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Human innate immune cell crosstalk induces melanoma cell senescence

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ABSTRACT

Mononuclear phagocytes and NK cells constitute the first line of innate immune defense. How these cells interact and join forces against cancer is incompletely understood. Here, we observed an early accumulation of slan+ (6-sulfo LacNaC) non-classical monocytes (slanMo) in stage I melanoma, which was followed by an increase in NK cell numbers in stage III. Accordingly, culture supernatants of slanMo induced migration of primary human NK cells in vitro via the chemotactic cytokine IL-8 (CXCL8), suggesting a role for slanMo in NK cell recruitment into cancer tissues. High levels of TNF-α and IFN-γ were produced in co-cultures of TLR-ligand stimulated slanMo and NK cells, whereas much lower levels were contained in cultures of slanMo and NK cells alone. Moreover, TNF-α and IFN-γ concentrations in slanMo/NK cell co-cultures exceeded those in CD14+ monocyte/NK cell and slanMo/T cell co-cultures. Importantly, TNF-α and IFN-γ that was produced in TLR-ligand stimulated slanMo/NK cell co-cultures induced senescence in different melanoma cell lines, as indicated by reduced melanoma cell proliferation, increased senescence-associated β-galactosidase expression, p21 upregulation, and induction of a senescence-associated secretory phenotype (SASP). Taken together, we identified a role for slanMo and NK cells in a collaborative innate immune defense against melanoma by generating a tumor senescence-inducing microenvironment. We conclude that enhancing the synergistic innate immune crosstalk of slanMo and NK cells could improve current immunotherapeutic approaches in melanoma.

Introduction

Despite recent success with T cell-based checkpoint inhibitor immunotherapies, a high number of melanoma patients still do not respond to current treatments. For these patients, an optimal stimulation of innate immune cell populations, representing the first line of innate immune defense against pathogens and tumors, might reestablish functional immune responses and reduce tumor growth.

Monocytes are an abundant blood leukocyte population that develop into macrophages in tissues and exert both homeostatic and inflammatory functions. slan+ monocytes (slanMo) are part of the non-classical monocyte (CD14dim/CD16+) subset in humans. They are defined by a carbohydrate modification on P-selectin glycoprotein-ligand 1 (PSGL-1), which can be specifically recognized by the monoclonal antibody M-DC8. slanMo share several characteristics with dendritic cells, which distinguish them from conventional CD14+ monocytes, including high cytokine production and efficient T cell priming. It was reported that tumor cells can activate slanMo and stimulate TNF-α production. slanMo can be detected at tumor-draining lymph nodes of patients with different solid tumors as well as non-Hodgkin lymphoma. The precise role and function of slanMo in cancer tissues is still under investigation, but recent reports have suggested the presence of a macrophage-like and a DC-like phenotype in tissues. slanMo can increase proliferation, expression of activation markers such as CD69, NKG2D, Nkp46, and CD95, IFN-γ cytokine production and cytotoxicity of NK cells. In turn, NK cells can promote slanMo maturation and IL-12 production, thereby facilitating adaptive immune responses. So far, it is unclear whether the crosstalk between slanMo and NK cells can result in an improved anti-tumor response.

The composition of the tumor microenvironment (TME) is dictated by several factors, including the need for appropriate chemotactic stimuli targeting the different immune subsets. In the murine system, patrolling monocytes were recently suggested to guide NK cell recruitment into tumors. In a mouse model of melanoma lung metastasis mice lacking patrolling monocytes (Nr4a1 super enhancer −/+) showed impaired NK cell recruitment and a reduced anti-metastatic activity. Human blood NK cells are attracted by various chemokines, including CCL1 (fractalkine), CCL2, CCL3, CCL4, CCL5, CXCL8 (IL-8), CXCL10. For instance, the IL-8 chemokine receptors CXCR1 and CXCR2 are expressed on CD56dimCD16+ NK cells and mediate recruitment into

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tissues.\textsuperscript{14,15} NK cells are known for exerting direct perforin/granzyme B-mediated cytotoxicity against tumor cells in the tissue. Alternatively, it was recently reported that NK cell cytokine secretion can arrest tumor cell growth through sensing of PDGF-DD via Nkp44 on NK cells.\textsuperscript{16} However, it is largely unknown which cell types are capable of recruiting human NK cells to tumor sites.

