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Chemerin partly mediates tumor-inhibitory effect of all-*trans* retinoic acid via CMKLR1-dependent natural killer cell recruitment

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Summary

Down-regulated chemerin expression has been reported to correlate with poor prognosis of several types of cancer including melanoma. All-*trans* retinoic acid (atRA) is a potent inducer of chemerin, and we previously reported that atRA inhibited murine melanoma growth through enhancement of anti-tumor T-cell immunity. Here, we aimed to investigate whether loss of endogenous chemerin accelerated melanoma growth and whether chemerin was involved in the melanoma-inhibitory effect of atRA. We demonstrated that chemerin was constitutively expressed in the skin, which was down-regulated during murine melanoma growth. *Rarres2*$^{-/-}$ mice, which are deficient in chemerin, exhibited aggravated tumor growth and impaired tumor-infiltrating natural killer (NK) cells that express CMKLR1, the functional receptor of chemerin. Topical treatment with atRA up-regulated skin chemerin expression, which was primarily derived from dermal cells. Moreover, atRA treatment significantly enhanced tumor-infiltrating NK cells, which was completely abrogated in *Rarres2*$^{-/-}$ mice and *Cmklr1*$^{-/-}$ mice, suggesting a dependency of NK cell recruitment on the chemerin–CMKLR1 axis in melanoma. Despite comparable melanoma growth detected in wild-type mice and *Cmklr1*$^{-/-}$ mice, lack of CMKLR1 partially abrogated the melanoma-inhibitory effect of atRA. This may be due to the inability to enhance tumor-infiltrating NK cells in *Cmklr1*$^{-/-}$ mice following atRA treatment. Collectively, our study suggests that down-regulation of chemerin could be a strategy used by cancers such as melanoma to impair anti-tumor NK cell immunity and identifies a new anti-tumor mechanism of atRA by up-regulating chemerin to enhance CMKLR1-dependent NK cell recruitment.

Keywords: all-*trans* retinoic acid; chemerin; CMKLR1; melanoma; natural killer cells.

Introduction

Chemerin is encoded by a gene named as retinoic acid receptor responder protein 2 (RARRES2) and was originally identified in psoriatic skin lesions treated with tazarotene, a synthetic retinoid.[1] Chemerin is widely expressed with the highest levels in adipose tissue, liver and placenta.[2] Chemerin is secreted as an inactive precursor that can be rapidly converted into active forms after proteolytic cleavage at its C-terminus by various proteases from different cell types.[3–5] The chemotactic activity of chemerin was first identified as mediating the migration of plasmacytoid dendritic cell and natural killer (NK) cells through its functional receptor CMKLR1 (also called ChemR23), which may be involved in the pathogenesis of autoimmune skin diseases such as psoriasis and cutaneous lupus erythematosus.[6–8] Further studies have revealed an anti-inflammatory role of chemerin in several mouse

Abbreviations: atRA, all-*trans* retinoic acid; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL-6, interleukin-6; MDSC, myeloid-derived suppressor cell; NK, natural killer; PCR, polymerase chain reaction; RARRES2, retinoic acid receptor responder protein 2; WT, wild-type

models of inflammatory disease by reducing the production of inflammatory cytokines and neutrophil infiltration, suggesting a context-dependent regulatory effect of chemerin on tissue inflammation.[9,10] A role of chemerin in tumor has recently been recognized.[11] Down-regulated chemerin expression was detected in most tumor types, including melanoma and liver cancer,[11–13] and overexpression of chemerin could inhibit tumor growth and metastasis through different mechanisms including direct inhibition of the proliferation and migration of tumor cells or modulation of the tumor microenvironment.[12–15] For example, our previous study demonstrated that chemerin inhibits the progression of orthotopic murine liver cancer by reducing tumor-promoting inflammation, and others found that chemerin inhibits the invasion of human liver cancer cells.[13,14]

Melanoma is a highly aggressive skin cancer, representing only 4% of skin cancers but accounting for 80% of deaths from dermatological cancer.[16] Melanoma has been recognized as one of the most immunogenic malignancies with abundant infiltration of various immune cells.[17] NK cells, T helper type 1 cells and cytotoxic $CD8^+$ T cells constitute the major anti-tumor immunity, but tumor-infiltrating myeloid cells, including macrophages and myeloid-derived suppressor cells (MDSCs) and $Foxp3^+$ regulatory T cells, suppress anti-tumor immunity.[18–20] Hence, tumor progression is influenced by the balance between pro-tumor immunosuppression and anti-tumor immunity. Pachynski *et al.*[12] have reported that overexpression of chemerin in murine melanoma B16F10 cells is able to inhibit melanoma growth by recruitment of NK cells in a CMKLR1-dependent way. However, they also found that CMKLR1 deficiency has no effect on murine melanoma growth. This may suggest that endogenous chemerin, which is down-regulated during tumor growth, may be not sufficient for NK cell recruitment. We speculated that loss of chemerin may accelerate melanoma growth by escaping immune surveillance, and hence the factors that maintain or up-regulate chemerin expression may be useful to inhibit melanoma.

All-*trans* retinoic acid (atRA), a natural metabolite of vitamin A, is a well-known anti-cancer drug that is used clinically to treat leukemia by inducing tumor cell differentiation.[21] It is also known to regulate T-cell immunity under different conditions.[22,23] Our previous study revealed a new immunological mechanism by which atRA inhibits melanoma growth by enhancing anti-tumor $CD8^+$ T-cell immunity.[24] Interestingly, epidemiological studies demonstrated that taking vitamin A supplements correlates with decreased risk of developing melanoma and vitamin A levels are positively associated with the number of circulating NK cells.[25,26] Given that atRA is a potent inducer of chemerin, we hypothesized that chemerin may be involved in the tumor-inhibitory effect of atRA through recruitment of NK cells.

