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NKG2D defines a subset of skin effector memory CD8 T cells with pro-inflammatory functions in vitiligo

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Abbreviations used: CCL: Chemokine (C-C motif) ligand, CXCR3: C-X-C motif chemokine receptor 3, DCs: dendritic cells, Mo-DCs: Monocyte-derived myeloid dendritic cells, IFN: Interferon, IL: Interleukin, ICOS: Inducible T-cell CO-Stimulator, JAK: janus kinase, mAbs: monoclonal antibodies, MICA-MICB: MHC class I chain-related protein A and B, NK: Natural killer, NKG2D: Natural killer group 2D, PBMC: Peripheral blood mononuclear cells, TCR: T cell receptor, TNF: tumor necrosis factor, VETF: Vitiligo European Task Force.

Abstract

Vitiligo is an autoimmune disease that results from the loss of melanocytes, associated with skin infiltration of CD8⁺ effector memory T (T_{EM}) cells with a Tc1 skewed immune response. Natural killer group 2D (NKG2D) is an activating receptor found on immune cells, in particular NK and activated CD8⁺ T cells, that is able to produce a high amount of IFN- γ . Here we found that NKG2D expression was increased in vitiligo skin CD8⁺ T_{EM} cells and was promoted by IL-15. Phenotypic and functional analyses showed that NKG2D⁺ CD8⁺ skin T_{EM} cells displayed an activated phenotype and produced elevated levels of both IFN- γ and TNF- α . Additional experiments revealed that vitiligo skin dendritic cells (DCs) expressed the NKG2D ligands MICA-MICB, and *in vitro* experiments showed that these ligands could be induced on DCs by IFN- α . Cultures of IFN- α stimulated DCs with skin NKG2D⁺ CD8⁺ T cells potentiated the production of type-1 cytokines, that was next inhibited by blocking the NKG2D/MICA-MICB interaction. These data show that NKG2D is a potential marker of pathogenic skin CD8⁺ T_{EM} cells during vitiligo. Therefore, targeting NKG2D could be an attractive strategy in vitiligo, a disease for which there is a strong need of innovative treatments.

INTRODUCTION

Vitiligo is a chronic inflammatory cutaneous disease characterized by the occurrence of patchy white lesions following the loss of epidermal melanocytes. Its pathogenesis involves complex combinatorial factors including dysregulation of both innate and adaptive immune responses (Boniface et al. 2018b; Ezzedine et al. 2015). Vitiligo skin is consistently infiltrated, at least at the margin of active lesions (perilesional site), by immune cells including T cells, with the predominance of CD8⁺ T cells located both in the dermis and epidermis close to melanocytes (van den Boorn et al. 2009; Oyarbide-Valencia et al. 2006). We and others have shown that vitiligo skin is characterized by the presence of resident memory T cells (T_{RM}) involved in flares (Boniface et al. 2018a; Boniface and Seneschal 2019; Cheuk et al. 2017; Richmond et al. 2019; Richmond et al. 2018). In addition, CD8⁺ T cells infiltrating vitiligo perilesional skin exhibit a skewed Tc1 profile characterized by expression of the chemokine receptor CXCR3 and an increased propensity to produce interferon (IFN)- γ and tumor necrosis factor (TNF)- α , while displaying moderate cytotoxic activity (Boniface et al. 2018a; Rashighi et al. 2014; Wang et al. 2016). Several studies have pointed to the role of IFN- γ in disease pathogenesis (Gregg et al. 2010; Harris 2015; Harris et al. 2012; Maouia et al. 2017). Depigmentation was significantly impaired in an autoimmune mouse model of vitiligo deficient for IFN- γ but not in mice deficient for perforin (Chatterjee et al. 2014), IFN- γ was found to be important for the accumulation of T cells in the skin (Harris et al. 2012). Its expression is upregulated in the skin of vitiligo patients and is associated with increased expression of IFN- γ -related genes (Reimann et al. 2012). Lastly, IFN- γ can directly impact the function and survival of melanocytes (Yang et al. 2015). As CD8⁺ T cells play a major role in vitiligo pathogenesis, the identification of a specific marker expressed by these cells that could be targeted to treat patients suffering from the disease is of interest.

Recently, an increased expression of the *KLRK1* (killer cell lectin-like receptor of the subfamily K member 1) gene, encoding the natural killer group 2D (NKG2D) molecule, was demonstrated in vitiligo perilesional skin (Yu et al. 2012). NKG2D is an activating receptor expressed on natural killer (NK) cells, NKT, $\gamma\delta$ and a subset of activated TCR $\alpha\beta$ CD8⁺ T cells (Wensveen et al. 2018). Engagement of NKG2D triggers cytotoxicity on NK cells, cellular proliferation and the production of pro-inflammatory cytokines, including IFN- γ , on T cells, which are important for tissue immune surveillance (Kavazović et al. 2017). However, the role of NKG2D-expressing CD8⁺ T cells in vitiligo remains elusive. Here, we report an increased expression of NKG2D and its ligands MICA-MICB in vitiligo perilesional skin and especially in patients with active disease, CD8⁺ T_{EM} cells being the main cell subset expressing NKG2D in the skin. Interestingly, NKG2D expression was maintained following T cell activation and could be induced by interleukin (IL)-15. Skin-infiltrating NKG2D⁺ CD8⁺ T_{EM} are characterized by an activated phenotype and a high capacity to produce the Tc1-related cytokines IFN- γ and TNF- α . Ligation of NKG2D with its cognate ligands MICA-MICB expressed by dendritic cells (DCs) potentiates the production of pro-inflammatory cytokines IFN- γ and TNF- α by CD8⁺ T_{EM} cells, which could be partially reversed by a NKG2D-blocking antibody. Altogether, these data suggest that NKG2D plays a role in the progression of vitiligo and could be an interesting target to treat it.

