HHS Public Access

Author manuscript

Nat Med. Author manuscript; available in PMC 2013 July 25.

Published in final edited form as:

Nat Med. 2012 August ; 18(8): 1248–1253. doi:10.1038/nm.2856.

Robust tumor immunity to melanoma mediated by interleukin 9

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Abstract

Interleukin-9 is a T cell cytokine that acts through a γC-family receptor on target cells. We determined that T cells from mice deficient in the T_H17 pathway genes ROR-γ and IL-23R produced abundant IL-9, and observed significant growth inhibition of B16F10 melanoma tumor in these mice. IL-9 blocking antibodies reversed this tumor growth inhibition, and enhanced tumor growth in normal mice. *IL9R*$^{-/-}$ mice showed accelerated tumor growth, while administration of rIL-9 to tumor bearing mice inhibited tumor growth. Adoptive transfer of tumor antigen-specific T_H9 cells blocked tumor growth; this was reversed by anti-IL-9. Exogenous rIL-9 inhibited tumor growth in *Rag1*$^{-/-}$ mice, but not in mast cell deficient mice, suggesting a T cell independent process. Finally, we found T_H9 cells in normal human skin and blood, and low IL-9 production from melanoma tumor infiltrating lymphocytes. These results suggest a role for IL-9 in tumor immunity, and suggest therapeutic strategies.

Introduction

Recent studies suggest that immunologic targeting of melanoma is a promising strategy[1,2]. An important role for $CD4^+$T cells in tumor immunity has emerged from several studies using murine models [3–6]. Adoptive transfer of tumor specific $CD4^+$T cells has been shown to eradicate large established and metastatic melanomas[5,67]. The role of T_H17 cells in tumor immunity is controversial, with apparently contradictory results having been published[8–13]. We explored another approach to studying the role of T_H17 cells in tumor immunity, using mice whose T cells were deficient in the transcription factor retinoid-related orphan receptor-gamma (ROR-γ). ROR-γt, an isoform of ROR-γ, is a lineage specific transcription

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Author contributions

RP designed the study, performed and analyzed the experiments and wrote the manuscript. SX and HSK performed experiments. WE, AMJ, SJK, and VKK discussed the data, provided reagents and edited the manuscript. XJ provided reagents. CS, RAC and RCF performed the human T cell experiments, discussed the data and edited the manuscript. TSK designed the study, analyzed the experiments, and wrote the manuscript.

factor critical for the development of T_H17 cells [14,1516] whose deficiency abrogates the development of IL-17A secreting T_H17 cells. Increased expression of ROR-γ is reported in autoimmune and inflammatory diseases [14,17]. Using ROR-γ deficient mice (*RORc*$^{-/-}$), we observed significant growth inhibition of B16F10 melanoma. Transcriptional profiling experiments revealed an unexpected increase in the expression of IL-9 in ROR-γ^{-}CD4^{+}T cells. We therefore explored the role of IL-9 in anti-tumor immunity, and demonstrate significant tumor growth inhibition mediated by IL-9 and T_H9 cells. We also demonstrated T_H9 memory T cells in human tissue, suggesting a role for this pathway in tumor immunity.

Results

Deficiency of ROR-γ is associated with inhibited melanoma growth and increased tumor lymphocytic infiltration

To examine the role of ROR-γ in tumor immunity, we used a B16F10 murine melanoma model. *RORc*$^{-/-}$ mice do not develop secondary lymph nodes and have fewer CD4^{+} and CD8^{+}T cells as compared to *RORc*$^{+/+}$ mice [18]. We therefore generated ROR-γ deficient chimeric mice (*RORc*$^{-/-}$ch) and ROR-γ normal wild type chimeric mice (*RORc*$^{+/+}$ch) by administering bone marrow cells from *RORc*$^{-/-}$ or *RORc*$^{+/+}$ mice into sublethally irradiated *Rag1*$^{-/-}$ C57BL/6 mice[14,16]. After full reconstitution of T cells and restoration of the intact immune system (8–10 weeks), mice were used for tumor growth experiments (Supplementary Fig. 1). B16F10 melanoma cells were injected subcutaneously into *RORc*$^{+/+}$ch and *RORc*$^{-/-}$ch mice, and tumor growth was monitored over time. Melanoma growth was strongly inhibited in *RORc*$^{-/-}$ch mice, and survival of the *RORc*$^{-/-}$ch mice was significantly increased compared to control mice (Fig. 1a–b). We examined the infiltration of lymphocytes in tumors removed from these mice. Melanomas from *RORc*$^{-/-}$ch mice contained 3–fold higher numbers of CD4^{+}T cells and CD8^{+}T cells as compared to *RORc*$^{+/+}$ch controls (Fig. 1c). T cells from draining lymph nodes of *RORc*$^{-/-}$ch melanoma bearing mice secreted negligible IL-17A, and increased amounts of IFN-γ as well as TNF-α (Fig. 1d). There was no difference in the number of melanoma tumor-infiltrating CD4^{+}CD25^{+}FoxP3^{+}T cells (T-regulatory cells) in *RORc*$^{+/+}$ch (7.2% ± 0.4) and *RORc*$^{-/-}$ch (5.9% ± 0.7).

Increased IL-9 expression in ROR-γ^{-} T cells

To further explore the mechanism of melanoma growth inhibition in *RORc*$^{-/-}$ch mice, we performed transcriptional profiling analysis using CD4^{+}T cells from *RORc*$^{-/-}$ and *RORc*$^{+/+}$ mice differentiated under T_H17 polarizing conditions. As expected, expression of IL-17A, IL-17F and IL-23R in ROR-γ^{-}CD4^{+}T cells was much lower compared to ROR-γ^{+}CD4^{+}T cells (Fig. 2). However, expression of IL-9 in ROR-γ^{-}CD4^{+}T cells was dramatically increased as compared to ROR-γ^{+}CD4^{+}T cells (Fig. 2, Supplementary Fig. 2). Granzyme-B and Granzyme-C expression were also significantly enhanced. Because of the striking upregulation of IL-9 under these conditions, we focused our subsequent experiments on the potential role of IL-9 in tumor immunity.