Cellular senescence in tumors is a common response to oncogene-mediated transformation, termed oncogene-induced senescence.\textsuperscript{17} However, other forms of senescence induction are possible, including homeostatic triggers such as telomere-mediated replicative senescence or the DNA-damage response. Those triggers lead to activation of the p53/p21 (CDKN1A) or the Retinoblastoma (Rb)/p16\textsuperscript{nk4a} pathways, resulting in a persistent growth arrest.\textsuperscript{18,19} Recent reports suggest that the proinflammatory cytokines TNF-\(\alpha\) and IFN-\(\gamma\) can induce senescence, termed cytokine-induced senescence.\textsuperscript{20–23} In a \(\beta\)-cancer mouse model, it was demonstrated that antigen-specific T cells were able to limit tumor progression via TNF-\(\alpha\)/IFN-\(\gamma\)-dependent senescence induction.\textsuperscript{24} This mechanism was reliant on both TNF-\(\alpha\) and IFN-\(\gamma\) mediated signaling, as the individual cytokines alone were not sufficient to induce senescence. The contribution of innate immunity to tumor senescence is still incompletely understood.

In this study, we investigated how slanMo and NK cells collaboratively affect melanoma growth. Since the slanMo/NK cell crosstalk relies on those cells being present in the TME, we initially analyzed patient samples for the presence of slanMo and NK cells. Additionally, we performed migration studies in order to elucidate a potential chemotactic mechanism for R848-activated slanMo recruiting NK cells to the TME. Finally, we determined the mechanism of melanoma growth arrest by inflammatory cytokines produced in slanMo/NK co-cultures. Taken together, the results gained here are of important relevance for therapies targeting innate anti-tumor immunity.

Results

\textit{slanMo and NK cells co-localize in the melanoma immune microenvironment}

To investigate a contribution of a potential slanMo/NK cell crosstalk in anti-melanoma immune responses, we determined the presence and distribution of slanMo and NK cells in formalin-fixed paraffin embedded (FFPE) samples from all four stages of melanoma and in healthy skin samples (Figure 1(a)). Using anti-slan staining with the monoclonal antibody DD2, we frequently found slanMo in stage I melanoma lesions, whereas their numbers were reduced in stage II and remained decreased over the rest of disease progression, with the exception of stage III cutaneous melanoma (Figure 1(a,b)). NK cells, identified by CD56, were present in relatively low numbers in stages I and II, but increased in frequency in stage III (Figure 1(a,c)). Frequencies of NKT cells that express CD56, but also CD3, were very low compared to NK cells in melanoma tissues (data not shown). Of note, CD56\textsuperscript{+} NK cells could be clearly distinguished by morphology from melanoma cells that occasionally expressed low levels of CD56 (Fig. S1). We could not detect NK cells and rarely slanMo in skin from healthy donors (Figure 1(a-c)). When focusing on stage III lymph node metastasis (III LN), we observed similar numbers of slanMo and NK cells with a significant positive correlation (Figure 1(a)).

\textit{slanMo induce NK cell migration via IL-8 production}

Since high numbers of slanMo in melanoma stage I preceded the accumulation of NK cells in melanoma stage III, we asked whether slanMo are capable of recruiting NK cells. We therefore analyzed the expression and secretion of chemokines in supernatants from slanMo, non-stimulated or stimulated with the TLR7/8 agonist R848. Our results show that several chemokines were expressed by slanMo and were upregulated after R848 stimulation, most notably CCL3, CCL4, and IL-8 (Figure 2(a,b)). In order to quantify specific migration of NK cells in response to slanMo-derived chemokines, we established an \textit{in vitro} migration assay, in which R848-activated slanMo-conditioned medium (slanMo CM) was provided in the bottom chamber. NK cells showed significant-specific migration (9% of total input cells normalized to control) toward R848-stimulated slanMo CM (Figure 2(c)), which was strongly reduced using supernatant from slanMo left unactivated (Fig. S2A). R848 alone was not sufficient to induce NK cell migration (Fig. S2A). Next, we tested the ability of NK cells to migrate in response to slanMo-associated CCL3, CCL4, and IL-8 (Fig. S2A). Only very few NK cells responded to CCL3 and CCL4, whereas IL-8 triggered a dose-dependent specific NK cell migration comparable to SDF-1 (CXCL12), which is known to be a potent inducer of NK cell migration (Fig. S2B). Since IL-8 was highly present in the chemotactic slanMo CM, we neutralized IL-8 during NK cell migration. This resulted in a significant reduction in NK cell migration in response to slanMo CM (Figure 2(c)). IL-8 is known to bind to the chemokine receptors CXCRI and CXCRII, mediating internalization and directed migration.\textsuperscript{25,26} Consistent with previous findings, we observed that NK cells expressed CXCRI and CXCRII (Fig. S2C). Migration toward slanMo CM significantly reduced expression of both CXCRI and CXCRII (Figure 2(d)). Moreover, IL-8 neutralization in the slanMo CM significantly abolished receptor down-regulation (Figure 2(d,e)). These data demonstrate that slanMo are capable of recruiting NK cells via IL-8.