RESULTS

Increased expression of NKG2D in CD8⁺ T_{EM} cells in active vitiligo skin

We first analyzed the expression of NKG2D on freshly isolated skin cells. NKG2D expression was significantly increased both in stable and active vitiligo compared to healthy controls (Figure 1a). This result was then confirmed *ex-vivo* by showing upregulated expression of NKG2D on vitiligo perilesional skin sections, especially in active patients (Figure 1b). NKG2D was mainly expressed on NK, NKT and CD8⁺ T_{EM} cells while, as expected, limited or no expression was found on CD4⁺ T_{EM} cells (Figure 1c). We next assessed the relative proportion of NK, NKT, and T cell subsets in vitiligo perilesional skin and show that within these subsets, T cells represented the main cell subset infiltrating vitiligo perilesional skin (Figure 1d and Supplementary Figure S1). In addition, the frequency of NKG2D⁺ CD8⁺ T_{EM} cells was significantly higher in the perilesional skin of vitiligo patients with active disease (Figure 1e and Supplementary Figure S2). This observation was confirmed by immunofluorescence analyses on vitiligo perilesional skin sections, which revealed an increased infiltration of NKG2D⁺ CD8⁺ T cells in active vitiligo in comparison to stable vitiligo or healthy skin from unaffected individuals (Figure 1f-g). In addition, the number of CD8 T cells expressing NKG2D was increased both in patients with stable and active disease in comparison to healthy skin from unaffected individuals (Figure 1h). Altogether, these results show that the increase in NKG2D in active vitiligo skin resulted not only from a more important infiltration of CD8⁺ T cells, but also from an increase in the frequency of CD8⁺ T cells expressing NKG2D. In contrast, perilesional skin of patients with stable disease did not show an increase of CD8 T cell infiltrate; however, a higher number of these cells expressed NKG2D. The presence of NKG2D⁺ CD8⁺ T cells in vitiligo skin was significantly correlated with disease activity (Figure 1i). However, no correlation was observed between the proportion of NKG2D⁺ CD8⁺ T cells and the body surface area involved (data not shown).

Therefore, NKG2D expression on CD8⁺ T_{EM} defines a subset of CD8⁺ T cells possibly involved in disease progression.

Vitiligo skin NKG2D⁺ CD8⁺ T_{EM} cells display an activated phenotype and are directed against melanocyte antigens

Following the observation that vitiligo perilesional skin contained an increased proportion of NKG2D⁺ CD8⁺ T_{EM} cells, we performed a thorough multiparametric flow cytometry analysis to characterize and compare the phenotype of vitiligo perilesional skin NKG2D⁺ and NKG2D⁻ CD8⁺ T_{EM} cells. As shown in Figure 2a and 2b, both the frequency and intensity of expression (MFI) of Ki67 (a cell cycle antigen specific for recent or ongoing proliferation) was significantly increased on NKG2D⁺ CD8⁺ T_{EM} cells. While non-significant, MFI and expression of the T-cell activation-associated markers CD69, ICOS, and HLA-DR tended to increase in vitiligo skin NKG2D⁺ CD8⁺ T_{EM} cells compared to NKG2D⁻ CD8⁺ T_{EM} cell subset. Therefore, owing to the well-known heterogeneity of human samples, we decided to compare the increase in the expression of these markers on NKG2D⁺ skin CD8⁺ T_{EM} cell subset for each patient (compared to NKG2D⁻ cell subset), which revealed a significant increase in the frequency and intensity of expression of CD69 and ICOS in the NKG2D⁺ subset, together with an increase in the frequency of HLA-DR expressing cells (Figure 2c-d). In subsequent experiments, cell surface expression of various regulation (CD25, CTLA-4, GITR, PD-1, KLRG1), migration, skin-homing and residency markers (CXCR3, CCR6, CXCR5, CCR10, CD103, CCR4, CLA) and cytotoxic-associated markers (CD107a, FasL, CRTAM) was analyzed and compared between vitiligo skin NKG2D⁺ and NKG2D⁻ CD8⁺ T_{EM} cell subsets. While displaying high expression of skin-homing receptors such as CLA, CXCR3, CD103, CCR4 and/or CCR6, no major difference was observed in the frequency or expression intensity of these markers between CD8⁺ T_{EM} NKG2D⁺ and NKG2D⁻ cells subsets (Figure 2e

and Supplementary Figure S3). Interestingly, the frequency of cells expressing cytotoxic- or regulation- associated markers was similar and low in the two subsets.

We next examined the expression of NKG2D on auto-reactive melanocyte-specific skin CD8⁺ T_{EM} cells in HLA-A2⁺ vitiligo patients with stable and active disease using melanocyte-antigen specific pentamers (Tyrosinase, MART-1, or gp100). As shown in Figure 2f-g, more than 60% of melanocyte-specific CD8⁺ T_{EM} skin cells expressed NKG2D, but there was no significant difference regarding the level of disease activity. Although non-significant, the frequency of NKG2D-expressing cells tended to be higher in melanocyte-specific CD8⁺ T_{EM} cells (Figure 2g). The presence of melanocyte-specific NKG2D⁺ CD8⁺ T_{EM} cells in the skin of patients with stable disease suggests their possible involvement in the maintenance of depigmentation and/or flare-up of the disease.

