Vitiligo, reactive oxygen species and T-cells

Steven J. GLASSMAN
Division of Dermatology, Department of Medicine, University of Ottawa, 737 Parkdale Avenue, Ottawa, Ontario, Canada K1Y 1J8

ABSTRACT

The acquired depigmenting disorder of vitiligo affects an estimated 1% of the world population and constitutes one of the commonest dermatoses. Although essentially asymptomatic, the psychosocial impact of vitiligo can be severe. The cause of vitiligo remains enigmatic, hampering efforts at successful therapy. The underlying pathogenesis of the pigment loss has, however, been clarified to some extent in recent years, offering the prospect of effective treatment, accurate prognosis and rational preventative strategies. Vitiligo occurs when functioning melanocytes disappear from the epidermis. A single dominant pathway is unlikely to account for all cases of melanocyte loss in vitiligo; rather, it is the result of complex interactions of biochemical, environmental and immunological events, in a permissive genetic milieu. ROS (reactive oxygen species) and H₂O₂ in excess can damage biological processes, and this situation has been documented in active vitiligo skin. Tyrosinase activity is impaired by excess H₂O₂ through oxidation of methionine residues in this key melanogenic enzyme. Mechanisms for repairing this oxidant damage are also damaged by H₂O₂, compounding the effect. Numerous proteins and peptides, in addition to tyrosinase, are similarly affected. It is possible that oxidant stress is the principal cause of vitiligo. However, there is also ample evidence of immunological phenomena in vitiligo, particularly in established chronic and progressive disease. Both innate and adaptive arms of the immune system are involved, with a dominant role for T-cells. Sensitized CD8⁺ T-cells are targeted to melanocyte differentiation antigens and destroy melanocytes either as the primary event in vitiligo or as a secondary promotive consequence. There is speculation on the interplay, if any, between ROS and the immune system in the pathogenesis of vitiligo. The present review focuses on the scientific evidence linking alterations in ROS and/or T-cells to vitiligo.

INTRODUCTION

Vitiligo is a common skin disorder characterized by the acquired loss of constitutional pigmentation manifesting as white macules and patches caused by a loss of functioning epidermal melanocytes. Extent of involvement is highly variable, ranging from focal to generalized, and the onset can be abrupt or gradual. Although essentially asymptomatic, the psychosocial impact of vitiligo can be devastating, and affected...
persons are often desperate for effective therapy [1]. As of 2010, this goal has not yet been reached, as the underlying pathomechanisms in vitiligo are still incompletely understood, despite intense scrutiny. Three recurring themes emanate from clinical and scientific analysis of melanocyte loss in vitiligo: genetic, immune and biochemical. The dominant theorem describes an autoimmune process occurring in a genetically susceptible host and triggered by a variety of host and environmental factors [2]. The aim of the present review is to summarize the role of ROS (reactive oxygen species) and T-cells in the pathogenesis of vitiligo.

Vitiligo occurs worldwide, with an estimated prevalence rate of about 1%. A large population-based survey found a prevalence rate of 0.38% in Denmark [3], and a clinic-based survey in Martinique found a prevalence rate of 0.34% [4]. Other surveys have reported rates from 0.15% to 8.8%, with the highest rates occurring in Indian locales [5]. Onset is before the age of 20 years in about half the cases, and three quarters have occurred by the age of 30 years. Sexes are equally affected, but there might be a female preponderance owing to reporting bias [6]. Two major subtypes of vitiligo are described, segmental and non-segmental. Segmental vitiligo tends to have an earlier onset, with the rapid evolution of unilateral depigmentation in the distribution of a dermatome, suggesting neural involvement, or corresponding to Blaschko’s lines, suggesting a form of genetic mosaicism. Segmental vitiligo mostly affects the face and tends to be stable once fully developed. The area of depigmentation is sometimes not as well demarcated as in other types of vitiligo [7]. Non-segmental vitiligo is, by far, the commoner type and is also known as generalized vitiligo or vitiligo vulgaris. Most work relating to pathogenesis has focused on this form of vitiligo exclusively. Onset is usually gradual with depigmented macules evolving into well-demarcated patches with convex or scalloped borders, in a symmetrical distribution in sites of predilection. These include regions which are normally slightly hyperpigmented, such as the face, groin, axillae, areolae and genitalia, or those prone to mechanical injury and repeated friction, such as the ankles, knees and elbows. Macules can have indistinct borders, and other morphological variants include trichrome vitiligo, where three shades in concentric zones are noted: normal, intermediate depigmentation and full depigmentation, and inflammatory vitiligo, where there is an erythematous, raised border to the lesion, with associated itching. Ordinary vitiligo can also present with pruritus. Mixed patterns, including segmental and non-segmental types can occur. A hyperpigmented rim can sometimes be seen at the outer margin of a vitiligo lesion, especially after sun exposure. A loss of hair pigment in affected areas is considered a late sign of vitiligo, with some prognostic significance. Therefore, it appears that epidermal melanocytes are lost before those in the hair bulb [6]. Non-segmental vitiligo is a slow and progressive disease, marked by occasional remissions and exacerbations, corresponding presumably to triggering factors. Full resolution of generalized vitiligo has never been reported, emphasizing the relentless chronicity of the condition. Depigmented areas are surprisingly tolerant of sun exposure and relatively resistant to photoaging and skin cancer. Up-regulation of wild-type p53 could be the reason [8]. The diagnosis is clinical, but occasionally, skin biopsy is required for confirmation: melanocytes and melanin are absent. Examination of the affected areas with Wood’s light can help distinguish active vitiligo from several disorders characterized by varying degrees of hypopigmentation by showing fluorescence owing to the presence of oxidized pteridines [9]. The degree of fluorescence depends on the skin phototype, and fluorescence is not seen in postinflammatory hypopigmentation. The differential diagnosis of vitiligo includes piebaldism, nevus depigmentosus, hypomelanosis of Ito, lichen sclerosus, pityriasis versicolor, idiopathic guttate hypomelanosis, progressive macular hypomelanosis, hypopigmented mycosis fungoides, indeterminate leprosy, pityriasis alba and many other forms of postinflammatory hypopigmentation, such as that following resolution of psoriasis. Particularly challenging can be the total depigmentation which can follow repeated excoriation of the skin from atopic dermatitis and psychogenic excoriations, and inflammation from connective tissue diseases like lupus erythematosus and systemic sclerosis. Depigmentation due to exposure to certain toxins can mimic vitiligo exactly and is termed chemical leukoderma. There can be shared pathomechanisms with ordinary vitiligo. Depigmentation around melanocytic nevi (halo nevi) can resemble vitiligo, and the two conditions can be associated [10]. A regressing melanoma can also demonstrate vitiligo-like depigmentation. An accurate history is often the only way to distinguish pseudovitiligo from the real entity. To improve the accuracy of diagnosis of vitiligo, and to monitor the response to treatment, the Vitiligo European Task Force has created an assessment tool, which incorporates a staging system [11]. The treatment of vitiligo encompasses preventative measures, camouflage makeup, medical and physical agents and surgical procedures. Prevention focuses on avoiding trauma, friction, sunburn and emotional stress. The friction from everyday activities like bathing and drying the skin has probably been overlooked. Tight clothing and sports apparel are also to be avoided when practical. Stable, inactive vitiligo, especially segmental vitiligo responds quite well to surgical repigmentation [12]. Evidence-based scrutiny supports treatment of vitiligo with UV light, especially narrow-band UVB, topical corticosteroids, topical calcineurin inhibitors and surgical techniques [13–15]. Repigmentation takes two main forms, follicular and diffuse, and is usually incomplete. In
the former, tiny islands of pigmentation, corresponding to hair follicles, appear in the patch and gradually increase in diameter and coalesce. In the latter, there is homogeneous repigmentation of the patch, starting with a pale hue and becoming darker. Certain sites, such as the hands and feet, are very resistant to therapy. Following successful treatment, relapse is very common at all sites. Very extensive vitiligo is sometimes best managed with depigmentation of remaining islands of normal skin. Psychological interventions should be offered to help patients with vitiligo cope with their disease. Patients can have significant co-morbidities like autoimmune thyroid disease, and ophthalmological and auditory effects from the loss of pigment have been reported [16]. The clinical features and management of vitiligo have been reviewed recently [17–20].

