Pro-opiomelanocortin
PPO	Pro-phenoloxidase
RT-PCR	Real-time polymerase chain reaction
RT	Room temperature
SCF	Stem cell factor
SSM	Superficial spreading melanoma
TIR	Toll-IL-1 receptor
TRAF	TNF receptor-associated factor
TNF	Tumor necrosis factor
TRP1	Tyrosinase-related protein
UV	Ultraviolet rays

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This work concludes my journey thorugh the pathways of the Italian school education system, started around 30 years ago. Still uncertain of where opportunities will lead me over the next decades, I am sure that the cultural background I gained along the way will support me till the end of my days. Namely, the last four years, partly spent in the completion of this Ph.D. program, allowed me to improve my knowledge in benchside research and to consider basic science applied to skin Biology not a sneaky enemy to be afraid of, but rather a giant's shoulder to stand on.

"Besides love, nothing is deeper than the skin."

Fyodor Dostoyevsky, *The Gambler* (1867)

Appendix – Tables and Figures

Table 1 – Demographic, clinical and histologic features of the patients selected for the study

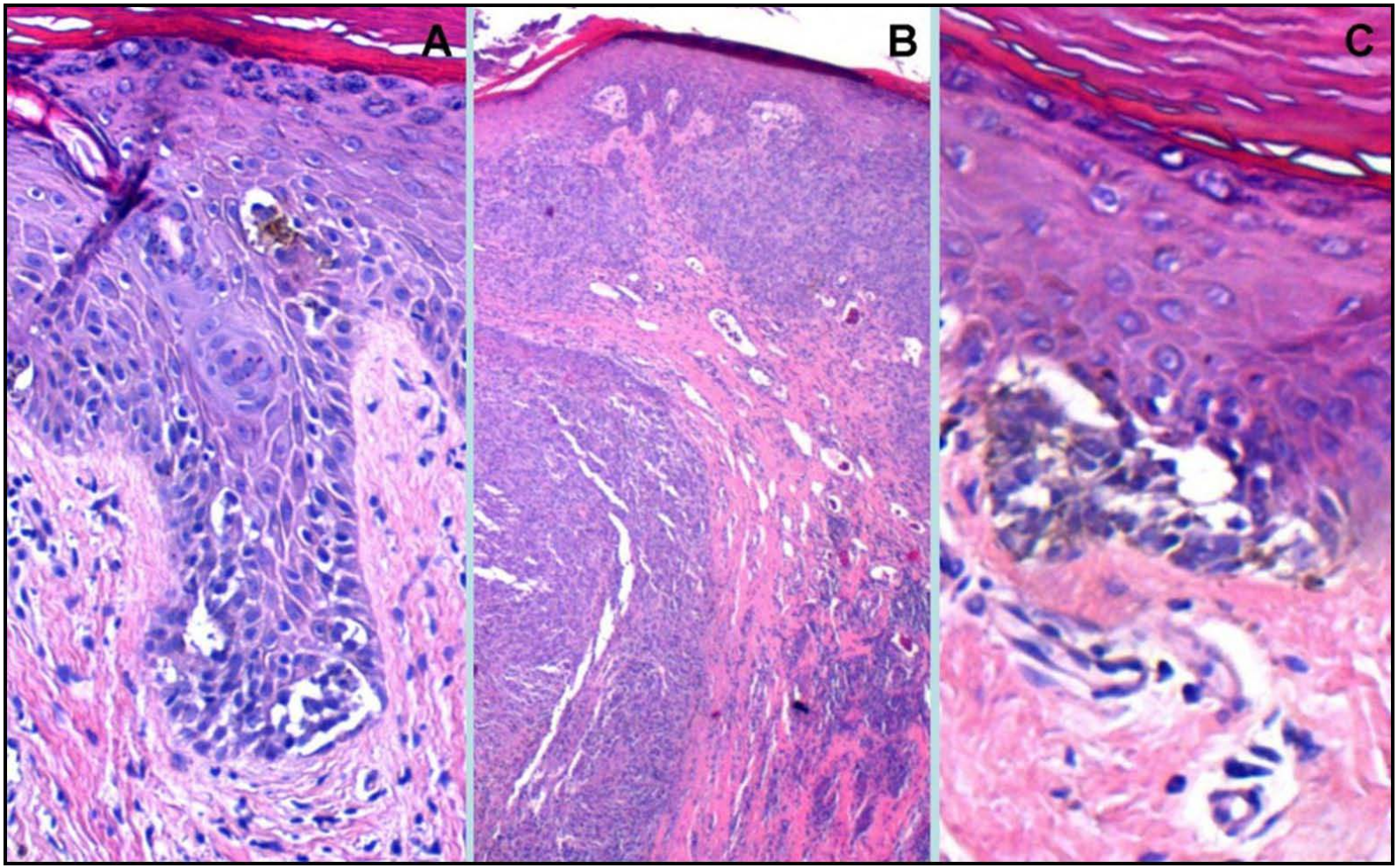
Patient	***Age***	***Sex***	***Location***	***Histological diagnosis***	***Notes***
1	68	F	Sole (heel)	Malignant melanoma, acral lentiginous type	*In situ* neoplasm
2	34	F	Sole, NOS	Malignant melanoma, acral lentiginous type	*In situ* neoplasm
3	72	F	Lateral sole	Malignant melanoma, acral lentiginous type	Invasive neoplasm (Breslow depth 2.67 mm)
4	41	F	Medial sole	Acral compound melanocytic nevus	Junctional pattern
5	6	M	Sole, NOS	Acral compound melanocytic nevus	Junctional pattern
6	73	F	Sole, NOS	Dermal fibrosis	Re-excision on a previously operated site for a proven benign lesion
7	34	M	Sole, NOS	Healthy acral skin	Free margins of a specimen containing a benign non-melanocytic lesion

F: female; M: male; NOS: not otherwise specified.