\textit{slanMo and NK cells synergistically elicit a cytokine-induced growth arrest in melanoma cells}

We next investigated whether the cytokine milieu generated by slanMo and NK cells affects melanoma cells, as it could occur in the TME. We incubated the melanoma cell line SK-Mel-28 for a period of 3–4 d with conditioned medium (CM) harvested from co-cultures of R848-stimulated slanMo and NK cells. After culture in CM, the melanoma cells were replated and equivalent cell numbers were cultured in the absence of CM. We observed greatly impaired growth of melanoma cells initially exposed to CM, whereas cells cultured in normal medium or with R848 (corresponding to concentrations used for
slanMo/NK cell co-culture assays) grew exponentially (Figure 3(a) and Fig. S3A). In contrast, incubation with medium harvested from individual slanMo or NK cell cultures had minor or no influence on cell growth (Figure 3(a)). Thus, the observed growth arrest required the combination of slanMo and NK cells for the production of soluble factors mediating the melanoma growth-limiting effect.

To investigate potential factors mediating the growth arrest, we analyzed the cytokine secretion profile in the CM of slanMo/NK cell co-cultures relative to mono- and co-cultures of other mononuclear cells that can be present in the TME. We observed that R848-stimulation and LPS-stimulation strongly upregulated TNF-α, IL-1β, and IL-12 secretion in slanMo mono-cultures (Figure 3(b)), exceeding the cytokine concentrations reached in corresponding CD14+ monocyte (CD14+ Mo) mono-cultures. In contrast, NK cells, as well as T cells, alone produced only negligible cytokine levels <200 pg/ml (data not shown). slanMo/NK cell co-cultures contained higher TNF-α, IFN-γ, and IL-12 levels compared to CD14+ monocyte (CD14+ Mo)/NK cell co-cultures (Figure 3(b)). Moreover, the slanMo/NK cell co-culture yielded higher levels of IFN-γ, TNF-α, and IL-12 compared to slanMo/T cell as well as CD14+ Mo/T cell co-cultures, highlighting the pro-inflammatory cytokine production by the slanMo/NK cell crosstalk. Despite the fact

Figure 1. slanMo and NK cells infiltrate human melanoma. (A) slanMo (slan – green) and NK cells (CD56 – red) were analyzed in FFPE samples of melanoma patients from stage I, stage II, stage III cutaneous lesions (III cutan.), stage III Lymphnode (III LN) metastasis, stage IV distant metastasis, and healthy skin (hSkin) (n = 7). Based on 3 images (200x magnification) of double-stained (slan + CD56) samples, the density of cells/mm² was analyzed and densities of slanMo were correlated with CD56+ NK cells. Density of (B) slanMo (slan) and (C) CD56+ NK cells displayed as cells/mm² over all stages of melanoma development and in healthy skin. Number of patients for stage I (n = 10), stage II (n = 16), stage III (n = 10), stage III LN (n = 10), stage IV (n = 8), and healthy skin (n = 7). Original magnification x100 (left column), x200 (middle column), x630 (right column).
that both slanMo and NK cells are capable of producing TNF-α, we observed that the TNF-α produced in our co-cultures predominantly derived from slanMo, as evidenced by intracellular staining (Fig. S3B). Since TNF-α and IFN-γ were expressed at particularly high levels in TLR-activated slanMo/NK cell co-cultures, we incubated melanoma cells with recombinant TNF-α and IFN-γ. Indeed, exposure of melanoma cells to TNF-α and IFN-γ significantly inhibited proliferation in several melanoma cell lines (Figure 3(c) and 3C). Neutralization of TNF-α and IFN-γ in slanMo/NK cell CM completely abolished the growth arrest and restored exponential cell growth (Figure 3(d)).

Growth inhibition induced by CM was quantified by analyzing the proliferative capacity of melanoma cells with Ki67 Immunofluorescence staining. The percentage of Ki67-positive cells was reduced by ~35% when the cells were cultured in CM (Figure 4(a)). Similar results were obtained when using recombinant TNF-α and IFN-γ (Figure 4(b)). This was supported by a ³H-Thymidine incorporation assay showing that proliferation was reduced to 66% of the untreated control (Figure 4(c)). The results were dependent on CM from co-cultures and consistent for multiple melanoma cell lines (Fig. S3D and S3E). As expected, the growth reduction was confirmed by using recombinant TNF-α and IFN-γ (Figure 4(d)). In both proliferation methods, a reversal of the anti-proliferative effect upon TNF-α and IFN-γ neutralization was observed (Figure 4(a,b,c)). Together, our data demonstrate that TNF-α and IFN-γ produced in slanMo/NK cell co-cultures affect melanoma cell growth by significantly reducing cell proliferation.