MELANIN

It is interesting to reflect that one's skin colour is not a 'permanent coat' but the result of regular replenishing or 'repainting' according to the turnover of the epidermis. Melanin pigments in the form of eumelanin (brown-black shades) and phaeomelanin (red-yellow shades) are transferred from melanocytes in the basal layer to associated keratinocytes via the so-called epidermal melanin unit. One melanocyte is in contact with approximately 36 surrounding keratinocytes [21]. Granules of melanin are transferred to keratinocytes via melanosomes in a process of phagocytosis. Because melanocytes represent only 8–10% of all epidermal cells [22], most of the skin's pigmented colour is determined by melanin in the keratinocytes, and only at localized collections of melanocytes, as occurs in melanozytic nevi or 'moles', is the colour due to the melanocytes themselves. Dark skin has a higher content of eumelanin, with larger melanosomes, and lighter skin has a higher proportion of phaeomelanin, with smaller melanosomes. Skin colour is not determined by the number or size of melanocytes. Melanin is thought to filter out UV radiation and scavenge ROS, thereby limiting UV damage to other cutaneous cells. The supranuclear melanin cap structure in keratinocytes presumably minimizes photodamage to the nucleus [23,24]. Melanocytes are also thought to play a role in the skin immune system, secreting a wide range of signal molecules and responding to growth factors and cytokines. Melanocytes can phagocytize and eliminate exogenous antigens, which have penetrated the skin barrier [25], and they can process and present antigen in the form of peptides with HLA (human leucocyte antigen) class II molecules to T-cells, triggering an adaptive immune response. Melanosomal proteins are involved in this antigen processing [26]. Activation of T-cells by melanocytes is shown by the secretion of co-stimulatory molecules like ICAM (intercellular adhesion molecule)-1 and LFA (leucocyte fusion-associated molecule)-3 [25,27]. When melanocytes die, migrate or stop functioning, skin reverts to its unpigmented form. This default colour is best exemplified in the single gene disorder of albinism, where melanin production is severely curtailed or completely absent. An acquired, patchy form of melanocyte failure occurs in vitiligo, with a fully depigmented area having the same colour seen in albinism. Onset is usually gradual, as several epidermal turnovers are required before all remaining pigment in keratinocytes is lost in the process of keratinization and desquamation. It seems reasonable to conclude that the skin colour disappears in a process of fading, taking several weeks to months. The specific dynamics of this process are difficult to study in vivo. Melanocytes in hair follicles seem to be more robust, so that normal hair colour often persists in the white patch [28]. Greying and eventual whitening of scalp and body hair in the condition of poliosis is not considered a form of vitiligo, but there may be some shared pathomechanisms; early greying of the hair can be seen in some vitiligo lesions [29]. New studies on the control of melanogenesis and constitutional skin colour point to roles for cytokines like SCF (stem cell factor), ET-1 (endothelin-1) and granulocyte macrophage colony stimulating factor [30].

VITILIGO AETIOPATHOGENESIS: HYPOTHESES

The subject of vitiligo aetiopathogenesis has been reviewed by several groups recently [31–35], but despite tremendous progress in molecular biology and genetics, there is still no universally accepted hypothesis. It could well be that vitiligo represents a 'syndrome' rather than one disease, with numerous different but not mutually exclusive pathways leading to melanocyte failure or disappearance. Genetic factors probably determine which particular pathway predominates in a specific patient. This was first proposed in the 'convergence’ theory of Le Poole et al. [36] in 1993 and later refined by Schallreuter et al. [34] in 2008. One proposed schema is as follows: increased endogenous or exogenous phenol/catechol concentrations around the melanocyte compete with tyrosine for tyrosinase binding sites in the melanocyte, generating aberrant substrates and curtailing melanin production. These aberrant substrates generate reactive quinones, especially in the presence of ROS and disturbed redox balance, which impair cell functions and bind covalently to the catalytic centre of tyrosinase, impairing or inactivating the enzyme and further reducing melanogenesis [31]. Similar detrimental effects from excessive ROS occur with other peptides and enzymes. Another proposal emphasizes the immunogenicity of melanocytes and melanosomal proteins, with aberrant T-cell attack.
resulting in melanocyte destruction by apoptosis. Vitiligo represents an autoimmune disease under this proposal [24]. Within these polar views of vitiligo pathogenesis, there is speculation that ROS and the immune system might somehow interact synergistically, so that both mechanisms might be relevant. Further hypotheses focus on neural dysregulation, hormones, cytokines, infections, calcium imbalance and melanocytorrhagy in the causation of vitiligo [37,38]. The molecular control of melanogenesis has been more accurately defined in the past decade: new hypotheses regarding vitiligo have emerged from this work, in particular, the role of SCF/KIT protein interactions, and the downstream effector, MITF-M (melanocyte-specific microphthalmia-associated transcription factor) [30,39,40].

VITILIGO GENETICS

The genetics of generalized vitiligo was reviewed by Spritz in 2008 [41]. Genes are thought to play a role in all aspects of vitiligo pathogenesis. There is a positive family history in about 20% of cases and similar concordance in identical twins. Vitiligo is considered a complex trait, involving the interaction of multiple genes with non-genetic factors, both environmental and host. At present, there is strong support for relatively few susceptibility genes, including certain HLA genes, PTPN22, NALP1 and perhaps CTLA-4 [HLA (cytotoxic T-lymphocyte) antigen-4]. These are all associated with a tendency to autoimmunity. HLA molecules present peptides to T-cells, and it has been proposed that certain HLA haplotypes confer more efficient presentation of cognate autoantigen, thereby predisposing to autoimmunity; an example is HLA-DQB1*0301 [28]. NALP1 is involved in the innate immune response to pathogens. Recent fine-mapping studies showed associations with chromosomes 7 and 9 [42]. Numerous other candidate genes and susceptibility loci bear ongoing scrutiny, including CAT, GST, COMT, ACE, mannose-binding lectin 2 and XBP1 [43–46]. A recent genome-wide association study by Jin et al. [47] using European white subjects and controls showed significant associations of generalized vitiligo with the following loci, which have been previously linked with autoimmune diseases: HLA class I and II molecules, PTPN22, LPP, IL2RA, UBASH3A and C1QTNF6. Two additional immune-related loci identified were RERE and GZMB. The HLA class I association occurred in the regions between HLA-A and HCG9, consistent with previous reports of strong associations with the HLA-A*02 allele, and the HLA class II gene association occurred in the region between HLA-DRB1 and HLA-DQAI, in keeping with known associations to the HLA-DRB1*04 allele. With the exception of PTPN22, the associations were similar whether patients had vitiligo alone or vitiligo as well as another autoimmune disease. An important association with a non-immune-related gene was identified: SNPs (single nucleotide polymorphisms) in the gene encoding tyrosinase, TYR. Tyrosinase is a melanocyte enzyme that catalyzes the rate-limiting step in melanin biosynthesis and is a putative target autoantigen in vitiligo. Interestingly, certain TYR SNPs are associated with melanoma risk, and some of these are in linkage disequilibrium with vitiligo TYR SNPs shown in this study. Vitiligo TYR SNPs could be more antigenic than melanoma TYR SNPs, thereby conferring protection from melanoma through immune surveillance [47,48].

MELANOCYTE LOSS

It is generally accepted that vitiligo results from loss of melanocytes rather than reduced functioning alone. This is supported by histological, ultrastructural and immunohistochemical techniques showing that vitiligo skin is devoid of melanocytes, and by the fact that melanocytes are almost never able to be cultured from affected skin. Whether melanocytes in vitiligo are eliminated by necrosis or apoptosis has been debated. Histological and ultrastructural studies support apoptosis by revealing nuclear shrinkage, vacuolization, loss of dendrites and detachment. Known cytotoxins like phenolic compounds, which can induce leukoderma similar to that seen in vitiligo, cause melanocyte loss with plasma membrane blebbing and DNA fragmentation suggestive of apoptosis; similar mechanisms might occur in vitiligo. Cytokine changes and immune reactions can initiate apoptosis too, and these are thought to be relevant in melanocyte loss in vitiligo [49].

ROS AND AUTOIMMUNITY

The potential role of oxygen free radicals in human autoimmune disease was reviewed by Ahsan et al. [50] in 2003. Free radicals are atoms or molecules which can exist independently despite having one or more unpaired electrons. This makes them more reactive than the corresponding non-radical forms. Free radicals derived from oxygen include four moieties: O$_2$•$^-$ (superoxide anion radical), $^1$O$_2$ (singlet oxygen), $^\cdot$OH (hydroxyl radical) and HO$_2$• (perhydroxyl radical). Together, these are termed ROS. ROS are routinely generated during many cellular biochemical and metabolic chemical reactions. Superoxide anion is thought to be the first free radical generated, primarily via the electron transport chain in mitochondria. Hydroxyl and perhydroxyl radicals are formed directly from superoxide. H$_2$O$_2$ is not as reactive as free radical-derived ROS, but it is an important oxidant, which can cross biological membranes and generate highly reactive hydroxyl radicals through an interaction with transition metal ions like Fe$^{2+}$ and

© The Authors Journal compilation © 2011 Biochemical Society
Cu$^+$ in the Fenton reaction. H$_2$O$_2$ can, therefore, be considered a marker of potential ROS. H$_2$O$_2$ is formed when superoxide anion undergoes a dismutation reaction catalysed by SOD (superoxide dismutase). Hydroxyl radical can also be generated from the interaction of superoxide anion radical and hydrogen peroxide in the Haber–Weiss reaction. Hydroxyl radical seems to be the most potent and damaging radical in biological systems, capable of interacting with all macromolecules like lipids, proteins, nucleic acids and carbohydrates. Polyunsaturated fatty acids are particularly susceptible to hydroxyl radicals, whereby removal of a hydrogen atom from the fatty acid starts the process of lipid peroxidation in a type of chain reaction. Aldehydes of lipid peroxidation can react with amino acids, altering their biological activity. Membrane structure and function are affected. Protein oxidation results in cross-linking, fragmentation and the addition of aldehyde groups, all affecting structure and function of the protein. Nucleic acids are damaged by single-strand breaks, cross-linking and changes to individual nucleotide bases. DNA is damaged by single-strand breaks, base modifications, conformational changes and DNA–protein cross-links. Thymine and guanine are more susceptible to damage than cytosine and adenine. The reaction product between superoxide anion and NO is OONO$^-$ (peroxynitrite), which is also a strong oxidant for proteins, lipids and nucleic acids, causing cell damage [51]. ROS can also be generated by exogenous stimuli like UV radiation and environmental chemicals. ROS have beneficial effects too, notably in the ‘respiratory burst’ of neutrophils during the phagocytosis of bacteria. Host cells have developed protective mechanisms to counter the deleterious effects of ROS and other free radicals in the form of free radical scavengers and antioxidant chemicals and reactions. An imbalance between production and removal of ROS leads to oxidant stress or redox imbalance, and this phenomenon has been implicated in numerous disease states as well as the process of normal aging. ROS can modulate the expression of a variety of immune and inflammatory molecules, leading to tissue damage. Aberrant immune reactions have been related to oxidative imbalance, and antioxidant functions have been linked to anti-inflammatory and immunosuppressive properties. If ROS-induced damage to cells is severe enough, programmed cell death or apoptosis occurs. Apoptotic debris can be highly immunogenic, and efficient removal by proteasomal degradation is necessary to prevent autoimmune reactions [52–56]. Antioxidant systems have evolved to control the redox balance. In the skin, these include antioxidant enzymes like catalase, thioredoxin reductase, glutathione peroxidase and superoxide dismutase, and small molecules like methionine, glutathione, vitamin C and vitamin E. Oxidatively damaged proteins are repaired by MSRs (methionine sulfoxide reductases) [57,58,122].