NKG2D-expressing CD8⁺ T_{EM} cells infiltrating vitiligo perilesional skin display a skewed pro-inflammatory Tc1 profile

Having defined the phenotype of the NKG2D⁺ CD8⁺ T_{EM} cell subset in vitiligo skin, we analyzed their cytokine-production profile. In line with the modest expression of surface cytotoxic markers (Figure 3a), production of granzyme B and perforin was moderate ~~and similar~~ between the NKG2D⁺ and NKG2D⁻ CD8 T_{EM} subsets (Supplementary Figure S4a). In addition, among the cells expressing NKG2D, T cells, which are the main cell subset infiltrating vitiligo skin, displayed moderate cytotoxic activity compared to the NK and NKT cell subsets (Supplementary Figure S4b). We also measured and compared the frequencies of NKG2D⁺ and NKG2D⁻ CD8⁺ T_{EM} cell subsets producing IFN- γ , TNF- α , IL-17, IL-4, IL-9, IL-22 in vitiligo skin following unspecific stimulation with PMA and ionomycin. NKG2D⁺ CD8⁺ T_{EM} cells were characterized by a Tc1-skewed immune response associated with high production of IFN- γ and TNF- α , while their propensity to secrete IL-17, IL-22, IL-9 and IL-4

remained low (Figure 3a). While NKG2D⁺ and NKG2D⁻ subsets were defined in vitiligo skin by a Tc1 bias in their cytokine profile (Figure 3a-b), NKG2D expressing CD8⁺ T_{EM} cells showed a higher capacity to produce IFN- γ or both IFN- γ and TNF- α in comparison to their negative counterpart (Figure 3a-b). Interestingly, a positive correlation between intracellular IFN- γ production and NKG2D expression in vitiligo skin CD8⁺ T_{EM} cells was observed (Figure 3c). The increase in the frequency of IFN- γ -producing cells in NKG2D⁺ skin CD8⁺ T_{EM} cell subset was further confirmed following TCR activation (Figure 3d). The activated vitiligo skin NKG2D⁺ CD8⁺ T_{EM} cell subset produced higher levels of Tc1-related cytokines compared to the NKG2D⁻ T_{EM} cell population (Figure 3e-f). Altogether, these data show that expression of NKG2D on skin CD8⁺ T_{EM} cells defines a highly functional melanocyte-specific CD8⁺ T cell subset important for the pathogenesis of vitiligo. Furthermore, a comprehensive overview of Tc1 and Tc17 cytokines expression in NKG2D⁺ skin CD8⁺ T cells was obtained by flow cytometry using a nonlinear dimensionality reduction algorithm with stochastic neighbor embedding. As shown in Figure 3g, clusters of TNF- α and IFN- γ producing cells were present in NKG2D⁺ cells, while IL-17 producers segregated in CD8⁻ NKG2D⁻ cells, likely being Th17 cells.

NKG2D expression by skin CD8⁺ T_{EM} cells is not affected following TCR activation and is promoted by IL-15

Surface expression of the TCR $\alpha\beta$ subunits is important for T-cell activation. Therefore, we next investigated the intensity of TCR $\alpha\beta$ levels on vitiligo skin NKG2D⁺ and NKG2D⁻ CD8 T_{EM} cell subsets. As shown in Figure 4a, vitiligo perilesional skin NKG2D⁺ CD8⁺ T_{EM} cells showed increased expression of TCR $\alpha\beta$ compared to the NKG2D⁻ compartment, suggesting that the NKG2D⁺ CD8⁺ T_{EM} cell population is prompted to be strongly activated following cognate stimulation. Indeed, some T-cell surface markers were previously found to be

downregulated following TCR activation and/or depending on the cytokine environment (San José et al. 2000). As shown in Figure 4b, TCR activation did not regulate the frequency of NKG2D-expressing skin CD8⁺ T_{EM} cells. Similar results were found following TCR stimulation for 36, 48 and 72h in the presence of cytokines or chemokine ligands known to be elevated in vitiligo perilesional skin (i.e. CXCL9, CXCL10, IFN- γ or TNF- α) (Figure 4c and Supplementary Figure S5). These data suggest that NKG2D expression on activated CD8⁺ T_{EM} cells remains stable in vitiligo skin and that this surface marker is an accessible potential target for vitiligo management.

In contrast to the increased skin infiltration of NKG2D⁺ CD8⁺ T_{EM} cells in active vitiligo, we failed to detect any significant difference in the proportion of NKG2D⁺ CD8⁺ T_{EM} cells in peripheral blood between stable, active vitiligo patients and healthy control donors (data not shown), suggesting that a skin factor could be responsible for the promotion of NKG2D expression. Peripheral blood mononuclear cells (PBMC) were cultured *in vitro* with type-1 pro-inflammatory: cytokines (IFN- γ , TNF- α , or IFN- α) or chemokines (CXCL9, CXCL10). These soluble factors displayed a limited or no effect in regulating NKG2D expression. Conversely, IL-15 promoted NKG2D expression on CD8⁺ T_{EM} cells (Figure 4 d-e). Interestingly, low doses of IL-15 significantly increased NKG2D expression in vitiligo peripheral CD8⁺ T_{EM} cells but not in CD4⁺ T_{EM} cells (Figure 4f). Finally, IL-15 upregulated the intensity of NKG2D expression not only in active CD8⁺ T cells but also in NK and NKT cells in vitiligo patients' blood. Furthermore, IFN- α showed a capacity to induce expression of NKG2D in NKT cells and more slightly in CD8⁺ T_{EM} cells (Figure 4g).