Table 2 – Histopathological features of the specimens considered for the study

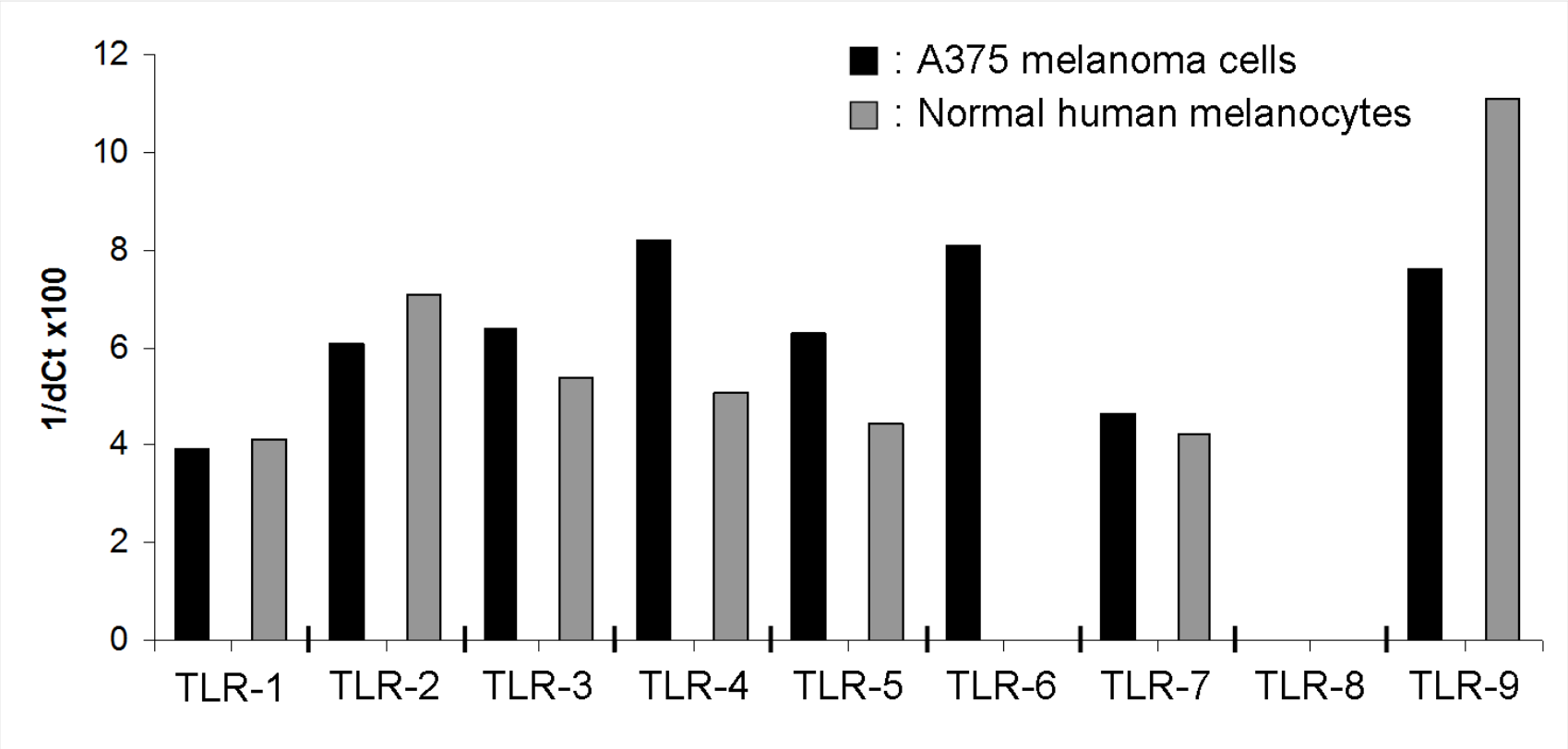
Specimen	Epidermal findings	Dermal findings
1	- Hyperkeratosis consistent with the site and focal parakeratosis - Proliferation of atypical melanocytes arranged in nests and in a pagetoid pattern	- Fibrosis - Scant lichenoid response
2	- Hyperkeratosis consistent with the site and focal parakeratosis - Proliferation of atypical melanocytes, individual or arranged in small nests	- Scattered pigmented macrophages - Collection of melanocytic nests
3	- Focal ulceration and moderate acanthosis - Proliferation of numerous nests of atypical melanocytes	- Invasion of the superficial and mid dermis with a vertical growth phase
4	- Hyperkeratosis consistent with the site and focal parakeratosis - Proliferation of melanocytes, individual or arranged in small nests	
5	- Hyperkeratosis consistent with the site and focal parakeratosis - Proliferation of melanocytes, individual or arranged in small nests	
6	- Normal	- Fibrosis
7	- Normal, with slight basal hypermelanosis	- Normal

Figure 1 – Histopathologic features of some specimens considered for the study.



A: *In situ* acral malignant melanoma. Note the small neoplastic cells at the dermoepidermal junction of a *crista profunda intermedia* (passed through by an eccrine duct). **B:** Invasive acral malignant melanoma. **C:** Acral acquired junctional melanocytic nevus. Note the nest of nevus cells in a *crista profunda limitans*. The eccrine duct visible in the dermis derives from the adjacent ridge.

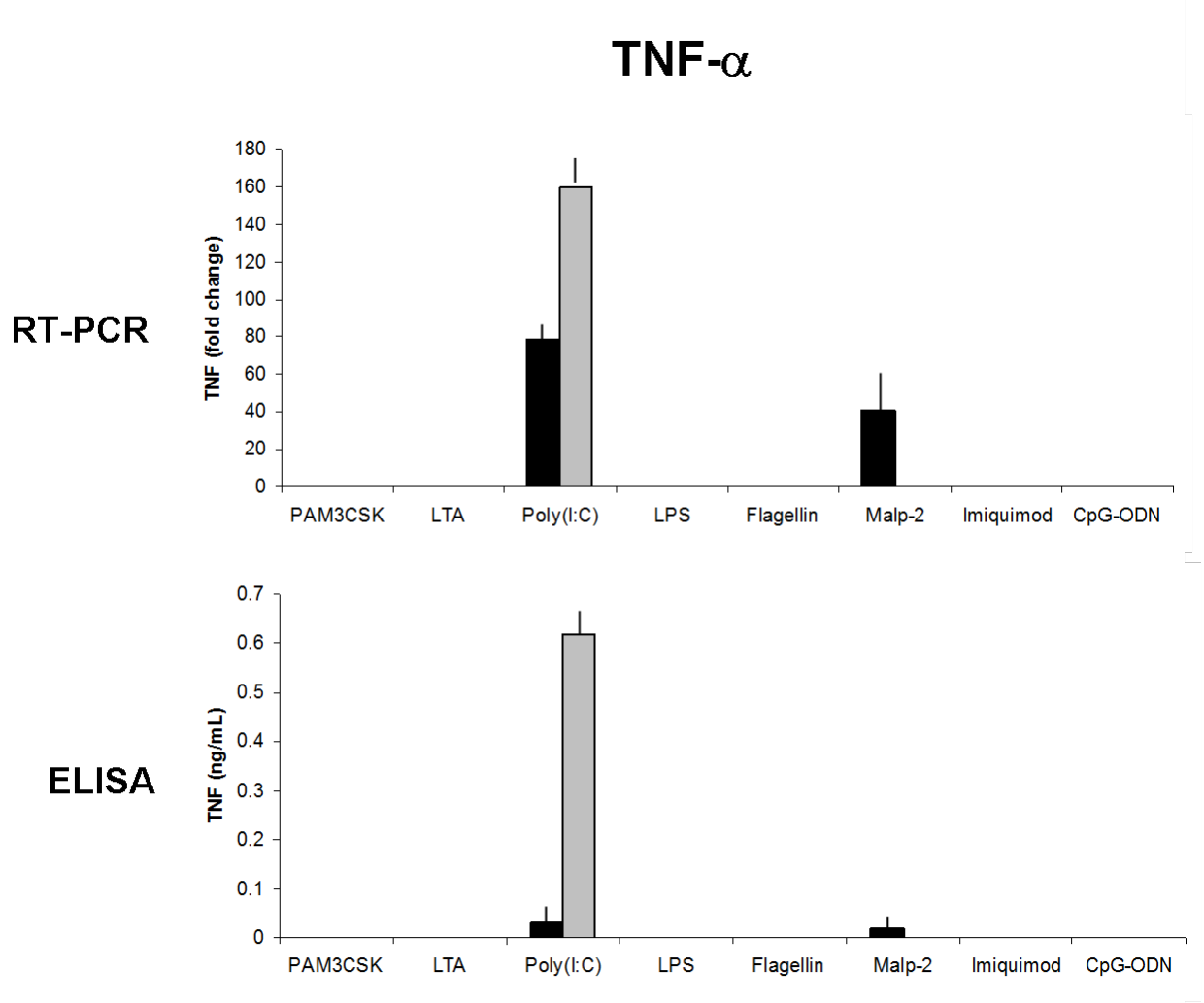
Figure 2 – *In vitro* expression of Toll-like receptors by the tested cells.