**ROS IN VITILIGO**

Human skin serves as an interface between the environment and the body. It is constantly exposed to a broad array of physical, chemical and biological agents, many of which are either inherent oxidants or catalyse the generation of ROS. ROS can denature proteins, alter apoptotic pathways, damage nuclear and mitochondrial DNA and mediate release of proinflammatory cytokines [59,60]. ROS are believed to be involved in the pathogenesis of inflammatory skin diseases, carcinogenesis, photoaging and hair greying [61,62]. Mitochondria are the most important endogenous source of ROS, but they are also a target of ROS-mediated damage. Thus, ROS can lead to mitochondrial dysfunction, reduced efficiency and more ROS in a vicious cycle of oxidant imbalance [59]. Several compelling lines of research have shown evidence of oxidative stress throughout the epidermis of patients with vitiligo, attributed to massive amounts of H$_2$O$_2$ in the 10$^{-3}$–10$^{-4}$ M range [63]. Impetus for this research came from the finding of low catalase levels in the epidermis of patients with vitiligo, first reported in 1991 [64]. Catalase removes H$_2$O$_2$ by converting it into water and oxygen. Generation of H$_2$O$_2$ is a physiological reaction in all cells via several metabolic pathways. There are also numerous exogenous direct and indirect sources of epidermal H$_2$O$_2$. While low concentrations, of the order of 10$^{-6}$ M, are necessary for cell signalling and transcription, high concentrations can have deleterious effects. Ultrastructural changes suggestive of lipid peroxidation have been demonstrated in melanocytes, keratinocytes and Langerhans cells in the skin of patients with vitiligo, both in affected and perilesional areas [65–68]. High levels of epidermal H$_2$O$_2$ have been demonstrated in vitro in vitiligo using FT (Fourier Transform) Raman spectroscopy [34,63]. This augmented previous findings of increased H$_2$O$_2$, which were in vitro, based on cell culture and skin biopsies [63]. FT Raman spectroscopy also revealed oxidation of l-tryptophan in epidermal albumin, and HPLC showed the presence of allantoin in the epidermis, confirming the presence of oxidative stress in vitiligo [69,70]. Oxidative destruction of polyunsaturated fatty acids of phospholipids is referred to as lipid peroxidation. It is one of the hallmarks of oxidative stress. MDA (malondialdehyde) is an end-product of lipid peroxidation, and elevated serum levels of MDA have been documented in patients with vitiligo [71–73].

**Sources of epidermal H$_2$O$_2$**

There are numerous sources of H$_2$O$_2$ in the normal epidermis. NADPH oxidase activity in neutrophils and macrophages generates H$_2$O$_2$ [74]. TNF-α (tumour necrosis factor-α) leads to the formation of H$_2$O$_2$ indirectly,
by inducing manganese superoxide dismutase [39]. Other cytokines have been reported to generate H$_2$O$_2$: TGF-β (transforming growth factor-β), EGF (epidermal growth factor) and PDGF (platelet-derived growth factor) [75]. Monoamine oxidase A activity in the epidermis generates H$_2$O$_2$ [76]. Nitric oxide synthases create H$_2$O$_2$ in an environment deficient in l-arginine [77]. XO (xanthine oxidase) catalyses the conversion of purine bases into uric acid, and generates H$_2$O$_2$ as a by-product [70]. Oxidation of aromatic phenols like 17β-oestradiol to catechols by an NADPH-dependent CYP (cytochrome P450) yields superoxide anion, which disproportionates to H$_2$O$_2$; this diffuses into the epidermis [78]. Photo-oxidation of epidermal 6-biopterin and sepiapterin yields H$_2$O$_2$ [80]. The enzymes tyrosine hydroxylase and phenylalanine hydroxylase also produce H$_2$O$_2$ as by-products. External phenols, ortho- and para-quinols, UVA and UVB, and X-rays also generate H$_2$O$_2$ in the mM range [63, 77]. Metabolism of xenobiotics, principally by CYP enzymes, generates ROS and H$_2$O$_2$, through toxic quinone and semiquinone intermediates [56].

Several of these epidermal sources of H$_2$O$_2$ are shown to be augmented in vitiligo, providing the presumed source for the elevated levels, which have been documented, both in affected and normal skin in patients with vitiligo (Table 1). Increased epidermal TNF-α levels have been shown [39]. Increased photo-oxidation of epidermal 6-biopterin and sepiapterin has been demonstrated [80]. Impaired recycling of the essential cofactor 6BH$_4$ [(6R)-l-erythro-5,6,7,8-tetrahydrobiopterin] by elevated H$_2$O$_2$ causes accumulation of H$_2$O$_2$ in the epidermis and affects all cofactor-dependent mechanisms. 6BH$_4$ is an essential electron donor in the hydroxylation of the aromatic amino acids, l-phenylalanine, l-tyrosine and l-tryptophan. These amino acids are substrates for melanogenesis, and thus, 6BH$_4$ is an essential component of the pigmentary system [79]. Inducible nitric oxide synthase levels in vitiligo epidermis are elevated, producing both H$_2$O$_2$ and peroxynitrite [8]. Homocysteine oxidation also causes elaboration of ROS, and elevated serum homocysteine levels have been reported in vitiligo patients [81]. Increased monoamine oxidase A activity has been found in the epidermis of patients with vitiligo, elaborating H$_2$O$_2$ [76]. Elevated SOD activity would seem a likely source of H$_2$O$_2$ in vitiligo, but results have been contradictory. Both normal and elevated serum and tissue SOD activity has been shown [71, 82–85]. XO catalyses the oxidative hydroxylation of hypoxanthine to xanthine, and xanthine to uric acid as part of purine degradation. These reactions generate H$_2$O$_2$, and XO is considered a major biological source of ROS, leading to oxidative stress in many organs. XO contains two non-haem iron atoms in its structure, which can react with H$_2$O$_2$ to produce OH$^•$, the most potent ROS with respect to DNA damage. Because H$_2$O$_2$ also oxidizes uric acid to allantoin, this metabolite is a useful marker of oxidative stress. XO activity has been shown in skin, and elevated plasma levels have been measured in patients with vitiligo. Recently, XO activity in melanocytes and keratinocytes was confirmed, with H$_2$O$_2$ regulating enzyme activity in a concentration-dependent fashion: low levels (10$^{-6}$ M) up-regulated activity, whereas high levels were suppressive. Oxidation by H$_2$O$_2$ of tryptophan and methionine residues in XO is thought to be the mechanism for this effect. Allantoin was detected in the epidermis of acute vitiligo but not in control skin, further supporting a role for ROS in vitiligo [70].

### Table 1 ROS in vitiligo: sources and effects

<table>
<thead>
<tr>
<th>Source/Effect</th>
<th>Level</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O$_2$</td>
<td>↑</td>
<td>[63]</td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>↑</td>
<td>[8]</td>
</tr>
<tr>
<td>NADPH oxidase</td>
<td>↑</td>
<td>[74]</td>
</tr>
<tr>
<td>TNF-α</td>
<td>↑</td>
<td>[39]</td>
</tr>
<tr>
<td>Oxidized pterins</td>
<td>↑</td>
<td>[80]</td>
</tr>
<tr>
<td>6BH$_4$ recycling</td>
<td>↓</td>
<td>[79, 80]</td>
</tr>
<tr>
<td>iNOS</td>
<td>↑</td>
<td>[8]</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>↑</td>
<td>[81]</td>
</tr>
<tr>
<td>Monoamine oxidase A</td>
<td>↑</td>
<td>[76]</td>
</tr>
<tr>
<td>SOD</td>
<td>n/↑</td>
<td>[71, 82–85]</td>
</tr>
<tr>
<td>Thioredoxin reductase</td>
<td>↓</td>
<td>[106]</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>↑</td>
<td>[70]</td>
</tr>
<tr>
<td>Catecholamines</td>
<td>↑</td>
<td>[33]</td>
</tr>
<tr>
<td>GTP-cyclodrolase I</td>
<td>↑</td>
<td>[98, 99]</td>
</tr>
<tr>
<td>Catalase</td>
<td>↓</td>
<td>[64, 85]</td>
</tr>
<tr>
<td>GSH-Px</td>
<td>n/↓</td>
<td>[73, 83–85]</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>↓</td>
<td>[72, 73]</td>
</tr>
</tbody>
</table>

