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**Graphical abstract**
Type-1 cytokines regulate matrix metalloprotease-9 production and E-cadherin disruption to promote melanocyte loss in vitiligo

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N.B, C.J., F.X.B., J.S., and K.B. are inventors on the patent application “New therapies in the prevention or treatment of a depigmenting disorder”: EP16306719.2, Filing date: December 16th 2016.
Abstract:

Loss of melanocytes is the pathological hallmark of vitiligo, a chronic inflammatory skin depigmenting disorder induced by exaggerated immune response, including autoreactive CD8 T cells producing high levels of type-1 cytokines. However, the interplay between this inflammatory response and melanocyte disappearance remains to be fully characterized. Here, we demonstrate that vitiligo skin contains a significant proportion of suprabasal melanocytes, associated with disruption of E-cadherin expression, a major protein involved in melanocyte adhesion. This phenomenon is also observed in lesional psoriatic skin. Importantly, apoptotic melanocytes were mainly observed once cells were detached from the basal layer of the epidermis, suggesting that additional mechanism(s) could be involved in melanocyte loss. The type-1 cytokines IFNγ and TNFα induce melanocyte detachment through E-cadherin disruption, and the release of its soluble form, partly due to the matrix metalloproteinase MMP-9. MMP-9, whose levels are increased in vitiligo skin and patients’ sera, is produced by keratinocytes in response to IFNγ and TNFα. Inhibition of MMP-9 or the JAK/STAT signaling pathway prevents melanocyte detachment in vitro and in vivo. Therefore, stabilization of melanocytes in the basal layer of the epidermis by preventing E-cadherin disruption appears promising to prevent the depigmentation occurring in vitiligo and during chronic skin inflammation.
**Introduction**

Vitiligo is the prototype of a depigmenting disease due to an exaggerated skin immune response. However, depigmentation is not restricted to vitiligo and may occur during the course of other chronic skin inflammatory disorders, such as psoriasis (1). Vitiligo impacts the quality of life of patients dramatically and therapies so far remain limited (2). Its pathological hallmark is the loss of epidermal melanocytes, the cells located in the basal layer of the epidermis and responsible for pigmentation and skin phototype. Vitiligo is a complex multifactorial disease combining genetic predisposition and environmental triggers (e.g. friction, chemicals), together with metabolic, immunologic and inflammatory abnormalities (2, 3). Depigmentation in vitiligo is consistently associated with infiltration of immune cells, in particular CD8 T cells, in close apposition to the remaining melanocytes, at least during disease progression (4–6). Importantly, vitiligo should be viewed as an immune memory skin disease, as recently highlighted by the role of resident memory T cells (T\textsubscript{RM}) during pathogenesis (7–9). A prominent type-1 cytokines skewed immune profile is characteristic of vitiligo patients’ skin, as the majority of melanocyte-specific skin CD8 T\textsubscript{RM} cells express the chemokine receptor CXCR3 and produce elevated levels of the type 1-related cytokines IFN\textgreek{y} and TNF\textalpha (7, 10). The CXCR3/ IFN\textgreek{y} / TNF\textalpha axis has been shown to be involved in the process leading to depigmentation *in vitro* and *in vivo* in a disease-prone mouse autoimmune model of vitiligo (11–14), and IFN\textgreek{y} and TNF\textalpha are involved in epidermal pigmentation homeostasis, inhibiting melanocyte function, phenotype and melanogenesis (4–6, 15, 16). However, although previous studies identified the role of these cytokines in the pathomechanism of the disease, the interplay between inflammation and the melanocyte loss characterizing vitiligo remains elusive.
The leading hypothesis is that melanocyte loss is mediated by autoreactive CD8 T cells (3, 17–19). Indeed, in vitro studies reported that CD8 T cells isolated from vitiligo patients’ skin induced the apoptosis of autologous melanocytes (17, 20, 21). Nonetheless, clear evidence of the presence of apoptotic cells in vivo is still lacking and we recently showed that CD8 T cells from vitiligo skin display moderate cytotoxic activity comparable to that found in healthy skin and the lesional skin of psoriasis patients (7), suggesting that additional disease mechanism could be involved in melanocyte disappearance. Moreover, depigmentation in mouse models of vitiligo depends rather on IFNγ signaling, while perforin is dispensable (11, 13), suggesting that the release of pro-inflammatory cytokines such as IFNγ in the skin is important in the process leading to depigmentation.

Melanocyte disappearance could also result from their detachment (22, 23). The stabilization of melanocytes in the basal layer of the epidermis is dependent on the adhesion protein E-cadherin (24, 25). Interestingly, impaired cell-surface E-cadherin expression was recently shown in the non-lesional skin of vitiligo patients (26, 27). Several mechanisms could be involved in such disruption, such as inhibition of its expression, internalization in the endosomal structure, or cell surface cleavage into a soluble form (soluble E-cadherin). E-cadherin cleavage may be induced by several proteases, including matrix metalloproteases such as MMP-3, MMP-7, MMP-9, A disintegrin and MMP domain-containing protein 10 (ADAM10), which are all known to be involved in extracellular matrix remodeling and cell migration in various physiologic and pathologic processes (28, 29).

MMPs are a family of Zn-dependent proteases with common functional and structural properties. MMP-9, also known as 92kDa gelatinase/type IV collagenase, is constitutively expressed primarily in leukocytes, while most other cell types including keratinocytes express MMP-9 in
response to various pro-inflammatory cytokines such as IFNγ, TNFα, IL-1β, IL-6 (30, 31). Initially recognized for its collagen-remodeling function, MMP-9 activity is now known for its role in the control of immune responses, such as the migration of immunocompetent cells into and out of peripheral tissues, and the regulation of the activation of both CD4 and CD8 T cells (32–34).

Here we report that vitiligo perilesional skin is characterized by the basal detachment of melanocytes associated with the disruption of E-cadherin surface distribution and the release of soluble E-cadherin; melanocyte apoptosis was mainly noticed following their detachment from the basal epidermal layer. Strikingly, this phenomenon is not restricted to vitiligo disease but may also occur in psoriasis. We further show that the type-1 cytokines IFNγ and TNFα induce melanocyte detachment in both in vitro and in vivo models, and that this effect is dependent on the inhibition of E-cadherin gene expression, internalization of E-cadherin, and its cleavage through the release of MMP-9 by keratinocytes. Strikingly, MMP-9 inhibitors and JAK inhibitors prevented the release of soluble E-cadherin, leading to the stabilization of melanocytes in the basal layer of the epidermis. Our results highlight an additional disease mechanism leading to the loss of melanocytes in vitiligo and more generally in skin inflammation, and suggest the need for further exploration of therapeutic strategies aiming to both dampen the inflammation and maintain melanocyte stability in the basal layer of the epidermis of vitiligo patients.
Results

Detachment of basal melanocytes is a hallmark of vitiligo and psoriasis

We first used immunofluorescence to examine the distribution of melanocytes in the epidermis in the context of skin inflammation. We examined the perilesional skin of patients with both vitiligo and psoriasis, and the lesional skin of psoriasis patients (Figure 1, A and B, patient characteristics are displayed in Supplemental Tables 1 and 2). Psoriasis was used as the archetype of a skin inflammatory disease. Collagen VII staining was used to identify the basal layer of the epidermis. While melanocytes were located in the basal layer of the epidermis in the control skin of unaffected individuals, a significant number of melanocytes were found in the suprabasal layers in vitiligo perilesional skin, not only in patients with stable or active disease but also in the acanthotic epidermis of the perilesional skin of patients with concomitant psoriasis and vitiligo. Strikingly, similar observations were made in the lesional skin of patients only with psoriasis. The remaining melanocytes in vitiligo perilesional skin were unable to proliferate irrespective of their localization within the epidermis, unlike the proliferating melanocytes observed in lesional psoriatic skin, as revealed by Ki67 expression (Supplemental Figure 1). This suggests that while the detachment of melanocytes is a common process seen in vitiligo and psoriasis, vitiligo is characterized by the absence of melanocyte regeneration, unlike psoriasis. Importantly, cleaved caspase 3 staining or TUNEL assay showed that only few basal melanocytes were apoptotic in these conditions. This was in contrast with the epidermal cell death observed in cutaneous lupus erythematosus and toxic epidermal necrolysis, two skin diseases associated with strong inflammation and epidermal cell death (Figure 1, C and D and Supplemental Figure 2). Apoptotic suprabasal melanocytes were evidenced only in some patients with active vitiligo (Figure 1, C and D). This suprabasal localization of melanocytes in vitiligo and psoriasis skin seems to be the consequence of a defect
in their adhesion to keratinocytes, as shown by the disrupted distribution of the major adhesion molecule mediating melanocyte adhesion to keratinocytes, E-cadherin (25), in both melanocytes and keratinocytes (Figure 1E).