In this study, we investigated the effect of chemerin deficiency on tumor growth by using *Rarres2*$^{-/-}$ mice and a murine melanoma model and further explored whether chemerin mediated the tumor-inhibitory effect of atRA by topical administration of tretinoin ointment (with atRA as the active ingredient) to tumor-bearing wild-type (WT) and *Cmklr1*$^{-/-}$ mice.

Materials and methods

Mice and cell lines

The generation of *Rarres2*$^{-/-}$ mice has been described previously.[13] *Cmklr1*$^{-/-}$ mice were generated by Cyagen Company (Shanghai, China) using a TALEN knockout strategy. Briefly, the Exon 3 of murine *Cmklr1* gene was selected as target site and TALEN mRNAs generated by *in vitro* transcription were then microinjected into fertilized eggs for knockout mouse production. The mice were genotyped by polymerase chain reaction (PCR) followed by DNA-sequencing analysis (see Supplementary material, Fig. S1a). We also confirmed the absence of CMKLR1 at protein level in *Cmklr1*$^{-/-}$ mice (see Supplementary material, Fig. S1b). *Rarres2*$^{-/-}$ and *Cmklr1*$^{-/-}$ mice have not shown altered fertility and body weight compared with WT mice. Six- to eight-week-old female C57BL/6 WT mice were purchased from the Chinese Academy of Sciences (Shanghai, China). All mice were maintained in a specific pathogen-free environment. All animal experiments were conducted according to protocols approved by the Animal Care and Use Committee at Shanghai Medical College, Fudan University. The murine melanoma B16F10 cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in complete RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, Grand Island, NY).

Animal tumor experiments

Mice were anesthetized, shaved, and inoculated subcutaneously into the right flanks with 5×10^5 cells/mouse of B16F10 cells in 100 μl of phosphate-buffered saline. Tumor sizes were measured every 2 days using calipers. The tretinoin ointment (0·15 g; PanGeo Pharma Inc., Montreal, QC, Canada) or vaseline as control was topically rubbed on the skin every other day starting from 1 day before subcutaneous inoculation of B16F10 melanoma cells till tumors were palpable on day 4, and then every day until the mice were killed. The tumor volume was calculated using the following formula:

$$V = (\text{larger diameter}) \times (\text{smaller diameter})^2/2.$$

Flow cytometry

Tumor-bearing mice were killed, and subcutaneous tumors were collected. Tumors were then minced and digested with Collagenase IV (1 mg/ml) and DNAase I (5 U/ml) at 37° for 20 min and passed through a 100-μm nylon mesh. A single-cell suspension was blocked with Fc block (anti-CD16/32; eBioscience, San Diego, CA). The following anti-mouse antibodies were used (Becton Dickinson, Franklin Lakes, NJ): CD45 (30-F11), CD11b (M1/70), F4/80 (BM8), Gr-1 (RB6-8C5), CD3 (17A2), CD8 (53-6.7), CD4 (GK1.5), NK1.1 (PK136), IFN-γ (AN-18) and isotype antibodies. CMKLR1 (477806) and its isotype antibody were from R&D Systems (Minneapolis, MN). Intracellular staining of interferon-γ (IFN-γ) was fixed and permeabilized with the intracellular staining kit (Cat#560409; Becton Dickinson). Samples were acquired using Cyan instrument (Beckman Coulter, Miami, FL) and analyzed with FLOWJO software (Treestar Inc., San Carlos, CA).

ELISA

Skin samples were obtained, minced and mechanically homogenized in phosphate-buffered saline containing protease inhibitor. The supernatants were harvested after centrifuging at 13 800 *g* for 10 min and then normalized based on protein concentration as described by BCA assay (Sigma, St Louis, MO). Skin chemerin protein levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (DuoSet; R&D Systems) according to the manufacturer's instructions.

RNA extraction and quantitative real-time PCR

Total RNA was extracted by TRIZOL reagent (Ambion, Austin, TX); then, cDNA was generated with a high-capacity cDNA Reverse Transcription kit (Takara, Shiga, Japan). Quantitative real-time PCR (qPCR) was performed using an SYBR green Gene Expression Assay (Takara). The specific primers of all genes for PCR were used as previously reported.[13,24] The relative quantities of mRNA per sample were calculated using the previous methods.[24]

Statistical analysis

All data were expressed as mean ± SEM. We used two-tailed Student's *t*-tests to compare significance between two groups. For multiple groups, the comparison was performed with one-way or two-way analysis of variance using GRAPHPAD PRISM version 7 (GraphPad Prism Software, Inc., San Diego, CA). Significant difference was determined as P value < 0·05.