#### Effects

- Tyrosinase: ↓ [121]
- TRP-1: ↓ [122]
- MSR: ↓ [57, 125]
- Catalase: ↓ [64, 85, 106]
- Thioredoxin reductase: ↓ [106]
- Tyrosine hydroxylase: ↓ [79, 80]
- POMC peptides: ↓ [109–111]
- L-Phenylalanine: ↑ [103]
- Acetylcholine: ↑ [106, 107]
- Calmodulin, furin: ↓ [103, 113, 114]
- Albumin (epidermal): ↓ [112]
- Malondialdehyde: ↑ [71–73]
- Methionine sulfoxide: ↑ [34, 57, 125]
- Allantoin: ↑ [70]

**Exogenous ROS**

The role of exogenous oxidants in vitiligo is highlighted by the conditions of chemical leukoderma and contact vitiligo, as shared mechanisms might elucidate trigger...
factors and reasons for progression and chronicity in idiopathic vitiligo. Chemical leukoderma refers to acquired depigmentation at sites of contact with certain chemicals; contact vitiligo starts in the same manner, but depigmentation then spreads to distant sites, in the same way as generalized idiopathic vitiligo. Depigmentation in both cases occurs from loss of melanocytes in the epidermis. Chemicals involved are mostly phenolic and catecholic derivatives, which resemble tyrosine and can occupy the catalytic centre of tyrosinase as surrogate substrates in the melanin synthesis pathway [86]. They include hydroquinone, MBEH (monobenzyl ether of hydroquinone) and 4-TBP (4-tertiary butyl phenol). These are oxidized by tyrosinase or tyrosinase-related protein to more reactive α-quinones, with the generation of ROS, which contribute to oxidative stress [87]. In the presence of excess H₂O₂, this process is accelerated. Melanin synthesis is reduced because the intermediate dopaquinone is not synthesized, and melanocyte viability can be compromised. 4-TBP, a potent cause of contact vitiligo, is cytotoxic to cultured human melanocytes in a dose-dependent manner, with melanocyte loss attributed to apoptosis. There is marked variation in individual susceptibility to chemical leukoderma and contact vitiligo, emphasizing the key role of genetic factors in determining melanocyte sensitivity to these stimuli. Highly reactive α-quinones can react with nucleophilic groups on proteins to create antigens and stimulate an immune response. Phenols in well-known contact allergens, like poison ivy, might also be oxidized by similar mechanisms to form antigens on keratinocytes. This could be the link between ROS and an altered immune response in vitiligo. Interestingly, the clinical picture of occupational contact vitiligo is similar to allergic contact dermatitis, with itching, redness and scaling. When MBEH is used to remove remaining pigment in patients with vitiligo universalis, a similar reaction is seen, but only in the pigmented areas, suggesting involvement of melanocytes rather than keratinocytes. Patients with established generalized idiopathic vitiligo can be exquisitely sensitive to exogenous phenols and catechols [38,88–91]. Generation of a reactive α-quinone from MBEH via tyrosinase was confirmed recently in vitro, with isolation of several by-products with potential relevance to melanocyte toxicity [92]. Cytotoxic experiments have also confirmed recently that both 4-TBP and MBEH induce melanocyte death, but by different pathways: 4-TBP activates the caspase cascade and causes DNA fragmentation with apoptosis, while MBEH induces release of Mobility Group Box-1 protein, which causes necrosis rather. This is confirmed by ultrastructural studies of MBEH-treated melanocytes [93]. Many drugs are potential exogenous sources of ROS, especially when metabolized by CYP enzymes, as they produce reactive quinones and semiquinones [56]. Interestingly, proton pump inhibitors were recently shown to reactivate vitiligo, and the mechanism might also be through generation of free radicals [94]; pH changes relating to melanogenic enzymes could also play a role [95].

**Endogenous ROS: sources and effects**

**Catechols**

Endogenous catechols are a source of ROS: elevated plasma and urine catecholamines like norepinephrine, epinephrine and dopamine and their metabolites have been documented in vitiligo patients [96]. Keratinocytes possess β-2 adrenoceptors and synthesize and degrade catecholamines, and melanocytes synthesize norepinephrine. Patients with vitiligo have markedly elevated GTP-cyclohydrolase I activity, which leads to excessive de novo production of 6BH₄, leading to increased synthesis of catecholamines in the epidermis [96–99]. Catecholamines also compete preferentially with tyrosine for tyrosinase active binding sites, becoming hydrolysed in the process and generating H₂O₂ [33]. Norepinephrine-induced vasoconstriction in vitiligo skin could cause hypoxia and predispose to oxidative stress by this mechanism [38]. These phenomena, among others, are the basis for the ‘neural’ theory of vitiligo aetiopathogenesis. Patients often report increased emotional stress prior to onset of vitiligo or concurrent with a flare of disease activity [100]. Estrogens and progesterone can generate H₂O₂ in vitiligo, contributing to quinone-mediated DNA damage in peripheral blood lymphocytes [78].

**BH₄ (tetrahydrobiopterin)**

BH₄ cofactor is essential for various enzyme activities and is present in probably all cells. Six and seven isoforms of BH₄ are synthesized de novo from GTP, but regeneration is crucial to adequate functioning, and requires two enzymes, pterin-4a-carbinolamine dehydratase and dihydropteridine reductase. The latter oxidizes both 6- and 7-BH₄ to 6- and 7-biopterin. This is the reason for fluorescence, which can be seen in vitiligo patches under Wood’s light. Thus, the homoeostasis of this important cofactor is compromised [79,101,102]. Some of the enzymes that depend on BH₄ are phenylalanine hydroxylase, tyrosine hydroxylase and tryptophan hydroxylase, as well as all nitric oxide synthase isofoms. Thus, BH₄ deficiency affects melanin, catecholamine, serotonin and NO synthesis.

1-phenylalanine levels would be expected to rise in this setting, and in fact, increased epidermal phenylalanine levels have been documented in patients with vitiligo by in vivo FT Raman spectroscopy [103]. Increased de novo synthesis of 6-BH₄ in vitiligo contributes to elevated norepinephrine levels and up-regulates monoamine oxidase A and catechol-O-methyl transferase in the
Epidermis. These result in increased epidermal H₂O₂ [74,104,105].

**Acetylcholine**

High epidermal levels of acetylcholine have been reported in vitiligo, and this is attributed to almost absent epidermal AchE (acetylcholinesterase) and BchE (butyrylcholinesterase) activities due to the effect of high levels of H₂O₂. While low levels of H₂O₂ (10⁻⁶ M) activate AchE, high concentrations (10⁻³ M) deactivate the enzyme. This regulation of enzyme activity by H₂O₂ is seen with several other enzymes. Molecular modelling of AchE suggests that the inhibition is due to H₂O₂-mediated oxidation of tryptophan and methionine residues in the protein, causing disorientation of the active-site histidine residue. The tetramerization domain and calcium-binding domains in BchE are also affected by high levels of H₂O₂ [106–108].

**POMC (pro-opiomelanocortin)**

Oxidative stress via H₂O₂ directly affects POMC peptides in the epidermis of patients with vitiligo. POMC peptides have a key role in melanogenesis. Prohormone convertases 1 and 2, which cleave POMC, are oxidized by H₂O₂, and POMC-derived peptides like ACTH, α-MSH (α-melanocyte-stimulating hormone) and β-endorphin are also altered directly by oxidation of their methionine residues. Reduced epidermal levels of α-MSH and β-endorphin have been demonstrated in vitiligo both in vitro and in vivo. These changes are reversed by the reduction of H₂O₂ levels with narrow-band UVB-activated pseudocatalase PC-KUS. Oxidation of α-MSH can also be ameliorated in the presence of abundant 6BH₄; this cofactor becomes deficient in the presence of abnormal levels of H₂O₂. Regulation of tyrosinase, the key enzyme for pigmentation, by α- and β-MSH via 6BH₄ and 7BH₄ has also been proposed, another link between ROS and dyspigmentation [109–111].

**Tryptophan**

Epidermal 1-tryptophan is oxidized by H₂O₂ in vitiligo, as shown in vivo by FT Raman spectroscopy. Albumin contains a tryptophan residue in its sequence, and oxidation could explain low levels of epidermal albumin, which have been reported in vitiligo. Several other amino acid residues in albumin, such as methionine, are also prone to oxidation. Some oxidation products of tryptophan like 5-OH-Trp are generated from H₂O₂ by Fenton chemistry. Albumin plays a key role in calcium homoeostasis, and reduced albumin levels might account, in part, for impaired calcium uptake, which has been described in vitiligo. H₂O₂ also affects all four calcium EF-hand-binding domains of calmodulin, and calmodulin-ATPase activity is low in vitiligo skin. The uptake of l-phenylalanine, which is defective in vitiligo, is also a calcium-dependent process. Epidermal furin is a calcium-dependent prohormone convertase, which plays a role in the cleavage of POMC. Loss of a calcium-binding site in furin because of oxidation from H₂O₂ has been shown in progressive vitiligo skin [103,112–114].