Melanocytes were classified into three types according to the distribution of cell surface E-cadherin staining, as previously described (26): homogeneous (type 1), heterogeneous (type 2) and absence of E-cadherin labelling (type 3). Compared to healthy control skin in which melanocytes stained homogeneously for E-cadherin, melanocytes from vitiligo perilesional skin, particularly in the active phase of the disease, and lesional psoriasis skin displayed a discontinuous cell-surface E-cadherin expression (Figure 1E and Supplemental Figure 3A). In addition, soluble E-cadherin levels were significantly higher in stable and active vitiligo patients’ sera compared to those of healthy controls (Figure 1F). These findings suggest that pro-inflammatory factors released by immune and epidermal cells during skin inflammation are able to regulate the distribution of E-cadherin on melanocytes and are responsible for their detachment from the basal layer of the epidermis.

**Type-1 cytokines IFNγ and TNFα induce detachment of melanocytes and disrupt E-cadherin distribution**

The immune response of vitiligo is predominantly associated with Th1/Tc1 cells infiltrating the skin together with an elevated production of both IFNγ and TNFα (3). This immune bias is also found to a lesser extent in psoriasis compared to the strong Th17 skewed immune profile (35).

We next used an *in vitro* 3D model of reconstructed pigmented human epidermis (RHPE) containing both keratinocytes and melanocytes to investigate whether TNFα and IFNγ could be involved in the E-cadherin disruption observed in patients. We found that the combination of
TNFα and IFNγ induced the detachment of more melanocytes from the basal layer than each cytokine alone. Such detachment was also observed in vitro on cocultures of melanocytes and keratinocytes (Supplemental Figure 3, B and C). The process was mediated partly by an altered E-cadherin distribution in melanocytes (Figure 2, A and B and Supplemental Figure 3D). It was not associated with prominent melanocyte cell death, as observed by TUNEL assay staining (Figure 2C) or by assessing melanocyte viability in response to increasing concentrations of these cytokines (Supplemental Figure 4). In addition, only the combination of TNFα and IFNγ was able to downregulate significantly the expression of the E-cadherin encoding gene CDH1. This inhibition was specific to melanocytes as CDH1 transcript levels were not regulated in keratinocytes (Figure 2D). The decrease was dose- and time-dependent (Figure 2, E and F). TNFα and IFNγ also decreased transcript levels of the melanocyte adhesion-related genes CCN3 and DDR1, albeit more weakly, while no significant regulation of the β-catenin-encoding gene CTNNB1 was noted (Supplemental Figure 5). DDR1 was recently reported to stabilize the membrane localization of E-cadherin (36). Importantly, the two cytokines induced E-cadherin relocalization in melanocytes, as evidenced by the presence of E-cadherin in vesicular structures positive for LAMP-1 staining, a known marker for lysosomes and late endosomes (Figure 2G). Furthermore, levels of soluble E-cadherin were also increased in cell-free supernatants of RHPE treated by both TNFα and IFNγ (Figure 2H), suggesting their ability to induce cleavage of E-cadherin indirectly.

**MMP-9 levels are increased in the circulation and skin of vitiligo patients**

Consequently, we explored which major protease(s) could be involved in the cleavage of E-cadherin and lead to destabilization of melanocytes. Serum levels of both zymogen and active
forms of MMP-9 were higher in active vitiligo and psoriasis patients than in healthy controls (Figure 3, A and B), while MMP-3, MMP-7, and ADAM10 serum levels were similar in all groups (Supplemental Figure 6, A-C). MMP-9 levels were significantly higher in vitiligo patients with active disease (Figure 3, A and B), and a positive correlation between total and active MMP-9 was observed in vitiligo patients (Supplemental Figure 6D). Interestingly, MMP-9 levels correlated positively with soluble E-cadherin levels in vitiligo patients’ serum, as well as with the body surface area involved (Figure 3, C and D, and Supplemental Figure 6, E and F). An increase in MMP-9 expression was observed both at the gene and protein levels in active and stable vitiligo perilesional skin and lesional psoriasis skin in comparison to healthy skin (Figure 3, E and F, and Supplemental Figure 6G). This was in contrast with MMP-3, MMP-7, and ADAM10 gene and protein expression, which remained lower and not significantly different between healthy control skin, vitiligo and psoriasis skin in comparison to strong MMP-9 upregulation (Supplemental Figure 6, H-J). We then compared the inflammatory transcriptome profile of the perilesional skin of patients with stable or active vitiligo using the nCounter® inflammation panel (248 genes) to identify the genes that were the most differentially regulated. We found that the MMP9 gene was among the top ten genes upregulated in active vitiligo skin (Figure 3G). The interactomic network of the upregulated transcripts showed MMP-9 to be at the core of the molecular signature identified, together with genes previously shown to be involved in depigmentation such as TNF and CXCL9 (Figure 3H). Lastly, TNFα and IFNγ strongly induced the expression and production of MMP-9 by epidermal cells, mainly by keratinocytes (Figure 3, I-K), while they had little or no effect on MMP-3, MMP-7 and ADAM10 expression (Supplemental Figure 7, A and B).

MMP-9 (also known as gelatinase B) belongs to the gelatinase subgroup of MMPs, which also includes MMP-2 (also known as gelatinase A). We therefore investigated whether MMP-2 was
deregulated in our experimental setting. In contrast to MMP-9, levels of MMP-2 were decreased in vitiligo patients’ sera and a similar, although not significant, tendency was observed in psoriasis serum (Supplemental Figure 8A). Nonetheless, MMP-2 serum levels did not correlate negatively with MMP-9 serum levels or the body surface area involved in vitiligo patients (Supplemental Figure 8, B and C). In accordance with these findings, TNFα and IFNγ reduced MMP-2 levels in keratinocytes. MMP-2 expression was not detected in melanocytes (Supplemental Figure 8, D and E and data not shown).

**MMP-9 is involved in melanocyte detachment induced by type-1 cytokines in vitro and in vivo**

We next explored the contribution of MMP-9 to melanocyte destabilization in RHPE and observed that active MMP-9 induced a dose-dependent increase in the proportion of suprabasal melanocytes, associated with an increase in soluble E-cadherin levels (Figure 4, A-C). Subsequent experiments using the gelatinase inhibitor SB-3CT or a selective inhibitor of MMP-9 (ab142180) investigated the impact of MMP-9 inhibition in RHPE treated with TNFα and IFNγ. We observed a modest and robust dose-dependent inhibition of melanocyte detachment induced by type-1 cytokines with both of the inhibitors tested (Figure 4, D and E). This effect was associated with a decrease in soluble E-cadherin levels (Figure 4F). To fully confirm this mechanism in vivo, both TNFα and IFNγ were injected intradermally into C57BL/6 wild-type mice every day for 6 days (Figure 4G). Owing to the low number of melanocytes in mouse skin, the mouse tail was used for injection, because melanocytes are located in the basal layer of epidermis, thus reproducing human pigmentation. Consistent with human data, concomitant intradermal injection of IFNγ and TNFα induced a significant detachment of melanocytes associated with disruption of E-cadherin
expression (Figure 4, H and I and Supplemental Figure 9). In support of our results obtained *in vitro*, MMP-9 inhibition with SB-3CT was associated with a significant stabilization of epidermal melanocytes in the basal layer of the epidermis (Figure 4, H and I), thus providing further evidence of the involvement of MMP-9 in the process leading to melanocyte loss.