Black bars: A375 melanoma cells.

Grey bars: normal human melanocytes.

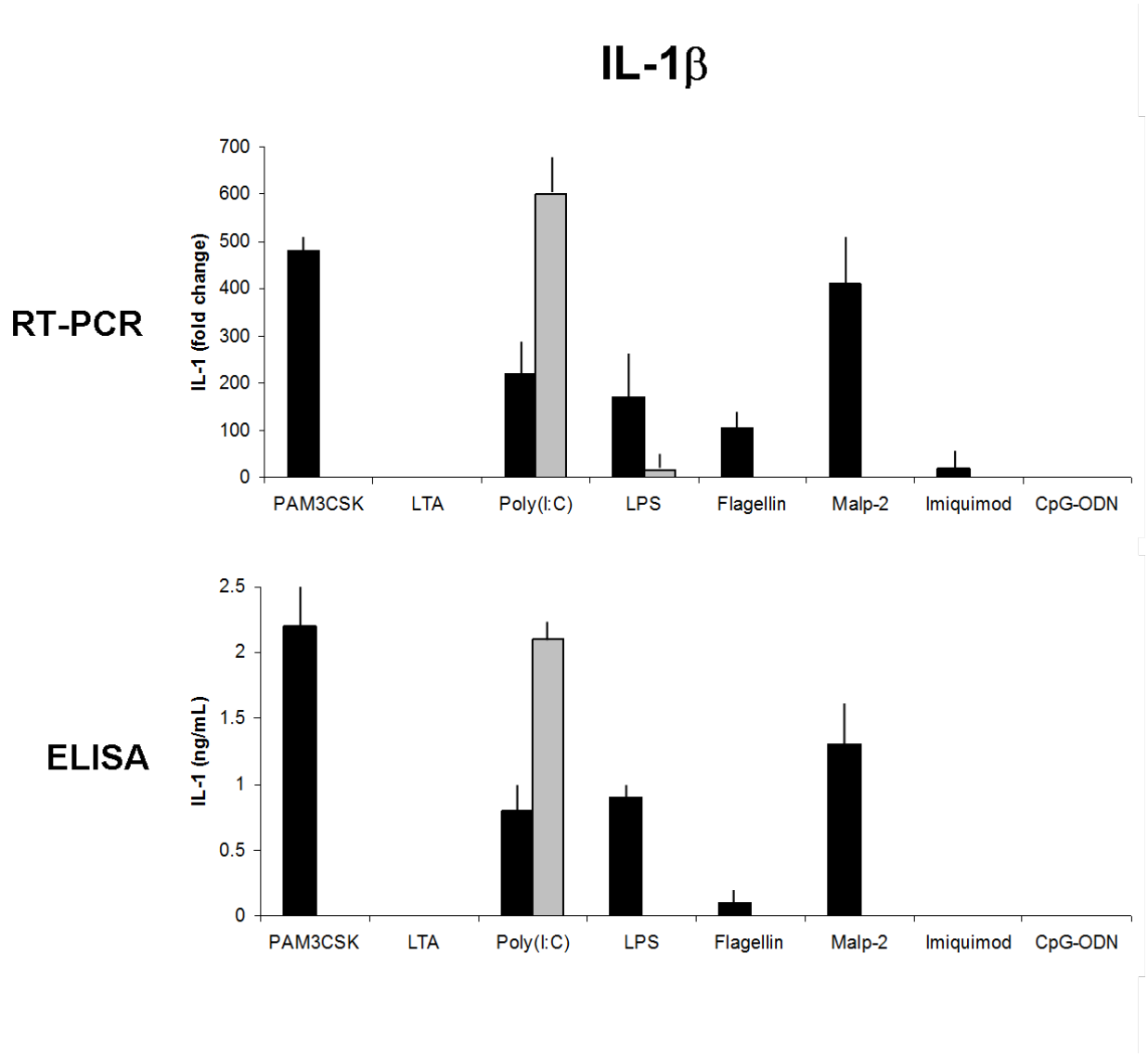
Figure 3 – Expression of tumor necrosis factor-α upon stimulation of the tested cells with Toll-like receptor agonists.



Black bars: A375 melanoma cells.

Grey bars: normal human melanocytes.

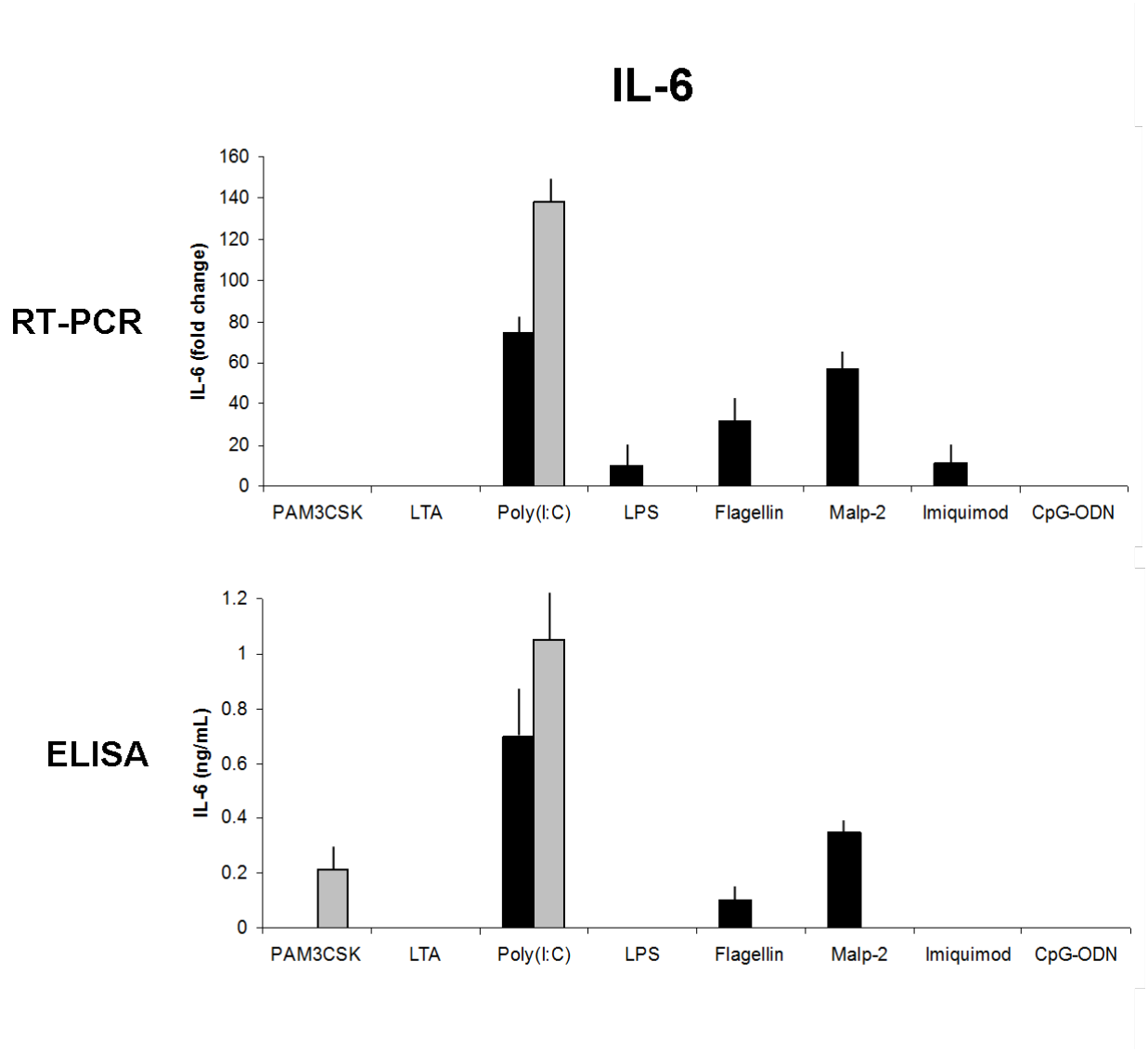
Figure 4 – Expression of interleukin 1-β upon stimulation of the tested cells with Toll-like receptor agonists.