**slanMo and NK cells induce senescence in melanoma cells**

To investigate the cellular characteristics of the growth arrest, we determined tumor cell growth by microscopy after culture in CM harvested from slanMo/NK cell co-cultures. We observed the accumulation of morphologically distinct
melanoma cells, displaying enlarged, flattened, and often multinucleated adherent cell features (Fig. S4A inlet). Since these characteristics are reminiscent of a senescence phenotype, we analyzed senescence-associated β-galactosidase (SA β-Gal), which is a common marker for senescent cells. After culture of melanoma cells with co-culture CM, the fraction of SA β-Gal+ cells was increased ~6 fold compared to untreated controls, whereas no increase was observed upon culture with CM from slanMo or NK cell monocultures (Figure 5a, S4B). TNF-α and IFN-γ neutralization significantly reduced SA β-Gal activity, whereas addition of recombinant TNF-α/IFN-γ resulted in higher cell numbers positive for SA β-Gal (Figure 5a, b).

To substantiate the senescent phenotype, we analyzed expression of p21 (CDKN1A) as a downstream target of p53 in the p53 senescence pathway. While treatment of melanoma cells with CM significantly upregulated p21 expression, TNF-α and IFN-γ neutralization normalized p21 levels (Figure 5c). Recombinant TNF-α and IFN-γ induced p21 in different melanoma cell lines such as SK-Mel-28, Ma-Mel-86b, SK-Mel-25, and SK-Mel-30 (Figure 5d), indicating that p21 represents a common regulator of non-proliferative, senescent states in melanoma cells. Moreover, p21 was detected by immunofluorescence after fixation of CM-treated melanoma cells grown on microscopic slides. p21 increased after exposure to CM and recombinant TNF-α and IFN-γ and could be reverted by neutralization of TNF-α and IFN-γ in the CM (Figure 5e, f). When staining p21 in stage II and stage III cutaneous melanoma samples by immunohistochemistry, we observed increasing p21 positive cells with incremental tumor progression (Fig. S4C).

In summary, these data demonstrate that the growth arrest observed in response to slanMo/NK cell co-cultures is linked to a TNF-α/IFN-γ dependent senescence state in melanoma cells characterized by high p21 levels.

**Melanoma cells display a senescence-associated secretory phenotype upon exposure to slanMo/NK cell co-cultures**

Senescence is known to induce changes in the cells’ secretome, termed senescence-associated secretory phenotype (SASP). After treatment with slanMo/NK cell co-culture CM, upregulation of SASP factors such as IL-6, IL-8, IL-1α, IL-1β was detected by qPCR analysis (Figure 6a). Other significantly upregulated markers included the matrix metalloproteinases (MMPs) 1, 2, 3, 9, the serpins PAI-1 and PAI-2, the chemokines CXCL1, CXCL2, CCL2, and CCL7, the growth factor VEGF-A, TGF-β, and ICAM-1. In all cases except for CXCL1 and TGF-β, the mRNA levels were significantly reduced upon TNF-α and IFN-γ neutralization.

Furthermore, we analyzed the expression of IL-6, IL-8, IL-1α, IL-1β at protein level. In line with the mRNA results, IL-6, IL-8, and IL-1β were significantly upregulated after exposure to co-culture CM, for IL-8 even above the maximal detection limit of the assay (Figure 6b). This effect was abolished or reduced after neutralization of TNF-α and IFN-γ. Together, our data provide evidence that slanMo and NK cells cooperate in a synergistic feedback loop that generates high levels of TNF-α and IFN-γ that drive melanoma cells into senescence and trigger the secretion of senescence-associated factors with the potential to further shape the tumor microenvironment.

**Discussion**

Here, we discovered that TLR-ligand stimulated slanMo and NK cells interact in a synergistic crosstalk generating high levels of pro-inflammatory TNF-α and IFN-γ that are able to induce melanoma cell senescence. Thereby, we uncover a novel senescence eliciting role for innate immunity extending the previous concept of T helper 1 (Th1) cell-mediated cytokine-induced senescence. The slanMo/NK cell-induced melanoma senescence phenotype manifested in severely reduced proliferation, increased SA β-Gal expression, and upregulation of common SASP factors. Additionally, our results reveal induction of p21 in senescent melanoma cells. We further demonstrate the relevance of the melanoma senescence phenotype by detecting slanMo and NK cells in the TME in all stages of melanoma development and by suggesting an IL-8-mediated
recruitment mechanism for migration of NK cells toward TLR-activated slanMo.