To be certain that the T cells from *RORc*$^{-/-}$ch mice did not have an intrinsic property that predisposed them to IL-9 production; we examined CD4^{+}T cells from these mice. There was

increased expression of IL-9 in ROR-γ^{-}CD4^{+}T cells (Supplementary Fig. 3a–b) and memory CD4^{+}T cells (Supplementary Fig. 3 c–e). However, negligible IL-9 expression was observed in naïve CD4^{+}T cells, and importantly there was no difference in IL-9 expression between ROR-γ^{+} and ROR-γ^{-}naïve CD4^{+}T cells (Supplementary Fig. 3f). T_H2 and T_H9 cells both secrete IL-9[19,20]; there was increased IL-9 expression in ROR-$\gamma^{-}T_H2$ cells. However, ROR-γ^{-}CD4^{+}T cells polarized under T_H9 conditions showed similar IL-9 production when compared to ROR$\gamma^{+}T_H9$ cells (Supplementary Fig. 3g–h).

To determine if the melanoma growth inhibitory response in *RORc*$^{-/-}$ ch mice could be attributed to IL-9, melanoma cells were injected subcutaneously into *RORc*$^{-/-}$ch mice. Tumor growth in these mice lagged significantly behind that of *RORc*$^{+/+}$ch mice. Mice were next treated with neutralizing antibodies to IL-9. As shown in Figure 3a, the impaired melanoma growth in *RORc*$^{-/-}$ch mice was significantly, but not completely, reversed by neutralization of IL-9. Therefore, we conclude that the melanoma growth inhibition observed in *RORc*$^{-/-}$ch mice was partially dependent on IL-9.

The role of IL-9 in melanoma immunity in *IL-23R*$^{-/-}$ mice

The most strongly downregulated gene in the transcriptional profiling experiments on ROR-γ^{-}CD4^{+}T cells was *IL23R*. We therefore explored melanoma tumor growth in *IL23R*$^{-/-}$ mice as well. We differentiated naïve CD4^{+}T cells from *IL23R*$^{+/+}$ and *IL23R*$^{-/-}$ mice under T_H17 polarizing conditions and re-stimulated (anti-CD3/CD28 mAbs) these cells. After 48h, supernatant was collected and IL-9 and IL-17 were measured. As expected, IL-23R^{-}CD4^{+}T cells produced significantly less IL-17A (Fig. 3b). However, there was increased IL-9 expression compared to IL-23R^{+}CD4^{+}T cells (Fig. 3b).

Next, melanoma cells were injected into *IL23R*$^{+/+}$ and *IL23R*$^{-/-}$ mice. Melanoma growth was greatly impaired in *IL23R*$^{-/-}$ mice. We next asked what role IL-9 played in this tumor growth inhibition. Neutralization of IL-9 also led to enhanced melanoma tumor growth in *IL23R*−/− and *IL23R*$^{+/+}$ mice (Fig. 3c), suggesting that the production of IL-9 by TIL's (Fig. 3d) was partially effective in tumor growth suppression. In addition, tumor site-draining lymph node cells (LNCs) from *IL23R*$^{+/+}$ and *IL23R*$^{-/-}$ mice were isolated and stimulated (anti-CD3/CD28 mAbs) for 48 h. There was increased IL-9 secretion by IL-23R^{-}T cells compared to IL-23R^{+}T cells (Fig. 3e).

T_H9 cells inhibit melanoma growth

Since IL-9 is mainly produced by CD4^{+}T cells, we examined the role of effector subsets of CD4^{+}T cells in melanoma immunity. T_H1, T_H2, T_H9 and T_H17 cells were generated from naïve CD4^{+}T cells of OT-II mice. CD4^{+}T cells from OT-II mice express a transgenic TCR that recognizes residues 323-339 from ovalbumin in the context of I-A^{b}. B16F10-ova melanoma cells express the full-length ovalbumin molecule. Before adoptive transfer into mice, *in vitro* cytokine polarized CD4^{+}T cells were analyzed for their cytokine expression (Supplementary Fig. 5a). Differentiated T_H cells were transferred into syngeneic immunocompetent mice, and, B16F10-ova melanoma cells were injected subcutaneously. Mice treated with T_H9 cells showed the greatest resistance to melanoma growth (Fig. 4a).

To determine if Th9 cells could inhibit the melanoma development independent of endogenous T cells, we transferred T_H0 and T_H9 cells generated from OT-II mice into immunocompromised hosts. In this setting, Th9 cells inhibited B16F10-ova induced melanoma growth (Fig. 4b), and survival of T_H9-treated mice was significantly increased (Fig. 4c). This data suggest that T_H9 cells are capable of inhibiting melanoma development, even in the absence of $CD8^+$T cells. Finally, IL-9 blockade accelerated melanoma growth in T_H9 treated mice, suggesting partial dependence of this effect directly on IL-9 (Fig. 4b).

To determine whether T_H9 cells could directly kill melanoma cells, we co-cultured B16F10-ova cells with OT-II T_H9 cells for 24 h. At that point, B16F10-ova cells were stained with 7-AAD. T_H9 cells alone were capable of inducing apoptosis in melanoma cells, whereas T_H0 and T_H17 cells were much less effective in this regard (Supplementary Fig. 6a). Next, the mechanism of T_H9 cell direct cytotoxicity was assessed. We measured a limited profile of effector molecules in T_H9 cells (Supplementary Fig. 5b). We observed increased expression of granzyme-B in T_H9 cells. Inhibition of granzyme-B significantly attenuated the T_H9 cell cytotoxic activity (Supplementary Fig. 6b). To further explore the direct cytotoxic effects of Th9 cells, we used another cytotoxic assay[5]. We incubated OT-II T_H9 cells with CFSE-labeled B16F10-ova cells and CFSE-labeled EL-4 cells for 36 h. There were dose dependent effects of T_H9 cells on tumor cell lysis (Supplementary Fig. 6c). More importantly, OT-II T_H9 cells killed B16F10-ova cell but not a tumor cell line that did not express Ova (Supplementary Fig. 6c).