Black bars: A375 melanoma cells.

Grey bars: normal human melanocytes.

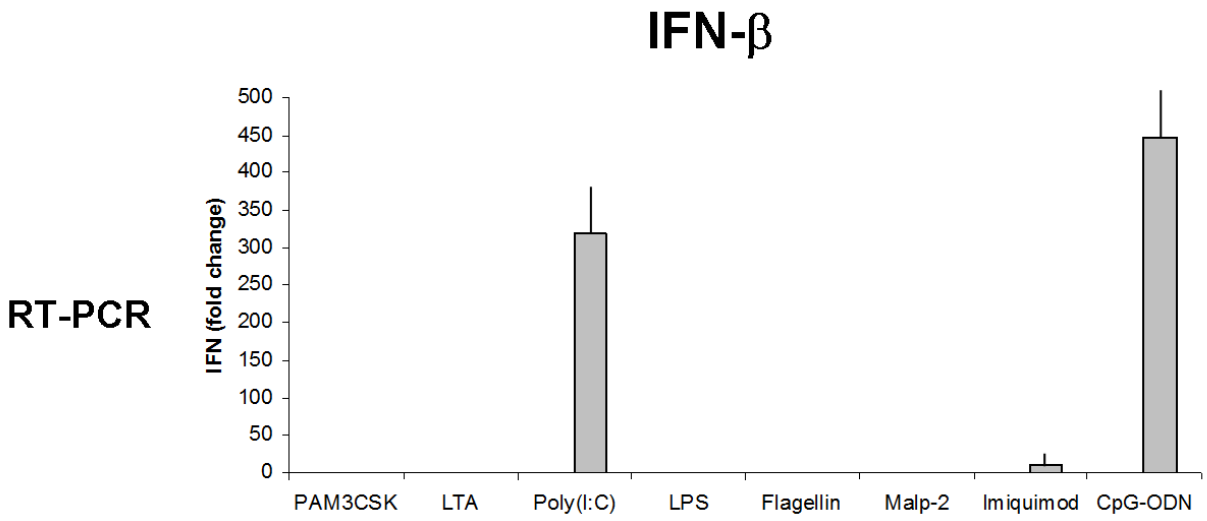
Figure 5 – Expression of interleukin 6 upon stimulation of the tested cells with Toll-like receptor agonists.



Black bars: A375 melanoma cells.

Grey bars: normal human melanocytes.

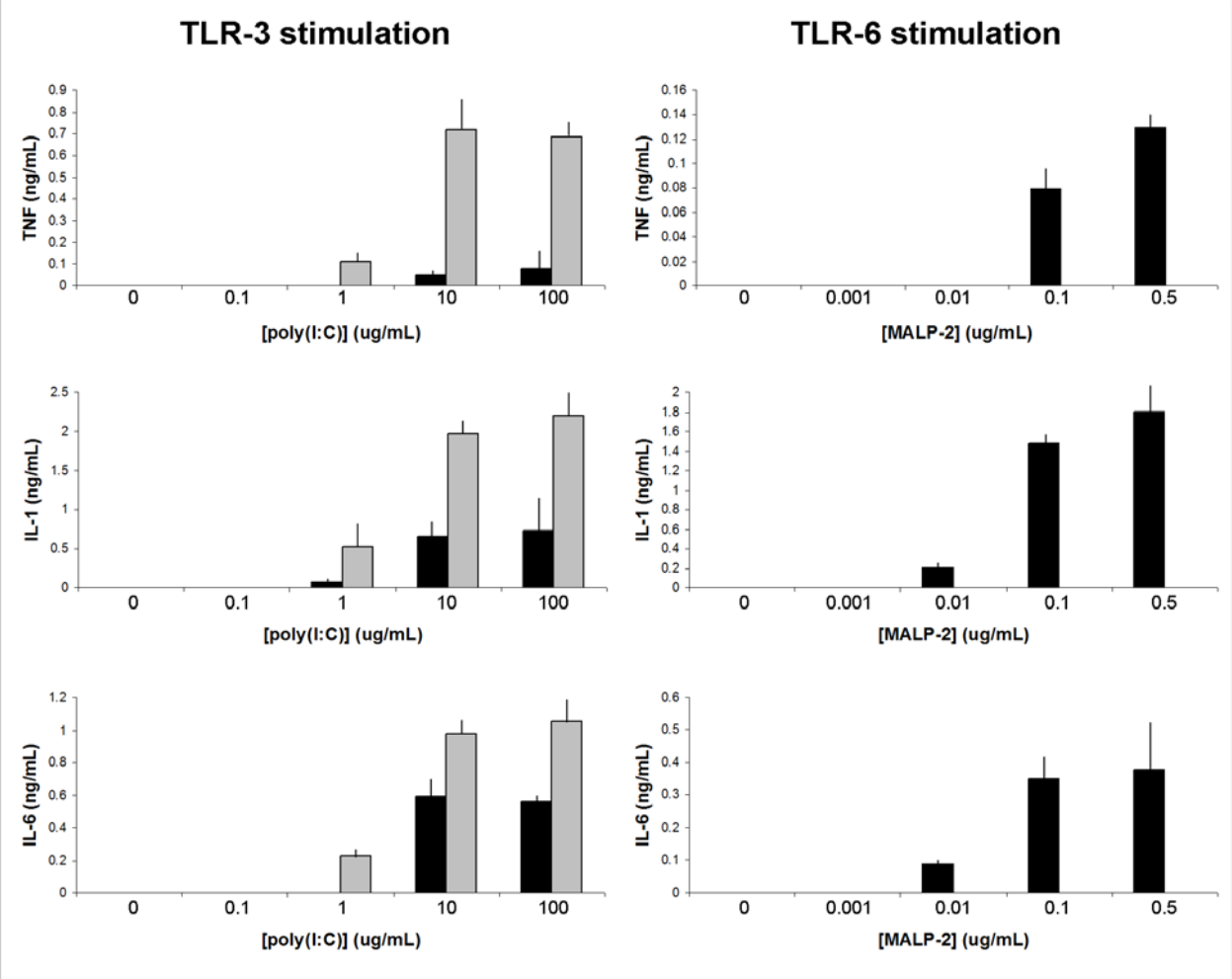
Figure 6 – Expression of interferon β upon stimulation of the tested cells with Toll-like receptor agonists.



Black bars: A375 melanoma cells.

Grey bars: normal human melanocytes.