Our histological analysis revealed that slanMo and NK cells can infiltrate primary and metastatic melanoma. We observed a high density of slanMo, particularly in stage I melanoma, which cannot be considered a homeostatic cutaneous condition since healthy skin was virtually devoid of slanMo. These results extend previous reports that described high numbers of slanMo in metastatic tumor-draining lymph nodes of carcinoma patients but absence of slanMo in the primary carcinoma. Increased slanMo numbers in early melanoma development suggest immediate recruitment to the tumor site. This would appear consistent with the reported patrolling function of human slanMo as a subpopulation of non-classical monocytes. In this model, the local chemokine production by slanMo could trigger the recruitment of other immune cells, such as NK cells. NK cells can be detected in various human tissues, such as LN, lung, liver, or kidney, and are known to traffic to sites of inflammation. In our histological analysis, NK cells were sparse in stages I and II, but increased in numbers in stage III for both satellite tumors and LN metastasis. This strong NK cell influx in stage III was accompanied by a significant correlation of slanMo and NK cell numbers, suggesting a connected migration mechanism. This is intriguing when considering the hierarchical migration routes described by Hanna et al., where Ly6C<sup>low</sup> monocytes recruited NK cells after tumor encounter. We observed substantial migration of human NK cells toward conditioned medium from R848-activated slanMo, which we found to be mostly dependent on IL-8 being produced in particularly high levels by slanMo. This mechanism is consistent with the literature and our results, where expression of the IL-8 receptors CXCR1 and CXCR2 is reported for CD56<sup>dim</sup> NK cells coupled to the ability to migrate toward IL-8 derived from different sources such as dendritic cells. In contrast, there is evidence that CD56<sup>bright</sup> NK cells are the major NK cell population in tumor tissue. However, with limited knowledge about how CD56 expression changes after extravasation from the blood into tissue, it is difficult to compare migration of blood NK cells with cells detected in the tissue. On the other hand, IL-8 has been shown to have pro-tumorigenic effects through recruitment of immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs) or neutrophils. The slanMo-mediated recruitment of other immune population could be the subject of future studies. The discovery of a novel potential innate migration axis is highly relevant for the understanding and initiation of immune responses in tissues. In this context, Böttcher et al. demonstrated that melanoma infiltrating NK cells recruit cDC1 via XCL1 and CCL2, which facilitated the initiation of the adaptive immune responses through...
recruitment and priming of T cells. In this line of thought, our results showing early slanMo infiltration followed by subsequent increase in NK cell numbers could suggest a sequence, in which slanMo extravasate at the site of newly formed tumors or metastasis and then recruit NK cells to initially limit tumor progression and foster adaptive immune responses.

The TME generally plays an important role in tumor growth, progression, and metastasis. We observed that the CM generated...
from TLR-activated slanMo and NK cell co-cultures reduced the proliferation of melanoma cell lines. Together with TNF-α and IFN-γ neutralization experiments, this demonstrated that the slanMo/NK cell co-culture is required to facilitate IFN-γ and TNF-α production-reaching levels capable of limiting melanoma growth. This is consistent with the established concept of the synergistic slanMo/NK cell crosstalk, in which slanMo-derived IL-12 stimulates IFN-γ production by NK cells and vice versa, which generates a positive feedback loop reaching very high levels of IL-12, TNF-α, and IFN-γ. When investigating the cellular basis for the growth arrest, we discovered that slanMo/NK cell co-culture CM upregulated the senescence marker p21 in melanoma cells, suggesting activation of the p53/p21 pathway. Similarly, p21 was also increased in stage III cutaneous melanoma samples compared to stage II, which mirrored the augmented influx of slanMo and NK cells in stage III. Although p21 staining of melanoma samples in situ by itself offers only limited information with respect to senescence in the tumor tissue, our finding suggests that a slanMo/NK cell crosstalk cytokine milieu might drive p21 upregulation and possibly senescence in melanoma in situ. Of note, in Th1-mediated cytokine-induced senescence, senescence was triggered by the p16INK4a/Rb pathway. However, melanoma cells regularly carry mutations in the CDKN2A locus, which is encoding for p16INK4a, arguing for the possibility of a p21-mediated alternative mechanism. p21 acts downstream of p53; however, it can also function in a p53-independent manner.19,39,40 Since also p53 is commonly mutated in melanoma cell lines and in particular in the cell lines SK-Mel-28 and SK-Mel-30 used in this study (Broad Institute Cancer Cell Line Encyclopedia), it appears that p21 is upregulated independent of p53 in our TNF-α + IFN-γ-triggered melanoma senescence model.

Oncogene-induced senescence is established as an important counter-mechanism against initial mutagenic events by limiting proliferation of tumorigenic clones. This cell-intrinsic mechanism is propagated by SASP-mediated release of factors triggering senescence in neighboring cells. Moreover, chemotactic SASP factors recruit innate immune cells supporting the clearance of senescent tumor cells and increased secretion of inflammatory mediators such as IL-1β potentially stimulates anti-tumor immune responses. However, senescence in tumor cells can also be associated with tumor-promoting functions mediated by pro-tumorigenic SASP factors. We discovered that the TNF-α/IFN-γ induced senescence increased the secretion of common SASP factors, most prominently IL-8, IL-6, IL-1β, in addition to different MMPs and chemokines (Figure 6a,b)). Importantly, tumors can utilize SASP-associated chemokines to recruit immune regulatory myeloid cells to the TME, which dampen local tumor-specific immune responses. Additionally, the increased mRNA levels for MMPs might skew the melanoma cells toward higher metastatic potential.