Results

Rarres2$^{-/-}$ mice exhibit aggravated murine melanoma growth

A previous study demonstrated that overexpression of chemerin in B16 melanoma cells inhibited tumor growth whereas knockout of CMKLR1 had no effect on tumor growth.[12] In this study, we attempted to investigate whether endogenous chemerin played a protective role in the growth of melanoma. We first examined the chemerin levels in skin over the melanoma growth. ELISA analysis of homogenates from skin overlying tumors showed significantly decreased chemerin concentrations compared with those from normal skin (Fig. 1a). Moreover, much lower concentrations of chemerin were detected in homogenates of skin overlying tumors from day 14 than those from day 6 following tumor inoculation, suggesting an inhibitory effect of tumor growth on skin chemerin expression (Fig. 1a). We then directly examined the influence of endogenous chemerin on melanoma growth *in vivo* by using *Rarres2*$^{-/-}$ mice that are deficient in chemerin. We found that tumor grew faster and larger in *Rarres2*$^{-/-}$ mice than in WT mice (Fig. 1b). *Rarres2*$^{-/-}$ mice had significantly higher tumor weight than WT mice on day 14 when the mice were killed (Fig. 1c,d). These results demonstrate that endogenous chemerin protects against murine melanoma growth.

Chemerin deficiency inhibits tumor-infiltrating CMKLR1-expressing NK cells and causes an immunosuppressive microenvironment

Chemerin has been shown to regulate tissue inflammation and tumor growth by directly or indirectly influencing immune cell infiltration. We therefore examined the effect of the lack of endogenous chemerin on the composition of immune cells in the tumor microenvironment by flow cytometry. We found that *Rarres2*$^{-/-}$ mice had markedly decreased proportions of tumor-infiltrating NK cells (CD3$^-$ NK1.1$^+$) and activated IFN-γ^+ NK cells compared with WT mice (Fig. 2a,b). This is consistent with the finding of Pachynski *et al.*[12] showing that overexpression of chemerin promotes NK cells infiltration in murine melanoma. Moreover, we detected significantly decreased fluorescence intensity of CMKLR1 in tumor-infiltrating NK cells from *Rarres2*$^{-/-}$ mice compared with those in WT mice (Fig. 2c), suggesting that endogenous chemerin may maintain CMKLR1 expression in NK cells. Consistent with decreased NK cells, a more immunosuppressive tumor microenvironment was detected in *Rarres2*$^{-/-}$ mice, as there were significantly increased proportions of tumor-infiltrating CD11b$^+$ Gr-1$^+$ MDSCs and CD11b$^+$ F4/80$^+$ tumor-associated macrophages but significantly decreased tumor-infiltrating CD4$^+$ and CD8$^+$ T cells in *Rarres2*$^{-/-}$