IFN- α upregulates expression of the NKG2D ligands MICA-MICB in antigen-presenting dendritic cells and promotes IFN- γ production by vitiligo skin NKG2D⁺ CD8⁺ T_{EM} cells

Engagement and activation of the NKG2D receptor depend on the interaction with its cognate ligands, such as membrane bound MICA-MICB (Dhar and Wu 2018). Therefore, we wondered whether these ligands could be involved in the activation of vitiligo NKG2D⁺ CD8⁺ T_{EM} cells. Flow cytometry was used to assess the frequency of vitiligo patients' circulating cell subsets able to express MICA and MICB. We observed that MICA/MICB⁺ cells were restricted to the CD11c⁺DR⁺ myeloid DCs subset (Figure 5a). Using immunofluorescence, we confirmed the expression of MICA-MICB on vitiligo skin CD11c⁺ DCs, with limited or no expression by epidermal cells, while such expression remained low in healthy skin sections (Figure 5b). Primary cultures of human melanocytes and keratinocytes expressed low basal levels of NKG2D ligands, and their expression was not induced in response to pro-inflammatory soluble factors known to be important in vitiligo pathogenesis (Supplementary Figure S6). In contrast, MICA and MICB expression was increased on the surface of monocyte-derived dendritic cells Mo-DCs in response to IFN- α (Figure 5c-e).

We next investigated the functional consequence of membrane expression of MICA-MICB by DCs on T cells. To this end, generated Mo-DCs pre-treated or not with IFN- α were co-cultured for four days with autologous vitiligo skin CD8⁺ T_{EM} cells in the presence or absence of anti-NKG2D blocking mAb. Intracellular cytokine production was evaluated by flow cytometry. As shown in Figure 5f-g, IFN- α -stimulated Mo-DCs potentiated IFN- γ production by vitiligo skin NKG2D⁺ CD8⁺ T cells in comparison to unstimulated Mo-DCs, while no significant changes were observed for IL-17 and TNF- α production. This increased production of IFN- γ was significantly inhibited in the presence of NKG2D blocking mAb, suggesting that NKG2D could be an attractive therapeutic target to limit activation of IFN- γ producing CD8⁺ T_{EM} cells in vitiligo.

DISCUSSION

Our findings demonstrate that NKG2D expression on CD8⁺ T_{EM} cells defines a highly functional memory CD8⁺ T-cell subset specific for melanocyte antigens and producing elevated levels of IFN- γ and TNF- α in vitiligo. As we previously reported that almost all CD8⁺ T cells infiltrating vitiligo perilesional skin express the Tc1 marker CXCR3 (Boniface et al. 2018a), NKG2D⁺ CD8⁺ T_{EM} cells could be highly involved in disease progression. This hypothesis is further reinforced by the correlation between NKG2D expression on skin CD8⁺ T_{EM} cells and disease activity. Moreover, we show that skin-infiltrating antigen-presenting cells can express the NKG2D ligands MICA-MICB upon inflammation and could represent a cell subset able to promote activation of NKG2D⁺ CD8⁺ T_{EM} cells in the skin. This is in line with previous reports showing an increased infiltration of skin DCs in vitiligo (Wang et al. 2011). DCs are essential for CD8⁺ T_{EM} cell activation and cytokine production. Interestingly, the type I IFN signature that characterizes vitiligo progression (Bertolotti et al. 2014) is associated with increased expression of NKG2D ligands on skin APCs, as previously observed in other models (Jinushi et al. 2003), and is in line with our previous work showing the involvement of IFN- α in the progression of the disease (Jacquemin et al. 2017).

Vitiligo skin-infiltrating NKG2D⁺ CD8⁺ T_{EM} cells produced elevated levels of the Tc1 cytokines IFN- γ and TNF- α but displayed moderate cytotoxic activity in comparison to other cell subsets expressing NKG2D, such as NK and NKT cells. Moreover, our data show that among the NKG2D-expressing cell subsets, NKG2D⁺ CD8⁺ T_{EM} cells are the most abundant cell population in vitiligo skin. These data reinforce the observation made on mouse models showing that vitiligo is predominantly dependent on IFN- γ and does not require cytotoxic molecules to develop (Chatterjee et al. 2014).

Additionally, we observed a stable expression of NKG2D following TCR activation and in response to various pro-inflammatory type-1-related cytokines and chemokines that are

upregulated in vitiligo skin, suggesting that NKG2D might be a reliable molecular target for future therapies. NKG2D is currently being investigated as a target in other chronic conditions such as inflammatory bowel diseases (e.g. Crohn's disease). Interestingly, a neutralizing anti-NKG2D mAb used in Crohn's disease induced a significant decrease in IFN- γ production and reduced disease activity (Allez et al. 2017; Cox et al. 2015). Importantly, we identified IL-15 as being a soluble factor important for promoting NKG2D in vitiligo CD8⁺ T_{EM} cells. Recently, interest has increased in the role of IL-15 in chronic inflammatory skin diseases, including vitiligo. IL-15 can be released by epidermal cells, such as keratinocytes (Blauvelt et al. 1996), and can be trans-presented by the IL-15 receptor alpha subunit CD215 to promote memory T-cell activation. In this line, it was recently shown that epidermal cells expressed higher amounts of CD215 in lesional vitiligo skin compared to non-lesional skin (Chen et al. 2019; Richmond et al. 2018). Moreover, trans-presentation of IL-15 by CD215-expressing keratinocytes promoted memory T-cell function and IFN- γ production. Another line of evidence for the involvement of the IL-15/IL-15 receptor in vitiligo is the durable and significant repigmentation observed in response to IL-15 signaling blockade in a vitiligo mouse model (Richmond et al. 2018). Therefore, the activation and function of the NKG2D⁺ CD8⁺ T_{EM} cell subset might also be affected by such inhibition.