Besides SASP-mediated non-cell-autonomous functions, senescence in tumors can act autonomously through affecting the tumorigenic and invasive potential of cancer cells. This suggests that the innate immune cell-induced senescence phenotype observed in our study might promote tumor progression and invasiveness through cell-autonomous and non-cell-autonomous mechanisms. Of note, NK cells were reported to recognize and eliminate senescent cells. This is enabled through expression of stress-induced ligands such as DNAX accessory molecule-1 (DNAM-1) ligands or UL16 binding proteins (ULBP) on the surface of senescent tumor cells. In line with this, the slanMo/NK cell crosstalk may synergize with controlling melanoma cells in two ways: By inducing senescence-mediated growth arrest of melanoma cells and by augmentation of the melanoma-directed cellular cytotoxicity.

Despite the likely presence of immunogenic cell death products like nuclear acid fragments or nuclear proteins like HMGB1 that can trigger TLR ligands and activate monocytes, it can be assumed that slanMo are not always optimally stimulated in the TME. This is intriguing in the light of TLR 7/8 ligand therapeutics such as imiquimod, which is therapeutically applied for cutaneous melanoma lesions and metastasis. The use of topically applied imiquimod has been successfully studied for the treatment of primary superficial melanoma and for cutaneous melanoma metastasis. Since slanMo express TLR 7 and 8, imiquimod therapy can potentially activate those cells optimally in the TME and thereby initiate a senescence-inducing slanMo/NK cell crosstalk. This is relevant for current clinical trials examining whether checkpoint inhibitor therapy resistance can be overcome by combination treatment with TLR agonists, e.g., for treatment of tistotolimod with ipilimumab in PD-1 refractory melanoma patients (NCT03445533).

Taken together, our results uncover an innate immune cross-talk as inducer of cytokine-induced senescence of melanoma cells. Our data support a concept that TLR 7/8 ligand therapies (e.g., imiquimod) in melanoma patients might strongly activate slanMo, fuel the recruitment of NK cells and lead to the formation of a slanMo/NK cell synergistic crosstalk that effectively hampers tumor progression through TNF-α/IFN-γ-mediated senescence induction.

**Materials and methods**

**Cell lines**

The human melanoma cell lines SK-Mel-28 (ATCC), Ma-Mel-86 (from Dr. Annette Paschen, University Clinics Essen), SK-Mel-25 (ATCC), SK-Mel-30 (ATCC), were cultured in RPMI
1640 (Biochrom) supplemented with 10% FCS (Sigma-Aldrich), 2 mM L-glutamine, and 1% penicillin/streptomycin. Cultured cells were tested negative for mycoplasma contamination (Multiplexion GmbH) and cell line identity was confirmed by MCA assay (Multiplexion GmbH) in 2017 for SK-Mel-28, Mel-86b, and in 2019 again for SK-Mel-30, SK-Mel-25. All cell lines were cultured for 1 week prior to experiments.

**Tissue specimen**

Formalin-fixed paraffin-embedded tissue sections of melanoma patients from all stages (I–IV) were retrieved from the National Center for Tumor Diseases. Characteristics of patients are presented in table S1. Skin samples from healthy donors (n = 7) were included as control tissue. Patient’s written informed consent was obtained prior to sample acquisition and use of samples was reviewed by the Ethic Board of the University of Heidelberg (S-091/2011; S-306/2010).

**Immunohistochemistry (IHC)**

Paraffin-embedded samples were cut into sections of 2–5 microns. After rehydration, antigen retrieval was performed by boiling the samples for 30 minutes in citrate buffer pH 6 (Dako), followed by blocking with 5% goat serum and incubation with the primary anti-human antibodies: slan (Miltenyi, clone DD2, individual hybridoma supernatant, 50 µg/ml), CD56 (Thermo Scientific, clone 123c3, 1:75), CD3 (Abcam, clone SP7, 1:250), CDKN1A/p21 (abcam, clone EPR362, 1:200). Single color stainings were developed using the Dako Real Detection System (Dako) according to the manufacturer’s instructions. For double color immunohistochemistry, after staining the first antigen as described for single staining, the slides were blocked for endogenous peroxidase activity for 5 min with 3% H2O2; then, the biotin-Streptavidin sites from the first reaction were blocked with the Avidin/Biotin Blocking Kit (Vector Laboratories), followed by overnight incubation with purified anti-slan or anti-CD3. The mouse IgM antibody clone DD2 was then detected with a goat anti-mouse IgM-specific detection system and rabbit anti-human CD3 with a goat anti-rabbit specific system (Vector Laboratories). This was followed by chromogen development with Histogreen (Linaris), hematoxylin counterstain, and embedding in xylene-based solution.