**Cytokines**

SCF, a paracrine cytokine produced by keratinocytes, has a major role in promoting melanogenesis and in melanocyte survival. SCF interacts with its receptor on melanocytes, KIT protein, produced by the c-kit gene. KIT protein interacts with MITF-M, which serves as a transcription factor regulating expression of tyrosinase mRNA. In response to SCF/KIT protein signalling, MITF-M is activated by phosphorylation through a MAP kinase, whereafter it is translocated to the nucleus to activate several genes. MITF-M also interacts with Bcl-2 to prevent apoptosis of the melanocyte [115]. ET-1 is also an important regulator of melanin production, via its ET₃R receptor [116]. SCF and ET-1 were not shown to be deficient in vitiligo lesions, suggesting that abnormal paracrine secretion by keratinocytes is not the cause of the hypopigmentation. At the edge of a vitiligo lesion, there are still melanocytes expressing tyrosinase, ET₃R and S100α, albeit at slightly lower levels than unaffected skin, but KIT protein and MITF-M are markedly reduced. Melanocytes expressing tyrosinase, S100α, ET₃R, KIT protein and MITF-M protein were absent at the centre of the vitiligo lesion. This suggests that reduced expression of KIT protein, and its downstream effectors like MITF-M, could explain melanocyte loss and/or dysfunction in vitiligo [30]. It is interesting that oxidative stress like excessive H₂O₂ leads to down-regulation of MITF-M expression in cultured human melanocytes. Thus, ROS could be the cause of the cytokine abnormality seen in vitiligo [117].

**Trauma**

Trauma to the skin, UV radiation and other sources of inflammation probably contribute to the epidermal pool of H₂O₂ in a non-specific manner via NADPH oxidase stimulation. This could explain the prominent Koebner phenomenon seen in vitiligo, especially in active stages of the disease [118,119]. In particular, UVB induces generation of free radicals, which damage DNA. This can be ameliorated by vitamin D, which up-regulates expression of metallothionein, a free radical scavenger protein with photoprotective properties [120].

**Tyrosinase**

High levels of H₂O₂ (0.5–5.0 × 10⁻³ M) have been shown to deactivate tyrosinase, and this effect is compounded by increased 6BH₄ [121]. Recent work on the mechanism of senile hair greying has provided a possible explanation for the inhibition of tyrosinase by H₂O₂. It was shown that methionine 374, at the enzyme active site, is oxidized by H₂O₂, disrupting enzyme activity, especially when
methionine sulfoxide repair by MSRA and MSRB is also affected by the same ROS. A similar scenario evidently occurs in vitiligo [62]. Abnormal expression of TRP-1 (tyrosinase-related protein-1) has been reported following oxidative stress to cultured melanocytes from the advancing border of vitiligo lesions. This leads to early cell death, possibly through an interaction with calnexin [122].

**Peroxynitrite**

Recently, significantly elevated levels of 8-oxoguanine were reported in plasma and skin of patients with vitiligo, an indication of DNA damage. Together with this, there was up-regulation of epidermal wild-type p53 and enhanced short-patch base-excision repair. In addition, high epidermal levels of iNOS (inducible nitric oxide synthase) were demonstrated, with corresponding elevations of 3-nitrotyrosine and nitrated p53. This implies increased epidermal ONOO−, a reactive nitrogen species, which can be added to the list of radicals involved in the pathogenesis of vitiligo. H2O2 was shown to enhance the DNA binding capacity of p53, while ONOO− completely inhibited this binding. Interestingly, H2O2 at a concentration of 10−3 M abolished this deleterious effect of ONOO−. Thus, H2O2 appears to be protective in the sense of improving DNA repair via enhanced p53. This could partly explain the relative absence of photoaging and skin cancer in chronic lesions of vitiligo [8].

**Antioxidants**

Elevated levels of H2O2 and other oxidants would ordinarily be countered by antioxidant defenses and free radical scavengers. However, several of these processes are actually inhibited by H2O2 itself. H2O2 in the mM range deactivates catalase by oxidizing its porphyrin ring and methionine and tryptophan residues in the structure of the enzyme-active site and the cofactor NADPH-binding site. This explains why catalase levels are low in vitiligo, despite unchanged mRNA expression for the enzyme [106]. Low tissue levels of catalase in patients with vitiligo were recently confirmed, with lower levels in active rather than stable disease [85,123]. Several heterogeneous SNPs in the catalase gene, observed in vitiligo patients, might render these catalase variants particularly susceptible to deactivation by H2O2, thereby predisposing to vitiligo [43,124]. Another antioxidant enzyme system, thioredoxin reductase, is impaired by high H2O2 levels through oxidation of the enzyme-active site and the NADPH cofactor-binding site. Low activities of this enzyme have been shown in vitiligo [99]. Oxidation of MSRA and MSRB by H2O2 leads to reduced functioning of this important protein-repair mechanism. Low levels of MSR have been shown in vitiligo. Furthermore, in vitro down-regulation of MSRA in normal cultured melanocytes rendered them very susceptible to oxidative stress [57,125].

GSH-Px (glutathione peroxidase) converts H2O2 and other peroxides into water and oxygen, utilizing glutathione, glutathione reductase and NADPH. Selenium is a cofactor for some isozymes of GSH-Px. Variable levels of GSH-Px and selenium have been found in tissue and blood of patients with active vitiligo [73,83–85,126–128]. GST (glutathione transferase) is a superfamily of broadly expressed isoenzymes involved in defence against oxidative stress. Polymorphisms in the GST gene have been identified, which confer risk of vitiligo [45]. Vitamin E levels were reported as low in vitiligo patients, who also showed elevated blood levels of MDA, as evidence of lipid peroxidation [72,73]. Total blood antioxidant status using the Randox kit was increased in vitiligo patients in one study [128].

Melatonin has a strong antioxidant effect, and an aberration of melatonin receptors was once proposed as a mechanism for the development of vitiligo [129]. Melatonin is synthesized in the skin yielding potent free radical scavengers like Nα-acetyl-Nω-formyl-5-methoxykynuramine, which also stabilize the mitochondrial electron transport chain [130].

Antioxidants react in tandem with proteins like Bcl-2 and caspas to regulate apoptosis, which can result from oxidative challenge [131]. For example, Bcl-2 expression prevents cell death from H2O2 and menadione [132]. Bcl-2 is expressed by melanocytes and keratinocytes and is crucial to their survival. Melanocytes from individuals with vitiligo have lower levels of Bcl-2 than normal, presumably contributing to melanocyte fragility [91]. Elevated levels of Bcl-2 have also been reported in vitiligo [8], as well as normal levels [133], and the role of melanocyte apoptosis in vitiligo is still unclear.

Further proof of the role of H2O2 and ROS requires evidence of improvement in lesions with antioxidant therapies. Topical pseudocatalase (PC-KUS) is a narrow-band UVB-activated bis-MnIII(EDTA)2−(HCO3−)2 complex that is applied to the entire skin as a lotion, followed by low-dose UVB exposure. It functions as a synthetic catalyst to oxidize H2O2 into O2 and H2O, thus mimicking natural catalase. This regimen causes rapid repigmentation of even long-standing vitiligo [134,135]. Consequent with this is a marked reduction in epidermal H2O2 as measured in vivo with FT Raman spectroscopy. Bathing in Dead Sea water also had a pseudocatalase effect, though milder, probably from its high content of transition metals like manganese [135]. A randomized controlled trial comparing narrow-band UVB with or without a pseudocatalase cream did not confirm additional benefit from the cream, but the methodology was different, and the pseudocatalase was not the same as PC-KUS [136]. A catalase of vegetable origin, combined with SOD in a microsphere formulation, showed equivalent efficacy to topical betamethasone,
when combined with sunlight exposure in the treatment of vitiligo [137]. Oral vitamin E appeared to confer some additional benefit to narrow-band UVB therapy of vitiligo, with a corresponding decrease in plasma MDA in the vitamin E group versus the control [138]. ROS/H$_2$O$_2$ sources, effects and interactions in vitiligo are summarized in Figure 1.

**VITILIGO AND AUTOIMMUNITY**

Generalized vitiligo is widely considered an autoimmune disease, with involvement of humoral and cellular components of the innate and adaptive immune system. The belief is supported by the following lines of epidemiological, clinical and investigational research: an association with other autoimmune disorders; chronic relapsing and remitting course so typical of autoimmune disorders; possible response to immunosuppressive therapies like UV phototherapy, topical and oral corticosteroids, and topical calcineurin inhibitors; circulating anti-melanocyte antibodies; T-cell infiltrates in perilesional skin; anti-melanocyte cytotoxic T-cells in the skin and circulation and proinflammatory cytokine patterns of a Th-1 type response. Autoimmunity might be the triggering event in vitiligo, but it could function instead as a promoter of disease progression and chronicity [26,139].