**Inhibition of JAK signaling stabilizes melanocytes in the basal layer of the epidermis by MMP-9 reduction**

To further decipher the mechanism involved in melanocyte destabilization, we studied the impact of JAK signaling using tofacitinib (a JAK1/3 inhibitor) or ruxolitinib (a JAK1/2 inhibitor) in our *in vitro* and *in vivo* models of melanocyte detachment induced by TNFα and IFNγ. Both JAK inhibitors led to significant melanocyte stabilization in RHPE treated with TNFα and IFNγ (Figure 5, A and B), associated with a decrease in levels of soluble E-cadherin and active MMP-9 (Figure 5, C and D). A decrease in MMP9 levels was also observed *in vitro* on vitiligo perilesional epidermis explants following tofacitinib treatment (Figure 5E). Inhibition of JAK signaling also prevented melanocyte detachment induced by type-1 cytokines *in vivo* (Figure 5 F and G), and no significant difference was noted between the two inhibitors tested in our models.
Discussion

Our findings shed light on a so far unknown mechanism regarding the interplay between the inflammatory response and melanocyte loss in vitiligo. We demonstrate that destabilization of melanocytes from the basal layer of the epidermis is an important event leading to their loss. This destabilization results from alteration of membrane E-cadherin in response, at least in part, to the upregulated production of active MMP-9 by epidermal cells, especially keratinocytes, induced by the type-1 cytokines IFNγ and TNFα, which are two pro-inflammatory cytokines involved in vitiligo. This phenomenon could be reproduced by administering active MMP-9 or prevented by using MMP-9 inhibitors or by inhibiting the JAK/STAT signaling pathway with JAK inhibitors (Figure 6).

Importantly, the suprabasal localization of melanocytes within the epidermis is not restricted to vitiligo, and the mechanism hereby identified could occur in the hypopigmentation associated with other causes of chronic skin inflammation, as in psoriasis. Indeed, we recently reported that hypopigmentation could occur in 10% of patients with psoriasis and was observed in areas previously affected by the disease (37). However, in contrast to vitiligo, complete and durable depigmentation was not observed in psoriasis, perhaps due to 1/ the proliferative capacity of melanocytes, which is greater in lesional psoriatic skin than in vitiligo perilesional skin and 2/ to apoptosis of suprabasal melanocytes in vitiligo, as also previously reported (26) and which could involve the recently identified innate lymphocyte-induced CXCR3B-mediated apoptosis (38). Another explanation could be that melanocytes in vitiligo patients have intrinsic abnormalities that cause a regenerative deficiency (2, 3). Indeed, the activation, maturation, proliferation and recruitment of melanocyte precursors in vitiligo skin are defective (39), explaining the persistence of white patches. This contrasts with lesional psoriatic skin which harbors a high proportion of
proliferating melanocytes, explaining not only the absence of complete depigmentation in patients despite melanocyte detachment from the basal layer of the epidermis, but also the fact that depigmented lesions are able to recover rapidly under therapies. This is consistent with previous reports showing an increased number of melanocytes both in psoriasis lesions and in resolved psoriasis skin (40, 41).

Our data suggest that the death of melanocytes, especially in vitiligo patients, could occur following their detachment and relocalization to the outer layers of the epidermis (melanocytorrhagy), as previously observed (26). Vitiligo is characterized by clinically undetectable and pathologically mild inflammation with infiltration of melanocyte-specific resident memory CD8 T cells producing elevated levels of pro-inflammatory cytokines, while production of cytotoxic markers was similar to healthy skin and lesional psoriasis skin (7). Interestingly, it was recently demonstrated that a melanocyte antigen can trigger auto-immunity in psoriasis with skin infiltration of epidermal melanocyte-specific CD8 T cells producing pro-inflammatory cytokines involved in the development of psoriasis, such as IL-17 and IFN\(\gamma\) (42). However, while granules containing granzyme B were identified in a psoriasis skin CD8 T cell subset, the authors failed to detect any signs of cell death in melanocytes. In view of our findings, we hypothesize that IFN\(\gamma\) and TNF\(\alpha\) produced by resident memory T cells in vitiligo skin contributes to melanocyte destabilization. Our observations contrast with what may be observed in other inflammatory skin disorders, such as the group of lichenoid dermatitis associated with cutaneous lupus disease and lichen planus. These diseases are characterized by strong immune infiltration of the basal layer leading to the destruction of epidermal cells such as melanocytes, the release of melanin in the dermis (incontinence) and to definitive cicatricial pigmentary changes (43).
Our data reveal a previously unknown role of MMP-9 during depigmentation and highlight its prominent role in inducing melanocyte destabilization by the shedding of E-cadherin and the release of its soluble form. Besides type 1 cytokines, additional cytokines upregulated in vitiligo patients’ blood and/or skin and known to increase MMP-9 expression could also be involved in its upregulation, including IL-1β, IL-17, or IL-6 (30, 31, 44). In line with a study suggesting the low production of MMP-9 by melanocytes in vitiligo patients (45), we found that keratinocytes were the main epidermal source of MMP-9. In contrast, another gelatinase, MMP-2, was found to be decreased in vitiligo patients’ sera and its production downregulated by IFNγ and TNFα. MMP-2 is known to be involved in the migration of melanocyte precursors for their optimal epidermal replenishment (46). MMP-2 reduction, together with the increase in MMP-9 production, could therefore impact simultaneously the stabilization of epidermal melanocytes and their replenishment from the melanocyte precursor reservoir, thus leading to durable depigmentation in vitiligo. However, while we could not identify any increased expression of some other proteases (e.g. MMP-3, MMP-7, and ADAM10) known to play a role in E-cadherin cleavage, we cannot rule out the possibility that other proteases not evaluated in this study are involved in this process. This is supported by the fact that the treatment of RHPE with active MMP-9 induced a significant but lower melanocyte detachment than that observed in response to IFNγ and TNFα. Additionally, it would be relevant in psoriasis to test the role of Th17-related cytokines alone or in combination with Th1-related cytokines in this phenomenon.