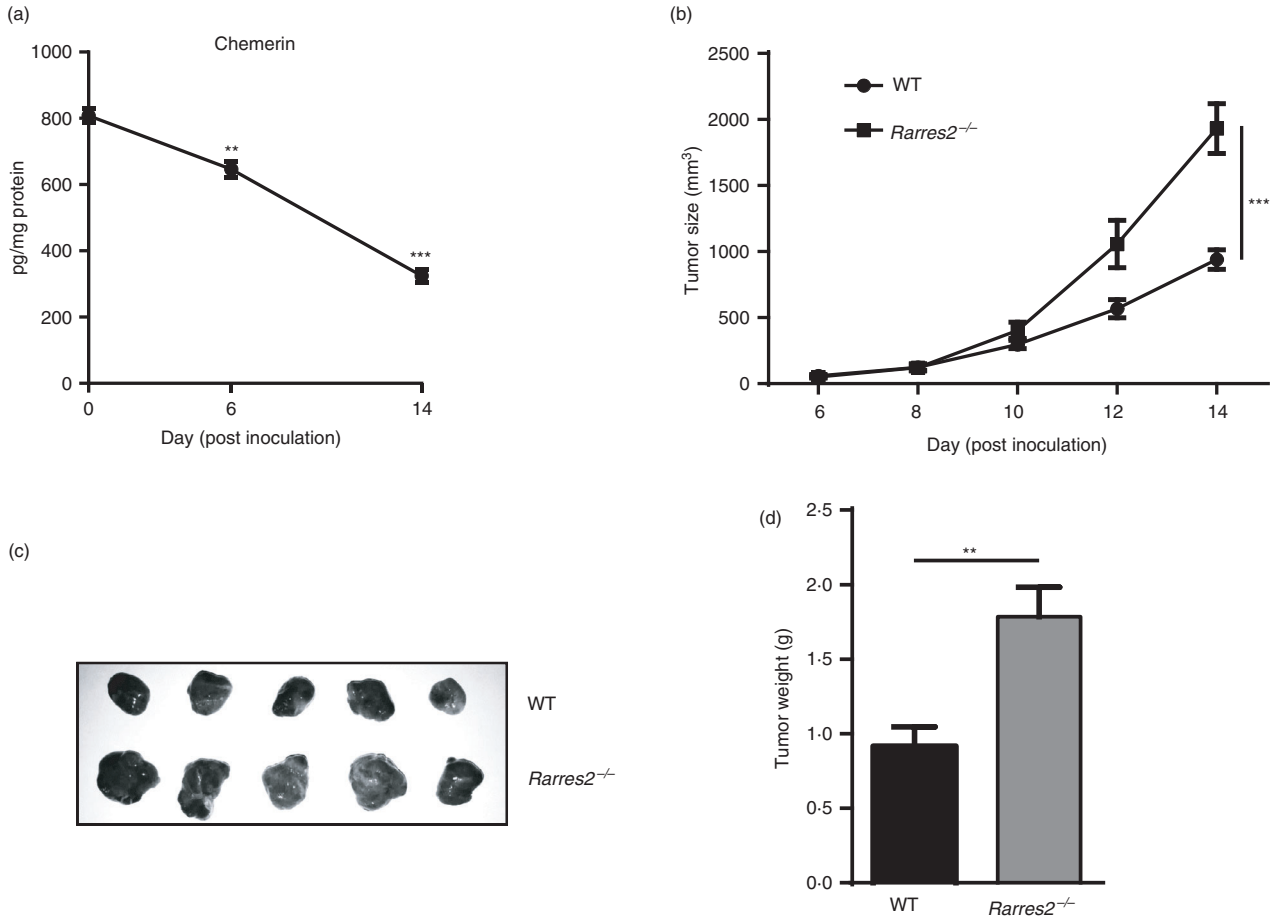

Figure 1. Lack of endogenous chemerin aggravates the growth of murine melanoma. (a) ELISA analysis of chemerin concentrations in homogenates of normal skin (indicated at 0) or skin overlying the tumors at indicated time-points. (b) Tumor growth was measured at indicated time-points post inoculation. Wild-type (WT) and *Rarres2*$^{-/-}$ mice were inoculated subcutaneously into the right flanks with B16F10 cells (5×10^5 in 100 µl phosphate-buffered saline). (c,d) Representative images of melanoma collected on day 14 (c) and the tumor weight (d). The columns and error bars represent mean ± SEM ($n = 5$ to $n = 7$ per group). $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}001$. Data are representative from three independent experiments.

mice compared with those from WT mice (Fig. 2d). Moreover, lack of endogenous chemerin caused significantly reduced gene expression of several critical anti-immune molecules including *Gzmb*, *Ifng* and *Prf1*, which are known to be primarily derived from NK cells and CD8$^+$ T cells (Fig. 2e). Interestingly, there were no differences in gene expression of granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin-6 (IL-6) in murine melanoma of *Rarres2*$^{-/-}$ mice and WT mice (Fig. 2e), although our previous study demonstrated that chemerin inhibited GM-CSF and IL-6 expression in orthotopic murine liver cancer,[13] suggesting that chemerin may not influence tumor-inducing MDSC generation in melanoma. We also found significantly increased gene expression of T-cell chemokines, including CXCL9 and CXCL10, but significantly decreased chemokines for MDSC recruitment, including CXCL1 and CXCL2 (Fig. 2e). Collectively, these data demonstrate that lack of endogenous chemerin causes an immunosuppressive tumor microenvironment, which may be due to impaired tumor infiltration of CMKLR1$^+$ NK cells.

Topical treatment of atRA induces skin expression of chemerin, which mediates tumor infiltration of NK cells

We previously showed that topical administration of atRA inhibited murine melanoma growth partly by potentiating the anti-tumor T-cell response through up-regulation of major histocompatibility complex class I expression on tumor cells.[24] Given that the gene encoding chemerin is defined as a responder to RA, we speculated that chemerin could partly mediate the melanoma-inhibitory effect of atRA through the enhancement of tumor-infiltrating NK cells. To test the hypothesis, we first examined whether topical administration of atRA could up-regulate