Abrogation of IL-9/IL-9R signaling promotes melanoma development, and treatment with rIL-9 inhibits melanoma development

To analyze the role of IL-9 in tumor growth more directly, melanoma cells were injected into *IL9R*$^{-/-}$mice, and tumor growth was monitored. Melanoma growth was accelerated in *IL9R*$^{-/-}$mice compared to *IL9R*$^{+/-}$ mice (Fig. 5a). We next asked if administration of rIL-9 could protect mice against melanoma growth and progression. Treatment of melanoma bearing mice with recombinant IL-9 (rIL-9) both impaired melanoma growth (Fig. 5b) and increased the survival of mice (data not shown). To determine if rIL-9 could potentiate the anti-melanoma response in mice already vaccinated against melanoma, we vaccinated mice with 10^6 irradiated melanoma cells. Live B16F10 melanoma cells were injected 7 days later, and mice were treated with rIL-9. Again, rIL-9 treated mice showed significantly reduced melanoma growth (Supplementary Fig. 4a). We asked if rIL-9 could inhibit an unrelated tumor—Lewis Lung Carcinoma (LLC). LLC-1 tumor development was significantly suppressed in rIL-9 treated mice compared with a control group (Fig. 5c), However, rIL-9 had no effect on EL-4 lymphoma growth (data not shown).

Mast cells, but not adaptive immune cells, are required for the anti-tumor effect of IL-9

We next investigated the mechanism of IL-9 mediated tumor immunity. Melanoma cells were injected into *Rag1*$^{-/-}$C57BL/6 mice, which lack T and B cells, and mice were treated with rIL-9. Treatment with rIL-9 still significantly inhibited melanoma growth (Fig. 5d), suggesting that the target of the rIL-9 effect was not a T cell or B cell. Because IL-9 is also known to promote mast cell development and function, we injected B16F10 melanoma and LLC-1 cells, respectively, into mast cell deficient mice (Kit W-sh/HNihrJaeBsmJ) and then

measured tumor growth in mice treated systemically with rIL-9 (B16F10: Fig 5e, LLC-1: Fig 5f). Strikingly, under these conditions, there was no significant difference in tumor development. These data suggest that tumor growth inhibition mediated by rIL-9 administration depends upon the presence of mast cells, but not on the presence of T cells or B cells.

IL-9 producing T cells (T_H9 cells) can be found in normal human skin and peripheral blood, and in metastatic melanoma lesions

For this work to be relevant to patients with melanoma, we felt it important to attempt to identify T_H9 cells in human tissues. Specifically, we asked if T_H9 cells can be identified in humans, and if so, whether they showed a phenotype comparable to murine T_H9 cells. Murine T_H9 cells are defined as a distinct population of $CD4^+$T cells that produce IL-9 but not IFN-γ, IL-4 or IL-17. We were able to demonstrate that human memory T cells isolated from peripheral blood contained a distinct population of IL-9 producing T cells that do not produce IFN-γ, IL-4 and IL-17. T cells with an identical phenotype, and in even greater abundance, were also found in resident T cells (T_{RM}) isolated from healthy skin (Fig. 6a–b). Finally, we looked for the presence of IL-9 producing T cells in TIL's extracted from patients with Stage IV metastatic melanoma (metastasis to lung (n=4), skin (n=2), adrenal gland (n=1), and bone (n=1)). We observed detectable IL-9 producing T cells in 6 out of 8 biopsies; however, T_H9 cells were less abundant in TIL's from melanoma lesions, and the level IL-9 production was significantly lower in these T cells melanomas compared to memory T cell populations from healthy donors (Fig. 6c).

Discussion

In this study, we report that mice deficient in pathways related to T_H17 development ($RORc^{-/-}$ and $IL23R^{-/-}$) show significant resistance to melanoma growth in a fashion that is largely IL-9 dependent. In addition, T_H9 cells inhibit tumor growth in an IL-9 dependent fashion. Treatment of tumor bearing mice with exogenous rIL-9 inhibits tumor growth, and this effect requires the presence of mast cells but not T and B cells. Finally, we showed that memory T_H9 T cells can be identified in normal human blood and skin, and are present, albeit at reduced levels, in metastatic melanoma lesions. These findings have not been previously reported.

At the outset of the study, our goal was to assess the role of T_H17 cells in melanoma immunity; thus, we used *RORc*$^{-/-}$ ch mice and *IL23R*$^{-/-}$ mice, and showed impaired melanoma in both models, suggesting an important role for the T_H17 pathway in tumor immunity. However, the role of other pathways remained an open question. Transcriptional profiling analysis of ROR-γ^-CD4^+T cells revealed a striking increase in IL-9 expression. At present, little is known about the regulation of IL-9. Recent studies have suggested that IL-25 and IL-21 enhance IL-9 expression in mice and humans, respectively[21,22]. We do not know the precise mechanism by which the absence of ROR-γ promotes IL-9 expression in T cells, although analysis of the IL-9 promoter does not reveal an ROR-γ binding site (VK Kuchroo, unpublished observations). Our data demonstrates greatly impaired IL-23R expression on ROR-γ^-CD4^+T cells in concert with increased IL-9 expression in

IL-23R^{-}CD4^{+}T cells. Therefore, inhibition of signaling via IL-23R expression in ROR-γ^{-}CD4^{+}T cells might be one of the factors responsible for increased in IL-9 expression.

Since IL-9 is primarily a product of CD4^{+}T cells, we explored the role of T_H9 cells in tumor immunity. Mice treated with adoptively transferred T_H9 cells showed profound resistance to melanoma growth. To our knowledge, this is the first report demonstrating an anti-tumor effect of T_H9 cells. Previous studies have reported that adoptive transfer of T_H9 cells can induce colitis and peripheral neuritis in lymphopoenic hosts[23], consistent with significant immune effector functions mediated by these cells. Similar to previous reports[7,9,24], in our experiments T_H2 and T_H17 cells, but not T_H0 and T_H1 cells inhibited melanoma growth. However the melanoma growth inhibitory properties of T_H9 cells were superior to those of T_H17 cells.

To determine independently whether IL-9 has melanoma growth inhibitory properties, tumor growth was studied in *IL9R*$^{-/-}$ mice. Melanoma tumor growth was reproducibly enhanced in *IL9R*$^{-/-}$ mice. Also, treatment with exogenous IL-9 reproducibly suppresses the growth of B16F10 melanoma and LLC-1 but not EL-4 tumor. To rule out the unlikely possibility that this was a direct effect of IL-9 on the tumor cells, we studied IL-9R expression on tumor cells and examined the direct effects of IL-9 on tumor cell growth (Supplementary Fig. 4b–d). B16F10 cells and LLC-1 cells show negligible IL-9R expression and IL-9 does not affect the growth of these cells *in vitro*, suggesting that IL-9 mediated anti-tumor effects on melanoma cells and on lung carcinoma cells are indirect. However, we observed increased IL-9R expression on EL-4 cells (Supplementary Fig. 4c). This suggests that the presence of IL-9R on EL-4 cells, and previously reported growth and survival promoting properties of IL-9 on T cells, may be accountable for negligible anti-tumor effect of IL-9 in this tumor model[25]. Therefore, these data indicates that potential tumor targets of rIL-9 therapy should be selected with care: specifically, lymphomas and other cells known to express receptors for this cytokine may not be appropriate candidates for treatment. However, taken together, these data are consistent with the idea that IL-9 is an independent factor that influences tumor growth.