Autoimmune conditions associated with vitiligo include autoimmune polyendocrine syndrome types 1 and 2, pernicious anaemia, Type 1 diabetes, Addison’s disease, Graves’ disease, alopecia areata, systemic lupus erythematosus, rheumatoid arthritis, psoriasis and myasthenia gravis. A recent survey of 2600 vitiligo patients showed increased frequencies of autoimmune thyroid disease, Addison’s disease, systemic lupus erythematosus and pernicious anaemia, with about 30% of patients having at least one of these disorders. In addition, family members who did not have vitiligo still had a tendency to the same autoimmune conditions, pointing to a genetic risk for a specific cluster of autoimmune diseases. Other studies report true associations only with thyroid dysfunction and thyroid antibodies, regarding the other conditions as random concomitant events. Psoriasis or lichen planus occurring in vitiligo lesions has also been reported, with a shared pathogenesis postulated. Organ-specific autoantibodies are recorded with increased frequency in vitiligo patients, often in the absence of clinical symptoms. There is probably an increased risk of developing clinical or subclinical disease later [139–142]. Several immunogenetic factors predispose patients to autoimmune diseases, and some of these are associated with vitiligo, adding to the evidence that vitiligo may have an autoimmune basis. Various HLA class II alleles have been associated with vitiligo, in particular, HLA-DR4 [143]. The particular haplotype association varies according to ethnic origin. Genes involved in antigen presentation and processing have been associated with autoimmune diseases and in some cases with vitiligo. These include LMP-1 and -7 (low-molecular-mass polypeptide-2 and -7) and TAP-1 and -2 (transporter associated with antigen processing protein-1 and -2) [144]. Homozygous or heterozygous complement
2 and 4 deficiency is associated with autoimmunity, and this has been described in vitiligo [145]. The CTLA-4 gene product down-regulates T-cell activation and controls T-cell apoptosis. Certain CTLA-4 polymorphisms predispose to vitiligo in patients who already have other autoimmune conditions [146]. Autoimmune polyendocrine syndrome type 1, which often includes vitiligo, is due to mutations in the autoimmune regulator gene, AIRE [147]. A missense mutation in the PTPN22 gene, which encodes LYP (lymphoid protein tyrosine phosphatase), has been linked to several autoimmune diseases including vitiligo [148]. Loci on chromosomes 1, 7 and 8 have been linked with autoimmune polyendocrine syndrome type 1, which often includes vitiligo [146]. Autoimmune diseases predispose to vitiligo in patients who already have other autoimmune conditions [146]. Autoimmune diseases predispose to vitiligo in patients who already have other autoimmune conditions [146]. Autoimmune diseases predispose to vitiligo in patients who already have other autoimmune conditions [146]. Autoimmune diseases predispose to vitiligo in patients who already have other autoimmune conditions [146]. Autoimmune diseases predispose to vitiligo in patients who already have other autoimmune conditions [146]. Autoimmune diseases predispose to vitiligo in patients who already have other autoimmune conditions [146].

T-cells in vitiligo

Animal models of vitiligo show prominent roles for anti-melanocyte antibodies. Some of these cross-react with mammalian TRP-1 [150]. Antibodies to melanocytes have been found in the circulation of patients with vitiligo [151]. These antibodies correlate with disease activity and extent [152]. Targets of these antibodies include a variety of melanocyte and melanosomal antigens. The frequency of each antibody in the vitiligo population is relatively low, and a dominant antigen has not been found. Antibodies might trigger vitiligo as a primary event, but they could also arise secondary to melanocyte damage or serve to perpetuate the disease. Whatever their role in vitiligo, these antibodies have the capacity to injure pigment cells in vivo and in vitro. Even if they are not pathogenic, study of melanocyte antibodies and target antigens might refine the diagnostic and prognostic testing of vitiligo, reveal putative T-cell targets and add to the therapeutic armamentarium [153]. Circulating anti-parietal cell, thyroid and adrenal antibodies have been detected in vitiligo patients, as well as antinuclear antibodies and rheumatoid factor, again suggesting an autoimmune pathomechanism for the disease [151]. Autoimmune diseases are the result of complex interactions between T and B cell subpopulations. A recent flow cytometric study of these cells in vitiligo did not show a pathological distribution of B cells, suggesting that T-cells might have a more dominant role. Circulating immune complexes were not elevated either [154]. Cases of generalized vitiligo in recipients of bone marrow transplants from donors with vitiligo have been reported, illustrating the role of bone marrow-derived cells in disease pathogenesis [155].

Table 2 T-cell changes in vitiligo

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral [156–163]</td>
<td></td>
</tr>
<tr>
<td>Total T-cell</td>
<td>n/↓</td>
</tr>
<tr>
<td>CD4+CD8+</td>
<td>n/↓/↑</td>
</tr>
<tr>
<td>CD45RA+</td>
<td>↓</td>
</tr>
<tr>
<td>CD45RO+</td>
<td>↑</td>
</tr>
<tr>
<td>Total NK T-cell</td>
<td>↓</td>
</tr>
<tr>
<td>Tissue [179–193]</td>
<td></td>
</tr>
<tr>
<td>Total T-cell</td>
<td>↑</td>
</tr>
<tr>
<td>CD8+</td>
<td>↑</td>
</tr>
<tr>
<td>CD45RO+</td>
<td>↑</td>
</tr>
<tr>
<td>IL-2 receptor</td>
<td>↑</td>
</tr>
<tr>
<td>CLA+</td>
<td>↑</td>
</tr>
<tr>
<td>Cytokine</td>
<td></td>
</tr>
<tr>
<td>Peripheral [164–174]</td>
<td></td>
</tr>
<tr>
<td>IL-1</td>
<td>↑</td>
</tr>
<tr>
<td>IL-6</td>
<td>↑</td>
</tr>
<tr>
<td>IL-8</td>
<td>n/↑</td>
</tr>
<tr>
<td>TNF-α</td>
<td>n/↑/↓</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>↓</td>
</tr>
<tr>
<td>TGF-β</td>
<td>↓</td>
</tr>
<tr>
<td>IL-2 receptor</td>
<td>↑/↓</td>
</tr>
<tr>
<td>TNF receptor</td>
<td>n</td>
</tr>
<tr>
<td>Tissue [165–174]</td>
<td></td>
</tr>
<tr>
<td>IL-2 receptor</td>
<td>↑</td>
</tr>
<tr>
<td>IL-6</td>
<td>↑</td>
</tr>
<tr>
<td>TNF-α</td>
<td>↑</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>↑</td>
</tr>
<tr>
<td>IL-10</td>
<td>↑</td>
</tr>
</tbody>
</table>

STUDIES FOUND NORMAL TOTAL LEVELS OF T CELLS BUT A DECREASED CD4+/CD8+ RATIO AND NORMAL OR INCREASED TOTAL NK CELLS. THERE WAS NO PARTICULAR CORRELATION NOTED WITH DISEASE ACTIVITY [156–160]. Abdel-Naser et al. [161] found normal levels of total T-cells, CD4+ T-cells and total NK cells, but a decreased proportion of CD45RA+ (naïve CD4+) T-cells and increased HLA-DR expression, suggesting the presence of activated T-cells. Mahmoud et al. [162] found decreased total T-cells, CD45RA+ cells and NK T-cells, with increased CD45RO+ (memory phenotype) T-cells in severe disease. Basak et al. [163] found normal total T-cells with a decreased CD4+/CD8+ ratio, as well as increased monocytes, CD45RO+ T-cells and total NK cells. Total T-cells were relatively higher in generalized than in acral/larrafacial vitiligo, and CD45RO+ T-cells were higher in acral/larrafacial disease. Unlike former studies, Pichler et al. [154] found an increased CD4+/CD8+ ratio in peripheral blood, but noted that the ratio in tissues could be different.

T CELLS IN VITILIGO

Peripheral blood

Lymphocyte analysis in peripheral blood of patients with vitiligo has yielded variable results (Table 2). Earlier
Cytokines

Cytokine studies of peripheral blood and skin in patients with vitiligo have also yielded variable results, but with a trend to proinflammatory T-cell patterns. Earlier work showed increased IL (interleukin)-6 and IL-8 but decreased TNF-α and IFN (interferon)-γ in serum, with elevated soluble IL-2 receptor in blood and tissue [164,165]. Moretti et al. [166] found increased IL-6, TNF-α and minimal TGF-β in tissue, and Tu et al. [167] found increased IL-6 but normal IL-1β, IL-8 and TNF-α in blood. Franczuk et al. [168] reported decreased soluble IL-2 receptor in blood. Grimes et al. [169] noted increased tissue TNF-α, IFN-γ and IL-10, and Zailaie [170] detected increased IL-1, IL-6, IL-8 and TNF-α in blood, while Birol et al. [171] found elevated tissue TNF-α but normal blood levels of this cytokine. It was recently noted that imiquimod often causes vitiligo-like depigmentation when used to treat superficial basal cell carcinoma. Imiquimod binds Toll-like receptors 7 and 8 and evokes a Th-1 response with the production of IFN-α, TNF-α and IL-12. Imiquimod also causes increased IL-6, IL-8 and IL-10. Similar cytokines might be involved in vitiligo [172]. Taher et al. [173] noted that topical tacrolimus, used successfully to treat vitiligo, increases tissue IL-10, which is an immunosuppressive Th-2 cytokine. This suggests that vitiligo might be a Th-1 type of autoimmune disease. Pichler et al. [154] recently found normal blood levels of TNF receptor with slightly elevated IL-6, while Basak et al. [174] reported significantly decreased serum levels of TGF-β in vitiligo, with potential inhibition of regulatory T-cell function.