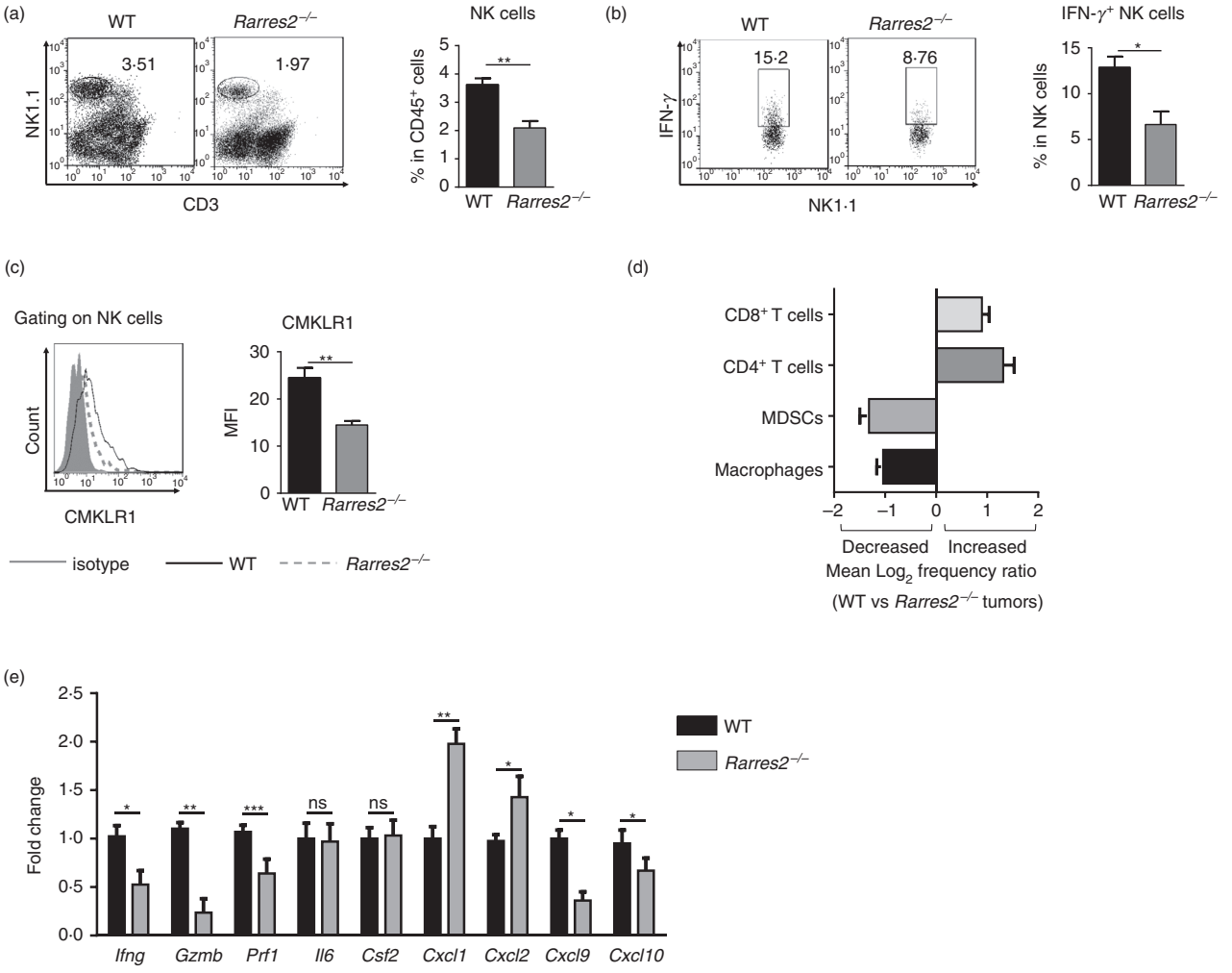

Figure 2. Chemerin deficiency inhibits CMKLR1-expressing natural killer (NK) cell infiltration and causes an immunosuppressive tumor environment. (a–c) Representative flow cytometry data and averaged percentages of NK cells ($CD3^-$ $NK1.1^+$) in $CD45^+$ cells (a), IFN-γ^+ cells in NK cells (b), mean fluorescence intensity (MFI) of CMKLR1 on NK cells (c). (d) Log_2 ratio of $CD4^+$ T cells, $CD8^+$ T cells, myeloid-derived suppressor cells (MDSCs; $CD11b^+$ $Gr\text{-}1^+$) and macrophages ($CD11b^+$ $F4/80^+$) in wild-type (WT) versus *Rarres2*$^{-/-}$ tumors as determined by FACS analysis. (e) Quantitative RT-PCR analysis of gene expression of *Ifng*, *Gzmb*, *Prf1*, *ll-6*, *Csf2*, *Cxcl9*, *Cxcl10*, *Cxcl1* and *Cxcl2* in melanoma of WT and *Rarres2*$^{-/-}$ mice. Columns and error bars represent mean ± SEM (n = 5 to n = 7 per group). *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$ and ns = no significance. Data are representative from three independent experiments.

chemerin expression in the skin. The skin was locally treated for 1, 3 and 5 days. Treatment with atRA markedly increased the protein levels of chemerin in the skin over time when compared with those on day 0 (the basal levels; Fig. 3a). The up-regulation of chemerin was also dose-dependent, as tretinoin ointment at all concentrations significantly increased chemerin levels, reaching the highest levels at the highest concentration of 0·15 g when compared with those without tretinoin ointment (Fig. 3b). Furthermore, we demonstrated that dermis constitutively expressed chemerin at high protein levels, which were up-regulated by atRA treatment, whereas epidermis constitutively expressed chemerin at very low protein levels, which were not influenced by atRA treatment (Fig. 3c). We next explored whether up-regulated chemerin expression by topical treatment of atRA could enhance tumor infiltration of NK cells. To this end, atRA or vaseline as control was topically administered on the skin every day, starting from 1 day before subcutaneous inoculation of B16F10 melanoma cells, until the mice were killed. As expected, atRA treatment up-regulated chemerin protein levels in the skin overlying the tumors on day 6 post inoculation (Fig. 3d). Notably, this was accompanied by obvious increases in tumor infiltration of NK cells and fluorescence intensity of CMKLR1 on tumor-infiltrating NK cells (see Supplementary material, Fig. S2a,b). In contrast, atRA treatment had no effect on the frequencies of other immune cells (including $CD11b^+$ myeloid cells, $CD8^+$ T

cells and $CD4^+$ T cells) in tumors (see Supplementary material, Fig. S2c) at this early stage. Accordingly, significantly increased frequencies of NK cells and fluorescence intensity of CMKLR1 on these cells were observed in tumors treated with atRA compared with those treated with vaseline on day 14 post inoculation (Fig. 3e,f). In contrast, topical treatment of atRA failed to cause increased tumor-infiltrating NK cells and fluorescence intensity of CMKLR1 on these cells in *Rarres2*$^{-/-}$ mice (Fig. 3g,h). These data together suggest that topical treatment of atRA increases tumor-infiltrating NK cells in a chemerin-dependent way.

Melanoma-inhibitory effect of atRA is partly mediated by CMKLR1-dependent tumor infiltration of NK cells

We next attempted to determine the contribution of chemerin-mediated tumor-infiltrating NK cells to the tumor-inhibitory effect of atRA. As lack of endogenous chemerin aggravated tumor growth but lack of CMKLR1 had no such effect (Figs 1b and 4a), we compared the therapeutic effect of atRA on tumors of WT and *Cmklr1*$^{-/-}$ mice. The atRA treatment significantly slowed down tumor growth and reduced tumor weight in *Cmklr1*$^{-/-}$ mice.