Based on three representative 100× magnification images of areas with immune infiltrate and tumor cell interactions of double-stained (sln + CD56) FFPE samples, the density of cells/mm² was analyzed blindly by two independent scientists and correlated for slanMo and CD56⁺ NK cells. p and r² values were calculated using linear regression in Graph Pad Prism. The density of p21 positive cells was quantified analogously with p21 single-stained sections.

**Isolation of slanMo, NK cells, CD14 monocytes, and T cells**

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy adult donors by density gradient purification with Ficoll (Biochrom). slanMo were isolated as described previously.³ Briefly, PBMCs were incubated in diluted M-DC8 hybridoma supernatant for 15 minutes at 4°C. After washing, the cells were incubated with rat anti-mouse IgM microbeads (Miltenyi) for 15 minutes at 4°C, washed again and then slanMo were purified by positive selection with an autoMACS (Miltenyi). Untouched NK cells from the remaining PBMCs were further purified using the human NK cell Isolation Kit (Miltenyi Biotech) according to the manufacturer’s instructions via autoMACS negative selection. The purity of slan⁺CD3⁺ slanMo and of CD56⁺CD3⁻ NK cells was ≥95%. Freshly isolated slanMo and NK cells were cultured in complete RPMI 1640 containing 2 mM L-glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), and 10% heat-inactivated pooled human AB serum (Sigma-Aldrich).

CD14 monocytes were isolated with CD14 microbeads (Miltenyi) by positive selection according to the protocol. T cell isolation was performed with the CD4 + T cell isolation kit (Miltenyi) by following the instructions and a negative selection autoMACS protocol.

**Immune cell co-cultures for supernatant generation and cytokine screening**

Freshly purified slanMo were seeded in 6-well or 12-well plates at a concentration of 5 × 10⁶ cells/ml and a density of 3 × 10⁵ cells/cm². After 6 hours of incubation at 37°C and 5% CO₂, purified NK cells were added to the well at 1 × 10⁶ cells/ml and 6 × 10⁵ cells/cm², resulting in a final ratio of 1:2 slanMo:NK cells. At the time of NK cells seeding, the co-culture was stimulated with 1 µg/ml R848 (Invitrogen). Twenty-four hours after slanMo seeding, the supernatant was removed, cleared of cellular parts by centrifugation and then either used directly or stored at −80°C. Alternatively, slanMo were cultured alone and stimulated with 1 µg/ml R848 after 6 h for harvesting of supernatants after 24 hours complete incubation.

For analysis of cytokine production, slanMo or CD14 monocytes were seeded in the same densities as described before for slanMo, and then co-cultured with NK cells or T cells, seeded as described for NK cells. Co-cultures were stimulated 6 hours after slanMo/CD14 monocyte seeding with 1 µg/ml R848 or 100 ng/ml LPS. Supernatants were removed after 24 hours total culture and stored at −20°C.

**Cytokine assays**

The supernatants of different co-cultures were screened for cytokine levels using the Legendplex pro-inflammatory Chemokine panel, human Cytokine panel 2, and anti-virus response panel (Biolegend).

**NK cell migration assay**

Purified NK cells were incubated overnight and then used in a 96 well migration system with a pore size of 5 µm (Neuroprobe). First, conditioned medium from slanMo R848-stimulated or unstimulated mono-cultures (slanMo CM), or recombinant R848 (1 µg/ml), was added to the bottom well. Anti-IL-8 neutralizing antibody (R&D, clone 6217, 20 µg/ml)
or control mouse IgG1 isotype control (20 μg/ml, BioXcell) was added directly to the slanMo CM. After fixation of the membrane, 5 × 10^5 NK cells were added on the membrane and incubated for 2.5 hours. The cells that migrated to the bottom chamber were transferred and measured via total ATP content with the Cell Titer Glo Luminescent cell viability assay (Promega). To calculate cell numbers, a standard curve was generated by measuring defined numbers of NK cells. Specific migration was defined as:

\[ \frac{(\text{Number of cells [sample]} - \text{number of cells [control]})}{\text{number of cells [input]}} 	imes 100 \]

Alternatively, 2 × 10^5 NK cells were seeded on a 24 well-permeable support and the slanMo CM with anti-IL-8 antibody or isotype control (each 10 μg/ml) was added as chemottractant. After 4 hours of incubation at 37°C, the migrated cells from the bottom well were resuspended, stained for CXCR1 (clone 8F1, Biolegend) and CXCR2 (clone 5E8, Biolegend), and analyzed by flow cytometry.