Interestingly, we found no difference between normal and *Rag1*$^{-/-}$ mice in the degree of tumor inhibition mediated by exogenous IL-9, suggesting that beneficial effects of IL-9 are mediated by other immune cells. Both human[26] and mouse[27] mast cells are well known targets of IL-9. Recently mast cells have been implicated in anti-tumor activity[28]. Indeed, we observed that rIL-9 treatment had no measureable inhibitory effect on tumor growth in mast cell deficient hosts, suggesting that mast cells play key role in IL-9 mediated anti-tumor activity. Studies are underway to delineate how IL-9 modulates the mast cell anti-tumor activity (survival and function).

Finally, there has been some controversy as to whether T_H9 cells are solely murine phenomenon, and their relevance to human disease has been questioned. To the contrary, we could readily detect T_H9 cells in populations of human skin resident memory T cells, as well as in the memory T cell population of peripheral blood mononuclear cells. Importantly, we could also detect the presence of memory T_H9 TIL's, but at a lower abundance than memory T_H9 cells in either normal skin or blood. Our finding of low levels of T_H9 T cells in human

metastatic melanoma is interesting, but should be interpreted with caution. It may be that T_H9 cells are part of the normal human immune response to melanoma, and thus augmenting their activity, or providing additional IL-9, may be therapeutically advantageous in this setting.

The role of IL-9 in melanoma immunity has not been previously studied. Interestingly, however, single nucleotide polymorphisms in IL-9 gene were found to be associated with increased risk of cutaneous malignant melanoma (CMM)[29]. The role of IL-9 in tumor immunity in general is somewhat controversial with at least one recent study suggesting IL-9 promotes T_{reg} and inhibits tumor immunity[30]. In our experiments, however, blockade of endogenous IL-9 invariably accelerated tumor development in the melanoma model, and the absence of IL-9 signaling (i.e., *IL9R*$^{-/-}$mice) also enhanced melanoma growth. While our extensive experiments, using a large number of different variables, were consistent with a distinct anti-tumor effect of IL-9 in two different tumor models, further studies will determine how generalizable these finding are to tumor immunity and growth in general.

Our findings suggest that strategies that favor generation of IL-9 mediated immune responses may have an important role in the treatment of melanoma and other solid tumors. Other γc chain cytokines, such as IL-2, IL-15, and IL-21 [31–33], have been used in the treatment of human melanoma. In addition, adoptive therapy of melanoma antigen-specific T cells, including TIL's, has been used extensively in patients with metastatic melanoma[9,34–38]. Our data suggests that IL-9 and T_H9 cells may also have role in treatment of this challenging malignancy.

Methods

Mice

WT C57BL/6, *Rag1*$^{-/-}$ C57BL/6 and *IFNG*$^{-/-}$ mice were obtained from Jackson Laboratories. *IL9R*$^{-/-}$ [39], *IL23R*$^{-/-}$ and *RORc*$^{-/-}$ [17] and their control mice (*RORc*$^{+/+}$, and *IL9R*$^{-/+}$) were used, Mice were housed in conventional, pathogen-free facilities at the animal facility of Harvard Medical School.

In vitro T cell differentiation

$CD4^+CD25^-CD62Lhigh$ cells from *RORc*$^{-/-}$ mice or *RORc*$^{+/+}$ controls were sorted by $CD4^+CD62L^+$ isolation kit II from Miltenyi Biotech (USA) according to manufacturer's protocol. Purity of $CD4^+CD25^-CD62Lhigh$ was >95%.

Sorted $CD4^+CD25^-CD62Lhigh$ cells were differentiated into T_H1 (IL-12: 10 ng ml^{-1}), T_H2 (IL-4: 10 ng ml^{-1}), T_H9 (TGF-β plus IL-4: 1 ng ml^{-1} and 10 ng ml^{-1}), and T_H17 (TGF-β plus IL-6: 1 ng ml^{-1} and 10 ng ml^{-1}) in presence with plate bound anti-CD3 (1 μg ml^{-1}) and irradiated splenocytes (1:5 ratio). After 48 h cells were fed with IL-2 (10 ng ml^{-1}) containing fresh media and split into two parts, if needed. On day 5, cells were harvested and processed for cytokine analysis at RNA or protein level by real-time RT-PCR, flow cytometry and ELISA.

For adoptive transfer experiments, $CD4^+T_H$ cells differentiation was carried out using above mentioned protocol with few modifications. Plate bound antibodies to CD3 (2 μg ml^{-1}) and CD28 (1 μg ml^{-1}) was used in place of irradiated splenocytes. In addition to above mention polarization condition, anti-IFN-γ mAb (10 μg ml^{-1}) was added into bv9 cultures and anti-IFN-γ mAb (10 μg ml^{-1}) plus anti-IL-4 mAb (10 μg ml^{-1}) was added into T_H17 cultures.

Tumor (Melanoma, Lewis lung carcinoma and thymic lymphoma) induction, *In vivo* T cell transfer and IL-9 neutralization

Melanoma cell lines (B16F10 cells or B16F10-ova cells), T cell lymphoma (EL-4) and Lewis lung carcinoma (LLC-1) were grown in RPMI1640 supplemented with 10% FBS, and penicillin/streptomycin. B16F10 cells (2–4 × 10^5 cells 150 μl^{-1}), EL-4 (2×10^5 cells 150 μl^{-1}), or LLC1 (5×10^5 cells 150 μl^{-1}) were injected subcutaneously into the right or left flank of the mice and tumor development was monitored over time. Tumor volume was calculated by following formula: (major circumference X minor circumference^2)/2. Mice were sacrificed when there was external necrosis or/and tumor volume reached no greater than 2 cm in any direction.