We now need to focus on two major goals in vitiligo therapy: first, dampening the activation of the immune response responsible for the loss of melanocytes and the maintenance of depigmentation; and second, maintaining melanocytes in the basal layer and promoting their regeneration from melanocyte precursors. Like other MMPs, MMP-9 is known to be upregulated
in autoimmune and inflammatory disorders and to play a role in modulating the innate and adaptive immune response, the production of chemokine ligands and cytokine activity (47). Therefore, in vitiligo and other chronic depigmenting disorders associated with inflammation, the inhibition of MMP-9 activity with topical or systemic agents could dampen both the immune response and help to stabilize melanocytes in the basal layer of the epidermis. Moreover, our study provides evidence that targeted therapies such as topical or systemic JAK inhibitors, which have shown promising results in vitiligo (48–51), could inhibit the type II IFN response, thereby maintaining melanocytes in the basal layer of the epidermis. Hence, the association of JAK inhibitors together with MMP-9 inhibitors could be a way to lower the dose of each component for either systemic or topical applications, ideally in combination with a therapy stimulating melanocyte regeneration such as phototherapy. While our study provides new insights into the targeting of MMP-9 in vitiligo, it is now critical to confirm this strategy in preclinical models of vitiligo. Mouse models of skin depigmentation following infiltration of melanocyte-specific CD8 T cells are available (57) and could be used to fully validate a strategy to inhibit melanocyte detachment through targeting of MMP-9. Showing that the use of a MMP-9 inhibitor alone or in combination with immunomodulating agents could either prevent depigmentation and/or induce repigmentation would be of great interest before going to clinical trials. We now need to set the therapeutic objective of stabilizing melanocytes in the basal layer of the epidermis with specific therapies able to restore the expression of membrane E-cadherin or to inhibit its cleavage.
Methods

Subjects, skin and serum samples. Human peripheral blood and skin punch biopsy samples were obtained from patients with vitiligo and psoriasis from the Department of Dermatology in Bordeaux University Hospital, France. None of the patients included in this study had received any treatments/immunosuppressive therapies during the six months preceding inclusion. Patients’ clinical characteristics are presented in Supplementary Tables 1 and 2. Vitiligo patients were classified according to the Vitiligo European Task Force (VETF) scoring system, as described previously (52), notably for disease activity. Briefly, they were classified using Wood’s lamp examinations, as previously reported (53, 54). Patients with a total spreading score ≥3 according to the VETF scoring system and/or the presence of hypomelanotic lesions with poorly defined borders and/or confetti-like lesions were considered active, while those with a total spreading score ≤1 and/or the absence of new lesions over the past 12 months were considered stable. Skin biopsies (4mm diameter) were obtained from the perilesional area. Lesional psoriatic skin biopsies were obtained as a control of skin inflammatory disorder. Paraffin-embedded skin sections from patients with cutaneous lupus and toxic epidermal necrolysis were obtained from the Department of Pathology at Bordeaux University Hospital, France. Unaffected control skin was obtained as discarded human tissue from cutaneous plastic surgery (Bordeaux University Hospital, France). Blood from unaffected subjects was obtained from volunteers exempt of autoimmune or inflammatory disorders.

Cell cultures and cytokine treatment. Primary Human Melanocytes were isolated from healthy children’s foreskin as previously described (55) and maintained in melanocyte growth medium (MGM) supplemented with 1 ng/ml basic recombinant human fibroblast growth factor, 0.5 μg/ml
hydrocortisone, 4 μg/ml bovine pituitary extract, 5 μg/mL recombinant human insulin (all from Promo Cell), 100 U/ml penicillin and 100 μg/ml streptomycin (Eurobio). Primary human keratinocytes were obtained from surgical samples of healthy breast skin as previously described (56). Cells were cultivated in keratinocyte serum-free medium (KSFM, Life Technologies) supplemented with 25 μg/ml bovine pituitary extract, 0.25 ng/mL epidermal growth factor (Life Technologies), 100 U/ml penicillin and 100 μg/ml streptomycin (Eurobio). Cells were maintained in a humidified atmosphere 5% CO₂ at 37°C and starved in growth factor-free medium prior to stimulation with indicated concentrations of TNFα and IFNγ (R&D Systems), alone or in combination. Cell-free supernatants were harvested for ELISA and remaining cells were lysed for RNA quantification or Western blot analysis. In vitro reconstructed human pigmented epidermis (RHPE) containing both melanocytes and keratinocytes was generated from surgical samples of pediatric foreskins on polycarbonate culture inserts and were from Bioalternatives Laboratories (Bioalternatives, Gençay, France). RHPE were cultured at the air-liquid interphase for 10 days in a humidified atmosphere 5% CO₂ at 37°C and then treated for 24 h with or without 10 ng/ml of TNFα and IFNγ, alone or in combination (R&D Systems). When appropriate, indicated concentrations of active MMP-9 (Sigma), SB-3CT, selective Ab142180 MMP-9 inhibitor (Abcam), tofacitinib (Sigma), or ruxolitinib (Stemcell) were added. Perilesional Skin explants were incubated 3 h at 37°C with trypsin-EDTA to isolate the epidermis. Epidermal sheets were then treated 24 h with or without 1 μM of tofacitinib. Cell-free supernatants were harvested for ELISA.

Animal studies. C57BL/6j wild-type mice were purchased from Charles River Laboratories and were housed in an animal facility at Bordeaux University under conventional conditions with
constant temperature and humidity. Mice were injected intradermally daily (from day 0 to day 6) with PBS and/or 1 µg of TNFα and IFNγ (Peprotech) and/or 1mM of tofacitinib (Sigma), 1mM ruxolitinib, or 1.25 mg/ml of SB-3CT (Abcam), in the base of the tail. On day 7, mice were sacrificed, the tail was fixed in formalin, and paraffin-embedded sections were prepared to perform immunofluorescence studies.

**Cell proliferation analysis.** Cell proliferation was measured using the premix WST-1 cell proliferation assay system (*Takara Bio*). Cells were treated with increasing concentrations of TNFα and/or IFNγ ranging from 0.2 to 200 ng/ml) for 24, 48, and 72 h at 37 °C. Ten microliters of WST-1 were added 4 h before measurement of the absorbance at 450 and 650 nm to determine proliferation and background absorbance, respectively (*Multiskan FC microplate reader, Thermo Fisher Scientific*).

**NanoString.** The nCounter® technology was performed by the “Groupe de Recherche en Immunologie Clinique” (GRIC) at Bordeaux University Hospital. RNA was extracted from formalin-fixed, paraffin-embedded (FFPE) skin sections of patients with stable (n=3) or active (n=6) vitiligo using the High Pure FFPEt RNA isolation kit (Roche). RNA purity and concentrations were determined using nanodrop and Bioanalyzer 2100 (Agilent RNA 6000 Nanokit). Samples were processed with Nanostring CodeSet technology and the NanoString nCounter Human Inflammation panel v2 mRNA Expression Assay (NanoString Technologies). Results were analyzed using the nSolver Analysis Software 4.0 (NanoString). The software STRING (Search Tool for the Retrieval of Interacting Genes, v.10.5 web server, http://strindb.org/, under a 'Creative Commons BY 4.0' license), with Homo Sapiens as the reference organism, was
used to generate an association network of potentially interacting proteins, with a minimum required interaction score of 0.4. The network was built following the automatic enrichment in STRING, based on the information provided by several databases such as KEGG, Ensembl, and BioCyc.