IL-15 is a member of the common cytokine receptor gamma-chain family that induces activation of the janus kinase (JAK)-1 and JAK3 signaling pathway after interaction with its cognate receptor. JAK inhibitors are showing promise in various chronic inflammatory disorders, as recently reported in vitiligo (Craiglow and King 2015). An increasing number of reports are showing repigmentation in patients receiving tofacitinib, a JAK1 and JAK3 inhibitor (Liu et al. 2017). Other reports have highlighted the potential benefit of using JAK1 and JAK2 inhibitors (e.g. ruxolitinib), thereby blocking the consequence of IFN- γ signaling on melanocyte loss in vitiligo (Harris et al. 2016; Rothstein et al. 2017).

Lastly, our findings suggest that NKG2D could be a potential specific target associated with disease activity and pro-inflammatory type-1 cytokine-producing cells that are restricted to the skin. Future clinical trials will undoubtedly identify which pathways will prove more suitable for the management of vitiligo, a disease for which there is still no efficient therapy.

MATERIALS AND METHODS

Patients

Fifty-four vitiligo patients seen in the Department of Dermatology at the University Hospital in Bordeaux were included in the study and signed an informed consent form approved by the local institutional ethics committee. None of them had received any treatments/immunosuppressive therapies in the previous six months. They were classified according to the Vitiligo European Task Force (VETF) scoring system (Taïeb et al. 2007) and using Wood's lamp examination to assess the presence of confetti-like lesions or trichome lesions, as previously reported (Benzekri et al. 2013; Sosa et al. 2015). Patients with a total spreading score +3, +4 or +5, according to the VETF scoring system and/or the presence of hypomelanotic lesions with poorly defined borders or confetti-like lesions were considered active and those with a total spreading score <1 or the absence of new lesions over the previous 12 months were considered stable. Their characteristics are summarized in supplementary Table S1. A 4-mm skin biopsy (48 patients) or peripheral blood (6 patients) was harvested.

Healthy donor skin samples were obtained from reconstructive skin surgery performed at the University Hospital in Bordeaux. Blood from healthy subjects was obtained from volunteers who were free of autoimmune or inflammatory disorders.

Processing of samples

PBMC were isolated by density gradient centrifugation through Ficoll (density 1077; Eurobio, Les Ulis, France). Skin T cells were isolated from three-week-old explant cultures maintained with IL-2 and IL-15 (R&D systems, Minneapolis, MN), as described in the literature (Clark et al. 2006b; Clark et al. 2006a). When indicated, a 4-mm skin biopsy from healthy donors or vitiligo patients was extensively minced and freshly isolated cells were obtained by filtering

skin through a 40- μ m cell strainer. When studying the impact of TCR activation, peripheral or skin T cells were activated in the presence of plate-bound anti-CD3 (1 μ g/ml, clone OKT3, Biolegend) and soluble anti-CD28 mAb (3 μ g/ml, clone CD28.2, Biolegend). For some experiments, 20 ng/ml of recombinant human CXCL9, CXCL10, IFN- γ , TNF- α , IL-15 (R&D systems), or IFN- α (2000U/ml) (Miltenyi Biotec) were added to the cultures.

Generation of Mo-DCs and co-culture

CD14⁺ monocytes were purified from vitiligo patients' PBMC using anti-CD14 MicroBeads (Miltenyi Biotec). Mo-DCs were generated following stimulation of monocytes with recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF) (25 ng/ml) and recombinant human IL-4 (10 ng/ml) (Miltenyi Biotec,) for 4 days. For *in vitro* Mo-DCs:T cells co-culture assay, Mo-DCs were first stimulated with or without 2000 UI of IFN- α (Miltenyi Biotec) for 36h. Mo-DCs were then co-cultured for 4 days with vitiligo skin CD8⁺ T cells (Mo-DCs:T cells ratio 1:1) in pre-coated plate-bound anti-CD3 mAb (1 μ g/ml, Biolegend). When indicated, T cells were first pre-treated for 30min with neutralizing anti-NKG2D mAb (1 μ g/ml, clone: 1D11, Biolegend).

Flow Cytometry

To analyze cell surface proteins, cells were stained with the appropriate fluorochrome-labeled monoclonal antibodies (mAbs). The Fc Receptor (FcR) blocking step (Miltenyi Biotec, Cambridge, MA) was first performed when using PBMC. For intracellular staining such as granzyme B, perforin and Ki67 expression, cells were surface-stained, fixed, permeabilized, and stained with appropriately labeled Abs following the manufacturer's instructions (Cytofix/Cytoperm Plus Kit, BD Biosciences, Billerica, MA). When assessing cytokine production, cells were first stimulated for 4 hours with 50 ng/ml phorbol 12-myristate 13-

acetate, 500 ng/ml ionomycin (Sigma Aldrich, Dorset, UK), and monensin (Golgi-Stop, BD Biosciences). All mAbs used are listed in supplemental Table S2. For freshly isolated skin cells, dead cells were stained with 4',6-diamidino-2-phenylindole (DAPI, Biolegend) and gated out during analysis. To assess the frequency of melanocyte antigen-specific T cells, we used phycoerythrin-conjugated HLA-A0201 restricted pentamers loaded with MART-1 (26-35) (ELAGIGILTV), tyrosinase (369-377) (YMDGTMSQV), gp100 (154-162) (KTWGQYWQV) made by Proimmune (Oxford, UK) using Pro5 MHC Class I pentamer technology. Vitiligo skin T cells were collected and analyzed as previously described. Data were acquired on a Canto II or Fortessa cytometer (BD Biosciences) and analyzed with DIVA (BD Biosciences) or FlowJo (Tree Star, Ashland, OR) software.