To investigate the role of effector subsets of T_H cells on melanoma and thymic lymphoma growth, 2-million differentiated cells (T_H1, T_H2, T_H9 and T_H17) from $CD45.1^+CD45.2^-$OT2 TCR transgenic mice were injected (*i.v.*) into WT-C57BL6 mice or *Rag1*$^{-/-}$ (C57BL6 background) mice and, on the same day tumor cells (B16F10-ova cells: 3×10^5 cells 150 μl^{-1}) were injected subcutaneously. Tumor growth was monitored over time.

IL-9 activity *in vivo* was neutralized by injecting (*i.p.*) 100 μg neutrilizing antibody to IL-9 mAb (clone: MM9C1, a generous gift by Jacques van Snick from Ludwig Institute, Belgium) for 4 times on day 0, day 3, day 6 and day 10. Melanoma cells were injected on day 0 and melanoma growth was monitored over time.

rIL-9 (5 μg: from Cell Sciences, USA) was injected (*i.p.*) from day 0 and every third day till the termination of the experiment. Unlike Cell Science rIL-9 (source: E.Coli), rIL-9 from RnD systems is glycosylated and therefore has stronger biological activity. Thus, we used 100 times less rIL-9 from RnD systems (i.e. 50 ng on every alternate day till the termination of experiment) in LLC-1 tumor model experiments compared to melanoma model experiments. In addition, we also treated melanoma bearing mice with rIL-9 from RnD systems, USA which produced similar results as were observed with rIL-9 from Cell Sciences (data not shown).

Cytotoxicity Assay

For the cytotoxicity assay, CFSE labeled B16F10-ova cells (5×10^5 cells 0.5ml^{-1}) were cultured with differentiated T_H cells (OT2-T_H0, OT2-T_H9 and OT2-T_H17) in several different ratios. After 24 h of co-culture, cells (gate on CFSE labeled B16F10-ova cells) were analyzed for 7-AAD staining, a sensitive indicator of apoptotic cell death by flow cytometry. In addition, we used another cytotoxicity assay as described before [5].

Growth curve assay

Effect of rIL-9 on growth of B16F10 cells were studied by growth curve assay. B16F10 cells were seeded with rIL-9. After each incubation period cells were fixed (10% acetic acid in 10% ethanol). Cells were subsequently stained with 0.4% crystal violet in 10% ethanol for 30 min. Subsequently, 200 μl 10% acetic acid was added. After 30 min, 100 μl solution was transferred into 96-well plate and OD was measured at 595-wavelength.

Measurement of cytokines by intracellular cytokine staining, CBA, ELISA and quantitative RT-PCR

Intracellular cytokines by Lymph node (LNs) cells, splenocytes, TILs or *in vitro* differentiated T_H cells were quantified after restimulation with PMA plus ionomycin in presence of GolgiStop for 6 h as described previously[40].

Cytokines were quantified in cell free culture supernatants by cytometric bead array (CBA by BD Biosciences) or by ELISA (eBioscience) according to the manufacturer's instructions.

RNA was extracted with High pure RNA isolation kit (Roche), cDNA was made by First strand cDNA synthesis kit (BioRad) and quantitative RT-PCR was done using multiplex kit (BioRad) on iCycler (BioRad) according to the manufacturer's instructions. IL-9R PCR was carried out by using IL-9R specific taqman probes and AB Biosystem PCR machine.

Cell purification, sorting, Intracellular cytokine staining and cytokine quantification in supernatants (Human study)

PBMCs were isolated from buffy coats of healthy donors by density centrifugation. Memory $CD4^+$T cells were purified from freshly isolated PBMCs by negative selection using a Memory $CD4^+$T cells Isolation Kit (Miltenyi Biotech, Germany) and stimulated with anti-CD3/CD2/CD28 beads (Milyenyi) in presence of TGF-β (3 ng ml^{-1}).

Normal human skin samples were obtained as discarded material after cosmetic surgery according to Institutional Review Board of Partners Human Research Committee. Total skin T cells from healthy donors and T cells of melanoma metastasis were isolated from explant cultures grown with IL-2 and IL-15 as previously[41] described.

Memory T cells ($CD4^+CD45RO^+$) from peripheral blood after stimulation with anti-CD3/CD28 mAbs plus TGF-β and skin-resident T cells isolated by skin-explant culture of healthy donors were restimulated for 5 h in the presence of PMA plus ionomycin (Sigma-Aldrich) in presence of golgistop. After incubation, $CD4^+$T cells were stained for IFN-γ (anti-IFN-γ: B27), IL-4 (anti-IL-4: MP4-25D2), IL-9 (antiIL-9: MH9A4) and IL-17 (anti-IL-17: eBio64DEC17,) using intracellular staining and analyzed by flow cytometry[40].

Memory T cells from blood, skin T cells from healthy donors and tumor-infiltrating lymphocytes of subjects with melanoma metastasis were stimulated at 10^6 cells ml^{-1} with beads coated with antibodies to CD3/CD2/CD28 (bead: T cell ratio: 1:2 from Miltenyi Biotech) in the presence of IL-2 (50 IU ml^{-1}) and TGF-β (3 ng ml^{-1}) for 2 days. IL-9 in

culture supernatants was measured by Luminex bead-based multiplex assays using a custom-made Luminex bead assay as described previously[42].

Statistical analysis

Student t-test (two tailed) was performed for the data analysis using GraphPad Prism software program. A paired t-test was used in Supplementary Fig 2h, and 2J. The p value <. 005, .025 and .05 are represented as ***, ** and * respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by National Institutes of Health to TSK (RO1 AI-41707; P50 CA 093683), RAC (R01-AR-056720) and to AMJ (Z01-ES-101586). The authors thank Dr. Kevin Gerrish (National Institute of Health) with his help with the microarray analysis and Dr. Jean-Christophe Renauld [39] (Ludwig Institute, Belgium) for providing *IL9R*$^{-/-}$ and their control mice (*IL9R*$^{-/+}$). Salary support for C.S. was provided by the Swiss National Science Foundation and the Foundation Rene Touraine