Infiltrates

Earlier histopathological studies of vitiligo focused on degenerative changes in melanocytes and keratinocytes, particularly at the perilesional location. Vacular alteration of the basal layer was noted, with scattered necrotic keratinocytes reminiscent of a lichenoid reaction. Electron microscopy showed intracellular oedema, cytoplasmic vacuolation and dilation of organelles within degenerating melanocytes and keratinocytes in normal-appearing skin adjacent to amelanotic skin. In some cases, degeneration was limited to melanocytes, sparing keratinocytes. Little attention was paid to mononuclear cell infiltrates, but these were, in fact, noted, being in a perivascular distribution and also within the epidermis. The infiltrate was considered to comprise lymphocytes and histiocytes, and while generally of a mild intensity, denser infiltrates were observed in vitiligo of more recent onset and in active rather than stable disease [66,175–178]. Lichenoid-type inflammation implies a T-cell-mediated autoimmune reaction [179], and in fact, many of the mononuclear cells in the perilesional infiltrate of ordinary generalized vitiligo were shown to be T-cells by immunoperoxidase techniques. CD8+ T-cells predominated in the epidermis, while in the dermis, there was a normal ratio of CD4+/CD8+ T-cells [180]. Activation of cell-mediated immunity was also suggested by the early finding of abnormal expression of HLA class II molecules and ICAM-1 on perilesional melanocytes in cases of vitiligo [181]. Highly significant overall increases in CD3+, CD4+ and CD8+ T-cells were also noted by Badri et al. [182] in the margins of depigmented skin. Many of the T-cells also expressed HLA class II molecules and IFN-γ, CD45RO+ memory phenotype predominated, with many T-cells expressing the skin-homing marker CLA (cutaneous lymphocyte-associated antigen), which binds to E-selectin on dermal endothelial cells [182]. Mononuclear infiltrates in perilesional vitiligo skin were noted to be IL-2 receptor- and IFN-γ-positive, suggesting the presence of activated T-cells [178]. CLA+ T-cells clustered around disappearing melanocytes were described by van den Wijngaard et al. [183]: most of the T-cells were CD8+, and those interacting with melanocytes were perforin and granzyme B-positive, suggesting a cytotoxic phenotype. There was focal increased expression of HLA-DR and ICAM-1 in the epidermis at sites of interaction with T-cells [183]. Melanocytes are sensitive to the granule exocytosis pathway of apoptosis (perforin/granzyme) but resistant to the Fas ligand-mediated pathway [184]. Melanocyte death in vitiligo has generally been attributed to apoptosis rather than necrosis, and cytokines like IL-1, IFN-γ or TNF-α, released by lymphocytes, keratinocytes and melanocytes, can initiate apoptosis of both melanocytes and keratinocytes. Fas/Fas ligand interactions may also be involved in the apoptosis of keratinocytes in vitiligo [185,186].

Cellular infiltrates were particularly noted in rare cases of ‘inflammatory’ vitiligo, characterized by elevated, reddish, scaly borders. Histopathological analysis of the border often showed a lichenoid pattern of lymphocytic infiltration, even a few centimeters away towards normal skin [187,188]. Lymphocytes were often juxtaposed to remaining melanocytes, suggesting a role in their destruction. Dense perivascular infiltrates were noted in the upper dermis, with exocytosis of lymphocytes into the epidermis. Vacular degeneration of the basal layer, with necrotic keratinocytes and colloid bodies were noted. Immunophenotyping of inflammatory vitiligo also confirmed the presence of many T-cells, particularly within the epidermis. Epidermal T-cells were either CD8+ or CD4+, while dermal T-cells were mainly CD4+. T-cells showed a memory subtype, being CD45RO+. Keratinocytes from the inflamed border were HLA DR+, and ICAM-1 staining was increased in one study. T-cells showed increased IL-2 receptor expression, suggesting activation, and many T-cells were CLA+ [189,190]. Thus, both ordinary and inflammatory vitiligo had similar patterns of T-cell infiltration.

In vitro characteristics of infiltrating T-cells in ordinary vitiligo were reported for the first time by
Wankowicz-Kalinska et al. [191] in 2003: biopsies of perilesional skin in four patients with active and one with stable vitiligo confirmed the presence of T-cell infiltrates, mainly CD8+ T-cells, but with many CD4+ T-cells as well. These were often noted in apposition to residual melanocytes. Normal skin usually shows an equal distribution of CD4+ to CD8+ subsets, mostly in a dermal perivascular location. The predominance of CD8+ over CD4+ T-cells in vitiligo, as well as their proximity to melanocytes, could represent the effector phase of an anti-melanocyte response, as seen in the Smyth chicken model of vitiligo [192]. CD8+ T-cells are known to infiltrate organs like the pancreas and thyroid gland in other autoimmune disorders like diabetes mellitus and Hashimoto’s thyroiditis, respectively [193,194]. A novel finding by Wankowicz-Kalinska et al. [191] in the study was the presence of T-cell infiltrates in normal-appearing skin remote from the site of vitiligo, with microscopic loss of melanocytes termed ‘microdepigmentation’. These could well represent sites of future vitiligo. T-cells were generated by mitogenic expansion and tested for cytokine elaboration and antigen specificity. Phenotypes generated were similar to those observed in situ. In all four patients with active vitiligo, a high proportion of both CD8+ and CD4+ cells produced IFN-γ and TNF-α, with relatively little IL-4, IL-5 and IL-13. This pattern was not seen in T-cells derived from normal skin in these patients. Thus, the T-cells in vitiligo are polarized to a Th-1-like profile. TNF-α, and IFN-γ, might act synergistically to up-regulate HLA Class II molecules and ICAM-1, as noted previously [191]. Melanocytes can process and present antigenic peptides to antigen-specific CD4+ cells after pretreatment with IFN-γ; therefore, elevated IFN-γ and TNF-α could predispose melanocytes to immune surveillance [195]. The patient with stable vitiligo had a different cytokine profile, more Th-2-like. It is interesting that multiple sclerosis shows a Th-1 type response in active disease and a Th-2-dominant response in quiescent stages; perhaps a similar phenomenon occurs in vitiligo [196]. Several CD8+ T-cell clones were able to lyse autologous cultured melanocytes in an HLA-restricted manner, the first time that T-cells from tissue rather than blood of patients with vitiligo have shown this effect. Blocking with anti-HLA class I antibody prevented the lysis, confirming a specific, HLA-restricted CD8+ cytotoxic T-cell response rather than an NK-like response [191].