Immunofluorescence

Three- μ m sections were prepared from formalin-fixed, paraffin-embedded skin biopsies. Sections were deparaffinized and subjected to a heat-induced epitope retrieval step. Anti-NKG2D, anti-CD8, anti-MICA-MICB primary Abs were used. After subsequent washing, the sections were mounted with Prolong Gold antifade reagent with DAPI (Thermo Fisher Scientific, Cambridge, MA) and covered with a coverslip. Cells were counted per three high-power fields with an original magnification of $\times 400$, and the mean number was calculated. All Abs used are listed in supplementary Table S2.

Statistics

Statistical analysis was performed with the unpaired Mann-Whitney *U* test or the Wilcoxon matched-pairs signed-rank test, using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). Correlation analysis was determined using the Spearman rank correlation test. Data are presented as mean \pm SEM and a *P* value of less than 0.05 was considered significant.

Data availability

The authors confirm that the data supporting the findings of this study are available in the article and its supplementary materials.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: CJ, JS, KB; Data Curation: CJ, JS, KB; Formal Analysis: CJ, JS, KB; Funding Acquisition (ATIP-AVENIR, ANR JCJC): JS, KB; Investigation: CJ, CM, FL, DT, JS, KB; Methodology: CJ, JS, KB; Project administration: JS, KB; Resources: AT, JS, KB; Software: CJ, JS, KB; Supervision: JS, KB; Validation: AT, JS, KB; Visualization: CJ, JS, KB; Writing - Original Draft Preparation: CJ, JS, KB; Writing - Review and Editing: CJ, JS, KB. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1. NKG2D-expressing cells in perilesional skin of vitiligo patients. (a) The frequency of NKG2D-expressing cells was assessed by flow cytometry on freshly isolated skin cells (gated on all skin live cells as shown in Supplementary Figure S1) in active and stable vitiligo patients' perilesional skin and healthy control skin. (b) Representative immunofluorescence analysis showing NKG2D expression (red) and DAPI (blue) in control healthy skin, stable and active vitiligo perilesional skin. Dashed lines represent dermo-epidermal layer. Scale bars represent 50µm (upper panel) and 20µm (lower panel). (c) Representative flow cytometry analysis of NKG2D expression in vitiligo skin NK (CD56⁺CD3⁻), NKT (CD56⁺CD3⁺), CD4⁺ and CD8⁺ T_{EM} cell subsets. (d) Flow cytometry analysis of the proportion of NK, NKT and T cells in control healthy skin, stable and active vitiligo perilesional skin (gated on all skin live cells and exclusion of CD3⁻/CD56⁻ cells, as shown in Supplementary Figure S1). (e) The frequency of NKG2D-expressing CD8⁺ T cells was assessed by flow cytometry in healthy control and patients with stable or active vitiligo. (f) Representative immunofluorescence analysis showing NKG2D (red) and CD8 (green) expression in control healthy skin, active and stable vitiligo perilesional skin. Dashed lines represent dermo-epidermal layer. Scale bars represent 50µm. (g,h) Quantification of skin NKG2D⁺ CD8⁺ T cells. (i) Spearman's rho correlation (two-tailed) between the frequency of NKG2D-expressing cells in vitiligo skin CD8⁺ T cells (determined by flow cytometry in e) and disease activity score in vitiligo patients. Data in a,c,d,e,i were obtained from freshly isolated skin cells. Data are means ± SEMs. * $P < .05$, ** $P < .01$, and **** $P < .0001$, using a two-tailed Mann-Whitney U test.

Figure 2. Phenotype of vitiligo skin NKG2D⁺ and NKG2D⁻ CD8⁺ T_{EM} cells. T cells were isolated from vitiligo skin explants as described in the method's section. (a-d) Flow cytometry analysis of the frequency (%) (a) and mean fluorescence intensity (MFI) (b) of CD69, ICOS, HLA-DR, and Ki67 expression in vitiligo skin NKG2D⁺ (red columns) and NKG2D⁻ (black columns) CD8⁺ T_{EM} cell subsets. The ratio of the frequency (c) or MFI (d) of each marker in NKG2D⁺ CD8⁺ T_{EM} compared to NKG2D⁻ CD8⁺ T_{EM} subset was determined for each patient (showed as a fold increase). (e) The expression of indicated regulation, migration, skin-homing, residency or cytotoxic-associated markers was assessed by flow cytometry in vitiligo perilesional skin NKG2D⁺ and NKG2D⁻ CD8⁺ T_{EM} skin cell subsets. Both the frequency (upper panel) and MFI (lower panel) are shown for each marker. (f,g) HLA-A2 pentamers loaded with the melanocyte peptides tyrosinase (Tyr), MART-1, or gp100 were used to identify HLA-A2 restricted melanocyte-specific CD8⁺ T_{EM} cells (f). Flow cytometry analysis of the frequency of NKG2D-expressing cells in pentamer⁺ (melanocyte-specific T cells for Tyr, MART-1, and gp100, red circles) and pentamer⁻ (Tyr-, MART-1-, gp100-, black squares) CD8⁺ T_{EM} cells isolated from perilesional skin of patients with stable or active vitiligo. Data are means ± SEMs. * $P < .05$, ** $P < .01$, using a two-tailed Mann-Whitney U test.