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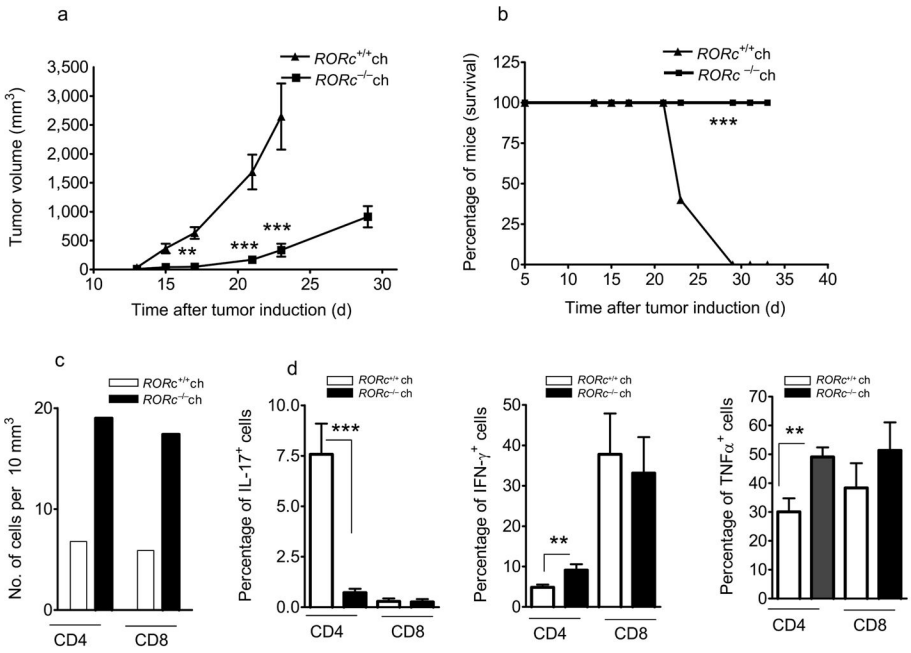

Figure 1. Deficiency of ROR-γ is associated with inhibited melanoma growth and increased tumor lymphocytic infiltration

B16F10 melanoma cells were injected subcutaneously into *RORc*$^{-/-}$ ch and *RORc*$^{+/+}$ ch mice. Tumor growth (**a**) and mice survival (**b**) was monitored over time. (**c**) Tumor infiltrating lymphocytes (TILs) were recovered and counted as described in methods. (**d**) Expression of IL-17A, IFN-γ, and TNF-a was analyzed in CD4^{+} and CD8^{+}T cells of tumor draining LNs by flow cytometry. Data is represented as Mean ± SEM in **a**–**b** (n= 8 mice per group (p<. 005: ***), in **c** (TILs from 4 mice were pooled and analyzed) and in **d** (n=8 mice per group, p<. 005: ***, p<. 025: **). Two-three additional independent experiments provided similar results.

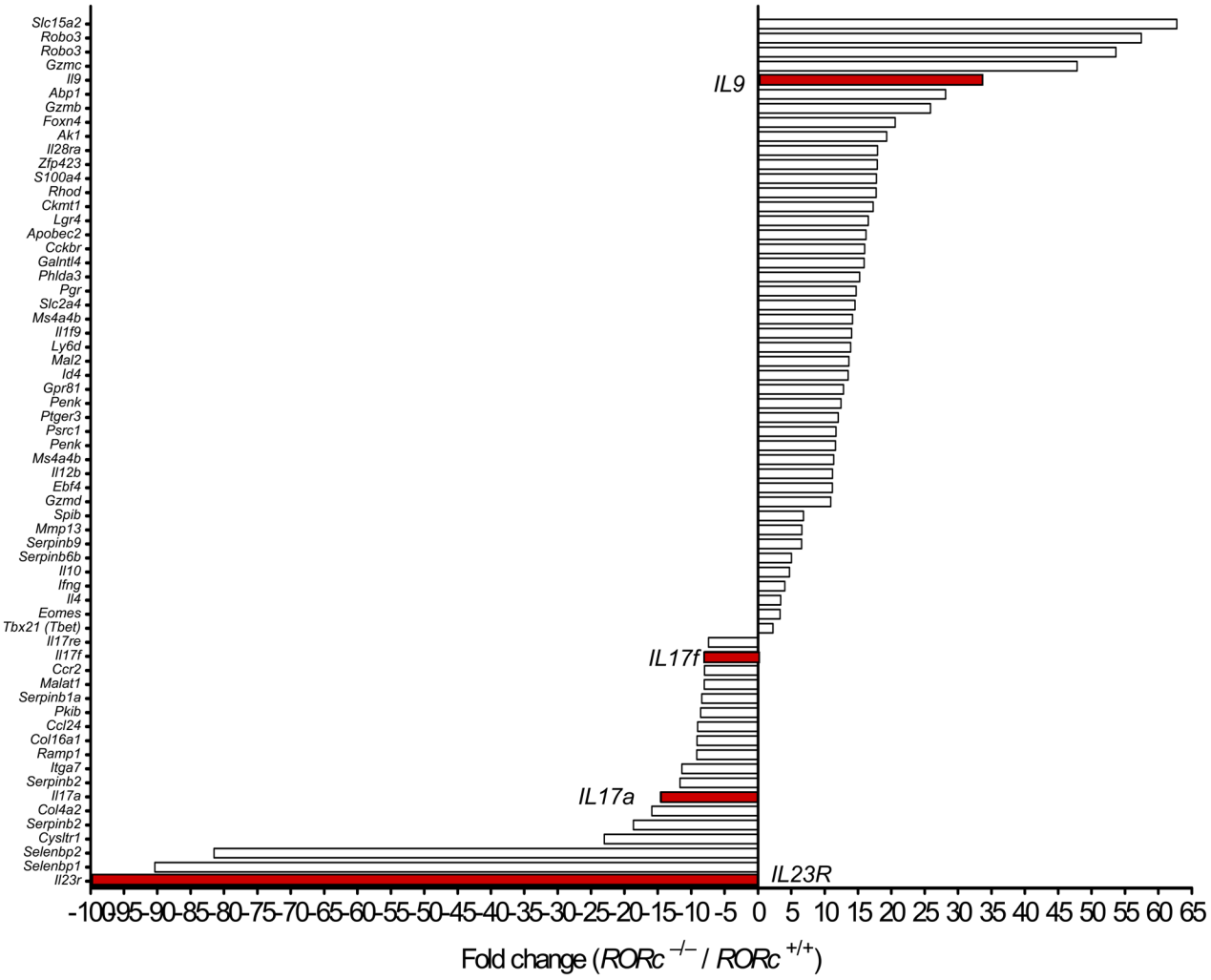

Figure 2. Increased IL-9 expression in ROR-γ⁻T cells

Sorted naïve T_H cells ($CD4^+CD25^-CD62L^{high}$) from *RORc*$^{+/+}$ and *RORc*$^{-/-}$ mice were differentiated under T_H17 polarizing conditions. After 4 days, cells were harvested for transcriptional profiling experiments. Gene expression analysis was performed and expression of a set of genes is depicted as fold change (*RORc*$^{-/-}$ vs. *RORc*$^{+/+}$). Two additional microarray analyses provided similar results.