**Antigen-specific T-cells**

The study of antigen-specific T-cells in vitiligo benefited from work on the cell-mediated immune response to melanoma, shared melanocyte differentiation antigens with melanoma and a simpler technique to detect antigen-specific T-cells, known as soluble tetramer staining. Vitiligo is more frequent in patients with metastatic melanoma than the general population [197–200] and is associated with increased survival. Vitiligo-like depigmentation has been reported after successful immunotherapy of melanoma, including high dose IL-2 therapy, infusions of peptide-pulsed DCs (dendritic cells) and Melan-A/MART-1-specific cytotoxic T-cell clones [201–206]. During adoptive transfer therapy, infused melanocyte-specific T-cells accumulated in the periphery of sites of incipient depigmentation. Depigmentation was not observed in non-responding patients or those with unrelated malignancy [203]. Cytotoxic T-cells generated from melanoma recognize melanocyte differentiation antigens expressed by normal melanocytes as well [203,207,208]. Clonally expanded T-cells with identical T-cell receptor Vβ regions were simultaneously demonstrated in the depigmented halo around a regressing melanoma and within the melanoma [209]. The possibility that melanocyte differentiation antigens represent the targets of cell-mediated autoimmunity in vitiligo has been investigated recently. These antigens include melanosomal proteins like Melan-A/MART-1, tyrosinase and gp100, involved in the synthesis of melanin [197]. High numbers of Melan-A-specific cytotoxic T-cells have been observed in the peripheral blood of melanoma patients with concurrent vitiligo, but not in those without vitiligo [210]. Regression of metastatic melanoma and the simultaneous development of vitiligo have occurred after immunization with Melan-A peptide, associated with oligoclonal expansion of Melan-A-reactive cytotoxic T-cells [211]. Utilizing the technique described by Altman et al. [212], multimeric soluble complexes of known peptides with HLA-A2 molecules could be used to identify peptide–antigen-specific T-cells in HLA matched hosts. These peptide–HLA-A2 complexes bind more than one T-cell receptor on a specific T-cell, allowing a slower dissociation rate and enabling successful immunological staining [212]. Utilizing this technique, Ogg et al. [213] showed for the first time a high frequency of Melan-A-specific CD8+ T-cells in the circulation of HLA-A2+ patients with vitiligo. These cells were able to lyse A2-matched melanoma cells in vitro. They were not observed in A2-negative vitiligo patients or in A2-positive control patients who did not have vitiligo. The T-cells also expressed high levels of skin-homing CLA [213]. Lang et al. [214] confirmed these findings, with levels of Melan-A-specific CD8+ T-cells correlating with disease activity. Four peptides from Melan-A were used. Gp100 and tyrosinase-specific CD8+ T-cells were also demonstrated, using five peptides from gp100 and two from tyrosinase. Intracellular IFN-γ was elevated in the CD8+ T-cells, confirming the active state shown by the enzyme-linked immunospot technique [214]. Wankowicz-Kalinska et al. [191] also showed Melan-A specificity of cloned T-cells from perilesional skin by the tetramer technique, revealing a high proportion of Melan-A-specific CD8+ T-cells in two of five HLA-A2.01+ patients with vitiligo.
Interestingly, these two patients failed to show similar CD8+ cells in the peripheral circulation, emphasizing the importance of testing both peripheral and tissue sites, if possible [191]. Mandelcorn-Monson et al. [215] in an ex vivo study utilized peripheral blood of patients with vitiligo to isolate antigen-specific CD8+ T-cells, without additional in vitro culture, and revealed a high proportion of gp100-positive CD8+ T-cells, unlike the other studies. Two epitopes of gp100 were used to increase sensitivity. Utilizing recognized epitopes from Melan-A and tyrosinase, specific T-cells were not isolated. One patient showed Melan-A and tyrosinase specificity after in vitro re-stimulation [215]. Palermo et al. [216] utilized the tetramer technique with peripheral blood of patients with vitiligo or melanoma and showed the presence of Melan-A-specific cytotoxic T-cells at similar levels in both HLA-A2-matched groups. However, only vitiligo cells showed T-cell-receptor down-regulation levels in both HLA-A2-matched groups. However, only vitiligo cells showed T-cell-receptor down-regulation and IFN-γ production after exposure to HLA-matched melanoma cells, suggesting a qualitative difference in receptor affinity in the two groups. Vitiligo has been considered the effective variant of melanoma immunity [216,217]. Van den Boorn et al. [218] expanded on all these findings in 2009: their study showed a prominent role for T-cells in vitiligo pathogenesis, being causative rather than just a consequence of other stimuli. Perilesional T-cells were easily identified in nine HLA-A2+ patients with progressive vitiligo, cultured and subjected to flow cytometric analysis using HLA-A2/peptide tetramers for tyrosinase-(369–377), gp100-(280–288), gp100-(292–217) and MART-1-(26–35). A control antigen in the form of influenza virus-(58–66) was employed. Compared with healthy donor skin-residing cells, significantly increased levels of T-cells recognizing all the melanocyte antigens were found in the perilesional T-cell population. Similar proportions of these T-cells were also found in the peripheral blood, showing that the presence of melanocyte antigen-specific T-cells in the skin usually coincides with similar increased levels of these cells in the blood. Functional activation of cultured perilesional T-cells upon exposure to melanocyte differentiation antigens was tested for the first time. T-cells were stimulated with the pool of HLA-A2-binding peptides in five patients and three controls. In order to exclude bias from prolonged in vitro culture, only those samples that grew sufficient cells within 14 days were analysed. Activation of T-cells was measured by CD-69 expression for early T-cell activation, CD137 for CD8+ T-cell activation and CD154 for CD4+ T-cell activation. Cytolytic action was measured by granzyme-B production and cytoxic degranulation by CD107a expression. IL-4, a humoral response promoter, and the proinflammatory cytokines IL-17, TNF-α and IFN-γ were also measured. Results showed that after melanocyte antigen exposure, perilesional CD8+ T-cells became activated (CD69+, CD137+, granzyme-B+ and CD107a+); they were not activated by influenza controls. The magnitude of the CD8+ T-cell response was proportional to the extent of vitiligo or the presence of poliosis. Perilesional CD4+ T-cells showed bystander activation, with CD69 and CD154 expressed but not granzyme-B. This demonstrates an antigen-independent activation of these CD4+ T-cells in this HLA class-I-restricted stimulation setup. There was a variable increase in TNF-α and IFN-γ by both perilesional CD4+ and CD8+ T-cells. One patient, who also had halo nevi, demonstrated elevated IL-17. Donor control skin-residing T-cells did not show a cytotoxic response to the melanocyte antigens. Therefore, perilesional T-cells in vitiligo are primed to react against melanocyte antigens unlike healthy skin-residing T-cells. To examine the functional capacity of these T-cells to kill melanocytes, a skin explant model, originally developed to predict GVHD (graft versus host disease) in bone marrow transplantation, was used. Perilesional T-cells were co-cultured for 2 days with non-lesional, normally pigmented autologous skin; CD8+ -enriched and -depleted populations were also tested. Explants were subsequently analysed for the presence of infiltrated T-cells, melanocytes and apoptosis. Results in all cases showed infiltration of explants, with induction of apoptosis of melanocytes as indicated by cytoplasmic active caspase-3 staining. Other epidermal cells in the suprabasal layers, probably keratinocytes, also underwent apoptosis. CD8+ -depleted samples showed infiltration but minimal apoptosis, while CD8+ enriched samples had the most apoptosis of melanocytes and keratinocytes, corresponding to sites of epidermal T-cell infiltration. Keratinocyte apoptosis did not occur in lesional skin devoid of melanocytes, and is thus considered a bystander effect from proinflammatory cytokines rather than a specific cytotoxic effect of T-cells on keratinocytes. Finally, the same explant experiment was performed, but utilizing purified melanocyte antigen-specific T-cell populations. Gp100-(280–288)-specific CTLs were co-cultured with autologous skin and demonstrated infiltration of the explant, with extensive disruption of the skin tissue. CLSM (confocal laser scanning microscopy) showed CTLs infiltrating the dermis and epidermis, causing damage to the dermo-epidermal junction and basal melanocytes. When these CTLs were stimulated in vitro with the same gp100-(280–288) antigen, they produced large amounts of IFN-γ. The same cells did not produce IFN-γ after stimulation with irrelevant tyrosinase epitopes, or if there was no additional peptide stimulation, indicating the antigen-specific nature of the activation. Tyrosinase-(369–377) and MART-1-(26–35)-specific CD8+ T-cell clones were also co-cultured with explant skin, also demonstrating infiltration and apoptosis of melanocytes. Keratinocyte apoptosis was not observed in these samples. No apoptosis was found when these T-cells were incubated.
with lesional (melanocyte-free) skin explants, showing that activation was antigen-specific and dependent on the presence of melanocytes [218].

With respect to tyrosinase as a potential autoimmune target in vitiligo, it is interesting that the recent genomewide association study confirmed a significant association with several TYR SNPs, in particular rs1393350. Significant epistasis was observed between this SNP and the HLA-A SNP rs12206499, and it is postulated that this combination presents the TYR polypeptide more efficiently to the immune system by HLA-A*02 [47].

### Regulatory T-cells

Although T-cells are considered integral to the onset of vitiligo, it is acknowledged that anti-melanocyte antibodies could play an important role in maintaining the disease [219]. CTLs alone are probably necessary but not sufficient for perpetuating generalized vitiligo, as regulatory mechanisms that prevent autoaggression by the immune system need to be overcome first [220].

Regulatory T-cells play a key role in removing self-reactive T-cells that have escaped the process of clonal deletion by the thymus [221]. Some regulatory T-cells have been shown in vitro to suppress activated T-cells in a contact-dependent process, utilizing CD25 and forkhead and winged-helix family factor FOXP3 (forkhead box P3) [33].

Regulatory T-cells may be stimulated by immature DCs in a Tol–DC regulatory loop [222]. DCs also have the capacity to augment melanocyte apoptosis in certain circumstances: melanocytes exposed to 4-TBP generate HSP-70 (heat shock protein-70), which although partly protective, also induces TRAIL (TNF-related apoptosis-inducing ligand) receptor expression on their cell surface. This activates DC effector functions and results in melanocyte death [223]. Tumour and self-directed CD8+ T-cells are augmented by CD4+ T-cells and suppressed by regulatory T-cells [224].

Regulatory T-cells can inhibit CD8+ and CD4+ T-cells and NK cells, via the cytokine TGF-β [219]; decreased TGF-β levels in peripheral blood of vitiligo patients were, in fact, recently reported, pointing towards impaired regulatory T-cell functioning [174]. Regulatory T-cells also control conversion of naïve CD4+ T-cells (CD45RA+) into Th-17 T-cells, producing IL-17. In the absence of regulatory T-cells, naïve CD4+ T-cells become Th-1 cells [225]. IL-17 is proinflammatory, acting synergistically with IL-6, IL-1β and TNF-α. IL-17 is increased in several chronic autoimmune diseases including psoriasis and systemic sclerosis [226]. Increased levels of IL-17-producing T-cells were reported in one patient with vitiligo [218], but serum IL-17 was not elevated in another study of vitiligo patients [174]. Dysregulation of CTLA-4 can also predispose to autoreactive T-cells, as this molecule decreases T-cell responsiveness and raises the threshold for T-cell activation. Increased CD8+ self-reactive function has been reported in the presence of defective CTLA-4, and CD4+ T-cells are also involved in this process [227]. CTLA-4 polymorphisms predispose to vitiligo in patients who already have other autoimmune conditions [146].

### CONCLUSIONS

A complex interplay of genetic, immunological, environmental and biochemical factors occurs in the lead up to generalized vitiligo. The hierarchy of events is still unknown, but evidence points to a major role for ROS and T-cells, probably acting in concert to destroy or incapacitate melanocytes in a vicious cycle of cellular fatigue, cytokine imbalance, immune attack and apoptosis. Melanocytes produce melanin, which has antioxidant properties, but its synthesis predisposes to ROS generation, and melanocytes, therefore, operate in a potentially hostile environment. Melanocytes seem to be uniquely fragile in people with a tendency to vitiligo. Numerous endogenous and exogenous sources of H2O2 and other ROS have been described in vitiligo, which are deleterious to a variety of melanocytic cellular processes, particularly in the context of impaired cellular antioxidant defence. The resultant protein and lipid damage could be sufficient, on its own, to initiate melanocyte failure, but another effect of oxidation could be to initiate melanocyte failure and apoptosis leading to uptake by Langerhans cells or DCs. If these Langerhans cells or DCs become activated, they may trigger a melanocyte-reactive immune response that can eradicate melanocytes in the skin, leading to depigmentation. This immune response principally involves cytotoxic T-cells. Failure of regulatory T-cell mechanisms allows the process to continue indefinitely, in keeping with the chronic, relentless course of generalized vitiligo. Further research into the pathogenesis of this common and distressing disorder will offer the potential for better therapies and might even facilitate effective strategies for primary prevention in genetically susceptible individuals.

### REFERENCES


Received 23 November 2009/26 August 2010; accepted 26 August 2010
Published on the Internet 20 October 2010, doi:10.1042/CS20090603