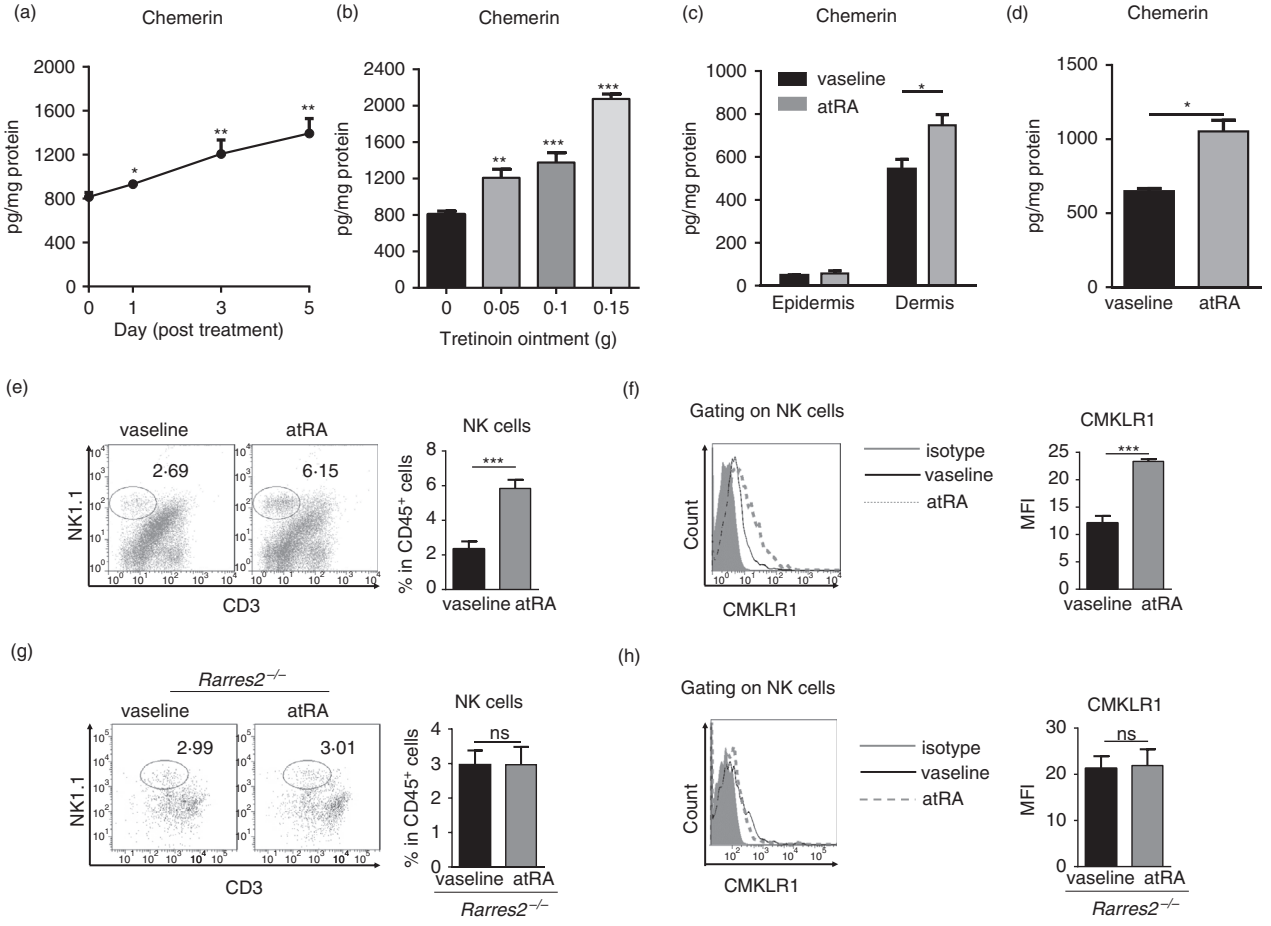

Figure 3. Topical administration of all-*trans* retinoic acid (atRA) enhances skin expression of chemerin, which mediates accumulation of tumor-infiltrating natural killer (NK) cells. (a–c) ELISA analysis of chemerin concentrations in homogenates of skin topically treated with tretinoin ointment (0·1 g) with atRA as the active ingredient for 1, 3 and 5 days (a), and in homogenates of skin topically treated with different dosages of tretinoin ointment on day 3 (b), as well as in epidermis and dermis after topical treatment of atRA or vaseline as control on day 3 (c). (d–h) The tretinoin ointment (0·15 g) or vaseline as control was topically rubbed on the skin every day starting from 1 day before subcutaneous inoculation of B16F10 melanoma cells until the mice were killed. (d) Chemerin protein expression in homogenates of skin overlying tumors in WT mice on day 6 post inoculation. (e,f) Representative flow cytometry data and averaged percentages of NK cells ($CD3^-$ $NK1.1^+$) in $CD45^+$ cells (e), as well as mean fluorescence intensity (MFI) of CMKLR1 on NK cells (f) in tumor-bearing WT mice on day 14 post inoculation. (g,h) Representative flow cytometry data and averaged percentages of NK cells ($CD3^-$ $NK1.1^+$) in $CD45^+$ cells (g), as well as mean fluorescence intensity (MFI) of CMKLR1 on NK cells (h) in tumor-bearing *Rarres2*$^{-/-}$ mice on day 14 post inoculation. In (a) and (b), the data on different time-points or different doses were compared with day 0 or 0 g tretinoin ointment, respectively. Columns and error bars represent mean ± SEM (n = 5 to n = 7 per group). *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ and ns = no significance. Data are representative from three independent experiments.