**Quantitative real-time PCR analysis.** Total RNA was isolated from primary human epidermal keratinocytes or melanocytes using NucleoSpin RNA and Nucleospin RNA XS kits (Macherey-Nagel), respectively. RNA from tissue samples and RHPE were extracted using TRIzol reagent (Invitrogen Life Technologies). Two hundred ng to 1 μg RNA were then retro-transcribed with 1X first-strand buffer, 0.25 μg/μl of random primers, 0.02 μM of dNTP, 0.03 μM of dithiothreitol, and 100 U of SuperScript II Reverse Transcriptase (Invitrogen), according to the manufacturer’s instructions. Quantitative real-time PCR was carried using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix kit (Agilent Technologies), according to the manufacturer’s instructions, together with gene-specific primers for *ADAM10, CCN3, CDH1, CTNNB1, DDR1, MMP2, MMP3, MMP7*, and *MMP9*. *GAPDH* was used as housekeeping gene. Following an initial step at 95°C for 3 min, cDNA was amplified for 40 cycles (15 s at 95°C, 22 s at 60 or 64°C) in a MX3000P Stratagene thermocycler (Agilent Technologies). Human primer sequences are as follows: *ADAM10*, 5’-CCTGAAGTGGAGCGAGAGGG-3’ (sense) and 5’-CATACTGACCTCCCATCCCCG-3’ (antisense); *CCN3*, 5’-CGGCTGCTCATGCTGTCTGG-3’ (sense) and 5’-TTATCTCCCTCTACCGCCGTGC-3’ (antisense); *CDH1*, 5’-ATCCTCCGATCTTCAATCCCACCAC-3’ (sense) and 5’-TTATCTCCCTCTACCGCCGTGC-3’ (antisense); *CTNNB1*, 5’-GTACCACATTCCGTCACTGCTACGTG-3’ (antisense); *CTNNB1*, 5’-GGCCTGTAGAGTTGCTGGAG-3’ (sense) and 5’-ACAAGCAAGGCTAGGGTGGT-3’;
**Immunohistochemistry / Immunofluorescence studies.** Four-μm sections were prepared from FFPE skin biopsies. Sections were deparaffinized and subjected to a heat-induced epitope retrieval step. Slides were rinsed in cool running water and washed in Tris-buffered saline (pH 7.4) before incubation overnight at 4°C with relevant primary antibodies. Primary antibodies used for immunofluorescence experiments were as follows. For human skin tissues: mouse monoclonal anti-Melan-A antibody (A103, M7196, Dako, 1/100), mouse monoclonal anti-MITF antibody (D5, M3621, Dako, 1/100), rabbit polyclonal anti-E-cadherin (ab15148, Abcam, 1/100), rabbit polyclonal anti-collagen-VII (ab93350; Abcam, 1/100), rabbit monoclonal anti-Ki67 (SP6, ab16667, Abcam, 1/100), rabbit polyclonal cleaved-caspase 3 (Asp175, Cell Signaling, 1/100), mouse monoclonal anti-LAMP-1/CD107a Antibody (5E7, NBP2-52721, Novus Bio, 1/50). For mouse skin tissues: mouse monoclonal anti-E-cadherin (M168, ab76055, Abcam, 1/200), and rabbit monoclonal anti-Melan-A (A19-P, NovusBio, 1/2000). Secondary antibodies used: Alexa
555 goat anti-mouse IgG (A21422, Life technologies, 1/1000), Alexa 488 goat anti-rabbit IgG (H+L) (ab150077, Abcam, 1/200). Following subsequent washing, the sections were mounted with Prolong Gold antifade reagent with DAPI (ThermoFisher Scientific, Cambridge, MA). Appropriate isotype-matched controls were included. TUNEL assay was performed using a commercially available kit (In-situ cell death detection, Roche) according to the manufacturer’s protocol. For immunohistochemistry (IHC), slides were incubated overnight at 4°C with relevant primary antibodies: rabbit polyclonal anti-MMP-3 antibody (NB100-91878, Novus Bio, 1/50); mouse monoclonal anti-MMP-7 Antibody (MM0022-4C21, NB110-60988, Novus Bio, 1/200); rabbit polyclonal anti-MMP-9 antibody (NB600-1217, Novus Bio, 1/2000); rabbit polyclonal anti-ADAM10 antibody - Cytoplasmic domain (ab39177, Abcam, 1/2000). Secondary labeling was performed using the LSAB2 kit (DAKO). Stained slides were analyzed independently by two individuals in a blinded manner. Expression of MMP-3, MMP-7, MMP-9, and ADAM10 was scored using a semi-quantitative method (0: no expression; 1: weak expression; 2: fair expression; 3: strong expression). Images were acquired using an epifluorescence microscope (Leica) or LSM 510 META confocal laser scanning microscope (Carl Zeiss).

**Western Blot analysis.** To perform immunoblotting, keratinocytes were lyzed in a cell lysis buffer (Cell Signaling) supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma) according to the manufacturer’s instructions. Protein concentrations were measured using a BCA protein assay kit (Pierce). Fifteen μg of protein lysates were separated by 10% SDS-PAGE gel electrophoresis and transferred to a PVDF membrane (GE Healthcare). MMP-9 and GAPDH were detected using rabbit anti-MMP-9 antibody (E11, sc-393859, Santa-Cruz, 1/250) and rabbit anti-GAPDH antibody (14C10, 2118, Cell Signaling, 1/1000) respectively, and peroxidase-conjugated
goat anti-rabbit antibody (7074, Cell Signaling, 1/2000), according to the manufacturer’s instructions. Detection was carried out using the ECL detection system (Bio-Rad) and a chemiluminescent image analyzer (LAS-3000, Fujifilm).

ELISA. Concentrations of soluble E-cadherin, MMP-2, MMP-7, MMP-3, MMP-9 (all from R&D systems), and ADAM10 (Elab Science Biotechnology) in patients’ sera and/or cell-free culture supernatants were measured according to the manufacturers’ instructions. Levels of the active form of MMP-9 were quantified using the Fluorokine Human Active MMP-9 kit (R&D Systems) according to the manufacturer’s instructions, and 4-aminophenylmercuric acetate (APMA) was added to samples.

Statistics. All statistical analyses were performed using GraphPad Prism 6 software. Data are presented as mean ± s.e.m. unless otherwise indicated. Spearman’s rank correlations were used for all correlation coefficients. Non-parametric (Mann-Whitney or Wilcoxon tests) two-tailed paired and unpaired t-tests were used to compare two groups at 95% confidence interval. To determine statistical differences between controls, vitiligo and psoriasis groups, the normal distribution and the homogeneity of variances were first tested using the Shapiro-Wilk and Bartlett tests, respectively. When the variables fit with normal distribution and were equal, the one-way ANOVA was used. In other cases, a Kruskal-Wallis test was performed. Differences were considered statistically significant when P ≤0.05.

Study approval. All studies involving human tissues were approved by the Ethics Committee and the Commission Nationale de l’Informatique et des Libertés (no.1545937). All subjects
provided written informed consent for inclusion in this study. Experimental procedures on animals were conducted in compliance with the guidelines of the ethical committee of Bordeaux University and the national French animal welfare laws, guidelines and policies (APAFIS 7406).
Authors’ contributions: J.S. and K.B. conceived and supervised the study. N.B., C.M., F.M., F.X.B., J.S, and K.B designed the experiments. N.B., A.S.D., C.M., J.R., C.B., J.G., C.D., C.J., D.T., and F.L. performed the experiments. N.B., C.M., J.S, and K.B. analyzed data. A.S.D., K.E, A.T., and J.S. contributed to clinical samples. N.B., C.M., J.S., and K.B. prepared the figures. J.S. and K.B. wrote the manuscript. All authors revised the manuscript.

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Figures:

Figure 1. Melanocyte loss results from defective adhesion of melanocytes and not from apoptosis. (A) Representative immunofluorescence analysis showing melanocytes (red, Melan-A) and basal layer of epidermis stained with an anti-Collagen VII antibody (green) in control healthy skin, vitiligo perilesional skin, skin obtained from patients with concomitant vitiligo and psoriasis, and lesional psoriatic skin. Dashed lines represent dermo-epidermal layer. Arrows show suprabasal melanocytes in different conditions. Scale bars represent 50µm (upper panel) and 20µm (lower panel). (B) Proportion of suprabasal melanocytes in control healthy skin (n=5), stable or active vitiligo perilesional skin (n= 18; black squares: stable vitiligo, red squares: active vitiligo), perilesional skin of patients with association of vitiligo and psoriasis (n=4) and lesional psoriatic skin (n=4). (C) Representative analysis of epidermal cell death using cleaved caspase 3 antibody (green). Melanocytes were stained with anti-MITF antibody (red) in control healthy skin, stable and active vitiligo perilesional skin, or lesional skin from psoriasis, cutaneous lupus erythematous, and toxic epidermal necrolysis. Dashed lines represent dermo-epidermal layer. Scale bar represents 20µm. (D) Proportion of cleaved caspase 3+ MITF+ basal (●) or suprabasal (▲) melanocytes in control healthy skin (n=3), stable (n=4) or active vitiligo perilesional skin (n=6), lesional skin of psoriasis (n=6), cutaneous lupus erythematous (n=6) and toxic epidermal necrolysis (n=3). (E) Representative immunofluorescence analysis of expression of E-cadherin (green) and melanocytes (red, Melan-A staining) in control healthy skin, perilesional stable or active vitiligo skin and lesional psoriatic skin. Staining is representative of 10 independent patients. Dashed lines represent dermo-epidermal layer. Arrows identify suprabasal melanocytes. Scale bars represent 50µm (right) and 10µm (left). (F) Assessment by ELISA of soluble E-cadherin levels in sera from healthy
controls (n=18) or patients with stable (n=37), progressive (n=38) vitiligo, or psoriasis (n=20). Data show mean ± SEM. *P < 0.05, **P < 0.01; calculated with a Kruskal-Wallis test.