Figure 3. Cytokine profile of NKG2D⁺ CD8⁺ T_{EM} cells in vitiligo perilesional skin. T cells were isolated from vitiligo skin explants as described in the method's section. (a) Flow cytometry intracellular analysis of IFN- γ -, TNF- α -, IFN- γ /TNF- α -, IL-17-, IL-4-, IL-9-, IL-22-, granzyme B-, and perforin-producing cells in NKG2D⁺ (red bars) and NKG2D⁻ (black bars) skin infiltrated CD8⁺ T_{EM} cells in vitiligo patients. Results from each patient is shown together with the mean. (b) The increase in the frequency of IFN- γ -, TNF- α -, and IFN- γ /TNF- α -producing-cells in skin CD8⁺ T_{EM} NKG2D⁺ subset compared to NKG2D⁻ subset was

evaluated for each patient (showed as a fold increase). (c) Spearman's rho correlation (two-tailed) between vitiligo perilesional skin NKG2D⁺ CD8⁺ T_{EM} cells and IFN- γ -producing, skin-infiltrating CD8⁺ T_{EM} cells. (d-g) Skin T cells were activated 4 days with anti-CD3 and anti-CD28 mAbs. Flow cytometry analysis of intracellular IFN- γ , TNF- α and IL-17 production by skin-infiltrating NKG2D⁺ (red) and NKG2D⁻ (black) CD8⁺ T_{EM} cells following PMA/ionomycin stimulation. The frequency (d) and the MFI (e,f) are shown. Data from 4 independent vitiligo patients (#1 to #4) are shown. (g) t-SNE map of vitiligo skin CD3⁺ T cells according to the expression level of the indicated markers. The right axis represents minimum and maximum values of the color scale. The same scales were used between all markers. *** $P < .001$, using a two-tailed Mann-Whitney U test.

Figure 4. IL-15 is involved in upregulation of NKG2D expression by CD8⁺ T cells. (a-c)

T cells were isolated from vitiligo skin explants as described in the method's section. (a) Flow cytometry analysis of TCR $\alpha\beta$ expression in NKG2D⁺ (red) and NKG2D⁻ (black) CD8⁺ T_{EM} cell subsets isolated from vitiligo perilesional skin. The ratio between the TCR $\alpha\beta$ MFI in NKG2D⁺ CD8⁺ T_{EM} cells / MFI in NKG2D⁻ CD8⁺ T_{EM} cells is shown. (b,c) Surface expression of NKG2D on CD8⁺ T_{EM} cells isolated from healthy control skin was analyzed following T-cell stimulation with anti-CD3 and anti-CD28 mAbs for 36, 48 and 72h in the absence (b) or presence (c) of "vitiligoïd" pro-inflammatory chemokines (CXCL9, CXCL10) or cytokines (IFN- γ , TNF- α) (n=4). (d,e) Vitiligo patients' PBMCs were stimulated in the presence of the indicated cytokines or chemokines for 36h and NKG2D expression on CD8⁺ T cells was evaluated (n=6). Representative staining is shown in d. (f) NKG2D expression on CD8⁺ and CD4⁺ T cells was evaluated in vitiligo patients' PBMC stimulated with increasing concentrations of IL-15 (n=4). Data are means \pm SEMs. * $P < .05$, using a nonparametric two-

tailed paired Wilcoxon test. (g) NKG2D MFI was studied in indicated circulating cell subsets in response to various cytokines and chemokines (n=4).

Figure 5. Expression of NKG2D ligands MICA-MICB by myeloid dendritic cells in vitiligo patients. (a) Representative flow cytometry analysis of vitiligo patients' circulating positive cells for MICA-MICB (red plot) in CD3, CD11c and HLA-DR cells (gray plot) (n=4). (b) Immunofluorescence microscopy analysis of MICA-MICB (red) and CD11c (green) expression in skin biopsy samples from healthy control and patients with active vitiligo. Dashed lines represent dermo-epidermal layer. Scale bars represent 50µm. (c) representative flow cytometry analysis and (d) cumulative experiments assessing MICA-MICB expression in vitiligo Mo-DCs stimulated with or without IFN- α for 36h. (e) Assessment of MICA-MICB expression on vitiligo Mo-DCs following stimulation with increasing concentrations of IFN- α (n=3). (f,g) Vitiligo Mo-DCs were first stimulated for 36h with or without IFN- α and were then co-cultured for 4 days with anti-CD3 activated vitiligo perilesional skin T cells (pre-treated or not with blocking anti-NKG2D mAb). Analysis of IFN- γ , TNF- α , and IL-17 production by skin NKG2D⁺ CD8⁺ T_{EM} cells as performed by flow cytometry after PMA/ionomycin stimulation. Data are means \pm SEMs. * P <.01, using a two-tailed Mann-Whitney U test and using a nonparametric two-tailed paired Wilcoxon test for Mo-DCs:T-cell co-culture analyses.