However, they were still significantly higher than those in WT mice treated with atRA (Fig. 4a–c), suggesting that CMKLR1 is partly required by the tumor-inhibitory effect of atRA. Accordingly, atRA treatment induced significantly fewer tumor-infiltrating NK cells and IFN-γ^+ NK cells in *Cmklr1*$^{-/-}$ mice compared with WT controls, although both had comparable tumor-infiltrating NK cells following vaseline treatment (Fig. 4d,e). These data together demonstrate that the ability of atRA to up-regulate skin chemerin levels contributes to the tumor-inhibitory effect of atRA through enhancement of CMKLR1-dependent tumor infiltration of NK cells.

Discussion

We here demonstrate that chemerin deficiency aggravates murine melanoma growth, which could be, at least partially, due to fewer tumor-infiltrating NK cells, thereby creating an immunosuppressive microenvironment. Moreover, we identify a new mechanism of tumor-inhibitory effect of atRA by up-regulating expression of chemerin to mediate tumor recruitment of NK cells in a CMKLR1-dependent way.

Although the majority of studies have been focused on the ability of chemerin to modulate tissue inflammation or metabolism,[27–29] more attention has recently been paid to the role of chemerin in tumor. Clinical studies suggest the correlation of down-regulated systemic and/or tumoral chemerin expression with the progression of several types of tumor including melanoma,[11] liver cancer[13] and adrenocortical carcinoma.[15] Several mechanisms have been shown to be involved in the tumor-inhibitory effect of chemerin, including the promotion of anti-tumor immunity through the recruitment of NK cells or the inhibition of MDSC generation,[12,13] or direct inhibition of tumor cell growth or migration,[14,15] depending on the context of tumor. Our study demonstrated that lack of endogenous chemerin aggravated tumor growth, which is consistent with the previous study showing that overexpression of chemerin inhibited melanoma growth. We further found that lack of endogenous chemerin caused fewer tumor-infiltrating NK cells and T cells but more myeloid cells, including MDSCs and tumor-associated macrophages, indicating an immunosuppressive microenvironment. We previously demonstrated that lack of chemerin caused higher levels of GM-CSF and IL-6, leading

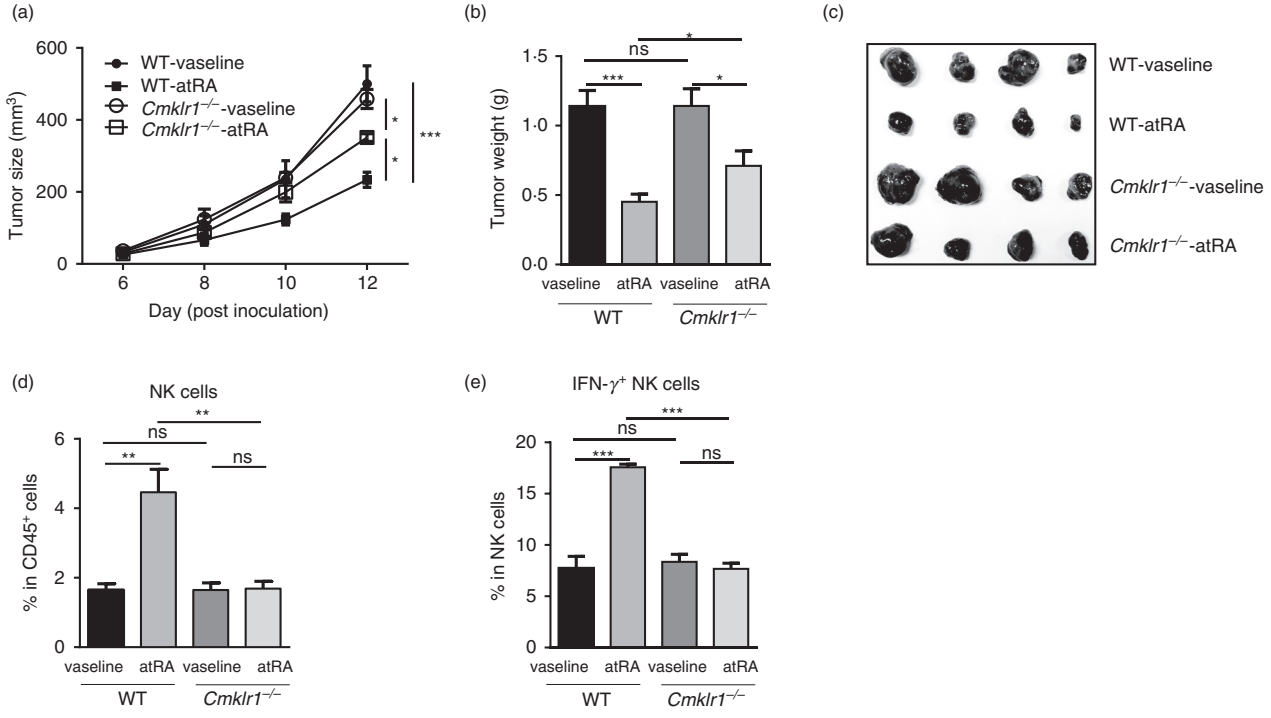

Figure 4. CMKLR1 deficiency completely inhibits all-*trans* retinoic acid (atRA) -induced tumor accumulation of natural killer (NK) cells and partly impairs the tumor-inhibitory role of atRA. The tretinoin ointment (0·15 g) or vaseline as control was topically rubbed on the skin of wild-type (WT) and CMKLR1-deficient (*Cmklr1*$^{-/-}$) mice every day starting from 1 day before subcutaneous inoculation of B16F10 melanoma cells until day 14 when the mice were killed. (a) Growth of tumor was measured at indicated time-points post inoculation. (b) Tumor weight. (c) Representative images of melanoma. (d,e) Representative flow cytometry data and averaged percentages of NK cells (CD3$^-$ NK1.1$^+$) in CD45$^+$ cells (d), and IFN-γ^+ cells in NK cells (e). Columns and error bars represent mean ± SEM (n = 4 to n = 6 per group). *P < 0·05, **P < 0·01, ***P < 0·001 and ns = no significance. Data are representative from three independent experiments.