**Melanoma cell growth inhibition with conditioned-medium (CM)**

SK-Mel-28, Ma-Mel-86b, SK-Mel-25, and SK-Mel-30 were seeded at a density of 1 × 10^4 cells/cm^2 and 2.5 × 10^4 cells/ml in diluted CM (1:5), to reduce potential growth-inhibiting effects originating from already exhausted co-culture medium. In order to prevent premature confluency of the control cultures during the passages, the overall growth was limited by using RPMI containing 5% instead of 10% FCS. Neutralizing antibodies against human TNF-α (AbVie, Adalimumab, 10 μg/ml) and human IFN-γ (BioXcell, clone B133.5, 10 μg/ml) or the respective isotype controls hIgG1 (BioXcell, 10 μg/ml) and mlG1 (BioXcell, MOPC-21, 10 μg) were added to the CM. As control, the cells were also incubated without stimulus, with recombinant TNF-α (PromoKine, 10 ng/ml) and IFN-γ (ImmunoTools, 100 ng/ml), and with recombinant R848 (1 μg/ml). After 4 d of culture, the melanoma cells were harvested and either seeded as before for prolonged culture or seeded at 2 × 10^4 cells/cm^2 and 4 × 10^4 cells/ml in appropriate plates for downstream analysis.

Alternatively, SK-Mel-28 cells were seeded in 96 well flat bottom plates and treated with CM + TNF-α/IFN-γ neutralization as described before. The plate was imaged every 2 h over a period of 4 d with 100x magnification.

**3H-thymidine assay, senescence-associated β-galactosidase (SA β-Gal) assay**

For 3H-Thymidine proliferation assay, the cells were treated as described before and 1 d after reseeding in 96 well plates, the adherent cells were pulsed for 12 hours with 3H Thymidine solution (Hartmann Analytic). After incubation, the cells were transferred to a membrane Filtermat (Perkin Elmer). The membrane was washed, developed with Betaplate Scint solution (Perkin Elmer) and measured in a Luminescence Counter (MicroBeta TriLux, Perkin Elmer).

SA β-Gal staining was performed on melanoma cells reseeded for 1 d after CM exposure with the SA β-Gal staining kit (United States Biological) according to the manufacturer’s instructions.

**Quantitative real-time PCR (qRT-PCR)**

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific), following manufacturers’ instructions. RNA was treated with RNase-free DNase I, followed by EDTA deactivation for 10 min at 65°C (both from Thermo Fisher Scientific). First-strand cDNA was synthesized from 2000 ng of total RNA with random hexamer primers, using dNTP mix and RevertAid H Minus Reverse transcriptase (all from Thermo Fisher Scientific). qRT-PCR was performed in a Stratagene Mx3500P qPCR machine (Agilent) using SYBR Select Master Mix containing SYBR GreenE dye (Applied Biosystems). The relative quantity of cDNA was estimated using the ΔΔCT method and data were normalized to GAPDH as a housekeeping gene. The following human forward and reverse primers, purchased from Metabion, were used for qRT-PCR (Table S2).

**Immunofluorescence (IF) staining of CM-treated melanoma cells**

CM-treated melanoma cells were seeded on glass diagnostic slides (Menzel) at 2 × 10^4 cells/cm^2. After 1 d of culture, the cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature and then permeabilized with 0.1% (w/v) Triton-X 100 for 10 minutes at room temperature. The primary antibodies CDKN1A/p21 (abcam, clone EPR362, 1:500) and Ki67 (Dako, clone MIB-1, 1:75) were incubated overnight at 4°C, followed by the secondary goat anti-mouse A647 and goat anti-rabbit A488 (both Dianova). Ki67 and p21 positive cells were identified and counted based on fluorescence intensity with the software ImageJ.

**Western blot**

After 4 d of culture in CM, melanoma cells were lysed by heating in hot lysis buffer (10 mM Tris-HCl pH 8.0, 1% SDS, 1 mM Na-Orthovanadate) for 15 minutes in a heat block at 100°C with regular mixing. The solution was centrifuged at 14,000 rpm at 4°C and the supernatant was analyzed by BCA assay (Thermo Scientific) for protein concentration. The protein samples were diluted in SDS buffer and run on a 15% SDS gel. After blocking in 5% BSA, the membrane was incubated overnight with anti-p21 (abcam, EPR362, 1:2000) primary antibody, followed by incubation with secondary goat anti-rabbit HRP antibody. Protein levels were normalized to β-actin.

**Statistical analysis**

Data were analyzed in GraphPad Prism and are presented as mean ± SEM. p Values were assessed using one-way ANOVA with Bonferroni’s multiple comparison post hoc test for groups of 3 and paired t-test for groups of 2, unless otherwise indicated. *p < .05, **p < .01, ***p < .001.
References