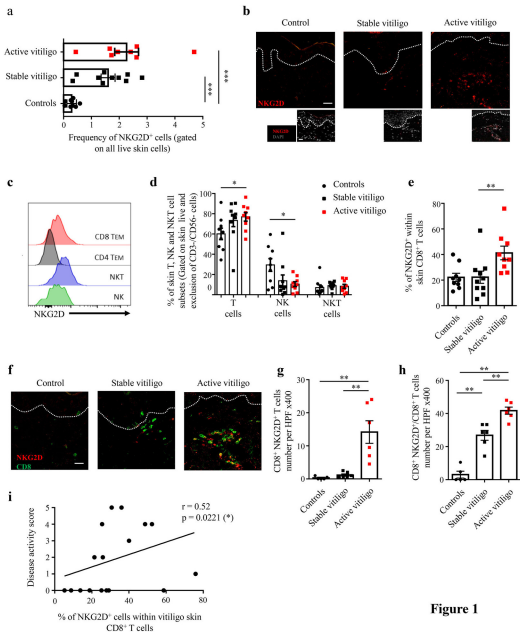


Figure 1

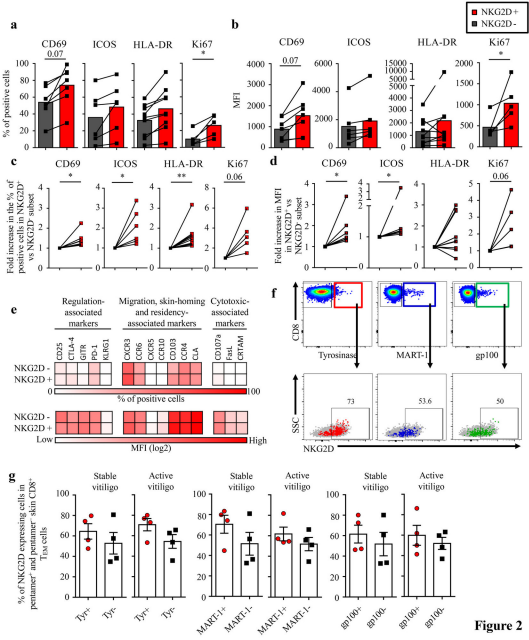


Figure 2

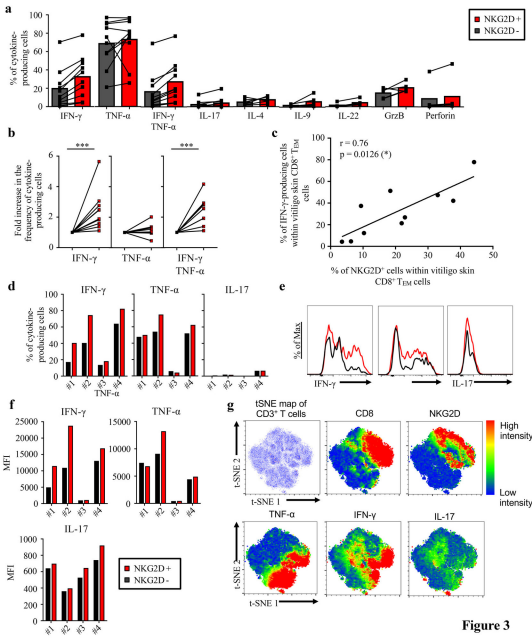


Figure 3

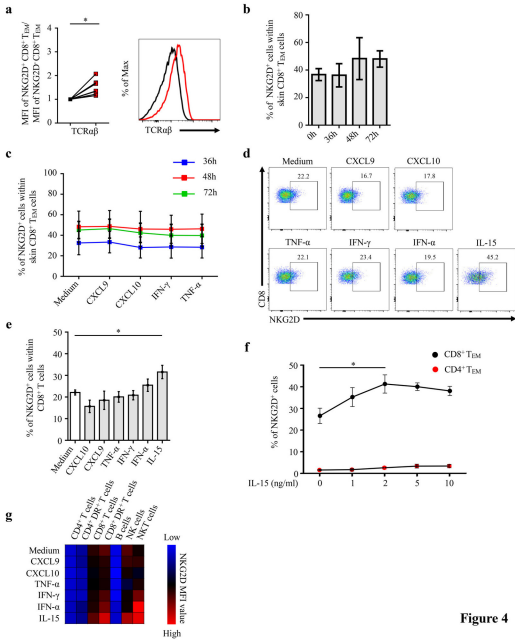


Figure 4

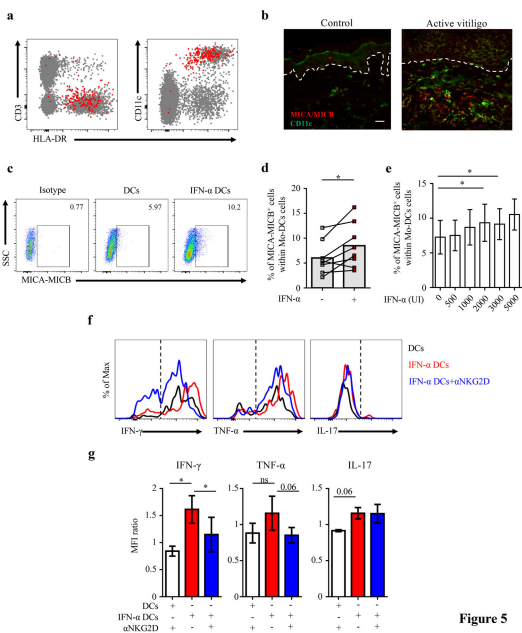


Figure 5