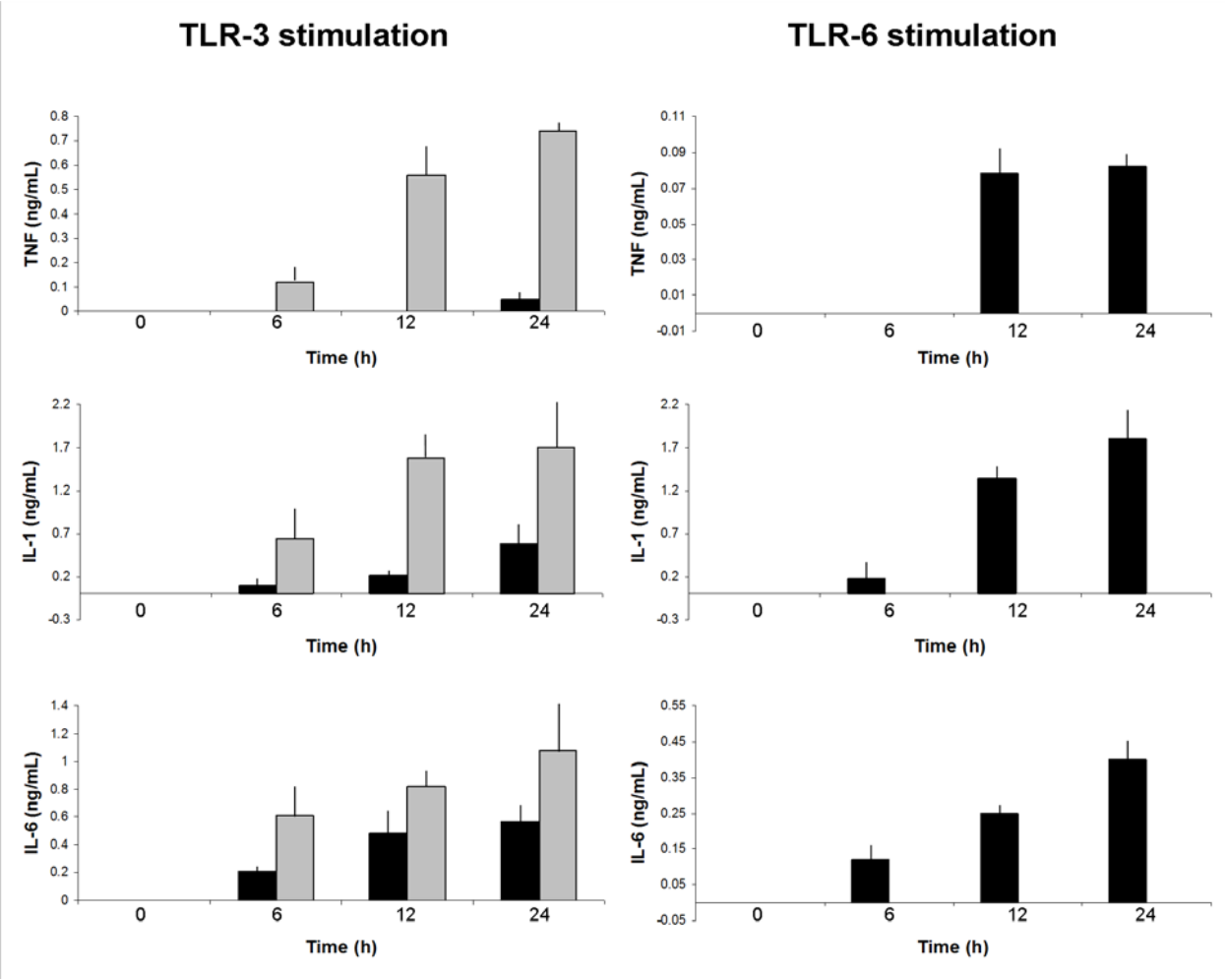
Figure 7 – Dose dependency of the production of tumor necrosis factor-α, interleukin 1 and interleukin 6 upon stimulation of the tested cells with selected Toll-like receptor agonists.



Black bars: A375 melanoma cells.

Grey bars: normal human melanocytes.

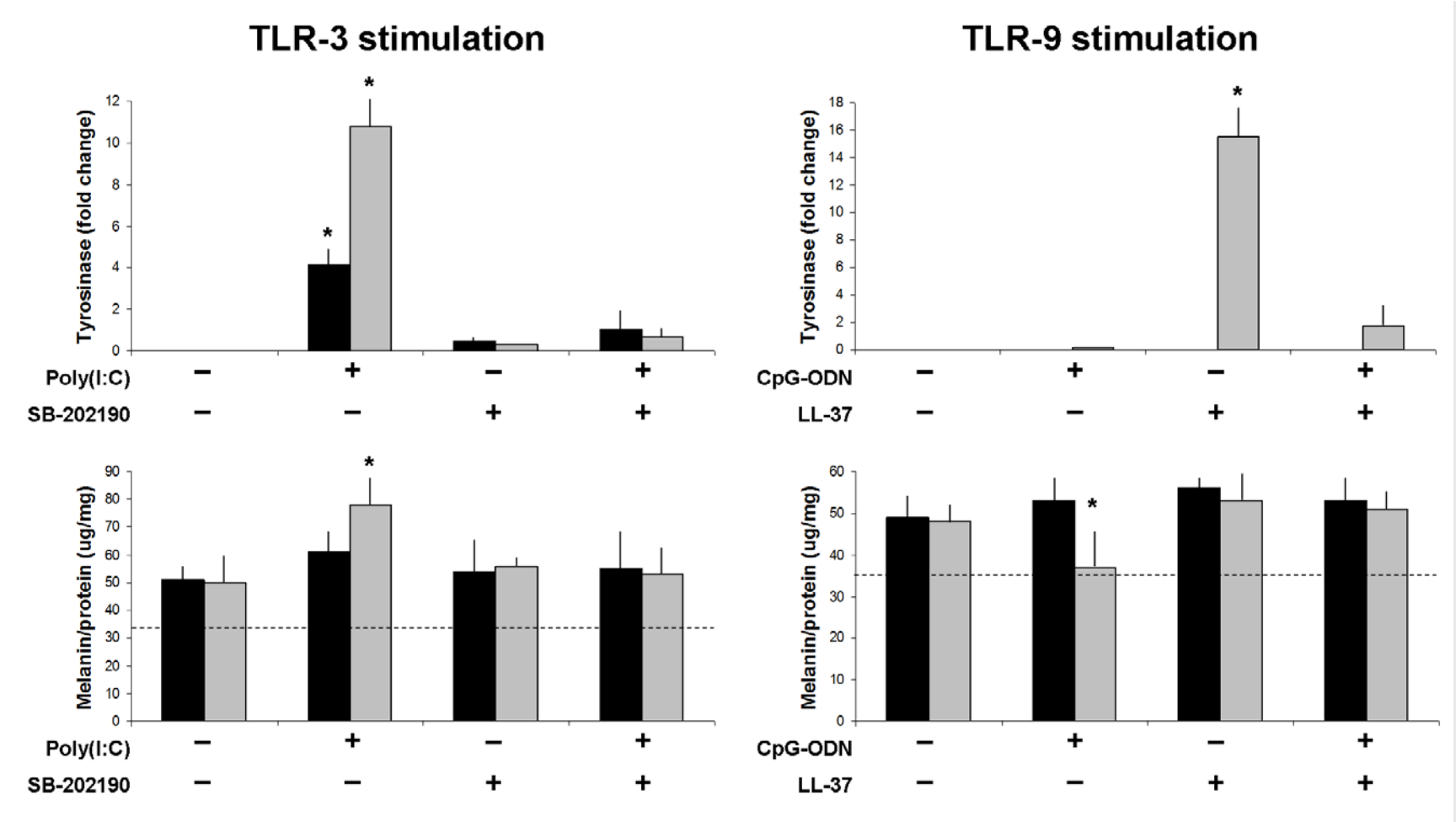
Figure 8 – Time dependency of the production of tumor necrosis factor-α, interleukin 1 and interleukin 6 upon stimulation of the tested cells with selected Toll-like receptor agonists.