**Figure 2. Combined activity of IFNγ and TNFα reproduces human vitiligo features in a 3D model of reconstructed pigmented epidermis in vitro.** (A-C) Reconstructed human pigmented epidermis (RHPE) containing melanocytes were stimulated for 24h in the presence or absence of 10ng/ml of TNFα and IFNγ alone or in combination. (A) Representative immunofluorescence analysis of Melan-A (red) and E-cadherin (green) expression. Right panel shows alteration of E-cadherin expression around melanocytes. Dashed lines represent dermo-epidermal layer. Scale bars represent 20µm (left panel) and 10µm (right panel). (B) Proportion of suprabasal melanocytes in the different conditions (n=4). (C) Representative analysis of epidermal cell death using a TUNEL assay (green), melanocytes were stained with anti-Melan-A antibody (red). Dashed lines represent the dermo-epidermal layer. Scale bar represents 20µm. Staining is representative of three independent experiments. (D) Primary cultures of normal human epidermal keratinocytes (NHEK, left panel, n=10) or melanocytes (NHEM, right panel, n=11) were treated for 24h with 20ng/ml of IFNγ and/or TNFα. CDH1 gene expression in epidermal cells was analyzed by real-time PCR. Results are shown as the % of change compared to the control culture. (E) Dose-response study of CDH1 gene expression in NHEM following 24h stimulation with TNFα and/or IFNγ. (F) Kinetic analysis of CDH1 gene expression in NHEM in response to 20 ng/ml of TNFα and/or IFNγ. Results from one experiment are shown in E and F panels and are representative of four independent experiments with four independent donors. GAPDH was used as a housekeeping gene. (G) Confocal microscopy analysis of RHPE treated in the presence or absence of the combination of 10 ng/ml of TNFα and IFNγ for 24h. Sections were stained for E-cadherin (green).
and LAMP-1 (a marker for lysosomes and late endosomes, red). Merge shows the presence of E-cadherin molecule into LAMP-1 vesicle structures. Scale bars represent 20µm and 10µm. Stainings are representative of three independent experiments. (H) Assessment by ELISA of soluble E-cadherin levels in cell-free supernatants of RHPE treated for 24h in the presence or absence of 10 ng/ml of TNFα and IFNγ. Data in B, D, H show mean ± SEM. *P < 0.05, **P < 0.01; calculated with two-tailed Mann–Whitney (B) or Wilcoxon (H) tests.

**Figure 3. MMP-9 levels are increased in of vitiligo patients and correlate with soluble E-cadherin levels and surface of depigmentation.** (A) ELISA levels of MMP-9 in sera of healthy controls (n=18), stable (n=37) or active (n=37) vitiligo, and psoriasis patients (n=20). (B) Active MMP-9 levels in sera of healthy controls (n=22), stable (n=30) or active (n=39) vitiligo, and psoriasis patients (n=19). (C) Spearman’s rho correlation (two-tailed) between MMP-9 and soluble E-cadherin levels in vitiligo patients’ sera (n=73). (D) Spearman’s rho correlation (two-tailed) between serum active MMP-9 and body surface area (BSA) involved in vitiligo patients (n=59). (E) Representative IHC staining of MMP-9 expression in healthy control skin, perilesional skin of stable and active vitiligo, and lesional psoriatic skin. Scale bar represents 100µm. (F) Semi-quantitative analysis of MMP-9 expression in skin from healthy controls (n=5), perilesional skin of vitiligo patients with stable (n=11) or active (n=10) disease, and lesional psoriatic skin (n=8). (G-H) Inflammatory transcriptomic profile of perilesional skin of stable (n=3) and active (n=6) vitiligo patients was assessed using NanoString technology. (G) The most upregulated genes are shown. Results show the change in gene expression between the two groups. (H) Predicted protein-protein interaction networks for upregulated genes using STRING online tool. The thickness of edges represents the strength of data support. The thicker the edge between two proteins, the more
these proteins are linked based on the enrichment evidenced by STRING. (I-J) RHPE, NHEK, and NHEM were stimulated for 24h in the absence or presence of TNFα and IFNγ. (I) Real-time PCR analysis of MMP9 gene expression in RHPE (n=7), NHEK (n=9) and NHEM (n=7). Data are shown as fold increase above the control culture. GAPDH was used as housekeeping gene. (J) Levels of MMP-9 in cell-free culture supernatants of RHPE (n=13, left panel) and NHEK (n=9, right panel). (K) Western blot analysis of MMP-9 expression in NHEK treated for 24h in the presence or absence of 20 ng/ml of TNFα and IFNγ. Data in A, B, F, I, J show mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001; calculated with Kruskal-Wallis (A, B, F) or Wilcoxon tests (I, J).

**Figure 4. MMP-9 inhibition allows melanocyte stabilization both in vitro and in vivo.**

(A-C) RHPE were treated for 24h in the presence or absence of increasing concentrations of active MMP-9. (A) Representative immunofluorescence staining of Melan-A (red) and E-cadherin (green). Dashed lines represent the dermo-epidermal layer. Arrows show suprabasal melanocytes in the different conditions. Scale bar represents 20µm. (B) Proportion of suprabasal melanocytes in the different conditions. (C) Assessment by ELISA of soluble E-cadherin levels in cell-free supernatants. (D-F) RHPE were treated for 24h in the presence or absence of 10 ng/ml of TNFα and IFNγ and/or 1, 10 or 100 µM of MMP-9 inhibitors Ab142180 or SB-3CT. (D) Representative immunofluorescence staining of Melan-A (red) and E-cadherin (green) Dashed lines represent the dermo-epidermal layer. Arrows show suprabasal melanocytes in the different conditions. Scale bar represents 20µm. (E) Proportion of suprabasal melanocytes in the different culture conditions. (F) Assessment by ELISA of soluble E-cadherin levels in cell-free supernatants. (G-I) The base of C57BL/6 mouse tail was treated daily for 6 days with intradermal injections of saline buffer (control), or the combination of 1 µg of TNFα and IFNγ, and/or 1.25 mg/ml of SB-3CT. (G) *In vivo* schema of C57BL/6 mice treatment. (H) Representative immunofluorescence analysis of
melan-A (red) and E-cadherin (green) staining in the different groups. Dashed lines represent the dermo-epidermal layer. Arrows show suprabasal melanocytes. Scale bars represent 20µm. (I) Proportion of suprabasal melanocytes was assessed in the different groups (n=7-9). Data in B,C,E,F,I shown mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001; ****P < 0.0001, calculated with two-tailed Mann–Whitney test.