to more MDSC generation and tumor infiltration, but no effect on NK cell infiltration in the murine orthotopic liver cancer. Considering abundant NK cells in normal liver, chemerin-mediated NK cell recruitment may be dispensable in the liver. In contrast, chemerin deficiency did not affect the expression of GM-CSF and IL-6, but decreased tumor-infiltrating NK cells in melanoma. As T cells do not express CMKLR1, and chemerin was originally identified as a chemoattractant of NK cells, these data suggest that lack of endogenous chemerin impairs anti-tumor NK cell migration into melanoma, which could lead to more immunosuppressive tumor microenvironment, aggravating tumor growth. These results together stress the distinct mechanism that chemerin uses to inhibit tumor growth in different types of cancer.

We found that chemerin was constitutively expressed in the skin, which was down-regulated over the murine melanoma growth. Interestingly, Pachynski *et al.*[12] showed that CMKLR1 deficiency had no effect on melanoma growth but impaired the effect of chemerin overexpression on NK cell recruitment and tumor inhibition. We confirmed the comparable melanoma growth between WT and *Cmklr1*$^{-/-}$ mice. This suggests that the down-regulation of chemerin could be a strategy used by melanoma to impair the CMKLR1-dependent recruitment NK cells. Hence, some factors that are able to up-regulate chemerin expression may exert anti-tumor activity through promotion of NK cell recruitment. We demonstrated that topical treatment with atRA up-regulated chemerin expression in the skin of naive mice and tumor-bearing WT mice. We further demonstrated that dermis, but not epidermis, constitutively expressed chemerin, which was further up-regulated by atRA treatment. Chemerin was reported to be produced by keratinocytes, fibroblasts and adipocytes.[6,30–32] Our data suggest that fibroblasts or adipocytes may be the source of chemerin in the dermis. Moreover, atRA treatment caused significantly more tumor-infiltrating NK cells in a chemerin–CMKLR1 axis-dependent manner, as lack of chemerin or CMKLR1 completely abrogated the increases in tumor-infiltrating NK cells. Importantly, the inhibitory effect of atRA treatment on melanoma growth was partially impaired in *Cmklr1*$^{-/-}$ mice, which was accompanied by reduced tumor-infiltrating NK cells. Hence, our study reveals that the ability of atRA to up-regulate chemerin partly contributes to its inhibitory effect on melanoma by promoting tumor infiltration of CMKLR1^{+} NK cells.

In the current study, we applied atRA 1 day before subcutaneous inoculation of B16F10 cells obviously increased tumor infiltration of NK cells. However, this seems to contrast with our previous study demonstrating that atRA applied when a tumor was palpable had no effect on tumor infiltration of NK cells, although it was able to inhibit established melanoma growth through the enhancement of anti-tumor T-cell immunity. The explanation for this discrepancy could be that the up-regulation in chemerin levels caused by atRA treatment fails to compensate for the reduction in chemerin levels caused by tumor growth, making it insufficient to promote recruitment of NK cells into tumor. Hence, our data indicate that atRA treatment as early as possible during tumor progression is more likely to cause more tumor-infiltrating NK cells to enhance anti-tumor immunity through regulation of chemerin levels. Interestingly, we noted that chemerin could up-regulate the expression levels of CMKLR1 in tumor-infiltrating NK cells, as lack of endogenous chemerin significantly decreased, but atRA treatment significantly increased fluorescence intensity of CMKLR1 in tumor-infiltrating NK cells. Hence, chemerin is likely to enhance its chemoattractive activity by up-regulating CMKLR1 expression in NK cells.

In summary, our study demonstrates that melanoma down-regulates local chemerin levels, which contributes to tumor progression with impaired NK cell infiltration, and increased chemerin levels induced by atRA treatment partially mediates the melanoma-inhibitory effect of atRA through recruitment of CMKLR1^{+} NK cells into the tumor microenvironment. Hence, our study suggests that strategies that can up-regulate chemerin expression could be promising to strengthen therapeutic efficacy in some tumors such as melanoma.

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Disclosures

None of the authors has a conflicting financial interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Genotyping of *Cmklr1*$^{-/-}$ mice

Figure S2. Related to Fig. 3. (a,b) Representative flow cytometry data and averaged percentages of natural killer (NK) cells ($CD3^-$ $NK1.1^+$) in $CD45^+$ cells (a), as well as mean fluorescence intensity (MFI) of CMKLR1 on NK cells (b) in tumor-bearing wild-type (WT) mice on day 6 post inoculation. (c) Averaged percentages of $CD11b^+$, $CD4^+$ T and $CD8^+$ T cells in $CD45^+$ cells in tumors treated with vaseline or all-*trans* retinoic acid (atRA) on day 6 post inoculation.