Black bars: A375 melanoma cells.

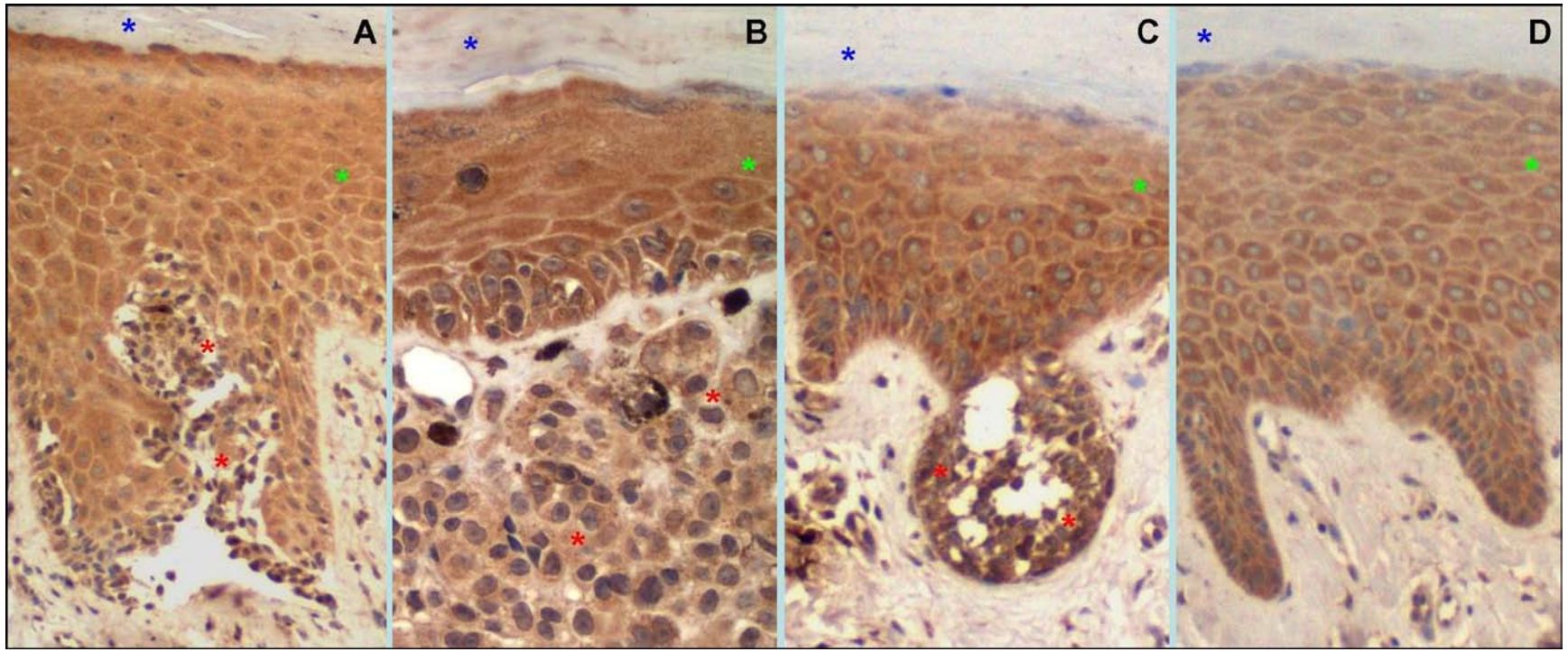
Grey bars: normal human melanocytes.

Figure 9 – Influence of selected Toll-like receptor agonists on melanogenesis.



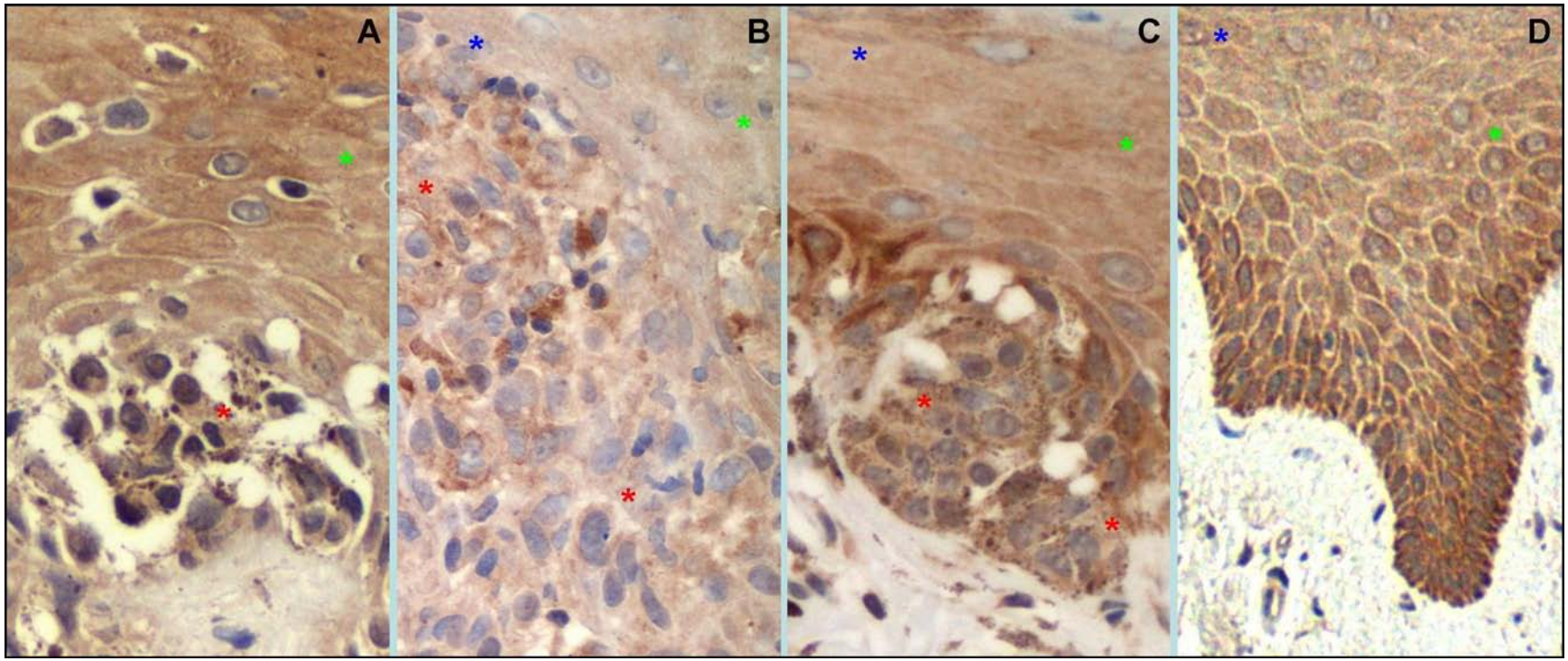
Black bars: A375 melanoma cells. Grey bars: normal human melanocytes. Horizontal lines: background melanin content of unstimulated cells at time 0. Asterisks: $P < 0.05$ (statistically significant).