**Figure 5. Inhibition of JAK prevents type-1 cytokine-mediated melanocyte detachment both in vitro and in vivo.** (A-D) RHPE were treated for 24h in the presence or absence of 10 ng/ml of TNFα and IFNγ and/or 0.1 or 1 µM of tofacitinib or ruxolitinib. (A) Representative immunofluorescence staining of Melan-A (red) and E-cadherin (green). Dashed lines represent the dermo-epidermal layer. Arrows show suprabasal melanocytes in the different conditions. Scale bar represents 20µm. (B) Proportion of suprabasal melanocytes in the different culture conditions. (C-D) Assessment by ELISA of (C) soluble E-cadherin or (D) active MMP-9 levels in cell-free supernatants. (E) Vitiligo perilesional epidermis were treated for 24h in the presence or absence of 1µM of tofacitinib. MMP-9 levels were assessed by ELISA in cell-free supernatants (n=3). (F-G) C57BL/6 mouse tails were treated by intradermal injection of saline solution (control), 1µg TNFα and IFNγ, and/or 1mM of tofacitinib or ruxolitinib, according to the same protocol described in Figure 4F. (F) Representative immunofluorescence analysis of melan-A (red) and E-cadherin (green) staining in the different groups. Dashed lines represent the dermo-epidermal layer. Arrows show suprabasal melanocytes. Scale bars represent 20µm. (G) Proportion of suprabasal melanocytes was assessed in the different groups (n=6-10). Data in B,C,D,G show mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001; ****P < 0.0001, calculated with two-tailed Mann–Whitney test.
Figure 6. Putative model of primary event leading to loss of melanocytes in depigmenting disorders. Type-1 cytokines TNFα and IFNγ produced by activated TRM cells induce an E-cadherin defect in melanocytes. TNFα and IFNγ induce the production of MMP-9 by epidermal cells, especially keratinocytes, that cleave E-cadherin (E-cad) to release its soluble form. E-cadherin cleavage leads to melanocyte destabilization. This effect is inhibited in the presence of MMP-9 or JAK inhibitors.
**Figure 1**

**A**

| Control | Vitiligo | Vitiligo and Psoriasis | Psoriasis |
|---------|----------|------------------------|-----------|

**B**

% of suprabasal melanocytes

**C**

| Control | Stable Vitiligo | Active Vitiligo | Psoriasis | Lupus | Toxic Epidermal Necrolysis |
|---------|-----------------|-----------------|-----------|-------|---------------------------|

% of cleaved caspase 3+

**D**

% of cleaved caspase 3+ MITF+ melanocytes

**E**

| Control | Stable Vitiligo | Active Vitiligo | Psoriasis |
|---------|-----------------|-----------------|-----------|

Soluble E-cadherin (pg/ml)

**F**

$p=0.089$
Figure 1. Melanocyte loss results from defective adhesion of melanocytes and not from apoptosis. (A) Representative immunofluorescence analysis showing melanocytes (red, Melan-A) and basal layer of epidermis stained with an anti-Collagen VII antibody (green) in control healthy skin, vitiligo perilesional skin, skin obtained from patients with concomitant vitiligo and psoriasis, and lesional psoriatic skin. Dashed lines represent dermo-epidermal layer. Arrows show suprabasal melanocytes in different conditions. Scale bars represent 50µm (upper panel) and 20µm (lower panel). (B) Proportion of suprabasal melanocytes in control healthy skin (n=5), stable or active vitiligo perilesional skin (n=18; black squares: stable vitiligo, red squares: active vitiligo), perilesional skin of patients with association of vitiligo and psoriasis (n=4) and lesional psoriatic skin (n=4). (C) Representative analysis of epidermal cell death using cleaved caspase 3 antibody (green). Melanocytes were stained with anti-MITF antibody (red) in control healthy skin, stable and active vitiligo perilesional skin, or lesional skin from psoriasis, cutaneous lupus erythematosus, and toxic epidermal necrolysis. Dashed lines represent dermo-epidermal layer. Scale bar represents 20µm. (D) Proportion of cleaved caspase 3* MITF* basal (●) or suprabasal (▲) melanocytes in control healthy skin (n=3), stable (n=4) or active vitiligo perilesional skin (n=6), lesional skin of psoriasis (n=6), cutaneous lupus erythematosus (n=6) and toxic epidermal necrolysis (n=3). (E) Representative immunofluorescence analysis of expression of E-cadherin (green) and melanocytes (red, Melan-A staining) in control healthy skin, perilesional stable or active vitiligo skin and lesional psoriatic skin. Staining is representative of 10 independent patients. Dashed lines represent dermo-epidermal layer. Arrows identify suprabasal melanocytes. Scale bars represent 50µm (right) and 10µm (left). (F) Assessment by ELISA of soluble E-cadherin levels in sera from healthy controls (n=18) or patients with stable (n=37), progressive (n=38) vitiligo, or psoriasis (n=20). Data show mean ± SEM. *P < 0.05, **P < 0.01; calculated with a Kruskal-Wallis test.
Figure 2

Panel A: Images showing the expression of E-cadherin and Melan-A under different conditions.

Panel B: Bar graph showing the percentage of suprabasal melanocytes under different treatments.

Panel C: Images illustrating the effects of TNFα, IFNγ, and TNFα + IFNγ on E-cadherin and Lamp-1 expression.

Panel D: Graph showing the percentage change in CDH1 expression compared to control in NHEK and NHEM.

Panel E: Graph showing the percentage change in CDH1 expression compared to control with different concentrations of TNFα and IFNγ.

Panel F: Graph showing the percentage change in CDH1 expression compared to control with different concentrations of TNFα, IFNγ, and TNFα + IFNγ.

Panel G: Images showing the expression of E-cadherin and Lamp-1 under different conditions.

Panel H: Graph showing the soluble E-cadherin levels under different treatments.
Figure 2. Combined activity of IFNγ and TNFα reproduces human vitiligo features in a 3D model of reconstructed pigmented epidermis in vitro. (A-C) Reconstructed human pigmented epidermis (RHPE) containing melanocytes were stimulated for 24h in the presence or absence of 10ng/ml of TNFα and IFNγ alone or in combination. (A) Representative immunofluorescence analysis of Melan-A (red) and E-cadherin (green) expression. Right panel shows alteration of E-cadherin expression around melanocytes. Dashed lines represent dermo-epidermal layer. Scale bars represent 20µm (left panel) and 10µm (right panel). (B) Proportion of suprabasal melanocytes in the different conditions (n=4). (C) Representative analysis of epidermal cell death using a TUNEL assay (green), melanocytes were stained with anti-Melan-A antibody (red). Dashed lines represent the dermo-epidermal layer. Scale bar represents 20µm. Staining is representative of three independent experiments. (D) Primary cultures of normal human epidermal keratinocytes (NHEK, left panel, n=10) or melanocytes (NHEM, right panel, n=11) were treated for 24h with 20ng/ml of IFNγ and/or TNFα. CDH1 gene expression in epidermal cells was analyzed by real-time PCR. Results are shown as the % of change compared to the control culture. (E) Dose-response study of CDH1 gene expression in NHEM following 24h stimulation with TNFα and/or IFNγ. (F) Kinetic analysis of CDH1 gene expression in NHEM in response to 20 ng/ml of TNFα and/or IFNγ. Results from one experiment are shown in E and F panels and are representative of four independent experiments with four independent donors. GAPDH was used as a housekeeping gene. (G) Confocal microscopy analysis of RHPE treated in the presence or absence of the combination of 10 ng/ml of TNFα and IFNγ for 24h. Sections were stained for E-cadherin (green) and LAMP-1 (a marker for lysosomes and late endosomes, red). Merge shows the presence of E-cadherin molecule into LAMP-1 vesicle structures. Scale bars represent 20µm and 10µm. Stainings are representative of three independent experiments. (H) Assessment by ELISA of soluble E-cadherin levels in cell-free supernatants of RHPE treated for 24h in the presence or absence of 10 ng/ml of TNFα and IFNγ. Data in B, D, H show mean ± SEM. *P < 0.05, **P < 0.01; calculated with two-tailed Mann–Whitney (B) or Wilcoxon (H) tests.
Figure 3
Figure 3. MMP-9 levels are increased in vitiligo patients and correlate with soluble E-cadherin levels and surface of depigmentation. (A) ELISA levels of MMP-9 in sera of healthy controls (n=18), stable (n=37) or active (n=37) vitiligo, and psoriasis patients (n=20). (B) Active MMP-9 levels in sera of healthy controls (n=22), stable (n=30) or active (n=39) vitiligo, and psoriasis patients (n=19). (C) Spearman’s rho correlation (two-tailed) between MMP-9 and soluble E-cadherin levels in vitiligo patients’ sera (n=73). (D) Spearman’s rho correlation (two-tailed) between serum active MMP-9 and body surface area (BSA) involved in vitiligo patients (n=59). (E) Representative IHC staining of MMP-9 expression in healthy control skin, perilesional skin of stable and active vitiligo, and lesional psoriatic skin. Scale bar represents 100µm. (F) Semi-quantitative analysis of MMP-9 expression in skin from healthy controls (n=5), perilesional skin of vitiligo patients with stable (n=11) or active (n=10) disease, and lesional psoriatic skin (n=8). (G-H) Inflammatory transcriptomic profile of perilesional skin of stable (n=3) and active (n=6) vitiligo patients was assessed using NanoString technology. (G) The most upregulated genes are shown. Results show the change in gene expression between the two groups. (H) Predicted protein-protein interaction networks for upregulated genes using STRING online tool. The thickness of edges represents the strength of data support. The thicker the edge between two proteins, the more these proteins are linked based on the enrichment evidenced by STRING. (I-J) RHPE, NHEK, and NHEM were stimulated for 24h in the absence or presence of TNFα and IFNγ. (I) Real-time PCR analysis of MMP9 gene expression in RHPE (n=7), NHEK (n=9) and NHEM (n=7). Data are shown as fold increase above the control culture. GAPDH was used as housekeeping gene. (J) Levels of MMP-9 in cell-free culture supernatants of RHPE (n=13, left panel) and NHEK (n=9, right panel). (K) Western blot analysis of MMP-9 expression in NHEK treated for 24h in the presence or absence of 20 ng/ml of TNFα and IFNγ. Data in A, B, F, I, J show mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001; calculated with Kruskal-Wallis (A,B,F) or Wilcoxon tests (I,J).
Figure 4