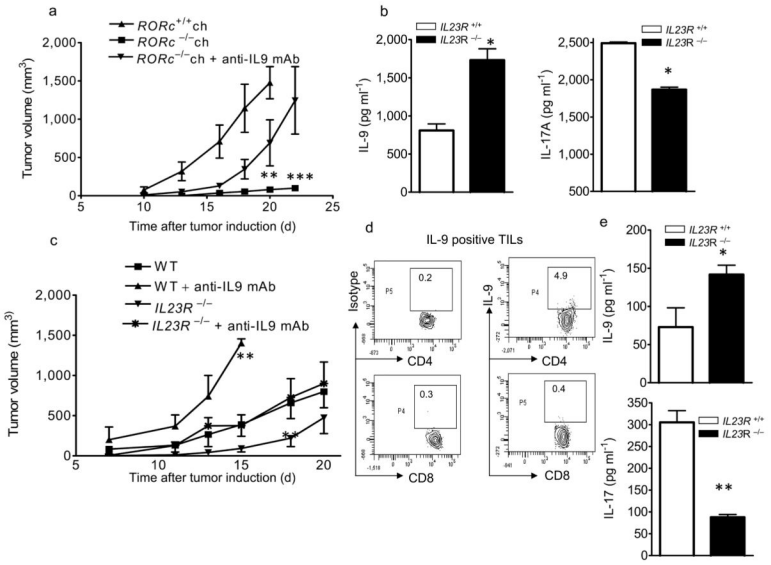

Figure 3. The role of IL-9 in melanoma immunity in *IL23R*$^{-/-}$ mice

(**a**) B16F10 melanoma cells were injected subcutaneously into *RORc*$^{-/-}$ ch, and control mice (*RORc*$^{+/+}$ ch). Anti-IL-9 neutralizing antibody was administered to *RORc*$^{-/-}$ch mice. (**b**) *in vitro* differentiated T_H17 cells from *IL23R*$^{+/+}$ and *IL23R*$^{-/-}$ mice were restimulated and IL-9 and IL-17A were estimated by ELISA. (**c**) B16F10 melanoma cells were injected subcutaneously into *IL23R*$^{-/-}$and their controls. Neutralizing antibody to IL-9 was administered to both WT and *IL23R*$^{-/-}$mice. Melanoma growth was monitored over time. (**d**) TILs were isolated from tumor growing in WT mice. IL-9 was stained and quantified by flow cytometry. (**e**) IL-9 and IL-17 were estimated by ELISA in supernatants from tumor draining LNCs. Data is represented as Mean ± SEM and statistically significant differences were observed compared to controls (p<. 005: ***, p<. 025: **, p<.05: *). At least two additional independent experiments produced similar results.

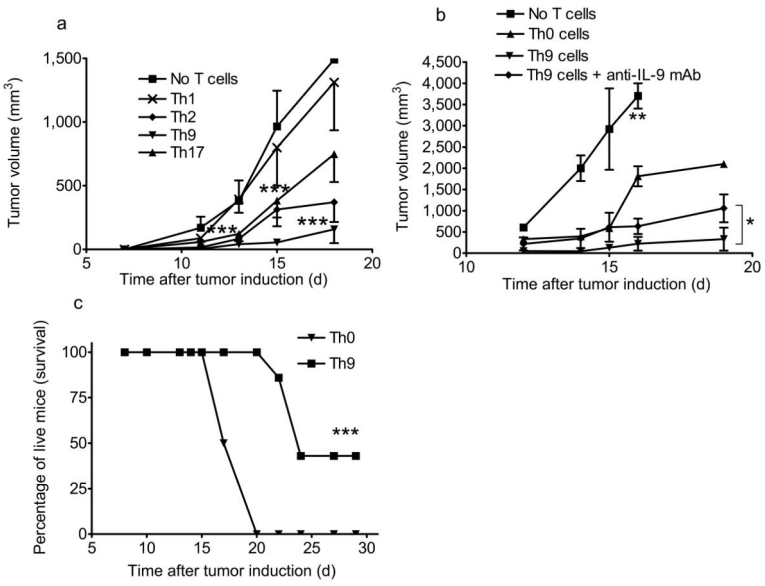

Figure 4. T_H9 cells inhibit melanoma growth

Differentiated-T_H cells from OT-II mice were generated and transferred into WT mice (WT-C57BL/6: **a**) or *Rag1*$^{-/-}$C57BL/6 mice (**b**–**c**). On the same day, B16F10-ova cells were injected subcutaneously. Melanoma growth (**a**–**c**) was monitored overtime. Neutralizing antibodies to IL-9 or isotype was given to T_H9 treated mice (**b**). Data is represented as Mean ± SEM (**a**–**c,** n=6 mice per group) and statistically significant differences were observed compared to No-T cells group (**a**), and as depicted (**b**–**c**) (p<. 005: ***, p<. 025: **, p<.05: *).