Figure 10 – Expression of Toll-like receptor 2 in representative portions of the specimens considered for our study.



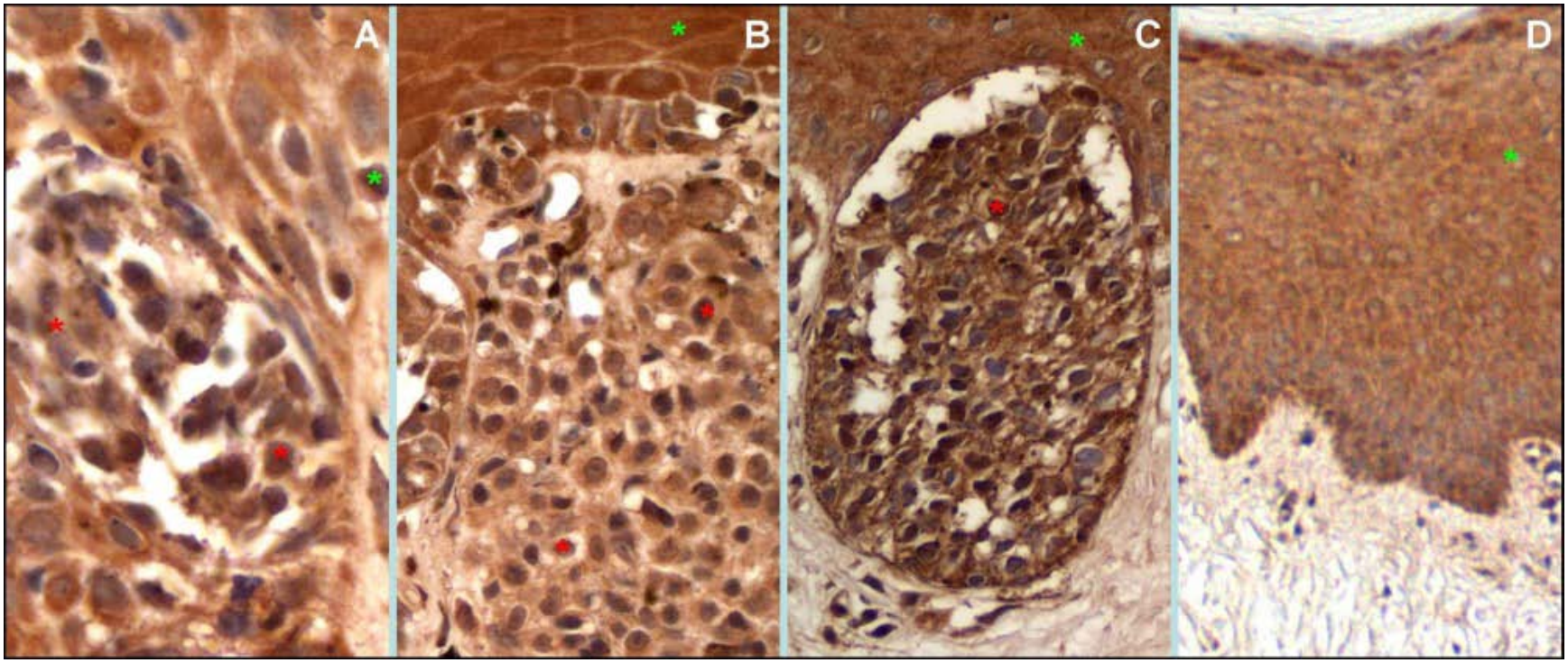
A: *In situ* acral malignant melanoma (ALM). Neoplastic nests at the dermoepidermal junction of a *crista profunda intermedia* express Toll-like receptor 2 (TLR2) similarly to the surrounding epidermis. **B:** Invasive ALM. Dermal neoplastic aggregates of neoplastic melanocytes show a weaker expression of TLR2 when compared with the overlying epidermis. **C:** Acral acquired junctional melanocytic nevus. A junctional nest of nevus cells expresses TLR2 similarly to the overlying epidermis. **D:** Normal acral skin. The epidermis is full-thickness TLR2-positive. Blue *: horny layer; green *: noncornified epidermis; red *: melanocyte aggregates.

Figure 11 – Expression of Toll-like receptor 3 in representative portions of the specimens considered for our study.



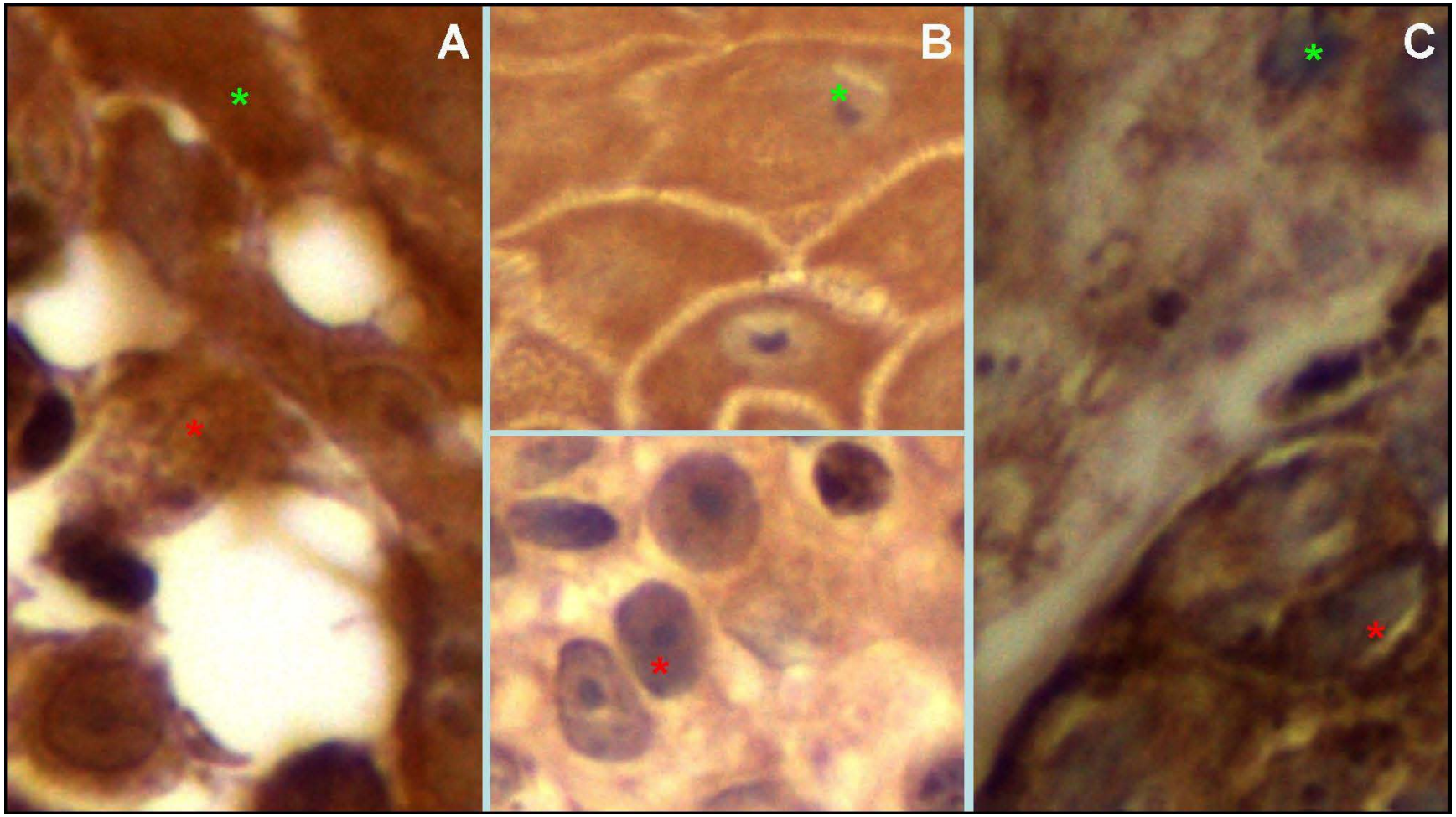
A: *In situ* acral malignant melanoma (ALM). Neoplastic nests at the dermoepidermal junction express Toll-like receptor 3 (TLR3) similarly to the surrounding epidermis. **B:** Invasive ALM. Dermal neoplastic aggregates of neoplastic melanocytes show a weaker expression of TLR3 when compared with the overlying epidermis. Darker brown spots likely represent melanin accumulation. **C:** Acral acquired junctional melanocytic nevus. A junctional nest of nevus cells expresses TLR3 similarly to the overlying epidermis. **D:** Normal acral skin. The epidermis is full-thickness TLR3-positive. Green *: noncornified epidermis; red *: melanocyte aggregates.