C57BL/6 ID tail injection

Assessment of melanocyte detachment

E-Cadherin
Melan-A

% of suprabasal melanocytes

TNFα + IFNγ + Ab142180 100µM
SB-3CT 100µM

Soluble E-cadherin (µg/ml)

** p=0.0589

G

C57BL/6

Assessment of melanocyte detachment

SB-3CT

% of suprabasal melanocytes

TNFα + IFNγ + SB-3CT

Figure 4
Figure 4. MMP-9 inhibition allows melanocyte stabilization both in vitro and in vivo. (A-C) RHPE were treated for 24h in the presence or absence of increasing concentrations of active MMP-9. (A) Representative immunofluorescence staining of Melan-A (red) and E-cadherin (green). Dashed lines represent the dermo-epidermal layer. Arrows show suprabasal melanocytes in the different conditions. Scale bar represents 20μm. (B) Proportion of suprabasal melanocytes in the different conditions. (C) Assessment by ELISA of soluble E-cadherin levels in cell-free supernatants. (D-F) RHPE were treated for 24h in the presence or absence of 10 ng/ml of TNFα and IFNγ and/or 1, 10 or 100 μM of MMP-9 inhibitors Ab142180 or SB-3CT. (D) Representative immunofluorescence staining of Melan-A (red) and E-cadherin (green). Dashed lines represent the dermo-epidermal layer. Arrows show suprabasal melanocytes in the different conditions. Scale bar represents 20μm. (E) Proportion of suprabasal melanocytes in the different culture conditions. (F) Assessment by ELISA of soluble E-cadherin levels in cell-free supernatants. (G-I) The base of C57BL/6 mouse tail was treated daily for 6 days with intradermal injections of saline buffer (control), or the combination of 1 μg of TNFα and IFNγ, and/or 1.25 mg/ml of SB-3CT. (G) In vivo schema of C57BL/6 mice treatment. (H) Representative immunofluorescence analysis of melan-A (red) and E-cadherin (green) staining in the different groups. Dashed lines represent the dermo-epidermal layer. Arrows show suprabasal melanocytes. Scale bars represent 20μm. (I) Proportion of suprabasal melanocytes was assessed in the different groups (n=7-9). Data in B,C,E,F,I shown mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001; ****P < 0.0001, calculated with two-tailed Mann–Whitney test.
Figure 5

A

Control

TNFα+IFNγ

Tofacitinib

Ruxolitinib

TNFα+IFNγ + Tofacitinib 0.1µM

TNFα+IFNγ + Tofacitinib 1µM

TNFα+IFNγ + Ruxolitinib 0.1µM

TNFα+IFNγ + Ruxolitinib 1µM

B

% of suprabasal melanocytes

Tofacitinib (µM)

Ruxolitinib (µM)

C

Soluble E-cadherin (ng/ml)

Tofacitinib (µM)

Ruxolitinib (µM)

D

Active MMP-9 (ng/ml)

Tofacitinib (µM)

Ruxolitinib (µM)

E

MMP9 (pg/ml) / mg of epidermis

Control

Tofacitinib

F

Control

Tofacitinib

Ruxolitinib

TNFα+IFNγ

TNFα+IFNγ + Tofacitinib

TNFα+IFNγ + Ruxolitinib

G

% of suprabasal melanocytes

TNFα+IFNγ

Tofacitinib

Ruxolitinib

p=0.0649

p=0.0848

p=0.0848

p=0.0848

Figure 5
Figure 5. Inhibition of JAK prevents type-1 cytokine-mediated melanocyte detachment both in vitro and in vivo. (A-D) RHPE were treated for 24h in the presence or absence of 10 ng/ml of TNFα and IFNγ and/or 0.1 or 1 µM of tofacitinib or ruxolitinib. (A) Representative immunofluorescence staining of Melan-A (red) and E-cadherin (green). Dashed lines represent the dermo-epidermal layer. Arrows show suprabasal melanocytes in the different conditions. Scale bar represents 20µm. (B) Proportion of suprabasal melanocytes in the different culture conditions. (C-D) Assessment by ELISA of (C) soluble E-cadherin or (D) active MMP-9 levels in cell-free supernatants. (E) Vitiligo perilesional epidermis were treated for 24h in the presence or absence of 1µM of tofacitinib. MMP-9 levels were assessed by ELISA in cell-free supernatants (n=3). (F-G) C57BL/6 mouse tails were treated by intradermal injection of saline solution (control), 1µg TNFα and IFNγ, and/or 1mM of tofacitinib or ruxolitinib, according to the same protocol described in Figure 4F. (F) Representative immunofluorescence analysis of melan-A (red) and E-cadherin (green) staining in the different groups. Dashed lines represent the dermo-epidermal layer. Arrows show suprabasal melanocytes. Scale bars represent 20µm. (G) Proportion of suprabasal melanocytes was assessed in the different groups (n=6-10). Data in B,C,D,G show mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001; ****P < 0.0001, calculated with two-tailed Mann–Whitney test.
Figure 6. Putative model of primary event leading to loss of melanocytes in depigmenting disorders. Type-1 cytokines TNF\(\alpha\) and IFN\(\gamma\) produced by activated TRM cells induce an E-cadherin defect in melanocytes. TNF\(\alpha\) and IFN\(\gamma\) induce the production of MMP-9 by epidermal cells, especially keratinocytes, that cleave E-cadherin (E-cad) to release its soluble form. E-cadherin cleavage leads to melanocyte destabilization. This effect is inhibited in the presence of MMP-9 or JAK inhibitors.