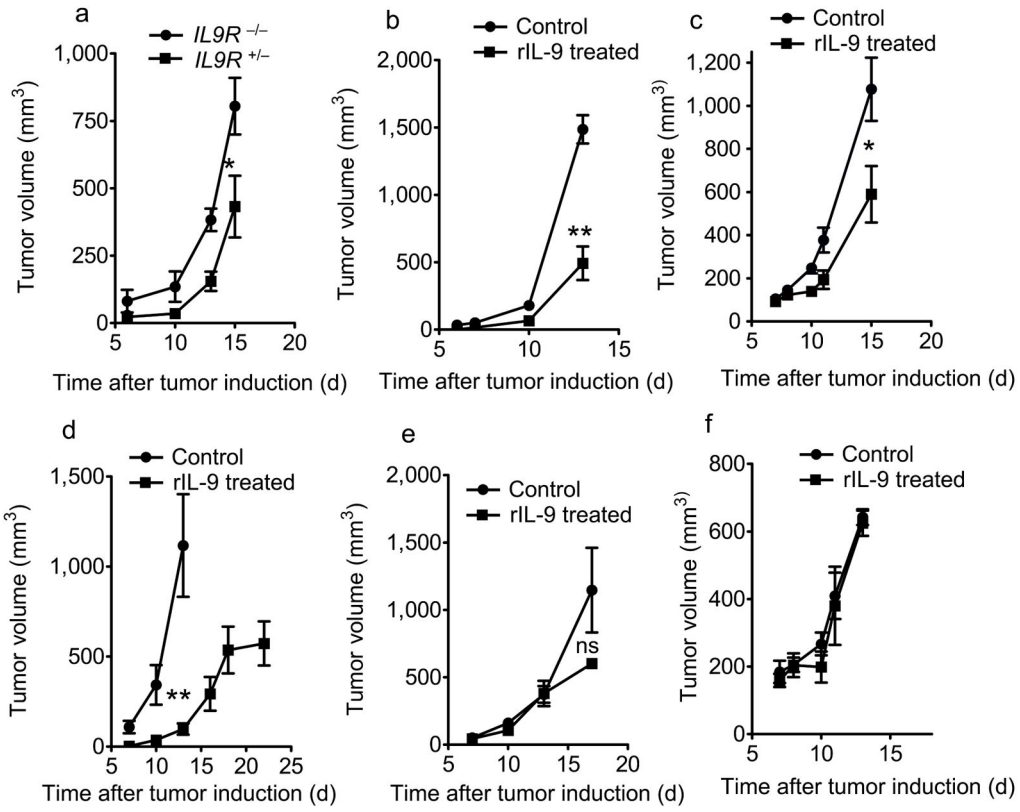

Figure 5. Abrogation of IL-9/IL-9R signaling promotes melanoma development, and treatment with rIL-9 inhibits melanoma development

(**a**, **b**, **d** & **e**) B16F10 melanoma cells were injected subcutaneously into *IL9R*$^{-/-}$ and controls (*IL9R*$^{-/+}$) mice (**a**), normal WT mice (**b**) and *Rag1*$^{-/-}$ mice (**d**) and Kit W-sh (mast cell deficient) mice (**e**). (**c** & **f**) Lewis lung carcinoma cells were injected subcutaneously into normal WT mice (**c**) and Kit W-sh (mast cell deficient) mice (**f**). Where indicated, rIL-9 was administered. Control mice received PBS. Tumor growth was monitored over time. Data is represented as Mean ± SEM (n=4 mice per group, 2–4 additional independent experiment produced similar results, and statistically significant differences were observed compared to controls (p<. 025: **, p<.05: *, ns: not significant).

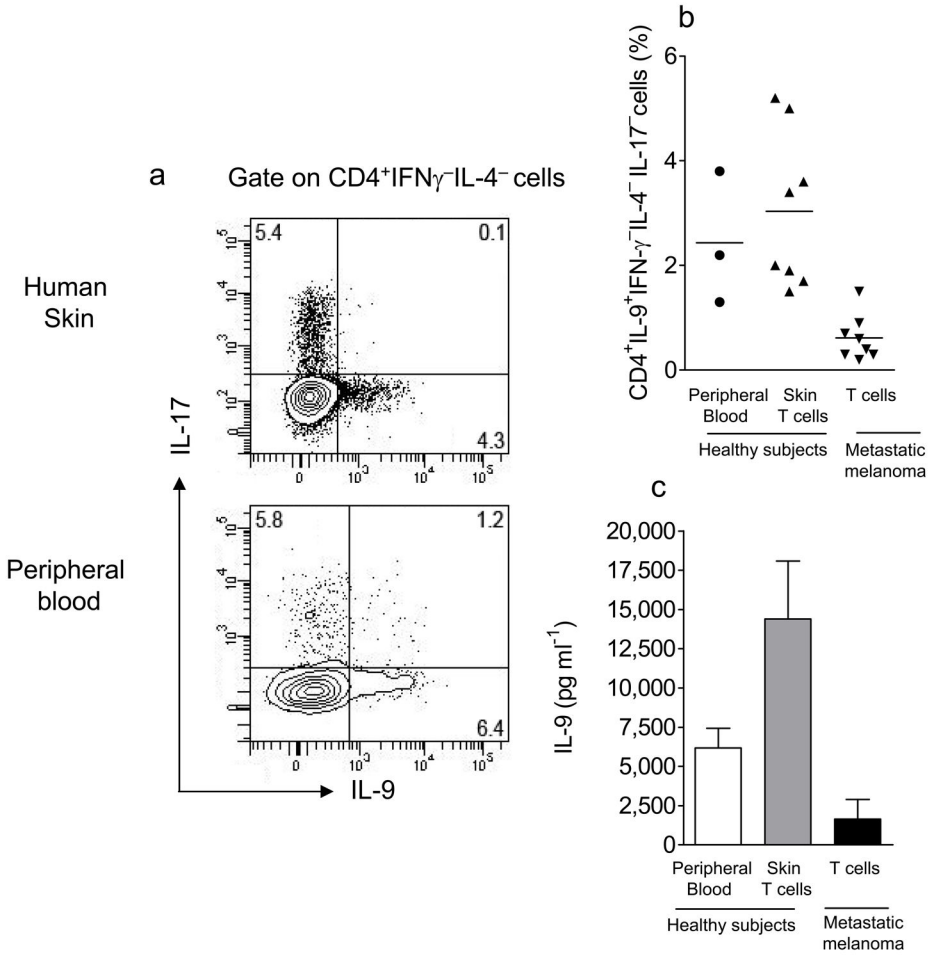

Figure 6. Presence of T_H9 cells in human skin and peripheral blood

(**a**–**b**) Memory T cells from peripheral blood and skin-resident T cells of healthy donors were stained for IL-9, IFN-γ, IL-17 and IL-4 and analyzed by flow cytometry. (**c**) IL-9 production was measured in cell free supernatants by luminex assay from memory T cells of peripheral blood from healthy donors, skin T cells from healthy donors and tumor-infiltrating lymphocytes of subjects with metastatic melanoma. Data is represented as Mean ± SEM (skin: n=8, PBMCs: n=3, MM: n=8).