Figure 12 – Expression of Toll-like receptor 4 in representative portions of the specimens considered for our study.



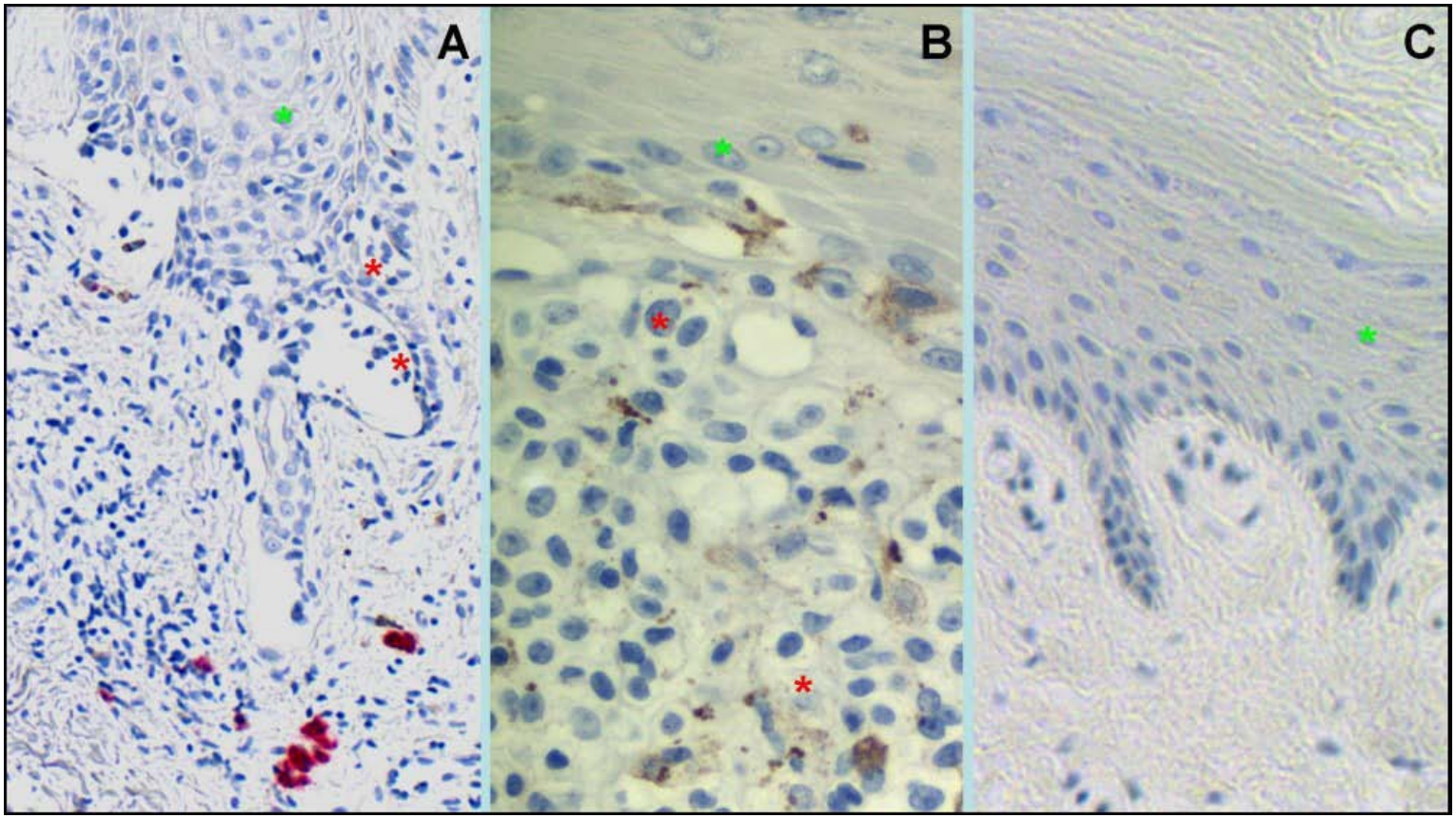
A: *In situ* acral malignant melanoma (ALM). Neoplastic nest at the dermoepidermal junction expressing less Toll-like receptor 4 (TLR4) than the surrounding epidermis. **B:** Invasive ALM. Dermal neoplastic aggregates of neoplastic melanocytes show a weaker expression of TLR4 when compared with the overlying epidermis. **C:** Acral acquired junctional melanocytic nevus. A junctional nest of nevus cells expresses TLR4 similarly to the overlying epidermis. **D:** Normal acral skin. The epidermis is full-thickness TLR4-positive. Green *: noncornified epidermis; red *: melanocyte aggregates.

Figure 13 – Expression of Toll-like receptor 9 in representative portions of the specimens considered for our study.



A: *In situ* acral malignant melanoma (ALM). Neoplastic cells expressing less Toll-like receptor 4 (TLR9) than keratinocytes (KCs). **B:** Invasive ALM. Neoplastic melanocytes (bottom box) show a weaker expression of TLR9 when compared with KCs (top box). **C:** Acral acquired melanocytic nevus. Melanocytes express TLR9 similarly to KCs. Green *: KC; red *: melanocyte.

Figure 14 – Expression of tumor necrosis factor-α in representative portions of the specimens considered for our study.



A: *In situ* acral malignant melanoma (ALM). Some cells infiltrating the dermis are **tumor necrosis factor** (TNF)-α positive (red staining). **B:** Invasive ALM. Neoplastic melanocytes are TNF-α negative. **C:** Normal acral skin. No TNF-α expression is detectable. Green *: noncornified epidermis; red *: melanocyte aggregates.